COMMON GENETIC VARIANTS IN CHOLINE, FOLATE, AND VITAMIN D METABOLIC PATHWAYS INFLUENCE METABOLISM IN WOMEN OF REPRODUCTIVE AGE MEETING NUTRIENT INTAKE RECOMMENDATIONS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Ariel Bacaner Ganz

August 2017

COMMON GENETIC VARIANTS IN CHOLINE, FOLATE, AND VITAMIN D METABOLIC PATHWAYS INFLUENCE METABOLISM IN WOMEN OF REPRODUCTIVE AGE MEETING NUTRIENT INTAKE RECOMMENDATIONS

Ariel Bacaner Ganz, Ph.D.

Cornell University 2017

Nutrient needs are a population-wide distribution, and can vary dramatically from person to person. Factors known to influence individual nutrient needs include common genetic polymorphisms as well as pregnancy and lactation, physiological states that are generally associated with an increased nutrient requirement. This doctoral dissertation examines the effects of common genetic variants in choline, folate, and vitamin D metabolic pathways on metabolism at recommended nutrient intakes. Interactions between genetic and reproductive factors that influence metabolism were also examined. While the genetic variants examined herein have been studied in conditions of nutrient deprivation or among non-pregnant women, these studies extend prior work to conditions of nutrient adequacy that are relevant to the population at large and to include pregnancy and lactating individuals, who have an increased risk for nutrient inadequacy, and much to gain from adequacy. We identified that common genetic variants impairing folate enzymes increase the metabolic use of choline for phosphatidylcholine production. In addition, variants in choline metabolizing enzymes altered choline dynamics and partitioning. Lastly, vitamin D binding protein rs7041 genotype altered vitamin D metabolism in pregnant women. Comprehensively, these findings provide evidence for differences in metabolism and nutrient needs at recommended intakes, and suggest personalized recommendations may be necessary to achieve optimal health and nutrition.

BIOGRAPHICAL SKETCH

Ariel graduated cum laude with a bachelor's degree in biology from Brandeis University in 2012. After a working in academic laboratories at the Scripps Institute of Oceanography at the University of California, San Diego, and the University of Minnesota, Ariel matriculated to Cornell in 2013, where her doctoral research has focused on examining genetic and biochemical differences in one-carbon metabolism and vitamin D metabolism. As part of an NIH T32 Training grant for translational research, Ariel was co-advised by Dr. Marie Caudill and Dr. Patrick Stover. Ariel has also been active in academic outreach throughout her time at Cornell, mentoring undergraduate students, co-teaching a weekly computer science course to a local high school chapter of the National Society of Black Engineers (NSBE) Jr., and co-taught a five-week mini-course on experiments in chemistry and biology to local elementary school students. She additionally served as a teaching assistant for undergraduate nutrition laboratory courses for 3 semesters.

Dedicated to the pursuit of truth

ACKNOWLEDGMENTS

Thank you to my advisors, Dr. Marie Caudill and Dr. Patrick Stover, for your constant support, and for giving me the freedom to find my own path. Engraved in the stone benches on libe slope, overlooking Lake Cayuga, are the words, "The Cornell tradition… allows a maximum of freedom and relies so confidently upon the sense of personal responsibility for making a good use of it." These words, from Professor Carl Becker, have defined my PhD experience; I am so grateful to have had this opportunity to develop my personal responsibility. Despite the freedom I've been given, doctoral degrees are a team effort, and I am truly grateful to everyone that has participated and supported me along the way. Thank you to my my lab mates in the Stover and Caudill Labs, Olga Malysheva, and Dr. Martha Field, as well as my committee members, Dr. Julia Finkelstein, Dr. Yimon Aye, Dr. Hening Lin, and Dr. Frank Schroeder for your mentorship. Thank you to Laure Conklin Camp, for keeping me on track and encouraging me to graduate. Thank you to my family for supporting me and providing me with a model of excellence. Thank you to my grandparents, Hadassah and Bucky Bacaner, for reminding me to believe in myself, always; my grandfather, George Backus, for impressing upon me the importance of rigor and precision; and my grandmother, Varda Backus, for teaching me to aim high, but not for perfection. Thank you to my father, Eric Ganz, for imparting upon me a sense of right and wrong, and to my mother, Nina Bacaner, for telling me to enjoy the sunshine. Thank you to my sister, Lily Ganz, my cousin, Leslie Ruff, my friends, and everyone in Ithaca that has made Cornell feel like a home.

Research reported in this publication was in part supported by the National Institutes of Health under award T32-DK007158. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) or the National Institutes of Health.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

LIST OF ABBREVIATIONS

AI	adequate intake
BHMT	betaine homocysteine methyltransferase
CDP	cytidine diphosphate
CHKA	choline kinase-α
CHDH	choline dehydrogenase
CI	confidence interval
DBP	vitamin D binding protein
dbSNP	Single Nucleotide Polymorphism Database
DHA	docosahexaenoic acid
DMG	dimethylglycine
FMO3	Flavin monooxygenase isoform 3
GC	gas chromatography
(in the context of MS)	
GC	the vitamin D binding protein gene
LC	liquid chromatography
MS	mass spectrometry
MTHFD1	methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
NAFLD	Non-alcoholic fatty liver disease
NCBIU.S.	National Center for Biotechnology Information
NP	non-pregnant

NTD	neural tube defect
OMIM	Online Mendelian Inheritance in Man
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase
PC	phosphatidylcholine
PEMT	phosphatidylethanolamine N-methyltransferase
QE	Q Exactive
RefSeq	U.S. Reference Sequence Database
REV	SNP is identified on the reverse strand
RS	reproductive state
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SNP	single nucleotide polymorphism
SLC44A1	solute carrier 44A1
THF	tetrahydrofolate
WT	wildtype

CHAPTER 1

Genetic Impairments in Folate Enzymes Increase Dependence on Dietary Choline for Phosphatidylcholine Production At the Expense of Betaine Synthesis[*]

Abstract

Although single nucleotide polymorphisms (SNPs) in folate-mediated pathways predict susceptibility to choline deficiency during severe choline deprivation, it is unknown if effects persist at recommended intakes. Thus, we employed stable isotope LCMS/MS methodology to examine the impact of candidate SNPs on choline metabolism in a long-term randomized controlled feeding trial among pregnant, lactating, and non-pregnant women consuming 480 or 930 mg choline/d (22% as choline-d_9) and meeting folate intake recommendations. Variants impairing folate metabolism, *MTHFR* rs1801133 (c.677T); *MTR* rs1805087 (wildtype c.66A); *MTRR* rs1801394 (c.2756G); and *MTHFD1* rs2236225 (c.1958A), influenced choline dynamics, frequently through interactions with reproductive state and choline intake, with fewer genotypic alterations observed among pregnant women. Women with these variants partitioned more dietary choline towards phosphatidylcholine (PC) biosynthesis via the CDP-choline pathway at the expense of betaine synthesis even when use of betaine as a methyl donor was increased. Choline intakes of 930 mg/d restored partitioning of dietary choline between betaine and CDP-PC among non-pregnant (*MTHFR* rs1801133

---

[*]Ganz AB, Shields K, Fomin VG, Lopez Y[#], Mohan S, Lovesky J, Chuang JC, Ganti A, Carrier B, Yan J, Taeswuan S, Cohen VV, Swersky CC, Stover JA, Vitiello GA, Malysheva OV, Mudrak E, and Caudill MA. Genetic Impairments in Folate Enzymes Increase Dependence on Dietary Choline for Phosphatidylcholine Production at the Expense of Betaine Synthesis FASEB J 2016, 30(7).

[#]Deceased

and *MTR* rs1805087 wildtype) and lactating (*MTHFD1* rs2236225) women with "risk" genotypes. Overall our findings indicate that loss-of-function variants in folate-metabolizing enzymes strain cellular PC production, possibly via impaired folate-dependent PEMT-PC synthesis, and suggest that women with these "risk" genotypes may benefit from choline intakes exceeding current recommendations.

Introduction

Single nucleotide polymorphisms (SNPs) can alter metabolism and utilization of the micronutrients folate and choline.[1,2] Inadequacy of these nutrients are risk factors for cancer[3] and birth defects,[4,5,6] which are pathologies that result from complex interactions between genetic and environmental risk factors. Methylenetetrahydrofolate reductase (*MTHFR*) rs1801133, methionine synthase (*MTR*) rs1805087, methionine synthase reductase (*MTRR*) rs1801394, and Methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolatecyclohydrolase-formyltetrahydrofolate synthetase (*MTHFD1*) rs2236225, are common SNPs in enzymes involved in folate-mediated one-carbon metabolism that predict increased risk of birth defects as well as risk of choline deficiency and onset of fatty liver disease in humans deprived of choline.[6,7]

The relationship between folate enzyme SNPs and choline requirement may arise from the overlapping roles of folate and choline in methionine and phosphatidylcholine (PC) biosynthesis. While folate is primarily responsible for the remethylation of homocysteine to methionine, which generates the methyl donor *S*-adenosylmethionine (SAM), the choline oxidation product betaine also contributes to methionine biosynthesis, particularly in the liver[8]. An increased contribution by choline-derived betaine to methionine biosynthesis may

spare folate cofactors for nucleotide biosynthesis. Furthermore, while choline is used for phosphatidylcholine (PC) synthesis via the cytidine diphosphate (CDP)-choline pathway, PC produced by the phosphatidylethanolamine-*N*-methyltransferase (PEMT) uses SAM as a methyl donor and is therefore dependent upon both folate- and choline-derived methyl groups.[9,10] Genetic risk factors that increase the nutritional requirement for choline may contribute to the pathogenesis of diseases related to one-carbon metabolism.[vi,11]

Physiological factors such as reproductive state can similarly influence choline requirements. The demand for choline is uniquely high during pregnancy and lactation due to rapid membrane biogenesis, methylation of placental and fetal DNA, and brain development. Experimental data in humans have shown altered choline partitioning that indicates an increased requirement for choline during pregnancy and lactation.[12,13,14] For example, compared to non-pregnant women, pregnant women use more choline to synthesize PC through both the CDP-choline and PEMT pathways and use more of choline-derived betaine as a methyl donor.[12] Furthermore, maternal choline intakes exceeding current dietary recommendations appear to have favorable effects on several physiologic outcomes during pregnancy, including placental functioning[15] and the fetal response to stress.[16]

At present, it is unknown whether the increased need for choline during pregnancy and lactation can be compounded by genetic risk. Furthermore, although genetic variation modifies disease risk and choline requirements during severe choline deprivation, and the *MTHFR* rs1801133 variant is known to alter the metabolic flux of choline under conditions of folate inadequacy,[17] it is unknown whether these variants are functionally relevant under conditions of nutrient sufficiency. Thus, we sought to evaluate the influence of "risk

genotypes" in folate enzymes on choline metabolism among women of differing reproductive states and meeting dietary choline and folate intake recommendations (**Figure 1**).

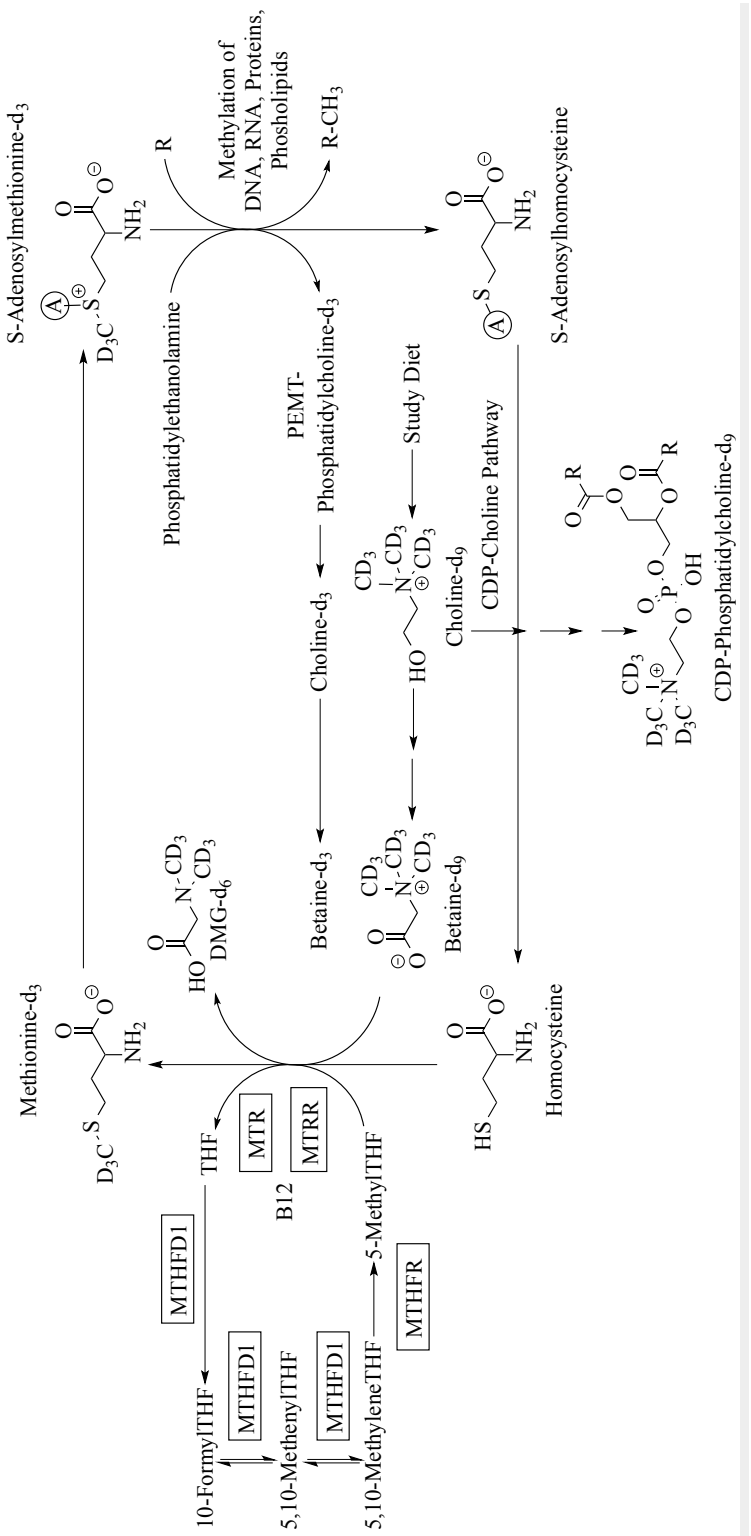


Figure 1. **Overview of the metabolic fate of isotopically labeled choline-d_9 consumed by study participants.** SNPs, in squared enzymes, were examined: MTHFD1 [dbSNP: rs2236225; c.1958 G > A; p.Arg653Gln; RefSeq NT_026437.13], MTHFR (dbSNP: rs1801133; c.677 C > T; p.Ala223Val; RefSeq NT_021937.17), MTR (dbSNP: rs1805087; c.2756 A > G; p.Asp919Gly; RefSeq NG_008959.1), MTRR (dbSNP: rs1801394; c.66 A > G; p.Ile22Met; RefSeq NG_008856.1). Note that a deuterated methyl group from DMG-d_6 could be added to tetrahydrofolate (THF) by MTHFD1 to produce 5,10-methenyl-THF-d_2 (loss of a deuterium occurs during addition), which might be converted to 5-methyl THF-d_2 and used to methylate homocysteine to produce methionine-d_2. Alternatively, the 5,10-methenyl-THF-d_2 might be converted to 10-formyl-THF-d_1 (loss of additional deuterium) and ultimately to methionine-d_1. However, these scenarios result in methionine-d_2 or methionine-d_1 rather than methionine-d_3 and therefore, do not confound our analysis of methionine-d_3 and its downstream metabolites.

Participants and Methods

A. Participants and Study Design

A long-term randomized controlled feeding study was carried out among women in different reproductive states, as described by Yan et al.[18] Briefly, healthy non-pregnant, lactating, and third-trimester pregnant women were recruited from the Ithaca, NY area and assessed for eligibility using a blood chemistry profile, complete blood count, and health history questionnaire. Exclusion criteria included chronic disease, alcohol or tobacco use, and indication of liver, kidney, or pregnancy complications.[18] Eligible participants (pregnant n=26, non-pregnant n=21, lactating n=28) consumed the study diet[18] for 10-12 weeks, which provided 380 mg choline/d and either 100 or 550 mg choline/d from supplemental choline chloride (Balchem). In addition, all participants consumed a daily prenatal multivitamin (Pregnancy Plus; Fairhaven Health, LLC) containing 600 μg folic acid, a daily docasahexanoic acid supplement (200 mg, Neuromins; Nature's Way), and a thrice-weekly potassium and magnesium supplement (General Nutrition Corp). During study weeks 6-10 (lactating women) or 6-12 (pregnant and non-pregnant women), participants consumed 22% of their total choline in the form of choline chloride-(trimethyl-d_9) (Cambridge Isotope Laboratories, 98%). The study was approved by the Institutional Review Boards at Cornell University and Cayuga Medical Center and was registered at clinicaltrials.gov as NCT01127022. All participants provided informed consent.

B. Sample Collection and Processing

Fasting blood (10-h) and breast milk samples were collected and processed as previously described.[13,18] Briefly, blood was collected into EDTA-coated tubes at baseline and study

weeks 3, 6, 9, 10, and 12 and placed on ice immediately. After centrifugation (2200 rpm, 15 minutes, 4°C), plasma and buffy coat were dispensed into 1.5 μL centrifuge tubes. Similar to blood, breast milk samples were placed on ice, processed without delay, and stored at −80°C until analysis. One lactating participant provided week 9 samples at week 8. Another provided samples at week 9.5 that were used for week 9 measurements in plasma and week 10 measurements in breast milk.

C. Genotyping

DNA for genotyping was extracted from blood sample buffy coats using the commercially available DNeasy Blood and Tissue kit (Qiagan). Genotyping of *MTR* (dbSNP: rs1805087; c.2756 A>G; p.Asp919Gly; RefSeq NG_008959.1; OMIM 156570) and *MTRR* (dbSNP: rs1801394; c.66 A>G; p.Ile22Met; RefSeq NG_008856.1; OMIM 602568), was performed using Endpoint Genotyping on a LightCycler480 (Roche) in our facility. Briefly, a commercially available mix (Applied Biosystems® TaqMan® Genotyping Master Mix and Assay Mix from Life Technologies) utilizing a dual hydrolysis probe with VIC and FAM dyes was used to quantify the fluorescence and presence of the variant and familial alleles. High fluorescence of VIC or FAM was considered to be homozygous variant or homozygous wild type (WT), respectively, while samples with moderate fluorescence in both VIC and FAM channels were considered heterozygous. Samples were run in duplicate with in-run standards and a negative water control. Cycling conditions were 95°C for 10 minutes, followed by 34 cycles of 92°C for 15s and 62°C for 90s.

Genotyping of *MTHFD1* (dbSNP: rs2236225; c.1958 G>A; p.Arg653Gln; RefSeq NT_026437.13; OMIM 172460) and *MTHFR* (dbSNP: rs1801133; c.677 C>T; p.Ala223Val;

RefSeq NT_021937.17; OMIM 607093) was performed by Sanger-DNA genotyping PCR as previously described[19] in Cornell's Life Sciences Core Laboratories Center. The forward and reverse primers used for *MTHFD1* rs2236225 were 5'GCATCTTGAGAGCCCTGAC3' and 5'CACTCCAGTGTTTGTCCATG3', respectively (Invitrogen).[20] Cycling condition were 95°C for 5 min; 35 cycles (95°C for 30 s, 50°C for 30 s, 72°C for 30 s); 72°C for 10 min, and 4°C hold. The *MTHFR* forward and reverse primers were 5'AGGACGGTGCGGTGAGAGTG3' and 5'TGAAGGAGAAGGTGTCTGCGGGA3', respectively (Invitrogen).[21] Cycling conditions were 95°C for 5 min; 30 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30s); 72°C for 10 min, 4°C hold. Double-stranded DNA obtained from PCR was purified using a commercially available QIAquick PCR Purification kit (Qiagen) and sequenced using an Applied Biosystems Automated 3730 DNA analyzer. Internal standards for each genotype were included and all results were duplicated.

D. Enrichment of Choline Metabolites

Metabolite enrichments of choline-d_9, choline-d_3, betaine-d_9, betaine-d_3, and DMG-d_6 as well as PC-d_3, PC-d_6, and PC-d_9 were measured at study week 9 for plasma and study week 10 for breast milk. Enrichments were measured on our TSQ Quantum Access triple quadrupole LCMS system (Thermo) operated in positive-ion mode using electrospray ionization as previously described.[12,17] Internal standards were not added to samples designated for enrichment measurements in order to allow for the measurement of isotopic enrichments. Enrichments were calculated by dividing the amount of each isotopically labeled choline metabolite by the total amount of all isotopomers and multiplying by 100%.

E. Enrichment of Methionine-d_3

Week 9 acidified plasma (250 µL) was thawed and 150 µL of 25 mM ammonium acetate buffer was added to each sample. Proteins were precipitated with 125 µL of 1 M perchloric acid, and samples were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was transferred to a new 1.5 µL eppendorf tube and neutralized with 90 µL of 10 M ammonium hydroxide (final pH ~ 4.5). Neutralized samples were applied to C18 solid-phase extraction (SPE) columns (200mg, 3cc, Sep-Pak® Vac, Waters) with a monofunctionally bonded silica phase (preconditioned with 1 mL methanol, 750 µL of 0.1N NaOH, and 1 mL H_2O) on a vacuum manifold (GAST). Milli-Q water (1 mL) was used to wash the samples before elution with 1.5 mL of 0.1% formic acid in 85:15 H_2O: methanol. Samples were evaporated in a speed vacuum and subsequently resuspended in 50 µL water, diluted with 50 µL acetonitrile/methanol (1:1, v/v), and centrifuged (20,000 g, 4 °C, 5 min) before injection into the LC-MS.

F. LCMS for Methionine-d_3

Enrichment measurements of methionine-d_3 were performed on a Unimate 3000 UPLC (Dionex) and Q Exactive-Mass spectromer (QE-MS, Thermo Scientific) in the Locasale Laboratory (Savage Hall, Cornell University). Methods were adapted from Kim et al[22]. The purified extracts containing methionine-d_3 were injected (5 µL) into an Xbridge amide column (100 x 2.1 mm i.d., 3.5 µm; Waters) and separated by elution (in acetonitrile and 10 mM ammonium acetate and 2.5 mM ammonium hydroxide in water with 3% acetonitrile, pH 8.6) at a rate of 150µL/min from 0 to 7.9 min and 19 to 20 min, and 180 µL/min from 8 to 10.6 min. The linear elution gradient was as follows: 0 min, 85% B; 1.5 min, 85% B; 10.6

min, 35% B; 12.5 min, 10% B, 14 min, 10% B, 15 min, 85% B, and 20 min, 85% B. All solvents were LC-MS grade and purchased from Fisher Scientific. The QE-MS is equipped with a HESI probe, and the relevant parameters are as follows: heater temperature, 120 °C; sheath gas, 30; auxiliary gas, 10; sweep gas, 3; spray voltage, 3.6 kV for positive mode and 2.5 kV for negative mode. The capillary temperature was set at 320 °C, and the S-lens was 55. The maximum injection time (max IT) was 200 ms. Automated gain control (AGC) was targeted at 3 × 106 ions and isolation width was 2.0. The QE-MS was operated in positive full scan mode with a resolution of 70,000 and a scan range of 70 to 900 m/z and ions were monitored at m/z 150.058 and 153.077, for identification of methionine and methionine-d_3, respectively.

G. Quantification of Choline Metabolites

Total choline, betaine, DMG, and PC pool sizes were quantified at baseline and study weeks 6, 9, and 10 for plasma and urine, and at study week 10 for breast milk on our TSQ Quantum Access triple quadrupole LCMS system (Thermo) operated in positive-ion mode using electrospray ionization as previously described.[12,17] Serum methionine pool sizes were quantified at baseline, study week 6, and study end by gas chromatography-mass spectrometry.[18]

H. Metabolic Flux Calculations

When an isotopically labeled metabolic precursor is converted to an isotopically labeled metabolic product (precursor→ product), the isotopic enrichment percentages of a metabolic precursor and a metabolic product, can be calculated by equations **1** and **2**, respectively.

$$Enrichment_{Precursor} = \frac{labeled\ precursor \times 100\%}{labeled + unlabeled\ precursor} \quad (\mathbf{1})$$

$$Enrichment_{Product} = \frac{labeled\ product \times 100\%}{labeled + unlabeled\ product} \quad (\mathbf{2})$$

In the present study, plasma choline metabolite pools were at steady state by study week six[18], when labeled choline was introduced into the study diet. Under conditions of steady state, labeled and unlabeled metabolites within a given pool are turned over at the same rate. Thus, $Enrichment_{Product}$ can be calculated from $Enrichment_{Precursor}$ given $Rate$ (the net conversion of precursor to product, in μmoles of metabolite product per liter of plasma, over the study period) and $Pool\ Size_{Product}$ (the plasma pool size in μmoles of metabolite product per liter of plasma) (Equation **3**). We used Equation **3** to derive the rate of conversion of metabolic precursors → products in μmols/L/study period, over the three-week period of label exposure (Equation **4**).

$$Enrichment_{Product} = \frac{Enrichment_{Precursor} \times Rate}{Pool\ Size_{Product}} \quad (\mathbf{3})$$

$$Rate = \frac{Enrichment_{Product} \times Pool\ Size_{Product}}{Enrichment_{Precursor}} \quad (\mathbf{4})$$

I. Statistical Analysis

We examined six metabolic outcomes in plasma as primary response variables. These outcomes were chosen to reflect partitioning *between* metabolic pathways (enrichment ratios of betaine-d_9/PC-d_9, PC-d_{3+6}/PC-d_9) or flux *through* metabolic pathways (metabolic flux calculations for turnover of betaine → methionine, betaine → DMG, and choline → betaine within the study period). A secondary analysis of individual metabolites in plasma, urine, and

breast milk was used to support interpretation.

The effect of genetic variation on choline metabolites was assessed using linear models. Heterozygous and homozygous variant individuals were grouped together to examine the effect of variant allele presence. Outcomes were modeled as a function of SNP genotype. Reproductive status and choline intake group (480 or 930 mg choline/d) were included as covariates. A backwards selection was used in which BMI was retained at an α-cutoff of 0.05 and interactions were retained at an α-cutoff of 0.1. The 0.1 cutoff for interactions was selected to ensure that interactions were detected and prevent the interpretation of main effects in the presence of an interaction. SNPs with a low presence of the variant allele were evaluated for the effect of minor allele presence (a two-level categorical variable for presence or absence of minor allele) on each outcome (**Table 1**). Standard diagnostic methods were used to assess model assumptions and the fit of the model to the data. Reported p-values include Bonferroni corrections for multiple comparisons. When evaluating the three-way interaction in *MTHFD1* models, several groups had only one participant, which precluded the use of statistical tests for several comparisons. To avoid pair-wise comparisons with such groups, for this one genotype, we chose to compare representative individuals to 95% confidence intervals (CIs) constructed around groups with adequate sample size. This approach did not allow for the determination of statistical significance of these comparisons, but was a conservative and statistically valid alternative given the circumstances. In such cases, we report whether the representative individual had metabolite enrichment levels outside or inside the 95% constructed around groups with adequate n to which we wished to draw comparisons. Two lactating participants had choline-d_9 enrichment values greater than 2 standard deviations from the mean and were excluded from the entire analysis. All statistical

analysis was performed using the lsmeans package[21] in the R statistical programming environment, available from CRAN 2014.[23] Data are presented as predicted least-squared means, unless otherwise noted. Tables and figures show values for every genotype, reproductive state, and choline intake group, while data in the text report average over choline intake groups when there is not a choline intake by genotype interaction and averages over reproductive states when there is not a reproductive state by genotype interaction.

Table 1. Genotype distribution (# of participants) between reproductive states and choline intake groups.

	480 mg Choline/d			930 mg Choline/d		
# Of Variant Alleles	**0**	**1**	**2**	**0**	**1**	**2**
***MTHFR* rs1801133**						
Lactating	4	7	1	5	6	1
Non-Pregnant	3	4	3	6	5	0
Pregnant	5	8	0	6	5	2
***MTR* rs1805087**						
Lactating	8	4	0	8	4	0
Non-Pregnant	6	3	1	8	3	0
Pregnant	10	3	0	8	5	0
***MTHFD1* rs2236225**						
Lactating	4	7	1	1	8	3
Non-Pregnant	1	7	2	2	8	1
Pregnant	6	6	1	4	5	4
***MTRR* rs1801394**						
Lactating	6	3	3	3	7	2
Non-Pregnant	2	6	2	5	3	3
Pregnant	2	9	2	2	7	4

Notes: Because of the relatively low prevalence of the variant allele, heterozygous and homozygous variant individuals were combined to examine the effect of variant allele presence on metabolic outcomes.
Abbreviations: MTHFD1, methylenetetrahydrofolate dehydrogenase methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase.

Results

MTHFR (dbSNP: rs1801133; c.677 C>T; p.Ala223Val; RefSeq NT_021937.17)

Genotype interacted with reproductive state to influence the partitioning of dietary choline between betaine synthesis and CDP-PC synthesis (p = 0.02) as well as the metabolic flux of choline → betaine (p = 0.01) (**Figure 2, Table 2, Supplemental Table 1**). Among non-pregnant women, variant women exhibited a lower betaine-d_9/PC-d_9 enrichment ratio compared to non-variant women ($0.8^{+/-0.03}$ vs. $0.9^{+/-0.04}$; p = 0.01) and a lower turnover of choline → betaine ($38^{+/-5}$ vs. $56^{+/-5}$ μM betaine/study period; p = 0.05) (**Table 2**).

Table 2. *MTHFR* rs1801133 genotype alters plasma choline metabolite partitioning and flux.

***MTHFR* rs1801133**	*480 mg Choline/d*		**p-value**	*930 mg Choline/d*		p-value
	Wildtype	**Variant**		**Wildtype**	**Variant**	
Betaine-d_9/PC-d_9 *RS x Gene interaction; p = 0.02**						
Lactating	0.79 +/- 0.04	0.82 +/- 0.03	> 0.99	0.93 +/- 0.03	0.96 +/- 0.03	> 0.99
Non-Pregnant	0.87 +/- 0.04	0.73 +/- 0.03	0.01**	1.0 +/- 0.03	0.87 +/- 0.03	0.01**
Pregnant	0.66 +/- 0.03	0.65 +/- 0.03	> 0.99	0.79 +/- 0.03	0.79 +/- 0.03	> 0.99
Choline → Betaine *RS x Gene interaction; p = 0.01***						
Lactating	34 +/- 6	44 +/- 5	0.6	41 +/- 6	51 +/- 5	0.6
Non-Pregnant	42 +/- 7	24 +/- 5	0.05*	71 +/- 6	52 +/- 6	0.05*
Pregnant	11 +/- 6	14 +/- 5	> 0.99	18 +/- 5	20 +/- 5	> 0.99
Betaine → DMG *Main effect; p = 0.04***						
Lactating	4.4 +/- 1.4	6.8 +/- 1.2	0.04**	6.0 +/- 1.3	8.4 +/- 1.2	0.04**
Non-Pregnant	6.9 +/- 1.4	9.3 +/- 1.2	0.04**	8.5 +/- 1.3	10.9 +/- 1.3	0.04**
Pregnant	2.8 +/- 1.3	5.1 +/- 1.1	0.04**	4.4 +/- 1.2	6.8 +/- 1.2	0.04**

Notes: Values are least-squared means +/- standard errors. Betaine-d_9/PC-d_9 values are ratios, choline → betaine values are in μM betaine/study period, and betaine → DMG values are in μM DMG/study period. p-values represent the highest order interaction or main effect.
Abbreviations: DMG, dimethylglycine, MTHFR, methylenetetrahydrofolate reductase; PC, phosphatidylcholine; RS, reproductive state.

Notably, non-pregnant variant women in the higher choline intake group (930 mg/d) exhibited metabolic profiles reminiscent of non-pregnant non-variant women at the lower (recommended) choline intake (Table 3). Specifically, differences in betaine-d_9/PC-d_9 enrichment ratios and turnover of choline → betaine were not observed between variants in the higher intake group and non-variants in the lower intake group ($p > 0.99$) (Table 3).

Table 3. Comparison of plasma betaine-d_9/PC-d_9 partitioning and choline → betaine turnover between non-pregnant *MTHFR* rs1801133 and MTR rs1805087 "non-risk" genotype women consuming 480 mg choline/d and "risk" genotype women consuming 930 mg choline/d.

Non-Pregnant	**480 mg Choline/d** *"Risk" Genotype*	**930 mg Choline/d** *"Non-Risk" Genotype*	**p-value**
***MTHFR* rs1801133**	***Non-Variant***	***Variant***	
Betaine-d_9/PC-d_9	0.87 $^{+/- 0.04}$	0.87 $^{+/- 0.03}$	> 0.99
Choline → Betaine	42 $^{+/- 7}$	52 $^{+/- 6}$	> 0.99
***MTR* rs1805087**	***Variant***	***Non-Variant***	
Betaine-d_9/PC-d_9	0.86 $^{+/- 0.04}$	0.90 $^{+/- 0.03}$	> 0.99

Notes: Values are least-squared means +/- standard errors. Betaine-d_9/PC-d_9 values are ratios, and choline → betaine values are in μM betaine/study period.
Abbreviations: MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; PC, phosphatidylcholine; RS, reproductive state.

Finally, across reproductive states, variant women exhibited a greater flux of betaine → DMG within the study period than non-variant women ($7.9^{+/-0.7}$ vs. $5.5^{+/-0.9}$ μM DMG/study period; $p = 0.04$) (**Figure 2, Table 2**).

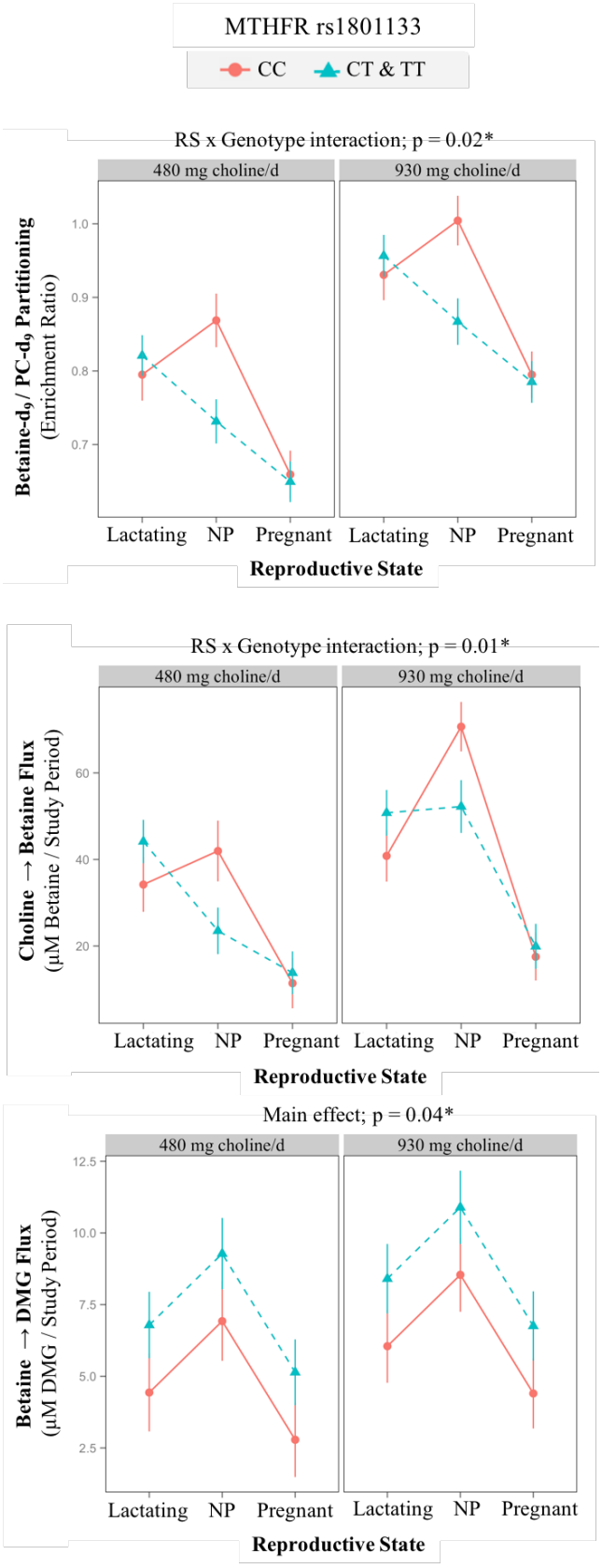


Figure 2. Effect of *MTHFR* (dbSNP: rs1801133; c.677 C>T; p.Ala223Val; RefSeq NT_021937.17) genotype on the metabolic flux and partitioning of plasma choline metabolites.

MTR rs1805087 (dbSNP: rs1805087; c.2756 A>G; p.Asp919Gly; RefSeq NG_008959.1)

MTR non-variant women exhibited metabolic profiles similar to women with the *MTHFR* "risk" genotype.

First, genotype had a near-significant interaction with reproductive state to influence the partitioning of dietary choline between betaine synthesis and CDP-PC synthesis ($p = 0.06$) (Figure 3, Table 4). Though multiple comparisons diminished significance, non-pregnant non-variant women exhibited a lower betaine-d_9/PC-d_9 enrichment ratio as compared to non-pregnant variant women ($0.8^{+/-0.03}$ vs. $0.9^{+/-0.04}$; $p = 0.07$) (**Figure 3, Table 4**).

Secondly, genotype interacted with reproductive state and choline intake to influence the flux of choline → betaine ($p = 0.008$) (**Figure 3, Table 4**). Genotypic differences were not observed in the lower choline intake group. However, within the higher choline intake group, non-pregnant non-variant women exhibited a lower flux of choline → betaine than non-pregnant variant women ($50.5^{+/-5}$ vs. $94^{+/-9}$ μM betaine/study period; $p = 0.0008$) (**Figure 3, Table 4**). Furthermore, different responses to increased choline intake were detected between variant and non-variant women. While non-pregnant non-variant women did not display differences in choline → betaine turnover as a function of choline intake, non-pregnant variant women used more dietary choline for betaine synthesis in the higher intake group as compared to the lower intake group ($94^{+/-9}$ vs. $23^{+/-7}$ μM betaine/study period; $p = 5.8 \times 10^{-7}$) (**Table 5**). Like the *MTHFR* "risk" genotype, non-pregnant women without the *MTR* variant in the higher choline intake (930 mg/d) exhibited betaine-d_9/PC-d_9 enrichment ratios reminiscent of *MTR* variant women in the lower (recommended) intake group ($p > 0.99$) (**Table 3**).

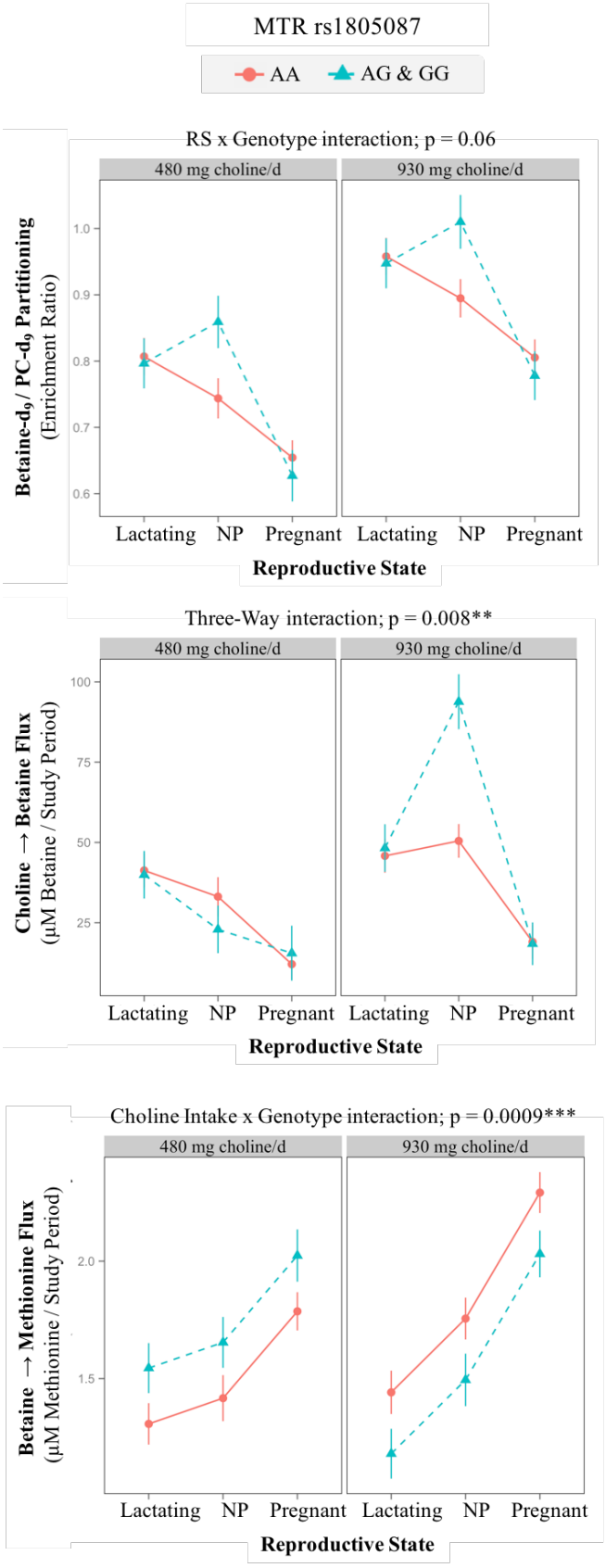


Figure 3. Effect of *MTR* (dbSNP: rs1805087; c.2756 A>G; p.Asp919Gly; RefSeq NG_008959.1) genotype on the metabolic flux and partitioning of plasma choline metabolites.

Table 4. *MTR* rs1805087 genotype alters plasma choline metabolite partitioning and flux.

***MTR* rs1805087**	*480 mg Choline/d*		**p-value**	*930 mg Choline/d*		**p-value**
	Wildtype	**Variant**		**Wildtype**	**Variant**	
Betaine-d_9/PC-d_9 *RS x Gene interaction; p = 0.06*						
Lactating	0.81 $^{+/- 0.03}$	0.80 $^{+/- 0.04}$	> 0.99	0.96 $^{+/- 0.03}$	0.95 $^{+/- 0.04}$	> 0.99
Non-Pregnant	0.74 $^{+/- 0.03}$	0.86 $^{+/- 0.04}$	0.07	0.90 $^{+/- 0.03}$	1.01 $^{+/- 0.04}$	0.07
Pregnant	0.65 $^{+/- 0.03}$	0.63 $^{+/- 0.04}$	> 0.99	0.81 $^{+/-0.03}$	0.78 $^{+/- 0.04}$	> 0.99
Choline → Betaine *3-way interaction; p = 0.008***						
Lactating	41 $^{+/- 5}$	40 $^{+/- 7}$	> 0.99	46 $^{+/- 5}$	48 $^{+/- 7}$	> 0.99
Non-Pregnant	33 $^{+/- 6}$	23 $^{+/- 7}$	> 0.99	50 $^{+/- 5}$	94 $^{+/- 9}$	< 0.001***
Pregnant	12 $^{+/- 5}$	16 $^{+/- 9}$	> 0.99	19 $^{+/- 5}$	18 $^{+/- 7}$	> 0.99
Betaine → Methionine *Cho x Gene interaction; p = 0.001****						
Lactating	1.31 $^{+/- 0.09}$	1.54 $^{+/- 0.11}$	0.1	1.44 $^{+/- 0.09}$	1.18 $^{+/- 0.11}$	0.05*
Non-Pregnant	1.42 $^{+/- 0.10}$	1.65 $^{+/- 0.11}$	0.1	1.76 $^{+/- 0.09}$	1.49 $^{+/- 0.11}$	0.05*
Pregnant	1.79 $^{+/- 0.08}$	2.02 $^{+/- 0.11}$	0.1	2.29 $^{+/- 0.09}$	2.03 $^{+/- 0.10}$	0.05*

Notes: Values are least-squared means $^{+/-}$ standard errors. Betaine-d_9/PC-d_9 values are ratios, choline → betaine values are in μM betaine/study period, and betaine → methionine values are in μM methionine/study period. p-values represent the highest order interaction or main effect. Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: Cho, choline intake; MTR, methionine synthase; PC, phosphatidylcholine; RS, reproductive state.

Finally, genotype interacted with choline intake to influence the metabolic flux of betaine → methionine (p = 0.001). *MTR* non-variant women and *MTR* variant women again displayed different responses to increased choline intake. Non-variant women in the higher intake group exhibited a greater turnover of betaine → methionine ($1.8^{+/-0.06}$ μM methionine/study period) as compared to *MTR* non-variant women in the lower intake group ($1.5^{+/-0.06}$ μM methionine/study period; p = 0.0008), as well as compared to variant women in

the higher intake group ($1.6^{+/-0.08}$ μM methionine/study period; p = 0.05) (**Figure 3, Tables 4 and 5**). Conversely, variant women did not display differences in betaine → methionine turnover as a function of choline intake (p = 0.6).

Table 5. Comparison of choline → betaine and betaine → methionine turnover between choline intake groups among *MTR* rs1805087 variant and non-variant women.

MTR rs1805087	**480 mg Choline/d**	**930 mg Choline/d**	**p-value**
Choline → Betaine			
Non-Pregnant Wildtype	$33^{+/-6}$	$50^{+/-5}$	> 0.99
Non-Pregnant Variant	$23^{+/-7}$	$94^{+/-9}$	$< 0.001^{***}$
Betaine → Methionine			
Wildtype	$1.5^{+/-0.06}$	$1.8^{+/-0.06}$	$< 0.001^{***}$
Variant	$1.7^{+/-0.08}$	$1.6^{+/-0.08}$	0.6

Notes: Values are least-squared means $^{+/-}$ standard errors in μM product/study period. Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: MTR, methionine synthase.

MTRR rs1801394 (dbSNP: rs1801394; c.66 A>G; p.Ile22Met; RefSeq NG_008856.1)

MTRR genotype interacted with reproductive state and choline intake to influence the metabolic flux of choline → betaine (p = 0.07) (**Figure 4, Table 6**). While genotypic differences were not detected within choline intake groups (p > 0.16), a differential response to choline intake was observed among non-pregnant women with and without the variant, similar to *MTR* "risk" genotype carriers. Specifically, while differences in choline → betaine were not observed as a function of choline intake among those without the variant (p > 0.99), those with the variant had a greater turnover of choline → betaine in the higher intake group as compared to the lower intake group ($73^{+/-6}$ vs. $49^{+/-7}$; p = $6x10^{-6}$) (**Tables 6 and 7**).

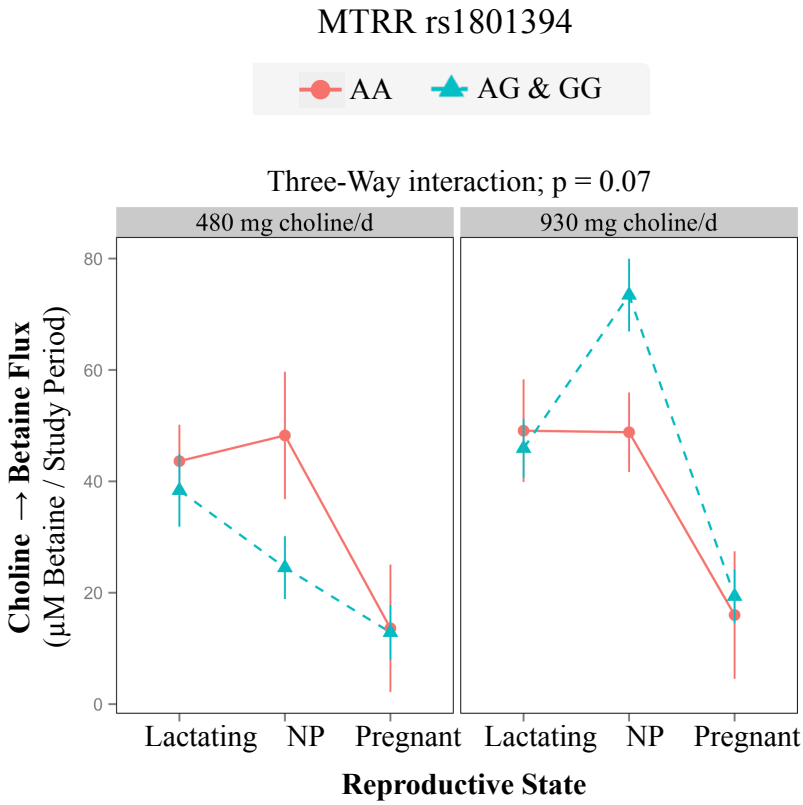


Figure 4. Effect of *MTRR (dbSNP: rs1801394; c.66 A>G; p.Ile22Met; RefSeq NG_008856.1)* genotype on the metabolic flux and partitioning of plasma choline metabolites.

Table 6. The effect of MTRR rs1801394 genotype on the metabolic flux of choline → betaine

***MTRR* r18011394**	*480 mg Choline/d*		**p-value**	*930 mg Choline/d*		**p-valu**
	Non-Variant	**Variant**		**Non-Variant**	**Variant**	
Choline → Betaine *3-way interaction; p = 0.07*						
Lactating	44 $^{+-7}$	38 $^{+/-7}$	> 0.99	49 $^{+/-9}$	46 $^{+/-5}$	> 0.9
Non-Pregnant	48 $^{+/-11}$	25 $^{+/-6}$	0.8	49 $^{+/-7}$	73 $^{+/-7}$	0.2
Pregnant	14 $^{+/-11}$	13 $^{+/-5}$	> 0.99	16 $^{+/-11}$	19 $^{+/-5}$	> 0.9

Notes: Values are least-squared means $^{+/-}$ standard errors in µM betaine/study period.
Abbreviations: MTR, methionine synthase.

Table 7. Comparison of choline → betaine turnover between choline intake groups among MTRR rs1801394 variant and non-variant women.

MTRR r18011394 *Choline → Betaine*	**480 mg Choline/d**	**930 mg Choline/d**	**p-value**
Variant			
Lactating	38 $^{+/-7}$	46 $^{+/- 5}$	> 0.99
Non-Pregnant	25 $^{+/-6}$	73 $^{+/- 7}$	< 0.001***
Pregnant	13 $^{+/- 5}$	19 $^{+/- 5}$	> 0.99
Non-Variant			
Lactating	44 $^{+-7}$	49 $^{+/- 9}$	> 0.99
Non-Pregnant	48 $^{+/-11}$	49 $^{+/- 7}$	> 0.99
Pregnant	14 $^{+/- 11}$	16 $^{+/- 11}$	> 0.99

Notes: Values are least-squared means $^{+/-}$ standard errors in µM betaine/study period. Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: MTR, methionine synthase.

MTHFD1 (dbSNP: rs2236225; c.1958 G>A; p.Arg653Gln; RefSeq NT_026437.13)

Much like women with the *MTHFR* and *MTR* "risk" genotypes, the *MTHFD1* variant influenced the partitioning of dietary choline between the CDP-choline pathway and betaine synthesis.

Genotype interacted with reproductive state and choline intake to influence enrichment of betaine-d_9/PC-d_9 ($p = 0.01$) (**Figure 5, Table 8**). Among non-pregnant women in the lower intake group, variant women had a betaine-d_9/PC-d_9 enrichment ratio well below that of the representative non-variant individual (*variant least-squares mean:* 0.73, *non-variant least-squares mean:* 1.07); importantly, the variant's 95% CI (0.66 - 0.79) did not include the non-variant (1.07) (**Table 8**). Genotypic differences were not observed within the higher choline intake group ($p > 0.99$) (**Figure 5, Table 8**). However, non-pregnant and

lactating variant women exhibited higher ($p < 0.003$) betaine-d_9/PC-d_9 enrichment ratios in the higher intake group ($0.96^{+/-0.03}$ and $0.96^{+/-0.03}$, respectively) than in the lower intake group ($0.73^{+/-0.03}$ and $0.79^{+/-0.03}$, respectively) (**Table 9**).

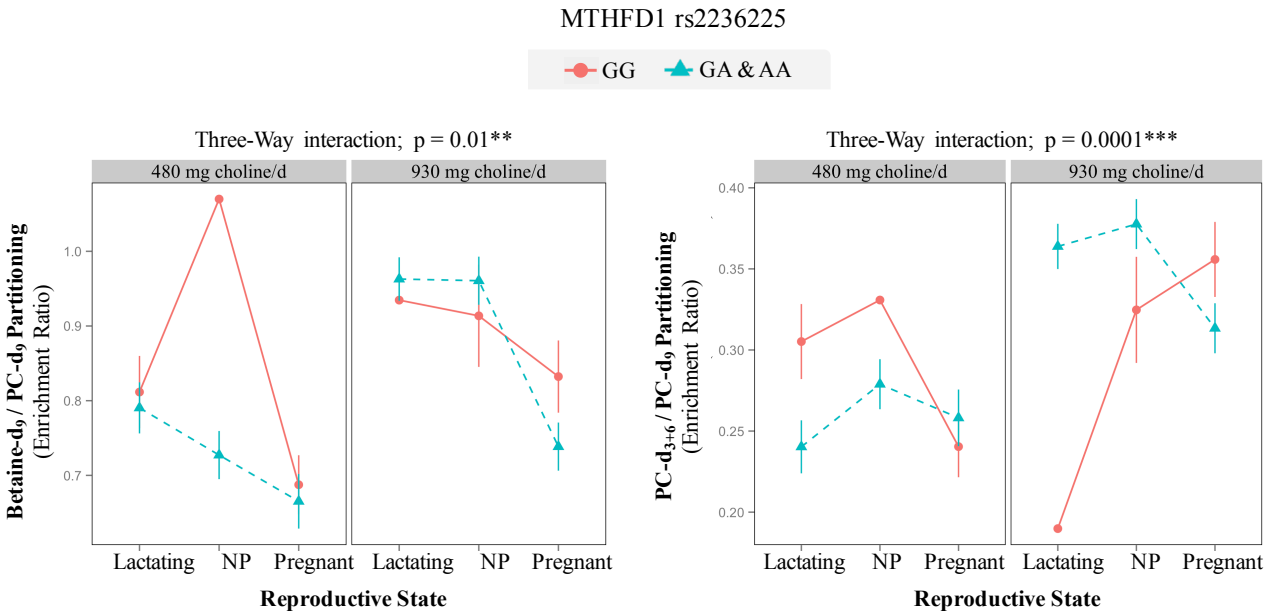


Figure 5. Effect of *MTHFD1* (dbSNP: rs2236225; c.1958 G>A; p.Arg653Gln; RefSeq NT_026437.13) genotype on the metabolic partitioning of plasma choline metabolites.

Furthermore, within the higher intake group, both non-pregnant and lactating women with the variant exhibited a greater betaine-d_9/PC-d_9 enrichment ratio (both $0.96^{+/-0.03}$) than variant pregnant women ($0.74^{+/-0.03}$; $p < 0.001$). Though non-pregnant and lactating women with the variant (both $0.96^{+/-0.03}$) did not differ ($p = 0.2$) from non-variant non-pregnant women ($0.83^{+/-0/05}$) or the representative non-variant woman (0.93).

Genotype also interacted with reproductive state and choline intake to influence the partitioning of dietary choline between PEMT-PC (PC-d_{3+6}) and CDP-PC (PC-d_9) ($p = 0.0001$) (**Figure 5, Table 8**). Among non-pregnant women consuming the lower choline

intake, variants had a PC-d_{3+6}/PC-d_9 enrichment ratio below that of the non-variant individual (*variant least-squares mean*: $0.24^{+/-0.02}$, *non-variant least-squares mean:* 0.31); importantly, the variant's 95% CI (0.21–0.27) did not include the non-variant (0.19) (**Figure 5**). Among lactating women consuming the higher choline intake, variants had a PC-d_{3+6}/PC-d_9 enrichment ratio nearly double that of the non-variant individual ($0.36^{+/-0.01}$ vs. 0.19); importantly, the variant's 95% CI (0.34–0.39) did not include the non-variant (0.19) (**Figure 5, Table 8**).

Table 8. *MTHFD1* r2236225 genotype alters plasma choline metabolite partitioning.

MTHFD1 **rs2236225**	*480 mg Choline/d*		**p-value**	*930 mg Choline/d*		**p-value**
	Non-Variant	**Variant**		**Non-Variant**	**Variant**	
Betaine-d_9/PC-d_9 *3-way interaction; p = 0.01***						
Lactating	0.81 +/- 0.05	0.79 +/- 0.03	> 0.99	0.93 NA	0.96 +/- 0.03	in CI
Non-Pregnant	1.07 NA	0.73 +/- 0.03	**ϕ**	0.91 +/- 0.07	0.96 +/- 0.03	> 0.99
Pregnant	0.69 +/- 0.04	0.67 +/- 0.04	> 0.99	0.83 +/-0.05	0.74 +/- 0.03	> 0.99
PC-d_{3+6}/PC-d_9 *3-way interaction; p = 0.0001****						
Lactating	0.31 +/- 0.02	0.24 +/- 0.02	0.2	0.19 NA	0.36 +/- 0.01	**ϕ**
Non-Pregnant	0.33 NA	0.28 +/- 0.02	**ϕ**	0.32 +/- 0.03	0.38 +/- 0.02	> 0.99
Pregnant	0.24 +/- 0.02	0.26 +/- 0.02	> 0.99	0.36 +/- 0.02	0.31 +/- 0.02	> 0.99

Notes: Values are ratios as least-squared means +/- standard errors. NA signifies a single participant. **ϕ** signifies that the least-square mean of the representative individual falls outside the 95% confidence interval (CI) of the comparing group. p-values represent the highest order interaction or main effect. Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: MTHFD1, methylenetetrahydrofolate dehydrogenase methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase; PC, phosphatidylcholine.

In addition, non-pregnant and lactating women with the variant and non-variant pregnant women exhibited increased PC-d_{3+6}/PC-d_9 as a function of choline intake, displaying

a greater PC-d_{3+6}/PC-d_9 enrichment ratio at the higher choline intake as compared to the lower intake (*NP variant:* $0.38^{+/-0.02}$ vs. $0.28^{+/-0.02}$; p = 0.0002) (*lactating variant:* $0.36^{+/-0.01}$ vs. $0.24^{+/-0.02}$; p = $3x10^{-6}$) (*pregnant non-variant:* $0.36^{+/-0.02}$ vs. $0.24^{+/-0.02}$*; p = 0.002)* (**Table 9**). Pregnant women with the variant also exhibited a greater PC-d_{3+6}/PC-d_9 enrichment ratio in the higher choline intake group as compared to the lower intake group, but this difference was no longer significant after adjusting for multiple comparisons ($0.31^{+/-0.02}$ vs. $0.26^{+/-0.02}$*; p = 0.2)* (**Table 9**)

Table 9. Comparison of betaine → methionine turnover between choline intake groups among *MTHFD1* rs2236225 variant and non-variant women.

***MTHFD1* rs2236225 Variant**	**480 mg Choline/d**	**930 mg Choline/d**	**p-value**
Betaine-d_9/PC-d_9			
Lactating	$0.79^{+/-0.03}$	$0.96^{+/-0.03}$	0.003^{**}
Non-Pregnant	$0.73^{+/-0.03}$	$0.96^{+/-0.03}$	$< 0.001^{***}$
Pregnant	$0.67^{+/-0.04}$	$0.74^{+/-0.03}$	> 0.99
PC-d_{3+6}/PC-d_9			
Lactating	$0.24^{+/-0.02}$	$0.36^{+/-0.01}$	$< 0.001^{***}$
Non-Pregnant	$0.28^{+/-0.02}$	$0.38^{+/-0.02}$	$< 0.001^{***}$
Pregnant	$0.26^{+/-0.02}$	$0.31^{+/-0.02}$	0.2

Notes: Values are least-squared means $^{+/-}$ standard errors in ratios. Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: MTHFD1, methylenetetrahydrofolate dehydrogenase methenyltetrahydro folate cyclohydrolase-formyltetrahydrofolate synthetase; PC, phosphatidylcholine.

Discussion

The present study is one of few to explore gene by nutrient interactions using stable isotope methodology, particularly within the context of a controlled feeding study. The use of an isotope label allows for the detection of *dynamic* changes in metabolite flux, providing an

advantage over commonly used *static* measures, which do not reflect kinetic changes.[24] In fact, Zeisel and colleagues found that plasma concentrations of choline, betaine, and PC did not correlate with clinical signs of choline deficiency in humans deprived of choline.[25] Conversely, although our post-hoc design allowed us the benefit of a controlled environment, the original study did not consider genotype during study enrollment, leading to unequal genotype distribution that lessened our ability to evaluate pair-wise comparisons within some higher order interactions. To address this limitation, and avoid over-interpretation of any single comparison with a small sample size, we focused on the identification of broad patterns across genes and, metabolites, and tissues that arose from many separate comparisons. This approach also mitigates the effect of myriad unexamined genetic factors that may be unequally distributed within the study population. Overall, several themes common to "risk" genotypes emerged from our data:

Variants impairing folate enzymes increase the use of dietary choline for phosphatidylcholine biosynthesis

Adequate PC is critical for the structural integrity of cell membranes and cell survival. Both the CDP-choline pathway, which uses choline as a substrate, and the PEMT-PC pathway, which uses SAM as a methyl donor, have a high metabolic priority. Indeed one of the primary uses of SAM is for PEMT-PC-synthesis.[26] Given that PEMT-PC comprises a substantial portion of cellular PC pools (~30% based on data from rat liver extracts),[27] impairments in folate-dependent SAM synthesis may decrease the contribution of folate-derived methyl groups for cellular PC production and increase the metabolic burden on choline-dependent PC synthesis. Our results, described below, support this notion by demonstrating that women with common genetic variants (*MTHFR* rs1801133, *MTR* rs1805087 wildtype, and *MTHFD1*

rs2236225) impairing folate-dependent enzymes display increased use of dietary choline for PC synthesis, primarily through the CDP-choline pathway.

MTHFR rs18011133

The *MTHFR* rs1801133 variant encodes a thermolabile enzyme with reduced capacity to bind its cofactor, FAD.[28] This translates to impaired production of 5-methylTHF, the substrate for the folate-dependent remethylation of homocysteine to methionine. Under conditions of folate deprivation, the variant is associated with decreased folate status and increased use of the choline derivative betaine for methyl donation.[17] Our data extend these findings to include women consuming a high folate diet.

Women with the rs1801133 variant used more betaine as a methyl donor, as indicated by a greater turnover of betaine → DMG within the study period. Notably, despite the increased turnover of betaine, non-pregnant variant women preferentially partitioned choline to the CDP-choline pathway at the expense of betaine synthesis, as indicated by decreased turnover of choline → betaine and a lower betaine-d_9/PC-d_9 enrichment ratio (**Figure 2, Table 2**). The enhanced partitioning of choline to the CDP-choline pathway despite greater use of betaine as a methyl donor may be a response to impaired PC synthesis via the PEMT pathway, which is dependent upon folate-derived SAM. Although choline-derived methyl groups can also be used for SAM synthesis, SAM is used for many different reactions in the cell while the CDP-choline pathway is a more direct method of PC production. Among non-pregnant variant women, higher choline intakes (930 mg/d) restored the rate of conversion of choline → betaine and the partitioning between betaine and the CDP-choline pathway to levels observed among non-variant women in the 480 mg choline/d group (**Figure 2, Table 3**). Given the low presence of homozygous variants within our sample, these findings largely

reflect the effect of one copy of the variant, and the burden on dietary choline for PC synthesis may be further compounded in women with two copies of the variant.

MTR rs1805087

MTR catalyzes the folate-dependent conversion of homocysteine to methionine.[29] The *MTR* rs1805087 variant encodes an aspartate to glycine amino acid change located in the region of the enzyme that binds accessory proteins to facilitate the regeneration of the active enzyme when oxidized.[30] The variant has been associated with decreased plasma homocysteine concentration, indicating it may provide a gain of function (i.e. an increased use of folate-derived methyl groups for the remethylation of homocysteine to methionine).[31,32] Thus, although the variant has been associated with disease risk,[33,34] within the context of choline requirement, the wildtype genotype may constitute a "risk" genotype" given the comparatively reduced efficiency of folate-dependent homocysteine remethylation.

Indeed, our findings for the *MTR* wildtype genotype support this notion and parallel those observed with the *MTHFR* rs1801133 "risk" genotype, indicating a prioritization of dietary choline for PC production when folate-dependent SAM synthesis is impaired. Specifically, within the higher choline intake group, non-pregnant women with the *MTR* wildtype "risk" genotype partitioned more choline to the CDP-choline pathway at the expense of betaine synthesis, as indicated by a decreased turnover of choline → betaine, and a lower betaine-d_9/PC-d_9 enrichment ratio, as compared to *MTR* variant women (**Figure 3, Table 4**). Even under conditions of folate adequacy, non-pregnant *MTR* wildtype women displayed an increased use of choline as a methyl donor in response to increased dietary choline, as indicated by a greater turnover of betaine → methionine (**Figure 3, Table 5**). In contrast, although non-pregnant *MTR* variant women exhibited an increased flux of choline → betaine

with increased choline intake, they did not increase their use of betaine as a methyl donor, instead accumulating plasma betaine, which manifested as larger plasma betaine pools among variant women as compared to wildtype women in the higher choline intake group ($53^{+/-5}$ vs. $35^{+/-3}$ μM betaine; p = 0.003). This supports the notion that non-pregnant women with the variant may have a decreased need for dietary choline as compared to wildtype women in conditions of folate adequacy. In addition, women with the *MTR* wildtype "risk" genotype may benefit from choline intakes exceeding current recommendation given the restoration of choline partitioning among wildtype women in the higher choline intake group to levels observed in the *MTR* variant women in the lower intake group (**Figure 3, Table 3**).

MTRR rs1801394

Methionine Synthase Reductase (MTRR) is a diflavin enzyme that participates in methionine biosynthesis by regenerating the oxidized MTR-Co(II) to active enzyme via a reductive methylation with *S*-adenosylmethionine[35]. Some, but not all, studies have identified moderate associations between the *MTRR* rs1801394 variant and neural tube defects[36] and elevated homocysteine concentrations, particularly in the presence of the *MTHFR* rs1801133 variant and low vitamin B12 status.[37] Due to power limitations, we did not consider vitamin B12 status or *MTHFR* rs1801133 genotype and therefore may not have fully captured its metabolic consequences. We did, however, detect a different response among variant individuals to increased choline intake (**Figure 4, Table 7**). Although non-variants did not use additional choline for betaine synthesis, variants exhibited increased turnover of choline → betaine in the higher choline intake group, suggesting that variants may have a greater need for choline derived methyl groups.

MTHFD1 rs2236225

The *MTHFD1* rs2236225 polymorphism encodes a thermolabile enzyme with a ~50% reduction in enzymatic activity[38] and appears to favor thymidylate biosynthesis at the expense of folate-dependent homocysteine remethylation. The variant also increases susceptibility to choline deficiency among premenopausal women deprived of choline.[7] Our findings suggest that the *MTHFD1* rs2236225 variant may increase susceptibility to choline and PC inadequacy, even at choline intakes that align with current recommendations. Non-pregnant variant women consuming 480 mg choline/d preferentially partitioned choline towards the CDP-choline pathway at the expense of betaine synthesis (i.e., a lower betaine-d_9/PC-d_9 enrichment ratio was observed among variant women as compared to the representative non-variant woman), which signifies a strain on cellular PC production (**Figure 5**). In addition, pregnant and lactating women exhibited increased CDP-PC synthesis across genotypes at the lower intake level, indicating a greater need for choline-dependent PC production during these reproductive states (**Figure 5**).

Nonetheless, our data showed favorable metabolic changes among non-pregnant and lactating women with the *MTHFD1* rs2236225 variant when choline intake exceeded current recommendations. Both non-pregnant and lactating variant women displayed greater betaine-d_9/PC-d_9, suggesting that increased dietary choline among variant carriers may decrease strain on PC production capacity (**Figure 5, Table 8**). Notably, at the higher choline intake level, non-variant women at all three reproductive states, as well non-pregnant and lactating women *with* the variant, exhibited similar partitioning between betaine and the CDP-choline pathway (**Figure 5**). This suggests that intakes of 930 mg/d are sufficient to restore partitioning among pregnant and lactating women *without* the variant to levels observed in non-pregnant women,

as well as partitioning among non-pregnant and lactating women *with* the variant to levels observed among non-variants. Variant pregnant women, on the other hand, exhibited preferential partitioning to the CDP-choline pathway even at the higher choline intake level, indicating that pregnant women with the variant may have a comparatively greater requirement for dietary choline in order to support PC production.

A second favorable metabolic change observed among variant non-pregnant and lactating women consuming higher choline intakes was the greater relative presence of PEMT-derived metabolites in circulation. Both non-pregnant and lactating women with the variant exhibited greater PC-d_{3+6}/PC-d_9, indicating that a greater portion of the dietary choline converted to PC was synthesized through the PEMT-PC pathway (**Figure 5, Tables 8 and 9**). In addition, among lactating women, higher choline consumption yielded greater enrichments of favorable PEMT-derived metabolites in plasma and breast milk compared to the representative non-variant lactating woman in the higher intake group, as well as compared to lactating variant women in the lower intake group (**Figure 6; Tables 10 and 11**). PEMT-PC and its lipid derivatives are enriched with long-chain polyunsaturated fatty acids such as DHA,[39] which beneficially influence brain development.[40] Improved plasma and breast milk PEMT metabolite composition may therefore provide an evolutionary basis for the high prevalence of this SNP in populations consuming high-choline diets despite its association with increased disease risk and evidence for negative selection in low choline environments.[41]

Table 10. *MTHFD1* r2236225 genotype alters plasma and breast milk choline metabolite partitioning among lactating women.

MTHFD1 r2236225	*480 mg Choline/d*		In CI?	*930 mg Choline/d*		In CI?
	Non-Variant	**Variant**		**Non-Variant**	**Variant**	
Plasma						
PC-d_3/PC-d_9	0.29 (0.26-0.32)	0.23 (0.21-0.25)	No	0.18 (NA)	0.33 (0.31-0.36)	No
Choline-d_3/Choline-d_9	0.32 (0.27-0.36)	0.24 (0.22-0.26)	No	0.18 (NA)	0.34 (0.30-0.36)	**ϕ**
Betaine-d_3/Betaine-d_9	0.25 (0.20-0.30)	0.18 (0.17-0.20)	No	0.09 (NA)	0.23 (0.20-0.25)	No
Breast Milk						
PC-d_3/PC-d_9	0.23 (0.18-0.29)	0.18 (0.17-0.19)	Yes [X]	0.13 (NA)	0.23 (0.21-0.26)	No
Choline-d_3/Choline-d_9	0.26 (0.24-0.28)	0.22 (0.20-0.25)	Yes [*]	0.14 (NA)	0.29 (0.26-0.34)	No
Betaine-d_3/Betaine-d_9	0.80 (0.63-0.97)	0.51 (0.37-0.66)	Yes [*]	0.33 (NA)	0.71 (0.61-0.80)	No

Notes: [X] Student's t-test failed to reject the null hypothesis ***** Significantly different by student's t-test. Values are group means and (95% CIs). Not available (NA) signifies a single participant. **ϕ** signifies that the least-square mean of the representative individual falls outside the 95% confidence interval of the comparing group. Although non-overlapping confidence intervals indicate a significant difference, overlapping confidence intervals do not necessarily exclude a significant difference. In cases where confidence intervals were overlapping, a student's t-test was performed using the Welch–Satterthwaite equation to calculate degrees of freedom.

Abbreviations: CI, confidence interval; MTHFD1, methylenetetrahydrofolate dehydrogenase methenyltetrahydro folate cyclohydrolase-formyltetrahydrofolate synthetase; PC, phosphatidylcholine.

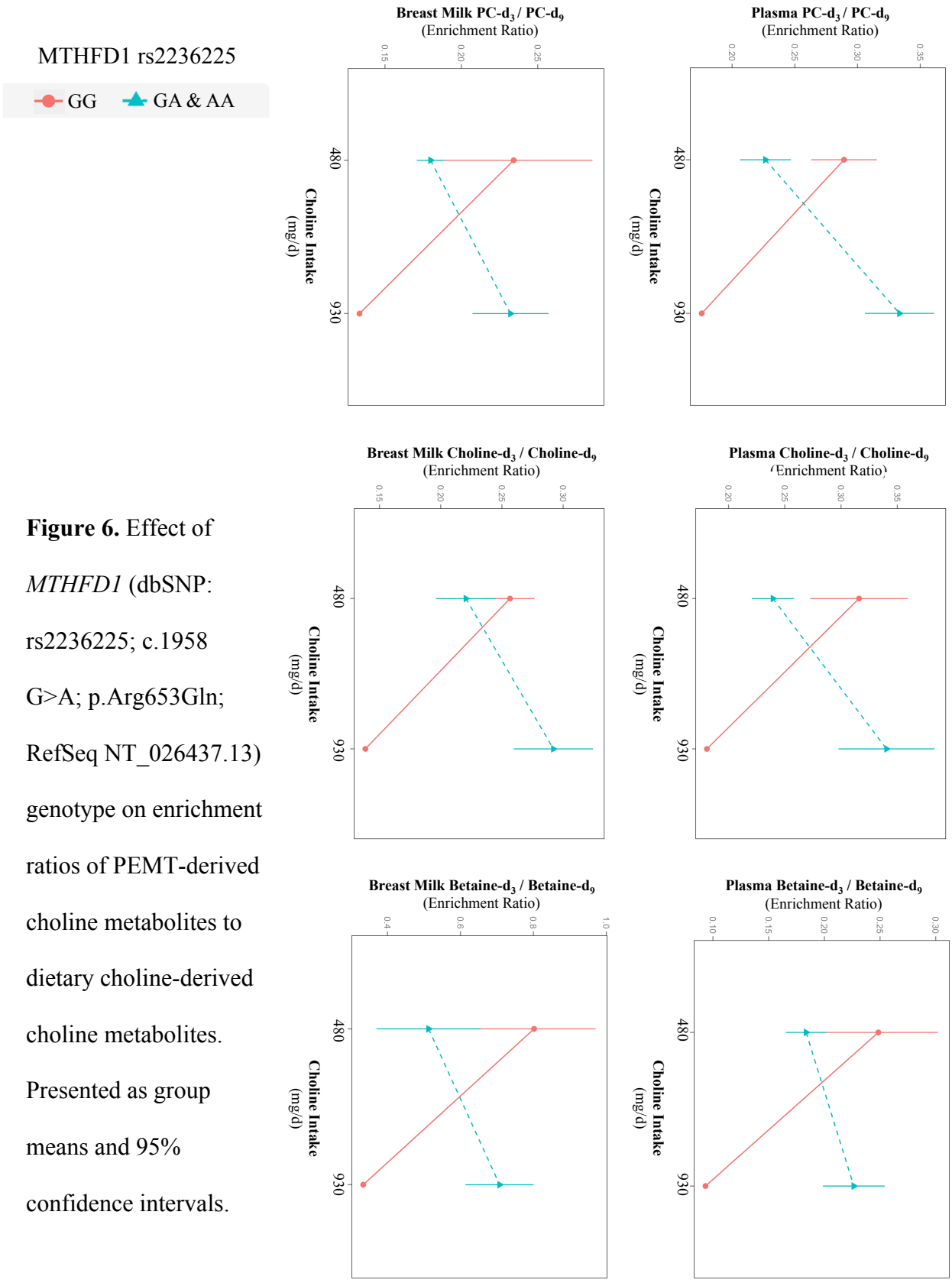


Figure 6. Effect of *MTHFD1* (dbSNP: rs2236225; c.1958 G>A; p.Arg653Gln; RefSeq NT_026437.13) genotype on enrichment ratios of PEMT-derived choline metabolites to dietary choline-derived choline metabolites. Presented as group means and 95% confidence intervals.

Table 11. Comparison of d_3/d_9 metabolite partitioning between choline intake groups among *MTHFD1* rs2236225 lactating women consuming 480 or 930 mg choline/d.

***MTHFD1* rs2236225 Variant**	**480 mg Choline/d**	**930 mg Choline/d**	**In CI?**
Plasma			
PC-d_3/PC-d_9	0.23 (0.21-0.25)	0.33 (0.31-0.36)	No
Choline-d_3/Choline-d_9	0.24 (0.22-0.26)	0.34 (0.30-0.36)	No
Betaine-d_3/Betaine-d_9	0.18 (0.17-0.20)	0.23 (0.20-0.25)	No
Breast Milk			
PC-d_3/PC-d_9	0.18 (0.17-0.19)	0.23 (0.21-0.26)	No
Choline-d_3/Choline-d_9	0.22 (0.20-0.25)	0.29 (0.26-0.34)	No
Betaine-d_3/Betaine-d_9	0.51 (0.37-0.66)	0.71 (0.61-0.80)	Yes*

Notes: *Significantly different by student's t-test. Values are group means of ratios with 95% confidence intervals. In cases where confidence intervals were overlapping, a student's t-test was performed using the Welch–Satterthwaite equation to calculate degrees of freedom.
Abbreviations: CI, confidence interval; MTHFD1, methylenetetrahydrofolate dehydrogenase methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase; PC, phosphatidylcholine.

Pregnant women exhibit few alterations in choline metabolism as a function of SNPS impairing folate enzymes

Given that the demand for choline is higher during pregnancy and lactation, and that genotypic effects are often more pronounced under conditions of nutrient inadequacy, we expected pregnant and lactating women to exhibit greater genotype-specific differences in choline partitioning than their non-pregnant counterparts. However, the metabolic flux of choline among pregnant women (and in some cases lactating women) was similar across genotypes, perhaps because the "risk" genotypes altered choline dynamics in ways that mirror changes observed during pregnancy. Thus, the physiological changes that alter choline metabolism in pregnancy (and to a lesser extent lactation) seemingly outweigh the effect of

genotype in many of the examined polymorphisms and indicate a greater choline requirement for this reproductive state as a whole.

Consideration of genotype in defining choline requirements

Our results indicate that genotype influences choline dynamics, even at and above the choline AI. Women with SNPs that impair folate-mediated methionine biosynthesis (i.e. *MTHFR* rs1801133, *MTR* rs1805087 wildtype, and *MTHFD1* rs2236225) displayed a pattern of preferential partitioning of dietary choline to the CDP-choline pathway, despite using more betaine for methionine biosynthesis (i.e. *MTHFR* rs1801133, *MTR* rs1805087 wildtype). This indicates a greater burden on dietary choline for PC synthesis, perhaps due to impaired folate-dependent PC production through the PEMT pathway. Notably, across these "risk" genotypes, increased choline intake appeared to restore dietary choline partitioning between betaine and the CDP-choline pathway to levels observed in those without the "risk" allele (Table 3). These findings collectively suggest that even in conditions of folate adequacy, women of reproductive age with SNPs impairing folate metabolism may benefit from choline intakes exceeding current recommendations.

Conclusions

In sum, these data provide compelling evidence that common SNPs in folate-metabolizing enzymes modulate choline dynamics in women of reproductive age consuming choline and folate intakes relevant to the population at large. Carriers of SNPs that alter cellular methylation capacity may rely more on choline-dependent PC synthesis as observed among *MTR* rs1805087 wildtype, and *MTHFR* rs1801133, *MTHFD1* rs2236225, and possibly *MTRR* rs1801394, variant women consuming 480 mg choline/d. Choline partitioning was restored

with higher choline intakes (930 mg/d). The metabolic strain on choline for PC synthesis may be particularly pronounced in the general population as American women consume on average 260 mg choline/d, well below the lower study intake level and choline AI[42]. The present findings also suggest that the partitioning of dietary choline between betaine and the CDP-choline pathway is a sensitive indicator of subclinical differences in choline requirements. These metabolic differences, while not severe enough to present as acute muscle or liver pathologies, may have long-term consequences on human health and chronic disease. Further studies with greater sample size are needed to confirm these findings and identify whether such metabolic differences have clinical implications.

Supplemental Information

Supplemental Table 1. Effect of SNP genotype (p-values) on plasma choline metabolite partitioning and flux.

	Gene	**G*RS**	**G*Cho**	**Three-way**
***MTHFR* rs1801133**				
Betaine-d_9/PC-d_9	0.1	0.022*	-	-
PC-d_{3+6}/PC-d_9	0.3	-	-	-
Choline → Betaine	0.6	0.01*	-	-
Betaine → DMG	0.04*	-	-	-
Betaine → Methionine	0.3	-	-	-
***MTR* rs1805087**				
Betaine-d_9/PC-d_9	0.4	0.06	-	-
PC-d_{3+6}/PC-d_9	0.5	-	-	-
Choline → Betaine	0.3	0.2	0.04	0.008**
Betaine → DMG	0.9	-	-	-
Betaine → Methionine	0.9	-	0.001***	-
***MTHFD1* rs2236225**				
Betaine-d_9/PC-d_9	0.02	0.7	0.2	0.01**
PC-d_{3+6}/PC-d_9	0.8	0.8	0.08	0.0001***
Choline → Betaine	0.2	-	-	-
Betaine → DMG	0.4	-	-	-
Betaine → Methionine	0.6	-	-	-
MTRR rs1801394				
Betaine-d_9/PC-d_9	0.7	-	-	-
PC-d_{3+6}/PC-d_9	0.2	-	-	-
Choline → Betaine	0.6	0.6	0.05	0.07
Betaine → DMG	0.96	-	-	-
Betaine → Methionine	0.2	-	-	-

Notes: Significance at an α-cutoff of: *0.05, **0.01, ***0.001.
Abbreviations: Cho, choline intake level; DMG, dimethylglycine; G, Gene; PC, phosphatidylcholine; RS, reproductive state.

REFERENCES

[1] Da Costa KA, Kozyreva OG, Song J, Galanko JA, Fischer LM, Zeisel SH. 2006. Common genetic polymorphisms affect the human requirement for the nutrient choline. FASEB 20: 1336–44.

[2] Guinotte CL1, Burns MG, Axume JA, Hata H, Urrutia TF, Alamilla A, McCabe D, Singgih A, Cogger EA, Caudill MA. 2003. Methylenetetrahydrofolate reductase 677C-->T variant modulates folate status response to controlled folate intakes in young women. J Nutr. 133: 1272–80.

[3] Glunde K, Bhujwalla ZM, Ronen SM. 2011. Choline metabolism in malignant transformation. Nature Reviews Cancer 11: 835-48

[4] MRC Vitamin Study Research Group. 1991. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. Lancet 338: 131–137.

[5] Pitkin RM. 2007. Folate and neural tube defects. Am J Clin Nutr 85: 285S–288S.

[6] Shaw GM, Lu W, Zhu H, Yang W, Briggs FBS, Carmichael SL, Barcellos LF, Lammer EJ, Finnell RH. 2009. 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Medical Genetics 10: 49.

[7] Da Costa KA, Corbin, KD, Niculescu MD, Galanko JA, Zeisel SH. 2014. Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. FASEB 28: 2970-2978.

[8] Caudill MA, Gregory JF III, Miller J, Shane B. 2012. Folate, choline, vitamin B-12 and vitamin B-6. In: Stipanuk MH, Caudill MA. Biochemical, Physiological and Molecular Aspects of Human Nutrition, 3e. MO: Elsevier Saunders. 565-608 p.

[9] Niculescu MD, Zeisel S. 2002. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr.132: 2333S-5S

[10] Fox, JT, & Stover PJ. 2008. Folate-mediated one-carbon metabolism. Vitamins and hormones. Vol. 79.

[11] Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. 2004. Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. Am J Epidemiol 160: 102-109.

[12] Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Brenna JT, Stabler SP, Allen RH, Gregory JF, Caudill MA. 2013. Pregnancy alters choline dynamics : results of a randomized trial using stable isotope methodology in pregnant and nonpregnant women. AJCN 133: 1272-80.

[13] Davenport C, Yan J, Taesuwan S, Shields K, West AA, Jiang X, Perry CA, Malysheva OV, Stabler SP, Allen RH, Caudill MA. 2015. Choline intakes exceeding recommendations during human lactation improve breast milk choline content by increasing PEMT pathway metabolites. J Nutr Biochem 26: 903-911.

[14] Jiang X, West AA, Caudill MA. 2015. Maternal choline supplementation: a nutritional approach for improving offspring health? Trends in Endocrinology & Metabolism 25: 263-273.

[15] Jiang X, Jones S, Andrew BY, Ganti A, Malysheva OV, Giallourou N, Brannon PM, Roberson MS, Caudill MA. 2014. Choline inadequacy impairs trophoblast function and vascularization in cultured human placental trophoblasts. J Cell Physiol 229: 1016-1027.

[16] Jiang X, Yan J, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Caudill MA. 2012. Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. FASEB 26: 3563-3574.

[17] Yan J, Wang W, Gregory JF, Malysheva O, Brenna JT, Stabler SP, Allen RH, Caudill MA. 2011. MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming d9-choline. Am J Clin Nutr 93: 348-355.

[18] Yan J, Jiang X, West AA, Perry C A, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Stabler SP, Allen RH, Caudill MA. 2012. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am J Clin Nutr 95: 1060-1071.

[19] West AA, Yan J, Jiang X, Perry CA, Innis SM, Caudill, MA. 2013. Choline intake influences phosphatidylcholine DHA enrichment in nonpregnant women but not in pregnant women in the third trimester 1 – 3. Am J Clin Nutr 97: 718-27.

[20] Hol FA, van der Put NMJ, Geurds MPA, Heil SG, Trijbels FJM, Hamel BCJ, Mariman ECM, Blom HJ. 1998. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD(methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-

cyclohydrolase,formyltetrahydrofolate synthetase) in patients with neural tube defects. Clin Gent 53: 119-125.

[21] Frosst P, Blom HJ, Milos R, Goyette P, Sheppard, CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10: 111-3.

[22] Kim JK, Harada K, Bamba T, Fukusaki E, Kobayashi A. 2005. Stable isotope dilution-based accurate comparative quantification of nitrogen-containing metabolites in Arabidopsis thaliana T87 cells using in vivo (15)N-isotope enrichment. Biosci Biotechnol Biochem 69: 1331-1340.

[23] R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

[24] Chew TW, Jiang X, Yan J, Wang W, Lusa AL, Carrier BJ, West AA, Malysheva OV, Brenna JT, Gregory JF III, and Marie A. Caudill. 2011. Folate Intake, Mthfr Genotype, and Sex Modulate Choline Metabolism in Mice. J Nutr 141:1475-1481.

[25] Fischer LM, Da Costa KA, Kwock L, Stewart PW, Lu TS, Stabler SP, Allen RH, Zeisel SH. 2007. Sex and menopausal status influence human dietary requirements for the nutrient choline. Am J Clin Nutr 85: 1275-85.

[26] Jacobs RL, Stead LM, Devlin C, Tabas I, Brosnan ME, Brosnan JT, Vance DE. 2005. Physiological regulation of phospholipid methylation alters plasma homocysteine in mice. J Biol Chem 280: 28299-305.

[27] Reo NV, Adinehzadeh M, Foy BD. 2002. Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine biosynthesis using 13C NMR spectroscopy. Biochim Biophys Acta - Molecular and Cell Biology of Lipids 1580: 171-188.

[28] Kang SS, Wong PW, Susmano A, Sora J, Norusis M, Ruggie N. 1991. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 48: 536-545.

[29] Leclerc D, Campeau E, Goyette P, Adjalla CE, Christensen B, Ross M, Eydoux P, Rosenblatt DS, Rozen R, Gravel RA. 1996. Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. Hum Mol Genet 5: 1867-1874.

[30] Chen LH, Liu M, Hwang H, Chen L, Korenberg J, & Shane, B. 1997. Human Methionine Synthase. Biochemistry 272: 3628-3634.

[31] Harmon DL, Shields DC, Woodside JV, McMaster D, Yarnell JWG, Young IS, Peng K, Shane B, Evans AE, Whitehead AS. 1999. Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. Genet Epidemiol 17: 298-309.

[32] Silaste ML, Rantala M, Sampi M, Alfthan G, Aro A, Kesäniemi YA. 2001. Polymorphisms of key enzymes in homocysteine metabolism affect diet responsiveness of plasma homocysteine in healthy women. J Nutr 131:2643-2647.

[33] Galbiatti aLS, Ruiz MT, Raposo LS, Maniglia JV. 2010. 5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (MTR) and risk of head and neck cancer. Brazilian Journal Of Medical And Biological Research 43: 445-450.

[34] Akbari MT, Naderi A, Saremi L, Sayad A, Irani S, Ahani A. 2015. Methionine synthase A2756G variation is associated with the risk of retinoblastoma in Iranian children. Cancer Epidemiol 39:1023-5.

[35] Olteanu H, Munson T, Banerjee R. 2002. Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between the common polymorphic variants of human methionine synthase reductase. Biochemistry, 41: 13378-13385.

[36] Boyles AL, Billups AV, Deak KL, Siegel DG, Mehltretter L, Slifer SH, Bassuk AG, Kessler JA, Reed MC, Nijhout F, George TM, Enterline DS, Gilbert JR, Speer MC, and the NTD Collaborative Group. 2006. Neural tube defects and folate pathway genes: Family-based association tests of gene-gene and gene-environment interactions. Environmental Health Perspectives, 114:1547-1552.

[37] Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, Gravel RA, Rozen R. 1999. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab. 67:317-323.

[38] Christensen KE, Rohlicek CV, Andelfinger GU, Michaud J, Bigras JL, Richter A, MacKenzie RE, Rozen R. 2009. The MTHFD1 p.Arg653Gln variant alters enzyme function and increases risk for congenital heart defects. Hum Mutat 30: 212-220

[39] Pynn CJ, Henderson NG, Clark H, Koster G, Bernhard W, Postle AD. 2011. Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed in vivo. J Lipid Res 52: 399-407.

[40] Innis SM. 2008. Dietary omega 3 fatty acids and the developing brain. Brain Res 1237: 35-43.

[41] Silver MJ, Corbin KD, Hellenthal G, da Costa KA, Dominguez-Salas P, Moore SE, Owen J, Prentice AM, Hennig BJ, Zeisel SH. 2015. Evidence for negative selection of gene variants that increase dependence on dietary choline in a Gambian cohort. FASEB 29: 3426-35.

[42] Chester DN, Goldman JD, Ahuja JK, Moshfegh AJ. 2011. Dietary Intakes of Choline: What We Eat in America, NHANES 2007-2008. Food Surveys Research Group Dietary Data Brief No. 9. Available at: http://ars.usda.gov/Services/docs.htm?docid=19476.

CHAPTER 2

Genetic Variation in Choline-Metabolizing Enzymes Alters Choline Metabolism in Young Women Consuming Choline Intakes Meeting Current Recommendations[*]

Abstract

Single nucleotide polymorphisms (SNPs) in choline metabolizing genes are associated with disease risk and greater susceptibility to organ dysfunction under conditions of dietary choline restriction. However, the underlying metabolic signatures of these variants are not well characterized and it is unknown whether genotypic differences persist at recommended choline intakes. Thus, we sought to determine if common genetic risk factors alter choline dynamics in pregnant, lactating, and non-pregnant women consuming choline intakes meeting and exceeding current recommendations. Women (n = 75) consumed 480 or 930 mg choline/day (22% as a metabolic tracer, choline-d9) for 10–12 weeks in a controlled feeding study. Genotyping was performed for eight variant SNPs and genetic differences in metabolic flux and partitioning of plasma choline metabolites were evaluated using stable isotope methodology. *CHKA* rs10791957, *CHDH* rs9001, *CHDH* rs12676, *PEMT* rs4646343, *PEMT* rs7946, *FMO3* rs2266782, *SLC44A1* rs7873937, and *SLC44A1* rs3199966 altered the use of choline as a methyl donor; *CHDH* rs9001 and *BHMT* rs3733890 altered the partitioning of dietary choline between betaine and phosphatidylcholine synthesis via the cytidine diphosphate (CDP)-choline pathway; and *CHKA* rs10791957, *CHDH* rs12676, *PEMT* rs4646343, *PEMT* rs7946 and *SLC44A1* rs7873937 altered the distribution of dietary choline between the CDP-choline and phosphatidylethanolamine *N*-methyltransferase (PEMT) *de novo* pathway. Such metabolic differences may contribute to disease pathogenesis and

[*]Ganz. et al. Genetic Variation in Choline-Metabolizing Enzymes Alters Choline Metabolism in Young Women Consuming Choline Intakes Meeting Current Recommendations. Int. J. Med. Sci. 2017 18(2).

prognosis over the long-term.

Introduction

Choline is an essential micronutrient with critical roles in a wide-array of physiologic processes [1]. As a source of methyl groups, choline supports cellular methylation reactions, including genomic methylation, which influences gene expression and DNA stability. Choline also serves as the substrate for the formation of acetylcholine, a neurotransmitter and non-neuronal cell-signaling molecule. Quantitatively, the primary metabolic fate of choline is biosynthesis of phosphatidylcholine (PC), the most abundant phospholipid in cell membranes [2]. Phosphatidylcholine adequacy is critical for cell membrane integrity and the export of fat from the liver by very low density lipoproteins (VLDL) [1]. Choline contributes to PC synthesis through two distinct pathways. Either choline can be phosphorylated, entering the cytidine diphosphate (CDP)-choline pathway where it is converted directly to PC, or, its methyl groups can be used in the triple-methylation of phosphatidylethanolamine (PE) to PC, which occurs by the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway [3]. This pathway is also responsible for endogenous choline production as PC can be produced through the PEMT pathway using PE and folate or methionine-derived methyl groups in the absence of dietary choline. The efficiency of endogenous choline production varies from person to person, and is greater among pre-menopausal women because PEMT is up regulated by estrogen. However, choline itself is used to support the PEMT pathway and is considered an essential dietary requirement because endogenous production capacity is not enough to support biological choline requirements [4–6]. Dietary choline restriction causes acute muscle and liver dysfunction and choline must be obtained from the diet to prevent deficiency [7]. However,

there is a large inter-individual variation in dietary choline requirement that depends upon genetic and physiological factors [4].

Considering the critical metabolic and structural roles of choline, it is not surprising that genetic variants that alter choline metabolism have been linked to increased risk for acute skeletal muscle and liver pathologies under conditions of dietary choline deprivation [8], as well as birth defects and other diseases in the general population (Table 1). However, the underlying metabolic signatures of these variants are not well characterized, and it is unknown whether genotypic differences persist among women meeting choline intake recommendations. Moreover, little is known about the effect of gene–nutrient interactions on choline metabolism and functional outcomes in reproductive states (i.e., pregnancy and lactation) that increase the metabolic use of choline [9]. We have previously shown that genetic variants in folate-metabolizing genes are associated with differences in choline dynamics and partitioning among women meeting current intake recommendations [9]. In the present study, we used isotopically labeled dietary choline to characterize differences in the metabolic flux and partitioning of dietary choline among carriers of genetic polymorphisms in choline metabolizing enzymes (Figure 1).

Table 1. Summary of examined SNPs and their connections to disease

Gene	Function	SNP	Choline Deficiency Risk	Disease Associations	References
CHKA	Phosphorylates choline, first step in CDP-choline pathway	rs10791957	↓ risk organ dysfunction *	↓ risk type 2 diabetes	[10,11]
CHDH	First step in oxidation of choline to betaine	rs9001	↓ risk organ dysfunction	↑ arsenic methylation	[10,12]
		rs12676	↑ risk organ dysfunction **	↑ breast cancer risk ↓ sperm ATP and altered sperm motility	[13,14]
BHMT	Converts homocysteine to methionine using betaine as a methyl donor	rs3733890	-	↓ breast cancer mortality ↑ orofacial cleft ↑ spina bifida (mixed results)	[10,15–18]
PEMT	Uses SAM to triply methylate PE to form PC (endogenous choline synthesis)	rs4646343	↑ risk organ dysfunction	↑ PEMT expression in adipose ↑ waist to hip ratio	[19]
		rs7946	-	↑ Non-alcoholic fatty liver disease	[20,21]
FMO3	Converts TMA to TMAO	rs2266782	-	↑ trimethylaminuria	[22]
SLC44A1	Transports choline across the cellular and mitochondrial membranes	rs7873937	↑ risk muscle damage		[10]
		rs3199966	↑ risk muscle damage		[10]

* Among women; ** among pre-menopausal women. ↑ increased, ↓ decreased.

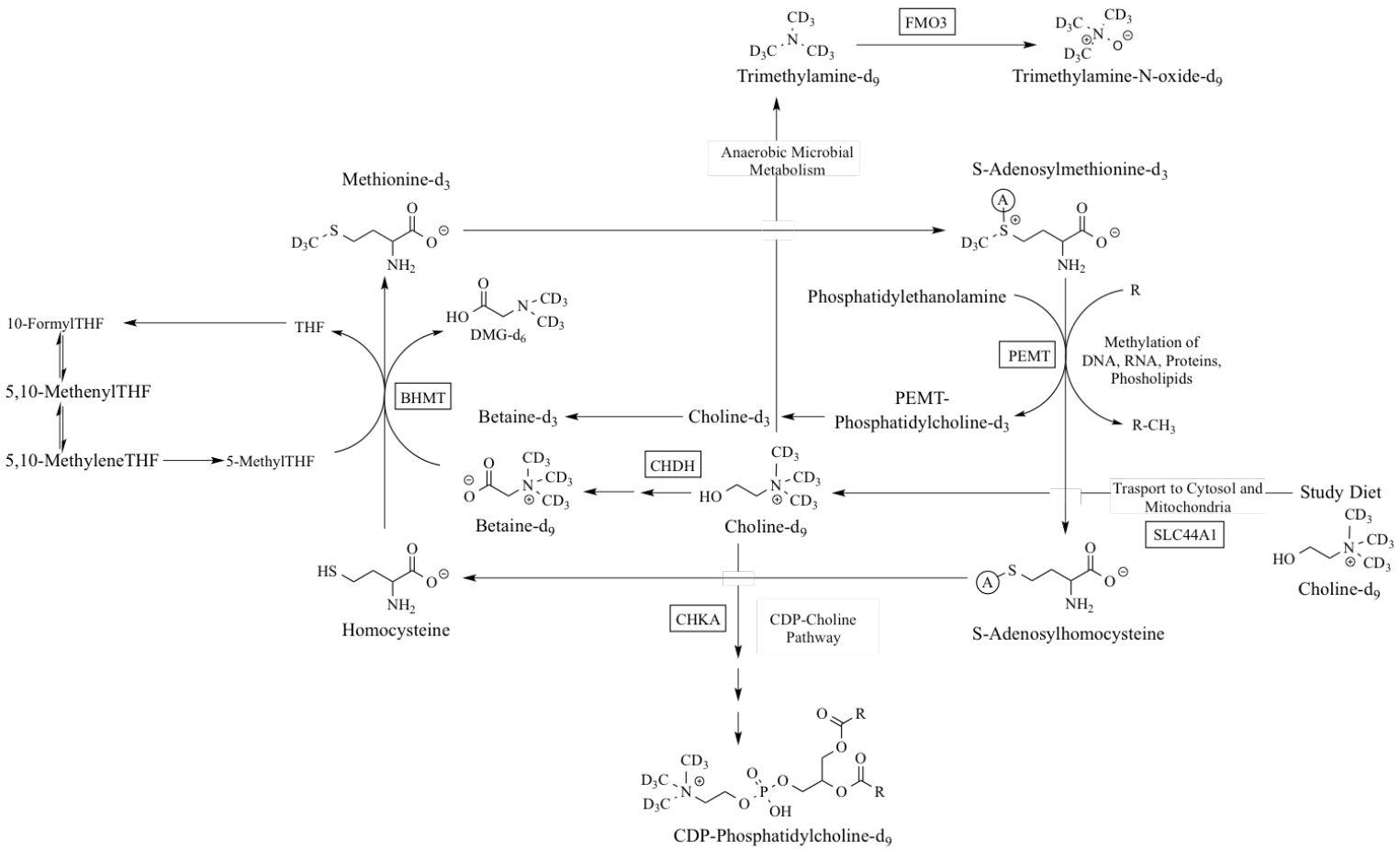


Figure 1. An overview of the metabolic fate of the isotopically labeled choline-d$_9$ consumed by study participants. SNPs in squared enzymes were examined. Choline kinase-α (*CHKA*) rs10791957; choline dehydrogenase (*CHDH*) rs9001, *CHDH* rs12676; betaine homocysteine methyltransferase (*BHMT*) rs3733890; phosphatidylethanolamine *N*-methyltransferase (*PEMT*) rs7946, *PEMT* rs4646343; solute carrier 44A1 (*SLC44A1)* rs7873937, *SLC44A1* rs3199966; and flavin monooxygenase isoform 3 (*FMO3)* rs2266782).

Participants and Methods

A. Participants and Study Design

This study was a follow-up investigation of a long-term randomized controlled feeding study conducted among women of reproductive age by Yan et al. [6]. Healthy third-trimester pregnant (n = 26), lactating (n = 28), and non-pregnant (n = 21), women consumed the study diet containing 380 mg choline/day, and either 100 or 550 mg choline/day from supplemental choline chloride (Balchem) for 10–12 weeks [6]. All participants also consumed a daily prenatal multivitamin (Pregnancy Plus; Fairhaven Health, LLC, Bellingham, WA, USA) containing 600 µg folic acid, a daily docasahexanoic acid supplement (200 mg, Neuromins; Nature's Way, Perris, CA, USA), and a potassium and magnesium supplement (General Nutrition Corp, Pittsburgh, PA, USA) thrice weekly. Beginning at week 6, participants consumed 22% of their total choline in the form of choline chloride-(trimethyl-d_9) (Cambridge Isotope Laboratories, Tewksbury, MA, USA, 98%). Fasting blood (10 h) was collected at study baseline and throughout the study and processed as previously described [6]. All samples were stored at −80 °C until analysis. The study was approved by the Institutional Review Boards at Cornell University and Cayuga Medical Center and was registered at clinicaltrials.gov as NCT01127022. All participants provided informed consent.

B. Genotyping

DNA was extracted from buffy coat for genotyping of PEMT rs7946, PEMT rs4646343, CHDH rs9001, CHDH rs12676, CHKA rs10791957, SLC44A1 rs7873937, SLC44A1 rs3199966, BHMT rs3733890, and FMO3 rs2266782 SNPs using the Qiagen DNeasy Blood

and Tissue on a LightCycler480 (Roche, Indianapolis, IN, USA). Endpoint genotyping was carried out as previously described using participant DNA and two commercially available products, (Applied Biosystems TaqMan Genotyping Master Mix and Thermo Fisher Scientific Assay Mix, Waltham, MA, USA) on a LightCycler 480 (Roche) in our facility [9].

C. *Enrichment of Choline Metabolites*

Choline metabolites were extracted from blood and enrichments of choline-d9, choline-d3, betaine-d9, betaine-d3, and DMG-d6 as well as PC-d3, PC-d6, and PC-d9, were measured using a TSQ Quantum Access triple quadrupole LCMS system (Thermo) operated in positive-ion mode using electrospray ionization as previously described in detail [9]. Enrichments of methionine-d3 were measured by gas chromatography-mass spectrometry (GC-MS) [9]. Enrichment percentages were calculated by dividing the area of each isotopically labeled choline metabolite by the total area of all isotopomers and multiplying by 100% (Equation (1)).

$$Enrichment_{metabolite} = \frac{labeled\ metabolite \times 100\%}{labeled + unlabeled\ metabolite} \quad (1)$$

D. Statistical Analysis

Seven metabolic outcomes in plasma were examined as primary response variables. Two of these outcomes were chosen to reflect partitioning between metabolic pathways (enrichment ratios of betaine-d_9/PC-d_9 and PC-d_{3+6}/PC-d_9). The other five outcomes were chosen to reflect flux through metabolic pathways and included turnover of choline → betaine, choline → CDP-PC, betaine → DMG, betaine → methionine, and choline-derived methionine → PEMT-

PC within the study period. Metabolic flux was defined as the rate of turnover of metabolic precursors → products in μmols/L/study period over the three-week period of label exposure (Equation (2)), where $Enrichment_{Product}$ and $Enrichment_{Precursor}$ are enrichments (percentages) of the product and precursor, and $Pool\ Size_{Product}$ is the plasma pool size in μmoles of metabolite product per liter of plasma.

$$Rate_{turnover} = \frac{Enrichment_{Product} \times Pool\ Size_{Product}}{Enrichment_{Precursor}} \quad (2)$$

The effect of genetic variation on each of these seven outcomes as a function of SNP genotype was assessed using linear models. Due to limited variant allele presence within our sample (**Table 2**), heterozygous and homozygous variant individuals were grouped together to examine the effect of variant allele presence. Reproductive status, choline intake group (480 or 930 mg choline/day), and possible interactions were included as covariates. A backwards selection was used in which BMI was retained at an α-cutoff of 0.05 and interactions were retained at an α-cutoff of 0.1 (the higher interaction cutoff was selected to prevent the interpretation of main effects in the presence of interactions). Model assumptions and the fit of the model to the data were assessed with standard diagnostic methods. Two lactating participants with choline-d_9 enrichment values greater than 2 standard deviations from the mean were excluded from the entire analysis. All statistical analysis was performed using the lsmeans package in the R statistical programming environment, available from CRAN 2014 [48]. Data are presented as predicted least-squared means, unless otherwise noted. Reported *p*-values include Bonferroni corrections for multiple comparisons and were considered significant at an α-cutoff of 0.05.

Results

A. Genotype Distribution

The distribution of genotypes within our cohort (n = 75) is depicted in Table 2. Because of the relatively low prevalence of the variant allele, heterozygous and homozygous variant individuals were combined to examine the effect of variant allele presence on metabolic outcomes. The number of participants in each sub-group analysis varies by gene and metabolic outcome. For example, if there are no interactions, there are more participants in each group because we are comparing all of the women of a certain genotype against all of the women of the other. On the other hand, when interactions are present, the groups are stratified by the interacting factor (e.g., reproductive status and/or choline intake), which decreases the number of participants per group.

Table 2. Genotype distribution (# of participants) among reproductive states and choline intake groups.

Group	**480 mg Choline/day**			**930 mg Choline/day**		
# Of Variant Alleles	0	1	2	0	1	2
CHKA rs10791957						
Lactating	2	5	6	2	4	7
Non-pregnant	2	5	3	2	7	2
Pregnant	2	6	5	1	3	9
CHDH rs9001						
Lactating	11	1	1	10	2	1
Non-pregnant	6	4	0	9	2	0
Pregnant	10	2	1	10	2	1
CHDH rs12676						
Lactating	5	7	1	9	3	1
Non-pregnant	7	1	2	5	6	0
Pregnant	6	6	1	8	4	1
BHMT rs3733890						
Lactating	5	6	2	3	8	2
Non-pregnant	4	6	0	9	1	1
Pregnant	8	4	1	6	6	1
PEMT rs4646343						
Lactating	6	4	3	6	6	1
Non-pregnant	5	5	0	6	4	1
Pregnant	5	5	3	6	4	3
PEMT rs7946						
Lactating	2	2	9	2	2	9
Non-pregnant	2	5	3	3	5	3
Pregnant	0	6	7	2	3	8
FMO3 rs2266782						
Lactating	7	5	1	3	8	2
Non-pregnant	6	3	1	4	4	3
Pregnant	6	4	3	7	4	1
SLC44A1 rs7873937						
Lactating	11	2	0	11	2	0
Non-pregnant	8	2	0	9	2	0
Pregnant	9	3	1	11	2	0
SLC44A1 rs3199966						
Lactating	10	3	0	11	2	0
Non-pregnant	8	2	0	8	3	0
Pregnant	8	4	1	9	4	0

A. CHKA rs10791957

Genotype influenced the production of PEMT-PC and the partitioning of dietary choline between PEMT-PC and CDP-PC (**Tables 3 and S1**). Specifically, variant women exhibited a lower turnover of choline-derived methionine → PEMT-PC ($8840^{\pm 291}$ vs. $11512^{\pm 664}$ μM PC/study period; $p = 0.0005$) over the study period and tended to have lower PC-d_{3+6}/PC-d_9 enrichment ratios than non-variants ($0.30^{\pm 0.007}$ vs. $0.33^{\pm 0.015}$; $p = 0.09$) (**Table 3, Figure 2**).

Table 3 *CHKA* rs10791957 genotype alters plasma choline metabolite partitioning and flux.

Metabolic Outcome	WT	Variant	*p*-Value
Choline-Derived Methionine → PEMT-PC	$11512^{\pm 664}$	$8840^{\pm 291}$	0.0005
PC-d_{3+6}/PC-d_9	$0.33^{\pm 0.015}$	$0.30^{\pm 0.007}$	0.09

Notes: Values are least-squared means ± standard errors for each group. PC-d_{3+6}/PC-d_9 values are ratios, choline-derived methionine → PEMT-PC values are in μM PEMT-PC/study period.
Abbreviations: CHKA, choline kinase-α; PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; RS, reproductive state; WT, wildtype.

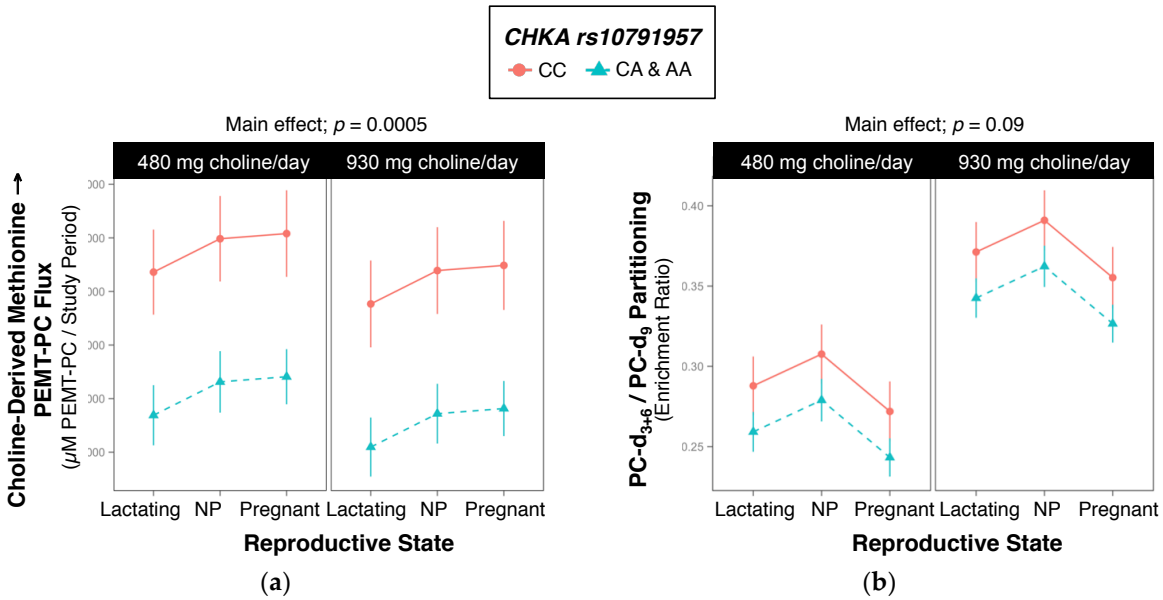


Figure 2 Effect of the *CHKA* rs10791957 variant on the metabolic flux and partitioning of dietary choline. (a) Choline-derived methionine → PEMT-PC flux; (b) PC-d_{3+6}/PC-d_9 partitioning.

B. CHDH rs9001

Genotype interacted with reproductive state to influence the turnover of choline → CDP-PC (p = 0.04) (**Table 4, Figure 3a**). While differences in choline → CDP-PC flux were not observed among pregnant or non-pregnant women, among lactating women, variants tended to exhibit a greater turnover of choline → CDP-PC as compared to non-variants (3355 $^{\pm 295}$ vs. 2541 $^{\pm 167}$ μM PC/study period; p = 0.09) (**Table 4**).

Table 4. Comparison of choline → CDP-PC turnover between genotypes by reproductive state

Metabolic Outcome and Group	WT	Variant	*p*-Value
Choline → CDP-PC			
RS × Gene Interaction; p = 0.04			
Lactating	2542 $^{\pm 167}$	3355 $^{\pm 324}$	0.09
Non-pregnant	2875 $^{\pm 185}$	2836 $^{\pm 295}$	>0.99
Pregnant	4279 $^{\pm 159}$	3804 $^{\pm 290}$	0.5

Notes: Values are least-squared means ± standard errors for each group in µM CDP-PC/study period.
Abbreviations: CDP, cytidine diphosphate; PC, phosphatidylcholine; RS, reproductive state; WT, wildtype.

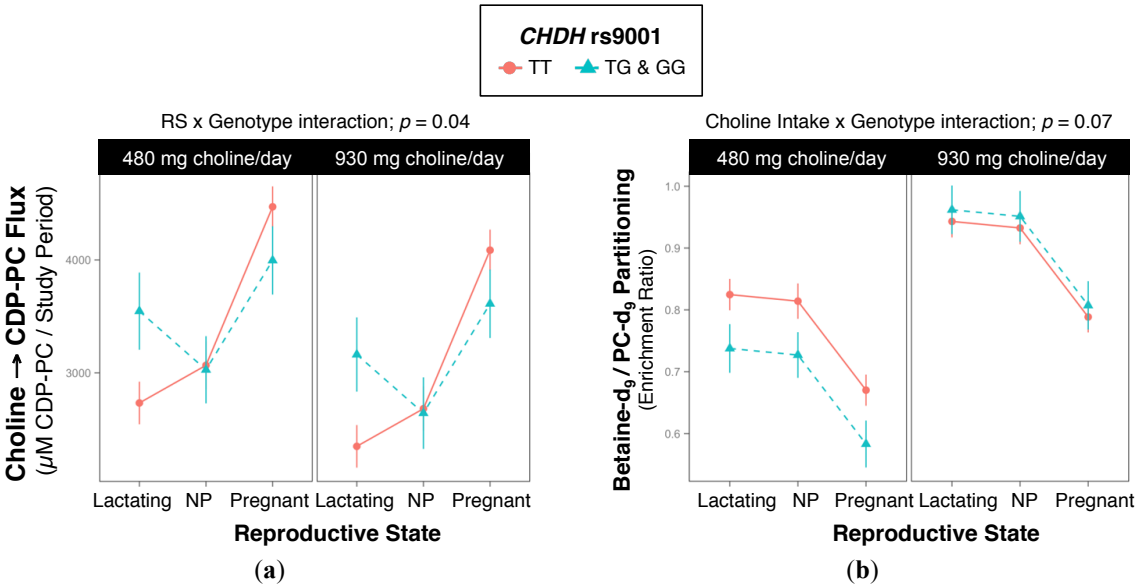


Figure 3. Effect of the *CHDH* rs9001 variant on the metabolic flux and partitioning of dietary choline. (**a**) Choline → CDP-PC flux; (**b**) Betaine-d_9/PC-d_9 partitioning

In addition, genotype tended to interact with choline intake to predict betaine-d_9/PC-d_9 enrichment (p = 0.07) (**Table 5, Figure 3b**). Within the lower choline intake group, variant women exhibited lower betaine-d_9/PC-d_9 enrichment ratios than non-variants (0.68 $^{\pm 0.03}$ vs. 0.77 $^{\pm 0.02}$; p = 0.06) (**Table 5**). Genotypic differences were not observed among women consuming the higher choline intake.

Table 5. Comparison of betaine-d_9/PC-d_9 enrichment ratios between *CHDH* rs9001 genotypes by choline intake group.

Metabolic Outcome and Group	WT	Variant	*p*-Value
Betaine-d_9/PC-d_9			
Cho × Gene Interaction; p = 0.07			
480 mg Choline/day	$0.77^{\pm 0.02}$	$0.68^{\pm 0.03}$	0.06
930 mg Choline/day	$0.89^{\pm 0.02}$	$0.91^{\pm 0.04}$	>0.99

Notes: Values are least-squared means ± standard errors in ratios.
Abbreviations: CDP, cytidine diphosphate; PC, phosphatidylcholine; RS, reproductive state; WT, wildtype.

C. CHDH rs12676

Genotype influenced the partitioning of dietary choline between PEMT-PC and CDP-PC (Figure 4). Specifically, variant individuals tended to have greater PC-d_{3+6}/PC-d_9 enrichment ratios as compared to non-variants ($0.32^{\pm 0.009}$ vs. $0.30^{\pm 0.008}$; $p = 0.055$) (**Table 6, Figure 4a**).

Table 6. *CHDH* rs12676 genotype alters plasma choline metabolite partitioning.

Metabolic Outcome	WT	Variant	*p*-Value
PC-d_{3+6}/PC-d_9	$0.30^{\pm 0.008}$	$0.32^{\pm 0.009}$	0.055

Notes: Values are least-squared means ± standard errors in ratios.
Abbreviations: CHDH, choline dehydrogenase; PC, phosphatidylcholine; WT, wildtype.

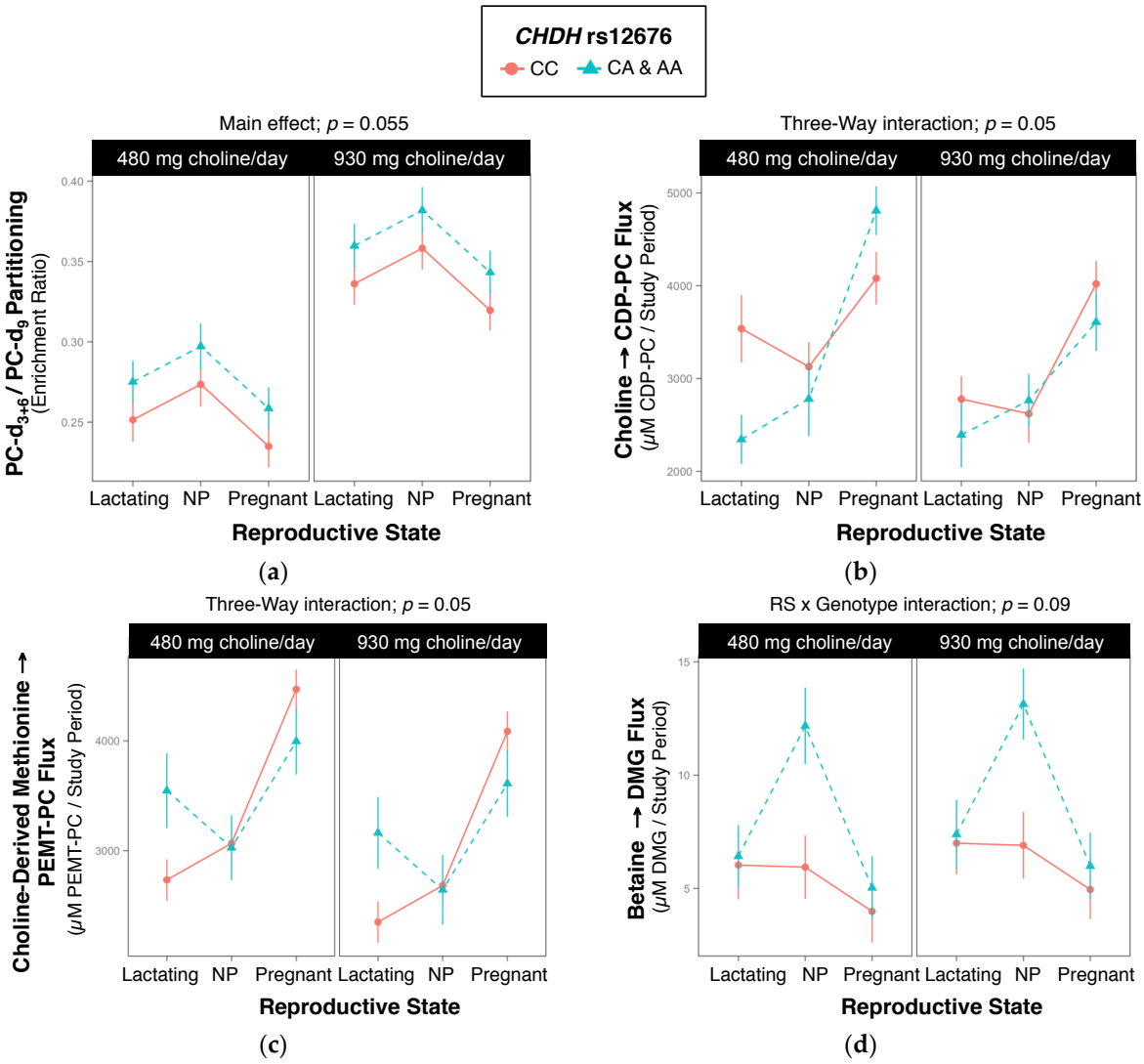


Figure 4. Effect of the *CHDH* rs12676 variant on the metabolic flux and partitioning of dietary choline. (**a**) PC-d_{3+6}/PC-d_9 partitioning; (**b**) Choline → CDP-PC flux; (**c**) Choline-derived methionine → PEMT-PC flux; (**d**) Betaine → DMG flux.

In line with this finding, genotype interacted with reproductive state and choline intake to influence the flux of choline → CDP-PC ($p = 0.05$) and choline-derived methionine → PEMT-PC ($p = 0.05$) (**Table S1**). The only detectable differences were among lactating women in the lower choline intake group. Within this subset, variants exhibited a lower turnover of both choline → CDP-PC ($2345^{\pm 264}$ vs. $3538^{\pm 364}$ μM PC/study period; $p = 0.06$) and choline-derived

methionine → PEMT-PC (7182 ± 799 vs. 12358 ± 1130 μM PC/study period; p = 0.003) as compared to non-variants (**Table 7, Figure 4b,c**).

Table 7. *CHDH* rs12676 genotype alters the metabolic flux of plasma choline metabolites.

Metabolic Outcome	480 mg Choline/day WT	480 mg Choline/day Variant	*p*-Value	930 mg Choline/day WT	930 mg Choline/day Variant	*p*-Value
Choline → CDP-PC 3-Way Interaction; p = 0.05						
Lactating	3538 ± 364	2345 ± 264	0.06	2779 ± 246	2394 ± 350	>0.99
Non-pregnant	3127 ± 266	2782 ± 403	>0.99	2621 ± 312	2764 ± 285	>0.99
Pregnant	4080 ± 285	4809 ± 263	0.39	4021 ± 248	3609 ± 313	>0.99
Choline-Derived Methionine → PEMT-PC 3-Way Interaction; p = 0.05						
Lactating	12358 ± 1130	7182 ± 799	0.003	9151 ± 799	8467 ± 1304	>0.99
Non-pregnant	9334 ± 854	10451 ± 1304	>0.99	9338 ± 1010	9272 ± 922	>0.99
Pregnant	9668 ± 922	10782 ± 854	>0.99	8728 ± 799	7929 ± 1010	>0.99

Notes: Values are least-squared means ± standard errors. Choline → CDP-PC and choline-derived methionine → PEMT-PC values are in μM PEMT-PC/study period. *p*-values represent the highest order interaction or main effect and pairwise comparisons between genotypes within intake groups.
Abbreviations: CDP, cytidine diphosphate; CHDH, choline dehydrogenase; PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

Finally, genotype tended to interact with reproductive state to influence the metabolic flux of betaine → DMG (p = 0.09). Non-pregnant variant women exhibited greater turnover of betaine → DMG as compared to non-pregnant non-variant women (6.9 ± 1.3 vs. 6.5 ± 1.3 μM DMG/study period; p = 0.01) (**Table 8, Figure 4d**).

Table 8. Comparison of betaine → DMG turnover between *CHDH* rs12676 genotype by reproductive state.

Metabolic Outcome and Group	WT	Variant	*p*-Value
Betaine → DMG			
RS × Gene Interaction; p = 0.09			
Lactating	$6.5^{\pm 1.3}$	$6.9^{\pm 1.3}$	>0.99
Non-pregnant	$6.4^{\pm 1.3}$	$12.7^{\pm 1.5}$	0.01
Pregnant	$4.5^{\pm 1.2}$	$5.5^{\pm 1.3}$	>0.99

Notes: Values are least-squared means ± standard errors in μM DMG /study period. *p*-values represent the highest order interaction or main effect and pairwise comparisons between genotypes.
Abbreviations: CHDH, choline dehydrogenase; PC, phosphatidylcholine; WT, wildtype.

D. BHMT rs3733890

Genotype influenced the metabolic flux of choline → betaine ($p = 0.03$) and choline → CDP-PC ($p = 0.03$) and accordingly, the partitioning of dietary choline between betaine and CDP-PC ($p = 0.07$) (**Table S1, Figure 5**). Variant women exhibited a greater turnover of choline → CDP-PC ($3440^{\pm 122}$ vs. $3063^{\pm 122}$ μM PC/study period; $p = 0.03$) and a non-significantly lower turnover of choline → betaine ($32^{\pm 3}$ vs. $38^{\pm 3}$ μM betaine/study period; $p = 0.2$) (Although the model effect of genotype was significant for the metabolic flux of choline → betaine, the comparison between the two genotypes was not, the difference being that the model effect does not account for the presence of a reproductive state by choline intake interaction.) (**Table 9, Figure 5a,b**). Consistent with these findings, variant individuals tended to have lower betaine-d_9/PC-d_9 enrichment ratios as compared to non-variants ($0.77^{\pm 0.02}$ vs. $0.82^{\pm 0.02}$; $p = 0.07$) (**Table 9, Figure 5c**).

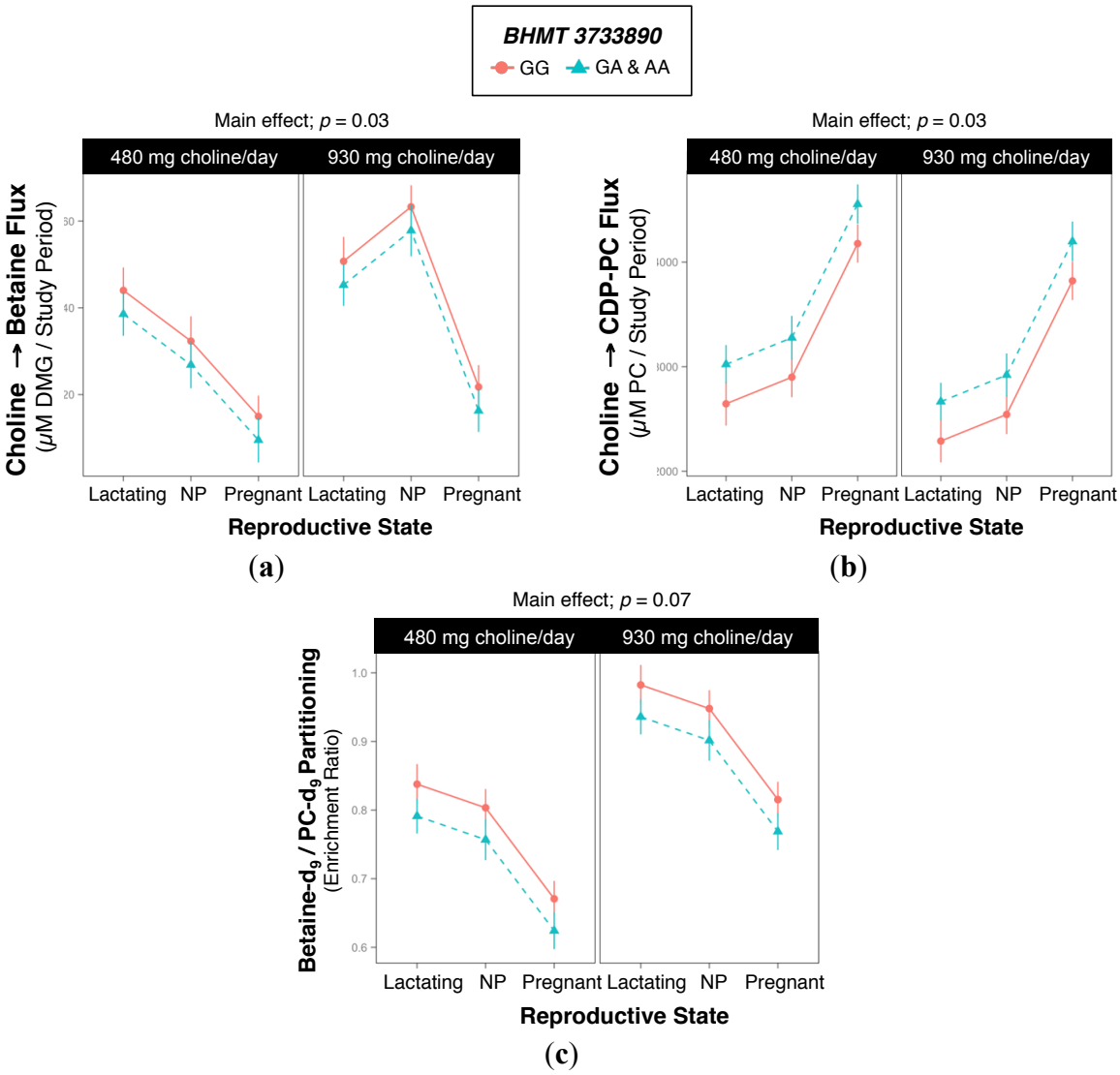


Figure 5. Effect of the *BHMT* rs3733890 variant on the metabolic flux and partitioning of dietary choline. (**a**) Choline → betaine flux; (**b**) Choline → CDP-PC flux; (**c**) Betaine-d_9/PC-d_9 partitioning.

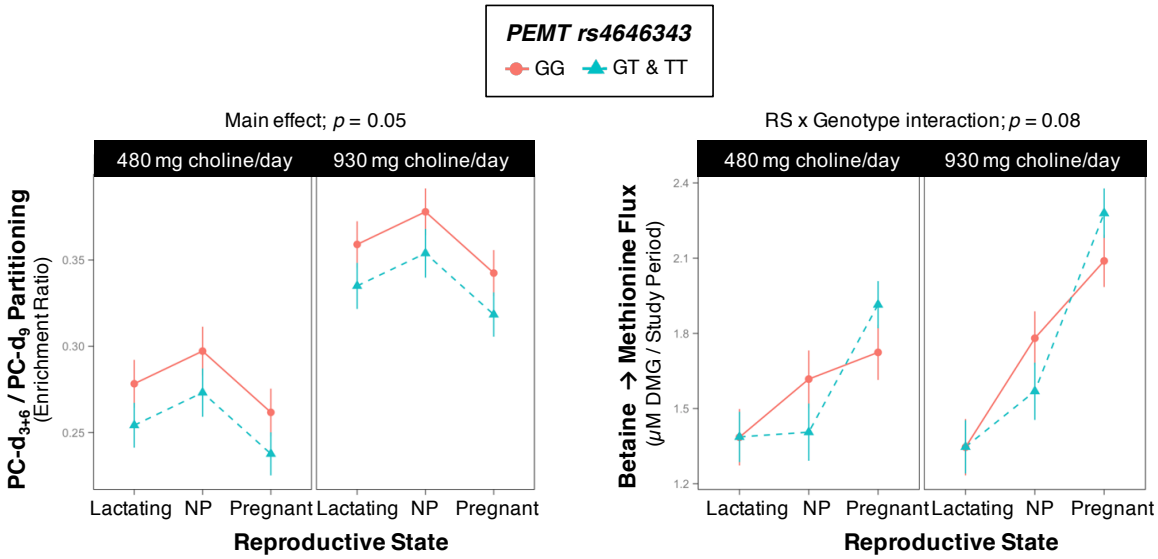
Table 9. *BHMT* rs3733890 genotype alters plasma choline metabolite partitioning and flux.

Metabolic Outcome	WT	Variant	*p*-Value
Choline → Betaine	38 $^{\pm 3}$	32 $^{\pm 3}$	0.2
Choline → CDP-PC	3063 $^{\pm 122}$	3440 $^{\pm 122}$	0.03
Betaine-d_9/PC-d_9	0.82 $^{\pm 0.02}$	0.77 $^{\pm 0.02}$	0.07

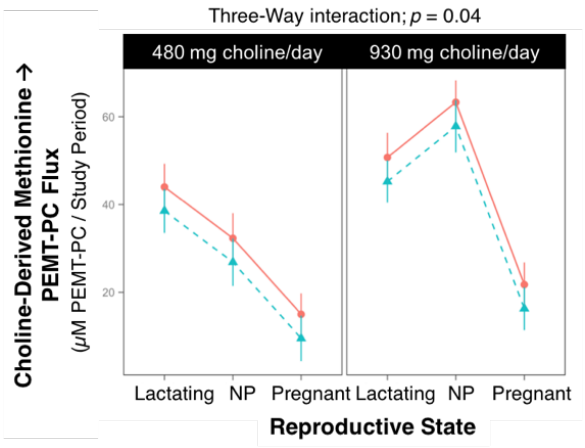
Notes: Values are least-squared means ± standard errors for each group. Betaine-d9/PC-d9 values are ratios, choline → betaine values are in µM betaine/study period and choline → CDP-PC values are in µM PC/study period.
Abbreviations: BHMT, betaine homocysteine methyltransferase; CDP, cytidine diphosphate; PC, phosphatidylcholine; WT, wildtype.

E. PEMT rs4646343

Genotype influenced partitioning of dietary choline between PEMT-PC and CDP-PC (**Table S1, Figure 6**). Women with the variant allele exhibited lower PC-d_{3+6}/PC-d_9 enrichment ratios as compared to women without (0.30 $^{\pm 0.008}$ vs. 0.32 $^{\pm 0.009}$; $p = 0.05$) (**Table 10, Figure 6a**).



(a) **(b)**



(**c**)

Figure 6. Effect of the *PEMT* rs4646343 variant on the metabolic flux and partitioning of dietary choline. (**a**) PC-d_{3+6}/PC-d_9 partitioning; (**b**) Betaine → methionine flux; (**c**) Choline-derived methionine → PEMT-PC flux.

Table 10. *PEMT* rs4646343 genotype alters plasma choline PC-d3+6/PC-d9 enrichment ratios. Values are least-squared means ± standard errors for each group. Values are ratios.

Metabolic Outcome	WT	Variant	*p*-Value
PC-d_{3+6}/PC-d_9	0.32 $^{\pm 0.009}$	0.30 $^{\pm 0.008}$	0.05

Notes: Values are least-squared means ± standard errors for each group in ratios.
Abbreviations: PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

Genotype also interacted with reproductive state to influence the metabolic flux of betaine → methionine ($p = 0.08$), though individual effects of genotype were not detectable after stratifying by reproductive state. Finally, genotype interacted with choline intake and reproductive state to influence the metabolic flux of choline-derived methionine → PEMT-PC ($p = 0.04$), though individual effects of genotype were not detectable after stratifying by choline intake (**Figure 6b,c**).

F. PEMT rs7946

Genotype interacted with reproductive state to influence the partitioning of dietary choline between PEMT-PC and CDP-PC (p = 0.097) (**Table S1, Figure 7a**). While genotypic differences in PC-d_{3+6}/PC-d_9 enrichment ratios were not observed among pregnant and non-pregnant women, lactating variant women exhibited lower PC-d_{3+6}/PC-d_9 enrichment ratios as compared to lactating non-variant women ($0.29^{\pm 0.01}$ vs. $0.37^{\pm 0.03}$; p = 0.03) (**Table 11**).

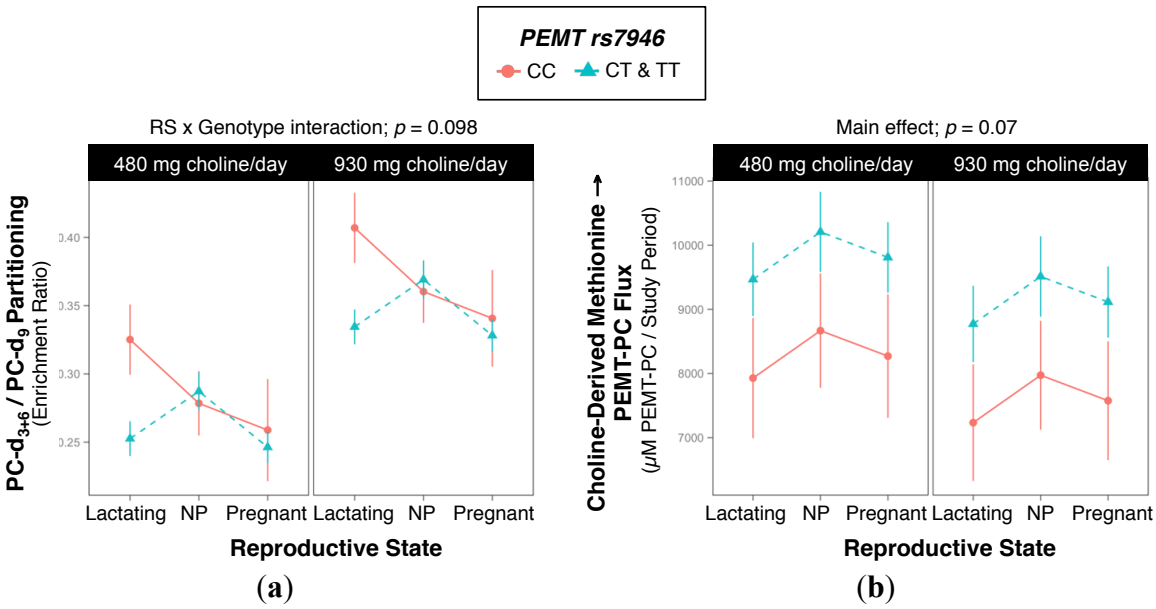


Figure 7. Effect of the *PEMT* rs7946 variant on the metabolic flux and partitioning of dietary choline. (**a**) PC-d_{3+6}/PC-d_9 partitioning; (**b**) Choline-derived methionine → PEMT-PC flux.

Table 11. Comparison of PC-d3+6/PC-d9 enrichment ratios between *PEMT* rs7946 genotype by reproductive state.

Metabolic Outcome and Group	WT	Variant	*p*-Value
PC-d_{3+6}/PC-d_9			
RS × Gene Interaction; p = 0.09			
Lactating	$0.37^{\pm 0.03}$	$0.29^{\pm 0.01}$	0.03
Non-pregnant	$0.32^{\pm 0.02}$	$0.33^{\pm 0.01}$	>0.99
Pregnant	$0.30^{\pm 0.04}$	$0.29^{\pm 0.01}$	>0.99

Notes: Values are least-squared means ± standard errors for each group in ratios.
Abbreviations: PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

In addition, genotype influenced the metabolic flux of choline-derived methionine → PEMT-PC (Table S1, Figure 7b). Variant women had non-significantly greater turnover of choline-derived methionine → PEMT-PC as compared to non-variants ($9481^{\pm 312}$ vs. $7942^{\pm 765}$ μM PC/study period; $p = 0.07$) (**Table 12**).

Table 12. *PEMT* rs7946 genotype alters the metabolic flux of choline-derived methionine → PEMT-PC in plasma.

Metabolic Outcome	WT	Variant	*p*-Value
Choline-Derived Methionine → PEMT-PC	$7942^{\pm 765}$	$9481^{\pm 312}$	0.07

Notes: Values are least-squared means ± standard errors for each group in μM PC/study period.
Abbreviations: PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

G. FMO3 rs2266782

Variant women had greater turnover of betaine → methionine ($1.6^{\pm 0.05}$ vs. $1.8^{\pm 0.05}$ μM methionine/study period; $p = 0.03$) and a greater turnover of choline-derived methionine → PEMT-PC ($9761^{\pm 384}$ vs. $8609^{\pm 433}$ μM PC/study period; $p = 0.05$) as compared to non-variants (**Table 13, Figure 8**).

Table 13. *FMO3* rs2266782 genotype alters the metabolic flux of plasma choline metabolites.

Metabolic Outcome	WT	Variant	*p*-Value
Betaine → Methionine	1.8 ± 0.05	1.6 ± 0.05	0.03
Choline-Derived Methionine → PEMT-PC	8609 ± 433	9761 ± 384	0.05

Notes: Values are least-squared means ± standard errors for each group. Betaine → methionine values are in µM methionine/study period and choline-derived methionine → PEMT-PC values are in µM PC/study period.
Abbreviations: FMO3, Flavin monooxygenase isoform 3; PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

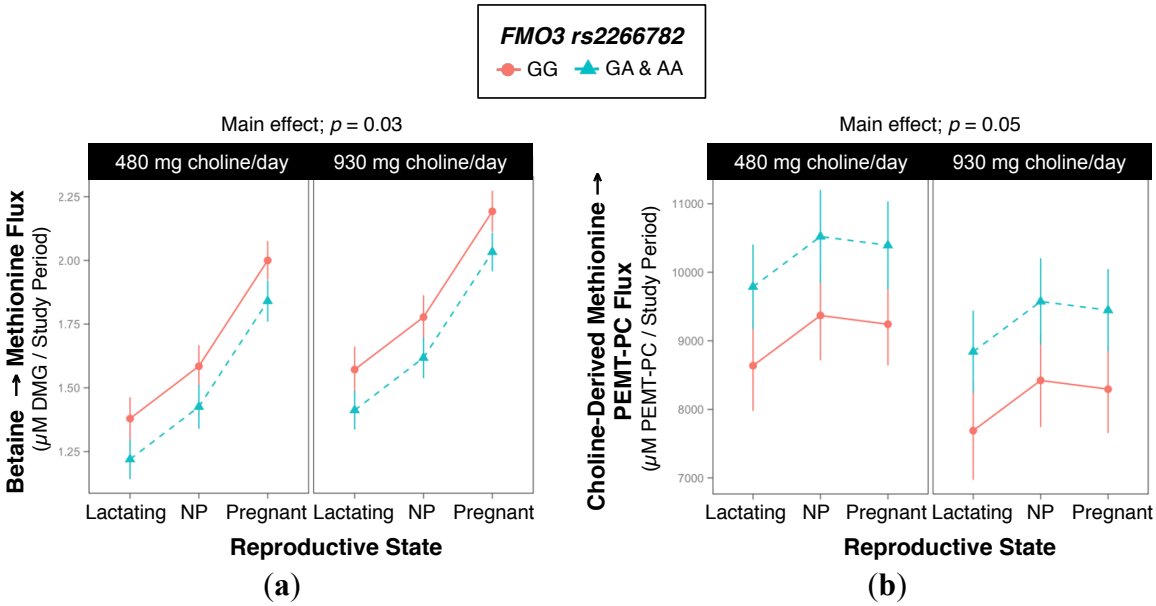


Figure 8. Effect of the *FMO3* rs3733890 variant on the metabolic flux and partitioning of dietary choline. (**a**) Betaine → methionine flux; (**b**) Choline-derived methionine → PEMT-PC flux.

H. SLC44A1 rs7873937

Genotype interacted with choline intake to influence the turnover of betaine → methionine (p = 0.06) (**Table S1, Figure 9a**). Although non-variants did not exhibit differences in the turnover of betaine → methionine as a function of choline intake, variant women exhibited greater

betaine → methionine turnover in the higher choline intake group as compared to the lower intake group (1.95 $^{\pm 0.11}$ vs. 1.51 $^{\pm 0.12}$ μM methionine/study period; $p = 0.03$) (**Table 14**).

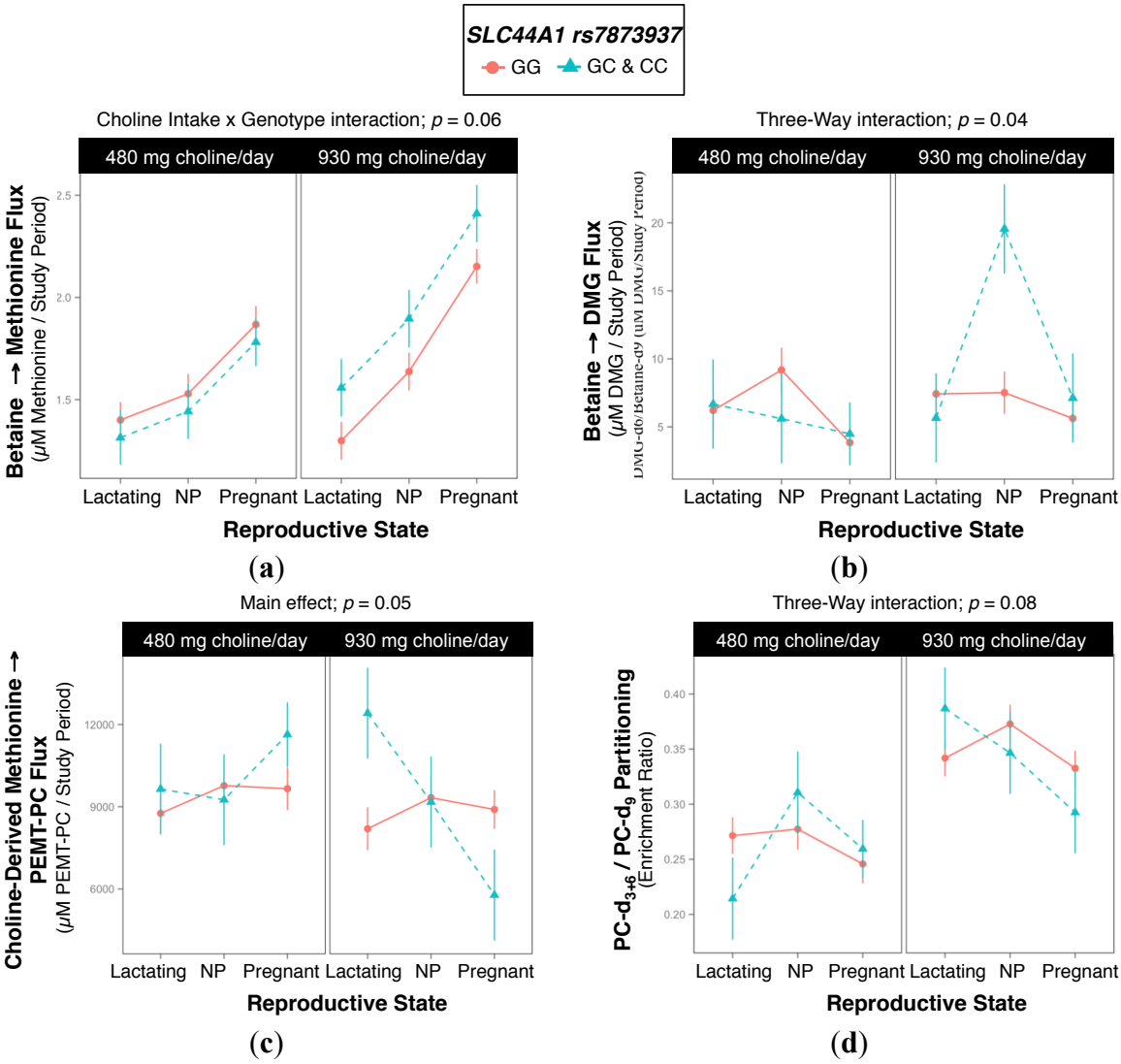


Figure 9. Effect of the *SLC44A1* rs7873937 variant on the metabolic flux and partitioning of dietary choline. (**a**) Betaine → methionine flux; (**b**) Betaine → DMG flux; (**c**) Choline-derived methionine → PEMT-PC flux; (**d**) PC-d_{3+6}/PC-d_9 partitioning.

Table 14. Comparison of choline → CDP-PC turnover between *SLC44A1* rs7873987 genotypes by choline intake group.

Metabolic Outcome and Group	480 mg Choline/day	930 mg Choline/day	*p*-Value
Betaine → Methionine			
Cho × Gene Interaction; p = 0.06			
WT	1.60 ± 0.06	1.70 ± 0.06	0.9
Variant	1.51 ± 0.12	1.95 ± 0.11	0.03

Notes: Values are least-squared means ± standard errors in μM methionine/study period.
Abbreviations: SLC44A1, solute carrier 44A1; WT, wildtype.

In addition, genotype interacted with reproductive state and choline intake to influence the metabolic flux of betaine → DMG (p = 0.04) (**Table S1, Figure 9b**). While genotypic differences were not observed in the lower choline intake group, within the higher intake group, non-pregnant variant women exhibited a greater turnover of betaine → DMG (19.6 ± 3.3 vs. 7.5 ± 1.5 μM DMG/study period; p = 0.02) (**Table 15**). Furthermore, similar to betaine → methionine turnover, although non-variants did not display differences in betaine → DMG as a function of choline intake (p > 0.99), variant non-pregnant women exhibited increased betaine → DMG in the higher choline intake group as compared to the lower intake group (19.6 ± 3.3 vs. 5.6 ± 3.3 μM DMG/study period; p = 0.05) (**Table 16**).

Table 15. *SLC44A1* rs7873987 genotype alters the metabolic flux of betaine → DMG.

Metabolic Outcome and Group	480 mg Choline/day WT	480 mg Choline/day Variant	*p*-Value	930 mg Choline/day WT	930 mg Choline/day Variant	*p*-Value
Betaine → DMG *3-Way Interaction; p = 0.04*						
Lactating	6.2 ± 1.5	6.7 ± 3.3	>0.99	7.4 ± 1.5	5.7 ± 3.3	>0.99
Non-pregnant	9.2 ± 1.6	5.6 ± 3.3	>0.99	7.5 ± 1.5	19.6 ± 3.3	0.02
Pregnant	3.8 ± 1.5	4.5 ± 2.3	>0.99	5.6 ± 1.4	7.1 ± 3.3	>0.99

Notes: Values are least-squared means ± standard errors in μM DMG/study period. *p*-values represent the highest order interaction and pairwise comparisons between genotypes within intake groups.
Abbreviations: DMG, dimethylglycine; SLC44A1, solute carrier 44A1; WT, wildtype.

Genotype also interacted with reproductive state and choline intake to influence the metabolic flux of choline-derived methionine → PEMT-PC ($p = 0.05$) (**Table S1 and Figure 9c)**. Genotypic differences were not detected within intake groups, however, variant and non-variant women responded differently to increased choline intake in a manner that depended upon reproductive state (**Table 16**). Specifically, only pregnant women with the variant exhibited different metabolic flux of choline-derived methionine → PEMT-PC between intake groups with lower flux in the higher intake group as compared to the lower intake group (5780 ± 1658 vs. 11635 ± 1172 μM PEMT-PC/study period; $p = 0.07$).

Table 16. *SLC44A1* rs7873987 genotype alters the metabolic flux and partitioning of dietary choline.

Metabolic Outcome and Group	480 mg Choline/day WT	930 mg Choline/day WT	*p*-Value	480 mg Choline/day Variant	930 mg Choline/day Variant	*p*-Value
Betaine → DMG *3-Way Interaction; p = 0.04*						
Lactating	6.2 ± 1.5	7.4 ± 1.5	>0.99	6.7 ± 3.3	5.7 ± 3.3	>0.99
Non-pregnant	9.2 ± 1.6	7.5 ± 1.5	>0.99	5.6 ± 3.3	19.6 ± 3.3	0.05
Pregnant	3.8 ± 1.5	5.6 ± 1.4	>0.99	4.5 ± 2.3	7.1 ± 3.3	>0.99
Choline-Derived Methionine → PEMT-PC *3-Way Interaction; p = 0.05*						
Lactating	8759 ± 741	8197 ± 782	>0.99	9648 ± 1658	12417 ± 1658	>0.99
Non-pregnant	9772 ± 829	9331 ± 782	>0.99	9257 ± 1658	9172 ± 1658	>0.99
Pregnant	9660 ± 782	8900 ± 707	>0.99	11635 ± 1172	5780 ± 1658	0.07
PC-d_{3+6}/ PC-d_9 *3-Way Interaction; p = 0.08*						
Lactating	0.27 ± 0.02	0.34 ± 0.02	0.05	0.21 ± 0.04	0.39 ± 0.04	0.02
Non-pregnant	0.28 ± 0.02	0.37 ± 0.02	0.005	0.31 ± 0.04	0.35 ± 0.04	>0.99
Pregnant	0.25 ± 0.02	0.33 ± 0.02	0.006	0.26 ± 0.03	0.29 ± 0.04	>0.99

Notes: Values are least-squared means ± standard errors. Betaine → DMG values are in μM DMG/study period. *p*-values represent the highest order interaction and pairwise comparisons between genotypes within intake groups.
Abbreviations: DMG, dimethylglycine, SLC44A1, solute carrier 44A1; PC, phosphatidylcholine; PEMT, Phosphatidylethanolamine N-methyltransferase; WT, wildtype.

Finally, genotype interacted with reproductive state and choline intake to influence the partitioning of dietary choline between PEMT-PC and CDP-PC (p = 0.08) (**Figure 9d**). Genotypic differences were not detected within intake groups; however, variant and non-variant women responded differently to increased choline intake in a manner that depended upon reproductive state (**Table 16**). Specifically, across reproductive states, women without the variant exhibited greater PC-d_{3+6}/PC-d_9 enrichment ratios in the higher choline intake group as compared to the lower ($p \leq 0.05$). Among women with the variant however, only lactating women exhibited increased PC-d_{3+6}/PC-d_9 enrichment ratios in the higher choline intake group as compared to the lower (p = 0.02) (**Table 16**).

I. SLC44A1 rs3199966

Genotype interacted with choline intake to influence the turnover of betaine → methionine (p = 0.08). While non-variants did not exhibit differences in the turnover of betaine → methionine as a function of choline intake, variant women exhibited greater betaine → methionine turnover in the higher choline intake group as compared to the lower intake group ($1.90^{\pm 0.11}$ vs. $1.52^{\pm 0.096}$ μM methionine/study period; p = 0.04) (**Table 17, Figure 10**).

Table 17. Comparison of betaine → methionine turnover between *SLC44A1* rs3199966 genotypes by choline intake group.

Metabolic Outcome and Group	480 mg Choline/day	930 mg Choline/day	*p*-Value
Betaine → Methionine			
Cho × Gene Interaction; p = 0.08			
WT	$1.60^{\pm 0.06}$	$1.70^{\pm 0.06}$	>0.99
Variant	$1.52^{\pm 0.10}$	$1.90^{\pm 0.11}$	0.04

Notes: Values are least-squared means ± standard errors in μM methionine/study period. *p*-values represent the highest order interaction and pairwise comparisons between intake groups within genotypes.
Abbreviations: SLC44A1, solute carrier 44A1; WT, wildtype.

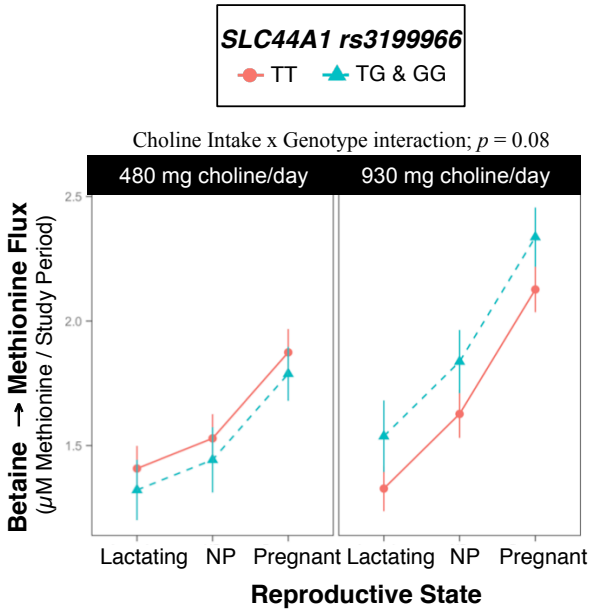


Figure 10. Effect of the *SLC44A1* rs3199966 variant on the metabolic flux and partitioning of dietary choline.

Discussion

These results demonstrate that common genetic variants in choline metabolizing genes alter the metabolic signature of choline in three ways: (i) the use of dietary choline as a methyl donor (*CHKA* rs10791957, *CHDH* rs9001, *CHDH* rs12676, *PEMT* rs4646343, *PEMT* rs7946, *FMO3* rs2266782, *SLC44A1* rs7873937 and *SLC44A1* rs3199966); (ii) the partitioning of dietary choline between betaine and CDP-PC synthesis (*CHDH* rs9001 and *BHMT* rs3733890); and (iii) the distribution of dietary choline between PEMT-PC and CDP-PC (*CHKA* rs10791957, *CHDH* rs12676, *PEMT* rs4646343, *PEMT* rs7946 and *SLC44A1* rs7873937). Such metabolic differences may contribute to disease pathogenesis and prognosis over the long-term.

A. CHKA (dbSNP: rs10791957; NC_000011.10: g.68100081 C > A)

Choline kinase-α catalyzes the cytosolic phosphorylation of choline to phosphocholine, which comprises the first step of the CDP-PC pathway. The rs10791957 SNP is located in the first intron, a possible enhancer region, and is associated with a decreased risk of organ dysfunction in women deprived of choline, as well as a decreased risk of type 2 diabetes [10,11]. Although the rate-limiting step in the CDP-PC pathway is considered to be the nucleotidyl transfer of CDP to phosphocholine, catalyzed by phosphocholine cytidylytransferase, differences in *CHKA* expression influence cellular PC production [23]. *CHKA* expression contributes to the regulation of cellular proliferation and apoptosis and *CHKA* overexpression and phosphocholine accumulation are associated with increased proliferation and oncogenesis [24,25]. Furthermore, tissue-specific *CHKA* expression (modulated in part by the circadian clock), has been proposed as a possible regulatory mechanism for the CDP-PC pathway [26]. Our data support a role for the *CHKA rs10791957* variant as an additional factor that may modulate PC homeostasis. Specifically, the variant appears to decrease the use of dietary

choline for PEMT-PC synthesis relative to CDP-PC synthesis. Variant individuals displayed decreased turnover of choline-derived methionine → PEMT-PC over the study period, indicating decreased activity of PEMT relative to women without the variant, and also tended to exhibit lower relative PEMT-PC/CDP-PC enrichment as compared to non-variants. These differences may be direct (arising from altered CHKA activity) or indirect (arising from changes in downstream signaling that regulate phospholipid metabolism) consequences of differences in *CHKA* expression. Notably, the decreased PEMT activity observed among variant women may provide a metabolic basis for the decreased risk of type 2 diabetes among variant individuals given that PEMT knockout mice are protected from high-fat diet induced obesity and insulin resistance (though not protected from hepatic steatosis) [27]. Additional studies are needed to determine whether *CHKA rs10791957* genotype distribution influences the relationship between diet, obesity and insulin resistance.

B. CHDH (dbSNP: rs9001; c.119 A > C; p.Glu40Ala) and (dbSNP: rs12676; c.233T > G; p.Leu78Arg)

CHDH is a flavin-dependent, mitochondrial enzyme that oxidizes choline to betaine aldehyde. The *CHDH* rs9001 variant is associated with a decreased risk of choline deficiency, while the rs12676 variant is associated with an increased risk among pre-menopausal women, which suggests opposing effects of these variants on CHDH activity [28].

Our results suggest that women with the rs9001 variant partition dietary choline to the CDP-choline pathway at the expense of betaine synthesis. Specifically, rs9001 variant lactating women exhibited increased turnover of choline → CDP-PC as well as non-significantly decreased betaine-d_9/PC-d_9 enrichment ratios in the lower intake group (**Figure 3**). They also

exhibited slightly and non-significantly lower PC-d_{3+6}/PC-d_9 enrichment ratios ($0.29^{\pm 0.01}$ vs. $0.31^{\pm 0.01}$; $p = 0.1$), further supporting the notion that women (particularly lactating women) with the rs9001 variant may relatively favor CDP-PC synthesis over PEMT-PC synthesis. This relatively greater use of choline for CDP-PC synthesis as compared to PEMT synthesis among CHDH rs9001 variants may conserve choline stores. For example, while each unit of choline directed to the CDP-choline pathway yields one unit of PC, choline converted to betaine (and eventually SAM) has many metabolic fates besides PEMT-PC production and requires three choline-derived methyl groups to yield just one additional unit of PC. In direct contrast, *CHDH* rs12676 variant women appear to favor the use of dietary choline for PEMT-PC synthesis relative to CDP-PC. rs12676 variant women exhibited higher PC-d_{3+6}/PC-d_9 enrichment ratios, non-pregnant rs12676 variant women exhibited a greater use of choline as a methyl donor, and lactating rs12676 variant women exhibited greater use of choline for PEMT-PC synthesis and lower use of choline for CDP-PC synthesis within the study period as compared to non-variants (**Figure 4**).

Importantly, our findings identify opposite metabolic differences for these variants, which is consistent with a decreased and increased risk of choline deficiency, respectively as previously reported by others [10,14]. In some ways, however, our data appear to contrast previous work, possibly due to a different effect of these variants by tissue, sex (the *CHDH* gene is under the control of an estrogen promoter), diet and other environmental factors [29]. Specifically, the rs12676 variant confers a relative loss of function in male sperm as variant men exhibit decreased sperm ATP and dyspmorphic mitochondrial structures similar to *Chdh*$^{-/-}$ mice, as well as decreased *CHDH* protein in sperm [14]. The mechanism however is unclear and it is unknown whether this effect is due to increased expression of *CHDH* or decreased

degradation. Additionally, a previous study identified increased dimethyl-arsenic:mono-methyl arsenic in *CHDH* rs9001 variant individuals exposed to arsenic, indicating increased efficiency of arsenic methylation [12]. The authors postulated that this increased efficiency of arsenic methylation (which facilitates detoxification) among rs9001 variant individuals may be due to a gain of function in *CHDH* activity that increases the conversion of choline to betaine, increasing SAM availability. Another possible interpretation is that the variant comprises a loss of *CHDH* activity. Decreased conversion of choline to betaine might increase the availability of choline for CDP-PC synthesis, reducing the burden on SAM for PC synthesis (PEMT is one of the main consumers of SAM in the liver), and increasing the availability of SAM for arsenic methylation. Overall, our results highlight that the *CHDH* rs9001 and *CHDH* rs12676 SNPs exert opposing metabolic effects, not only among individuals deprived of choline or exposed to one-carbon stressing conditions, but also among healthy women consuming choline intakes relevant to the general population.

C. BHMT (dbSNP: rs3733890; c.716 G > A, also known as c.742 G > A; p.Arg239Gln)

Betaine homocysteine methyltransferases (BHMT) is a zinc-dependent enzyme that uses betaine to remethylate homocysteine to methionine. It acts primarily in the liver, but is also present in the kidney and optic lens. The *BHMT* rs3733890 variant encodes an arginine to glutamine change at amino acid 239, which results in a lower K_m (roughly half) for both betaine and homocysteine [30]. The *BHMT* rs3733890 polymorphism has been associated with reduced all-cause mortality in breast cancer patients and a number of developmental outcomes including a decreased risk of orofacial cleft and an increased risk of spina bifida, particularly with high maternal folic acid intake, but results have been mixed [15–18,31]. There is some evidence that the effect of this variant is modulated by *MTHFR* rs1801133 variant allele presence, however

the relationship remains unresolved [18]. Given the scarcity of *MTHFR* rs1801133 and *BHMT* rs3733890 homozygous variant individuals in our cohort, this study was unable to examine this interaction. When evaluated independently, the *BHMT* variant allele was associated with non-significantly decreased turnover of choline → betaine, and increased turnover of choline → CDP-PC as well as a (non-significantly, $p = 0.07$) lower betaine-d_9/PC-d_9 enrichment ratio. Together, these results indicate that the variant favors the use of dietary choline for CDP-PC synthesis at the expense of betaine synthesis. These findings can be understood in the context of the effect of this SNP on enzyme kinetics. A lower K_m for both betaine and homocysteine, results in increased affinity of the enzyme for these substrates, meaning less betaine is needed among variants to maintain maximal BHMT activity. Partitioning away from betaine, therefore, may conserve dietary choline among variant individuals.

D. PEMT (dbSNP: rs4646343; c.2768 C > A REV) and (dbSNP: rs7946; c.5465 G > A REV; p.Val175Met)

Phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the de novo synthesis of choline via the triple methylation of PE to form PC [32]. The *PEMT* rs7946 variant encodes a valine to methionine substitution at amino acid 175, which results in decreased enzymatic activity in vitro and may increase susceptibility to non-alcoholic fatty liver disease (NAFLD) [20,21]. Previous work has found a 92% overlap of the intronic *PEMT* rs4646343 SNP with the functional rs12325817 SNP, which is located in the promoter region of the *PEMT* gene, near the estrogen response element, and impedes its estrogen-mediated up-regulation [33]. This impairment leads to an increased susceptibility to organ dysfunction in variant individuals [10]. Although we did not observe direct indications of decreased PEMT activity, the observed

decreased PEMT-PC/CDP-PC in rs4646343 variant individuals is consistent with decreased PEMT activity and an impaired estrogen response among variant individuals. Decreased PEMT-PC/CDP-PC was also observed in *PEMT* rs7946 variant lactating women.

Overall, these data suggest a relatively decreased contribution of PEMT-PC relative to CDP-PC in PC pools with both *PEMT* rs4646343 and rs7946 variants. Impaired PEMT activity may compromise PC-DHA supply to extra-hepatic tissue including vital reproductive organs during pregnancy and lactation [34]. Therefore, given that these data support the notion that both PEMT variants lead to functional changes in PC homeostasis at choline intakes meeting current recommendations, these SNPs deserve further study to determine whether such effects are associated with negative clinical outcomes among the general population, whose intakes are well below current recommendations [35].

E. FMO3 (dbSNP: rs2266782; c.472 G > A; p.Glu158Lys)

FMO3 is a (largely) hepatic enzyme that converts trimethylamine, a breakdown product of choline produced by anaerobic intestinal microbiota, to trimethylamine *N*-oxide (TMAO) [36]. The rs2266782 SNP is a common polymorphism that encodes a glutamate to lysine amino acid change (E158K) in flavin monooxygenase isoform 3 (FMO3) [37]. This variant is associated with a relative loss-of-function and, when in *cis* with other common variants, can cause mild trimethylaminuria (due to a relative excess of trimethylamine), which has largely unknown metabolic consequences [22]. FMO3 is activated by insulin, and knockout in insulin resistant mice prevents hyperglycemia, hyperlipidemia, and atherosclerosis [38]. FMO3 is suppressed by testosterone and up regulated by bile acids, which also stimulate hepatic cholesterol absorption [39].

Differences in TMAO metabolism are known to alter cholesterol transport and influence risk for cardiovascular disease [40,41]. TMAO supplementation in mice has been shown to increase macrophage cholesterol accumulation, which subsequently increases risk for atherosclerosis [41]. More recently, Brown and colleagues identified FMO3 itself (rather than TMAO) as a direct regulator of cholesterol balance, lipid metabolism, and inflammation in mice. FMO3 knockdowns had decreased hepatic cholesterol production, decreased intestinal absorption, and increased hepatic inflammation along with activation of liver X receptor (LXR)-stimulated macrophage reverse cholesterol transport [42]. While a previous study from our group suggested that the variant might be associated with increased use of choline as a methyl donor in men (based on increased DMG pool size) [43], results from the present study, indicate that women with the variant actually use choline *less* as methyl donor. Variant women tended to have a lower turnover of betaine → methionine over the study period. In addition, variant women exhibited a greater turnover of choline-derived methionine → PEMT-PC over the study period, which is consistent with previous findings from our lab that have identified lower methionine excretion among variant individuals (i.e., a greater use of methionine may reduce excretion) [43]. While it is not clear how these findings relate to data in mice, our results strengthen previous evidence of a relationship between FMO3 and phospholipid metabolism and demonstrate that this SNP exerts an effect on the metabolic use of dietary choline.

F. SLC44A1 (dbSNP: rs7873937; NC_000009.11:g.108089321 G > C) and SLC44A1 (dbSNP: rs3199966; c.1930 T > G; p.Ser644Ala)

Solute Carrier 44A1 (SLC44A1), also referred to as choline transporter-like protein 1 (CTL1), is a transmembrane protein that mediates choline transport across the mitochondrial

and plasma membranes [44–46]. It is expressed in four major splice variants throughout the brain and central nervous system including the spinal cord, motor neurons, and oligodendrocytes during and after myelination, as well as in the colon and lung. While the majority of people present with liver dysfunction in response to choline deprivation, Zeisel and colleagues noted that some individuals present first with muscle dysfunction, and they tend to carry mutations in the *SLC44A1* gene [10]. Though not exonic, the *SLC44A1* rs7873937 variant allele is associated with an increased susceptibility to muscle dysfunction in humans deprived of choline [10]. *SLC44A1* rs7873937 may exist in a regulatory region that responds to choline status, or may be in linkage disequilibrium with a functional SNP. The rs3199966 polymorphism confers a serine to alanine amino acid substitution, and like rs7873937, is associated with increased susceptibility to muscle dysfunction in humans deprived of choline [10]. For each of these SNPs, rs7873937 and rs3199966, we identified an interaction between genotype and choline intake that governed the use of choline as a methyl donor. The finding that the effect of genotype depends on choline intake aligns with in vitro evidence that dietary choline is known to modulate expression of the *SLC44A1* gene [47]. Specifically, for both SNPs, variant (but not non-variant) individuals exhibited greater turnover of betaine → methionine in the higher choline intake group as compared to the lower, suggesting that additional choline was used for methyl donation. For *SLC44A1* rs7873937, this effect was partially mirrored in a greater turnover of betaine → DMG at the higher choline intake among non-pregnant women. While we expect that betaine → DMG and betaine → methionine turnover would be identical, our results are not exactly the same, and this is likely due to differences in choline partitioning and sequestration throughout the various pools in the body.

G. Study Limitations

Given the post-hoc design, which did not preemptively evenly distribute participants across reproductive states, choline intake groups, and genotypes, some comparisons that involved two- and three-way interactions ended up with small sample sizes. Other limitations include possible unequal distribution of other functional variants within the study population, and the fact that circulating metabolites may not be in equilibrium with all tissues. Nonetheless, the present study benefitted from a highly controlled study environment and the precision afforded from the use of an isotopic tracer.

Conclusion

These data provide compelling evidence that common SNPs modulate choline partitioning in women of reproductive age consuming intakes that are relevant to the population at large. These metabolic differences may contribute to disease pathogenesis and prognosis over the long-term given relationships between methyl group and PC homeostasis and disease. Therefore, these SNPs deserve further study in a clinical and epidemiological context.

Supplemental Information

Table S1. Effect of SNP genotype and interaction (*p*-values) on plasma choline metabolite partitioning and flux.

Outcome	Gene	G*RS	G*Cho	Three-Way
***CHKA* rs10791957**				
Betaine-d_9/PC-d_9	0.6	-	-	-
PC-d_{3+6}/PC-d_9	0.09	-	-	-
Choline → Betaine	0.8	-	-	-
Choline → CDP-PC	0.3	-	-	-
Betaine → DMG	0.5	-	-	-
Betaine → Methionine	0.6	-	-	-
Methionine → PEMT-PC	0.0005	-	-	-
***CHDH* rs9001**				
Betaine-d_9/PC-d_9	0.2	-	0.07	-
PC-d_{3+6}/PC-d_9	0.1	-	-	-
Choline → Betaine	0.6	-	-	-
Choline → CDP-PC	0.7	0.04		
Betaine → DMG	0.2	-	-	-
Betaine → Methionine	0.9	-	-	-
Methionine → PEMT-PC	0.9	-	-	-
***CHDH* rs12676**				
Betaine-d_9/PC-d_9	0.2	-	-	-
PC-d_{3+6}/PC-d_9	0.055	-	-	-
Choline → Betaine	0.3	-	-	-
Choline → CDP-PC	0.2	0.08	0.9	0.05
Betaine → DMG	0.03	0.09	-	-
Betaine → Methionine	0.2	-	0.1	-
Methionine → PEMT-PC	0.2	0.02	0.8	0.05
***BHMT* rs3733890**				
Betaine-d_9/PC-d_9	0.066	-	-	-
PC-d_{3+6}/PC-d_9	0.3	-	-	-
Choline → Betaine	0.03	-	-	-
Choline → CDP-PC	0.03	-	-	-
Betaine → DMG	0.1	-	-	-
Betaine → Methionine	0.95	-	-	-
Methionine → PEMT-PC	0.15	-	-	-

REFERENCES

1. Caudill, M.A.; Miller, J.W.; III, J.F.G. Folate, choline, vitamin B12 , and vitamin B6. In *Stipanuk MH and Caudill MA. Biochemical, Physiological and Molecular Aspects of Human Nutrition*; Elsevier Saunders: St. Louis, MO, USA, 2012; pp. 565–608.
2. Li, Z.; Vance, D.E. Phosphatidylcholine and choline homeostasis. *J. Lipid Res.* **2008**, *49*, 1187–1194.
3. Delong, C.J.; Shen, Y.; Michael, J.; Cui, Z. Molecular Distinction of Phosphatidylcholine Synthesis between the CDP-Choline Pathway and Pathway Molecular Distinction of Phosphatidylcholine Synthesis between the CDP-Choline Pathway and Phosphatidy. *J. Biol. Chem*. **1999**, *274*, 29683–29688.
4. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and C. *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*; National Academies Press (US): Washington, DC, USA, 1998.
5. Yan, J.; Wang, W.; Iii, J.F.G.; Malysheva, O.; Brenna, J.T.; Stabler, S.P.; Allen, R.H.; Caudill, M.A. MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming. *Am. J. Clin. Nutr*. **2011**, *93*, 348–355.
6. Yan, J.; Jiang, X.; West, A.A.; Perry, C.A.; Malysheva, O.V.; Devapatla, S.; Pressman, E.; Vermeylen, F.; Stabler, S.P.; Allen, R.H.; et al. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. *Am. J. Clin. Nutr.* **2012**, *95*, 1060–1071.
7. Fischer, L.M.; DaCosta, K.A.; Kwock, L.; Stewart, P.W.; Lu, T.S.; Stabler, S.P.; Allen, R.H.; Zeisel, S.H. Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am. J. Clin. Nutr.* **2007**, *85*, 1275–1285.
8. Da Costa, K.-A.; Kozyreva, O.G.; Song, J.; Galanko, J.A.; Fischer, L.M.; Zeisel, S.H. Common genetic polymorphisms affect the human requirement for the nutrient choline. *FASEB J.* **2006**, *20*, 1336–1344.
9. Ganz, A.B.; Shields, K.; Fomin, V.G.; Lopez, Y.S.; Mohan, S.; Lovesky, J.; Chuang, J.C.; Ganti, A.; Carrier, B.; Yan, J.; et al. Genetic impairments in folate enzymes increase dependence on dietary choline for phosphatidylcholine production at the expense of betaine synthesis. *FASEB J.* **2016**, *30*, 3321–3333.
10. Da Costa, K.A.; Corbin, K.D.; Niculescu, M.D.; Galanko, J.A.; Zeisel, S.H. Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. *FASEB J.* **2014**, *28*, 2970–2978.
11. Jeff, J.M.; Armstrong, L.L.; Ritchie, M.D.; Denny, J.C.; Kho, A.N.; Basford, M.A.; Wolf, W.A.; Pacheco, J.A.; Li, R.; Chisholm, R.L.; et al. Admixture mapping and subsequent fine-mapping suggests a biologically relevant and novel association on chromosome 11 for type 2 diabetes in African Americans. *PLoS ONE* **2014**, *9*, e86931.
12. Schläwicke Engström, K.; Nermell, B.; Concha, G.; Strömberg, U.; Vahter, M.; Broberg, K. Arsenic metabolism is influenced by polymorphisms in genes involved in one-carbon metabolism and reduction reactions. *Mutat. Res.* **2009**, *667*, 4–14.

13. Xu, X.; Gammon, M.D.; Zeisel, S.H.; Lee, Y.L.; Wetmur, J.G.; Teitelbaum, S.L.; Bradshaw, P.T.; Neugut, A.I.; Santella, R.M.; Chen, J. Choline metabolism and risk of breast cancer in a population-based study. *FASEB J.* **2008**, *22*, 2045–2052.
14. Johnson, A.R.; Lao, S.; Wang, T.; Galanko, J.A.; Zeisel, S.H. Choline dehydrogenase polymorphism rs12676 is a functional variation and is associated with changes in human sperm cell function. *PLoS ONE* **2012**, *7*, e36047.
15. Mostowska, A.; Hozyasz, K.K.; Wojcicki, P.; Dziegelewska, M.; Jagodzinski, P.P. Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. *J. Med. Genet.* **2010**, *47*, 809–815.
16. Xu, X.; Gammon, M.D.; Wetmur, J.G.; Bradshaw, P.T.; Susan, L.; Neugut, A.I.; Santella, R.M.; Chen, J. NIH B-vitamin Intake, One-carbon Metabolism and Survival among a Population-based Study of Women with Breast Cancer. *Biomarkers* **2009**, *17*, 2109–2116.
17. Shaw, G.M.; Lu, W.; Zhu, H.; Yang, W.; Briggs, F.B.S.; Carmichael, S.L.; Barcellos, L.F.; Lammer, E.J.; Finnell, R.H. 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. *BMC Med. Genet.* **2009**, *10*, 49, doi:10.1186/1471-2350-10-49.
18. Boyles, A.L.; Billups, A.V.; Deak, K.L.; Siegel, D.G.; Mehltretter, L.; Slifer, S.H.; Bassuk, A.G.; Kessler, J.A.; Reed, M.C.; Nijhout, H.F.; et al. Neural tube defects and folate pathway genes: Family-based association tests of gene-gene and gene-environment interactions. *Environ. Health Perspect.* **2006**, *114*, 1547–1552.
19. Sharma, N.K.; Langberg, K.A.; Mondal, A.K.; Das, S.K. Phospholipid biosynthesis genes and susceptibility to obesity: Analysis of expression and polymorphisms. *PLoS ONE* **2013**, *8*, e65303.
20. Song, J.; da Costa, K.A.; Fischer, L.M.; Kohlmeier, M.; Kwock, L.; Wang, S.; Zeisel, S.H. Polymorphism of the *PEMT* gene and susceptibility to nonalcoholic fatty liver disease (NAFLD). *FASEB J.* **2005**, *19*, 1266–1271.
21. Zeisel, S.H. People with fatty liver are more likely to have the *PEMT* rs7946 SNP, yet populations with the mutant allele do not have fatty liver. *FASEB J.* **2006**, *20*, 2181–2182.
22. Phillips, I.R.; Shephard, E.A. *Primary Trimethylaminuria*; University of Washington: Seattle, WA, USA, 1993.
23. Gibellini, F.; Smith, T.K. The Kennedy pathway-de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* **2010**, *62*, 414–428.
24. Trousil, S.; Lee, P.; Pinato, D.J.; Ellis, J.; Dina, R.; Aboagye, E.O.; Keun, H.C.; Sharma, R. Alterations of choline phospholipid metabolism in endometrial cancer are caused by choline kinase α overexpression and a hyperactivated deacylation pathway. *Cancer Res.* **2014**, *74*, 6867–6878.
25. Ramírez de Molina, A.; Gallego-Ortega, D.; Sarmentero-Estrada, J.; Lagares, D.; Gómez del Pulgar, T.; Bandrés, E.; García-Foncillas, J.; Lacal, J.C. Choline kinase as a link connecting phospholipid metabolism and cell cycle regulation: Implications in cancer therapy. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 1753–1763.
26. Gréchez-Cassiau, A.; Feillet, C.; Guérin, S.; Delaunay, F. The hepatic circadian clock regulates the choline kinase **α** gene through the BMAL1-REV-ERB α axis. *Chronobiol. Int.* **2015**, *32*, 774–784.

27. Jacobs, R.L.; Zhao, Y.; Koonen, D.P.Y.; Sletten, T.; Su, B.; Lingrell, S.; Cao, G.; Peake, D.A.; Kuo, M.-S.; Proctor, S.D.; et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine *N*-methyltransferase-deficient mice are protected from diet-induced obesity. *J. Biol. Chem.* **2010**, *285*, 22403–22413.
28. Zeisel, S.H. Gene response elements, genetic polymorphisms and epigenetics influence the human dietary requirement for choline. *IUBMB Life* **2007**, *59*, 380–387.
29. Wang, Z.; Dahiya, S.; Provencher, H.; Muir, B.; Carney, E.; Coser, K.; Shioda, T.; Ma, X.-J.; Sgroi, D.C. The Prognostic Biomarkers HOXB13, IL17BR, and CHDH Are Regulated by Estrogen in Breast Cancer. *Clin. Cancer Res.* **2007**, *13*, 6327–6334.
30. Li, F.; Feng, Q.; Lee, C.; Wang, S.; Pelleymounter, L.L.; Moon, I.; Eckloff, B.W.; Wieben, E.D.; Schaid, D.J.; Yee, V.; et al. Human betaine-homocysteine methyltransferase (BHMT) and BHMT2: Common gene sequence variation and functional characterization. *Mol. Genet. Metab.* **2008**, *94*, 326–335.
31. Morin, I.; Platt, R.; Weisberg, I.; Sabbaghian, N.; Wu, Q.; Garrow, T.A.; Rozen, R. Common variant in betaine-homocysteine methyltransferase (BHMT) and risk for spina bifida. *Am. J. Med. Genet. A* **2003**, *119A*, 172–176.
32. Ridgway, N.D.; Vance, D.E. Kinetic mechanism of phosphatidylethanolamine *N*-methyltransferase. *J. Biol. Chem.* **1988**, *263*, 16864–16871.
33. Resseguie, M.E.; da Costa, K.-A.; Galanko, J.A.; Patel, M.; Davis, I.J.; Zeisel, S.H. Aberrant estrogen regulation of PEMT results in choline deficiency-associated liver dysfunction. *J. Biol. Chem.* **2011**, *286*, 1649–1658.
34. Pynn, C.J.; Henderson, N.G.; Clark, H.; Koster, G.; Bernhard, W.; Postle, A.D. Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed in vivo. *J. Lipid Res.* **2011**, *52*, 399–407.
35. Wallace, T.C.; Fulgoni, V.L. Assessment of Total Choline Intakes in the United States. *J. Am. Coll. Nutr.* **2016**, *35*, 108–112.
36. Craciun, S.; Balskus, E.P. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 21307–21312.
37. Koukouritaki, S.B.; Poch, M.T.; Cabacungan, E.T.; McCarver, D.G.; Hines, R.N. Discovery of novel flavin-containing monooxygenase 3 (FMO3) single nucleotide polymorphisms and functional analysis of upstream haplotype variants. *Mol. Pharmacol.* **2005**, *68*, 383–392.
38. Miao, J.; Ling, A.V.; Manthena, P.V.; Gearing, M.E.; Graham, M.J.; Crooke, R.M.; Croce, K.J.; Esquejo, R.M.; Clish, C.B.; Torrecilla, E.; et al. Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis. *Nat. Commun.* **2015**, *6*, 6498, doi:10.1038/ncomms7498.
39. Bennett, B.J.; Vallim, T.Q.A.; Wang, Z.; Shih, D.M.; Meng, Y.; Gregory, J.; Allayee, H.; Lee, R.; Graham, M.; Crooke, R.; et al. Trimethylamine-N-Oxide, a Metabolite Associated with Atherosclerosis, Exhibits Complex Genetic and Dietary Regulation. *Changes* **2012**, *29*, 997–1003.
40. Tang, W.H.W.; Wang, Z.; Levison, B.S.; Koeth, R.A.; Britt, E.B.; Fu, X.; Wu, Y.; Hazen, S.L. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* **2013**, *368*, 1575–1584.

41. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* **2013**, *19*, 576–585.
42. Warrier, M.; Shih, D.M.; Burrows, A.C.; Ferguson, D.; Gromovsky, A.D.; Brown, A.L.; Marshall, S.; McDaniel, A.; Schugar, R.C.; Wang, Z.; et al. The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. *Cell Rep.* **2015**, *10*, 326–338.
43. Cho, C.E.; Taesuwan, S.; Malysheva, O.V.; Bender, E.; Yan, J.; Caudill, M.A. Choline and one-carbon metabolite response to egg, beef and fish among healthy young men: A short-term randomized clinical study. *Clin. Nutr. Exp.* **2016**, *10*, 1–11.
44. PB|CTL1–5 (Plasma Membrane). Available online: http://www.reactome.org/PathwayBrowser/#/R-HSA-425366&SEL=R-HSA-444452 (accessed on 14 December 2016).
45. SLC44A1; Choline Transporter-Like Protein 1. Available online: https://www.nextprot.org/entry/NX_Q8WWI5/ (accessed on 14 December 2016).
46. Traiffort, E.; O'Regan, S.; Ruat, M. The choline transporter-like family SLC44: Properties and roles in human diseases. *Mol. Asp. Med.* **2013**, *34*, 646–654.
47. Michel, V.; Singh, R.K.; Bakovic, M. The impact of choline availability on muscle lipid metabolism. *Food Funct.* **2011**, *2*, 53–62.
48. R Core Team. *R: A Language and Environment for Statistical Computing, 2014*; R Foundation for Statistical Computing: Vienna, Austria, 2016.

CHAPTER 3

Vitamin D Binding Protein rs7041 Genotype Alters Vitamin D Metabolism in Pregnant Women[*#]

Abstract

Vitamin D inadequacy is highly prevalent among pregnant and lactating women worldwide. Previous research has identified reduced circulating 25(OH)D and DBP in individuals with the *GC* rs7041 (c.1296T>G) T allele in the vitamin D binding protein gene (i.e. *GC*). We aimed to further characterize the effects of *GC* rs7041 on vitamin D metabolism and to examine whether this genotype modulates the metabolic changes that occur during pregnancy and lactation. In a controlled feeding study that provided 2 doses of choline, healthy third-trimester pregnant (n=26), lactating (n=28), and non-pregnant (n=21) women consumed a single amount of vitamin D (511 IU/d: 311 IU/d from diet and 200 IU/d as supplemental cholecalciferol) and related nutrients for 10-12 weeks. Vitamin D biomarkers were measured in blood and placenta, and osteocalcin, a biomarker of bone formation, was measured in umbilical cord blood. Participants were genotyped for *GC* rs7041 (GG n=18, GT n=36, TT n=20). We found lower serum 25(OH)D among GT (85% of GG, $p = 0.1$) and TT (80% of GG, $p = 0.05$) individuals. In addition, while GG pregnant women exhibited greater DBP (216%, $p<0.0001$) than GG non-pregnant women, among GT women, this difference was dampened (157%, $p = 0.001$), and TT pregnant women did not exhibit greater DBP than TT

[*]Ganz[#], Park[#] et al. A common genetic variant in vitamin D binding protein GC rs7041 alters metabolism and the metabolic response to pregnancy. (Planned Submission July 2017)

[#] Co-first authorship

non-pregnant women (p = 0.9). Furthermore, unlike GG pregnant women (140% of GG non-pregnant women), TT pregnant women did not exhibit greater placental $25(OH)D_3$:$24,25(OH)_2D_3$ ratios than TT non-pregnant and exhibited lower osteocalcin in the cord blood of their neonates (24% of GT, p=0.02). Collectively these data reveal that the *GC* rs7041 genotype modifies the effects of pregnancy on maternal and placental vitamin D metabolism, with possible functional consequences for fetal bone development and infant health.

Introduction

Mounting evidence suggests that vitamin D inadequacy during pregnancy and lactation is associated with adverse maternal, fetal, and infant bone health outcomes, as well as other adverse maternal outcomes such as preeclampsia, gestational diabetes, obstructed labor, and infectious disease.[1,2] There have also been reports of adverse fetal outcomes including aberrations in growth and developmental programming, as well as adverse infant health outcomes.[1,2] Despite increasing awareness of these health risks, vitamin D inadequacy, defined as serum 25-hydroxyvitamin D [25(OH)D] levels less than 50 nmol/L by the National Academy of Medicine, remains highly prevalent among pregnant and lactating women, with estimates ranging from 30-96% in different regions worldwide.[3]

Serum 25(OH)D concentrations can be influenced by many factors including UV exposure, seasonal variation, dietary vitamin D intake, BMI, age, and race/ethnicity. Genetic factors may also affect serum 25(OH)D levels, and several genome-wide studies have demonstrated that common genetic variants in vitamin D metabolic components are significantly associated with serum 25(OH)D concentrations.[4,5] Thus, it is of interest to

ascertain whether genetic factors modify alterations in vitamin D metabolism that occur during pregnancy and lactation, or contribute to individual differences in vitamin D requirements in these reproductive states.

GC rs7041 c.1296T>G is a single nucleotide polymorphism in the vitamin D binding protein (DBP) gene that encodes an aspartate to glutamate amino acid change that alters the affinity of DBP for 25(OH)D. The T allele of this genotype has been associated with lower serum 25(OH)D in some but not all studies.[4,6–9] Although previous studies have identified differences in circulating 25(OH)D by rs7041 genotype in non-pregnant individuals, the effect of the variant on vitamin D biomarkers including 25(OH)D in pregnancy and lactation has not been extensively examined.[10]

Taking advantage of a highly controlled long-term feeding study previously conducted by our research group,[11] the present study sought to evaluate the effects of the *GC* rs7041 variant on vitamin D metabolism in pregnant, lactating, and non-pregnant/non-lactating women consuming vitamin D intakes approaching the 600 IU RDA.

Participants and Methods

A. Participants and Study Design

As part of a 10-12 week controlled feeding study designed to assess the effect of increased choline intake during pregnancy and lactation, healthy third-trimester pregnant women (n=26), lactating women 5 weeks post-partum (n=28), and non-pregnant women (n=21) were randomized to either 480 mg choline/d or 930 mg choline/d. Throughout the controlled feeding period, all participants consumed identical intakes of vitamin D (511 +/- 48 IU/d: 311 +/- 48 IU/d from diet and 200 IU/d as supplemental cholecalciferol) and related nutrients (1.6

+/- 0.4 g Ca/d and 1.9 +/- 0.3 g P/d). Details on the study protocol and diet can be found in our previous publication.[11] The study was approved by Institutional Review Boards at Cornell University (Ithaca, NY, USA) and Cayuga Medical Center (Ithaca, NY, USA) and registered at clinicaltrials.gov as NCT01127022 and NCT03051867[11–13] All participants provided informed consent.

B. Sample Collection and Processing

Fasting blood samples (10 h) were collected at study week 10 into a serum separator tube (used for serum vitamin D metabolite quantification) and EDTA-coated tubes. Samples were placed on ice immediately, and EDTA tubes were centrifuged (2200 rpm, 15 min, 4°C) to separate plasma (used for quantification of vitamin D metabolites) from buffy coat (used for genotyping). Samples were stored at -80°C until use. Placentas (n=24) were collected and processed within 90 minutes of delivery, as previously described.[11] Fetal cord blood samples (n = 23) were collected at delivery into EDTA-coated tubes and processed accordingly. Two placentas and three cord blood samples were not obtained because two participants gave birth at home, and one participant gave birth without notifying the research team.[11–13]

C. Genotyping

The commercially available DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) was used to extract DNA from buffy coat samples. Genotyping of GC rs7041 was performed in our facility using Endpoint Genotyping on a LightCycler 480 (Roche, Indianapolis, IN, USA) and a commercially available mix (Applied Biosystems TaqMan Genotyping Master Mix and Assay Mix; Thermo Fisher Scientific, Waltham, MA, USA). Samples were run in duplicate with in-run standards and a negative water control. Cycling conditions were as follows: 95°C

for 10 min, followed by 34 cycles of 92°C for 15 s and 62°C for 90 s.

D. Quantification of Vitamin D Metabolites

25(OH)D, 3-epi 25(OH)D_3, & 24,25$(OH)_2$D

Serum 25(OH)D (the sum of 25(OH)D_2 and 25(OH)D_3), serum 3-epi 25(OH)D_3, and plasma 24,25$(OH)_2$D were quantified using an isotope dilution LCMS/MS method with modifications based on our instrumentation, as previously described.[13–15] This same methodology was used for placental vitamin D metabolites. Assay precision and accuracy were assessed using the National Institute of Standards and Technology SRM and the Vitamin D External Quality Assessment Scheme.

1,25$(OH)_2$D & DBP

ELISA kits were used to measure plasma 1,25$(OH)_2$D (Immunodiagnostic Systems, Inc., Scottsdale, AZ) and DBP (R&D Systems, Minneapolis, MN).

Free 25(OH)D

Free 25(OH)D was estimated using an equation as previously described.[13]

25(OH)D_3 and 24,25$(OH)_2D_3$

Placental 25(OH)D_3 and 24,25$(OH)_2D_3$ were extracted from homogenized placental tissues as previously described (Park et al., submitted 2017). 1mL of methanol was added to 0.4 g of homogenized placental tissues along with 600µL of acetonitrile, 1mL of MTBE, and 25µL of internal standard containing 215mol/L d_3-25(OH)D_3 (IsoSciences) and 1.7mol/L d6-24,25(OH)2D3 (Toronto Research Chemicals Inc.) A liquid-liquid extraction was followed by a solid-phase extraction on Oasis HLB cartridges (3cc/60mg), and subsequent derivatization with 50µL of 0.1mg/mL DMEQ-TAD.[16,17] Finally, extracts were resuspended in 110µL of

60:40 methanol:water and injected onto a PFP column (PFP Accucore 2.1 x 100, 2.6 mm) with matching guard column at 45°C, and separated by our LC-MS/MS, a Surveyer HPLC system (pump and autosampler) and a TSQ Quantum Ultra mass spectrometer, operated with XCalibur (2.2 SP1.48) software (ThermoElectron Corp).[12]

Biochemical marker of bone formation in neonates (cord blood)

Osteocalcin (OC) was measured in cord plasma obtained at delivery using an ELISA kit (R&D Systems, Minneapolis, MN).[12]

E. Statistical Analysis

The effect of GC rs7041 genotype on vitamin D metabolism was assessed using linear models. Vitamin D metabolites in blood and placenta were modeled as a function of rs7041 genotype. Reproductive status, BMI, season, and ethnicity were included as covariates in the blood metabolite models, and age was considered as a co-variate and retained in the model at an α-cutoff of 0.05. Choline intake level was considered as a covariate but was not significant in any model. We used a two-level ethnicity/race outcome, white and non-white (including Latino, Black, Asian, and mixed race/other), due to the sparsity of non-white participants, though grouping these ethnicities did not change our results. Interactions between genotype and reproductive state were retained at an α-cutoff of 0.1. Analysis with and without ethnicity/race in the model yielded the same results. Given that little is known about the factors that influence vitamin D outcomes in the placenta and umbilical cord, the change method was used to identify co-variates that influenced outcomes and to construct placental and umbilical cord blood models. BMI, season, ethnicity (white and non-white), age, infant sex, parity, and vitamin supplementation status at baseline were evaluated as possible co-

variates by examining one at time whether they changed the estimate of genotype by more than ten percent as compared to a bivariate model of genotype with the outcome of interest. Co-variates that passed this test were assembled into an initial model, and a backwards selection was carried out where the co-variate with the highest p-value was eliminated until all co-variates included in the model had a p-value less than or equal to 0.2. Standard diagnostic methods were used to assess model assumptions and the fit of the models to the data. Some models had outliers and not perfectly normal distributions. $24,25(OH)D_3$ met normality criteria after log transformation. ANOVA was determined to be robust enough to deal with all other models, and assessment of models that had outliers without their outliers yielded normal models with the same results qualitatively in terms of significance. Statistical analysis was performed using the lsmeans package in The R Project for Statistical Computing environment, available from Comprehensive R Archive Network (CRAN) 2014.[18] T-tests were used for pairwise comparisons, and reported p-values include adjustments for multiple comparisons using the Tukey method and were considered significant at an α-cutoff of 0.05.

Results

A. Characteristics of the participants

Baseline characteristics by genotype are displayed in ***Table 1****.*

Table 1. Baseline Characteristics

	All n=74	GG n=18	GT n=36	TT n=20
BMI, kg/m^2	$24.8^{+/-5.1}$	$23.3^{+/-3.4}$	$26.1^{+/-6.1}$	$23.9^{+/-3.7}$
Ethnicity, *n. (%)*				
White	49 (66%)	13 (72%)	23 (64%)	13 (65%)
Black	4 (5%)	1 (6%)	1 (3%)	2 (10%)
Latina	9 (12%)	3 (17%)	4 (11%)	2 (10%)
Asian	6 (8%)	1 (6%)	2 (6%)	3 (15%)
Other	6 (8%)	0 (0%)	6 (17%)	0 (0%)
Season at study entry, *n.*				
April-September	41	10	19	12
October-March	33	8	17	8
Age, *y.*	$28.4^{+/-5.6}$	$30.3^{+/-5.2}$	$27.1^{+/-6.2}$	$29.1^{+/-4.0}$
Non-pregnant Women, *n.*	21	6	9	6
Lactating Women, *n.*	27	3	16	8
Pregnant Women, *n.*	26	9	11	6
Parity, *primiparas/multiparas*	12/14	5/4	3/8	4/2
Infant sex, *male/female*	19/7	6/3	7/4	6/0
Multivitamin supplement use before study entry, *n.*	49/74	10/18	26/36	13/20

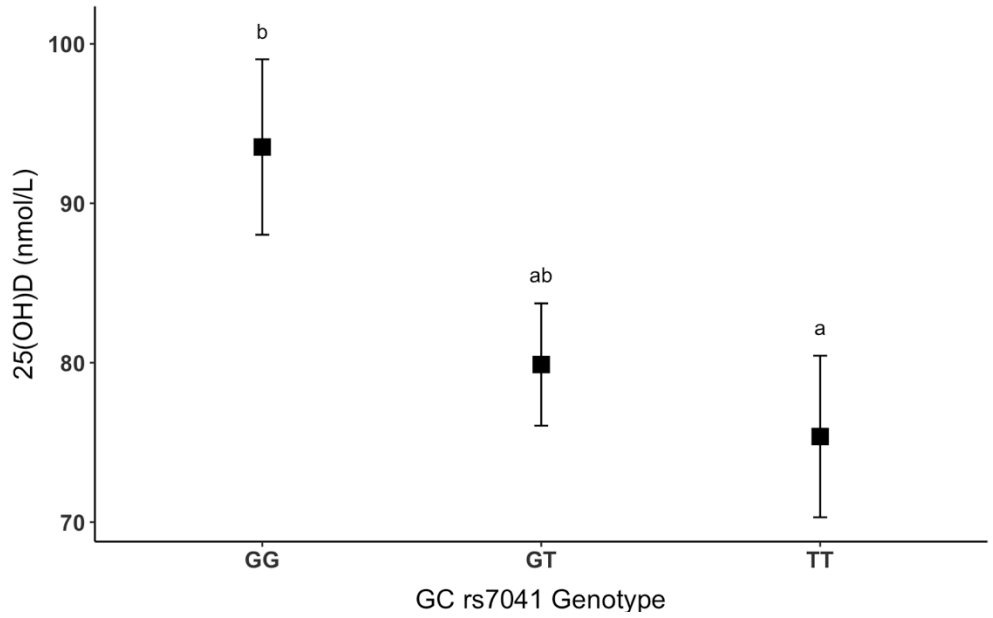
Notes: Pregnant women self-reported pre-pregnancy BMIs. Averages are reported +/- standard deviations
Abbreviations: y, year; n, number.

B. 25(OH)D

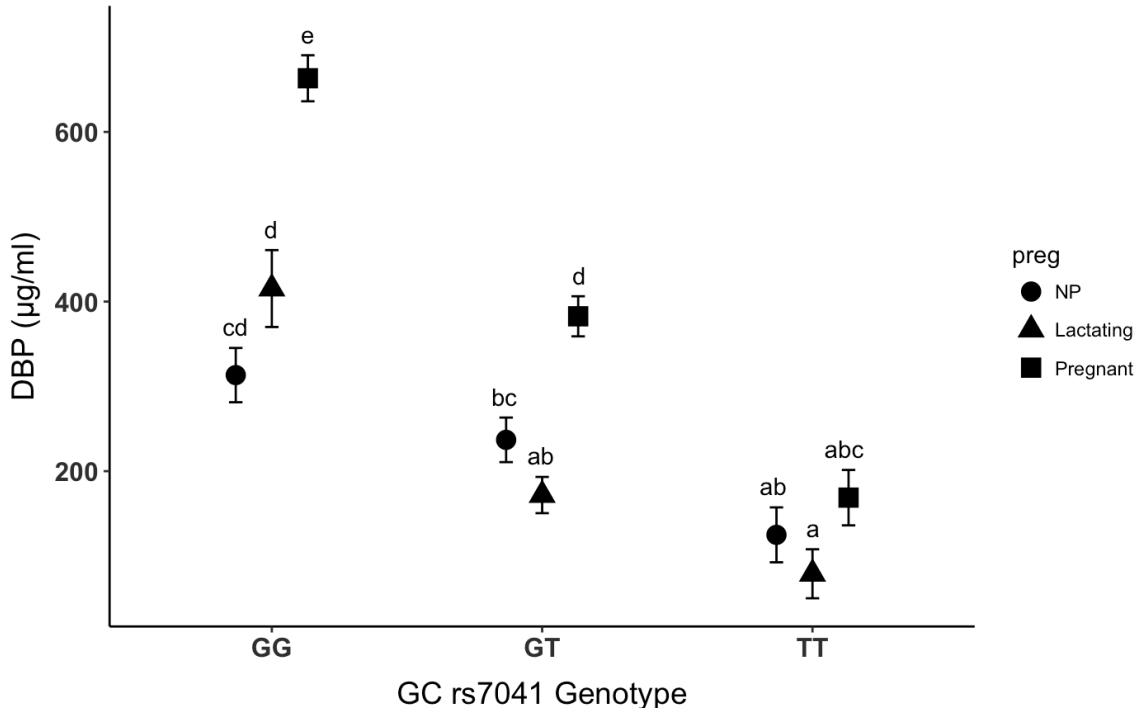
In line with previous findings, GC rs7041 genotype predicted total 25(OH)D_{2+3}, main effect, p = 0.04. The effect of genotype on 25OHD was independent of reproductive state. Individuals with one or two copies of the T allele tended to exhibit lower serum 25OHD_{2+3}. The rs7041 GG genotype had a total 25(OH)D level of $93.5^{+/- 5.5}$ nmol/L, vs. heterozygotes, GT, at $79.9^{+/- 3.8}$ nmol/L (p = 0.1) and homozygous TT, at $75.4^{+/- 5.1}$ nmol/L (p = 0.04) (**Figure 1a, Table 2**).

This effect of genotype on 25(OH)D was most prominent in the D_3 component, main effect, p = 0.07. Individuals with one or two copies of the T allele tended to exhibit lower serum 25(OH)D_3. rs7041 GG individuals had a level of $91.8^{+/-5.5}$ nmol/L, vs. GT heterozygotes at $78.5^{+/-3.8}$ nmol/L (p = 0.1) and homozygous TT at $74.2^{+/-5.1}$ nmol/L (p = 0.05) (**Supplemental Figure 1b, Table 2**). Although rs7041 genotype did not significantly predict serum 25(OH)D_2, p = 0.5, women with the T allele did exhibit non-significantly lower levels **(Supplemental Figure 1a, Table 2**).

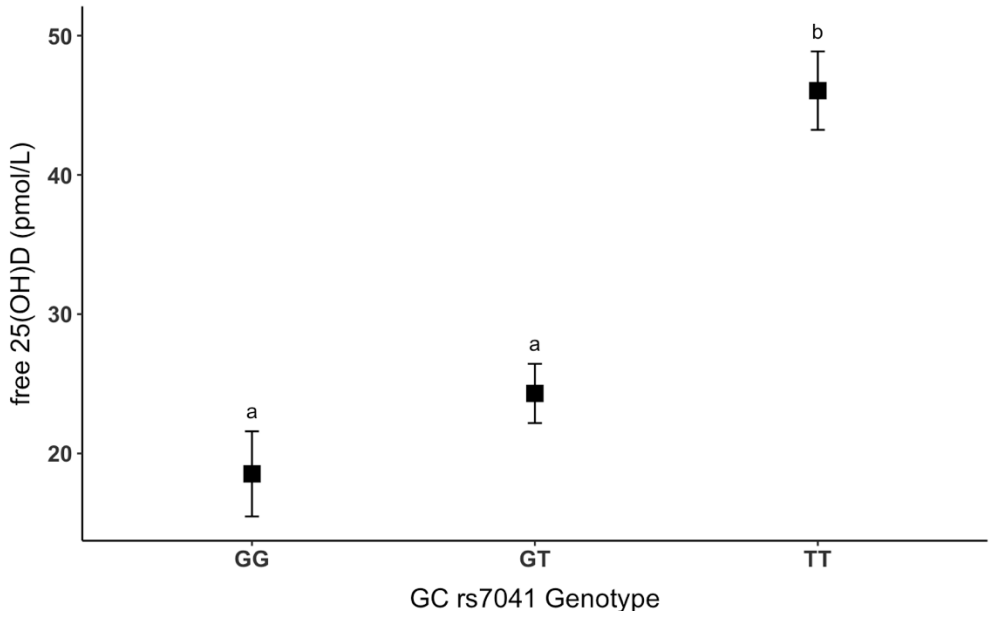
(a)



(b)


DBP (µg/ml)
600
400
200
e
d
cd
d
bc
ab
ab
a
abc
GG
GT
TT
GC rs7041 Genotype
preg
NP
Lactating
Pregnant

(c)


free 25(OH)D (pmol/L)
50
40
30
20
b
a
a
GG
GT
TT
GC rs7041 Genotype

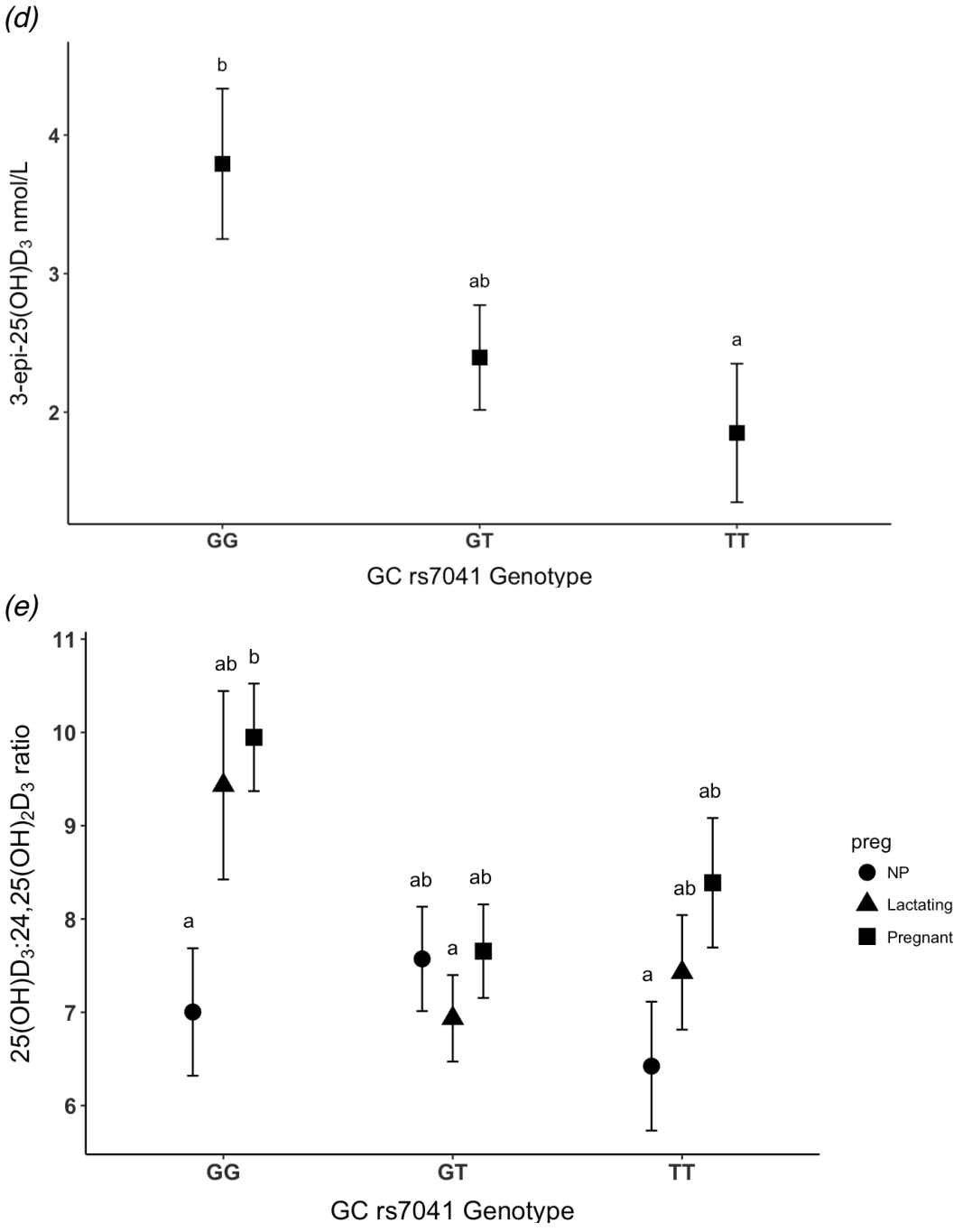


Figure 1. Effect of the *GC* rs7041 genotype on circulating maternal vitamin D metabolites at study week 10. Total 25(OH)D was calculated as the sum of $25(OH)D_2$ and $25(OH)D_3$. Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group: GG (n=18), GT (n=36), TT (n=20). Groups that are not labelled with any of the same letters are significantly different at an α-cutoff of 0.05. DBP and the $25(OH)D_3$:$24,25(OH)_2D_3$ ratio exhibited interactions between *GC* rs7041 genotype and reproductive state. Number of participants in each group for these two outcomes: Lactating (GG n=3, GT n=16, TT n=8); Non-Pregnant (GG n=6, GT n=9, TT n=6), Pregnant (GG n=9, GT n=11, TT n=6).

Table 2. Circulating vitamin D metabolites among pregnant, lactating, and non-pregnant women (combined) at study week 10

	GG	**GT**	**TT**	p-value GG vs. GT	p-value GG vs. TT	p-value GT vs. TT
Total 25(OH)D, *nmol/L* *Main effect; p = 0.04*	$93.5^{+/- 5.5}$	$79.9^{+/- 3.8}$	$75.4^{+/- 5.1}$	0.1	0.05	0.8
25(OH)D_2, *nmol/L* *Main effect; p = 0.5*	$1.7^{+/-0.4}$	$1.4^{+/-0.2}$	$1.2^{+/-0.3}$	0.8	0.5	0.8
25(OH)D_3, *nmol/L* *Main effect; p = 0.05*	$91.8^{+/-5.5}$	$78.5^{+/-3.8}$	$74.2^{+/-5.1}$	0.1	0.05	0.8
DBP, *µg/ml* *Genotype x RS interaction; p = 0.0002*						
Non-Pregnant	$313^{+/-32}$	$237^{+/-26}$	$125^{+/-32}$	0.6	0.003	0.2
Lactating	$415^{+/-45}$	$172^{+/-21}$	$79^{+/-29}$	0.0004	<0.0001	0.2
p-value vs. Non-Pregnant	0.7	0.6	0.97			
Pregnant	$663^{+/-27}$	$383^{+/-24}$	$169^{+/-33}$	<0.0001	<0.0001	0.0001
p-value vs. Non-Pregnant	<0.0001	0.003	0.99			
Free 25(OH)$D_{2,3}$, *pmol/L* *Main effect; p = $2x10^{-9}$*	$18.5^{+/-3.1}$	$24.3^{+/-2.1}$	$46.0^{+/-2.8}$	0.3	<0.0001	<0.0001
3-epi-25(OH)D_3, *nmol/L* *Main effect; p = 0.03*	$3.8^{+/-0.5}$	$2.4^{+/-0.4}$	$1.9^{+/-0.5}$	0.09	0.02	0.7
25(OH)D_3: *24,25(OH)$_2D_3$* Ratio *Genotype x RS interaction; p = 0.1*						
Non-Pregnant	$7.0^{+/-0.7}$	$7.6^{+/-0.6}$	$6.4^{+/-0.7}$	>0.99	>0.99	0.9
Lactating	$9.4^{+/-1.0}$	$6.9^{+/-0.5}$	$7.4^{+/-0.6}$	0.4	0.7	>0.99
p-value vs. Non-Pregnant	0.5	0.99	0.97			
Pregnant	$9.9^{+/-0.6}$	$7.7^{+/-0.5}$	$8.4^{+/-0.7}$	0.07	0.8	0.99
p-value vs. Non-Pregnant	0.04	>0.99	0.6			
1,25(OH)$_2D_3$	$208^{+/-29}$	$228^{+/-21}$	$204^{+/-26}$	0.8	0.99	0.8
24,25(OH)$_2D_3$	$10.2^{+/-1.3}$	$10.1^{+/-1.0}$	$10.2^{+/-1.2}$	0.99	>0.99	0.99

Notes: Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group: GG (n=18), GT (n=36), TT (n=20). Total 25(OH)D was calculated as the sum of 25(OH)D_2 and

$25(OH)D_3$. No significant interaction was detected between genotype and reproductive state for each vitamin D metabolite unless otherwise indicated. Study week 10 DBP and $25(OH)D_3$:$24,25(OH)_2D_3$ ratios exhibited interactions between *GC* rs7041 genotype and reproductive state and comparisons are also presented within genotypes across reproductive states. Number of participants in each group for these two outcomes: Lactating (GG n=3, GT n=16, TT n=8); Non-Pregnant (GG n=6, GT n=9, TT n=6), Pregnant (GG n=9, GT n=11, TT n=6).
Abbreviations: DBP, vitamin D binding protein; 3-epi-$25(OH)D_3$, C3 epimer of 25-hydroxyvitamin D_3; Free 25(OH)D, free 25-hydroxyvitamin D; $25(OH)D_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

C. DBP

The rs7041 polymorphism influenced DBP levels in our cohort in a manner that depended upon reproductive state (RS x genotype interaction, $p = 0.0002$).

Within each reproductive state, the T allele was associated with lower levels of DBP ($p<0.003$) (**Table 2**), with the exception of heterozygous non-pregnant women, who were not significantly different than GG non-pregnant women. Furthermore, the difference in DBP associated with pregnancy varied by rs7041 genotypes. Among the women with the GG genotype, GG pregnant women exhibited more than 2x greater DBP than GG non-pregnant women ($663^{+/-27}$μg/mL vs. $313^{+/-32}$ μg/mL, $p < 0.0001$). However, this difference was dampened among the GT heterozygotes to roughly 1.6x greater DBP levels in GT pregnant women than GT non-pregnant women ($383^{+/-24}$ μg/mL vs. $237^{+/-26}$ μg/mL, $p = 0.003$). Moreover, no difference in DBP was observed between homozygous TT pregnant women and non-pregnant TT women ($169^{+/-33}$ μg/mL vs. $125^{+/-32}$ μg/mL, $p = 0.99$), suggesting that TT women do not increase DBP during pregnancy (**Figure 1b, Table 2**).

D. Free 25(OH)D

GC rs7041 genotype was a significant predictor of free circulating 25(OH)D, $p = 2 \times 10^{-9}$.

This effect was independent of reproductive state. Individuals with the TT genotype exhibited greater levels ($46.0^{+/-2.8}$ pmol/L) of free 25(OH)D than those with the GG ($18.5^{+/-3.1}$ pmol/L, $p < 0.0001$) and GT genotypes ($24.3^{+/-2.1}$ pmol/L, $p < 0.0001$) (**Figure 1c, Table 2**).

E. 3-Epi-25(OH)D_3

In addition, genotype predicted 3-epi-25(OH)D_3 ($p = 0.03$). This effect was independent of reproductive state. Additional copies of the T allele were associated with successively lower levels of 3-epi-25(OH)D_3. Women with the GG genotype exhibited levels of $3.8^{+/-0.5}$ nmol/L, which were greater than GT ($2.4^{+/-0.4}$ nmol/L, $p = 0.09$) and TT ($1.9^{+/-0.5}$ nmol/L, $p = 0.02$) individuals (**Figure 1d, Table 2**).

F. 25(OH)D_3:24,25$(OH)_2D_3$ ratio

A genotype by reproductive state interaction tended to predict the 25(OH)D_3: 24,25$(OH)_2D_3$ ratio ($p = 0.1$). Genotypic differences were not observed among non-pregnant and lactating women. However, among pregnant women, GG individuals tended to exhibit greater 25(OH)D_3:24,25$(OH)_2D_3$ ratios than those with the GT genotype ($9.9^{+/-0.6}$ vs. $7.7^{+/-0.5}$, $p = 0.07$) **(Figure 1e, Table 2)**.

In addition, the metabolic response to pregnancy appeared to differ between genotypes. Among GG individuals, pregnant women tended to exhibit greater 25(OH)D_3:24,25$(OH)_2D_3$ ratios than non-pregnant women. GG pregnant women displayed 25(OH)D_3:24,25$(OH)_2D_3$ ratios of ($9.9^{+/-0.6}$, $p = 0.04$), which were significantly greater, as compared to non-pregnant GG women ($7.0^{+/-0.7}$, $p = 0.04$).

In contrast, pregnant women with the GT and TT genotypes did not exhibit significantly greater 25(OH)D_3:24,25$(OH)_2D_3$ ratios than non-pregnant women with the same

genotype. (**Figure 1e, Table 2**).

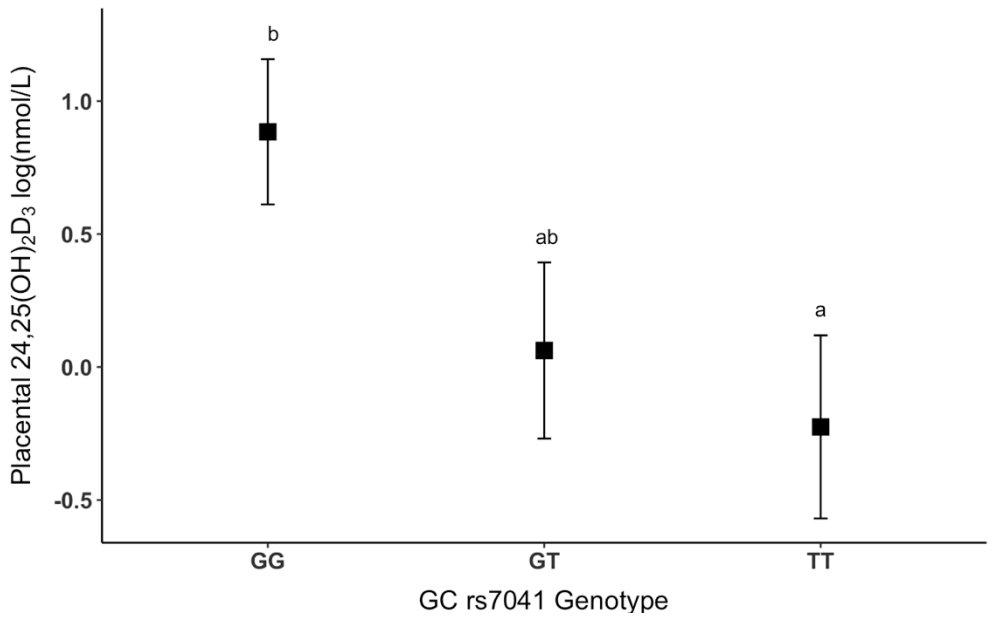
G. 1-25$(OH)_2$D and 24,25$(OH)_2D_3$

Genotypic differences in 1-25$(OH)_2$D and *24,25$(OH)_2D_3$* were not detected (**Table 2**).

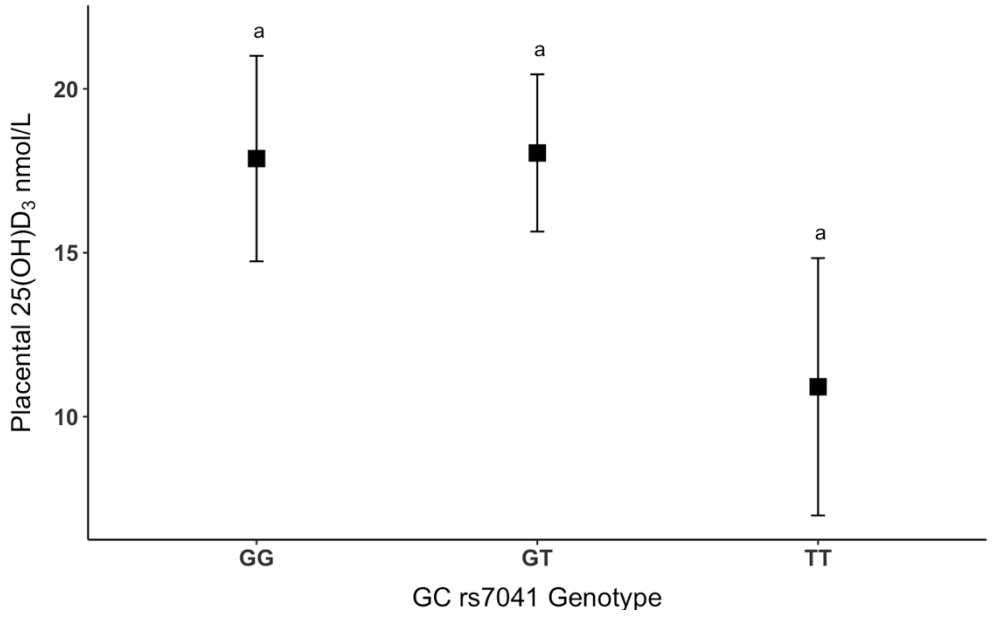
H. Placental 24,25$(OH)_2D_3$

In the placenta, genotype predicted 24,25$(OH)_2D_3$ (p = 0.04). Additional copies of the T allele were associated with lower levels of 24,25$(OH)_2D_3$. Women with the GG genotype exhibited levels of $0.88^{+/-0.27}$ log(nmol/L), which were greater than GT ($0.06^{+/-0.33}$ log(nmol/L), p = 0.1) and TT ($-0.23^{+/-0.34}$ log(nmol/L), p = 0.04) individuals (**Figure 2a, Table 3**).

(a)



(b)



(c)

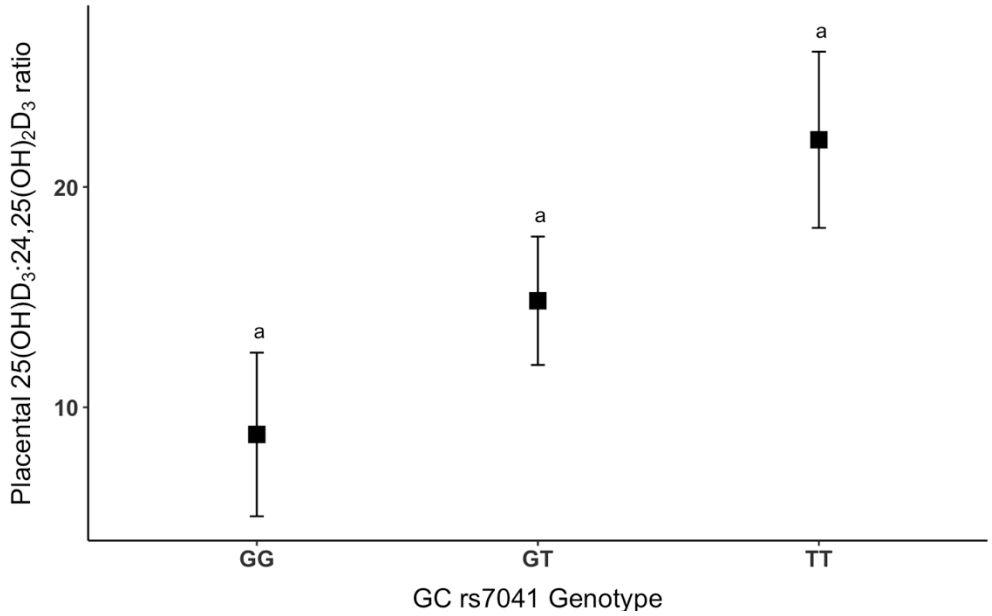


Figure 2. Effect of *GC* rs7041 genotype on placental vitamin D metabolites. Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group: GG (n=8), GT (n=10), TT (n=6). Groups that are not labelled with any of the same letters are significantly different at an α-cutoff of 0.05

I. Placental $25(OH)D_3$

Genotypic differences in placental $25(OH)D_3$ were not detected. However, homozygous TT individuals exhibited non-significantly lower placental $25(OH)D_3$ (**Figure 2b, Table 3**).

J. Placental $25(OH)D_3$/Placental $24,25(OH)_2D_3$ Ratio

Genotype predicted the $25(OH)D_3$: $24,25(OH)_2D$ ratio ($p = 0.02$). Individuals with the homozygous TT genotype tended to exhibit greater placental $25(OH)D_3$:$24,25(OH)_2D_3$ ratios than those with the GG genotype ($22.1^{+/-4.0}$ vs. $8.8^{+/-3.7}$, $p = 0.06$) (**Figure 2c, Table 3**).

Table 3. Placental vitamin D metabolites and biochemical marker of bone formation in neonatal cord blood at delivery.

	GG	**GT**	**TT**	p-value GG vs. GT	p-value GG vs. TT	p-value GT vs. TT
Placental $24,25(OH)_2D_3$, *log(nmol/L)* *Main effect; p = 0.04*	$0.88^{+/- 0.27}$	$0.62^{+/- 0.33}$	$-0.24^{+/- 0.34}$	0.1	0.04	0.8
Placental 25(OH)D_3, nmol/L *Main effect; p = 0.8*	$17.9^{+/-3.1}$	$18.0^{+/-2.4}$	$10.9^{+/- 3.9}$	>0.99	0.4	0.3
Placental $25(OH)D_3$: *$24,25(OH)_2D_3$* Ratio *Main effect; p = 0.02*	$8.8^{+/-3.7}$	$14.8^{+/-2.9}$	$22.1^{+/-4.0}$	0.3	0.07	0.4
Umbilical cord plasma OC *ng/ml* *Main effect; p = 0.02*	$49.0^{+/-12.3}$	$79.4^{+/-11.9}$	$18.8^{+/-15.3}$	0.2	0.3	0.02

Notes: Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group for: placental outcomes GG (n=8), GT (n=10), TT (n=6). Number of participants in each group for cord blood plasma OC: GG (n=8), GT (n=8), TT (n=6).
Abbreviations: $25(OH)D_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25 dihydroxyvitamin D_3, OC, osteocalcin.

K. Cord OC

GC rs7041 genotype predicted cord OC (p = 0.02). GG individuals had levels of $49.0^{+/-12.3}$ ng/ml, GT individuals had $79.4^{+/-11.9}$ ng/ml, and TT individuals had $18.8^{+/-15.3}$ ng/ml (p = 0.02 as compared to GT) (**Figure 3, Table 3**).

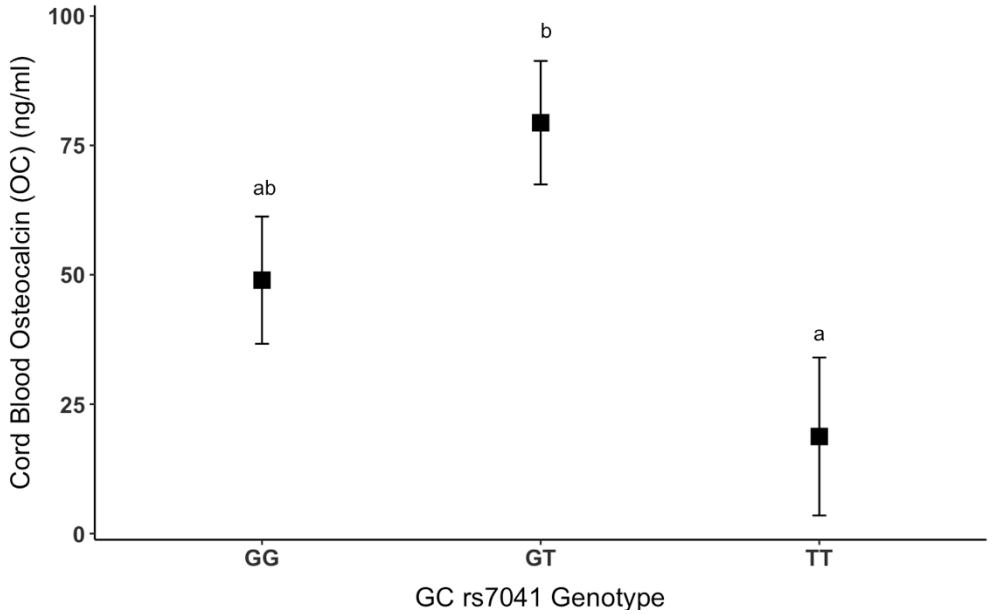


Figure 3. Effect of *GC* rs7041 genotype on cord blood osteocalcin. Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group: GG (n=8), GT (n=8), TT (n=6). Groups that are not labelled with any of the same letters are significantly different at an α-cutoff of 0.05.

Discussion

This study evaluated the effects of the *GC* rs7041 variant on vitamin D metabolism in pregnant, lactating, and non-pregnant/non-lactating women consuming vitamin D intakes approaching the RDA. Three main findings emerged: (i) the GC rs7041 alters vitamin D metabolites in pregnant, lactating, and non-pregnant women, (ii) the GC rs7041 T allele is associated with an altered metabolic response to pregnancy, and altered placental vitamin D

metabolism, and (iii) neonates of women with the TT genotype exhibited lower levels of OC, a biochemical marker of bone formation.

A. The GC rs7041 alters vitamin D metabolism in pregnant, lactating, and non-pregnant women

Within our cohort, the *GC* rs7041 T allele was associated with significantly lower serum total 25(OH)D, independent of reproductive state. Specifically, we observed lower serum 25(OH)D among heterozygous GT (85% of GG, $p = 0.1$) and homozygous TT (80% of GG, $p = 0.05$) individuals across reproductive states (**Figure 1a, Table 2**). This finding confirms previous reports of lower levels of 25(OH)D in non-pregnant women with the rs7041 T allele[4,6–9], and expands on previous results to include pregnant and lactating women. Serum 25(OH)D is the primary indicator of vitamin D status and women with the rs7041 T allele may be at a greater risk for poor vitamin D status as compared to women with the G allele. Within our cohort, this genotypic effect appeared to be driven by $25(OH)D_3$ as we did not detect significant differences in $25(OH)D_2$ (**Supplemental Figure 1, Table 2**). This is not surprising in our cohort given that the study diet provided cholecalciferol (vitamin D_3) as a dietary supplement, which comprised the major source of vitamin D for these women. We also observed lower levels of 3-epi-$25(OH)D_3$ in GT (63% of GG, $p = 0.09$) and TT women (50% of GG, $p = 0.02$), consistent with lower 25(OH)D (**Figure 1d, Table 2**).

In addition, we observed lower DBP concentrations among individuals with the rs7041 T allele. Non-pregnant TT women displayed lower levels of DBP than non-pregnant GG women; lactating GT and TT women displayed lower levels than lactating GG women; and pregnant GT and TT women displayed lower DBP levels that pregnant GG women.

(**Figure 1b, Table 2**).

Finally, homozygous TT women exhibited higher free 25(OH)D levels, 1.9x GT women and 2.5x GG women (**Figure 1d, Table 2**). Along with the evidence of lower total 25(OH)D concentrations (comprised of free and DBP-bound forms) in TT women, this data supports lower levels of DBP-bound 25(OH)D among women with the variant T allele. These findings also appear to align with *in vitro* studies that report differential affinities of 25(OH)D for different DBP isoforms.[9,19]

B. The GC rs7041 T allele is associated with an altered metabolic response to pregnancy

Women with the *GC* rs7041 T allele appear to have a distinct metabolic response to pregnancy as compared to women with the G allele. Previously, DBP levels have been reported to increase by 7-152% during pregnancy.[2,20–22] Within our cohort, the increase in DBP during pregnancy appeared to differ by rs7041 genotype. Specifically, GG pregnant women in our cohort exhibited 2.1x higher plasma DBP than GG non-pregnant women. However, this pregnancy-associated increase in DBP appeared to be dampened among heterozygotes, as GT pregnant women exhibited only 1.6x higher plasma DBP than GT non-pregnant women, and differences in DBP were not detected between TT pregnant and TT non-pregnant women. Overall, these data suggest that the DBP metabolism is modulated by the rs7041 genotype during pregnancy, with no increase in TT pregnant women (**Figure 1b, Table 2**). A differential increase in DBP during pregnancy by rs7041 genotype may account for some of the variability between previous reports of increases in DBP during pregnancy. This result should be confirmed in a longitudinal study that compares DBP levels during pregnancy to either pre- or post-pregnancy levels in the same individual. Although it is not

clear why TT women fail to increase DBP levels during pregnancy, the finding indicates that TT women have a different metabolic response to pregnancy than GG women, and may also differ in the mechanism by which they source vitamin D to the placenta.

The mechanism by which vitamin D enters the placenta is currently unknown, however, there are several possibilities. Placental uptake of vitamin D metabolites could occur preferentially via megalin-cubilin receptors, by simple diffusion in the free form, or in some combination of the two. DBP of maternal origin has been detected in the human placenta (22), providing evidence that the placenta does in fact take up DBP, though is not known whether this constitutes the major source of placental vitamin D. Moreover, the placental membrane expresses megalin-cubilin receptors (23,24) (the same receptors that the kidney uses to internalize DBP-bound 25(OH)D), as well as all the necessary components to make the active form of vitamin D (25–27), providing further rationale for placental uptake of DBP-bound 25(OH)D. Among GG individuals, the increase in DBP during pregnancy (a known phenomenon), may transpire to increase the uptake of DBP-bound 25(OH)D by the tissues that express megalin-cubilin receptors, including the placenta. DBP-bound 25(OH)D is more stable and can be taken up across a concentration gradient, and thus may be favored by the placenta. In contrast, among TT women, the lack of increase in DBP, along with the increase in free 25(OH)D, suggests that in an environment with compromised DBP levels, free 25(OH)D may be a more biologically important form of vitamin D for this genotype. Thus, the functional link of vitamin D with DBP, and the predominant mechanism of placental uptake of vitamin D during pregnancy, may differ by *GC* rs7041 genotype. Isotope labelling studies are needed to further investigate this notion.

In addition to the lack of increase in DBP, we also observed differences in the blood

$25(OH)D_3$:$24,25(OH)_2D_3$ ratio that depended upon genotype and reproductive state. While GG pregnant women exhibited higher $25(OH)D_3$:$24,25(OH)_2D_3$ ratios than GG non-pregnant women, we did not observe the same phenomenon in TT pregnant women as compared to TT non-pregnant women **(Figure 1e, Table 2)**. Given that the $25(OH)D_3$:$24,25(OH)_2D_3$ ratio is an emerging status indicator that accounts for inter-individual metabolic differences in catabolizing 25(OH)D to $24,25(OH)_2D$, this finding is a second indication (backing up 25(OH)D data) that pregnant women with the T allele may have lower status, or more aptly may not increase their vitamin D status during pregnancy like GG women. No such differences were detected in the metabolic response to lactation.

C. The GC rs7041 T allele is associated with altered placental vitamin D metabolism

Interestingly, in line with the notion that TT individuals may source vitamin D differently to the placenta, they also exhibited differences in placental vitamin D metabolites. TT individuals exhibited non-significantly lower concentrations of placental $25(OH)D_3$, significantly lower $24,25(OH)_2D_3$, and tended to exhibit greater placental $25(OH)D_3$:$24,25(OH)_2D_3$ ratios as compared to GG individuals (**Figure 2, Table 3**). Substantially higher (nearly 3x GG) placental $25(OH)D_3$:$24,25(OH)_2D_3$ among TT individuals indicates comparatively less vitamin D catabolism relative to 25(OH)D. A reduction in vitamin D catabolism relative to 25(OH)D among TT individuals may transpire to secure the placental 25(OH)D pool as a compensatory response to the overall reduced presence and availability of vitamin D in TT individuals.

D. The GC rs7041 TT genotype is associated with reduced presence of a biochemical marker of bone formation in neonates

Despite high placental $25(OH)D_3$:$24,25(OH)_2D_3$ ratios, the neonates of TT women exhibited lower cord OC as compared to GT individuals (**Figure 3, Table 3)** suggesting that this genetic variant may have functional consequences for fetal bone health and bone development. We did not detect differences between TT and GG individuals. This could be due to a heterozygote effect, or alternatively could represent inadequate power. Notably, our entire population was highly vitamin D adequate. The effect of genotype may be more pronounced among women with vitamin D inadequacy. Interestingly, a recent study reported that lower DBP during the third trimester of pregnancy is associated with an increased risk of type 1 diabetes in offspring. [23] The study did not consider BMI, ethnicity, or *GC* genotypes

and found only a marginal association, but their finding is nonetheless intriguing in the context of our results. We observed no increase in DBP among pregnant women with the TT genotype and found lower osteocalcin in the cord blood of their neonates. Given that osteocalcin is a hormone that regulates glucose metabolism and promotes glucose tolerance, lower cord blood osteocalcin might contribute to the previously observed linkage between low DBP and type I diabetes. Furthermore, the rs7041 TT genotype has been found to modify the association of 25(OH)D levels with diabetes among white Americans but not black Americans. Specifically, among white Americans, low 25(OH)D was associated more strongly with diabetes in individuals with the rs7041 T allele. [24] These findings, together with our findings, suggest that the rs7041 T allele (most likely in the context of the Gc2 isoform of DBP that is the more common form among whites with the T allele) may modify the association between 25(OH)D levels in utero and risk of diabetes in offspring. Further studies are needed to clarify these relationships and determine the effect of the TT genotype and Gc isoforms on diabetes and bone health outcomes in infancy and early childhood.

E. Study Limitations

Concerns have been raised about whether the monoclonal antibody-based assay we used to measure DBP may have distinct immunoreactivity against the different isoforms of DBP that result from different genotypes. (5) Specifically, previous studies that have used this monoclonal assay have found lower DBP in certain *Gc* types, while studies that use polyclonal assays or direct measurement by proteomic methods and mass spectrometry do not find differences by race or Gc type. (30–32) A differential affinity of the assay for the TT genotype as compared to the GG genotype limits the extent to which we can draw conclusions

from comparisons of DBP levels between genotypes. However, it would not be expected to interfere with *comparisons* within genotypes. Importantly, the finding regarding the apparent lack of increase in DBP during pregnancy among TT individuals is based on comparisons between reproductive groups *within* genotypes. In particular, we compared TT pregnant women to TT non-pregnant women. Furthermore, given that *GC* polymorphisms are known to differ across racial and ethnic groups, we considered ethnicity in all our statistical models. We think this method is sufficient for our purposes and unlikely to qualitatively alter our main conclusions for the reasons described above; however, a direct measurement of DBP as well as free 25(OH)D. would have been preferable.

In addition, these findings represent our study population and cannot necessarily be extrapolated to all individuals with rs7041 T allele. Combinations of this rs7041 (c.1296T>G; p.Asp416Glu) polymorphism and an additional SNP, rs4588 (c.1307C>A; p.Thr420Lys) result in three common isoforms of the vitamin D binding protein: Gc1F (rs7041 T and rs4588 C); Gc1S (rs7041 G and rs4588 C); and Gc2 (rs7041 T and rs4588 A). The rs7041 G allele generally accompanies the rs4588 C allele and generates the Gc1S isoform, whereas the rs7041 T allele can generate either the Gc1F or Gc2 isoform, depending on the individual's rs4588 genotype. The prevalence of these isoforms varies across racial groups. The Gc1F isoform is more prevalent among black and Asian populations; the Gc1S isoform is most common in whites, but among whites with the *GC* rs7041 T allele, the Gc2 isoform is more common than the Gc1F isoform. (19,32,33) Given that our study population was primarily white, it is likely that individuals with the T allele primarily had the Gc2 isoform rather than the Gc1f isoform. Because we did not have data for rs4588 (or a large enough sample size to include additional groups), this homogeneity in race/ethnicity helped our analysis by limiting

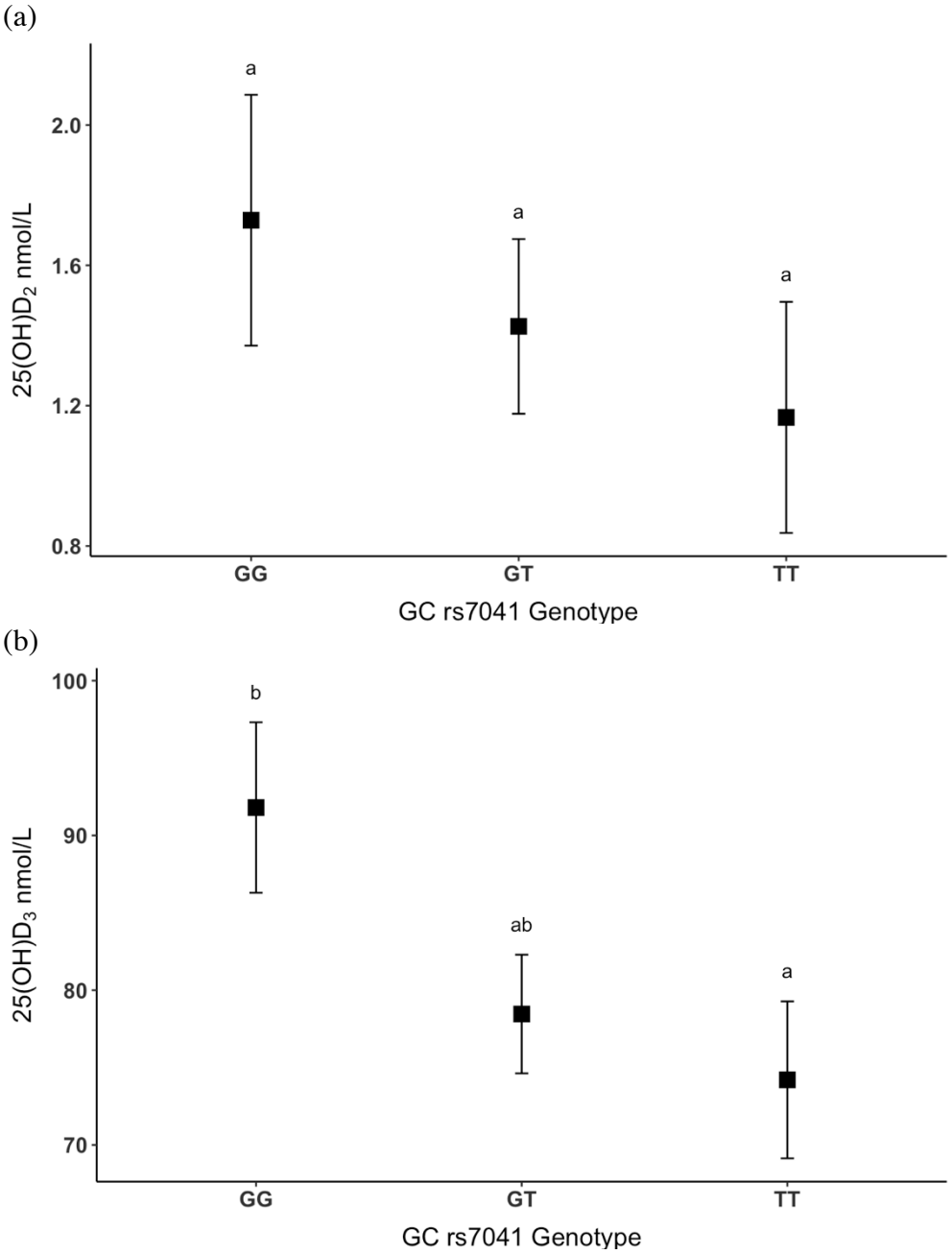
the variability; however, it also limits the extent to which these results can be extrapolated to other Gc types. It is worth mentioning that all TT pregnant women all carried female infants. This was considered in all our statistical models, and infant sex did not have a significant bivariate relationship with any of the placental outcomes or cord OC.

Although the rs7041 T allele appears to be a risk allele in our study and previous studies that have identified lower 25(OH)D or increased risk of diabetes (29), one study by Chun et al. identified a protective effect of the rs7041 T allele during pregnancy. They reported that *GC* rs7041 modifies the effect of low 25(OH)D on infant birth weight. Specifically, low maternal serum 25(OH)D predicted risk of low birth weight among pregnant women carrying the *GC* rs7041 G allele, but not among pregnant women homozygous for the T allele. (34) What is beneficial for one health outcome can be disadvantageous for another, and it is possible that the rs7041 T allele is a risk allele in some disease contexts, and protective in others. It is also pertinent to consider that the rs7041 T allele in the Chun et al. study may be more representative of the Gc1f isoform than the Gc2 isoform. Chun et al. did not report information on Gc types of their participants or their ethnicities, but their study was conducted in a South Korean population, and the Gc1f isoform is more common in Asian populations. Notably, the Gc isoforms have been found to have differential affinity for vitamin D metabolites, with Gc1F displaying the highest affinity and Gc2 the lowest. (19) Therefore, it is also possible that the rs7041 T allele represents a risk allele in the context of the Gc2 isoform, but is protective in the context of the Gc1f isoform. Studies that examine these outcomes in greater depth and in relation to the Gc types themselves will surely provide further clarity.

Conclusion

Our study demonstrates that the *GC* rs7041 genotype alters vitamin D metabolism, as well as the metabolic response to pregnancy, and a marker of bone formation in neonates. Notably, although DBP is known to increase markedly during pregnancy, this phenomenon was observed only in individuals with rs7041 GG or GC genotypes; pregnant TT women in our cohort did not exhibit higher DBP than non-pregnant women. The genotypic differences in DBP status during pregnancy may have implications for placental vitamin D transport and availability of vitamin D to the mother and fetus. Future studies with larger sample size are needed to comprehensively characterize the effects of the three common Gc isoforms on vitamin D metabolism during pregnancy and the consequences of these metabolic differences for maternal, fetal, and infant health.

Supplemental Information



Supplemental Figure 1. Effect of the *GC* rs7041 genotype on serum 25(OH)D_2 & serum 25(OH)D_3. Values are least-squared means $^{+/-}$ standard errors. Number of participants in each group: GG (n=18), GT (n=36), TT (n=20). Groups that are not labelled with any of the same letters are significantly different at an α-cutoff of 0.05

REFERENCES

1. **De-Regil LM, Palacios C, Lombardo LK, Peña-Rosas JP.** Vitamin D supplementation for women during pregnancy. Pe?a-Rosas JP, ed. *Cochrane Database Syst. Rev.* 2016;2016(1):CD008873.
2. **Brannon PM, Picciano MF.** Vitamin D in Pregnancy and Lactation in Humans. *Annu. Rev. Nutr.* 2011;31(1):89–115.
3. **Saraf R, Morton SMB, Camargo CA, Grant CC.** Global summary of maternal and newborn vitamin D status - a systematic review. *Matern. Child Nutr.* 2016;12(4):647–668.
4. **Ahn J, Yu K, Stolzenberg-Solomon R, Claire Simon K, McCullough ML, Gallicchio L, Jacobs EJ, Ascherio A, Helzlsouer K, Jacobs KB, Li Q, Weinstein SJ, Purdue M, Virtamo J, Horst R, Wheeler W, Chanock S, Hunter DJ, Hayes RB, Kraft P, Albanes D.** Genome-wide association study of circulating vitamin D levels. *Hum. Mol. Genet.* 2010;19(13):2739–2745.
5. **Wang TJ, Zhang F, Richards JB, Kestenbaum B, Van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, De Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouhi NG, Loos RJF, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasan RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Foroud T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Järvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD.** Common genetic determinants of vitamin D insufficiency: A genome-wide association study. *Lancet* 2010;376(9736):180–188.
6. **McGrath JJ, Saha S, Burne THJ, Eyles DW.** A systematic review of the association between common single nucleotide polymorphisms and 25-hydroxyvitamin D concentrations. *J. Steroid Biochem. Mol. Biol.* 2010;121(1–2):471–477.
7. **Bu FX, Armas L, Lappe J, Zhou Y, Gao G, Wang HW, Recker R, Zhao LJ.** Comprehensive association analysis of nine candidate genes with serum 25-hydroxy vitamin D levels among healthy Caucasian subjects. *Hum. Genet.* 2010;128(5):549–556.
8. **Jolliffe DA, Walton RT, Griffiths CJ, Martineau AR.** Single nucleotide polymorphisms in the vitamin D pathway associating with circulating concentrations of vitamin D metabolites and non-skeletal health outcomes: Review of genetic association studies. *J. Steroid Biochem. Mol. Biol.* 2016;164:18–29.
9. **Arnaud J, Constans J.** Affinity differences for vitamin D metabolites associated with

the genetic isoforms of the human serum carrier protein (DBP). *Hum. Genet.* 1993;92(2):183–8.
10. **Medlej-Hashim M, Jounblat R, Hamade A, Ibrahim JN, Rizk F, Azzi G, Abdallah M, Nakib L, Lahoud M, Nabout R.** Hypovitaminosis D in a Young Lebanese Population: Effect of *GC* Gene Polymorphisms on Vitamin D and Vitamin D Binding Protein Levels. *Ann. Hum. Genet.* 2015;79(6):394–401.
11. **Yan J, Jiang X, West AA, Perry CA, Malysheva O V, Devapatla S, Pressman E, Vermeylen F, Stabler SP, Allen RH, Caudill MA.** Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans 1 – 4. 2012;(1):1060–1071.
12. **Park H, Brannon PM, West AA, Yan J, Jiang X, Perry CA, Malysheva O, Mehta S, Caudill MA.** Maternal vitamin D biomarkers are associated with maternal and fetal bone turnover among pregnant women consuming controlled amounts of vitamin D, calcium, and phosphorus. *Bone* 2017;95:183–191.
13. **Park H, Brannon PM, West AA, Yan J, Jiang X, Perry CA, Malysheva O V., Mehta S, Caudill MA.** Vitamin D Metabolism Varies among Women in Different Reproductive States Consuming the Same Intakes of Vitamin D and Related Nutrients. *J. Nutr.* 2016;146(8):1537–1545.
14. **Kaufmann M, Gallagher JC, Peacock M, Schlingmann K-P, Konrad M, DeLuca HF, Sigueiro R, Lopez B, Mourino A, Maestro M, St-Arnaud R, Finkelstein JS, Cooper DP, Jones G.** Clinical Utility of Simultaneous Quantitation of 25-Hydroxyvitamin D and 24,25-Dihydroxyvitamin D by LC-MS/MS Involving Derivatization With DMEQ-TAD. *J. Clin. Endocrinol. Metab.* 2014;99(7):2567–2574.
15. **Schleicher RL, Encisco SE, Chaudhary-Webb M, Paliakov E, McCoy LF, Pfeiffer CM.** Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. *Clin. Chim. Acta* 2011;412(17–18):1594–1599.
16. **Kaufmann M, Gallagher JC, Peacock M, Schlingmann K-P, Konrad M, DeLuca HF, Sigueiro R, Lopez B, Mourino A, Maestro M, St-Arnaud R, Finkelstein JS, Cooper DP, Jones G.** Clinical Utility of Simultaneous Quantitation of 25-Hydroxyvitamin D and 24,25-Dihydroxyvitamin D by LC-MS/MS Involving Derivatization With DMEQ-TAD. *J. Clin. Endocrinol. Metab.* 2014;99(7):2567–2574.
17. **Lipkie TE, Janasch A, Cooper BR, Hohman EE, Weaver CM, Ferruzzi MG.** Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* 2013;932:6–11.
18. **R Core Team.** R: A language and environment for statistical computing. 2014. Available at: https://www.r-project.org/. Accessed December 14, 2016.
19. **Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G.** Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol. Rev.* 2016;96(1):365–408.
20. **Wilson SG, Retallack RW, Kent JC, Worth GK, Gutteridge DH.** Serum free 1,25-dihydroxyvitamin D and the free 1,25-dihydroxyvitamin D index during a longitudinal

study of human pregnancy and lactation. *Clin. Endocrinol. (Oxf).* 1990;32(5):613–22.
21. **Bikle DD, Gee E, Halloran B, Haddad JG.** Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *J. Clin. Invest.* 1984;74(6):1966–71.
22. **Emerson DL, Werner PA, Cheng MH, Galbraith RM.** Presence of Gc (vitamin D-binding protein) and interactions with actin in human placental tissue. *Am. J. Reprod. Immunol. Microbiol.* 1985;7(1):15–21.
23. **Christensen EI, Birn H.** Megalin and cubilin: multifunctional endocytic receptors. *Nat. Rev. Mol. Cell Biol.* 2002;3(4):258–268.
24. **Lundgren S, Carling T, Hjälm G, Juhlin C, Rastad J, Pihlgren U, Rask L, Åkerström G, Hellman P.** Tissue Distribution of Human gp330/Megalin, a Putative Ca^{2+} -sensing Protein. *J. Histochem. Cytochem.* 1997;45(3):383–392.
25. **Ma R, Gu Y, Zhao S, Sun J, Groome LJ, Wang Y.** Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas from normal and preeclamptic pregnancies. *AJP Endocrinol. Metab.* 2012;303(7):E928–E935.
26. **Díaz L, Sánchez I, Avila E, Halhali A, Vilchis F, Larrea F.** Identification of a 25-Hydroxyvitamin D3 1α-Hydroxylase Gene Transcription Product in Cultures of Human Syncytiotrophoblast Cells. *J. Clin. Endocrinol. Metab.* 2000;85(7):2543–2549.
27. **Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM, Hewison M.** The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. *Am. J. Pathol.* 2002;161(1):105–14.
28. **Sorensen IM, Joner G, Jenum P, Eskild A, Brunborg C, Torjesen PA, Stene LC.** Vitamin D-binding protein and 25-hydroxyvitamin D during pregnancy in mothers whose children later developed type 1 diabetes. *Diabetes. Metab. Res. Rev.* 2016;32(8):883–890.
29. **Reis JP, Michos ED, Selvin E, Pankow JS, Lutsey PL.** Race, vitamin D-binding protein gene polymorphisms, 25-hydroxyvitamin D, and incident diabetes: the Atherosclerosis Risk in Communities (ARIC) Study. *Am. J. Clin. Nutr.* 2015;101(6):1232–1240.
30. **Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, Powe NR, Thadhani R.** Vitamin D?Binding Protein and Vitamin D Status of Black Americans and White Americans. *N. Engl. J. Med.* 2013;369(21):1991–2000.
31. **Kamboh MI, Ferrell RE.** Ethnic variation in vitamin D-binding protein (GC): a review of isoelectric focusing studies in human populations. *Hum. Genet.* 1986;72(4):281–93.
32. **Chun S-K, Shin S, Kim MY, Joung H, Chung J.** Effects of maternal genetic polymorphisms in vitamin D-binding protein and serum 25-hydroxyvitamin D concentration on infant birth weight. *Nutrition* 2017;35:36–42.