INFLUENCE OF CHOLINE INTAKE DURING PREGNANCY ON MATERNAL
AND FETAL GENOMIC MARKERS IN HUMANS

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Doctor of Philosophy

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INFLUENCE OF CHOLINE INTAKE DURING PREGNANCY ON MATERNAL AND FETAL GENOMIC MARKERS IN HUMANS

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Choline is an essential nutrient, which functions in cellular membrane structure, neurotransmission and methyl group donation. The need for choline increases substantially during pregnancy. An Adequate Intake (AI) for choline has been established at 450mg/d for pregnant women. This thesis was focused on assessing the influence of choline intake exceeding the current AI during the third trimester of pregnancy on maternal and fetal genomic readouts in humans.

A 12-week choline controlled feeding study was conducted among third trimester pregnant and nonpregnant control women. The participants were randomized to either 480mg choline/d, which is slightly above the AI, or 930mg/d. Maternal blood samples were retrieved at the study beginning (wk-0) and end (wk-12). Placental biopsies, and maternal and cord blood samples were retrieved at delivery. Epigenetic marks and transcriptomes were assessed.

Genomic markers in fetal derived tissues were responsive to maternal choline intake. Specifically, the higher maternal choline intake group (930 vs 480 mg/d) had higher placental global DNA and histone methylation ($P=0.02$). The placental promoter DNA methylation of cortisol regulating genes corticotropin releasing hormone ($CRH$) ($P=0.05$) and glucocorticoid receptor ($NR3C1$) ($P=0.002$) were also
higher among women consuming 930 vs 480 mg choline/d, which was consistent with decreased CRH gene expression ($P=0.05$) in the placenta and lower cortisol in cord blood ($P=0.07$) in the 930 mg choline/d group. Analysis of the placental transcriptome revealed that the higher maternal choline intake group had lower expression of the anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFLT1) ($P=0.05$), a marker that predicts preeclampsia. Similar decreases ($P=0.04$) were detected in maternal blood sFLT1 protein concentrations. The effect of choline on decreasing sFLT1 was confirmed in a trophoblast cell line HTR-8/SVneo. Additionally, in this cell line, suboptimal choline concentrations in the culture medium induced apoptosis, elevated oxidative stress, increased expression of angiogenic and inflammatory genes, and impaired in vitro angiogenesis. Inhibition of protein kinase C rescued the effects of low choline on angiogenesis and apoptosis indicating that choline deficiency perturbs this signaling pathway.

In sum, a maternal choline intake exceeding current recommendations may beneficially program offspring stress reactivity and mitigate the production of proteins associated with preeclampsia.
BIOGRAPHICAL SKETCH

Xinyin Jiang was born on November 12, 1985 in Guangzhou, Guangdong, China. After graduating from Zhixin High School in Guangzhou, she entered Fudan University in Shanghai in September, 2004. She majored in Biological Sciences in the College of Life Science at Fudan University and worked as an undergraduate research assistant in the lab of Dr. Daming Ren for two years. She conducted enzymological assays on isolated sea ice bacteria, and studied the use of attenuated *Salmonella* as a gene vector to deliver tumor suppressive genes to target melanoma tissues in mice, which she focused her undergraduate honors thesis on. She got her bachelor’s degree in July, 2008. Because of her interest in the interaction between diet and health, she entered the graduate program in Nutritional Sciences at Cornell University in August, 2008, and joined the laboratory of Dr. Marie Caudill. Xinyin participated in a human feeding study that investigated the influence of maternal choline intake during the third trimester of pregnancy on maternal and fetal health outcomes, and her specific dissertation project investigated the genomic readouts of the maternal-fetal dyads. Xinyin was enrolled in the dietetic internship program at Cornell in August, 2012 and expect to complete her supervised practice in June, 2013.
Dedicated to my parents and Mr. Siwei Zhang
ACKNOWLEDGMENTS

I would like to thank Dr. Marie Caudill for always providing me with support and guidance, for giving me independence to pursue my own interests, and for setting an example of a wonderful mentor and a terrific teacher.

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I am always grateful to my family, with whose love I become who I am, a person with trust, courage and happiness. I am also very fortunate to have my 4-year roommates and long-time friends Liu and Cheng, who helped me survive the snowy winters in Ithaca.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOGRAPHICAL SKETCH ................................................................. iii</td>
</tr>
<tr>
<td>DEDICATION ......................................................................................... iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS ........................................................................... v</td>
</tr>
<tr>
<td>LIST OF FIGURES .............................................................................. vii</td>
</tr>
<tr>
<td>LIST OF TABLES ............................................................................... ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS .................................................................. x</td>
</tr>
<tr>
<td>PREFACE ............................................................................................. xv</td>
</tr>
<tr>
<td>CHAPTER 1: Maternal choline intake alters the epigenetic state of fetal cortisol regulating genes in humans ........................................................... 1</td>
</tr>
<tr>
<td>CHAPTER 2: A higher maternal choline intake among third trimester pregnant women lowers placental and circulating concentrations of the anti-angiogenic factor fms-like tyrosine kinase-1 (sFLT1) .......................................................... 45</td>
</tr>
<tr>
<td>CHAPTER 3: Pregnancy induces transcriptional activation of the peripheral innate immune system and increases oxidative DNA damage among healthy third trimester pregnant women ................................................................. 81</td>
</tr>
<tr>
<td>AFTERWORD ......................................................................................... 114</td>
</tr>
<tr>
<td>APPENDIX A: Choline affects placental development and vascular function in a dose response manner .............................................................. 118</td>
</tr>
<tr>
<td>APPENDIX B: Copy right approval forms from The FASEB Journal .......... 157</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Section</th>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREFACE</td>
<td>Figure P.1</td>
<td>xviii</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>Figure 1.1</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Figure 1.2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Figure 1.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Figure 1.4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Figure 1.5</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure S1.1</td>
<td>43</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>Figure 2.1</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Figure 2.2</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Figure 2.3</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure S2.1</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure S2.2</td>
<td>80</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>Figure 3.1</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Figure 3.2</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Figure 3.3</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Figure 3.4</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Figure 3.5</td>
<td>106</td>
</tr>
</tbody>
</table>
APPENDIX A

Figure A.1 .......................................................... 128
Figure A.2 .......................................................... 131
Figure A.3 .......................................................... 134
Figure A.4 .......................................................... 135
Figure A.5 .......................................................... 136
Figure A.6 .......................................................... 139
Figure A.7 .......................................................... 141
Figure A.8 .......................................................... 143
Figure A.9 .......................................................... 145
Figure A.10 ......................................................... 146
## LIST OF TABLES

### CHAPTER 1

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>15</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>16</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>25</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>29</td>
</tr>
<tr>
<td>Supplemental Table S1.1</td>
<td>40</td>
</tr>
<tr>
<td>Supplemental Table S1.2</td>
<td>41</td>
</tr>
<tr>
<td>Supplemental Table S1.3</td>
<td>42</td>
</tr>
</tbody>
</table>

### CHAPTER 2

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>58</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>61</td>
</tr>
<tr>
<td>Supplemental Table S2.1</td>
<td>78</td>
</tr>
</tbody>
</table>

### CHAPTER 3

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>90</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>94</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>95</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>99</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>103</td>
</tr>
<tr>
<td>Table 3.6</td>
<td>105</td>
</tr>
</tbody>
</table>

### APPENDIX A

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemental Table SA.1</td>
<td>156</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

5mdC, 5-methyl-2′-deoxycytidine
ACHE, acetylcholine esterase
ADORAS, adenosine A3 receptor
AI, adequate intake
CAMP, cathelicidin
BADH, betaine aldehyde dehydrogenase
B-cell linker protein (BLNK)
BHMT, betaine-homocysteine S-methyltransferase
BLNK, B-cell linker protein
C1QB, complement component 1, q subcomponent, B chain
C2, complement component 2
CAMP, cathelicidin
CASP3, caspase 3
CCR, chemokine (C-C motif) receptor
CD14, CD molecule 14
CDP-choline, cytidine 5′-diphosphocholine
CGB, chorionic gonadotropin
CHAT, choline acetyltransferase
CHDH, choline dehydrogenase
CHRM4, cholinergic receptor, muscarinic 4.
CK, choline kinase
COL8A1, Collagen, type VIII, alpha 1
CpG, cytosine phosphate guanine dinucleotide
CR1, complement component (3b/4b) receptor 1
CRH, corticotropin releasing hormones
CT, choline phosphate cytidylyltransferase
D3-SAM, deuterium labeled methyl SAM
D3-5mdC, deuterium labeled 5-methyl-2'-deoxycytidine
D4-SAM, 4 deuterium labeled SAM
D9-choline, deuterium labeled trimethyl choline
DAG, diacylglycerol
DEFA, alpha defensin
DHA, docosahaexinoic acid
DMG, dimethylglycine
DMR, differentially methylated region
DNMT1, DNA methyltransferase A
DNMT3A, DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B, DNA (cytosine-5-)-methyltransferase 3 beta
DRI, dietary reference intake
EHMT2, euchromatic histone-lysine N-methyltransferase 2
ELANE, elastase
ELN, elastin
ENG, endoglin
ERRFI1, ERBB report feedback inhibitor 1
FBS, Fetal bovine serum
FDR, false discovery rate
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GHRL, ghrelin
GLM, general linear model
GNAS, guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1
GO, gene ontology
GUSB, beta glucuronidase
H3K27me3, trimethylated histone H3 at lysine 27
H3K4me2, dimethylated histone H3 at lysine 4
H3K9me2, dimethylated histone H3 at lysine 9
Hcy, Homocysteine
HPA, hypothalamic-pituitary-adrenal
IGF2, insulin-like growth factor 2
IL10, interleukin 10
IL6, interleukin 6
IL1B, interleukin 1 beta
IUGR, intrauterine growth restriction
JNK, c-JUN N-terminal kinase
JUN, jun proto-oncogene
KDR, kinase insert domain receptor
LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry
LEP, leptin
LTF, lactoferrin
MIAME, minimum information about a microarray experiment
MKI67, monoclonal antibody Ki-67
MMP14, matrix metalloproteinase 14
MPO, myeloperoxidase
MYD88, myeloid differentiation primary response gene 88
MYH7, myosin, heavy chain 7, cardiac muscle, beta
NCF, neutrophil cytosolic factors
NFkB, nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGFI-A, nerve growth factor inducible protein A
NPY5R, neuropeptide Y receptor Y5
NR3C1, nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
OXTR, oxytocin receptor
PBMC, peripheral blood mononuclear cell
PCho, phosphorylcholine
PE, phosphatidylethanolamine
PEMT, phosphatidylethanolamine methyltransferase
PGF, placental growth factor
PKC, protein kinase C
PTK2B, tyrosin kinase 2 beta
RBP4, retinol binding protein
ROS, reactive oxygen species
SAH, S-adenosylhomocysteine
SAM, S-adenosylmethionine
SE, standard error
sENG, soluble endoglin
sFLT1, fms-like tyrosine kinase-1
SUV39H1, suppressor of variegation 3-9 homolog 1
TGF-B, transforming growth factor beta
TLR, toll-like receptor
TNFα, tumor necrosis factor alpha
TP53, tumor protein P53
UL, tolerable upper intake level
VEGF, vascular endothelial growth factor
VLDL, very low density lipoprotein
INTRODUCTION AND SIGNIFICANCE:

Choline, an essential nutrient often grouped with the B vitamins, is a precursor of the membrane constituent phosphatidylcholine, the neurotransmitter acetylcholine, and the methyl donor betaine (1). These choline biomolecules participate in various biological processes including growth and development, neurotransmission and one-carbon metabolism (2, 3).

The demand for choline increases substantially during pregnancy as both placental expansion and fetal development require a large amount of choline to support membrane biosynthesis and accelerations in one-carbon metabolism (4). Babies are born with up to five times higher plasma free choline concentrations than their mothers (5), which facilitates choline uptake by the developing brain (6). In rodents, provision of extra choline (i.e., 4 times higher than normal) during the last half of gestation improves spatial memory and prevents age-related cognitive deficits in the adult offspring (7). Maternal choline supplementation of the dam diet during gestation can also delay the progression of chemically-induced breast cancer in the female offspring (8).

The dietary requirement for humans is unknown and recommendations are provided in the form of adequate intakes (AIs). The choline Adequate Intake (AI) for pregnant women of 450 mg/d was designed to prevent liver dysfunction (9) which arises from insufficient amounts of choline to synthesize very low density lipoproteins. Given the many other roles of choline in human health, studies are needed to address the impact of maternal choline intake during pregnancy on a wider array of metabolic markers and physiologic
Metabolic and physiological alterations often involve changes in the genomic landscape and prior work has shown that choline intake can affect several genomic endpoints including epigenetic modifications (e.g. DNA and histone methylation) (10, 11), DNA damage (12) and genome-wide gene expression (13). As such, it is important to understand the genomic implications of varied maternal choline intake during pregnancy as it may have lasting effects on maternal health as well as fetal development and metabolic functioning. Ultimately, these data can be used to scientifically inform development of dietary choline intake recommendations that support the health and well-being of both mother and child.

To facilitate the discovery of metabolic and physiologic processes influenced by maternal choline intake, the primary goal of my dissertation was to investigate the response of genomic markers to maternal choline intake. A secondary goal was to investigate the effect of pregnancy on genomic markers. To achieve these goals we conducted a controlled feeding study in which pregnant (n=26, gestational week 26-29) and nonpregnant (n=21) women were randomized to choline intakes of 480 mg/d (slightly above the AI) or 930 mg/d. The 7-day rotational diet, consumed by all women, provided 380 mg choline/d. Supplemental choline chloride, 100 or 550 mg/d, was used to achieve the target choline intake levels. Blood was collected throughout the feeding phase from both pregnant and nonpregnant women. Maternal and cord blood samples along with the placenta were obtained at delivery (14).
SPECIFIC AIMS:

Aim 1: To test the hypothesis that maternal choline intake during the third trimester of pregnancy influences epigenetic and epigenomic markers in fetal derived tissues. This aim was achieved by measuring placental and cord blood global DNA and histone methylation, as well as site specific methylation patterns of genes with key roles in fetal development and are regulated by DNA methylation.

Results are presented in chapter 1.

Aim 2. To test the hypothesis that maternal choline intake during the third trimester of pregnancy influences the placental transcriptome. This hypothesis was tested by assessing the placental gene expression changes in response to maternal choline intake using genome-wide expression microarray. Genes and physiological processes of particular interest were further analyzed at protein level and in a trophoblast culture model.

Results are presented in chapter 2.

Aim 3. To test the hypothesis that maternal choline intake and/or pregnancy affects maternal blood leukocyte genomic markers. This aim was achieved by measuring DNA damage, global DNA and histone methylation, and genome-wide gene expression patterns in blood samples obtained from pregnant and nonpregnant women.

Results are presented in chapter 3.
This dissertation research yielded three published peer-reviewed articles (Chapters 1 - 3) and one original research manuscript, which will be submitted for publication within the next few months (Appendix A).
BACKGROUND AND RATIONALE:

Choline metabolism and function

The three major metabolic fates of choline are synthesis of phosphatidylcholine, oxidation to betaine and acetylation to acetylcholine. Through these metabolic pathways, choline participates in various physiological functions throughout the body (Figure P.1).

Figure P.1. Choline metabolism and function. There are three major metabolic fates of choline - synthesis of acetylcholine, betaine and phosphatidylcholine. Key enzymes involved in the choline metabolic pathways are shown next to the arrows which indicate chemical reactions. Major functions of the choline metabolites are listed in the boxes.

Abbreviations: BHMT, betaine homocysteine S-methyltransferase; CHAT, choline acetyltransferase; CHDH, choline dehydrogenase; CT, CTP:phosphocholine cytidylyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; SAM, S-adenosylmethionine.
Phosphatidylcholine

Phosphatidylcholine is synthesized via the cytidine diphosphate-choline (CDP-choline) pathway from an intact choline molecule or via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway from phosphatidylethanolamine and SAM derived methyl groups (15). CTP:phosphocholine cytidylyltransferase (CT) and PEMT are the rate limiting enzymes of these pathways, respectively. Although the PEMT pathway is considered a de novo pathway for choline biosynthesis, it is notable that PEMT uses choline derived methyl groups to make a new choline moiety (16) and that a higher choline intake can upregulate this pathway in nonpregnant women (West et al. in press). As such, maternal choline intake can affect the synthesis of phosphatidylcholine via both biosynthetic pathways and therefore its availability for the biological processes outlined below.

As a major phospholipid in cellular membranes, phosphatidylcholine is essential for cell division and tissue expansion (17, 18) as well as membrane integrity. A diminished presence of phosphatidylcholine in mitochondrial membranes leads to the leakage of free radicals, oxidative stress and DNA damage (19, 20). Phosphatidylcholine is also an indispensable component of very low density lipoproteins (VLDLs) and phosphatidylcholine deficiency results in fatty liver due to the inability to export fat (21). Finally, phosphatidylcholine is a source of several signaling molecules (e.g., diacylglycerol, phosphocholine, phosphatidic acid and glycerophosphocholine) that can influence a wide range of physiologic processes including proliferation, differentiation, cell cycle and apoptosis (22).
Betaine

Choline is oxidized to betaine via a two-step reaction initiated by choline dehydrogenase (CHDH). The oxidation of choline to betaine diverts choline towards the one carbon cycle, which mediates the transfer of methyl groups (23). Specifically, in liver and kidney, one of the labile methyl groups from betaine is transferred to homocysteine by betaine: homocysteine S-methyltransferase (BHMT), forming methionine. The methionine produced by the reaction is then converted to S-adenosylmethionine (SAM), the universal methyl donor required for methylation reactions (24). As such, choline derived methyl groups are used to synthesize over 60 metabolites including phosphatidylcholine (via PEMT) and to methylate DNA and histones (25).

Acetylcholine

The acetylation of choline to acetylcholine is catalyzed by choline acetyltransferase (CHAT). Acetylcholine can serve as a signaling molecule in neuronal and non-neuronal tissues by binding to nicotinic or muscarinic acetylcholine receptors (26, 27). In the neuronal system, acetylcholine serves as a neurotransmitter for cholinergic neurons. In some non-neuronal organs, such as the placenta, acetylcholine is present in large amounts and may function in vascular dilation, amino acid transport and placental differentiation (27, 28).

Interactions between choline and genomic biomarkers

Epigenetic modifications

Epigenetic marks (e.g., DNA and histone methylation) are heritable modifications on the
genome that do not change DNA sequences (29). The most common form of DNA methylation is the addition of a methyl group to the C-5 position of cytosine within the context of a cytosine phosphate guanine (CpG) dinucleotide. Although 60-90% of all CpG sites in mammal genomes are methylated (30), the promoter region of most housekeeping genes contains a high frequency of CpG sites that are usually not methylated (i.e. CpG islands), which allows for transcription factor access and transcription procession. Often but not always, CpG island methylation leads to transcription suppression. Histone modification, the alkaline proteins which DNA wraps around, is another common epigenetic mark which can influence accessibility of transcription factors to their binding sites (31) and ultimately gene expression. For example, methylation of histone 3 lysine 9 (H3K9) associates with heterochromatin formation and the suppression of DNA transcription (31).

The epigenetic state of the human genome is modulated by environmental exposures (e.g., nutrients) especially during critical windows of development like the prenatal period. Epigenetic modifications occurring during the prenatal period can permanently modify physiological functions with subsequent effects on chronic disease risk including Type 2 diabetes (32), hypertension (33) and cancer (34).

Maternal choline intake during pregnancy has been shown to affect the fetal epigenome presumably through its role as a methyl donor. For example, prenatal choline deficiency decreased hippocampal DNA and histone methylation (35, 36), which resulted in reduced hippocampal angiogenesis. In addition, prenatal choline supplementation decreased DNA methylation of a tumor suppressor gene and delayed the progression of chemically induced breast cancer in the female offspring (8).
DNA damage

Lesions that compromise the integrity of DNA molecules include single or double strand breaks, base modification, and DNA-protein crosslinks (37). Unresolved DNA damage decreases genomic stability, which results in aberrant gene expression, apoptosis and/or necrosis. One of the major causes of DNA damage is oxidative stress, a condition that arises when the amount of reactive oxygen species (ROS) released by mitochondria exceeds the balancing capabilities of the cellular antioxidant system. Under these conditions, surplus ROS react with DNA, causing its damage (38). In turn, the level of DNA damage indicates the severity of oxidative stress. Pregnant women have a higher susceptibility to oxidative stress mostly because that the human placenta is enriched with mitochondria (39). As such, DNA damage may serve as a sensitive genomic marker to advance understanding of redox balance during normal pregnancy.

In addition, given choline’s role in maintaining the integrity of the mitochondrial membrane, DNA damage may serve as a functional indicator of choline status. In this regard, studies in hepatic cell lines and rodents have consistently demonstrated that choline deficiency increases DNA strand breaks and cellular apoptosis of hepatocytes (20). In humans, consumption of a choline deplete diet for 42-d increased the level of lymphocyte DNA damage and apoptosis as compared to baseline values (19). Similarly, consumption of a higher choline intake (i.e, 2200 versus 550 mg/d) among folate compromised men lowered the level of lymphocyte DNA damage (10).
**Messenger RNA synthesis**

Transcription of DNA to messenger RNA can influence gene expression with downstream effects on physiological processes. Choline intake may influence mRNA synthesis via its role as a methyl donor and/or signaling molecule. Notably, consumption of a choline deficient diet for 6 weeks by healthy men and women altered lymphocyte transcription of genes related to cell cycle, methylation, apoptosis and cell growth as compared to baseline values (13). Additionally, supplementing the rodent maternal diet with extra choline during pregnancy altered the transcriptome of the female offspring with a profile consistent with better tumor prognosis (8).

**Conclusions**

There is a wealth of evidence from animal studies (and a few human studies) that choline intake can influence genomic readouts. Nonetheless, the effect of maternal choline intake during human pregnancy, a critical window of development, on genomic marks is unknown. As part of a controlled feeding study in third trimester pregnant women, this dissertation employed targeted and non-targeted approaches to examine the impact of maternal choline intake on DNA and histone methylation, mRNA abundance, and DNA damage in maternal and fetal derived tissues. Data generated from this dissertation are expected to guide the refinement of choline intake recommendations during pregnancy and ultimately improve human health.
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CHAPTER 1

Maternal choline intake alters the epigenetic state of fetal cortisol regulating genes in humans


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ABSTRACT

The *in utero* availability of methyl donors, such as choline, may modify fetal epigenetic marks and lead to sustainable functional alterations throughout the lifecourse. The hypothalamic-pituitary-adrenal (HPA) axis regulates cortisol production and is sensitive to perinatal epigenetic programming. As an extension of a 12-week dose-response choline feeding study conducted in third trimester pregnant women, we investigated the impact of maternal choline intake (930 versus 480 mg/d) on the epigenetic state of cortisol regulating genes, and their expression, in placenta and cord venous blood. The higher maternal choline intake yielded: higher placental promoter methylation of the cortisol regulating genes, corticotropin releasing hormone (*CRH*) (*P*=0.05) and glucocorticoid receptor (*NR3C1*) (*P*=0.002); lower placental *CRH* transcript abundance (*P*=0.04); lower cord blood leukocyte promoter methylation of *CRH* (*P*=0.05) and *NR3C1* (*P*=0.04); and 33% lower (*P*=0.07) cord plasma cortisol. In addition, placental global DNA methylation and dimethylated histone H3 at lysine 9 (H3K9me2) were higher (*P*=0.02) in the 930 mg choline/d group as was the expression of select placental methyltransferases. These data collectively suggest that maternal choline intake in humans modulates the epigenetic state of genes that regulate fetal HPA axis reactivity as well as the epigenomic status of fetal derived tissues.
INTRODUCTION

Nutrients such as choline, betaine and folate provide methyl groups for cellular methylation reactions. In animal models, the maternal consumption of these methyl donors modifies fetal epigenetic marks (e.g. promoter region DNA and histone methylation) (2-5) and leads to sustainable functional alterations throughout the lifecourse (6, 7). Nevertheless, evidence of fetal epigenetic modification by maternal consumption of methyl donors in humans is lacking.

Our research group recently conducted a 12-wk dose-response choline feeding study in which pregnant women (gestational age wk 26-29) were randomized to choline intakes of 480 [approximating the Adequate Intake (AI) for choline] or 930 mg/d throughout their third trimester. We found that a higher maternal choline intake increased the use of choline as a methyl donor as evidenced by elevations in maternal and fetal plasma dimethylglycine, the metabolite produced when choline is used as a methyl donor (9). The increased use of choline as a methyl donor may alter the epigenetic state of genes regulated by methylation as previously shown in animal studies (3, 4).

The methylation state of the cortisol regulating genes, corticotropin releasing hormone (CRH) and nuclear receptor subfamily 3, group C, member 1 (NR3C1), may be particularly sensitive to prenatal and early postnatal exposures (1, 2, 10, 11). These genes encode components of the hypothalamic-pituitary-adrenal (HPA) axis, which have important roles in stress response, immunity and glucose metabolism. In response to stress, the hypothalamus produces CRH which stimulates the secretion of adrenocorticotropic hormone from the anterior pituitary and subsequently glucocorticoids (e.g. cortisol) from the adrenal gland.
NR3C1 encodes the glucocorticoid receptor which plays a critical role in central HPA inhibition via glucocorticoid negative feedback (12). The programming of the HPA axis through promoter region methylation of CRH and NR3C1 may have implications for susceptibility to stress-related (i.e., autoimmune disorders and mental health disturbances) (13, 14) and metabolic (e.g., hypertension and diabetes) diseases later in life (15, 16).

As an extension of our 12-week dose-response choline feeding study conducted in third trimester pregnant women (9), this study investigated the influence of maternal choline intake (480 vs. 930 mg/d) on the epigenetic state of cortisol regulating genes, and their expression, in placenta and cord venous blood. As varied maternal choline intake may systematically affect a wide-array of placental genes regulated by DNA and histone methylation, four additional genes for site specific methylation measurements were targeted: insulin-like growth factor 2 (IGF2), leptin (LEP), guanine nucleotide binding protein, alpha stimulating, antisense transcript (GNAS-AS1), and interleukin 10 (IL10). Selection of these genes was based on prior evidence of methylation regulation, susceptibility to nutritional exposures, and associations with chronic disease risk (17, 18). Finally, given that prenatal choline exposure alters epigenomic readouts in animal models (3, 5), global DNA and histone methylation were measured.
MATERIALS AND METHODS

Study Participants

Healthy third trimester (wk 26-29 gestation) singleton pregnant women aged ≥ 21y were recruited from Ithaca, New York, and surrounding areas between January 2009 and October 2010. During the screening phase, all study volunteers completed a questionnaire which queried participants on their age, education, work status, ethnicity/race, pre-pregnancy body mass index, parity, health history, medication and nutritional supplement use, and physical activity. Entry into the study was contingent upon good health status (i.e., no chronic diseases, normal kidney and liver function, and non-anemic). Additional inclusion criteria included: no tobacco or alcohol product use, and a willingness to comply with the study protocol. For those who were included in the study, information on gestational weight gain, health insurance, mode of delivery, obstetrical complications, and newborn sex and health characteristics were obtained from medical charts after delivery. Twenty-six of the 29 pregnant women who started the study completed it. Of the 26 women completing the study, placental samples were retrieved from 24 participants (n = 12/ choline intake group) and cord blood samples were retrieved from 23 participants. The study protocol was approved by the IRB for Human Study Participant Use at Cornell University and at Cayuga Medical Center (Ithaca, NY, USA). Written informed consent was obtained from all the participants prior to study entry. The study was registered at clinicaltrials.gov as NCT01127022. Additional information regarding the study participants is described elsewhere (9).

Study Design and Diet

This was a 12-wk controlled feeding study in which pregnant women were randomized to
either 480 (approximating the choline adequate intake (AI); n=12) or 930 (n=12) mg choline/d. The choline was derived from the diet (380 mg/d) plus supplemental choline chloride (either 100 or 550 mg choline/d for the 480 or 930 mg/d intake levels, respectively). During the last 6-wk of the study, ~20% of the total choline intake was provided as deuterium labeled trimethyl d9-choline (Cambridge Isotope Laboratories, Andover, MA, USA). Throughout the 12-wk study, the participants consumed a 7-day cycle menu as detailed in Yan et al. (9). All food and beverage were provided by the investigators and one meal per day was consumed on-site throughout the week. All other meals and beverages were provided as take-aways. In addition to the supplemental choline, a prenatal multivitamin (Pregnancy Plus, Fairhaven Health, LLC, Bellingham, WA, USA) and DHA (200 mg; Neuromins, Nature's Way Products, Lehi, UT, USA) were provided daily; a potassium and magnesium supplement (General Nutrition Corp, Pittsburgh, PA, USA) was provided three times per week. The supplements were consumed with the on-site meal under the supervision of the investigators. At the end of the study, participants continued on their choline supplement until the delivery of their babies.

Sample collection

Fasting (10 h) venous blood samples were obtained at baseline (wk 0) and at the end of the study (wk 12) in 10mL EDTA tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). A maternal EDTA-blood sample was also obtained within 24 h of delivery and cord venous EDTA-blood was collected immediately after delivery, processed and stored as previously described(9). The placenta samples (n=21) were processed within 10-30 minutes after the delivery of the babies, except for three cases (n=2 in the 930 and n=1 in the 480 mg
choline/d groups, 60-90 minutes after delivery) in the hospital. Samples collected 60-90 minutes after delivery did not alter sample quality, e.g. RNA integrity. After removing the amniotic sac, the placenta was visually divided into four quadrants. Full thickness biopsies (0.5cm*0.5cm*placenta depth) were obtained from the center of each quadrant, rinsed with PBS and either flash frozen in liquid nitrogen or immersed in RNAlater (Qiagen, Valencia, CA, USA). The samples were stored at -80C until subsequent analysis. For the DNA, metabolite and histone measurements, one tissue biopsy from each quadrant was pooled and ground in liquid nitrogen. For RNA extraction, one tissue biopsy from one of the quadrants was homogenized. As the placenta is a heterogeneous tissue, the results obtained from our experiments represent an average effect of maternal choline intake on a subset of the placental cells.

Analytical Measurements

Cortisol concentration

The relative intensity of maternal and umbilical cord plasma cortisol was initially measured by LC-MS as part of a metabolite panel by a diagnostics and services company Metabolon (Durham, NC, USA). The actual concentrations of cortisol (μg/dL) were later quantified in house using a commercially available ELISA kit (Diagnostic Automation, Calabasas, CA, USA) according to the manufacturer’s instructions.

Site specific methylation

Site specific methylation was analyzed using base-specific cleavage and mass spectrometry (19). The loci of interest included regions in the promoters of CRH, GNAS-ASI, LEP and IL10 (17); the 5’ untranslated exon 1F (and flanking regions) of NR3CI (1); and a
part of the differentially methylated region 0 (DMR0) of *IGF2* (20, 21). Genomic DNA was extracted via the DNeasy blood & tissue kit (Qiagen) and 1 μg extracted DNA was bisulfite-treated using the EZ 96-DNA methylation kit (Zymo, Irvine, CA, USA). DNA sequences of interest were PCR amplified using the bisulfite-treated DNA as the template and published primers (1, 17) that incorporate the T7 tag (Supplemental Table S1.1). The amplification products were analyzed with the MassArray EpiTYPER system (Sequenom Inc, San Diego, CA, USA) at the Cornell Life Sciences Core Laboratories Center (Cornell University). Briefly, the PCR products were *in vitro* transcribed by T7 polymerase, digested by RNase A and measured via matrix assisted laser desorption ionization/time of flight mass spectrometry. Percent methylation of the cytosine residues was determined for each cytosine-phosphate-guanine (CpG) unit, which may contain one or multiple adjacent CpG dinucleotides (CpG sites) that cannot be resolved individually by EpiTYPER. The CpG sites belonging to a particular CpG unit of each gene are shown in Supplemental Table S1.2. Only CpG units with measurement success rates of > 75% were included in the final analyses (Supplemental Table S1.3) (17).

**RNA extraction and quantitative real-time PCR**

RNA was extracted via a commercially available kit (RNeasy Mini kit, Qiagen) from placental tissues. The concentration and purity of the isolated RNA were assayed with NanoDrop® ND-1000 instrumentation (Thermo, Wilmington, DE, USA). All of the samples had RNA concentrations >300 ng/μL and A260/280 ratios of >2.0, indicating adequate quantity and purity, respectively. The quality of RNA samples was verified by agarose gel electrophoresis which showed discreet 18S and 28S ribosomal RNA bands. Reverse
transcription was performed via the ImProm-II Reverse Transcription System™ (Promega, Madison, WI, USA). The reaction conditions were: 25 °C for 10 min, 42 °C for 40 min, 95 °C for 5 min and quenching at 4 °C for 5 min. Gene transcript abundance of CRH and NR3C1, as well as certain DNA and histone methyltransferases [i.e., DNA (cytosine-5-) methyltransferase 3 alpha (DNMT3A), DNA (cytosine-5-) methyltransferase 3 beta (DNMT3B), DNA (cytosine-5-) methyltransferase 1 (DNMT1), euchromatic histone-lysine N-methyltransferase 2 (EHMT2) and suppressor of variegation 3-9 homolog 1 (SUV39H1)] were analyzed by quantitative real-time PCR with the SYBR® Green system in a Roche LightCycler480® machine (Roche, Basel, Switzerland). The reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles with 15 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Data were expressed as the fold change of the gene of interest relative to the housekeeping gene (22), glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers were designed using GeneRunner Version 3.01. The primers are:

**CRH, F 5’-TGAAACATAGAGGGAGAGG-3’,**

R: 5’-ACCTGGAAACGGAAACGGAATAACCGGAGGAGG-3’;

**NR3C1, F: 5’-TACCTAAGCCTATTTTTTGCA-3’,**

R: 5’-AGCTGTATCTGGAATCAGCGGT-3’;

**DNMT3A, F: 5’-ACGGCAAATTCTCAGTGGTG-3’,**

R: 5’-GGACCTCGTAGATGGCTTTG-3’;

**DNMT3B, F: 5’-CAAACCAACACACCCACACACAGCAAC-3’,**

R: 5’-CAGCTGTGCCTCCTCGAGTC-3’;

**DNMT1, F: 5’-GTGTGGGAAATGTAAGCCTGC-3’,**
Global DNA methylation

Global DNA methylation was measured in placental tissues, cord blood leukocytes and maternal leukocytes (wk 0 and wk 12) via LC-MS/MS as described by Song et al. (23) with modifications based on our instrumentation (24). Briefly, DNA was extracted with the DNeasy blood & tissue kit (Qiagen) and digested with nuclease P1 (Sigma-Aldrich, St.Louis, MO, USA), phosphodiesterase 1 (Sigma-Aldrich) and alkaline phosphatase (Sigma-Aldrich). Hydrolyzed DNA was separated and analyzed with a LC-MS/MS system consisting of a TSQ Quantum mass spectrometer with electrospray ionization source operated in positive ion mode (Thermo, ), a refrigerated Accela autosampler (Thermo), and an Accela pump with degasser (Thermo). Global DNA methylation [5-methyl-2'-deoxycytidine (5mdC)] is expressed as a percentage of 2’-deoxyguanosine (representing total 2’-deoxycytidine). Isotopic enrichment (deuterium labeled d3-5mdC) is expressed as a percentage of total 5mdC (labeled and unlabeled metabolite). Natural isotopic enrichment and background noise was addressed by subtracting the signal obtained from control placental samples that were not exposed to labeled choline.
Global histone methylation

Global histone methylation marks di-methylated histone H3 at lysine 4 (H3K4me2), di-methylated histone H3 at lysine 9 (H3K9me2) and tri-methylated histone H3 at lysine 27 (H3K27me3) were measured using western blot analysis. Histone proteins were extracted from 100 mg frozen placental tissue and 200 μL of maternal buffy coat. Samples were incubated in 1 mL lysis buffer (40 mM sodium citrate, 1% Triton X-100) for 10 min and then centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in 0.2 N hydrochloric acid at a cell density of $4 \times 10^7$/mL and incubated at 4°C overnight. Samples were centrifuged again. Total protein concentrations of the supernatant were quantified by the Bradford assay (Thermo), and histone bands were visualized by SDS-PAGE and coomassie blue staining to ensure the presence of histones before western blot analysis. The extracts were separated by SDS-PAGE and transferred to Immobilon-FL polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA, USA). Membranes were blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) and incubated overnight with anti-H3K4me2 (GeneTex, Irvine, CA, USA), anti-H3K9me2 (Abcam, Cambridge, MA, USA), anti-H3K27me3 (GeneTex) and anti-Histone 3 (Abcam) antibodies. Secondary antibodies were either IRDye 800CW Goat Anti Rabbit or IRDye 680 Goat Anti mouse (LI-COR), depending on the primary antibodies. Target protein bands were visualized and quantified using the LI-COR Odyssey® imaging system (LI-COR) and expressed as the ratio of intensity of the histone epigenetic mark to total histone 3 protein (5).

$S$-adenosylmethionine (SAM) and $S$-adenosylhomocysteine (SAH) measurements

SAM and SAH measurements were performed using the method described by Kim et al.
(25) with modifications based on our LC-MS/MS instrumentation. Briefly, 50 mg of placental tissue was mixed with d3-SAM (CDN isotopes, Pointe-Claire, Quebec, Canada) and d4-SAH (Cayman Chemical, Ann Arbor, MI, USA) as internal standards and homogenized in 300μL of 25mM ammonium acetate. The homogenate was treated with 250μL of 1N perchloric acid and centrifuged at 12,000×g for 10 min at 4°C. An aliquot of the supernatant (550μL) was neutralized with 300μL of 1M ammonium hydroxide. The neutralized samples were applied to the Sep-Pak® Vac C18 cartridge (Waters, Milford, MA, USA) preconditioned with 1mL methanol, 750μL of 0.1N NaOH and 1mL water. The samples were washed with 1mL water and eluted with 1 mL 85:15 water: methanol (vol: vol) with 0.1% formic acid. The eluent was dried in a speed vacuum and re-dissolved in 150μL 0.05M ammonium formate with 0.1% formic acid. Analyses were conducted with the LC-MS/MS instrumentation described in the global DNA methylation section. Compounds of interest were separated by HPLC using a Luna C18(2) column (250×4.6mm, 5μm) (Phenomenex, Torrance, CA, USA) with matching guard column (4×3mm). The mobile phase containing acetonitrile and 0.1% formic acid in 0.05M ammonium formate was run in a rate of 500μL/min with 2% acetonitrile for 4 min, a linear gradient from 2% to 80% acetonitrile for 7 min, followed by a return to 2% acetonitrile over the next 4 min. The mass spectrometer was operated in positive-ion mode using electrospray ionization. The metabolites of interest were detected in a multiple reaction monitoring mode with the following transitions: SAM, m/z 399→250; d3-SAM 402→250; d4-SAH, m/z 385→136; SAH-d4, m/z 389→136. Quantification of SAM and SAH was performed by comparing samples with the signals obtained from the standards using Xcalibur software (Thermo).
**Statistical analysis**

Student’s t-tests (for continuous dependent variables) and Chi-square tests (for categorical variables) were performed to test for differences between the choline intake groups at baseline and at delivery.

General linear models (GLMs) were constructed to assess the effects of maternal choline intake (independent variable) on the dependent variables (e.g. cord plasma cortisol concentration, placental gene transcript abundance, and global DNA and histone methylation).

For the site specific CpG methylation data, GLMs were constructed to analyze the effects of maternal choline intake on the average CpG methylation of each gene (i.e., the mean methylation levels of all the CpG units). To explore the effects of maternal choline intake on individual CpG units, mixed models were run. The mixed models included choline intake, all the CpG units (e.g. CpG unit A, CpG unit B, etc) within the region of interest, and the two-way interaction between the CpG units and choline intake as fixed factors. Participant identifier was included as a random factor. The analyses were followed by individual contrasts assessing the effect of choline on each CpG unit.

Candidates for entry as covariates into the GLMs and mixed models are listed in Table 1.1 and Table 1.2. To adjust for the potential influence of circadian rhythm, time of delivery was included as an additional covariate. The covariates not achieving a statistical significance of $P < 0.1$ were removed from the models in a stepwise process. Baseline measures (wk 0) were also entered into the models as covariates for maternal DNA and histone methylation. To account for batch effect, batch was included in the models as a covariate if the
measurements of a variable were done in multiple runs. All two-way interactions between the
covariates and maternal choline intake were tested in the models. Covariates retained in the
final statistical models are specified following the presentation of the $P$ values.

In addition, paired t-tests were employed to assess differences in global DNA
methylation among tissues. Pearson’s correlation coefficient ($r$) was used to examine the
association of the methylation levels among the different tissues and between the different
epigenomic marks.

Plots and histograms of the residuals were used to assess normality and variance
homogeneity in the models. Dependent variables deviated from the normal distribution (e.g.
placental gene transcript abundance) were logarithmically transformed to meet the
assumption of normality. Differences between choline intake groups were considered to be
significant at $P \leq 0.05$; $P < 0.10$ was considered to be indicative of trends. Values are
presented as means ± SEM. If covariates were retained in the final model, the values
presented are predicted means; $P$ values are two-tailed. All analyses were performed using
SPSS (release 18.0 for Windows, SPSS Inc, Chicago, IL).
## Table 1.1
Baseline characteristics of singleton third trimester pregnant women prior to randomization to 480 or 930 mg choline/d

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>480 mg/d</th>
<th>930 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Age, yr (range)</td>
<td>29 (25-33)</td>
<td>28 (22-34)</td>
</tr>
<tr>
<td>Pre-pregnant BMI, kg/m² (range)</td>
<td>23.6 (20.2-31.9)</td>
<td>23.4 (19.9-29.8)</td>
</tr>
<tr>
<td>Parity, primiparas/ multiparas&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/4</td>
<td>3/9</td>
</tr>
<tr>
<td>Gestational age, week</td>
<td>27 (26-29)</td>
<td>27 (26-28)</td>
</tr>
<tr>
<td>Ethnicity, Caucasian/African American/ Latino/ Asian/ Other</td>
<td>8/0/2/1/1</td>
<td>6/1/2/3/0</td>
</tr>
<tr>
<td>Physical activity, usual daily activity/ exercise ≥ 3 times per wk/ unknown</td>
<td>4/8/0</td>
<td>1/9/2</td>
</tr>
<tr>
<td>Education, high school and college/ university level</td>
<td>1/11</td>
<td>4/8</td>
</tr>
<tr>
<td>Work status, not employed/ employed</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Health Insurance, government subsidized/ private/ unknown&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2/8/2</td>
<td>4/8/0</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05, Chi-square test; <sup>b</sup>This parameter served as a surrogate of income. Eligibility for government subsidized insurance implied a relatively lower income.
RESULTS

Mother and neonate characteristics

At baseline, maternal characteristics of the third trimester pregnant women did not differ ($P = 0.14 – 0.99$) between the choline intake groups (i.e., 930 versus 480 mg/d) with the exception of parity (i.e., there were more primiparous women in the 480 mg choline/d intake group; $P = 0.04$) (Table 1.1). All babies were delivered without major complications and were apparently healthy. Mode of delivery, gestational age at birth, infant sex, infant birth weight and Apgar score did not differ ($P = 0.11 – 0.79$) between the choline intake groups (Table 1.2).

Table 1.2

Maternal and neonatal characteristics at delivery for pregnant women consuming 480 or 930 mg choline/d throughout the third trimester

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>480 mg/d</th>
<th>930 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode of delivery, caesarean section/ vaginal</td>
<td>3/9</td>
<td>2/10</td>
</tr>
<tr>
<td>Gestational age at birth, wk (range)</td>
<td>40 (39-42)</td>
<td>40 (39-40)</td>
</tr>
<tr>
<td>Infant sex, female/ male</td>
<td>3/9</td>
<td>4/8</td>
</tr>
<tr>
<td>Infant birth weight, kg</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Infant Apgar score</td>
<td>8-9; 9</td>
<td>8-9; 9</td>
</tr>
</tbody>
</table>

Cord plasma cortisol

Cord plasma cortisol concentrations of both choline intake groups (range: 5.9 – 56.3 μg/dL) were consistent with previous observations in uncomplicated pregnancies (26). Both vaginal birth ($P = 0.004$) and primiparous pregnancy ($P = 0.04$) positively affected the concentration
Figure 1.1. Effect of maternal choline intake (930 versus 480 mg/d) on maternal and fetal cortisol. A) For neonates born vaginally, cord plasma cortisol tended to be lower ($P = 0.07$ controlling for parity; $n = 7-8$ per treatment group) in the 930 versus 480 mg choline/d maternal intake group. B) No differences ($P = 0.17; n = 10-11$ per treatment group) were detected in maternal plasma cortisol at delivery between the choline intake groups. C) No correlation (Pearson’s correlation $r = 0.13; P = 0.60$) was detected between cord and maternal plasma cortisol concentrations. For A) and B), dashed lines represent predicted mean or mean cortisol concentrations for each choline intake group, respectively.
of cord plasma cortisol. Because the number of women giving birth through caesarean section was small (n = 2-3/choline intake group), only cases of vaginal birth were further analyzed. In this subset of samples, cord plasma cortisol concentrations tended to be 33% lower ($P = 0.07$, controlling for parity) in babies born to mothers consuming 930 ($20.9 \pm 3.6 \mu g/dL$, n=8) versus 480 ($31.1 \pm 3.6 \mu g/dL$, n=7) mg choline/d (**Figure 1.1A**). Maternal plasma cortisol did not differ ($P = 0.17$) between the choline intake groups (930 mg/d: $46.1 \pm 8.3$ vs. $61.1 \pm 6.4 \mu g/dL$) (**Figure 1.1B**), nor did it correlate with cord plasma cortisol (Pearson’s correlation $r = 0.13$, $P = 0.60$) (**Figure 1.1C**). As expected (26, 27), maternal plasma cortisol was higher ($P < 0.001$) than cord plasma cortisol.

*Site specific CpG methylation*  
*CRH* and *NR3C1*

We assessed CpG methylation of the proximal promoter region of *CRH*. This region interacts with several transcription factors that regulate *CRH* expression (**Figure 1.2A**) (8). Five CpG units in this region met our quality control criteria and were included in the final analyses. In placental tissue, the higher maternal choline intake (930 vs. 480 mg/d) yielded higher ($P = 0.05$, controlling for infant sex) average CpG methylation of the *CRH* promoter region (**Figure 1.2B**). In addition, although statistical significance was not achieved, methylation of each individual CpG unit was consistently higher in the 930 mg choline/d group (**Figure 1.2C**). The higher maternal choline intake also resulted in lower ($P = 0.04$) placental *CRH* gene transcript abundance (**Figure 1.2D**). In cord leukocytes, the higher maternal choline intake yielded lower ($P = 0.05$) average promoter methylation of *CRH* (**Figure 1.2E**) and lower ($P = 0.02$) methylation of CpG unit A (containing CpG site 1)
Maternal choline intake did not affect maternal blood leukocyte average CpG methylation of CRH (480 mg/d group: 62.4 ± 0.7%; 930 mg/d group: 61.8 ± 0.8%; \( P = 0.39 \)).
Figure 1.2. Effect of maternal choline intake (930 versus 480 mg/d) on CRH promoter region CpG methylation and gene expression. A) Sequence map of the individual CpGs analyzed by Epityper and the binding sites of select transcription factors (8). B) Average placental CRH CpG methylation. C) Placental CRH CpG methylation of the individual CpG units. D) Placental CRH gene transcript abundance relative to the 480 mg/d group. E) Average cord leukocyte CRH CpG methylation. F) Cord leukocyte CRH CpG methylation of the individual CpG units. Gray bar, 480 mg choline/d group; black bar, 930 mg choline/d group. CpG units A, B, H, I, J cover CpG sites 1, 2, 8, 9, 10, respectively (Supplemental Table S1.2). Only CpG units that achieved a measurement success rate >75% were included in the analyses and were shown in C) and F). Values represent means ± SEM, except for B) and C) which are predicted means controlling for infant sex; n = 11-12 per treatment group. * P ≤ 0.05 between choline intake groups.
We also examined CpG methylation of the CpG-rich region of *NR3C1* including exon 1F (Figure 1.3A). In rats, hippocampal CpG methylation of the homolog of this region (*Nr3c1* exon 1γ) is altered by maternal care with downstream effects on HPA axis reactivity (10). As rat *Nr3c1* exon 1γ (and presumably human *NR3C1* exon 1F) contains a binding site for nerve growth factor inducible protein A (NGFI-A), the methylation state of this region may modify NGF1-A binding with subsequent effects on *NR3C1* expression. In the present study, 15 CpG units within this region were included in the analyses. The higher maternal choline intake (930 versus 480 mg/d) yielded higher (*P* = 0.002) average placental *NR3C1* promoter methylation (Figure 1.3B), as well as higher placental methylation of CpG unit C (containing CpG sites 5-7) (*P* = 0.01), F (containing CpG site 12) (*P* = 0.001), M (containing CpG site 29) (*P* = 0.003) and N (containing CpG sites 30-32) (*P* = 0.05) (Figure 1.3C). In cord leukocytes, the higher maternal choline intake yielded lower (*P* = 0.04) average *NR3C1* promoter methylation (Figure 1.3D), as well as lower (*P* = 0.003) methylation of CpG unit E (containing CpG sites 10, 11) (Figure 1.3E). However, maternal choline intake did not alter methylation of placental or cord leukocyte CpG unit R (CpG sites 37, 38) (*P* = 0.55 and 0.99, respectively), where the predicted NGFI-A binding site resides; nor did it alter placental *NR3C1* transcript abundance (*P* = 0.44). Maternal choline intake did not affect maternal blood leukocyte average CpG methylation of *NR3C1* (480 mg/d group: 5.0 ± 0.2%; 930 mg/d group: 5.5 ± 0.3%; *P* = 0.11).
Figure 1.3. Effect of maternal choline intake (480 versus 930 mg/d) on NR3C1 promoter region CpG methylation. A) Sequence map of individual CpGs analyzed by Epityper in NR3C1 exon 1F (1). NR3C1 contains multiple non-translated exon 1s. Translation starts from exon 2. The exon 1F contains a predicted NGFI-A binding site (underlined). B) Average NR3C1 placental CpG methylation. C) Placental NR3C1 CpG methylation of the CpG units. CpG unit R represents the NGFI-A binding site. D) Average NR3C1 cord leukocyte CpG methylation. E) Cord leukocyte NR3C1 CpG methylation of the CpG units. A CpG unit may contain one or multiple CpG sites (Supplemental Table S1.2). Only CpG units had measurement success rates >75% were included in the analyses. Gray bar, 480 mg choline/d group; black bar, 930 mg choline/d group. Values represent means ± SEM; n = 8 - 12/treatment group. ** P < 0.01 or * P ≤ 0.05 between choline intake groups.
**GNAS-AS1, IGF2, IL10 and LEP**

To further investigate the influence of maternal choline intake on site specific DNA methylation, the promoter CpG methylation of 4 additional genes involved in different physiological pathways was assessed. For *GNAS-AS1*, average placental and cord blood leukocyte CpG methylation were not altered by maternal choline intake (*P* = 0.17 and 0.88 respectively); however, CpG unit C (containing CpG site 5) of *GNAS-AS1* displayed lower (*P* = 0.02) placental methylation in the 930 versus the 480 mg choline/d intake group (Supplemental Figure 1). The average and individual CpG methylation of *IGF2, IL10* and *LEP* were not altered (*P* = 0.20 – 0.87) by maternal choline intake in either placental tissue or cord blood leukocytes (Table 1.3 and Supplemental Figure S1.1). However, our ability to detect subtle alterations in DNA methylation within these genes was precluded by the study’s small sample size.
Table 1.3
Percent DNA methylation of selected genes in the placental and cord leukocyte samples obtained from the 480 and 930 mg choline /d maternal intake groups

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Placenta</th>
<th>Cord leukocyte</th>
<th>P value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>480 mg/d</td>
<td>930 mg/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNAS-AS1</td>
<td>Growth regulation</td>
<td>50.5 ± 1.0</td>
<td>48.0 ± 1.4</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>Growth regulation</td>
<td>36.5 ± 1.5</td>
<td>33.5 ± 1.6</td>
<td>0.21</td>
<td>35.4 ± 0.8</td>
</tr>
<tr>
<td>IL10</td>
<td>Anti-inflammation</td>
<td>79.0 ± 0.5</td>
<td>77.6 ± 0.6</td>
<td>0.20</td>
<td>22.6 ± 1.2</td>
</tr>
<tr>
<td>LEP</td>
<td>Energy metabolism</td>
<td>24.5 ± 1.6</td>
<td>23.9 ± 1.7</td>
<td>0.78</td>
<td>14.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Modified from Talens et al.(17). bThe statistical analysis controlled for parity. Values presented are predicted means. cThe statistical analysis controlled for infant sex. Values presented are predicted means. Data without notation are presented as mean ± SEM.
Global DNA methylation

Placental global DNA methylation was 22% higher \( (P = 0.02) \) in the 930 mg choline/d maternal intake group \( (4.4 \pm 0.2\%) \) compared to the 480 mg choline/d group \( (3.6 \pm 0.2\%) \) (Figure 1.4A). Because the study participants consumed 20% of their total choline intake as trimethyl-d9 choline chloride (i.e., methyl groups of the choline molecule are labeled with deuterium) throughout the last half of the study, we were able to examine placental isotopic enrichment percent of 5mdC, i.e. d3-5mdC / total 5mdC * 100. Remarkably, placental enrichment of 5mdC was detected in both choline intake groups \( (480 \text{ mg/d: } 0.14 \pm 0.02 \text{ vs. } 930 \text{ mg/d: } 0.21 \pm 0.03\%) \) and tended to be higher \( (P = 0.09) \) in the 930 mg choline/d group (Figure 1.4B). This finding demonstrates unequivocally that dietary choline derived methyl groups are used for DNA methylation and support the putative role of choline as an epigenetic regulator of gene expression.

Leukocyte global DNA methylation in maternal \( (480 \text{ mg/d: } 5.2 \pm 0.3 \text{ vs. } 930 \text{ mg/d: } 5.0 \pm 0.2\%; P = 0.50) \) and cord \( (480 \text{ mg/d: } 5.0 \pm 0.1 \text{ vs. } 930 \text{ mg/d: } 4.9 \pm 0.1\%; P = 0.60) \) blood was not altered by maternal choline intake.

As previously reported by others (28), global DNA methylation percent was lower \( (P < 0.001) \) in placental tissue than cord and maternal leukocytes. No correlations in global DNA methylation were observed among maternal leukocytes, cord leukocytes and placental tissue.

Global histone methylation

Placental H3K9me2, a transcription repression and heterochromatin marker, was 20% higher \( (P = 0.02) \) among women consuming 930 versus 480 mg choline/d (Figure 1.4C) and was positively correlated with placental global DNA methylation (Pearson’s correlation \( r = \)).
0.40, $P = 0.05$). Maternal ($P = 0.48$) and cord leukocyte ($P = 0.58$) H3K9me2 did not differ between the choline intake groups, nor was placental H3K4me2 ($P = 0.91$), a transcription activation marker or H3K27me3 ($P = 0.48$), a transcription repression marker altered by maternal choline intake (Figure 1.4B).

Figure 1.4. Effect of maternal choline intake (930 versus 480 mg/d) on epigenomic markers and methyltransferase gene transcript abundance. A) Percent global DNA methylation in placenta, cord leukocytes and maternal leukocytes. B) Deuterium labeled methylated cytosine enrichment (percent d3-5mdC to 5mdC) in placenta. C) Fold difference of placental global H3K9me2, H3K4me2 and H3K27me3. D) Fold difference of placental gene transcript abundance of $DNMT1$ and $EHMT2$. Gray bar, 480 mg choline/d group; black bar, 930mg choline/d group. Values represent means ± SEM; n = 12 /treatment group. * $P \leq 0.05$, (*) $P < 0.1$ between choline intake groups.
Gene expression of DNA and histone methyltransferases

To further clarify the mechanisms by which varied maternal choline intake may modulate epigenetic marks, the expression of several methyltransferases was examined. In placenta, the higher maternal choline intake (930 vs 480 mg/d) yielded higher (36%, \( P = 0.02 \)) gene transcript abundance of \( EHMT2 \), the histone methyltransferase that mediates H3K9 dimethylation (Figure 1.4D). In addition, the gene transcript abundance of \( DNMT1 \), the DNA methyltransferase that functions in DNA methylation maintenance, tended to be higher (33%, \( P = 0.09 \)) in the 930 mg/d group (Figure 1.4D). The expression levels of DNA methyltransferases that mediate \textit{de novo} DNA methylation establishment (e.g. \( DNMT3A \) and \( DNMT3B \)) and histone \( SUV39H1 \) which mediates H3K9 methylation, were not altered (\( P = 0.45 - 0.61 \)) by maternal choline intake (data not shown).

Placental SAM and SAH

SAM is the direct methyl donor for methylation reactions. SAH is formed when the methyl group of SAM is transferred to a methyl acceptor. Despite the changes in global DNA and histone methylation, placental concentrations of SAM, SAH and the SAM/SAH ratio did not differ (\( P = 0.38 - 0.97 \)) between the maternal choline intake groups (\textbf{Table 1.4}). We suggest that these static measures may not adequately capture the enhanced flow of SAM through the methionine cycle (i.e, homeostatic mechanisms are engaged to prevent alterations in metabolite concentrations).
Table 1.4

Placental $S$-adenosylmethionine (SAM) and $S$-adenosylhomocysteine (SAH) concentrations in the 930 versus 480 mg choline /d maternal intake groups

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>480 mg/d</th>
<th>930 mg/d</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM, nmol/g</td>
<td>5.92 ± 0.32</td>
<td>5.90 ± 0.38</td>
<td>0.97</td>
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<tr>
<td>SAH, nmol/g</td>
<td>1.82 ± 0.11</td>
<td>1.92 ± 0.08</td>
<td>0.53</td>
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<tr>
<td>SAM/SAH</td>
<td>3.31 ± 0.16</td>
<td>3.10 ± 0.17</td>
<td>0.38</td>
</tr>
</tbody>
</table>
DISCUSSION

This study provides compelling evidence that maternal choline intake during the third trimester of human pregnancy can modify global and site-specific epigenetic marks in fetal derived tissues. Alterations in fetal epigenetic marks by maternal choline intake may have long lasting functional effects as demonstrated in animal studies (6, 7). The modified epigenetic state of cortisol regulating genes in response to varied prenatal choline exposure is particularly noteworthy as it implies an effect of maternal choline intake on programming of the HPA axis.

*Maternal choline intake alters the epigenetic profile of cortisol regulating genes*

CRH is a main regulator of HPA axis reactivity and cortisol production. During pregnancy, large amounts of CRH are produced by placenta (expressed in high primate species only), which stimulate the HPA axis in both the maternal and fetal compartments to produce cortisol (29, 30). In the present study, promoter methylation of placental *CRH* was higher, and its transcript abundance lower, in women consuming 930 versus 480 mg choline/d. The observed alteration in CpG methylation may have modified the expression of *CRH* by changing accessibility of transcription factors to their regulatory elements in this region (8). The decrease in placental *CRH* expression in the higher maternal choline intake group may attenuate fetal HPA axis reactivity and is consistent with the ~33% lower cord plasma cortisol concentrations in babies of mothers consuming 930 versus 480 mg choline/d.

*NR3C1* encodes the glucocorticoid receptors and is regulated by promoter region methylation (1, 2, 10). In the placenta, glucocorticoids bind to glucocorticoid receptors which stimulate *CRH* expression via a feedforward mechanism (unlike the central HPA axis where
glucocorticoids inhibit *CRH* by a negative feedback mechanism) (8). The increased methylation of the placental glucocorticoid receptor observed in the present study may decrease its expression and attenuate placental expression of *CRH*. Nonetheless, we were unable to detect a change in the transcript abundance of placental *NR3C1*.

The change in the epigenetic state of the cortisol regulating genes was not limited to placenta. However, in contrast to placenta, cord leukocyte *CRH* and *NR3C1* methylation were lower in the 930 versus 480 mg choline/d intake group. The lower CpG methylation of cord leukocyte *CRH* and *NR3C1* in the 930 mg/d group may represent a secondary response to the altered epigenetic state of the placental HPA axis genes and to the lower circulating cortisol. Decreased promoter methylation of *NR3C1* in the central HPA axis is associated with increased sensitivity to cortisol stimuli and improved feedback inhibition (10). While the value of leukocyte *NR3C1* promoter methylation as a proxy of hypothalamic *NR3C1* and/or HPA reactivity has yet to be ascertained, a cross-sectional study reported a direct relationship between cord leukocyte *NR3C1* CpG methylation in the NGFI-A binding site and salivary cortisol concentrations in babies at 3 months of age (1).

These data collectively suggest that a higher maternal choline (i.e., 930 versus 480 mg choline) may lower fetal/neonatal circulating cortisol by altering the methylation state of cortisol regulating genes in both the placental and fetal compartments. Notably, abnormal increases in placental CRH are associated with obstetric complications such as preeclampsia and intrauterine growth restriction (IUGR) (30), and IUGR neonates exhibit elevated cord blood cortisol (31). Furthermore, a heightened fetal HPA reactivity as a result of early life exposures (e.g. prenatal glucocorticoid overexposure and maternal anxiety), has been shown
to enhance lifelong vulnerability to stress induced illness and chronic conditions such as hypertension and insulin resistance (13, 15, 16). As such, the study findings raise the exciting possibility that prenatal choline supplementation may be employed therapeutically in cases where excess maternal stress (i.e., anxiety, depression) and/or other prenatal conditions might adversely affect fetal HPA axis reactivity and increase vulnerability to stress-related diseases.

**Maternal choline intake affects placental global DNA and histone methylation**

The higher maternal choline intake (930 vs. 480 mg/d) yielded higher placental genome wide DNA methylation and H3K9me2, as well as increased expression of *DNMT1* and *EHMT2*. These results are consistent with studies in rodents showing that *in utero* choline exposure altered epigenomic marks and methyltransferase expression in several fetal tissues (3, 5). As maintenance of DNA methylation is an important strategy to ensure genomic integrity and H3K9me2 is a marker of transcription repression and heterochromatin formation, the increase of these two epigenetic markers in placenta by a higher maternal choline intake may systematically affect genome stability and expression. In turn, a wide array of placental functions, including endocrine regulation, may be influenced by maternal choline intake which merits further investigation.

**Placental tissue is highly susceptible to epigenetic modification**

The role of the placenta as a mediator of fetal programming is increasingly recognized (11, 32). More specifically, the placenta acts on behalf of the fetus as both a sensory and effector organ to facilitate the incorporation of environmental information (i.e, nutrient exposure) into the developmental process (33). In the present study, the epigenetic state of the placenta was highly sensitive to varied maternal choline intake during the third trimester of
pregnancy with nearly all the examined placental epigenetic marks (global and site specific DNA methylation, global histone methylation) showing some degree of alteration. On the contrary, the leukocyte epigenetic state of the mothers, who received the choline treatment directly, was relatively stable. The plasticity of the placental epigenome in response to maternal nutrition may provide a mechanism through which the maternal environment can alter placental function and ultimately fetal development.

Figure 1.5. A proposed mechanism by which a higher maternal choline intake may modulate the epigenetic and epigenomic marks of fetal derived tissues. Solid arrows, process enhanced by a higher maternal choline intake; dashed arrows: process attenuated by a higher maternal choline intake.
Proposed mechanism by which maternal choline intake modulates epigenetic and epigenomic marks

Maternal choline intake may alter the epigenetic and epigenomic marks of fetal derived tissues by influencing the supply of methyl donors (Figure 1.5). The use of choline as a methyl donor transpires predominately in the liver where the choline derivative, betaine, provides a methyl group for the conversion of homocysteine to methionine in a reaction catalyzed by betaine-homocysteine S-methyltransferase (BHMT) (34). Following the activation of methionine to SAM, the choline-derived methyl group may be used in cellular methylation reactions including the methylation of DNA and histones. As the placenta does not express BHMT, the choline-mediated placental epigenetic and epigenomic modifications that were observed in the present study, depend on the maternal supply, and placental uptake, of methionine and SAM, both of which can transport choline/betaine derived methyl groups from the maternal liver to extrahepatic tissues including the placenta and fetus.

As shown in Figure 1.5, we propose that the higher maternal choline intake increased the use of choline as a methyl donor for methionine and SAM production in the maternal compartment. Uptake of methionine and SAM by the placental tissue yielded greater placental global DNA methylation and H3K9me2, as well as greater CRH promoter CpG methylation, either directly (increased placental uptake and utilization of methionine and SAM) and/or indirectly via upregulation of DNMT1 and/or EHMT2. A higher CRH methylation diminished CRH transcription and the entry of CRH into the fetal compartment. In turn, stimulation of the fetal central HPA axis and adrenal production of cortisol was attenuated thereby lowering circulating concentrations of cortisol. This reduction in
circulating fetal cortisol concentrations may have triggered other regulatory events that resulted in a lowering of CRH and NR3CI CpG methylation.

Conclusions

In summary, the study findings suggest that maternal choline intake throughout the third trimester of pregnancy can alter placental epigenomic marks as well as the epigenetic state of key modulators of placental and fetal HPA axis reactivity. As such, the study findings raise the exciting possibility that a higher maternal choline intake may counter some of the adverse effects of prenatal stress on behavioral, neuroendocrine and metabolic development in the offspring. The investigation of the influence of genetic variants in one-carbon metabolizing genes on epigenetic and epigenomic responses to varied maternal choline intake are planned. Additional studies in larger independent cohorts are needed to replicate these results and explore the long-term functional consequences of these choline-induced epigenetic alterations.
REFERENCES


5. Davison JM, Mellott TJ, Kovacheva VP, Blusztajn JK. Gestational choline supply regulates methylation of histone H3, expression of histone methyltransferases G9a (Kmt1c) and Suv39h1 (Kmt1a), and DNA methylation of their genes in rat fetal liver and brain. Journal of Biological Chemistry 2009;284(4):1982-9.


Res 2003;31(9):e47.


Supplemental Table S1.1. Information of the gene promoter regions where CpG methylation was measured and primers used

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Chromosome</th>
<th>Length</th>
<th>No. of CpG sites</th>
<th>Forward primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRH</strong></td>
<td>chr8:67,090,692-67,091,132</td>
<td>441bp</td>
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<td>5'TGGTTGTTGTTTTTTTGTAGG 3'</td>
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<td><strong>GNAS-AS1</strong></td>
<td>chr20:57,425,81-57,426,119</td>
<td>305bp</td>
<td>19</td>
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<td><strong>IGF2</strong></td>
<td>chr11:2,169,459-2,169,796</td>
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<td>5'TGGATAGGAGATTGAGGAGAAGA 3'</td>
<td>5'AAACCCCAACAAAAACCACT 3'</td>
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<td><strong>IL10</strong></td>
<td>chr1:206,946,011-206,946,339</td>
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<td>chr7:127,881,054-127,881,410</td>
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<td><strong>NR3C1</strong></td>
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<sup>a</sup>Primers were previously published by Talens et al (17) and Oberlander et al (1). The 5’ ends of the forward primers are connected with a 10-mer tag: 5’ AGGAAGAGAG + forward primer 3’; the 5’ ends of the reverse primers are connected with a T7 tag: 5’

CAGTAATACGACTCATAAGGAGAAGGCT + reverse primer 3’. <sup>†</sup>This pair of primers was used for placental and cord leukocyte samples. Primers for maternal leukocytes were F: 5’ GATTTGGTTTTTTTGGG 3’; R: 5’ TCCCTTCCCTAAAACCT 3.
Supplemental Table S1.2. The corresponding CpG sites of each CpG unit

<table>
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<th>CpG unit</th>
<th>Corresponding CpG sites</th>
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<sup>a</sup>For placental and cord leukocyte samples; <sup>b</sup>for maternal leukocyte samples
### Supplemental Table S1.3. Selection of CpG units for final analyses

<table>
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<tr>
<th>Gene symbol</th>
<th>CpG units included</th>
<th>CpG units excluded&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
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<td>CRH</td>
<td>A, B, H~J</td>
<td>C, D (SN); E (H_mass); F, G (D)</td>
</tr>
<tr>
<td>GNAS-AS1</td>
<td>A~K</td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>A-D, F, G</td>
<td>E (NS)</td>
</tr>
<tr>
<td>IL10</td>
<td>A~C</td>
<td></td>
</tr>
<tr>
<td>LEP</td>
<td>A, C~N, P, Q</td>
<td>B, R (H_mass); O (NS)</td>
</tr>
<tr>
<td>NR3C1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A-G, K, M, N, P~R, T, U</td>
<td>H, J, O, S (L_mass); I, L, V(H_mass)</td>
</tr>
<tr>
<td>NR3C1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>E, F, H<del>J, L, N</del>P, R, T</td>
<td>A, G, K, M, S (H_mass); B, Q (L_mass); C, D (NS)</td>
</tr>
</tbody>
</table>

<sup>a</sup>CpG units were included if 75% samples had successful measurements; Reasons for exclusion were indicated in parentheses. NS: not specified; SN: high signal to noise ratio; H_mass: the CpG unit had high mass and was not reliably detected; L_mass: the CpG unit had low mass and was not reliably detected; D: duplicate CpG unit (e.g. two CpG units being of the same mass).<sup>b</sup>For placental and cord leukocyte samples. <sup>c</sup>For maternal leukocyte samples.
Supplemental Figure S1.1. Effects of maternal choline intake (930 versus 480 mg choline /d) on the placental (A-D) and cord leukocyte (E-H) CpG methylation of the individual CpG units of GNAS-AS1, IGF2, LEP and IL10. Gray bar: 480 mg choline /d group; black bar: 930 mg choline /d group. Values represent means ± SEM, except for (B) and (G), which are predicted means controlling for parity and infant sex, respectively; n = 11-12 per treatment group. * P ≤ 0.05 between choline intake groups.
CHAPTER 2

A higher maternal choline intake among third trimester pregnant women lowers placental and circulating concentrations of the anti-angiogenic factor fms-like tyrosine kinase-1 (sFLT1)*


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ABSTRACT

This study investigated the influence of maternal choline intake on the human placental transcriptome, with a special interest in its role in modulating placental vascular function. Healthy pregnant women (n=26, wk 26-29 gestation) were randomized to 480 mg choline/d, an intake level approximating the adequate intake of 450 mg/d, or 930 mg/day for 12-wk. Maternal blood and placental samples were retrieved at delivery. Whole genome expression microarrays were conducted in a subset of samples (n=6 / maternal choline intake group) to identify placental genes and biological processes impacted by maternal choline intake. Maternal choline intake influenced a wide-array of genes (n=166) and biological processes (n=197) including those related to vascular function. Of special interest is the anti-angiogenic factor and preeclampsia risk marker fms-like tyrosine kinase-1 (sFLT1). Quantitative real time PCR conducted in all placental samples confirmed the 30% downregulation ($P=0.05$) of sFLT1 in the placenta tissues obtained from the 930 versus 480 mg choline/d intake group. Similar decreases ($P=0.04$) were detected in maternal blood sFLT1 protein concentrations. The downregulation of sFLT1 by choline treatment was confirmed in a human trophoblast cell culture model and may be related to enhanced acetylcholine signaling. These findings indicate that supplementing the maternal diet with extra choline may improve placental angiogenesis and mitigate some of the pathological antecedents of preeclampsia.
INTRODUCTION

The human placenta is a highly vascularized organ that plays a critical role in nutrient transport, endocrine homeostasis and immune function. Vasculogenesis and subsequent angiogenesis are required for proper placental development and function and are regulated by angiogenic factors produced by placental cells (e.g., trophoblasts) (1).

Excess placental production of the anti-angiogenic factors, soluble fms-related tyrosine kinase-1 (sFLT1) and soluble endoglin, play a key role in the pathogenesis of preeclampsia (2, 3) and possibly other diseases of pregnancy (1). These placental anti-angiogenic factors are released into the maternal circulation where they can sequester pro-angiogenic factors [vascular endothelial growth factor (VEGF), placental growth factor (PGF), and/or transforming growth factor beta], thereby inhibiting normal maternal endothelial cell signaling (2). Disturbances in maternal endothelial cell signaling cause endothelial cell dysfunction and result in hypertension, proteinuria, and other systemic manifestations of preeclampsia. Thus treatments that restore placental angiogenic balance may reduce the risk of diseases caused by placental dysfunction (2, 4, 5).

Choline is an essential nutrient that functions in phospholipid metabolism, neurotransmission, and as a methyl donor (6). Several biological processes are modified by choline supply including inflammation (7), apoptosis (8), and angiogenesis (9). During pregnancy, the demand for choline increases substantially to support the growth and development of the placenta and fetus (6, 10-12). Notably, supplementing the maternal rodent diet with extra choline during pregnancy yields lasting improvements in offspring cognitive functioning (13) and reduces vulnerability to mammary tumors in the female offspring (14).
The lasting beneficial effects of maternal choline intake on offspring cognitive and physiologic functioning are mediated in part by epigenetic mechanisms, e.g. altering DNA and histone methylation (15). Notably, the epigenome of the human placenta is especially responsive to maternal choline intake. Specifically, we showed that a higher maternal choline intake (930 versus 480 mg/d) yielded 22% higher placental genome-wide methylation and altered the methylation state, and expression, of placental corticotropin-releasing hormone (16). In addition, we observed an elevation in placental acetylcholine, which plays a critical role in placenta development and function, in the 930 versus 480 mg/d choline intake group (11). These data collectively indicate that maternal choline intake may alter several biological processes in the placenta including its vascular development.

As an extension of our previous work (11), this study investigated the effects of maternal choline intake on the placental transcriptome, with a special interest in its role in modulating placental vascular function. To achieve these aims, we conducted whole genome expression microarrays on placental tissues previously obtained from third trimester pregnant women randomized to either 480 mg choline/d, an intake level slightly above the adequate intake of 450 mg/d, or 930 mg/day for 12-weeks (11). Biological processes of particular interest, such as angiogenesis, were further investigated with a human placental cell culture model.
MATERIALS AND METHODS

Study participants

Healthy third trimester (weeks 26-29 gestation) singleton pregnant women aged ≥ 21 y were recruited from Ithaca, New York, and surrounding areas between January 2009 and October 2010. Entry into the study was contingent upon good health status as assessed by a blood chemistry profile, complete blood count and medical history questionnaire. Additional inclusion criteria included no tobacco or alcohol use, no history of chronic disease, normal kidney and liver function, and a willingness to comply with the study protocol (11). Exclusion criteria included the use of prescription medications known to affect liver function, multiple pregnancy, or pregnancy associated complications, e.g., preeclampsia, gestational diabetes.

The screening and experimental procedures were approved by the Institutional Review Boards for Human Participants at Cornell University and Cayuga Medical Center, and written informed consent was obtained prior to study entry. The study was registered at ClinicalTrials.gov as NCT01127022 (http://clinicaltrials.gov/) and the CONSORT flowchart has been reported (11).

Study design, diet and supplements

This was a 12-wk controlled feeding study in which pregnant women (n = 26) were randomized to either 480 (approximating the adequate intake level; n=13) or 930 (n=13) mg choline/d as previously described (11). The choline was derived from the diet (380 mg/d) plus supplemental choline chloride (either 100 or 550mg choline/d for the 480 or 930 mg/d intake levels, respectively). Throughout the 12-wk study, the participants consumed a 7-day cycle menu (11). All food and beverage was provided by the investigators and one meal per day
was consumed on-site throughout the week. All other meals and beverages were provided as take-away.

In addition to the supplemental choline (Balchem, New Hampton, NY, USA), study participants consumed a daily 200 mg docosahexaenoic acid supplement (Neuromins, Nature’s Way Products, Springville, UT, USA), a daily over-the-counter prenatal multivitamin supplement (Pregnancy Plus®, Fairhaven Health, LLC, Bellingham, WA, USA), and a thrice weekly potassium/magnesium supplement (General Nutrition Corp., Pittsburgh, PA, USA) in order to achieve recommended nutrient intake levels not met with the study diet. When eating on-site, participants consumed supplements under the supervision of study personnel. Otherwise, supplements were provided in plastic bags along with take-away meals and participants were instructed to consume the supplements with a meal of their choice. At the end of the study, participants continued on their choline supplement until the delivery of their babies (11). The study design, diet composition and supplements, and participant compliance were fully described in Yan et al, 2012 (11).

At delivery, placental and maternal blood samples were retrieved from 24 of the 26 participants (n = 12/ choline intake group). Information regarding the health status of the study participants, the delivery, and the health parameters of the newborns were obtained from medical charts.

Sample collection

Fasting (10-h) venous serum samples were obtained at study baseline (week 0) and within 24 hours before delivery at the hospital in a 10mL serum separator gel and clot-activator tube (SST Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (11). Placental
samples were processed at the hospital within 90 minutes of delivery (16). After removal of the amnion, the placenta was visually divided into four quadrants and full thickness biopsies were taken from each quadrant. The biopsies were immediately rinsed with phosphate buffered saline and stored in tubes with RNAlater® (Qiagen, Valencia, CA, USA) which was added to prevent RNA degradation. All samples were stored at -80°C. As the placenta is a heterogeneous tissue, the results obtained from the study represent an average effect of maternal choline intake on a subset of placental cells. The difference in the placental biomarkers observed in the two maternal choline intake groups could be attributed to the differential production of these biomarkers in a specific cell type, changes in placental cell population composition, and/or changes in cell numbers.

HTR-8/SVneo cell culture

HTR-8/SVneo is a stable cell line derived from human first trimester placental extravillous trophoblast and immortalized with the large T-antigen SV-40 (17). The cells, kindly provided by Dr. Charles Graham (Queens University, Kingston, Ontario, Canada), were maintained in RPMI1640 culture medium (Mediatech. Inc, Manassas, VA, USA) with 2 mM L-glutamine and 5% fetal bovine serum (FBS) in 5% CO₂-95% air at 37°C. For experiments, customized RPMI1640 medium (Life Technologies, Grand Island, NY, USA) which contained no choline, 2 mM L-glutamine and 1.25% FBS were used. The FBS provided the cells with 8 μM total choline (including free choline, phosphocholine, lysophosphatidylcholine, glycerophosphocholine, phosphatidylcholine and sphingomyelin) as measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in our laboratory (11). Choline chloride was added to the media in the amounts of 0, 5 and 20
μM, yielding total choline concentrations of 8, 13 and 28 μM, and generating a low, medium and high choline group, respectively. Cells were seeded in the media containing the different choline concentrations at a starting amount of $1.39 \times 10^6$ cells (passages 5-20) per 100×20 mm dish (BD Biosciences). The cells were cultured for 96-h before harvest. Cell counts were performed with a TC10™ automatic cell counter (Bio-Rad, Hercules, CA, USA). Cell viability was measured by Trypan blue dye exclusion. RNA and protein were extracted as described below.

**RNA extraction**

Total RNA was extracted from the placental tissue and HTR-8/SVneo cell samples via commercially available kits [RNeasy Mini kit (Qiagen) for the placental tissue and PerfectPure RNA cultured cell kit (5 Prime Inc, Gaithersburg, MD, USA) for the cells]. The integrity of the RNA samples prepared for the microarray was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA Integrity Number >8.0 (n = 6 per choline intake group) were used for the microarray analysis. Agarose gel electrophoresis was used to ensure intact ribosomal bands in the trophoblast RNA.

**Microarray preparation and data analysis**

Microarrays were conducted in samples (n = 6/ maternal choline intake group) that met the RNA integrity cut off noted above. Antisense RNA amplification and cyanine-3 labeling were performed with an Amino Allyl MessageAmp II aRNA amplification kit (Ambion Inc., Grand Island, NY, USA). Microarray hybridization was performed at 65°C for 17-h in an Agilent Microarray Hybridization oven. Microarray scanning was performed in an Agilent
Scanner (G2505C) and the images were extracted using Agilent Feature Extraction Software 10.5. Data normalization was performed using log$_2$ transformation and median normalization. An extension of the Laplace approximation EM Microarray Analysis (LEMMA) package based on the R software platform (18) developed by Bar et al. (19, 20) was used to detect differentially expressed genes. The version used here (Bar H.Y., unpublished observations) can sensitively detect differentially expressed genes, as it takes into account not only mean differences, but also variational differences between the two treatment groups. The differentially expressed genes were declared by controlling the false discovery rate (FDR) of < 0.2 and an expression fold difference of > 1.5. Differentially expressed genes were classified according to their gene ontology using High-Throughput GoMiner (21). All data is compliant with the Minimum Information About a Microarray Experiment (MIAME) guideline. The data have been deposited in the NCBI's Gene Expression Omnibus database (22) as detailed on MGED Society website (http://www.mged.org/Workgroups/MIAME/miame.html), and are accessible through GEO Series accession number GSE39290. Microarray results of select genes were verified using quantitative real-time PCR.

Reverse transcription and quantitative real-time PCR

Reverse transcription and quantitative real-time PCR were performed in all placental samples (n = 12 / maternal choline intake group) as previously described (23). All primers were designed using GeneRunner Version 3.01 (http://www.softpedia.com/). The primers for FLT1 transcripts [sFLT1, membrane bound FLT1 (mFLT1) and total FLT1], the choline metabolizing genes [i.e., phosphate cytidylyltransferase 1, choline, alpha (PCYT1A),
phosphatidylethanolamine N-methyltransferase (PEMT), choline dehydrogenase (CHDH)],
the cholinergic receptor, muscarinic 4 (CHRM4), and the housekeeping genes [i.e.,
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta glucuronidase (GUSB)] are:

CHDH, F: 5'-GCAAGGAGGTGATTCTGAGTG-3';
    R:5'-GGATGCCAGTTTCTTGAGGTC-3'

CHRM4, F: 5'-TGTGCTGATCTCATCATAGGC-3';
    R:5'-TCACCACGTAGTCCAGGGC-3'

mFLT1, F: 5'-AGGGGAAGAAATCCTCCAGAAG-3';
    R: 5'-GCATGACAGTCTAAAGTGTTG-3'
sFLT1, F: 5'-AGGGGAAGAAATCCTCCAGAAG-3';
    R: 5'-GTGGTACAATCATCCTCCTTGCT-3'

Total FLT1, F: 5’- CACATGACTGAAGGAGGAGGAGC-3’;
    R: 5'-GTCCCAAGATTATGCGTTTCCA-3'

GAPDH, F: 5'-TGTTGCCATCAATGACCCCTT-3';
    R: 5'-CTCCACGACGTACTCAGCG-3'

GUSB, F: 5'-CTCTTGGTATCACGACTACGGG 3';
    R: 5'-CAATCGTTTCTGCTCCATACTC-3'

PCYT1A, F: 5'-CAGAAGGTGGAGGAAAAAGCA-3';
    R: 5'-TATGTTTCTAGTGCTCCTCCCG-3'

PEMT, F: 5'-GGGGGTTCGCTGGAACTTTC-3';
    R: 5'-GCCCAAGGTAGTGGCTGTG-3'

Data were expressed by the delta delta Ct method in which the expression level of the gene of
interest was normalized by the expression level of housekeeping gene as fold change before comparison between samples (24). GAPDH and GUSB were used as the housekeeping gene for the placental tissue or the cell culture model, respectively.

*sFLT1 measurement*

Circulating concentrations of sFLT1 in maternal serum samples obtained at study baseline and at delivery were measured using an ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

*FLT1 promoter cytosine-phosphate-guanine (CpG) methylation*

Site specific promoter methylation of FLT1 was analyzed using base-specific cleavage and mass spectrometry (25). Briefly, genomic DNA was extracted via the DNeasy blood & tissue kit (Qiagen) and bisulfite-treated using the EZ DNA methylation kit (Zymo, Irvine, CA, USA). The FLT1 promoter region was PCR amplified using the bisulfite-treated DNA as the template and published primers (26). The T7 tag and 10 mer tag were incorporated into the reverse and forward primers respectively. The primers were:

Forward: 5’ aggaagagagGGTGGAGGGAGTTTGTAAGGA 3’

Reverse: 5’ cagtaatcgactcactatagggagaaggt CCTCCCCACCTACCCTCTTC 3’

The amplification products were analyzed with the MassArray EpiTYPER system (Sequenom Inc, San Diego, CA, USA) at the Cornell Life Sciences Core Laboratories Center (Cornell University). Percent methylation of the cytosine residues was determined for each CpG unit, which may contain one or multiple adjacent CpG dinucleotides (CpG sites) that cannot be resolved individually by EpiTYPER. Only CpG units with measurement success rates of > 75% were included in the final analyses (27).
**Cellular sFLT1 protein measurements**

sFLT1 protein concentrations in the HTR-8/SVneo cells were measured with western blot assays. The protein was extracted with PhosphoSafe™ Extraction Reagent and protease inhibitor cocktail set III (EMD Millipore, Billerica, MA, USA), subjected to SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride membrane. Rabbit anti-human sFLT1 primary antibody (Life Technologies) and reference protein primary antibody (mouse anti-human β-actin, Cell Signaling Technology, Danvers, MA, USA) were added overnight at 4°C, after which secondary antibodies, IRDye 800CW Goat Anti Rabbit and IRDye 680 Goat Anti mouse (LI-COR, Lincoln, NE, USA), were added. Target protein bands were visualized and quantified by the LI-COR Odyssey® imaging system (LI-COR) and expressed as the ratio of intensity of sFLT1 to the reference protein (β-actin).

**Statistical analysis**

Student’s t-tests (for continuous dependent variables) and Chi-square tests (for categorical variables) were performed to test for difference in the dependent variables between the choline intake groups at study baseline and at delivery. General linear models (GLMs) were used to assess the effect of maternal choline intake on the dependent variables (e.g. placental mRNA abundance, circulating sFLT1). Candidates for entry as covariates into the GLMs are listed in Table 2.1. All 2-way interactions between the covariates and maternal choline intake were tested in the models. The covariates and interactions not achieving a statistical significance of $P < 0.05$ were removed from the models in a stepwise process. Covariates retained in the final statistical models are specified following the presentation of the $P$ values.

One-way ANOVA followed by post-hoc Fisher’s LSD tests were performed to assess the
effects of choline treatment on cell count and sFLT1 expression in the cell culture model. The maternal circulating sFLT1 and quantitative PCR data were log-transformed to meet the normality criteria (i.e. residuals were normally distributed). All analyses were performed using SPSS (release 18.0 for Windows, SPSS Inc, Chicago, IL). Differences were considered to be significant at $P \leq 0.05$; $P < 0.10$ was considered to be indicative of trends. Values are presented as means +/- standard error (SE). $P$-values are two-tailed.
Table 2.1. Baseline characteristics of the third trimester pregnant women randomized to 930 or 480 mg choline/d and the neonatal outcomes of their children

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Maternal baseline characteristics</th>
<th></th>
<th>Delivery and neonatal characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>930 mg/d, n = 12</td>
<td>480 mg/d, n = 12</td>
<td>930 mg/d, n = 12</td>
</tr>
<tr>
<td>Age, y (range)</td>
<td>28 (22-34)</td>
<td>29 (25-33)</td>
<td>28 (22-34)</td>
</tr>
<tr>
<td>Pre-pregnancy BMI, kg/m² (range)</td>
<td>23.4 (19.9-29.8)</td>
<td>23.6 (20.2-31.9)</td>
<td>23.4 (19.9-29.8)</td>
</tr>
<tr>
<td>Parity, primiparas/multiparas</td>
<td>3/9</td>
<td>8/4*</td>
<td>3/9</td>
</tr>
<tr>
<td>Ethnicity, Caucasian/African American/ Latino/ Asian/Other</td>
<td>6/1/2/3/0</td>
<td>8/0/2/1/1</td>
<td>6/1/2/3/0</td>
</tr>
<tr>
<td>Education, high school/college</td>
<td>3/9</td>
<td>1/11</td>
<td>3/9</td>
</tr>
<tr>
<td>Work status, not employed/employed</td>
<td>2/10</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Serum sFLT1, pg/mL</td>
<td>3275 ± 439</td>
<td>2928 ± 494</td>
<td>3275 ± 439</td>
</tr>
<tr>
<td>Mode of delivery, caesarean section/ vaginal</td>
<td>2/10</td>
<td>3/9</td>
<td>2/10</td>
</tr>
<tr>
<td>Gestational age at birth, wk (range)</td>
<td>40 (39-40)</td>
<td>40 (39-42)</td>
<td>40 (39-40)</td>
</tr>
<tr>
<td>Infant birth weight, kg</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Infant Apgar score</td>
<td>8-9; 9</td>
<td>8-9; 9</td>
<td>8-9; 9</td>
</tr>
</tbody>
</table>

aData are presented as mean ± SE or (range)

bData were analyzed with student’s t tests for continuous data and Chi-square tests for categorical data; *P < 0.05 between choline intake group.
RESULTS

Maternal and neonatal characteristics

Twenty-four women were included in the final analyses (n = 12 for each choline intake group). Baseline characteristics of these third trimester pregnant women did not differ (P > 0.14) between the choline intake groups (i.e., 930 versus 480 mg/d) with the exception of parity (i.e., there were more primiparous women in the 480 mg choline/d intake group; P = 0.04) (Table 2.1) (16). Mode of delivery, gestational age at birth, infant sex, infant birth weight, and Apgar score did not differ (P > 0.11) between the choline intake groups (Table 2.1) (16). No complications (e.g., hypertension, gestational diabetes, preeclampsia) were observed during pregnancy, and all babies were delivered at term and were apparently healthy.

Placental genes and biological processes modified by maternal choline intake

The whole genome expression microarrays were conducted in a subset of placental samples (n=6/ maternal choline intake group), which suggested that 43 placental genes were upregulated and 123 genes were downregulated in the 930 versus 480 mg choline/d intake group (Supplemental Figure S2.1). Similarly, the gene ontology (GO) analysis suggested that 197 biological processes in the placenta were altered (P < 0.05) by a higher maternal choline intake (930 vs 480 mg/d) (Supplemental Table S2.1).

Of particular interest to our research group was the differential expression of genes related to circulatory system development (GO: 0072359) and blood circulation (GO: 0008015) between the two choline intake groups (Table 2.2). The majority of these differentially expressed genes were downregulated by the higher choline intake (930 vs. 480 mg/d).
mg/d) including FLT1, the VEGF receptor. FLT1 mediates normal endothelial signaling (26). However, the soluble form of this receptor (sFLT1), a splice variant lacking the transmembrane and cytoplasmic domains, acts as a potent VEGF and PGF antagonist and is elevated in women with preeclampsia (3, 28, 29).
Table 2.2. Differentially expressed placental vascular function genes in the 930 versus 480 mg choline/d maternal intake group identified by microarray

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Gene function</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP4</td>
<td>Retinol binding protein 4, plasma</td>
<td>Circulatory system development</td>
<td>0.12</td>
</tr>
<tr>
<td>ADORA3</td>
<td>Adenosine A3 receptor</td>
<td>Vascular permeability regulation</td>
<td>0.32</td>
</tr>
<tr>
<td>OXTR</td>
<td>Oxytocin receptor</td>
<td>Vascular tone regulation</td>
<td>0.38</td>
</tr>
<tr>
<td>COL8A1</td>
<td>Collagen, type VIII, alpha 1</td>
<td>Extracellular matrix, vascular remodeling</td>
<td>0.41</td>
</tr>
<tr>
<td>ERRFI1</td>
<td>ERBB receptor feedback inhibitor 1</td>
<td>Circulatory system development</td>
<td>0.48</td>
</tr>
<tr>
<td>FLT1</td>
<td>Fms-related tyrosine kinase 1</td>
<td>VEGF receptor</td>
<td>0.49</td>
</tr>
<tr>
<td>MYH7</td>
<td>Myosin, heavy chain 7, cardiac muscle, beta</td>
<td>Circulatory system development; Blood circulation</td>
<td>0.52</td>
</tr>
<tr>
<td>PTK2B</td>
<td>PTK2B protein tyrosine kinase 2 beta</td>
<td>Endothelial sprouting</td>
<td>0.53</td>
</tr>
<tr>
<td>GHRL</td>
<td>Ghrelin/obestatin prepropeptide</td>
<td>Angiogenesis</td>
<td>1.61</td>
</tr>
<tr>
<td>NPY5R</td>
<td>Neuropeptide Y receptor Y5</td>
<td>Vascular smooth muscle cell proliferation</td>
<td>1.70</td>
</tr>
<tr>
<td>ELN</td>
<td>Elastin</td>
<td>Arterial morphogenesis</td>
<td>2.03</td>
</tr>
</tbody>
</table>

aThe data were derived from the genome wide microarray and were analyzed with the LEMMA statistical package.

bFold difference is the average mRNA abundance in the 930 mg/d maternal choline intake group divided by the average mRNA abundance in the 480 mg/d maternal choline intake group in the microarrays.
To explore the possible effects of maternal choline intake on placental sFLT1 expression, we measured the placental abundance of the sFLT1 transcript as well as maternal circulating sFLT1 protein concentrations at delivery in all placental samples (n=12/ maternal choline intake group). Maternal serum sFLT1 concentrations ranged from 2745-18555 pg/mL which is similar to the concentrations reported by Reddy et al. (i.e., 1600-16,200 pg/mL) among normal pregnancies (30). We found lower placental \( sFLT1 \) mRNA abundance \((P = 0.05, \text{controlled for baseline circulating sFLT1})\) and lower circulating sFLT1 concentrations at delivery \((P = 0.04, \text{controlled for baseline circulating sFLT1})\) (Figure 2.1A and B) in the 930 versus 480 mg choline/d intake group. In addition, we observed a strong positive correlation \((r=0.70; P<0.001)\) between placental \( sFLT1 \) mRNA abundance and maternal circulating sFLT1 (Figure 2.1C) suggesting that the lower placental expression of sFLT1 contributed to the lower circulating concentrations of sFLT1 in maternal blood.

As the results from a previous study (31) suggested that parity may influence sFLT1 concentrations, we stratified the data by parity. However, as there were only 2 circulating sFLT1 samples available from primiparous women in the 930 mg/d group, statistical analysis was performed among the multiparous women only. Consistent with the analyses of the entire dataset, placental \( sFLT1 \) expression \((P = 0.08, \text{controlled for baseline circulating sFLT1})\) (Figure 2.1D) and maternal circulating sFLT1 \((P = 0.07, \text{controlled for baseline circulating sFLT1})\) (Figure 2.1E) tended to be lower among the multiparous women in the 930 versus the 480 mg choline/d intake group.

We also measured the placental transcript abundance of total \( FLT1 \) and the membrane bound
functional FLT1 (mFLT1). Total FLT1 tended to be lower (fold change $= 0.73; P = 0.08$) in the 930 versus 480 mg choline/d group, but mFLT1 expression did not differ (fold change $= 0.94; P = 0.49$) between the 930 versus 480 mg choline/d group. The responsiveness of sFLT1 transcript abundance, but not mFLT1, to extra choline implies a selective effect of maternal choline intake on sFLT1 expression.
Figure 2.1. Placental sFLTI mRNA abundance and maternal circulating sFLT1 concentrations in the 480 and 930 mg choline/d maternal intake groups at delivery. A) placental sFLTI mRNA abundance; B) maternal serum sFLT1 concentrations (pg/mL); C) relationship between placental sFLTI mRNA abundance and maternal serum sFLT1 concentrations; D) placental sFLTI mRNA abundance among multiparous women; and E) maternal serum sFLT1 concentrations among multiparous women. White bar: 480 mg choline/d intake group. Black bar: 930 mg choline/d intake group. Values are mean ± SE,
The relationship between sFLT1 and other biomarkers of choline metabolism

To investigate the mechanism by which choline may be modulating placental sFLT1 expression, we examined the correlations of placental and maternal circulating sFLT1 with the placental concentrations of several choline metabolites [e.g. choline, betaine, acetylcholine, published previously by Yan et al. 2012 (11)] and the placental mRNA abundance of several choline metabolizing genes (e.g. PCYT1A, PEMT, CHDH, CHRM4). We found that placental sFLT1 mRNA abundance \(r = -0.42, P = 0.05\) and maternal circulating sFLT1 concentrations \(r = -0.38, P = 0.08\) correlated negatively with placental acetylcholine concentrations (Figure 2.2A and B, respectively). Similarly, placental sFLT1 mRNA abundance \(r = -0.46, P = 0.02\) and maternal circulating sFLT1 concentrations \(r = -0.38, P = 0.08\) correlated negatively with placental mRNA abundance of CHRM4, a receptor for acetylcholine signaling (Figure 2.2C and D, respectively). In addition, CHRM4 expression tended to be higher (Fold difference = 1.5, \(P = 0.06\)) in the 930 versus the 480 mg choline/d maternal intake group which is consistent with more active signaling through the acetylcholine pathway in the higher maternal choline intake group.

Effect of maternal choline on FLT1 promoter methylation

Secondary to choline’s role as a methyl donor and the presence of CpG dinucleotides within the proximal promoter region of FLT1 (26), we examined the CpG methylation status of this region. No differences in CpG methylation were detected between the two choline intake groups (480 mg/d: 5.6 ± 0.4% vs. 930 mg/d: 5.0 ± 0.4%, \(P = 0.29\)) (Supplemental Figure S2.2).
Figure 2.2. Association of placental sFLT1 mRNA abundance and maternal circulating sFLT1 concentrations with placental acetylcholine concentrations and CHRM4 mRNA abundance. A) placental sFLT1 mRNA abundance (Pearson’s correlation $r = -0.42$, $P = 0.05$) and B) maternal circulating sFLT1 concentrations (Pearson’s correlation $r = -0.38$, $P = 0.08$) were negatively associated with placental acetylcholine concentrations. C) placental sFLT1 mRNA abundance (Pearson’s correlation $r = -0.46$, $P = 0.02$) and D) maternal circulating sFLT1 concentrations (Pearson’s correlation $r = -0.38$, $P = 0.08$) were negatively associated with placental CHRM4 mRNA abundance. n = 22 (including both the 480 and 930 mg choline/d intake groups). The data of mRNA abundance were log-transformed. Data were analyzed with Pearson’s correlation.
The effect of choline treatment on sFLT1 expression in the HTR-8/SVneo cell model

The immortalized placental trophoblast cell line HTR-8/SVneo was utilized to verify the human data of downregulation of sFLT1 expression by extra choline. The cells cultured in low choline concentrations (8 μM) had significantly lower ($P < 0.001$, 30%) viable cell counts than the cells cultured in the medium (13 μM) and high (28 μM) choline concentrations; the cells in the medium and higher groups did not differ in cell counts (Figure 2.3A). Consistent with the human study, the higher choline concentrations in the cell culture media yielded a dose response decrease ($P < 0.001$) in $sFLT1$ mRNA abundance (Figure 2.3B) and sFLT1 secretion to the cell culture medium (Figure 2.3C). Intracellular sFLT1 protein concentrations were also lower in the high group as compared to the medium ($P = 0.001$) or low ($P = 0.05$) groups (Figure 2.3D).
Figure 2.3. Effects of choline treatment on viable cell number, sFLT1 mRNA abundance, and sFLT1 protein concentrations in the HTR-8/SVneo cells. A) viable cell counts measured with trypan blue exclusion; B) sFLT1 mRNA abundance; C) sFLT1 protein secreted to the cell culture medium; and D) intracellular sFLT1 protein concentrations. The HTR-8/SVneo cells were cultured in RPMI1640, 2mM L-glutamine and 1.25% fetal bovine serum supplemented with three different doses of choline to final total choline concentrations of 8 (low), 13 (medium) and 28 (high) μM. Cells were cultured at 5% CO₂-95% air at 37°C for 96-h. The same experiment was repeated three times. Lowercase letters that differ denote significant difference. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed post-hoc Fisher’s LSD tests.
DISCUSSION

In this controlled choline feeding study involving third trimester pregnant women, we employed an untargeted approach to identify genes and biological processes within the placenta that may be affected by maternal choline intake. We subsequently focused our analysis on the impact of maternal choline intake on the placental production of the preeclampsia risk marker, sFLT1.

*A higher maternal choline intake suppresses placental expression of sFLT1*

We show that maternal choline intake alters placental mRNA abundance and maternal circulating protein concentrations of sFLT1 with lower levels in the 930 versus the 480 mg/d choline intake group. The strong correlation between placental sFLT1 expression and circulating sFLT1 protein suggests that choline modifies maternal protein sFLT1 via its effect on placental sFLT1 production. Notably, the modulatory role of choline on placental production of sFLT1 was confirmed in our human placenta cell culture model which demonstrated 20-70 % lower sFLT1 transcript and protein abundance in cells treated with 28 versus 8 μM choline.

Excess sFLT1 is considered to be a contributing factor in the development of placental dysfunction and preeclampsia (28, 32). Administration of exogenous sFLT1 produces several of the clinical and pathological symptoms (e.g., hypertension, proteinuria and glomerular endotheliosis) of preeclampsia in rats (28, 32) and neutralizing antibodies directed against sFLT1 can rescue angiogenic deficits in cell culture models (33). In addition, placental expression and circulating concentrations, of sFLT1 are elevated in preeclamptic women (34) and increased maternal serum sFLT1 predicts preeclampsia several weeks before the onset of
the disease (3). As such, supplementing the maternal diet with extra choline to lower placental production of sFLT1 may be a therapeutic approach to improve placental function and to prevent/mitigate some of the pathological changes that accompany preeclampsia.

*Acetylcholine signaling may be involved in the altered sFLT1 expression by maternal choline intake*

The placenta is a nonneuronal tissue that accumulates large amounts of acetylcholine, a signaling molecule that can affect cellular proliferation, amino acid transport, vasodilation, and parturition (35). Notably, the higher maternal choline intake (930 versus 480 mg/d) enhanced placental production of acetylcholine (11) and expression of CHRM4, a cholinergic receptor that mediates acetylcholine signaling among pregnant women consuming 930 versus 480 mg/d choline. Moreover, both acetylcholine signaling components (i.e., acetylcholine and CHRM4) were inversely related to placental sFLT1 transcript abundance and maternal circulating sFLT1. Collectively, these data suggest that acetylcholine signaling may play a regulatory role in the placental production of sFLT1.

Another mechanism by which maternal choline intake can affect angiogenesis is through epigenetic mechanisms (9). Like other angiogenic factors, sFLT1 expression is regulated by the methylation state of CG dinucleotides residing in its promoter region (i.e., FLT1 gene) (26). Nonetheless, maternal choline intake did not alter FLT1 methylation in the present study suggesting that the observed changes in placental sFLT1 production were independent of this epigenetic mark.
Maternal choline intake is associated with the altered expression of a wide array of angiogenic genes in the placenta

Despite our focus on sFLT1, it is noteworthy that the whole genome expression microarray identified many other genes with roles in circulatory system development and angiogenesis that were differentially altered by maternal choline intake. The majority of the genes altered in the GO of circulatory system development were downregulated in the 930 versus 480 mg choline/d group including OXTR which regulates vascular tone (36), PTK2B which is involved in endothelial sprouting (37), and COL8A1 and ELN, components of the extracellular matrix that mediate vascular morphogenesis (38, 39). Overall, the microarray data are consistent with a modulatory role of maternal choline intake on the transcription of placental genes with key roles in vascular development. In turn, these data can be used to guide additional “proof of principle” studies that seek to confirm a causal role of choline in modulating the expression of genes with critical roles in placental vascular function.

Conclusion

A higher maternal choline intake throughout the third trimester of human pregnancy suppresses the placental production of sFLT1, an anti-angiogenic factor linked to placental dysfunction and preeclampsia. Therefore supplementing the maternal diet with extra choline may be a strategy for improving maternal and placental vascular function and/or reducing the risk of conditions associated with placental dysfunction. Nonetheless, additional studies that supplement with choline across the gestational period, and involve both healthy and complicated pregnancies, are required to more thoroughly understand the putative benefits of maternal choline supplementation on placental function in humans.
REFERENCES


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21. Zeeberg, B. R., Qin, H., Narasimhan, S., Sunshine, M., Cao, H., Kane, D. W., Reimers,


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Supplemental Figure S2.1. Differentially expressed placental genes in the 930 vs. 480 mg choline/d maternal intake group identified by genome wide microarray. A) Hierarchical clustering of differentially expressed genes. Color scheme: yellow, upregulated in the 930 mg/d group; blue, downregulated in the 930 mg/d group. B) Fold difference of differentially expressed genes in the 930 vs. the 480 mg/d group. Select genes (indicated by asterisks) were verified by quantitative real-time PCR.

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Supplemental Figure S2.2. CpG methylation of the proximal promoter region of FLT1. A: Sequence map of the amplified region. Result of a specific CpG unit was included in the analysis if measurement success rate > 75%. CpG units 1-4 were included in final analysis. B: CpG methylation of individual CpG units that were included in the analysis. C: Average CpG methylation across the 4 CpG units. White bar: 480 mg choline/d group. Black bar: 930 mg choline/d group. Values are mean ±SE. n=12/choline group. Data were analyzed with general linear models. CpG, cytosine-phosphate-guanine; FLT1, fms-like tyrosine kinase-1
CHAPTER 3

Pregnancy induces transcriptional activation of the peripheral innate immune system and increases oxidative DNA damage among healthy third trimester pregnant women*


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ABSTRACT

Pregnancy induces physiological adaptations which may involve, or contribute to, alterations in the genomic landscape. Pregnancy also increases the nutritional demand for choline, an essential nutrient that can modulate epigenomic and transcriptomic readouts secondary to its role as a methyl donor. Nevertheless, the interplay between human pregnancy, choline and the human genome is largely unexplored.

As part of a controlled feeding study, we assessed the influence of pregnancy and choline intake on maternal genomic markers. Healthy third trimester pregnant (n=26, wk 26-29 gestation) and nonpregnant (n=21) women were randomized to choline intakes of 480 mg/day, approximating the Adequate Intake level, or 930 mg/day for 12-weeks. Blood leukocytes were acquired at study week 0 and study week 12 for microarray, DNA damage and global DNA/histone methylation measurements. A main effect of pregnancy that was independent of choline intake was detected on several of the maternal leukocyte genomic markers. Compared to nonpregnant women, third trimester pregnant women exhibited higher \( (P<0.05) \) transcript abundance of defense response genes associated with the innate immune system including pattern recognition molecules, neutrophil granule proteins and oxidases, complement proteins, cytokines and chemokines. Pregnant women also exhibited higher \( (P<0.001) \) levels of DNA damage in blood leukocytes, a genomic marker of oxidative stress. No effect of choline intake was detected on the maternal leukocyte genomic markers with the exception of histone 3 lysine 4 di-methylation which was lower among pregnant women in the 930 versus 480 mg/d choline intake group.

Pregnancy induces transcriptional activation of the peripheral innate immune system and increases oxidative DNA damage among healthy third trimester pregnant women.
INTRODUCTION

Pregnancy induces several physiological adaptations to meet the needs of the developing fetus and the health requirements of the mother. Many of these pregnancy-induced physiologic adaptations may involve, or contribute to, alterations in the genomic landscape. Nevertheless, very few studies have examined the interplay between pregnancy and the human genome.

A prominent change during pregnancy is the modulation of the immune system to accommodate the presence of a semiallogeneic fetus (1). In normal pregnancy, there is a shift in the balance of T lymphocytes, mediators of the adaptive cellular immune response, towards a Th2 (immunosuppressive) phenotype with an anti-inflammatory cytokine profile. However, there is also a need to defend against pathogens for the mother-fetus dyad (2). Neutrophils, mediators of the innate immune response that play a key role in the first line of defense against pathogens, increase in peripheral blood during pregnancy (3). Nevertheless, it is unclear as to whether these neutrophils are activated. In vitro studies showed that neutrophils from pregnant women exhibited reduced microbial killing and chemotaxis (4, 5). However, flow cytometry studies showed that several neutrophil surface markers were higher in the pregnant state indicating neutrophil activation (6, 7). Thus, studies that profile the transcriptome of blood leukocytes are needed to better understand the status of the immune system during human pregnancy.

A greater susceptibility to oxidative stress, mostly because of the mitochondria-rich placenta (8), is another characteristic of human pregnancy. Oxidative stress can cause DNA damage (9), which in turn can lead to aberrant gene expression and apoptosis. Although higher levels of DNA damage are detected among women with complicated pregnancies (9, 10), it is unclear whether DNA damage is elevated in normal pregnancy. Studies that examine indicators of DNA damage are needed to advance understanding of redox balance during
normal pregnancy and to inform nutritional therapeutic opportunities.

Pregnancy is also associated with an increased demand for methyl donors to maintain epigenetic marks in the expanding maternal and fetal tissues (11). DNA and histone methylation play a fundamental role in regulating chromatin structure, stability and gene expression (12, 13). A major source of methyl groups for DNA and histone methylation is the essential micronutrient choline, which is recommended at an intake level of 450 mg/d during pregnancy (14). Notably, we previously demonstrated in third trimester pregnant women that consumption of choline at approximately two times the current intake recommendation (i.e., 930 versus 480 mg/d) substantially altered epigenetic marks (15) and transcriptome readouts in fetal derived tissues (i.e., placental and cord blood leukocytes). Nonetheless, it remains to be determined if a maternal choline intake exceeding current recommendations can alter genomic marks in the mothers themselves.

The aims of the current study were to investigate the influence of both pregnancy and maternal choline intake on leukocyte genomic markers. To accomplish these aims, we examined genome-wide gene expression, DNA damage, and global DNA/histone methylation in blood leukocytes obtained from third trimester pregnant women and nonpregnant control women enrolled in a 12-week controlled feeding study (16).
MATERIALS AND METHODS

Study Participants

Healthy third trimester (week 26-29 gestation) singleton pregnant women and nonpregnant control women aged \( \geq 21 \text{y} \) were recruited from Ithaca, New York, and surrounding areas between January 2009 and October 2010. Entry into the study was contingent upon good health status, no tobacco or alcohol use, and a willingness to comply with the study protocol. Twenty-six of the 29 pregnant women and 21 of the 22 nonpregnant women who started the study completed it. Information regarding the study participants and the CONSORT flowchart have been reported previously (16).

Description of procedures

Study Design and Diet

This was a 12-week controlled feeding study in which pregnant (n=26) and nonpregnant (n=21) women were randomized to either 480 (an intake level that approximates the choline adequate intake, AI) or 930 mg choline/d. The choline was derived from the diet (380 mg/d) plus supplemental choline chloride (either 100 or 550 mg choline/d for the 480 or 930 mg/d intake levels, respectively). Throughout the 12-wk study, the participants consumed a 7-day cycle menu. Choline-free prenatal multivitamins and docosahexaenoic acid supplements were provided daily as detailed in Yan et al (16). The fact that all participants consumed the same diet and supplements makes this study particularly suitable to examining the effects of pregnancy on a wide-array of genomic and metabolic endpoints.

Sample collection

Blood samples were obtained at study-baseline (week 0) and study-end (week 12). Fasting (10-h) peripheral blood mononuclear cells (PBMC, including lymphocytes and monocytes) samples were retrieved with Vacutainer CPT tubes (BD, Franklin Lakes, NJ); whole blood, leukocyte and plasma samples were retrieved with EDTA tubes (BD) as
previously described (16, 17).

**Analytical measurements**

Complete blood counts were conducted with 1mL whole blood using an AcT diff 2 hematology analyzer (Beckman Coulter, Brea, CA) according to the manufacturer’s instructions.

Total RNA purification was performed with a commercially available kit (RNeasy Mini kit, Qiagen, Valencia, CA) as previously detailed (15). The integrity of the RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples of an RNA Integrity Number (RIN) >8.0 were included for microarray. There were 12 sets (a set includes both a wk-0 and a wk-12 measurement) of samples from pregnant participants (n= 6/choline intake group) and 10 sets of samples from nonpregnant participants (n = 5/choline intake group) that passed the RIN cutoff.

Gene expression profiling was performed using the Whole Human Gene Expression Microarray 4×44K (Agilent) in the samples that met the RIN cutoff. Antisense RNA amplification and cyanine-3 labeling was performed with an Amino Allyl MessageAmp II aRNA amplification kit (Ambion Inc., Grand island, NY). Microarray hybridization was performed at 65°C for 17-h in an Agilent Microarray Hybridization oven. Microarray scanning was performed in an Agilent Scanner (G2505C) and the images were extracted using Agilent Feature Extraction Software 10.5. Data normalization was performed using log2 transformation and median normalization. An extension of the Laplace approximation EM Microarray Analysis (LEMMA) package based on the R software platform (http://www.R-project.org) (18) developed by Bar et al. (19, 20) was used to detect differentially expressed genes. The version used here (unpublished data) can sensitively detect differentially expressed genes, as it takes into account not only mean differences, but also variational differences between the comparison groups. Using this method we were able
to increase the number of discoveries of differentially expressed genes, relative to other methods that do not account for differential variation. The differentially expressed genes were declared by controlling the false discovery rate (FDR) with Benjamini-Hochberg (BH) correction <0.05 and expression fold difference >2. Differentially expressed genes were classified according to their gene ontology (GO) using High-Throughput GoMiner (21). Hierarchical clustering was conducted with MultiExperiment Viewer (22, 23). Microarray results of select genes were verified using quantitative real-time PCR. All data is MIAME compliant. The data has been deposited in a MIAME compliant database NCBI's Gene Expression Omnibus (24) as detailed on MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html, and are accessible through GEO Series accession number GSE36532 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36532).

Reverse transcription and quantitative real-time PCR were performed as previously described in the samples used for microarrays (25). Data are expressed using the delta delta Ct method (26) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as the housekeeping gene.

Circulating tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6) were measured in EDTA plasma with commercially available ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer’s instructions.

Peripheral blood mononuclear cell (PBMC) DNA damage was measured in duplicate via the alkaline version of single-cell gel electrophoresis (COMET assay). Sample preparation was performed as described previously (17). DNA migration from the nucleus was visualized (75 cells/sample) with an Olympus BX-50 light microscope and a high resolution QImaging Retiga EXi cooled CCD camera. Photos were acquired using the MetaMorph Premier (ver. 7.0) software (Molecular Devices, Sunnyvale, CA) after staining with SYBR Gold.
fluorescent dye (Invitrogen, Grand Island, NY). Percent tail DNA (defined as the proportion of DNA that has migrated from the nucleus) was used as an index of DNA damage and was calculated using the software Komet 6.0 (Andor Technology, South Windsor, CT).

Global DNA methylation was measured in leukocytes using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described by Song et al. (27) with modification based on our instrumentation (17). Global DNA methylation [5-methyl-2'-deoxycytidine (5mC)] is expressed as a percentage of 2’-deoxyguanosine (representing total 2’-deoxycytidine).

Global histone methylation was measured with western blots as described previously (15). Leukocyte samples (200 μL) were suspended in 0.2N hydrochloric acid at 4°C overnight. The extracts were separated by SDS-PAGE and transferred to Immobilon-FL polyvinylidene fluoride transfer membranes. Membranes were incubated with corresponding primary and secondary antibodies (15). Target protein bands were quantified with the LI-COR Odyssey® imaging system (LI-COR, Lincoln, NE) and expressed as the ratio of intensity of the histone epigenetic mark to the reference total histone 3 proteins.

Ethics

The study was approved by the Cornell University and Cayuga Medical Center Institutional Review Boards for Human Participants and written informed consent was obtained from every participant prior to study entry. The study was registered at clinicaltrials.gov as NCT01127022.

Statistical analysis

The effect of pregnancy on dependent variables (e.g. DNA damage, epigenetic marks, gene expression) was assessed at study-baseline (wk-0) and study-end (wk-12) separately with general linear models (GLMs). Pregnancy status and choline intake were included as independent variables and all two-way interactions were tested. The effect of choline intake
on the genomic markers was assessed at study-end in a similar manner with study-baseline data included as a covariate. All of the analyses included leukocyte sub-population (e.g. the percentage of granulocytes) as a covariate with the exception of the DNA damage variable which only contains PBMCs. Additional candidates for entry as covariates into the statistical models are listed in Table 3.1. Covariates and interaction terms that did not achieve a statistical significance of \( P \leq 0.05 \) were removed from the models in a stepwise process.

Plots and histograms of the residuals were used to assess normality in the models. Dependent variables that deviated from the normal distribution (e.g. global histone methylation and quantitative PCR data) were logarithmically transformed to meet the assumption of normality. Differences were considered to be significant at \( P \leq 0.05 \); \( P < 0.10 \) was considered to be indicative of trends. Values are presented as means ± SEM. If covariates were retained in the final models, the values presented are predicted means; \( P \) values are two-tailed. All analyses were performed using SPSS (release 18.0 for Windows, SPSS Inc, Chicago, IL).
Table 3.1. Baseline (study week 0) characteristics of third trimester pregnant women and nonpregnant women randomized to either the 480 or 930 mg/d choline intake group<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Pregnant 480 mg/d (n = 13)</th>
<th>930 mg/d (n = 13)</th>
<th>Nonpregnant 480 mg/d (n = 10)</th>
<th>930 mg/d (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr (range)</td>
<td>29 (25-33)</td>
<td>28 (22-34)</td>
<td>28 (21-37)</td>
<td>29 (21-40)</td>
</tr>
<tr>
<td>Ethnicity, Caucasian/African American/ Latino/Asian/Other</td>
<td>9/0/2/1/1</td>
<td>7/1/2/3/0</td>
<td>8/1/1/0/0</td>
<td>6/1/1/1/2</td>
</tr>
<tr>
<td>BMI&lt;sup&gt;b&lt;/sup&gt;, kg/m&lt;sup&gt;2&lt;/sup&gt; (range)</td>
<td>23.6 (20.2 – 31.9)</td>
<td>23.6 (19.9 – 29.8)</td>
<td>23.6 (19.6 – 27.3)</td>
<td>23.5 (18.2 – 29.8)</td>
</tr>
<tr>
<td>Education, high school/college</td>
<td>1/12</td>
<td>3/10</td>
<td>3/7</td>
<td>3/8</td>
</tr>
<tr>
<td>Physical activity, usual daily activity/exercise ≥ 3 times per wk/unknown</td>
<td>5/7/1</td>
<td>1/10/2</td>
<td>2/8/0</td>
<td>1/9/1</td>
</tr>
<tr>
<td>Vitamin supplement consumption, yes/no&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11/2</td>
<td>11/2</td>
<td>2/8</td>
<td>5/6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detailed in Yan et al, 2012 [16].

<sup>b</sup>For pregnant women, this parameter represented pre-pregnancy BMI

<sup>c</sup>P < 0.01 between pregnant and nonpregnant women, Chi-square test
RESULTS

Participant characteristics

The characteristics of the study population at study-baseline (wk-0) are shown in Table 3.1. Third trimester pregnant and nonpregnant women did not differ in their age, ethnicity/race, pre-pregnancy BMI, education, or physical activity. However, vitamin supplement consumption was greater ($P < 0.01$) among pregnant women. No differences ($P > 0.13$) in the baseline characteristics were detected among the choline intake groups (Table 3.1). All pregnant women delivered their babies at term without major complications and their babies were apparently healthy (16).

The effects of choline

No main effects of choline ($P > 0.05$) were detected for any of the dependent variables. However, choline intake interacted ($P = 0.03$) with pregnancy status to affect H3K4me2. After stratifying by pregnancy status, H3K4me2 was higher ($P = 0.04$, controlling for the leukocyte sub-populations) in the 930 versus 480 mg choline/d group among pregnant women (Figure 3.1). This relationship did not achieve statistical significance among nonpregnant women ($P = 0.12$). No additional choline × pregnancy interactions ($P > 0.05$) were detected.
The effects of pregnancy

A main effect of pregnancy was detected for several of the dependent variables at study-baseline and study-end (values taken after controlled feeding and choline randomization) as described in subsequent text. The pregnancy effect was not modified by choline intake (i.e., no significant pregnancy × choline interactions were detected for any of the variables) at study-end indicating an effect of pregnancy that is independent of choline intake.

Peripheral blood leukocyte counts

Granulocytes and monocytes are important components of the innate immune system. Third trimester pregnant women exhibited higher ($P < 0.01$) granulocyte counts (the majority
of which was neutrophils) and borderline higher \((P < 0.09)\) monocyte counts than nonpregnant women at both study-baseline and study-end. Lymphocyte count was not altered by pregnancy (Table 3.2).

**Leukocyte genome wide expression**

There were 1068 upregulated and 244 downregulated genes at study-baseline, and 1048 upregulated and 280 downregulated genes at study-end, in pregnant versus nonpregnant women (Figure 3.2). Of these \(\sim1300\) differentially expressed genes, 932 were altered at both study-baseline and study-end (Supplemental Table S3.1, available at http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0046736#s5). The differential expression of select genes was verified by quantitative PCR (Table 3.3). Gene expression did not differ between study-end and study-baseline, suggesting that the leukocyte gene expression profile was stable throughout the third trimester of pregnancy.

Figure 3.2. Venn diagram of differentially expressed genes by pregnancy. This figure presents the number of genes differentially expressed in third trimester pregnant versus nonpregnant women at study-baseline (circle on the left) and study-end (circle on the right). The number of genes altered both at study-baseline and study-end are presented in the intersecting area of the two circles. Color scheme: blue represents low expression and yellow represents high expression. \(n=12\) for pregnant women; \(n=10\) for nonpregnant women. Analyzed with the LEMMA statistical package.
Table 3.2. Peripheral blood leukocyte counts in third trimester pregnant women and nonpregnant women at the beginning and end of the controlled feeding study.

<table>
<thead>
<tr>
<th></th>
<th>Study-baseline</th>
<th>Study-end</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pregnant (n=26)</td>
<td>Nonpregnant (n=21)</td>
</tr>
<tr>
<td>Leukocytes (× 10^3/μL)</td>
<td>9.8 ± 0.5</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Lymphocytes (× 10^3/μL)</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Monocytes (× 10^3/μL)</td>
<td>0.3 ± 0.03</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Granulocytes (× 10^3/μL)</td>
<td>7.7 ± 0.4</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Granulocyte (%)</td>
<td>78.2 ± 0.9</td>
<td>65.4 ± 1.4</td>
</tr>
</tbody>
</table>

Data were analyzed using general linear models.
Table 3.3 Quantitative PCR verification of microarray results of genes differentially expressed in third trimester pregnant women (n=12) and nonpregnant women (n=10) at the end of the controlled feeding study.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Total transcript abundance</th>
<th>Transcript abundance /percent granulocyte</th>
<th>Transcript abundance /granulocyte count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fold change</td>
<td>P value</td>
<td>Fold change</td>
</tr>
<tr>
<td><strong>OLFM4</strong></td>
<td>Olfactomedin 4</td>
<td>Cell adhesion</td>
<td>64</td>
<td>&lt;0.01</td>
<td>49</td>
</tr>
<tr>
<td><strong>LTF</strong></td>
<td>Lactoferrin</td>
<td>Host defense</td>
<td>39</td>
<td>&lt;0.01</td>
<td>30</td>
</tr>
<tr>
<td><strong>ELANE</strong></td>
<td>Elastase</td>
<td>Virulence factor</td>
<td>34</td>
<td>&lt;0.01</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>degradation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DEFA4</strong></td>
<td>Defensin, alpha 4</td>
<td>Host defense</td>
<td>25</td>
<td>&lt;0.01</td>
<td>20</td>
</tr>
</tbody>
</table>

Data were analyzed with general linear models. Fold change was calculated as the average transcript abundance in pregnant women/ the average transcript abundance in nonpregnant women. Samples were the same as the ones used for microarray. Microarray and qPCR results are consistent.
The GO analyses conducted at study-baseline and study-end showed similar biological themes: thus, only the GO results at study-end are presented. At study-end, 246 molecular function categories were altered ($P < 0.01$) (Supplemental Table S3.2, available at http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0046736#s5), ~40% of which was related to immune response. The GO of defense response (GO: 0006952) showed the most significant alteration. Of the 112 defense response genes altered, 105 were upregulated in third trimester pregnant women as compared to nonpregnant women (Figure 3.3). To evaluate whether the elevated expression of defense response genes was mediated at least in part by the activation of granulocytes, we used leukocyte sub-population as a covariate in the statistical models. After adjusting for leukocyte sub-populations, the greater expression of the defense response genes among pregnant women was maintained (examples are shown in Table 3.3 and 3.4), suggesting that the higher expression of defense response genes was not solely a function of higher granulocyte counts, but also due to enhanced activation of the granulocytes.
Figure 3.3. Hierarchical clustering of differentially expressed immune defense genes (GO: 0006952) in pregnant (n=12) versus nonpregnant (n=10) women.

This figure presents the hierarchical clustering of 112 differentially expressed immune defense genes in third trimester women versus nonpregnant women (reference group) at the beginning and end of the controlled feeding study. Color scheme: blue represents low expression and yellow represents high expression. n=12 for pregnant women; n=10 for nonpregnant women. Analyzed with Euclidean distances using MultiExperiment Viewer.
Table 3.4. Select list of genes involved in defense response that were upregulated in third trimester pregnant women at the end of the controlled feeding study.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Category</th>
<th>Fold change (unadjusted)</th>
<th>Fold change (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>CD14 molecule</td>
<td>Surface marker</td>
<td>2.30</td>
<td>1.77</td>
</tr>
<tr>
<td>TLR1</td>
<td>Toll-like receptor 1</td>
<td>TLR signaling pathway</td>
<td>2.16</td>
<td>1.79</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
<td>TLR signaling pathway</td>
<td>2.47</td>
<td>1.94</td>
</tr>
<tr>
<td>TLR5</td>
<td>Toll-like receptor 5</td>
<td>TLR signaling pathway</td>
<td>3.07</td>
<td>2.43</td>
</tr>
<tr>
<td>TLR6</td>
<td>Toll-like receptor 6</td>
<td>TLR signaling pathway</td>
<td>2.51</td>
<td>1.97</td>
</tr>
<tr>
<td>MYD88</td>
<td>primary response gene (88)</td>
<td>TLR signaling pathway</td>
<td>2.02</td>
<td>1.51</td>
</tr>
<tr>
<td>ELANE</td>
<td>Elastase, neutrophil expressed</td>
<td>Azurophil granule</td>
<td>11.05</td>
<td>13.77</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
<td>Azurophil granule</td>
<td>7.25</td>
<td>7.44</td>
</tr>
<tr>
<td>AZU1</td>
<td>Azurocidin 1</td>
<td>Azurophil granule</td>
<td>2.94</td>
<td>2.93</td>
</tr>
<tr>
<td>CTSG</td>
<td>Cathepsin G</td>
<td>Azurophil granule</td>
<td>7.50</td>
<td>7.03</td>
</tr>
<tr>
<td>DEFA1</td>
<td>Defensin, alpha 1</td>
<td>Azurophil granule</td>
<td>4.76</td>
<td>3.72</td>
</tr>
<tr>
<td>DEFA3</td>
<td>Defensin, alpha 3, neutrophil-specific</td>
<td>Azurophil granule</td>
<td>7.53</td>
<td>4.97</td>
</tr>
<tr>
<td>DEFA4</td>
<td>Defensin, alpha 4, corticostatin</td>
<td>Azurophil granule</td>
<td>26.95</td>
<td>25.81</td>
</tr>
<tr>
<td>LTF</td>
<td>Lactotransferrin</td>
<td>Specific granule protein</td>
<td>35.51</td>
<td>26.33</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cathelicidin antimicrobial peptide</td>
<td>Specific granule protein</td>
<td>17.48</td>
<td>15.59</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Pathway</td>
<td>Fold Change 1</td>
<td>Fold Change 2</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------</td>
<td>---------------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>C1QB</td>
<td>Component 1, q subcomponent, B</td>
<td>Complement system</td>
<td>3.35</td>
<td>2.62</td>
</tr>
<tr>
<td>CR1</td>
<td>Component (3b/4b) receptor 1</td>
<td>Complement system</td>
<td>3.20</td>
<td>2.50</td>
</tr>
<tr>
<td>C2</td>
<td>Component 2</td>
<td>Complement system</td>
<td>3.58</td>
<td>2.80</td>
</tr>
<tr>
<td>IL1B</td>
<td>Interleukin 1, beta</td>
<td>Cytokine signaling</td>
<td>2.78</td>
<td>2.14</td>
</tr>
<tr>
<td>IL1R1</td>
<td>Interleukin 1 receptor, type I</td>
<td>Cytokine signaling</td>
<td>2.42</td>
<td>1.77</td>
</tr>
<tr>
<td>IL8RB</td>
<td>Interleukin 8 receptor, beta</td>
<td>Cytokine signaling</td>
<td>2.86</td>
<td>2.23</td>
</tr>
<tr>
<td>IL8RA</td>
<td>Interleukin 8 receptor, alpha</td>
<td>Cytokine signaling</td>
<td>2.81</td>
<td>2.19</td>
</tr>
</tbody>
</table>

*P* values < 0.05 with or without adjusting for leukocyte sub-populations as analyzed with general linear models or the LEMMA statistical package, respectively. Fold change was calculated as the average transcript abundance in pregnant women / the average transcript abundance in nonpregnant women in the microarrays.
Among pregnant women, several components of the toll-like receptor (TLR) signaling pathway were upregulated, including the genes encoding TLR1, 4, 5, 6; the CD molecule 14 (CD14), a major surface marker of neutrophils and monocytes that interacts with TLR4; and myeloid differentiation primary response gene 88 (MYD88). The TLRs are pattern recognition molecules on immune cells (e.g., neutrophils) that recognize pathogenic elements (e.g., lipopolysaccharides) and illicit a signaling cascade involving MYD88 that results in the increased production of proinflammatory cytokines such as interleukin 1β (IL1β) and the chemokine interleukin 8 (IL8) (28, 29). The increased IL8 production favors chemotaxis and survival of the neutrophils (30).

Third trimester pregnant women also exhibited higher expression of genes encoding: (i) the microbicidal proteins stored in the neutrophil granules [e.g. the azurophil proteins elastase (ELANE), alpha-defensins (DEF4A) and specific granule proteins lactoferrin (LTF) and cathelicidin (CAMP)]; (ii) the complement proteins involved in innate humoral immune response [e.g. complement component 1, q subcomponent, B chain (C1QB), complement component 2 (C2) and complement component (3b/4b) receptor 1 (CR1)]; (iii) proteins involved in neutrophil superoxide anion production [e.g. the NADPH oxidase complex neutrophil cytosolic factors (NCF), and myeloperoxidase (MPO)]; and (iv) the proinflammatory cytokines (e.g. IL1B). The GO of TNF (GO:0032640) and IL6 (GO:0032675) production were also upregulated.

Only a few (~8) immune response genes were downregulated among pregnant women after controlling for the leukocyte sub-populations. The repressed genes were mostly associated with the adaptive immune response (Table 3.5) and included: (i) the transmembrane glycoprotein CD1 family members CD1c and CD1e molecules, which present primary lipid and glycolipid antigens to T cells (31); (ii) the chemokine (C-C motif) receptors CCR6 and CCR9, which are preferably expressed by T cells (32, 33); (iii) CD70 molecule, a surface
antigen on activated T and B lymphocytes (34) and (iv) B-cell linker protein (BLNK), which plays an important role in B cell development and receptor signaling (35).

**Plasma TNFα and IL6**

Circulating concentrations of TNFα were higher among pregnant women at study-end (1.18 ± 0.09 vs. 0.94 ± 0.07 pg/mL, \( P = 0.05 \)) but not at study-baseline (1.19 ± 0.16 vs. 0.94 ± 0.09 pg/mL, \( P = 0.19 \)). IL6 was elevated among pregnant women at both study-baseline (1.22 ± 0.19 vs. 0.73 ± 0.07 pg/mL, \( P = 0.02 \)) and study-end (1.50 ± 0.15 vs. 0.73 ± 0.10 pg/mL, \( P < 0.01 \)) (Figure 3.4). The higher circulating concentrations of TNFα and IL6 among pregnant women at study-end corresponded with the microarray GO results showing upregulation of TNF and IL6 production.

![Figure 3.4. Plasma TNFα and IL6 concentrations. Plasma concentrations of TNFα and IL6 in third trimester pregnant versus nonpregnant women at study-baseline (A) and study-end (B). White bar: nonpregnant women (n=21); black bar: pregnant women (n=26). Values are means ± SEM. Analyzed with general linear models.](image-url)
Table 3.5. Genes involved in adaptive immune response that were downregulated in third trimester pregnant women at the end of the controlled feeding study.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Fold change (unadjusted)</th>
<th>Fold change (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD70</td>
<td>CD70 molecule</td>
<td>Activated T and B cell marker</td>
<td>0.47</td>
<td>0.61</td>
</tr>
<tr>
<td>CD1C</td>
<td>CD1c molecule</td>
<td>Antigen presentation</td>
<td>0.46</td>
<td>0.63</td>
</tr>
<tr>
<td>CD1E</td>
<td>CD1e molecule</td>
<td>Antigen presentation</td>
<td>0.42</td>
<td>0.55</td>
</tr>
<tr>
<td>CCR6</td>
<td>chemokine (C-C motif) receptor 6</td>
<td>T cell recruitment</td>
<td>0.34</td>
<td>0.54</td>
</tr>
<tr>
<td>CCR9</td>
<td>chemokine (C-C motif) receptor 9</td>
<td>T cell recruitment</td>
<td>0.50</td>
<td>0.47</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker</td>
<td>B cell receptor signaling</td>
<td>0.38</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*p values < 0.05 with or without adjusting for leukocyte sub-populations as analyzed with general linear models or the LEMMA statistical package, respectively. Fold change was calculated as the average transcript abundance in pregnant women / the average transcript...
Leukocyte percent tail DNA

Third trimester pregnant women had significantly higher ($P < 0.01$) percent tail DNA than nonpregnant women at study-baseline and study-end (Table 3.6). The higher percent tail DNA represents greater DNA strand breakage due to oxidative stress.

Leukocyte global histone and DNA methylation

Third trimester pregnant women (versus nonpregnant women) had lower levels of the transcription activation histone mark H3K4me2 ($P < 0.01$), and the transcription repression mark H3K9me2 ($P = 0.05$) at study-baseline and study-end (not controlling for leukocyte sub-populations) (Figure 3.5, A and C). However, after controlling for leukocyte sub-populations, the effect of pregnancy disappeared (Figure 3.5, B and D), suggesting that the alterations in leukocyte H3K9me2 and H3K4me2 during pregnancy were a function of the shift in the leukocyte sub-populations and that different leukocyte cell types may have a different abundance of these histone modifications. Global DNA methylation (study-baseline or study-end) was not altered ($P > 0.38$) by pregnancy (Table 3.6).
Table 3.6. Leukocyte DNA damage and global DNA methylation in third trimester pregnant women and nonpregnant women consuming 480 or 930 mg choline/d at the beginning and end of the controlled feeding study.

<table>
<thead>
<tr>
<th></th>
<th>Pregnant</th>
<th></th>
<th>Nonpregnant</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>480 mg/d (n=13)</td>
<td>930 mg/d (n=13)</td>
<td>Total (n=26)</td>
<td>480 mg/d (n=10)</td>
</tr>
<tr>
<td><strong>Percent tail DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study-beginning</td>
<td>44.9 ± 3.6</td>
<td>43.5 ± 4.8</td>
<td>44.3 ± 2.9</td>
<td>26.4 ± 2.9</td>
</tr>
<tr>
<td>Study-end</td>
<td>33.2 ± 4.6</td>
<td>43.5 ± 4.8</td>
<td>38.3 ± 3.4</td>
<td>20.6 ± 1.9</td>
</tr>
<tr>
<td><strong>DNA methylation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study-beginning</td>
<td>5.1 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Study-end</td>
<td>5.2 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
</tbody>
</table>

Data were analyzed with general linear models. *P < 0.01 between pregnant and nonpregnant women.
Figure 3.5. Peripheral blood leukocyte histone modification marks H3K4me2, H3K9me2 and H3K27me3. (A) and (C), histone modifications in third trimester pregnant versus nonpregnant women without controlling for percent granulocytes at study-baseline and study-end, respectively (B) and (D), histone modifications in third trimester pregnant versus nonpregnant women controlling for percent granulocytes at study-baseline or study-end, respectively. White bar: nonpregnant women (n=21), black bar: pregnant women (n=26). Data are predicted means ± SEM. Analyzed with general linear models.
DISCUSSION

Genomic markers were investigated among healthy third trimester pregnant and nonpregnant control women to advance the understanding of pregnancy induced alterations in immune response, redox balance, and epigenomic stability. The effect of maternal choline intake on these genomic markers was also investigated secondary to the role of choline as a methyl donor. Under both uncontrolled (study-baseline) and controlled (study-end) dietary conditions, our data demonstrate that healthy third trimester pregnant women experience transcriptional activation of the peripheral innate immune system and elevated oxidative stress as indicated by higher DNA damage. Our data also indicate that a choline intake exceeding current dietary recommendations does not alter blood leukocyte genomic markers, except for H3K4me2 among third-trimester pregnant women.

The effects of pregnancy

Immune Function

Pregnant women experienced an elevation in the transcript abundance of 105 leukocyte defense response genes as compared to nonpregnant control women. The majority of upregulated defense genes were neutrophil-associated genes including neutrophil surface markers (e.g. CD14), pattern recognition molecules, cytokines, chemokines, neutrophil granule proteins, complement proteins and neutrophil oxidases. Importantly, the greater transcription of these host defense genes among third trimester pregnant women persisted after controlling for the leukocyte sub-populations, indicating transcriptional activation, rather than suppression, of individual neutrophils. The activation of peripheral neutrophils (and the innate immune system) during the last third of human pregnancy may serve as a compensatory mechanism to protect the maternal-fetal dyad against pathogens when the adaptive immune response is suppressed. Overall our data provide a blueprint of pregnancy induced changes in the peripheral blood transcriptome and elucidate new pathways and
molecules that function in the modulation of the immune system during the third trimester of human pregnancy.

**Oxidation stress and DNA damage**

Leukocyte DNA damage was approximately two times greater among third trimester pregnant women than nonpregnant control women. This elevation in DNA damage among pregnant women may arise from increases in oxidative stress particularly as pregnancy advances (9). Healthy pregnant women were previously thought to overcome oxidative stress through upregulation of antioxidation machinery (36, 37). However, our study suggests that DNA damage commonly occurs throughout the last third of uncomplicated pregnancies.

It is noteworthy that oxidative stress and immune activation are interrelated. Neutrophils are both activated by, and a producer of, reactive oxygen species. TLR4 signaling is enhanced by reactive oxygen species, leading to sustained production of proinflammatory proteins, which in turn maintain oxidative stress (38). In addition, activated neutrophils are a significant source of reactive oxygen species via the activity of NADPH oxidase and myeloperoxidase (MPO) (39), which were both upregulated among pregnant women in our study. Although the increased oxidative stress appears to be a normal event of pregnancy, the DNA damage by reactive oxygen species can lead to genomic instability, aberrant gene expression and apoptosis (40, 41). Additional studies are thus needed to address the health effects of pregnancy-induced leukocyte DNA damage and whether the DNA damage resolves after delivery.

**The effects of choline intake**

A higher maternal choline intake increased dimethylation of H3K4, which is indicative of active transcription (42). To the best of our knowledge, this is the first report in humans that choline intake affected an epigenetic mark of histones obtained from leukocytes of pregnant women. Nevertheless, we did not observe a corresponding change in leukocyte gene
expression by choline intake within the time frame of this study and no other genomic marks were affected by maternal choline intake. Thus, unlike the genomic marks (e.g., DNA methylation (15) and transcriptome profiles (unpublished data) of developing fetal-derived tissues (i.e., placenta and cord blood leukocytes) which are highly responsive to maternal choline intake, genomic markers of maternal-derived tissue may be relatively unresponsive to changes in maternal choline intake.

Limitations

Our study has two major limitations. First, data collection was limited to the third trimester of pregnancy; thus, additional studies are needed to address the effects of pregnancy and maternal choline intake on maternal genomic marks in earlier stages of gestation as well as in the postnatal period. Second, genomic marks from peripheral blood sampling do not necessarily reflect genomic marks in other tissues. Nonetheless, blood transcript profiling is considered a robust tool for assessing the status of the human immune system (43-45).

Conclusions

In summary, the last third of human pregnancy is characterized by transcriptional activation of the neutrophils and elevations in maternal leukocyte oxidative DNA damage. These findings are consistent with an upregulation of the peripheral innate immune system which plays a central role in protecting the maternal and fetal dyad against pathogens. Additional studies are needed to delineate the long-term functional consequences of these genomic alterations and to investigate the relationship between these genomic marks and complicated pregnancy.
REFERENCES


The findings of my dissertation advance current understanding of the impact of maternal choline intake on genomic readouts in humans and suggest that a choline intake exceeding current recommendations may enhance maternal and fetal wellbeing. In particular, we show robust effects of maternal choline intake on the genomic landscape of the placenta with evidence of functional consequences on the neonates’ response to stress and the vasculature of the placenta. In addition, the study findings illustrate the importance of employing an untargeted exploratory approach to unveil physiologic processes previously unconnected to choline.

Maternal choline intake can influence fetal epigenetic markers

Animal studies have demonstrated that gestational choline intake can influence the epigenome of the offspring, which contributes at least in part to functional improvements in neurogenesis, angiogenesis and cancer prognosis (1, 2). Our study is the first to show that maternal choline intake also affects fetal epigenetic programming in humans. The epigenetic alterations in genes that regulate the HPA axis and cortisol production were especially responsive to maternal choline intake and provide mechanistic insights as to how a higher maternal choline intake (930 versus 480 mg choline/d) can attenuate stress reactivity and cortisol production in the fetal compartment. In addition, the epigenetic alteration in cortisol regulating genes implies that maternal choline intake may beneficially program the HPA axis. Based on prior work (3-5), a dampening of the HPA axis in response to stress would be
expected to reduce susceptibility to stress induced illness and chronic conditions such as hypertension and insulin resistance later in life.

**Maternal choline intake may influence placental vascular function**

Previous animal and human studies have shown that choline intake altered the transcriptome, leading to changes in pathways related to growth and development, methylation and apoptosis (2, 6). Our study is the first to show that choline intake during human pregnancy can alter the placental transcript abundance of genes related to vascular functioning, including the risk marker of preeclampsia – sFLT1. Consumption of 930 versus 480 mg choline/d reduced placental sFLT1 expression and circulating maternal sFLT1 concentrations. Importantly, these results were confirmed in a “proof of principle” study employing a human trophoblast cell culture model. These results collectively suggest that a maternal choline intake exceeding current recommendations may improve placental function and reduce the risk of diseases arising from placental dysfunction including preeclampsia (7).

**The response to choline is tissue specific**

In our feeding study conducted in third trimester pregnancy women, the epigenetic state and the transcriptome of the placenta were highly sensitive to maternal choline intake. The plasticity of the placental genomic markers in response to maternal nutrition may provide a mechanism through which the maternal environment can alter placental function and ultimately fetal programming and development (8, 9).

On the contrary, we did not observe changes in either DNA methylation or gene
expression in the peripheral blood leukocytes of the pregnant women within the time frame of this study. Thus, unlike the genomic markers of the developing fetal-derived tissues which are highly responsive to maternal choline intake (8), genomic markers of the pregnant women themselves may be relatively stable to moderate changes in dietary choline intake.

Limitations and future directions

Our study examined the impact of maternal choline intake during the last third of pregnancy only. As most women would consume a prenatal supplement throughout gestation, additional studies that administer the supplemental choline from an early timepoint are needed. In addition, this thesis examined the “short term” effects of maternal choline intake on fetal derived tissues. As epigenetic changes may have long lasting functional effects, exploring the long-term functional consequences of these choline-induced alterations is warranted. Lastly, as placental angiogenesis starts from early gestation, further studies encompassing earlier stages of gestation are needed to more thoroughly understand the potential benefits of choline supplementation on maternal vascular health.
REFERENCES


APPENDIX A

Choline affects placental development and vascular function in a dose response manner*  

*This paper has not been submitted for publication.
ABSTRACT

Maternal choline intake during gestation may influence placental function and fetal outcome. We previously showed that a choline intake exceeding current recommendations during the third trimester of human pregnancy reduced the placental expression of fms-like tyrosine kinase-1 (sFLT1), an anti-angiogenic factor and marker of preeclampsia risk. Down-regulation of sFLT1 by choline was confirmed in a human trophoblast cell culture model. The current study aimed to quantify the effect of choline on a wider array of biomarkers related to trophoblast development and vascular function. Immortalized HTR-8/SVneo trophoblasts were cultured in different choline concentrations (28, 13 and 8 μM) for 96 hours and markers of angiogenesis, inflammation, apoptosis, and blood vessel formation were examined. Decreases in choline concentrations led to a graded increases (P<0.01) in angiogenic factors [e.g. vascular endothelial growth factor (VEGF)] and in proinflammatory markers (e.g., interleukin 1 beta [IL1B]). Diminished choline availability (i.e. 8 vs. 28 μM) also induced apoptosis, enhanced oxidative stress, impaired in vitro angiogenesis, and increased the protein kinase C (PKC) isoforms δ and ε. These PKC isoforms are activated by diacylglycerol, an intermediate of phosphatidylcholine synthesis that accumulates under conditions of choline insufficiency. Notably, the addition of a PKC inhibitor rescued the impaired angiogenesis, attenuated apoptosis, and partially normalized the gene expression profile. In conclusion, choline availability plays an important role in trophoblast development and placental vasculature. The influence of choline on the trophoblast may be partially mediated by PKC signaling.
INTRODUCTION

Trophoblasts are placental cells of fetal origin which function in nutrient transport, immune response and endocrine regulation. Malfunctioning of trophoblast cells leads to elevations in inflammation (1), oxidative stress (2), and apoptosis (3), as well as a dysregulated angiogenic profile (4, 5). Cytokines and apoptotic debris produced by malfunctioning trophoblasts adversely impact adjacent endothelial cells, resulting in abnormal placental vasculature (6). The trophoblast induced endothelial dysfunction leads to the onset of several pregnancy complications including intrauterine growth restriction (IUGR) as well as preeclampsia, a disease characterized by maternal hypertension and proteinuria (6, 7). Restoring trophoblast function may alleviate the endothelial cell dysfunction of these pregnancy complications.

Choline is an essential nutrient that functions in phospholipid metabolism, neurotransmission, and methyl group donation (8). The availability of choline influences various biological processes including inflammation (9), apoptosis (10) and angiogenesis (11). Our group recently demonstrated that a choline intake exceeding current recommendations of 450 mg/d reduced placental mRNA expression and maternal circulating concentrations of the anti-angiogenic factor and preeclampsia risk marker soluble fms-like tyrosine kinase-1 (sFLT1) among third trimester pregnant women (12). In addition, a higher maternal choline intake (930 versus 480 mg) increased genome-wide and site specific methylation (13), as well as acetylcholine concentrations, in the placenta (14). These findings suggest that intrauterine choline availability may profoundly influence placental development and vasculature in humans.
The aim of this study was to analyze the effects of choline on a broader array of biomarkers related to trophoblast development and vascular function. Immortalized HTR-8/SVneo trophoblast cells were cultured in different choline concentrations and readouts of angiogenesis, inflammation, apoptosis, and blood vessel formation were assessed. To delineate the mechanism by which choline availability influences trophoblast function, we examined the modulatory role of a choline-related signaling pathway (i.e. diacylglycerol and protein kinase C [PKC]) on these same biomarkers.
MATERIALS AND METHODS

HTR-8/SVneo cell culture and treatments

HTR-8/SVneo is a stable cell line derived from human first trimester placental extravillous trophoblast and immortalized with the large T-antigen SV-40 (15). The cells, kindly provided by Dr. Charles Graham (Queens University, Kingston, Ontario, Canada), were maintained in RPMI1640 culture medium (Mediatech. Inc, Manassas, VA, USA) with 2mM L-glutamine and 5% fetal bovine serum (FBS) in 5% CO₂-95% air at 37°C. For experiments, customized RPMI1640 medium (Life Technologies, Grand Island, NY, USA) which contained no choline, 2mM L-glutamine and 1.25% FBS were used. The FBS provided the cells with 8 µM total choline as measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in our laboratory (14). Choline chloride was added to the media to concentrations of 0, 5, 20, 40, and 100μM, yielding total choline concentrations of 8, 13, 28, 48 and 108 µM, respectively. As standard RPMI1640 medium contains 21µM of choline, the range of choline used in the present study was chosen to simulate conditions of both choline insufficiency and excess. Cells were seeded in the media containing the different choline concentrations at a starting amount of 1.39×10⁶ cells (passages 5-20) per 100×20 mm dish. The cells were cultured for 96-h before harvest. The antioxidant N-Acetyl-L-cysteine (NAC) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the cell culture at 72-h at a concentration of 125µM. The tumor protein P53 (TP53) inhibitor pifithrin-α (Cayman Chemical, Ann Arbor, Michigan, USA) was added to cell culture at 48-h at a concentration of 30 µM. To assess the effect of PKC inhibition, 1µM of a broad spectrum PKC inhibitor GF 109203X (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) was added to the cells at
72-h of culture. To inhibit c-JUN N-terminal kinase (JNK), 5µM of the JNK inhibitor SP600125 (Fisher Scientific, Pittsburgh, PA, USA) was added at 48-h.

**Human umbilical vein endothelial cells (HUVECs) culture and treatments**

HUVECs were purchased from Life Technologies and maintained in medium 200 (Life technologies) with low serum growth supplements (Life technologies). *In vitro* angiogenesis experiments were performed in cells of less than 16 population doubling.

**Cell count and viability**

Cell counts were performed with a TC10™ automatic cell counter (Bio-Rad, Hercules, CA, USA). Cell viability was measured by Trypan blue dye exclusion.

**RNA extraction**

At 96-h of culture, the attached cells were trypsinized and collected. Total RNA was extracted via the commercially available PerfectPure RNA cultured cell kit (5 Prime Inc, Gaithersburg, MD, USA). Agarose gel electrophoresis was used to ensure intact ribosomal bands in the trophoblast RNA.

**Reverse transcription and quantitative real-time PCR**

Reverse transcription and quantitative real-time PCR were performed as previously described (16). All primers were designed using GeneRunner Version 3.01 ([http://www.softpedia.com/](http://www.softpedia.com/)) (Supplemental Table SA.1). Data were expressed by the delta delta Ct method in which the expression level of the gene of interest was normalized by the expression level of housekeeping gene as fold change before comparison between samples (17). Beta glucuronidase (*GUSB*) was used as the housekeeping gene.
Choline measurements

Measurements of the choline derivatives were performed with LC-MS/MS using the method described previously (14).

Membrane fluidity test

Plasma membrane fluidity was assessed with the fluorescence polarization anisotropy value using the dye 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) (Marker Gene Technologies, Eugene, OR, USA) which was integrated into cellular membranes (18). Attached cells were suspended in a concentration of 2×10^5/mL. TMA-DPH dye (2 mM) was added (1mL) to an equal volume of cells and incubated at 4°C for 10 min. The anisotropy value was assessed with a PTI spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) and calculated with the formula r = (IVV-G×IVH)/(IVV+2G×IVH). In this formula, r represents the anisotropy value, IVV is the fluorescence intensity recorded with excitation and emission polarization in vertical position, IVH is the intensity with excitation in vertical and emission in horizontal position, and G is the ratio of sensitivities of the detection system for the vertically and horizontally polarized light, with the formula G=I_{HV}/I_{HH}. The lower anisotropy represents the higher membrane fluidity.

Reactive oxygen species (ROS) measurement

The intracellular ROS was measured with the cell-permeable 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA) dye (Cayman) (19). The harvested cells were resuspended at a concentration of 1×10^6/mL and incubated with 4μM H2DCFDA at 37°C for 45 min. The cells were then centrifuged, resuspended in 1mL PBS and measured
with a Coulter Epics XL-MCL flow cytometer (Global Medical Instrumentation, Inc, Ramsey, MN, USA) using the FL1 channel.

**Oxidative DNA damage**

Oxidative DNA damage was measured via the alkaline version of single-cell gel electrophoresis (the COMET assay) as previously described (20). DNA migration from the nucleus after electrophoresis was visualized under a light microscope after staining with SYBR Gold fluorescent dye (Life Technologies). Percent tail DNA (defined as the proportion of DNA that has migrated from the nucleus) was used as an index of DNA damage and was calculated using the software Komet 6.0 (Andor Technology, South Windsor, CT).

**Apoptosis assay**

Cellular apoptosis was measured with a Multiple Dye Apoptosis assay kit (Cayman) according to the manufacturer’s instructions. Briefly, both attached and detached cells were collected at 96-h of culture. The FITC-conjugated Annexin V and 7-AAD dyes were added to $5 \times 10^5$ cells in 250µL binding buffer and incubated at room temperature for 15 minutes. The cells were centrifuged and resuspended in 500µL binding buffer. The fluorescence intensity was measured with flow cytometry. The FITC and 7-AAD signals were detected using the FL1 or FL3 channels, respectively. Mitochondria membrane potential was measured using the tetramethylrhodamine (TMRE) dye, which was incubated with $5 \times 10^5$ cells at 37°C for 30 min. The fluorescence signals were measured with flow cytometry using the FL2 channel. The data was analyzed by the WinMDI 2.9 software (http://www.mybiosoftware.com).

**Western blotting**

The relative intensity of proteins of interest was measured with western blot assays. For
total protein extraction, proteins were extracted with PhosphoSafe™ Extraction Reagent and protease inhibitor cocktail set III (EMD Millipore, Billerica, MA, USA). For membrane bound protein extraction, the cells were first lysed by a protein extraction buffer (50mM Tris-Cl pH7.5, 150mM NaCl, 1mM EDTA) without adding detergent. The samples were centrifuged at 800 × g for 5 min to discard the unlysed cells and the supernatant was transferred to a new microfuge tube and centrifuged at 20000 × g for 30 min. The supernatant contained the cytosolic proteins and the pellet contained the membrane-bound proteins. The pellet was dissolved in the protein extraction buffer with 2% IPEGAL (Sigma). For nuclear protein extraction, a hypotonic buffer was used to make the cells swell, after which 10% IPEGAL was applied to lyze the cells for 30 seconds. After centrifuging at full speed for 20 min, the pellets were resuspended in a nuclear protein extraction buffer. The protein extracts were subjected to SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride membrane. Primary antibodies used were caspase 3 (CASP3), β-actin, nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB) 50kDa subunit (Cell Signaling Technology, Danvers, MA, USA); and TP53, PKC delta isoform (PRKCD), PKC epsilon isoform (PRKCE) and P84 (Gene Tex, Irvine, CA, USA). Primary antibody were added overnight at 4°C, after which secondary antibodies, IRDye 800CW Goat Anti Rabbit and IRDye 680 Goat Anti mouse (LI-COR, Lincoln, NE, USA), were added. Target protein bands were visualized and quantified by the LI-COR Odyssey® imaging system (LI-COR) and expressed as the ratio of intensity of a target protein to the reference proteins (β-actin for total and membrane-bound proteins and P84 for nuclear proteins).
**In vitro angiogenesis**

*In vitro* angiogenesis was measured with a commercially available kit (Millipore) according to manufacturer’s instruction. Briefly, after HTR-8/SVneo cells were cultured in RPMI1640 media of differing choline concentrations for 96-h, the cell culture media were collected. ECMatrixTM gel was plated in a 96-well tissue culture plate. After the gel was solidified, $2 \times 10^4$ HUVECs were seeded in each well with 25μL medium 200 with growth supplement and 125μL of the RPMI1640 media previously collected. After 12-h, HUVECs connected with each other and formed tube- like structures. For each well, 10 random view-fields were selected for imaging. Branch points and total capillary tube length were measured in these view-fields. The more branch points and longer total capillary tube length suggested more vigorous angiogenesis.

**Statistical analysis**

One-way ANOVA followed by post-hoc Bonferroni tests was performed to assess the effects of choline treatments. Data not meeting the normality assumption (i.e. residuals were normally distributed) were log-transformed. All analyses were performed using SPSS (release 18.0 for Windows, SPSS Inc, Chicago, IL). Differences were considered to be significant at $P \leq 0.05$. Values are presented as means +/- standard error (SE). $P$-values are two-tailed.
RESULTS

Suboptimal choline elevated angiogenic and inflammatory gene expression in the HTR-8/SVneo cell culture model

At 96-h of culture, HTR-8 cells in the lowest choline concentration (8μM) exhibited a lower cell count ($P < 0.01$) and were more sparsely situated in the culture dishes than the other choline groups (13 – 108μM ) which did not differ in cell counts (Figure A.1A-E). Markers of cellular proliferation, (mRNA abundance of antigen identified by the monoclonal antibody Ki-67 [MKI67]), and cellular differentiation, (mRNA abundance of the β subunit of human chorionic gonadotropin [CGB]) did not differ among any of the choline treatment groups (Figure A.1F).
Figure A.1. Effects of choline treatment on cell number, proliferation and differentiation of the HTR-8/SVneo cells. 

A-E) Cells were more sparse in the lowest choline group (8\(\mu\)M) than the higher choline groups (13-108 \(\mu\)M). 

F) mRNA abundance of the proliferation marker \textit{KI67} and differentiation marker \textit{CGB} in different choline treatment groups. The HTR-8/SVneo cells were cultured in RPMI1640, 2mM L-glutamine and 1.25% fetal bovine serum supplemented with five different doses of choline to final total choline concentrations of 8 - 108 \(\mu\)M. Cells were cultured at 5% CO\(_2\)-95% air at 37\(^\circ\)C for 96-h. The same experiment was repeated three times. Lowercase letters that differ denote significant difference (\(P < 0.05\)); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
We then measured the mRNA abundance of key angiogenic factors, such as vascular endothelial growth factor A (*VEGFA*). VEGF is an angiogenic factor that mediates normal endothelial development and its abundance increases in preeclamptic and IUGR placentas (5, 21). As choline concentration decreased from 28 to 8 μM in our cell culture model, the mRNA abundance of *VEGFA* increased (*P* < 0.01) dose dependently (**Figure A.2 A**). We have previously reported a similar increase in *sFLT1*, which is an anti-angiogenic factor and VEGF signaling inhibitor, as choline concentrations decreased (12). However, choline concentrations greater than or equal to 28μM did not yield significant differences in the expression of either gene.

The results described hereafter focus on the effects of choline treatment concentrations of 8 to 28 μM on trophoblast function. The groups with lower choline concentrations (i.e. 8 and 13 μM) were considered to be cultured in environments of suboptimal choline availability based on their increased angiogenic factor expression. As key cellular functions such as cellular proliferation or differentiation were not affected in either the 8 or 13μM choline groups, and the cell count of the 13μM group was not diminished, these two treatment groups, 8 and 13μM choline, were deemed to represent moderate and mild choline restriction, respectively. Choline treatment did not alter mRNA abundance of the pro-angiogenic placental growth factor (*PGF*) (*P* = 0.12), kinase insert domain receptor (*KDR*) (*P* = 0.12), a major VEGF receptor (Figure A.2B) or endoglin (*ENG*) (*P* = 0.36). Soluble ENG (sENG) is a sensitive anti-angiogenic factor, yet its secretion from the HTR-8/SVneo cells was below the concentration that can be reliably quantified. However, matrix metalloproteinase 14 (*MMP14*), a proteinase that cleaves ENG and releases sENG under the condition of preeclampsia (22),
was upregulated as choline concentrations decreased (Figure A.2B, \( P < 0.01 \)) indicating a route to increase sENG production under conditions of choline insufficiency.

Choline treatment influenced the mRNA abundance of several proinflammatory markers. Increases \((P < 0.01)\) in \( \text{NFKB1} \), which encodes the 50kDa subunit of the transcription factor NFκB; v-rel reticuloendotheliosis viral oncogene homolog A (\( \text{RELA} \)), which encodes the 65kDa subunit of NFκB; and the proinflammatory cytokines interleukin 6 (\( \text{IL6} \)) and interleukin 1, beta (\( \text{IL1B} \)) with decreases in choline concentration (Figure A.2C). The nuclear localization of the 50kDa NFKB1 protein, a sign of NFκB activation, was also elevated as choline concentrations decreased (Figure A.2D).
Figure A.2. Effects of choline treatment on the expression of angiogenic and pro-inflammatory factors in HTR-8/SVneo cells.  

A) mRNA abundance of angiogenic factors \( VEGF\) and \( sFLT1 \) in cells treated with 5 different concentrations of choline (8-108\( \mu \)M) for 96-h.  

B) and C) mRNA abundance of angiogenic factors \( PGF, ENG, KDR, MMP14 \) and proinflammatory factors \( NFKB1, RELA, IL6, IL1B \) in cells treated with 3 different concentrations of choline (8, 13, 28\( \mu \)M) for 96-h.  

D) The relative abundance of the active NFKB1 protein 50 kDa subunit localized to the nuclear in the 3 different choline treatment groups (8, 13, 28\( \mu \)M). Lowercase letters that differ denote significant difference \( (P < 0.05) \); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed post-hoc Bonferroni tests.
Suboptimal choline increased trophoblast apoptosis

One of the consequences of trophoblast dysfunction is cell death. Uncontrolled cell death diminishes trophoblast invasion, exacerbates inflammatory cytokine production and leads to trophoblast dysfunction and is observed in complicated pregnancies (23). We observed that the percentage of cells detached from the culture dish increased ($P < 0.01$) dose dependently as choline concentrations decreased (Figure A.3A). These data suggest that deprivation of choline in culture medium inhibited the growth and/or increased trophoblast apoptosis. We then examined whether apoptosis and/or necrosis occurred in these trophoblasts under the condition of suboptimal choline concentrations. Apoptosis was analyzed with annexin V/7-AAD staining and flow cytometry combining the attached and detached cells. The percentage of annexin V positive and 7-AAD negative cells which indicates early apoptosis did not differ ($P = 0.39$) among the choline treatment groups, whereas the percentage of annexin V and 7-AAD positive cells which indicates late apoptosis or necrosis increased ($P < 0.01$) dose dependently as choline concentrations lowered (Figure A.3B-E). Lowering choline concentrations led to a dose response loss ($P < 0.01$) in mitochondria potential, as demonstrated by the negative staining of the mitochondria specific TMRE dye (Figure A.3F-G). The mRNA abundance of the apoptosis executor CASP3 increased ($P < 0.01$) in the lower versus high choline group (Figure A.5H). Western blotting also detected a dose response increase in cleaved CASP3, a sign of CASP3 activation, in the lower choline groups (Figure A.3H-I), which confirmed the induction of apoptosis by the suboptimal choline concentrations.
Figure A.3. Effects of choline treatment on cell death of the HTR-8/SVneo cells. 

A) Percent cells detached from the dishes after choline treatments (8, 13, 28μM) for 96-h. 

- E) Cellular apoptosis in the three choline treatment groups. Both attached and detached cells were collected at 96-h of culture. Annexin V positive and 7-AAD negative cells were early apoptotic; anexitin V and 7-AAD positive cells were late-apoptotic/ necrotic. 

F) Relative fluorescence intensity of TMRE dye in the three choline groups. Red line, 28μM choline; blue line, 13μM choline; grey shape, 8μM choline. 

G) Mitochondria potential of attached cells. 

H) CASP3 mRNA abundance and active cleaved CASP3 protein abundance in the three choline groups. 

I) Example western blotting of CASP3 species. Lowercase letters that differ denote significant difference (P < 0.05); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
As P53 activation is often involved in the apoptotic process, we examined mRNA abundance and protein concentrations of TP53, both of which were higher \( (P < 0.05) \) in the lower choline groups (Figure A.4A-B). However, the addition of a P53 inhibitor (pifithrin-\(\alpha\)) did not resolve apoptosis (Figure A.4C), despite that P53 inhibition rescued/partially rescued the increase in mRNA abundance of \(IL6\), \(P53\), \(VEGF\) and \(IL1B\) (Figure A.4D).

Figure A.4. P53 activation and the effects of addition of the P53 inhibitor pifithrin-\(\alpha\) on the three choline treatment groups (8, 13, 28\(\mu\)M). A) mRNA abundance and B) intracellular protein levels of P53. C) Percent apoptotic cells after the addition of P53 inhibitor pifithrin-\(\alpha\) 30\(\mu\)M after 48-h of culture. D) mRNA abundance of angiogenic and proinflammatory genes after the addition of piththrin-\(\alpha\). Lowercase letters that differ denote significant difference \( (P < 0.05) \); NS, not significant. Values are mean ± SE, \(n=3\) per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
Suboptimal choline inhibited in vitro angiogenesis

It has been proposed that dysregulated trophoblast cytokine secretion and apoptosis impair normal endothelial cell functioning, thereby inhibiting angiogenesis (6). To examine whether the difference in choline concentrations and the secretory profiles of the HTR-8 cells affect angiogenesis, the culture media obtained from different choline treatment groups at 96-h of HTR8 culture were incubated with HUVECs seeded on matrix gels. The 8μM choline group had significantly lower ($P < 0.01$) endothelial tube branching and shorter ($P < 0.01$) total tube length, which indicate impaired angiogenesis, compared to the higher choline groups (Figure A.5 A-D). These results confirmed that suboptimal choline availability impaired endothelial function.
Figure A.5. Influence on *in vitro* angiogenesis of HTR8S/SVneo cells in the three choline treatment groups (8, 13, 28μM). A)- C) Development of endothelial tube networks in HUVEC cells. After HTR-8/SVneo cells were cultured in RPMI1640 media of different choline concentrations for 96-h, the cell culture media were collected. ECMatrixTM gel was plated in a 96-well tissue culture plate. After the gel was solidified, 2 × 10^4 HUVECs were seeded in each well with 25μL medium 200 with growth supplement and 125μL of the RPMI1640 media previously collected. After 12-h, the tube formation network was viewed. D). Branch points and tube lengths of HUVEC cells incubated with media from different HTR8 choline treatment groups. Lowercase letters that differ denote significant difference (*P* < 0.05); NS, not significant. Values are mean ± SE, *n*=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
Suboptimal choline altered phosphatidylcholine metabolism

To delineate through which pathway(s) choline treatment influences trophoblast function, we measured the alterations in choline metabolite concentrations and gene expression (Figure A.6A). Lowering choline concentrations in culture media led to a decrease \( (P < 0.01) \) in the concentrations of phosphatidylcholine, glycerophosphocholine, phosphocholine, and free choline (Figure A.6B-G). However, betaine and sphingomyelin concentrations were not altered \( (P = 0.4 - 0.6) \) and acetylcholine was not detected in the HTR8/SVneo cells.

The decrease in choline availability led to higher \( (P < 0.01) \) expression of phosphatidylethanolamine \( N \)-methyltransferase \( (PEMT) \), the gene encoding the enzyme that mediates \textit{de novo} phosphatidylcholine synthesis (Figure A.6H). Choline kinase, isoform alpha \( (CHKA) \) and phosphate cytidylyltransferase 1, choline, alpha \( (PCYT1A) \), the genes encoding enzymes for the CDP-choline pathway of phosphatidylcholine synthesis were also higher \( (P < 0.05) \) in the lowest \( (8\mu M) \) versus the highest \( (28\mu M) \) choline group. Choline dehydrogenase \( (CHDH) \), which oxidizes choline to betaine, was not altered. Betaine homocysteine \( S \)-methyltransferase \( (BHMT) \), which donates a methyl group from betaine to remethylate homocysteine, was not expressed in these cells (data not shown). These data collectively show that when choline is limited, the cells engage mechanisms to compensate for diminished phosphatidylcholine by upregulating the enzymes in both pathways of phosphatidylcholine synthesis.
Figure A.6. Effects of choline treatment on choline metabolism in the HTR-8/SVneo cells.  

A) Choline metabolic pathways and key enzymes in trophoblasts. Abbreviations: CHDH, choline dehydrogenase; CK, choline kinase (encoded by CHKA); CT, phosphate cytidylyltransferase encoded by (PCYT1A); GPC, glycerophosphocholine; PC, phosphatidylcholine; PCHO, phosphocholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; SAM, S-adenosylmethionine.  

B) - F) Intracellular concentrations of choline metabolites in HTR8/SVneo cells treated with three different choline concentrations (8, 13, 28 μM) for 96-h.  

H) The mRNA abundance of choline metabolic enzymes in the three choline treatment groups. Lowercase letters that differ denote significant difference (P < 0.05); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
The altered phosphatidylcholine metabolism may increase cellular membrane fluidity and ROS production

Disrupted metabolism of choline derived phospholipids (e.g. phosphatidylcholine) has been shown to decrease cellular membrane integrity (i.e., increased membrane fluidity), which increases the mitochondrial leakage of ROS in hepatocytes (24, 25). ROS leakage causes oxidative stress, which is a known stimulator of anti-angiogenic factor production and apoptosis in trophoblasts (26, 27). We examined the membrane fluidity and ROS production in the HTR-8/SVneo cells treated with different concentrations of choline. Cellular membrane anisotropy was used as a marker of membrane fluidity, and the higher anisotropy indicates lower membrane fluidity. The anisotropy value was lower ($P = 0.02$) in the 8 versus 28μM choline group, suggesting elevated membrane fluidity under the condition of suboptimal choline concentrations (Figure A.7A). Consistently, intracellular ROS increased ($P < 0.01$) as the choline concentrations in the cell culture media decreased (Figure A.7B). As a consequence, oxidative DNA damage increased ($P < 0.01$) dose dependently as choline concentrations lowered (Figure A.7C). These results confirmed that lowering choline availability diminished membrane integrity and increased oxidative stress in the HTR-8/SVneo cells. However, adding the antioxidant NAC to the 8μM choline group during cell culture did not resolve either the increased intracellular ROS or angiogenic and proinflammatory gene expression (Figure A.7D and E).
Figure A.7. Effects of choline treatment and the addition of the antioxidant NAC on membrane fluidity and oxidative stress markers in the HTR-8/SVneo cells.  

A) Membrane fluidity of cells cultured in 3 different choline concentrations (8, 13, 28μM) for 96-h. Cells were incubated with 2mM TMA-DPH dye at 4°C for 10 min and fluorescence anisotropy was measured with a fluorometer. The higher anisotropy represents lower membrane fluidity.  

B) Intracellular production of ROS in the three choline treatment groups. Cells were collected and incubated with 4μM H2DCFDA at 37°C for 45 min. Fluorescence was measured by a flow cytometer. 

C) DNA damage of the three choline treatment groups measured by the Comet assay. The higher DNA damage suggests higher oxidative stress. 

D) Intracellular production of ROS and mRNA abundance of VEGF, NFKB1 and TP53 after the addition of the antioxidant NAC 125μM at 72-h of culture in the lowest (8μM) choline group. Lowercase letters that differ denote significant difference ($P < 0.05$); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
The JNK/c-Jun pathway is a potential mediator of intracellular ROS production, as previous studies suggest that the mitogen activated protein kinase (MAPK) JNK activates the jun proto-oncogene (JUN), which can subsequently bind to DNA and modulate the transcription of an array of genes (e.g., VEGF (28), and is involved in P53 activation and ROS generation (29). Thus, we examined the mRNA abundance of \( \text{JNK} \) and \( \text{JUN} \), both of which increased \((P < 0.01 \text{ for } \text{JNK} \text{ and } P = 0.05 \text{ for } \text{JUN})\) under conditions of reduced choline concentrations (Figure A.8A and B). The phosphorylation of JNK at Tyr183 and 185 is required for the activation of this MAPK. We found that phosphorylated JNK concentrations increased \((P < 0.01)\) in the lower choline treatment groups, consistent with JNK activation (Figure A.8C). The addition of the JNK inhibitor SP600125 at 48-h of culture resolved the increased ROS production (Figure A.8D). However, JNK inhibition did not resolve apoptosis (data not shown) and did not improve the gene expression profile, except for \( \text{IL1B} \) (Figure A.8E). These results suggest that rescuing the oxidative stress alone cannot normalize the adverse consequences induced by suboptimal choline concentrations and that other upstream mechanisms may be involved.
Figure A.8. Effects of the JNK/s-JUN pathway on the HTR8/SVneo cells treated with 3 different concentrations of choline (8 and 13 μM). A) and B), mRNA abundance of JNK and JUN in the choline treatment groups. C) relative abundance of the phosphorylated JNK. D), intracellular production of ROS and mRNA abundance after the addition of the JNK inhibitor SP600125 of 5μM at 48-h. Lowercase letters that differ denote significant difference ($P < 0.05$); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
The activation of PKCs might contribute to the trophoblast dysfunction related to suboptimal choline availability

Previous studies in mice have shown that a choline deficient diet elevated intermediates of phosphatidylcholine synthesis, such as diacylglycerol (DAG), which are major activators of PKCs (30, 31). As the PKCs have been shown to regulate angiogenic genes (32), ROS production (33) and apoptosis (31), we examined whether the altered expression of PKC was involved in the trophoblast dysfunction induced by suboptimal choline availability. The mRNA abundance of the DAG regulated PKC δ and ε isoforms (PRKCD and PRKCE) both increased ($P < 0.01$) in the low choline group (8μM) (Figure A.9A and B). On the contrary, the non-DAG regulated atypical PKC ξ (PRKCZ) was not altered by choline treatment ($P = 0.33$) (Figure A.9C). As the activation of PKCs involves the translocation of the enzymes to the cellular membrane, we then measured the cytosolic and membrane-bound PRKCD and E protein concentrations. Consistent with the mRNA abundance, PRKCE increased under suboptimal choline environments both in the cytosolic and the membrane fractions (Figure A.9D). However, PRKCD protein concentrations were not altered in the choline treatment groups in either cellular fractions (Figure A.9E).

Adding a PKC inhibitor GF109203X resolved the heightened apoptosis (Figure A.10A) and impaired in vitro angiogenesis of the lowest choline group (8μM) (Figure A.10B). This inhibitor also partially resolved the increased mRNA abundance of sFLT1, IL1B and TP53 (Figure A.10C), and secretion of sFLT1 to cell culture medium (Figure A.10D).
Figure A.9. The activation of PKCs in the HTR8/SVneo incubated in three choline concentrations (8, 13, 28μM) for 96-h. A) - C) mRNA abundance of PRKCD, PRKCE and PRK CZ of the three choline treatment groups at 96-h of culture. D) and E) cytosolic and membrane bound protein abundance of PRKCD and PRKCE in the three choline treatment groups. Lowercase letters that differ denote significant difference (P < 0.05); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed by post-hoc Bonferroni tests.
Figure A.10. PKC inhibition rescued some adverse consequences induced by suboptimal choline concentrations (8 and 13 μM) in culture of the HTR8/SVneo cells. 

A) Apoptosis, B) in vitro angiogenesis, C) mRNA abundance and D) sFLT1 secretion after the addition of the broad brand PKC inhibitor GF 109203X. The 1μM of GF 109203X was added to the cells at 72-h of culture. Lowercase letters that differ denote significant difference ($P < 0.05$); NS, not significant. Values are mean ± SE, n=3 per choline treatment group per experiment. Data were analyzed with one-way ANOVA followed post-hoc Bonferroni tests.
DISCUSSION

This study investigated the influence of choline availability on trophoblast functioning. Our results show that mild to moderate choline restriction in the HTR-8/SVneo trophoblast culture model did not affect cellular proliferation and differentiation, yet resulted in trophoblast dysfunction, as demonstrated by increased expression of angiogenic and proinflammatory genes, heightened oxidative stress, apoptosis and/or necrosis, which subsequently led to impaired endothelial angiogenesis. These choline mediated effects were partially resolved by inhibiting PKC, suggesting the involvement of the phospholipid signaling pathway.

Suboptimal choline resulted in trophoblast dysfunction and impaired angiogenesis

A common characteristic of placentas from preeclamptic and IUGR pregnancies is abnormal trophoblast production of angiogenic factors such as VEGF, sFLT1, sENG and PGF (21, 34). The increased trophoblast secretion of anti-angiogenic factors (e.g. sFLT1 and sENG) sequesters placental and maternal endothelial growth factors (e.g. VEGF), resulting in vascular dysfunction (7, 35). In our cell culture model, VEGFA, a vascular factor that mediates blood vessel development, increased as choline concentrations in the culture decreased. The upregulation of VEGFA is consistent with observations in preeclamptic placentas, and may be an attempt of the stressed placenta to counter endothelial dysfunction and placental ischemia (21). sENG and sFLT1 are two major anti-angiogenic factors, which inhibit normal vascular development and are upregulated in preeclampsia(27). We previously found that sFLT1 increased as choline concentrations decreased (12). Although ENG
transcript was not altered in the present study, the increase in *MMP14* which converts ENG to the anti-angiogenic sENG, suggests its potential inhibitory effect on angiogenesis. Overall, the altered angiogenic panel under conditions of suboptimal choline is consistent with a shift towards an anti-angiogenic profile which may adversely affect angiogenesis.

Enhanced inflammation is another manifestation of trophoblast dysfunction (36-38). In our study, transcripts of several pro-inflammatory markers (i.e., *IL1B*, *IL6*, *NFKB1* and *RELA*) were increased. The higher nuclear localization of the P50 subunit of NFKB1 in the suboptimal choline groups is also consistent with activation of proinflammatory pathways. It may also reflect a compensatory attempt of the cells to prevent choline deficiency induced apoptosis (39).

In addition to the transcriptional responses, reducing choline availability also led to cell death which appeared to involve both apoptosis and necrosis. Although apoptosis is a normal phenomenon which occurs during trophoblast development, uncontrolled apoptosis that turns into necrosis (i.e. aponecrosis) can result in the release of “uncoated” cell debris to adjacent tissues and maternal circulation, which triggers a systemic inflammatory response and endothelial dysfunction (3). The higher proportion of late apoptotic/necrotic cells and the presence of activated apoptosis executor CASP3 observed in the low choline groups indicate that reducing choline resulted in aponecrosis.

The *in vitro* angiogenesis assay confirms that the disturbance in trophoblast functioning due to suboptimal choline availability eventually impaired the angiogenic process of endothelial cells. Taken together, these data suggest that suboptimal choline availability may influence the development of pregnancy complications by adversely affecting trophoblast
functioning and placental vasculature.

*PKC signaling may be involved in the suboptimal choline induced trophoblast dysfunction*

The mechanism by which choline affects trophoblast functioning and angiogenesis is ill-defined. In the current study, several choline metabolites related to phosphatidylcholine synthesis were diminished in a suboptimal choline environment. The impact of phosphatidylcholine on trophoblast functioning can be exerted via disturbance in membrane integrity, which leads to increased oxidative stress (25). Although both diminished plasma membrane integrity and increased oxidative stress were observed as choline concentrations decreased, neither the addition of an antioxidant NAC nor rectifying oxidative stress by a JNK inhibitor restored the cytokine expression profile or inhibited cell death, suggesting that oxidative stress may be a downstream, rather than an upstream, consequence of choline inadequacy.

Phosphatidylcholine may also influence trophoblast functioning via phospholipid signaling. Previous studies have shown that choline deficiency leads to an increase in DAG, an intermediate of the CDP-choline pathway as well as a by-product of phosphatidylcholine catabolism (40, 41). The gene expression profile of the choline metabolizing enzymes in the present study is most consistent with accumulation of DAG in an attempt to synthesis more phosphatidylcholine via the CDP-choline pathway. The increase in DAG can activate PKCs, a group of proteins that are involved in multiple signaling pathways. The PKCs are functionally diverse; some isoforms promote growth (e.g. PRKCA, PRKCE) while other isoforms are either pro-apoptotic or have mixed effects on survival (e.g. PRKCD, PRKCZ)
We detected that both PRKCD and E were increased as choline concentrations decreased. For PRKCE, membrane localization increased, which is a sign of activation. Importantly, after we applied a PKC inhibitor, both apoptosis and the inhibitory effects of choline insufficiency on angiogenesis were completely rescued and trophoblast dysfunction was alleviated. The restoration of trophoblast function by inhibiting PKC suggests that phospholipid and PKC signaling is an important route through which choline is involved in placental development and vasculature. As such, PKC may serve as a molecular target for diagnosis and treatment (via inhibition) of placental related complications. However, it should be noted that not all aspects of trophoblast function were rescued by the inhibition of PKCs. Additional studies which further delineate the complex signaling network regulated by choline availability in trophoblasts are warranted.

Conclusion

In conclusion, our study revealed the importance of adequate choline nutrition for maintaining normal placental trophoblast function and preventing its adverse influence on the endothelial system. Additional studies are needed to more fully delineate the mechanism by which choline availability impacts trophoblast functioning. Studies are also needed to explore the clinical efficacy of choline supplementation in reducing the risk of pregnancy complications that arise from impaired placental function and vascularization.
REFERENCES


151


152


**Supplemental Table SA1. Primers used for quantitative real-time PCR**

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