TITLE: Effects of Choline Supplementation on Fetal Growth in Gestational Diabetes Mellitus

BACKGROUND AND SIGNIFICANCE

Macrosomia resulting from gestational diabetes mellitus (GDM) has long-lasting influence on health

GDM, characterized by glucose intolerance that develops during pregnancy, is a major public health problem affecting up to 15% of pregnancies in some populations(1). The prevalence of GDM has increased by 10 – 100% in different populations in recent years, partly due to the obesity epidemic (20, 21). GDM increases direct medical cost by over 40% compared to healthy pregnancies (22, 23). A major adverse birth outcome of GDM is macrosomia, defined as a birth weight higher than 4kg, occurring in 20% of GDM versus 10% of non-GDM pregnancies (22). Macrosomic neonates are prone to childhood obesity, hypertension, and diabetes across the life-course (2-6). ***Addressing macrosomia at birth could be one of the most cost-effective ways to mitigate the fetal origin of cardio-metabolic diseases in adulthood.***

Calorie control and weight management alone cannot effectively improve fetal growth outcomes

Medical Nutrition Therapy (MNT) is a cornerstone for GDM management. The American Diabetes Association guidelines of MNT for GDM are focused on caloric intake and weight management of the pregnant women to improve their glycemic control (24). However, in studies among 600 – 1,500 pregnancies, using lifestyle interventions based on the current guidelines failed to reduce macrosomia despite improvements in appropriate gestational weight gain and dietary glycemic load of the pregnant women (8-12). These results ***imply that therapy targeted to improve maternal health is not always sufficient to benefit the fetus.*** Moreover, approximately 50% of pregnant women are not able to achieve appropriate weight gain during pregnancy (25) and approximately 20 % (26) of pregnant women with GDM are not able to make blood glucose under control due to obesity, excessive maternal weight gain, lifestyle factors, and other reasons. These concerns warrant a ***simple regimen that can be easily adopted by expecting mothers and specifically targeted to normalize fetal growth, so as to minimize the risk of fetal overgrowth and excess adiposity at birth.***

Restoring placental transport is critical for normalizing fetal growth in GDM

The current perception suggests that adverse pregnancy outcomes arise from a malfunctioning placental-fetal unit, since an array of pregnancy complications including GDM in

mothers disappear after delivery (7). As the interface between maternal and fetal circulation, the placenta dictates nutrient transport from mothers to fetuses (7). The co-I's study in GDM mice (18, 19) as well as research from others (27, 28) demonstrate elevated transport of macronutrients such as glucose and fat through the placenta in GDM, which leads to excessive macronutrient accretion by the fetus, resulting in macrosomia (29). Moreover, this excess macronutrient transport to the fetus seems to **disproportionally increase fetal adiposity** in both humans and rodents (30-32). ***Normalizing placental fat and glucose transport highlights a fundamental solution to fetal overgrowth and long-term cardio-metabolic consequences associated with GDM.***

Potential effect of choline on placental transport and fetal growth in GDM

Choline is an essential nutrient and its derivatives phosphatidylcholine (PC) and betaine play important roles in modulating macronutrient metabolism and energy homeostasis(15). PC is an essential component of cellular membrane and lipoproteins. Choline deficiency has been robustly demonstrated to result in fatty liver associated with compromised lipoprotein and bile secretion both in C57BL/6 mice and healthy participants receiving a choline depleted diet for 42 days (33-36). Choline **when oxidized to betaine**, ***serves as a methyl donor*** that influences the epigenetic control of various genes, including DNA methylation of genes in the pathways of lipid and glucose metabolism such as peroxisome proliferator-activated receptor gamma (PPAR-γ) and the LDL receptor (37, 38). **The Co-I's study in GDM mice** ***demonstrated for the first time that maternal choline supplementation (MCS) of 4 times the standard intake (2 g/kg diet) normalized placental fat and glucose transport and prevented fetal overgrowth in high-fat feeding-induced GDM mice at mid-gestation (E12.5) (Figure 1A)(18)***. These effects of choline appeared to be related ***to the inhibition of AKT/mTOR*** (protein kinase B/ Mechanistic target of rapamycin) signaling which was known to enhance placental growth and nutrient transport (39-41) (Figure 1B-C). In late gestation, MCS prevented excess total body adiposity and fat accumulation in the fetal liver in the GDM-affected pups (Figure 1D-E). Furthermore, the MCS offspring demonstrated better blood glucose control into their early adulthood when they were faced with an obesogenic environment (HF feeding), underscoring that the influence of MCS on offspring metabolic health is long-lasting (Figure 1F).

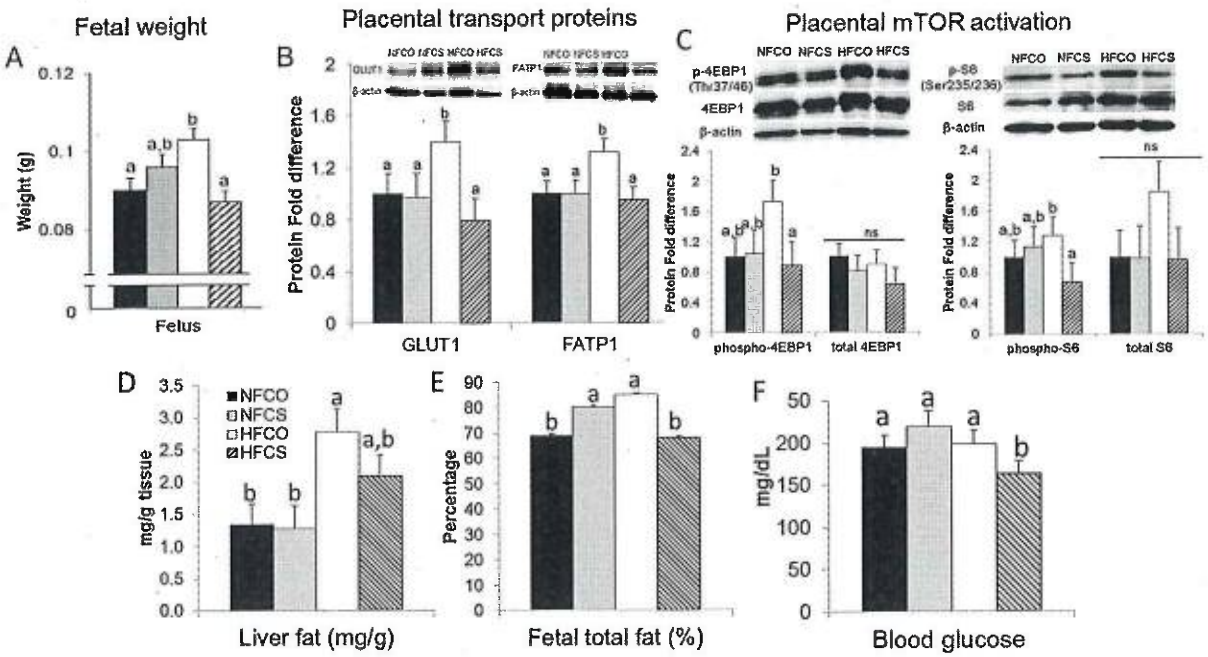

Figure 1. (A) E12.5 fetal weight; (B) Placental glucose and fatty acid transporter expression; (C) mTOR activation (4EBP1 and S6 protein phosphorylation are markers of mTOR activation); (D) E17.5 liver fat content and (E) Total adiposity; and (F) Offspring blood glucose levels after postnatal high-fat feeding in C57BL/6J mouse dams fed control (NF) or high-fat (HF, GDM) diets, with (CS) or without (CO) choline supplementation before and during pregnancy. Fatp: fatty acid transporter; Glut1: glucose transporter; IGF: insulin-like growth factor; 4EBP1: 4E binding protein 1. Different letters indicate statistical significance. $P < 0.05$

Later, we also verified in the human choriocarcinoma cell line BeWo that MCS decreased glucose and fatty acid uptake into the placental cells, suggesting that the impact of choline on placental transport likely apply to humans as well (**Figure 2**) (42). Moreover, we have conducted a pilot observational study in human participants with GDM and found that choline metabolite and enzyme concentrations in the human placenta were associated with several glucose and fat transporters in the placenta (**Table 1**). ***With these premises, an intervention to validate the influence of choline on placental transport and fetal growth in GDM human pregnancies is warranted.***

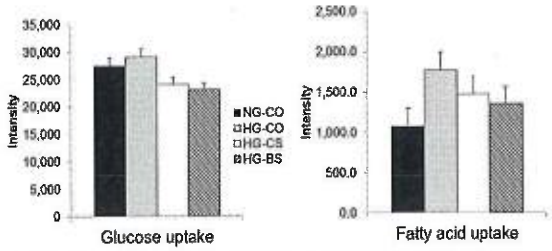

Figure 2. Glucose and fatty acid uptake in BeWo cells. BeWo cells were treated with 1mM choline (CS), 1mM betaine, a choline oxidation product (BS), or saline control and with 30 mM glucose (HG) or 30mM mannose (NG). a, b: any two groups with no overlapping characters have a statistically significant difference ($P < 0.05$) between them.

Table 1. Correlations between placental choline markers and placental transporter expression

	GLUT1		GLUT3		FATP1		FATP4	
	r	p	r	p	r	p	r	p
PEMT	0.92	0.001	0.26	0.47	0.39	0.16	0.85	0.01
CHDH	0.23	0.44	0.25	0.5	-0.16	0.96	-0.34	0.9
PCYT1A	0.14	0.6	0.3	0.44	0.99	0.01	0.99	0.01
Glycerophosphocholine	0.38	0.2	0.08	0.8	0.78	0.01	0.72	0.01
Betaine	-0.1	0.8	0.74	0.02	-0.38	0.2	-0.36	0.2

PEMT: phosphatidylethanolamine methyltransferase; CHDH, choline dehydrogenase; PCYT1A: phosphocholine citidyltransferase; r: correlation coefficient; $p < 0.05$ is considered significant

Choline and epigenetic programming

The long-term influence of maternal exposures (e.g. obesity and GDM) and fetal overgrowth on cardio-metabolic health in adulthood is mainly mediated through the epigenetic mechanism. Epigenetic modifications lead to gene expression changes without altering the gene sequence. DNA methylation is one of the major epigenetic modifications that often lead to downregulation of genes. Maternal obesity in rodents persistently alter the epigenome of offspring in adulthood, including DNA methylation on genes such as leptin (*Lep*), insulin like growth factor (*Igf*), and peroxisome proliferator-activated receptor gamma (*Pparg*) that have important roles in lipid accretion and metabolism (43, 44). Choline is a methyl group donor, which provides the substrate methyl group for DNA methylation. Maternal supplementation of 4 times normal choline intake in mice alters the offspring's DNA methylation on *Igf2*, a-grow promoting gene critical for placental expansion and fetal growth (45). The Co-I's work using the HF-feeding induced-GDM mouse model also demonstrates that MCS increased global DNA methylation in the fetal liver. Moreover, site-specific DNA methylation of the *Srebp1C* gene was increased with its gene expression decreased accordingly. SREBP1 is an important transcription factor that promotes lipogenesis. Its downregulation is consistently with the lower fat accumulation in MCS offspring at birth (Figure 1 D-E) and highlights the involvement of epigenetics in mediating the effect of MCS on offspring adiposity. Moreover, in a controlled-feeding study in healthy women, 2 x recommended intake of choline (930 versus 480 mg/d

choline) was sufficient to lead to global upregulation in placental DNA methylation and alterations in DNA methylation of components in the stress response pathway in both the placenta and fetal cord blood (46). **These premises in animals and human studies justify an intervention to determine whether MCS is effective on modifying offspring DNA methylation of growth and obesity related genes, such as *SREBP1C*, *LEP*, and *IGF2* in human GDM pregnancies, thereby reducing the susceptibility of the children to cardio-metabolic diseases in adulthood.**

In summary, there is no current effective approach to prevent GDM-related fetal overgrowth. We demonstrated that choline sufficiency was critical for restoring normal placental transport and preventing fetal overgrowth in GDM mice. If similar effects of choline could be validated in humans, the practical impact would be remarkable because currently less than 10% of pregnant women in the U.S. consume the recommended 450 mg/d Adequate Intake of choline (47). Choline is readily available in common foods such as eggs, beans, and meat and is easy and safe to provide via a choline-rich diet or choline supplements. When used in clinical trials, choline supplementation was well tolerated and was not associated with any serious adverse events.

STUDY OBJECTIVES

Specific Aim 1: Determine the effect of MCS during pregnancy on birth weight in GDM.

Specific Aim 2: Determine the influence of MCS on macronutrient transport and epigenetic modifications in the placenta and cord blood.

HYPOTHESIS

Aim 1: We hypothesize that the choline group would have lower birth weight and fewer LGA neonates than the non- choline group.

Aim 2: We hypothesize that the choline group would reduce placental transport and increase DNA methylation of metabolic genes.

STUDY DESIGN

Subjects: A convenient sampling methodology will be adopted. We will recruit pregnant women diagnosed with GDM during the second trimester of pregnancy on a rolling basis. Block randomization will be conducted based on treatment methods (i.e. insulin therapy, oral hypoglycemics, or diet only) to randomly assign participants to the intervention and control groups. Recruitment will be conducted using the following methods: (i) study flyers will be posted

at the prenatal clinic; (ii) the principle investigator and her fellows will identify pregnant women who are potentially eligible by reviewing medical records. All these patients are routinely seen by members of the MFM team fellows. The PI or co-I will introduce the study to potential participants.

Eligibility Criteria: The inclusion criteria include GDM pregnant women before gestational week 28. GDM is diagnosed with the 2-step process: blood glucose higher than the 140 mg/dL cut-off value after 50 g non-fasting 1-hr and 100 g 3-hr oral glucose challenges. Pregnant women are qualified to participate if they are English- or Spanish- speaking, over 18 years of age, having singleton pregnancy, intending to deliver their babies at Maimonides and are without any of the conditions listed in the exclusion criteria. Exclusion criteria include pre-existing hyperglycemia, diabetes, cardiovascular conditions and liver disease prior to pregnancy. Patients may choose to participate in the study and not donate a biopsy of their placenta or umbilical cord. Patients who desire stem cell donation or storage will not be able to donate a biopsy of their placenta or umbilical cord. These patients will not be excluded from the primary analysis.

Design and Data Collection Procedures:

All interaction with participants will occur at Maimonides while sample analyses will occur at Brooklyn College (where the co-I's lab is located). The PI or co-I will obtain written informed consent from the pregnant women before study entry. Randomization will be done by pharmacy after patient signs consent. It will be block randomization of 10 patients. After randomization, the PI or co-I will provide the participant in the intervention group with choline supplement pills containing 500 mg of choline bitartrate (235 mg choline, Douglas Lab). The participant will consume 2 pills per day to obtain 470 mg choline for 8 weeks. The recommended intake of choline in the form of Adequate Intake (AI) is 450 mg/d for pregnant women and the Tolerable Upper Intake Level (UL) is 3500 mg/d. Since less than 10% pregnant women reach the AI of choline intake and the upper quartile of choline intake is below 900 mg/d (48). The dosage we provide plus the habitual intake of choline in these pregnant women will fall between the AI and UL, i.e. sufficient but not exceeding the limit. The participants in the control group will receive a placebo that contains corn oil. Both groups will also receive standard prenatal multivitamins and 200 mg/d DHA provided by the study group. We will provide the participants with 5 weeks supply of supplements and we will verify their compliance during the time with daily check-off logs of supplement consumption and leftover pill count during their next visit.

The participants will come in for a total of 3 visits at times of routine clinical care. The first visit will be at 28 weeks of gestation when we enroll the participants, the second visit will be at

32 weeks of gestation, and the third visit will be at 36 weeks. There are additional routine clinic visits (e.g., at 32 weeks) during which information can be obtained if a patient missed a study visit. We will collect a baseline questionnaire at the first visit (approximately 20 minutes), conduct 3-day dietary recalls after the first visit (approximately 20 minutes, each), and check current weight and collect 20 mL blood samples at all visits. At delivery, we will collect placenta samples and cord blood from the participants (**Figure 3**).

Baseline questionnaire: The questionnaire includes demographic and medical information such as age, due date, ethnicity, parity, marital status, education level, occupation, household income, medical insurance, substance use, medication use, nutrition supplement, family history of chronic diseases, and self-reported pre-pregnancy weight.

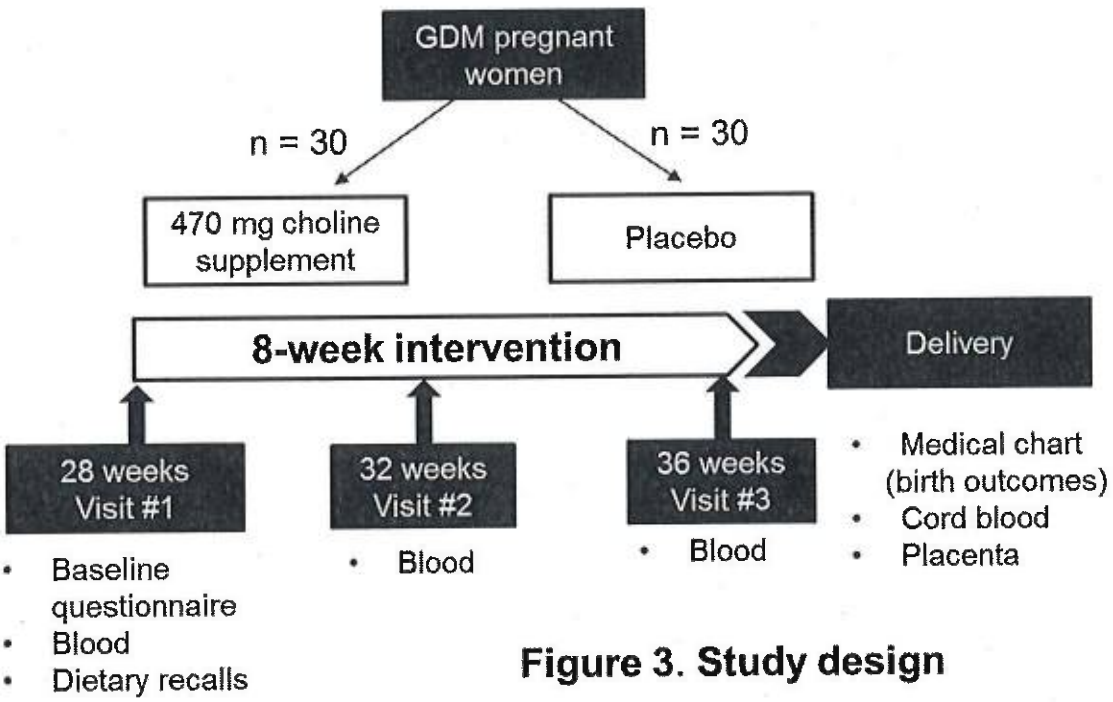

Figure 3. Study design

Dietary assessment: Three 24-hr dietary recalls, 2 on weekdays and 1 on a weekend day, will be obtained from each participant by a trained research assistant (RA) via phone calls following the first visits to quantify dietary choline intakes. The multi-pass method of Harnack and colleagues will be used (49). This method is widely used in dietary recall data collection for research (e.g. National Health and Nutrition Examination Survey) and **validated for its accuracy in assessing dietary intake (50).** The RA will record food identification, quantity, ingredient specification, preparation method and enter data into the Nutrition Data System for Research (NDSR) software to analyze choline intake. The RA will also ask participants about their **physical activity** (PA) during the past 24 hours using the PA recall instrument (51).

Anthropometric measurements and blood collection: We will measure the participants' weight and height using a medical weighing scale with mechanical height rods during the visits. To minimize food-related concentration differences in plasma biomarkers, participants will be instructed not to consume food for at least 4 hours before their blood draw in the morning. This is a standard recommendation for prenatal visits and is considered as safe (52, 53). We will also have preventative measures such as an examination bed to rest in and juices, food, and water should the women need them. About 20 mL of fasting venous blood will be drawn from each participant during the visits by a fellow/resident/nurse. Blood samples will be collected into one EDTA blood collection tube and one serum separator tube and centrifuged to obtain plasma, buffy coat, and serum. Samples will be processed and stored at -80°C at Brooklyn College until being used for analytical measurements.

Blood glucose management: We will obtain information about fasting glucose, hemoglobin A1C, medication and insulin therapy from medical charts of the participants.

Birth information and sample collection at delivery: The participants will notify the PI or co-I when they are admitted to the birth center by phone. We will also send them reminders as their due dates are approaching. In addition, **the birth center medical staff will be given a list of participant names, thus they will also contact the PI or co-I if a participant checks in when none of the study staff is on site.** The research team will be available 24/7. Once the baby is delivered, we will collect two tubes of cord blood to retrieve serum, plasma, and buffy coat. We will measure the size and weight of the placenta and process the placenta using the following method: full-thickness placental biopsies will be obtained using a 6-mm Keyes punch and stabilized in RNAlater® (for RNA), or frozen in liquid nitrogen (for other purposes). Samples will be transported to the co-I's lab for long-term storage at -80C before use.

Data Analysis: ***Specific Aim 1:*** **Determine the effect of maternal choline supplementation (MCS) during pregnancy on birth weight in GDM.** To achieve this aim, we will compare the birth weight as well as incidence of LGA between the intervention versus control group. We will also assess the influence of MCS on blood and placenta choline metabolite status, as well as the correlation between total choline intake (diet + supplement) or choline metabolite status and birth weight.

Dietary intake assessment: As mentioned above, dietary intake data collected from 3-day dietary recalls will be analyzed by the NDSR software (54, 55). Daily intakes of total choline will be calculated by multiplying the frequency of consumption by the sum of choline derivatives including free choline, phosphatidylcholine (PC), glycerophosphocholine (GPC), phosphocholine, and sphingomyelin of each food item(56). Daily intakes of betaine (the oxidation product of choline) will be calculated by multiplying consumption frequency and

betaine content of each food. Average daily intakes will be calculated as the average daily consumption of total choline and betaine over the 3 days of dietary recalls.

Measurements of choline metabolites: Free choline, betaine and other choline derivatives [e.g. PC, GPC, lysophosphatidylcholine, dimethylglycine, trimethylamine oxide (TMAO)] will be measured in maternal plasma using liquid chromatography (LC)-mass spectrometry (MS) /MS (57, 58) as was conducted in previous studies. ***To ensure the accuracy and reproducibility of data***, stable isotope labeled standards will be used in extraction, all samples will be quantified based on a 6-point standard curve, and each sample will be run in duplicate. Samples with a coefficient of variation (CV) % higher than 5% within run or 10% between run will be rerun.

Specific Aim 2: Determine the influence of MCS on macronutrient transport and epigenetic modifications in the placenta and cord blood. To achieve this aim, we will determine expression of fat and glucose transporters, as well as the upstream regulating pathways AKT/mTOR in placental biopsies from the participants. We will measure the site-specific DNA methylation of key growth and metabolism related genes in the placenta and cord blood.

Analyses of macronutrient transport markers in placental biopsies: To analyze how choline metabolism may be related to placental fat and glucose transport, we will use real-time PCR to quantify **mRNA abundance** of fat and glucose metabolic genes and transporters, such as glucose transporters GLUT1 and GLUT3, fatty acid metabolism-related genes lipoprotein lipase (LPL), fatty acid translocase (CD36), FATPs, and amino acid transporters SNAT2 and SNAT4. Abundance of respective proteins, as well as phosphorylation of AKT, and mTOR targets 4EBP1 and S6K (indicative of activation of the pathway) will also be measured by **western blotting** that is routinely conducted in the co-I's lab, which includes SDS-PAGE, membrane transfer, incubation with antibodies and visualization with a chemiluminescence imaging system (18). All primer efficiency and antibody quality will be tested before being used. Both real-time PCR and western blot will be run in triplicate.

Epigenetic regulation of growth and metabolism: we will examine **site-specific DNA methylation** of genes that are published by previous studies as susceptible to prenatal exposures and mediating long-term growth and metabolic programming of the offspring using bi-sulfite sequencing as was previously conducted by the PI(58). Briefly, genomic DNA will be treated with bisulfite reagent, after which the target genomic region will be amplified by PCR. A MassArray EpiTyper system (available at CUNY Epigenetics Core, fee for service) will be used to quantify methylated sequences. Genes of interest include *LEP*, *SREBP1C*, and *PPARG* which mediates fat metabolism, *IGF2* which promotes growth, corticotropin releasing hormone (*CRH*) which promotes cortisol secretion, and glucocorticoid receptor (*GR*) which receives cortisol signals.

Sample Size: We anticipate a 10% dropout rate due to reasons such as participants moving out of the city, pre-term deliveries, or missed placental collection. Therefore, we expect to have at least 27 sets of complete data in each group. A statistical power analysis was performed for sample size estimation accounting for rates of dropout and missing data, based on data from a randomized controlled trial of choline supplementation which demonstrates that 2 x recommended intake of choline for 12 weeks is sufficient to increase plasma choline and alter placental global DNA methylation. The initial sample size of n=30/ group will allow detection of a >20% difference in birth weight, plasma choline and placental DNA methylation at $\alpha < 0.05$ and power = 80%.

Expected Outcomes: The successful completion of this study will provide valuable insights into the use of choline supplement as a modifier of placental macronutrient homeostasis in GDM to improve fetal growth outcomes.

Timetable:

	Quarter 1	Quarter 2	Quarter 3	Quarter 4
Participant Recruitment	n = 20	n = 20	n = 20	
Sample and Data Collection				
Choline measurements		Aim 1	Aim 1	Aim 1
Gene expression and epigenetic measurements		Aim 2	Aim 2	Aim 2
Data analysis				Both Aims
Result dissemination				Both Aims

We expect to complete recruitment by the end of the third quarter and complete dietary record, clinical information, and biosample collection in the middle of the fourth quarter. We will start analytical measurements once we obtain at least a third of the samples. We expect to complete analytical measurements by the end of the year. We will analyze and disseminate data in the last quarter.

Data Safety Committee Team Plans:

Randomization is a block randomization of 5 patients. After every block of randomization, the research team with a pharmacist and at least one external physician outside of the research team [Dr. Shoshana Haberman] will analyze the data to ensure that there are no unanticipated adverse effects via ultrasound fetal growth patterns. If unanticipated effects are found, we will report to IRB as per policy.

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ATTACHMENTS

List any attachments (contracts, participant materials, data collection tools, etc.)