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Original Research Article

Total sulfur amino acid requirements are higher during late gestation compared with early gestation in healthy Canadian pregnancies in a repeated-measures trial

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A B S T R A C T

Background: Dietary Reference Intake (DRI) Recommendations for total sulfur amino acids (TSAAs; methionine + cysteine) during pregnancy are based on factorial calculations using data from adult males. To date, no data exist on TSAA requirements obtained directly during pregnancy.

Objectives: The objective of this study was to examine whether TSAA requirements during early (11–20 wk) and late (31–40 wk) gestation in healthy females with singleton pregnancies are different than current recommendations, and different between early and late gestation using the indicator amino acid oxidation (IAAO) technique.

Methods: Twenty-five females 20–40 y with a healthy singleton pregnancy were studied using the IAAO technique in a repeated measures design for a total of 70, 8-h d. On each study day a methionine test intake (range: 0–40 mg·kg^{-1}·d^{-1}) was provided in 8 hourly, isonitrogenous and isocaloric meals with cysteine excluded from the diet. Breath samples were collected at baseline and isotopic steady state of orally provided L-1-^{13}C-Phenylalanine for measurement of phenylalanine oxidation. The requirement was determined using biphasic linear regression crossover analysis to identify a breakpoint in $^{13}CO_2$ production, representing the estimated average requirement (EAR).

Results: The TSAA requirement in healthy pregnant participants in early gestation was 11.1 mg·kg^{-1}·d^{-1} {R^2_m = 0.79, R^2_c = 0.79; 95% confidence interval [CI] (8.9, 13.3 mg·kg^{-1}·d^{-1})} and 15.0 mg·kg^{-1}·d^{-1} (R^2_m = 0.72, R^2_c = 0.79; 95% CI [13.0, 17.0 mg·kg^{-1}·d^{-1}]) in late gestation. The difference between confidence intervals of the 2 breakpoints was = −3.9 ± 3.0, and statistically different.

Conclusions: We directly measured TSAA requirements in healthy pregnant mothers, and our findings suggest that requirements are lower than current DRI recommendations of 20 and 25 mg·kg^{-1}·d^{-1}, as the EAR, and Recommended Dietary Allowance, respectively. Late gestation TSAA needs are significantly different and increased 35% compared with early gestation. Recommendations for TSAA intake need to be tailored for gestational stage. This clinical trial was registered at clinicaltrials.gov as NCT04326322.

Keywords: pregnancy, sulfur amino acids, one-carbon metabolism, requirements, IAAO, methionine

Abbreviations: APE, atoms percent excess; BCCHRI, British Columbia's Children's Hospital Research Institute; CI, confidence interval; DCI, difference between confidence intervals; DRI, Dietary Reference Intake; EAR, estimated average requirement; F$^{13}CO_2$, rate of appearance of (13C)-labeled carbon dioxide in breath; GSH, glutathione; IAA, indispensable amino acid; IAAO, indicator amino acid oxidation; RDA, Recommended Dietary Allowance; REE, resting energy expenditure; RM, re-methylation; SAH, S-adenosyl homocysteine; SAM, S-adenosylmethionine; TM, transmethylation; TS, transsulfuration; TSAA, total sulfur amino acids (methionine + cysteine).

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Introduction

Adequate nutrition during pregnancy is important for the optimal growth of the fetus and for health in later life of both mother and child. The relationship between proteins and their constituent amino acids and pregnancy outcomes was established in early studies showing a positive relationship between birth weight and maternal protein intake [1]. However, requirements for amino acids during pregnancy have been less studied and the current recommendations are based on factorially derived estimates and theoretical models of protein accretion in the developing fetus and maternal adnexa [2]. The use of the factorial approach during pregnancy has been debated due to the many assumptions used in the formation of these estimates [2]. Recommendations for amino acids during pregnancy are also static, with one recommendation serving throughout the entirety of pregnancy. This approach does not reflect the dynamic, evolving state of metabolism during pregnancy. These gaps in knowledge have resulted in a call for a more systematic evaluation of amino acid and protein needs during pregnancy [3].

Dietary one-carbon metabolism nutrients that are consumed during pregnancy can alter DNA methylation patterns, therefore altering the expression of genes and ultimately having the potential to adversely affect pregnancy outcomes [4]. The indispensable amino acid methionine (Figure 1) is the most important methyl donor in vivo via its metabolite, S-adenosylmethionine (SAM) and plays a crucial role in methylation of DNA, RNA, histones, and proteins [5]. Cysteine can be endogenously synthesized from methionine via the transsulfuration pathway. Both amino acids contain a sulfur atom and are collectively referred to as the total sulfur amino acids (TSAAs), with cysteine being conditionally indispensable when adequate methionine is not present in the diet [6].

Recently, protein and amino acid requirements during both early (11–20 wk) and late (31–40 wk) gestation were determined experimentally using the indicator amino acid oxidation (IAAO) technique [7–10]. Mean early and late gestation requirements were found to be different for protein (1.22 and 1.52 $g \cdot kg^{-1} \cdot d^{-1}$), lysine (37 and 50 $mg \cdot kg^{-1} \cdot d^{-1}$), phenylalanine (15 and 21 $mg \cdot kg^{-1} \cdot d^{-1}$), and total aromatic amino acids – phenylalanine + tyrosine (44 and 50 $mg \cdot kg^{-1} \cdot d^{-1}$) [7–10]. These values are also different from the current pregnancy estimated average requirements (EARs) for protein (1.1 $g \cdot kg^{-1} \cdot d^{-1}$), lysine (41 $mg \cdot kg^{-1} \cdot d^{-1}$), and phenylalanine + tyrosine (36 $mg \cdot kg^{-1} \cdot d^{-1}$) providing evidence that current Dietary Reference Intake (DRI) recommendations are inadequate. Together, these results indicate that factorially derived estimates during pregnancy are not appropriate. Current EAR and Recommended Dietary Allowance (RDA) for TSAA – methionine + cysteine during pregnancy are 20 and 25 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively [1], and have not been directly determined.

This study aimed to determine dietary TSAA requirements during 2 stages of pregnancy – early (11–20 wk) and late (31–40 wk), with no cysteine in the diet. We hypothesized that TSAA requirements during pregnancy would differ from current DRI recommendations and that requirements would differ between early and late pregnancy [1].

Methods

Participants

Between October 2020 and October 2022, 25 healthy, pregnant, females ($n_{early} = 12$, 30 observations; $n_{late} = 13$, 40 observations) participated in this study at the British Columbia Children's Hospital Research Institute (BCCHRI) in Vancouver, Canada (Supplemental Figure 1). Participants were recruited between September 2020 and September 2022 from the Vancouver area through a mixture of physical recruitment advertisements and virtual advertisements on popular social media sites. Inclusion criteria included: 20–40 y, singleton pregnancy, between 11 and 20 or 31 and 40 wk of gestation, free of chronic/acute disease, and with a

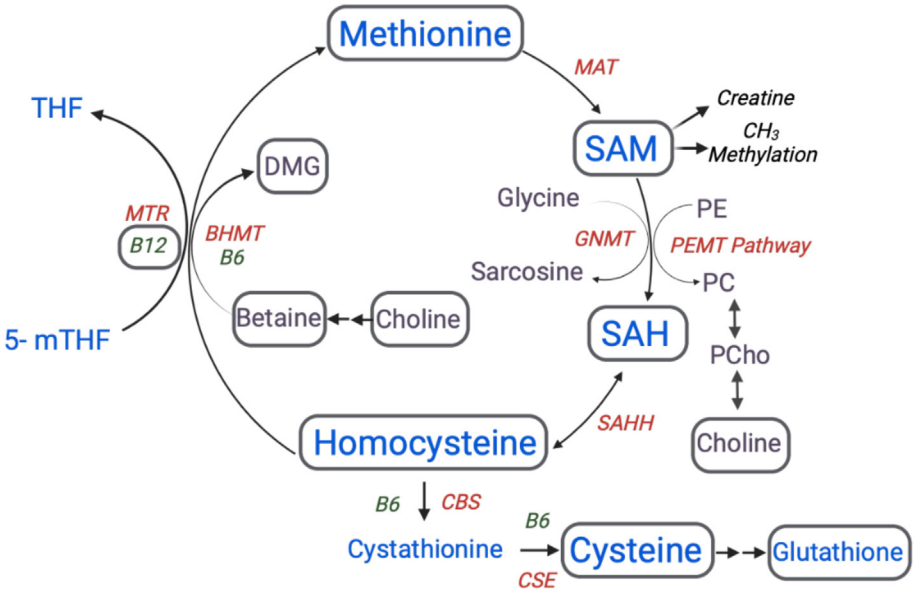

FIGURE 1. Simplified schematic of methionine metabolism. Metabolites measured from study days' samples (plasma/erythrocytes) are highlighted within boxes. BHMT, betaine-homocysteine S-methyltransferase CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; DMG, dimethylglycine; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MTR, methionine synthase; PEMT, phosphatidylethanolamine N-methyltransferase; SAH, *S*-adenosylhomocysteine; SAHH, *S*-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate; 5-mTHF, methylfolate. Created with BioRender.

prepregnancy body mass index (BMI) between 19 and 28 kg/m^2. Exclusion criteria included: multiple gestation; history of spontaneous abortion or pre-term birth; recent pregnancy ($\leq$ 6 mo); and metabolic, cardiovascular, genetic, or immune disorders, including gestational diabetes, pre-eclampsia, anemia, or jaundice. Any participants who were substance dependent, <20 y or >40 y, had severe nausea/vomiting, experienced recent weight loss (~1.5 kg or more during their current pregnancy), or had a fasting blood glucose screen > 5.3 mmol/L were excluded. All participants were asked about current medication and supplement use. Throughout the study, all participants were confirmed to be taking a prenatal vitamin containing folic acid. Informed, written consent was obtained for each participant at the Clinical Research Evaluation Unit located on-site at BCCHRI. Transit or parking costs were covered for all participants. Compensation was provided to participants at the end of each completed study visit. The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board approved this study (H20-00383), and the study was registered on Clinicaltrials.gov (NCT04326322).

Experimental design

The minimally invasive IAAO protocol was used with L-[1-^{13}C] phenylalanine as an indicator amino acid. Subjects were studied with methionine intakes ranging from 0 to 40 mg·kg^{-1}·day^{-1} as previously described [7–11].

Preliminary assessment of participants

To assess eligibility, participants were screened during a preliminary assessment at a minimum of 10 wk and maximum of 19 wk gestation (early) and a minimum of 30 wk and maximum of 39 wk gestation (late). Participants provided information about current and previous pregnancies and answered a health and diet questionnaire focused on acute and chronic diseases of metabolism and/or pregnancy. Weight was measured to the nearest 0.1 kg using a digital patient weighing scale (Healthometer Professional), height was measured to the nearest 0.1 cm using a stadiometer (Seca, model 213) and skinfold measurements were taken at triceps, biceps, and subscapular sites using a Harpenden Skinfold Body Fat Caliper (Baty International). Fat mass was calculated using equations specific to pregnancy that include: age, gestational stage, sex, and the mean of the 3 skinfold measurements [12–14]. Blood glucose was measured after 10–12 h of overnight fasting using a finger prick glucometer and blood glucose test strips (OneTouch Ultra 2 Lifescan). Fasting blood glucose cutoffs were set at $\leq$5.3mmol/L to screen for gestational diabetes [15]. Chemstrip7 urinalysis strips (Roche Diagnostics) were used to assess for the presence of glucose or protein in the urine to further screen for potential gestational diabetes as well as possible risk of pre-eclampsia [16]. Resting energy expenditure (REE, kcal/d) was determined using an open-circuit indirect calorimeter (Vmax Encore, VIASYS). Participants were asked to complete detailed 2-d diet records indicating the type and amount of food and drink consumed, providing details of brand name where applicable. These diet records were obtained and reviewed at the pre-assessment. Participants were given detailed instructions on how to improve the accuracy and detail of their 2-d diet records in preparation for upcoming oxidation day study visits. Participants were prescribed an adequate protein diet (1.5 g·kg^{-1}·d^{-1}) [17] for the 2 d leading up to each oxidation study day, based on each participant's foods preferences as assessed by the 2-d diet records. Participants were instructed to continue taking prenatal supplements throughout the study to ensure micronutrient intake was adequate during the study period. COVID-19 questionnaires were administered upon arrival and COVID-19 protocol, as stipulated by the BC Children's Hospital, was followed during the entirety of the study. Any participants known or suspected to have active, or recent (within 14 d) COVID-19 were excluded from the study day.

Study day diets

Participants arrived at the Clinical Research and Evaluation Unit at BCCHRI after a 10–12 h overnight fast. Before consuming any experimental diets, height, weight, fasting blood glucose, details about any changes in health or medication, and a urine test strip were administered to assess changes in health.

Participants consumed 8 hourly, isocaloric and isonitrogenous meals on each day with randomly assigned methionine intake (0, 5, 10, 15, 20, 30, 35, and 40 mg·kg^{-1}·d^{-1}). Methionine intakes were randomly assigned by the study administrator pulling an intake number from a bag, individual subjects did not repeat the same intake >1 time, and a minimum of 5 d separated each experimental study day. Experimental diets given in the form of protein shakes consisted of orange-flavored, protein-free liquid formula that contained protein-free powder to provide essential vitamins and minerals (PFD1: Mead Johnson Nutrition), orange flavored sugar crystals (Tang and Kool-Aid: Kraft Canada), corn oil (Mazola: ACH Food Companies), and water. The protein content was added by measuring exact values of crystalline L-amino acids (Ajinomoto) calculated by modeling the amino acid composition of egg protein. Alanine was used to keep the diet isonitrogenous. Dietary tyrosine was provided in excess (61.095 mg·kg^{-1}·d^{-1}) to minimize phenylalanine retention in body tyrosine pools as previously described by Elango et al. [18].

The range of intakes for methionine were chosen based on previously determined requirement studies done on adult males [19] and data from previous protein requirement studies in pregnancy that suggested increased demands during the late stages of pregnancy [7].

Each of the 8 meals contained 1/12th of the participants' daily requirement for energy and nutrients. Protein in the diet was provided at 1.5 g·kg^{-1}·d^{-1} with total caloric intake (energy) provided at 1.7 × REE, based on indirect calorimetry, to ensure dietary energy was not limiting. Protein-free cookies were given alongside each experimental protein shake to provide a portion of the daily energy requirement and allow participants to consume a small amount of solid food on the day of the study. Macronutrient distribution on the day of study was ~50% carbohydrates, 35% fat, and 15% protein. All diets were prepared at BCCHRI. Other than water, no other food or drink was consumed on oxidation study days until the experiment was finished and all data were collected.

Isotope protocols

Priming oral doses of L-[1-^{13}C]phenylalanine (3.048 mg/kg; 99% atom percent excess (APE), Cambridge Isotope Laboratories) and NaH^{13}CO$_3$ (0.176 mg/kg; 99% APE, Cambridge Isotope Laboratories) were administered at the fifth meal, with hourly oral doses of L-[1-^{13}C] phenylalanine (1.958 mg·kg^{-1}·h^{-1}) administered during the remaining 3 hourly meals. Dietary phenylalanine for the last 4 meals was adjusted to obtain a total intake of 34 mg·kg^{-1}·d^{-1}.

Sample collection and analysis

A total of 9 breath samples were taken throughout the length of the 8-h study day by asking participants to exhale into a breath bag (EasySampler Collection Device, QuinTron, Terumo Medical) that served as a tool to prevent room air from entering evacuated exetainer tubes (Labco) for breath collection. Three baseline samples were

collected 45, 30, and 15 min before the administration of oral isotope (meal 5) and 150, 180, 195, 210, 225, and 240 min after isotope administration, representative of isotopic steady state (Supplemental Figure 2). Samples were stored at room temperature and analyzed using a continuous flow isotope ratio mass spectrometer (CF-IRMS IsoPrime100) for $^{13}CO_2$ in expired breath. Isotopic $^{13}CO_2$ was quantified in APE over a reference carbon dioxide standard. Isotopic enrichment was calculated by subtracting baseline $^{13}CO_2$ from steady state enrichment $^{13}CO_2$.

One venous blood sample (4 mL) was collected in a 4-mL dipotassium EDTA tube at the 6th hour of the study day to assess plasma amino acid and various circulating metabolite concentrations in response to changes in methionine intakes (Supplemental Figure 2). Blood was processed for analysis or storage (as described below) between 2 and 8 min of sample collection. Of this 4 mL sample, 200 µL of whole blood that remained at room temperature was used to measure hematocrit concentration using a Micro-Hematocrit Capillary Tube Reader (Oxford Labware, Sherwood Medical). Whole blood (100 µL) remained at room temperature after collection and was combined with 1 mL of 0.4% (w/v) ascorbic acid solution, incubated at 37°C for 90 min, then stored at −80°C until analysis for erythrocyte folate concentration by chemiluminescent microparticle immunoassay (Architect, I 2000 SR analyzer, Abbott) and corrected for hematocrit and plasma contribution. Another 500 µL of whole blood was used to test for erythrocyte glutathione concentrations as previously described [20]. In brief, N-ethylmaleimide was added after the removal of plasma to prevent the dimerization of glutathione (GSH), erythrocytes were separated and lysed by adding methanol and zinc sulfate, supernatant was removed and stored at −80°C until further analysis. Samples were sent to The Hospital for Sick Children in Toronto, Canada, for further analysis of GSH concentration using an Orbitrap mass spectrometer (Orbitrap MS, Thermo Q-Exactive; Thermo Fisher Scientific) operated in positive ionization mode with the Heated Electrospray Ionization probe source (HESI-II, operated at 3.5 KV) and coupled to an ultra-performance liquid chromatography system (UPLC, Thermo Scientific Ultimate 3000; Thermo Fisher Scientific). The areas under the peaks were integrated using Thermo Scientific Xcalibur 4.5 Quan software (version 4.2). GSH concentrations were determined using an external standard curve and the ratio of the analyte to the internal standard (γ-glutamyl-leucine) [20]. The remainder of the sample had plasma isolated by centrifugation (2000 × *g*, 4°C, 10 min; Beckman Coulter Allegra X-22 stored in 4°C cold room or Beckman Coulter J6-MI) and stored at −80°C until further analysis for total vitamin B-12, plasma folate, estradiol, and progesterone by chemiluminescent microparticle immunoassay (Architect, I 2000 SR analyzer, Abbott) and SAM, S-adenosylhomocysteine (SAH), methionine, cysteine, homocysteine, betaine, choline, and dimethylglycine by liquid chromatography-mass spectrometry (LC-MS; Acuity UPLC H-Class, Waters Corporation; Xevo TQ-XS Triple Quadrupole Mass Spectrometer, Waters Corporation) by the Analytical Core for Metabolomics and Nutrition at BCCHRI.

One 30 mL urine sample was collected at the end of the study period (8 h after study start), then frozen at −80°C until further analysis. Urinary sulfate was analyzed using a 96-well plate method involving the use of sodium rhodizonate combined with barium chloride as previously described [21]. Briefly, when combined with sodium rhodizonate, barium chloride produces a known concentration of barium rhodizonate solution with an orange/pink color. In the presence of sulfate, barium sulfate will form leading to a decrease in barium rhodizonate thus lowering the color intensity. A spectrophotometer (uQuant Microplate Spectrophotometer; Bio-Tek) and Microplate Data Collection & Analysis Software (Gen5; Bio-Tek) was used to measure the absorbance of known sulfate samples to generate a standard curve, which was used to determine the concentration of unknown inorganic sulfate in urine samples. For urinary creatinine, a 96-well plate assay based off Jaffe's reaction, where an orange color forms when creatinine is treated with alkaline picrate was used [22]. Urinary sulfate concentrations were standardized to creatinine concentrations.

Calculations

The rate of ^{13}C-phenylalanine tracer oxidation ($F^{13}CO_2$, µmol·kg^{-1}·h^{-1}) was calculated using the following equation:

$$F^{13}CO_2 = \frac{(FCO_2)(ECO_2)(44.6)(60)}{W(0.82)(100)}$$

Where FCO_2 represents the production of carbon dioxide (mL/min, measured by open-circuit indirect calorimeter; Vmax Encore, VIASYS); ECO_2 represents the $^{13}CO_2$ enrichment in breath at isotopic steady state (APE), W is the weight (kg) of the participant, 44.6 (µmol/L) and 60 (min/h) are constants used to convert FCO_2 to µmol/h, 0.82 is the correction factor for carbon dioxide retained by the body due to bicarbonate fixation [23], and 100 is used to convert APE to a fraction.

Statistical analysis

The Kolmogrov–Smirnov test was used to compare the distribution of data to test for normality (IBM SPSS Statistics for Windows, Version 28.0., IBM Corp). TSAA requirement was estimated by applying a biphasic linear mixed-effects model to the $F^{13}CO_2$ data [24]. The mean amino acid requirement (EAR) is defined as the change-point or breakpoint in the regression line, the point at which the $F^{13}CO_2$ data plateaus (slope of zero) and is no longer influenced by increasing intake of the test amino acid. The model accounts for the correlation among observations made for the same participant by treating the participant as a random effect term. The change-point is estimated at the amino acid intake where the mean squared error is minimized.

To evaluate the model's goodness of fit, we calculated the marginal and conditional R-squared (R^2_m and R^2_c) based on the method by Nakagawa and Schielzeth [25]. The marginal R^2 represents the variance explained by the fixed effects, as in a typical ordinary least square method [25,26]. In contrast, the conditional R^2 includes variance explained by both the fixed and random effects. Both R^2 computations offer a unique interpretation of the data, and the difference between the R^2_m and R^2_c values reflects the amount of variability explained by random effects.

We used parametric bootstrap [27], to estimate the variance around the breakpoint and calculate the 95% confidence interval [CI] as follows: 95% CI = BP ± 1.96 × SE. The difference between the confidence intervals (DCIs) defines the amount of overlap, and therefore statistical significance between early and late gestation. If the DCI does not contain zero, the null hypothesis can be rejected, indicating a statistical significance between the 2 groups. Statistical analyses were performed using R (version 4.0.5) for Windows. Values are presented as means ± SDs. Statistical significance was set at $P \leq 0.05$.

Descriptive data were analyzed using SPSS (Version 28; IBM SPSS Statistics for Windows, Version 28.0., IBM Corp). When obtaining a blood sample was not possible due to a collapsed vein or sample processing error (blood) and flushed sample (urine), missing data were addressed pairwise to use all information observed.

Results

Subject characteristics

In total, 25 participants (Table 1) completed a total of 70 oxidation study days (Table 2) ($n_{early} = 30$ oxidation days, $n_{late} = 40$ oxidation days). Each completed oxidation study day represents 1 complete graded experimental intake of methionine. In early pregnancy, 1 participant completed 7 experimental intakes, 1 participant completed 4 experimental intakes, 3 participants completed 3 experimental intakes, 3 participants completed 2 experimental intakes, and 4 participants completed 1 experimental intake (Supplemental Table 1). In late pregnancy, 1 participant completed 7 experimental intakes, 2 participants completed 6 experimental intakes, 2 participants completed 4 experimental intakes, 2 participants completed 3 experimental intakes, 1 participant completed 2 experimental intakes, and 5 participants completed 1 experimental intake. One participant came back to participate in late-stage pregnancy trial after completing 2 experimental intakes in early-stage pregnancy trials. No participants reported the use of alcohol, smoking, or illicit drugs during pregnancy. One participant reported the use of ranitidine, 1 reported the use of levothyroxine and 150 mg elemental iron, 1 participant reported occasional use of fluticasone (as needed), 2 reported the use of low-dose acetylsalicylic acid, and 1 reported the use of pyridoxine/doxylamine. However, no participants consumed any of the reported medications throughout the oxidation study day. None of the participants reported pregnancies in the 6 mo before joining the study. Gestational age for each participant was recorded based on self-report of the first day of last menstrual cycle. Based on measured height at time of preliminary assessment and self-reported prepregnancy weight, BMI range was 18.4 and 28.8 kg/m^2 (Table 1). The age of participants was between 27 and 39 y, and all experienced an appropriate amount of gestational weight gain compared with current recommendations [28]. There were no observations of abnormal glucose or protein in urine during the preliminary assessment or on the oxidation study days for any of the participants involved. One participant withdrew from the study after consenting and before completing an experimental study day due to parturition/delivery (Supplemental Figure 1).

Tracer oxidation

In early pregnancy (Figure 2), $F^{13}CO_2$ production decreased with increasing methionine intakes, until intake of 10 mg·kg^{-1}·d^{-1}. Further increase in methionine intakes did not result in a change to phenylalanine oxidation. Biphase linear regression crossover analysis of early pregnancy IAAO data suggest a breakpoint (EAR or mean requirement) of 11.1 mg·kg^{-1}·d^{-1} ($R^2_m = 0.79$, $R^2_c = 0.79$; 95% CI [8.9, 13.3 mg·kg^{-1}·d^{-1}]). In late pregnancy (Figure 3), $F^{13}CO_2$ production decreased with increasing methionine intakes, until 15 mg·kg^{-1}·d^{-1}. Biphasic linear regression crossover analysis of late pregnancy $F^{13}CO_2$ data suggest a breakpoint (EAR or mean requirement) of 15.0 mg·kg^{-1}·d^{-1} ($R^2_m = 0.72$, $R^2_c = 0.79$; 95% CI [13.0, 17.0 mg·kg^{-1}·d^{-1}]). The null hypothesis of no difference between breakpoint estimates was rejected, as the estimation of overlap in the CI of the breakpoints (DCI) did not contain an interval of zero (DCI = −3.9 ± 3.0). Therefore, the mean TSAA requirement in late gestation is significantly higher than the mean TSAA requirement in early gestation.

TABLE 1
Baseline characteristics[1] of participants.

Characteristics	Early gestation	Late gestation
Participants[2], n	12	13
Age at baseline, y	33.1 ± 4.0	32.5 ± 3.8
Prepregnancy BMI[3], kg/m^2	23.0 ± 3.4	22.6 ± 3.1
Fasting blood glucose, mmol/L	5.0 ± 0.4	4.5 ± 0.3
Fat mass[4], %	25.8 ± 3.6	24.8 ± 1.8
Resting energy expenditure[5], kcal/d	1357 ± 174	1483 ± 192

Abbreviation: BMI, body mass index.
[1] Values are mean, ± SD.
[2] One female participated in both early and late gestation groups.
[3] Based on self-reported prepregnancy weight.
[4] Determined by skinfold measurements (Harpenden Skinfold Caliper, Baty International).
[5] Determined by open-circuit indirect calorimetry (Vmax Encore, Viasys).

TABLE 2
Oxidation study day characteristics[1] of participants.

Characteristics	Early gestation	Late gestation
Oxidation study days	30	40
Gestational age[2], wk	17.3 ± 2.6	35.4 ± 2.2
Fasting blood glucose, mmol/L	4.8 ± 0.4	4.6 ± 0.4
Weight, kg	71.4 ± 12.4	77.3 ± 13.0
Protein intake prior to study day,[3] g·kg^{-1}·d^{-1}	1.45 ± 0.31	1.36 ± 0.26
Methionine intake prior to study day,[3] mg·kg^{-1}·d^{-1}	29.5 ± 8.79	27.3 ± 5.79
Habitual supplement intake[4]		
Folate, μg	890 (600–1000)	600 (600–1000)
Vitamin B_6, μg	2400 (1900–5250)	1900 (1400–5000)
Vitamin B-12, μg	2.8 (2.6–9.0)	2.8 (2.6–2.8)

[1] Values are mean, ± SD.
[2] Based on self-reported first day of last menstrual cycle.
[3] Based on 2-d dietary intake record.
[4] Based on self-reported daily prenatal vitamin intake; values are median (IQR).

Plasma and urinary metabolites

Table 3 presents measured plasma and erythrocyte metabolites during early and late gestation. Erythrocyte folate concentration (nmol/L) was 844 ± 207 (mean ± SD) in early pregnancy and 879 ± 235 in late pregnancy. In total, 38% (25 of 65) of blood samples had erythrocyte folate concentrations above 906 nmol/L [29]. Plasma concentrations of vitamin B-12 ranged from 148 to 220 or >220 pmol/L in both early and late gestation, with no deficiencies in vitamin B-12 (<148 pmol/L) found in any participants [30,31].

In both early and late pregnancies, circulating methionine concentrations increased in a linear fashion ($R^2 = 0.88$ and 0.86, respectively) in response to graded methionine intakes (Figure 4A, C). There was no correlation of graded dietary methionine intake on circulating cysteine (Figure 4B, D) or homocysteine ($R^2_{early} = 0.12$, $R^2_{late} = 0.10$; figure not shown). In both early ($R^2 = 0.94$) and late ($R^2 = 0.88$) pregnancy, plasma SAM concentrations had a positive linear association (Figure 5A, C) with graded dietary methionine intake. Plasma SAH concentrations also increased in a linear fashion in both early ($R^2 = 0.62$) and late ($R^2 = 0.42$) gestation (Figure 5B, D). There was no statistical difference in plasma homocysteine or glutathione concentrations with increasing methionine intakes in either early or late pregnancy. Plasma-free choline in early pregnancy averaged 8.6 ± 1.2 μmol/L (median = 8.6 μmol/L; IQR: 7.94–9.29 μmol/L). The distribution of free choline was skewed in late pregnancy, with a median concentration of 9.86 μmol/L (IQR: 9.00–11.2 μmol/L). Plasma betaine concentrations were 18.6 μmol/L ± 3.00 in early pregnancy and 16.7

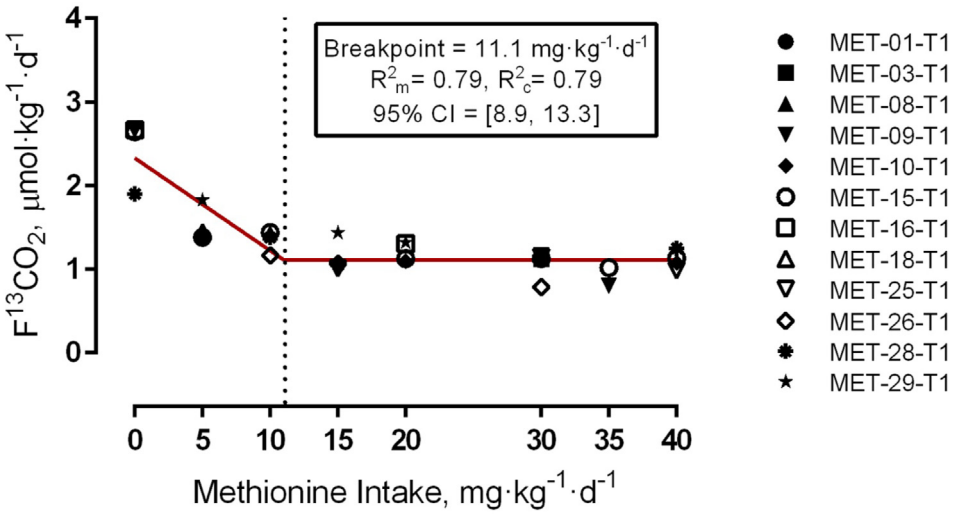

FIGURE 2. Estimated average requirement of TSAA in early gestation using the indicator amino acid oxidation method in healthy pregnant females. Biphasic linear regression crossover analysis of l-[1-^{13}C]phenylalanine tracer oxidation ($F^{13}CO_2$, µmol kg^{-1} h^{-1}) was used to determine the TSAA requirement. Statistical analyses were performed using R (version 4.0.5) for Windows TSAA requirement was determined to be 11.1 mg kg^{-1} d^{-1} ($R^2_m = 0.79$, $R^2_c = 0.79$; 95% CI [8.9, 13.3 mg·kg^{-1}·d^{-1}] n = 12, individual study days = 30). Dotted line indicates the mean requirement. Statistical analysis was performed using R (version 4.0.5) for Windows; $n = 30$. TSAA, total sulfur amino acids (methionine + cysteine).

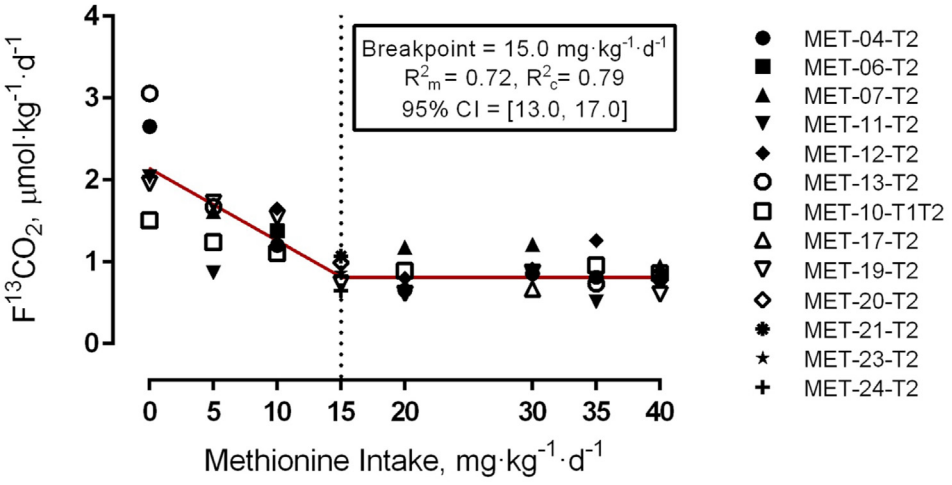

FIGURE 3. Estimated average requirement of TSAA in late gestation using the indicator amino acid oxidation method in healthy pregnant females. Biphasic linear regression crossover analysis of l-[1-^{13}C]phenylalanine tracer oxidation ($F^{13}CO_2$, µmol kg^{-1} h^{-1}) was used to determine the TSAA requirement. Statistical analyses were performed using R (version 4.0.5) for Windows TSAA requirement was determined to be 15.0 mg·kg^{-1}·d^{-1} ($R^2_m = 0.72$, $R^2_c = 0.79$; 95% CI [13.0, 17.0 mg·kg^{-1}·d^{-1}] n = 13, individual study days = 40). Dotted line indicates the mean requirement. Statistical analysis was performed using R (version 4.0.5) for Windows; $n = 40$. TSAA, total sulfur amino acids (methionine + cysteine).

µmol/L ± 2.63 in late pregnancy. Dimethylglycine (DMG) concentrations were 1.23 µmol/L ± 0.43 and 1.51 µmol/L ± 0.59 in early and late pregnancy, respectively.

Urinary sulfate concentrations, standardized to creatinine, had a moderate linear association with methionine intake in both early (Supplemental Figure 3A; $n = 29$) and late (Supplemental Figure 3B; $n = 37$) gestation ($R^2_{early} = 0.49$, $R^2_{late} = 0.33$).

Discussion

To best of our knowledge, this is the first study to directly investigate TSAA requirements (in the absence of dietary cysteine) during pregnancy in early (11–20 wk) and late (31–40 wk) gestation. The mean TSAA requirement during early stages of pregnancy was found to be 11.1 mg·kg^{-1}·d^{-1} with a population-safe (95% CI) intake of 13.3 mg·kg^{-1}·d^{-1}. In late pregnancy, the mean TSAA requirement and population-safe intake were found to be 15.0 and 17.0 mg·kg^{-1}·d^{-1}, respectively. This suggests that late-stage requirements are 35% higher compared with early gestation requirements. The increase in TSAA needs between early gestation and late gestation, is in line with previous evidence that amino acid requirements during pregnancy are dynamic and necessitate independent recommendations based on gestational stage [8–10].

Based on our hypothesis we found that TSAA needs differ compared with current recommendations, and that early compared with late gestation TSAA needs would be different. Interestingly, the current

TABLE 3
Circulating nutrients, metabolites, and hormones at early and late gestation[1].

Plasma	Early gestation	Late gestation
Cysteine, μmol/L	206 (195−219)	190 (178−204)
Homocysteine, μmol/L	4.68 (4.04−5.25)	5.02 (3.87−6.18)
Plasma-free choline, μmol/L	8.60 (7.9−9.3)	9.86 (9.00−11.2)
Betaine, μmol/L	18.4 (16.5−20.5)	17.2 (15.1−18.0)
Dimethylglycine, μmol/L	1.31 (0.99−1.31)	1.57 (0.94−1.95)
Vitamin B-12[2], pmol/L	319 (239−446)	216 (193−405)
Folate, nmol/L	33.3 (31.2−35.9)	34.3 (32.2−37.1)
Erythrocyte folate, nmol/L	798 (682−986)	871 (684−1043)
Glutathione[3], mmol/L	2.5 (2.3−2.7)	2.9 (2.2−3.6)
Progesterone, ng/mL	47.3 (36.3−54.3)	156 (124−183)
Estradiol[4], pg/mL	4045 (3355−4985)	14 565 (11 055−17 835)

[1] Values are median (IQR).
[2] Total Vitamin B-12.
[3] Erythrocyte glutathione; $n_{early} = 21$; $n_{late} = 24$.
[4] 17β-Estradiol.

study TSAA requirements were lower than the current recommended EAR and RDA for TSAA of 20 and 25 mg·kg^{-1}·d^{-1}, respectively, during pregnancy. Current recommendations are factorially derived estimates, based on the theoretical model of increased protein requirements during pregnancy. The current EAR for protein during pregnancy is set at 0.88 g·kg^{-1}·d^{-1}, which is a factor of 1.33 above the adult nonpregnant EAR of 0.66 g·kg^{-1}·d^{-1} [1]. The EAR and RDA for TSAA (methionine + cysteine) for nonpregnant adults is 15 and 20 mg·kg^{-1}·d^{-1}, which, by including the factor of 1.33 provides the pregnancy TSAA recommendations of 20 and 25 mg·kg^{-1}·d^{-1}. Based on the results from the current study the recommendations are overestimated by ~47% in early pregnancy, and 32% in late pregnancy. These findings add to our series of studies in human pregnancies that suggest although protein requirements increase throughout pregnancy, the increase in amino acid demand is not proportionally equal for each individual amino acid [7–10,32]. Because regulation of one-carbon metabolites by its substrates and products is complex, this allows the pathway to conserve methionine to maintain a sufficient level of methyl group availability and remove unwanted homocysteine [4]. It is likely that obligatory losses for amino acids are not the same and conserved differentially. Additionally, recent work done by Paoletti et al. [24] has shown that TSAA requirements in older adults (≥60 y) is higher for males than females, suggesting a possible sex difference, likely due to differential demands for methionine in the sulfur amino acid metabolic pathway. Currently, requirements of young, nonpregnant females are unknown. It is possible that the requirement for TSAA of healthy young males of 12.6 mg·kg^{-1}·d^{-1}, are different and likely lower in young females, similar to elderly females [24]. Our current study is the first of the experimentally determined indispensable amino acid needs that suggest a lower requirement than current recommendations, suggesting methionine is conserved within the one-carbon metabolite pathway, and recycled as needed, resulting in a lower number of

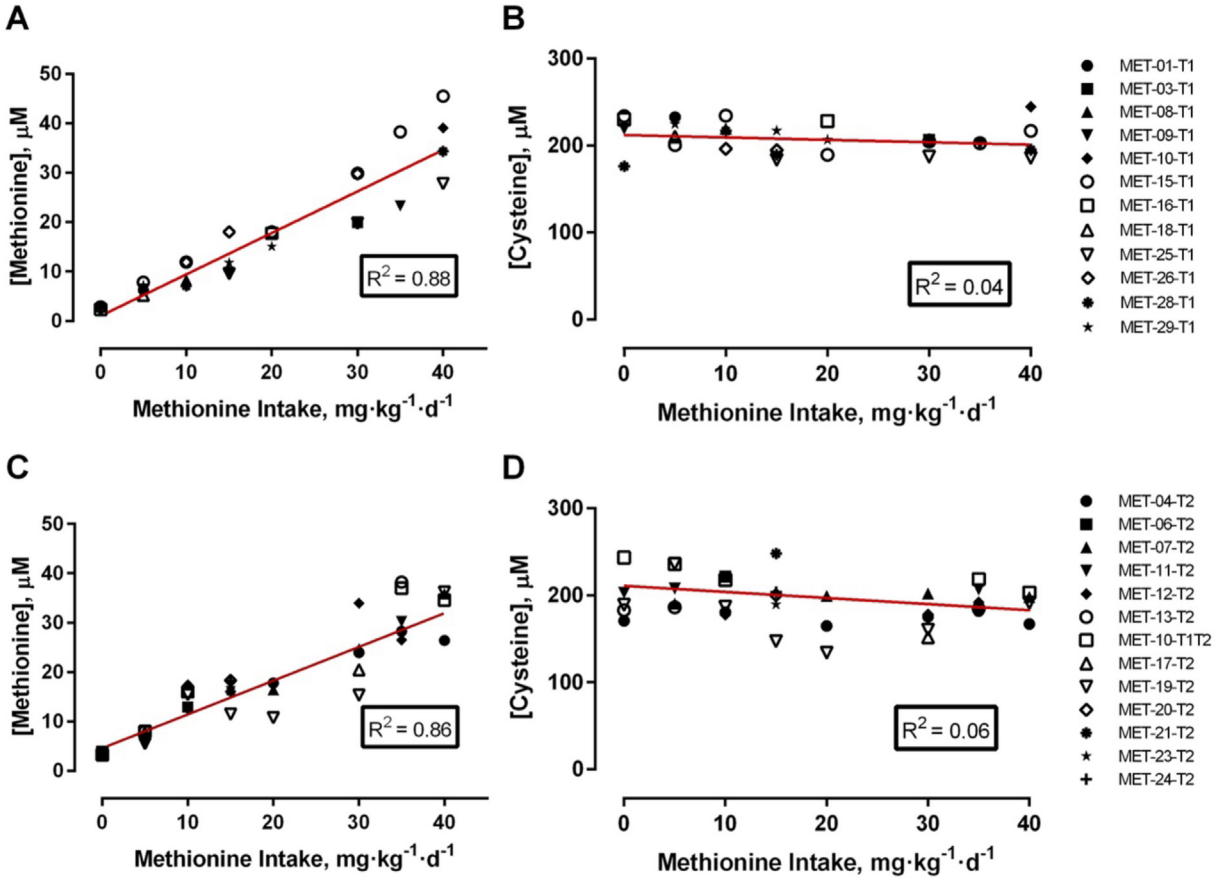

FIGURE 4. Plasma metabolite concentration based on graded methionine intake in the diet in early vs. late gestation. (A) Plasma methionine concentration (μM) in early pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.88$). (B) Plasma cysteine (μM) in early pregnancy in response to graded dietary intakes of ($R^2 = 0.04$). (C) Plasma methionine (μM) in late pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.86$). (D) Plasma cysteine (μM) in late pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.06$). $n_{early} = 29$; $n_{late} = 37$. Abbreviations: SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

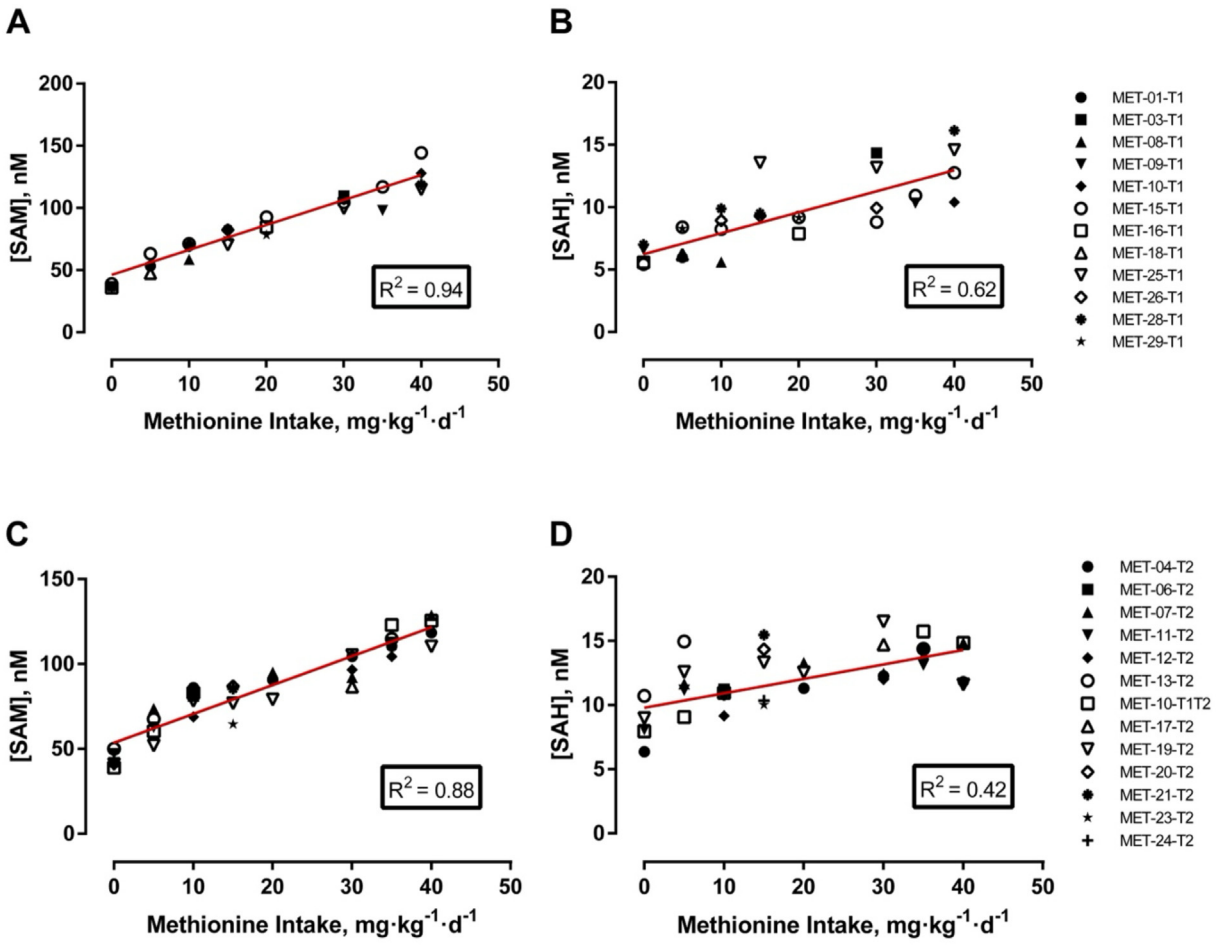

FIGURE 5. Plasma metabolite concentration based on graded methionine intake in the diet in early vs. late gestation. (A) Plasma SAM concentration (nM) in early pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.94$). (B) Plasma SAH (nM) in early pregnancy in response to graded dietary intakes of ($R^2 = 0.62$). (C) Plasma SAM concentration (nM) in late pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.88$). (D) Plasma SAH (nM) in late pregnancy in response to graded dietary intakes of methionine ($R^2 = 0.42$). $n_{early} = 29$; $n_{late} = 37$. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

obligatory losses, and therefore lower requirement than expected in our well-nourished pregnant participants.

The human fetal liver has been suggested to lack the enzyme cystathionase, responsible for the conversion of cystathionine to cysteine [33–38]. Without the ability to synthesize cysteine from an endogenous supply of maternal methionine, fetal tissues become reliant on a supply of maternal circulating cysteine. For this reason, it is possible that the increase in TSAA needs in late gestation could be partially due to an increase in cysteine requirements and the fetal inability to synthesize cysteine during the dynamic increase in protein deposition needs throughout the later stage of pregnancy. The regulatory enzyme that catalyzes the reaction of homocysteine to cysteine, cystathionine β-synthase has increased activity when concentrations of circulating SAM are high, essentially shunting homocysteine toward the transsulfuration (TS) pathway [39]. Either of these theories could explain why plasma SAM and methionine concentrations increases with methionine intakes, whereas homocysteine and cysteine concentrations do not show any pattern of increase or change. However, plasma concentrations are not reflective of intracellular metabolism, and needs to be confirmed with isotopic tracer–based studies. Whether the TSAA needs increase in late gestation is due to a higher need of methionine is also unclear. During pregnancy, various SAM-dependent methylation reactions (TM) may be increased to support physiologic processes and could place a high demand on methionine [40–42]. It has been shown earlier using stable isotope-labeled methionine that TS rates are higher in early pregnancy compared with later pregnancy in well-nourished American women, whereas transmethylation (TM) and re-methylation (RM) rates increase progressively through gestation stages [43]. However, it is important to note that "early" pregnancy in our study is defined as 11–20 wk, and early pregnancy in the mentioned study is defined as first trimester with women recruited and studied before 13 wk gestation (mean ± SD; 10.4 ± 2.5 wk), with no significant difference in TS kinetics found during second trimester, a time point that would more accurately align with the participants studied in our "early" pregnancy population (mean ± SD; 17.3 ± 2.6 wk). In contrast, a study on a pregnant Indian population demonstrated that TS, TM, and RM rates do not change with stages of pregnancy. It is worth noting that these women were not consuming adequate protein during pregnancy with the authors of this study suggesting that lower dietary protein consumption might explain the different results between the 2 populations [44]. Furthermore, protein and methionine intakes correlated with RM and TM rates in these Indian women and thus, it is likely that the minimum methionine (methionine in the presence of adequate dietary cysteine) needs are likely elevated during pregnancy and needs to be determined. Determining the minimum (obligatory) methionine requirement in early and late stages of healthy, human pregnancy might

give a better insight into whether the increase in dietary TSAA intake is due to an increased need for dietary methionine, dietary cysteine, or both.

Recently it has been suggested based on a population-based case–control study that optimizing all methyl donors in the maternal diets would reduce the incidence of infants with neural tube defects [45,46]. In the current study our participants were adequate for folate and vitamin B-12 (Table 3), based on plasma concentrations being above-recommended cutoffs of 15.9 nmol/L [29] and 148 pmol/L [31,47–49], respectively. Similarly, plasma-free choline, betaine, and dimethylglycine concentrations (Table 3) in the current study were similar to earlier pregnancy reported values [31,50]. Both estrogen and progesterone concentrations are known to increase throughout pregnancy [51], as seen in our current study, suggesting progression of pregnancy stages were adequate from a hormonal perspective. Thus, our study design under sufficient protein, energy, and other methyl donors determines the requirement for methionine to meet the demand for cysteine and protein synthesis during 2 distinct stages of human pregnancy. Under conditions of deficiency of protein or other one-carbon donors it is likely that methionine needs could be altered, especially during pregnancy even in well-nourished females, as shown by our earlier study in females receiving 0.8 g/kg protein/day; a dietary need for glycine arises in later stages of pregnancy [32]. Lastly, glutathione is a valuable storage molecule for cysteine [52,53], and it is therefore possible that glutathione may buffer the protein synthetic needs of cysteine during our study period.

We are aware of limitations of this study, which include the short-term nature of adaptation and study days, small sample size, and the inability of a full repeated-measures design due to progressive nature of the life stage, and some participants unable to participate more than once. Additionally, maternal plasma metabolites are not indicative of intracellular metabolite status, nor are they indicative of concentrations present in fetal tissues. Our objective was to compare patterns of response for related one-carbon metabolites between early and late gestation, and to identify deficiencies, if any. Some of the strengths of the study include the first, to the best of our knowledge, to determine TSAA requirements directly in pregnant participants, continuing previous work that has investigated protein, lysine, phenylalanine, and tyrosine requirements during pregnancy [7–10]. Participants were studied at 2 different gestational stages, allowing insight into amino requirements of a highly dynamic and metabolically active state such as pregnancy. It is important to note that although sample size calculations were not determined, the statistical model used (biphase linear regression) is reliant on the total number of observations per breakpoint, with the corresponding R^2 providing a representation of the goodness of fit. The R^2_C (conditional R^2, that takes into account variance due to both fixed and random effects) in this study (early gestation $= 0.79$ and late gestation $= 0.79$) are substantially higher than previous studies investigating protein, lysine, total aromatic requirements, and phenylalanine requirements in early and late pregnancy [7–10] when using the IAAO method. The high coefficients of determination highlight the robustness of the data from the current sample size. The results of this study are generalizable only to healthy pregnant females in the Vancouver area who have no apparent nutrient deficiencies and are of adequate energy and protein status.

In conclusion, our study determined the TSAA requirements, as an EAR for healthy pregnant females, 11.1 and 15.0 $mg \cdot kg^{-1} \cdot d^{-1}$, in early and late stages of gestation, respectively. Late gestation requirement for TSAA increases by 35% and is significantly different from the requirement for early gestation (DCI= -3.9 ± 3.0), although both gestation stage requirements are considerably lower than current DRI recommendations, EAR and RDA of 20 and 25 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively. These findings are important for future dietary intake recommendations for optimizing maternal diets and suggest that gestation stage–specific recommendations might be necessary in healthy human pregnancy.

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Author contributions

The authors' responsibilities were as follows – KS, BFR, ROB, PBP, GC-M, RE: designed research; KS: conducted research; GC-M: provided essential reagents; KS, BL, DK: analyzed data and performed statistical analysis; KS: wrote the article; RE: had primary responsibility for final content; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data Availability

Data described in the manuscript will be made available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2024.07.034.

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