HHS Public Access

Author manuscript

Pediatr Obes. Author manuscript; available in PMC 2023 May 01.

Published in final edited form as:

Pediatr Obes. 2022 May ; 17(5): e12876. doi:10.1111/ijpo.12876.

Infant Intakes of Human Milk Branched Chain Amino Acids are Negatively Associated with Infant Growth and Influenced by Maternal Body Mass Index

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Abstract

Background: Branched-chain amino acids (BCAAs: isoleucine, leucine, and valine) and aromatic amino acids (AAAs: phenylalanine and tyrosine) are hypothesized to influence early-life obesity risk.

Objective: To assess HM free amino acid (AA) concentrations and infant intakes of HM AAs from women with obesity (OB) compared to those with normal weight (NW) and determine the relationships between HM AA consumption and infant growth.

Methods: HM samples were collected at 0.5 (n=151), 2 (n=129), and 6 (n=93) months postpartum from mothers with NW (body mass index (BMI) =18.5–24.9 kg/m^2) and OB (BMI>30 kg/m^2). HM AAs were quantified via mass spectrometry. Infant HM intake, anthropometrics and body composition were assessed. Linear mixed-effects models (LMEM) examined the relationships between maternal BMI and HM AA intakes, and HM AA intake and infant growth over the first 6 months postpartum after adjusting for maternal and infant characteristics.

Results: Maternal BMI was positively associated with infant intakes of isoleucine, leucine, and AAAs across timepoints. HM AA intakes were positively associated with weight-for-length z-score, fat mass index, and fat free mass index in infants ($p<0.05$).

Conclusions: Maternal BMI led to differences in HM AA composition, which was associated with infant body composition.

Keywords

BCAA; aromatic amino acids; breastmilk; essential amino acids; postnatal programming

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Conflict of Interest: The authors have no conflict of interest.

Clinical Trial Registry Numbers: www.clinicaltrials.gov, ID# NCT01131117 and ID# NCT02125149

Introduction

Early-life growth and development are shaped by genetics [1,2] as well as both *in-utero* [3] and postnatal [4] environmental exposures. Human milk is a complex biological fluid that is considered the gold standard for infant nutrition [5] and is a likely source of postnatal nutritional programing. Varied biological environments affect human milk composition and volume, which may influence the delivery of optimal amounts of nutritive and nonnutritive components to the infant. To help optimize infants' well-being, it is imperative to define the nutritive and nonnutritive factors that are associated with early-life growth and development that may program life-long patterns in health and disease.

Free amino acids are amongst the most variable metabolites in human milk across all lactation phases [6,7] and can vary significantly between mothers [8,9]. Free amino acids, such as branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) and aromatic amino acids (AAA: phenylalanine and tyrosine) are increased in both the plasma and human milk of women with obesity (OB) and are negatively associated with metabolic health[10-12]. Studies examining the relationship between elevated human milk BCAAs and infant growth and development have not been carried out for term infants; however, a positive association was found between human milk BCAA content and faster growth in preterm infants [13]. Furthermore, infant intakes of human milk free BCAAs, AAAs, His, Thr, and Met have been shown to correlate significantly with the respective infant plasma concentrations [14], supporting the potential for a relationship between these free amino acids and infant physiology. Importantly, it remains to be determined whether or not infants fed by mothers with OB also consume a greater amount of these amino acids, contributing to altered growth patterns.

Circulating BCAAs and AAAs are involved in cell signaling [15,16], are hypothesized to take part in obesogenic metabolic programming (the early protein hypothesis [17,18]), and some have insulin stimulating properties [19]. Although less research exists for the pediatric population, plasma BCAA levels are reportedly associated with adolescent BMI [20] and are linked to insulin resistance during puberty [21]. Compared to breastfed infants, plasma BCAAs are increased in formula fed babies [18] which may negatively impact metabolism via impaired beta-oxidation [22] leading to increased growth velocity and adiposity [23]. To date, studies testing the effect of early-life protein consumption on metabolic programming have focused on formula protein type or protein content [22,24,25]. However, whether specific human milk protein and free amino acid concentrations influence infant metabolism and growth remain largely unknown.

In this study, we first set out to determine the free amino acid concentrations of human milk from women with normal weight (NW) and OB and the subsequent infant intakes of BCAAs and AAAs. Thereafter, we characterized the relationship between human milk free amino acid consumption, infant growth, and body composition in the first 6 months of life. We hypothesized that infants born to OB mothers would consume more BCAAs and AAAs. We further hypothesized that BCAA and AAA intakes would be positively associated with infants' growth and adiposity [26].

Subjects and Methods

Participants

This was a secondary analysis of healthy breastfeeding participants (169 mother-child pairs, Supplemental Figure 1) with NW (body mass index (BMI) 18.5-24.9 kg/m^2) or OB (BMI 30-65 kg/m^2) enrolled in two parent longitudinal studies (www.clinicaltrials.gov, ID# NCT01131117 and ID# NCT02125149). Enrollment criteria for NCT01131117 have been previously described in detail elsewhere [27,28]. The women in this secondary analysis were recruited prior to pregnancy or during their first trimester and attended their first study visit prior to pregnancy (mean = 10 weeks pre-pregnancy, SD = 12 weeks) or during their first trimester (mean = 10 weeks of gestation, with a range of 4-14 weeks). Exclusion criteria included preexisting or ongoing medical conditions (e.g., diabetes mellitus, hypertension), use of medications during pregnancy that are known to influence fetal growth, smoking, or alcohol consumption. Additional enrollment criteria for NCT02125149 also required participants to be sedentary and cleared for an exercise program by their physician. The study procedures were in accordance with the ethical standards of the Institutional Review Board of the University of Arkansas for Medical Sciences.

Maternal Anthropometrics and Gestational Weight Gain

During the first study visit, maternal weight and height were measured with a standing digital scale (Tanita Corporation, Tokyo, Japan) and a wall-mounted stadiometer (Perspective Enterprises, Portage, Michigan), respectively. BMI was calculated as kg/m^2. Gestational weight gain was calculated based upon the differences in measured weights between the participant's first study visit and week 36 of gestation. We have previously demonstrated that there are very few variations between pre-pregnancy or first trimester measures in categorizing women [27]. The 2009 Institute of Medicine (IOM) guidelines for gestational weight gain (GWG) based on BMI [29] were used to evaluate the number of women categorized as having inadequate (less than the recommended weight gain for BMI category), appropriate (within the recommended weight gain for BMI category), and excessive (exceeding the recommended weight gain for BMI category) weight gain.

Infant Body Composition

At each postnatal visit (0.5, 2, and 6 months), infant weight and length were measured using a tared scale (Seca, Hamburg, Germany) and a length board with a sliding foot piece (Perspective Enterprises, Michigan, USA), respectively. Appropriately trained and highly experienced research assistants performed these measures. Of note, the Lin's concordance coefficient for inter-rater reliability was 0.999 for length measurements presented in this analysis. Infants' gestational weight-for-age categories (small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA)) were calculated using the updated US-based birth weight for gestational age reference [30]. Weight-for-length (WLZ) and weight-for-age (WAZ) Z-scores were calculated based on the WHO Child Growth Standards [31]. Infant fat (FM) and fat free (FFM) mass were determined by quantitative nuclear magnetic resonance (EchoMRI-AH, Echo Medical Systems, Houston, Texas) as previously described [32]. Fat mass index (FMI) and fat free mass index (FFMI) were calculated as kg/m^2.

Self-reported outcomes

Maternal race and age were self-reported. At 0.5 months postpartum, mothers reported their infants' race, sex, birth weight, and birth length as well as their delivery mode. Gestational age was calculated using the mother's last menstrual period and the child's date of birth.

Human Milk Collection

Participants were asked to collect a human milk sample at the second feeding of the day (or before 9 am) by fully expressing one breast (manually or using a pump [electrical or manual] at home or during their study visit) at postnatal age 0.5, 2, and 6 months. All milk samples were stored at −70°C until analysis. At each visit, women reported their frequency of breastfeeding and completed weighed food records (for expressed human milk and additional formula feeding). Once the participant discontinued breastfeeding completely, they could no longer participate in this secondary analysis (see Supplemental Figure 1 for details).

Infant Daily Intake

A single test weigh combined with nursing frequencies was used to estimate daily infant intakes of human milk [33]. Infants were fasted for at least 2 hours prior to the test weighing procedure. Infant weights were measured immediately before and after a nursing session to determine the volume of human milk consumed at 0.5, 2, and 6 month visits. Mothers recorded their nursing frequency over 3 days (2 week and 1 weekend day) or weighed the expressed milk fed to their infant prior to the corresponding study visit. Human milk volume was estimated from the average daily nursing frequency, single test weight or bottle weighing for expressed milk to estimate daily human milk intake. Infants were categorized as either exclusively breastfed or mixed fed based upon the infant's formula intake. If at any visit the infant received more than 100 mL of formula per day, they were then considered a mixed feeder from that visit onwards. Data from visits that lacked dietary intake data were not used in the daily intake analyses, but human milk amino acid concentrations were still included in the study analysis.

Human Milk Free Amino Acid Concentrations Analysis

Concentrations of free amino acids; arginine (Arg), alanine (Ala), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), cystine (C-C), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), selenocysteine (Se-C), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Try), and valine (Val) were quantified in human milk samples. Dried extracts were reconstituted in 100 μL of mobile phase (A:B 1:2) just prior to analysis. Briefly, an EZ:faast amino acid analysis kit was purchased from Phenomenex (Torrance, CA). Se-C and Cys standards (1 mg/mL in H_2O) were obtained from Sigma Aldrich (St. Louis, MO) and were added to the calibration mixtures at 20, 100, and 200 nmol/mL. All other reagents used were of optima grade from Fisher Scientific (Pittsburgh, PA). Standards and samples (100 μL) were processed in accordance with the manufacturers' instructions.

Chromatographic separation was performed on an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA) fitted with the Phenomenex EZ:faast column (250 x 3 mm,

4 μm) kept at 35 °C. A flow rate of 500 μL/min and injection volume of 5 μL was used. Mobile phases consisted of 10 mM ammonium formate in water (A) and 10 μM ammonium formate in methanol (B) with a 20-minute elution gradient as follows: ramp from 68 to 86% B over 13 min, return to 68% B in 0.01 min and hold 68% B for 7 min. Identification was carried out on a SCIEX 4000 QTRAP (Framingham, MA) mass spectrometer with data acquisition and analysis performed using *Analyst 1.7* software. Data were acquired by multiple reaction monitoring (MRM) in positive Turbo spray ionization mode. Nitrogen as curtain, CAD, GS1, and GS2 gas was set at 10, medium, 10, and 10 units, respectively. Ion spray voltage and source temperature were at 5500 V and 425 °C. MRM parameters such as declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) are supplied in supplemental data (Supplemental Table 1). Due to the high-throughput analysis, a mixture of all standard amino acids was injected every 15 samples to monitor the column degeneration. The amino acid intensities of each of the 15 samples were normalized by the standards in the same batch. All samples were assayed in duplicate. The sample coefficient of variances (Supplemental Table 2) were used to determine the validity of each measurement.

Statistical Analysis

Descriptive statistics (mean, standard deviation, counts, and percent) were calculated for demographic data, clinical characteristics (Table 1, Supplemental Table 3), infant feeding patterns (Supplemental Table 4), human milk free amino acid concentrations (Table 2), and daily infant intake of free amino acids (Table 3). T-tests and Pearson's Chi-squared tests were used to compare values between groups (NW and OB) for continuous and categorical data, respectively. Significance was set at alpha ≤ 0.05. Linear mixed-effects models with Bonferroni-corrected post hoc pairwise comparisons were constructed to investigate any differences between the human milk free amino acid composition (nmol/mL) of NW and OB mothers while adjusting for maternal race, maternal age, and infant sex (Figure 1). Linear mixed-effects models were also constructed to determine: 1) the associations between maternal BMI and normalized free amino acid intakes (μmol/day/kg infant weight) over time (repeated measures at 0.5, 2, and 6 months, Table 4), 2) the associations between human milk free amino acid intake (μmol/day) on infant body composition (FMI and FFMI) after adjusting for infant age, infant birth weight, infant sex, maternal BMI, gestational weight gain, maternal race, delivery mode (vaginal or C-section), and breastfeeding status over time (repeated measures at 0.5, 2, and 6 months, Table 5) and 3) the associations between human milk free amino acid intake (μmol/day) on infant growth (WAZ and WLZ) after adjusting for infant age, infant birth weight, maternal BMI, gestational weight gain, maternal race, delivery mode (vaginal or C-section) and breastfeeding status over time (repeated measures at 0.5, 2, and 6 months). Linear mixed-effects models were fitted with all available data and confounders were chosen *a priori*, where subject identification number was considered as a random effect. Statistical analyses were conducted in R (version 3.6.0) and IBM SPSS® software version 27.

Results

Participant characteristics and infant feeding

Of the 169-breastfeeding mother-infant pairs studied (Table 1), 83 mothers had NW (mean BMI = 22.3 ± 1.7 kg/m^2) and 86 mothers had OB (mean BMI = 36.4 ± 5.7 kg/m^2). The number of participants who provided human milk at each postnatal visit (0.5, 2, and 6 months) are listed in Supplemental Figure 1 and Table 1. Women were, on average, 30.1 ± 4.0 years of age at the time of delivery (39.2 ± 1.5 weeks gestation). The majority of women were Caucasian (78%) and had vaginal deliveries (66%). However, compared to women with NW, a greater proportion of women with OB were non-Caucasian (29% vs. 14%, $p = 0.017$) and delivered via C-section (43% vs. 24%, $p = 0.007$). Women with OB gained significantly less weight during gestation compared to women with NW (8.7 ± 5.2 kg vs. 12.6 ± 2.8 kg, $p < 0.001$), as recommended by IOM guidelines [29]. However, the majority of women with OB (45%) were categorized as gaining an excessive amount of weight during pregnancy based on IOM categorizations [29], while a greater proportion of women with NW gained an inadequate amount of weight as compared to women with OB (31% vs. 22%, $p < 0.001$).

Similar proportions of male and female infants were included in this study (Table 1). There were no differences in sex, birth weight, birth length, or weight-for-gestational-age category between infants born to women with NW vs. those with OB (Table 1). Infants born to women with OB had 16.6% greater FM ($p = 0.001$) and 17.8% greater FMI ($p < 0.001$) at 0.5 months postpartum compared to those born to women with NW (Supplemental Table 3). However, there were no observed differences in adiposity at later time points (2 and 6 months), Supplemental Table 3. The FFMI of infants born to women with OB was slightly, but significantly greater at 0.5 (3.1%, $p = 0.023$) and 6 months (6.0%, $p = 0.001$) compared to those born to women with NW (Supplemental Table 3). There were no differences in the total amount of milk consumed (human milk plus formula) or the total amount of human milk consumed by infants born to OB women compared to NW women after normalizing intake to infant body weight (kg) at any timepoint measured in this study (Supplemental Table 4). However, the proportion of women who supplemented their infants' intake with formula was significantly greater for women with OB than for women with NW at both 0.5 months (21.7% vs. 3.6%, $p = 0.005$) and 2 months postpartum (15.2% vs. 3.7%, $p = 0.048$) (Supplemental Table 4).

Human milk free amino acid concentrations are altered by maternal BMI and time

Sixteen of the 22 targeted free amino acid concentrations were detected in human milk at 0.5, 2, and 6 months postpartum (Table 2). BCAAs (Ile (53.5%) and Leu (31.1%)) and AAAs (Phe (27.5%) and Tyr (42.3%)) were significantly increased in human milk from women with OB at all timepoints, while the essential amino acid His was significantly decreased by ~35% compared to human milk from women with NW. At 2 and 6 months postpartum, Asn (39%), Gln (33%), Glu (11%), Gly (20%), and Ser (19%) concentrations were significantly lower, while Asp (39%) was significantly higher in human milk from women with OB vs. NW. Human milk concentrations of C-C at 0.5 and 2 months were, on average, 27.0% significantly lower in human milk from women with OB compared to women with NW.

Linear mixed-effects models were developed to test the effects of maternal OB on free amino acid concentrations in human milk, adjusting for maternal race, maternal age, infant age, and infant sex (Figure 1). The majority of free amino acid concentrations (Ala, Asn, Asp, C-C, Gln, Glu, Gly, Phe, and Ser) significantly increased in human milk over time (0.5 – 6 months). However, Pro and the positively charged free amino acids, His and Lys, decreased significantly as lactation progressed, while Ile, Leu, and Tyr did not change (Figure 1). There was a significant group effect (NW vs. OB) on 13 of the 16 free amino acid concentrations (Asn, Asp, C-C, Gln, Glu, Gly, His, Ile, Leu, Phe, Ser, Tyr, and Val), where women with OB had significantly lower Asn, C-C, Gln, Glu, Gly, and Ser concentrations at later timepoints (2 and/or 6 months) and decreased His levels at each timepoint measured. Higher BCAA (Ile, Leu, and Val), AAA (Phe and Tyr), and Asp concentrations were observed in milk from women with OB compared to women with NW.

Daily infant intakes of human milk free amino acids change over time and are altered by maternal BMI

Actual intakes of human milk free amino acids were estimated at 0.5, 2, and 6 months of age to better understand how differences in human milk free amino acid concentrations translate to infant nutrition and growth (Table 3). Contrary to what was observed for human milk free amino acid concentrations, normalized infant consumption of human milk free amino acids decreased for the majority of free amino acids measured (Ala, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Tyr, and Val) over the first 6 months of life. However, infant intakes of human milk Gln tended to increase over time (Table 3). There were only a few differences detected in infants' daily human milk free amino acids intakes after normalizing to infant weight (kg) among infants born to women with NW or OB (Table 3). At 0.5 months, the daily intakes of His were numerically lower (~24% lower, $p = 0.064$) while Ile intakes were numerically higher (~39% higher, $p = 0.083$) in infants born to women with OB. The daily intakes of Asn (~52%), C-C (~32%), Gln (~23%), Gly (~25%), and His (~33%) were significantly lower whereas Ile (~50%) and Leu (~25%) were significantly higher in infants born to women with OB compared to those with NW at 2 months postpartum. The daily intake of human milk His was the only free amino acid intake that differed at 6 months of age, and was ~33% lower in infants born to women with OB compared to the NW group.

Linear mixed-effects models (Table 4) revealed that as maternal BMI increases (1 kg/m^2 BMI), the daily infant human milk intakes (μmol/kg/day) of Asn ($\beta = -0.04$, $p = 0.03$), C-C ($\beta = -0.03$, $p = 0.05$), and His ($\beta = -0.07$, $p = 0.004$) decreases after adjusting for infant age, infant sex, maternal race, gestational weight gain, and breastfeeding status (exclusive or mixed). On the other hand, daily infant human milk intakes (μmol/kg/day) of BCAAs, Ile ($\beta = 0.04$, $p = 0.001$) and Leu ($\beta = 0.05$, $p = 0.08$), and AAAs, Phe ($\beta = 0.03$, $p = 0.01$) and Tyr ($\beta = 0.05$, $p = 0.002$), were associated with a significant increase as maternal BMI increased (per 1 kg/m^2 BMI) after controlling for the same covariates.

Normalized daily infant free amino acid intakes are associated with infant FMI and FFMI over the first 6 months of age

To test the possible associations between infant intakes of free amino acids and infant growth (Table 5), linear mixed-effects models were constructed after adjusting for infant

age, infant sex (FMI and FFMI models only), infant birth weight, maternal race, gestational weight gain, and breastfeeding status and considering the trajectories of the repeated measurements. The individual daily intakes for each free amino acid were modeled to associate with infant WLZ, WAZ, FMI, and FFMI over the first 6 months of life (repeated measures at 0.5, 2, and 6 month visits). Infant intakes of free amino acids were not associated with WAZ. For every μmol/day increase of free Asp (trend, $p = 0.093$), C-C (trend, $p = 0.088$), Glu ($p = 0.042$), and His ($p = 0.039$) consumed, child WLZ increases by 0.0024 kg/m^2, 0.0103 kg/m^2, 0.0004 kg/m^2, and 0.0080 kg/m^2, respectively. For every μmol/day increase of free Asp ($p = 0.011$), C-C ($p = 0.006$), Gln ($p < 0.001$), Glu ($p = 0.005$), His ($p = 0.008$), and Ser ($p = 0.002$) consumed, child FMI increases by 0.0011 kg/m^2, 0.0038 kg/m^2, 0.0174 kg/m^2, 0.0011 kg/m^2, 0.0005 kg/m^2, 0.0106 kg/m^2, 0.0185 kg/m^2, and 0.0029 kg/m^2, respectively.. On the other hand, free Asp ($\beta = 0.0034$ kg/m^2, $p = 0.011$), C-C ($\beta = 0.0163$ kg/m^2, $p = 0.004$), Gln ($\beta = 0.0007$ kg/m^2, $p = 0.009$), Glu ($\beta = 0.0004$ kg/m^2, $p = 0.026$), His ($\beta = 0.0088$ kg/m^2, $p = 0.015$), and Ser ($\beta = 0.0014$ kg/m^2, $p = 0.088$) intakes were positively associated with infant FFMI. Together, these data suggest that differences in the daily consumption of several human milk free amino acids is associated with infant body composition.

Discussion

As increased numbers of breastfeeding mothers develop overweight or obesity, it is important to define the impact of maternal BMI on human milk composition and on infant intakes of various human milk constituents. From this study, three key observations were made. 1) Women with obesity produce human milk that differs significantly from women of normal weight in terms of free amino acid composition. Of interest, changes in human milk free amino acid content does not directly translate to altered infant free amino acid consumptions, suggesting that accurate estimation of infant intakes is crucial in understanding nutritional exposures during infancy. 2) Free BCAAs and AAAs concentration were significantly higher in human milk from women with obesity and maternal BMI was positively associated with Ile, Leu, Phe, and Tyr concentrations over the first 6 months postpartum. However, infants born to women with obesity only consumed greater quantities of these free amino acids, relative to infant body weight, at 2 months of age. 3) The daily intakes of several free amino acids were positively associated with FMI and FFMI over the first 6 months of life. None of the BCAA or AAA intakes were significantly associated with infant growth or infant body composition. These results suggest that specific human milk free amino acid consumption positively associates with infant body composition.

The concept of early-life metabolic programming has been widely demonstrated in both clinical and preclinical studies. Specific to the postnatal period, faster weight gain during infancy is linked to obesity risk later in life [34] and augmented consumption of amino acids can increase weight gain velocity in formula fed infants [17,35], supporting the early protein hypothesis [18]. Accordingly, the early protein hypothesis is well adopted in formula fed infants and predicts that the risk for obesity increases with augmented protein consumption in early life via an elevation in circulating BCAAs and subsequent activation of insulin and insulin-like growth factor 1 pathways [22,36]. In a randomized clinical trial examining

the effects of feeding infant formula containing low (1.7 g/100 kcal) vs. high protein (2.9 g/100 kcal) compared to exclusively breastfed infants on growth, Koletzko *et. al.* found that the 24-month WAZ of infants fed the lower protein milk formula was significantly lower than that of the higher protein group, but not different than that of the breastfed reference group [35]. These findings suggest that lower protein infant formulas may normalize weight gain velocity in formula fed infants to that of breastfed infants, decreasing their potential for future obesity risk. Interestingly, the potential impact of variations in human milk protein compositions on infant growth and metabolism are still relatively unknown. In our previously published work [28], we observed that lactating women with overweight and obesity have a greater concentration of total protein in their milk at 5 months postpartum and that the daily average infants' intake of total protein is significantly greater at both 1 and 6 months of age compared to infants born to women with normal weight. Furthermore, daily protein intake was positively associated with an increase in length-for-age z score and WAZ after adjusting for infant feeding mode and infant sex, but negatively associated with both FMI and FFMI [28]. Conversely, Young *et. al.* found decreased total protein concentrations in human milk from women with overweight and obesity at 2 weeks after giving birth [37], suggesting that the timing of human milk sampling is an important consideration.

Excess circulating BCAAs can promote growth through activation of the insulin and insulin-like growth factor 1 pathways that are upstream of the mammalian target of rapamycin (mTOR) signaling network [38,39]. Amino acids also directly stimulate mTOR independent of insulin, and adequate amounts of amino acids are essential for muscle growth during the neonatal period [40,41]. Conversely, through the insulin pathways of mTOR activation, adipogenic signaling is thought to be activated [39] leading to increased susceptibility to obesity [23]. We therefore hypothesized that with increased consumption of free BCAAs, infants born to women with obesity would have increased growth and/or adiposity. Our data indicate that there is not an association between the intake of free BCAAs and infant growth over the first 6 months of age.

It is possible that because human milk free amino acids provide only around 3-5% of the amino acids required to perform metabolic activities in the infant [36], that total human milk amino acid intakes (free amino acids + amino acids in proteins) may provide a more comprehensive perspective on infant growth. We recently published that the protein concentration in HM from women with overweight was higher than in women with NW only at 5 and 6 months postpartum, and the offspring's protein intake was negatively associated with both FMI and FFMI [28]. Further, free amino acids are rapidly absorbed in the intestine, and circulating and intracellular essential amino acid availabilities are primary regulators of muscle protein synthesis [42,43]. This absorption and regulation highlights the significance of determining the association between HM free amino acid consumption and infant growth. Of interest, HM concentrations of several essential amino acids were elevated in the OB vs. NW group (Table 2; BCAAs, Phe), but this did not always translate to higher daily infant intakes of these amino acids when normalized to infant weight. On the other hand, the essential amino acid His was significantly lower in OB vs. NW HM, leading to a lower daily normalized infant intake (Table 3). Of interest, intake of the conditionally essential amino acids, Glu, Tyr and C-C, were all positively related to FFMI, together with His and Val, highlighting their importance in growth.

It remains to be determined if other mechanisms of metabolic programming are associated with this greater exposure to human milk free BCAAs. Hellmuth et al. found a significant positive association between human milk protein content and infant lyso-phosphatidylcholine levels [44], a compound that has been linked to increased risk of childhood obesity [45]. Although these data do not speak specifically to the effects of human milk BCAAs on metabolic programming, they do suggest that human milk proteins may play a role in nonhereditary nutritional programming. Future studies should focus on molecular markers of metabolic risk in the infants and longer-term growth outcomes.

Human milk free amino acid content changes dynamically over lactation, where some amino acids increase while others decrease or stay relatively unchanged (recently reviewed here [46]) and are in-line with those observed in the current study. Interestingly, the plasma pools of BCAAs, AAAs, as well as basic and neutral amino acids in lactating women were found to be 1- to 15-fold higher than those found in human milk [47], suggesting that there is a selectivity in amino acid transport at the level of the mammary epithelium during lactation [46]. The mammary gland expresses BCAA aminotransferase and the branched-chain α-ketoacid dehydrogenase complex that are responsible for the two-step breakdown of BCAAs [48]. Although only demonstrated in species other than human, the lactating mammary gland is known to take up copious amounts of BCAAs and extensively degrades them for the synthesis of other non-essential amino acids, such as Glu [48,49]. Throughout the body, catabolism of BCAAs may be an important means of dealing with a surplus in supply [38]. Accordingly, in non-lactating individuals with metabolic disease, BCAA plasma concentrations increase as a function of impaired BCAA catabolism [38]. We theorize that the dysregulation of BCAA catabolic enzymes in mammary epithelial cells from women with obesity may therefore contribute to elevations in human milk BCAA content and lower Glu levels associated with maternal obesity.

Herein, we have presented a longitudinal assessment of human milk free amino acid concentrations using state of the art technologies in one of the largest, well-characterized cohorts of mother-infant dyads representing both women with normal weight and with obesity to date. Furthermore, we have presented infant human milk intakes of free amino acids, estimated with validated methods, which were then used to assess the relationship between infant exposure to human milk free amino acids and infant growth. However, it is imperative to interpret these data within the scope of their limitations. While one-feed test weighing is a validated method for measuring human milk intake, it is not as reliable as 24 hour test-weighing [33]. However, this method, compared to 24 hour test-weighing, provides a significantly decreased burden on participants and is financially favorable for a large population such as the one presented here. To date, the nutritive and non-nutritive importance of human milk free amino acids has not been completely established. Although our data suggest that there are associations between infant intake of human milk free amino acids and infant body composition, we cannot conclude that this association results from a direct cause-and-effect relationship. However, infant intake of human milk free BCAAs, AAAs, His, Thr, and Met have been shown to correlate significantly with the respective infant plasma concentrations [14], supporting the potential for a more direct relationship between human milk amino acid intake and the impact on infant physiology. Additionally, although we observed clear differences in various free amino acid concentrations in human

milk from women with normal weight compared to those with obesity, these data are observational, and we do not have maternal dietary intake to rule out the potential influence of dietary amino acids on human milk composition. Therefore, conclusions regarding the mechanisms driving these differences cannot be made.

In summary, we have demonstrated a strong relationship between maternal BMI and human milk free amino acid composition over the first 6 months postpartum. Additionally, daily infant intake of human milk free amino acids did not always mimic the corresponding differences in concentrations, indicating the importance for estimating infant intakes to accurately determine infant nutritional exposure. Finally, although our data indicate increased consumptions of BCAAs and AAAs in infants born to women with obesity, BCAA and AAA intakes were not associated with infant growth. However, infant intakes of human milk Asp, C-C, Gln, Glu, His, and Ser showed associations with infant body composition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

AA designed the research; CRS, JLS, LP, and RL conducted the research; CRS and JLS analyzed the data; JLS, CRS, LP, RL, EB, and AA wrote and edited the manuscript; AA has primary responsibility for the final content. We thank the participants and the clinical research team at ACNC for their dedication and hard work in producing and collecting the samples and data presented in this manuscript.

Sources of Support:

USDA ARS Project # 6026-51000-010-05S and 6026-51000-012-06S. AA, EB and CRS are partially supported by NIH R01 DK107516.

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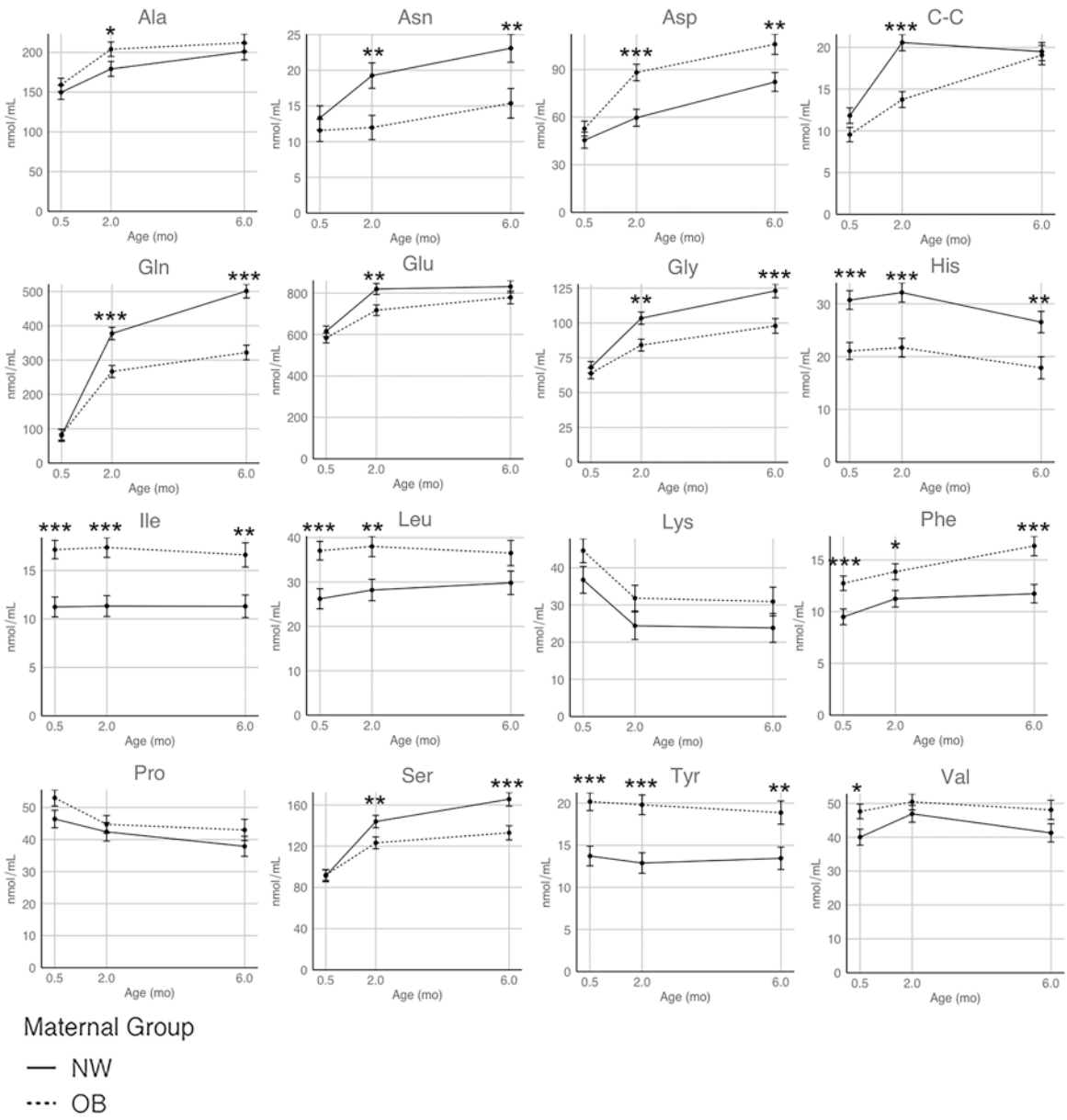

Figure 1. The Effect of Maternal Obesity and Time on Human Milk Amino Acid Concentrations.

Linear mixed-effects models were constructed to test the associations between maternal BMI group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and human milk amino acid concentrations (nmol/mL). Models were adjusted for potential confounding factors: maternal age, maternal race, and infant sex. All data are presented as the model estimated marginal mean amino acid concentrations over three timepoints: 0.5 months (n = 151), 2 months (n = 129), and 6 months (n = 93). Solid line = NW group, dotted line = OB group.

Table 1.

Maternal and Infant Characteristics.

Maternal and infant clinical and demographic characteristics are summarized as N (%) or mean ± SD. Comparisons were made between NW and OB groups where t-tests were performed for continuous data and Chi-squared tests were used for categorical data. NW = normal weight, OB = obese, GWG = gestational weight gain, IOM = Institute of Medicine, LGA = large for gestational age, AGA = appropriate for gestational age, SGA = small for gestational age

N (%) or mean ± SD	All	NW	OB	P - Value
Number of Participants	**169**	**83**	**86**	0.473
0.5M, N (%)	151 (100%)	74 (49%)	77 (51%)	
2M, N (%)	129 (100%)	66 (51%)	63 (49%)	
6M, N (%)	93 (100%)	53 (57%)	40 (43%)	
Maternal Age at Delivery (years)	30.1 ± 4.0	30.4 ± 3.4	29.9 ± 4.5	0.376
Race				**0.017**
Caucasian, N (%)	132 (78%)	71 (86%)	61 (71%)	
Non-Caucasian, N (%)	37 (22%)	12 (14%)	25 (29%)	
Maternal BMI (kg/m^2)	29.5 ± 8.3	22.3 ± 1.7	36.4 ± 5.7	**<0.001**
GWG (kg)	10.6 ± 4.6	12.6 ± 2.8	8.7 ± 5.2	**<0.001**
GWG (IOM Category)				**<0.001**
Inadequate, N (%)	45 (27%)	26 (31%)	19 (22%)	
Adequate, N (%)	71 (42%)	46 (56%)	25 (29%)	
Excessive, N (%)	48 (28%)	9 (11%)	39 (45%)	
Unknown, N (%)	5 (3%)	2 (2%)	3 (4%)	
Gestational Age at Delivery (weeks)	39.2 ± 1.5	39.3 ± 1.3	39.2 ± 1.6	0.714
Delivery Method				**0.007**
Vaginal, N (%)	112 (66%)	63 (76%)	49 (57%)	
C-Section, N (%)	57 (34%)	20 (24%)	37 (43%)	
Infant Sex				0.153
Female, N (%)	77 (46%)	34 (41%)	43 (40%)	
Male, N (%)	92 (54%)	49 (59%)	43 (50%)	
Birth Weight (kg)	3.5 ± 0.55	3.5 ± 0.51	3.5 ± 0.59	0.643
Birth Length (cm)	50.7 ± 3.0	50.8 ± 3.0	50.6 ± 2.9	0.697
Weight-for-Gestational Age category				0.478
LGA, N (%)	29 (17%)	12 (15%)	17 (20%)	
AGA, N (%)	134 (79%)	67 (81%)	67 (78%)	
SGA, N (%)	6 (4%)	4 (5%)	2 (2%)	

Table 2.
Human Milk Amino Acid Concentrations.

Human milk amino acid concentrations (nmol/mL) from 0.5, 2, and 6 months postpartum are presented as mean ± SEM. Independent samples t-tests were performed where $p < 0.05$ was considered significant. NW = normal weight, OB = obese, Arg = arginine, Ala = alanine, Asn = asparagine, Asp = aspartic acid, C-C = cystine, Glu = glutamic acid, Gln = glutamine, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Phe = phenylalanine, Pro = proline, Ser = serine, Tyr = tyrosine, and Val = valine.

	0.5 Months			2 Months			6 Months		
	NW (N = 74)	**OB (N = 77)**	**P - Value**	**NW (N = 66)**	**OB (N = 63)**	**P - Value**	**NW (N = 53)**	**OB (N = 40)**	**P - Value**
Branched-chain Amino Acids (nmol/mL)									
Ile	10.3 ± 1.0	16.7 ± 1.2	**<0.001**	10.4 ± 0.6	16.3 ± 1.0	**<0.001**	10.3 ± 0.5	14.6 ± 1.0	**<0.001**
Leu	25.5 ± 2.6	36.7 ± 2.7	**0.003**	27.5 ± 1.2	37.0 ± 2.3	**<0.001**	29.2 ± 1.1	33.5 ± 1.9	**0.044**
Val	40.7 ± 2.2	48.5 ± 2.6	**0.023**	47.7 ± 2.2	49.6 ± 1.9	0.517	42.8 ± 2.0	44.9 ± 2.5	0.502
Aromatic Amino Acids (nmol/mL)									
Phe	10.1 ± 0.6	13.2 ± 0.8	**0.004**	11.9 ± 0.7	14.3 ± 0.8	**0.020**	12.3 ± 0.7	16.2 ± 1.1	**0.004**
Tyr	13.6 ± 0.9	20.3 ± 1.4	**<0.001**	12.9 ± 0.9	18.9 ± 1.2	**<0.001**	13.2 ± 0.9	17.3 ± 1.2	**0.006**
Other Amino Acids (nmol/mL)									
Ala	158.8 ± 8.3	165.4 ± 8.3	0.556	188.6 ± 8.7	209.7 ± 10.6	0.122	211.1 ± 8.2	210.9 ± 11.6	0.988
Asn	13.2 ± 1.4	11.5 ± 1.4	0.397	19.0 ± 21.7	11.6 ± 1.4	**0.001**	23.0 ± 2.2	14.2 ± 2.2	**0.006**
Asp	44.7 ± 3.1	52.9 ± 3.3	0.070	58.2 ± 4.2	87.4 ± 5.9	**<0.001**	81.2 ± 7.0	104.3 ± 8.8	**0.041**
C-C	12.9 ± 0.7	10.2 ± 0.8	**0.013**	21.6 ± 1.0	14.5 ± 0.8	**<0.001**	20.9 ± 1.1	19.7 ± 1.5	0.512
Gln	108.6 ± 11.4	102.7 ± 10.9	0.711	406.7 ± 21.5	286.2 ± 18.7	**<0.001**	535.1 ± 21.8	337.9 ± 26.3	**<0.001**
Glu	645.8 ± 25.4	606.3 ± 24.5	0.265	851.5 ± 26.6	743.0 ± 23.5	**0.003**	866.2 ± 24.8	789.9 ± 25.5	**0.038**
Gly	70.6 ± 3.5	66.1 ± 3.2	0.348	105.9 ± 4.9	86.5 ± 4.0	**0.003**	126.5 ± 5.0	98.5 ± 5.6	**<0.001**
His	32.2 ± 1.7	22.1 ± 1.9	**<0.001**	33.5 ± 1.7	22.7 ± 1.6	**<0.001**	28.9 ± 1.4	17.7 ± 1.7	**<0.001**
Lys	33.8 ± 3.2	44.1 ± 4.3	0.059	21.6 ± 2.2	24.9 ± 2.7	0.348	19.8 ± 1.9	21.0 ± 2.2	0.691
Pro	44.4 ± 3.1	52.1 ± 3.2	0.084	40.7 ± 1.9	42.0 ± 2.3	0.672	36.0 ± 1.4	38.6 ± 1.9	0.265
Ser	94.9 ± 4.7	94.9 ± 4.0	0.997	147.3 ± 6.0	125.1 ± 4.7	**0.004**	171.8 ± 7.7	132.9 ± 7.0	**<0.001**

Table 3.
Daily Infant Intakes of Human Milk Amino Acids, Normalized to Infant Weight.

Daily infant intakes of amino acids (μmol/day) were normalized to infant body weight (kg) and are presented as mean ± SEM. Independent samples t-tests were performed where $p < 0.05$ was considered significant. NW = normal weight, OB = obese, Arg = arginine, Ala = alanine, Asn = asparagine, Asp = aspartic acid, C-C = cystine, Glu = glutamic acid, Gln = glutamine, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Phe = phenylalanine, Pro = proline, Ser = serine, Tyr = tyrosine, and Val = valine.

	0.5 Months			2 Months			6 Months		
	NW (N = 56)	**OB (N = 46)**	**P - Value**	**NW (N = 54)**	**OB (N = 46)**	**P - Value**	**NW (N = 51)**	**OB (N = 27)**	**P - Value**
Branched-chain Amino Acid Intake (μmol/kg/day)									
Ile	1.8 ± 0.2	2.5 ± 0.3	0.083	1.2 ± 0.1	1.8 ± 0.2	**0.002**	0.9 ± 0.1	1.2 ± 0.2	0.152
Leu	4.6 ± 0.5	5.1 ± 0.5	0.492	3.2 ± 0.2	4.0 ± 0.3	**0.049**	2.5 ± 0.2	2.8 ± 0.5	0.567
Val	7.1 ± 0.5	7.5 ± 0.8	0.685	5.8 ± 0.5	5.9 ± 0.6	0.933	3.6 ± 0.6	3.7 ± 0.6	0.975
Aromatic Amino Acid Intake (μmol/kg/day)									
Phe	1.8 ± 0.2	2.2 ± 0.3	0.273	1.4 ± 0.1	1.7 ± 0.2	0.114	1.0 ± 0.1	1.3 ± 0.2	0.213
Tyr	2.4 ± 0.2	3.0 ± 0.3	0.134	1.5 ± 0.2	2.2 ± 0.2	**0.013**	1.2 ± 0.1	1.4 ± 0.3	0.324
Other Amino Acid Intake (μmol/kg/day)									
Ala	27.2 ± 2.3	27.9 ± 2.8	0.829	23.3 ± 2.0	24.9 ± 2.4	0.621	19.0 ± 1.8	20.0 ± 3.5	0.763
Asn	2.4 ± 0.3	1.7 ± 0.4	0.195	2.7 ± 0.4	1.3 ± 0.2	**0.002**	2.0 ± 0.3	1.5 ± 0.5	0.295
Asp	7.2 ± 0.7	8.2 ± 1.0	0.425	7.6 ± 0.9	9.8 ± 1.2	0.154	7.4 ± 0.9	7.4 ± 1.0	0.961
C-C	2.3 ± 0.2	1.9 ± 0.2	0.263	2.8 ± 0.3	1.9 ± 0.2	**0.007**	1.8 ± 0.2	1.9 ± 0.4	0.680
Gln	18.8± 2.7	20.4 ± 3.2	0.698	53.4 ± 4.4	41.1 ± 4.7	0.057	47.4 ± 4.2	34.8 ± 7.3	0.115
Glu	114.7 ± 8.9	108.3 ± 11.3	0.655	107.4 ± 7.7	92.0 ± 8.0	0.168	74.6 ± 5.9	66.2 ± 9.4	0.433
Gly	12.5 ± 1.0	11.6 ± 1.5	0.613	13.7 ± 1.1	10.3 ± 0.9	**0.024**	10.7 ± 0.9	8.8 ± 1.5	0.276
His	5.4 ± 0.5	4.1 ± 0.6	0.064	4.2 ± 0.4	2.8 ± 0.4	**0.009**	2.4 ± 0.2	1.6 ± 0.3	**0.034**
Lys	6.1 ± 0.7	5.8 ± 0.8	0.732	2.5 ± 0.3	2.8 ± 0.5	0.536	1.7 ± 0.2	1.9 ± 0.4	0.720
Pro	7.8 ± 0.7	8.2 ± 0.9	0.680	4.9 ± 0.4	4.7 ± 0.4	0.735	3.0 ± 0.3	3.1 ± 0.5	0.920
Ser	16.5 ± 1.3	16.0 ± 1.9	0.827	18.4 ± 1.4	15.9 ± 1.5	0.225	14.8 ± 1.3	12.0 ± 2.2	0.244

Table 4.

Maternal BMI is Associated with Daily Infant Intakes of Human Milk Amino Acids Over the First 6 Months of Life.

Linear mixed-effects models (n = 169 subjects) included human milk amino acid intakes (μmol/kg/day) as the dependent variables and maternal BMI (kg/m^2) at first visit as the fixed effect. Model covariates included: infant age, infant sex, maternal race, gestational weight gain, and breastfeeding status (exclusive or mixed). Asn = asparagine, C-C = cystine, His = histidine, Ile = isoleucine, Leu = leucine, Phe = phenylalanine, and Tyr = tyrosine.

	All BMI	
Amino Acid Intake (μmol/kg/day)	**β**	**P Value**
Ile	0.04	**0.001**
Leu	0.05	0.08
Phe	0.03	**0.01**
Tyr	0.05	**0.002**
Asn	−0.04	**0.03**
C-C	−0.03	0.05
His	−0.07	**0.004**

Table 5.

Daily Infant Intakes of Human Milk Amino Acids are Associated with Infant Body Composition at 0.5 - 6 Months of Age.

Linear mixed-effects models included infant weight-for-length z-score (WLZ), fat mass index (FMI), and fat free mass index (FFMI) over the first 6 months of life (time points 0.5, 2, and 6 months) as the dependent variables and infants' intakes of human milk amino acids (μmol/day) as the fixed effects. Model covariates included: infant age, infant birth weight, infant sex (FMI and FFMI models only), maternal BMI, gestational weight gain, maternal race, delivery mode (vaginal or C-section), and breastfeeding status. Random effects included: participant identification number, to account for repeated measures. Asp = aspartic acid, C-C = cystine, Gln = glutamine, Glu = glutamic acid, His = histidine, and Ser = serine.

	Weight-for-Length Z-score		Fat Mass Index (kg fat mass / m^2)		Fat Free Mass Index (kg fat free mass / m^2)	
Amino Acid Intake (μmol/day)	**β**	**p-value**	**β**	**p-value**	**β**	**p-value**
Asp	0.0024	0.093	0.0038	**0.011**	0.0034	**0.011**
C-C	0.0103	0.088	0.0174	**0.006**	0.0163	**0.004**
Gln	0.0004	0.15	0.0011	**<0.001**	0.0007	**0.009**
Glu	0.0004	**0.042**	0.0005	**0.005**	0.0004	**0.026**
His	0.0080	**0.039**	0.0106	**0.008**	0.0088	**0.015**
Ser	0.0011	0.23	0.0029	**0.002**	0.0014	0.088