Research Paper

Longitudinal plasma amino acid profiling with maternal genomic background throughout human pregnancy

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Abstract Amino acid metabolism in pregnancy is an important regulatory mechanism for fetal growth and maturation. We aimed to clarify pregnancy-induced changes in amino acid concentrations in maternal plasma by frequent sampling throughout pregnancy and to test the effects of genomic backgrounds on amino acid concentrations. We recruited 19 pregnant women and collected plasma samples monthly during pregnancy and one month after delivery. Amino acid concentrations during pregnancy were analyzed with linear models that considered both individual differences and gestational age. Maternal genotyping was used to evaluate the effects of single-nucleotide variants on the levels of specific amino acids. Significant increases in the amount of threonine, histidine, glutamic acid, aspartic acid and proline and decreases in tryptophan, arginine, valine, and leucine in plasma concentrations of pregnant women were observed as gestation proceeded. Total amino acid concentrations during pregnancy were decreased relative to post-partum concentrations, whereas the ratio of essential to non-essential amino acids was increased across the sampling period. The effects of alternative alleles for three missense SNVs on the concentrations of glycine, asparagine and proline were confirmed in pregnant women and were consistent with a previous report for a non-pregnant population. Principal component analysis of the relative amino acid profiles indicated that principal component (PC) 1 reflected advancing gestation, whereas PC2 was dominated by the difference between pregnant and non-pregnant states. The change in relative compositions of circulating amino acids was a distinctive marker of pregnancy and genomic background dominantly affected maternal metabolic changes in gestation.

Key words: pregnancy, amino acid, mass spectrometry, longitudinal analysis, single nucleotide variant

Introduction

Pregnancy-related maternal adaptations are widely recognized as critical processes for inheritance across generations[1)]. Failure of these regulatory systems can result in various maternal and fetal complications, such as hypertensive disorders of pregnancy[2)], premature labor[3)], and fetal growth restriction[4)].

Amino acid metabolism in pregnancy is an important

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regulatory mechanism for fetal growth and maturation[5]. During pregnancy, amino acids in maternal circulation are mainly regulated by food intake and the feto-placental unit. Fetal demands for rapid cellular proliferation and differentiation require marked energy consumption from maternal circulation through the placenta. Placental transport systems play important roles in supplying essential factors, including amino acids[6]. Accumulating evidence suggests that more than 20 amino acid transporters are expressed in the human placenta[7–9]. These active transport systems are controlled by a variety of transporter proteins encoded by specific regulatory genes[10,11]. Amino acid concentrations in maternal plasma decrease during pregnancy compared with non-pregnant conditions and this decrease begins early in pregnancy and persists until delivery[12], although changes in amino acid composition as pregnancy proceeds are not well characterized in part because changes in amino acid levels during pregnancy are relatively small compared to variations in amino acid levels among individuals.

High-throughput technologies, such as liquid chromatography followed by mass-spectrometry (LC-MS) and nucleic magnetic resonance (NMR), are now used extensively to investigate metabolic effects of pregnancy on plasma concentrations of amino acids in studies having a cross-sectional[13,14] or longitudinal design[15–17]. Cross-sectional studies often cannot detect small changes in amino acid concentrations because pregnancy-related effects can be confounded by variations between individuals. Meanwhile, longitudinal studies involving large cohorts can identify changes in amino acids levels that are linked to gestational age, although the number of samples obtained per participant is typically three, or once per trimester, and this low number of sampling points can limit the evaluation of how gestational age, rather than differences among individuals, contributes to variations in amino acid levels during pregnancy.

Amino acid levels can be perturbed by critical mutations in genes encoding key enzymes involved in amino acid synthesis or uptake that are associated with genetic diseases that significantly affect overall health[18] or alter plasma amino acid concentrations in healthy individuals. Recent integrations of genome analysis and metabolome measurements in large-scale cohorts allowed the identification of gene variants that are associated with specific amino acid concentrations[19,20]. Notably, Koshiba et al. performed a genome-wide association study (GWAS) of a Japanese cohort by integrating whole genome sequencing and NMR plasma metabolomics to identify five missense single nucleotide variants (SNVs) that affect phenylalanine, glycine, asparagine, proline, and formate levels[19]. Although many variations in metabolite amounts can be explained by these nonsynonymous variants, how these mutations affect plasma amino acid concentrations during pregnancy remains unclear.

In the present study, we undertook frequent plasma amino acid profiling of pregnant women to elucidate the effect of gestational age and individual differences on amino acid concentrations. We performed a longitudinal analysis of plasma amino acid profiles for 19 pregnant women by sampling plasma monthly during pregnancy and one month after delivery. The variations in amino acid concentrations during pregnancy were divided into contributions from gestational age and individual differences using linear models. Our analysis identified those amino acids for which levels significantly changed with gestational age and also evaluated the relative contributions of gestational age and individual differences among the study participants. We also demonstrated that overall amino acid concentrations were decreased in pregnant women relative to non-pregnant states, whereas the amounts of several amino acids were retained during pregnancy. Furthermore, we used maternal genotyping to investigate the effect of missense SNVs that can influence specific amino acid concentrations. These results bring new insights into individual regulation of amino acid profiles in normal pregnancy.

Material and Methods

Subjects

Twenty-two pregnant women were recruited from Tohoku University Hospital, Japan, after providing satisfactory written informed consent. At study entry, all women had singleton pregnancies, took no medications, and had no history of cardiovascular or endocrine disease or tobacco use. Each participant received monthly routine obstetric care that included a maternal weight check, analysis of urine and plasma, measurement of blood glucose levels, self-reported record of time since the last meal and measurement of fetal development by ultrasound echography. Three women were excluded due to hypertensive disorders of pregnancy, premature labor or gestational diabetes. A total of 19 women were further analyzed in this study. Clinical information was collected from obstetric and neonatal

medical records. Ethical approval was obtained from the Ethical Committee of the Tohoku University Graduate School of Medicine (2012-1-443 and 2014-10).

Sample collection and measurement of plasma metabolites

Plasma samples were collected from each participant at each hospital visit during pregnancy. Plasma samples were also collected one month after delivery (hereafter referred to as ADM), if possible. To eliminate the effect of elevated blood glucose and time since the last meal, we excluded those plasma samples that were collected at times when the blood glucose levels exceeded 120 mg/dL or those for which the time since the last meal was less than 3 hours. Samples were collected between April 2013 and April 2014. Plasma samples were sent to a qualified clinical laboratory and amino acid concentrations were measured using LC-MS. The protocol of amino acids analysis was followed by the previous report[21]. After the extraction of analytes from plasma by means of the deproteinization of acetonitrile precipitation, the analytes were derivatized by the regent of 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDSTAG® Wako Eluent, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)[22]. The LCMS was performed by LC-MS2020 (Shimadzu, Kyoto, Japan), and the APDS-derivatized analytes were detected by multiple reaction monitoring mode with the separation using a column (Inertsil ODS-3, 2 μm, 100 mm×2.1 mm, I.D., GL Science, Tokyo, Japan). The concentrations of 20 standard amino acids and three additional amino acids that are nearly always present in plasma (taurine, ornithine, and citrulline) were analyzed in this study.

Linear model to evaluate the effect of gestational age and individual differences

We used a linear model to evaluate the contribution of gestational age and individual differences to the plasma concentration of each amino acid:

$$y=\beta_{ID}x_{ID}+\beta_{day}x_{day}+\varepsilon \quad (1)$$

where y is the amino acid concentration in the plasma sample of a participant, β_{ID} is a 19-dimensional vector, wherein each dimension represents the weight for each of the 19 participants and x_{ID} is another 19-dimensional vector, in which only one element corresponding to the participant is unity and the other elements are zero, β_{day} is the weight for gestational age in days, x_{day} is the gestational age on the day the sample was collected, and ε is the residual error of the model. Positive and negative β_{day} values for amino acids with a P-value$<$0.05/23 (i.e., the number of amino acids tested) were considered to be significant increases and decreases with gestational age.

Contribution of individual differences and gestational age to amino acid concentration

To calculate the contribution of each of the two factors in Eq. 1 to the concentration of each amino acid, two additional linear models using individual differences (*ID*) alone or gestational day (*day*) alone were used. The contribution of *ID* and *day* was calculated as the difference in the variance explained (R-squared) by the linear model in Eq. (1) and that by the linear model using the other single variable, *day* or *ID*, respectively.

Comparison of amino acid concentrations during and after pregnancy

We obtained plasma samples at ADM from seven study participants and compared amino acid concentrations during pregnancy relative to those at ADM, which served as a reference for non-pregnant conditions. We averaged the plasma concentrations of a given amino acid from a single participant over the course of pregnancy and divided the averaged values by the concentration of the amino acid from the same individual at ADM. Relative concentrations above and below unity indicated an increase and decrease, respectively, of amino acids in pregnancy compared to ADM.

Genotyping participants for genome-wide significant SNVs

The genotypes of missense SNVs that are known to be associated with specific amino acid concentrations were obtained by whole exome sequencing of participant samples. Briefly, blood samples for exome sequencing were collected at the first health checkup for each participant. Genomic DNA was isolated using SureSelect Human All Exon V4 Kits (Agilent Technologies, Santa Clara, CA). Sequencing was performed on a HiSeq2500 instrument (Illumina, San Diego, CA) with 101 bp paired end reads. Variant identification was performed as described previously[23].

Genetic factors and amino acid concentrations

Although the participants have number of SNVs by means of the exome sequencing, our sample size was too small to statistically support novel associations between SNVs and amino acid concentrations. Therefore, we selected the previously identified SNVs. We examined the combinations of three SNVs and amino acid levels in plasma of pregnant women: i) rs1047891, chr2.g211540507.C>A, *CPS1* Thr1406Asn and glycine; ii) rs8012505, chr14.g104571054.C>G, *ASPG* Ser344Arg and asparagine; and iii) rs5747933, chr22.g18910355.G>T, *PRODH* Thr275Asn and proline (genomic coordinates are shown in the GRCh37/hg19 reference genome)[19]. These missense mutations are significantly associated with and are likely to have causal effects on the corresponding plasma amino acid levels, based on careful consideration of the amino acid substitutions using three-dimensional structures of the enzymes, which are involved in the metabolic pathways of the three amino acids[19]. For a linear model of a particular amino acid (Eq. (1)), we considered the elements of β_{ID} that represent the plasma concentration in the corresponding participants, who were classified by genotypes for each SNV as either homozygous to the reference allele (ref) or heterozygous with the reference and alternative alleles (het). Differences in the plasma concentration of amino acids, or elements of β_{ID} in Eq. (1), between the "ref" and "het" genotype participants for the respective SNV were tested using a Wilcoxon ranked sum test.

Principal component analysis of amino acid profiles

To evaluate changes in amino acid profiles during pregnancy and avoid effects associated with the decrease in total amino acid concentration throughout pregnancy, we focused on the relative rather than the absolute amounts of amino acids. The concentrations (in μM) of nineteen standard amino acids except aspartic acid, which had a concentration that was too low to allow consistent measurement, were normalized to relative values by dividing by the sum of the concentrations of nineteen amino acids in the same sample. These nineteen-dimensional, relative amino acid profiles of pregnant and ADM samples were subjected to principal component analysis, after centering and scaling for each amino acid level. As a reference for the non-pregnant conditions in the general population, we obtained plasma amino acid profiles for 5,093 Japanese individuals determined by NMR that were collected and analyzed by the Tohoku Medical Megabank Organization (ToMMo)[19]. The profiles were grouped by sex and age and the average of the profiles was calculated for the subgroups. The average profiles for each sex and age subgroup were also mapped in the principal component plots.

Table 1. Summary of characteristics of the 19 participants

Basic data	Median	Range
Age (year)	37	26–44
Pre-pregnancy BMI (kg/m^2)	20.4	17.1–35.8
Delivery data		
Gestational weeks at delivery	39 w 5 d	37 w 5 d–41 w 5 d
Birth weight (g)	3,069	2,298–3,978
Fetal sex	Male	Female
	10	9
Parity	Nullipara	Para
	10	9

Results

Participant and sample summary

The basic and obstetric background of participants is summarized in Table 1. The median age of participants at the time of study entry was 37 (26–44) years old. The median BMI was 20.5 (17.0–35.8), with three underweight (BMI<18.5), 14 in the normal range (BMI 18.5 to 25), one overweight (BMI 25 to 30) and one obese (BMI over 30). All participants gave birth to a single child, totaling ten males and nine females.

Plasma samples were taken at weeks 9 and 40 of gestation. The number of samples collected from each participant ranged from six to nine, and among the 168 plasma samples collected during pregnancy, 120 met the criteria of blood glucose level<120 mg/dL and>3 h since the last meal. The concentrations of nineteen standard amino acids and those of three metabolites, taurine, ornithine, and citrulline, were successfully measured for all 120 pregnant samples. Meanwhile, aspartic acid levels were much lower than those for the other amino acids and were measurable in only 80/120 samples during gestation. ADM plasma samples were also obtained for 16/19 participants, and of these, 7 met the criteria for blood glucose and time since the last meal.

Table 2. Trends for amino acid concentrations with gestational age calculated using linear models
(Eq. (1)). The estimated slopes and SDs are given as μM/day.

Amino acid	Estimate	Std. Error	t value	Pr(>|t|)
Threonine	**0.40**	**0.036**	**10**	**7.0E-19**
Tryptophan	**−0.045**	**0.0068**	**−6.6**	**1.6E-09**
Arginine	**−0.039**	**0.0071**	**−5.5**	**3.0E-07**
Histidine	**0.049**	**0.010**	**4.8**	**6.2E-06**
Glutamic acid	**0.053**	**0.013**	**4.0**	**0.00014**
Aspartic acid	**0.0061**	**0.0015**	**4.0**	**0.00016**
Proline	**0.084**	**0.024**	**3.5**	**0.00072**
Valine	**−0.11**	**0.032**	**−3.5**	**0.00080**
Leucine	**−0.056**	**0.017**	**−3.4**	**0.0011**
Asparagine	0.020	0.0066	3.0	0.0036
Methionine	0.0082	0.0033	2.5	0.014
Isoleucine	−0.026	0.011	−2.4	0.019
Alanine	0.12	0.052	2.4	0.020
Total	0.42	0.21	2.0	0.047
Glutamine	−0.073	0.047	−1.5	0.12
Serine	0.015	0.013	1.2	0.23
Cystine	−0.0054	0.0048	−1.1	0.27
Taurine	0.0096	0.012	0.83	0.41
Lysine	0.016	0.023	0.70	0.48
Tyrosine	−0.0055	0.0079	−0.70	0.49
Glycine	−0.012	0.020	−0.59	0.56
Phenylalanine	0.0027	0.0062	0.43	0.67
Citrulline	0.00035	0.0051	0.069	0.94
Ornithine	−0.00018	0.0049	−0.037	0.97

Correlations of amino acid concentration and gestational age

We first determined whether plasma amino acid concentrations changed with advancing gestational age using linear models of plasma samples from 19 participants (Eq. (1)). The number of plasma samples used for regression was 120 for the standard amino acids (except aspartic acid) and for citrulline, ornithine, and taurine. A total of 80 samples were considered for aspartic acid. After separating the effect of individual differences, five positive and four negative correlations with gestational age were found (Table 2, Fig. 1, Fig. S1). Amino acids having a positive correlation with gestational age were threonine, histidine, glutamic acid, aspartic acid and proline, whereas those with negative correlations were tryptophan, arginine, valine, and leucine (Fig. 1). Total amino acid concentrations were unchanged throughout pregnancy (Fig. S2).

Contribution of gestational age and individual differences to variances in amino acid concentrations

The fractions of variances in amino acid concentration explained by individual differences and gestational age are shown in Table 2. Variations in the levels of most amino acids could be explained by individual differences that ranged from 0.21 to 0.80, whereas the fractions of variances that could be explained by gestational age were lower for most of the amino acids. The nine amino acids that exhibited significant changes with gestational age had a larger fraction that could be explained by gestational age compared to the other amino acids. Variations in the levels of threonine and tryptophan, which had the largest significant increase and decrease in concentration, respectively, with gestational age had contributions from gestational age of 0.39 and 0.16, respectively, whereas for most of the other amino acids variations due to gestational age were less than 0.1.

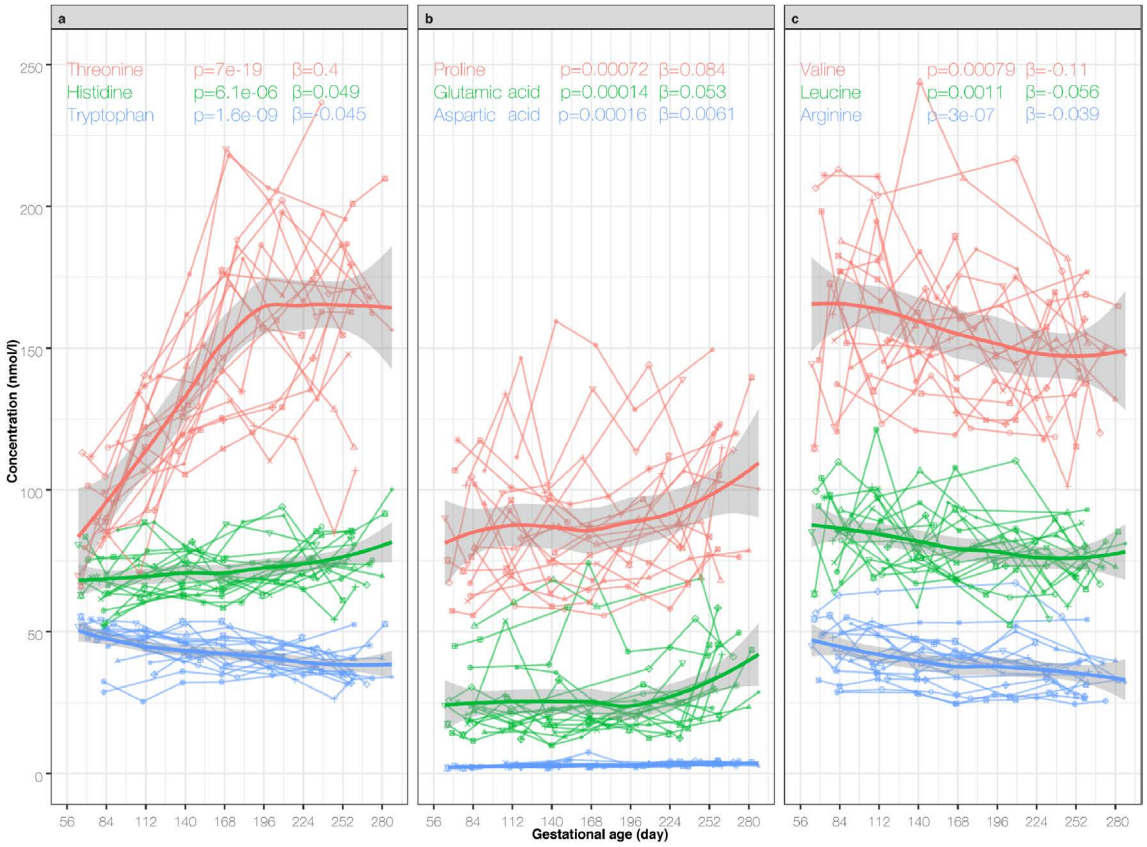

Fig. 1. The relationship between gestational age and plasma concentrations of amino acids that showed significant changes with gestational age using linear models of Eq. (1).

(a) Threonine (red), histidine (green) and tryptophan (blue). (b) Proline (red), glutamic acid (green) and aspartic acid (blue). (c) Valine (red), leucine (green) and arginine (blue).

The time series of the 19 subjects are represented by separate thin lines and data points. Values represented by thick lines were calculated using a locally weighted scatterplot smoothing (LOESS) method that included all sample points, which were treated as independent from each other; grey regions indicate confidence intervals.

Changes in amino acid concentrations during and after pregnancy

To evaluate changes in amino acid concentrations during and after pregnancy, the average concentration of amino acids throughout pregnancy relative to those at ADM were compared (Fig. 2). The total amino acid concentration was decreased during pregnancy by 27% compared to ADM. Levels of most individual amino acids, except threonine, also decreased during pregnancy relative to ADM, which suggests a blood dilution effect during gestation. Notably, levels of essential amino acids remained relatively stable compared to that for the total amino acid concentration. The concentrations of some non-essential amino acids, such as aspartic acid, asparagine, glutamic acid, alanine, taurine and glutamine, were also increased compared to the total amino acid concentrations. Taurine is also an essential amino acid for the fetus[24] and had a higher median value than that for total amino acids. On the other hand, concentrations of ornithine, glycine, arginine, citrulline and tyrosine were decreased compared with that of total amino acids.

Relationship between amino acid concentration trends during pregnancy and changes in pregnancy relative to ADM

We examined the relationship between the trends for amino acid levels during pregnancy (quantified by β_{day} in Eq. (1) normalized by standard deviation) and their relative level in pregnancy to that for ADM. There was a modest positive correlation between these two values, with Pearson's r=0.50 (P=0.01) and Spearman's ρ=0.46 (P=0.03) . The amino acids that perverted this trend were tryptophan, valine, leucine, cysteine (decreased during gestation and retained pregnant/ADM ratio) and proline (increased during gestation and suppressed pregnant/ADM ratio).

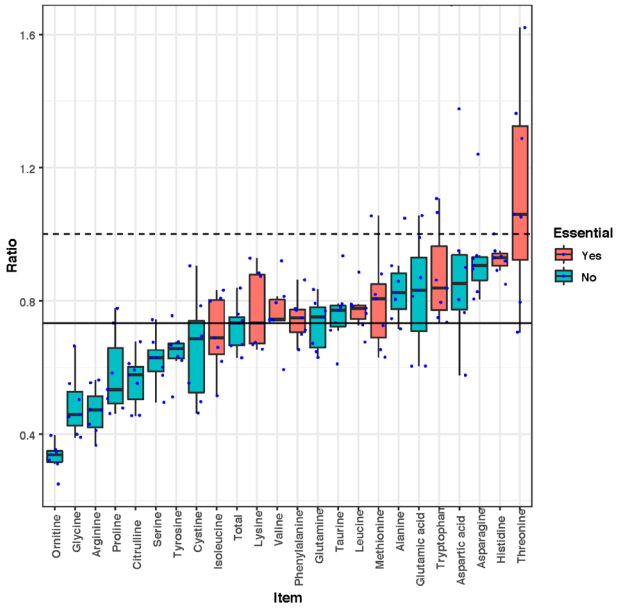

Fig. 2. Variations in plasma amino acid concentration changes during pregnancy compared to ADM.
The relative concentration of an amino acid in pregnancy was calculated as the ratio of the average concentration over the entire pregnancy and that at ADM for each individual. The values for the seven individuals are plotted in box and jitter plots. Red and blue colors indicate essential and non-essential amino acids, respectively. Solid and dotted horizontal lines indicate the median value for total amino acid (0.73) and unity, respectively. Asterisks indicate significant change ($P<0.05/23$, paired *t*-test) in the distribution of the relative concentrations of total amino acids and those for each amino acid.

Genetic factors and amino acid concentrations

Six, five and three participants were heterozygous for genome-wide significant SNVs (Het) in *CPS1*, *ASPG* and *PRODH*, respectively, whereas the other participants were homozygous for the reference allele (Ref) for these SNVs (Table 3). Thus, we examined how the *CPS1*, *ASPG*, and *PRODH* genotypes affected changes in glycine, asparagine, and proline concentrations in this pregnancy cohort.

Among the effects of SNVs on amino acid concentrations, participants heterozygous for *CPS1* had a 44% higher median plasma glycine concentration than those homozygous to the reference *CPS1* (increase of 46 nmol/L; Fig. 3a, b). Participants heterozygous for *ASPG* had 30% higher median asparagine concentrations than those homozygous to the reference *ASPG* (increase of 10 nmol/L; Fig. 3c, d). Participants heterozygous for *PRODH* had slightly (21%) higher median proline concentrations than those homozygous to the reference *PRODH* (increase of 16 nmol/L; Fig. 3e, f). Differences in glycine concentration between the *CPS1* genotypes were significant, whereas differences in asparagine and proline concentrations between the *ASPG* and *PRODH* genotypes, respectively, were not. These outcomes could be associated with the relatively small size of this cohort. Although the median concentrations of glycine (55%), asparagine (11%), and proline (47%) decreased during pregnancy compared with those at ADM (Fig. 2), we observed increasing trends for the plasma concentrations of these three amino acids that were associated with the presence of an alternative allele for the corresponding SNVs, which is consistent with the increasing trend in amino acid concentrations seen in a non-pregnant Japanese population when alternative alleles were present[19]. Interestingly, the amounts of these three amino acids showed a large variance that could be explained by individual differences, and represented 0.80, 0.71, and 0.58 of the variances explained for glycine, asparagine, proline, respectively (Table 4).

Table 3. Study participant genotypes for the three SNVs that affect specific amino acid levels

Genotypes[a]	Ref	Het
Glycine	13	6
Asparagine	14	5
Proline	16	3

[a]Genotypes were determined for *CPS1* Thr1406Asn (rs1047891) for glycine, *ASPG* Ser344Arg (rs8012505) for asparagine, and *PRODH* Thr275Asn (rs5747933) for proline. Het: heterozygous; Ref: reference allele.

Dynamic shift in amino acid profiles in pregnancy

The changes in relative amino acid profiles throughout pregnancy were determined by PCA of 120 pregnant and seven ADM samples (Fig. 4a). Clustering of pregnant (subclassified as T1, T2 and T3) and ADM samples from the same individuals along PC1 indicated that it partly reflects differences between individuals (Fig. 4b). On the other hand, PC2 clearly separated pregnant from ADM samples (Fig. 4c) and located ADM samples in a large negative PC2 region. Non-pregnant controls measured by NMR in a Japanese population[19] clustered with the large negative PC2 values, which was similar to that for ADM samples and supports the ability of PC2 to discriminate pregnant from non-pregnant values. Furthermore, PC1 separated male and female samples in a Japanese population, whereas PC2 correlated with decreasing age for both sexes. The relative

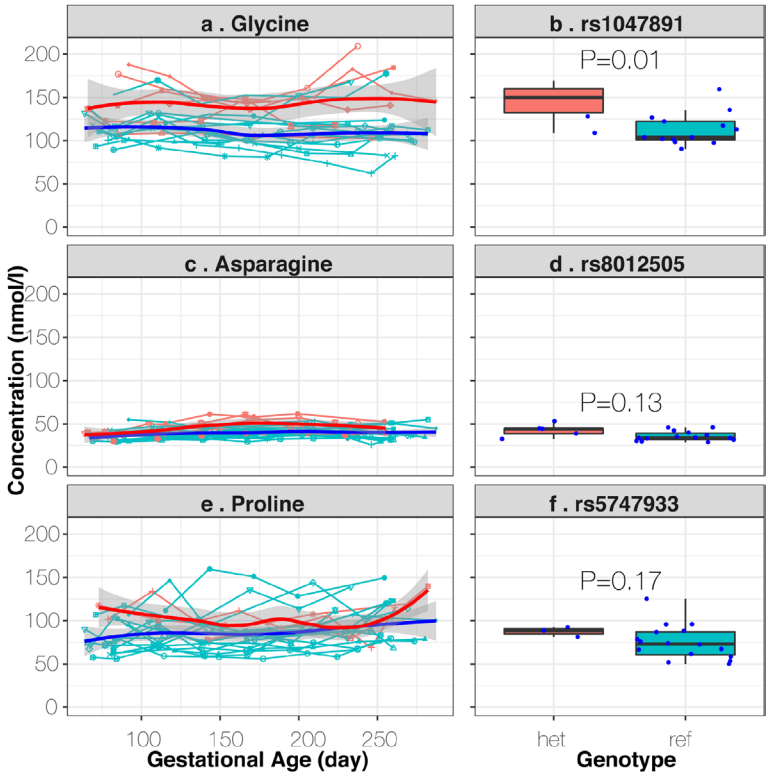

Fig. 3. Effects of SNVs on amino acid concentrations.
(a) and (b) *CPS1* Thr1406Asn (rs1047891) for glycine, (c) and (d) *ASPG* Ser344Arg (rs8012505) for asparagine, and (e) and (f) *PRODH* Thr275Asn (rs5747933) for proline. (a), (c) and (e) The time series for the 19 subjects is represented by separate lines. Red and blue colors indicate heterozygous (Het) and reference (Ref) genotypes, respectively. (b), (d) and (e) Individual differences for the participants (β_{ID} in Eq. (1)) are shown in boxplots for Het and Ref genotypes.

amino acid profiles of pregnant women usually moved in the direction of large negative PC1 and small negative PC2 (Fig. 4a grey arrow), indicating that PC1 partly described changes with advancing gestation. The PC1 loadings showed large positive values for the four amino acids that showed decreasing values (valine, leucine, arginine and tryptophan, Fig. 1) and large negative values for threonine, which showed the largest increase (Fig. 1), indicating that PC1 is correlated with advancing gestation. Meanwhile, PC2 was characterized by positive essential amino acids and negative non-essential amino acids, similar to the trend seen for the change during pregnancy relative to ADM, with proline, tyrosine and arginine values having the largest difference (Fig. 4e).

Notably, the PCA of absolute amino acid profiles in our pregnant and ADM samples indicated that half of the variance could be explained by PC1, which represents the decrease in the concentrations of all amino acids that likely can be attributed to blood dilution during pregnancy (Fig. S3). The PC2 of the absolute amino acid profiles is similar to that of PC1 for the relative amino acid profiles (Fig. 4d, Fig. S2e) that had negative values for branched chain amino acids. Although PC1 of the absolute profiles reflected blood dilution that occurs during pregnancy, PC2 of the relative profiles was better able to discriminate pregnant and non-pregnant samples than the PC1 of the absolute profiles (Fig. 4e and Fig. S2b), indicating that the change in the relative amino acid profile can be another marker of pregnancy in addition to the absolute dilution of blood.

Discussion

In the present study we performed frequent (monthly) profiling of plasma amino acids in a longitudinal cohort of pregnant women to clarify changes in amino acid concentrations during pregnancy and with gestational age, as well as the effects of genetics as determined by exome sequencing. Compared to previous reports of maternal plasma profiling reports[13–17], we performed more frequent sampling of plasma per individual during gestation, which enabled us to quantify relative contributions of individual difference and

Table 4. Variances explained by individual differences and gestational age. Asterisks indicate amino acids that exhibited significant increases or decreases with gestational age

Amino acid	Individual difference	Gestational age	Number of significantly different individuals[a]	Significant change with gestational age[b]
Glycine	0.80	0.00067	8.83 (±3.90)[3]	
Serine	0.74	0.0035	6.60 (±3.31)	
Asparagine	0.70	0.023	5.08 (±3.28) [3]	
Ornithine	0.67	0.0000046	4.38 (±3.25)	
Arginine	0.66	0.063	7.13 (±3.43)	*
Glutamic acid	0.62	0.052	4.29 (±4.06)	*
Tyrosine	0.59	0.0019	2.88 (±2.95)	
Proline	0.58	0.0456	4.13 (±3.40)[3]	*
Glutamine	0.58	0.010	2.46 (±2.87)	
Citrulline	0.57	0.000020	2.58 (±2.34)	
Alanine	0.53	0.025	1.83 (±2.33)	
Total	0.52	0.019	1.75 (±2.38)	
Lysine	0.52	0.0024	1.46 (±2.70)	
Methionine	0.47	0.030	1.25 (±1.94)	
Histidine	0.45	0.10	1.25 (±1.94)	*
Phenylalanine	0.42	0.0011	0.88 (±1.15)	
Tryptophan	0.38	0.16	1.42 (±2.47)	*
Valine	0.38	0.066	0.92 (±1.14)	*
Isoleucine	0.34	0.035	0.46 (±0.72)	
Leucine	0.25	0.074	0.29 (±0.46)	*
Taurine	0.25	0.0051	0 (±0)	
Aspartic acid	0.23	0.16	0 (±0)	*
Cystine	0.22	0.0095	0 (±0)	
Threonine	0.21	0.39	0.71 (±1.08)	*

[a]Each of the 19 participants was selected as a reference individual, and the average and standard deviation of the number of individuals who showed significantly ($P<0.05/(23*19)$) different amino acid concentration from the reference individual by the linear model (Eq. (1)) was calculated; [b]significant change with gestational day; [3]amino acids with genome-wide significant SNV.

gestational time on plasma amino acid concentrations (Table 4). The variances in amino acid concentrations explained by gestational time were<0.1 for most amino acids (except threonine and tryptophan), suggesting that the amino acid concentrations are strongly regulated compared with e.g. lipid levels, which significantly increase in late pregnancy. We also classified the participants by their genotypes for the GWAS-significant SNVs on amino acid concentrations and demonstrated their effect on amino acid concentrations in maternal plasma (Fig. 3). In addition, the absolute quantification methods for amino acid concentrations with LC-MS enabled us to calculate the abundance of each amino acid relative to total, and this relative amino acid profile was useful to discriminate plasma from pregnant participants (Fig. 4). As amino acid metabolism is carried out by complex networks involving multiple enzymes, linking perturbations in amino acid concentrations with the formation and progress of gestation is difficult. However, several implications for amino acid metabolism during pregnancy were apparent from our findings.

First, we observed a decrease in total amino acid concentration of about 27% during pregnancy. Considering that the net increase in circulating blood volume during pregnancy is estimated to be 30−50% over that of the non-pregnant state[25], the absolute amount of amino acids in maternal circulation would also increase relative to the non-pregnant (ADM) condition and this increase would be beneficial for meeting the needs of the fetus.

We also compared the relative amount of amino acids in pregnant women to the non-pregnant state and with advancing gestation and found that the plasma amino acid composition was altered in pregnant women. During pregnancy,

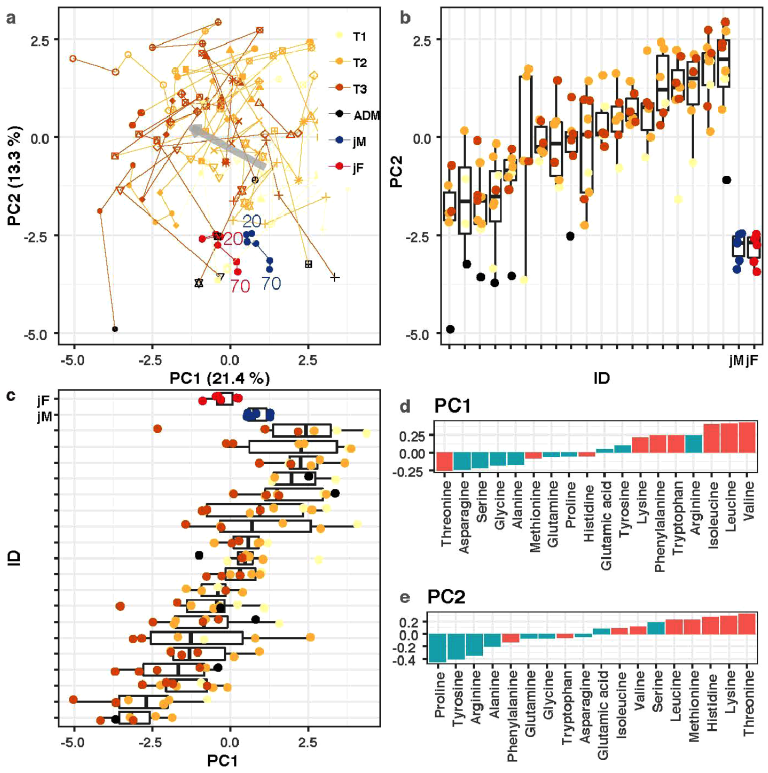

Fig. 4. Principal Component Analysis (PCA) of relative amino acid profiles.
(a) PCA of relative amino acid profiles for pregnant and ADM samples. Pregnant and ADM samples from the same individual are identified by separate point types and joined by lines. Pregnant samples collected during the first (T1), second (T2) and third (T3) trimesters and ADM are colored light brown, medium brown, dark brown and black, respectively. The grey arrow joining the first and last sample points during pregnancy was averaged over the 19 participants. As reference data for amino acid profiles for non-pregnant people, we used plasma amino acid profiles from 5,093 Japanese individuals measured by NMR that were collected and analyzed by the Tohoku Medical Megabank Organization (ToMMo) [19]. These profiles were divided into 12 subgroups by sex and age (20s, 30s, 40s, 50s, 60s and 70s) and average relative amino acid profiles were calculated for each subgroup. Male (jM) and Female (jF) groups are colored blue and red, respectively, and joined by separate lines with increasing age from 20s (denoted as 20) to 70s (denoted as 70). (b), (c). Box and scatter plots of (b) PC1 and (c) PC2 scores for pregnant and ADM samples and the ToMMo general cohort age and sex subgroups. Sample points are colored as in Panel A. (d), (e). (d) PC1 and (e) PC2 loadings of the 19 amino acids, which are arranged in ascending order. Essential and non-essential amino acids are colored in red and blue, respectively.

amino acids are transported from the maternal plasma to the fetal circulation by amino acid transporters in the placental syncytiotrophoblast[8,26,29]. Due to this active amino acid transport, amino acid concentrations are higher in the placental intervillous space and in the umbilical vein than in maternal plasma[27]. Amino acid infusion experiments[28] and measurement of arteriovenous differences in amino acid concentration[29] showed that most of the essential amino acids are actively transported from maternal blood through the placenta to the fetus. Enrichment of essential amino acids in the plasma of pregnant women may reflect differences in transport activities among amino acids, or arise from other maternal adaptations. We also observed significantly pronounced decreases in urea-cycle intermediates, e.g., arginine, ornithine and citrulline, during pregnancy as compared with the decrease in total amino acid concentrations. Amino acids transferred from maternal plasma to the fetus through the placenta are used either to synthesize proteins for the fetus or as an oxidized energy source wherein the amide group is converted to urea[11] and fetal amino acid consumption can decrease amounts of maternal urea cycle intermediates.

Using linear models applied to longitudinal plasma amino acid profiling, we found that threonine, histidine, glutamic acid, aspartic acid and proline showed significantly increased concentrations and arginine, tryptophan, valine and leucine had significantly decreased concentrations with increasing gestational age (Fig. 1). These results

were consistent with previous cross-sectional or longitudinal reports that involved a relatively small number of samplings per individual- usually once per trimester[13–17]. Thus, trends for amino acid concentrations with gestational age appear to be reproducible despite differences in ethnicities and other genetic and environmental backgrounds of the cohorts.

Although the metabolic and biological mechanisms that underlie these changes in amino acid concentrations await further characterization, several trends were apparent. Threonine is metabolized to acetyl-CoA and glycine by either threonine oxidase or serine dehydratase and increases in this amino acid may be associated with changes in the threonine oxidation state during pregnancy[30]. Decreases in tryptophan concentrations between T2 and T3 were previously shown to be accompanied by decreases in the amounts of some metabolites in the tryptophan metabolism pathway, such as 5-hydroxytryptophan, and could be associated with pregnancy-induced depression[14]. Arginine is involved in the urea cycle and decreases in arginine are consistent with decreases in urea production and excretion in late pregnancy[31]. Although levels of two additional amino acids that are important for the urea cycle, ornithine and citrulline, did not decrease significantly with gestational age (Fig. S1), overall arginine, ornithine, and citrulline concentrations were strongly decreased during pregnancy compared to the non-pregnant state (Fig. 3), suggesting that systemic nitrogen balance in pregnancy may influence levels of these amino acids. Arginine is also involved in the synthesis of nitric oxide, which plays a crucial role in vasodilation during pregnancy[32]. In addition, degradation of arginine by fetal arginase-2 is important to maintain maternal immunosuppression toward the fetus[33,34]. These multiple factors can contribute to the decrease in arginine concentration seen with advancing gestation. We observed consistent pregnancy-related decreases in branched-chain amino acids (valine, leucine, and isoleucine). Branched-chain amino acids are essential amino acids and consumption of fish oil during pregnancy can compensate for decreases in plasma concentrations of these amino acids in late gestation[17]. Meanwhile, we observed significant increases in the concentrations of glutamic acid and aspartic acid. These two amino acids, which are directly converted to α-ketoglutaric acid and oxaloacetic acid, respectively, both act as intermediate metabolites in the tricarboxylic acid (TCA) cycle. The plasma levels of these TCA cycle intermediate metabolites, including citric acid, isocitric acid, and α-ketoglutaric acid are increased in later trimesters[15], and could reflect the increased metabolism of the fetus, and explain the observed increase in aspartic acid and glutamic acid with gestational age.

In this study, we did not observe significant changes in concentrations of several amino acids that were reported in previous studies[17–19]. These differences between studies may be due to differences in genetic and environmental factors of the participants, such as ethnicity, age, and diet. We assumed that most changes in amino acid concentrations with advancing gestational age were small compared to variations resulting from differences between individuals or within individuals (Table 4) that could complicate the detection of trends during pregnancy.

Although the MS-based amino acids analysis, the quantified levels of the analyte, i.e. aspartic acid were too low to allow consistent measurement because of the limitation of derivatization efficiency. Therefore, the quantified levels of the amino acids should be concerned for comparing with the other analytical results when the values were used for the prediction of disease expression as the plasma biomarkers[35].

We confirmed the effect of genotypes for genome-wide significant SNVs on the concentrations of three amino acids, glycine, asparagine and proline (Fig. 3). Interestingly, the large variance for these three amino acids could be explained by individual differences, representing 0.80, 0.71, and 0.58 of the variances explained for glycine, asparagine, proline, respectively (Table 4). These results indicate that the genetic background of the participants likely influences amino acid concentrations during pregnancy and the importance of longitudinal analysis for identifying effects of individual differences on pregnancy-related changes in parameters such as amino acid concentrations.

Maternal pre-pregnancy BMI and fetal sex were associated with the concentrations of several amino acids. We tested the effect of pre-pregnancy BMI, fetal sex and mothers' parity on amino acid concentrations (β_{ID} in Eq. (1)) using Pearson's correlation coefficient or Wilcoxon-rank sum test. We found no significant correlations after adjustment for multiple testing (data not shown). In a previous study that compared maternal plasma samples from normal and obese groups, concordant decreases in most amino acids in the obese group were reported[36]. Male fetus is reported to be a possible risk for gestational diabetes mellitus[37,38]. Due to the small number of subjects enrolled for

this study, it was difficult to evaluate the effect of these clinical factors for the participants, but they could be confounding factors for changes in amino acid concentrations during pregnancy.

Conclusions

Amino acid profiles were changed significantly by pregnancy and with advancing gestation. The amounts of essential amino acids remained relatively consistent during pregnancy, although levels of urea cycle components were decreased relative to post-delivery, which supported that changes in amino acid profiles do occur during pregnancy. An effect of genotypes for three genome-wide significant SNVs on the concentrations of glycine, asparagine, and proline in pregnant women was observed, indicating that genetic variations can dominate changes in amino acid concentrations. Together our results indicated that relative amino acid profiles reflect the formation and progression of gestation in pregnant women.

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Declarations

Compliance with Ethical Standards

This study was approved by the Tohoku University Ethics Committee (authorization numbers, 2012-1-443 and 2014-10). Written informed consent was obtained from all participants.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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