Integrated Profiling of Metabolites and Trace Elements Reveals a Multifaceted Malnutrition in Pregnant Women from a Region with a High Prevalence of Congenital Malformations

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Abstract:

Considerable evidence from epidemiological and clinical studies demonstrated that maternal nutritional status is closely associated with placental, embryonic, fetal growth and development, and ultimately pregnancy outcomes. In recent years, there has been a growing interest in the study of disorders of pregnancy using a metabolomic profiling approach. In this study, we presented an integrated comprehensive profiling approach to assess maternal nutritional status through measuring a wide variety of small-molecule metabolites and trace elements in serum of pregnant women. A total of 56 pregnant women with normal pregnancy outcomes were enrolled from Lvliang prefecture of Shanxi province, the area with the highest prevalence of congenital anomalies in China, and 40 pregnant women with normal pregnancies were recruited from Huairou county of Beijing city, the region representing a national average level. As compared with the national average level, these pregnant women from Lvliang region shown distinct metabolic phenotypic variations as revealed by the depleted serum concentrations of folate and vitamin B_{12}, lower concentrations of carbohydrates, lipids, Se, Zn, and Cu, as well as higher concentrations of amino acids, urea-cycle metabolites, Sr, Cd, and Pb. Our results offer an improved understanding of severe multifaceted malnutrition in the pregnant women from a population with a high prevalence of congenital anomalies, highlighting the potential of a panel of critical nutrients as markers for aiding the diagnosis, prevention, and intervention of pregnancy complications.

Article:

INTRODUCTION

Emerging evidence from epidemiological and clinical studies demonstrates that maternal malnutrition during pregnancy can be detrimental to embryonic and fetal development because

maternal environment alters the epigenetic state of the fetal genome through cell signaling occurred in the placenta (Zhang et al. 2008; Martin-Gronert and Ozanne 2006; Wu et al. 2004; Harding 1999). Failure to supply sufficient amount of nutrients mandatory for embryonic and fetal development before and during pregnancy will result in a suboptimal intra-uterine environment for embryonic and fetal growth and development (Keen et al. 2003; Harding and Johnston 1995). The immediate response of a fetus to undernourishment is, through utilizing alternative energy supply or altering its own metabolic rate, to adapt itself to maximize the opportunity for postnatal survival (Martin-Gronert and Ozanne 2006; Harding and Johnston 1995). The adaptation of a fetus during fetal development, unfortunately, increases the risk of permanent changes in fetal structure or body function e.g., congenital anomalies (Forchielli et al. 1994).

Congenital anomalies are severe physical malformations, deformations, and chromosomal abnormalities (Q00–Q99, codes of the International Classification of Diseases) that are present at birth (Christianson et al. 2006). Congenital anomalies affect one in every 33 babies born in the United States annually (Rynn et al. 2008) and approximately 7.9 million infants in the world (6% of worldwide births) (Christianson et al. 2006). Congenital anomalies are also a leading cause of infant deaths, accounting for over 20% of all infant deaths in the United States (Martin et al. 2008). The birth prevalence of congenital malformations in the Chinese population varies because of regional differences in socioeconomic status, dietary habits, lifestyles,environmental factors, and medical care etc. (Berry et al. 1999; Li et al. 2009; Zhang et al. 2008; Zheng et al. 2007). For instance, the prevalence of congenital malformations in Beijing city, the capital of the People's Republic of China, is 175.6/10,000 (Li et al. 2009), whereas our recent epidemiological investigation reported that the prevalence of congenital malformations in a rural area—Lvliang preference of Shanxi province is approximately 844.2/10,000, which is among the highest in the world (Zheng et al. 2007).

For decades, remarkable progress has been made to prevent and improve disorders of pregnancy such as intrauterine growth restriction (IUGR), small for gestational age (SGA) or large for gestational age (LGA), stillbirth, congenital anomalies and so forth through regulating maternal nutritional status (CDC 1992; Berry et al. 1999; De Wals et al. 2007; King 2006). It has been suggested that vitamin D supplementation has beneficial effect on preeclampsia, SGA risk, birth weight, and postnatal growth, however, the clinical effect of dietary vitamin D supplementation during pregnancy has not been fully evaluated by more rigorous and well-designed randomized clinical trials (Brannon and Picciano 2011; Leffelaar et al. 2010; Robinson et al. 2011). Food fortification with folic acid significantly reduces the risk of neural tube defects (NTDs), one of the most common congenital malformations, in the pregnant women (CDC 1992; Berry et al. 1999; De Wals et al. 2007). Nevertheless, maternal folate deficiency is not necessarily the sole recognized risk factor for NTDs occurrence because DNA methylation relies on not only cofactors such as folic acid, vitamin B_3, and B_{12}, but also dietary methyl donors including glycine, choline, and serine in biological system (Koury and Ponka 2004; Mosley et al. 2009; Steegers-Theunissen 1995; Zeisel and da Costa 2009; Zhu et al. 2009). Accumulating evidence supports that dietary vitamin C, alone or in combination with vitamin E, during pregnancy does not reduce the risk of preeclampsia, early pregnancy failure, fetal loss, and SGA infant (Klemmensen et al. 2009; McCance et al. 2010; Polyzos et al. 2007). With increasing awareness of the importance and challenge of embryonic and fetal development, it is becoming evident and

crucial to assess overall maternal nutritional status such that the underlying association of maternal nutrition with the potential risk for pregnancy complications could be uncovered.

To date, ultrasonography has been extensively used in obstetrics and gynecology to examine embryos or fetuses during routine prenatal care as this technique allows clinicians and prenatal specialists to monitor health status of a fetus and to diagnose potential pregnancy complications at an early gestation stage (ACOG Practice Bulletin No. 58. Ultrasonography in pregnancy 2004; McIntire et al. 1999). In clinical practice, however, screening ultrasonography does not improve pregnancy outcomes in terms of two key metrics i.e., the increased number of live births and the reduced number of perinatal morbidity (Crane et al. 1994; Ewigman et al. 1993; Bucher and Schmidt 1993). One possible explanation of this phenomenon is that once a fetus is diagnosed as phenotypic or physical anomalies using ultrasonography, the homeostatic state of the epigenetic networks and their interactive surroundings including gene expression, proteins, and small-molecule metabolites present in biological system have been disrupted substantially and can no longer be retrieved to the normal physiological condition (Zhu et al. 2009; Martin-Gronert and Ozanne 2006; Gallou-Kabani and Junien 2005). Thus, it is of imperative importance to develop guidelines and protocols that can assist clinicians and physicians in early diagnosis and management of pregnant subjects with abnormal pregnancy outcomes.

Metabolomics/metabonomics has been rapidly evolved and become a robust and powerful tool that is capable of quantitatively assessing the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification through simultaneously measuring a set of metabolic end-point small-molecule metabolites (metabolome) in biological matrices (Fiehn *2002*; Nicholson et al. 1999). As metabolome is closest to phenotype of the specific biological system, there is a growing research interest in using metabolomics/metabonomics approach to study pregnancy complications. Metabolic profiling of amniotic fluid from a rat model of maternal diet restriction to mimic famine condition demonstrated a close association of prenatal malnutrition with abnormal fetal neurodevelopment (Shen et al. 2008). Studies employing mass spectrometry-based metabolomics observed metabolic profile changes in placental explants cultured under different oxygen tensions (Dunn et al. 2009; Heazell et al. 2008). Horgan et al. applied metabolomic technology to investigate the differences between small for gestational age (SGA) and normal pregnancies under different oxygen conditions (Horgan et al. 2010). Tissot van Patot et al. demonstrated metabolic differences in placental tissue from pregnancies at low and high altitude using nuclear magnetic resonance (NMR)-based metabolomics strategy (Tissot van Patot et al. 2010). Metabolomics has also been used to differentiate women suffering from preeclampsia and those with normal pregnancies (Kenny et al. 2010; Odibo et al. 2011; Turner et al. 2007). We have recently reported that metabolic signature of pregnant women with neural tube defects (NTDs) in offspring can be characterized by the impaired mitochondrial respiration, neurotransmitter γ-aminobutyric acid, and methionine cycle (Zheng et al. 2011), which was supported by metabolic profile changes in a mouse model of di-*n*-butyl phthalate (DBP)-induced teratogenesis (Xia et al. 2011).

As reported in our previous epidemiological surveillance and investigation, insufficient intake of vegetables, fruits, meat, milk, and certain micronutrients including folic acid, zinc, and selenium in pregnant women from Lvliang preference of Shanxi province may be the key diet factor for

high prevalence of congenital anomalies in this area (Zhang et al. 2008). The ultimate goal of this study was to gain an improved understanding of the association between maternal nutritional status resulting from dietary insufficiency or imbalance of nutrients and potential risk for high prevalence of pregnancy complications. We proposed an integrated profiling strategy to quantitate a broad range of small-molecule metabolites and trace elements in pregnant women using immunoassay analyzer, gas chromatography time-of-flight mass spectrometry (GC–TOF MS), and inductive coupled plasma–mass spectrometry (ICP–MS). A comparative study was conducted on the pregnant women with normal pregnancies from two typical populations: one has the highest birth prevalence of congenital malformations in China while the other represents a national average level. Multivariate statistical technique was employed to afford a global view of similarities and separation trend of maternal nutritional status of the two populations. The fold changes and *P* values of the detected metabolites and trace elements were attained by Mann–Whitney test. Correlation matrix map (CMM) and hierarchical cluster analysis (HCA) were performed to explore the interrelationship of metabolites and/or trace elements most correlated with the characteristic metabolic profile in Lvliang area.

SUBJECTS AND METHODS

Subjects, and inclusion/exclusion criteria

Serum samples of pregnant women with normal pregnancy outcomes (n = 54) were collected from Lvliang prefecture of Shanxi province, a region with the highest prevalence of congenital malformations in China (Zheng et al. *2007*). A comparative group of women with normal pregnancies (n = 40) was recruited from Huairou region of Beijing city that represents a national average prevalence of congenital malformations (Li et al. *2009*). The basic characteristics and dietary information from all of the participants are provided in Table 1.

Table 1: Descriptive characteristics of all of the subjects

Groups[a]	Descriptive statistics[b]	Shanxi group	Beijing group
Maternal age (years)	Mean (95% C.I.)	25.7 (24.6–26.9)	26.8 (25.3–28.0)
	Median (range)	25.0 (18–37)	26.0 (21–39)
Gestational age[c] (weeks)	Mean (95% C.I.)	21.6 (19.5–23.6)	16.4 (14.4–18.4)
	Median (range)	20.0 (5–38)	15.5 (7–27)
Total energy (kcal)		2414.3	2814.7
Protein (g/day)		85.2 (12.1%)	94 (15.44%)
Fat (g/day)		67.2 (20.8%)	83 (30.92%)
Carbohydrates (g/day)		467.5 (67.1%)	324 (53.64%)

[a]Shanxi group (n = 54) and Beijing group (n = 40) are the groups of the pregnant women with normal pregnancies from Shanxi and from Beijing
[b]Mean (95% C.I.) is mean value of each group with 95% confidence interval (C.I.) and Median (range) is median value of each group with the range from minimum to maximum of the group
[c]Gestational age (weeks) was estimated starting either from the first day of the last woman's last menstrual period or 14 days before conception (if the conception is known)

The participants with normal pregnancy outcomes were confirmed by one-year follow-up examination using standard operational protocol (SOP) in our surveillance database developed jointly by Institute of Population Research/WHO Collaborating Center for Reproductive Health and Population Science at Peking University (Beijing, P. R. China). Subjects bearing diseases such as cardiovascular diseases, metabolic disorders (obesity, diabetes or diabetic complications), malignant tumors, taking anti-folate medication, or in influenza/inflammation with fever within 2 weeks before specimen collection were excluded. Blood sample collection, storage, and delivery were in compliance with SOP developed by Capital Institute of Pediatrics

(CIP, Beijing, P. R. China) and implemented by the trained physicians or nurses at the both sites. The overnight (minimal 10 h to maximal 14 h) fasting blood sample was collected in the morning and placed in BD Vacutainer® Blood Collection Tubes (Franklin Lakes, NJ) at room temperature for 30 min allowing coagulation. The clotted blood samples were rimmed or ringed with an applicator stick if necessary, and centrifuged for 10 min at 3,000 rpm. Each 100 μl of supernatant fluid (serum) was aliquoted in 2 ml non-glass plastic Eppendorf centrifuge tube for multiple platform analysis and stored at −20°C until shipping. The samples were delivered to the research laboratory with dry ice and stored in −80°C freezer prior to sample preparation. The written informed consent and dietary questionnaires were gathered from each subject at the time of sampling. The study followed the Declaration of Helsinki and was approved by the Institutional Review Board at CIP.

Immunoassay

Folate (vitamin B_9) and vitamin B_{12} are two fundamental nutrients for embryonic and fetal development, but they are not feasible for GC–TOF MS analysis due to their high polarity and molecular weight. We determined their concentrations in the two groups: Shanxi group (n = 54) and Beijing group (n = 40) using an immunoassay analyzer (IA) ARCHITECT i2000 (Abbott Laboratories Inc., Abbott Park, IL) following the manufacturer's procedures (Abbott 2011).

Untargeted profiling of small-molecule metabolites

An unbiased metabolic profiling was performed by measuring a variety of small-molecule metabolites including amino acids, fatty acids, organic acids, carbohydrates etc. in serum from all of the participants. The sample preparation followed our previously published methods (Qiu et al. 2009; Wang et al. 2009). In brief, two internal standard solutions (10 μl of L-2-chlorophenylalanine in water, 0.3 mg/ml; 10 μl of heptadecanoic acid in methanol, 1 mg/ml) were spiked with 100 μl of serum sample, to which 300 μl of organic mixture (methanol/chloroform = 3:1, v/v) was added for protein precipitation and small-molecule metabolite exaction. After vortexing and centrifugation, each aliquot of 300-μl supernatant was evaporated to dryness under vacuum at room temperature in a 2-ml sample vial. Subsequent to vacuum dryness, each 80 μl of methoxyamine (15 mg/ml in pyridine) was added to the vial and the reaction was maintained at 30°C for 90 min. The trimethylsilyl derivatization was conducted at 70°C for 60 min by the addition of 80 μl of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (containing 1% trimethylchlorosilane). After centrifugation, each 1-μl aliquot of the supernatant was injected into a hyphenated Agilent 6890N gas chromatography (Agilent Technologies, Santa Clara, CA) coupled with a Pegasus HT time-of-flight mass spectrometer (Leco Corp., St Joseph, MI) in a splitless mode. To minimize systematic variations during sample analysis, the study samples were analyzed in the order of "Shanxi group subject 1 → Beijing group subject 1 → Shanxi group subject 2…etc." and the subject of each group was selected randomly. A DB-5 ms capillary column (Dimension: 30 m × 250 μm I.D. 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA) was used for metabolite separation. The helium was used as carrier gas with a constant flow rate of 1.0 ml/min. The temperatures of the front inlet, transfer line, ion source, and quadruple were set to 270, 290, 200, and 150°C, respectively. The GC temperature program was set to 2 min isothermal heating at 80°C, followed by 10°C/min and oven temperature ramped to 180°C, 5°C/min to 240°C, and 25°C/min to 290°C, and a final 9 min maintaining at 290°C. Electron impact ionization at a full scan mode (m/z 30–600) was used with an acquisition rate of 20 spectra/sec.

Quantification of minerals and trace elements

A total of 16 macrominerals and trace elements including cobalt (Co), copper (Cu), zinc (Zn), selenium (Se), magnesium (Mg), phosphorus (P), chromium (Cr), arsenic (As), strontium (Sr), cadmium (Cd), tellurium (Te), barium (Ba), lead (Pb), tin (Sn), caesium (Cs), and erbium (Er) were quantitated using an Agilent 7500ce inductively coupled plasma–mass spectrometer (ICP–MS, Agilent Tech., Tokyo, Japan) (Zhao et al. 2009). The serum samples were used from those subject (n = 19 per group) with sufficient volume of serum remained after GC–TOF MS and IA measurement. Each 0.1 g of serum sample was weighed and placed into a 15-ml PFA-coated pressure vessel, to which 150 µl of HNO_3 (ultrapure grade, 65% v/v, Merck KGaA, Darmstadt, Germany) and 150 µl of H_2O_2 (ultrapure grade, 30% v/v, Merck KGaA, Darmstadt, Germany) were added. The digestion was carried out in a Mars-5 microwave digestion system (CEM Corp., Matthew, NC) with 40 reactors in a cycle using the following program: (i) 5 min at 1,600 W power and 115°C (ramp 10 min), (ii) 5 min at 1,600 W power and 150°C (ramp 4 min) and (iii) 5 min at 1,600 W power and 185°C (ramp 4 min). The resulting solution was diluted into about 1.3 g using 18.2 MΩ ultrapure water from Q-POD Element unit with LC-Pak filter (Millipore, Billerica, MA). The diluted samples were stored at 4°C refrigerator pending ICP–MS analysis. The internal standard solution was prepared by mixing the single-element standard solutions of Sc, In, and Re (Merck KGaA, Darmstadt, Germany) and introduced by peristaltic pump into ion source at an approximate concentration of 25 µg/l with an on-line mode. The instrument was tuned to an optimal condition across mass scan range using a tuning solution containing 10 µg/l Li, Y, Tl and Ce in 2% HNO_3 (w/v) (Agilent Technologies, Tokyo, Japan). Data were acquired by Agilent Chemstation E.03.07 at a full-quantitation mode.

Each of 16 single-element standard stock solutions at a concentration of 1,000 ppm (ultrapure grade, Alfa Aesar China, Beijing, P. R. China) was mixed and used for quantitation. The mixed solution of the 16 elements was diluted into a series of concentrations with 2% HNO_3 to establish quantitation curves. The quantitative concentration range, linear equations, and coefficients of determination (R^2) were attained by Agilent Chemstation software (Supplemental Table 1).

Data pretreatment

The raw data files from GC–TOF MS analysis were exported to NetCDF format by ChromaTOF software v4.40 (Leco Corp., St Joseph, MI) and processed by our previously developed software package in which automated peak detection, deconvolution, alignment, and library search were conducted (Jiang et al. 2010). The resulting data set was organized in a Microsoft Excel spreadsheet as a format of sample information (subject identity code), variable identities (retention time–unique mass pairs), and peak areas. Initial compound annotation was conducted by searching against commercial mass spectral databases such as NIST library 2008 (National Institute of Standards and Technology, Gaithersburg, MD) and LECO/Fiehn Metabolomics Library (Leco Corp., St Joseph, MI). Further validation of compound annotation was performed by comparing the mass spectral data and retention time of each tentatively assigned metabolite with those in our internal metabolite library encompassing over 500 endogenous metabolite standards commonly detected in biological matrix. The quantitative report of each sample generated from ICP–MS was stored as a separate csv format file by Agilent Chemstation E.03.07. In order to combine and compare the results from all of the files, the custom scripts compiled in MATLAB software (The MathWorks, Inc., Natick, MA) were used to extract

information including sample information (subject identity code), variable identities (element symbols), and element concentrations. The exported data set was organized in a Microsoft Excel spreadsheet.

Statistical analysis

The metabolome data derived from both GC–TOF MS and IA was imported into SIMCA-P+ 12.0.1 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. To minimize statistical bias resulted from the concentration variations of different metabolites, the data set was mean-centered and scaled to unit variance prior to principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) (Umetrics 2011; Wold 1987; Wold et al. 2001). Unsupervised PCA was performed to visualize intrinsic variations of nutritional status in pregnant women from the two regions at a global fashion. A more sophisticated discriminant technique PLS-DA was further applied to achieve global profile separation between inter-groups through maximizing systematic variations between the two groups. For PLS-DA modeling in SIMCA-P+ software, a default 7-fold (Leave–1/7th Samples–Out) cross–validation procedure was carried out to avoid model over-fitting (Trygg et al. 2007; Umetrics 2011). The values of the two key parameters, R^2Y and Q^2Y, approaching 1.0, imply a reliable mathematical model with satisfactory predictability, while the PLS-DA models with Q^2Y value ≥ 0.40 are acceptable in practical applications (Trygg et al. 2007; Ni et al. 2008; Qiu et al. 2009).

A non-parametric Mann–Whitney test in PASW Statistics 18.0 software package (The SPSS Inc., Chicago, IL) was used to determine if a significant difference of each metabolite or element exists between the Beijing group versus the Shanxi group. The critical *P* value was set as 0.05 in this study. The fold change (FC) was defined as the ratio of the mean rankings of metabolite signal response (peak areas) or trace element concentrations in the Shanxi group versus the Beijing group.

With the aim of exploring the interrelationship of metabolites and/or trace elements potentially associated with pregnancy malnutrition status, CMM and HCA were employed in this study. HCA creates a hierarchy of clusters to measure the similarities and dissimilarities of the variables (herein referred to metabolites and/or elements) using a tree structure (dendrogram), in which the root of the tree consists of a single cluster containing all the variables and the leaves correspond to individual variable. CMM describes the interrelationship of these metabolites and/or elements using Pearson's correlation coefficients. In CMM, each cell represents the correlation of the two given variables and the diagonal cells (correlations of variables with themselves) are always equal to 1.0. Both HCA and CMM were performed on R environment (R 2011).

RESULTS

Assessment of global metabolic profiles

A three-dimensional (3D) PCA scores plot, which was constructed using a total of 343 metabolite signals analyzed by GC–TOF MS and IA, depicted a clear separation trend of the global metabolic profiles between the Shanxi group and the Beijing group without any outliers detected (Fig. 1a). The metabolic profile variations between the two groups were further validated and presented by a three-component PLS-DA model with satisfactory modeling and predictive abilities ($R^2Y = 0.863$ and $Q^2Y = 0.706$) (Fig. 1b).

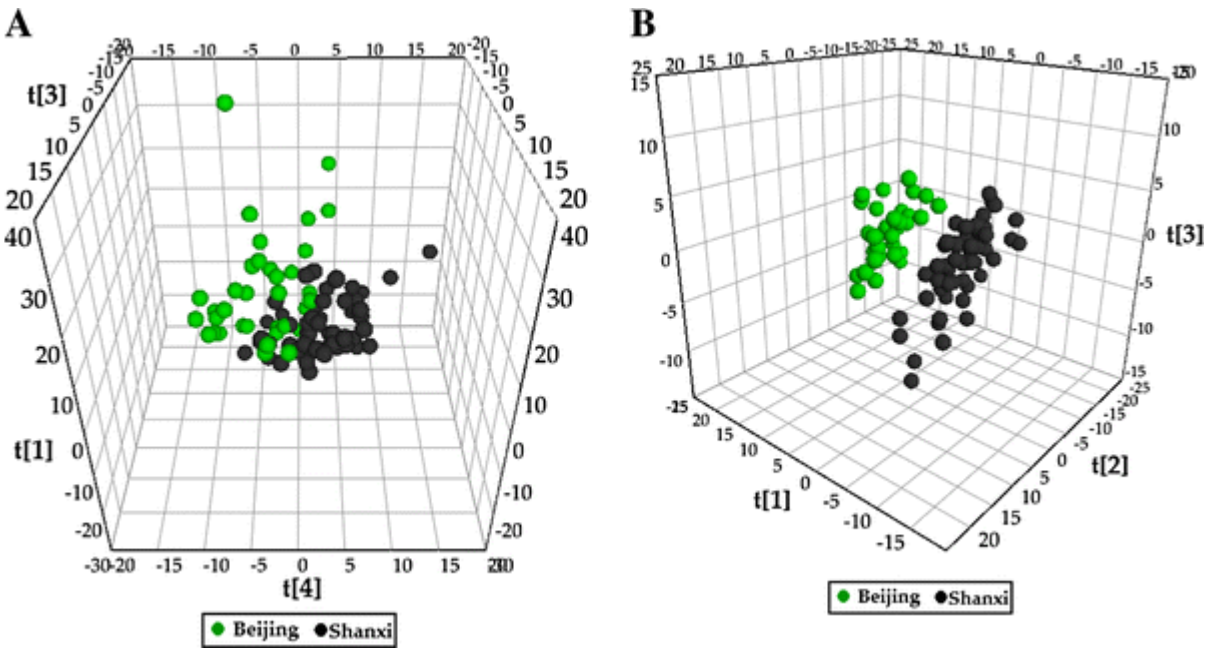

Figure 1: Visualization of overall maternal nutritional status. **a** shows a 3-D PCA scores plot generated by a total of 343 metabolite signals analyzed by GC–TOF MS and IA. It presents a separation trend of maternal nutritional status in the pregnant women with normal pregnancies from Shanxi and Beijing regions. **b** A more intrinsic metabolic variation between the two groups was further validated and presented by a three-component PLS-DA model ($R^2Y = 0.863$ and $Q^2Y = 0.706$). In PCA and PLS-DA scores plot, each *dot symbol* represents an individual subject and the spatial distribution of these dots reveals the variations of maternal metabolic status

Folate and vitamin B12

According to the established criteria for nutritional disorders (serum folate <7 nmol/l and serum vitamin B_{12} < 145 pmol/l) (Johnson 2007), 22 subjects (40.7%) and 47 subjects (87.0%) in the Shanxi group were considered folate deficiency and vitamin B_{12} deficiency, respectively; whereas only three subjects (7.5%) and eight subjects (20%) in the Beijing group shown deficiency in the two nutrients, respectively. As illustrated in Figs. 2a, b, serum concentrations of folate and vitamin B_{12} were significantly lower ($P < 0.001$) in the Shanxi group than the Beijing group. The other noteworthy finding is that the serum concentrations of the two essential nutrients were consistently lower at each pregnancy trimester in the Shanxi group than in the Beijing group.

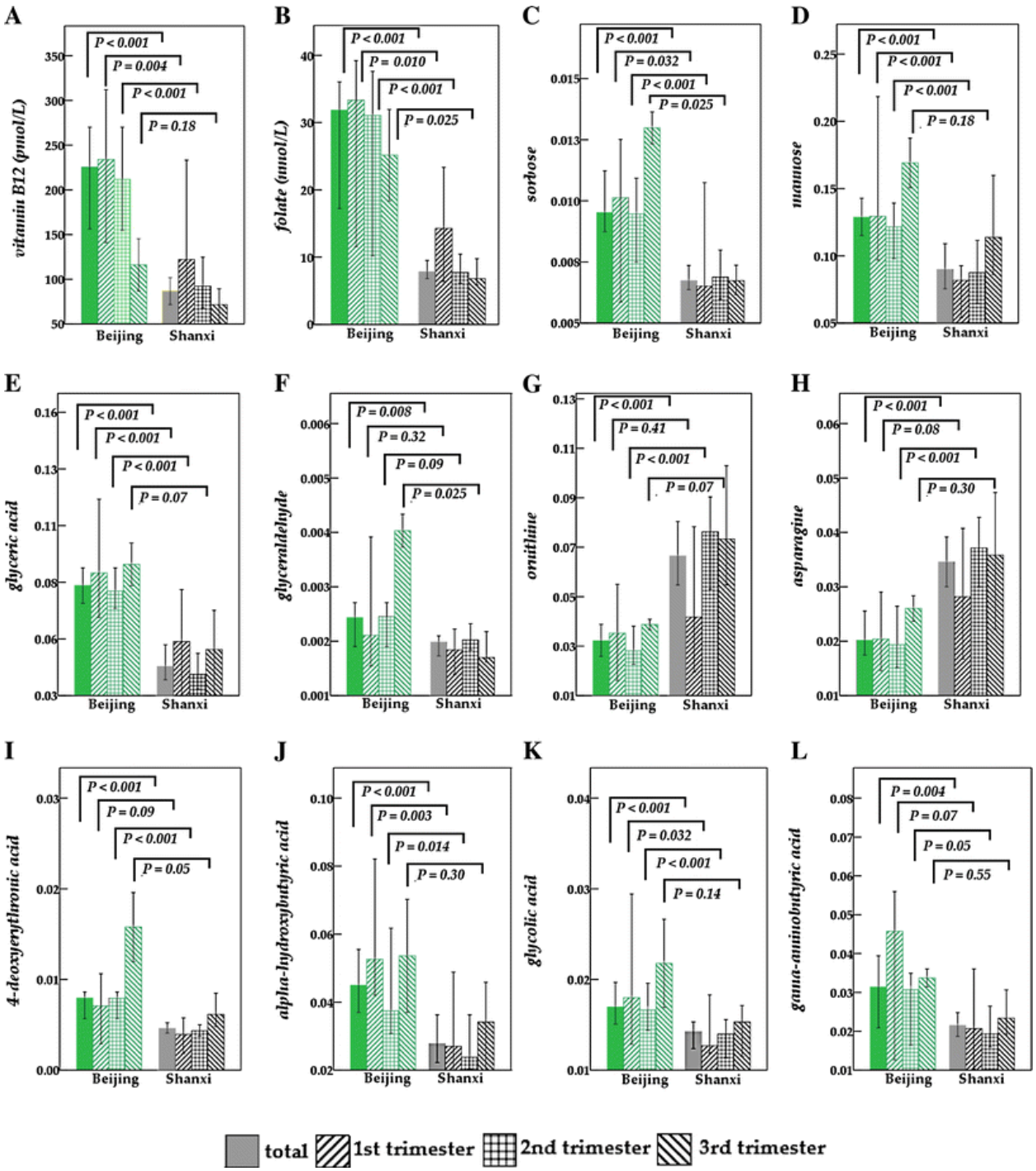

Figure 2: Serum concentrations of representative metabolites. **a** – **l** show the concentrations of the representative metabolites that are most dysregulated in serum of the pregnant women from Shanxi group in comparison with that from the Beijing group. The dynamic metabolic variations are present at three different pregnancy trimesters, where the first, second, and third trimester are defined as the 0–13th week, 14th–26th week, and 27th–41st week of gestation, respectively. The *bar* is the median concentration of metabolites for each group and the error bar represents a 95% confidence interval (95% CI). The *P* values were obtained by Mann–Whitney test

Metabolite analysis

Using a threshold of $P < 0.05$ from Mann–Whitney test, we obtained a list of 51 differentially expressed metabolites in the Shanxi group in relative to the Beijing group (Supplemental Table 2). Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg) pathway database and human metabolome database (HMDB, www.hmdb.ca) were employed to aid interpretation of pathophysiological molecular mechanisms by linking the differential metabolites with their biological function in metabolic regulatory network. As seen in Table 2, the representative metabolites were classified and sorted out by their regulatory pathways. In this study, significantly lower concentrations of carbohydrates and lipids while significantly higher concentrations of amino acids and urea-cycle metabolites were present in the Shanxi group in relative to the Beijing group.

Table 2: A representative list of most dysregulated small-molecule metabolites between the normal pregnancy women from Shanxi versus those from Beijing

Metabolite[a]	**Metabolic pathway**	**Shanxi versus Beijing**	
		FC[b]	***P*** [c]
Glucose[d]	Carbohydrates	0.71	4.61E−03
Mannose[d]	Carbohydrates	0.63	5.84E−05
Galactose[d]	Carbohydrates	0.67	8.77E−04
Glucurono-6,3-lactone	Carbohydrates	0.42	9.86E−13
Sorbose	Carbohydrates	0.59	4.14E−06
Caproic acid	Lipid metabolism	0.67	6.70E−04
Dodecanoic acid[d]	Lipid metabolism	0.77	4.45E−02
Stearic acid[d]	Lipid metabolism	0.77	2.62E−02
Linoleic acid[d]	Lipid metabolism	0.71	7.25E−03
Oleic acid[d]	Lipid metabolism	0.77	1.98E−02
Petroselinic acid[d]	Lipid metabolism	0.71	5.51E−03
Glyceraldehyde[d]	Lipid metabolism	0.71	8.00E−03
Glyceric acid[d]	Lipid metabolism	0.45	9.06E−11
Cholesterol[d]	Lipid metabolism	0.77	4.62E−02
Phenylalanine[d]	Amino acid	1.90	2.90E−06
Leucine[d]	Amino acid	1.40	1.07E−02
Isoleucine[d]	Amino acid	1.40	5.87E−03
Proline[d]	Amino acid	1.60	3.29E−04
Histidine[d]	Amino acid	1.60	6.70E−04
Serine[d]	Amino acid/one carbon metabolism	2.00	6.54E−07
Methionine[d]	Amino acid/one carbon metabolism	1.90	4.67E−06
Glycine[d]	Amino acid/glutathione/one carbon metabolism	1.30	2.91E−02
Cystine[d]	Amino acid/glutathione/one carbon metabolism	0.67	8.51E−04
Glutamic acid[d]	Amino acid/glutathione metabolism	2.10	6.26E−08
Pyroglutamic acid[d]	Glutathione metabolism	1.50	1.36E−03
Ornithine[d]	Amino acid/urea cycle	2.20	2.84E−08
Asparagine[d]	Amino acid/urea cycle	2.00	7.12E−07
Aspartic acid[d]	Amino acid/urea cycle	2.10	1.24E−07
Citrulline[d]	Amino acid/urea cycle	1.40	1.58E−02
Urea[d]	Urea cycle	1.50	4.05E−03
Lactic acid[d]	Glycolysis	2.00	6.00E−07
Uric acid[d]	Nuclear acid metabolism	0.63	2.10E−04
Hypoxanthine[d]	Nuclear acid metabolism	1.90	2.10E−06
Dihydrouracil[d]	Nuclear acid metabolism	1.80	1.78E−05

[a]These metabolites were annotated by NIST mass spectral database and selected for biological interpretation purpose. The complete list of differential metabolites is provided in Supplemental Table S2

[b] Fold change (FC) was calculated by the ratio of mean rankings between two groups using Mann–Whitney test and the [c] *P* values were obtained accordingly. The critical *P* value was set as 0.05 across the study

[d] These metabolites were validated by our metabolite library created by the standard compounds available

Dynamic changes of metabolites during pregnancy trimesters

As gestational age of the pregnant women at sampling could influence the maternal metabolic profiles, we investigated the serum metabolic variations across the three different pregnancy trimesters, where the first, second, and third trimester are defined as the 0–13th week, 14th–26th week, and 27th–41st week of gestation, respectively (Zheng et al. 2011). As expected, the concentrations of the representative differential metabolites varied across the three different pregnancy trimesters but the change trend among the pregnancy trimesters was highly consistent (Fig. 2). For instance, the concentrations of vitamin B_{12}, folate, sorbose, and γ-aminobutyric acid were lower in the Shanxi group at each pregnancy trimester than in the Beijing group while the concentrations of ornithine and asparagine were higher in the Shanxi group at all of the three pregnancy trimesters.

Trace element quantitation

Lower serum concentrations of Cu (FC = 0.77), Zn (FC = 0.80), Se (FC = 0.58), P (FC = 0.55), and Mg (FC = 0.77), while higher concentrations of Sr (FC = 1.41), Cd (FC = 1.25), and Pb (FC = 1.28), were observed in the Shanxi group than in the Beijing group (Fig. 3 and Supplemental Table 3).

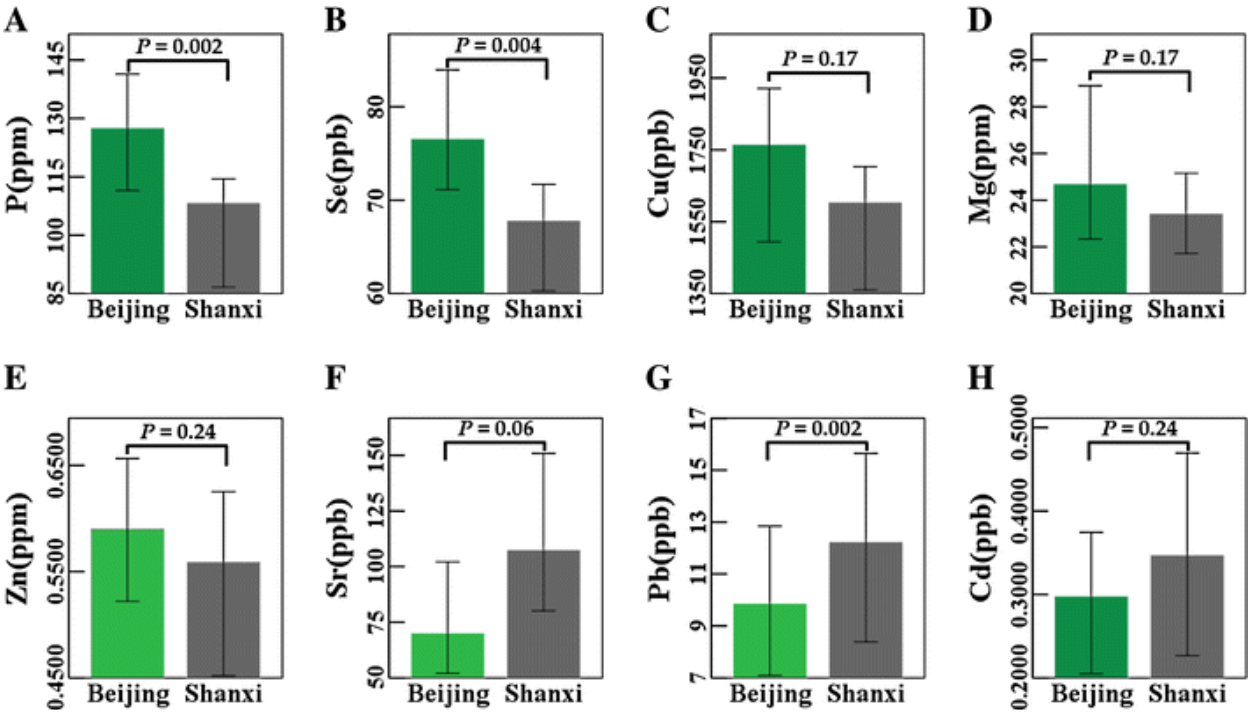

Figure 3: Serum concentrations of representative trace elements. **a** – **h** show the eight elements most dysregulated in serum of pregnant women from the Shanxi group in comparison with that from the Beijing group. The *bar* is the median concentration of trace elements for each group and the error bar represents a 95% CI. The *P* values were obtained by Mann–Whitney test

Interrelationship of metabolites and/or elements

A subset of 53 metabolites and 8 elements were selected for HCA and CMM analysis by *P* values and fold changes obtained from Mann–Whitney test. We conceived that these metabolites and elements were most correlated with the characteristic metabolic profile in Lvliang area. As seen in Fig. 4, certain essential nutrients such as folate, vitamin B_{12}, Cu, Mg, and P are positively correlated with each other in CMM and they tend to cluster in HCA. This finding implicated that deficiency of certain nutrients may result in a synergistic impact on abnormal pregnancy outcomes. However, discussion of the intrinsic relation of metabolites and elements is beyond the scope of this study.

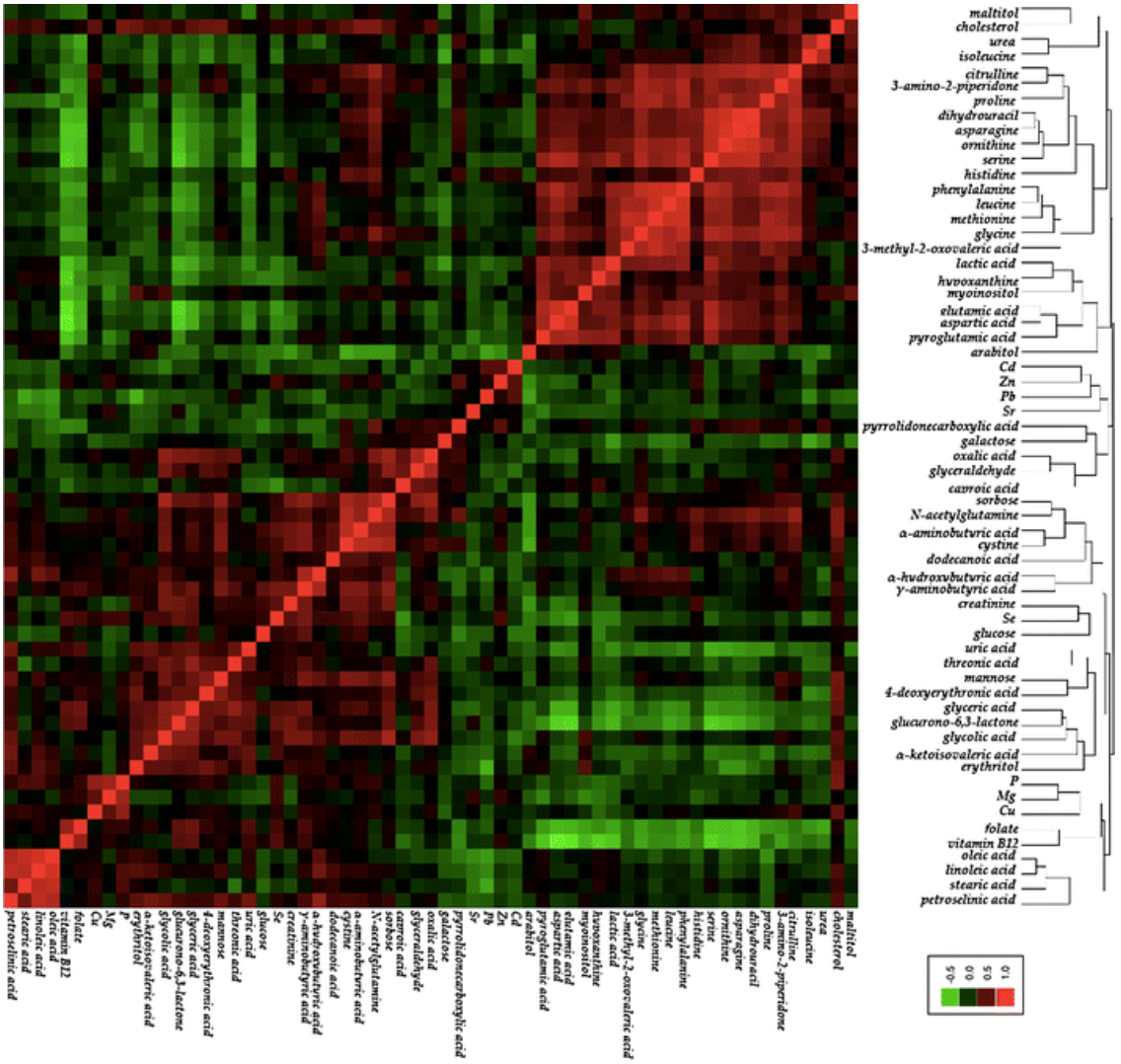

Figure 4: Interrelationship of metabolites and/or elements using correlation matrix map (CMM) and hierarchical clustering analysis (HCA). This figure was produced by the 53 metabolites and 8 elements most dysregulated in serum of pregnant women from Shanxi region versus that from Beijing region. The HCA uses average distance for clustering. In CMM, each cell represents a Pearson's correlation coefficient that was calculated by each of two metabolites and/or elements.

The negative values suggest an inverse correlation between the two given metabolites or elements while the positive values indicate a proportional relation between them

DISCUSSION

There is a considerable interest that maternal malnutrition can induce an impaired embryonic and fetal development, leading to ultimate impact on the fetus such as low birth weight, intrauterine growth restriction (IUGR), miscarriage, congenital malformations and so forth (CDC 1992; Gallou-Kabani and Junien 2005; Hadden and McLaughlin 2009; Harding 1999; Martin-Gronert and Ozanne 2006; McIntire et al. 1999; Wu et al. 2004). In this study, we profiled a broad range of small-molecule metabolites and trace elements in serum of pregnant women from two independent populations with high and average birth prevalence of congenital anomalies in offspring. In addition to severe deficiency of folate and vitamin B_{12}, a distinct disturbance of many other essential small-molecule metabolites including carbohydrates, fatty acids, amino acids, intermediates of urea cycle, and certain trace elements was also recognized as an indicator of high prevalence of congenital malformations in Lvliang preference of Shanxi province.

In this study, the significantly lower concentrations of folate ($P < 0.001$) and vitamin B_{12} ($P < 0.001$) were observed in the pregnant women in the Shanxi group than in the Beijing group (Fig. 2a, b). Folate and vitamin B_{12} play crucial roles in DNA synthesis and regulation, rapid cell division, and one-carbon metabolism in biological system, thus these two vitamins are fundamental to fetal growth and development during pregnancy (Berry et al. 1999; De Wals et al. 2007; Mosley et al. 2009; Zheng et al. 2011; Zhu et al. 2009). In the case of the role of the two nutrients, our results implicated that deficiency in these two nutrients at early gestation stage and/or during pregnancy is potentially associated with abnormal pregnancy outcomes in offspring. This finding was also evidenced by the fact that there was significantly insufficient intake of dark green vegetables, fruits, meat, milk, and certain micronutrients including folic acid, zinc, selenium etc. in pregnant women from Lvliang area when compared with the national average level (Zhang et al. 2008).

The most striking feature of this work is that we captured the characteristics of maternal metabolic phenotype during normal pregnancy in a region with the highest prevalence of congenital malformations in China, which is beyond a deficient status of folate and vitamin B_{12}. In Fig. 1, both PCA and PLS-DA scores plots clearly demonstrated a distinct metabolic profile of pregnant women under malnutrition status in Shanxi area as compared to the Beijing group, although both of the two groups had normal pregnancy outcomes in offspring. Using Mann–Whitney test with the threshold of *P* value less than 0.05, we obtained a list of 51 differentially expressed metabolites in the Shanxi group in relative to the Beijing group (Supplemental Table 2, 3). There is a significant decrease in carbohydrates including glucose, mannose, galactose, glucuronolactone etc. and seven fatty acids whereas a significant increase in 14 amino acids and intermediates of urea cycle such as ornithine, citrulline, and urea in the serum samples from the Shanxi group as compared to the pregnant women from Beijing. Pregnancy has a remarkable effect on the maternal metabolism and physiological state (Hadden and McLaughlin 2009; Martin-Gronert and Ozanne 2006; King 2006). The concentrations of carbohydrates and lipids fluctuate substantially during pregnancy in order to maintain a continuous supply of nourishment for the growing fetus (Hadden and McLaughlin 2009; Martin-Gronert and Ozanne

2006; King 2006). The significantly decreased concentrations of saccharides and lipids in maternal serum implicated either a reduced supply of these metabolites or an elevated consumption of them by fetal development process. From the dietary information present in Table 1, daily consumption of carbohydrates was markedly higher (about 44%) in the pregnant women from the Shanxi group than those from the Beijing group whereas fat intake per day was moderately lower (about 19%) in the Shanxi group than the Beijing group. Therefore, it is more likely that the significantly low concentration of maternal serum saccharides in the Shanxi group was resulted from an increased consumption of saccharides during fetal growth. However, it is difficult to determine which of these factors i.e., insufficient dietary fat supply or enhanced fat consumption by fetal growth and development, is dominant because they are highly interacted. Pregnant women normally sustain an elevated blood glucose level and undergo fat storage for fetal growth and development from their nongravid state and early pregnancy towards middle and late pregnancy (King 2006). The lower concentrations of saccharides and lipids in the Shanxi group suggested that their glucose metabolism and fat storage are suboptimal, which is detrimental to fetal development. In addition, as maternal saccharides and lipids are profoundly low, protein catabolism in maternal hepatic and adipose tissues will be activated to subsidize and preserve energy needs by fetus (Hadden and McLaughlin 2009; King 2006; Wu et al. 2004). As a consequence of protein catabolism, an increased concentration of amino acids was observed in maternal serum (Table 2). The enhanced amino acid oxidation could lead to an increased urea synthesis and urea nitrogen level in maternal blood and urine (Table 2). As urea concentration in maternal and fetal blood is nearly same through the exchange of urea in both of the circulations (Hutchinson et al. 1962), an unusual accumulation of maternal urea cycle metabolites due to pathophysiological change, may lead to an increase of these metabolites in fetal blood. Unfortunately, the significant elevation of urea excretion in a fetus causes chronic neurotoxicity to the fetus (Matsuoka and Igisu 1993). To this end, the disrupted energy supply from carbohydrate, lipid, and amino acid metabolism and subsequently urea over-excretion seem to be interrelated and present in pregnant women from a region with a high prevalence of severe congenital malformations. Additionally, we observed that several amino acids involved in one-carbon metabolism, including serine, methionine and glycine were unexpectedly higher in serum of pregnant women from Lvliang region. We suspected that the synthesis from homocysteine to methionine was inhibited due to the depleted folate and vitamin B_{12} and as a consequence, one-carbon metabolism donors including serine, methionine, and glycine in upstream can be excessive in serum samples from Lvliang region.

Finally, we determined the concentrations of 16 representative elements in serum of the two groups of pregnant women. As illustrated in Fig. 3a–h, a relatively lower serum concentration of Se, Cu, Zn, Mg, and P while a higher concentration of Sr, Cd, and Pb were detected in the pregnant women from the Shanxi group, as compared to the subjects from the Beijing group. It is well-documented that essential trace elements e.g., selenium and copper play an indispensable role in various biological functions such as being a cofactor with enzymes in regulating cell signaling pathways (Rotruck et al. 1973). Trace element selenium is critical for cellular functions in most mammals and acts predominantly as a cofactor for the reduction of antioxidant enzymes such as glutathione peroxidases because it forms the bioactive center of certain enzymes (Rotruck et al. 1973). Essential element copper is often involved in maintaining the normal enzyme conformation of superoxide dismutase (SOD) and functions of the central nervous system (CNS). Thus, deficiency in these elements was linked with the occurrence or severity of

congenital structural malformation in diverse human populations (Keen et al. 2003). Macrominerals such as cadmium and lead are believed to be teratogenic to normal biological system and exposure to those harmful elements during the embryonic developmental period will lead to an increased potential risk of congenital malformations (Ferm 1976, 1969).

CONCLUDING REMARKS

In our study, the pregnant women from Shanxi region demonstrated a multifaceted malnutrition status characterized by depleted serum concentrations of folate and vitamin B_{12}, lower concentrations of Se, Zn, Cu, and higher concentrations of Sr, Cd, and Pb, significantly down-regulated carbohydrate and lipid metabolism in combination with significantly increased amino acid and urea metabolism. The multiple metabolic variants as potential risk factors of congenital malformations suggest that malnutrition status in Shanxi area is multifaceted. Given the complexity of events that are required for impaired embryonic and fetal growth and development, it is very likely that congenital malformations result from disruptions in multiple developmental metabolic pathways. There is growing consensus that many metabolic dysregulations can be characterized by aberrant epigenetic programming that is associated with malnutrition-induced metabolic disturbances during fetal and postnatal development (Gallou-Kabani and Junien 2005; Zeisel and da Costa 2009). DNA methylation patterns can be altered by many environmental factors including long-term malnutrition in prenatal or postnatal stages, and such epigenetic effects can be passed on to successive generations (Sinclair et al. 2007; Hanson and Gluckman 2008; Godfrey et al. 2011). We speculate that a significant disparity in the overall nutrition status in Shanxi group has modified one's epigenetic marks, which may be an important mechanism for the high prevalence of phenotypic development of congenital anomalies in this region. If this hypothesis is true, our population-based prevention and improvement of prenatal congenital malformations should take into consideration two key components—a comprehensive dietary intervention to normalize multi-pathway metabolic dysregulations, and a long-term strategy to facilitate a gradual epigenetic normalization for fetal development, perhaps, through several generations.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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