Five serum fatty acids are associated with subclinical hypothyroidism in a Chinese pregnant population

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Subclinical hypothyroidism (SCH) is a common endocrine disorder affecting women of reproductive age. Although SCH and abnormal fatty acid composition are often associated with adverse pregnancy outcomes and metabolic syndrome later in maternal and fetal life, the longitudinal relationship between SCH and serum fatty acids during pregnancy has rarely been studied. Therefore, the aim of this study was to investigate the association between SCH and maternal serum fatty acids throughout gestation. A total of 240 women enrolled in the Complex Lipids in Mothers and Babies (CLIMB) study in Chongqing, China were included in our study. Clinical information and maternal serum samples were collected at three time points during pregnancy: 11–14th, 22–28th, and 32–34th weeks of gestation. Twenty serum fatty acids were quantified using gas chromatography-mass spectrometry (GC-MS) analysis. A majority of the 20 serum fatty acids increased as gestation progressed in women with a normal pregnancy and women experiencing SCH. Levels of arachidic acid, docosahexaenoic acid, and eicosenoic acid were significantly higher in the serum of women with SCH when compared to women with a normal pregnancy, in the second trimester. On the other hand, the levels of eicosadienoic acid and octadecanoic acid were significantly higher in SCH in the third trimester. Our findings demonstrate that serum fatty acid composition during the second and third trimesters was significantly associated with SCH in pregnant Chinese women.

Subclinical hypothyroidism (SCH) is a common endocrine disorder that can manifest in pregnant women. This complication of pregnancy is diagnosed when a mildly elevated serum thyroid-stimulating hormone (TSH) level above the upper limit of the trimester-specific reference is detected in combination with a normal free thyroxine (FT4) concentration during pregnancy1,2. SCH occurs in approximately 2–5% of all pregnant women3–5. In China, the incidence of SCH in pregnancy has reached epidemic proportions, affecting between 2.8% and 7.2% of all pregnancies6–8. SCH during pregnancy can lead to various maternal and fetal complications such as pregnancy loss, preterm birth, placental abruption, severe preeclampsia, gestational diabetes, and impaired offspring neurodevelopment9–13.

Several clinical studies have indicated that patients with SCH have altered serum lipid profiles and a higher prevalence of metabolic syndrome14–16. Elevated plasma free fatty acid (FFA) levels have been reported in patients with SCH regardless of age, sex, or menopausal status14–17. Fluctuations in fatty acid levels also have been associated with metabolic syndromes such as dyslipidemia, type-2 diabetes, and obesity18. Fatty acids play a crucial role in the support and regulation of a healthy pregnancy and normal infant development19. However, there is a lack of evidence directly relating the changes in serum fatty acids with SCH in pregnancy.

Considering the importance of fatty acids for both maternal and offspring health, as well as an increasing prevalence of SCH during pregnancy, our study aimed to investigate the changes in serum fatty acids in women with SCH, throughout pregnancy. We analyzed 20 fatty acids in maternal serum across the three trimesters of

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pregnancy in order to unravel their possible association with SCH for the identification of potential biomarkers for diagnosis or therapeutic intervention.

Results
Clinical characteristics. All clinical characteristics for the study population are listed in Table 1. Only the TSH levels were significantly higher in SCH women when compared to normal women (3.30 uIU/ml vs. 1.19 uIU/ml; \( p < 0.001 \)). Other maternal characteristics including age, education, gravidity, parity, BMI, blood pressure, gestational age at sampling and FT4 exhibited no significant differences between case and control groups.

Differences in the levels of serum fatty acids across trimesters. A total of 20 serum fatty acids were absolutely quantified in the maternal serum of the participants in their first, second, and third trimesters, as shown in Tables 2–4 and Fig. 1. Fatty acids generally increased in both SCH women and normal women as gestation progressed, particularly from the first to second trimester. However, this trend did not occur between the second and third trimesters. In contrast, eicosapentaenoic acid (EPA) was reduced in both SCH and normal groups from the second to the third trimester, while docosapentenoic acid (DPA) and \( \gamma \)-linolenic acid were only reduced in the normal women from the second to the third trimester.

Differences in the levels of serum fatty acids between women diagnosed with SCH and women with a normal pregnancy. Although the majority of serum fatty acids were detected at higher concentrations in SCH women compared to the normal women, none of the fatty acids in the first trimester significantly discriminated women diagnosed with SCH from normal groups (Table 2). Interestingly, we observed a significantly higher level of three fatty acids (arachidic acid, docosahexaenoic acid (DHA), and eicosanoic acid) in women with SCH in the second trimester, while docosapentenoic acid (DPA) and \( \gamma \)-linolenic acid were only reduced in the normal women from the second to the third trimester.

Discussion
To our knowledge, this is the first study to investigate the association between serum fatty acids and SCH among Chinese women across the three trimesters of pregnancy. In this study we observed that maternal serum fatty acid levels increased in both SCH and control groups as pregnancy progressed. In addition, we found a significantly higher concentration of three serum fatty acids (arachidic acid, DHA and eicosanoic acid) in women with SCH in
Table 3. The concentrations, AUC, OR and statistical tests of maternal serum fatty acids between SCH and normal pregnancies between the second trimester and higher concentration of another two serum fatty acids (eicosadienoic acid and octadecanoic acid) in the third trimester, when compared to normal pregnant women.

The observations of increasing levels of serum fatty acids across the first two trimesters and to a lesser extent in the third trimester in both SCH and normal pregnant women, have been found previously in studies based on normal pregnancy. Al et al. analyzed plasma essential fatty acids (EFA) at eleven different time points.

| Fatty acids          | Fatty acids concentrations (mg/L) | AUC   | OR (95%CI) | P value |
|----------------------|-----------------------------------|-------|------------|---------|
|                      | Normal (n = 222)                  | SCH (n = 18) |          |         |
| Arachidonic acid     | 6.14 ± 1.73                       | 6.35 ± 1.93 | 0.48     | 1.07 (0.80,1.37) | 0.63 |
| Arachidonic acid     | 176.31 ± 52.16                    | 175.96 ± 66.18 | 0.57     | 1.00 (0.99,1.01) | 0.98 |
| Docosahexaenoic acid | 70.55 ± 22.35                     | 80.98 ± 38.71 | 0.45     | 1.01 (1.00,1.03) | 0.08 |
| Docosanoic acid      | 14.45 ± 4.41                      | 15.12 ± 5.53 | 0.49     | 1.03 (0.93,1.13) | 0.54 |
| Docosapentenoic acid | 11.25 ± 4.43                      | 12.45 ± 6.81 | 0.48     | 1.05 (0.95,1.14) | 0.30 |
| Docosatetraenoic acid| 6.55 ± 2.31                       | 6.52 ± 3.42  | 0.57     | 0.99 (0.79,1.19) | 0.96 |
| Eicosadienoic acid   | 10.08 ± 3.84                      | 9.87 ± 4.58  | 0.54     | 0.99 (0.85,1.11) | 0.82 |
| Eicosapentenoic acid | 10.26 ± 8.51                      | 12.42 ± 9.61 | 0.56     | 0.97 (1.01,1.06) | 0.32 |
| Eicosatrienoic acid  | 74.49 ± 33.57                     | 78.80 ± 69.3 | 0.59     | 1.00 (0.99,1.01) | 0.64 |
| Eicosanoic acid      | 5.50 ± 2.41                       | 5.00 ± 2.00  | 0.56     | 0.90 (0.86,1.11) | 0.39 |
| Hexadecanoic acid    | 469.15 ± 113.21                   | 493.68 ± 167.37 | 0.47     | 1.00 (1.00,1.01) | 0.39 |
| Hexadecanoic acid    | 24.7 ± 12.69                      | 31.63 ± 35.53 | 0.51     | 1.02 (0.99,1.04) | 0.10 |
| Lignoceric acid      | 10.68 ± 3.64                      | 10.78 ± 5.06 | 0.54     | 1.01 (0.88,1.13) | 0.92 |
| Linoleic acid        | 752.88 ± 177.04                   | 756.70 ± 187.03 | 0.53     | 1.00 (1.00,1.00) | 0.93 |
| Octadecanoic acid    | 150.47 ± 33.29                    | 161.49 ± 42.01 | 0.57     | 1.01 (1.00,1.02) | 0.19 |
| Octadecanoic acid    | 375.63 ± 92.41                    | 382.86 ± 134.38 | 0.53     | 1.00 (1.00,1.01) | 0.76 |
| Tetraconoic acid     | 36.07 ± 13.14                     | 38.28 ± 17.58 | 0.50     | 1.01 (0.98,1.04) | 0.50 |
| Tetraenoic acid      | 12.96 ± 8.08                      | 14.59 ± 14.60 | 0.53     | 1.02 (0.96,1.06) | 0.45 |
| γ-Linolenic acid     | 29.09 ± 14.25                     | 26.44 ± 13.41 | 0.58     | 0.98 (0.94,1.02) | 0.44 |
| γ-Linolenic acid     | 8.69 ± 5.96                       | 9.39 ± 11.18  | 0.58     | 1.02 (0.94,1.08) | 0.66 |

Table 2. The concentrations, AUC, OR and statistical tests of maternal serum fatty acids between SCH and normal pregnancies in the first trimester. Data are presented as mean ± SD, *p < 0.05. Abbreviation: SCH, subclinical hypothyroidism; AUC, area under the curve; OR, odds ratios; CI, confidence intervals.
during pregnancy (at 10, 14, 18, 22, 26, 30, 32, 34, 36, 38 and 40 weeks) in 110 healthy pregnant women in the Netherlands; Otto et al.\textsuperscript{21} evaluated plasma EFA at three different periods (before 18 weeks and at 22 and 32 weeks of gestation) in 239 healthy pregnant women from the Netherlands, Hungary, Finland, England, and Ecuador; Stewart et al.\textsuperscript{22} assessed the fatty acid composition of the erythrocyte membranes in each trimester of pregnancy (mean gestational weeks of 12.5, 26.1 and 35.5) in 47 pregnant Australian women without any metabolic complications; and Pinto et al.\textsuperscript{23} examined serum fatty acid concentrations in the 5–13, 20–26 and 30–36 weeks of gestation in 146 healthy pregnancies in Brazil. The possible underlying reason for all of the described studies to have observed a similar increasing trend of fatty acid levels from the first to the second trimester, may be due to the accumulation of maternal fat reserves that occurs to meet the anabolic storage phase in the early and middle periods of gestation. This phenomenon is mediated by the progressive increase in insulin level, promoting lipogenesis and suppression of lipolysis via progesterone and cortisol\textsuperscript{24,25}. Meanwhile, the observed trend of a lesser increase or sustained levels of fatty acids in the third trimester may occur as a result of upregulated lipolysis in maternal fat.

Figure 1. The concentrations of serum fatty acids in the first, second, and third trimesters collected from normal pregnancies and pregnancies with SCH. Red triangles represent serum fatty acid levels collected from women with SCH. Blue circles represent serum fatty acid levels from normal pregnancies. Red asterisks (*) indicate fatty acids with significantly different levels in SCH and normal pregnancies (p-values less than 0.05).
Methods
Study participants. All participants were selected from the Complex Lipids in Mothers and Babies (CLIMB) study. The CLIMB study was conducted at the First Affiliated Hospital of Chongqing Medical University and Chongqing Health Centre for Women and Children in China from September 2015 to June 2017. The details of the CLIMB study have been published previously. A total of 1,500 women were recruited into the CLIMB study.

Table 4. The concentrations, AUC, OR and statistical tests of maternal serum fatty acids between SCH and normal pregnancies in the third trimester. Data are presented as mean ± SD. *p < 0.05. Abbreviation: SCH, subclinical hypothyroidism; AUC, area under the curve; OR, odds ratios; CI, confidence intervals.
For fatty acids analysis, 750 women were selected at random using randomly generated numbers. We excluded women lost to follow up at birth (n = 24) and those who had other adverse pregnancy outcomes (n = 486) such as gestational diabetes mellitus (GDM), and pregnancy-induced hypertension (PIH). This resulted in 240 women who were eligible for inclusion in the study; 1) 222 normal women without any pregnancy complications and 2) 18 women who were diagnosed with SCH based on the criteria used in China (Supplementary Table 1) which included having TSH > the upper limit of reference during pregnancy (97.5th percentiles) and a normal FT4.

A flowchart of the study participants included in this study is shown in Fig. 3. All procedures performed in this study were in accordance with the principles in the Declaration of Helsinki 1964 and the International Conference on Harmonisation Good Clinical Practice E6 (ICH-GCP). The study was approved by the Ethics Committee of Chongqing Medical University (2014034). Written informed consent was obtained from all participants included in the study at enrollment.

Clinical information and sample collection. Clinical information, demographic factors (maternal age, total years of schooling, last menstrual period (LMP), gravidity, parity), gynecologic and obstetric history (gravidity, delivery, abortion, infertility) were collected at enrollment. Maternal anthropometry (body mass index (BMI) and blood pressure (BP)) and maternal blood samples were collected at three visits during pregnancy (11–14, 22–28, and 32–34 gestational weeks) by trained nurses. Maternal blood samples were collected from participants in the morning after an overnight fast. Fasted blood samples were collected from the antecubital vein into vacutainer tubes containing separator gel and separated by centrifugation twice (3000 rpm at 4 °C for 10 min, then 4000 rpm at 4 °C for another 10 min). The supernatant was transferred into cryotubes (Micronic, Lelystad, The Netherlands) then stored at −80 °C until further processing. Pregnancy outcomes were diagnosed and managed by experienced obstetricians. Throughout the pregnancy, maternal gestational week was determined by LMP and mid-trimester ultrasound information (if the two estimates differed by more than seven days, gestational age was based on the obstetric ultrasound data).
Reagents and fatty acid standards. Twenty one fatty acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Supplementary Table 2). Methanol (chromatography grade) and n-hexane (chromatography grade) were purchased from Merck-Chemicals, KGaA (Germany). Hydrochloric acid and Milli-Q pure water were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Stock solutions of the 20 fatty acids and the internal standard (heptadecanoic acid) were prepared at 250 mg/mL in n-hexane. Working solutions were made up with methanol at concentrations of 0.10, 0.50, 2.5, 25, 100, 250 mg/L. All standard solutions were stored at −20 °C until required. The internal standard working solution was a 20 mg/L heptadecanoic acid n-hexane solution.

Sample preparation and gas chromatography-mass spectrometry (GC-MS) analysis. A 250 µL aliquot of thawed serum from each participant was spiked with 250 µL of the internal standard (IS) working solution, followed by the addition of 1 mL hydrochloric acid/methanol and a mix using a vortex mixer. The sample was then incubated at 90 °C for 3 h using an electric blast drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd.). After cooling, 2 mL of n-hexane was added to extract the fatty acid methyl ester products and the sample was vortexed. The supernatant was isolated after centrifugation and then evaporated to dryness under nitrogen. Following the addition of 400 µL n-hexane to dissolve the fatty acid methyl esterification product, the suspension was mixed and then transferred to a sample vial (Agilent Technologies, USA) prior to mass spectrometry analysis. Gas Chromatography-Mass Spectrometry (GC-MS) was performed using an Agilent 7890B gas chromatograph coupled to a 5977 A mass spectrometer (Agilent Technologies, USA). The fatty acid separation was performed on the DB-23 capillary column (20 m × 0.18 mm × 0.20 µm, Agilent Technologies, USA). Helium was used as the carrier gas with a constant flow rate of 0.78 ml/min. The 1 µL of derivatized sample was injected into the inlet set at 230 °C, using the splitless inlet mode. Free fatty acid methyl esters were separated using the following oven temperature program: (1) 50 °C hold for 1 min; (2) increased to 175 °C at 25 °C/min; (3) reached 223 °C at 4 °C/min; and (4) hold at 223 °C for 8 min. The ion-trap mass spectrometer was operated in full scan (Scan) and selected ion scan (SIM) monitoring mode (mass range: 60.00–450.00 m/z). The transfer line was maintained at 230 °C, the source temperature was set at 220 °C, and solvent delay time was set at 10 min.

Fatty acid quantification and statistical analysis. The chromatographic height of each of the fatty acids was extracted using Agilent ChemStation (version 2.6). Levels of individual serum fatty acids were first normalized by the internal standard and then quantified to absolute concentration using calibration curves derived from the corresponding chemical standard. A student’s t-test and non-parametric Mann-Whitney U test were executed in R to compare clinical characteristics between women with a normal pregnancy and women diagnosed with SCH. Prior to metabolomic statistical analysis, all fatty acid concentrations were corrected to a Gaussian distribution through logarithmic transformation and Pareto scaling. The area under the receiver operating characteristic (ROC) curve was analysed using the pROC R-package37. Odds ratios (OR) were calculated to assess associations between the levels of fatty acids and the occurrence of SCH. Tukey’s HSD test was implicated to account for multiple comparisons. A p-value <0.05 was considered statistically significant. Data were described as mean ± SD or median (IQR) for continuous variables. Boxplots were illustrated using ggplot2 R-based packages38.

Figure 3. Flowchart of study participants.
Data availability

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

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Author contributions
Y.Y.X., T.L.H., H.Z. and P.B. conceived and designed research; T.Z., Y.Y.X. and H.Z. recruited the patients and collected the samples. Y.Y.X., T.L.H. and H.Z. performed research and analyzed data. T.Z. wrote the manuscript text and prepared the figures. Y.Y.X., T.H., H.Z and P.B. revised the manuscript. T.L.H. and H.Z. provided funding resources. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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