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Changes of serum homocysteine levels during pregnancy and the establishment of reference intervals in pregnant Chinese women

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ABSTRACT

Backgroud: Reference intervals (RIs) of clinical laboratory indexes are important basis for interpretation of corresponding test results. While elevated homocysteine (HCY) level is a risk factor of some severe gestational diseases, HCY RIs for pregnant women have not been reported so far. The current use of HCY RIs established for general population in pregnant women may challenge clinicians' judgment. This study aims to investigate the changes of serum HCY levels during pregnancy and establish the RIs of serum HCY in healthy pregnant Chinese women to provide valuable data to clinicians and enable the provision of more appropriate therapy.

Methods: 354 healthy pregnant Chinese women were randomly selected and divided into three groups according to gestational age: 114 in first trimester (1–13 week), 120 in second trimester (14–27 week) and 120 in third trimester (≥28 week). 120 healthy non-pregnant Chinese women were randomly selected as the non-pregnant control group. Serum HCY levels were determined on automatic biochemical analyzer with enzymatic cycling method. The RIs of serum HCY for healthy pregnant women were established using a nonparametric method.

Results: the RIs of serum HCY for healthy pregnant women is 5.79–11.86 μmol/L in first and second trimester (combined) and 6.13–16.75 μmol/L in third trimester. Besides, the RIs of serum HCY for healthy non-pregnant women is 8.25–22.92 μmol/L.

Conclusions: Rigorously according to CLSI C28-A3 guidelines, the authoritative document of RIs establishment, the RIs of serum HCY for healthy pregnant Chinese women were established, which will provide a valuable reference for clinical work and laboratory researches.

1. Introduction

Homocysteine (HCY) is a non-protein amino acid which generated from methionine that lost its terminal methyl group [1,2]. HCY metabolism is at the crossroad of two pathways: the remethylation pathway generating methionine which requires folates and vitamin B12 and the pathway of transsulfuration to cysteine that requires vitamin B6 [3,4].

Maintaining the balance of HCY metabolism and keeping the volume of blood HCY at an appropriate level are of great significance. Researches around the world have already demonstrated that elevated serum HCY level is not only a risk factor for both vasculopathy and cardiovascular diseases [2,5,6] but also a risk factor of some severe gestational diseases [6], which threaten the lives of both pregnant women and their babies. It has been reported that preeclampsia patients have higher levels of serum HCY than healthy pregnant women [7], which can be detected at early trimester in pregnant women. Hyperhomocysteinemia (HHCY) is regarded as an independent risk factor for preeclampsia and HCY level is capable to reflect how severe preeclampsia is [2,8,9]. In addition, HHCY, due to the deficiency of folate and vitamin B12 in pregnant women, probably inhibits the closure of baby's neural tube, which is regarded as the major risk factor for Neural tube defects [6]. Furthermore, the influence of elevated HCY in pregnant women on normal labor and baby's birth weight have also been investigated. High levels of HCY in follicular fluid will cause impaired placental function, leading to early pregnancy loss, placental abruption (PA), and intrauterine growth restriction [10]. Apart from these, elevated HCY levels are as well as risk factors for Down syndrome [11,12]. It is best illustrated by the fact that 56% of Down syndrome mothers carried four or more of folate-HCY pathway gene variants, while the percentage in control mothers is 11%. Investigators have also discovered that HHCY has been formed in many polycystic ovary syndrome patients, which may be associated with the injury of endothelial cells [13,14].

Reference intervals (RIs) of clinical laboratory indexes are

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important basis for interpretation of corresponding test results. They help defining whether human bodies are in normal condition or not and thus are essential for clinical decision-making. Pregnant women are always in a very special condition in which biochemistry indexes present physiological changes and RIs for these biochemistry indexes may be different from general population. However, most current HCY RIs used in domestic and foreign countries are established for general population, HCY RIs for pregnant women have not been reported so far. In consequence, it is not clear what extent of the changes of serum HCY during pregnancy can be considered abnormal and the current use of HCY RIs established for general population in pregnant women may challenge clinicians' judgment

This study aims to investigate the changes of serum HCY levels during pregnancy and establish RIs of serum HCY in healthy pregnant Chinese women to provide valuable data to clinicians and enable the provision of more appropriate therapy.

2. Materials and methods

2.1. Subjects

The information of pregnant women who were admitted to the Xiangya Hospital of Central South University from September 2017 to March 2018 for prenatal check-up or delivery was collected. A total of 354 healthy pregnant Chinese women with a mean age of 29.5 ± 8.5 years were randomly selected. They were then divided into three groups according to gestational age: 114 in first trimester (1–13 week), 120 in second trimester (14–27 week), and 120 in third trimester (≥28 week). Besides, 120 healthy non-pregnant Chinese women with a mean age of 28.7 ± 9.2 years were randomly selected as the non-pregnant control group. Table 1 shows the baseline characteristics for reference population.

This study was approved by the ethics committee of the Second Xiangya Hospital of Central South University and all participants have signed the informed consent form. The selection of healthy pregnant and non-pregnant women was based on the exclusion criteria from CLSI C28-A3 guidelines:

1. excessive drinking (> 30 g per day) or smoking (> 20 cigarettes per day)
2. high blood pressure (either systolic pressure ≥ 140 mmHg or diastolic pressure ≥ 90 mmHg) for > 3 years
3. obesity (body mass index ≥ 28 kg/m2) or underweight (body mass index ≤ 18.5 kg/m2)
4. blood transfusion or donation within 6 months, or surgery during the previous 4 months of pregnancy
5. history of inherited diseases
6. medicine intake within 2 weeks or antibiotic abuse
7. a diagnosis of hepatic diseases, endocrine diseases, impaired renal function, cardiac diseases, cancer, or other chronic diseases
8. heavy exercise or laborious work
9. any structural abnormality of heart, liver, lungs, and kidneys found though ultrasound examination, or abnormal electrocardiogram
10. laboratory test results: RBC $\geq 5.0 \times 10^{12}$/L or $\leq 3.0 \times 10^{12}$/L, HGB ≤110 g/L, WBC $\geq 12.0 \times 10^9$/L or $\leq 4.0 \times 10^9$/L, PLT $\leq 100 \times 10^9$/L; triglyceride ≥ 2.26 mmol/L, total cholesterol ≥6.22 mmol/L, fasting blood glucose ≥7.0 mmol/L; any positive results of hepatitis B surface antigen, anti-hepatitis C virus, and anti-HIV; or abnormal urinalysis

    Further exclusion criteria include factors affecting HCY levels: Serum folate ≤ 3.1 ng/mL or ≥20.5 ng/mL and vitamin B12 ≤ 187 pg/mL or ≥883 pg/mL

Table 1
baseline characteristics for reference population.

n	Total	Non-pregnant women	Pregnant women
	474	120	354
Age (years)	29.2 ± 8.7	28.7 ± 9.2	29.4 ± 8.5
BMI (kg/m^2)	24.6 ± 2.1	24.5 ± 2.7	24.6 ± 1.9
SBP (mmHg)	132.5 ± 16.4	126.2 ± 13.8	134.6 ± 16.6
DBP (mmHg)	76.3 ± 11.2	73.2 ± 9.6	77.4 ± 11.5
Glu (mmol/L)	5.25 ± 0.62	5.21 ± 0.58	5.26 ± 0.63
TC (mmol/L)	4.97 ± 0.39	4.82 ± 0.36	5.02 ± 0.39
TG (mmol/L)	1.19 ± 0.45	1.21 ± 0.58	1.19 ± 0.39
HGB (g/L)	115.4 ± 9.7	118.7 ± 8.7	114.3 ± 9.8
Serum folate (ng/mL)	9.5(3.2, 20.0)	9.5(3.6, 20)	9.3(3.2, 18.0)
Vitamin B12 (pg/mL)	312(187,799)	415(243, 755)	277(187,799)

Values are mean ± SD or median (range) unless otherwise indicated. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Glu, glucose; TC, total cholesterol; TG, triglycerides; HGB, hemoglobin.

2.2. Laboratory methods

All of the candidate participants were asked to maintain their normal lifestyle and avoid strenuous physical exercise within 3 days of physical examination and laboratory testing. They were also asked to refrain from alcoholic beverages for at least 1 day prior to the examination and testing. The candidate participants fasted overnight for at least 8 h and sat for at least 30 min before specimen collection. About 5 mL of blood was collected in a separation gel vacutainer (BD Biosciences, New Jersey, USA) between 8 am to 10 am. Blood samples were kept at room temperature for 30 min followed by centrifugation at 2500 to 3000 rpm. Serum HCY were analyzed on HITACHI 7600-DDP/7600-020 automatic biochemical analyzer (Hitachi, Japan) with HCY reagent (Medical System, China) by enzyme cycling method within 2 h.

All of the tests were performed according to standard operating procedures recommended by NCCLS. For quality control, two control concentrations of HCY were tested. The precision and accuracy were demonstrated according to the document EP 15A8 recommended by CLSI. The precision was expressed as the total coefficient of variation (CV), and the accuracy was expressed as recovery. The estimated total CV (< 5%) and recovery (range 95%–105%) met the requirement of the manufacturer. For participants in the comparison between laboratories organized by the NCCLS, the bias of HCY was < 5%, and the result was satisfactory.

2.3. Statistical analyses

All data were analyzed using SPSS 22.0 Statistical Software (SPSS Inc., Chicago, USA). The distribution of the data was detected using Kolmogorov-Smirnov test. Outliers were excluded using Dixon test. For non-normal distribution, Mann-Whitney *U* test were used to compare variables between two groups and Kruskal-Wallis H test was applied to compare variables among multiple groups. Differences at $P < .05$ were considered statistically significant. According to the recommendations of CLSI C28-A3 document, a non-parametric test was used to calculate serum HCY RIs. Reference range were expressed between the 2.5th and 97.5th percentiles and 90% confidence intervals (CIs) of lower and upper reference limits were calculated.

3. Results

3.1. HCY levels in healthy non-pregnant women and healthy pregnant women

Komlogorov-Smimov test demonstrated serum HCY levels were not in normal distribution in both healthy non-pregnant women and healthy pregnant women group ($P < .05$). Outliers were removed by Dixon's test. *U* test showed that serum HCY levels were significantly

Table 2
Serum HCY levels in healthy non-pregnant women and healthy pregnant women (μmol/L).

Group	n	Median	2.5th percentile	97.5th percentile
Non-pregnant	120	11.15	8.25	22.92
Pregnant[a]	354	8.66	5.85	14.53

[a] Comparison of serum HCY: has significant difference from non-pregnant group ($p < .05$).

different between healthy non-pregnant women and healthy pregnant women group ($P < .05$). serum HCY RIs for healthy non-pregnant and pregnant women should be established separately (Table 2).

3.2. HCY levels in healthy pregnant women in different trimesters

According to CLSI C28-A3 guidelines, healthy pregnant women were divided into three groups according to gestational age: first trimester (1–13 week), second trimester (14–27 week), and third trimester (≥28 week) group to calculate RIs.

Komlogorov-Smimov test demonstrated that serum HCY levels were not in normal distribution in all first, second, and third trimester group ($P < .05$). Outliers were removed by Dixon's test. H test confirmed that serum HCY levels for healthy women in first, second, and third trimester group were not all significantly different ($p > .05$). *U* tests were thus performed for pairwise comparisons and serum HCY levels were significantly different between first and third trimester group as well as second and third trimester group ($P < .05$). Nevertheless, there was no significant difference between first and second trimester group ($P > .05$). Healthy pregnant women in first and second trimester could be combined into one group to calculate serum HCY RIs and serum HCY RIs for healthy pregnant women in third trimester should be established separately (Table 3).

3.3. HCY RIs and 90% CI for healthy non-pregnant women and healthy pregnant women

According to CLSI C28-A3 guidelines, the RIs of serum HCY and 90% CI for healthy non-pregnant women and healthy pregnant women were calculated (Table 4).

Serum HCY RI is 8.25–22.92 μmol/L for healthy non-pregnant women. For healthy pregnant women, serum HCY RIs are 5.79–11.86 μmol/L in first and second trimester (combined) and 6.13–16.75 μmol/L for in third trimester.

4. Discussion

HCY plays an important role in the health of both pregnant women and their babies. Clinical studies have suggested that elevated maternal HCY levels are able to result in severe gestational diseases. Firstly, HHCY may be a consequence of ageing, oxidative stress, hypertension, diabetes and dyslipidemia [6], which increases the risk of preeclampsia as well. There are two aspects of reasons. On one hand, free radicals originating from oxidation of HCY are toxic to vascular endothelium [5,14]. on the other hand, high level of HCY disturbs coagulation cascade and endothelium which is predominantly anti-thrombotic under normal circumstances becomes more thrombotic [10]. In addition, low follicular fluid HCY level is related to the birth of a healthy baby [6]. Low folate and vitamin B12 always results in a decrease in the conversion of HCY to methionine [12]. While results in an elevated level of circulating HCY, it also results in lower methionine and S-adenosyl methionine (SAM)—the methyl donors for methylation reactions. Many genes regulate actin dynamics, cell adhesion, electron transport and DNA damage repair. As methylation of these genes are usually stop signals for gene expression, hypomethylation due to the deficiency of folate and vitamin B12 will finally cause reprogramming and overexpression of these genes, which could advance to neural tube defects in fetuses. Moreover, it has also been postulated that high level of serum HCY may affect vascular endometrium and contribute to the development of placental microvascular diseases, which adversely affect embryo implantation and maternal-fetal circulation [7]. Early pregnancy loss, placental abruption (PA), and intrauterine growth restriction happen under the circumstance [13].

Concerning about the negative impacts of elevated maternal serum HCY, establishing a reliable RIs of serum HCY for pregnant women is crucial for clinicians to correctly evaluate the physiological status of both pregnant women and their babies and provide appropriate therapy to avoid poor prognosis. However, the RIs of serum HCY we use currently for pregnant women are the same RIs for general population which have not considered the unique physiological changes during pregnancy.

This study was performed rigorously according to CLSI C28-A3 guidelines, the authoritative document of RIs establishment, and we successfully established the RIs of serum HCY for healthy pregnant Chinese women. The RIs of serum HCY for healthy pregnant women is 5.79–11.86 μmol/L in first and second trimester (combined) and 6.13–16.75 μmol/L in third trimester. Besides, the RIs of serum HCY for healthy non-pregnant women is 8.25–22.92 μmol/L.

As we can see from the results, it is obvious that the level of serum HCY is lower in healthy pregnant women than in non-pregnant women. From first and second trimester to third trimester, serum HCY levels seem to bounce back but still remain at a low level (Fig. 1). Several authors have also proposed that HCY levels tends to decrease during pregnancy. Julie et al. reported that serum HCY is 6.84 μmol/L and 12.4 μmol/L (median) respectively during pregnancy and after delivery. In Francien et al.'s study, plasma HCY was 9.42 μmol/L, 7.28 μmol/L, 7.33 μmol/L, 7.11 μmol/L, 6.89 μmol/L, 7.17 μmol/L, and 7.60 μmol/L (geometrical mean) respectively in gestational week 9, 16, 20, 24, 28, 32, and 36. Michelle et al. demonstrated that plasma HCY was 8.17 μmol/L, 6.48 μmol/L, 5.22 μmol/L, and 5.16 μmol/L (geometrical mean) respectively before pregnancy and in gestational week 8, 20, and 32. Mark et al.'s study proved that plasma HCY was 7.9 μmol/L, 5.6 μmol/L, 4.3 μmol/L, and 5.6 μmol/L (geometrical mean) respectively in non-pregnant women and 8–16, 20–28, 36–42 gestational week. The variation tendency in these researches are approximately coordinate with our findings. Slight discrepancy on values probably due to different race, region, and sample type. Some of the studies analyzed variation tendency using geometrical mean instead of median can be a reason as well [10].

Maternal serum HCY is a major determinant of fetal serum HCY

Table 3
Serum HCY levels in healthy pregnant women in different trimesters (μmol/L).

group	n	Median	2.5th percentile	97.5th percentile
First trimester (1–13 week)	114	8.17	5.95	11.42
Second trimester (14–27 week)	120	8.34	5.32	12.27
Third trimester (≥28 week)[a,b]	120	9.53	6.12	16.75

[a] Comparison of serum HCY: has significant difference from first trimester group ($p < .05$).
[b] Comparison of serum HCY: has significant difference from second trimester group ($p < .05$).

Table 4
The RIs of serum HCY and 90% CI for healthy non-pregnant women and healthy pregnant women (μmol/L).

group	n	Median	2.5th percentile and 90%CI	97.5th percentile and 90%CI	Reference intervals
Non-pregnant	120	11.15	8.25 (8.24–9.06)	22.92 (20.73–22.95)	8.25–22.92
First and second trimester (14–27 week)	234	8.26	5.79 (5.31–6.25)	11.86 (11.43–11.91)	5.79–11.86
Third trimester (≥28 week)	120	9.53	6.12 (6.12–6.55)	16.75 (14.53–16.77)	6.13–16.75

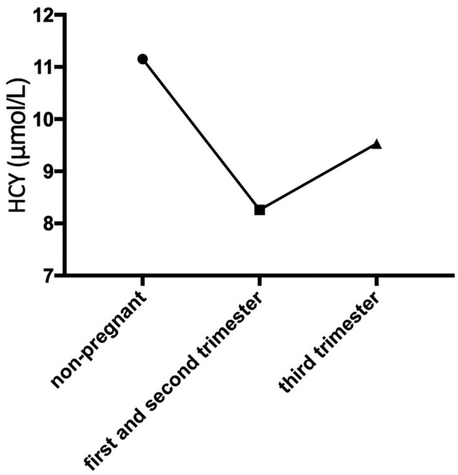

Fig. 1. Changes of serum HCY levels (median) in healthy non-pregnant woman and healthy pregnant woman in different trimesters.

levels. In a certain observation, changes of fetal serum HCY levels present a totally opposite pattern to maternal serum HCY levels. During pregnancy, fetal serum HCY levels increase slowly to a peak value and then decrease until delivery [15]. It is supposed that the decreasing trend of mother's serum HCY during pregnancy is due to the physiological demand of babies. Moreover, 70% of HCY in human serum exists through binding with albumin. As serum albumin levels fall progressively over the duration of pregnancy, decreased serum albumin may lead to low levels of HCY. Ubeda et al. also investigated the possible reason for low serum HCY levels in pregnancy and reported that it is mainly endocrine-based, especially due to higher estrogen status [16]. Besides, there are some assumptions concerning the increase of protein anabolism and folic acid supplementation during pregnancy. Further researches are needed to unveil the mechanism of decreased levels of HCY throughout pregnancy [10].

The limitation of our study was the lack of multicenter RIs of serum HCY levels for healthy pregnant women worldwide. The participants in our study were pregnant Chinese women. Our results are only applicable to pregnant women in Chinese population. Further studies should include multicenter RIs of serum HCY levels to acquire the RIs of serum HCY which can be universally applicable to population worldwide. Moreover, HCY in serum is stable after removal of the blood cells. No changes were observed among serum samples kept at room temperature, in the refrigerator, or at 20 °C. However, Serum, even if optimally prepared, yields slightly higher values than plasma [17]. Our results can be applied to serum kept on ice but not EDTA plasma. Further studies can focus on the establishment of RIs of plasma HCY levels.

In summary, rigorously according to CLSI C28-A3 guidelines, the authoritative document of RIs establishment, we established the RIs of serum HCY for healthy pregnant Chinese women. The results of this study will provide a valuable reference for clinical work and laboratory researches.

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