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Reference intervals of homocysteine in apparently healthy Chinese Han ethnic adults

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Abstract

Objectives: The aim of this study was to establish reference intervals (RIs) of homocysteine (Hcy) in healthy Chinese Han ethnic adults according to the Clinical and Laboratory Standard Institute (CLSI) CA28-A3.

Methods: After filtering, serum Hcy values in 20,810 healthy subjects from a middle area of China (Wuhu, Anhui province) were measured. The non-parametrical percentile method was used to establish RIs and the 90% confidence intervals of lower and upper limits were calculated. The relationship between Hcy and age was analyzed by using Spearman's approach. Besides, the risk of HHcy in males and females was examined by logistic regression analysis.

Results: The RIs of Hcy were 9.10–20.20 $\mu\text{mol/L}$ for males, 6.10–15.90 $\mu\text{mol/L}$ for females and 8.00–19.80 $\mu\text{mol/L}$ for total subjects from 20 to 90 years old. The serum Hcy level was significantly correlated with age both in males ($r=0.2159$, $p<0.0001$) and females ($r=0.2955$, $p<0.0001$). In males, the prevalence and the risk of HHcy were higher than females of all ages ($p<0.001$).

Conclusions: Through the analysis of a large dataset from healthy population, it showed that the variations in different age- and sex-related RIs of Hcy were significant. It suggested that establishing more specific age- and sex-related RIs for Hcy in China is necessary.

Keywords: adult; China; homocysteine; reference intervals; serum level.

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that cannot be obtained from the diet, the main function of Hcy in the human body is acting as an important intermediate metabolite in the metabolism of methionine and cysteine [1, 2]. The concentration of Hcy in plasma could be affected by many factors, such as vitamin B12, vitamin B6, folate, and the mutations involved in the Hcy metabolic pathway [3–5]. In addition, it has been shown that sex differences could also affect Hcy levels, with higher concentration in males than females [6].

Hyperhomocysteinemia (HHcy) refers to a condition caused by abnormally elevated levels of Hcy in plasma. Generally, enzyme defects related to Hcy metabolism are considered to be the most universal causes of HHcy, including Cystathionine beta-synthase (CBS) deficiency, Methylene tetrahydrofolate reductase (MTHFR) deficiency and Methionine synthase (MS) deficiency, etc [7–9]. HHcy is associated with a large category of diseases that are characterized by variable presentation affecting many organs, it is worthy to pay more attention to monitoring the changes of serum Hcy levels in patients, which is positively significant for the therapy and prognosis of diseases in clinical.

Elevated levels of Hcy in plasma are considered to be an independent risk factor for many kinds of diseases. Previous clinical research confirmed that there is a direct relationship between HHcy and the risk of cardiovascular diseases morbidity [10, 11]. Hcy has great potential as a biomarker for neurological disorders, diabetes, kidney dysfunction, metabolic syndrome and digestive system diseases [12–15]. A few studies pointed that high Hcy levels were also related to pregnancy complications such as preeclampsia [16]. Therefore, determination of Hcy and judgment of whether sample levels were within “normal range” are critical, the normal reference range is a basic parameter for clinical practice, it might be helpful to predict and prevent the early occurrence of diseases in clinical applications. The distribution of the healthy subjects will help to decide the cutoff and the clinical decision in diagnosis and screening.

However, there are few research data about Hcy RIs from Chinese population, most of the data has been from

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different ethnicities in western countries. Furthermore, the majority of laboratories in China directly use the RIs provided by the reagent manufacturer, which ignored the variabilities of Hcy levels across different laboratories and assays. Additionally, the determined levels of serum Hcy were influenced by ethnic differences, age, sex and other factors. Based on this, this study was aimed to establish more specific and reliable RIs for serum Hcy in healthy Han ethnic Chinese people according to the CLSI CA28-A3 guidelines [17].

Materials and methods

Subjects

Between January 2019 and May 2021, a total of 30,000 subjects were recruited from the center of health checkups in the First Affiliated Hospital of Wannan Medical College (Wuhu, Anhui province, China) in this study. The exclusion criteria were: (1) cardiovascular disease, neurological diseases, renal disease or other systemic diseases; (2) diabetes, hypertension, cancers, other acute or chronic diseases; (3) pregnancy; (4) recent medication history of folate, vitamin B12 or vitamin B6 that could alter the levels of Hcy. Each of these participants had filled in the detailed health questionnaire and underwent a full physical examination.

Finally, 20,810 apparently healthy Chinese Han adults (15,486 males and 5,324 females) were selected after screening in the study. The mean age of total subjects was 51.30 ± 12.91 years old (age range 20–90 years old). This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Wannan Medical College and all participants provided written informed consent.

Detection of serum Hcy

In this study, after fasting venous blood collection, the samples were centrifuged at 3,000 rpm for 10 min and then detected within an hour. The Hitachi 7,600 series automatic biochemical analyzer and the original reagent (Hitachi, Ltd, Tokyo, Japan) were used to measure Hcy levels by enzymatic cycling method based on continuous monitoring assay. The detection range of the reagent was 0.5–50 $\mu\text{mol/L}$. When the measured concentration of the samples exceeded the upper limit, dilution and remeasure were required. The total coefficient of variation (CV%) and the recovery were less than 5% and more than 95%, respectively. The entire process must follow the standard operating protocol (SOP) and regular internal quality control (IQC) procedures were operated every day in the laboratory. The normal Hcy level is ranging between 5 and 15 $\mu\text{mol/L}$, while HHcy was defined as plasma Hcy concentration above 15 $\mu\text{mol/L}$ [18].

Statistical analysis

Descriptive statistics are expressed as mean $\pm$ standard deviation (SD) for continuous variables, or as median if significant deviation from normal distribution. For all Hcy data in this study, the D'Agostino-

Pearson test for normal distribution and the non-parametrical percentile method (95% double-sided) were used to calculate RIs following the CLSI C28-A3 guidelines, and the outliers' removal procedure was abandoned due to a large apparently healthy population. Also for the 90% confidence intervals (CI) of the reference limits the CLSI guidelines are followed and conservative confidence intervals are calculated using integer ranks. The age-related RIs (a reference interval that varies with the subjects' age) and the 2.5th and 97.5th percentiles were calculated. Spearman's correlation was used to assess the degree of association between Hcy concentration and age. One-way analysis of variance (ANOVA) test and Mann-Whitney U test were used to determine trending age-groups (20–40, 41–60, 61–90) and different sex groups, respectively. *p*-Values less than 0.05 were considered statistically significant. Binary logistic regression was used to examine the odds ratios (OR) and 95% CI to predict HHcy risk in relation to age and gender. Statistical analysis was performed using MedCalc Version 18.2.1 (MedCalc Software bvba, Ostend, Belgium) and IBM SPSS 22.0 (SPSS Inc. Chicago, USA).

Results

The D'Agostino-Pearson test showed that all Hcy data in this study were not in normal distribution ($p < 0.0001$, Figure 1). Totally, the Hcy levels of 20,810 subjects were analyzed, including 15,486 males (74.4%) and 5,324 females (25.6%), the RIs for Hcy in different age- and sex-groups were calculated by non-parametrical percentile method. Figure 2 has shown the serum of Hcy for different age-groups in males and females, and Tables 1–3 have listed the RIs of serum Hcy in age- and sex-groups.

Spearman's correlation revealed that the values of Hcy were significant associated with age both in males ($r = 0.2159$, $p < 0.0001$), females ($r = 0.2955$, $p < 0.0001$) (Figure 3). In combination with visual inspection of the data, the proportion criteria (applicable to both Gaussian and non-Gaussian distributions) described by Lathi et al. was applied for partitioning into age and sex subgroups [19, 20]. Therefore, it was logical to divide the participants into different age groups: 20–40, 41–60 and 61–90 years old (Tables 1–3). Mann-Whitney U test showed the significant difference between males and females in different age-groups ($p < 0.001$), and the median values of Hcy consistently increased in age-groups (ANOVA test, $p < 0.001$) for trend both in males and females (Tables 1 and 2).

In order to indicate the change of Hcy levels with age, the percentiles lines were drawn to show continuous RIs of Hcy concentration with age-related (Figure 4). There was an approximate turning point at the age of 50 and a relative upward tendency of Hcy values over 50 years old, which means it was reasonable to establish RIs between the age range 20–50 and above 50 years of age. The RIs were: 8.70–19.80 $\mu\text{mol/L}$ in age-group 20–50 and 9.50–20.50 $\mu\text{mol/L}$

Figure 1: Histograms of homocysteine (Hcy) and age in subjects. The unit of Hcy is $\mu\text{mol/L}$.

for more than 50 years old for males; 4.90–15.30 $\mu\text{mol/L}$ in age-group 20–50 and 7.70–16.18 $\mu\text{mol/L}$ for over 50 years old for females.

The proportion of HHcy in males was significantly higher than in females in each age range ($p < 0.001$). The prevalence of HHcy was higher in males at all age ranges, with the lowest proportion (22.2%) from 20 to 40 years of age; and in females, the prevalence of HHcy remained at a low level in all age ranges, the highest value was 10.6%, at

61–90 years of age. By logistic regression analysis, males have a higher risk of HHcy than females of all ages (OR: 7.233, 95% CI: 6.400–8.174, $p < 0.001$). Table 4 showed the association between HHcy and ages in males and females. Among males, there was a high risk of HHcy for those aged between 41 and 60 years and those aged 61 years and older compared to those aged from 20 to 40 years old, with an odds ratio of 1.288 (1.167–1.422), 2.849 (2.549–3.183), respectively. For females, the OR of HHcy aged 61 years and

Figure 2: Box-plot of the serum level of homocysteine (Hcy) for different age-groups. The whiskers of the box plot were 1.5 times the interquartile range from the box.

Table 1: Reference intervals of Hcy for apparently healthy adult population.

Sex group	n	Median	Lower limit (90% CI)	Upper limit (90% CI)
Male	15,486	13.60	9.10 (9.00–9.20)	20.20 (20.10–20.40)
Female	5,324	11.10	6.10 (5.90–6.40)	15.90 (15.70–16.00)
Total	20,810	12.90	8.00 (7.90–8.10)	19.80 (19.70–19.90)

95% Reference interval, Double-sided. Non-parametric percentile method (CLSI CA28-A3). CI, confidence interval. The unit of Hcy is $\mu\text{mol/L}$.

older compared to those aged between 20 and 40 years was 3.199 (2.220–4.609), while there was no significant HHcy risk at age 41–60 years in females, compared to those aged between 20 and 40 years.

Table 2: Reference intervals of Hcy for the different age-group and sex.

Age-group	Male n	Median	Lower limit (90% CI)	Upper limit (90% CI)	Female n	Median	Lower limit (90% CI)	Upper limit (90% CI)	p-Values ^b
20–40 ^a	2,918	12.80	8.40 (8.10–8.60)	19.90 (19.50–20.20)	1,119	10.40	4.30 (3.70–4.60)	15.40 (15.10–15.80)	0.000
41–60 ^a	9,271	13.40	9.10 (9.00–9.30)	19.90 (19.80–20.10)	3,007	11.10	6.62 (6.50–6.90)	15.60 (15.30–15.80)	0.000
61–90 ^a	3,297	14.70	10.00 (9.90–10.20)	20.90 (20.70–21.10)	1,198	12.40	8.40 (8.00–8.60)	16.30 (16.10–16.50)	0.000
20–50	6,916	13.00	8.70 (8.60–8.80)	19.80 (19.60–20.00)	2,438	10.50	4.90 (4.60–5.20)	15.30 (15.10–15.40)	0.000
51–90	8,570	14.00	9.50 (9.40–9.70)	20.50 (20.30–20.60)	2,886	11.70	7.70 (7.30–7.90)	16.18 (16.00–16.20)	0.000
p-Values ^a for trend	0.000				0.000				

95% reference interval, double-sided. Non-parametric percentile method (CLSI CA28-A3). CI, confidence interval. ^aOne-way analysis of variance (ANOVA) test was used to determine Hcy for trending age-groups. ^bMann-Whitney U test was used to determine Hcy for different sex-groups. The unit of Hcy is $\mu\text{mol/L}$.

Table 3: Reference intervals of Hcy for different age-group in total subjects.

Age-group	n	Median	Lower limit (90% CI)	Upper limit (90% CI)
20–40 ^a	4,037	12.30	6.30 (5.90–6.70)	19.30 (19.00–19.60)
41–60 ^a	12,278	12.80	8.10 (8.00–8.20)	19.40 (19.30–19.60)
61–90 ^a	4,495	14.00	9.30 (9.10–9.50)	20.60 (20.40–20.80)
20–50	9,354	12.40	7.20 (6.90–7.40)	19.30 (19.00–19.40)
51–90	11,456	13.30	8.70 (8.60–8.80)	20.10 (20.00–20.20)
p-Values ^a for trend	0.000			

95% Reference interval, Double-sided. Non-parametric percentile method (CLSI CA28-A3). CI, confidence interval. ^aOne-way analysis of variance (ANOVA) test was used to determine Hcy for trending age-groups. The unit of Hcy is $\mu\text{mol/L}$.

Discussion

As an important human health parameter, serum Hcy has been widely used in clinical nowadays, and many studies have analyzed the level of Hcy in diseases and its correlation with other biochemical indicators [21–24]. In this study, according to the CLSI CA28-A3 documents, we established RIs of serum Hcy for different sex- and age-groups in a great amount of apparently healthy individuals in Wuhu area. To the best of our knowledge, this present study is the first to establish RIs for Hcy in such a large sample size of Chinese Han population.

Due to the result of D'Agostino-Pearson test being non-Gaussian distribution for Hcy, non-parametric statistical method was used to calculate RIs in this study. However, the RIs of serum Hcy were expressed as means ($\pm$ SD) since the data was normal distribution in other studies (7.34 ± 1.95 , 6.04 ± 2.02 $\mu\text{mol/L}$ in males and females from 10 to 19 years old for Arab adolescents, respectively) [25]. Our results show that the reference values of Hcy were higher in males (median 13.60 $\mu\text{mol/L}$,

Figure 3: Scatter diagram showing variation of the serum level of homocysteine (Hcy) with age. The unit of Hcy is $\mu\text{mol/L}$.

range 9.10–20.20 $\mu\text{mol/L}$) than females (median 11.10 $\mu\text{mol/L}$, range 6.10–15.90 $\mu\text{mol/L}$). In a recent study about age and sex differences for Hcy concentration in the general population of China, the results showed that Hcy levels were significantly higher in males than females in different age groups, this is generally consistent with our study and which were previously reported in Korean and Arab people [25–28]. Moreover, Hcy levels also showed racial and ethnic differences, the values of plasma Hcy for our subjects tended to be higher compared with the geometric mean values in the study for Norway and the U. S. NHANES report [29, 30]. Thus, the data of Hcy in the studies indicated that the RIs of Hcy for Caucasians or Blacks in western countries may not be applicable for Chinese population.

Pregnant women are always in a very special condition in which biochemistry indexes present physiological changes and RIs for these biochemistry indexes may be

Figure 4: Continuous reference intervals for homocysteine (Hcy). The blue line represents the 50th percentile and the lower and upper red lines represent the 2.5th and 97.5th percentiles. The unit of Hcy is $\mu\text{mol/L}$.

different from the general population. A few studies have shown that the level of Hcy was lower than non-pregnant women, and there was a decreasing trend of Hcy levels

Table 4: Logistic regression analyses of the risk of HHcy between ages in sex.

Age-group	Male			Female		
	HHcy	OR (95% CI)	p	HHcy	OR (95% CI)	p
20–40	22.2%	Reference		3.6%	Reference	
41–60	26.8%	1.288 (1.167–1.422)	0.000	4.3%	1.199 (0.835–1.722)	0.325
61–90	44.8%	2.705 (2.442–2.997)	0.000	10.6%	3.199 (2.220–4.609)	0.000

OR, odds ratio; CI, confidence interval. HHcy (hyperhomocytinemia) was defined as serum Hcy levels >15 $\mu\text{mol/L}$.

during pregnancy [31, 32]. Besides, recently published data shows that the Hcy level is significantly higher in pre-eclampsia patients than in normal pregnant women [33]. The above studies showed that establishing a suitable reference interval for pregnant women is warranted, so as to better monitor Hcy levels and provide valuable data to clinicians and enable the provision of more appropriate therapy.

Another finding in this study is that serum Hcy of males and females were weakly correlated with age and there is an increasing trend at age 20–40, 41–60, 61–90. The trend differs from the recent study in Chinese population (first decreased and then increased, being lowest at age 30–50 and significantly increased after 50 years of age) [28], but is consistent with the white and black populations in the United States. The RIs of Hcy were 8.40–19.90, 9.10–19.90, 10.00–20.90 $\mu\text{mol/L}$ in males and 4.30–15.40, 6.62–15.60, 8.40–16.30 $\mu\text{mol/L}$ in females at age 20–40, 41–60, 61–90, respectively in this study. For European populations, there is a previous study about Hcy levels in healthy Swiss senior citizens, the RIs were 10.50–14.80, 11.60–16.70 and 12.80–19.90 $\mu\text{mol/L}$ for age range 60–69, 70–79, ≥ 80 , respectively [34]. Moon et al. estimated the RIs of Hcy using two automated immunoassays (AxSym and ADVIA centaur) in a Korean population, they found that Hcy levels were closely related to the detection assays in addition to age and sex [27]. Another study on plasma Hcy levels in Asian populations showed that the RIs (means $\pm$ SD) were 7.34 ± 1.95 and 6.04 ± 2.02 $\mu\text{mol/L}$ in males and females from 10 to 19 years old, respectively [25]. By comparing with the previous studies on the RIs of Hcy, we found that the variation tendency on sex and age are almost coordinate with our findings, but the distinctions also exist, possibly because of the different employed assays, geographic location, lifestyles and ethnicity, etc.

According to the analysis of Hcy levels in males and females in this study, the results revealed definite differences between sexes. Compared with females, Hcy levels were higher in males at each age range, and the trend did not diminish with age. The prevalence of HHcy was higher than that of females in each age range and the risk of HHcy

was significantly different according to age groups in males and females, this is consistent with the results reported by Xu et al. [28], but different from the study in the Korean population, which indicated there was no significant prevalence of HHcy in ages possibly because the subjects were mainly middle-aged adults [26]. Apart from the above, the significant effect of age on Hcy concentration was also confirmed in this study. Over 60 years old, the Hcy levels are higher than in other age groups whether in males nor females. We speculate that the reasons for higher levels of Hcy in the elderly are as follows: vitamin digestion and absorption dysfunction, which affects Hcy metabolism; decrease in the activity of enzymes involved in Hcy metabolism and decreased liver and renal function resulting in increased serum Hcy concentration.

It has been reported that the metabolism of Hcy would be influenced by endogenous sex hormones, the levels of Hcy were higher in menopausal women and the difference with females diminishes [35]. And bad habits such as smoking and drinking are also responsible for elevated Hcy levels in males [36]. In addition to these, it is well known that vitamin B12 deficiency is a major contributor to elevated Hcy levels. And it was significantly correlated with gender, with lower mean serum vitamin B12 concentrations in males. The values of serum vitamin B12 in this study were not recorded, which limits the current study. The previous study has been reported that there were significant regional and seasonal variations in Hcy levels, and these variations could be explained by the differences in the vitamin status [37]. Nutritional deficiencies in vitamin B12, vitamin B6 and folate are the reason for enzymatic defects in the process of Hcy metabolism (remethylation and transsulfuration) and the cause of elevated plasma Hcy levels. Methylene tetrahydrofolate reductase (MTHFR) mutation is the main cause of hereditary abnormal methylation, which reduces serum folate concentration and increases plasma Hcy levels. And some studies have confirmed that 10 percent of people over the age of 60 will be affected by vitamin B12 and folic acid deficiencies, which leads to an increase in serum Hcy concentration [38, 39].

However, there are several analytical techniques of Hcy determination in the laboratory which could affect the RIs, such as fluorescence polarization immunoassay (FPIA), chromatographic methods, chemiluminescence immunoassay (CLIA) and enzyme immunoassay (EIA) [40, 41]. The sensitivity and specificity of detection methods should be considered especially in diagnostic tests.

The present study contains some limitations. First, we only used one detection assay and didn't analyze the determinants of serum Hcy level, such as vitamin B12, vitamin B6 and folate. Second, we couldn't guarantee the subjects are completely healthy and the undetected diseases may make impact on the results. Third, the data was from a single center and area, it's a bit lacking in representation. Therefore, follow-up multi-center and more extensive studies are needed to confirm our findings.

In conclusion, we have established the RIs of serum Hcy using a large sample of Chinese healthy people. In comparison of other studies, the causes of different RIs for Hcy could be considered as differences between ethnicity and detection systems. And the laboratories had better establish continuous RIs by age that meets the characteristics of the local population. We speculate that this study will be positive to promote the establishment of reference intervals for Hcy in China.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the Institutional Ethics Committee of the First Affiliated Hospital of Wannan Medical College.

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