

# SERUM HOMOCYSTEINE CONCENTRATIONS IN PORTUGUESE YOUNG ADULTS

## Reference Interval

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### SUMMARY

**Introduction and objectives:** Hyperhomocysteinemia has been shown to be an independent risk factor for cardiovascular disease. Assessing the reference intervals for homocysteine among young adults is a critical step in the process of preventing cardiovascular diseases later in life. This study aimed to identify reference intervals for total serum homocysteine concentration in young adults.

**Methods:** The sample was composed of 469 (74.4% female) young adults (mean age 20.4  $\pm$  2.1) who participated in an ongoing longitudinal study of apparently healthy undergraduate students registered in different academic years and different scientific areas in a Portuguese University, and coming from all regions of the country. Total homocysteine concentrations were determined and the data were analyzed employing the Hoffmann approach. This approach method has been widely used to evaluate reference intervals for various analytical parameters. Reference intervals (5<sup>th</sup> and 95<sup>th</sup> percentiles) for the total homocysteine concentration were first estimated by gender and since male and female reference intervals were similar, one reference interval for total homocysteine was estimated irrespective of gender.

**Results:** Reference intervals showed to be similar for both genders. The reference range, for homocysteine in young Portuguese adults, was 6.2-11.6  $\mu$ mol/l, regardless of gender. Above 11.6  $\mu$ mol/l surveillance should be considered in young adults.

**Conclusions:** This is the first known study providing reference intervals for total homocysteine in young adults, based on a sample of Portuguese university students. The identification of a reference interval for total serum homocysteine concentration at these ages may help to identify those with a higher cardiovascular disease risk. Values above 11.6  $\mu$ mol/L should lead physician concern and surveillance in adulthood.

### RESUMO

#### CONCENTRAÇÕES SÉRICAS DE HOMOCISTEÍNA EM ADULTOS JOVENS

##### Intervalo de Referência

**Introdução e objectivos:** A hiperhomocisteinemia tem demonstrado ser um factor de risco independente para doenças cardiovasculares. Avaliar os intervalos de referência para a homocisteína entre adultos jovens é fundamental no processo de prevenção das doenças cardiovasculares em fases tardias da vida. Este estudo teve como objectivo identificar os intervalos de referência para a concentração sérica total de homocisteína em adultos jovens.

**Métodos:** A amostra foi composta por 469 adultos jovens (74,4% mulheres), com uma idade média de 20,4 ( $\pm$  2,1), que participaram num estudo longitudinal de estudantes de graduação numa universidade Portuguesa, matriculados em diversos anos lectivos, em diferentes áreas científicas e provenientes de todas as regiões do país. As concentrações

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de homocisteína total foram determinadas e os intervalos de referência (percentis 5 e 95) para a concentração de homocisteína total foram estimados através da aplicação da abordagem de Hoffmann, um método largamente utilizado para estimar intervalos de referência de variados parâmetros analíticos. Numa primeira fase os intervalos de referência foram calculados separadamente para homens e mulheres. Sendo estes intervalos semelhantes, foi estimado um intervalo de referência para a homocisteína total independentemente do sexo.

Resultados: Os intervalos de referência foram semelhantes para ambos os sexos. O intervalo de referência, para a homocisteína em adultos jovens portugueses, independentemente do sexo, foi 6,2-11,6  $\mu\text{mol}$ , sendo que, acima de 11,6  $\mu\text{mol/l}$  é recomendada a vigilância médica em indivíduos adultos jovens.

Conclusões: Em Portugal, este é o primeiro estudo conhecido que fornece intervalos de referência para a homocisteína total em adultos jovens, com base numa amostra de estudantes universitários Portugueses aparentemente saudáveis. A identificação de um intervalo de referência para a concentração sérica total de homocisteína nessas idades pode ajudar a identificar aqueles com maior risco de desenvolver doença cardiovascular. Valores acima de 11,6  $\mu\text{mol} / \text{L}$  devem conduzir a preocupação por parte do médico e vigilância na idade adulta.

## INTRODUCTION

Homocysteine is a nonessential sulphur-containing amino acid formed from the demethylation of an essential amino acid, methionine<sup>1</sup>. It has been implicated as a potential risk factor for cardiovascular diseases<sup>2,3</sup>. Total homocysteine is defined as the sum of all homocysteine species in plasma or serum, including free and protein bound forms<sup>4</sup>.

Genetic abnormalities in enzymes<sup>5,6</sup> and a deficiency of vitamins B involved in homocysteine metabolism<sup>7</sup> lead to elevated total homocysteine. Vitamins B can be supplied by dietary intake. Insufficient intake or mal-absorption can result in chronic vitamins B deficiency.

Approximately 10% of the population's risk of coronary artery disease is attributable to total homocysteine<sup>8</sup>. The main cause of death in Portugal is diseases of the circulatory system<sup>9</sup>.

In the general population, hyperhomocysteinemia is an established independent risk factor for cardiovascular disease<sup>10</sup>, and may result from genetic alterations in enzymes involved in the metabolism of methionine or homocysteine<sup>11</sup> and/or dietary<sup>12</sup>. Since 1969, McCully suggested that moderate levels of hyperhomocysteinemia might be associated with atherosclerosis<sup>13,14</sup>.

Folic acid, pyridoxine (vitamin B<sub>6</sub>), and cobalamin (vitamin B<sub>12</sub>) reduce homocysteine levels and may help to reverse endothelial injury associated with elevated total homocysteine<sup>12</sup>.

According to the Centre Disease Control of Prevention, and described in the Laboratory Procedure Manual

of Abbott AxSYM System<sup>15</sup> the normal concentration of total homocysteine varies between 4.6 and 8.1  $\mu\text{mol/l}$ , for subjects aged under 30 years without regard to gender, however moderate hyperhomocysteinemia is considered for values greater than 16  $\mu\text{mol/l}$ . These values are confirmed in studies reviewed by Kilmer McCully and also in his book *The Heart Revolution*<sup>13,14</sup> where hyperhomocysteinemia is classified as: moderate – between 15 and 30  $\mu\text{mol/l}$ ; intermediate – between 30 and 100  $\mu\text{mol/l}$ ; and severe – over 100  $\mu\text{mol/l}$ <sup>15</sup>.

Most laboratories use 15  $\mu\text{mol/L}$  as the cutoff point, between normal and abnormal values, without considering the age of the patient. However, a study conducted from 1991 to 1994 found that the reference range for serum total homocysteine concentration increased with age even among adults<sup>16</sup>.

The reference values usually considered in Portugal are based on the information leaflet on hyperhomocysteinemia used by the pathology laboratories namely the Hospital de São Sebastião, which differentiates between the sexes: >16.0  $\mu\text{mol/l}$  for men, >20.4  $\mu\text{mol/l}$  for women.

The Hoffmann approach<sup>17</sup> is a simple statistical technique that can be applied in the determination of reference interval for any normal values in Medicine when measurements are available and mathematical assumptions are reasonable. Comparatively with the classical percentile approach, the Hoffmann approach is advantageous when it is not possible to guarantee that all the participants are healthy and that some of them were not taking any medication that could interfere with homocysteine concentrations, not having elevated levels of total cholesterol and

no tobacco consumption. Using this approach outliers and the possibly *sick* population are removed statistically, providing reference intervals comparable to those obtained with healthy individuals using the percentile approach<sup>17,18</sup>. According to international criteria the number of individuals per subgroup to establish reference values should be 120 or more<sup>19</sup>.

Data was obtained as part of a project currently in progress at a Portuguese University and developed to characterize the health of young university students and to characterize the distribution of risk factors for cardiovascular disease among university students<sup>20</sup>. Those students are young adults, individuals in the transition to adulthood. These data present an excellent opportunity to develop population reference intervals for serum total homocysteine concentration for young adults, in a South European country. In this region of Europe with a predominant Mediterranean diet, the dietary vitamin content differs from that in Northern Europe or North America<sup>21,22</sup>. Assessing the reference intervals for total homocysteine among young adults is a critical step in the process of preventing Cardiovascular Diseases later in life. The aim of this study was to determine gender-specific young adult reference intervals for total homocysteine.

## MATERIALS AND METHODS

### Subjects

The study population was composed of young adults. Four hundred and sixty nine participants (120 men and 349 women) aged 18-30 years (mean age  $20.4 \pm 2.1$ ) were recruited.

Data was obtained as part of an on going longitudinal study of undergraduate students in the Aveiro University (Central Region, Portugal) registered in the 2005 to 2008 academic years. A stratified random sample was drawn based on major areas of degree courses. This methodology is described in greater detail elsewhere<sup>20</sup>.

The information about demographic characteristics was retrieved from an online structured anonymous questionnaire.

### Blood analysis

Collection of two blood samples between 8 and 10 am after 12 hours fasting was performed by trained nursing students on a single occasion under the supervision of their teachers. The students were trained in order to reduce the inter and intra observer variability. Blood was placed in a centrifugation tube and spun at 2000 g for 10 minutes. The blood samples were transferred to the Pa-

thology Laboratory of S. Sebastião Hospital in Santa Maria da Feira in tanks containing ice packs so as to maintain a temperature of 3-4° C. One aliquot was used for homocysteine measurements on the same day of collection. Serum total homocysteine was evaluated with a commercial standardized kit (AxSYM Homocysteine) from Abbott Laboratories (Abbott Diagnostic Division, Wiesbaden, Germany).

### Demographic information

Participants were asked to complete the questionnaires on line at our Web site (<http://www2.cs.ua.pt/QEEU>). In order to assure confidentiality, an identification number was first provided to each participant. After registration in the Web site with this identification number, a password was provided to the student. The questionnaire data completed by each participant was automatically inserted into a data base.

### Vitamins B intake

Dietary information was assessed using a detailed and validated semiquantitative food frequency questionnaire<sup>23</sup>. Participants were asked to record the frequency of consumption of specified portions of each selected food item during the previous year.

An adequate intake of vitamins B6 and B12 and folic acid was considered when contained less than 400 µg/day of folic acid, and at least 1.3 mg/day of vitamin B6 and 2.4 µg/day of vitamin B12, according to the recommendations intakes of the World Health Organization<sup>24</sup> and the UN Food and Agriculture Organization<sup>25</sup> for a healthy population aged between 19-30 years.

### Homocysteine Assay

A method widely used for total homocysteine determination in Portuguese laboratories is the Abbott's AxSYM. This method uses reduction of protein-bound homocysteine by dithiothreitol, conversion of homocysteine to S-adenosylhomocysteine in the presence of adenosine catalyzed by hydrolase enzyme and detection of S-adenosylhomocysteine by fluorescence polarization immunoassay. The AxSYM assay does not require sample pre treatment, and serum or plasma may be used<sup>4,26</sup>.

### Ethical Procedure

Information about the aims of the study was provided to all participants. All participants agreed to act as subjects for the study after reading and signing an informed consent form. The informed consent was carried out in compliance with the Helsinki Declaration. Ethical approval

for the study was granted by the S. Sebastião Hospital ethics committee (CE-056/05). All individual blood analysis results were given to each participant in a closed envelope.

**Statistical analysis**

Statistical analysis was performed with SPSS (version 16.0) and Excel (version 9.0) and a two-sided significance level of 5% was used throughout.

For the characterisation of the study population, a descriptive analysis was done. Qualitative variables were described as percentages and quantitative variables as arithmetic means or geometric means when necessary.

The total homocysteine was converted to a natural logarithm to normalize its skewed distribution and allow the estimation of the reference intervals based on the Hoffmann approach. In this approach outliers and the *sick* population are removed statistically by plotting the analyses value *versus* % cumulative frequency<sup>18</sup>. As a first step, data was separated by gender in order to compare the mean and the variance of the logarithmic transformation of the total homocysteine values. Means were compared with the *t*-test, variances were compared with the Levene’s test. Due to a marginally significant difference of the variances, data was separated by gender in order to estimate the reference intervals for total homocysteine separately for male and female subjects. Since male and female reference intervals’ were similar, one reference interval for total homocysteine was estimated irrespective of gender.

The reference intervals for total homocysteine were estimated using the Hoffmann approach<sup>17,18</sup>. The cumulative distribution of the logarithmic transformation of the total homocysteine values were plotted, in order to verify the required normal (Gaussian) distribution. Then a straight line was adjusted to the central 50% (25-75<sup>th</sup> percentile) of the data using the method of least squares. With the extrapolation of this linear equation that relates the cumulative percentage and the logarithm of the total homocysteine, the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles were calculated. The exponential conversion of these vales will be the reference interval for the total homocysteine.

The total homocysteine was compared between subjects with recommended and non-recommended intakes of folate, vitamins B6 and B12 with the *t*-test.

**RESULTS**

A total of 469 students (120 men and 349 women) from the Aveiro University, with mean age 20.4 (± 2.1) years (18 to 30 years) comprised the sample.

Students came from all regions of the country, including the Islands (Azores and Madeira). Two hundred and eighty three (60.3%) of the participants had not been born in Aveiro. One hundred and thirty eight (29.4%) participants were students in the first academic year.

The mean value of the logarithmic transformation of the total homocysteine values were similar between male and female students (P = 0.494), however a significant difference between the variances was observed (P = 0.033). Since this difference could be reflected in different reference intervals for total homocysteine for male and female subjects, as a first step male and female subjects were analysed separately (Table 1, Fig. 1).

Since the reference interval for total homocysteine for male and female subjects were very similar (Table 1), a reference interval obtained from 469 subjects was calculated irrespective of gender. This reference interval is 6.2 to 11.6 µmol/l (Figure 2).

Table 1 – Total homocysteine by gender

	Male (n = 65)	Female (n = 116)	P value
Ln (Homocysteine)			
Mean	2.13	2.10	0.494
Variance	0.097	0.048	0.033
Homocysteine (µmol/l)			
Geometric Mean	8.40	8.17	
CI 95%	7.77 to 9.08	7.85 to 8.51	
Reference Interval	5.74 to 11.03	5.98 to 11.18	
	5.89 to 11.17 <sup>a</sup>		

<sup>a</sup> males and females combined.

No statistically significant difference was found for total homocysteine between subjects with recommended and non-recommended intakes of folate, vitamins B6 and B12<sup>15,25</sup> (P = 0.438).

**DISCUSSION**

This is the first known study providing reference intervals for total homocysteine in young adults, based in a sample of Portuguese university students.

This study aimed to determine normal values for total homocysteine in young adults. However the reference intervals determined separately for male and fe-

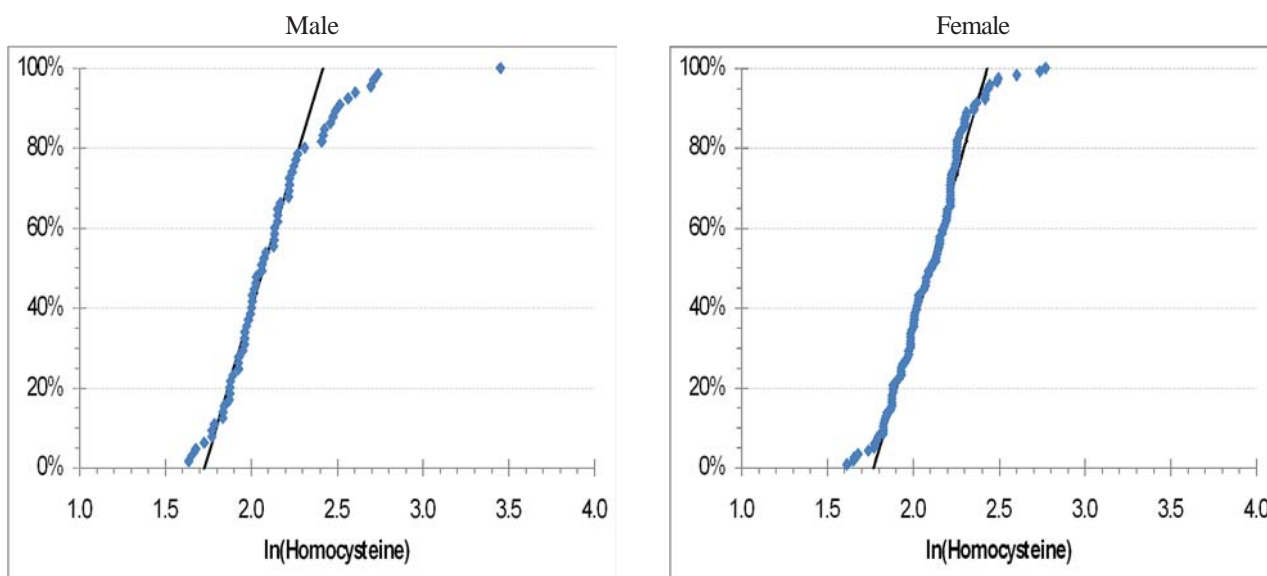


Fig. 1 – Cumulative distribution of the logarithmic transformation of the total homocysteine values and the line fitted using the Hoffmann approach, by gender

male were similar, and results in females and males were combined for homocysteine reference interval irrespective of gender (6.2 to 11.6  $\mu\text{mol/l}$ ). These values are not in accordance with homocysteine reference intervals previously published in some studies, namely the CDC<sup>15</sup> and the information on the leaflet of the assay used in this study.

Reference intervals were estimated using the Hoffmann approach<sup>17,18</sup> based in a sample of 469 subjects (120 men and 349 women) according to the international criteria that the number of individuals per subgroup to establish reference values should be 120 or more<sup>19</sup>. This simple statisti-

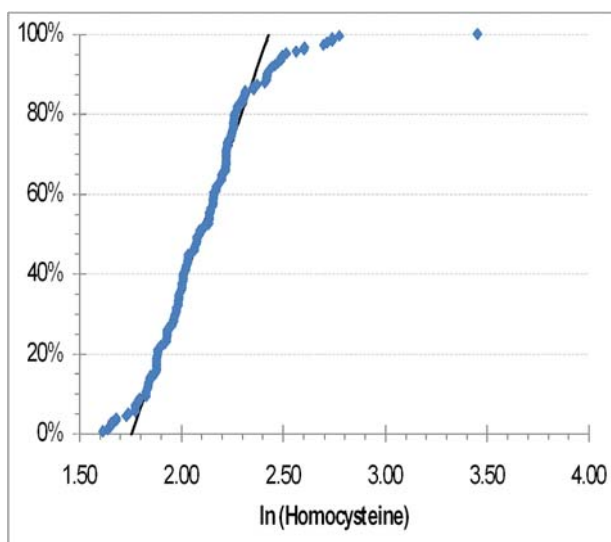


Fig. 2 – Cumulative distribution of the logarithmic transformation of the total homocysteine values and the line fitted using the Hoffmann approach, irrespective of gender

cal technique can be applied in the determination of any normal values in Medicine when measurements are available and mathematical assumptions are reasonable<sup>17,18</sup>. Since using this approach outliers and the possibly *sick* population are removed statistically, the Hoffmann approach<sup>17,18</sup> is advantageous when it is not possible to guarantee that all the participants are healthy and that some of them were not taking any medication that could interfere with homocysteine concentrations, not having elevated levels of total cholesterol and no tobacco consumption<sup>17,18</sup>. Due to the asymmetrical distribution of homocysteine, the Hoffmann approach was applied to the logarithmic transformation of the total homocysteine values using the method of least squares. The Hoffmann approach<sup>17</sup> has been used widely by Soldin and co-workers to evaluate reference intervals for various analytical parameters in the paediatrics. In addition, this approach is well accepted among clinicians as it is of great importance in differentiating between the *healthy* and the *diseased* populations<sup>18</sup>.

Elevated homocysteine levels ( $>12 \mu\text{mol/L}$ ) are considered cytotoxic and are found in 5% to 10% of the general population and in up to 40% of patients with vascular disease and in up to 40% of patients with vascular disease<sup>27</sup>. Since some of the Portuguese Laboratories consider above the normal range only results higher than 16 for men and higher than 20.44  $\mu\text{mol/L}$  for women, values less than those limits but higher than around 12  $\mu\text{mol/L}$  would not lead to physician concern and surveillance. The identification of reference intervals for homocysteine in young adults may help to identify those with a higher

cardiovascular disease risk, so that preventive strategies may be considered.

According to Anderson, total homocysteine seems to be dependent on age, gender, and in women, possibly menopausal status<sup>28</sup>. Our study suggests that normal values for total homocysteine in young adults are similar for both genders. Similar results were found in another study with apparently healthy teenagers in the same geographical area<sup>21</sup>.

The role of the three components folate, vitamins B6 and B12 in the metabolism of homocysteine is well described in the literature<sup>7,13,29</sup>. However when comparing subjects with recommended and non-recommended intakes of folate, vitamins B6 and B12, no statistically differences were found concerning total homocysteine. There could be a sampling bias in the study group since data correspond to a group of students studying in the same environment with a (partly) shared life style, and it is well known that homocysteine values depend on vitamin status. However the university students evaluated came from all regions of the country and 29.4% of the participants are in the 1<sup>st</sup> academic year. Since collection of data took place during the first semester of each year, probably there was not enough time for the acquisition of new habits for those students. Since the remaining students were enrolled in different academic years and different scientific areas their life styles could have had different influences from the university environment. For this reason, there seem to be enough heterogeneity among this group to make them a unique opportunity to develop population reference intervals for serum total homocysteine concentration in young adults.

A Portuguese study suggested hyperhomocysteinemia as a risk factor for cerebral vascular accident<sup>30</sup>. In this study hyperhomocysteinemia was considered when total homocysteine values were greater than 13.1  $\mu\text{mol/L}$  above the limit suggested in the current study (11.6  $\mu\text{mol/L}$ ).

In our study the reference intervals were obtained from results of Portuguese university students. The reference interval published in this study should be helpful to clinicians attempting to assess cardiovascular disease risk in young adults. These reference values provide a basis for preventive strategies, as well as monitoring strategies of an important risk factor for cardiovascular diseases, among young adults, which is an important public health concern.

This article provides reference intervals for serum total homocysteine levels similar for male and female young adults between 6.2 and 11.6  $\mu\text{mol/L}$ . Special attention

should be considered for 11.6  $\mu\text{mol/L}$  as the possible cut-off point between normal and abnormal values for serum total homocysteine for young adults. Serum total homocysteine concentrations above 11.6  $\mu\text{mol/L}$  in subjects in transition to adulthood may increase the risk for future development of Cardiovascular Diseases. A reference interval for total homocysteine in these ages may help to identify those with a higher disease risk. Values above 11.6  $\mu\text{mol/L}$  should lead physician concern and surveillance in adulthood.

## CONCLUSIONS

- Some Portuguese Laboratories of Clinical Pathology consider above the normal range for hyperhomocysteinemia only results higher than 16 for men and higher than 20.44  $\mu\text{mol/L}$  for women;
- The scientific literature referred that elevated homocysteine levels (> 12  $\mu\text{mol/L}$ ) are considered cytotoxic;
- The reference intervals, determined separately for male and female in this study, were similar, and results in females and males were combined for homocysteine reference interval irrespective of gender (6.2 to 11.6  $\mu\text{mol/L}$ );
- The limit suggested in the current study for hyperhomocysteinemia for young adults is 11.6  $\mu\text{mol/L}$ ;
- The identification of reference intervals for homocysteine in young adults may help to identify those with a higher risk for cardiovascular disease, so that preventive strategies may be considered.
- The reference interval published in this study should be helpful to clinicians attempting to assess cardiovascular disease risk in young adults.

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