British Journal of Nutrition (2006), **96**, 719–724
DOI: 10.1079/BJN20061882

Reference range of total serum homocysteine level and dietary indexes in healthy Greek schoolchildren aged 6–15 years

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(Received 27 December 2005 – Revised 10 May 2006 – Accepted 18 May 2006)

Elevated total serum homocysteine (tHcy) may be a possible risk factor for CVD. A 5 μmol/l increase in tHcy is associated with an approximately 70 % increase in relative risk of CVD in adults. Data for children and adolescents are, however, limited. The purpose of the present study was to provide a reference range for tHcy and investigate any relationship between tHcy and nutritional indexes in a Greek paediatric population. tHcy, folate, vitamin B_{12} levels and dietary indexes were measured in 520 healthy schoolchildren (274 boys, 246 girls) aged 6–15 years. As in adults, the tHcy distribution skewed to the right, with a geometric mean for both genders of 7·4 (range 3·4–29 μmol/l). Concentrations were lower in young children and increased with age. No statistically significant difference in tHcy level was observed between gender. The 95th percentiles for the three age groups were as follows: 6–9 years, 9·98 μmol/l; 10–12 years, 10·62 μmol/l; 13–15 years, 14·4 μmol/l. Using Pearson's coefficient analysis, tHcy level was correlated with age, serum folate, BMI and systolic blood pressure. Dietary analysis showed that folate, vitamin B_{12} and fibre intake were inversely related with tHcy; conversely, sugar and fat were positively associated with tHcy. However, in multiple linear regression analysis, only age (odds ratio 0·246, $P<0·05$) and folate (odds ratio −0·346, $P<0·05$) were significantly and independently associated with tHcy. This study provides age-specific reference data regarding tHcy concentration in a Greek paediatric population. tHcy levels increased as a function of age. Serum folate levels were significantly and independently associated with tHcy levels.

Homocysteine: Folate: Diet: Children: Greece

Homocysteine (Hcy) is a sulphur amino acid and derives from methionine during numerous transmethylation reactions. Further metabolism of this amino acid is dependent on the cofactors folate, vitamin B_{12}, vitamin B_6 and riboflavin (Selhub *et al.* 1993, 1999; Pancharuniti *et al.* 1994; Jacques *et al.* 2002). Hyperhomocysteinaemia is also associated with age, gender, renal function, disease states, hormones, antifolate medications and genetic variations (Selhub *et al.* 1993; Kluijtmans *et al.* 2003; Molloy, 2004). A C → T substitution at nucleotide 677 in the methylenetetrahydrofolate reductase gene is associated with increased levels of Hcy (Canepa *et al.* 2003). In addition, the folic acid and B vitamins required for Hcy metabolism and a low intake from the diet may affect the circulating Hcy concentration (Selhub *et al.* 1993).

An elevated level of total Hcy (tHcy) is an independent risk factor for CVD (Boushey *et al.* 1995; Nygard *et al.* 1995), stroke (Cardo *et al.* 1999) and venous thrombosis (Koch *et al.* 1999). However, other studies have provided conflicting results (Toole *et al.* 2004; Bonna *et al.* 2006; Heart Outcomes Prevention Evaluation 2 Investigators, 2006). Heart disease is one of the leading causes in adult death in Greece (Chimonas, 2001). Obesity, hypertension, diabetes and lack of exercise are well-known risk factors for CVD in Greece (Kapantais *et al.* 2004; Magkos *et al.* 2005); a possible high tHcy level may also count as another risk factor for CVD.

tHcy concentration in children has been investigated by many authors. Tonstad *et al.* (1996) reported tHcy concentration in Norwegian children aged 6–12 years. In another study from New Orleans, Reddy (1997) published reference values for tHcy in children. Vilaseca *et al.* (1997) reported Hcy concentrations in 195 Spanish children aged 2–18 years, and Ganji & Kafai (2005) have published data for tHcy in 6461 American children.

The purpose of the present study was to provide age-specific data regarding tHcy level and to determine the relationship between tHcy and folate, vitamin B_{12}, age, BMI, blood pressure and diet in healthy schoolchildren aged 6–15 years.

Subjects and methods

Data collection

A total of 520 children (274 boys, 246 girls) aged 6–15 years participated in the study from various schools in Thessaloniki, Northern Greece. Permission was obtained from the Ethical Committee of Aristotle University of Thessaloniki, Greece. Written, informed consent was also obtained from the parents of each child who participated in the study. A detailed medical history (questionnaire) was taken, and children with a past

Abbreviations: Hcy, homocysteine; tHcy, total homocysteine.

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history of renal disease, thyroid dysfunction, hormone therapy, liver disease or medication usage were excluded from the study.

The dietary history report was obtained by a registered dietitian. A 3 d validated food record (Schroder *et al.* 2001) was completed by the parents after they had been informed how to measure and report the food items. The dietary record was analysed using a software program (Sciencetech Diet 200A; Science Technologies, Athens, Greece) that consisted of more than 2500 Greek food items (Trichopoulou, 1992).

Anthropometric parameters

We measured height (cm) and weight (kg) in all children. BMI was calculated as weight (kg)/height^2 (m^2). Blood pressure was obtained in a supine relaxed position using a Hg sphygmomanometer; three measurements were performed at intervals of 2–5 min, and the mean of the three values was considered to be the blood pressure.

Blood sampling

Serum tHcy was measured early in the morning after subjects had fasted overnight. Blood samples were drawn by venepuncture into 10 ml empty evacuated tubes without EDTA, heparin or clot activators. The tubes were centrifuged within the next half an hour at 2000 ***g*** for 15 min. The serum then was separated and analysed for tHcy, folate and B_{12} measurements. tHcy levels were measured using an ABBOT IMx Analyzer (Axis-shield, Dundee, UK), which uses the fluorescence polarisation immunoassay method (Ueland *et al.* 1993; Frantzen *et al.* 1998; Pernet *et al.* 2000; Refsum *et al.* 2004) with an interassay coefficient of 6·8 %. Folate and B_{12} levels were measured by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany; Erler, 1998; Gutierrez Revilla *et al.* 2004; Lindblad *et al.* 2005) with interassay coefficients of 5 % and 6·2 %, respectively.

Statistical analysis

We performed statistical analysis with SPSS 11.5 software (SPSS Inc., Cary, NC, USA). Variables that were found not to be normally distributed were log-transformed. Unpaired two-tailed *t* tests were calculated to detect significant differences between independent groups. ANOVA was used to test for differences of variance in the three age groups. For *post hoc* comparisons of means, the Bonferroni test was used. Correlation between variables was analysed by Pearson's method. In addition, the effect of several variables on Hcy concentration was considered using multiple linear regression analysis. In the regression model, we verified Hcy as a dependent variable and included age, BMI, folate and vitamin B_{12} as independent variables. In statistical analysis, values of $P<0{\cdot}05$ were regarded as statistically significant.

Results

A total of 520 healthy schoolchildren participated in the study (274 boys, 246 girls).The geometric mean of age of the participating children was 11·4 (range 6–15) years. Boys had higher geometric mean serum tHcy levels than girls, at 7·7 (range 3·48–24·2) *v.* 7·2 (range 3·9–29·0) μmol/l, but this difference was not statistically significant. We observed no statistical significant differences in serum folate, vitamin B_{12}, BMI, waist circumference or systolic blood pressure between gender.

Serum tHcy level was statistically significantly ($P<0{\cdot}05$) lower in young children compared with older ones and increased progressively with age (Table 1). Serum folate levels were statistically significantly different ($P<0{\cdot}05$) between the first (6–9 years) and the third (13–15 years) age groups, and also between the second (10–12 years) and third (13–15 years) age groups (Table 1). Serum vitamin B_{12} level was found to be statistically significantly different ($P<0{\cdot}05$) between all three age groups. In addition, BMI was found to increase with age and was statistically significantly different ($P<0{\cdot}05$) between all three age groups (Table 1). Table 1 also presents data for systolic and diastolic blood pressure. It should also be noted that ninety (17%) children out of the 520 were hypertensive for their age ($>$95th percentile). Of these ninety children, thirty-five (38.8%) were overweight and nineteen (21%) were obese according to International Obesity Task Force criteria (data not shown).

Table 2 shows the percentile distribution (25/50/75/85/95) of serum tHcy level by age. Serum tHcy concentration was lower in younger children and increased with age. In the first age group (6–9 years), the 95th percentile lay at

Table 1. Characteristics of children in the three age groups (Geometric means and (ranges))

	Age 6–9 years (first group)	Age 10–12 years (second group)	Age 13–15 years (third group)
	(*n* 111)	(*n* 143)	(*n* 266)
BMI (kg/m^2)	17·1 (9·6–31·4)*	19·1 (10·7–31·1)*	20·5 (13·3–41·1)*
Waist (cm)	57·7 (21·0–90·0)*	67·8 (49·0–100·0)*	74·2 (56·0–111·0)*
Systolic blood pressure (mmHg)	104 (59–164)†	120 (78–181)†	124 (86–168)†
Diastolic blood pressure (mmHg)	69 (47–113)†	74 (55–126)†	74 (54–123)†
Folate (ng/ml)	11·80 (4·66–20·00)‡	10·00 (1·82–20·00)‡	7·50 (0·99–20·00)‡
Vitamin B_{12} (pg/ml)	1048 (117–2000)*	805 (296–2000)*	700 (214–2000)*
Total homocysteine (μmol/l)	6·50 (3·48–11·29)*	7·10 (4·12–22·19)*	8·60 (3·90–29·03)*

* Mean values were significantly different between the first and second age groups, the second and third age groups, and the first and third age groups: $P<0{\cdot}05$.

† Mean values were significantly different between the first and second age groups, and the first and third age groups: $P<0{\cdot}05$.

‡ Mean values were significantly different between the first and third age groups, and the second and third age groups: $P<0{\cdot}05$.

For details of subjects and procedures, see p. 719.

Table 2. Percentile distribution of total homocysteine (tHcy) among children, by age

	6–9 years (*n* 111)	10–12 years (*n* 143)	13–15 years (*n* 266)
Percentiles	tHcy (μmol/l)	tHcy (μmol/l)	tHcy (μmol/l)
25	5·57	6·28	7·15
50	6·42	7·19	8·32
75	7·32	8·30	9·86
85	7·62	8·58	10·35
95	9·98	10·62	14·4

For details of subjects and procedures, see p. 719.

9·98 (range 3·48–11·29) μmol/l. Of the 115 children in this group, six (5 %) were hyperhomocysteinaemic (tHcy > 9·98 μmol/l). In the second age group (10–12 years), the 95th percentile of tHcy was 10·62 (range 4·12–22·19) μmol/l. In this group, seven (4·8 %) children out of 143 were hyperhomocysteinaemic (tHcy > 10·62 μmol/l). In the third age group (13–15 years), the 95th percentile of tHcy level jumped to 14·4 (range 3·9–29·03) μmol/l. Of the 266 children of this group, thirteen (4·8 %) were hyperhomocysteinaemic (tHcy > 4·4 μmol/l).

The daily dietary intakes according to gender and age are presented in Tables 3 and 4, respectively, after adjusting for total energy intake (not all data shown). The data show that boys consume greater amounts of lipids, sugar, fibre, folate and vitamin B_{12} than do girls ($P<0·05$). In addition, both genders were found to consume higher levels of saturated fat and cholesterol, and less fibre, than recommended by the National Cholesterol Education Program. The levels of consumption of folate and vitamin B_{12} were above the recommended dietary reference intakes (Institute of Medicine of National Academies of Science, 2002).

Using a Pearson's correlation test, tHcy levels were significantly correlated with age (r3,13, $P<0·01$), serum folate (r[subs] −0·335, $P<0·01$), serum vitamin B_{12} (r[subs] −0·217, $P<0·01$), BMI (r[subs] 0·191, $P<0·01$), systolic blood pressure (r[subs] 0·124, $P<0·01$), sugar intake (r[subs] 0·082, $P<0·01$) and dietary folate (r[subs] −0·073, $P<0·01$). Dietary B_{12} and fibre were not correlated with tHcy level. However, in multiple linear regression analysis, the only variables significantly and independently associated with tHcy level were age (odds ratio 0·246, $P<0·05$) and folate (odds ratio −0·346, $P<0·05$) (Table 5).

Discussion

In this study, we found that serum tHcy level was independently related to age and serum folate level. tHcy distribution was skewed to the right, as in adults. tHcy levels were found to be lower in the children in the present study than in adults (Boushey *et al.* 1995). The findings show that tHcy levels were relatively similar to those given in other studies from Greece (Papoutsakis *et al.* 2005) and some Mediterranean countries (Vilaseca *et al.* 1997; Mainou *et al.* 2002; Canepa *et al.* 2003). They were, however, higher in comparison with studies by some other authors (Tonstad *et al.* 1996; de Laet *et al.* 1999; Rauh *et al.* 2001; Bates *et al.* 2002; Must *et al.* 2003; van Beynum *et al.* 2005). These differences may be due to genetic, nutritional and/or environmental factors (Selhub *et al.* 1993; Kluijtmans *et al.* 2003).

The 95th percentiles in our subjects for the three age groups of 6–9, 10–12 and 13–15 years were 9·98, 10·62 and 14·4 μmol/l, respectively. Using this cut-off point as a definition, hyperhomocysteinaemia in our sample corresponded to concentrations of above 9·98 μmol/l for the first age group, 10·62 μmol/l for the second group and 14·5 μmol/l for the third. The hyperhomocysteinaemic children in the present study had higher mean values of BMI and blood pressure, and lower intakes of dietary folate and fibre compared with the children with normal tHcy levels. Thus, hyperhomocysteinaemia in these children could emerge as a possible additional risk factor for CVD in later life. de Laet *et al.* (1999) reported 95th percentile values of 10·2 μmol/l in Belgian children aged 10–14 years, whereas Osganian *et al.* (1999) described corresponding values of 8·5 μmol/l in children aged 13–14 years in the USA.

Papoutsakis *et al.* (2005) reported 95th percentile values of 11·5 μmol/l in 186 Greek children aged 11–12 years. The authors of this study examined in depth the most common genetic defect of tHcy: the 677C → T mutation in the gene for 5,10-methylenetetrahydrofolate reductase. Unfortunately, we did not measure this in the present study. However, Papoutsakis *et al*. did not study a comprehensive paediatric age range, as we did. In addition, the nutritional data provided

Table 3. Gender differences in daily dietary intakes of the study group (adjusted for total energy intake) (Geometric means and (ranges))

Nutrient	Males (*n* 274)	Females (*n* 246)	Total (*n* 520)	*P* value*
Fat (g)	96·0 (11·3–218·0)	97·6 (45–230)	96·2 (11·3–230·0)	0·000
MUFA (g)	44·8 (10·5–112·0)	45 (21–99)	44·9 (10·5–112·0)	0·000
PUFA (g)	11·8 (2·0–85·0)	11·9 (4·0–27·6)	11·9 (2·0–85·0)	0·003
Saturated fatty acids (g)	32·5 (4·6–84·0)†	33·3 (10·0–105·0)†	32·7 (4·6–105·0)	0·000
Cholesterol (mg)	255·7 (72·0–850·0)†	253 (73–888)†	254·0 (72·5–888·0)	0·000
Sugar (g)	3·5 (0·0–64·0)	3·1 (1·0–54·0)	3·4 (0·0–64·0)	0·009
Fibre (g)	12·4 (1·5–35·5)‡	12·0 (4·3–28·0)‡	12·3 (1·5–35·5)	0·004
Folate (μg)	395 (75–660)	390 (79–701)	392·5 (75·0–760·0)	0·000
Vitamin B_{12} (μg)	4·2 (1·0–54·4)	3·9 (0·1–17·1)	4·1 (0·1–54·4)	0·029

* Mean values were statistically significant between boys and girls: $P<0·05$.
† Values are higher than the National Cholesterol Education Program (NCEP II) recommendations.
‡ Values are lower than the NCEP II recommendations.
For details of subjects and procedures, see p. 719.

Table 4. Daily dietary intakes according to three age groups (adjusted for total energy intake) (Geometric means and (ranges))

	Age 6–9 years (*n* 111)	Age 10–12 years (*n* 143)	Age 13–15 years (*n* 266)
Fat (g)	95·1 (11·3–180·0)	91·7 (35·0–230·0)	99·2 (19·8–218·0)
MUFA (g)	44·6 (13·0–89·0)	42·1 (10·5–99·0)*	46·5 (12·5–112·0)*
PUFA (g)	11·9 (2·4–31·0)†	11·4 (3·3–51·5)†	12 (2–85)†
Saturated fatty acids (g)	33·4 (8·4–61·7)	31·2 (11·0–105·0)	33·2 (4·6–84·4)
Cholesterol (mg)	254 (86–498)	256·5 (72·0–888·0)	255·3 (75·0–650·0)
Sugar (g)	3·5 (0·0–64·0)	3·3 (1·0–33·0)	3·5 (1·0–54·0)
Fibre (g)	12·9 (4·5–30·0)	11·6 (1·5–35·5)	12·5 (3·1–29·0)
Folate (μg)	385 (75–750)‡	388 (82–710)‡	391 (88–760)‡
Vitamin B_{12} (μg)	4·2 (1·3–17·0)‡	4·1 (1·2–31·0)‡	4·2 (0·1–54·4)‡

* Mean values were significantly different between the second and third age groups: $P<0·05$.
† Mean values were significantly different between first and second age groups, and between the second and third age groups: $P<0·05$.
‡ Values are higher than dietary reference intakes.
For details of subjects and procedures, see p. 719.

Table 5. Multiple linear regression analysis of the association between total homocysteine as a dependent variable and age, folate, BMI and vitamin B_{12} status as independent variables

	Odds ratio	95 % CI	*P* value*
Total homocysteine (R^2 0·24)			
Age	0·246	0·158, 0·362	0·000
Serum folate	−0·346	−0·206, −0·118	0·000
BMI	0·093	−0·029, 0·154	0·167
Serum vitamin B_{12}	0·075	0·000, 0·001	0·128

* Mean values were significantly different at $P<0·05$.
For details of subjects and procedures, see p. 719.

by the authors of that trial should be under consideration as it was based on two 24 h recalls and analysed using software that did not correspond to Greek food standards. It should be noted, however, that the 95th percentile we found for the same age group is slightly lower (10·62 *v.*11·5 μmol/l). This is probably due to the fact that we included 10-year-olds in our group, which was not done in the other study.

Reddy (1997) did not find an association between tHcy level and age, although we have shown a strong association in the present study. The data from the present study are in agreement with those reported by others (Tonstad *et al.* 1996; de Laet *et al.* 1999; Rauh *et al.* 2001; Must *et al.* 2003; Ganji & Kafai, 2005). This finding may be related to an increased synthesis of creatinine due to a higher muscle mass in boys (Brattstrom *et al.* 1994). An association between tHcy level and gender was not confirmed, although we did find a slightly higher tHcy levels in boys.

In the present study, serum folate and vitamin B_{12} levels were inversely associated with tHcy concentrations. These observations are in agreement with data from other authors (Tonstad *et al.* 1996; de Laet *et al.* 1999; Osganian *et al.* 1999; Bates *et al.* 2002). However, the influence of vitamin B_{12} on tHcy was less marked than the influence of folate. We also demonstrated that the concentrations of folate and vitamin B_{12} decreased markedly with increasing age (de Laet *et al.* 1999; Papaoutsakis *et al.*, 2005). This inverse relationship between tHcy and vitamin B_{12} suggests that an optimal vitamin B status level is globally important in order to achieve lower tHcy concentrations.

Findings from the present study also show a positive correlation between tHcy level and BMI. Osganian *et al.* (1999) and Papoutsakis *et al.* (2005) published a weak association between tHcy and BMI, but de Laet *et al.* (1999) found BMI to be significant positively associated with tHcy level. This should be a concern as BMI naturally increases with age, and a possible high BMI may predispose to childhood obesity, which has been found to be related to hyperhomocysteinaemia (Narin *et al.* 2005).

The results of the present study show that dietary folate, vitamin B_{12}, sugar and fibre are associated with tHcy level. Diakomopoulou *et al.* (2005) reported an inverse relationship between plasma tHcy level and weekly consumption of fruits and vegetables. A high intake of fruits and vegetables is associated with higher plasma folate and lower plasma Hcy levels (Lasheras *et al.* 2003).

We did not find any association between tHcy level and dietary fat intake, whereas there was a weak inverse correlation between tHcy level and dietary fibre. Conversely, a positive association between dietary fat and tHcy level was found by Oshaug *et al.* (1998). The authors attributed these findings, however, to the lower intake of essential vitamins.

The present study is limited by the lack of measurement of the methylenetetrahydrofolate reductase polymorphism. Nevertheless, we provided age-specific data on tHcy level in a large sample of Greek children aged 6–15 years. In our study, age and serum folate were strongly related to tHcy level. Considering the growing interest in tHcy as a risk factor for CVD, it is important to establish tHcy references values and explore their predictors.

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