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Homocysteine concentrations in a German cohort of 500 individuals: Reference ranges and determinants of plasma levels in healthy children and their parents

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Summary. Elevated plasma homocysteine is a risk factor for cardiovascular disease and a sensitive marker of inadequate vitamin B12 and folate status. We studied 257 pupils (120 boys, 137 girls, aged 6–17 years) and their parents (88 males, 172 females, aged 26–50 years). Our measurements were part of a national Bavarian health and nutrition examination survey evaluating cardiovascular risk factors. A mild hyperhomocysteinemia (Hcys $>15\,\mu$mol/l) occurred in 7% of the adults, but in none of the children. Men had significantly higher Hcys levels than women ($p < 0.0001$), boys and girls had comparable concentrations. For adults and children, Hcys correlated inversely with vitamin B12 and folate and positively with the lean body mass and creatinine in serum, but not with cystatin C. Genetic and nutritional factors are determinants of Hcys metabolism. The correlation of Hcys and serum creatinine is dependent on the metabolic link between Hcys production and creatine synthesis.

Keywords: Amino acids – Cobalamin – Cystatin C – Folate – Healthy population – Homocysteine – Reference values

Introduction

In general, homocysteine derives from the essential amino acid methionine. Through vitamin B12 and folic acid-dependent pathways it may be reconverted to methionine or degraded via a vitamin B6-dependent route.

Multiple studies have shown that a moderately elevated plasma homocysteine concentration is an independent risk factor for atherosclerosis and thrombosis, most likely through endothelial injury via reactive oxygen

species (Welch and Loscalzo, 1998). Hyperhomocysteinemia is typically either caused by genetic defects of the enzyme cystathionine β-synthase or by nutritional deficiencies of vitamin cofactors. It can also be detected in individuals suffering from leukemia and proliferating tumors (Vilaseca et al., 1997 and 1998). Testing for hyperhomocysteinemia may therefore be useful to assess the nutritional status in humans. It has been described by others, that total homocysteine in plasma is inversely correlated with plasma folate and vitamin B12 levels in adults (Clarke et al., 1998). Heterozygosity for cystathionine β-synthase deficiency can be detected by the ratios of total plasma homocysteine to cysteine and folate (Boddie et al., 1998).

Total homocysteine is composed of free and protein-bound forms of homocysteine. Within-person variability of plasma homocysteine concentrations in adults is relatively low, a single measurement characterizes the average concentration in plasma over at least one month and plasma homocysteine concentrations in adult men are significantly higher than in women (Garg et al., 1997). In children aged 2 months – 18 years homocysteine values are independent of gender and increase with age (Vilaseca et al., 1998).

Screening for mild hyperhomocysteinemia in a large pediatric population has rarely been reported (Vilaseca et al., 1997, De Laet et al., 1999).

We therefore measured total plasma homocysteine, serum vitamin B12, serum folate, creatinine and serum cystatin C, respectively, which is a determinant of glomerular filtration rate and independent of age and height (Bökenkamp et al., 1998).

Methods

Study design

The present data are part of a prospective intervention study (designated as Family Intervention Trial, FIT study) evaluating nutritional status, health and cardiovascular risk factors in a large cohort of first grade pupils, their older siblings and parents. Information concerning health status was obtained by means of questionnaires, including detailed information concerning family history and medical history, diet and life-style habits. This part of the survey was planned as a cross-sectional prevalence study. All first-grade pupils of the term 1997/1998 from 10 schools in the Bavarian town Erlangen were included after informed consent was obtained. The study protocol was reviewed by the local ethics committee and school authorities.

Furthermore, another purpose of this study is to evaluate risk profiles in children and their families and to improve their status by intervention and education programs in a randomized and prospective manner.

Venous blood samples were obtained in the early morning hours from first-grade pupils and their older siblings (n = 257; 120 boys, 137 girls, aged 6–17 years) and their parents (n = 260; 88 males, 172 females, aged 26–50 years) after an overnight fast. The plasma samples were anticoagulated with citrate, chilled on ice and immediately carried to the laboratory together with the serum probes, where they were centrifuged and stored as aliquots at $-20°C$. In this part of the study, the following parameters were analyzed: total plasma homocysteine, serum vitamin B12, serum folate, creatinine, serum cystatin C.

Biochemical measurements

Plasma total homocysteine was measured by a modified HPLC method with fluorescence detection (Vester and Rasmussen, 1991). Briefly, to 150 μl plasma 50 μl 0.1 M potassium borate buffer (pH 9.5, 2 mM EDTA) was added. Reduction was carried out for 30 min at 4°C (Reduction agent, Chromsystems, Munich, Germany). Deproteinization was performed with 125 μl 10% perchloric acid containing 1 mmol/l EDTA. After incubation at room temperature for 30 min the tubes were centrifuged at 13,000 RPM (15,500 g) for 10 min. 100 μl of the supernatant was mixed with 200 μl 2 mM potassium borate (pH 10.5, 5 mM EDTA) and 50 μl SBD-F derivatisation reagent (1,0 g/l ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (Wako, Kyoto, Japan) in 2 mM potassium borate buffer, pH 9.5). After incubation for 60 min at 60°C tubes were chilled on ice. A 20 μl volume of the derivatised sample was injected using an autosampler system (Gilson, Villiers Le Bel, France). The derivatives were separated isocratically by reversed-phase HPLC using a Nucleosil 100-5C18 (250 × 3 mm) column (Macherey-Nagel, Düren, Germany) as stationary phase. The mobile phase consisted in a 15 mM phosphate buffer, pH 6.0, containing 16 ml/l methanol, flow rate was 0.7 ml/min. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. Calibrators were obtained by spiked pool plasma (pool plasma from healthy volunteers, added with 12.5, 25, 50, 100 μmol/l Hcys). External calibration was performed daily, the between-day coefficients of variation (CV) were between 5% (for 20,3 μmol/l) and 10% (for 9,3 μmol/l).

Vitamin B12 and folate were measured simultaneously in the serum aliquot using Elecsys 2010 electrochemiluminescens analyzer (Roche, Basel, Switzerland) according to the manufacturer's instructions. CV were between 7–9% for vitamin B12, 9–18% for folate, respectively. Serum creatinine was determined enzymatically (PAP method), using Hitachi 911 (Roche, Basel, Switzerland). Between-day CV was 1,7%. Serum cystatin C was analyzed using a commercially available kit (DAKO, Copenhagen, Denmark) for a rapid automated particle-enhanced turbidimetric method, which was applied on a Hitachi 704 (Roche, Basel, Switzerland) with a CV between 3–7%.

Analysis of body composition

Body weight, percentage of body fat, fat mass and lean body mass were determined using the BIA method (Biological Impedance Analysis) according to the manufacturer's instructions (Bodyfat-Analyzer TBF-305, TANITA, Tokyo, Japan). Briefly, the impedance of the body refers to its electrical resistance. Among the body components the musculature easily transports electricity because of its high water content, whereas the adipose tissue has almost non-conductivity. In our system, a sinus wave current with a frequency of 50 kHz and a virtual value of 0.8 mA is applied via detector electrodes on both feet of the volunteer, and the voltage drop compared with the heel electrodes is estimated. The body composition is then calculated automatically.

Statistical analysis

All data are expressed as means, standard deviation, percentiles. Correlation was tested using Spearman's correlation coefficient. A p value <0.05 was considered to be significant.

Results

In Table 1 range, means, standard deviation and percentiles (P 2.5, P 97.5) for children (girls and boys) and adults (females and males) are presented for the

Table 1. Range, means and standard deviation and percentiles (P 2.5; P 97.5) for children (137 girls and 120 boys) and adults (172 females and 88 males) are presented for the following parameters: Homocysteine, vitamin B12, folate, creatinine, cystatin C, lean body mass

Parameter	Girls	Boys	Women	Men
Homocysteine [μmol/l]	0.6–11.4 (5.5 ± 1.6) 2.7; 9.2	1.9–11.0 (5.7 ± 1.7) 3.1; 10.5	2.4–30.3 (9.2 ± 3.3) 4.8; 18.0	2.7–40.3 (11.4 ± 4.5) 4.2; 22.5
Vitamin B12 [pg/ml]	262.5–1658.0 (750.1 ± 245.8) 414.1; 1351.4	316.7–1473.0 (719.4 ± 245.6) 353.4; 1350.6	203.0–978.1 (469.7 ± 143.5) 262.7; 801.3	199.3–891.6 (447.4 ± 133.8) 240.2; 741.4
Folate [ng/ml]	4.3–23.8 (10.5 ± 3.7) 4.4; 17.8	3.0–18.0 (11.0 ± 3.7) 3.8; 17.8	2.3–27.4 (7.3 ± 3.4) 3.2; 16.4	2.6–19.5 (6.3 ± 2.6) 2.9; 13.7
Creatinine [mg/dl]	0.3–0.8 (0.5 ± 0.1) 0.3; 0.7	0.2–1.0 (0.5 ± 0.1) 0.3; 0.9	0.4–1.0 (0.7 ± 0.1) 0.5; 1.0	0.6–1.2 (0.9 ± 0.1) 0.7; 1.2
Cystatin C [mg/l]	0.3–2.0 (1.3 ± 0.2) 0.8; 1.7	0.3–2.0 (1.3 ± 0.2) 0.8; 1.8	0.3–1.7 (1.2 ± 0.2) 0.7; 1.6	0.3–1.7 (1.2 ± 0.3) 0.4; 1.6
Lean Body Mass [kg]	15.2–44.0 (21 ± 5.8) 15.4; 40.3	17.4–73.4 (26.5 ± 11.0) 18.0; 62.2	34.4–58.4 (43.0 ± 3.9) 37.2; 53.0	47.4–78.6 (63.8 ± 5.9) 50.6; 77.0

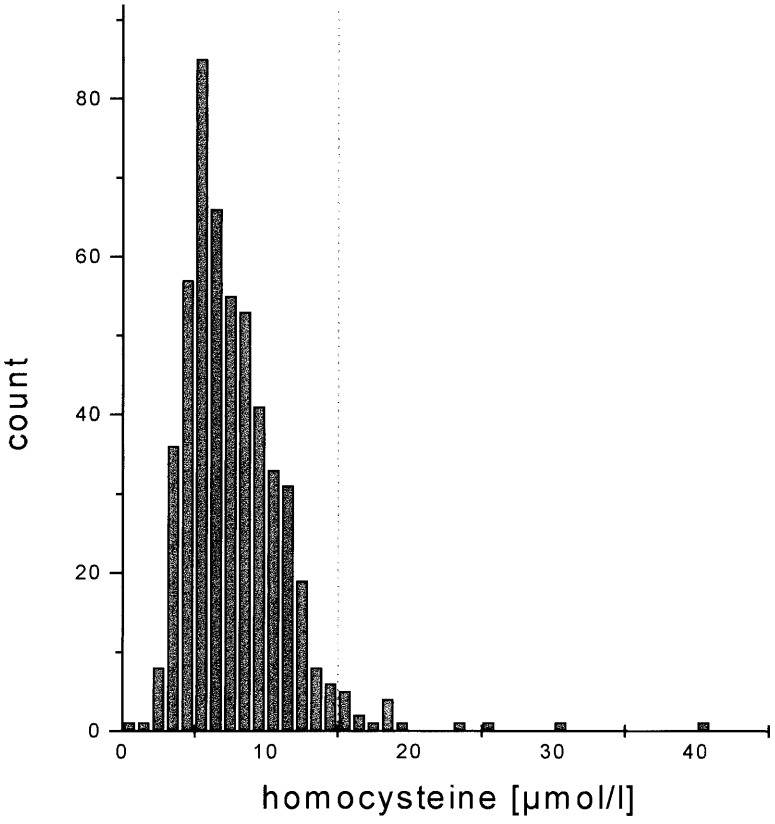

Fig. 1. Frequency distribution curve of total plasma homocysteine levels [μmol/l] is shown for the whole study group (n = 517)

following parameters: Hcys, vitamin B12, folate, creatinine, cystatin C, lean body mass.

A mild hyperhomocysteinemia (Hcys $>15\mu$mol/l) was detected in 7% of the adults (Fig. 1). None of the children had Hcys concentrations above 12μmol/l. If the same percentage of mild hyperhomocysteinemia would be expected in the pediatric population, the cut-off level had to be lower ($<8\mu$mol/l). Adults had significantly higher Hcys levels than children ($p < 0.0001$), and Hcys levels correlated positively with age ($r = 0.67$, $p < 0.0001$). Adult men had significantly higher Hcys plasma concentrations than women ($p < 0.001$), between boys and girls there were no significant differences (Fig. 2).

Plasma Hcys concentrations of the total study group correlated positively with creatinine and the lean body mass ($r = 0.68$, $p < 0.0001$ and $r = 0.70$, $p < 0.0001$, respectively), but not with cystatin C ($r = -0.10$, n.s.), (Table 2). In general, high Hcys concentrations were usually associated with low vitamin concentrations. There was an inverse correlation between Hcys and serum vitamin B12 or folate concentrations ($r = -0.56$ and $r = -0.49$, respectively, $p < 0.0001$), (Fig. 3). Serum folate and cobalamin levels were significantly higher for children than for adults ($p < 0.0001$; Fig. 4, 5).

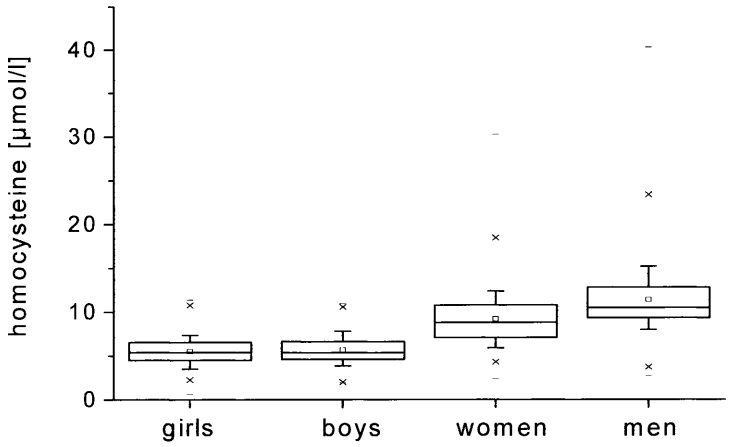

Fig. 2. Plasma homocysteine levels [μmol/l] in children (137 girls and 120 boys) and their parents (172 women and 88 men) are presented: Mean (square symbol), 0th, 1st, 5th, 25th, 50th, 75th, 95th, 99th and 100th percentiles. Men had significantly higher levels than women ($p < 0.001$)

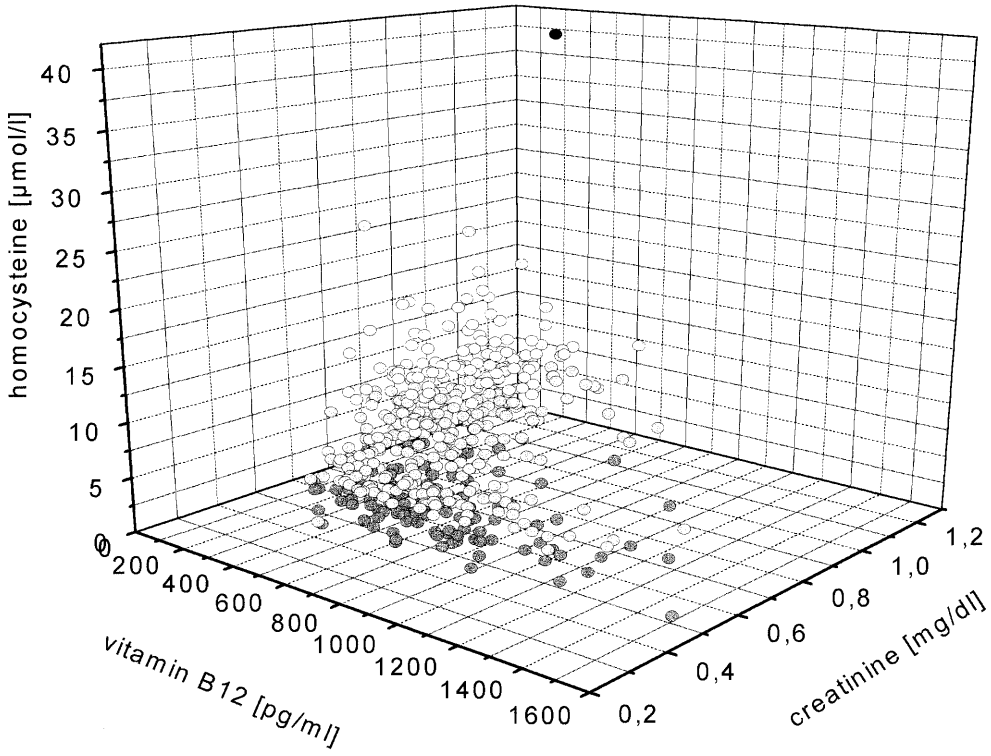

Fig. 3. The relationships between plasma homocysteine levels [μmol/l] and creatinine levels [mg/dl] and between plasma homocysteine and cobalamin levels [pg/ml] are shown for the whole study group (n = 517)

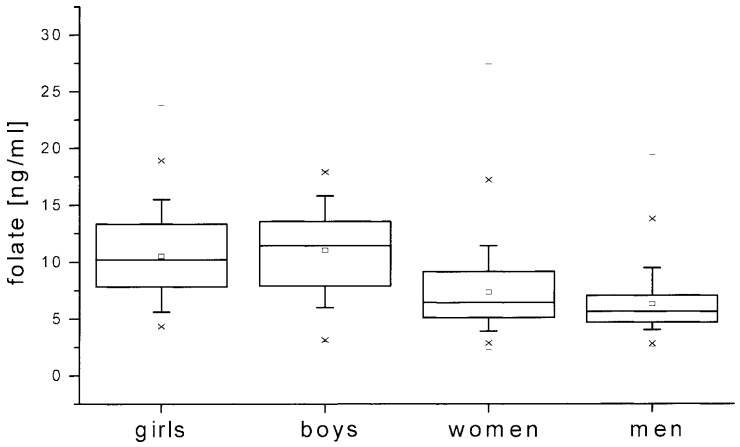

Fig. 4. Serum folate levels [ng/ml] in children (137 girls and 120 boys) and their parents (172 women and 88 men) are shown: Mean (square symbol), 0th, 1st, 5th, 25th, 50th, 75th, 95th, 99th and 100th percentiles. Adults had significantly lower folate levels than children ($p < 0.0001$)

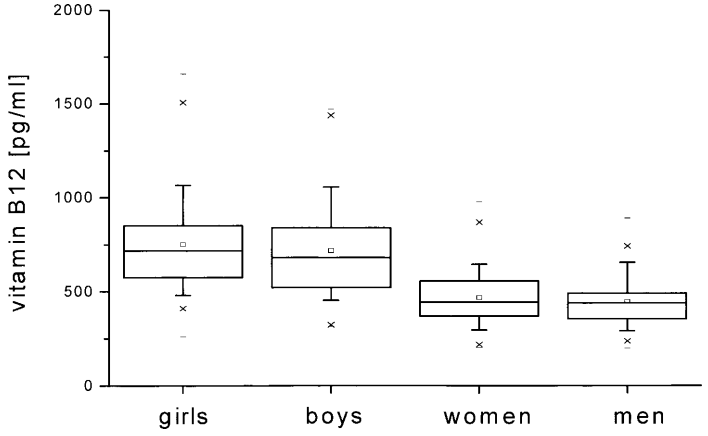

Fig. 5. Serum cobalamin levels [pg/ml] in children (137 girls and 120 boys) and their parents (172 women and 88 men) are shown: Mean (square symbol), 0th, 1st, 5th, 25th, 50th, 75th, 95th, 99th and 100th percentiles. Adults had significantly lower cobalamin levels than children ($p < 0.0001$)

Table 2. Spearman's correlation coefficients between plasma homocysteine levels and the following parameters are presented: vitamin B12, folate, creatinine, cystatin C, lean body mass. The data are shown for children (n = 257), adults (n = 260) and totals (n.s. = not significant)

Homocysteine	Vitamin B12	Folate	Creatinine	Cystatin C	Lean Body Mass
Children	−0.21 ($p < 0.001$)	−0.18 ($p < 0.005$)	0.19 ($p < 0.005$)	n.s.	0.25 ($p < 0.0001$)
Adults	−0.26 ($p < 0.0001$)	−0.24 ($p < 0.0001$)	0.29 ($p < 0.0001$)	n.s.	0.28 ($p < 0.0001$)
Totals	−0.56 ($p < 0.0001$)	−0.49 ($p < 0.0001$)	0.68 ($p < 0.0001$)	−0,10 ($p < 0.05$)	0.70 ($p < 0.0001$)

Cystatin C levels were independent of age ($r = -0.16$), whereas creatinine concentrations correlated significantly with age ($r = 0.78$, $p < 0.0001$). For cystatin C, cobalamin and folate there were no significant differences for males and females, neither for children nor for adults.

Discussion

Hyperhomocysteinemia is a well known graded and independent cardiovascular risk factor. In accordance with previously published data (Welch and Loscalzo, 1998), we found elevated Hcys levels in 7% of the adults in our group. Life-style (e.g. smoking habits) and subtle vitamin deficiencies of vitamin B12 and folate or otherwise impaired metabolism are possible reasons for increasing Hcys levels in life, solely genetic factors are less likely, because none of their children had Hcys concentrations above 12 μmol/l.

Hcys levels were independent of gender in pediatric study group, but men and women had significantly higher concentrations than children ($p < 0{,}001$, respectively). As reported earlier (Norlund et al., 1998) the differences between the sexes disappeared when men and women were stratified by their serum creatinine concentrations. We assume that sex dependent differences of plasma Hcys concentrations are the result of different creatine turnover rates with increased creatine production in men. The link between creatine biosynthesis and homocysteine metabolism is the enzyme guanidinoacetate methyltransferase. Because muscle mass and therefore demand for creatine biosynthesis in men is usually greater than in woman, more homocysteine could be produced at the same time. Additionally, Hcys levels are dependent on renal function. In our healthy cohort there was no correlation between cystatin C and Hcys neither for the children group, nor for the adult group. Cystatin C is a parameter for glomerular filtration and therefore renal function (Helin et al., 1998). It is a 13 kD basic protein produced by all nucleated cells, independent of age, gender, height, and body composition. In children as well as in adults with chronic renal failure, plasma homocysteine levels are elevated and negatively correlated with glomerular filtration rate. Hyperhomocysteinemia can be demonstrated in children with renal insufficiency before end-stage renal disease has developed and may persist even after renal transplantation (Lilien et al., 1999). A known genetic cause for hyperhomocysteinemia is the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene. In homozygote affected individuals elevated fasting and postprandial Hcys concentrations are described (Candito et al., 1999). Since there are still conflicting data and Hcys plays an increasing role both in prevention and disease, further investigations are of interest.

Taken together we have defined reference ranges for a representative German population consisting of 257 children (6–17 years) and 260 adults (26–50 years). Validity of plasma Hcys determinations is dependent on careful

laboratory standardization. The Hcys levels obtained have to be interpreted with regard to age, gender, renal function and vitamin status of the individuals. Suboptimal vitamin levels of B12 and folate promote increased homocysteine levels. An augmentation of the vitamin status should be discussed.

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