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Folic acid, vitamin B12, and homocysteine levels during fasting and after methionine load in patients with Type 1 diabetes mellitus

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ABSTRACT. ***Aims:*** **To assess plasma concentrations of folic acid, vitamin B12, and total plasma homocysteine (tHCY) during fasting and after methionine load in young patients with Type 1 diabetes mellitus (T1DM).** ***Methods:*** **We enrolled 41 young patients with T1DM without any sign of microvascular complications and 123 healthy controls in a 1:3 case-control study. Fasting and post-methionine load (PML) tHCY, folic acid, and vitamin B12 levels were measured in both groups. Data regarding chronological age, metabolic control (assessed by mean values of glycated hemoglobin in the last 12 months) and disease duration were also recorded.** ***Results:*** **Fasting and PML tHCY levels were significantly lower in patients than in controls: 7.3±2.7 μmol/l *vs* 8.3±2.5 μmol/l (p=0.01), and 16.7±5.8 μmol/l *vs* 17.3±4.3 μmol/l (p=0.01), respectively. No correlation was found between fasting and PML tHCY levels and chronological age, disease duration, metabolic control, and insulin requirement. Patients had significantly higher vitamin B12 levels compared to controls: 767±318 pg/ml *vs* 628±236 pg/ml (p=0.003), while folic acid turned out to be lower in patients than in controls: 5.3±1.9 nmol/l *vs* 7.5±2.6 nmol/l (p<0.0001).** ***Conclusions:*** **Adolescents and young adults with T1DM without microvascular complications showed lower tHCY both during fasting and after methionine load. Lower folate concentrations in these patients might benefit from food fortification.**

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INTRODUCTION

Homocysteine (HCY) is a sulfur amino acid whose metabolism stands at the intersection of two pathways: remethylation to methionine, which requires folate and vitamin B12 (or betaine in an alternative reaction), and trans-sulfuration to cystathionine, which requires pyridoxal-5'-phosphate (1). The two pathways are coordinated by *S*-adenosylmethionine, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase reaction and as an activator of cystathionine β-synthase (1). Severe hyper-homocysteinemia (HHCY) is due to rare genetic defects resulting in deficiency of cystathionine β-synthase, methylenetetrahydrofolate reductase, or other enzymes involved in methyl-B12 synthesis and HCY methylation. Fasting mild HHCY could be due to impairment in the methylation pathway, while post-methionine load (PML) HHCY could be due to heterozygous cystathionine β-synthase defect or vitamin B6 deficiency (2). Moreover, HHCY is associated with coronary artery disease, atherothrombosis, and cardiovascular mortality in the general population (3), but the mechanisms regulating HCY metabolism in humans remain largely undefined. Vascular complications are the leading cause of morbidity and mortality in adults with Type 1 diabetes mellitus (T1DM) (4); however, studies regarding HHCY in T1DM patients yielded conflicting results (5-9). In a previous study we already demonstrated that, in children with T1DM without complications, total plasma HCY (tHCY) levels during fasting were lower than in controls (10). The aim of this study was to assay plasma concentrations of folic acid, vitamin B12, and HCY during fasting and after methionine load in young patients with T1DM.

PATIENTS AND METHODS

Forty-one young patients with T1DM were matched with 123 healthy subjects in a 1:3 case-control study to provide at least 80% power (two-tailed α of 0.05).

Patients were all Caucasian (21 males; 51.2% and 20 females; 48.8%), aged 9 to 31 yr with T1DM in regular follow-up at the Regional Pediatric Diabetes Center, University of Genoa, G. Gaslini Institute, Genoa, Italy. Diabetes mellitus was diagnosed according to the 1997 American Diabetes Association Criteria (11). Inclusion criteria were absence of microvascular complications i.e.: persistent microalbuminuria [defined as albumin excretion rate (AER) >20 μg/min in 2 of 3 overnight urine collections in a period of 6 months] or retinopathy (diagnosed after a detailed ophthalmologic examination) and absence of other risk factors for vascular disease (smoking, hypertension, estroprogestinic therapy, high cholesterol levels). Moreover, autoimmune disorders like celiac disease and autoimmune thyroid disease were excluded. None of the patients had been treated with any other drug except insulin for 30 days. The control group included 123 healthy Caucasian subjects (63 males; 51.2% and 60 females; 48.8%), aged 6.4 to 22 yr. These subjects (all non-smokers and not on estroprogestinic therapy), age- and sex-matched, were already described in our previous paper (10). All the subjects were tested for fasting and PML tHCY, vitamin B12, and folic acid levels. In T1DM patients, age at clinical onset, dura-

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tion of disease, metabolic control [mean glycated hemoglobin (HbA_{1c}) levels in the last 12 months] and daily insulin requirement (U/kg/day) were also collected. Informed consent, according to guidelines approved by the Ethics Committee, was obtained from the parents or guardians of every child participating in the study.

Biochemical analysis

After an overnight fasting, venous blood samples were collected between 09:00 and 10:00 h and tHCY levels, red blood cell folate, and serum vitamin B12 levels were evaluated. A second blood sample for tHCY assay was obtained 4 h after oral methionine load (0.1 g/kg body weight). Plasma was separated immediately after sampling, while for serum samples whole blood was left to clot for 30 min at room temperature, then centrifuged at 3000 rpm for 10 min. Samples were stored at 4 C for analysis on the same day. tHCY was determined by HPLC with fluorescence detection. Assays were performed as previously described (12). Normal values for fasting tHCY are <12.7 µmol/l for males and <11.5 µmol/l for females and PML cut-off is 28 µmol/l for both genders (12). Both vitamin B12 and folic acid levels were measured by means of a competitive chemiluminescent enzyme immunoassay (IMMULITE 2000, Siemens Medical Solution Diagnostics).

Statistical analysis

Results are expressed as mean and SD for continuous variables and absolute and relative frequencies for categorical variables. Some variables were transformed to normalise the data. Parameters of the two groups were compared using t-Student test for continuous variables and χ^2 or Fisher exact test for categorical variables. Pearson's correlation coefficient was used to evaluate the relationship between fasting or PML concentration of tHCY and HbA_{1c} and disease duration. A p-value <0.05 was considered statistically significant, and all p-values were based upon two-tailed tests. Statistical analyses was performed using SPSS for Windows (SPSS Inc, Chicago, Illinois USA).

RESULTS

Baseline characteristics of the enrolled subjects are reported in Table 1. Patients' mean age was 16.7±5.7 yr at enrolment, mean age at T1DM clinical onset was 8.3±3.4 yr, and mean disease duration was 8.4±4.9 yr. Mean tHCY plasma concentrations during fasting and PML were lower in patients than in controls [7.3±2.7 µmol/l *vs* 8.3±2.5 µmol/l (p=0.01) and 16.7±5.8 µmol/l *vs* 17.3±4.3 µmol/l (p=0.01), respectively]. Patients had significantly higher vitamin B12 levels compared to the control group (767±318 pg/ml *vs* 628±236 pg/ml, p=0.003), while folic acid concentrations were lower in patients than in controls (5.3±1.9 nmol/l *vs* 7.5±2.6 nmol/l, p<0.0001) (Table1). HHCY occurred in 2 patients (4.9%) (basal in both and PML in 1) and 11 controls (8.9%) (basal in 10 and PML in 2) (Table 2). No significant correlation was found between fasting or PML tHCY and HbA_{1c} levels, disease duration, insulin dose and age at onset even if analyzed separately according to gender. A significant negative correlation was found between basal tHCY and serum folate levels in T1DM patients. The analysis was split by gender: fasting tHCY, PML tHCY, and tHCY delta (i.e., post-methionine minus fasting). tHCY levels were significantly lower in female patients than in female controls (6.6±1.8 µmol/l *vs* 7.9±2.3 µmol/l, p= 0.009), while no significant difference was detected in male controls (7.9±3.1 µmol/l *vs* 8.7±2.6 µmol/l, p=0.91) (Table 3). Folate values were significantly lower in both female and male patients than in controls [5.3±1.8 nmol/l *vs* 8.1±2.9 nmol/l (p<0.0001) and 5.3±1.8 nmol/l *vs* 6.9±2.1 nmol/l (p=0.002), respectively]. Vitamin B12 levels were significantly higher only in male patients than in male controls (832±328 pg/ml *vs* 641±250 pg/ml, p<0.006).

Table 1 - *Characteristics of the study groups. Data were expressed as mean±SD.*

	DM patients No.=41	Controls No.=123	p
Fasting tHCY (µmol/l)	7.3±2.7	8.3±2.5	0.01
tHCY after load (µmol/l)	15.7±5.8	17.3±4.3	0.01
Delta (µmol/l)	8.4±3.7	9.4±3.5	0.09
Vitamin B12 (pg/ml)	767±318	628±236	0.003
Folic acid (nmol/l)	5.3±1.8	7.5±2.6	<0.0001
HbA_{1c} (%)	8.5±1.3	-	
Daily insulin dose (U/kg/24h)	0.81±0.2	-	
Duration of diabetes (yr)	8.4±4.9	-	

tHCY: total plasma homocysteine; HbA_{1c}: glycated hemogobin; DM: diabetes mellitus.

Table 2 - *Hyperhomocysteinemia (HHCY) in patients and controls.*

	DM patients No.=41	Controls No.=123	p
Hyperhomocysteinemia	2 (4.9%)	11 (8.9%)	0.52
Fasting HHCY	2 (4.9%) (2 M)	10 (8.1%) (7 F, 3M)	0.73
HHCY after load	1 (2.4%) (1 M)	2 (1.6%) (1 F, 1M)	0.74

DM: diabetes mellitus; M: males; F: females.

DISCUSSION

This study demonstrated that mean tHCY concentrations – both fasting and PML – were significantly lower in pa-

Table 3 - *Homocysteine, folic acid, and vitamin B12 levels (split by gender). Data were expressed as mean±SD.*

	DM patients	Controls	p
Female	No.=20	No.=60	
Fasting tHCY (µmol/l)	6.6±1.8	7.9±2.3	0.009
tHCY after load (µmol/l)	14.4±3.5	17.3±4.2	0.003
Delta (µmol/l)	7.9±2.6	10±3.2	0.004
Vitamin B12 (pg/ml)	698±298	617±225	0.19
Folic acid (nmol/l)	5.3±1.8	8.1±2.9	<0.0001
Male	No.=21	No.=63	
Fasting tHCY (µmol/l)	7.9±3.1	8.7±2.6	0. 91
tHCY after load (µmol/l)	16.7±7	17.1±4.4	0.71
Delta (µmol/l)	8.7±4.5	8.7±3.8	0.94
Vitamin B12 (pg/ml)	832±328	641±250	0.006
Folic acid (nmol/l)	5.3±1.8	6.9±2.1	0.002

tHCY: total plasma homocysteine.

tients than in controls. Cronin et al. (6) found similar levels of tHCY in adults with T1DM without microangiopathic complications and in age- and sex-matched healthy controls; however, they reported a significant difference in male patients. In our study tHCY resulted significantly lower in female patients: this difference between Cronin's data and ours could be explained by the different age population.

Furthermore, the observation of lower values of tHCY in patients compared to controls still lacks a clear explanation. Plasma tHCY values are determined by genetic and nutritional factors. Various studies showed an inverse correlation between folic acid and HCY levels (13, 14). Unexpectedly, our patients showed lower folic acid values compared to healthy controls, even though their tHCY concentration was lower than in controls. Chiarelli et al. observed increased plasma HCY concentrations both during fasting and PML in adolescents and young adults with T1DM and microvascular complications; they also reported that HCY levels increase with AER and found a positive correlation between plasma HCY concentrations and HbA_{1c} values (15). The authors hypothesized that HCY causes damage to vessels exposed to advanced glycation and products and, by this mechanism, contributes to microvascular damage in eyes and kidneys. Furthermore, other authors found a correlation between low levels of tHCY and glomerular hyperfiltration occurring in diabetic patients (16-18). This condition might determine an increase in amino-acid catabolism and therefore tHCY decrease in plasma. Nowadays, mild or latent HHCY has emerged as a new risk factor for cardiovascular diseases (3). While its role in Type 2 diabetes mellitus has been clearly defined (19), no conclusive results have been reported in T1DM up to now. Wotherspoon F et al. found mild HHCY in microalbuminuric DM patients (20). Interestingly, in our study population, lacking any sign of microvascular complications, only a small percentage of patients (1/41, 2.4%) showed tHCY values above the normal range (4.9% fasting and 2.4% PML). These patients and those with mild HHCY could therefore represent a population to be carefully followed to lower their risk of developing late macroangiopathic complications (21).

For this purpose, we recommend to perform yearly tHCY screening both during fasting and after methionine load to identify patients with HHCY during fasting and the few patients with only PML HHCY.

It has been stated that high folic acid intake could prevent atherosclerotic vascular disease by reducing tHCY levels (22): for this reason, food fortification with folate could be proposed for T1DM patients.

However, oral folic acid supplementation must be started early, because it successfully lowers plasma tHCY levels but is not able to improve endothelial function nor to reduce oxidant stress (23).

Clinical trials to evaluate the efficacy of prevention treatment in these subjects could contribute to reduce the risk of developing arteriosclerotic vascular complications of diabetes mellitus.

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