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Homocysteine and its relation to B-vitamins in Graves' disease before and after treatment: effect modification by smoking

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Abstract. Nedrebø BG, Hustad S, Schneede J, Ueland PM, Vollset SE, Holm PI, Aanderud S, Lien EA (University of Bergen; Haukeland University Hospital, Bergen; and Haugesund Hospital, Haugesund; Norway). Homocysteine and its relation to B-vitamins in Graves' disease before and after treatment: effect modification by smoking. *J Intern Med* 2003; **254:** 504–512.

Objectives. To investigate plasma total homocysteine levels and its relation to B-vitamins and smoking in Graves' disease before and after antithyroid therapy.

Design. A longitudinal study taking place at four hospitals in Norway.

Methods and subjects. Plasma total homocysteine, serum folate, serum cobalamin and riboflavin, flavin mononucleotide and flavin adenine dinucleotide in plasma were investigated in 182 patients with hyperthyroidism before treatment. The same parameters were reinvestigated in 112 of these patients after attaining euthyroid state.

Results. In hyperthyroidism, plasma total homocysteine was low, and inversely related to folate, cobalamin and riboflavin, and positively related to serum creatinine and age. Following antithyroid therapy, total homocysteine increased and the concentration of folate, cobalamin, riboflavin, flavin mononucleotide and flavin adenine dinucleotide decreased significantly. The most pronounced reduction (35%) was observed for flavin mononucleotide. In the hyperthyroid state, smokers had lower levels of folate and flavin mononucleotide than non-smokers. After restoration of euthyroidism, both folate and riboflavin were significantly lower in smokers than non-smokers. Plasma total homocysteine increased according to decreasing quartiles of B-vitamins. For riboflavin, this relation was confined to smokers.

Conclusion. Plasma total homocysteine changes according to thyroid status. These changes may be partly attributable to altered folate, cobalamin but also riboflavin status, particulary in smokers.

Keywords: folate, homocysteine, hyperthyroidism, riboflavin, smoking.

Introduction

Homocysteine is an intermediary sulphur-containing metabolite generated by demethylation of the essential amino acid methionine [1]. An elevated concentration of fasting plasma total homocysteine (tHcy) is a risk factor for a diversity of pathological conditions, including cardiovascular disease, cognitive impairment, Alzheimer's disease and adverse pregnancy outcomes [2–4].

Plasma tHcy is determined by genetic and physiological traits, like age and gender and by several nutritional and lifestyle factors [5]. Folate status is an independent and strong predictor of plasma tHcy in all age groups, whereas cobalamin is an important predictor particularly in the elderly

[6]. In addition, it has been recently reported that plasma riboflavin is an independent determinant of plasma tHcy in healthy subjects [7–9].

Amongst the lifestyle factors, smoking is a strong predictor of plasma tHcy. In the Hordaland Homocysteine Study, tHcy increased markedly with daily number of cigarettes in all age groups, and the smoking effect was particularly strong in women [10]. Smoking is associated with low folate and cobalamin levels [11–13], and low intake of fruit and vegetables [14], but whether or not impaired B-vitamin status fully accounts for the elevated tHcy concentration in smokers is debated [5, 10].

Plasma tHcy is also influenced by renal failure and thyroid dysfunction [15, 16]. Notably, there are consistent reports on elevated plasma tHcy in hypothyroidism [16–21] and low levels in hyperthyroidism [16, 20, 21]. The effect of thyroid function on plasma tHcy has been partly explained by altered renal function and folate status [19, 20, 22], but altered riboflavin metabolism has been considered as well [23].

In addition to a pronounced effect on homocysteine and B-vitamin status, smoking influences the relapse rate after medical treatment, ophthalmopathy and thyroid volume in Graves' disease [24, 25]. Thus, published data indicate that smoking influences the course of Graves' disease, and may also modify the B-vitamin status previously described in patients with thyroid dysfunction. This motivated the present study of tHcy and related B-vitamins according to thyroid and smoking status. A total of 182 patients with hyperthyroidism were investigated before treatment and 112 of these patients after restoration of euthyroidism.

Materials and methods

Subjects and study design

Between January 1997 and July 1999, 218 consecutive patients (214 Caucasian, four Asian) between 16 and 75 years of age were enrolled in a randomized trial of Graves' disease. The inclusion and exclusion criteria have been described in detail elsewhere [25]. The patients were recruited at four hospitals in Norway. The diagnosis was based on the clinical signs of hyperthyroidism combined with suppressed serum thyrotropin and positive thyrotropin receptor antibodies or ophthalmopathy. Euthyroidism was defined as free thyroxine $\leq$20 pmol L^{-1}. Blood samples were available from 182 patients before treatment and 112 patients after restoration of euthyroidism. Only one patient with complete follow-up data reported intake of vitamin supplement containing folate and/or cobalamin. No patients used gastric acid inhibitors, and three patients with diabetes received insulin treatment. None used oral antidiabetic drugs.

The patients were randomized to two different antithyroid therapeutic regimens. Half of the patients received high-dose antithyroid drugs, with the addition of L-thyroxine when normal serum levels of free thyroxine were achieved. The other half was given a titration regimen, whereby drug doses were adjusted according to the free thyroxine levels [25]. Carbimazole was the primary antithyroid drug and was used in an initial mean dose of 29.5 mg day^{-1}, except in six patients who received propylthiouracil at a daily dose of 200–400 mg.

Patients with mild drug reactions to carbimazole were switched to propylthiouracil if they did not refuse to use antithyroid drug. Clinical and biochemical assessments were carried out before treatment, and after 1.5, 3 and 6 months.

The Regional Ethics Committee, University of Bergen approved the study protocol, and the study subjects gave their written informed consent prior to participation in the study.

Blood sampling

Blood samples were collected into Venoject tubes (Terumo Europe N.V., Leuven, Belgium) after overnight fasting. Ethylenediaminetetraacetic acid (EDTA) blood sample was immediately put on ice and centrifuged within 30 min to obtain plasma. Plain silicon-coated tubes were centrifuged within 1 h to obtain serum. Serum and EDTA plasma samples were stored at −20 °C for about 1 year and later stored at −80 °C until analysis.

Biochemical analysis

Plasma tHcy was analysed by high-performance liquid chromatography and fluorescence detection [26]. Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were determined in EDTA plasma by a modification [7] of the method described by Hustad *et al.* [27]. Serum

folate was determined by a *Lactobacillus casei* microbiological assay [28], and serum cobalamin by a *Lactobacillus leichmannii* microbiological assay [29]. Both the folate and cobalamin assays were adapted to a microtitre plate formate and carried out by a robotic workstation (Microlab AT plus 2, Hamilton Bonaduz, Switzerland). The within- and between-run variations were 6.0 and 6.3% for serum folate and 5.4 and 6.7% for cobalamin. Serum creatinine was determined using the Technicon Chem 1 system (Technicon Instruments, Terrytown, NY, USA). Serum thyrotropin and free thyroxine were measured using the AutoDELFIA hTSH Ultra kit and AutoDELFIA Free Thyroxine (FT4) kit, respectively (Wallac Oy, Turku, Finland). Thyrotropin receptor antibodies was determined by radioimmunoassay (DLD Diagnostika GmbH, Hamburg, Germany). Thyrotropin receptor antibodies was defined as positive above 13 U L^{-1}.

Statistical analysis

Biochemical characteristics of the patients before and during treatment are given as medians and fifth to 95th percentiles. Differences between smokers and non-smokers were assessed using the chi-square test and Mann–Whitney *U*-test, as appropriate. Wilcoxon matched pairs signed rank sum test was used to explore changes within groups during treatment. The correlation between serum folate, serum cobalamin, plasma riboflavin, plasma FMN, plasma FAD, serum creatinine, serum free thyroxine, age and plasma tHcy were assessed using Spearman's rank correlation test.

Multiple linear regression models were used to assess the simultaneous relationship between the various predictors and plasma tHcy levels. The regression coefficients estimated the difference in mean tHcy between the reference quartile and the other quartiles for each predictor. tHcy concentrations across quartiles of each predictor were tested for linear trend. In the multiple regression models, effect modification was assessed by including product terms (e.g. smoking × riboflavin and smoking × folate). The statistical tests were two-tailed, and *P*-values of <0.05 were considered statistically significant.

All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA), version 11.0.

Results

Characteristics of patients

There were more women ($n = 157$, 86%), than men ($n = 25$) in the study group. Forty-three per cent of the patients were current tobacco smokers. The median age (range) was 43 (17–73) years. For the whole study population ($n = 182$), median (5–95th percentiles) concentration for plasma tHcy [8.1 (5.7–14.3) μmol L^{-1}], serum folate [10.5 (5.4–31.0) nmol L^{-1}], plasma riboflavin [9.7 (4.0–35) nmol L^{-1}], plasma FMN [8.6 (3.7–24.4) nmol L^{-1}], plasma FAD [60.5 (43.5–90.8) nmol L^{-1}], serum cobalamin [401 (216–708) pmol L^{-1}], serum creatinine [58 (42.3–78.8) μmol L^{-1}], and serum free thyroxine [62.0 (26.2–77.0) pmol L^{-1}] were obtained. Plasma tHcy was higher, and serum folate and plasma FMN were significantly lower in smokers than in non-smokers. The concentrations of the other blood indices were not different in smokers as compared with non-smokers. The characteristics before treatment were essentially the same in the subgroup with complete data sets ($n = 112$) (Table 1).

Bivariate correlation before treatment

Plasma tHcy showed a strong, inverse relationship to serum folate and serum cobalamin, a moderate inverse relation to plasma riboflavin and serum free thyroxine, and a positive relation to both age and creatinine. There was a strong and positive relation between plasma riboflavin and plasma FMN, and a moderate relation between age and creatinine. The levels of the vitamins, cobalamin, folate and riboflavin, were positively correlated (Table 2).

Changes during treatment

Euthyroidism was obtained in 81 patients (73%) after 1.5 months, in 18 patients (16%) after 3 months and in the remaining 11 patients after 6 months of antithyroid treatment.

Treatment of hyperthyroid patients with antithyroid drugs was associated with a moderate (about 12%) increase in plasma tHcy and serum creatinine (Fig. 1, Table 1). After treatment there was a marked (35%) reduction in plasma FMN, a moderate (20%) reduction in plasma folate and a

Table 1 Blood indices in non-smokers and smokers, before and after restoring the euthyroid state in patients with data before and after treatment

	Hyperthyroid			Euthyroid		
	All subjects ($n = 112$)	Non-smokers ($n = 66$)	Smokers ($n = 46$)	All subjects ($n = 112$)	Non-smokers ($n = 66$)	Smokers ($n = 46$)
Plasma tHcy (μmol L^{-1})	8.3 (5.7–15.2)	8.0 (5.4–13.9)	8.8 (5.9–17.7)*	9.2 (6.1–17.7)§	8.9 (5.9–18.3)§	10.3 (6.3–19.3)§,*
Serum folate (nmol L^{-1})	9.9 (4.9–23.0)	11.0 (4.9–29.7)	8.7 (4.6–22.5)†	8.1 (4.9–15.7)§	8.6 (5.2–15.9)§	7.2 (4.6–13.5)§,†
Plasma riboflavin (nmol L^{-1})	9.8 (3.8–31.9)	10.8 (4.5–34.1)	9.2 (3.1–28.1)	7.8 (3.0–31.4)§	9.6 (4.1–35.9)	6.4 (2.7–17.0)§,*
Serum cobalamin (pmol L^{-1})	397 (217–649)	386 (217–691)	412 (199–649)	338 (192–576)§	339 (196–620)§	334 (180–581)§
Plasma FMN (nmol L^{-1})	8.3 (4.1–19.2)	9.0 (4.3–19.6)	7.7 (3.0–22.0)*	5.3 (2.5–12.4)§	5.4 (2.5–13.8)§	4.5 (2.3–11.3)§
Plasma FAD (nmol L^{-1})	62 (44.2–91.0)	63.2 (44.6–93.4)	61.5 (43.3–84.5)	57.7 (42.7–80.4)§	58.5 (42.6–83.5)§	56.6 (43.0–77.7)‡
Serum creatinine (μmol L^{-1})	56.5 (41.0–73.7)	56.0 (41.4–82.8)	58 (37.0–72.8)	70.0 (51.6–88.6)§	70.5 (48.5–88.7)§	69.0 (54.2–88.3)§
Free thyroxine (pmol L^{-1})	65 (23.2–77.0)	65.5 (24.8–77.0)	61.0 (17.7–77.0)	13.0 (5.0–20.0)§	13.5 (6.3–20.0)§	13.0 (4.0–20.00)§

tHcy, total homocysteine; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.
Values are given as median and fifth to 95th percentiles.
For comparison between non-smokers and smokers (Mann–Whitney *U*-test), *$P < 0.05$, †$P < 0.005$.
For comparison between hyperthyroid and euthyroid state (Wilcoxon matched pairs signed rank sum test), ‡$P < 0.05$, §$P < 0.005$.

slight (<20%) reduction in serum cobalamin, plasma riboflavin and plasma FAD (Fig. 1). All blood indices changed significantly following treatment, except riboflavin in non-smokers (Table 1).

After antithyroid treatment, smokers still had significantly higher tHcy, and lower serum folate levels than non-smokers (Table 1), and also plasma riboflavin was now significantly lower in smokers than non-smokers (Table 1). The concentrations of tHcy and B-vitamins after treatment did not differ according to the two antithyroid regimens (data not shown). Only 10 of 112 patients (8.9%) had a tHcy concentration above 15 μmol L^{-1} after they attained euthyroidism.

Plasma tHcy according to vitamin and creatinine levels by multiple regression

The changes in plasma tHcy were determined according to B-vitamin and creatinine levels in hyperthyroid patients and after restoration of euthyroidism. High creatinine and low levels of riboflavin, folate and cobalamin were associated with elevated plasma tHcy, corresponding to a difference from 0.9 to 3.7 μmol L^{-1} when comparing the highest and lowest quartiles before treatment in a simple model (adjustment for sex and age). These relations were observed both before and after treatment, but the strength of the associations was somewhat reduced after multiple adjustments (Table 3).

The changes in tHcy according to concentrations of B-vitamin and creatinine were also determined separately according to smoking habits. For cobalamin, creatinine, folate, and in particular for riboflavin, the associations with plasma tHcy were stronger in smokers than in non-smokers (Table 4). Accordingly, a statistically significant interaction between smoking and riboflavin both before ($P = 0.043$) and after treatment ($P = 0.028$) was demonstrated. No significant interaction between smoking and folate was observed.

Discussion

This study, including 182 patients, is the largest published study on homocysteine and B-vitamin status in hyperthyroidism. Longitudinal data on the same parameters were obtained in 112 patients receiving therapy with antithyroid

Table 2 Spearman correlation coefficients before treatment

	tHcy	Folate	Cobalamin	Riboflavin	FMN	FAD	Creatinine	Age
Folate	−0.439*							
Cobalamin	−0.421*	0.246*						
Riboflavin	−0.190**	0.166**	0.292*					
FMN	−0.140	0.261*	0.257*	0.578*				
FAD	−0.064	0.059	−0.007	0.153**	0.239*			
Creatinine	0.314*	0.040	−0.151	−0.031	0.034	0.030		
Age	0.306*	0.196*	−0.117	0.116	0.045	0.131	0.260**	
Free thyroxine	−0.245*	0.085	0.379*	0.276*	0.321*	−0.06	−0.429*	−0.128

tHcy, total homocysteine; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.
*$P < 0.01$, **$P < 0.05$.

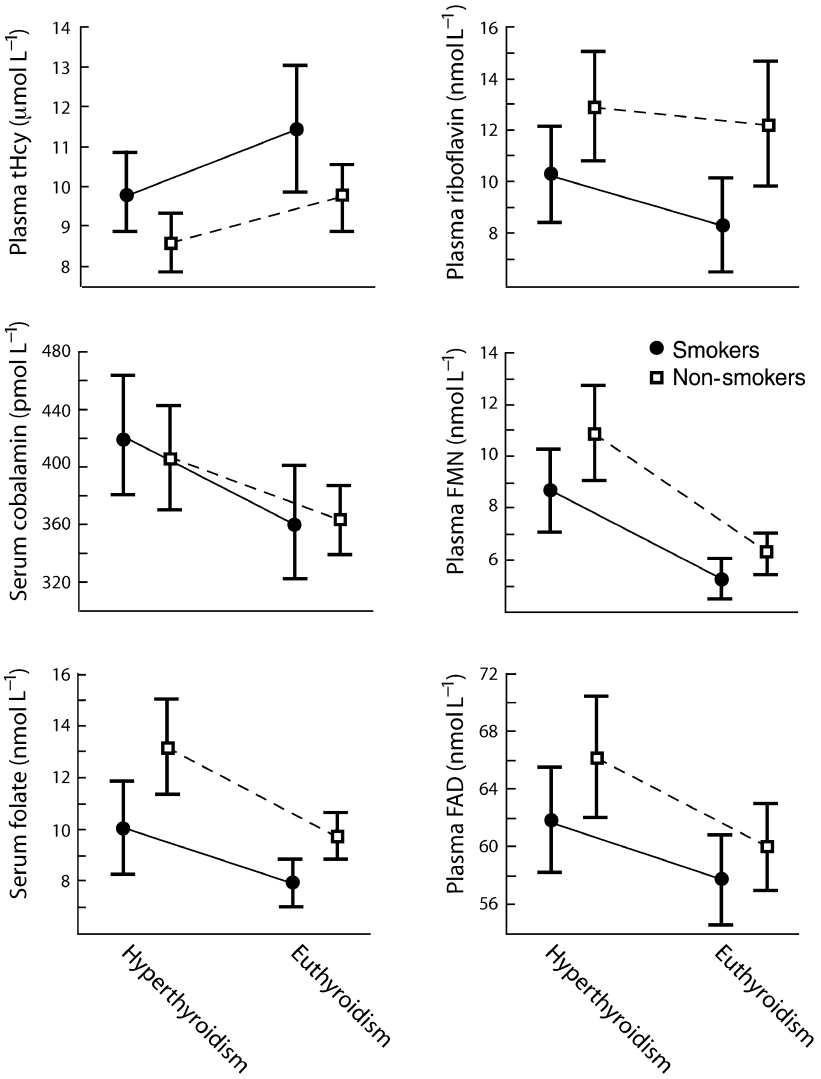

Fig. 1 Change in plasma tHcy and related B-vitamins following treatment of hyperthyroid patients with antithyroid therapy. Data presented are from 112 patients for which complete data set were obtained in the hyperthyroid and euthyroid state. Data are shown separately for smokers and non-smokers. Error bars represent mean and 95% confidence intervals.
tHcy, total homocysteine; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

drugs. Normalization of thyroid hormone levels was associated with a significant increase in plasma tHcy, a marked (35%) reduction in plasma FMN, a moderate (20%) reduction in plasma folate, whereas decreases in riboflavin and cobalamin levels were modest. The plasma/serum levels of riboflavin,

Table 3 Plasma tHcy across quartiles of B-vitamins and creatinine in patients with Graves' disease before and after treatment

	Estimated change in tHcy (μmol L^{-1})			
	Simple adjustment[a]		Multiple adjustment[b]	
	Before treatment ($n = 182$)	After treatment ($n = 112$)	Before treatment ($n = 182$)	After treatment ($n = 112$)
Plasma riboflavin (nmol L^{-1}) (versus highest quartile, Q4)				
Q3	0.8	0.6	0.4	1.2
Q2	0.9	1.2	1.1	1.9
Q1	1.8	3.3	1.1	2.8
P for trend	0.002	0.007	0.042	0.044
Serum folate (nmol L^{-1}) (versus highest quartile, Q4)				
Q3	0.7	0.7	0.9	0.5
Q2	1.4	1.5	1.2	1.4
Q1	3.7	4.7	3.7	4.8
P for trend	<0.001	<0.001	<0.001	<0.001
Serum cobalamin (pmol L^{-1}) (versus highest quartile, Q4)				
Q3	0.7	0.9	0.6	0.9
Q2	1.8	1.6	1.1	1.5
Q1	2.4	2.6	1.6	0.4
P for trend	<0.001	0.025	0.003	0.522
Serum creatinine (μmol L^{-1}) (versus lowest quartile, Q1)				
Q2	0.6	0.6	0.6	1.4
Q3	1.2	0.7	1.3	2.0
Q4	0.9	2.9	1.2	3.1
P for trend	0.083	0.040	0.027	0.009

tHcy, total homocysteine.
[a]Adjusted for sex and age.
[b]Adjusted for sex, age, riboflavin, folate, cobalamin, creatinine and total triiodothyronine.

folate, and cobalamin were strong determinants of plasma tHcy both in the hyperthyroid and euthyroid state, but for plasma riboflavin in particular, this association was confined to smokers. Smokers had a less favourable B-vitamin status and higher plasma tHcy than non-smokers.

The strength of this study is the relatively large study population and its prospective, longitudinal design. This allows comparison of tHcy and vitamin status in the same patients both in hyperthyroid and euthyroid state. The patients were followed during therapy for 1.5, 3 and 6 months, and were regarded as euthyroid after normalization of free thyroxine.

The study assesses folate and cobalamin levels and renal function (in terms of serum creatinine), indices known to be major determinants of plasma tHcy [30]. In addition, the riboflavin status was included, which recently has been established as an independent determinant of tHcy [7–9]. This parameter may be particularly important because of the documented effects of thyroid hormones on riboflavin metabolism [31, 32].

The variations in vitamin status according to smoking habits led to separate investigation of 46 smokers and 66 non-smokers, for whom data were obtained before and after treatment. The limited number of smokers gave small subgroups defined by quartiles of vitamin concentrations (Table 4). Thus, estimates of tHcy changes according to vitamin levels in this subgroup show trends, but lack statistical power to provide significant results.

Plasma tHcy in hyperthyroid patients was positively related to creatinine and age, and inversely related to serum folate and serum cobalamin (Table 2). Similar observations were made after therapy (Table 3). These findings confirm existing data on tHcy determinants in humans [6, 10, 15, 33]. In addition, tHcy was related to plasma riboflavin, but showed a weaker association with plasma FMN and essentially no relation to plasma FAD. Such relations between tHcy and vitamin B2 indices have recently been observed in healthy adults [7, 8].

Table 4 Plasma tHcy across quartiles of B-vitamins and creatinine in patients with Graves' disease (non-smokers versus smokers) before and after medical treatment

	Estimated change in tHcy (μmol L^{-1})							
	Simple adjustment[a]				Multiple adjustment[b]			
	Before treatment		After treatment		Before treatment		After treatment	
	Non-smokers ($n = 104$)	Smokers ($n = 78$)	Non-smokers ($n = 66$)	Smokers ($n = 46$)	Non-smokers ($n = 104$)	Smokers ($n = 78$)	Non-smokers ($n = 66$)	Smokers ($n = 46$)
Plasma riboflavin (nmol L^{-1}) (versus highest quartile, Q4)								
Q3	0.4	1.2	1.6	0.7	0.9	0.1	2.2	1.4
Q2	0.5	1.9	0.2	3.0	0.7	0.9	0.5	1.5
Q1	0.7	3.2	1.4	5.9	0.3	2.2	1.3	3.0
P for trend	0.342	<0.001	0.456	0.009	0.926	0.008	0.556	0.259
Serum folate (nmol L^{-1}) (versus highest quartile, Q4)								
Q3	−0.1	1.3	0.8	0.5	0.2	1.1	1.1	−0.2
Q2	−0.1	2.0	1.2	1.6	−0.2	1.0	1.7	−1.4
Q1	2.6	5.3	2.1	5.9	2.8	5.1	2.8	5.7
P for trend	<0.001	<0.001	0.065	0.021	0.003	<0.001	0.037	0.044
Serum cobalamin (pmol L^{-1}) (versus highest quartile, Q4)								
Q3	1.2	0.8	1.2	1.6	0.8	1.5	−0.4	1.9
Q2	2.1	1.7	0.0	1.6	1.8	0.5	−1.4	2.9
Q1	2.0	3.9	1.8	6.7	1.7	2.1	0.9	5.3
P for trend	0.008	<0.001	0.236	0.008	0.045	0.107	0.580	0.211
Serum creatinine (μmol L^{-1}) (versus lowest quartile, Q1)								
Q2	0.5	0.1	0.9	0.2	1.1	0.7	2.9	0.7
Q3	0.9	1.5	0.2	1.7	1.3	0.5	0.4	1.8
Q4	1.1	1.4	0.8	5.1	0.8	1.3	2.2	5.7
P for trend	0.150	0.094	0.729	0.043	0.143	0.078	0.343	0.112

tHcy, total homocysteine.
[a]Adjusted for sex and age.
[b]Adjusted for sex, age, riboflavin, folate, cobalamin, creatinine and total triiodothyronine.

Normalization of thyroid status in hyperthyroid patients resulted in increased plasma tHcy and reduction in serum/plasma levels of B-vitamins, in particular FMN and folate (Fig. 1). This observation is consistent with previous reports on increased tHcy and lower folate levels in hypothyroid patients, and low tHcy and adequate vitamin status in hyperthyroid patients [19–21, 34]. The underlying mechanisms could be related to effects of thyroid hormones on folate metabolizing enzymes, including methylenetetrahydrofolate reductase [35]. Likewise, thyroid hormones have a marked effect on riboflavin metabolism, mainly by stimulating flavokinase, and thereby the synthesis of FMN and FAD [31, 32]. This effect on flavokinase may also explain the marked reduction in plasma FMN as compared with the modest change in plasma riboflavin following treatment. Taken together, the present study shows that thyroid status influences on the concentration or function of several B-vitamins which all converge on homocysteine metabolism.

In hyperthyroid patients, smokers had higher tHcy and lower serum folate and plasma FMN levels than non-smokers (Table 1), whereas after antithyroid treatment, smoking was associated with low serum folate and plasma riboflavin. These observations are in agreement with published data on poor dietary habits and impaired B-vitamin status in smokers [5].

In most regression models, the changes in tHcy according to B-vitamin quartiles were stronger in smokers than in non-smokers (Table 4). This effect of smoking may be explained by a stronger relation between tHcy and B-vitamins in the lower vitamin concentration ranges prevailing in smokers. For plasma riboflavin, the effect modification by smoking was statistically significant. Taken together, our data highlight the importance of riboflavin status for

the marked plasma tHcy changes according to thyroid status observed in smokers (Table 1).

The higher plasma tHcy concentration across vitamin quartiles in smokers compared to non-smokers may also point to impaired vitamin status in tissues exposed to cigarette smoke, which is not reflected in blood levels of vitamins [36]. A recent investigation on the role of diet and specific micronutrients in the etiology of oral carcinoma also suggests an interaction between tobacco smoking and riboflavin [37].

In conclusion, this study demonstrates changes in plasma tHcy, folate and creatinine according to thyroid function, consistent with results from several published studies [16–21]. Independent of thyroid status, higher tHcy and lower B-vitamin levels were demonstrated in smokers compared with non-smokers. The novel and most important findings are a marked reduction in plasma FMN following treatment of hyperthyroid patients, and an effect modification of the tHcy-riboflavin relation by smoking. Thus, data presented here further emphasize the negative effect of smoking on the metabolic balance in patients with thyroid dysfunction [25].

Conflict of interest statement

No conflict of interest was declared.

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