

Impact of Renal Function or Folate Status on Altered Plasma Homocysteine Levels in Hypothyroidism

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Abstract. Hyperhomocysteinemia is an independent risk factor for coronary, peripheral and cerebrovascular diseases. Moderately elevated total homocysteine (tHcy) levels have been reported in patients with overt hypothyroidism. Plasma tHcy concentration is affected by several physiological factors and is elevated under conditions of impaired folate and cobalamin status and in renal failure. The aim of this study was to assess plasma tHcy concentrations and to evaluate the role of potential determinants of plasma tHcy levels in hypothyroid patients. Fasting plasma tHcy, serum homocysteine-related vitamins folate and vitamin B₁₂, serum cystatin C (CysC) and creatinine, were determined in 22 hypothyroid patients and compared with 25 healthy control subjects. Creatinine clearance (CCr) was calculated using the Cockcroft-Gault formula. Plasma tHcy levels were determined by HPLC with fluorescence detection and serum CysC by automated particle enhanced immunoturbidimetry. Plasma tHcy, creatinine levels were significantly higher, and serum CysC levels, and creatinine clearance values were lower in hypothyroid patients than in control subjects. Folate levels were lower in hypothyroid group compared to the control group. There were no differences in vitamin B₁₂ levels between hypothyroid and control groups. Positive correlation was noted between tHcy and creatinine levels in hypothyroid patients ($r = 0.596$); however, an inverse correlation was found between tHcy and folate levels ($r = -0.705$) in hypothyroid patients. In conclusion, tHcy was increased in hypothyroidism, and this increase was more strongly associated with changes in serum folate than in serum creatinine and CysC, suggesting an altered folate status.

Key words: Hypothyroidism, Homocysteine, Cystatin C, Renal function, Folate status

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THYROID status has a profound influence on a variety of biochemical processes, some of which may have secondary effects on homocysteine metabolism. Elevated plasma total homocysteine (tHcy) levels have been reported in patients with overt hypothyroidism and hypothyroidism is associated with an increased risk for arteriosclerotic coronary artery disease [1]. Homocysteine (Hcy) is a sulphur-containing nonproteinogenic amino acid biosynthesized during the conversion of methionine to cysteine. Hcy is metabolized by one

of two pathways: remethylation or transsulfuration. In the remethylation pathway, reaction is catalyzed by the enzyme, methionine synthetase, and requires both B12 as a necessary cofactor and 5-methyltetrahydrofolate, derived from folate, as a methyl donor [2].

The mechanism by which hypothyroidism causes hyperhomocysteinemia is not clear. Experimental studies have indicated that thyroid hormones affect folate metabolism [3]. The observations that methylenetetrahydrofolate reductase is increased in hyperthyroidism and decreased in hypothyroidism [4] may be relevant for the relation between plasma homocysteine levels and thyroid status. On the other hand, both animal and human studies have demonstrated that hypothyroidism is associated with low and hyperthyroidism with high glomerular filtration rate, which in turn is closely re-

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lated to plasma tHcy concentrations [5, 6].

Kidney is the major site for the removal and metabolism of tHcy [7]. The pathogenesis of hyperhomocysteinemia in hypothyroidism likely involves a reduction in glomerular filtration rate (GFR) [8]. In clinical practice, serum creatinine and creatinine clearance (CCr) determinations are the most widely used indexes for the noninvasive assessment of GFR. Recent investigations have indicated that serum cystatin C (CysC) is superior to serum creatinine for the detection of early decreases in GFR [9–13]. CysC comprises one non-glycosylated polypeptide chain with 120 amino acid residues, having a molecular weight of 13 kD. Recently, several studies demonstrated that thyroid dysfunction has a major impact on Cys C levels [14–18].

The aim of this study is to assess plasma tHcy concentration and to evaluate the impact of renal function or folate status on plasma Hcy levels in hypothyroidic patients.

Materials and Methods

Patients

This study included 22 patients (5 males, 17 females, ages 35–66 yr) with primary hypothyroidism, who received care at the Department of Endocrinology of Celal Bayar University Hospital. The study was approved by the local Ethics Committee and all subjects were informed about the nature of the study and their consent was obtained. These patients had elevated serum thyrotropin (TSH) levels and had low free T₃ (FT₃) and free T₄ (FT₄) levels. The cause of hypothyroidism was chronic autoimmune thyroiditis (n = 11), subacute thyroiditis (n = 5), thyroidectomy (n = 3), ¹³¹I treatment (n = 2) and prolonged overdose of thiamazol (n = 1).

The control group consisted of 25 euthyroid healthy subjects (7 males, 18 females, ages 42–60 yr). Euthyroid subjects had serum concentrations of TSH, FT₃ and FT₄ within normal reference ranges. None of the patients and controls were on a special diet or used nutritional supplements; they did not use any medication known to interfere with thyroid hormone or Hcy metabolism.

Blood samples were taken from the subjects after 12–14 h overnight fasting for the analysis of clinical chemical parameters including plasma tHcy, serum CysC, creatinine, folate, vitamin, B₁₂, FT₃, FT₄ and TSH.

Methods for assay

Serum CysC levels were measured using latex particle-enhanced turbidimetry (PET) kits (DAKO, Cystatin C PET kit, Glostrup, Denmark) on the Hitachi 704 automatic analyzer (Boehringer Mannheim GmbH, Mannheim, Germany).

For Hcy analyses, blood was collected in EDTA containing tubes, the plasma were separated within 15–20 minutes after collection and stored at –20°C. THcy in plasma was measured by HPLC and fluorescence detection as described elsewhere [19]. A Shimadzu LC 10A HPLC system (Shimadzu Corp., Kyoto, Japan), consisting of a Shimadzu LC-10AD pump, a Shimadzu SIL-10AXL autoinjector with a 20 µL loop and a Shimadzu RF-10AXL fluorescence detector, was used. The system was controlled through a Shimadzu CBM-10A communication module and a personal computer. The column was EC 150/4.6 Nucleosil 100-5 C18 5 µm (Macherey-Nagel, Duren, Germany). Tri-n-butylphosphine (TBP) in dimethyl formamide (DMF) was applied for reduction of disulfide-bound Hcy and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBDF) served as a derivatization agent. Cysteamine hydrochloride was added as an internal standard.

Serum creatinine was determined by a kinetic Jaffe reaction (Technicon Dax-48, Bayer Diagnostics, Toshiba, Tokyo, Japan). Since measurement of CCr requires the collection of urine over a 24 h period, which is inconvenient for patients and often leads to inaccurate collections, we preferred the Cockcroft-Gault formula [20]: CCr (ml/min) = 140-age (years) × weight (kg)/0.81 × creatinine (µmol/L). For females, the value obtained by this equation was multiplied by 0.85.

Folate and vitamin B₁₂ in serum were measured by chemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics GmbH, Mannheim, Germany).

Thyroid hormones, FT₃, FT₄, and TSH were determined by chemiluminescent method (Immulite 2000, Diagnostic Products Corp. Los Angeles, CA, USA).

Statistical analysis

For statistical analysis the results were subjected to nonparametric tests (Mann-Whitney U Test) and data are expressed as median (min-max), because of the skewed distribution of data. Correlations were tested by the Spearman correlation test. Furthermore, multiple regression analysis was performed for identifying

independent determinants of Hcy in each study group. After this procedure, covariance analysis was performed to determine the sole group effect on the dependent variable Hcy. All statistical procedures were performed using the SPSS (Statistical Package for Social Sciences) Windows version 10.0, (SPSS Inc., Chicago, IL, USA).

Results

Clinical and biochemical characteristics of the patients and control group are summarized in Table 1. Values are expressed as median (min-max).

Age and sex distributions of the hypothyroid patients and controls were similar. Plasma tHcy, serum CysC, serum creatinine concentrations and CCr values showed significant differences between the two study groups. Plasma Hcy and serum creatinine concentrations were significantly higher in hypothyroid patients than in the controls. Serum CysC concentrations and creatinine clearance values of the hypothyroid patients were lower than the healthy controls.

While there were no differences in serum vitamin B₁₂ levels between hypothyroid patients and the control group, folate levels of the hypothyroid patients were significantly lower than control group.

Positive correlation was observed between tHcy and creatinine levels ($r = 0.596$, $p = 0.002$); however, a significant inverse correlation was found between tHcy and folate levels ($r = -0.705$, $p = 0.0001$). There were no correlations between plasma Hcy and CCr values and serum CysC levels in hypothyroid patients. For control subjects, positive correlation was observed between folate and vitamin B₁₂ levels ($r = 0.683$, $p =$

0.0001); however, there were no other correlations.

For each independent variable (CysC, folate, vitamin B₁₂, creatinine and CCr) the degree of contribution to plasma Hcy levels was determined by multiple regression analysis. None of the parameter had significant effect on the control group. However, all parameters, except vitamin B₁₂, were effective significantly. According to the standardized coefficient values, folate showed the strongest effect (beta value -0.664), which was followed by, in decreasing order, CCr (beta value -0.436), creatinine (beta value 0.419) and CysC (beta value -0.406). Covariance analysis showed that Hcy values were significantly ($p = 0.004$) different between controls and hypothyroid patients after the correction. The uncorrected and corrected means are shown in Table 2.

Discussion

Several studies have demonstrated elevated Hcy levels in hypothyroidism with improvement after T₄ replacement [21–26]. However, three case-control studies [27–29] have reported no differences in Hcy levels

Table 2. The uncorrected and corrected means for plasma homocysteine

	Plasma Homocysteine Concentration (μmol/L)			
	Uncorrected		Corrected	
	Mean	SD	Mean	Std. Error
Control subjects	9.94	1.86	12.5	0.849
Hypothyroid patients	19.65	6.06	16.96	0.874

Table 1. Clinical and laboratory characteristics of the patients and control groups [median (min-max)]

	Control subjects (n = 25)	Hypothyroid patients (n = 22)	Statistical significance
Males n (%)	7 (28%)	5 (22%)	NS
Females n (%)	18 (72%)	17 (78%)	NS
Age (year)	50 (42–60)	51 (35–66)	NS
FT ₃ (pmol/L)	4.62 (3.85–6.16)	2.65 (1.35–3.86)	P = 0.0001
FT ₄ (pmol/L)	15.48 (10.32–20.64)	7.30 (4.80–9.92)	P = 0.0001
TSH (mU/L)	1.3 (0.8–2.2)	34.60 (27.30–38.40)	P = 0.0001
Folate (nmol/L)	10.3 (6.5–17.2)	7.22 (5.40–13.10)	p = 0.0001
Vitamin B ₁₂ (pmol/L)	378 (160–486)	368 (248–420)	NS (P = 0.170)
Hcy (μmol/L)	9.4 (6.8–11.6)	15.42 (9.80–31.72)	P = 0.0001
Cys C (mg/L)	1.19 (0.87–1.37)	0.78 (0.60–1.28)	P = 0.0001
Creatinine (μmol/L)	83.36 (52.54–107.25)	98.28 (64.36–136.42)	P = 0.002
Creatinine clearance (ml/min)	92.0 (68.0–137.0)	72.0 (58.0–122.0)	P = 0.002

between individuals with subclinical hypothyroidism and euthyroid controls. Furthermore, Christ-Crain *et al.* found no significant change in Hcy levels after treatment of subclinical hypothyroidism [25].

In our study, plasma Hcy and serum creatinine levels were high and creatinine clearance values were low and plasma Hcy concentrations correlated with serum creatinine ($r = 0.596$), but no relation was found between plasma Hcy and CCr values in hypothyroid patients. Confirming our results, Diekman *et al.* observed higher plasma Hcy concentrations and creatinine levels and lower CCr values in hypothyroidic patients. They found a linear relationship between the changes in Hcy and creatinine, but an inverse correlation between Hcy and CCr. The changes in Hcy correlated better with creatinine ($r = 0.61$) than with creatinine clearance ($r = -0.54$) [30]. Lien *et al.* observed a close relation between plasma tHcy and serum creatinine in iatrogenic hypothyroidism [21]. In addition, Montenegro *et al.* concluded that CCr was slightly decreased in patients with hypothyroidism [31]. Both the Hcy and creatinine responses can be explained by the hypodynamic circulation in hypothyroidism [32].

A slight decrease in GFR has been found in hypothyroidism. Thyroid hormones have significant effects on renal hemodynamics, renal handling of salt and water, and the active tubular transport. It is possible that tubular creatinine secretion is diminished in hypothyroidism, thereby increasing serum creatinine concentrations independently of GFR [5]. Therefore, it has been suggested that serum creatinine could not be considered as an accurate and reliable determinant of GFR in hypothyroid patients [33]. However, it has also been reported that GFR determinations using creatinine levels show results comparable to gold standard approaches such as ^{125}I -iodomate or para-aminohippurate in thyroid dysfunction [34, 35], and so changes in creatinine production or tubular handling will not be a significant confounder. Existing literature suggests that it is creatinine that is providing the correct assessment of GFR changes [6] since a definitive method to assess GFR in thyroid disease has yet to be determined.

A study in rats has identified the kidney as a major site for the removal and metabolism of tHcy [7]. Two different mechanisms may be involved. The first is the fact that the main source of Hcy is the adenosyl-methionine-dependent methylation of guanidoacetate to form creatine and its anhydride creatinine [36]. Sec-

ondly, renal function plays a central role for the clearance of both creatinine and Hcy [37, 38]. The pathogenesis of hyperhomocysteinemia in hypothyroidism likely involves a reduction in GFR, given the strong, independent association between Hcy concentrations and GFR throughout the normative range of renal function [8] and the well-established effects of hypothyroidism on the kidney [5]. Furthermore, GFR is strongly predictive of plasma Hcy levels; even mild reductions in GFR lead to increased levels of Hcy [38]. The limitations of serum creatinine as a marker of the GFR are widely appreciated. CCr may be more sensitive, but it requires a timed urine collection, which is imprecise and inconvenient. Besides, both CCr and serum creatinine values are affected by dietary protein and muscle mass. Recently, serum CysC has been introduced as a more sensitive marker for mildly impaired GFR, compared to creatinine and also as an independent determinant of plasma Hcy concentrations [9, 10]. Norlund *et al.* suggested that the increase of plasma Hcy concentrations with advancing age may be due to age-related decline in renal function. It has also been shown that cystatin C shows a higher predictive value for Hcy concentrations than age or serum creatinine [39]. Recently, the impact of thyroid dysfunction on serum CysC levels has been investigated in a few studies. Four studies have demonstrated that in untreated patients with hypothyroidism serum CysC levels were significantly lower than those in euthyroid subjects; similarly, in untreated patients with hyperthyroidism serum CysC levels were significantly higher than those in euthyroid subjects [14–17].

In agreement with the previous studies, our data indicate that serum CysC levels in patients with hypothyroidism were significantly lower than in healthy control subjects, but there were no correlations between serum CysC and tHcy, creatinine levels and CCr values. The reason for these changes in serum CysC levels remain speculative. CysC is considered to be produced in all nucleated cells at a constant rate. It was proposed that thyroid hormones may alter its production rate in the context of a changed cell turnover and/or metabolic rate in thyroid dysfunction [14–18]. Thyroid function has to be considered when CysC is used as a marker of kidney function. The inverse changes in CysC and serum creatinine seen in hypothyroid patients suggests that the effects of thyroid hormone on GFR are overridden by the effects of thyroid hormones on CysC.

Nedrebo *et al.* found that plasma Hcy, serum folate, total cholesterol, HDL-cholesterol and creatinine levels were significantly higher in patients with hypothyroidism than patients with hyperthyroidism. During treatment of both patient groups, plasma Hcy showed a strong relation with serum cholesterol and creatinine whereas there was no relation to serum folate and cobalamin. From these observations, they explained that changes in renal function, rather than vitamin status, might account for variations in plasma tHcy [22]. Contradicting this data, Barbe *et al.* found lower serum folate in the hypothyroid compared with the hyperthyroid state, and they observed the usual inverse relationship between folate and Hcy [23]. From this observation, they inferred that the changes in homocysteine may be explained by altered folate status or by a modification of the activity of folate-metabolizing enzymes, as has previously been suggested [24]. Like Barbe *et al.*, Lien *et al.* found lower serum folate during short-term iatrogenic hypothyroidism, but they observed that the changes in plasma homocysteine concentrations during the hypothyroid phase were more strongly associated with changes in serum creatinine than in folate, suggesting a renal mechanism [21]. Our

data presented here confirm the study of Barbe *et al.* [23]. In our study, folate levels were found significantly lower in hypothyroid subjects whereas vitamin B₁₂ were similar to the control group. Additionally, tHcy was inversely and strongly correlated with folate ($r = -0.705$). Our results suggested that increased Hcy concentrations in hypothyroidism may be due to the low folate levels, possibly reflecting altered folate status or modification of the activity of folate-metabolizing enzymes.

In conclusion, this study confirm, that the hypothyroid state is associated with an elevation in plasma Hcy concentration. In addition, we observed higher folate and creatinine levels and lower serum CysC levels and CCr values in hypothyroid patients than euthyroid healthy subjects. In patients with hypothyroidism, serum creatinine concentrations could be influenced by effects of thyroid hormones on the renal tubular cells, and serum CysC concentrations could be influenced by the effects of thyroid hormones on CysC production. These results support the observation that an altered folate status, rather than a renal mechanism, are behind the Hcy response.

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