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ORIGINAL ARTICLE

Hyperhomocysteinemia in acute iatrogenic hypothyroidism: the relevance of thyroid autoimmunity

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Abstract

Purpose Hyperhomocysteinemia is a known cardiovascular risk factor and a key player in the inflammatory activation of autoimmune diseases. Hashimoto's thyroiditis (HT) is the leading cause of hypothyroidism which, in itself, has been associated with a significant raise of homocysteine (Hcy) levels and increased cardiovascular risk. Our aim was to assess the impact of HT on Hcy levels in patients with acute hypothyroidism.

Methods We prospectively enrolled 121 patients (mean age: 46 years, F/M = 102/19) with acute post-surgical hypothyroidism. Based on the presence of anti-thyroid antibodies and the histological description of an inflammatory infiltrate, 26 and 95 patients were classified as HT and non-HT, respectively. Several parameters including thyroid-stimulating hormone (TSH), levels of serum free T3 and free T4, weight, glucose levels, total cholesterol, creatinine, vitamin B_{12}, ferritin and erythrocyte sedimentation rate were obtained from all patients and correlated with Hcy levels.

Results Median Hcy level in the whole cohort was 16.8 µmol/L (normal values: < 12 µmol/l). Among all parameters analysed, only Hcy levels were significantly different between HT and non-HT patients (median Hcy = 19.7 vs 16.2 µmol/L, respectively; $p = 0.018$, Mann–Whitney U test). Analysis of covariance showed the presence of HT to be the strongest predictor of Hcy levels (coefficient = 0.25534, $p = 0.001$). Serum TSH was not significantly associated with Hcy levels ($p = 0.943$).

Conclusion In patients with iatrogenic hypothyroidism, those with HT have significantly higher Hcy levels than those without HT. The increase of Hcy levels appears to be mainly determined by the HT-related immune-inflammatory condition.

Keywords Homocysteine · Hashimoto disease · Autoimmune thyroiditis · Hypothyroidism · Systemic inflammation · Cardiovascular risk

Introduction

Homocysteine (Hcy) is a non-proteinogenic, sulphur-containing amino acid derived from the metabolism of methionine. Intracellular metabolism of Hcy is regulated by enzymes using B vitamins and folates as cofactors, and

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its excess is poured into plasma [1]. Hyperhomocysteinemia is a strong independent cardiovascular risk factor producing endothelial dysfunction and atherogenesis, and is emerging as a key player in the inflammatory activation of autoimmune diseases [2]. Chronic inflammation leads to the development of oxidative stress, which depletes anti-oxidants and oxidation-sensitive substances, including B vitamins and folates, thereby producing hyperhomocysteinemia [3]. Furthermore, Hcy acts as a pro-inflammatory molecule by enhancing monocyte chemotaxis into the arterial wall and by inducing transcription of several cytokines and pro-inflammatory mediators [2].

Increased levels of serum Hcy have been linked with several disorders, including thyroid diseases [1]. In particular, overt hypothyroidism has been associated with a significant raise of Hcy levels, which has not been univocally demonstrated in subclinical hypothyroidism [4, 5]. Decreased glomerular filtration rate and urinary excretion are thought to be the main determinants of Hcy increase in overt hypothyroidism [4, 6, 7]. Reduced activity of key enzymes and cofactors involved in its metabolism might also explain Hcy elevation in hypothyroidism [8, 9]; however, contrasting data have been reported about levels of vitamin B_{12} and folates in this context, and no clear consensus has been reached so far [6, 7].

Hashimoto's thyroiditis (HT) is the leading cause of hypothyroidism in adult patients and represents the most common autoimmune disease [10]. Developments of endothelial dysfunction and increased cardiovascular risk have been demonstrated in patients with positive anti-thyroid antibodies and in patients with confirmed HT [11–15]. Nonetheless, the link between hyperhomocysteinemia and autoimmune thyroid disease has been seldom investigated. Two previous studies showed higher Hcy levels in patients with increased serum thyroid autoantibodies compared to healthy controls [16, 17], while another paper reported no differences in Hcy levels between euthyroid patients with or without thyroid autoimmunity [18]. However, the role of the concurrent presence of autoimmune thyroid disease and overt hypothyroidism on plasma Hcy levels has not been previously assessed.

The present study was designed to assess the impact of HT on plasma Hcy levels in patients with acute post-surgical hypothyroidism.

Materials and methods

Patients

Between February 2012 and February 2014, 140 patients with differentiated thyroid cancer (DTC), referred to our institution for radioiodine remnant ablation after total thyroidectomy, were prospectively enrolled in the present study. All patients gave a written informed consent to participate. As per current standard in our institution, levothyroxine was withheld 40 days before radioiodine ablation and patients were supplemented with triiodothyronine (20 µg three times/day) until 15 days before ablation. A low-iodine diet was recommended starting 10 days before ablation.

Euthyroid patients who underwent radioiodine ablation after recombinant TSH stimulation were excluded from the final analysis, as well as patients with known alterations of the Hcy metabolism or severe vitamin B_{12} defects. Patients treated with folic acid and/or vitamin B_{12} were also excluded from the study. Due to the difficulty to control all exogenous factors potentially interfering with Hcy levels, especially smoke, coffee and alcohol consumption, patients under common medications with possible indirect effect on Hcy, such as metformin [19], proton pump inhibitors [20] and statins [21] were allowed to participate.

Laboratory tests

On the day of admission for radioiodine treatment, venous blood samples were obtained after an overnight fasting. Standard routine tests included complete blood count and complete thyroid function assessment including anti-thyroglobulin (anti-Tg) and anti-thyroperoxidase (anti-TPO) antibodies. Patient's weight, blood glucose, total cholesterol and creatinine levels, as well as Hcy, vitamin B_{12} ferritin and erythrocyte sedimentation rate (ESR) were also obtained from all patients. All blood samples were processed according to our laboratory's standards; in particular, Hcy plasma levels were determined by chemiluminescence immunoassay (normal reference value: < 12 µmol/l) (Immulite, Bayer Centaur®, Abbott AxSYM).

Diagnosis of autoimmune thyroid disease

Anti-thyroid antibodies, ultrasonography of the thyroid gland before surgery and histopathological thyroid reports were available for all patients. A chronic inflammatory thyroid infiltrate is not uncommon in presence of DTC, though it does not necessary reflect an autoimmune disease of the thyroid gland. For this reason, diagnosis of HT requires the presence of elevated levels of anti-thyroid antibodies [22, 23]. Hence, diagnosis of HT was defined by the combination of clinical and histological criteria. To be classified as HT, patients were required to have: (1) anti-TPO and/or anti-Tg antibodies levels ≥ 2-fold higher than the upper normal limits with or without characteristic ultrasound features (i.e. non-homogeneous pattern with diffuse reduction of echogenicity [24]) and (2) histological description of an inflammatory infiltrate.

The histological definition of Hashimoto-like thyroiditis (HLT) was based on the presence of progressive loss of thyroid follicular cells, replacement of the gland by lymphocytes and formation of germinal centres with concomitant fibrosis. Non-Hashimoto-like lymphocytic thyroiditis (NHLT) was defined as the presence of varying degrees of thyroid infiltration by lymphocytes and other inflammatory cells—with or without infiltration of the tumour—but with none of the typical features of autoimmune thyroiditis, such as oxyphilic metaplasia, atrophy and formation of germinal centres [22, 23]. A figure of the association between levels of anti-thyroid autoantibodies and the patterns of thyroid infiltrate in our cohort is available as supplementary material.

Statistical analysis

Descriptive statistics was based on unpaired Student's *t* test or Mann–Whitney *U* test, depending on sample distribution. The Kruskal–Wallis test was used when comparison between three groups was performed. Normal distribution was assessed with D'Agostino–Pearson omnibus normality test. Fischer's exact test was applied when appropriate to describe differences between groups.

To investigate the strength of the association between Hcy and other variables, an analysis of covariance (ANCOVA) was performed with Hcy as dependent variable. Age, weight and blood chemical parameters were entered as continuous predictors, while the presence of an autoimmune condition was entered as dichotomous variable (HT vs non-HT). Preliminary versions of this analysis revealed that the employment of Hcy led to a violation of the normality assumption in the ANCOVA model. Hence, the final analysis was run with the natural logarithmic transformation of Hcy. Statistical analysis began including all covariates, the grouping variable (HT vs non-HT) and their interactions. The model was then simplified by means of Akaike's information criterion [25]. One observation was removed following the Bonferroni outlier test [26]. Level of significance was set at two-tailed $p < 0.05$. Statistical analysis was performed with GraphPad Prism 7.03 and R version 3.11.

Results

Patients' characteristics

Seventeen patients, 12 (71%) female and 5 (29%) male, who underwent remnant thyroid ablation after recombinant TSH stimulation, were excluded. One patient with a severe vitamin B_{12} defect due to atrophic body gastritis and one patient with known hyperhomocysteinemia under acid folic treatment were also ruled out from the final analysis. Therefore, the final cohort consisted of 121 patients, 102 (84.3%) female and 19 (15.7%) male, with a mean age of 46 years.

Figure 1 gives a detailed description of the variations of Hcy levels in the patient cohort. According to the serological and pathological criteria described above, 95 and 26 patients were classified as non-HT and HT, respectively. Consumption of metformin, statins, proton pump inhibitors or a combination thereof was equally prevalent between non-HT and HT patients ($p = 1$, Fisher's exact test, data not shown).

Overall, Hcy values were above the normal ranges (i.e., > 12 µmol/L) in 98 (81%) patients and within normal limits (< 12 µmol/L) in 23 (19%) patients. The resulting median concentration of Hcy in the whole sample was above the normal range (16.8 µmol/L).

Among the patients' characteristics and available blood test parameters, only Hcy levels were significantly different between HT and non-HT patients (median Hcy = 19.7 vs 16.2, respectively; $p = 0.018$, Mann–Whitney *U* test). Characteristics of the study cohort as well as of the two subgroups of HT and non-HT patients are shown in Table 1.

Analysis of covariance

After the model selection process described above, the following variables retained a significant association with Hcy levels: patient's weight, ESR, vitamin B_{12}, and autoimmune condition. Notably, the presence of an autoimmune thyroid disease resulted to be the strongest predictor of Hcy levels (coefficient = 0.25534, $p = 0.001$) (Table 2 and Fig. 2). All other variables showed no significant association with Hcy levels and were excluded from the model (full data not shown). In particular, TSH was not significantly associated with Hcy levels (coefficient = 0.00005, $p = 0.943$).

Discussion

The objective of the present study was to assess the relevance of autoimmune thyroid disease on the variations of plasma Hcy levels during acute iatrogenic hypothyroidism, which represents a unique and well reproducible human model of hypothyroidism. Hypothyroidism produces a significant increase of atherogenic risk factors, including Hcy [27, 28]. Additional metabolic conditions are characterised by an elevation of plasma Hcy levels; as an example, recent reports showed that patient's weight and metabolic syndrome are associated with raising Hcy levels in both children and adults [29–31]. In our setting, both study groups, i.e. HT and non-HT patients, share the same metabolic condition which, in itself, produces hyperhomocysteinemia. To our knowledge, no previous studies have investigated on the

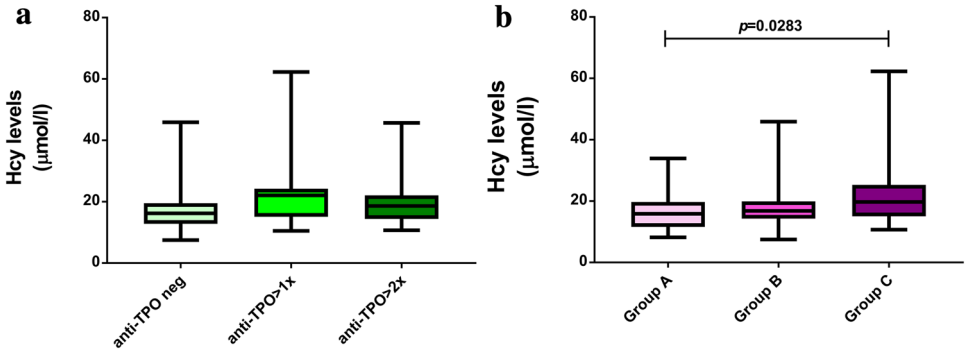

Fig. 1 Variation of Hcy levels in the study population. Left panel (**a**): box plot showing the variation of plasma Hcy levels in patients classified according to the titre of anti-TPO antibodies only (normal reference values: < 35 UI/mL). Differences of Hcy levels between the three groups are not statistically significant ($p = 0.0748$, Kruskal–Wallis test). Right panel (**b**): box plot showing the variation of plasma Hcy levels in patients classified according to combined serological and histological criteria. Antibody positivity is defined as anti-TPO and/or anti-Tg titre twofold higher than the upper normal limit (normal values: < 35 and < 40 UI/mL for anti-TPO and anti-Tg, respectively). Patients are grouped as follows: (1) Group A ($n = 52$), including patients with or without autoantibody positivity but no thyroid infiltration, (2) Group B ($n = 43$) including patients with any type of thyroid infiltrate but no autoantibody positivity, and (3) Group C ($n = 26$), including patients with both autoantibody positivity and presence of any type of thyroid infiltrate (i.e. HT patients). Median Hcy values (µmol/L) were 15.8, 16.8 and 19.7 in Group A, B and C, respectively ($p = 0.0283$, Kruskal–Wallis test). Results of Tukey's test for multiple comparisons were the following: significant Hcy differences were found only between Group A and Group C ($p = 0.0228$), while differences between Group A and Group B, as well as differences between Group B and Group C were not significant ($p = 0.8753$ and $p = 0.2613$, respectively). This demonstrates that, among patients with thyroid cancer and post-surgical acute hypothyroidism, those with HT, defined as the combined presence of autoantibody positivity and thyroid infiltration, have higher Hcy levels than those with autoantibody positivity only. Patients with thyroid infiltration alone might represent an intermediate-risk group with respect to Hcy elevation. These figures and corresponding p values were obtained after the removal of one outlier from the patient cohort

Table 1 Characteristics of the overall study cohort ($n = 121$ patients) and of the non-autoimmune (non-HT = 95 patients) and autoimmune (HT = 26 patients) groups

	Overall cohort ($n = 121$)	Non-HT ($n = 95$)	HT ($n = 26$)	p value
Gender (F/M)	102/19	79/16	23/3	0.76
Age (years)	46 (± 12.5)	47 (± 12.5)	42.5 (± 11.5)	0.11
Weight (kg)	68 (45–125)	68.5 (45–125)	67 (50–90)	0.91
Hb (12–16 g/dl)	13.8 (± 1.2)	13.8 (± 1.3)	13.6 (± 1.2)	0.56
TSH (0.35–4 µIU/ml)	82 (16–265)	81 (16–265)	88.5 (39.3–228)	0.35
fT3 (1.71–3.74 pg/ml)	1.455 (< 1–2.56)	1.525 (< 1–2.46)	1.355 (< 1–2.56)	0.47
fT4 (0.70–1.48 ng/dl)	0.5 (< 0.4–0.5)	0.5 (< 0.4–0.5)	0.5 (< 0.4–0.5)	>0.99
ESR (2–25 mm/h)	13 (0–59)	13 (0–47)	13 (4–59)	0.62
Glycemia (70–110 mg/dl)	79 (58–152)	80 (58–152)	78.5 (67–119)	0.95
Total cholesterol (120–220 mg/dl)	293.5 (179–447)	293.5 (179–439)	286.5 (219–447)	0.96
Hcy (< 12 µmol/L)	16.8 (7.49–91.9)	16.2 (7.49–91.9)	19.7 (10.7–62.3)	0.018*
Vitamin B_{12} (197–866 pg/ml)	396 (117–988)	396 (117–988)	392 (159–637)	0.40
Creatinine (0.57–1.11 mg/dl)	1 (0.6–1.4)	1 (0.6–1.4)	1.01 (0.9–1.2)	0.42
Ferritin (11–336 ng/ml)	30.9 (1.9–984)	34.6 (1.9–984)	28.4 (4.3–181.8)	0.22

Diagnosis of HT required (1) the presence of anti-TPO and/or anti-Tg antibodies levels ≥ 2-fold higher than the upper normal limits with or without characteristic ultrasound features and (2) the histological description of an inflammatory infiltrate. For each variable, normal laboratory ranges are reported between brackets. Data are reported as mean (± standard deviation) or median (range) in case of normally and non-normally distributed variables, respectively. p values are reported for comparisons between groups according to Fisher's exact test, unpaired Student's t test or Mann–Whitney U test, depending on sample distribution

*Indicates that statistical significance was reached

Table 2 Summary of the results of the analysis of covariance (ANCOVA)

Variable	Coefficient	Standard error of coefficient	*p* value
Weight	0.00562	0.00218	0.011
ESR	0.00849	0.00335	0.012
Vitamin B_{12}	− 0.00067	0.00022	0.003
Autoimmunity	0.25534	0.08027	0.001

The table shows the minimal adequate model including patient's weight, ESR, vitamin B12 and the grouping based on the presence of an autoimmune thyroid disease (HT vs non-HT, reference level: non-HT)

interaction between HT and plasma Hcy levels in patients with similar characteristics.

Hcy proved to have a systemic and local pro-inflammatory activity in the course of autoimmune diseases [2]. HT represents the most frequent autoimmune disorder and the leading cause of hypothyroidism which, in turn, has been associated with cardiac and cerebrovascular events [11, 32]. Hyperhomocysteinemia on its own has shown a positive association with ischemic cardiovascular events and stroke, which seems to be independent from other cardiovascular risk factors [33, 34]. Therefore, the combination of HT and hyperhomocysteinemia may confer an increased cardio- and cerebrovascular risk which, due to the high prevalence of

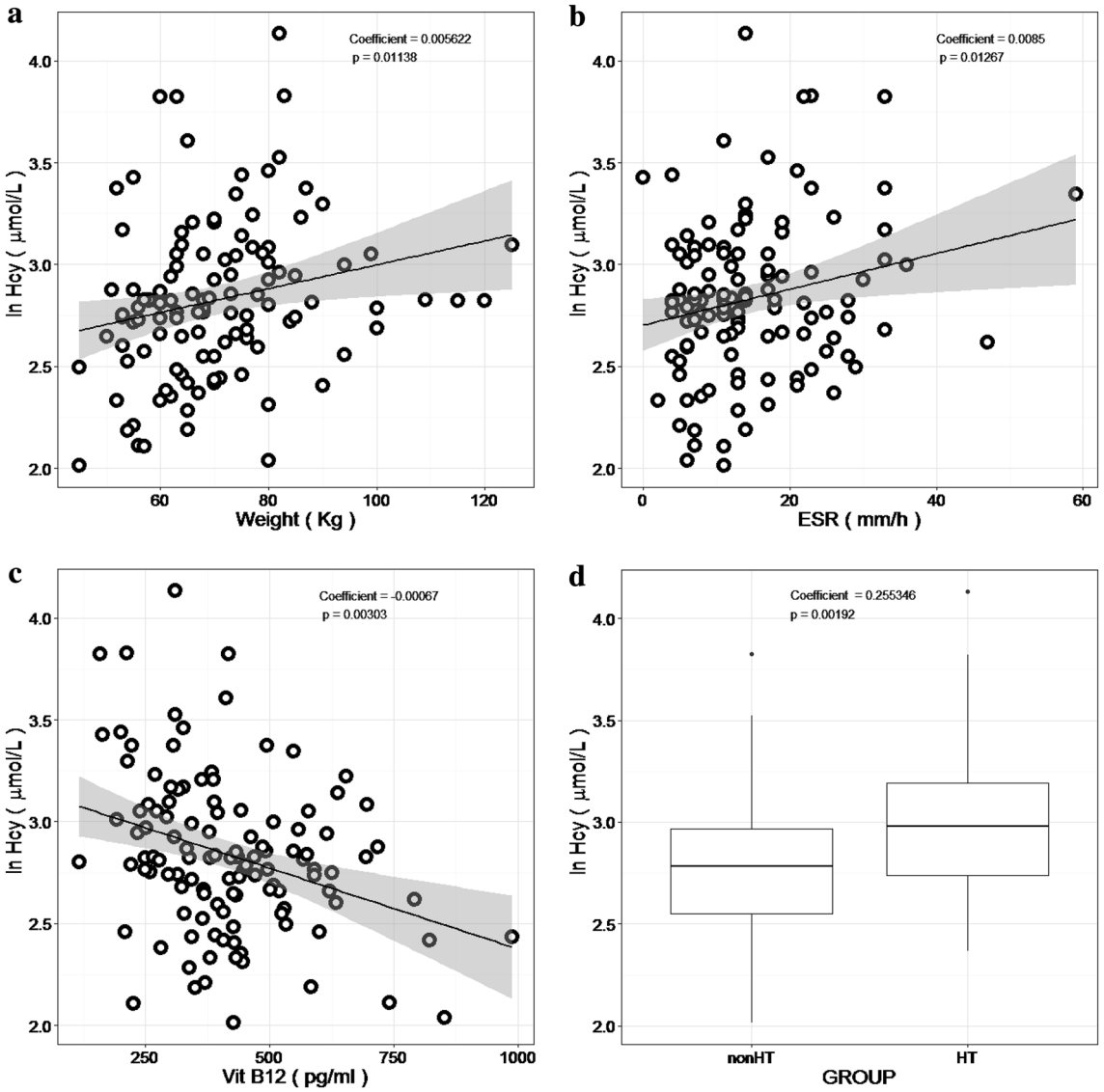

Fig. 2 Results of analysis of covariance. Scatter plots showing variations of Hcy levels with patient's weight (**a**), erythrocyte sedimentation rate (**b**) and vitamin B_{12} (**c**). A box plot showing differences of Hcy levels between HT and non-HT patients is represented in panel (**d**). For each variable, the coefficient and *p* values are reported

thyroid disorders in the general population, might be of particular relevance [35].

In our study, median serum Hcy levels were significantly elevated in the whole cohort, confirming that hypothyroidism increases Hcy levels [4]. As expected, TSH and total cholesterol were also increased, whereas FT3 and FT4 levels were below normal values. Among all biochemical parameters analysed, only serum Hcy levels were different between groups. The relevance of the autoimmune status for Hcy elevation was confirmed by the ANCOVA analysis: among all variables analysed, the presence of HT showed the strongest association with Hcy levels. ESR elevation and vitamin B_{12} reduction, which are constant features of inflammation [2], were also weakly, but significantly, associated with Hcy levels. Similarly, a weak but significant correlation with Hcy was found for patient's weight, which has a well-known linkage with low-grade systemic inflammation [36]. None of the other parameters analysed, including TSH, free thyroid hormone fractions and serum creatinine levels were significantly different between the two groups, as well as none correlated with Hcy levels.

Taken together, these results suggest that, in overt hypothyroidism, a systemic immune-inflammatory activation is the main determinant of the increase of Hcy levels. On the other hand, in absence of overt hypothyroidism, the autoimmune condition itself might not suffice to increase Hcy levels above normal ranges, as previously shown by others [5, 16–18] and confirmed in this study by the presence of normal Hcy values in 4 euthyroid HT patients who received radioiodine after recombinant TSH stimulation, and were excluded from the final analysis (median Hcy: 9.55 µmol/L, range 5–10.3 µmol/L).

The reciprocal interactions between hypothyroidism, hyperhomocysteinemia and thyroid autoimmunity described here suggest a possible higher cardio- and cerebrovascular risk for hypothyroid patients with autoimmune thyroid disease. Indeed, we identified a subset of hypothyroid patients with HT with significant Hcy elevation, which might confer them an increased risk compared to hypothyroid patients without autoimmunity [11–15, 17, 32–34].

The question may arise as to whether hyperhomocysteinemia deserves treatment during thyroid hormones washout prior to radioiodine treatments in these higher-risk patients, taking into account that even a transient increase of Hcy might have adverse vascular effects [37].

This study has some limitations that deserve to be acknowledged. First of all, not all the potential variables affecting Hcy were recorded, such as smoke, coffee and alcohol consumption, and common medications with known interactions with Hcy were allowed. However, in our opinion, the absence of strict exclusion criteria allows the extension of our results to the general population, in which all these variables are hardly controlled. In addition, C-reactive protein would have been a better marker of systemic inflammation than ESR, and serum folate levels could have complemented the information given by vitamin B_{12}; unfortunately, these parameters were not included in our biochemical assessment. Similarly, body mass index should have better been used instead of patient's weight. Furthermore, we did not check the profile of Hcy levels over time in our patients after initiation of levothyroxine replacement. However, a recent study showed that levothyroxine replacement reverses the Hcy elevation in acute post-surgical hypothyroid DTC patients [38]. Finally, the two patient groups (i.e., HT and non-HT) were numerically unbalanced, due to the low prevalence of HT in our population of patients with thyroid cancer [22].

In summary, we demonstrated that, in overt iatrogenic hypothyroidism, only serum Hcy levels are significantly different between patients with and without HT. In addition, the increase of Hcy levels appears to be mainly determined by a preexisting immune-inflammatory condition. Having knowledge of the association between autoimmune thyroiditis and hyperhomocysteinemia might help to identify patients with increased cardiovascular risk in a highly prevalent clinical condition such as hypothyroidism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in the study were in accordance with the standards of the institutional ethical committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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