Homocysteinemia in relation to anemia in hypothyroid patients

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Background Anemia and hypothyroidism are both common diseases in the community. Homocysteine (HCY) levels are increased in patients with hypothyroidism and methylenetetrahydrofolate reductase (MTHFR) deficiency is the most common genetic cause of hyperhomocysteinemia.

The aim of the present study was to evaluate the level of serum HCY in patients with hypothyroidism and to study the relation of associated anemia with the serum level of HCY and MTHFR gene in patients with hypothyroidism.

Patients and methods The study was conducted on 60 adult women attending the Endocrinology Outpatient Clinic of Al-Zahraa Hospital between September 2014 and June 2015 for proper diagnosis and management. Individuals of the study were divided into two main groups: group I (GI) with 30 hypothyroid patients, where 13 of them were postsurgical cases, and group II (GII) with 30 euthyroid individuals as a control group. Diagnosis was based on thyroid-stimulating hormone level reference values. Patients in GI were further classified into two subgroups: mild hypothyroid (subgroup I) and overt hypothyroid (subgroup II). Patient and control groups also were classified into anemic and nonanemic subgroups according to hemoglobin levels. The selected hypothyroid patients were women under thyroid hormone replacement therapy. Blood sample was obtained for proper investigations. Complete blood count, routine blood chemistry, serum iron level, thyroid function tests, vitamin B_{12} level, serum homocysteine (HCY), and MTHFR were performed. We performed a pilot study on *MTHFR* gene polymorphism. *The C677T MTHFR* gene mutation was detected in three of 10 patients and in two of 10 controls. No evidence of *TT MTHFR* gene mutation was observed in both patient and control groups. IBM SPSS statistics (version 23.0, USA, 2015) was used for data analysis.

Results revealed the presence of anemia according to hemoglobin level (<12 g/dl). In patients group (GI), 50% (15/30) as compared with 13.3% (4/30) in the control group (GII) had anemia. Serum iron level in patients group (GI) was deficient in 40% (11/30), whereas deficient in 16.7% (5/30) in control group (GII). Vitamin B_{12} deficiency was found to be 44% (11/25) in patients group (GI), whereas in the control group (GII) was 6.7% (2/30). Analysis by Wilcoxon's rank sum test, homocysteine (HCY) serum level showed a highly significant increase among patients (GI) as compared with control (GII). Ranked Spearman's correlation test for the patients (GI) and control (GII) showed a significant negative correlation between homocysteine (HCY) and MTHFR serum levels, whereas the correlation with red cell indices parameters was insignificant. Serum iron and B_{12} levels were significantly correlated in patient group (GI). Pearson χ^2 tests were done between both patients and control groups for the presence of anemia, iron deficiency, and elevated serum homocysteine (HCY) level and all revealed statistically significant results.

Conclusion There is no significant correlation between homocysteinemia and anemia. However, the strong association between anemia and hypothyroidism is attributed mainly owing to combined iron and vitamin B_{12} deficiencies. This might explain the decreased response to treatment among the selected hypothyroid patients.

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Introduction

Hypothyroidism is one of the most common functional disorder of the thyroid gland, and its main clinical feature is a low basal metabolic rate caused by the insufficient synthesis, secretion, or biological effect of thyroid hormones [1,2]. A decrease of T3, T4, or thyrotropin-releasing hormone accompanied by an increase in thyroid-stimulating hormone (TSH) is considered as the assessment criteria of hypothyroid patients [3].

Hypothyroidism has been reported to be associated with mild hyperhomocysteinemia [4,5,30]. Plasma levels of homocysteine (HCY), which is a sulfhydryl amino acid derived from the metabolic conversion of methionine, are closely dependent on nutritional and genetic factors and the B vitamins such as folate (B_9), vitamin B_{12}, and vitamin B_6. The etiology by which hypothyroidism causes mild hyperhomocysteinemia is poorly understood, but altered remethylation is likely to be involved [6]. Methylation is carried out by an enzyme methylenetetrahydrofolate reductase (MTHFR), which

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converts 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, and this reaction provides a methyl for homocysteine (HCY) and converts it into methionine [7,8].

MTHFR deficiency is the most common genetic cause of elevated levels of homocysteine (HCY) in the plasma (hyperhomocysteinemia) [9]. In humans, the *MTHFR* gene resides on chromosome 1 'p36.3' and was originally described as containing 11 exons [9]. A common mutation in the *MTHFR* gene, involving a *C-to-T* substitution at nucleotide *677 (C677T)*, results in the conversion of alanine to valine at position 226 of the protein [10]. The *C677T* mutation reduces the specific activity of MTHFR, increases its thermolability, and has been reported to induce hyperhomocysteinemia [11].

Anemia is a common, although frequently underestimated, clinical condition accompanying thyroid diseases. Despite the fact that anemia and thyroid dysfunction often occur simultaneously, the causative relationship between the two disorders remains ambiguous [12]. Mild or moderate hyperhomocysteinemia may result from a relative deficiency of folic acid and vitamin B_{12} and homozygosity for the common polymorphism *677CT* in the *MTHFR* gene [10]. Accordingly, the aim of the present study was to evaluate the level of serum homocysteine (HCY) in patients with hypothyroidism and to study the relation of associated anemia with the serum level of homocysteine (HCY) and *MTHFR* gene in patients with hypothyroidism.

Patients and methods

The study was conducted on 60 women attending the endocrinology outpatient clinic at Al-Zahraa Hospital between September 2014 and June 2015, who were classified into two main groups: 30 hypothyroid patients (GI) and 30 euthyroid control group (GII).

Patient group (GI) consisted of 30 females clinically diagnosed as having primary hypothyroidism of different etiology (postthyroidectomy and nonsurgical hypothyroidism). The selected patients had an average age of 40.8±9.5 years, ranging from 25 to 65 years. They were subjected to thyroid hormone replacement therapy by levothyroxine (L-T4) (1.6–1.8 μg/kg body weight) for a duration ranging from 12 to 84 months.

Of these 30 hypothyroid patients, according to free thyroxine (FT4) levels; the patients were further subdivided into two subgroups: 14 patient in first subgroup with normal FT4 and mild elevations in the TSH levels (TSH ≤10 mIU/ml), and 16 patient in the second subgroup with decreased FT4 levels and marked elevations in the TSH levels.

Control group (GII) comprised 30 apparently healthy individuals age, sex, and socioeconomic matched with the patients. Their age ranged from 25 to 65 years, with an average age of 41.6±6.7 years. They were referred to the Endocrinology Outpatient Clinic for the suspicion of thyroid disease and were found to be euthyroid and not having the disease.

The study was approved by the Clinical Pathology Review Board as well as the head of Endocrinology Department Faculty of Medicine for Girls at Al-Azhar University. Informed oral consent was obtained from all studied patients and controls before enrollment in the study.

All participants were subjected to full clinical examination, including detailed history and physical examination such as body weight measurement. General laboratory tests [complete blood count, serum iron, liver and kidney function tests, FT4, triiodothyronine (FT3), and TSH) were done to all participants. Serum B_{12} examination was done for only 25 patients and 30 control to determine the type and possible causes of anemia if present. Specific laboratory tests (homocysteine (HCY) and MTHFR enzyme assay) were done for all participants. Pilot samples (10 patients and 10 controls) were used to study the *MTHFR* gene polymorphism on selected patients and controls.

Presence of comorbid conditions including hypertension, diabetes, coronary artery disease, and renal disease was excluded from the study.

Sampling

Fasting samples of about 7–10 ml of venous blood from all patients and controls were withdrawn under complete aseptic condition. Two milliliters of the blood was collected into EDTA tube. EDTA samples were mixed well for estimation of complete blood count and *MTHFR* gene mutation.

Complete blood picture

Were done on the Autoanalyzer (Sysmex, KX-21N).

Reference range for Hemoglobin (F:-12:15/M:13:17g/dl), anemia is defined as hemoglobin levels lower than 12 g/dL in women and 13 g/dL in men.

The rest were collected in a plain tube. Samples of the plain tube were left to clot for 30 min, and serum was

separated by centrifugation at 3500*g* for 5 min. Serum estimation for routine biochemical profile and iron level were done on a clinical chemistry auto-analyzer. The remaining serum samples were stored at −20°C as three separate aliquots for each sample until assays of the HCY, MTHFR enzyme, and vitamin B_{12} by electrochemiluminescence method.

Vitamin B_{12} analysis was done by electrochemiluminescence immunoassay intended for use on Elecsys and Cobas E601 immunoassay analyzers (This only available).

Vitamin B_{12} kits were provided by Roche Diagnostics (Ref; 07212771/190). Vitamin B_{12} assay employs a competitive test principle using intrinsic factor specific for vitamin B_{12}.

Vitamin B_{12} reference range was 187–883 pg/ml.

Determination of homocysteine

Analysis was performed in Health Radiation Research Department, National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt. Method of determination was enzyme-linked immunosorbent assay (ELISA).

ELISA kits were supplied by Uscn Life International Inc. (catalog no: E0772Ge). The reagents in the kit were prepared following the standard protocol provided with the kit.

Test principle

The microliter plate provided was precoated with an antibody specific to homocysteine (HCY). Standards or samples were added to the appropriate microliter plate wells with a biotin-conjugated polyclonal antibody specific for HCY, and avidin conjugated to horseradish peroxidase was added to each well. The enzyme substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450±2 nm. The concentration of HCY in the samples was determined by comparing the optical density of the samples with the standard curve.

Its detection range was 0.78–50.0 μmol/l.

Reference range was 5–15 μmol/l.

Determination of methylenetetrahydrofolate reductase

Its analysis was performed in Health Radiation Research Department, National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt.

The method of determination was ELISA.

Human methylenetetrahydrofolate reductase

Kits were supplied by Uscn Life International Inc. (catalog no: E6325h). The reagents in the kit were prepared following the standard protocol provided with the kit.

Principle

The microliter plate provided was precoated with an antibody specific to MTHFR. Standards or samples were added to the appropriate microliter plate wells with a biotin-conjugated polyclonal antibody preparation specific for MTHFR, and avidin conjugated to horseradish peroxidase was added to each microliter plate wells and incubated. Then a TMB substrate solution was added to each well. The enzyme substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450±2 nm. The concentration of MTHFR in the samples was then determined by comparing the optical density of the samples to the standard curve.

Detection Range for MTHFR wasRR was 0.78–50 ng/ml.

Methylenetetrahydrofolate reductase mutation analysis

The analysis was done in Serum and Vaccine Center in Doki.

Polymerase chain reaction (PCR) was performed in an AB 2400 thermocycler (ABI, USA). Genomic *DNA* was isolated from nucleated blood cells using a high pure *DNA* extraction kit (Roche Diagnostics). DNA samples were kept at −80°C till analysis.

The *C677T MTHFR* gene mutation was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). analysis using HinfI restriction analysis of a 198-bp PCR amplified fragment in the gene for *MTHFR* [10]. The restriction enzymes were obtained from Promega Corporation (USA).

In brief, ∼50 to 80 ng of DNA samples was amplified in a final volume of 25 μl containing 1×PCR buffer with 1.5 mmol/l $MgCl_2$ and 2 U of Taq DNA polymerase. The PCR profile consisted of an initial melting step of 2 min at 94°C, followed by 35 cycles of

30 s at 94°C, 30 s at 61°C, and 30 s at 72°C, with a final elongation step of 7 min at 72°C.

The restriction enzyme HinfI (Promega, UK) was used to distinguish the *C677T* polymorphism, and the gain of a HinfI restriction site occurs in the polymorphic allele. The products of the HinfI digestion were electrophoresed on 1% agarose gel.

Determination of triiodothyronine, free thyroxine, and thyroid-stimulating hormone levels

Their analysis done by an electrochemiluminescence immunoassay on Cobas E411 in AL-Zahraa Hospital (Al-Azhar University).

Reference range for TSH was 0.27–4.2 mIU/ml, FT3 was 3.1–6.8 pmol/l, and FT4 was 12–22 pmol/l.

Serum iron levels

Their assessments were done in Al-Zahraa Hospital Al-Azhar University by photometric method on Cobas Integra 400/800.

Reference range of serum iron was 59–158 μg/dl.

Statistical methods

The collected data were arranged and tabulated and statistically analyzed using IBM SPSS (version 23.0, 2015; IBM Corp., USA). Data were expressed as mean ±SD for quantitative parametric measures in addition to median percentiles for quantitative nonparametric measures and both number and percentage for categorized data. χ^2 test was used for qualitative variables. *P* value less than 0.05 was considered statistically significant.

Results

All patients underwent complete blood count, serum iron level, vitamin B_{12} level, and serum HCY measurements, which revealed the presence of anemia hemoglobin (Hb) level (<12 g/dl) in patients group (GI), with 50% (15/30), compared with its level in the control group (GII), with 13.3% (4/30). Serum iron level in patient group (GI) was lower than 59 μg/dl (Reference range, 59–158/dl) in 40% (11/30), whereas in the control group (GII), it was lower in 16.7% (5/30). Vitamin B_{12} deficiency was found to be present in 44% (11/25) of the patient group (GI), whereas in the control group (GII), it was seen in 6.7% (2/30).

Table 1 shows the comparative analysis of the median ±25% and 75% of the represented parameters between patients and control groups, which revealed that TSH, free FT3, and FT4 were highly significant among patient group (GI) compared with control group (GII). Serum homocysteine (HCY) was significantly higher among patient group (GI) compared with controls (GII). MTHFR by the student *t* test showed no significant differences in patients group (GI) compared with the control group (GII).

The Hb and red cell width distribution (RDW) levels were statistically significant and also the hemoglobin-hematocrit ratio (Hb/Hct) was statistically highly significant among patient and control groups, as shown in Table 2.

On comparison between the two subgroups by the Wilcoxon's rank sum test, iron, homocysteine (HCY), and B_{12} showed nonsignificant difference between the first and second subgroups (Table 3). Blood picture parameters such as Hb, RDW, mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration between both subgroups were compared by the student *t* test and showed a significant difference in RDW (Table 4). According to anemia status, group I (GI) was divided into anemic and nonanemic patients, as shown in Table 5. On comparison between the anemic and nonanemic subclasses by Student's *t* test, Hb and MCV showed a highly significance difference, whereas RDW showed a significant difference (Table 5). In comparative analysis between anemic and nonanemic subclasses by Wilcoxon's rank sum test, serum HCY showed no significant difference, serum (B_{12}) showed a significant difference, and iron showed a highly significant difference (Table 6).

Ranked Spearman's correlation showed a strong negative correlation between homocysteine and MTHFR in the patients (GI) (r=−0.73, P<0.0001). There was a significant positive correlation between B_{12} and iron among the patients (GI) (r=0.41, P<0.038). There was no significant correlation between TSH and T4 among patients (GI), but was significantly negatively correlated among controls (GII) (r=−0.44, P<0.1). Pearson's χ^2 test was done between both patient (GI) and control (GII) groups for the presence of anemia, iron deficiency, B_{12} deficiency, and elevated serum HCY level, and all revealed significant results. MTHFR serum level was insignificant between GI GII (Tables 7–10). Table 7 and Fig. 1 shows anemia in the main groups.

Table 10 and Fig. 2 represent homocysteinemia in the main groups. Table 11 show the sensitivity, the

Table 1 Comparative analysis of the patient (GI) and control (GII) groups by Wilcoxon's rank sum test: methylenetetrahydrofolate reductase by Student's *t* test

	Patients (GI)	Control (GII)	25% patients (GI)	25% control (GII)	75% patients (GI)	75% control (GII)	*P*	Significance
Serum TSH (mIU//ml)								
Median	12.5	1.15	8.3	0.8	49	1.7	0	HS
Range	4.5–100	0.5–3.7						
Serum FT3 (pmol/l)								
Median	3	4.3	2.5	3.9	3.5	5	0	HS
Range	1.7–3.8	3.2–5.7						
Serum FT4 (pmol/l)								
Median	11.4	16	7.6	14.9	12.3	16.9	0	HS
Range	2.5–19.5	13.2–19.0						
Serum B_{12} (pg/ml)								
Median	262.5	343.5	51	264	541	536	0.1	NS
Range	30–2000	192–756						
Serum iron (μg/dl)								
Median	62	71	46.5	60.5	72.2	79.2	0.1	NS
Range	34–192	35–155						
Serum HCY (μmol/l)								
Median	12.9	8.8	10.15	7.1	17.5	9.3	0	HS
Range	8–25	7.1–9.3						
MTHFR serum level (ng/ml)[a]								
Mean ±SD	7.18±1.46	7.2±1.49					0.8	NS
Range	4.6–9.5	5.5–8.8						

FT3, triiodothyronine; FT4, free thyroxine; HCY, homocysteine; HS, highly significant; MTHFR, methylenetetrahydrofolate reductase; TSH, thyroid-stimulating hormone.

Table 2 Comparative analysis of patient (GI) and control (GII) groups by Student's *t* test

	Patients (GI) [mean±SD (range)]	Controls (GII) [mean±SD (range)]	*P* value	Significance
HB (g/dl)	11.9±1.2 4.8–13.5	12.6±0.6 (11.6–13.6)	0.02	S
RDW %	13.3±1.6 (11.3–16.5)	12.4±1.1 (11.5–13.5)	0.029	S
MCV (fl)	81.2±6.0 (65–90.0)	83.2±3.6 (76.4–95.0)	0.13	NS
MCH (pg)	26.8±3.0 (18.5–30.2)	26.5±3.4 (25.2–30.3)	0.7	NS
Hct (%)	36.6±3.4 (33.2–40.0)	37.3±1.9 (35.4–39.2)	0.29	NS
Hb/Hct ratio	0.32±0.017 (0.303–0.337)	0.33±0.008 (0.312–0.338)	0.01	HS

Hb/Hct, hemoglobin-hematocrit ratio; HS, highly significant; MCH, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red cell width distribution; S, significant.

specificity, the positive predictive value, the negative predictive value, and the efficacy of HCY in the present study at cutoff of 9.9 between cases and controls, and cutoff of 11.1 between anemic and nonanemic. Diagnostic Validity Test was done for both iron and B_{12} levels, which revealed no valid best cutoff for both (no cutoff having both specificity and sensitivity of more than 50%).

Receiver operating characteristic curve (Fig. 3) analysis was done for homocysteine (HCY) serum concentration for discriminating hypothyroid patients from euthyroid control. We performed a pilot study for *MTHFR* mutation. The *C677T MTHFR* gene mutation was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis on 10 selected cases and 10 controls.

CT MTHFR gene mutation was detected in three patients and two controls. No evidence of TT *MTHFR* gene mutation was observed in both patients and controls (Table 12).

Discussion

Homocysteine (HCY) is a sulfur amino acid, and its metabolism requires folate, vitamin B_6, and vitamin B_{12} [13]. Having a deficiency of any of these three vitamins can lead to an increase in homocysteine (HCY). MTHFR is an enzyme that is vital for remethylation of homocysteine (HCY) to methionine. Methylation is necessary for most of the body systems. Pollutants, poor nutrition, and stress are putting increased demands on methylation processes, as the body works to preserve itself. Homocysteine (HCY) is a marker for inflammation, such that high levels are associated with high risk of autoimmune

Table 3 Comparative analysis between patient subgroups by Wilcoxon's rank sum test

	Median and IQR group I	Median and IQR group II	25% group I	25% group II	75% group I	75% group II	*P*	Significance
Serum TSH (mIU/ml)	9.2 (5.87–11.6)	46.5 (17.92–71.75)	5.87	17.92	11.6	71.25	0	HS
Serum FT3 (pmol/l)	3.55 (3.45–3.95)	2.6 (2.22–2.95)	3.45	2.22	3.95	2.95	0	HS
Serum FT4 (pmol/l)	12.35 (11.67–14.52)	7.75 (6.32–9.7)	11.67	6.32	14.52	9.7	0	HS
Serum HCY (μmol/l)	12.6 (8.97–17.5)	13.05 (10.82–17.35)	8.97	10.82	17.5	17.35	0.454	NS
Serum B_{12} (pg/ml)	319 (121–680.7)	108.5 (49.5–455.5)	121	49.5	680.7	455.5	0.328	NS
Serum iron (μg/ml)	67 (48.75–82.5)	44–70.5	48.75		82.5	70.5	0.212	NS
Serum MTHFR [mean±SD (range)]	7.23±1.45 (5.78–8.68)	7.13±1.50 (5.63–8.63)					0.849	NS

FT3, triiodothyronine; FT4, free thyroxine; HCY, homocysteine; HS, highly significant; IQR, interquartile range; MTHFR, methylenetetrahydrofolate reductase; TSH, thyroid-stimulating hormone.

Table 4 Comparison of blood picture parameter between patient subgroups

	Mean±SD (range) in group I	Mean±SD (range) in group II	*P*	Significance
HGB (g/dl)	12.06±1.05 (11.01–13.11)	11.85±1.39 (10.46–13.24)	0.636	NS
RDW %	12.95±1.14 (11.81–13.99	13.84±1.83 12.01–15.67)	0.032	S
MCV (fl)	82.09±4.50 (77.0–86.69	80.37±7.24 (73.13–87.61)	0.437	NS
MCH (pg)	27.14±1.96 (25.18–29.9)	26.56±3.79 (22.77–30.35)	0.535	NS

MCH, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red cell width distribution; S, significant.

Table 5 Comparative analysis to anemic/nonanemic groups by Student's *t* test

	Mean±SD anemic (15 cases)	Mean±SD nonanemic (15 cases)	*P*	Significance
Age (years)	40.2±9.59	41.4±9.81	0.737	NS
HGB (g/dl)	10.95±0.95	12.94±0.29	0	HS
RDW%	13.92±1.99	12.59±0.83	0.028	S
MCV (fl)	78.26±7.10	84.09±2.81	0.008	HS
MCH (pg)	25.13±3.35	28.53±1.32	0.002	HS
MTHFR (ng/ml)	7.41±1.21	6.94±1.68	0.392	NS
Weight (kg/body weight)	73.3±8.54	79.1±7.38	0.057	NS

HS, highly significant; MCH, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MTHFR, methylenetetrahydrofolate reductase; RDW, red cell width distribution; S, significant.

disease, that is, thyroid disorder and cardiovascular disease.

Taking synthetic levothyroxine (L-T4) or natural thyroid hormone might not completely normalize homocysteine (HCY) levels in people with hypothyroidism, and if this is the case, then other factors which affect homocysteine (HCY) need to be addressed.

The aim of the present study was to evaluate the level of serum homocysteine (HCY) in patients with hypothyroidism and to study the relation of associated anemia with the serum level of homocysteine (HCY) and *MTHFR* gene in patients with hypothyroidism. The diagnosis was based on TSH above reference value [14]. All selected patients and controls were women, because the prevalence of subclinical hypothyroidism and primary hypothyroidism is constantly increasing, especially in women [15]. The association between HCY and hypothyroidism has been demonstrated in several studies; however, the conclusion is controversial. Several studies reported that plasma HCY concentrations elevated in patients with overt hypothyroid compared with those healthy participants [16,17]. Another study revealed that plasma HCY is associated with the severity of hypothyroidism, and L-T4 treatment is good for patients with hypothyroidism to reduce the plasma HCY levels [18]. In the present study, the patients were of average age of 40.8±9.5 years, ranging from 25 to 60 years, who were subjected to thyroid hormone replacement therapy by levothyroxine (L-T4), with the duration of treatment ranging from 12 to 84 months. The patient group (GI) was further subdivided according to their FT4 levels into two subgroups: the first subgroup with normal FT4 level and TSH levels ≤10 m IU/ml, comprising (14/30) 46.7% of the patients, and the second subgroup with low FT4 levels and TSH more than or equal to 10 mIU/ml, comprising (16/30) 53.3% of the patient group. The mean values of serum total homocysteine (tHCY) were significantly higher in patient group (GI) by 30% compared with the control group (GII). By Wilcoxon's rank sum test, the result was statistically

Table 6 Comparative analysis of the anemic and nonanemic patients by Wilcoxon's rank sum test

	Median anemic	Median nonanemic	25% anemic/nonanemic	75% anemic/nonanemic	*P*	Significance
Serum TSH (mIU/ml)	13	12	9.5/6.2	75/47	0.52	NS
Serum FT3 (pmol/l)	2.8	3.3	2.5/2.7	3.5/3.6	0.27	NS
Serum FT4 (pmol/l)	7.8	11.5	6.3/8.8	12.3/14.3	0.11	NS
Serum HCY (μmol)	11.7	13.7	10/10.2	14.5/17.5	0.60	NS
Serum B_{12} (pg/ml)	56	486	46/312	189/560	0.01	S
Serum iron (μg/dl)	47	72	38/68	55/88	0	HS

FT3, triiodothyronine; FT4, free thyroxine; HCY, homocysteine; TSH, thyroid-stimulating hormone; S, significance.

Table 7 Pearson's χ^2 test for anemia between patient (GI) and control (GII) groups

Anemia	Patients	Control	Total
Nonanemic [*n* (%)]	15 (50.0)	26 (86.7)	41 (68.3)
Anemic [*n* (%)]	15 (50.0)	4 (13.3)	19 (31.7)
Total [*n* (%)]	30 (100)	30 (100)	60 (100)
	Value	*P*	Significance
Pearson's χ^2	9.320[a]	0.002	S

S, significant.

Table 8 Pearson's χ^2 test for iron deficiency between patient (GI) and control (GII) groups

	Patients (GI)	Controls (GII)	Total
Iron deficiency [*n* (%)]	12 (40.0)	5 (16.7)	17 (28.3)
No iron deficiency [*n* (%)]	18 (60.0)	25 (83.3)	43 (71.7)
Total [*n* (%)]	30 (100)	30 (100)	60 (100)
	Value	*P*	Significance
Pearson's χ^2	4.022[a]	0.045	S

S, significant.

Table 9 Pearson's χ^2 test for B_{12} deficiency between patient (GI) and control (GII) groups

	Patients (GI)	Controls (GII)	Total
B_{12} deficiency [*n* (%)]	11 (44.0)	2 (6.7)	13 (23.6)
No B_{12} deficiency [*n* (%)]	14 (56.0)	28 (93.3)	42 (76.4)
Total [*n* (%)]	25 (100)	30 (100)	55 (100)
	Value	*P* (MTHFR)	Significance
Pearson's χ^2	10.530[a]	0.001	HS

HS, highly significant.

highly significant ($P\leq0.00$). This observation is in line with [19] and with other studies [16,17] and was not in agreement with [20] who observed that HCY levels were statistically nonsignificantly higher in the patient group (GI) than the control group (GII).

Consistent reports demonstrated that thyroid status is an important determinant of the plasma/serum concentration of tHCY [21]. Inconsistent with results of the present study, no statistically significant difference was found between the patients and control for HCY [5,22]. It was suggested that the elevation of plasma HCY level can be explained by impaired renal clearance or reduced urinary excretion in hypothyroidism [16]. Thyroid hormones have important effects in the kidney, affecting renal growth, renal hemodynamics, and salt and water metabolism [23]. However, in our study, patients with liver and renal impairment were excluded.

Methylenetetrahydrofolate reductase (MTHFR) enzyme activity serum level (Student's *t* test) showed no significant differences between the patient group (GI) and control group (GII). This finding is not in agreement with experimental studies that implied MTHFR, a key enzyme in folate metabolism, decreased in patients with hypothyroidism [17]. As the major determinant of plasma HCY, folate level decreases in patients with hypothyroidism.

Methylenetetrahydrofolate reductase (*MTHFR*) gene variants have been investigated most extensively in relation to their effect on the tHCY concentration. The prevalence of these polymorphisms is relatively high in the general population; the prevalence of homozygosity (*677TT*) is 5–15% in most white populations [24].

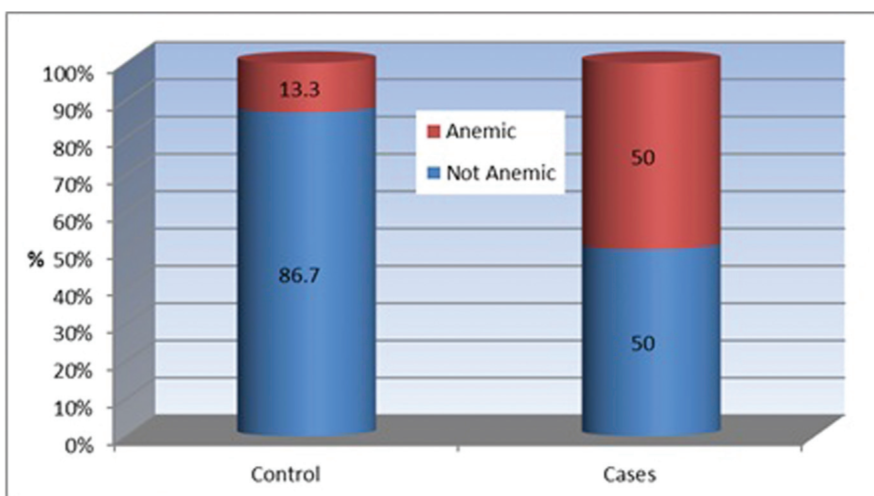
It is caused by a point mutation leading to the substitution of valine for alanine, which results in a thermolabile and less active form of the enzyme. Patients who carry the mutation appear to have an exaggerated hyperhomocysteinemic response to the depletion of folic acid [25,26]. Both the homozygote (*677TT*) and heterozygote (*677CT*) genotypes result in elevated plasma HCY levels. In a large cohort of male patients with coronary artery disease, the mean plasma HCY concentrations in the *677TT* and *677CT* groups were 2.8 and 0.8 μmol/l higher than in the 677CC group [27]. The 677TT genotype is a modest risk factor for neural tube defects and possibly for coronary artery disease [28]. In our study, we performed a pilot study on MTHFR gene polymorphism. The *C677T*

Table 10 Pearson's χ^2 test for increased homocysteine level between patient (GI) and control (GII) groups

	Patients (GI)	Control (GII)	Total
Increased HCY [*n* (%)]	9 (30.0)	0 (0.0)	9 (20.0)
Not increased HCY [*n* (%)]	21 (70.0)	15 (100.0)	36 (80.0)
Total [*n* (%)]	30 (100)	15 (100)	45 (100)
	Value	*P*	Significance
Pearson χ^2	5.625[a]	0.018	S
AUC			
Cases vs. control	0.931		
Anemic vs. nonanemic	0.645		

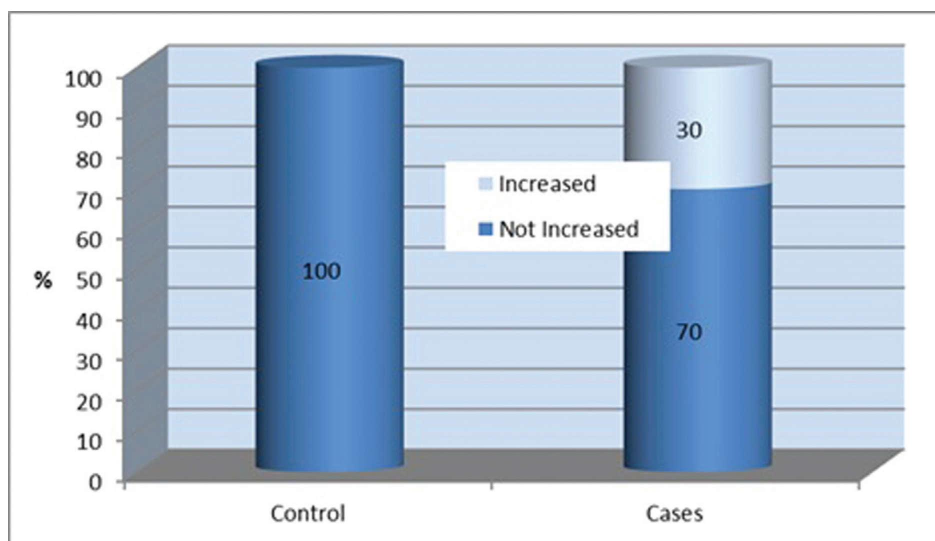
AUC, area under the curve; HCY, homocysteine; S, significant.

Figure 1



The percentage of anemia in control and case groups.

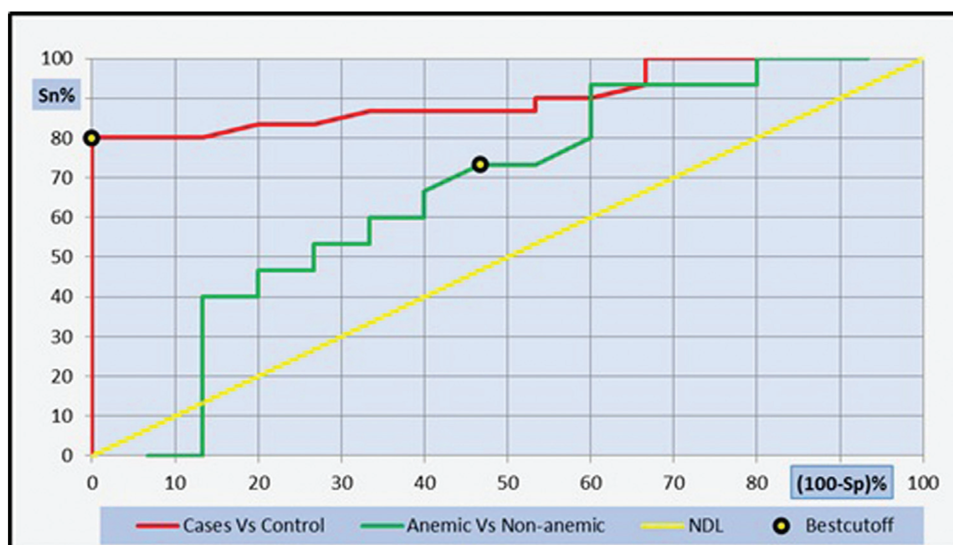
Figure 2



The percentage of elevated homocysteine level in control and case groups.

MTHFR gene mutation was detected by PCR-RFLP analysis in 10 selected patients and 10 controls of the study. The results showed no presence of the homozygous form (*TT*) of the polymorphism in both patient group (GI) and control group (GII), and only the heterozygous form (*CT*) was detected in 3/10 in patients and in 2/10 of the controls. The absence of TT genotype in the present study might exclude *MTHFR* as the causative factor for increased level of HCY. This may prove that hematopoietic

Figure 3



Receiver operating characteristic curve analysis for homocysteine serum concentration for discriminating hypothyroid patients from control euthyroid.

Table 11 The sensitivity, the specificity, the positive predictive value, the negative predictive value, and the efficacy of homocysteine

	%SP	%SN	%NP	%PP	%EFF
Cases/controls	100	80	71.4	100	86.7
Anemia/nonanemia	53.3	73.3	66.7	61.1	63.3

SN, specificity; SP, sensitivity; NP, negative predictive value; PP, positive predictive value; EFF, efficacy.

Table 12 Methylenetetrahydrofolate reductase gene mutation analysis by PCR-RFLP

	MTHFR patients			MTHFR control		
	CC	CT	TT	CC	CT	TT
1	+			+		
2		+			+	
3	+			+		
4	+			+		
5	+			+		
6	+			+		
7	+			+		
8		+		+		
9		+		+		
10	+				+	

MTHFR, methylenetetrahydrofolate reductase.

system is one of the systems affected by hypothyroidism and anemia is the most common manifestation. Numerous mechanisms are involved in the pathogenesis of these anemias, which can be microcytic, macrocytic, and normocytic [29]. The most frequently encountered anemia type is normochromic normocytic anemia. The most frequent reason of this is the bone marrow repression owing to thyroid hormone deficiency, which also causes defective erythropoietin production [12].

Results of this study showed statistically insignificant decreased levels of MCV, mean corpuscular hemoglobin concentration, and Hct in the patient group (GI) than the control group (GII), but the Hb, Hb/Hct, and RDW were of statistical significant values, with a P value of 0.02 for Hb, P value of 0.01 for Hb/Hct, and a P value of 0.02 for the RDW, which reveal the relation between thyroid dysfunction and different hematological parameters [3]. Only the RDW showing significant difference between both subgroups of this study, denoting thyroid hormone influences the size variability of circulating red blood cells. These abnormalities might reflect the presence of micro/macrocytosis owing to combined iron and vitamin B_{12} deficiencies [27]. In this study, the serum iron level was below normal level (59 μg/dl). In 5/30 (16.7%) individuals in the control (GII) and 40% (12/30) in the patient group (GI), P value was 0.045, suggesting a significant relation between presence of hypothyroidism and iron-deficiency. Ranked Spearman's correlation in this study revealed HCY serum level was not significantly correlated with red cell indices, whereas a significant correlation (r=0.41, P=0.038) was found between serum iron level and B_{12}. The present study showed the presence of vitamin B_{12} deficiency to be seen in 6.7% (2/30) of the control group (GII), whereas it was found to be 44% (11/25) in the patient group (GI), showing a highly significance difference, with a P value of 0.001, which shows a relation between hypothyroidism and vitamin B_{12} deficiency. A recent study showed that vitamin B_{12} deficiency was found in 5.3% of the hypothyroid patients [16]. Vitamin B_{12} deficiency mostly occurs as

a result of malabsorption owing to pernicious anemia accompanying hypothyroidism. Insufficient intake, absorption change arising from deceleration in intestinal motility, intestinal wall edema, and bacterial infiltration are blamed among other reasons causing vitamin B_{12} deficiency [29, 30]. Most people with hypothyroidism have digestive insufficiency especially low gastric acid secretion. Thus, low thyroid activity may alter nutrient absorption, and iron deficiency is one of the many deficiencies likely to occur [16].

Conclusion

Our study observed an association between elevated HCY levels and hypothyroidism and found no correlation between homocysteinemia and anemia. The study also revealed a strong association between anemia and hypothyroidism, mainly owing to combined iron and vitamin B_{12} deficiencies.

We recommend screening hypothyroid patients for this polymorphism, and the patient food and nutrient needs should be considered more essential than treating the thyroid dysfunction.

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Conflicts of interest

None declared.

References

1 Yen PM. Physiological and molecular basis of thyroid hormone action. *Physiol Rev* 2001; **81**:1097–1142.

2 Jahromi MK, Ahmadi A, Samedanifared SH, Doostmohamadian S. The association between anemia and hypothyroidism: a clinical study. *Int J Hematol Oncol Stem Cell Res* 2010; **2012**:6–9.

3 Omar S, Hadj-Taeib S, Kanoun F, Hammami MB, Kamoun S, Ben Rneta N. Erythrocyte abnormalities in thyroid dysfunction. *Tunis Med* 2010; **88**:783–788.

4 Yang N, Yao Z, Miao L, Liu J, Gao X, Fan H, *et al.* Novel clinical evidence of an association between homocysteine and insulin resistance in patients with hypothyroidism or subclinical hypothyroidism. *PLoS One* 2015; **10**: e0125922.

5 Turhan S, Sezer S, Erden G, Guctekin A, Ucar F, Ginis Z, *et al.* Plasma homocysteine concentrations and serum lipid profile as atherosclerotic risk factors in subclinical hypothyroidism. *Ann Saudi Med* 2008; **28**:96–101.

6 Den Heijer M, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *J Thromb Haemost* 2005; **3**:292–299.

7 Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, *et al.* Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc Natl Acad Sci U S A* 1998; **95**:3059–3064.

8 Martinez-Frias ML, Perez B, Desviat LR, Castro M, Leal F, Rodriguez L, *et al.* Maternal polymorphisms 677C-T and 1298A-C of MTHFR, and 66A-G MTRR genes: is there any relationship between polymorphisms of the folate pathway, maternal homocysteine levels, and the risk for having a child with Down syndrome? *Am J Med Genet A* 2006; **140**:987–997.

9 Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, *et al.* Gene structure of human and mouse methylenetetrahydrofolate reductase(MTHFR). *Mamm Genome* 1998; **9**:652–656.

10 Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, *et al.* A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**:111–113.

11 Abd-Elmawla MA, Rizk SM, Youssry I, Shaheen AA. Impact of genetic polymorphism of methylenetetrahydrofolate reductase C677T on development of hyperhomocysteinemia and related oxidative changes in Egyptian β-thalassemia major patients. *PLoS One* 2016; **11**:e0155070.

12 Szczepanek-Parulska E, Hernik A, Ruchała M. Anemia in thyroid diseases. *Pol Arch Intern Med* 2017; **127**:352–360.

13 Selhub J, Morris MS, Jaques PF. In vitamin B12 deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentration. *PNAS* 2007; **104**:19995–20000.

14 Lippi G, Montagnan AM, Salvagno GL, Guidi GC. Should women with abnormal serum thyroid stimulating hormone undergo screening for Anaemia? *Arch Pathol Lab Med* 2008; **132**:321–322.

15 Bashir H, Hayat Bhat M, Shoib S, Farooq R, Hamid R, Majid S. Comparison of hematological parameters in untreated and treated subclinical hypothyroidism and primary hypothyroid patients. *Med J Islam Repub Iran* 2012; **26**:172–178.

16 Gunduz M, Gunduz E, Kircelli F, Okur N, Ozkaya M. Clinical study: role of surrogate markers of atherosclerosis in clinical and subclinical thyroidism. *Int J Endocrinol* 2012; **109797**: 1–6.

17 Lien EA, Nedrebø BG, Varhaug JE, Nygård O, Aakvaag A, Ueland PM. Plasma total homocysteine levels during short-term iatrogenic hypothyroidism. *J Clin Endocrinol Metab* 2000; **85**:1049–1053.

18 Zhou Y, Chen Y, Cao X, Liu C, Xie Y. Association between plasma homocysteine status and hypothyroidism: a meta-analysis. *Int J Clin Exp Med* 2014; **7**:4544–4553.

19 19.Bamashmoos SA, Al-Nuzaily MA, Al-Meeri AM, Ali FH. Relationship between total homocysteine, total cholesterol and creatinine levels in overt hypothyroid patiens. *Springerplus* 2013; **30**:423.

20 Orzechowska-Pawilojc A, Sworczak K, Lewczuk A, Babinska A. Homocysteine, folat and cobalamin levels in hypothyroid women before and after treatment. *Endocr J* 2007; **54**:471–476.

21 Nedrebø BG, Nygård O, Ueland PM, Lien EA. Plasma total homocysteine in hyper- and hypothyroid patients before and during 12 months of treatment. *Clnical Chem* 2001; **47**:1738–1741.

22 Luboshitzky R, Herer P. Cardiovascular risk factors in middle-aged women with subclinical hypothyroidism. *Neuro Endocrinol Lett* 2004; **25**:262–266.

23 Vargas F, Moreno JM, Rodriguez-Gomez I, Wangensteen R, Osuna A, Alvarez-Guerra M, Garcia-Estan J. Vascular and renal function in experimental thyroid disorders. *Eur J Endocrinol* 2006; **154**:197–212.

24 24.Brattstrom L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. *Circulation* 1998; **98**:2520–2526.

25 25.Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, *et al.* Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 1996; **93**:7–9.

26 Van der Put NMJ, Van de Heuvel LP, Steegers-Teunissen RPM, Trijbels FJM, Eskes TKAB, Mariman ECM, *et al.* Decreased methylenetetrahydrofolate reductase activity due to the C677T mutation in families with spina bifida offspring. *J Mol Med* 1996; **74**:691–694.

27 Montagnana M, Lippi G, Targher G, Salvagno GL, Guidi GC. The redblood cell distribution width is associated with serum levels of thyroid stimulating hormone in the general population. *Int J Lab Hematol* 2009; **31**:581–582.

28 28.Kluijtmans LAJ, Kastelein JJC, Lindemans J, Boers GHJ, Heil SG, Bruschke AVG, *et al.* Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. *Circulation* 1997; **96**:2573–2577.

29 Kaferle J, Strzoda CE. Evaluation of Macrocytosis. *Am Fam Physician* 2009; **79**:203–208.

30 Morris MS, Bostom AG, Jacques PF, Selhub J, Rosenberg IH. Hyperhomocysteinemia and hypercholesterolemia associated with hypothyroidism in the third US National Health and Nutrition Examination Survey. *Atherosclerosis* 2001; **155**:195–200.