HORMONES 2014, 13(3):389-397

Research paper

The associations of polymorphisms of TSH receptor and thyroid hormone receptor genes with L-thyroxine treatment in hypothyroid patients

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ABSTRACT

OBJECTIVE: To investigate the possible association between response to levothyroxine (L-T4) doses in hypothyroid patients and variation in thyroid stimulating hormone receptor *(TSHR)* gene and thyroid hormone receptor (*THRα*) gene. DESIGN: This cross-sectional correlation study included 228 patients with primary hypothyroidism who were using L-T4 replacement therapy. Thyroid function test was performed using standard techniques. Genotyping of rs939348 of the *THRα* gene, and rs2268458 and rs2239610 of the *TSHR* gene was performed using the polymerase chain reaction-based restriction fragment length polymorphism assay (PCR-RFLP). Patient history of illness, medication and compliance data were collected using the patients' medical files. RESULTS: The *THRα* rs939348 polymorphism was associated with L-T4 replacement doses in hypothyroid patients and in central obesity. No significant correlation was detected between the examined SNPs to *TSHR* and L-T4 doses or the different clinical and biochemical parameters. Finally, L-T4 dose was associated with lower BMI, waist circumference and TSH, and higher free T4 (fT4) among hypothyroid patients. CONCLUSIONS: Whereas the two tested *TSHR* polymorphisms were not associated with the dose of T4, the *THRα* rs939348 polymorphism was associated with L-T4 dose and central obesity among hypothyroid patients. T4 dose was also associated with multiple beneficial effects among hypothyroid patients.

Key words: Hypothyroidism, Levothyroxine, Jordan, Thyroid hormone, TSH receptor

INTRODUCTION

Hypothyroidism is one of the most common endocrine disorders.[1] It is characterized by underproduction of thyroid hormone[2,3] which plays an essential physiologic role in the development of the individual and body metabolism.[4] Hypothyroidism is associated with a wide spectrum of signs, symptoms and long-term complications such as skin manifestations, obesity, hyperlipidemia, bradycardia, fatigue and depression.[1-3,5,6] Diagnosis and monitoring of hypothyroid patients are based on both clinical evaluation and thyroid function testing.[2,7] Thyroid stimulating

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Received 14-10-2013, Accepted 14-12-2013

hormone (TSH or thyrotropin) is the most sensitive and valuable test of thyroid function and treatment response among patients.[2]

Oral levothyroxine (L-T4) is the drug of choice in the treatment of hypothyroidism.[2,3,8-10] The goal of therapy is to resolve clinical symptoms and to restore TSH, free T4 (fT4) and free T3 (fT3) to normal ranges.[2,3] The average daily L-T4 replacement dose that is needed to control hypothyroid adults is 1.7 mcg/kg of body weight (ideal body weight for obese patients).[2,3,11,12] Several factors can affect achievement of the goals with L-T4 therapy, including age, gender, other medical conditions, concurrent medications, patient compliance and genetic background.[2,3,9,11-15]

Thyroid hormones exert their action by binding to two subtypes of nuclear receptors, which are known as thyroid hormone receptors (THR) α and β.[16,17] These receptors are regulated by two genes: *THRα*, which is located on chromosome 17, and *THRβ*, which is located on chromosome 3.[18,19] Rs939348 is a *THRα* single nucleotide polymorphism that was found to be associated with increased systolic blood pressure and incidence of hypertension.[20] On the other hand, thyroid gland secretion and growth are regulated by the action of TSH, which exerts its role through binding to TSH receptor (TSHR).[21-23] Many studies have investigated the role of mutations in gene encoding TSHR in the development of different thyroid diseases.[5,24-26] An association between *TSHR* gene SNP (rs2268458), which is located in intron 1, and higher incidence of Graves' disease was found by two studies conducted by Dechairo BM and colleagues and Yin X and colleagues.[24,25,27] Gu LQ and colleagues have investigated the role of *TSHR* SNP (rs2239610) in thyroid diseases and have found CC genotype to be associated with higher serum concentrations of fT4.[28] In this study, we investigated the possible association between L-T4 doses in hypothyroid patients and *THRα* rs939348 SNP, and *TSHR* rs2268458 and rs2239610 SNPs.

SUBJECTS AND METHODS

Subjects

This is a cross-sectional study that was approved by Jordan University of Science and Technology Institutional Review Board (IRB) and was carried out in accordance with the Helsinki Declaration of 1975, with all amendments and revisions. A total of 228 unrelated patients with primary hypothyroidism were recruited from diabetes and endocrinology clinics at Ibn Al-Nafees Hospital and King Abdullah University Hospital. Patients included in this study are those who had completed puberty and had already been on stable doses of L-T4 for the previous 3 months. Excluded from this study were those with active neoplasm or history of neoplasm, severe liver dysfunction, severe-renal failure, diseases of the pituitary gland or hypothalamus including secondary hypothyroidism, major surgery within 2 weeks of enrolment, a severe psychiatric condition not related to hypothyroidism symptoms, GIT malabsorption diseases, pregnancy, alcohol abuse, concurrent medication that may interfere with thyroid hormone absorption or activation, critically ill patients, Hashimoto's thyroid disease and thyroidectomized patients on L-T4 treatment. Data collection was carried out by trained individuals and using information obtained either from subjects or their files. Written informed consents were obtained from all patients in accordance with the requirements of the IRB of Jordan University of Science and Technology.

Measurements of body mass index (BMI) and blood pressure

BMI was calculated as (weight in Kgs) / (height in meters)^2 as previously described.[29] For each patient, the ideal body weight (IBW) and adjusted body weight (ABW) were calculated. The study population was divided into two groups: the low-dose group, which represented patients who were controlled on $<$1.7mcg/kg/day, and the high-dose group, which represented patients who were controlled on $\geq$1.7mcg/kg/day. This cut point was chosen based on previous studies[30] and because it represents the average L-T4 dose that is required by most hypothyroid patients.[31] Blood pressure was measured by a trained healthcare professional using an arm cuff and a mercury column sphygmomanometer on the left arm after the patient had been in a resting period of at least 10 minutes in the supine position. Three measurements at one visit was averaged to evaluate the systolic and diastolic blood pressure.

Sample Collection and Handling

Blood samples were collected from patients who matched the study criteria by a specialized licensed nurse. Blood (5 mL) was withdrawn and distributed into anticoagulant – free plain tube (3 mL) and evacuated EDTA tube (2 mL). The blood sample in the plain tube was centrifuged after 30 minutes of sampling and serum was isolated and stored at -20˚C and sent to Ibn Alnafees Hospital laboratory for biochemical analysis. Assays include TSH, fT3 and fT4. Assays were carried out using the AxSYM instrument (Abbott Laboratories, IL, U.S.A.) and commercially available diagnostic kits (Abbott Laboratories). Samples were assayed in duplicate and the mean of the paired results was determined. The blood collected in the EDTA tubes was used for DNA extraction.

MOLECULAR ANALYSIS

DNA extraction

The venous blood, which was collected in the evacuated EDTA tubes, was used for DNA extraction that was performed using the Promega wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the standard protocol provided by the manufacturer.

PCR-RFLP

Reference sequence and details of SNPs, PCR primers' design and restriction enzymes were obtained by searching the UCSC Genome Bioinformatics Site, Primer3 program and NEBcutter program, respectively.[32-36] The location for the three SNPs, Rs939348 on the *THRα* gene, and Rs2268458 and Rs2239610 on the *TSHR* gene, is described in Table 1. The genomic DNA was amplified using the following steps: denaturation of double stranded genomic DNA at 94˚C for 5 minutes, DNA amplification using 30 cycles. Each cycle consists of: denaturation at 94˚C for 30s, annealing for 30s, extension at 72˚C for 40s, final elongation at 72˚C for 7 minutes, and ending reaction at 4˚C. Table 1 summarizes restriction enzymes and restricted fragments for all SNPs. The restriction enzyme digestion was carried out in 20 μl containing 1 unit of enzyme and 10 μl of PCR product and incubated at 37˚C for overnight. All enzymes were obtained from Fermentas (GmbH, St. Leon-Rot, Germany). PCR products and digested fragments were detected using electrophoresis on 2% agarose.

Statistical Analysis

Data was analyzed using the SPSS version 17 package (SPSS Inc, Chicago, USA) for Windows. Continuous variables were expressed as mean (standard deviation) and the differences were accomplished by comparison via student's unpaired 2-sided t-test or one way ANOVA as appropriate. Discrete variables were expressed as counts and frequencies and were compared using the chi-square test. If $N<5$, exact Fisher statistic was used. The genotype distributions of SNPs were analyzed in agreement with the Hardy-Weinberg equilibrium. A significant difference is considered at $P<0.05$.

RESULTS

The mean age of patients was 39.4 years, male to female ratio was 1:8 and the mean BMI (kg/m^2) was 29.3. Of the 228 participants, 23.3% were dyslipidemic, 17.5% were diabetic and 23.2% were hypertensive (Table 2). The average duration of disease was 4.8 years. About 44% of the participants had a family history of thyroid disorders (Table 2).

Table 1. The PCR primers, restriction enzymes and sizes of the amplified and digested fragments of the examined SNPs

SNP	PCR product (bp)	Primers	Restriction enzyme	Restricted fragments (bp)
Rs939348	190	F: 5'CCTGTGTCTCCCAGCTTAGG '3 R: 5'CCACCAGACTCACAGCCTCT '3	MesI	C allele 190 T allele 48 & 142
Rs2268458	162	F: 5'CTAACCAGCAGAGGGAGCAC'3 R: 5'CCACTGCTTAAAGCCCAGAT '3	AluI	T allele 162 C allele 100 & 62
Rs2239610	172	F: 5'CCAGAGATCAAGGGCATCTG '3 R: 5'CCAAGTGTGGGCGATTAAGT '3	NlaIV	G allele 172 C allele 142 & 30

Table 2. Characteristics of the hypothyroid patients (N=228)

Variable		Number of hypothyroid patients N (%) or mean (SD)	
Age: mean (SD)		39.439	(14.071)
Gender	Male	15	(6.6%)
	Female	213	(93.4%)
Smoker	Yes	20	(8.8%)
	No	208	(91.2%)
BMI (kg/m^2): mean (SD)		29.334	(6.515)
BMI categories:	Normal weight	56	(24.6%)
	Overweight	75	(32.9%)
	Obese	55	(24.1%)
	Severely obese	42	(18.4%)
Waist circumference (cm): mean (SD)		90.766	(18.355)
Central obesity:	Yes	119	(52.2%)
	No	109	(48.8%)
Dyslipidemia	Yes	54	(23.7%)
	No	174	(76.3%)
Hypertension	Yes	53	(23.2%)
	No	175	(76.8%)
Diabetes Mellitus	Yes	40	(17.5%)
	No	188	(82.5%)
Coronary Heart Disease	Yes	14	(6.1%)
	No	214	(93.9%)
Osteoporosis	Yes	24	(10.5%)
	No	204	(89.5%)
Family history of Diabetes Mellitus	Yes	139	(61%)
	No	89	(39%)
Family history of Thyroid Disorders	Yes	101	(44.3%)
	No	127	(55.7%)
Family history of Coronary Heart Disease	Yes	76	(33.3%)
	No	152	(66.7%)
Duration of illness (years): mean (SD)		4.843	(5.177)
	<10 years	188	(82.5%)
	≥10 years	40	(17.5%)
TSH (mIU/L): mean (SD)		2.129	(1.263)
fT3 (pg/mL): mean (SD)		2.458	(0.597)
fT4 (ng/dL): mean (SD)		1.148	(0.324)
L-T4 daily dose (mcg/kg/day): mean (SD)		1.381	(0.655)
SBP (mmHg): mean (SD)		117.697	(12.187)
DBP(mmHg): mean (SD)		75.351	(8.544)

The genotype and allele frequencies of examined SNPs were determined. For rs939348 (C/T), the frequency of wild type C allele was 70%, whereas for rs2268458 (T/C), the frequency of the wild type T allele was 76% and for rs2239610 (G/C) the wild type G allele frequency was 74%. The distribution of rs939348 genotypes was 50.9% for CC, 37.3% for CT and 11.8% for TT, whereas for rs2268458, the distribution was 59.2% for TT, 33.3% for TC and 7.5% for CC. For rs2239610, the distribution of the genotypes was 58.3% for GG, 32.3% for GC and 9.4% for CC.

The clinical and biochemical characteristics of rs939348, rs2268458 and rs2239610 genotypes were studied (Tables 3-5). There was no significant cor-

Table 3. Means and standard deviations of total hypothyroid patients according to genotypes and alleles of Rs939348

Variable	THRα rs939348			P-value one way ANOVA	Alleles		P-value un-paired t-test
	CC N=116	CT N=85	TT N=27		C N=317	T N=139	
Age, years Mean (SD)	40.48 (13.96)	39.64 (12.78)	33.89 (15.11)	0.079	40.25 (13.61)	37.4 (13.91)	0.413
BMI Mean (SD)	29.74 (7.02)	29.6 (5.86)	26.6 (5.63)	0.071	30.10 (6.44)	29.00 (5.59)	0.072
Waist circum, cm Mean (SD)	91.65 (19.16)	92.4 (16.65)	81.63 (17.90)	**0.021**	91.84 (18.47)	88.21 (17.81)	0.510
Duration of illness, years Mean (SD)	4.62 (4.72)	4.8 (5.33)	5.61 (6.46)	0.671	4.69 (4.88)	5.15 (5.76)	0.377
TSH (mIU/L)	2.16 (1.27)	2.13 (1.20)	1.96 (1.42)	0.759	2.15 (1.25)	2.06 (1.28)	0.490
fT3 (pg/mL)	2.45 (0.57)	2.48 (0.64)	2.36 (0.57)	0.654	2.46 (0.59)	2.44 (0.61)	0.755
fT4 (ng/dL)	1.15 (0.35)	1.12 (0.27)	1.17 (0.35)	0.707	1.14 (0.33)	1.14 (0.30)	0.995
L-T4 daily dose (mcg/kg/day)	1.30 (0.59)	1.45 (0.73)	1.49 (0.57)	0.167	1.31 (0.62)	1.48 (0.67)	**0.014**
SBP (mmHg)	117.33 (11.54)	118.29 (13.28)	116.67 (11.43)	0.783	117.58 (12.0)	117.66 (12.5)	0.952
DBP (mmHg)	74.87 (8.37)	75.88 (8.66)	75.37 (9.08)	0.710	75.14 (8.44)	75.68 (8.76	0.534

Table 4. Means and standard deviations of total hypothyroid patients according to genotypes and alleles of Rs2268458

Variable	TSHR rs2268458			P-value one way ANOVA	Alleles		P-value Un-paired t-test
	TT N=135	TC N=76	CC N=17		T N=346	C N=110	
Age, years Mean (SD)	39.3 (13.73)	39.46 (14.22)	39.76 (12.7)	0.990	39.33 (13.8)	39.55 (13.6)	0.883
BMI Mean (SD)	29.09 (6.28)	29.90 (7.17)	28.47 (5.04)	0.590	29.27 (6.48)	29.46 (6.58)	0.789
Waist circum, cm Mean (SD)	90.87 (18.59)	91.54 (18.58)	86.18 (15.47)	0.550	91.01 (18.53)	89.88 (17.72)	0.573
Duration of illness, years Mean (SD)	4.7 (4.60)	4.6 (5.44)	6.14 (7.79)	0.553	4.73 (4.78)	5.1 (6.21)	0.482
TSH (mIU/L)	2.19 (1.25)	1.93 (1.27)	2.27 (1.31)	0.464	2.14 (1.25)	2.07 (1.28)	0.617
fT3 (pg/mL)	2.47 (0.63)	2.43 (0.566)	2.35 (0.466)	0.684	2.46 (0.61)	2.41 (0.53)	0.374
fT4 (ng/dL)	1.18 (0.33)	1.09 (0.31)	1.09 (0.306)	0.152	1.16 (0.32)	1.09 (0.31)	0.063
L-T4 daily dose (mcg/kg/day)	1.38 (0.65)	1.32 (0.611)	1.64 (0.779)	0.192	1.37 (0.64)	1.42 (0.67)	0.441
SBP (mmHg)	117.85 (13.26)	117.04 (10.71)	118.24 (11.85)	0.877	117.67 (12.62)	117.4 (10.61)	0.843
DBP (mmHg)	75.33 (9.16)	74.93 (7.68)	76.76 (7.27)	0.728	75.24 (8.83)	75.5 (7.54)	0.786

Table 5. Means and standard deviations of total hypothyroid patients according to genotypes and alleles of Rs2239610

Variable	TSHR rs2239610			P-value one way ANOVA	Alleles		P-value Un-paired t-test
	GG N=133	GC N=73	CC N=22		G N=339	C N=117	
Age, years Mean (SD)	39.44 (13.58)	39.66 (14.85)	38.14 (11.44)	0.900	39.45 (13.8)	39.08 (13.60)	0.784
BMI Mean (SD)	29.09 (6.37)	29.97 (7.10)	28.49 (5.22)	0.533	29.28 (6.52)	29.41 (6.46)	0.846
Waist circum, cm Mean (SD)	91.35 (18.89)	90.34 (17.40)	88.36 (18.5)	0.761	91.13 (18.54)	89.59 (17.72)	0.435
Duration of illness, years Mean (SD)	4.91 (5.09)	4.22 (4.627)	6.73 (6.98)	0.226	4.7 (4.98)	5.03 (5.66)	0.628
TSH (mIU/L)	2.15 (1.269)	2.10 (1.263)	2.06 (1.273)	0.930	2.14 (1.26)	2.08 (1.25)	0.683
fT3 (pg/mL)	2.43 (0.59)	2.51 (0.629)	2.35 (0.575)	0.498	2.45 (0.598)	2.45 (0.60)	0.986
fT4 (ng/dL)	1.16 (0.33)	1.12 (0.319)	1.09 (0.307)	0.486	1.15 (0.329)	1.11 (0.312)	0.197
L-T4 daily dose (mcg/kg/day)	1.40 (0.66)	1.28 (0.621)	1.54 (0.668)	0.219	1.38 (0.65)	1.38 (0.64)	0.964
SBP (mmHg)	117.86 (13.17)	117.05 (10.43)	117.95 (11.61)	0.895	117.68 (12.60)	117.3 (10.79)	0.824
DBP (mmHg)	75.3 (8.87)	75.27 (8.03)	75.45 (8.57)	0.996	75.29 (8.67)	75.34 (8.16)	0.959

relation between rs2268458 and rs2239610 genotypes and L-T4 dose or any of the studied biochemical and clinical parameters (Tables 4-5). A significant difference between the *THRα* rs939348 genotypes and waist circumference (P=0.021) was detected, where the TT genotype was associated with less waist circumference (Table 3). In addition, a significant correlation between L-T4 daily dose (mcg/kg/day) and the two alleles of rs939348 was found (P=0.014), where T allele carrier patients required significantly higher doses of L-T4 for control of hypothyroidism (Table 3).

The patients were also divided into two groups based on current replacement L-T4 dose: low dose group (<1.7 mcg/kg/day, n = 154) and high dose group (≥1.7 mcg/kg/day, n = 74). The dose of T4 was associated with lower BMI (P=0.034), WC (P=0.018) and TSH (P=0.001), and higher fT4 levels (P=0.001; Figure 1). Other parameters including age, presence of dyslipidemia, hypertension, diabetes mellitus, systolic and diastolic blood pressures, and the distribution of the genotypes of the three examined *SNPs* were not different between the two dosing groups.

DISCUSSION

In this study, we hypothesized that there might be an association between polymorphisms in the *THR* and *TSHR* genes and the replacement doses of L-T4. The role of thyroid hormones receptors (THRα and THRβ) in mediating the functions of the thyroid gland is well documented.[16,17] In addition, thyroid gland functions are regulated by the action of TSH, which exerts its role through binding to TSHR.[21-23] Many studies have investigated the role of mutations in genes encoding TSHR and THR in the development of different thyroid diseases.[5,24-26]

This study showed no association between *TSHR* SNPs rs2268458 and rs2239610, and the replacement doses of L-T4. Additionally, the current study found no correlation between these polymorphisms and the studied biochemical and clinical markers including thyroid function tests (TSH, fT3 and fT4). A previous study by Gu LQ and colleagues found that rs2239610 CC genotype was associated with higher serum concentrations of fT4.[28] Another study by Louwerens et al. detected a modest effect for TSHR-Asp727Glu polymorphism on fatigue in patients with differentiated thyroid carcinoma.[5]

In our study, we found a positive correlation between the *THRα* rs939348 polymorphism and both L-T4 replacement doses and central obesity. On the other hand, our study showed lack of association

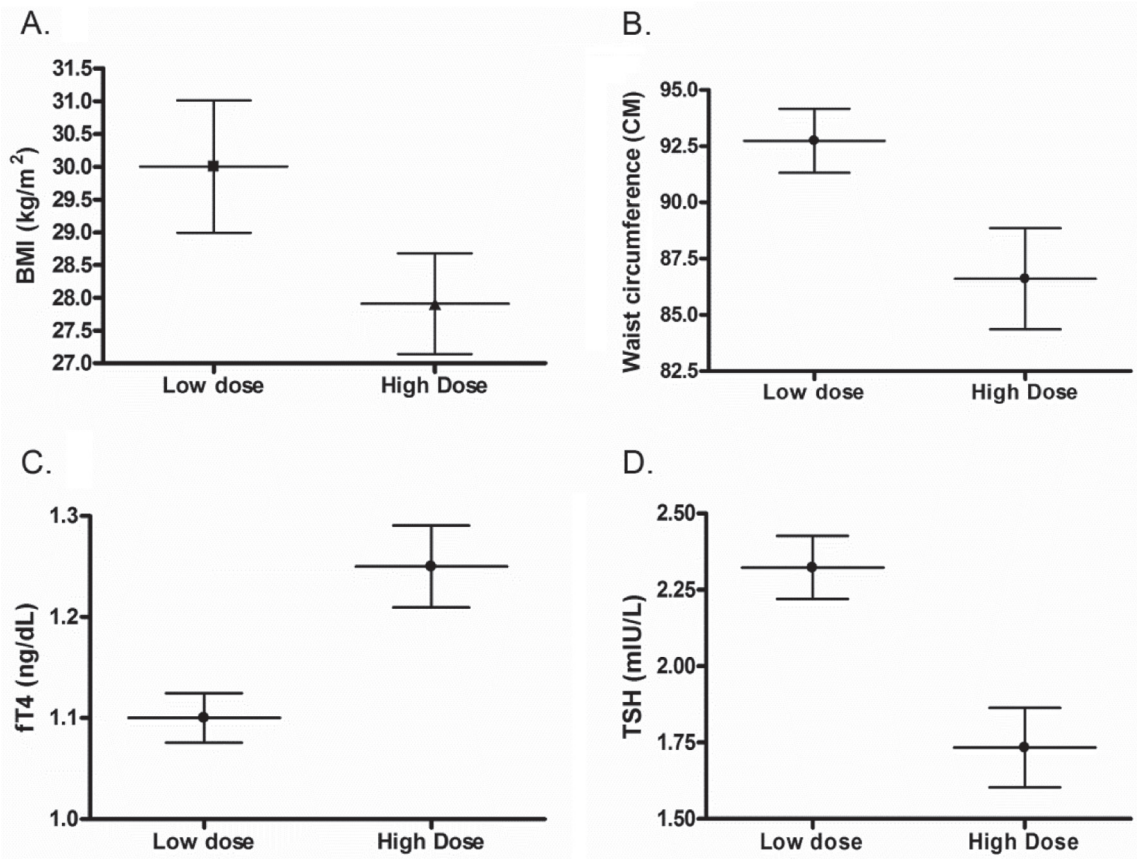

Figure 1. Patients were divided into two groups: low-dose group, which represented patients who were controlled on <1.7mcg/kg/day, and high-dose group, which represented patients who were controlled on ≥1.7mcg/kg/day. Levothyroxine (L-T4) dose was associated with lower BMI (A), waist circumference (B), and TSH (D) and higher free T4 (fT4; C) among hypothyroid patients.

between *THRα* rs939348 and elevated blood pressure, yet an association was shown in Goumidi L and colleagues' study among US populations.[20] This could be due to the small number of hypertensive patients included in the present study (n = 53).

In the current study, a positive association that was found between central obesity and *THRα* rs939348 polymorphism suggests that this SNP could have a role in development of metabolic syndrome, which is known to be a risk factor for coronary heart disease.[15,37] The rs939348 SNP was studied in addition to a wide range of other SNPs by Arnaud-Lopez L. and colleagues and a positive correlation was detected among its variations and TSH serum level.[24]

Factors other than genetic variations were shown to be associated with the dose of L-T4. For example, high L-T4 dose was associated with lower BMI, lower body weight and TSH and higher levels of fT4. These results are in agreement with the previous knowledge about L-T4 treatment among hypothyroid patients[38] and generally support our genetic findings.

The clinical consequence of this study is the proposal that hypothyroidism patients with rs939348 TT genotypes might need to be identified prior to treatments, as they are expected to require higher doses of L-T4. More studies are required in other populations to generalize the present finding.

In conclusion, hypothyroid patients with rs939348 TT genotype had more central obesity and those who carry the T allele needed higher replacement doses of L-T4. We therefore suggest that this SNP might have a role in control of both hypothyroidism, of metabolic syndrome and its associated risk and of resistance to L-T4 therapy.

FUNDING

The study was funded by grant no. 168/2010 from the Deanship of Research of the Jordan University of Science and Technology.

CONFLICT OF INTEREST

None to declare.

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