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THRA RS939348 POLYMORPHISM AND L-THYROXINE RESPONSE IN IRAQI HYPOTHYROID FEMALES

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ABSTRACT

Hypothyroidism is a prevalent endocrine disorder, particularly affecting women and older adults, with potentially serious health impacts if left untreated. Levothyroxine is the standard therapy for primary hypothyroidism; however, its efficacy may be influenced by genetic polymorphisms. This study investigates the influence of the THRA rs939348 T>C polymorphism on L-thyroxine treatment response in Iraqi females with primary hypothyroidism. A cross-sectional study was conducted from September 2023 to July 2024, including 100 hypothyroid female patients on levothyroxine therapy (≥4 months) and 50 healthy female controls. Clinical data and treatment outcomes were gathered through questionnaires and medical records. DNA genotyping for THRA rs939348 was performed using Restriction Fragment Length Polymorphism-PCR (RFLP-PCR), and biochemical analyses assessed thyroid function, glucose, insulin, and lipid profiles. Patients were grouped by THRA rs939348 genotype (TT, TC, CC) and categorized as responders or non-responders based on achieving normalized TSH levels (0.5-5μU/ml). Key findings indicated that among responders, 33% had the TT genotype, while 20% had TC/CC genotypes. Conversely, among non-responders, only 11% had the TT genotype, and 36% had TC/CC genotypes, showing a significant association between the mutant allele and poorer treatment response ($P<0.0001$). Responders with the TT genotype demonstrated stable TSH levels similar to healthy controls, whereas non-responders with the TC/CC genotypes had TSH levels six times higher than responders and healthy individuals. These results underscore the clinical relevance of THRA rs939348 polymorphism testing, as it may help identify patients at risk for suboptimal treatment response, potentially guiding personalized hypothyroidism management strategies.

KEYWORDS: hypothyroidism, L-thyroxin, TSH, THRA1 polymorphism.

INTRODUCTION

Hypothyroidism is a condition characterized by thyroid hormone deficiency, which can lead to serious health complications, including mortality, if untreated. It is primarily diagnosed through elevated thyroid stimulating hormone (TSH) and low free thyroxine (FT4) levels indicate overt hypothyroidism while elevated TSH along with normal FT4 levels signifies subclinical hypothyroidism [1,2]. Hypothyroidism is classified into primary hypothyroidism caused by thyroid gland dysfunction, secondary hypothyroidism that characterized by pituitary dysfunction, or tertiary hypothyroidism caused by hypothalamic dysfunction. Globally, hypothyroidism affects approximately 5–10% of the population, with significant geographical variability in prevalence due to dietary iodine intake, autoimmune disorders, and environmental factors [3]. Women are predominantly affected, with an incidence rate 8–9 times higher than in men, particularly between the ages of 30 and 50 [4,5]. The clinical presentation of hypothyroidism varies with age, sex, and the timing of diagnosis, and often include, dry skin, cold sensitivity, fatigue, muscle cramps, voice changes, constipation, heavy menstrual periods, and weight gain [4,5]. Cardiovascular complications are significant, with increased vascular resistance, lower cardiac output, impaired left ventricular function, and higher rates of myocardial damage and pericardial effusions [6]. Metabolic syndrome, such as dyslipidemia, elevated waist circumference, and hypertension are also common among hypothyroid patients [7,8]. Severe untreated hypothyroidism can lead to myxedema, a life-threatening condition that may result in coma [9]. Levothyroxine (L-thyroxine) is a synthetic form of T4, and it is a dominant treatment for hypothyroidism [10,11]. L-thyroxine treatment, generally at 1.6μg/Kg body weight, is influenced by many factors such as malabsorption, pH changes, and drug interactions [12]. For instance, proton pump inhibitors can reduce levothyroxine absorption by increasing stomach pH [13,14]. Despite its efficacy, variability in treatment response remains a challenge, with genetic factors like polymorphisms influencing drug effectiveness [15]. The human TRalpha1 gene encodes the thyroid receptor alpha 1 and located on chromosome 17 is crucial for heart development [8,15–17]. Variations in this gene have been linked to alterations in thyroid hormone receptor activity and associated with conditions such as higher blood pressure and dyslipidemia [17–22]. For example, THRA gene polymorphisms like rs939348 were found to correlate with variations in lipid

profiles and body mass index (BMI) among patients with hypothyroidism, indicating a protective effect of certain alleles against dyslipidemia [23–25]. Additionally, the importance of THRA gene polymorphism in regulating thyroid hormone functions may be relevant in the context of neurodegenerative diseases, particularly Alzheimer's disease (AD). Therefore, understanding the effects of thyroid hormones on brain functions and development by investigating the relationship between THRA and AD risk becomes a critical area of research [26–28]. Given the variability in hypothyroidism prevalence worldwide and the potential role of genetic factors like THRA rs939348 in influencing treatment outcomes, this study focuses on understanding these genetic effects in Iraqi females. By investigating the association between the THRA rs939348 T>C polymorphism and treatment responses to L-thyroxine, we aim to identify genotype-specific variations that could guide tailored dosing strategies and improve therapeutic outcomes [29,30].

PATIENTS AND METHODS

Patients

This study was performed from September 2023 to July 2024. A total of 150 female patients were enrolled in this cross-sectional study when they visit a private clinic to get medication and advice about their cases. All the patients were already diagnosed with primary hypothyroidism. The patients were receiving levothyroxine for at least 4 months. The study was approved by the College of Pharmacy's Scientific and Ethical Committee at the University of Kerbala. Following an explanation of the study's purpose and design, each patient signed an informed consent form. Patients were excluded from this study whose course of treatment is shorter than four months so who is under 40 years old, also the patients were given any medicine that affects the expression or activity of TRA1 or interacts with levothyroxine and the patient had a thyroidectomy. The sample size was performed based on practical considerations, including resource viability, the prevalence of hypothyroidism in the local population and recruitment feasibility within the study time line. In addition, this study was designed based on previous genetic cross-sectional studies aimed to detect statistically significant differences in treatment outcomes and genotype frequencies.

Clinical Data Collection

All subjects completed a written consent form outlining the goal of the research and were asked to complete a specially created questionnaire before being officially included age, weight, family history, smoking, Hypothyroidism complication, Duration of treatment of levothyroxine, Drug side effect, Other disease, Other medication, and blood pressure.

Inclusion Criteria

Female patients with hypothyroidism aged 40 years or older who have been receiving levothyroxine for four months or more.

Exclusion Criteria

patients whose treatment duration was less than four months, those under 40 years of age. Patients who were taking medications that affect the expression or activity of TRA1 or interact with levothyroxine, and those who had undergone thyroidectomy.

Blood Sample Collection

Following an overnight fast and vein puncture, patients' 5 ml of blood were drawn; these samples were separated into two portions, the first of which, 2 ml, was stored in an EDTA tube for the extraction of DNA, and the remainder of which Second part: 3 ml were placed in a gel tube for the purpose of extracting sera, which was utilized to evaluate the other biochemical test and thyroid function tests

GENOTYPING

Genotyping Methodology

This study investigated a single nucleotide polymorphism (SNP), rs939348, in the thyroid hormone receptor alpha1 (THRA1) gene, which encodes a receptor crucial for the binding and response of thyroid hormones. DNA was extracted from collected samples at the Laboratory of Molecular Biology, College of Pharmacy, University of Kerbela, using the gSYNC™ Genomic DNA Extraction Mini-Kit (Taiwan). DNA concentration and purity were assessed using spectrophotometry, and the extracted DNA was stored at -20 °C until use. The RFLP-PCR method was employed for genotyping the THRA1 rs939348 SNP. This technique utilized the restriction enzyme MseI (purchased from American BioLabs), selected based on its ability to specifically recognize and cleave the sequence containing the rs939348 polymorphism. Specific primers for this SNP, designed using Primer-BLAST software and synthesized by Oligo (Korea), are listed in Table 1.

Primer Preparation

Each primer was dissolved in nuclease-free water to prepare a stock solution at a concentration of 100 pmol/µl. Working solutions (10 pmol/µl) were prepared by diluting 10 µl of stock solution with 90 µl of nuclease-free water.

PCR Amplification
PCR was performed in a total reaction volume of 25 µl containing:
50 ng of genomic DNA
1X PCR buffer
1.5 mM $MgCl_2$
0.2 mM dNTPs
0.5 µM of each primer
1 U Taq polymerase
The thermocycling conditions were as follows:
Initial denaturation at 95 °C for 3 minutes
32 cycles of:
Denaturation at 95 °C for 35 seconds
Annealing at 62 °C for 45 seconds
Extension at 72 °C for 55 seconds
Final extension at 72 °C for 5 minutes
Gel Electrophoresis and Visualization:
Amplified PCR products were separated using 1.5% agarose gel electrophoresis stained with ethidium bromide. A 100–1500 base pair DNA ladder was used to estimate the fragment sizes. Bands were visualized and photographed under a UV trans-illuminator.
Limitations of the RFLP-PCR Method:
The RFLP-PCR method has limitations, including the potential for incomplete digestion of PCR products, which can result in genotype misclassification. Additionally, its sensitivity may be affected by DNA quality, requiring high-purity DNA for accurate results. Advanced techniques like real-time PCR or next-generation sequencing could offer improved sensitivity and specificity but were not feasible in this study due to resource constraints.

Biochemical Analysis
An immunoassay was used to quantify thyroid hormones. TSH was quantified using electrochemiluminescence immunoassay (ECLIA) designed for the Cobase immunoassay analyzer. Briefly, two different monoclonal antibodies specific for TSH to form a sandwich complex. Microparticles are magnetically attracted to the ekectrode surface, where chemiluminescent emission is induced by applied voltage and measured by a photomultiplier. Total and free T4 and T3 were measured using competitive chemiluminescence immunoassays. The catalog numbers for the thyriod function test kits were 130203001M, 130203003M, 130203005M, 130203002M, and 130203004M, respectively. Fasting serum insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit based on the sandwich principle. The kit was manufactured Mindray Corporation (China), catalog number 130205002M. Blood glucose, cholesterol, triglycerides, HDL, LDL and VLDL plasma levels were measured photometrically using lab kits manufactured by Mindray Corporation (China). The respective catalog numbers were GLU0102, TC0102, TG0102, HDL0102, LDL0102, and VLDL0102.

Clarifying Cut-off Determination for Responders and Non-Responders
The classification of hypothyroid patients as responders or non-responders to levothyroxine (L-thyroxine) treatment was based on their thyroid function test results and clinical improvement after at least four months of treatment. Responders were defined as patients achieving normalized TSH levels within the reference range (0.4–4.0 µIU/mL) accompanied by clinical symptom resolution. Non-responders were defined as those with persistently elevated TSH levels (>4.0 µIU/mL) despite adherence to treatment or those reporting incomplete symptom resolution. These criteria align with guidelines for managing hypothyroidism and reflect a combination of biochemical and symptomatic assessment.

Statistical Analysis
The collected data of the present study were entered from patients' sheets and analyzed through the Statistical Package for the Social Sciences (SPSS version 26). The data were presented as frequencies and percentages or mean and standard deviation in appropriate tables and graphs. The distribution of alleles and genotypes in accordance with Hardy-Weinberg equilibrium was examined using the goodness of fit test, followed by a Chi-square analysis. The P value of 0.05 or less was regarded as statistically significant.

RESULTS

Demographic Data of The Study's Participants:
The demographic and clinical analyses of the study participants all of whom were females including 50 healthy and 100 patients with hypothyroidism undergoing L-thyroxine treatment was presented in the Table 2. No significant difference was found in age between the two groups, with a mean age of 51.40 ± 8.776 years for healthy controls and 2.76 ± 9.358 years for the patients (P=0.3932). Highly significant differences were noticed in body weight and BMI. The mean wight of hypothyroid patients was significantly higher (84.88 ± 15.96 Kg) than the mean weight of healthy controls (71.66 ± 9.983Kg,

P<0.0001). Similarly, the BMI of hypothyroid patients (33.26 ± 5.929 kg/m²) was dramatically higher than in healthy individuals (28.38 ± 3.600, P value <0.0001). HT prevalence was much more prevalent hypothyroid patients with (66%, n=66) compared to (6%, n=3, P=0.0272) in the healthy group. While (94%, n=47) of healthy women did not have hypertension, only 34%(n=34) of patients were without hypertension. Blood pressure measurement was further supported these findings: the mean SBP was significantly higher in the patients with hypothyroidism (133.8±15.80mmHg) compared to controls (124.6±7.549mmHg, P<0.0001). DBP was also elevated significantly in patients (84.61±7.184mmHg) compared to healthy people (81.00±5.425mmHg, P=0.0021). DM was significantly higher in hypothyroid patients (37%) compared to healthy individuals (16%, P=0.048). conversely, 84% of healthy group and 63% of the patients did not have DM. The duration of L-thyroxine treatment among patients was varied as follow: 42% of the patients had been treated for 1-5 years, 44% for 6-10 years, 8% for 11-15 years, 3% for 16-20 years, and 3% for 21-25 years. The L-thyroxine dosage ranged from 25mcg to 250mcg, as shown in Table 2.

Analyses of Plasma Thyroid Hormones and Glucose Hemostasis in Patients and Healthy Control Groups:

The plasma thyroid hormone levels and metabolic biomarkers in patients with hypothyroidism and healthy individuals were presented in the Figure 1A-H. Interestingly, the data revealed significant differences between the two groups. Thyroid stimulating hormone (TSH) levels were significantly higher in hypothyroid patients (4.300±4.708 µU/ml) compared to healthy group (2.130 ± 0.8125, P<0.0015, and a 95% CI 0.8421 to 3.497). Conversely, FT4 levels were significantly lower in the patient with hypothyroidism (13.38±2.283 pmol/L) compared to healthy individuals (14.60±1.804 pmol/L, P<0.001 and a 95% CI -1.942 to -0.5014). FT3 plasma levels were slightly lower, but statistically significant in patients (5.108 ± 0.979 pmol/L) compared to healthy controls (5.450 ± 0.997 pmol/L, P=0.0483 and a 95% CI 0.002551 to 0.6814), with a P value of 0.0483. However, we did not notice statistically significant differences in total triiodothyronine (TT3) and total thyroxine (TT4) plasma levels between the groups as shown in the Figure 1A-H. Glucose hemostasis analyses revealed elevated plasma insulin levels in hypothyroid patients with a P value of <0.0001. Fasting plasma glucose (FPG) were also significantly higher in hypothyroidism patients (119.4±41.22 mg/dl) compared to healthy controls (99.13±12.97 mg/dl, P=0.004 and 95% CI 8.628 to 31.82). Furthermore, the hemostatic model assessment for insulin resistance (HOMA-IR) was significantly elevated in hypothyroid patient (5.166±3.186) compared to healthy individuals (2.120±0.981, P<0.0001 and a 95% CI 2.133 to 3.959).

Genotyping of THRA rs939348 T>C Gene:

the Figure 2, the RFLP-PCR yielded a clear 190 bp and 142 bp amplicons, which were comparison with a 100-1500 bp DNA ladder. Lane M refers to the DNA ladder for size reference. Lanes 1-12 refer to the individuals (1-3) represent TT genotype (wild alleles), lanes (4 and 7) refer to the mutant genotype (CC, homozygote), and (5 and 10) indicate the mutant genotypes (CT/TC, heterozygote). The electrophoresis gel was conducted at 45V.

Examine the Association of THRA rs939348 T>C Genotypes with Hypothyroid Risk:

Our findings revealed that the frequency distribution of the THRA rs939348 T>C genotypes had a significant difference between healthy individuals and hypothyroid patient as presented in the Table 3. The TT genotype (wild alleles) was significantly more common in healthy control (80%) compared to patients (33%), suggesting a promising protective effect (P<0.001). In contrast, the CC genotype (mutant homozygote) was more prevalent in patients (48%) compared to healthy group (10%), indicating a strong correlation with increased risk of hypothyroid P<0.001. The CT genotype (mutant heterozygote) showed a higher frequency in patients (19%) than in healthy people (10%), but this difference was not statistically significant P=0.199, indicating it might not be a major risk factor for the disease. The pairwise comparison of allele frequencies for the THRA rs939348 gene, using Chi-square (χ^2) tests, revealed a substantial difference between the wild (TT) and mutant (CT and CC) genotypes (P<0.001), as shown in Table 4.

Table 5 and Figure 3 present the pairwise comparisons of alleles frequencies for the THRA rs939348 T>C polymorphism among healthy individuals, using chi-square (χ^2) tests. The TT (wild type) and CT (mutant heterozygous) genotypes yielded a chi-square value of 54.44 with a p-value of <0.0001, indicating a highly significant difference. Similarly, the TT vs. CC (mutant homozygous) comparison also showed a chi-square value of 54.44 with a p-value of <0.0001, demonstrating a highly significant difference. The comparison between the CT and CC genotypes resulted in a chi-square value of 0 with a p-value of 1, indicating no significant difference between these two genotypes. These results indicating that although the wild alleles (TT) significantly differ in frequency from both mutant genotypes (CT and CC) in healthy individuals, the frequencies of the mutant genotypes do not differ significantly from each other.

Analyses of THRA Rs939348 T>C Genotype Frequencies in Hypothyroid Patient and Healthy Individuals Using Hardy-Weinberg Equilibrium:

The genotype frequencies of THRA rs939348 T>C in hypothyroid patient were analyzed using HWE. The calculation involved of 100 sample size and comparing observed frequencies and those expected under HWE. In the patients the observed genotype frequencies were 42.5% for the TT genotype and 57.5% for the C allele. The expected frequencies were 18.1% for the T allele, 33.1% for the C allele, and 48.9% for the TC/CT genotype. A significant deviation from HWE expectation was found with a P-values of 0.0002 for the TT genotype, 0.0001 for the CT genotype, and 0.001 for the CC genotype, indicating a statistically relevant difference between the observed and expected frequencies (Table 6 and Figure 4).

Healthy control group of 50 individuals showed that the observed genotype frequencies were 85% for the TT genotype and 15% for the allele C. HWE equilibrium calculations yielded 72.25% for the T allele, 2.25% for the allele C and 25.5% for the TC/CT genotype. Interestingly Fischer exact test results showed a significant deviation with a P values of 0.0002 for the TT genotype, 0.001 for the CC genotype, and 0.001 for the CT/TC genotype as presented in the (Table 7 and Figure 4). These results suggest a meaningful difference between the observed and expected frequencies in both healthy control group.

Compare Demographic and Clinical Characteristics of Participants with THRA Rs939348 T>C Genotype:

The demographic analyses of the THRA rs939348 T>C genotypes showed a notable difference between healthy control group (n=50) and hypothyroid patient (n=100), as presented in the Table 8. The mean age was slightly higher in patients with all genotypes (TT: 51.82±8.897, TC: 52.68±8.159, CC: 53.44±10.2) compared to healthy participants (TT: 52.25±8.602, TC: 51.8±10.45, CC: 44.2±6.38) Figure 5A. Age distribution revealed a higher percentage of older individuals among patients with CC genotype Figure5B. BMI and mean body weight were statistically higher in the patients with all the genotypes compared to the healthy individuals. However, within each group either healthy individuals or patients, no statistical differences were observed based on the genotypes see Figure 5C-F. BMI analysis showed statistically significant differences in the distribution of normal weight (TT: 10%, TC: 0%, CC: 3%) and overweight individuals (TT: 46%, TC: 6%, CC: 8%) between the patients and control group Table 8. HT prevalence was significantly higher in patients (TT: 20%, TC: 14%, CC: 32%) compared to healthy participants (TT: 6%, TC: 0%, CC: 0%) Table 9. SBP was significantly elevated in the hypothyroid patient with TT genotype compared to the healthy group with TT genotype whereas no statistically differences were noticed in the other genotypes. DBP in the other hand was constant and did not show significant differences between the two groups as described in the Figure 5G-H.

Intriguingly, DM was more common in the patients compared to the healthy participants ($\chi 2=11.67$, 5, $P<0.0396$) see Table 10. The duration and dose of L-thyroxin treatment were recorded only for patients, with the many requiring higher doses over time. These results suggesting that THRA rs939348 T>C polymorphism are associated with various demographic and clinical parameters, underscore the need for personalized management strategies in hypothyroid patient with these genotypes.

Analyses of Plasma Thyroid Hormones in Patients and Healthy Individuals with THRA Rs939348 T>C Genotypes:

We aimed to investigate the idea of genetic variations at the THRA rs939348 T>C locus influences plasma thyroid hormone levels and metabolic markers in both healthy individuals and hypothyroid patients. Interestingly, patients with CC-mutant type showed two folds higher plasma TSH levels (4.74±3.636) compared to the healthy people with CC genotype (2.132±0.9426, $P<0.01$ with a 95% CI -0.3801 to 6.854) as shown in the Figure 6A-C and detailed in the Table 10. However, TSH levels were relatively consistent across all the other genotypes between healthy and hypothyroid patients. TT3 levels were relatively consistent across genotypes, while comparing patients to controls, there were significantly differences in FT3 levels across different genotypes as explained in the Table 10 and presented in the Figure 6E-G. However, within each group (patients or controls), FT3 levels showed no astatically differences, which may indicate the effect of gene variations on the FT3 levels is crucial in the context of comparing patients to controls but not in the same group. We did not notice statistically important differences between the two groups in the level of TT4 as shown in the Figure 6H. Not surprisingly, healthy people with TT, TC and CC variations presented higher levels of FT4 compared to hypothyroid patients Figure 6I. This is consistent with the hypothesis that genetic variations significantly impact thyroid hormone levels in the context of comparing patients to controls, rather than within each group (patients or controls). Hence the results revealed significant variations in thyroid hormone levels between patients and healthy control groups, indicating the influence of THRA rs939348 T>C polymorphism on thyroid physiological functions.

Analyses of Glucose Hemostasis and Blood Lipid Profiles in Patients and Healthy Individuals with THRA rs939348 T>C Genotypes:

Glucose hemostasis profiles of individuals with THRA rs939348 T>C gene variations revealed notable differences between healthy people and patients. Hypothyroid patients showed more than two folds increased in insulin levels for both wild and mutant genotypes compared to healthy individuals see Table

11 and Figure 7A. Although insulin level was significantly higher in the patients compared to healthy controls, it was remained relatively constant within both patient and control groups, as shown in the Figure 7B-C. This indicates that THRA rs939348 T>C gene polymorphism significantly impact insulin stability in hypothyroid patients compared to healthy controls but does not cause notable differences within the same groups. FPG is a critical parameter used to study glucose hemostasis because it provides a baseline measurement of blood glucose levels without the influence of recent food intake, reflecting the ability of body to blood glucose through endogenous insulin production and response to glucose regulation mechanisms. Interestingly, our results showed elevated of FPG in hypothyroid patients with wild type TT genotype and mutant type CC genotype ($P<0.01$) compared to healthy individuals as shown in the Figure 7D. However, no notable differences were observed within both patient and healthy control groups as shown in the Figure 7E-F. HOMA-IR was considerably higher in patients across all genotypes compared to healthy people, as shown in the Figure 7G-I. Patients with wild type TT genotype showed significant higher blood cholesterol level compared to healthy individuals as detailed in the Table 12 and Figure 8A. Cholesterol levels were relatively similar between patients and healthy controls in the other genotypes as shown in the Figure 8B-C. TG blood levels were significantly higher in hypothyroid patients with TT and CC genotypes compared to the healthy females with a P-value<0.01 Figure 8D. Healthy people with mutant CC genotype showed the lowest level of TG compare to other gene variations Figure 8E, while TG levels remained constant across the patient groups. In addition, HDL, LDL and vLDL levels were consistent and showed no significant differences between patients and healthy individuals, and they remained constant within both groups as well.

THRA rs939348 T>C Genotype and Response to L-Thyroxine Treatment Hypothyroid Patients:
We examined whether the THRA rs939348 T>C genotypes influences the response to L-thyroxine treatment in patient with hypothyroidism. Table 12 describes the duration of L-thyroxine treatment in patients with different THRA rs939348 T>C genotypes (TT, TC, CC). For patients treated for five years or less, 12% had wild genotype (TT), 9% were heterozygous mutant (TC) and 21% had the homozygous mutant genotype (CC). For patients who treated for more than 5 years, 21% had the wild genotype (TT), 10% were mutant genotype (heterozygous) and 27% had the homozygous mutant genotype (CC). There were no statistical differences were found in duration of treatment among the genotypes ($P=0.1496$), nor there were differences in genotype distribution between the two treatment durations (P-value 0.0721). Therefore, it is worthwhile to study the correlation between the duration of the treatment, the genotypes, and TSH levels to gain a better understanding of their interplay and influence on treatment outcomes. To determine whether the duration of L-thyroxine treatment is affected by THRA rs939348 T>C polymorphism, which may influence thyroid hormone and metabolic response to L-thyroxine, we analyzed TSH plasma levels across treatment durations. Interestingly, for patients treated for five years or less, TSH were significantly higher in the TC genotype compared to patients with the same genotype treated for more than five years (Table 13 and Figure 9A). In contrast, TSH levels was slightly higher but not statistically significant for patients with CC and TT genotypes treated for ≤5 compared to those who treated for >5 years (Table 13 and Figure 9A). TT3 levels were relatively similar across genotypes for both treatment durations, with a notable increase in the CC genotype for >5 years Figure 9B. FT3 levels remained fairly consistent across all genotypes and treatment durations. FT3, TT4 and FT4 plasma levels were relatively similar across all genotypes for ≤5 compared to patients treated for >5years, as showed in the Figure 9C-E. These results indicate that prolonged L-thyroxine treatment did not lead to improve thyroid hormone levels, especially in the TT and TC mutant genotypes, suggesting that alpha thyroid gene variations may strongly correlated to the effectiveness of the treatment in hypothyroid patients and should be considered in their care and management.

Impact of L-Thyroxine Treatment Duration on Clinical Parameters in Hypothyroid Patient with THRA rs939348 T>C Genotypes:
Thyroid hormones in particular T4 and T3 play a crucial regulation role in glucose hemostasis and lipid profiles. Hypothyroidism often leads to increase insulin resistance, elevated FPG, and alter blood lipid profiles. Therefore, we aimed to study the impact of L-thyroxine treatment duration on clinical biomarkers in hypothyroid patient with THRA rs939348 T>C gene polymorphism to assess whether genetic variations influence treatment outcomes. Interestingly, our study did not reveal notable differences in glucose hemostasis parameters-including insulin, FPG and HOMA-IR between patients treated with L-thyroxin for 5 years or less and those treated for more than 5 years across all genotypes as detailed in the Table 14 and presented in the Figure 10A-H. These data did not show a clear pattern of effect of L-thyroxine treatment on the clinical parameters across different genotypes. This may suggest either a lack of response to the treatment or THRA gene variations, in particular rs939348 T>C polymorphism, influence responses to treatment in ways that not evident in these patients.

Measuring the Impact of THRA rs939348 Mutation on The TSH Levels in Hypothyroid Patient and Healthy People:
In this experiment, we interested to measure TSH levels by categorizing of wild genotypes (TT) vs mutant genotypes (TC, CC) instead of the specific genotypes. This approach increased the sample size for each group by enhancing the power of the statistical calculations. This would help the researchers to

understand the impact of the mutation that providing a basis for more detailed studies if significant differences were found. Interestingly, as detailed in (Table 15 and Figure 11) hypothyroid patient with wild genotypes showed no significant differences in TSH plasma levels compared to the healthy individuals with wild genotypes. However, patients with mutant genotypes exhibited fourfold higher levels of plasma TSH compared to the healthy individuals with mutant genotypes with ($P<0.01$, 95% CI -5.780 to -0.06452). Additionally, Patients with mutant genotypes showed significantly higher levels of TSH compared to healthy controls with wild genotypes. These results indicate that the THRA rs939348 T>C mutation has a strong impact on TSH levels in hypothyroid patients. Hypothyroid patient with mutant genotypes (TC or CC) showed significantly higher TSH levels compared to the healthy participants with the same genotypes, suggesting a potential genetic influence of the mutant allele on the severity of the hypothyroidism and responses to L-thyroxine. In contrast, there is no clinically relevant differences between patients with wild genotypes and healthy individuals with the same genotype, indicating that the wild type does not strongly influence TSH levels in the context of hypothyroidism.

Impact of THRA rs939348 T>C Polymorphism on The Treatment Responsiveness in Patients with Hypothyroidism:

The results of the previous experiment heightened responsiveness among patients with wild genotype (TT). Therefore, in the next experiment we measured TSH levels in wild type genotype based on the duration of L-thyroxine treatment aiming to examine whether genotype influence the efficacy of treatment by regulating TSH levels. Table 16 and Figure 12 presented plasma TSH levels in hypothyroid patient and healthy participants according to the THRA rs939348 mutant and wild genotypes, categorized by the duration of L-thyroxine treatment. Not surprisingly, for patient with wild type genotypes, TSH levels were consistent and no significant changes were noticed between patients who treated for 5 years or less and patients who treated for more than 5 years compared to compared to healthy individuals. In contrast, patients with mutant genotypes exhibited higher TSH levels regardless of treatment duration compared to healthy individuals. These findings strongly indicate that THRA rs939348 T>C mutation may contribute to elevated TSH levels in hypothyroid patients and influence their response to the therapy.

Effect of L-Thyroxine Dose Variations on TSH Plasma Levels in Hypothyroid Patient with THRA rs939348 T>C Polymorphism Compared to Healthy Individuals:

This experiment was conducted based on our observations that patients with wild alleles responded effectively to the therapy. We aimed to examine whether this responsiveness varies according to different doses of L-thyroxine. Consistently, patients with THRA rs939348 T>C wild genotype exhibited consistent and stable TSH plasma levels compared to the healthy individuals with the same genotypes, ranging from 4.253 ± 2.336 μU/ml to 3.390 ± 1.854 μU/ml, with no data available for dosages below 50mcg or ≥200mcg (Table 17 and Figure 13A-G). In contrast, patients with mutant genotypes (TC or CC) showed significant elevated in the levels of TSH compared to the healthy participants with the same genotypes. These results indicate that genotype specific response to L-thyroxine treatment among hypothyroid patient are significant and underscoring the importance of gene polymorphism testing in therapeutic strategies.

Impact of THRA rs939348 T>C Genotype Mutation on L-Thyroxine Response Based on TSH Levels in Hypothyroid Patients:

The hypothesis of this study is to investigate whether gene variations in the THRA gene influence the response to treatment in hypothyroidism, potentially leading to either effectively response or non-response to therapy. Accordingly, hypothyroid patient was grouped based on THRA rs939348 T>C genotypes and defined as responders and non-responders depending on the plasma TSH levels, normalized to the levels observed in the healthy individuals ranged (0.5-5μU/ml). Among responders, 33% were wild genotype and 20% were mutant, while among non-responders, 11% were wild and 36% were mutant. Statistically, non-responder patient proportions with mutant genotype were significantly higher than responder patients with wild genotype as detailed in the Table 18, indicating a strong association between the THRA rs939348 T>C polymorphism and the response to the therapy. Interestingly, TSH levels were close to normal (2.019 ± 1.09μU/ml) in responder patients with the wild-type genotype compared to the healthy controls, while TSH dramatically elevated in non-responder patients with the wild-type genotype $P<0.0001$ compared to responder patients with the same genotype. Among patients with mutant genotypes (TC or CC), responders showed significantly higher levels of TSH compared to healthy individuals ($P<0.02$) whereas non-responder patients with mutant genotype showed a six-fold elevation in TSH levels compared to the responders or healthy individuals (Table 19 and Figure 14). These findings suggest that the responsiveness to L-thyroxine treatment significantly impacts TSH levels in hypothyroid patients. Patients with wild-type showed better treatment outcomes than those with mutant-type, indicating the necessary of considering THRA rs939348 T>C polymorphism in treating hypothyroidism.

Impact of THRA Rs939348 T>C Polymorphism on BMI and Response to L-Thyroxine in Hypothyroid Patients:

BMI is a significant parameter for assessing the response to hypothyroidism treatment. Normal BMI measurements often correlate with a positive response to L-thyroxine treatment, indicating adequate thyroid hormone replacement therapy. By Investigating how gene variations impact BMI and treatment outcomes, scientists can potentially identify genetic markers that predict treatment response and help enhance the specificity and precision therapies description for individual patients. Table 20 presents the data of 100 hypothyroid patients and 50 healthy individuals. Patients were categorized based on THRA rs939348 T>C genotypes into wild-type genotype and mutant genotypes and further classified into responders and non-responders according to TSH levels normalized to those of healthy individuals. For patients with the wild genotype, responders had a mean of BMI 31.569 ± 2.402 Kg/m2, while non-responders had a mean of BMI 31.047 ± 6.664 Kg/m2; both groups showed no significant differences compared to the healthy controls (Figure 15). In contrast, among patients with mutant genotypes, for both responder and non-responder patients reported significantly increased in BMI levels ($P<0.001$) compared to the healthy individuals with the same genotypes. Additionally, there was no statistically significant difference in BMI between responder and non-responder groups among mutant patients see Figure 15. These data suggest that patients with the wild-type THRA rs939348 genotype had BMI levels similar to healthy controls, indicating a promising treatment response. While mutant patients exhibited significantly higher BMI levels regardless of treatment response status.

DISCUSSION

Hypothyroidism is a condition affects a significant percentage of the global population, with L-thyroxine being the dominant prescribed treatment [31]. Despite its widespread use, the treatment response to L-thyroxine varies among people due to several unknown factors [32,33]. Recently, scientists suggest that genetic variations in particular gene related to thyroid stimulating hormone may play a crucial role in impacting individual response to hypothyroidism therapy. The study compared demographic data collected from female participants grouped to 100 hypothyroid patients undergoing L-thyroxine treatment and 50 healthy individuals. Interestingly, the patients showed significantly higher BMI, body weight, hypertension prevalence, and blood pressure values compared to the healthy controls. These findings align with previous research indicating that hypothyroidism often leads to overweight, obesity, alter metabolic index and increased cardiovascular disease risk [34,35]. For instance, a study by Smith and his team found similar trends in hypothyroid patients, indicating the importance of monitoring and care improving during L-thyroxine therapy [36]. Additionally, scientists observed that L-thyroxine did not significantly improve hypothyroidism symptoms or fatigue in patients with hypothyroidism condition compared to a placebo group. Therefore, it is necessary to understand that individual responses to treatment can vary, necessitating further research to elucidate the underlaying mechanisms [36–39]

The data of this study revealed that serum TSH levels in hypothyroid patients significantly higher than healthy individuals. However, the mean levels of TSH in hypothyroid patients remained within normal established reference range. This may be because hypothyroid patients undergoing L-thyroxine treatment. These data align with many previous studies suggesting that TSH serum levels are the most sensitive and specific marker of systemic thyroid status [40–44] Additionally, the data showed that FT3 plasma levels were significantly elevated in hypothyroid patients compared to healthy ($P<0.04$). In contrast, FT4 was statistically lower in patients with hypothyroidism (13.38±2.283) compared to healthy individuals (14.60±1.804, $P<0.001$). Thyroid disorder can be confusing by virtue of being discordant with the clinical symptoms like inability of L-thyroxine to suppress (TSH;thyrotropin) in hypothyroidism, or because using many different assays that contradict each other such as raised thyroid hormones levels without suppress TSH or low thyroid hormones levels with inappropriately normal or low TSH [45–48]. There are many possible causes of anomalous thyroid function in hypothyroid patients undergoing L-thyroxine including: genetic variation in TSH receptor, inappropriate administration of L-thyroxine, malabsorption of L-thyroxine, change the treatment dosages, resistance to L-thyroxine and poor compliance [45,47–49]. On the other hand, scientists suggested that to avoid thyrotoxicosis or resistance to L-thyroxine treatment, L-thyroxine should be substitute with T3 supplement, which can be done without reducing or interfering with FT3 levels, there by restoring FT4/FT3 ratio in most patients [50]. Not surprisingly, in some patients with elevated FT3 and normal FT4 levels, and excessive L-thyroxine intake typically results in a higher FT4/FT3 ratio compared to spontaneously occurring hyperthyroidism [51].

Moving on to the metabolic biomarkers, the data analyses revealed that hypothyroid patients had insulin plasma levels that were twice as high $P<0.0001$ compared to healthy controls. In addition, patients with hypothyroidism showed significantly elevated FPG $P<0.004$ compared to the healthy individuals. Similarly, the hemostasis model assessment of insulin levels was significantly elevated ($P<0.0001$) in hypothyroid patients compared to the healthy controls. These data align with previous findings, by their scientists indicated that thyroid hormones play crucial roles in both glucose metabolism and consumption [52–54]. Thyroid disorder is closely corelated with diabetes mellitus development or vice versa [55]. Thyroid hormone affects glucose hemostasis by impacting pancreatic βcells, and regulate glucose metabolism through several organs including skeletal muscle, liver and central nervous system. Hypothyroidism may be the result of insulin resistance of peripheral tissues [56,57]. Maratou et al.

suggested that patients with hypothyroidism present with decreased expression of insulin-stimulated glucose transport in monocytes, resulting from impaired translocation of glucose transporter, which is essential for glucose utilization [58]. Furth more, reduced rate of blood flow in peripheral tissues is associate with the development of insulin resistance, as previously described by Dimitriadis and his research team [59].

Association of THRA Rs939348 T>C Genotypes with Hypothyroid Risk:

Molecular biology analysis of THRA rs939348 T>C gene polymorphism in hypothyroidism showed significant differences in genotype distribution between healthy individuals and hypothyroid patients. For instance, the TT genotype (wild allele) was the most common in healthy controls, indicating a protective effect against hypothyroidism. Conversely, the CC genotype (mutant homozygote alleles) was more prevalent in patients, which may indicate a strong association with increased risk of hypothyroidism. Although, the CT genotype (mutant heterozygous alleles) showed higher proportion in patients compared to healthy controls, the differences were not statistically significant. Chi-square calculation for pairwise comparisons revealed substantial differences between wild-type and mutant genotypes $P<0.001$, with no significant differences between the mutant genotypes themselves. Interestingly, the findings indicated that the mutation alleles (CT and CC) were significantly shifted in hypothyroid patients compared to the wild-type, while in healthy controls, the wild-type was the most common frequency than both the mutant alleles and the corresponding frequencies in patients. These results aligned with most of the studies, which also observed significant differences in genotype distribution, even the exact proportions varied [60–62]. Furthermore, most of the studies compared the genotypes proportion within the patient groups, whereas out study included a comparison with healthy individuals to understand the baseline of alleles frequencies in the general population. Hence, these findings suggest that mutation strongly associate with the risk of hypothyroidism. Moreover, both patients and healthy groups exhibited deviation from HWE, which possibly due to several factors including genetic and epigenetic modifications, environmental factors, sample size, risk factors and geographical distributions [63–67]. The deviations from HWE observed in our analysis may be attributed to the unique population structure or sub-population stratification within our study cohort. The population of Karbala, Iraq, where the study was conducted, is known to have distinct genetic characteristics influenced by historical and geographical factors. Such localized variations could affect allele frequencies and genotype distributions, potentially explaining the observed deviations from HWE.

In the context of disease-associated studies, deviations from HWE in the patient group are not uncommon and may reflect the genetic association between specific genotypes and disease susceptibility. However, the significant deviation observed in the healthy control group warrants further consideration. This finding could be influenced by factors such as sample size limitations or population-specific allele frequency dynamics, which might differ from broader or more heterogeneous populations.

Demographic and Clinical Characteristics of Participants with THRA rs939348 T>C Genotype:

The data of this study reported several notable demographic and clinical differences between healthy group and hypothyroid patients based on THRA rs939348 T>C gene variations. BMI and mean body weight were significantly higher in hypothyroid patients compared to healthy individuals regardless the genotype groups, although no differences were found within each group based on genotype. Studies have adjusted for BMI and body weight to isolate the effect of gene polymorphisms on hypothyroidism, counting that overweight and obesity are a potential risk factors for this condition [68]. Clearly, our results showed the differences of body weight and BMI were more pronounced between healthy and patients independent THRA rs939348 T>C gene variations. Our data align with Al-Azaam et al, who explored the impact of THRA gene polymorphism, specially rs939348, rs2268458 and rs2239610 on L-thyroxine treatment response in hypothyroid patients and they found no differences in BMI and body weight among genotype groups [65,66]. In contrast, several studies on animal models have shown that gene variations such as FTO gene polymorphism influence metabolic rate and energy expenditure [69].

SBP was notably elevated in hypothyroid patents with wild genotype, while DBP remain stable and constant across both groups. Prevalence of DM was significantly high among patients $P<0.0396$. Furthermore, hypothyroid patients required longer and higher doses of L-thyroxine treatment. Goumidi et al for the first time reported that a significant and potential associations between the TT allele of the THRA rs939348 SNP and elevated SBP in two population-based studies, and these findings align with our results [70]. It is worth to mention that we are the first to report association between THRA rs939348 T>C gene polymorphism and BP in Iraqi population selected from Kerbala city. These results are agreeing with the known links between thyroid dysfunction and BP although the underlying mechanisms are unclear [60,71,72]. Additionally, the same effect was also observed for DBP, and consequently this allele was significantly associated with higher risk of hypertension [73]. BP regulated by many physiological factors. Atrial and brain natriuretic peptides are directly controlled by thyroid hormones and may play a role in the process of blood pressure stasis. Autonomic nervous system providing an alternate possible mechanism depends on the action of TT3 and FT3 [74–76]. Catecholamine secretion are highly elevated in hypothyroidism in particular plasma noradrenaline, which may play a crucial role in elevated blood pressure [77–79]. Thyroid gland dysfunction is associated with an increased sympathetic influence on the autonomic cardiovascular system [80,81]. Finally, studies using animal

models suggested that thyroid hormone receptor alpha functions to activate parasympathetic nervous system signaling pathway [70].

Analyses of Plasma Thyroid Hormones in Patients and Healthy Individuals with THRA rs939348 T>C Genotypes:

Next, we explored the influence of THRA rs939348 T>C locus on plasma thyroid hormones levels and metabolic biomarkers in hypothyroid patients compared to healthy individuals. Clearly, patients with mutant-homozygous CC genotype exhibited plasma TSH levels two folds higher than of healthy individuals with the same genotype $P<0.01$. In contrast, TSH levels remained stable and constant across other genotypes between healthy and hypothyroid groups. Although TT3 levels were consistent across genotypes, significant differences in FT3 plasma levels were observed between patients and healthy controls, but not within each group indicating THRA rs939348 T>C polymorphism is more pronounced when comparing patients to healthy group. Interestingly, there were no clear differences were observed in TT4 levels between the two groups across all the genotypes. FT4 on the other hand was significantly higher in healthy group compared to the patients across all genotypes (TT, TC and CC), supporting the hypothesis that THRA rs939348 T>C gene variations impact thyroid hormone levels in the context of hypothyroidism. These findings line with previous studies examined the association between THRA rs939348 T>C gene polymorphism and thyroid hormones and metabolic biomarkers. For instance, Goumedi et al found that hypothyroidism patients with rs939348 T allele had higher systolic blood pressure, suggesting a potential impact of this locus [70]. Clinical studies suggested that mutation in either of thyroid hormone receptor (THRA or THRβ) can lead to resistance to thyroid hormone, and these mutations can result in variable phenotypes both same receptor isoform and between the two isoforms. Therefore, patients with thyroid hormone resistance can present with unexplained elevated of plasma FT4, high levels of TSH and decreased the ratio of FT4/FT3 [70,82]. Hence these explanations were similar to our observations.

Analyses of Glucose Hemostasis and Blood Lipid Profiles in Patients and Healthy Individuals with THRA Rs939348 T>C Genotypes:

Advance in areas of cell imaging, cell biology such as autophagy, gene expression and metabolomics have generated solid evidences that thyroid hormones play important role in hepatic lipid regulation [83–85]. Indeed, hypothyroidism strongly associated with dyslipidemia, increased blood lipid cholesterol and triglycerides as well as NAFLD. Thyroid hormone regulates lipid metabolism through different pathways such as autophagy, βoxidation, cholesterol synthesis and activate cholesterol transporter system [86]. Not surprisingly, scientists suggested using thyroid analogues for treatment of metabolic diseases involving the liver, such as hypercholesterolemia and NAFLD [86–88]. This why in this study we explored the effects of THRA rs939348 T>C gene variations on lipid profiles, as blood lipid levels may serve as important indicators for evaluating the response to treatment in hypothyroidism and should be taken into account.

Interestingly, cholesterol levels were significantly elevated in patients with wild-type TT genotype compared to healthy group with the same genotype, while TG levels were elevated in hypothyroidism patients with TT and CC genotypes compared to healthy controls with the same genotypes. HDL and LDL remained constant and stable between the two groups across all genotypes. We noticed an increase in vLDL levels in healthy participants, with significant increase observed in healthy individuals with TT genotype compared to the patients with the same genotype. It is noteworthy to mention that this is the first time to study the effect of THRA rs939348 SNP on blood lipid profiles.

Mostly, scientists focused on THRB gene variations because it is the major form express in the liver, while THRA is commonly expresses in the other tissues like heart and bone [89–91]. Generally, THRs are nuclear receptor owning nucleo-cytoplasmic shuttling, in spite of some of the residual available in the cytoplasm. This feature enables THRs to bind directly to their target gene on the promotor area of DNA, forming co-repressor complex with histone deacetylase activity to repress positively regulated transcription genes in response to thyroid hormone elements. Upon ligand binding, co-repressors are released lead to conformation changes in the THR, and activate histone acetyltransferase expression which activate other target gene promotor to trigger transcription [92,93]. Furthermore, thyroid hormone can regulate several transcription factors play a role in lipid metabolism such as (such as activation of forkhead box protein O1 (FOXO1)), modulating cell-signaling cascades through protein–protein interactions (such as the regulation of phosphoinositide 3-kinase (PI3K) by THRβ) or binding to proteins other than THRs (such as binding to αvβ3 integrin) [94].

Previously, scientists demonstrated the correlation between THRA rs939348 gene polymorphism and SBP and increased risk of cardiovascular disease to 25% [70]. Other study reported that THRA variations increased waist circumference in hypothyroid patients specially in those who had T allele. These studies agree with our results, as patients with TT genotype had higher levels of blood cholesterol and TG [65,66]. The strength of our study lies in comparing these results to healthy individuals rather than just to other genotypes within the patient group, which solidify the evidence and concludes that people with T alleles are more vulnerable to developing resistance to hypothyroidism treatment.

The analyses of glucose hemostasis revealed significant differences between patients and healthy individuals. Insulin levels were significantly higher in hypothyroid patients with TT and CT genotypes

compared to healthy controls indicating a potential impact of THRA rs939348 T>C gene polymorphism on insulin stasis. Similarly, FPG levels were elevated in hypothyroid patients with both TT and CC genotypes compared to healthy participants with the same genotypes. HOMA-IR was dramatically elevated in the patients compared to the healthy individuals across all genotypes. Impairment of glucose metabolism in hypothyroidism patients is supported by many studies, but the uniquely in this study the patients undergoing L-thyroxine which should improve thyroxine hormones and improve glucose hemostasis. Therefore, these results may indicate resistance to the therapy due to gene variations in THRA in particular rs939348 T>C locus. This is consistent with previous studies suggesting THRA polymorphisms influence insulin sensitivity and resistance [70,82].

Impact of THRA rs939348 Mutation on The TSH Levels and Treatment Responsiveness in Hypothyroid Patient and Healthy People:

Several observational studies recommended that TSH is an important indicator of the biochemical response to L-thyroxine, while FT3, FT4, TT3 and TT4 are an additional hypothyroidism treatment target [95–98]. Since we did not find a noticeable change in the levels of thyroid hormones, we focused on exploring the impact of THRA rs939348 gene polymorphism on hypothyroidism treatment response based on the measurement plasma TSH levels. Indeed, THRα gene plays a crucial role in regulating thyroid hormone physiological and biological functions. Variations in this gene such rs939348 SNP may affect the gens functions and consequently influence the severity of the disease and the patient's response to treatment [99–101].

Interestingly, no notable differences in the TSH levels were observed in hypothyroid patients with wild-type TT genotype compared to healthy individuals. While patients with mutant alleles either heterozygous or homozygous showed significantly higher TSH levels compared to healthy individuals with the same genotypes, indicating that THRA rs939348 mutation potentially impact hypothyroidism severity and L-thyroxine responsiveness. Additionally, TSH levels in patients with wild allele were stable and constant regardless treatment duration or L-thyroxine dosage, with no significant differences compared to healthy individuals. In contrast, patients with mutant genotypes (CT and/or CC) exhibited elevated TSH levels regardless of treatment term or dosage, suggesting a less effective response to L-thyroxine therapy.

THRA rs939348 T>C polymorphism showed a notable difference between responders and non-responders in terms of genotype distribution. Wild-type individuals exhibited better treatment outcomes, with near normal TSH plasma levels, while those with mutant genotypes showed a poorer response based on significantly elevated plasma TSH levels. Previously scientists found that THRA rs939348 T>C gene polymorphism is potentially correlated with significant changes in TSH levels and treatment response efficiently. They found patients with mutant genotypes CC alleles had a higher risk of poor response to treatment along with our findings that wild-type TT genotype is associated with better treatment response and lower TSH levels [102]. In contrast, Zhang et al., found no significant differences in treatment outcomes based on the THRA rs939348 T>C gene variations, though they acknowledge the impact of gene variations on TSH variability [60,103]. Our study demonstrated that mutant genotypes of THRA rs939348 T>C were associated with higher TSH levels in hypothyroid patients undergoing L-thyroxine treatment. This finding suggests that patients carrying mutant genotypes may exhibit a suboptimal biochemical response to the standard treatment protocol. Elevated TSH levels in these patients could potentially translate into persistent hypothyroid symptoms, such as fatigue, weight gain, and diminished quality of life, even when receiving therapy. To further elucidate the clinical impact of these findings, future studies should include longitudinal evaluations of both biochemical and clinical outcomes, such as symptom resolution, quality of life measures, and comorbidities like dyslipidemia and insulin resistance. Investigating these aspects could provide critical insights into the necessity and efficacy of personalized treatment approaches based on THRA genotypes.

CONCLUSION

The THRA rs939348 gene polymorphism is potentially associated with hypothyroidism, influencing treatment response as well as lipid and glucose homeostasis. These findings suggest that genetic screening for THRA polymorphisms could be a valuable tool in optimizing L-thyroxine treatment for hypothyroid patients, allowing for personalized treatment strategies. However, further research is needed to fully elucidate the mechanisms underlying these associations and to explore how genotype-specific treatment protocols could improve therapeutic outcomes. Future studies should aim to investigate the impact of THRA polymorphisms on both biochemical and clinical parameters over longer treatment durations, as well as explore the potential benefits of adjusting L-thyroxine dosages based on genetic variations. By incorporating genetic data into clinical decision-making, we may be able to better tailor treatments, improve patient outcomes, and enhance the management of hypothyroidism.

LIMITATIONS

Our study has several limitations that should be acknowledged. The relatively small sample size may limit the generalizability of the findings, and the cross-sectional design prevents establishing causal relationships between the THRA rs939348 polymorphism and treatment outcomes. Additionally, the focus on a specific population restricts the applicability of the results to other ethnic or genetic groups.

Unaccounted variables, such as lifestyle factors, comorbid conditions, and medication adherence, may have influenced the outcomes. While the findings suggest potential for personalized treatment, further research is needed to address clinical implementation challenges, including cost, accessibility, and integration into healthcare settings.

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Author Contributions
Nabaa Zuhair Miran contributed to the conceptiolization of the study, data collection, and drafting of the manuscript. Zahraa Abed Al-kareem contributed in the data analysis, interpretation of the results, helped in the revieweing and revised the manuscript. Shaima Jabbar was responsible for supervising the experimental design, coodinating the clinical aspects of the study, and participated to the manuscript writing. Qusay Baqer Alzajaji helped with clinical data collection, provided clinical expertise, supervised the biochemical and genetic analyses, and contributed to data interpretation. All authors reviewed and approved the final version of the manuscript.

REFERENCES
1. Cooper DS, Biondi B. Subclinical thyroid disease. In: The Lancet. 2012.
2. Ross I, Omengan DB, Huang GN, Payumo AY. Thyroid hormone-dependent regulation of metabolism and heart regeneration. Vol. 252, Journal of Endocrinology. 2022.
3. Chiovato L, Magri F, Carlé A. Hypothyroidism in Context: Where We've Been and Where We're Going. Vol. 36, Advances in Therapy. 2019.
4. Wilson SA, Stem LA, Bruehlman RD. Hypothyroidism: Diagnosis and Treatment. Am Fam Physician. 2021;103(10).
5. Wassner AJ. Pediatric Hypothyroidism: Diagnosis and Treatment. Pediatric Drugs. 2017;19(4).
6. Gao X, Liu M, Qu A, Chen Z, Jia Y, Yang N, et al. Native magnetic resonance T1-mapping identifies diffuse myocardial injury in hypothyroidism. PLoS One. 2016;11(3).
7. Tiller D, Ittermann T, Greiser KH, Meisinger C, Agger C, Hofman A, et al. Association of Serum Thyrotropin with Anthropometric Markers of Obesity in the General Population. In: Thyroid. 2016.
8. Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. Physiol Rev. 2014;94(2).
9. Wall CR. Myxedema coma: Diagnosis and treatment. Vol. 62, American Family Physician. 2000.
10. Handbook of Pharmaceutical Granulation Technology. Handbook of Pharmaceutical Granulation Technology. 2021.
11. Parikh DM. Handbook of pharmaceutical granulation technology. Handbook of Pharmaceutical Granulation Technology. 2016.
12. Duntas LH, Jonklaas J. Levothyroxine Dose Adjustment to Optimise Therapy Throughout a Patient's Lifetime. Vol. 36, Advances in Therapy. 2019.
13. Virili C, Trimboli P, Centanni M. Novel thyroxine formulations: a further step toward precision medicine. Vol. 66, Endocrine. 2019.
14. Virili C, Antonelli A, Santaguida MG, Benvenga S, Centanni M. Gastrointestinal malabsorption of thyroxine. Vol. 40, Endocrine Reviews. 2018.
15. Osmak-Tizon L, Poussier M, Cottin Y, Rochette L. Non-genomic actions of thyroid hormones: Molecular aspects. Arch Cardiovasc Dis. 2014;107(4).
16. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Vol. 31, Endocrine Reviews. 2010.
17. Pantos C, Mourouzis I. Thyroid hormone receptor α1 as a novel therapeutic target for tissue repair. Ann Transl Med. 2018;6(12).
18. Chaves C, Kay T, Anselmo J. Early onset obesity due to a mutation in the human leptin receptor gene. Endocrinol Diabetes Metab Case Rep. 2022;2022(1).
19. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Vol. 31, Endocrine Reviews. 2010.
20. Stamatouli A, Bedoya P, Yavuz S. Hypothyroidism: Cardiovascular Endpoints of Thyroid Hormone Replacement. Vol. 10, Frontiers in Endocrinology. 2020.

21. Mazzaferri EL. Thyroxine-Triiodothyronine Combination Therapy Versus Thyroxine Monotherapy for Clinical Hypothyroidism: Meta-Analysis of Randomized Controlled Trials. Yearbook of Endocrinology. 2007;2007.
22. Grozinsky-Glasberg S, Fraser A, Nahshoni E, Weizman A, Leibovici L. Thyroxine-triiodothyronine combination therapy versus thyroxine monotherapy for clinical hypothyroidism: Meta-analysis of randomized controlled trials. Journal of Clinical Endocrinology and Metabolism. 2006;91(7).
23. Sinha S, Kar K. Single nucleotide polymorphism of thyroid hormone receptor α gene can be a risk of atherogenic dyslipidemia. Journal of Clinical and Diagnostic Research. 2018;12(9).
24. Neves JS, Vale C, von Hafe M, Borges-Canha M, Leite AR, Almeida-Coelho J, et al. Thyroid hormones and modulation of diastolic function: a promising target for heart failure with preserved ejection fraction. Vol. 11, Therapeutic Advances in Endocrinology and Metabolism. 2020.
25. Bianco AC, Kim BW. Deiodinases: Implications of the local control of thyroid hormone action. Vol. 116, Journal of Clinical Investigation. 2006.
26. Chang YS, Wu YH, Wang CJ, Tang SH, Chen HL. Higher levels of thyroxine may predict a favorable response to donepezil treatment in patients with Alzheimer disease: A prospective, case-control study. BMC Neurosci. 2018;19(1).
27. Dolatshahi M, Salehipour A, Saghazadeh A, Sanjeari Moghaddam H, Aghamollaii V, Fotouhi A, et al. Thyroid hormone levels in Alzheimer disease: a systematic review and meta-analysis. Endocrine. 2023;79(2).
28. Goumidi L, Flamant F, Lendon C, Galimberti D, Pasquier F, Scarpini E, et al. Study of thyroid hormone receptor alpha gene polymorphisms on Alzheimer's disease. Neurobiol Aging. 2011;32(4).
29. Pearce EN. Thyroid hormone and obesity. Vol. 19, Current Opinion in Endocrinology, Diabetes and Obesity. 2012.
30. van Mullem A, van Heerebeek R, Chrysis D, Visser E, Medici M, Andrikoula M, et al. Clinical Phenotype and Mutant TRα1. New England Journal of Medicine. 2012;366(15).
31. Staff MC. Hypothyroidism (Underactive Thyroid). Women's Health. 2021;
32. Kaur H, Chakwop Ngassa H, Elmenawi KA, Anil V, Gosal H, Mohammed L. Hypothyroidism-Related Cardiac Tamponade. Cureus. 2021;
33. Efstathiadou Z, Bitsis S, Milionis HJ, Kukuvitis A, Bairaktari ET, Elisaf MS, et al. Lipid profile in subclinical hypothyroidism: Is L-thyroxine substitution beneficial? Eur J Endocrinol. 2001;145(6).
34. Fadeev VV. Comments to guidelines for the treatment of hypothyroidism prepared by the American thyroid association task force on thyroid hormone replacement. Clinical and experimental thyroidology. 2015;10(4).
35. Jonklaas J, Bianco AC, Bauer AJ, Burman KD, Cappola AR, Celi FS, et al. Guidelines for the treatment of hypothyroidism: Prepared by the American thyroid association task force on thyroid hormone replacement. Thyroid. 2014;24(12).
36. Mooijaart SP, Du Puy RS, Stott DJ, Kearney PM, Rodondi N, Westendorp RGJ, et al. Association between Levothyroxine Treatment and Thyroid-Related Symptoms among Adults Aged 80 Years and Older with Subclinical Hypothyroidism. JAMA - Journal of the American Medical Association. 2019;322(20).
37. Akirov A, Fazelzad R, Ezzat S, Thabane L, Sawka AM. A systematic review and meta-analysis of patient preferences for combination thyroid hormone treatment for hypothyroidism. Vol. 10, Frontiers in Endocrinology. 2019.
38. Meier C. TSH-Controlled L-Thyroxine Therapy Reduces Cholesterol Levels and Clinical Symptoms in Subclinical Hypothyroidism: A Double Blind, Placebo-Controlled Trial (Basel Thyroid Study). Journal of Clinical Endocrinology & Metabolism. 2001;86(10).
39. Meier C, Staub JJ, Roth CB, Guglielmetti M, Kunz M, Miserez AR, et al. TSH-controlled L-thyroxine therapy reduces cholesterol levels and clinical symptoms in subclinical hypothyroidism: A double blind, placebo-controlled trial (basel thyroid study). Journal of Clinical Endocrinology and Metabolism. 2001;86(10).
40. Sinha RA, Singh BK, Yen PM. Thyroid hormone regulation of hepatic lipid and carbohydrate metabolism. Vol. 25, Trends in Endocrinology and Metabolism. 2014.
41. Portman MA. Thyroid hormone regulation of heart metabolism. Vol. 18, Thyroid. 2008.
42. Ross I, Omengan DB, Huang GN, Payumo AY. Thyroid hormone-dependent regulation of metabolism and heart regeneration. Vol. 252, Journal of Endocrinology. 2022.
43. Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. Physiol Rev. 2014;94(2).
44. Sharma H, Kakadiya J. Different novel biomarkers involved in diagnosing hypothyroidism. Egypt J Intern Med. 2023;35(1).
45. Vaidya B, Pearce SHS. Management of hypothyroidism in adults. Vol. 337, BMJ. 2008.
46. Dave JA, Klisiewicz A, Bayat Z, Mohamed NA, Stevens Z, Mollentze WF, et al. SEMDSA/ACE-SA guideline for the management of hypothyroidism in adults. Vol. 83, SA Pharmaceutical Journal. 2016.
47. Sharma A, Stan MN. Thyrotoxicosis: Diagnosis and Management. Vol. 94, Mayo Clinic Proceedings. 2019.

48. Vaidya B, Pearce SHS. Diagnosis and management of thyrotoxicosis. Vol. 349, BMJ (Online). 2014.
49. Levy MJ, Koulouri O, Gurnell M. How to interpret thyroid function tests. Clin Med (Lond). 2013;13(3).
50. Santini F, Ceccarini G, Pelosini C, Giannetti M, Ricco I, Querci G, et al. Treatment of Hypothyroid Patients With L-Thyroxine (L-T4) Plus Triiodothyronine Sulfate (T3S). A Phase II, Open-Label, Single Center, Parallel Groups Study on Therapeutic Efficacy and Tolerability. Front Endocrinol (Lausanne). 2019;10.
51. Parvazinia P. Development of hyperthyroidism in a hypothyroid patient. Aust J Gen Pract. 2022;51(7).
52. Martinez B, Ortiz RM. Thyroid hormone regulation and insulin resistance: Insights from animals naturally adapted to fasting. Vol. 32, Physiology. 2017.
53. Vito P, Candelotti E, Ahmed R, Luly P, Davis P, Incerpi S, et al. Role of Thyroid Hormones in Insulin Resistance and Diabetes. Immunol Endocr Metab Agents Med Chem. 2015;15(1).
54. Fazio E, Bionda A, Chiofalo V, Crepaldi P, Lopreiato V, Medica P, et al. Adaptive Responses of Thyroid Hormones, Insulin, and Glucose during Pregnancy and Lactation in Dairy Cows. Animals. 2022;12(11).
55. Eom YS, Wilson JR, Bernet VJ. Links between Thyroid Disorders and Glucose Homeostasis. Vol. 46, Diabetes and Metabolism Journal. 2022.
56. Mun E, Lee HA, Choi JE, Lee R, Kim KH, Park H, et al. Association between Thyroid Function and Insulin Resistance Indices in Korean Adolescents: Findings from the 2014–2015 Korea National Health and Nutrition Examination Survey. Children. 2024;11(3).
57. Ha J, Lee J, Lim DJ, Lee JM, Chang SA, Kang M Il, et al. Association of serum free thyroxine and glucose homeostasis: Korea national health and nutrition examination survey. Korean Journal of Internal Medicine. 2021;36.
58. Maratou E, Hadjidakis DJ, Peppa M, Alevizaki M, Tsegka K, Lambadiari V, et al. Studies of insulin resistance in patients with clinical and subclinical hyperthyroidism. Eur J Endocrinol. 2010;163(4).
59. Dimitriadis G, Mitrou P, Lambadiari V, Boutati E, Maratou E, Panagiotakos DB, et al. Insulin action in adipose tissue and muscle in hypothyroidism. Journal of Clinical Endocrinology and Metabolism. 2006;91(12).
60. Wibowo RA, Hastuti P, Susanti V. Polymorphism of thyroid hormones receptor, angiotensin-converting enzyme, and high blood pressure in childbearing age women with hyperthyroidism. Open Access Maced J Med Sci. 2021;9(A).
61. Pastor S, Akdi A, González ER, Castell J, Biarnés J, Marcos R, et al. Common genetic variants in pituitary–thyroid axis genes and the risk of differentiated thyroid cancer. Endocr Connect. 2012;1(2).
62. Onda M, Li D, Suzuki S, Nakamura I, Takenoshita S, Brogren CH, et al. Expansion of microsatellite in the thyroid hormone receptor-α1 gene linked to increased receptor expression and less aggressive thyroid cancer. Clinical Cancer Research. 2002;8(9).
63. Alhawari H, Jarrar Y, Zihlif M, Wahbeh A, Alshelleh S, Alhabahbeh R, et al. The Association of M235T Genetic Polymorphism in Angiotensinogen Gene and Other Non-Genetic Factors with Essential Hypertension among Jordanian Patients. J Pers Med. 2024;14(3).
64. Efe JOJ, Chris-Ozoko LE, Taniyohwo EM, Aforkogene UJ, Okolie EI, Onyesom I. Hardy-Weinberg Equilibrium Study of Some Morphogenetic Traits in a Nigerian Population. International Journal of Anatomy and Research. 2024;12(1).
65. S.I. AA, K.H. A, O. K, O. AA. The associations of polymorphisms of TSH receptor and thyroid hormone receptor genes with L-thyroxine treatment in hypothyroid patients. Hormones. 2014;13(3).
66. Al-Azzam SI, Alzoubi KH, Khabour O, Al-Azzeh O. The associations of polymorphisms of TSH receptor and thyroid hormone receptor genes with L-thyroxine treatment in hypothyroid patients. Hormones. 2014;13(3).
67. Hansen PS, Van Der Deure WM, Peeters RP, Iachine I, Fenger M, Sørensen TIA, et al. The impact of a TSH receptor gene polymorphism on thyroid-related phenotypes in a healthy Danish twin population. Clin Endocrinol (Oxf). 2007;66(6).
68. Qiu Y, Liu Q, Luo Y, Chen J, Zheng Q, Xie Y, et al. Causal association between obesity and hypothyroidism: a two-sample bidirectional Mendelian randomization study. Front Endocrinol (Lausanne). 2023;14.
69. Wang L, Yi Q, Yao H, He L, Fang B, Xu W, et al. Correlations between FTO Gene Polymorphisms and TSH Level in Uyghur Chinese Patients with Type 2 Diabetes. Biomed Res Int. 2021;2021.
70. Goumidi L, Gauthier K, Legry V, Mayi TH, Houzet A, Cottel D, et al. Association between a thyroid hormone receptor-α gene polymorphism and blood pressure but not with coronary heart disease risk. Am J Hypertens. 2011;24(9).
71. Saito I, Saruta T. Hypertension in thyroid disorders. Vol. 23, Endocrinology and Metabolism Clinics of North America. 1994.
72. Berta E, Lengyel I, Halmi S, Zrínyi M, Erdei A, Harangi M, et al. Hypertension in thyroid disorders. Vol. 10, Frontiers in Endocrinology. 2019.
73. Danzi S, Klein I. Thyroid hormone and blood pressure regulation. Vol. 5, Current Hypertension Reports. 2003.

74. Li Y, Wei B, Liu X, Shen XZ, Shi P. Microglia, autonomic nervous system, immunity and hypertension: Is there a link? Vol. 155, Pharmacological Research. 2020.
75. Zhao Y, Zheng Y, Ma X, Qiang L, Lin A, Zhou M. Low-Intensity Resistance Exercise Combined With Blood Flow Restriction is More Conducive to Regulate Blood Pressure and Autonomic Nervous System in Hypertension Patients—Compared With High-Intensity and Low-Intensity Resistance Exercise. Front Physiol. 2022;13.
76. Mancia G, Grassi G. The autonomic nervous system and hypertension. Vol. 114, Circulation Research. 2014.
77. Moghetti P, Castello R, Tosi F, Zenti MG, Magnani C, Bolner A, et al. Glucose counterregulatory response to acute hypoglycemia in hyperthyroid human subjects. J Clin Endocrinol Metab. 1994;78(1).
78. Moghetti P, Castello R, Tosi F, Zenti MG, Magnani C, Bolner A, et al. Glucose counterregulatory response to acute hypoglycemia in hyperthyroid human subjects. Journal of Clinical Endocrinology and Metabolism. 1994;78(1).
79. Levey GS, Klein I. Catecholamine-thyroid hormone interactions and the cardiovascular manifestations of hyperthyroidism. Am J Med. 1990;88(6).
80. Cacciatori V, Gemma ML, Bellavere F, Castello R, De Gregori ME, Zoppini G, et al. Power spectral analysis of heart rate in hypothyroidism. Eur J Endocrinol. 2000;143(3).
81. Chechekhin V, Ivanova A, Kulebyakin K, Sysoeva V, Naida D, Arbatsky M, et al. Alpha1A- and Beta3-Adrenoceptors Interplay in Adipose Multipotent Mesenchymal Stromal Cells: A Novel Mechanism of Obesity-Driven Hypertension. Cells. 2023;12(4).
82. Singh BK, Yen PM. A clinician's guide to understanding resistance to thyroid hormone due to receptor mutations in the TRα and TRβ isoforms. Clin Diabetes Endocrinol. 2017;3(1).
83. Souza LL, Nunes MO, Paula GSM, Cordeiro A, Penha-Pinto V, Neto JFN, et al. Effects of dietary fish oil on thyroid hormone signaling in the liver. Journal of Nutritional Biochemistry. 2010;21(10).
84. Sinha RA, You SH, Zhou J, Siddique MM, Bay BH, Zhu X, et al. Thyroid hormone stimulates hepatic lipid catabolism via activation of autophagy. Journal of Clinical Investigation. 2012;122(7).
85. Kube I, Jastrow H, Führer D, Zwanziger D. Thyroid Hormone Deficiency Modifies Hepatic Lipid Droplet Morphology and Molecular Properties in Lithogenic-Diet Supplemented Mice. Experimental and Clinical Endocrinology and Diabetes. 2021;129(12).
86. Sinha RA, Singh BK, Yen PM. Direct effects of thyroid hormones on hepatic lipid metabolism. Vol. 14, Nature Reviews Endocrinology. 2018.
87. Cordeiro A, Souza LL, Einicker-Lamas M, Pazos-Moura CC. Non-classic thyroid hormone signalling involved in hepatic lipid metabolism. Vol. 216, Journal of Endocrinology. 2013.
88. Wang JJ, Zhuang ZH, Shao CL, Yu CQ, Wang WY, Zhang K, et al. Assessment of causal association between thyroid function and lipid metabolism: a Mendelian randomization study. Chin Med J (Engl). 2021;134(9).
89. Moran C, Agostini M, Visser WE, Schoenmakers E, Schoenmakers N, Offiah AC, et al. Resistance to thyroid hormone caused by a mutation in thyroid hormone receptor (TR)α1 and TRα2: Clinical, biochemical, and genetic analyses of three related patients. Lancet Diabetes Endocrinol. 2014;2(8).
90. Chamba A, Neuberger J, Strain A, Hopkins J, Sheppard MC, Franklyn JA. Expression and function of thyroid hormone receptor variants in normal and chronically diseased human liver. Journal of Clinical Endocrinology and Metabolism. 1996;81(1).
91. Chamba A, Neuberger J, Strain A, Hopkins J, Sheppard MC, Franklyn JA. Expression and function of thyroid hormone receptor variants in normal and chronically diseased human liver. J Clin Endocrinol Metab. 1996;81(1).
92. Luo Z, Qing X, Benda C, Huang Z, Zhang M, Huang Y, et al. Nuclear-cytoplasmic shuttling of class IIa histone deacetylases regulates somatic cell reprogramming. Cell Regeneration. 2019;8(1).
93. Kawata M. Subcellular steroid/nuclear receptor dynamics. Vol. 64, Archives of Histology and Cytology. 2001.
94. Singh BK, Sinha RA, Zhou J, Tripathi M, Ohba K, Wang ME, et al. Hepatic FOXO1 target genes are co-regulated by thyroid hormone via RICTOR protein deacetylation and MTORC2-AKT protein inhibition. Journal of Biological Chemistry. 2016;291(1).
95. Midgley JEM, Larisch R, Dietrich JW, Hoermann R. Variation in the biochemical response to l-thyroxine therapy and relationship with peripheral thyroid hormone conversion efficiency. Endocr Connect. 2015;4(4).
96. Hennessey J V. Levothyroxine Monotherapy: What Works Better for the Individual With Hypothyroidism? Vol. 29, Endocrine Practice. 2023.
97. Andersen S, Andersen SL. Response to Fitzgerald et al. re: "Thyroid Stimulating Hormone and Thyroid Hormones (Triiodothyronine and Thyroxine): An American Thyroid Association-Commissioned Review of Current Clinical and Laboratory Status". Vol. 34, Thyroid. 2024.
98. Trimboli P, Piticchio T, Dadda Z, Stramazzo I, Gamarra E, Ruinelli L, et al. Analysis of dose–TSH response effect of levothyroxine soft-gel formulation. Front Endocrinol (Lausanne). 2024;15.
99. Paisdzior S, Schuelke M, Krude H. What is the Role of Thyroid Hormone Receptor Alpha 2 (TRα2) in Human Physiology? Experimental and Clinical Endocrinology and Diabetes. 2022;130(5).

100. Bochukova E, Schoenmakers N, Agostini M, Schoenmakers E, Rajanayagam O, Keogh JM, et al. A Mutation in the Thyroid Hormone Receptor Alpha Gene. New England Journal of Medicine. 2012;366(3).
101. Korkmaz O, Ozen S, Ozdemir TR, Goksen D, Darcan S. A novel thyroid hormone receptor alpha gene mutation, clinic characteristics, and follow-up findings in a patient with thyroid hormone resistance. Vol. 18, Hormones. 2019.
102. Kim TH, Kim KW, Ahn HY, Choi HS, Won H, Choi Y, et al. Effect of seasonal changes on the transition between subclinical hypothyroid and euthyroid status. Journal of Clinical Endocrinology and Metabolism. 2013;98(8).
103. Pappa T, Refetoff S. Resistance to Thyroid Hormone Beta: A Focused Review. Vol. 12, Frontiers in Endocrinology. 2021.

Table 1. Primers sequences of THRA1 (T>C) (rs939348) genetic polymorphism

Primers		Sequence	Product size (bp)
Primers sequences of 939348 T > C	**F**	CCTGTGTCTCCCAGCTTAGC	200
	R	CCACCAGACTCACAGCCTCT	

Table 2: Demographic analyses of the study participants

Parameters	Healthy, n=50	Patients, n=100	P value
Age/year, mean±SD	51.40 ± 8.776	52.76 ± 9.358	0.3932
40-49, (n)%	(22), 44	(35), 35	
50-59, (n)%	(16), 32	(38), 38	
60-69, (n)%	(12), 24	(22), 22	
70-79, (n)%	-	(4), 4	
80-89, (n)%	-	(1), 1	
Wight/Kg, mean±SD	71.66 ± 9.983	84.88 ± 15.96	<0.0001
BMI/(kg/m2), mean±SD	28.38 ± 3.600	33.26 ± 5.929	<0.0001
Underweight, (n)%	-	-	0.424
Normal, (n)%	(5), 10	(5), 5	
Overweight, (n)%	(30), 60	(27), 27	
Obese, (n)%	(15), 30	(68), 68	
HT			
Yes, (N)%	(3), 6%	(66), 66%	0.0272
No, (N)%	(47), 94%	(34), 34%	
SBP, mean±SD	124.6±7.549	133.8±15.80	0.0001
DBP, mean±SD	81.00±5.425	84.61±7.184	0.0021
DM			
Yes, (N)%	(8), 16	(37), 37	0.048
No, (N)%	(42), 84	(63), 63	
Duration of L-thyroxin treatment/Years, (N)%			
1-5	-	(42), 42%	-
6-10	-	(44), 44%	-
11-15	-	(8), 8%	-
16-20	-	(3), 3%	-
21-25	-	(3), 3%	-
Dose of L-thyroxin mcg, (N)%			
25	-	(3), 3%	
50	-	(15), 15%	
75	-	(12), 12%	
100	-	(56), 56%	
125	-	(4), 4%	
150	-	(6), 6%	
200	-	(2), 2%	
225	-	(1), 1%	
250	-	(1), 1%	
Data is presented as mean±SD or percentages. P<0.05 is considered significant. SBP refers to Systolic blood pressure, DBP refers to Diastolic blood pressure, BMI refers to body mass index, DM refers to diabetes mellitus.			

Table 3: Frequency distribution of THRA rs939348 T>C in healthy individuals and hypothyroid patient

Genotypes	Healthy, n=50	Patients, n=100	P value
Wild (TT), (n)%	(40), 80	(33), 33	<0.001
Mutant heterozygous (CT), (n)%	(5), 10	(19),19	0.199
Mutant homozygous (CC), (n)%	(5), 10	(48), 48	<0.001
Chi-square was used to find the differences between healthy and patients genotypes. Data was presented as number and frequencies.			

Table 4: Pairwise comparison of THRA rs939348 T>C allele frequencies in hypothyroid patient

Pairwise comparisons	Chi-Square (χ^2)	p-value
TT vs. CT	83.12	<0.001
TT vs. CC	343.09	<0.001
CT vs. CC	496.75	<0.001

Table 5: Pairwise comparison of THRA rs939348 T>C allele frequencies in healthy subjects.

Pairwise Comparison	Chi-Square (χ^2)	p-value
TT vs. CT	54.44	<0.0001
TT vs. CC	54.44	<0.0001
CT vs. CC	0	1

Table 6: Analyses of THRA rs939348 T>C genotype frequencies: Hardy-Weinberg and Fischer exact test calculations in hypothyroid patients.

Genotype/observed % n=100		Hardy–Weinberg equilibrium/ expected %			Fischer exact test	P < 0.05
T	C	T	C	TC/CT	TT/observed vs TT/expected	0.0002
0.425	0.575	0.180625	0.330625	0.48875	CT/observed vs CT/expected	0.0001
					CC/observed vs CC/expected	0.001
P <0.05 considered significant, data was showed as percentage.						

Table 7: Analyses of THRA rs939348 T>C genotype frequencies: Hardy-Weinberg and Fischer exact test calculations in healthy control people.

Genotype/observed % n=50		Hardy–Weinberg equilibrium/ expected %			Fischer exact test	P < 0.05
T	C	T	C	TC/CT	TT/observed vs TT/expected	0.0002
0.85	0.15	0.7225	0.0225	0.255	CT/observed vs CT/expected	0.0001
					CC/observed vs CC/expected	0.0001
P <0.05 considered significant, data was showed as percentage.						

Table 8: Differences among demographic analyses in the study's participants with THRA rs939348 T>C genotype

Parameters	Healthy, n=50			Patients, n=100		
	TT	TC	CC	TT	TC	CC
Age/year, mean±SD	52.25±8.602	51.8±10.45	44.2±6.38	51.82±8.897	52.68±8.159	53.44±10.2
40-49, (n), %	(15), 30	(3), 6	(4), 8	(12), 12	(6), 6	(17),17
50-59, (n), %	(14), 28	(1), 2	(1), 2	(14), 14	(8),8	(16),16
60-69, (n), %	(11), 22	(1), 2		(6), 6	(5),5	(11),11
70-79, (n), %				(1), 1		(3),3
80-89, (n), %						(1),1

Wight/Kg, mean±SD	72.1±10.37	71.6±9.555	68.2±7.95	82.48±16.97	86.42±13.51	85.92±16.26
BMI/(kg/m2), mean±SD	28.32±3.651	30.47±3.544	26.82±2.778	32.48±5.822	33.62±5.163	33.64±6.335
Normal, (N), %	(5), 10	-	(1), 2	(2), 2	-	(3), 3
Overweight, (N), %	(23), 46	(3), 6	(3), 6	(11), 11	(5), 5	(8), 8
Obese, (N), %	(12), 24	(2), 4	(1), 2	(20), 20	(14), 14	(37), 37
HT						
Yes, (N)%	(3), 6	-	-	(20), 20	(14), 14	(32), 32
No, (N)%	(37), 74	(5), 10	(5), 10	(13), 13	(5), 5	(16), 16
SBP, mean±SD	124.3±7.982	124.8±7.155	126.2±4.658	134.2±17.47	134.5±12.99	134.4±16.01
DBP, mean±SD	80.98±5.855	81.6±2.608	79.2±2.49	85.15±6.195	83.16±6.644	85.38±7.789
DM						
Yes, (N)%	(7), 14	(1), 2	(),	(10), 10	(4), 4	(23), 23
No, (N)%	(33), 66	(4), 8	(5), 10	(23), 23	(15), 15	(25), 25
Duration of L-thyroxin/Years, (N)%						
1-5	-	-	-	(12), 12	(9), 9	(21), 21
6-10	-	-	-	(15), 15	(8), 8	(21), 21
11-15	-	-	-	(5), 5	(1), 1	(2), 2
16-20	-	-	-	(1), 1	-	(2), 2
21-25	-	-	-	-	(1), 1	(2), 2
Dose of L-thyroxin mcg, (N)%						
25	-	-	-	-	(1), 1	(2), 2
50	-	-	-	(4), 4	(2), 2	(9), 9
75	-	-	-	(6), 6	(4), 4	(2), 2
100	-	-	-	(19), 19	(11), 11	(26), 26
125	-	-	-	(1), 1	(1), 1	(2), 2
150	-	-	-	(3), 3	-	(3), 3
200	-	-	-	-	-	(2), 2
225	-	-	-	-	-	(1), 1
250	-	-	-	-	-	(1), 1

Table 9: Differences among clinical parameters in the study's participants with THRA rs939348 T>C genotype

Parameters	**Healthy, n=50**			**Patients, n=100**				
HT	**TT**	**TC**	**CC**	**TT**	**TC**	**CC**	**Chi-square, df**	**P value**
Yes, (n)%	(3), 6	-	-	(20), 20	(14), 14	(32), 32	$\chi2$=49.34, 5	<0.0001
No, (n)%	(37), 74	(5), 10	(5), 10	(13), 13	(5), 5	(16), 16		
DM								
Yes, (N)%	(7), 14	(1), 2	(),	(10), 10	(4), 4	(23), 23	$\chi2$=11.67, 5	0.0396
No, (N)%	(33), 66	(4), 8	(5), 10	(23), 23	(15), 15	(25), 25		
BMI/(kg/m2)								
Normal, (N), %	(5), 10	-	(1), 2	(2), 2	-	(3), 3	$\chi2$=6	0.0419

Overweight, (N), %	(23), 46	(3), 6	(3), 6	(11), 11	(5), 5	(8), 8		
Obese, (N), %	(12), 24	(2), 4	(1), 2	(20), 20	(14), 14	(37), 37		

Table 10: Plasma thyroid hormones in patients and healthy people with THRA rs939348 T>C genotypes

Parameters	**Healthy, means±SD N=50**			**Patients, means±SD N=100**		
	TT	**TC**	**CC**	**TT**	**TC**	**CC**
Insulin, µU/ml	8.905 ± 3.541	6.294 ± 1.701	8.149 ± 3.189	16.13 ± 9.238	17.50 ± 8.410	17.39 ± 10.76
FPG, mg/dl	101.0±13.22	92.60±6.877	93.29±12.80	125.5±58.40	114.5±38.90	117.1±25.22
HOMA-IR	2.060±0.8242	2.550±1.747	1.743±0.9396	5.024±2.672	5.258±3.874	5.227±3.280
Cholesterol	157.5±23.86	183.0±63.13	159.6±19.9	187.9±37.23	188.1±44.14	189.6±39.88
TG	110.0±29.37	141.6±60.03	78.86±34.05	139.5±70.93	144.7±61.36	142.5±61.15
HDL	49.28±12.39	48.80±15.75	38.40±12.52	49.27±9.149	53.37±12.27	50.13±12.07
LDL	99.5±22.42	122.8±28.54	100.4±31.62	104.9±31.09	111.2±33.73	113.2±38.97
VLDL	34.34±11.9	34.58±9.702	34.04±9.942	25.99±9.785	28.04±11.9	28.32±12.35
Data presented as mean±SD, P value≤0.05 considered significant. FPG=fasting plasma glucose, TG=triglyceride, HDL=high density lipoprotein, LDL=low density lipoprotein, VLDL=very low-density lipoprotein.						

Table 11: Metabolic profiles in patients and healthy individuals with THRA rs939348 T>C genotypes.

TSH, µU/ml mean±SD			
Wild/Patients	**Wild/Healthy**	**Mutant/Patients**	**Mutant/Healthy**
4.441±4.471	2.228±0.801	4.490±4.4882	1.568±0.6799
Data presented as mean±SD, P value≤0.05 considered significant.			

Table 12: Duration of L-thyroxin treatment in patients with THRA rs939348 T>C genotypes.

Time/years	**Patients%, N=100**			**P value**	
	TT	**TC**	**CC**		
≤5 years	12	9	21	Row Factor	0.1496
>5 years	21	10	27	Column Factor	0.0721
Data presented as percentage, P value≤0.05 considered significant.					

Table 13: Effect the duration of L-thyroxine on plasma thyroid hormones in Hypothyroid patient with THRA rs939348 T>C genotypes.

Parameters	**Duration of L-thyroxine treatment**					
	≤5 Years mean±SD			**>5 Years mean±SD**		
Genotypes	**TT**	**TC**	**CC**	**TT**	**TC**	**CC**
TSH, µU/ml	5.53 ± 5.11	7.47 ± 9.65	5.37 ± 4.23	3.35 ± 3.98	2.16 ± 1.83	3.47 ± 3.00
TT3 ng/ml	1.714±0.222	1.674±0.251	1.570±0.1924	1.610±0.1722	1.629±0.2733	2.166±3.369
FT3 pmol/L	5.735±0.782	5.241±1.102	5.426±0.788	5.632±1.16	5.189±0.9971	5.471±1.1276
TT4 nmol/L	137.38 ± 33.76	135.68 ± 29.83	131.39 ± 138.68	145.50 ± 22.7	139.65 ± 20.46	138.68 ± 24.640
FT4 pmol/L	12.54 ± 2.38	14.64 ± 1.920	12.57 ± 2.55	14.07 ± 2.4	13.33 ± 0.96	13.45 ± 2.17
TSH=thyroid stimulating hormone, TT3=total thyroid hormone, FT3=free thyroid hormone, TT4=total thyroid hormone, FT4=free thyroid hormone. Data presented as mean±SD, P value≤0.05 considered significant.						

Table 14: Effect the duration of L-thyroxine on clinical parameters in Hypothyroid patient with THRA rs939348 T>C genotypes

Parameters	Duration of L-thyroxine treatment					
	≤5 Years mean±SD			>5 Years mean±SD		
	TT	TC	CC	TT	TC	CC
Insulin, µU/ml	14.604 ± 7.682	17.141 ± 11.373	16.700 ± 5.613	17.009 ± 10.094	16.821 ± 5.986	14.294 ± 5.760
FPG, mg/dl	140.25 ± 80.424	122.667 ± 51.171	121.762 ± 33.241	117.048 ± 41.162	107.1 ± 23.914	113.182 ± 17.440
HOMA-IR	4.5 ± 1.665	6.144 ± 5.053	5.095 ± 2.101	5.324 ± 3.105	4.46 ± 2.414	4.009 ± 1.699
CHO	182.750 ± 53.916	198.889 ± 52.999	190.190 ± 38.196	182.619 ± 34.254	178.400 ± 34.336	181.370 ± 46.474
TG	149.833 ± 104.616	166.889 ± 63.686	151.381 ± 55.999	130.583 ± 43.471	124.700 ± 54.663	135.519 ± 65.065
HDL	50.083 ± 11.301	51.889 ± 14.435	50.329 ± 12.628	48.810 ± 7.941	54.700 ± 10.563	49.978 ± 11.862
LDL	106.183 ± 32.353	124.544 ± 30.085	119.057 ± 32.785	104.100 ± 31.125	99.140 ± 33.633	108.730 ± 43.237
vLDL	30.475±21.716	30.711±12.85	28.929±10.471	26.53±9.44	25.64±11.078	27.848±14.191
Data presented as mean±SD, P value≤0.05 considered significant. TG=triglyceride, HDL=high density lipoprotein, LDL=low density lipoprotein, VLDL=very low-density lipoprotein.						

Table 15: Plasma TSH levels in wild type and mutant THRA rs939348 T>C genotypes among hypothyroid patient and healthy people.

L-thyroxin duration/Year	TSH, µU/ml mean±SD			
	Wild/Patients	Wild/Healthy	Mutant/Patients	Mutant/Healthy
≤5 years	5.369±4.233	2.132±0.9425	6.262±7.300	2.130±0.808
>5 years	3.469±3.003	2.132±0.9425	3.124±3.513	2.130±0.808
Data presented as mean±SD, P value≤0.05 considered significant.				

Table 16*: TSH plasma levels in Hypothyroid patient with THRA rs939348 T>C genotypes compared to healthy individuals considering the duration of L-thyroxin treatment

L-thyroxin mcg	TSH, µU/ml mean±SD			
	Wild/Patients	Wild/Healthy	Mutant/Patients	Mutant/Healthy
25	-	2.228 ± 0.8010	4.507 ± 1.786	1.647 ± 0.6986
50	4.253 ± 2.336	2.228 ± 0.8010	7.339 ± 7.793	1.647 ± 0.6986
75	3.140 ± 2.255	2.228 ± 0.8010	3.637 ± 2.503	1.647 ± 0.6986
100	2.917 ± 2.039	2.228 ± 0.8010	3.155 ± 1.850	1.647 ± 0.6986
125	1.693 ± 0.2274	2.228 ± 0.8010	4.065 ± 3.566	1.647 ± 0.6986
150	3.390 ± 1.854	2.228 ± 0.8010	4.280 ± 3.897	1.647 ± 0.6986
≥200	-	2.228 ± 0.8010	12.03 ± 6.398	1.647 ± 0.6986
Data presented as mean±SD, P value≤0.05 considered significant.				

Table 17: The effect of L-thyroxine on TSH plasma levels in Hypothyroid patient with THRA rs939348 T>C genotypes compared to healthy individuals.

TSH, µU/ml mean±SD					
Wild patients-Responders	Wild patients-Non responders	Wild Healthy	Mutant patients-Responders	Mutant patients-Non responders	Mutant Healthy
2.019 ± 1.09	8.394 ± 5.628	2.228 ± 0.801	2.243 ± 1.220	6.662 ± 5.889	1.398 ± 0.562
Data presented as mean±SD, P value≤0.05 considered significant.					

Table 18: Response of hypothyroid patient to L-thyroxine based on THRA rs939348 T>C genotypes

Genotypes	BMI=R/S^2, mean±SD			
	Responders	Healthy Individuals	Non-responders	Healthy Individuals
Wild	31.569 ± 2.402	28.317 ± 3.651	31.047 ± 6.664	28.317 ± 3.651
Mutant	33.088 ± 5.895	28.649 ± 3.566	34.452 ± 6.141	28.649 ± 3.566
Data was presented as mean±SD, P<0.05 was considered significant.				

Table 19: Plasma TSH levels in Hypothyroid patient undergoing L-thyroxine based on THRA rs939348 T>C genotypes, categorized by responders and no responders.

Response to L-thyroxin	Patients %			
	Wild	Mutant	Chi-square (χ^2) statistic	p-value
Responders	33	20	13.730	0.000211
Non-responders	11	36		
Data was presented as percentage and the statistical values was calculated by Chi-square				

FIGURE LEGENDS

Figure 1: Presents the differences in the plasma thyroid hormone and metabolic markers in patients and control groups. A) TSH=thyroid stimulating hormone, B) Plasma TT3=total thyroid hormone, C) FT3=free thyroid hormone, D) TT4=total thyroid hormone, E) FT4=free thyroid hormone, F) Plasma insulin, G) FPG=fasting plasma glucose. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01, **P<0.001, ***P<0.0001.

Figure 2: Detection of THRA rs939348 T>C genetic polymorphism by using RFLP- PCR. Lane M refers to the DNA ladder for size reference. Lanes 1-12 refer to the individuals (1-3) represent TT genotype (wild alleles), lanes (4 and 7) refer to the mutant genotype (CC, homozygote), and (5 and 10) indicate the mutant genotypes (CT/TC, heterozygote). The electrophoresis gel was conducted at 45V.

Figure 3: Demonstrates of Pairwise comparison of THRA rs939348 T>C allele frequencies in healthy people and hypothyroid patients. data was presented as percentage, **P<0.001, ***P<0.0001

Figure 4: Shows THRA rs939348 T>C genotype frequencies: Hardy-Weinberg and Fischer exact test calculations for healthy control group and hypothyroidism patients. The data was presented as percentages, *P<0.05, **P<0.001, ***P<0.0001

Figure 5: Distribution of the patients and healthy control groups based on the THRA rs939348 T>C genotype. A) Age/years. B) Age groups. C-D) Mean of the Body weight. E-F) refers to the BMI. G) Systolic blood pressure (SBP). H) refers to the Diastolic blood pressure (DBP). *P<0.01, **P<0.001, ***P<0.0001, ns=no significant. the data presented as mean±SD or percentage.

Figure 6: Analyses of plasma thyroid hormones in patients and healthy females with THRA rs939348 T>C genotypes. A) TSH=thyroid stimulating hormone. B) TSH within patient groups. C) TSH within healthy control groups. D) TT3=total thyroid hormone. E) FT3=free thyroid hormone. F) FT3 within patient groups. G) FT3 within healthy control groups. H) TT4=total thyroid hormone. I)FT4=free thyroid hormone. J) FT4 plasma levels within healthy control groups. K) FT4 plasma levels within patient groups. Data presented as mean±SD. P<0.05 considered significant. *P<0.01, **P<0.001, ***P<0.0001.

Figure 7: Analyses of glucose hemostasis in patients and healthy females with THRA rs939348 T>C genotypes. A) Plasma Insulin µU/ml comparison among different genotypes in healthy (B) and Hypothyroid patient in (C). D) FPG refers to fasting plasma glucose mg/dl. E) FPG in healthy group. F) FPG in patients. G) HPMA-IR comparisons based on different genotypes. H) HOMA-IR in healthy control and I) HOMA-IR differences in hypothyroidism patients. Data presented as mean±SD. P<0.05 considered significant. *P<0.01, **P<0.001, ***P<0.0001.

Figure 8: Analyses of blood lipid profiles in patients and healthy controls with THRA rs939348 T>C genotypes. A) CHOL Cholesterol mg/dl µU/m comparison among different genotypes in healthy and Hypothyroidism patients. B) CHOL in healthy group. C) CHOL in hypothyroidism patients. D) TG triglycerides in the two different groups. E) TG in healthy controls. F) TG in patients. G) High density lipoprotein HDL in both comparable groups. H) HDL in the healthy control. I) HDL in the hypothyroidism patients. J) low density lipoprotein LDL in control and patient groups. K) LDL in healthy individuals. L) LDL in hypothyroidism patients. M) very low-density lipoprotein vLDL in the both groups. N) vLDL in healthy controls. O) in the hypothyroidism patients. Data presented as mean±SD. P<0.05 considered significant. *P<0.01, **P<0.001, ***P<0.0001.

Figure 9: Describes duration L-Thyroxine treatment on plasma thyroid hormones in Hypothyroid patient with THRA rs939348 T>C genotypes. A) TSH=thyroid stimulating hormone. B) TT3=total thyroid hormone. C) FT3=free thyroid hormone. D) TT4=total thyroid hormone. E) FT4=free thyroid hormone. Data presented as mean±SD, P value≤0.05 considered significant, ns refers to non-significant.

Figure 10: Describes the impact of L-thyroxine treatment duration on clinical parameters in Hypothyroid patient with THRA rs939348 T>C genotypes (TT, TC and CC). A) Insulin. B) FPG fasting plasma glucose. C) HOMA-IR. D) CHOL refers to cholesterol. E) TG refers to Triglycerides. F) HDL means high density lipoprotein. G) LDL means low density lipoprotein. H) vLDL refers to very low-density lipoprotein. Data presented as mean±SD. P<0.05 considered significant. *P<0.01, **P<0.001, ***P<0.0001.

Figure 11: Describes the impact of THRA rs939348 mutation on the TSH levels in Hypothyroid patient and healthy people. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01.

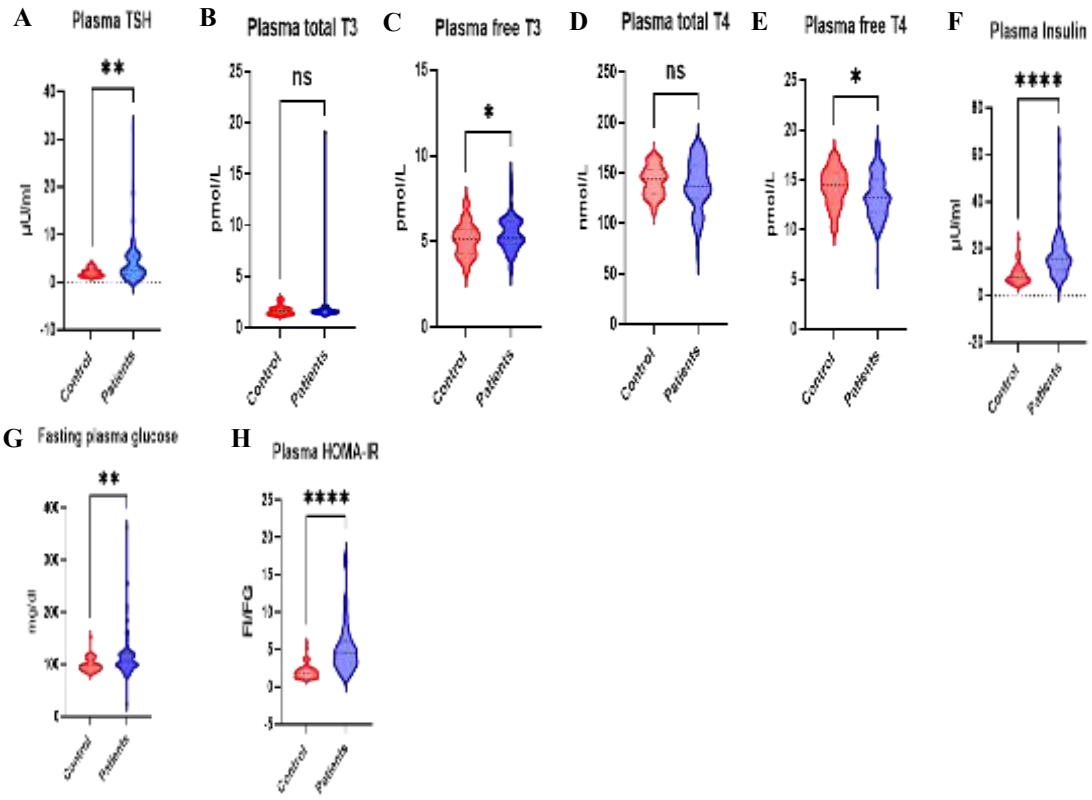
Figure 12: Presents the impact of THRA rs939348 T>C on the treatment responsiveness in hypothyroidism patients. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01, **P<0.001, P<0.0001.

Figure 13: Describes the statistical differences of L-thyroxine dose variations on TSH plasma levels in Hypothyroid patient with THRA rs939348 T>C genotypes compared to healthy individuals. L-thyroxine dosages ranged from 25mcg-≥200mcg as presented in the figure respectively A-G. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01, **P<0.001, P<0.0001.

Figure 14: Describes the Impact of THRA rs939348 T>C genotypes mutation on L-thyroxine response based on TSH plasma levels in Hypothyroid patient compared to healthy controls. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01, **P<0.001, P<0.0001, ns=non-significant.

Figure 15: Describes BMI variations among Hypothyroid patient by THRA rs939348 T>C polymorphism and treatment response. Data presented as mean±SD, P value≤0.05 considered significant. *P<0.01, **P<0.001, P<0.0001, ns=non-significant.

Figure 1



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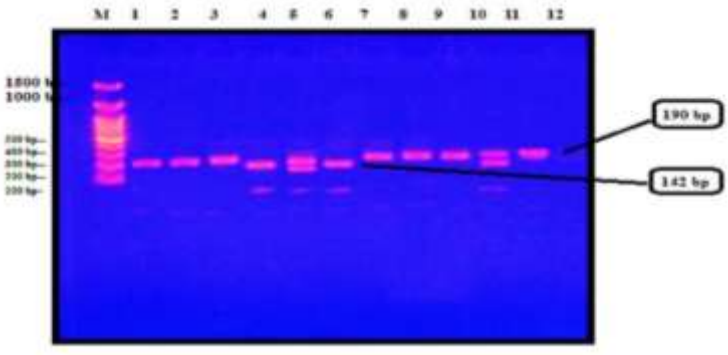

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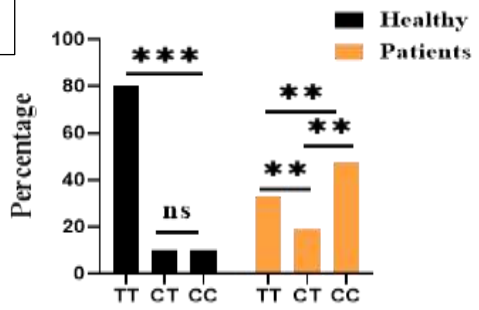

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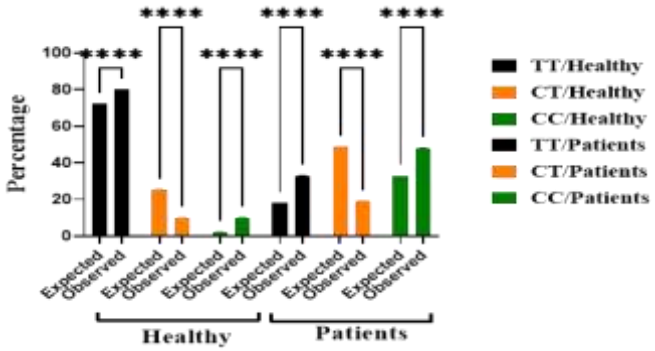

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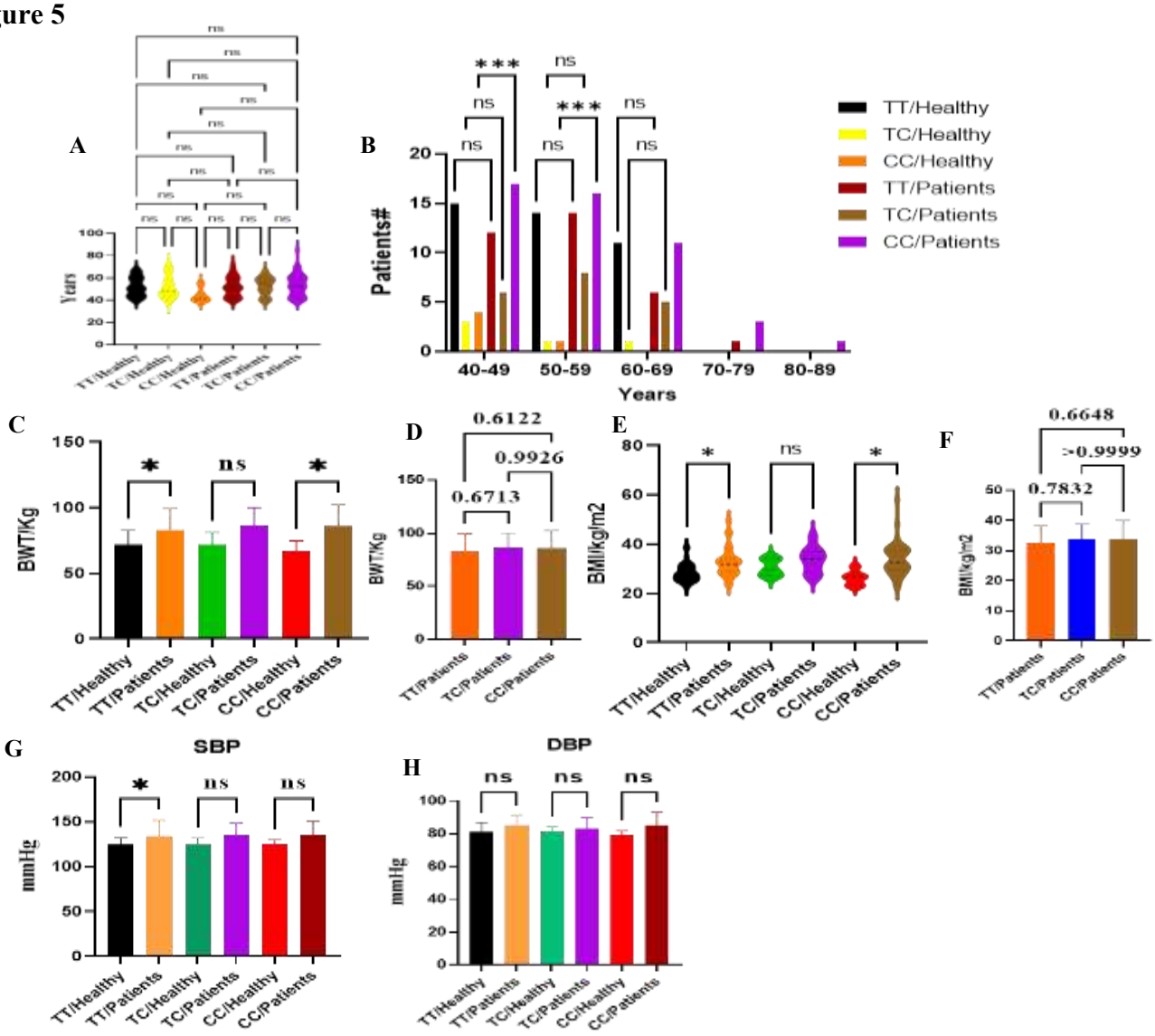

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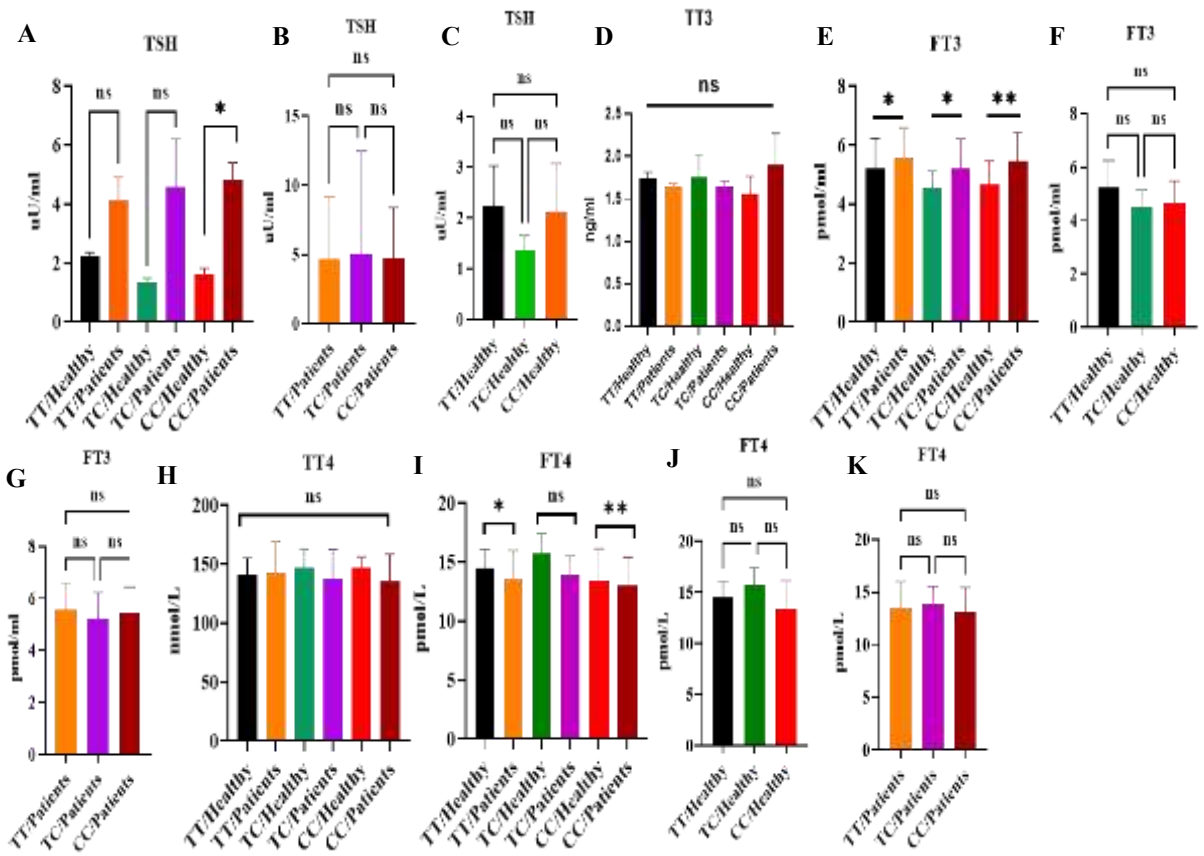

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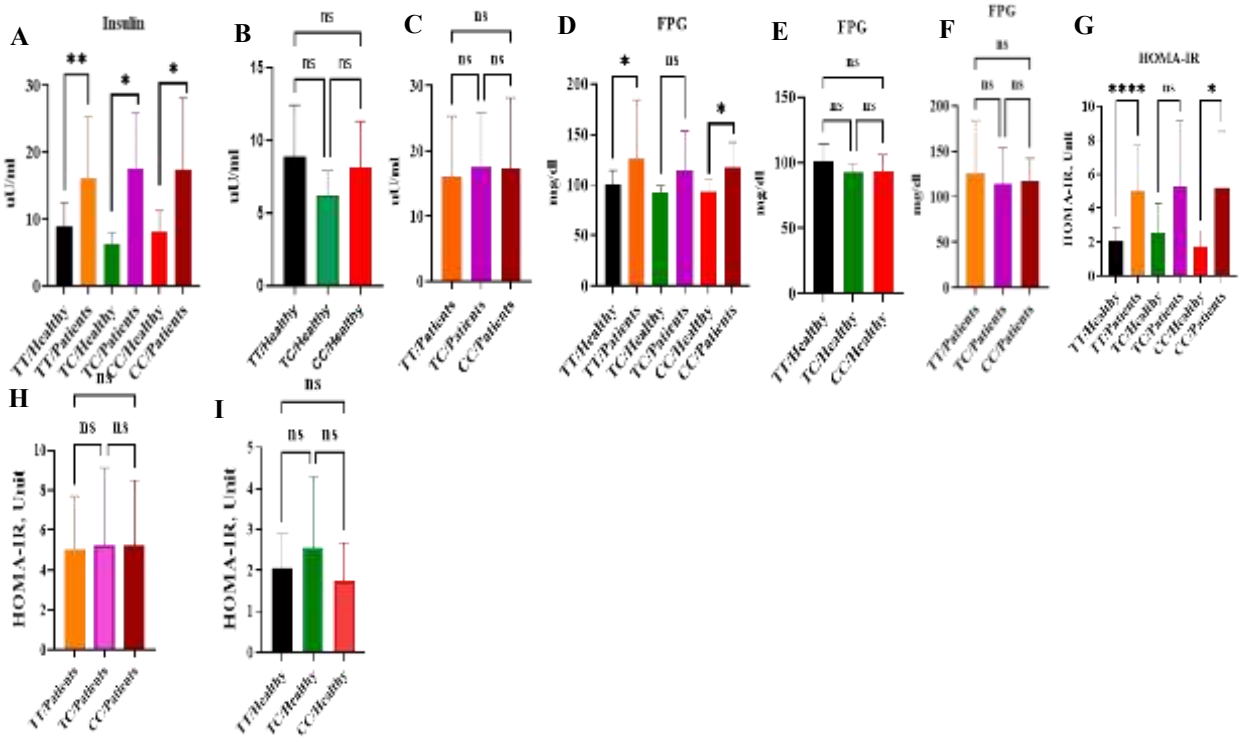

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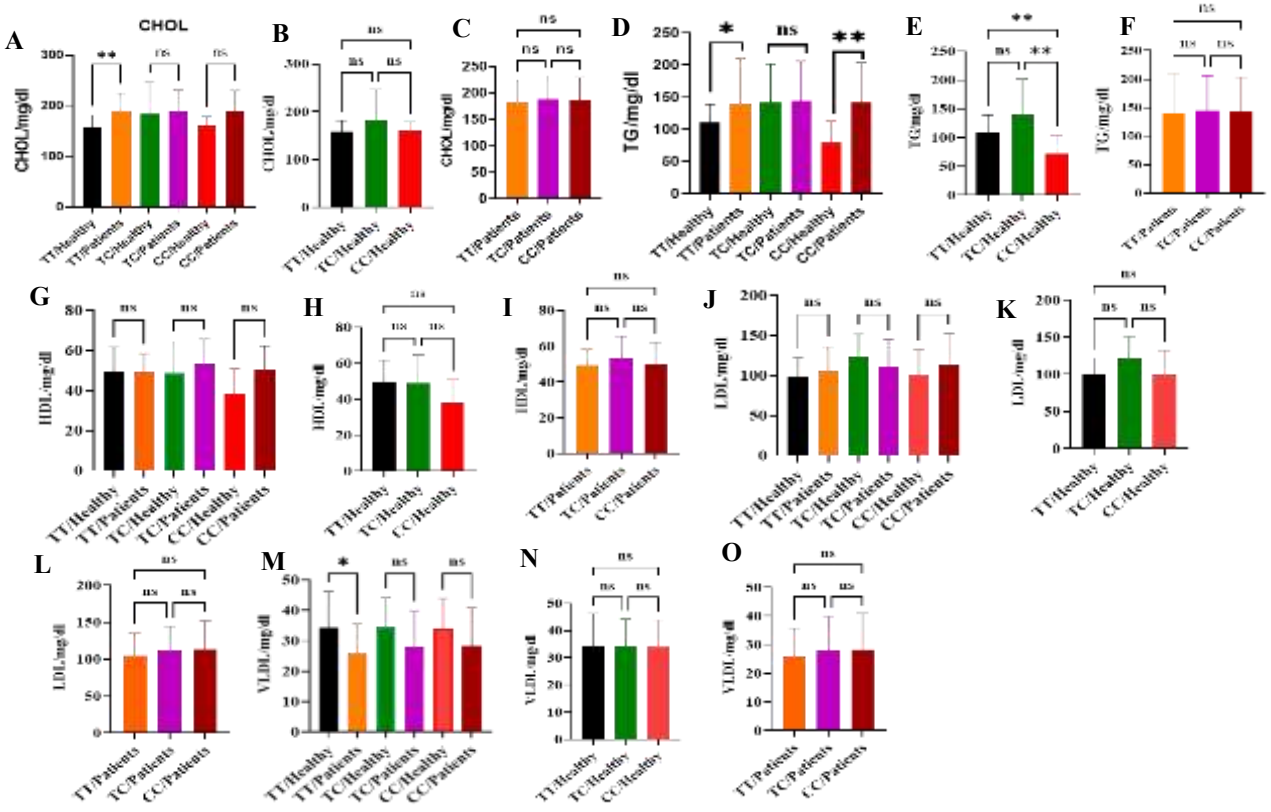

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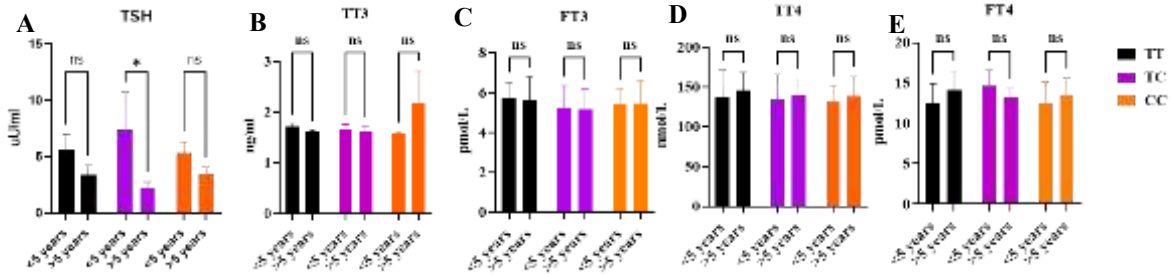

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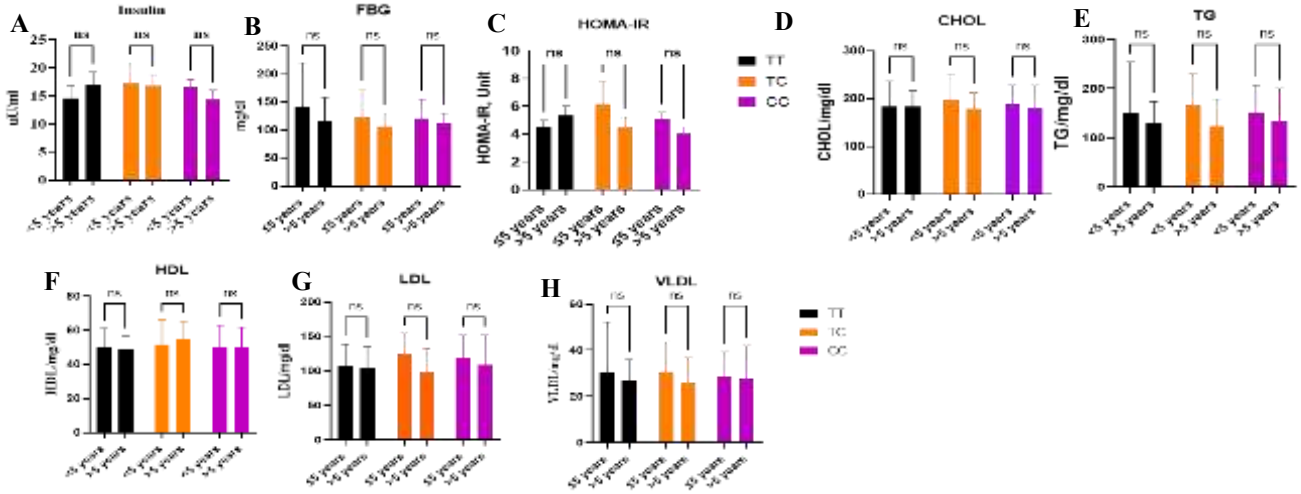

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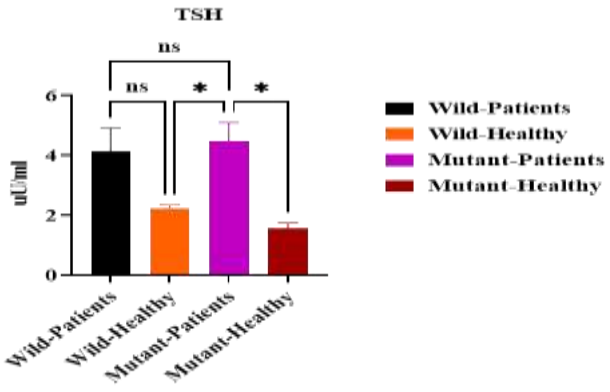

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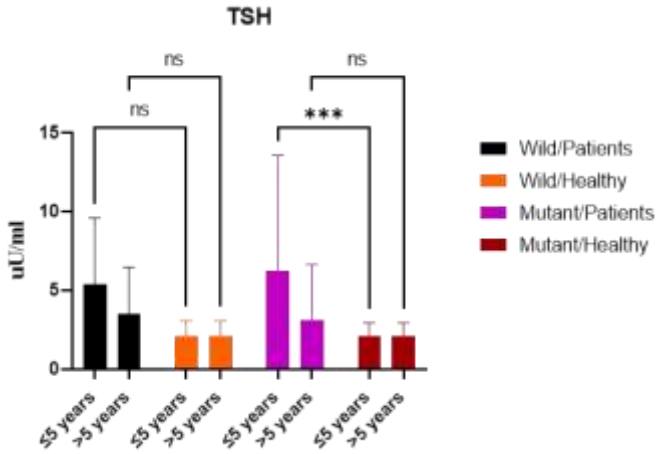
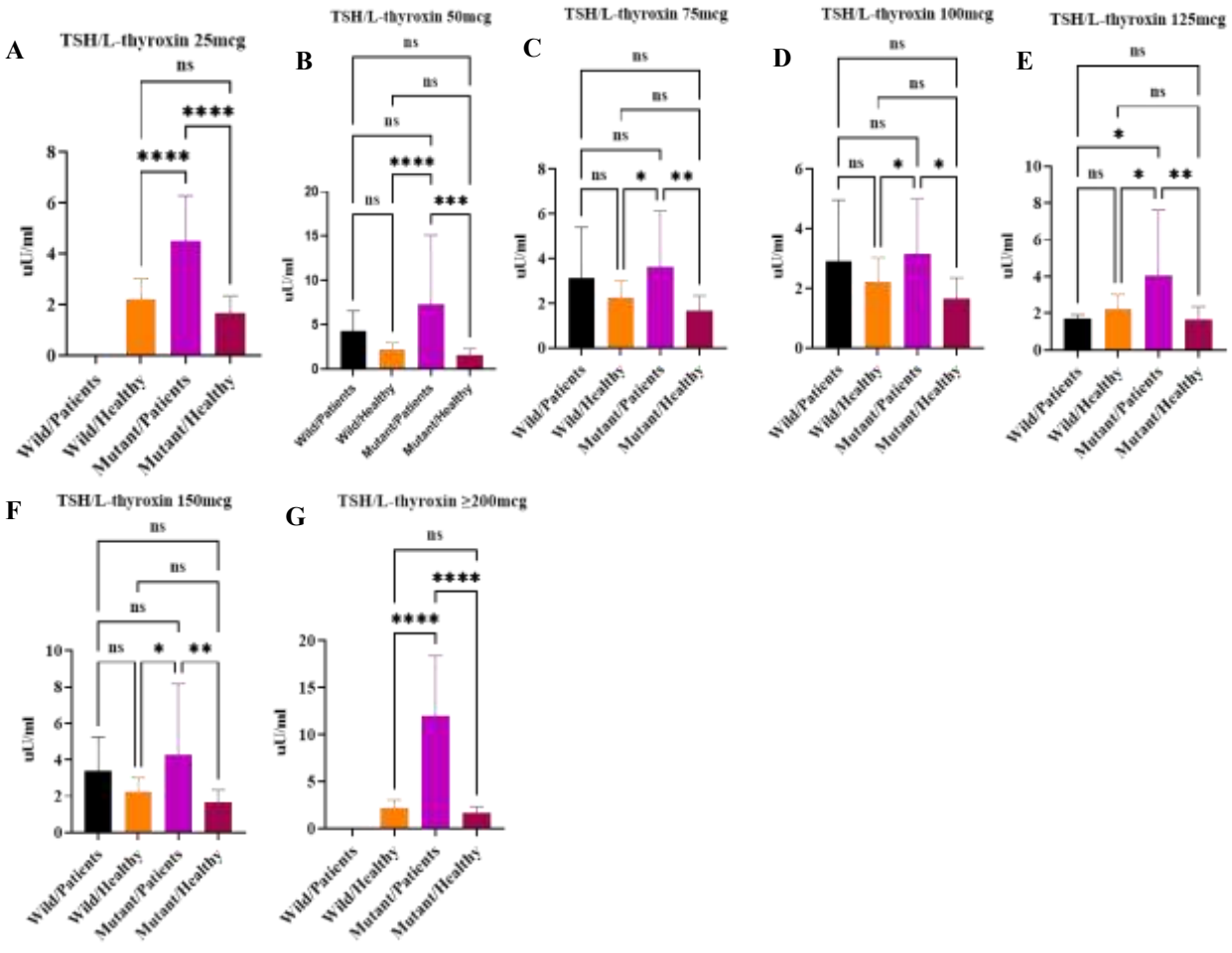


Figure 13



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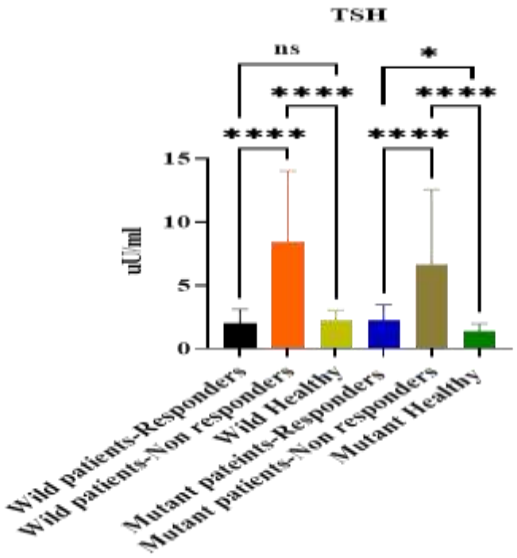

Figure 15