



Association of DIO1 rs2235544 Polymorphism with Thyroid Hormone Profile and Levothyroxine Dose Requirement in Iraqi Women with Primary Hypothyroidism

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Abstract: Type 1 iodothyronine deiodinase (DIO1) plays an important role in peripheral thyroid hormone metabolism, and genetic variation in DIO1 may be associated with differences in thyroid hormone profiles and levothyroxine (LT4) dosing. The present study investigated the association of DIO1 rs2235544 polymorphism with thyroid hormone parameters and the current prescribed LT4 dose in Iraqi women with primary hypothyroidism. A cross-sectional study was conducted among 76 women receiving LT4 monotherapy. Serum thyroid-stimulating hormone (TSH), free triiodothyronine (FT3) and free thyroxine (FT4) levels were tested, and the FT3/FT4 ratio was calculated. DIO1 rs2235544 polymorphism was genotyped using real-time PCR-based probe allelic discrimination. Demographic, biochemical and treatment-related variables were compared across the genotype groups. Multiple linear regression analysis was used to evaluate the factors associated with the current prescribed LT4 dose. Significant differences in body mass index (BMI) and LT4 dose between the genotype groups (both $P < 0.001$) were identified. The mean BMI was 28.43, 26.89, and 25.81 kg/m² in the homozygous (AA), heterozygous (CA) and homozygous (CC) genotypes, respectively, while the mean LT4 dose was 126.79, 124.07, and 79.76 µg/day, respectively. No significant differences were observed in TSH ($P = 0.552$), FT3 ($P = 0.462$), FT4 ($P = 0.975$), or the FT3/FT4 ratio ($P = 0.785$). These findings suggest an association between DIO1 rs2235544 and the current prescribed LT4 dose in Iraqi women with primary hypothyroidism.

1. Introduction

The thyroid gland is an endocrine organ that produces the thyroid hormones thyroxine (T4) and triiodothyronine (T3), which are essential for normal metabolic and physiological functions [1, 2]. Primary hypothyroidism is among the most prevalent endocrine disorders and arises when the thyroid gland does not produce sufficient thyroid hormones [3]. It is significantly more prevalent among women than men and may impair numerous physiological processes such as metabolic balance, thermoregulation, cardiovascular function, and neurocognitive performance [1, 2]. Thyroid hormones are vital for growth, metabolism and the functioning of several organs [4]. Therefore, insufficient thyroid hormone production can have negative consequences on overall health and quality of life [5]. Levothyroxine (LT4) is a synthetic form of T4 used to replace deficient thyroid hormone and is the gold standard

replacement treatment in primary hypothyroidism because of its efficacy, general tolerability, suitability for long-term use, and relatively low cost [6, 7].

Treatment focuses mainly on the restoration of euthyroidism with the serum thyroid-stimulating hormone (TSH) being an important marker for dose adjustment [7, 8]. However, the dose of LT4 needed to establish effective biochemical control varies substantially across individuals, yet LT4 is widely used [9]. In some patients, very low doses are sufficient to provide good management, while others require greater doses to maintain proper thyroid function [9, 10].

LT4 requirements vary according to several clinical parameters including age, body weight, body mass index (BMI), severity of the disease, adherence to treatment, pregnancy and related medical problems [10]. Typically, an initial estimate of about 1.6 µg/kg/day is utilized in eligible individuals receiving complete substitution, but modification is frequently customized [11]. Moreover, due to the half-life of LT4 being roughly seven days, thyroid function is usually re-evaluated several weeks after initiating therapy and other changes in dose [12, 13].

Thyroid hormones undergo many metabolic routes such as glucuronidation, sulfation and deiodination [14]. The deiodinase type 1 (DIO1) gene encodes the DIO1 enzyme, which participates in thyroid hormone metabolism and in the peripheral conversion of T4 to the physiologically active T3 [15]. Deiodination is vital for the regulation of the activation and inactivation of thyroid hormones. This mechanism is mediated by three iodothyronine deiodinases: type 1 (DIO1), type 2 (DIO2), and type 3 (DIO3) [16]. Clinical features alone cannot explain all of the variability observed in LT4 requirements [17]. Therefore, genetic differences in thyroid hormone metabolism have increasingly drawn attention as potential contributors to this variability [18].

Genetic polymorphisms, particularly single nucleotide polymorphisms (SNPs), can be detected using molecular techniques such as probe-based real-time PCR with allelic discrimination. DIO1 polymorphisms may alter the expression or activity of the DIO1 enzyme, potentially affecting peripheral T4-to-T3 conversion. This variation may contribute to differences in thyroid hormone levels and individual responsiveness to LT4 replacement therapy [19, 20]. DIO1 SNP has been increasingly investigated due to a reported association with free triiodothyronine (FT3) / free thyroxine (FT4) ratio, circulating thyroid hormone concentrations and daily LT4 dose [19, 21, 22].

Although DIO1 rs2235544 has been investigated in other populations, evidence regarding its association with thyroid hormone levels and LT4 dose requirements in Middle Eastern populations remains limited. To our knowledge, no previous study has specifically evaluated these associations in Iraqi patients with primary hypothyroidism. We hypothesized that DIO1 rs2235544 genotypes may be associated with differences in thyroid hormone levels and current prescribed LT4 dose. Therefore, the current study aimed to determine the distribution of DIO1 rs2235544 genotypes and alleles, additionally the study explored their relationships with serum thyroid hormone levels and the current prescribed daily LT4 dose in Iraqi women with primary hypothyroidism.

2. Materials and Methods

2.1. Study Design and Registration

Eligible participants were women aged ≥18 years who had been receiving LT4 monotherapy for at least four months before enrollment. The current prescribed LT4 dose was verified using the participants' available medical records, compared with previous records when available. However, a predefined minimum duration of dose stability was not documented.

The exclusion criteria included pregnancy or lactation, central hypothyroidism, active malignancy or a history of malignancy, severe liver dysfunction, chronic kidney disease stage 4 or higher, gastrointestinal malabsorption disorders, severe psychiatric illness unrelated to hypothyroidism, and the use of drugs known to disturb thyroid hormone absorption or metabolism. Disease/treatment duration, medication adherence, relevant medical history, and concomitant medication use were assessed during participant screening. The minimum treatment duration required for eligibility was four months, while adherence was assessed based on participant self-reporting rather than a validated adherence scale.

Exact disease duration and quantitative adherence measures were not systematically recorded as analytical variables and therefore were not included as covariates in the regression analyses.

The study was authorized by the Medical Ethics Committee of the College of Medicine, University of Kufa, Iraq (reference number: MEC-225; approval date: 23 September 2025). All participants provided written informed consent before enrollment.

2.2. Sample Size Calculation

Prior to recruitment, sample-size estimation was performed using G*Power based on a one-way analysis of variance (ANOVA) according to the three genotype groups, a significance level of 0.05, and 80% statistical power [23]. Under medium ($f = 0.25$) and large ($f = 0.40$) effect-size assumptions, the estimated sample size ranged from approximately 66 to 159 participants. A total of 76 eligible participants were ultimately recruited during the predefined study period.

2.3. Blood Sample Collection and Biochemical Measurements

Peripheral venous blood (5 mL) was taken from each participant. Two milliliters were transferred into an EDTA-containing tube for DNA extraction and genotyping, while the remaining 3 mL was collected in a gel tube for serum separation and biochemical analysis.

Serum TSH, free triiodothyronine (FT3), and free thyroxine (FT4) concentrations were measured using an automated electrochemiluminescence immunoassay system (Cobas e 411, Roche Diagnostics, Germany), according to the manufacturer's instructions. The FT3/FT4 ratio was calculated for subsequent statistical analysis.

2.4. DNA Extraction and Quantification

Genomic DNA was isolated from EDTA-anticoagulated whole blood using the HiPure Blood DNA Mini Kit (Magen Biotechnology, China) following the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop 8000 spectrophotometer before genotyping.

2.5. Genotyping of DIO1 rs2235544

The DIO1 rs2235544 SNP was genotyped using a probe-based allelic discrimination real-time polymerase chain reaction (PCR) assay. The primers and allele-specific probes were custom designed and synthesized by Alpha ADN (Canada). The C-allele-specific probe was labeled with FAM at the 5' end and BHQ-1 at the 3' end, whereas the A-allele-specific probe was labeled with HEX and BHQ-1. The primer and probe sequences used for genotyping are presented in Table 1.

Table 1: Primer and allele-specific probe sequences used for DIO1 rs2235544 genotyping.

Type of Oligonucleotide	Name of primer and Probe	Sequence (5'→3')	Labeling
Primer	rs44-F	ACCCTGCAAGAGAAACGAATCAG	Unlabeled
	rs44-R	GCAACTAACCTCCAGACCTACA	Unlabeled
Probe	rs44-C	CATTCCCAACTTGTAAAGGA	5'-FAM / 3'-BHQ-1
	rs44-A	CATTCCCAACTTTTAAGGAT	5'-HEX / 3'-BHQ-1

Real-time PCR was performed using SolisAcura™ Probe Genotyping qPCR Mix and the Light-Cycler 96 Real-Time PCR System (Roche Diagnostics, Germany). Each reaction had a final volume of 20 µL, consisting of 4.0 µL of 5× SolisAcura™ Probe Genotyping qPCR Mix, 0.6 µL of forward primer, 0.6 µL of reverse primer, 0.3 µL of the FAM-labeled C-allele probe, 0.3 µL of the HEX-labeled A-allele probe, 3.0 µL of genomic DNA, and 11.2 µL of nuclease-free water. A no-template control was included by replacing the genomic DNA with nuclease-free water [24]. Each DNA sample was analyzed once in a single reaction. The no-template control showed no amplification, indicating the absence of detectable contamination.

The thermal cycling protocol consisted of initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Endpoint fluorescence

signals were analyzed using LightCycler 96 software for allelic discrimination. The participants were classified as belonging to one of the homozygous (AA), heterozygous (CA), and homozygous (CC) genotype groups according to the observed fluorescence pattern [24, 25]. For independent validation, a subset of randomly selected samples was re-genotyped using the Mx3005P Real-Time PCR System at a separate laboratory. The repeated genotype calls were concordant with the initial results.

2.6. Statistical Analysis

Continuous variables were presented as mean $\pm$ standard deviation (SD) and categorical variables as frequencies and percentages. Differences between the three DIO1 rs2235544 genotype groups were compared using ANOVA when the homogeneity of variance assumption was met. Levene's test was used to assess the homogeneity of the variances. Pairwise comparisons were performed using Tukey's honestly significant difference (HSD) test post hoc when equal variances were assumed. When the homogeneity of variance assumption was violated, Welch's ANOVA using the Games–Howell post hoc test was utilized. The Hardy–Weinberg equilibrium was tested using the Chi-square goodness-of-fit test. Pearson correlation analysis was done to assess the associations between the demographic, clinical and biochemistry factors. Factors associated with current prescribed LT4 dose were evaluated using multiple linear regression analysis. LT4 dose ($\mu\text{g}/\text{day}$) was the dependent variable and age, BMI, TSH, FT3/FT4 ratio were included as clinical covariates.

In addition to the primary analysis, sensitivity analyses were performed using recessive (CC vs. AA/CA), dominant (CA/CC vs. AA), and additive (0, 1, and 2 C alleles) genetic models. Because the genetic model was not prespecified before the analysis, comparisons across these models are considered exploratory. Additional sensitivity analyses were performed after excluding TSH from the regression model because TSH may be influenced by the current prescribed LT4 dose. Regression coefficients (B), standardized coefficients (β), 95% confidence intervals (CI), and P values were reported. Multicollinearity was assessed using variance inflation factors, and the regression diagnostics included residual plots and Cook's distance. Statistical analyses were carried out using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) and Microsoft Excel 365 and Statistical significance was defined as two-sided $P < 0.05$.

3. Results

This cross-sectional study consisted of a sample of 76 Iraqi women with primary hypothyroidism. All participants had complete biochemical and genotyping data. The study subjects were recruited from November 2025 to March 2026 from private clinics and the Specialized Center of Endocrinology and Diabetes in Najaf, Iraq. The mean age of the participants was 39.16 ± 10.43 years (range: 22–60 years), while the mean BMI was 27.16 ± 2.10 kg/m^2 (range: 23–33 kg/m^2).

3.1. Genotype and Allele Distribution of DIO1 rs2235544

The genotype distribution deviated from the Hardy–Weinberg equilibrium ($\chi^2 = 6.10$, $P = 0.014$). The genotype and allele distribution of DIO1 rs2235544 among the study participants are shown in Figure 1. The distribution of the DIO1 rs2235544 polymorphism among the study participants showed that the AA genotype was the most frequent genotype (36.8%), followed by CA (35.5%) and CC (27.6%). Allele frequency analysis demonstrated that the A allele represented 54.6%, while the C allele represented 45.4% of the total alleles.

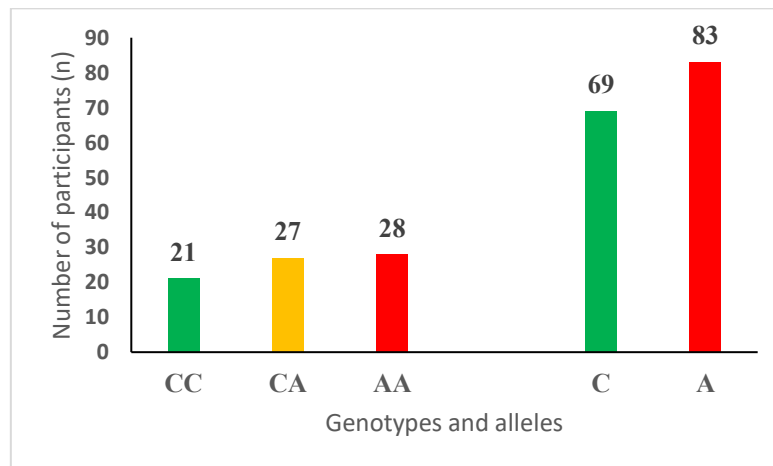


Figure 1: Distribution of DIO1 rs2235544 genotype and allele frequencies among the study participants.

3.2. Association between DIO1 rs2235544 Genotypes and the Demographic, Clinical, and Biochemical Variables

The demographic, clinical, and biochemical characteristics of the participants were compared across the three DIO1 rs2235544 genotype groups (AA, CA, and CC). Group differences were assessed using one-way ANOVA when the homogeneity of variance assumption was satisfied, and Welch's ANOVA when this assumption was not met. A significant difference in BMI was observed among the DIO1 rs2235544 genotype groups ($P < 0.001$). Participants carrying the AA genotype had the highest mean BMI ($28.43 \pm 2.28 \text{ kg/m}^2$), followed by those with the CA genotype ($26.89 \pm 1.65 \text{ kg/m}^2$), while the CC genotype showed the lowest mean BMI ($25.81 \pm 1.33 \text{ kg/m}^2$). Daily LT4 dose also differed significantly among the DIO1 rs2235544 genotype groups ($P < 0.001$). The mean LT4 dose was higher in participants with the AA ($126.79 \pm 43.00 \text{ } \mu\text{g/day}$) and CA ($124.07 \pm 45.19 \text{ } \mu\text{g/day}$) genotypes than in those with the CC genotype ($79.76 \pm 21.82 \text{ } \mu\text{g/day}$). The results are presented in Table 2. In contrast, no significant differences were observed among the genotype groups concerning age ($P = 0.810$), TSH ($P = 0.552$), FT3 ($P = 0.462$), FT4 ($P = 0.975$), or FT3/FT4 ratio ($P = 0.785$).

Table 2: Demographic, clinical, and biochemical characteristics according to DIO1 rs2235544 genotype.

Variable	AA (n=28)	CA (n=27)	CC (n=21)	F Test	P-value
Age (years)	39.00 $\pm$ 11.25	40.11 $\pm$ 10.35	38.14 $\pm$ 9.78	0.211	0.810
BMI (kg/m ²)	28.43 $\pm$ 2.28	26.89 $\pm$ 1.65	25.81 $\pm$ 1.33	12.740**	<0.001
TSH (mIU/L)	6.00 $\pm$ 6.94	4.87 $\pm$ 6.78	7.14 $\pm$ 7.87	0.599	0.552
FT3 (pmol/L)	4.31 $\pm$ 1.10	4.67 $\pm$ 1.06	4.61 $\pm$ 1.31	0.781	0.462
FT4 (pmol/L)	14.88 $\pm$ 4.29	14.76 $\pm$ 2.74	14.65 $\pm$ 2.96	0.025	0.975
FT3/FT4 ratio	0.307 $\pm$ 0.098	0.321 $\pm$ 0.070	0.323 $\pm$ 0.103	0.242	0.785
LT4 dose ($\mu\text{g/day}$)	126.79 $\pm$ 43.00	124.07 $\pm$ 45.19	79.76 $\pm$ 21.82	10.324**	<0.001

Values are presented as mean $\pm$ SD. One-way ANOVA was used when the homogeneity of variance assumption was satisfied, while Welch's ANOVA was used when this assumption was violated.

The overall genotype-group differences were significant for both BMI and daily LT4 dose (both $P < 0.001$; Table 3). The Games–Howell post hoc comparisons showed that BMI differed between AA and CA ($P = 0.016$), AA and CC ($P < 0.001$), and CA and CC ($P = 0.040$), with AA having the highest mean BMI and CC the lowest. For LT4 dose, Tukey HSD showed no difference between AA and CA ($P = 0.965$), whereas CC had a lower dose than AA and CA ($P < 0.001$ for each comparison). The results are presented in table 3.

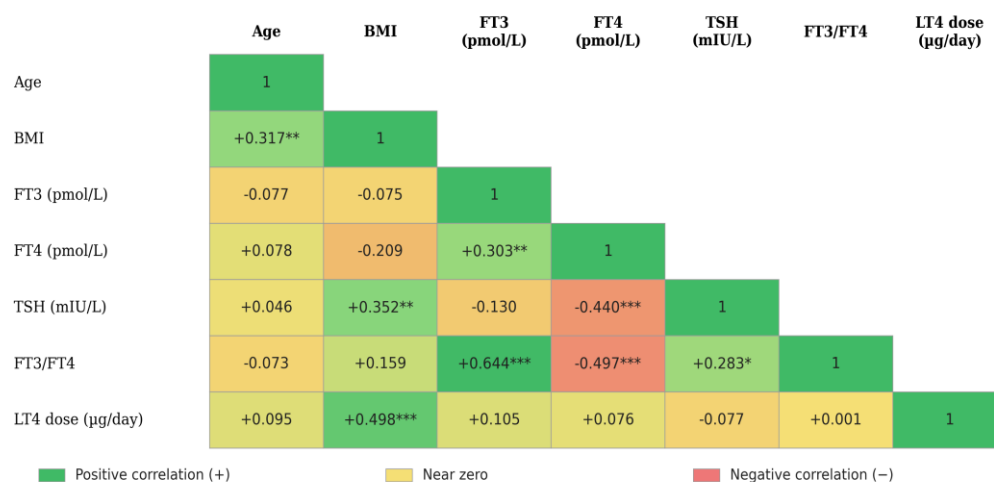
Table 3: Post hoc comparisons of BMI and daily levothyroxine dose according to the DIO1 rs2235544 genotypes.

Genotype	BMI (kg/m ²)	LT4 dose (µg/day)
AA (n = 28)	28.43 ± 2.28 ^a	126.79 ± 43.00 ^a
CA (n = 27)	26.89 ± 1.65 ^b	124.07 ± 45.19 ^a
CC (n = 21)	25.81 ± 1.33 ^c	79.76 ± 21.82 ^b
Overall P-value	<0.001***	<0.001***

Values are presented as mean ± SD. The p-values indicate the overall comparison among the genotypes. Within each column, means sharing a superscript letter do not differ significantly; means with different letters in same column were significantly different as $P < 0.05$. The Games–Howell post hoc test was used for BMI and Tukey HSD for LT4 dose. *** $P < 0.001$.

3.3. Pearson Correlation Analysis

Pearson correlation analysis was performed for age, BMI, FT3, FT4, TSH, the FT3/FT4 ratio, and the daily LT4 dose in 76 participants (Figure 2). A strong positive correlation was observed between FT3 and the FT3/FT4 ratio ($r = 0.644$, $P < 0.001$). Moderate positive correlations were found between BMI and LT4 dose ($r = 0.498$, $P < 0.001$), BMI and TSH ($r = 0.352$, $P = 0.002$), age and BMI ($r = 0.317$, $P = 0.005$), and FT3 and FT4 ($r = 0.303$, $P = 0.008$). Moderate negative correlations were found between FT4 and the FT3/FT4 ratio ($r = -0.497$, $P < 0.001$) and between FT4 and TSH ($r = -0.440$, $P < 0.001$). TSH had a weak positive correlation with the FT3/FT4 ratio ($r = 0.283$, $P = 0.013$). The other variable pairs did not reach statistical significance ($P \geq 0.05$).

**Figure 2:** Heatmap illustrating the Pearson correlation coefficients among the demographic, clinical, and biochemical variables.

Positive values trend toward green, near-zero values appears yellow, and negative values trend toward red. Asterisks mark two-sided significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 76$). The numerical value and direction of r do not establish statistical significance alone.

3.4. Linear Regression Analysis of the Factors Associated with the Current Prescribed LT4 Dose

Multiple linear regression analysis was performed with the current prescribed LT4 dose (µg/day) as the dependent variable and age, BMI, TSH, FT3/FT4 ratio, and DIO1 rs2235544 genotype (CC vs. AA/CA) as the independent variables. BMI had the largest positive standardized association with the current prescribed LT4 dose among the variables included in the model (standardized $\beta = 0.498$, $P < 0.001$). The CC genotype was associated with a lower prescribed LT4 dose compared with AA/CA after adjustment for age, BMI, TSH, and the FT3/FT4 ratio (standardized $\beta = -0.250$, $P = 0.024$). TSH was also negatively associated with prescribed dose (standardized $\beta = -0.219$, $P = 0.047$). Age and the FT3/FT4 ratio were not statistically significant after adjustment ($P = 0.500$ and $P = 0.921$, respectively). This indicates insufficient evidence of independent associations within this sample, rather than evidence of no association. Both variables were retained as adjustment covariates.

Overall, the model explained 37.6% of the variation in the current prescribed LT4 dose ($R^2 = 0.376$; adjusted $R^2 = 0.331$). The model was not externally validated; therefore, its suitability for predicting or

selecting an individual patient's LT4 dose has not been established. The regression results are presented in Table 4. The results of the additional genetic model and sensitivity analyses are presented in Table 5.

Table 4: Multiple linear regression analysis of the factors associated with the current prescribed levothyroxine (LT4) dose.

Variable	B	Standardized β	t	p-value
Constant	-141.752	—	-2.167*	0.034
TSH (mIU/L)	-1.352	-0.219	-2.023*	0.047
BMI (kg/m ²)	10.389	0.498	4.087***	<0.001
Age (years)	-0.288	-0.069	-0.677	0.500
CC genotype (vs. AA/CA)	-24.411	-0.250	-2.312*	0.024
FT3/FT4 ratio	-4.835	-0.010	-0.099	0.921

Model statistics: $R^2 = 0.376$; Adjusted $R^2 = 0.331$; $F = 8.422$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 5: Additional genetic model and sensitivity analyses for the current prescribed LT4 dose.

Analysis	Genetic model	B	Standardized β	95% CI for B	P-value
Including TSH	Dominant (CA/CC vs. AA)	6.344	0.070	-14.633 to 27.321	0.548
	Additive (0, 1, 2 C alleles)	-6.502	-0.119	-19.704 to 6.701	0.329
Excluding TSH	Recessive (CC vs. AA/CA)	-30.263	-0.310	-50.947 to -9.578	0.005**
	Dominant (CA/CC vs. AA)	1.691	0.019	-19.925 to 23.307	0.876
	Additive (0, 1, 2 C alleles)	-10.604	-0.194	-23.711 to 2.503	0.111

B, unstandardized regression coefficient; β , standardized regression coefficient; CI, confidence interval. Models including TSH were adjusted for age, BMI, TSH, and FT3/FT4 ratio. Models excluding TSH were adjusted for age, BMI, and FT3/FT4 ratio. All analyses included 76 participants. The recessive model including TSH is presented in Table 4. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Regression diagnostics showed no evidence of substantial multicollinearity (variance inflation factors < 2). Visual inspection of the normal P–P plot and the standardized residuals-versus-predicted-values plot indicated generally acceptable residual behavior, although Case 47 was identified as a potential outlier with a standardized residual exceeding 4. Cook's distance remained below 1. In a sensitivity analysis excluding Case 47, the recessive model association remained statistically significant ($B = -27.369$, $\beta = -0.323$, 95% CI: -44.614 to -10.124, $P = 0.002$; $n = 75$).

4. Discussion

The goal of the present study was to analyze the distribution of DIO1 rs2235544 polymorphism and its association with thyroid hormone parameters and the current prescribed LT4 dose in Iraqi women with primary hypothyroidism.

The frequency of the A allele is in agreement with the study by Panicker *et al.* [21], who reported a frequency of the A allele of ~52.9% in the WATTS cohort of patients who were on T4 replacement treatment. However, the genotype distribution differed between the two populations, as the heterozygous CA genotype was the most common genotype in the WATTS cohort, whereas AA was the most common genotype in the present investigation. In contrast, Al-Ejilat *et al.* [19] reported a different distribution among Jordanian patients with hypothyroidism and stable TSH levels, in whom the CA genotype was the most frequent (42.1%), followed by CC (39.5%) and AA (18.4%). The C allele was also more frequent than the A allele in the same population (60.5% vs. 39.5%). Differences between the Iraqi and Jordanian cohorts may reflect ethnic and geographic variation, population genetic structure, differences in participant selection, or sampling variability. However, because the present study included only patients with hypothyroidism and lacked a healthy control group, the observed genotype distribution cannot establish an association between a particular genotype and susceptibility to hypothyroidism.

The distribution of the genotypes significantly deviated from the Hardy–Weinberg equilibrium ($\chi^2 = 6.10$, $P = 0.014$). This discrepancy could be due to the disease-specific sampling scheme, small sample size, population structure, selection variables or genotyping-associated factors. However, independent

re-genotyping of a randomly selected subset of samples using a separate real-time PCR system showed concordant genotype calls with the initial analysis, reducing the likelihood that the observed HWE deviation was solely attributable to genotyping error. Thus, the genetic relationships reported in the present investigation should be viewed with caution and need to be validated in larger independent populations.

In this study, a significant difference in BMI was observed among the DIO1 rs2235544 genotype groups. This finding suggests an association between DIO1 rs2235544 genotype and BMI in the current study population. The association between DIO1 rs2235544 and BMI has not been adequately established in earlier investigations. Panicker *et al.* [21] revealed that rs2235544 was highly related with the FT3/FT4 ratio, while the C allele was determined to be associated with a pattern consistent with enhanced DIO1 activity. But the authors state that a very high sample size would be required to fully detect the expected effect of this variation on BMI. Moreover, a study from Jordan by AlEjilat *et al.* [19] investigated BMI as one of the clinical characteristics of hypothyroid patients, and not the association of rs2235544 with BMI, but the LT4 dose required. Hence, the significant variation in BMI identified among the genotypes in the present study should be interpreted with caution and requires confirmation in larger populations.

A significant difference in daily LT4 dose among DIO1 rs2235544 genotypes indicated an association between rs2235544 genotype and the current prescribed LT4 dose in the present study population.

The present observation is consistent with earlier findings suggesting an association between DIO1 rs2235544 and LT4 dose requirements. Al-Ejilat *et al.* [19] identified rs2235544 as a predictor of LT4 dose requirements in patients with primary hypothyroidism. However, the direction of the genotype-dose association differed between the two studies. The observed association may be considered in the context of the established role of DIO1 in peripheral thyroid hormone metabolism. Panicker *et al.* [21] demonstrated an association of the C allele of rs2235544 with a larger FT3/FT4 ratio, higher FT3 and lower FT4, which is a hormonal pattern consistent with increased DIO1-mediated T4-to-T3 conversion. Nevertheless, the present observational findings cannot establish that rs2235544 itself directly caused the differences in the current prescribed LT4 dose.

BMI and LT4 dose differed significantly between the DIO1 rs2235544 genotype groups, although TSH, FT3, FT4 and the FT3/FT4 ratio did not. The lack of a strong correlation with TSH is in agreement with Panicker *et al.* [21], who showed that rs2235544 was more strongly linked with peripheral thyroid hormone conversion than circulating TSH concentrations. These findings may be consistent with a greater association of this variant with peripheral thyroid hormone metabolism than with circulating TSH concentrations. Nevertheless, the absence of significant differences in FT3, FT4, and the FT3/FT4 ratio is contrary to the findings reported by Panicker *et al.* [21], who found higher FT3 levels, lower FT4 levels, and an increased FT3/FT4 ratio in association with the C allele. The current results were also different in part from those of Al-Ejilat *et al.* [19], who reported the CC genotype as an independent predictor of T3 concentration in their multivariable analysis. Several factors may explain these differences, including the relatively small sample size of the present study, the ethnic and genetic differences between populations, variability in LT4 dose and treatment status, differences in thyroid disease severity, and other clinical characteristics of the recruited contributors [26, 27, 21].

The correlation analysis showed associations among the clinical and biochemical variables, as illustrated in the correlation heatmap. BMI was positively correlated with daily LT4 dose ($r=0.50$), indicating that a higher BMI was associated with a higher current prescribed LT4 dose. This finding is consistent with the established association between body size and LT4 dosing [28]. The heatmap also indicated a positive correlation between FT3 and the FT3/FT4 ratio ($r=0.644$) and an inverse correlation between FT4 and the FT3/FT4 ratio ($r=-0.497$). These relationships are expected because FT3 and FT4 are components of the calculated FT3/FT4 ratio, and should therefore be interpreted cautiously [29].

The findings suggest that the association between the CC genotype and lower current prescribed LT4 dose remained statistically significant after adjustment for BMI and the other variables included in the regression model. However, the standardized coefficient of BMI was greater than that of genotype, showing that body size remained substantially associated with the current prescribed LT4 dose in this

population (Table 4). The recessive coding employed in the regression is a statistical comparison and should not be regarded as definitive evidence that rs2235544 is biologically recessive [30, 31].

The additional genetic-model analyses showed that the association with the current prescribed LT4 dose was observed under the recessive model, whereas the dominant and additive models were not statistically significant. Because the genetic model was not prespecified, these findings should be considered exploratory. Importantly, the recessive model association remained statistically significant after excluding TSH from the regression model and in the sensitivity analysis excluding the identified outlier. These findings suggest that the observed association was not solely dependent on the adjustment for TSH or on a single influential observation; confirmation in a larger independent cohort is required.

The direction of this association differed from that reported by Al-Ejilat *et al.* [19], who found that the CC genotype was associated with a higher weight-adjusted LT4 dose. An important methodological difference is that the present study evaluated absolute LT4 dose ($\mu\text{g}/\text{day}$), whereas the Jordanian study evaluated weight-adjusted dose ($\mu\text{g}/\text{kg}/\text{day}$). This distinction is particularly relevant because BMI differed significantly among the genotype groups in the present cohort. Differences in population characteristics, treatment stability, adherence, and other clinical covariates may also have contributed to the contrasting findings [10]. In comparison, Santoro *et al.* [32]. reported no significant effect due to DIO1 rs2235544 on the T4 dose requirement in patients with differentiated thyroid cancer. However, a direct comparison should be made cautiously because their participants were thyroidectomized patients receiving TSH-suppressive therapy rather than conventional replacement therapy for primary hypothyroidism [33].

Taken together, the present findings suggest an association between DIO1 rs2235544 and the current prescribed LT4 dose in Iraqi women with primary hypothyroidism. However, the small sample size, deviation from the Hardy–Weinberg equilibrium, cross-sectional design, and differences from previous populations warrant caution when interpreting the findings. Individual body weight was not recorded as a study variable; therefore, the weight-adjusted LT4 dose ($\mu\text{g}/\text{kg}/\text{day}$) could not be calculated. The LT4 dose analyzed in this study represents the current dose prescribed by the treating physician documented in the participants' medical records. In addition, the exact disease duration, detailed disease etiology, quantitative adherence measures, and predefined duration of LT4 dose stability were not systematically recorded as analytical variables and therefore could not be included as covariates in the multivariable analysis. Although medication adherence, relevant medical history, and concomitant medication use were assessed during participant screening, residual confounding due to unmeasured clinical and treatment-related factors could not be excluded. The relatively small sample size should also be considered a limitation. Although a larger sample was targeted, recruitment was constrained by the limited number of eligible participants available during the predefined study period. Consequently, the final sample was comprised of 76 participants, which may have limited the statistical power when it comes to detecting moderate or small genetic effects. Larger independent studies incorporating weight-adjusted LT4 dose and more complete evaluations of the clinical variables are needed to confirm the observed association.

5. Conclusions

The present study identified an association between DIO1 rs2235544 polymorphism and the current prescribed LT4 dose in Iraqi women with primary hypothyroidism. Women carrying the CC genotype received lower than the current prescribed LT4 doses than AA and CA carriers, whereas no clear genotype-related differences were observed in the measured thyroid hormone parameters. BMI was also associated with the current prescribed LT4 dose. Given the cross-sectional design, relatively small sample size, deviation from the Hardy–Weinberg equilibrium, absence of weight-adjusted LT4 dosing, and the incomplete measurement of some clinical confounders, these findings should be considered exploratory and require confirmation in larger independent populations.

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