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PPARG and *FTO* Gene Variants and Their Association with Type 2 Diabetes in The Kurdish Population

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ABSTRACT

Type 2 Diabetes (T2D) is considered the most common form of diabetes; it develops when pancreatic cells produce an excess amount of insulin, which leads to insulin resistance by peripheral cells. T2D is an illness caused by interactions between genetics and environmental factors. Genetic factors are involved in the evolution of T2D, and several genetic variants increase the risk of developing T2D. The SNPs rs1801282 in the *PPARG* gene and rs9939609 in the *FTO* gene were found to be associated with increasing the risk of T2D in different populations. We aimed to discover if rs1801282 in the *PPARG* gene and rs9939609 in the *FTO* gene are responsible for increasing the risk of T2D in the Kurdistan population. In the current study, DNA from 200 unrelated samples (100 T2D and 100 non-diabetic control) individuals were genotyped using Allele-specific PCR for both SNPs. The PCR methods were validated by the Sanger sequencing method.

The association analysis for the rs1801282 variant (adjusted by sex, age, and BMI) showed significant differences between the case and the control groups; individuals with genotypes (GG and GC) had a higher risk of the disease (*p-value* = 0.0045, *OR* = 3.96, 95%CI: 1.31-11.94) than genotype (CC). On the contrary, there were no significant differences (*p-value*= 0.39) between the case and control groups for the rs9939609 variant.

Our finding suggested that the variant rs1801282 in the *PPARG* gene was a suspectable SNP in T2D in the Kurdish population, while SNP rs9939609 in the *FTO* gene was not associated with T2D. Further investigations with larger number of samples are required to validate our findings.

Keywords: T2D; Allele-specific PCR; Association study; SNPs; FTO; PPARG.

1. Introduction

More than 400 million people are affected by diabetes worldwide[1]. Almost 90% of the reported diabetic cases are Type 2 Diabetes (T2D)[2, 3]. T2D is a disorder in insulin usage by the body, characterized by increased blood glucose concentration and resistance to insulin[4]. Interactions between genetic, epigenetic, and environmental factors are powerful causes of T2D. In addition, family history is considered a risk factor. Environmental factors include obesity, lifestyle, weight at birth, stress smoking, and age[5, 6]. The strongest risk factor for T2D is increased fat cells[7, 8]. Obesity tends to be related to T2D, and is responsible of an increase in death rates around the world; losing weight in individuals that have prediabetes (early stages of T2D) can prevent the disease[9]. A healthy diet, regular exercise, maintaining a normal body weight, and quitting smoking can aid in preventing diabetes or delaying it[10].

Diabetes Mellitus is a fast-growing epidemic disease. The prevalence rates of diabetes around the world indicate that it is higher in the Middle East, which includes Kurdistan Region[11]. In 2018 the worldwide estimation of affected individuals with diabetes was 405.6 million, and this number is expected to increase to 510.8 million in 2030[12]. Furthermore, diabetes cause more than 1 million deaths per year, which makes diabetes the ninth causative reason of death[13].

Genetic factors play a significant role in the evolution of T2D and raise the risk of developing the disease. The risk of developing T2D for a person who has a parent with the disease is 40%, and this will increase to 70% if both parents are affected[14]. According to Genome-Wide Association Studies (GWAS), T2D is a polygenic disease that is susceptible to variations in many genes, such as those that are responsible for insulin secretion (*TCF7L2, CDKAL1, IGF2BP2.JAZF1, KCNQ1, HHEX/IDE),* and other genes that are responsible for insulin sensitivity

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(*PPARG* and *FTO*)[2, 15-17]. GWAS suggested that 63 % of T2D patients could be under the control of genetic mutations (variations) in the form of Single Nucleotide Polymorphisms (SNPs)[17].

The Peroxisome Proliferator-Activated Receptors gamma *(PPARs)*, also called NR1C3 (nuclear receptor subfamily 1, group C, member 3) is found in the adipose tissue, colon, and immune cells (macrophages)[18]. It regulates the transcription of various genes[19]. The *PPARG* is involved in the storage of fatty acid in adipose tissues and glucose usage inside the body and increases sensitivity to insulin by promoting the storage of fatty acid in adipose tissue and by releasing adiponectin from adipose tissue[20-22]. *PPARG* gamma is an important gene due to its effect on the evolution of diseases like T2D, obesity, cardiovascular diseases, and cancer[23]. The SNP in the *PPARG* gene results in a switching of proline to alanine at codon 12[24]. This exchange is called (Pro12Ala) rs1801282, and this mutation was reported to be linked with insulin resistance and a higher risk of T2D[25].

Fat mass and obesity-associated protein *(FTO)* is an enzyme controlled by the *FTO* gene found on chromosome 16[26]. Expression of the *FTO* gene has been found to be linked with food intake and development of obesity[27, 28]. GWAS studies have confirmed that *FTO* gene variants are related to obesity and T2D[29-30]. The rs9939609 SNP occurs in the intronic region of the *FTO* gene that, leads to obesity, and increases the risk of having T2D[32]. The SNP rs9939609 was associated with high levels of fasting insulin, sugar, triglyceride, and lowe the high-density lipoprotein (HDL)[33]. Around 16% of individuals with the rs9939609 variant are found to be weighing 3 kilograms more when compared with individuals without the variant[34].

Genome-wide association studies showed that the pathogenesis of T2D is associated with variations in *PPARG* and *FTO* genes; both SNPs rs1801282 and rs9939609 are considered as an increased risk factor for T2D in different populations[26, 32]. It is very important to study the genetic background of patients with T2D to understand the risk factors for this illness and identify the frequency of each SNP in the Kurdistan population. The effect of these genetic variants on T2D is different among different populations, hence this study aimed to discover if rs1801282 in the *PPARG* gene and rs9939609 in the *FTO* gene are associated with the risk of T2D in the Kurdistan population.

2. Materials and methods

2.1 Sample collection

In this study, 100 unrelated patients with T2D and 100 non-diabetic controls who visited two laboratories in Erbil city (Smart Lab and Pharma Lab) were enrolled. Venous blood samples were collected and stored at -80ºC deep freezer for DNA extraction. The patients and controls were selected depending on the HbA1c, FBS, insulin, and clinical history of the patients. The characteristics and biochemical tests of the collected samples were also recorded (data not shown), including mean of (Age ±51.05, BMI ±30.01, insulin ±22.55, RBS ±145.2, HbA1c ±6.81).

2.2 DNA Extraction and Genotyping by Allele-Specific PCR

DNA samples were extracted from 200µl of whole blood using an available commercial kit, according to the manufacturer's instructions (WizPrep™ gDNA Mini Kit (Blood); WizBio Solutions, Korea) and stored at -80ºC. To evaluate the quantity and quality of the extracted DNA, 1µl of samples were determined using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All the samples were genotyped using allele-specific PCR for two SNPs, rs1801282 in the *PPARG* gene and rs9939609 in the *FTO* gene. For the rs1801282 SNP, we have designed four primers using Tetra ARMS - PCR Primer Design Tool[36]. Two outer and two inner primers, the forward outer primer S11-3'-CTCCTAATAGGACAGTGCCAGCCA-5' and reverse outer primer S12-3'-TTTAAATGAACGCGATAGCAACGAG-5', were used as a control with a product size of 453bp. The forward inner primer S09- 3'-GAAACTCTGGGAGATTCTCCTATTGTCC-5' worked with the reverse outer primer (S12) and gave a product size of 238 bp for the (C) allele, and the reverse inner primer S10- 3'-ATCAGTGAAGGAATCGCTTTCAGC-5' worked with the forward outer primer (S11) and gave a product size of 267 bp for the (G) allele. The PCR mixture contained 10µl master mix (Add *Taq* Master; Add bio, Korea), 1µl of each primer (10µM), 1µl DNA sample (10ng/µl), and sterilized distilled water was added to obtain a total volume of 20 µl. The PCR steps were an initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 1 minute, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes.

The SNP rs9939609 in the *FTO* gene was genotyped similarly to rs1801282 by using allele-specific PCR. We have designed four primers using Tetra ARMS - PCR Primer Design Tool[36], two outer and two inner primers. The forward outer primer S07-3'-TTCCAGTCATTTTTGACAGCATGG-5', and reverse outer primer S08-3'- AGCCCAAGGATGGTGTTTCTAAGG-5' were used as a control with a product size of 473bp. The forward inner primer S05-3'-TCCTTGCGACTGCTGTGAATCTT-5' worked with the reverse outer primer (S08) and gave a product size of 242bp for the T allele, and reverse inner primer S06-3'AACAGAGACTATCCAAGTGCATCGCT5' worked with forward outer primer (S07) and gave a product size of 280bp for the A allele. The PCR mixture was similar to the rs1801282 PCR mixture. Conditions of the PCR reactions were an initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 1 minute, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. All the PCR products were resolved on agarose gel electrophoresis, stained with safe stain (Add bio, Korea), and visualized with a UV transilluminator.

2.3 DNA sequencing

Samples from both SNPs were selected for sequencing by the Sanger method (Applied Biosystems 3730xl). For rs1801282 in the *PPARG* gene, four samples with different genotypes were sequenced, including two heterozygotes (CG), one homozygote (CC), and one homozygote (GG). Similarly, for SNP rs9939609 in the *FTO* gene, two heterozygous (TA), one homozygous (AA), and one homozygous (TT) were sequenced.

2.4 Statistical analysis

The FBS, HbA1c, insulin, and BMI data were obtained from (Pharma and Smart Labs) in Erbil city. The genotype and allele frequencies were calculated by the SNPStats web tool[39]. Hardy-Weinberg equilibrium (HWE) was assessed for T2D individuals and control groups by using Chi-square test[37, 38]. The Logistic regression model was used for the association study between each SNP and T2D in the case and control groups. To analyze allele-specific risk, the Odd Ratios (OR) with associated 95% confidence intervals (CIs) were assessed. To choose the best mode of inheritance for the SNPs, the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were assessed[39].

3. Results and Discussions

3.1 Samples characteristics

In the current study, a total of 200 samples were enrolled, including T2D (49% males and 51% females) and non-diabetic controls (52% male and 48% females). The characteristics and biochemical tests of the collected samples were as follows: including the mean of (Age ±51.05, BMI ±30.01, insulin ±22.55, RBS ±145.2, HbA1c ±6.81).

3.2 Genomic DNA

Genomic DNA was extracted from 200µl of whole blood samples in EDTA tubes using a DNA extraction kit (WizPrep™ gDNA Mini Kit (Blood); WizBio Solutions, Korea). The extracted DNA concentration ranged from 8.5-346 ng/µl, and the purity ranged from 1.79-2.1, determined by NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The concentrations were unified to 10ng/µl.

3.3 Allele-Specific PCR and DNA sequencing

All the samples were subjected to allele-specific PCR (ARMS-PCR) for both variants (rs1801282 and rs9939609). The PCR products of rs1801282 (in the *PPARG* gene) were resolved on agarose gel electrophoresis to identify the heterozygous (CG) and homozygous (GG/CC) genotypes. All the samples were genotyped successfully for SNP rs1801282 (figure 1). Similarly, the PCR products of rs9939609 in the *FTO* gene were resolved by agarose gel electrophoresis to identify the heterozygotes (AT) and homozygotes (AA/TT) genotypes (figure 2). To validate the accuracy of the PCR method used for genotyping rs1801282 in the *PPARG* gene, 4 PCR products were sequenced by the Sanger sequencing method (Applied Biosystems 3730xl). Samples 145 and 79 were heterozygote CG, sample 150 was homozygous GG, and sample 188 was homozygous CC. Similarly, to validate the accuracy of the PCR method used for genotyping rs9939609, 4 PCR products were sequenced by the Sanger sequencing method (Applied Biosystems 3730xl), and the genotypes of samples number 116, 113, and 112 were TA, AA, and TT, respectively; and the genotype of sample 117 was TA. All the sequencing results matched our PCR reaction products (Figure 3).

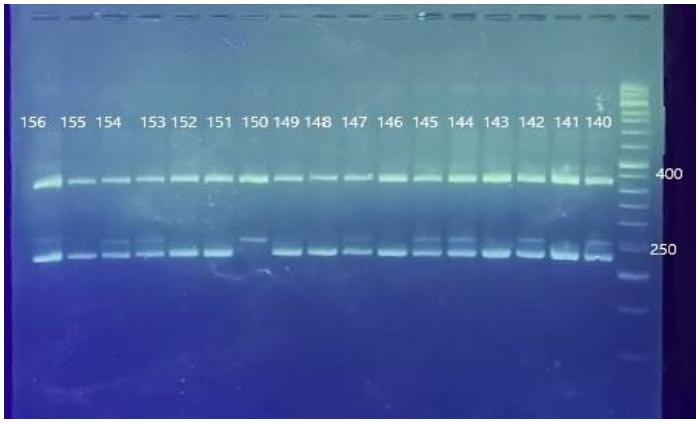

Figure 1: PCR Products of rs1801282, shows three genotypes GG, CC, and CG. The product size for the C allele is 238bp, and for the G allele is 267bp. The outer primers product size is 453 bp was used as a control. The first lane is a 50 bp ladder. Sample 150 is homozygous for GG (risk allele), Samples 141-143-146-148-149-151-152-155 were homozygous CC, and samples 140-142-144-145-147-153-154-156 heterozygous CG. The PCR products were resolved on 3% agarose, and ran for 1 hour at 95 volts.

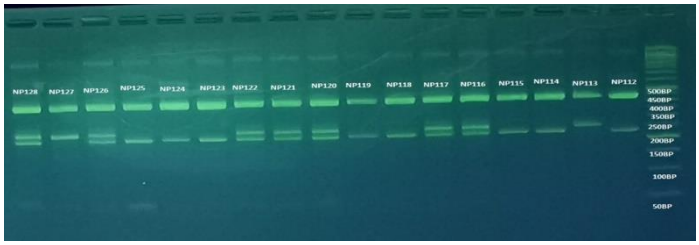

Figure 2: PCR products rs9939609 (FTO gene) shows three genotypes TT, TA, and AA. The product size for the T allele is 242 bp, and for the A allele 280 bp. The outer primers product size is 473 bp. The first lane is a 50 bp ladder. Samples number113 and 127 homozygous AA, samples number 116, 117, 120, 121, 122, 126 and 128 were heterozygous TA., finally samples number 112, 118, 119, 123, 124 and 125 were homozygous for TT allele. The PCR products were resolved on 2.5% agarose, and ran at 110 volts for 1 hour.

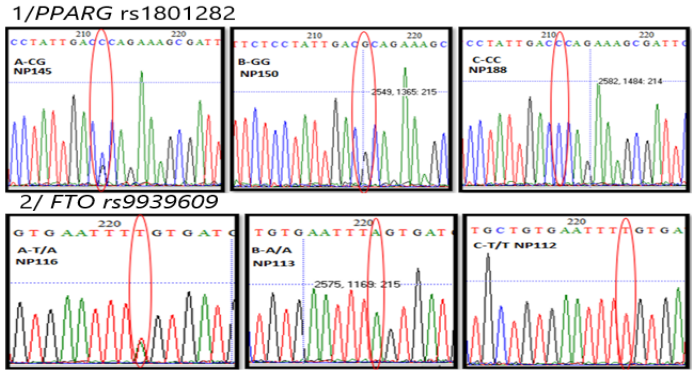

Figure 3: PPARG rs1801282, Sanger sequencing traces of three genotypes matched the PCR method. (A) shows sample NP145 in which the genotype is CG, (B) shows sample NP150 in which the genotype is the risk alleles GG. (C) shows NP188 in which the genotype is CC. All three genotypes are indicated by red circles. 2/FTO rs9939609, Sanger sequencing traces of three genotypes that matched the PCR method, (A) shows sample NP116 in which the genotype is TA, (B) shows sample NP113 in which the genotype is the risk alleles AA, and (C) shows NP 112 in which the genotype is TT. All three genotypes are indicated by red circles.

3.4 Genotype and allele frequencies

Allele and genotype frequencies for SNPs rs1801282 and rs9939609 are listed in (Table 1). The genotype frequency for SNP rs9939609 was in Hardy-Weinberg Equilibrium (HWE) for case and control groups (P>0.05). The genotype frequency of SNP rs1801282 was in HWE for the cases (P> 0.05), while there was a slight deviation from the HWE in the control group (*p-value* = 0.039).

Table 1: Genotype and allele frequencies of SNPs rs1801282 and rs9939609 in the case and control groups. The p-value of Hardy Weinberg Equilibrium of both SNPs is also listed in the table.

z	Genotype (n (%))			Allele (n (%))		HWE (*P-value*)
SNP rs1801282	C/C(freq)	CG(freq)	GG(freq)	C(freq)	G(freq)	
All status	180 (0.9)	17 (0.08)	3 (0.02)	377 (0.94)	23 (0.06)	0.019
Case	96 (0.96)	4 (0.04)	0 (0)	196 (0.89)	4 (0.02)	1
Control	84 (0.84)	13 (0.13)	3 (0.3)	181 (0.9)	19 (0.1)	0.039
SNP rs9939609	T/T (freq)	T/A (freq)	A/A (freq)	T (freq)	A (freq)	
All status	76 (0.38)	85 (0.42)	39 (0.2)	237 (0.59)	163 (0.41)	0.11
Case	37 (0.37)	43 (0.43)	20 (0.02)	117 (0.58)	83 (0.42)	0.3
Control	39 (0.39)	42 (0.42)	19 (0.19)	120 (0.6)	80 (0.4)	0.22

3.5 Association analysis of SNPs rs1801282 and rs9939609 with T2D

In the current study, the association of both SNPs with T2D was conducted using logistic regression models. For each SNP, the association test was adjusted by covariates (sex, age, and BMI). Table (2 and 3) shows the association analysis results for SNPs (rs1801282 and rs9939609) before and after adjustment by the covariates. Different modes of inheritance (co-dominant, dominant, recessive, over-dominant, and log-additive) were compared together. To choose the best mode of inheritance that fit the data compared to the most common one (co-dominant), the lowest AIC (Akaike information criterion) and BIC (Bayesian information criterion) were used[40]. The best mode of inheritance for SNP rs1801282 was the log-additive model after adjustment by sex, age, and BMI. On the other hand, for the SNP rs9939609, the best mode of inheritance after adjustment by same covariates was over-dominant. The association analysis for SNP rs1801282 showed significant differences between the case and control groups (*p-value*=0.0045), (*OR*=3.96, 95%*CI*:1.31-11.94). However, there were no significant differences between the case and control groups for the SNP rs9939609 (*p-value*= 0.39, *OR* = 0.77, 95%*CI*: (0.42-1.40).

Table 2: Different mode of inheritance model, analysis for the SNP rs1801282 in PPARG gene between T2D and control groups.

				Before adjustment by (Age, Sex, and BMI)		After adjustment by (Age, Sex, and BMI)	
Mode of inheritance	Genotype	Case	Control	Or (95%CI)	p-value	Or (95%CI)	P-value
Co-dominant	C/C C/G G/G	96% 4% 0%	84% 13% 3%	1 3.71 (1.17-11.83)	0.0068	1 3.52 (1.07-11.54)	0.015
Dominant	C/C C/G- G/G	96% 4%	84% 16%	1 4.57 (1.74-14.21)	0.0035	1 4.42 (1.33-13.52)	0.0072
Recessive	C/C-C/G G/G	100% 0%	97% 3%	1 NA (0.00-NA)	0.04	1 NA (0.00-NA)	0.062
Over-dominant	C/C- G/G C/G	96% 4%	87% 13%	1 3.59 (1.13-11.41)	0.02	1 3.41 (1.04-11.18)	0.03
Log-additive	-------	--------	--------	4.22 (1.44-12.35)	0.002	3.96 (1.31-11.94)	0.0045

Table 3: Different mode of inheritance models analysis for the SNP rs9939609 in FTO gene between T2D and control groups.

				Before adjustment by (Age, Sex, and BMI)		After adjustment by (Age, Sex, and BMI)	
Mode of inheritance	Genotype	Case	Control	OR (95%CI)	P-value	OR (95%CI	P-value
Co-dominant	T/T T/A A/A	37% 43% 20%	39% 42% 19%	1 0.93 (0.50-1.72) 0.90 (0.42-1.95)	0.96	1 0.74 (0.38-1.43) 0.89 (0.39-2.00)	0.66
Dominant	T/T T/A-A/A	37% 63%	39% 61%	1 0.92 (0.52-1.63)	0.77	1 0.78 (0.43-1.44)	0.43
Recessive	T/T-T/A A/A	80% 20%	81% 19%	1 0.94 (0.47-1.89)	0.86	1 1.04 (0.50-2.16)	0.91

Over-dominant	T/T-A/A T/A	57% 43%	58% 42%	1 0.96 (0.55-1.68)	0.89	1 0.77 (0.42-1.40)	0.39
Log-additive	-------	------	-------	0.95 (0.65-1.38)	0.77	0.91 (0.61-1.36)	0.64

3.6. Discussion

This study aimed to identify the association of variants rs1801282 and rs9939609 (in *PPARG* and *FTO* genes, respectively) with T2D in Kurdistan Region/Iraq. For this purpose, 200 samples were enrolled, 100 were T2D, and 100 were non-diabetic controls. The analysis demonstrated that the variant rs1801282 was associated with the risk of T2D. Furthermore, under the control of the log-additive model GG genotype has double the risk of the heterozygous GC genotype; nevertheless, rs9939609 was not associated with the risk of T2D in the Kurdistan Region.

A robust and accurate genotyping method is an important step to proceed with case-control association studies; one way to identify the genotyping error is to test for HWE in the study samples, specifically the control groups[41]. The case and control groups for SNP rs9939609 were in HWE, indicating an accurate genotyping method[42]. However, the Chi-square test for SNP rs1801282 showed a slight deviation from the HWE in the control group (*p-value* =0.039), which might indicate a loss of heterozygosity. Nevertheless, this possibility was excluded in the current study for several reasons. First, the p-value threshold for a SNP that is not in HWE ranges between 0.001- 5.7 x 10^{-7}[43, 44]. Second, duplicate samples were genotyped, and all gave the same results. Third, the PCR methods for both variants were validated using the Sanger sequencing method (see figure 3 and figure); all the sequenced samples were consistent with the genotyping method by the allele-specific PCR. Finally, the error rate in this study is very low, in addition to the fact that our sample size is small; hence it is not an indication of a genotyping error in our study[45].

In this study, both SNPs were selected and analyzed in the Kurdish population due to their importance in increasing the risk of T2D in different populations worldwide[35, 46-48]. The association analysis in this study demonstrated that the SNP rs1801282 was associated with the risk of T2D (*p-value* = 0.0045, OR= 3.96, 95%CI1.31-11.94). Our analysis indicated that the rs1801282 allele increases the risk of T2D in the Kurdistan population, similar to Hong Kong, Indian, Pakistani and Finnish populations[49-52]. On the other side, there were few studies that concluded that rs1801282 is not associated with T2D, such as a study in the South India population, due to their small sample size[53]. As well as a study conducted in the Bangladesh population showed no association between rs1801282 and T2D due to the small sample size used in their study, lack of research funds, and insufficient laboratory facilities[54]. For the SNP rs9939609, our analysis showed no significant differences between the cases and control groups (*p-value*= 0.39). Similar to east Asian populations[53]. While several studies found that rs9939609 was associated with the risk of T2D in different populations[54-56]. The lack of association between rs9939609 in the *FTO* gene and T2D in our study might be due to the small sample size, or this variant does not affect the risk of T2D in the Kurdish population due to different genetic background than the other studied populations. These discrepancies in the relations between SNPs and disease among different populations might be due to the diversity in the genetic background of each population and exposure to different environmental factors[57].

Conclusion

In conclusion, this study showed that the variant rs1801282 in the *PPARG* gene increases the risk of T2D in the Kurdish population. Whereas the variant rs9939609 in the *FTO* gene was not associated with T2D in the Kurdish population. Further analysis is required with a larger number of samples to clarify the effect of these two variants in the Kurdish population.

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Conflict of interests

The authors declare no conflict of interest.

Author contributions

Study design Nzar A. A. Shwan; Sample and data collection Sally K. Baqer; Both authors contributed to the analytical tool, methodology, and data analysis. Both authors contributed the manuscript preparation.

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