

## Association of interleukin-6 level and DNA polymorphism in type 2 diabetic patients in Kirkuk city

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### ABSTRACT

The basic inflammatory factor interleukin-6 plays an important role in the inflammatory response. It has been shown that IL-6 has a role in insulin resistance and T2DM. The IL-6 gene is found on chromosome 7p15-p21 in humans, it works via a receptor composed of two components, the IL-6 receptor, and gp130. Single nucleotide polymorphisms in the regulatory areas of IL-6R genes influence their expression levels and are linked to a higher risk of T2DM. There were 80 T2DM patients and 80 healthy people enrolled in the present work. Fasting blood glucose levels, HbA1C, and IL-6 concentrations were estimated in both groups. The allelic frequencies of 2 single nucleotide polymorphisms (rs2229238, rs4845625) were identified by PCR-SSP. In this study, we found that the fasting blood sugar levels, HbA1c, and IL-6 levels were statistically highly significant in patients in comparison with controls. In regarding the association of genotypic (CC, CT, TT) and allelic frequencies of rs2229238, and rs4845625 polymorphism of the gene IL6R, and prevalence of diabetes mellitus. The results of PCR-SPP products of rs2229238 (C/T) of the IL6R gene indicated the significant correlation the rs4845625 IL6R polymorphism with T2DM, where no significant association was observed between these genotypes (CC, CT, TT) and allelic (T) frequencies of rs2229238 as a risk factor and T2D.

**Keywords:** Single nucleotide polymorphism, IL-6R gene, rs2229238 C/T, rs4845625 C/T, T2DM

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### 1. Introduction

Diabetes mellitus is a common illness that causes high morbidity and mortality across the world [1]. Diabetes affects about 400 million people worldwide [2]. Diabetes can cause long-term deterioration, and dysfunction in several organs; eyes retinopathy, renal disease, Diabetes-related peripheral neuropathy, diabetic foot difficulties, as well as cardiovascular disorders[3, 4]. T2DM diabetes, which represents over 90% of all diabetes occurrences globally, has been characterized by inadequate insulin or maybe a malfunction of this hormone, or its receptor [5, 6]. The pathophysiology of T2DM has been linked to prolonged inflammation as well as immune system response. T2DM patients had greater levels of inflammatory markers such as interleukin-6 (IL-6), plasminogen activator inhibitor-1, tumor necrosis factor- $\alpha$ , intercellular adhesion molecule-1, and C reactive protein [7, 8]. Insulin resistance is influenced by genetics [9]. Genetics is thought to be responsible for 30–70 percent of the risk of T2DM. The Interleukin-6 gene is one of the potential cytokine genes that are involved in the pathogenesis of T2DM. The IL-6 gene is found on chromosome 7p15-p21, which has seven exons, spans around 12.8 kb of genomic DNA, and is responsible for the formation of these cytokines [10]. The rs1800795 (also known as -174G/C) was first identified as a frequent single nucleotide polymorphism in the promoter of IL-6 [11]. Single nucleotide polymorphisms (SNPs) in the regulatory regions of genes can influence the inflammatory cytokines release [12, 13]. Many research has looked into the link between IL-6, TNF- $\alpha$ , and IL-10 gene variations, as well as metabolic disorders [14, 15]. IL-6 functions through the receptor assembly made up of two active proteins found in membranes; a ligand-binding IL-6R of and a signaling transducer of gp130 [16]. Normal serum and synovial fluids include soluble and active versions of the IL-6R's subunit, that created



by variable splicings of the IL-6R mRNA and protein breakdown. The majority of IL-6 activity may be regulated by membrane bound IL-6Rs. In several investigations, IL-6R gene variations have been related to metabolic syndrome, insulin sensitivity, and the risk of diabetes [17]. There are few researches, employed molecular methods which describe the entire IL-6R gene diversity, and the findings were inconsistent among communities. Furthermore, There is less information available regarding how IL-6R mutations change circulating IL-6 levels [18, 19]. Single nucleotide polymorphisms of the IL-6 gene may affect the amount of the IL-6 protein produced. Numerous SNPs of the IL-6 gene have been discovered in the promoter, including rs1800797, and rs1800795. Two single-nucleotide polymorphisms (rs4845625 and rs2229238) were related to a variety of immune-related illnesses, including type 2 diabetes, cardiovascular disease, chronic renal failure, and sub-clinical atherosclerosis [20, 21]. The impact of IL6R polymorphisms on possibility of developing type 2 diabetes is poorly defined in Iraqi people. In the current study, we evaluate if there were any relationships between interleukin-6 receptor (IL-6R) genes polymorphisms (rs2229238 C/T and rs4845625 C/T) and type 2 diabetic Mellitus in Kirkuk city.

## 2. Material and methods

Eighty diabetes patients, with 80 healthy individuals were included in this study. The age in patients and controls varied from 28-70, 33-68 years respectively. The patients were selected from the diabetes mellitus unit/Azadi teaching hospital/Kirkuk city during October 2018 to March 2019. Fasting blood glucose levels, and HbA1C were estimated in both groups. Five milliliters of venous blood were taken from patients and controls. Three milliliters of the obtained blood were kept at room temperature for 30 minutes, and the sera were separated and stored at -20 °C for estimation of IL-6 concentration. The remaining 2 ml blood samples were placed in an anti-coagulant tube containing sodium citrate, and the buffy coat was collected following centrifugation of whole blood at 1500 rpm for 10 minutes and plasma removal. The buffy coat was then kept in 70% ethanol at -20 °C for genetic analysis. Fasting blood glucose was determined according to the glucose oxidase method (Giese® Diagnostics, Italia) with GenoTEK Chemistry Analyzer. The cases of diabetes mellitus were defined as Fasting blood glucose  $\geq 126$  mg/dl. HbA1c measurement was performed using Giese® Diagnostics kit.

### 2.1. Determination of IL-6 concentration in serum by ELISA

Serum IL-6 levels were determined using a commercially available ELISA kit (Quantikine® ELISA, Catalog No. D6050, R&D SYSTEMS a biotechne brand, USA). The assay was performed according to the manufacturer's guidelines.

### 2.2. Genomic DNA Extraction protocols

In the current study, a blood DNA extraction kit (Jena Bioscience, Germany) has been used to extract genomic DNA from buffy coats. After DNA extraction, the DNA concentration was estimated by UV spectrophotometric absorbance at 260 nm, then the eluted DNA was stored at -20 °C for the PCR method. Furthermore, samples were subjected to gel electrophoresis using 1% agarose to check for genomic DNA fragments between 500 - 23,000 bp (28).

## 3. PCR amplification protocols

### 3.1. Single nucleotide polymorphisms genotyping

For genotyping the SNP of interleukin-6 receptor (IL-6R) genes, 2 single nucleotide polymorphisms rs2229238 (C/T) and rs4845625 (C/T) were used in this study, the sequence-specific primers used in PCR-SSP listed in table 1.

Table 1. Set of specific primer sequences used in PCR-SSP

Primers	Sequence 5'→3'	T <sub>m</sub> °C	Size (bp)
rs2229238	Fw: CCTGGACCCTGTGGATGTC	58	321
	Fm: CCTGGACCCTGTGGATGTT	58	
	R: AGCAGCTTCTCCACACCGA	58	
rs4845625	Fw: GGAACCAGCATAACCAGTCTC	60	234
	Fm: GGAACCAGCATAACCAGTCTT	60	
	R: AGTTCTGGAGCTACCTCCTC	60	

The amplification processes were carried out in 20µl that contain; 2µl DNA template, 0.5µM/primer, 4µl of hot start red load, and 5x Master Mix (3.5mM MgCl<sub>2</sub> final concentration), with distilled water, was filled to a capacity of 20 microliters.

The PCR settings were as follows: a 2-minute initial incubation at 95 °C, after that 29 cycles of reaction, denaturation for 20 seconds at 95 °C, annealing for 45 seconds at 59 °C, and elongation at 72 °C for 1.5 minutes, then final extension for 5-minute at 72 °C. A 1.7 percent agarose gel was then used for the evaluation the PCR products for amplification.

### 3.2. Statistical analysis

The data was statistically analyzed using the SPSS Software 22. (SPSS, Chicago, IL, USA). The Student's t-test was used to compare variables between the two groups. P<0.05 has been used as a statistical level of significance. The odds ratio (OR), and 95 percent confidence intervals based logistical regression method, were used to examine the relationship of rs2229238 and rs4845625 with T2D. P≤ 0.05, is regarded statistically for being significant in all the tests.

## 4. Results

Eighty T2DM patients and 80 healthy individuals involved in this work, the mean age ± SD of patients and controls were 53.88±11.49 and 50.30±10.62 respectively, which is statistically insignificant. In this study, we evaluated fasting blood sugar levels, HbA1c, and IL-6 levels in both patients and controls. The mean value of fasting blood sugar in patients (169.83±19.05mg /dl) was significantly higher (P = 0.000) when compared with a control group (90.5±4.94 mg /dl). Regarding HbA1c value, the mean value of HbA1c in diabetes mellitus patients (9.77±1.40) was statistically highly significant (P = 0.000) than the control group (4.16±0.69). Findings of this study indicated high significant difference (P = 0.000) in the mean value of serum IL-6 in diabetes mellitus cases (7.21±5.20 pg/ml) than the control group (1.94±1.23 pg/ml), as shown in Table (2).

Table 2. Comparison of age, fasting blood sugar, HbA1c and serum IL-6 between the Diabetes Mellitus cases and controls.

Parameters	Diabetes (n = 80) (mean ± SD)	Control (n = 80) (mean ± SD)	P- value
Age (in years)	53.88±11.49	50.30±10.62	0.249
Fasting Blood Sugar (mg/dl)	169.83±19.05	90.5±4.94	0.000
Hb A1c (%)	9.77±1.40	4.16±0.69	0.000
IL-6 (pg/ml)	7.21±5.20	1.94±1.23	0.000

### 4.1. Single-nucleotide polymorphism (SNP) genotyping

In the present study, we screened the association of genotypic (CC, CT, TT) and allelic frequencies of rs2229238, and rs4845625 polymorphism of the gene IL6R, and prevalence of diabetes mellitus. The results of PCR products of rs2229238 (C/T) of the IL6R gene revealed that genotype frequency of CC, CT, and TT among diabetic patients was 72.5 %, 21.25 %, and 6.25 % respectively while in the control group was as follow: 78.75 %, 17.5%, and 3.75 respectively (Figure 1, 2, Table 3). Statically here have been no substantial differences in genotype (CC, CT, TT), and allele (C, T) dispersion of rs2229238 between patients and controls, and no association was observed between these genotypes (CC, CT, TT) and allelic (T) frequencies of rs2229238 as a risk factor and T2D, as shown in Figure 1, 2, and Table 3.

In regarding to frequency distribution of rs4845625 (T/C) genotypes of the IL6R gene in patients and controls, these results of the current research shown that the frequencies of TT, TC, and CC genotypes in T2DM patients were: 45 %, 30 %, and 25 % where in controls were: 61.25 %, 17.5 %, and 21.25 % (Table 3, Figure 1, 2,). There were statistical differences in the distribution of TC genotype (OR:2.333, 95% CI:1.062-5.126, P-value:0.0349). The results of TC+CC of rs4845625 showed significant association differences between groups (OR:1.932, 95% CI:1.029-3.626, P-value:0.0404). Furthermore, there were considerable differences identified in regarding to allele C distribution between diabetic group and control group (1.621, 95% CI:1.022-2.572, P-value: 0.0402). These findings revealed the relationship of rs4845625 variation of IL6R with type 2 diabetes mellitus (Table 3, Figure 3). These results showed that here have been significant variations in distribution of genotype TC+CC, TC, and allele C of rs4845625 polymorphisms between patients and controls indicating that rs4845625 polymorphisms is risk factor for type 2 diabetics.

Table 3. Distribution of IL6R - Polymorphism genotypic and allelic patterns in diabetic and control individuals.

IL-6R Polymorphisms	T2DM, No. (%)	Control. No. (%)	OR (95%CI)	P. value	z statistic
rs2229238 (C/T) Genotype					
CC	58 (72.5 %)	63 (78.75 %)			
CT	17 (21.25 %)	14 (17.5 %)	1.319(0.597-2.9126)	0.4934	0.685
TT	5 (6.25 %)	3 (3.75 %)	1.81(0.414-7.914)	0.4303	0.789
CT+TT	22 (27.5 %)	17 (21.25 %)	1.406(0.679-2.907)	0.3583	0.909
Allele					
C	133 (83.13 %)	140 (87.5 %)			
T	27 (16.87 %)	20 (12.5%)	1.4211(0.761-2.655)	0.2705	1.102
rs4845625 (T/C) Genotype					
TT	36 (45 %)	49 (61.25 %)			
TC	24 (30 %)	14 (17.5 %)	2.333(1.062-5.126)	0.0349	2.11
CC	20 (25 %)	17 (21.25 %)	1.601(0.737-3.482)	0.235	1.188
TC+CC	44 (55 %)	31 (38.75 %)	1.932(1.029-3.626)	0.0404	2.05
Allele					
T	94 (58.75 %)	112 (70 %)			
C	66 (41.25 %)	48 (30 %)	1.621(1.022-2.572)	0.0402	2.052

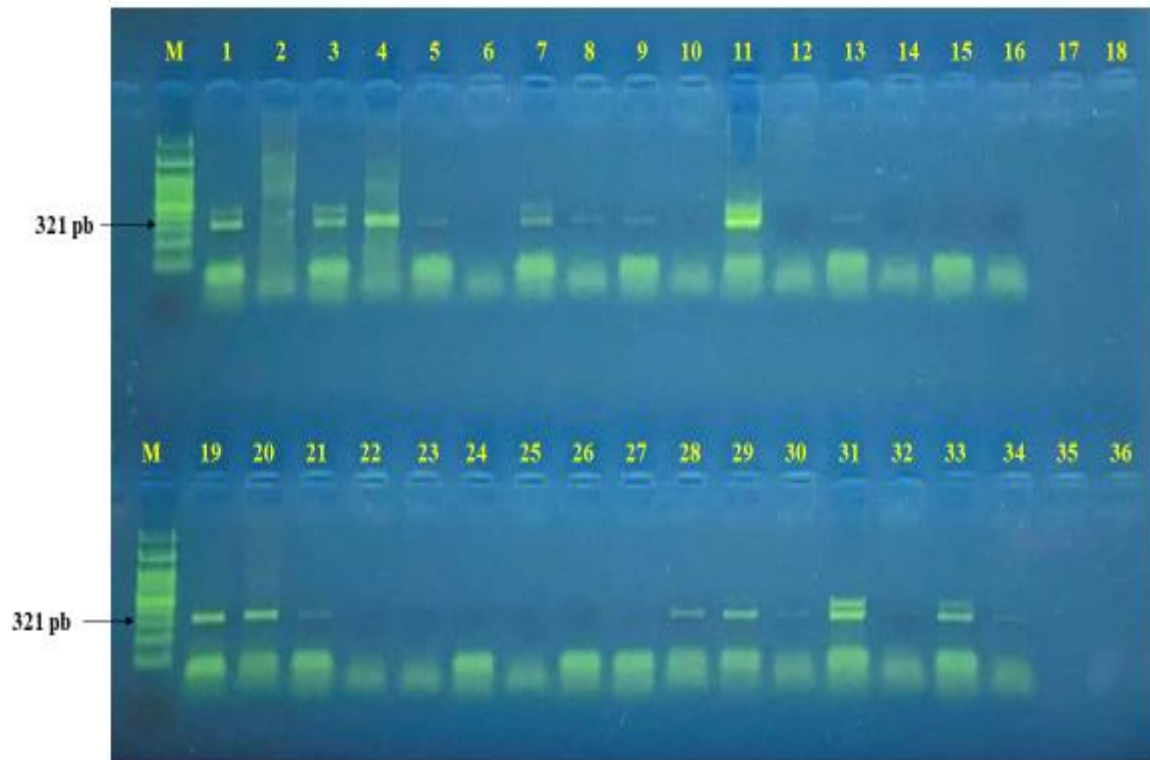


Figure 1. Gel electrophoresis showing PCR product for rs2229238 gene amplicons (321 bp) using agarose gel (1.7 %). Lane 1-36 are diabetic DNA samples and Lane (M) is DNA ladder = 100 base pair.

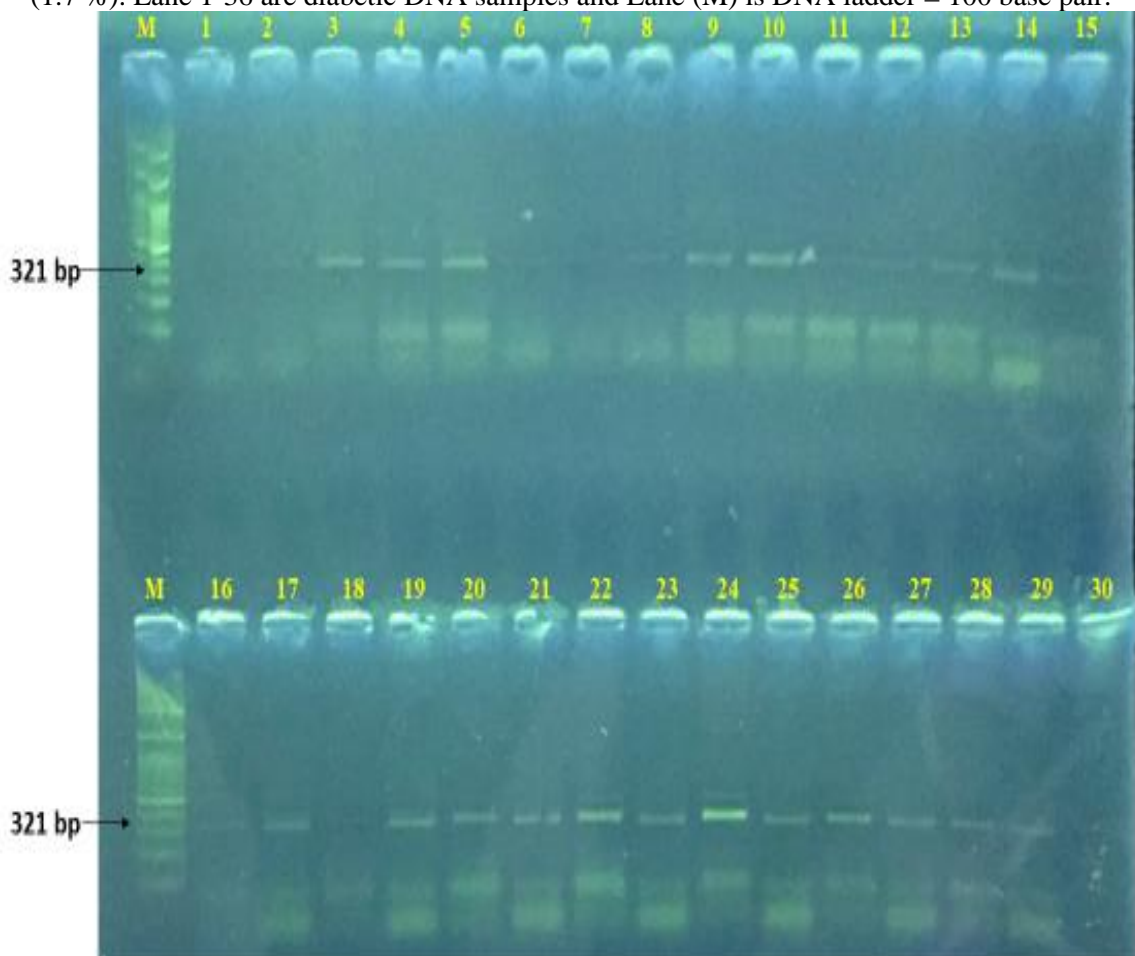


Figure 2. Gel electrophoresis showing PCR product for rs2229238 gene amplicons (321 bp) using agarose gel (1.7 %). Lane 1-30 are DNA samples in control group and Lane (M) is DNA ladder = 100 base pair.

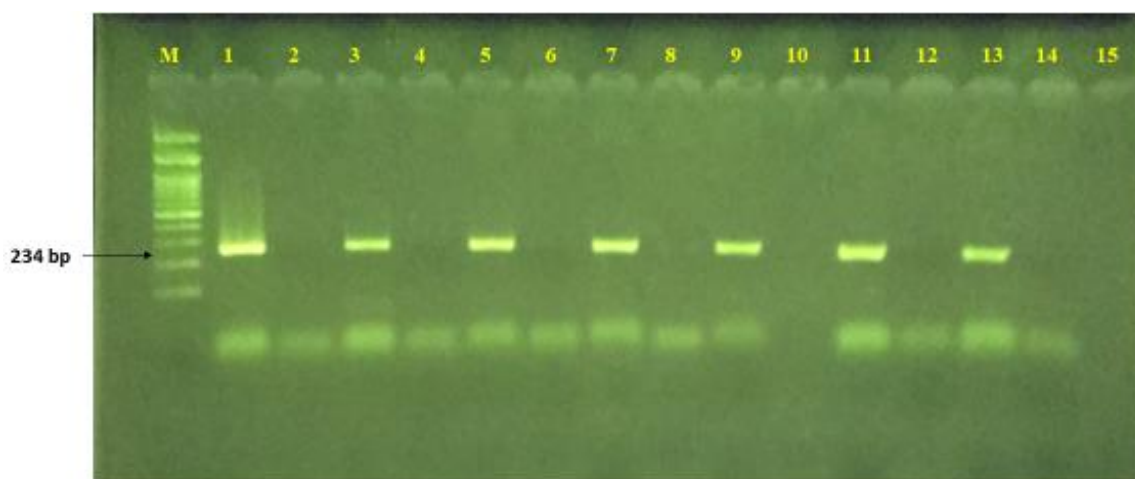


Figure 3. Gel electrophoresis showing PCR product for rs4845625 gene amplicons (234 bp) using agarose gel (1.7 %). Lane 1-15 are diabetic DNA samples and Lane (M) is DNA ladder = 100 base pair.

## 4.2. Discussion

T2DM is a complex disease collection of hyperglycemia-related metabolic diseases caused by either absolute insulin shortage or a decline in insulin biological activity [22]. This study reported that there was a highly significant increase in fasting blood sugar (FBS) in T2DM patients in comparison to the controls, these results are agreement with previous studies [23, 24]. An elevation in fasting blood sugar is an indicator of diabetes includes biochemical changes. T2DM patients suffer chronic hyperglycemia as a result of diminished sensitivity of the tissues to the effect of insulin or resistance to insulin. The resistance to insulin occurs when insulin in the circulation is unable to phosphorylate the target cell's substrate receptors, resulting in a decrease in glucose uptake into the cell [25]. Alteration in glucose metabolism is the main cause of T2DM. In mammalian species, plasma glucose levels of up to 100 mg/dL assist physiological functioning [26].

The level of HbA1c was significantly higher in Type 2 DM patients when compared to the control group. A measurement of 6.5% hemoglobin A1C has also been used as a T2D indication [27]. This result corresponds with the results of other studies [28, 29]. Results of the current study revealed that serum IL-6 levels are highly significant ( $p = 0.000$ ) among T2DM patients than in the control group. The results in agreement with results of other investigations which reported that T2D patients have remarkably elevated IL-6 values than controls [30, 31]. Devaraj, et al 2005 concluded that in high glucose condition, monocytes secrete greater quantities of IL-6 via up - regulation of PKC- $\alpha$ , PKC- $\beta$ , NF- $\kappa$ B, and p38MAPK activity, resulting in enhanced IL-6 expression and release [32]. Beyond the immune system, interleukin-6 is a multifunctional cytokine that have an essential function in both immune regulation and non-immune activities in a range of cell and tissues types [33]. The intracellular signaling cascade that leads to the inflammatory process is activated when IL-6 binds to either membrane or soluble interleukin-6 receptor (IL-6R), [34]. Over than 1240 gene loci have been related to diabetes in humans [35]. Incidence to complex cases of T2DM is linked to a variety of polymorphisms that affect the activity of genes that are involved in the same or separate causative pathways [36, 37]. In the present study, two single-nucleotide polymorphisms (rs4845625 and rs2229238) of the IL-6R gene were examined in T2DM patients. These single-nucleotide polymorphisms were linked to a variety of immune-related illnesses, including type 2 diabetes, coronary heart disease, chronic kidney disease, and subclinical atherosclerosis [21, 38, 39]. The results of the current study indicated no significant differences in (CC, CT, TT) genotypes, and (C, T) alleles distribution of rs2229238 between Type 2 diabetes patients and controls. These findings are agreement with other studies, when the researchers looked into single nucleotide polymorphisms in European Caucasians, they found no evidence of an elevated risk of T2DM [40]. Also, The IL6R rs2229238 polymorphism was examined in Pima Indians by Wolford et al., who recognized that rs2229238 has no significant correlation in this community [18]. In contrast, another research, they studied rs2229238 and rs4845625 IL-6R gene variations in an Iranian sample and discovered a substantial correlation between rs4845625 and rs2229238 polymorphisms and type 2 diabetes mellitus [21]. The findings of present study indicated to the significant correlation of rs4845625 polymorphism of IL-6R gene, and T2DM, these are consistent with results that detected a significant correlation between rs4845625 polymorphisms and T2DM [21, 38]. The importance of IL6R SNPs on the risk of T2DM is poorly defined. The interleukin-6 gene found on the 7p21 chromosome. The gene, which has 7 exons, spans around 12.8 kb of genetic material. The rs1800795 (also known as -174G/C) was first identified



as a frequent SNP in the promoter of IL-6 gene [41]. Many epidemiological studies have shown relationships between IL-6 genetic polymorphisms and diabetes risk. According to findings of another study, the rs1800795 genotype has a significantly substantial correlation with type 2 diabetes mellitus [42]. In SardiNIA research, which included 6145 individuals, showed a relationship between SNPs in the IL-6 levels, and also a relationship between a variation in the IL6R gene, which expresses the IL-6 receptor [43]. A polymorphism in the IL-6R gene (rs4537545) was shown to be linked to serum levels of the both IL-6 and IL-6R [44]. These indicators were also found to be associated with a variety of metabolic features, such as diabetes, and to be slightly associated with other diseases such as cardiovascular disease and asthma. Another study demonstrated that IL-6 levels are more closely linked to IL-6R variations than to IL-6 polymorphisms [45]. The IL-6R is based on substantial findings supporting the importance of IL-6 in the etiology of resistance to insulin hormone in obesity and type 2 diabetes patients [46, 47]. The essential contribution of IL-6R in function of interleukin 6, and its position of the IL- on chromosome 1q21 in an area of highly repeated T2DM linkage. Seventeen SNPs have been discovered, two of which change transmembrane amino acids. D358A believed to modify IL-6R splicing to sIL6R, potentially altering the response to IL-6 in non-hepatic tissues [48]. In the promoter region of the IL-6 gene, the single nucleotide polymorphism (SNP) rs1800795, often known as -174 G>C, could influence IL-6 expression. The pro-inflammatory G allele, not the C allele, is the main cause of high IL-6 plasma levels in healthy people [49]. This previous research suggests that IL-6 genetic polymorphism causes metabolic and cytokine regulation [50], and these modifications could be the primary cause of T2DM. The rs1800-795 and the reduced risk of T2DM were initially demonstrated in the United States, Spanish Caucasians, and Pima Indians [13]. The rs2229238 IL6 variations may combine with C-Reactive Protein (CRP) and predict the risk of diabetes. Other investigations also indicating that IL-6R gene variations are substantially related to serum CRP levels [51], indicating that in adults, both the CRP gene and the IL-6 gene effect on CRP level in serum, insulin resistance, obesity condition or other metabolic disorders [52]. The disparity across studies can be related in part to demographic variability, differing confound structures, and varying research quality. Many susceptibility genes are thought to predispose to diabetes, although individual genes are estimated to have a moderate role [53, 54]. Despite that our findings imply that IL6R variations are not directly related to diabetes risk, they are useful in explaining the complex relations between IL-6R polymorphisms, serum IL-6 levels, and complications of diabetes.

## 5. Conclusions

The current work was studied the relation between interleukin 6 receptor gene (rs2229238, rs4845625) variants and the risk of T2DM. The rs2229238 variants were not associated with diabetes risk, whereas rs4845625 interleukin 6 receptor gene variants were significantly associated with T2DM.

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