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## Insulin resistance and the PC-1 gene sequence (ENPP1) (rs1044498) in people with Diabetes mellitus type- 2

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### Abstract

The research study was carried out between December and May 2023 at the Biotechnology Research Unit of Thi-Qar at Mazaya University College. the current study aimed to identify the genetic polymorphisms of 120 samples to investigate the possible association of *PC-1* gene polymorphism with the diabetes mellitus. The observed PCR amplicons were subjected to the DNA sequencing procedure. The discovered variants were then localized based on where they were located within the reference genomic DNA sequences. The recent findings showed that the samples under investigation had genetic variations that were unevenly distributed. Besides, the rs1044498, A248C, also the high frequency variant is usually detected in three polymorphic modes, AA, AC, CC. Homozygous AA pattern discovered in the majority of patients (62.5%) and normal specimens (60%). However, each observed SNP was taken a particular pattern of variation differ from its mate in terms of the investigated samples. Ultimately, a statistical analysis is required to evaluate the possible association of the SNPs is tested samples, with the diabetes mellitus. As a result, the PC1 polymorphism was found to be associated with type 2 diabetes mellitus and insulin resistance in the current investigation.

### 1-Introduction

The chronic illnesses and health difficulties brought on by diabetes mellitus (DM) are a concern for people all over the world. A rise in hyperglycemia often known as high blood sugar is the metabolic condition known as diabetes mellitus (DM). By 2030, more than 280 million adults will have diabetes worldwide, and by 2050, that number will rise to more than 400 million (Shaw *et al.*, 2010). Diabetes is commonly divided into categories (Thomas and Philipson ., 2015). These include autoimmunemediated type 1 diabetes causes insulin deficiency; which develops as a result of pancreatic injury; diabetes linked to particular genetic disorders and a general category known as

diabetes type 2, in which insulin production is impaired and resistance to insulin actions is typically present but not always (American, 2020).

Diabetes type 1 results in insulin deficiency. Growing understanding of significant variances in the physiological mechanisms underpinning these different categories has the potential to improve outcomes by making some therapeutic modalities more accessible (American, 2021). Body's incapacity to create or react to insulin is what distinguishes the metabolic syndrome, which includes diabetes type 2 (T2DM). In addition to hypertension, dyslipidemia (low lipid levels), and insulin resistance, which is directly related to the development of T2DM, a major risk factor for metabolic syndrome (Zafa *et al.*, 2019). Attacks on the brain and other heart and circulatory (CVD) illnesses (Martins *et al.*, 2019).

Estimates of the high prevalence of a metabolic disorder and insulin resistance range from 10% to 40% in younger individuals and developing nations (Ansarimoghaddam *et al.*, 2018). In comparison to other T2DM diabetes has the highest prevalence and accounts to over 90% of the worldwide diabetes prevalence (Santoro *et al.*, 2021). In the event that insulin production levels fall for whatever cause or cells develop resistance to insulin, T2D disease will result (Chaudhury *et al.*, 2017). But because they contribute to insulin resistance and type 2 diabetes (T2D), genetic and environmental variables that control how cells respond to insulin are also crucial to be studied (de Veciana *et al.*, 2017). According to the International Diabetes Federation (IDF), diabetes is an endocrine system illness that will affect more than 693 million persons worldwide by the year 2045, up from 50% in 2017 (Cho *et al.*, 2018).

Diabetes of type 2 is a clinical syndrome that causes high blood sugar levels, which is due to genetic and environmental risk factors. Diabetes is one of the biggest challenges facing scientific health (Ogurtsova *et al.*, 2017). Unhealthy changes in biological clock and Additionally contributing to metabolic syndrome and insulin resistance is lifestyle. Genetic variables also influence a person's likelihood of developing insulin resistance and the metabolic syndrome (Brown and Walker, 2016).

Ectonucleotide Pyrophosphatase-Phosphodiesterase 1 (ENPP1) is another name for the PC1 gene. It is found on the long arm of chromosome 6 (6q23.2) which has 25 exons and 24 introns, and it encodes for a protein that influences the sensitivity of the body to insulin. It is an endogenous transmembrane glycoprotein that can be found in the endoplasmic reticulum (ER) as well as on the plasma membrane. It is possible for the (PC1) to suppress the autophosphorylation of the insulin receptor (IR) by directly engaging with the insulin receptor subunit, hence preventing further downstream signaling, despite the fact that its function is not fully understood. (Vickers, 2017; Pappalardo *et al.*, 2017).

## 2 . MATERIAL AND METHODS

### 2.1. Ethical statement

Before the start of the study, institutional approval was obtained, Nasiriyah Teaching Hospital, Thi-Qar, Iraq.

## 2.2. DNA extraction

Genomic DNA was isolated from whole blood using a Geneaid Blood DNA extraction Kit (of Korean origin) after blood was collected in EDTA tubes. in accordance with industrial requirements. Primer-based Polymerase Chain Reaction (PCR) was used.

(F \ 5-TCAGAGTGGCCATGGTAGTG-3; R \ 5-TGTAAAGCCCCGCTAAGACG-3) rs1044498, a polymorphism in PC1. Digested products and marker DNA (350 bp ladder) were resolved on a 2% agarose gel electrophoresis for scale interpretation.

No.	Steps	Temperature	Time	No. of cycle	Size (bp)
1	Initial denaturation	95	10 min.	1	350
2	Denaturation	95	30 sec.	30	
	Annealing	62	30 sec.		
	Extention	72	30 sec.		
3	Final extention	72	10 min.	1	

## 2.3. DNA amplicon sequencing for PCR

According to the manuals provided by the sequencing business (Macrogen Inc. Geumchen, Seoul, South Korea), the PCR amplicons were commercially sequenced from one direction, forward direction. To confirm that the annotation and variances are not the result of PCR or sequencing artifacts, subsequent analysis was limited to clean chromatographs acquired from ABI sequence files. The virtual locations and additional information of the obtained PCR fragments were identified by comparing the observed DNA sequences of local samples with the retrieved DNA sequences.

## 2.4. analysis of sequencing data

Using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA), the sequencing results of the PCR products of the targeted samples were edited, aligned, and assessed along with the corresponding sequences in the reference database. Each sequenced sample's detected changes were given a number in both its PCR amplicon and in the appropriate place in the referring genome.

## 2.5. the rs1044498 SNPs are examined

To verify their originality, the observed SNP was entered into the dbSNP database. The location of each specific SNP in the reference genome was marked. Then, by looking at its corresponding dbSNP

site, it was possible to determine whether a previous SNP was present. The dbSNPs location for the discovered SNP was then recorded.

## 2.6. Results and discussion

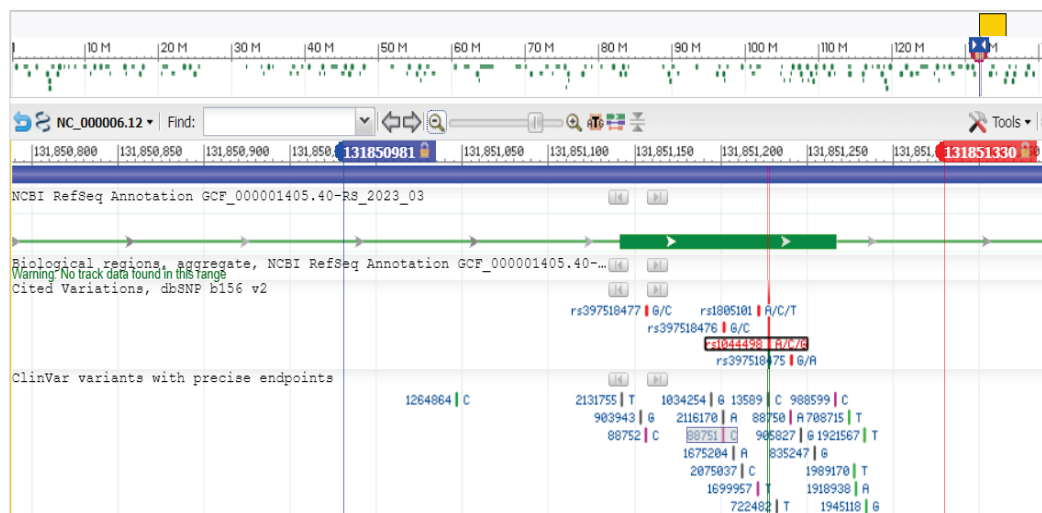
### Sequencing of the 350 bp region within the *PC-1* gene

within the area investigated in the current study, the *PC-1* genetic sequencing was analyzed in eighty patient samples (assigned from S1 to S80), and forty control samples (C1 to C40). Following the NCBI blastn technique (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), sequencing reactions revealed the precise identify of the genetic segment that is presently being increased. The *PC-1* gene was largely covered by this engine, which demonstrated more than 99% sequence symmetry between amplified samples and intended reference target sequences. The precise locations and other specifics of the retrieved PCR amplifications were discovered by comparing the observed DNA differences of the currently being examined samples with the retrieved DNA sequences (Gen Bank acc. NC\_000006.12) (Fig. 1).

#### Homo sapiens chromosome 6, GRCh38.p14 Primary Assembly

NCBI Reference Sequence: NC\_000006.12

[GenBank](#) [FASTA](#)



**Figure 1** the precise location of the 350 bp amplicon that was recovered and partially encompassed a section of the *PC-1* gene on chromosome 6 (Gen Bank acc no. NC\_000006.12). This amplicon's beginning point is indicated by the cyan arrow, while its ending point is indicated by the red arrow.

Following the placing of the 350 bp amplified amplicons, regions on chromosome no. 6 were highlighted in terms of the placement of both the forward and reverse primers of the amplicon (Table 1).

**Table 1.** The location and dimensions of the 350 bp PCR amplicons that were utilized to amplify a segment of the PC-1 gene on chromosome 6 (Gen Bank accession number NC\_000006.12). The positions of the reverse and forward primers were indicated by the gray-hued sequences.

Amplicon	Referring locus sequences (5' - 3')	Length
<p><b>DNA sequences within the <i>pc-1</i> gene</b></p>	<p>*ACCTGGCCAAGGAATCTGTGAGGAAAAGAAAGAT  CAAATGGAAAATCAAGGTACATGACACCAGAAGAC  CTACATGTTACTTCAAACCTTTTCTTCCTCATGAACC  ATTAAAATAGAGCATAACTCTTCTGGCAGCTGTACA  TATGTTCATAAATACATGATATTGACCCATAGCATA  GCAGCTCTGCTCAGCTTCTAACAAGTAAGAATGAAA  AGAGGACATGGTCTTTAGGAACATGAATTTCTGCCC  TTCCATTTTCTTCAGAAGGAGAGATTCTTCTATGA  CCTCATTGGGGGCGGAAATTTAACCAAAATGGTGT  CACCCCTGAACCCACTGCGACACGCCACGTAAGTGA  CCACAGAAGGAGAAAAGCCCTATAAAAAGAGAGAC  GATAGCGCTACATTTTGTCCATCTCATAGCAGGCAC  AAACTCATCCATCCCCAGTTGATTGGAAGAAACAAC  GATGACTCCTGGGAAGACCTCATTGGTGGTGAGTCC  TGC ACTAACGTGCGATGCTCTTGC **</p>	<p>350bp</p>

\*Indicates the sequences of the front primer (placed in a front orientation)

\*\* Refers to reverse primer sequences (placed in reverse direction)

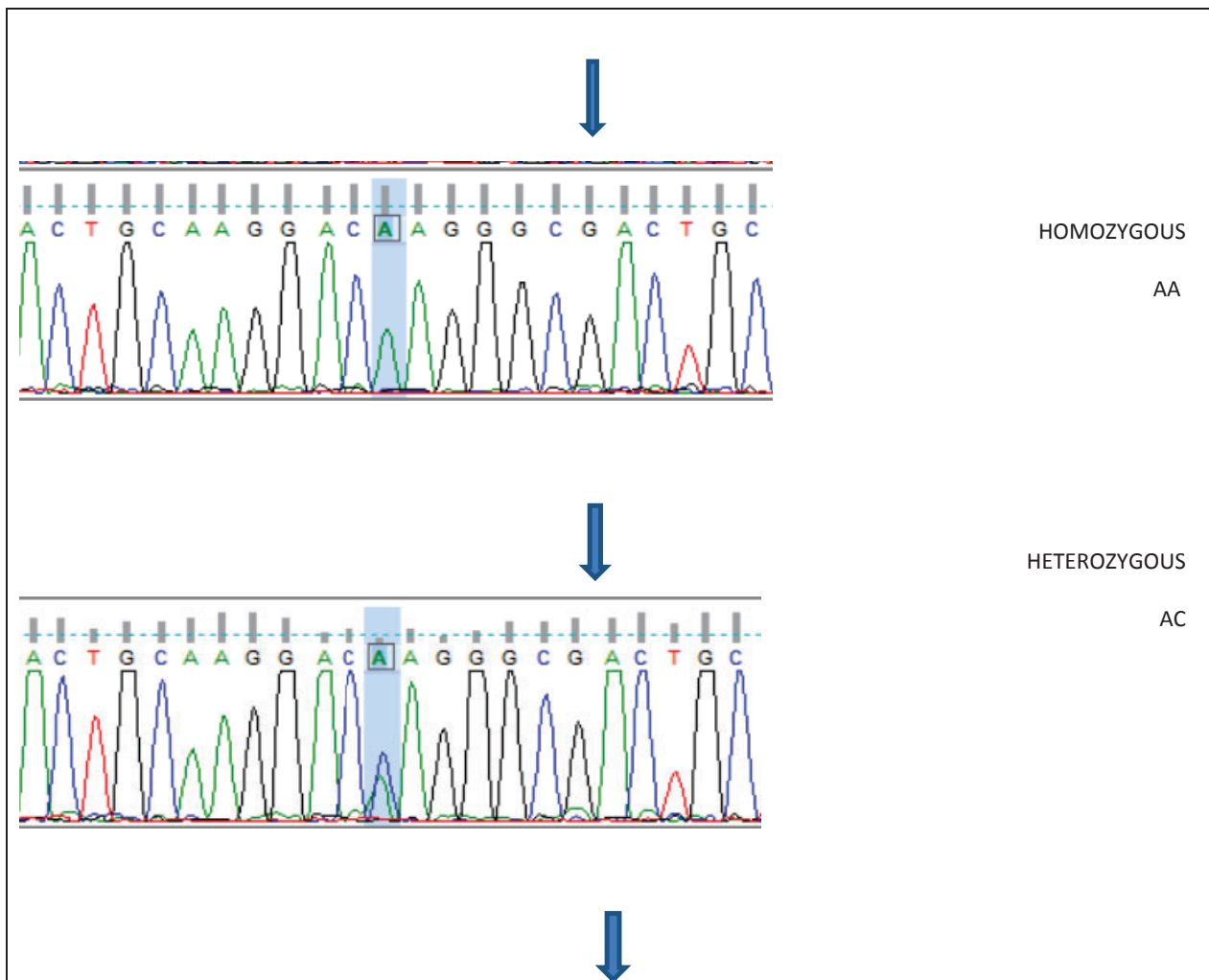
The results of the alignment of 350bp samples revealed the presence of single nucleotide polymorphisms (SNPs) throughout the analyzed samples in comparison with the referring reference DNA sequences .

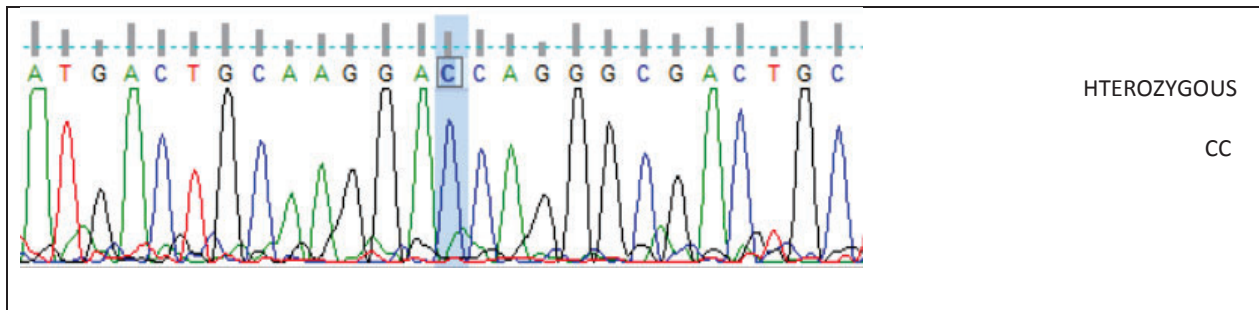
DNA sequences alignment of 120 samples with their corresponding reference sequences of the 350bp amplicons of the *PC-1* genetic DNA sequences. The symbol “ref” refers to the NCBI referring sequence; and the letters S1 to S80 refer to the samples.

The sequence chromatogram the identified variation, along with its detailed explanations, is recorded and shown in the amplified version in accordance with its matching dwelling polymerase chain reaction fragments 350 basis points (Fig. 3).

The target high-frequency SNP was detected in the tested samples, where nucleotides A were replaced by nucleotides C at position 248 of amplified polymerase chain reaction fragments, namely 248A>C, or A248C. This high-frequency variable is usually detected in three polymorphic modes, AA, AC,CC, AG, and GG and the chromatograms these sequences were shown according to their positions in the PCR amplicons.

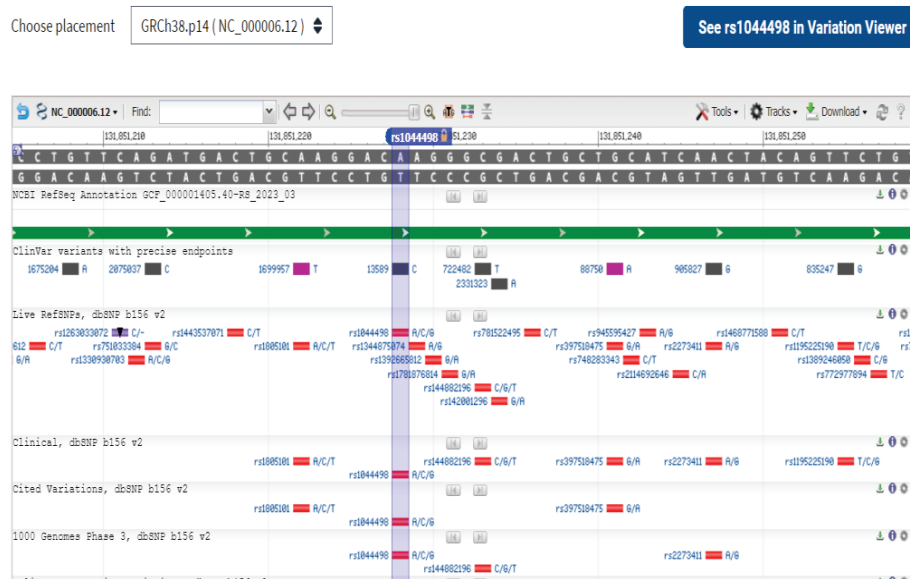
Homozygous A/A status was found in the majority of cases' (50/ 80) and controls' (24/ 40) samples, but the heterozygous A/C pattern was only found in a small number of cases' (2/ 80) and controls' (0/40) samples. It's interesting to note that homozygous CC patterns were found in patients (28/80) and controls (16/40), but AG and GG patterns were not found in the examined samples, as shown in table (2).





**Figure 3.** The pattern of the detected SNPs within the DNA chromatogram of the targeted 350bp amplicons of the *PC-1* gene. The identified SNPs were highlighted according to their positions in the PCR amplicons.

The detected polymorphic position was shown to show diffusion within the samples analyzed. As a result, it was required to investigate this variant in greater depth at the locations where it appeared in the genomic DNA sequences of the *PC-1* gene that were included in the dbSNP database. The matching position of the *PC-1* gene was obtained from the dbSNP website (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) in order to discover the genomic positions of the target variant in relation to its deposited SNP database of the sequenced 350bp segments. Additionally, using the Gen Bank Account No. NC\_000006.12, graphical representations of the discovered SNP were displayed in reference to the *PC-1* dbSNP database on chromosome No. 6. A evaluation of the dbSNP engine revealed that the genome already had the newly identified A248CSNP (NC\_000006.12: rs1044498. A>C), which was deposited in Fig. (4).



**Fig. (5):** The exact position of the retrieved 350bp amplicon that partially covered a portion of the *PC-1* gene within chromosome no. 6 (GenBank acc no. Nc\_000006.12).

### 2.7. Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium (HWE) principle holds that, in the absence of unfavorable circumstances, genetic variation in a population will remain constant from generation to generation. The genotype and allele frequency of the *PC-1*, A>C SNP are provided in Tables 2, 3, and 4 to compute HWE. This study's findings are consistent with HWE, with a significant result (P=0.0001).

The AA genotype frequency was higher in diabetes mellitus patients (62.5%) compared to healthy controls (60%), the difference between patient and control groups were statistically non-significant (OR=1.1, P. value= 0.8); while heterozygous AC genotype frequency was detected only in diabetes mellitus patients (2.5%), the CC genotype was detected in case (35%) and control (40%), as shown in table (2).

**Table 2: Genotype frequency among diabetes mellitus and control**

<i>PC-1</i> ,248 A>C	Case=80	Control=40	OR	P.V
AA	50( 62.5%)	24(60%)	1.1	0.8
AC	2(2.5%)	0(0%)	2.5	0.5
CC	28(35%)	16(40%)	0.8	0.5
AG	0	0	1	1
GG	0	0	1	1

The allele frequency of *PC-1* gene, A>C SNP among cases and control was statistically, no a significant relationship (p. value 0.1) between *PC-1* gene and 248 A>C polymorphisms, Table (3) .

**Table 3. Allele frequency of *PC-1*, 248 A>C SNP among diabetes mellitus and control**

	Case NO:80	Control:40	OR	p. value
A	102	48	1	0.1
C	58	16		

The present results of HWE of *PC-1* gene, the A248C SNP among the control, recorded that closely relation between expected genotype and observed genotype and a significant relationship (p. value 0.0001) as shown in table (4).



**Table (4) Hardy-weinberg equilibrium in *PC-1A>C* among control**

genotype	AA	AC	CC
Observed genotype	24	0	16
Expected genotype	<b>14.4</b>	<b>19.2</b>	<b>6.4</b>
H-W Freq.	<b>(36%)</b>	<b>(48%)</b>	<b>(16%)</b>

- P= 0.0001
- Allele Frequency **0.6** p
- Allele Frequency **0.4** q

The distribution of *PC-1*, A>C SNP in this study population under recessive and dominant model table (5) and table (6) showed non significantly results, so appeared the recessive model (AA+AC), (OR=1.2), more sensitive infected in diabetic mellitus than dominant model.

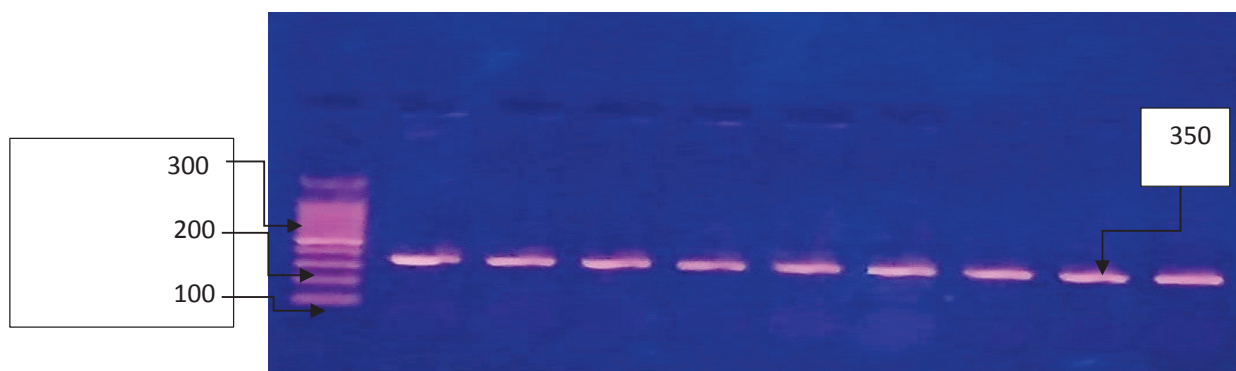
**Table 5: Distribution of *PC-1* A>C SNP in the population under study using a recessive model**

genotype	Case NO:80	Control:40	OR	p. value
AA+ AC	<b>52</b>	<b>24</b>	1.2	0.6
CC	<b>28</b>	<b>16</b>		

**Table 6: Distribution of *PC-1* A>C SNP in the population of this study using the dominant model.**

genotype	Case NO:80	Control:40	p. value	p. value
AA	<b>28</b>	<b>16</b>	0.8	0.6
AC+CC	<b>52</b>	<b>24</b>		

### Molecular study 2.8.



**Figure 6.** Show Electrophoresis of PCR products on 2% agarose gel. *PC-1* gene and bundle appearance at the base pair 350bp

### 2.9.Submission in NCBI

LC768831, LC768832, LC768833, LC768834, LC768835. LC768836, LC768837 LC768838, LC768839, LC768840, LC768841, LC768842, LC768843.

### 3.Discussion

The results of the current study based on the sequencing analysis of the (*PC-1*)To elucidate the positions of the targeted SNPs with regard to their deposited SNP database of the sequenced 350bp fragment, the corresponding positions of the PC-1 gene was retrieved from the dbSNP server. To find out the nature of these SNPs, a graphical representation was performed concerning the PC-1dbSNP database within chromosome no. 6 (Gen Bank Acc. NC\_000006.12).).By reviewing all the detected fourteen SNPs in the dbSNP engine.

More than 180 million people worldwide suffer from diabetes mellitus, which reaches pandemic proportions. Global projections for 2010 call for an additional growth of about 50%, with the largest increases expected in the developing nations of Africa, Asia, and South America (Zimmet *et al.*, 2011 ).

It was also known as Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) and Plasma Membrane Glycoprotein 1 (PC-1). It was situated on the long arm of chromosome 6 (6q23.2) and encoded for the protein that affected insulin sensitivity, among other things. Both the endoplasmic reticulum (ER) and the plasma membrane had a grade II transmembrane glycoprotein.

The molecular mechanism underlying the phenomenon was initially linked to a single synonymous SNP, K121Q17, which allowed the Q allele to have a "gain-of-function" effect that was several times more potent than the K allele in reducing insulin stimulation of IR autophosphorylation, IR substrate-1 phosphorylation, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cell proliferation (Costanzo *et al.*,2001).

Though, the role of *PC-1* was not fully understood, it was able to inhibit the autophosphorylation of insulin receptors by interacting directly with  $\alpha$ -subunit insulin receptors, thereby blocking subsequent downstream signals (Johnson, 2006; Pappalardo *et al.*, 2017).

The results of current study recorded that an association between PC-1 248 A>C SNP polymorphism with diabetes mellitus patients (P. value= 0.0001).

But it has been shown that excessive PC-1 expression inhibits the action of insulin receptors in tyrosine kinase and, as a result, cellular communication in a number of cells. There is still some confusion regarding the PC-1 protein's functions (Maddux and Goldfine, 2000).

The results of current study were C>T (rs1044498) polymorphism significantly associated with diabetes mellitus; The present results incorporated with different studies documented the polymorphism is linked to metabolic syndrome, T2DM, and insulin resistance. (Bhatti *et al.*, 2010; Daoming, Marchenko *et al.*, 2018). However, other research has not confirmed a genetic link between the PC1 K121Q polymorphism and insulin resistance (Rasmussen *et al.*, 2000). According to Sortica *et al.* (2015), the PC1 K121Q polymorphism was a misleading functional alteration since the 121Q variant more strongly binds insulin receptors than the 121K variant does (Costanzo *et al.*, 2001). Additionally, genetic association studies have confirmed and shown that ENPP1 polymorphisms are linked to a risk for T2D, obesity, and a number of metabolic disorders. (Zhao *et al.*, 2011).

Failed to replicate a positive association between rs1044498, and T2D in the Chinese Han population (Zhao *et al.*, 2011). The positive associations were often reported from White (Abate *et al.*, 2005; Moore *et al.*, 2009) and African (Chandalia *et al.*, 2007) populations; while the negative results were found in Moroccan (El Achhab *et al.*, 2009) Chinese, (Chen *et al.*, 2006) Japanese (Keshavarz *et al.*, 2006) and Korean (Seo *et al.*, 2008) populations. Indeed, demographic variation needs to be taken into account, particularly in the case of a complex metabolic condition. Furthermore, T2D and obesity should be attributed to multifactorial etiology, rather than a single gene disease, as a result of changes in several gene products.

In present results of genotype distribution of PC-1 gene documented that the AA genotype was identified in the majority of case samples (62.5%), and controls (60%), while AC pattern was detected only in case samples (2.5%). The present results were incorporated with results of . Research done in China also revealed that T2DM or any of the characteristics of the metabolic syndrome have no correlation with PC-1 K121Q polymorphotype distribution (Daoming *et al.*, 2006). The present findings differed from those of Abdo Albegali *et al.* (2019), who found a correlation between PC-1 and the incidence of type 2 diabetes in the Punjabi population of Pakistan. While other studies have showed no link of this allele with illness risk (Rasmussen *et al.*, 2000), prior research has demonstrated a correlation between the Q allele of PC1 (rs1044498) and insulin resistance (Gu *et al.*, 2000). Very few reports about the role of PC1 risk alleles in disease have come from the South Asian continent (Prakash *et al.*, 2013).

Positive findings and proven genetic connections were found in a meta-analysis of the PC1 K121Q polymorphism and T2DM risk (Abate et al., 2005; Hamaguchi et al., 2004) , that the precise molecular mechanism of this gene's risk factor is still unknown (Abdo Albegali et al., 2019).

Many diseases, including different genetic polymorphisms and risk alleles in this gene have been linked to coronary heart disease, polycystic ovarian disease, obesity, risk of diabetic kidney disease, coronary artery calcification, ischemia of the heart, and T2DM. (Di et al., 2018; Li et al., 2016; Pappalardo et al., 2017).

Some studies recorded relation between the same rs1044498 and other disease that detectesuch as: (Zhang *et al.*, 2021) Hemodialysis patients in the participants recruited from one hospital in China showed the variations of ENPP1 (rs1044498). According to a review, T2DM patients in Asia were the basis for the rs1044498 variation.

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