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## **Phosphatidylinositol 3-Kinase and Insulin Receptor Substrate-1 Gene Polymorphism / Adipocytokines Interaction in Pathogenesis of Insulin Resistance among Egyptian Patients: A Pilot Study**

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### **Abstract:**

*Background and Objectives: This study was designed to elucidate a possible impact of gene polymorphism of M326I variant of the regulatory subunit p85 $\alpha$  of phosphatidylinositol 3-kinase and/or G972R variant of IRS-1 in correlation with serum leptin and visfatin on development of insulin resistance among Egyptian patients.*

*Material and methods: The study included 116 Egyptian patients (30 men and 86 women) divided into 3 groups, T2DM lean (group II, n=32), T2DM obese (group III, n=44) and metabolic syndrome (T2DM, obese, and hypertensive) patients (group IV, n=40). The control group comprised 40 healthy nonobese volunteers with euglycemic state and having body mass index (BMI)  $\leq 25$  Kg/m<sup>2</sup> was considered group I.*

*The serum concentrations of visfatin, leptin and IL-6 were assayed by enzyme-linked immunosorbent assay kits. All subjects were genotyped for the M326I of the regulatory subunit p85 $\alpha$  of phosphatidylinositol 3-kinase and IRS-1 Gly972Arg polymorphisms. Body mass index, serum glucose, insulin, insulin sensitivity index HOMA (homeostasis model assessment) and serum lipoproteins were estimated.*

*Results and Conclusions: The frequency of genotypes associated with polymorphism of insulin receptor substrate-1 (IRS-1) gene at G972R is not discriminative among our Egyptian diabetics as well as the normal controls while, PI3K p85 regulatory subunit gene polymorphism may participate in pathogenesis of IR characterizing T2DM and metabolic syndrome.*

*Elevated serum levels of visfatin, leptin and IL-6 in patients with the metabolic syndrome suggest that these adipokines are involved in the pathogenesis of this syndrome*

**Keywords:** *PI-3 kinase, IRS-1, Insulin resistance, Metabolic syndrome, Adipokines, leptin, visfatin.*

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### **1. INTRODUCTION**

Metabolic syndrome (MetS) constitute multiple risk factors for atherothrombotic cardiovascular disease (CVD) and diabetes in association with obesity, hypertension, and/or dyslipidemia [1]. It is a major cause of mortality (6-7% of the general mortality) of CVD (12-17%), and of diabetes (30-52%) [2]. Early manifestations of MetS and the sequence of its features are not settled.

Insulin resistance is a constant feature of MetS. As insulin binds to its receptor  $\alpha$ -subunit, multiple transphosphorylation reactions of tyrosine residues occur. Phosphorylation reactions include insulin receptor substrate family [3]. Downstream signal transduction, depends on the integrity of the regulatory subunit p85 $\alpha$  of phosphatidylinositol 3-kinase (PIK3) [4]. Insulin stimulated increase of PIK3 mRNA level has been totally impaired in patients with type 2 diabetes (T2DM) compared with lean and obese subjects in spite of proper insulin tolerance [5]. In insulin receptor substrate-1 (IRS-1), polymorphism at codon 972 causing Gly $\rightarrow$  Arg (G972R) is associated with increased risk for T2DM when combined with obesity [6]. G972R, variant of IRS-1, is located between two potential sites of tyrosine phosphorylation involved in binding the p85 $\alpha$  subunit of PI-3 kinase [7]. This variant impairs insulin signaling [8]. On the other hand, the decreased IRS-1-associated PI3K activity and the subsequent reduced activation of the Ser/Thr kinase-Akt, result in defective activation of multiple biological functions of insulin as a result of impaired signaling transduction[9].

With insulin resistance (IR), there is an increase in lipolysis and release of free fatty acids (FFAs) from the adipose tissue (AT). FFAs are diverted to the liver, muscle, heart and pancreatic  $\beta$ -cells [10]

causing lipotoxicity of these organs owing to fatty acid accumulation. In addition, enhanced lipogenesis and defective lipid export, despite FFAs oxidation and increased insulin levels, results in hepatic steatosis. This indicates that hepatic steatosis, can be a cause and result in hepatic IR. Changes in plasma lipid profile ensue; favoring elevation of triacylglycerol-containing VLDL and cholesterol-containing LDL [10].

Adipose tissue secretes several hormones and cytokines that play a role in the pathogenesis of IR [11]. Among these adipocytokines are leptin and visfatin and interleukin-6 (IL-6) [12]. Visfatin has insulin-mimetic actions might be via activating the insulin receptor at a site distinct from that of insulin [13]. The proinflammatory properties of leptin and IL-6 may contribute to atherogenesis in obesity [14].

This work was planned to elicit a possible impact of gene polymorphism of M326I variant of the regulatory subunit p85 $\alpha$  of phosphatidylinositol 3-kinase and/or G972R variant of IRS-1 in correlation with serum leptin, visfatin and IL-6 on development of insulin resistance among Egyptian patients. It is also, a trial to follow the sequence with which the features of MetS develop.

## 2. SUBJECTS AND METHODS

This cross-sectional study was conducted in the Medical Biochemistry Department, Faculty of Medicine, Cairo University in the period between May 2011 to May 2012. This study was approved by the Ethical Committee of Kasr Al Ainy Medical Hospital and was in accordance with the principles of Helsinki Declaration, and all participants provided written informed consent.

One hundred and fifty six Egyptian subjects participated in the present study. One hundred and sixteen consecutive patients attending El Kasr Al Ainy Hospital, Tropical Medicine Department and 40 unrelated healthy controls with euglycemic state and having body mass index (BMI)  $\leq 25$  Kg/m<sup>2</sup>. (Group I, n= 40) with similar demographic characteristics. According to criteria for MetS advocated by *Alberti, et al* [15], three groups were included; T2DM lean (group II, n= 32), T2DM obese (group III, n= 44) and metabolic syndrome (T2DM, obese, and hypertensive) patients (group IV, n= 40).

It has been known since decades that a simple obesity is associated with insulin resistance in spite of apparent euglycemia, hence it seems to be valueless to include obese normoglycemic as a second control group in our work.

All subjects underwent complete full history taking, clinical examination (anthropometric measurement, weight, height to calculate body mass index (BMI) [16] and routine laboratory investigations. Subjects were defined as being lean if their BMI  $\leq 25$  Kg/m<sup>2</sup> and obese if their BMI  $\geq 30$  Kg/m<sup>2</sup> [17].

For each patient, infectious, endocrine, toxic and autoimmune diseases were excluded. None of the patients was receiving medication affecting insulin sensitivity or having metabolic interactions.

## 3. METHODS

**Sample collection:** After over-night fast, venous blood samples were collected in a sterile EDTA and plain tubes. Sera were divided into aliquots. Subjects took lemon-flavored 75 g glucose in solution, blood re-sampling withdrawn 2.00 hours later for postprandial blood glucose estimation.

Serum lipid profile (TAG, cholesterol-total, LDL-C and HDL-C), were estimated using commercially available kits. Atherogenic index of plasma (AIP), calculated as Log TAG/HDL-C, expressing TAG and HDL-C values in molar units [18]. Other serum aliquots were stored at -80°C till used in estimation of fasting insulin, leptin, visfatin and IL-6.

ELISA kits were used for estimation of fasting insulin (DRG® Highway East, Mountainside, NJ USA) [19], visfatin (RayBiotech, Norcross, Georgia, USA) [13], leptin (DRG instruments GmbH, division of DRG International, Inc. Marburg, Germany) [20], and IL-6 (R&D Systems, Minneapolis, Minnesota, USA) [21].

Insulin resistance was assessed by using the homeostasis model assessment (HOMA) [22, 23] that was calculated using the formula:

Fasting insulin ( $\mu$ IU /mL) \* Fasting glucose (mmol/L)/22.5. Subjects were considered as insulin resistant when HOMA  $\geq 2.6$ .

EDTA whole blood samples were used for DNA extraction using BDtract™ Genomic DNA Isolation Kit (Maxim Biotech, Inc. San Francisco, USA). DNA yield was determined from the concentration of DNA in elutes, measured by absorbance at 260 nm [24].

The extracted DNA was stored at -80°C to study gene polymorphism of codon 326 of p85 $\alpha$  regulatory subunit of PI-3 kinase and the codon 972-IRS-1.

PI3 kinase-326 (M/I) and IRS-1- 972 (G/R) genotypes were identified by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) methods.

PCR was accomplished by Taq PCR Master Mix (Qiagen, Valencia, CA, USA). The primers for the detection of PI3 kinase-326 (M/I) polymorphism, were designed Forward primer: (5'CCAACAACGGTATGAATAACCATAT 3'), Reverse primer: (5'CGAGATATCTCCCCAGTACC 3') and the Forward (5'-CTTCTGTCAGGTGCCATCC-3'), Reverse (5'-TGGCGAGGTGTCCACG TAGC-3') , for the detection of IRS-1- 972 (G/R) polymorphism.

For PI3 kinase-326 (M/I) genotyping, PCR was performed in a final volume of 50  $\mu$ L, in which 1  $\mu$ L of each primer, 2  $\mu$ L of extracted DNA were added to 25  $\mu$ L of Master Mix then the volume is completed with 21  $\mu$ L of nuclease free water [25].

For IRS-1- 972 (G/R) genotyping, PCR was carried out in a reaction volume of 50  $\mu$ l containing 1  $\mu$ l (0.5  $\mu$ g) DNA, 25  $\mu$ l master mix (HotStar Taq Master Mix Kit (250U) (Qiagen), 3 x 0.85 ml HotStar Taq Master Mix containing 250 units HotStar Taq DNA polymerase total, 2 x 1.7 ml distilled water), and 0.4  $\mu$ M of each primer.

PCR amplification was achieved using Thermal Cycler (TECHNE, TC-3000) with the following thermal profile: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at (53°C for PI3 kinase and 60 °C for IRS 1) for 1 min and extension at 72 °C for 1 min and final extension step at 72°C for 10 min.

PCR products were checked by DNA 3% agarose gel electrophoresis for regulatory subunit p85 $\alpha$  of phosphatidylinositol 3-kinase gene and 2% for IRS-1 gene.

#### **4. PIK3- P85A REGULATORY SUBUNIT GENOTYPING BY RESTRICTION ENZYME ANALYSIS**

Restriction enzyme digestion was carried out at 37°C for at least 3 hours. 1  $\mu$ L of 10x NEB buffer 4 (New England Biolabs Inc) was added to 20 $\mu$ L of the secondary PCR product and at least 20 U of the restriction enzyme NdeI (New England Biolabs Inc). The fragments were analyzed by 3% high-resolution agarose gel electrophoresis. Digestion patterns obtained in homozygous wild-type, homozygous mutation and heterozygous mutation carriers are shown in Figures (1). In wild genotype (2 bands smaller than 65bp), mutant heterozygous type (one band at 65bp and 2 smaller bands), and homozygous mutant genotype (one large band at 65bp).

#### **5. IRS-1 (G972R) GENOTYPING BY RESTRICTION ENZYME ANALYSIS**

Restriction enzyme digestion was carried out at 60°C for 12 hours in 15  $\mu$ L reaction buffers containing 10  $\mu$ L of the PCR product, 1.5  $\mu$ L of 10x NEB buffer 2 (New England Biolabs Inc. Ipswich, MA, USA), and 10 U of the restriction enzyme BstNI (New England Biolabs Inc.). Restriction enzyme products were loaded with loading dye in ratio 1:4 on 4% agarose gel and analyzed by Cleaver horizontal electrophoresis. Gel imaging and documentation was performed by Digi Doc-It™ imaging system utilizing UVP life science software. Digestion patterns obtained in wild-type (158, 81, 23 bp), 5 bands sized 159, 108, 81, 51 and 23 bp for G972/R972 heterozygous and 4 bands sized 108, 81, 51 and 23 bp for R972 homozygous figure (2). [26]

**Statistical Analysis** Data was coded and entered using the statistical package SPSS version 16. Data was summarized using mean, standard deviation and rang (minimum and maximum) for quantitative variables. Ratio and proportion for qualitative variables comparison between groups was done using ANOVA (analysis of variants) followed by dunett test to compare the three pathological groups to control healthy group. Correlations were done using Pearson's correlation (r) test, to show the relation between quantitative variables. P-values < 0.05 were considered as statistically significant.

#### **6. RESULTS**

A significant difference was found in BMI, atherogenic index and other biochemical parameters (total-cholesterol, LDL-C, HDL-C, FBS and PPBS) among the studied groups.

**Table1.** Mean ± SD of Biochemical, Demographic and anthropometric Data among Studied Groups

Variable	Group I N=40	Group II N=32	Group III N=44	Group IV N=40	P <sub>2</sub> value
Age(Years) P <sub>1</sub> value	45.2±9.2	50.9±10.4 0.081	49.5±7.4 0.111	49.7±8.4 0.117	0.214
BMI(Kg/m <sup>2</sup> ) P <sub>1</sub> value	22.9±2.6	24.98±0.913 0.491	37.83±8.5 <0.0001*	39.06±8.6 <0.0001*	<0.0001*
AIP	2.66±0.13	0.15±0.13 0.001*	0.17±0.14 <0.001*	0.39±0.17 <0.001*	<0.0001
T-Ch(mmol/L) P <sub>1</sub> value	4.1±0.62	4.77 ± 0.8 0.006*	5.11± 0.31 <0.0001*	4.28±1.08 0.0002*	<0.0001*
TAG(mmol/L) P <sub>1</sub> value	1.25±0.31	1.56±0.34 0.005*	1.52±0.31 0.022*	2.16±0.86 <0.0001*	<0.0001*
HDL-C(mmol/L) P <sub>1</sub> value	1.27±0.12	1.06±0.14 <0.0001*	0.94±0.24 <0.0001*	0.83±0.14 <0.0001*	<0.0001*
LDL-C(mmol/L) P <sub>1</sub> value	2.28±0.48	2.98±0.72 0.001*	3.44±0.39 <0.0001*	3.44±0.915 <0.0001*	<0.0001*
FBG (mmol/L) P <sub>1</sub> value	4.49±0.56	10.54±3.14 <0.0001*	10.76±4.08 <0.0001*	10.02±3.28 <0.0001*	<0.0001*
PPBG(mmol/) P <sub>1</sub> value	6.33±0.83	14.37±3.93 <0.0001*	14.80±4.95 <0.0001*	14.09±4.50 <0.0001*	<0.0001*

\* Statistical significance, P<sub>1</sub>: Patients groups vs group I (control), P<sub>2</sub>: among the 4 groups. AIP: Atherogenic index of plasma, BMI: Body mass index, TAG: Triacylglycerol, HDL-C: High density lipoprotein-Cholesterol, LDL-C: Low density lipoprotein-Cholesterol, FBG: Fasting blood glucose, PPBG: 2 hours postprandial blood glucose.

### 7. SERUM INSULIN AND ADIPOKINES

Serum Insulin, visfatin and IL-6 levels were significantly increased in patient groups versus the control one. Leptin highest concentration was in diabetic obese group and lowest concentration in diabetic lean group with statistically significant difference between each patient group (groups II, III and IV) and the control group (p<0.0001 for each) and among all 4 groups (p<0.0001), table (2).

**Table2.** Mean levels of serum fasting insulin, visfatin, leptin and IL-6 among studied groups

Variable	Group I N=40	Group II N=32	Group III N=44	Group IV N=40
Insulin (µIU/ml)	10.9±3.4	14.9±2.8 <sup>a</sup>	16.1±3.7 <sup>a,c</sup>	22.47±3.6 <sup>a,c</sup>
Visfatin(ng/ml)	15.4±4.5	28.5±5.3 <sup>a,b</sup>	34±6.6 <sup>a,b,c</sup>	39.6±3.45 <sup>a,c</sup>
Leptin (ng/ml)	18.7±6.5	7.2±2.2 <sup>a,b,d</sup>	59.7±16.2 <sup>a,b,c</sup>	39.4±11.1 <sup>a,c,d</sup>
IL-6 (pg/ml)	8.3±3.2	16.6±3.4 <sup>a,b</sup>	19.8±3.9 <sup>a,b,c</sup>	21.1±6.1 <sup>a,c</sup>

<sup>a</sup>P Group I (control group) Vs each patient group (<0.05).

<sup>b</sup>P Group II Vs group III

<sup>c</sup>P Group III Vs group IV

<sup>d</sup>P Group II Vs group IV

Highly significant difference between each two groups was found in serum leptin levels. Also the difference in serum visfatin and IL-6 was highly significant between group II and each of group III and IV.

#### Calculated insulin resistance and sensitivity

HOMA-IR (Homeostatic model assessment) level showed a statistically significant difference between the control group and patient groups (p<0.0001), and among all 4 groups (p<0.0001 for each).

**Table3.** Mean ± SD of calculated IR results

Variable	Group I	Group II	Group III	Group IV
HOMA-IR	2.20±0.87	6.93±1.95 <sup>a</sup>	7.10±4.06 <sup>a</sup>	5.69±2.677 <sup>a</sup>

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The difference between each two groups was insignificant, while it was significant between each group and the control one.

<sup>a</sup>P Group 1 (control group) Vs each patient group (<0.001). <sup>b</sup>P Group II Vs group III

<sup>c</sup>P Group III Vs group IV . <sup>d</sup>P Group II Vs group IV

**Calculated Insulin Resistance and Sensitivity**

HOMA-IR (Homeostatic model assessment) level showed a statistically significant difference between the control group and patient groups (p<0.0001), and among all 4 groups (p<0.0001 for each). The difference between each two groups was insignificant.....

**8. CORRELATIONS**

A significant positive correlations could be detected between insulin and both FBG and IL-6 (r= 0.330; p=0.003, and r=0.265; p=0.019 respectively). Another significant positive correlations were found between visfatin and both leptin and IL-6 (r=0.395; p=0.000, and r=0.750; p=0.000 respectively). Significant positive correlations could be detected between leptin and each of: BMI, T-Ch., LDL-C, PPBG, and IL-6 (r = 0.873; p=0.001, r= 0.314; p=0.005, r= 0.408; p=0.000, r= 0.223; p=0.049, r=0.379; p=0.001 sequentially), but a negative significant correlation was found with HDL-C (r=-0.366; p=0.001). No significant correlations could be detected between leptin and each of FBG or insulin. Regarding IL-6, significant positive correlations could be detected between IL-6 and each of: BMI, T-cholesterol, LDL-C, and FBS (r=0.311; p=0.006, r= 0.356; p=0.001, r=0.383; p=0.001, and r= 0.415; p=0.001), but a negative significant correlation was found between IL-6 and HDL-C (r=-0.342; p=0.002).

There was a significant positive correlation between IL-6 and HOMA-IR, but it was lacking with leptin and visfatin.

**Table4.** Genotype frequency of M326I polymorphism of the p85α regulatory subunit of phosphatidylinositol 3-kinase

M326I Genotype	Group I (n= 40)	Group II (n=32)	Group III (n= 44)	Group IV (n= 40)	Total cases (n= 116)	P value
Homozygous wild (M/M)	35 (87.5%)	22 (68.75%)	17 (38.636%)	11 (27.5%)	50 (43.103%)	
Heterozygous (M/I)	5 (12.5%)	7 (21.875%)	7 (15.91%)	0 (0%)	14 (12.07 %)	
Homozygous mutant (I/I)	0 (0%)	3 (9.375%)	20 (45.454%)	29 (72.5%)	52 (44.827%)	
(M/I)+ (I/I)	5/40(12.5%)	10/32(31.25%)	27/44(61.364%)	29/40(72.5%)	66/116(56.897%)	<0.001

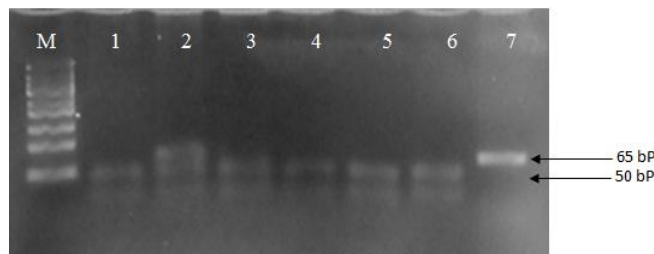
Mutant genotype (M/I)+ (I/I) of the p85α-regulatory subunit of phosphatidylinositol 3-kinase was almost more frequent in diabetic lean, diabetic obese and metabolic syndrome patient than in controls (30%, 61.6%, 71.3% versus 11.8 %, respectively), Using the Chi-Square Tests, the difference was significant (P<0.001). No significant difference in genotypes between each two groups, only the difference was significant between group II and III versus the control group (p = 0.001; OR 15; 95 % CI 2.41–93.0 and p = 0.001; OR 15; 95 % CI 2.41–93.0 respectively) OR (95 % CI) = odds ratio (95 % confidence interval)

**Table5.** Investigated variables in M326I polymorphism of phosphatidylinositol 3-kinase p85α regulatory subunit genotypes

Variables	M/M	M/I	I/I	P
Age (years)	41.9±12.15	43.5±10.85	52.9±6.42	.004
BMI (kg/m <sup>2</sup> )	28.6±7.04	26.1±2.1	39.3±9.4	0.000
T- Ch (mmol/L)	4.57±0.98	4.72±0.94	5.27± 0.70	0.043
TAG (mmol/L)	1.57±0.69	1.43±0.37	1.93±0.76	NS
HDL-C (mmol/L)	1.08±0.28	1.07±0.25	0.88±0.23	0.040
LDL-C (mmol/L)	2.72±0.85	2.85±0.66	3.06±0.66	NS
FBG (mmol/L)	8.09±4.33	10.39±5.66	8.52±1.93	NS
PPBG (mmol/L)	11.35±5.89	14.25±6.67	11.67±2.85	NS
Insulin (µIU/ml)	11.8±3.48	16.0±3.5	12.6±5.54	0.049

Visfatin (ng/ml)	23.7±9.6	31.1±4.4	28.0±14.9	0.032
Leptin (ng/ml)	25.24±19.8	23.45±14.4	48.28±18.8	0.001
IL-6 (pg/ml)	13.4±6.5	15.5±8.1	17.2±5.4	NS
HOMA-IR	4.4±2.9	7.5±6.17	4.6±1.11	0.086

A significant difference was found between different genotypes in some assessed parameters as serum visfatin, leptin, insulin, HDL-C and total cholesterol while the difference in other parameters was insignificant.



**Figure1.** Restriction products of p85a regulatory subunit of PI<sub>3</sub> kinase gene with the restriction enzyme. Lane 1, 3, 4, 5, and 6 shows the homozygous wild genotype (2 bands smaller than 65bp). Lane 2 shows the heterozygous type (one band at 65bp and 2 smaller bands). Lane 7 shows the homozygous mutant genotype (one band at 65bp, which mean no action of restriction enzyme).

**Table6.** Frequency of genotypes and alleles of IRS-1(G972R) gene polymorphism in studied groups

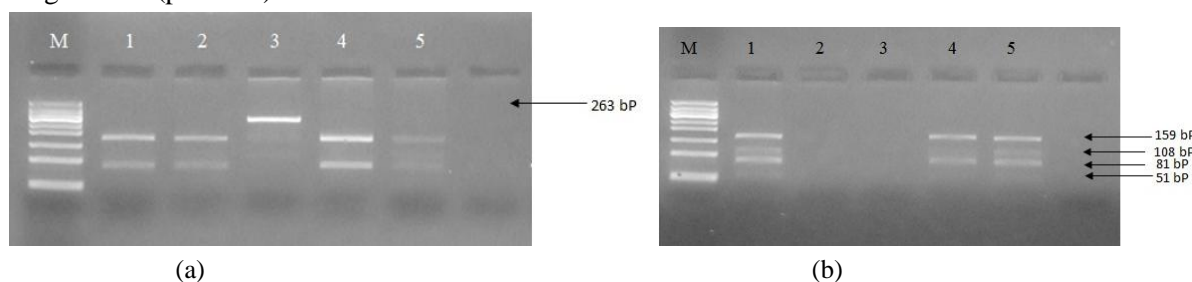
Allele frequency		Frequency	Genotype		
R(mutant)	G (normal)		RR	GG	GR
10.2%	89.7%		n=0 (0.0 %)	n=140 (89.7%)	n=16 (10.2%)

**Table7.** Percentage of mutant and wild gene polymorphism among control and other studied groups

IRS-1 genotype	Group I (n= 40)	Group II (n=32)	Group III (n= 44)	Group IV (n= 40)	Total cases (n= 116)	P-Value
Wild (GG)	34 (85%)	32 (100%)	40 (90.91%)	34 (85%)	106 (91%)	0.38
Mutant (GR)	6 (15%)	0 (0%)	4 (9.09%)	6 (15%)	10 (9%)	

No cases of RR mutation was observed

No significant difference was detected in the genotype of IRS-1 gene between the different studied groups and the control group (p= 0.38). Also the difference between each two groups was insignificant (p > 0.05).



**Figure2.** Restriction products of IRS-1 gene. Lane M: Ladder (50 bp), lanes 1&5(b) show heterozygous mutation: 5 bands (159, 108, 81, 51, 23 bp), lanes 1, 2 & 4(a) show wild type: 3 bands (159, 81, 23 bp). Band 23bp is too small and not seen. Lane 3(a) show the PCR product (263bp)

**Table8.** Investigated variables in genotypes of IRS-1(G972R) gene polymorphism in studied groups

Variable	Wild (GG)	Mutant (GR)	P value
Age(years)	46.1±11.17	44.38±12.29	0.681
BMI(kg/m <sup>2</sup> )	32.4±9.3	31.59±7.25	0.803
AIP	0.18±0.2	0.16±0.19	0.738
T-Ch(mmol/L)	4.9±0.88	4.25±0.75	0.051
TAG(mmol/L)	1.62±0.64	1.49±0.22	0.600
HDL-Ch(mmol/L)	1.04±0.24	1.06±0.32	0.763
LDL-Ch(mmol/L)	2.96±0.75	2.53±0.48	0.123
FBG(mmol/L)	9.28±4.10	6.20±1.54	<0.001
PPBG(mmol/L)	12.80±5.31	8.86±2.34	0.002

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Insulin( $\mu$ IU/ml)	12.7 $\pm$ 3.4	14 $\pm$ 4.8	0.347
Visfatin(ng/ml)	27.1 $\pm$ 8.7	25.9 $\pm$ 10.1	0.707
Leptin (ng/ml)	31.4 $\pm$ 23.3	35.3 $\pm$ 15.3	.531
IL-6(pg/ml)	15.05 $\pm$ 6.03	14 $\pm$ 7.4	0.664
HOMA-IR	5.6 $\pm$ 3.4	4 $\pm$ 1.93	0.209

Fasting and postprandial serum glucose levels were significantly less with mutant alleles. Lipid profile variables as well as HOMA-IR were lesser with mutant alleles though at insignificant degrees.

### 9. DISCUSSION

Metabolic syndrome (MetS) has many pathoclinical components, namely; diabetes, obesity, hypertension, and dyslipidemia in different combinations. Insulin resistance (IR) is identified as a common contributing element. Fat deposited in otherwise subcutaneous aspect is referred to be ectopic fat [27]. This ectopic fat distribution may be more of a predictive factor for cardiovascular risk than obesity [27]. One may suppose that the pathoclinical criteria of MetS are not necessarily associated with obesity, but rather with ectopic fat deposition.

Visfatin and leptin were selected to be studied in correlation with gene polymorphism of PI3-kinase regulatory unit and IRS-1 as mediators of insulin signal transduction supposed to be responsible for IR-associated MetS.

IR pathogenesis is based on a defect in insulin signal transduction. Phosphatidylinositol-3 kinase [28] and insulin-receptor substrate-1 play crucial role in this respect [29].

This pilot study was conducted on four groups, Group I (healthy controls), Group II (lean diabetics), Group III (obese diabetics), and Group IV (patients with MetS).

Serum leptin were significantly elevated in groups III and IV compared to groups II and I. A significant positive correlation was detected between serum leptin and BMI. Similar results were obtained by other investigators [30,31]. *Stringer et al.*, [30] failed to find a difference in leptin levels in diabetic regardless of obesity but *Yared et al.*, [32] noticed higher serum leptin in MetS patients. There was significant increase in the levels of serum visfatin in patient groups versus the control group ( $p = 0.000$ ).

Visceral adipose tissue pools its venous drainage into the liver, containing high concentrations of FFAs, and adipocytokines, viz. leptin, IL-6, TNF- $\alpha$ , resistin, visfatin, adiponectin and others. These factors would promote hepatic insulin resistance [33]. IL-6 and TNF- $\alpha$  have been shown to induce suppressor of cytokine signaling-3 (SOCS3) [34], a protein interfering with tyrosine phosphorylation of the insulin receptor and IRS-1 and to cause ubiquitination and proteasomal degradation of IRS-1 [35]. This, in turn, impairs translocation of the insulin-responsive glucose transporter, GLUT-4, to the plasma membrane. In addition, SOCS3 stimulates phosphorylation of the enzyme nitric oxide synthase (NOS) so, it reduces NO generation [36], promoting pathogenesis of hypertension. Leptin is a pro-inflammatory and platelet pro-aggregatory factor; contributing for the inflammatory state in obesity and the consequent atherogenicity [37]. Leptin functions, similar to IL-6, in stimulating expression of apolipoprotein B (apoB). Insulin retained its inhibitory effect on apoB secretion in presence of leptin [38]. Insulin regulates hepatic apo-B availability for synthesis of VLDL mediated by activation of phosphatidylinositol 3-kinase via insulin receptor substrates [39]. With development of hepatic insulin resistance, the suppressive effect of insulin on apoB secretion is lost [40], and the incremental increase in apoB may be one possible mechanism responsible for hepatic hypersecretion of apoB-containing lipoproteins; VLDL. Hepatic VLDL production can be further augmented by increased fatty acid delivery to the liver released from adipose tissue lipolysis [41] with consequent hypertriglyceridemia. IL-6 increases hepatic apoB mRNA. Therefore, dyslipidemia characterizing IR might be secondary to proinflammatory adipokines, and fluxed FFA into the liver, secondary to augmented lipolysis. Hepatic steatosis results from increased lipogenesis and limited lipid export [42].

Insulin resistance is often associated with mitochondrial dysfunction [43] that promotes generation of ROS, resulting in potentiation of insulin resistance [44]. Leptin is able to modulate the expression of heat shock protein 60 (HSP60) [45] that maintains mitochondrial integrity to guard against extramitochondrial leakage of reactive oxygen species [46], suggesting promotion of cellular oxidation stress. This is a contributing factor in development of IR (44).

Serum visfatin in diabetic groups were significantly higher than the control group. *Hammarstedt et al. and Chen et al.* [47, 48] demonstrated a significant elevation of visfatin in diabetics regardless of anthropometric parameters. Other investigators had different findings [49, 50, 51]. Possible explanations for the observed discrepancies among the various studies may include differences in age, sex, ethnic background, duration of diabetes, and concomitant drug therapy. In consistence with our results, *Zhong et al.* [52] reported increased serum visfatin in MetS compared with healthy control, which was proportionate to the number of MetS components [53]. Other investigators [54, 55] denied these observations. The exact mechanism underlying the elevated visfatin in patients with the MetS is unclear. Role of visfatin in the pathogenesis of the MetS are not well-established. Although *Chen et al. and Zahorska-Markiewicz et al.* [48, 56] claimed positive correlation between circulating visfatin and fasting insulin, we found this correlation is lacking between visfatin and either serum insulin or HOMA-IR in all studied groups. Experimentally, visfatin activates insulin receptor, and exerts insulin-like effects in cultured cells and lowers glucose levels in mice [13]. *Dogru et al. and Kamińska et al.* [57, 58] obtained similar findings to ours. Visfatin has been identified as a proinflammatory cytokine [59]. Its levels were positively correlated with that of IL-6 in all groups suggesting their co-regulation. IL-6 and visfatin serum levels might be correlated because of the presence of both negative and positive feedback loops. IL-6 has been shown to induce SOCS-3, [34], attributing its share in pathogenesis of IR.

The prevalence of T2DM was assessed in individuals whose DNA had been genotyped for the polymorphism at codon 326 phosphatidylinositol 3-kinase p85 regulatory subunit gene [60]. We found that this mutation is more prevalent in diabetic lean, diabetic obese and metabolic syndrome patient than in controls (30%, 61.6%, 71.3% versus 11.8 %, respectively), and the difference was highly significant ( $P < 0.0001$ ). Another study revealed lesser incidence of such mutation among T2DM [28].

Regarding the relevance of this polymorphism for insulin sensitivity, we observed that the M326I substitution in the phosphatidylinositol 3-kinase p85 regulatory subunit gene was significantly related to a higher fasting insulin and HOMA-IR and also to a more atherogenic lipid profile and atherogenic index, as well as to a higher prevalence of T2DM and metabolic syndrome. Therefore, PI3K p85 regulatory subunit gene polymorphism may participate in pathogenesis of IR characterizing T2DM and metabolic syndrome.

Normal IRS-1 phosphorylation, following insulin/receptor binding, leads to IRS-1 interaction with the PI3-K p85 regulatory subunit. PI3-K subsequently activates endothelial NO synthase (eNOS) and enhances eNOS transcription [61]. Endothelial dysfunction with reduced eNOS activity is a pivotal step in the pathogenesis of atherosclerosis, a feature of insulin-resistance [62]. This of particular importance in presence of IL-6 induced SOCS-3 as mentioned before. Hyperinsulinemia-associated IR might exert potentially dangerous mitogenic effects on smooth muscle cells [63], an additional atherogenic factor.

The relationships of variants of p85 $\alpha$  with diabetes and relative phenotypes have been analyzed in several studies with inconsistent results [64]. In Danish Caucasians, *Hansen et al.* [65] found no association of the M326I polymorphism with the risk of diabetes. By contrast, *Baier et al.*, [60] found that the I326I genotype was associated with a decreased prevalence of diabetes and higher acute insulin response in Pima women. Studies in Japanese and Swedish Caucasian populations [ 66] did not support this polymorphism as a susceptibility variation for T2DM.

Genetic heterogeneity across races and ethnicities might partially explain the discrepancy of these studies, also the difference in sample size besides environmental factors accounts for disagreement of these studies. The frequency of the M326I allele in our pilot observational study was relatively high (56.7% in cases and 11.7% in control).

Lean and obese diabetics (groups II & III) are lacking the full-blown picture of metabolic syndrome [67]. The frequency of gene mutation is least in group II, rises in group III, and is highest in group IV. This suggests that this type of gene mutation might be predictive for development of MetS in either apparent healthy, lean diabetics (type II), and/or obese diabetics. This may suggest that the full picture of MetS begin with T2DM, followed by obesity and other features of MetS come sequentially.

It remains to be determined whether this variant might be the causative polymorphism or in linkage with an unidentified etiological variables; genetic, acquired or environmental. This might be



important in the view the recent issue of “immunometabolism,” which connects control systems developed for regulation of immunity with those that control metabolism. The macrophages invading the adipose tissue develops the so-called "adipose tissue remodeling"[68]. The circumstances of such process need further clarification. Large scale study may be necessary for better evaluation and explanation.

In the current study, searching for the frequency of genotypes associated with polymorphism of insulin receptor substrate-1 (IRS-1) gene at G972R is not discriminative among our Egyptian diabetics as well as the normal controls. We observed percentage of occurrence of this mutation was 9 % and 15 % among diabetic patients and control subjects respectively. Fasting and postprandial serum glucose levels were significantly less with mutant alleles. Lipid profile components as well as HOMA-IR were lesser with mutant alleles though at insignificant degrees. Low number of investigated subjects, besides other genetic, acquired or environmental contributing factors may account for these findings.

Existence of mutation in the control group may suggest that phenotype features of glucose intolerance might not be developed due to epigenetic elements or subjective factors. In obese adults, however, G972R appears to increase insulin resistance in its heterozygous form [69]. G972R variant is not associated with insulin resistance in adults with T2DM but is possibly associated with higher insulin and glucose levels [70]. This variant gene might be associated with mild impairment of glucose tolerance rather than overt diabetes because the variant itself is apparently insufficient for the development of T2DM [71]. Similar findings were observed among Japanese [72], and Scandinavian people [73]. Mechanism of IR mediated G972R polymorphism might be reduction of phosphorylation of IRS-1 and inhibition of the insulin receptor kinase [74]. G972R, a common variant of IRS-1, lies between two potential sites of tyrosine phosphorylation involved in binding the p85 subunit of PI-3 kinase. This variant impairs insulin signaling [8].

Expression of the R972 variant of IRS-1 causes a defect in binding of the p85 regulatory subunit of PI3K to the IRS-1, resulting in a decrease in IRS1-associated PI3K activity and subsequent decreased activity of the Ser/Thr kinase Akt, a key enzyme linking PI3-K activation to multiple biological functions of insulin [75].

Because of the relatively low frequency of the R972 allele, well constructed large population studies are required to assess the role of this variant in pathogenesis of IR in a future study.

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