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ASSOCIATION OF NEUROPEPTIDE-Y -399 (T/C) GENE POLYMORPHISM AND ITS LEVEL WITH THE RISK OF TYPE-2 DIABETES MELLITUS

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ABSTRACT

Neuropeptide-Y (NPY) is widely distributed centrally and peripherally and plays an important role in regulating glucose metabolism, energy metabolism and vascular function. Several studies had found that NPY is involved in the pathophysiology of type-2 diabetes Mellitus (T2DM). The aim of this study was to evaluate the association of *NPY* -399 (T/C) gene polymorphism and its serum level with susceptibility to T2DM. Seventy diabetic patients and seventy age and gender matched healthy controls were enrolled in this study. Serum NPY was measured by ELISA and *NPY* rs16147(-399 T/C) polymorphism was analyzed using the TaqMan allelic discrimination assay technique**.** Results showed significant statistical differences between the two studied groups regarding serum NPY level and *NPY* rs16147 (-399T/C) genotype distribution with increased serum NPY level and increased frequency of the CC and TC genotype in patients with diabetes mellitus. **Conclusion:** CC genotypes of *NPY* (-399 T/C) gene polymorphism and its associated high serum NPY level might be a genetic risk factor for T2DM.

Keywords: Type-2 diabetes mellitus, neuropeptide-Y, insulin resistance, gene polymorphism.

INTRODUCTION

Type-2 diabetes Mellitus (T2DM) is a multifactorial disorder characterized by chronic hyperglycemia, insulin resistance and impaired insulin secretion and/or action (**Patel et al., 2016**).

T2DM is a complex metabolic disease interplay of genetic and environmental factors. The development of diabetes involved several

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pathogenic processes which are autoimmune destruction of the β-cells of the pancreas with consequent insulin secretion abnormalities that result in resistance to insulin action and caused by alterations in several gene products. Identification of the genes responsible for the pathogenesis of the disease is very important (**Hasibe Cingilli Vural, 2017**). The hormonal dysfunctions are sometimes referred to either as primary or secondary causes of T2DM (Shpakov et **al., 2015).**

Neuropeptide-Y (NPY) is a 36-amino acid neuromodulator secreted by the central and peripheral nervous systems. The *NPY* gene is located on chromosome 7, it is about 8 kb in length with four exons separated by three introns . The effect of NPY is mediated by multiple G protein coupled receptors (Y1-Y5), these receptors are found in many tissues including those involved in metabolism like adipose tissue and liver and this explains its action on metabolism **(Pedragosa-Badia et al., 2013)**. Also, NPY is known to play a role in the regulation of satiety, body weight, and insulin release, and it has been associated with loss of beta-cell mass in type-2 diabetes. There is an evidence for a strong correlation between structural and promoter polymorphisms of *NPY* gene and with susceptibility to T2DM and alteration of lipid metabolism. -339T/C (rs16147) polymorphism of *NPY* was found to be associated with T2DM patients **(Patel et al., 2016**).

Elevated central neuropeptide Y level promotes food intake and reduces energy expenditure, thereby increasing adiposity. Neuropeptide Y is co-localized with noradrenaline in central and sympathetic nervous systems. As a major peripheral vascular contractive neurotransmitter, through interactions with its receptors, neuropeptide Y has been implicated in the pathology and progression of diabetes, by promoting the proliferation of endothelial cells and vascular fibrosis, which may contribute to diabetes-induced cardiovascular disease **(Sun et al., 2017).**

The aim of the present study is to evaluate the association of *NPY* rs16147 (-399 T/C) gene polymorphism and its protein serum level with susceptibility to T2DM.

MATERIALS AND METHODS

This study was carried out in Medical Biochemistry and Molecular Biology department and Internal Medicine out patient

clinic, Faculty of Medicine, Menoufia University. 140 subjects were included in this study; they were 70 diabetic patients (39 males and 31 females) with mean age of 54.6±7.6 years and 70 age and gender matched healthy controls (42 males and 28 females) with mean age of 54.1±7.2 years. The diagnosis was based on the American Diabetes Association (ADA) criteria **(Standards of medical care in diabetes, 2014)**. The study was approved by ethical committee of Faculty of Medicine, Menoufia University.

A written consent was obtained from all subjects before the study. All studied subjects were subjected to complete history taking, physical examination including anthropometric measurements. Estimation of body mass index (BMI) was done by dividing body weight in kilograms by the square of body height in meter **(Enyioma et al., 2002).** Laboratory investigations included estimation of lipid profile [serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDLc) and low density lipoprotein cholesterol (LDLc)]. Determination of fasting and 2 hours post prandial blood glucose levels, glycated hemoglobin (HbA1c), fasting serum insulin and serum neuropeptide Y (NPY) by ELISA. Insulin resistance was assessed by homeostatic model assessment of insulin resistance (HOMAIR) **(Matthews et al., 1985)** and genotyping of neuropeptide Y (NPY) -399T/C (rs 16147) using the TaqMan Allelic Discrimination assay technique (real time PCR).

After 12 hours overnight fasting, 9 ml of venous blood were withdrawn from every subject by sterile vein-puncture and divided into three tubes.

4 ml of blood were transferred into two EDTA tubes: one of them was used for quantitative colorimetric determination of glycated hemoglobin as percent of total hemoglobin using kits supplied by Teco diagnostics, USA (**Gonen, 1987**), the other EDTA tube was preserved in -20°C for DNA extraction and further molecular analysis.

One ml of blood was transferred into sodium fluoride tube and another sample of blood was collected after 2 hours for enzymatic colorimetric determination of blood glucose. Blood glucose was determined by enzymatic colorimetric test, using Spinreact kit, SPAIN **(Trinder, 1969**).

4 ml of blood were transferred into a plain tube and allowed to clot at 37°C, centrifuged for 10 minutes at 4000 r.p.m. The clear

supernatant serum was separated and kept frozen at -80°C until determination of serum HDL (**Gordon et al., 1977**), TC (**Rifai et al., 2006**), TG **(Fossati et al., 1982)**, LDL **(Friedewald et al., 1972)**, serum level of NPY by ELISA using **(SinoGeneClon Biotech**) and fasting serum insulin level **(Judzewitch et al., 1982)**. Serum TC and TG were determined by enzymatic colorimetric test, using Spinreact kit, SPAIN. Serum HDL-c was determined by colorimetric method, using Human kit, GERMANY. LDL-c was calculated from TC concentration, HDL-c, TG according to Friedewald equation (**Friedewald et al., 1972**). Serum insulin was determined by enzyme linked immunosorbent assay method using DRG® Insulin ELISA kit, GERMANY **(Judzewitch et al., 1982)**. Assessment of insulin resistance was done by homeostatic model assessment (HOMA) according to **(Matthews et al., 1985).** HOMA-IR = fasting glucose (mg/dl) x fasting insulin (µIU/mL) / 405. Genotyping of NPY -399T/C (rs16147) polymorphism: DNA extraction using Zymo Research Quick-g DNA mini prepGenomic DNA purification kit (USA). DNA was eluted and stored at -20 C for further PCR procedure.

NPY gene was genotyped using allelic discrimination assay by real time PCR technique using Taq Man probe, Applied Biosystems, USA. The maxima probe qPCR Master Mix (40X), primers and probes were supplied from Thermo Fisher Scientific; the forward primer was 5´- GCTTCCTACTCCGGCACCCAGTGGG 3´ and the reverse primer was 5´-TGGTAGTCCTGTTGGCAGGAGACAA 3´10 µl of master mix was added to 1.25 µl of the genotyping assay of primer/ probe mix and 3.75 µl of DNAase-free water. 5 µl of genomic DNA extract for every sample and 5 µl of DNAase-free water for the negative control reaction were applied.. The following cycling conditions were used: Initial denaturation was done at 95°C for 10 minutes, followed by 40 cycles of: denaturation at 94°C for 15 seconds, primer annealing at 50°C for 60 seconds then extension at 72°C for 2 minutes and the last extension at 72°C for 1 minute. Analysis of data was completed using 7500 Real-Time PCR instrument, version 2.0.1, Applied Bio systems.

Statistical analysis:

The data collected was tabulated and analyzed by IBM personal computer and statistical package SPSS version 20. Chi-

square test is used to study the association between two qualitative variables. For comparison between more than two groups Kruskal–Wallis test for not normally distributed variables and one way analysis of variance test (ANOVA) for quantitative variables were used. Student's t test was used to assess the statistical significance of parametric data. Spearman's correlation was used for skewed distributed quantitative variables. Multiple regression analysis was performed to calculate the effects of risk factors as independent. Values less than 0.05 were considered significant.

RESULTS

The study included 70 diabetic patients and 70 age and gender matched apparently healthy individuals. There were significant statistical differences regarding the BMI, FBG, 2 hr pp, serum insulin, HOMA-IR, lipid profile (serum TC, TG, LDLc, HDLc) (P<0.0001) and a significant increase in the serum level of NPY was found in diabetic patients (180.6±21.8ng/l) compared to controls (52.1±5.8ng/l) (P<0.0001) as shown in **(table1).**

As regards *NPY* rs16147 (T/C) genotype distribution between the two studied groups; it showed a significant statistical difference, with increased frequency of the CC and TC genotypes and C allele in diabetic group and increased TT genotype and T allele frequency in the control group **(P <0.001; Table 2)** .

The results also showed that the CC genotype of *NPY* rs16147 (T/C) increases the risk of diabetes by 14.9 fold and TC genotype increases the risk by 8.3 fold, while the C allele increases the risk by 6.8 fold, as shown in **(Table 2)**. CC genotype of *NPY* rs16147 (T/C) polymorphism is associated with high BMI, TC, TG and LDL-c, FBG, 2hr pp and low level of serum HDL-c (P<0.001) **(table 3)**. CC genotype of *NPY* rs16147 (T/C) polymorphism is also associated with high level of serum level of NPY and HOMAIR as shown in **(table 3)**.

Multivariate logistic regression for risk of diabetes showed that the most common risk factor is serum NPY OR; 149.6 (4.6-890.3), followed by BMI OR; 148 (5.4-418.1), HOMA-IR OR; 109.7 (2.8-320.6), CC genotype of *NPY* rs16147 (T/C) polymorphism OR; 89.2 (1.8-296.5), TAG OR; 56.8 (1.5-176.4), serum insulin OR; 5.9 (0.576-63.4), TC genotype of *NPY* rs16147 (T/C) polymorphism OR; 5.3 (0.623-53.8), **(Table 4)**.

There was significant correlation between serum NPY level and HOMA-IR (r =0.39 , p value 0.001) **(figure 1). Figure 2** showed the allelic discrimination plot of the TT, TC and CC genotypes of NPY rs16147 (T/C).

Table 1: Demographic and clinical characteristics in DM (group1) and control (group2)

	Patients N=70	Control N=70	P value
Age (years)	54.6±7.6	54.1±7.2	0.987
Sex			
Male	39 (55.7%)	42 (60%)	0.608
Female	31 (44.3%)	28 (40%)	
BMI (kg/m^2)	27.7±2.6	21±1.7	<0.001*****
SBP (mm.Hg)	140.6±11.5	112.7±9.3	<0.001*****
DBP (mm.Hg)	92.2±7.5	77.3±7.8	<0.001*****
FBS (mg/dl)	214.4±32.7	90.3±8.3	<0.001*****
2h pp (mg/dl)	242.7±34.2	89±9.1	<0.001*****
HbA1c (%)	7.7±1	5.1±0.8	<0.001*****
HDLc (mg/dl)	32.9±1.4	49.3±1.3	<0.001*****
T.cholesterol (mg/dl)	210.9±27.1	170.9±10.1	<0.001*****
LDLc (mg/dl)	145.9±27.1	103.5±9.4	<0.001*****
TG (mg/dl)	166±9.3	93.4±4.1	<0.001*****
F.insulin (uIU/ml)	21.9±2.7	5±0.4	<0.001*****
HOMA-IR	11.1±2.9	0.9±0.2	<0.001*****
Serum NPY(ng/l)	180.6±21.8	52.1±5.8	<0.001*****

BMI:body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, FBS: fasting blood sugar, 2HPP: two hour post prandial, HbA1C: glycated hemoglobin, TG: triglycerides, LDLc: low density lipoprotein cholesterol, HDLc: high density lipoprotein cholesterol, F. insulin: fasting insulin, NPY: neuropeptide-Y, *: X^2 chi square test.

Table 2: Comparison of NPY rs16147 genotypes between the studied groups.

	Patients N=70	Control N=70	P value	OR (95% CI)
***NPY* rs16147**				
CC	31 (44.3%)	8 (11.4%)		14.9 (5.5-40.03)
TC	26 (37.1%)	12 (17.1%)	<0.001*****	8.3 (3.3-20.8)
TT*	13 (18.6%)	50 (71.4%)		
***NPY* rs16147 allels**				
C	88 (62.9%)	28 (20%)	<0.001*****	6.8 (3.9-11.6)
T*	52 (37.1%)	112 (80%)		

*****: X^2 chi square test, OR: odd's ratio, CI: confedence interval.

Table 3: Biochemical parameters of the studied diabetic patients in different genotypes of *NPY* rs16147.

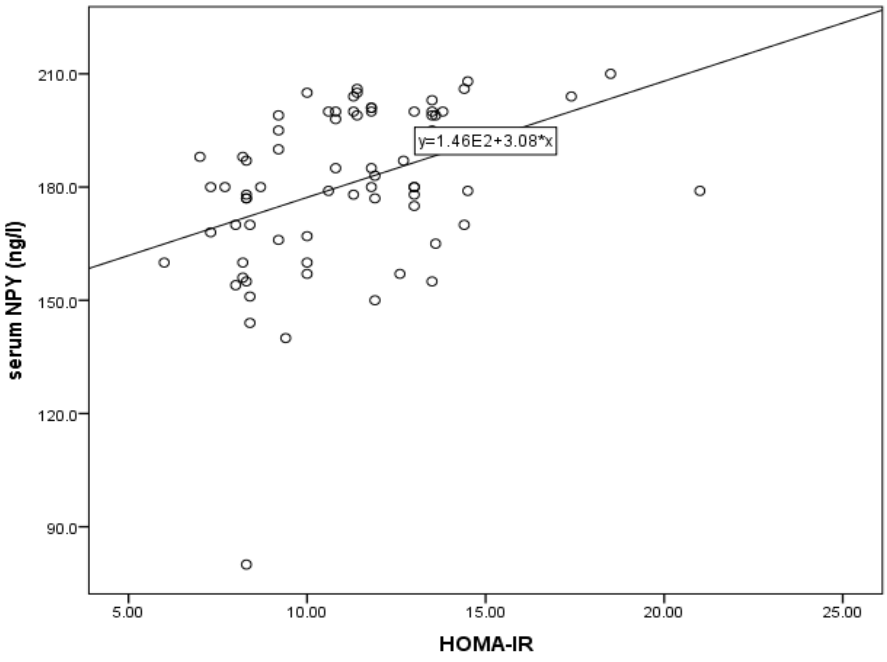
	CC	TC	TT	P value
Age (years)	54.6±7.2	54.5±8.3	54.6±7.5	0.997#
BMI (kg/m^2)	26.4±3.5	25.8±4.2	22.1±2.9	<0.001#
SBP (mm.Hg)	131.7±15.1	133.2±18.1	119.4±15.9	<0.001#
DBP (mm.Hg)	87.3±9.2	87.9±11.6	81.3±10.1	0.002#
FBS (mg/dl)	188.9±59.5	176.2±64.9	113.2±50.3	<0.001*
2hr pp (mg/dl)	211.8±72.8	193.3±76.5	120.9±64.4	<0.001*****
HbA1c (%)	7.2±1.6	6.9±1.4	5.7±1.2	<0.001*****
HDLc (mg/dl)	35.1±6.6	37.1±8.1	45.1±6.6	<0.001*****
T.cholesterol (mg/dl)	203.9±31.4	199.5±31.9	177.7±17.2	<0.001*****
LDLc (mg/dl)	137.9±31.9	134.4±32.8	110.8±17.3	<0.001*****
TG (mg/dl)	173.5±6.4	162.9±5.4	154.4±4.9	<0.001*****
F.insulin (uIU/ml)	17.9±7.3	15.6±8.5	7.1±6.3	<0.001*****
HOMA-IR	9.3±4.9	7.9±5.5	2.7±1.8	<0.001*****
Serum NPY (ng/l)	164.7±61.3	138.7±59.1	73±42.5	<0.001*****

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, FBS: fasting blood sugar , 2hr pp: two hour post prandial, HbA1C: glycated hemoglobin, TG: triglycerides, LDLc: low density lipoprotein cholesterol, HDLc: high density lipoprotein cholesterol, NPY: neuropeptide-Y, #F: ANOVA test and *K: kruskal wallis test.

Table 4: Multivariate logistic regression for risk of DM.

	B	P value	OR	95% CI
Genotype				
CT	1.7	0.217	5.3	0.623-53.8
CC	3.8	0.001	89.2	1.8-296.5
HDLc (mgldl)	-4.4	0.014	0.012	0.001-0.191
T.cholesterol (mgldl)	0.668	0.632	1.95	0.122-8.5
LDLc (mgldl)	1.4	0.312	3.9	0.259-19
TG (mgldl)	2.7	0.02	56.8	1.5-176.4
Serum insulin (uIU/ml)	1.8	0.242	5.9	0.576-63.4
HOMA-IR	4.7	0.012	109.7	2.8-320.6
Serum NPY (ng/l)	5.008	0.005	149.6	4.6-890.3
BMI (kg/ m^2)	4.99	0.003	148	5.4-418.1

HDLc: high density lipoprotein cholesterol, LDLc: low density lipoprotein cholesterol, TG: triglycerides, NPY: neuropeptide-Y, BMI: body mass index.



R=0.39 p value 0.001

Figure 1: Correlation coefficient between serum NPY & HOMA-IR in group I.

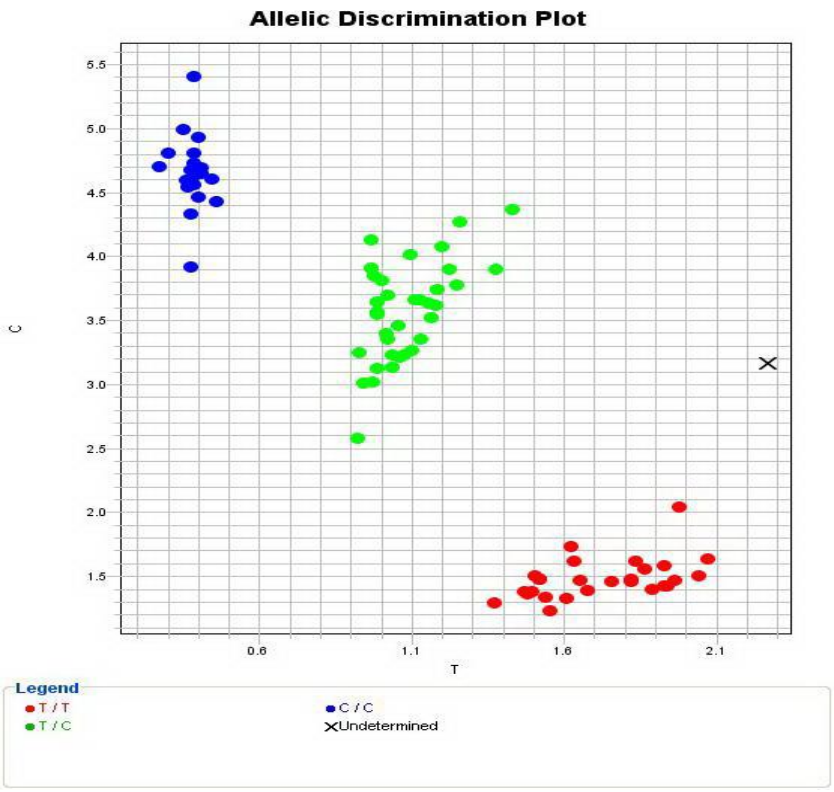


Figure 2: Allelic discrimination plot showing TT, TC and CC genotypes of *NPY* rs16147 (T/C).

DISCUSSION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels **(Babikr et al., 2016)**. Diabetes-induced atherosclerotic cardiovascular disease is the leading cause of death of diabetic patients. Neuronal regulation plays a critical role in glucose metabolism and cardiovascular function under physiological and pathological conditions, among which, neurotransmitter neuropeptide Y has been shown to be closely involved in these two processes **(Sun et al., 2017)**.

In the present study, There was a statistically significant difference between the two studied groups regarding serum FBG, HBA1C, SBP, DBP, 2HPP, TC, TG, LDLc, HDLc, fasting insulin, HOMA-IR and BMI. This result was in agreement with **Bhambhani et al., 2015** who reported that hyperlipidemia is the commonest complication of diabetes mellitus and it predisposes to premature atherosclerosis and macrovascular complications. Common lipid abnormalities in diabetes are raised serum triglycerides, LDL and cholesterol levels and low serum HDL level.

Wamique et al., 2016 reported that in diabetes mellitus insulin deficiency causes higher metabolization of free fatty acid and can cause disorder in lipid metabolism. As compared to non-diabetic control group, type 2 diabetes mellitus have high triglycerides, low HDL-c levels. and associated with increased risk of cardiovascular disease. Also **Khalid et al., 2015** reported that the diabetic patients had higher BMI, serum triglyceride and HbA1c values whereas, had lower HDL level when compared to non-diabetic subjects. This study revealed that obesity and dyslipidemia were high among diabetic patients.

Shimodaira et al., 2014 reported that elevated TG and decreased HDL-C levels are known to be associated with the development of T2DM. These atherogenic lipid abnormalities often precede T2DM by several years, indicating that altered lipoprotein metabolism is an early event in the development β-cell dysfunction.

In this study serum neuropeptide-Y level was significantly higher in T2DM than control group which in agreement with several studies who had been reported that the levels of NPY were increased in diabetes mellitus (**Ilhan et al., 2010**, **Milewicz et al., 2000 and Satoh et al., 1999**).

Sun et al., 2017 reported that increased level of peripheral NPY not only causes dysregulation in glucose metabolism, insulin resistance (IR) and abnormal lipolysis but also promotes abnormal proliferation of vascular smooth muscle cells (VSMCs) in heart and enhances function of macrophage and platelets. As all these factors are involved in developing diabetic complications including atherosclerosis.

Long et al., 2015 demonstrated that high dose of NPY can inhibit basal, insulin stimulated glucose uptake and consumption in

adipose tissue and that NPY Y5 receptor antagonist can specifically reversed this inhibition. So, they suggested that the use of these antagonists might be pharmacologically beneficial in treatment of some diseases caused by NPY disorders. They attributed the insulin resistant associated with high NPY level is caused by modulation of phosphorylation of PI3K, AKT and GSK3 in adipose tissue and adipocytes.

In the current study there was significant difference regarding the *NPY* rs16147 (T/C) genotype distribution between the two studied groups, with increased frequency of the CC and TC genotypes and C allele in the patient (T2DM) group and increased TT genotype and T allele frequency in the control group.

The result also showed that the CC genotype of *NPY* rs16147 (T/C) increases the risk of T2DM by 14.9 fold and TC genotype increases the risk by 8.3 fold, while the C allele increases the risk by 6.8 fold, with highest serum NPY level in CC genotype, this result was in agreement with **Patel et al., 2016** who reported that there is an evidence for a strong correlation between structural and promoter polymorphisms of *NPY* gene and with susceptibility to T2DM and altering the lipid metabolism. The promoter -339T/C polymorphism of *NPY* was found to be significantly associated with T2DM patients when comparing patients with controls.

Zhou et al., 2008 reported that SNP (rs16147) -399 T/C in *NPY* gene changes its *in vitro* expression and may be responsible for *in vivo* alteration in mRNA expression levels. Also **Sommer et al., 2010** stated that this polymorphism exhibits differences in DNA structure and thereby elevate the expression levels of NPY and in turn the NPY serum level.

In a finding that illustrates a direct epigenetic mechanism for regulation of NPY expression and function, **Melas et al., 2013** reported that a SNP in a rat *NPY*-gene promoter (C/T;rs105431668) affects in vitro transcription and DNA-protein interactions. They stated that the presence of the C-allele enables binding of a transcription factor (CREB2) and a histone acetyltransferase (Ep300) and this correlates with increased hippocampal levels of *NPY* mRNA and H3K18 acetylation, a gene-activating histone modification maintained by Ep300. This explains the increased NPY with the C allele.

Current study revealed that there was significant positive correlation between serum NPY and HOMA-IR in patients. In agreement of this result it was reported that in an experimental model of strees induced obesity, induction of NPY in fat was found to be correlated with insulin resistence that could be attenuated by blockage of the Y2 receptor **(Kuo et al., 2008).** Also**, Sun et al., 2017** reported that NPY contributed to the development of stress-induced obesity accompanied with impaired glucose metabolism and IR.

In addition, NPY activation of the parasympathetic nervous system and the abundance of autonomic nerves in adipose tissue may explain partly the occurance of insulin resistance in adipose tissue and decreased glucose uptake in adipocytes (**Long et al., 2015**).

Lastly, Chronic hyperglycemia has been shown to impair β cell function (glucotoxicity), this glucotoxicity stimulates macrophages to secret proinflammatory cytokines such as interleukin-1 beta (IL1B). IL1B causes not only apoptosis of the β cells and loss of function of these cells **(Imai et al., 2014)** but also it stimulates the synthesis and release of NPY **(Rosmaninho-Salgado et al., 2009)** and both aggrevate the insulin resistance associated with T2DM.

Conclusion:

From this study, it could be concluded that the C allele is associated with a higher serum level of NPY and it may be genetic risk factor for T2DM. Also, serum NPY level was positively correlated with insulin resistance commonly present in T2DM.

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الملخص العربى

علاقة التعدد الجينى-٣٩٩ (C/T) للنيوروبيبتيد Y و مستوى البروتين بالمصل و خطورة حدوث مرض البول السكرى من النوع الثانى

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نيوروبيبتيد Y موزع على نطاق واسع مركزيا و طرفيا و يلعب دورا مهما في تنظيم عمليه التمثيل الغذائي للجلوكوز و الطاقة و وظائف الأوعية الدموية , و قد وجدت العديد من الدراسات أن نيوروبيبتيد Y له دور فى التسبب المرضى لمرض البول السكرى من النوع الثانى. ان **الهدف من هذه الدراسة** هو تقييم نسبة الأشكال الجينية للنيوروبيبتيد Y (T/ C) -٣٩٩ ومستوى البروتين فى مصل الدم في المرضى الذين يعانون من مرض البول السكرى من النوع الثانى. وقد أجريت هذه الدراسة على ١٤٠ شخص مقسمين إلى مجموعتين: ٧٠ مريضا بالبول السكرى من النوع الثاني وهم (المجموعة الأولى) و ٧٠ شخصا أصحاء بمثابة المجموعة الضابطة (المجموعة الثانية). وقد تم قياس النيوروبيبتيد Y في مصل الدم بإستخدام الإيليزا بالاضافة الي دراسة التعدد الجيني للنيوروبيبتيد Y (16147T/ C) بواسطه التفاعل التسلسلي للبلمره الزمنيه. و قد أظهرت النتائج وجود فروق ذات دلالة إحصائية بين المجموعة الاولي والمجموعة الثانيه فيما يتعلق بزيادة مستوى النيوروبيبتيد Y في مصل الدم فى مجموعة المرضى عن المجموعة الضابطة بالاضافة الي التعدد الجيني للنيوروبيبتيد Y (16147T/ C) مع زيادة تواتر النمط الوراثي CC و TC في المرضى المصابين بمرض البول السكري من النوع الثاني وزيادة النمط الوراثي TT في المجموعه الضابطه. **الإستنتاج:** يمكن إعتبار التعدد الجيني CC لجين النيوروبيبتيد Y وإرتفاع مستوى البروتين في مصل الدم المصاحب لهذا التعدد الجينى كعامل خطر فى مرضي البول السكري من النوع الثانى.