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Association of Carnosinase-2 Single Nucleotide Polymorphism (rs 6566810) with Serum Carnosine and Carnosinase-2 Levels in Type 2 Diabetics with Cardiovascular Diseases in Iraq

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Abstract

Carnosine has several important biological activities, as implicated by its effects on several diseases such as cardiovascular disease, diabetes mellitus and cancer and can play an important role in improving the functional capability during ischemic circumstances. Carnosine levels in diabetes patients may be affected by genetic variants in the carnosinase-2 gene, which may also be linked to the development of cardiovascular problems. The purpose of this study was to examine the impact of the Carnosinase-2 gene polymorphism SNP (rs 6566810) on serum levels of carnosine and carnosinase-2 and their relationship to CVD in type 2 diabetes (T2DM) patients with and without CVD compared to healthy controls. Method: The serum concentrations of carnosine and carnosinase-2 were determined using ELISA-specific kits. The carnosinase-2 gene (CN2) was subjected to the high-resolution melt technique (HRM) to identify gene polymorphisms. Results: Carnosinase-2 levels were considerably raised in the T2DM with CVD group, but serum carnosine levels were significantly decreased in both groups of diabetic's. The polymorphisms of SNP (rs 6566810) have no effect on both serum carnosine & carnosinase-2 levels, but increases risk of CVD through (AC) hetrogenotype. Conclusion: polymorphisms of SNP (rs6566810) of the carnosinase-2 has no role in CVD as a complication of T2DM.

Keywords: Carnosine, Carnosinase -2, CVD, Polymorphism, T2DM.

Introduction

The metabolic disorders of diabetes mellitus (DM) are mostly related to persistent hyperglycemia as a result of a reduction in beta-cell insulin production, which is usually associated with a deterioration of the response to insulin[(1)]. Diabetic complications and increased risk for many diseases are a result of damage to a variety of biological systems, including blood vessels, eyes, kidneys, heart, and nerves[(2,3)].Type 2 diabetes mellitus (T2DM) is a significantly more frequent type of DM, doubles or quadruples the risk of death from cardiovascular disease or stroke, and is associated with both micro- and macro-vascular complications, such as accelerated atherosclerosis leading to severe peripheral diseases[(4,5)]. Previous studies have ensured carnosine's role in treatment and protection against many diseases like Alzheimer, Parkinsonism, neuropathy, and nephropathy and also that it can alleviate aging-related vascular diseases, such as atherosclerosis in T2DM and related complications, i.e., carnosine may prevent the development of cardiovascular disease linked to vascular calcification and atherosclerosis, including diabetes and chronic renal failure[(6,7)]. Carnosine is a naturally occurring dipeptide (beta-alanyl-L-histidine) expressed in both the central nervous system and the periphery in both vertebrate and invertebrate organisms. Also, it's found in several tissues, most notably in muscle, as an appreciable fraction of the total water-soluble nitrogen-containing compounds[(8)]. Carnosine degradation by the carnosinase enzyme takes place in serum and tissues; it's a homologous enzyme. There are two human isoforms of the enzyme carnosinase: serum carnosinase-1 (CN1) (EC 3.4.13.20). Highly active and abundant CN1, which is found in serum and brain tissues, catalyzes the degradation of both carnosine and homocarnosine, while carnosinase-2 (CN2) (EC 3.4.13.18), a Mn2+-dependent CN2, is expressed mainly in tissues and described as "cytosol nonspecific dipeptidase." High activity and selectivity of serum CN1 result in fast degradation of circulating carnosine[(9)].

Some gene polymorphisms, especially single nucleotide polymorphisms (SNPs), may lead to many diseases like rheumatoid arthritis (RA), nephrotic syndrome, and decreased treatment response[10,11]. Whereas, genetic polymorphisms related to enzymes that affect serum levels of related proteins like carnosinease-2 and carnosine level could lead to increased risk of many diseases, hence being related to macrovascular complications like in the case of T2DM[12]. In this research, a novel single nucleotide polymorphism (rs6566810) in the coding gene for the carnosinase-2 gene, located on chromosome 18q22.3, which is related to one of the enzymes that are involved in carnosine metabolism, was studied to investigate the possibility of being related to the development of CVD in T2DM in relation to serum carnosine.

Materials and Methods

This case-controlled study was conducted as a multicenter study in Baghdad, Iraq, including the National Diabetic Center and Ibn Al-Bitar Center for Cardiac Surgery, during the period from April 2022 to October 2022. Ethical approval with the number (RECAUBCP9112021A) was obtained on November 29, 2021, from the Scientific and Ethical Committee of the College of Pharmacy, University of Baghdad. A total of 150 Iraqi adult subjects (age range of 35–65 years) of both sex were enrolled in the study and were divided into three groups. The diabetic patients, who were already on anti-diabetic treatment, had been divided into two groups, with 50 patients in each group. The diabetic patients (100) were diagnosed by a specialized physician (at least 5 years ago) based on the American Diabetes Association (ADA) criteria[13]: Group 1: included (50) patients with T2DM without complications; Group 2: included (50) patients with T2DM and cardiovascular complications (angina, myocardial infarction, stroke), with an ECG-based diagnosis, supported by information from their medical records and family history of cardiovascular diseases (CVD)[14]. In addition to the fifty apparently healthy subjects in Group-3 to serve as control, subjects with chronic kidney or liver disease were not included, nor were those with autoimmune diseases or vegetarian subjects.

Inclusion criteria

Diabetic patients had been diagnosed with type 2 DM for at least 5 years ago by a specialized physician based on the American Diabetes association (ADA criteria) [13]. These patients are already on anti-diabetic treatment.

Exclusion criteria

Patients diagnosed with other types of diabetes like T1DM, gestational diabetes; T2DM with complications other than CVD; patients having chronic kidney and liver disease; patients having autoimmune disease; vegetarian patients.

Specimen collection and handling

About three milliliters of venous blood samples were obtained from each participant after about 12 hours of fasting. One ml of the collected blood was transferred to an ethylene diamine tetra-acetic acid (EDTA) tube and stored at (+2 to +8℃) for analysis of HbA1c and, 1 milliliter of blood was collected into another EDTA tube for DNA extraction and genotyping by High Resolution Melt (HRM) real time PCR. The remaining blood sample was transferred to a gel containing tube to collect serum after clotting and centrifugation at (3000 rpm) for 5 minutes. Serum was stored as aliquots at -20℃ for measuring of carnosine, carnosine synthase-1 by enzyme-linked immunosorbent assay (ELISA)[15,16].

Polymorphism study by (HRM) steps

Genomic DNA extraction

Genomic DNA was extracted from the peripheral blood leukocytes (frozen EDTA blood samples) by Easy Pure Blood Genomic DNA Kit and then measurement the concentration and purity of DNA. By a Nano drop UV spectrophotometer by which the optical density of DNA (1.5 µl) was measured at two wavelengths (260 and 280 nm)[17]. In most samples, DNA preparation gave A260/A280 ratio between 1.8 and 2.0, which is considered to be suitable for further analysis in detection gene polymorphisms. The measurement of DNA concentration for most of the samples was in the range of (25-118) ng/ml.

The primers preparation

The primers applied in this study are listed in Table 1, were lyophilized, and they were liquefied in the nuclease-free water to give a final concentration of 100 pmol/µl as stock solution. The stock was kept at -20˚C, to prepare 10 pmol/µl concentrations as work primer suspended, 10 µl of the stock solution in 90 µl of nuclease free water to reach a final volume of 100 µl, the primers were supplied by Alpha DNA company/USA.

Table1. The Primers of rs(6566810) in Carnosinase-2 SNP for HRM Technique

SNP	Primer Sequence	Tm(℃)
rs6566810	F-´5 TGACGTCACCTTCCGCAC 3´ R-´5 CCCACCAATCCAGCCACA 3´	54

HRM =High-Resolution Melt analysis, SNP= single nucleotide polymorphism

High-resolution melting technique

Three of the genetic variations were chosen to investigate their relationships with Iraqi T2DM Patients. These SNP (rs6566810) detections were achieved by using HRM real-time PCR.

Thermal profile of HRM technique

The annealing degree was 54 and using EVA green dye, the reaction took about 120 minutes as showed in Figure-1.

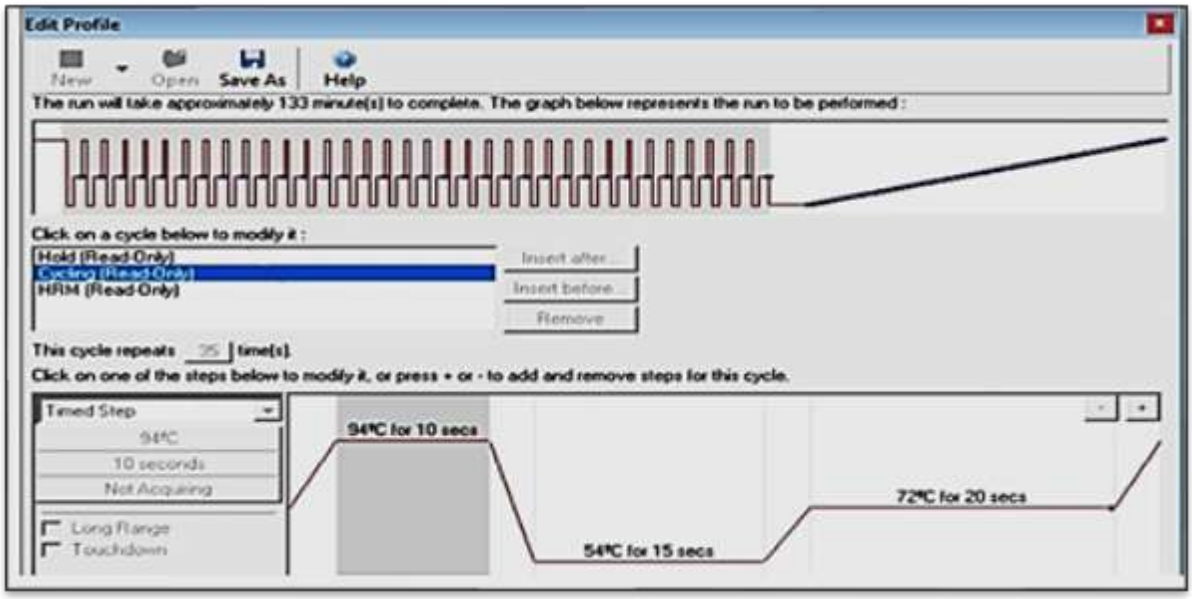

Figure 1. The Thermal Profile for HRM Analysis in Real-Time P

Carnosine and Carnosinase-2

Measurement

Assay Principle

The Human Carnosine, Carnosinase-2 solid-phase sandwich ELISA (enzyme-linked immunosorbent assay) was used to determine the quantity of target Ag bound by a matched antibody pair. In the wells of the supplied microplate, a target-specific antibody has been pre-coated. In these wells, samples, standards, or controls bind to the immobilized (capture) antibody. After that the second (detector) antibody was added, and then a substrate solution was added, which interacts with the enzyme antibody-target complex to create a quantifiable signal. This signal's strength is proportional to the concentration of the target contained in the original material[15,16].

Statistical analysis

Version 11.63 of the WINPEPI computer program was used to evaluate the statistical significance of the P values calculated with Fisher's exact test as well as the odds ratio that was assessed by a special $\chi 2$ formula (Abramson, 2011). Analysis of variance (ANOVA) were used to compare differences between means. Means followed by different (a,b,c) letters are significantly different according to Dunca n's multiple range comparisons (DMRTs), while means followed by the same letter are not significantly different. A Chi-square test was used to compare non-continuous variables. Pearson correlation was performed, and the correlation coefficient (r) was used to calculate the association between parameters. P-values equal to 0.05 and 0.01 are used to characterize significant differences.

Results and Discussion

. According to the current study's results, there are significant differences ($p<0.01$) in the carnosine serum level in two patient groups, and there are substantial differences ($p<0.01$) in the carnosinase-2 measured in the serum between the patient and control groups.as illustrated in Table 3.

Table2. Descriptive Data for Participants Included in the Study

Parameters	(Group-1) T2DM without complications	(Group-2) T2DM with CVD	(Group-3) Healthy control
Number	50	50	50
Sex (F/M)	25/25	25/25	25/25
Age(years)	44.00 ± 6.52 [a]	44.04± 6.59[a]	45.84 ± 7.89[a]
BMI(Kg/M^2)	27 ± 4.25 [a]	26.36 ± 3.20 [a]	27.26 ± 1.7 [a]
FSG(mg/dl)	233.34 ± 65.33 [a]	230.56 ± 59.67 [a]	97.74 ± 9.99 [b]
HbA_{1C} (%)	9.59 ± 2.15 [b]	10.72 ± 2.25 [a]	5.32 ± 0.50 [c]
TG (mg/dl)	141.50 ± 29.31[b]	375.8 ± 96.39[a]	119.8± 44.19[c]
VLDL(mg/dl)	30.04± 17.35[b]	65.70 ± 19.22[a]	23.66 ± 12.89[b]
LDL(mg/dl)	102.51 ± 25.09[b]	128.94 ± 19.21[a]	92.52 ± 29.08[c]

Data are presented as Mean ± SD, Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different, FSG=Fasting serum glucose,HbA1C=Glycated Hemoglobin ,TG=Triglycerides ,VLDL=Very low density lipoprotein ,LDL=Low density Lipoprotein, a,b,c meaning significantly differences in mean of serum levels of markers between groups depending ANOV

Table 3.Serum carnosine and carnosinase-2 isoform

Groups	Carnosine (ng/ml)	Carnosinase-2 (CN2)(ng/ml)
(Group-1)T2DM without CVD	0.23 ± 0.10 [b]	2.08 ±0.69 [a]
(Group-2)T2DM with CVD	0.19 ± 0.13 [b]	1.97 ±0.67 [a]
(Group-3) Healthy control	1.14 ± 0.43 [a]	0.49±0.36 [b]
p-value	0.001	0.001

Means followed by different letters (a,b,c) are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different.

The ROC curve analysis measured in this study has different sensitivity and specificity percentages that show the efficiency of markers and dependence on them as an indicator of disease, as shown in Figures 2 and 3.

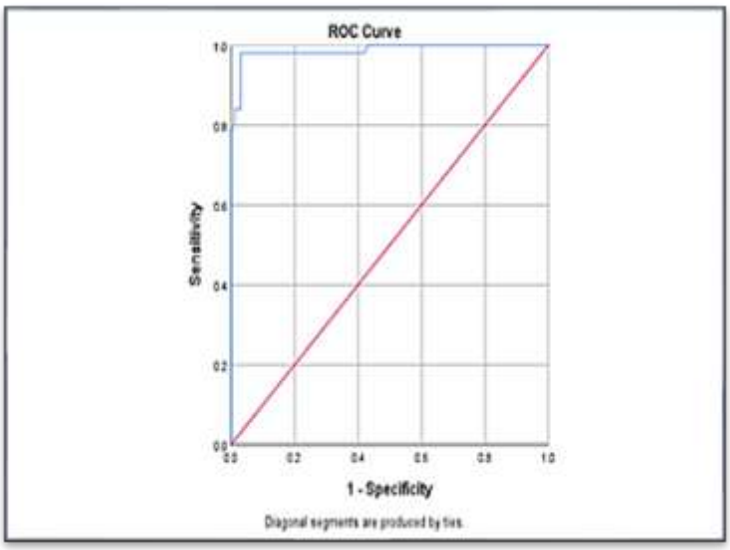

Figure 2.Receiver Operating Characteristic curve of serum level of Carnosine, AUC=0.98 & p value = 0.001 that mean sensitivity 98% while specificity 97% excellent biomarkers use in diagnosis of diabetics and its complications

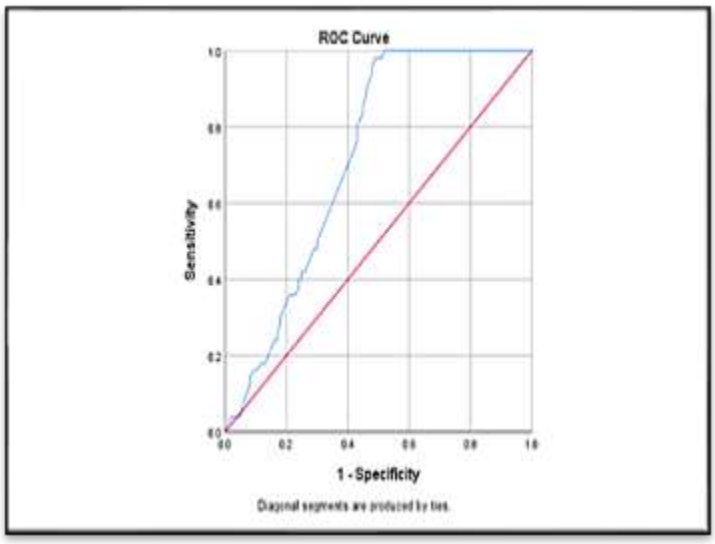

Figure 3. Receiver Operating Characteristic curve of serum level of carnosinase-2 AUC=0.71 & p value = 0.001 that mean sensitivity 98% while specificity 52% not very good biomarkers use in diagnosis of diabetics and its complications

Genotyping results of (T2DM patients without CVD & control) group show that (AA) wild genotype the highly significant difference (p-value 0.001) between two groups , while (AC) hetero genotype also show highly significant difference but odds ratio less than 1, on the other hands (CC) mutant genotype show non- significant difference in subjects in (T2DM patients without CVD) group in comparison with control group, as in table-4 below. Furthermore, the outcome of (T2DM with CVD)group genotyping showed that the wild type (AA) genotype was non-significantly different between patients and control, also both the heterozygous genotype (AC) and homozygous genotype (CC) showed non-significant difference (p-values 0.2 and 0.5, respectively) between patients and control subjects as listed in table-5. While the results of (T2DM patients with and without CVD) genotyping showed that the significant difference p-value 0.04 (AA) genotype and p-value 0.05 for (AC) genotype as in table-6.

Table4. Genotypes & Allele Frequency of Carnosinase -2 SNP (rs6566810) in T2DM (without CVD) Control

Genotypes Group rs 6566810	Study Group		Odds Ratio	CI 95%	P-value
	Patients	Control			
AA	5(10.00%)	22 (44.00%)	1.00	-----	0.001
AC	40(80.00%)	24(48.00%)	0.13636	0.046 -0.408	0.001
CC	5(10.00%)	4(8.00%)	0.18182	0.035 - 0.932	0.74
Alleles Distribution					
A	50(50.00%)	68(68.00%)	1	-----	0.01
C	50(50.00%)	32(32.00%)	0.47	0.265 -0.836	0.01

P ≤ 0.01 = highly significant differences, and P> 0.01= non-significant

Table 5. Genotypes & Allele Frequency of Carnosinase -2 SNP (rs6566810) in T2DM Patients with CVD & Control

Genotypes Group rs10	Study Group		Odds Ratio	CI 95%	P-value
	Patients	Control			
AA	13(26.00%)	22(44.00%)	1.00	------	
AC	31(62.00%)	24(48.00%)	0.45748	0.192 -1.09	0.2
CC	6(12.00%)	4(8.00%)	0.39394	0.093-1.661	0.5
Alleles Distribution					
A	57(57.00%)	68(68.00%)	1.00	-------	0.1
C	43(43.00%)	32(32.00%)	0.6238	0.35 - 1.111	0.1

P ≤ 0.01 = highly significant differences, and P> 0.01= non-significant

Table 6. Genotypes and Alleles Frequency of Carnosinase-2 SNP (rs6566810) in T2DM Patients with and without CVD

Genotypes Group rs10	Study Group		Odds Ratio	CI 95%	P-value
	T2DM	T2DM-CVD			
AA	5(10.00%)	13(26.00%)	1.00	--------	------
AC	40(80.00%)	31(62.00%)	0.29	0.096 - 0.926	0.03
CC	5(10.00%)	6(12.00%)	0.46	0.096 - 2.224	0.33
Alleles Distribution					
A	50(50.00%)	57(57.00%)	1.00	———	-------
C	50(50.00%)	43(43.00%)	0.75	0.43 to 1.32	

**=P ≤ 0.01 = highly significant differences, NS= P> 0.01= non-significant

In table-7 showed highly significant differences in all groups of study with all variant genotypes and showed significance in serum level of CAR in comparison with the patients groups (T2DM with and without CVD) with control group ,while non- significance in comparison between two patients groups, the results also showed non-significant differences in comparison between variant genotypes in each groups.

Table 7.Serum Carnosine levels (ng/ml) with variant genotypes of Carnosinase-2 SNP(rs6566810)

Genotype		Serum Carnosine levels (ng/ml)			p-value
		T2DM	T2DM-CVD	Control	
rs10	AA	0.26 ^{b} ±0.07	0.21 ^{b} ±0.16	1.04 ^{a} ±0.38	0.001**
	AG	0.23 ^{b} ±0.10	0.20 ^{b} ±0.12	1.21 ^{a} ±0.47	0.001**
	GG	0.21^{b} ±0.06	0.13 ^{b} ±0.06	1.22 ^{a} ±0.47	0.001**
p-value		0.7	0.47	0.41	

Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different , Data are presented as Mean ± SD, **= highly significant difference (p ≤0.01).

While the Table-8 clarified the effect of SNP on serum levels of CN2 showed highly significant differences in all groups of study with all variant genotypes and showed significance in serum level of CN2 in comparison between patients groups (T2DM with and without CVD) with control group, while non-significance in comparison between two patients groups, the results also showed non-significant differences in comparison with variant genotypes in each groups.

Table 8.Serum Levels of Carnosinase-2 with Variant Genotypes of Carnosinase-2 SNP (rs 6566810)

Genotype		Serum Carnosine levels (ng/ml)			p-value
		T2DM	T2DM-CVD	Control	
rs10	AA	2.41 ^{a} ±0.48	2.12 ^{a} ±0.53	0.50 ^{b} ±0.11	0.001**
	AG	2.09 ^{a} ±0.70	1.85 ^{a} ±0.71	0.49 ^{b} ±0.52	0.001**
	GG	1.65 ^{a} ±0.69	2.30 ^{a} ±0.64	0.43 ^{b} ±0.13	0.001**
p-value		0.9	0.2	0.2	

Type 2 diabetes increases the risk of many diseases as the complications like nephropathy, retinopathy, and cardiovascular disease[19]. Previous studies showed that carnosine has several important biological activities through its impact on age-related diseases such as cardiovascular disease, DM, cancer, and neurological problems and plays an important role in improving functional capability in ischemic events [20,21]. Carnosine prevents CVD by different mechanisms through its anti-inflammatory, antiglycating, and antioxidant properties[22]. In addition to other risk factors for CVD associated with diabetes, such as metabolic disorders and insulin resistance that characterize type 2 diabetes, the rise in oxidative stress and inflammatory factors in hyperglycemia may be the primary cause of the deterioration of T2DM and its related complications, such as an increase in serum levels of fasting glucose, HbA1c, TG, VLDL, and LDL (Table 2). Several previous studies have established the role of carnosine in normalizing plasma glucose levels and

reducing insulin levels after oral glucose intake and proven that carnosine lowered fasting glucose levels, serum levels of TG, enhanced lipid metabolism, and improved glycemic control (decreased HbA1c, insulin resistance, and increased insulin secretion)[23,24]. The results clarified a highly strong inverse correlation between serum levels of carnosine and carnosinase-2 isoform levels may be increased the risk of CVD[25,26,27]. Clinical risk factors and glycemic control alone cannot predict the development of vascular complications; numerous genetic studies have demonstrated a clear genetic component to both diabetes and its complications or other diseases. This study clarified the effect of the SNP (rs 6566810) on serum levels of carnosine and carnosinase-2 and their role in T2DM complications, especially CVD, the results showed no direct effect of SNP on CVD, while that insure the effect of this SNP in deterioration of CVD as complication of T2DM may be through change in concentration or activity of enzymes related with carnosine. On the other hand, the SNP has not effect on serum level of carnosine &carnosinase-2 directly, may be the SNP effect on other enzymes related with disease by the effect on serum levels or activity, while the increase in the level of carnosinase-2 may occur as a result of other factors like overexpression caused by effect of other SNP, no previous study reported or disagree with the result making it novel study. The current study still represents only the first steps toward a better understanding of the genetic factors that influence the development of CVD as one of the serious complications affecting T2DM patients, so further studies in this regard are necessary before the implementation of research findings into practice.

Study limitations

This study has some limitations, lack similar studies. Therefore, care must be taken when extrapolating the finding of this study to the entire nation. Additionally, environmental factors, different medications and adherence to treatment, and dietary practices that influence how diabetes manifests and the consequences it causes. Because of the combined effects of these factors, the importance of the studied polymorphism in defining the phenotype may be over-or under- estimated.

Conclusion

The SNP (rs 6566810) of the carnosinase-2 gene has no role in CVD as a complication of T2DM.

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Conflicts of Interest

No conflict interest of this study.

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Ethics Statements

Ethical approval with number (RECAUB CP9112021A) on 29 Nov, 2021, was obtained from the Scientific and Ethical Committee in College of Pharmacy / University of Baghdad.

Author Contribution

The authors confirm contribution to the paper as follows: study conception and design: H. S.Sh., S. H A., A. M. R. ; data collection: H. S. Sh.. Author; analysis and interpretation of results: H. S. Sh. . Author.; draft manuscript preparation: H. S. Sh., Sh. H A. , Dr. A. M. R. All authors reviewed the results and approved the final version of the manuscript.

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علاقة تعدد الاشكال الجينية للكارنوسينيز-٢ جين(rs 6566810) مع مستويات الكارنوسين وانزيم الكارنوسينيز-٢ في مصل الدم لدى مرضى السكري من النوع الثاني المصابين بأمراض القلب والاوعية الدموية في العراق

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الخلاصة

للكارنوسين العديد من الأنشطة البيولوجية المهمة، كما يتضح من تأثيراته على العديد من الأمراض مثل أمراض القلب والأوعية الدموية والسكري والسرطان ويمكن أن يلعب دورا هاما في تحسين القدرة الوظيفية أثناء الظروف الإقفارية. قد تتأثر مستويات الكارنوسين لدى مرضى السكري بالمتغيرات الجينية في جين الكارنوسينيز -٢، والتي قد تكون مرتبطة أيضًا بتطور مشاكل القلب والأوعية الدموية. كان الغرض من هذه الدراسة هو دراسة تأثير تعدد الأشكال الجيني Camosinase-2 SNP (rs 6566810) على مستويات مصل الدم و علاقتها بأمراض القلب والأوعية الدموية لدى مرضى السكري من النوع الثاني المصابين بأمراض القلب والأوعية الدموية وبدونها. طرق العمل: تم تحديد تركيز كل من الكارنوسين والكارنوسينيز-٢ في مصل الدم باستخدام المقايسة الامتصاصية المناعية للانزيم المرتبط. كما تم إخضاع جين كارنوسينيز -٢ لتقنية الذوبان عالي الدقة لغرض تحديد تعدد أشكال الجينية. النتائج: ارتفاع مستويات كارنوسينيز-٢ بشكل كبير في مجموعة المرضى مع مجموعة الأمراض القلبية الوعائية، ولكن مستويات الكارنوسين في المصل انخفضت بشكل ملحوظ في كلا المجموعتين من مرضى السكري. تعدد الأشكال من (rs) SNP 6566810 ليس لها تأثير على كل من مستويات الكارنوسين والكارنوسينيز -٢في المصل، ولكنه يزيد من خطر النمط التغايري للأمراض القلبية الوعائية (AC). الاستنتاج: تعدد أشكال SNP (rs6566810) لجين الكارنوسينيز-٢ليس لها دور في حدوث امراض القلب الوعائية كمضاعفات لداء السكري من النوع الثاني.

الكلمات المفتاحية: كارنوسين, كارنوسينيز-٢, امرض قلبية وعائية, تعدد الاشكال الجينية, داء السكري من النوع الثاني

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