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Research article

Association of Carnosinase-1 Gene Polymorphism with Serum Carnosine and Carnosinease-1 Isoform Levels in Type 2 Diabetics with Cardiovascular Diseases in Iraq

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Abstract

Background: Genetic variations in the carnosinase-1 gene, which may also be associated with cardiovascular issues and result in a higher serum carnosinease-1 level, may affect the levels of carnosine and carnosine synthase in diabetes patients. ***Objective***: To examine the impact of the Carnosineas-1 gene polymorphism SNP (rs 2887) on blood levels of carnosine and carnosine synthase-1 and their relationship to CVD in diabetes. ***Method***: The serum concentrations of carnosine and carnosineas-1 were determined using ELISA-specific kits. The carnosineas-1 gene (CN1) was subjected to the high-resolution melt technique (HRM) with the purpose of identifying gene polymorphisms. ***Results***: Carnosinase-1 levels were considerably raised in the T2DM with CVD group, but serum carnosine levels were significantly higher in both groups. SNPs had little impact on serum carnosine levels, whereas polymorphisms had a big impact on carnosinase-1 levels. ***Conclusion***: By raising serum levels of carnosenease-1, which in turn increases carnosine breakdown, the SNP (rs2887) of the carnosinase-1 gene contributes indirectly to the development of CVD in T2DM.

Keywords: Carnosine, Carnosinase isoform-1, Carnosinase -1 SNPs, T2DM.

ارتباط تعدد الأشكال الجيني للنارنوسينيز-1 مع مستويات مصل الكارنوسين والكارنوسينيز -1 في مرضى السكري من النوع 2 المصابين بأمراض القلب والأوعية الدموية في العراق

الخلاصة

الخلفية: الاختلافات الجينية في جين كارنوسيناز-1 ، والتي قد تترافق أيضا مع مشاكل القلب والأوعية الدموية وتؤدي إلى ارتفاع مستوى كارنوزسنيز-1 في الدم، قد تؤثر على مستويات الكارنوسين وسينسيز الكارنوسينيز-1 في مرضى السكري. **الهدف**: دراسة تأثير تعدد الأشكال الجيني Carnosineas-1 SNP (rs 2887) على مستويات الدم من الكارنوسين والكارنوسينسيز-1 وعلاقتهما بالأمراض القلبية الوعائية لدى مرض السكري. **الطريقة**: تم تحديد تركيزات مصل الكارنوسين والكارنوسينيز-1 باستخدام تقنية ELISA وتعريض الموروث carnosineas-1 (CN1) لتقنية الذوبان عالية الدقة بهدف تحديد تعدد الأشكال الجينية. **النتائج**: ارتفعت مستويات كارنوسينيز-1 بشكل كبير في مرضى السكري بالأمراض القلبية الوعائية، لكن مستويات كارنوسين في المصل كانت أعلى بكثير في كلا المجموعتين. كان ل SNPs تأثير ضئيل على مستويات كارنوسين المصل، في حين كان لتعدد الأشكال الوراثية تأثير كبير على مستويات كارنوسينيز-1. **الأستنتاج**: من خلال رفع مستويات كارنوسينيز-1، والذي بدوره يزيد من انخفاض الكارنوسين، يساهم SNP (rs2887) لجين carnosinase-1 بشكل غير مباشر بزيادة احتمال حدوث الأمراض القلبية الوعائية في مرضى النوع الثاني من داء السكري.

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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 confirmed carnosine's role in treatment and protection against many diseases like Alzheimer's, Parkinson's, 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]. 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 muscle, as an appreciable fraction of the total water-soluble nitrogen-containing compounds [7]. Carnosine degradation by the carnosinase, a homologous enzyme, takes place in serum and tissues. 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 (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 [8]. Carnosine has functional properties that are specific to muscle and excitable tissues and acts as a quencher for advanced glycation end-products and advanced lipid peroxidation end-products (AGE/ALE) precursors, the Reactive Carbonyl Species (RCS), which are highly reactive aldehydes derived from oxidative and non-oxidative modifications of sugars and lipids [9]. Other studies have shown the anti-diabetic effect of carnosine supplementation and an endogenous antioxidant. In vitro studies with human dermal fibroblasts and microvascular-endothelial cells have shown that carnosine increases cell viability in the presence of high glucose; such effects and others depend on its role as an antioxidant and a precursor for histamine synthesis, which provide evidence for a possible therapeutic use of carnosine in diabetic wound healing [10,11]. Some gene polymorphisms, especially single nucleotide polymorphisms (SNPs), may lead to many diseases like rheumatoid arthritis (RA), nephrotic syndrome, and decreased treatment response [12,13], whereas genetic polymorphisms related to enzymes that affect serum levels of related proteins like carnosinease-1 and carnosine level could lead to increased risk of many diseases, hence being related to macrovascular complications like in the case of T2DM [14]. In this research, the novel single nucleotide polymorphism (rs2887) in the coding gene for the carnosinase-1 gene located on chromosome 18q22.3, which is related to one of the enzymes that are related to carnosine metabolism, was studied to indicate the possibility of being related to the development of CVD in T2DM.

METHODS

Study design

This case-controlled study was conducted as a multicenter study in Baghdad, Iraq, including the National Center for Diabetic Research and the 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. An overall total of 150 Iraqi adult subjects (age range of 35–65 years) of both genders 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 [15]: Group 1: included patients were T2DM without complications; Group 2: included T2DM with 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) [16]. In addition to the fifty apparently healthy subjects in Group-3 to serve as controls (Table 1), 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) [15]. These patients are already on anti-diabetic treatment.

Table 1: Descriptive data for participants included in the study

Parameters	T2DM without complications (*n*=50)	T2DM with CVD (*n*=50)	Healthy control (n=50)
Gender (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]	37.58±96.39[a]	11.98±44.19[b]
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; values with different superscripts (a,b,c) are significantly different according to Duncan's multiple range comparisons (DMRTs); FSG= Fasting serum glucose, HbA1c= Glycated hemoglobin; TG= Triglycerides; VLDL= Very low density lipoprotein; LDL= Low density lipoprotein.

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 diseases; patients having autoimmune diseases, and 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°C 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 [Figure 1]. The remaining blood sample was transferred to a gel tube to collect serum after clotting and centrifugation at 3000 rpm for 5 minutes. Serum was stored as aliquots at -20ºC for measuring of carnosine, carnosine synthase-1 by enzyme-linked immunosorbent assay (ELISA) [17,18].

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 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) [19]. 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 detecting 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 2. They were lyophilized and 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 concentration as work primer, 10 µl of the stock solution was suspended in 90 µl of free deionized distilled water to reach a final volume of 100 µl, the primers were supplied by Alpha DNA Co., USA.

Table 2: The Primers of rs(2887) in carnosinease-1 gene for HRM technique

SNP	Primer Sequence	T (°C)
rs2887 in (CN-1) gene	F-´5 GCATATCTCCAACCTTGCAATT 3´ R-´5 TGTCACGAGCACTTGAGGAC 3´	58

CN-1= carnosinease-1 gene, HRM =High-Resolution Melting analysis

High-resolution melting technique

Three of the genetic variations were chosen to investigate their relationships with Iraqi T2DM patients. Detection of these SNPs (rs2778) were achieved by using HRM real-time PCR.

Assay principle

As a logical progression from the real-time monitoring of PCR reactions, high-resolution melting (HRM) analysis is a homogeneous, incredibly robust method for genotyping, sequence, and mutation scanning in DNA samples [20]. The HRM method was enabled not only to provide its apparatus or software but also to provide third-generation dyes that made this method more accurate, such as the fluorescent Eva green dye, which can bind to dsDNA. One of the advantages of this dye is that it is non-toxic to cells, has no trans-membrane permeability, and is non-mutagenic. It can be used in large quantities to obtain the largest percentage of saturation of dsDNA and thus more brilliance and greater accuracy in the measured signals [21]. High-Resolution Melting (HRM) analysis for genetic analysis (Polymorphism analysis) was performed using a Rotor Gene Q Real-time CYTO PCR System(QIAGEN), scaling temperature for amplification of DNA from 55 to 95°C, then HRM analysis was done with 0.1°C within a wavelength 470-510 nm [Figure 1]. The 2xTransStart Tip Green qPCR SuperMix was used for SNPs sequences determination. qPCR-HRM was utilized on triple

synthetic controls to determine allelic differences, and differential curves (DC) and, normalized melting curves (NMC) were created using the HRM tool included in the integrated software (Rotor gene 4.4).

Reaction components of HRM

The reaction components of HRM analysis was shown in Table 3.

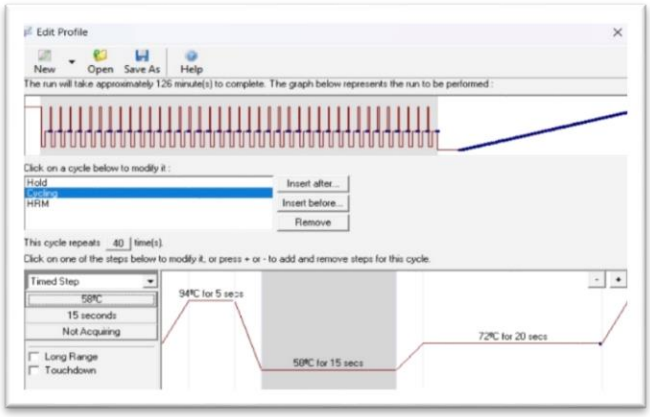

Figure 1: The thermal profile for HRM analysis in a RealTime PCR Device.

Table 3: The reaction components of HRM analysis for genotype using quantitative real-time PCR

Reaction Components	Volume (µl)
2x TransStart®Tip Green qPCR	10
Super Mix 10	5
Nuclease free water	1
Forward primer	1
Reverse primer	1
DNA	3
Final volume	20

HRM =High-Resolution Melting analysis

Thermal profile of HRM technique

The details of HRM technique were illustrated in Table 4. The annealing degree was 58 and using EVA green dye, the reaction took about 120 minutes.

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.

Table 4: Thermal profile of the HRM technique

Step	Temperature (°C)	Time (Sec)	Cycle number
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	58	15	40
Extension	72	20	40
HRM	65-95	0.1 C for 2 sec	1

HRM=high resolution melt

Unpaired t-test, least significant difference (LSD) test, and analysis of variance (ANOVA) were used to compare differences between means. Means followed by different letters are significantly different according to Duncan'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

The results of the current study show highly significant differences (p<0.01) in carnosine and carnosinase-1 isoforms measured in the serum of patients and control groups, as illustrated in Table 5.

Table 5: Serum carnosine and carnosinase-1 isoform

Groups	Carnosine	Carnosinease-1 isoform1
(Group-1)T2DM without CVD	0.23 ± 0.10^{b}	2.15 ± 0.71^{a}
(Group-2)T2DM with CVD	0.19 ± 0.13^{b}	1.97 ± 0.65^{a}
(Group-3) Healthy control	1.14 ± 0.43^{a}	0.40 ± 0.38^{b}

Values with different superscripts (a,b) are significantly different according to Duncan's multiple range comparisons (DMRTs).

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 Table 6 and Figures 2 and 3.

Table 6: Receiver Operating Characteristic curve data of the studied parameters

Parameters	AUC	Explanation	*p*-value	The best Cut off	Sensitivity %	Specificity%
Carnosine	0.98	Excellent	0.001	0.4785	98	97
CN1	0.78	Good	0.001	1.0820	96	51

CN1= carnosinease-1 isoform.

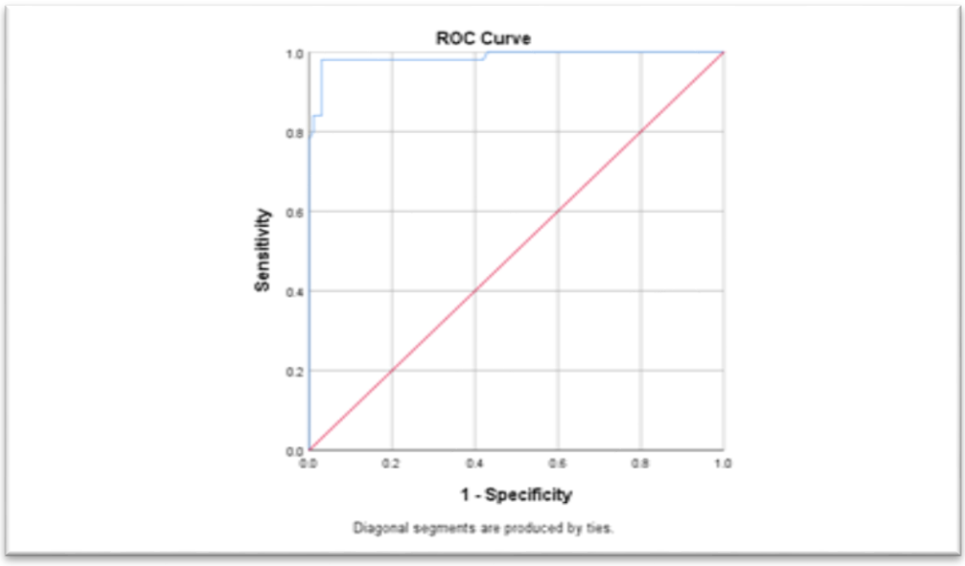

Figure 2: Receiver Operating Characteristic curve of serum level of Carnosine.

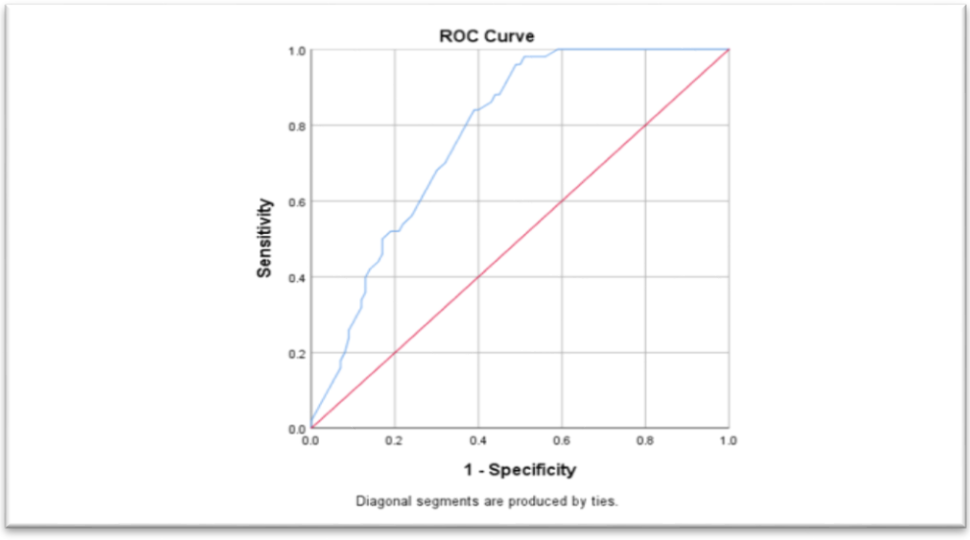

Figure 3: Receiver Operating Characteristic curve of serum level of carnosineas-1

The results clarified a highly strong inverse correlation between serum levels of carnosine and carnosenease-1 isoform levels, as shown in Table 7.

Table 7: Correlations of studied parameters

Parameters		Carnosine	Carnosinase-1
Carnosine	*r*-value	1	-0.728**
	p-value		0.000
Carnosinease-1	*r*-value	-0.728**	1
	p-value	0.000	

**= highly significant difference ($p<0.01$).

Genotyping results of T2DM patients without CVD and control group showed highly significant differences ($p<0.01$) in AA wild genotype between the two groups, and may have protection effect in those subjects (78.1%), while AG hetro-genotype shows significant difference and increase the risk of CVD that reach to 69.4% and odds ratio of 3.27. On the other hand, GG mutant genotype shows non-significant difference in those subjects and alleles distribution demonstrated the important role of G allele in increased risk of disease in T2DM patients without CVD group compared with control group as shown in Table 8.

Table 8: Genotypes and allele frequency of carnosinase-1 SNP (rs2887) in T2DM and control groups

Genotypes group rs2887	Study group		Odds Ratio	CI 95%	Fisher's exact probability	Etiological fraction (%)	Prevented Fraction (%)
	Patients	Control					
AA	14 (28)	32(64)	0.22	0.09-0.51	0.0001		78.1
AG	24 (48)	11 (22)	3.27	1.36-7.96	0.007	69.4	
GG	12 (24)	7 (14)	1.94	0.69-5.70	0.2	48.4	
Total	50	50					
Alleles Distribution							
A	52 (52)	75 (75)	0.36	0.20 - 0.66	0.001		63.9
G	48 (48)	25 (25)	2.77	1.52 - 5.07	0.001	63.9	

Furthermore, the outcome of T2DM with CVD group genotyping showed that AA genotype wild type showed a highly significant difference ($p<0.01$) and gave a protection effect in carrying subjects until 78.1%, and AG hetero genotype showed a significant difference ($p<0.01$) and an odds ratio of 2.57 that increased the risk of disease in carrying subjects to 61.1%. While the GG mutant genotype showed a significant difference ($p<0.01$) between subjects with an odds ratio of 3.16, and an increase in the risk of CVD to 68.4%, allele distribution results appear to indicate the important role of the G allele in increasing CVD complications in patients with T2DM in the CVD group in this study, as clarified in Table 9.

Table 9: Genotypes and allele frequency of carnosinase-1 SNP (rs2887) in T2DM patients with CVD and control

Genotypes group Rs87	Study group		Odds Ratio	CI 95%	Fisher's exact probability	Etiological fraction (%)	Prevented Fraction (%)
	Patients	Control					
AA	12(24)	32(64)	0.18	0.07-0.43	0.0001		82.2
AG	21(42)	11(22)	2.57	1.07-6.28	0.04	61.1	
GG	17(34)	7(14)	3.16	1.18-8.95	0.02	68.4	
Total	50	50					
Alleles Distribution							
A	45(45)	75(75)	0.27	0.15-0.50	0.0001		72.7
G	55(55)	25(25)	3.67	2.01-6.72	0.0001	72.7	

The results of the current study showed that there is no observed effect of SNPs on carnosine serum levels, which means non-significant differences (p>0.05) between the three genotypes in each group, as shown in Table 10.

Table 10: Serum levels of carnosine with variant genotypes of carnosinase-1 SNP (rs 2887)

Groups	rs2887	Mean±SD	No.	*p*-value
T2DM	AA	0.22 ± 0.06	19	0.7
	AG	0.24±0.138	22	
	GG	0.25±0.06	9	
T2DM with CVD	AA	0.15±0.08	17	0.2
	AG	0.20±0.14	15	
	GG	0.23±0.14	18	
Control	AA	1.21±0.50	20	0.4
	AG	1.12±0.36	26	
	GG	0.93±0.51	4	

While there is a highly significant difference (p<0.01) in the mean serum level of carnosenase-1 between various genotypes in T2DM and the CVD group, especially in subjects carrying the GG mutant genotype, significant differences in the T2DM group lead to increased serum levels of the enzyme, as shown in Table 11.

Table 11: Level of serum carnosinase-1 isoform with variant genotype carnosinase -1 SNP (rs 2887)

Groups	rs2887	Mean±SD	No.	*p*-value
T2DM	AA	1.88±0.63	19	0.04*
	AG	2.21±0.72	22	
	GG	2.57±0.63	9	
T2DM with CVD	AA	0.32±0.08	17	0.001**
	AG	0.38±0.09	15	
	GG	1.03±1.30	18	
Control	AA	1.99±0.65	20	0.3
	AG	1.77±0.56	26	
	GG	2.12±0.70	4	

DISCUSSION

Type 2 diabetes increases the risk of many diseases like nephropathy, retinopathy, and cardiovascular disease [22]. 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 [23]. Carnosine prevents CVD by different mechanisms through its anti-inflammatory, antiglycating, and antioxidant properties [24]. The increase in oxidative stress and inflammatory factors in hyperglycemia could be the leading cause of the deterioration of T2DM and consequent complications, besides other risk factors for CVD associated with diabetes that arise as metabolic disorders and insulin resistance that characterize type 2 diabetes, such as an increase in serum levels of fasting glucose, HbA1c, TG, VLDL, and LDL (Table 1). 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) [25,26]. 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 and other diseases. This study clarified the effect of the SNP (rs 2887) on serum levels of carnosine and carnosineas-1 and their role in T2DM complications, especially CVD. The results in Table 8 showed an increase in the chance of CVD in subjects carrying the heterogeneous genotype AG by 69.4%, and the G allele distribution increased the chance of CVD by 63.9% in the T2DM without CVD group, while in Table 9, the results of genotyping in T2DM with CVD showed an increase in the chance of CVD in subjects carrying the heterogeneous genotype AG by 61.1%, the mutant genotype GG by 68.4%, and the presence of the G allele by 72.7%. The result showed the role of a SNP (rs2887) on the CN1 gene (a protein-coding gene located in 18q22.3) in the pathogenesis of CVD in T2DM with CVD more than in T2DM without CVD. As shown in Tables 11 and 12, there are no similar studies on CVD and its relationship with the carnosenease-1 polymorphism SNP (rs 2887), but many studies confirm the role of the carnosinease-1 polymorphism (and other rs SNPs) in T2DM complications like nephropathy [27]. Other studies showed the polymorphism in the carnosinase-1 gene in T2DM, resulting in reduced carnosine degradation activity and a significant DN risk reduction, played a protective role [28]. A gene polymorphism of SNP (rs2887 on the carnosine-1 gene (protein-coding gene)) was chosen in this study to investigate its role in the development of CV complications in T2DM, which has not been studied before. 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

The study has some limitations, just like most association studies do. The small size and narrow

focus on a single facility in a single city (Baghdad) are study's limitations. Therefore, care must be taken when extrapolating the finding of this study to the entire nation. Additionally, interactions among different risk alleles, environmental factors, adherence to treatment, and dietary practices influence how diabetes manifests and the consequences it causes. Because of the combined effects of these several factors, the importance of a studied polymorphism in defining the phenotype may be over- or under-estimated.

Conclusion

By raising serum levels of carnosenease-1, which in turn increases carnosine breakdown, the SNP (rs2887) of the carnosinase-1 gene contributes indirectly to the development of CVD in T2DM.

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Conflict of interests

The author declares no conflict of interests.

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Data sharing statement

Data can be provided based on a reasonable request to the corresponding author.

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