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EVALUATION OF SERUM LEVELS OF NETRIN-1 AS A POTENTIAL BIOMARKER FOR EARLY PREDICTION OF PREDIABETES

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ABSTRACT

Objectives: Prediabetes is a candidate status for type 2 diabetes mellitus, typically identified glycemic values range above normal but below diabetes thresholds. Early and accurate diagnosis of this period would be helpful to prevent the diabetes and its consequences. Netrin-1 is a potential metabolic biomarker that has been associated with diabetes. Previous studies are scarce and have yielded contrasting results on serum netrin-1 levels in diabetes. The aim of this study was to investigate the correlation between serum netrin-1 levels and prediabetes.

Methods: Sixty-nine participants with prediabetes and 35 healthy controls were included in this study. Serum netrin-1 levels were determined using the Enzyme Linked Immunosorbent Assay (ELISA) method. Correlation analysis were done to assess the relationship between serum netrin-1 levels and biochemical parameters.

Results: Serum netrin-1 levels were significantly decreased in the prediabetes group compared to the healthy control group ($P<0.0001$). A significant negative correlation was observed between serum netrin-1 levels and age, hemoglobin A1c (HbA1c), sedimentation, fasting blood glucose (FBG) and C-reactive protein (CRP) ($r = -0.2738$, 0.2886, $r = -0.3180$; $P<0.01$, $r = -0.3439$ and $r = -0.3987$; $P<0.01$), respectively.

Conclusion: Serum netrin-1 significantly negative-correlated with prediabetes and also with FBG and HbA1c. These results suggested that netrin-1 could be used as a biomarker for the early detection or screening of prediabetes.

Introduction

Prediabetes is identified as an intermediate stage of hyperglycemia, between normoglycemia and diabetes [1]. Prediabetes is a condition in which glycemic values range from normal to diabetes mellitus (DM) and is used to define the high-risk group for developing diabetes. 5-10% of prediabetics turn into overt type 2 diabetes mellitus (T2DM) per year and at the same rate it turns into normoglycemia. As a result of the rapidly aging population and lifestyle changes in developing countries, the incidence of diabetes is rapidly increasing universally. The incidence of prediabetes is increasing all over the world. It is estimated that more than 470 million people will be monitored for prediabetes in the 2030s [2]. Prediabetes is generally characterized by isolated impaired fasting glucose (IFG), isolated impaired glucose tolerance (IGT), isolated elevated hemoglobin A1c (HbA1c) levels between 5.7% and 6.5%, or combinations of these conditions [3]. In the last few years, a clear link between prediabetes and cardiovascular disease has been revealed. Studies have shown that prediabetics can suffer from coronary artery disease and diastolic heart failure even before they progress to diabetes [4]. This information has led to the necessity of identifying new biomarkers for early diagnosis of prediabetes and taking appropriate measures to optimize glycemic control.

The word "Netrin" came from the Sanskrit word "netr," meaning one who guides [5]. Netrins are a highly conserved family of extracellular proteins that direct cells and axons to the ventral midline of the

developing nervous system during embryogenesis [6]. Netrins were first discovered in 1990 in *Caenorhabditis elegans*, a nematode. In terms of the evolutionary history of gene organization, the first netrin reported was UNC-6. The first homolog of the UNC-6 form in mammals was discovered in 1994 and has been reported to be a vital guide for the commissural axon found in the spinal cords of rodents [7, 8]. Expressions of five types of netrin have been reported in mammals. These are netrin-1, netrin-3, netrin-4, netrin G1, and netrin G2. While netrin-1, 3, and 4 are secreted from the membrane, netrins G1 and G2 are linked to the cell membrane by two glycosylphosphatidylinositols (GPI) [6]. Netrin-1 has been shown to play an important role in the development of the nervous system by regulating axon quidance and neuronal migration [9, 10]. Besides its role in the nervous system, Netrin-1 is involved in various processes in the development of epithelial tissues such as adhesion, motility, proliferation and differentiation of cells [11]. Netrin-1 has also been found to be effective for kidney, intestinal, prostate, cardiovascular, and muscle diseases, and cancer [12-15].

The use of netrin-1 levels as a biomarker in the diagnosis of complicated diseases has also led netrin-1 to research in diabetics. The fact that the idea that netrin-1 may be a new indicator of subclinical inflammation is becoming more dominant day by day leads to research on this subject [16]. In a recent study, it was observed that the netrin-1 level was significantly higher in diabetics compared to the control group. However, low levels of netrin-1 are also thought to have a protective role in pancreatic islet cells by delaying the progression of the disease [17]. Netrin-1 is thought to be an important factor in the pathogenesis, diagnosis, and treatment protocol of diabetes. Therefore, further research is needed in this area. In the current study, we were aimed to investigate serum netrin-1 levels and the relationship with other biochemical parameters in prediabetics.

Materials and Methods

Ethical Approval

This study was approved by University of Health Sciences Antalya Training and Research Hospital Clinical Research Ethical Committee (2019-215/04.07.2019) and all subjects were informed about this study, and written consent of each patient was received.

Subjects

A total of 104 subjects (84 women and 20 men) who applied to University of Health Sciences Antalya Training and Research Hospital (Antalya, Turkey) Internal Medicine outpatient clinic was included in the study. Of these, 35 were the healthy controls, and 69 were the individuals with prediabetes. Prediabetes was defined according to the American Diabetes Association guidelines. Participants are considered to have prediabetes if they have one or more of the following; impaired oral glucose tolerance test (OGTT 75 g 2h 140-200 mg/dL) and/or Impaired fasting blood glucose (FBG 100-125 mg/dL) and/or impaired hemoglobin A1c (HbA1c) (%) 5.7-6.5 [3]. The following patients were excluded from the study. Patients with a history of connective tissue diseases, neurological disease, smokers, diagnosed liver failure, patients with a previously diagnosed malignancy, patients with known comorbid disease influence on vasculopathy such as, systemic hypertension (defined as systolic blood pressure exceeding 140 mm Hg and\or a diastolic blood pressure exceeding 90 mm Hg or taking an anti-hypertensive medication), cardiovascular disease, etc., Body Mass Index (BMI) >35 and participants using anti-inflammatory drugs were not included in the study. Thirty-five healthy volunteers, who were selected among healthy individuals with FBG and OGTT within normal values, similar to the age and BMI distribution of the prediabetes group, were taken as the control group.

Measurement of Clinical and Biochemical Parameters

Basic clinical and demographic data were collected from medical records for the participants in all groups.

Blood samples were drawn into serum separator tubes by venipuncture from each subject after fasting for 8-12 h. The samples were separated by centrifugation at 4000 ×g for 10 min at 4 °C. Serum samples were carefully separated and stored at −80 ° C to study serum netrin-1 levels until the day of study.

Biochemical parameters including Glucose, C-reactive protein (CRP), serum triglyceride (TG), total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein (LDL-c), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, sedimantation and blood urea nitrogen (BUN) levels were determined using an autoanalyzer (Beckman AU5800; Beckman Coulter Diagnostics, Brea, CA). Fasting insulin (FINS) and thyroid stimulating hormone (TSH) levels were measured using a chemiluminescent assay (AccessDxI800; Beckman Coulter Inc., Brea, CA), and HbA1c levels were determined using commercially available kits and high-performance liquid chromatography (Tosoh HLC 723 G8, Tosoh Bioscience, Tokyo, Japan). Furthermore, insulin resistance was estimated using homeostatic model assessment (HOMA-IR). The HOMA index was calculated using formula; (Fasting Plasma Glucose (mmol/L) × Fasting Insulin (μIU/mL))/22.5 [18].

Measurement of Serum Netrin-1 levels

The netrin-1 levels in control and prediabetic serum samples were measured using the Human Netrin-1 ELISA Kit (E1277Hu, Bioassay Technology Laboratory, Birmingham, UK). Briefly, the standard stock solution (4800 pg/mL) was diluted with assay diluent in 2-fold dilution series and generated the standard wells in duplicate in 96-well ELISA plate. Then, each plasma sample (40 μL) was added to test wells and mixed with netrin-1 antibody (10 μL). Following, the HRP-Streptavidin solution (50 μL) was infused into this test wells, and the plate was covered with a seal and incubated at 37˚C for 60 minutes. Thereafter, each well was washed 5-times with the wash buffer. After washing, the substrate A (50 μL) and the substrate B (50 μL) were added to each well, concomitantly. Once again, the plate was covered with a new seal and incubated at 37˚C for 10 minutes in the dark. Then, HRP reaction of each well was interrupted rapidly by adding stop solution (50 μL) and the optical density (OD) value of each well was measured with MultiscanGO spectrophotometer (ThermoFisher Sci, Massachusetts, USA) at 450 nm. The calculation of netrin-1 (pg/mL) amount of each sample was performed by using elisa analysis software according to the standards.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 9.0.2. The Data were expressed as mean ± standard deviation (SD) or median (interquartile range). The Kolmogorov–Smirnov test was used to evaluate the distribution of variables. Unpaired t-test was used for variables with normal distribution, and Mann-Whitney U test was used for non-normally distribution. Spearman correlation analysis were used to analyze the relationship of serum netrin-1 levels with other biochemical parameters. Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of serum netrin-1 with maximum sensitivity and specificity. The P-value < 0.05 was considered significant.

Results

The present study comprised of total of 104 subjects (69 prediabetes and 35 healthy controls). The female-to-male ratio among the prediabetes group was 56/13 (4.3/1) and the female-to-male ratio of the control group was 28/7 (4/1). The mean age was 48.3±10.4 in prediabetes group and 34.3±11.9 in control group. The prediabetes group showed significantly higher age, FBG, HBA1c, CRP, sedimentation, HDL, LDL, TC and TG values than control group (P<0.0001, P<0.0001, P<0.0001, P=0.0003, P=0.0002, P=0.0115, P=0.0076, P=0.0455, P=0.0194, respectively). No significant differences in OGTT, FINS, HOMA-IR, Creatinine, ALT, AST, BUN and TSH levels were determined between the two groups. The demographic and clinical characteristics of the prediabetes and control groups

are listed in Table 1.

The mean values (±SD) of netrin-1 levels were defined as 819.4 (±723.2) pg/mL in prediabetes group and 1265 (±979.1) pg/mL in control group (Table 1). The difference in serum netrin-1 level between two groups were evaluated statistically significant by using Mann-Whitney U test (*P*<0.0001). In prediabetes group, the mean of serum netrin-1 levels was found to be significantly lower than in control group (Figure 1). The area under receiver operating characteristic (ROC) curve (AUC) was evaluated to check the accuracy of the Netrin-1 ELISA system and how well the test differentiated the two different groups (Figure 2). The AUC was calculated as 0.767 (95% CI=0.6715-0.8634; P < 0.0001). A cut-off value of <615.5 pg / mL was determined for serum netrin-1 with a sensitivity of 71.01% and a specificity of 85.71% to determine prediabetes.

Spearman correlation analysis indicated that the serum netrin-1 level was negatively correlated with age, HbA1c, sedimentation, FBG and CRP (r = -0.2738, r=0.2886, r = -0.3180; P<0.01, r = -0.3439 and r = -0.3987; P<0.001, respectively), but was not significantly correlated with OGTT, FINS, HOMA-IR, Creatinine, ALT, AST, HDL-C, LDL-C, TC, TG, BUN, or TSH (Table 2).

Discussion

Netrins, first defined as axonal guide cues during embryonic development, are a class of laminin-like proteins. Netrin-1's DCC (Deleted in Colorectal Cancer) and UNC5 (uncoordinated-5) receptors in cells other than neurons have been discovered. This suggests that netrin-1 may play a role outside the central nervous system. Recent research showed that netrin 1 is involved in many physiological responses to inflammation, thus making it a potential therapeutic target [19]. Since the angiogenic, regenerative, and anti -inflammatory properties of netrin-1 have been reported in different studies [20-23], its effects on β-cell islet and glucose homeostasis have been discussed in various preclinical and clinical studies to date [24].

Table 1. Demographic and clinical characteristics of the prediabetes and control groups.

Parameters	Prediabetes (mean±SD)	Controls(mean±SD)	*P*-value
Gender F/M	56/13	28/7	
Age (years)	48.3 ± 10.4	34.3± 11.9	**<0,0001**
FBG (mg/dL)	101.76 ± 12.30	91.53 ± 8.28	**<0,0001**
OGTT (mg/dL)	122 ± 32.18	101.6± 26.58	0,1869
FINS (uIU/mL)	9.72± 8.49	6.35± 3.28	0,1532
HbA1c (%)	6.06± 0.44	5.21± 0.28	**<0,0001**
HOMA-IR (%)	3.71± 9.52	1.56± 0.88	0,1329
CRP (mg/L)	3.99 ± 4.42	1.74 ± 1.98	**0,0003**
Sedimentation	10.57± 6.47	5.52± 3.49	**0,0002**
Creatinine (mg/dL)	0.85± 0.16	0.83± 0.12	0,7060
ALT (U/L)	21.15± 13.43	21.62± 12.83	0,7287
AST (U/L)	20.98± 7.31	20.13± 6.89	0,7373
HDL-C (mg/dL)	56.57± 12.79	64.36± 13.90	**0,0115**
LDL-C (mg/dL)	127.36± 34.24	106.44± 24.22	**0,0076**
TC (mg/dL)	208.98± 42.98	189.56± 30.05	**0,0455**
TG (mg/dL)	128.39± 75.31	93.27± 34.81	**0,0194**
BUN (mg/dL)	13.48± 3.99	12.12± 3.06	0,2600
TSH (uIU/mL)	1.85± 1.02	2.13± 1.36	0,4858
Netrin-1 (pg/mL)	819.4±723.2	1265±979.1	**<0,0001**

FBG, fasting blood glucose; OGTT, oral glucose tolerance test; FINS, fasting insülin; HbA1c, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-reactive protein; ALT, alanin-aminotransferase; AST, aspartate-aminotransferase; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; TG , total triglyceride; BUN, blood urea nitrogen; TSH, thyroid stimulating hormone. All parameters were expressed as mean ± standart deviation (SD) values unless otherwise stated. P < 0.05 was accepted as the level of significance.

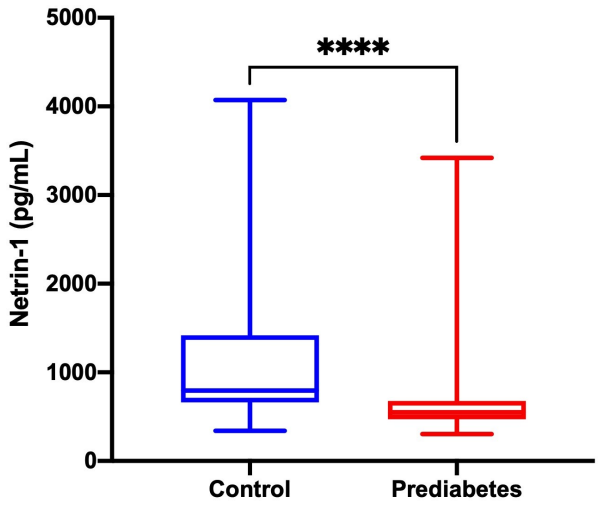

Figure 1. Serum netrin-1 levels of the study groups. Serum netrin-1 levels of the study groups. The box and whisker plots showed that serum netrin-1 levels were significantly decreased in prediabetes group as compared with healthy controls (****p < 0.0001). The center box and the middle line of each graph represent values from the bottom to the upper quartile (25th - 75th percentile) and the median, respectively. Horizontal lines represent the minimum and maximum values.

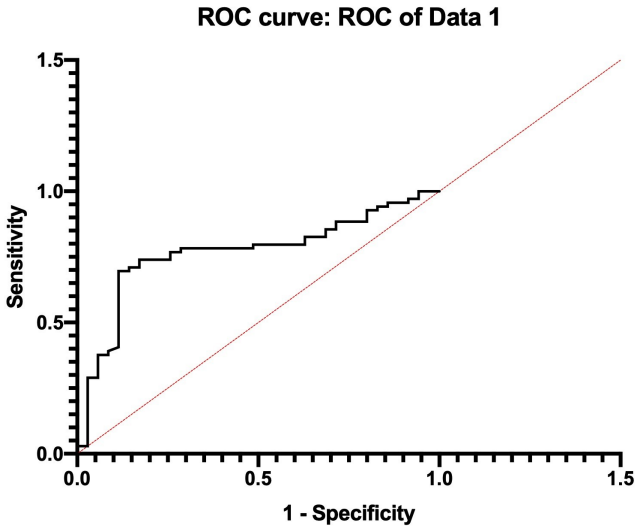

Figure 2. The ROC curve for serum netrin-1 levels in prediabetes and healthy controls. The AUC was 0.767 (95% CI=0.6715-0.8634; P < 0.0001). The cut-off value was <615.5, and sensitivity and specificity were determined to be 71.01 and 85.71, respectively.

Most of the studies that have been done to explain the relationship between netrin-1 and diabetes so far consist of healthy controls and patients with T2DM. Prediabetes is regarded as an intermediate hyperglycemia state with a high risk for T2DM. Every year, 5-10% of prediabetics lead turn into overt T2DM, and the same rate turns into normoglycemia [2]. Therefore, in evaluating whether netrin-1 could be used as a biomarker for diabetes, it is important to clarify the relationship between prediabetes and netrin-1. This study hypothesize that serum netrin-1 levels can serve as an early biomarker of prediabetes and thus the development of T2DM.

The result of this study showed that the levels of serum netrin-1 were significantly low in the prediabetes group compared to the healthy control group. In addition, the level of serum netrin-1 was found to correlate negatively with age, FBG, HbA1c, CRP, and sedimentation. Liu et al. [17] measured plasma netrin-1 levels in 30 newly diagnosed T2DM patients and 26 healthy controls, and found the levels to be significantly lower in T2DM patients. In addition, researchers reported that netrin-1 level was negatively correlated with HOMA-IR, FBG, PBG, fasting insulin, and HbA1c by logistic regression analysis. Similarly, Nedeva et al. [25] showed that netrin-1 levels were significantly decreased in obesity patients, prediabetes and diabetes patients compared to the control group. They reported negative correlation with serum netrin-1 levels and BMI, waist, waist-to-height ratio (WSR) and LDL and positive correlation with sudomotor function with correlation analysis. Findings including changes in netrin-1 levels in these two studies supported the results of our study. Also, in the study of Liu et al. [17], consistent with our results, a negative correlation was found between netrin-1 levels and FBG, HbA1c.

On the contrary, Ay et al. [26] showed that plasma netrin -1 levels increased in diabetic patients with microalbuminuria. They also found a significant positive correlation between the netrin-1 and HbA1c, and a significant negative correlation the netrin-1 between eGFR (Epidermal Growth Factor Receptor). Another recent study by Yim et al. [16] reported that serum netrin-1 levels increased significantly in subjects with T2DM or IFG compared to the control group. The researchers also found a significant positive correlation with netrin-1 levels with fasting glucose, HbA1c, and HOMA-IR. The above available clinical studies reported conflicting findings concerning netrin-1 levels and diabetes. This contradiction might be explained by the

Table 2. Correlations between Netrin-1 levels and biochemical parameters.

Variables	*r*	*P*-value
Age (years)	-0.2738	**0.0061****
FBG (mg/dL)	-0.3439	**0.0005*****
OGTT (mg/dL)	-0.1710	0.2506
FINS (uIU/mL)	0.005908	0.9624
HbA1c (%)	-0.2886	**0.0099****
HOMA-IR (%)	-0.02600	0.8345
CRP (mg/L)	-0.3987	**0.0006*****
Sedimentation	-0.3180	**0.0048****
Creatinine (mg/dL)	-0.1624	0.1638
ALT (U/L)	-0.1518	0.1815
AST (U/L)	0.2109	0.0844
HDL-C (mg/dL)	0.1144	0.3155
LDL-C (mg/dL)	-0.1313	0.2518
TC (mg/dL)	-0.1143	0.3191
TG (mg/dL)	-0.1044	0.3567
BUN (mg/dL)	-0.1472	0.3182
TSH (uIU/mL)	-0.02207	0.8459

FBG, fasting blood glucose; OGTT, oral glucose tolerance test; FINS, fasting insülin; HbA1c, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-reactive protein; ALT, alanin-aminotransferase; AST, aspartate-aminotransferase; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; TG , total triglyceride; BUN, blood urea nitrogen; TSH, thyroid stimulating hormone. Significant correlations are indicated: **P<0.01, ***P<0.001. r=Spearman correlation coefficient.

different characteristics of the study population, the number of patients, and the presence of additional findings related to diabetes.

The means (SD) of serum netrin-1 concentrations of 69 prediabetes and 35 healthy controls were 819.4 (723.2) pg/ml and 1265 (979.1) pg/ml, respectively, and the results showed that the difference in serum netrin-1 levels between healthy control and prediabetic groups was statistically significant ($P < 0.0001$). The method we used to determine the Netrin-1 level was evaluated with AUC to check how well it separated the two different groups. The AUC was 0.767 (95% CI = 0.6715-0.8634; P < 0.0001). A cut-off value of <615.5 pg / mL was determined for serum netrin-1 with a sensitivity of 71.01% and a specificity of 85.71% to determine prediabetes. An AUC of less than 0.5 is considered no discrimination (the ability to diagnose with and without the disease based on testing), 05-0.6 bad, 0.7-0.8 good, 0.8-0.9 very good, and 0.9-1.0 excellent [27]. Since this study's AUC value is in the range of 0.7-0.8, the serum netrin-1 ELISA method can be considered as a "good" method in distinguishing individuals with prediabetes from normal individuals. These results suggest that netrin-1 can be used as a potential biomarker for the detection or screening of prediabetes.

In conclusion, the findings suggested that decreased serum netrin-1 is associated with prediabetes and serum netrin-1 may be a potential biomarker for prediabetes. However, a well-controlled and much larger clinical study involving various populations is needed to precisely claim that netrin-1 can be used as a biomarker for prediabetes.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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