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Investigation of new biomarkers that can be used for the diagnosis of asymptomatic diabetic peripheral artery disease (PAD): Serum netrin-1 levels can be a potential biomarker for the diagnosis of PAD

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Abstract

Diabetic peripheral arterial disease (PAD) is strongly associated with cardiovascular and cerebrovascular diseases, contributing to a high risk of mortality and morbidity. Diagnostic tests that can predict the development of diabetic PAD in the asymptomatic period are inadequate, and new potential biomarkers are needed. This study was conducted to investigate the diagnostic values of serum netrin-1, endothelin-1, oxidative stress parameters, and L-arginine/methylarginine derivatives for diabetic asymptomatic PAD. Three hundred and eighty-six type-2 diabetes mellitus patients who were clinically asymptomatic for PAD were screened for PAD by ankle-brachial index (ABI) measurement. At the same time, forty patients with PAD were included in the study group, and forty-one patients without PAD were included in the control group. Ready-to-use commercial kits were used to measure serum netrin-1, endothelin-1, and oxidative stress markers. Serum netrin-1 median values were found to be significantly higher in the group with diabetic PAD than in the group without (OR [CI 95%]: 1.434 [1.136-1.964], p: 0.036). While a significant positive correlation relationship was detected between total oxidative status and netrin-1 and LDL, a negative significant correlation relationship was detected between ADMA and eGFR. No significant difference was detected between the groups regarding serum endothelin-1, oxidative stress parameters, and L-arginine/methylarginine derivatives. As a result, this study showed that serum netrin-1 levels are high in asymptomatic diabetic PAD patients and can be a possible biomarker in the detection of asymptomatic PAD.

Keywords: diabetes mellitus, PAD, netrin-1, oxidative stress, endothelin-1, ADMA

1. Introduction

Peripheral artery disease (PAD) is generally defined as progressive narrowing or blockage of peripheral arteries, especially regarding lower extremity arteries. Diabetes mellitus (DM) is among the most critical risk factors for PAD (1, 2). One of the most essential features of diabetic PAD is that it may be associated with macroangiopathic complications such as cerebrovascular and cardiovascular diseases that cause high morbidity and mortality (3, 4). The prevalence and clinical significance of PAD is high, but the angiographic findings required for definitive diagnosis are not observed until the late stages of PAD (5-7). Today, there is no diagnostic biomarker that can be used to detect asymptomatic PAD in diabetic patients, and there is a need for non-invasive, inexpensive, and practical biomarkers that can help in the diagnosis of asymptomatic PAD. In this study, new potential biomarkers that could detect asymptomatic PAD early in diabetic patients were investigated. For this purpose, serum netrin-1, endothelin-1, oxidative stress parameters such as total anti-oxidative status (TAS), total oxidative status (TOS) and oxidative stress index (OSI), and L-Arginine, asymmetric dimethylarginine (ADMA), NG-monomethyl-L-arginine (L-NMMA), symmetric dimethylarginine (SDMA) and citrulline, which are involved in the nitric oxide metabolic pathway, levels were evaluated between groups with and without PAD in type 2 DM patients.

Netrin-1 is a protein that exerts its effect mainly through receptors and serves as a critical guidance cue in the guidance of neurons and axonal growth cones (8). Netrin-1 plays

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physiological or pathophysiological roles in conditions such as cardiovascular disease, renal disease, and malignancy (9-12). In addition, netrin-1 is closely associated with angiogenesis (13, 14), hypoxic conditions (15), and the development of atherosclerosis due to increased surveillance of lipid-laden macrophages (16).

Endothelin-1 (ET-1), secreted by the vascular endothelium and has vasoconstrictor properties, is a peptide containing 21 amino acids (17, 18). Vascular endothelial dysfunction in the diabetic population is associated with increased ET-1 production (19). ET-1 contributes to endothelial dysfunction by antagonizing endothelium-derived vasodilators, such as NO, with strong vasoconstrictor properties (20, 21).

TAS and TOS are considered functional laboratory parameters to estimate antioxidant and oxidant balance. Using OSI, obtained by proportioning these two parameters, gives general information about oxidative balance without the need to measure all components of oxidative-antioxidative capacity separately (22). In diabetic patients, the oxidant-antioxidant balance changes in an oxidative direction (23).

L-arginine is an essential amino acid precursor to NO, which has significant vasodilator effects on the cardiovascular system (24, 25). L-arginine reduces oxidative stress (26, 27) and prevents endothelial dysfunction (28) by controlling the decrease of antioxidants at the cellular level in DM. ADMA, L-NMMA, and SDMA amino acid derivatives are all methylated arginine derivatives. These proteins competitively inhibit L-arginine transport across the cell membrane and lead to a decrease in intracellular concentrations of L-arginine (29). It is thought that all these proteins involved in the L-arginine-NO metabolic pathway are closely related to endothelial dysfunction and other clinical consequences that develop in DM (30, 31).

In this study, we tested the usability of serum netrin-1, endothelin-1, oxidative stress parameters, and L-arginine/methylarginine derivatives as biomarkers as possible candidates to detect diabetic PAD in the asymptomatic period. None of these potential biomarkers have previously been compared between groups with and without PAH in diabetic patients. Our results present the comparison of these biomarkers between diabetic asymptomatic PAD and control groups for the first time.

2. Materials and Methods

2.1. Study design

The design of this study was planned as a cross-sectional study. An informed consent form prepared by the World Medical Association's Declaration of Helsinki was obtained from each participant to be included in the study. Three hundred eighty-six individuals between the ages of 40 and 65, diagnosed with T2DM according to the American Diabetes Association (ADA) criteria (32) and clinically asymptomatic for PAD, were screened for the presence of PAD by ABI measurement. The PAD patients included in the study were collected over a period of approximately 6 months, while the screening of diabetic patients was collected. According to ABI measurement, PAD was detected in 40 patients, and these patients were included in the PAD group. Among the patients without PAD, 41 patients aged 40-65 were selected for the control group, having demographic and clinical characteristics similar to the PAD group. Since it is known that the prevalence of PAD in the general population is high over the age of 40 and that calcification in vascular structures can develop at ancient ages (33, 34), individuals between the ages of 40 and 65 were included in this study. After patients were assigned to study groups according to ABI measurement values, venous blood samples were taken from the patients for laboratory examinations. Those with active infection, those diagnosed with any malignancy, pregnant patients, those with exercise or rest claudication, those with foot ulcers, diabetic feet, calluses or varicose veins on physical examination, patients with genetic thrombophilia, patients with antiphospholipid antibody syndrome, those with past or active deep vein thrombosis, and smokers were excluded from the study. Those with hypertension, hyperlipidemia, or coronary artery disease were considered positive for the presence of comorbidities. The estimated glomerular filtration rate (eGFR) measurement was calculated online from the website of the Turkish Nephrology Society according to the Modification of Diet in Renal Disease (MDRD) formula (35).

2.2. Ankle-Brachial Index Measurement

After 10 minutes of rest, the DM patients lay in the supine position while the cuffs of the Huntleigh brand automatic ABI measurement device were connected to the four extremities. The patient was told not to move or talk during the measurement. After the device automatically calculated the measurement results, they were recorded in the patient's file. According to this measurement method, after the monitoring and occlusion cuffs were placed appropriately around the lower and upper extremities, the pressure waveforms from the tibial and brachial arteries were recorded with an oscillometric technique. The device automatically calculated the ABI value obtained by dividing the ankle systolic blood pressure by the brachial systolic blood pressure. Cases with an ABI value of 0.4-0.90 were assigned to the PAD group. Patients with ABI values between 1.1 and 1.3 were selected for the control group (36). Studies evaluating the value of ABI in diagnosing PAD using angiography have reported that a cut-off level of 0.9 for ABI has a diagnostic value of over 90% in both specificity and sensitivity. ABI measurement is a cost-effective, practical, non-invasive, and sensitive screening test for diagnosing PAD (36). Cases with ABI values lower than 0.4 or higher than 1.4 were considered advanced late-stage PAD and calcified vascular structure, respectively, and were not included in this study (34, 37).

2.3. Analysis of Blood Samples

Daily, monthly, and quarterly standard maintenance and calibrations of the device to measure blood parameters were

performed regularly. Blood samples were taken from the patients in the study into gel biochemistry tubes from the brachial vein for serum samples after an 8-hour fast. Blood samples were kept at room temperature for 30 minutes and then centrifuged at 3000 rpm for 10 minutes. For plasma samples, venous blood samples were taken into plastic tubes containing EDTA and centrifuged at 5000xg for 10 minutes. Hemoglobin A1c, insulin, C-peptide, complete blood count, creatinine, and C-reactive protein were analyzed from serum samples obtained after centrifugation. After these examinations, the remaining serum and plasma samples were portioned into Eppendorf tubes and stored at -80°C until the study day. After the pieces were thawed at room temperature approximately three months after the freezing date, they were vortexed, and all other specific examinations of the study were performed on the same day.

2.3.1. Arginine and its derivatives

L-arginine, ADMA, SDMA, L-NMMA, and citrulline analyses were carried out on the ABSCIEX API 3200 tandem mass spectrometry (MS) (high-performance liquid chromatography [HPLC]/MS) device using a Phenomenex Luna C18HPLC column in a positive mode with Turbo IonSpray Electrospray (ESI). Two mobile phases were used during the analysis. A gradient was created with a flow of HPLC-grade water containing 0.1% formic acid in pump A and methanol containing 0.1% formic acid in pump B. It was made based on the Gradient B pump. During the study, isotope (internal standard) was used to prevent analyte losses in pre-analysis processes. Analyte calculations were made from the calibration chart obtained from the criteria using the internal standard (38).

2.3.2. TAS, TOS and OSI

RelAssay Diagnostics brand commercial kits (Rel Assay®-Diagnostics kits-Mega Tip-Gaziantep-Turkey) were studied with the colorimetric method after calibration-control procedures on the Abbott Architect C16000 (Japan) device.

TAS measurement method: Antioxidants in the sample reduced the dark blue-green colored 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical to the colorless reduced ABTS form. The absorbance change at 660 nm is related to the total antioxidant level of the sample. The test was calibrated with a stable antioxidant standard solution traditionally called Trolox equivalent, an analog of vitamin E. Measurements were made according to the kit recommendations. Results were calculated in mmol Trolox Equiv/L.

TOS measurement method: The oxidants present in the sample oxidized the iron ion-chelator complex to iron ion. The oxidation reaction was prolonged by enhancer molecules abundant in the medium. Ferric ions formed a colored complex with chromogen in an acidic environment. The color intensity measured spectrophotometrically was related to the sample's total amount of oxidant molecules. The experiment was calibrated with hydrogen peroxide. Measurements were made according to the kit recommendations. Results were calculated in μmol H2O2 Equiv/L.

OSI calculation method: OSI level, accepted as an indicator of oxidative stress, is defined as the percentage of TOS levels and TAC ratio. In the OSI calculation, TAS levels are converted to μmol. The unit of OSI is Arbitrary Units (AU).

2.3.3. Netrin-1

Netrin-1 was analyzed using the ELISA method on a Rayto-2100C Microplate Reader (India). The analytical unit of the kit is pg/mL. By the quantitative immunoassay method, serum netrin-1 levels were calculated using an ELISA kit (catalog number: PN0484, BIOTANG, Massachusetts, USA). Serum samples and standards for Netrin-1 were added to micro-ELISA plate wells and incubated with specific antibodies for approximately 1.5 hours at 37°C. After adding biotin-rich human-specific detection antibodies for Netrin-1 and Avidin-Horseradish Peroxidase (HRP), the samples were incubated for 30 minutes at 37°C. Optical density grades, which indirectly indicate human netrin-1 concentrations, were measured using a spectrophotometric microplate reader with a wavelength of 450 nm. Standard curves of optical density values were used to calculate serum human netrin-1 levels. The sensitivity of the test for Netrin-1 ranged from 31.25 to 3000 pg/mL. The assay variability for Netrin-1 was <10% interassay and <10% intraassay.

2.3.4. Endothelin-1

Human ET-1 was analyzed using the enzyme-linked immunosorbent assay (ELISA) method on a Rayto-2100C microplate reader (India). Human ET-1 plasma levels were measured using a commercially available ET-1 ETA (Endothelin ETA Kit- Catalog No: 583151-Cayman-Chemical Company-Michigan) kit according to the manufacturer's instructions. The kit analytical unit is pg/mL. The variability of ET-1 was 2.7% intraassay and 17.2% interassay.

2.4. Statistical analysis

Version 22.0 Statistical Packages for the Social Sciences package program was used for statistical analysis. Normality distributions of continuous variables were tested with Shapiro/Wilk or Kolmogorov/Smirnov tests and probability/histogram plots. Descriptive statistical analyzes were shown as mean and standard deviation (mean±SD) for normally distributed variables, and median (interquartile range [IQR], [25%-75%]) for non-normally distributed variables. Pairwise comparisons were made with the Independent Samples T test for normally distributed variables, and with the Mann-Whitney U test for non-normally distributed variables. Spearman correlation analysis was used to calculate correlation tests between continuous variables. Comparisons between multiple groups were made after Bonferroni correction, and the One-way Anova post hoc Tukey test was used for quantitative variables with normal distribution, and the Independent Samples Kruskal Wallis test was used for quantitative variables without normal distribution. Fisher and Chi-square tests were

used to compare categorical variables. Binary logistic regression and linear regression analysis tests were used to evaluate the regression analyzes of the variables. The upper limit considered to be significant was taken as P<0.05 statistical level.

3. Results

A comparison of demographic and clinical characteristics and laboratory parameters between the study groups is shown in Table 1. PAD was detected in 40 (10.4%) of 386 T2DM patients who underwent ABI. A total of 40 patients, 28 women, and 12 men, were included in the PAD group, and 41 patients, 24 women, and 17 men were included in the control group. Age, gender, body mass index, presence of comorbid diseases, and diabetes duration were similar between the study groups (p>0.05). Netrin-1 median values were found to be significantly higher in the PAH group (1897.4 [1780.3-1940.2]) than in the control group (1624.1 [1542.6-1797]) (p: 0.035). Urine albumin/creatinine ratio (UACR) median values were found to be significantly higher in the PAH group (337.8 [265-376.9]) than in the control group (180.8 [120.7-212.3]) (p: 0.038). No significant difference was detected between the PAH group and the control group in terms of endothelin-1, L-arginine, ADMA, SDMA, L-NMMA, citrulline, total methylarginine, arginine/ADMA ratio, TAS, TOS and OSI (p>0.05). High-density lipoprotein (HDL) median values were found to be significantly lower in the PAH group (39.8 [32.7-47.6]) than in the control group (44.3 [39.2-53.4]) (p: 0.040).

The binary logistic regression analysis model created between the dependent variables of the PAD and control group and the independent variables of age, UACR, HDL and netrin-1 showed that the netrin-1 (OR [CI 95%]:1.434 [1.136-1.964], p:0.036), UACR (OR [CI 95%]:1.196 [1.014-2.936], p:0.040) ve HDL (OR [CI 95%]:1.O13 [1.005-3.007], p:0.041) median values were significantly different between the groups. The independent variables included in the model were selected from the variables shown in Table 1 and causing significant differences between groups in pairwise comparisons.

Table 1. Comparison of demographic and clinical characteristics and laboratory parameters between study groups

	PAD group	Control group	P-Value
n (total)	40	41	-
Gender female/male, n	28/12	24/17	.282
Age, mean ± SD (years)	57.73 ± 5.36	58.36 ± 3.75	.521
Body mass index, mean ± SD.	34.02 ± 8.11	31.96 ± 4.33	.140
Diabetes duration, median (IQR) [yıl]	10.5 (7.2-12.6)	10.2 (6.9-11.8)	.914
Presence of hypertension, n (%)	14 (%35)	12 (%29.2)	.689
Hypertension duration, median (IQR) [yıl]	5.4 (2.7-7.4)	5.6 (2.9-7.7)	.852
Presence of hyperlipidemia, n (%)	11 (%27.5)	13 (%31.7)	.678
Duration of hyperlipidemia, median (IQR) [years]	6.3 (4.6-8.1)	6.1 (4.2-7.8)	.791
Presence of coronary artery disease	7 (17.5)	6 (14.6)	.725
Fasting Glucose, median (IQR) [mg/dl]	124 (105-146)	116 (94-135)	.242
LDL, mean ± SD [mg/dl]	122.09±37.6	122.9±35.5	.913
HDL, median (IQR) [mg/dl]	39.8 (32.7-47.6)	44.3 (39.2-53.4)	**.040**
HbA1C, median (IQR) [%]	7.6 (5.8-9.1)	7.3 (5.4-8.7)	.312
C-peptide, median (IQR) [ng/mL]	2.9 (2.2-3.8)	2.5 (1.9-3.6)	.609
Insulin, median (IQR) [µlU/mL]	12.6 (10.2-14.1)	10.3 (8.6-13.8)	.103
Creatinine, median (IQR) [mg/dL]	.92 (.74-1.1)	.81 (.69-.98)	.376
eGFR, median (IQR) [ml/min/1.73 m^2]	86 (70-98)	79 (64-92)	.125
Uric acid, median (IQR) [mg/dL]	5.1 (4.2-6.9)	4.8 (3.8-5.9)	.346
Albumin, median (IQR) [g/dL]	4.4 (3.8-4.7)	4.6 (4.1.2-4.9)	.373
CRP, median (IQR) [mg/L]	3.1 (1.8-4.6)	3.4 (2.0-4.5)	.507
UACR, median (IQR) [mg/g]	337.8 (265-376.9)	180.8 (120.7-212.3)	**.038**
Netrin-1, median (IQR) [pg/ml]	1897.4 (1780.3-1940.2)	1624.1 (1542.6-1797)	**.035**
Endothelin-1, median (IQR) [pg/ml]	279.6 (262.3-321.9)	295.4 (278-330.2)	.109
ADMA, median (IQR) [umol/L]	.38 (.32-.47)	.37 (.30-.44)	.848
SDMA, median (IQR) [umol/L]	.41 (.36-4.5)	.39 (.34-4.2)	.601
L-NMMA, mean ± SD (IQR) [umol/L]	.07±.005	.07±.003	.671
L-arginine, median (IQR) [mol/L]	298.7 (276.2-346.2)	284.1 (267.3-320)	.403
Citrulline, median (IQR) [umol/L]	15.1 (12.8-19.5)	16.6 (13.4-21.8)	.471
Total Methylarginine, median (IQR) [umol/L]	.88 (.56-97)	.85 (.55-.92)	.702
L-arginine/ADMA ratio, median (IQR)	894.8 (639.1-1180.4)	810.2 (580.9-1124.1)	.419
TAS, median (IQR) [mmoltrolox Equlv./L]	1.8 (1.5-2.1)	1.9 (1.7-2.2)	.502
TOS, median (IQR) [µmol H202 Equlv./L]	112 (78.9-130.6)	92 (67.3-110.4)	.193
OSI, median (IQR) [AU]	5.84 (4.02-7.9)	5.69 (3.98-7.21)	.714

LDL: Low-density lipoprotein, HDL: High-density lipoprotein, HbA1C: Hemoglobin A1C, Egfr: Estimated glomerular filtration rate, UACR: Urine albumin creatinine rate, CRP: C-reactive protein, ADMA: Asymmetric Dimethylarginine, SDMA: Symmetric dimethylarginine, L-NMMA: L-NG-monomethyl-arginine, TOS: Total oxidative status, TAS: Total anti-oxidative rate, OSI: Oxidative stress index

A comparison of some specific laboratory tests according to the presence of drugs used in the medical treatment of T2DM and accompanying comorbidities is shown in Table 2. Median values of netrin-1, ADMA, endothelin-1, and OSI were found

to be similar between those who received any medical treatment and those who did not receive the same treatment (p>0.05).

Table 2. Comparison of some specific laboratory tests according to the drugs used in medical treatment

		n	Netrin-1	P-value	ADMA	P-value	Endothelin-1	P-value	OSI	P-value
Metformin	Available	70	1732 (1430-1852)	.641	.36 (.31-.39)	.870	282 (240-319)	.823	6.1 (5.2-7.8)	.403
	No	11	1750 (1564-1881)		.38 (.33-.41)		288 (248-326)		6.7 (5.5-8.7)	
Sulfonylurea	Available	54	1742 (1580-1860)	.427	.37 (.31-.40)	.361	272 (239-318)	.914	6.5 (5.2-8.5)	.519
	No	27	1711(1418-1781)		.33 (.25-.37)		270 (235-312)		6.2 (4.7-7.9)	
Pioglitazone	Available	48	1712 (1530-1832)	.312	.31 (.27-.36)	.316	271 (218-306)	.427	5.9 (4.8-7.4)	.249
	No	33	1786 (1665-1894)		.37 (.32-.43)		285 (228-312)		6.6 (5.5-8.4)	
DPP-4 inh	Available	39	1692 (1512-1780)	.279	.33 (.28-.39)	.443	265 (208-308)	.229	5.7 (4.6-7.7)	.162
	No	42	1759 (1640-1856)		.36 (.30-.43)		290 (219-335)		6.8 (5.2-8.5)	
Glp-1 analogue	Available	8	1718 (1569-1755)	.410	.31 (.27-.38)	.286	268 (211-336)	.462	5.2 (4.5-6.9)	.128
	No	73	1757 (1626-1821)		.35 (.29-.41)		291 (214-348)		6.5 (5.4-7.7)	
Insulin	Available	36	1734 (1526-1885)	.395	.36 (.32-.41)	.783	276 (242-326)	.380	6.7 (5.4-8.2)	.532
	No	45	1688 (1498-1701)		.34 (.26-.39)		261 (233-319)		6.4 (4.9-7.9)	
ACE inhibitors	Available	23	1695 (1450-1756)	.192	.32 (.27-.36)	.247	264 (236-311)	.176	6.2 (5.3-7.5)	.603
	No	58	1780 (1664-1898)		.39 (.32-.43)		293 (266-352)		6.5 (5.4-8.2)	
ARB	Available	19	1702 (1480-1809)	.402	.34 (.30-.37)	.410	262 (239-315)	.105	6.0 (4.9-7.6)	.195
	No	62	1738 (1519-1865)		.37 (.32-.40)		290 (252-347)		6.8 (5.7-8.9)	
Statin	Available	24	1737 (1542-1816)	.608	.33 (.29-.38)	.397	274 (208-299)	.474	6.1 (5.0-7.5)	.430
	No	57	1759 (1637-1871)		.36 (.31-.41)		296 (234-332)		6.5 (5.6-7.9)	
Acetylsalicylic acid	Available	45	1747 (1635-1842)	.352	.35 (.30-.42)	.311	287 (221-329)	.499	6.6 (5.3-8.4)	.237
	No	36	1677 (1509-1771)		.33 (.27-.37)		269 (211-312)		5.9 (4.8-7.6)	

DPP4 inh: Dipeptidyl peptidase-4 inhibitor, Glp-1: Glucagon-like peptide-1, ACE: Angiotensin-converting enzyme, ARB: Angiotensin-receptor blocker

The results of pairwise Spearman correlation analysis between some continuous variables of the study in which all groups were included are shown in Table 3. A low-level positive correlation was detected between TOS and Netrin-1 (r: 0,235, p: 0,031), while a moderate-level positive correlation was detected between TOS and low density lipoprotein (LDL) (r: 0,322, p<0,01). A low-level negative correlation was detected between ADMA and eGFR (r: -0.232, p: 0.034).

Linear regression analysis models showed the existence of a significant relationship between TOS and netrin-1 (β:0.325, CI 95%: 0.296-1.695, p:0.025) and LDL LDL (β:0.436, CI 95%: 0.349-1.486, p:0.012), as well as between ADMA and GFR (β:0.305, CI 95%: 0.192-1.987, p:0.039). The independent variables included in the model were selected from the variables with a significant correlation in Table 3.

Table 3. Binary Spearman correlation analysis results between some continuous variables of the study

r (p)	ADMA	ET-1	TAS	TOS	Diabetes duration	eGFR	UACR	LDL	HDL	HbA1c
Netrin-1	.143 (.195)	-.025 (.819)	.069 (.553)	**.235 (.031)**	.144 (.190)	-.206 (.064)	.040 (.717)	.170 (.172)	-.154 (.143)	.167 (.130)
ADMA	-	-.047 (.671)	.175 (.11)	.031 (.180)	.021 (.51)	**-.232 (.034)**	-.111 (.31)	.081 (.462)	-.102 (.351)	.055 (.618)
ET-1	-.047 (.671)	-	-.101 (.36)	-.032 (.775)	.075 (.447)	-.070 (.526)	-.088 (.42)	.018 (.874)	-.052 (.642)	.061 (.582)
TAS	.175 (.110)	-.101 (.360)	-	.156 (.158)	-.110 (.319)	-.116 (.295)	.078 (.481)	.014 (.898)	.183 (.096)	-.117 (.267)
TOS	.031 (.180)	-.032 (.775)	.156 (.158)	-	.015 (.895)	.230 (.068)	.100 (.367)	**.322 (<.01)**	-.210 (.072)	.119 (.281)

ET-1: Endothelin-1, LDL: Low-density lipoprotein, HbA1C: Hemoglobin A1C, eGFR: Estimated glomerular filtration rate, ADMA: Asymmetric Dimethylarginine, SDMA: Symmetric dimethylarginine, TOS: Total oxidative status, TAS: Total anti-oxidative status

4. Discussion

In this study, we tested the predictive values of serum netrin-1, endothelin-1, oxidative stress parameters, and L-arginine/methylarginine derivatives as biomarkers as possible candidates to detect PAD in the asymptomatic period in T2DM patients. Our study showed a significant relationship between PAD, high serum netrin-1, and albuminuria levels. Additionally, our results revealed a low-level positive correlation between TOS and netrin-1, a moderate-level positive correlation between TOS and LDL, and a low-level negative correlation between ADMA and eGFR. However, our study also showed that serum levels of endothelin-1, ADMA, SDMA, L-NMMA, citrulline, total methylarginine, arginine/ADMA ratio, TAS, TOS, and OSI did not change in the presence of asymptomatic diabetic PAD.

Our study results showed that serum netrin-1 levels were higher in T2DM in the presence of asymptomatic PAD than in controls. Although the relationship of netrin-1 with PAD developing in T2DM is unknown, a study with a low PAD sample number (n: 11) showed the presence of higher serum netrin-1 levels in the non-diabetic population, independent of

smoking, in those with PAD compared to those without (39). It has been shown that the development of endothelial dysfunction and atherosclerosis due to the increase in LDL, oxidative stress, and inflammation mediators CRP and tumor necrosis factor-alpha (TNF-α) molecules play a role in the pathophysiology of diabetic PAD (40). Previous studies have reported that these factors involved in the pathophysiology of diabetic PAD are related to netrin-1. One of these studies determined that macrophage foam cells responsible for the development of human coronary artery atheroma plaques expressed increased netrin-1 and its receptor UNC5B. It has been reported that netrin-1 inhibits macrophage migration, and the mechanism responsible for this is netrin-1-dependent myeloid cell arrest areas within the atheroma plaque. It is also argued that netrin-1 causes an increase in macrophage adhesion and surveillance (9). A study conducted by Ramkhelawon et al. determined that under hypoxic conditions, netrin-1 and its receptor UNC5B synthesis in macrophages were up-regulated and protected macrophages from apoptosis (41). Some studies have shown that hypoxic runs cause increased netrin-1 release (15, 41).

Studies report that high netrin-1 expression supports defective adipose tissue storage and contributes to the progression of metabolic dysfunction with increased chronic inflammation and insulin resistance (42). Consistent with these studies, our results found that serum netrin-1 levels were higher in the presence of diabetic PAD than in controls, and a significant correlation relationship was found between TOS levels and netrin-1. Although current data provide important clues about the possible roles netrin-1 in the development of diabetic PAD, this area needs to be clarified with further and numerous studies.

Our study results showed no significant difference in plasma ET-1 levels between the diabetic PAD group and the controls. Although ET-1 plasma levels have not been previously compared between groups with and without PAD in T2DM, it has been reported that there is a close relationship between increased production of ET-1, a potent vasoconstrictor and pro-inflammatory peptide, and diabetic vascular endothelial dysfunction (43). Similarly, some studies have shown that ET-1 plays a role in the pathogenesis of diabetic macrovascular complications (44, 45). On the other hand, similar to our results, there are also studies showing that there is no increase in plasma ET-1 levels in diabetic patients compared to healthy controls (46, 47) and that plasma ET-1 levels do not change in the presence of hypertension (48, 49), angiopathy (50) and microalbuminuria (51) accompanying diabetes. An explanation for why plasma ET-1 levels do not increase in diabetic angiopathic complications in our study and similar studies is that ET-1 is essentially a paracrine hormone and can produce pathophysiological effects independent of plasma levels (52). However, further studies at the molecular level are needed to better understand the pathophysiological roles of ET-1 in the development of diabetic PAD.

Our study showed diabetic asymptomatic PAD patients had L-arginine, ADMA, SDMA, L-NMMA, total methylarginine arginine/ADMA ratio, and citrulline levels similar to controls. Although none of these molecules have been previously compared between groups with and without PAH in T2DM, it has been shown that the increase in L-arginine, citrulline, and arginine/ADMA ratio represents an increase in NO levels, which is a vasodilator and regulator of the vascular endothelial system. It is known that an increase in ADMA, SDMA, L-NMMA, and total methylarginine levels represents a decrease in NO levels (31, 53). Studies conducted in non-diabetic PAD patients have shown that as the severity of lower extremity claudication symptoms increases, serum ADMA and SDMA levels increase. At the same time, L-arginine and L-arginine/ADMA ratios decrease (30). Available data suggest that the balance in L-arginine and methylarginine derivatives may represent an essential pathophysiological step in the development of microangiopathic complications of DM. However, our study results showed that the serum levels of this molecule did not change in the presence of asymptomatic diabetic PAD. Obtaining such a result may have been influenced by the fact that individuals in the PAD group did not have advanced diabetic PAD or by selecting homogeneous individuals with similar demographic and clinical characteristics to the study and control groups. Investigating L-arginine and methylarginine derivatives in more heterogeneous and advanced-stage diabetic PAD groups may contribute to a better understanding of the clinical importance of these molecules in the development of diabetic PAD.

In our study, albuminuria level was found to be significantly higher in the diabetic PAD group than in the control group. Our results also showed a significant correlation between serum ADMA levels and eGFR. Similar to our results, previous studies report that as the albuminuria level increases in DM, the risk of PAD increases, and the ABI value decreases (54, 55). It is also accepted that albuminuria constitutes an independent risk factor for coronary artery disease (56). Consistent with our results, studies are showing that ADMA is an independent prognostic marker for diabetic nephropathy (31). Previous studies have shown a negative relationship between eGFR and ADMA (57) and that ADMA levels increase in diabetic nephropathy (58). Our results confirmed previous studies showing close relationships between albuminuria-PAD and ADMA-nephropathy.

Our study showed that TAS, TOS, and OSI levels did not change, and HDL levels were significantly lower in the asymptomatic diabetic PAD group compared to the control group. In addition, in our study, the existence of a significant positive correlation between serum TOS and LDL was determined. Although TAS, TOS, and OSI have not been previously studied in groups with or without diabetic PAH, oxidative stress is associated with the etiopathogenesis of diabetes (59), long-term complications (60), and endothelial dysfunction (61). LDL, which turns into an oxidized form due

to oxidative stress in diabetes, induces the release of cytokines such as interleukin-1 or TNF-α by macrophages and thus contributes to the development of the inflammatory atherosclerotic process (62). It is known that the decrease in HDL levels creates a risk for significant atherosclerosis (63) and predisposition to PAD (64). Our study results supported previous studies showing the atherosclerosis-promoting effects of LDL due to oxidative stress (62) or the role of low HDL levels in the development of diabetic macroangiopathy (65, 66). As an explanation for the similar detection of oxidative status indicators between groups with and without diabetic PAD in our study, factors such as the fact that individuals in the PAD group are probably in the early stages of asymptomatic diabetic PAD or that the groups are homogeneously composed of individuals with similar demographic and clinical characteristics can be cited. Further studies in advanced and heterogeneous diabetic groups will contribute to our better understanding of the roles of oxidative stress in the development of diabetic PAD.

Limitations of this study include not using angiography or ultrasonographic findings such as carotid intima-media thickness to support the diagnosis of PAD, selection bias of individuals in the control group, and possible effects of agents used in medical treatment on laboratory studies. Additionally, another limitation of our study is the small sample size of the groups. However, this study showed that serum netrin-1 levels can be a potential biomarker in detecting asymptomatic diabetic PAH patients. Additionally, this study supported the known roles of HDL, LDL, albuminuria level, oxidative stress, and ADMA in diabetic complications.

Serum netrin-1 levels increase in the presence of asymptomatic diabetic PAD and can be a potential biomarker for early detection of PAD. Low HDL and high albuminuria, ADMA, and TOS levels are associated with micro- or macroangiopathic complications of diabetes.

Conflict of interest

All authors involved in the study declare no conflicts of interest.

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Authors' contributions

Concept: A.K., L.K., F.Ç., Design: A.K., L.K., F.Ç., Data Collection or Processing: A.K., A.A., S.B., S.H.I., L.K., K.M., A.B.I., F.A., B.Ö., A.Ü., Analysis or Interpretation: A.K., Literature Search: A.K., T.B.K., Writing: A.K, L.K.

Ethical Statement

This study was approved by the Selçuk University Faculty of Medicine Ethics Committee with decision number 2014-322. All applicable legal study procedures, the Declaration of Helsinki, and the Principles of Good Clinical Practice were followed at all stages of this study.

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