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Neopterin and asymmetric dimethylarginine levels in patients with type 2 diabetic retinopathy

Alpaslan Karabulut[1] - Tayfun Şahin[2] - Mustafa Şahin[3]

1. Hitit University Faculty of Medicine, Department of Internal Medicine, Çorum, Turkey
2. Hitit University Faculty of Medicine, Department of Ophthalmology, Çorum, Turkey
3. Hitit University Faculty of Medicine, Department of Medical Biochemisty, Çorum, Turkey

Correspondence
Mustafa Şahin, Hitit University Erol Olçok Training and Research Hospital, Çepni Street, 19100, Çorum, Turkey.

e-mail
mustafaistanbulx@hotmail.com

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ORCID ID of the author(s):
AK: 0000-0003-2893-692X
TŞ: 0000-0003-2319-0807
MŞ: 0000-0001-6073-563X

Abstract

Objective: We aimed at determining the relationship between asymmetric dimethyl arginine (ADMA) and neopterin levels and diabetic retinopathy (DR) in type 2 diabetes mellitus (T2DM).

Materials and methods: In our study, we included 41 T2DM patients with DR, 21 T2DM patients without DR, and 20 healthy controls. We measured HbA1c, fasting blood glucose, creatinine, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, neutrophil-lymphocyte ratio, neopterin, and ADMA levels by taking fasting serum and plasma samples. In healthy controls, we evaluated ADMA and neopterin levels in T2DM patients with DR, and T2DM patients without DR. We evaluated the relationship between ADMA levels and HbA1c, fasting blood glucose, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, and neopterin.

Results: There was no statistically significant difference in ADMA and neopterin levels between the DR group, the non-DR group, and the control group. There was no significant relationship found between ADMA and neopterin levels and the severity of retinopathy. In our study, we only found a moderate positive correlation between ADMA and neopterin ($\rho=0.453$ $p<0.001$).

Conclusions: Serum ADMA and neopterin levels did not differ significantly between groups at all stages of DR. Regardless of macrovascular disease, we think that serum neopterin and ADMA levels are not associated with retinopathy in patients with T2DM. Further studies are needed to determine whether ADMA and neopterin have an effect on the pathogenesis.

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Introduction

With the global surge of diabetes mellitus (DM) to epidemic levels, vascular complications from diabetes have become a major health concern (1). Serious complications of microvascular nature, including retinopathy, nephropathy, and neuropathy, are prevalent among chronic diabetic patients, leading to significant morbidity and mortality (2). Extensive clinical studies such as the Diabetes Control and Complication Trial and the United Kingdom Prospective Diabetes Study have indicated that rigorous management of blood glucose and/or blood pressure can mitigate the risk of these microvascular complications (3). However, even with effective glycemic control, a notable portion of diabetic patients may still advance to end-stage renal disease and blindness (1-3). This underlines the necessity to better comprehend the fundamental mechanisms underlying microvascular complications.

The endothelium, serving as the primary facilitator of glucose transport from vascular channels to the interstitial space, is a key target for damage in hyperglycemic conditions (4). A significant body of research points towards the critical involvement of endothelial dysfunction in the onset and progression of diabetes-related microvascular complications (4). Asymmetric dimethyl arginine (ADMA), a natural inhibitor of nitric oxide synthetase (NOS), presents at elevated levels in individuals with endothelial dysfunction or associated risk factors (5). It's been demonstrated that ADMA disrupts endothelial function by impeding vasodilation, along with instigating endothelial aging and apoptosis (6). Furthermore, ADMA concentrations are markedly increased in diabetic individuals (5).

ADMA is recognized as a predictor of impending cardiovascular incidents in various diseases, including type 2 diabetes mellitus (T2DM) patients (7). It's linked to microvascular complications in diabetes, along with macrovascular complications. Oxidative stress significantly contributes to the development of these diabetic microvascular complications (8), and has been shown to correlate positively with ADMA in diabetic patients, thereby increasing ADMA production (9,10).

Neopterin, a pteridine derivative, is produced by activated monocytes, macrophages, dendritic cells, and endothelial cells from guanosine triphosphate. Smaller quantities are also produced by renal epithelial cells, fibroblasts, and vascular smooth muscle cells stimulated by interferon-gamma (11,12). Neopterin is an autonomous marker of immune activation, and its role as a critical predictive marker of cardiovascular risk is increasingly acknowledged (13). Development of chronic complications in various conditions, such as malignancies, neurodegenerative diseases, and T2DM, is linked with raised serum neopterin concentrations (13). The precise function of neopterin, despite its crucial role in the activation of immunological and inflammatory responses, remains unclear. There is ongoing debate regarding whether neopterin's effects are beneficial or detrimental to the organism, leading to a focused interest in the activity of the neopterin molecule (14).

Diabetic retinopathy (DR), the most prevalent microvascular complication of diabetes, stands as the primary cause of blindness in working-age adults. After prolonged hyperglycemia, retinopathy linked to diabetes can be observed in nearly all type 1 DM patients and the majority of T2DM patients. DR progresses from retinal vascular cell dysfunction to non-proliferative diabetic retinopathy, and eventually, to proliferative diabetic retinopathy. Initial changes in DR include microvascular endothelial dysfunction and increased permeability of the retinal vascular cell layer, followed by vision-impairing conditions like retinal hemorrhages, macular edema, and exudate formation (15).

In this study, serum ADMA and neopterin levels of patients diagnosed with DR were compared with T2DM patients without retinopathy and a healthy control group. As a result, we aimed to determine whether ADMA and neopterin have an effect on the etiopathogenesis of DR.

Materials and methods

This study was planned as a prospective, case-control study. In the study, the Declaration of Helsinki was adhered to, ethical approval was obtained from the Hitit University Clinical Research Ethics Committee (2019/67) and written informed consent forms were received from all participants.

Our study was carried out in cooperation with the eye clinic and internal medicine clinic. Between December 2019 and November 2020, 41 diabetic patients with DR, 21 diabetic patients without DR, and 20 participants without disease, who applied to the eye clinic of our hospital, were included in the study. All patients were questioned in terms of systemic diseases and ocular diseases. Patients with macrovascular complications such as coronary artery disease (CAD), peripheral artery disease (PAH), cerebrovascular event (CVE), diabetic foot, and patients with proteinuria (to exclude nephropathy) in complete urinalysis were excluded from the study. The presence of macrovascular complications in T2DM was determined according to clinical examination and laboratory results. According to the history, physical examination, and routine laboratory results, patients younger than 18 years of

age, older than 80 years of age, having signs of kidney, liver, and heart failure, pregnant, and using steroids and nitrates were excluded from the study.

All patients and the healthy control group were referred to the ophthalmology clinic. All participants underwent complete ophthalmological examinations including visual acuity, slit-lamp examination findings, eye pressure, and fundus examination findings. Fundus Fluorescein Angio was performed in patients with DR findings (microaneurysm, hemorrhage, exudate, etc.) in the fundus examination. Patients with DR were divided into two groups the group with DR and the group without DR. Patients with retinal laser, intraocular injection, cataract surgery in the last 6 months, eye surgery other than cataract surgery, glaucoma, and retinal pathology other than DRP were excluded from the study.

Body mass index (BMI) was calculated by dividing weight by the square of height. After resting for 15 minutes, the blood pressure of the patients was measured and recorded. Those with resting blood pressure values > 140/90 mmHg and those using antihypertensive drugs were defined as patients with arterial hypertension.

Laboratory analysis: Fasting blood glucose, creatinine, HbA1c, serum lipids, direct bilirubin, indirect bilirubin, and neutrophil-lymphocyte ratio (NLR) were calculated for the patient and healthy group.

The remaining blood samples after routine examinations were centrifuged at 3500 rpm for 10 minutes, their serums were separated and stored in an eppendorf tube at -80°C. Kits and serum samples were kept at room temperature (+25 °C) for 30 minutes so that enzyme levels could be checked. ADMA and neopterin levels were studied in the sera of the participants using the ELISA method.

Serum ADMA levels were measured in mmol/L using Human ADMA ELISA kits (Bioassay Technology Laboratory, Shanghai, China Catalog No: E1887Hu). Serum neopterin levels were measured in nmol/L using Human Neopterin ELISA kits (Bioassay Technology Laboratory, Shanghai, China Catalog No: E3155Hu). ADMA and neopterin levels were compared between the groups.

Statistical analysis

The statistical evaluations were conducted using SPSS software (Version 22.0, SPSS Inc., Chicago, IL, USA). Normal distribution of the groups was assessed through Shapiro-Wilk and Kolmogorov-Smirnov tests. For groups exhibiting normal distribution, continuous variables were expressed as mean ± standard deviation. For those that did not adhere to normal distribution, continuous variables were represented as the median. The comparison between patient and control groups employed the Student's t-test for those normally distributed, whereas the Mann-Whitney U-test was utilized for non-normally distributed groups. For the comparison among three or more groups, one-way ANOVA was used for normally distributed data, and the Kruskal-Wallis test was used for data not normally distributed. Correlation analysis was conducted using the Spearman correlation analysis. A p-value of less than 0.05 was deemed statistically significant.

Results

A total of 82 patients, consisting of patients and healthy volunteers, were included in the study and were divided into three groups. Group A consisted of 41 T2DM patients with DR; Group B consisted of 21 T2DM patients without DR; and Group C consisted of 20 healthy volunteers.

Demographic data of the groups, age, gender distribution, BMI, systolic and diastolic blood pressures were similar in all groups (**Table 1**).

When we examined the biochemical data of the groups, FBG and HbA1c levels in groups A and B were significantly higher than those in group C [for FBS; for p <0.001 and HbA1c; p <0.001]. Triglyceride, total cholesterol, HDL, and LDL cholesterol values were similar in all groups. In addition, serum creatinine

Table 1: Demographic data of the groups

	Group with retinopathy (A) (n:41)	Group without retinopathy (B) (n:21)	Robust control group (C) (n:20)	*p*-value
Gender (Female/Male)	21/20	14/7	13/7	p=0.448
Age (Year)	60.14±8.82	60.19±10	57.10±6.95	p=0.400
BMI (kg/m^2)	30±4.6	31.8±5.6	28.3±3.4	p=0.069
SBP (mmHg)	127.6±12.3	126.5±10.4	125.4±9.6	p=0.424
DBP (mmHg)	78.1±8.8	76.3±7.8	75.6±7.4	p=0.321

Table 2: Biochemical data of the groups

	Group with retinopathy (A) (n:41)	Group without retinopathy (B) (n:21)	Robust control group (C) (n:20)	*p*-value
HbA1c (%)	9.30±2.54	8.14±1.77	5.17±0.34	(B-C) p<0.001 (A-C) p<0.001
FBG (mg/dL)	203±102	177±71	98±10	(B-C) p<0.001 (A-C) p<0.001
T.bilirubin (mg/dL)	0.63±0.24	0.64±0.32	0.69±0.31	p=0.754
D.bilirubin (mg/dL)	0.31±0.10	0.60±0.11	0.53±0.11	p=0.818
I.bilirubin (mg/dL)	0.52±0.21	0.52±0.26	0.57±0.26	p=0.710
T. Cholesterol (mg/dL)	222±59	207±41	220±37	p=0.540
LDL (mg/dL)	131±43	120±32	138±31	p=0.334
HDL (mg/dL)	49±28	53±13	51±7	p=0.401
Triglyceride (mg/dL)	203±171	169±63	152±71	p=0.316
NLR	2 ±0.80	2.02±0.74	1.85±0.57	p=0.718
Creatinine (mg/dL)	0.77±0.16	0.74±0.19	0.78±0.17	p=0.772

values, total bilirubin, indirect bilirubin and direct bilirubin values, and neutrophil-lymphocyte ratio (NLR) were found to be similar in all groups (**Table 2**).

ADMA levels were similar in all groups (**Table 3**). Group A had an overall median ADMA of 0.17 mmol/L (min-max: 0.13-0.65 mmol /L); group B had a median ADMA of 0.17 mmol/L (min-max: 0.13-1.10 mmol/L); and group C had a median ADMA of 0.16 mmol /L(min-max: 0.14-0.66 mmol/L).

In our study, while 28 of our 41 patients with DR were found to have non-proliferative DR, we found proliferative DR in 13 of them.

According to the results of our study, we found that there was no relationship between ADMA level and the severity of retinopathy (p=0.173).

Neopterin levels were also found to be similar in all groups (Table 3). The group A had an overall median neopterin of 1.42 nmol /L (min-max: 0.46-10.44 nmol /L); group B had a median neopterin of 1.23 nmol /L (min-max: 0.46-18.16 nmol /L); and group C had a median neopterin of 1.76 nmol /L (min-max: 1.25-10.60 nmol /L).

We detected higher levels of neopterin in patients with diabetic retinopathy. There was also no significant relationship between the level of neopterin and the severity of retinopathy (p=0.931).

We evaluated the correlation of ADMA with variables such as age, FBG, HbA1c, T. cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and neopterin. In our study, we found only a moderate positive correlation between ADMA and neopterin (ρ=0.453 p<0.001) (**Table 4**).

Table 3: ADMA and neopterin data of the groups

	Group with retinopathy (A) (n:41)	Group without retinopathy (B) (n:21)	Robust control group (C) (n:20)	p-value
ADMA (mmol /L)	0.17(0.13-0.65)	0.17(0.13-1.10)	0.16(0.14-0.66)	0.139
Neopterin (nmol/L)	1.42(0.46-10.44)	1.23(0.46-18.16)	1.76(1.25-10.60)	0.262

ADMA: Asymmetric Dimethyl Arginine, median (min-max): minimum-maximum *Mann-Whitney U-test. p<0.05: Statistically significant*

Table 4: Correlation of ADMA with other variables

	ρ*	P
Age (Year)	-0.064	0.566
FBG (mg/dL)	0.146	0.191
HbA1c (%)	0.216	0.051
T. Cholesterol (mg/dL)	0.083	0.456
LDL (mg/dL)	0.021	0.851
HDL (mg/dL)	-0.086	0.441
Triglyceride (mg/dL)	0.191	0.205
Neopterin (nmol/L)	0.453	<0.001

** Spearman Correlation Test*

Discussion

Recent studies have drawn attention to the potential role of neopterin and ADMA in the evolution of vascular complications of diabetes. The present study was specifically designed to evaluate whether serum levels of ADMA and neopterin are associated with DR. In our study, no statistically significant difference was found between the groups in serum levels of ADMA and neopterin. In addition, it was determined that there was no correlation between ADMA and the severity of DR. However, we found a moderately positive significant correlation between ADMA and neopterin in the entire study cohort.

T2DM is a chronic disease and the prevalence of microvascular complications such as neuropathy and retinopathy increases as the duration of diabetes increases (16). It is known that ADMA reduces NO synthesis as a result of competitive inhibition of the NOS enzyme (7). Free ADMA synthesis occurs in the cell as a result of the methylation of arginine residues in proteins by protein arginine methyl transferase (PRMT) enzymes and then proteolysis. The synthesized ADMA is degraded intracellularly by dimethylarginine dimethylaminohydrolase (DDAH) 1-2 enzymes. A large part of the circulating ADMA is taken into the cells in many tissues, especially the liver and kidney, and metabolized by DDAH enzymes. Most of the circulating ADMA is excreted by the kidneys (17).

ADMA, a potent and independent determinant of endothelial dysfunction, has become a focus of increasing interest. Abhary et al. (18) stated that the plasma level of ADMA was significantly increased in patients with advanced diabetic retinopathy. Malecki et al. (19) showed that ADMA levels were significantly increased in patients with diabetic retinopathy compared to individuals without retinopathy.

Nephropathy, a microvascular pathology of diabetes characterized by a decrease in glomerular filtration rate and protein leakage in the urine, is one of the main causes of chronic renal failure. It has become the strongest determinant of mortality in diabetes (20). ADMA is a crucial stimulus for oxidative stress, which is essential for the initiation and progression of diabetic nephropathy (21). It has been found that circulating ADMA values were elevated in patients with diabetic nephropathy. Proteinuria is a conventional indicator of kidney damage in diabetes. It has also been shown that high ADMA level is associated with severe proteinuria (22). Some studies have shown that high ADMA levels are important in patients with DR. However, some other studies have reported that plasma ADMA level cannot be considered as an independent predictor for DRP. Tarnow et al. (23) reported that there was no statistically significant difference between plasma ADMA levels in type 1 diabetic patients with and without DR. In the same study, ADMA levels were found to be higher in type 1 diabetic patients with a history of myocardial infarction (MI) compared to those without a history of MI. In our study, patients with macrovascular complications were not included in the study. Therefore, there may not have been a significant difference between the groups. Yonem et al. (24) also reported that there was no significant difference between plasma ADMA levels in diabetic patients with and without DR. In addition, Krzyzanowska et al. (25) showed that there is no relationship between ADMA and the progression of DR.

Due to these different results from the studies, it is not clear whether ADMA has a relationship with the etiopathogenesis of diabetic retinopathy. Sugai et al. showed that ADMA level in aqueous humor is mostly related to DR. However, in that study, it was also shown that the ADMA concentration in the aqueous humor was not related to the circulating ADMA level (26). Therefore, we may not have found a significant difference in serum ADMA levels between the groups. ADMA with a high rate in the eye, may be pathophysiologically significant in the formation of DR.

Neopterin serves as a general marker for the activation of cellular immunity. Variations in neopterin concentrations can aid in forecasting the severity and hence the prognosis of a disease. In some diseases, heightened levels of neopterin in serum and urine have been employed as indicators for diagnosis or prognosis. Numerous pathological conditions, including systemic infections, renal failure, autoimmune disorders, glomerulonephritis, kidney transplant rejection, , atherosclerosis ,ischaemic heart

disease, obesity insulin resistance and diabetes have been associated with increased neopterin levels. Neopterin is not only viewed as a preliminary marker of inflammation but also as a predictive element in the progression of diseases (27-30).

There are also a few studies that found the opposite of these results. Anwaar et al. found no significant difference in serum neopterin levels between the groups in patients with T2DM, as in our study (31). In another study, it was determined that there was no relationship between circulating neopterin levels and microvascular complications (including DR) in type 1 diabetic patients (32). In our study, we included only T2DM patients with DR from microvascular complications. Our study is one of the rare studies in the literature evaluating neopterin levels in DR patients.

The limitations of this study are the absence of a group with macrovascular complications in our study, the inability to measure ADMA and neopterin levels in the aqueous humor, the lack of measurements of serum NO levels, and the small number of patients.

The difference in our study from other studies is that in other studies, patients with T2DM were included in the study without discrimination whether they had macrovascular complications or not. Therefore, ADMA and neopterin levels may be higher in patients with DR. Since only T2DM patients with DR without macrovascular complications were included in our study, ADMA, and neopterin levels may not differ between the groups. According to the results of our study, while ADMA and neopterin can be used as a biomarker in patients with macrovascular complications, we cannot use them as a marker in T2DM in the occurrence of microvascular (including DR) complications.

Conclusions

In the current study, serum ADMA and neopterin levels did not differ significantly between the groups at all stages of DR. However, based on the conclusions of our study we think that serum neopterin and ADMA levels are not associated with retinopathy in patients with T2DM, regardless of macrovascular disease. In order to determine whether ADMA and neopterin have an effect on the pathogenesis of DR, future studies with a group with macrovascular complications, measuring ADMA and neopterin levels in aqueous humor, and a larger number of patients are needed.

Conflict of interest

The authors report no conflict of interest.

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Hitit University Faculty of Medicine scientific research project was applied for this study.

Ethical approval:

Ethical approval was obtained from the Hitit University Clinical Research Ethics Committee (2019/67) and written informed consent forms were obtained from all participants.

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Contributions

Research concept and design: **AK**
Data analysis and interpretation: **AK, TŞ**
Collection and/or assembly of data: **AK, TŞ, MŞ**
Writing the article: **AK, TŞ, MŞ**
Critical revision of the article: **AK, MŞ**
Final approval of the article: **AK, TŞ, MŞ**

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