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Angiostatin levels in diabetes mellitus patients receiving insulin treatment: associations with laboratory findings, comorbidities, and medications

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Background/aim: The clinical effect of angiostatin in diabetes mellitus (DM) patients receiving insulin is a meaningful gap in the literature. In this study, we aimed to show the levels and the clinical significance of angiostatin in DM patients receiving insulin.

Materials and methods: This is a case-control study. Serum angiostatin levels were determined by ELISA. A total of 83 people consisting of healthy subjects (n = 36) and patients with a diagnosis of DM receiving insulin therapy (n = 47) were included in this study.

Results: The mean angiostatin levels of the DM group were significantly higher than those of the control group (86.0 ± 68.1 ng/mL and 58.0 ± 22.4 ng/mL, respectively; P = 0.011). Significantly lower angiostatin levels were determined in the DM patients receiving metformin with respect to those not receiving metformin (97.2 ± 74.4 ng/mL and 49.3 ± 7.0 ng/mL, respectively; P = 0.021). Significantly higher levels of angiostatin were observed among the DM patients using a beta-blocker (BB) than the DM patients not using a BB (115.5 ± 78.71 ng/mL and 73.44 ± 60.08 ng/mL, respectively; p = 0.029).

Conclusion: This is the first study evaluating and demonstrating the serum angiostatin levels in DM patients receiving insulin. Further studies are required to understand the effect of angiostatin in diabetics and the effect of medications on angiogenesis in these patients.

Key words: Diabetes mellitus, angiostatin, insulin, metformin

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that requires continuous medical care, where the organism cannot adequately benefit from carbohydrates, fats, and proteins due to inherited and/or acquired deficiency in the production of insulin by the β-cells of the pancreas, the ineffectiveness of the produced insulin, or both (1). According to the International Diabetes Federation (IDF) Atlas in 2017, there are about 425 million people with DM, of which one-third are people older than 65 years (https://www.idf.org/e-library/epidemiology-research/diabetes-atlas.html). According to the World Health Organization (WHO), the number of people with DM increased from 108 million to 422 million between 1980 and 2014 (http://www.who.int/news-room/fact-sheets/detail/diabetes). Additionally, according to the IDF, over 6.694 million individuals suffer from DM in Turkey and the number of deaths due to DM is approximately 46,300 (2). If left untreated, DM can lead to varying degrees of disability and long-term complications that are not only major health issues but also can generate a number of social and economic problems. Around the world, up to 80% of deaths in patients with DM are closely associated with vascular disease, and in a study evaluating management of DM and DM policies in Turkey, cardiovascular complications were found to account for 32.6% of the total costs of DM, followed by 25% for nephrological complications, 6.4% for eye complications, and 6% for neurological complications (3).

Angiostatin, a 38-kDa protein naturally occurring by fragmentation of plasminogen, is a potent endogenous inhibitor of proliferation of endothelial cells and angiogenesis (4). Although its mechanism of action has not yet been completely elucidated, there are many studies in the literature that have focused on the enhancing effect of angiostatin on endothelial cell apoptosis (5–7). The binding of angiostatin to plasma membrane-localized ATP synthetase suppresses the endothelial surface's ATP metabolism and thus downregulates endothelial cell proliferation and migration (8).

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Currently, increased attention is focused on the development of new directions for the treatment of DM complications based on the fundamentals of the antiinflammatory and antiangiogenic approach (9). As indicated before, diabetic microvascular complications are considered to be influenced by angiogenic and antiangiogenic factors. Antiangiogenic factors, which inhibit the growth of new blood vessels (angiostatin, endostatin, IL-4), are considered to be not only biomarkers but also therapeutic targets in diabetic complications. However, studies reporting the association among circulating angiostatin levels and the clinical information of patients with DM are very limited. From this point of view, we hypothesized that increased angiostatin formation could contribute to the impaired angiogenesis in DM patients and correlate with other variables and treatments of these patients.

2. Materials and methods

2.1. Subjects

The protocol of this study was approved by the Ethics Committee of the Gülhane Faculty of Medicine Hospital, Ankara, Turkey, and the study was conducted according to the Declaration of Helsinki. Informed consent was obtained from each individual.

This cross-sectional study was conducted with a total of 83 subjects consisting of patients with a diagnosis of type I or II DM (n = 47) patients, all of whom were receiving insulin treatment, and healthy controls (n = 36) who applied. All were endocrine or internal medicine outpatients of the Gülhane Training and Research Hospital, Ankara, Turkey. A flowchart of the study is demonstrated in the Figure.

Patients with any acute or chronic inflammatory disease or clinical signs of infection, high C-reactive protein (CRP) (>5 mg/L), and/or known malignancy were excluded from the study.

2.2. Clinical examination

All patients underwent a medical examination and a structured interview to investigate any family history of vascular diseases and cancer, smoking, and use of medications. Body mass index (BMI, kg/m^2) was calculated as weight (in kilograms) divided by height (in meters) squared and used as an index of body fat.

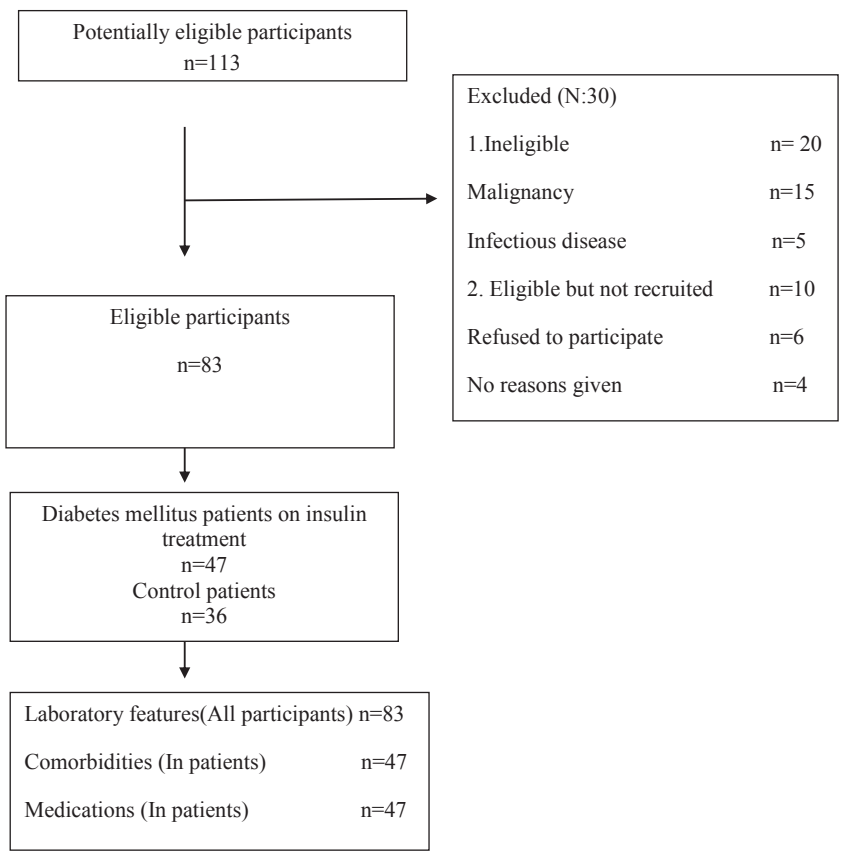

Figure. Flow of participants during the study.

2.3. Sample collection and laboratory measurements

After overnight fasting, baseline blood samples of each subject were drawn from their antecubital vein and collected in BD Vacutainer venous blood collection tubes containing a clot activator and a gel for serum separation. The serum samples were separated by centrifugation at 2000 × *g* for 10 min. Subsequently, analysis of the biochemical parameters was performed without freezing while 2 mL of serum samples were aliquoted and immediately frozen at –80 °C until examination for future analysis of the angiostatin.

Total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting blood glucose (FBG), postprandial plasma glucose (PPG), total protein, albumin, urea, and creatinine levels were also measured by the enzymatic and colorimetric methods with an Olympus AU5800 (Beckman Coulter, USA) using reagents from Olympus Diagnostics. Serum sodium and potassium levels were analyzed by the ion-selective electrode method. Low-density lipoprotein cholesterol (LDL-C) was calculated by Fridewald's formula (10). CRP level was determined in serum using the immunoturbidimetric fixed-rate method with an Olympus AU-5800 autoanalyzer (Beckman Coulter). HbA1c levels were measured with a Premier Hb9210 analyzer (Trinity Biotech, Bray, Ireland) using a boronate affinity chromatography-based high-performance liquid chromatography (HPLC) system for the measurement of glycated hemoglobin. The glomerular filtration rate (GFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (11).

Serum angiostatin levels (Cat. No. CK-E90461) were measured using quantitative ELISA kits (Hangzhou Eastbiopharm Co., Ltd., China). Measurements were carried out using the ELISA plate reader Bio-Tek Synergy HT (Bio-Tek Instruments Inc., Winooski, VT, USA). Intraassay CV and interassay CV were <10% and <12%, respectively, while the minimum detectable dose of angiostatin was less than 20 ng/mL.

2.4. Statistical analyses

The SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to conduct the statistical analyses. For descriptive statistics, discontinuous variables were shown as numbers and percentages (%); continuous variables were shown as mean ± standard deviation or median (25th–75th interquartile range) as appropriate. Normality of the data was evaluated with the Kolmogorov–Smirnov test.

Comparisons between two groups were assessed for continuous variables with the unpaired t-test and Mann–Whitney test, as appropriate. Correlations were performed by Spearman rank test. All of the reported P-values were two-tailed, and those less than 0.05 were considered to be statistically significant. Metformin and beta-blocker are the categorical structure variables. Therefore, the effects of these medications on angiostatin were analyzed by two-way ANOVA test.

3. Results

The differences between patients with DM and the control group were analyzed and the demographics and clinical characteristics of the study population together with the laboratory findings are presented in Table 1. The mean ages of patients with DM and the controls were 69 ± 9 years and 70 ± 11 years, respectively. There was no statistically significant difference between groups in terms of BMI, AST, ALT, total protein, total cholesterol, triglycerides, HDL-C, and LDL-C levels. HbA1c, FBG, and PPG levels were significantly higher in patients with DM than controls, as expected ($P < 0.001$ for all). In addition, serum sodium, GFR, and albumin levels were significantly lower in DM patients than controls ($P = 0.002$, $P = 0.001$, and $P < 0.001$, respectively). On the other hand, serum angiostatin, potassium, CRP, urea, and creatinine levels were significantly higher in DM patients than controls ($P = 0.011$, $P = 0.050$, $P < 0.001$, $P < 0.001$, and $P = 0.001$, respectively).

In correlation analysis, serum angiostatin levels were positively correlated with PPG ($r = 0.239$, $P = 0.045$) (Table 2).

Significantly higher levels of angiostatin were observed among DM patients with beta-blocker therapy than DM patients without beta-blocker therapy (115.5 ± 78.71 ng/mL and 73.44 ± 60.08 ng/mL, respectively; $P = 0.029$), while significantly lower angiostatin levels were determined in DM patients receiving metformin therapy versus without therapy (97.2 ± 74.4 ng/mL and 49.3 ± 7.0 ng/mL, respectively; $P = 0.021$). Two-way ANOVA was performed considering the effects of metformin and beta-blocker therapy on angiostatin. The interaction of these two variables was not coeffective on angiostatin ($P = 0.618$). The mean angiostatin levels of patients with comorbidities such as coronary artery disease (CAD), hypertension (HT), heart failure (HF), chronic kidney disease (CKD), and microvascular end-organ damage such as retinopathy and nephropathy were not significantly higher than those of patients without these comorbidities and end-organ damage (respectively $P = 0.960$, $P = 0.456$, $P = 0.076$, $P = 0.377$, $P = 0.254$, $P = 0.914$). The mean angiostatin levels of patients using medications such as ACE inhibitor, ARB, spironolactone, ASA, clopidogrel, CCB, insulin glargine, insulin lispro, insulin aspart, insulin regular, and statin were not significantly higher than those of the patients not using these medications (respectively $P = 0.097$, $P = 0.258$, $P = 0.322$, $P = 0.714$, $P = 0.647$, $P = 0.865$, $P = 0.274$, $P = 0.066$, $P = 0.530$, $P = 0.374$, $P = 0.483$) (Table 3).

Table 1. Comparison of anthropometric and laboratory features of patients with diabetes mellitus and controls.

Variable	Diabetes mellitus (n = 47)	Controls (n = 36)	P
Sex (M/F)	15/32	15/21	0.359
Age (years)	69 ± 9	70 ± 11	0.531
Duration of DM (years)	15.34 ± 9.232		
BMI (kg/m^2)	30.8	27.3	0.088
Angiostatin (ng/mL)	86.0 ± 68.1	58.0 ± 22.4	0.011
Sodium (mEq/L)	138 ± 3	140 ±2	0.002
Potassium (mEq/L)	4.6 ± 0.6	4.4 ± 0.4	0.050
Urea (mmol/L)	11.2 ± 9.5	5.5 ± 1.4	<0.001
Creatinine (μmol/L)	141.4 ± 119.3	77.8 ± 19.4	0.001
GFR (mL/min/1.73 m^2)	54 ± 25	76 ± 16	<0.001
AST (U/L)	22 ± 9	23 ± 6	0.355
ALT (U/L)	20 ± 10	21 ± 8	0.586
Albumin (g/L)	38 ± 9	43 ± 3	<0.001
Total protein (g/L)	74 ± 14	78 ± 10	0.155
HbA1c (%) (mmol/mol)	9.2 ± 2.6 77 ± 16	5.3 ± 0.4 34 ± 1.4	<0.001
FBG (mmol/L)	10.7 ± 4.8	5.2 ± 0.5	<0.001
PPG (mmol/L)	13.0 ± 5.64	7.1 ± 0.9	<0.001
T. Cholesterol (mmol/L)	4.76 (2.22–7.78)	5.07 (1.81–7.42)	0.482
LDL-C (mmol/L)	2.87 ± 1.04	2.90 ± 0.93	0.916
HDL-C (mmol/L)	1.09 ± 0.26	1.19 ± 0.31	0.105
Triglycerides (mmol/L)	1.82 ± 1.10	1.84 ± 0.76	0.924
CRP (mg/L)	3.24 ± 1.76	1.50 ± 1.35	<0.001

Data are expressed as the mean ± SD or median (25th–75th interquartile range) as appropriate. P-values were calculated using independent samples t-test or Mann–Whitney U test.
ALT, Alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus, ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate; FPG, fasting blood glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PPG, postprandial plasma glucose; T. Cholesterol, total cholesterol.

4. Discussion

To the best of our knowledge, this is the first case-control study evaluating and demonstrating the serum angiostatin levels in DM patients receiving insulin treatment. The primary novel findings of this study indicate serum levels of angiostatin are slightly increased in DM patients compared to healthy controls. Correlation results suggest that there is a positive correlation between PPG and angiostatin values. In addition, significantly lower angiostatin levels were determined in the DM patients receiving metformin therapy versus those without therapy.

Angiostatin has been the focus of intensive study for the treatment of cancer where it has been shown to be an effective antiangiogenic agent and there are a number of data concerning the involvement of angiostatin in angiogenesis (6,12). On the other hand, there are studies demonstrating the alterations of angiostatin levels in the setting of DM and these studies were mostly based on assessing its levels in various tissues. Some studies have reported that the expression of angiostatin was significantly elevated in the diabetic myocardium, atrial tissue, skeletal muscle, and vessels (internal mammary artery) compared to nondiabetics (13–16). However, there is a lack of studies demonstrating changes in systemic concentrations of angiogenic factors in patients with DM. Considering the role of angiostatin in inhibiting angiogenesis in DM

Table 2. Correlation between serum angiostatin levels and laboratory parameters in diabetes mellitus patients.

	Angiostatin level (n = 83)	
Variable	r	P
Age	0.067	0.605
Duration of DM	0.250	0.091
Potassium	0.108	0.333
Urea	0.145	0.190
Creatinine	0.195	0.077
GFR	–0.104	0.349
Albumin	–0.209	0.058
HbA1c	0.197	0.075
FBG	0.040	0.720
PPG	0.221	0.045
Duration of DM	0.250	0.091
Triglyceride	–0.079	0.598
LDL-C	0.580	0.700
HDL-C	–0.192	0.196
Total cholesterol	–0.119	0.425
CRP	–0.024	0.833

Correlation analysis among variables was performed using Pearson's correlation test. $P < 0.05$ was considered significant.
CRP, C-reactive protein, DM, diabetes mellitus, FPG, fasting blood glucose; GFR, glomerular filtration rate; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol, LDL-C, low-density lipoprotein cholesterol, PPG, postprandial plasma glucose.

patients and demonstrating increased serum angiostatin levels in DM patients in the present study, we think that it could be an important physiopathological molecule in DM patients receiving insulin treatment.

Impaired angiogenesis is an important factor leading to the development of DM-associated vascular complications. Although there is no study evaluating the direct effects of metformin administration on serum/plasma angiostatin levels, the efficacy of metformin in reducing other antiangiogenic agents and improving the angiogenic factors has been demonstrated many times before. In a recent study by Bakhashab et al., metformin was shown to improve the angiogenic potential of human $CD34^+$ cells coincident with downregulating tissue inhibitor of metalloproteinase 1 (*TIMP1*) gene expression and increasing VEGF-A (16). Metformin was also determined to reduce the angiogenic inhibitors, chemokine ligand 10, and *TIMP1* mRNAs, which were upregulated under hyperglycemia (16). Consistent with these studies and based on significantly lower angiostatin levels in DM patients receiving metformin therapy versus those without therapy in our patient group, it can be suggested that the use of metformin may improve angiogenesis via reducing angiostatin levels. On the other hand, in the same patient group, there was no effect of insulin administration on angiostatin levels.

Another important finding of the medication–angiostatin relationship in our diabetic subjects was the relationship with the use of beta-blockers. Significantly higher levels of angiostatin were observed among the DM patients with beta-blocker therapy than those without. As previously demonstrated, some antihypertensive agents and particularly beta-blockers have been reported to impair glucose tolerance. Previous studies also revealed that the side effects of beta-blockers in patients with DM included increased insulin resistance with worsening glycemic control (17). On the other hand, there are many studies about the effect of beta-blockers on angiogenesis (18–20). One of the most interesting of these addressed the effect of propranolol in infantile hemangioma (18). Childers et al. also reported that beta-blockers have beneficial effects in breast cancer cases by their antiangiogenic effect (19). However, there are limited animal experimental data in the literature regarding the effects of beta-blockers on angiogenesis in DM patients (21). On the basis of increased angiostatin levels, we assume that the use of beta-blockers may reduce angiogenesis by increasing angiostatin in DM patients receiving insulin treatment. Additionally, users of beta-blockers have higher levels of FPG and PPG and the use of beta-blockers may not affect the synthesis or expression of angiostatin in these patients directly. This may be one of the antiangiogenic and apoptotic mechanisms of beta-blockers. The two variables of metformin and beta-blocker medications were not coeffective on angiostatin according to the two-way ANOVA study. On the other hand, considering the angiogenesis in DM patients receiving metformin and beta-blocker treatment, the statistical analyses revealed that angiostatin may be included in this process. Further clinical and experimental studies should be done in this regard.

We assume that these patients are advanced DM patients and have uncontrollable blood sugar levels with insulin treatment. Moreover, our results reveal that hyperglycemia, mostly increased PPG, is associated with increased antiangiogenic signaling, specifically implicating angiostatin. As shown in Table 2, serum angiostatin levels were found to be positively correlated with PPG in the correlation analysis. These results show the importance of glycemic control, which may degrade angiogenesis by reducing angiostatin levels. Further investigations are needed from this point of view.

Table 3. Comparison of angiostatin levels with comorbidities and medications in patients with diabetes mellitus.

		Angiostatin (ng/mL)		
	N	Mean ± SD	P1	P2
Control	36	58.0 ± 22.4		
CAD (-) CAD (+)	36 11	86.3 ± 69.3 85.1 ± 67.5	0.960	0.393
HT (-) HT (+)	22 25	68.8 ± 51.0 101.1 ± 78.2	0.456	0.133
HF (-) HF (+)	26 21	75.7 ± 66.6 98.7 ± 69.5	0.076	0.028
CKD (-) CKD (+)	37 10	80.8 ± 65.3 105.0 ± 78.4	0.377	0.099
Retinopathy (-) Retinopathy (+)	27 20	85.4 ± 72.1 86.8 ± 64.2	0.254	0.046
Nephropathy (-) Nephropathy (+)	38 9	81.4 ± 64.2 105.4 ± 84.3	0.914	0.321
ACE inhibitor (-) ACE inhibitor (+)	41 6	91.4 ± 71.4 49.3 ± 9.1	0.097	0.408
ARB (-) ARB (+)	36 11	80.3 ± 63.4 104.6 ± 82.2	0.258	0.092
Spironolactone (-) Spironolactone (+)	43 4	89.1 ± 70.4 52.4 ± 10.3	0.322	0.822
ASA (-) ASA (+)	35 12	89.5 ± 72.9 75.6 ± 53.2	0.714	0.490
Clopidogrel (-) Clopidogrel (+)	43 4	87.5 ± 70.9 70.2 ± 20.4	0.647	0.241
Beta Blocker (-) Beta Blocker (+)	33 14	73.4 ± 60.1 115.5 ± 78.7	0.029	0.013
CCB (-) CCB (+)	39 8	84.9 ± 69.8 91.2 ± 63.6	0.865	0.301
Insulin glargine (-) Insulin glargine (+)	13 34	73.7 ± 53.9 90.7 ± 73.0	0.274	0.074
Insulin lispro (-) Insulin lispro (+)	39 8	89.4 ± 69.7 69.4 ± 61.1	0.066	0.584
Insulin aspart (-) Insulin aspart (+)	31 16	77.5 ± 59.5 102.4 ± 81.9	0.530	0.145
Insulin regular (-) Insulin regular (+)	28 19	86.5 ± 71.2 85.2 ± 65.3	0.374	0.100
Metformin (-) Metformin (+)	36 11	97.2 ± 74.4 49.3 ± 7.0	0.021	0.366
Statin (-) Statin (+)	37 10	89.2 ± 69.1 74.1 ± 66.4	0.483	0.709

Values are given as ±standard deviation. $P < 0.05$ was considered significant.
P1: Comparison of the accompanying diseases or medications used in the DM group,
P2: Comparison of the accompanying diseases or medications with the control group.
ACE, Angiotensin-converting-enzyme; ARB, angiotensin II receptor blocker; ASA, acetylsalicylic acid; CAD, coronary artery disease; CCB, calcium channel blocker; CKD, chronic kidney disease; CVA, cerebrovascular accident; DM, diabetes mellitus; ESRD, end-stage renal disease; HF, heart failure; HT, hypertension.

The mean angiostatin levels of DM patients with CKD and nephropathy were not significantly different than those of patients without. In addition, there was no significant result of the correlation analyses between angiostatin and GFR of the patients. Based on these results, we believe that the level of angiostatin in the DM group was not affected by GFR.

According to the study by Yamahara et al., the level of angiostatin in patients with ischemic cardiomyopathy was significantly higher than that of the control group (22). In the present study, the mean angiostatin level of DM patients with CAD as a macrovascular complication was not significantly different than those of DM patients without CAD and control subjects. We did not have patients as participants with other macrovascular complications such as cerebrovascular occlusion or peripheral artery disease. This may be due to a limited number of cases in the groups. According to the literature, animal studies have shown that angiostatin inhibits retinal neovascularization and reduces retinal vascular permeability in diabetic retinopathy (23). Spranger et al. reported that the decrease of angiostatin in the diabetic kidney might contribute to pathologic changes of diabetic nephropathy (24). These studies suggest a possible therapeutic potential of angiostatin for microvascular complications. In the literature, targeting neovascularization has been presented as a novel approach to retard the progression of diabetic microvascular complications. In the present study, the mean angiostatin levels of DM patients with retinopathy and nephropathy as microvascular complications were higher than in patients without these complications but they were not statistically different. We think that significant results might be found in a study performed in a larger group of participants. Also, our findings cannot be extrapolated to all DM patients.

The present study does pose some limitations. First, the present study is limited to analysis of only one antiangiogenic agent, angiostatin, which makes it difficult to evaluate the imbalance of angiogenic/antiangiogenic factors in patients with DM since no angiogenic factor was evaluated concurrently. However, we think that the present study is meaningful and valuable because this topic has not been studied before in the literature and it is a clinical study. Second, this study is based on a limited number of patients and thus cannot ascertain whether these findings apply to other patients with DM. Accordingly, larger clinical studies will be necessary for confirmation of these findings.

In conclusion, circulating angiostatin levels are higher in DM patients receiving insulin treatment. Metformin may have a positive effect on angiogenesis in DM patients by decreasing and beta-blockers may have a negative effect on angiogenesis by increasing angiostatin levels via effects on angiogenesis and apoptosis. Finally, we report novel associations among angiostatin, metformin, and beta-blocker medications. Larger clinical studies will be necessary for confirmation of these findings.

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