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Correlation between Circulating Endothelial Progenitor Cell Markers, Vitamin D, and Iron Levels in Diabetic Nephropathy

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Abstract

Objective: Circulating endothelial progenitor cells (EPCs) play an essential role in endothelial repair and neovascularization. Vitamin D deficiency may contribute to EPC depletion and endothelial dysfunction in patients with type 2 diabetes. In addition, iron overload is closely related to the development of diabetes and its various chronic complications. This study was designed to determine the relationship between EPC markers (CD34, CD133), vitamin D, and iron in patients with diabetic nephropathy.

Materials and Methods: This case-control study was conducted on 67 diabetic patients with or without nephropathy. Blood pressure and all biochemical parameters were measured and compared. Serum concentrations of insulin, vitamin D, CD34, and CD133 were measured using ELISA. Serum iron concentration was measured using atomic absorption spectrometry.

Results: Body mass index *(P*= 0.006), diastolic pressure (*P*= 0.018), insulin level (*P*= 0.028), Creatinine (*P*= 0.013), duration of diabetes, uric acid, and glomerular filtration rate (GFR) were significantly different between the two groups (each *P*= 0.0001).The vitamin D (*P*= 0.034), CD34 (*P*= 0.0001), and CD133 (*P*= 0.025) levels decreased, and Iron (*P*= 0.0001) increased in the case group. Also, CD34 has a significant direct relationship with insulin, insulin resistance, and CD133. The results showed that vitamin D, iron, CD34, and CD133 had a significant relationship with the severity of nephropathy (*P*= 0.0001, each).

Conclusion: Increased iron levels and decreased vitamin D, CD34, and CD133 levels are associated with the severity of nephropathy. This result indicates that diabetic nephropathy may directly reduce CD34 and CD133 levels in the body, increasing the incidence of secondary complications in these patients.

Keywords: Diabetic nephropathy, Vitamin D, CD34 antigen, CD133 antigen, Iron

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Introduction

Diabetes mellitus (DM) is a type of disease caused by the inability of pancreatic islet β-cells to produce insulin, or insulin resistance (1), and is usually manifested by hyperglycemia. According to the International Diabetes Federation (IDF), 537 million people worldwide are reported to have diabetes, causing 6.7 million deaths in 2021. In addition, in countries with low or middle incomes, 3 out of every 4 adults have diabetes (2). In Iran, between 2014 and 2020, it was estimated that 15.0% of adults over 35 years of age had diabetes (3).

One of the most common complications of diabetes mellitus is diabetic nephropathy (DNP). DNP is a metabolic disorder caused by chronic hyperglycemia, which leads to various dysfunctions in kidney cells and ultimately to complete loss of kidney function (4). It is one of the main factors influencing end-stage renal disease (ESRD) and has severe effects on mortality and quality of life. In nephropathy with diabetes, the probability of death is 17 times more than non-diabetics (5). Kidney function is determined by glomerular filtration rate evaluation (eGFR). The normal GFR is approximately 100 ml /min for an adult man (6). Kidney problems in diabetic patients can be diffuse or nodular glomerulosclerosis, which is usually accompanied by albumin excretion (albuminuria) (7).

In DNP, many factors, such as hyperglycemia, hyperlipidemia, high blood pressure, and proteinuria, are associated with the development of renal failure (8,9).

In addition, in recent years, much research has been conducted to investigate the possible effect of vitamin D on the pathophysiology of DM (10-12). Some studies have demonstrated that low vitamin D levels in a person with diabetes are significantly associated with more severe complications, especially in DNP (13-15). Nevertheless, this connection remains in a state of ambiguity.

However, during the last five years, several limited studies have been conducted on iron accumulation in the kidneys of patients with DNP. This hypothesis was reinforced by an animal study that found more iron deposition in the kidneys of the group with DNP than in the healthy group (16).

One of the cells influencing the inflammatory process in the body is progenitor cells (PC), which are very sensitive to ischemia and vascular interference (17). The occurrence of vascular accidents in diabetes is one of the main complications of this disease, which can cause limited ischemia and increase the rate of PC. This increase may become a crucial factor in the occurrence of DNP by increasing the rate of vascular destruction in individuals with diabetes (18). One of the main markers of PC is CD133. The presence of these antigens is almost entirely caused by the presence of these cells, and antigen levels in the patient's serum are usually checked to measure the activity of these cells (18). During vascular damage, special cells are used for endothelial repair or angiogenesis (19). The CD34 antigen is a specific marker for these cells and is used to measure the activity of endothelial progenitor cells (19). Given that vascular lesions occur as a complication of diabetes-induced nephropathy, these cells may attempt to repair the lesions. Therefore, their levels in the serum of individuals with diabetes may be reduced (11,20).

Considering the significant difference between these parameters and their relationship with DNP and the lack of a single study that examines these markers in the same group of patients with diabetes, this study aimed to evaluate the serum levels of CD34, CD133, vitamin D, and iron in patients with DNP compared with those in patients with diabetes.

Material and methods

This Case-Control study included 67 individuals with type 2 diabetes mellitus (T2DM) who were referred to a hospital in Mashhad, Iran. Individuals with T2DM were

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divided into two groups based on urine proteinuria (21): diabetic nephropathy (without other complications who had albumin to creatinine ratio more than 30 mg/dL or 24-hour urine protein greater than 150 mg/dL), and non-diabetic nephropathy. Therefore, we selected 37 individuals with diabetic nephropathy as the case group and 30 individuals without nephropathy as the control group. The control group was selected from diabetic patients without nephropathy by matching age and sex. Inclusion criteria include being over 25 years old and having T2DM with or without nephropathy. Exclusion criteria included pregnant women, patients with liver disease, hypothyroidism, hyperthyroidism, acute coronary syndromes, strokes, cardiovascular patients, diabetic neuropathy, cancer, and patients with any blood disorder.

After written informed consent was obtained from all study participants, demographic information, FBS, HbA1c, blood pressure, creatinine, uric acid, urinary albumin, lipid profile (TG, Cholesterol, LDL, and HDL), age, weight, duration of diabetes, and possible medications were collected.

Preparation of the samples

Five milliliters of blood was taken from the patient's veins after 8 h of fasting, and blood serum was separated by centrifugation (at 2000 rpm for 20 min) and stored at -20° C for further studies.

Insulin and Insulin Resistance Measurements

Serum levels of insulin for all subjects were measured using an ELISA Abcam kit, UK (ab100578), according to the manufacturer's instructions. In this study, insulin resistance (IR) was measured using the HOMA-IR formula (22): [HOMA-IR= [IF (μU/ mL)× GF (mmol/ L)]/ 22/5; (IF=fasting insulin, GF= fasting glucose)].

Iron measurement

In the isolated serum sample, the iron content was measured by atomic absorption spectroscopy (Perkin Elmar AAS-700 Ueberlinger, Germany).

Serum iron concentration was analyzed using flame atomic absorption spectrophotometry. Commercial Fe calibrators were used as standards (1.000 mg/L) by serial dilutions, and samples were evaluated according to a standard curve.

Vitamin D measurement

The 25-hydroxyvitamin D kit (PishtazTEB Diagnostics, Iran) was used for ELISA. This kit is based on the principle of competitive binding. In this method, the wells are coated with a monoclonal antibody against vitamin D (23). Absorption was measured at 450 nm using an ELISA reader (model elx800, BioTek Company, USA). A standard curve was obtained by plotting the standard concentration against the amount of light absorbed.

CD34 and CD133 measurements

The sandwich ELISA method was used for measuring, according to the instructions given in the brochure of the ZellBio Company.

-Human Cluster of Differentiation 133 (CD133), ELISA Kit: ZellBio GmbH (Germany) Cat. No: ZB-15170C-H9648.

-Human Cluster of Differentiation 34 (CD34) ELISA Kit: ZellBio GmbH (Germany) Cat. No: ZB-10219C-H9648.

Glomerular filtration rate (GFR) calculate

The Cockcroft– Gault formula used to estimate the GFR (eGFR):

eGFR= (140- age* weight (kg))/ (Serum level of Creatinine*72)* (0.85 if the patient is female), the GFR was calculated in (ml/min/ a.73m^2) (24). According to the 2011 Diabetic Nephropathy Guidelines (25), the severity of nephropathy in patients is classified according to the GFR level into five stages: the first stage: normal or high (GFR≥ 90), the second stage of slight reduction (60≤ GFR< 89), the

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third stage was divided into two groups: mild to moderate (45≤ GFR≤ 59) and moderate to severe (30≤ GFR≤ 44), stage four is severe reduction (15≤ GFR≤ 29), and stage five is renal failure (GFR< 15).

Subjects were compared for iron, CD34, CD133, and vitamin D levels based on the severity of nephropathy.

Statistical Analysis

Appropriate statistical tables and indicators such as mean and standard deviation, were used for the statistical description of the data. Data were first tested for normality using the Shapiro-Wilk test. If abnormal data are observed, the Mann-Whitney test was done, and for confirmation of normality, appropriate parametric methods such as Student's T-test were used. A chi-square test was done to analyze the data on a nominal scale. The Pearson correlation coefficient was used to examine the relationship between variables.

The software used in this study was SPSS v.24, and the significance level of the tests was considered to be less than 5%.

Ethical considerations

All procedures performed in studies involving human participants were performed according to the ethical standards of the Ethics Committee of Medical Sciences, Islamic Azad University, Mashhad, Iran. (Date 2020.9/No. IR.IAU.MSHD.REC.1399.133. And IR.IAU. MSHD.REC.1399.134).

Results

Thirty-seven diabetic individuals with nephropathy (DNP or Case group) with a mean age of 58.76 (± 11.72) years, including 54.1% women, and 30 patients with diabetes without nephropathy (Control group or CTL) with a mean age of 53.90 (± 10.38) years, including 53.3% women, were studied (*P*= 0.081) (Table 1).

Blood indices

A comparison of blood indices showed a significant difference between the two groups regarding FBS (*P*= 0.0001), HbA1c (*P*= 0.0001), LDL (*P*= 0.0001), Creatinine (*P*= 0.013), uric acid (*P*= 0.0001), and insulin (*P*= 0.028) (Table 2).

Distribution of CD133

The mean serum level of CD133 in the control group was 3.84 (±0.72) ng/ml and in the DNP group was 3.04 (±1.97) ng/ml (*P*= 0.025) (Table 2).

Severity of nephropathy

The severity of nephropathy in the DNP group, two individuals (4.5%) with slight renal impairment, 13 individuals (35.1%) with mild renal impairment, 20 individuals (54.1%) with moderate to severe decrease, and two individuals (5.4%) with severe renal impairment were associated.

Correlation of iron with the index

In the control group, iron has a significant direct relationship with vitamin D (*P*= 0.012, r= 0.455), and uric acid (*P*= 0.010, r= 0.465) and a significant inverse relationship with FBS (*P*= 0.005, r= -0.496), HbA1c (*P*= 0.021, r= -0.420), and total cholesterol (*P*= 0.024, r= -0.410). In the DNP group, iron had a significant inverse relationship with the duration of the disease (*P*= 0.013, r= -0.405).

Correlation of vitamin D levels with the

Table 1. Comparison of demographic indices between diabetic nephropathy (DNP), and diabetic without nephropathy (Control) groups

Variable	DNP Group Mean (±SD)	CTL Group Mean (±SD)	*P*-value
Age (years)	58.76 (± 11.72)	53.90 (± 10.38)	0.081
BMI (kg/m2)	27.88 (± 1.49)	30.19 (± 4.10)	0.006
Diastolic pressure (mmHg)	85.49 (± 2.47)	80.67 (± 9.44)	0.018
Systolic pressure (mmHg)	140.38 (±3.44)	138.77 (12.47)	0.497
Diabetes duration (years)	4.76 (± 1.86)	2.70 (± 1.68)	0.0001

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index

The results showed that vitamin D had a significant inverse correlation with FBS (*P*= 0.0001, r= -0.680) and HbA1c (*P*= 0.0001, r= -0.920) in the control group. In the DNP group, there was a significant direct relationship between vitamin D and HbA1c (*P*= 0.019, r= 0.3884) and a significant inverse relationship with HDL (*P*= 0.027, r= -0.363). In general, vitamin D has a significant inverse association with FBS (*P*= 0.001, r= -0.413) and HbA1c (*P*= 0.003, r= -0.353).

Correlation of CD34 with the index

The results showed that in the control group, CD34 had a significant direct relationship with insulin (*P*= 0.019, r= 0.425), and IR (*P*= 0.017, r= 0.433). In the DNP group, CD34 had a significant direct relationship with CD133 (*P*= 0.011, r= 0.411). In general, CD34 has a significant inverse relationship with FBS (*P*= 0.0001, r= -0.425), HbA1c (*P*= 0.001, r= -0.384), LDL (*P*= 0.046, r= -0.245), and uric acid (*P*= 0.0001, r= -0.466). In addition, CD34 has a significant direct relationship with Insulin (*P*= 0.001, r= 0.382), IR (*P*= 0.011, r= 0.308), and CD133 (*P*= 0.001, r= 0.399).

Correlation of CD133 with the index

In the control group, CD133 had a significant inverse relationship with GFR (*P*= 0.043, r= -0.372). In the DNP group, CD133 had a significant direct correlation with age (*P*= 0.037, r= 0.344), and disease duration (*P*= 0.025, r= 0.368).

Association of nephropathy severity with iron, vitamin D, CD34, and CD133 levels

The results showed that iron, vitamin D, CD34, and CD133 levels were significantly associated with the severity of nephropathy (*P*= 0.0001, each) (Table 3).

Discussion

Table2. Comparison of blood indices between diabetic nephropathy (DNP) and diabetic without nephropathy (Control) groups.

Variable	DNP Group Mean(±SD)	CTL Group Mean(±SD)	*P*-value
FBS (mg/dL)	195.89 (± 13.42)	128.33 (±17.92)	0.0001
HBA1c (%)	8.54 (±0.51)	7.42 (± 0.60)	0.0001
Cholesterol (mg/dL)	196.65 (± 12.34)	180.37 (± 49.59)	0.089
Triglyceride (mg/dL)	183.95 (± 11.37)	166.10 (± 85.06)	0.263
HDL (mg/dL)	39.55 (± 1.73)	42.77 (± 13.42)	0.202
LDL (mg/dL)	129.95 (± 5.27)	97.83 (± 32.95)	0.0001
Creatinine (mg/dL)	1.96 (± 0.34)	1.61 (± 0.66)	0.013
Uric Acid (mg/dL)	8.34 (± 0.38)	5.92 (± 0.59)	0.0001
Insulin (µU/mL)	6.75 (± 0.86)	12.18 (± 2.83)	0.028
Insulin Resistance (HOMA- Score)	3.26 (± 0.45)	3.46 (± 1.25)	0.738
GFR (mL/minute/1.73 cubic meters)	42.41 (± 10.04)	65.57 (± 24.68)	0.0001
Iron (µg/dL)	64.45 (±34.04)	39.27 (±10.12)	0.001
Vitamin D (ng/mL)	24.16 (±10.05)	29.51 (±9.98)	0.034
CD34 (ng/mL)	1.86 (±0.77)	2.74 (±0.74)	0.001
CD133 (ng/mL)	3.04 (±1.97)	3.84 (±0.72)	0.025

Table 3. Relationship between Iron, vitamin D, CD34, and CD133 with nephropathy severity

Variable / Group		Mean (±SD)	*P*-value
Iron	Mild to moderate reduction in kidney function	56.93 (±31.73)	0.0001
	Moderate to severe reduction in kidney function	69.57 (±35.32)	
Vitamin D	Mild to moderate reduction in kidney function	23.17 (±10.24)	0.0001
	Moderate to severe reduction in kidney function	24.85 (±10.10)	
CD34	Mild to moderate reduction in kidney function	2.08 (±0.89)	0.0001
	Moderate to severe reduction in kidney function	1.70 (±0.66)	
CD133	Mild to moderate reduction in kidney function	3.27 (±2.23)	0.0001
	Moderate to severe reduction in kidney function	2.87 (±1.81)	

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Nephropathy is one of the most important complications of diabetes, which begins with a decrease in kidney function due to vascular damage to the renal vessels and eventually leads to many complications in patients (26).

In this case-control study, the mean BMI was significantly increased in the control group compared with the case group. The results showed that only the mean diastolic blood pressure was significantly increased in the case group compared with the control group. High blood pressure is prevalent in people with diabetes, especially in T2DM. However, blood pressure depends on several factors, including obesity; therefore, this difference could be due to a significant difference in BMI and duration of diabetes between the two groups. Furthermore, in this study, serum FBS and HbA1c levels in the control group were significantly lower than those in the case group.

The results showed that the mean IR obtained according to the HOMA-IR formula was insignificant between the two groups. However, in terms of insulin level, the difference was significant, with a higher value in the control group than in the case group.

Moreover, in our study, the mean cholesterol, triglycerides, and LDL levels were higher in the DNP group than in the control group. However, only the difference in LDL levels between the two groups was significant. There is evidence that hyperlipidemia may contribute to the development and progression of kidney disease and diabetes.

The results showed that the mean creatinine level in the DNP group was significantly higher than that in the control group. A study by Luo et al. in China showed that the mean GFR of the DNP group was significantly lower than that of the control group (27), which is consistent with our results.

In addition, our results showed that the mean serum level of uric acid in the case group was significantly higher than that in the control group. The results of Razi et al. (2018) are consistent with our results. Their study showed that the amount of serum uric acid is related to a decrease in GFR (28).

Our results showed that the mean vitamin D level in the case group was significantly lower than that in the control group. These results are consistent with those of Balla et al. (29). In this study conducted in 2018, Balla et al. investigated the relationship between serum vitamin D levels with glucose control and complications of nephropathy in T2DM. 40 DNP patients with 40 diabetic individuals without nephropathy and 40 healthy individuals were compared for the main variables. They concluded that nephropathy and FBS were inversely related to vitamin D levels in patients. In our study in the DNP group, vitamin D had a significant direct relationship with HbA1c and a significant inverse relationship with HDL. These results indicate that this decrease in vitamin D levels is significantly associated with the incidence of diabetes complications, particularly nephropathy. The study conducted by Xiao et al. in 2016 also showed that with the progression of kidney disease in diabetic patients, the average level of vitamin D decreases significantly (30).

In this study, the mean serum iron level in the case group was significantly higher than that in the control group. The relationship between serum iron levels and other variables in the two groups showed that in diabetic nephropathy patients, serum iron levels had a significant direct relationship with vitamin D and uric acid and a significant inverse relationship with FBS, HbA1c, and total cholesterol. Furthermore, it has been shown that in the case group, serum iron levels have an inverse significant relationship with disease duration.

Excess iron and oxidative stress are involved in the pathogenesis and increase the risk of T2DM and related problems. Iron, even when not in large amounts, is known to influence glucose metabolism. Some studies have shown that the body's iron stores are involved in developing glucose tolerance in T2DM and gestational diabetes (31). Oxidative stress and

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inflammatory cytokines extend and exacerbate these disorders (32).

Tuleub et al. evaluated serum iron levels in DNP patients. 24 healthy individuals as a control group, along with 30 DNP patients and 30 patients with T2DM without nephropathy, were selected. The results of this research showed that the iron level in T2DM patients without nephropathy was significantly higher than that in other groups, in contrast to the results of our study (33). Iron plays an essential role in kidney injury and the progression of kidney disease (34,35). Therefore, it has been hypothesized that reducing excessive iron levels may contribute to the prevention of diabetes complications such as nephropathy.

Moreover, our results showed that the mean serum CD34 and CD133 levels in the control group were significantly higher than those in the case group.

The results showed that CD34 had a significant direct relationship with CD133 in the DNP group. In general, CD34 has a significant inverse relationship with HbA1c, FBS, LDL, and uric acid levels. There is also a significant direct relationship with insulin, IR, and CD133levels.

In Rigato et al. (36), the level of the CD133 marker was measured and analyzed in patients with and without nephropathy. However, similar to our results, the level of CD133 in DNP patients was higher than in another group, but this difference was not statistically significant. In the study by Dessapt et al. (37), the level of CD133 was measured in patients with and without microalbuminuria. However, this value was not explicitly reported; only the ratio of CD34 to CD133 between the two groups was reported, implying that this ratio was significant. This result also provides secondary support for our finding, showing a relationship between CD34 and CD133 levels and the occurrence of microalbuminuria and consequently nephropathy in diabetic patients.

In the study by Fadini et al. (38), CD34 levels were measured in diabetic patients with or without vascular disease and in a control group that included healthy individuals. This result was inconsistent with our results, showing that the level of CD34 in T2DM patients with vascular disease was significantly lower than that in diabetic patients without vascular disease and in the control group. A recent study showed that CD34 was significantly reduced in diabetic patients compared with that in non-diabetic patients (11). A study by Boscari et al. (39) in 2021 confirmed the reduction of hematopoietic stem/progenitor cells in T1DM. They concluded that long-term diabetes reduces the potential for hypoglycemic stimulatory effects on hematopoietic stem/progenitor cells.

This study showed that iron, vitamin D, CD34, and CD133 levels were significantly associated with the severity of nephropathy. We found no similar studies examining the relationship between the severity of nephropathy and CD34 and CD133 levels.

In addition to monitoring the blood level of vitamin D and some trace elements such as iron, manage endothelial progenitor cells, such as CD34 and CD133, and prevent their decline by improving their growth status and preventing their apoptosis to study their effects for the treatment of nephropathy diabetic or even diabetes, is the main result of this study, which should be seriously pursued.

Conclusions

This study determined the relationship between serum levels of vitamin D, CD34, CD133, and iron in patients with T2DM divided into two groups with or without nephropathy.

Serum iron levels were increased in the DNP group compared with the control group. Additionally, the mean levels of vitamin D, CD34, and CD133 in the case group were significantly lower than those in the control group.

Iron and vitamin D, CD34, and CD133 levels were significantly associated with the severity of nephropathy. Therefore, increasing the severity of nephropathy may increase

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serum iron levels and decrease serum levels of vitamin D, CD34, and CD133.

Thus, controlling these parameters can improve the complications of T2DM, particularly nephropathy.

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Conflict of Interest

The authors declare no competing interests.

Authors' contributions

V. P: Conceived and designed the analysis, contributed data or analysis tools and wrote the paper.

M. J: Collected the data and performed the analysis.

M. M: Collected the data and performed the analysis.

T. RN: Collected the data and wrote the paper.

M. HT: Conceived and designed the analysis and performed the analysis.

All authors have accepted responsibility for the entire content of this manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and approved the version to be published.

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