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The Role of Nitric Oxide, Lipocalin-2, and Proinflammatory Cytokines on Proteinuria and Insulin Resistance in Type 2 Diabetes Mellitus Subgroups

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Background: Nitric oxide (NO) is a bioactive signaling molecule that mediates various physiological and biological processes. Type 2 diabetes mellitus (T2DM) can be categorized into several subgroups according to fasting plasma glucose (FPG) and hemoglobin A1c (HbA1c) levels. Few studies have closely examined the effect of NO and lipocalin-2 on albuminuria and insulin resistance in T2DM subgroups. This study investigated the role of NO, lipocalin-2, and proinflammatory cytokines on the development of proteinuria and insulin resistance in patients with T2DM subgroups.

Methods: A total of 256 subjects, including 191 patients with T2DM and 65 non-diabetic healthy individuals, were evaluated. NO metabolites (NOx), lipocalin-2, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels were measured. Patients with T2DM were classified into three subgroups: patients with FPG-defined diabetes (PG-DM), those with HbA1c-defined diabetes (HA-DM), and those who met the criteria for both FPG and HbA1c (PG/HA-DM). The albumin-to-creatinine ratio (ACR) and the homeostasis model assessment of β-cell function (HOMA-B) and insulin resistance (HOMA-IR) were calculated.

Results: NOx, lipocalin-2, and TNF-α levels were significantly higher in patients with T2DM than in healthy individuals. Patients with PG/HA-DM had significantly higher NOx levels than those with PG-DM or HA-DM. Of the patients with high NOx levels, patients with lipocalin-2 elevation exhibited higher ACR and HOMA-IR than those without lipocalin-2 elevation. NOx was positively correlated with lipocalin-2, ACR, HOMA-IR, and TNF-α but not with HOMA-B and IL-6. The upper quartile of NOx levels led to a 1.2-fold increase in the risk of albuminuria (odds ratio: 1.215; 95% CI: 1.012–2.418; $p < 0.001$).

Conclusion: NO plays a crucial role in proteinuria and insulin resistance by collaborating with lipocalin-2 and TNF-α, showing significantly higher levels in patients with PG/HA-DM than in those with PG-DM or HA-DM.

Keywords: nitric oxide, lipocalin-2, proteinuria, insulin resistance, diabetes subgroups

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia, elevated hemoglobin A1c (HbA1c), insulin resistance, and inadequate insulin secretion.[1] Prolonged hyperglycemia causes endothelial dysfunction, which induces micro- and macro-vascular complications, such as diabetic nephropathy, ischemic heart disease, and stroke.[2] In patients with T2DM, vascular inflammation and endothelial dysfunction usually precede the onset of microalbuminuria.[3] Vascular endothelial cells secrete diverse biological mediators, including nitric oxide (NO) and endothelin-1.[4] Hyperglycemia enhances NO production, which is closely related to T2DM with cardiovascular complications.[5]

NO is a highly reactive free radical that plays a key role in various physiological processes, such as vasodilation, neurotransmission, immune responses, and apoptosis.[6] NO participates in the pathogenesis of inflammation, acting as

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a pro-inflammatory facilitator in pathological conditions and as an anti-inflammatory mediator in physiological conditions.[7] Proinflammatory cytokines induce expression of inducible NO synthase (iNOS) in macrophages and granulocytes, and activated iNOS promotes an approximately 1000-fold increase in NO production.[8] NO is produced in the pancreas and can affect insulin synthesis and secretion, contributing to the development of T2DM.[9]

NO also plays an important role in lipocalin-2 production.[10] Lipocalin-2 is a member of the lipocalin superfamily and serves as a pleiotropic mediator of various physiological and pathological processes.[11] Lipocalin-2 also regulates glucose and lipid homeostasis, insulin secretion, and energy metabolism.[12] Lipocalin-2 is an acute-phase protein involved in regulating host responses to inflammation; thus, it has been regarded as an inflammatory marker.[13] Recently, lipocalin-2 was reported to play a vital role in the development of diabetic complications.[14]

Patients with T2DM can be categorized into several subgroups, such as patients diagnosed by fasting plasma glucose (FPG) alone (PG-DM), those with isolated elevated hemoglobin A1c (HbA1c) levels, such as HbA1c-defined diabetes (HA-DM), and those who met the criteria for both FPG and HbA1c (PG/HA-DM). NO production in T2DM patients has been widely studied; however, to date, no studies have closely examined the differences in the effects of NO and lipocalin-2 on proteinuria and insulin resistance between T2DM subgroups.

Therefore, this study investigated the role of NO, lipocalin-2, and proinflammatory cytokines on the development of insulin resistance and proteinuria in patients with T2DM subgroups. This study also evaluated the difference in NO metabolites (NOx) levels among T2DM subgroups.

Materials and Methods

Subject Populations

A total of 256 subjects aged 43 to 72 years (mean age: 58.4 years), including 191 patients with T2DM and 65 non-diabetic healthy individuals, were evaluated. In this study, only the patients with newly diagnosed T2DM who had no history of antihyperglycemic treatment or lipid-lowering and antihypertensive therapies were included. As the control group, age-matched healthy individuals without evidence of T2DM, renal dysfunction, inflammation, or medication were enrolled. T2DM was diagnosed based on the criteria of the American Diabetes Association.[15] Patients with T2DM were categorized into three subgroups: patients with PG-DM (FPG $\geq$ 126 mg/dL and HbA1c $<$ 6.5%), those with HA-DM (HbA1c $\geq$ 6.5% and FPG $<$ 126 mg/dL), and those with PG/HA-DM (FPG $\geq$ 126 mg/dL and HbA1c $\geq$ 6.5%). The following patients were excluded from the study: (a) those receiving surgery or medication; (b) those with a history of type 1 DM, thyroid diseases, stroke, or hemoglobinopathies; (c) those who were pregnant; (d) those with acute blood loss or transfusion; and (e) those with fasting times less than eight hours. Information on smoking habits, alcohol consumption, and physical activity was obtained. The study protocol was approved by the institutional review board of Inha University Hospital (approval number: 2023–06-005), and informed consent was obtained from participants. This study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Measurement of Laboratory Parameters

Blood samples were collected from patients after fasting for at least eight hours. All laboratory measurements were performed using samples obtained prior to treatment. Lipocalin-2 levels were measured by fluorescence immunoassay using a Triage lipocalin-2 test kit (Alere Inc., San Diego, CA, USA). A high lipocalin-2 level was defined as over 150 ng/mL.[16] The NOx levels in the serum of the participants were measured by an enzyme-linked immunosorbent assay using a total nitric oxide and nitrate/nitrite kit (R&D Systems, Minneapolis, MN, USA). Nitrate was converted to nitrite using nitrate reductase, and total nitrite was measured. Elevated NOx levels were defined as $>$ 53.1 μmol/L, which was a provisional cutoff level based on median of NOx levels in T2DM patients. The concentrations of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured using enzyme-linked immunosorbent assay kits (BD Inc., San Diego, CA, USA and R&D Systems, Minneapolis, MN, USA). HbA1c levels were analyzed using high-performance liquid chromatography (G8 Glycohemoglobin analyzer; Tosoh Bioscience, Tokyo, Japan). Serum insulin levels were measured by an immunoradiometric assay using an insulin IRMA kit (Beckman Coulter, Fullerton, CA, USA). FPG, albumin, creatinine, and high-sensitivity C-reactive protein (hsCRP) levels were measured using

a chemical analyzer (Cobas 8000 C702; Roche, Mannheim, Germany). An elevated hsCRP level was defined as greater than or equal to 0.5 mg/dL and was considered laboratory evidence of inflammation.[17] The estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) method.[18] An eGFR less than 75 mL/min/1.73 m^2 was defined as a decreased eGFR and an eGFR greater than or equal to 90 mL/min/1.73 m^2 was considered normal.[19] The albumin-to-creatinine ratio (ACR) was calculated using the following formula: ACR (μg/mg) = (urine albumin [μg/mL]/urine creatinine [mg/dL]) × 100.[20] Normoalbuminuria and micro- and macroalbuminuria were defined as an ACR less than 30 μg/mg, an ACR of between 30 and 300 μg/mg, and an ACR equal to or greater than 300 μg/mg, respectively.[21] The homeostasis model assessment of insulin resistance (HOMA-IR) and β-cell function (HOMA-B) were estimated using the following equation: HOMA-IR = (fasting serum insulin [μIU/mL] × FPG [mg/dL]) / 405 and HOMA-B = fasting serum insulin (μIU/mL) × 360 / (FPG [mg/dL] – 63).[22] Insulin resistance was defined as a HOMA-IR value equal to or greater than 2.56.[23] Current smokers were defined as people who had smoked more than 100 cigarettes in their lifetime and who had smoked in the last four weeks.[24] A person who consumed alcohol two or more times per week was considered a drinker with high-frequency drinking.[25] A regular exerciser was defined as an individual who performed 20 minutes or more of vigorous-intensity physical activity three or more days per week or 30 minutes or more of light to moderate-intensity physical activity five or more days per week.[26]

Categorization of Patients

Patients were categorized into several groups according to the median and quartiles of the NOx levels: those with elevated NOx levels (> 53.1 μmol/L, n = 95) and non-elevated NOx levels (≤ 53.1 μmol/L, n = 96), based on the median of the NOx levels; and those in the upper quartile of the NOx levels (> 97.5 μmol/L, n = 48) and the lower quartile of the NOx levels (< 32.4 μmol/L, n = 48). Patients with elevated NOx levels were further stratified into two groups based on lipocalin-2 levels: those with high lipocalin-2 levels (> 150 ng/mL, n = 53) and low lipocalin-2 levels (≤ 150 ng/mL, n = 42). To evaluate the NOx levels according to HOMA-IR, hsCRP, eGFR, and ACR, patients were divided into two groups based on each cutoff level of the corresponding parameters.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD), median (interquartile range; IQR), or frequency (percentage). Continuous variables with normal distribution or non-normally distributed data were analyzed using a Student's *t*-test or a Mann–Whitney *U*-test, respectively. To identify the normality of the data, the Shapiro–Wilk test was used. Categorical variables were analyzed using a χ^2 test. Relationships between NOx, lipocalin-2, proinflammatory cytokines, ACR, HOMA indices, and glycemic parameters were evaluated using multivariate linear regression analyses with adjustment for potential confounders, such as age, sex, body mass index (BMI), systolic blood pressure (SBP), current smoking, alcohol consumption, and physical activity. The association between NOx elevation and the prevalence of albuminuria was assessed using a multivariate logistic regression analysis. Statistical analyses were performed using SPSS (version 26; IBM SPSS Statistics, Armonk, NY, USA) and MedCalc (version 20; MedCalc Software Ltd., Ostend, Belgium). A p value of less than 0.05 was considered statistically significant.

Results

Clinical and Laboratory Characteristics of Patients

Of the 191 T2DM patients, 46 (24.1%) had PG-DM, 59 (30.9%) had HA-DM, and 86 (45.0%) had PG/HA-DM. Albuminuria was observed in 22.0% of the patients, with 20.4% having microalbuminuria and 1.6% having macroalbuminuria. The NOx and lipocalin-2 levels were significantly higher in patients with T2DM than in healthy individuals (53.1 μmol/L and 162.5 ng/mL vs 24.7 μmol/L and 71.5 ng/mL, respectively, $p < 0.001$). The mean disease duration was 1.4 years (Table 1).

Table 1 Clinical and Laboratory Characteristics of Subject Populations

Parameters	Patients with T2DM (n = 191)	Healthy Individuals (n = 65)	p value
Anthropometric parameters			
Age (year)	58.4 ± 10.2	58.6 ± 9.3	0.879
Sex (male; n, %)	96 (50.3)	34 (52.3)	0.781
BMI (kg/m^2)	24.9 ± 3.8	22.6 ± 2.7	< 0.001
SBP (mmHg)	128.6 ± 9.2	120.4 ± 7.1	< 0.001
Disease duration (year)	1.4 ± 0.3	NA	NA
Current smoker (n, %)	41 (21.5)	12 (18.5)	0.582
Alcohol drinker (n, %)	87 (45.5)	28 (43.1)	0.687
Regular exerciser (n, %)	76 (39.8)	27 (41.5)	0.854
Glycemic parameters			
FPG (mg/dL)	148.2 ± 34.5	91.7 ± 5.9	< 0.001
HbA1c (%)	7.1 ± 0.8	5.4 ± 0.3	< 0.001
T2DM subgroups (n, %)			
PG-DM	46 (24.1)	NA	NA
HA-DM	59 (30.9)	NA	NA
PG/HA-DM	86 (45.0)	NA	NA
Biological mediators			
NOx (μmol/L)	53.1 (32.4–97.5)	24.7 (12.3–46.2)	< 0.001
Lipocalin-2 (ng/mL)	162.5 (94.3–231.8)	71.5 (48.2–141.5)	< 0.001
Proinflammatory cytokines			
TNF-α (pg/mL)	60.4 (21.5–98.2)	15.8 (6.2–27.9)	< 0.001
IL-6 (pg/mL)	19.2 (8.3–31.6)	6.7 (2.5–15.4)	< 0.001
Urinary albumin excretion			
ACR (μg/mg)	16.1 (9.7–30.9)	5.3 (2.8–12.5)	< 0.001
Microalbuminuria (n, %)	39 (20.4)	NA	NA
Macroalbuminuria (n, %)	3 (1.6)	NA	NA
Insulin secretion			
Insulin (μIU/mL)	13.2 ± 6.4	9.2 ± 3.6	< 0.001

Notes: Data are expressed as the mean ± SD, median (IQR), or frequency (%). In healthy individuals, the disease duration was not measured; thus, it was expressed as NA (not applicable).

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; PG-DM, diabetes diagnosed by the FPG criteria; HA-DM, diabetes diagnosed by the HbA1c criteria; PG/HA-DM, diabetes diagnosed by both the FPG and HbA1 criteria; NOx, nitric oxide metabolites; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ACR, albumin-to-creatinine ratio; NA, not applicable.

NOx, Lipocalin-2, and TNF-α Levels in Patients with T2DM Subgroups

Inflammatory parameters and biological mediators were examined according to the T2DM subgroups. NOx and lipocalin-2 levels were significantly higher in patients with PG/HA-DM than in those with PG-DM or HA-DM (64.5 μmol/L and 195.7 ng/mL vs 39.8 μmol/L and 132.4 ng/mL, respectively, $p < 0.001$) (Figure 1). Patients with PG/HA-DM had 1.8- and 2.3-fold higher TNF-α and hsCRP levels, respectively, than patients with PG-DM or HA-DM ($p < 0.001$) (Table 2).

Lipocalin-2, TNF-α, and ACR According to Median and the Quartiles of NOx Levels

Lipocalin-2, TNF-α, and ACR levels were evaluated according to the median and quartiles of the NOx levels. Lipocalin-2, TNF-α, and ACR levels of patients with NOx elevation did not differ from those of patients without NOx elevation, which was based on median of NOx levels. However, patients ranked in the upper quartile of NOx levels had significantly higher TNF-α, ACR, and lipocalin-2 levels and more prevalent proteinuria than patients ranked in the lower quartile of NOx levels (Table 3).

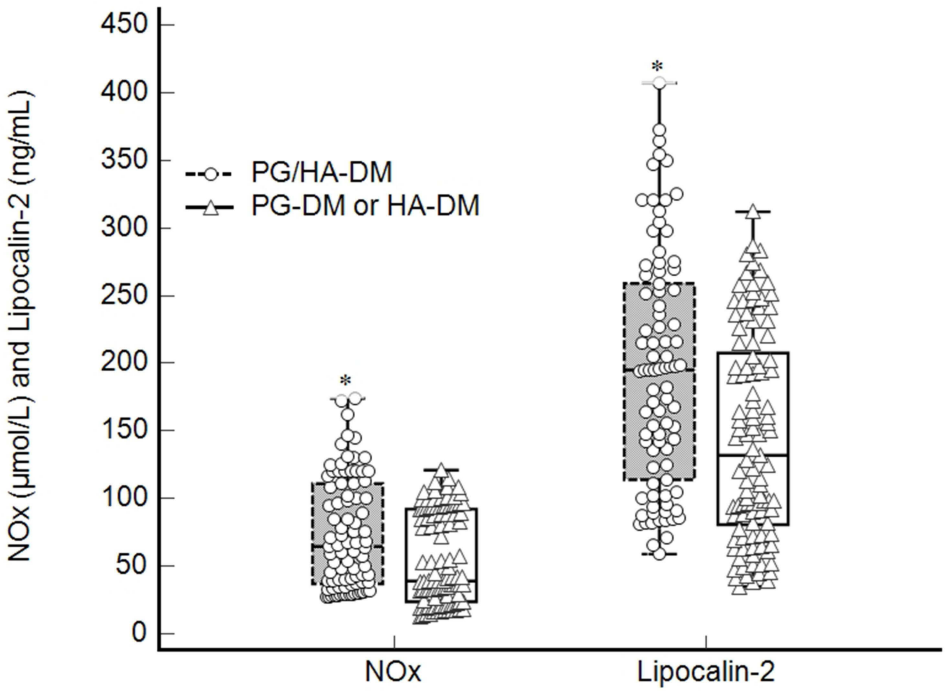

Figure 1 NOx and lipocalin-2 levels in T2DM patients according to T2DM subgroups. NOx and lipocalin-2 levels are significantly higher in patients with PG/HA-DM than in those with PG-DM or HA-DM (64.5 μmol/L and 195.7 ng/mL vs 39.8 μmol/L and 132.4 ng/mL, respectively). *p < 0.001. Units for parameters: NOx (μmol/L), lipocalin-2 (ng/mL).
Abbreviations : NOx, nitric oxide metabolites; PG-DM, diabetes diagnosed by the FPG criteria; HA-DM, diabetes diagnosed by the HbA1c criteria; PG/HA-DM, diabetes diagnosed by both the FPG and HbA1 criteria.

ACR and HOMA-IR in Patients with Elevated NOx and Lipocalin-2 Levels

ACR and HOMA-IR levels were evaluated according to NOx and lipocalin-2 levels. Of the patients with elevated NOx, those with high lipocalin-2 levels exhibited significantly higher ACR and HOMA-IR than those with low lipocalin-2 levels. In contrast, there was no significant difference in HOMA-B between the two groups (Table 4).

NOx Levels in Relation to HOMA-IR, eGFR, hsCRP, and Microalbuminuria

NOx levels were examined in relation to insulin resistance, kidney function, microalbuminuria, and inflammation severity. NOx levels were significantly higher in patients with high HOMA-IR, elevated hsCRP, and decreased eGFR levels than in those with low HOMA-IR, non-elevated hsCRP, and normal eGFR levels. However, patients with microalbuminuria did not differ from those with normoalbuminuria regarding the NOx levels (Table 5).

Relationship Between NOx, Lipocalin-2, ACR, and HOMA Indices

The correlations between NOx and lipocalin-2, cytokines, ACR, HOMA indices, and glycemic parameters were evaluated. NOx was significantly correlated with lipocalin-2 ($r = 0.261$), TNF-α ($r = 0.274$), and ACR ($r = 0.239$) following adjustment for potential confounders. However, no significant correlations between NOx and HOMA-B, IL-6, and glycemic parameters were observed (Table 6). A scatter plot of the relationship between NOx and lipocalin-2 in patients with T2DM is shown in Figure 2.

Odds Ratio of NOx Elevation for the Prevalence of Proteinuria

The association between NOx elevation and the prevalence of proteinuria was assessed using a multivariate logistic regression analysis. After adjusting for potential confounders, the upper quartile of the NOx levels led to a 1.2-fold increase in the risk of albuminuria (odds ratio: 1.215; 95% CI: 1.012–2.418; $p < 0.001$) (Table 7).

Table 2 Proinflammatory Cytokines, Kidney Function, and Inflammatory and Glycemic Parameters in the T2DM Subgroups

Parameters	PG/HA-DM (n = 86)	PG-DM or HA-DM (n = 105)	p value
Clinical parameters			
Age (year)	59.5 ± 9.8	58.3 ± 11.2	0.121
Sex (male; n, %)	45 (52.3)	51 (48.6)	0.672
BMI (kg/m^2)	25.2 ± 3.9	24.5 ± 3.5	0.136
Current smoker (n, %)	19 (22.1)	22 (20.9)	0.854
Alcohol drinker (n, %)	40 (46.5)	47 (44.8)	0.872
Regular exerciser (n, %)	32 (37.2)	44 (41.9)	0.413
Proinflammatory cytokines			
TNF-α (pg/mL)	76.2 (31.4–156.8)	42.6 (12.5–84.3)	< 0.001
IL-6 (pg/mL)	21.4 (9.3–45.1)	16.2 (7.2–35.6)	0.258
Kidney function			
ACR (μg/mg)	20.1 (10.5–41.9)	12.1 (5.2–23.7)	0.010
eGFR (mL/min/1.73 m^2)	86.2 ± 9.8	89.3 ± 11.2	0.046
Inflammatory parameters			
hsCRP (mg/dL)	1.06 (0.21–2.34)	0.47 (0.13–1.95)	< 0.001
Elevated hsCRP (n, %)	49 (57.0)	41 (39.0)	0.015
Glycemic parameters			
FPG (mg/dL)	172.5 ± 32.6	126.1 ± 9.8	< 0.001
HbA1c (%)	7.9 ± 1.4	6.5 ± 0.7	< 0.001

Notes: Data are expressed as the mean ± SD, median (IQR), or frequency (%).
Abbreviations: BMI, body mass index; NOx, nitric oxide metabolites; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; PG-DM, diabetes diagnosed by the FPG criteria; HA-DM, diabetes diagnosed by the HbA1c criteria; PG/HA-DM, diabetes diagnosed by both the FPG and HbA1 criteria.

Discussion

This study examined the relationship between NOx, lipocalin-2, proteinuria, and insulin resistance in patients with T2DM, especially according to T2DM subgroups. The main findings of this study were as follows: (a) NO was closely associated with proteinuria and insulin resistance; (b) PG/HA-DM patients had significantly higher NOx levels; (c)

Table 3 Lipocalin-2, HOMA Indices, and the Prevalence of Albuminuria According to the Median and Quartiles of the NOx Levels

Parameters	Median of the NOx Levels		Quartiles of the NOx Levels	
	Elevated (n = 95)	Non-Elevated (n = 96)	Upper Quartile (n = 48)	Lower Quartile (n = 48)
Biological mediators				
Lipocalin-2 (ng/mL)	174.5 (134.5–258.2)	150.3 (119.2–237.5)	194.5 (164.5–295.3)*	129.7 (92.3–206.8)
TNF-α (pg/mL)	68.3 (32.1–107.6)	53.2 (14.3–93.4)	78.5 (39.5–116.7)*	43.2 (12.9–81.5)
IL-6 (pg/mL)	21.2 (9.3–45.8)	18.6 (8.2–31.9)	23.1 (10.2–46.1)	17.2 (7.4–30.8)
Urinary albumin excretion				
ACR (μg/mg)	18.6 (8.5–34.2)	13.2 (5.6–28.7)	21.7 (9.6–41.5)*	10.4 (5.1–21.6)
Albuminuria (n, %)	26 (27.4)	16 (16.7)	18 (37.5)*	7 (14.6)
HOMA indices				
HOMA-IR	4.8 ± 3.5	4.5 ± 3.2	4.9 ± 3.6	4.3 ± 3.4
HOMA-B (%)	58.3 ± 34.1	63.1 ± 37.4	56.7 ± 32.5	65.2 ± 39.7

Notes: Data are expressed as the mean ± SD, median (IQR), or frequency (%). *p < 0.05, compared with the lower quartile group.
Abbreviations: TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ACR, albumin-to-creatinine ratio; HOMA-IR and HOMA-B, homeostasis model assessment of insulin resistance and β-cell function; NOx, nitric oxide metabolites.

Table 4 Proinflammatory Cytokines, Kidney Function, and HOMA Indices in Patients with Increased NOx Levels and High Lipocalin-2 Levels

Parameters	Patients with NOx Elevation		p value*
	High Lipocalin-2 (n = 53)	Low Lipocalin-2 (n = 42)	
Proinflammatory cytokines			
TNF-α (pg/mL)	91.6 (43.2–142.5)	45.7 (21.6–95.3)	< 0.001
IL-6 (pg/mL)	29.4 (13.7–52.8)	12.1 (7.5–30.4)	< 0.001
Kidney function			
eGFR (mL/min/1.73 m^2)	85.4 ± 9.7	89.9 ± 10.2	0.031
ACR (μg/mg)	25.3 (14.6–42.5)	12.1 (4.3–21.6)	< 0.001
Albuminuria (n, %)	20 (37.7)	6 (14.3)	0.025
HOMA indices			
HOMA-IR	5.6 ± 3.4	4.1 ± 2.9	0.024
HOMA-B (%)	57.2 ± 35.1	65.3 ± 38.7	0.289

Notes: *Analyzed using the Student's *t*-test, Mann–Whitney *U*-test, or χ^2 test. Data are expressed as the mean ± SD, median (IQR), or frequency (%).
Abbreviations: TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; eGFR, estimated glomerular filtration rate; ACR, albumin-to-creatinine ratio; HOMA-IR and HOMA-B, homeostasis model assessment of insulin resistance and β-cell function; NOx, nitric oxide metabolites.

Table 5 NOx Levels in Relation to HOMA-IR, hsCRP, Microalbuminuria, and Kidney Function

Categorization*	NOx Levels (μmol/L)	p value
Insulin resistance		
High HOMA-IR	62.8 (39.1–115.3)	0.027
Low HOMA-IR	43.4 (25.2–92.6)	
Inflammation		
Elevated hsCRP	64.1 (42.8–126.4)	< 0.001
Non-elevated hsCRP	41.7 (23.7–89.5)	
Urinary albumin excretion		
Microalbuminuria	56.2 (32.5–112.8)	0.425
Normoalbuminuria	50.3 (27.3–95.7)	
Kidney function		
Decreased eGFR	63.9 (41.5–123.5)	0.013
Normal eGFR	42.5 (24.1–90.4)	

Notes: *High HOMA-IR (≥ 2.56), elevated hsCRP (≥ 0.5 mg/dL), microalbuminuria (ACR: 30–300 μg/mg), and decreased eGFR (< 75 mL/min/1.73 m^2). Data are expressed as the median (IQR).
Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; NOx, nitric oxide metabolites.

patients with elevated NOx and lipocalin-2 levels exhibited higher ACR and HOMA-IR; and (d) high NOx levels increased the odds ratio for albuminuria.

There have been many conflicting results on NOx levels in patients with T2DM. For instance, in some studies, NOx levels were significantly elevated in patients with T2DM compared with healthy subjects.[27,28] In contrast, in other studies, NOx levels were significantly lower in T2DM patients than in non-diabetic individuals.[29,30] In our study, serum NOx levels were significantly higher in T2DM patients than in healthy subjects. Interestingly, in the subgroup analysis, NOx and hsCRP levels were significantly elevated in patients with PG/HA-DM compared to those with PG-DM or HA-DM. These results suggest that NO production is increased in T2DM patients; however, NOx levels vary according to the T2DM subgroups, which may explain the conflicting NO results reported in many previous studies. Considering the

Table 6 Relationships Between NOx Levels and Biological Mediators, Glycemic Parameters, and HOMA Indices

Parameters	Correlation with NOx Levels	
	Standardized β*	p value
TNF-α	0.274	< 0.001
IL-6	0.105	0.168
Lipocalin-2	0.261	< 0.001
hsCRP	0.281	< 0.001
ACR	0.239	< 0.001
FPG	0.116	0.131
HbA1c	0.121	0.104
HOMA-IR	0.205	0.005
HOMA-B	–0.102	0.172

Notes: *Analyzed using multivariate linear regression analyses with adjustment for potential confounders, such as age, sex, BMI, SBP, current smoking, alcohol consumption, and physical activity. Standardized β indicates the regression coefficients resulting from a regression analysis.

Abbreviations: TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; hsCRP, high-sensitivity C-reactive protein; ACR, albumin-to-creatinine ratio; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HOMA-IR and HOMA-B, homeostasis model assessment of insulin resistance and β-cell function; NOx, nitric oxide metabolites.

increased hsCRP levels in patients with PG/HA-DM, the enhanced NO production in PG/HA-DM patients may be due to systemic inflammation observed in this subtype. These assumptions are supported by the results of a study showing that NO was a critical regulator of inflammation in T2DM patients.[31]

Lipocalin-2 has been regarded as a proinflammatory adipokine, which is up-regulated in obese persons.[32] Lipocalin-2 is closely related to NO and its related enzyme activity. Gómez et al demonstrated that NO plays a main role in lipocalin-2 expression and strongly provokes lipocalin-2 production.[33] In this study, lipocalin-2 levels were examined according to NOx levels in T2DM patients. There were no significant differences in lipocalin-2 levels between patients with and without increased NOx, which was based on the median of NOx levels. However, patients within the upper quartile of NOx levels exhibited significantly higher lipocalin-2 levels than those within the lower quartile of NOx levels. Furthermore, the multivariate regression analysis revealed that NOx levels were positively and significantly correlated with lipocalin-2 levels. These results suggest that lipocalin-2 levels may not be affected by a mild increase in NOx but are affected when NOx rises to moderate to high levels. These findings are consistent with the results of a previous study, which demonstrated that NO regulates the lipocalin-2 expression of β-cells in inflammatory conditions.[34]

Numerous studies have been conducted on NO production in relation to albuminuria. Several studies have reported that NOx levels are significantly higher in patients with microalbuminuria than those with normoalbuminuria.[35,36] Moreover, one study reported that enhanced NO production contributed to the development of microalbuminuria.[37] In contrast, other studies have demonstrated that decreased NO activity is closely related to increased albuminuria.[38] In an experimental research, endothelial NOS (eNOS)-deficient diabetic mice showed marked albuminuria.[39] In the present study, NOx levels were positively correlated with ACR, and high NOx levels significantly increased the risk of albuminuria. The difference in NOx levels was more evident when based on the presence of reduced eGFR than on the presence of microalbuminuria. These findings imply that NOx levels are closely linked to the prevalence of albuminuria, and active NO production occurs at a more advanced stage of renal impairment than at the early stage of microalbuminuria.

Hyperglycemia induces the production of oxidative free radicals, which causes damage to endothelial cells and subsequently leads to inflammation of the blood vessels.[40] Hyperglycemia can also activate NO production in patients

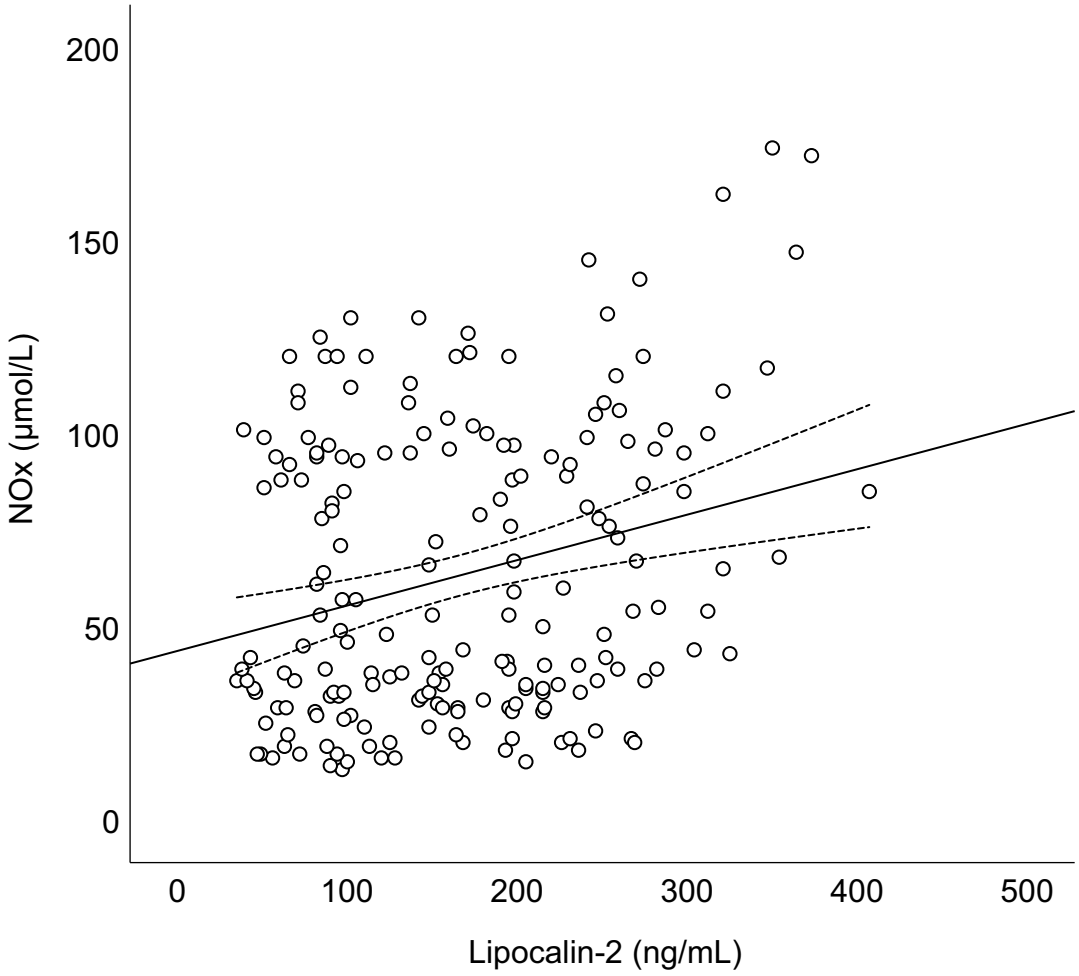

Figure 2 Scatter plots of the relationship between NOx and lipocalin-2 levels in patients with T2DM. The NOx levels are positively and significantly correlated with lipocalin-2 levels ($y = 0.121x + 43.256$; $r^2 = 0.072$; $p < 0.001$).
Abbreviation: NOx, nitric oxide metabolites.

with T2DM.[41] Several studies have shown a positive correlation between serum NOx levels and blood glucose and HbA1c levels.[28,42] Adela et al reported that high blood glucose levels were strongly responsible for enhanced NO production in endothelial cells.[43] In the present study, the relationship between NOx levels and glycemic parameters was

Table 7 Associations Between NOx Levels and the Prevalence of Albuminuria in T2DM Patients

Upper Quartile of NOx Levels	Prevalence of Albuminuria	
	Odds Ratio (95% confidence interval)	p value
Unadjusted	2.134 (1.109–4.161)	< 0.001
Adjusted for		
Age and sex	2.061 (1.103–4.023)	< 0.001
Age, sex, BMI, and SBP	1.742 (1.081–3.572)	< 0.001
Age, sex, BMI, SBP, and smoking habits	1.681 (1.065–3.124)	< 0.001
Age, sex, BMI, SBP, current smoking, and alcohol consumption	1.329 (1.054–2.637)	< 0.001
Age, sex, BMI, SBP, current smoking, alcohol consumption, and physical activity	1.215 (1.012–2.418)	< 0.001

Abbreviations: NOx, nitric oxide metabolites; BMI, body mass index; SBP, systolic blood pressure.

evaluated. There were no significant associations between NOx levels and FPG and HbA1c levels. However, NOx levels were positively correlated with TNF-α and hsCRP levels. Additionally, patients with high NOx levels showed significantly elevated TNF-α levels. Therefore, it appears that NO production is enhanced in hyperglycemic conditions accompanied by systemic inflammation. Overall, it is estimated that hyperglycemia induces oxidative stress, which increases NO synthesis, particularly in coexisting inflammatory conditions. Subsequently, high NO levels cause glomerular hyperfiltration and hyperperfusion, escalating urinary albumin excretion in patients with T2DM.

Vascular inflammation and endothelial dysfunction are closely related to the development of microalbuminuria in T2DM.[44] NO is synthesized in endothelial cells by eNOS, and NO plays an important role in the evolution of inflammation.[7] Chronic inflammation is associated with insulin resistance and β-cell dysfunction, consequently increasing the risk of T2DM.[3] In this study, NOx levels were approximately 1.4-fold higher in patients with high HOMA-IR than in those with low HOMA-IR. Additionally, NOx levels were positively correlated with HOMA-IR, although they were not significantly correlated with HOMA-B. Therefore, it appears that elevated NOx levels are closely related to insulin resistance in patients with T2DM. These findings are in accordance with the results of one study demonstrating that NO generation was significantly associated with insulin resistance in T2DM patients.[45]

This study has several limitations. An oral glucose tolerance test was not conducted; thus, the prevalence of T2DM might be underestimated. Diet habits, total energy intake, and the types of alcoholic beverages in the patients could not be assessed, which may have affected the results of this study. As this study was a cross-sectional study, the evidence of a cause-and-effect relationship between NO production and T2DM was limited. In this study, the sample size was small, especially in the subgroup analysis. Despite these limitations, to the best of our knowledge, this is the first study to report the relationships between NOx levels, lipocalin-2, proinflammatory cytokines, insulin resistance, and proteinuria, especially in relation to T2DM subgroups. Further studies are warranted to verify the findings of this study in larger randomized prospective trials.

Conclusions

In conclusion, this study demonstrated that NOx levels were significantly elevated in T2DM patients, particularly in patients with PG/HA-DM subgroup, and closely associated with TNF-α and hsCRP levels and the prevalence of albuminuria. Patients with elevated NOx and lipocalin-2 levels exhibited significantly higher ACR and HOMA-IR. These results suggest that NO may play a pivotal role in the development of albuminuria and insulin resistance, particularly in connection with lipocalin-2 and TNF-α. NO production in T2DM patients differs depending on the T2DM subgroups, and the elevated NOx levels in patients with PG/HA-DM may be due to the systemic inflammation observed in this subtype.

Abbreviations

NOx, nitric oxide metabolites; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ACR, albumin-to-creatinine ratio; PG-DM, diabetes diagnosed by the FPG criteria; HA-DM, diabetes diagnosed by the HbA1c criteria; PG/HA-DM, diabetes diagnosed by both the FPG and HbA1 criteria.

Data Sharing Statement

All data relevant to this study are included in the article. The data used for this paper can be provided to credible investigators with verification for patient confidentiality upon a reasonable request to the corresponding author.

Ethics Approval and Consent to Participate

This study was approved by the institutional review board of Inha University Hospital (approval number: 2023-06-005), and informed consent was obtained from participants. This study was performed in accordance with the guidelines of the Declaration of Helsinki.

Author Contributions

All authors contributed to the work reported, whether in the conception, study design, execution, data acquisition, analysis, or interpretation. All authors took part in drafting, revising, and critically reviewing the manuscript and gave their final approval of the version to be submitted. Authors agree to take responsibility and be accountable for the contents of this article.

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Disclosure

The authors declare that they have no conflicts of interest.

References

1. Karalliedde J, Gnudi L. Diabetes mellitus, a complex and heterogeneous disease, and the role of insulin resistance as a determinant of diabetic kidney disease. *Nephrol Dial Transplant*. 2016;31(2):206–213. doi:10.1093/ndt/gfu405
2. Funk SD, A Y Jr, Orr AW. Hyperglycemia and endothelial dysfunction in atherosclerosis: lessons from type 1 diabetes. *Int J Vasc Med*. 2012;2012:569654. doi:10.1155/2012/569654
3. Scurt FG, Menne J, Brandt S, et al. Systemic inflammation precedes microalbuminuria in diabetes. *Kidney Int Rep*. 2019;4(10):1373–1386. doi:10.1016/j.ekir.2019.06.005
4. Krüger-Genge A, Blocki A, Franke RP, Jung F. Vascular endothelial cell biology: an update. *Int J Mol Sci*. 2019;20(18):4411. doi:10.3390/ijms20184411
5. Pandolfi A, De Filippis EA. Chronic hyperglycemia and nitric oxide bioavailability play a pivotal role in pro-atherogenic vascular modifications. *Genes Nutr*. 2007;2(2):195–208. doi:10.1007/s12263-007-0050-5
6. Delledonne M, Polverari A, Murgia I. The functions of nitric oxide-mediated signaling and changes in gene expression during the hypersensitive response. *Antioxid Redox Signal*. 2003;5(1):33–41. doi:10.1089/152308603321223522
7. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. *Inflammopharmacology*. 2007;15(6):252–259. doi:10.1007/s10787-007-0013-x
8. Andrabi SM, Sharma NS, Karan A, et al. Nitric oxide: physiological functions, delivery, and biomedical applications. *Adv Sci*. 2023;10(30):e2303259. doi:10.1002/advs.202303259
9. Gheibi S, Ghasemi A. Insulin secretion: the nitric oxide controversy. *EXCLI J*. 2020;19:1227–1245. doi:10.17179/excli2020-2711
10. Gu Y, Sun W, Xu ZH, et al. Neutrophil gelatinase-associated lipocalin 2 accelerates hypoxia-induced endothelial cell injury via eNOS/NRF2 signaling. *Cell J*. 2021;23(4):435–444. doi:10.22074/cellj.2021.7167
11. Kjeldsen L, Cowland JB, Borregaard N. Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim Biophys Acta*. 2000;1482(1–2):272–283. doi:10.1016/S0167-4838(00)00152-7
12. Jun LS, Siddall CP, Rosen ED. A minor role for lipocalin-2 in high-fat diet-induced glucose intolerance. *Am J Physiol Endocrinol Metab*. 2011;301(5):E825–E835. doi:10.1152/ajpendo.00147.2011
13. Bhusal A, Lee WH, Suk K. Lipocalin-2 in diabetic complications of the nervous system: physiology, pathology, and beyond. *Front Physiol*. 2021;12:638112. doi:10.3389/fphys.2021.638112
14. Guo H, Jin D, Zhang Y, et al. Lipocalin-2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice. *Diabetes*. 2010;59(6):1376–1385. doi:10.2337/db09-1735
15. American Diabetes Association. Classification and diagnosis of diabetes: standards of medical care in diabetes-2018. *Diabetes Care*. 2018;41(Suppl 1):S13–S27. doi:10.2337/dc18-S002
16. Choi JW, Fujii T, Fujii N. Corrected neutrophil gelatinase-associated lipocalin (NGAL) level adjusted by the scoring system of an inflammation index for screening renal dysfunction in patients with systemic inflammation. *Ann Clin Lab Sci*. 2015;45(3):248–255.
17. Choi JW, Lee MH, Fujii T. Association between neutrophil gelatinase-associated lipocalin and fetal hemoglobin levels in patients with type 2 diabetes mellitus. *Biomed Res Int*. 2021;2021:8383875. doi:10.1155/2021/8383875
18. Chen LI, Guh JY, Wu KD, et al. Modification of diet in renal disease (MDRD) study and CKD epidemiology collaboration (CKD-EPI) equations for Taiwanese adults. *PLoS One*. 2014;9(6):e99645. doi:10.1371/journal.pone.0099645
19. Delanaye P, Glassock RJ, Pottel H, Rule AD. An age-calibrated definition of chronic kidney disease: rationale and benefits. *Clin Biochem Rev*. 2016;37(1):17–26.
20. Fisher H, Hsu CY, Vittinghoff E, Lin F, Bansal N. Comparison of associations of urine protein-creatinine ratio versus albumin-creatinine ratio with complications of CKD: a cross-sectional analysis. *Am J Kidney Dis*. 2013;62(6):1102–1108. doi:10.1053/j.ajkd.2013.07.013
21. Toto RD. Microalbuminuria: definition, detection, and clinical significance. *J Clin Hypertens (Greenwich)*. 2004;6(Suppl 11):2–7. doi:10.1111/j.1524-6175.2004.4064.x
22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412–419. doi:10.1007/BF00280883
23. Yun KJ, Han K, Kim MK, et al. Insulin resistance distribution and cut-off value in Koreans from the 2008-2010 Korean National Health and Nutrition Examination Survey. *PLoS One*. 2016;11(4):e0154593. doi:10.1371/journal.pone.0154593
24. Ryan H, Trosclair A, Gfroerer J. Adult current smoking: differences in definitions and prevalence estimates--NHIS and NSDUH, 2008. *J Environ Public Health*. 2012;2012:918368. doi:10.1155/2012/918368

25. Kang M, Kim JH, Cho WH, Park EC. The gender-specific association between age at first drink and later alcohol drinking patterns in Korea. *PLoS One*. 2014;9(3):e90713. doi:10.1371/journal.pone.0090713
26. Han S, Jang HD, Choi S, et al. Changes in physical activity and risk of fracture: a Korean nationwide population-based cohort study. *Sci Rep*. 2020;10(1):16266. doi:10.1038/s41598-020-73495-1
27. Maejima K, Nakano S, Himeno M, et al. Increased basal levels of plasma nitric oxide in type 2 diabetic subjects: relationship to microvascular complications. *J Diabetes Complications*. 2001;15(3):135–143. doi:10.1016/S1056-8727(01)00144-1
28. Assmann TS, Brondani LA, Bouças AP, et al. Nitric oxide levels in patients with diabetes mellitus: a systematic review and meta-analysis. *Nitric Oxide*. 2016;61:1–9. doi:10.1016/j.niox.2016.09.009
29. Tessari P, Cecchet D, Cosma A, et al. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. *Diabetes*. 2010;59 (9):2152–2159. doi:10.2337/db09-1772
30. Ghosh A, Sherpa ML, Bhutia Y, Pal R, Dahal S. Serum nitric oxide status in patients with type 2 diabetes mellitus in Sikkim. *Int J Appl Basic Med Res*. 2011;1(1):31–35. doi:10.4103/2229-516X.81977
31. Guo H, Zhang Q, Yuan H, et al. Nitric oxide mediates inflammation in type 2 diabetes mellitus through the PPARγ/eNOS signaling pathway. *PPAR Res*. 2020;2020:8889612. doi:10.1155/2020/8889612
32. Liu JT, Song E, Xu A, et al. Lipocalin-2 deficiency prevents endothelial dysfunction associated with dietary obesity: role of cytochrome P450 2C inhibition. *Br J Pharmacol*. 2012;165(2):520–531. doi:10.1111/j.1476-5381.2011.01587.x
33. Gómez R, Scotece M, Conde J, et al. Nitric oxide boosts TLR-4 mediated lipocalin-2 expression in chondrocytes. *J Orthop Res*. 2013;31 (7):1046–1052. doi:10.1002/jor.22331
34. Chang SY, Kim DB, Ko SH, Jang HJ, Jo YH, Kim MJ. The level of nitric oxide regulates lipocalin-2 expression under inflammatory condition in RINm5F beta-cells. *Biochem Biophys Res Commun*. 2016;476(1):7–14. doi:10.1016/j.bbrc.2016.05.110
35. Chiarelli F, Cipollone F, Romano F, et al. Increased circulating nitric oxide in young patients with type 1 diabetes and persistent microalbuminuria: relation to glomerular hyper-filtration. *Diabetes*. 2000;49(7):1258–1263. doi:10.2337/diabetes.49.7.1258
36. Jankar JS, Harley KN, Mohod KM. Study of serum nitric oxide (NO) in type 2 diabetes mellitus patients having normoalbuminuria and microalbuminuria in Central India. *Int J Clin Biochem Res*. 2018;5(1):5–11.
37. Prabhakar SS. Role of nitric oxide in diabetic nephropathy. *Semin Nephrol*. 2004;24(4):333–344. doi:10.1016/j.semnephrol.2004.04.005
38. Ott C, Schneider MP, Delles C, Schlaich MP, Schmieder RE. Reduction in basal nitric oxide activity causes albuminuria. *Diabetes*. 2011;60 (2):572–576. doi:10.2337/db09-1630
39. Takahashi T, Harris RC. Role of endothelial nitric oxide synthase in diabetic nephropathy: lessons from diabetic eNOS knockout mice. *J Diabetes Res*. 2014;2014:590541. doi:10.1155/2014/590541
40. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes: the role of reparatory mechanisms. *Diabetes Care*. 2011;34(Suppl 2):S285–S290. doi:10.2337/dc11-s239
41. Cosentino F, Hishikawa K, Katusic ZS, Lüscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation*. 1997;96(1):25–28. doi:10.1161/01.CIR.96.1.25
42. Shahid SM, Mahboob T. Correlation between glycosylated hemoglobin (HbA1c) and serum nitrite oxide (NO). *Aust J Basic Appl Sci*. 2009;3 (2):1323–1327.
43. Adela R, Nethi SK, Bagul PK, et al. Hyperglycemia enhances nitric oxide production in diabetes: a study from South Indian patients. *PLoS One*. 2015;10(4):e0125270. doi:10.1371/journal.pone.0125270
44. Festa A, D'Agostino R, Howard G, Mykkänen L, Tracy RP, Haffner SM. Inflammation and microalbuminuria in non-diabetic and type 2 diabetic subjects: the insulin resistance atherosclerosis study. *Kidney Int*. 2000;58(4):1703–1710. doi:10.1046/j.1523-1755.2000.00331.x
45. Muniyappa R, Quon MJ. Insulin action and insulin resistance in vascular endothelium. *Curr Opin Clin Nutr Metab Care*. 2007;10:523–530. doi:10.1097/MCO.0b013e32819f8ecd