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Full Length Research Paper

Asymmetrical dimethylarginine (ADMA) and nitric oxide as potential cardiovascular risk factors in type 2 diabetes mellitus

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Hyperglycemia affects biochemical parameters and influences the progression of coronary heart disease and mortality rates in diabetic patients. L- arginine is the substrate used by NO synthase to produce the vasodilator NO. However, in patients with type 2 diabetes mellitus (T2DM), there is an increase in serum levels of methylated L -arginines, such as ADMA, which is a recently identified potent cardiovascular risk factor. The aim of this study was designed to determine both risk factors (ADMA and NO) in type 2 diabetic patients with and without cardiovascular disease and to evaluate whether there is an association between ADMA and glycosylated hemoglobin (HbA1c) on the one hand and nitric oxide on the other hand. The study included 3 groups of subjects; Group I (Control group); comprising 20 healthy subjects; the mean age 48 ± 1.6 years; Group II: 20 diabetic patients without cardiovascular complications; the mean age 51.0 ± 1.96 years and Group III; 20 diabetic patients with evidence of cardiovascular complications; the mean age 54.0 ± 2.1 years. Fasting and postprandial serum glucose, HbA1c, lipid profile (total cholesterol, triacylglycerol, HDL -c and LDL-c), ADMA and serum NO metabolite level, were determined. Serum glucose (fasting and postprandial), HbA1c and ADMA levels showed significant increase in diabetic patients type 2 with and without cardiovascular complications compared to healthy normal control. Total cholesterol, triacylglycerol and LDL-c manifested significant elevations, while HDL-c level showed insignificant change in both groups in compared to non diabetic healthy subjects. Serum NO metabolite level was significantly reduced in the both diabetic patient groups compared with controls. No correlation between ADMA level and studied parameters in diabetic patients without evidence of cardiovascular complications, whereas in cardiovascular complications group, the ADMA level was positively correlated with both postprandial serum glucose and HbA1c, but there was a negative correlation between ADMA levels and NO. Also, NO was negatively correlated with postprandial serum glucose and HbA1c. In conclusion, ADMA and NO may serve as predictors for future cardiovascular events in type 2 diabetic patients. So, early diagnosis and good glycemic control are more effective in reducing the cardiovascular complications.

Key words: Type 2 diabetes, cardiovascular disease, ADMA, nitric oxide.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a progressive and complex metabolic disorder characterized by chronic hyperglycemia resulting from impaired insulin secretion and/or insulin action, the lack of effective insulin leads to disturbances in carbohydrate, lipid and protein metabolism. It is a proinflammatory, hypercoagulable state that predisposes patients to develop cardiovascular disease. It is also associated with risk factors for atherosclerosis, including dyslipidemia, hypertension, inflammation and altered hemostasis, (Granberry and Fonseca, 2005). Patients with T2DM tend to have a characteristic dyslipidemia (increased concentrations of

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LDL-c and decreased concentrations of HDL-c), likely responsible for their being 2 to 4 times more inclined to develop cardiovascular disease than those without T2DM (Haffner et al., 1998). In fact, patients with T2DM are twice as likely as those without T2DM to have elevated triacylglycerol levels and decreased HDL-c concentrations (Garg and Grundy, 1990).

Cardiovascular disease remains the most important cause of morbidity and mortality in diabetes mellitus (Haffner et al., 1998), accounting for approximately 65% of total mortality in patients with type 2 diabetes mellitus (Stamler et al., 1993), in whom hyperglycaemia is one of the main metabolic abnormalities (Yasuda et al., 2006). Aggressive risk factor management is an important mean for reducing the cardiovascular morbidity in this patient group, which implies accurate risk stratification. Poorly controlled blood pressure and hyperglycemia seem to be significantly involved in the development process of cardiovascular disease in patients with type 2 diabetes (Selvin et al., 2004).

Beside traditional risk markers, the nitric oxide (NO), which is synthesized by NO synthases, is an important antiatherogenic molecule (Moncada, 1999). As initially described by Vallance et al. (1992) an endogenous inhibitor of NO synthase, asymmetric dimethylarginine (ADMA), occurs in significant amounts in peripheral blood. Since this discovery, numerous clinical studies have been performed that found elevated circulating ADMA concentrations in humans suffering from diseases associated with increased cardiovascular risk. Different prospective cohort studies in selected patient groups suggested ADMA as an independent predictor of cardiovascular morbidity (Mittermayer et al., 2006). In a cross-sectional study, ADMA was associated with macrovascular disease in patients with type 2 diabetes (Krzyzanowska et al., 2006). However, the predictive role of ADMA for the occurrence of cardiovascular events in type 2 diabetes has not been examined prospectively until now.

The aim of this study was designed to determine both risk factors (ADMA and NO) in type 2 diabetic patients with and without cardiovascular disease and to evaluate whether there is an association between ADMA and glycosylated hemoglobin (HbA1c) on the one hand and nitric oxide on the other hand.

SUBJECTS AND METHODS

Subjects

The study was performed on forty outpatients with type 2 diabetes and 20 healthy subjects. All patients were selected from outpatients Clinic of National Institute of Diabetes and Endocrinology (NIDE), Cairo, Egypt. Type 2 DM was diagnosed according to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2006) . Cardiovascular disease was defined as a positive medical history for myocardial infarction, angina, coronary artery bypass graft and stroke, and ischemic changes in electrocardiogram. Type 2 diabetic patients were taking the same oral therapy. Patients with any history of smoking and alcohol habits, respiratory disorder and showed any clinical or laboratory signs of liver disease, or thyroid function impairments, renal dysfunction, chronic inflammatory and clinically significant infectious diseases were excluded. The healthy control subjects, which matched with age and sex as patients with type 2 diabetes, had no recognizable diseases and clinically free from any abnormality. They were not receiving any medications and represented the control group. Demographic data was recorded for each subject using self -made questionnaire. Approval had been taken from the research ethics committee of General Organization of Teaching Hospitals and Institutes. An informed consent was obtained from all patients and healthy subjects that described the aim of the study and the procedures that would be required from them. The study included 3 groups of subjects, Group I: control group (n = 20), the mean age 48 ± 1.6 years; Group II: type 2 diabetic patients without cardiovascular complications (n = 20), the mean age 51.0 ± 1.96 years, the mean duration of the disease was 5.1 ± 0.37 years and Group III: type 2 diabetic patients with evidence of cardiovascular complications (n = 20), the mean age 54.0 ± 2.1 years, the mean duration of the disease was 8.1 ± 0.75 years.

Methods

Blood samples were collected into vacutainer tube without additive after 12 h overnight fasting from healthy subjects and diabetic patients, this was followed by the ingestion of meals and a further blood samples were collected 2 h later (postprandial) . Blood was then centrifuged at 3000 rpm for 10 minutes at 4°C. Serum was rapidly separated; subdivided into aliquots and were stored at -80°C until the measurements of lipid profile, ADMA and nitrite /nitrate metabolites (NO). Another part of collected blood was taken on EDTA for determination of HbA1c level. Hemolysed samples were excluded. Serum glucose concentration (fasting and postprandial) was assayed at once by glucose oxidase method according to Barham and Trinder (1972). Serum total cholesterol was determined by the enzymatic method (Allain et al., 1974). Triacylglycerol was assayed by peroxidase-coupled method (McGowan et al., 1983). HDL-c was measured by enzymatic method after precipitation of other lipoproteins with $MgCl_2$ and dextran sulphate (Finley et al., 1978), LDL-c was calculated according to Friedewald et al. (1972) and HbA1c level was done according to the method of Grey et al. (1996) using an immunoturbidimetric assay on Dimension RxL Max (Dade Behring). Serum ADMA concentration was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits based on the method of Schulze et al. (2004). Nitric oxide decomposes rapidly in aerated solutions to form stable nitrate/nitrite products, so NO production was evaluated by measuring the serum concentration of nitrate/nitrite products via a Griess reaction in accordance with Moshage et al. (1995).

Statistical analysis

Data was expressed as the mean ± S.D. Statistical analysis was performed with Statistical Package for the Social Science for Windows (SPSS, version 10.0, 1999, Chicago, IL, USA). Differences between groups were analyzed by one-way analysis of variance (ANOVA). Post-hoc testing was performed by the Bonferroni test to compare the difference among the groups studied as reported by Altman (1991). Pearson's correlation analysis was performed to determine the relationships between

Table 1. Demographic and biochemical characteristics of control and diabetic patients (Mean ± SD).

Parameters	Group I	Group II	Group III
Age (Years)	48.0 ± 1.6	51.0 ± 1.96	54.0 ± 2.1
Gender (F/M)	12/8	14/6	11/9
Duration of diabetes (Years)	-------	5.1 ± 0.37	8.1 ± 0.75 [c]
BMI (Kg/m^2)	26.19 ± 0.3	25.87 ± 0.37	25.14 ± 0.44
SBP (mmHg)	116.6 ± 9.7	114.5 ± 11.3	124.4 ± 4.1
DBP (mmHg)	69.1 ± 4.8	74.0 ± 6.7	74.2 ± 7.4
Fasting serum glucose (mg/dl)	80.4 ± 9.1	191.9 ± 32.0 [a]	219.4 ± 45.5 [a,c]
Postprandial serum glucose (mg/dl)	109 ± 9.9	269.9 ± 41.6 [a]	350.7 ± 66.9 [a,d]
HbA_{1c} (%)	5.9 ± 0.47	9.17 ± 1.07 [a]	11.37 ± 0.94 [a,d]

SBP, Systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index
[a]$p < 0.0001$ Vs. Group I, [c]$p < 0.029$ Vs. group II, [d]$p < 0.0001$ Vs. group II.

Table 2. Serum lipid profile in control and diabetic patients (Mean ± SD).

Parameters	Group I	Group II	Group III
Cholesterol (mg/dl)	177.3 ± 40.0	202.7 ± 30.3 [a]	279.4 ± 25.8 [b, c]
Triacylglycerol (mg/dl)	121.1 ± 29.6	209.9 ± 39.1 [e]	283.9 ± 87.9 [b, d]
HDL-c (mg/dl)	44.7± 16.6	40.2 ± 12.16	42.6 ± 11.6
LDL-c (mg/dl)	113.0 ± 31.2	134.1 ± 24.14 [a]	190.0 ± 24.5 [b, c]

[a]$p < 0.05$ group 1, [b]$p < 0.0001$ Vs. group I, [c]$p < 0.0001$ Vs. group II, [d]$p < 0.001$ Vs. group II, [e]$p < 0.0001$ Vs. group I.
Group I: control group, Group II: type 2 diabetic patients without cardiovascular complications.
Group III: type 2 diabetic patients with evidence of cardiovascular complications.

ADMA, NO and other cardiovascular risk variables. P-value < 0.05 was accepted to indicate statistical significance.

RESULTS

Demographic and some biochemical characteristics of the patients and controls are presented in Table 1. No differences were found between diabetic groups and controls with respect to age, gender and BMI ($P > 0.05$). In the diabetic group, the mean duration of diabetes with and without vascular complications was 5.1 ± 0.37 and 8.1 ± 0.75 respectively. Serum glucose (fasting and postprandial) and glycosylated Hemoglobin (HbA1c) showed significant increase in diabetic patients type 2 with and without cardiovascular complications compared to healthy normal control. Vascular complications in type 2 diabetic patients produced pronounced increase in serum fasting glucose, postprandial serum glucose and HbA1c concentrations when compared to diabetic patients without vascular complications ($p < 0.029$, $p < 0.0001$, $p < 0.0001$ respectively).

Table 2 demonstrates the changes of lipid profile of diabetic patients type 2. Total cholesterol (TC), triacylglycerol (TG) and LDL-c manifested significant elevations ($p < 0.05$, $p < 0.0001$, $p < 0.05$ respectively) without cardiovascular complications and with cardiovascular complications ($p < 0.0001$ for each) when compared to normal control subjects, while HDL-c level showed insignificant change in both groups in compared to non diabetic healthy subjects. TC, TG and LDL-c represented pronounced increases in diabetic patients with vascular complications ($p < 0.0001$, $p < 0.001$, $p < 0.0001$ respectively) compared to diabetic patients without vascular complications.

Table 3 illustrates the effects of diabetes mellitus type 2 on asymmetrical dimethylarginine (ADMA) and nitric oxide (NO). The level of ADMA represented significant elevation ($P < 0.0001$ for each) in both groups in comparison to normal subjects but the vascular complications in type 2 diabetic patients produced pronounced increase ($P < 0.0001$ for each) when compared to diabetic patients without vascular complications.

Serum NO metabolite level (nitrate/nitrite) was significantly reduced ($P < 0.0001$) in the both diabetic patient groups compared with controls. However the diabetic patients with vascular complications showed pronounced increase of NO ($P < 0.0001$) when compared to diabetic patients without vascular complications (Table 3).

Pearson's correlation analyses revealed that, no correlation was observed between ADMA level and studied parameters in diabetic patients without evidence of cardiovascular complications, whereas in cardiovascular complications group, the ADMA level was positively correlated with both postprandial serum glucose and HbA1c

Table 3. Serum levels of asymmetrical dimethylarginine (ADMA) and nitric oxide (NO) in control and diabetic patients (Mean ± SD).

Parameters	Group I	Group II	Group III
ADMA (mol/L)	0.41± 0.092	1.31 ± 0.45 [a]	2.34 ± 0.59 [a, b]
NO (mol/L)	274.4 ± 34.3	222.2 ± 8.8 [a]	125.9 ± 48.3 [a, b]

[a]P < 0.0001 Vs. group I, [b]P < 0.0001 Vs. group II.
Group I: control group, Group II: type 2 diabetic patients without cardiovascular complications.
Group III: type 2 diabetic patients with evidence of cardiovascular complications.

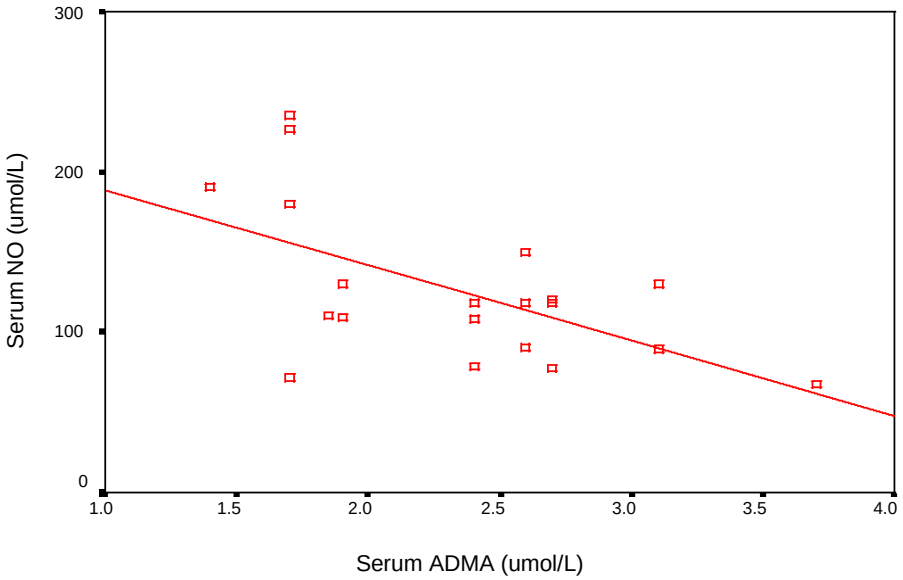

Figure 1. Correlation between serum ADMA and NO in type 2 diabetic patients with evidence of cardiovascular complications (r = - 0.580, p = 0.007).

(r = 0.593, p = 0.006; r = 0.496, p = 0.026, respectively) as represented in Figures 2 and 3. On the other hand, there was a negative correlation between ADMA levels and NO (r = - 0.580, p = 0.007) (Figure 1). Also NO was negatively correlated with postprandial serum glucose and HbA1c (r = - 0.497, p = 0.026; r = - 0.460, p = 0.041, respectively) (Figures 4 and 5).

DISCUSSION

Cardiovascular disease is the major cause of morbidity and mortality in patients with type 2 diabetes mellitus T2DM (Haffner et al., 1998), in whom hyperglycaemia is one of the main metabolic abnormalities (Yasuda et al., 2006). Blood glucose control occupies the centre stage in T2DM management (Grundy et al., 1999) . Patients with diabetes can have many complications including elevated levels of VLDL-c, LDL-c triacylglycerol and low levels of HDL-c (Haffner, 1998). These patients have a preponderance of abnormalities in the composition of smaller, denser particles, which increase atherogenicity even if the absolute concentration of LDL-c is not significantly increased (Haffner, 1998). In our study, Table 2 elucidates that total cholesterol, triacylglycerol and LDL-c levels showed pronounced increases in diabetic patients with evidence of cardiovascular complications compared with control group, while no change in HDL-c level in diabetic groups compared with control group. These findings are in agreement with the previous studies which suggest that lipoprotein abnormalities are higher in diabetics than in non-diabetics (Idogun et al., 2007; Albrki et al., 2007).

Our finding that increased ADMA and decreased NO in type 2 diabetic subjects is significant because endothelial dysfunction associated with increased ADMA concentrations seems to begin before the detectable vascular damage in type 2 diabetic patients. Additionally, type 2 diabetic patients had increased levels of ADMA leading to endothelial damage, even if hypertension or hyperlipidemia does not exist. Measurement of ADMA and NO as markers of endothelial dysfunction may

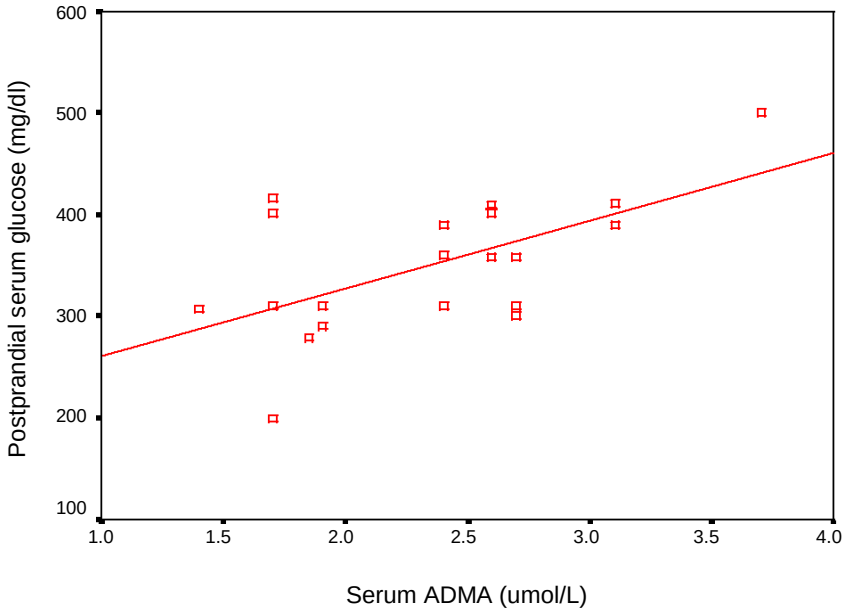

Figure 2. Correlation between serum ADMA and postprandial serum glucose in type 2 diabetic patients with evidence of cardiovascular complications (r = 0.593, p = 0.006).

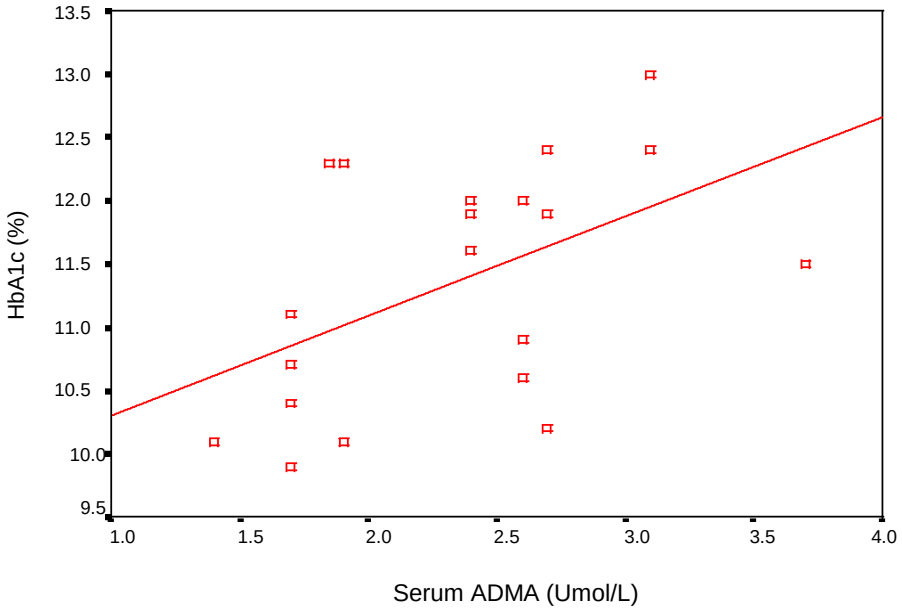

Figure 3. Correlation between serum ADMA and HbA1c (%) in type 2 diabetic patients with evidence of cardiovascular complications (r = 0.496, p = 0.026).

provide an opportunity for the prevention of irreversible endothelial damage in these patients (Altinova et al., 2007).

There are some explanations about the interaction between hyperglycemia and the L-arginine-NO system. Hyperglycemia-induced activation of protein kinase C, increased superoxide anion production from glucose autoxidation and accumulation of advanced glycation end product due to nonenzymatic cross-linking of proteins via oxidative stress can reduce the bioavailability of

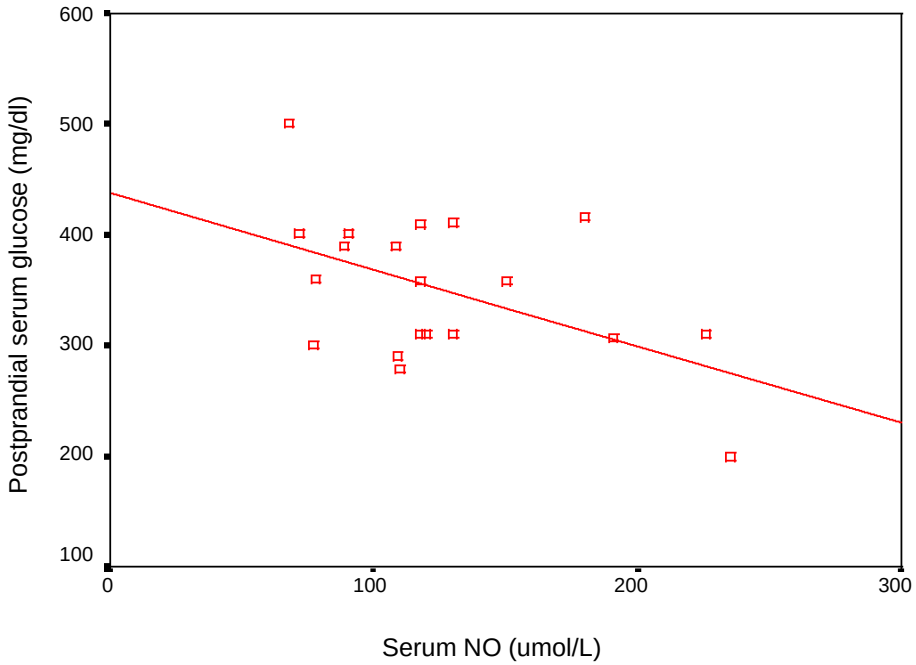

Figure 4. Correlation between serum NO and postprandial serum glucose in type 2 diabetic patients with evidence of cardiovascular complications (r = - 0.497, p = 0.026).

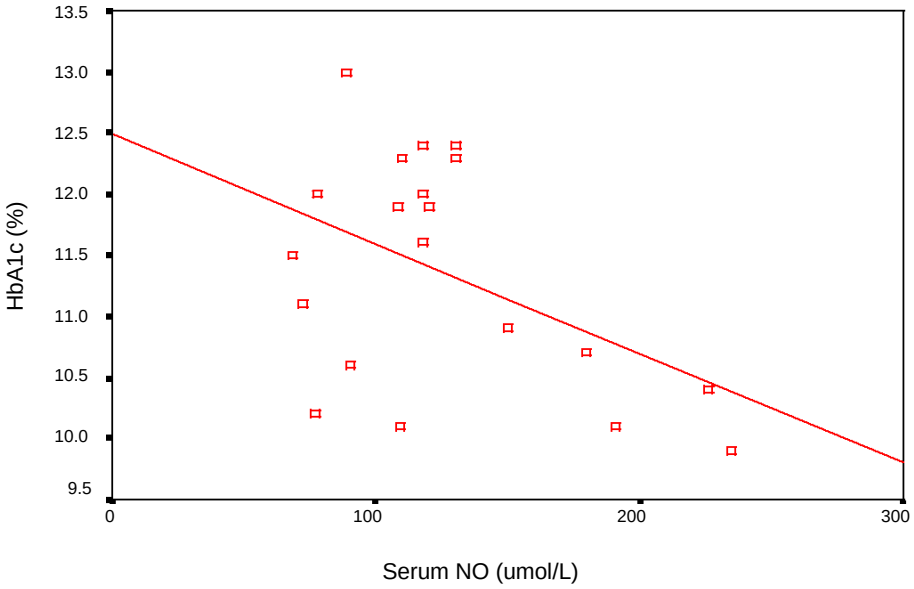

Figure 5. Correlation between serum NO and HbA1c (%) in type 2 diabetic patients with evidence of cardiovascular complications (r = - 0.460, p = 0.041).

NO and activation of the polyol pathway, which increases the use of nicotinamide adenine dinucleotide phosphate can reduce the biosynthesis of NO (Chan and Chan, 2002). However, the exact mechanism of how hyperglycemia influences circulating ADMA concentrations in T2DM is not fully known. One possible mechanism has been suggested in an animal study that hyperglycemia-induced oxidative stress increases ADMA by impairing the dimethylarginine dimethylaminohydrolase (DDAH), which is involved in the metabolic degradation of ADMA (Lin et al., 2002). Furthermore, Sorrenti et al. (2006) reported that exposure to high glucose in

endothelial cells increases oxidative stress, reduces DDAH-2 and leads to a NOS imbalance. Although renal clearance is the first mechanism for the elimination of ADMA (Zoccali et al., 2001), enzymatic degradation of ADMA by DDAH has recently gained substantial importance. DDAH degrades ADMA to dimethylamine and L-citrulline and DDAH activity is found in almost all tissues, especially in kidney and liver (Kimoto et al., 1995). One of the allelic isoforms of this enzyme, DDAH- 2, is mainly present in vascular tissues that coexpress endothelial NOS (Leiper, et al., 1999). Another mechanism for the increase in ADMA concentrations in hyperglycemic media may be associated with the enzyme arginine methyltransferase, which synthesizes ADMA, because hyperglycemia-induced oxidative stress up-regulates the expression of arginine methyltransferases (Maas, 2005).

One of the most critical vasoactive mediators synthesized by the vascular endothelium is nitric oxide (NO), previously known as endothelium -derived relaxing factor (Palmer et al., 1987). Nitric oxide is synthesized from L-arginine by NO synthase. Endothelium-derived NO is a powerful endogenous vasodilator and also has important roles in the maintenance of vascular homeostasis. For instance, NO inhibits platelet aggregation, leucocyte migration and cellular adhesion to the endothelium and attenuates vascular smooth muscle cell proliferation and migration. In addition, it inhibits activation and expression of adhesion molecules and the production of superoxide anions (Vallance and Chan, 2001). In type 1, type 2 diabetes and insulin resistance syndrome, there is evidence that the release and/or bioavailability of NO are diminished (Chan et al., 2000). Despite the heterogeneous nature of these conditions, they all share the same feature of increased plasma glucose concentrations which could affect the L- arginine: NO pathway. Nitric oxide bioavailability can be reduced due to increased oxidative stress which can result from increased superoxide anions production from glucose autoxidation. Hyperglycaemia-induced activation of protein kinase C (PKC) followed by that of phospholipase A2, results in increased production of arachidonic acid metabolites which also have potent oxidizing effects. In contrast, reduced NO synthesis can result from activation of the polyol pathway which increases the utilization of nicotinamide adenine dinucleotide phosphate (NADPH), an important cofactor in the biosynthesis of NO. Furthermore, accumulation of advanced glycation end product AGE due to non-enzymatic crosslinking of proteins, could quench NO, further reducing its bioavailability. In addition to these mechanisms, the endogenous NO synthase inhibitor, asymmetric dimethylarginine (ADMA), has emerged as a key factor in determining NO biosynthesis (Chan et al., 2000).

ADMA is derived from the catabolism of proteins containing methylated arginine residues and is released as the proteins are hydrolysed. These proteins are predominately found in the nucleus and are involved in RNA processing and transcriptional control (Najbauer et al., 1993). The synthesis of ADMA and NG-monomethyl-L-arginine (L-NMMA) requires the enzyme protein arginine methyltransferase type I (PRMT I) which methylates arginine residues (Ghosh et al., 1988). Protein arginine methyltransferase type II (PRMT II) forms symmetric dimethylarginine (SDMA). SDMA is a stereoisomer of ADMA and has no direct inhibitory effect on NO synthase. All three methylarginines (ADMA, SDMA and L-NMMA) enter endothelial cells through the cationic amino acid transporters known collectively as the y^+ transporter. The activity of this transporter was found to co-locate with caveolin-bound NO synthase which suggests that the y^+ transporter activity could be an important determinant of the local concentrations of methylarginines (McDonald et al., 1997). The three methylarginines compete with each other and with arginine for transport into the cell (Leiper and Vallance, 1999). Hence high concentrations of ADMA could potentially interfere with intracellular transport of L-arginine resulting in a decrease in NO synthesis. The transport system concentrates methylarginines within the endothelial cells such that intracellular concentrations are greater than circulating concentrations. In this regard, a defective y^+ transporter system could result in a higher concentration of circulating ADMA leading to decreased NO biosynthesis. Hence the y^+ transporter system could be a potential site of defect in disease states (Toutouzas et al., 2008).

We observed that our diabetic patients did not show a good glycemic control. Therefore, the present study showed a positive correlation between serum ADMA and HbA1c levels. It is likely to be a major effect of tight blood glucose control on increased serum ADMA concentrations in diabetic patients.

Our study showed no correlation between ADMA level and studied parameters in diabetic patients without evidence of cardiovascular complications, whereas in patients with cardiovascular complications, the ADMA level was positively correlated with both postprandial serum glucose and HbA1c, but there was a negative correlation between ADMA levels and NO. Also, NO was negatively correlated with postprandial serum glucose and HbA1c. This agrees with the previously reported data by Schernthaner and Krzyzanowska (2008) that indicate ADMA is directly related to the serum glucose level. Besides, Yasuda et al. (2006) reported that intensive control of serum glucose levels led to a decrease in ADMA level in hospitalized patients with type 2 DM. No correlation was observed between ADMA and lipid parameters including HDL-c and LDL- c in patients with type 2 diabetes. This is in accordance with previous researches (Krzyzanowska et al., 2006; Paiva et al., 2003).

In conclusion, ADMA and NO may serve as predictors for future cardiovascular events in type 2 diabetic patients.

So, early diagnosis and good glycemic control are more effective in reducing the cardiovascular complications. Further studies would be required to clearly establish the utility of decreasing ADMA levels or increasing NO in the treatment of type 2 diabetic patients.

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