



## Role of Asymmetric Dimethylarginine and Nitric Oxide in Type 2 Diabetes Mellitus and its Cardiovascular Complications

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AN ENDOGENOUS inhibitor of nitric oxide synthase, asymmetric dimethylarginine (ADMA), is elevated in patients with type 2 diabetes mellitus (T2DM) and has been related to atherosclerotic disease. The study aims at investigating the status of ADMA and nitric oxide (NO) and their possible correlation in patients with T2DM with and without cardiovascular complications in comparison to normal controls. Forty patients with T2DM and 20 age, sex and body mass index (BMI)-matched healthy controls were included in the study. The studied individuals were divided into Group 1: healthy controls (n= 20), Group 2: T2DM patients without cardiovascular complications (n= 20) and Group 3: T2DM patients with evidence of cardiovascular complications (n= 20). Serum ADMA levels were determined by enzyme linked Immunosorbent Assay (ELISA). Serum nitric oxide was measured as stable end product, nitrite. Insulin was measured by immunoradiometric method. Serum ADMA levels showed a significant elevation while serum NO levels were significantly reduced in diabetic patients groups in comparison to controls. Diabetic patients with vascular complications showed highly significant increase in ADMA levels and pronounced decrease in NO compared to those without complications. In the group of vascular complications, the ADMA level was positively correlated with postprandial serum glucose and HbA1c, but there was a negative correlation between ADMA levels and NO. ADMA and NO may serve as predictors for future cardiovascular events in T2DM patients. Further studies are required to establish the utility of decreasing ADMA levels or increasing NO in the management of T2DM patients.

**Keywords:** Asymmetric dimethylarginine, Type 2 diabetes mellitus, Nitric oxide, Cardiovascular complications.

### Introduction

Asymmetric dimethylarginine (ADMA) is a naturally occurring component of human blood plasma. It is formed as a metabolic by-product of continuous protein turnover in all cells of the body. More than one decade ago, ADMA was first reported to exert biological effects by inhibiting nitric oxide (NO) synthesis (Böger et al., 1998). NO is a well-recognized anti-atherogenic factor; it inhibits the inflammatory-proliferative processes in atherosclerosis. Indeed, endothelial dysfunction due to reduced synthesis and/or bioavailability of NO is thought to be an early step in the course of atherosclerotic cardiovascular disease (Cooke & Dzau, 1997). NO is synthesized from L-arginine via the action of NO synthase (NOS), which is

known to be blocked by endogenous L-arginine analogues such as ADMA (Valkonen et al., 2001). Recently, it has been demonstrated that plasma levels of ADMA are elevated in patients with diabetes. These findings suggest that the elevated ADMA in diabetes could contribute to acceleration of atherosclerosis (Anderssohn et al., 2010).

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

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to develop cardiovascular disease (Granberry & Fonseca, 2005). It is also associated with risk factors for atherosclerosis, including dyslipidemia, hypertension, inflammation and altered hemostasis. Patients with T2DM tend to have a characteristic dyslipidemia (increased concentrations of low density lipoprotein cholesterol (LDL-c) and decreased concentrations of high density lipoprotein cholesterol (HDL-c)), likely responsible for their being 2 to 4 times more inclined to develop cardiovascular disease than those without T2DM (Haffner, 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 & Grundy, 1990).

Cardiovascular complications are the major cause of mortality and morbidity for the individuals world wide afflicted by T2DM (Hsu et al., 2014). Endothelial dysfunction is a common feature in diabetic patients (van de Ree et al., 2001) and may contribute to cardiovascular morbidity (Suwaidi et al., 2000). Mechanisms of diabetes-induced endothelial dysfunction include the production of prostanoid vasoconstrictors and the increased oxidative degradation of NO. Deficiency of NO increases vascular resistance and promotes atherogenesis (Tsefamariam et al., 1995). In addition to its increased oxidative degradation, another possible mechanism for NO deficiency and cardiovascular morbidity is reduced NO synthesis caused by ADMA (Valkonen et al., 2001). The aim of this study is to investigate the status of ADMA, nitric oxide (NO) and their possible correlation in patients with T2DM with and without cardiovascular complications in comparison to normal controls.

### **Materials and Methods**

A total of 40 consecutive unrelated adult T2DM patients were recruited from the outpatient diabetes clinics in Kaser el Einy hospital, Cairo University. Clinical and epidemiological data, as well as family history were collected for all patients according to a standardized questionnaire. T2DM was diagnosed according to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (ECDCDM, 2003). Cardiovascular disease was defined as a positive medical history for hypertension, myocardial infarction, angina, coronary artery bypass graft and stroke and ischemic changes in

electrocardiogram. Each subject was screened by a complete history, physical examination and laboratory analysis. Patients with any history of respiratory disorder or showed any clinical or laboratory signs of liver disease, or thyroid function impairment, renal dysfunction, chronic inflammatory and clinically significant infectious diseases were excluded. 20 healthy control subjects, who matched in age and socioeconomic status as patients with T2DM were included in our study. They had no recognizable diseases and clinically free from any abnormality. They were not receiving any medications. The studied individuals were divided into 3 groups, Group 1: control group (n= 20) (age 47.3± 8.2 years, gender: 11male/ 9female), Group 2: T2DM patients without cardiovascular complications (n= 20) (age 50.3± 1.96 years, gender: 12male/ 8female), and Group III: T2DM patients with evidence of cardiovascular complications (n= 20) (age 53.1± 5.5 years, gender: 12male/ 8female). All procedures performed were according to the ethical standards of the institutional and national research committee given in the Declaration of Helsinki 1964, as revised in 2013. A written informed consent of participation in the study was taken from all subjects. This study was approved by the committee of ethics and research of the university hospital.

- Serum glucose concentration (fasting and postprandial) was measured by glucose oxidase method (Beckman glucose analyzer, Fullerton, CA).
- Serum fasting total cholesterol, triglycerides, HDL-c and LDL-c were measured by automated enzymatic methods using the commercial available kits (Boehringer Mannheim, Germany).
- The concentration of insulin was measured by immunoradiometric assay kit, DIA source INS-IRMA kit, supplied by DIA source ImmunoAssays S.A. (Belgium).
- Serum ADMA levels were determined by enzyme linked Immunosorbent Assay (ELISA) technique using the kit provided from Immunodiagnostic AG, Bensheim, Germany (Nijveldt et al., 2003).
- Serum Nitric oxide was measured as the stable end product, nitrite, according to

the method of Miranda et al., based on the reduction of nitrate by vanadium trichloride combined with detection by acidic Griess reaction (Miranda et al., 2001).

*Statistical analysis*

The results were expressed as means± SD in different groups. The statistical difference between groups was analyzed using student t-test. Pearson’s correlation analysis was performed to determine the relationships between variables. The results were considered significant whenever P values <0.05 were observed. The statistical calculations were performed using statistica (version 6) program.

**Results**

Serum glucose (fasting and postprandial) and glycosylated Hemoglobin (HbA1c) showed a significant increase in diabetic patients type 2 with and without cardiovascular complications (P< 0.0001 for each) compared to healthy normal control. They also showed significant increase in the diabetic patients with evidence of cardiovascular complications when compared with patients without cardiovascular complications (P< 0.05, P< 0.0001, P< 0.0001, respectively). Systolic blood pressure showed a significant difference in patients with vascular

complications compared to both healthy controls and patients without vascular complications (P< 0.001, P< 0.0001). Diastolic blood pressure and insulin levels showed non-significant difference between the studied groups (Table 1).

Total cholesterol, triglycerides and LDL-c manifested significant elevations in diabetic patients with (P< 0.0001 for each ) and without (P< 0.05, P< 0.0001, P< 0.05, respectively) cardiovascular complications when compared to normal control subjects, they also showed 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. While HDL-c level showed a significant decrease in diabetic patients with cardiovascular complications compared to both healthy controls (P< 0.0001) and diabetic patients without vascular complications (P< 0.001) (Table 2).

The level of ADMA represented significant elevation (P< 0.05, P< 0.0001, respectively) in both diabetic patient groups in comparison to normal subjects, but the vascular complications in T2DM patients produced pronounced increase (P< 0.0001) when compared to diabetic patients without vascular complications (Table 3).

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

Parameter	Group 1	Group 2	Group 3
Age (years)	47.3± 8.2	50.3± 1.96	53.1± 5.5
Gender (M/F)	11/9	12/8	12/8
Duration of diabetes (years)	-----	5.7± 0.74	8.6± 0.8
BMI (Kg/m <sup>2</sup> )	25.94± 0.7	25.8± 0.3	25.1± 0.4
SBP (mmHg)	118.6± 1.2	116.5± 8.5	125.4± 7a **b***
DBP (mmHg)	79.3± 5.3	84± 6.2	84.2± 7.4
Insulin (pmol/L)	6.5± 4.3	6.9± 3.3	5.8± 3.7
Fasting serum glucose (mg/dl)	80.3± 7.4	192.5± 30.1 a***	220.4± 42 a*** b*
Postprandial serum glucose (mg/dl)	108± 11.2	260.6± 38.2 a***	340.6± 66.9 a*** b***
HbA1c (%)	5.9± 0.45	8.87± 2 a***	11.4± 0.5 a*** b***

When P value < 0.05, it is statistically significant.

a: indicate the statistical difference between patients and control.

b: indicate the statistical difference between patients with and without cardiovascular.

\*: means P< 0.05.

\*\*: means P< 0.001.

\*\*\*: means P< 0.0001.

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

	Group 1 N= 20	Group 2 N= 20	Group 3 N= 20
Cholesterol (mmol/L)	4.55± 0.83	5.31± 0.72 a*	7.09± 0.72 a*** b***
Triglycerides (mmol/L)	1.36± 0.34	2.26± 0.45 a***	3.17± 1.02 a*** b**
HDL (mmol/L)	1.09± 0.22	0.99± 0.19	0.75± 0.25 a*** b**
LDL (mmol/L)	2.63± 0.72	3.24± 0.66 a*	4.88± 0.66 a*** b***

When P value < 0.05, it is statistically significant.

a: indicate the statistical difference between patients and control.

b: indicate the statistical difference between patients with and without cardiovascular.

\*: means P < 0.05.

\*\*: means P < 0.001.

\*\*\*: means P < 0.0001.

HDL-c: high density lipoprotein-cholesterol, LDL-c: low density lipoprotein-cholesterol.

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

	Group 1 N= 20	Group 2 N= 20	Group 3 N= 20
ADMA (micromol/L)	0.45± 0.14	0.63± 0.15 a*	1.7± 0.65 a*** b***
NO (micromol/L)	49.87± 8.44	42.34± 2.32 a*	28.14± 6.40 a*** b***

When P value < 0.05, it is statistically significant.

a: indicate the statistical difference between patients and control.

b: indicate the statistical difference between patients with and without cardiovascular.

\*: means P < 0.05.

\*\*: means P < 0.001.

\*\*\*: means P < 0.0001.

Serum NO metabolite level (nitrate/nitrite) was significantly reduced ( $P < 0.05$ ,  $P < 0.0001$ , respectively) in the both diabetic patient groups compared with controls. However the diabetic patients with vascular complications showed a pronounced decrease of NO ( $P < 0.0001$ ) when compared to diabetic patients without vascular complications (Table 3).

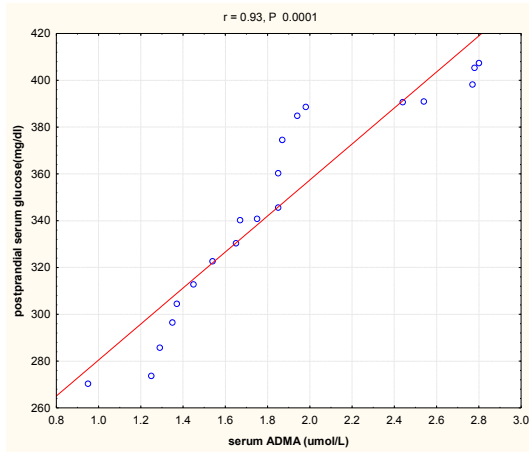
In cardiovascular complications group, the ADMA level was positively correlated with both postprandial serum glucose and HbA1c ( $P < 0.0001$ ). On the other hand, there was a negative correlation between ADMA levels and NO ( $P < 0.0001$ ) (Fig. 1-3).

## Discussion

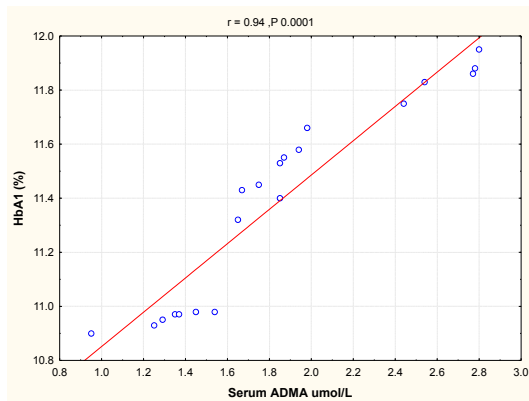
T2DM is associated with an increased risk of cardiovascular disease, Hyperglycaemia is associated with endothelial dysfunction both *in-vivo* (Kawano et al., 1999) and *in-vitro* (Cosentino et al., 1997), therefore endothelial dysfunction is

an early feature in the development of vascular complications in people with diabetes (Chan et al., 2003). Blood glucose control occupies the center stage in T2DM management (Grundy et al., 1999).

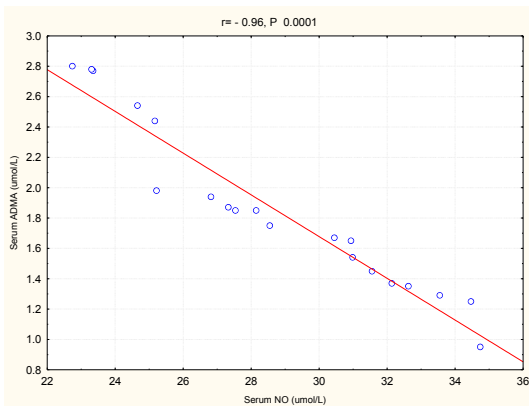
In the present study, total cholesterol, triacylglycerol and LDL-c levels showed pronounced increases in diabetic patients with evidence of cardiovascular complications compared with control group, while a decrease in HDL-c level in diabetic group with cardiovascular complications compared with control group was also shown. These findings are in agreement with the previous studies which suggest that lipoprotein disorders are higher in diabetics than in non-diabetics (Idogun et al., 2007; Albrki et al., 2007). Haffner (1998), reported that patients with diabetes can have elevated levels of triacylglycerol, very LDL-c, LDL-c, and low levels of HDL-c. These patients have abnormalities in the composition of smaller, denser particles, which increase atherogenicity even if the absolute concentration of LDL-c is not significantly increased.



**Fig. 1. Correlation between serum ADMA and postprandial serum glucose in type 2 diabetic patients with evidence of cardiovascular complications.**



**Fig. 2. Correlation between serum ADMA and HbA1c (%) in type 2 diabetic patients with evidence of cardiovascular complications.**



**Fig. 3. Correlation between serum ADMA and NO in type 2 diabetic patients with evidence of cardiovascular complications.**

The obtained results showed significant increased ADMA and decreased NO in both groups of T2DM subjects with and without

cardiovascular complications. There are controversial data from the studies investigated ADMA levels in T2DM. Krzyzanowska et al. (2006) reported increased ADMA concentration and its relation with macrovascular complications in T2DM. In another study (Mahfouz et al., 2009), ADMA levels showed a significant increase in diabetic patients type 2 with and without cardiovascular complications compared to healthy normal control. Serum NO metabolite level was significantly reduced in the diabetic patient groups compared with the controls. The ADMA level was positively correlated with both postprandial serum glucose and HbA1c. In a meta-analysis study to assess the association between ADMA and T2DM, it showed that increased ADMA is the predictive bio-marker of the endothelial dysfunction and cardiovascular complications in T2DM (Hisalkar et al., 2017).

Konya et al. (2015), reported that the cases of T2DM with a high level of ADMA could have diabetes mellitus cardiovascular complications in the future within five years. The clinical acceptance of this parameter will rely on the availability of therapies to immediately reduce ADMA such as incretin-based drugs.

In contrast, Päivä et al. (2003) showed decreased ADMA levels in T2DM patients. A high glomerular filtration rate and poor glycemic control were suggested to be responsible for the decrease in ADMA levels in these patients, but the mechanism related the decrease of ADMA levels could not be clearly defined in their study.

Altinova et al. (2007) found that increased ADMA and decreased l-arginine to ADMA ratio in uncomplicated type 1 diabetic subjects is significant, these patients had increased levels of ADMA leading to endothelial damage, even if hypertension or hyperlipidemia does not exist. Measurement of ADMA and l-arginine to ADMA as markers of endothelial dysfunction may provide an opportunity for the prevention of irreversible endothelial damage in these patients.

In a recent study, aimed to evaluate asymmetric dimethylarginine levels in young patients with Type 1 diabetes mellitus according to diabetes duration, the results are in striking contrast with the results of previous adult studies. In this study, ADMA concentrations decreased with age and duration of diabetes and is associated with



worsening measures of cardiovascular risk and poorer diastolic function (Ersoy et al., 2018).

There are some explanations about the interaction between hyperglycemia and the L-arginine-NO system. Hyperglycaemia-induced activation of protein kinase C (PKC), then phospholipase A2, results in increased production of arachidonic acid metabolites which have potent oxidizing effects. In contrast, reduced NO synthesis can result from activation of the polyol pathway that increases the utilization of nicotinamideadenine dinucleotide phosphate (NADPH), an important cofactor in the biosynthesis of NO (Chan & Chan, 2002). However, the exact mechanism of how hyperglycemia affects circulating ADMA concentrations in T2DM is not fully known.

It 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).

Endothelium-derived NO is the most potent endogenous vasodilator known, exerting its effect via stimulation of soluble guanylate cyclase to produce cyclic guanosine monophosphate (Murad, 1995). NO is critical modulator of blood flow and blood pressure (Cooke et al., 1991). It is released by the endothelium in response to shear

stress and plays an important role in flow-mediated vasodilation (Pohl et al., 1986). Endothelial release of NO opposes the vasoconstrictor effects of norepinephrine, endothelin, angiotensin II, and serotonin (Cooke & Dzau, 1997). Pharmacological inhibition or a genetic deficiency of endothelial NOS impairs endothelium-dependent vasodilatation and increases vascular resistance in patients with coronary artery disease (Huang et al., 1995). An impairment of NO activity may contribute to ischemic syndromes (Nabel et al., 1990). In addition to maintaining a vasodilator tone, NO inhibits platelet aggregation and adhesion and modulates smooth muscle cell proliferation and has been implicated in a number of cardiovascular diseases and virtually every risk factor for these appears to be associated with a reduction in endothelial generation of NO (Moncada & Higgs, 2006).

ADMA is an endogenous and competitive inhibitor of NOS. Plasma levels of this inhibitor are elevated in patients with atherosclerosis and in those with risk of atherosclerosis. In these patients, plasma ADMA levels are correlated with the severity of endothelial dysfunction and atherosclerosis. By inhibiting the production of NO, ADMA may impair blood flow, accelerate atherogenesis, and interfere with angiogenesis (Cooke, 2000).

NO levels might be reduced as a result of a combination of decreased NO production because of decreased activity or reduced expression of endothelial NOS, or its increased degradation by reactive oxygen species (ROS) or increased production of super oxide ions. Accordingly, treatment of endothelial cells with antioxidants has been demonstrated to restore the activity of DDAH, leading to a normalization of cellular ADMA levels and endothelial NO production (Stühlinger et al., 2001). The imbalance between NO and ROS resulting in oxidative stress has been implicated in the pathophysiology of hypertension. NO deficiency can precede the development of hypertension. ADMA can inhibit NOS and regulate local NO/ROS balance. Emerging evidence supports the hypothesis that ADMA-induced NO-ROS imbalance is involved in the development and progression of hypertension (Tain & Huang, 2014). Also, Taner et al. (2013) indicated that serum ADMA may play a role in both the pathophysiology and screening of masked hypertension in diabetic subjects.

Accordingly, future therapeutic strategies that could be used to target NOS (Daiber et al., 2019) and/or DDAH (Jarzebska et al., 2019) activity could be promising in the management of cardiovascular and other vascular complications of diabetes mellitus.

The current 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 postprandial serum glucose and HbA1c. On the other hand, there was a negative correlation between ADMA levels and NO. This agrees with the previously reported data by Schernthaner & Krzyzanowska (2008), Tariq et al. (2017) and Mahfouz et al. (2009) who indicated that ADMA is directly related to the serum glucose level. However, Hsu et al. (2014) reported that ADMA was not correlated with serum HbA1c level, and in diabetic patients with well glycemic control, namely HbA1c  $\leq$  6.5%, elevated ADMA level was no longer associated with increased risk of long-term prognosis.

### **Conclusion**

ADMA and NO may serve as predictors for future cardiovascular events in T2DM patients. Hence, 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 management of T2DM patients.

*Conflict of Interest:* All authors declare that they have no conflict of interest.

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## دور ثنائي ميثيل أرجينين غير المتماثل وأكسيد النيتريك في داء السكري من النوع الثاني ومضاعفات القلب والأوعية الدموية الخاصة به

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لقد وجد ان ثنائي ميثيل أرجينين غير المتماثل (ADMA) و هو المثبط الداخلي لإنزيم تصنيع أكسيد النيتريك ، يرتفع في مرضى داء السكري من النوع الثاني (T2DM) وأن له علاقة بمرض تصلب الشرايين. تم تصميم هذه الدراسة لفحص حالة ADMA، أكسيد النيتريك (NO) وعلاقتها الممكنة في مرضى داء السكري من النوع الثاني المصحوب وغير المصحوب بمضاعفات القلب والأوعية الدموية مقارنة بالأصحاء. تم قياس مستويات ADMA و NO في مصل الدم ل 40 مريض بداء السكري من النوع الثاني و مقارنتها بمجموعة من الأصحاء (20) بالإضافة إلى التحاليل الروتينية.

تم تقسيم المشاركين في البحث إلى ثلاث مجموعات : المجموعة الأولى من الأصحاء والمجموعة الثانية : مرضى السكر بدون مضاعفات والمجموعة الثالثة مرضى السكر المصحوب بمضاعفات القلب والأوعية الدموية. تم قياس ADMA باستخدام تقنية الأليزا وقياس الأنسولين بالإختبار الأشعاعي المناعي وقياس النيترات كناتج نهائي ثابت لل NO . أظهر مستوى ADMA ارتفاعا ذو دلالة إحصائية في كلتا مجموعتي مرضى السكر مقارنة بالأصحاء ولكن مضاعفات الأوعية الدموية في مرضى النوع الثاني لمرض السكر أظهر زيادة ملحوظة عند مقارنتها بمرضى السكر بدون مضاعفات الأوعية الدموية. مستوى نواتج أيض NO (نيترات/ نيتريت) في مصل الدم إنخفض انخفاضاً ذو دلالة إحصائية في كلتا مجموعتي مرضى السكر مقارنة بالأصحاء. بينما مرضى السكر ذوي مضاعفات الأوعية الدموية أظهروا انخفاضاً ملحوظاً ل NO عند مقارنتهم بمرضى السكر بدون مضاعفات الأوعية الدموية. في مجموعة مضاعفات القلب والأوعية الدموية، وجدت علاقة إيجابية بين مستوى ADMA وكل من الجلوكوز في مصل الدم بعد الأكل بساعتين والهيوجلوبين السكري، ولكن على الجانب الآخر، وجدت علاقة سلبية بين مستوى ADMA و NO. يستنتج من هذه الدراسة أن ADMA و NO ربما يكونا بمثابة منبئان للإصابة بأمراض القلب والأوعية الدموية في المستقبل في مرضى داء السكري من النوع الثاني. لذلك فإن التشخيص المبكر والتحكم الجيد في نسبة السكر في الدم يكونا أكثر فاعلية في الحد من مضاعفات القلب والأوعية الدموية. ستكون هناك حاجة إلى مزيد من الدراسات لتثبيت بوضوح فائدة خفض مستوى ADMA أو زيادة NO في علاج مرضى داء السكري من النوع الثاني.