Platelet-Derived NO in Subjects Affected by Type 2 Diabetes with and without Complications: Is there any Relationship with their Offspring?

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ABSTRACT

Aims Macro- and microvascular complications are currently the principal causes of morbidity and mortality in patients with diabetes mellitus. Aim of this study was to determine if type 2 diabetic patients with nephropathy and coronary artery disease showed altered platelet-derived nitric oxide (NO) production, compared with diabetic subjects without complications, and if this alteration is also present in their diabetic offspring.

Methods In this case-control observational study, platelet NO and peroxynitrite content was determined on plasma from 60 male adult type 2 diabetic patients and 60 male offspring type 2 diabetic patients. Plasmatic levels of homocysteine were also determined in the same individuals. Moreover, Western blot analysis of platelet lysates was performed with specific monoclonal antibody for endothelial (eNOS) and inducible (iNOS) nitric oxide synthase.

Results Our study showed a lower piastrinic production of NO in the group of parents without complications (ADH), compared with the group of offspring without complications (YDH) and with the groups of parents with complications. Furthermore, we observed a lower synthesis of peroxynitrite in platelets from the ADH group than in the groups of patients with complications, and in the YDH group compared with all other groups. Subjects from YDH group also showed lower iNOS expression, compared with all other groups.

Conclusions Our data suggest that alterations in nitric oxide metabolism may represent potential risk factors in type 2 diabetes complications, such as nephropathy and cardiovascular diseases, leading to development of new therapeutic strategies in order to delay and prevent the onset of such complications.

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Introduction

Macro- and microvascular complications are currently the principal causes of morbidity and mortality in patients with type 1 and type 2 diabetes mellitus [1].

Several studies have shown that hypercholesterolemia, systemic hypertension, smoking, diabetes, congestive heart failure, pulmonary hypertension, estrogen deficiency, hyperhomocysteinemia, and the aging process itself, are associated with impaired functions of the endothelium [2].

Endothelial dysfunction includes a number of functional alterations in the vascular endothelium, such as impaired regulation of vasodilation and vasoconstriction, impaired or excessive angiogenesis, decreased barrier function and increased inflammatory activation, all of which are associated with cardiovascular disease [3].

Maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF) [4]. A defect in NO production or activity has been proposed as a major mechanism of endothelial dysfunction, an early event in the development of atherosclerosis [5].

At least 3 Nitric Oxide Synthase (NOS) isoforms have been characterized: endothelial (eNOS) and neuronal (nNOS) isoenzymes are constitutive and calcium/calmodulin-dependent, while inducible

Table 1 Study sample characteristics.

	ADH	ADC	ADN	YDH	YDC	YDN
Number of subjects	20	20	20	20	20	20
HbA_1c % (mmol/mol)	8.2 ± 1.4 (66.0 ± 15.3)	8.6 ± 1.6 (70.0 ± 17.5)	8.8 ± 1.5 (73.0 ± 16.4)	8.3 ± 1.2 (67.0 ± 13.1)	8.5 ± 1.3 (69.0 ± 14.2)	8.7 ± 1.2 (72.0 ± 13.1)
Age	61.6 ± 10.5	60.9 ± 7.7	59.2 ± 4.8	38.6 ± 9.5	39.4 ± 4.5	40.6 ± 8.6
Sex (M/F)	12/8	8/12	10/10	10/10	12/8	12/8
Body mass index (BMI, kg/m^2)	28.16 ± 4.43	28.63 ± 3.56	27.45 ± 4.20	26.74 ± 3.98	27.40 ± 4.20	26.97 ± 3.39
Fasting glucose (mmol/l)	7.1 ± 0.4	7.2 ± 0.6	7.8 ± 1.0	7.3 ± 1.2	7.7 ± 0.5	7.5 ± 0.7
Serum creatinine (µmol/l)	99.9 ± 9.7	105.2 ± 13.1	162.7 ± 14.8	89.3 ± 7.1	99.0 ± 10.3	100.8 ± 12.1
Total cholesterol (mmol/l)	4.78 ± 0.31	4.73 ± 0.37	4.94 ± 0.32	4.65 ± 0.43	4.58 ± 0.36	5.04 ± 0.57
LDL cholesterol (mmol/l)	2.59 ± 0.20	2.51 ± 0.15	2.53 ± 0.23	2.43 ± 0.19	2.33 ± 0.16	2.72 ± 0.29
HDL cholesterol (mmol/l)	1.24 ± 0.13	1.22 ± 0.14	1.29 ± 0.10	1.16 ± 0.17	1.19 ± 0.15	1.22 ± 0.09
Triglycerides (mmol/l)	2.03 ± 0.15	2.15 ± 0.21	2.20 ± 0.19	2.18 ± 0.18	2.21 ± 0.17	2.27 ± 0.20

ADH = Adult type 2 diabetic patients without complications; ADC = Adult type 2 diabetic patients with coronary artery disease; ADN = Adult type 2 diabetic patients with diabetic nephropathy; YDH = Offspring of ADH; YDC = Offspring of ADC; YDN = Offspring of ADN

NOS (iNOS) is calcium/calmodulin independent and is expressed after stimulation by endotoxins and cytokines [6]. Specifically, eNOS-derived NO possesses multiple antithrombotic and anti-atherosclerotic properties, including inhibition of platelet aggregation and adhesion, prevention of leukocyte adhesion and migration into the vascular wall, inhibition of LDL oxidation, and inhibition of vascular smooth muscle cell proliferation [7].

Therefore, endothelial cell eNOS plays a pivotal role in maintaining a healthy vasculature and, in the presence of hyperglycemia, a dysfunctional uncoupled eNOS becomes a major contributor to the pathophysiology of micro- and macroangiopathy [8].

Furthermore, it has been proven that in strong oxidative conditions, NO reacts with superoxide anion to form a highly reactive oxidant, peroxynitrite ($ONOO^-$), which is found to be a source of platelet damage in diabetes [9].

In previous works from our group we showed that NOS activity was significantly reduced in diabetic patients, in a good metabolic control also, compared with control subjects; such decrease might play a role in the pathogenesis of diabetic vascular complications [10].

Based on the evidence herein reported, the aim of this study was to determine if type 2 diabetic patients with nephropathy and CAD showed altered platelet-derived NO and peroxynitrite production, compared with diabetic subjects without complications, and if this alteration is also present in their diabetic offspring. We also investigated, in the same subjects, the levels of plasma homocysteine, as hyperhomocysteinemia is considered an independent risk factor for arterial thrombosis and has been shown to be associated with complications in type 2 diabetic patients [11, 12] and increased risk of cardiovascular disease.

Patients and Methods

Study design and participants

This observational case-control study was performed on 60 adult type 2 diabetic patients (mean age 60.6 ± 7.8 years) and 60 offspring type 2 diabetic patients (mean age 39.5 ± 7.6 years). Among the 60 adult type 2 diabetic patients: 20 did not show cardiac or renal complications (ADH), 20 suffered from diabetic nephropathy (ADN), 20 suffered from coronary artery disease (ADC).

According to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative, diabetic nephropathy was defined as a spot urine albumin/creatinine ratio (ACR) ≥ 30 mg/g and an estimated glomerular filtration rate (GFR) < 60 ml/min/1.73 m^2 [13]. Coronary artery disease was defined as a ≥ 50 % stenosis in a major coronary vessel. Among 60 offspring type 2 diabetic patients: 20 were offspring of ADH (YDH), 20 were offspring of ADN (YDN) and 20 were offspring of ADC (YDC). To avoid potential sources of bias, patients were matched for sex, body mass index (BMI), duration of disease and glycated haemoglobin (HbA1c).

Clinical characteristics of all recruited subjects are summarized in ▶ **Table 1**.

Participants were recruited from the Diabetology Unit of Italian National Research Centre on Aging "U. Sestilli", IRCCS-INRCA, Ancona, Italy between September 2014 – March 2015. Selected

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patients were non-smokers, consumed Mediterranean diet, none of them had an alcohol abuse history or took any drugs known to lower lipids and interfere with the coagulation and antioxidant systems. Diet was slightly hypocaloric (1800 Kcal/die; 22 % of proteins, 26 % of lipids, 52 % of carbohydrates). All of the subjects took antihypertensive drugs; 53 subjects showed control on their blood pressure, taking only one drug (36) or using a combination of 2 drugs (17); 7 subjects (7 from ADC group and 3 from ADN group) did not have adequate control on their blood pressure despite treatment. None of the patients had infectious comorbidities.

The study was performed according to the guidelines laid down in the Declaration of Helsinki as revised in 2001 and all procedures involving subjects were approved by the Institutional Review Board of Università Politecnica delle Marche.

Written informed consent was obtained from all subjects after the procedures had been fully explained.

Sample collection

Peripheral venous blood was drawn after overnight fasting, and immediately mixed with Anticoagulant Citrate Dextrose (ACD) (36 ml citric acid, 5 mM KCl, 90 mM NaCl, 5 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 6.8).

Determination of blood parameters

Blood concentrations of the main parameters of lipo- and glyco-metabolic balance (total cholesterol, HDL cholesterol, triglycerides, fasting glucose, creatinine) were measured by standard procedures. Glycosylated haemoglobin was assayed by cation exchange chromatography and spectrophotometric detection (Diamat Analyser, Bio-Rad).

Platelet isolation

Platelets were isolated by differential centrifugation according to Rao [14]. The method involved a preliminary centrifugation step (200 × g for 10 min) to obtain platelet-rich plasma (PRP). Platelets were then washed 3 times in the ACD buffer and centrifuged as above. PRP was centrifuged again at 200 × g for 10 min to remove any residual red cell and finally centrifuged at 2 000 × g for 20 min to isolate the platelets. The platelet pellet was washed twice in phosphate buffered saline PBS (containing NaCl 135 mM, KCl 5 mM, EDTA 10 mM, Na2PO4 8 mM, NaH2PO4 H2O 2 mM, pH 7.2) and immediately used for the experiments.

NO production

NO released by the cells was measured in the intact platelets suspension as nitrite/nitrate, by the Griess reaction as previously described [15]. Briefly, equal amounts of 1 % sulphanylic acid and 0.1 % N-(1-naphthyl) ethylene diamine were added to the samples and the resulting absorbance was measured at 543 nm. Blank (background) was determined in each experiment utilizing medium incubated without cell. The amount of NO in each sample was determined using a standard curve generated with known concentrations of NO; protein concentration was determined with the Bradford BioRad protein assay using serum albumin as a standard [16] and NO concentration was expressed as nmol NO/mg protein.

Preparation of DCF-free base

DCFDA-free base was prepared daily, by mixing 0.05 ml of 10 mM/l DCFDA with 2 ml of 0.01 N NaOH, at room temperature for 30 min. The mixture was neutralized with 18.0 ml of 25 mmol/l phosphate-buffered saline (PBS) pH 7.4. This solution was maintained on ice in the dark until use [17].

Peroxynitrite production

Peroxynitrite production was evaluated using the fluorescence probe 2,7-dichlorofluorescein diacetate (DCFDA) as previously described [18]. Briefly, platelets were incubated for 15 min with 5 µM DCFDA-free base at 37 °C. Then, the DCFDA-treated samples were incubated with or without addition of L-arginine 100 mM and NG-monomethyl-L-arginine (L-NMMA) 100 mM for 15 min at 37 °C in the dark. After washing in PBS pH 7.4, cells were broken by sonication. The mixture was then centrifuged at 1 000 rpm 5 min and the fluorescence was measured in the supernatant in a Perkin-Elmer LS-50B spectrofluorometer, at an excitation wavelength of 475 nm and emission wavelength of 520 nm.

Western blotting

Washed platelets were lysed in RIPA lysis buffer containing 1 × PBS, 1 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 mg/mL PMSF, aprotinin, 100 mM sodium orthovanadate and 4 % protease inhibitor cocktails by microcentrifugation at 10,000 × g for 10 min at 4 °C. The supernatants were collected and treated with an equal volume of sample application buffer (125 mmol/L Tris–HCl, pH 6.8, 2 % SDS, 5 % glycerol, 0.003 % bromophenol blue, 1 % β-mercaptoethanol). The mixture was boiled for 5 min; 15 µL of each sample was applied to each well of an 8 % SDS polyacrylamide gel and electrophoresed for 1 h at 130 V along with a set of molecular weight markers (Broad Range, Sigma Chemical Co., St. Louis, MO). The resolved protein bands were then transferred onto PVDF membranes at 100 V for 60 min using a transfer buffer of 25 mmol/L Tris base, 192 mmol/L glycine, and 20 % methanol. The blots were blocked overnight at 4 °C with blocking buffer (5 % nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1 % Tween 20). The blocking buffer was decanted and blots were incubated for 1 h at room temperature with primary antibody: rabbit anti endothelial NOS (eNOS, 1:1 000, Chemicon, CA, USA) and rabbit anti inducible-NOS (iNOS, 1:1 000, Chemicon, CA, USA) diluted in blocking buffer. Positive controls were included in all experiments as provided by the manufacturer to confirm antibody specificity. As an internal control, blots were reprobed with an anti-β-actin antibody (Sigma Chemical Co., St. Louis, Mo). Blots were then washed using TTBS washing buffer (10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1 % Tween 20) and incubated with horseradish peroxidase-conjugated antirabbit immunoglobulin G (IgG) (1:5 000; Sigma Chemical Co., St. Louis, Mo) for 1 h at room temperature following washes in TTBS. Peroxidase activity was revealed using 3,3'- diaminobenzidine (Sigma Chemical Co., St. Louis, Mo) as a substrate.

Optical density of the bands was evaluated using AMERSHAM Image Master 1D software.

Plasma homocysteine assay

Plasma homocysteine was measured by HPLC according to Minniti et al. [19].

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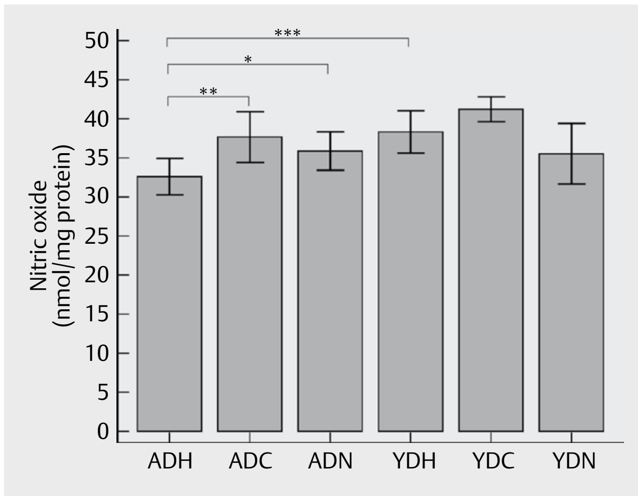
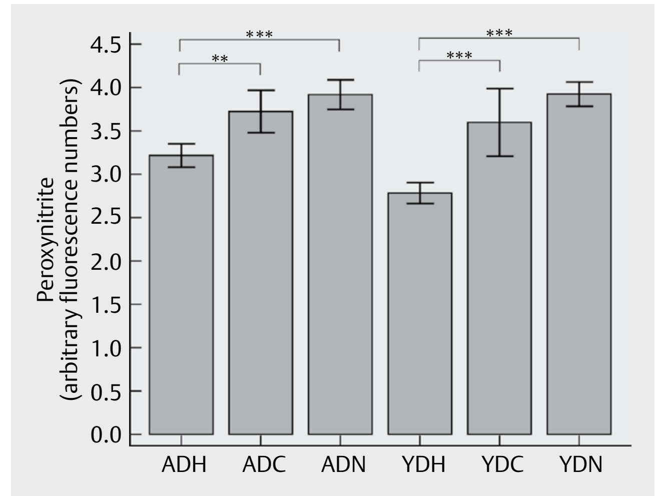


▶ **Fig. 1** Piastrinic production of nitric oxide (NO). Data are expressed as mean ± SD. ADH vs. ADC, ** p < 0.001; ADH vs. AND, * p < 0.01; ADH vs. YDH, *** p < 0.0001.



▶ **Fig. 2** Piastrinic production of peroxynitrite. Data are expressed as mean ± SD. ADH vs. YDH, *** p < 0.0001; ADH vs. ADC, ** p < 0.001; ADH vs. ADN, *** p < 0.0001; YDH vs. YDC and YDN, *** p < 0.0001.

Statistical analysis

Statistical analysis was performed using the SAS statistical package (Statistical Analysis System Institute, Cary, NC). All experiments were carried out in duplicate and were usually repeated 3 times. Results are expressed as mean ± SD. The Kolmogorov-Smirnov test (K-S test) was used to determine whether the data were random samples from a normal distribution. Because all variables were found to be normally distributed, parametric tests were applied in all further analyses. Differences in values among groups were tested by analysis of variance (one-way ANOVA) followed by Tukey post hoc test for multiple comparisons. Differences were considered significant with $p < 0.05$.

Results

Data from 60 male adult type 2 diabetic patients and 60 male offspring type 2 diabetic patients were included in this study. ▶ **Table 1** summarizes characteristics of each group of patients. There were no missing data for the baseline covariates (age, sex, BMI, duration of disease and HbA1C). No significant difference in metabolic control among groups was reported.

Platelet nitric oxide production

The measurement of platelet NO levels showed a significantly lower NO production in ADH subjects than their offspring (YDH) (32.6 ± 2.3 vs. 38.2 ± 2.7 nmol/mg respectively, $p < 0.0001$) and subjects with complications (ADC = 37.2 ± 3.4 nmol/mg, $p < 0.001$; ADN = 35.8 ± 2.5 nmol/mg, $p < 0.01$) (▶ **Fig. 1**).

No significant difference was found between the other groups (diabetic patients with coronary artery disease and diabetic patients with nephropathy) ($p > 0.05$).

Platelet peroxynitrite production

A lower peroxynitrite production has been shown in YDH subjects than the one observed in ADH subjects (2.71 ± 0.13 vs. 3.18 ± 0.13 arbitrary fluorescence numbers respectively, $p < 0.001$) and in all the other groups of patients ($p < 0.001$). ▶ **Fig. 2** shows peroxynitrite production levels in each group.

Furthermore, significant differences were found in peroxynitrite production among platelets of the 3 groups of parents (ADC = 3.77 ± 0.44 arbitrary fluorescence numbers, $p < 0.001$; ADN = 3.93 ± 0.18 arbitrary fluorescence numbers, $p < 0.0001$) as well as among the platelets of the 3 groups of offspring (YDC = 3.64 ± 0.40 arbitrary fluorescence numbers, $p < 0.0001$; YDN = 3.87 ± 0.18 arbitrary fluorescence numbers, $p < 0.0001$).

Western blot analysis of iNOS and eNOS

The lower levels of peroxynitrite in YDH subjects were fully compatible with the findings from the Western blot analysis of the inducible isoform of NOS (iNOS). iNOS protein was found at 130 kDa in washed platelets (▶ **Fig. 3a**). One-way ANOVA on densitometric analysis of bands [$F(5,114) = 8.227$, $p < 0.001$], followed by post hoc Tukey test indicated that the YDH group was the only one that didn't show a relevant expression of the iNOS, which accounts for peroxynitrite production. Furthermore, iNOS expression in ADH group was significantly lower compared to the groups of patients with complications and their offspring ($p < 0.01$, ▶ **Fig. 3b**).

At last, determination of the expression of eNOS showed that the constitutive form of NOS is always expressed in human platelets (▶ **Fig. 4a**). One-way ANOVA on densitometric analysis yelded significative variations among groups [$F(5,114) = 7.637$, $p < 0.001$]. Post hoc Tukey test showed that eNOS expression in ADC and YDC groups was significantly higher, compared to all other groups ($p < 0.001$, ▶ **Fig. 4b**).

Plasma homocysteine levels

Measurements of plasma homocysteine levels showed high data dispersion. Specifically, in all examined groups range of homocysteine levels varied from 5.2 to 21.6 µmol/l with no significant differences among the groups.

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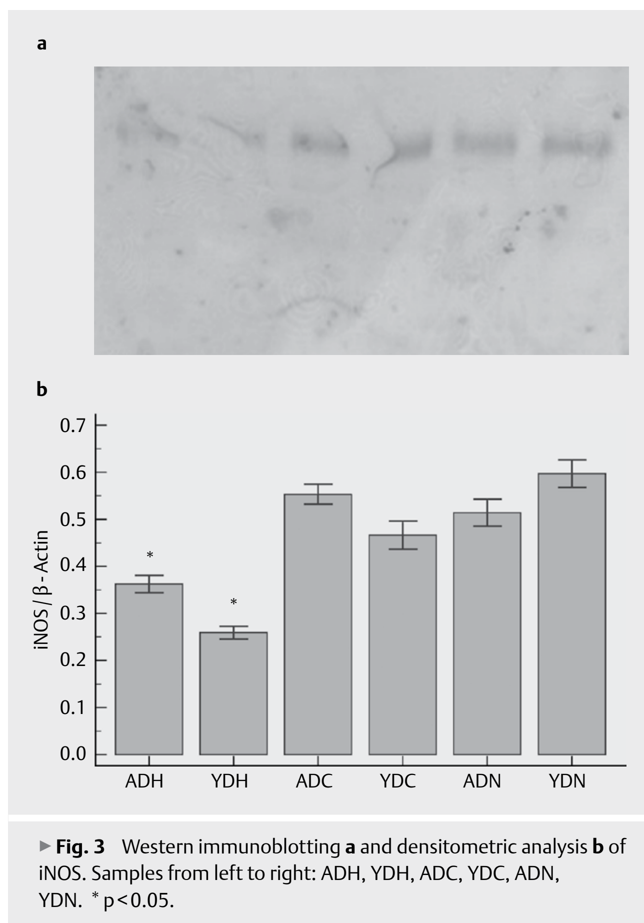
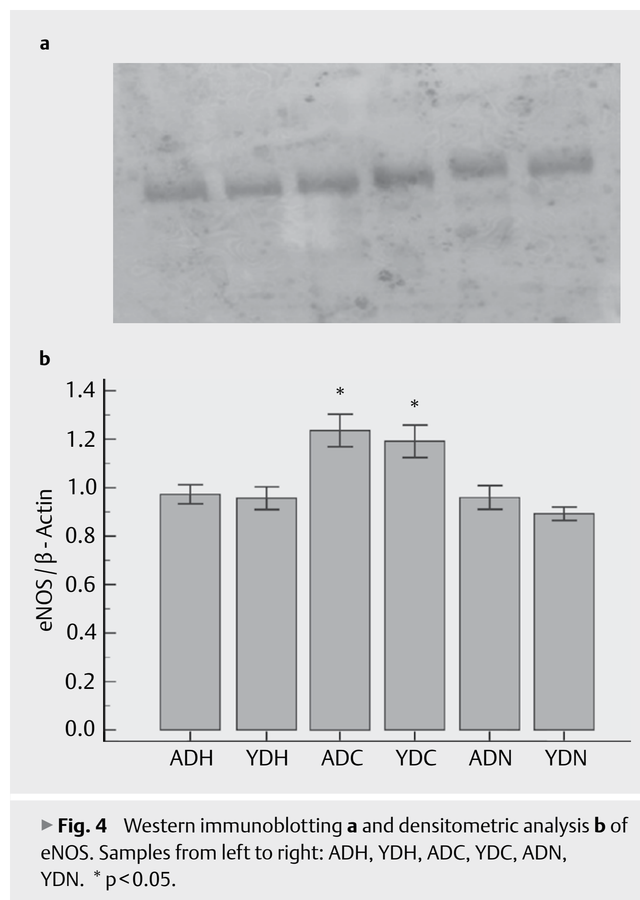


▶ **Fig. 3** Western immunoblotting **a** and densitometric analysis **b** of iNOS. Samples from left to right: ADH, YDH, ADC, YDC, ADN, YDN. * $p < 0.05$.



▶ **Fig. 4** Western immunoblotting **a** and densitometric analysis **b** of eNOS. Samples from left to right: ADH, YDH, ADC, YDC, ADN, YDN. * $p < 0.05$.

Statistical analysis didn't show any significant association between plasma homocysteine levels and piastrinic NO and/or peroxynitrite production.

Discussion

The unbalance between the production of oxidizing agents (ROS) and the efficiency of antioxidant biohumoral systems, as well as alterations of endothelial-dependent vasodilation and platelet aggregation, have been considered as determining factors in the onset of type 2 diabetes organ complications. Since NO plays an important role in all of these processes, there is growing interest on the enzymatic system responsible for its synthesis and on the implications of its alterations in micro- and macroangiopathy. Specifically, we focused on the endothelial (eNOS) and inducible (iNOS) NOS isoforms, which are both expressed in platelets.

In our study, we analyzed platelets from type 2 diabetic patients with or without chronic complications (coronary artery disease or nephropathy), to determine if there is an impairment of NO production or expression of the 2 NOS isoforms in type 2 diabetes complications.

It has been proven that macro- and microangiopathic type 2 diabetes complications are mainly due to prolonged exposure to hyperglycemia, which interacts with other known risk factors, such as arterial hypertension, dyslipidemia and genetic susceptibility [20]. The trigger by which high glucose concentrations alter vascular function is the unbalance between NO bioavailability and ROS accumulation [21]. In fact, hyperglycemia-induced superoxide anion production inactivates NO by converting it in peroxynitrite. Peroxynitrite then inactivates several antioxidant enzymes by nitrosylation, such as eNOS.

Our study showed a significantly lower piastrinic production of NO in the group of parents without complications (ADH), compared both with the group of offspring without complications (YDH) and with the groups of parents with complications (with nephropathy, ADN, or coronary artery disease, ADC). No significant difference was observed for all the other groups considered.

Our data agree with previous evidences showing that early stages of diabetic nephropathy are characterized by increased NO levels. This enhanced production leads to hyperfiltration and microalbuminuria characterizing early diabetic nephropathy [22, 23]. Progression towards end-stage kidney disease is accompanied by a decrease in NO bioavailability, mainly due to excessive accumulation of advanced glycation end products [24, 25].

The finding of a lower NO piastrinic production in ADH than in the groups of patients with complications is consistent with the results from Western blotting, which showed a greater iNOS expression in the groups of patients with complications than in the groups of patients without complications. The group of YDH and the ones of patients with complications seem to be more protected from thromboembolic events.

Whereas on one hand NO exerts several protective effects, including prevention of endothelial dysfunction, on the other hand, the pro-oxidant status of diabetes may contribute to promote NO

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interaction with superoxide radical (O_2^-) leading to peroxynitrite formation and thus to nitrosative stress.

Hence, we can suppose that the enhanced NO production in platelets from patients with complications may be linked to the greater iNOS expression, which might also cause an increase in the synthesis of peroxynitrite, strongly associated to the organ damage observed in type 2 diabetes.

In fact, it is well demonstrated that iNOS, whose production in endothelium and platelets is induced by inflammatory mediators, is the main producer of peroxynitrite [26]. This, in turn, has been considered as the main responsible for the detrimental effects of increased NO levels. The findings from our study agree with data from Tannous et al. [9], who showed the expression of iNOS and the increased production of peroxynitrite in platelets from diabetic patients.

Our study pointed out a lower synthesis of peroxynitrite in platelets from the ADH group than the groups of patients with complications and in the YDH group compared with all other groups. Our data therefore suggest that iNOS is involved in peroxynitrite production: that may be considered as a risk factor in phenotypical manifestation of micro- and macroangiopathic type 2 diabetes complications, such as respectively nephropathy and coronary artery disease.

A further result from our study is that the offspring without complications (YDH) do not express iNOS and show a significantly lower peroxynitrite production compared with all other groups. We can therefore speculate for an important role of age in the production of free radicals. This finding agrees with literature data showing that superoxide anion formation increases with age. It has been demonstrated that several age-related disorders, such as type 2 diabetes, are characterized by increased ROS production, whose signaling triggers the pathway responsible for apoptosis.

This study has some limitations which need to be pointed out. Metabolic control was assessed by HbA1c values, which show the average level of blood glucose over the previous 3 months. Our findings may be therefore influenced by the short platelet lifetime, which is about 7 to 10 days. However, it would have been difficult to perform in vivo determinations on human endothelium. For this reason, substances with a shorter half-life time period, such as fructosamine, may be more appropriate for the assessment of metabolic control. Moreover, our determinations on platelet could be affected by undiagnosed minor infections occurring at the time of recruitment, although patients with severe infections were excluded. Given the nature of the study, it is impossible to determine whether changes in NO and peroxynitrite platelet content might account for development of diabetic complications.

In conclusion, our data suggest that alterations in nitric oxide metabolism, resulting in production of the harmful compound peroxynitrite and in a higher iNOS expression in platelets from diabetic patients, may represent potential risk factors in chronic type 2 diabetes complications, such as nephropathy and cardiovascular diseases.

The identification of new risk factors may lead to the development of new therapeutic strategies in order to delay and prevent the onset of type 2 diabetes complications. As an instance, previous studies showed that in case of uncoupling between NADPH and arginine, even the eNOS enzyme could induce ROS formation. It is believed that a lack of the cofactor tetrahydrobiopterin (BH_4) could be responsible for this uncoupling [27, 28]. In fact, it has been demonstrated that it is possible to preserve the functionality of the eNOS enzyme by integrating the diet of diabetic subjects with folic acid [29]. It is well known that folic acid has multiple anti-inflammatory actions, inhibits intracellular formation of superoxide (thus extending NO half-life) [30], and increases omega-3 fatty acid concentrations, which seems to intensify eNOS dependent NO synthesis.

Further investigations are needed to clarify the role of NO metabolism in the complications of diabetes and, specifically, to determine if the alterations observed in the present study may represent risk factors appearing before the development of diabetes complications or simply are epiphenomena linked to other modifications developing in the same complications. The answer to this question could open new strategies in pharmacologic and dietetic prevention of chronic complications of diabetes.

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Conflict of interest

None.

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