

Platelet Isoprostane Overproduction in Diabetic Patients Treated With Aspirin

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Aspirin modestly influences cardiovascular events in patients with type 2 diabetes mellitus (T2DM), but the reason is unclear. The aim of the study was to determine whether in T2DM patients aspirin enhances platelet isoprostanes, which are eicosanoids with proaggregating properties derived from arachidonic acid oxidation by platelet NOX2, the catalytic subunit of reduced NAD phosphate oxidase. A cross-sectional study was performed comparing T2DM patients, treated ($n = 50$) or not treated ($n = 50$) with 100 mg/day aspirin, with 100 nondiabetic patients, matched for age, sex, atherosclerosis risk factors, and aspirin treatment. A short-term (7 days) treatment with 100 mg/day aspirin also was performed in 36 aspirin-free diabetic and nondiabetic patients. Higher platelet recruitment, platelet isoprostane, and NOX2 activation was found in diabetic versus nondiabetic patients and in aspirin-treated diabetic patients versus nontreated patients ($P < 0.001$). Platelet thromboxane (Tx) A₂ ($P < 0.001$) was inhibited in all aspirin-treated patients. In the interventional study, aspirin similarly inhibited platelet TxA₂ in diabetic and nondiabetic patients ($P < 0.001$). Platelet recruitment, isoprostane levels, and NOX2 activation showed a parallel increase in diabetic patients ($P < 0.001$) and no changes in nondiabetic patients. These findings suggest that in aspirin-treated diabetic patients, oxidative stress-mediated platelet isoprostane overproduction is associated with enhanced platelet recruitment, an effect that mitigates aspirin-mediated TxA₂ inhibition. *Diabetes* 61:1626–1632, 2012

Accelerated atherosclerosis is a typical feature of type 2 diabetes mellitus (T2DM). Thus, patients with T2DM have a two- to fourfold increased risk of cardiovascular diseases (coronary artery disease) and a two- to sixfold increased risk of stroke (1–3).

Platelets play a major role in the etiology of atherosclerotic disease, as shown by the significant decrease of cardiovascular events in patients treated with aspirin, an inhibitor of cyclooxygenase (COX1) that prevents platelet thromboxane (Tx) A₂ formation (4,5). Platelet TxA₂ overproduction, combined with a significant decrease after aspirin administration, has been demonstrated in diabetic patients (6). Despite this, interventional trials with aspirin in diabetic patients failed to show a beneficial effect in primary prevention; reasons for this lack of response still are uncertain (7).

Isoprostanes are a family of eicosanoids derived from arachidonic acid interaction with reactive oxidant species (ROS). Thus, ROS generated by NOX2, the catalytic subunit

of reduced NAD phosphate (NADPH) oxidase, play a crucial role in platelet isoprostane formation (8). In contrast to TxA₂, isoprostanes are chemically stable compounds that serve to propagate platelet activation, amplifying platelet response to common agonists via glycoprotein (Gp)IIb/IIIa activation (9). Accordingly, patients with hereditary deficiency of NOX2 showed impaired isoprostane formation and GpIIb/IIIa activation, as well as a subnormal propagation of platelet thrombus (9).

It previously has been demonstrated that COX1 inhibition determines a shift in arachidonic acid metabolism toward other pathways, such as the lipoxygenase system (10). We speculated that COX1 inhibition also could be associated with an increased conversion of arachidonic acid to isoprostanes in platelets. The increase of platelet isoprostanes would counterbalance the inhibition of TxA₂, therefore hampering the antiplatelet effect of aspirin. To explore this hypothesis, we performed a cross-sectional study comparing the behavior of platelet isoprostanes and TxA₂ and their interplay with platelet NOX2 in diabetic and nondiabetic patients treated or not with aspirin. Platelet activation tests, including arachidonic acid-induced platelet aggregation, which is dependent upon TxA₂ formation (11), and platelet recruitment, which is dependent upon ROS and isoprostane formation (9), were determined. Analysis of these variables was repeated in a prospective, short-term study of diabetic and nondiabetic patients treated for 7 days with low-dose aspirin.

RESEARCH DESIGN AND METHODS

Study design

Cross-sectional study. The study was performed in consecutive T2DM patients attending our metabolic outpatient clinic who were taking ($n = 50$) or not taking ($n = 50$) low-dose (100 mg/day) aspirin. T2DM was diagnosed according to the American Diabetes Association definition (12). As a control group, we selected nondiabetic outpatients taking ($n = 50$) or not taking ($n = 50$) low-dose aspirin who were matched to the diabetic group in terms of age, sex, and history of vascular disease.

Low-dose aspirin treatment was defined as a self-reported daily intake of 100 mg acetylsalicylic acid at least in the previous month. Exclusion criteria were 1) recent history (<3 months) of acute vascular events, 2) clinical diagnosis of type 1 diabetes (diagnosis of diabetes and insulin use before the age of 35 years), 3) serum creatinine level >2.5 mg/dL, 4) active infection or malignancy, 5) cardiac arrhythmia or congestive heart failure, and 5) use of nonsteroidal anti-inflammatory drugs, vitamin supplements, or other antiplatelet drugs, such as clopidogrel, in the previous 30 days. All participants provided written informed consent. The local ethical committee approved the study protocol (approval no. Prot. 403/09-Rif. 1621/07.05.09). Diabetic patients received different antidiabetes treatments: metformin ($n = 57$), subcutaneous insulin ($n = 25$), sulfonylureas ($n = 14$), glinides ($n = 6$), glitazones ($n = 3$), and dipeptidyl peptidase-4 inhibitor ($n = 16$).

Interventional study. We tested the effect of short-term treatment with 100 mg/day aspirin in diabetic ($n = 18$) and nondiabetic ($n = 18$) patients, not currently under aspirin treatment and with no clinical history of vascular diseases, who were matched for sex, age, and atherosclerotic risk factors. Aspirin was given after dinner between 8:00 and 8:30 P.M., and adherence was assessed by the pill-count method. Blood samples were collected before

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Received 5 September 2011 and accepted 1 February 2012.

DOI: 10.2337/db11-1243. Clinical trial reg. no. NCT01250340, clinicaltrials.gov. R.C. and P.P. contributed equally to this work.

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aspirin ingestion and after 3 and 7 days of treatment. The study was registered in August 2010 at clinicaltrials.gov (clinical trial reg. no. NCT01250340).

Laboratory analyses. All materials were from Sigma-Aldrich, unless otherwise specified. Blood analyses were performed in a blinded manner. All blood samples were taken after a 12-h fast. Between 8:00 and 9:00 A.M., subjects underwent routine biochemical evaluations, including fasting total cholesterol and glucose.

Serum and platelet-poor plasma sampling. After overnight fasting (12 h) and supine rest for at least 10 min, blood samples were taken into tubes containing either 3.8% sodium citrate (ratio 9:1) or anticoagulant-free tubes and centrifuged at 300g for 10 min to obtain supernatant (plasma or serum), which was stored at -80°C until use.

Platelet-rich plasma preparation. To obtain platelet-rich plasma (PRP), samples were centrifuged for 15 min at 180g. To avoid leukocyte contamination, only the top 75% of the PRP was collected; leukocyte contamination was verified as reported below.

Washed-platelet procedure. Platelet pellets were obtained by double centrifugation (2×5 min, 300g) of PRP after the addition of acid/citrate/dextrose (1:7 vol/vol) to avoid cell activation during processing. Samples were suspended in HEPES buffer in the presence of 0.1% albumin, pH 7.35 (2×10^5 platelets/mL, unless otherwise noted).

Platelet sizing. To assess if there were differences between T2DM patients and control subjects in platelet size and function, we stratified in two portions (50% upper phase, 50% lower phase) the top 75% of the PRP. After the washing procedure of the two portions, we analyzed TxA_2 and 8-iso-PGF 2α -III production.

Cytometric analysis of platelets and leukocytes. Leukocyte content in the top 75% of the PRP was analyzed using the specific fluorescein isothiocyanate-labeled monoclonal antibody (Mab) anti-CD4 (BD International). Platelets were analyzed using the specific phycoerythrin-labeled monoclonal antibodies anti-CD61 (Mab) (BD International).

All assays included samples to which an irrelevant isotype-matched antibody (fluorescein isothiocyanate-labeled IgG1 or phycoerythrin-labeled IgG1) was added. Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon laser at 488 nm/L. For every histogram, 5×10^4 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity is reported as mean fluorescence intensity and percentage (%) of positivity.

Platelet NOX2, TxA_2 , 8-iso-PGF 2α -III, and ROS. Platelet suspension was activated with arachidonic acid (1 mmol/L), and the supernatant was treated with butylhydroxytoluene and stored at -80°C . In some experiments, samples were treated with or without NOX2-blocking peptide (gp91^{phox} ds-tat-blocking peptide, 50 $\mu\text{mol/L}$) (10 min 37°C) before activation.

Extracellular levels of soluble NOX2-derived peptide (sNOX2-dp), a marker of NADPH oxidase activation, were detected by enzyme-linked immunosorbent assay, as previously described by Pignatelli et al. (13). The peptide was recognized by the specific monoclonal antibody against the amino acidic sequence (224–268) of the extra membrane portion of NOX2, which was released in the medium upon platelet activation. Values were expressed as picograms per milliliter; intra-assay and interassay coefficients of variation were 5.2 and 6%, respectively.

Platelet TxB_2 was measured by enzyme-linked immunosorbent assay (Amersham Pharmacia, Biotech, Little Chalfont, U.K.) and expressed as picomoles per liter. TxB_2 levels in all experiments were expressed as defined in the following formula: (TxA_2 in stimulated samples) – (TxA_2 in unstimulated samples). Intra- and interassay coefficients of variation were 4.0 and 3.6%, respectively.

The platelet isoprostane 8-iso-PGF 2α -III was measured by the enzyme immunosorbent assay method, as previously described (9), and expressed as picomoles per liter. In a subgroup of patients ($n = 20$), platelet isoprostane levels also were measured by gas mass chromatography (7890A GC-MS; Agilent Technologies) to validate the method (14). Intra- and interassay coefficients of variation were 5.8 and 5.0%, respectively.

To evaluate ROS formation, cell suspension was incubated with 2',7'-dichlorofluorescein diacetate (5 $\mu\text{mol/L}$) for 15 min at 37°C . ROS production was evaluated by flow cytometric analysis and expressed as stimulation index (mean level of fluorescence in stimulated cells/mean level of fluorescence in unstimulated cells; stimulation index), as previously described (11). Intra- and interassay coefficients of variation were 4%.

Platelet aggregation. Platelet aggregation was induced by arachidonic acid (1.0 mmol/L) and measured as light transmission (LT%) difference between PRP and platelet-poor plasma, as previously described (11).

Platelet recruitment. Platelet recruitment was performed with a method modified from that described by Krötz et al. (15). Collagen (2 $\mu\text{g/mL}$)-induced platelet aggregation was measured for 10 min, then an equal portion of untreated platelets was added to each tube, causing a reduction in light transmission. This method consists of two phases, the first one depending on the

agonist (collagen) and the second one depending on the release of molecules discharged by activated platelets (ADP, isoprostanes, etc.). Thus, the second phase (recruitment) is independent from the agonist (collagen) initially added to the first phase of aggregation because collagen is fully taken up by platelets during the first phase (16). Aggregation of the newly added platelet portion in the presence of an existing aggregate was then measured for 5 min and expressed as a percentage (REC%) of the aggregation that had been initially reached, according to Pignatelli et al. (9). In some experiments, PRP samples were incubated (30 min at 37°C) with or without TxA_2 /isoprostane receptor inhibitor (SQ29548, 0.1 $\mu\text{mol/L}$) or control before collagen stimulation.

Flow cytometry analysis of GpIIb/IIIa activation (pituitary adenylate cyclase-activating peptide receptor 1 [PAC1] binding). Platelet GpIIb/IIIa activation was measured by PAC1 binding on platelet membranes, as previously reported, and expressed as mean fluorescence intensity (17). GpIIb/IIIa activation was analyzed after platelet suspension incubation with scalar doses of 8-iso-PGF 2α (25–450 pmol/L) with or without the TxA_2 /isoprostane receptor antagonist SQ29548 (0.1 $\mu\text{mol/L}$), ADP (0.01–10 $\mu\text{mol/L}$) with or without 8-iso-PGF 2α (300 pmol/L), and thrombin receptor agonist peptide (TRAP) (0.01–10 $\mu\text{mol/L}$).

Statistical analysis. The minimum sample size was computed with respect to a two-tailed Student *t* test for independent groups, considering 1) relevant difference in isoprostane levels to be detected between groups $|\delta| \geq 90$ pmol/L, 2) SDs homogeneous between the groups (SD = 80 pmol/L), and 3) type I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$. This resulted in $n = 17$ per group.

Categorical variables were reported as counts (percentage) and continuous variables were expressed as means \pm SD, unless otherwise indicated. Differences between percentages were assessed by the χ^2 test or Fisher exact test. Student unpaired *t* test and Pearson product moment correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney *U* test and Spearman rank correlation test [R_s]) were used for all the other variables. Multiple linear regression analysis was performed to further quantify the relationship between the variables studied.

Interventional study data were analyzed performing a MANOVA with one between-subject factor (group: diabetic vs. nondiabetic patients) and one within-subject factor (time: baseline, 3 days, and 7 days after initiation of treatment). As covariates, we considered the possible random differences in age, sex, BMI, smoking habits, and hypertension between the two groups. Pairwise comparisons were performed using Bonferroni correction. Probability values < 0.05 were regarded as statistically significant. All calculations were made with Statistica 7 software for Windows (StatSoft, Tulsa, OK).

RESULTS

Cross-sectional study. Diabetic and nondiabetic patients had a similar distribution of risk factors, with the exception of BMI, which was higher in diabetic patients (Table 1). Statin therapy was more common in diabetic patients ($P = 0.015$). Diabetic and nondiabetic patients had similar platelet TxB_2 values (Fig. 1A) and platelet aggregation percentages (95 ± 3 vs. 96 ± 2 LT%, respectively; $P = 0.864$). Diabetic patients had significantly higher platelet isoprostanes, platelet recruitment, and sNOX2-dp compared with nondiabetic patients (Fig. 1B–D). In diabetic patients, HbA_{1c} significantly correlated with sNOX2-dp ($r = 0.40$, $P < 0.0001$) and platelet isoprostanes ($r = 0.39$, $P < 0.0001$).

Diabetic patients treated or not with aspirin had similar clinical characteristics (Table 1) and received equally distributed different antidiabetes treatment (data not shown). Platelet TxB_2 was significantly lower in aspirin-treated diabetic patients; conversely, aspirin-treated diabetic patients had higher platelet isoprostanes, platelet recruitment, and NOX2 activation (Fig. 1).

In nondiabetic patients, aspirin treatment significantly lowered platelet TxB_2 compared with aspirin-untreated patients. However, no significant differences were found in platelet isoprostanes, platelet recruitment, and sNOX2-dp levels between nondiabetic patients treated or not with aspirin (Fig. 1). Similar results were observed comparing control subjects with impaired fasting glucose ($n = 22$) taking ($n = 10$) or not taking ($n = 12$) aspirin (data not shown). Platelet aggregation was completely suppressed in aspirin-treated diabetic and nondiabetic patients (not shown).

TABLE 1
Clinical and anthropometric characteristics of study participants

	Diabetic patients (n = 100)				Nondiabetic patients (n = 100)				P*
	All	ASA treatment			All	ASA treatment			
		No (n = 50)	P	Yes (n = 50)		No (n = 50)	P	Yes (n = 50)	
Age (years)	66 ± 10	0.934	66 ± 10	65 ± 14	67 ± 10	0.138	63 ± 17	0.560	
Male (%)	57	1.000	56	50	42	0.161	58	0.395	
Smoking habit (%)	18	0.795	16	12	18	0.121	6	0.322	
BMI (kg/m ²)	29.4 ± 5.0	0.903	29.3 ± 4.8	27.1 ± 4.1	27.7 ± 4.0	0.116	26.5 ± 4.1	<0.001	
Previous coronary artery disease (%)	31	0.02	46	22	12	0.028	32	0.200	
Previous stroke (%)	8	1.000	8	4	0	0.117	8	0.373	
Arterial hypertension (%)	81	0.308	86	72	82	0.044	62	0.182	
Total cholesterol (mg/dL)	188.6 ± 43.0	0.177	182.6 ± 36.8	189.8 ± 34.2	192.8 ± 40.8	0.371	186.7 ± 25.9	0.335	
HDL cholesterol (mg/dL)	50.2 ± 19.0	0.942	50.4 ± 21.3	51.5 ± 15.7	52.5 ± 20.3	0.531	50.5 ± 9.0	0.606	
Triglycerides (mg/dL)	147.8 ± 82.1	0.700	144.6 ± 85.8	136.2 ± 50.1	131.2 ± 50.9	0.332	141.1 ± 49.2	0.227	
Blood glucose (mg/dL)	142.0 ± 40.7	0.537	144.3 ± 47.0	93.5 ± 5.7	93.4 ± 9.5	0.952	93.6 ± 13.4	<0.001	
HbA _{1c} (%)†	7.1 ± 1.3	0.927	7.1 ± 1.2	5.7 ± 0.4	5.8 ± 0.3	0.168	5.6 ± 0.4	<0.001	
Statin therapy (%)	55	1.000	56	30	30	1.000	30	0.001	
ACE/angiotensin receptor blockers (%)	67	0.202	74	62	72	0.063	52	0.555	
Antidiabetes drugs (%)	81	0.125	88	0	0	1.000	0	<0.001	

Data are means ± SD, unless otherwise indicated. ASA, aspirin. *Diabetic vs. nondiabetic patients. †International Federation of Clinical Chemistry and Laboratory Medicine HbA_{1c} (mmol/mol) = [Diabetes Control and Complications Trial HbA_{1c} (%) - 2.15] × 10.929.

Correlation analysis. In the overall population, platelet isoprostanes significantly correlated with platelet recruitment ($r = 0.43$, $P < 0.001$) and sNOX2-dp ($r = 0.49$, $P < 0.001$). To further define the predictors of the platelet isoprostane level, multiple regression analysis including clinical and laboratory characteristics, presence of diabetes, and concomitant use of aspirin and statins was performed. Stepwise linear regression yielded a model in which only T2DM ($\beta = 0.276$, $P < 0.001$), aspirin treatment ($\beta = 0.158$, $P = 0.009$), and sNOX2-dp ($\beta = 0.229$, $P < 0.001$) predicted platelet isoprostane levels, independently from the other included variables.

Interventional study. Clinical and anthropometric characteristics of interventional study participants are reported in Table 2.

At baseline, platelets from diabetic and nondiabetic patients showed similar platelet TxB₂ values (Fig. 2) and platelet aggregation percentages (94 ± 2.2 vs. 97 ± 3 LT %; $P = 0.640$). Conversely, platelet recruitment, platelet isoprostanes, platelet ROS, and sNOX2-dp were significantly higher in diabetic compared with nondiabetic patients. Platelet TxB₂ formation (Fig. 2A) and platelet aggregation (not shown) were significantly inhibited after aspirin intake in both groups. Platelet isoprostanes, sNOX2-dp, platelet ROS, and platelet recruitment increased after aspirin intake only in diabetic patients (Fig. 2).

MANOVA analysis showed a significant influence of aspirin treatment in diabetic patients on platelet isoprostanes ($F = 11.9$, $P < 0.001$), platelet ROS production ($F = 171.6$, $P < 0.001$), sNOX2-dp ($F = 32.5$, $P < 0.001$), and platelet recruitment ($F = 9.6$, $P < 0.001$). No significant interaction with covariates was found. Incubation of platelets from aspirin-treated diabetic patients with the NOX2-blocking peptide was associated with a significant inhibition of ROS (-3.3 S.I. at 3 days, $P = 0.006$, and -7.9 S.I. at 7 days, $P = 0.003$, respectively) and platelet isoprostane (-134 pmol/L at 3 days, $P < 0.001$, and -137 pmol/L at 7 days, $P = 0.005$) production. Conversely, in aspirin-treated nondiabetic patients, NOX2-blocking peptide had a less significant effect on platelet ROS (-0.8 stimulation index at 3 days, $P = 0.023$, and -0.9 S.I. at 7 days, $P = 0.021$) and isoprostane (-6 pmol/L at 3 days, $P = 0.048$, and -7 pmol/L at 7 days, $P = 0.043$) production.

The Tx/isoprostane inhibitor SQ29548 significantly inhibited recruitment of platelets from aspirin-treated diabetic patients but not from aspirin-treated nondiabetic patients (Fig. 2E and F).

In vitro study

Effect of 8-iso-PGF2 α on platelet activation. TRAP (0.01–10 μ mol/L) dose-dependently increased GpIIb/IIIa activation (Fig. 3). Compared with TRAP, GpIIb/IIIa activation by 8-iso-PGF2 α (25–350 pmol/L) was weaker and significantly inhibited by platelet coinubation with SQ29548. GpIIb/IIIa activation by ADP also was dose dependent; it almost was undetectable with doses <0.1 μ mol/L and became detectable with values >1 μ mol/L. The incubation of 8-iso-PGF2 α (300 pmol/L) with lower ADP doses (0.01–0.1 μ mol/L) amplified GpIIb/IIIa activation with values similar to those achieved with higher ADP doses.

Influence of platelet size in eicosanoids formation. The top 75% of the PRP of diabetic and control subjects was stratified in two portions (50% upper and 50% lower phases). Arachidonic acid-induced platelet TxB₂ and isoprostanes were measured in both phases. Platelets from diabetic patients were greater in the upper 50% (Fig. 4A

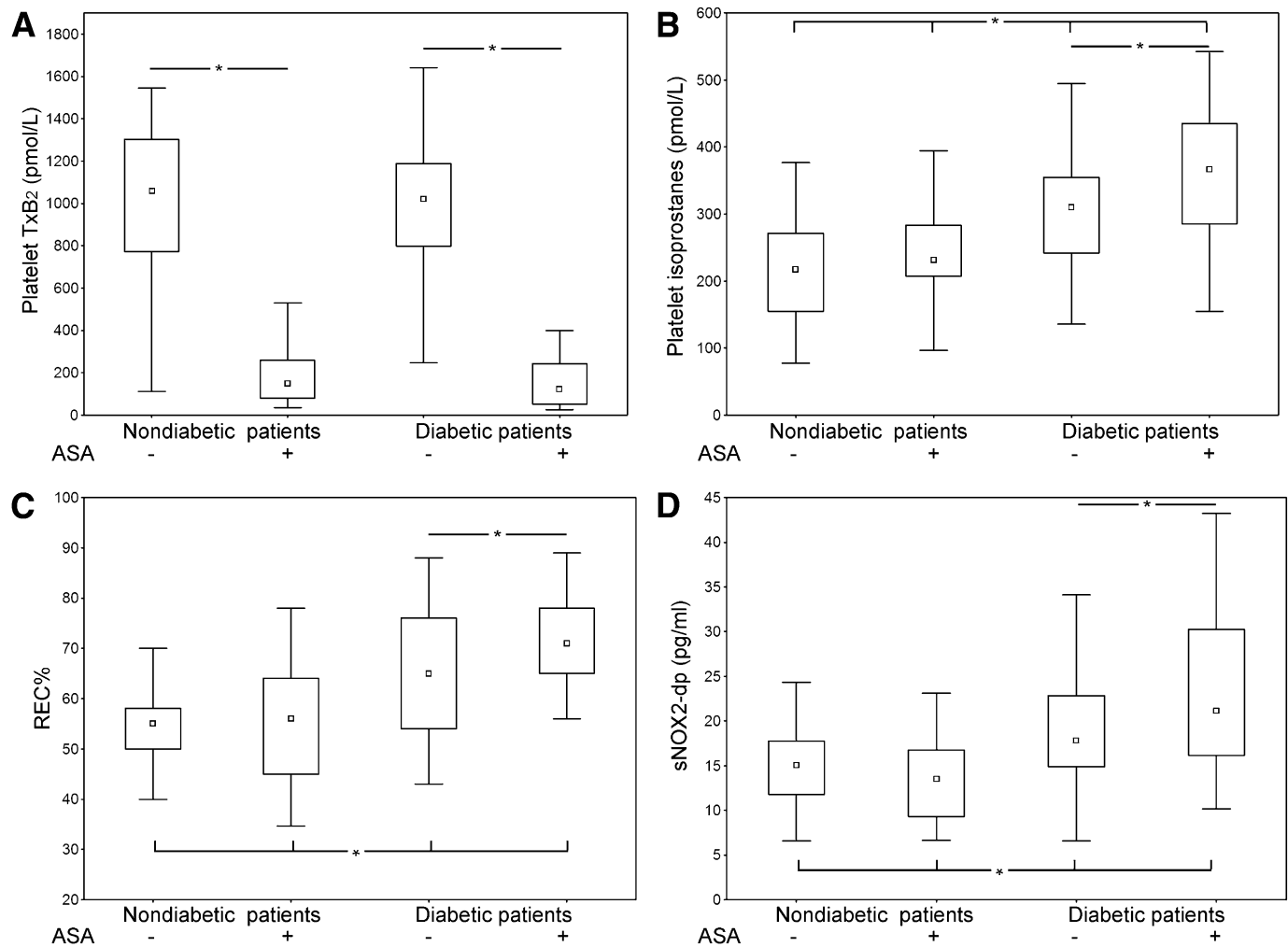


FIG. 1. TxB₂ (A) and isoprostane formation (B), platelet recruitment (REC%) (C), and sNOX₂-dp (D) from arachidonic acid-activated platelets in nondiabetic and diabetic patients taking (ASA+) or not (ASA-) 100 mg acetylsalicylic acid daily in the previous month. **P* < 0.001.

and B), whereas no difference was detected in the lower phase. The increase in eicosanoids formation between diabetic patients and control subjects persisted in the two phases, suggesting that platelet size was not a determinant in the above-reported differences (Fig. 4C and D).

Leukocyte contamination. To ensure the quality of platelet preparation, samples also were tested for leukocyte contamination. The contamination found was <1% in all samples (an exemplificative image of flow cytometric analysis is shown in Fig. 4A and B)

TABLE 2
Clinical and anthropometric characteristics of interventional study participants

	Diabetic patients	Nondiabetic patients	<i>P</i>
<i>n</i>	18	18	
Age (years)	62.3 ± 9.4	61.6 ± 10.1	0.886
Male (%)	61	56	0.735
BMI (kg/m ²)	27.3 ± 4.3	27.0 ± 2.8	0.789
Arterial hypertension (%)	77	72	0.700
Previous coronary artery disease (%)	0	0	1.000
Previous stroke (%)	0	0	1.000
Total cholesterol (mg/dL)	188.1 ± 36.4	186.8 ± 37.2	0.385
HDL cholesterol (mg/dL)	51.4 ± 16.6	50.8 ± 15.8	0.472
Triglycerides (mg/dL)	144.5 ± 63.2	134.2 ± 51.8	0.239
Blood glucose (mg/dL)	140.2 ± 35.4	91.5 ± 9.8	<0.001
HbA _{1c} (%)	7.1 ± 1.4	5.5 ± 0.4	<0.001
Statin therapy (%)	11	6	0.546
ACE/angiotensin receptor blockers (%)	67	61	0.729
Antidiabetes drugs (%)	89	0	<0.001

Data are means ± SD, unless otherwise indicated.

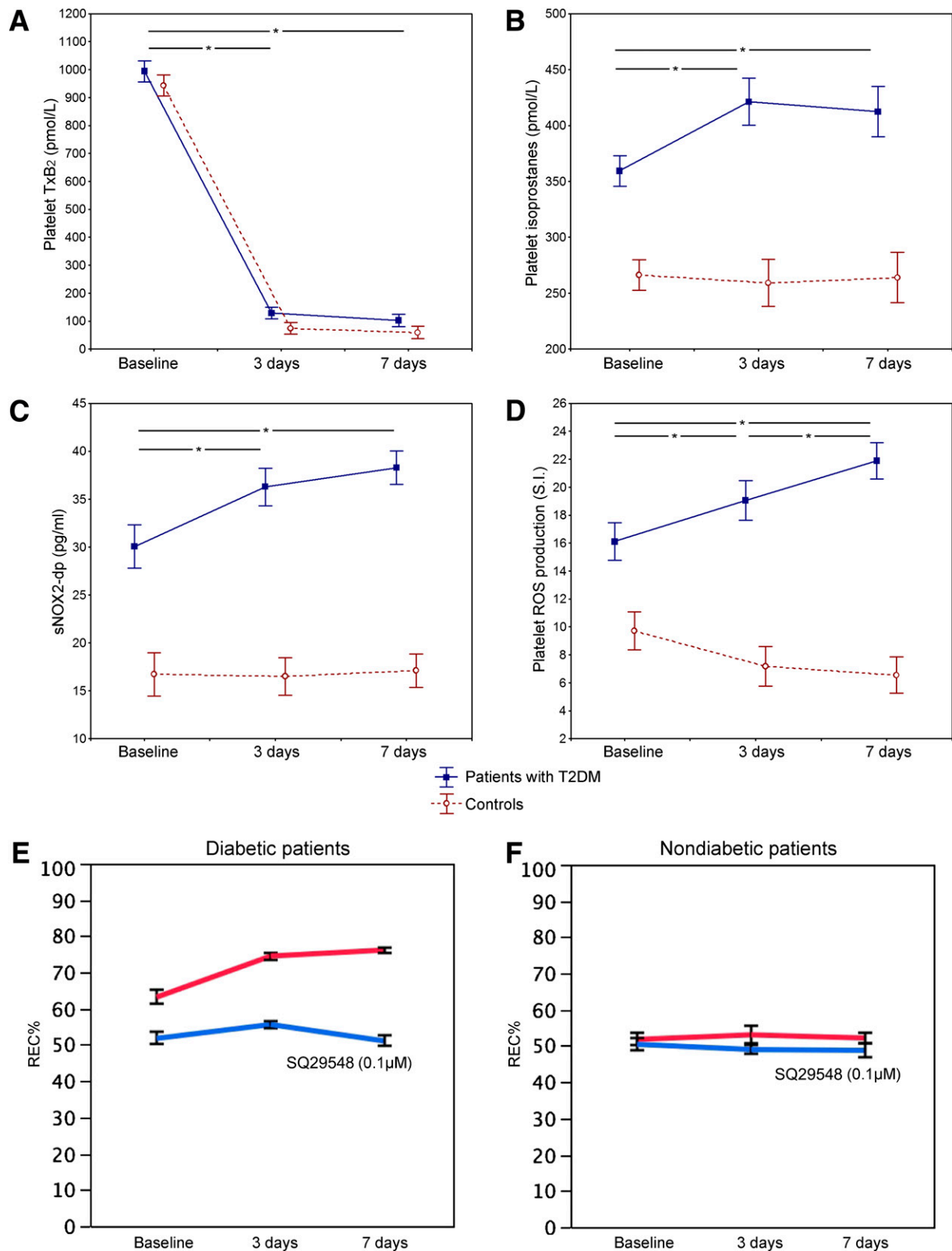


FIG. 2. TxB₂ (A) and isoprostane (B) formation, sNOX2-dp release (C), and ROS production (D) from arachidonic acid-activated platelets in nondiabetic and diabetic patients at baseline and after 3 and 7 days of aspirin (100 mg) treatment. E and F: Platelet recruitment (REC%) in diabetic and nondiabetic patients at baseline and after 3 and 7 days of low-dose aspirin treatment in the presence or not of SQ29548 (0.1 μ M). **P* < 0.001.

DISCUSSION

This study provides evidence that, in diabetic patients treated with low-dose aspirin, platelets overproduce isoprostanes, an effect that is dependent upon enhanced

formation of ROS generated by NOX2 activation. Platelet ROS plays a central role in the propagation of platelet aggregation by inactivating platelet nitric oxide, releasing ADP, and producing isoprostanes (9,18). Previous studies

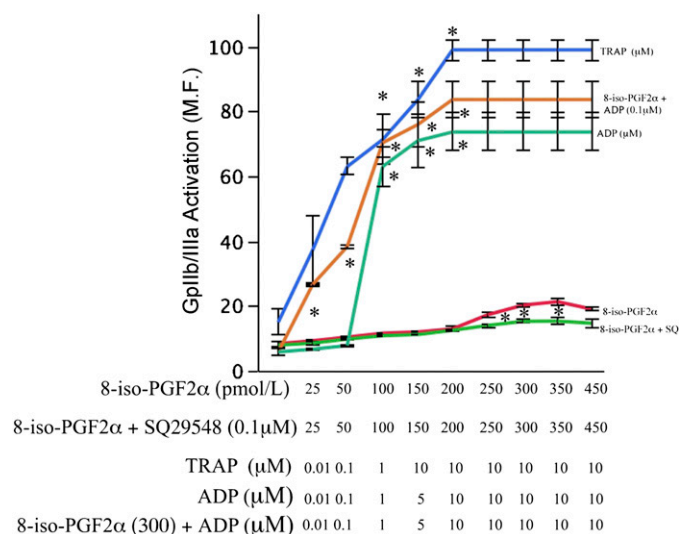


FIG. 3. GpIIb/IIIa activation in platelets treated with a scalar dose of 8-iso-PGF2α (25–450 pmol/L) treated or not with SQ29548 (0.1 μmol/L), ADP (0.01–10 μmol/L) treated or not with 8-iso-PGF2α (300 pmol/L), and TRAP (0.01–10 μmol/L) (values are mean of experiments performed in five healthy subjects). **P* < 0.001.

showed that diabetic patients have a systemic isoprostane overproduction (19,20), but the role of platelets has never been examined. The cross-sectional study showed enhanced platelet isoprostane formation and platelet recruitment along with NOX2 activation in diabetic versus nondiabetic patients without aspirin treatment.

Comparing diabetic and nondiabetic patients treated or not with aspirin, a great behavioral difference between the two platelet eicosanoids was detected. Thus, in nondiabetic patients, aspirin inhibited platelet TxB₂ without affecting platelet isoprostanes. Conversely, diabetic patients on aspirin treatment showed platelet TxB₂ inhibition but a higher isoprostanes production compared with aspirin-untreated diabetic patients. The increase in isoprostane levels was associated with enhanced platelet NOX2 activation. This reinforces the hypothesis that NOX2 is crucial for the ROS overproduction observed in T2DM (21) and suggests a role for NOX2 in platelet isoprostane overproduction in T2DM. Furthermore, our data reinforce previous reports showing that NOX2 upregulation occurs in diabetes not only in the endothelial cells but also in platelets (22). Hyperglycemia is likely to play a major role as also indicated by the direct correlation between HbA_{1c} and sNOX2-dp, but the underlying mechanism still is controversial (23).

The different effect of aspirin on TxB₂ and isoprostanes likely accounts for the divergent behavior of platelet function tests in aspirin-treated diabetic patients. Although arachidonic acid-induced platelet aggregation was inhibited, platelet recruitment was enhanced. To further support these findings, we measured platelet TxB₂ and isoprostanes in diabetic and nondiabetic patients before and after short-term aspirin treatment. Although both groups disclosed a similar platelet TxB₂ inhibition, platelet isoprostanes showed a different behavior. In nondiabetic patients, platelet isoprostane formation was not influenced by aspirin, and in diabetic patients isoprostanes increased 3 and 7 days after aspirin treatment. This change in isoprostane levels was coincidental with enhanced platelet ROS formation and NOX2 activation, suggesting a cause-and-effect relationship between NOX2-generated ROS and platelet

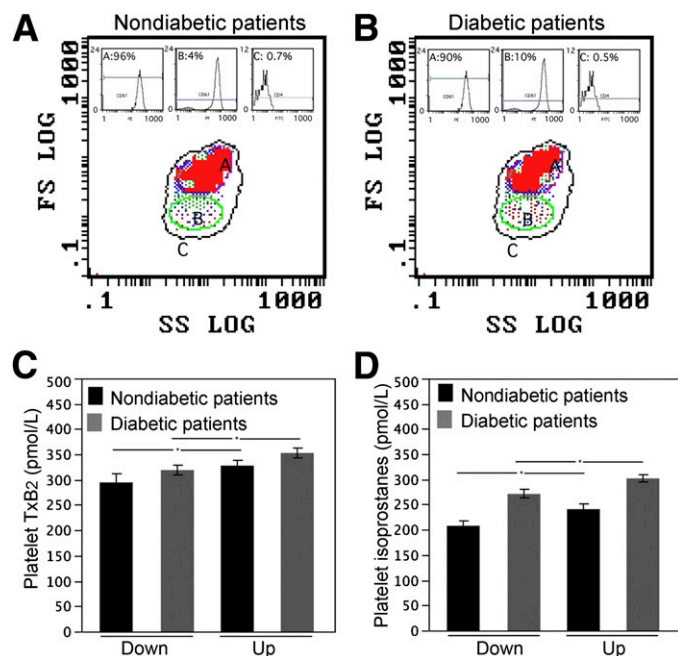


FIG. 4. Platelet sizing and leukocyte contamination in the top 75% of the PRP in nondiabetic patients (A) and in diabetic patients (B). Evaluation of TxB₂ (C) and isoprostanes (D) production in the stratified two portions of the top 75% of the PRP (50% upper phase, 50% bottom phase; up and down, respectively). Values are means of experiments performed in five nondiabetic patients and five diabetic patients. **P* < 0.001.

isoprostane overproduction. This hypothesis was supported by ex vivo experiments where platelets were incubated with a specific NOX2 inhibitor. The NOX2 inhibitor significantly inhibited platelet ROS and isoprostanes in both diabetic and nondiabetic patients with a reduction of the two variables that was, however, much higher in T2DM patients.

A crucial issue of the study was to establish if platelet isoprostane increase observed after aspirin treatment was functionally relevant in diabetic patients (i.e., it was associated with platelet activation). We found no changes in platelet recruitment in nondiabetic but an increase in diabetic patients. Of note, platelet incubation with the TxA₂/isoprostane receptor antagonist prevented platelet recruitment increase in diabetic but not in nondiabetic patients. We speculated that this different behavior could depend upon the 8-iso-PGF2α concentration expressed by platelets from the two groups. To evaluate this, we conducted in vitro experiments to analyze the interplay between isoprostane concentration and GpIIb/IIIa activation (9). A dose-response curve with scalar concentration of 8-iso-PGF2α demonstrated that GpIIb/IIIa was activated in a range of 250–350 pmol/L, which is close to the level of isoprostanes produced by platelets from aspirin-treated diabetic patients (~300 pmol/L) but higher than that expressed by platelets from aspirin-treated nondiabetic patients (~200 pmol/L). Together, these data indicate that in diabetic patients aspirin enhances the platelet production of isoprostanes up to functionally relevant concentrations, so enhancing platelet recruitment via GpIIb/IIIa activation.

The study has pathophysiologic and clinical implications. The upregulation of NOX2 in T2DM has important functional consequences, as it is associated with enhanced ROS and isoprostane production and ultimately with platelet activation. This finding supports and extends previous data indicating that platelet ROS formation are implicated in the

thrombus formation process (18) and provides further insight into the association between T2DM and thrombosis.

The upregulation of NOX2-generated ROS is likely to have a negative impact on the antiplatelet effect of aspirin in diabetic patients. In T2DM, the inhibition of COX1 by aspirin could favor a shift of arachidonic acid toward other metabolic pathways with ensuing enhancement of ROS-mediated nonenzymatic oxidation of arachidonic acid and subsequent isoprostane overproduction. Accordingly, previous studies showed that arachidonic acid activates NADPH oxidase via interaction with p47^{phox}, a subunit of NADPH oxidase (24,25).

Such effect represents a plausible explanation for the lower efficacy of aspirin in preventing cardiovascular events in diabetic patients, as the divergent effect on platelet TxA₂ and isoprostanes has a different impact on platelet activation. The suppression of the early phase of platelet aggregation by TxA₂ may be counterbalanced by the increased propagation of platelet aggregation elicited by isoprostane overproduction. The inhibition of this last effect by *in vitro* addition of TxA₂/isoprostane receptor antagonist suggests a potential benefit of drugs that antagonize TxA₂/isoprostane receptors in patients with T2DM. Prevention of isoprostane overproduction via inhibition of NOX2-generated ROS is another attractive option to be considered. Statins have been reported to downregulate systemic isoprostanes with a mechanism that may involve inhibition of NADPH oxidase (13,26). Therefore, it could be interesting to examine if statins improve the antiplatelet effect of aspirin via inhibition of platelet isoprostanes.

A limitation of the study is the lack of analysis of aspirin compliance in the cross-sectional study. However, both diabetic and control groups had similar values of platelet TxB₂, indicating that a scarce adherence to aspirin treatment, if any, was well balanced between the two. Furthermore, platelet function analysis was performed *in vitro* and *ex vivo*, which may not reflect platelet activation *in vivo*. Pharmacologic study with an inhibitor of isoprostane receptors could be useful to explore our study hypothesis *in vivo*.

In conclusion, we provide evidence that, in T2DM patients, low-dose aspirin enhances platelet isoprostanes as a consequence of NOX2-generated ROS upregulation. This effect mitigates the antiplatelet effect of aspirin and may account for its lower clinical efficacy in T2DM compared with other atherosclerotic settings.

ACKNOWLEDGMENTS

This study was supported by a grant from "Fondazione Roma" 2009.

No potential conflicts of interest relevant to this article were reported.

R.Car., C.N., and M.P. researched data. R.Can. and P.P. wrote the manuscript and researched data. F.A. and D.L. reviewed and edited the manuscript. S.B. researched data. F.V. designed the study and wrote the manuscript. F.V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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