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## **DARK CHOCOLATE INHIBITS PLATELET ISOPROSTANES VIA NOX2 DOWN-REGULATION IN SMOKERS**

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**Running title:** NOX2 and platelet inhibition: effect of chocolate.

**Disclosures:** None.

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**Abstract:**

**Background:** Dark chocolate is reported to decrease platelet activation but the underlying mechanism is still undefined. Dark chocolate is rich of polyphenols that could exert an antiplatelet action via inhibition of oxidative stress. Aim of the study was to assess if dark chocolate inhibits platelet reactive oxidant species (ROS) formation and platelet activation.

**Methods:** Twenty healthy subjects (HS) and 20 smokers were randomly allocated to receive 40 g of dark (cocoa >85%) or milk chocolate (cocoa <35%) in a cross-over, single blind study. There was an interval of 7 days between the two phases of the study. At baseline and 2 hours after chocolate ingestion platelet recruitment (PR), platelet ROS, platelet isoprostane 8-ISO-prostaglandin F<sub>2</sub>α (8-iso-PGF<sub>2</sub>α), Thromboxane (TxA<sub>2</sub>) and platelet activation of NOX2, the catalytic sub-unit of NADPH oxidase, and serum epicatechin were measured.

**Results:** Compared to HS, smokers showed enhanced PR, platelet formation of ROS and eicosanoids and NOX2 activation. After dark chocolate platelet ROS (-48%, p<0.001), 8-iso-PGF<sub>2</sub>α (-10%, p<0.001) and NOX2 activation (-22%, p<0.001) significantly decreased; dark chocolate did not affect platelet variables in HS. No effect of milk chocolate was detected in both groups. Serum epicatechin increased after dark chocolate in HS (from 0.454±0.3nM to 118.3±53.7nM) and smokers (from 0.5±0.28 nM to 120.9±54.2 nM). Platelet incubation with 0.1-

10µM catechin significantly reduced PR, platelet 8-iso-PGF2α and ROS formation and NOX2 activation only in platelets from smokers.

**Conclusions:** Dark chocolate inhibits platelet function by lowering oxidative stress only in smokers; this effect seems to be dependent on its polyphenolic content.

**Key words:** atherosclerosis, cocoa, oxidative stress, platelets.

## **INTRODUCTION**

Epidemiologic studies demonstrated that a diet rich in polyphenols reduces cardiovascular events in general population and in patients at risk of cardiovascular disease [1-6]. The clinical effects included reduction of cardiovascular mortality, myocardial infarction and stroke [1-6].

Platelets play a key role in the athero-thrombotic process as shown by significant reduction of cardiovascular events by antiplatelet drugs in acute and chronic coronary syndromes and in patients with acute and chronic cerebro-vascular disease [7]. Several nutrients containing polyphenols have been investigated in human to assess if they possess antiplatelet effects. Polyphenol-rich cocoa or dark chocolate consistently showed an acute inhibitory effect on agonist-induced platelet aggregation: thus, among 9 interventional trials only one showed no effect on platelet aggregation [8-17]. However, the mechanism accounting for the inhibitory effect of cocoa and dark chocolate on platelet function was not clarified [8-17].

There are several evidences that polyphenols possess antioxidant property that may be implicated in the antiplatelet effect of cocoa or dark chocolate. Thus, polyphenols inhibit NADPH oxidase-dependent platelet reactive oxygen species (ROS) formation or enhance nitric oxide (NO) generation and/or bioactivity [18] ; both of these activities may be involved in the inhibition of

platelet function [18]. Furthermore, ROS generated by NADPH oxidase are involved in the formation of isoprostane 8-ISO-prostaglandin F<sub>2</sub>α (8-iso-PGF<sub>2</sub>α), which play a pivotal role in the amplification of the platelet response to the agonists via activation of Glicoprotein IIb/IIIa [19]. Therefore, we aimed to investigate if acute ingestion of polyphenol-rich dark chocolate or milk chocolate, that has lower polyphenol content [20, 21], differently affected platelet-derived oxidative stress and platelet activation. At this end we compared the effect of dark or milk chocolate in healthy subjects and in smokers, who are known to have enhanced oxidative stress and platelet activation [22, 23]. Furthermore, we performed in vitro study to evaluate if epicatechin, one of the polyphenol commonly found in the human circulation after dark chocolate administration [20], affected platelet function with an oxidative stress-mediated mechanism.

## **MATERIALS AND METHODS**

Twenty healthy subjects (HS) and twenty smokers, matched for age and gender (Table 1) agreed to participate in the study, which was performed between October and December 2010. Each smoker consumed an average number of cigarettes per day of 11 (range 6 to 28). All study participants did not take any medications. They were randomly allocated to a treatment sequence with 40 g of dark chocolate ( $\geq 85\%$  cocoa) or milk chocolate ( $\leq 35\%$  cocoa) in a cross-over, single-blind design. There was a one week-washout between the two phases of the study.

Commercially available chocolate was used for this study; compared to milk chocolate (40 g), dark chocolate (40 g) has a higher content of fiber (3 vs 0 g) and a lower content in cholesterol (0 vs 10 mg), sodium (20 vs 40 mg) and sugar (5 vs 20 g). Furthermore, there weren't significant differences in calorific content between dark (Calories 230) and milk (Calories 220) chocolate.

Platelet activation and oxidative stress, as assessed by recruitment, ROS production, soluble NOX2 derived peptide (sNOX2-dp), 8-iso-PGF2 $\alpha$ , nitric oxide (NO) generation and thromboxane formation were assessed at baseline, after 24 hours' abstinence from food rich in polyphenols, and two hours after ingestion of chocolate. Participants were studied after a fasting period of eight hours and, for smokers, after a smoke-free interval of at least 2 hours before each experiment.

All subjects underwent a full medical history and physical examination. Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL), acute cerebro-vascular disease, acute myocardial infarction, dyslipidemia, diabetes, hypertension, or were taking antioxidants or any drug known to interfere with platelet function.

### **Randomization and Blinding**

An individual not involved in the study, assigned codes to the study treatments, randomly allocated the participants to a treatment sequence with dark or milk chocolate, and kept the key in a sealed envelope. The randomization was carried out by a procedure based on a random numeric sequence. The authors and laboratory technicians were unaware of the treatment allocation.

### **Platelet preparation**

To obtain platelet rich plasma (PRP), samples were centrifuged 15 min at 180g. To avoid leukocyte contamination, only the top 75% of the PRP was collected. Platelet pellet was suspended in HEPES buffer, pH 7.4 ( $2 \times 10^8$  platelets/mL, unless otherwise noted). Platelets were activated with or without arachidonic acid (AA) (0.5mM) for 10 minutes at 37°C and supernatant stored at -80° unless otherwise indicated.

### **Platelet ROS production**

Cell suspension was incubated with 2',7'-dichlorofluorescein diacetate (5  $\mu\text{mol/L}$ ) for 15 minutes at 37°C. After incubation, platelets were activated with AA. Platelet ROS production was expressed as mean fluorescence and ROS production in stimulated cells was expressed as stimulation index (mean level of fluorescence in stimulated cells/mean level of fluorescence in unstimulated cells) (S.I.).

Fluorescence intensity was analyzed on an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 510-550 nm (green). For every histogram, 50,000 platelets were counted to determine the proportion of positive platelets. The fluorescent signal generated by the probe was expressed as mean fluorescence intensity (S.I.). Intra-assay coefficient of variation was 5%.

### **Platelet 8-iso-PGF2 $\alpha$ assays**

To measure the platelet isoprostanone 8-iso-PGF2 $\alpha$  cell platelet suspension was activated with AA. The supernatant was stored at -80 C° until measurement. Quantification of isoprostanes was performed measuring 8-iso-PGF2 $\alpha$  by a previously described and validated EIA assay method [24] and was evaluated in vivo and in vitro study. Intra-assay and inter-assay coefficients of variation were 5.8% and 5.0% respectively.

### **ELISA detection of platelet sNOX2-dp**

NOX2- derived peptide, a marker of NADPH oxidase activation, was detected in vivo and in vitro study by ELISA method as previously described by Pignatelli et al. [25]. The peptide was recognized by a specific monoclonal antibody against the amino acidic sequence (224-268) of the extra membrane portion of NOX2. To measure sNOX2-dp platelet suspension was activated with

AA and 100µl of the supernatant stored at -80 C° until measurement. Values were expressed, as pg/ml, intra-assay and inter-assay coefficients of variation were 5.2% and 6% respectively.

### **Platelet recruitment**

Platelet recruitment was performed with a method modified from that described by Krotz et al. [26]. PRP samples were incubated (30 minutes, at 37°C) with or without scalar doses of epicatechin (0.1-10µM), catechin (0.1-10µM), epicatechin + catechin (0.1-10µM) or NOX2ds-tat (a specific inhibitor of NADPH oxidase) (0.1-10µM), before activation with collagen (6µg/ml). Solvents were used as controls. In some experiments catechin and epicatechin were re-moved from the supernatant after 30 min incubation to evaluate the recovery of platelet reactivity. Collagen-induced platelet aggregation was measured for 10 minutes. Then, an equal portion of untreated platelets was added to each tube, which increased the density of the solution and hence caused a reduction in light transmission. Aggregation of the newly added platelet portion in the presence of an existing aggregate was then measured for 5 minutes and expressed as a percentage of the aggregation that had been initially reached [18]. Platelet aggregation was measured according to Born's method [18] and calculated as light transmission difference (LT%) between PRP and platelet poor plasma (PPP) as described previously [27].

### **Platelet NOx**

A colorimetric assay kit (Tema Ricerca, Italy) was used to determine the nitric oxide metabolites nitrite and nitrate (NOx) in 100µl of the unstimulated platelet suspension maintained under stirring condition 10 min at 37°C. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7% respectively.

## **Platelet TxA<sub>2</sub>**

AA(0.5mM)- activated PRP samples were centrifuged and the supernatant stored at -80°C. Platelet TxA<sub>2</sub> was analyzed as previously described [27, 28]. Briefly, platelet TxA<sub>2</sub> was measured by evaluating its stable metabolite TxB<sub>2</sub> by an EIA commercial kit (Amersham Pharmacia, Biotech, Little Chalfont, UK) and expressed as pg/10<sup>8</sup> cells or ng/ml respectively. Intra- and inter-assay coefficients of variation for TxB<sub>2</sub> EIA Kit were 4.0% and 3.6%, respectively.

## **Sample preparation and epicatechin evaluation in HPLC**

One hundred µl of serum was extracted twice with 0.8ml followed by 0.5ml of ethyl acetate by vortexing for 1 min. in 1ml vial. After centrifuging the mixture for 5min at 3500g, the top layers comprising ethyl acetate were removed, pooled and evaporated to dryness under nitrogen [29]. The dried samples was dissolved in methanol (0.1ml) before analysis in HPLC.

The determination of epicatechin (EC) was carried out using an Agilent 1200 Infinity series HPLC system equipped with an Eclipse plus C<sub>18</sub> column (4.6 x 100mm). All determinations were undertaken at 25°C. An isocratic mobile phases consisting of water-methanol (80:20) was used; running at a flow rate of 1.2 ml/min and the UV detection were carried out at 280nm. The sample injection volume was 20µl. Chromatographic peaks of analytes were identified by comparing their retention times with those of the standard [30].

## **In vitro study**

In vitro study was performed in blood taken from 5 healthy subjects and 5 smokers matched for age and gender (6 males and 4 females, mean age 32±4 years) who had not taken dark or milk chocolate. We analyzed the effect of epicatechin, catechin, epicatechin plus catechin or an inhibitor of NADPH oxidase (NOX2ds-tat) on platelet ROS production, 8-iso-PGF<sub>2</sub>α formation, platelet

recruitment and sNOX2-dp after platelet activation. Platelets were incubated 10 min at 37C° with 0.1-10µM epicatechin, 0.1-10µM catechin, 0.1-10µM epicatechin plus 0.1-10µM catechin or 0.1-10µM NOX2ds-tat before stimulation with the agonists.

## **STATISTICAL ANALYSIS**

### **Sample size determination**

We computed the minimum sample size with respect to a two-tailed one-sample Student t test with Welch correction, considering a (i) difference for platelet sNOX2-dp variation in smokers to be detected between dark and milk chocolate treatments  $|\delta| \geq 10$ , (ii) standard deviation of the paired differences SD= 5, (iii) type I error probability  $\alpha=0.05$  and power  $1-\beta=0.90$ .

This resulted in n=12 patients, which was increased to n=20.

### **-Statistical methods**

Continuous variables are reported as means±SD unless otherwise indicated. Comparisons between smokers and HS were carried out by Student's t-test and were replicated as appropriate with nonparametric tests (Kolmogorov-Smirnov (z) test in case of non-homogeneous variances as verified by Levene's test).

The cross-over study data were analysed for the assessment of treatment and period effects, by performing a split-plot ANOVA with one between-subject factor (treatment sequence) and two within-subject factors (period 1 vs 2; pre vs post treatment) [31]. The full model was considered, allowing for the assessment of all main effects and two- and three-way interactions. Pairwise comparisons were corrected by the Bonferroni. In vitro experiments were analysed by ANOVA. A value of  $p < 0.05$  was considered statistically significant. All analyses were carried out with SPSS-18.0 software (SPSS Inc.).

## RESULTS

### Interventional study

Clinical characteristics of smokers and HS are reported in table 1. There were no differences between the two groups with the exception of smoking habit.

At baseline, compared to HS, smokers had higher platelet production of ROS, sNOX2-dp, 8-iso-PGF2 $\alpha$  (Figure 1 A-C) and lower platelet NOx (Figure 1 Panels E); platelet TxB<sub>2</sub> production was weakly increased in smokers with borderline significance (**p=0.048**) (Figure 1 Panel D). Furthermore, platelet recruitment was higher in smokers compared to HS (Figure 1 Panel F); epicatechin serum levels did not differ between groups (Figure 2).

After dark chocolate intake, platelet oxidative stress changed in smokers but not in HS. Thus, 2 hours after dark chocolate intake, smokers showed lower platelet ROS and NOX2 activation and enhanced platelet NOx (Figure 1 Panels A, B and E) compared to baseline. Platelet production of eicosanoids was differently modified by dark chocolate as platelet 8-iso-PGF2 $\alpha$  decreased while platelet TxB<sub>2</sub> did not change (Figure 1 Panels C and D). Dark chocolate administration reduced platelet recruitment in smokers but not in HS (Figure 1 Panel F). Milk chocolate did not affect platelet oxidative stress, eicosanoid production and platelet function in either smokers or HS (Figure 1).

Blood epicatechin concentration was measured at baseline and 2 hours after dark or milk chocolate administration. Blood epicatechin increased significantly only after dark chocolate in either smokers or HS (Figure 2).

### **In vitro study**

The basal values of platelet ROS production (Mean Fluorescence  $1.3 \pm 0.2$  vs  $3.6 \pm 0.3$ ,  $p=0.004$ , respectively) (Figure 3 Panels A and B), sNOX2-dp levels ( $8.8 \pm 1.9$  pg/ml vs  $14.5 \pm 4.6$  pg/ml,  $p=0.02$ , respectively) (Figure 3 Panels C and D), 8-iso-PGF2 $\alpha$  ( $14.1 \pm 4.9$  pmol/L vs  $20.1 \pm 6.1$  pmol/L,  $p=0.03$ , respectively) (Figure 3 Panels E and F) and platelet recruitment ( $4.15 \pm 1.17\%$  vs  $7.1 \pm 1.5\%$ ,  $p=0.01$ , respectively) (Figure 3 Panels G and H) were significantly higher in smokers as compared to HS.

AA-stimulated platelet incubated with epicatechin, epicatechin plus catechin or NOX2ds-tat resulted in a significant inhibition of platelet oxidative stress and function including platelet ROS, NOX2 activation, 8-iso-PGF2 $\alpha$  formation and platelet recruitment. Epicatechin or catechin alone dose-dependently inhibited oxidative stress and platelet function with an effect which was higher when used in combination. The inhibitory effect of polyphenols was observed in platelets from smokers but not in platelets from HS (Figure 3). Conversely inhibition of oxidative stress and platelet function by NOX2ds-tat was detected in both smokers and non smokers.

### **DISCUSSION**

The study provides evidence that in smokers dark chocolate inhibits platelet 8-iso-PGF2 $\alpha$  via down-regulation of platelet NOX2-induced ROS generation.

ROS serve as second messenger of platelet activation via modulation of several intra-signaling mechanisms including activation of PLA<sub>2</sub>, inactivation of Nitric Oxide (NO) and formation of 8-iso-PGF2 $\alpha$  [18,19,27,32]. Isoprostanes are chemically stable eicosanoids that derive from ROS interaction with arachidonic acid [33]; they are pro-aggregating molecules that favor propagation of platelet aggregation via activation of Glycoprotein IIb/IIIa [19]. NOX2 plays a key role in platelet isoprostane formation as suggested by low 8-iso-PGF2 $\alpha$  production in platelets taken from patients

with hereditary deficiency of NOX2 [34]. The first novel finding of the present study is the up-regulation of NOX2 in platelets from smokers reinforcing the concept that smoking habit is associated with oxidative stress [22]. The increase of NOX2 was coincident with enhanced ROS, 8-iso-PGF2 $\alpha$  formation and platelet recruitment suggesting a link between ROS generated by NOX2 and 8-iso-PGF2 $\alpha$  -induced platelet activation. Smokers had a weak increase of platelet TxB<sub>2</sub>, that is apparently in contrast with previous study showing no difference in serum TxB<sub>2</sub> between smokers and non smokers [35]. However, the difference between smokers and non smokers was of borderline significance (p=0.05), therefore we are not certain that such weak COX-1 up-regulation is of biologic relevance.

In the present study we tested the hypothesis that a polyphenol-rich nutrient down-regulates platelet NOX2 and in turn reduces platelet activation via lowering 8-iso-PGF2 $\alpha$  formation. This study hypothesis was supported by the results of the interventional study as dark chocolate but not milk chocolate was associated with down-regulation of ROS generated by platelet NOX2, platelet 8-iso-PGF2 $\alpha$  formation and platelet recruitment. The reduction of oxidative stress was likely to account for the enhanced platelet NO generation as oxygen free radicals are known to inactivate NO or reduce NO synthase activation [18]. The antioxidant and antiplatelet effects were observed in smokers but not in non smokers suggesting that baseline generation of ROS is a prerequisite to lower oxidative stress by compounds with antioxidant property. This findings is apparently in contrast with a previous report from our groups showing that Vitamin E, an antioxidant molecule that inhibits the formation of lipid peroxides [36] and platelet aggregation in healthy subjects [36]; however vitamin E possesses other properties that may affect platelet function with mechanisms independent from its antioxidant action [37].

Differently from 8-iso-PGF2 $\alpha$ , dark chocolate had scarce influence on platelet TxB2 indicating that COX1 activation was not affected by a polyphenol-rich nutrient. This is consistent with previous data showing that oxidative stress has a neutral action on COX1 activation [19].

To explore the mechanism through which dark chocolate inhibited 8-iso-PGF2 $\alpha$  formation and platelet activation, in vitro experiments were conducted by incubating platelets with epicatechin. Thus, after dark chocolate epicatechin was detected in the peripheral circulation of both smokers and non smokers at concentration similar to that previously detected after administration of dark chocolate or cocoa [38]. Conversely, blood epicatechin did not increase after milk chocolate likely because its content of polyphenol is much lower compared to dark chocolate [21] or to the fact that the antioxidant effect of cocoa is attenuated if milk is added.

The experiments demonstrated that epicatechin, at concentration found in the peripheral blood after dark chocolate administration, possess antioxidant property as its incubation with platelets these patients resulted in lowered NOX2 activation and ROS generation. This effect was associated with reduced 8-iso-PGF2 $\alpha$  formation and platelet recruitment suggesting down-regulation of platelet ROS as a mechanism potentially impairing activation of platelets via reduced 8-iso-PGF2 $\alpha$  production. Of note, such changes were observed only in platelets from smokers while no effect of epicatechin was detected in platelets from non smokers. This would imply that the rate of intracellular ROS generation could represent a “conditio sine qua non” to lower oxidative stress by molecules with antioxidant properties. However, it is interesting to note that platelet incubation with an inhibitor of NOX2 resulted in down-regulation of oxidative stress and platelet activation also in healthy subjects. Therefore it cannot be excluded that other pharmacologic approach with polyphenol-rich nutrients may results in oxidative stress and platelet activation inhibition also in healthy subjects.

The present study corroborates and extends previous reports showing that polyphenol-rich nutrients such as dark chocolate exert an antiplatelet effect with an antioxidant effect. Herewith we show for the first time that such antiplatelet activity is mediated by NOX2 down-regulation and ultimately lowered 8-iso-PGF2 $\alpha$  formation [38]. The inhibition of NOX2 activation by epicatechin is consistent with other studies showing that polyphenols possess antioxidant effects via inhibition of ROS generated by NOX2 [39]. Even if epicatechin per se may account for the inhibitory effect of dark chocolate, it is possible that a synergism among polyphenols contributes to the antiplatelet effect in vivo. Thus in vitro combination of epicatechin with catechin, another polyphenol contained in dark chocolate, potentiated the antioxidant and antiplatelet effect achieved by a single polyphenol. This is in accordance with our previous studies showing that polyphenols synergize in inhibiting platelet NADPH oxidase [18] and would imply that the antiplatelet effect of dark chocolate could be attributed not only to epicatechin but also to other polyphenols contained in dark chocolate.

The study has implications and limitations that should be acknowledged. Despite the intrinsic difficulties to perform a double-blind study with dark and milk chocolate, we tried to mitigate this bias by performing laboratory analyses in blind. We did not analyze how long the antiplatelet effect of dark chocolate lasted, therefore further study is necessary to explore this issue. Hence, this study should be considered a proof-of-concept study with no implication for clinical purpose but suggests to analyze if the antiplatelet effect is detectable also after chronic administration of dark chocolate. The lack of antiplatelet effect in non smokers reinforces the concept that nutrients with antioxidant property are unlikely to counteract oxidative stress in healthy subjects and should not be used in general population [40].

In conclusion the study demonstrates that dark chocolate down-regulates platelet ROS generated by NOX2 and ultimately inhibits platelet activation via inhibition of platelet 8-iso-PGF2 $\alpha$ . Such effect seems to be related to the antioxidant property of its polyphenol content as platelet incubation with epicatechin and catechin reproduced the findings achieved in vivo. The results warrant further study to see if similar effect can be obtained by chronic administration of dark chocolate.

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## Figure legends

### Figure 1

Platelet ROS (Panel A), platelet sNOX2-dp (Panel B), platelets 8-iso-PGF2 $\alpha$  (Panel C), and TxB<sub>2</sub> (Panel D), platelet NOx levels (Panel E) and platelet recruitment (Panel F) before and 2 hours after intake of dark or milk chocolate in smokers and HS. Data are presented as means+SE. (**\*p<0.05**).

### Figure 2

Serum levels of epicatechin before and 2 hours after intake of milk (Panel A) or dark (Panel B) chocolate in smokers and HS. Data are presented as means+SE. (**\*p<0.05**).

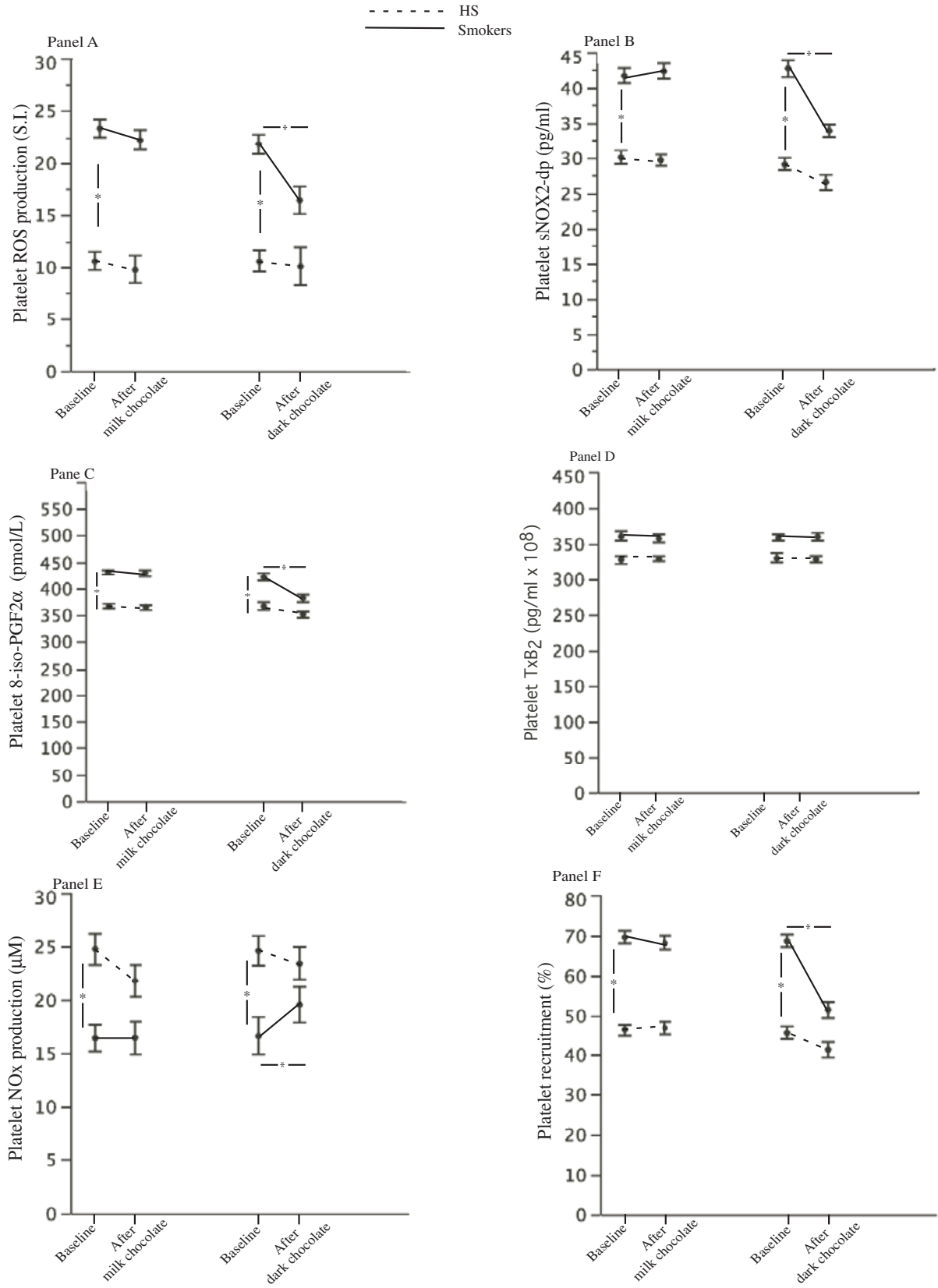
### Figure 3

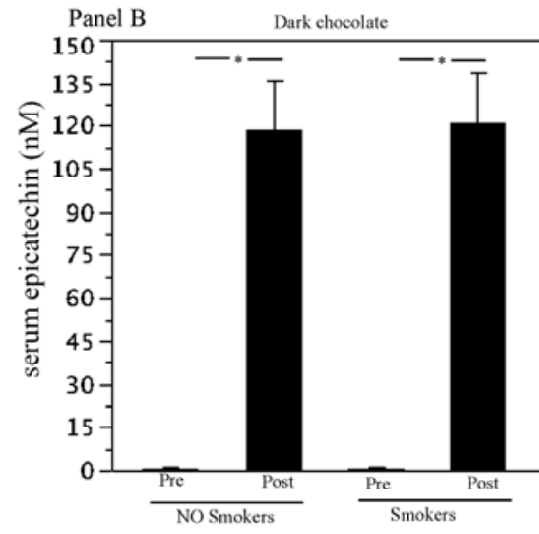
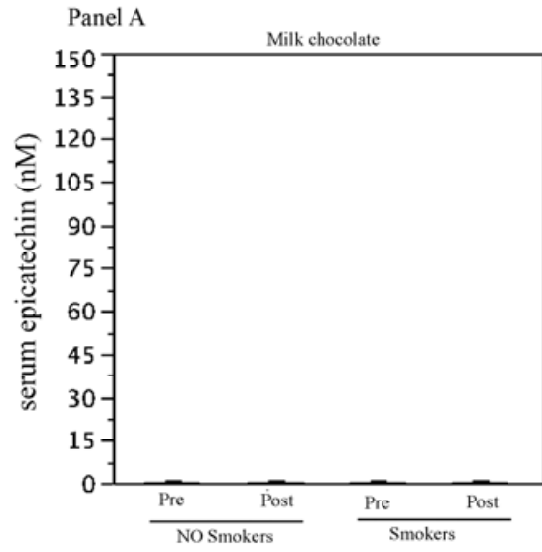
Effect of scalar doses of epicatechin (0.1-10 $\mu$ M), catechin (0.1-10 $\mu$ M), epicatechin plus catechin (0.1-10 $\mu$ M) or NOX2ds-tat (0.1-10 $\mu$ M) on platelet ROS (Panel A and B), platelet sNOX2-dp (Panel C and D), platelet 8-iso-PGF2 $\alpha$  formation (Panel E and F) and platelet recruitment (Panel G and H). The experiments were performed in blood taken from 5 smokers and 5 HS. \*p<0.001.

**Table 1.** Clinical characteristics of smokers and HS.

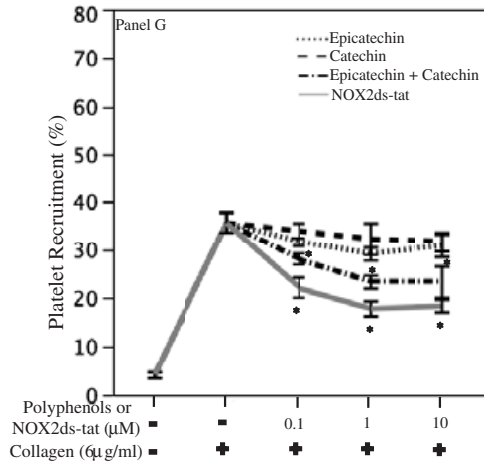
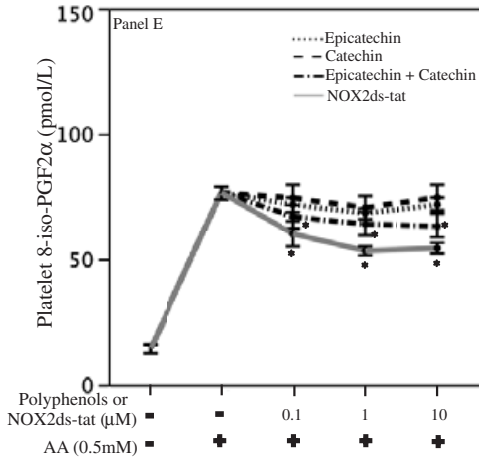
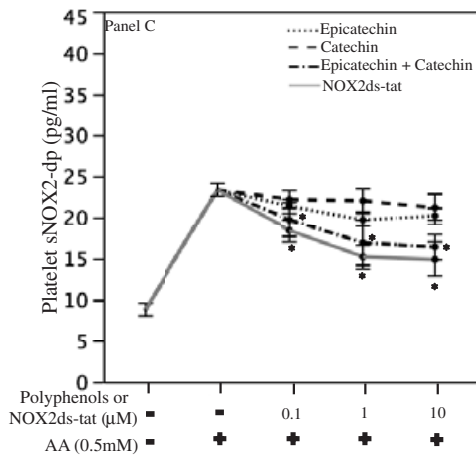
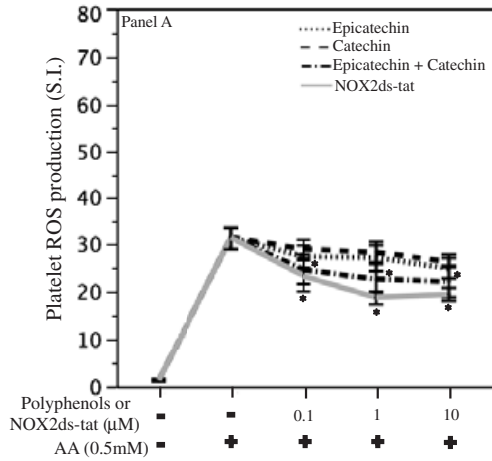
<b>Variables</b>	<b>HS</b>	<b>Smokers</b>	<b><i>P value</i></b>
	(n=20)	(n=20)	
Mean age (yr)*	33±11	33±11	0.954
Males/Females	7/13	7/13	1.0
High (cm)*	168.7±8.5	168.7±10.1	1.0
Weight (Kg)*	65.5±9.8	64.75±9.5	0.808
BMI*	23±2.8	22.8±3.3	0.775
Systolic blood pressure (mmHg)*	113±10	114±12	0.977
Diastolic blood pressure*	68±6	68±8	0.983
Cigarettes/day*	0	11±5.2	<0.001

\*Data are expressed as mean ± SD.





## HS



## SMOKERS

