RESEARCH

Cardiovascular Therapeutics

Ascorbic Acid Infusion Blunts CD40L Upregulation in Patients Undergoing Coronary Stent

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Ascorbic acid; CD40L; Oxidative stress; Percutaneous coronary interventions, PCI; Platelets.

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SUMMARY

Objectives: To reduce the increase of oxidative stress and the upregulation of CD40L during stenting procedure using ascorbic acid infusion. **Background:** CD40L upregulation occurring after coronary Percutaneous Coronary Intervention predicts vascular events but the underlying mechanism is still unclear. **Methods:** Fifty-six patients undergoing elective coronary stenting were randomly allocated to intravenous infusion of the antioxidant ascorbic acid or placebo. Platelet CD40L and plasma levels of soluble CD40L and of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress, were measured before and after coronary stenting. *In vitro* study was also done to measure reactive oxidant species and CD40L expression in platelets exposed to anoxia-reoxygenation. **Results:** Placebo-treated patients showed a significant increase of platelet CD40L, soluble CD40L and 8-hydroxy-2′-deoxyguanosine compared to baseline values. Patients given ascorbic acid showed no change of soluble CD40L and platelet CD40L but a significant decrease of 8-hydroxy-2′-deoxyguanosine. After 60 and 120 min, soluble CD40L, platelet CD40L and 8-hydroxy-2′-deoxyguanosine were significantly lower in the ascorbic acid-treated group compared to the placebo-treated one. A significant correlation between platelet CD40L and soluble CD40L and between soluble CD40L and 8-hydroxy-2′-deoxyguanosine was observed. Platelets, *in vitro* exposed to anoxia-reoxygenation, had a burst of ROS and an upregulation of CD40L that were inhibited by ascorbic acid or apocynin, an inhibitor of NADPH oxidase. **Conclusions:** This study shows that in patients undergoing coronary stenting CD40L is upregulated with a mechanism which is likely mediated by oxidative stress.

Introduction

Despite intracoronary stents improved survival in patients with coronary heart disease who underwent percutaneous coronary intervention (PCI), stent thrombosis (2.03%), and vascular events such as myocardial infarction, stroke, and vascular death (11.9%) are still observed in a high percentage of patients undergoing PCI [1–3]. Platelets have a key role in such poor outcomes as shown by the fact that reduction of vascular outcomes is related to the rate of platelet inhibition [3–5]. Knowledge of the mechanisms of coronary stent-related platelet activation is still unclear.

CD40L is a cytokine of the tumor necrosis factor alpha (TNF)α super-family primarily identified in the immunosystem cells [6]. CD40L is expressed on platelet surface upon agonist stimulation and is released in the circulation as soluble form (sCD40L) [7]; more than 95% of the circulating CD40L stems from platelet activation [8]. CD40L is a potent inflammatory and procoagulant molecule that is over-expressed in a series of clinical settings characterized by accelerated atherosclerosis, such as

hypercholesterolemia [9], diabetes [10], and smoking [11]. Prospective studies have shown that CD40L is predictive of poor vascular outcome in patients with acute vascular events or at risk of both thromboembolic and atherothrombotic diseases [12–14]. Furthermore, in patients undergoing PCI the increase of CD40L immediately after stenting procedure seems to be associated with restenosis [5,15]. Thus comprehension of the mechanism leading to the increase of CD40L could be relevant not only to provide a mechanistic insight but also to eventually develop novel therapeutic strategies. We, as well as others, have reported that oxidative stress plays a major role in the upregulation of CD40L [16–18]. Interestingly, in patients undergoing PCI, a significant increase of oxidative stress has been reported as a likely consequence of the ischemia-reperfusion process occurring in this setting [19–22]. We, therefore, speculated that the increase of oxidative stress may be pertinent in the upregulation of CD40L and that the use of an antioxidant during stenting procedure could minimize this phenomenon. To explore the validity of such hypothesis we planned a pilot study consisting in measuring CD40L before and after coronary stenting in patients treated or not with ascorbic acid, a known antioxidant that scavenges superoxide radicals [23]. This approach was based on the results of a previous study demonstrating that intravenous infusion of 1 g ascorbic acid is associated with a significant decrease of circulating sCD40L [24]. As ischemia-reperfusion phenomenon could be a part of the injury linked to PCI, we performed *in vitro* study to see if anoxia-reoxygenation of platelets was associated with oxidative-stress mediated CD40L upregulation.

Materials and Methods

Interventional Study

Study Population and Study Design

This prospective randomized pilot study included patients ≥18 years of age with clinically stable class I or II effort angina, a positive functional study for myocardial ischemia and a single *de novo* lesion in a native coronary artery referred to Department of the Heart and Great Vessels "Attilio Reale," University of Rome "La Sapienza," Italy between June 2008 and January 2009.

Angiographic inclusion criteria were all type A or type B coronary lesions, as described by the American College Cardiology (ACC)/American Heart Association (AHA) Task Force [25], a target vessel reference diameter of 2.5–3.5 mm, a lesion length ≤33 mm, which would be covered with a single medicated stent, diameter stenoses ≥70% and <100%, and a Thrombolysis In Myocardial Infarction grade >1 flow.

Procedural exclusion criteria were a target vessel diameter <2.5 mm or >4.0 mm (14 patients), type C lesions (four patients) and bifurcation lesions (four patients). Clinical exclusion criteria were: contraindication to aspirin or clopidrogel (two patients), previous myocardial infarction (three patients), graft vessel disease (four patients), platelet count $<100{,}000/mm^3$ (two patients), history of bleeding diathesis (two patients), renal dysfunction (creatinine levels >1.5 mg/dL) (one patient).

The study was approved by the University of Rome "La Sapienza" Ethical Committee, and written informed consent was obtained from all patients.

Fifty-six consecutive patients [47 male and 9 female, mean ages 67 (50–84) years] who met inclusion and exclusion criteria entered the study.

The PCI was carried out according to international guidelines, using a standard technique, through the femoral route [25]. Systematic stent implantation was achieved in all patients. The sheath was removed immediately at the end of the procedure in all cases. Routine care before and after the procedure was undertaken for all patients, including pretreatment clopidogrel (600-mg initial bolus) 12 h before the procedure followed by 75 mg daily for at least 1 year. In addition, all patients received aspirin, 100 mg daily, for at least 1 week before, with a dose administered 12 h before stenting.

Serum cardiac-Troponin I (cTpI) was measured at baseline before the procedure, every 6 h over the next 2 days and thereafter if abnormal values were present. Left ventricular ejection fraction (LVEF) was calculated by the biplane Simpson's rule, as recommended by the American Society of Echocardiography at baseline and at 72 h after PCI by operators unaware of the treatment allocation.

Angioplasty Procedure

Preparation and percutaneous access were performed according to standard hospital procedures. After percutaneous access was obtained, an intravenous bolus of 5000 U of unfractionated heparin was administered, with sufficient supplements (if necessary) to maintain an activated clotting time (ACT) ≥250 seconds during interventions. A baseline angiography of the involved vessel was performed in at least two near orthogonal views that showed the target lesion free of foreshortening or vessel overlap, using a 6 F diagnostic catheter. The angiograms included at least 2 cm of catheter to allow for accurate quantitative coronary angiographic measurements.

Selective angiography was performed with an automatic injector (ACIST HD101, Eden Prairie, Minnesota), by using a total volume of 10 mL iopromide (Ultravist 370, Schering AG, Berlin, Germany), at a rate of 2.0 mL/s for left coronary arteries, and a total volume of 8 mL

iopromide at a rate of 1.0 mL/s for right coronary arteries, at 450 PSI. Following identification of the target lesion that meets all eligibility criteria, those patients who continue to meet eligibility criteria were randomized to placebo or ascorbic acid administration and received a unique study identification code. Thus, after baseline collection of blood samples a sealed, opaque envelopes containing a computer-generated random sequence were used for randomization to an intravenous infusion of ascorbic acid (1 g/L at 24 mg/min) or placebo (saline solution).

The target lesion was crossed with a 0.014 exchange-length guide wire and a single predilatation with an appropriately sized balloon was performed by inflating the balloon to the nominal pressure over a 15 sec period. Within 1 min from balloon dilatation a sirolimus-eluting stent (SES; Cypher^{TM}, Cordis, Johnson & Johnson) was implanted. Stent deployment was achieved with high pressure balloon inflation (more than 15 atm) lasting 10 sec without any post-dilatation. No direct stenting was performed.

Laboratory Analysis

To ensure blind analysis, cardiologists sent tube identified by numerical code to the laboratory where biologists perform analytical tests. The randomization list was unveiled after that the analytical phase was terminated.

Blood Sampling Protocol for Platelet Isolation

Blood samples were drawn from an antecubital vein with a 21-gauge needle and then mixed in a tube with 0.13 mM/l sodium citrate (ratio 9:1) for plasma preparation. Samples were collected before and 60, 120 min after the procedure. To obtain platelet rich plasma (PRP) sample was centrifuged (15 min at 180 *g*) as previously reported [26].

sCD40L, 8-hydroxy-2′-deoxyguanosine (8OH-dG), high sensitivity C-reactive protein (hs-CRP), TNFα, and Troponin.

Blood samples were immediately centrifuged at 300 *g* for 20 min at 4°C, and the supernatant was collected and stored at −80°C until measurement. Plasma levels of sCD40L, 8OH-dG and serum levels of hs-CRP were measured with a commercial immunoassay (Tema Ricerche s.r.l., Bologna, Italy). Intra-assay and interassay coefficients of variation were 5 and 7% for sCD40L, 2.1 and 4.5% for 8OH-dG, 8.3 and 7.8% for hsPCR, respectively. TNF$_\alpha$ (Quantikine R&D Systems, Minneapolis, MN, USA) were measured with a commercial immunoassay (Intra-assay and interassay coefficients of variation were 5.3 and 6.8%). cTp I levels were measured using an automated enzyme immuno-assay system (Dimension RXL MAX, Siemens Healthcare diagnostic, Germany) with upper limit of normal being 0.05 ng/mL in our laboratory.

Flow Cytometric Analysis of Platelet CD40L Expression

CD40L expression on platelet membranes was analyzed with specific fluorescein isothiocyanate-labeled monoclonal antibodies (mAbs; anti-CD40L antibody by Beckman Coulter). The binding of the primary antibody was detected with a secondary antibody (anti-mouse IgG-FITC by Beckman Coulter). In all assays, an irrelevant isotype-matched antibody was used as a negative control.

Blood samples were centrifuged to obtain PRP and immediately fixed with tromboFix platelet stabilizer (Beckman Coulter) (30 min at room temperature before any other treatment). After fixing procedure, twenty microliters of mAb were added to 200 μL of platelet suspension (2×10^8/mL). The unbound mAb was removed by addition of 0.1% bovine serum albumin–phosphate buffered saline (PBS) and centrifugation at 300 *g* for 3 min (twice). Fluorescence intensity was analyzed by an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 488 nm. For every histogram, 50,000 platelets were counted to determine the proportion of positive platelets. Antibody reactivity is reported as mean fluorescence. Intra-assay coefficient of variation was 5%. To test the effect of fixing procedure on the functional integrity of platelet unfixed and fixed samples were incubated after centrifugation with mAB anti-PAC1-FITC (Beckman Coulter) (an antibody that recognizes an epitope on the glycoprotein IIb/IIIa of activated platelets) showing that PAC1 binding did not change before and after centrifugation.

In Vitro Study

Blood Sampling Protocol and Platelet Preparation

Five non-smoking healthy volunteers (three males and two females, mean age 60 ± 3 years) who had not ingested any drug known to interfere with platelet function for at least 15 days, after having given informed consent, were enrolled for the study. All subjects had fasted for 12 h before venipuncture and blood samples were drawn between 8:00 and 9:00 am. Platelet suspension was obtained as described earlier with a final platelet concentration of 2×10^8/mL. Platelets were incubated with apocynin (25, 50, and 100 μM), an inhibitor of NADPH oxidase, Vitamin C (25, 50, and 100 μM) and apocynin (25, 50, and 100 μM) plus Vitamin C (25, 50, and

100 μM). To keep platelets in anaerobic conditions, samples were purged with a gentle stream of pure nitrogen gas up to 20 min. Platelets were reoxygenated by re-exposure to atmospheric conditions. Platelets exposed to a gentle stream of room air were used as control.

Flow Cytometric Analysis of Platelet Reactive Oxidant Species (ROS) Formation and CD40L

To detect the platelet formation of ROS, the property of DCF-DA was used. This molecule rapidly diffuses across cell membranes and is then trapped within the cell via a deacetylation reaction. In the presence of hydrogen peroxide, this compound is oxidized to the highly fluorescent dichlorofluorescein (DCF). DCFH-DA was added to platelet suspension (final concentration: 40 μmol/L) immediately after the anoxia phase; 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. CD40L expression on platelet membrane was analyzed as earlier described.

Intra-assay coefficient of variation was 5%.

Statistical Analysis

Two-sided *t*-test was used to compare means. Results were confirmed by nonparametric tests as Mann-Whitney *U*-test. Pearson chi-square test was used to compare proportions. Friedman testing was performed for evaluating differences over time within the group. Differences between paired data were tested by Wilcoxon rank testing. The Spearman correlation coefficient was calculated to assess the correlation of absolute change from the baseline of sCD40L plasma levels (ΔsCD40L), 8-OHdG plasma levels (Δ8-OHdG), platelet CD40L expression (Δplatelet CD40L expression), and LVEF (ΔLVEF). Data are presented as mean (1 SD) or as median and interquartile range (IQR) (25th, 75th percentile). Statistical significance was defined at $P < 0.05$. Statistical analysis was performed with SPSS 13.0 software for Windows.

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 (1) difference for CD40L variation to be detected between the patients treated or not with Vitamin C $|\delta| \geq 1.5$, (2) standard deviations SD = 1 for the control group and 1.2 for the Vitamin C group, (3) type I error probability $\alpha = 0.05$ and power $1\text{-}\beta = 0.90$. This resulted in a minimum sample size of n = 10 for control and n = 12 for Vitamin C group, which are increased to n > 20.

Results

Characteristics of the Study Population

Table 1 shows baseline demographic and clinical characteristics of included patients. Angiographic and procedural data are shown in Table 2. The two groups assigned to ascorbic acid or placebo did not differ in terms of cardiovascular risk factors and pharmacological therapy. The implantation of single stent was successful in all patients with complete covering of the vessel lesion length and without visual residual stenosis within the stent. Furthermore, angiographic images obtained following stent deployment did not show signs of endothelial dissection in the areas proximal or distal to the stent struts. No side effects were observed during or after ascorbic acid or placebo infusion.

Baseline mean values of LVEF were similar in both Vitamin C and control groups (52.3 ± 4.3% vs. 53.7% ± 3.9%, P = ns).

Ascorbic acid assigned group showed, at the baseline, similar values of sCD40L (2.3 ± 1.4 vs. 2.3 ± 1.2 ng/mL, $P = 0.959$), 8-OHdG (3.7 ± 1.3 vs. 3.7 ± 1.1 ng/mL, $P = 0.373$), hs-CRP [Median (IQR):1.0 (0.71–1.90) vs. 1.25 (0.80–2.00) mg/L, $P = 0.451$) and TNF-α [42.5 (35.0–50.0) vs. 40.0 (40.0–50.0) pg/mL, $P = 0.735$) compared to placebo group. The platelet CD40L expression was similar the in two groups (4.2 ± 0.88 MF in ascorbic acid assigned group and 4.3 ± 0.78 MF in placebo group, $P = 0.0724$), the apparent trend in differences between

Table 1 Baseline clinical characteristics

	Placebo (n = 28)	*P*-value	Vitamin C (n = 28)
Age (years)	68 ± 9	>0.05	66 ± 8
Male, n (%)	23 (82)	>0.05	24 (86)
Familiarity, n (%)	17 (61)	>0.05	14 (50)
Hypertension, n (%)	20 (71)	>0.05	20 (71)
Dyslipidemia, n (%)	20 (71)	>0.05	19 (68)
Current smoker, n (%)	13 (46)	>0.05	11 (39)
Diabetes mellitus, n (%)	15 (54)	>0.05	18 (64)
Pharmacological therapy			
Oral hypoglycaemic, n (%)	12 (43)	>0.05	11 (39)
Insulin treatment, n (%)	2 (7)	>0.05	3 (10)
Statins, n (%)	19 (68)	>0.05	16 (57)
Nitrates, n (%)	13 (46)	>0.05	16 (57)
ACE-inhibitors, n (%)	15 (54)	>0.05	12 (43)
β-blockers, n (%)	9 (32)	>0.05	10 (36)
Ca-antagonists, n (%)	7 (25)	>0.05	6 (21)

Table 2 Angiographic characteristics and quantitative angiographic measurements of included subjects

		Placebo (n = 28)	*P*-value	Vitamin C (n = 28)
Target vessel	Left anterior descending artery, n (%)	18(64)	>0.05	15 (54)
	Left circumflex artery, n (%)	3 (11)	>0.05	6 (21)
	Right coronary artery, n (%)	7 (25)	>0.05	7 (25)
Procedural data	Diameter stenosis (%)	80.1 ± 9.7	>0.05	83.5 ± 11.5
	Stent diameter (mm)	3.0 ± 0.39	>0.05	3.1 ± 0.37
	Stent length (mm)	19.8 ± 7.1	>0.05	20.8 ± 7.5
	Balloon pressure (atm)	16.5 ± 1.4	>0.05	16.9 ± 1.1

the two groups is well under the coefficient of variation of the method.

There were no adverse events during hospitalization in both Vitamin C and placebo group.

Interventional Study

Serum cardiac-Troponin I and Left Ventricular Ejection Fraction

The median absolute increase after PCI in serum cTpI level was similar between controls and ascorbic acid group [Median (IQR): 0.027 (0.05–0.032) vs. 0.008 (0.02–0.013) ng/mL, respectively, $P = 0.0832$].

After the intervention, LVEF improved in both groups with a higher increase in Vitamin C treated patients (58.3 ± 2.9%) compared to the placebo one (54.1 ± 4.7%) ($P < 0.03$). Vitamin C-treated patients showed a greater improvement of LVEF after procedure compared to that observed in the control group ($P < 0.01$).

sCD40L Plasma Levels

Compared to baseline, no statistically significant changes of sCD40L was found after 60 (2.2 ± 1.1 ng/mL, $P = 0.269$) and 120 min (2.4 ± 1.0 ng/mL, $P = 0.738$) of Vitamin C infusion; conversely, a significant increase of sCD40L was observed after 60 (3.2 ± 1.5 ng/mL, $P = 0.0007$) and 120 min (3.4 ± 1.7 ng/mL, $P = 0.0003$) of placebo infusion (Figure 1, Panel A). Comparing sCD40L values at 60 and 120 min after balloon inflation, the ascorbic acid-treated group showed a significant lower value of sCD40L in respect to placebo group ($P = 0.0057$ and $P = 0.016$, respectively) (Figure 1, Panel A). Similar results were obtained in the subgroup of diabetic patients (n = 33; 60 min: $P = 0.01$; 120 min: $P = 0.06$).

Platelet CD40L Expression

Compared to baseline, platelet expression of CD40L did not significantly differ at 60 (3.8 ± 1.3 MF, $P = 0.077$) and 120 min (3.8 ± 1.1 MF, $P = 0.307$) after balloon inflation in Vitamin C treated group (Figure 1, Panel B) (Figure 2, Panel A). On the other hand, in the placebo group, at 60 (5.1 ± 1.3 MF, $P = 0.005$) and 120 min (5.4 ± 1.2 MF, $P = 0.0002$) after balloon inflation, platelet CD40L expression was higher than baseline (Figure 1, Panel B) (Figure 2, Panel B). A significant difference between groups was observed at 60 ($P = 0.0008$) and 120 ($P < 0.0001$) min after balloon inflation (Figure 1, Panel B). Similar results were obtained in the subgroup of diabetic patients (n = 33; 60 min: $P = 0.036$; 120 min: $P = 0.001$).

8-hydroxy-2′-deoxyguanosine, hs-CRP, and TNF-α

Compared to baseline, 8-OHdG plasma levels significantly decreased at 60 (2.6 ± 1.1, ng/mL, $P < 0.0001$) and 120 min (2.7 ± 0.87 ng/mL, $P = 0.0003$) after balloon inflation in Vitamin C treated group (Figure 1, Panel C). In contrast, the placebo group at 60 (4.2 ± 1.1 ng/mL, $P = 0.045$) and 120 min (4.6 ± 1.0 ng/mL, $P = 0.003$) after balloon inflation, showed 8-OHdG plasma levels higher than baseline (Figure 1, Panel C). A significant difference between groups was observed at 60 ($P < 0.0001$) and 120 min ($P < 0.0001$) after balloon inflation (Figure 1, Panel C).

Hs-CRP [Vitamin C: 1.0 (0.67–1.60) and 1.37 (1.0–2.0) mg/L, $P > 0.05$; Placebo: 1.30 (0.75–2.0) and 1.25 (0.95–2.10) mg/L, $P > 0.05$] and TNF-α [Vitamin C: 45.0 (40.0–60.0) and 43.5 (36.5–58.5) pg/mL, $P > 0.05$; Placebo: 40.0 (40.0–50.0) and 46.5 (40.0–58.0) pg/mL, $P > 0.05$] did not show any significant changes in both groups of treatment at 60 and 120 min after PCI.

Bivariate Analysis

Simple linear regression analysis showed an overall correlation between sCD40L plasma levels and platelet CD40L expression, sCD40L and 8-OHdG, and between 8-OHdG and CD40L platelet expression (Figure 1, Panels D–F).

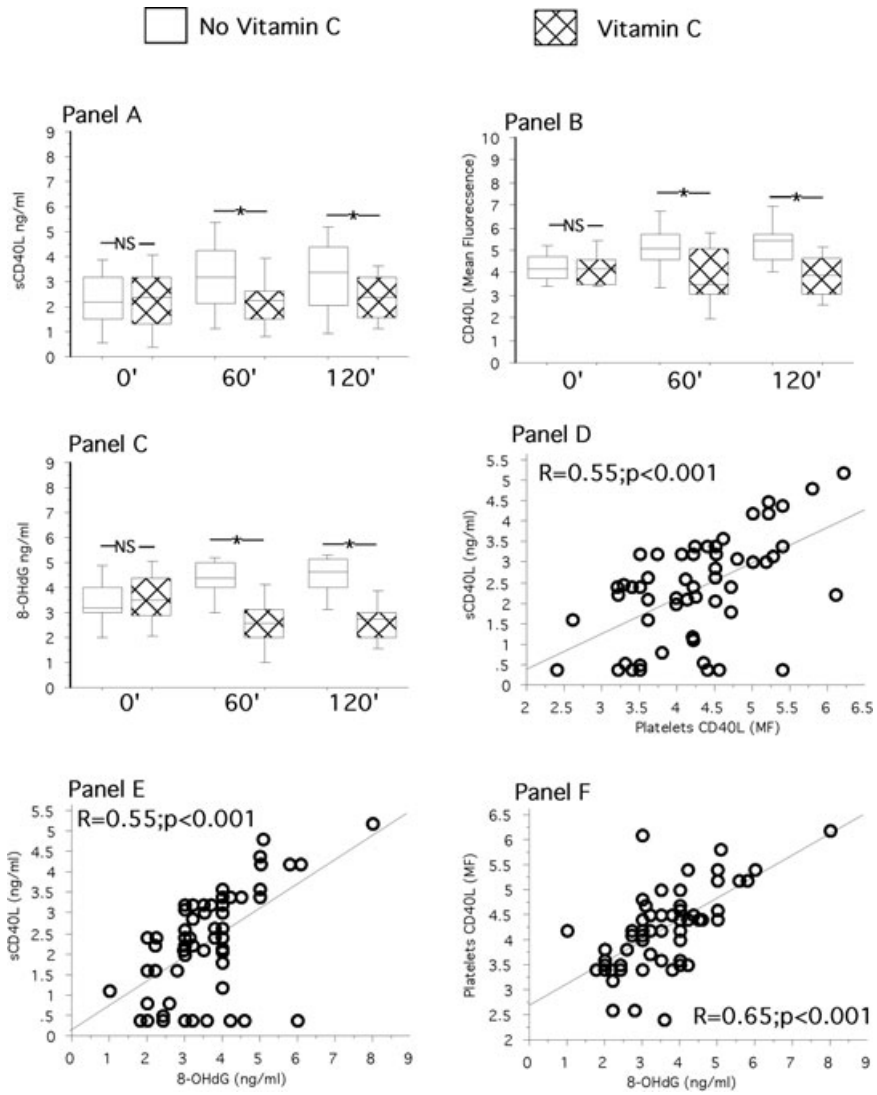

Figure 1 Plasma levels of sCD40L (Panel **A**), expression of platelet CD40L (Panel **B**), plasma levels of 8OHdG (Panel **C**) in patients treated with (black box) or without (white box) Vitamin C infusion. Simple linear correlation between platelet and soluble CD40L (Panel **D**), 8OHdG and both soluble and platelet CD40L (Panels **E** and **F**) before and after 10, 60, and 120 min after coronary stenting $^*P < 0.05$; data are expressed as mean ± SE.

These correlations have been observed at each study time (data not shown). Hs-CRP and TNF-α did not show any *statistically significant* correlations with sCD40L plasma levels, platelet CD40L expression and 8-OHdG serum levels at each study time (data not shown).

We further analyzed the correlation between absolute change from the baseline in plasma concentrations of sCD40L, 8-OhdG, and platelet CD40L expression. After 60 min of Vitamin C or Placebo infusion, absolute change from the baseline of plasma concentrations of sCD40L correlated positively with both Δ8-OHdG plasma levels (Spearman R = 0.44, $P = 0.0006$) and Δplatelet CD40L expression (Spearman R = 0.33, $P = 0.014$); thus, Δ8-OHdG plasma levels correlated positively with Δplatelet CD40L expression (Spearman R = 0.36, $P = 0.006$). Similar data was observed after 120 min of Vitamin C or Placebo administration. Δ8-OHdG plasma levels directly correlated with ΔsCD40L plasma levels (Spearman R = 0.40, $P = 0.002$) and Δplatelet CD40L expression (Spearman R = 0.48, $P = 0.0002$). ΔsCD40L plasma levels did not significantly correlate with Δplatelet CD40L expression (Spearman R = 0.22, $P = 0.102$) after 120 min of experimental drugs.

ΔsCD40L plasma levels, measured after 120 min of Vitamin C or Placebo administration, showed a statistically significant inverse correlation with ΔLVEF evaluated 72 h after coronary stenting (Spearman R = −0.35, $P < 0.02$).

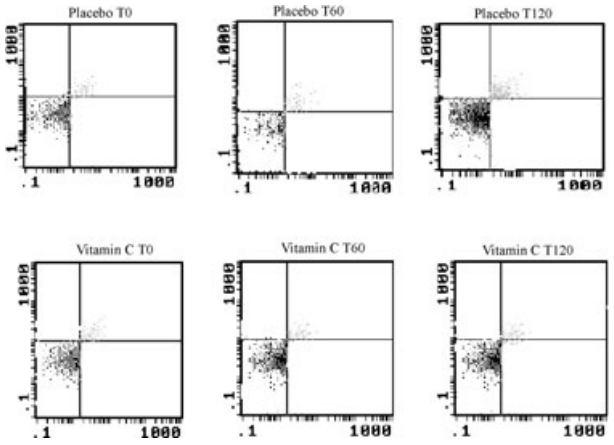

Figure 2 A representative flow cytometric analysis of CD40L from placebo group (panel A) or Vitamin C treated patients (panel B).

In Vitro Study

Flow Cytometric Analysis of Platelet ROS Formation and CD40L Expression

Platelets that underwent anoxia-re-oxygenation showed higher values of ROS compared to control platelets (time 0). The burst of platelet ROS depended on the time of anoxia exposure (5, 10, 20 min). Incubation with scalar doses, singularly added or together, of ascorbic acid (25, 50, and 100 μM) and apocynin (25, 50, and 100 μM) dose dependent and sinergically blunted anoxia-re-oxygenation induced platelet ROS formation (Figure 3).

Similarly to ROS formation, platelet expression of CD40L significantly increase in anoxic condition compared to control platelets (time 0). The CD40L platelet expression depended on the time of anoxia exposure (5, 10, 20 min). Treatment with scalar doses, singularly added or together, of ascorbic acid (25, 50, and 100 μM) and apocynin (25, 50, and 100 μM) dose dependent and sinergically inhibited anoxia-re-oxygenation induced platelet CD40L expression (Figure 4).

Discussion

The study provides evidence that oxidative stress is implicated in upregulating CD40L expression in patients undergoing coronary stenting.

Previous studies have shown that sCD40L increased after angioplasty [27] and suggested that this reflects platelet activation occurring after interventional procedure [5]. This change was usually observed 24 h after coronary angioplasty; therefore, it was unclear if sCD40L increase was an early or a late phenomenon.

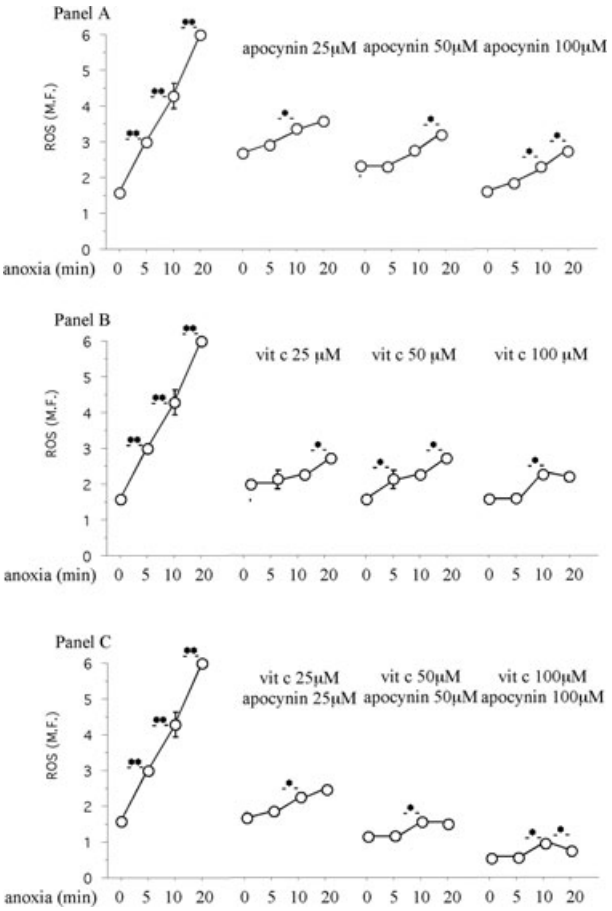

Figure 3 ROS production in platelets that underwent anoxia-re-oxygenation (0, 5, 10, and 20 min) pretreated or not with apocynin (25, 50, 100 μM), Vitamin C (25, 50, 100 μM) or a combination of the two. $^{**}P < 0.001$ versus platelets kept at room air; $^{*}P < 0.001$ versus untreated platelets exposed to a same anoxic time. Data are expressed as mean $\pm$ SE.

Our study shows that as early as 60 min after coronary stenting, sCD40L and platelet CD40L had a significant increase. Platelet CD40L and sCD40L were significantly correlated 60 min but not 120 min after coronary stenting; this might suggest that the late increase of sCD40L may not only be platelet-related but also involve other cellular sources of CD40L, even if to a minor extent.

The increase of sCD40L after coronary angioplasty has been consistently associated with deleterious outcomes [5,15]. This association has been interpreted as a consequence of the inflammatory and endothelial cell proliferation effect of CD40L [5]. Thus, knowledge of the mechanisms eliciting PCI-induced CD40L upregulation may be useful to eventually counteract its deleterious effects.

We, along with others, have previously shown that oxidative stress is implicated in CD40L upregulation with a mechanism involving the activation of NADPH

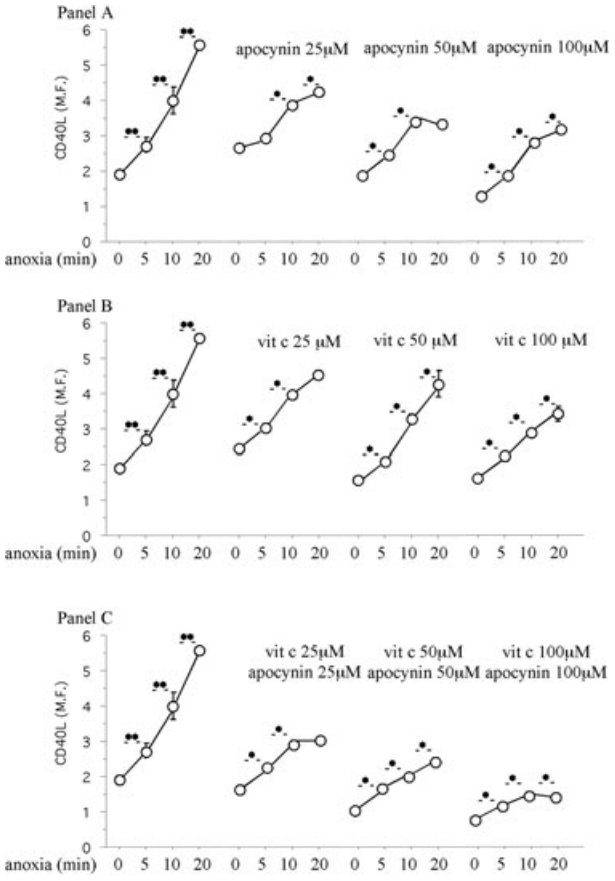

Figure 4 CD40L expression in platelets that underwent anoxia-re-oxygenation (0, 5, 10, and 20 min) pretreated or not with apocynin (25, 50, 100 μM), Vitamin C (25, 50, 100 μM) or a combination of the two. $^{**}P < 0.001$ versus platelets kept at room air; $^{*}P < 0.001$ versus untreated platelets exposed to a same anoxic time. Data are expressed as mean ± SE.

oxidase [28,29]. Therefore, in patients lacking this enzyme, platelet CD40L was down-regulated and normal platelets, incubated *in vitro* with apocynin, an inhibitor of NADPH oxidase, showed lower expression of CD40L [28]. Also, intravenous infusion of 1 g ascorbic acid resulted in a significant reduction of both platelet CD40L and sCD40L [24].

Using different markers of oxidative stress, previous studies showed that oxidative stress increased after PCI as a likely consequence of the burst of ROS occurring after ischemia-reperfusion [19–22]. Based on these data we planned a pilot study to see if ascorbic acid infusion was able to counteract the increase of CD40L occurring after PCI. We observed that such treatment was able to prevent both sCD40L and platelet CD40L expression occurring after coronary stenting with a mechanism that was likely dependent upon its antioxidant effect. Thus intravenous infusion of ascorbic acid was associated with a significant reduction of 8-OHdG plasma levels. Even if it has been underscored that ascorbic acid may exert antioxidant effect *in vivo* only at millimolar concentration [30], it is noteworthy that such concentration can be achieved *in vivo* when ascorbic acid is intravenously given [31]. So, it is plausible that the decrease of serum 8-OHdG might be attributable to its antioxidant effect.

Together, these data lead to hypothesize that the burst of ROS occurring after coronary stenting may trigger platelets CD40L upregulation. This hypothesis is consistent with the fact that sCD40L and platelet CD40L showed a significant correlation with 8-OHdG levels.

To further corroborate the role of ROS in upregulating platelet CD40L, the ischemia-reperfusion phenomenon occurring after coronary stenting was mimicked *in vitro* by exposing platelets to anoxia-re-oxygenation. This phenomenon has been proved to be associated with NADPH oxidase-dependent ROS formation [32,33]. Through this experiment we demonstrated that platelets CD40L upregulation occurs after platelet exposure to anoxia-re-oxygenation and that such effect was likely mediated by NADPH oxidase-generated platelet ROS. Thus, an inhibitor of NADPH oxidase as well as ascorbic acid, prevented both platelet ROS and CD40L upregulation elicited by anoxia-re-oxygenation in a dose dependent and sinergical manner. As NADPH oxidase is the most important cellular producer of superoxide anion [34] and ascorbic acid is an antioxidant able to scavenge it [24], it is conceivable that anoxia-re-oxygenation-induced platelet CD40L upregulation is mediated by a burst of superoxide anion.

The study has limitations and implications. Even if we postulate that NADPH oxidase- generating platelet ROS is responsible for enhanced oxidative stress and in turn platelet CD40L upregulation, our study did not provide evidence that this enzymatic pathway is actually activated after coronary stenting. Despite the fact that experimental study seems to support such hypothesis [35], further investigation in humans is necessary to explore the role of NADPH oxidase in the ROS formation occurring after coronary stenting.

The lack of correlation between hs-CRP and CD40L observed in the present study could be explained by a different pattern of platelet activation and inflammation in the early phase after coronary stenting. In fact CRP serum levels reach peak values at mean interval of 49 h after coronary stenting [36] while oxidative stress and CD40L have a much earlier increase.

We observed that LVEF, a good predictor of stent thrombosis [37], significantly and inversely correlated with plasma sCD40L after PCI and was moderately improved in Vitamin C treated group. Nevertheless, a longer follow-up and a larger study need to be planned to

confirm the clinical validity of ascorbic acid infusion in this setting.

As we used a standardized treatment including dual antiplatelet treatment with clopidogrel and aspirin, the enhanced level of sCD40L after stenting procedure suggests that such therapeutic approach is insufficient to counteract PCI-induced platelet CD40L release and that. ascorbic acid may represent a simple and cheap approach to counteract it.

In conclusion, we provide evidence that platelet CD40L upregulation occurring after coronary stenting could be oxidative stress-mediated and could be dependent upon NADPH oxidase activation. Interventional trials with ascorbic acid will be needed to evaluate if intravenous infusion of ascorbic acid could reduce vascular outcomes in patients undergoing coronary stenting.

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Author Contributions

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Serena Di Santo: laboratory experimental procedures.
Lorenzo Loffredo: statistical analysis.
Andrea Celestini: paper preparation, data elaboration.
Marco Proietti: clinical data collection and elaboration.
Priscilla Tovaglia: clinical data collection and elaboration.
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Conflict of Interest

None.

References

1. Katritsis DG, Meier B. Percutaneous coronary intervention for stable coronary artery disease. *J Am Coll Cardiol* 2008;**52**:889–893.
2. Cohen DJ, Bakhai A, Shi C, et al.; SIRIUS Investigators. Cost-effectiveness of sirolimus-eluting stents for treatment of complex coronary stenoses: Results from the Sirolimus-Eluting Balloon Expandable Stent in the treatment of patients with *de novo* native coronary artery lesions (SIRIUS) trial. *Circulation* 2004;**110**:508–514.
3. Wiviott SD, Braunwald E, McCabe CH, et al.; TRITON-TIMI 38 Investigators. Intensive oral antiplatelet therapy for reduction of ischaemic events including stent thrombosis in patients with acute coronary syndromes treated with percutaneous coronary intervention and stenting in the TRITON-TIMI 38 trial: A subanalysis of a randomised trial. *Lancet* 2008;**371**:1353–1363.
4. Eisenstein EL, Anstrom KJ, Kong DF, et al. Clopidogrel use and long-term clinical outcomes after drug-eluting stent implantation. *JAMA* 2007;**297**:159–168.
5. Cipollone F, Ferri C, Desideri G, et al. Preprocedural level of soluble CD40L is predictive of enhanced inflammatory response and restenosis after coronary angioplasty. *Circulation* 2003;**108**:2776–2782.
6. Noelle RJ, Ledbetter JA, Aruffo A. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. *Immunol Today* 1992;**13**:431–433.
7. Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;**391**:591–594.
8. Freedman JE. CD40-CD40L and platelet function: Beyond hemostasis. *Circ Res* 2003;**92**:944–946.
9. Sanguigni V, Ferro D, Pignatelli P, et al. CD40 ligand enhances monocyte tissue factor expression and thrombin generation via oxidative stress in patients with hypercholesterolemia. *J Am Coll Cardiol* 2005;**45**:35–42.
10. Santilli F, Davì G, Consoli A, et al. Thromboxane-dependent CD40 ligand release in type 2 diabetes mellitus. *Am Coll Cardiol* 2006;**47**:391–397.
11. Harding SA, Sarma J, Josephs DH, et al. Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. *Circulation* 2004;**109**:1926–1929.
12. Ferro D, Loffredo L, Polimeni L, et al. Soluble CD40 ligand predicts ischemic stroke and myocardial infarction in patients with nonvalvular atrial fibrillation. *Arterioscler Thromb Vasc Biol* 2007;**27**:2763–2768.
13. Varo N, de Lemos JA, Libby P, et al. Soluble CD40L: Risk prediction after acute coronary syndromes. *Circulation* 2003;**108**:1049–1052.
14. Heeschen C, Dimmeler S, Hamm CW, Van Den Brand MJ, Boersma E, Zeiher AM, Simoons ML; CAPTURE Study Investigators. Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 2003;**348**:1104–1111.
15. Yan JC, Ma GS, Zhu J, et al. The clinical implications of increased coexpression of CD40-CD40 ligand system and C-reactive protein in patients after percutaneous coronary intervention. *Clin Chim Acta* 2006;**374**:140–141.
16. Rizvi M, Pathak D, Freedman JE, Chakrabarti S. CD40–CD40 ligand interactions in oxidative stress, inflammation and vascular disease. *Trends Mol Med* 2008;**14**:530–538.
17. Pignatelli P, Sanguigni V, Lenti L, Loffredo L, Carnevale R, Sorge R, Violi F. Oxidative stress-mediated platelet

CD40 ligand upregulation in patients with hypercholesterolemia: Effect of atorvastatin. *J Thromb Haemost* 2007;**5**:1170–1178.
18. Schönbeck U, Gerdes N, Varo N, et al. Oxidized low-density lipoprotein augments and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors limit CD40 and CD40L expression in human vascular cells. *Circulation* 2002;**106**:2888–2893.
19. Inoue T, Kato T, Hikichi Y, et al. Stent-induced neutrophil activation is associated with an oxidative burst in the inflammatory process, leading to neointimal thickening. *Thromb Haemost* 2006;**95**:43–48.
20. Berg K, Wiseth R, Bjerve K, et al. Oxidative stress and myocardial damage during elective percutaneous coronary interventions and coronary angiography. A comparison of blood-borne isoprostane and troponin release. *Free Radic Res* 2004;**38**:517–525.
21. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): Short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation* 2004;**109**:3164–3170.
22. Iuliano L, Praticò D, Greco C, Mangieri E, Scibilia G, FitzGerald GA, Violi F. Angioplasty increases coronary sinus F2-isoprostane formation: Evidence for *in vivo* oxidative stress during PTCA. *J Am Coll Cardiol* 2001;**37**:76–80.
23. Schneider MP, Delles C, Schmidt BM, Oehmer S, Schwarz TK, Schmieder RE, John S. Superoxide scavenging effects of *N*-acetylcysteine and vitamin C in subjects with essential hypertension. *Am J Hypertens* 2005;**18**:1111–1117.
24. Pignatelli P, Sanguigni V, Paola SG, Lo Coco E, Lenti L, Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Radic Biol Med* 2005;**38**:1662–1666.
25. Smith SC Jr., Feldman TE, Hirshfeld JW Jr., et al.; American College of Cardiology/American Heart Association Task Force on Practice Guidelines; ACC/AHA/SCAI Writing Committee to Update the 2001 Guidelines for Percutaneous Coronary Intervention. ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/SCAI Writing Committee to Update the 2001 Guidelines for Percutaneous Coronary Intervention). *J Am Coll Cardiol* 2006;**47**:e1–e121.
26. Sanguigni V, Pignatelli P, Caccese D, et al. Increased superoxide anion production by platelets in hypercholesterolemic patients. *Thromb Haemost* 2002;**87**:796–801.
27. Wexberg P, Jordanova N, Strehblow C, et al. Time course of prothrombotic and proinflammatory substance release after intracoronary stent implantation. *Thromb Haemost* 2008;**99**:739–748.
28. Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, Violi F. gp91phox-dependent expression of platelet CD40 ligand. *Circulation* 2004;**110**:1326–1329.
29. Ha YJ, Lee JR. Role of TNF receptor-associated factor 3 in the CD40 signaling by production of reactive oxygen species through association with p40phox, a cytosolic subunit of nicotinamide adenine dinucleotide phosphate oxidase. *J Immunol* 2004;**171**:231–239.
30. Jackson TS, Xu A, Vita JA, Keaney JF Jr. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res* 1998;**83**:916–922.
31. Padayatty SJ, Sun H, Wang Y, et al. Vitamin C pharmacokinetics: Implications for oral and intravenous use. *Ann Intern Med* 2004;**140**:533–537.
32. Leo R, Praticò D, Iuliano L, et al. Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated. *Circulation* 1997;**95**:885–891.
33. Liu JQ, Zelko IN, Folz RJ. Reoxygenation-induced constriction in murine coronary arteries: The role of endothelial NADPH oxidase (gp91phox) and intracellular superoxide. *J Biol Chem* 2004;**279**:24493–24497.
34. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 2006;**8**:691–728.
35. Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T, Pagano PJ. Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery. *Circ Rec* 2003;**92**:637–643.
36. Ohlmann P, Jaquemin L, Morel O, et al. Prognostic value of C-reactive protein and cardiac Troponin I in primary percutaneous interventions for ST-elevation myocardial infarction. *Am Heart J* 2006;**152**:1161–1167.
37. Iakovou I, Schmidt T, Bonizzoni E, et al. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *JAMA* 2005;**293**:2126–2130.