High preprocedural non-HDL cholesterol is associated with enhanced oxidative stress and monocyte activation after coronary angioplasty: possible implications in restenosis

F Cipollone, M Fazia, A Iezzi, B Pini, F Costantini, D De Cesare, L Paloscia, G Materazzo, E D’Annunzio, T Bucciarelli, J Vecchiet, F Chiarelli, F Cuccurullo, A Mezzetti

Objective: To investigate whether enhanced oxidant stress in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) is associated with a higher concentration of non-high density lipoprotein (HDL) cholesterol at baseline, and whether this contributes to the inflammatory reaction and luminal renarrowing after PTCA.

Design: An ex vivo and in vitro study of 46 patients who underwent PTCA and who had repeat angiograms after six months. Blood samples were collected immediately before PTCA, and at 24 hours, 48 hours, and 15 days after.

Setting: Tertiary referral centre.

Subjects: 46 patients (30 male, 16 female; mean (SD) age, 62 (5) years) with stable or unstable angina who underwent elective PTCA.

Main outcome measures: Continuous variable luminal loss as defined by change in minimum lumen diameter during follow up, normalised for vessel size; lag phase of low density lipoprotein to in vitro oxidation; plasma fluorescent products of lipid peroxidation (FPLP); plasma vitamin C and E; interleukin (IL) 1β secretion from unstimulated monocytes; plasma C reactive protein (CRP).

Results: Restenosis occurred in 12 patients (26%). Oxidant stress after PTCA was greater (p < 0.0001 at 15 days) in the patients with restenosis and showed a significant correlation with the preprocedural concentration of non-HDL cholesterol (p < 0.001). Inflammatory reaction (as reflected by IL-1β) and late lumen loss were linearly correlated (p < 0.001) with lag phase and FPLP throughout the study, and inversely (p < 0.05) with vitamin C and E measured at two and 15 days after PTCA.

Conclusions: This study provides evidence for the critical role of cholesterol dependent oxidant stress in the pathophysiology of restenosis after PTCA. The findings raise the possibility that drugs capable of modulating oxidant stress might provide a novel form of adjuvant treatment in patients with hypercholesterolaemia undergoing PTCA.

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) is still a major clinical problem.1 Recent studies have suggested a promising role for probucol, a lipid lowering agent with powerful antioxidant properties, in the prevention of restenosis.2 However, the underlying mechanisms of its benefit remain controversial.

Raised total cholesterol and low high density lipoprotein (HDL) cholesterol concentrations in serum are associated with coronary artery disease,3 and with increased production of reactive oxygen species (ROS).4 Notably, immunisation with homologous oxidised low density lipoprotein (LDL) reduces neointimal formation after balloon injury in hypercholesterolaemic rabbits.5 Furthermore, the newly discovered ACAT inhibitor F-1394 prevented intimal hyperplasia induced by balloon injury in rabbits.6 Finally, LDL apheresis, when applied before and after PTCA, is effective in preventing restenosis of coronary artery lesions in patients with coronary artery disease and hypercholesterolaemia.7

The generation of ROS and oxidation of lipids have profound and wide ranging effects that can dramatically increase vascular toxicity and initiate a cascade of molecular and cellular responses. In particular, there is now substantial evidence that oxidative modification of LDL may play an important role in restenosis by inducing endothelial dysfunction and enhancing the recruitment, intimal retention, and phenotypic transformation of monocytes.8 In addition, Liao and colleagues have recently proposed mechanisms whereby lipid peroxidation products—such as those generated during the oxidative modification of LDL—may induce expression of inflammatory mediator genes by activating nuclear factor (NF) κB-like transcription factors in vascular cells.4 In fact, oxidised LDL (oxLDL) may induce expression of adhesion molecules and cytokines such as interleukin (IL) 1β in circulating monocytes and arterial wall cells.9 It is assumed that IL-1β is an important determinant of intimal hyperplasia, amplifying and sustaining the proliferative response to vessel injury.9 Furthermore, it has been reported that plasma from ischaemic and reperfused hearts stimulates the expression of IL-1β in leucocytes from healthy donors.10

In humans, the association between hypercholesterolaemia and oxidative modifications of LDL has been well documented.
using both in vitro and in vivo markers of lipid peroxidation. Moreover, treatment with probucol has been reported to be effective in reducing plasma cholesterol and preventing LDL oxidation, IL-1β secretion, and smooth muscle cell growth. However, although atherosclerosis has long been associated with oxidation of LDL and lipoprotein, the relative importance of oxidative stress in mediating the proinflammatory effects of hypercholesterolaemia in the setting of coronary angioplasty is still unclear.

We speculated that enhanced oxidant stress in patients with higher concentrations of atherogenic cholesterol could induce the generation of inflammatory cytokines, and that these compounds might in turn contribute to restenosis after PTCA. In the present study, therefore, we investigated whether treatment with probucol has been reported to be effective in reducing plasma cholesterol and preventing LDL oxidation, IL-1β secretion, and smooth muscle cell growth. However, although atherosclerosis has long been associated with oxidation of LDL and lipoprotein, the relative importance of oxidative stress in mediating the proinflammatory effects of hypercholesterolaemia in the setting of coronary angioplasty is still unclear.

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Table 1  Baseline characteristics of the study patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>No restenosis (n=34)</th>
<th>Restenosis (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62 (5)</td>
<td>62 (4.5)</td>
</tr>
<tr>
<td>Male/female</td>
<td>22/12</td>
<td>8/4</td>
</tr>
<tr>
<td>Stable angina/unstable angina</td>
<td>11/23</td>
<td>4/8</td>
</tr>
<tr>
<td>Total cholesterol [mmol/l]</td>
<td>5.85 (0.31)</td>
<td>7.10 (0.35)</td>
</tr>
<tr>
<td>HDL cholesterol [mmol/l]</td>
<td>1.32 (0.23)</td>
<td>1.14 (0.21)</td>
</tr>
<tr>
<td>Non-HDL cholesterol [mmol/l]</td>
<td>4.53 (0.26)</td>
<td>5.96 (0.41)</td>
</tr>
<tr>
<td>Triglycerides [mmol/l]</td>
<td>1.63 (0.49)</td>
<td>1.71 (0.59)</td>
</tr>
<tr>
<td>Patients with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Previous PTCA or CAGB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Family history of IHD</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Hypertension</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smokers</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are mean (SD) or n. *p < 0.05.
CAGB, coronary artery bypass grafting; HDL, high density lipoprotein; IHD, ischaemic heart disease; LCX, left anterior descending coronary artery; LAD, left anterior descending coronary artery; PTCA, percutaneous transluminal coronary angioplasty; RCA, right coronary artery.

METHODS

Patients

The study population consisted of 46 of 96 consecutive patients with stable or unstable angina pectoris who underwent elective PTCA on a single, non-occluded, stenosed coronary vessel. There were no differences between the patients with respect to any of the classic cardiovascular risk factors. Patients with factors that could influence the lipid profile (such as alcohol abuse, thyroid disease, kidney disease, diabetes, and so on) were excluded from the study. None of the participating subjects was taking vitamins, dietary supplements, or drugs with known antioxidant activity. All patients received aspirin (100 mg daily) for the entire study period. The baseline characteristics of the study subjects are given in table 1.

Informed consent was obtained from each subject. The study protocol was approved by the institutional ethics committee.

Angioplasty procedure and follow up evaluation

Balloon angioplasty was undertaken using standard techniques. All patients received an intracoronary bolus of glyceryl trinitrate (1 mg) before the procedure. Patients were discharged with aspirin and any other necessary drugs. They were specifically asked not to take additional vitamins and mineral supplements and they were instructed in the American Heart Association step 1 diet. After successful angioplasty, patients returned for clinical follow up after 15 days and at one, three, and six months. They were readmitted for follow up coronary angiography six months after the angioplasty.

Quantitative coronary angiography

The four coronary arteriograms (obtained before the procedure, immediately afterwards, 15 minutes afterwards, and at the six months follow up visit) were analysed by an experienced cardiologist using the Philips H 4000 quantitative coronary analysis system (Philips Co, Eindhoven, Netherlands). Measurements were made in a single projection showing the most severe stenosis. Whenever possible, the same projection was used in all four arteriograms to allow more accurate comparisons. The variation among repeated measurements of the percentage of stenosis was 7% when frames recorded one to six months apart were analysed.

Definitions

Restenosis in a previously successfully dilated lesion was defined as recurrent lumen diameter stenosis of more than 50% at follow up, as determined by quantitative coronary angiography.

The continuous variable luminal loss was defined as the change in minimum lumen diameter (MLD) during follow up normalised for vessel size, according to the following equation:

relative loss = [(postintervention MLD − follow up MLD)/ vessel size] × 100%,

and reflects the degree of luminal renarrowing. Vessel size was the value of the reference diameter function at the minimal position of the obstruction.

Blood collection and biochemical measurements

Blood samples were collected from each patient immediately before PTCA and 24 hours, 48 hours, and 15 days afterwards. Blood was drawn into dark pyrogen-free test tubes containing EDTA (2.7 mM) as an anticoagulant and separated within one hour after sampling (2000 g for 20 minutes at 4°C). Immediately after separation, the plasma vitamin C concentration was measured spectrophotometrically at 595 nm, as described previously. In our laboratory, the intra-assay coefficient of variation for this method was 6.5%. In addition, total cholesterol concentration was determined by an enzymatic method, and HDL cholesterol was measured after phosphotungstic acid/MgCl2 precipitation of fresh plasma. Finally, aliquots of the plasma in EDTA were promptly stored at −80°C until LDL isolation and oxidisability analysis. Previous studies have shown that plasma storage and freezing/thawing does not affect LDL isolation or its major chemical characteristics.

The LDL fraction was isolated by single vertical spin ultracentrifugation using a discontinuous NaCl/KBr density gradient, as previously reported. After dialysis against phosphate buffered saline (PBS), pH 7.4, at 4°C, LDL protein and cholesterol concentrations were determined by established

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methods. LDL oxidation (0.2 mg LDL cholesterol per millilitre) was triggered by the addition of 5 µM CuSO₄ in PBS, pH 7.4, at 37°C. The lag phase preceding the formation of conjugated dienes and the LDL oxidisability as reflected by fluorescent products of lipid peroxidation (FPLP) was calculated as previously described.

Finally, plasma vitamin E content was determined by high performance liquid chromatography.

IL-1β release
The isolation and culture of peripheral blood monocytes from the study patients was done by an established method. The capacity of monocytes to produce IL-1β in vitro was determined as previously described by Pietersma and colleagues. Concentrations of IL-1β in supernatants were determined in triplicate by an enzyme immunoassay (Quantikine, R&D, Minneapolis, Minnesota, USA). The intra-assay and interassay coefficients of variation were less than 7%.

C reactive protein assay
C reactive protein (CRP) was assayed by an automated monoclonal antibody solid phase sandwich type enzyme immunoassay on an Abbott Imx instrument (Abbott Laboratories, Chicago, Illinois, USA), as previously described by Buffon and colleagues.

Statistical analysis
For the clinical data, variables were compared using the χ² test. An analysis of variance for repeated measures followed by a multiple comparison test (Sheffe test) was undertaken to test the changes in biochemical variables measured over time. Differences in biochemical variables between the restenotic and non-restenotic groups at each collection time were analysed by Student’s unpaired t test. The correlation between biochemical variables was assessed by the Spearman rank correlation test. The strength of the association of late lumen loss with each of the potential biological risk factors described in the previous sections was assessed by linear regression analysis.

Data are expressed as proportions or as mean (SD). Significance was assumed at a probability value of p < 0.05. All calculations were made using the SPSS 10.0.5 computer program.

RESULTS
Clinical course
No patient developed total acute obstruction at the angioplasty site immediately after the procedure or underwent urgent bypass surgery. None developed myocardial infarction and there were no sudden deaths during the study. There were no bleeding complications requiring blood transfusion. Restenosis occurred in 12 of the 46 patients (26%).

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Comparison of oxidant status between patients with or without restenosis

The susceptibility of LDL to in vitro oxidation was significantly increased after PTCA. The lag phase did not differ at baseline between the restenotic and non-restenotic groups (mean (SD), 59 (3) v 61 (4) minutes), but was significantly shorter (p < 0.0001) in the patients with restenosis at one day (43 (2) v 50 (4)), two days (34 (2) v 46 (5)), and 15 days (37 (7) v 54 (4)) after PTCA (fig 1). Likewise, plasma FPLP concentrations were significantly raised after the procedure (fig 1). There was no difference between the two groups before PTCA (17 (6) v 16 (2) URF (units of relative fluorescence)/ml); however, in the samples collected after the procedure there was a relatively stable increment in LDL oxidisability in the patients with restenosis and less consistent changes in those without restenosis. Overall, FPLP generation was significantly greater (p < 0.0001) in the patients with restenosis during the first (29 (1) v 20 (2)), second (32 (2) v 21 (2)), and 15th day (34 (4) v 18 (2)) of the study (fig 1).

The time courses of the plasma antioxidant activities measured in the study are shown in fig 2. Antioxidant activity as reflected by vitamins C and E was significantly reduced after PTCA. Vitamin C did not differ at baseline (34 (4) v 36 (4) µmol/l), but was significantly lower in the patients with restenosis during the first (24 (4) v 28 (5) µmol/l; p < 0.02), second (21 (4) v 27 (2) µmol/l; p = 0.002), and 15th day (24 (5) v 36 (4) µmol/l; p < 0.0001) after PTCA. Similarly, vitamin E concentration was comparable in the two groups before PTCA (28 (4) v 30 (5) µmol/l), but declined significantly in the restenotic group at one day (24 (3) v 28 (4) µmol/l; p = 0.008), two days (21 (3) v 26 (4) µmol/l; p = 0.002), and 15 days (19 (3) v 33 (3) µmol/l; p < 0.0001) after the procedure.

Relation between non-HDL cholesterol and oxidant stress

To analyse whether preprocedural concentration of atherogenic cholesterol can influence oxidative response after PTCA, we studied the correlation between non-HDL cholesterol measured at baseline and oxidant stress measured after the procedure. In the samples collected one, two, and 15 days after PTCA, we found correlations between the concentration of preprocedural non-HDL cholesterol and the following variables: lag phase (ρ = −0.574, p < 0.0001; ρ = −0.471, p = 0.001; and ρ = −0.622, p < 0.0001, respectively); FPLP (ρ = 0.460, p = 0.001; ρ = 0.488, p = 0.001; and ρ = 0.481, p = 0.001); vitamin C (ρ = −0.247, NS; ρ = −0.327, p < 0.003; and ρ = −0.584, p < 0.0001); and vitamin E (ρ = −0.252, NS; ρ = −0.427, p = 0.003; and ρ = −0.681, p < 0.0001).

Variables predictive of late lumen loss

To investigate whether redox state could contribute to luminal renarrowing after PTCA, we assessed the association between oxidative burden determined at one, two, and 15 days after PTCA and the degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment.

Figure 3 Scatterplots showing association between lag time of in vitro LDL oxidation, plasma fluorescent products of lipid peroxidation (FPLP), plasma vitamin C, and plasma vitamin E measured in patients with and without restenosis (box) 15 days after coronary angioplasty and the degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment.
between the two groups before PTCA (1286 (283) pg/10^6 monocytes). There was no notable difference particularly enhanced after PTCA in the patients with restenosis. In the samples collected one, two, and 15 days after coronary angioplasty (PTCA), the degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment (upper panel), and with preprocedural non-HDL cholesterol (lower panel) in the 12 patients with restenosis.

Relation between oxidant stress and IL-1β production
Finally, to investigate whether the cholesterol dependent redox state could influence restenosis by inducing an inflammatory reaction after PTCA, we analysed the correlation between non-HDL cholesterol, oxidant stress, and IL-1β production in the group of patients who developed late restenosis. In the samples collected one, two, and 15 days after PTCA, we found a significant association between IL-1β and the following variables: non-HDL cholesterol (p = 0.641, p < 0.01; p = 0.62, p < 0.03; and p = 0.734, p = 0.007, fig 5, respectively); lag phase (p = −0.741, p < 0.005; p = −0.565, p < 0.01; and p = −0.762, p = 0.004); FPLP (p = 0.714, p < 0.01; p = −0.59, p < 0.05; and p = 0.678, p < 0.03); vitamin C (p = −0.367, p < 0.05; p = −0.459, p < 0.05; and p = −0.759, p = 0.004); and vitamin E (p = −0.393, p < 0.03; p = −0.462, p < 0.03; and p = −0.727, p = 0.007) (fig 6).

DISCUSSION
In this study we tested the hypothesis that a component of the restenosis process following coronary angioplasty is dependent on the enhanced and long lasting oxidant stress that is related to baseline non-HDL cholesterol concentration, and would have the capacity to regulate inflammatory reactions and vascular smooth muscle cell proliferation after reperfusion.

Although Nunes and colleagues demonstrated persistently increased O_2^- generation in injured vessels after experimental PTCA in animals, studies in humans have shown only transient increases in LDL oxidisability after PTCA. In fact,
these studies have analysed only the period (a matter of minutes) immediately following myocardial reperfusion, and they have not compared oxidant status in subsets of patients with different outcomes after PTCA. Thus an original finding of the present study was that patients undergoing PTCA experienced a persistent increase in systemic oxidative stress, and this was significantly more obvious and prolonged in those with restenosis. Concurrent drug treatments are unlikely to have contributed to such different levels of lipid peroxidation as the treatment was similar in the two groups. Oxidant stress increased significantly in both groups 24 hours after PTCA; however, while it returned to baseline at 15 days in the patients without restenosis, in the patients with restenosis the changes persisted throughout the study. Thus the differences in oxidative burden between the two groups became apparent 24 hours after PTCA and remained significant until the final day of the study.

Our second finding was that enhanced oxidant stress after PTCA was related to the preprocedural concentration of non-HDL cholesterol, as suggested by the significant correlation between the two. Because oxidant stress has biological effects on vascular and monocyte function, enhanced production of oxidant compounds might mediate some of the effects of hypercholesterolaemia on important determinants of vascular occlusion, as suggested by the striking correlation between redox state and late lumen loss. Thus the ability of cholesterol dependent oxidant stress to induce and amplify monocyte activation may be relevant in settings where inflammation and enhanced lipid peroxidation coincide, such as PTCA.

Our final original finding was that the activation status of monocytes after PTCA—as reflected by spontaneous IL-1β release—was persistently higher in the group of patients who developed restenosis. That an increased oxidative burden after PTCA might have an effect on cytokine biosynthesis in influencing vascular healing was also suggested by the significant correlation between the circulating levels of indices of oxidative burden and the rate of IL-1β biosynthesis.

At least two alternative explanations could be considered for the mechanisms underlying the more impressive oxidant stress and monocyte activation following PTCA in the patients with restenosis. First, the diversity may simply have reflected differences in the degree of ischaemia/reperfusion during PTCA. However, this seems unlikely for the following reasons: all the procedures were done by the same experienced cardiologist according to standard techniques; the two groups had similar clinical and angiographic profiles at baseline; there were no significant differences in the total period of vessel occlusion during the procedure (table 1); and the differences in oxidant status between the two groups remained significant in the samples collected 15 days after PTCA. Alternatively, our data are consistent with the hypothesis that the high interindividual variability in the oxidative burden after revascularisation—which was critically related to preprocedural concentrations of non-HDL cholesterol—might, by governing early inflammatory events, contribute at least in part to the differences observed in the processes of healing, remodelling, and late lumen loss after PTCA in the presence of similar angiographic profiles.

In this study, a pronounced and long lasting systemic oxidant burden in the patients with restenosis was always characterised by a concomitant increase in oxidants and a decrease in antioxidant substances. We speculate that the reduction in vitamins C and E after PTCA in this group of patients may have reflected consumption resulting from...
interference of these vitamins with the pathways responsible for superoxide anion (O$_2^-$) production or perhydroxy radical formation.

The striking effect of PTCA in increasing lipid peroxidation raises the question of the origin of this oxidant burden. Macrophages and smooth muscle cells, the major types of cell left in the atherosclerotic lesion after PTCA, can both elaborate active oxygen species. Furthermore, granulocytes can also produce large amounts of O$_2^-$ and other oxidants. Interestingly, it has been reported that inflammatory cells may accumulate after PTCA at sites of acute tissue trauma and thrombosis, not only in the injured intima but also in the adventitial layer surrounding the artery, which is increasingly recognised as a site of scarring and luminal constriction in restenotic lesions.

There is increasing evidence to suggest that oxygen species might contribute to restenosis, including smooth muscle cell migration, replication, and accumulation and remodelling of the extracellular matrix. In particular, many vascular biologists now ascribe a central role to augmented transcription of several atherosclerosis related genes by the oxidant sensitive regulatory pathway involving NF-κB.

Exposure to oxidant stress activates the NF-κB regulatory complex in vascular cells and triggers the transcription of genes that encode certain leucocyte adhesion molecules, chemoattractant cytokines, and enzymes that can influence extracellular matrix metabolism. Moreover, it has been proposed that the early acute cytokine generation in macrophages, which are a major source of IL-1β, could evoke a secondary cytokine and growth factor response from other types of cell in the lesion, including smooth muscle cells and endothelial cells. This would establish a positive, self stimulatory autocrine and paracrine feedback loop, amplifying and sustaining the proliferative response. The positive results of probucol (a lipid lowering drug with powerful antioxidant properties) in slowing down the proliferative response. The positive results of probucol (a lipid lowering drug with powerful antioxidant properties) in slowing down the proliferative response of porcine coronary arteries.

We believe that our findings have implications for both a fundamental standpoint because they demonstrate the critical role of cholesterol dependent oxidant stress in the pathophysiology of restenosis after PTCA. From a practical standpoint, the findings raise the possibility that drugs capable of modulating oxidant status might provide a novel form of adjuvant treatment in patients with hypercholesterolemia who undergo PTCA.

**References**


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