Enhanced Levels of Oxidized Low-Density Lipoprotein Prime Monocytes to Cytokine Overproduction via Upregulation of CD14 and Toll-Like Receptor 4 in Unstable Angina


Objectives—The purpose of this study was to establish whether oxidized low-density lipoprotein (oxLDL) contributes to cytokine overproduction via upregulation of CD14 and toll-like receptor-4 (TLR-4) expression on circulating monocytes of unstable angina (UA) patients.

Methods and Results—Expression of CD14 and TLR-4 on circulating monocytes, and the concentration of plasma oxLDL, (interleukin [IL]-6, IL-1 beta, IL-8, tumor necrosis factor (TNF)-alpha, monocyte chemoattractant protein-1 (MCP-1) were measured in 27 control (C) subjects, 29 patients with stable angina (SA), and 27 with UA. CD14 and TLR-4 expression on monocytes and circulating IL-6, IL-1 beta, and oxLDL were higher in UA than in SA and C subjects (P<0.001). In in vitro experiments, oxLDL increased CD14 and TLR-4 expression (P<0.001) in control monocytes as well as IL-6, IL-1 beta, and at a lower extent TNF-alpha and MCP-1 levels in the supernatant (P from ≤0.05 to <0.001). The preincubation of sera derived from UA patients but with control monocytes also induced a significant increase of CD14 and TLR-4 expression (P<0.001) and of IL-6 and IL-1 beta production (P<0.001) in the supernatant.

Conclusions—In UA patients oxLDL may contribute to monocyte overproduction of some cytokines by upregulating CD14 and TLR-4 expression. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: oxidized LDL, IL-6, IL-1 beta, CD14, toll-like receptors, monocytes, unstable angina
This study was aimed to evaluate whether in UA patients oxLDL contributes to monocyctic overproduction of cytokines via upregulation of TLR-4 and CD14.

### Methods

#### Study Population

The study was approved by the hospital ethical committee and informed consent was obtained from all the patients before their enrolment.

Major requirements for the enrolment in all the groups were: absence of infectious or acute/chronic inflammatory diseases, known malignancy, absence of acute/chronic renal failure, or hepatic failure.

Three groups of patients were studied: control (C) subjects (patients without clinical history of CAD, who underwent coronary angiography for a presurgical evaluation, because affected by valvular or congenital heart disease), patients with stable angina (SA), and patients with UA. The subjects were enrolled on the basis of previously established criteria.

For both SA and UA patients exclusion criteria were: prior coronary artery bypass grafting (CABG), recent (<6 months) myocardial infarction (MI), recent (<6 months) percutaneous coronary intervention (PCI), congestive heart failure (CHF), coronary tree free of significant coronary artery disease (CAD: defined as at least one stenosis with minimal luminal diameter >70% of the arterial lumen by visual estimate) detected at coronary angiography.

The following data were obtained from all the patients: age, sex, presence of CAD risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, family history of CAD), use of medications, previous MI, previous revascularization (PCI or CABG).

#### Blood Samples

Venous blood samples were obtained from SA patients and C subjects the morning after the day of admission together with the routine laboratory tests. In UA patients samples were drawn within 24 hours from the index event (the last episode of anginal pain or admission if angina occurred previously). Blood was collected from each patient and drawn into pyrogen-free blood collection tubes (patients without clinical history of CAD, who underwent coronary angiography for a presurgical evaluation, because affected by valvular or congenital heart disease), patients with stable angina (SA), and patients with UA. The subjects were enrolled on the basis of previously established criteria.

For both SA and UA patients exclusion criteria were: prior coronary artery bypass grafting (CABG), recent (<6 months) myocardial infarction (MI), recent (<6 months) percutaneous coronary intervention (PCI), congestive heart failure (CHF), coronary tree free of significant coronary artery disease (CAD: defined as at least one stenosis with minimal luminal diameter >70% of the arterial lumen by visual estimate) detected at coronary angiography.

The following data were obtained from all the patients: age, sex, presence of CAD risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, family history of CAD), use of medications, previous MI, previous revascularization (PCI or CABG).

#### Peripheral Blood Mononuclear Cells and Monocyte Isolation

Peripheral blood mononuclear cells (PBMCs) were separated as previously described. A detailed description of the method is available in the supplement materials (available online at http://atvb.ahajournals.org).

#### Stimulation of Whole Blood With LPS and Measurement of IL-6 and IL-1 Beta

Aliquots of 1-mL of heparinized whole blood (with or without LPS) were placed in sterile 1.5-mL centrifuge tubes and placed on a rotator and incubated under sterile conditions at 37°C in an atmosphere containing 5% CO₂. The samples were treated with 1 ng/mL of LPS (Sigma Chemical) for 4 hours. The supernatant was then removed and stored at −80°C for the measurement of IL-6 and IL-1 beta.

### Stimulation of Monocytes With LPS and Measurement of IL-6 and IL-1 Beta

Freshly isolated monocytes (5×10⁶) were incubated for 4 hours at 37°C in RPMI-1640 supplemented with 8% fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 mmol/L L-glutamine with or without LPS (1 ng/mL). Then the culture supernatant was removed and stored at −80°C for the measurement of IL-6 and IL-1 beta.

#### Flow Cytometry and Western Blotting Analysis

CD14 and TLR-4 protein expression was analyzed in blood samples and in pools of separated monocytes by flow cytometry and Western blotting analysis. A detailed description of the methods is available in the supplement materials.

#### Circulating Plasma oxLDL Measurement

Circulating plasma oxLDL levels were measured with the enzyme-linked immunosorbent assay Merckodia Oxidized LDL ELISA (Merckodia AB) as previously described. Cu²⁺-modified LDL ranging from 50 to 500 ng/mL was used as a standard solution.

#### LDL Isolation and Oxidation

LDL and Cu²⁺-modified LDL were prepared as reported.

#### Real-Time Quantitative RT-PCR Analysis and Protein Expression of CD14 and TLR-4 in Separated Monocytes

A detailed description of the method is available in the supplement materials.

#### oxLDL-Dependent Expression of CD14/TLR-4 in Monocytes and Production of IL-6, IL-1 Beta, TNF-α, and MCP-1

Purified monocytes (3×10⁶/mL, 200 μL/well) from healthy donors were cultured in RPMI 1640 with L-glutamine (GIBCO) for 20 hours at 37°C with increasing amounts of oxLDL (from 20 to 40 μg/mL medium), CD14 and TLR-4 expression and IL-6, IL-1 beta, TNF-α, and MCP-1 concentration in the supernatant were measured as described. In some experiments, blocking anti-CD14 monoclonal antibody (mAb; 20 μg/mL) or control mouse IgG (50 μg/mL) were also added.

#### Serum-Dependent Expression of CD14/TLR-4 in Monocytes and Production of IL-6, IL-1 Beta, TNF-α, and MCP-1

Purified monocytes (3×10⁶/mL, 200 μL/well) from healthy volunteers were cultured in RPMI 1640 with L-glutamine (GIBCO) for 20 hours at 37°C with 40% serum from: (1) 10 UA patients with the lowest oxLDL levels, (2) 10 UA patients with the highest oxLDL levels, (3) 10 C subjects with the lowest oxLDL levels, and (4) 10 C subjects with the highest oxLDL levels. For processing of serum, 80 μL of serum from each patient or C subject was added to monocyte culture immediately after thawing, at the start of the culture period. As a further control, monocytes were also incubated with the corresponding UA lipoprotein-deprived serum (LPDS) in which all the lipoproteins were taken away by ultracentrifugation at 80°C for 80 hours at 37°C in RPMI-1640 supplemented with 8% fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 mmol/L L-glutamine with or without LPS (1 ng/mL). Then the culture supernatant was removed and stored at −80°C for the measurement of IL-6 and IL-1 beta.

#### Endotoxin contamination of cell cultures involving the use of oxLDL, serum, or LPDS was routinely excluded with the chromogenic Limulus amebocyte lysate assay (Sigma).

#### Statistical Analysis

A detailed description of the statistical methods is available in the supplement materials.
Results

Baseline Characteristics of the Patients

Along a period of 14 months, 102 patients were enrolled in the study; of these only 82 (27 C, 29 with SA, and 27 with UA) fully satisfied the enrolling criteria. Baseline clinical characteristics of the patients are described in supplemental Table I.

Laboratory Data

Data on total, LDL, HDL cholesterol, triglycerides, glucose plasma levels, and on WBC number and CRP are shown in Table I. Total WBC number and CRP were higher in UA patients than in SA or C patients (P < 0.001). Data on IL-6, IL-1 beta, TNF-α, IL-8, and MCP-1 and on the concentrations of IL-6 and IL-1 beta stimulated in blood and in separated monocytes with LPS are indicated in the Table. There were no significant differences in TNF-α, IL-8, and MCP-1 plasma levels; both basal and stimulated IL-6 and IL-1 beta levels were higher in UA patients than in SA or C patients (P < 0.001).

CD14 and TLR-4 Expression on Circulating Monocytes

Figure 1A shows the mean expression of CD14 and TLR-4 in peripheral blood monocytes of the 3 groups of patients as measured by flow cytometry. CD14 and TLR-4 were higher in UA patients than in SA and in C subjects (P < 0.001). A representative histogram of the flow cytometry analysis is presented in supplemental Figures I and II. Data on CD14 and TLR-4 were confirmed by Western blotting analysis on separated monocytes (Figure 1B).

Circulating Plasma oxLDL

oxLDL differed significantly among groups (Figure 2A). In particular, post-hoc tests revealed that oxLDL levels were higher in UA than in SA and C subjects and in SA than in C subjects (P from <0.001 to <0.01).

In the whole population of subjects studied, a statistically significant nonlinear sigmoidal regression (r² = 0.983; P < 0.001) was detected between plasma oxLDL and TLR-4 (Figure 2B). oxLDL levels also correlated linearly with CD14 (r = 0.81, P < 0.001; data not shown).

oxLDL-Dependent Expression of CD14 and TLR-4 and Cytokine Production in Monocytes Derived From Healthy Volunteers

Different amounts of Cu²⁺-oxLDL but not of native-LDL (n-LDL) dose-dependently increased cell mRNA and protein expression of CD14 and TLR-4 as evaluated by flow cytometry and Western analysis (data on n-LDL not shown) in monocytes derived from healthy volunteers (P < 0.001; supplemental Figure IIA through IIC).

Similarly increasing amounts of oxLDL but not of n-LDL dose-dependently increased the production of IL-6, IL-1 beta, TNF-α and MCP-1 (P < 0.001; Figure IVA through IVD). The effect of oxLDL on TNF-α and MCP-1 was much lower than on IL-6 and IL-1 beta (P < 0.001 for any amount of oxLDL). On average with the highest dose of oxLDL (40 μg/mL), IL-6 and IL-1 beta increased respectively 23.3 ± 3.1 and 10.2 ± 1.8 times whereas TNF-α and MCP-1 respectively 2.6 ± 0.31 and 3.2 ± 0.41 times when compared with control. The presence of anti-CD14 blocking mAb significantly reduced the oxLDL-dependent increase of IL-6, IL-1 beta, TNF-α, and MCP-1 (P from <0.01 to <0.001; supplemental Figure IVA through IVD).

Serum-Dependent Expression of CD14 and TLR-4 and Cytokine Production in Monocytes

Monocytes from healthy volunteers were evaluated for CD14 and TLR-4 expression after they were cultured for 20 hours in medium supplemented with either 40% serum from UA patients with the highest (n = 10) and lowest (n = 10) oxLDL plasma concentrations (respectively 36.4 ± 3.25 and 26.4 ± 3.21 μg/mL) or 40% serum from C subjects with the highest (n = 10) and lowest (n = 10) oxLDL levels (respectively 17.7 ± 2.7 and 9.73 ± 3.1 μg/mL). The incubation of monocytes with the sera derived from UA patients with the highest and lowest oxLDL levels induced a significant increase in the expression of CD14 and TLR-4 compared with the corresponding sera derived from the C subjects (Figure 3A and 3B as evaluated by flow cytometry and Western analysis respectively; P < 0.001). The incubation of monocytes with the LPDS of UA patients with the highest oxLDL levels (oxLDL undetectable) induced a reduced increase in CD14 and TLR-4 when compared with the corresponding sera in toto (Figure 3A and 3B; P < 0.001). The

Data on Cytokines and Chemokines in the Three Groups of Patients

<table>
<thead>
<tr>
<th></th>
<th>C (n = 27)</th>
<th>SA (n = 29)</th>
<th>UA (n = 26)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6, pg/ml</td>
<td>0.72 (0.2–12)</td>
<td>1.18 (0.82–4.44)</td>
<td>7.22 (4.21–31.1)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-1 beta, pg/ml</td>
<td>0.14 (0.04–0.25)</td>
<td>0.21 (0.12–0.42)</td>
<td>0.62 (0.22–1.11)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-alpha, pg/ml</td>
<td>4.97 (2.61–12.41)</td>
<td>4.11 (1.96–10.52)</td>
<td>4.19 (2.90–10.21)</td>
<td>NS</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>141.2 (82.3–181.4)</td>
<td>153.3 (96.6–204.6)</td>
<td>145.8 (102.3–202.7)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-8, pg/ml</td>
<td>15.88 (2.44–40.58)</td>
<td>18.42 (7.32–48.53)</td>
<td>17.92 (3.47–39.62)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6 response to LPS in blood, pg/ml</td>
<td>634 (186–1430)</td>
<td>938 (189–1942)</td>
<td>3457 (1510–8609)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 response to LPS in monocytes, pg/ml</td>
<td>447 (126–1094)</td>
<td>655 (189–1751)</td>
<td>2318 (998–4601)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-1 beta response to LPS in blood, pg/ml</td>
<td>659 (82–1211)</td>
<td>905 (156–1600)</td>
<td>2103 (609–4311)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-1 beta response to LPS in monocytes, pg/ml</td>
<td>190 (80–742)</td>
<td>382 (120–1091)</td>
<td>1612 (620–1950)*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median (ranges).

*UA vs SA and C. C indicates controls; SA, stable angina; UA, unstable angina; LPS, lipopolysaccharide.
concentrations of IL-6, IL-1 beta, TNF-α, and MCP-1 were significantly higher in the monocytes incubated with the sera of UA patients with highest and lowest oxLDL levels than in those incubated with the corresponding sera of C subjects ($P<0.001$; Figures 4A, 4B, 5A, and 5B). The effect on TNF-α and MCP-1, however, was negligible, and post-hoc test revealed that the changes of IL-6 and IL-1 beta were much higher than those of TNF-α and MCP-1 ($P<0.001$). The presence of anti-CD14 blocking mAb significantly reduced the sera-dependent increase of IL-6 and IL-1 beta ($P<0.001$), but not of TNF-α and MCP-1 (Figures 4A, 4B, 5A, and 5B). In the LPDS-stimulated monocytes, only the production of IL-6 and IL-1 beta was lower than that induced by UA sera in toto ($P<0.001$; Figures 4A, 4B, 5A, and 5B).

**Discussion**

The results of this study show that during unstable phases of angina, UA patients exhibited a greatly enhanced concentration of IL-6 and IL-1 beta in plasma as well as in whole blood and separated monocytes after LPS stimulation. The mechanisms leading to this rather selective increase of cytokines and to the abnormal production of IL-6 and IL-1 beta after LPS challenge in UA patients are unclear. Here we considered the possibility that oxLDL may contribute to the cytokine overproduction by upregulating CD14 and TLR-4 expression in circulating monocytes of UA patients.

In our study there was an expansion of CD14- and TLR-4-positive monocytes in UA patients and very interestingly statistically significant correlations were found between the plasma levels of circulating oxLDL and the circulating concentration of CD14 and TLR-4 indicating that oxLDL may be implicated in CD14 and TLR-4 expression in vivo.
The in vitro experiments definitely support this hypothesis because the amounts of oxLDL that increased the expression of CD14 and TLR-4 in monocytes were in the same order of concentrations found in the plasma of UA patients. The upregulation in CD14 and TLR-4 expression induced by oxLDL was associated with an increased production of IL-6 and IL-1 beta and at a lower extent of TNF-alpha and MCP-1.

The in vitro experiments definitely support this hypothesis because the amounts of oxLDL that increased the expression of CD14 and TLR-4 in monocytes were in the same order of concentrations found in the plasma of UA patients. The upregulation in CD14 and TLR-4 expression induced by oxLDL was associated with an increased production of IL-6 and IL-1 beta and at a lower extent of TNF-alpha and MCP-1.
reduced the cytokine concentration in the supernatant of monocytes, may indicate that the cytokine increase and above all that of IL-6 and IL-1 beta is, at least partially, related to the binding of oxLDL to this receptor and therefore to TLR-4 engagement. This conclusion is in agreement with previous results showing that oxLDL induced actin polymerization and spreading of macrophages by binding to CD14 and signaling through the LPS receptor complex composed of CD14 and MD-2. At variance with these results, Walton et al showed that CD14 did not appear to mediate the action of oxidized 1-palmitoyl-2- arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) on IL-8 transcription in endothelial cells because it was not blocked by anti-CD14 neutralizing antibodies. These differences may be attributable to the fact that oxLDL is a mixture of possible ligands, including various proteins, cholesterol, and oxidized phospholipids, whereas ox-PAPC only contains oxidized phospholipids. Our conclusion is also in contrast with the results of Miller et al who found that the induction of IL-6 by oxLDL was not dependent on TLR-4. These contrasting results may be attributable to the different oxLDL preparations or alternatively to differences in cell types. 

Starting from these results we then evaluated the effect of sera derived from UA patients and C subjects on CD14 and TLR-4 expression in monocytes derived from healthy volunteers. The results show that the sera of UA patients increased at a much higher extent the expression of CD14 and TLR-4 in monocytes derived from healthy volunteers than in those of C subjects. Similarly to the results obtained with oxLDL where there was a prevalent increase of IL-6 and IL-1 beta, the overexpression of CD14 and TLR-4 caused by sera of UA patients, was associated with a clear-cut increase of IL-6 and IL-1 beta whereas the rise of TNF-alpha and MCP-1 was negligible. The reasons why oxLDL and sera of UA patients with the highest oxLDL circulating levels produced slight variations of TNF-alpha and MCP-1 are unclear. However, it is known that TRL engagement results in activation of mitogen-activated protein kinase (MAPKs), which, together with the NF-kB pathway, transduces extracellular signals to cellular responses. Negative regulation of MAPK activity is affected primarily by MAPK phosphatase-1 (MKP-1), a member of the MAPK phosphatase family, which has been recently reported to regulate the temporal response of pro- and antiinflammatory cytokines by TLRs. oxLDL has been demonstrated to activate MKP-1, and even though its role in TLR-mediated immune responses in vivo is far from being fully elucidated, a tentative explanation of our results could be that in our experimental conditions MKP-1 may have repressed to a greater degree some cytokines than others. Of course on the basis of present results we cannot exclude that other suppressing mechanisms that are normally evoked by TLR activation may have contributed to the different cytokine production. Furthermore different serum components may have reduced the secretion of TNF-alpha and MCP-1 induced by the sera of UA patients. For instance, a C-C chemokine called regulated on activation normally T-cell expressed and secreted (RANTES), which has been reported to be high in acute coronary syndrome, has been shown to modulate the TLR4-induced cytokine secretion in human peripheral blood monocytes. These hypothetical counteracting mechanisms and others unknown may have therefore depressed not all but just some cytokines/chemokines and explain at least partially why basal circulating TNF-alpha and MCP-1 were not elevated in UA patients. In addition, the known very short half-life of some cytokines/chemokines...
and in particular of TNF-α may have contributed to the results.29

Because a number of circulating agents could potentially have increased monocyctic CD14 and TLR-4 expression as well cytokine production, the LPDS of UA patients was then used to evaluate the magnitude of lipoprotein effect. In these conditions, ie, in absence of lipoproteins and with no detectable oxLDL, the increase in monocyctic expression of CD14/TLR-4 and in the concentration of supernatant IL-6 and IL-1 beta were significantly lower than that obtained with the complete sera. These results of course have limitations because they have not been shown for oxLDL specifically because in LPDS all the lipoproteins and not only oxLDL were removed. Furthermore, because a considerable amount of TLR4/CD14 expression and of IL-6 and IL-1 beta production was not abolished by the absence of lipoproteins in the sera, it is likely that many other parameters and not just oxLDL or other lipoproteins may differ between the 2 groups and contribute to the results. These unknown parameters may have also been important in determining the slight increase of TNF-α and MCP-1 because there was no difference in the results obtained with sera in toto and LPDS.

Taken together these results suggest that the increased plasma levels of IL-6 and IL-1 beta in patients with UA may be at least in part related to the effect of circulating oxLDL on monocyte expression of CD14 and TLR-4 and provide new insights into the inflammatory origin of atherosclerosis.

Sources of Funding

This work was supported in part by grants from the Ministry of University and Scientific Research of Italy.

Disclosures

None.

References

Enhanced Levels of Oxidized Low-Density Lipoprotein Prime Monocytes to Cytokine Overproduction via Upregulation of CD14 and Toll-Like Receptor 4 in Unstable Angina

Arterioscler Thromb Vasc Biol. published online June 28, 2007;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2007/06/28/ATVBAHA.107.142695.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/08/22/ATVBAHA.107.142695.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMCs) and monocyte isolation
Whole blood was layered onto a sterile aqueous medium containing ficoll and sodium diatrizoate at a predetermined density of 1.007 g/ml at 25° C. Gentle centrifugation at room temperature resulted in the separation of PBMCs at the blood/ficoll interface, with the other white and red blood cells passing through the interface. Monocytes were then isolated from PBMCs by negative selection with a cocktail of hapten-conjugated antibodies and magnetic microbeads coupled to an anti-hapten monoclonal antibody (No touch monocyte isolation kit, Miltenyi Biotec) and depletion on a column in a magnetic field (VarioMACS, Miltenyi Biotec). Monocyte purity was greater than 97% as assessed by flow cytometry (data not shown).

Flow cytometry
Blood samples were stained within 30 minutes after sampling to prevent artificial activation of the cells. The cells were labelled with mouse anti-CD14 (UCH-M1, Santa Cruz Biotechnology, Inc.) and mouse anti-TLR-4 (HTA 125, Santa Cruz Biotechnology, Inc) antibodies as previously described (14). In order to distinguish positive antibody interactions from background fluorescence, control antibodies matching the immunoglobulin class and isotype of the primary antibodies were used (Santa Cruz Biotechnology, Inc). Immunofluorescence staining was analysed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA).

Western blotting analysis
Monocyte lysates were prepared by using T-PER lysis reagent (25 mM Bicine, 150 mM NaCl, pH 7.6) (Pierce) with a mortar and pestle. Samples were centrifuged at 10,000 rpm for 5 min to pellet cell debris, and supernatants were collected, aliquoted, and stored at –80°C. Protein concentrations were determined
by the BCA protein assay (Pierce). TLR-4 was immunoprecipitated from 1 mg of each protein lysate with a mouse monoclonal anti-TLR4 antibody (HTA 125; Santa Cruz Biotechnology). Immune complexes were captured with protein A/G-Sepharose beads (Pierce) for 2 h at 4°C, and the beads were washed four times with 100 mM NaCl. To ensure the specificity of protein-antibody interaction, lysates were incubated with beads in the absence of antibody as well as with an irrelevant P3 immunoglobulin G1 isotype control (Caltag). Proteins were resolved by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and electrotransferred to nitrocellulose membrane in Tris-glycine buffer, with 20% methanol. Immunoblots were washed with 1x phosphate-buffered saline-0.05% Tween 20 and blocked in 5% milk for 1 h at room temperature. TLR-4 was then detected by probing immunoprecipitates with rabbit polyclonal anti-TLR-4 antibody H-80 (Santa Cruz Biotechnology), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Reactive antigens were visualized with Supersignal chemiluminescence substrate (Pierce). As positive control for TLR-4, the PC-3 lysate derived from human prostate adenocarcinoma was used (ProSci Incorporated, San Diego, CA, USA).

**Real-Time quantitative RT-PCR analysis and protein expression of CD14 and TLR-4 in separated monocytes**

Total RNA was extracted with a RNeasy Mini Kit (Qiagen) and was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was conducted by iCycler thermocycler (Bio-Rad, Hercules, CA) using IQSYBR Green PCR SuperMix (Bio-Rad, Hercules, CA) and 300 pmol/ml each primer pair. Primer design and optimization in regard to primer dimmers, self priming formation, miss priming and amplicon length was done with the Beacon Design 4.0 (PREMIER Biosoft International, Palo Alto, CA, USA) primer design software. All primers were optimized to an equal annealing temperature of 60°C, a similar GC content and supplied by MWG Biotech (Ebersberg, Germany). The results were quantified as Ct values, where Ct is defined as the threshold
cycle of the polymerase chain reaction at which the amplified product is first
detected, and expressed as the ratio of target to control (β-actin).

**Statistical analysis**
Continuous data are expressed as mean ± SD values if normally distributed.
Median (interquartile range) was used for variables not normally distributed.
Normal distribution of the data was determined using Shapiro-Wilk test.
Differences between continuous data were analysed by two-tailed unpaired
Student’s t-test. Statistical comparison between 3 groups was performed by one-
or two-way ANOVA and post-hoc multiple comparison using Student-Newmann-
Keuls’ test, if a parametric distribution was assessed. If the data were not
parametric, ANOVA on ranks and post-hoc Dunn’s test was used. Relationship
between variables was assessed by linear and non-linear regression. A probability
value (p) <0.05 was considered to be statistically significant. All data were
analyzed with SPSS 11.04 for Macintosh (SPSS, Chicago, III.)
Fratta Pasini et al., Data supplement 1

Table I. Baseline clinical characteristics of the three groups of patients.

<table>
<thead>
<tr>
<th></th>
<th>C (n=27)</th>
<th>SA (n=29)</th>
<th>UA (n=27)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66±11</td>
<td>61±9</td>
<td>61±9</td>
<td>NS</td>
</tr>
<tr>
<td>Females</td>
<td>9 (33%)*</td>
<td>3 (10%)</td>
<td>3 (11%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Risk factors:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td>10 (37%)</td>
<td>19 (66%)</td>
<td>10 (38%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoke</td>
<td>5 (18%)</td>
<td>8 (28%)</td>
<td>12 (46%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>11 (41%)*</td>
<td>23 (79%)</td>
<td>18 (69%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (48%)</td>
<td>23 (79%)</td>
<td>16 (61%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (7%)</td>
<td>4 (14%)</td>
<td>4 (15%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Current therapy:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ace-inhibitors</td>
<td>17 (63%)</td>
<td>17 (59%)</td>
<td>9 (35%)</td>
<td>NS</td>
</tr>
<tr>
<td>Statins</td>
<td>3 (11%)</td>
<td>7 (24%)</td>
<td>3 (11%)</td>
<td>NS</td>
</tr>
<tr>
<td>Aspirin</td>
<td>8 (30%)</td>
<td>22 (76%)†</td>
<td>13 (50%)†</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Clinical history:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous ACS</td>
<td>-</td>
<td>19 (65%)‡</td>
<td>8 (31%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>-</td>
<td>3 (11%)‡</td>
<td>2 (8%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are expressed as numbers and percent. * = C vs SA and UA, † = SA vs C, ‡ = SA vs UA. C = controls; SA = stable angina; UA = unstable angina; ACS = acute coronary syndrome; PCI = percutaneous coronary intervention.
Table II. Laboratory data of the three groups of patients.

<table>
<thead>
<tr>
<th></th>
<th>C (n=27)</th>
<th>SA (n=29)</th>
<th>UA (n=26)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mg/dl)</strong></td>
<td>206.1±25.9</td>
<td>206.4±23.4</td>
<td>215.0±27.1</td>
<td>NS</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mg/dl)</strong></td>
<td>133.0±26.4</td>
<td>136.5±4.5</td>
<td>139.9±27.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mg/dl)</strong></td>
<td>49.9±9.42</td>
<td>46.8±10.9</td>
<td>44.4±8.50</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dl)</strong></td>
<td>135.5±69.4</td>
<td>139.9±49.6</td>
<td>155.0±66.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Glucose (mg/dl)</strong></td>
<td>106.3±25.6</td>
<td>97.3±18.0</td>
<td>100.8±16.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>White blood cells (10^9/L)</strong></td>
<td>6.75±1.57</td>
<td>6.51±1.81</td>
<td>9.07±1.63*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>CRP (mg/dl)</strong></td>
<td>0.43 (0.08-0.83)</td>
<td>0.51 (0.08-1.21)</td>
<td>0.98 (0.25-2.01)*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD or median (ranges).
* UA vs SA and C
C = controls; SA = stable angina; UA = unstable angina.
Fratta Pasini et al., Data supplement 3 and 4

**Figure I**

[Histogram showing CD14 (MFI)]

**Figure II**

[Histogram showing TLR-4 (MFI)]
Fratta Pasini et al., Data supplement 5

**Figure III A**
Real-time PCR

![Real-time PCR graph](image)

**Figure III B**
Flow cytometry: mean values

![Flow cytometry graph](image)

**Figure III C**
Western blotting analysis

![Western blotting analysis](image)
Fratta Pasini et al., Data supplement 6

**Figure IV A**

**Figure IV B**

**Figure IV C**

**Figure IV D**
FIGURE LEGENDS

Figure I and II. Representative analysis of the expression of CD14 and toll-like receptor-4 (TLR-4) in peripheral blood monocytes from control subjects (C), stable angina patients (SA) and unstable angina patients (UA). As controls, isotype antibodies for CD14 and TLR-4 were used (ISOTYPE ANTIBODY). Data are expressed as mean fluorescence intensity (MFI).

Figure III. Oxidized (Ox)- and native (n)-LDL-dependent CD14 and toll-like receptor-4 (TLR-4) mRNA and protein expression in monocytes derived from healthy donors. Increasing amounts of ox-LDL and a fixed amount of native (n)-LDL were incubated with monocytes for 20 hours. CD14 and TLR-4 mRNA (A) and protein expression as evaluated by flow cytometry (B) and Western analysis (data on CD14 and on n-LDL not shown) (C) were then measured. mRNA was quantified as Ct value, where Ct is defined as the threshold cycle of the polymerase chain reaction at which the amplified product is first detected, and expressed as the ratio of target to control (β-actin). Results of flow cytometry are expressed as mean fluorescence intensity. Data on Western analysis are expressed as density in arbitrary units (AU). Results are the means±SD of experiments performed in triplicate in six separated occasions. *p<0.001 versus control (ox-LDL= 0 µg/ml); †p<0.001 versus ox-LDL 20 µg/ml; ‡p<0.001 versus ox-LDL 30 µg/ml.

Figure IV. Interleukin (IL)-6, IL-1 beta, tumor necrosis factor-alpha (TNF-alpha) and monocyte chemoattractant protein-1 (MCP-1) production induced by increasing amounts of oxidized (ox)-LDL and a fixed amount of native (n)-LDL in monocytes derived from healthy volunteers. Increasing amounts of ox-LDL and a fixed amount of n-LDL were incubated with monocytes for 20 hours. IL-6 (A), IL-1 beta (B), TNF-alpha (C) and MCP-1 were then measured in the supernatant. In some experiments, anti CD14 antibody (Ab) (20µg/ml) or control IgG (50µg/ml) were also added to cell culture. Results are the
means±SD of experiments performed in triplicate in six separate occasions. *p<0.001 versus none; †p<0.001 versus anti CD14 Ab-; ‡p<0.001 versus ox-LDL 20; §p<0.001 versus ox-LDL 30.