Inflammation impairs eNOS activation by HDL in patients with acute coronary syndrome

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Aims
The aim of the present study was to evaluate the high-density lipoprotein (HDL) structure and endothelial NO synthase (eNOS) activation capacity in ST-elevation myocardial infarction (STEMI) patients with different acute-phase inflammatory response (APR).

Methods and results
Forty-five STEMI patients were stratified in quartiles according to the delta CRP level, calculated by subtracting the CRP value at admission from the CRP peak value (APR peak). The HDL structure and HDL capacity to stimulate NO production were evaluated at admission and at APR peak. STEMI patients with a low APR had a completely preserved HDL structure and HDL ability to activate eNOS and promote NO production, which did not change during STEMI. On the contrary, HDL from STEMI patients developing a significant APR had compromised ability to stimulate eNOS and promote NO production, and underwent a significant particle remodelling during STEMI. The defective capacity to stimulate NO production of HDL isolated from STEMI patients with high APR was explained, at least in part, by the reduced PON-1 and S1P content. The HDL ability to promote cell cholesterol efflux through different pathways was preserved in ACS patients independently of the inflammatory response.

Conclusion
The present results extend previous studies reporting an impaired eNOS-activating capacity of HDL from ACS patients, showing that only a subset of patients undergoing STEMI, and in particular those developing an important inflammatory response, have circulating HDL defective in stimulating endothelial eNOS and NO production.

Keywords
High-density lipoproteins • Acute inflammatory response • Acute myocardial infarction • Nitric oxide

1. Introduction
High-density lipoproteins (HDL) exert multiple protective effects against atherosclerosis through several mechanisms.¹ The most relevant function of HDL is to promote the removal of cholesterol from peripheral cells, including macrophages within the arterial wall and shuttle it to the liver for excretion in the bile and faeces. This process, called reverse cholesterol transport (RCT), results in a net mass transport of cholesterol from the arterial wall into the bile, preventing arterial cholesterol accumulation.² Besides their major role in RCT, HDL can contribute to the maintenance of vascular endothelium function, through a variety of effects on vascular tone, inflammation, and endothelial cell homeostasis and integrity.³ In cultured endothelial cells, HDL increase endothelial NO synthase (eNOS) protein abundance, by acting at both transcriptional and post-translational levels,⁴ ⁵ and promote eNOS activation. This latter effect is mediated by the binding of apoA-I to SR-BI, which leads to the activation of the phosphatidyl-inositol 3-kinase (PI3K)/Akt signalling pathway and the subsequent phosphorylation of eNOS at multiple sites.⁷ ⁸ In addition, HDL can mediate eNOS activation through the interaction with sphingosine-1-phosphate (S1P) receptors.⁹ In agreement with in vitro data, HDL protect against the endothelial dysfunction induced by high-cholesterol diet in mice,¹⁰ and, more importantly, the infusion of synthetic HDL restores the compromised endothelial function in low-HDL subjects.¹¹

A recent paper has shown that HDL from patients with an acute coronary syndrome (ACS) are unable to stimulate endothelial NO production, partly due to a reduced HDL-associated paraoxonase-1 (PON-1) activity.¹² HDL isolated from ACS patients have also impaired antioxidant capacity,¹³ but retain the ability to promote cell cholesterol efflux.¹² ¹⁴ The role of inflammation in ACS is well established; the
inflammatory response observed in ACS is characterized by elevated levels of acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) that are secreted by the liver. During an acute-phase response (APR), HDL undergo dramatic modifications in structure and composition,15,16 which can affect their atheroprotective capacity.17–20 We have recently studied a series of ACS patients (with ST-elevation myocardial infarction, STEMI) who developed an APR of different intensity despite a comparable STEMI severity.17 In the present study, a systematic characterization of the HDL structure and subclass distribution was performed and combined with the evaluation of HDL function in order to assess the role of APR in affecting HDL atheroprotective functions during ACS.

2. Methods

2.1 Patients

Patients enrolled into the present study have been previously described.21 The 45 STEMI patients were stratified in quartiles of delta CRP, calculated by subtracting the value at admission from the peak value achieved between admission and discharge (APR peak). The APR peak was reached at 72 h for the majority of the patients.21 STEMI severity was assessed by measuring serum creatine kinase (CK), CK-myocardial band (CK-MB), and troponin I (TnI) levels.21 A detailed clinical history was collected for each subject. The study conformed to the guidelines set out in the Declaration of Helsinki and was approved by the Ethics Committee of the Ospedali Riuniti of Trieste; all enrolled patients gave written informed consent for participation in the study.

2.2 Plasma lipids and apolipoproteins

Blood was collected at admission, after 4, 8, 12, 24, 48, 72, and 96 h, and at hospital discharge, and plasma and serum prepared by low-speed centrifugation.21 For the present study, only samples collected at admission and at APR peak were evaluated. Plasma levels of total and HDL cholesterol, and triglycerides were determined by certified enzymatic techniques. LDL cholesterol (LDL-C) was calculated by the Friedewald's formula. Apolipoprotein A-I (apoA-I), A-II, and B levels were determined by immunoturbidimetry, using commercially available polyclonal antibodies.

2.3 HDL purification

HDL (d = 1.063–1.21 g/mL) were purified from the plasma collected at admission and at APR peak by sequential ultracentrifugation. Purified lipoproteins were dialysed against sterilized saline immediately before use. To obtain sufficient amounts of HDL to perform the in vitro experiments, plasma samples from two or three patients belonging to the same quartile were pooled before ultracentrifugation; five pools of HDL were prepared for each group. HDL concentrations are expressed as mg of protein/mL. Control-HDL (d = 1.063–1.21 g/mL) were isolated from fasting plasma of five healthy subjects (blood donors) and pooled before use.

2.4 HDL subclass distribution

The plasma concentrations of HDL particles containing only apoA-I (LpA-I) and of particles containing both apoA-I and apoA-II (LpA-I/A-II) were determined by electroimmunodiffusion in agarose gel (Sebia Italia). HDL distribution according to size was determined by non-denaturing polyacrylamide gradient gel electrophoresis (GGE) of the d < 1.21 g/mL plasma total lipoprotein fraction; the protein-stained gels were scanned with an imaging densitometer; and the relative protein content of HDL subclasses was determined by dividing the HDL profile into three size intervals, small (diameter 7.2–8.2 nm), medium (diameter 8.2–8.8 nm), and large HDL (diameter 8.8–12.7 nm).22 HDL subclasses were also analysed by non-denaturing two-dimensional (2D)-electrophoresis followed by immunodetection with commercial polyclonal antibodies against human apoA-I or apoA-II (Calbiochem),22 and with a polyclonal antibody against paroxonase-1 (PON-1, kindly provided by Dr Richard James, University of Geneva). The serum content of preβ-HDL was expressed as the percentage of total apoA-I. The content of PON-1 in HDL was expressed as PON-1/apoA-I signals. HDL protein composition was assessed by SDS––PAGE followed by Coomassie Blue R250 staining and quantitation of protein bands by densitometry. The SAA content of the plasma total lipoprotein fraction (d < 1.21 g/mL) was assessed by western blot analysis with an antibody against human SAA (BioSource International, Camarillo, USA).21 The concentration of S1P in HDL was measured with a commercial competitive ELISA kit (Echelon Biosciences).

2.5 Studies on endothelial cells

The ability of HDL to promote eNOS expression and activation in endothelial cells was evaluated as previously described.22 Primary cultures of human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Lonza, Milano). Experiments were performed in M199 with 0.75% bovine serum albumin (BSA) and 1% foetal calf serum (FCS), and HDL were used at the protein concentration of 1.0 mg/mL in all experiments. To evaluate nitric oxide (NO) production, endothelial cells were incubated with HDL for 30 min and the NO levels were measured by fluorescence using a diacetate derivative of 4,5-diaminofluorescein (DAF-2 DA, Sigma-Aldrich Chemie, Steinheim, Germany). For each sample, fluorescence was normalized by the protein concentration of the total cell lysate. To evaluate eNOS expression, HUVECs were incubated overnight with HDL. Cells were harvested and proteins were separated by SDS––PAGE and transferred on a nitrocellulose membrane. Membranes were developed against total HDL (BD Biosciences), stripped and reprobed with an antibody against β-actin (Sigma–Aldrich Chemie). eNOS activation by phosphorylation was evaluated in HUVEC harvested after 10 min of incubation with HDL. Proteins were separated by SDS––PAGE and then transferred on a nitrocellulose membrane. Membranes were developed against phosphorylated eNOS (Ser1177; Cell Signalling Technology), stripped, and reprobed with an antibody against the total eNOS. Bands were visualized by enhanced chemiluminescence (GE Healthcare Biosciences). Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyser software (Bio-Rad Laboratories).

2.6 Cell cholesterol efflux studies

Cholesterol efflux mediated by the ATP-binding cassette A1 (ABCA1) transporter was measured using J774 macrophages stimulated with cpt-cAMP.23 Cells were incubated for 24 h in medium containing [3H]cholesterol ([3H]C, 4 μCi/mL) in the presence of 1% FCS and 2 μg/mL of an ACAT inhibitor, washed and then incubated with 0.2% BSA, with or without 0.3 mM cpt-cAMP for 18 h. After this incubation, some wells were extracted with 2-propanol to provide baseline (time 0) values for the total [3H]C content. Stimulated and unstimulated monolayers containing [3H]C were incubated in the presence of 2% (v/v) serum or 12.5 μg/mL of HDL for 4 h. Radioactivity in the supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as (cpm in medium at 4 h/cpm at time 0) × 100. The ABCA1-mediated cholesterol efflux was calculated as the difference between the percentage efflux from stimulated J774 macrophages and unstimulated J774 cells.

Cholesterol efflux mediated by the ATP-binding cassette G1 (ABCG1) transporter was measured using CHO-K1 cells stably expressing human ABCG1.23 Parent and hABCG1-expressing cells were labelled for 24 h with [3H]C, washed, and equilibrated for 90 min in serum-free medium, then incubated with BSA (1 mg/mL) in the presence of 1% (v/v) serum or 12.5 μg/mL of HDL for 6 h. Cells and media were assayed for radioactivity and cholesterol efflux in medium samples calculated as a percentage of total cholesterol (cells plus medium) in the culture. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and parent cells.
Cholesterol efflux mediated by the scavenger receptor class B type I (SR-BI) was measured using Fu5AH rat hepatoma cells \(^2\); cell labelling with \(^{3}H\)C and efflux experiments were performed as described for unstimulated J774 macrophages. Cholesterol efflux was calculated as (cpm in medium at 4 h/cpm at time 0) \(\times 100\).

### 2.7 Statistical analyses
Categorical variables are presented as frequency and percentage, numerical variables as mean \(\pm\) SEM. Variations in the parameters during STEMI were expressed as delta, calculated by subtracting the value at admission from the value achieved at the APR peak. The association of baseline values of all variables with delta CRP was tested by Spearman correlation (continuous variables) or by Mantel–Haenszel \(x^2\) for trend (categorical variables). The difference among quartiles was tested by covariance analysis adjusting for age and gender. The association of delta CRP with delta of other parameters was assessed by Spearman correlation; adjustment for potential confounders (age, gender, and admission values) was carried out by computing Pearson partial correlations. All tests were two-sided and the \(P\)-values below 0.05 were considered as significant. All analyses were performed by using the SAS Statistical package v. 9.2 (SAS Institute, Inc., Cary, NC, USA).

### 3. Results

#### 3.1 Clinical features of patients
STEMI patients in the four quartiles of delta CRP were comparable for age, gender distribution, and BMI (Table 1). The prevalence of common risk factors for cardiovascular disease, such as smoking habits, hypercholesterolaemia, hypalpahlipoproteinaemia, diabetes, and obesity were similar in the four quartiles, while hypertension was positively correlated with delta CRP (Supplementary material online, Table). Pharmacological treatments prior to and upon admission were similar in the four quartiles (Supplementary material online, Table). STEMI severity was also similar in the four quartiles (CK, \(P = 0.24\); CK-MB, \(P = 0.15\); Tnl, \(P = 0.96\)).

#### 3.2 Impact of inflammation on lipids/lipoproteins
Plasma levels of total and LDL cholesterol, triglycerides, and apoB were comparable in the four quartiles; HDL cholesterol, apoA-I, and apoA-II levels correlated significantly and negatively with delta CRP, while LpA-I:A-II concentrations correlated positively with delta CRP (Table 1). No association was observed between delta CRP and delta in plasma lipids (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride) (Table 2). Delta CRP was significantly and negatively associated, with a linear trend, with delta apoA-I, delta apoA-II, delta LpA-I:A-II, delta IL-6, and delta SAA (Table 2). The correlation between delta CRP and delta apoA-II, delta IL-6, and delta SAA remained significant after adjustment for age, gender, and admission values (Table 2).

#### 3.3 Impact of inflammation on HDL structure
HDL structural properties, including small, medium, and large HDL content, and preβ-HDL content, were comparable in the four quartiles of delta CRP (Table 3). Inflammation had a high impact on HDL structure (Figures 1 and 2A); indeed, delta CRP correlated significantly and negatively with delta small HDL and delta preβ-HDL, and significantly and positively with delta large HDL (Table 4). The shift towards large HDL particles was associated with a selective loss of the smallest apoA-II-containing particles observed in patients belonging to the fourth quartile of delta CRP (Figure 2B). The loss of apoA-II was concomitant with a marked enrichment of HDL in SAA, which becomes on average the 25.4% of total HDL proteins in this subset of patients (range 14.3–52.0%).

#### 3.4 Impact of inflammation on HDL ability to activate eNOS
HDL capacity to activate eNOS was significantly different among groups and a significant inverse correlation was observed between delta CRP

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### Table 1 Baseline lipid profile of STEMI patients stratified according to delta CRP

<table>
<thead>
<tr>
<th>Delta CRP quartiles</th>
<th>First (n = 11)</th>
<th>Second (n = 11)</th>
<th>Third (n = 12)</th>
<th>Fourth (n = 11)</th>
<th>(P) trend*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta CRP (mean, range)</td>
<td>8.2 (0–15.3)</td>
<td>23.5 (15.6–37.5)</td>
<td>41.1 (38.3–44.6)</td>
<td>81.8 (48.7–118.1)</td>
<td>[0.221</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>8/3</td>
<td>9/2</td>
<td>7/5</td>
<td>6/5</td>
<td>0.221</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.1 ± 3.2</td>
<td>61.6 ± 2.3</td>
<td>64.1 ± 2.9</td>
<td>67.3 ± 1.8</td>
<td>0.077</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.2 ± 0.9</td>
<td>26.7 ± 1.1</td>
<td>24.3 ± 0.9</td>
<td>26.3 ± 1.1</td>
<td>0.237</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>209.5 ± 7.3</td>
<td>213.6 ± 9.1</td>
<td>213.9 ± 11.4</td>
<td>199.2 ± 6.7</td>
<td>0.473</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>165.2 ± 23.8</td>
<td>148.6 ± 16.5</td>
<td>111.2 ± 19.0</td>
<td>160.0 ± 24.3</td>
<td>0.554</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>145.5 ± 6.7</td>
<td>146.9 ± 8.7</td>
<td>153.6 ± 12.5</td>
<td>126.1 ± 5.6</td>
<td>0.201</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>31.0 ± 2.3</td>
<td>37.0 ± 4.9</td>
<td>38.1 ± 3.2</td>
<td>41.1 ± 5.2</td>
<td>0.034</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dL)</td>
<td>85.1 ± 3.6</td>
<td>105.2 ± 10.1</td>
<td>99.8 ± 4.5</td>
<td>104.9 ± 9.4</td>
<td>0.030</td>
</tr>
<tr>
<td>Apolipoprotein A-II (mg/dL)</td>
<td>25.7 ± 1.2</td>
<td>29.1 ± 2.1</td>
<td>29.2 ± 1.7*</td>
<td>31.5 ± 2.1*</td>
<td>0.028</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>128.5 ± 6.3</td>
<td>123.6 ± 8.2</td>
<td>133.0 ± 11.8</td>
<td>109.0 ± 6.7</td>
<td>0.207</td>
</tr>
<tr>
<td>LpA-I (mg/dL)</td>
<td>49.4 ± 3.3</td>
<td>58.3 ± 5.3</td>
<td>49.2 ± 3.7</td>
<td>52.0 ± 7.0</td>
<td>0.599</td>
</tr>
<tr>
<td>LpA-I:A-II (mg/dL)</td>
<td>34.1 ± 3.0</td>
<td>46.9 ± 5.6</td>
<td>50.6 ± 2.9*</td>
<td>52.9 ± 5.9*</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Data are expressed as mean \(\pm\) SEM. BMI: body mass index; LpA-I, lipoprotein containing apoA-I; LpA-I:A-II, lipoprotein containing apoA-I and apoA-II.

*\(P<0.05\) vs. first quartile.

*Computed by Spearman correlations.
and the capacity of patient HDL collected at admission to activate eNOS (Table 3, Figure 3A). The HDL ability to stimulate NO production was also significantly different among quartiles, and again a significant inverse correlation was observed between delta CRP and the ability of HDL collected at admission to promote NO production (Table 3, Figure 3B). The HDL isolated from patients belonging to the first and second quartiles of delta CRP showed a normal ability to activate eNOS and to promote NO production (reference values for control second quartiles of delta CRP showed a normal ability to activate eNOS and to promote NO production (reference values for control second quartiles of delta CRP showed a normal ability to activate eNOS and to promote NO production). Indeed, a significant inverse correlation was observed between delta CRP and S1P and PON1 content of HDL collected at admission (Table 3). In agreement with the absence of effect of APR on the HDL ability to activate eNOS and promote NO production, no effects of APR were observed on HDL S1P and PON1 content (Figure 4, Table 4). The HDL ability to induce eNOS expression was similar in the four groups and unaffected by APR (Tables 3 and 4).

### Table 2 Correlations between delta CRP and changes of lipid and inflammatory parameters during STEMI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Delta CRP</th>
<th>R</th>
<th>P-value</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta total cholesterol</td>
<td>−0.26</td>
<td>0.086</td>
<td>0.136</td>
<td></td>
</tr>
<tr>
<td>Delta triglycerides</td>
<td>−0.15</td>
<td>0.323</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>Delta LDL cholesterol</td>
<td>−0.21</td>
<td>0.169</td>
<td>0.124</td>
<td></td>
</tr>
<tr>
<td>Delta HDL cholesterol</td>
<td>0.12</td>
<td>0.462</td>
<td>0.564</td>
<td></td>
</tr>
<tr>
<td>Delta apolipoprotein A-I</td>
<td>−0.39</td>
<td>0.009</td>
<td>0.132</td>
<td></td>
</tr>
<tr>
<td>Delta apolipoprotein A-II</td>
<td>−0.47</td>
<td>0.001</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Delta apolipoprotein B</td>
<td>−0.18</td>
<td>0.261</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td>Delta LpA-I</td>
<td>0.16</td>
<td>0.325</td>
<td>0.591</td>
<td></td>
</tr>
<tr>
<td>Delta LpA-I-I-A-II</td>
<td>−0.29</td>
<td>0.066</td>
<td>0.637</td>
<td></td>
</tr>
<tr>
<td>Delta interleukin 6</td>
<td>0.84</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Delta SAA</td>
<td>0.66</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, and admission values.

### Table 3 Baseline HDL structural and functional properties in STEMI patients stratified according to delta CRP

<table>
<thead>
<tr>
<th>Delta CRP quartiles</th>
<th>First (n = 11)*</th>
<th>Second (n = 11)</th>
<th>Third (n = 12)</th>
<th>Fourth (n = 11)</th>
<th>P trend#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta CRP (mean, range)</td>
<td>8.2 (0–15.3)</td>
<td>23.5 (15.6–37.5)</td>
<td>41.1 (38.3–44.6)</td>
<td>81.8 (48.7–118.1)</td>
<td></td>
</tr>
<tr>
<td>Small HDL (% of total HDL)</td>
<td>34.5 ± 3.9</td>
<td>25.4 ± 1.9</td>
<td>23.6 ± 2.8*</td>
<td>26.9 ± 3.0</td>
<td>0.241</td>
</tr>
<tr>
<td>Medium HDL (% of total HDL)</td>
<td>32.4 ± 1.8</td>
<td>32.3 ± 1.0</td>
<td>32.0 ± 2.0</td>
<td>32.5 ± 1.9</td>
<td>0.841</td>
</tr>
<tr>
<td>Large HDL (% of total HDL)</td>
<td>33.1 ± 2.5</td>
<td>42.3 ± 2.1</td>
<td>44.4 ± 3.0</td>
<td>40.6 ± 4.2</td>
<td>0.207</td>
</tr>
<tr>
<td>Preβ-HDL (% of total apoA-I)</td>
<td>11.2 ± 0.8</td>
<td>12.6 ± 0.8</td>
<td>12.7 ± 1.5</td>
<td>11.9 ± 0.5</td>
<td>0.468</td>
</tr>
<tr>
<td>eNOS activation (fold increase)</td>
<td>1.92 ± 0.14</td>
<td>1.86 ± 0.12</td>
<td>1.57 ± 0.13</td>
<td>1.41 ± 0.22*</td>
<td>0.006</td>
</tr>
<tr>
<td>NO production (fold increase)</td>
<td>1.49 ± 0.04</td>
<td>1.53 ± 0.06</td>
<td>1.35 ± 0.04*</td>
<td>1.21 ± 0.03**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eNOS expression (fold increase)</td>
<td>1.56 ± 0.07</td>
<td>1.51 ± 0.07</td>
<td>1.52 ± 0.16</td>
<td>1.42 ± 0.16</td>
<td>0.271</td>
</tr>
<tr>
<td>S1P content (pmol/mg HDL protein)</td>
<td>204.9 ± 7.1</td>
<td>196.1 ± 12.5</td>
<td>166.1 ± 15.8</td>
<td>116.1 ± 5.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1 content (PON1/apoA-I ratio)</td>
<td>0.21 ± 0.05</td>
<td>0.22 ± 0.06</td>
<td>0.14 ± 0.04</td>
<td>0.09 ± 0.02</td>
<td>0.051</td>
</tr>
<tr>
<td>ABCA1-mediated cholesterol efflux (%)</td>
<td>6.4 ± 0.6</td>
<td>7.2 ± 0.3</td>
<td>7.0 ± 0.4</td>
<td>5.3 ± 0.4</td>
<td>0.060</td>
</tr>
<tr>
<td>ABCG1-mediated cholesterol efflux (%)</td>
<td>7.1 ± 0.7</td>
<td>7.0 ± 0.3</td>
<td>5.9 ± 0.4</td>
<td>7.9 ± 0.6</td>
<td>0.744</td>
</tr>
<tr>
<td>SR-BI-mediated cholesterol efflux (%)</td>
<td>5.5 ± 0.5</td>
<td>5.5 ± 0.4</td>
<td>5.2 ± 0.3</td>
<td>5.6 ± 0.5</td>
<td>0.638</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. For NO production, eNOS activation, eNOS expression, and S1P content, n = 5.

*P < 0.05 and **P < 0.005 vs. first quartile.

*Computed by Spearman correlations.

**3.5 Impact of inflammation on HDL-mediated cholesterol efflux**

To determine whether the impaired capacity of HDL from patients with a high inflammatory response to stimulate endothelial NO production is associated with changes in the HDL ability to promote cell cholesterol efflux, we evaluated the ability of patients’ sera to remove cholesterol from different cell types. The capacity to promote cell cholesterol efflux through the different pathways was similar for all the tested sera (Table 3). APR had no significant effects on ABCA1- and SR-BI-mediated cholesterol efflux (Table 4). A significant and positive correlation was observed between delta CRP and delta ABCG1-mediated cholesterol efflux (Table 4), in agreement with the shift towards large HDL, the best acceptor of cell cholesterol through this pathway. Very similar results were obtained when cholesterol efflux experiments were run with isolated HDL (not shown).
The present study demonstrates that only HDL from ACS patients who will develop an important inflammatory response during STEMI: (i) have an impaired capacity to stimulate eNOS and (ii) undergo a significant particle remodelling during ACS. The availability of cohort of patients with comparable STEMI severity but with a variable inflammatory response allowed to differentiate the effects of STEMI from those of the consequent APR. The results demonstrate that STEMI patients with a low APR had a completely preserved HDL structure and HDL ability to activate eNOS, which did not change during STEMI. On the contrary, STEMI patients developing a significant APR had HDL depleted in S1P and in PON-1 and with compromised ability to stimulate eNOS, which underwent a significant particle remodelling during STEMI. The HDL ability to promote cell cholesterol efflux through different pathways was preserved in ACS patients independently of the inflammatory response.

The HDL ability to stimulate eNOS-dependent NO production represents a potential anti-atherogenic property of HDL and indeed reduced endothelial NO bioavailability contributes to development and progression of atherosclerosis. In cultured endothelial cells, HDL increase endothelial eNOS protein abundance, by acting at both transcriptional and post-translational levels, and promote eNOS activation. This latter effect requires the binding of apoA-I to SR-BI, which leads to the activation of the PI3 K/Akt signalling pathway and the subsequent phosphorylation of eNOS at multiple sites. ApoA-I is necessary but not sufficient for eNOS stimulation, since no activation is observed with lipid-free apoA-I. The interaction of HDL with SR-BI for endothelial binding has been shown to be preserved in ACS patients. In line with this evidence, the present study shows that HDL isolated from ACS patients maintain the ability to promote cell cholesterol efflux via SR-BI independently from the degree of inflammation, supporting the concept that the impaired ability to activate eNOS observed in HDL from high APR patients is related to intracellular signalling rather than...
activating eNOS-Thr495 phosphorylation, resulting in attenuated NO
decreased activating eNOS-Ser1177 phosphorylation and increased in-
sites.27 The activation of protein kinase C-
phorylated at its stimulatory site and dephosphorylated at its inhibitory
tyrosine residues.27 To generate NO efficiently eNOS must be phos-
 transcriptional mechanisms including protein–protein interactions,
The enzymatic activity of eNOS is regulated by a variety of post-
ificance during STEMI have significantly impaired capacity to induce eNOS phosphoryl-
ation and have a dramatic reduced PON1 content. We did not measure
PKC- activation in cultured endothelial cells and consequent decreased activating eNOS-Ser1177 phosphorylation and increased in-
avidating eNOS-Thr495 phosphorylation, resulting in attenuated NO

Figure 3 Effects of HDL from STEMI patients on eNOS activation
and NO production in HUVEC. (A). Densitometric analysis of eNOS
phosphorylation by HDL at admission (open bars) and at APR peak
(full bars). Data are expressed as fold of increase of the phosphor-
lated/total eNOS ratio in treated vs. untreated cells. Data are mean ± SEM, n = 5 in each group. (B). Induction of NO production
by HDL at admission (open bars) and at APR peak (full bars). Data
are expressed as fold of increased fluorescence in treated vs. untreated
cells. Data are mean ± SEM, n = 5 in each group.
to a major impairment in SR-BI binding or cholesterol efflux capacity.
The enzymatic activity of eNOS is regulated by a variety of postranscriptional mechanisms including protein–protein interactions,
subcellular localization, and phosphorylation of serine, threonine, and
tyrosine residues.27 To generate NO efficiently eNOS must be phos-
phorylated at its stimulatory site and dephosphorylated at its inhibitory
sites.27 The activation of protein kinase C-βII (PKC-βII), which inhibits
Akt-activating phosphorylation, is known to decrease NO production
by direct effects on eNOS phosphorylation sites.28 HDL-associated
PON1 has been suggested to have major impact on endothelial function
by modulation of PKC-βII.12 PON1 inactivation in HDL results in greater
PKC-βII activation in cultured endothelial cells and consequent decreased activating eNOS-Ser1177 phosphorylation and increased in-
avidating eNOS-Thr495 phosphorylation, resulting in attenuated NO
production. Furthermore, HDL from PON1-deficient mice fail to
stimulate endothelial NO production and the supplementation of
HDL isolated from these mice with purified PON1 restores the protec-
tive function.12 The present study demonstrates that HDL isolated from
ACS patients who develop an important inflammatory response during
STEMI have significantly impaired capacity to induce eNOS phosphoryl-
ation and have a dramatic reduced PON1 content. We did not measure
PON1 activity, but the reduced PON1 content is likely to be associated
with a reduced enzymatic activity.27 The reduced PON1 content in HDL
from ACS patients who develop an important inflammatory response
might be related to the structural properties of patient’s HDL, in particu-
lar to the apolipoprotein composition. ApoA-I appears of major impor-
tance in defining serum PON1 activity and stability, likely establishing the
architecture of the HDL particle that optimally binds PON1;30 the higher
apoA-II content in HDL and the predominance of LpA-I:A-II particles
observed in ACS patients undergoing high APR may thus explain the
reduced HDL-associated PON1 content.

It has been shown that S1P, one of the bioactive sphingolipids carried
by HDL, can increase the HDL ability to activate eNOS, since the
absence of its receptor S1P3 reduced HDL-mediated vasodilation by
60%.7,9 In the present study, HDL-associated S1P was significantly
reduced in ACS patients developing an important inflammatory re-
ponse, in agreement with a previous paper reporting reduced S1P
content in HDL from STEMI patients.31 The reduced S1P content
could be related to the abnormal HDL subclass distribution, since differ-
ent HDL subclasses differently bind to S1P3 or to a reduced content of
apoM, recently identified as a carrier of S1P in HDL.33

Structural and compositional alterations in HDL during myocardial in-
farction have been described in earlier studies;15 the present study
extends the previous findings showing that the structural and compos-
utional alterations in HDL are related to the APR driven by the myocar-
dial infarction and are indeed similar to those observed in APR driven by
acute inflammation.34,35 HDL are known to undergo dramatic modificati-
structure and composition as a result of the concerted actions of
APR and inflammation.14,16 The major APR-mediated changes in the
HDL subclass distribution include a shift towards large HDL particles,
a significant decrease in small preβ-HDL, and a selective depletion of
LpA-I:A-II particles.21 Interestingly, we show here that the decrease in
LpA-I:A-II particles is due to a selective loss of small apoA-II containing
HDL, likely due to the displacement of apoA-II by SAA, which preferen-
tially binds to HDL3;36

The present study has some limitations. First, only a small number of
patients with STEMI have been recruited, which limits generalization of
present findings to other ACS. Moreover, some minor differences in
drug treatment have been observed among patients belonging to the dif-
ferent quartiles of delta CRP; however, the inflammatory response to
STEMI goes in the opposite direction as expected, with patients on
statin treatment displaying greater inflammatory response than those
without.

In conclusion, the present results extend recent data reporting an
impaired eNOS-activating capacity of HDL from ACS patients,12
showing that only a subset of patients undergoing STEMI, and in particu-
lar those developing an important inflammatory response subsequent to
the event, have circulating HDL defective in stimulating endothelial
eNOS and NO production. Moreover, our studies confirm the impor-
tance of HDL-associated PON1 in defining HDL vasodilation properties,
since only HDL with compromised eNOS activation capacity have
reduced PON1 content.
Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

References


Figure 4 HDL content of S1P and PON-1. (A). The concentration of S1P was measured in HDL isolated from the plasma of STEMI patients at admission (open bars) and at APR peak (full bars) by competitive ELISA. (B). Densitometric analysis of PON-1 content in HDL at admission (open bars) and at APR peak (full bars), calculated as the ratio of the apoA-I signal. Data are mean ± SEM, n = 5 in each group.


