Clinical research

C-reactive protein levels determine systemic nitric oxide bioavailability in patients with coronary artery disease

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Aim Elevated C-reactive protein (CRP) levels are associated with impaired endothelial vasoreactivity in patients with coronary artery disease (CAD). Because inflammatory cytokines experimentally reduce basal and stimulated endothelial nitric oxide (NO) release, we hypothesised that patients with elevated CRP-levels are characterised by a systemic impairment in NO bioavailability.

Methods and results Forearm blood flow (FBF) responses were measured by venous occlusion plethysmography in 75 male patients with documented CAD. Inhibition of NO synthesis by infusion of L-NMMA significantly reduced baseline FBF (2.2 ± 0.5 vs. 1.9 ± 0.5 mL/min/100 mL of forearm tissue, \( P < 0.001 \)) and acetylcholine-stimulated FBF responses (AUC: 35.0 ± 16.0 vs. 25.9 ± 11.9; \( P < 0.001 \)). CRP serum levels were inversely correlated with L-NMMA-induced decreases in baseline as well as acetylcholine-stimulated FBF responses. Co-infusion of the oxygen-derived free radical scavenger vitamin C significantly increased baseline FBF from 2.0 ± 0.5 to 2.5 ± 0.7 (mL/min/100 mL forearm tissue (\( P < 0.001 \))) and acetylcholine-stimulated FBF responses in patients with elevated CRP, but not in patients with low CRP serum levels. Vitamin C-induced increases in baseline FBF and in acetylcholine-stimulated FBF responses were significantly correlated with CRP serum levels. By multivariable analysis, CRP serum levels remained the only statistically significant independent predictor of NO bioavailability in the systemic circulation of patients with CAD.

Conclusions In patients with CAD, low grade systemic inflammation is associated with increased in vivo oxidative stress leading to impaired systemic bioavailability of NO, which might significantly contribute to the transition from stable coronary artery disease to acute coronary syndromes.

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KEYWORDS
Endothelium;
Nitric oxide;
Inflammation;
CRP;
Coronary artery disease

Introduction

Atherosclerosis and chronic risk factors for coronary artery disease are associated with impaired endotheliat
um-dependent vasodilator function. Importantly, endothelial vasodilator dysfunction contributes to coronary artery (CAD) disease progression and cardiovascular event rates.1,2,3 Likewise, systemic markers of inflammation have been shown to be of significant prognostic relevance for assessing the risk of atherosclerotic disease progression.4 We have previously demonstrated that elevated C-reactive protein (CRP) levels in patients with CAD are associated with an attenuated systemic endothelial vasodilator function.5 However, the mechanisms involved in endothelial dysfunction linking a systemic marker of inflammation to atherosclerotic disease progression are unclear at present.

Recent experimental studies suggest that CRP might directly interfere with endothelial nitric oxide (NO) bioavailability by decreasing endothelial NO synthase expression6 and increasing the production of reactive oxygen species, which might inactivate NO.7 Because NO protects against atherothrombosis,8 we hypothesised that CRP serum levels determine the systemic bioavailability of endothelium-derived NO in patients with established CAD. We therefore prospectively tested whether basal and stimulated NO bioavailability correlates with CRP serum levels. In addition, we investigated whether scavenging reactive oxygen species might restore NO bioavailability.

Methods

Patients

A total of 75 male patients with stable CAD were studied. None of these patients was included in our previous report describing an association between impaired vasodilator function and CRP serum levels.9 All patients had angiographically documented atherosclerotic coronary artery lesions, but no elevation of troponin T levels (<0.1 ng/mL). Patients with inflammatory disease or malignancy, ejection fraction <50%, clinical evidence of heart failure, or patients with angina > Braunwald class II were excluded. Vasoactive medications including calcium channel blockers, ACE-inhibitors and long-acting nitrates were withheld at least 24 h prior to the study. All patients were on chronic aspirin (100 mg/day) and chronic β-blocker therapy. All patients gave written informed consent. The study protocol was approved by the Ethical Committee of the Johann Wolfgang Goethe-University of Frankfurt/Main.

Study protocol

Venous occlusion plethysmography of the forearm for measurement of the forearm blood flow (FBF) was performed in the morning in a quiet and temperature-controlled (22 °C) laboratory as previously described.9 For assessment of endothelium-dependent vasodilatation, acetylcholine (Ciba Vision GmbH, Wessling) was infused intra-arterially in increasing doses from 10 to 50 μg/min. Patients were randomly selected for the two different protocols. For assessment of vascular oxidative stress, vitamin C (sodium ascorbate; Wörwag Pharma, Germany) was infused into the brachial artery in 40 patients at a constant dose of 25 mg/min (1 mL/min) as described.9 For the assessment of NO bioavailability, N’-monomethyl-L-arginine (L-NMMA; Alexis Biochemicals, Germany) was infused into the brachial artery in 35 patients at a rate of 4 μmol/min for 10 min as described.10 To assess endothelium-independent vasodilatation, sodium nitroprusside (Schwarz Pharma, Mohnheim) was infused in increasing doses from 2 to 8 μg/min. After cannulation of the brachial artery, NaCl (0.9%) was infused intra-arterially for 20 min in order to achieve stable baseline FBF measurements prior to acetylcholine and SNP. After FBF returned to baseline values during continuous NaCl (0.9%) infusion for 20 min, vitamin C or L-NMMA was infused for 10 min and thereafter the acetylcholine and SNP infusion protocol was repeated with continuous co-infusion of vitamin C or L-NMMA.

Laboratory analysis

At the time of the FBF study, blood samples were collected, and serum and plasma were aliquoted and stored at −80 °C until analysed for the measurement of high sensitive CRP (hs-CRP). The hs-CRP serum levels were measured with a highsensitive CRP test (N Latex CRP mono; Behring). The measurement range was 0.02–1.1 mg/dL (for 1:20 dilution; higher concentrations were determined after appropriate dilution) with intra-assay co-efficients of variation of 1.7–2.5% and interassay co-efficients of variation of 1.7–3.6%.

Statistical analysis

Data are expressed as mean±SD value. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test and compared by 1-way ANOVA. Categorical variables were compared by the χ² test and the Fisher exact test. In the case of non-normal distribution, the Mann–Whitney U test was used. All the tests were two-sided. Because CRP levels have a skewed distribution, logarithmically transformed CRP values were also used to relate CRP levels with FBF data. Forearm blood flow responses to the drug infusions were examined using a general linear model procedure (GLM) with repeated measures for the different doses of each drug. Based on the hypothesis that the vitamin C-induced improvement of ACH-mediated forearm blood flow responses as well as the L-NMMA-induced decrease are dependent on systemic CRP levels, we generated a formal analysis using a term of interaction (CRP and vitamin C or L-NMMA) and correlated this term with the FBF response to vitamin C (r = 0.44, P = 0.002) and to L-NMMA (r = 0.39, P = 0.007), respectively. FBF responses to acetylcholine and sodium nitroprusside were calculated as area under the curve (AUC) and expressed in arbitrary units. Associations were assessed by linear regression analysis and non-parametric bivariate correlation (Spearman rank correlation coefficient r). The interaction between ACH-induced FBF, CRP, and clinical and procedural parameters was examined by multivariable analysis using the multiple stepwise linear regression model. CRP, LDL and HDL-cholesterol serum levels were taken as continuous variables in the model, whereas the clinical parameters and the concurrent medication were used as categorical variables. Statistical significance was assumed at P < 0.05. All statistical analyses were performed using SPSS for Windows 11.0.

Results

The characteristics of the study populations receiving either L-NMMA or vitamin C are summarised in Table 1.

Elevated CRP levels are associated with reduced bioavailability of NO

Inhibition of NO synthesis by infusion of L-NMMA significantly reduced both baseline FBF (2.2±0.5 vs. 1.9±0.5
mL/min/100 mL of forearm tissue, \( P < 0.001 \) and acetylcholine-stimulated FBF responses (AUC: 35.0 ± 16.0 vs. 25.9 ± 11.9; \( P < 0.001 \)). As illustrated in Fig. 1, there was a significant correlation between CRP serum levels and L-NMMA-induced decreases in baseline FBF (panel A) as well as in acetylcholine-stimulated FBF responses (panel B). When patients were dichotomised (according to the median of CRP) into those with low (<0.5 mg/dL) and elevated CRP serum levels (≥0.5 mg/dL), L-NMMA significantly \( (P = 0.02) \) reduced baseline FBF from 2.2 ± 0.6 to 1.8 ± 0.5 (mL/min/100 mL forearm tissue) in patients with low CRP serum levels, whereas baseline FBF remained essentially unchanged in patients with elevated CRP serum levels (1.9 ± 0.3 vs. 1.8 ± 0.5 (mL/min/100 mL forearm tissue) 1.8 ± 0.5 (mL/min/100 mL forearm tissue) in patients with low CRP serum levels, whereas baseline FBF remained essentially unchanged in patients with elevated CRP serum levels (1.9 ± 0.3 vs. 1.8 ± 0.4 mL/min/100 mL forearm tissue) (\( P = 0.12 \)). Likewise, in patients with low CRP serum levels, co-infusion of L-NMMA profoundly reduced acetylcholine-stimulated FBF responses to the level of patients with elevated CRP serum levels, whereas no effect was observed in patients with elevated CRP serum levels (Fig. 2). Co-infusion of L-NMMA had no effect on SNP-stimulated FBF responses (AUC: 30.6 ± 7.3 vs. 32.4 ± 8.7; \( P = 0.13 \)). Thus, the impairment in responsiveness to L-NMMA directly correlating with elevated CRP serum levels indicates the presence of an impairment in both, basal and stimulated NO bioavailability associated with elevated CRP serum levels in the systemic circulation of patients with coronary artery disease.

**Vitamin C normalises FBF responses in patients with elevated CRP**

As illustrated in Fig. 3, co-infusion of the oxygen-derived free radical scavenger vitamin C significantly increased both, baseline FBF from 2.0 ± 0.5 to 2.5 ± 0.7 mL/min/100 mL forearm tissue (\( P < 0.001 \)) and acetylcholine-stimulated FBF responses in patients with elevated CRP, whereas no significant improvement by co-infusion of vitamin C was observed in patients with low CRP serum levels. Co-infusion of vitamin C did not affect FBF responses to SNP. Notably, in patients with elevated CRP serum levels, the concomitant infusion of vitamin C normalised both, baseline FBF as well as acetylcholine-stimulated FBF responses to the level of patients with low CRP serum levels (Fig. 3(a/b)). Importantly, the vitamin C-induced increases in both baseline FBF (Fig. 4(a)) as well as in acetylcholine-stimulated FBF responses (Fig. 4(b)) were significantly correlated with CRP serum levels. Since patients with diabetes and smokers may have elevated levels of oxygen-derived free radicals, which are not related to vascular inflammation, we repeated our analysis excluding these patients. However, the results were identical. Thus, concomitant vitamin C infusion normalised both the impaired acetylcholine-stimulated FBF responses as well as the reduced baseline FBF associated with elevated CRP serum levels, suggesting that the impairment of NO bioavailability correlating with elevated CRP serum levels is, to a large extent, mediated by oxygen-derived free radicals.

**CRP serum level is an independent predictor of systemic NO bioavailability**

In order to establish an independent role for CRP serum levels to determine systemic basal NO bioavailability in patients with CAD, we performed a multivariable analysis relating the L-NMMA inhibitable component of baseline FBF to classical risk factors for CAD including CRP serum levels. As illustrated in Table 2, CRP serum levels remained the only statistically significant independent predictor of the NO bioavailability in the systemic circulation of patients with documented CAD. Importantly, CRP serum levels were also independently associated with the capacity to increase baseline FBF by acutely scavenging oxygen-derived free radicals with vitamin C (Table 3), thus, further supporting a prominent role of increased oxidative stress to mediate the inflammation-associated impairment in systemic NO bioavailability.

**Table 1 Baseline clinical characteristics of the study population**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>L-NMMA</th>
<th>Vitamin C</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study protocol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( N )</td>
<td>35</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.2±7.3</td>
<td>55.7±5.2</td>
<td>0.63</td>
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<tr>
<td>Total serum cholesterol (mg/dL)</td>
<td>203.3±66.9</td>
<td>196.6±40.9</td>
<td>0.59</td>
</tr>
<tr>
<td>Low density lipoprotein-cholesterol (mg/dL)</td>
<td>126.7±32.1</td>
<td>122.1±32.5</td>
<td>0.69</td>
</tr>
<tr>
<td>High density lipoprotein-cholesterol (mg/dL)</td>
<td>43.1±11.8</td>
<td>41.6±13.2</td>
<td>0.64</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mmHg)</td>
<td>90.5±12.2</td>
<td>86.0±15.1</td>
<td>0.19</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (11.4%)</td>
<td>6 (15.0%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Current smoking</td>
<td>5 (14.3%)</td>
<td>12 (30.0%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Hypertension</td>
<td>18 (51.4%)</td>
<td>21 (60.0%)</td>
<td>0.56</td>
</tr>
<tr>
<td>Concurrent medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \beta )-Blocker</td>
<td>35 (100%)</td>
<td>40 (100%)</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>35 (100%)</td>
<td>40 (100%)</td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>2 (5.7%)</td>
<td>3 (7.5%)</td>
<td>0.56</td>
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<tr>
<td>ACE-inhibitors</td>
<td>15 (42.3%)</td>
<td>21 (60.0%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Statins</td>
<td>21 (60.0%)</td>
<td>30 (66.7%)</td>
<td>0.13</td>
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<tr>
<td>Calcium channel blocker</td>
<td>1 (2.8%)</td>
<td>0</td>
<td>0.47</td>
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</table>

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Discussion

The results of the present study demonstrate that the CRP serum level is an important independent predictor of both basal and stimulated NO bioavailability in the systemic circulation of patients with established CAD. Mechanistically, increased oxidative stress appears to be a major contributor to the reduced NO bioavailability associated with elevated CRP serum levels. Given the pivotal atheroprotective function of preserved NO bioavailability, these data considerably extend our previous observation demonstrating a direct association between elevated CRP serum levels and blunted endothelium-mediated dilator responses, in order to link atherosclerotic disease progression with a systemic marker of inflammatory activity in patients with CAD.

The present study is the first to document that basal systemic NO bioavailability correlates with CRP serum levels in patients with established coronary artery disease. Acetylcholine-stimulated vasodilator responses...
are mediated by a variety of endothelium-derived medi-
ators like NO, endothelium derived hyperpolarising fac-
tors and both vasodilating and vasoconstricting prostanoids.11 Atherosclerosis disturbs the balance be-
tween endothelium-derived relaxing and constricting
factors.3 As such, acetylcholine-stimulated vasodilation
comprises the disturbed balance of a variety of media-
tors associated with atherosclerosis. In contrast, analys-
ing the LL-NMMA inhibitable component of basal blood
flow enables the specific component of NO bioavailability
to contribute to endothelial vasodilator function to be
disclosed. Whereas a previous study reported an associa-
tion between the effects of LL-NMMA and CRP serum levels
in healthy volunteers,12 the results of the present study
demonstrate for the first time that endothelial activa-
tion, associated with elevated CRP serum levels, is char-
acterised by an impaired systemic bioavailability of NO in
patients with coronary artery disease.

The two fundamental mechanisms for impaired bio-
availability of NO are reduced synthesis and increased
oxidative inactivation by reactive oxygen species. Intra-
arterial infusion of the oxygen radical scavenger vitamin
C completely normalised reduced baseline, as well as acetylcholine-stimulated, blood flow responses in patients with elevated CRP serum levels. Most importantly, the effect of the oxygen-derived free radical scavenger vitamin C on basal blood flow was directly related to CRP serum levels and superimposed on the effects of other risk factors for CAD, as evidenced by the multivariable analysis identifying CRP serum levels as an independent predictor of vitamin C-mediated increases in basal blood flow. Thus, enhanced oxygen-derived free radicals may, at least in part, contribute to the impaired systemic bioavailability of NO in patients with elevated CRP serum levels. Obviously, the present clinical study cannot discern whether vitamin C enhanced endothelial NO bioavailability either via reducing direct inactivation of NO by oxygen-derived free radicals or in a tetrahydrobiopterin-dependent manner. Oxidative modification of tetrahydrobiopterin was recently shown to contribute importantly to uncoupling of the endothelial NO synthase (eNOS) resulting in significant oxygen radical production by the eNOS itself. Thus, we cannot exclude that vitamin C might have improved the substrate availability of the eNOS leading to increased basal and stimulated NO production. Moreover, we do not know whether vitamin C infusion interferes with the activity of important antioxidative enzyme systems like ecSOD.

At first glance, the results of the present clinical study do not support experimental data demonstrating that inflammatory cytokines like TNF-alpha, as well as CRP itself, directly reduce eNOS protein levels via shortening of the mRNA half-life. If such a mechanism is operative, one would not expect vitamin C to be capable of acutely normalising the impaired systemic NO bioavailability. However, this apparent discrepancy might well be reconciled by the fact that the forearm circulation, which was tested in the present study, does not develop overt atherosclerotic lesions. Indeed, Lüscher and co-workers elegantly documented that the endothelial cell layer overlying atherosclerotic lesions exhibits reduced expression of eNOS coinciding with a blunted NO release. Thus, elevated levels of circulating CRP do not appear to downregulate eNOS expression in the endothelium of the forearm vasculature, although localised inflammatory processes within the atherosclerotic plaque may well do so in the overlying endothelium.

Nevertheless, elevated CRP levels are well established as the characterising feature of the transition from stable

Table 2 Multivariate analysis including risk factors for CAD

<table>
<thead>
<tr>
<th></th>
<th>Univariate P</th>
<th>Percent decrease of L-NMMA-induced baseline forearm blood flow</th>
<th>Standardised co-efficient</th>
<th>P-value</th>
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<tbody>
<tr>
<td>CRP (mg/dL)</td>
<td>0.02</td>
<td>0.48</td>
<td></td>
<td>0.03</td>
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<tr>
<td>Low density lipoprotein-cholesterol (mg/dL)</td>
<td>0.06</td>
<td>–0.23</td>
<td>0.09</td>
<td>0.67</td>
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<tr>
<td>High density lipoprotein-cholesterol (mg/dL)</td>
<td>0.82</td>
<td>–0.11</td>
<td>0.06</td>
<td>0.75</td>
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<tr>
<td>Hypertension</td>
<td>0.75</td>
<td>–0.09</td>
<td>0.16</td>
<td>0.76</td>
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<tr>
<td>Diabetes</td>
<td>0.46</td>
<td>0.23</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Current smoking</td>
<td>0.49</td>
<td>0.32</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>0.75</td>
<td>0.23</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Statins</td>
<td>0.75</td>
<td>0.23</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Adjusted R²</td>
<td></td>
<td>0.39</td>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

P-values in bold indicate a significant difference. ANOVA, analysis of variance.

Table 3 Multivariate analysis including risk factors for CAD

<table>
<thead>
<tr>
<th></th>
<th>Univariate P</th>
<th>Percent increase of Vitamin C-induced baseline forearm blood flow</th>
<th>Standardised co-efficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/dL)</td>
<td>0.003</td>
<td>0.46</td>
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<td>0.008</td>
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<tr>
<td>Low density lipoprotein-cholesterol (mg/dL)</td>
<td>0.58</td>
<td>0.27</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>High density lipoprotein-cholesterol (mg/dL)</td>
<td>0.53</td>
<td>–0.09</td>
<td>0.54</td>
<td>0.91</td>
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<tr>
<td>Hypertension</td>
<td>0.93</td>
<td>–0.08</td>
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<tr>
<td>Diabetes</td>
<td>0.31</td>
<td>–0.02</td>
<td>0.25</td>
<td>0.17</td>
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<td>Current smoking</td>
<td>0.94</td>
<td>–0.19</td>
<td>0.32</td>
<td>0.17</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>0.13</td>
<td>–0.24</td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>Statins</td>
<td>0.75</td>
<td>0.23</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Adjusted R²</td>
<td></td>
<td>0.14</td>
<td></td>
<td>0.03</td>
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</tbody>
</table>

P-values in bold indicate a significant difference. ANOVA, analysis of variance.
coronary artery disease to acute coronary syndromes, \cite{18,19} which are in most cases secondary to formation of a platelet-rich thrombus following plaque rupture or erosion. \cite{20} NO inhibits platelet aggregation. \cite{5} Indeed, a recent study demonstrated that impaired platelet production of NO predicts the presence of an acute coronary syndrome, which coincides with elevated CRP levels. \cite{21} In vivo, in the intact circulation, platelet activation and recruitment are tightly regulated by endothelium-derived NO, especially in endothelium-injured arteries. \cite{22} Thus, the impaired systemic bioavailability of NO associated with elevated CRP levels might indeed link progression of atherosclerosis and the occurrence of acute coronary syndromes with endothelial activation. In addition, the demonstration of impaired NO bioavailability and enhanced reactive oxygen generation in the systemic circulation associated with the elevation of CRP serum levels, a systemic marker of inflammation, further supports the concept of widespread endothelial activation resembling a pathogenetic component of the vulnerable patient with coronary artery disease. \cite{23} Indeed, Heitzer and coworkers previously demonstrated that the vitamin C-recruitable component to improve endothelial vasodilator dysfunction in the forearm circulation predicts the risk of cardiovascular events in patients with coronary artery disease. \cite{9}

In summary, in patients with stable coronary artery disease, low grade systemic inflammation is associated with increased in vivo oxidative stress leading to impaired systemic bioavailability of NO, which might importantly contribute to the transition from stable coronary artery disease to acute coronary syndromes. Thus, improving NO bioavailability might represent a suitable target for therapy.

Acknowledgement

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References