Platelet Nitric Oxide Responsiveness
A Novel Prognostic Marker in Acute Coronary Syndromes

Scott R. Willoughby, Simon Stewart, Andrew S. Holmes, Yuliy Y. Chirkov, John D. Horowitz

Objectives—Nitric oxide (NO) is critically important in the regulation of vascular tone and the inhibition of platelet aggregation. We have shown previously that patients with acute coronary syndromes (ACS) or stable angina pectoris have impaired platelet responses to NO donors when compared with normal subjects. We tested the hypotheses that platelet hyporesponsiveness to NO is a predictor of (1) cardiovascular readmission and/or death and (2) all-cause mortality in patients with ACS (unstable angina pectoris or non-Q-wave myocardial infarction).

Methods and Results—Patients (n = 51) with ACS had evaluation of platelet aggregation within 24 hours of coronary care unit admission using impedance aggregometry. Patients were categorized as having “normal” (≥32% inhibition of ADP-induced aggregation with the NO donor sodium nitroprusside; 10 μmol/L; n = 18) or “impaired” (<32% inhibition of ADP-induced aggregation; n = 33) NO responses. We then compared the incidence of cardiovascular readmission and death during a median of 7 years of follow-up in these 2 groups. Using a Cox proportional hazards model adjusting for age, sex, index event, postdischarge medical treatment, revascularization status, left ventricular systolic dysfunction, concurrent disease states, and cardiac risk factors, impaired NO responsiveness was associated with an increased risk of the combination of cardiovascular readmission and/or death (relative risk, 2.7; 95% CI, 1.03 to 7.10; P = 0.041) and all-cause mortality (relative risk, 6.3; 95% CI, 1.09 to 36.7; P = 0.033).

Conclusions—Impaired platelet NO responsiveness is a novel, independent predictor of increased mortality and cardiovascular morbidity in patients with high-risk ACS. (Arterioscler Thromb Vasc Biol. 2005;25:2661-2666.)

Key Words: nitric oxide • platelets • acute coronary syndrome • nitric oxide resistance

It is currently considered that elements in the pathophysiology of acute coronary syndromes (ACS), such as acute myocardial infarction (AMI) and unstable angina pectoris (UAP), include antecedent inflammatory states1 and rupture of coronary atheromatous plaques,2 followed by activation of platelet adhesion/aggregation, vasospasm, and thrombus formation.3 There is also considerable evidence that one of the factors predisposing to the development of coronary atheroma and the emergence of ACS is “endothelial dysfunction,” generally defined as an impaired vasodilator response4-5 or a paradoxical constrictor response to endothelium-dependent agents, such as acetylcholine.6

More recently, it has been recognized that patients with endothelial dysfunction may also exhibit attenuated responses to endothelium-independent vasodilators, such as nitroglycerin (NTG)7-10 and sodium nitroprusside (SNP).11,12 This impaired vasodilator response within the coronary circulation is predictive of poor long-term outcomes.9,13

Evidence is also emerging that circulating blood platelets exhibit a range of functional abnormalities in patients with coronary risk factors14-16 and with overt ischemic heart disease.17-19 We have previously demonstrated the phenomenon of platelet resistance to the antiaggregatory effects of nitric oxide (NO) and prostacyclin in patients with stable angina pectoris.20,21 In addition, we have recently documented that the presence of ACS is associated with more marked impairment of platelet responses to NO than are present in patients with stable angina pectoris.18,22

Resistance to NO-induced inhibition of platelet aggregation is mediated both by increased concentrations of superoxide anion and by partial inactivation of soluble guanylate cyclase.21 Furthermore, a number of agents, such as angiotensin-converting enzyme (ACE) inhibitors,23 the antiangiinal agent perhexiline,22 acute insulin therapy,24 and possibly statins,25 ameliorate platelet NO resistance.

Although impaired antiaggregatory responses to NO might theoretically predispose subjects to the occurrence of acute coronary events, there is currently no evidence indicating any prognostic impact of this anomaly. The current study set out to test the hypothesis that the presence of severe impairment of platelet responsiveness to NO is an independent correlate of adverse medium term outcome in a cohort of patients with high-risk ACS.
Methods

Patient Population

Fifty-one patients (31 men and 20 women) with ACS without evidence of transmural myocardial infarction (UAP or non-Q-wave myocardial infarction) were recruited between July 1997 and May 1999. All had been admitted to the coronary care unit (CCU) of the Queen Elizabeth Hospital in the preceding 24 hours with acute symptomatic myocardial ischemia at rest, and all had experienced ongoing pain despite pharmacotherapy including aspirin, intravenous heparin, intravenously infused NTG, plus orally administered prophylactic antianginal agents as indicated in Table 1. In view of these ongoing symptoms, 61% of patients underwent cardiac catheterization during the first 72 hours after CCU admission, with revascularization [by percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG)] undertaken on the basis of symptomatic ischemia in those with appropriate anatomy (47% of patients underwent revascularization procedures within the first 30 days).

Platelet aggregation studies were undertaken before initiation of incremental therapy with perhexiline or ACE inhibitors; no patients received glycoprotein IIb/IIIa inhibitors or ADP receptor antagonists before assessment of platelet function. All of the patients remained in the CCU for 72 hours after initiation of the study. The study was approved by the Ethics of Research Committee of the Queen Elizabeth Hospital. Written informed consent was obtained before study entry.

Blood Sampling and Platelet Aggregation

Blood samples were drawn by venipuncture of an antecubital vein. Blood was collected into plastic tubes containing 1:10 volume of acid citrate (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate) for whole blood platelet aggregation studies. All of the aggregation studies were performed using a dual-channel impedance lumiaggregometer (Model 560, Chrono-Log) as described previously. In all of the cases, aggregation was induced with ADP.
(final concentration of 1 μmol/L), and responses were recorded for electrical impedance, in Ohms via a computer interface system (Aggrolink, Chrono-Log). Responses to the NO donor SNP rather than NTG were used as an index of platelet responsiveness to NO to avert any potential effect of nitrate tolerance on the assessment of platelet function. SNP (final concentration of 10 μmol/L) was added to samples 1 minute before ADP. The inhibition of aggregation by SNP was evaluated as a percentage of maximal aggregation in the absence of SNP.

We have previously reported platelet responses to this concentration of SNP in a normal population as ~65±17% (mean±SD) inhibition of aggregation. It was known that mean responses to SNP would be impaired in an ACS cohort; we now tested the hypothesis that severe NO resistance (SNP response >2SDs below the normal mean) would be a marker of poor long-term outcome. Thus, for the purpose of the current study, an SNP response of <32% inhibition of aggregation was categorized as an “impaired” platelet NO response. Conversely, an SNP response of ≥32% inhibition of aggregation was categorized as a “normal” platelet NO response.

Chemicals
ADP sodium salt and SNP were obtained from Sigma.

Follow-Up
All of the patients were followed up for a median of 7 years (range, 6 to 8 years) after the index admission via a linked mortality/morbidity dataset using each patient’s unique hospital record number. No patient was lost to follow-up. A total of 36 demographic or clinical parameters were collected for each patient. All of the information regarding potential cardiovascular events was obtained from source data, including charts of hospital stays and discharge letters.

The following events were assessed during long-term follow-up. Death from any cause was documented. Cardiovascular death was defined as death because of a myocardial or cerebral infarction or documented sudden cardiac death. Myocardial infarction was defined as death because of prolonged chest pain together with an elevation of creatine kinase >2 times the upper limit or new ST elevation in ≥2 contiguous ECG leads. Recurrent UAP was defined as hospitalization because of prolonged ischemic chest pain occurring at rest without diagnostic elevation of creatine kinase levels.

Statistical Analysis
In view of the anticipated high-cardiovascular risk of the patient cohort over medium-term follow-up, the principal end point of the study was the relationship between platelet NO response and the composite end point of total mortality and cardiovascular readmission rates. Secondary end points were total mortality, cardiovascular mortality, and cardiovascular readmission rates.

Differences between the groups were analyzed by Student unpaired t test or Fisher exact test, where appropriate. Cumulative event rates were estimated by Kaplan-Meier survival curves for categorical data. P values were determined by the use of log-rank statistics. Cox regression analysis was used to examine the potential relationships between continuous variables and events during the follow-up period. Multivariate analysis using Cox regression techniques was performed to examine potential interactions among the covariates. Variables entered in the models were NO response, age, sex, past myocardial infarction, index event, baseline platelet aggregation, discharge medical treatment, revascularization status, left ventricular systolic dysfunction (LVSD), concurrent disease states, and cardiac risk factors. All of the data were analyzed using SPSS (version 12.0). Data are expressed as mean±SEM unless otherwise stated.

Results

Patient Characteristics
Table 1 summarizes the clinical characteristics of the 51 patients examined, categorized according to platelet NO responsiveness. Overall, non-Q-wave infarction was present on admission in more than half of the cases. In addition, more than half of the patients had hemodynamically significant stenoses in ≥1 major coronary vessel, whereas most patients had well-preserved LV systolic function. Approximately half of the overall group underwent coronary revascularization (PCI or CABG) within 30 days of admission. The extent of concomitant disease burden as assessed by the Charlson index of comorbidity27 was not different between groups.

Mean platelet responsiveness to SNP for the total cohort was 29±3% inhibition of aggregation (Please see online at http://atvb.ahajournals.org). Among the patients with an impaired NO response, the mean value was 17±2% inhibition of aggregation compared with 51±3% inhibition of aggregation for the remainder of the cohort (P<0.001). Thus, mean platelet responses in this “normal” subgroup were ~15% less than normal population means described previously.18

Comparisons between patients with impaired (n=33) and normal (n=18) platelet NO responsiveness revealed that patients with an impaired platelet NO response were significantly older (Table 1). Consistent with this age differential, there were more men than women in this group. Patients with an impaired NO response were also more likely (although not statistically significant) to have previously experienced an AMI. However, there was no difference in the rate of revascularization procedures (PCI or CABG) within 30 days of their initial presentation or the extent of LVSD. In addition, there was a trend (P=0.06) toward a greater frequency of renal insufficiency in those patients characterized as having an impaired NO response. There was no difference with regard to the median duration of hospital admission between each group (7±1 versus 8±1 days).

Pharmacotherapy on hospital discharge included aspirin, ACE inhibitors, and prophylactic antiangial agents as indicated in Table 1; there was no difference between subgroups regarding pharmacotherapy. In general, pharmacotherapy with perhexiline was ceased after coronary revascularization. Moreover, follow-up data in patients who survived beyond the year 2002 demonstrated that ~80% and 60% of patients were prescribed a statin and ACE inhibitor, respectively.

Patient Outcomes
Morbidity and mortality rates were high during the 6- to 8-year period of follow-up. A total of 30 patients (59%) required emergency hospitalization for a recurrent cardiovascular event. The most common reason being recurrent ACS (~75% of cases). Moreover, 17 patients (33%) died. Causes of death were cardiovascular related in 11 of the 17 cases (see Table 2 for profile of these events). Cardiovascular events occurred in 16 (62%) and 14 (54%) patients with and without early revascularization, respectively. In total, 32 patients (63%) were either hospitalized with an acute cardiac event and/or died within the prolonged follow-up period.

Outcomes According to Platelet NO Response
Table 3 categorizes patient outcomes according to platelet NO response at the time of index admission. Patients with severely impaired platelet NO responsiveness tended to have more cardiovascular-related hospitalizations (66% versus 44%), although this difference was not statistically significant.
These patients were significantly more likely to experience the combined end point of death or cardiovascular-related hospitalization ($P=0.046$; see Figure a) or death ($P=0.013$; see Figure b). All of the cardiovascular-related deaths occurred in those patients with an impaired NO response ($P<0.005$), irrespective of revascularization status. Multivariate analysis showed that after adjusting for all of the other variables (including age, extent of coronary artery disease, comorbidity, LVSD, past MI, CABG, and cardiac risk factors), impaired platelet NO response was independently correlated with the combined end point of cardiovascular-related readmission or death. Those patients with an impaired NO response had an $≈3$-fold increased risk [relative risk (RR) 2.69; 95% CI, 1.03 to 7.10; $P=0.041$] of such an event occurring. Age (RR, 1.04; 95% CI, 1.00 to 1.09; $P<0.05$) and extent of platelet aggregation (RR, 1.09; 95% CI, 1.01 to 1.17; $P<0.05$) were also independently associated, albeit weakly, with the combined end point. Revascularization via CABG was not associated with the combined end point (RR, 1.24; 95% CI, 0.52 to 2.93; $P=0.63$). More importantly, impaired platelet NO responsiveness was an independent correlate of all-cause mortality. Those patients with an impaired platelet NO response had an $≈6$-fold increased risk of death ($P=0.033$) during long-term follow-up (Table 4).

### Discussion

The current study demonstrates that impaired platelet responsiveness to NO at admission is an independent predictor of poor outcomes (death/cardiovascular related readmission) in a high-risk population of ACS patients during prolonged follow-up. These patients were defined as being at high risk for future events because of ongoing symptomatic ischemia despite extensive pharmacotherapy. Almost half of the cohort underwent early (<30 days) revascularization. Of the total population, many were unsuitable for either cardiac surgery or coronary angioplasty (primarily because of diffuse coronary artery disease), and, in several cases, revascularization was known to be incomplete. Over the 6- to 8-year follow-up period, 33% of patients died, and 59% had a major cardiovascular morbid event. This very high incidence of ongoing morbidity/mortality permitted the evaluation of major determinants of outcome, despite the small patient number (51 patients in total).

The hypothesis tested was that severely impaired platelet responsiveness to SNP (and, thus, NO) would be an independent predictor of poor outcomes within this patient population. In fact, the majority of patients in this cohort had SNP responses $≥2$ SD below that of a normal population, and, thus, had “severely impaired” platelet NO responses. Among

### TABLE 2. Causes of Death During Follow-Up

<table>
<thead>
<tr>
<th>Cause</th>
<th>Normal Platelet NO Response</th>
<th>Impaired Platelet NO Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sudden cardiac event</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>AMI</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Progressive heart failure</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>CVA</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Progressive renal failure</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Terminal malignancy</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Chronic airways limitation</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

CVA indicates cerebrovascular aneurysm.

### TABLE 3. Summary of End Points

<table>
<thead>
<tr>
<th>End Point</th>
<th>Normal Platelet NO Response</th>
<th>Impaired Platelet NO Response</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular readmission</td>
<td>8 (44%)</td>
<td>22 (66%)</td>
<td>1.50 (0.85 to 2.65)</td>
</tr>
<tr>
<td>Cardiovascular readmission/death</td>
<td>8 (44%)</td>
<td>24 (73%)</td>
<td>1.64 (1.00 to 2.65)*</td>
</tr>
<tr>
<td>All cause mortality</td>
<td>2 (11%)</td>
<td>15 (46%)</td>
<td>4.1 (1.05 to 15.9)†</td>
</tr>
<tr>
<td>Cardiovascular mortality</td>
<td>0 (0%)</td>
<td>11 (33%)‡</td>
<td></td>
</tr>
</tbody>
</table>

*P=0.046; †P=0.013; ‡P<0.005.
such patients, the mortality rate was 46% (versus 11% for the “normal” NO responder group); there was also a significant excess of the combined end point of hospitalization plus mortality. Thus, severe impairment of platelet responsiveness to NO is a sufficiently strong correlate of outcome to emerge as highly significant in a small study of this type. Although other analogous studies have shown that vascular responsiveness to NO also predicts the risk of future events, it is possible that platelet dysfunction is a stronger, as well as a more direct, predictor. The other determinants of mortality emerging in this multivariate analysis (age, extent of coronary disease, extent of comorbidity, and LVSD) have been shown previously to be associated with poor outcomes, and, therefore, these results were not unexpected.

This is the first study to demonstrate that decreased platelet NO responsiveness is associated with adverse outcomes. However, other investigators have previously reported reduced platelet production of NO in patients with ACS. Freedman et al. demonstrated that platelets from ACS patients produced less NO when compared with patients with stable angina or normal volunteers. Furthermore, lower platelet-derived NO levels have also been shown in chronic cigarette smokers compared with normal volunteers. In a more recent study, Katoh et al. demonstrated that in patients with cardiovascular disease the amount of platelet-derived NO decreases as the number of coronary risk factors increases and that the decrease in platelet-derived NO was significantly correlated with a reduction in endothelium-derived NO. Analogously, Schafer et al. demonstrated that systemic inhibition of NO production causes marked platelet activation, which was reversed by an exogenous NO donor.

There may be a partial common underlying mechanism between impaired platelet NO responsiveness and decreased platelet NO production, because both of these parameters are modulated by rates of clearance of NO, largely by superoxide. Thus, both parameters may relate in part to levels of oxidant stress. Thus, if severe platelet hyporesponsiveness to NO indeed predicts future coronary events in a high-risk population of this type, the mechanism of association might not purely represent a propensity toward coronary thrombotic events.

Limitations of the Study
As stated above, this was a small study, involving high-risk ACS patients. Therefore, the results may not apply to the full spectrum of ACS patients, nor to those in whom revascularization can be “complete.” It is possible that long-term pharmacotherapy may alter the prognostic impact of platelet dysfunction, whether by augmenting NO responses (eg, ACE inhibitors and possibly statins) or by more effectively inhibiting aggregation directly (eg, ADP receptor antagonists). However, because of the strength of the association (poor NO response and all-cause mortality), a large prospective study addressing the above issues is warranted.

Conclusion
The current study is the first to demonstrate that impairment of platelet responses to NO is a strong and independent predictor of adverse outcomes in high-risk ACS patients. In addition, it provides further evidence that impairment of tissue responsiveness to NO is likely to be important prognostically and that platelets can be used to categorize subject status. However, the current data suggest that impairment of platelet NO responsiveness is a more sensitive prognostic marker than “traditional” measures of endothelial dysfunction. Larger studies using platelet NO responsiveness are needed to define more precisely the impact of such a phenomenon.

Acknowledgments
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References


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Figure I. Comparison of mean platelet Nitric Oxide (NO) response.

Legend:
Mean platelet NO response are shown for the total patient cohort (All, n=51). Patients were subsequently categorized into those having an impaired (Impaired, < 32% inhibition of ADP-induced aggregation) or normal (Normal, ≥ 32% inhibition of ADP-induced aggregation) platelet NO response. Platelet NO responses for the impaired group were significantly lower than the normal group (p < 0.001, Student’s unpaired t-test).