Aims
Heart failure is associated with deficient endothelial nitric oxide (NO) production as well as increased oxidative stress and accelerated NO degradation. The aim of this study was to evaluate platelet NO biosynthesis and superoxide anion (O$_2^-$) production in patients with heart failure.

Methods and results
In platelets from patients with heart failure due to idiopathic dilated cardiomyopathy ($n=16$) and healthy control subjects ($n=23$), NO synthase (NOS) activity was evaluated by L-[3H]-arginine to L-[3H]-citrulline conversion, cGMP was determined by radioimmunoassay, vasodilator-stimulated phosphoprotein (VASP: total and serine-239-phosphorylated) was assessed by western blotting, and O$_2^-$ production and O$_2^-$ scavenging capacity were measured by pholasin-enhanced chemiluminescence. In platelets from patients with heart failure, basal NOS activity was higher than in those from controls; furthermore, whereas platelet NOS activity increased as expected in response to albuterol or collagen in controls, no increase occurred in platelets from heart failure subjects. Despite this, basal intraplatelet NO-attributable cGMP was lower in heart failure than in control subjects, as was serine-239 phosphorylation of VASP, suggesting a decrease in bioactive NO. Platelets from heart failure subjects exhibited higher basal and collagen-stimulated O$_2^-$ production and impaired O$_2^-$ scavenging capacity, resulting in higher oxidative stress, consistent with the observed decrease in bioactive NO.

Conclusion
In heart failure, despite activation of NOS, platelets produce less bioactive NO, probably as a result of NO scavenging due to increased O$_2^-$ production. This functional defect in the platelet L-arginine/NO/guanylyl cyclase pathway could contribute to the platelet activation observed in heart failure.

Keywords
Nitric oxide • Superoxide anion • Oxidative stress • Platelets • Heart failure

1. Introduction
Despite the considerable recent advances made in the treatment of heart failure, its prevalence is increasing, the quality of life of patients remains impaired and its mortality rate remains high.$^1$ Much attention has focused on the role of endothelium-derived nitric oxide (NO) in this condition, since it plays an important role in regulation of vascular tone,$^{2,3}$ modulation of inflammation,$^4$ inhibition of vascular remodeling,$^{5,6}$ suppression of platelet adhesion to endothelium,$^7$ aggregation, and thrombus formation as well as leucocyte adhesion to vessel wall.$^8$ Traditional cardiovascular risk factors such as hypercholesterolemia, smoking, diabetes, and obesity are associated with impaired bioactive NO, an important characteristic of endothelial dysfunction, and independently of these risk factors, heart failure in itself may cause endothelial dysfunction.$^9$–$^{11}$

Despite reduced bioactive NO, conditions such as diabetes and hypertension have been reported to be associated with increased endothelial NO synthase type 3 (NOS3) activity and/or expression in the vasculature, but also with increased vascular superoxide anion (O$_2^-$) production.$^{12}$–$^{14}$ O$_2^-$ can react with NO very rapidly to form peroxynitrite (ONOO$^-$), a potent oxidant and nitrating agent, thereby both reducing NO availability and increasing oxidative and nitrative stress.

Although vascular NOS3 is mainly localized to the endothelium, platelets have also been reported to possess a functional L-arginine/NO pathway and to express NOS3.$^{15}$–$^{19}$ In the context of heart...
failure, platelets may play a pivotal role in much of the morbidity and mortality associated with the condition. Patients with heart failure are at increased risk of stroke and sudden cardiac death. These thrombosis-related complications have been attributed to a pro-thrombotic state in heart failure, the exact cause of which is unclear. 20

The pathophysiological role of NO in heart failure remains the subject of much controversy. There are contradictory reports in the literature concerning NO production in patients with heart failure, some suggesting increased production, 21–23 others no change, 24 and yet others reduced production. 25 Very little is currently known about platelet-derived NO and O2•− production and O2•− scavenging capacity.

2. Methods

2.1 Subjects

The study conforms with the Declaration of Helsinki. The King’s College Hospital Research Ethics Committee granted approval for the study and all subjects gave written informed consent. Sixteen patients with heart failure were recruited from the heart failure clinics at St. Thomas’ Hospital and King’s College Hospital, London, UK. All of these had heart failure diagnosed on clinical and echocardiographic criteria, with clinical features of heart failure coupled with a measured ejection fraction <40% on echocardiography, and were free of significant coronary atherosclerosis as determined by coronary angiography. Based on exclusion of other known causes of heart failure, the diagnosis in all cases was of idiopathic dilated cardiomyopathy. Other exclusion criteria were a history of hypertension, current blood pressure >140 mm Hg systolic and/or >90 mm Hg diastolic, evidence of other cardiovascular disease, use of recreational or other non-prescribed drugs, and the presence of any significant co-morbidities. Healthy control subjects of similar age and sex were recruited from the database of healthy volunteers held by the Department of Clinical Pharmacology, King’s College London, London, UK. They were free of any cardiovascular risk factors, were not on any anti-platelet drugs in the 7 days prior to the study. Subject characteristics are shown in Table 1. Patients with heart failure were on a variety of medications, which are shown in Table 2.

Blood was drawn in the fasting state for determination of full blood count, biochemical profile, lipid profile, glucose, glycated haemoglobin (HbA1C), high-sensitivity C-reactive protein (CRP), and homocysteine (Table 1). These measurements were carried out by the Departments of Hematology and Clinical Chemistry, St. Thomas’ Hospital. Additionally, von Willebrand Factor antigen (vWF:Ag) was measured by the Department of Hemostasis and Thrombosis, St Thomas’ Hospital.

2.2 Preparation of platelets

Subjects attended the Department of Clinical Pharmacology in the morning, having fasted overnight, and refrained from alcohol and caffeine since the previous evening. One hundred millilitres of blood was drawn from a large antecubital vein using a 19G butterfly needle, collected into tri-sodium citrate (0.38% final concentration), and centrifuged (200 g, 10 min, room temperature) to obtain platelet-rich plasma (PRP). Gel-filtered platelets were obtained by eluting PRP through a Sepharose gel column as previously described. 26

<table>
<thead>
<tr>
<th>Table 1 Subject characteristics</th>
<th>Heart failure subjects (n = 16)</th>
<th>Controls (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51.3 ± 4.1</td>
<td>50.9 ± 3.9</td>
</tr>
<tr>
<td>Males/females</td>
<td>12/4</td>
<td>17/6</td>
</tr>
<tr>
<td>NYHAII/III/IV</td>
<td>4/6/42</td>
<td>–</td>
</tr>
<tr>
<td>Caucasian/African/Asian</td>
<td>12/3/1</td>
<td>13/4/6</td>
</tr>
<tr>
<td>Pulse (beats/min)</td>
<td>70 ± 3</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127 ± 3</td>
<td>130 ± 4</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>74 ± 2</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.5 ± 3.0*</td>
<td>26.4 ± 4.1</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>13.8 ± 0.5</td>
<td>14 ± 0.3</td>
</tr>
<tr>
<td>WCC (×10³/μL)</td>
<td>6.7 ± 0.7</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>240.3 ± 20.8</td>
<td>260.1 ± 9.8</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>7.3 ± 0.2*</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>139.9 ± 1.2</td>
<td>139.1 ± 0.5</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.3 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>7.8 ± 1.1*</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>97.4 ± 7.0*</td>
<td>79.8 ± 3.2</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>72.0 ± 5.0*</td>
<td>90.0 ± 5.0</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.1 ± 0.2*</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>2.4 ± 0.2*</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.0 ± 0.1*</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2 ± 0.2</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6.1 ± 0.3*</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>21.7 ± 3.3</td>
<td>18.9 ± 1.6</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>3.5 ± 1.0</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>vWF:Ag (IU/dL)</td>
<td>214.8 ± 14.6*</td>
<td>165.0 ± 26.0</td>
</tr>
<tr>
<td>Beta-thromboglobulin (ng/mL)</td>
<td>32.1 ± 7.6*</td>
<td>18.9 ± 6.5</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>3275.0 ± 1621.1*</td>
<td>58.3 ± 9.4</td>
</tr>
</tbody>
</table>

BMI, body mass index; WCC, white cell count; MPV, mean platelet volume; eGFR, estimated glomerular filtration rate; vWF:Ag, von Willebrand Factor antigen; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1C, glycated haemoglobin; hs-CRP, high-sensitivity C-reactive protein; NYHA, New York Heart Association class; NT-proBNP, N-terminal pro-B-type natriuretic peptide; LVEF, left ventricular ejection fraction; LVEV, left ventricular end-diastolic volume; LVEDV, left ventricular end-diastolic diameter; LVMDs, left ventricular internal diameter in systole; LVMDd, left ventricular internal dimension in diastole; *P < 0.05 vs. controls.

2.3 Platelet NOS and cGMP measurement

NOS activity was measured from the rate of conversion of L-[³H]-arginine to L-[³H]-citrulline, and cGMP was determined by radioimmun assay, as previously described. 26 cGMP assays were all done in the presence of 3-isobutyl-1-methylxanthine 500 µmol/L as described. 26 NOS activity was measured as the difference in values in the absence and presence of the NOS inhibitor N⁵-monomethyl-¹-arginine (L-NMMA, 100 µmol/L). Similarly, the amount of cGMP produced in response to NO (NO-attributable
cGMP was determined as the difference in measured intraplatelet cGMP in the absence and presence of l-NMMA (100 μmol/L).

2.4 Measurement of platelet $O_2^-$ production and antioxidant capacity
Platelet-derived $O_2^-$ was measured by pholasin-enhanced chemiluminescence, as described in detail in the Supplementary material online.

2.5 Measurement of serum arginine, symmetric and asymmetric dimethylarginine levels, and arginase activity
Serum levels of arginine, symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) were measured by high-performance liquid chromatography following extraction using a solid-phase cation exchange column, as previously described.27

Serum arginase activity was determined as described by Sopi et al.28 Briefly, 50 μL serum was pre-activated by addition of 10 mmol/L MnCl2 followed by heating at 55°C in a total volume of 100 μL. An equal volume of 250 mmol/L L-arginine was added to samples (yielding a final concentration of 125 mmol/L L-arginine), and incubated for 30 min at 55°C. The arginase reaction was stopped by addition of 1 mL diaetyl monoxime/acid solution prepared as follows: 1 mL diaetyl monoxime reagent (3% in absolute ethanol) was added to 29 mL of an acid mixture containing $H_2$SO4, $H_2$PO4, and $H_2$O (1:3:7 v/v). Samples were heated at 100°C for 30 min and maintained in the dark for 10 min, before the absorbance of the urea (formed from arginine by the action of arginase) was measured spectrophotometrically at 490 nm and the concentration determined from a standard urea concentration calibration curve. One unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min. Data were expressed in U/L serum.

2.6 Determination of serine-239 phosphorylation of vasodilator-stimulated phosphoprotein
The degree of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at serine-239 was measured in platelet lysates by western blotting, as previously described.29

2.7 Statistical analysis
All data were expressed as mean $\pm$ SEM. Since data demonstrated a non-Gaussian distribution, within-group and between-group changes were analyzed by the non-parametric Friedman and Kruskal–Wallis tests, respectively, with Dunn’s post-hoc testing where significance was found. In all cases, $P < 0.05$ (two-tailed) was considered to be significant. Data analysis was performed using GraphPad Prism version 5.

3. Results
3.1 Subject characteristics
Patients and healthy controls were well matched in terms of age, sex, and race distribution. Heart failure subjects had higher body mass index in comparison to healthy controls. Mean platelet volume (MPV) was higher in heart failure subjects in comparison to healthy controls, as were vWF:Ag, β-thromboglobulin and HbA1c (known diabetes was one of the exclusion criteria for the study). Urea and creatinine were higher, and estimated glomerular filtration rate (calculated using the abbreviated Modification of Diet in Renal Disease Study equation) was lower, in heart failure subjects, suggesting that they had impaired renal function as compared with healthy controls, as might be expected. Total and low-density lipoprotein cholesterol, as well as triglycerides, were lower in heart failure subjects in comparison to healthy controls, and this is most likely attributable to the high usage of statin therapy in these patients. As expected, N-terminal pro-B-type natriuretic peptide levels were higher in heart failure subjects than controls.

3.2 Platelet NOS activity and bioactive NO
Basal platelet NOS activity was considerably higher in heart failure subjects in comparison to healthy controls (Figure 1A). Despite this, we found intraplatelet NO-attributable cGMP to be markedly less in the former group compared with the latter: 0.7 ± 0.6 vs. 121.4 ± 35.4 fmol cGMP/10⁸ platelets, respectively, ($P < 0.05$). In healthy subjects, platelet NOS activity significantly increased from baseline, upon stimulation with albuterol (10⁻⁵ mol/L) or collagen (0.8 μg/ml), although these agents are known to stimulate platelet NOS,18,26,30 in the present study the levels of platelet NOS activity reached after stimulation with either agent did not reach that found basally in subjects with heart failure (Figure 1A). In platelets from subjects with heart failure, no further increase was elicited in NOS activity in response to either albuterol or collagen. Platelet NOS activity, both basal and agonist-stimulated, and NO-attributable cGMP were not different between heart failure patients receiving or not receiving anti-platelet medications, warfarin, digoxin or statins. Platelet expression of NOS3 and of soluble guanylyl cyclase were not different between platelets from heart failure and control subjects, whilst neither NOS1 nor NOS2 expression were detectable, by western blotting (data not shown).

To confirm that the decrease in bioactive NO, as assessed by cGMP levels, in platelets from heart failure subjects gives rise to a functional consequence, we measured serine-239 phosphorylation of VASP in platelet lysates by western blotting, since phosphorylation of this residue is highly dependent on the activity of cGMP-dependent protein kinase. We found that, in platelets from heart failure subjects, serine-239 phospho-VASP was markedly reduced as compared with platelets from control subjects (Figure 1B).

Arginine levels were lower, and ADMA levels higher, in serum from heart failure as compared with control subjects (Table 3), with no difference seen in serum SDMA. Serum arginase activity was not different between the two groups (Table 3).

### Table 2 Medications taken by heart failure subjects (n = 16)

<table>
<thead>
<tr>
<th>Medication</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Blocker</td>
<td>15</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>1</td>
</tr>
<tr>
<td>Angiotensin II receptor blocker</td>
<td>3</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor</td>
<td>14</td>
</tr>
<tr>
<td>Statin</td>
<td>5</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>1</td>
</tr>
<tr>
<td>Warfarin</td>
<td>10</td>
</tr>
<tr>
<td>Furosemide</td>
<td>13</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>9</td>
</tr>
<tr>
<td>Digoxin</td>
<td>9</td>
</tr>
</tbody>
</table>
3.3 Platelet $O_2^-$ production and scavenging capacity

Baseline platelet $O_2^-$ production was increased in subjects with heart failure as compared with healthy controls (Figure 2). In both groups, incubation with collagen increased platelet $O_2^-$ production and collagen-induced $O_2^-$ production was greater in heart failure than in control subjects, when measured after 5 min of collagen co-incubation (Figure 2). This was reflected also by total platelet $O_2^-$ production over 30 min after addition of collagen, as assessed by the area under the curve of luminescence vs. time over this period (97 620 + 48 900 vs. 1 306 000 + 674 000 (luminescence units/10^8 platelets) × min in healthy vs. heart failure subjects, respectively, $P < 0.0001$).

Addition of platelets to Na-tyrode containing HRP and pholasin resulted in a decrease in light signal. This might be expected to occur simply due to the physical presence of platelets, but additionally as a result of $O_2^-$ scavenging by both enzymatic and non-enzymatic antioxidant systems present in platelets. Although a decrease in signal was found following addition of platelets from either group, the decrease in signal was attenuated with platelets from heart failure subjects as compared with those from healthy subjects (Figure 3). Since the data were normalized for number of platelets, this difference suggests a true difference in $O_2^-$ scavenging capacity.

4. Discussion

In the present study, we examined l-arginine/NO signalling in platelets from subjects with heart failure. Platelet-derived NO inhibits several aspects of platelet function, including their aggregation and recruitment following aggregation. In a mouse model deficient in platelet-derived NO, bleeding time is reduced, underlining its importance in modulation of platelet function and thrombus formation. Therefore, deficient platelet NO production in heart failure may contribute to the increased thrombotic tendency seen in this condition.

Elevated MPV has previously been found to be a marker of platelet activation. We found that MPV was higher in heart failure subjects, consistent with platelet activation in these subjects. This is supported by the concomitant elevation of vWF:Ag and more specifically of $\beta$-thromboglobulin in this group. We also found that HbA1c was higher in subjects with heart failure, which may indicate underlying impaired glucose tolerance or insulin resistance (even though diabetic...
Platelet nitric oxide and heart failure

Figure 3  

Platelet nitric oxide and heart failure

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subjects were specifically excluded from this study). β-blocker therapy is now routine in the modern management of patients with heart failure, but their long-term use is associated with increased incidence of diabetes.35

We found that platelet NOS activity was higher in heart failure subjects in comparison to healthy controls, suggesting that platelet NO biosynthesis is increased. Most studies that have assessed NO production in heart failure have either measured total body NO production (not specific to the site of NO production) or assessed different vascular beds, in both human and animal models. We have studied NO production only from platelets and the literature to date has very few studies of platelet NO production in heart failure. Similar to the findings of Drexler et al. and Habib et al., we found that, despite markedly increased platelet NO production in heart failure subjects at baseline, there was no further rise in response to stimulation by standard NOS agonists. Our findings suggest that in heart failure subjects, platelet NOS activity is already at maximum, with no further increase possible. The overall picture, therefore, is of an increase in NO biosynthesis in platelets from heart failure subjects, despite a decrease in plasma concentration of the principal substrate for NOS3 (L-arginine) and an increase in the plasma concentration of the principal endogenous inhibitor of NOS3 (ADMA), which is likely due to an increase in NOS3 activity despite unchanged levels.

On the other hand, despite elevated NOS activity, basal intraplatelet NO-attributable cGMP was reduced in subjects with heart failure, suggesting that, despite increased NO biosynthesis, there is accelerated NO clearance in these subjects. That this decrease in bioactive NO translates to a decreased functional response was confirmed by the observation that phosphorylation of VASP on serine-239, which is highly dependent on cGMP-dependent protein kinase, and which regulates filamentous actin formation with resultant effects on cell adhesion and motility, was markedly reduced in the platelets of patients with heart failure.

One of the most important means by which NO is degraded is by reaction with O$_2^-$. We therefore postulated that the apparent increase in NO clearance (and hence decrease in bioactive NO, as manifested by NO-attributable cGMP) in platelets might be explained by increased platelet O$_2^-$ generation, in heart failure. We measured platelet O$_2^-$ generation by pholasin-enhanced chemiluminescence in the absence and presence of Tiron. Tiron is a superoxide dismutase-mimetic and a cell membrane-permeable-specific scavenger of O$_2^-$; therefore, the difference in luminescence in the absence and presence of Tiron is a specific index of O$_2^-$ generation. We found that, indeed, platelets from heart failure subjects exhibit increased basal O$_2^-$ production in comparison to those from healthy subjects. Upon incubation with collagen, O$_2^-$ production increased in both heart failure and healthy subjects, but to a considerably greater extent in the former. Collagen was chosen as the stimulus, since platelet exposure to collagen is a major stimulus for platelet activation and thrombosis locally at sites of vascular damage. Increasing evidence suggests that generation of reactive oxygen species (ROS), including O$_2^-$, is increased in heart failure, and in particular O$_2^-$, can directly inactivate NO and cGMP,9 the second messenger of NO. O$_2^-$ reacts rapidly with NO to form peroxynitrite, which can uncouple NOS3,3 thereby resulting in a further decrease in NO production and a further increase in O$_2^-$ production, setting up a vicious cycle of decreased NO and increased O$_2^-$. Although the observed increase in oxidative stress is likely to explain the apparent reduction in bioactive NO in platelets from heart failure subjects in this study, we cannot exclude the possibility that the observed decrease in NO-attributable cGMP is in part caused by deficient production of cGMP (due to a reduction in activity of soluble guanylyl cyclase) and/or increased degradation of cGMP (due to increased phosphodiesterase activity). The latter possibility is unlikely, since in all our experiments cGMP measurements were performed in platelets pre-incubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. However, the possibility remains that soluble guanylyl cyclase activity is reduced in platelets from patients with heart failure; this in turn could conceivably arise as a result of oxidative damage to soluble guanylyl cyclase, although existing evidence suggests that soluble guanylyl cyclase oxidation, if anything, causes its activation rather than inhibition.36 Nevertheless, this possibility merits further study.

Various enzymatic and non-enzymatic sources of platelet ROS production have been characterized. It has been shown that collagen-induced platelet aggregation is associated with a burst of H$_2$O$_2$, a reactive oxygen species, which produces OH$^-$ through a Fenton-type reaction. H$_2$O$_2$ is also known to activate arachidonic acid metabolism, through phospholipase A$_2$ stimulation. De et al. have suggested that increased platelet O$_2^-$ in patients with heart failure is mediated by tumour necrosis factor-α, via activation of arachidonic acid metabolism and the NADPH-oxidase pathway. Platelets exposed to anoxia-reoxygenation generate O$_2^-$ and OH$, which in turn activate arachidonic acid metabolism via phospholipases A$_2$ and C, resulting in production of further O$_2^-$. Yamagishi et al. have shown that hyperglycaemia potentiates collagen-induced platelet activation through mitochondrial O$_2^-$ production. Our finding of increased HbA1c in heart failure subjects may therefore potentially create an environment for platelets to generate more O$_2^-$ upon stimulation with collagen.

An excess of O$_2^-$ may result from either increased O$_2^-$ production or impairment in O$_2^-$ scavenging capacity, or both, resulting in increased oxidative stress. Platelets are known to have reducing and antioxidant capacity, and to scavenge O$_2^-$ when co-incubated with stimulated polymorphonuclear cells generating an oxidative burst. We found that platelets from heart failure subjects have

![Figure 3](image-url)
impaired $\mathrm{O}_2^-$ scavenging capacity in comparison to those from healthy subjects. Impairment of antioxidant enzyme systems has been demonstrated in platelets from patients with coronary artery disease, as well as in myocardial cells in animal models of heart failure. The major antioxidant enzymes present in platelets are superoxide dismutase, catalase, and glutathione peroxidase. Whether the observed reduction in platelet $\mathrm{O}_2^-$ scavenging capacity results from a deficiency of one or more of these enzyme systems, or in other non-enzymatic antioxidant systems, remains to be determined.

An important limitation of this study is that all subjects with heart failure, unlike the controls, were on medications (often multiple). Indeed, 15 of 16 heart failure subjects were on $\beta$-blocker therapy, and all were either receiving an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker (and, in the case of one subject, both). Additionally, all heart failure subjects received one or more diuretics. Therefore, we cannot exclude the possibility that our results were influenced by use of these medications. It is possible, for example, that bradikynin release in response to angiotensin-converting enzyme inhibition may cause stimulation of NOS activity in platelets, and may even be a source of increased reactive oxygen species. However, for ethical reasons, it was not possible to study patients after a period of stopping these drugs. As for the other medications, we found that platelet NOS activity, both basal and agonist-stimulated, and NO-attributable cGMP were not different between heart failure patients receiving or not receiving anti-platelet medications, warfarin, digoxin, or statins. Although it remains possible that medications may have influenced our results, the existing literature suggests that most heart failure medications have little if any effect on platelet function (reviewed by Malinin et al.).

Additionally, we did not study any possible differences in platelet uptake of l-arginine in the present work. Platelet l-arginine uptake occurs purely through system y$^+$L and not system y$^-$. To date, no literature exists on platelet l-arginine transport in heart failure in humans, although very recently Matsuura et al. have reported no change in platelet l-arginine transport in a rat model of doxorubicin-induced heart failure (a widely used model of human dilated cardiomyopathy). Moreover, in erythrocytes from humans with heart failure, it has been reported that, whilst overall arginine uptake is increased, this is due to an increase in transport via system y$^+$L with no change in transport via system y$^-$. We therefore consider it unlikely that platelet l-arginine transport is altered in heart failure, although we did not specifically examine this in the present work since this was not the primary focus of the study.

Another limitation is that the present experiments do not allow us to distinguish between an increase in platelet $\mathrm{O}_2^-$ generation, a decrease in platelet antioxidant capacity, or both. To do this will require further experiments to measure individual antioxidant systems, both enzymatic and non-enzymatic, as well as to determine chemiluminescence signals in the presence of specific inhibitors of O$_2^-$-generating pathways: mitochondrial electron transport, NADPH oxidase, and indeed NOS3 itself. Further work is also required to confirm that the increase in platelet generation of NO in parallel with the increase in its inactivation by $\mathrm{O}_2^-$ translates to an increase in nitrosative stress, as manifested by an increase in nitrotyrosine, as well as to explore alternative mechanisms influencing local activity of ROS.

In conclusion, we have demonstrated that, in platelets from subjects with heart failure, there is impaired bioactive NO despite increased NOS activity, which can be explained by an increase in platelet oxidative stress and resultant increased $\mathrm{O}_2^-$ generation. Further studies are needed to evaluate the source of increased platelet $\mathrm{O}_2^-$ production, as well as to examine the precise nature of the defect in platelet $\mathrm{O}_2^-$ scavenging capacity, in heart failure.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Acknowledgement**

We are grateful to Professor Neil Dalton (Professor of Paediatric Biochemistry, King’s College London) for performing the ADMA, SDMA, and arginine assays reported here.

**Conflict of interest:** none declared.

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**References**


