Biological efficacy of low against medium dose aspirin regimen after coronary surgery: Analysis of platelet function

Jacqueline Cornelissen*, Stephen Kirtland*, Eric Lim, Martin Goddard, Sarah Bellm, Kate Sheridan, Stephen Large, Alain Vuylsteke
Department of Clinical Pharmacology and the Cardiothoracic Anaesthetic and Surgical Units of Papworth Hospital NHS Trust, Cambridge, United Kingdom

Summary
The failure of aspirin to inhibit platelet function has been documented in patients undergoing coronary artery bypass graft (CABG) surgery, but the causes of “aspirin-resistance” remain uncertain. The aim of this study was to investigate the efficacy of aspirin in patients undergoing CABG surgery receiving either 100 mg or 325 mg of oral aspirin for 5 days. Platelet function was tested the day before surgery and on day+1 and day+5, and evaluated by changes in collagen-induced thromboxane-A2 (TxA2) release and platelet aggregation following stimulation with collagen, ADP and epinephrine. In all patients, baseline platelet aggregation was significantly inhibited by pre-incubation with in vitro aspirin (150 µmol/l), with a mean reduction in TxA2-release of ≥95.5% (82.3, 99.1). After 5 days of oral aspirin, platelet aggregation was significantly inhibited, and was not further inhibited by in vitro aspirin. Oral aspirin was also associated with a >99.5% (97.8, 99.7) reduction in TxA2-release, and with the reversal of the second-phase of ADP-induced aggregation which is TxA2-dependent. In addition a single-dose of 325 mg aspirin on the first post-operative morning may have a greater inhibitory effect on collagen-induced aggregation than 100 mg aspirin. Western blot analysis provided no evidence for the presence of COX-2 in platelets, while the up-regulation of p38-MAPK following platelet-stimulation and surgery was seen. The inhibition of COX-2 (NS398) or p38-MAPK (SB203580) activity did not affect platelet aggregation and TxA2-release on day+5. In summary, there was no evidence for inherent or acquired aspirin-resistance in this surgical population, or for the involvement of either COX-2 or p38-MAPK.

Keywords
CABG surgery, aspirin, platelets, thromboxane A2

Introduction
The administration of aspirin following CABG surgery reduces mortality, ischaemic complications (1) and vascular occlusions (2). We have previously compared the effects of low (100 mg) and medium (325 mg) dose aspirin and clopidogrel (75 mg) following CABG surgery, and platelet aggregation following stimulation with collagen, ADP and epinephrine. In all patients, baseline platelet aggregation was significantly inhibited by pre-incubation with in vitro aspirin (150 µmol/l), with a mean reduction in TxA2-release of ≥95.5% (82.3, 99.1). After 5 days of oral aspirin, platelet aggregation was significantly inhibited, and was not further inhibited by in vitro aspirin. Oral aspirin was also associated with a >99.5% (97.8, 99.7) reduction in TxA2-release, and with the reversal of the second-phase of ADP-induced aggregation which is TxA2-dependent. In addition a single-dose of 325 mg aspirin on the first post-operative morning may have a greater inhibitory effect on collagen-induced aggregation than 100 mg aspirin. Western blot analysis provided no evidence for the presence of COX-2 in platelets, while the up-regulation of p38-MAPK following platelet-stimulation and surgery was seen. The inhibition of COX-2 (NS398) or p38-MAPK (SB203580) activity did not affect platelet aggregation and TxA2-release on day+5. In summary, there was no evidence for inherent or acquired aspirin-resistance in this surgical population, or for the involvement of either COX-2 or p38-MAPK.

Thromb Haemost 2006; 95: 476–82

*These authors contributed equally to this manuscript.

Financial support:
This study was supported by the Papworth Hospital NHS Trust and Papworth Surgeons Research Fund.

Received October 3, 2005
Accepted after resubmission January 5, 2006
Prepublished online February 10, 2006 DOI 10.1160/TH05–10–0649
and may be potentially aspirin-resistant (14). Evidence that COX-2 is not a source of thromboxane A2 (TxA2) in platelets that are insensitive to aspirin was also recently described, despite the potential blood loss frequently encountered during CABG surgery (13).

Stress-stimulated platelet p38-mitogen activated protein kinase (MAPK) activity may also play a role in aspirin-resistance. Epinephrine-induced platelet activation was shown to cause the COX-1 independent activation of cytosolic phospholipase A2 (cPLA2), through p38-MAPK, and to cause the reversal of aspirin inhibition in experimental models (15, 16). Platelet p38-MAPK has an important role in the regulation of cPLA2 and the subsequent liberation of arachidonic acid for conversion to TxA2 (15, 17), and stress upregulation of this pathway may potentially lead to platelet activation which is insensitive to aspirin.

In a randomised trial comparing the efficacy of low (100 mg) against medium (325 mg) dose oral aspirin in patients undergoing CABG surgery, we previously demonstrated that both doses were effective in inhibiting collagen-induced platelet aggregation on the 5th post-operative day, by 36% and 37% respectively (4). The generality of this response was unexpected considering the high proportion of non-responders reported by others. In this extension we therefore describe the changes in TxA2-release and its relationship with platelet aggregation, to further assess the efficacy of aspirin and the incidence of aspirin-resistance. The possible role of COX-2 and p38-MAPK expression in the behaviour of platelets to aspirin was also examined.

**Methods**

**Clinical trial protocol and study population**

Study data and samples were collected as part of a Local Research Ethics Committee approved prospective double-blind randomised trial (4). Patients undergoing elective primary CABG surgery were invited to participate, and excluded if they did not stop anti-platelet therapy a week prior to surgery; had contraindications to aspirin or clopidogrel; were on medications that interacted with aspirin or clopidogrel; if surgery was performed without cardiopulmonary bypass; if platelet transfusion was administered; and if extubation was not achieved within the first 24-hours. On the first post-operative morning, patients were randomised to receive one of the following identically encapsulated treatments, daily for the first 5 post-operative days: aspirin 100 mg, aspirin 325 mg, or clopidogrel 75 mg. Protocol compliance was monitored, and no other NSAIDs were administered to the study population during this 5-day period. Patients did not receive morphine, paracetamol and tramadol as part of their routine postoperative pain management.

Data was collected from 72 aspirin patients (66 males; mean age 66; range 46–79), and analysed by combining the two aspirin groups, as no significant differences in platelet aggregation were found between the two doses (4). Comparison samples were collected from the 18 patients receiving clopidogrel (17 males; mean age 64; range 44–77) and used as a negative control, as clopidogrel was shown to be ineffective at inhibiting platelet aggregation following CABG (3). Volunteer positive control samples (n=19) were also collected (10 male; mean age 38; range 21–58), with 5 subjects treated with 75 mg aspirin for 5-days.

**Platelet response ex vivo**

Blood samples were taken on the day prior to operation (day–1, baseline), then prior to and at 2-hours post-drug administration on day+1 (post-operative morning) and day+5. Assessment of platelet aggregation was undertaken using the technique of Born (18), and determined turbidimetrically using the Platelet Aggregation Profiler® PAP-4 (BioData Corporation, USA). Venous blood (30 ml) was collected into 3.8% trisodium citrate monovettes (Sarstedt), gently inverted, and centrifuged within 30 minutes of venipuncture at 1000 r.p.m for 15 minutes to obtain platelet rich plasma (PRP). PRP samples (225µl) were stimulated with 25 µl of agonist for 4.5 minutes, using at least four different concentrations of freshly prepared ADP (Sigma; final concentration range of 0.25–5.0 µmol/L), Horm collagen (Axis-Shield Diagnostics; range 0.11–4.4 µg/ml) and epinephrine (Sigma; range 0.125–5.0 µmol/L). Platelet aggregometry readings for each agonist were converted to EC50 (concentration required to cause 50% of maximal aggregation) using curve-fitting software (Curve Expert 1.34; Daniel Hyams, USA). Single percentage aggregation readings to collagen (1.1 µg/ml), epinephrine (0.5 µmol/L) and ADP (1.0 µmol/L) were also presented in the analysis to enable direct comparison to others studies.

**Platelet response to in vitro aspirin**

The response of collagen-stimulated platelets to aspirin in vitro was determined by the addition of 25 µl of aspirin solution [aspirin (Sigma) was freshly dissolved in sodium bicarbonate 0.1%w/v]. The aspirin solution was added to the PRP 1 minute before the addition of collagen to give a final concentration 150 µmol/L. For vehicle control experiments, 25 µl of the sodium bicarbonate solution was used.

**TxA2 levels**

TxA2 levels (measured as TxB2 by ELISA, Cayman Chemicals) were estimated in 61 aspirin treated patients, 8 clopidogrel patients, and 12 healthy volunteer samples. Following aggregation with 1.1 µg/ml collagen (+/− in vitro aspirin) platelet samples were immediately centrifuged (6000rpm, 1 minute) and indomethacin added to the supernatant (final concentration 10 µg/ml). The detection range was 0.2–200.0 µg/ml (with dilution), and individual patient samples were batch tested in duplicate.

**Platelet response to in vitro COX-2 and p38-MAPK inhibition**

Prior to the addition of a single concentration of collagen (1.1 µg/ml), platelet samples were incubated for 1 minute at 37°C with 1 µl of COX-2 inhibitor NS398 (Cayman Chemical) and p38-MAPK inhibitor SB203580 (CN Biosciences) [dissolved in DMSO/saline], to give final concentrations of 10 µmol/L and 0.1 µmol/L respectively. For vehicle controls, 1 µl of DMSO/saline solution was used.

**COX-1, COX-2 and pp38-MAPK levels**

Immediately following platelet aggregation with a single concentration of agonist [collagen (1.1 µg/ml) or epinephrine (0.5
µmol/L), samples were quenched with 1ml ice-cold isotonic Tris-saline solution [pH7.35; 0.12 mol/L NaCl, 0.03 mol/L Tris, 0.006 mol/L EDTA], pelleted (6000rpm 1 min) and resuspended with ice-cold HEPES extraction buffer (19). Western blotting was performed as previously described (19) with goat-anti-human COX-1 (1:2000; Santa Cruz Biotechnology); goat-anti-human with ice-cold HEPES extraction buffer (19). Western blotting was performed as previously described (19) with goat-anti-human COX-1 (1:2000; Santa Cruz Biotechnology); goat-anti-human COX-2 (1:1000; Biosource Europe). The mean EC50 for collagen covered more than a ten-fold range across the quartiles, and the individual values strongly correlated with percentage aggregation to a fixed dose of collagen (1.1 µg/ml) and the EC50 values for epinephrine or ADP-induced aggregation (Kendall’s tau, all <0.001). Baseline platelet responsiveness to in vitro aspirin, as measured by the significant increase in EC50 for collagen (Wilcoxon p<0.001) (Fig. 1), also varied across the quartiles (Table 1). With 1st quartile patients showing a greater increment in EC50 compared to patients with higher baseline EC50 levels.

Day-1 platelet sensitivity to collagen also corresponded with TxA2-release, with notably more TxA2 produced in the 1st, 2nd, and 3rd quartiles compared to the 4th quartile (Table 1). This suggests that the differences in sensitivity to collagen depend upon TxA2-synthesis. The addition of aspirin in vitro caused a substantial inhibition of TxA2-release across all patients, with a mean inhibition of ≥95.5% (82.3, 99.1) (Wilcoxon p<0.001) (Table 1). With patients showing a clear response at baseline to aspirin when added in vitro, it does not appear that these platelets display a pharmacodynamic aspirin-resistance.

**Table 1. Inhibition of agonist-induced platelet aggregation and TxA2-release by in vitro and oral aspirin.** Data presented as median (IQR).

<table>
<thead>
<tr>
<th>Day-1 (baseline):</th>
<th>Aspirin Patients (100mg or 325mg aspirin for 5-days)</th>
<th>Control Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st Quartile (n=19)</td>
<td></td>
</tr>
<tr>
<td>Collagen EC50 (µg/ml)</td>
<td>0.11 (0.11,0.11)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (with collagen 1.1µg/ml)</td>
<td>87.0 (83.0,90.0)</td>
<td></td>
</tr>
<tr>
<td>TxA2-release (µg/ml)</td>
<td>65.5 (42.7,115.0)</td>
<td></td>
</tr>
<tr>
<td>Collagen + in vitro aspirin EC50 (µg/ml)</td>
<td>0.62 (0.29,1.07)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (with collagen 1.1µg/ml)</td>
<td>66.0 (53.8,75.8)</td>
<td></td>
</tr>
<tr>
<td>TxA2-release (µg/ml)</td>
<td>4.8 (1.1,2.7)</td>
<td></td>
</tr>
<tr>
<td>ADP EC50 (µmol/L)</td>
<td>0.35 (0.03,0.41)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (1.0µmol/l)</td>
<td>90.0 (80.0,100.0)</td>
<td></td>
</tr>
<tr>
<td>Epinephrine EC50 (µmol/L)</td>
<td>0.34 (0.10,0.13)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (0.5µmol/l)</td>
<td>83.0 (77.0,94.0)</td>
<td></td>
</tr>
<tr>
<td>Day+5 (5-days oral aspirin):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen EC50 (µg/ml)</td>
<td>1.02 (0.89,1.46)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (with collagen 1.1µg/ml)</td>
<td>49.5 (35.8,58.5)</td>
<td></td>
</tr>
<tr>
<td>TxA2 production (µg/ml)</td>
<td>0.2 (0.2,0.3)</td>
<td></td>
</tr>
<tr>
<td>Collagen + in vitro aspirin EC50 (µg/ml)</td>
<td>1.45 (0.93,2.09)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (with collagen 1.1µg/ml)</td>
<td>40.8 (33.5,53.0)</td>
<td></td>
</tr>
<tr>
<td>ADP EC50 (µmol/L)</td>
<td>0.79 (0.65,1.08)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (1.0µmol/l)</td>
<td>52.0 (48.6,63.0)</td>
<td></td>
</tr>
<tr>
<td>Epinephrine EC50 (µmol/L)</td>
<td>0.97 (0.23,1.89)</td>
<td></td>
</tr>
<tr>
<td>% aggregation (0.5µmol/l)</td>
<td>41.5 (24.8,51.3)</td>
<td></td>
</tr>
</tbody>
</table>

**Results**

**Platelet sensitivity at baseline**

Kawasaki, et al. suggested that the enhanced response of platelets to low doses of collagen might be used to identify aspirin responders/non-responders (20). Therefore, for descriptive comparisons, platelet function measurements from the aspirin-treated patients were ranked according to baseline (day-1) EC50 values for collagen-induced aggregation, and divided into 4-quartiles (each quartile contained a similar number of low and medium dose aspirin patients) (Table 1). The control measurements from the clopidogrel patients and volunteers were also tabulated for comparison, and no significant differences across the baseline EC50s for all three agonists were found between the patient and control groups (Kruskal-Wallis, all NS).

On day-1, the mean EC50 for collagen covered more than a ten-fold range across the quartiles, and the individual values strongly correlated with percentage aggregation to a fixed dose of collagen (1.1 µg/ml) and the EC50 values for epinephrine or ADP-induced aggregation (Kendall’s tau, all <0.001). Baseline platelet responsiveness to in vitro aspirin, as measured by the significant increase in EC50 for collagen (Wilcoxon p<0.001) (Fig. 1), also varied across the quartiles (Table 1). With 1st quartile patients showing a greater increment in EC50 compared to patients with higher baseline EC50 levels.

Day-1 platelet sensitivity to collagen also corresponded with TxA2-release, with notably more TxA2 produced in the 1st quartile compared to the 4th quartile (Table 1). This suggests that the differences in sensitivity to collagen depend upon TxA2-synthesis. The addition of aspirin in vitro caused a substantial inhibition of TxA2-release across all patients, with a mean inhibition of ≥95.5% (82.3, 99.1) (Wilcoxon p<0.001) (Table 1). With patients showing a clear response at baseline to aspirin when added in vitro, it does not appear that these platelets display a pharmacodynamic aspirin-resistance.
Platelet response following oral aspirin

Following 5-days of oral aspirin there was a significant inhibition of TxA2-release \( \geq 99.5\% (97.8, 99.7) \) (Wilcoxon \( p < 0.001 \)) and platelet aggregation (Wilcoxon \( p < 0.001 \)) compared to baseline (Table 1, Fig. 1). Furthermore, exposure to in vitro aspirin caused little further increase in the EC50 for collagen (Table 1, Fig. 1), suggesting a high degree of pharmacodynamic response to oral aspirin in this surgical population. The overall level of patient responsiveness to oral aspirin is reflected by the control group measurements, with similar responses to the surgical population seen in the volunteer group. While the day+5 measurements for the clopidogrel patients remain comparable to baseline (Table 1).

Patients in the 1\textsuperscript{st} quartile again tended to show a greater increment in EC50, with similar EC50 levels on day+5 found across the quartiles for all three agonists (Table 1). Thus patients with an enhanced response to collagen at baseline do not appear to demonstrate an inherent resistance to oral aspirin. Though the surgical group has a wide response range to collagen on day+5, it is doubtful that the small residual levels of TxA2 can account for the small differences seen in the EC50 and percentage aggregation between quartiles on day+5.

ADP-induced aggregation

The responses to ADP based on changes in maximal aggregation within the 4.5 minute period were used to generate the EC50 measurements for the primary analysis (4) (Table 1). On day+5 the maximum aggregation with 1.0 \( \mu \)mol/L ADP was 50.0% (42.5, 58.5), compared to 72.0% (42.8, 87.8) at baseline (Wilcoxon \( p < 0.001 \)). When the second-phase response (or irreversible aggregation) was considered, oral aspirin was found to inhibit this TxA2-mediated response. Thus on day+5, the final (4.5 minute) aggregation readings were significantly reduced to 33.0% (16.5, 51.0), compared to 72.0% (42.8, 87.8) at baseline (Wilcoxon \( p < 0.001 \)).

Platelet aggregation across the dosing interval

On day+1 platelet function was determined before the dose of aspirin, and then again in a random selection of patients (\( n=37 \)) at 2-hours post-dose. It was found that EC50 responses to collagen (Fig. 1), ADP and epinephrine were significantly inhibited following a single dose of aspirin, indicating exposure to aspirin early after surgery [collagen, 0.25 (0.13, 0.84) vs. 0.9 (0.28, 1.32); ADP, 0.69 (0.43, 1.22) vs. 1.2 (0.68, 1.58); epinephrine, 0.26 (0.13, 0.85) vs. 1.1 (0.25, 2.54); respectively] (Wilcoxon, all \( p < 0.001 \)). When analysed separately, a single dose of either low (\( n=19 \)) or medium (\( n=18 \)) aspirin significantly reduced collagen-induced aggregation (Mann-Whitney, both \( p < 0.001 \)). However, the medium dose group had a mean post-dose/pre-dose EC50 for collagen ratio of 5.41 ± 3.98, compared to 2.19 ± 2.01 for low dose (Mann-Whitney \( p = 0.45 \)). This observation suggests that patients may achieve a greater exposure to aspirin if a single dose of 325 mg is given on the first post-operative morning. On day+5 no further differences were seen across the dosing interval for either of the regimes, with a mean pre/post ratio of 1.30 ± 0.33 (\( n=5 \)) for the medium dose and 1.90 ± 1.37 (\( n=7 \)) for low dose.

Platelet response to COX-2 and p38-MAPK inhibitors in vitro

At baseline and day+5, the inhibition of COX-2 with NS-398 in vitro did not significantly reduce collagen-induced aggregation \([-2.5% ± 14.1 (n=11) \] and \(+3.2% ± 18.4 (n=13) \), respectively\). However, a reduction in TxA2-release was seen at baseline, but...
not on day+5, in both patients [-32.0% ± 30.0 (n=8)] and volunteers [-32.3% ± 9.9 (n=3)]. This disparity is probably due to the limited selectivity of NS-398 for COX-2, reducing TxA2-release through COX-1 inhibition. Furthermore, both patient (n=47) and volunteer (n=4) were tested for platelet COX-2 expression and no quantities of protein were found (Fig. 2).

At baseline, Western blot analysis of both patient (n=47) and volunteer samples (n=4) showed the phosphorylation of p38-MAPK following collagen and epinephrine stimulation (Fig. 2). The up-regulation of p38-MAPK was also seen in day+1 and day+5 controls from both aspirin treatment groups, suggesting that platelets were highly stimulated following surgery. On day-1, inhibition of p38-MAPK with SB203580 in vitro, reduced platelet aggregation and TxA2-release following stimulation with collagen (n=7) [-24.4% ± 34.5 and –30.8% ± 46.1, respectively], and epinephrine (n=7) [-11.9% ± 33.2 and –31.7% ± 39.5, respectively]. Following exposure to oral aspirin, this inhibitory effect of SB203580 was no longer seen. Furthermore, the overall activation of p38-MAPK signalling in response to agonist was reduced on day+5 (Fig. 2), compared to baseline. These findings indicate reduced signalling through COX-1, and demonstrate that the up-regulation of p38-MAPK is not an important factor in the residual aggregation occurring in platelets on day+5.

Discussion

Aspirin resistance may impose limitations on the clinical usefulness of aspirin for the treatment of potential occlusive events including those that can occur after CABG (7, 8). It has been suggested that the effects of aspirin on the formation of TxA2 may provide a more acceptable pharmacological indicator of “true” aspirin resistance than its effects on aggregation or other functional changes (9). Considering this background, a notable feature of the present study is the fact that oral aspirin was effective in inhibiting platelet TxA2-release. Thus, in the 72 patients studied, aspirin caused ≥99.5% (97.8, 99.7) mean inhibition of collagen-induced TxA2-release on day+5 as compared with baseline, with a mean TxA2-release on day+5 of ≤0.2 μg/ml (0.3,0.7). Moreover, the production of TxA2 was substantially inhibited ≥95.5% (82.3,99.1) by in vitro aspirin on day–1, despite the use of a sub-maximal inhibitory dose. Aspirin was used in vitro in order to evaluate its pharmacodynamics in view of a report that there are a high proportion of pharmacodynamic non-responders among bypass patients (7, 8). It should be noted that the inhibition of aggregation by aspirin in vitro is enhanced by prolonged pre-incubation of the drug with platelets before the addition of agonist. This test is therefore detecting responsiveness to aspirin, but does not define its maximal effects. In summary, the overall data for collagen-induced TxA2-release in this group of patients does not support the concept of a high prevalence of aspirin “non-responders” among patients elected for bypass surgery.

Despite the widespread use of aspirin in CABG patients, its effect on the key COX-1 metabolite TxA2 has been little investigated and the findings are conflicting. Some have reported that oral aspirin blocks collagen-induced aggregation and TxA2-release (5, 6). These and the present findings contrast with the study by Zimmermann, et al., which reported a 30% inhibition of TxA2-release on day+5 following CABG surgery in 24 patients, but found no inhibition of collagen-induced aggregation up to day+10 (8). They concluded that CABG patients were aspirin-resistant in the early post-operative period, an observation that is difficult to reconcile with the present data. It is possible that some difference/s in bypass procedure may determine the platelet response to aspirin. Indeed, haemostatic changes during CABG surgery have been shown to depend on whether surgery is carried out on- or off-pump (21, 22), on the nature of the pump (23), on the anticoagulant regime (24) and on perfusion temperature (25). It has been suggested that patients elected for bypass surgery are predisposed to hyperactive and aspirin-insensitive aggregation (26), though there was no evidence for this in our patient population. The baseline EC50s for all three agonists and in vitro responses to aspirin were not significantly different between the patient and control groups. This conclusion though, has the reservation that the volunteers were not matched for all the variables that can affect platelet function.

The changes in aggregation across the dosing interval for aspirin on day+1 and day+5 also demonstrated general platelet responsiveness to aspirin. The differences between the EC50 responses between the pre- and post-dose tests are considered to reflect the appearance of aspirin in blood, and are indicative of responses to aspirin that depend on both its pharmacokinetics and pharmacodynamics. It is possible that on the first post-operative morning, a single dose of 325 mg aspirin may provide better early anti-thrombotic cover than a 100 mg dose, because of the higher post-dose/pre-dose EC50 for collagen ratios in the medium dose group. These differences between the two regimes were no longer present by day+5, and are most readily interpreted in terms of the penetration of aspirin into megakaryocytes, so that a proportion of circulating platelets already possess impaired COX-1 activity. It is unclear whether these inhibitory effects of oral aspirin on aggregation on day+5 are maximal, as most patients were discharged by day 5. Studies in healthy volunteers have shown the maximal inhibition of epinephrine and ADP-induced aggregation by day+3 and collagen-induced TxA2-release by day+7 (27). However, these timescales cannot be extrapolated to on-pump CABG patients in whom there is evidence of accelerated platelet production and turnover (21).

Kawasaki, et al. suggested that the platelets of subjects that are most sensitive to aggregation with collagen are more likely to be resistant to aspirin (20). To examine this possibility, the data was divided into quartiles based on the baseline EC50s to collagen. This showed that patients in the lowest EC50 quartile released more TxA2. However, patients in this quartile were not less sensitive to aspirin, and actually demonstrated a greater improvement in EC50 to collagen in the presence of in vitro or oral aspirin, with final day+5 EC50 to collagen readings similar to the other quartiles. Despite the sensitivity to aspirin of platelets from the first quartile, this group also showed the highest residual aggregation in the presence of oral aspirin. These patients could not be defined as “resistant” or as “non-responders” to aspirin, but do appear to possess more of a component of aggregation that was not blocked by aspirin. The most likely mediator of this residual aggregation is platelet ADP, which is an important mediator of aggregation in human PRP (28). Considering the quartiles, patients with a low EC50 to collagen were also more
responsive to ADP. In the present study, aspirin caused the reversal of second-phase ADP-induced aggregation, which is TXA2-dependent. It is possible that the high residual aggregation in those patients who are most responsive to agonists reflects their heightened response to ADP.

Prior to this study, there have been conflicting reports on the expression of COX-2 in platelets and its potential role in aspirin-resistance (12–14, 29–33), and the present work found no evidence for the existence of COX-2 in platelets of either volunteers or patients. Rocco, et al. also demonstrated near complete inhibition of TXA2-release with 50 µmol/L aspirin in vitro, even though COX-2 expression was estimated at 30–60% in these patients (14). Zimmermann, et al., also demonstrated a high expression of COX-2 in platelets from patient undergoing CABG surgery (13). These patients had high residual TXA2-release present at 5–10 days post-surgery, which was not attenuated by the in vitro aspirin or celecoxib. They concluded that COX-2 was not producing functionally relevant levels of TXA2 to be responsible for aspirin-resistance (13).

A possible reason for the discrepancies in platelet function and COX-2 measurements seen in the present study could be due to reduced blood loss and platelet depletion/turnover compared to previous studies. Newly formed COX-2 positive platelets would therefore be in lower abundance and Western blot detection may not have been sensitive enough (32). However, platelets counts (mean x10^3/µl ± SD) taken at baseline (221.2 ± 61.6), day+1 (179.2 ± 52.4) and day+5 (255.2 ± 68.1), were found to be comparable to those published by Zimmermann, et al. (17, 21). In addition, mean platelet counts on day+5 were not significantly different between the two aspirin dose groups.

The up-regulation of platelet p38-MAPK signalling following surgery was also suggested to have a potential role in aspirin-resistance, possibly through CPLA2-dependent release of arachidon-acid for conversion to TxA2 in platelets (15–17). Indeed, the up-regulation of p38-MAPK was seen in day+1 and day+5 control samples. However, the overall activation of p38-MAPK signalling in response to agonist was reduced following oral aspirin, and SB203580 had no further effect on TXA2-release. Thus, this pathway does not seem to be an important mediator of the day+5 residual aggregation levels either.

In the surgical population studied here, no evidence for resistance to aspirin was evident from the changes in collagen-induced aggregation and TXA2-release. Quartile responses based on sensitivity to collagen did identify high sensitivities to agonists in a number of patients, but these were equally responsive to aspirin overall. From a clinical standpoint the final EC50 response to oral aspirin may be more critical than the actual change induced with aspirin. In this regard, the present study has demonstrated a degree of residual aggregation in all patients that appeared quartile-dependent, but which was not associated with aspirin-insensitive TXA2-release. Other signalling pathways independent of COX-1, possibly involving enhanced responses to ADP (34), may contribute to this residual platelet aggregation in these patients.

Acknowledgments
We gratefully acknowledge the support of Dr Andrew Trull, Dr Kanchen Rege, Mr Tom Routledge, Mrs Helen Munday and Mr Ayyaz Ali from Papworth Hospital for project team assistance; Dr Linda Sharples and Dr Angela Wood from the MRC Biostatistics Unit, Cambridge; Dr Nick Morrell from the Department of Medicine, Addenbrooke’s Hospital, Cambridge for kindly donating COX-1/COX-2 positive control samples. We would also like to thank the assistance of staff from the R&D, Pharmacy and Phlebotomy Departments, and the nursing staff of the Surgical Unit of Papworth Hospital NHS Trust.

In memory of Dr. Andrew Trull, who sadly passed away on April 1st, 2004.

References