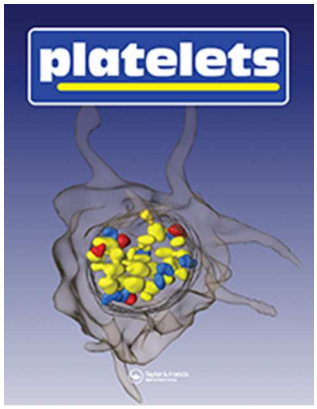

Changes in platelet function with inflammation in patients undergoing vascular surgery.

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TITLE PAGE

"Changes in platelet function with inflammation in patients undergoing vascular surgery."

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ABSTRACT

The role of platelets in ischaemic events is well established. Aspirin represents the default antiplatelet and blocks the metabolism of arachidonic acid (AA) at the cyclo-oxygenase enzyme (COX). AA is commonly used as a test of response to aspirin, but recent data raise uncertainty about the validity of this approach. Specifically, in some patients AA-induced clotting is not suppressed, but the level of COX-dependent AA metabolite, thromboxane B2 (TXB_2) is negligible. Furthermore, AA-induced whole blood clotting varies dynamically in individuals, who are aspirin responsive according to TXB_2 levels.

The aim of this study was to assess the level of AA-, ADP- and thrombin mediated platelet reactivity in patients on aspirin before, during and after major vascular surgery, which represents a model of on/off vascular inflammation. Firstly, we hypothesised, that in association with this inflammatory episode AA-, ADP- and thrombin-induced clotting would change in a dynamic manner. Secondly, that AA-induced clotting will be modified despite complete suppression of platelet TXB_2 production by aspirin throughout the periprocedural period, possibly via a lipoxygenase-mediated mechanism.

Fourty patients underwent major vascular surgery (open abdominal aortic aneurysm operation, infrainguinal bypass for subcritical limb ischaemia or peripheral aneurysm repair with bypass). They were all on 75 mg of aspirin prior to and throughout the perioperative period and received 5000 units of unfractionated heparin intraoperatively. AA-, ADP- and thrombin- induced clotting, AA metabolites (TxB_2 and 12-Hyroxyeicosatetraenoic acid (12-HETE)) and inflammatory markers (CRP, IL-6, TNF-α and CD40) were measured pre-procedure and at 2, 24, 48 hours, 3 to 5 days and 3 months after surgery. AA-, ADP- and thrombin- induced platelet reactivity was assessed using thrombelastography. TxB_2, 12-HETE, IL-6, TNF-α, CD40 were determined using the sequential competitive binding

Enzyme-Linked ImmunoAssay technique and CRP was determined using an immune-turbidimetric test on human serum.

There was a transient rise in inflammatory markers in the early perioperative period (CRP at 24, 48 hours and 3 to 5 days $p<0.001$ and IL-6 at 2, 24, 48 hours and 3 to 5 days $p<0.001$ as compared to baseline). Patients had negligible levels of TxB throughout, confirming a consistent therapeutic response to aspirin. There was a transient rise in thrombin-mediated clotting ($MA_{Thrombin}$ at 48 hours $p=0.001$ and 3 to 5 days $p<0.001$) and a fall in AA- and ADP-induced clotting in the early post op period (both MA_{AA} and MA_{ADP} $p=0.001$ at 2 hours). At 3 months, the level of AA- and ADP-induced clotting was significantly higher than at baseline ($p=0.008$ for MA_{AA} and $p=0.002$ for MA_{ADP}), hence demonstrating a rebound effect.

These data demonstrate a novel dynamic variation in platelet aggregation with acute vascular inflammation, including AA-induced whole blood clotting which is apparently COX-1 independent.

INTRODUCTION

The role of platelets in the pathophysiology of acute atherothrombotic events is well established. Aspirin represents the default antiplatelet agent in primary and secondary cardiovascular (CV) prevention strategies, based upon significant outcome advantage. (1) In particular, in patients presenting with acute coronary syndrome aspirin is considered essential for the prevention of reinfarction and stent thrombosis. (2, 3) In these patient populations, the conventional strategy is to commit patients to dual antiplatelet therapy (DAPT) that includes aspirin plus a $P2Y_{12}$ inhibitor for a limited period of time.

Despite the central role that aspirin plays in our treatment of patients after stent placement, there is a growing body of evidence that there are flaws in the "one size fits all" standard dosing that we employ for both aspirin and $P2Y_{12}$ inhibitors. Specifically, reports in the literature describe aspirin resistance between 10-90% in patients with CV disease. (4-12) These reports are often based upon platelet function tests (PFT) using arachidonic acid (AA) as the agonist. Recent data from this group and others raise important questions about the validity of assays employing AA as a test of the true functional activity of aspirin at its pharmacological target. (13-15) This activity inhibits production of thromboxane A_2 (TxA2) and the subsequent generation of its metabolite thromboxane B2 (TXB_2). Specifically, in patients treated with intracoronary stents and in populations with ischaemic stroke we have previously demonstrated a clear disconnect between apparent hyporesponsiveness/"resistance" to aspirin, as determined by AA-induced clotting using thrombelastography (TEG), and serum TXB_2 concentration, a true biochemical test of aspirin activity at its pharmacological target. (16-18) In these studies, AA-induced clotting was not significantly inhibited despite negligible concentrations of TXB_2: an outcome that would previously have been diagnosed as aspirin "resistance", based upon the AA-based test result, but is actually inconsistent with this conclusion based upon the biochemical evidence.

These data have raised two important questions. Firstly, is it now unsustainable to use AA-based PFT to assess the true clinical response to aspirin? The data described above suggest that this is indeed the case. Secondly, by what mechanistic pathway does AA induce whole blood clotting if not predominantly via the cyclooxygenase/TxA2 axis? Regarding the second question, we have previously demonstrated that in patients stopping clopidogrel 1 year after the insertion of drug-eluting stent(s), AA-induced clotting progressively increased over the next 2 weeks, despite complete suppression of TXB_2 production. (16) The implication of this observation is that there is a recruitable cyclooxygenase- and aspirin-independent pathway that metabolises AA and results in whole blood clotting. The nature of this alternative pathway is not yet explained, but production of AA metabolites via the lipoxygenase (LOX) pathway is one possible explanation that requires investigation. Thus, LOX`s stable metabolite 12-Hyroxyeicosatetraenoic acid (12-HETE) will be measured as part of our study to determine potential association with AA-mediated platelet activation and TXB_2 levels. Further, given the observation that non-cardiac inflammatory conditions, such as chest infection (19, 20), surgery for fractured neck of femur (21), exacerbations of rheumatoid conditions (22, 23) or psoriasis (24) can be temporally associated with acute coronary thrombotic events, we speculated that vascular inflammation could be a trigger for such a recruitable pathway for AA-induced clotting.

The aim of this study was to assess the level of AA-, ADP- and thrombin mediated platelet reactivity in patients taking aspirin before, during and after undergoing major vascular surgery (MVS), which represents an “on-off” model of intense vascular inflammation. (25, 26) Firstly, we hypothesised that in association with this inflammatory episode AA-, ADP- and thrombin-induced clotting would change in a dynamic manner. Secondly, that AA-induced clotting will be modified despite complete suppression of platelet TXB_2 production

by aspirin throughout the periprocedural period, possibly via a lipoxygenase-mediated mechanism.

METHODS:

Ethical approval

This study was sponsored by University Hospital Southampton NHS Foundation Trust Research and Development department, approved by the National Research Ethics Service Committee East of England – Essex (REC reference: 13/EE/0300, IRAS project ID: 111580) and registered on the National Institute for Health research portfolio database. All study participants provided written informed consent.

Study population

Our aim was to recruit 40 patients who were electively admitted to University Hospital Southampton for MVS. All patients were over 18 years old and were established on aspirin 75mg daily for at least 5 days preoperatively. Exclusion criteria consisted of: surgery in an emergency setting, current infection, liver failure, renal failure requiring dialysis, platelet count <150,000, medications including steroids, anticoagulants, non-steroidal anti-inflammatory drugs, COX-inhibitors and antiplatelets other than aspirin. In addition to this, participants were withdrawn from the study in pre-specified circumstances such as receipt of either platelets intraoperatively or more than two units of packed red cells perioperatively. In addition, the cessation of aspirin in the postoperative period was also part of the exclusion criteria.

Study design

All study participants were continued on 75mg of aspirin daily perioperatively, with administration in hospital witnessed and compliance strongly encouraged in the community.

As per standard surgical practice all patients were given periprocedural 5000 units of unfractionated heparin. Venesection was performed at 6 pre specified time points during the study period (T1-T6): a) T1- baseline sample - not more than 48 hours pre surgery, b) T2 – 2 hours after the operation, c) T3- 1 day after the operation, d) T4 – 2 days after the operation, e) T5- 3 to 5 days after the operation and f) T6 – more than 3 months after the initial procedure.

Blood sampling

Blood sampling was performed using 18-gauge needle either from the antecubital fossa or similar large superficial vein or from central venous/arterial line in those in the early post op period. The first 2 ml of blood collected was discarded (10 ml if using a CVC or arterial line) as per the manufacturer`s instructions. Subsequently, blood was collected into a 2 ml 3.2% sodium citrate vacutainer for thrombin channel analysis. Citrate chelates Ca2+ ions, thus inhibiting clotting activation, which was then reversed by the addition of 20uL 0.2M CaCl2 to the thrombin channel during TEG analysis. Final concentration of the prepared reagent was 555.56M/L CaCl2, as 1ml of citrated blood was added into kaolin and 340uL of this solution was mixed with 20uL of 0.2M CaCl2. For TEG platelet mapping channels, blood was collected into a 6ml lithium heparin (102 units) vacutainer. After 15 minutes, both blood samples were gently inverted 5 times before analysis. Further blood was collected into two serum separating tubes (SST) and after a period of 30 minutes centrifuged at 1000 x g for 15 minutes. Finally, separated serum samples were divided into 250µl aliquots and placed in a -80°C freezer for batch analysis.

Thrombelastography

TEG is an *ex vivo* whole blood clotting assay, which assess changes in viscoelasticity during blood clotting. These changes are conducted to the pin, which generates torque and produces an electrical signal of varying magnitude. (27) This signal is represented by a graphic visualisation, which consists of several parameters such as (i) R - reaction time, (ii) K

- speed of clot formation, (iii) α - rate of clot formation and (iv) Maximal amplitude (MA) – strength of the final clot.

TEG platelet mapping (Haemonetics Corp, Massachusetts, USA) was used according to the manufacturer's instructions, with the following channels: a) kaolin with heparinise - reverses the effect of heparin, b) activator F (reptilase and factor XIIIa) - activates fibrin formation without activating platelets, c) activator F and AA – activates platelets via TxB_2 production, pathway targeted by aspirin and d) activator F and ADP – activates platelets via $P2Y_1$ and $P2Y_{12}$ receptors, targeted by thienopyridines (i.e. clopidogrel). TEG has been shown to correlate well with light transmission aggregometry (LTA) and MA is proven to be a predictive tool for ischaemic events (28, 29). This group has previously described a novel TEG parameter called the Area Under the Curve (AUC15) which is a representation of both the speed and the strength of clot formation (30). It is calculated using the computer software National Instrument Labview 7.0 (Areafinder 2:1). AUC15 provides a rapid assessment of platelet reactivity, within 15 minutes, and has been shown to strongly correlate with MA. (31)

AA metabolites analysis

TxB_2 levels were determined using the sequential competitive binding Enzyme-Linked ImmunoAssay (ELISA) technique (R&D Systems, Abingdon, UK) as per the manufacturer's instructions. All samples, after a 2-fold dilution, were measured in duplicate and compared to known standards and maximum binding control. Previous studies have demonstrated that more than 98% inhibition of platelet COX-1 activity can be achieved with ingestion of 100mg of aspirin, which is related to serum TXB_2 concentration of <10 ng/ml. (32) Using the same principle we assumed that a TXB_2 concentration of less than 10ng/ml is an evidence of adequate therapeutic response to aspirin. (33)

12-HETE was measured after an 8-fold dilution using a commercially available ELISA kit (Enzo, Lause, Switzerland) as per the manufacturer`s instructions. Similarly to TXB_2, 12-

HETE final concentrations were calculated using four parameter logistic curve-fit in Microsoft Excel.

Inflammatory biomarkers

CRP levels were determined using an immune-turbidimetric test on human serum on Beckman Coulter AU analysers (High Wycombe, UK). Serum IL-6, sCD40-L and TNF-alpha levels were all measured using commercially available ELISA kits (R&D Systems, Abingdon, UK).

Statistical considerations and analysis

The CESSATION study (16) was used to calculate the sample size. This was done by a medical statistician using the G-Power software package (Version 3.1.3, Universitat Kiel, Germany, 2010). Assuming a normal distribution, a sample size of n=38 was deemed to be sufficient for a two-tailed, matched pairs t-test to detect, with 95% power, significant difference at the level of $p<0.05$, in AA-mediated platelet activation. However, based on a lack of normal distribution, the non-parametric Wilcoxon signed-rank matched pairs test, concluded a sample size of at least n=40 (effect size = 0.6, $\alpha = 0.05$, power = 95%) Normally distributed, continuous variables are shown as mean and standard deviation (SD) in tables and 95% confidence intervals (CI) in figures. Non-normally distributed data is presented as median and interquartile (IQR) range. Repeated-measures analysis of variance (ANOVA) was used to determine the difference in continuous variables over separate time points. Statistical significance was considered at $p<0.05$ at all times, with Bonferroni's adjustment used for multiple comparisons. Categorical variables are presented as frequencies (percentages). All analyses were performed using IBM SPSS Statistics software version 22 for Microsoft Windows.

RESULTS

Study participants and baseline characteristics

A total of 46 patients were recruited to our study. Six participants did not complete/were withdrawn from the study for the following reasons: a) death in the postoperative period, b) bed cancellation, c) blood transfusion of more than 2 units, d) withdrawal of consent after completing two study time points, e) aspirin not administered prior to operation and f) withdrawal of consent after first, baseline venesection. Therefore, we present data for 40 included patients. Twenty (50%) patients underwent open abdominal aortic aneurysm operation, 15 (37.5%) infrainguinal bypass for subcritical limb ischaemia and 5 (12.5%) peripheral aneurysm repair with bypass. In addition to this, two patients declined sampling at a single time point and one patient was on intravenous unfractionated heparin during two study time points. Twenty-five patients underwent 3-month follow-up, twelve politely declined, one patient was restarted on clopidogrel before three-month follow-up, one patient failed to attend outpatient follow-up and we were not able to contact one patient despite multiple attempts. Intraoperative blood loss was recorded in 38 patients, with a median of 550 ml (IQR=1160). Baseline demographics, medication use, procedural data and laboratory investigations are presented in Table 1.

Time from aspirin administration to venesection

All study participants were given aspirin at all time points. Time intervals between aspirin ingestion and prespecified venesection time points are demonstrated in Table 2. Variation in preoperative blood sampling can be explained by diverse admission times. The variation at 2 hours was due to the timing and length of the surgical procedure.

Platelet count and packed cell volume

Platelet count and packed cell volume (PCV) for all time points are illustrated as Figure 1. The overall difference between time points (T1 to T5) for both platelets and PCV was

statistically significant (p<0.001). The platelet count dropped postoperatively with the lowest values at T4 and recovery of the platelet count observed at the 3 months follow up. (p=0.113) Similarly, a decline in PCV was observed in the postoperative period with the lowest values at T5 and recovery to baseline at 3 months follow up (p=0.194). Details are demonstrated as supplementary tables 1 and 2.

Platelet reactivity

Variations in agonist-mediated platelet reactivity over all time points are demonstrated in Figure 2A (MA) and 2B (AUC15). Values for the agonist-mediated clotting and pairwise comparison between time points are shown in supplementary Tables 1 and 2 respectively.

Thrombin-mediated platelet reactivity

Both $MA_{Thrombin}$ and $AUC15_{Thrombin}$ showed significant variation between the pre specified time points (T1 to T5) with an initial significant increase in the postop period (p<0.001). Specifically, the most significant increase was observed at T5 in comparison with other time points. At the follow up appointment (T6) measurements were similar to baseline for both $MA_{Thrombin}$ and $AUC15_{Thrombin}$ (p=0.5767 and p=0.995 respectively).

ADP-mediated platelet reactivity

Statistically significant difference between in-hospital (T1 to T5) time points was observed for both MA_{ADP} (p<0.001) and $AUC15_{ADP}$ (p=0.008) with an initial significant reduction in the postop period. Firstly, at 2 hours, ADP-mediated platelet reactivity declined (p<0.001) and then recovered at T3 (p=0.919 for MA_{ADP}, p=0.277 for $AUC15_{ADP}$), T4 (p=1 for MA_{ADP}, p=1 for $AUC15_{ADP}$) and T5 (p=1 for MA_{ADP}, p=1 for $AUC15_{ADP}$) in comparison to the baseline. By 3 months, the ADP response was significantly higher than at baseline (p=0.002 for MA_{ADP} and p=0.008 for $AUC15_{ADP}$).

AA-mediated platelet reactivity

Statistically significant difference between all in-hospital time points (T1 to T5) was observed for both MA_{AA} and $AUC15_{AA}$ ($p<0.001$) with an initial significant reduction in the postop period. The initial drop from baseline to 2 hours (MA_{AA} $p=0.001$ and $AUC15_{AA}$ $p=0.031$) and at 1 day (MA_{AA} $p=0.024$, $AUC15_{AA}$ $p=0.272$) was followed by a rise back to baseline levels at T4 ($p=0.797$ for MA_{AA}, $p=1$ for $AUC15_{AA}$) and T5 ($p=1$ for MA_{AA}, $p=1$ for $AUC15_{AA}$). AA-mediated platelet reactivity is then higher than the baseline at the 3 months follow-up ($p=0.008$ for MA_{AA} and $p=0.12$ for $AUC15_{AA}$).

AA metabolites

Summarised TXB_2 and 12-HETE values and a pairwise comparison between time points (T1 to T5) are shown in supplementary Tables 3 and 4 respectively.

Thromboxane B2

TXB_2 levels were negligible throughout all the study time points in the vast majority of patients, thus confirming adequate inhibition of COX-1 pathway [Figure 3A]. Out of 219 analysed samples, 7 samples at various time points showed a subtherapeutic response. One patient expressed TXB_2 values of >10ng/ml (12.1ng/ml and 11.2ng/ml) at the time points T1 and T5, with readings of <10ng/ml between them (T2, T3 and T4) and this probably reflects the only true aspirin non-responder in our cohort. Two further individuals had a subtherapeutic response at baseline (11.1ng/ml and 17.4ng/ml), which subsequently changed into a therapeutic response perioperatively and remained suppressed at the 3 month follow-up. Samples analysed from two more patients showed values of >10ng/ml at a single time point (T2), 12.9ng/ml and 13.5ng/ml respectively. A further patient had a TXB_2 level over 10ng/ml at 3 to 5 days sampling (T5), 10.8ng/ml.

We observed a statistically significant change between the study time points (T1 to T5, $p<0.001$). It was driven by a decline in values at T3, T4 and T5 ($p=0.014$, $p=0.005$ and

p=0.014 respectively) in comparison to baseline readings. Subsequently, at 3 months follow up, TXB_2 levels recovered to baseline values (p=0.223).

12-Hyroxyeicosatetraenoic acid

We observed no statistically significant difference between all the in-hospital time points (p=0.052), as well as no significant difference between any of the study time points [Figure 3B]. Results at baseline and at 3 months follow up appointment were similar (p=0.907).

Inflammatory biomarkers

Summarised CRP, IL-6, TNF-α, CD40 values and a pairwise comparison between time points (T1 to T5) are shown in supplementary Tables 3 and 4 respectively.

C-reactive protein

CRP readings increased significantly perioperatively (differences between the study time points T1 to T5 (p<0.001) [Figure 3C]. At the 3 month follow up CRP levels had declined back to baseline levels (p=0.95).

Interleukin 6

A significant rise in IL-6 was observed (T1 to T5, p<0.001) [Figure 3D]. At T6 IL-6 levels had returned to baseline levels (p=0.223).

Tumour necrosis factor α

There was a significant change in TNF-α values between the in hospital time points (T1 to T5, p=0.005) [Figure 3E]. TNF-α levels at the 3 month follow up were no different to baseline values (p=0.704).

Cluster of differentiation 40

There was a significant difference in CD40 values between the in hospital time points (T1 to T5, p<0.001) [Figure 3F]. At the 3 month follow up CD40 values were back up to baseline levels. (p=0.387)

DISCUSSION

This study supports our hypothesis that AA-, ADP- and thrombin-induced clotting shows dynamic variation during acute vascular inflammatory episode after MVS and that AA-induced clotting is modified despite demonstrable therapeutic activity of aspirin at its primary pharmacological target. The main findings were as follows. Firstly, our model did indeed create an intense model of on-off vascular inflammatory response as evidenced by the changes in the levels of inflammatory markers. Secondly, that within this period of inflammation, AA-induced clotting dropped significantly between baseline and 2 and 24 hour time points, and then went back up to baseline levels at 3-5 days. Interestingly, we observed a higher level of AA-induced clotting at the 3 month time point as compared to baseline. This observation therefore represents a rebound. Third, throughout this period, TXB_2 levels indicated therapeutic activity of aspirin at its pharmacological target and fourthly, there was no corresponding increase in the AA-lipoxygenase metabolite, 12-HETE. Fifth, ADP-induced clotting was also significantly lower at 2 and 24 hours compared to baseline and also showed a significant rebound at the 3 month time point. Lastly, by contrast, thrombin-induced clotting significantly increased up to day 5 compared to baseline after surgery and then returned to baseline levels by 3 months. This observation related to thrombin negates the notion that perioperative changes seen with ADP- and AA- induced platelet activation are due to changes in platelet count/PCV and the potential influence of inflammatory leukocytes on platelet function, which should be recruited perioperatively.

These findings are seen in the context of previous literature that demonstrates discrepant results with regard to platelet reactivity after major surgery. For example, previous populations undergoing cardiac surgery have been reported to exhibit increased AA-induced platelet aggregation following surgery: in these studies, however, the patients were only established on therapeutic levels of aspirin in the few days post op. (34, 35) In populations

undergoing MVS specifically, previous data are again inconsistent. In contrast to our current data, Rajagopalan et al demonstrated an increase in AA-mediated clotting using the VerifyNow assay in patients on aspirin.(36) Another study found no difference in AA-mediated platelet activation using LTA in patients after carotid endarterectomy who, in the majority of cases, were on aspirin. (37) By contrast, 2 studies have demonstrated a similar reduction in AA-and ADP-induced blood clotting after cardiac surgery using TEG. (38, 39) The challenges involved in the interpretation of these variable data include the heterogeneity of the clinical circumstances and also the PFTs used: it is plausible that tests that look specifically at isolated platelet aggregation in response to a specific agonist do not reflect the effect of that agonist on whole blood clotting.

It is interesting that we have observed a significantly higher level of whole blood clotting to both AA and ADP at 3 months after surgery, thus representing a true rebound phenomenon. Since both AA- and ADP-induced clotting were enhanced at 3 months, it may be considered elevated global platelet phenotype, which is not specific for AA. However, such an effect was not seen in thrombin. As yet, we cannot explain this observation. Although, it adds weight to our concept that agonist-mediated clotting can be dynamic in a time-dependent manner. It is tempting to speculate that such a variability could contribute to the observed delayed link between an acute inflammatory event, such as a chest infection or emergency hip fracture surgery, and acute myocardial infarction. (20, 21) Certainly, further research into a mechanism for the association is warranted.

This study has also shown that, at the same time as AA- and ADP-induced clotting decreased in the days after vascular surgery, thrombin-mediated whole blood clotting increased, despite perioperative heparin and routine thromboprophylaxis. This result is compatible with previous data showing a similar hypercoagulability effect on TEG in patients undergoing major abdominal surgery. (40) A similar prothrombotic effect of vascular surgery

was demonstrated by Collins et al. who showed not only increased platelet aggregation using Ultegra point of care but also elevated levels of thrombin-antithrombin III complex (TAT). (41) In cardiac surgery, Parolari et al. confirmed increased levels of prothrombin fragment F1.2, TAT complex and D-dimer up to 30 days post both traditional and off pump coronary bypass surgery (CABG). (42) Li et al. demonstrated an interesting biphasic state with pro-thrombotic response up to 7 days and a peak in platelet reactivity and thrombin generation a week after CABG. (43) In addition to this, generally speaking surgery is linked with an increased level of fibrinogen and acute phase proteins as well as diminished quantities of natural anticoagulants such as protein C and antithrombin III. Noteworthy is the fact that thrombin is involved not only in platelet aggregation but also in fibrin formation. Our data indicate that platelet-mediated clotting is not generically altered by the vascular inflammatory process initiated by MVS but is agonist-specific. Aspirin has also several additional pleiotropic effects, which to a degree are independent of TXB_2 generation. (44) Firstly, aspirin has thrombin-lowering properties, with 29% of less thrombin produced after 7 days of aspirin ingestion. (45) Mechanisms by which aspirin reduces thrombin generation remain unproven with one potential, COX-1 independent explanation of changes in fibrin architecture and network. (46) Aspirin also through acetylation of fibrinogen (47) increases plasma clot permeability. (48) Additionally it has been suggested that aspirin`s antithrombotic effects are associated with specific allelic variants, i.e. FXIII Leu34 allele (49), but not Pro33 allele (50).

Regarding our secondary question, as to which aspirin-independent pathway mediates the dynamic changes in AA-induced clotting that we have observed in this population, we have failed to find any evidence that it utilises the lipoxygenase metabolic pathway. Previous studies suggested this pathway as a potential explanation of induced platelet activation in aspirin treated patients. For example, Frelinger et al. proved the existence of such an

additional pathway, which was proportional to platelet activity and partly mediated by ADP-induced platelet activation. (33) Furthermore, McMahon et al showed that heparin administration during vascular surgery caused a transient increase in platelet activation to AA and ADP, despite effective COX-1 inhibition with aspirin. (51) In addition to this, in patients treated with aspirin, heparin administration during vascular surgery generated AA that is metabolised to 12-HETE via the LOX pathway. (52) This observation was not reproduced in our cohort of patients. Further mechanistic investigation is therefore required.

This study has several limitations. Firstly, patients undergoing MVS were seen to exhibit a significant drop in platelet count in the perioperative period. This may have a multifactorial explanation, but could have contributed to our platelet-mediated clotting results, although the difference in the responses to thrombin in comparison with AA and ADP make this simple explanation less plausible. Secondly, there was a dropout rate from complete follow up of 15 patients, despite our efforts. Thirdly, our population, whilst all undergoing MVS, had a variety of different types of surgery and we have assumed that this provides a relatively consistent model of on-off inflammation. Fourthly, although medication compliance at discharge was strongly encouraged, we were not able to witness it or measure it objectively until the follow up visit. Finally, our TxB_2 readings were measured with no immediate incubation at body temperature before centrifuging and freezing. Whilst our methodology has been employed by several other groups around the world (53-57), there is some evidence that the assay should be performed on sample that is incubated for at least 30 minutes at 37^{o}C (58-62).

In conclusion, this study has proved its hypothesis that AA-induced clotting is modified by vascular inflammation despite demonstrable therapeutic activity of aspirin at its primary pharmacological target. This COX-1 independent pathway is not lipoxygenase-mediated and is associated with a rebound increase in AA-induced clotting at 3 months, a finding that is

mirrored by the responses to ADP, but not thrombin. These observations may offer insight into a delayed increase in platelet reactivity after vascular inflammation which could be relevant to the timing of some coronary thrombotic events. Further data are required.

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Table 1. Study cohort baseline demographics, medication use, procedural data and laboratory investigations.

Variable	Study Cohort (N=40)
Patient Demographics	
Gender, Male	35 (87.5%)
Age	67.4 (8.6)
Ethnicity, Caucasian	40 (100%)
BMI (kg/m^2)	26.4 (3.9)
Risk Factors	
Hypertension	29 (72.5%)
Hyperlipidaemia	34 (85%)
Diabetes	5 (12.5%)
Previous or Current Smoker	22 (55.0%)
Family History of premature CVD	7 (17.5%)
Cerebrovascular disease	2 (5.0%)
Ischaemic Heart Disease	16 (40.0%)
Previous Myocardial Infarction	10 (25.0%)
Previous CABG	5 (12.5%)
Previous PCI	10 (25.0%)
Medications	
Beta Blocker	16 (40.0%)
Angiotensin Converting Enzyme inhibitor	23 (57.5%)
Calcium Channel Blocker	19 (47.5%)
Proton Pump Inhibitor	14 (35.0%)
Oral Hypoglycaemic agent	4 (10.0%)
Insulin	3 (7.5%)
Statin	31 (77.5%)
Aldosterone antagonist	1 (2.5%)
Diuretic	5 (12.5%)
Surgical procedure	
Open AAA repair	20 (50%)
Infra-inguinal Bypass for subcritical limb ischaemia	15 (37.5%)
Peripheral aneurysm repair with bypass	5 (12.5%)
Laboratory results	
Haemoglobin (g / litre)	141.4 (13.8)
Platelet Count (x 10^9 / litre)	264.0 (73.5)
MCV (fL)	90.4 (5.1)
Urea (mmol / litre)	5.8 (2.1)*
Creatinine (µmol / litre)	84.1 (21.5)
Estimated Glomerular Filtration Rate (ml / min)	80.0 (27)*

BMI- body mass index, CVD – cardiovascular disease, CABG – coronary artery bypass grafting, PCI – percutaneous coronary intervention, AAA – abdominal aortic aneurysm, MCV – mean corpuscular volume

Table 2. Time intervals between pre-specified study time points.

Time point	N	Time interval in minutes Mean (SD) OR Median (IQR)*
Pre-operation	40 (34)	555.4 (258.28)
2 hours	39 (39)	579.2 (262)*
1 day	39 (38)	293.2 (253.8)*
2 day	38 (38)	290.9 (139.5)*
3-5 day	39 (38)	287.9 (160.1)
3 month	25 (13)	275.5 (127.5)*

SD – standard deviation, IQR – interquartile range

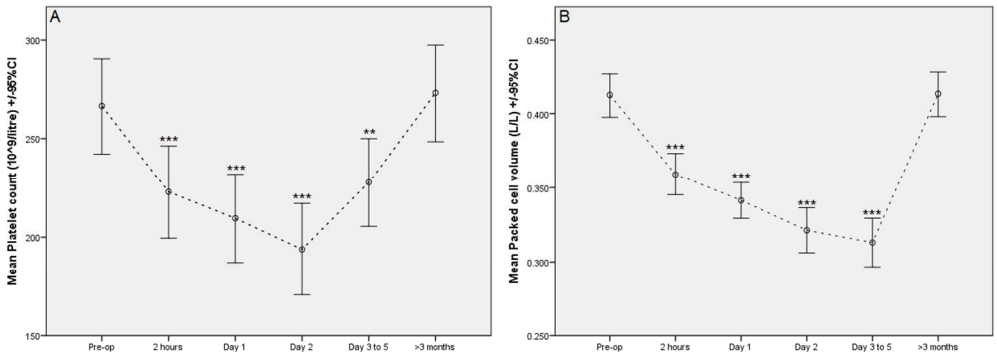

Figure 1. Change in platelet count (A) and packed cell volume (B) between time points (T1-T6). * - p<0.05, ** - p<0.01, *** - p<0.001 compared to baseline.

330x121mm (96 x 96 DPI)

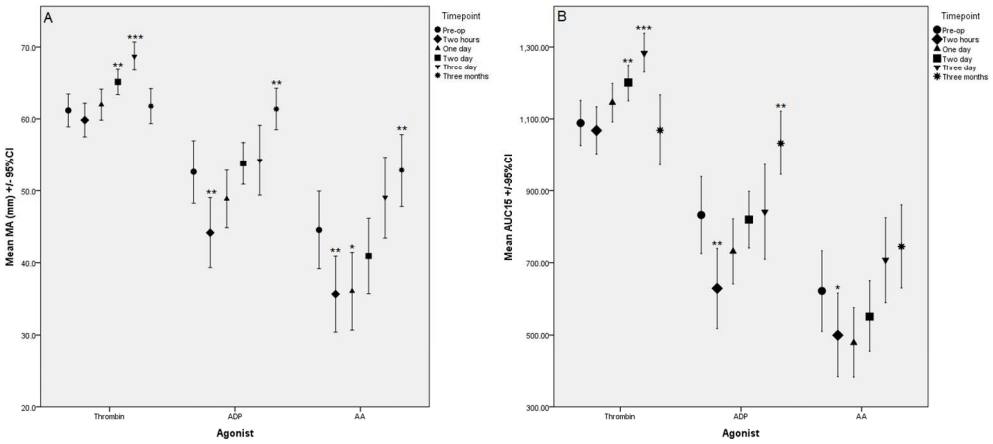

Figure 2. Variation in platelet reactivity for all agonists - MA (A) and AUC15 (B). * - p<0.05, ** - p<0.01, *** - p<0.001 compared to baseline.

382x179mm (96 x 96 DPI)

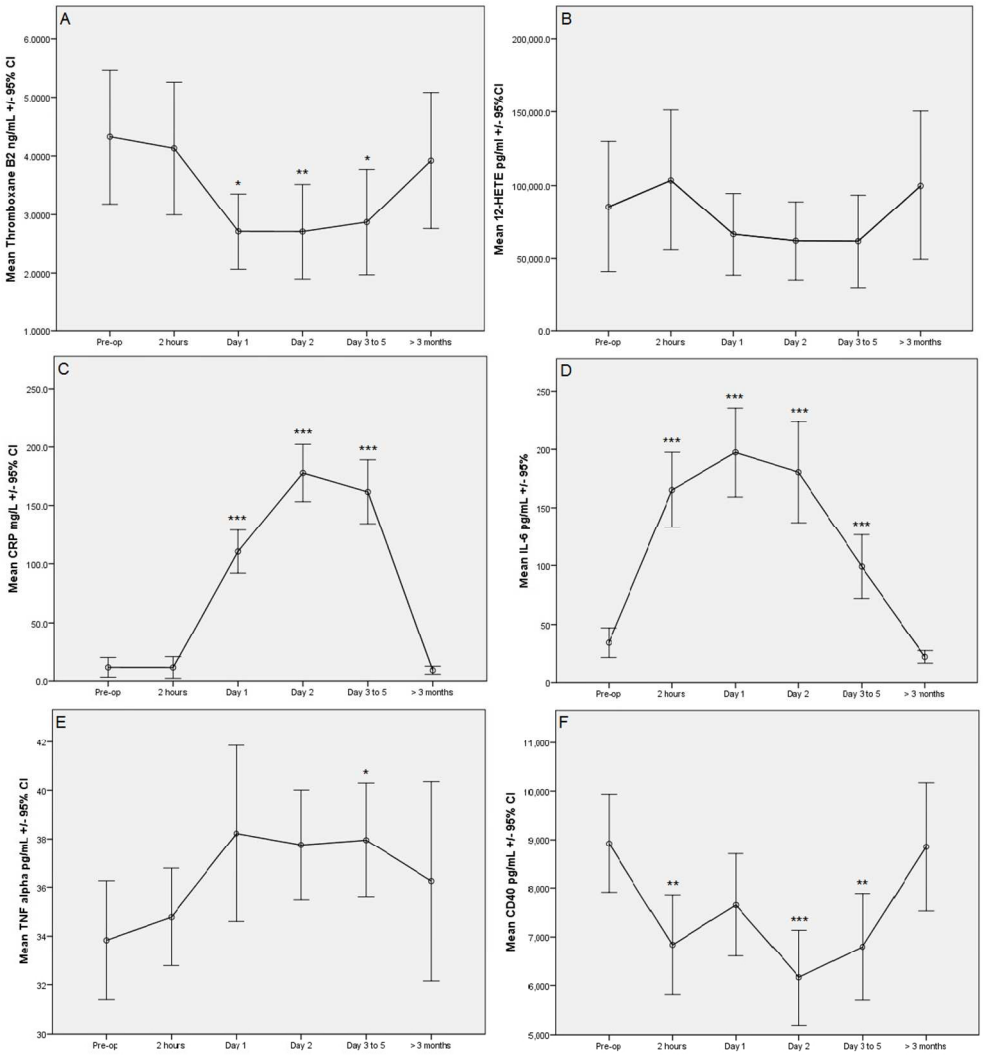

Figure 3. Variation in AA metabolites – TXB2 (A) and 12-HETE (B) and inflammatory biomarkers – CRP (C), IL-6 (D), TNF-α (E) and CD40 (F). * - p<0.05, ** - p<0.01, *** - p<0.001 compared to baseline.

12-HETE - 12-Hyroxyeicosatetraenoic acid, CRP – C-reactive protein, IL-6 – interleukin 6, TNF-α – tumour necrosis factor α, CD40 – Cluster of differentiation 40, CI 0 confidence interval

333x354mm (96 x 96 DPI)

Supplementary table 1. Values for platelet count, packed cell volume and all agonists (MA/AUC15) for all time points.

Parameter	Time point	N	Mean/Median*	SD/IQR*
Platelets (10^9/litre)	Preprocedure	38	266.3	73.8
	2 hours	34	208*	109*
	Day 1	40	211*	82*
	Day 2	35	186*	91*
	Day 3 to 5	37	227*	92*
	>3 months	24	249.5*	89*
Packed cell volume (L/L)	Preprocedure	38	0.412	0.046
	2 hours	34	0.359	0.04
	Day 1	40	0.341	0.038
	Day 2	35	0.315*	0.056*
	Day 3 to 5	37	0.302*	0.057*
	>3 months	24	0.413	0.036
$MA_{Thrombin}$ (mm)	Preprocedure	40	61.2	7
	2 hours	39	59.8	7.1
	Day 1	38	61.9	6.4
	Day 2	38	65	5.3
	Day 3 to 5	39	68.7	6.1
	>3 months	25	61.8	5.8
MA_{ADP} (mm)	Preprocedure	40	54.9*	12.8*
	2 hours	39	44.2	15
	Day 1	39	48.9	12.3
	Day 2	38	53.8	8.9
	Day 3 to 5	38	57.2*	20.6*
	>3 months	25	61.4	6.9
MA_{AA} (mm)	Preprocedure	39	44.6	16.6
	2 hours	39	35.7	16.2
	Day 1	39	36.1	16.6
	Day 2	38	43.4*	31.1*
	Day 3 to 5	39	49.1	17.4
	>3 months	25	52.8	12.2
$AUC15_{Thrombin}$	Preprocedure	40	1088.7	193.3
	2 hours	39	1068.4	199.4
	Day 1	38	1144.4	160.4
	Day 2	38	1199.5	152.4
	Day 3 to 5	39	1283.3	168.1
	>3 months	25	1113.3*	207.8*
$AUC15_{ADP}$	Preprocedure	40	832.4	332.4
	2 hours	39	629.0	343.2
	Day 1	39	731.5	278.7
	Day 2	38	819.6	238.3
	Day 3 to 5	39	923.23*	581.47*

	>3 months	25	1075.8*	193.03*
AUC15AA	Preprocedure	39	544.5*	582.0*
	2 hours	39	486.5*	476.3*
	Day 1	39	426*	506.5*
	Day 2	38	540.5*	608.63*
	Day 3 to 5	39	707.4	363.4
	>3 months	25	745.4	279.1

SD – standard deviation, IQR – interquartile range

Supplementary table 2. Pairwise comparison of platelet count, packed cell volume and platelet reactivity (MA/AUC15) for all agonists at in-hospital time points (T1 to T5).

Parameter	Time point	2hours	Day 1	Day 2	Day 3 to 5
Platelet count p<0.001	Pre-op	**<0.001**	**<0.001**	**<0.001**	**0.007**
	2 hours		1	**0.035**	1
	Day 1			**0.049**	1
	Day 2				**0.026**
Packed cell volume p<0.001	Pre-op	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	2 hours		1	**<0.001**	**<0.001**
	Day 1			**0.017**	**0.01**
	Day 2				1
MA$_{Thrombin}$ p<0.001	Pre-op	1	1	**0.001**	**<0.001**
	2 hours		0.129	**<0.001**	**<0.001**
	Day 1			**0.019**	**<0.001**
	Day 2				**0.006**
MA$_{ADP}$ p<0.001	Pre-op	**0.001**	0.919	1	1
	2 hours		0.25	**<0.001**	**<0.001**
	Day 1			**0.039**	0.055
	Day 2				1
MA$_{AA}$ p<0.001	Pre-op	**0.001**	**0.024**	0.797	1
	2 hours		1	0.432	**<0.001**
	Day 1			1	**0.001**
	Day 2				0.21
AUC15$_{Thrombin}$ p<0.008	Pre-op	1	0.383	**0.001**	**<0.001**
	2 hours		0.061	**<0.001**	**<0.001**
	Day 1			0.168	**<0.001**
	Day 2				**0.022**
AUC15$_{ADP}$ p<0.001	Pre-op	**0.001**	0.277	1	1
	2 hours		0.115	**<0.001**	**0.002**
	Day 1			0.133	0.225
	Day 2				1
AUC15$_{AA}$ p<0.001	Pre-op	**0.031**	0.272	1	1
	2 hours		1	1	**0.003**
	Day 1			1	**0.004**
	Day 2				**0.04**

MA – maximal amplitude, AUC – area under the curve

Supplementary table 3. Values for AA metabolites and inflammatory biomarkers for all time points.

Parameter	Time point	N	Mean/Median*	SD/IQR*
Thromboxane B2 (ng/ml)	Preprocedure	40	3.4*	3.7*
	2 hours	39	2.9*	4.6*
	Day 1	39	2.1*	2.8*
	Day 2	38	2.0*	2.3*
	Day 3 to 5	39	2.4*	2.4*
	>3 months	24	3.4*	2.8*
12-HETE (pg/ml)	Preprocedure	39	36451.1*	49022.9*
	2 hours	38	57876.1*	91137.4*
	Day 1	39	41023.8*	55885.8*
	Day 2	38	29109.2*	65742.9*
	Day 3 to 5	39	22944.5*	46342.9*
	>3 months	24	50296.2*	1413331.1*
CRP (mg/L)	Preprocedure	39	4.3*	7.2*
	2 hours	38	4.9*	6*
	Day 1	37	110.446	55.2
	Day 2	37	177.7	73.4
	Day 3 to 5	34	161.6	78.6
	>3 months	24	6.2*	9.8*
IL-6 (pg/mL)	Preprocedure	40	17.0*	28.2*
	2 hours	39	120.2*	170.0*
	Day 1	39	201.4*	206.4*
	Day 2	38	131.3*	224.0*
	Day 3 to 5	40	62.7*	103.6*
	>3 months	23	17.8*	18.4*
TNF-α (pg/mL)	Preprocedure	40	31.5*	6.1*
	2 hours	39	32.8*	5.8*
	Day 1	38	34.8*	6.5*
	Day 2	39	37.2*	4.9*
	Day 3 to 5	38	36.3*	5.9*
	>3 months	23	32.8*	7.3*
CD40 (pg/ml)	Preprocedure	40	8702.4*	5679.6*
	2 hours	39	5624.6*	4656.2*
	Day 1	38	6816.8*	4558.2*
	Day 2	37	5512.3*	4312.4*
	Day 3 to 5	39	5705.8*	4925.4*
	>3 months	22	8549.4*	5736.8*

12-HETE - 12-Hyroxyeicosatetraenoic acid, CRP – C-reactive protein, IL-6 – interleukin 6, TNF-α – tumour necrosis factor α, CD40 – Cluster of differentiation 40, SD – standard deviation, IQR – interquartile range

Supplementary table 4. Pairwise comparison of AA metabolites and inflammatory biomarkers for all agonists at in-hospital time points (T1 to T5).

Parameter	Time point	2 hours	Day 1	Day 2	Day 3 to 5
Thromboxane B2 p<0.001	Pre-op	1	**0.014**	**0.005**	**0.014**
	2 hours		**0.014**	0.097	0.177
	Day 1			1	1
	Day 2				1
12-HETE p=0.052	Pre-op	1	1	1	1
	2 hours		0.43	0.275	0.23
	Day 1			1	1
	Day 2				1
CRP p<0.001	Pre-op	**1**	**<0.001**	**<0.001**	**<0.001**
	2 hours		**<0.001**	**<0.001**	**<0.001**
	Day 1			**<0.001**	**0.001**
	Day 2				0.548
IL-6 p<0.001	Pre-op	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	2 hours		0.242	1	**<0.001**
	Day 1			1	**<0.001**
	Day 2				**<0.001**
TNF-α p=0.005	Pre-op	1	0.37	0.294	**0.015**
	2 hours		0.246	0.103	**0.006**
	Day 1			1	1
	Day 2				1
CD40 p<0.001	Pre-op	**0.004**	0.478	**<0.001**	**0.003**
	2 hours		1	1	1
	Day 1			**0.001**	1
	Day 2				1

12-HETE - 12-Hyroxyeicosatetraenoic acid, CRP – C-reactive protein, IL-6 – interleukin 6, TNF-α – tumour necrosis factor α, CD40 – Cluster of differentiation 40