

**Bleeding due to post-bypass haemostatic dysfunction:
An investigation of mechanisms, prevention and therapy.**

A thesis submitted for the degree of
Master of Surgery
in the University of London

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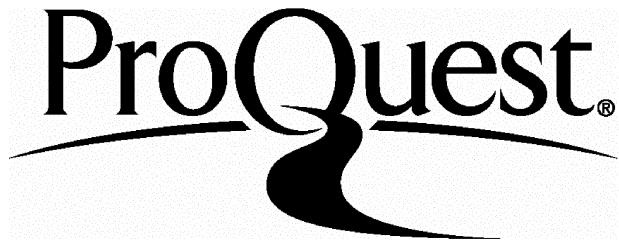
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Abstract

The morbidity and mortality following cardiopulmonary bypass (CPB) is significantly increased by excessive post-operative bleeding. Furthermore excessive bleeding drains our limited financial resources and supply of homologous blood products. In this thesis the mechanisms responsible for excessive bleeding following CPB are investigated and measures to prevent and to treat excessive bleeding are established.

The effect of aprotinin on post-operative blood loss was investigated in a prospective, randomised, placebo controlled, double blind clinical trial in 60 patients who bled in excess of 400 ml during the first three hours following CPB and who had **not** received prophylactic aprotinin in theatre. The principal conclusions of this trial are:

Post-operative aprotinin therapy

- 1) significantly reduced excessive post-operative bleeding,
- 2) was associated with a significantly higher Hb at the time of hospital discharge,
- 3) but did not reduce homologous blood transfusion.

Aprotinin therapy was associated with a significant

- 1) increase in surface expression of platelet GPIb adhesive receptors,
- 3) reduction in plasma tPA level,
- 4) increase in plasma fibrinogen levels compared to the placebo group.

Aprotinin treatment may have been associated with preservation of platelet vWF levels but without any alteration in platelet aggregation and vWF multimeric structure.

The consequences of continuation of pre-operative aspirin, in terms of altered haemostasis and increased bleeding, was defined in a prospective, randomised, placebo controlled, double blind clinical trial in 100 patients scheduled to undergo elective CABG. The principal conclusions of this trial are:

Pre-operative aspirin significantly increased

- 1) post-operative bleeding,
- 2) transfusion requirements of blood products, and
- 3) the risk of re-exploration for bleeding.

The principal effects of aspirin on platelet function were:

- 1) Reduction in platelet aggregation,
- 2) increase in bleeding time, but
- 3) without any significant effect on the components of platelet adhesion.

From the work described in this thesis practical conclusions can be drawn which influence clinical practice:

- 1) Pre-operative aspirin therapy should be discontinued one week prior to elective CABG in order to reduce post-operative bleeding and
- 2) in patients who bleed excessively in the first few hours following CPB aprotinin therapy is effective in reducing further blood loss.

Preface

The first re-sternotomy I performed for excessive post-operative bleeding was the most memorable experience of the early part of my cardiothoracic training. As soon as I saw the pericardial and pleural cavities full of blood I was convinced that the cause must have been catastrophic bleeding from a suture line. I became anxious and frustrated when I cleared all the blood from the pericardial sac without finding an obvious cause of bleeding. I was too worried to close the chest without correcting a "surgical cause" for the bleeding, as I was only too familiar with the maxim "all bleeding is surgical". It was even more frustrating to see the excessive blood loss continue on the intensive care unit after the re-sternotomy and not having a definite answer to the question being asked at the time ; "what did you find as the cause of bleeding when you re-explored the patient?" It was a great relief when the blood loss ceased after transfusing the patient with platelets. Experiences of this kind are familiar to all involved in cardiac surgery.

Further similar experiences, early on in my training, highlighted the importance of the CPB acquired haemostatic dysfunction and the major contribution of platelet dysfunction to this problem. Even in cases with an obvious surgical cause of the bleeding, the major blood loss and transfusion of homologous blood exacerbates this coagulopathy.

Excessive post-operative haemorrhage is also associated with a higher morbidity and mortality. The increased risk of transmitting the HIV virus as well as the hepatitis viruses to patients has also prompted increased efforts to attempt to reduce transfusion of blood products to our patients.

The initiating stimulus for the randomised aspirin versus placebo trial was provided by the two policies on the pre-operative use of aspirin in our unit at the time of this research project. Two of the consultants preferred their patients to continue taking aspirin until the day of operation because of a belief in its effect on pre-operative stability and higher post-operative graft patency rates. The remaining two surgeons, however, felt that the risk of post-operative bleeding outweighed the benefit of aspirin and discontinued aspirin 10 days prior to surgery. As two totally opposite policies were practised, I felt that there must be enough doubt and controversy on this issue that required further formal testing. Furthermore I felt there was no ethical dilemma in setting up an aspirin versus placebo controlled trial in order to establish the risk of pre-operative aspirin use and to investigate the mechanisms of platelet dysfunction during the critical early hours following CPB.

At the time of this research project the prophylactic use of aprotinin in our unit was reserved for those patients that were undergoing repeat valve operations, cardiac transplantation, aortic dissection and surgery for infective endocarditis. The reasons for this restricted use of

aprotinin, despite its documented efficacy, were concern for the effect of aprotinin on vein graft patency and the cost of widespread use as only a minority of patients bleed excessively. For these reasons I felt that the unit provided the appropriate environment to investigate the therapeutic effect of aprotinin, as distinct from the prophylactic effect, on excessive post-operative bleeding.

All these factors provided the necessary stimulus for me to embark on the investigation of the mechanisms of platelet dysfunction, which plays a central role in the CPB related haemostatic defect, during the critical first few hours following CPB. In addition I strived to establish measures to prevent and to treat excessive post-operative bleeding.

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Statement of Originality

The work on which this thesis is based is original and is submitted to the University of London in the knowledge that it has not been previously performed.

Aprotinin has been shown to be effective in preventing the reduction of platelet adhesive receptors (GPIb) during CPB and in reducing blood loss. This prophylactic use of aprotinin is well established but its use as therapy for excessive bleeding post-operatively had not been previously explored. In this thesis the effect of post-operative aprotinin therapy is defined, in a randomised, placebo controlled, double blind clinical trial, on the post-operative blood loss in a group of patients with excessive bleeding during the first few hours following CPB and who had **not** received prophylactic aprotinin.

The mechanism of action of aprotinin may be different if used only post-operatively for the treatment of excessive bleeding as compared to its prophylactic effect. Plasmin has been shown to reduce the number of platelet surface GPIb, *in vitro*, by internalising them to an intraplatelet pool. These receptors can be redistributed to the surface of platelets once the effect of plasmin is removed. Could this dynamic distribution of GPIb also be true *in vivo*? Could aprotinin, by inhibiting plasmin, effectively replenish the GPIb on the platelet surface and improve platelet adhesion? If this were true it would imply that

the adhesive capacity of platelets is not irreversibly damaged during CPB but could be "rescued" post-operatively in those patients who bleed excessively. The effect of aprotinin on the platelet surface GPIb receptors is elucidated. The effect of aprotinin on the relationship between platelet GPIb, vWF and ristocetin induced aggregation is defined with the concomitant effect on fibrinolysis during excessive haemorrhage in the immediate post CPB period. The effect of aprotinin on the multimeric composition of plasma and platelet vWF is examined.

In this thesis I also define the risk of continuation of pre-operative aspirin therapy up to the day of operation in a prospective, randomised, placebo controlled, double blind clinical trial. The superimposed effect of aspirin on the platelet dysfunction following CPB is explored and the aspects most crucial to haemostasis at the most critical time elucidated. The changes in plasma and platelet vWF and its multimeric pattern following CPB are investigated.

Glossary of abbreviations

ACT	Activated coagulation time
ADP	Adenosine diphosphate
AN51	Monoclonal antibody to platelet GPIb (IgG2a)
AP	Alkaline phosphatase
ASA	Aspirin (acetylsalicylic acid)
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BT	Bleeding time
CABG	Coronary artery bypass grafting
CD	Cluster of differentiation
CI	Confidence interval
CPB	Cardiopulmonary bypass
DDAVP	Desmopressin
DIC	Disseminated intravascular coagulation
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FDPs	Fibrin(ogen) degradation products
FFP	Fresh frozen plasma
FITC	Fluorescein isothiocyanate
FL1	Fluorescence 1
FSC	Forward scatter
GMP-140	α -granule membrane glycoprotein (PADGEM)
GP	Glycoprotein
GPIb	Glycoprotein Ib
GPIIb-IIIa	Glycoprotein IIb-IIIa
Hb	Haemoglobin
Hct	Haematocrit

HMW	High molecular weight
IMA	Internal mammary artery
KIU	Kallikrein inactivator unit
KPTT	Kaolin partial thromboplastin time
LMW	Low molecular weight
mAb	Monoclonal antibody
MESF	Molecules of equivalent standard fluorochrome
MPV	Mean platelet volume
MsIG	Mouse immunoglobulin
MTBE	Methyl tertiary butyl ether
MWU	Mann Whitney U test
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
Pct	Plateletcrit
PDW	Platelet distribution width
PK	Pyruvate kinase
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SSD	Side scatter
TBS	Tris buffered saline
TLC	Thin layer chromatography
tPA	Tissue plasminogen activator
TT	Thrombin time
uPA	Urine type plasminogen activator (urokinase)
vWF	von Willebrand Factor

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Summary and outline of research

The morbidity and mortality following CPB is significantly increased by excessive post-operative bleeding which drains upon our restricted financial resources and limited supply of blood products. In this thesis the mechanisms responsible for excessive bleeding following CPB are investigated and measures to prevent and treat excessive bleeding are established.

Prophylactic aprotinin has been shown to reduce blood loss and transfusion requirements. As it is not possible to reliably predict which patients are likely to bleed excessively most patients are treated unnecessarily. In addition there remains some concern, recently partly alleviated, that aprotinin may increase peri-operative myocardial infarction and vein graft occlusion. For these reasons the efficacy of aprotinin in a different clinical scenario was investigated.

The effect of aprotinin on post-operative blood loss was investigated in a prospective, randomised, placebo controlled, double blind clinical trial in 60 patients who bled excessively during the first three hours following CPB and who had not received prophylactic aprotinin in theatre.

The principal conclusions of this trial are:

- 1) Aprotinin significantly reduced excessive post-operative bleeding, but without reducing the requirement for

blood products. Aprotinin was associated, however, with a significantly higher Hb at the time of hospital discharge.

2) Aprotinin restored the surface expression of platelet GPIb receptors, as measured by flow cytometric analysis, towards the pre CPB levels.

3) Aprotinin significantly reduced tPA antigen plasma levels, as measured by an ELISA method.

4) Aprotinin significantly *increased* the plasma fibrinogen levels, as measured by the Clauss method.

5) Aprotinin may have been associated with preservation of platelet vWF levels, as measured by the ristocetin cofactor assay.

6) Aprotinin did not significantly affect platelet aggregation, in response to collagen, arachidonic acid and ristocetin as observed in platelet rich plasma.

7) Aprotinin did not influence the plasma vWF levels or the vWF multimeric composition.

As a result of this study we can conclude that reserving aprotinin use until excessive post-operative bleeding is detected is effective in reducing the total blood loss.

The most plausible mechanism of action, in this situation, seems to be inhibition of fibrinolysis and improvement in the components of platelet adhesion with no effect on platelet aggregation.

Based on the results of this thesis, as well as a review of the literature, the following recommendations can be made: Prophylactic aprotinin is more efficacious in that it reduces the requirement for blood products as well as blood

loss and should therefore be used for all patients considered to have a high risk of bleeding (Repeat valve surgery, infective endocarditis, aortic dissection, pre-operative use of streptokinase, transplantation and congenital disorders of haemostasis). In addition prophylactic aprotinin should be used in patients who are Jehovah's Witnesses in an effort to avoid exposure to blood products. For the remaining patients aprotinin can be reserved for post-operative use and if they happen to bleed excessively, they may receive aprotinin in addition to all the conventional treatment.

Several questions remain unanswered for future research. How long should the aprotinin infusion be continued for? Should it be for four to five hours or should it continue until the bleeding stops completely? What dose should we use? In this study the high dose regime as recommended by Royston and colleagues aimed at inhibiting Kallikrein was used. If the principal effect of aprotinin in the post-operative scenario is on fibrinolysis and plasmin, however, then a lower dose may still be effective. We know from theoretical calculations as well as other studies that only a quarter of the plasma concentration of aprotinin is required to inhibit the enzyme activity of plasmin as compared to plasma Kallikrein (50 versus 200 KIU/ml). As the effects of therapeutic aprotinin and desmopressin seem to be different they may have supplementary actions.

Aspirin has an established benefit in reducing the incidence of ischaemic events and vein graft occlusion. The risk of

continuation of pre-operative aspirin therapy, however, seemed to be in some doubt despite the fact that several studies have shown that it increases blood loss. These studies, however, were imperfectly designed in one of several ways: The trial was not randomised, not blind, the patients received only one aspirin the day before surgery and the control group consisted of patients undergoing valve replacement in comparison to patients undergoing CABG. In addition some studies failed to demonstrate an increase in post-operative bleeding. For these reasons argument has persisted in the literature about the benefit / risk equation of pre-operative aspirin therapy.

In this thesis the risk of continuation of pre-operative aspirin therapy up to the time of cardiac surgery is defined in a prospective, randomised, placebo controlled, double blind clinical trial in 100 patients scheduled to undergo elective CABG. Any prescribed aspirin and non-steroidal anti-inflammatory drugs were discontinued two weeks pre-operatively and these were replaced by a randomly assigned tablet of either aspirin 300 mg daily or placebo taken until the day of surgery. Patient compliance was confirmed by serum and urinary salicylate analysis. The two groups were similar in demographic characteristics, bypass time, number of grafts placed and number of internal mammary arteries used. All patients survived to be discharged home.

The principal conclusions of this trial are that pre-operative aspirin therapy significantly increased:

- 1) post-operative bleeding,

- 2) transfusion requirements of homologous blood products,
- 3) and the risk for re-exploration for bleeding.

The principal effects of aspirin on platelet function were:

- 1) reduction in platelet aggregation in response to collagen and arachidonic acid, as observed in platelet rich plasma,
- 2) increase in simple bleeding time,
- 3) but without any significant effect on the components of platelet adhesion (GPIb, vWF and multimeric composition).

As a result of this study and a literature review on the beneficial effect of aspirin on bypass graft patency rates the following practice has been adopted in our unit: In patients awaiting to undergo elective CABG, excluding patients with unstable angina and left main stem stenosis, aspirin should be discontinued one week pre-operatively. Six hours post-operatively all patients are given aspirin through a nasogastric tube or rectally, provided they are not bleeding excessively. This present practice maximises the benefit of pre-operative aspirin therapy and minimises the risk.

The main effects of CPB on platelet function were:

- 1 A reduction in platelet number and MPV,
- 2 an increase in PDW,
- 3 a prolongation of the bleeding time,
- 4 a diminution in platelet aggregation,
- 5 a decline in the expression of platelet surface GPIb,
- 6 a reduction in plasma vWF activity,
- 7 enhancement of the platelet vWF activity,

8 a reduction in plasma fibrinogen level,
9 and an increase in plasma FDPs.

CPB did not influence the plasma and platelet vWF multimeric composition. None of these haematological variables were predictive of post-operative blood loss using linear regression.

Chapter 1

Introduction and review of the literature

1.1a Introduction - coagulopathy

In spite of a steady improvement in the outcome of cardiac surgery we are still troubled by post-operative bleeding.

Most other aspects have become routine. In elective cases, and even in most emergencies, we expect to be able to discontinue bypass at the first time of asking and need inotropic support infrequently. We expect our patients to wake without clinically evident neurological problems.

Troublesome renal impairment is uncommon. Lung function is usually not a problem and other features of organ dysfunction such as jaundice or pancreatitis rarely trouble us in our routine work. So in many aspects risks are low and complications few, and yet in the first few hours after all operations, in which cardiopulmonary bypass (CPB) is employed, we are preoccupied with an anxiety about bleeding.

Bleeding appears in an analysis of incremental risk factors for post-operative mortality and morbidity (Kirklin and Barratt-Boyes 1986_b). The return of patients to the operating theatre for bleeding remains an important cause of morbidity, mortality and expense in all cardiac units. In 1991 there were 47 re-sternotomies for bleeding out of 816 operations (5.8%) in our unit (St George's Hospital audit), which is not dissimilar to world wide experience. Of these patients 20 subsequently died. In the remaining 27

patients there was an inevitable prolongation of ventilation, tissue injury of further surgery, compromised cardiac output and added transfusion requirement. These preliminary findings, from our unit audit, were confirmed in a larger retrospective study (Unsworth-White, Herriot, Valencia et al 1995).

There is another group of patients (10 %) who bleed excessively but do not require re-sternotomy because the bleeding is due to defective haemostasis rather than bleeding that can be controlled by surgical intervention. These patients, however, require large quantities of coagulation factors and platelets in order to reduce the bleeding. As it may be up to six hours before the situation is brought under control, numerous units of blood need to be transfused in order to keep up with the blood loss, and this inevitably exacerbates the coagulopathy. As a "surgical cause" for the haemorrhage can not be excluded, even in the presence of a documented concomitant coagulopathy, a proportion of these patients undergo a "futile" re-exploration because of concern of missing a "surgical cause" for the haemorrhage. In less severe cases there is still a need for excessive transfusion of homologous blood (3-6 units) that could be reduced.

Thrombolytic therapy has created another new problem. The resources required to combat the level of haemorrhage that can be expected in these cases are considerable and the risk of post-operative death is significantly higher than comparable cases (Lee, Mandell, Rankin et al 1988). In

addition many patients come to emergency coronary surgery or cardiac transplantation without the opportunity to discontinue warfarin or aspirin.

Even without these problems, there is an inevitable bypass acquired haemostatic defect that we encounter and which is well documented and characterised (Harker, Malpass, Branson et al 1980). There is a generalised reduction in all clotting factors revealed by assay. The platelets are degranulated with increased circulating levels of platelet factor 4 and β -thromboglobulin, which are contained in these granules (Zilla, Fasol, Groscurth et al 1989). The platelets are therefore, less haemostatically active and the bleeding time is prolonged even though the platelet count is normal, or within apparently acceptable limits. The fact that it is the function of the platelets that matters rather than the platelet count is well known (Copeland, Harker, Joist et al 1989). Despite the clear cut platelet function defect there are other factors which contribute to the coagulopathy.

Further evidence to substantiate the magnitude of this problem was offered by Buffalo and colleagues (Buffalo, Andrade, Branco et al 1990) who reported 593 cases of myocardial revascularisation without extracorporeal circulation. The most eloquent statement in their manuscript was that only a total of eight units of homologous blood were given, under 7 ml per case! Similarly bleeding following median sternotomy for excision of mediastinal tumours is much less frequent than following

median sternotomy for cardiac surgery with CPB.

It was shown by Duke in 1910 that the transfusion of fresh blood would halt haemorrhage in patients with a low platelet count and this was one of the first pieces of clinical evidence of the importance of thrombocytopenia in bleeding disorders. Cardiac surgeons used to resort to fresh blood drawn from volunteers such as, firemen and policemen, even though their haematological colleagues questioned the scientific basis of that practice. This is no longer acceptable because of the risk of transfusion related infection. There has also been a heightened awareness of the risk of transmission of disease with transfusion of blood products. This has been confirmed recently in patients with haemophilia and other disorders of haemostasis who require large quantities of coagulation factors. The viruses implicated are the human immunodeficiency virus and the hepatitis viruses (Anderson, Contreras, Barbara et al 1987; Marlink, Allan, McLane et al 1986).

Donated blood can only become more expensive in the future due to the increasing safety testing and a decreasing donor base. A single unit of platelets had increased by 120 % in price during 1992. This post-bypass haemostatic dysfunction, therefore, places an increasing burden on the limited resources of the blood transfusion service as well as on our progressively restricted financial resources.

1.1b Introduction - aspirin

In a prospective study of three different doses of aspirin, as used by different referring cardiologists, pre-operative aspirin was found to increase post-operative blood loss after coronary artery bypass grafting (Taggart, Siddiqui and Wheatley 1990). Aspirin is now administered, for its proven efficacy in reducing ischaemic events (Chesebro and Fuster 1986), to all patients with unstable angina, recent myocardial infarction and, as a means of secondary prevention, to patients who have had a myocardial infarction or an episode of unstable angina in the past. These patients come for surgery, often at short notice, still with aspirin affecting their platelets, and even when we have adequate notice it is arguable that we should continue the aspirin in the interests of both pre-operative stability and post-operative graft patency (Fuster and Chesebro 1986; Gavaghan, Gebski, Baron et al 1991 Sethi; Copeland, Goldman et al 1990).

This raises the question whether we should accept the increased post-operative blood loss associated with pre-operative aspirin as the price we have to pay for improved vein graft patency. On the other hand, if we could identify the subgroup of patients who are particularly sensitive to aspirin (Ferraris, Ferraris, Lough et al 1988) by studying their platelet function we could minimise this risk while maintaining the benefit. Unfortunately, routine pre-operative clotting screens are of limited value. Adult patients with detectable abnormalities (thrombocytopenia,

haemophilia) usually give a history of bleeding and have usually been identified as at risk already. Extremely few new cases of a coagulation disorder are thus detected. Platelet function is usually not tested even though platelet dysfunction probably causes us most of our problems in cardiac surgery.

This is a contentious area but it is a fact that we do not routinely perform the tests of platelet function which might help us. These are *in vivo*, the bleeding time, and *in vitro*, measurement of platelet aggregation and adhesion (Michelson, Morganroth, Torosian et al 1978). Mohr and colleagues (Mohr, Golan, Martinowitz et al 1986) studied the platelet aggregation response to adenosine diphosphate (ADP) and collagen of 20 patients, not on aspirin, before and after bypass. Six of the patients had incomplete aggregation response, three of whom had significant bleeding, whereas none of the patients with normal platelet aggregation response bled significantly.

The first standardised bleeding time was devised by Ivy and colleagues (Ivy, Nelson, Bucher et al 1941) and it was used more than twenty years ago to look at the effects of aspirin (Mielke, Kaneshiro, Maher et al 1969). These researches looked at bleeding times in those subjects taking aspirin or a placebo, randomised and double blind, and were able to compare both with their own control measurement. The tests were carried out two hours after a single dose of one gram aspirin or lactose as a control. One wonders about the completeness of absorption in such an acute experiment.

There is also convincing data that inactivation of cyclo-oxygenase is time dependent and further changes in platelet aggregation are seen after 90, 180 and 270 minutes of incubation at the lower plasma concentrations.

Nevertheless, Mielke and colleagues showed a highly significant prolongation of the bleeding time after aspirin. Reworking of the data using current non parametric statistics further emphasises this conclusion (Table 1.1).

Table 1.1 Non parametric analysis of original data from Mielke, Kaneshiro, Maher et al (1969).

Mielke's figures as presented			Non parametric analysis				
Group	mean	$\pm 2SD$	median	range	10 centile	90 centile	
Control	5	2.5-10	5	2.5-12	3.5	8	
Placebo	5.5	2.5-11	5	2.5-15.5	3.5	8	
Aspirin	9.5	4 - 21	11	4 - 15.5	5.5	14.5	

Looked at either way, about half the aspirin recipients had bleeding times outside the normal range. If these patients with prolonged bleeding time proved to be the patients who subsequently bled, it would help concentrate our attention on the "at risk" subgroup, in good time for earlier haematological intervention. It may be that the study of platelet function will enable the identification of these patients who are particularly sensitive to aspirin.

1.1c Introduction - aprotinin

Aprotinin, a drug with known potent inhibitory effects on

plasma proteases but which had failed to be of proven worth in the management of pancreatitis, was reported in 1987 by Royston and colleagues (Royston, Bidstrup, Taylor et al 1987) to reduce transfusion requirements in repeat cardiac surgery in a prospective randomised clinical trial. This news was welcomed with enthusiasm by cardiac surgeons and anecdotal reports supporting these findings quickly appeared (Markland, Sturridge, Hulfe et al 1988). These findings were confirmed by further trials (Alajmo, Calamai, Perna et al 1989).

Bidstrup and colleagues (Bidstrup, Royston, Sapsford et al 1989) reported more detailed results in a prospective randomised trial of patients undergoing elective, first time coronary surgery and in this case it seemed to be a beneficial effect on platelet number and action which reduced the bleeding tendency (Van Oeveren, Jansen, Bidstrup et al 1987). To gain this benefit the drug had been infused before bypass, throughout its course and continued until the chest was closed. Subsequent work suggested that the platelet membrane was stabilised the start of CPB (Van Oeveren, Eijsman, Rozendaal et al, 1988). It was observed anecdotally, by many people, that there was less bleeding during the preparatory dissection for repeat operations, before the bypass had even commenced or even before heparin was given.

More recent work suggests that during the initial part of CPB platelet function is altered by reduction in the number of adhesive (glycoprotein Ib) receptors which are preserved

by aprotinin (Van Oeveren, Harder, Rozendaal et al 1990). It is therefore of great interest to see what effect aprotinin has on these receptors if given post-operatively only and whether the number of receptors correlates with other platelet function tests and the amount of blood loss.

The report by Angelini and colleagues (Angelini, Cooper, Lamarra et al 1990) that aprotinin may be effective in stopping post-operative haemorrhage in a group of patients with life threatening post-operative bleeding was very encouraging. This, however, was based on six cases, following cardiac surgery, in an uncontrolled use of the drug under difficult clinical circumstances. Nevertheless the data were impressive.

Aprotinin is expensive and can cause allergic reactions. Use of a drug infusion from induction of anaesthesia, through into the post CPB period, for all patients is a major undertaking when haemorrhage of worrying proportions only affects a minority. Furthermore, there is remaining concern that some degree of coagulopathy is beneficial, at least in the first few hours and days after performing coronary anastomoses to small vessels individually carrying a low flow. If we achieve the levels of haemostasis that seem to be possible with aprotinin there might be progressive coagulation around the suture lines and areas of vascular trauma thus causing occlusion of grafts which might otherwise have become established as long lasting conduits. Surgeons were therefore selective, trying to predict from experience which patients were likely to bleed, including

re-operations, endocarditis, multiple valves and aortic dissection. Unfortunately, bleeding problems are sporadic and remain largely unpredictable.

1.2 Platelet function

In addition to the literature reviewed in this section I relied heavily on the textbooks by Colman and Rao (1990), by Page (1991), by Fuster and Vestræte (1992) and by Pittilo and Machin (1987). The structure and role of the **platelet GPIb-IX** complex was reviewed by Ruggeri (1991).

The main functions of platelets are adhesion to damaged vessel walls, aggregation to form a platelet plug and promotion of fibrin clot formation. **Platelet adhesion** is primarily mediated by the adhesive vWF molecule, which binds both to a specific receptor on the platelet surface glycoprotein (GP) Ib-IX complex and to exposed subendothelial components (reviewed recently by Roth 1992, including the role of shear in the development of affinity between platelet GPIb and vWF). **Platelet to platelet aggregation** is primarily mediated by fibrinogen binding to its receptor on the platelet surface GPIIb-IIIa complex (Shattil, Hoxie, Cunningham et al 1985). Normal circulating platelets are in a resting state and despite the presence of platelet surface GPIb-IX and GPIIb-IIIa complexes, they bind neither plasma vWF nor plasma fibrinogen. *In vitro*, the cationic antibiotic ristocetin induces binding of vWF to its receptor on GPIb (George, Nurden and Philips 1984), but *in vivo* the analogue of

ristocetin remains uncertain. Physiological platelet agonists such as thrombin induce exposure of the fibrinogen receptor on the platelet surface GPIIb-IIIa complex (Shattil, Hoxie, Cunningham et al 1985), stimulate platelets to change shape, to secrete the contents of their granules and to aggregate. Secreted thrombospondin binds to a receptor on the platelet surface membrane, as well as fibrinogen, and thus stabilising the platelet to platelet aggregates (Leung 1984). GMP-140, a component of the α -granule membrane of resting platelets is a very precise marker of platelet secretion (Stenberg, McEver, Shuman et al 1985). Although its physiologic role remains speculative, GMP-140 mediates in vitro adhesion of activated platelets to monocytes and neutrophils (Larsen, Celi, Gilbert et al 1989). In contrast to its effect on GMP-140 and the fibrinogen receptor on GPIIb-IIIa complex, thrombin downregulates the platelet surface expression of the vWF receptor on the GPIb-IX complex (George, Pickett, Saucerman et al 1986; Michelson and Barnard 1987). Thrombin induces rapid redistribution of GPIb-IX complexes within the surface connected membrane system, which is in opposite direction to the thrombin induced externalisation of internal pools of GPIIb-IIIa complexes and GMP-140 (Hourdillé, Heilmann, Combrié et al 1990). These observations were reviewed recently by Michelson (1992).

von Willebrand factor

The structure and function of vWF was extensively reviewed by Meyer and Girma (1993) and the function of platelet vWF and the effect of aspirin was reviewed by Gralnick and

colleagues (Gralnick, Williams, McKeown et al 1991). von Willebrand's disease was reviewed by Ruggeri and Zimmerman in 1987 and by Holmberg and Nilsson in 1992.

Platelet vWF is synthesised by the megakaryocyte and does not interchange with plasma vWF (Bowie, Solberg, Fass et al 1986). When released from the platelet α -granule on activation, it binds to platelet surface receptor GPIIb/IIIa (Parker and Gralnick 1986) and is the major factor in platelet binding to collagen-coated surfaces (Fressinaud, Baruch, Rothschild et al 1987). Clinically, in variants of the congenital bleeding disorder von Willebrand's disease, platelet vWF correlates better with bleeding time than the plasma level of vWF (Gralnick, Rick, McKeown et al 1986) and is important in replacement therapy (Mannucci, Moia, Rebulla et al 1987). One proposed model of platelet function is that plasma vWF / platelet GPIb interaction mediates initial subendothelial contact (platelet adhesion) and then platelet vWF / GPIIb-IIIa binding facilitates spreading and aggregation on subendothelium (Gralnick, Williams, McKeown et al 1991).

In 1986 Salzman and colleagues (Salzman, Weinstein, Weintraub et al 1986) reported that desmopressin (DDAVP) reduced blood loss after CPB. As patients with the most bleeding had relatively low levels of vWF before operation these authors speculated that the haemostatic benefit of desmopressin may be related to its effect on increasing plasma vWF, particularly the higher molecular weight multimers. The beneficial effect of desmopressin on blood

loss was also documented in another study of aspirin pre treated patients (Gratz, Koehler, Olsen et al 1992). Two other, smaller studies, however, did not confirm the beneficial effect of prophylactic desmopressin (Andersson, Solem, Tengborn et al 1990; Rocha, Llorens, Páramo et al 1988).

Czer and his group (Czer, Bateman, Gray et al 1987) targeted desmopressin treatment in patients bleeding excessively in the first two hours post CPB in a similar fashion to the aprotinin study described in this thesis. Their study, however, was neither randomised nor blind. Nevertheless these authors demonstrated a reduction in blood loss and transfusion requirements by desmopressin, which was associated with shortening of bleeding time and an increase in plasma levels of the factor VIII complex (procoagulant activity and vWF).

In this thesis therefore, by estimating platelet GPIb and vWF (levels and multimeric structure) as well as observing platelet aggregation in response to different agonists, major determinants of the effect of aspirin and CPB on primary haemostasis were examined.

1.3 The post-bypass haemostatic defect

In addition to the enormous literature concerning the haemostatic defect related to CPB I relied heavily on the review by Bick (1985) and the textbook by Ellison and Jobes (1988). The causes of bleeding following surgery requiring

CPB can be classified into three broad categories: 1) Pre-operative factors such as congenital haemostatic defects, aspirin or warfarin use, 2) Surgical causes such as bleeding from an anastomotic site or a side branch from a vein graft and 3) due to the post-bypass haemostatic defect. The first and the third causes can not be easily distinguished clinically, unless the pre-operative causes were documented, and can therefore be grouped together as "medical causes" as opposed to "surgical causes" of bleeding. Even bleeding secondary to surgical causes which has not been controlled adequately and promptly can in itself produce or exacerbate an existing coagulopathy because of excessive blood loss and transfusion of large quantities of blood. Conversely minor surgical bleeding points do not stop bleeding in the presence of coagulopathy whereas they may have stopped in the presence of normal haemostasis.

The haemostatic defect following CPB is multifactorial and includes hypothermia, haemodilution, unneutralised heparin (and heparin rebound, Gollub 1967), excessive protamine, thrombocytopenia, platelet dysfunction, contact activation and excessive fibrinolysis. Although hypothermia has been considered an important factor in the past (Valeri, Cassidy, Khuri et al 1987) the more recent use of normothermic CPB has made this more controversial. In a recent study of normothermic CPB (Mazer, Hornstein and Freedman 1995) it was demonstrated that normothermic CPB affects platelet activation and integrity in a similar way to hypothermic CPB. Thrombocytopenia, platelet dysfunction, contact activation and excessive fibrinolysis, the four most

important contributing factors, are considered further.

1.3a Thrombocytopenia

Thrombocytopenia has been widely reported in association with CPB (Harker, Malpass, Branson et al 1980; Martin, Daniel and Trowbridge 1987), but there is wide variability in the degree of thrombocytopenia in different series. This is most likely to be a reflection of differences in the synthetic materials of the CPB circuit, type of oxygenator, systemic temperature, bypass time, degree of haemodilution and pharmacological agents used. Haemodilution is generally considered to be one of the major factors that contribute to the thrombocytopenia during CPB. Other factors include removal of activated platelets from the circulation by adherence to the synthetic surfaces, formation of platelet aggregates, disseminated intravascular coagulation (DIC) and heparin induced thrombocytopenia.

Platelets adhere to the synthetic surfaces by binding to fibrinogen and other adhesive proteins which form an intermediate adhesive layer on the synthetic surfaces. The mechanisms by which platelets are initially activated are not completely clear, but possible causes include: direct surface contact, thrombin and ADP. Once platelet activation occurs it results in exposure of fibrinogen receptors on the GPIIb-IIIa complex and therefore permits binding to fibrinogen molecules previously absorbed onto the synthetic surface (Musial, Niewiarowski, Hershock et al 1985) and thus contributes to the platelet consumption

(Gluszko, Rucinski, Musial et al 1987). Gluszko and colleagues (Gluszko, Rucinski, Musial et al 1987) demonstrated that blood from patients with Glanzmann's thrombasthenia (an inherited deficiency of the GPIIb-IIIa complex) had less CPB induced thrombocytopenia, compared to patients with Bernard-Soulier syndrome (an inherited deficiency of GPIb, GPIX and GPV). Further evidence that platelet activation, adherence and clearance is important comes from studies using inhibitors of platelet activation during CPB leading to maintenance of near normal platelet counts during CPB (Addonizio, Macarak, Niewiarowski et al 1979; Walker, Davidson, Faichney et al 1981). In addition activated platelets are more likely to be removed by the reticuloendothelial system.

Bubble oxygenators, as compared to membrane oxygenators, result to greater platelet damage because of the direct blood gas interface (Van den Dungen, Karliczek, Brenken et al 1982). The cardiotomy sucker (Edmunds, Saxena, Hillyer et al 1978) and the formation of platelet aggregates (Dutton, Edmunds, Hutchinson et al 1974) further decrease the platelet counts. DIC although probably uncommon as distinct from primary fibrinolysis is another cause of thrombocytopenia following CPB.

Type I, heparin induced thrombocytopenia is transient, of immediate onset and mild degree that accompanies heparin therapy in approximately 5% of patients, probably related to the pro-aggregatory effect of heparin (Salzman, Rosenberg, Smith et al 1980). Type II occurs in approximately 0.6% of

patients receiving heparin and is delayed, severe and probably immune mediated thrombocytopenia that may occur in association with platelet activation, aggregation and occasionally with massive arterial thrombosis (Berndt, Chong, Andrews et al 1989).

Thrombocytopenia, the end result of multiple factors, is also an important contributing factor in the post CPB haemostatic dysfunction.

1.3b Platelet dysfunction

The acquired defect of platelet function plays a pivotal role in the post CPB haemostatic defect (Harker 1986). Heparin does not prevent adsorption or activation of platelets during CPB. Although the bleeding time is unaffected by heparinisation it is abruptly prolonged following initiation of CPB (Harker, Malpass, Branson et al 1980). The causes of platelet dysfunction are not entirely clear but include: exposure to synthetic surfaces, shear force (Addonizio, Colman and Edmunds 1978), hypothermia (Harker, Malpass, Branson et al 1980), oxygenators, plasmin and FDPs (Bick 1985), denatured plasma proteins, thrombin, ADP, heparin (Berndt, Chong and Andrews 1989), protamine (Ellison, Edmunds and Colman 1978), and sodium nitroprusside (Hines and Barash 1989). The presence of a circulating platelet inhibitor is unlikely because plasma from patients who had undergone a period on CPB does not inhibit the function of normal platelets. Circulating FDPs can interfere with platelet function (Kowalski, Kopec and

Wegrzynowicz 1963) and are present in the majority of patients who have undergone CPB (Bick 1985). There is poor correlation, however, between FDPs and the degree of platelet dysfunction following CPB (Bick, Schmalhorst and Arbegast 1976).

The mechanism of platelet dysfunction following CPB remains contentious but it is likely to be multifactorial. There are two main reasons for the variability in the reported defects in platelet function following CPB. Firstly it is a reflection in the differences in equipment and techniques mentioned previously and secondly it is a reflection of the difficulties in the in vitro methods used to characterise in vivo platelet function defects. It is well known that platelets are susceptible to membrane alterations and in vitro activation during their separation from whole blood (George, Thoi and Morgan 1981). Plasma assays of the secretion products of platelet α -granules (platelet factor 4 and β -thromboglobulin) have been widely used, but the problem of in vitro secretion is of concern because even a one per cent platelet secretion may cause as much as 30 fold in the plasma level of platelet factor 4 (Levine and Krentz 1977). Furthermore plasma assays of platelet factor 4 and β -thromboglobulin reflect activated platelets, lysed platelets and platelets adherent to synthetic surfaces.

In order to overcome some of these problems whole blood assays have been developed which do not involve separation or manipulation of platelets (George, Pickett, Saucerman et al 1986; Shattil, Cunningham and Hoxie 1987). George

and colleagues (George, Pickett, Saucerman et al 1986) directly measured platelet surface glycoproteins in immediately fixed whole blood samples with ¹²⁵Iodine labelled monoclonal antibody (mAb). These workers demonstrated changes consistent with membrane fragmentation without secretion: a decrease in platelet surface concentration of GPIb and GPIIb, no increase in platelet surface GMP-140 or thrombospondin and an increase in plasma concentration of membrane microparticles as a result of turbulence and shear stress. These findings are supported by other authors (Abrams, Ellison, Budzynski et al 1990; Shattil, Cunningham and Hoxie 1987) who used whole blood flow cytometry to demonstrate fragmentation and production of microparticles, but not of platelet secretion as determined by a GMP-140 specific mAb.

During and following CPB the platelet response to agonists such as collagen, ADP and adrenaline (Mammen, Koets, Washington et al 1985; Wachtfogel, Musial, Jenkin et al 1985) is reduced, most likely because of reduction of platelet membrane fibrinogen (Wenger, Lukasiewicz, Mikuta et al 1989) and adrenaline receptors (Wachtfogel, Musial, Jenkin et al 1985). After protamine administration there is a further decrease in ADP induced platelet aggregation (Mammen, Koets, Washington et al 1985; Holloway, Summaria, Sandesara et al 1988). Mammen and colleagues (Mammen, Koets, Washington et al 1985) reported that ristocetin induced platelet agglutination is relatively unchanged during CPB, but decreases after administration of protamine and remains abnormal for 24 hours post-

operatively. Although the protamine effects on platelets are well documented (Ellison, Edmunds and Colman 1978; Velders and Wildevuur 1986) the mechanism of these effects are uncertain. In vitro experiments have suggested that the effects are mediated by the protamine-heparin complex rather than by protamine alone (Ellison, Edmunds and Colman 1978). Another possible explanation for the protamine induced reduction in ristocetin and ADP induced platelet aggregation is that protamine leads to increase in plasmin (Bick, Arbegast, Crawford et al 1975; Ekert, Montgomery and Aberdeen 1971), which then leads to plasmin induced cleavage of platelet surface GPIb (Adelman, Michelson, Greenberg et al 1986; Adelman, Michelson, Loscalzo et al 1985) and GPIIb-IIIa complex (Stricker, Wong, Shiu et al 1986).

CPB leads to a primary fibrinolytic state resulting in generation of plasmin (Bick 1985). Adelman and associates (Adelman, Michelson, Greenberg et al 1986; Adelman, Michelson, Loscalzo et al 1985) have demonstrated that plasmin cleaves platelet surface GPIb in vitro, which is important both for platelet adhesion (vWF) and activation (via its thrombin receptor) (Takamatsu, Horne and Gralnick 1986). Mohr and colleagues (Mohr, Golan, Martinowitz et al 1986) reported that in all 20 patients studied after CPB there was impaired platelet response to ADP, collagen and ristocetin but only the ristocetin response correlated with clinical bleeding. As the vWF assays were normal it suggests that the abnormality in ristocetin induced platelet agglutination is the result of a defect in platelet surface GPIb. Redistribution of platelet surface GPIb to

intraplatelet stores (Michelson, Adelman, Barnard et al 1988) could account for the rapid decrease in platelet surface GPIb that occurs after CPB (van Oeveren, Harder, Rozendaal et al 1990). Aprotinin has been demonstrated to inhibit the plasmin mediated cleavage of platelet GPIb (Adelman, Michelson, Loscalzo et al 1985) and may account for the preservation of platelet surface GPIb during CPB (van Oeveren, Harder, Rozendaal et al 1990). As fibrinolytic activity is not enhanced in the early phase of CPB, redistribution of surface GPIb into an intraplatelet pool is a more likely explanation of the rapid decrease of surface GPIb.

The acquired platelet dysfunction, both adhesion and aggregation, has a central role in the post CPB haemostatic derangement.

1.3c Contact activation

During CPB factor XII (Hageman factor) and platelets are activated directly by contact with synthetic surfaces, air, blood and tissue interfaces. Activated factor XII initiates a series of cascade reactions involving the intrinsic coagulation pathway, the classic complement pathway, fibrinolysis, kallikrein and neutrophil activation. Anticoagulation with heparin only inhibits one component of this contact activation system, coagulation, while the rest of the cascades remain uninhibited and therefore culminate in a systemic inflammatory response (Kirklin, Westaby, Blackstone et al 1983). The effect of synthetic surfaces

on platelet activation and adsorption was discussed in the previous section.

1.3d Hyperfibrinolysis

The coagulation cascade and the fibrinolytic systems are interlinked dynamically. Their normal action controls clot formation at specific sites in a controlled manner and eventually leads to clot breakdown. This intricate control of the activity of the fibrinolytic system is achieved by activators and inhibitors.

The inactive plasminogen, upon activation by the tissue plasminogen activator (tPA) or by urokinase (uPA), is converted to plasmin which degrades fibrin into soluble fibrin degradation products (FDPs). tPA is continuously released by endothelial cells into the blood stream especially in response to acidosis, hypoxia and in response to agents such as bradykinin, thrombin, adrenaline and others. tPA exists in two forms: in a free single chain form and in an inactive form bound to plasminogen activator inhibitor type 1 (PAI-1). Both inactive tPA and uPA (single chain) can be activated (two chain) by small amounts of plasmin or kallikrein and inactivated by PAI. Free plasmin is rapidly inactivated by α_2 -antiplasmin.

The increased fibrinolysis in relation to CPB was recognised as early as 1968 by Bentall and Allwork. A variety of researchers have observed increased fibrinolysis during CPB as indicated by the shortening of by reduction in

plasminogen concentration, the euglobulin lysis time, of the dilute whole blood clot lysis time and the increased levels of FDPs (Holloway, Summaria, Sandesara et al 1988; Kucuk, Kwaan, Frederickson et al 1986; Stibbe, Kluft, Brommer et al 1984), which is suppressed by aprotinin (Van Oeveren, Jansen, Bidstrup et al 1987). This increase in fibrinolytic activity is most likely to be due to contact activation of prekallikrein and tPA release as discussed previously.

During CPB a marked shortening of euglobulin lysis time occurs, but if this activity is assayed on fibrin plates in the presence of an inhibitor which does not block the activity of tPA, only about one third of the fibrinolytic activity is quenched. The remaining activity can be completely blocked with tPA antibodies (Stibbe, Kluft, Brommer et al 1984). Approximately two thirds of the fibrinolytic activity during CPB can therefore be attributed to tPA. The fibrinolytic activity rapidly disappears at the end of CPB which approximates to the disappearance of tPA which has a half life of a few minutes.

What would account for the increased generation of plasminogen activators and generation of plasmin during CPB? Plasmin converts single chain tPA and uPA to their two chain active forms, releases fibrinopeptide^{84 15-42} from fibrinogen and forms complexes with its primary inhibitor, α_2 -antiplasmin. Free plasmin degrades fibrin, fibrinogen, factor V, factor VIII, vWF and platelet glycoproteins (Adelman, Michelson, Greenberg et al 1986; Adelman, Michelson, Loscalzo et al

1985). The increase of FDPs and the partial degradation of other coagulation factors and of platelet glycoproteins may not only be due to plasmin, but it could also be caused by proteases released from leucocytes and monocytes

(Wachtfogel, Kucich, Greenplate et al 1987). Van Oeveren, Harder, Rozendaal and colleagues demonstrated in 1990 that there is a decrease of approximately 30% of platelets surface GPIb within five minutes of the commencement of CPB.

It seems highly unlikely that the increased levels of circulating tPA or plasmin account for this early reduction.

Fibrinolytic activity, as measured in the circulating blood, is not very much enhanced in the early phase of bypass (Van Oeveren, Jansen, Bidstrup et al 1987). The nearly absent increase of circulating plasmin- α_2 -plasmin inhibitor complex formation further supports the absence of biologically important concentrations of free plasmin (Wachtfogel, Harpel, Edmunds et al 1989). However events occurring at platelet membranes or at the blood interface with immobilised proteins may not necessarily be reflected in circulating blood.

The majority of evidence, therefore, points towards increased fibrinolysis during CPB.

1.4 Historical review of aspirin

The analgesic properties of willow bark, which contains salicylates, was known by Hippocrates (460-355 B.C.) as well as by the Indians of North America. In 1763 the Reverend Edward Stone of Chipping Norton submitted a letter to the

Royal Society in which he described the benefit of willow bark for the treatment of ague (Stone 1763). His quotation "I have no other motives for publishing this valuable specific than that it may have a fair and full trial in all its variety of circumstances and situations, and that the world may reap the benefits accruing from it" is still being fulfilled in different ways. During the 18th century Willow bark, which contains salicin, was used for the treatment of fevers as a cheap substitute for imported cinchona (quinine) bark. It was tried because both willow trees and agues occurred in marshy places and because, according to the "doctrine of signatures" where a disease was found, so there would be a remedy nearby, provided by a beneficent nature (Laurence and Bennett 1980). In 1876 MacLagan described the use of salicin, the active ingredient of willow bark, for the treatment of acute rheumatism. Felix Hoffman at Bayer Industries, searched over a century ago for a less indigestible salicylic acid derivative for his father, who had rheumatoid arthritis, and as a result acetylsalicylic acid became available. In 1899 aspirin (acetylsalicylic acid) was introduced as a more palatable and apparently less toxic form of salicylic acid (Dreser 1899).

During the early 1940s, papers started appearing in the literature which highlighted some of the potential dangers of aspirin especially post-operative bleeding. The report by Craven in 1950, however, is particularly distinguished by a number of prophetic statements about the benefits and risks of aspirin which have since been substantiated.

Craven in 1950 reported in his paper entitled "Acetylsalicylic acid, possible preventive of coronary thrombosis" that for several years, during the 1940s, he prescribed one stick of Aspergum (3.5 g acetylsalicylic acid), before each meal and at bedtime, in order to enable his patients to eat and sleep after tonsillectomy.

Although pain was usually relieved many patients had serious post-operative haemorrhages which were difficult to control, and in each instance the coagulation time was prolonged. After investigating all the patients with severe haemorrhage Craven discovered that the patients had not only chewed the four sticks of Aspergum per day as ordered but purchased additional supply, consuming up to 20 sticks (70 g acetylsalicylic acid) daily. Craven concluded that large quantities of salicylates may be dangerous in the presence of a recent wound, unless its anticoagulant effect was counteracted by adequate amounts of vitamin K. Craven continued to prescribe aspirin but with two mg of 2-methyl-naphthoquinone daily with no serious post-operative haemorrhage. His observation of the prolonged coagulation time, can now be explained by the fact that aspirin in high doses, in excess of one gram daily, may exert an anticoagulant effect via impaired synthesis of clotting factors II, VII, IX and X by the liver (Webster 1983).

The last three paragraphs quoted from Craven's paper summarise some of the early impressions of the benefit / risk equation of aspirin, as well as some eloquent predictions, which have been proven to be correct in properly controlled studies and thus accomplishing Craven's

wish. "The detrimental effects of acetylsalicylic acid may also be noted in women with excessive menstrual bleeding who are unnecessarily subjected to radiation therapy or even surgical procedures in order to control bleeding which is actually the result of overdosage with salicylates. That the coagulation time of blood may become shorter as certain individuals grow older is suggested by the progressive decrease in nosebleeds as well as in bleeding from shaving wounds I, and no doubt other men, have experienced between youth and middle age". (Later documented by Jorgensen, Dyerberg, Olesen et al 1980). "The apparently lower incidence of coronary disease and thrombosis in undernourished nations and lower income groups suggests that this change in coagulation time is to some extent due to overconsumption of rich foods. However, only a small percentage of many overweight middle aged and elderly women in the American population die of coronary thrombosis. A possible explanation of this apparently contradictory evidence is that women frequently use aspirin to relieve minor discomforts while men hesitate to employ such allegedly effeminate methods. Of course, there are other factors involved in thrombosis and coronary disease."

"If a further study confirms the impression that acetylsalicylic acid prolongs coagulation time, it would appear that the drug might be of value as a preventive of vascular thrombotic conditions, including coronary thrombosis. During the past two years, I have advised all my male patients between the ages of 40 and 65 to take from 10 to 30 grains of acetylsalicylic acid daily as a possible

preventive of coronary thrombosis. More than 400 have done so, and of these, none has suffered a coronary thrombosis. From past experience, I should have expected at least a few thrombotic episodes in this group."

"There would appear to be enough evidence of antithrombotic action of acetylsalicylic acid to warrant further study under more carefully controlled conditions" (Craven 1950).

Aspirin has since been used for the treatment of fever, rheumatoid arthritis, osteoarthritis, headache, pre-eclampsia, to delay premature labour and for numerous indications in the treatment and prevention of cardiovascular disease. As a result, by the 1960s, aspirin had become the most widely used pharmaceutical product in the world. The British consumed about two thousand tons (>2 million kg) of aspirin yearly (6,000 million tablets or two tablets every week for every citizen (Laurence and Bennett 1980). Numerous aspirin containing compounds became commercially available (Leist and Banwell, 1974) and many patients were unaware that they were taking aspirin. As a result of the widespread use of aspirin and despite its therapeutic benefits, aspirin induced gastrointestinal bleeding became widely acknowledged as a serious public health problem. Concern that aspirin was nephrotoxic and evidence that Reye's syndrome was somehow related to the use of aspirin lead to the removal of aspirin from the World Health Organization's list of essential drugs in 1988.

The balance of the benefit versus the risk of aspirin,

therefore remains unresolved.

1.5 Mechanism of action of aspirin

In 1971 Vane reported that aspirin inhibited the production of prostaglandins by cells and tissues in guinea-pig lung. In the same issue of *Nature*, Smith and Willis (1971) reported that the inhibition of platelet aggregation by aspirin was due to the inhibition of prostaglandin synthesis by human platelets. Aspirin inhibits prostaglandin endoperoxide synthetase (Roth, Stanford and Majerus 1975; Smith and Willis 1971) in platelets and in normal vascular endothelial cells by irreversibly acetyloyating a single serine residue of the enzyme (Roth, Stanford and Majerus 1975, Roth and Majerus 1975) forming an N-acetyl serine at the NH₂ terminus of the cyclo-oxygenase portion of the enzyme. Since platelets can not synthesise new protein this irreversible inhibition of the enzyme lasts for the entire ten day life span of the platelets (Burch, Stanford and Majerus 1978). Small doses of aspirin may affect the synthesis of thromboxane by platelets and prostacyclin by endothelial cells differently (FitzGerald, Pedersen and Patrono 1983). This is explained by the short half life of acetylsalicylate which allows synthesis of new enzyme by endothelial cells whereas platelets are unable to replace their enzyme which has been irreversibly inactivated. During prostaglandin synthesis arachidonic acid is released from the hydrolysis of platelet membrane phospholipids by phospholipase A₂ (Smith, Araki and Lefer 1980). Arachidonic acid is converted to prostaglandin cyclic

endoperoxides, prostaglandin G_2 and prostaglandin H_2 , by prostaglandin endoperoxide synthetase. Prostaglandin cyclic endoperoxides are then converted to thromboxane A_2 in platelets and to prostacyclin in normal vascular endothelial cells. Thromboxane A_2 is finally converted to its stable metabolite thromboxane B_2 and prostacyclin to its stable metabolite 6-keto-prostaglandin $F1\alpha$.

Thromboxane A_2 causes platelet aggregation and constricts blood vessels (Hamberg, Svensson and Samuelsson 1975) while prostacyclin inhibits platelet aggregation and dilates blood vessels (Moncada and Vane 1979). These opposing actions maintain the delicate balance necessary for normal haemostasis. Congenital cyclo-oxygenase deficiency shifts the haemostatic balance in favour of haemorrhage rather than thrombosis (Pareti, Mannucci, D'Angelo et al 1980). This was confirmed by the work of Preston and colleagues who showed that the inhibition of both the platelet and vascular endothelium prostaglandin synthesis results in a bleeding diathesis (Preston, Greaves, Jackson et al 1982).

Aspirin has also been shown to acetylate proteins and other cellular components (Hawkins, Pinckard and Farr 1968) and to inhibit megakaryocyte cyclo-oxygenase in rats (Smith and Willis 1971). Aspirin has no effect on platelet adhesion (Weiss, Turitto and Baumgartner 1978) unless the calcium ion concentration in the platelets is reduced to unphysiological levels (Baumgartner 1979) or when the red blood cells are excluded from the system (Davies, Essien, Cazenave et al 1979).

The effect of aspirin on platelet aggregation is due to interference with the ability of platelets to synthesise prostaglandins (Smith and Willis 1971), thus inhibiting the conversion of arachidonic acid to cyclic endoperoxides and their derivatives (Roth, Stanford and Majerus 1975). There is controversy whether prostaglandin cyclic endoperoxides or thromboxane A₂ mediate the aggregatory activity of arachidonic acid (Bertele, Cerletti, Schieppati et al 1981; Vermylen, Defreyn, Carreras et al 1981). Originally it was felt that thromboxane A₂ was responsible but it is now felt that it is not necessary for platelet aggregation to occur (Bertele, Tomasiak, Falanga et al 1982). Formation of prostaglandin endoperoxides or other cyclo-oxygenase products may be sufficient to mediate aggregation induced by arachidonic acid when thromboxane A₂ synthesis is pharmacologically inhibited. Two selective inhibitors of thromboxane A₂ synthetase, dazoxiben and OKY1581, did not prevent arachidonic acid induced aggregation of human platelets. Instead prevention was obtained by combining thromboxane A₂ synthetase inhibitors with low concentrations of aspirin (Bertele, Falanga, Tomasiak et al 1983). The mechanism of action of aspirin has, therefore, been resolved but research continues for more selective inhibitors of prostaglandin synthesis.

1.6 Pharmacokinetics of aspirin

Aspirin is preferentially absorbed through the gastric mucosa because of the acidic pH unless it is given with an alkaline or if it is enterically coated. Aspirin has a

plasma half life of 15 minutes, being converted into salicylate (half life 4-6 hours) which is also therapeutically active. Salicylates are 50-80 % bound to plasma protein, they are partially conjugated in the body and they are excreted by the kidney unchanged and as conjugates. The proportion excreted as free salicylates varies with the urinary pH from 10-80 %. A reasonably steady plasma concentration can be maintained if salicylates are given orally six hourly which is about the half life of the drug at therapeutic doses, though this can also vary with urine pH (Laurence and Bennett 1980).

Numerous studies have been conducted in an attempt to determine the dose which will inhibit platelet aggregation while leaving prostacyclin formation unimpaired. Both low (Dybdahl, Daae, Eika et al 1981) and high dose aspirin (Rothschild 1979) have been shown to increase bleeding time. Some workers found a paradoxical shortening of the bleeding time following a large dose of aspirin (O'Grady and Moncada 1978), while others demonstrated a benefit in prevention of thromboembolic complications only after high doses (Olsson 1979). Maximum prolongation of bleeding time occurred on average at two and a half hours and reached basal levels within six days (Amezcua, O'Grady, Salmon et al 1979; Rothschild 1979).

Some workers have suggested that aspirin should be given daily (Bradlow and Chetty 1982; Jaffe and Weksler 1979) while others have advocated a dose every third or fourth day (Masotti, Galanti, Poggessi et al 1979; Patrono, Ciabattoni,

Pinca et al 1980) since this might cause the least possible inhibition of prostacyclin production and yet maintain adequate suppression of platelet activity. Animal studies have shown that the capacity of the vascular endothelium to synthesise prostacyclin is far in excess of that required to inhibit platelets adhesion and thrombus formation (Weksler, Ley and Jaffe 1978, Higgs, Moncada, Vane et al 1978).

Further studies have demonstrated a person to person variation in response to 40 mg aspirin (Hanley, Bevan, Cockbill et al 1981; O'Brien 1980), which inhibits human platelet thromboxane B₂ synthesis 98-100% after three daily doses (Preston, Greaves, Jackson et al 1982). A single dose of 40 mg aspirin has no effect on human venous prostacyclin synthesis (Hanley, Bevan, Cockbill et al 1981) while four daily doses inhibit prostacyclin synthesis 80-100% (Preston, Greaves, Jackson et al 1982) identical to that obtained two hours after a single large dose of 150 or 300 mg (Pareti, D'Angelo, Mannucci et al 1980; Webster 1983). This suggests that repeated doses of aspirin have a cumulative inhibitory effect on endothelial cell cyclo-oxygenase.

Jaffe and Weksler showed that cultured human endothelial cells began to regain their ability to produce prostacyclin as soon as aspirin was removed from the system (Jaffe and Weksler 1979). Other workers, however, found the rate of recovery to be considerably slower (Hanley, Bevan, Cockbill et al 1981). Bleeding time falls with age in men (Jorgensen, Dyerberg, Olesen et al 1980) and aspirin has a

greater antithrombotic effect in males than in females (Young, Giles, Pater et al 1980). This occurs even though female patients have higher salicylate levels than male patients following a fixed dose of aspirin, which indicates a difference in absorption (Kelton, Carter, Rosenfeld et al 1981). All these points may explain some of the differences in the findings of previous aspirin studies and emphasise the importance in matching the groups for age and sex as well as other relevant factors. Despite extensive research the most appropriate dose regime of aspirin remains controversial.

1.7 Benefits of pre-operative aspirin therapy

The benefit of pre-operative aspirin therapy in reducing ischaemic events is well established (Chesebro and Fuster 1986). Aspirin has been shown to reduce mortality in myocardial infarction (ISIS-2 Collaborative Group 1988), unstable angina (The RISC Group 1990) and in chronic stable angina pectoris (Juul-Möller, Edvardsson, Jahnmatz et al 1992). Furthermore pre-operative aspirin was shown to reduce early vein graft occlusion in a prospective randomised trial (Chesebro, Clements, Fuster et al 1982). Further evidence from clinical trials, as well as experimental evidence, suggests that there is an early post-operative phase of platelet thrombotic occlusion of vein grafts which can be prevented by peri-operative anti-platelet therapy (Fuster and Chesebro 1986). The benefit of pre-operative aspirin therapy in reducing pre-operative ischaemic events and in improving post-operative vein graft

patency rates is well established.

1.8 Risks of pre-operative aspirin therapy

Several studies, however, have shown that pre-operative aspirin therapy increases post-operative blood loss, transfusion requirements and the risk of re-exploration for excessive bleeding. None of these studies clinched the issue because of inadequate study design. These flaws included lack of randomisation (Taggart, Siddiqui and Wheatley 1990), the use of only one aspirin the day before surgery (Sethi, Copeland, Goldman et al 1990), lack of blinding (Ferraris, Ferraris, Lough et al 1988) and the comparison of patients undergoing valve replacement with patients undergoing coronary artery bypass surgery (CABG) who were on aspirin (Boldt, Knothe, Zickmann et al 1992). Furthermore other workers have failed to demonstrate significantly increased bleeding in patients given pre-operative aspirin (Rawitscher, Jones, McCoy et al 1991; Williams, Borgstein and Gallandat Huet 1989).

For these reasons argument has persisted in the literature (Gay 1990; Violaris and Angelini 1991) on the balance of the benefit versus the risk of pre-operative aspirin therapy. One school of thought is that the increased risk of bleeding is a small price to pay for improved vein graft patency. The other school of thought is that the increased morbidity and risks of avoidable blood transfusion outweigh the small advantage of improved vein graft patency.

Such was the divergence of opinion, on this benefit / risk equation, in our unit that two surgeons insisted that their patients remained on pre-operative aspirin until the day of operation and two surgeons preferred the aspirin to be discontinued ten days earlier. The respective groups accepted that the evidence to support their practices was lacking and were therefore prepared to randomise their patients for the purposes of the clinical trial. This clinical scenario provided the perfect environment for a prospective, randomised, placebo controlled, double-blind clinical trial with no ethical dilemmas.

1.9 Historical review of aprotinin

In addition to the review of the extensive literature in this chapter I relied heavily on the reviews by Royston (1990), Taylor (1993), and Vestraete (1985). Aprotinin was discovered in organs of cattle by Kraut, Frey and Werle in 1930 and was used clinically for the first time in acute pancreatitis by Frey in 1953. Although there were anecdotal reports of the beneficial effect of aprotinin in reducing blood loss after cardiac surgery as early as 1964 (Tice, Worth, Clauss et al), it was not until 1971 that Ambrus (Ambrus, Schimert, Lajos et al) reported this effect in a randomised double blind trial using low dose aprotinin (100,000 KIU/h, for four hours) during surgery. It took another 16 years for the efficacy of high dose aprotinin (2×10^6 KIU loading, 2×10^6 KIU in CPB and 0.5×10^6 KIU/h for the duration of surgery) to be reported (Royston, Bidstrup, Taylor et al 1987) in patients undergoing repeat cardiac

surgery. Further trials confirmed the efficacy of aprotinin in patients with sepsis (Bidstrup, Royston, Taylor et al 1988) and in patients taking aspirin pre-operatively (Bidstrup, Royston, McGuiness et al 1990; Murkin, Lux, Shannon et al 1994). Numerous studies, in a variety of centres, have since confirmed the effectiveness of prophylactic aprotinin in reducing blood loss after cardiac surgery.

Further studies have demonstrated the effectiveness of aprotinin in different clinical situations. Goldstein and colleagues (Goldstein, Seldomridge, Chen et al 1995) demonstrated a decrease in bleeding and blood use in a retrospective study of aprotinin in patients who required left ventricular assist devices. In another study Kesten and colleagues (Kesten, de Hoyas, Chaparro et al 1995) demonstrated a reduction in blood loss and blood transfusion in patients who underwent lung transplantation. This difference was only demonstrated in patients who required CPB during lung transplantation which emphasises once more the importance of CPB in post-operative bleeding.

Shinfeld and colleagues (Shinfeld, Zippel, Lavee et al 1995) demonstrated that aprotinin reduces bleeding and blood product requirement better than single donor platelet concentrate. This supports the concept that preservation of platelet function is more efficient means to improve haemostasis than by increasing platelet number by platelet transfusion. Platelet transfusion provides additional potentially active platelets but does not have any effect on

other factors such as fibrinolysis and platelet stimulation by thrombin and plasmin.

In early anecdotal reports and in subsequent studies, however, concern was expressed that there was a tendency for a higher incidence of peri-operative myocardial infarction in the aprotinin group (Cosgrove, Heric, Lytle et al 1992), even though the difference did not reach statistical significance. In addition, at post mortem examination, occluded vein grafts were only found in the aprotinin group. Subsequent investigation indicated that this finding may have been due to the maintenance of the customary level of activated clotting time (ACT) during bypass around 400 seconds, as was essential in a blind placebo controlled trial, rather than the now recommended 750 seconds in the aprotinin group (Hardy and Belisle 1993; Hunt, Segal and Yacoub 1992). Another study of the use of aprotinin in aortic surgery with hypothermic circulatory arrest suggested that aprotinin was associated with an increased incidence of myocardial infarction, renal failure and death (Sundt, Kouchoukos, Saffitz et al 1993). However, a review of aprotinin use in 671 cardiac patients in 41 centres in the UK (Bidstrup, Harrison, Royston et al 1993) did not substantiate this risk.

Furthermore a recent randomised, double blind study using magnetic resonance imaging did not demonstrate any increase in occlusion rates of coronary artery bypass grafts (Bidstrup, Underwood and Sapsford 1993). In another randomised study using ultrafast computed tomography to

assess early vein graft patency, in 216 patients there was no difference in patency rates, even though there was a tendency for a higher incidence of myocardial infarction in the aprotinin group as compared to the placebo group (8.9% vs 5.6%) (Lemmer, Stanford, Bonney et al 1994). These authors concluded that an adverse effect on early vein graft patency rates was not demonstrated, but the number of grafts assessed was insufficient for absolute conclusions to be drawn. In a more recent randomised, double blind study (Lab, Welz, Kochs et al 1995), early vein graft patency was assessed by angiography, there was no difference in vein graft patency but more internal mammary artery grafts were occluded in the aprotinin group.

Nevertheless, anxiety remains about the possible association of aprotinin with a higher incidence of peri-operative myocardial infarction, coronary bypass graft occlusion and renal failure. Prophylactic aprotinin has to be started prior to the commencement of surgery, and since excessive bleeding only affects a minority of patients, who cannot be reliably predicted pre-operatively, the majority of patients are treated unnecessarily. For these reasons some workers started looking at other ways of using aprotinin.

One of these was the post-operative use of aprotinin in order to avoid 100% exposure. Angelini and colleagues (Angelini, Cooper, Lamarra et al 1990) reported six cases of life threatening bleeding which failed to respond to all forms of conventional treatment but responded to the unorthodox use of aprotinin post-operatively (2×10^6 KIU

loading followed by an infusion of 0.5×10^6 KIU/h). Some workers tried to reduce the prophylactic dose (Carrel, Bauer, Laske et al 1991; Kawasuji, Ueyama, Sakakibara et al 1993) while Tatar and colleagues (Tatar, Çiçek, Demirkiliç, et al 1993) demonstrated that topical use of aprotinin (1×10^6 KIU) in the pericardial cavity before chest closure reduced blood loss significantly. This finding was confirmed by O'Regan and colleagues recently (O'Regan, Giannopoulos, Mediratta et al 1994).

For these reasons I decided to perform a randomised, double-blind, clinical trial to investigate the use of aprotinin in the post-operative period **restricted** to those patients who bled significantly and who had **not** received prophylactic aprotinin. The randomised nature of the trial enabled the study of haemostatic variables (Lu, Soria, Commin et al 1991) to identify the principal factors associated with the aprotinin effect. In particular, since platelet dysfunction is central to the CPB related bleeding diathesis, factors critical to platelet function in primary haemostasis were examined. Therefore, by measuring platelet GPIb and vWF together with plasma vWF, major determinants of primary haemostasis were examined. In addition the effect of aprotinin on the multimeric composition of plasma and platelet vWF was examined. As aprotinin is a non-specific serine protease inhibitor and anti-fibrinolytic tPA levels, a major initiator of fibrinolysis, as well as the final products of fibrinolysis (FDPs and D-dimers) were examined.

1.10 Structure of aprotinin

Aprotinin was discovered by Kraut, Frey and Werle in 1930 and is a basic polypeptide, isolated from bovine lung with a molecular weight of 6512. It contains 16 different amino acids in a chain consisting of a total of 58 amino acid radicals. The tertiary structure of aprotinin was elucidated by Huber, Kukla, Ruhlmann and colleagues in 1970 using X-ray analysis. The stability of the molecule is due to the three disulphide bridges linking the six cysteine members. The lysine(15)-alanine(16) sequence represents the active centre.

1.11 Biochemistry of aprotinin

Aprotinin inhibits human trypsin, plasmin, plasma kallikrein and tissue kallikreins by forming reversible stoichiometric enzyme inhibitor complexes. The inhibitory effect of aprotinin on the serine proteinases is due to formation of aprotinin-proteinase complexes by the active serine site of the enzyme. The stability of these complexes varies enormously with the trypsin complex being the most stable and with human plasma kallikrein the least stable. The dissociation constant with trypsin is 0.06 pmol/l (Lazdunski, Vincent, Schweitz et al 1974), with human plasmin is 1 nmol/l (Wiman 1980) and with human plasma kallikrein is 30 nmol/l (Philipp 1978).

Aprotinin will inhibit free plasmin and also the plasmin-streptokinase complex, which is the intermediate in

plasminogen activation formed during thrombolytic therapy with streptokinase (Alajmo and Calamai 1992; Efstradiadis, Munsch, Crossman et al 1991; Wiman 1980). The in "vitro" anticoagulant effect of aprotinin has long been difficult to understand (Amris 1966). The enigma was solved when Wuepper (1973) discovered that plasma kallikrein takes part in the contact activation process of coagulation factor XII through a positive feedback mechanism, which is inhibited by aprotinin. Aprotinin, however, inhibits only one of several pathways by which prothrombin is activated to thrombin and therefore does not influence the conversion of fibrinogen to fibrin. As aprotinin directly inhibits excessively activated plasmin it not only protects the direct substrate of plasmin, fibrin, against lysis but similarly protects fibrinogen and the coagulation factors V and VIII.

The half-life of the fall in aprotinin serum level is approximately one to two hours, one hour after injection and reaches five to ten hours, within the following nine hours (Kaller, Patzschke, Wegner et al 1978). The kidney plays a central role in the elimination of aprotinin and ligation of the renal vessels in animal experiments delays the fall in aprotinin blood level considerably (Kaller, 1968).

Aprotinin binds to epithelial cells of the proximal renal tubules and to a lesser degree, due to the affinity of the alkaline aprotinin molecule to the acid glycoproteins, to cartilaginous tissue. Aprotinin is metabolised to shorter peptides or amino acids by lysosomal activity in the kidneys and even after administration of 1×10^6 KIU no unchanged

aprotinin is excreted in the urine.

The addition of aprotinin to heparinised blood prolongs the ACT, which therefore provides no precise indication of the heparin levels when both agents are present. de Smet and colleagues (De Smet, Joen, Van Oeveren et al 1990) felt that this may be advantageous by allowing "under heparinisation" in the presence of aprotinin. In view of the potential thrombotic complications, however, an ACT greater than 750 seconds is now recommended during CPB when the patient has received high dose prophylactic aprotinin (Hunt, Segal, Yacoub 1992). This prolongation of ACT is probably due to Kallikrein inhibition limiting the activation of factor XII and thus the early phase of the intrinsic pathway, so that fewer coagulation factors through the coagulation cascade become activated. There is a risk of under heparinisation if the ACT is used to monitor anticoagulation during CPB as well as difficulties in reversal with protamine, which should be in doses relative to the heparin concentration and not relative to the ACT (Najman, Walenga, Fareed et al 1993).

1 KIU (kallikrein inactivator unit) is equivalent to 0.14 μ g of crystalline active aprotinin. The aprotinin vials used in this project were 50 ml vials containing 70 mg of aprotinin (0.5×10^6 KIU) in 0.9 % sodium chloride solution (Trasylol^R, Bayer UK Ltd, Newbury, Berks, UK).

1.12 Mechanism of action of prophylactic aprotinin

In addition to the review of the extensive literature in this chapter I relied heavily on the reviews by Hunt and Yacoub (1991) and by Royston (1990). Aprotinin is a relative non-specific serine protease inhibitor (SERPIN) which was found to, coincidentally, reduce post-operative bleeding when it was investigated for its effect on inhibiting kallikrein and the systemic inflammatory response to CPB (Royston, Bidstrup, Taylor et al 1987). Most researches have concentrated on three main areas of investigation in an attempt to elucidate its mechanism of action.

Inhibition of kallikrein

During CPB contact activation of factor XII by the synthetic surfaces leads to formation of kallikrein, from its inactive plasma precursor prekallikrein, which is important in initiating the inflammatory response. The high dose aprotinin regime was designed to inhibit kallikrein (Royston, Bidstrup, Taylor et al 1987) and therefore reduces the activation of complement, bradykinin, the inflammatory response, and also inhibits fibrinolysis and coagulation.

Inhibition of fibrinolysis

Aprotinin is an antifibrinolytic agent, its molar potency in vitro is 100 and 1000 times that of tranexamic acid and ϵ -aminocaproic acid respectively. tPA and factor XII are both activators of plasminogen and are formed in excess

during CPB. By inhibiting kallikrein, aprotinin indirectly inhibits the formation of factor XIIa and its release of tPA via bradykinin. In addition aprotinin directly inhibits any plasmin formed.

In a recent study of half-dose aprotinin it was demonstrated that aprotinin significantly reduced tPA activity and plasminogen levels during CPB and significantly ~~reduced~~ D-dimer concentrations compared to the ^{rise} placebo group (Liu, Tengborn, Larson et al 1995). Similar prevention of hyperfibrinolysis, by aprotinin, during CPB was confirmed in other studies (Havel, Teufelsbauer, Knöbl et al 1991 and Mastroroberto, Chello, Zofrea et al 1995). Further evidence of inhibition of fibrinolysis comes from animal studies (Erhardtzen, Bregengaard, Hedner et al 1994) which demonstrated the reduction of tPA induced bleeding in rats, by aprotinin, by inhibiting plasmin.

Preservation of platelet function

Aprotinin prevents prolongation of bleeding time after CPB without affecting the platelet count, suggesting a possible preservation of platelet function (Bidstrup, Royston, Sapsford et al 1989). This preservation of bleeding time was also demonstrated more recently (Liu, Tengborn, Larson et al 1995) who concluded that this may be an indirect effect of aprotinin on platelet function by inhibition of fibrinolysis. The maintenance of bleeding time closer to physiological levels could also be accounted for by the preservation of platelet surface GPIb receptors (van Oeveren, Harder, Roozendaal et al 1990).

In another study (Lu, Soria, Commin et al 1991) aprotinin prevented the reduction of ristocetin induced agglutination during CPB as well as the increase in FDPs. In addition this phenomenon was not related to hydrolysis of vWF or platelet GPIb. Further evidence comes from Huang and colleagues (Huang, Ding, Su et al 1993) who demonstrated both in vivo and in vitro that prophylactic aprotinin inhibits fibrinolysis and preserves the platelet surface GPIb receptors. In a very recent study (Boldt, Knothe, Zickmann et al 1993), prophylactic aprotinin blunted the negative effect of hypothermic CPB on platelet aggregation.

Royston and colleagues showed that aprotinin did not inhibit the release of prostacyclin or vWF from cultured human endothelial cells (Royston, Royston, Coade et al 1992). Another study (Lavee, Savion, Smolinsky et al 1992) demonstrated that prophylactic aprotinin significantly preserved platelet aggregation following CPB compared to the placebo group.

The evidence so far, seems to point in the direction of preservation of platelet function, which may be an indirect effect of inhibition of excessive fibrinolysis. Nevertheless, there may also be a superimposed effect of the inhibition of kallikrein. Despite extensive research the precise mechanism of action of prophylactic aprotinin remains elusive and, may well be different from the mechanism of action of the post-operative therapeutic use of aprotinin, specifically targeted towards patients who are bleeding excessively.

Chapter 2

Materials and methods

2.1 Introduction

The majority of assays and measurements were carried out by myself and the research assistant Miss D Cowans, who learned the methods from me and was under my supervision during her training period in the haemostasis laboratory of St George's Hospital. A team effort was required because of the large number of assays and platelet function tests that had to be carried out. Furthermore the bulk of the tests for the aprotinin trial were performed late at night or the early hours of the morning. For these reasons some of the coagulation screens were carried out by the staff in the haemostasis laboratory. The serum salicylic acid and urinary salicyluric acid were measured in the Analytical Unit of St George's Hospital Medical School under the direction of Dr DW Holt. In addition to the references quoted in each section I relied on the following textbooks for the theory, limitations and methods of the assays described in this chapter: Chanarin (1989), Harker and Zimmerman (1983) and Triplett (1978).

2.2 Aspirin trial protocol

Patients

One hundred patients scheduled for elective CABG were assessed two weeks pre-operatively and informed written

consent was obtained. All patients were instructed to stop their own aspirin and non-steroidal anti-inflammatory drugs two weeks pre-operatively. They were instructed not to take aspirin containing medications and were then randomised by minimisation (Pocock 1983) into two groups. This was done by computer generated number codes, rather than group A and B, so that the individual's group was not evident to any of the participants. The patients in the aspirin group were commenced on aspirin 300 mg (CP Pharmaceuticals), once a day, two weeks pre-operatively until the day of operation. The patients in the placebo group were commenced on placebo (Calcium lactate 300 mg, Evans Medical), once a day, two weeks pre-operatively until the day of operation. All tablets were dispensed by the hospital pharmacy according to the number codes so that both the patients and all participants were kept blind. The factors used for minimisation were; age, gender, surgeon, number of vessels involved and left ventricular function. The exclusion criteria were; second operation, another defined coagulopathy, a history of peptic ulceration and diabetes mellitus.

Methods

The day prior to surgery all patients had their serum salicylic acid and urinary salicyluric acid measured in order to check their compliance. These assays were performed by an independent worker in a different laboratory and the results were only revealed to the rest of the group when the codes were broken at the end of the project.

Anaesthesia for operation

The anaesthetic protocol used in this study was the same as the established protocol used at our institution. All cardiac medication was continued into the morning of the operation. The premedication was intramuscular papaveretum and scopolamine. All intravenous and intra-arterial access was obtained under local anaesthesia and sedation with intravenous diazepam. Induction of anaesthesia and endotracheal intubation was under full monitoring of the patient. This was achieved with alfentanil, thiopentone and suxamethonium followed by pancuronium. Maintenance of anaesthesia was achieved with intravenous papaveretum, enflurane and nitrous oxide. Blood pressure control was achieved with an infusion of glyceryl trinitrate which was continued in the intensive care unit, where the patients were allowed to gradually recover from their anaesthesia.

Cardiopulmonary bypass (CPB)

All patients had standard CPB with a Stockert Shiley roller pump. A Sorin Monolyth integrated membrane oxygenator (Sorin Biomedicus, Salugia, Italy) and a Dideco 40 μm arterial filter (Dideco, Mirandola, Italy) were used. The CPB circuit was primed with 2 l of Hartmann's solution, 10,000 U of sodium heparin (Leo laboratories Ltd, Bucks, UK) and 10 ml of sodium bicarbonate (10 mmol and 0.01% disodium edetate). Patients who received cardioplegia had 500 ml of Hartmann's solution of the circuit prime replaced with 500 ml of 10% mannitol. Systemic heparinisation with sodium heparin (3 mg/Kg) was carried out, through a central venous cannula, before aortic cannulation and the activated

clotting time (ACT) (Hemotec inc., Colorado, USA) was maintained above 400 seconds during CPB. After securing haemostasis and removal of the aortic cannula the heparin was reversed with protamine sulphate (1:1 ratio excluding the 10,000 units of heparin in the CPB prime fluid). Further supplements of 50 mg given until the ACT was within 20 sec of the pre-operative ACT). During bypass all the blood from the pericardial and pleural cavities was returned to the CPB via the cardiotomy suction and all remaining blood in the CPB reservoir was returned to the patient at the end of bypass. Bypass was only discontinued after full rewarming to 37°C was achieved.

Operative procedure

Following midline sternotomy, the left internal mammary artery was harvested when indicated and the long saphenous vein was dissected simultaneously. The pericardium was opened and the ascending aorta and right atrium cannulated in preparation for bypass. Myocardial protection was achieved with antegrade St Thomas's Hospital crystalloid cardioplegia (4°C), topical slush and systemic cooling to 28°C for two of the surgeons. The remaining two surgeons used systemic cooling to 31°C and intermittent aortic cross clamping with induced ventricular fibrillation. These two strategies of myocardial management were the strategies used in our institution at the time of the study.

The relevant coronary arteries were grafted, end-to-side using 6/0 prolene and 5/0 prolene for the aorto saphenous anastomoses. All coronary anastomoses were completed first

and the aorto saphenous anastomoses last by the two surgeons using cardioplegia. The remaining two surgeons, using intermittent ischaemic arrest, alternated distal and proximal anastomoses. On completion of the anastomoses, CPB was discontinued and decannulation undertaken. Once the surgeon involved was confident that there was no significant bleeding any pooled blood was sucked out of the pericardial and pleural cavities. The chest was closed with sternal wires over 2 or 3 chest drains, depending whether the pleural cavities were opened or not. Blood loss in theatre after decannulation was measured by weighing the swabs used and by measuring the volume of blood in the waste suction. The mediastinal chest drains of all patients were connected to a standard autotransfusion circuit (Baxter, Irvine, CA, USA) at the end of surgery and to a wall suction unit (10 kPa), according to the standard protocol of our institution.

Measurements

The following measurements were made pre-operatively and half an hour after the administration of protamine (all specimens were collected from a dedicated central venous line, which was kept heparin free, and all precautions were taken to avoid activation of platelets):

Platelet count, Platelet size distribution,
Haemoglobin (Hb), Haematocrit (Hct),
Activated Clotting Time (ACT),
Coagulation screen (PT, KPTT, TT),
Fibrinogen level and FDPs,
Bleeding time,

Platelet aggregation and stimulated nucleotide release,
Platelet vWF activity, antigen and multimeric analysis,
Plasma vWF activity, antigen and multimeric analysis,
Platelet GPIb surface receptors,

The following were also recorded:

Time from protamine to chest closure,
Total blood loss after protamine in theatre,
Hourly and total blood loss in the intensive care unit,
Bypass time,
Number of units of packed red cells transfused,
Number of units of haemostatic factors transfused,
Volume of colloid transfused,
Volume of autotransfused blood,
Volume of urine in the first 24 hours,
Clinical outcome and Hb on discharge,
ECG changes.

Guidelines for transfusion of haemostatic factors

The results of the coagulation screens were always interpreted as a group and in combination with the clinical picture and never in isolation. Conventional treatment in the form of protamine and coagulation factors were given to both groups depending on the results of the parameters measured and according to the following guidelines:

- ▶ If the KPTT/control ratio was >1.5 then 50 mg of protamine was given.
- ▶ If the PT/control ratio was >1.5 then 2 units of fresh frozen plasma (FFP) were transfused.
- ▶ If the platelet count was $< 100 \times 10^9 /l$ then 6 units of

platelets were transfused if the patient was still bleeding in excess of 200 ml/h and any abnormalities in the coagulation screen were corrected.

► If the Fibrinogen level was < 1 g/l then cryoprecipitate (5 ml / Kg = 1 U / 5 Kg) was transfused.

Guidelines for re-exploration

Surgical re-exploration was always an option according to the conventional guidelines under the clinical direction of the consultant in charge of the patient. The patients that had to be re-explored continued the protocol but the cause of bleeding at re-exploration was recorded. The criteria used for reoperation were those promulgated by Kirklin and Barratt-Boyes (1986_a) .

(1) More than 500 ml during the first hour.

More than 400 ml during each of the first 2 hours.

More than 300 ml during each of the first 3 hours.

More than 1,000 ml in total during the first 4 hours.

More than 1,200 ml in total during the first 5 hours.

(2) Excessive bleeding that restarts (indicating a possible surgical cause).

(3) Sudden massive bleeding.

Transfusion guidelines

During the first 24 hours the need for colloid transfusion was according to our standard protocol depending on the haemodynamic variables of the patient and regular measurement of the Hb and Hct. If the following indices were met then no colloid was added:

If the arterial blood pressure was > 110 mmHg ,

the central venous pressure was $> 8 \text{ cmH}_2\text{O}$,
the urinary output was $> 0.5 \text{ ml/Kg/h}$
and the patient's peripheries were warming.

If these indices were not met then blood or colloid was transfused until haemodynamic improvement occurred or up to a central venous pressure of $13 \text{ cmH}_2\text{O}$. Blood was transfused when the Hct was $<28\%$ and Haemaccel when the Hct was $>28\%$. After 24 hours blood transfusion was given if the Hb was $<8 \text{ g/dl}$.

2.3 Ethical considerations

Argument has persisted about the balance of the benefit versus the risk of pre-operative aspirin therapy. One school of thought is that the increased risk of bleeding is a small price to pay for improved vein graft patency. The other is that the increased morbidity and risks of avoidable blood transfusion outweigh the small advantage of improved vein graft patency. Such was the divergence of opinion, on this benefit / risk equation, in our unit that two surgeons insisted that their patients remained on pre-operative aspirin until the day of operation and two preferred the aspirin to be discontinued ten days earlier. The respective groups accepted that the evidence to support their practices was lacking. As 40% of our patients were on aspirin pre-operatively, they were prepared to randomise them to either policy for the purpose of the clinical trial. This provided the number of patients required for the aspirin study without any ethical implications as both policies were common practice at the time. This clinical

scenario provided the perfect environment for a prospective, randomised, placebo controlled, double-blind clinical trial with no ethical dilemmas. This study was approved by the St George's hospital ethical committee and informed written consent was obtained from all the patients randomised.

2.4 Aprotinin trial protocol

Entry criteria

Patients who had undergone a period of CPB during the course of cardiac surgery who reached the following limits for blood loss, from the mediastinal and pleural drains, after return to the intensive care unit:

a total of 200 ml - at the end of first hour,
or a total of 300 ml - at the end of second hour,
or a total of 400 ml - at the end of third hour.

These entry criteria were set on the basis of blood loss data from our unit audit process in order to select the 10% or so patients with heavier blood loss.

Exclusion criteria

Patients who had known previous exposure to aprotinin or who had received prophylactic aprotinin in theatre and those patients with renal failure.

Randomisation

All patients who met the entry criteria were allocated a trial number and blindly allocated a code number by randomisation to one of two groups: (The patients were only identifiable by their random code number held by the pharmacist so that the individual groups were not evident to

any of the participants).

Aprotinin Group received aprotinin (Trasylol^R, Bayer UK, Newbury, UK) 2×10^6 KIU as a loading dose over half an hour, after a 5 ml test dose, followed by an infusion of 0.5×10^6 KIU per hour for four hours.

Placebo Group received the same volume of normal saline in identical bottles as aprotinin, supplied by Bayer UK.

Patients Between August 1991 and July 1992, 800 patients were operated upon, who did not have the exclusion criteria. Sixty patients met the entry criteria for blood loss and were randomised. The factors used for randomisation by minimisation were age, gender, CPB time, type of operation, repeat operation and the use of autotransfusion.

Measurements

All patients had the following measurements at the time of randomisation and five hours at the completion of aprotinin/placebo infusion.

Haemoglobin (Hb), Haematocrit (Hct),
Platelet count, platelet size distribution,
Coagulation screen (PT, KPTT, TT),
Fibrinogen level, FDPs, D-dimers,
tPA antigen and tPA activity,
Plasma vWF antigen, activity and multimeric analysis,
Platelet vWF antigen, activity and multimeric analysis,
Platelet aggregation studies,
Platelet GPIb surface expression.

The following were also recorded:

Hourly blood loss,

Total blood loss in theatre if re-explored,
Total blood loss (when chest drains removed),
Number of units of packed red cells transfused,
Platelet and clotting factors transfused,
Volume of colloid transfused,
Volume of autotransfused blood,
Volume of urine in the first 24 hours,
Resternotomy for bleeding and cause of bleeding,
Hb and Hct on discharge,
Clinical outcome.

Guidelines for transfusion and re-exploration

The guidelines were the same as for the aspirin trial. The second blood specimen was taken before any coagulation factors or platelets were transfused.

2.5 Ethical considerations

This study was approved by the St George's hospital ethical committee. The committee, however felt that, unlike the aspirin trial, there was no need to obtain written consent from the patients as they were sedated and ventilated at the time. As it is not possible to predict pre-operatively which patients are likely to bleed excessively, over 800 patients would have had to be consented in order to randomise 60 patients for the trial. The ethical committee felt that this would have introduced unnecessary distress to a large number of patients and as the use of aprotinin in this clinical scenario was not established at the time the patients were not denied any of the conventional treatment.

All the patients were given all conventional treatment, the only difference between the two groups being the use of aprotinin or placebo post-operatively.

2.6 Haemoglobin (Hb) and haematocrit (Hct)

The Hb (g/dl) was measured using a Coulter-S-Plus counter in blood (5 ml) collected in EDTA. The Hct, which represents the volume of packed cells per unit volume of blood (ratio of litres per litre), was automatically calculated by the Coulter counter by multiplying the total number of red blood cells by the average volume of the red blood cells.

2.7 Platelet count

The platelet count (number of platelets $\times 10^9 / l$) was measured using a Coulter-S-Plus counter in blood (5 ml) collected in EDTA within ten minutes from the time of collection. The platelet count was calculated from the area under the platelet size distribution curve to a lognormal model. The count was corrected for count periods and any possible red cell masking and scaled by an output constant.

2.8 Platelet size distribution

The platelet size distribution was measured using a Coulter-S-Plus counter in blood (5 ml) collected in 3.2% trisodium citrate (9/1 volume) within ten minutes from the time of collection in order to minimise swelling of the platelets in vitro. The mean platelet volume (MPV) (fl) was calculated

from the log median value and the log standard deviation of the platelet size distribution curve and then scaled by the latex modal platelet volume of 9.3 fl. The plateletcrit (Pct), which is an indication of the platelet biomass, similar to the Hct, was derived from the product of the platelet count and the MPV. The platelet distribution width (PDW) was calculated by taking the antilog of the log standard deviation of the platelet size distribution curve, multiplying it by ten and then normalising it to ten (normal range 17-19, arbitrary units).

2.9 Coagulation screen

The preparation of plasma for the measurement of PT, KPTT, TT, fibrinogen, FDPs, D-dimers, plasma vWF activity, plasma vWF antigen and for plasma vWF multimeric analysis was the same. Venous blood (5 ml) was collected into 3.2% trisodium citrate in a ratio of nine parts blood to one part citrate. The collection tube was spun at 3000 rpm (150 g) for 15 minutes at 4°C. If the centrifuged blood was not used within 30 minutes the plasma was separated into a plastic tube, respun as above to ensure adequate removal of platelets, aliquoted for the appropriate batch analysis and then stored at -70°C.

The coagulation end points for the coagulation screens were determined in the KC 10 which is a 10 channel semi-automated coagulometer. The KC 10 uses a rotating ball, in an inclined tube, which rotates at a given place until it is carried away as the coagulation commences. The coagulation

end points are determined by displacement, by fibrin, of the rotating ball which generates an impulse in a magnetic sensor. This impulse automatically stops the times in the individual channels. The KC 10 was allowed to reach 37°C before the measurements were started. Once the samples were preheated the reagent was added and the coagulation time was indicated on the display.

2.9a Prothrombin time (PT)

The PT was measured by the method first described by Quick (1935). Brain tissue factor and calcium ions are added to plasma resulting in the activation of extrinsic clotting factors, thrombin generation and formation of fibrin clot. This method is sensitive in detecting coagulation defects of the extrinsic pathway (vitamin K dependent factors, II, VII and X). A prolonged PT can also be due to increased consumption of coagulation factors (DIC), dilution, high levels of heparin or very low levels of fibrinogen.

0.1 ml of rabbit brain thromboplastin (Diagen Diagnostics Reagents Ltd, Thame, Oxon, UK) was added in two glass tubes prewarmed at 37°C. 0.1 ml of normal control plasma was added to each tube and incubated for 60 seconds. 0.1 ml of 0.025 M CaCl_2 was added to each tube and the coagulation time was determined in the KC 10 as described previously. The PT in seconds was expressed as the mean of the times for the duplicate tubes (normal range 11-15 seconds). The previous steps were repeated with the test plasma.

2.9b Kaolin partial thromboplastin time (KPTT)

The KPTT was measured using the 10 minute kaolin thromboplastin time. Kaolin activates the contact factors and triggers the intrinsic coagulation pathway. The activated contact factors, in the presence of calcium ions and phospholipid (partial thromboplastin), in turn trigger a cascade of enzyme reactions culminating in the generation of thrombin and clot formation. A prolonged KPTT may be caused by deficiency of factors II, V, VIII, IX, X, XI, XII, prekallikrein, high molecular weight kininogen or fibrinogen. Other causes are increased consumption of the mentioned factors (DIC), dilution (massive transfusion) or anticoagulation with warfarin or heparin.

0.1 ml of normal control plasma was added in two glass tubes prewarmed at 37°C. 0.1 ml of Manchester activated partial thromboplastin time reagent (Manchester Comparative Reagents, Manchester, UK) and 0.1 ml of 0.25% kaolin (BDH Chemicals, Poole, UK) in Owrens buffer (5.875 g sodium barbitone, 7.335 g sodium chloride dissolved in distilled water, pH adjusted with 1 M HCl and volume adjusted to 1 litre) were added. These reagents were mixed and incubated at 37°C for 10 minutes and the kaolin was resuspended at two minute intervals. After 10 minutes 0.1 ml of 0.025 M CaCl₂ was added and mixed. The clotting time was recorded in the KC 10 as described previously. The KPTT was expressed as the mean of the times for the duplicate tubes (normal range 34-48 seconds). The previous steps were repeated with the test plasma.

2.9c Thrombin time (TT)

The TT was measured using the calcium thrombin time which measures the formation of a fibrin clot in plasma by the action of thrombin on fibrinogen. A prolonged TT may be caused by deficiency of fibrinogen, abnormal fibrinogen molecule, raised levels of FDPs or the presence of heparin.

In two glass tubes prewarmed at 37°C, 0.2 ml normal control plasma was added and incubated for 60 seconds. 0.1 ml of thrombin (4 IU/ml of CaCl₂/NaCl, 4/1 ratio, Immuno Coagulation Diagnostics, Near Seven Oaks, Kent, UK) was added and the clotting time was determined in the KC 10 as described previously. The TT was expressed as the mean of the times for the two times (normal range 11-15 seconds). The previous steps were repeated for the test plasma.

2.10 Fibrinogen

The fibrinogen levels were measured using the Clauss method, which uses different dilutions of standard plasma with known fibrinogen concentration and of test plasma which are clotted with excess of thrombin. As the relationship between fibrinogen and clotting time is linear over a certain range of concentrations the fibrinogen level can be assayed. The detection of the end point can be difficult if the fibrinogen level is low, with high dilutions and in the presence of very high heparin levels.

The 1/10 dilution represented the relative concentration of the standard plasma. Serial dilutions of standard plasma

(1/5, 1/10, 1/20, 1/40) were prepared in polystyrene tubes at room temperature using 0.05 M Imidazole (Glyoxaline) buffer (3.4 g Imidazole, 5.85 g NaCl, dissolved in distilled H₂O, the pH adjusted to 7.3 with 18.5 ml of M HCl and the volume adjusted to 1 litre). 0.2 ml of each dilution was then transferred into two glass tubes at 37°C and 0.1 ml of bovine thrombin (90 NIH U/ml, Dade Thrombin Baxter, Miami, Florida, USA) was added, mixed and the KC 10 clotting time was recorded. A double logarithm standard curve was then constructed of the clotting time against the dilution of the standard plasma. The previous steps were repeated for a 1/10 dilution of the test plasma. The test plasma clotting time obtained was used to convert it to the fibrinogen level, of the test plasma, from the standard curve (normal range 1.5-4.0 g/l).

2.11 Fibrin(ogen) degradation products (FDPs)

The FDPs were measured using the latex agglutination test which uses dilutions of serum mixed with latex particles coated with antibody to fibrinogen and FDPs on a slide. If fibrinogen, fibrin, or their derivatives are present, they bind to the antibody and cause agglutination of the latex, which can be read visually. In order to determine levels of degradation products it is necessary to remove all traces of fibrinogen and fibrin i.e. plasma must be clotted and the fibrin clot removed. This may be done by either collecting the blood into commercially available vacutainer tubes containing thrombin or clotting the citrated plasma with thrombin. Commercial tubes also contain an anti

fibrinolytic agent to prevent further degradation in vitro. The clinical relevance of raised FDPs measured by this technique is often difficult to determine. Mild elevations may be due to non specific breakdown of fibrin(ogen).

Citrated plasma (0.2 ml) was clotted with an equal volume of thrombin (10 IU/ml of CaCl₂, Immuno Coagulation Diagnostics, Near Seven Oaks, Kent, UK). The remaining reagents required for this assay were included in the FDP kit (Baxter, BM Browne Ltd, Reading, UK). The tube was incubated at 37°C for 10 minutes and the clot wrung against the side of the tube to expel the serum. A one in five dilution of the serum was made in buffer (Sorensen's glycine buffer pH 8.2) resulting in 1 in 10 dilution of the original plasma and from this doubling dilutions prepared. One drop (25 µl) of the first dilution was mixed with one drop (25 µl) of latex suspension (Latex beads coated with antibody sensitivity 0.8 mg/l) on a darkened slide and observed for agglutination for two minutes. If a positive result was obtained sequential dilutions were tested until a negative result (no agglutination), was obtained. The FDP concentration was calculated by using the formula:

$$Y \times 0.8 \text{ mg/l} = \text{mg/l}$$

The sensitivity of the latex is known to be 0.8 mg/l and the dilution which produced a positive result (1 in Y). Normal = <8 mg/l.

2.12 D-dimers

D-dimers in plasma were measured using the semiquantitative

Dimertest latex assay (Baxter, BM Browne Ltd, Reading, UK). During fibrin polymerisation, thrombin activates factor XIII to an active enzyme which then converts soluble fibrin to an insoluble fibrin clot by cross-linking adjacent fibrin molecules in the region of the D-domain. Plasmin lysis of such cross-linked fibrin yields derivatives (XDP) that contain the cross-linked D-domain (D-dimer) and are markers of fibrinolysis. A highly specific monoclonal antibody (mAb) to D-dimer is attached to latex beads and the presence of D-dimers leads to visible agglutination of the latex particles. The plasma concentration of D-dimers in normal subjects is typically less than 0.5 mg/l. The amount of D-dimer detected in a specimen will depend on several interrelated factors in vivo, such as the severity of the thrombotic episode, the rate of cross-linked fibrin formation and the time elapsed after the thrombotic event until the blood is drawn from the patient. The presence of rheumatoid factor may give false positive reactions.

Reagents (All included in the Baxter Dimertest kit)

Dimertest latex beads 0.4% suspension of latex particles coated with murine mAb specific for human D-dimer in derivatives of cross-linked fibrin, containing stabilisers and 0.01% sodium azide as a preservative (stored at 4°C).

Dimertest positive control lyophilised solution containing purified human D-dimer fragment, stabilisers and preservative. When reconstituted with 0.2 ml of Dimertest buffer solution gives a positive agglutination reaction with the Dimertest latex beads. Reconstituted material was

stored frozen for up to three months but was dissolved completely before use.

Dimertest buffer salts phosphate buffer salts with preservative. When reconstituted with 100 ml distilled water, a buffer solution of pH 7.3±0.2 was produced. Stored at 4°C or frozen for extended use.

Failure of latex beads to agglutinate with the positive control, agglutination with the negative control or evidence of microbial infection was taken as an indication of deterioration of the reagents. The Dimertest latex assay reagents and specimens were equilibrated at room temperature and the latex beads were mixed by rapidly inverting the bottle several times immediately prior to use. Ten μ l of undiluted plasma or positive or negative control were dispensed on the test slide. One drop (25 μ l) of the Dimertest latex leads was placed on the slide adjacent to each sample. The suspension was promptly mixed with the flat tip of a plastic stirrer until the reaction area was covered. The slide was then rotated gently for exactly three minutes before checking for agglutination. As positive agglutination was normally obtained with specimens containing more than approximately 0.5 mg of D-dimer/l of plasma, an estimate of higher levels of D-dimer in a sample was obtained by serial dilutions of the plasma in Dimertest buffer. The previous steps were repeated for the sample dilutions. The concentration of D-dimer in plasma (mg/l) was semiquantitatively assayed by determining the higher dilution titre which remained positive for a particular specimen (Table 2.1).

Table 2.1 D-dimer level corresponding to each dilution.

Approximate D-dimer range (mg/l)	specimen dilution			
	Undiluted	1:2	1:4	1:8
<0.5	-	-	-	-
0.5-1.0	+	-	-	-
1.0-2.0	+	+	-	-
2.0-4.0	+	+	+	-
>4.0	+	+	+	+

"+" = agglutination, "-" = no agglutination

2.13 Bleeding time

The bleeding time is defined as the time between making a small incision and the moment when the bleeding stops.

The original bleeding time test described by Duke (1910) is performed by making a puncture wound (2.5-3 mm deep and 1 mm wide) in the ear lobe with a lancet. Although many methods for performing the bleeding time have been developed since two of them are the most widely used. Ivy, Nelson and Bucher (1941) increased the sensitivity of the bleeding time by making puncture wounds on the forearm while maintaining a constant pressure of 40 mmHg on the arm by the use of a sphygmomanometer. The bleeding times performed with a lancet often give discrepant duplicates because the cuts are deep, thus increasing the possibility of hitting a deep vessel. In the 1950's several procedures for performing bleeding times using scalpel blade incisions were described and in 1969 Mielke and colleagues (Mielke, Kaneshiro, Maher et al) standardized the blade incisions by the use of a

template system. They used a standard surgical blade mounted in a plastic handle that allowed only a fixed portion of the blade to protrude. The blade was used with a plastic template containing a slit in the middle through which a one mm deep and five mm long incision is made.

The Simplate device (Organon teknika, Turnhout, Belgium) was used for measuring bleeding time. As numerous patient related factors affect the bleeding time; age, skin type, skin condition, vascularity and temperature, the normal range is quite wide (2.3-9.5 minutes). A muscular area on the volar aspect of the forearm, distal to the anticubital fossa and avoiding veins, scars and bruises, was selected. The selected area was cleaned with alcohol and allowed to dry for 30 seconds. The sphygmomanometer cuff was inflated to 40 mmHg and the Simplate device was placed firmly on the forearm (parallel to the long axis). The trigger was pressed and the device was removed one second later. At 30 seconds intervals the flow of blood was blotted with filter paper without touching the edge of the wound. This was repeated in similar manner every 30 seconds until the blood no longer stained the filter paper. During this time the pressure in the cuff was carefully maintained at 40 mmHg. The bleeding time was recorded to the nearest 30 seconds and the wound was cleaned and a bandage applied.

2.14 Platelet aggregation

In addition to the textbooks mentioned in section 2.1, I relied heavily on the reviews by Day and Rao (1986) and by

Yardumian, Mackie and Machin (1986). The method used to observe platelet aggregation was that described first by Born in 1962 and by O'Brien in 1962. Photometric recording of platelet aggregation is based upon changes occurring in the platelets dispersed in the plasma environment. Before the stimulation of aggregation, the platelets in the plasma act like particles in solution, being evenly distributed in the cuvette and create the initially recorded optical density by scattering or absorbing light passing through the solution. As aggregation begins, the even distribution of free platelets is disrupted, with clumps of increasing size being formed. With continued aggregation, increasing areas of plasma become platelet free and allow greater light transmittance through the aggregation suspension. The recorded changes in optical density correspond to the change in the number of free platelets.

20 ml of blood collected in 3.2% trisodium citrate was centrifuged at 800 rpm for 10 minutes at room temperature. The supernatant platelet rich plasma (PRP) was carefully removed and placed into a plastic container. The remaining sample was centrifuged at 3,000 rpm for a further 10 minutes at 4°C. The supernatant platelet poor plasma (PPP) was carefully removed and placed in a plastic container. The platelet count, MPV and PDW were measured and if necessary the platelet count was adjusted to $200 \times 10^9 /l$ by diluting with PPP. All precautions were taken from the time that the blood was drawn to the time of observing the platelet aggregation to minimise activation of the platelets.

0.5 ml of PRP was pipetted into each cuvette in the prewarmed wells of the PAP-4 platelet aggregometer (BIO/data Corporation, Hatboro PA 19040, USA) and 0.5 ml of PPP was pipetted into a cuvette. A teflon coated magnet was placed into each of the PRP cuvettes. Once the aggregometer temperature was at 37°C the PPP cuvette was placed in the reading channel to set the PPP reading. This was then removed and the PRP cuvette placed in the same channel to read zero % aggregation. Each of the agonists (Table 2.2) was then added and the platelet aggregation was observed and printed for five minutes. At this stage 50 µl of 0.1 M EDTA was added to each cuvette. All the cuvettes were labelled with the agonist concentration and kept until the end for processing for stimulated nucleotide release. All platelet aggregometry was completed within one hour of venesection.

Table 2.2 Volume and concentration of platelet agonists.

Agonists	Concentration	Volume
Collagen	200 mg/ml	10 µl
		20 µl
Ristocetin	20 mg/ml	30 µl
		40 µl
Arachidonic Acid	15 mg/ml	10 µl
		40 µl
Platelet activating factor	500 nM	1 µl
	100 nM	1 µl
	50 nM	1 µl
	25 nM	1 µl

Collagen (Immuno Coagulation Diagnostics, Near Seven Oaks, Kent, UK). Ristocetin (Forum Chemicals Ltd, Redhill, Surrey, UK). Arachidonic Acid and platelet activating

factor (Sigma Chemical Company Ltd, Poole, Dorset, UK).

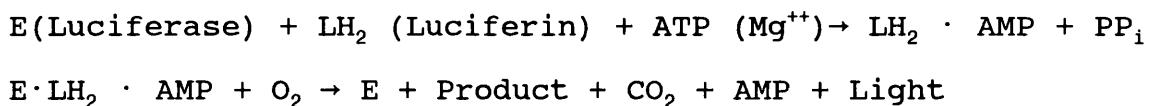
After preliminary studies of the dose response curves of the four agonists mentioned as well as ADP, adrenaline and thrombin, four were chosen in order to test all main pathways of platelet activation. For each agonist the volumes and concentrations chosen were both from the steep and the plateau part of the dose response curve.

The printout from the platelet aggregometer automatically generated the maximum aggregation (%) and the slope (degrees) of the aggregation curve for each agonist. The slope of the primary and secondary waves of aggregation represent the rate of reaction, with greater values indicative of higher rates. In biphasic aggregation the instrument recorded the sharpest slope of the fastest reacting phase. The lag or reaction time (seconds), the interval from the addition of the aggregatory agent to the initiation of a measurable aggregation response, was calculated manually.

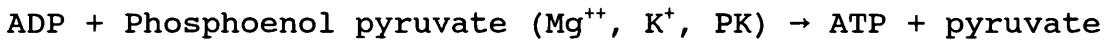
2.15 Analysis of the ATP and ADP content of platelets

In human platelets approximately two-thirds of adenine nucleotides are stored in dense granules and do not participate in metabolism. This compartmentalisation makes it difficult to measure the ATP and ADP solely in the granules when the entire platelet is extracted. These nucleotides, however, are secreted upon stimulation while the cytoplasmic pool is retained. The concentration of ATP and ADP in the dense granules can therefore be estimated in

supernatants of platelets having undergone maximal secretion. The kinetics of secretion of ATP can be monitored directly with the luminoaggregometer (Fukami, Bauer, Stewart et al 1978) but this only measures the ATP and since 80% of the platelet ADP is stored in the dense granule we chose the bioluminescence assay (McElroy, Seliger and White 1969). This assay measures the ATP in extracts of supernatants after secretion, employing a firefly lantern extract and a luminescence biometer. The firefly luciferin-luciferase reaction sequence is as follows:



When a micro-sample containing ATP is injected into a suitably buffered reaction mixture of the firefly reagents, the peak intensity of the resulting light flash is directly proportional to the concentration of ATP. The secreted ADP is converted to ATP by the pyruvate kinase (PK) system and the difference is measured from an unconverted sample, obtaining both the concentrations of ATP and ADP separately. Both of these reactions require a solution at pH 7.4.



The concentration of EDTA in the sample should not exceed that of the Mg⁺⁺ when added to the active buffer. Mg⁺⁺ is essential for the conversion of ADP to ATP and if it is complexed with the EDTA the assay will give false low concentrations. Some batches of the enzyme PK, although they appear to function well when measuring the stock ADP standard, fail to convert ADP to ATP when diluted in the

active buffer. As a check, ATP standards were run with the ADP standards to ensure the total conversion of ADP. The normal ranges are shown in table 2.3. Under conditions of maximal secretion, 30% of the ATP and 80% of the ADP are released (Fukami, Dangelmaier, Bauer et al 1980).

Table 2.3 Normal ranges of nucleotide content of platelets and maximum stimulated release to collagen (Our own lab).

nmol/10 ⁹ platelets	ATP	ADP	ATP/ADP
Total	45-120	25-75	1.2-2.4
Stimulated release	5-15	9-24	0.5-0.75

50 µl of 0.1 M EDTA was added to all the cuvettes used for aggregation, at the end of each aggregation, including the PPP cuvette but not the PRP cuvette. All the EDTA treated cuvettes were centrifuged at 3,000 rpm for 10 minutes at 4°C. 0.25 ml 0.1 M EDTA was added to the untreated PRP as well as 0.25 ml of 20% trichloroacetic acid. Ethanol (96%) was added to separate tubes so that the total amount of fluid in each tube was one ml (PRP + EDTA + agonist + ethanol). Once the specimens were spun the supernatant of each was tipped into the appropriately labelled tube containing ethanol. All tubes were vortex mixed and centrifuged (3000 rpm) at 4°C for 10 minutes. The supernatant was pipetted into plastic tubes, labelled with the specimen details and the agonist volume, which were then frozen (-70°C) for batch analysis. The frozen specimens were allowed to thaw and if cloudy they were centrifuged

again at 3,000 rpm at 4°C. The buffer solution consisted of 25 ml Tris-maleate buffer, 5.4 ml M NaOH, pH adjusted to 7.4 and the volume was made up to 100 ml with distilled water. The working buffer (10 ml buffer solution, 0.1 ml phosphoenol pyruvate and 6 μ l pyruvate kinase) (Sigma Ltd) was divided into two portions. One portion was heated at 80°C for ten minutes (inactive for ATP measurement) and then cooled. The other portion (active for ATP + ADP measurement) was left at room temperature.

Standard curve

20 μ l of working buffer was added to tubes two to five and ATP standard to tubes one and two. Tube two was vortex mixed and 20 μ l transferred to tube three. The process was repeated until tube five when the extra 20 μ l was discarded.

Stimulated platelet release

For each agonist concentration two luminometry tubes were labelled one and two and 20 μ l of the extract was added.

PPP

As for stimulated platelet release.

Total platelet nucleotides (PRP)

4.9 ml of the working buffer was added into a tube as well as 0.1 ml of PRP and inverted a few times. To each of two labelled tubes 20 μ l of the extract was added.

0.78 ml of inactive buffer was added to each of the standard curve tubes and to each of the tubes labelled one. The same volume of active buffer was added to all the tubes labelled two. All the tubes were vortex mixed and incubated at 37°C for 10 minutes and then at 80°C for six

minutes. The tubes were cooled for five minutes while the luminometer was calibrated. 0.2 ml of the ATP monitoring reagent (Labsystems UK Ltd, Basingstoke, Hampshire, UK) was added to each tube, vortex mixed and the luminometer reading taken (LKB Wallac 1250 Luminometer, Wallac, Oy, Finland). The same vial of monitoring reagent was used for all tubes otherwise the standard curve was repeated. If the standard curve was not linear the double dilution was repeated.

Calculations

The luminometer value that corresponded to 200×10^{-12} M (200 pmol) of ATP on the standard curve was read. This value was divided by 200 to give the value of mV/pmol = memory.

PPP

value

----- = $\times 10^{-7}$ M/l

memory

PRP

value

----- $\times 50 (\div$ platelet count) $\times 100 =$ nM/ 10^9 platelets

memory

Stimulated release

value - PPP value

----- $(\div$ platelet count) $\times 100 =$ nM/ 10^9 platelets

memory

2.16 Platelet surface GPIb - Flow cytometric analysis

Flow cytometry is a technology that allows the simultaneous

measurement of multiple physical characteristics of a single cell. The aim of this section is not to cover all the principles and applications of flow cytometry, but to review the main principles relevant to this thesis. As these have been extensively reviewed in the literature I relied heavily on the review by Macey (1988), on the textbook by Ormerod (1990) for the principles and practical approach and on the textbook by Macey (1994) for the clinical applications of flow cytometry. The methods available as well as a critical review of the advantages, disadvantages and limitations of each method were reviewed by Abrams and Shattil in 1991.

The flow cytometer consists of three functional units (figure 2.1). 1) A laser and a sensing system (sample chamber and optical assembly), 2) a hydraulic system which controls the passage of single particles through the sensing system and 3) a computer system which converts the optical signals, relayed from the sensing system, to proportionate electronic signals for computer analysis. Flow cytometry is a system for sensing single cells or particles as they move in a liquid stream through a laser beam past a sensing area, and for measuring the relative light scattering and colour discriminated fluorescence of the labelled cells or particles. As the cell passes through the laser beam, light is scattered in all directions, and that scattered in a forward direction is proportional to the size of the cell (figure 2.1). Light that enters the cell is reflected and refracted by the nuclear and granular contents of the cell. The light scattered at 90° is proportional to the granularity of the cell (figure 2.1). The cells may be

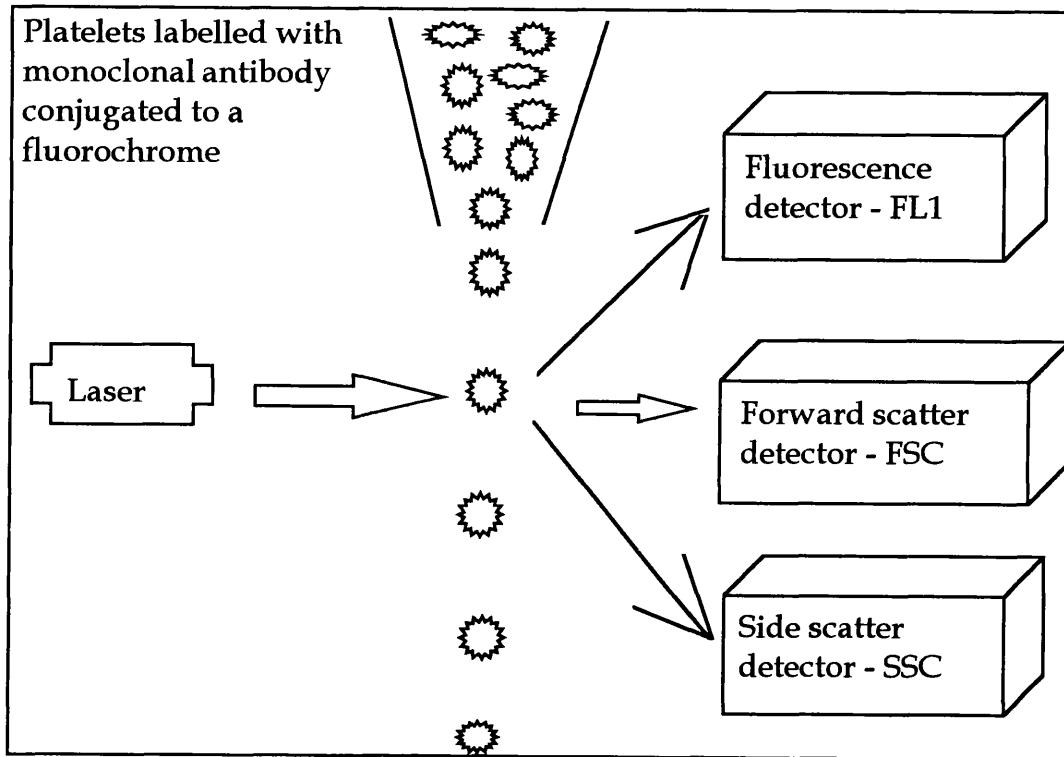


Figure 2.1 Schematic diagram illustrating the basic components of the flow cytometer. 1) a hydraulic system which controls the passage of single particles and 2) a laser and sensing system. The optical signals from the detectors are relayed to a computer system (not shown here). Light scattered in a forward direction (FSC) is proportional to the size of the cell and light scattered at 90° (SSC) is proportional to the granularity of the cell.

labelled with monoclonal antibodies (mAb) conjugated to a fluorochrome such as fluorescein isothiocyanate (FITC) which allows identification of different cell types. Analysis and differentiation of the cells is therefore based on size and granularity, and whether the cell is carrying a fluorescent marker. The simultaneous recording of several measurements on each cell enables the instrument to identify a cell population within a heterogeneous population.

Lasers have the advantage of producing an intense beam of monochromatic light which can be tuned to several different wavelengths. The fluorochrome is excited as cells, surrounded by sheath fluid, transverse the laser beam, and this fluorescence is collected by optics placed at 90° to the incident beam. A barrier filter blocks laser excitation illumination, while a dichroic mirror and appropriate filters are used to select the required wavelengths of fluorescence for measurement. The photons of light falling upon the detectors are converted and amplified by photomultiplier tubes (PMT) to an electrical impulse, and this signal is processed by an analog-to-digital converter, which changes the analog impulse to a digital signal. The quantity and intensity of the fluorescence are recorded by the computer system and displayed on a visual display unit as a frequency distribution, which may be a double or single parameter. Dual parameter histograms of forward angle light scatter and 90° light scatter allow identification of the different cell types within the preparation, based on size and granularity. Single parameter histograms usually convey information

regarding the intensity of fluorescence (FL1) and number of cells of a given fluorescent intensity (figure 2.2).

Platelet surface GPIb - PRP method

The surface expression of platelet GPIb (CD42b) was estimated using a specific mAb AN51 (IgG2a) directed at the surface receptor. The mAb was preconjugated to FITC, the intensity of which was measured using a Becton Dickinson FACScan flow cytometer (Becton Dickinson UK Ltd, Oxford, UK). Preliminary testing experiments with different quantities of mAb revealed that 5 μ l of mAb achieved saturating concentrations of the mAb for labelling purposes. Similar experiments using different concentrations of formol-saline fixative indicated that the optimal concentration was 1%. Preliminary studies also showed that the results were not affected by the route of sampling. The method used was a modification of the method used by the following groups: Adelman, Carlson, and Powers (1987), Adelman, Michelson, Hardin et al (1985) and Jennings, Ashmun, Wang et al (1986).

Five ml of blood collected in 3.2% trisodium citrate/ 1 mM EDTA (ratio of blood to anticoagulant 9/1) were centrifuged, immediately after collection, at 800 rpm (180 g) for 10 minutes at room temperature. The PRP was removed with a plastic pasteur pipette and was washed twice in 9 mM EDTA/ PBS by centrifuging at 1700 rpm (400 g) for 10 minutes at room temperature. The washed platelets were then incubated in PBS/ 1% BSA for five minutes at room temperature (to an approximate density of 100×10^6 /l).

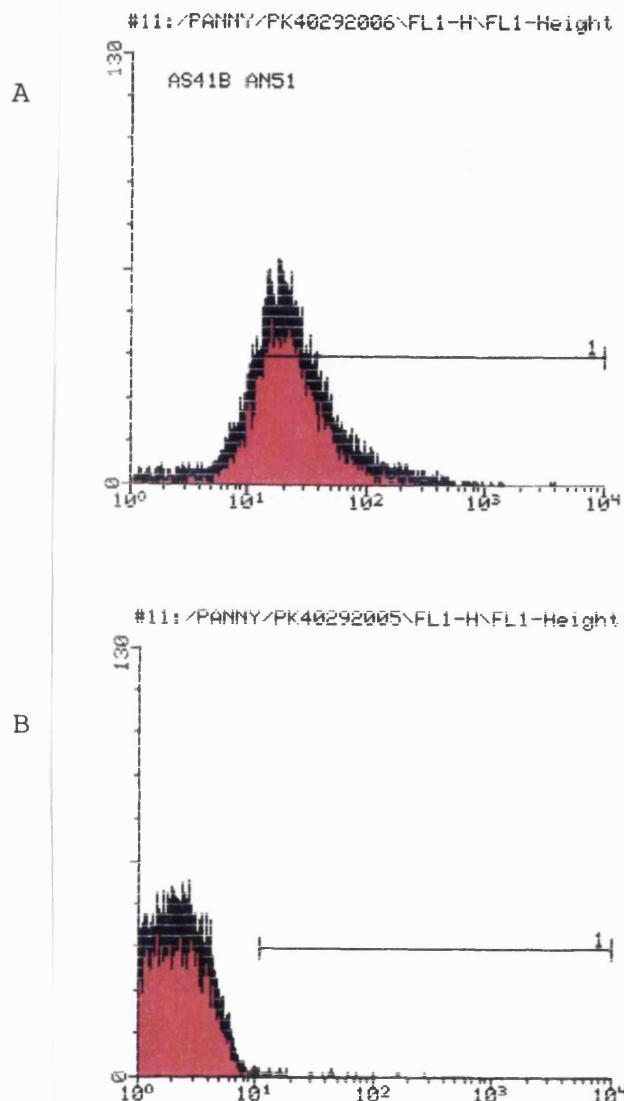


Figure 2.2A Printout from the flow cytometer of a frequency histogram of number of platelets (y axis) against log fluorescence (x axis). Increased binding of the FITC conjugated mAb shifts the log normal distribution to the right (i.e. increased fluorescence intensity). The peak (mean) fluorescence value is proportional to the mean number of receptors bound to the fluorescein conjugated mAb.

Figure 2.2B Non-specific binding (negative control) was excluded for each sample by using irrelevant mouse immunoglobulin. The fluorescence threshold value was defined for each sample, above which not more than 1% of non-specific binding occurred. AN51 labelled platelets with fluorescence greater than the threshold value were classed as positive and their peak fluorescence channel quantified by placing a marker outside the fluorescence threshold value.

50 μ l of this platelet suspension were pipetted into two tubes. In one tube 10 μ l of FITC conjugated irrelevant mouse immunoglobulin (MsIgG, IgG2a, DAKO Ltd, Bucks, UK) were added and in the other tube 10 μ l of AN51 (FITC) (DAKO Ltd) were added. These were then incubated for 30 minutes at room temperature. The platelets were then washed in 9 mM EDTA/ PBS and centrifuged at 1700 rpm for four minutes at room temperature. The platelets were then fixed with 800 μ l of 1% formol-saline and data acquisition was carried out immediately, even for samples obtained late at night. Once the data were acquired statistical analysis was carried out at a later stage using the software LYSYS II. Each sample was processed simultaneously with a sample from various normal subjects.

For data acquisition the forward, side scatter and the fluorescence amplifiers were set on logarithmic scale. A real time scatter plot of forward versus side scatter was generated.

Non-specific binding (negative control) was excluded for each sample by using irrelevant mouse immunoglobulin (figure 2.2). The fluorescence threshold value was defined for each sample, above which not more than 1% of non-specific binding occurred. AN51 labelled platelets with fluorescence greater than the threshold value were classed as positive and their peak fluorescence channel quantified by placing a marker outside the fluorescence threshold value. All the instrument settings were kept constant throughout the study period. Data acquisition on 10,000

platelets per sample were saved for subsequent statistical analysis. The peak fluorescence channel (arbitrary units) of the AN51 FITC labelled platelets was quantified using the geometric (logarithmic) statistics of the LYSYS II software.

Standard fluorescent beads (QuantumTM24, Flow Cytometry Standard Corp., PO Box 12621, NC 27709 USA) were employed, every week, for calibration of the flow cytometer as well as for checking the stability of the instrument over time (Vogt, Cross, Henderson et al 1989). The instrument settings were kept the same throughout the study period. One drop of the blank and one drop of each of the fluorescence standard microbeads were added to PBS just before use. A FITC fluorescence histogram was created by placing a real time gate around the singlet population on forward versus side scatter dot plot (figure 2.3 A). The forward, side scatter and the fluorescence amplifiers were set on logarithmic scale. The laser power, amplifier gains, PMT voltages and all instrument settings were kept the same throughout the study period. The peak channels of the five calibration microbeads were recorded (figure 2.3 B). The molecules of equivalent standard fluorochrome (MESF) (y-axis, log) and the peak channel (x-axis, linear) were plotted on semilog paper to construct the standard curve. From the fluorescence intensity histograms and the calibration curve the mean fluorescein equivalents per platelet were interpolated.

Platelet surface GPIb - Whole blood method

The method used was a modification of the method used by the

following groups: Jennings, Ashmun, Wang et al (1986), Michelson, Ellis, Barnard et al (1991) and Shattil, Cunningham and Hoxie (1987). Whole blood (100 μ l), collected as for the PRP method, was fixed immediately with 100 μ l of 0.25% formaldehyde in BSA. After a 30 minute incubation at room temperature 800 μ l of 9 mM EDTA/ PBS were added to an approximate density of 10^6 platelets / ml. 15 μ l of fixed whole blood and 5 μ l of FITC preconjugated AN51 (DAKO Ltd) were incubated for 30 minutes at room temperature. Similarly, in a separate tube, 15 μ l of fixed whole blood and 5 μ l of FITC preconjugated irrelevant mouse immunoglobulin (DAKO Ltd) were incubated for 30 minutes. 800 μ l of 9 mM EDTA/ PBS were then added and the samples were used for data acquisition on the FACScan immediately.

For data acquisition the forward, side scatter and the fluorescence amplifiers were set on logarithmic scale. Figures 2.4 - 2.6 illustrate the preparation of the flow cytometer for data acquisition. A real time dot plot of forward versus side scatter was generated and a gate placed around the platelet population as distinct from red cells and microparticles (figure 2.4). All the instrument settings were kept constant (for each of the two methods) throughout the study period. Non-specific binding was excluded for each sample by using irrelevant mouse immunoglobulin. The fluorescence threshold value was defined for each sample, above which not more than 1% of non-specific binding occurred. AN51 labelled platelets with fluorescence greater than the threshold value were classed as positive and their peak fluorescence channel quantified.

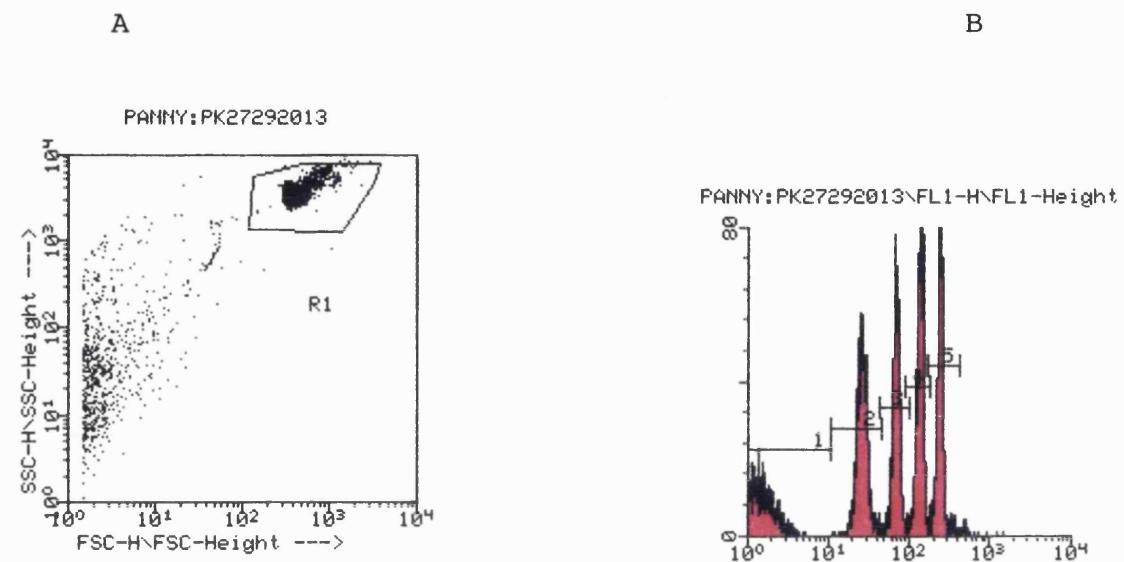


Figure 2.3A Calibration of the flow cytometer with standard fluorescent beads. A real time gate was placed around the singlet bead population on forward versus side scatter dot plot and the fluorescence estimated.

Figure 2.3B FITC fluorescence histograms were created for all five microbeads by placing markers around each peak.

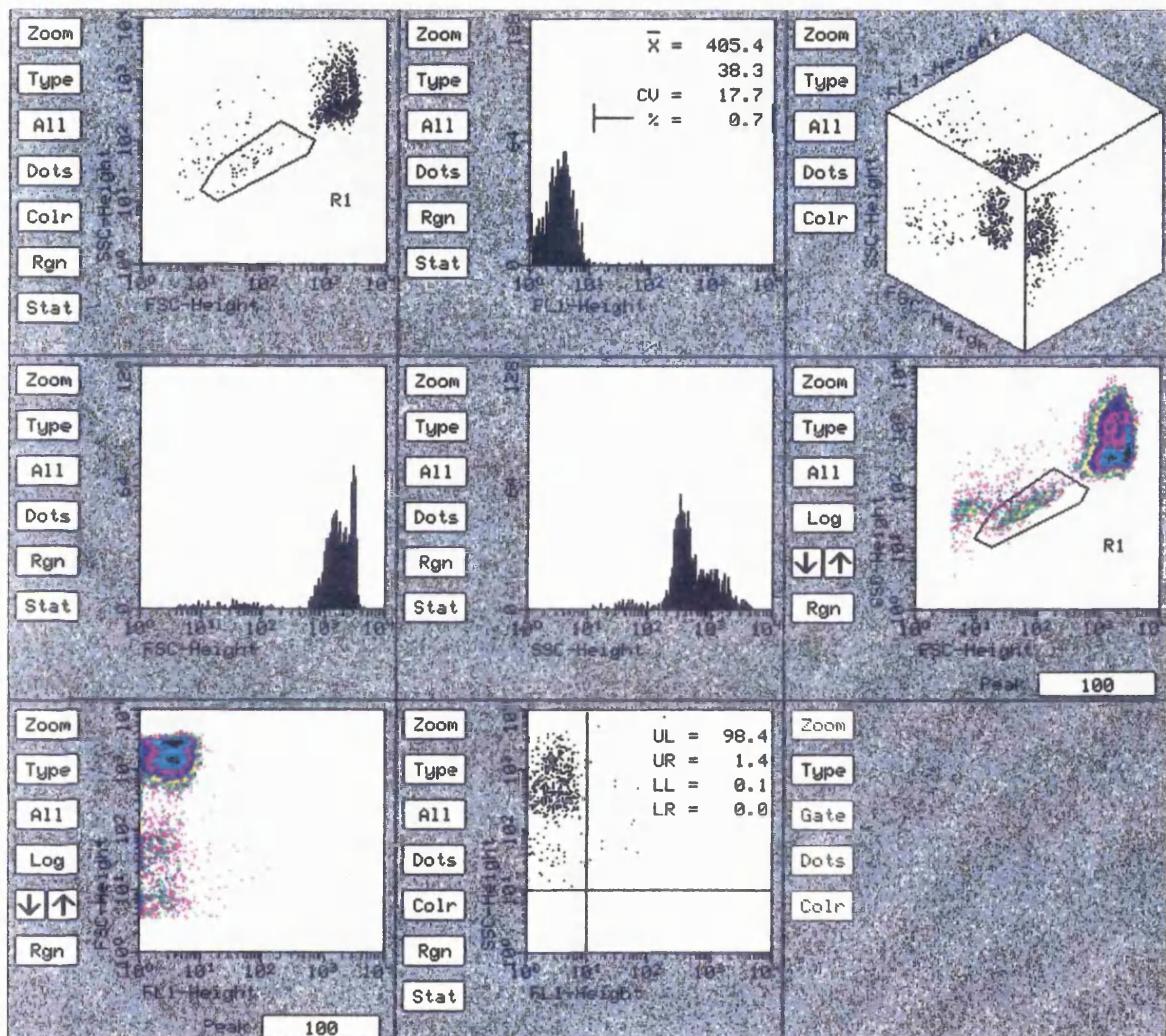


Figure 2.4 Printout from the flow cytometer illustrating the preparation of the instrument for data acquisition. A real time dot plot of forward versus side scatter was generated (right hand plot of the middle row). A gate (R1) was placed around the platelet population as distinct from red cells (population to the right of the gate) and microparticles (population to the left of the gate).

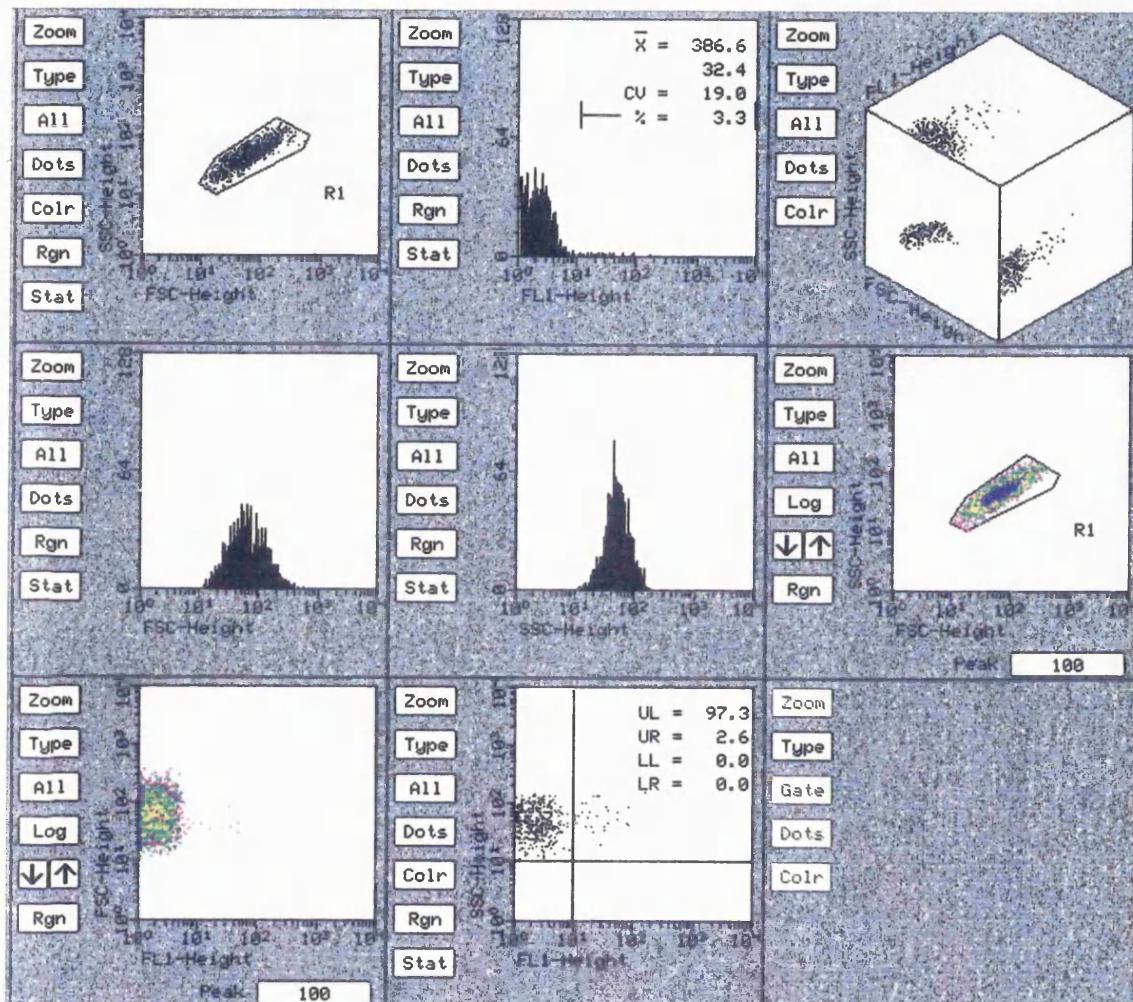


Figure 2.5 Printout from the flow cytometer illustrating the preparation of the instrument for data acquisition. Non-specific binding was excluded for each sample by using FITC conjugated irrelevant mouse immunoglobulin (MsIg) (middle plot or the upper row). All the fluorescence falls to the left of the 10^1 threshold unlike the fluorescence for AN51 which falls to the right of the 10^1 threshold (figure 2.6).

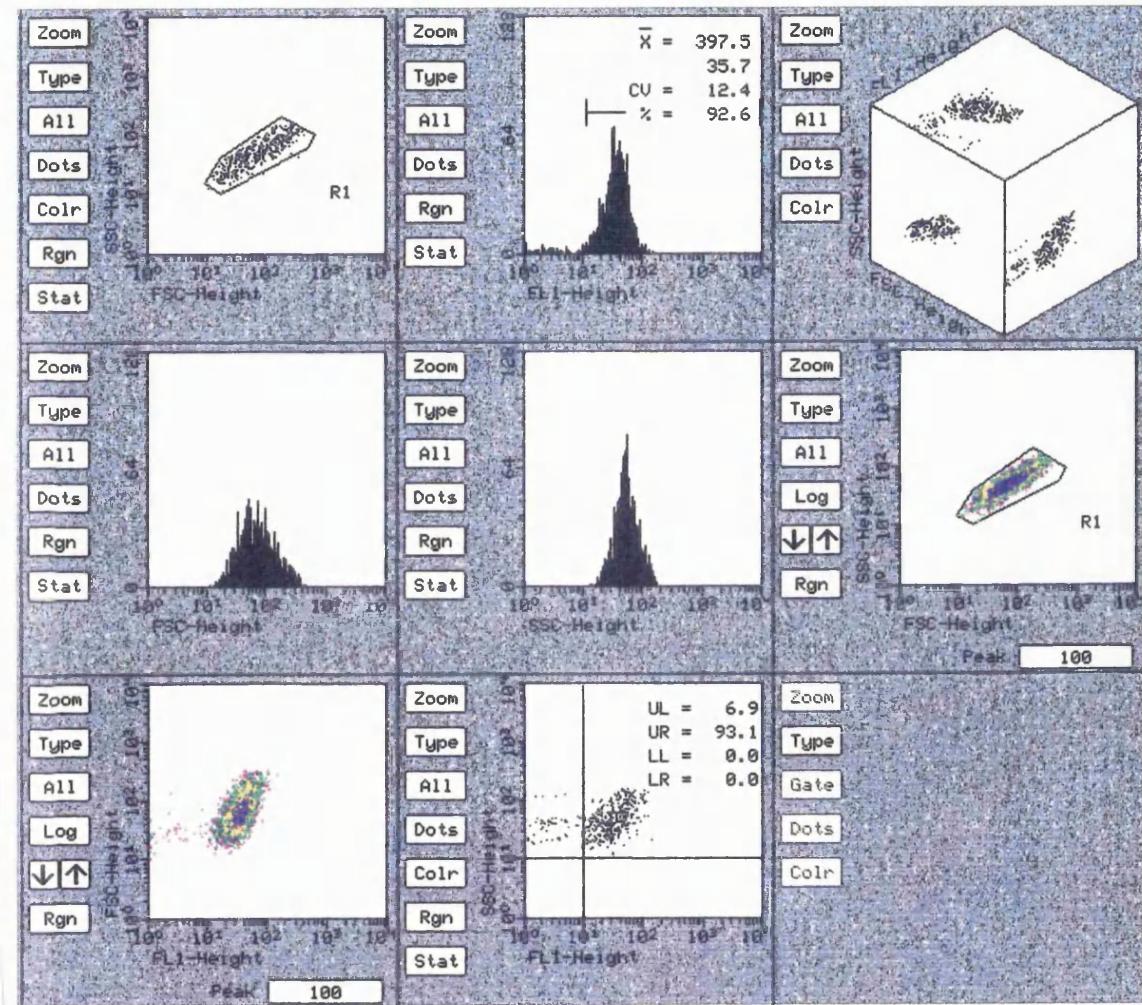


Figure 2.6 Printout from the flow cytometer illustrating the data acquisition of 5,000 platelets labelled with FITC conjugated AN51. All the fluorescence falls to the right of the 10^1 threshold (middle plot of the upper row) unlike the fluorescence for MsIg which falls to the left of the 10^1 threshold (figure 2.5).

by placing a marker outside the threshold value. Data acquisition on 5,000 platelets per sample were saved for subsequent analysis as described for the PRP method.

2.17 Plasma vWF activity

The ristocetin cofactor activity of plasma was determined in an assay using formalin fixed, washed platelets agglutinated in the presence of vWF using the antibiotic ristocetin (Ramsey and Evatt 1979). vWF is a very large protein of varying molecular size existing in plasma as a series of multimers of molecular weight $1-20 \times 10^6$ daltons. These multimers are built from protomers of 1×10^6 daltons which are in turn composed of subunits each of molecular weight of 2.3×10^5 . vWF mediates the adhesion and spreading of platelets to the subendothelium, particularly in the small vessels where there is a high shear rate. The importance of this action to haemostasis is reflected by the marked bleeding tendency seen in von Willebrand's disease, where it is congenitally absent or abnormal. vWF is synthesised in megakaryocytes (and thus circulates in platelets) and vascular endothelial cells. Each of these cell types is able to release vWF under suitable stimulation. The plasma factor VIII concentration is less than 10 mg/l.

Factor VIII^{historically} consisted of two entities (Koutts, Lavergne and Meyer 1977). One very high molecular weight (larger than 1×10^6 daltons) which is responsible for vWF activity (the ristocetin co-factor) and carries antigenic determinants which react with heterologous anti-sera against factor VIII

(F.VIII R:Ag). The other entity has a molecular weight of about 2×10^5 daltons and is responsible for the coagulant activity F.VIII :C. By using antiserum against F.VIII R:Ag, haemophilia can be distinguished from von Willebrand's disease (Meyer, Lavergne, Larrieu et al 1972). In haemophilia the level of F.VIII R:Ag is normal but the level of F.VIII :C is diminished or nil. In classic von Willebrand's disease, the three factor VIII activities (coagulant, antigenic and ristocetin co-factor) are simultaneously reduced.

The plasma vWF activity (normal range 50-150 IU/dl, our own laboratory) was measured in blood collected in 3.2% trisodium citrate and the plasma prepared as described for the coagulation screen. Pooled normal plasma from 20 normal individuals with equal sex distribution was also obtained and 500 μ l aliquots were stored at -70°C. The vWF activity was measured by the ristocetin cofactor assay, with formalin fixed donor platelets, with a platelet aggregometer (Model PAP-4, BIO/data Corporation, Hatboro PA 19040, USA). Preliminary studies in 18 samples were carried out in the presence or absence of proteolytic inhibitors (5 mM EDTA, 1 mM leupeptin, 2 mM n-ethylmaleimide, 200 KIU/ml aprotinin, 2 mM phenylmethylsulfonylfluoride) (Gralnick, Williams, McKewon et al 1985). No differences in vWF activity were detected when plasma or platelets were collected in the presence or absence of proteolytic inhibitors. Similar results were obtained with vWF antigen, multimeric analysis and platelet surface GPIb expression. As a result no proteolytic inhibitors were used with the anticoagulant for

the tests just mentioned.

Ristocetin cofactor assay

Reagents

Fixed platelet suspension.

Tris Buffered Saline (TBS) 0.05 M (pH 7.5).

Ristocetin 10 mg/ml in TBS.

Pooled normal plasma.

Control plasma.

The standard curve was generated first. A 1/2 dilution of normal pooled plasma made in TBS was used as the 100% reference point. Further double dilutions were made to obtain 50% and 25% reference points mixing thoroughly between dilutions. A 1/2 dilution of control plasma was made in TBS. The aggregometer blank was prepared by pipetting 0.25 ml of platelet suspension and 0.25 ml of TBS into an aggregometer cuvette, mixing thoroughly. The PAP-4 platelet aggregometer was switched on and allowed to reach 37°C after which the standard curve mode for the vWF assay was selected. 0.4 ml of platelet suspension was pipetted into each of four aggregometer cuvettes. 0.05 ml of ristocetin was added to each cuvette and mixed by gently tapping. The reagents were allowed to equilibrate for two minutes and a stir bar was added to each cuvette. The aggregometer blank was placed in each reading channel to set the blank reading. The cuvettes containing the platelet suspension and ristocetin were placed into the test wells and the zero percent baseline for each channel was set. 0.05 ml of plasma dilutions were added and when no further

increase in light transmission was observed the reaction was stopped by depressing the respective channel switch.

Once the standard curve had been generated and stored in the memory the vWF assay mode was selected to analyse the samples. Test plasma samples were diluted in TBS starting at 100% (1/2 dilution). Subsequent dilutions were made as required by double dilutions. For each sample to be assayed 0.4 ml of platelet suspension was pipetted into an aggregometer cuvette. 0.05 ml of ristocetin was added and was mixed by gentle tapping. After the aggregometer blank was set, the cuvettes containing the platelet suspension and ristocetin were placed into each test well and the zero percent baseline for each channel set. 0.05 ml of the test plasma dilutions were added and when no further increase in light transmission was observed the reaction was stopped. A log-log graph of the standard curve on which all test plasma slope values were plotted were generated followed by the percentage activity for each specimen assayed. The plasma vWF activity of the tested sample (IU/dl) was then calculated using the formula:

vWF activity of standard pooled plasma = 95 IU/dl

Test plasma vWF activity = % vWF activity x 100/95

For each run of batch analyses of the ristocetin cofactor assay a fresh (the same day) preparation of formalin fixed, washed platelets was obtained as follows: 200 ml of fresh blood was collected from the same normal individual into plastic tubes containing 3.2% trisodium citrate (volume of

blood to anticoagulant 9/1) ensuring constant mixing. The blood was centrifuged immediately at 800 rpm for 15 minutes at room temperature, the separated PRP was transferred into a plastic tube and was incubated in a water bath at 37°C for one hour. The PRP was then diluted with an equal volume of 2% formalin in normal saline using plastic containers. This mixture was placed at 4°C and was left undisturbed for 18 hours. The mixture was then centrifuged at 3000 rpm for 10 minutes at room temperature. The supernatant was then discarded and the platelets were resuspended in normal saline. Sedimentation and resuspension in normal saline was repeated three times. The final reconstitution was made in saline (pH 7.2) and the platelet count was adjusted to 200 - 250 $\times 10^9$ platelets/l. After reconstitution the platelets were examined for agglutinates both before and after the assay.

2.18 Platelet vWF activity

The platelet vWF activity (normal range 25-75 IU/dl/ 10^6 platelets, our own laboratory) was measured in blood (ratio of blood to anticoagulant 9/1) collected in 3.2% trisodium citrate / 1 mM EDTA. PRP was obtained by centrifuging 20 ml of the collected blood at 800 rpm for 10 minutes at room temperature. The platelets were separated from plasma on an arabinogalactan density gradient under centrifugation. The density gradient was prepared by using five ml of the 10% arabinogalactan (Aldrich Chemical Company, Milwaukee, WI, USA) into a round bottom polypropylene tube. Three ml of the 40% gradient (arabinogalactan) was carefully

introduced at the bottom of the tube with a pipette, thus achieving a discontinuous gradient. The tube was spun at 3000 rpm for 20 minutes (at 24°C) during which time the platelets settled at the top of the 40% gradient. The platelet layer was carefully removed with a plastic pipette taking care to avoid contamination by the PPP. The platelets were washed twice in buffer solution (pH 6.8), centrifuged at 3000 rpm at 24°C and resuspended in between washes. The platelets were resuspended in buffer (pH 7.4) and incubated for five minutes at 37°C. The platelet count was then measured and adjusted to 1000×10^9 platelets/l. The platelet suspension was lysed with 20% Triton (volume of Triton to platelets was 1/40) (Triton X 100, Sigma Ltd) and incubated for 30 minutes at 37°C. The lysate was frozen at -70°C, thawed and centrifuged prior to assay. The vWF activity was measured by the ristocetin cofactor assay, with formalin fixed donor platelets, with a platelet aggregometer (PAP-4 BIO/data Corporation, Hatboro PA 19040, USA). The platelet vWF activity of the test sample (IU/dl/ 10^6 platelets) was calculated using the formula: (standard pooled plasma used with 95 IU/dl activity as used for the plasma vWF activity)

% vWF activity $\times 100/95 \times 1000/\text{platelet count of test sample}$

2.19 Plasma vWF antigen

The plasma vWF antigen was estimated using an ELISA method. The ELISA technique allows the assay of larger numbers of samples and gives greater precision and reproducibility at

low concentrations of antigen. The plasma vWF antigen (normal range 45-160 IU/dl) was measured in blood collected in 3.2% trisodium citrate (ratio of blood to anticoagulant 9/1) and the plasma was obtained and stored as described for the coagulation screen.

Reagents

Low salt - coating buffer

0.0025 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

0.0075 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

0.145 M NaCl

Made to one litre, pH 7.2 and stored at 4°C.

High salt buffer - wash buffer

As low salt buffer but in addition containing

0.5 M NaCl

10 ml 0.2% Tween 20 (Sigma Ltd)

Made up to five litres.

Dilution buffer

High salt buffer but in addition containing

3% Polyethylene glycol (PEG) 8000 (Sigma Ltd)

Substrate buffer (0.1 M citric acid phosphate)

0.0347 M $\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$

0.0667 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

Made to one litre, pH 5.0 and stored at 4°C.

Colour reagent

1x10 mg tablet Orthophenylenediamine (OPD) (Sigma Ltd)

15 ml substrate buffer

0.007 ml 30% hydrogen peroxide (stored at 4°C)

Made fresh just before use and protected from light.

1.5 M Sulphuric acid

16.5 ml of concentrated acid added to 180 ml of water.

Coating antibody

Rabbit anti vWF (Dako Ltd).

Secondary antibody

Peroxidase conjugated rabbit immunoglobulin to vWF (DAKO).

100% reference plasma (Immunodiagnostics).

Abnormal control plasma (Uniplasmatrol Index P Company, Bio mérieux, Marcy, France).

Equipment

Nunc - Immuno Plate Maxisorp plates (Life Technologies, Glasgow, UK).

Microtitre plate reader (Molecular devices).

Repeating pipette (Accustep dilutor with 8 port manifold).

The Maxisorp plate was coated with 0.1 ml diluted primary antibody (low salt PBS buffer pH 7.2 to an optimal dilution of 1/500) and was kept covered with parafilm overnight at 4°C. The samples were diluted in the dilution before they were added to the plate to reduce non specific reactions taking place. The samples and standards were diluted in the dilution buffer to improve the sensitivity which would otherwise be approximately 1 to 5 ng/ml. The prolonged incubation time also improved sensitivity.

The plate was washed three times in high salt PBS buffer at three minute intervals and blot dried. 0.1 ml of dilution buffer was added in duplicate wells as a reagent blank.

The diluted plasma (0.1 ml) together with 0.1 ml standard

curve samples were placed into the designated duplicate wells and incubated at room temperature for three hours while the plate was covered with parafilm. The plate was then washed three times as before. 0.1 ml of the diluted secondary antibody (1/2000 dilution buffer) was applied to the wells and incubated at room temperature for three hours while the plate was covered with parafilm. The plate was then washed three times. The Orthophenylenediamine tablets (Dako Ltd) were dissolved in citric acid buffer, hydrogen peroxide was added and it was left for five minutes to check for the absence of discolouration. 0.1 ml of this substrate was then added to the wells and was protected from light until the colour had developed (approximately five minutes) and then the reaction was stopped with 0.1 ml of 1.5 M sulphuric acid. The optical density was then read in each well at 492 nm. The standard curve was plotted on semi log paper with the concentration on the x axis and the optical density on the y axis. The concentrations of the unknown samples were read and any necessary corrections for dilution were made. All the calculations were performed by the instrument, which took the average of two values.

2.20 Platelet vWF antigen

The platelet vWF antigen was estimated by using the same ELISA method as described for the plasma vWF antigen. The platelets were obtained from the aliquots of platelets prepared and stored as described for the platelet vWF activity (normal range 0.15-0.58 U/ml/10⁶ platelets, from Gralnick, Rick, McKeown et al 1986).

2.21 Plasma vWF multimeric analysis

It is possible to visualise the multimeric composition of vWF by diluting the plasma in urea and sodium dodecyl sulphate (SDS) to ensure that electrophoresis through a 1.6% agarose gel depends on molecular weight and not charge. The vWF:Ag can then be visualised by either autoradiography (Ruggeri and Zimmerman 1981) or by enzyme linked antibodies (Chow and Savidge 1985). Abnormalities of vWF multimer patterns can occur in the number of bands present, their density, mobility and presence of subsidiary bands and a change in pattern after certain treatments such as DDAVP.

The plasma vWF multimeric analysis was carried out in blood collected in 3.2% trisodium citrate (ratio of blood to anticoagulant 9/1) and the plasma obtained and stored as described for the coagulation screen. The standard pooled plasma aliquoted as described for the ristocetin cofactor assay was also used for a control. The method used was similar to that used by Dalton, Lasham and Savidge in 1988 and by Webb and McLellan in 1989. A uniform one mm thickness of a 1.6% running gel was achieved by pouring molten agarose into a "template". A strip of gel was removed from the top of the template and replaced by 0.8% stacking gel. When the agarose had set the template was dismantled to expose the entire gel. Plasma samples were diluted in urea and SDS buffer and bromophenol blue added as a small molecular weight marker. Wells were cut in the stacking gel and into these the diluted control and specimen plasma (50 μ l) samples pipetted. Electrophoresis was

carried out overnight until the marker dye had reached the opposite end of the plate.

Visualisation of the multimer pattern was done by Western blotting with alkaline phosphatase (AP) staining. Protein on the agarose gel was transferred onto a nitrocellulose membrane by sandwiching both between filters soaked in a 20% methanol buffer and passing an electric current through the "stack" (gel at cathode side, membrane at anode side). The membrane was incubated with 5% milk protein to prevent non specific binding then washed in PBS and incubated overnight in diluted rabbit anti vWF. After washing, the secondary antibody, alkaline phosphatase swine anti rabbit antibody, was applied to the plate for a further two hour incubation. A final wash was followed by staining in Nitro blue tetrazolium 5 bromo-4 chloro-3 indolyl phosphate. This procedure generally took three days.

Materials electrophoresis

Stock solutions	2 M Tris	(stored at 4°C)
	3 M HCl	
	0.01 M Na ₂ EDTA.	
Running buffer	37.5 ml 2 M Tris	
	4.45 ml 3 M HCl	
	made to 50 ml with H ₂ O and pH to 8.8	
	stored at 4° C for two weeks maximum.	
Stacking buffer	12.5 ml 2 M Tris	
	7.9 ml 3 M HCl	
	made to 50 ml with H ₂ O and pH to 6.8	
	stored at 4°C for two weeks maximum.	

Sample buffer 500 µl 2 M Tris
 10 ml stock EDTA
 made up to 100 ml with water
 stored at 4°C.

Sample diluent 9.61 g urea (BDH Chemicals, Poole, UK)
 0.4 g SDS (BioRad Laboratories, CA, USA)
 dissolved in sample buffer to 20 ml and
 pH to 8.0. (Used on day of preparation).

10% SDS 1 g SDS in 10 ml H₂O stored at 4°C and
 warmed to room temperature before use.

Electrophoresis 28.8 g Glycine (BDH, Poole, UK)
buffer 6.0 g Trizma base (Sigma Ltd)
 1.0 g SDS
 made to one litre in H₂O.

DNA grade agarose (Bio Rad, Hercules, CA, USA).

Gelbond plate (Flowgen Instruments Ltd, Sittingbourne, UK).

Materials - Western blotting and AP staining

1 M NaCl for use with car battery charger.
 1 M CuSO₄ "
 20% Methanol "
 Aluminium foil "
 Whatman 3MM paper (Downswood Products Ltd, Knebworth, UK).
 Phosphate buffered saline 1.95 g NaH₂PO₄.2H₂O
 (Low salt) 13.4 g Na₂HPO₄.12H₂O
 42.37 g NaCl
 made to 5 l, pH to 7.2.

5% milk powder (Sainsbury) in PBS ..

Rabbit anti vWF (Dako Ltd, High Wycombe, Bucks, UK).

Alkaline phosphatase conjugated swine anti rabbit antibody

(Dako Ltd, High Wycombe, Bucks, UK).

Staining reagents:

5-Bromo-4chloro-3-indolyl phosphate (Sigma Ltd)

4 mg/ml in 2:1 methanol/acetone (0.75 ml).

Nitro blue tetrazolium (Sigma Ltd)

1 mg/ml (5.0 ml).

Ethanolamine buffer 0.1 M, pH 9.6 (Sigma Ltd)

3.05 ml to 500 ml in H₂O (50 ml).

Magnesium chloride 1 M (made fresh each day) (Sigma Ltd)

0.95 g in 10 ml (0.2 ml).

Methods - electrophoresis

Agarose gel was cast onto the hydrophobic side of the gelbond by sandwiching the latter between two glass plates separated by a one mm spacer. The gel was made as follows:

Agarose	0.8 g
running buffer	12.5 ml
water	37.0 ml
10% SDS	0.5 ml.

Molten agarose was applied to the warmed template with a syringe. Any undissolved agar particles were removed with a filter needle and the gel was left to set at 4°C. By carefully sliding down the top glass plate a 1.5 cm deep strip exposed was removed using a scalpel. The plate was replaced in its original position and the 1.5 cm space filled with the stacking gel prepared as follows:

Agarose	0.16 g
stacking buffer	5.0 ml
water	14.8 ml

10% SDS 0.2 ml.

Once the stacking gel had set at 4°C the template was disassembled and the gel on its backing gelbond plate removed. Wells were cut in the stacking gel to accommodate the samples prepared as follows:

Sample diluent	250 μ l
plasma	50 μ l
1% bromophenol blue	5 μ l.

The plate was placed in the electrophoresis tank with the stacking gel at the cathode. Electrophoresis was initially set at 6 mA until the marker dye had moved out of the wells (approximately one hour) which were then filled with stacking gel. The electrophoresis was then resumed 4-5 mA for 18 hours or until the marker dye had reached the anode side of the plate.

Methods - transfer

The following transfer system was assembled:

Beaker containing melting ice.

Glass plate, one sheet foil and lead.

Five sheets CuSO₄ soaked filter paper.

Dialysis membrane (Pharmacia, Uppsala, Sweden).

Five sheets NaCl soaked filter paper.

Two sheets 20% methanol soaked filter paper.

Gel.

Nitrocellulose (Schleicher & Schuell, Dassel, Germany).

Two sheets 20% methanol soaked filter paper.

Five sheets NaCl soaked filter paper.

Three sheets foil and lead.

Cooling platten.

Filter papers were soaked in the appropriate solution at 4°C before use. Nitrocellulose was slid at an angle in the 20% methanol solution, to ensure thorough "wetting", and left for 20 minutes before use. The cooling platten was attached to a circulating water bath containing melting ice. "Blotting" took about 10 minutes on the 12V battery charger.

Methods - antibody incubation and AP staining

After blotting the nitrocellulose membrane was treated as follows:

Immersed in 5% milk powder in PBS	(30 minutes).
Washed in PBS	(3 x 5 minutes).
Immersed in primary antibody (anti vWF 60 µl in 50 ml PBS)	(overnight).
Washed in PBS	(3 x 5 minutes).
Immersed in secondary antibody (swine anti rabbit AP conjugated 50 µl in 50 ml PBS)	(2 hours).
Washed in PBS	(3 x 5 minutes).
Stained	30 minutes.
Rinsed in water and dried.	

Densitometric scanning of multimers

The method used was a modification of the method used by Mannucci, Moia, Rebulla et al in 1987. The gels were scanned with a ScanJet densitometer (Hewlett-Packard, Washington, USA), which resolved the multimeric structure

into a series of peaks (figure 2.7). The low molecular weight (LMW) multimers were arbitrarily defined as the area under the first three peaks from the left as viewed in figure 2.7. The high molecular weight (HMW) multimers were defined as the area to the right of the fifth peak. These two areas, as well as the whole area under all the peaks, were computed using the Macintosh software NIH image.

2.22 Platelet vWF multimeric analysis

The platelet vWF multimeric analysis was performed using the same method as described for plasma. The platelets were obtained from the aliquots of platelets prepared and stored as described for the platelet vWF activity. The standard platelets from the same normal individual were used as a control as described for the platelet vWF activity.

2.23 tPA activity

The SpectrolyseTM/fibrin tPA assay (Biopool, Umeå, Sweden), based on the colour change of a chromogenic substrate cleaved by plasmin, was used to measure the plasma tPA activity. This assay is based on the functional parabolic rate assay described by Rånby, Norrman and Wallén (1982) and on its adaptation to plasma samples as described by Wiman, Mellbring and Rånby (1983). The blood is rapidly acidified and acidified plasma obtained by centrifugation. This preserves tPA activity and destroys plasmin inhibitors especially α_2 -antiplasmin. The tPA activity is measured by adding Glu-plasminogen, chromogenic plasmin substrate and fibrin at neutral pH. In the presence of fibrin,

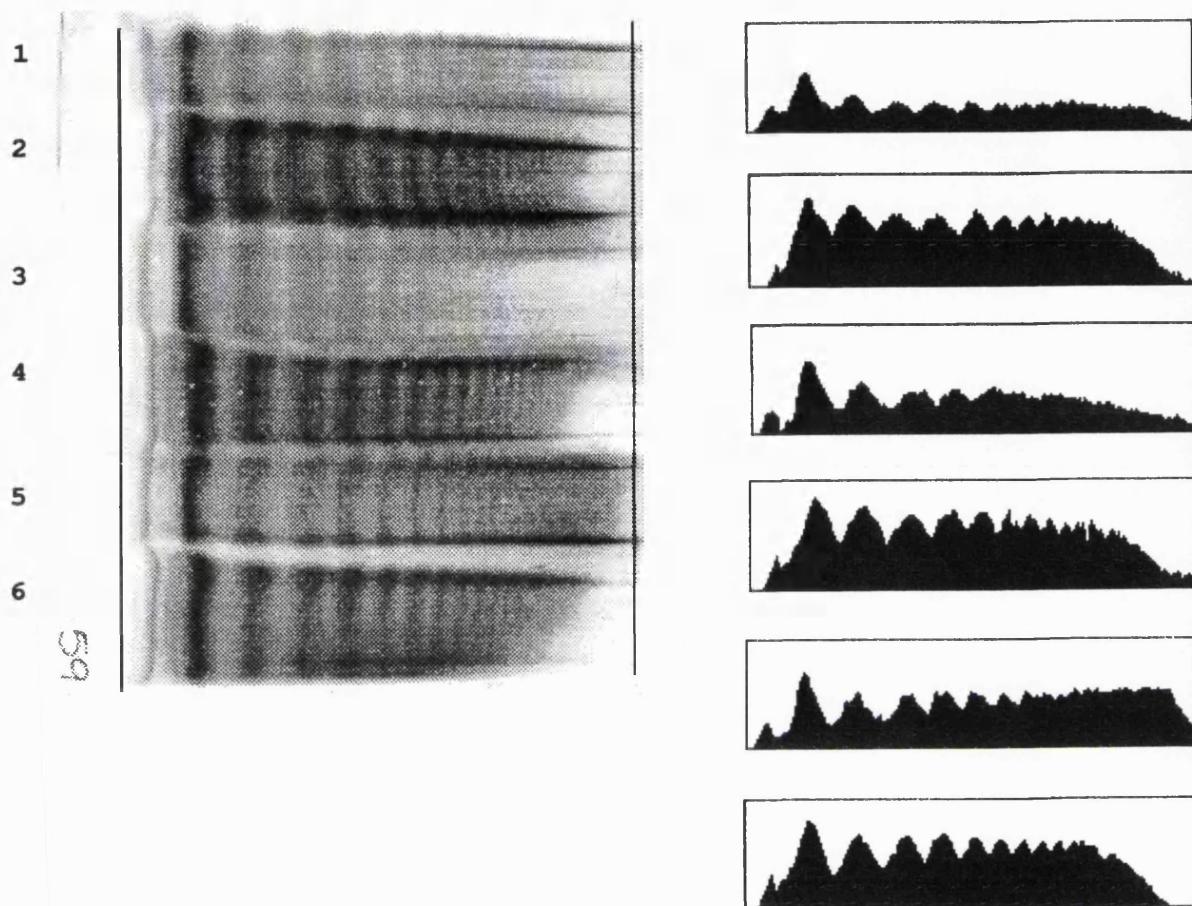


Figure 2.7 Printout from the densitometer illustrating the scanned gel plate of plasma and platelet vWF multimeric composition (left). The right hand diagram represents the densitometric scanning of the gel plate which resolved the multimeric structure into a series of peaks.

- 1 Post CPB platelet vWF multimeric structure.
- 2 Post CPB plasma vWF multimeric structure.
- 3 Pre CPB platelet vWF multimeric structure.
- 4 Pre CPB plasma vWF multimeric structure.
- 5 Normal control platelet vWF multimeric structure.
- 6 Normal control plasma vWF multimeric structure.

tPA converts plasminogen to plasmin, which subsequently cleaves the chromogenic substrate. The amount of colour developed during a given time is proportional to the tPA activity.

Glu-plasminogen (tPA & fibrin) → plasmin

D-But-CHT-Lys-pNA (plasmin) → free pNA (yellow)

The tPA chromogenic assay measures physiological tPA activity; tPA complexed with its inhibitor PAI does not react in the assay. The content of tPA activity in blood plasma varies greatly. For healthy humans the basal level is in the range 0.2 - 2 IU/ml. The tPA levels for healthy individuals following venous occlusion are reported to be 1.4 -14 IU/ml (Wiman, Mellbring and Rånby 1983). Abnormal levels of tPA have been observed in patients with venous thrombosis, in pregnancy and post-operatively.

Materials

PAR; 0.5 mg human Glu-plasminogen colyophilised with D-But-CHT-Lys-pNA (6 μ mol).

DESAFIB-XTM; stimulator for tPA, 2 mg solubilised desAA fibrinogen (fibrin).

tPA standard; 10 μ g human melanoma cell line derived single chain tPA.

tPA standard for external control from the National Institute for Biological Standards and Controls (South Mimms, Herts, UK).

Stop/fibrin (ready to use stop solution).

Spectrophotometer operable at 405 nm and 492 nm.

Blood was drawn into stabylite tubes, centrifuged immediately and the plasma frozen to -70°C before analysis. 50 μ l of acidified plasma was added to 2 ml 0.15 mol/l NaCl (plasma dilution 1:81). The diluted acidified plasma was diluted further with zero IU/ml tPA standard. tPA activity standards, for analysing tPA in plasma, were prepared by adding highly purified single chain tPA to acidified PAI depleted plasma and then diluting this in the same way as the samples. The standards were prepared as follows:

Acidified PAI depleted plasma was prepared and mixed with 300 μ l PAI depleted plasma and 300 μ l acetate and incubated for 20 minutes at 37°C. Diluted zero IU/ml tPA standard was prepared by mixing 200 μ l acidified PAI depleted plasma and 8 ml 0.15 mol/l NaCl. Diluted 40 IU/ml tPA standard was prepared by mixing 200 μ l acidified PAI depleted plasma, 100 μ l tPA and 7.9 ml 0.15 mol/l NaCl. Diluted 4 IU/ml tPA standard was prepared by mixing 150 μ l diluted 40 IU/ml standard and 1350 μ l diluted zero IU/ml tPA standard. Diluted 0.4 IU/ml tPA standard was prepared by mixing 50 μ l diluted 4 IU/ml standard and 450 μ l diluted zero IU/ml tPA standard. Additional diluted tPA standards were prepared for the three and 19 hour incubation (table 2.4).

The tPA assay was carried out, by adding 20 μ l diluted plasma sample or diluted tPA standard to the wells of the micro test plates. 200 μ l of PAR was added to the wells and the plate was then placed in the refrigerator for 15 minutes. 10 μ l of DESAFIB-X™ was added to each well with a repeating pipette (completed within two minutes) and the

Table 2.4 Dilutions for tPA standards.

<u>dil. tPA std</u> IU/ml	<u>add</u> μ l	<u>dil. tPA std</u> IU/ml	<u>to dil. 0</u> μ l	<u>IU/ml tPA std</u>
30	300	40	100	(3 h incubation)
20	200	40	200	
15	150	40	250	
10	100	40	300	
5	50	40	350	
3	300	4	100	
1	100	4	300	
0	-	-	400	
<hr/>				
3	300	4	100	(19 h incubation)
2	200	4	200	
1.5	150	4	250	
1.0	100	4	300	
0.5	50	4	350	
0.3	300	0.4	100	
0.1	100	0.4	300	
0	-	-	400	

plate was agitated for 10 seconds. The plate was then incubated for 3 or 19 hours depending on the tPA level measured. 25 μ l of Stop/fibrin was added, the plate agitated and the absorbance at 405 nm and 492 nm was measured 15 minutes later. Paranitroanilide absorbs light at 405 nm but not at 492 nm whereas the absorbance due to turbidity is approximately equal at 405 and 492 nm. The absorbance (A) difference (A_{405nm} - A_{492 nm}) therefore, corrects for background due to turbidity. A standard curve was then plotted for each incubation period (A₄₀₅ - A₄₉₂ for the standards on the ordinate against their tPA activity on the abscissa) on linear graph paper. The tPA activity of the tested samples was then read from the standard curve.

2.24 tPA antigen

The tPa antigen was measured using the biopool TintElizeTM tPA kit (Biopool, Umeå, Sweden), an enzyme linked immunosorbent assay (Rånby, Bergsdorf, Nilsson et al 1986) (normal range = 0.5-8.3 ng/ml, 20 normal controls).

Materials

Precoated 96-well microtest plate lyophilised with standard and control antibodies.

PET (PBS-EDTA-Tween) buffer substance for 1000 ml solution.

Conjugate; horseradish peroxidase labelled goat anti human tPA IgG.

Substrate (Orthophenylenediamine Dihydrochloride) with buffer salts.

Hydrogen peroxide 0.15%.

Sulphuric acid 3 mol/l.

Four disposable reagent reservoirs.

Microtest plate spectrophotometer operable at 492 nm.

The reagents were stored at 2-6 °C in protective foil bags.

The principle of the assay is as follows: Each sample is added into two adjacent wells: One contains 2 µg normal goat IgG (N) and the other contains 2 µg anti human tPA IgG (A). The difference in assay response between these two wells is tPA specific. During this incubation period tPA antigen in the sample binds to the anti tPA IgG. The conjugate (horseradish peroxidase labelled anti tPA IgG) is added to the wells. This will bind to free antigenic determinants on the tPA molecules present. After one hour incubation

unbound conjugate is washed away. The amount of enzyme, peroxidase, remaining in the wells is proportional to the amount of tPA antigen. The substrate is then added to the wells. The amount of colour developed from substrate catalytically oxidized by the enzyme, peroxidase, is proportional to the amount of tPA bound in the well.

100 μ l of buffer (PBS-EDTA-Tween) was added to each well taking care not to contaminate the N-wells (blue) with the contents of the A-wells (green). Column 1 and 2 both contained tPA standards (30, 24, 18, 12, 6, 3, 1.5 and 0 ng/ml from row A to row H). In addition column one contained normal goat IgG (N) and column two goat anti tPA IgG (A). Column 3-12 were used for samples. Odd numbered columns contained normal goat IgG (blue, N-wells) and even numbered columns contained goat anti human tPA IgG (green, A-wells). 20 μ l of plasma sample was added to two adjacent N and A wells. The plate was then incubated for three hours at 25°C on a micro-test plate shaker at 600 rpm. The contents were then discarded and the plate was then washed four times. All washings were performed by filling the wells with buffer (PBS-EDTA-Tween), emptying and then drying by hitting the plate five times face down against absorbing material. The substrate was prepared within 30 minutes prior to use by adding 22 ml water to the substrate vial and agitating it gently until dissolved. The hydrogen peroxide vial (2 ml) was emptied in the substrate and mixed. 200 μ l of substrate was then added to the wells and the plate incubated for 15 minutes at 25°C. The enzymatic reaction was then stopped by adding 50 μ l 3.0 mol/l H_2SO_4 in the same

order and speed as the substrate was added. The plate was then stored in the dark for ten minutes until measured. The "blank" of the micro-test plate was set against air and the absorbance was read at 492 nm. The difference between the N value and the corresponding A value (δA_{492}) was calculated for all the standards and the samples. The standard curve was obtained by plotting δA_{492} against the standards. This curve was a straight line and was only acceptable if the 30 ng/ml resulted in an absorbance at 492 (δA_{492}) of at least 0.8 absorbance units.

2.25 Salicylic acid

The high performance liquid chromatography (HPLC) assay for salicylic acid was established and standardised in the Analytical Unit of St George's Hospital Medical School. Calibration was by means of five standards of blank plasma spiked with salicylic acid (range 1-20 mg/l). 200 μ l of calibration standard, quality control, sample serum and internal reference standard (0-Anisic acid 10 mg/l, 100 μ l) were mixed with 100 μ l of 25% hydrochloric acid in appropriately labelled tubes. These were extracted by mixing with 2 ml of methyl tertiary butyl ether (MTBE) on a rotating mixer for ten minutes. The extract was centrifuged at 3,000 rpm for five minutes and the MTBE (supernatant) was transferred to a clean tube and re-extracted with 200 μ l of 0.1 M dipotassium hydrogen phosphate by mixing on a rotating mixer for five minutes. The extract was then centrifuged at 3,000 rpm for five minutes. The supernatant solvent was removed and discarded

while the aqueous layer was transferred to an autosampler vial. 50 μ l of the aqueous layer was injected in the high performance liquid chromatography assay system. The ratio of the area of the salicylic acid peak to the O-Anisic acid peak, from the detector printout, was proportional to the amount of salicylic acid present in the tested sample.

Reagents

Column; (Spherisorb S5, ODS2, 12.5 cm, Hichrom Chromatography, Reading, UK).

Mobile phase; (Acetic acid:sodium acetate [AnalaR grade]:water:methanol [HPLC grade] 4ml:16gm:800ml:200 ml).

AnalaR grade reagents; (Merck [BDH] Ltd, Poole, Dorset, UK).

HPLC grade reagents; (Rathburn Chemicals Ltd, Walkerburn, UK).

Pump; (Shimadzu LC6A, flow rate 1 ml/minute, Dyson Instruments Ltd, Tyne and Wear, UK).

Detector; (Jasco UVIDEC-100-VI spectrophotometer set at 300 nm, Biotech Instruments Ltd, Hertford, UK).

Injector; (Perkin Elmer ISS-100 auto sampler, Perkin Elmer, Beconfield, UK).

2.26 Salicyluric acid

The thin layer chromatography (TLC) assay for salicyluric acid was established and standardised in the Analytical Unit of St George's Hospital Medical School. One ml of sample urine was mixed with 25% of hydrochloric acid. These were then extracted with 4 ml of MTBE by mixing on a rotating mixer for 10 minutes. The extract was then centrifuged at

3,000 rpm for five minutes. The solvent was transferred to a clean tube containing 500 mg anhydrous sodium sulphate and mixed for five minutes. The extract was then centrifuged at 3,000 rpm for five minutes. The solvent layer was transferred to a clean tube and evaporated to dryness using the Savant SC200 SpeeVac[®] concentrator (Life Sciences International, Dunstable, UK). This was used in combination with an RT4104 refrigerated condensation trap (Life Sciences International operated at -108°C to trap the vapours of the organic solvent during vacuum evaporation). The extract was reconstituted in 20 µl of MTBE and streaked onto a TLC glass plate next to the standard (pure salicylic acid in MTBE) and the TLC plates were sprayed with Trinder's reagent. The TLC plate was visually inspected for the presence or absence of a spot the same colour and corresponding to the same position as the standard salicyluric acid.

Reagents

Solvent; (Toluene:Ethyl acetate : Glacial acetic acid (5:4:1], AnalaR grade, Merck [BDH] Ltd, Poole, UK).

TLC plates; (Silica Gel 60, F254, 0.25 mm thickness, Merck [BDH] Ltd, Poole, UK).

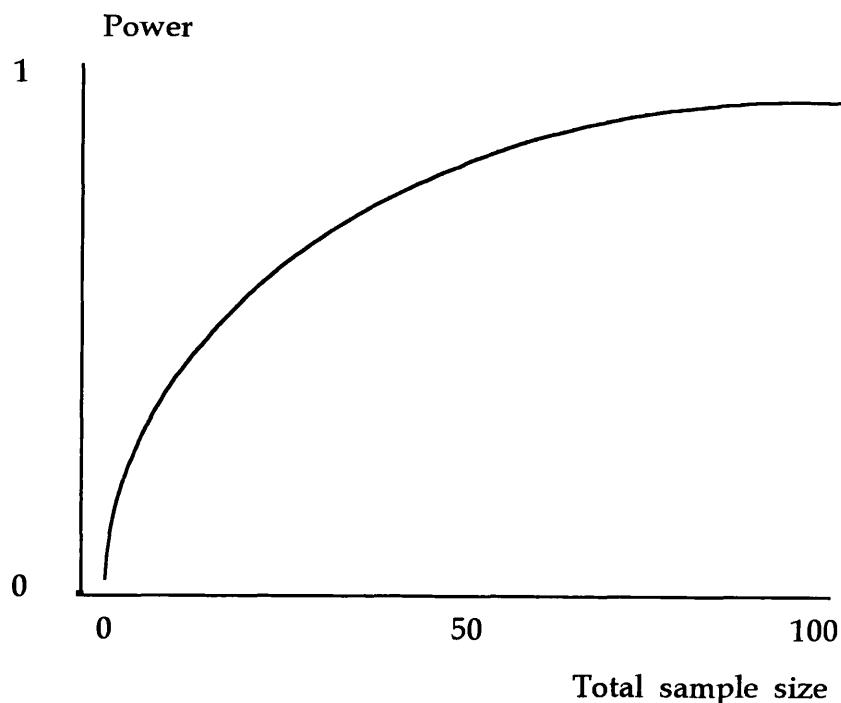
Location reagent; (Trinder's reagent: 4 g mercuric chloride, 4 g ferric nitrate, 12 ml hydrochloric acid diluted to 100 ml with water), (Merck [BDH] Ltd).

2.27 Statistical methods

In addition to the statistical advice from Dr Martin Bland and Dr Steven Gallivan I relied heavily on the textbook by Bland (1989). In estimating the sample size several factors were taken into account but blood loss was considered to be the most important. Assuming a standard deviation of 500 ml (from previous studies) and a clinically significant difference of 450 ml of blood the estimated sample size was 26 patients per treatment group (figure 2.8). Such a study would have a power of 0.9 and a 0.05 level of two sided significance. As illustrated in figure 2.8 increasing the sample size beyond 60 does not increase the power of the study to a great extent (Altman 1980). Similar power calculations on haematological assays revealed sample sizes 15 and 30 per group. For clinical outcome such as resternotomy, myocardial infarction, death etc. sample sizes in excess of 100 would be required. For these reasons as well as for logistical reasons we decided to study 60 patients in the aprotinin trial and 100 patients in the aspirin trial (discussion in section 2.27 critique of methods).

In the aspirin trial 100 patients who met the entry criteria were randomised by minimisation (minim software, Professor Steven Evans, The Royal London Hospital) according to the method documented by Pocock (1983). The factors used for minimisation were; age, gender, surgeon, number of vessels involved and left ventricular function.

A



B

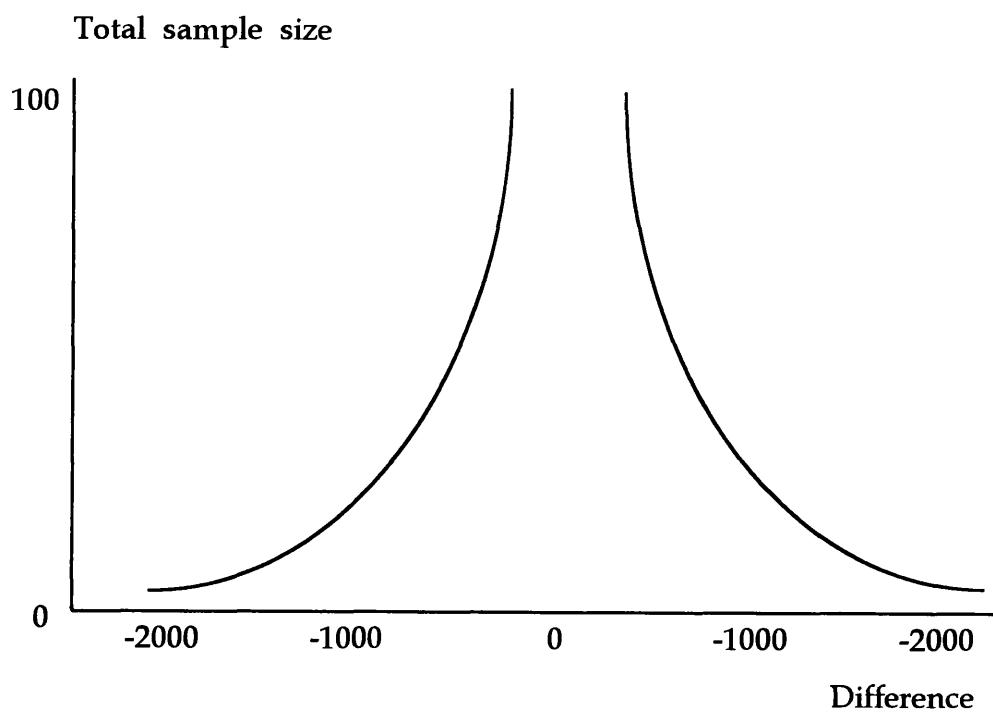


Figure 2.8A Plot of power of the study against sample size (SD=500, difference=450, $\alpha=0.05$ and $n_1/n_2=1$).

Figure 2.8B Total sample size plotted against difference (SD=500, $\alpha=0.05$, power=0.9 and $n_1/n_2=1$).

In the aprotinin trial 60 patients met the entry criteria for blood loss and were randomised by minimisation. The factors used for minimisation were age, gender, CPB time, type of operation, repeat operation and the use of autotransfusion.

The data were stored in microsoft excel for windows (version 4.0) and the statistical analyses were performed using excel, clinstat and the statistical package for social sciences (SPSS) for windows (version 6.0). The results are expressed as the mean \pm the 95% confidence interval when normally distributed (Gardner and Altman, 1990) and as the median with the interquartile range when not normally distributed (Altman and Bland 1994). Summary statistics in this thesis are described as "box and whiskers plots" (figure 2.9) produced in the statistical software SPSS and described by Wilson (1993).

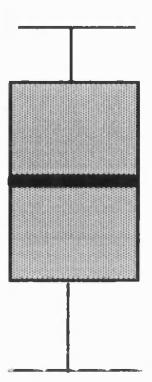
Statistical comparisons were made using the student's t-test for normally distributed data and the Mann-Whitney U test (MWU) and the Wilcoxon matched-pairs test for non-parametric data. The difference was considered statistically significant when p was < 0.05 .

2.28 Critique of methods

The critique of each of the laboratory methods is included, for convenience in each of the sections of this chapter. In this section the critique of the methods of trial design and randomisation by minimisation are included.

- * Values > 3 box-lengths from 75th percentile (extremes).
- Values > 1.5 box-lengths from 75th percentile (outliers).

Largest observed value that isn't outlier.



75th percentile.

Median.

25th percentile.

Smallest observed value that isn't outlier.

- Values > 1.5 box-lengths from 25th percentile (outliers).

- * Values > 3 box-lengths from 25th percentile (extremes).

Figure 2.9 Definitions of the box and whiskers plot used in this thesis.

During our discussions of study design we considered the relative importance of the two quite different objectives in clinical research. The first is a clinical trial to investigate the relative efficacy of one treatment versus another. The second is a study to investigate mechanisms of action. In clinical trials, where the main aim is to investigate efficacy of treatment as judged by the occurrence of discrete clinical outcome, much greater numbers may be required for the trial to have enough power to detect a difference. In studies of investigation of mechanisms of action smaller sample sizes are required in order to detect a difference, but the individual subjects may be studied more thoroughly in the search for explicative rather than clinically important end points. Furthermore, in a clinical trial it is important to include a reasonably wide range of cases to ensure that any findings are applicable to as full a range as possible of patients who might present with the disease in question. Studies of mechanisms are easier to conduct if as many variables as possible are excluded. Thus clinical trials and studies of mechanisms have different requirements which should be recognised when one is trying to do both at the same time.

We decided to study 100 patients in the aspirin trial which would have enough power to detect both a clinical end point and enough detail to explain mechanisms. In the aprotinin trial we set out to investigate 60 patients which would have enough power to detect differences in blood loss and mechanisms of action but not necessarily enough power to detect a difference in clinical outcome. There were many

logistical difficulties in recruiting more patients in the aprotinin trial as these patients were studied late at night and the tests performed in the middle of the night. In addition it could have taken another year to recruit another 40 patients who had met the entry criteria.

The minimisation method obviates the need for extensive overstratification, which is a considerable problem in small clinical trials, and ensures that the groups to be compared are matched for the most important patient characteristics. This method balances the "marginal treatment totals" for each level of each patient factor used for minimisation. For example once the first 30 patients have been allocated each of these patients appears five times in the table, once for each factor used for minimisation. Before the thirty first patient is allocated treatment the programme derives the "marginal treatment total" by adding together, for each treatment, the number of patients in the corresponding five rows of the table (factors used for minimisation). The minimisation programme allocates the next patient to the treatment with the smallest such sum of "marginal totals". If the "marginal total" for the two treatments were equal then the programme uses simple randomisation to assign treatment. In general minimisation is of greatest value in relatively small trials of less than 100 patients where several factors are known to be of prognostic importance.

Chapter 3

Results

Clinical endpoints of the aspirin trial

3.1 Introduction

The demographic characteristics of the patients in the aspirin and placebo groups were similar (table 3.1). The average age of the patients was 62 years and the average height and weight were similar. In addition the two groups were similar in CPB time, number of bypass grafts, number of internal mammary arteries (IMA) used, method of myocardial protection (systemic cooling) and operating surgeon. The immaculate matching of the two groups is a reflection of the study design and the randomisation by minimisation.

Table 3.1 Demographic characteristics of the patients.

Variable	Aspirin	Placebo
Male / Female	41/9	40/10
Mean age (years)	62	62
Mean height (cm)	169	170
Mean weight (kg)	76	75
Mean bypass time (min)	82	81
Median number of grafts	3.5	3.5
Median number of IMA	1	1

The serum and urinary salicylate analysis revealed that the compliance was 49 out of 50 patients in the placebo group,

in other words one patient had taken an aspirin containing medication despite our instructions. The compliance was 50 out 50 in the aspirin group. All the results were analysed on an intention to treat basis.

3.2 Post-operative bleeding

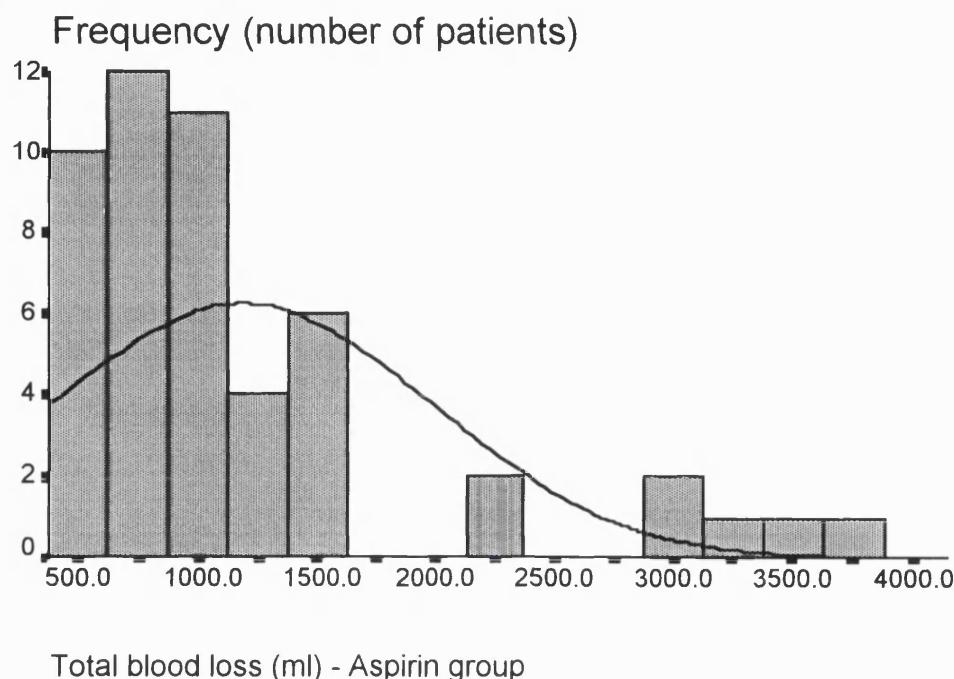
The tests of normality for the blood loss data are shown in figures 3.1 and 3.2. These data did not conform to a Gaussian distribution so they were analysed with non parametric methods (median, interquartile range and the two tailed Mann Whitney U t-test, table 3.2 and figure 3.3).

Table 3.2 Blood loss in theatre and the intensive care unit.

Median (ml)	aspirin	placebo	p value
Theatre	423	310	0.04
Interquartile range	280-581	224-450	
First four hours	373	300	0.003
Interquartile range	258-613	219-363	
Total	980	710	0.01
Interquartile range	649-1381	590-939	

The blood loss in theatre, from the time of protamine administration to the time of sternal closure, was significantly higher in the aspirin group as compared to the placebo group. In addition the blood loss in the first four post-operative hours was significantly higher in the aspirin group. The total blood loss, from the time of chest closure to the time of removal of the chest drains, was also significantly higher in the aspirin group.

A



B

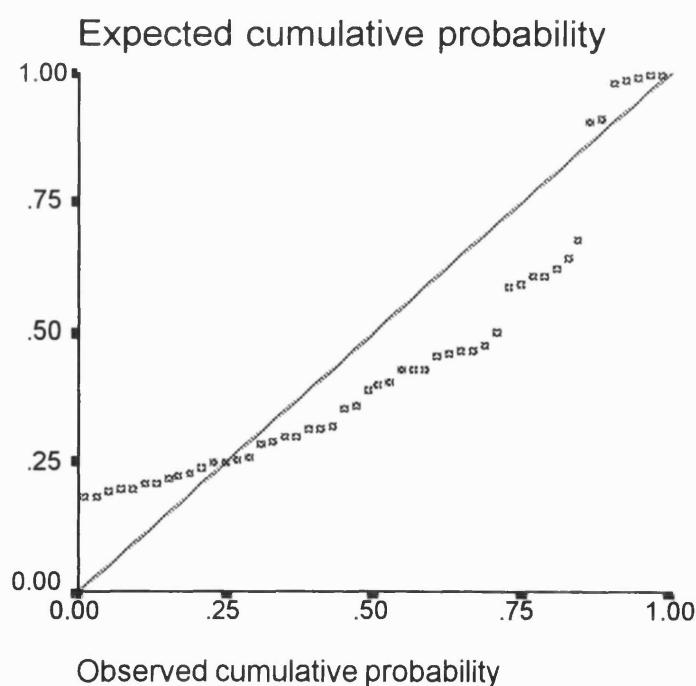


Figure 3.1A Histogram of the total post-operative blood loss (aspirin group) with a superimposed normal curve.

Figure 3.1B Normal plot of the same data demonstrating deviation from a straight line (hence non normal data).

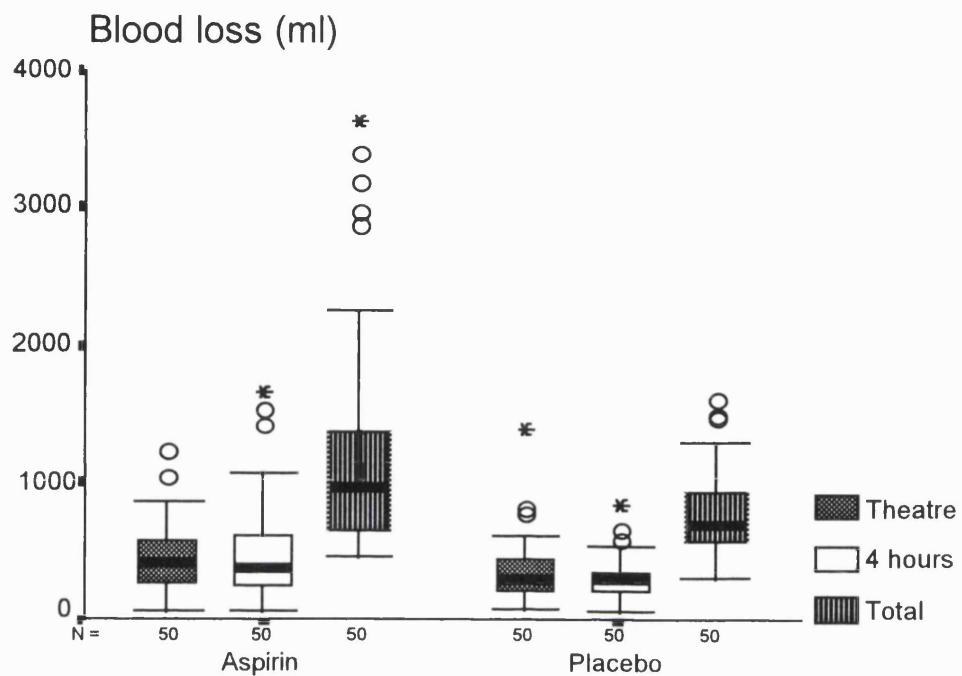


Figure 3.2 Box and whiskers plots for the operative, first four hours and the total post-operative blood loss of the patients in the aspirin and placebo groups (as described in the statistical methods, section 2.27).

The method of myocardial protection did not influence the volume of the post-operative blood loss. In the placebo group the median blood loss was 720 ml (625-860) for the cardioplegia group and 700 ml (585-1035) for the intermittent aortic cross clamping group ($p=0.9$, two tailed Mann Whitney U t-test). The respective median blood loss in the aspirin group was 785 ml (631-1378) and 1050 ml (656-1381) ($p=0.5$, two tailed Mann Whitney U test).

The median time between the administration of protamine and the approximation of the sternum, as an indication of the difficulty in achieving satisfactory haemostasis, was slightly longer in the aspirin group 32 minutes (22-40) as compared to the placebo group 25 minutes (19-37). This difference, however, did not reach statistical significance ($P=0.2$, two tailed Mann Whitney U test).

The impression of the operating surgeon whether the patient was on aspirin or placebo was also noted at the end of the procedure. For the aspirin group the surgeon was correct in 42%, of the cases wrong in 48% and not certain in 10% of the cases. The corresponding figures for the placebo group were 60%, 30% and 10%. Overall therefore the surgeon had a 51% chance of being correct, 39% chance being wrong and did not know in 10% of the cases.

3.3 Coagulation screen

The coagulation screens were similar in both groups both pre-operatively as well as post-operatively (table 3.3).

Table 3.3 Coagulation screens (seconds).

Median (interquartile range)	aspirin	placebo
TT preop	13 (12-14)	12 (12-14)
TT postop	15 (12-21)	17 (13-25)
PT preop	13 (12-14)	13 (12-14)
PT postop	16 (14-20)	17 (15-20)
KPTT preop	45 (39-48)	42 (40-47)
KPTT postop	52 (46-59)	54 (49-63)

3.4 Transfusion requirements

The patients in the aspirin group were transfused on average one unit of blood more than the patients in the placebo group ($p=0.02$, two tailed Mann Whitney U test, table 3.4). The volume of autotransfused blood was significantly higher in the aspirin group but the transfusion of colloid did not differ between the two groups (two tailed Mann Whitney U test table 3.4).

Table 3.4 Transfusion requirements.

Variable (Median)	aspirin	placebo	p value
Blood transfused (U)	3	2	0.02
Interquartile range	2-4	1-3	
Autotransfusion (ml)	445	320	0.01
Interquartile range	298-650	230-418	
Colloid transfused (ml)	1500	1500	0.1
Interquartile range	1500-2000	1000-1500	
FFP (number of patients)	8	0	0.01
Platelets (patients)	5	0	0.05
Cryoprecipitate (patients)	4	0	0.05
Re-sternotomy (patients)	4	0	0.05

Another striking result was the fact that no patients in the placebo group required blood products whereas 8 patients in the aspirin group required a total of 20 units of FFP ($p=0.01$, Fisher's exact test, table 3.4). Five patients in the aspirin group required a total of 30 units of platelets as compared to none in the placebo group ($p=0.05$, Fisher's exact test). Furthermore four patients in the aspirin group received cryoprecipitate and none in the placebo group ($p=0.05$, Fisher's exact test).

3.5 Pre-operative and discharge Hb

The haemoglobin data were normally distributed and therefore were analysed with parametric methods (mean \pm 95% confidence interval and the two tailed unpaired t-test).

The patients in the two groups had similar Hb levels prior to surgery (14 ± 0.4 g/dl versus 14.3 ± 0.4 g/dl, $p=0.3$, two tailed unpaired t-test). The aspirin patients, however, had significantly lower Hb levels on discharge from the hospital (10.8 ± 0.3 g/dl) as compared to the patients in the placebo group (11.5 ± 0.3 g/dl, $p=0.001$, two tailed unpaired t-test).

3.6 Re-exploration for excessive bleeding

Four patients in the aspirin group required re-sternotomy for excessive bleeding as defined in the standard guidelines for re-exploration. In the placebo group, however, no patients required re-exploration for excessive bleeding ($p=0.05$, Fisher's exact test).

The first patient was bleeding from two internal mammary artery side branches and the bleeding was classified as "surgical". The coagulation screen of the patient was normal and the platelet count was 120×10^9 platelets/l. The platelet surface GPIb expression had decreased from 55 arbitrary units pre-bypass to 25 arbitrary units post-bypass (55% reduction as compared to the average reduction of 30%).

The second patient was bleeding from a distal anastomosis and the bleeding was classified as "surgical". The coagulation screen was deranged and the platelet count was 74×10^9 platelets/l and both were corrected according to the standard guidelines. The platelet surface GPIb expression had decreased from 46 to 25 arbitrary units, a 46% reduction.

The third patient had diffuse bleeding and the cause of bleeding was classified as due to "haemostatic dysfunction". The coagulation screen was normal and the platelet count was 103×10^9 platelets/l. The platelet GPIb surface expression had decreased from 48 to 25 arbitrary units, a reduction of 48%.

The fourth patient was bleeding from a proximal anastomosis and the bleeding was therefore classified as "surgical". The coagulation screen and the platelet count were normal. The platelet surface expression of GPIb was not estimated.

3.7 Clinical outcome

During the two weeks of discontinuation of pre-operative aspirin and the ingestion of either aspirin or placebo, none of the 100 patients had any significant episodes of unstable angina or any other complications of note. The median intensive care stay was one day for the patients in both groups ($p>0.05$, two tailed Mann Whitney U test). The median hospital stay was seven days for the patients in both groups ($p>0.05$, two tailed Mann Whitney U test). All the patients survived to be discharged home and the only significant complication was the use of intra-aortic balloon counterpulsation in one patient, in the aspirin group, who subsequently made a good recovery. There were no significant pericardial effusions requiring drainage. Three patients in each group had ECG changes of peri-operative myocardial infarction.

3.8 Principal conclusions

1. Discontinuation of aspirin therapy two weeks pre-operatively, in patients scheduled to undergo elective CABG, did not have an adverse effect on the pre-operative, operative and early post-operative clinical outcome.
2. Continuation of pre-operative aspirin therapy up to the time of surgery, however, significantly increased operative and post-operative bleeding, transfusion of homologous blood and blood products and the risk of re-exploration for excessive bleeding.
3. The surgeon could not reliably predict which patients were taking aspirin or placebo pre-operatively.

Chapter 4

Results

The effect of aspirin and CPB on platelet function

4.1 Introduction

The clinical data presented in chapter three were collected from all 100 patients studied. The data in this chapter were collected from a subgroup of 71 patients, 35 from the aspirin group and 36 from the placebo group. The patients in this subgroup were also randomised by minimisation and were similar in their demographic characteristics as described in section 3.1. As these data did not conform to a normal distribution they were analysed using non parametric methods. The descriptive statistics used in this chapter are the median and the interquartile range and illustrated as box and whiskers plots (section 2.27). The data were analysed in two ways in order to elucidate both **the effect of pre-operative aspirin therapy** and **the effect of CPB on post-operative platelet function**.

The pre-operative aspirin therapy effect

Each pre-operative variable in the aspirin group was compared with the same pre-operative variable in the placebo group using the two tailed Mann Whitney U test (MWU). Similarly each post-operative variable in the aspirin group was compared with the same post-operative variable in the placebo group using the two tailed Mann Whitney U test.

The effect of CPB

The same data were analysed as matched pairs in order to elucidate the influence of CPB on the platelet function. Each pre-operative variable was compared to its equivalent post-operative variable, using the two tailed Wilcoxon matched pairs test, separately for the aspirin group as well as the placebo group. All the data affected by haemodilution were corrected, by using the change in haematocrit between the two specimens, before statistical analyses (Orchard, Goodchild, Prentice et al 1993).

4.2 Platelet count

Aspirin did not affect the platelet count pre-operatively, post-operatively (table 4.1) or at the time of discharge from the hospital (platelet count for the aspirin group was 252×10^9 (215-315) platelets/l and for the placebo group 259×10^9 (210-294) platelets/l, (p=0.5).

There was a highly significant decrease in the platelet count, following CPB, in both groups (table 4.1).

Table 4.1 Platelet count ($\times 10^9$ platelets/l).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	252	235	0.1
Interquartile range	216-307	210-285	
Post-operative	113	114	0.8
Interquartile range	84-147	95-127	
p (Wilcoxon)	0.001	0.001	

4.3 Platelet size distribution

Aspirin did not influence the mean platelet volume (MPV) (table 4.2), the platelet distribution width (PDW) (table 4.3) or the plateletcrit (Pct). The MPV decreased significantly (table 4.2) and the PDW increased significantly (table 4.3), following bypass, in both groups.

Table 4.2 Mean platelet volume (fl).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	7.4	7.5	0.3
Interquartile range	6.7-7.8	7.0-8.0	
Post-operative	7.0	7.1	0.9
Interquartile range	6.8-7.9	6.7-7.8	
p (Wilcoxon)	0.001	0.01	

Table 4.3 Platelet distribution width (arbitrary units).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	17.7	17.6	0.8
Interquartile range	17.2-18.2	17.2-18.0	
Post-operative	18.0	18.0	0.9
Interquartile range	17.4-18.8	17.7-18.3	
p (Wilcoxon)	0.001	0.003	

4.4 Bleeding time

The patients in the aspirin group had significantly longer bleeding times pre-operatively [6 (5.3-8) minutes] than the patients in the placebo group [5 (5-6) minutes, p=0.005, figure 4.1]. The patients in the aspirin group also had

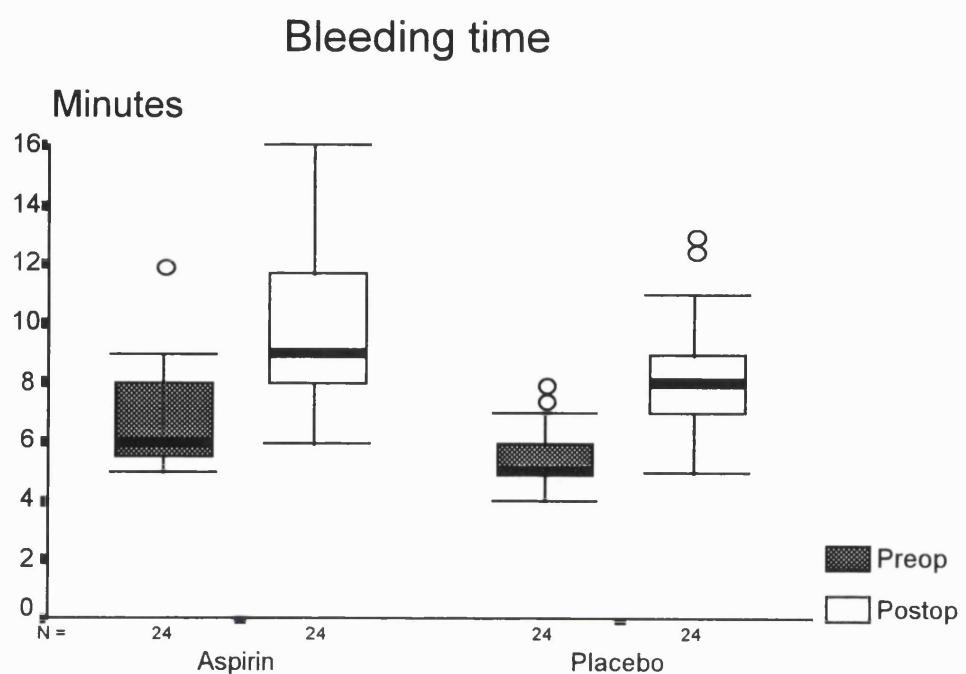


Figure 4.1 Box and whiskers plot of the bleeding times of the patients in the two groups pre and post-operatively.

significantly longer bleeding times post-operatively [9 (8-11.9) minutes] as compared to the patients in the placebo group [8 (7-9) minutes, $p=0.05$]. **Cardiopulmonary bypass** prolonged significantly the bleeding time in both groups of patients ($p<0.001$, figure 4.1).

4.5 Platelet aggregation

The pre-operative maximum platelet aggregation, in response to collagen (10 μ l of 200 mg/ml) as the agonist, was significantly lower in the **aspirin** group as compared to the placebo group (median 37% versus 77%, $p<0.001$, figure 4.2A). This difference in the platelet responsiveness to the collagen stimulus was also reflected in the maximum slope of the aggregation trace. The median slope of the trace for the aspirin group was 13 (9-19.5) $^{\circ}$ whereas the median slope for the placebo group was 33 (19.5-41) $^{\circ}$ ($p<0.001$). This sluggish platelet aggregatory response of the aspirin group, however, was not reflected in the reaction interval of the aggregation response. The median reaction time for the aspirin group was 48 (28-55) seconds and the median reaction time for the placebo group 45 (33-50) seconds ($p=0.1$).

The post-operative platelet aggregation, in response to collagen (10 μ l of 200 mg/ml), was significantly lower in the aspirin group as compared to the placebo group (median 34% versus 68%, $p<0.001$, figure 4.2A). This difference in the platelet responsiveness to the collagen stimulus was also echoed in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was

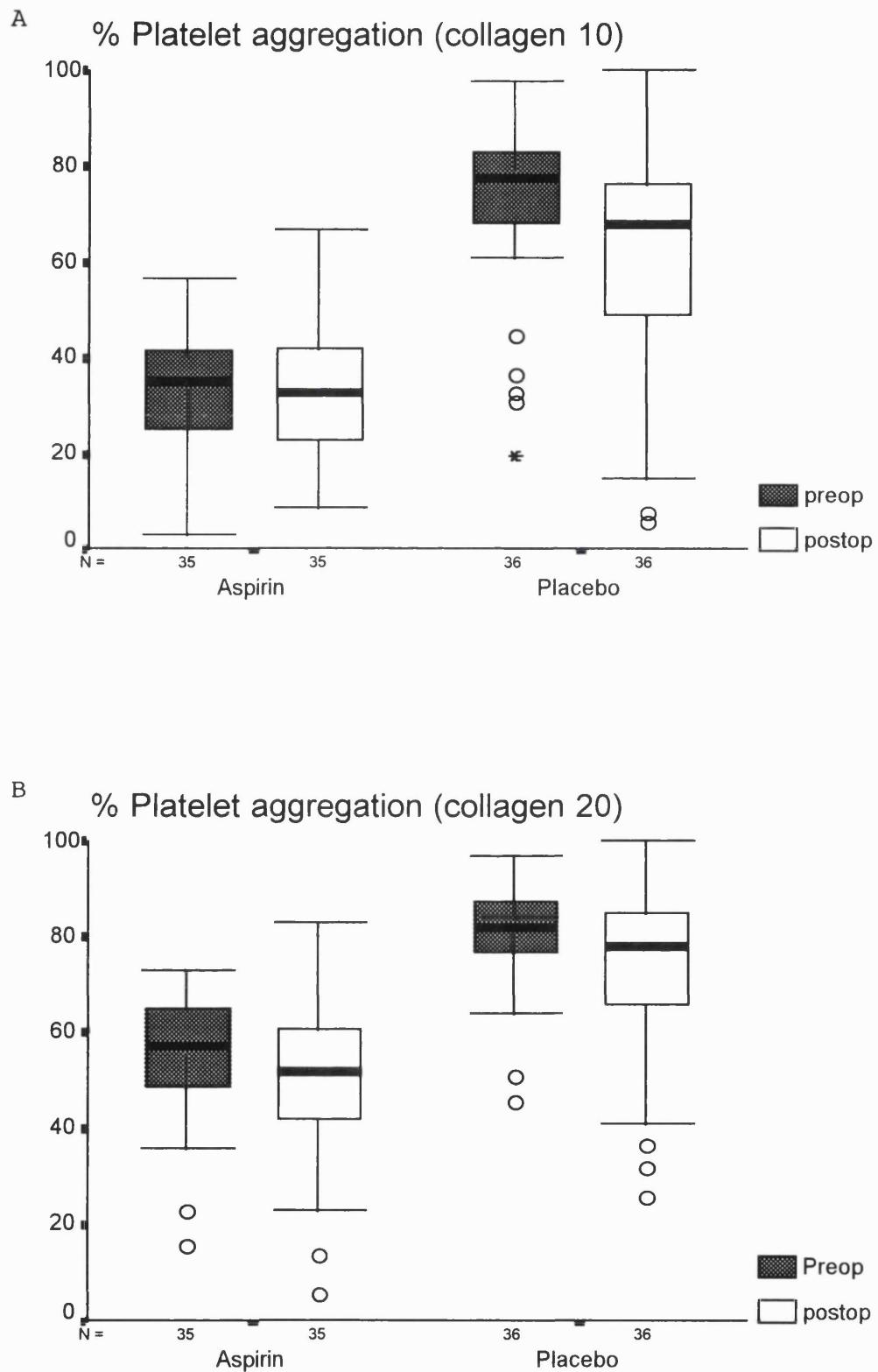


Figure 4.2 Platelet aggregation in response to 10 μ l(A) and 20 μ l(B) of collagen 200 (mg/ml) pre and post-operatively in the aspirin and placebo groups.

9 (4-12)° whereas the slope for the placebo group was 18 (9-27)° ($p<0.001$). This sluggish platelet aggregatory response of the aspirin group, however, was not observed in the reaction interval of the aggregation response. The reaction time for the aspirin group was 60 (26-129) seconds and for the placebo group 55 (33-83) seconds ($p=0.5$).

The platelet aggregation in response to 10 μ l of collagen (figure 4.2A) was reduced by **bypass**, in the placebo group ($p<0.001$), but not in the aspirin group ($p=0.7$) which was relatively low pre-operatively anyway. This diminished response, in the placebo group, was also observed in the slope ($p<0.001$) and the reaction time ($p<0.001$) of the aggregation curve.

The pre-operative platelet aggregation, in response to collagen (20 μ l of 200 mg/ml), was significantly lower in the **aspirin** group as compared to the placebo group (median 57% versus 82%, $p<0.001$, figure 4.2B). This difference in the platelet responsiveness to the collagen stimulus was also echoed in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was 22 (15-28)° whereas the slope for the placebo group was 38 (23-46)° ($p<0.001$). This sluggish platelet aggregatory response of the aspirin group, however, was not observed in the reaction interval of the aggregation response. The reaction time for the aspirin group was 35 (22-50) seconds and for the placebo group 30 (25-38) seconds ($p=0.3$).

The post-operative platelet aggregation, in response to

collagen (20 μ l of 200 mg/ml), was significantly lower in the aspirin group as compared to the placebo group (median 54% versus 80%, $p<0.001$, figure 4.2B). This difference in the platelet responsiveness to the collagen stimulus was also noticed in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was 11 (8-21) $^{\circ}$ whereas the slope for the placebo group was 27 (14-33) $^{\circ}$ ($p<0.001$). This sluggish platelet aggregatory response of the aspirin group, however, was not reflected in the reaction interval of the aggregation response. The reaction time for the aspirin group was 45 (35-64) seconds and for the placebo group 40 (23-65) seconds ($p=0.3$).

The platelet aggregation in response to 20 μ l of collagen (figure 4.2B) was reduced by **bypass** in the placebo group ($p=0.004$) and in the aspirin group ($P=0.05$). This diminished response, in both groups, was also echoed in the slope ($p<0.001$) and the reaction time ($p=0.01$) of the aggregation curve.

The pre-operative platelet aggregation, in response to arachidonic acid (10 μ l of 15 mg/ml) as the agonist, was significantly lower in the **aspirin** group as compared to the placebo group (median 5% versus 77%, $p<0.001$, figure 4.3A). This difference in the platelet responsiveness to the arachidonic acid stimulus was also observed in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was 3 (3-4) $^{\circ}$ whereas the slope for the placebo group was 3 (5-41) $^{\circ}$ ($p<0.001$). This sluggish platelet aggregatory response of the aspirin group was also

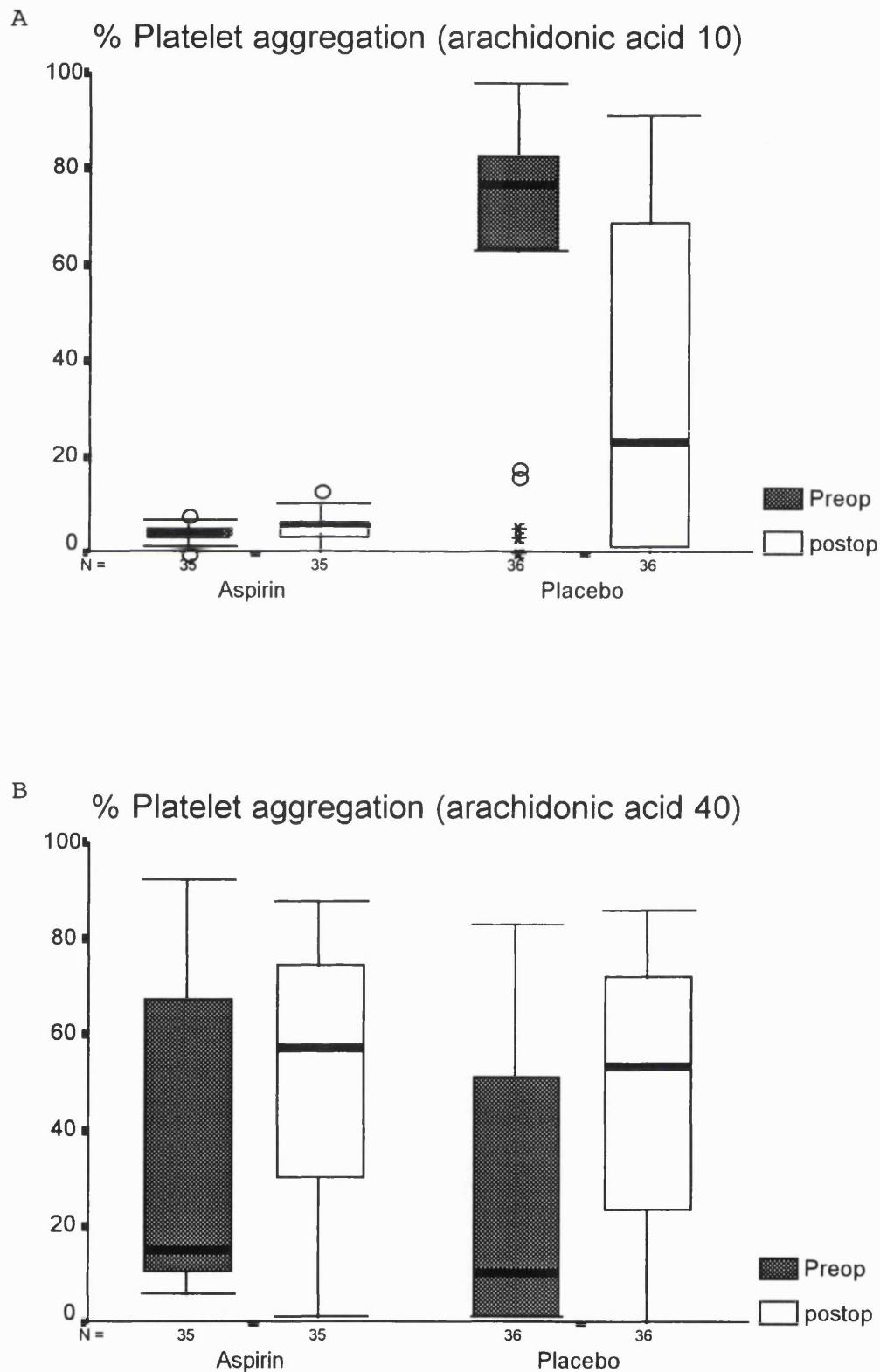


Figure 4.3 Platelet aggregation in response to 10 μ l (A) and 40 μ l (B) of arachidonic acid 15 (mg/ml) pre and post-operatively in the aspirin and placebo groups.

reflected in the reaction interval of the aggregation response. The median reaction time for the aspirin group was longer than 300 seconds because very little aggregation had taken place. As a result it was difficult to record accurately the reaction time for the aspirin group. The reaction interval for the placebo group 25 (10-300) seconds.

The post-operative platelet aggregation, in response to arachidonic acid (10 μ l of 15 mg/ml), was significantly lower in the aspirin group as compared to the placebo group (median 6% versus 23%, $p=0.01$, figure 4.3B). This difference in the platelet responsiveness to the arachidonic acid stimulus was also observed in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was 3 (3-5) $^{\circ}$ whereas the slope for the placebo group was 16 (6-33) $^{\circ}$ $p<0.001$. This sluggish platelet aggregatory response of the aspirin group was also echoed in the reaction interval of the aggregation response. The median reaction time for the aspirin group was longer than 300 seconds and for the placebo group 15 (10-300) seconds.

The platelet aggregation in response to 10 μ l of arachidonic acid (figure 4.3A) was diminished by **bypass**, in the placebo group ($p=0.005$), but not in the aspirin group ($P=0.7$) which was extremely low pre-operatively. This diminished response, in the placebo group, was also reflected in the slope ($p=0.01$) but not the reaction time ($p=0.9$) of the aggregation curve.

The pre and post-operative platelet aggregation, in response

to arachidonic acid ($40\mu\text{l}$ of 15 mg/ml) as the agonist, was not significantly altered by **aspirin** (figure 4.3B).

The platelet aggregation in response to $40\mu\text{l}$ of arachidonic acid (figure 4.3B) was enhanced by **bypass** in both the placebo group ($p<0.001$) and in the aspirin group ($P=0.01$). This increased response was also reflected in the slope ($p<0.001$) and in the reaction time ($p<0.001$) only in the placebo group.

The pre-operative platelet aggregation, in response to ristocetin ($30\mu\text{l}$ of 20 mg/ml) as the agonist, was significantly lower in the **aspirin** group as compared to the placebo group (median 82% versus 90%, $p=0.002$, figure 4.4A).

This difference in the platelet responsiveness to the ristocetin stimulus was also reflected in the maximum slope of the aggregation trace. The slope of the trace for the aspirin group was 37 ($30-48$)° whereas the slope for the placebo group was 43 ($30-50$)° ($p=0.05$). This difference in platelet aggregatory response, however, was not observed in the reaction interval of the aggregation response, as both groups had very fast reaction times (0-2 seconds).

The post-operative platelet aggregation, in response to ristocetin ($30\mu\text{l}$ of 20 mg/ml), did not differ significantly between the two groups (figure 4.4A).

The platelet aggregation in response to $30\mu\text{l}$ of ristocetin (figure 4.4B) was curtailed by **bypass** in the placebo group ($p<0.001$) and in the aspirin group ($P=0.003$).

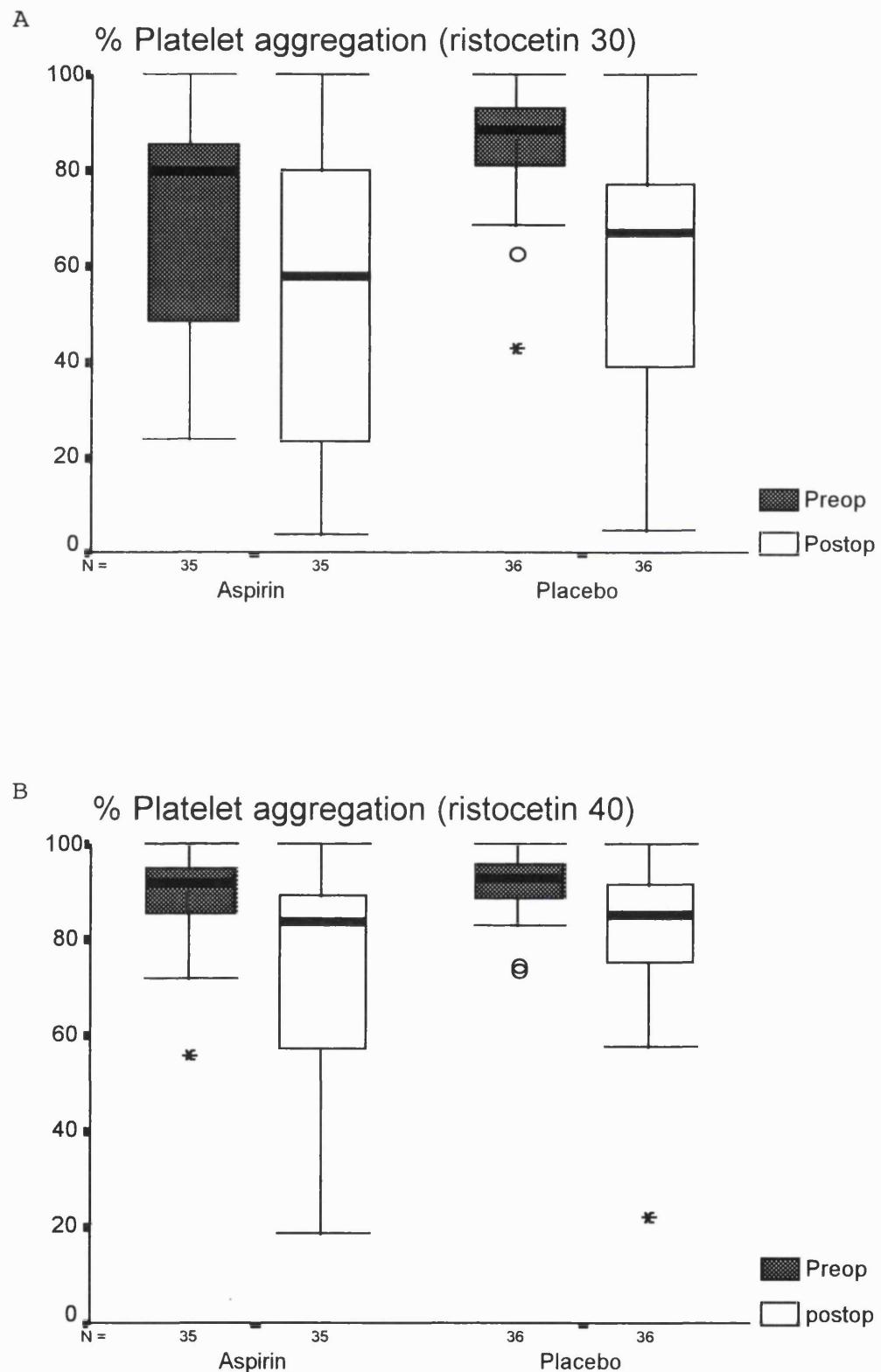


Figure 4.4 Platelet aggregation in response to 30 µl(A) and 40 µl(B) of ristocetin 20 (mg/ml) pre and post-operatively in the aspirin and placebo groups.

This diminished response was also reflected in the slope ($p<0.001$) and the reaction time ($p<0.001$) in both groups. Similar results were obtained when 40 μ l of ristocetin was used as the agonist (figure 4.4B).

The pre and post-operative platelet aggregation, in response to ristocetin (40 μ l of 20 mg/ml), was not altered by **aspirin** or by **bypass** (figure 4.4B). The main observation from the ristocetin aggregation data, however, was that maximum aggregation was greater, the slope was much steeper and the reaction time much shorter than with the other agonists.

4.6 Platelet ATP and ADP content and stimulated release

The total ADP content of the pre-operative platelet rich plasma (PRP) was 14 (10-25) nM/10⁹ platelets for the **aspirin** group and 17 (8-30) nM/10⁹ platelets for the placebo group ($p=0.7$, figure 4.5A). The total ADP content of the post-operative PRP was 21 (10-31) nM/10⁹ platelets for the aspirin group and 17 (1-26) nM/10⁹ platelets for the placebo group ($p=0.2$, figure 4.5A). The total ATP content of the pre-operative PRP was 86 (69-114) nM/10⁹ platelets for the aspirin group and 99 (85-118) nM/10⁹ platelets for the placebo group ($p=0.5$, figure 4.5B). The total ATP content of the post-operative PRP was 81 (70-107) nM/10⁹ platelets for the aspirin group and 100 (92-117) nM/10⁹ platelets for the placebo group ($p=0.5$, figure 4.5B).

The ADP content of PRP (figure 4.5A) was not significantly affected by **bypass** in either the aspirin ($p=0.4$) or the

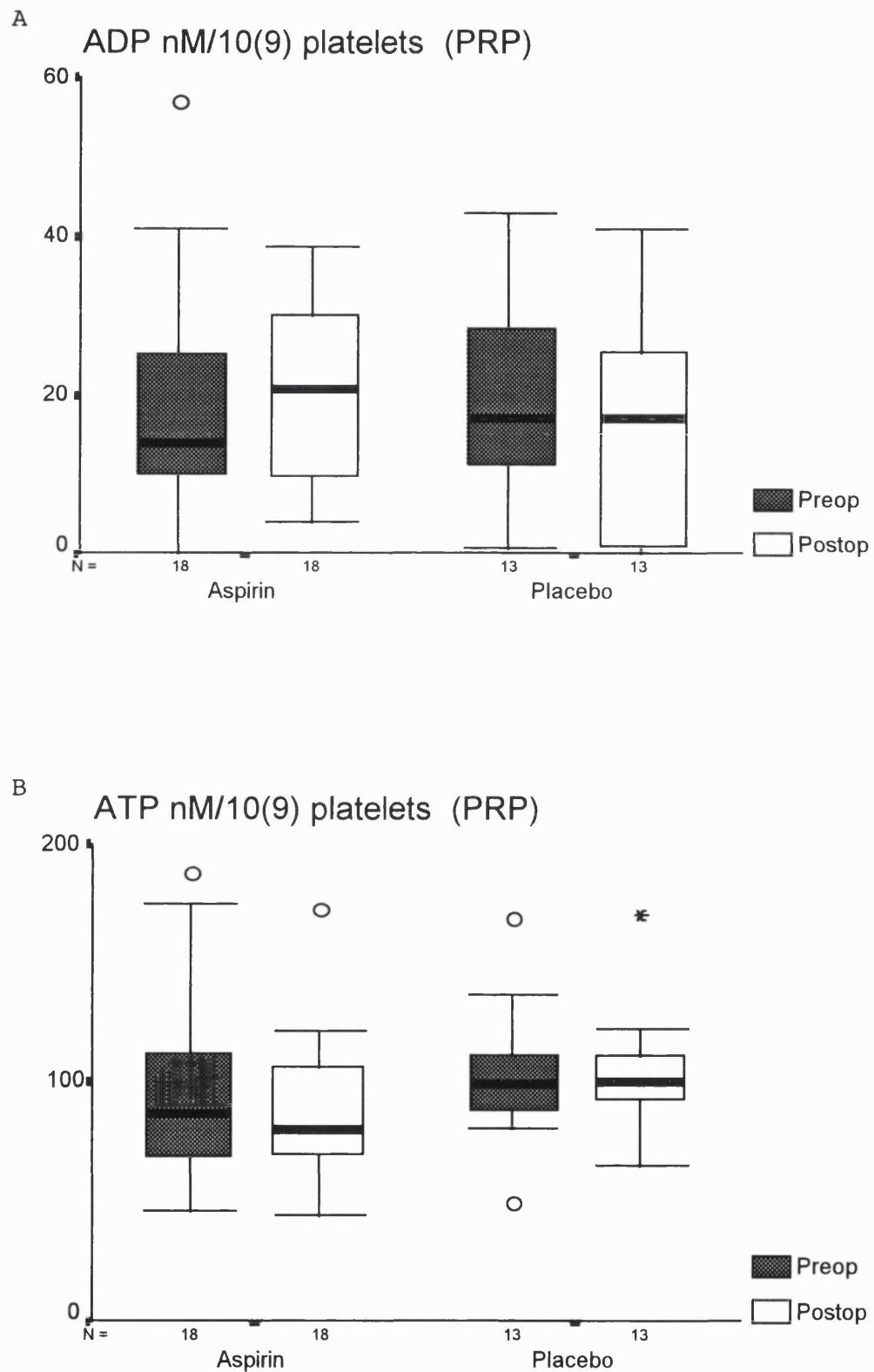


Figure 4.5 Box and whiskers plots of the total ADP and ATP content of platelets ($nM/10^9$ platelets) in both groups.

placebo group ($p=0.5$). Similarly the ATP content of PRP (figure 4.5A) was not altered by bypass in either group ($p=0.5$ and $p=0.2$).

The ADP and ATP content of the platelet poor plasma (PPP), both pre and post-operatively, was not affected by **aspirin** or **bypass** (figure 4.6).

The median quantity of ADP released on stimulation of the pre-operative PRP with collagen (10 μ l of 200 mg/ml) was 5.7 (4.8-8.9) nM/ 10^9 platelets for the **aspirin** group and 16.6 (13.1-22.2) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.7A). The median quantity of ADP released on stimulation of the post-operative PRP with collagen (10 μ l of 200 mg/ml) was 9.7 (7.6-13.7) nM/ 10^9 platelets for the aspirin group and 15.4 (12.2-23.7) nM/ 10^9 platelets for the placebo group ($p=0.003$, figure 4.7A).

The median quantity of ATP released on stimulation of the pre-operative PRP with collagen (10 μ l of 200 mg/ml) was 2.3 (1.9-3.5) nM/ 10^9 platelets for the aspirin group and 10.6 (7.9-15.4) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.7B). The median quantity of ATP released on stimulation of the post-operative PRP with collagen (10 μ l of 200 mg/ml) was 4 (3.5-5.1) nM/ 10^9 platelets for the aspirin group and 9.8 (5.9-12.2) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.7B).

The ADP stimulated release in response to 10 μ l of collagen (figure 4.7A) was not significantly altered by **bypass** in the

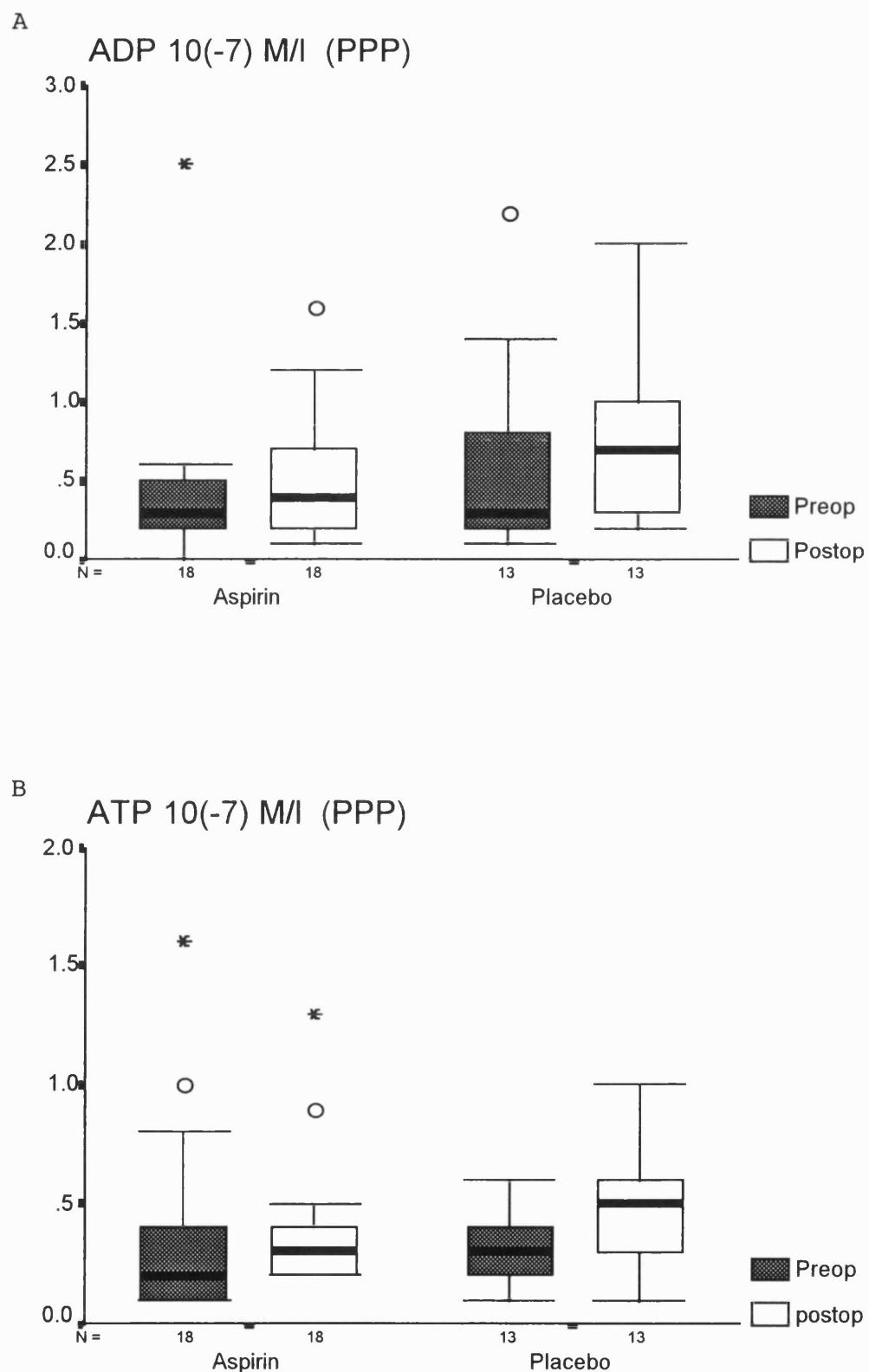


Figure 4.6 Box and whiskers plots of the total ADP and ATP content of platelet poor plasma (10^{-7}M/l) in both groups.

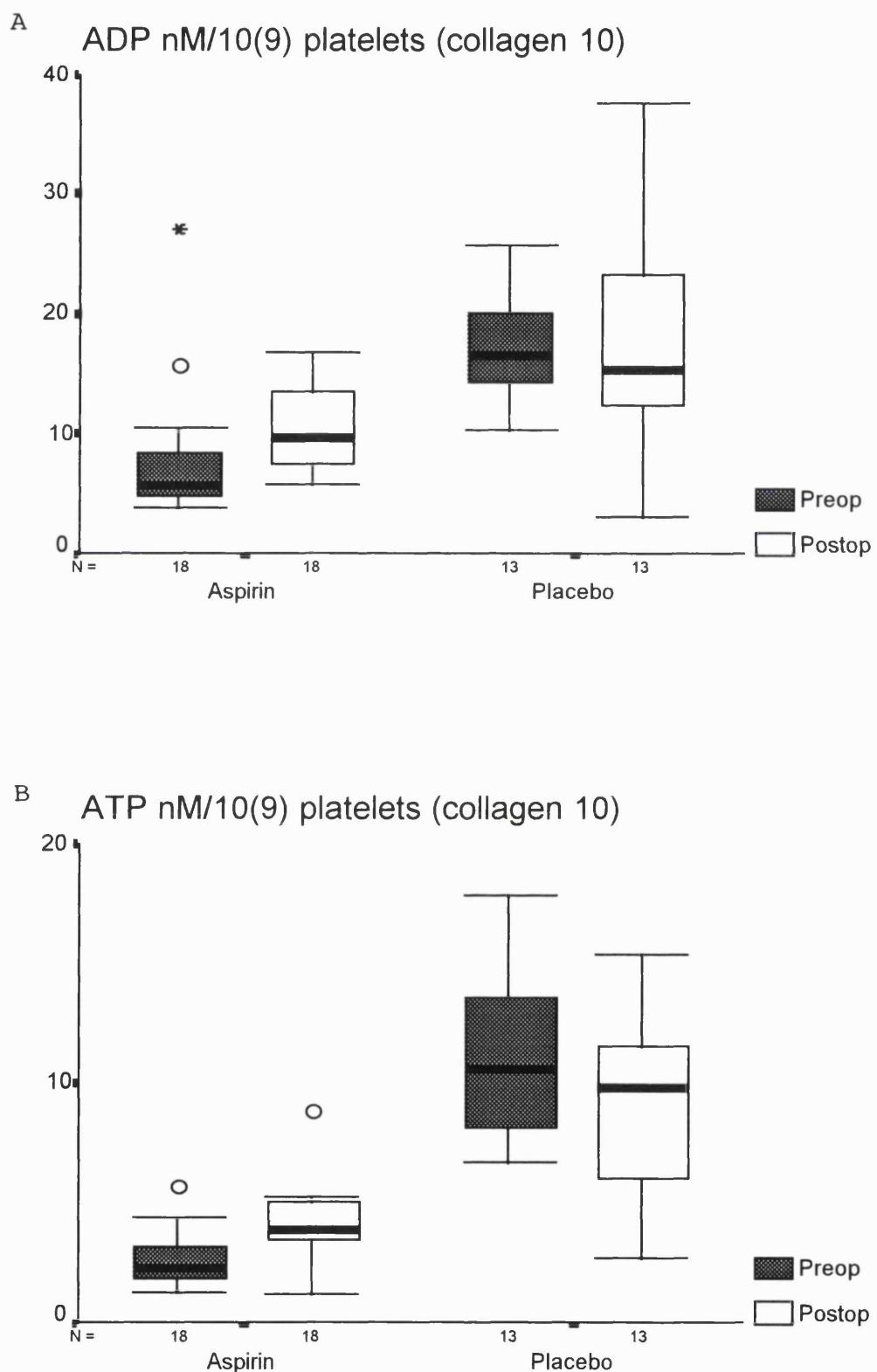


Figure 4.7 Box and whiskers plots of the ADP and ATP release by platelets (nM/10⁹ platelets), in response to collagen stimulation (10 μ l of 200 mg/ml), in both groups.

aspirin ($p=0.1$) or in the placebo group ($p=0.6$). The ATP stimulated release in response to 10 μ l of collagen (figure 4.7B) was increased, following bypass, in the aspirin group ($p=0.002$) but not in the placebo group ($p=0.4$).

The median quantity of ADP released on stimulation of the pre-operative PRP with collagen (20 μ l of 200 mg/ml) was 11.2 (8.2-14.1) nM/ 10^9 platelets for the **aspirin** group and 18.4 (16.1-28.2) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.8A). The median quantity of ADP released on stimulation of the post-operative PRP with collagen (20 μ l of 200 mg/ml) was 13.6 (11.1-17.3) nM/ 10^9 platelets for the aspirin group and 24 (18.2-28.1) nM/ 10^9 platelets for the placebo group ($p=0.003$, figure 4.8A).

The median quantity of ATP released on stimulation of the pre-operative PRP with collagen (20 μ l of 200 mg/ml) was 5.3 (3.9-6.7) nM/ 10^9 platelets for the aspirin group and 12 (10.1-16.2) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.8B). The median quantity of ATP released on stimulation of the post-operative PRP with collagen (20 μ l of 200 mg/ml) was 6.8 (6-8.3) nM/ 10^9 platelets for the aspirin group and 10.6 (9.4-15) nM/ 10^9 platelets for the placebo group ($p<0.001$, figure 4.8B).

The ADP stimulated release in response to 20 μ l of collagen (figure 4.8A) increased, following **bypass**, in the aspirin ($p=0.03$) but not in the placebo group ($p=0.3$). The ATP release (figure 4.8B) did not change, following bypass, in either the aspirin ($p=0.1$) or in the placebo group ($p=0.2$).

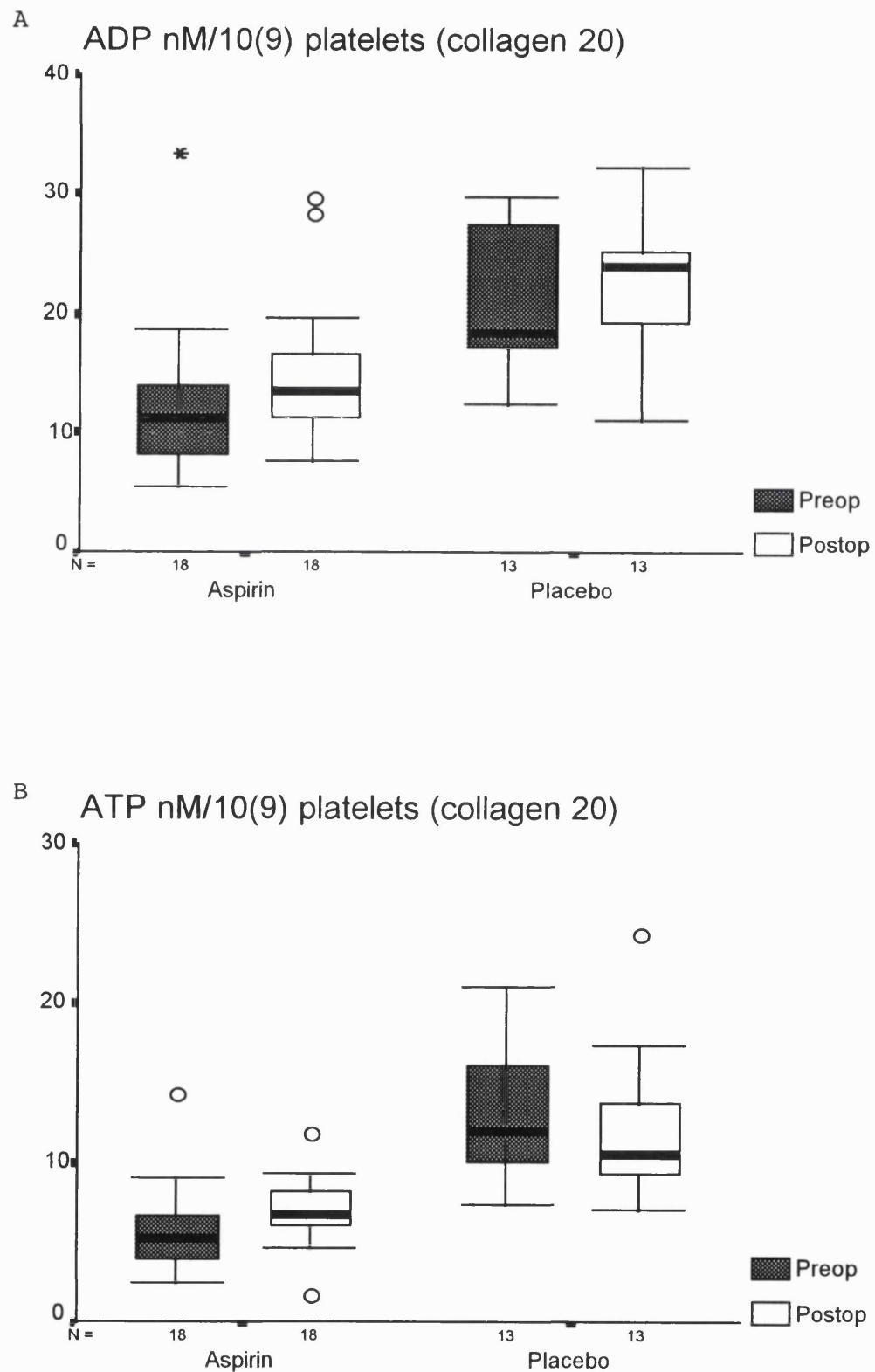


Figure 4.8 Box and whiskers plots of the ADP and ATP release by platelets ($nM/10^9$ platelets), in response to collagen stimulation ($20 \mu l$ of 200 mg/ml), in both groups.

The median quantity of ADP released on stimulation of the pre-operative PRP with arachidonic acid (10 μ l of 15 mg/ml) was significantly lower in the **aspirin** group than the placebo group ($p<0.001$, figure 4.9A). Similarly the median quantity of ADP released on stimulation of the post-operative PRP with arachidonic acid (10 μ l of 15 mg/ml) was significantly lower in the aspirin group than the placebo group ($p=0.003$, figure 4.9A).

The median quantity of ATP released on stimulation of the pre-operative PRP with arachidonic acid (10 μ l of 15 mg/ml) was significantly lower in the aspirin group than the placebo group ($p<0.001$, figure 4.9B). Similarly the median quantity of ATP released on stimulation of the post-operative PRP with arachidonic acid (10 μ l of 15 mg/ml) was significantly lower in the aspirin group than the placebo group ($p=0.02$, figure 4.9B).

The ADP stimulated release in response to 10 μ l of arachidonic acid (figure 4.9) did not change, following **bypass**, in the aspirin ($p=0.1$) but increased significantly in the placebo group ($p=0.02$). The ATP stimulated release in response to 10 μ l of arachidonic acid (figure 4.9) did not change significantly, following bypass, in the aspirin ($p=0.2$) but decreased in the placebo group ($p=0.02$).

The amount of nucleotide (ADP and ATP) released from both the pre and post-operative PRP, when stimulated with 40 μ l of arachidonic acid (15 mg/ml) did not differ significantly between the **aspirin** and the placebo group (figure 4.10).

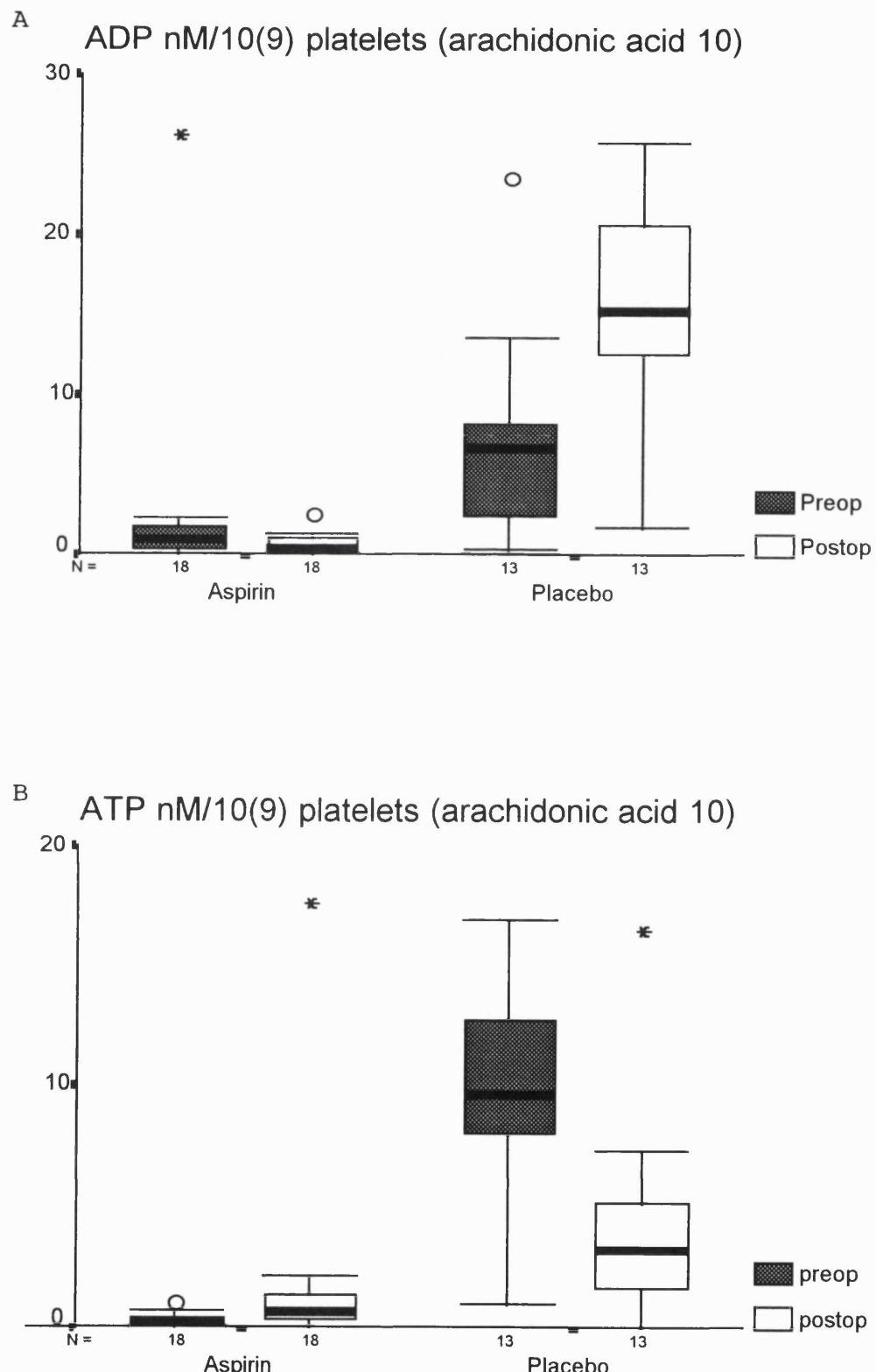


Figure 4.9 Box and whiskers plots of the ADP and ATP release by platelets ($nM/10^9$ platelets), in response to arachidonic acid stimulation ($10 \mu l$ of 15 mg/ml), in both groups.

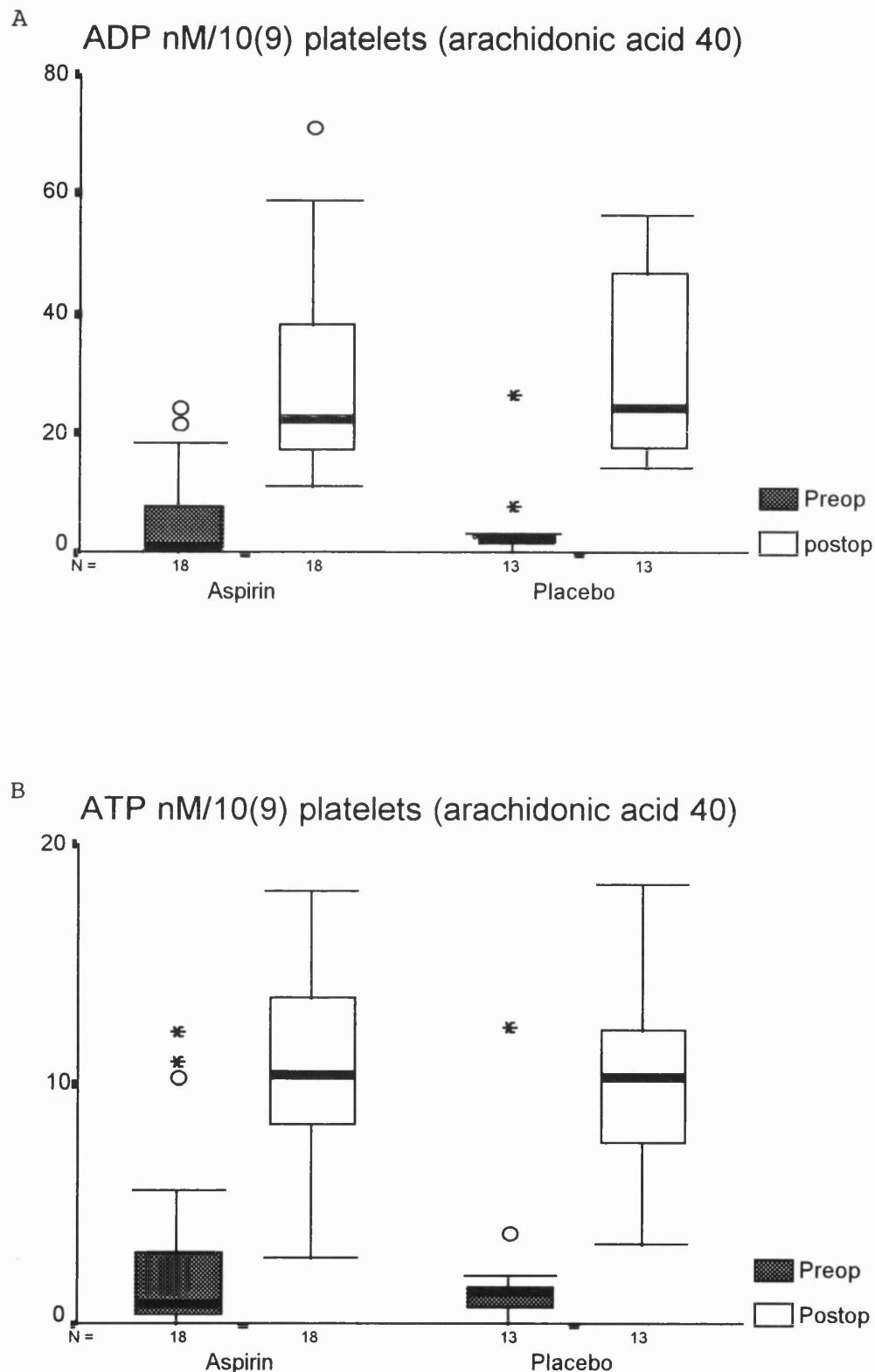


Figure 4.10 Box and whiskers plots of the ADP and ATP release by platelets ($nM/10^9$ platelets), in response to arachidonic acid stimulation ($40 \mu l$ of 15 mg/ml), in both groups.

Both the ADP and ATP stimulated release in response to 40 μ l of arachidonic acid (figure 4.10) were significantly enhanced, following **bypass**, in both groups ($p<0.001$).

The amount of ADP and ATP released from both the pre and post-operative PRP, when stimulated with 30 μ l of ristocetin (20 mg/ml) was significantly less in the **aspirin** group than the placebo group ($p<0.001$, figure 4.11).

The ADP stimulated release in response to 30 μ l of ristocetin (figure 5.11A) did not change, following **bypass**, in the aspirin group ($p=0.9$), as it was very low pre-operatively anyway, but decreased in the placebo group ($p<0.001$). The ATP stimulated release followed the same pattern (figure 5.11B).

Similarly the amount of both nucleotides released from both the pre and post-operative PRP, when stimulated with 40 μ l of ristocetin (20 mg/ml) was significantly less in the **aspirin** group than the placebo group ($p<0.001$, figure 4.12).

Both the ADP and ATP stimulated release in response to 40 μ l of ristocetin (figure 4.12) did not change, following **bypass**, in either group.

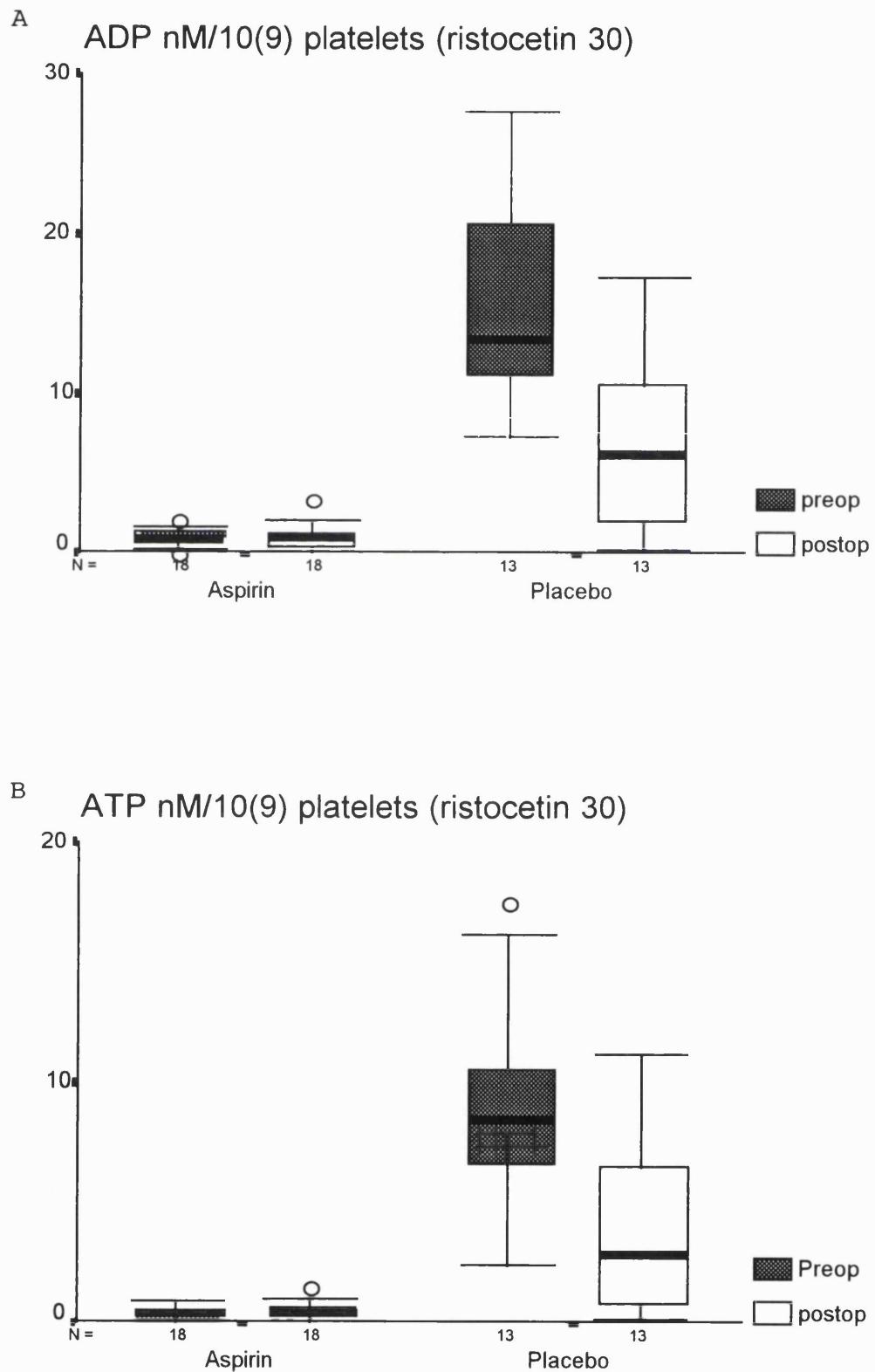


Figure 4.11 Box and whiskers plots of the ADP and ATP release by platelets ($nM/10^9$ platelets), in response to ristocetin stimulation ($30 \mu l$ of 20 mg/ml), in both groups.

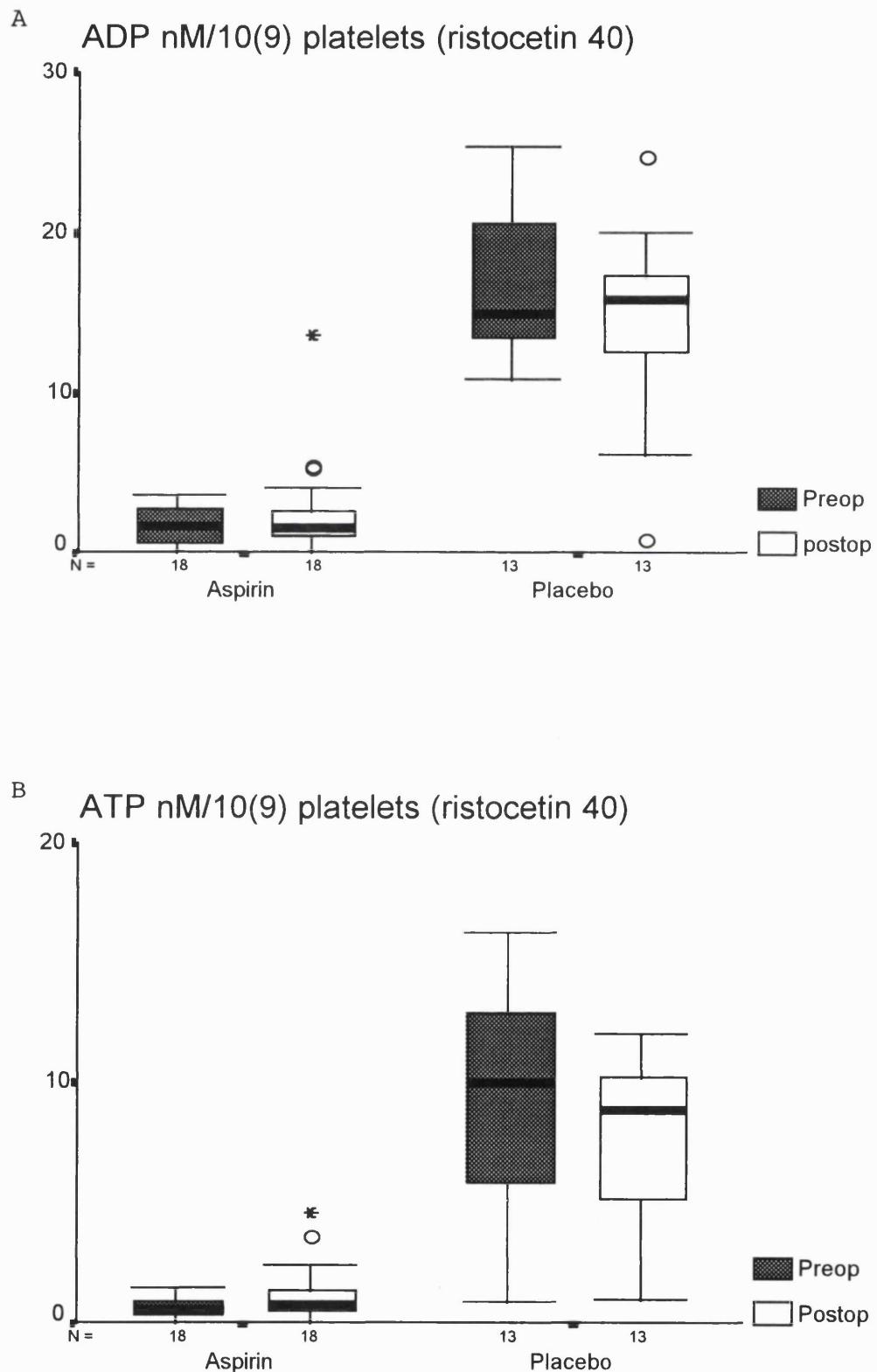


Figure 4.12 Box and whiskers plots of the ADP and ATP release by platelets ($nM/10^9$ platelets), in response to ristocetin stimulation ($40 \mu l$ of 20 mg/ml), in both groups.

4.7 Platelet surface GPIb expression

Aspirin did not influence significantly the platelet GPIb surface expression either pre-operatively ($p=0.4$) or post-operatively ($p=0.4$) (figure 4.13). The median peak fluorescence intensity was 46 (arbitrary units) before bypass and 27 following bypass in the aspirin group ($p<0.001$, figure 4.13). This significant decrease was also observed in the placebo group (42 versus 35, $p<0.001$). These data, from the PRP method of flow cytometry, are also expressed as molecules of equivalent standard fluorochrome (MESF) in table 4.4.

Table 4.4 Platelet GPIb surface expression (MESF) (PRP).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	4,400	4,100	0.4
Interquartile range	3,800-5,300	3,900-4,700	
Post-operative	2,800	3,400	0.4
Interquartile range	2,700-3,500	2,800-3,600	
p (Wilcoxon)	0.001	0.001	

Normal control 3,100 (3,700-4,100), n=32.

The changes in the platelet GPIb surface expression were similar when the whole blood method of flow cytometry was used. These results are depicted in figure 4.14 as fluorescence intensity and in table 4.5 as MESF.

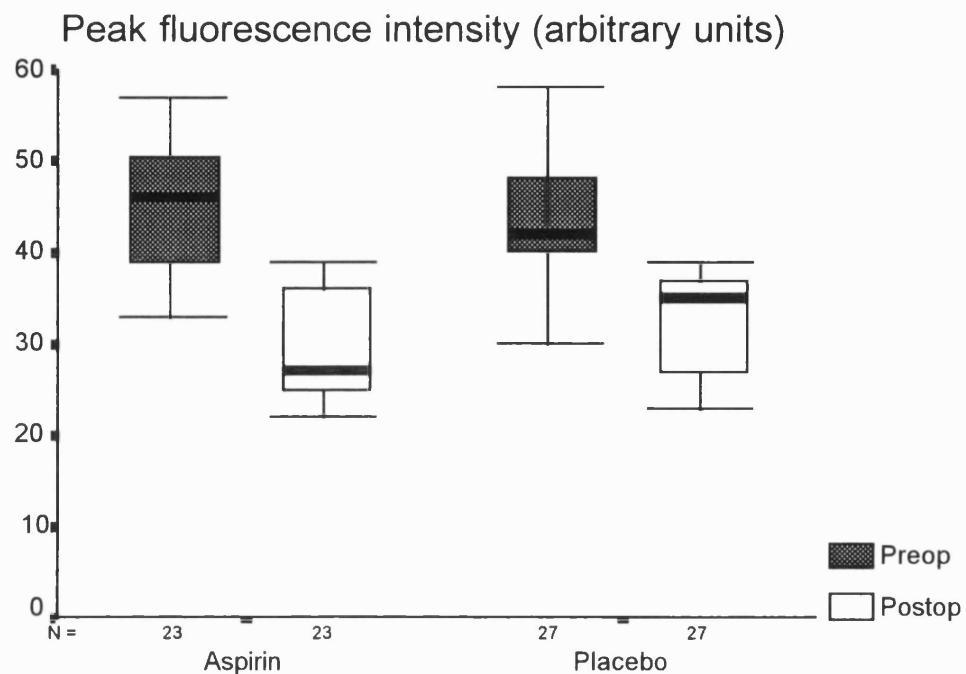


Figure 4.13 Surface expression of platelet GPIb, as estimated by using the platelet rich plasma method (peak fluorescence, arbitrary units). Normal control values in 32 subjects: 38 (31-42).

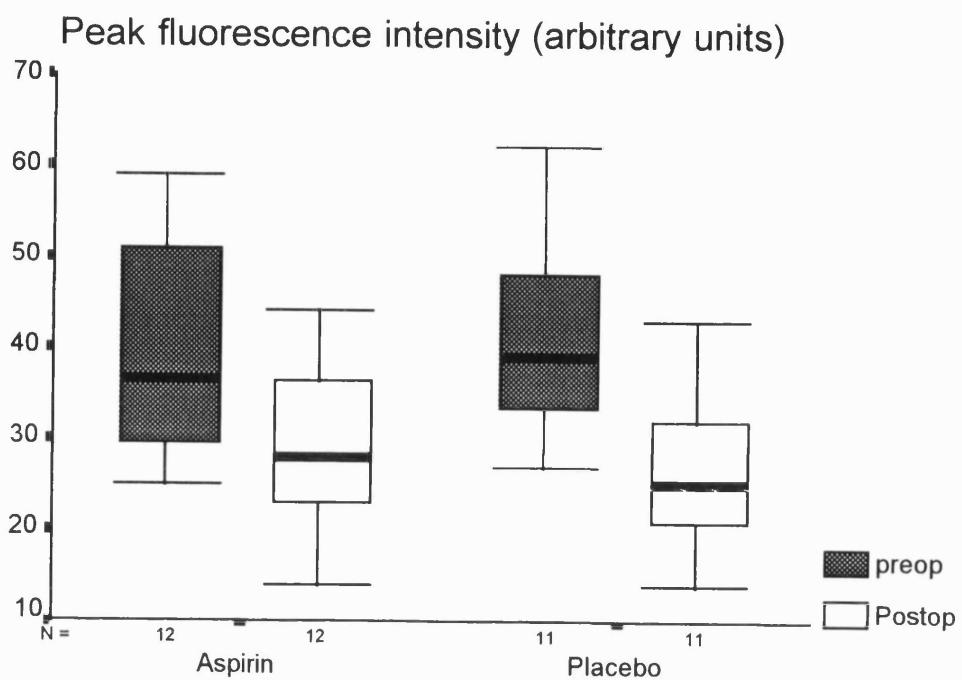


Figure 4.14 Surface expression of platelet GPIb, as estimated by using the whole blood method (peak fluorescence, arbitrary units). Normal control values in 14 subjects: 35 (29-39).

Table 4.5 Platelet GPIb surface expression (whole blood).

Median (MESF)	Aspirin	Placebo	p (MWU)
Pre-operative	13,000	14,000	0.7
Interquartile range	8,200-31,000	9,000-28,000	
Post-operative	7,800	6,500	0.5
Interquartile range	5,900-15,000	5,000-9,600	
p (Wilcoxon)	0.003	0.003	

Normal control 11,500 (8,000 - 15,000), n=14.

4.8 Plasma vWF activity

Aspirin did not alter significantly the plasma vWF activity either pre-operatively or post-operatively (Table 4.6).

The plasma vWF activity decreased significantly, following **bypass**, in the placebo group but not in the aspirin group.

Table 4.6 Plasma vWF activity (IU/dl).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	97	112	0.3
Interquartile range	64-143	88-162	
Post-operative	84	91	0.3
Interquartile range	66-111	77-116	
p (Wilcoxon)	0.02	0.01	

4.9 Plasma vWF antigen

Aspirin did not affect significantly the plasma vWF antigen either pre-operatively or post-operatively (Table 4.7).

The plasma vWF antigen was not altered by **bypass** in either group.

Table 4.7 Plasma vWF antigen (IU/dl).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	95	94	0.9
Interquartile range	64-122	78-115	
Post-operative	98	81	0.6
Interquartile range	76-125	62-99	
p (Wilcoxon)	0.7	0.2	

4.10 Platelet vWF activity

Aspirin did not affect significantly the platelet vWF activity either pre or post-operatively (Table 4.8).

Cardiopulmonary bypass enhanced the platelet vWF activity in the placebo group but not in the aspirin group .

Table 4.8 Platelet vWF activity (IU/dl/ 10^6 platelets).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	76	91	0.1
Interquartile range	61-130	70-132	
Post-operative	107	112	0.3
Interquartile range	56-181	69-176	
p (Wilcoxon)	0.1	0.04	

4.11 Platelet vWF antigen

Aspirin did not change significantly the platelet vWF antigen either pre or post-operatively (Table 4.9).

The platelet vWF antigen was not altered by **bypass** in either group.

Table 4.9 Platelet vWF antigen (IU/dl/10⁶ platelets).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	57	61	0.8
Interquartile range	40-87	38-88	
Post-operative	53	55	0.7
Interquartile range	41-76	37-73	
p (Wilcoxon)	0.3	0.2	

4.12 Plasma vWF multimeric composition

Neither **aspirin** nor **bypass** altered the ratio of high molecular weight (HMW) to low molecular weight (LMW) multimers (figure 4.15A, appendix). Similar results were obtained when the percentage changes between the pre and post-operative values were used for the HMW, LMW as well as for the whole area of the multimeric structure. This was also the case when the results were corrected for the amount of vWF antigen placed in each plate well and for variability of staining between plates by dividing each value by the equivalent normal control value on the same plate.

4.13 Platelet vWF multimeric composition

As illustrated in figure 4.15B (appendix), there was no significant alteration in platelet vWF multimeric patterns either by **aspirin** or by **bypass** when analysed as for section 4.12.

4.14 Plasma fibrinogen and FDPs

Aspirin did not influence the plasma fibrinogen levels but there was a highly significant reduction in plasma fibrinogen level following bypass (table 4.10).

Table 4.10 Plasma fibrinogen (g/l).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	3.1	3.1	0.7
Interquartile range	2.8-3.6	2.9-3.4	
Post-operative	1.8	1.8	0.2
Interquartile range	1.3-2.1	1.6-2.0	
p (Wilcoxon)	0.001	0.001	

Aspirin did not influence the plasma FDP levels and the increase in FDPs, following bypass, did not reach statistical significance in either group alone (table 4.11). When the data from all 71 patients were analysed together, however, this increase in FDPs reached statistical significance [preop 8 (8-12) and postop 16 (12-16), p=0.03].

Table 4.11 Plasma FDPs (mg/l).

Median	Aspirin	Placebo	p (MWU)
Pre-operative	8	8	0.1
Interquartile range	8-8	8-16	
Post-operative	12	12	0.1
Interquartile range	8-16	8-16	
p (Wilcoxon)	0.6	0.6	

4.15 Linear regression and prediction of blood loss

Linear regression analysis was used in an attempt to identify factors which may be predictive of post-operative blood loss. The variables used were bleeding time, platelet aggregation, platelet surface GPIb receptors, plasma and platelet vWF and multimeric analysis as described in sections 4.12 and 4.13. None of these individual variables were particularly predictive of blood loss (four hour or total blood loss). As there was no relationship between these variables and blood loss no correlation coefficients were calculated, as one can not test how close the relationship is when there is no relationship.

4.16 Principal conclusions

Pre-operative aspirin therapy:

- 1 Prolonged significantly the bleeding time both pre and post-operatively.
- 2 Diminished the platelet aggregatory response to collagen (10 and 20 μ l) both pre and post-operatively.
- 3 Reduced the platelet aggregatory response to arachidonic acid (10 μ l but not 40 μ l) both pre and post-operatively.
- 4 Slightly reduced the platelet aggregation to 30 μ l of ristocetin pre-operatively only with no effect on the aggregation secondary to 40 μ l of ristocetin.
- 5 Diminished the stimulated release of both the ADP and ATP, pre and post-operatively, in response to collagen (10 and 20 μ l).

- 6 Curtailed the stimulated release of both nucleotides, pre and post-operatively, in response to 10 μ l of arachidonic acid but not in response to 40 μ l of arachidonic acid.
- 7 Decreased the stimulated release of both nucleotides, pre and post-operatively, in response to 10 and 40 μ l of ristocetin.

Pre-operative aspirin therapy did not have a significant effect, pre or post-operatively, on the:

- 1 Platelet count and size distribution.
- 2 Total ADP and ATP content of platelets.
- 3 Surface expression of platelet GPIb.
- 4 Plasma vWF activity, antigen or multimeric structure.
- 5 Platelet vWF activity, antigen or multimeric structure.
- 6 Plasma fibrinogen or FDPs.

The main effects of CPB on platelet function were:

- 1 A reduction in platelet number and MPV.
- 2 An increase in PDW.
- 3 A prolongation of the bleeding time.
- 4 A diminution in platelet aggregation to all agonists except;
 - a) when the pre-operative aggregation was low (10 μ l of collagen and arachidonic acid) and
 - b) when 40 μ l of arachidonic acid was the agonist, in which case the platelet aggregation was enhanced.
- 5 The stimulated nucleotide release;
 - a) An increase in ATP (collagen 10 μ l) only in the aspirin group.

- b) An increase in ADP (collagen 20 μ l) only in the aspirin group.
- c) An increase in ADP and a decrease in ATP (arachidonic acid 10 μ l) only in the placebo group.
- d) An increase in both ADP and ATP (arachidonic acid 40 μ l) in both groups.
- e) A reduction in both ADP and ATP (ristocetin 30 μ l) only in the placebo group.

6 A decline in platelet GPIb surface expression.

7 A drop in plasma vWF activity.

8 Enhancement of the platelet vWF activity.

9 A reduction in plasma fibrinogen level.

10 An increase in plasma FDPs.

CPB did not influence:

- 1 The total nucleotide content of PRP or PPP.
- 2 The plasma vWF antigen or multimeric pattern.
- 3 The platelet vWF antigen or multimeric pattern.

No individual variable was predictive of blood loss.

Chapter 5

Results

Clinical endpoints of the aprotinin trial

5.1 Introduction

Sixty patients, drawn from 1,000 patients undergoing cardiac surgery during the same time frame, who met the entry criteria were randomised. The 100 patients included in the aspirin trial were excluded from the aprotinin trial. The two groups were similar in the demographic characteristics of the patients (table 5.1). In addition the two groups did not differ in the number of patients on aspirin pre-operatively, CPB time, the number of bypass grafts and the number of internal mammary arteries (IMA) used per patient.

Table 5.1 Demographic characteristics of the patients.

Variable	Placebo	Aprotinin
Number	31	29
Pre-operative aspirin	19	21
Male / female	30 / 1	27 / 2
Mean age (years)	64	64
Mean weight (Kg)	81	76
Mean Height (cm)	172	173
Median bypass time (min)	74	70
Median cross clamp time (min)	37	36
Median number of grafts	3	3
Median number of IMA	1	1

A similar number of patients did not receive autotransfused blood in the two groups (3 in the placebo and 2 in the aprotinin group). The types of operative procedures performed were also similar in the two groups (table 5.2). All of these procedures were first time except for one in each group, which were second time operations.

Table 5.2 Operative procedures performed.

	Placebo	Aprotinin
CABG	21	22
AVR	4	2
AVR & CABG	4	3
MVR	1	1
MVR & AVR	1	1
 TOTAL	 31	 29

CABG = Coronary Artery Bypass Grafting.

AVR = Aortic Valve Replacement.

MVR = Mitral Valve Replacement.

5.2 Post-operative bleeding

All the data were analysed on an intention to treat basis and patients that were re-explored for continued bleeding were included in the analysis as blood loss measurement was continued in theatre. The distribution of the blood loss

data was not normal, therefore these data were analysed using the two tailed Mann Whitney U test (Table 5.3).

Table 5.3 Non parametric analysis for blood loss.

Median blood loss (ml)	Placebo	Aprotinin	p value
Randomisation	350	340	0.84
5 hours	700	500	0.08
10 hours	870	615	0.04
Chest drain removal	1866	1220	0.01

Figure 5.1 shows the total blood loss at the time of randomisation, before the commencement of the aprotinin/placebo infusion, at five hours later, at ten hours later and at the time the chest drains were removed. The patients in the aprotinin group bled significantly less than the placebo group even though the two groups had similar blood loss at the time of randomisation.

5.3 Coagulation screen

The PT did not differ between the two groups either before ($p=0.3$, figure 5.2A) or after the infusion ($p=0.7$). The KPTT did not differ between the two groups either before ($p=0.4$, figure 5.2B) or after the infusion ($p=0.3$). The TT was also similar in the two groups before ($p=0.2$) and after the infusion ($p=0.6$).

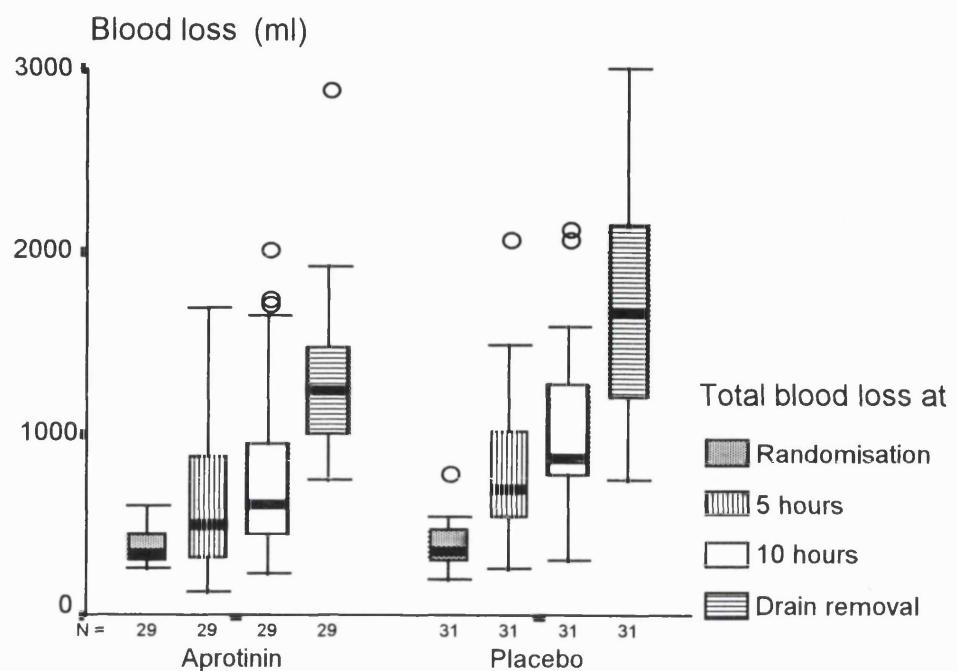


Figure 5.1 The total blood loss before the commencement of the aprotinin/placebo infusion, at 5 hours later, 10 hours later and at the time of chest drain removal.

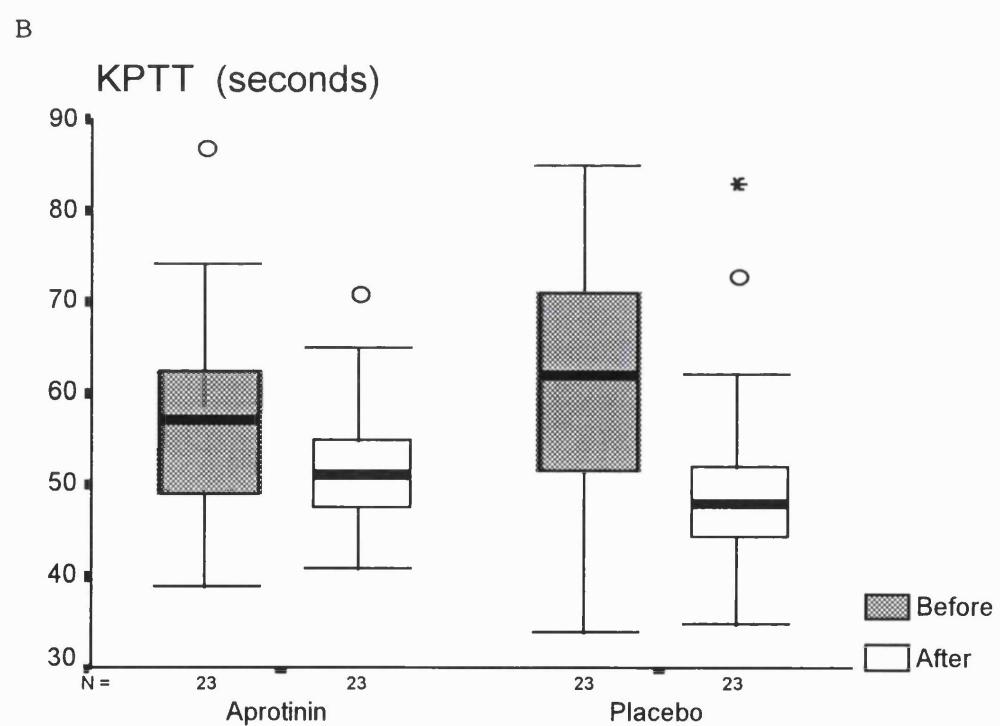
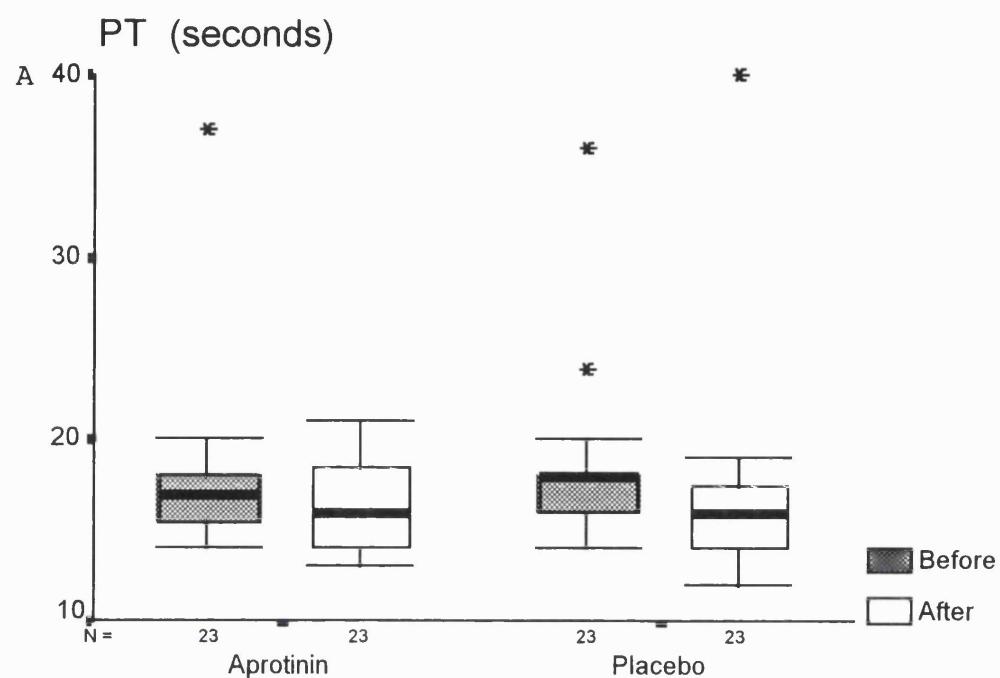


Figure 5.2A The PT in the two groups before and after the aprotinin/placebo infusion.

Figure 5.2B The KPTT in the two groups before and after the aprotinin/placebo infusion.

5.4 Transfusion requirements

The median number of units of homologous packed cells transfused, per patient, was four in both groups ($p=0.4$, figure 5.3). In addition there was no significant difference in the number of units of platelets transfused in each group. In the aprotinin group a total of 48 units of platelets were used for eight patients and in the placebo group a total of 42 units were used for seven patients ($p=0.7$). The FFP requirement was also similar in the two groups. In the aprotinin group a total of 28 units of FFP were used for 13 patients and in the placebo group a total of 30 units were used for 11 patients ($p=0.6$). One patient in each group received cryoprecipitate (12 Units).

The volume of colloid transfused per patient was 1500 (1500-2000) ml in the aprotinin group and 2000 (1500-2000) ml in the placebo group ($p=0.07$). The volume of mediastinal shed blood autotransfused was 595 (458-843) ml in the aprotinin group and 785 (615-930) in the placebo group ($p=0.02$).

5.5 Pre-operative and discharge Hb

Even though there was no significant difference in transfusion of homologous blood between the two groups, the patients in the aprotinin group had significantly higher discharge Hb levels compared to the placebo group. The pre-operative Hb levels of the two groups were similar (figure 5.4). The median pre-operative Hb for the placebo group was 14.6 g/dl and for the aprotinin group was 14.3 g/dl, ($p=0.5$). The median discharge Hb for the placebo

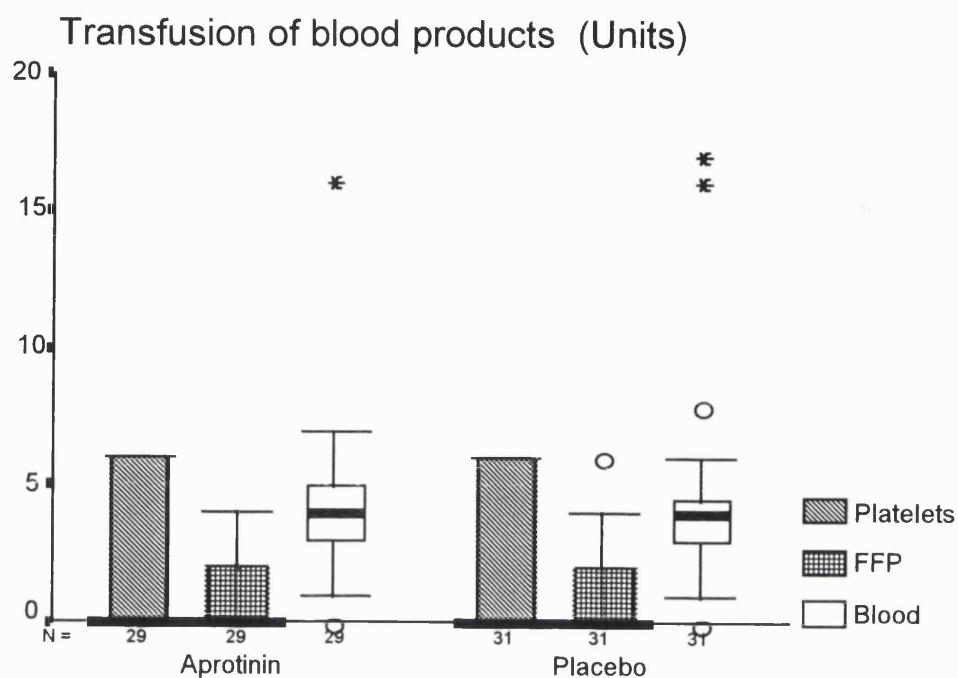


Figure 5.3 Transfusion of homologous blood and blood products (FFP= Fresh Frozen Plasma).

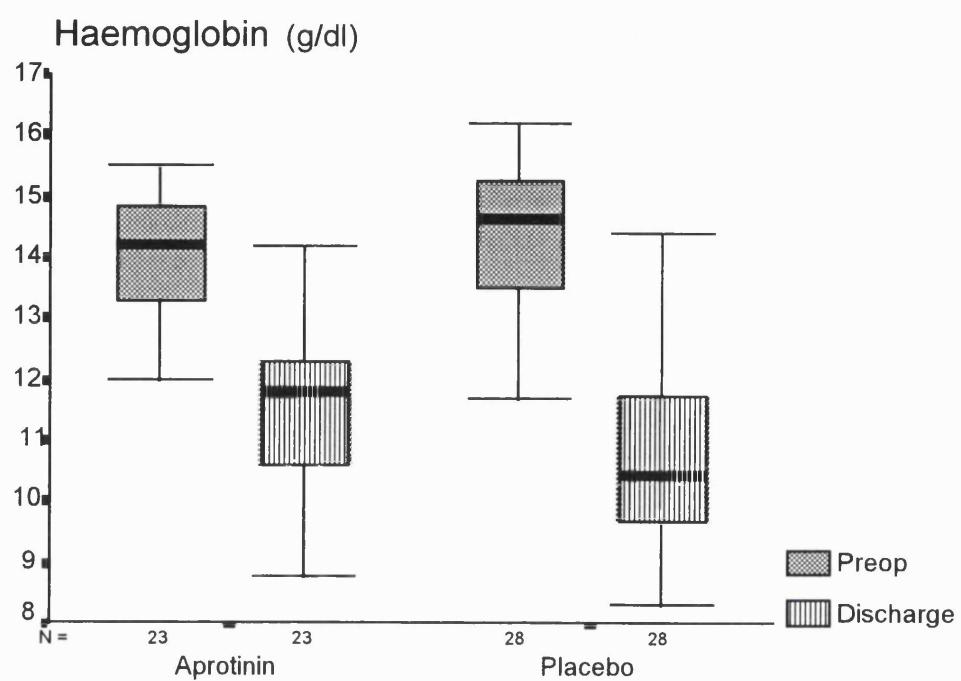


Figure 5.4 Haemoglobin levels pre-operatively and at the time of discharge of the patients from the hospital.

group was 10.5 g/dl and for the aprotinin group 11.8 g/dl ($p=0.04$). The median Hb drop was 4.1 g/dl in the placebo group and 2.5 g/dl in the aprotinin group.

5.6 Re-exploration for excessive bleeding

Three patients were re-explored for continued excessive bleeding in each group. The cause of bleeding was attributed to "a surgically preventable cause" in all of the three patients in the aprotinin group and in two of the three patients in the placebo group. All of these patients continued the trial protocol during re-exploration in theatre and the blood loss was measured in theatre. The analysis of the results was on an intention to treat basis.

5.7 Clinical outcome

The clinical outcome was similar in the two groups (table 5.4).

Table 5.4 Clinical outcome.

Number of patients /median	Placebo	Aprotinin
Deaths	1	1
Re-exploration for bleeding	3	3
Renal failure	0	2
Myocardial infarction	1	3
Intensive care stay (Days)	1	1
Hospital stay (Days)	7	7

Renal failure and myocardial infarction occurred more often in the aprotinin group, but the cases were so few that no statistically valid conclusion can be drawn. The intensive care and hospital stay were similar in the two groups.

One patient died in each group. A 79 year old man with end stage aortic stenosis died 48 hours post-operatively in the placebo group. He required inotropic support and intra-aortic balloon counterpulsation to be weaned from CPB and was re-explored for continued bleeding before he died from low cardiac output. In the aprotinin group a 73 year old man died after coronary artery bypass surgery and re-exploration for continued bleeding. He had a history of mesenteric angina and required resection of his small bowel for mesenteric ischaemia. He died from multisystem failure 16 days post-operatively. Histopathological examination did not reveal any evidence of thrombus or embolus in the mesenteric vessels.

5.8 Principal conclusions

Aprotinin use reserved until excessive bleeding is detected:

- 1 Reduced post-operative blood loss.
- 2 Maintained higher Hb levels at the time of discharge.
- 3 Did not reduce the requirement for blood products.
- 4 Did not influence re-exploration for bleeding.
- 5 Did not alter the coagulation screen.
- 6 Did not alter clinical outcome.

Chapter 6

Results

The effect of aprotinin on the measurements of haemostasis

6.1 Introduction

The measurements of haemostasis in this chapter were made before the aprotinin/placebo infusion was commenced (A) and at the end of the infusion five hours later (B). These measurements were made in a subgroup of 30 out of the 60 patients, whose demographic characteristics were similar to the group as a whole (section 5.1). None of these patients had received platelets or FFP before the last sample of blood was taken for haematological assays.

These data were not normally distributed and were therefore analysed using non parametric methods. The data were expressed as the median and the interquartile range. Each variable, before the infusion, in the aprotinin group was compared with its equivalent variable in the placebo group using the two tailed Mann Whitney U test. Each variable, after the infusion, in the aprotinin group was compared with its equivalent variable in the placebo group using the two tailed Mann Whitney U test.

6.2 Platelet count

The platelet counts did not differ between the two groups either before or after the infusion (table 6.1). Although the platelet count decreased slightly, within each group, during the study period this did not reach statistical significance.

Table 6.1 Platelet count and size distribution ($p>0.05$).

Median (interquartile range)		Aprotinin	Placebo
Platelets ($\times 10^9/1$)	A	139(82-176)	114(93-147)
Platelets ($\times 10^9/1$)	B	117(99-161)	110(75-132)
MPV (fl)	A	7.8(6.8-8.3)	7.6(7.1-8.2)
MPV (fl)	B	7.6(6.8-8)	7.3(7.1-7.6)
PDW (arbitrary units)	A	17.9(17-18.2)	18.1(17.5-18.5)
PDW (arbitrary units)	B	17.7(17.3-18)	18(17.6-18.6)

A: Before the aprotinin/placebo infusion.

B: After the aprotinin/placebo infusion.

6.3 Platelet size distribution

The median MPV did not differ between the two groups either before or after the aprotinin/placebo infusion (table 7.1). The platelet distribution width (PDW) was the same in the two groups before the infusion and remained the same during the study period (table 6.1).

6.4 Platelet aggregation

As shown in table 6.2 the platelet aggregatory response to different agonists was not altered by aprotinin. Although

there was a tendency of the platelet aggregation to decrease, within each group during the study period, this did not reach statistical significance. The platelet aggregation response to collagen and ristocetin are illustrated as box and whiskers plots in figure 6.1.

Table 6.2 Platelet aggregation.

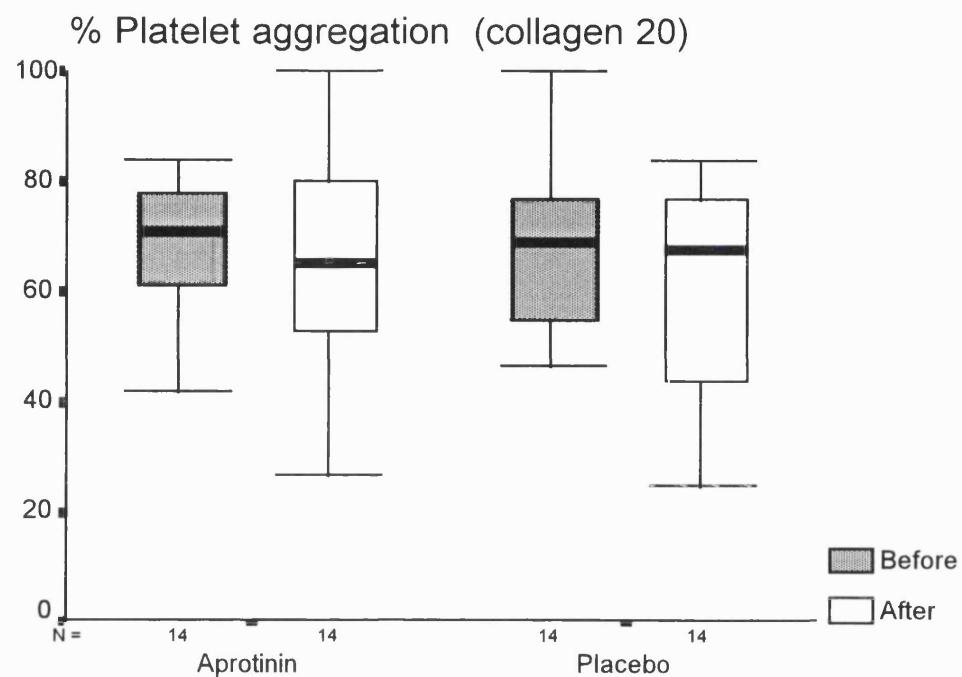
Median % maximum aggregation (interquartile range).

Agonist	Aprotinin	Placebo	p value
Collagen 20 (A)	71 (59-79)	69 (55-78)	0.9
Collagen 20 (B)	65 (51-81)	68 (44-78)	0.7
Arachidonic acid 40 (A)	38 (18-65)	68 (21-89)	0.1
Arachidonic acid 40 (B)	45 (31-74)	37 (14-76)	0.5
Ristocetin 40 (A)	88 (71-95)	86 (78-92)	0.8
Ristocetin 40 (B)	84 (58-94)	80 (67-91)	0.9
Ristocetin 30 (A)	49 (28-72)	72 (30-83)	0.3
Ristocetin 30 (B)	35 (12-58)	55 (32-67)	0.1

6.5 Platelet surface GPIb expression

The surface expression of platelet GPIb was the same in the two groups before the aprotinin/placebo infusion was commenced ($p=0.3$, figure 6.2), but increased significantly in the aprotinin group during the study period (two tailed Mann Whitney U test, $p=0.007$, table 6.3). Figure 6.3 shows a print out from the flow cytometer illustrating the increase in peak fluorescence intensity (i.e. GPIb surface expression) in an individual patient before and after aprotinin infusion (PRP method). Table 6.3 indicates the same changes when the values are expressed as MESF.

A



B

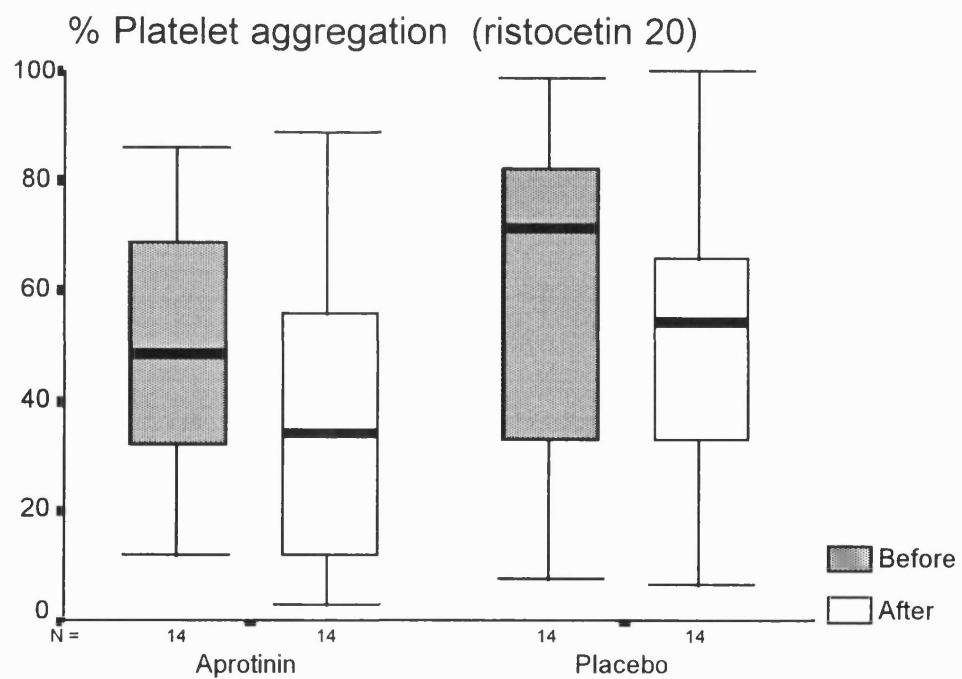


Figure 6.1A Platelet aggregation to collagen before and after the aprotinin / placebo infusion.

Figure 6.1B Platelet aggregation to ristocetin before and after the aprotinin / placebo infusion.

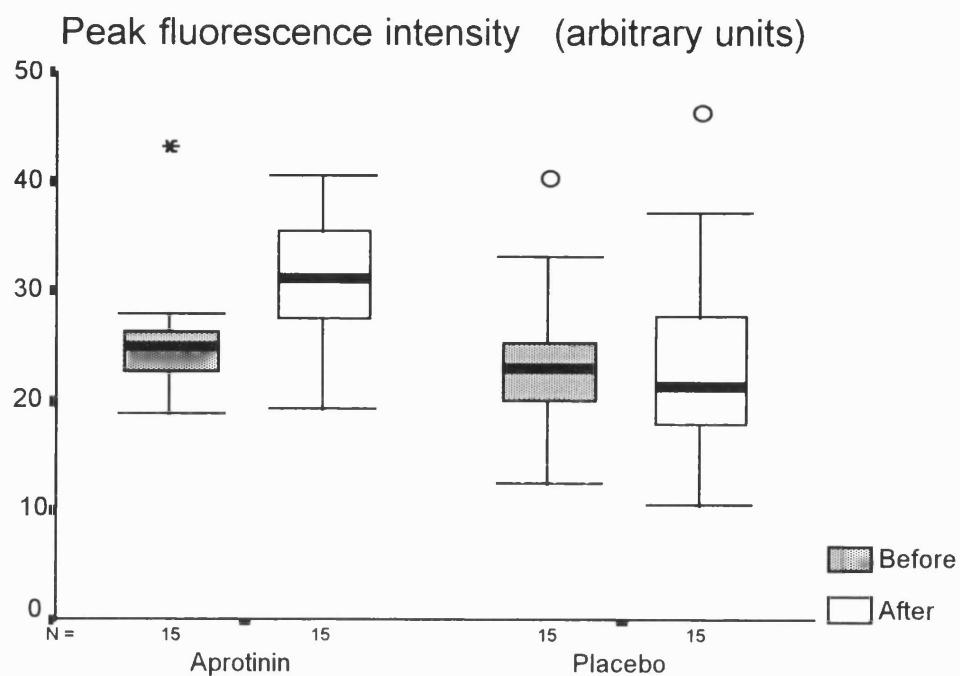


Figure 6.2 Platelet surface GPIb receptor expression before and after the aprotinin / placebo infusion.

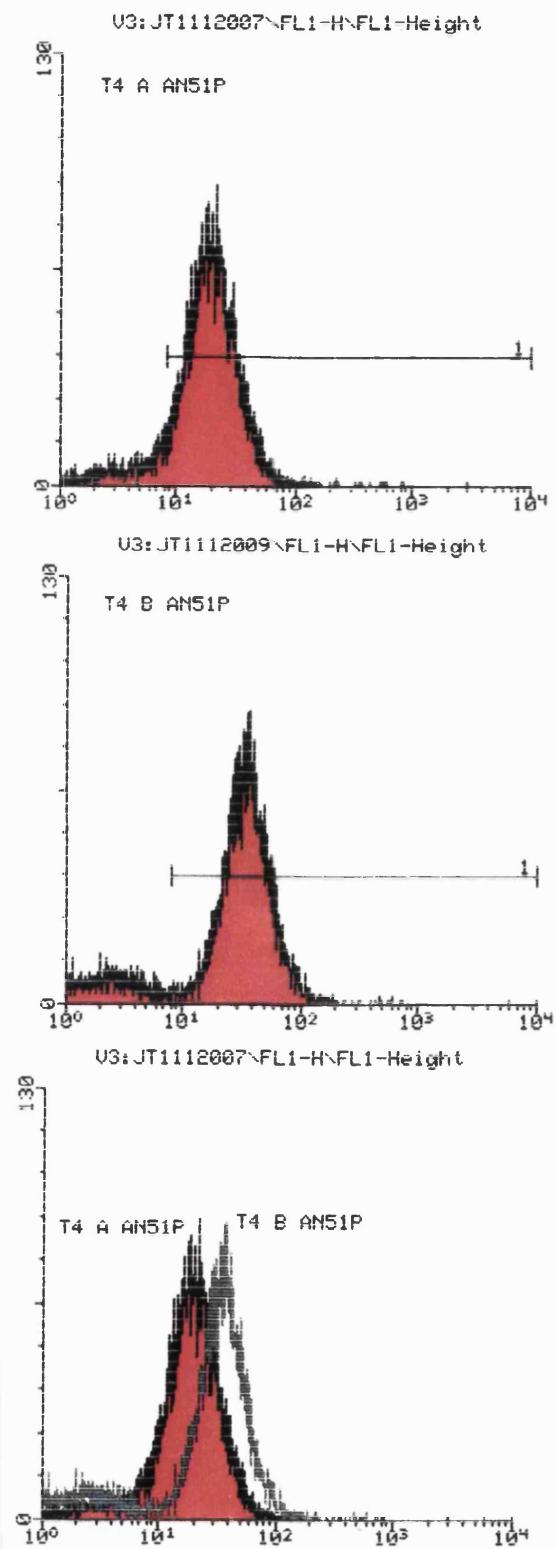


Figure 6.3 Flow cytometer print out illustrating the increase in peak fluorescence in a patient before and after the aprotinin infusion.

Table 6.3 Platelet surface GPIb expression (MESF).

Median	Aprotinin	Placebo	p value
Before (A)	2,700 (2,500-2,800)	2,600 (2,300-2,800)	0.3
After (B)	3,100 (2,800-3,500)	2,500 (2,200-3,000)	0.007

6.6 Plasma vWF activity

The plasma vWF activity was not significantly different in the two groups before or after the aprotinin/placebo infusion (table 6.4).

6.7 Plasma vWF antigen

The plasma vWF antigen was similar in the two groups before the start of the infusion and it was not altered by the aprotinin during the study period (table 6.4).

Table 6.4 Plasma and platelet vWF (p>0.05).

Variable (median)	Aprotinin	Placebo
Plasma vWF Activity (IU/dl) A	127	109
Interquartile range	105-177	93-163
Plasma vWF Activity (IU/dl) B	111	110
Interquartile range	89-151	99-155
Plasma vWF Antigen (IU/dl) A	106	110
Interquartile range	76-145	88-149
Plasma vWF Antigen (IU/dl) B	112	136
Interquartile range	76-202	117-172
Platelet vWF Antigen A	63	62
Interquartile range	45-128	53-74
Platelet vWF Antigen B	58	48
Interquartile range (IU/dl/10 ⁶ platelets)	48-74	33-61

6.8 Platelet vWF antigen

As shown in table 6.4 the platelet vWF antigen had not changed significantly during the study period.

6.9 Platelet vWF activity

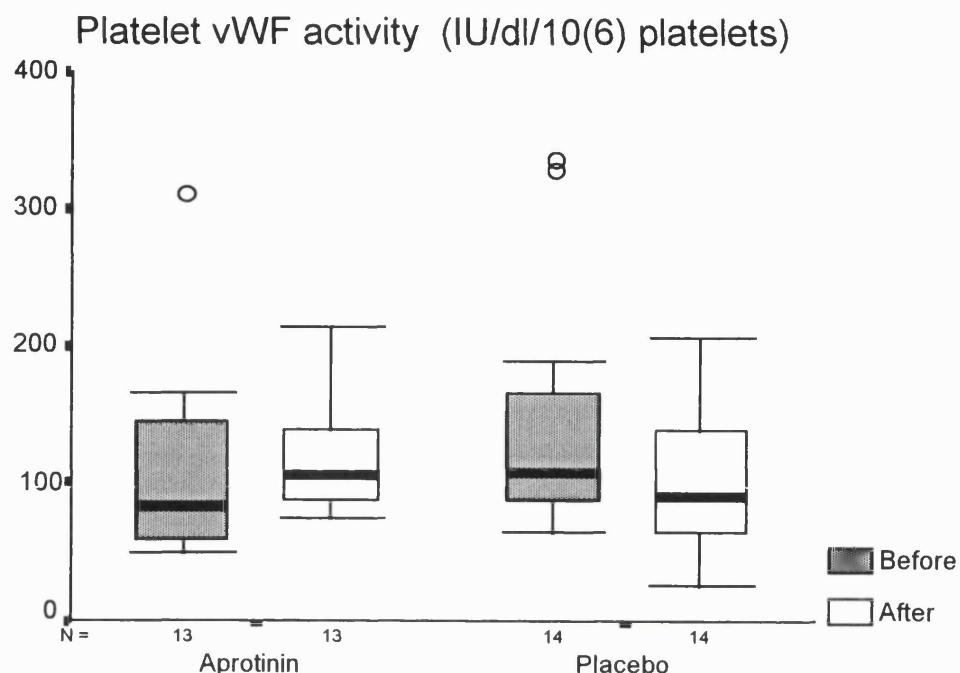
Figure 6.4A illustrates the changes in platelet vWF activity during the study period. The median platelet vWF activity in the aprotinin group increased from 83 to 106 IU/dl/ 10^6 platelets whereas the median activity in the placebo group decreased from 109 to 91 IU/dl/ 10^6 platelets. Using the two tailed Mann Whitney U test there was no significant difference between the two groups either before ($p=0.2$) or after the infusion ($p=0.2$).

Figure 6.4B shows a box and whiskers plot of the difference in platelet vWF activity, of each value, before (A) and after (B) the infusion (A-B). In the aprotinin group the median change was -25 and in the placebo group +28 IU/dl/ 10^6 platelets (i.e. the median increase was 25 in the aprotinin group and the median decrease was 28 in the placebo group). When the two differences were compared with the two tailed Mann Whitney U test failed to reach statistical significance ($p=0.06$)

6.10 Plasma and platelet vWF multimeric composition

The plasma and platelet multimeric patterns were analysed using the same methods as explained in section 4.12 but no significant alteration was noted in either of the groups.

A



B

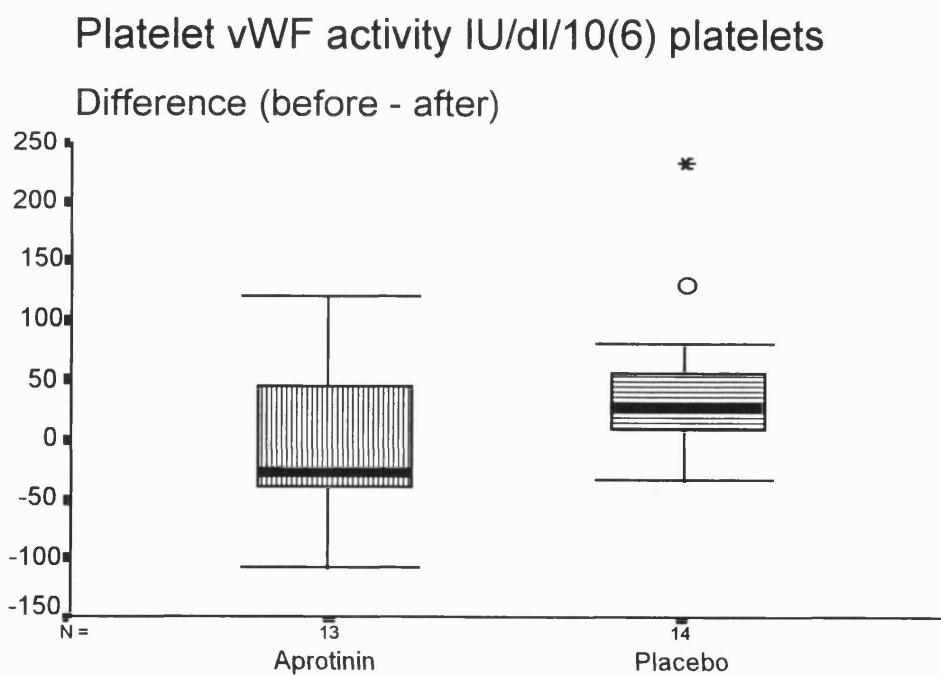


Figure 6.4A Platelet von Willebrand Factor activity before and after the aprotinin / placebo infusion.

Figure 6.4B Platelet von Willebrand Factor activity difference before and after the aprotinin / placebo infusion.

6.11 Fibrinogen

The median fibrinogen level before the infusion was 1.8 g/l in both groups (Figure 6.5, $p=0.3$). The median fibrinogen level after the infusion, however, increased in the aprotinin group and decreased in the placebo group ($p=0.03$).

6.12 Fibrinogen degradation products and D-dimers

As shown in table 6.5 there was no significant difference between the two groups in the levels of FDPs or D-dimers either before or after the infusion. Even though there was a tendency for the both levels to decrease in the aprotinin group and for both levels to increase in the placebo group the difference in values also failed to reach statistical significance.

Table 6.5 FDPs and D-dimers ($p>0.05$).

Variable (median)		Aprotinin	Placebo
FDPs (mg/l)	A	16	12
Interquartile range		8-32	8-32
FDPs (mg/l)	B	16	16
Interquartile range		8-20	14-32
D-dimers (mg/l)	A	2	1
Interquartile range		0.9-2	0.5-1
D-dimers (mg/l)	B	1	1
Interquartile range		0.5-1.4	0.5-2

A: Before the aprotinin/placebo infusion.

B: After the aprotinin/placebo infusion.

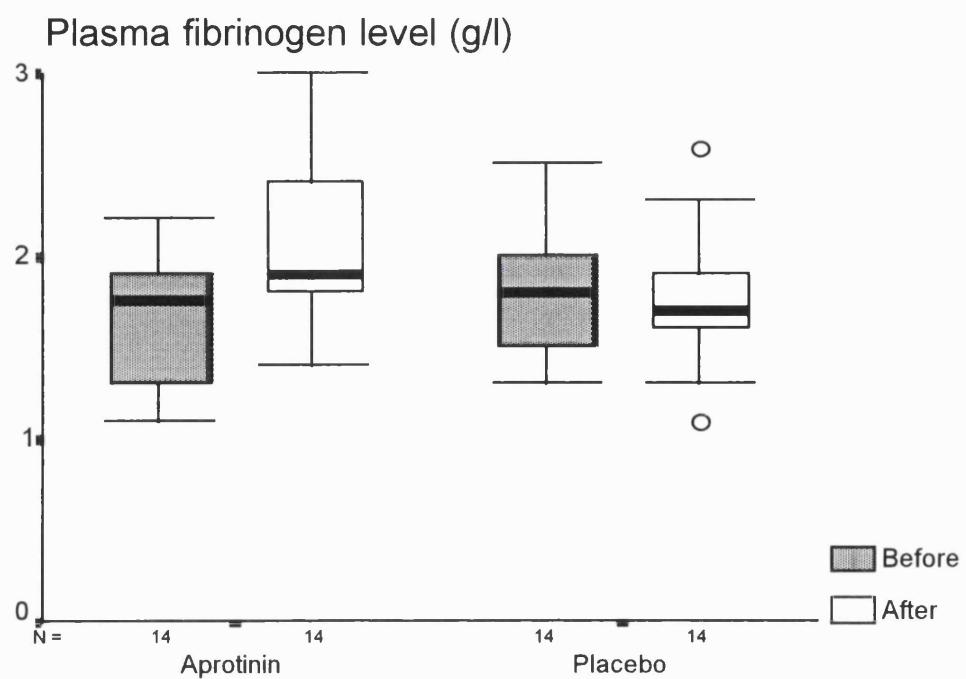


Figure 6.5 Fibrinogen level before and after the aprotinin / placebo infusion.

6.13 tPA activity

The tPA activity before the infusion was high in both groups compared to the normal range (figure 6.6). The tPA activity after the infusion was no different between the two groups ($p=0.1$). The tPA activity in the aprotinin group decreased dramatically during the study period whereas it increased slightly in the placebo group. The decrease, however, is probably due to the in vitro effect of aprotinin on this assay indicating plasma levels of aprotinin sufficient to inhibit plasmin in vitro and not necessarily in vivo (discussion section 7.4).

7.14 tPA antigen

For this reason the tPA antigen was also measured, which demonstrated similar changes to tPA activity, albeit to a lesser degree (figure 6.7A). The tPA antigen before the infusion was high in both groups compared to the normal range indicating excess fibrinolysis. The tPA antigen decreased slightly in the aprotinin group during the study period and increased in the placebo group, but only the difference (A-B) in the values (before and after the infusion) reached statistical significance ($p=0.05$, two tailed Mann Whitney test) (figure 6.7B).

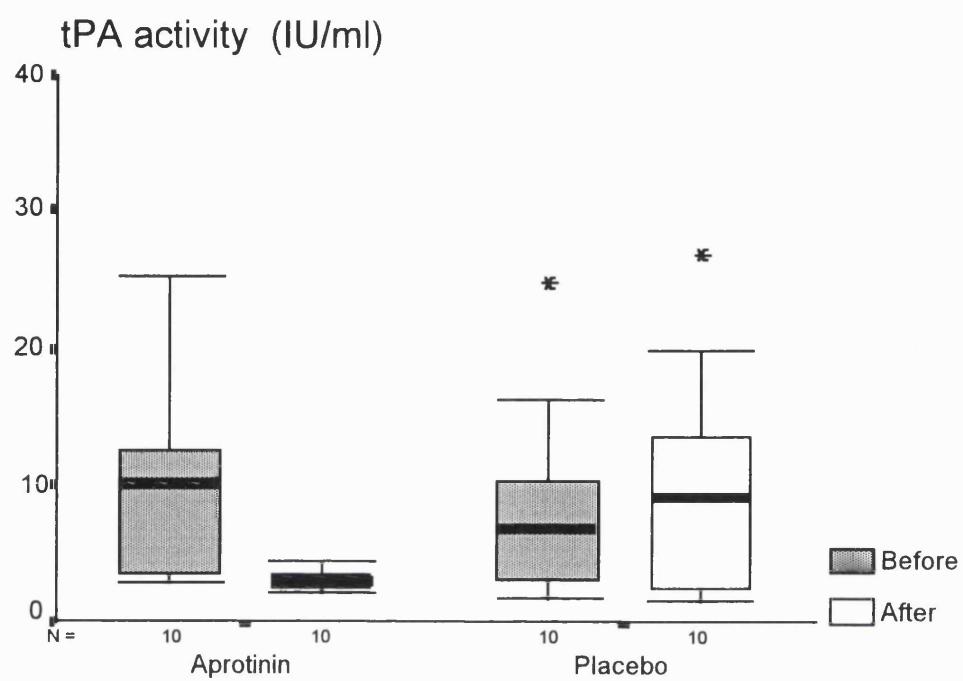


Figure 6.6 Tissue Plasminogen Activator activity before and after the aprotinin / placebo infusion.

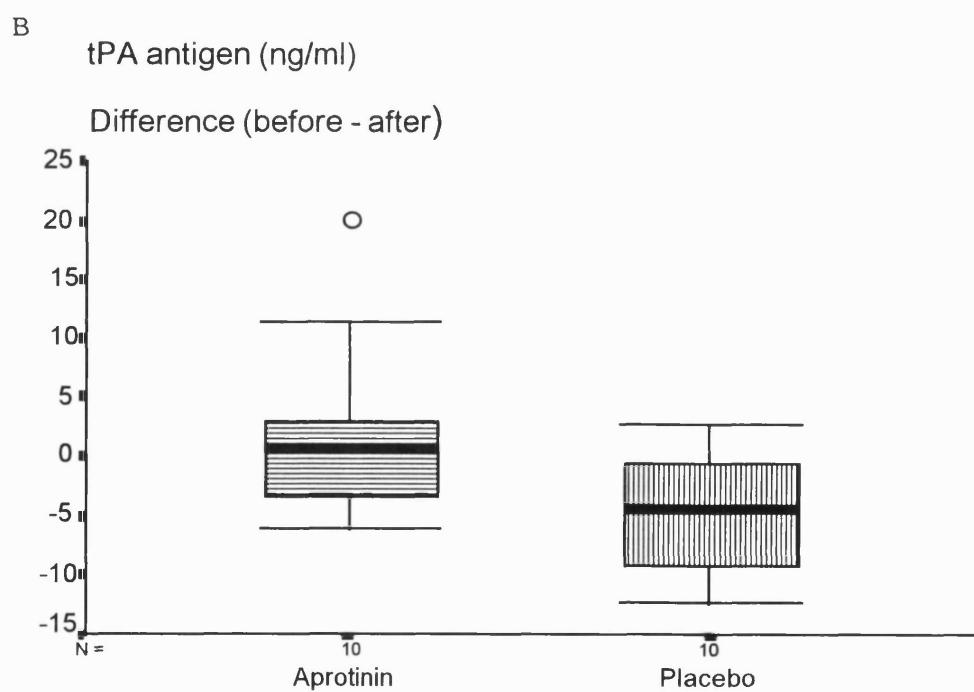
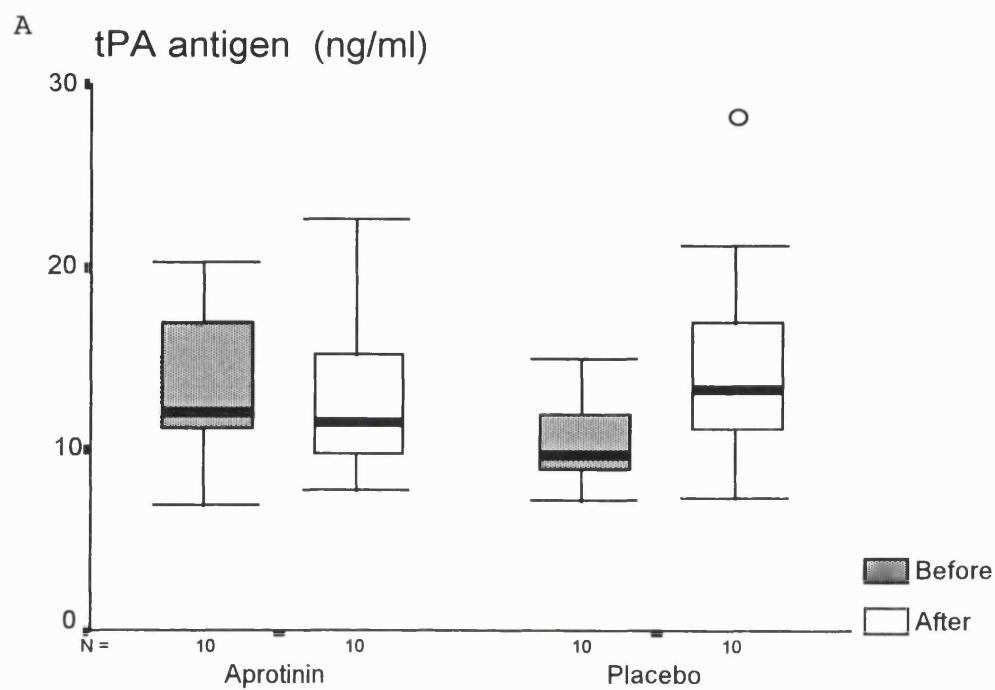


Figure 6.7A tPA antigen before and after the aprotinin / placebo infusion.

Figure 6.7B tPA antigen difference before and after the aprotinin / placebo infusion.

6.15 Principal conclusions

The main actions of aprotinin when used for the first time during excessive post-operative bleeding were:

- 1 Increase of platelet GPIb surface expression towards pre-bypass levels.
- 2 Augmentation of plasma fibrinogen levels.
- 3 A reduction in tPA antigen.
- 4 Probable maintenance of platelet vWF activity.

Aprotinin use in this clinical scenario did not influence:

- 1 The platelet count and size distribution.
- 2 The platelet aggregation response to collagen, arachidonic acid and ristocetin.
- 3 The plasma vWF activity, antigen and multimeric structure.
- 4 The platelet vWF antigen and multimeric structure.
- 5 The plasma FDPs and D-dimers.

Chapter 7

Discussion

7.1 Introduction

The morbidity and mortality following CPB is significantly increased by excessive post-operative bleeding (Kirklin and Barratt-Boyes 1986_b; Unsworth-White, Herriot, Valencia et al 1995) which drains upon our limited financial resources and supply of blood products. In this thesis the mechanisms responsible for excessive bleeding following CPB are investigated and measures to prevent and treat excessive bleeding are established. The effects of pre-operative aspirin and CPB on the post-operative platelet function are investigated. In addition the influence of therapeutic aprotinin on platelet function and fibrinolysis is investigated in patients who bled excessively post-operatively.

The word haemostasis, derived from the Greek word *aiματοστασις* (*aiμa* - blood and *στασις* - stop), is used to describe the process by which the loss of blood from a divided blood vessel is arrested by the interaction of many factors and cascades. This was recognised early on (Bizzozero 1882) who identified the importance of platelets and fibrin in this interaction. Knowledge of the complexity of this dynamic interplay of the various components of haemostasis has increased considerably since

that time. We now know that this dynamic interaction involves the wall of the damaged vessel, the platelet plug formation, the coagulation cascade and fibrinolysis.

Each process is complex in its own right and it involves the interaction of numerous factors. Any one study attempting to understand the mechanisms involved in haemostasis can only study a small fraction of this process. The aim would be to identify the clinical problem and to concentrate on the most relevant haemostatic factors involved. After an exhaustive search through the enormous literature, related to this field, haemostatic variables were chosen because:

- 1) they relate to platelet adhesion in high-shear rate vessels (GPIb and vWF), 2) they indicate the aspirin effect on platelets (aggregation and stimulated nucleotide release) and 3) they manifest the effect of plasmin and fibrinolysis (FDPs, D-dimers, fibrinogen and tPA).

7.2 The aspirin trial

The trial of continuation of aspirin or placebo until surgery demonstrated that aspirin significantly increased operative and post-operative bleeding, transfusion requirements for homologous blood products and re-exploration for excessive bleeding. Furthermore, despite the increased blood transfusion of the patients in the aspirin group, these patients had significantly lower Hb levels at the time of discharge from the hospital. As a result of these demonstrated differences this trial has confirmed the suspected risk of increased bleeding if

pre-operative aspirin is continued up to the time of CABG.

In this thesis the risk of continuation of pre-operative aspirin therapy up to the time of CABG is established in a randomised, prospective, double-blind clinical trial.

This assessment, however, is only one side of the benefit/risk equation. The benefit of continuation of pre-operative aspirin therapy up to the time of CABG, was only partly answered in this thesis, as this was not the main aim of the study. There was no significant difference in the incidence of myocardial infarction, intensive care unit stay, hospital stay or clinical outcome between the two groups. This result, however, may not be as certain as the assessment of the risk because the power of the study may not have been enough to detect a small difference in clinical outcome. Furthermore this study made no assessment of vein graft patency and longer term recurrence of angina. It is important, however, that there was no adverse effect in the discontinuation of pre-operative aspirin therapy in a group of patients awaiting to undergo elective CABG and without a positive indication for aspirin therapy other than for chronic stable angina.

The benefit of pre-operative aspirin therapy was not pursued in this study because several other recent studies examined this issue (Gavaghan, Gebski and Baron 1991). This was a randomised, double-blind study comparing the effect of pre-operative aspirin (12 hours) with early post-operative aspirin (one hour) on angiographically assessed vein graft patency. Gavaghan and co-workers demonstrated significant

improvement in early vein graft patency, at one week and one year, with 324 mg aspirin commenced at one hour post-operatively via a nasogastric tube without any significant increase in bleeding. In another study Goldman and colleagues (Goldman, Copeland, Moritz et al 1991) failed to demonstrate any difference in ten day graft patency between 325 mg of aspirin started 12 hours pre-operatively and six hours post-operatively. There was a tendency, although not significant, however, toward improved patency of sequential and internal mammary artery grafts with pre-operative aspirin. In another study there was no difference between the internal mammary artery and vein grafts at one year and aspirin had no effect on patency rates (Goldman, Copeland, Moritz et al 1990). Early post-operative aspirin seems to provide the benefit of reducing early vein graft occlusion but without increasing the risk of bleeding.

Findings in this study, together with the literature on the beneficial effect of aspirin on coronary artery bypass graft patency, indicate the following practice: In patients awaiting elective CABG (excluding patients with unstable angina and left main stem stenosis) aspirin should be discontinued one week pre-operatively. Six hours post-operatively all patients should be given aspirin through a nasogastric tube or rectally, provided they are not bleeding excessively. This practice maximises the benefit of pre-operative aspirin therapy and minimises the risk.

7.3 The aspirin effect

Platelet aggregation can be induced by at least three different pathways elucidated so far (Kinlough-Rathbone, Packam, Reimers et al 1977). The first pathway involves the release of ADP and the second involves the generation of prostaglandin endoperoxides and thromboxane A₂. Platelet activating factor may be the mediator of a third pathway (Chignard, Le Couedic, Tence et al 1979), which involves the aggregation in response to thrombin when the other two pathways have been blocked.

Pre-operative aspirin diminished the maximum platelet aggregatory response to collagen (10 and 20 μ l) both pre and post-operatively. This reduction in platelet aggregation was echoed with a diminished stimulated release of both the ADP and ATP, pre and post-operatively. The decrease in platelet aggregation and release is expected as low dose collagen causes liberation of arachidonic acid and thromboxane A₂ which is inhibited by aspirin. High dose collagen, however, can cause aggregation by different pathways.

Aspirin reduced the platelet aggregatory response to 10 μ l of arachidonic acid, but not 40 μ l, both pre and post-operatively. Similarly aspirin curtailed the stimulated release of both nucleotides, pre and post-operatively, in response to 10 μ l of arachidonic acid but not in response to 40 μ l of arachidonic acid. An interesting observation was the increase in platelet aggregation and stimulated

nucleotide release by 40 μ l of arachidonic acid in both groups following bypass. This may be explained by the findings of Di Minno, Silver and Murphy (1983) who discovered that the combination of arachidonic acid and collagen could produce full aggregation and nucleotide secretion when only 2.5% of aspirin-free platelets were present. These researches also found that the combination of arachidonic acid and collagen demonstrated early (four hour) entry into the circulation of platelets originating from megakaryocytes whose cyclo-oxygenase had not been inhibited.

Aspirin reduced slightly the platelet aggregation to 30 μ l of ristocetin pre-operatively only with no effect on the aggregation secondary to 40 μ l of ristocetin. Aspirin also decreased the stimulated release of both nucleotides, pre and post-operatively, in response to 10 and 40 μ l of ristocetin. An interesting observation was the reduction by bypass of both the aggregation and stimulated nucleotide release in response to 30 μ l of ristocetin, but not in response to 40 μ l, which may be due to platelets requiring more potent agonist stimulation following CPB.

The prolongation of the bleeding time both pre and post-operatively by aspirin is attributed to the reduction in platelet aggregation. The bleeding time is a physiological test of both platelet adhesion and aggregation but it is operator dependent. In addition in the context of cold peripheries, as a result of hypothermic CPB, bleeding time may not be reliable test. Nevertheless aspirin

significantly increased the bleeding time.

Pre-operative aspirin therapy did not have a significant effect, pre or post-operatively, on the platelet count, size distribution or the total ADP and ATP content of platelets. Aspirin did not influence the surface expression of platelet GPIb, the platelet and plasma vWF or multimeric patterns.

The mechanism by which aspirin alters the platelet function, in addition to the CPB induced platelet dysfunction, is predictably, a reduction of platelet aggregation pre and post-operatively mainly to arachidonic acid and partly to collagen and ristocetin. Aspirin also prolongs the bleeding time, both pre and post-operatively, but does not seem to have an effect on the components of platelet adhesion. Pre-operative aspirin, as an inhibitor of platelet aggregation, did not have a "protective effect" on platelet number and size distribution.

7.4 The bypass effect

Cardiopulmonary bypass reduced the in platelet number and mean platelet volume, as demonstrated by Martin, Daniel and Trowbridge in 1987, but increased the platelet distribution width. These changes can be explained by platelet consumption and fragmentation during CPB. Bypass also prolonged the bleeding time, regardless of the aspirin effect, which can be accounted for by the reduction in platelet count, GPIb, plasma vWF and platelet aggregation. Post-operative hypothermia, compared to pre-operative

normothermia, may also contribute to this finding as experimental studies in baboons subjected to hypothermia have demonstrated (Valeri, Cassidy, Khuri et al 1987).

A period on CPB also diminished the platelet aggregation to all agonists except 10 μ l of collagen and arachidonic acid. Platelet aggregation in response to low dose collagen and arachidonic acid was very low pre-operatively anyway and it is not possible to demonstrate a reduction in a value that is already very low. The platelet aggregation to 40 μ l of arachidonic acid was paradoxically enhanced following CPB which was echoed by an increased nucleotide release. The most plausible explanation for this finding could be that new platelets were released during bypass which were more responsive to high dose arachidonic acid than the pre-operative ones as discussed in the previous section.

Bypass had no effect on the nucleotide content of the PRP or PPP which argues against the platelet activation theory during CPB. The increase of nucleotide release in response to collagen following bypass in the aspirin group only indicates a certain "preservation" of the dense granule contents of platelets during bypass, by aspirin, which can be secreted by in vitro stimulation of arachidonic acid independent pathways.

In this study CPB reduced the expression of platelet surface GPIb as estimated by both the platelet rich plasma and the whole blood method. Argument has persisted in the literature on the explanation of this observation. Van

Oeveren and colleagues demonstrated this reduction in platelet GPIb using a PRP method of flow cytometric estimation (van Oeveren, Harder, Rozendaal et al 1990). This finding was not confirmed by Kestin and co-workers (Kestin, Valeri, Khuri et al 1993), who used a whole blood method of flow cytometric analysis, and who attributed the previous observation with the PRP method as artifactual because of in vitro activation of platelets intrinsic to the PRP method. It was postulated that this reduction may be due to thrombin activation of platelets leading to internalisation of surface GPIb (Hourdille, Heilmann, Combriè et al 1990). **The absence of evidence**, in the study by Kestin's group, **is not necessarily evidence of absence** as the results were based on only 14 patients (Altman and Bland 1995). This study therefore may not have had adequate power to detect a significant reduction in GPIb. Furthermore another recent study (Mazer, Hornstein and Freedman 1995) using the whole blood method demonstrated a significant reduction in platelet surface GPIb as shown by the use of both methods in this thesis.

This reduction in platelet surface GPIb expression could be accounted for by internalisation of surface GPIb by high plasmin levels during CPB. In addition to the evidence of excessive fibrinolysis presented in the section of literature review another recent study demonstrated increased levels of tPA and FDPs during CPB (de Haan, Schönberger, Haan et al 1993). As discussed in chapter one the majority of the fibrinolytic activity during CPB can be attributed to tPA (Stibbe, Kluft, Brommer et al 1984). In

the aspirin study the evidence for a degree of fibrinolysis during CPB was derived from the reduction of plasma fibrinogen and the increase in FDPs.

CPB reduced the plasma vWF activity and augmented the platelet vWF activity, which is a similar picture with that observed in "pseudo"-von Willebrand's disease (George, Nurden and Philips 1984). In this disorder the platelet membrane is intrinsically abnormal and platelets bind an increased amount of plasma vWF (which is entirely normal) with a greater affinity than normal, hence the other name for this disorder "platelet-type" von Willebrand's disease. The higher molecular weight vWF multimers, which are normally more reactive with platelets, are preferentially adsorbed and thereby depleted from plasma in "pseudo" von Willebrand's disease. In "pseudo" von Willebrand's disease it is assumed that the number or affinity of the von Willebrand factor receptor sites is increased. If the CPB haemostatic defect was similar to this disorder, the increased platelet binding of plasma vWF would have to be caused by an increased platelet receptor affinity for plasma vWF as the number of vWF receptors, at least the main type, is decreased during CPB.

In an endeavour to substantiate the theory of the CPB defect being similar to the "platelet-type" von Willebrand's disease, analysis of the VWF multimeric patterns were carried out before and after CPB. In this study the multimeric composition of plasma and platelet vWF, however, was not altered significantly by bypass, unlike the study by

Weinstein and colleagues (Weinstein, Ware, Troll et al 1988). These authors found that the high molecular weight multimers rose following CPB but their distribution was not altered by desmopressin.

7.5 The aprotinin trial

In this study, the reduction in blood loss by post-operative aprotinin was comparable to that seen with prophylactic use (section 1.9) but there was no significant reduction in transfusion of homologous blood. The patients in the aprotinin group, however, had Hb levels higher at the time of discharge from the hospital than the placebo group, even though the pre-operative Hb levels did not differ. The difference in the median Hb drop between the two groups was 1.6 g/dl in favour of the aprotinin group.

The failure to demonstrate a reduction in transfusion of homologous blood may have been due to the transfusion guidelines indicating transfusion at an unnecessarily high Hct. As a result of this trial the Hct threshold for the transfusion of homologous blood, in our unit, has been set at less than 25%. A possible explanation the failure to demonstrate a difference in transfusion of haemostatic factors is that the decisions for transfusion would have largely been made before any effect of the aprotinin could be clinically evident.

A similar rationale, as the aprotinin trial, was used in another study using desmopressin (Czer, Bateman, Gray et al

1987) as target therapy for excessive post-operative bleeding rather than given prophylactically to all the patients. These researches studied 16 consecutive patients in the control group and 23 consecutive patients in the treatment group. These patients were bleeding more than 100 ml/h more than two hours after bypass, which was less than the aprotinin trial in this thesis. Czer and colleagues demonstrated that desmopressin reduced the requirement of blood and blood product transfusion but without reducing post-operative bleeding. In addition desmopressin reduced re-operation for haemorrhage from 75% to 9%. The haemostatic benefit of desmopressin in this study was attributed, at least in part, to elevation of plasma vWF and shortening of the bleeding time.

7.6 The mechanism of action of aprotinin

In this thesis it was demonstrated that, in a clinical scenario of excessive post-operative bleeding following CPB, an infusion of aprotinin was associated with a greater surface expression of GPIb on platelets compared to the patients in the control group. There are several discussion points related to this effect. It is known from the aspirin study (using the MESF values), as well as previous studies (Mazer, Hornstein and Freedman 1995; van Oeveren, Harder, Rozendaal et al 1990), that the surface expression of platelet GPIb is decreased by 30-50% at the end of bypass, and gradually increases towards normal during the next 24 hours. The patients in the aprotinin study were randomised one to three hours post-bypass and started,

therefore, with a diminished level of platelet surface GPIb expression at the time of randomisation. Aprotinin significantly enhanced the surface expression of platelet GPIb starting from this low level, whereas the surface expression decreased slightly in the placebo group rather than the expected gradual increase towards normal.

As circulating platelets cannot manufacture new proteins and these patients had not received platelet transfusions between the two samples, how can this increase in GPIb receptors, starting from a subnormal level, be explained? New platelets, with higher density of surface GPIb receptors, could have been released from the bone marrow. If this was the only mechanism one would expect a similar increase in these receptors in the control group rather than the slight decrease observed.

It is known from in vitro studies that in the presence of fibrinolysis, due to high plasmin levels, these surface receptors are internalised into intra-platelet pools and when the effect of plasmin is removed they are redistributed to the platelet surface (Cramer, Lu, Caen et al 1991; Michelson and Barnard 1990). It may be that aprotinin, by inhibiting excessive fibrinolysis and reducing plasmin levels, allows redistribution of platelet GPIb receptors from intra-platelet pools to the platelet surface. Such a process would replenish the platelet surface with GPIb receptors thus making them available for platelet adhesion and improved haemostasis. It is important to emphasise that, if this theory was true, aprotinin did not increase

these surface receptors above normal levels but increased their surface expression from subnormal levels towards normal. It would also support the theory that these adhesive receptors are not irreversibly damaged by bypass but can be rescued with appropriate therapeutic interventions.

The slight reduction in platelet surface expression of GPIb in the placebo group, during the five hour study period, rather than the expected increase towards normal levels could also be explained by the excessive fibrinolysis theory. These patients started with a low GPIb surface expression, following bypass, and continued to bleed in the first three post-operative hours probably due a combination of this platelet dysfunction and excessive fibrinolysis (discussed later on in this chapter). As a result of continued presence of high levels of plasmin the GPIb receptors remained internalised and were not allowed to be redistributed to the platelet surface as would normally be expected or if the effect of plasmin was inhibited.

The discussion about the mechanism of action of prophylactic aprotinin continues in the literature. Orchard and colleagues (Orchard, Goodchild, Prentice et al 1993) demonstrated that the effect of prophylactic aprotinin was to inhibit fibrinolysis without an effect on platelet aggregation and GPIb, unlike the findings of other studies (van Oeveren, Harder, Rozendaal et al 1990). The same discussion applies to this argument as that in section 7.3. In addition, however, aprotinin being an inhibitor of a

number of serine proteases could have a different mechanism of action when used prophylactically as opposed to its therapeutic use in the different clinical situation in this thesis (discussion on fibrinolysis next page).

The platelet vWF activity was reduced during the study period in the placebo group but was maintained in the aprotinin group, but just failed to reach statistical significance. This reduction in the placebo group could be due to proteolysis or loss of the platelet vWF. The preliminary studies with proteolytic inhibitors (in the anticoagulant mixture) demonstrated that in vitro proteolysis did not significantly alter the VWF analyses, but this does not exclude a proteolytic effect *in vivo*. Aprotinin may have prevented this process in new platelets released from megakaryocytes, thus preserving another component of platelet adhesion, in the treatment group.

Although there was a dramatic reduction in the tPA activity in the aprotinin, and not in the placebo group, this is likely to have been as a result of the effect of aprotinin on plasmin *in vitro* on which this particular assay is based. Nevertheless the results of the tPA activity are still of interest for the following reasons: Firstly the patients in the placebo group had high tPA activity at the time of randomisation which continued to rise during the study period indicating increased stimulation of fibrinolysis. Secondly the aprotinin group had similar high levels of tPA activity at the time of randomisation as the placebo patients. Thirdly it indicates plasma levels of aprotinin

sufficient to inhibit plasmin in vitro. These patterns of results were further confirmed by the tPA antigen results, as estimated by an ELISA method which is not affected by aprotinin. These results suggest that, in this clinical scenario of excessive early post-operative bleeding, these patients had excessive stimulation of fibrinolysis, which continued to rise in the placebo group during the study period, but was reduced by aprotinin.

This study failed to demonstrate any statistically significant difference in FDPs and D-dimers unlike the study of Blauth and colleagues (Blauth, Gross, Necek et al 1991). This difference could be due to the fact that FDP levels depended on the rate of clearance by the reticuloendothelial system in the different clinical scenarios of the two studies. Nevertheless the average FDP levels were higher than normal (normal < 8 mg/l) indicating a degree of fibrinolysis in patients with significant bleeding. Although the FDP levels increased in the placebo group and decreased in the aprotinin group this did not reach statistical significance. Similarly the average levels of D-dimers were higher than normal (normal less than 0.5 mg/l). The fibrinogen levels increased significantly in the aprotinin group and decreased in the placebo group tending to support the theory of a reduction in fibrinolytic effect by aprotinin.

Another study (de Haan, Schönberger, Haan et al 1993) demonstrated that autotransfusion (retrasfusion of blood shed from the mediastinal drains) increased the blood loss

significantly because it increased fibrinolysis. This finding was particularly true for the patients with the heavier blood loss compared to the patients with low blood loss. As all, but five, of the patients in the aprotinin trial received autotransfused blood, as was the standard policy of our unit at the time, the clinical scenario in which the therapeutic effect of aprotinin was tested was one of excessive bleeding and fibrinolysis, with these effects possibly potentiated by the use of autotransfusion.

The platelet aggregation in response to different agonists (collagen, ristocetin and arachidonic acid) was not affected by aprotinin in agreement with the study by Orchard and colleagues (Orchard, Goodchild, Prentice et al 1993).

Three main questions remain unanswered. How long should the aprotinin infusion be continued for? Should it be for 4-5 hours or should it continue until the bleeding stops? What dose should one use? In this study the dose used was the high dose regime Royston and colleagues aimed at inhibiting Kallikrien. If the principal effect of aprotinin in the post-operative scenario is on fibrinolysis and plasmin, however, then a lower dose may still be effective. It is known from theoretical calculations as well as other studies that only a quarter of the plasma concentration of aprotinin is required to inhibit the enzyme activity of plasmin as compared to plasma Kallikrien 50 versus 200 KIU/ml (Fritz 1985; Fritz and Wunderer 1983).

7.7 Conclusion

Aspirin significantly increased post-operative bleeding, transfusion of homologous blood products and the risk of re-exploration for bleeding but did not affect the early clinical outcome. The principal effects of aspirin on the post-operative platelet dysfunction were a reduction in platelet aggregation and an increase in the bleeding time without any significant effect on the components of platelet adhesion.

CPB reduced the platelet number, platelet size, platelet aggregation by physiologic agonists. Bypass also reduced the platelet surface GPIb expression, thus reduced a major component of platelet adhesion. In addition bypass produces a picture similar to "pseudo" von Willebrand's disease with a reduction in plasma vWF activity and an enhancement of platelet vWF activity. This bypass induced reduction in platelet number, aggregation and the decline in the components of platelet adhesion resulted in a prolongation of bleeding time following a period on CPB. Furthermore, bypass produced in a reduction in plasma fibrinogen levels and an increase in plasma FDPs.

Reserving aprotinin use until excessive post-operative bleeding was detected was effective in reducing total blood loss. The most plausible mechanism of action in this situation seems to be inhibition of excessive fibrinolysis and improvement in the components of platelet adhesion with no effect on platelet aggregation.

Recommendations and changes in clinical practice

Based on the study of the therapeutic use of aprotinin and the literature of the prophylactic use of aprotinin the recommendations of this thesis are:

The prophylactic use of aprotinin is more efficacious, than its therapeutic use, in that it reduces blood loss as well as the requirement for blood products and should therefore be used for all the patients considered to be at increased risk of bleeding (repeat valve surgery, infective endocarditis, aortic dissection, pre-operative use of streptokinase, transplantation, Jehovah's witnesses and congenital disorders of haemostasis). For the remaining patients aprotinin can be reserved for post-operative use and if the patients happen to bleed excessively, they may receive aprotinin in addition to all the conventional treatment post-operatively. Our present practice may maximise the benefit of aprotinin in patients with the highest risk of haemorrhage and may minimise the risk of side effects in patients with the lowest risk of bleeding. The latter is contentious, however, as prophylactic aprotinin also reduces exposure to blood products and associated risks of transfusion related infection. The treatment of an individual patient, therefore, has to be tailored for each patient, depending on the balance of the benefit versus risk equation of aprotinin for the patient in question.

As a result of this study and a literature review on the

beneficial effect of aspirin on bypass graft patency rates we have adopted the following practice: In patients awaiting to undergo elective CABG, excluding patients with unstable angina and left main stem stenosis, aspirin is discontinued one week pre-operatively. Six hours post-operatively all patients are given aspirin through a nasogastric tube or rectally, provided they are not bleeding excessively. The rationale behind our present practice is to maximise the benefit of pre-operative aspirin therapy and to minimise the risk of pre-operative aspirin.

Future work and unanswered questions

Several questions remain unanswered for future research. How long should the aprotinin infusion be continued for? Should it be for four to five hours or should it continue until the bleeding stops completely? What dose should we use? In this study the high dose regime as recommended by Royston and colleagues aimed at inhibiting Kallikrien, was used. If the principal effect of aprotinin in the post-operative scenario is on fibrinolysis and plasmin, however, then a lower dose may still be effective. We know from theoretical calculations as well as other studies that only a quarter of the plasma concentration of aprotinin is required to inhibit the enzyme activity of plasmin as compared to plasma Kallikrien (50 versus 200 KIU/ml).

As the main effect of therapeutic aprotinin is on fibrinolysis and platelet GPIb and the effect of therapeutic desmopressin is on vWF, it would be of great interest to study the therapeutic use of a combination of aprotinin and desmopressin in patients who are bleeding excessively.

During the work for this thesis I became suspicious that the use of autotransfusion of shed blood from the mediastinal drains was inappropriate. The reasons for my suspicion were; in patients who did not bleed excessively there was not enough blood to retransfuse (thus a futile effort) and in patients that bled excessively the retransfusion of blood may have exacerbated the post CPB haemostatic defect by increasing fibrinolysis and platelet dysfunction further.

(thus prolonging post-operative bleeding). It would be of great clinical value to test both hypotheses, the clinical as well as the exacerbation of the haemostatic derangement.

This line of research did not exist in our department until this study was started. This project was initiated by myself with the help and support of the people mentioned in the introduction. This required raising funds from three main sources, purchasing new equipment, establishing new assays within our department and initiating collaboration between practising cardiac surgeons, haematologists and laboratory based personnel. This combination of expertise from the three key fields resulted in early success. As a result of the success of this prototype further projects have been carried out, with my active participation and fund raising. The cardiopulmonary bypass related defect of haemostasis has now been established as a long term research interest in the department. I hope that after this initiative the momentum and the enthusiasm will be maintained in order to answer, not only the aforementioned questions, but several more in the future.

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September 1991	British Heart Foundation research grant, £59,932 (BHF 91/130).
January 1992	Miscellaneous, £3,700.

List of publications

The following, peer-reviewed, published papers and abstracts are based on the studies presented in this thesis.

Original articles

Aprotinin inhibits fibrinolysis, improves platelet adhesion and reduces post-operative blood loss.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, D.H. Bevan, T. Treasure.

Eur J Cardiothorac Surg 1994;8;315-323.

Pre-operative aspirin decreases platelet aggregation and increases post-operative blood loss.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, D.H. Bevan, T. Treasure.

Eur J Cardiothorac Surg 1994;8;404-409.

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Aprotinin treatment rather than prophylaxis for excessive bleeding following cardiac surgery.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, T. Treasure, D.H. Bevan.

Blood 1992;80(10) Suppl;314.

Mechanisms of platelet dysfunction following cardiopulmonary bypass.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, D.H. Bevan, T. Treasure.

Br Heart J 1993;68(5, Suppl);85.

Pre-operative aspirin therapy increases post-operative blood loss following aorto-coronary bypass surgery.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, D.H. Bevan, T. Treasure.

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Preoperative aspirin therapy increases postoperative blood loss following CABG.

P. Kallis

ACCEL 1993:25(8), Side 4.

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Aprotinin therapy rather than prophylaxis for excessive bleeding following cardiopulmonary bypass.

P. Kallis, D.H. Bevan, A.J. Murday, E.E.J. Smith, D.J. Parker, T. Treasure.

Cardiac Surgical Research Club, Oxford, November 1992.
(Prize for best presentation).

Aprotinin treatment rather than prophylaxis for excessive bleeding following cardiac surgery.

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Aprotinin inhibits fibrinolysis, improves platelet adhesion and reduces post-operative blood loss following cardiac surgery.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, T. Treasure, D.H. Bevan.

International Society on Thrombosis and Haemostasis, New York, July 1993.

Pre-operative aspirin decreases platelet aggregation and increases post-operative blood loss.

P. Kallis, J.A. Tooze, S. Talbot, D. Cowans, D.H. Bevan, T. Treasure.

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European Association of Cardiothoracic Surgeons, Barcelona, September, 1993.

The following, peer-reviewed, published papers and abstracts are based on the continued research, along the same line, in our department.

A prospective randomised controlled trial of post-operative autotransfusion with and without a heparin-bonded circuit (original article).

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The effect of postoperative autotransfusion on primary haemostasis and homologous blood requirements in cardiac surgical patients.

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The abstract has been submitted to The Society of Thoracic Surgeons and the manuscript is in preparation.

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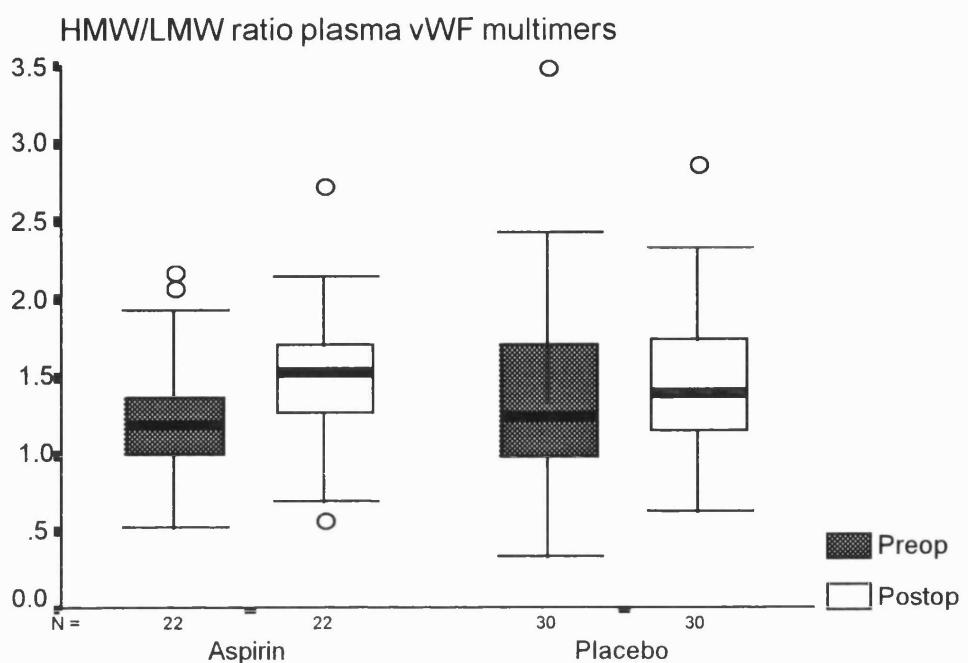
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Appendix

A



B

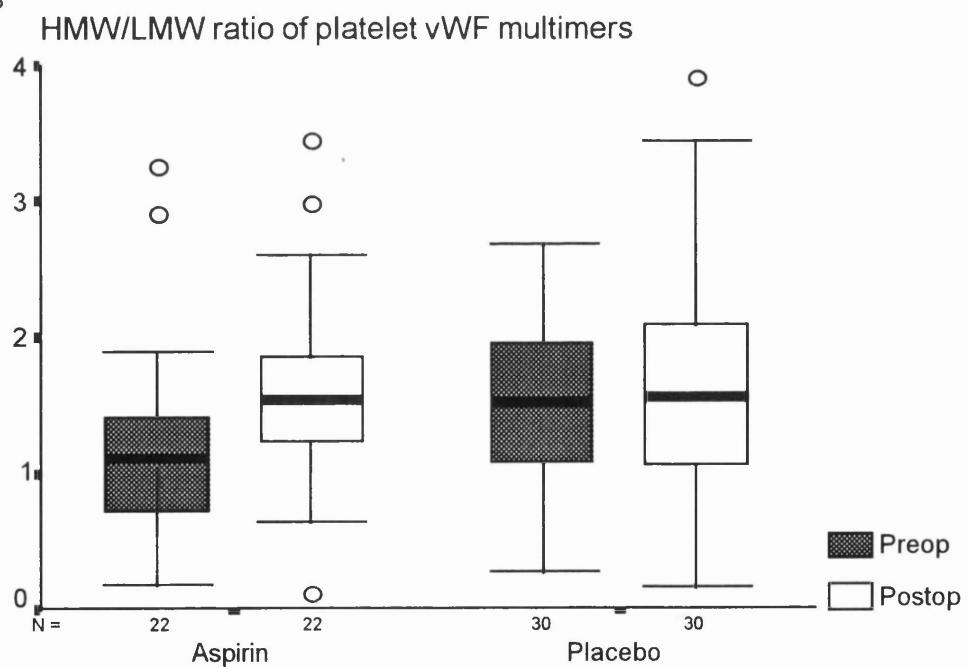


Figure 4.15 Ratio of high molecular weight / low molecular weight plasma vWF multimers (A) and platelet vWF multimers.

Pre-operative aspirin decreases platelet aggregation and increases post-operative blood loss – a prospective, randomised, placebo controlled, double-blind clinical trial in 100 patients with chronic stable angina

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Abstract. Aspirin has an established benefit in reducing the incidence of coronary events and vein graft occlusion. We have now assessed the risk of pre-operative aspirin in a prospective, randomised, double-blind clinical trial in 100 patients scheduled for elective coronary artery surgery. Any prescribed aspirin and non-steroidal anti-inflammatory drugs were discontinued 2 weeks pre-operatively and these were replaced by a randomly assigned tablet of either aspirin 300 mg daily or placebo taken until the day of surgery. Patient compliance was confirmed by serum and urinary salicylate analysis. The two groups were similar in demographic characteristics, bypass time, number of grafts placed and number of internal mammary arteries used. All patients survived to be discharged home (see Table). Aspirin decreases platelet aggregation to arachidonic acid and to collagen both pre- and post-operatively. The benefit of pre-operative aspirin has to be balanced against the risk of increasing post-operative blood loss, re-exploration for excessive bleeding and transfusion requirements.

[Eur J Cardio-thorac Surg (1994) 8: 404–409]

Variable (Mean)	Aspirin	Placebo	P value
Total blood loss (ml)	1185	791	0.001
Blood transfused (units)	3.1	2.1	0.01
Fresh frozen plasma (units)	0.4	0	0.01
Platelets (units)	0.5	0	0.05
Re-explorations (number)	4	0	0.05
Pre-operative Hb (g/l)	14	14.3	N/S
Discharge Hb (g/l)	10.8	11.5	0.001
% platelet aggregation (mean)			
Collagen pre-op	55	81	0.001
Collagen post-op	50	72	0.001
Ristocetin pre-op	90	92	0.14
Ristocetin post-op	74	81	0.06
Arachidonic acid pre-op	4.4	64	0.001
Arachidonic acid post-op	5.8	35	0.001

Key words: Aspirin – Cardiac surgery – Bleeding – Platelet function

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The benefit of pre-operative aspirin therapy in reducing ischaemic events is well established. Aspirin has been shown to reduce mortality in myocardial infarction [16], unstable angina [28] and in chronic stable angina pectoris [19]. Furthermore pre-operative aspirin was shown to reduce early vein graft occlusion in a prospective randomised trial [3]. Further evidence from clinical trials, as well as experimental evidence, suggests that there is an early post-operative phase of platelet thrombotic occlusion of

vein grafts which can be prevented by peri-operative anti-platelet therapy [7].

Several studies, however, have shown that pre-operative aspirin therapy increases post-operative blood loss, transfusion requirements and the risk of re-exploration for excessive bleeding. These studies have either not been randomised [27] or, if randomised, the patients only received one aspirin the day before surgery [25], or the trial was not blind [4], or the control group consisted of patients undergoing valve replacement in comparison to patients undergoing coronary artery bypass surgery (CABG) who were on aspirin [1]. Other workers failed to demonstrate significantly increased bleeding in patients given pre-operative aspirin [23, 31].

For these reasons argument has persisted in the literature [9, 29] about the balance of the benefit versus the risk of pre-operative aspirin therapy. One school of thought is that the increased risk of bleeding is a small price to pay for improved vein graft patency. The other is that the increased morbidity and risks of avoidable blood transfusion outweigh the small advantage of improved vein graft patency. Such was the divergence of opinion, on this benefit/risk equation, in our unit that two surgeons insisted that their patients remained on pre-operative aspirin until the day of operation and two preferred the aspirin to be discontinued 10 days earlier. The respective groups accepted that the evidence to support their practices was lacking. This clinical scenario provided the perfect environment for a prospective, randomised, placebo controlled, double-blind clinical trial with no ethical dilemmas.

The main clinical end points of the trial were volume of blood loss, homologous blood products used, re-exploration for bleeding and clinical outcome. In addition, we examined factors critical to platelet function in primary haemostasis since platelet dysfunction plays a pivotal role in the post cardiopulmonary bypass (CPB) bleeding diathesis [14, 15]. The release of arachidonic acid is stimulated by many platelet aggregating agents including collagen [5]. The effect of aspirin on platelet aggregation is due to interference with the ability of platelets to synthesise prostaglandins [26], by the irreversible acetylation of the platelet enzyme cyclooxygenase, thus inhibiting the conversion of arachidonic acid to cyclic endoperoxides and their derivatives [24]. Since platelets lack the ability to synthesise new protein, the inhibition lasts for the whole life-span of the platelet (10 days) [30].

Platelet von Willebrand Factor (vWF) is synthesised by the megakaryocyte and does not interchange with plasma vWF [2]. When released from the platelet α -granule on activation, it binds to platelet surface receptor glycoprotein (GP) IIb/IIIa [21] and is the major factor in platelet binding to collagen-coated surfaces [6]. Clinically, in variants of the congenital bleeding disorder von Willebrand's disease, platelet vWF correlates better with bleeding time than the plasma level of vWF [12] and is important in replacement therapy [18]. One proposed model of platelet function is that plasma vWF/platelet GPIb interaction mediates initial – subendothelial contact (platelet adhesion) and then platelet vWF/GPIIb/IIIa binding facilitates spreading and aggregation on subendothelium [13]. Therefore, by measuring platelet GPIb and vWF as well as ob-

serving platelet aggregation in response to three agonists, we examined major determinants of the effect of aspirin and CPB on primary haemostasis.

Material and methods

Patients

One hundred patients scheduled for elective CABG were assessed 2 weeks pre-operatively and informed written consent was obtained. All patients were instructed to stop their own aspirin and non-steroidal anti-inflammatory drugs 2 weeks pre-operatively. They were instructed not to take aspirin-containing medications and were then randomised by minimisation [22] into two groups. This was done by computer-generated number codes, rather than group A and B, so that the individual's group was not evident to any of the participants. The aspirin patients were commenced on aspirin 300 mg once a day 2 weeks pre-operatively until the day of operation. The placebo patients were commenced on placebo once a day 2 weeks pre-operatively until the day of operation. All tablets were dispensed by the hospital pharmacy according to the computer generated number codes so that both the patients and all participants were kept blind. The factors used for randomisation by minimisation were: age, gender, surgeon, number of vessels involved and left ventricular function. The exclusion criteria were: second operation, another defined coagulopathy, a history of peptic ulceration and diabetes mellitus.

Methods

The day prior to surgery all patients had their serum salicylic acid (HPLC assay) and urinary salicyluric acid (TLC assay) measured in order to check the compliance of the patients. These assays were performed by an independent worker in a different laboratory and the results were only revealed to the rest of the collaborators when the codes were broken at the end of the project.

All patients had standard anaesthesia and CPB with a Sorin Monolyth integrated membrane oxygenator and a Dideco 40 μ arterial filter. A Stockert Shiley roller pump was used and the circuit was primed with 2 l of Hartmans solution and 10 000 U of sodium heparin¹. Systemic heparinisation with sodium heparin¹ (3 mg/kg) was carried out before aortic cannulation and the activated clotting time (ACT) (Hemotec) was maintained above 400 s during CPB. After securing haemostasis and removal of the aortic cannula the heparin was reversed with protamine sulphate (1:1 ratio and until the ACT was within 20 s of the pre-operative ACT). During bypass all the blood from the pericardial and pleural cavities was returned to the CPB via the cardiotomy suction and all remaining blood in the CPB reservoir was returned to the patient at the end of bypass. Blood loss in theatre after decannulation was measured by weighing the swabs used and by measuring the volume of blood in the waste suction. All patients were connected to an autotransfusion circuit at the end of surgery according to the standard protocol of our institution.

Myocardial protection was achieved with antegrade St. Thomas' Hospital crystalloid cardioplegia (4 °C) and systemic cooling to 28 °C for two of the surgeons. The remaining two surgeons used systemic cooling to 31 °C and intermittent aortic cross-clamping with induced ventricular fibrillation. Cardiopulmonary bypass was only discontinued after full rewarming to 37 °C had been achieved.

Measurements

The following measurements were made pre-operatively and half-an-hour after the administration of protamine (all specimens were collected from a dedicated central venous line that was kept heparin free and all precautions were taken to avoid activation of plate-

¹ Leo Laboratories Ltd., Bucks, UK

lets): platelet count, platelet size distribution; haemoglobin (Hb), haematocrit (Hct); activated clotting time (ACT); clotting screen (PT = prothrombin time, KPTT = kaolin partial thromboplastin time, TT = thrombin time); fibrinogen level; bleeding time; platelet aggregation studies; platelet vWF activity and the number of GPIb platelet receptors. The following were also recorded: time from protamine to chest closure; total blood loss after protamine in theatre; hourly blood loss in the intensive care unit; bypass time; number of units of blood transfused; number of units of clotting factors transfused; clinical outcome and Hb on discharge, and ECG changes.

Guidelines for transfusion of haemostatic factors

Conventional treatment in the form of protamine and clotting factors were given to both groups depending on the results of the parameters measured and according to the following guidelines:

- If the KPTT/control ratio was >1.5 then 50 mg of protamine was given.
- If the PT/control ratio was >1.5 then 2 U of fresh frozen plasma (FFP) were transfused.
- If the platelet count was $<100 \times 10^9/l$ then 6 U of platelets were transfused if the patient was still bleeding in excess of 200 ml/h, and any abnormalities in the coagulation screen were corrected.
- If the fibrinogen level was $<1 \text{ g/l}$ then cryoprecipitate (5 ml/kg = 1 U per 5 kg) was transfused.

These results were always interpreted as a group and in combination with the clinical picture and never in isolation.

Guidelines for re-exploration

Surgical re-exploration was always an option according to the conventional guidelines under the clinical direction of the consultant in charge of the patient. The patients that had to be re-explored continued the protocol but the cause of bleeding at re-exploration was recorded.

The criteria used for re-operation were those promulgated by Kirklin and Barratt-Boyces [17].

1. More than 500 ml during the first hour. More than 400 ml during each of the first 2 h. More than 300 ml during each of the first 3 h. More than 1000 ml in total during the first 4 h. More than 1200 ml in total during the first 5 h.

2. Excessive bleeding that restarts (indicating a possible surgical cause).

3. Sudden massive bleeding.

Transfusion guidelines

During the first 24 h the need for colloid transfusion was according to our standard protocol depending on the haemodynamic variables of the patient and regular measurement of the Hb and Hct. If the following indices were met then no colloid was added: If the arterial blood pressure was $>110 \text{ mmHg}$, the central venous pressure was $>8 \text{ cm H}_2\text{O}$, the urinary output was $>0.5 \text{ ml/kg per h}$ and the patient's peripheries were warming. If these indices were not met then blood or colloid was transfused until haemodynamic improvement occurred or up to a central venous pressure of $13 \text{ cm H}_2\text{O}$. Blood was transfused when the Hct was $<28\%$ and Haemaccel when the Hct was $>28\%$. After 24 h blood transfusion was given if the Hb was $<8 \text{ g/dl}$.

Laboratory methods

Blood counts were measured using a Coulter-S-Plus counter in blood collected in Ethylenediaminetetraacetic acid (EDTA) and the platelet size distribution was measured in blood collected in 3.8% trisodium citrate (9/1 volume). Clotting screens were measured in blood

collected in 3.8% in trisodium citrate (9/1 volume). The PT was measured using the "Quick" method, the PTT using the 10-min Kaolin Thromboplastin Time and the TT using the calcium Thrombin Time. The fibrinogen levels were measured using the Clauss method. The Simplate² bleeding time (single blade) was measured on the volar surface of the forearm distal to the antecubital fossa taking care to avoid surface veins.

The platelet vWF activity (normal range 25–75 IU/dl per 10^6 platelets) was measured in blood collected in citrate/EDTA. Platelets were separated from plasma on an arabinogalactan density gradient under centrifugation. The platelet layer was washed in buffer solution before adjusting the platelet count to $1000 \times 10^9/l$. The platelet suspension was lysed with 20% triton, frozen, thawed and centrifuged prior to assay. The vWF activity was measured by the ristocetin co-factor activity, with formalin-fixed donor platelets, with a platelet aggregometer³.

Platelet-rich plasma was obtained by centrifugation, at 800 rpm at room temperature, of blood collected in 3.8% trisodium citrate (9/1 volume). Platelet aggregation was then observed straight away, with the platelet aggregometer [20]. The percent of platelet aggregation in response to three different agonists (collagen, arachidonic acid and ristocetin) was observed.

Platelet GPIb surface expression was estimated using the monoclonal antibody AN51 (DAKO) and the Becton Dickinson FACScan flow cytometer⁴. Platelet-rich plasma was prepared as described above from blood drawn into citrate/EDTA and platelets further separated from plasma by centrifuging at 1700 rpm for 10 min at room temperature. The platelets (50 μl) were then incubated in phosphate buffered saline/1% bovine serum albumin for 5 min at room temperature. The platelets (50 μl) were then labelled with FITC pre-conjugated monoclonal antibodies (10 μl), AN51 against GPIb (DAKO) and irrelevant mouse immunoglobulin (DAKO) and incubated for 30 min at room temperature. These were then washed in 9 mM EDTA/phosphate buffered saline (1700 rpm for 4 min at room temperature) and then fixed with 1% formal-saline before flow cytometric analysis.

Statistical methods

The results are expressed as the mean \pm the 95% confidence interval. Statistical comparisons were made using the Student's t-test for normally distributed data, the Mann-Whitney U test for non-parametric data and the Fisher's exact test. The difference was considered significant when P was less than 0.05.

This study was approved by the St. George's Hospital Ethical Committee.

Results

The demographic characteristics were similar in the two groups (Table 1). In addition, the two groups were similar in CPB time, number of bypass grafts, number of internal mammary arteries used, method of myocardial protection (and systemic cooling) and operating surgeon. The intensive care unit stay and the hospital stay were similar in the two groups. All patients survived to be discharged home and the only significant complication was the use of intra-aortic balloon counterpulsation in one patient who subsequently made a good recovery. There were no significant pericardial effusions requiring drainage. Three patients in each group had ECG changes of peri-operative myocardial infarction.

² Organon Teknica N.V., Turnhout, Belgium

³ PAP-4, BIO/data corporation, Hartboro, PA, USA

⁴ Becton Dickinson, Cowley, Oxford, UK

Table 1. Demographic characteristics of the patients ($P>0.05$)

Mean	Aspirin	Placebo
Male/Female	41/9	40/10
Age (years)	62	62
Height (cm)	169	170
Weight (kg)	76	75
Bypass time (min)	82	81
Number of grafts	3.5	3.4
Number of IMA	1	1.1
Intensive care stay	1.5	1
Hospital stay (days)	7.9	7.3

IMA, internal mammary artery

Table 2. Blood loss and transfusion requirements

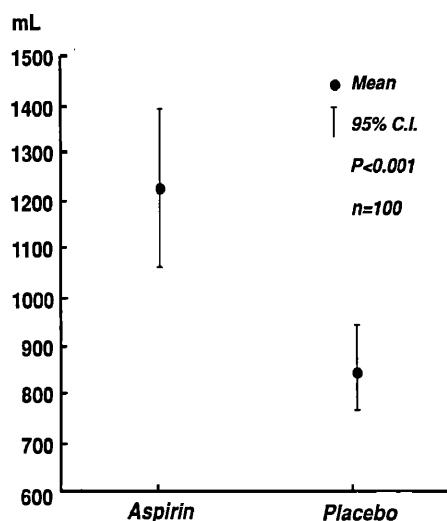
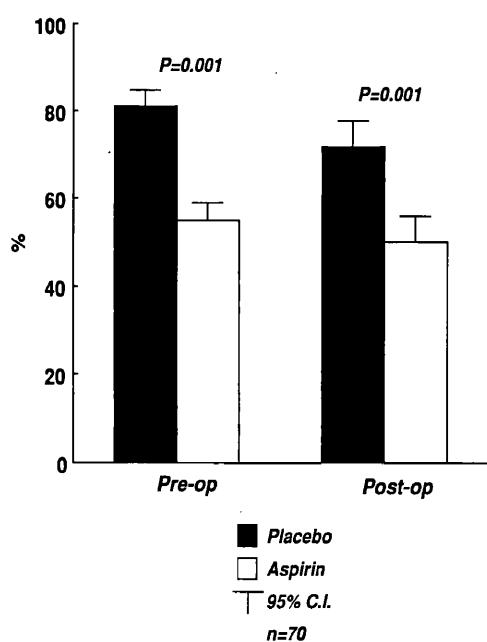
Variable (Mean)	Aspirin	Placebo	P value
Theatre blood loss (ml)	454	372	0.05
Closing time (min)	32	29	0.44
Blood transfused (U)	3.1	2.1	0.01
Colloid transfused (ml)	1610	1490	0.1
FFP (U)	0.4	0	0.01
Platelets (U)	0.5	0	0.05
Resternotomy	4	0	0.05
Pre-op Hb (g/l)	14	14.3	0.3
Discharge Hb (g/l)	10.8	11.5	0.001

FFP, fresh frozen plasma; Hb, haemoglobin

The theatre blood loss (after protamine) was significantly higher in the aspirin group, but the time required between the administration of protamine and the approximation of the sternum did not differ between the two groups (Table 2). The surgeon's impression of whether the patient was on aspirin or placebo was also noted at the end of the procedure. For the aspirin group the surgeon was correct in 42%, wrong in 48% and not certain in 10% of the cases. The corresponding figures for the placebo group were 60%, 30% and 10%. Overall, therefore, the surgeon had a 51% chance of being correct, 39% chance of being wrong and did not know in 10% of the cases.

The total post-operative blood loss was significantly higher in the aspirin group (Fig. 1), the difference being approximately one unit of blood. The patients in the aspirin group were transfused on average one unit of blood more than the patients in the placebo group (Table 2). Furthermore the patients in the two groups had similar Hb levels prior to surgery but the aspirin patients had significantly lower Hb levels on discharge from the hospital. Another striking result was the fact that no other blood products were used in the placebo group but an average of 0.4 U of FFP (eight patients) and 0.5 U of platelets (five patients) were used in the aspirin group. Four patients in the aspirin group received cryoprecipitate and none in the placebo group. In addition, four patients in the aspirin group required resternotomy for excessive bleeding but no patients required re-exploration in the placebo group.

The method of myocardial protection did not influence the volume of the post-operative blood loss. In the placebo group the mean blood loss was 770 ml for the cardioplegia patients and 810 ml for the intermittent aortic cross-

**Fig. 1.** The total post-operative blood loss of the patients in the two groups**Fig. 2.** The % platelet aggregation in response to collagen pre- and post-operatively in the two groups

clamping group ($P=0.9$). The respective blood losses in the aspirin group were 1160 ml and 1210 ml ($P=0.5$).

The serum and urinary salicylate analysis revealed that the compliance was 49 out of 50 patients in the placebo group, in other words one patient had taken an aspirin containing medication despite our instructions. The compliance was 50 out of 50 in the aspirin group.

The percent of platelet aggregation in response to collagen was significantly lower in the aspirin group both pre-operatively and post-operatively (Fig. 2). This difference between the two groups was more pronounced when arachidonic acid was the agonist (Fig. 3) but there was no significant difference in response to ristocetin (Fig. 4).

Table 3 shows that the aspirin patients had slightly higher platelet counts pre-operatively than the patients in the placebo group but similar platelet counts post-operatively. In both groups there was a significant reduction in platelet number post-operatively. Both groups had similar

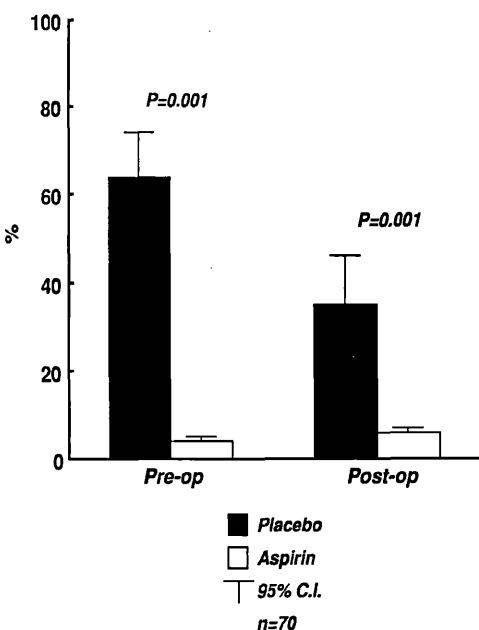


Fig. 3. The % platelet aggregation in response to arachidonic acid pre- and post-operatively in the two groups

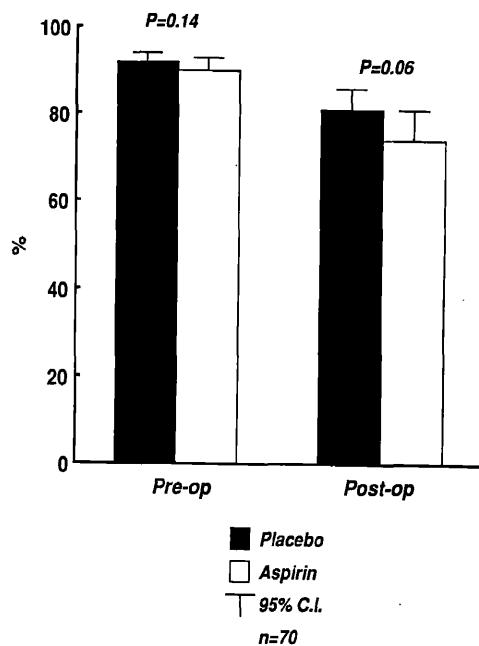


Fig. 4. The % platelet aggregation in response to ristocetin pre- and post-operatively in the two groups

platelet size distribution pre- and post-operatively. Both groups had similar platelet vWF activity and platelet surface GPIb expression but the latter decreased by 30% post-operatively. The aspirin patients had significantly longer bleeding times which were prolonged post-operatively in both groups.

Discussion

This trial of continuation of aspirin (300 mg) or placebo until surgery has demonstrated that aspirin significantly increases post-operative bleeding, transfusion requirements for homologous blood products and re-exploration

Table 3. Platelet function tests

Variable (mean \pm 95% CI)	Aspirin	Placebo	P value
Platelet count A ($10^9/l$)	214 \pm 14	185 \pm 12	0.01
Platelet count B ($10^9/l$)	111 \pm 11	105 \pm 8	0.4
MPV A (fl)	7.4 \pm 0.3	7.6 \pm 0.2	0.4
MPV B (fl)	7.3 \pm 0.2	7.2 \pm 0.2	0.6
Platelet vWF activity A	99 \pm 24	122 \pm 32	0.3
Platelet vWF activity B (IU/dl per 10^6 platelets)	149 \pm 41	153 \pm 52	0.9
Platelet GPIb A	45 \pm 3.9	43 \pm 3.8	0.5
Platelet GPIb B (Arbitrary units)	30 \pm 2.9	32 \pm 3.1	0.4
Bleeding time A (min)	6.7 \pm 0.6	5.5 \pm 0.6	0.01
Bleeding time B (min)	9.8 \pm 0.9	8.4 \pm 0.7	0.03

CI, confidence interval; A, pre-operatively; B, post-operatively; MPV, mean platelet volume; vWF, von Willebrand Factor

for excessive bleeding. In addition, there was no significant difference in intensive care unit stay, hospital stay or clinical outcome (but no assessment of vein graft patency and longer term recurrence of angina was made). Furthermore there was no adverse effect in the discontinuation of pre-operative aspirin therapy in a group of patients awaiting to undergo elective CABG and without a positive indication for aspirin therapy other than for chronic stable angina.

The mechanism by which aspirin alters the platelet function, in addition to the CPB-induced platelet dysfunction, seems to be a reduction of platelet aggregation pre- and post-operatively mainly to arachidonic acid and partly to collagen. Aspirin also prolongs the bleeding time both pre- and post-operatively, but seems not to have an effect on the components of platelet adhesion (platelet surface GPIb and platelet vWF). In addition the platelet number and size distribution were not altered by aspirin.

In our study we were able to establish the risk of continuation of pre-operative aspirin therapy in a randomised, prospective, double-blind clinical trial. There have been, however, several other recent studies that have re-assessed the possible benefit of pre-operative aspirin. These are randomised, double-blind studies comparing the effect of pre-operative aspirin (12 h) with early post-operative aspirin (1–6 h) on angiographically assessed vein graft patency in order to avoid the increased risk of bleeding. Gavaghan and coworkers [8] demonstrated significant improvement in early vein graft patency at 1 week and 1 year with 324 mg aspirin commenced at 1 h post-operatively via a nasogastric tube without any significant increase in bleeding. In another study Goldman and colleagues [11] failed to demonstrate any difference in 10-day graft patency between 325 mg of aspirin started 12 h pre-operatively and 6 h post-operatively. There was a tendency, although not significant, toward improved patency of sequential and internal mammary artery grafts with pre-operative aspirin. In another study, however, there was no difference between the internal mammary artery and vein grafts at 1 year and aspirin had no effect on patency rates [10]. Early post-operative aspirin seems to give benefits in reducing early vein graft occlusion but without increasing the risk of bleeding.

As a result of our study and a literature review on the beneficial effect of aspirin on bypass graft patency rates we have adopted the following practice: In patients waiting to undergo elective CABG (excluding patients with unstable angina and left main stem stenosis) aspirin should be discontinued 1 week pre-operatively. Six hours post-operatively all patients are given aspirin through a nasogastric tube or rectally, provided they are not bleeding excessively. We feel that our present practice maximises the benefit of pre-operative aspirin therapy and minimises the risk.

In conclusion, aspirin significantly increases post-operative bleeding, transfusion of homologous blood products and the risk of re-exploration for bleeding but does not affect the early clinical outcome. The principal effects of aspirin on the post-operative platelet dysfunction are a reduction in platelet aggregation and an increase in the bleeding time without any significant effect on the components of platelet adhesion.

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Aprotinin inhibits fibrinolysis, improves platelet adhesion and reduces blood loss

Results of a double-blind randomized clinical trial

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Abstract. The present recommendation is that aprotinin should be started before cardiac surgery, but as bleeding is only a problem in a minority, most patients are treated unnecessarily. In a prospective, randomised, double-blind trial we have studied the use of aprotinin, given only to the minority of patients who bled significantly post-operatively and who had not received prophylactic aprotinin. Sixty patients, who bled in excess of 400 ml in the first 3 h post-operatively were randomised to receive either aprotinin (2×10^6 KIU loading dose followed by an infusion of 0.5×10^6 KIU/h for 4 h) or placebo, in addition to conventional treatment. The demographic characteristics and the surgical procedures performed were similar in the two groups. Haematological variables were measured (A) before and (B) at the end of the infusion. Three patients were re-explored for excessive bleeding in each group and one patient died in each group. The patients in the aprotinin group bled significantly less and had higher haemoglobin levels on discharge than the patients in the placebo group. The tissue plasminogen activator antigen decreased and the fibrinogen level increased in the aprotinin group. In addition, aprotinin increased the number of surface GPIb platelet receptors as estimated by flow cytometry (36% versus 5%, $P < 0.01$) and maintained the platelet von Willebrand Factor activity (vWF). There was no significant difference in D-dimers, fibrin(ogen) degradation products, plasma vWF activity and antigen, platelet vWF antigen, platelet aggregation (to collagen, arachidonic acid, platelet activating factor and ristocetin), platelet count or transfusion of blood products between the two groups. Post-operative aprotinin reduces blood loss, inhibits fibrinolysis and replenishes platelet GPIb receptors and vWF activity, the components of platelet adhesion. [Eur J Cardio-thorac Surg (1994) 8:315–323]

Key words: Cardiac surgery – Aprotinin – Bleeding – Platelet function

Although there were anecdotal reports of the beneficial effect of aprotinin in reducing blood loss after cardiac surgery as early as 1964 [32], it was not until 1971 that Ambus [1] reported this effect in a randomised double-blind trial using low-dose aprotinin (100,000 KIU/h) during surgery. It took another 16 years for the efficacy of high-dose aprotinin to be reported [29] in patients undergoing repeat cardiac surgery. Further trials confirmed the efficacy of aprotinin in patients with sepsis [3] and in patients taking aspirin preoperatively [4]. Numer-

ous studies, in a variety of centres, have since confirmed the effectiveness of prophylactic aprotinin in reducing blood loss after cardiac surgery.

In early anecdotal reports and in subsequent studies, however, concern was expressed that there was a tendency for a higher incidence of peri-operative myocardial infarction in the aprotinin group [10], even though the difference did not reach statistical significance. In addition, at post mortem examination, occluded vein grafts were found only in the aprotinin group. Subsequent investigation indicated that this finding may have been due to the maintenance of the customary level of activated clotting time (ACT) during bypass around 400 s, as was essential in a blind placebo controlled trial, rather than the now recommended 700 s in the aprotinin group [17, 18]. Another study of the use of aprotinin in aortic surgery with hypothermic circulatory arrest suggested that aprotinin was associated with an increased incidence of myocardial infarction, renal failure and death [30].

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However, a review of aprotinin use in 671 cardiac patients in 41 centres in the UK [5] did not substantiate this risk. Furthermore a recent randomised, double-blind study using magnetic resonance imaging did not demonstrate any increase in occlusion rates of coronary artery bypass grafts [6].

Nevertheless, anxiety remains about the possible association of aprotinin with a higher incidence of peri-operative myocardial infarction, vein graft occlusion and renal failure. Prophylactic aprotinin has to be started prior to the commencement of surgery and, since excessive bleeding only affects a minority of patients, who cannot be reliably predicted pre-operatively, the majority of patients are treated unnecessarily. For these reasons some workers started looking at other ways of using aprotinin. One of these was the post-operative use of aprotinin in order to avoid 100% exposure. Angelini and colleagues [2] reported six cases of life-threatening bleeding which failed to respond to all forms of conventional treatment but responded to the unorthodox use of aprotinin post-operatively (2×10^6 KIU loading followed by an infusion of 500,000 KIU/h). Some workers tried to reduce the prophylactic dose [9, 20] while Tatar and colleagues [31] demonstrated that topical use of aprotinin (1,000,000 KIU) in the pericardial cavity before chest closure reduced blood loss significantly.

For these reasons we decided to perform a randomised, double-blind, clinical trial to investigate the use of aprotinin in the post-operative period restricted to those patients who bled significantly and who had not received prophylactic aprotinin. The main clinical end points of the trial were volume of blood loss, volume of homologous blood products used and re-exploration for bleeding. The randomised nature of the trial also allowed us to study haemostatic variables [22] to identify the principal factors associated with the aprotinin effect. In particular, we examined factors critical to platelet function in primary haemostasis since platelet dysfunction is central to the post cardiopulmonary bypass (CPB) bleeding diathesis. Platelet von Willebrand Factor (vWF) is synthesised by the megakaryocyte and does not interchange with plasma vWF [8]. When released from the platelet α -granule on activation, it binds to platelet surface receptor glycoprotein (GP) IIb/IIIa [26] and is the major factor in platelet binding to collagen-coated surfaces [12]. Clinically, in variants of the congenital bleeding disorder von Willebrand's disease, platelet vWF correlates better with the bleeding time than the plasma level of vWF [15] and is important in replacement therapy [23]. One proposed model of platelet function is that plasma vWF/platelet GPIb interaction mediates initial-subendothelial contact (platelet adhesion) and then platelet vWF/GPIIb/IIIa binding facilitates spreading and aggregation on subendothelium [16]. By measuring platelet GPIb and vWF together with plasma vWF, therefore we examined major determinants of primary haemostasis. As aprotinin is a non-specific serum protease inhibitor and anti-fibrinolytic, we also examined tissue plasminogen activator (tPA) levels, a major initiator of fibrinolysis, as well as the final products of fibrinolysis (fibrinogen degradation products (FDPs) and D-dimers).

Material and methods

This study was approved by the St. George's Hospital Ethical Committee.

Entry criteria

Patients who had undergone a period of CPB during the course of cardiac surgery and reached the following limits for blood loss (from the mediastinal and pleural drains) after return to the intensive care unit: a total of 200 ml at the end of the first hour, or a total of 300 ml at the end of the second hour, or a total of 400 ml at the end of the third hour. These entry criteria were set on the basis of blood loss data from our unit audit process in order to select the 10% or so patients with heavier blood loss.

Exclusion criteria

Patients who had known previous exposure to aprotinin or who had received prophylactic aprotinin in theatre and those patients with renal failure.

Randomisation

All patients who met the entry criteria were allocated a trial number and blindly allocated a code number by randomisation to one of two groups: (The patients were only identifiable by their random code number held by the pharmacist so that the individual groups were not evident to any of the participants).

The Aprotinin Group received aprotinin (Trasylol¹) 2×10^6 KIU as a loading dose followed by an infusion of 500,000 KIU/h for 4 h [2].

The Placebo Group received the same volume of normal saline in identical bottles as aprotinin, supplied by the manufacturers.

Patients. Between August 1991 and July 1992, 800 patients were operated upon who were eligible in that they did not have the exclusion criteria. Sixty patients met the entry criteria for blood loss and were randomised by minimisation [27]. The factors minimised for were age, gender, CPB time, type of operation, repeat operation and the use of autotransfusion.

Measurements

All patients had the following measured at the time of randomisation: haemoglobin (Hb), haematocrit (Hct), platelet count, platelet size distribution, clotting screen (PT = prothrombin time, KPTT = kaolin partial thromboplastin time, TT = thrombin time), fibrinogen level, FDPs, D-dimers, tPA antigen and tPA activity, plasma vWF antigen and activity, platelet vWF antigen and activity, platelet aggregation studies, platelet GPIb surface expression. All of these were measured again at 5 h after randomisation (at the completion of aprotinin/placebo infusion). The following were also recorded: hourly blood loss, total blood loss in theatre if re-explored, total blood loss (when chest drains were removed), blood transfused, platelet and clotting factors transfused, resternotomy for bleeding and cause of bleeding, Hb and Hct on discharge, clinical outcome.

¹ Bayer U.K., Newbury, England

Guidelines for transfusion of haemostatic factors

Conventional treatment in the form of protamine and clotting factors were given to both groups depending on the results of the parameters measured and according to the following guidelines:

- If the KPTT/control ratio has more than 1.5, then 50 mg of protamine was given.
- If the PT/control ratio more than 1.5, then 2 U of fresh frozen plasma (FFP) were transfused.
- If the platelet count was less than $100 \times 10^9/l$ or the patient had been on aspirin preoperatively, then 6 U of platelets were transfused provided the patient was still bleeding in excess of 200 ml/h and any abnormalities in the coagulation screen were corrected.
- If the fibrinogen level was less than 1 g/l then cryoprecipitate (5 ml/kg = 1 U/5 kg) was transfused.
- The second blood specimen was taken before platelets were transfused.

The results were always interpreted as a group and in combination with the clinical picture and never in isolation.

Guidelines for re-exploration

Surgical re-exploration was always an option, according to our usual guidelines, under the clinical judgement of the consultant in charge of the patient. The patients that had to be re-explored continued to protocol but the cause of bleeding at a re-exploration was recorded. The criteria used for reoperation were those promulgated by Kirklin and Barratt-Boyce [21].

1. More than 500 ml during the first hour.
- More than 400 ml during each of the first 2 h.
- More than 300 ml during each of the first 3 h.
- More than 1,000 ml in total during the first 4 h.
- More than 1,200 ml in total during the first 5 h.
2. Excessive bleeding that restarts (indicating a possible surgical cause).
3. Sudden massive bleeding.

Transfusion guidelines

During the first 24 h the need for colloid transfusion was established according to our standard protocol depending on the haemodynamic variables of the patient and regular measurement of Hb and Hct. If the following indices were met, then no colloid was added: If the arterial blood pressure more than 110 mm Hg, the central venous pressure more than 8 cm H₂O, the urinary output was more than 0.5 ml per hour and the patient's peripheries were warming. If these indices were not met then blood or colloid was transfused until haemodynamic improvement occurred or up to a central venous pressure of 13 cm H₂O. Blood was transfused when the Hct was less than 28% and Haemacel when it was more. After 24 h blood transfusion was given if the Hb was less than 8 g/dl.

Laboratory methods

Blood counts were measured using a Coulter-S-Plus counter in blood collected in ethylene diamine tetraacetic acid (EDTA) and the platelet size distribution was measured in blood collected in 3.8% trisodium citrate (9/1 volume). Clotting screens were measured in blood collected in 3.8% trisodium citrate (9/1 volume). The PT was measured using the "Quick" method, the PTT using the 10-min kaolin thromboplastin time and the TT using the calcium TT. The fibrinogen levels were measured using the Clauss method and the FDPs using the latex agglutination method. The D-dimers were measured using the Data-Fi Dimertest Latex Assay².

² Baxter, Irvine, CA

The tPA antigen was measured using the biopool Tint-Elize³ tPA kit, an enzyme linked immunosorbent assay (ELISA) [28] (normal range = 0.5–8.3 ng/ml) and the tPA activity using the Spectrolyse⁴/fibrin assay based on the colour change of a chromogenic substrate cleaved by plasmin [34] (normal range = 0.2–2 IU/ml). Blood was drawn into stabylite tubes, centrifuged and the plasma frozen to -70°C before analysis.

The platelet vWF activity (normal range 25–75 IU/dl per 10^6 platelets) was measured in blood collected in citrate/EDTA, and the plasma vWF activity (normal range 50–150 IU/dl) was measured in blood collected in citrate.

Platelets were separated from plasma on an arabinogalactan density gradient under centrifugation. The platelet layer was washed in buffer solution before adjusting the platelet count to $1000 \times 10^9/l$. The platelet suspension was lysed with 20% triton, frozen, thawed and centrifuged prior to assay. The vWF activity was measured by the ristocetin cofactor activity, with formalin-fixed donor platelets, with a platelet aggregometer⁵. The plasma and platelet vWF antigen was estimated using an ELISA method.

Platelet-rich plasma was obtained by centrifugation, at 800 rpm at room temperature, of blood collected in 3.8% trisodium citrate (9/1 volume). Platelet aggregation was then observed straight away, with the platelet aggregometer⁵ [25]. The percent platelet response to four different agonists was observed (collagen, arachidonic acid, platelet activating factor and ristocetin). Platelet GPIb surface expression was estimated using the monoclonal antibody AN51 (DAKO) and the Becton Dickinson FACScan flow cytometer. Platelet-rich plasma was prepared as described above from blood drawn into citrate/EDTA and platelets further separated from plasma by centrifuging at 1700 rpm for 10 min at room temperature. The platelets were then incubated in phosphate buffered in saline/1% bovine serum albumin for 5 min at room temperature. The platelets (50 μl) were then labelled with FITC pre-conjugated monoclonal antibodies (10 μl), AN51 against GPIb (DAKO) and irrelevant mouse immunoglobulin (DAKO) and incubated for 30 min at room temperature. These were then washed in 9 mM EDTA/phosphate buffered saline (1700 rpm for 4 min at room temperature) and then fixed with 1% formal-saline before flow cytometric analysis.

Statistical methods

The results are expressed as the mean \pm the 95% confidence interval. Statistical comparisons were made using the Student's t-test for normally distributed data and the Mann-Whitney U test and the Wilcoxon matched-pairs test for non-parametric data. The difference was considered statistically significant when P was < 0.05 .

Results

The two groups were similar in their demographic characteristics (Table 1) and did not differ in the number of patients on aspirin pre-operatively, CPB time, the number of bypass grafts and the number of internal mammary arteries used per patient. A similar number of patients did not receive autotransfused blood in the two groups (3 in the placebo and 2 in the aprotinin group). The types of operative procedures performed were also similar in the two groups (Table 2). Except for one in each group, these procedures were initial operations.

³ Porton Cambridge LTD, Cambridge, UK

⁴ Porton Cambridge LTD, Cambridge, UK

⁵ PAP-4 Bio/data Corp. Hatboro, PA, USA

Table 1. Demographic characteristics of the patients (mean \pm 95% confidence interval) ($P > 0.05$)

	Placebo	Aprotinin
Number	31	29
Pre-operative aspirin	19	21
Male/female	30/1	27/2
Age (years)	64 \pm 3.2	64 \pm 4
Weight (kg)	81 \pm 7.2	76 \pm 8.4
Height (cm)	172 \pm 6.2	173 \pm 3.1
Bypass time (min)	80 \pm 7.2	72 \pm 8.1
Cross-clamp time (min)	41 \pm 6	36 \pm 4.4
Number of grafts	3.2 \pm 0.3	3.1 \pm 0.3
Number of IMA	0.9 \pm 0.2	0.9 \pm 0.3

IMA, internal mammary artery

Table 2. Operative procedures performed

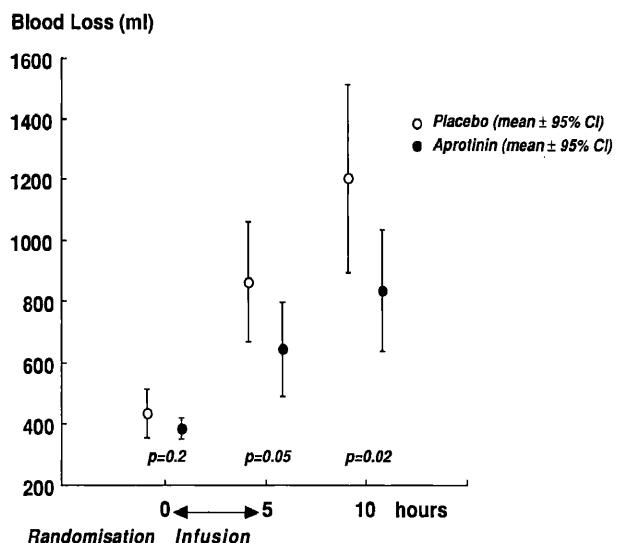
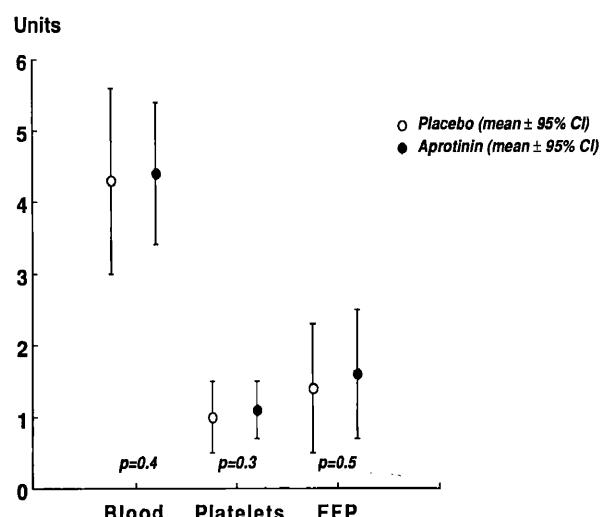
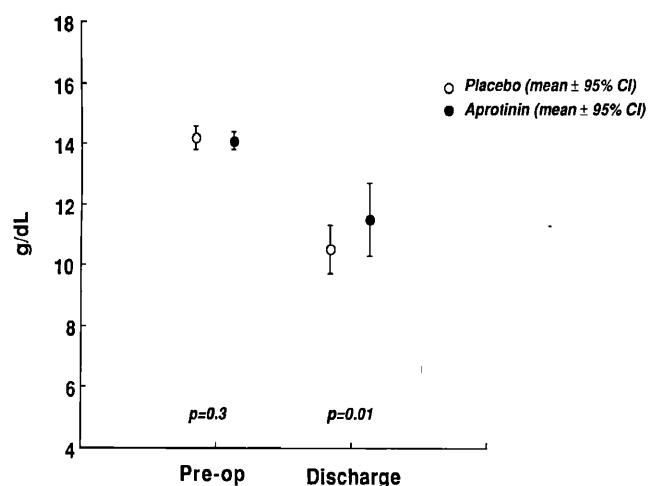
	Placebo	Aprotinin
CABG	21	22
AVR	4	2
AVR & CABG	4	3
MVR	1	1
MVR & AVR	1	1
Total	31	29

CABG, coronary artery bypass grafting; AVR, aortic valve replacement; MVR, mitral valve replacement

Table 3. Clinical outcome

	Placebo	Aprotinin
Deaths	1	1
Re-exploration for bleeding	3	3
Renal failure	0	2
Myocardial infarction	1	3
Intensive care stay (days)	1.1 \pm 0.1	1.7 \pm 0.3
Hospital stay (days)	7.1 \pm 0.2	7.3 \pm 0.3

The clinical outcome was similar in the two groups (Table 3). Renal failure and myocardial infarction occurred more often in the aprotinin group, but the cases were so few that no statistically valid conclusion could be drawn. Three patients were re-explored for continued bleeding in each group. The cause of bleeding was attributed to "a surgically preventable cause" in all of the three patients in the aprotinin group and in two of the three patients in the placebo group. A 79-year-old man with end-stage aortic stenosis died 48 h post-operatively in the placebo group. He required inotropic support and intra-aortic balloon counterpulsation to be weaned from CPB and was re-explored for continued bleeding before he died from low cardiac output. In the aprotinin group a 73-year-old man died after coronary artery bypass surgery and re-exploration for continued bleeding. He had a history of mesenteric angina and required resection

**Fig. 1.** The total blood loss before the commencement of the aprotinin/placebo infusion and at 5 and 10 h later**Fig. 2.** Transfusion of homologous blood and blood products (FFP fresh frozen plasma)**Fig. 3.** Pre-operative and discharge haemoglobin levels of the patients

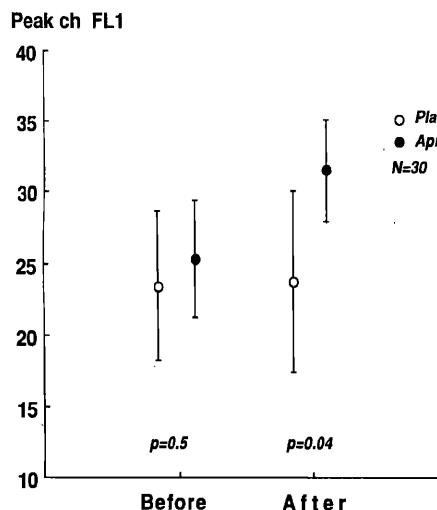


Fig. 4. Platelet GPIb surface receptor expression before and after the aprotinin/placebo infusion (*Peak ch FL1* peak fluorescence channel: arbitrary units)

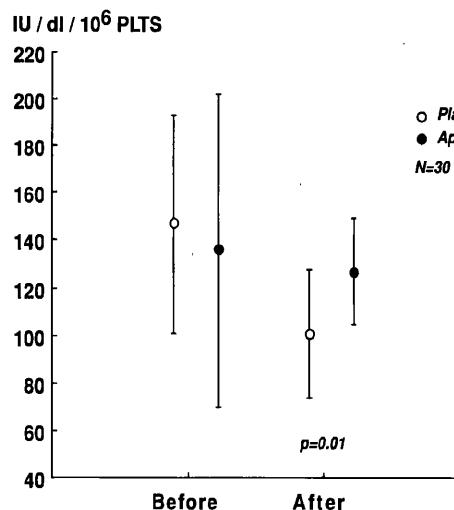


Fig. 5. Platelet (PLTS) von Willebrand Factor activity before and after the aprotinin/placebo infusion

of his small bowel for mesenteric ischaemia. He died from multisystem failure 16 days post-operatively. Histopathological examination did not reveal any evidence of thrombus or embolus in the mesenteric vessels.

Figure 1 shows the total blood loss before the commencement of the aprotinin/placebo infusion and at 5 and 10 h later. The patients in the aprotinin group bled significantly less, both at 5 h and 10 h, than the placebo group, even though the two groups had similar blood losses at the time of randomisation. The total blood loss (at the time of removal of the chest drains) was also significantly lower in the aprotinin group (1460 ± 350 ml versus 1938 ± 430 ml, $P < 0.05$). We did not find a significant difference between the two groups in terms of transfusion of homologous blood products (Fig. 2). The aprotinin patients, however, had significantly higher haemoglobin levels on discharge from the hospital than the placebo group, even though their admission haemoglobin levels had been similar (Fig. 3).

Post-therapy platelet surface expression of GPIb was significantly higher in the aprotinin group, whereas it remained unchanged in the placebo group (Fig. 4) ($P = 0.04$). The platelet vWF activity decreased significantly in the placebo group during the study period but was maintained in the aprotinin group ($P = 0.01$) (Fig. 5).

The tPA activity was high in the placebo group at the time of randomisation and it continued to rise during the study period (Fig. 6). The tPA activity in the aprotinin group was similar to that in the placebo group at the time of randomisation but it decreased during the study period. The last result is probably due to the in vitro effect of aprotinin on this assay indicating plasma levels of aprotinin sufficient to inhibit plasmin in vitro. For this reason we also measured tPA antigen, which demonstrated similar changes to tPA activity, albeit to a lesser degree. The tPA antigen increased in the placebo group during the study period and decreased in the aprotinin group, but only the difference in the values (before and after the

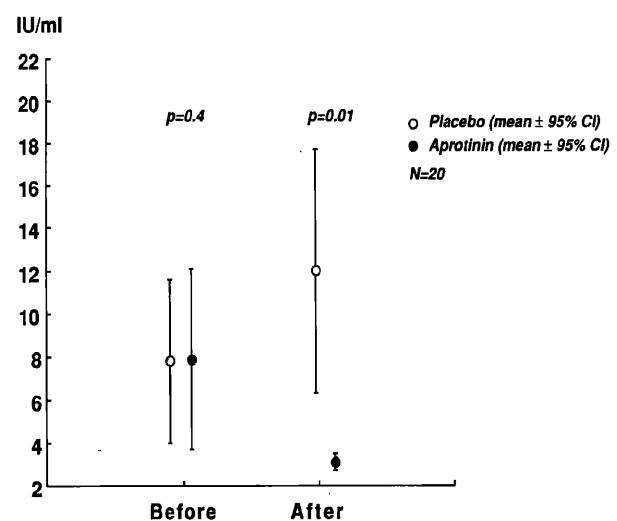


Fig. 6. Tissue plasminogen activator activity before and after the aprotinin/placebo infusion

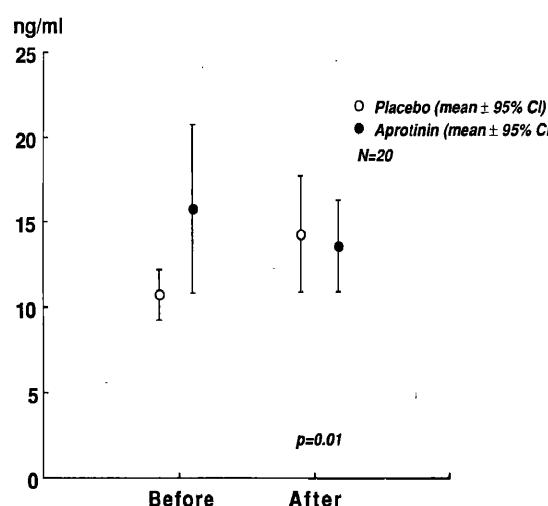


Fig. 7. Tissue plasminogen activator antigen before and after the aprotinin/placebo infusion

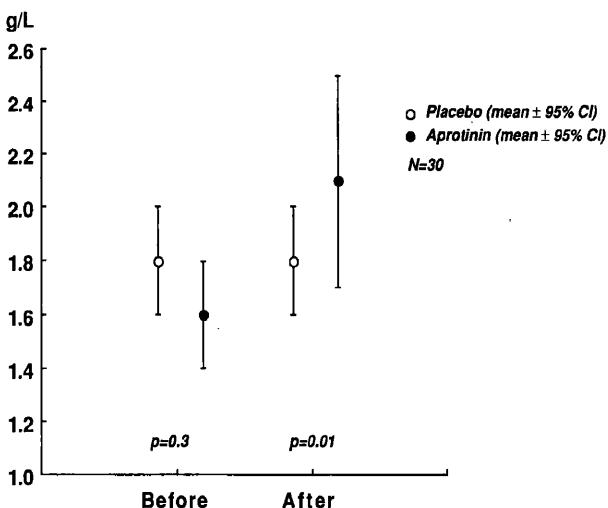


Fig. 8. Fibrinogen levels before and after the aprotinin/placebo infusion

Table 4. Haematological variables, mean \pm 95% confidence interval ($P < 0.05$)

Variable	Aprotinin	Placebo
Platelets ($\times 10^9/l$) A	133 \pm 27	117 \pm 16
Platelets ($\times 10^9/l$) B	122 \pm 20	109 \pm 20
FDPs (mg/l) A	18.9 \pm 5.6	18.8 \pm 8.4
FDPs (mg/l) B	16.5 \pm 4.8	21.1 \pm 7.8
D-dimers (mg/l) A	1.7 \pm 0.6	1.5 \pm 0.6
D-dimers (mg/l) B	1.5 \pm 0.8	1.6 \pm 0.5
Plasma vWF activity (IU/l) A	142 \pm 34	121 \pm 39
Plasma vWF activity (IU/l) B	135 \pm 21	126 \pm 25
Plasma vWF antigen (IU/l) A	121 \pm 31	119 \pm 19
Plasma vWF antigen (IU/l) B	130 \pm 31	146 \pm 27
Platelet vWF antigen A	86 \pm 32	68 \pm 13
Platelet vWF antigen B (IU/dl per 10^6 platelets)	73 \pm 25	49 \pm 12

FDPs = fibrin(ogen) degradation products, vWF = von Willebrand Factor

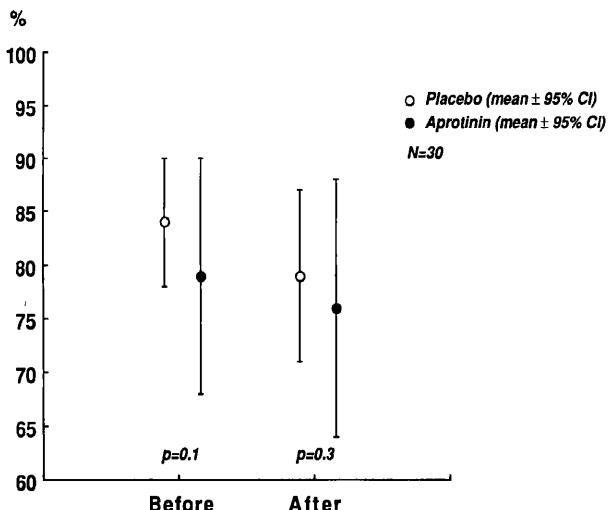


Fig. 9. Platelet aggregation to ristocetin before and after the aprotinin/placebo infusion

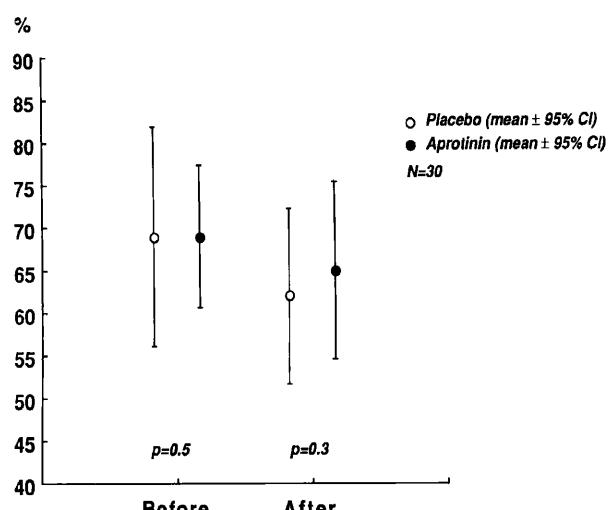


Fig. 10. Platelet aggregation to collagen before and after the aprotinin/placebo infusion

infusion) reached statistical significance ($P = 0.01$) (Fig. 7). The fibrinogen levels increased significantly in the aprotinin group (Fig. 8).

The percent platelet aggregation in response to ristocetin (Fig. 9) and collagen (Fig. 10) did not differ in the two groups. Similarly, there was no difference in aggregation in response to arachidonic acid and platelet activating factor. There was no significant difference in platelet count, FDP, D-dimers, plasma vWF activity and antigen and platelet vWF antigen between the two groups (Table 4).

Discussion

In this study, the reduction in blood loss by post-operative aprotinin is comparable to that seen with prophylactic use [4, 10] but we failed to demonstrate a significant reduction in transfusion of homologous blood. The patients in the aprotinin group, however, had 1 g/dl Hb levels higher on discharge from the hospital than the placebo group, even though the pre-operative Hb levels had not differed. Perhaps we failed to demonstrate a reduction in transfusion of homologous blood because of our transfusion guidelines indicating an unnecessarily high Hct. As a result of this trial we have subsequently lowered our Hct threshold for the transfusion of homologous blood, to less than 25%. A possible explanation for not demonstrating a difference in transfusion of haemostatic factors is that the decisions for transfusion would have largely been made before any effect of the aprotinin could be clinically evident.

The platelet surface expression of GPIb was significantly increased (36%) by aprotinin. As circulating platelets cannot manufacture new proteins and these patients had not received platelet transfusion between the two samples, how can this increase in GPIb receptors be explained? New platelets, with higher density of surface GPIb receptors, could have been released from the bone

marrow. If this was the only mechanism one would expect a similar increase in these receptors in the control group. We know from previous studies [19, 33] that the surface expression of platelet GPIb is decreased by 30–50% during CPB. We also know from in vitro studies that in the presence of active fibrinolysis due to high plasmin levels these surface receptors are internalised into intraplatelet pools and when the effect of plasmin is removed they are redistributed to the platelet surface [11, 24]. We would suggest that aprotinin, by inhibiting excessive fibrinolysis and reducing plasmin levels, allows replenishment of the platelet surface GPIb receptors from intraplatelet pools and makes them available for platelet adhesion.

The platelet vWF activity was reduced during the study period in the placebo group but was maintained in the aprotinin group. This reduction in the placebo group could be due to proteolysis or loss of the platelet vWF: aprotinin may have prevented this process in new platelets released from megakaryocytes, thus preserving another component of platelet adhesion, in the treatment group.

Although there was a dramatic reduction in the tPA activity in the aprotinin and not in the placebo group this was probably as a result of the effect of aprotinin on plasmin in vitro, on which this particular assay is based. Nevertheless the results of the tPA activity are still of interest for the following reasons: firstly the patients in the placebo group had high tPA activity at the time of randomisation which continued to rise during the study period, indicating increased stimulation of fibrinolysis; secondly, the aprotinin group had similar high levels of tPA activity at the time of randomisation to the placebo patients; thirdly, it indicates plasma levels of aprotinin sufficient to inhibit plasmin in vitro. These patterns of results are further confirmed by the tPA antigen results, as estimated by an ELISA method which is not affected by aprotinin.

In this study we failed to demonstrate any statistically significant difference in FDPs and D-dimers, unlike Blauth and colleagues [7]. This difference could be due to the fact that FDP levels depended on the rate of clearance by the reticuloendothelial system in the different clinical scenarios of the two studies. Nevertheless, the average FDP levels were higher than normal (normal being less than 8 mg/l) indicating excessive fibrinolysis in patients with significant bleeding. Although the FDP levels increased in the placebo group and decreased in the aprotinin group, this did not reach statistical significance. Similarly, the average levels of D-dimers were higher than normal (normal being less than 0.5 mg/l). The fibrinogen values were significantly higher ($P = 0.01$) in the aprotinin group, tending to support a reduction in fibrinolytic effect.

The platelet aggregation is response to four different agonists (collagen, ristocetin, arachidonic acid and platelet activating factor) did not differ in the two groups.

As a result of this study we can conclude that reserving aprotinin use until excessive post-operative bleeding is detected is effective in reducing total blood loss. The most plausible mechanism of action in this situation seems to

be inhibition of fibrinolysis and improvement in the components of platelet adhesion with no effect on platelet aggregation.

Two main questions remain unanswered. How long should the aprotinin infusion be continued for? Should it be for 4–5 h or should it continue until the bleeding stops? What dose should we use? In our study we used the high-dose regime Royston and colleagues aimed at inhibiting kallikrein [29]. If the principal effect of aprotinin in the post-operative scenario is on fibrinolysis and plasmin, however, then a lower dose may still be effective. We know from theoretical calculations, as well as other studies, that only a quarter of the plasma concentration of aprotinin is required to inhibit the enzyme activity of plasmin as compared to plasma kallikrein 50 vs 200 KIU/ml [13, 14].

In conclusion aprotinin used after excessive bleeding is detected following cardiac surgery reduced total blood loss. Its principal actions seem to be the inhibition of fibrinolysis, enhancement of platelet surface GPIb receptors, maintenance of platelet vWF levels and, thereby, improvement of primary haemostasis.

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Discussion

Mr. D. Wheatley (Glasgow, UK). That was a very interesting paper, very important, but I think from what you have said you are showing a saving of approximately half a liter of blood loss which, in the context of excessive bleeding, is perhaps not a lot. One has to counter that against the worry that one still has about the risk of thrombotic complications. Could I press you a little bit about the three myocardial infarcts and did you have any other, as I am sure you did, any other measures of postoperative myocardial infarction of this nature?

Mr. P. Kallis: In this study we only looked at clinical outcome and ECG evidence as end points because the number of patients was too small to detect a statistical difference in myocardial infarction. The problem with all the aprotinin trials is that they have all showed a tendency for a higher incidence of myocardial infarction in the treatment group but in none of them has this difference reached statistical significance. As to the question of the balance of the tendency for higher myocardial infarction in the treatment group against the saving of half a litre of blood I would like to emphasise that we used aprotinin in a different clinical scenario. We used it in only 60 patients, who happened to bleed excessively, rather than prophylactically in all of the 800 patients that were operated on in

our unit during the same time period. We have no hesitation, therefore, in using a drug that has been shown to reduce bleeding significantly in patients that are actively bleeding. Furthermore the recent data from Bidstrup and colleagues demonstrated that aprotinin does not influence aortocoronary bypass graft patency.

Dr. J. Bidstrup (Jeddah, Saudi Arabia). I have got a number of questions for you, *Dr. Kallis*. First of all, what about the rate of decrease of bleeding? Have you got any data on that to show whether the bleeding rate decreased more rapidly in the patients that were treated with the drug? Secondly, what about the relationship of the GP1b receptors? I noticed in your abstract you have quite low values when you compare them to some of the data that Alan Michelsen has recently published. Were you able to correlate the changes of GP1b receptor with the amount of blood loss in the patients that bled? Can you compare this with other data from one of your other studies that you have done with it, looking at the GPIb? You stated that all the patients received some form of conventional therapy. I noticed there was a marked change in fibrinogen levels. Can you tell us about the volume of, say, platelets and FFP that the patients received in each group?

Mr. P. Kallis: The answer to your first question is that although we recorded the hourly blood loss from the time of return to the inten-

sive care unit until the drains were removed, we only performed the statistical analysis at 5 and 10 h after randomization and at the time of removal of the chest drains, as presented here.

The answer to your second question is that the values of the GPIb receptors given are arbitrary values of peak fluorescent channel which are dependent on the calibration of the individual flow cytometer used. One cannot, therefore, compare these arbitrary values between different laboratories. In this particular study we did not correlate the concentration of the GPIb receptors with blood loss because the number of patients was too small. When we compare the receptors with the aspirin trial, that we presented 2 days ago, the number of receptors in the postoperative scenario were approximately 30% less than they were in the aspirin study, because the patients in the aprotinin trial were heavier bleeders than in the aspirin trial.

The answer to your last question is that the average number of units of platelets per patient was 1.6 in the aprotinin group and 1.4 in the placebo group. The corresponding figures for fresh frozen plasma (FFP) were 0.9 units per patient in the aprotinin group and 1.4 in the placebo group. I would like to emphasize that the second blood specimen for measurement of the haematological variables mentioned was drawn before any platelets of FFP were transfused.

Dr. B. Bidstrup: I think probably then the conclusion about fibrinogen, for instance, is by reducing the amount of bleeding you are reducing the consumption of fibrinogen.

Mr. P. Kallis: Precisely.

Dr. E. Gams (Homburg/Saar, Germany): Did you measure the aprotinin level during the postoperative phase?

Mr. P. Kallis: We did not measure the actual aprotinin level itself, but one of our assays, the tPA activity, which is based on the conversion of plasminogen to plasmin, demonstrated that the plasma aprotinin levels were adequate to inhibit plasmin totally in vitro. This was an indication, therefore, that we had given enough aprotinin to the treatment group.

Dr. E. Gams: Well, the second question refers to the split products of fibrinogen, e.g. D-dimers. Did you measure these? And can you comment on the use of aprotinin not only pre- and intra-operatively, but also post-operatively?

Mr. P. Kallis: The efficacy of prophylactic aprotinin has been established in several studies. Our study demonstrates, for the first time, that aprotinin can be reserved until the post-operative period and it is still effective in reducing excessive blood loss. One can not extrapolate from these two findings, in two different scenarios, and change the indication for aprotinin and use it pre-, intra- and post-operatively. The only way of answering this question is by performing a randomized trial using aprotinin as you suggested.

Dr. F. Fontan (Bordeaux, France): May I ask you what percentage this group of 60 patients represents in the total group of coronary patients operated upon during the same period of time?

Mr. P. Kallis: It's 60 patients out of a group of 800 that went through the unit. Now, out of those, there were about 60 to 80

patients who would have received prophylactic aprotinin and obviously were not eligible while the rest of the patients were eligible. So the percentage would be roughly 10% of all the patients that went through the unit during the time. So we only treated the patients that happened to bleed as opposed to everybody.

Dr. F. Fontan: You gave information on the preoperative and at discharge hemoglobin levels but you did not provide any information on the immediate postoperative hematocrit or hemoglobin levels, which are very important with regard to the preservation of the coagulation system. Could you clarify this situation.

Mr. P. Kallis: Obviously in a presentation of 5 min it is impossible to show all the data, but certainly at the time of randomization there was no difference between the groups in terms of hematocrit and hemoglobin levels.

Dr. F. Fontan: I don't mean differences. I asked for the levels.

I think it is very important to have, at the end of the bypass hematocrit and hemoglobin as close as possible to normal. We try to have as little hemodilution as possible, and since we have adopted this policy, the amount of bleeding has considerably decreased in our unit. So I would like to ask you about your policy with regard to hemodilution and what are the levels of hematocrit and hemoglobin at the end of the bypass and in the following hours, if you know that.

Mr. P. Kallis: I don't recall the exact average figures for hematocrit and hemoglobin, but I know that there was no significant differences between the two groups in terms of platelet count, hematocrit and hemoglobin at the time of randomization. In addition we had standard guidelines for the transfusion of blood and blood products according to our hematocrit and hemoglobin that were measured regularly during those 24 h.

Dr. A. Piwnica (Paris, France): I am sorry, that is not the question. What is the usual level that you accept in your unit?

Mr. P. Kallis: The actual level was 28%.

Dr. A. Piwnica: Okay, thank you.

Dr. F. Fontan: That would be a reason for excessive bleeding. I think that it is more important to have the patient's own individual coagulation system preserved than to add drugs like aprotinin. They can be very helpful, but first we have to make an effort to preserve the patient's system.

Mr. P. Kallis: Are you suggesting that the hematocrit should be higher than 28%?

Dr. F. Fontan: Yes.

Mr. P. Kallis: It is an important point and it would be interesting to investigate further, but that was the standard policy in our unit. In fact most units in Britain have changed to a lower hematocrit, between 22 and 25% which is even lower than our unit, in order to increase the proportion of patients that have cardiac surgery without any blood transfusion.