The Evaluation of Tirofiban Hydrochloride in a High Shear Rate Arterial Thrombosis Model in Baboons

By

Walter James Janse van Rensburg

Dissertation submitted in fulfilment of the requirements of the degree

M.Med.Sc in Haematology

At the Department of Haematology and Cell Biology, School of Medicine, Faculty of Health Sciences, University of the Free State, Bloemfontein.

November 2009

Study-leader: Prof. S.M. Meiring

Co-study-leader: Mr. J.P. Roodt

Declaration

I, Walter James Janse van Rensburg, declare that the dissertation hereby handed in for the qualification Master in Haematology at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at/in another University/faculty. I hereby also concede copyright of the dissertation to the University of the Free State.

____

WJ Janse van Rensburg

November 2009

Table of Contents

Chapter **Page**

List of Figures and tables.......... **v-vi**

Abbreviations.......... **vii**

Chapter 1 **Introduction**.......... **1-3**

Chapter 2 **Literature Review**

2.1 Haemostasis, platelets, atherosclerosis and acute coronary syndrome.......... **4-7**

2.2 Current medication available for acute coronary syndrome.......... **7-11**

2.3 The need for more effective and safe medication.......... **11-12**

2.4 Tirofiban hydrochloride.......... **12-14**

2.5 Experimental animals.......... **15-16**

2.6 Animal thrombosis models.......... **16-19**

2.7 Modified animal thrombosis model.......... **19**

2.8 Bleeding models.......... **19-20**

Aim.......... **20**

Chapter 3 **Method**

3.1 Experimental animals.......... **21**

3.2 Anaesthetic.......... **21-22**

3.3 Overview of experiment.......... **22-24**

3.4 Surgical procedure.......... **24-26**

3.5 Laboratory analysis.......... **27-32**

3.6 Post-operative care.......... **32**

3.7 Statistical analysis.......... **32**

Chapter 4 **Results**.......... **33-42**

Chapter 5 **Discussion**.......... **43-47**

Chapter 6 **Conclusion**.......... **48**

Chapter 7 **Summary**.......... **49-50**

Hoofstuk 7 **Opsomming**.......... **51-52**

References.......... **53-61**

Appendix A – Data tables.......... **62-76**

Appendix B – Roodt *et al*.......... ***77-101***

Acknowledgements.......... **102-103**

List of Figures and Tables

Figure/Table **Page**

Figure 1: Example of Thrombogenic device used in the Hanson model........ **18**

Figure 2: Schematic presentation of CFR formation........ **23**

Figure 3: Results of CFRs during a previous experiment, showing full inhibition after injection of 0.1 mg/kg of anti-platelet drug........ **25**

Figure 4: Experimental setup for the arterial thrombosis model in baboons........ **26**

Figure 5: Example of ADP induced platelet aggregation........ **29**

Figure 6: Schematic presentation of the TEG........ **30**

Table 1: Normal Full Blood Count – Caucasian Males........ **32**

Figure 7: A typical results graph of the efficacy study for tirofiban hydrochloride........ **33**

Figure 8: Dose effect on number of CFRs/30 minutes........ **34**

Figure 9: Dose effect on ADP platelet aggregation........ **34**

Figure 10: Dose effect on blood loss........ **35**

Figure 11: Dose effect on Bleeding Time........ **36**

Figure 12: Dose effect on PT and aPTT........ **36**

Figure 13: Dose effect on Haematological Parameters............................ **37**

Figure 14: Results for different TEG Parameters..................................... **38**

Figure 15: Example of efficacy study with clopidogrel.............................. **39**

Figure 16: Dose effect of clopidogrel on blood loss.................................. **39**

Figure 17a: SEM image of surface of an intact uninjured artery at 3000x magnification.. **40**

Figure 17b: SEM image of surface of an intact uninjured artery at 3600x magnification.. **40**

Figure 17c: SEM image of surface of an injured artery covered with aggregating platelets at 3000x magnification................... **41**

Figure 17d: SEM image of surface of an injured artery covered with aggregating platelets at 3600x magnification................... **41**

Figure 18: Compilation of efficacy study results of all 5 baboons............ **42**

Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
aPTT	Activated partial thromboplastin time
BT	Bleeding time
cAMP	Cyclic adenosine monophosphate
CFR	Cyclic flow reduction
CVD	Cardiovascular disease
ECG	Electrocardiogram
FBC	Full blood count
FXa	Activated Factor X
GP	Glycoprotein
Hb	Haemoglobin
HDB	High-dose bolus
LDL	Low-density lipoprotein
LMWH	Low molecular weight heparin
MCV	Mean corpuscular volume
PCI	Percutaneous coronary intervention
Plt	Platelet
PT	Prothrombin time
RGD	Arg-Gly-Asp
SEM	Scanning electron microscope
STEMI	ST-elevation myocardial infarction
TEG	Thromboelastography
vWF	Von Willebrand Factor
WBC	White blood cell count
WHO	World Health Organization

Chapter 1

Introduction

The definition of haemostasis according to Sherwood (2004) is “the arrest of bleeding from a broken vessel” (Sherwood, 2004). Under high shear conditions exposed subendothelium is first coated by von Willebrand Factor (vWF) by binding to the subendothelial collagen. Platelets then adhere to the exposed subendothelium, with vWF acting as a bridge. This stimulates the activation of the platelet that leads to firmer adherence of the platelets to the vWF. Platelets aggregate to form a platelet plug (thrombus) at the place of vessel injury by cross-linking. The cross-linkage results in firmer connection and further activation of platelets. Platelets therefore play a key role in haemostasis (Hoffbrand *et al*., 2006). Haemostasis is a balance between clot formation and bleeding. If the balance is shifted towards clot formation, as in atherosclerosis, it may lead to life threatening situations such as acute coronary syndrome (ACS).

Atherosclerosis is a progressive and degenerative disease of the arteries, and leads to a gradual occlusion of affected arteries. It is characterised by the development of atherosclerotic plaques (Sherwood, 2004). These plaques consist of a lipid-rich atheromatous core, covered by a hard collagen rich sclerotic tissue cap. The sclerotic cap can rupture, leading to the sudden exposure of the highly thrombogenic lipid core to the flowing blood (Falk *et al*., 1995). Platelets then aggregate upon this surface to form thrombi (Fenton, 2008). Disrupted plaques are the major cause of thrombosis in ACS cases (Falk *et al*., 1995).

ACS is a collective term used to describe coronary artery diseases, which include unstable angina, ST-elevation myocardial infarction (STEMI) and non-STEMI. The early presentation

and initial management of these diseases are frequently very similar (Achar *et al*., 2005). Unstable angina and non-STEMI is the main cause of mortality and morbidity in the Western world, and is responsible for roughly 2.5 million hospital admissions annually worldwide (Grech and Ramsdale, 2003). Thrombotic complications are also increasing in patients with HIV and AIDS in developing countries (Saif and Greenberg, 2001). In one study, the incidence of HIV in patients presenting with acute thrombosis was as high as 84% (Louw *et al*, 2008). The World Health Organisation estimates that by 2015 almost 20 million people will die of cardiovascular disease (CVD) annually, this includes ACS (WHO Factsheet No. 317, 2007).

The remaining high incidence of deaths world-wide due to ACS gives rise to the need to develop antithrombotic drugs with an improved therapeutic window, thus with better efficacy but with minimal side-effects. With the development of safer and more effective antithrombotic drugs, there is an ongoing need to develop safe and effective animal models to evaluate these antithrombotic agents. Lower mammals (e.g. rats, mice, rabbits etc.) are usually used as test subjects to screen for novel antithrombotic agents. After a promising antithrombotic agent is identified, the agent is further evaluated in more discriminating animal models. In these models, species are selected with similar blood coagulation, platelet adhesion and aggregation and fibrinolytic systems as that of humans. Non-human primates are thus the best suited animals taking these requirements into consideration (Mason *et al*., 1976).

A safe and effective anti-platelet drug for which little clinical data is available is tirofiban hydrochloride. This drug is used to help treat and prevent platelet thrombus formation during ACS. Some of the studies done on tirofiban hydrochloride however suggested that the recommended therapeutic dose is suboptimal, and this led to the misconception that it is not as effective as other anti-platelet drugs in preventing ischaemic events associated with ACS (Topol *et al*., 2001).

This study was therefore designed to evaluate the efficacy and safety of different dosages of tirofiban hydrochloride in our high shear rate arterial thrombosis model in baboons, and to compare these results with those from previous studies done on other anti-platelet drugs, viz clopidogrel, in the same model.

Chapter 2

Literature Review

2.1 Haemostasis, platelets, atherosclerosis and acute coronary syndrome

Haemostasis is the process where blood loss from the body is stopped, and is mainly attributed to fibrin generation and platelet aggregation. These two processes synergistically work together to cause haemostasis, with fibrin generation primarily responsible for haemostasis in the venous circulation and platelet aggregation mainly responsible for arterial haemostasis. If the process of haemostasis is pathological it is referred to as thrombosis. Both haemostasis and thrombosis are initiated by blood vessel lining (endothelium) damage, which is associated with the exposure of the thrombogenic subendothelium components (Kroll, 2001).

Platelets accomplish their haemostatic role by forming a platelet plug at the place of endothelium damage. Platelets achieve this via the processes of adhesion, activation, aggregation and recruitment. Firstly platelets adhere to the exposed subendothelium components (collagen, fibronectin, von Willebrand factor (vWF), laminin, vitronectin and thrombospondin) via special glycoprotein (GP) platelet membrane receptors. After adhesion to the subendothelium, platelets are activated and intracellular signalling leads to further expression of more functional GP receptors, like GP IIb/IIIa. Intracellular signalling also leads to granular secretion of substances, such as thrombin and vWF that enhance the adherence and aggregation of the platelets. GP IIb/IIIa receptors function as receptors for fibrinogen and vWF, which acts as a bridge between platelets. Aggregation thus takes place when fibrinogen and vWF cross-link neighbouring platelets via the GP IIb/IIIa receptors (Harker and Mann, 1992). During the normal haemostatic process, active GP IIb/IIIa receptors bind to fibrinogen

and bridges or cross-links platelets. This cross-linkage leads to conformational changes within the platelet membrane, that cause firmer connection and results in further platelet activation. An activated platelet GP IIb/IIIa complex also binds to vWF, which in turn is bound to exposed collagen in damaged subendothelium. This process helps to capture platelets at the site of vascular damage (Hoffbrand *et al.*, 2006). GP IIb/IIIa ($\alpha_{IIb}\beta_3$) is an integrin that contains an amino group, a carboxyl group and a disulfide group. The α-subunit has a molecular weight of 136 kD, and consists of a heavy and light chain. The light chain is membrane-bound and consists of a cytoplasmic tail, a transmembrane region and an extracellular domain. The heavy chain is completely extracellular. The β-subunit has a molecular weight of 92 kD, and is a membrane-bound single polypeptide consisting of 762 amino acids. The β-subunit has, similar to the α-light chain, a cytoplasmic tail, a transmembrane region and an extracellular domain. Roughly 50,000 copies of the GP IIb/IIIa receptor are present on each platelet, making it the most abundant integrin on the platelet surface. The GP IIb/IIIa receptors' recognition specificity is defined by two peptide sequences, namely the Arg-Gly-Asp (RGD) sequence and the Lys-Gln-Ala-Gly-Asp-Val sequence. Fibronectin, fibrinogen, vWF and vitronectin all contain the RGD sequence, with fibrinogen containing two per half molecule. The Lys-Gln-Ala-Gly-Asp-Val sequence are only found in fibrinogen at the carboxyl end of the γ-chain, and this is the main binding site of fibrinogen to the GP IIb/IIIa receptor (Lefkovits *et al.*, 1995).

Atherosclerosis is a very old disease and can be traced back to ancient civilizations, as the arteries of Egyptian mummies showed signs of atherosclerotic lesions (Khan, 2006). Atherosclerosis is an immuno-inflammatory disease of arteries and is characterised by the formation of atherosclerotic plaques which consist of a lipid-rich core covered with a fibrous cap. Atherosclerosis is the main cause of coronary artery-, carotid artery- and peripheral arterial diseases. Alone it does not commonly cause fatalities. Atherosclerosis associated thromboses are usually caused by rupture of the atherosclerotic plaques and subsequent exposure of the lipid-rich, thrombogenic core to the platelet containing blood. Platelets adhere to, and aggregate at this thrombogenic core, leading to obstruction of the artery and subsequent loss of blood flow through the artery. Thrombosis after plaque rupture causes life-threatening events such as acute coronary disease and stroke, and contributes to roughly 76%

of all fatal coronary thrombi. Risk factors for atherosclerosis include high plasma cholesterol levels, hypertension, diabetes, smoking, chronic inflammation and male gender (Falk, 2006). Atherosclerotic lesions firstly become visible as yellow dots or streaks on the interior surface of medium to large arteries. These dots/streaks mainly consist of lipid containing foam cells. Over the course of a few years these dots/streaks progress to form plaques, which, in addition to the foam cells, contain smooth muscle cells, as well as extracellular lipids. These plaques may be raised and may protrude into the artery cavity, causing partial obstruction in the artery. The extracellular lipid, which makes up the core of the plaque, is predominantly cholesterol, believed to be derived from plasma low-density lipoprotein (LDL) (Woolf and Davies, 1992). Macrophages help with the removal of lipids, mostly LDL, from the intima of the arterial wall, which leads to the formation of foam cells. Foam cells are macrophages laden with intracellular lipid. In people with high plasma cholesterol levels this removal mechanism is overwhelmed. Thus after these cells die and leave behind the lipid within the plaque core, the atherosclerotic plaque increases in size, hence further protruding into the arterial cavity (Falk, 2006). The cholesterol does not however have to be processed by macrophages in order to stay within the intima. LDL can bind to proteoglycans, be cleaved, and then deposit its cholesterol ester in the intima leading to further progression of atherosclerosis (Woolf and Davies, 1992). Endothelial cells, macrophages and smooth muscle cells die during atherosclerosis, mostly via apoptosis but also due to necrosis. The breakup of foam cells and loss of smooth muscle cells destabilize the lipid-rich core of the plaque and render the fibrous cap fragile and prone to rupture. Apoptosis also increases the tissue factor activity within the core that contributes to the thrombogenicity of the core. The lipid rich core is covered by a fibromuscular cap which contains both smooth muscle cells and collagen (Woolf and Davies, 1992). Atherosclerotic plaques may also calcify with age, but clinical evidence suggests that highly calcified plaques are more responsible for stable angina, than the softer less calcified plaques found with ACS. Rupture-prone plaques responsible for ACS are usually large, non-protruding plaques, which mean that the artery had undergone vascular remodelling to preserve a normal luminal diameter. On the other hand, plaques that cause stable angina are small, protruding plaques, associated with a narrower luminal diameter due to constrictive vascular remodelling (Falk, 2006). A ruptured plaque is the main underlying cause of ACS and is defined as: “A plaque with deep injury with a real defect or gap in the fibrous cap that had separated its lipid-rich

atheromatous core from the flowing blood, thereby exposing the thrombogenic core of the plaque" (Schaar *et al.,* 2004).

ACS is a result of atherosclerotic plaque disruption and the resulting platelet thrombus formation. ACS is a term introduced by the cardiac societies in the United States, Canada and United Kingdom in order to try and identify high risk patients with acute chest pain. These patients usually present with ST-elevation myocardial infarction (STEMI), non-STEMI and unstable angina. Roughly seven million Americans suffered from angina and eight million have had a myocardial infarction (heart attack) in 2006. It is estimated that by 2025 worldwide roughly 24 million people will annually die of cardiovascular disease (Khan, 2006). Annually coronary heart disease, which includes ACS, accounts for more than half of deaths caused by cardiovascular disease (AHA and ASA, 2006), thus ACS is a main cause of deaths worldwide. The following treatment regimens are currently recommended for patients presenting with symptoms of ACS: intravenous nitroglycerin to relieve chest pain, morphine to stop the pain, a beta-blocker to stop adverse effects of norepinephrine like increased heart rate, chewable aspirin to inhibit platelet aggregation, calcium antagonist (if beta-blocker is contraindicated), subcutaneous heparin/low molecular weight heparin to inhibit coagulation, a statin to keep LDL-cholesterol low, and clopidogrel and a glycoprotein IIb/IIIa inhibitor to inhibit arterial thrombosis in high risk patients undergoing angiography and/or angioplasty (Khan, 2006).

2.2 Current medication available for acute coronary syndrome

The most widely used antithrombotic drugs available for ACS treatment include Aspirin, clopidogrel (Plavix®), Aggrenox®, Heparin (Unfractionated), Fractionated Heparin, abciximab (Reopro®) and tirofiban hydrochloride (Aggrastet®).

Aspirin is an anti-platelet drug which inhibits platelet aggregation by irreversibly blocking the cyclooxygenase activity of the enzyme platelet prostaglandin G/H synthase 1. Prostaglandin G/H synthase catalysis the conversion of arachidonic acid to prostaglandin H_2; this is the first

step in prostaglandin synthesis. Prostaglandin G/H synthase 1 is expressed by most tissues as well as platelets, and is important in the production of prostaglandins and thromboxane. Aspirin causes irreversible loss of cyclooxygenase activity by acetylating the hydroxyl group of a single serine residue within platelet prostaglandin G/H synthase 1. This leads to a decrease in the conversion of arachidonic acid to prostaglandin G_2. Because prostaglandin H_2 and thromboxane A_2 are derived from prostaglandin G_2, production of these compounds is also decreased. Thromboxane A_2 causes irreversible platelet aggregation and is released by platelets following exposure to thrombin, collagen and adenosine diphosphate (ADP). Thromboxane A_2 thus helps to amplify the platelets' response to these stimuli. Therefore, a reduction in thromboxane A_2 causes a reduction in platelet aggregation (Patrono, 1994). Some of the side-effects of Aspirin are indigestion, nausea and vomiting caused by irritation of the stomach and intestines. Difficulty in breathing and intestinal bleeding are also side-effects, but occur less commonly (Vega, 2008). Some studies have also found that Aspirin can increase the risk of chronic renal failure, as well as impair effective blood-pressure control in hypertensive patients (Patrono, 1994). Resistance to aspirin therapy has also become a major cause of concern in the management of some patients (Maksoud *et al*., 2005).

Clopidogrel bisulphate (Plavix®) is an anti-platelet drug which inhibits ADP-induced platelet aggregation (http://products.sanofi-aventis.us/plavix/plavix.html). ADP plays a pivotal role in thrombosis and haemostasis. ADP activates platelets via the P2Y1-, P2Y12- and P2X1 purinergic receptors. P2Y1 is a G-protein receptor coupled with G_q. The P2Y1 receptor plays a role in platelet shape change and aggregation, thromboxane A_2 generation, procoagulant activity, adhesion of platelets to fibrinogen and platelet thrombus formation under high shear conditions. P2Y12 is also a G-protein receptor, but is coupled with G_i. The P2Y12 receptor has very similar functions to the P2Y1 receptor. In addition to the functions of P2Y1, P2Y12 also potentiates platelet activation caused by collagen, vWF and thromboxane A_2 (Murugappan and Kunapuli, 2006). P2Y12 has shown to play a role in dense granule secretion, fibrinogen-receptor activation and thrombus formation. The aggregation caused by this receptor is irreversible, and can be activated not only by ADP but also by thromboxane A_2 and the PAR1 selective peptide agonist SFLLRN. P2Y12 activation leads to G_i signalling that, in the presence

of high concentrations of ADP, can lead to platelet aggregation, or with concurrent stimulation of either the G_q or $G_{12/13}$ pathways can also lead to platelet aggregation. P2Y12 is also critical in ADP-mediated thromboxane A_2 generation. Thromboxane A_2 is also an important platelet activator. P2Y12 activation caused secretion of platelet α-granules that subsequently leads to expression of P-selectin on the surface of activated platelets. Interestingly, epinephrine can mimic all the functions of the P2Y12 receptor (Dorsam and Kunapuli, 2004). P2X1 is an ion channel that causes influx of calcium. Activation of P2X1 does not lead to platelet aggregation, but causes shape change and help with the activation of other agonists (Murugappan and Kunapuli, 2006). Clopidogrel directly inhibits the binding of ADP to the P2Y12 receptor, and thus inhibits the ADP-mediated activation of the platelet GP IIb/IIIa complex (http://products.sanofi-aventis.us/plavix/plavix.html). Stomach pain, muscle aches, dizziness, headache, easy bruising and nose bleeds are some of the most common side effects of clopidogrel. Special care should be taken with patients that have stomach ulcers, as clopidogrel can cause life threatening intestinal bleeding in these patients (Vega, 2008). Clopidogrel may also not be effective during stressful episodes, as its effect is reversed by infusion of 2.2 µg/kg/min adrenaline (epinephrine) (Roodt *et al.*, Appendix B). An infusion dose of 2-5 µg/min adrenaline is given therapeutically to maintain stroke volume, heart rate and cardiac output during some surgeries (Heidegger and Kreienbühl, 1998). Some patients have resistance to clopidogrel therapy, thus alternative drugs are needed to treat these patients (Maksoud *et al.*, 2005). Clopidogrel also reduces proliferation and inflammation in severely injured coronary arteries in pigs, a side effect that can potentially be implemented in humans to assist in the prevention of atherosclerotic progression in coronary arteries (Pels *et al.*, 2009). Dual anti-platelet therapy with clopidogrel and aspirin is currently the gold standard in treating patients with ACS to prevent ischaemic complications (Han *et al.*, 2009).

Aggrenox® is a combined anti-platelet drug, manufactured by Boehringer Ingelheim Pharmaceuticals, which contains both aspirin and extended-release dipyridamole (http://www.aggrenox.com). According to the FDA Professional Drug Information database, dipyridamole is an inhibitor of adenosine uptake into platelets, which leads to an increase in adenosine concentrations in the local vicinity of the platelets. The increased adenosine

concentrations activate the platelet A2-receptor, which then stimulates platelet adenylate cyclase to increase the platelet cyclic-adenosine monophosphate (cAMP) levels. The result is inhibition of platelet aggregation (stimulated by platelet activating factor, collagen and ADP) (http://www.drugs.com/pro/dipyridamole-tablets.html). Aggrenox® is available in a sugar-coated tablet form (contains 200 mg dipyridamole and 25 mg aspirin) which is orally administered (http://www.aggrenox.com). Patients using this medication might suffer from side-effects such as headache (±40% of patients), abdominal pain, indigestion and diarrhoea (Vega, 2008).

Unfractionated heparin (sulphated polysaccharide) is an anticoagulant which inactivates thrombin and activated factor X (FXa) by means of an antithrombin dependant system (Hirsh *et al*., 2001). Binding of heparin to antithrombin accelerates the inactivation of thrombin and FXa by almost a 1000 times (Weitz, 1997). Heparin and antithrombin bind to each other via a high-affinity pentasaccharide. Heparin must bind to thrombin and antithrombin simultaneously to inhibit thrombin, but this simultaneous binding is not necessary for FXa inhibition. By inhibition of thrombin, heparin prevents fibrin formation, and thrombin-induced platelet-, factor V- and factor VIII activation (Hirsh *et al*. 2001). The most common side-effects of heparin include abnormal bleeding and easy bruising. Allergic reactions and irritation at the site of injection can occur (Vega, 2008). Heparin has also been implicated in some cases as the cause of thrombocytopenia and osteopenia (Hirsh *et al*. 2001). Heparin therapy needs constant monitoring using the activated partial thromboplastin time (aPTT) (Vega, 2008).

Fractionated Heparin or low molecular weight heparin (LMWH) is about a third of the size of unfractionated heparin. LMWH only inhibits FXa and not thrombin, since it is not big enough to bind simultaneously to both thrombin and antithrombin (Weitz, 1997). LMWH has a lesser chance of causing thrombocytopenia and osteopenia, but the risk still remains (Hirsh *et al*. 2001). One advantage of LMWH is that the therapy does not have to be monitored. LMWH tends to accumulate in the blood of patients with impaired renal function, thus it is not advised to use LMWH in these patients. LMWH can cause skin irritation at the place of injection, as

well as nausea. Patients can also develop allergic reactions and skin rashes. These allergic reactions may manifest in swelling of the hands and lips, and impaired breathing. Like unfractionated heparin, LMWH may also cause abnormal bleeding (Vega, 2008).

Abciximab (Reopro®) is an anti-platelet antibody that blocks the platelet GP IIb/IIIa receptor and thus inhibits the binding of four soluble adhesive proteins, namely fibrinogen, vWF, fibronectin and vitronectin, to activated platelets (Mousa *et al.*, 2001). This prevents the interaction of fibrinogen with the GP IIb/IIIa receptor (final common pathway of platelet aggregation). Thus by blocking this interaction abciximab potently inhibits platelet aggregation. Abciximab has a higher affinity for the GP IIb/IIIa receptor than what fibrinogen has (fairly weak affinity). This higher affinity binding leads to more than 80% of GP IIb/IIIa receptors being blocked by abciximab. It has been reported that platelet aggregation decreases to less than 20% within 10 minutes post abciximab administration (http://www.reopro.com/about_reopro?product_information.jsp). Abciximab is also associated with a substantial increase in bleeding risk. Severe thrombocytopenia and allergic reactions (even fatal anaphylaxis) have also been described in patients on abciximab therapy (http://www.reopro.com/index.jsp). It has been reported that abciximab does not increase surgical mortality, but that preoperative exposure increases postoperative bleeding and transfusion requirements. It is also connected with a high frequency of postoperative re-entry (Bizzari *et al.*, 2001). Injection of 1.6 mg/kg abciximab was effective to inhibit arterial thrombosis in baboons, but it led to an almost 20 fold increase in blood loss compared to a control phase (Roodt *et al.*, Appendix B).

2.3 The need for more effective and safe medication

Four percent of patients undergoing cardiac surgery require re-entry into the operating theatre within the first 24 hours after surgery. In 50 – 60% of cases, reopening is due to nonsurgical bleeding. The increased risk in bleeding associated with potent anti-platelet drugs administered before surgical interventions remains a point of concern for surgeons (Bizzari *et al.*, 2001). Thus the remaining high incidence of ACS worldwide and the number of adverse

effects (especially bleeding complications) seen with current antithrombotic therapies, give rise to the need to develop antithrombotic drugs with a better therapeutic window, i.e. better efficacy with less adverse side effects. We hypothesize that tirofiban hydrochloride is such a drug.

2.4 Tirofiban hydrochloride

Tirofiban hydrochloride (USA brand name: Aggrastat®) inhibits platelet aggregation by being an antagonist to the platelet GP IIb/IIIa receptor. Tirofiban hydrochloride monohydrate is a non-peptide molecule ($C_{22}H_{36}N_2O_5S \cdot HCl \cdot H_2O$) with a molecular weight of 495.08 kD. In South Africa tirofiban hydrochloride is marketed by MSD (Pty) Ltd. under the brand name Aggrastet®, which is intravenously administered (www.rxlist.com/cgi/generic/tiro.htm). Fibrinogen binds to the GP IIb/IIIa receptor on platelets via the Arg-Gly-Asp (RGD) recognition sequence (Mousa *et al*., 2001). Tirofiban hydrochloride's design started with the RGD sequence and it mimics the RGD sequence to bind to the binding pocket on GP IIb/IIIa, thus inhibiting fibrinogen mediated platelet aggregation by competing with fibrinogen for the GP IIb/IIIa receptor (Shanmugam, 2005).

MK-383, the unrefined compound that finally lead to the development of tirofiban hydrochloride, was first tested in humans in 1993, and found to effectively inhibit *in vitro* and *ex vivo* fibrinogen-dependant platelet aggregation, as well as to prolong the bleeding time in healthy volunteers receiving this compound (Peerlinck *et al*., 1993). The *in vivo* anti-aggregatory and antithrombotic effect of MK-0383 was tested in arterial thrombosis models in dogs and found to effectively inhibit *in vivo* thrombus formation in a dose dependant manner. It was also effective in inhibiting ADP- and collagen induced platelet aggregation and had no effect on platelet counts (Lynch *et al*., 1995). Clinical dose finding studies done on tirofiban hydrochloride (MK-383) used *ex vivo* ADP-induced platelet aggregation studies to measure the pharmacodynamic effect of tirofiban hydrochloride. It was found that bolus doses of 5, 10 and 15 µg/kg tirofiban hydrochloride, together with maintenance doses of 0.05, 0.10 and 0.15 µg/kg/min respectively, inhibited ADP-induced platelet aggregation in a dose-dependent

manner. A slight increase in adverse bleeding events was also found at the highest dose, mainly at the vascular access site. A dosage regimen that was well tolerated in high risk angioplasty patients was established. Following this study, the therapeutic dose for adult humans undergoing angioplasty/artherectomy was established as a bolus dose of 10 µg/kg tirofiban hydrochloride, together with a maintenance infusion of 0.15 µg/kg/min tirofiban hydrochloride (Kereiakes *et al*., 1996).

Tirofiban hydrochloride has a plasma half-life of about 2 hours. GP IIb/IIIa inhibitors with a shorter half-life, such as tirofiban hydrochloride, are hypothesised to have improved safety, because platelet function returns to baseline more rapidly than those inhibitors with a longer half-life. The RESTORE trial showed that tirofiban hydrochloride is effective in preventing adverse cardiac events during coronary angioplasty in patients with acute coronary syndromes, and this effect was seen for another seven days post-operatively (The RESTORE Investigators, 1997). It was also found that tirofiban hydrochloride infusion together with aspirin resulted in lower rates of ischaemic events and similar bleeding side-effects than heparin and aspirin together (PRISM Study, 1998). When administered together with aspirin and heparin, tirofiban hydrochloride is more effective in preventing ischaemic events in patients with acute coronary syndrome than aspirin and heparin alone, but tirofiban hydrochloride alone is associated with a high rate of mortality seven days post-operatively (PRISM-PLUS Study Investigators, 1998). From the few studies done to analyse the incidence of post-operative bleeding in patients on tirofiban hydrochloride therapy, no excessive post-operative bleeding was observed. Bleeding tendencies in some of these studies were not enhanced and in other cases even reduced (Shanmugam, 2005). It was also reported that tirofiban hydrochloride infusion immediately before a surgical intervention does not have any adverse effect on postoperative bleeding, with haemoglobin and platelet levels remaining stable (Bizzari *et al*., 2001). Furthermore, tirofiban hydrochloride significantly lowers the incidence of heart attack after angioplasty, as was seen in patients suffering from coronary artery disease, who have shown a poor response to oral anti-platelet drugs such as clopidogrel and aspirin (Valgimigli and Verheugt, 2008).

Another major advantage of tirofiban hydrochloride is that an antidote strategy is not needed, because platelet function rapidly recovers after tirofiban hydrochloride administration (50% decrease in inhibition and return to normal bleeding time after 4 hours). This is due to the short plasma half life and rapid dissociation of tirofiban hydrochloride. It is also hypothesized that tirofiban hydrochloride may have a platelet-sparing effect during bypass surgery, as it prevents platelet consumption by inhibiting platelet thrombus formation (Shanmugam, 2005). Tirofiban hydrochloride is also much more cost effective than abciximab. In the TARGET trial it was found that tirofiban hydrochloride is not as effective as abciximab in preventing major ischaemic events. The lack of efficacy was attributed to a possible suboptimal dosage of tirofiban hydrochloride. The tirofiban hydrochloride group also gave similar major bleeding side-effects as the abciximab group, but less minor bleeds and thrombocytopenia. All the patients taking part in the trial also received 250 to 500 mg of aspirin, unfractionated heparin (≤70 U/kg), and if possible, a loading dose of 300 mg clopidogrel (Topol *et al*., 2001). Bleeding complications seen in combination studies of tirofiban and heparin can however not be excluded as being caused by too high doses of heparin (Kralisz *et al*., 2004). Schneider *et al*. suggested a higher bolus dose of 25 µg/kg tirofiban hydrochloride, together with a maintenance dose of 0.15 µg/kg/min to be used. Such a dose provides enhanced inhibition of *ex vivo* platelet aggregation and is safe, with no increased risk of major bleeding (Schneider *et al*., 2002, Danzi *et al*., 2003). Tirofiban hydrochloride was not only safer than abciximab at this dose, but it also compared to the efficacy of abciximab in preventing ischaemic events (Danzi *et al*., 2004). It has also been reported that a high bolus dose of tirofiban hydrochloride can be safely and effectively used in high-risk coronary angioplasty (Danzi *et al*, 2006 and Valgimigli *et al*, 2004). However, in the Facilitated Angioplasty with Tirofiban or Abciximab (FATA) in ST-elevation myocardial infarction trail, the high-dose bolus (HDB) did not compare with abciximab in achieving complete ST-segment resolution during percutaneous coronary intervention (Marzocchi *et al*, 2008). This is in contrast with results found in the MULTISTRATEGY trial, where HDB of tirofiban hydrochloride did compare with abciximab in a similar patient group (Valgimigli *et al*, 2008). Thus, many questions relating to the optimal dosage regimen for tirofiban hydrochloride in the treatment and prevention of ACS remain to be answered.

2.5 Experimental animals

It is known that *in vitro* tests can not accurately predict the haemodynamic, cellular and molecular interactions which occur during *in vivo* thrombus formation, since some drugs have unexpected side-effects such as hypotension or thrombocytopenia *in vivo*. Therefore after laboratory testing, all novel antithrombotic drugs need to be tested in animal models of thrombosis before they can undergo clinical trials. Non-human primate thrombosis models should however be reserved for drugs that have successfully achieved the efficacy and safety standards of rodent and rabbit models (Leadley *et al*., 2000).

Our non-human primate of choice is the baboon, because evidence from previous studies showed that the arterial thrombotic processes in baboons and humans are similar. Similarities between human and baboon coagulation systems are well known and were first described in 1966 (Hampton and Matthews, 1966). The haemostatic mechanism of baboons and humans are also similar, since concentrations of clotting factors, bleeding time, platelet- count, volume and morphology, as well as platelet aggregation show great similarities (Hanson *et al*., 1985). Furthermore, baboons show the same physiological properties as humans, such as the tendency to develop high blood cholesterol levels and atherosclerosis when placed on high fat content diets. These characteristics have an important part in common diseases in humans. The broad structure of the baboon and human genomes are also fundamentally the same (Rogers and Hixon, 1997). The close evolutionary relationship between baboons and humans has been shown with the genetic similarities of the overall DNA sequence of the two species (Caccone and Powell, 1989). This evolutionary connection has been shown to be significant since the establishment of the scientific discipline of pharmacogenetics, where the close relationship between genotype and drug response were determined (Abbott, 2003).

A baboon thrombosis model is an effective model to test and evaluate new anti-thrombotic drugs prior to clinical trials, due to the similarities between the human and non-human primate blood clotting systems (Leadley *et al*., 2000). Another advantage of using baboons is that environmental and dietary conditions can be carefully monitored and controlled to suit

experimental purposes (Rogers and Hixon, 1997). Due to the size of the animal, the acquisition of blood samples is also easy and has no serious adverse effects on the animal and multiple samples can also be drawn with minimal discomfort for the animal and without compromising the haemodynamic system (Leadley *et al*., 2000).

2.6 Animal thrombosis models

Professor John D. Folts introduced a model of measuring arterial blood flow in coronary arteries of dogs. This model simulates thrombus formation during cardiovascular disease by mimicking diseased arteries that are partially occluded because of atherosclerosis and superimposed intimal damage (Coller *et al*., 1989). He was the first person to describe a phenomenon known as cyclic flow reductions (CFRs), in which there is a periodic decrease in coronary blood flow due to the thrombus formation followed by a sudden return to control flow. He determined that this loss of blood flow was caused by platelet thrombi that are formed in a narrowed arterial lumen. The return of blood flow is caused by the dislodgement of the thrombi (Folts *et al*., 1982).In the Folts model, a throracotomy is performed through the fifth intercostals space to expose the heart. Part of the proximal left circumflex coronary artery is exposed and the adventitia is stripped away. A Statham electromagnetic flow probe is then placed on this exposed part of the artery. A plastic constricting cylinder is placed distal to the flow probe to produce constriction. These plastic cylinders (constructed of Lexan) are selected to produce a stenosis that reduces the luminal diameter by 60-80%. The average coronary blood flow is not significantly reduced by this amount of stenosis (Folts *et al*., 1982). The stenosis is produced to simulate the turbulent flow that is produced at sites of atherosclerotic plaques where arteries are partially occluded. At these sites platelets are activated under the high shear stress and aggregate (Folts *et al*, 1976). A recorder is used to record control aortic and coronary blood pressure and flow, as well as surface electrocardiograms (ECGs). If platelet aggregates do not break loose spontaneously to recover blood flow, the vessel must be carefully pinched or poked with a surgical instrument to mechanically dislodge the platelet-rich thrombus (Folts *et al*., 1982).

A variation of this model was described by Coller *et al*. (1989) for non-human primates. In this model a 2-3 cm portion of the carotid artery is exposed. Blood flow through the artery is measured with an electromagnetic flow probe. The artery is clamped several times with a hemostat just distal to the flow probe to produce intimal damage. External stenosis of 70-75% is produced by placing a constricting cylinder around the damaged artery. CFRs are also observed in this model. Unlike in the dog model, thrombi that do not dislodge spontaneously are mechanically dislodged by shaking the constricting cylinder (Coller *et al*., 1989). It is possible to determine the efficacy of anti-platelet drugs in eliminating thrombus formation at high shear rates using this model.

One major disadvantage of these models is the fact that a very invasive technique is used that involves some of the most important blood vessels in the body, i.e. the coronary artery supplies the heart muscle with oxygen and nutrients and the carotid artery supplies the brain with oxygen and nutrients. A mistake on the part of the operator can very quickly cause major heart- or brain damage to the animal or even death. The coronary and carotid arteries are also not easily accessible, and a vast amount of expertise is needed for surgery to be done safely. The animals also need to be intubated and ventilated during the procedure, which is another aspect that needs specific expertise in being performed (Folts *et al*., 1991).

Another arterial thrombosis model that is used is the Hanson model in primates, which was developed by Professor Stephen R. Hanson in 1985. This model quantifies *in vivo* arterial thrombosis formation, free of uncontrolled variables (Hanson *et al*., 1985). In this model a chronic arteriovenous shunt is surgically inserted between the femoral artery and vein. A dacron vascular graft serves as a synthetic thrombogenic surface in this model. An uncrimped knitted dacron vascular graft (thrombogenic device) is then built into the shunt (Cadroy *et al*., 1989). A Doppler ultrasonic flowmeter, whose transducer probe fits around the silastic tubing of the shunt, is used to measure mean blood flow rates through the vascular graft segment (Hanson *et al*., 1985). Autologous baboon platelets are radioactively labelled, reinfused in the baboon, and allowed to distribute in the vasculature for one hour. The thrombogenic device is

then inserted in the shunt and exposed to the blood. A gamma scintillation camera is used to measure the accumulation of labelled platelets within the thrombogenic device. The data acquired is analysed using a computer-assisted image processing system interfaced with the camera. The amount of deposited platelets is determined by dividing the amount of deposited platelet radioactivity per minute by the whole blood platelet radioactivity (counts/minute/millilitre) and then multiplying it by the circulating platelet count (platelets/millilitre) (Cadroy *et al.*, 1989). A variation on this method exists where a teflon tube with an expanded diameter is placed directly distal to the dacron graft thrombogenic device. Within this tube blood is allowed to circulate and have a degree of stasis in order to simulate venous thrombosis. The amount of fibrin formation within this model is measured by radioactively labelling homologous baboon fibrinogen and injecting it intravenously in the baboon. The dacron graft thrombogenic device serves to simulate arterial thrombosis and the fibrin and red cell enriched expanded tube serves as a simulation of venous thrombosis (Figure 1). Images are acquired by a gamma scintillation camera and analysed for both regions separately (Hanson *et al.*, 1993).

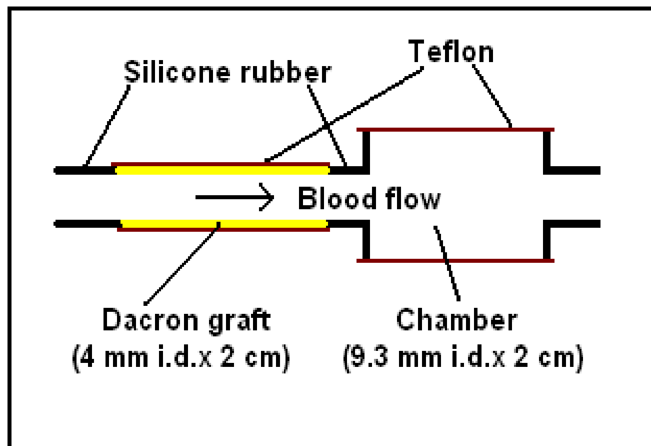


Figure 1. Example of Thrombogenic device used in the Hanson model (Hanson *et al.*, 1993)

This model is used to measure the efficacy of an anti-platelet drug to inhibit platelet thrombus formation on a thrombogenic surface, as well as measure the efficacy of an anti-coagulant to inhibit fibrin formation. One major disadvantage of this method is the use of a synthetic thrombogenic surface (Dacron graft) that is not completely representative of *in vivo* conditions.

The use of radioactive reagents is also dangerous and a suitable environment for safe handling of these compounds is not always available.

2.7 Modified animal thrombosis model

Due to the shortcomings of the methods described by Folts and Hanson, a need existed to develop a more effective and safe animal thrombosis model. We developed a high shear rate thrombosis model in baboons that combines different components of the models described by Folts and Hanson. We simulate the Folts model by inducing CFRs via injury and stenosis of the artery to mimic diseased arteries, but we limit the invasiveness of the procedure by performing the procedure on the femoral vessels, as first described in baboons by Wu *et al.* (Wu *et al.*, 2002), and using a shunt as described in the Hanson model (Fontayne *et al.*, 2008). By limiting the invasiveness of the procedure and the use of the artery-wall as thrombogenic surface we address and rectify the disadvantages and shortcomings and/or technical difficulties of the previous models.

2.8 Bleeding Models

To evaluate the safety of anti-thrombotic drugs in terms of bleeding caused we used the skin template bleeding time (using a Surgicutt® device) and an incision bleeding method (Benedict *et al.*, 1991; Thiagarajan and Benedict, 1997). In the skin template bleeding time a standard incision is made usually on the forearm, but some studies indicate that this incision can also be made on the tongue of research animals (Björkman *et al.*, 2007). The time it takes for bleeding to cease is then measured. This is an indication of the adequacy of the number and ability of platelets to form a haemostatic plug (Laffan and Manning, 2006). However, several studies demonstrated that a prolonged bleeding time in man does not necessarily predict clinical bleeding (Lind, 1991). The incision bleeding method, which measures blood loss from a well defined wound, might be more predictable as it more closely reflects surgical tissue damage. In the incision method, gauze swabs were inserted in a 2cm long, 0.8 cm deep wound and the weight of blood lost was measured by weighing the swabs. Blood loss was then determined as

a ratio to the saline phase. As this method mimics surgical interventions, it might better predict surgical bleeding than the template bleeding time, however a thorough characterisation of this method is lacking.

Aim of study

The aim of our study was therefore to evaluate the efficacy of different dosages of tirofiban hydrochloride to inhibit arterial thrombosis in our high shear rate arterial thrombosis model in baboons, as well as to test its safety in two different bleeding models.

Chapter 3

Method

3.1 Experimental Animals

Five male Cape Chacma baboons (*Papio ursinus*), weighing between 13.4 kg and 16.4 kg were used as test subjects. All the baboons were kept in holding cages for at least one month before the procedure, and were disease free. The baboons neither received any medication nor were subjected to any other intervention for at least one month prior to experiment. This project was approved by the Control Committee for Animal Experimentation at the Faculty of Health Sciences, University of the Free State (Animal experiment NR 18/08).

Each individual baboon was sedated with Ketamine (10 mg/kg intramuscular) at the primate facility on the Western Campus of the University of the Free State, and promptly transported via vehicle to the Animal Research Facility of the University of the Free State. This facility contains an operating theatre where the surgical procedure was performed. The baboons were numbered W01 to W05.

3.2 Anaesthetic

Our anaesthetic of choice was Ketamine [Ketamine-2-(2-chhlorophenyl)-2-methylaminocyclohexanone hydrochloride]. Ketamine is lipophilic and contains benzethonium chloride as a preservative. Ketamine is usually administered intravenously, but anaesthesia can also be induced via the intramuscular or rectal routes. Ketamine causes a rapid onset of anaesthesia, and can be used for maintenance of anaesthesia. Ketamine is an analgesic

(pain-suppressing) agent even at sub-anaesthetic doses; this is in contrast to other induction agents. Ketamine is especially useful for animal experiments taking into consideration that it maintains muscle tone, preserves spontaneous respiration and has a profound analgesic and amnesia effect. Furthermore, ketamine leads to an increase in heart rate, blood pressure, cardiac output and myocardial consumption, and is therefore perfectly suited to use in this experiment where high shear forces are needed. Ketamine also helps with the management of pain during the procedure by reducing central sensitivity after tissue injury and secondary hyperalgesia (Davies and Cashman, 2006).

3.3 Overview of Experiment

A high shear rate arterial thrombosis model in baboons was used to determine efficacy in preventing acute arterial thrombosis. Briefly, a 40 cm long silicone shunt was placed between the femoral artery and femoral vein, the femoral artery was injured with a forceps, and a constriction applied with an adjustable clamp to obtain high shear rates. This was to simulate atherosclerotic conditions. A platelet rich thrombus resulted, which caused a decrease in flow as measured on the flow meter and then dislodged mechanically, resulting in cyclic flow reductions (CFRs). One CFR is the time between stenosis and complete occlusion of the artery. This process is described in figure 2. Blood flow was allowed to stabilize, and then we injected saline into the shunt to serve as an internal control. Tirofiban hydrochloride was then injected intravenously and the effect on CFRs studied. Tirofiban hydrochloride was administered via an intravenous bolus injection into the shunt between the femoral vein and femoral artery. According to the dosage instructions of Aggrastet®, the therapeutic dose for angioplasty/atherectomy is a 10 µg/kg bolus followed by a maintenance infusion of 0.15 µg/kg/min. Following an initial dose of 3.0 µg/kg tirofiban hydrochloride, the baboons were injected every 30 minutes with an increasing dose of tirofiban hydrochloride; 10 µg/kg, 30 µg/kg, 90 µg/kg and 270 µg/kg. A maintenance infusion of 0.015 times dose per minute was given as according to Aggrastet® dosage instructions. After administration of an effective dose of the tirofiban hydrochloride, a new injury was applied to determine whether the drug was able to inhibit platelet deposition on a fresh lesion. Epinephrine was injected after the highest dose to further test the potency of tirofiban hydrochloride.

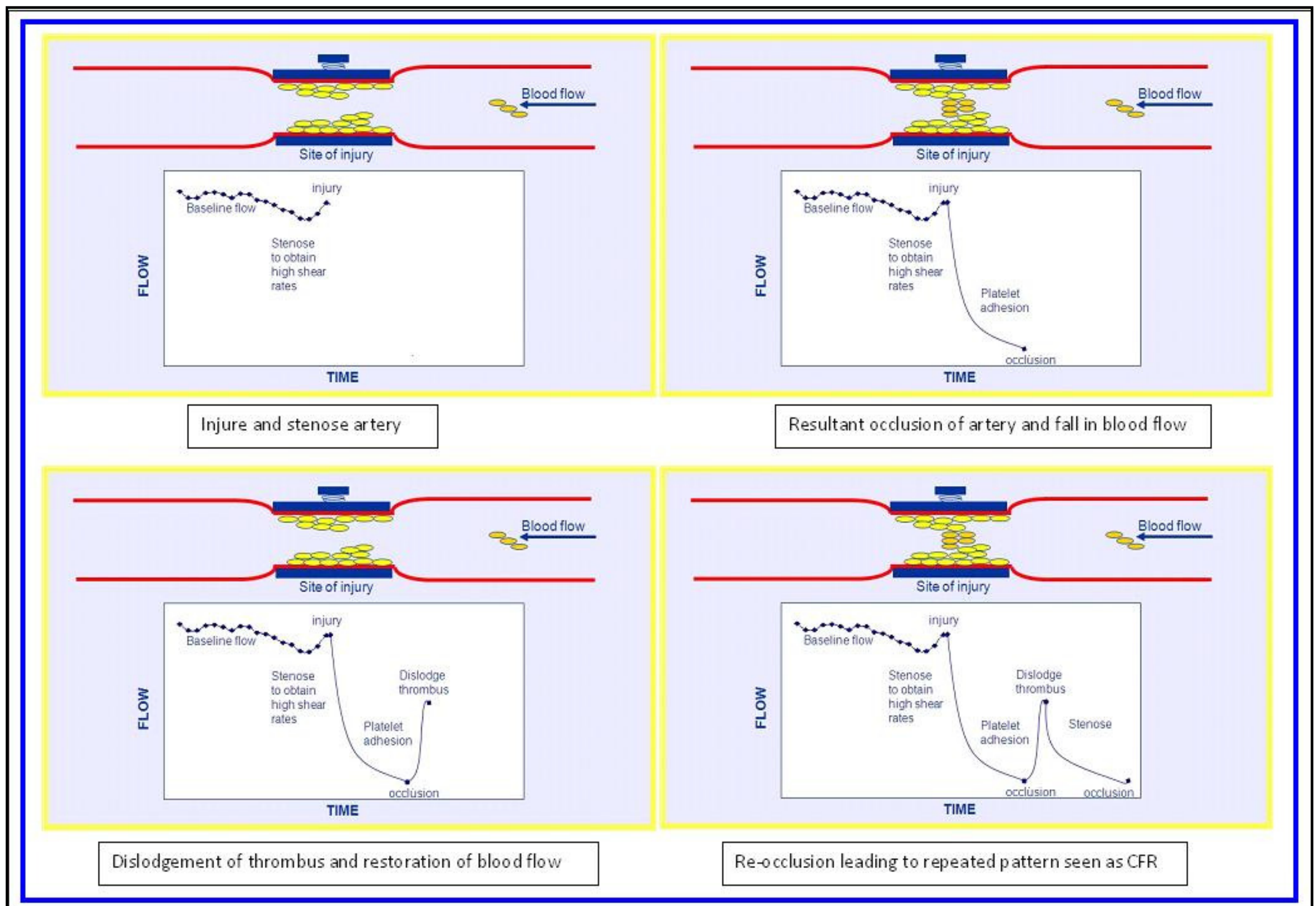


Figure 2. Schematic presentation of CFR formation

Two different bleeding analysis were performed: the skin bleeding time (using a Surgicutt® device) and an incision bleeding method (Benedict *et al.*, 1991; Thiagarajan and Benedict, 1997). The skin bleeding time however fails to accurately predict a bleeding episode, and thus lacks clinical benefit (Peterson *et al.*, 1998). The incision bleeding method might be more predictable of adverse bleeding events during surgery. In this method, swabs are inserted in a well defined wound and the weight of blood lost is measured by weighing the swabs. As this method mimics surgical interventions, it might predict surgical bleedings. A thorough characterisation of this method is however still lacking.

To ensure the safety and comfort of the animals, they were post-operatively followed up for 7 days for any signs of discomfort, adverse effects (excessive post-operative bleeding or bruising) or infection and given prophylactic antibiotics as an infection control measure.

3.4 Surgical Procedure:

A 4-5 cm segment of the femoral vessels was exposed by blunt dissection after an incision was made in the skin. All nearby branches of the femoral artery and femoral vein were ligated. A small incision was made in the femoral artery and femoral vein and vessel tips inserted and secured with surgical silk. Silicone tubing was attached to the vessel tips to shunt arterial blood into the femoral vein. Shunting directly from the arterial circulation and bypassing the capillaries increases the blood flow 3 to 5 fold, resulting in flow rates of between 150 and 300ml/minute. There is also a concomitant increase in shear rates. The increase in shear rate is important, as von Willebrand factor, which serves to capture and localise platelets at a place of vascular injury, only gets activated at high shear rates. A flow probe (Transonic systems TS410, probe: ME3PXL1OO8) was attached to the silicone tubing and flow was allowed to stabilise for about 15 minutes. Blood flow was measured with a flow meter and the resulting flow chart was recorded on a computer.

After 15 minutes, two overlapping injuries were made on the femoral artery using a Martin needle holder (Hegar-Baumgartner TC Gold 14 cm) by pressing down on the artery for 10 seconds at a time. The injuries were made to simulate the thrombogenic event of plaque disruption, by exposing the subendothelium. An adjustable plastic clamp was then placed around the place of injury to stenose the artery and to reduce the blood-flow to roughly 30 ml/min (80% – 90% stenosis). A blood-flow of ± 30 ml/min was used as the baseline flow. The stenosis simulates the occluded state of a diseased artery. The injury and stenosis lead to thrombus formation, resulting in a fall in blood-flow. At a blood-flow rate of ± 5 ml/min the thrombus was mechanically dislodged by first slightly closing the clamp and then opening it up to restore baseline flow of 30 ml/min. After restoring baseline flow, the process of thrombus formation was repeated. This repeated process was seen as CFRs. After a 30-minute control

period of reproducible CFRs, the shunt was flushed and a vehicle (saline) administered as an internal control. CFRs were followed for 30 more minutes. After this period, tirofiban hydrochloride was administered via an intravenous bolus injection, together with a maintenance dose infusion. Monitoring continued for up to 30 minutes after drug administration. This procedure was repeated for several times with escalating doses of the tirofiban hydrochloride. The antithrombotic effect was quantified by comparing the amount of CFRs/30 minutes before and after drug administration. After injection of an effective dose of tirofiban hydrochloride, a new injury was applied to determine whether inhibition was a strong inhibition able to inhibit platelet aggregation on a fresh injury. At the end of the experiment, Epinephrine (2.2 µg/kg/min) was injected in order to distinguish between a weak and a strong inhibition of the CFRs. Figure 3 is an example of a successful experiment, with full platelet inhibition.

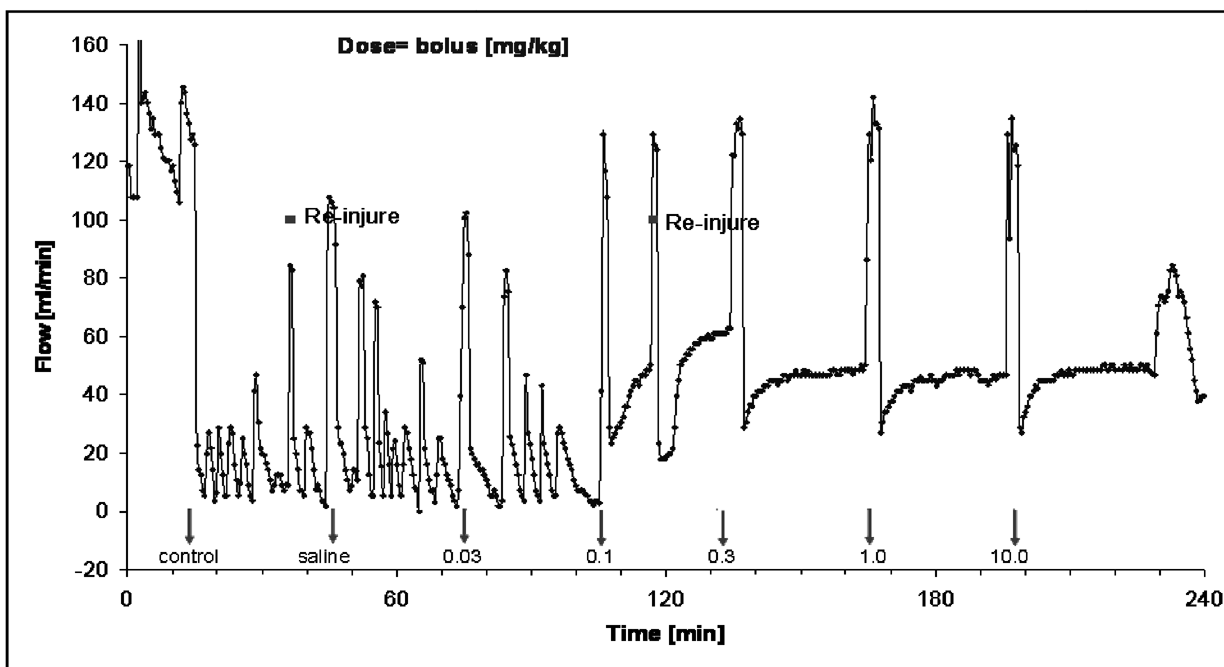


Figure 3: Results of CFRs during a previous experiment, showing full inhibition after injection of 0.1 mg/kg of an anti-platelet drug

Ten minutes after each dose of tirofiban hydrochloride, blood samples (6 ml) were taken for laboratory analysis to monitor the full blood count (FBC), prothrombin time (PT), activated partial thromboplastin time (aPTT), ADP induced platelet aggregation and Thromboelastography (TEG).

At the end of the procedure of baboon W05, a 1 cm segment of the injured section of the artery, as well as a 1 cm segment of an uninjured section of the artery was removed for scanning electron microscopy at the Centre for Microscopy, Faculty of Natural and Agricultural Sciences, University of the Free State. This was done to evaluate the nature of the injury and to compare it with intact vessel-wall. Arteries were processed for scanning electron microscopy using a method similar to the one described by Wong *et al*. (Wong *et al*., 2000).

The skin bleeding time was measured 10 minutes after each dose of tirofiban hydrochloride. A control bleeding time was measured 10 minutes after injury to the artery. In the second blood loss analysis, a pre-weighed gauze swab was inserted in a wound 2 cm long and 0.8 cm deep in the upper thigh muscle and replaced every 30 minutes just before each new dose of tirofiban hydrochloride was given. The amount of blood loss for each dose was determined by weighing the gauze swabs before and after placement. This is expressed relative to the amount of blood loss in the control gauze (during the saline injection period). The experimental setup of the whole experiment is illustrated in figure 4.

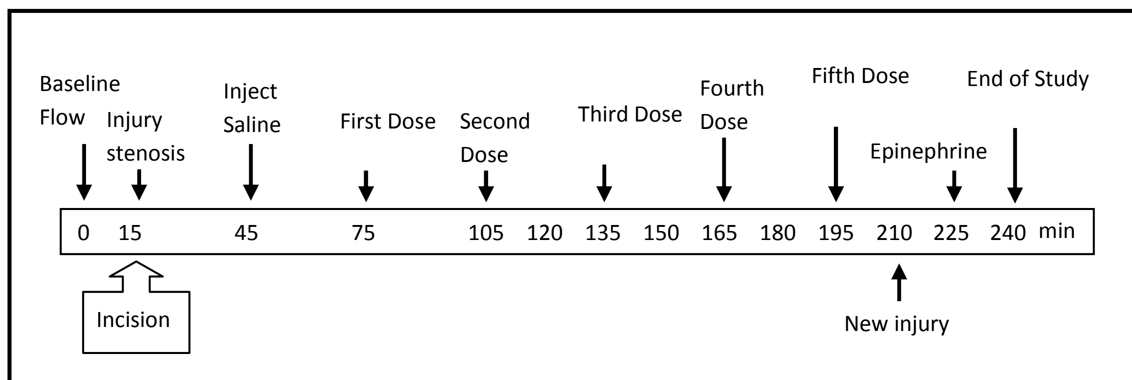


Figure 4: Experimental setup for the arterial thrombosis model in baboons

3.5 Laboratory analysis

1 ml EDTA and 5 ml sodium-citrate anti-coagulated blood was drawn following administration of each dose for laboratory analyses. A Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) were done to analyze the effect of the drug on clotting proteins. A full blood count (FBC) was done to measure the effect of the drug on the different whole blood components. An ADP induced platelet aggregation study was done to test the effect of the drug on platelet aggregation. The Thromboelastograph (TEG) was used to measure the effect of the drug on platelet inhibition. All laboratory testing was done by staff at the NHLS laboratory at Universitas Tertiary Hospital, and formal reports were provided. These results were noted on EXCEL spreadsheets for further analysis.

3.5.1 Coagulation screening tests:

These tests include the Prothrombin time (PT) and the activated partial Thromboplastin time (aPTT), which are designed to measure the different clotting proteins involved in blood coagulation. The samples used for these coagulation tests were sodium-citrate anti-coagulated. Sodium-citrate binds to calcium-ions in the blood, which are necessary for coagulation to proceed. Coagulation is thus halted until addition of reagent which replaces the calcium-ions for coagulation to proceed. The sodium-citrate anti-coagulated whole blood samples were centrifuged at 2000 x g for 15 minutes to obtain plasma that is poor in platelets. The platelet poor plasma was then used to determine the PT and aPTT. Both the PT and aPTT tests were done on the ACL Top® automated coagulation monitor (supplied by Beckman Coulter, South Africa). This instrument works on a photo-optical principle. It determines the time it takes for a clot to form by measuring the change in optical density of the plasma after addition of the different coagulation initiators.

3.5.1.1 Prothrombin Time (PT):

The PT measures the time it takes for plasma to form a clot in the presence of an optimal concentration of tissue factor. This is suggestive of the overall efficacy of the extrinsic clotting

system. This test measures the activity of Factors II, V, VII and X, as well as the concentration of fibrinogen in the plasma. In this method, plasma is incubated for 60 seconds at 37℃, after which a reagent that contains an optimal concentration of tissue factor is added for clotting to take place. The PT is always done in duplicate and the results are expressed in seconds as the mean of the duplicate values (Laffan and Manning, 2006). The normal value for humans at the NHLS laboratory at Universitas Tertiary Hospital in Bloemfontein is 10-14 seconds. The reagent used is a recombinant human tissue factor from Instrument Laboratories (supplied by Beckman Coulter South Africa).

3.5.1.2 Activated Partial Thromboplastin Time (aPTT):

The aPTT measures the time it takes for plasma to form a clot after the activation of the contact factors but without adding tissue factor. This is suggestive of the overall efficacy of the intrinsic clotting system. In the method used, plasma is first incubated with a contact activator (ellagic acid) for 3 minutes at 37℃, this allows FXII to be activated, which then leads to FXI activation. At this point calcium is needed for coagulation to proceed. The plasma is recalcified after 3 minutes and clotting is allowed to proceed. This test measures the activity of the contact factors (High molecular weight kininogen and prekallikrein/kallikrein) and Factors II, V, VIII, IX, X, XI, XII, as well as the concentration of fibrinogen in the plasma. The aPTT is always done in duplicate and the results are expressed in seconds as the mean of the duplicate values (Laffan and Manning, 2006). The normal value for humans at the NHLS laboratory at Universitas Tertiary Hospital in Bloemfontein (the laboratory we use) is 26-35 seconds. The reagents used are manufactured by Instrument Laboratories (supplied by Beckman Coulter South Africa).

3.5.2 ADP induced platelet aggregation:

The ADP induced platelet aggregation tests were performed on a Chrono-log platelet aggregometer. Platelet function can be tested by adding adenosine 5-diphosphate (ADP) as agonist and recording the aggregation by measuring light absorbance with an aggregometer. The less light absorbed by the plasma, the more the aggregation. Aggregation is thus inversely

proportional to light absorbance. Low concentrations of ADP cause the release of Ca^{2+} ions by binding to a membrane receptor on the platelet surface. This causes a reversible complex to form with extracellular fibrinogen. This complex causes platelets to undergo a shape change which can be visually observed by a slight increase in light absorbance in the aggregometer. The bound fibrinogen then helps with the cell-to-cell contact, and reversible aggregation takes place. At higher concentrations of ADP, dense and α-granules release their content which leads to irreversible aggregation via the arachidonic acid pathway of platelet aggregation (Laffan and Manning, 2006). Figure 5 shows results of a normal control (Blue line – 88% aggregation) and a patient with decreased ADP induced platelet aggregation (Black line – 36% aggregation).

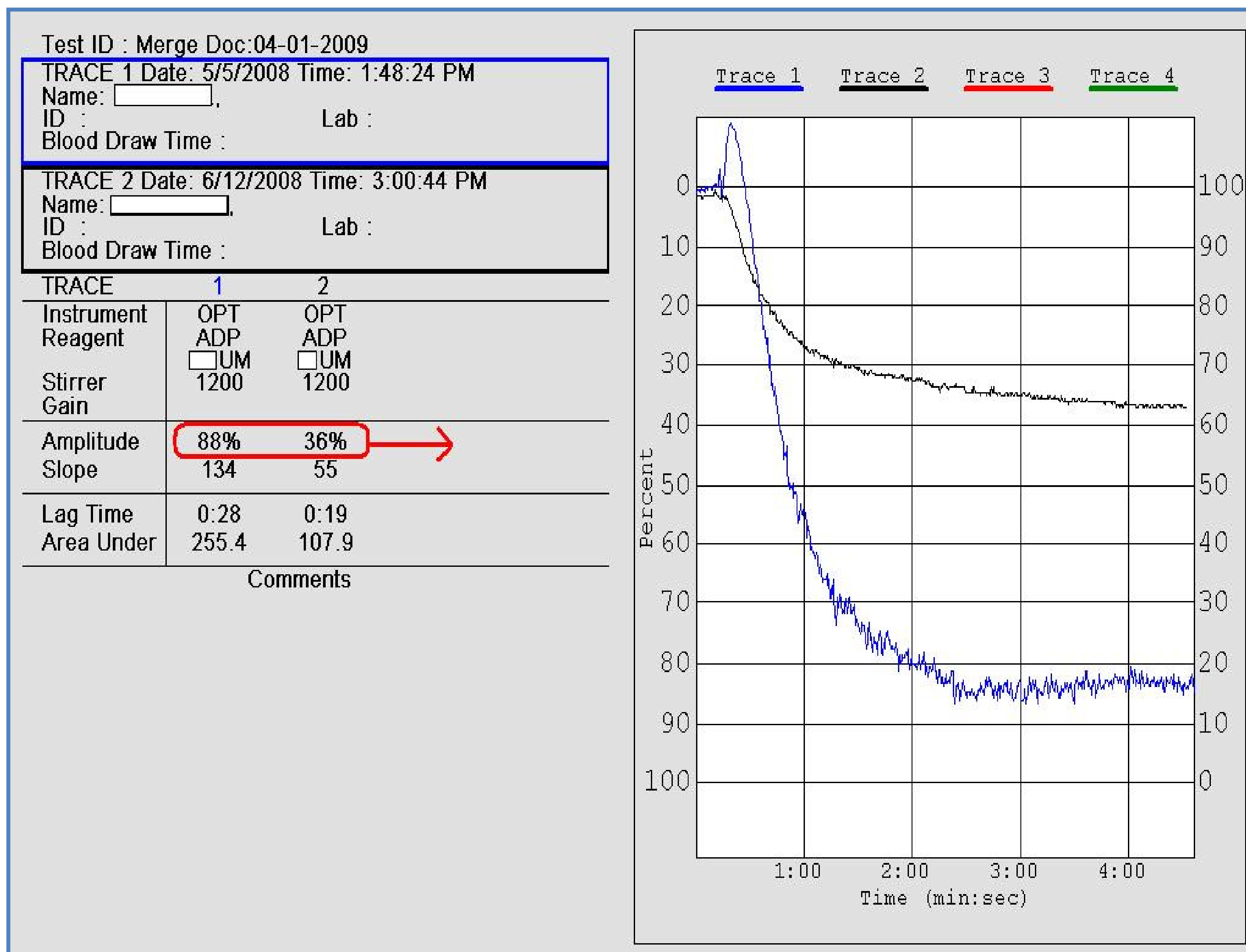


Figure 5: Example of ADP induced platelet aggregation

3.5.3 Thromboelastography (TEG):

As fluid blood starts to clot, the blood becomes thicker and stickier, this increased stickiness is detected by the TEG. With a TEG one can determine the overall characteristics of a blood clot (Gorton and Lyons, 1999). TEG determines the time for an initial clot to form (**R**), the rate at which it forms (**α Angle**), the time it takes to reach a certain strength (**K**), the overall strength of the clot (**MA** - mainly due to platelet aggregation) and fibrinolysis of the clot (**LY30** – amount of lysis after 30 minutes). It thus gives you a good idea of the overall effect an antithrombotic drug has on blood coagulation (TEG® 5000 User Manual, 2007). The TEG produces a real-time graphical display (Figure 6) of the formation and lysis of the blood clot. The TEG is most often used in liver transplant and cardiothoracic surgery to examine coagulopathy and the effect of antithrombotic agents (Gorton and Lyons, 1999).

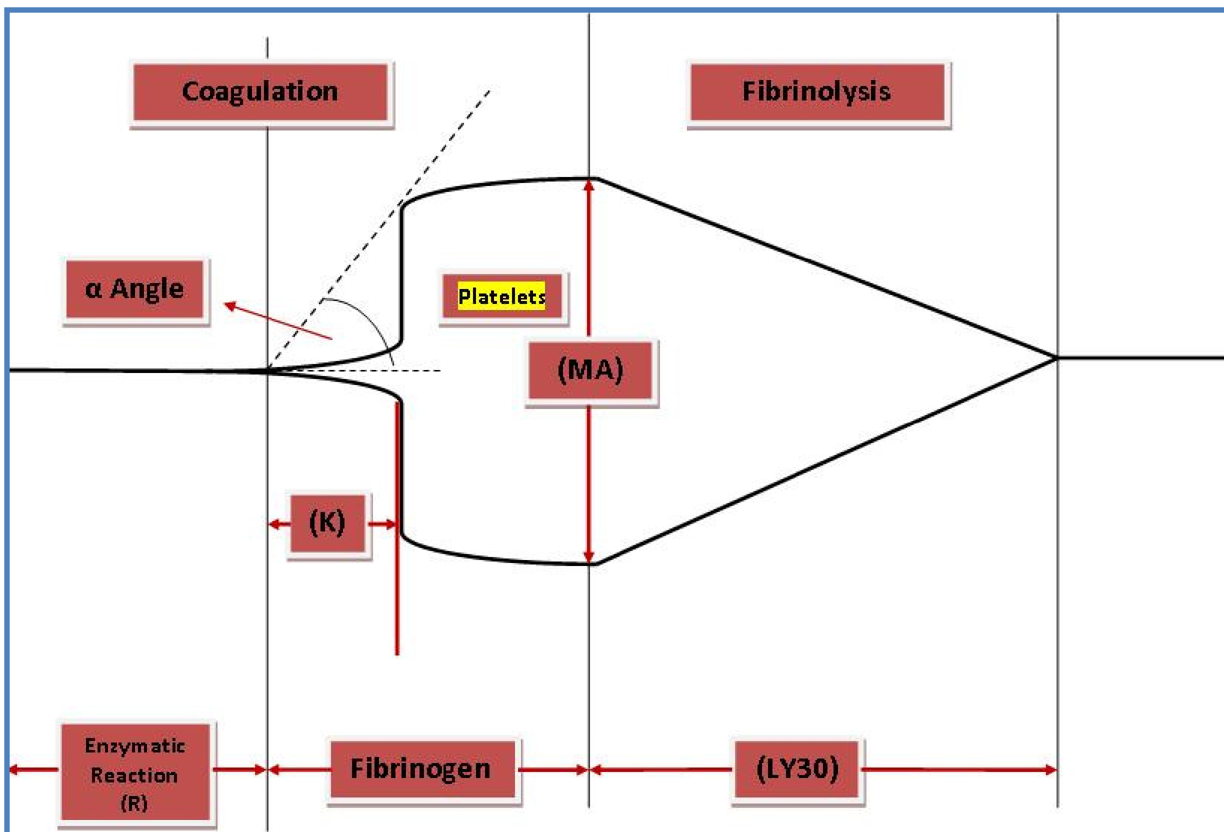


Figure 6: Schematic presentation of the TEG

3.5.4 Scanning Electron Microscopy:

Scanning electron microscopy (SEM) is the process where images are formed using electrons instead of light (http://mse.iastate.edu/microcopy/whatsem.html). The electrons are used to illuminate an object. This process makes it possible to create images at a much higher resolution than that with light microscopy, as electrons have a much smaller wavelength (50 trillionth of a meter) than the smallest wavelength of visible light (40 millionths of a meter) (http://encarta.msn.com/text_761562052___0/Microscope.html). This higher resolution makes it possible to examine the surface of objects at very high magnification (http://mse.iastate.edu/microcopy/whatsem.html). Scanning electron microscopes have the ability to magnify objects 100 000 times or more. They work by focussing electrons in a beam on the object, then constructing an image with the pattern of electron emission from the surface. They can hereby construct detailed three-dimensional images of the object surfaces (http://encarta.msn.com/text_761562052___0/Microscope.html). The artery was fixed overnight in a 3% glutaraldehyde 0.1M phosphate buffer solution (pH = 7.0). The tissue was then transferred to and fixed in 2% osmiumtetraoxide. The tissue was then dehydrated in a series of different alcohol solutions as follows: 50%, 70%, 95% and 100% (once per solution for 15 minutes, twice in 100% for 1 hour). After dehydration the tissue was dried with critical point drying in carbondioxide under high pressure in a Sampdri-795 apparatus. The tissue was then mounted on a small metal plate and coated with gold in the Biorad SEM coating system. Images were then taken with the Shimadzu SSX-550 Superscan Scanning Electron Microscope.

3.5.5 Full blood count (FBC):

A blood cell analyser determines the numbers and concentrations of the various components of blood. The parameters we were interested in were the haemoglobin (Hb) concentration (indicative of number of red blood cells in blood), mean corpuscular volume (MCV - size of cell), platelet (Plt) count and white blood cell count (WBC; indicative of infection). Normal FBC values for adult Caucasian males are shown in Table 1 (NHLS - Universitas Tertiary Hospital).

Parameter	Normal Value
Haemoglobin	14.5-18.5 g/dL
MCV	80-100 fl
Platelet Count	150-400 x 10^9/L
White Blood Cell Count	4.0-11 x 10^9/L

Table 1: Normal Full Blood Count – Caucasian Males

3.6 Post-operative care

The animals received 10 mg/kg Baytril, an antibiotic drug, after the procedure to prevent infection. Tengesic (0.3 mg/10 kg) was administered intramuscularly as a long-lasting analgesic after the procedure. Animals were housed in holding cages at the primate facility on the main campus of the UFS. Animals were monitored at least 3 times a day by staff working at the primate facility. An animal welfare sheet was filled in once a day to monitor the animals for 7 days post-operatively. Any adverse effects noted on the animal welfare sheet were put in the final report of this study. This was a further step to establish the safety of the drug.

3.7 Statistical analysis

Different parameters were compared using the Student t-test for paired data, with differences considered significant when $p < 0.05$. All results were put in an EXCEL spreadsheet for further analysis.

Chapter 4

Results

Figure 7 is an example of a results graph obtained in the efficacy study. No significant effect was seen after injection of up to 3 µg/kg (plus 0.05 µg/kg/min maintenance dose) tirofiban hydrochloride. We saw a momentary inhibition after injection of 10 µg/kg (plus 0.15 µg/kg/min maintenance dose) tirofiban hydrochloride, but this was soon abolished and a return of CFRs was observed. Thus 10 µg/kg (plus 0.15 µg/kg/min maintenance dose) tirofiban hydrochloride had no significant effect on the number of CFRs. Full inhibition was seen after injection of 30 µg/kg (plus 0.45 µg/kg/min maintenance dose) tirofiban hydrochloride, but this was abolished by re-injuring the artery. This was therefore not a true strong inhibition. This can be interpreted that at this dose tirofiban hydrochloride is able to inhibit further platelet deposition on a stable thrombus, but not on a fresh lesion. Only after injection of 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) we observed full inhibition that was not reversed by re-injury. This is a dose nine times higher than is stated in the Aggrastet® package insert as the therapeutic dose for adults during angioplasty. Inhibition was not reversed by infusion of epinephrine.

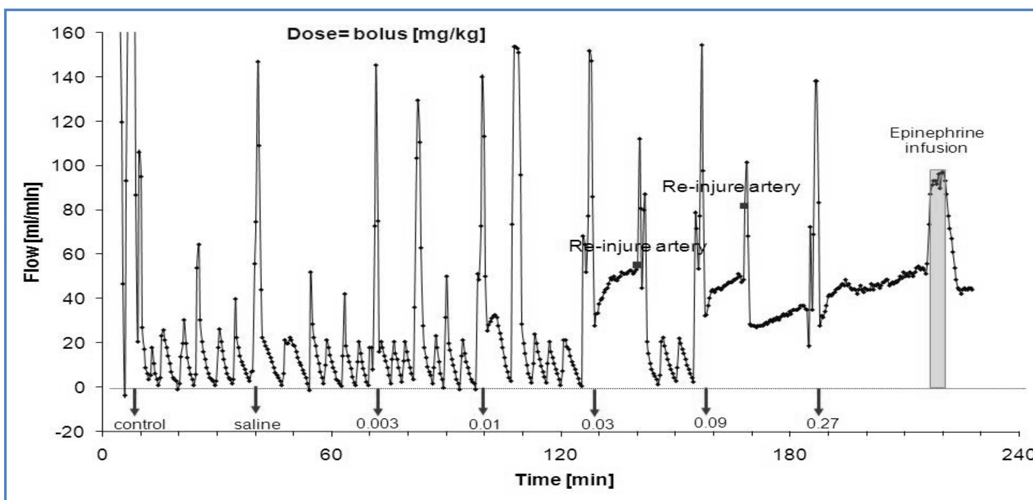


Figure 7: A typical results graph of the efficacy study for tirofiban hydrochloride

Figure 8 shows the mean CFRs for each dose of all five animals used in this study. A significant decrease in CFRs was only observed after injection of 30 µg/kg (plus 0.45 µg/kg/min maintenance dose) tirofiban hydrochloride. Injection of 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) caused complete inhibition of CFRs in all animals, and this inhibition persisted for all the escalating doses and was not reversed by either re-injury or 2.2 µg/kg/min epinephrine infusion.

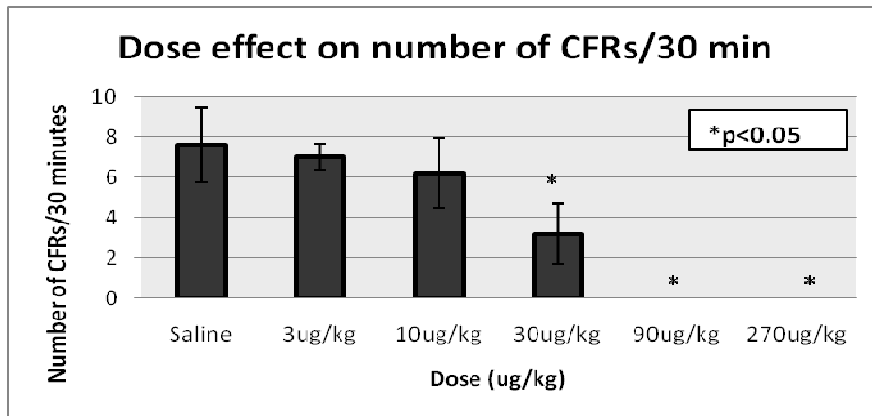


Figure 8: Dose effect on number of CFRs/30 minutes

Figure 9 shows the effect of the escalating doses of tirofiban hydrochloride on ADP platelet aggregation. A significant decrease was already observed after injection of 3 µg/kg (plus 0.05 µg/kg/min maintenance dose), with full inhibition observed after injection of 10 µg/kg (plus 0.15 µg/kg/min maintenance dose) and all escelating doses thereafter.

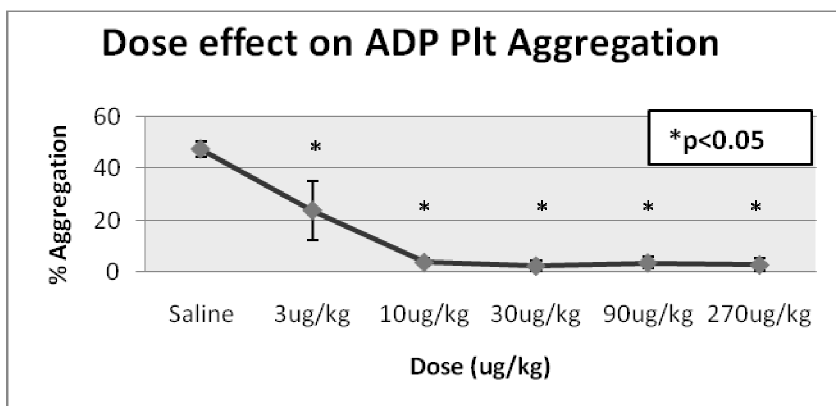


Figure 9: Dose effect on ADP platelet aggregation

Figure 10 shows results of the incision bleeding model. Blood loss was expressed as a ratio to the amount of blood lost during the saline phase. A significant increase in bleeding was only seen after injection of 270 µg/kg (plus 4.05 µg/kg/min maintenance dose) tirofiban hydrochloride, which is a dose 27 times higher than the stated therapeutic dose. A mean 2.7 fold increase in blood loss was seen after injection of 90 µg/kg (plus maintenance dose) tirofiban hydrochloride, which was the effective dose. The maximum mean increase in blood loss was 3.4 fold after injection of 270 µg/kg (plus 4.05 µg/kg/min maintenance dose) tirofiban hydrochloride.

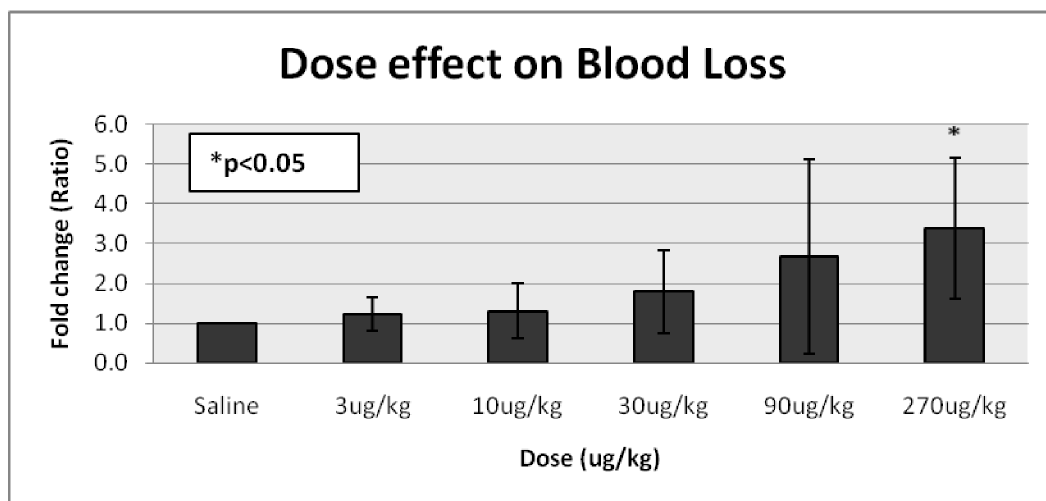


Figure 10: Dose effect on blood loss

Figure 11 shows results for the bleeding time (BT). A gradual increase in BT was seen with escalating doses of tirofiban hydrochloride. A significant prolongation in bleeding time was observed after injection of 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) tirofiban hydrochloride, and it remained significantly prolonged for the remaining dose. In three of the five animals BT was longer than 15 minutes at 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) tirofiban hydrochloride.

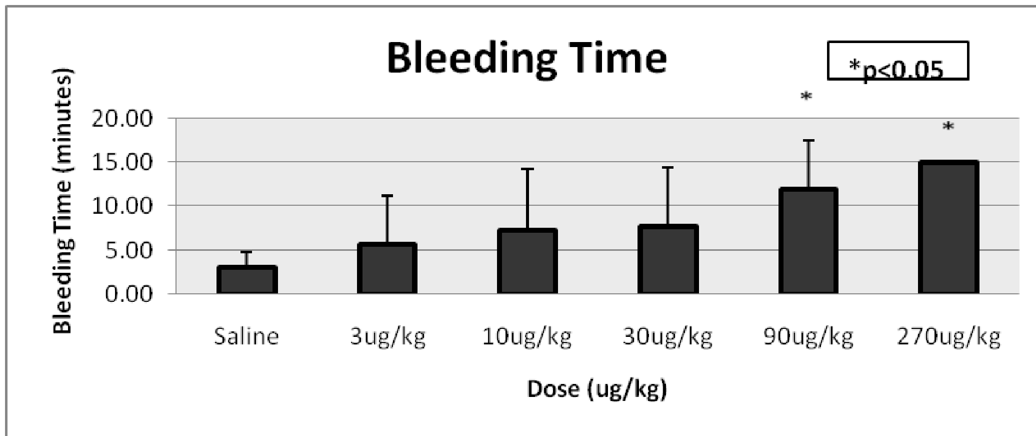


Figure 11: Dose effect on Bleeding Time

Figure 12 shows the results for the coagulation parameters. There was a significant prolongation of the Prothrombin Time (PT) after injection of 270 µg/kg (plus 4.05 µg/kg/min maintenance dose) tirofiban hydrochloride. The Activated Partial Thromboplastin Time (aPTT) showed significant prolongation after injection of 10 µg/kg (plus 0.15 µg/kg/min maintenance dose) and all the following doses. Even though they were statistically significant, it was clinically irrelevant as each value still was within the very narrow normal ranges of the different tests, i.e. 10-14 second for PT and 26-35 seconds for aPTT.

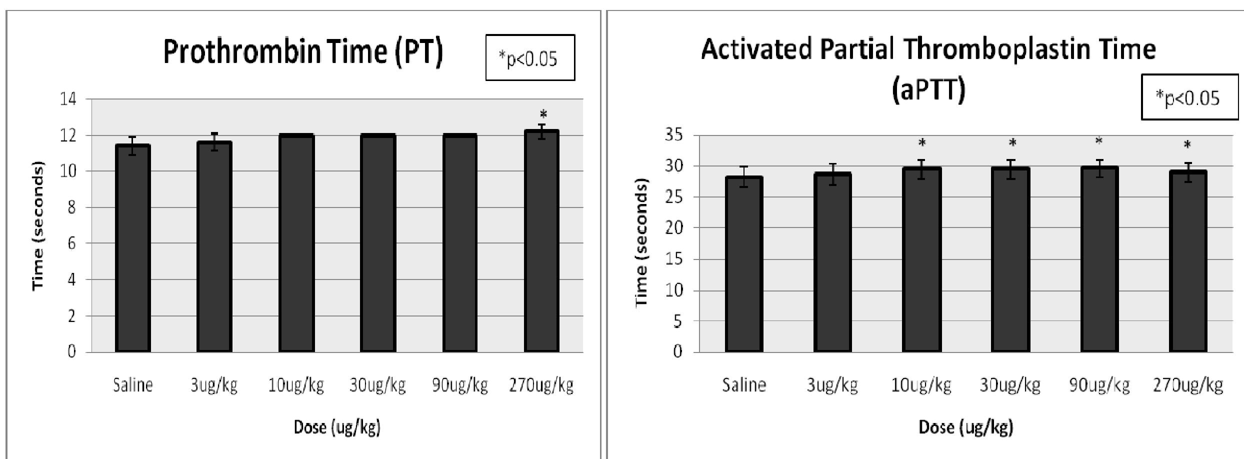


Figure 12: Dose effect on PT and aPTT

Figure 13 shows results of the different haematological parameters. A gradual increase in the white cell count (WBC) was observed, but none of the increases were significant. This increase in WBC is usually seen in surgical interventions, and is caused by the intervention. We saw a steady decrease in Haemoglobin concentration (Hb), with a significant decrease observed after injection of 30 µg/kg (plus 0.45 µg/kg/min maintenance dose) tirofiban hydrochloride. This significant decrease was observed for all the following doses. A decrease in Hb also happens during surgical interventions and continued venesection, as in this study. Platelet counts (Plt) decreased slightly after injection of 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) tirofiban hydrochloride, possibly due to the continuous venesection in the study. Although the decrease in platelet count was significant, the values remained well within normal range. No significant change was observed in the mean corpuscular volume (MCV) of the red blood cells.

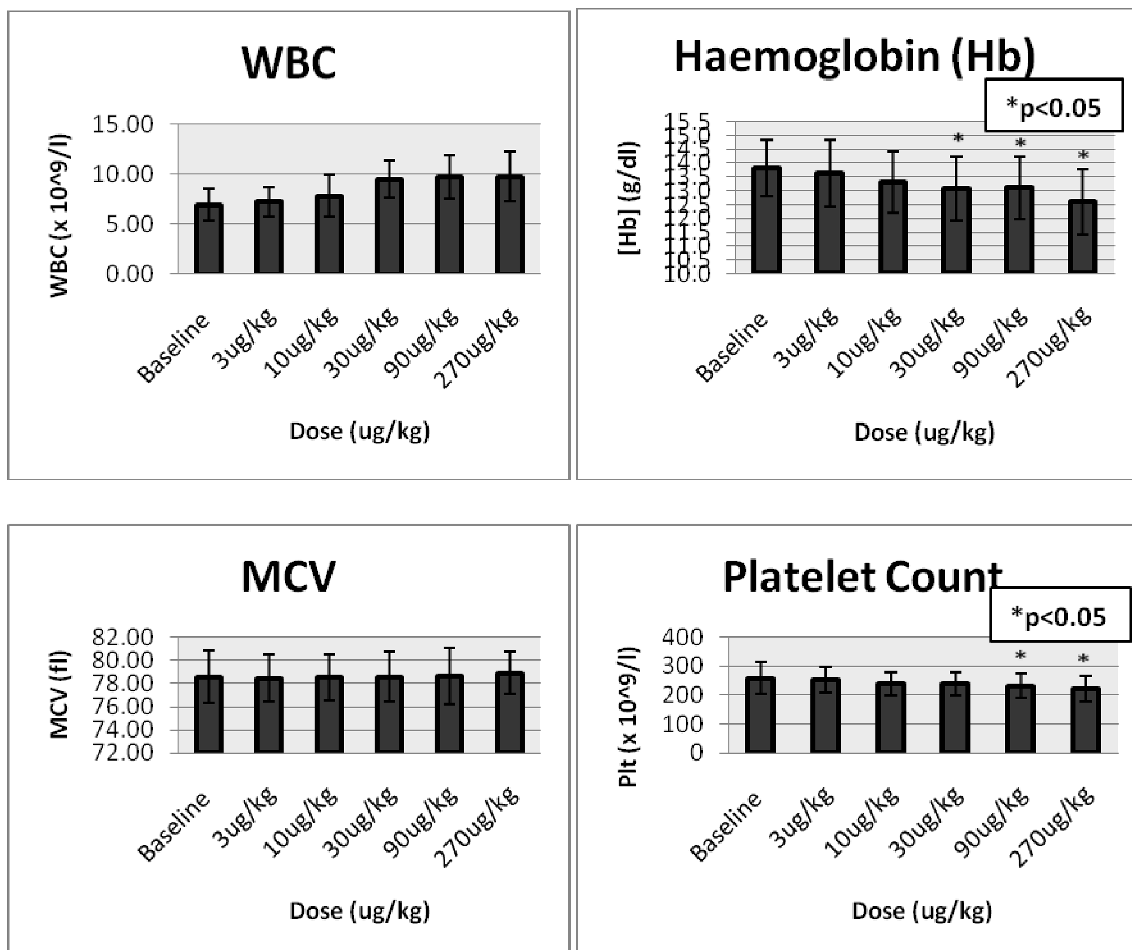


Figure 13: Dose effect on Haematological Parameters

Figure 14 shows results obtained with the thromboelastograph (TEG). A gradual increase in the time it took for the initial clot to form (**R**) was observed, with a significant increase after injection of 90 µg/kg (plus 1.35 µg/kg/min maintenance dose) tirofiban hydrochloride, as well as with the following dose. The rate at which the initial clot formed (**α Angle**) showed a significant decrease after injection of 10 µg/kg (plus 0.15 µg/kg/min maintenance dose) tirofiban hydrochloride. A significant increase in the time it took for the clot to reach a certain strength (**K**) was observed after injection of 30 µg/kg (plus 0.45 µg/kg/min maintenance dose) tirofiban hydrochloride, as well as with the following doses. In three of five of the studies the clot failed to reach that certain strength after injection of 270 µg/kg (plus 4.05 µg/kg/min maintenance dose) tirofiban hydrochloride. For calculation purposes, these values were taken as 15 minutes. A significant decrease in clot strength (**MA**) was observed after injection of 30 µg/kg (plus 0.45 µg/kg/min maintenance dose) tirofiban hydrochloride, as well as with all the higher doses. In this study we did not measure the fibrinolysis of the clot (**LY30**), as we were only interested in the anti-platelet effect of the drug, which is measured by the other four parameters.

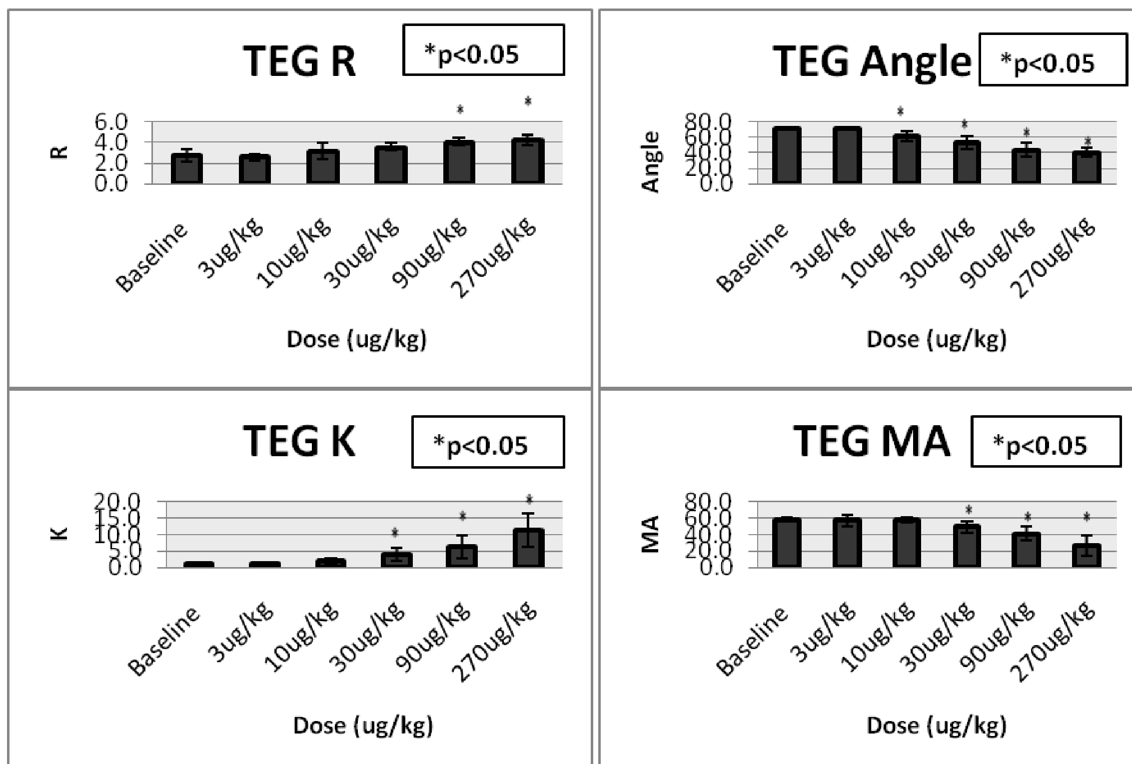


Figure 14: Results for different TEG Parameters

Figure 15 shows an example of an efficacy study previously done with clopidogrel in the same model at the University of the Free State (Roodt *et al.*, Appendix B). Inhibition was observed after injection of 1.5 mg/kg clopidogrel, but it was reversed by re-injury of the artery, thus it was not true inhibition, a new injury was applied to determine whether inhibition was a strong inhibition able to inhibit platelet aggregation on a fresh injury. Full inhibition was observed after injection of 2.5 mg/kg clopidogrel, as well with all the following doses. This inhibition was not reversed by re-injury. After injection of 10 mg/kg clopidogrel, the infusion of 2.2 µg/kg/min epinephrine did, however, reverse inhibition and caused CFRs to return. This is in contrast to results found with tirofiban hydrochloride where epinephrine infusion did not reverse platelet inhibition and is a sign of weak platelet inhibition. Blood loss at the effective dose (2.5 mg/kg) increased 4.25 fold when compared to the amount lost during the saline phase. After injection of double the effective dose, this blood loss further increased to nearly 8 (7.98) fold to that during the saline phase (Fig. 16). This was the maximum increase in blood loss seen, as blood loss stabilised with the following doses.

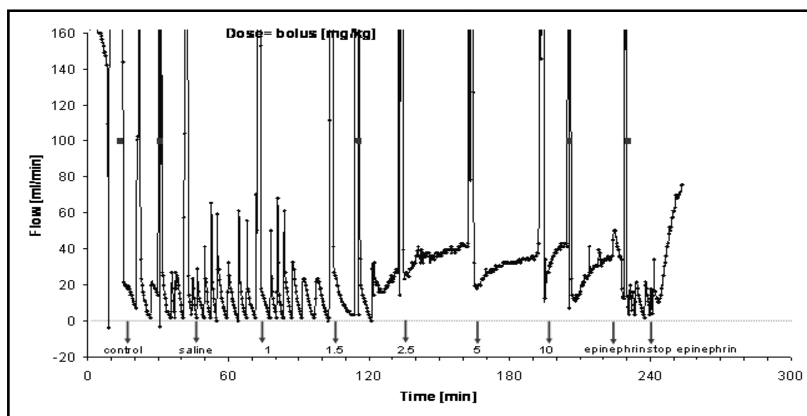


Figure 15: Example of efficacy study with clopidogrel

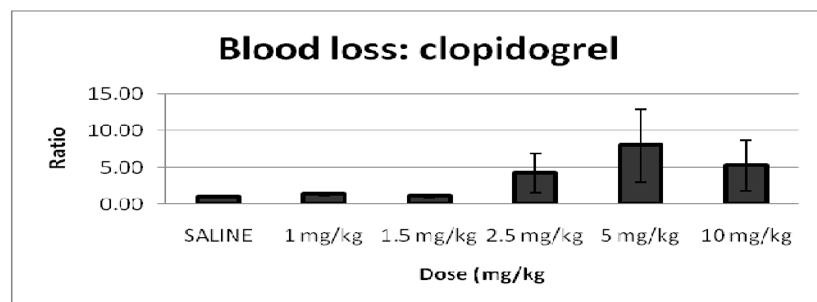


Figure 16: Dose effect of clopidogrel on blood loss

Figure 17a and 17b is scanning electron microscopy (SEM) images of the surface of an intact uninjured artery at 3000x and 3600x magnification respectively, and figures 17c and 17d is SEM images of the surface of the injured artery covered by aggregating platelets at 3000x and 3600x magnification respectively. These images illustrate the difference between an injured and uninjured artery, and that the method we used to injure the artery was effective in exposing the thrombogenic subendothelium for thrombus formation to take place.

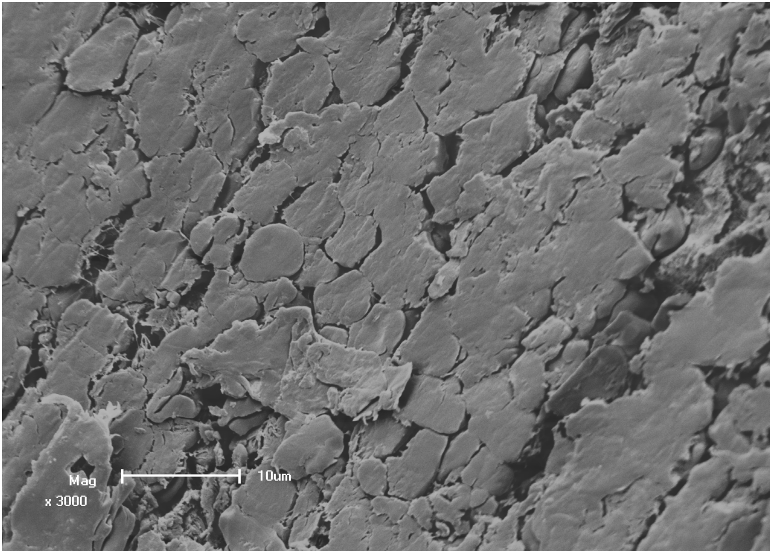


Figure 17a: SEM image of surface of an intact uninjured artery at 3000x magnification

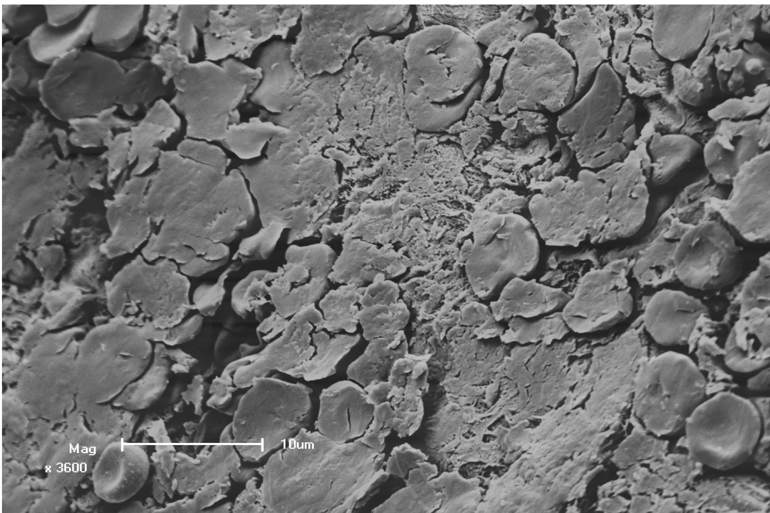


Figure 17b: SEM image of surface of an intact uninjured artery at 3600x magnification

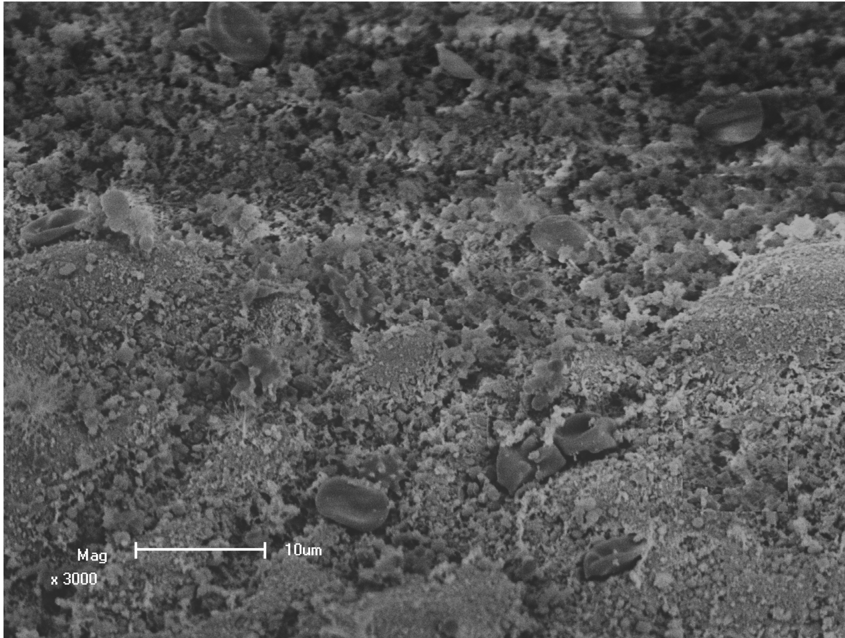


Figure 17c: SEM image of surface of an injured artery covered with aggregating platelets at 3000x magnification

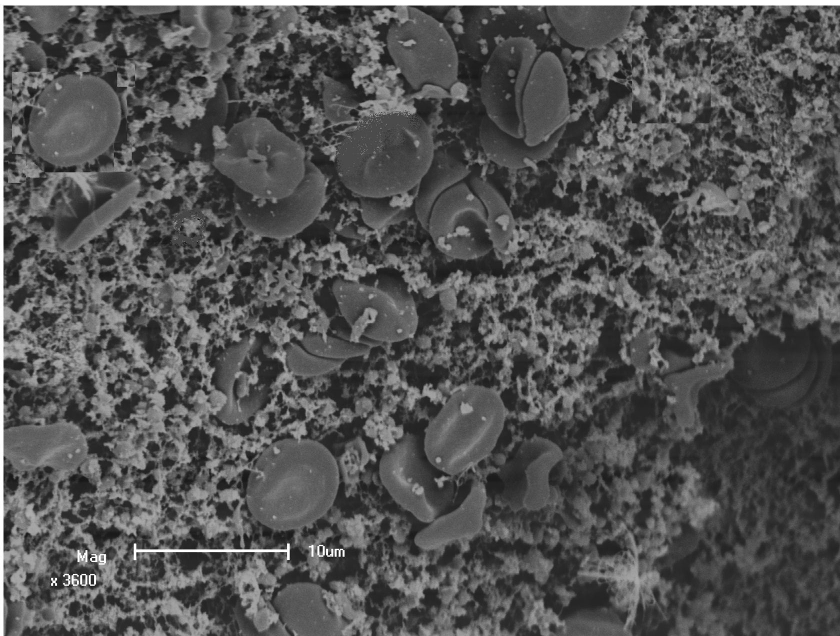


Figure 17d: SEM image of surface of an injured artery covered with aggregating platelets at 3600x magnification

Figure 18 is a compilation of the results obtained in the efficacy studies of all five baboons, and shows the good reproducibility achieved in these studies.

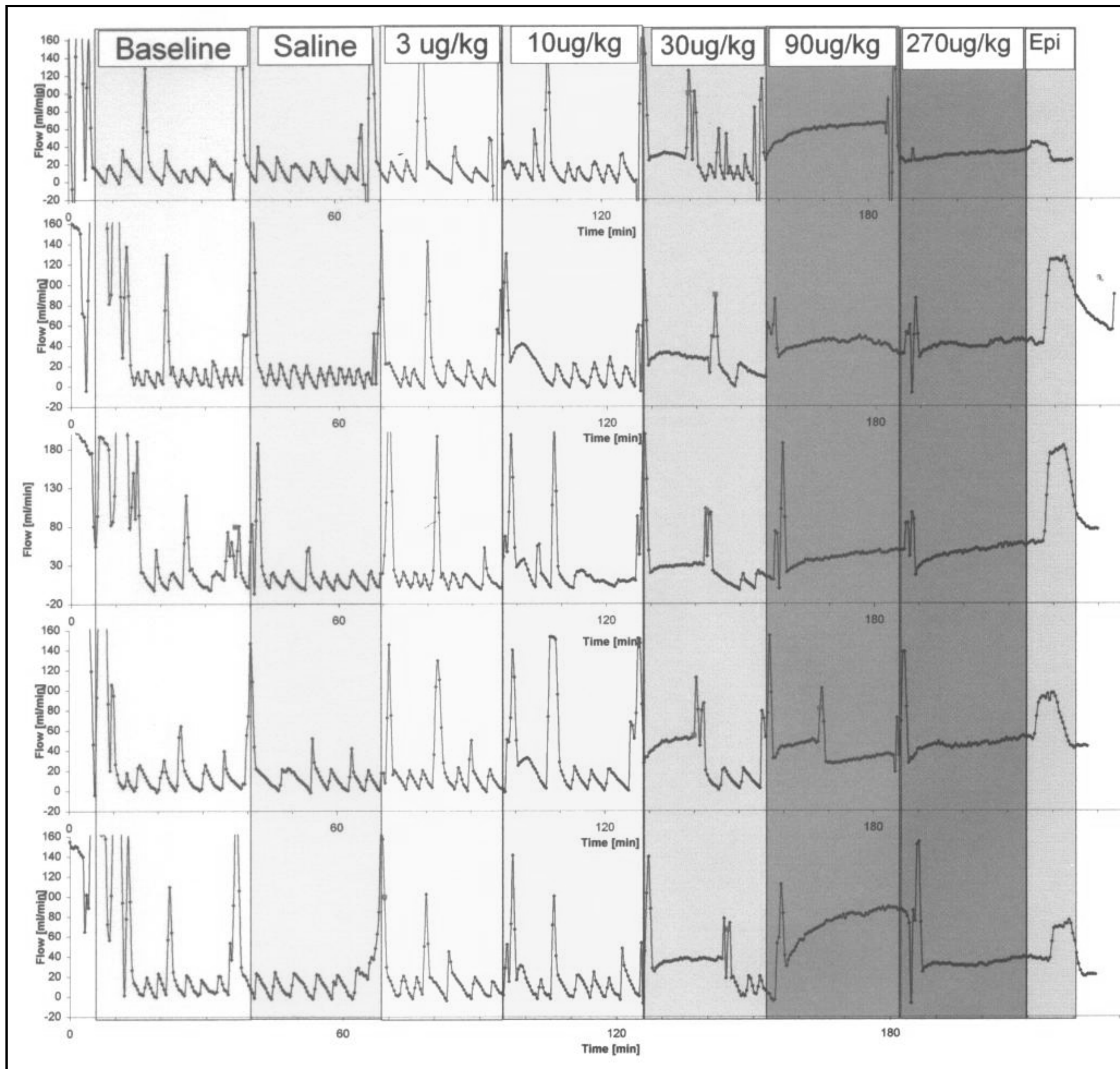


Figure 18: Compilation of efficacy study results of all 5 baboons

Chapter 5

Discussion

When a patient presents with symptoms of acute coronary syndrome, the American College of Cardiology and the American Heart Association recommend that the patient be given aspirin and heparin immediately to prevent further thrombosis formation. It is further recommended to administer clopidogrel to patients who are unable to take aspirin due to hypersensitivity or gastrointestinal intolerance, as well as to patients where a surgical intervention is not planned. The guideline further states that tirofiban hydrochloride should be given, together with aspirin and heparin, if a surgical intervention is planned, and clopidogrel stopped five to seven days prior to surgery (Braunwald *et al.*, 2002).

Aspirin and clopidogrel cannot be effectively given to all patients, as some patients show evidence of aspirin and/or clopidogrel resistance. However, aspirin therapy remains the standard in the management of unstable angina (PRISM Study, 1998). Administering tirofiban hydrochloride to patients with aspirin and/or clopidogrel resistance decreases periprocedural myocardial infarction during elective percutaneous coronary intervention (PCI) (Valgimigli and Verheugt, 2008). Taking these facts into consideration we selected clopidogrel as the drug of choice to compare tirofiban hydrochloride to.

Taking into account all the similarities between baboon and human blood clotting, especially similarities in the mechanisms of clot initiation, prothrombin activation, production of thromboplastin and fibrin formation and stability (Hampton and Matthews, 1966), it made the selection of the baboon as our research animal of choice a practical and rational one.

We found that an injection of 90 µg/kg tirofiban hydrochloride, together with a maintenance infusion of 1.35 µg/kg/min, is effective to inhibit thrombus formation in our high shear rate arterial thrombosis model in baboons. This inhibition is not reversed by either re-injury or 2.2 µg/kg/min epinephrine infusion. The effect can thus be seen as true, as well as strong inhibition (not reversed by epinephrine infusion). The importance of re-injuring the artery was shown in this case where full inhibition was seen after injection of 30 µg/kg tirofiban hydrochloride (plus maintenance dose of 0.45 µg/kg/min), but inhibition was reversed after re-injury. This was a pattern observed in all the baboons. The fact that full inhibition was only observed after injection of 90 µg/kg (plus maintenance dose of 1.35 µg/kg/min) tirofiban hydrochloride is a worrying one, as this is nine times above the recommended adult human therapeutic dose (10 µg/kg bolus plus 0.15 µg/kg/min maintenance infusion) for angioplasty/artherectomy.

Suboptimal dosage of tirofiban hydrochloride is a known problem, and was first described by the TARGET (The Do Tirofiban and Reopro Give Similar Efficacy Outcome Trial) investigators. They found that a 10 µg/kg bolus of tirofiban hydrochloride, together with a maintenance infusion of 0.15 µg/kg/min, was much less effective than abciximab in preventing ischaemic events (Topol *et al.*, 2001). This dose was determined by measuring the pharmacodynamic effect of tirofiban hydrochloride on *ex vivo* platelet aggregation studies. In this dose-ranging study, ADP-induced platelet aggregations were done on samples collected in 3.8% sodium citrate (in contrast with the recommended 3.2%), and by using 5 µM ADP (in contrast with using 20 µM as used for other anti-platelet agents) as agonist (Kereiakes *et al.*, 1996). Analysis has shown that this combination of a high concentration of citrate together with low concentration ADP leads to an overestimation of aggregation inhibition by tirofiban hydrochloride (Moliterno and Topol, 2002 and Soffer *et al.*, 2003). In the study done by Soffer *et al.* in 2003, the 3.8% sodium citrate was replaced with 3.2% sodium citrate, and 20 µM ADP was used as agonist, instead of 5 µM ADP. They found that the mean inhibition of platelet aggregation was 63 ±25%, where only 32% of patients achieved inhibition of more than 80% (>80% is seen as optimal inhibition) (Soffer *et al.*, 2003). In the study conducted by Valgimigli, an increased bolus of 25 µg/kg tirofiban hydrochloride was given together with the standard

infusion of 0.15 µg/kg/min. They found that at this dose it was an effective treatment to reduce the risk for myocardial infarction, but it did not show any difference in major or minor bleeding compared to a placebo group (Valgimigli and Verheugt, 2008). Other studies still found increased doses were inadequate when compared to the efficacy of abciximab (Marzocchi *et al*, 2008). It must be noted that the increased dose was only determined by *ex vivo* platelet aggregation studies (Schneider *et al*., 2002). This all correlates well with our findings of a failure to inhibit *in vivo* thrombus formation, but effective *ex vivo* platelet inhibition at the therapeutic dose (Figure 8 and 9). In all the baboons we noted a very short temporary inhibition after injection of 10 µg/kg (plus maintenance dose of 0.15 µg/kg/min) tirofiban hydrochloride. This inhibition was rapidly and spontaneously reversed and thrombus formation continued. This dose also brought significant changes in the aPTT, ADP aggregations and TEG angle. The most significant difference was seen with the ADP platelet aggregation tests, where full inhibition of platelet aggregation was seen at this dose. Taking the temporary inhibition of CFRs and the full inhibition of ADP platelet aggregations into account, one can postulate that an increase in maintenance dose will result in sustained platelet inhibition *in vivo*.

The first significant increase in blood loss in the incision bleeding model was only seen after injection of 270 µg/kg (plus maintenance dose of 4.05 µg/kg/min) tirofiban hydrochloride. This was a 3.4 fold increase in blood loss compared to the saline phase. This dose was 27 times higher than the one stated as the therapeutic dose, and three times higher than the dose in which we found to be effective. This is less than the bleeding caused by clopidogrel where an 8.4 fold increase in bleeding was seen at twice the therapeutic dose. The template bleeding time was significantly prolonged after injection of 90 µg/kg (plus maintenance dose of 1.35 µg/kg/min). In three of the five animals a prolongation of more than 15 minutes was observed, but we found that the template bleeding model was not representative of the clinical bleeding observed during the study.

In the TARGET trial the tirofiban hydrochloride group gave similar major bleeding complications as the abciximab group, but less minor bleeding and thrombocytopenia. It must however be noted that all the patients also received 250 to 500 mg of aspirin, unfractionated heparin (≤70 U/kg), and if possible a loading dose of 300 mg clopidogrel (Topol *et al.*, 2001). Clopidogrel is known to synergistically enhance the anti-platelet effect of other anti-platelet drugs (Hotline Editorial, 2002), and on its own is known to cause severe bleeding (Vega, 2008). Kralisz *et al.* noted that the bleeding complications seen in tirofiban-heparin combination studies cannot be excluded as being caused by too high doses of heparin. They also noted that PCI could be done safely and effectively with lower doses of heparin, given that the GP IIb/IIIa inhibitors are at an effective dose to inhibit platelet aggregation (Kralisz *et al.*, 2004).

Observation of the animals post-operatively showed no adverse effects in the 7 days following the operation. Two animals did show worrying signs of bleeding just after completion of the operation, but bleeding ceased within an hour. No signs of vomiting, haematomas, abnormal stool or bleeding were observed, and the animals returned to normal feeding on the same day of operation. Results of the haematological parameters showed no unexpected results. The slight increase in white cell count and decrease in haemoglobin concentration and platelet count are all phenomena normally seen during surgical interventions, and were not clinically significant. Even though significant differences were measured within the PT and aPTT, these results all fell within the normal ranges of the different tests, and can thus be contributed to normal physiological variation and not due to tirofiban hydrochloride. Taking into consideration that the strength of the clot (measure by the TEG - MA) decreases significantly after injection of 30 µg/kg (plus maintenance dose of 0.45 µg/kg/min), together with the full inhibition of ADP platelet aggregations and partial inhibition of CFRs at lower doses than the one we found to give full true CFR inhibition, a case for a lower bolus injection (than 90 µg/kg) together with a higher maintenance dose (than 0.15 µg/kg/min) can be made. The decrease in clot strength in itself is a cause of concern, as this can possibly lead to an increased risk of embolisation of the thrombi, and subsequent damage to distal tissue. At 90 µg/kg (plus maintenance infusion of 1.35 µg/kg/min) tirofiban hydrochloride compares favourably with clopidogrel in terms of

efficacy. Clopidogrel however caused 1.55 times more blood loss than tirofiban hydrochloride at an effective dose. Taking maximum blood loss into consideration clopidogrel also caused 4.58 times more blood loss at twice its effective dose than tirofiban hydrochloride at three times its effective dose. Thus tirofiban hydrochloride causes much less bleeding than clopidogrel, and is therefore a safer drug to use. The fact that epinephrine infusion reversed inhibition in the clopidogrel study, but not in the tirofiban hydrochloride study, showed that tirofiban hydrochloride caused strong platelet inhibition, in contrast with the weak inhibition caused by clopidogrel.

The similar results obtained for all five animals, as shown in fig. 18, is an illustration of the good reproducibility achieved with this arterial thrombosis model. The scanning electron microscope images reveal that we are successful in stimulating arterial thrombosis by exposing the thrombogenic subendothelium via injury. By using the animals own artery as thrombogenic surface we believe that we are successful in generating a true reflection of thrombogenic conditions *in vivo*.

Chapter 6

Conclusion

We conclude that tirofiban hydrochloride is an effective inhibitor of arterial thrombus formation in a high shear rate arterial thrombosis model in baboons, but at a dose nine times higher than the one recommended as therapeutic dose in adult humans. Our findings correlate well with clinical findings and are thus a true reflection of the efficacy and safety of the drug in the clinical setting.

The efficacy of tirofiban hydrochloride also compares favourably to clopidogrel, but with less bleeding complications and stronger inhibition during stressful situations. We recommend that further *in vivo* testing should be done to determine at which dose platelet inhibition is sustained. We further feel that higher dose clinical trials can be done with confidence, as even the much higher dose of 270 µg/kg bolus plus a maintenance dose of 4.05 µg/kg/min did not cause as much bleeding as the commonly used drugs that are available, and that even though a increase in bleeding is seen, it still remains safer than the drugs that are currently used.

We were successful in creating conditions conducive to arterial thrombosis as shown with scanning electron microscopy. We were also able to show the high level of reproducibility of our model. We can therefore also conclude that our model is an effective platform to evaluate the efficacy of an anti-platelet drug to inhibit and prevent platelet thrombus formation on a thrombogenic surface at high shear rates.

Chapter 7

Summary (English)

Keywords: *Acute coronary syndrome • Anti-platelet drugs • Arterial thrombosis • Atherosclerosis • GP IIb/IIIa inhibitor • Non-human primates • Tirofiban hydrochloride*

Background: Acute coronary syndrome (ACS) is a major cause of mortality and morbidity world-wide, and is responsible for roughly 2.5 million hospital admissions world-wide annually. ACS is commonly associated with platelet thrombus formation on disrupted atherosclerotic plaques, therefore effective and safe anti-platelet drugs are needed to help treat and prevent ACS. The current most popular anti-platelet drugs are associated with increased bleeding risk and reduced efficacy, thus drugs with a wider therapeutic window (more efficacy with less bleeding) need to be developed. Tirofiban hydrochloride is a small, short half-life molecule that inhibits platelet aggregation by antagonising the glycoprotein IIb/IIIa receptor on platelets preventing fibrinogen and von Willebrand factor to cross-link platelets, thereby inhibiting the final pathway of platelet aggregation. Tirofiban hydrochloride was believed to be a very promising drug due to its short half-life, as an antidote strategy is not needed to reverse adverse bleeding events, but it soon fell out of favour when it was found not to be as effective as for example abciximab in preventing ischaemic events. This was possibly due to the recommended dose being suboptimal.

Methods and Results: We studied the efficacy of tirofiban hydrochloride to inhibit platelet thrombus formation on an injured and partially occluded artery by evaluating the effect of escalating doses on cyclic flow reduction (CFR) formation in a high shear arterial thrombosis model in baboons, and also evaluated its safety in two different bleeding models. We then compared our results to results found in the same model using clopidogrel. A significant effect on the number of CFRs was only observed after injection of three times (30 µg/kg bolus plus 0.45 µg/kg/min infusion) the therapeutic dose tirofiban, but it was a weak inhibitor at this dose. Only after injection of nine times (90 µg/kg bolus plus 1.35 µg/kg/min infusion) the recommended therapeutic dose, a strong complete inhibition was observed. A further dose of

27 times (270 µg/kg bolus plus 4.05 µg/kg/min infusion) the recommended therapeutic dose was given to evaluate the effect of an overdose on the bleeding tendency. A significant prolongation in bleeding time (3.05 minutes to 11.90 minutes) was observed after injection of nine times the therapeutic dose, an average 2.7 ± 2.44 fold increase in blood loss was also observed at this dose. A maximum increase in blood loss of an average of 3.4 ± 1.77 fold was seen after injection of 27 times the therapeutic dose. The efficacy of tirofiban hydrochloride was comparable to that of clopidogrel found in earlier studies, but the blood loss was much less when compared to the average 4.3 ± 2.6 fold increase with clopidogrel at 2.5 mg/kg and 8.0 ± 5.0 fold increase at 5 mg/kg.

Conclusion: Tirofiban hydrochloride is an effective anti-platelet drug, but only offers adequate protection against arterial thrombosis at a dose between three and nine times the recommended therapeutic dose. However, it still remains safer in terms of bleeding than the most common anti-platelet drugs used today. We recommend that further *in vivo* studies be done to determine the optimal dose for tirofiban hydrochloride treatment, and that new clinical trials be done with higher dose tirofiban hydrochloride.

Hoofstuk 7

Opsomming (Afrikaans)

Sleutelwoorde: *Akute koronêre sindroom • Anti-plaatjie middels • Arteriële thrombose • Aterosklerose • GP IIb/IIIa inhibitor • Nie-menslike primate • Tirofiban hidrochloried*

Agtergrond: Akute koronêre sindroom (AKS) is die hoof-oorsaak van mortaliteit en morbiditeit wêreld-wyd, en is verantwoordelik vir ongeveer 2.5 miljoen hospitaal-toelatings per jaar in die wêreld. AKS word in die algemeen geassosieer met plaatjie thrombus-vorming op ontwrigte aterosklerotiese plake, dus is effektiewe en veilige anti-plaatjie-middels noodsaaklik vir die behandeling en voorkoming van AKS. Huidiglik word die mees populêre anti-plaatjie-middels geassosieer met ‘n verhoogde risiko vir bloeding en verlaagde effektiwiteit, dus is dit noodsaaklik om middels met ‘n beter terapeutiese venster (hoër effektiwiteit met minder bloeding) te ontwikkel. Tirofiban hidrochloried is ‘n klein, kort half-leeftyd molekuul wat plaatjie aggregasie inhibeer deur die glikoproteïen IIb/IIIa reseptor op plaatjies te antagoniseer. Dit verhoed dat fibrinogeen en von Willebrand faktor die plaatjies kruisverbind, en inhibeer so die finale pad van plaatjie-aggregasie. Daar is gedink dat tirofiban hidrochloried ‘n baie belowende middel is as gevolg van sy korter half-leeftyd, want in so ‘n geval word ‘n omkeer strategie nie benodig indien ‘n ongewenste bloeding sou voorkom nie. Dit het egter vinnig uit guns geval toe daar gevind is dat dit nie so effektief soos abciximab was om ischemiese gebeure te voorkom nie. Dit was moontlik as gevolg van die aanbevole dosis wat suboptimaal was.

Metodes and Resultate: Ons het die effektiwiteit van die middel om plaatjie-trombus vorming op ‘n beseerde en vernoude arterie te inhibeer bestudeer, deur die effek van verskillende verhogende dosisse op sikliese vloei reduksie (SVR) vorming in ‘n hoë skuifkrag arteriële trombose-model in bobbejane te evalueer. Ons het ook die veiligheid in twee verskillende bloedingsmodelle geëvalueer. Ons het toe ons resultate vergelyk met resultate gevind met clopidogrel in dieselfde model. ’n Statisties betekenisvolle effek op die hoeveelheid SVRe is eers na toediening van drie maal (30 µg/kg bolus plus 0.45 µg/kg/min infusie) die aanbevole terapeutiese dosis tirofiban hidrochloried waargeneem, maar dit is gevind om swak inhibisie te

wees. Eers na toediening van nege maal (90 µg/kg bolus plus 1.35 µg/kg/min infusie) die aanbevole terapeutiese dosis is ‘n sterk, volledige inhibisie waargeneem. Ons het nog ‘n dosis van 27 maal (270 µg/kg bolus plus 4.05 µg/kg/min infusie) die aanbevole terapeutiese dosis gegee om die effek van ‘n oordosis op die bloedingsneiging te evalueer. ‘n Statisties betekenisvolle verlenging in bloeityd (3.05 minute tot 11.90 minute) is waargeneem na toediening van nege maal die aanbevole terapeutiese dosis, ‘n gemiddelde 2.7 ± 2.44 maal toename in bloedverlies is ook by dié dosis waargeneem. ‘n Maksimum verhoging in bloeding van gemiddeld 3.4 ± 1.77 maal is na toediening van 27 maal die aanbevole terapeutiese dosis waargeneem. Die effektiwiteit van tirofiban hidrochloried was vergelykbaar met dié van clopidogrel, maar die bloedverlies was baie minder in vergelyking met die gemiddeld 4.3 ± 2.6 maal toename met clopidogrel by 2.5 mg/kg dosis en die 8.0 ± 5.0 maal toename by ‘n 5 mg/kg dosis.

Gevolgtrekking: Tirofiban hidrochloried is ‘n effektiewe anti-plaatjie-middel, maar verskaf slegs genoegsame beskerming teen arteriële trombose teen ‘n dosis tussen drie en nege maal die aanbevole terapeutiese dosis. Dit bly egter steeds veiliger in terme van bloeding as die mees algemene anti-plaatjie-middels in gebruik vandag. Ons stel voor dat verder *in vivo* studies gedoen word om te bepaal teen watter dosis tirofiban hidrochloried terapeuties gebruik kan word, asook dat nuwe kliniese proewe op hoër dosis tirofiban hidrochloried gedoen word.

References

- Abbott A. With your genes? Take one of these, three times a day. *Nature* 2003; 425:760-762.
- Achar SA, Kundu S, Norcross WA. Diagnosis of Acute Coronary Syndrome. *American Family Physician* 2005; 72:119-126.
- Aggrenox. [Online] Available: http://www.aggrenox.com [Accessed 28 November 2008].
- Aggrastat (Tirofiban) drug description – FDA approved labeling for prescription drugs. [Online] Available: www.rxlist.com/cgi/generic/tiro.htm [Accessed 11 August 2008].
- American Heart Association and American Stroke Association. Heart Disease and Stroke Statistics, 2006 – Update. [Online] Available: http://www.americanheart.org/downloadable/heart/1140534985281Statsupdate06book.pdf [Accessed 27 March 2009].
- Benedict CR, Ryan J, Wolitzky B, Ramos R, Gerlach M, Tijburg P, Stem D. Active site-blocked factor IXa prevents intravascular thrombus formation in the coronary vasculature without inhibiting extravascular coagulation in a canine thrombosis model. *Journal of Clinical Investigation* 1991; 88:1760-1765.
- Bizzari F, Scolletta S, Tucci E, Davoli G, Toscano T, Neri E *et al*. Perioperative use of Tirofiban hydrochloride (Aggrastat) does not increase surgical bleeding after emergency or urgent coronary artery bypass grafting. *The Journal of Thoracic and Cardiovascular Surgery* 2001; 122:1181-1185.
- Björkman JA, Van Giezen JJJ, Zachrisson H. Reduced bleeding time prolongation for the P2Y12 antagonist compared with the Thienopyridine, clopidogrel, in both a rat and dog model of combined thrombosis and hemostasis. *Journal of Thrombosis and Haemostasis* 2007; 5 Supplement 2: P-M-279.
- Braunwald E, Antman EM, Beasley JW, Califf RM, Cheitlin MD, Hochman JS *et al*. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice

Guidelines (Committee on Management of Patients With Unstabel Angina). *Circulation* 2002; 106:1893-1900.

- Caccone A and Powell JR. DNA divergence among hominoids. *Evolution* 1989; 43:925-942.
- Cadroy Y, Houghten RA, Hanson SR. RGDV Peptide Selectively Inhibits Platelet-dependant Thrombus Formation In Vivo. *The Journal of Clinical Investigation* 1989; 84:939-944. DOI: 10:1172/JCI114256.
- Coller BS, Folts JD, Smith SR, Scudder LE, Jordan R. Abolition of in vivo platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. Correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors. *Circulation* 1989; 80:1766-1774.
- Danzi GB, Capuano C, Sesana M, Baglini R. Preliminary experience with a high bolus dose of tirofiban during percutaneous coronary intervention. *Current Medical Research and Opinion* 2003; 19:28-33.
- Danzi GB, Capuano C, Sesana M, Baglini R. Safety of a High Bolus Dose of Tirofiban in Patients Undergoing Coronary Stent Placement. *Catheterization and Cardiovascular Interventions* 2004; 61:179-184.
- Danzi GB, Sesana M, Capuano C, Mauri L, Predolini S, Baglini R. Downstream administration of a high-dose tirofiban bolus in high-risk patients with unstable angina undergoing early percutaneous coronary intervention. *International Journal of Cardiology* 2006; 107:241-246.
- Davies NJH and Cashman JN. Ketamine. In: Lee's Synopsis of Anaesthesia. 13th Edition. *United Kingdom: Elsevier Butterworth Heinemann*; 2006: 162-164. ISBN: 0-7506-8834-3.
- Dipyridamole. [Online] Available: http://www.drugs.com/pro/dipyridamole-tablets.html [Accessed 12 August 2008].
- Dorsam RT and Kunapuli SP. Central role of the $P2Y_{12}$ receptor in platelet activation. The *Journal of Clinical Investigation* 2004; 113:340-345.
- Falk E, Shah PK, Fuster V. Coronary Plaque Disruption. *Circulation* 1995; 92:657-671.
- Falk E. Pathogenesis of Atherosclerosis. *Journal of the American College of Cardiology* 2006; 47:C7-C12. DOI: 10.1016/j.jacc.2005.09.068.

- Fenton DE. Acute Coronary Syndrome. eMedicine. Updated Oct 10, 2008. [Online] Available: http://emedicine.medscape.com/article/756979 [Accessed 2 February 2009].
- Fontayne A, Meiring M, Lamprecht S, Roodt J, Demarsin E, Barbeaux P, Deckmyn H. The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear rate arterial thrombosis model in baboons. *Thrombosis and Haemostasis* 2008; 100:670-677.
- Folts JD, Crowell EB, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation* 1976; 54:365-370.
- Folts JD, Gallagher K, Rowe GG. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation? *Circulation* 1982; 65:248-255.
- Folts JD, Stamler J, Loscalzo J. Intravenous nitroglyserine infusion inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries. *Circulation* 1991; 83:2122-2127.
- Gorton H and Lyons G. Is it time to invest in a Thromboelastograph? *International Journal of Obstetric Anesthesia* 1999; 8:171-178.
- Grech ED and Ramsdale DR. Acute coronary syndrome: unstable angina and non-ST segment elevation myocardial infarction. *British Medical Journal* 2003; 326:1259-1261.
- Hampton JW and Matthews C. Similarities between baboon and human blood clotting. *Journal of Applied Physiology* 1966; 21:1713-1716.
- Han Y, Wang B, Li Y, Xu K, Wang S, Jing Q, Wang Z, Wang D, Ma Y, Wang G. A high maintenance dose of clopidogrel improves short-term clinical outcomes in patients with acute coronary syndrome undergoing drug-eluting stent implantation. *Chinese Medical Journal* 2009; 122:793-797.
- Hanson SR, Griffin JH, Harker LA, Kelly AB, Esmon CT, Gruber A. Antithrombotic Effects of Thrombin-induced Activation of Endogenous Protein C in Primates. *The Journal of Clinical Investigation* 1993; 92:2003-2012. DOI: 10:1172/JCI116795.
- Hanson SR, Kotze HF, Savage B, Harker LA. Platelet interactions with Dacron vascular grafts. A model of acute thrombosis in baboons. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1985; 5:595-603.

- Harker LA and Mann KG. Thrombosis and Fibrinolysis. In: Fuster V and Verstraete M. Thrombosis in Cardiovascular Disorders. *Philadelphia: W.B. Saunders Company*, 1992: 1-16. ISBN: 0-7216-4012-5.
- Heart attack and acute coronary syndrome. [Online] Available: http://adam.about.com/reports/Heart-attack-and-acute-coronarysyndrome.htm [Accessed 2 February 2009].
- Heidegger T and Kreienbühl G. Unsuccessful Resuscitation Under Hypotensive Epidural Anesthesia During Elective Hip Arthroplasty. *Anesthesia and Analgesia* 1998; 86:847-849.
- Hirsh J, Anand SS, Halperin JL, Fuster V. Mechanism of Action and Pharmacology of Unfractionated Heparin. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2001; 21:1094-1096. DOI: 10.1161/hq0701.093686.
- Hoffbrand AV, Moss PAH, Pettit JE. Platelets, blood coagulation and haemostasis. In: Essential Haematology. Fifth Edition. *Oxford: Blackwell Publishing*; 2006: 265-269. ISBN: 1-4051-3649-9.
- Khan MG. Angina and Atherosclerosis/Atheroma. In: Encyclopedia of Heart Diseases. *Oxford: Elsevier Academic Press*; 2006: 35-55 and 117-130. ISBN: 0-12-406061-7.
- Kereiakes DJ, Kleiman NS, Ambrose J, Cohen M, Rodriguez S, Palabrica T *et al*. Randomized, double-blind, placebo-controlled dose-ranging study of tirofiban (MK-383) platelet IIb/IIIa blockade in high risk patients undergoing coronary angioplasty. *Journal of the American College of Cardiology* 1996; 27:536-542.
- Kralisz P, Dobrzycki S, Nowak K, Kochman W, Gajewska-Bachórzewska H, Gugala K *et al*. Platelet inhibition by increased tirofiban dosing during primary coronary angioplasty for ST elevation myocardial infarction. *Kardiologia Polska* 2004; 60:464-467.
- Kroll MH. Mechanisms of Coagulation. In: Manual of Coagulation Disorders. *Massachusetts: Blackwell Science*, 2001: 1-8. ISBN: 0-86542-446-2.
- Laffan M and Manning R. Investigation of haemostasis. In: Lewis SM, Bain BJ, Bates I, editors. Dacie and Lewis Practical Haematology. Tenth Edition. *Philadelphia: Churchill Livingstone Elsevier*; 2006: 379-441. ISBN: 0-443-06660-4.

- Leadley RJ, Chi L, Rebello SS, Gagnon A. Contribution of in vivo models of thrombosis to the discovery and development of novel antithrombotic agents. *Journal of Pharmacological and Toxicological Methods* 2000; 43:101-116. DOI: 10.1016/s1056-8719(00)00095-2.
- Lefkovits J, Plow EF, Topol EJ. Platelet Glycoprotein IIb/IIIa Receptors in Cardiovascular Medicine. *The New England Journal of Medicine* 1995; 332:1553-1559.
- Lind SE. The bleeding time does not predict surgical bleeding. *Blood* 1991; 77:2547-2552.
- Louw S, Jacobson BF, Büller H. Human Immunodeficiency Virus Infection and Acute Deep Vein Thrombosis. *Clinical and Applied Thrombosis/Hemostasis* 2008; 14:352-355. DOI: 10.1177/1076029607304411.
- Lynch JJ, Cook JJ, Sitko GR, Holahan MA, Ramjit DR, Mellott MJ *et al*. Nonpeptide Glycoprotein IIb/IIIa Inhibitors. 5. Antithrombotic Effects of MK-0383. *The Journal of Pharmacology and Experimental Therapeutics* 1995; 272:20-32.
- Maksoud A, Candipan RC, Wilson DB, Vacek JL. Aspirin and Clpoidogrel: Efficacy, Treatment, and Resistance in Coronary Artery Disease. *International Journal of Angiology* 2005; 14:109-114. DOI: 10.1007/s00547-005-2024-z.
- Marzocchi A, Manari A, Piovaccari G, Marrozzini C, Marra S, Magnavacchi P *et al*. The FATA Investigators. Randomized comparison between tirofiban and abciximab to promote complete ST-resolution in primary angioplasty: results of the facilitated angioplasty with tirofiban or abciximab (FATA) in ST-elevation myocardial infarction trial. *European Heart Journal* 2008: 29:2972-2980.
- Mason RG, Wolf RH, Zucker WH, Shinoda BA, Mohammad SF. Effects of Antithrombotic Agents Evaluated in a Nonhuman Primate Vascular shunt model. *American Journal of Pathology* 1976; 83:557-568.
- Microscope. Microsoft® Encarta® Online Encyclopedia 2008. [Online] Available: http://encarta.msn.com/text_761562052___0/Microscope.html [Accessed 2 April 2009].
- Moliterno DJ and Topol EJ. Hotline Editorial. The TARGET trial: hit or miss? *European Heart Journal* 2002; 23:835-837. DOI: 10.1053/euhj.2001.3133.

- Mousa SA, Bozarth JM, Naik UP, Slee A. Platelet GPIIb/IIIa binding characteristics of small molecule RGD mimetic: distinct binding profile for Roxifiban. *British Journal of Pharmacology* 2001; 133:331-336.
- Murugappan S and Kunapuli SP. The role of ADP receptors in platelet function. *Frontiers in Bioscience* 2006; 11:1977-1986.
- Patrono C. Aspirin as an Antiplatelet Drug. *The New England Journal of Medicine* 1994; 330:1287-1294.
- Peerlick K, De Lepeleire I, Goldberg M, Farrell D, Barret J, Hand E, Panebianco D, Deckmyn H, Vermylen J, Arnout J. MK-383 (L-700,462), a selective nonpeptide platelet glycoprotein IIb/IIIa antagonist, is active in man. *Circulation* 1993; 88:1512-1517.
- Pels K, Schwimmbeck PL, Rosenthal P, Loddenkemper C, Dang-Heine C, Rauch U *et al*. Long-term clopidogrel administration following severe coronary injury reduces proliferation and inflammation via inhibition of nuclear factor-kappaB and activator protein 1 activation in pigs. *European Journal of Clinical Investigation* 2009; 39:174-182. DOI: 10.1111/j.1365-2362.2009.02089.x.
- Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock WA, Triplett DA, Brandt JT. The Preoperative Bleeding Time Test Lacks Clinical Benefit. *Archives of Surgery* 1998; 133:134-139.
- Plavix – clopidogrel bisulfate tablets. [Online] Available: http://products.sanofi-aventis.us/plavix/plavix.html [Accessed 11 August 2008].
- The Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. A Comparison of Aspirin plus Tirofiban with Aspirin plus Heparin for Unstable Angina. *The New England Journal of Medicine* 1998; 338:1498-1505.
- The Platelet Receptor Inhibition in Ischemic Syndrome Management In Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators. Inhibition of the Platelet Glycoprotein IIb/IIIa Receptor with Tirofiban in Unstable Angina and Non-Q-Wave Myocardial Infarction. *The New England Journal of Medicine* 1998; 338:1488-1497.
- ReoPro (Abciximab) for Cardiac Ischemic Complications. [Online] Available: http://www.reopro.com/index.jsp [Accessed 13 August 2008].

- ReoPro (Abciximab) GP Receptor Inhibitor Product Information. [Online] Available: http://www.reopro.com/about_reopro?product_information.jsp [Accessed 11 August 2008].
- The RESTORE Investigators. Effects of Platelet Glycoprotein IIb/IIIa Blockade With Tirofiban on Adverse Cardiac Events in Patients With Unstable Angina or Acute Myocardial Infarction Undergoing Coronary Angioplasty. *Circulation* 1997; 96:1445-1453.
- Rogers J and Hixon JE. Insights from model systems. Baboon as an Animal Model for Genetic Studies of Common Human Disease. *The American Journal of Human Genetics* 1997; 61:489-493.
- Saif MW and Greenberg B. HIV and Thrombosis: A review. *AIDS Patient Care* 2001; 15:15-24.
- Schaar JA, Muller JE, Falk E, Virmani R, Fuster V, Serruys PW *et al*. Terminology for high-risk and vulnerable coronary artery plaques. *European Heart Journal* 2004; 25:1077-1082. DOI: 10.1016/j.ehj.2004.01.002.
- Schneider DJ, Herrmann HC, Lakkis N, Aguirre F, Wan Y, Aggarwal A, Kabbani SS, DiBattiste PM. Enhanced Early Inhibition of Platelet Aggregation With an Increased Bolus of Tirofiban. *The American Journal of Cardiology* 2002; 90:1421-1423.
- The SEM – What is the S.E.M? [Online] Available: http://mse.iastate.edu/microcopy/whatsem.html [Accessed 1 April 2009].
- Shanmugam G. Tirofiban and emergency coronary surgery. *European Journal of Cardio-thoracic Surgery* 2005; 28:546-550. DOI: 10.1016/j.ejcts.2005.07.008.
- Sherwood L. Atherosclerosis. In: Human Physiology: From Cells to Systems. Fifth Edition. *Belmont, USA: Brooks/Cole, a Division of Thomson Learning, Inc.*; 2004: 333-334. ISBN 0-534-39536-8.
- Sherwood L. Platelets and Hemostasis. In: Human Physiology: From Cells to Systems. Fifth Edition. *Belmont, USA: Brooks/Cole, a Division of Thomson Learning, Inc.*; 2004: 402. ISBN 0-534-39536-8.
- Soffer D, Moussa I, Karatepe M, Harjai KJ, Boura J, Dixon SR *et al*. Suboptimal Inhibition of Platelet Aggregation Following Tirofiban Bolus in Patients Undergoing

Percutaneous Coronary Intervention for Unstable Angina Pectoris. *The American Journal of Cardiology* 2003; 91:872-875.

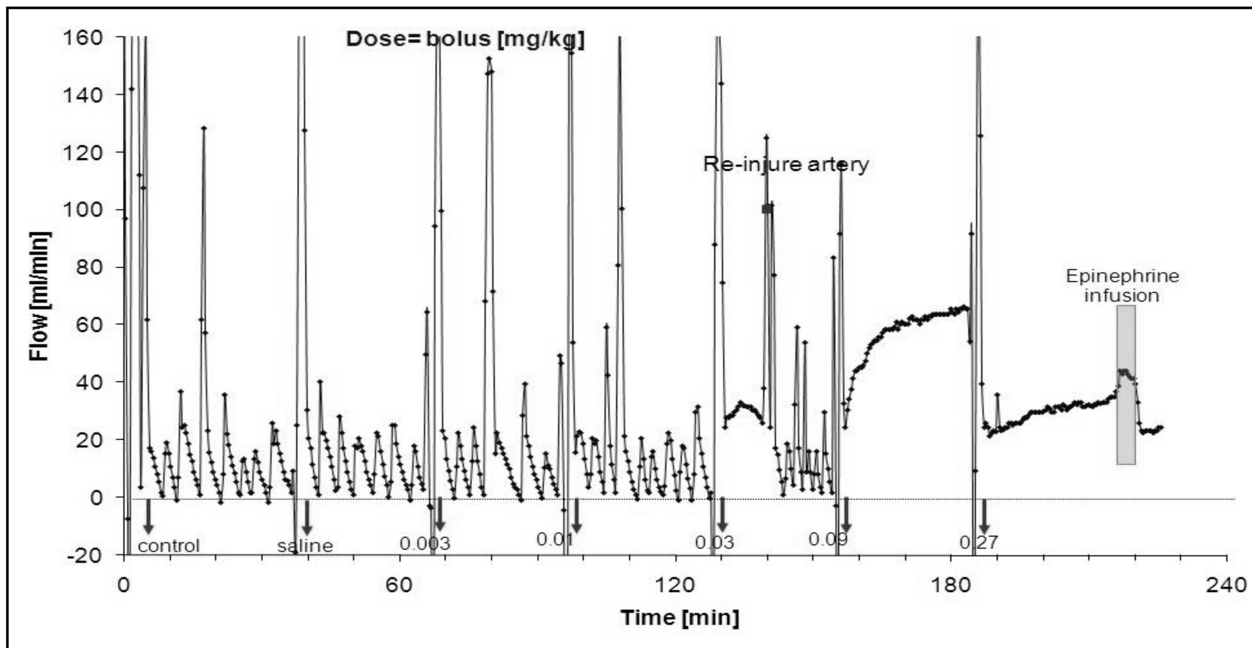
- TEG® 5000 Thromboelastograph® Hemostasis System - User Manual. Haemoscope Corporation: Niles, USA. Copyright ©1999-2007. Contact at: info@haemoscope.com
- Thiagarajan P, Benedict CR. Inhibition of arterial thrombosis by recombinant Annexin V in a rabbit carotid artery injury model. *Circulation* 1997; 96:2339-2347.
- Topol EJ, Moliterno DJ, Herrmann HC, Powers ER, Grines CL, Cohen DJ *et al*. Comparison of Two Platelet Glycoprotein IIb/IIIa Inhibitors, Tirofiban and Abciximab, for the Prevention of Ischemic Events with Percutaneous Coronary Revascularization. *The New England Journal of Medicine* 2001; 334:1888-1894.
- Valgimigli M, Campo G, Percoco G, Bolognese L, Vassanelli C, Colangelo S *et al*. The MULTISTRATEGY Investigators. Comparison of Angioplasty With Infusion of Tirofiban or Abciximab and With Implantation of Sirolimus-Eluting or Uncoated Stents for Acute Myocardial Infarction: The MULTISTRATEGY Randomized Trial. *JAMA* 2008; 299:1788-1799.
- Valgimigli M, Precoco G, Barbieri D, Ferrari F, Guardigli G, Parrinello G *et al*. The additive value of tirofiban administered with the high-dose bolus in the prevention of ischemic complications during high-risk coronary angioplasty. The ADVANCE Trial. *The Journal of the American College of Cardiology* 2004; 44:14-19.
- Valgimigli M and Verheugt F. 2008. Main Results of the Tailoring Treatment with Tirofiban in patients showing Resistance to aspirin or Resistance to clopidogrel study (3T/2R). *ESC2008 Congress-Reports*, Munich, Germany, 30 Aug-3 Sep 2008. Available: http://www.escardio.org/CONGRESSES/ESC2008/CONGRESS-REPORTS [Accessed 5 February 2009].
- Vega J. Side Effects of Coumadin, Plavix and Other Blood Thinners. [Online] 9 Mar 2008. Available: http://stroke.about.com/od/caregiverresources/a/blood_thinners.htm [Accessed 8 August 2008].
- Weitz JI. Low-molecular-weight Heparins. *The New England Journal of Medicine* 1997; 337:688-698.
- WHO Fact sheet No. 317. Cardiovascular Diseases. [Online] Feb 2007. Available: www.who.int/mediacentre/factsheets/fs317/en/print.html [Accessed 8 August 2008].

- Woolf N and Davies MJ. Interrelationship between atherosclerosis and thrombosis. In: Fuster V and Verstraete M. Thrombosis in Cardiovascular Disorders. *Philadelphia: W.B. Saunders Company*, 1992: 41-73. ISBN: 0-7216-4012-5.
- Wong PC, Crain EJ, Knabb RM, Meade RP, Quan ML, Watson CA *et al*. Nonpeptide Factor Xa Inhibitors II. Antithrombotic Evaluation in a Rabbit Model of Electrically Induced Carotid Artery Thrombosis. *The Journal of Pharmacology and Experimental Therapeutics* 2000; 295:212-218.
- Wu D, Vanhoorelbeke K, Cauwenberghs N, Meiring M, Depraetere H, Kotze HF, Deckmyn H. Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. *Blood* 2002; 99:3623-3628.

Appendix A – Data Tables

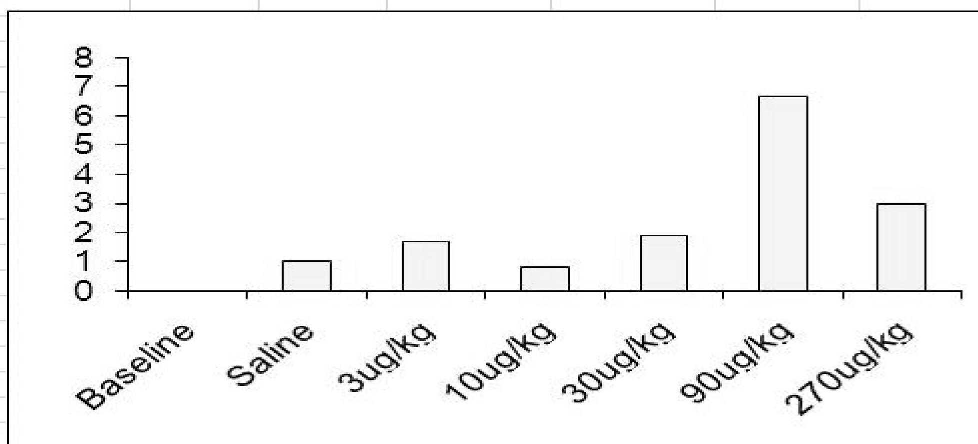
Baboon	W01							
Sex	Male							
Mass	16.4							
Drug	Aggrestet							
Date	04/05/2009							
Action	**Time**	**Remarks**	**Flow**	**Start CFR**	**End CFR**	**Length**	**length seconds**	**Comments**
Start operation	08h39							
Start recording	09h31		224					
Flush lines	6:00		250					
Make incision	6:00							
Injure artery	7:55		29.0	09:55	13:05	3:10		
Ketamine	6:45		28.7	13:50	15:50	2:00		
			35.2	17:10	21:05	3:55		
Draw blood	21:20		24.0	22:50	25:20	2:30		BT=1:45
			30.0	26:40	29:40	3:00		
			26.3	30:20	32:05	1:45		
			25.9	32:55	36:35	02:40		
			35.3	37:10	41:00	3:50		
Flush lines	41:20							
Change gauze	42:30							
Inject 5ml saline	42:50		29.1	44:35	46:35	02:00		
			31.5	47:35	50:50	3:15		
			32.2	51:35	55:35	4:00		
			28.8	55:25	58:50	3:25		
			28.9	59:20	1:02:05	2:45		
Ketamine	1:00:45		29.8	1:03:15	1:06:40	3:25		
			29.2	1:07:40	1:10:00	2:20		
Flush lines	1:10:20							
Change gauze	1:12:10							
Inject 3ug/kg Aggrastet	1:12:40		34.3	1:13:55	1:16:25	2:30		
			32.3	1:17:05	1:20:00	2:55		
			28.6	1:20:50	1:23:05	2:15		
Draw blood	1:24:00		33.8	1:25:25	1:30:35	5:10		BT=1:45
Ketamine	1:27:20		29.6	1:31:55	1:35:25	3:30		
			29.1	1:36:00	1:39:10	3:10		
Flush lines	1:39:40							
Change gauze	1:41:10							
Inject 10ug/kg Aggrastet	1:41:40		27.0	1:42:50	1:45:55	3:05		
			32.4	1:47:00	1:49:10	2:10		
			27.0	1:50:15	1:51:40	1:25		
Draw blood	1:52:00		28.9	1:53:10	1:55:55	2:45		BT=2:15
Ketamine	1:58:35		30.3	1:56:55	1:58:40	1:45		
			30.3	1:59:10	2:01:45	2:35		
			33.4	2:03:00	2:04:50	1:50		
			28.2	2:05:55	2:07:55	2:00		
			30.2	2:09:35	2:11:50	2:15		
Flush lines	2:12:10							
Change gauze	2:14:00							
Inject 30ug/kg Aggrastet	2:14:20		30.0	2:15:00	-			
Draw blood	2:24:00							BT=2:00
Re-injure artery	2:25:00		26.0	2:26:20	2:28:00	1:40		
			29.2	2:28:55	2:30:25	1:30		
			32.6	2:31:00	2:32:25	1:25		
			28.6	2:33:10	2:34:30	1:30		
			28.5	2:35:00	2:36:20	1:20		
			28.9	2:36:55	2:38:35	1:40		
Flush lines	2:39:00							
Change gauze	2:40:15							
Inject 90ug/kg Aggrastet	2:41:00		34.5	2:41:35	-			
Draw blood	2:51:00							BT=2:00
Ketamine	2:54:00							
Flush lines	3:09:00							
Change gauze	3:10:00							
Inject 270ug/kg Aggrastet	2:51:04							
Ketamine	3:14:00							
Draw blood	3:20:00							BT>15:00
Ketamine	3:34:20							
Start EPI	3:40:00							
Change gauze	3:40:00							
Stop EPI	3:44:00							
Stop recording	3:50:00							

Raw results Efficacy study: W01



Efficacy study: W01

Mass Gauze	0.4					
	# Tubes	# Gauze	Mass full	Mass empty	Blood loss	Fold incease
Baseline	1	1	14.63	12.88	1.35	
Saline	1	1	13.61	12.85	0.36	1.0
3ug/kg	1	1	13.9	12.89	0.61	1.7
10ug/kg	1	1	13.59	12.9	0.29	0.8
30ug/kg	1	1	13.95	12.87	0.68	1.9
90ug/kg	1	2	16.07	12.87	2.4	6.7
270ug/kg	1	1	14.37	12.9	1.07	3.0
				Total	6.76	
				Bloodvolume		



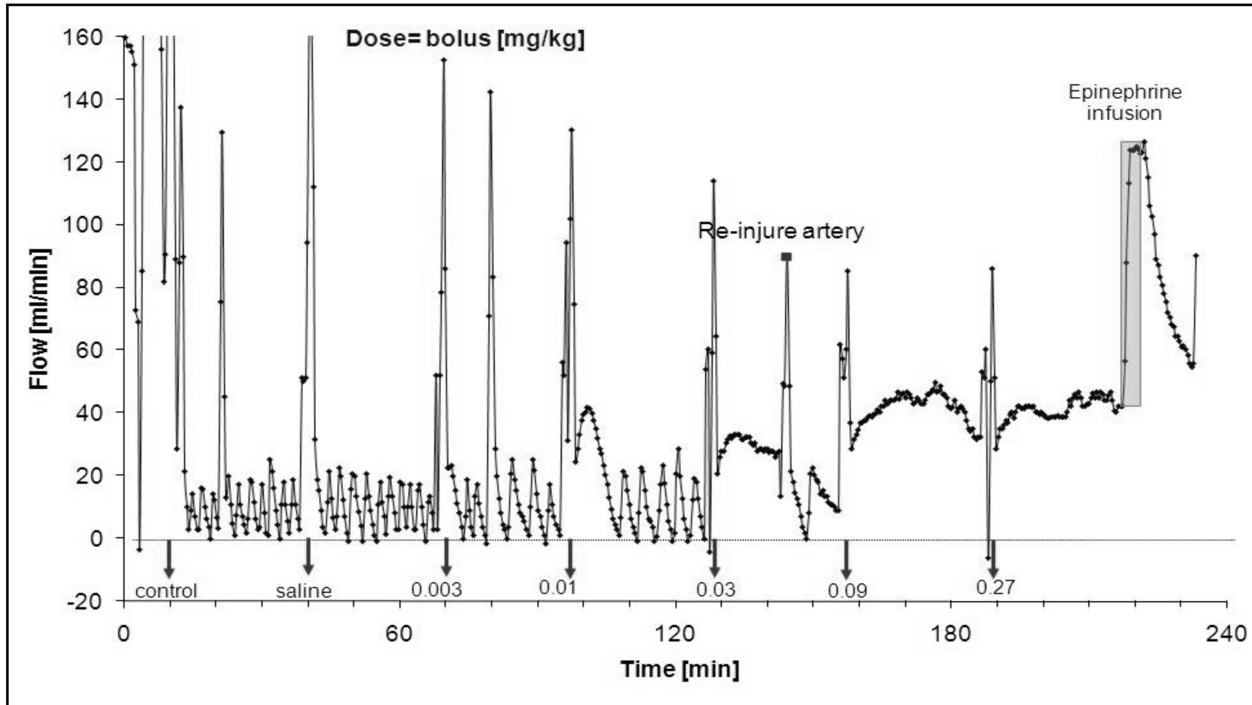
Blood Loss: W01

	Baseline	3ug/kg	10ug/kg	30ug/kg	90ug/kg	270ug/kg	Mean excl Base		% Difference
WBC x 10^9/l	7.23	8.49	11.53	10.99	10.6	11.11	10.544	145.84	45.84
RBC x 10^{12}/l	5.62	5.43	5.59	5.34	5.32	5.35	5.406	96.19	3.81
haemoglobuin g/dl	12.9	12.5	12.8	12.4	12.3	12.1	12.42	96.28	3.72
haematocrit l/l	0.423	0.410	0.423	0.403	0.399	0.410	0.409	96.69	3.31
MCV fl	75.3	75.5	75.7	75.5	75.0	76.6	75.66	100.48	0.48
MCH pg	23.0	23.0	22.9	23.2	23.1	22.6	22.96	99.83	0.17
MCMC g/dl	30.5	30.5	30.3	30.8	30.8	29.5	30.38	99.61	0.39
plt x 10^9/l	262	243	246	252	237	244	244.4	93.28	6.72
PT (sec)	11	12	12	12	12	12	12	109.09	9.09
aPTT (sec)	27	28	29	29	29	28	28.6	105.93	5.93
ADP Aggr (%)	45	17	5	5	5	6			88.89
				Increase	Decrease		Max inhibition		

FBC: W01

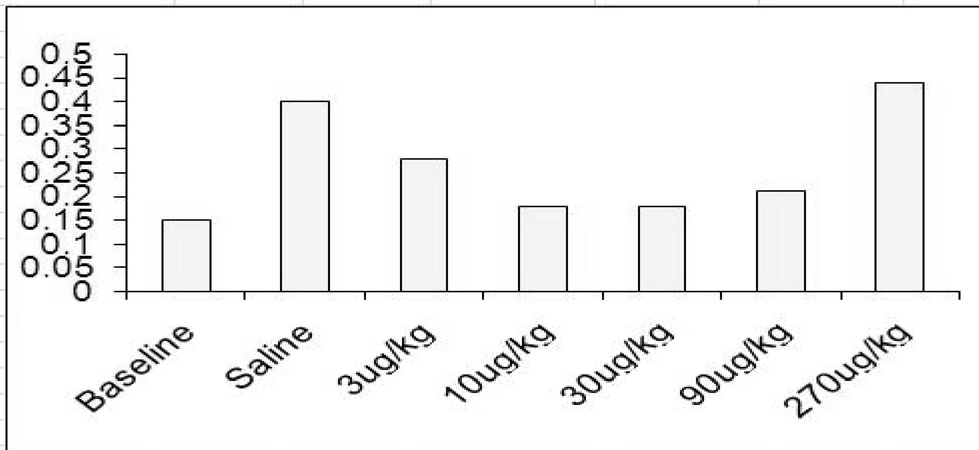
Baboon	W02							
Sex	Male							
Mass	15.5							
Drug	Aggrestet							
Date	05/05/2009							
Action	**Time**	**Remarks**	**Flow**	**Start CFR**	**End CFR**	**Length**	**length seconds**	**Comments**
Start operation	09h00							
Start recording	09h50		180					
Flush lines	2:46		190					
Flush lines	8:35		210					
Make incision	9:30							
Injure artery	11:20		25.5	13:30	14:45	1:15		
Ketamine	14:30		28.0	15:00	16:20	1:20		
			29.1	17:05	19:00	1:55		
			31.1	19:30	21:10	1:40		
Draw blood			32.0	22:50	24:25	1:35		BT=2:30
Ketamine	26:00		28.1	25:00	27:00	2:00		
			28.6	28:00	29:45	1:45		
			29.3	30:20	31:25	1:05		
			30.0	32:15	34:05	1:50		
			32.4	34:45	36:30	1:45		
			32.0	36:55	38:45	1:50		
Flush lines	39:10							
Change gauze	40:40							
Inject saline	40:50		28.0	42:15	44:15	2:00		
Ketamine	43:30		33.7	44:45	46:30	1:45		
			33.1	47:10	49:10	2:00		
			30.2	50:20	52:10	1:50		
			31.6	53:00	55:10	2:10		
			31.8	55:45	57:25	1:40		
			29.1	58:10	59:35	1:25		
			28.5	1:00:30	1:01:50	1:20		
			30.4	1:02:15	1:03:40	1:25		
			28.2	1:04:20	1:05:50	1:30		
			28.8	1:06:15	1:07:45	1:30		
Flush lines	1:08:15							
Change gauze	1:09:30							
Inject 3ug/kg Aggrastet	1:09:50		29.2	1:10:55	1:14:10	3:15		
Ketamine	1:13:00		32.2	1:14:45	1:16:05	1:20		
Draw blood	1:19:30		30.0	1:16:45	1:19:15	2:30		BT=4:15
Rompun	1:22:40		31.0	1:21:05	1:23:40	2:35		
			31.2	1:24:45	1:28:30	3:45		
			28.5	1:29:40	1:31:55	2:15		
			28.1	1:32:40	1:35:20			
Flush lines	1:35:40							
Change gauze	1:36:00							
Inject 10ug/kg Aggrastet	1:36:20		32.1	1:38:50	1:48:10	9:20		
Ketamine	1:40:00		33.8	1:48:50	1:51:35	2:45		
Draw blood	1:44:40		31.5	1:53:00	1:55:40	1:40		BT=1:45
			33.0	1:57:20	1:59:30	2:10		
			33.0	2:00:50	2:03:00	2:10		
			28.0	2:04:25	2:06:30	2:05		
Flush lines	2:07:00							
Change gauze	2:08:10							
Inject 30ug/kg Aggrastet	2:08:10		27.2	2:09:25	-			
Ketamine	2:10:16							
Draw blood	2:18:20							BT=2:45
Rompun	2:17:50							
Re-injure artery	2:23:45		29.8	2:25:00	2:28:50	3:50		
Flush lines			29.7	2:29:50	-			
Change gauze	2:39:30							
Inject 90ug/kg Aggrastet	2:39:40		34.1	2:38:30	-			
Ketamine	2:44:34							
Draw blood	2:50:00							BT=12:30
Rompun	2:57:00							
Flush lines	3:08:10							
Change gauze	3:09:00							
Inject 270ug/kg Aggrastet	3:09:20		33.2	3:09:40				
Ketamine	3:13:30							
Draw blood	3:19:00							BT>15
Rompun	3:27:00							
Ketamine	3:32:30							
Start EPI	3:37:30							
Change gauze	3:37:40							
Stop EPI	3:41:30							Minor gingival bleed
Stop recording	3:54:00							

Raw Results Efficacy study: W02



Efficacy Study: W02

Mass Gauze	0.4					
	# Tubes	# Gauze	Mass full	Mass empty	Blood loss	Fold incease
Baseline	1	1	13.43	12.88	0.15	
Saline	1	1	13.67	12.87	0.4	1.0
3ug/kg	1	1	13.56	12.88	0.28	0.7
10ug/kg	1	1	13.48	12.9	0.18	0.4
30ug/kg	1	1	13.48	12.9	0.18	0.4
90ug/kg	1	1	13.49	12.88	0.21	0.5
270ug/kg	1	1	13.79	12.95	0.44	1.1
				Total	1.84	
				Bloodvolume	1085	



Blood Loss: W02

	Baseline	3ug/kg	10ug/kg	30ug/kg	90ug/kg	270ug/kg	Mean excl Base		% Difference
WBC x 10^9/l	5.52	5.73	6.46	8.6	8.75	8.73	7.654	138.66	38.66
RBC x 10^{12}/l	5.75	5.58	5.18	5.2	5.11	4.79	5.172	89.95	10.05
haemoglobuin g/dl	14.3	13.8	12.9	12.9	12.7	12.1	12.88	90.07	9.93
haematocrit l/l	0.465	0.450	0.417	0.420	0.415	0.387	0.4178	89.85	10.15
MCV fl	80.9	80.6	80.5	80.8	81.2	80.8	80.78	99.85	0.15
MCH pg	24.9	24.7	24.9	24.8	24.9	25.3	24.92	100.08	0.08
MCMC g/dl	30.8	30.7	30.9	30.7	30.6	31.3	30.84	100.13	0.13
plt x 10^9/l	268	265	229	228	226	205	230.6	86.04	13.96
PT (sec)	11	11	12	12	12	12	11.8	107.27	7.27
aPTT (sec)	27	28	28	28	28	28	28	103.70	3.7
ADP Aggr (%)	51	21	3	0	4	1			100.00

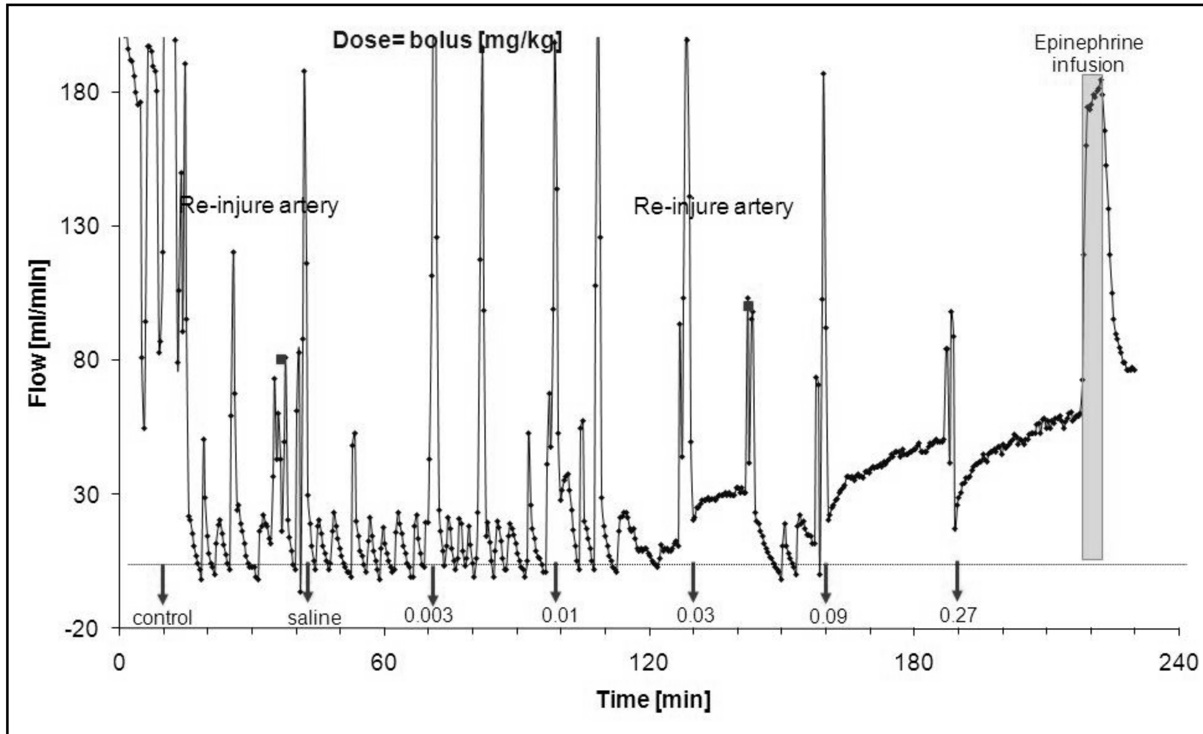
Increase | Decrease

Max inhibition

FBC: W02

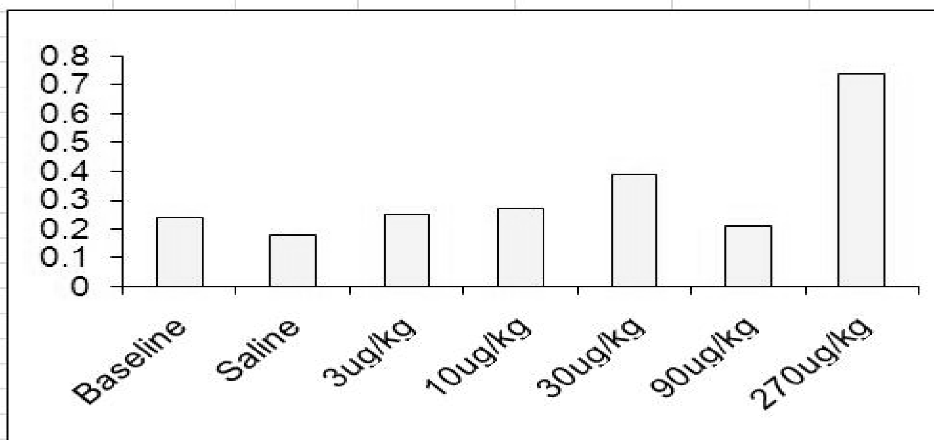
Baboon	W03							
Sex	Male							
Mass	14.9							
Drug	Aggrestet							
Date	07/05/2009							
Action	**Time**	**Remarks**	**Flow**	**Start CFR**	**End CFR**	**Length**	**length seconds**	**Comments**
Start operation	08h46							
Ketamine	09h07							
Start recording	09h24		232					
Flush lines	5:30		220					
Flush lines	9:10		270					
Make incision	10:30							
Injure artery	13:00							
Ketamine	10:30		28.3	16:00	18:30	2:30		
			31.8	19:45	21:40	1:55		
			31.0	22:50	25:15	2:25		
Draw blood	25:45		31.8	26:50	31:05	4:15		BT=1:15
Ketamine	09h57		33.8	32:30	-			
Re-injure artery	36:40		32.6	38:00	40:15	2:15		Remove excess tissue
Flush lines	40:40							
Change gauze	41:30							
Inject saline	41:50		30.3	43:05	44:45	1:40		
			29.5	45:25	47:55	2:30		
			27.4	49:00	52:30	3:30		
			27.5	53:55	56:10	2:15		
Ketamine	57:30		32.5	57:00	58:55	1:55		
			30.1	59:35	1:02:05	2:30		
			30.0	1:03:05	1:06:10	3:05		
			25.3	1:07:35	1:09:30	1:55		
Flush lines	1:10:00							
Change gauze	1:11:00							
Inject 3ug/kg Aggrastet	1:11:20		31.6	1:12:30	1:14:10	1:40		
Ketamine	1:15:50		28.3	1:15:05	1:16:25	1:20		
			33.5	1:17:20	1:18:35	1:15		
			28.5	1:19:30	1:20:30	1:00		
			29.4	1:21:30	-			
Draw blood	1:22:00		30.6	1:23:25	1:24:55	1:30		BT=2:00
			28.7	1:25:50	1:27:30	1:40		
			28.0	1:28:15	1:32:00	3:45		
			32.5	1:33:15	1:37:05			
Flush lines	1:38:00							
Change gauze	1:39:10							
Inject 10ug/kg VWF-0001	1:39:30		31.0	1:39:50	1:44:40	4:50		
			28.7	1:45:20	1:48:00	2:40		
Draw blood			30.8	1:49:30	1:52:55	3:25		BT=2:15
Ketamine	1:51:00		29.0	1:53:40	-			
Ketamine	2:05:15							
Flush lines	2:07:20							
Change gauze	2:08:30							
Inject 30ug/kg VWF-0001	2:09:00		28.3	2:10:00	-			
Draw blood	2:19:00							BT=4:14
Re-injure artery	2:22:30		28.7	2:24:30	2:30:05	5:35		
Ketamine	2:25:00		31.6	2:31:00	2:33:30	2:30		
			29.3	2:34:20				
Flush lines	2:38:00							
Change gauze	2:39:15							
Inject 90ug/kg VWF-0001	2:40:00		28.8	2:40:43				
Ketamine	2:42:00							
Draw blood	2:49:00							BT>15
Flush lines	3:08:00							
Change gauze	3:09:15							
Inject 270ug/kg VWF-0001	3:09:30		33.7	3:09:40				
Ketamine	3:12:20							
Draw blood	3:19:00							BT>15
Ketamine	3:30:00							
Start EPI	3:38:00							
Change gauze	3:38:00							
Stop EPI	3:42:00							
Stop recording	3:44:00							

Raw Results Efficacy Study: W03



Efficacy Study: W03

Mass Gauze	0.4					
	# Tubes	# Gauze	Mass full	Mass empty	Blood loss	Fold incease
Baseline	1	1	13.49	12.85	0.24	
Saline	1	1	13.53	12.95	0.18	1.0
3ug/kg	1	1	13.6	12.95	0.25	1.4
10ug/kg	1	1	13.54	12.87	0.27	1.5
30ug/kg	1	1	13.72	12.93	0.39	2.2
90ug/kg	1	1	13.51	12.9	0.21	1.2
270ug/kg	1	1	14.06	12.92	0.74	4.1
				Total	2.28	
				Bloodvolume	1043	



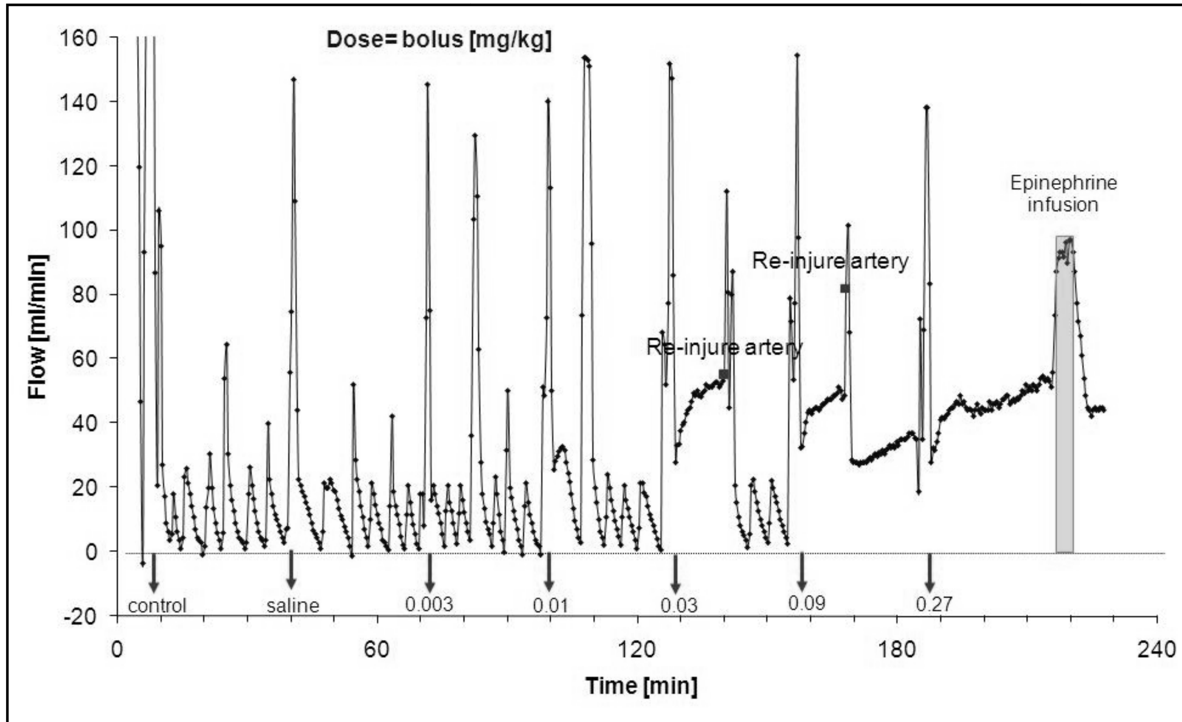
Blood Loss: W03

	Baseline	3ug/kg	10ug/kg	30ug/kg	90ug/kg	270ug/kg	Mean excl Base		% Difference
WBC x 10^9/l	9.02	8.92	7.97	9.46	10.77	10.65	9.554	105.92	5.92
RBC x 10^{12}/l	5.29	5.28	5.15	4.88	5.07	4.88	5.052	95.50	4.5
haemoglobuin g/dl	13.4	13.3	12.8	12.5	12.7	12.2	12.7	94.78	5.22
haematocrit l/l	0.425	0.424	0.413	0.392	0.408	0.393	0.406	95.53	4.47
MCV fl	80.3	80.3	80.2	80.3	80.5	80.5	80.36	100.07	0.07
MCH pg	25.3	25.2	24.9	25.6	25.0	25.0	25.14	99.37	0.63
MCMC g/dl	31.5	31.4	31.0	31.9	31.1	31.0	31.28	99.30	0.7
plt x 10^9/l	317	307	288	286	288	278	289.4	91.29	8.71
PT (sec)	12	12	12	12	12	12	12	100.00	0
aPTT (sec)	31	32	32	32	32	32	32	103.23	3.23
ADP Aggr (%)	50	24	4	2	0	5			100.00
				Increase	Decrease		Max inhibition		

FBC: W03

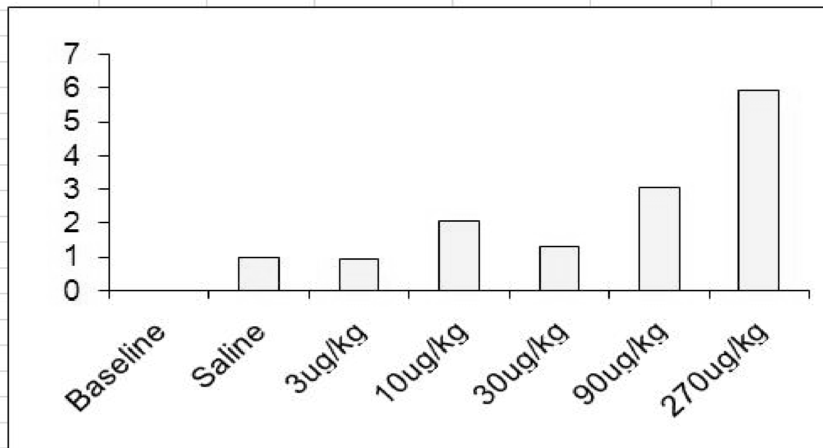
Baboon	W04							
Sex	Male							
Mass	13.4							
Drug	Aggrestet							
Date	08/05/2009							
Action	**Time**	**Remarks**	**Flow**	**Start CFR**	**End CFR**	**Length**	**length seconds**	**Comments**
Start operation	08h30							
Start recording	09h04		199					
Flush lines	6:00		210					
Make incision	6:40							
Injure artery	8:50							
Ketamine	9:45		27.6	11:00	12:40	01:40		
			29.1	13:05	15:00	1:55		
			32.0	16:10	19:50	3:40		
Draw blood	24:45		31.8	21:40	24:15	2:35		BT=5:30
			31.7	25:50	29:40	3:50		
			31.3	30:55	34:15	3:25		
			31.9	35:15	39:05	2:50		
Flush lines	39:25							
Change gauze	40:25							
Inject saline	40:40		30.7	41:40	47:15	5:35		
Ketamine	43:00		28.7	48:10	54:10	6:00		
			28.5	55:20	58:20	3:00		
			29.2	58:50	1:02:45	3:55		
			28.0	1:03:45	1:06:40	2:55		
			32.9	1:07:15	1:10:00	2:45		
Flush lines	1:10:30							
Change gauze	1:11:30							
Inject 3ug/kg Aggrastet	1:11:50		30.0	1:12:40	1:15:50	3:10		
Ketamine	1:17:30		31.3	1:16:20	1:18:50	2:30		
			30.0	1:19:20	1:21:55	2:35		
Draw blood	1:22:30		29.9	1:24:10	1:26:45	2:35		BT>15
			29.4	1:27:30	1:29:35	2:35		
			29.9	1:30:30	1:33:40	3:10		
			30.6	1:34:30	1:37:55	3:25		
Flush lines	1:38:20							
Change gauze	1:39:15							
Inject 10ug/kg Aggrastet	1:39:40		31.5	1:40:40	1:49:30	8:50		
Ketamine	1:44:00		31.2	1:50:10	1:52:50	2:40		
Draw blood	1:53:10		32.1	1:53:15	1:56:30	3:15		BT>15
			31.5	1:56:50	2:00:30	3:40		
			31.8	2:01:05	2:05:55	4:50		
Flush lines	2:06:15							
Change gauze	2:07:40							
Inject 30ug/kg Aggrastet	2:08:00		30.9	2:09:00	-			
Ketamine	2:13:30							
Draw blood	2:19:00							BT>15
Re-injure artery	2:20:50		30.2	2:22:40	2:26:00	3:20		
			33.0	2:26:40	2:30:25	3:45		
			30.1	2:31:05	2:35:15	4:10		
Flush lines	2:35:40							
Change gauze	2:36:50							
Inject 90ug/kg Aggrastet	2:37:10		32.7	2:37:50	-			
Draw blood	2:46:30							BT>15
Re-injure artery	2:48:30		34.0	2:49:40	-			
Ketamine	2:46:00							
Flush lines	3:05:35							
Change gauze	3:06:40							
Inject 270ug/kg Aggrastet	3:07:00		32.5	3:08:00				
Draw blood	3:17:00							BT>15
Ketamine	3:14:30							
Start EPI	3:36:00							
Change gauze	3:35:33							
Stop EPI	3:40:00							
Stop recording	3:50:00							

Raw Results Efficacy Study: W04



Efficacy Study: W04

Mass Gauze	0.4					
	# Tubes	# Gauze	Mass full	Mass empty	Blood loss	Fold incease
Baseline	1	1	13.57	12.89	0.28	
Saline	1	1	13.42	12.88	0.14	1.0
3ug/kg	1	1	13.41	12.88	0.13	0.9
10ug/kg	1	1	13.55	12.86	0.29	2.1
30ug/kg	1	1	13.46	12.88	0.18	1.3
90ug/kg	1	1	13.71	12.88	0.43	3.1
270ug/kg	1	1	14.11	12.88	0.83	5.9
				Total	2.28	
				Bloodvolume	938	



Blood Loss: W04

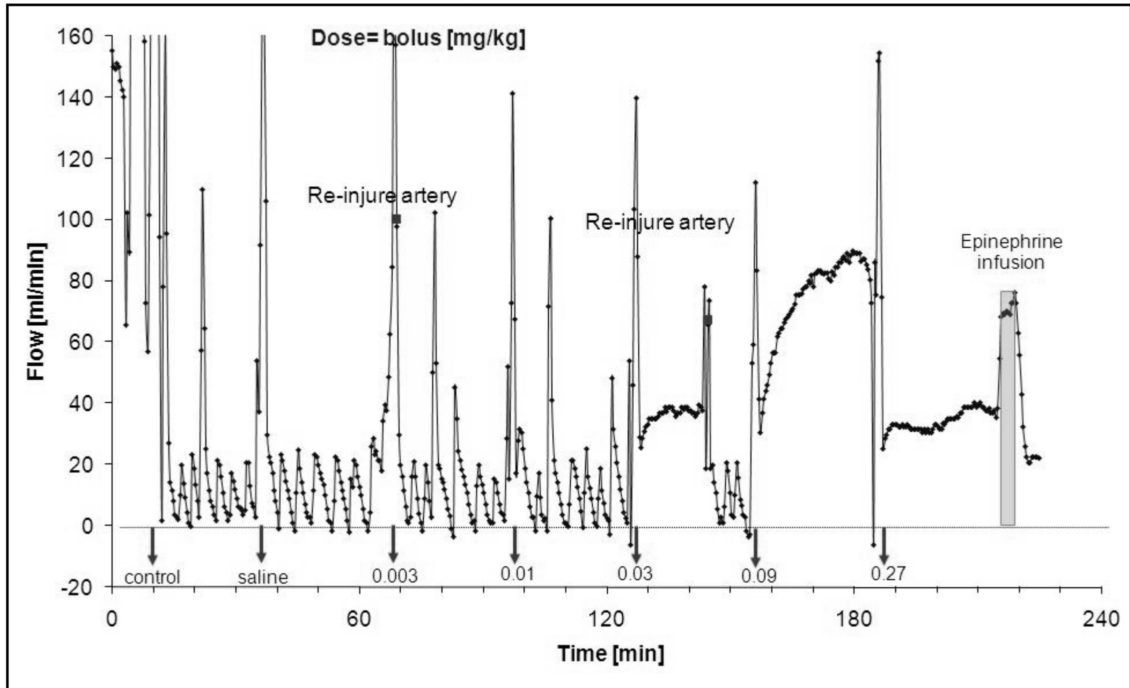
	Baseline	3ug/kg	10ug/kg	30ug/kg	90ug/kg	270ug/kg	Mean excl Base		% Difference
WBC x 10^9/l	7.70	7.31	7.00	6.96	6.37	5.96	6.72	87.27	12.73
RBC x 10^{12}/l	5.23	5.13	5.08	5.00	4.99	4.74	4.988	95.37	4.63
haemoglobuin g/dl	13.1	13.0	12.7	12.5	12.7	11.9	12.56	95.88	4.12
haematocrit l/l	0.406	0.399	0.394	0.389	0.387	0.369	0.3876	95.47	4.53
MCV fl	77.6	77.8	77.6	77.8	77.6	77.8	77.72	100.15	0.15
MCH pg	25.0	25.3	25.0	25.0	25.5	25.1	25.18	100.72	0.72
MCMC g/dl	32.3	32.6	32.2	32.1	32.8	32.2	32.38	100.25	0.25
plt x 10^9/l	168	185	180	181	172	158	175.2	104.29	4.29
PT (sec)	12	12	12	12	12	13	12.2	101.67	1.67
aPTT (sec)	29	28	30	30	30	29	29.4	101.38	1.38
ADP Aggr (%)	46	43	2	3	5	2			95.65

Increase | Decrease | Max inhibition

FBC: W04

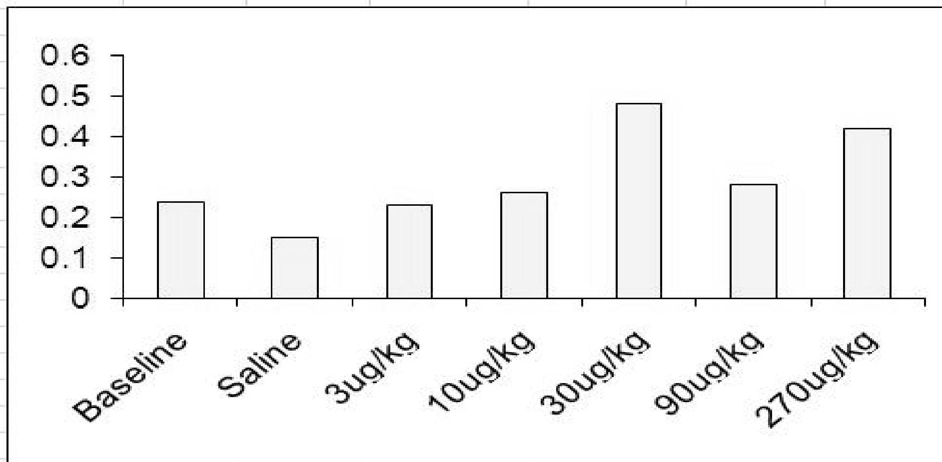
Baboon	W05							
Sex	Male							
Mass	14.2							
Drug	Aggrestet							
Date	11/05/2009							
Action	**Time**	**Remarks**	**Flow**	**Start CFR**	**End CFR**	**Length**	**length seconds**	**Comments**
Start operation	08h25							
Start recording	08h58		170					
Flush lines	4:00		190					
Flush lines	8:20		220					
Make incision	9:30							
Injure artery	11:40		28.7	13:50	16:35	2:45		
Ketamine	10:45		29.5	16:55	19:20	2:25		
			26.7	19:55	21:30	1:35		
Draw blood	21:45		30.5	22:50	25:20	2:40		BT=4:15
Ketamine			30.0	26:00	28:30	2:30		
			28.5	29:05	32:15	3:10		
			32.1	33:00	35:05	2:05		
Flush lines	35:30							
Change gauze	36:20							
Inject saline	36:30		29.0	37:55	40:40	2:45		
Ketamine	37:25		30.5	41:20	44:25	3:05		
			29.4	45:20	48:40	3:20		
			30.7	49:30	53:25	3:55		
			30.6	54:35	57:30	2:55		
			29.6	58:50	1:02:00	3:10		
Re-injure artery	1:06:20		32.2	1:03:35	-			
Flush lines	1:07:05							
Change gauze	1:08:00							
Inject 3ug/kg Aggrastet	1:08:20		28.4	1:10:00	1:12:30	2:30		
Ketamine	1:09:10		29.0	1:13:20	1:15:30	2:10		
			29.9	1:16:05	1:17:45	1:40		
Draw blood	1:16:10		28.8	1:19:05	1:22:30	3:25		BT=5:45
			30.8	1:23:50	1:28:10	4:20		
			29.1	1:28:40	1:32:20	3:40		
			29.7	1:32:50	1:35:30	2:40		
Flush lines	1:36:10							
Change gauze	1:36:55							
Inject 10ug/kg Aggrastet	1:37:15		29.2	1:38:15	1:42:50	4:35		
Ketamine	1:40:00		30.9	1:43:20	1:45:40	2:20		
Draw blood			30.4	1:47:00	1:50:45	3:45		BT>15
			30.2	1:51:30	1:54:20	2:50		
			29.7	1:55:15	1:57:45	2:30		
			30.3	1:58:20	2:00:45	2:25		
			29.8	2:02:15	2:05:15	3:00		
Flush lines	2:05:40							
Change gauze	2:06:40							
Inject 30ug/kg Aggrastet	2:07:10		31.0	2:08:20	-			
Ketamine	2:08:40							
Draw blood	2:17:00							BT>15
Re-injure artery	2:24:00		29.4	2:25:45	2:28:25	2:40		
Ketamine	2:26:30		29.4	2:29:00	2:30:35	1:35		
			30.0	2:31:40	2:34:00	2:20		
Flush lines	2:34:50							
Change gauze	2:35:50							
Inject 90ug/kg Aggrastet	2:36:30		30.4	2:37:10	-			
Ketamine	2:40:00							
Draw blood	2:47:00							BT>15
Flush lines	3:04:40							
Change gauze	3:05:40							
Inject 270ug/kg Aggrastet	3:06:00		31.5	3:07:10				
Ketamine	3:07:30							
Draw blood	3:16:00							
Ketamine	3:30:00							
Start EPI	3:34:45							
Change gauze	3:34:20							
Stop EPI	3:38:45							
Stop recording	3:45:00							

Raw Results Efficacy Study: W05



Efficacy Study: W05

Mass Gauze	0.4					
	# Tubes	# Gauze	Mass full	Mass empty	Blood loss	Fold incease
Baseline	1	1	13.5	12.86	0.24	
Saline	1	1	13.39	12.84	0.15	1.0
3ug/kg	1	1	13.49	12.86	0.23	1.5
10ug/kg	1	1	13.52	12.86	0.26	1.7
30ug/kg	1	1	13.76	12.88	0.48	3.2
90ug/kg	1	1	13.57	12.89	0.28	1.9
270ug/kg	1	1	13.72	12.9	0.42	2.8
				Total	2.06	
				Bloodvolume	994	



Blood Loss: W05

	Baseline	3ug/kg	10ug/kg	30ug/kg	90ug/kg	270ug/kg	Mean excl Base		% Difference
WBC x 10^9/l	5.26	5.85	6.26	11.66	12.12	12.37	9.652	183.50	83.5
RBC x 10^{12}/l	6.03	6.22	6.1	6.1	5.92	5.83	6.034	100.07	0.07
haemoglobuin g/dl	15.4	15.6	15.3	15.1	15.1	14.7	15.16	98.44	1.56
haematocrit l/l	0.475	0.487	0.480	0.479	0.467	0.460	0.4746	99.92	0.08
MCV fl	78.8	78.3	78.7	78.5	78.9	78.9	78.66	99.82	0.18
MCH pg	25.5	25.1	25.1	24.8	25.5	25.2	25.14	98.59	1.41
MCMC g/dl	32.4	32.0	31.9	31.5	32.3	32.0	31.94	98.58	1.42
plt x 10^9/l	282	257	259	248	235	227	245.2	86.95	13.05
PT (sec)	11	11	12	12	12	12	11.8	107.27	7.27
aPTT (sec)	27	27	28	28	29	28	28	103.70	3.7
ADP Aggr (%)	45	14	5	2	4	0			100.00

Increase | Decrease

Max inhibition

FBC: W05

Appendix B – Roodt *et al.*

Manuscript submitted for publication

A novel complete high shear rate arterial thrombosis and bleeding model in baboons

Roodt JP, Lamprecht S, Silence K*and Ulrichts H*.

Department of Haematology and Cell Biology, University of the Free state and NHLS Tertiary Laboratory, Universitas Hospital, Bloemfontein, South Africa

* Ablynx NV, Technologiepark 4, 9052 Zwijnaarde, Belgium

Draft
Roodt et al-

Corresponding author: Johannes Petrus Roodt

Address: Department of Haematology and Cell Biology,
School of Medicine,
Faculty of Health Sciences
University of the Free State
Box 339 (G2)
Bloemfontein, 9300
South Africa

Tel. +27 51 405 3043

Fax +27 51 444 1036

Mobile +27 72 395 0268

e-Mail gnhmjpr.md@ufs.ac.za

Running title: A high shear rate arterial thrombosis model in baboons.

Word Count:

Abstract: 320

Body text: 4268

Legend to figures: 393

References: 1438

Total number of figures: 4

Draft
Roodt et al-

Abstract

Aim of the study. Given the remaining high incidence of cardiovascular events, there is a high need for safer and more effective antithrombotic therapies. These newly developed drugs have to be tested in predictive and relevant animal models.

The aim of this study was to develop a complete, technically uncomplicated arterial thrombosis and bleeding model in baboons representative for the human clinical situation.

Methods. The femoral artery of baboons was dissected free and was shunted to the femoral vein, increasing blood flow. The artery was mechanically injured and an external stenosis was applied. The resulting in thrombus formation was measured by decrease in blood flow. Thrombi were mechanically dislodged causing a pattern of cyclic flow reductions (CFRs). Escalating doses of aspirin, clopidogrel, heparin or abciximab were infused in the animals and the effect of this treatment on the CFRs was measured. The effect of these drugs on an incisional and a template bleeding model was assessed.

Results. Aspirin and heparin failed to prevent thrombosis in stenosed, endothelium-injured femoral arteries in baboons while clopidogrel and abciximab efficiently prevented CFRs. Inhibition of CFRs was sustained upon infusion of epinephrine in baboons treated with effective doses of abciximab. CFR's returned in animals treated with effective doses of clopidogrel. Template bleeding time was unaffected in animals treated with heparin or aspirin but was severely prolonged in animals treated with clopidogrel or abciximab. Blood loss from a well defined wound was increased 4- and 15-fold for animals treated with respectively an effective dose of clopidogrel or abciximab but was unaffected upon treatment with aspirin.

Conclusion. The efficacy of the tested drugs our model closely matches the clinical situation in man. A similar bleeding tendency profile compared to the clinical situation is observed using the incision bleeding model. These results suggest that our technically uncomplicated and reproducible arterial thrombosis and bleeding model approaches that of the human situation.

Key words: Thrombosis, baboon, antithrombotic, high shear rate, platelet adhesion.

Draft Roodt et al.

Introduction

Atherothrombotic diseases such as coronary heart diseases, stroke and peripheral artery diseases are prevalent in 34% of the American population and are as the underlying cause of 1 in every 2.7 deaths in the United States[1]. Similar figures are found in other Western and developing countries. Clearly, this represents a serious social and economical burden for the society and therefore, much effort is being put in the development of effective and safe drugs.

Antiplatelet agents such as aspirin, clopidogrel and the integrin $\alpha_{IIb}\beta_3$ blocker abciximab have a beneficial effect on outcomes in patients with cardiovascular diseases[2-9]. Intravenous unfractionated heparin is presently the most frequently used anticoagulant during cardiovascular interventions [10]. However, there is still need for more effective therapies, given the remaining high incidence of cardiovascular events despite the current drugs. More specifically, there is a strong need for a better therapeutic window, i.e. a wide span between the therapeutic efficacy and side-effects of the drug, with bleeding risk being an important concern.

Draft Roodt et al.

Several animal thrombosis models have been developed [11] in which the efficacy of novel antithrombotics are assessed. These models should reflect the human pathological conditions as much as possible in order to efficiently predict the clinical outcome of the drug tested. Of particular interest is the model developed by Folts[12,13] as it combines two key factors which trigger thrombus formation in atherosclerotic arteries in the patient, namely platelet exposure to subendothelial components and a high shear stress environment. The original model entails the opening of the chest cavity of anesthetized dogs and exposure of the heart. One of the coronary arteries is dissected free from the surrounding tissue and the blood flow is measured by an electromagnetic flowmeter. Using a vascular constrictor, a fixed amount of stenosis (60-80%) is produced in the coronary artery which resulted in reduction of the blood flow to near 0 mL/min due to thrombus formation. This process is followed by a sudden return of the blood flow to near baseline levels due to spontaneous embolisation of the thrombus in the distal circulation. Following this embolisation, thrombus formation restarts. This repetitive pattern of periodic thrombosis and blood flow variations is portrayed as cyclic flow reductions (CFRs). The original model mimics the human pathological situation of unstable angina and this model has since been further adapted and used in different vascular beds and animals[14-18].

Besides determining the antithrombotic efficacy of drugs, *in vivo* models should be able to detect bleeding risk as clinical bleeding represents the major side-effect of antithrombotic therapy and limits its overall clinical benefit. Clinical bleedings comprise surgical, intracranial or gastro-intestinal bleedings with the latter two difficult to assess in animal models. The commonest method for evaluating the bleeding risk of antithrombotic drugs in *in vivo* models is the template bleeding time. However, several studies demonstrate that a prolonged bleeding time in man does not necessarily predict clinical bleedings[19,20]. The incisional bleeding method, which measures blood loss from a well defined wound [21,22] might be more predictable as it more closely reflects surgical tissue damage. In this method, a gauze is inserted in a well defined wound and the weight of blood lost is measured by weighing the gauze. As this method mimics surgical interventions, it might predict surgical bleedings. However a thorough characterisation of this method is lacking.

Draft
Roodt et al.

The aim of this study was to develop a reproducible, technical uncomplicated and predictive thrombosis and bleeding model which closely mimics the human situation. We have previously developed a modified Folts' model in the femoral artery of baboons [23,24]. This model was further extended and optimised using an arterio-venous shunt to increase blood flow reflecting more closely a pathological high shear environment. The newly developed model was characterised by determining the effect of the most currently used antiplatelet agents aspirin, clopidogrel, abciximab and the anticoagulant heparin comparing the results with the human clinical situation. A dose-escalating study was performed and the antithrombotic potency upon infusion of epinephrine was analysed. These studies allow full analysis of the efficacy of the drug. The effect of these drugs on the template bleeding time and the incisional bleeding method was verified in order to identify if the second test might be more representative.

Materials and Methods

Materials

Unfractionated heparin and epinephrine were purchased from Intramed (Port Elizabeth, South Africa). Aspirin (Aspegic™) was obtained from Sanofi-Aventis (Paris, France). Clopidogrel (Plavix™), purchased from Bristol-Myers Squibb/Sanofi Pharmaceuticals (New York, NY), was pulverised and suspended in methanol (150 mg/mL) as previously described[14]. This solution was further diluted in saline to a concentration of 10 mg/mL and passed through a 0.22 µm filter. Oral administration does not allow the performance of baseline measurements or the use of stepping doses and as the active metabolite was not available from Sanofi, we decided to perform the procedure described by Yao et al[14], which was done with the co-operation of the Sanofi Research department. Abciximab (Reopro™) was obtained from Lilly/Centocor (Indianapolis, IN). Ketamine hydrochloride (Anaket-V™) was acquired from Premier Pharmaceutical Company (Bryanston, South Africa).

Draft Roodt et al.

Thrombosis model

In this study 24 arteries from 17 healthy baboons (*Papio ursinus*) of either sex were injured and an external stenosis was applied after which CFRs were measured. The animals weighed 8.1-17.6 kg and were disease-free for at least 2 weeks prior to use. The study was approved by the Ethics Committee for Animal Research of the University of the Free State.

This study was performed as described by us earlier[25]. Baboons were anaesthetized with ketamine (10 mg/kg IM/30 minutes or when needed to maintain general anesthesia). Body temperature was maintained at 37°C with a heating table. A 4-5 cm segment of the femoral vessels was gently dissected free from surrounding tissue. All nearby branches in the femoral artery and femoral vein were ligated. A small incision was made in the femoral artery and femoral vein and vessel tips were inserted and secured with surgical silk. Silicone tubing was attached to the vessel tips to shunt arterial blood into the femoral vein. The direct shunting from the arterial to the venous circulation, while bypassing the capillaries, increased blood flow to 150-300 ml/minute. A tube-type ultrasonic flow probe (Transonic Systems Inc, Maastricht, The Netherlands) was attached to the silicone tubing and blood flow was allowed to stabilize for about 20 minutes. The mean and phasic blood flow was measured continuously throughout the experiment. The shunt was used for drug administration as well as for blood sampling.

Draft
Roodt et al-

The endothelium of the femoral artery was injured proximally to the vessel tip with a Martin needle holder (Hegar-Baumgartner TC Gold 14 cm Product code 20.634.14) by pressing hard on the endothelium for 10 seconds at maximum depression. Two overlapping injuries were made and an adjustable plastic constrictor was placed over the injury site to reduce blood flow to 10 to 20% of baseline value. A gradual decline in blood flow was observed due to thrombus formation. When blood flow was reduced to ≤ 5 mL/min, the constrictor was opened to dislodge the platelet-rich thrombus. The external stenosis was applied again and the process of thrombus formation restarted. The repetitive pattern of decreasing blood flow following mechanical restoration was referred to as cyclic flow reductions (CFRs). The number of CFRs as a function of time was measured.

After a 30 minutes control period, saline was injected and CFRs were monitored for a further 30 minutes. The antithrombotic effect of the different compounds tested was quantified by measuring the number of CFRs

before and after drug administration. When full inhibition of CFRs was observed, a new injury was applied in order to confirm that the inhibition was an effect of treatment and not of a natural healing phenomenon. In earlier exploratory studies we have observed a sudden loss of CFR's in long term (>6 hours) control studies, which was reversed by re-injury of the artery. Baboons were treated with either saline (n=4), aspirin (n=3), heparin (n=3), clopidogrel (n=4), abciximab (n=3), or with a combination of aspirin, heparin and clopidogrel (n=7). Repeated administration of saline or escalating doses of test substances were administered at 30 min intervals while recording the CFRs.

Heparin and aspirin were injected as a bolus to obtain cumulative doses of 15-240 IU/kg and 1-40 mg/kg respectively. Clopidogrel was administered intravenously as a bolus injection at a cumulative dose of 1-20 mg/kg according to previous studies[14]. Abciximab was injected as a bolus at cumulative doses of 20-920 µg/kg.

Draft
Roodt et al.

At the end of each dose phase, the shunt was flushed with saline before the next dose was injected. After the cumulative dose, i.e. when complete inhibition of CFRs was observed, epinephrine was infused at a dose of 2.2 µg/kg/min for 20 minutes and CFRs were measured again. Epinephrine alone does not cause platelet aggregation in baboons but can restore the abolished cyclic flow variations by enhancing other platelet aggregation factors[25].

Bleeding measurements

Two methods were used to asses bleeding upon drug administration. In a first method, the skin template bleeding time was determined on the surface of the fore-arm. A pressure cuff was applied around the arm and inflated at 40 mm Hg, after which a wound was induced with the Surgicut device (ITC, Edison, NJ). The skin bleeding time was defined as the time between the induction of the wound and visual cessation of bleeding. The blood was carefully dabbed every 15 s with filter paper whilst not touching the wound. Measurements were stopped when the skin bleeding time exceeded 900 s and were considered as 900 s.

In a second method, the blood loss from an incision was assessed as described before[21,22]. A 2cm x 0.8cm incision was made in the groin. Pre-weighed gauze swabs were inserted and replaced at the end of each 30 minute dose infusion period or when it was saturated with blood. All gauzes were weighed at the end of the study to assess the amount of blood loss. The value for each dose was expressed as a ratio of the total blood loss in the saline control phase gauze.

Draft Roodt et al.

Results

Antithrombotic effect of Aspirin, heparin, Clopidogrel and Abciximab in a baboon thombosis model.

In a first part, 17 arteries from 12 baboons were injured and an external stenosis was applied after which CFRs were measured for 30 minutes to determine the baseline values. The baboons were randomized and were treated with saline (n=4), aspirin (n=3), heparin (n=3), clopidogrel (n=4) or abciximab (n=3). There was individual variation in the duration of CFR's between animals ranging between an average of 1 and 5 minutes, therefore we decided to do a 30 minutes baseline study followed by a 30 minute observation following the infusion of saline. This saline phase was used a baseline to compare the effect of the drug to.

Draft Roodt et al.

In the negative control group, saline was repeatedly administered every 30 min and CFRs were measured (Fig.1A). These results demonstrated that infusion of saline did not affect CFRs compared with the baseline value, as expected. The repetitive pattern of CFRs was highly stable and reproducible during the experiment. This procedure of stable and reproducible CFRs could be maintained for 4hours.

In the drug treatment groups, saline was administered after setting the baseline values and CFRs were measured for 30 min. Subsequently, escalating doses of the drugs were injected every 30 min and CFRs were assessed (Fig. 1). A representative read-out is shown in figure 2.

Aspirin failed to affect CFRs in this model, even at a cumulative dose of 40 mg/kg. With heparin there was a slight decrease in the number of CFR's. This was caused by partial or complete inhibition of CFR's that were always reversed by re-injuring the artery. We had seven occurences of this phenomenon in the three studies and was seen in one study at doses as low as 15IU/kg. This phenomenon was not seen with any of the other drugs tested. In contrast, clopidogrel and abciximab completely abolished CFRs: a complete inhibition of thrombus formation was observed at a cumulative dose of 5 mg/kg clopidogrel or of 420 µg/kg abciximab. When

full inhibition of CFRs was observed, a new injury was applied in order to confirm that the inhibition was an effect of the treatment and not of a natural healing phenomenon. When the effective dose of clopidogrel or abciximab was reached, epinephrine was subsequently infused and the CFRs were measured for 20 min (Fig.1). The CFRs returned in the animals treated with clopidogrel. However, no CFRs reappeared with the epinephrine infusion in the animals treated with abciximab, demonstrating a strong antithrombotic effect of this compound.

In a second part, we verified if aspirin, heparin and clopidogrel synergistically inhibit arterial thrombus formation. Seven femoral arteries of five baboons were treated with a loading dose of 5 mg/kg aspirin and 60 IU/kg heparin and used in the same femoral artery thrombosis model as described above. After a control and saline phase, increasing cumulative doses of clopidogrel (1-2 mg/kg) were infused and CFRs were measured (Fig. 1). These results demonstrated that a combination of aspirin, heparin and clopidogrel was able to fully inhibit arterial thrombus formation. Epinephrine, however, caused re-appearance of CFRs (Fig. 1F).

Draft
Roodt et al.

Bleeding tendency

Bleeding tendency was determined using either skin bleeding time measurements or measurement of blood loss in a well-defined incision in the groin.

The template bleeding time was stable in the control saline group over the time of the experiment. Contrary to findings of other researchers, no major changes in bleeding time were observed for the animals treated with aspirin or heparin when the bleeding time is performed on the forearm with the Surgicut bleeding time device. We have no explanation for this. However, the template bleeding time was significantly prolonged (> twice the bleeding time at pre-dose level) upon administration of a cumulative dose of 2.5 mg/kg clopidogrel or 170 µg/kg abciximab. This effect even persisted for over 24hours. Similarly, template bleeding time was significantly prolonged in baboons treated with a combination of aspirin (5 mg/kg), heparin (60 IU/kg) and a cumulative dose of 2 mg/kg clopidogrel.

In a second set-up, the effect of the drug on blood loss from a well defined wound was determined. The relative amount of blood loss compared to the loss in the control gauze was stable for animals treated with Aspirin (data not shown). A mild bleeding tendency was observed for heparin treated animals, more specifically at the dose of 240 IU/kg where a twofold increase in blood loss was measured (data not shown). For animals treated with clopidogrel or abciximab however, a significant bleeding tendency was observed, which increased with increasing drug doses (Fig. 4). A 4- or 15-fold increase in blood loss was measured for animals treated with doses from 20 mg/kg clopidogrel or 420 µg/kg abciximab respectively. Similarly, a 5-fold increase in blood loss was observed for animals treated with a loading dose of 5 mg/kg Aspirin and 60 IU/kg heparin and a cumulative dose of 2 mg/kg clopidogrel (data not shown).

Draft
Roodt et al.-

Discussion

The use of appropriate *in vivo* thrombosis models is essential to verify the efficacy and safety of novel antithrombotic strategies. It is of key importance that the experimental model reflects as much as possible the human situation and hence that an effective and safe therapy in this model will be as efficacious and safe as in the human pathological situation. The goal of this study was to identify a reproducible, technically uncomplicated and highly representative *in vivo* arterial thrombosis and bleeding model.

The model was developed in non-human primates as they are phylogenically the closest animals to humans. We chose the baboon as test animal as their hemostatic apparatus, platelet receptor profile and vasculature is extensively researched and closely resembles that of humans[26-28]. The molecular homology between baboons and humans is high which increases the likelihood that drugs designed by molecular engineering to be highly effective in humans will show a similar *ex vivo* profile in baboons.

Draft
Roodt et al.

In our model, the blood flow from the femoral artery was shunted to the femoral vein, which resulted in an increase of blood flow to 150-300 ml/min and a concomitant increase in wall shear stress. In agreement with others, the shear rate at peak flow is likely to exceed 200 dynes/cm^2 when the shunted artery was stenosed[28] which is similar to the shear rate in stenotic vessels[31].

In a first part of the study, we investigated the potency of aspirin, heparin, clopidogrel and abciximab in preventing arterial thrombosis. Our results demonstrated that intravenous injection of clopidogrel and abciximab completely eliminated CFRs in stenosed, shunted and endothelium-injured femoral arteries in baboons while aspirin and heparin failed to do so. The mean effective dose of clopidogrel was 5 mg/kg and of abciximab 420 µg/kg. We saw several partial or complete inhibitions of CFR’s that were reversed by re-injury of the artery after infusion of heparin. This was not seen with any of the other agents and probably indicates that the beneficiary role of heparin is healing of the injury, although the mechanism is not clear at this stage. The activated partial thromboplastin time in plasma samples prepared during the study were significantly prolonged from a cumulative dose of 60 IU/kg (data not shown).

Previous studies also demonstrated that clopidogrel efficiently inhibits CFRs in stenosed and damaged femoral arteries in baboons [14] and as well in pigs [16] at similar doses. Similarly, other studies showed that blocking the platelet receptor $\alpha_{IIb}\beta_3$ in the baboon or monkey, abolishes CFRs in stenosed and endothelium-injured femoral arteries at again comparable doses of antagonists[24,28,32].

Our results demonstrate that aspirin fails to inhibit CFRs in stenosed, shunted and endothelium-injured baboon femoral arteries. This is in line with previous studies which demonstrated that high shear forces can overcome the inhibitory effect of aspirin[34,35]. Moreover, CFRs in stenosed coronary arteries of some patients with unstable angina were observed even when these patients received aspirin[29]. Our data could explain these specific observations in patients. Our data could explain these specific observations but do not completely reflect the overall *in vivo* efficacy of Aspirin in preventing vascular events such as stroke or myocardial infarction[36,37].

Draft
Roodt et al.

In order to distinguish between weaker and stronger antiplatelet compounds, we verified whether prevention of CFRs was sustained upon infusion of epinephrine. Upon administration of effective doses of abciximab, thrombus formation remained inhibited when epinephrine was infused in agreement with previous studies[32]. However, when epinephrine was infused in baboons treated with an effective dose of clopidogrel, CFRs returned. While ADP signaling has been shown *in vitro* to be essential for shear-induced platelet aggregation[35,39,40], blockage of the ADP receptor $P2Y_{12}$ by clopidogrel or other drugs does not affect epinephrine-induced signalisation in platelets[41,42]. Therefore, in our model characterized by the high shear stress, the inhibitory effect of Clopidogrel on thrombus formation might be overcome by a synergistic action of epinephrine with the high shear stress on platelet activation/aggregation.

Our results demonstrate that a combination of aspirin and clopidogrel potently inhibits thrombus formation in stenosed and endothelium-denuded femoral vessels in baboons. CFRs returned however when epinephrine was infused, demonstrating that even the combination therapy is not sufficient to prevent thrombus formation induced by a combination of vascular damage and stenosis with an infusion of catecholamines.

Our results demonstrate that clopidogrel is more potent than aspirin in preventing arterial thrombosis, while abciximab is even more potent. This antithrombotic effect of aspirin, clopidogrel and abciximab in the here-described baboon model closely correlates with the clinical situation in man. Indeed, in patients at risk for ischemic events, clopidogrel was more efficient than aspirin in reducing the combined risk of ischaemic stroke, myocardial infarction, or vascular death[7]. Moreover, clinical studies have demonstrated an impressive beneficial effect for parenteral integrin $\alpha_{IIb}\beta_3$ blockers in reducing ischemic complications following percutaneous intervention, and a more modest beneficial effect in the treatment of patients with acute coronary syndromes compared to conventional antithrombotic management [43].

In a second part of the study, we investigated the safety of aspirin, heparin, clopidogrel and abciximab in terms of bleeding. We measured the effect of these drugs on the template skin bleeding time and on blood loss from a well defined wound. No significant effect of aspirin or heparin was found in these bleeding models although strong variability was observed in the skin bleeding time test. Skin bleeding time was similarly and severely impaired upon administration of clopidogrel or abciximab, even at doses below the effective dose and this effect persisted for over 24h. Similar studies also demonstrated a severely prolonged skin bleeding time in baboons upon administration of high doses of clopidogrel[44] or of abciximab[45]. Similarly, the bleeding from a well defined incision was increased upon administration of Clopidogrel or Abciximab. However, blood loss was much more severe upon infusion of abciximab compared with clopidogrel. Although the contrary is often assumed, skin bleeding time measurements in man have no predictive value to indicate the risk of hemorrhage in the patient upon treatment [19,20]. The same might hold true in *in vivo* animal models. In patients receiving clopidogrel on top of aspirin, an increase in major bleedings was observed[2]. An even higher relative risk in bleeding was observed in patients receiving parenteral integrin $\alpha_{IIb}\beta_3$ blockers[46]. A similar profile was observed in the incision bleeding model: an increase in blood loss was observed when clopidogrel was administered and an even higher bleeding was observed when abciximab was infused. In contrast, no different effects were observed for clopidogrel and abciximab in the template bleeding model. Moreover, the template bleeding time measurements were more variable than the blood loss measurements and more prone to experimenter's bias Therefore, the incision bleeding model is more reproducible and likely more indicative for the *in vivo* situation.

Draft Roodt et al.

In conclusion, we have developed a novel *in vivo* arterial thrombosis model characterised by a pathological high shear stress.

Our model has essential advantages over other more established models in other animals: (i) the model is characterised by less noise and mechanical interference in the flow readings, (ii) the model is highly reproducible and technically uncomplicated (iii) animals can be used twice and failure to dislodge the thrombus does not lead to a study termination as the thrombus can be flushed out (iv) the model allows a full analysis of the efficacy: a dose escalating study can be performed and the antithrombotic effect upon a new injury can be analysed. In each animal, the saline pre-dose phase can serve as an internal control (v) by infusion of catecholamines in this model, we were able to discriminate between weak and strong antiplatelet agonists (vi) because the animals do not have to terminated at the end of the study long term testing like immunogenicity can be determined in the same animal. Finally, we promote the incision bleeding model as more predictive for the human clinical situation than the template bleeding model. The observed potency and safety of the drugs tested in the here-described baboon model closely correlate with the human clinical situation, rendering this technically uncomplicated thrombosis and bleeding model highly representative.

Draft Roodt et al.

Figure legends

Figure 1: Effect of saline, aspirin, heparin, clopidogrel or abciximab on CFRs in the stenosed, damaged femoral artery in baboons. Femoral arteries of baboons were exposed, damaged with a forceps and stenosed externally. When blood flow was decreased to 5 mL/min, the platelet thrombus was dislodged and the external stenosis was reapplied. CFRs were quantified by counting the number of times blood flow decreased to 5 mL/min for 30 min at pre-dose level or upon infusion of consecutive doses of saline (A), aspirin (B), heparin (C), clopidogrel (D), abciximab (E), or clopidogrel after a loading dose of 2 mg/kg aspirin and 60 IU/kg heparin (F). In D-F: when complete inhibition of CFRs was observed, epinephrine was infused and CFRs were quantified for 20 min.

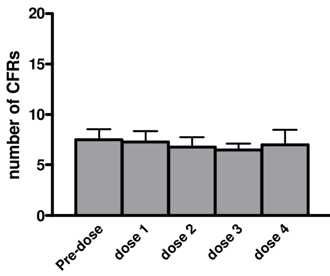
Draft
Roodt et al.

Figure 2: Representative recording of flow velocity in the stenosed, damaged femoral artery in baboons treated with clopidogrel. A representative of four independent experiments is shown. Femoral artery was damaged and externally stenosed after which CFRs were measured. CFRs were recorded for 30 min at pre-dose level and after infusion of saline or consecutive increasing doses of clopidogrel (mg/kg, indicated with arrows). When inhibition of CFRs was observed, a new injury was applied in order to confirm that the inhibition was an effect of the treatment but not of a natural healing phenomenon. When complete inhibition of CFRs was observed, epinephrine was injected for 20 min.

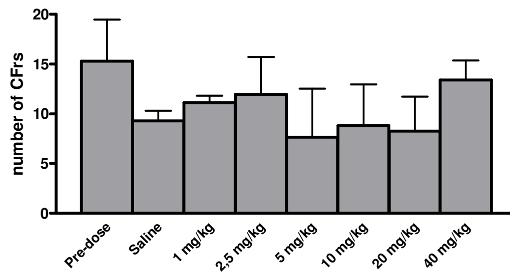
Figure 3: Effect of Aspirin, heparin, clopidogrel or abciximab on bleeding tendency in baboons as measured by skin bleeding time. Template bleeding time was measured using the surgicut device. Measurements were stopped when the skin bleeding time exceeded 900 s and were considered as 900 s. Skin bleeding time was measured at pre-dose level and 10 min after infusion of consecutive doses of saline (A), aspirin (B), heparin (C), clopidogrel (D), abciximab (E) or clopidogrel after a loading dose of 5 mg/kg aspirin and 60 IU/kg heparin (F).

Figure 4: Effect of clopidogrel or abciximab on bleeding tendency in baboons as measured by blood loss from a well defined wound. An incision was made in the upper thigh of baboons and a gauze was inserted. The amount of blood loss was measured for 30 min upon infusion of saline and upon infusion of consecutive doses of clopidogrel (A) or abciximab (B). Data are represented as fold-changes from the blood loss upon saline infusion.

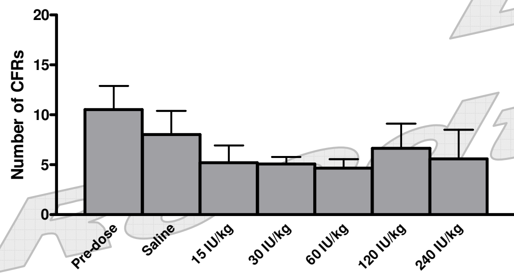
A



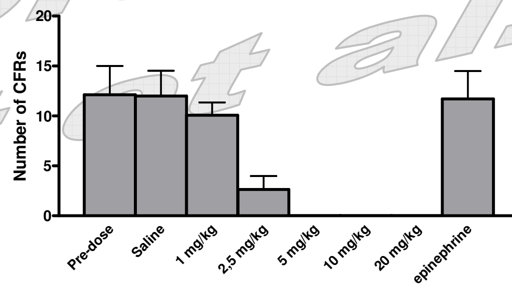
B



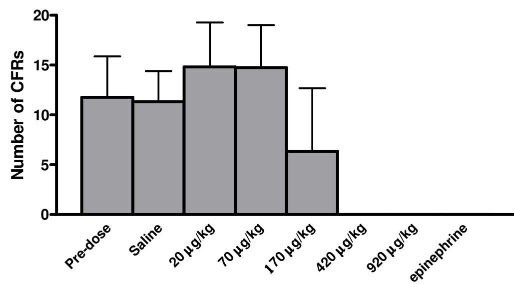
C



D



E



F

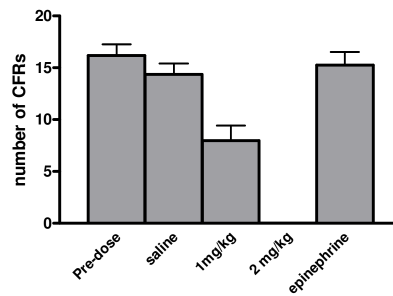
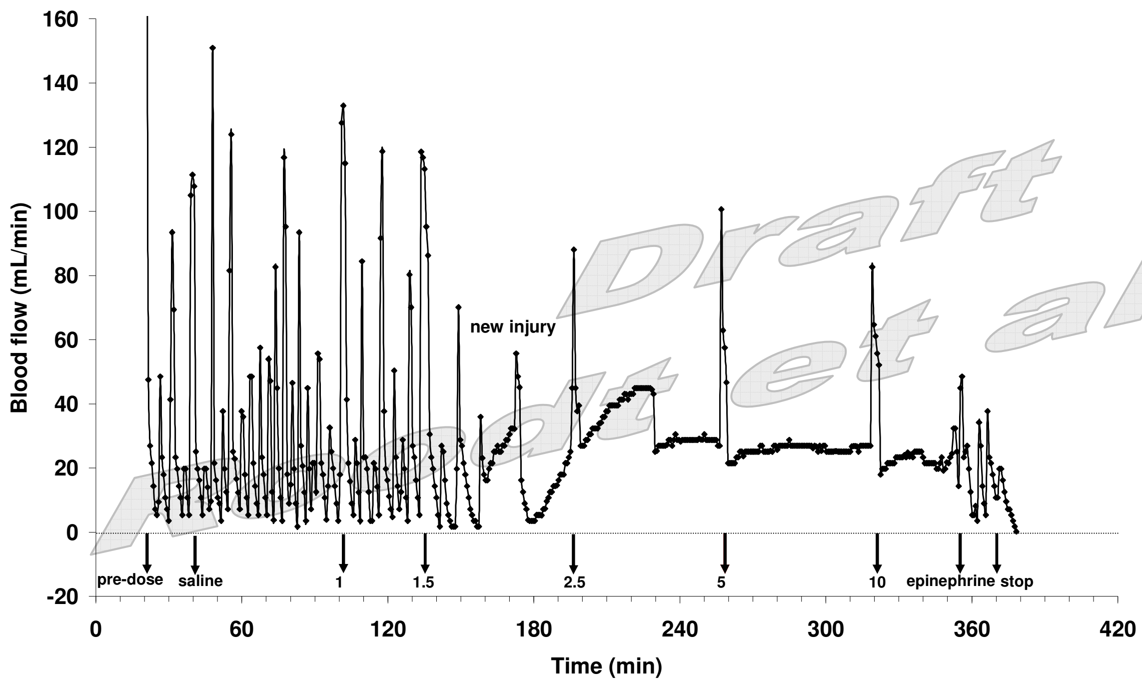
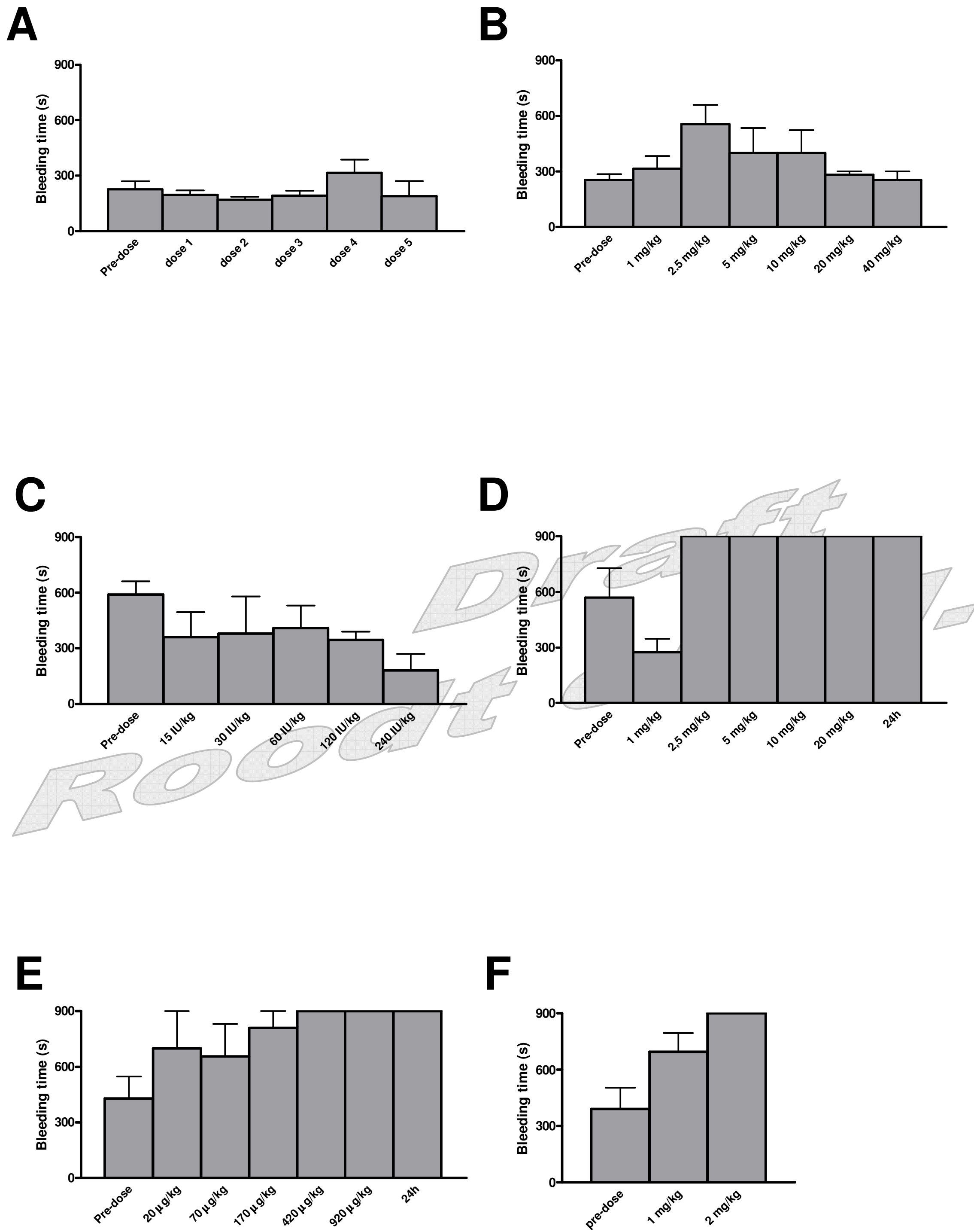


Figure 1



Draft Roodt et al.

Figure 2



Draft ... Roodt

Figure 3

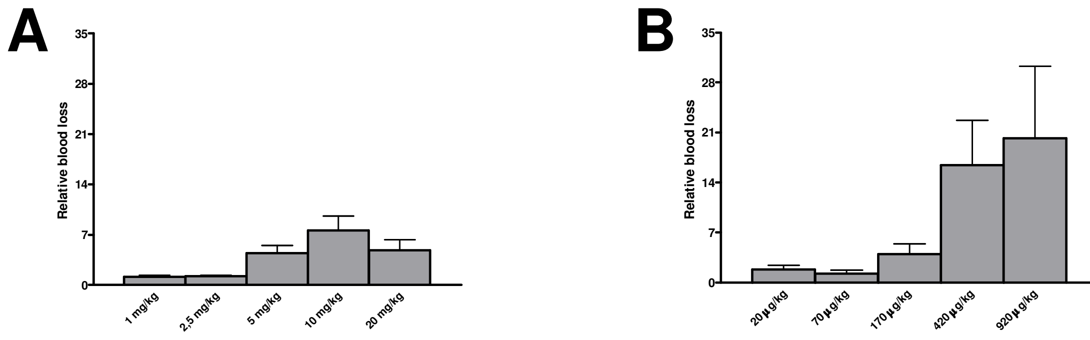


Figure 4

Draft
Roodt et al.-

References

1. Thom T, Haase N, Rosamond W, Howard VJ, et al. Heart disease and stroke statistics--2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2006;113:e85-151

2. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001;345:494-502

3. Steinhubl SR, Berger PB, Mann JT, 3rd, Fry ET, et al. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. Jama. 2002;288:2411-2420

4. Sabatine MS, Cannon CP, Gibson CM, et al. Effect of clopidogrel pretreatment before percutaneous coronary intervention in patients with ST-elevation myocardial infarction treated with fibrinolytics: the PCI-CLARITY study. Jama. 2005;294:1224-1232

5. Sabatine MS, Cannon CP, Gibson CM, et al. Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med. 2005;352:1179-1189

6. Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet. 2005;366:1607-1621

7. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet. 1996;348:1329-1339

8. Diener HC, Bogousslavsky J, Brass LM, et al. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. Lancet. 2004;364:331-337

9. Casserly IP, Topol EJ. Glycoprotein IIb/IIIa antagonists--from bench to practice. Cell Mol Life Sci. 2002;59:478-500

10. Spyropoulos AC. Pharmacologic therapy for the management of thrombosis: unfractionated heparin or low-molecular-weight heparin? Clin Cornerstone. 2005;7:39-48

11. Andre P, Bal dit Sollier C, Bonneau M, et al. Which experimental model to choose to study arterial thrombosis and evaluate potentially useful therapeutics? Haemostasis. 1996;26 Suppl 4:55-69

12. Folts JD, Crowell EB, Jr., Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. Circulation. 1976;54:365-370

13. Folts J. An in vivo model of experimental arterial stenosis, intimal damage, and periodic thrombosis. Circulation. 1991;83:IV3-14

Draft Roodt et al.

14. Yao SK, McNatt J, Cui K, Anderson HV, et al. Combined ADP and thromboxane A2 antagonism prevents cyclic flow variations in stenosed and endothelium-injured arteries in nonhuman primates. Circulation. 1993;88:2888-2893

15. Fattorutto M, Tourreau-Pham S, Mazoyer E, et al. Recombinant activated factor VII decreases bleeding without increasing arterial thrombosis in rabbits. Can J Anaesth. 2004;51:672-679

16. Samama CM, Bonnin P, Bonneau M, et al. Comparative arterial antithrombotic activity of clopidogrel and acetyl salicylic acid in the pig. Thromb Haemost. 1992;68:500-505

17. Poletti LF, Bird K, Harris RB, et al. Prevention of arterial thrombosis using a novel heparin with enhanced antiplatelet activity and reduced anticoagulant activity. J Vasc Surg. 1997;26:366-372

18. Daykin HJ, Sturgeon SA, Jones C, et al. Arterial antithrombotic effects of aspirin, heparin, enoxaparin and clopidogrel alone, or in combination, in the rat. Thromb Res. 2006

19. Lind SE. The bleeding time does not predict surgical bleeding. Blood. 1991;77:2547-2552

20. Rodgers RP, Levin J. A critical reappraisal of the bleeding time. Semin Thromb Hemost. 1990;16:1-20

21. Benedict CR, Ryan J, Wolitzky B, et al. Active site-blocked factor IXa prevents intravascular thrombus formation in the coronary vasculature without inhibiting extravascular coagulation in a canine thrombosis model. J Clin Invest. 1991;88:1760-1765

22. Thiagarajan P, Benedict CR. Inhibition of arterial thrombosis by recombinant annexin V in a rabbit carotid artery injury model. Circulation. 1997;96:2339-2347

23. Wu D, Vanhoorelbeke K, Cauwenberghs N, et al. Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. Blood. 2002;99:3623-3628

24. Wu D, Meiring M, Kotze HF, et al. Inhibition of platelet glycoprotein Ib, glycoprotein IIb/IIIa, or both by monoclonal antibodies prevents arterial thrombosis in baboons. Arterioscler Thromb Vasc Biol. 2002;22:323-328 25. Anfossi G, Trovati M. Role of catecholamines in platelet function: pathophysiological and clinical significance. Eur J Clin Invest. 1996;26:353-370

25. Fontayne A, Meiring M, Lamprecht et al. The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. Thrombosis and Haemostasis 2008:100:670-677.

26. Hampton JW, Matthews C. Similarities between baboon and human blood clotting. J Appl Physiol. 1966;21:1713-1716

27. Hanson SR, Harker LA, Ratner BD, et al. In vivo evaluation of artificial surfaces with a nonhuman primate model of arterial thrombosis. J Lab Clin Med. 1980;95:289-304

Draft Roodt et al.

28. Harker LA, Kelly AB, Hanson SR. Experimental arterial thrombosis in nonhuman primates. Circulation. 1991;83:IV41-55

29. Eichhorn EJ, Grayburn PA, Willard JE, et al. Spontaneous alterations in coronary blood flow velocity before and after coronary angioplasty in patients with severe angina. J Am Coll Cardiol. 1991;17:43-52

30. Sunamura M, di Mario C, Piek JJ, et al. Cyclic flow variations after angioplasty: a rare phenomenon predictive of immediate complications. DEBATE Investigator's Group. Am Heart J. 1996;131:843-848

31. Kroll MH, Hellums JD, McIntire LV, et al. Platelets and shear stress. Blood. 1996;88:1525-1541

32. Coller BS, Folts JD, Smith SR, et al. Abolition of in vivo platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. Correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors. Circulation. 1989;80:1766-1774

33. Leadley RJ, Jr., Kasiewski CJ, Bostwick JS, et al. Inhibition of repetitive thrombus formation in the stenosed canine coronary artery by enoxaparin, but not by unfractionated heparin. Arterioscler Thromb Vasc Biol. 1998;18:908-914

34. Maalej N, Folts JD. Increased shear stress overcomes the antithrombotic platelet inhibitory effect of aspirin in stenosed dog coronary arteries. Circulation. 1996;93:1201-1205

35. Moake JL, Turner NA, Stathopoulos NA, et al. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. Blood. 1988;71:1366-1374

36. Collaborative overview of randomised trials of antiplatelet therapy--I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration. Bmj. 1994;308:81-106

37. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Bmj. 2002;324:71-86

38. Wagner CT, Kroll MH, Chow TW, et al. Epinephrine and shear stress synergistically induce platelet aggregation via a mechanism that partially bypasses VWF-GP IB interactions. Biorheology. 1996;33:209-229

39. Cattaneo M, Lombardi R, Bettega D, et al. Shear-induced platelet aggregation is potentiated by desmopressin and inhibited by ticlopidine. Arterioscler Thromb. 1993;13:393-397

40. Roald HE, Barstad RM, Kierulf P, et al. Clopidogrel--a platelet inhibitor which inhibits thrombogenesis in non-anticoagulated human blood independently of the blood flow conditions. Thromb Haemost. 1994;71:655-662

41. Geiger J, Brich J, Honig-Liedl P, et al. Specific impairment of human platelet P2Y(AC) ADP receptor-mediated signaling by the antiplatelet drug clopidogrel. Arterioscler Thromb Vasc Biol. 1999;19:2007-2011

Draft Roodt et al.

42. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A. 1998;95:8070-8074

43. Bhatt DL, Topol EJ. Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. Jama. 2000;284:1549-1558

44. Harker LA, Marzec UM, Kelly AB, et al. Clopidogrel inhibition of stent, graft, and vascular thrombogenesis with antithrombotic enhancement by aspirin in nonhuman primates. Circulation. 1998;98:2461-2469

45. Kohmura C, Gold HK, Yasuda T, et al. A chimeric murine/human antibody Fab fragment directed against the platelet GPIIb/IIIa receptor enhances and sustains arterial thrombolysis with recombinant tissue-type plasminogen activator in baboons. Arterioscler Thromb. 1993;13:1837-1842

46. Boersma E, Harrington RA, Moliterno DJ, et al. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet. 2002;359:189-198

Draft
Roodt et al.

Acknowledgements

I would like to thank the following people who have made an impact on this study, and without whom it would not have been possible to successfully perform this study:

- Mister Jan Roodt: For all the advice and hard work that you put into this study, but most of all thank you for giving me the chance to become part of the primate research team and for having the confidence in me to grow on my own as part of the team.
- Professor Philip Badenhorst: For being a brilliant leader and a man that I can look up to. Thank you for giving me the opportunity and privilege to become part of this great department. Your door was always open and you were never too busy to listen and give sound advice. You are not only my role model professionally and academically, but also in my personal life. You are truly my second father.
- Professor Muriel Meiring: For all the advice, insight and patience throughout the study.
- Mister Seb Lamprecht: For teaching me the finer points of working with the animals and for having the confidence in me that I could do this project with a model that you perfected.
- The Department of Haematology and Cell Biology: For all the support and help with the processing and performance of all the laboratory tests, and also for all the moral support, it is great to be part of this family.
- Professor Pieter van Wyk and Miss Beanélri Janecke: For the support with the Scanning Electron Microscopy and being patient with me even though I was a novice in your field.

- All my family and friends: For the moral support and the help to keep my life balanced. You are too many to name, but know that each one of you has made an impact on my life.
- My wife, Elize Janse van Rensburg: For always being there, for sharing my enthusiasm and for having belief in my abilities. Your strength helped to carry me through this study.

  - I dedicate this dissertation to my wife and best friend, Elize Janse van Rensburg.

"Gloria in Excelsis Deo" - Glory to God in the highest