THROMBOLYSIS AND HYPERCOAGULABILITY:

RELEVANCE TO POST-THROMBOLYTIC EVENTS

AND PROCEDURES

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

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March 2005

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DEDICATION

This thesis is dedicated to my wife Dee

and also to the late

Professor David de Bono

STATEMENT

This thesis submitted for the degree of Doctor of Medicine entitled "Thrombolysis And Hypercoagulability: Relevance To Post-Thrombolytic Events And Procedures" is based on work conducted by the author in the Department of Cardiology of the University of Leicester during the period between June 1996 and December 1998.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. All blood samples and assay procedures were performed by the author unless otherwise stated.

None of the work has been submitted for another degree in this or any other University.

ACKNOWLEDGEMENTS

This study would not have been possible without the assistance, guidance, support and help from a considerable number of people.

First and foremost I would like to thank Professor David de Bono whose initial ideas formed the basis of this thesis. Despite ill health throughout this study he provided continued support, encouragement and constructive criticism. His mental prowess was truly stunning and inspiring. His untimely death came prior to completion of this work, but his contribution pervades the thesis.

Next I would like to thank Dr. Patrick Gaffney at the National Institute of Biological Standards and Control (NIBSC) who helped me with a considerable amount of assay work. In addition, he also provided advice and encouragement throughout. A World expert in his field, he has put me in touch with other World-renowned scientists who have provided additional support. Trips to the NIBSC were always great fun thanks to Patrick's wit and companionship. Thanks are also due to his team who toiled alongside me and on my behalf, especially Colin Whitton.

Thanks are also due to Professor Alison Goodall here in Leicester who saved me and this thesis from going under more than once. Despite not being formally responsible for me in any way, she has always freely given of her time. My debt to her is immense.

Special thanks are also due to Liz Spring who guided me with much of the assay and laboratory work in Leicester. Always expected to be in more than one place at once, she has been a tremendous help.

My gratitude is also due to Professor Wilhelm Nieuwenhuizen and Dr Rogier Bos from the Gaubius Laboratory, Leiden, The Netherlands, who arranged for the intact fibrinogen and in-house soluble fibrin assays to be performed in their laboratory. I would also like to thank Dr Laszlo Bajzar at Hamilton Civic Hospitals Research Centre in Ontario who arranged for assays of TAFI to be performed employing his own in-house ELISA. Thanks are also due to Professor Mike Nesheim at Queens University, Ontario for his constructive criticisms and advice.

I would also like to thank all the staff on the Coronary Care Unit at Derbyshire Royal Infirmary, especially Maggie, Sara, Mike and Vicki. Through very long nights and interminable days, their companionship, help, support and everything else was truly life-

saving. I would also like to acknowledge the support of the consultant physicians at Derbyshire Royal Infirmary who allowed their patients to be enrolled into the study: Dr M Millar-Craig, Dr AS Scott, Dr KA McLean, Dr I Peacock, Dr B Norton, Dr WJ Windebank, Dr GK Holmes, Dr J Gorman and Dr M Mishra. Thanks are also due to the patients who, under very difficult circumstances, kindly entered the study. I would also like to thank the consultant cardiologists at The Glenfield Hospital, Leicester who allowed their PCI patients to be enrolled: Dr JD Skehan, Dr AH Gershlick, Dr PJB Hubner and Professor NJ Samani. Again, thanks are also due to the patients.

This research was fully funded by a generous grant from the British Heart Foundation to whom I am indebted.

Last, but certainly not least, I would like to thank Dee. Despite my being most difficult, bad-tempered, tired, irrational, unpleasant, and solitary, this wonderful woman still married me during this study. Without her I wouldn't be where I am today, and this piece of work would never have been completed. Pampered and tolerated through long hours at my computer, accompanied to the flow cytometer and cold room at all hours of the day and night - I am forever in her debt, and will love her always.

ABBREVIATIONS

ACS	acute coronary syndrome
ACT	activated clotting time
ADP	adenosine diphosphate
AMI	acute myocardial infarction
AP	antiplasmin
APC	activated protein C
APSAC	anisoylated plasminogen streptokinase activator complex
AT III	antithrombin III
ATP	adenosine trisphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CABG	coronary artery bypass grafting
CHD	coronary heart disease
CHOL	cholesterol
CK	creatine kinase
CPB	carboxypeptidase B
CPN	carboxypeptidase N
CX	circumflex (coronary) artery
DIAG	diagonal (coronary) artery
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid (anticoagulant)
ELISA	enzyme-linked immunosorbent assay
FDP	fibrin degradation products
FITC	fluoroscein isothiocyanate conjugated
FPA	fibrinopeptide A
FPB	fibrinopeptide B
GP Ib	glycoprotein Ib
GP IIb/IIIa	glycoprotein IIb/IIIa
HCl	hydrochloric acid
HMW	high molecular weight
LAD	left anterior descending (coronary) artery
LBBB	left bundle branch block
LMWH	low molecular weight heparin
LV	left ventricular/ ventricle
Mab	monoclonal antibody
mP-selectin	membrane P-selectin
n-PA	novel plasminogen activator [lanoteplase]
NIBSC	National Institute of Biological Standards and Control
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
NSTEMI	non-ST elevation myocardial infarction
PAI-1	plasminogen activator inhibitor type-1
PAR-1	protease-activated receptor 1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PF4	platelet factor 4
PGI_2	prostaglandin I_2

PCI	percutaneous coronary intervention
PEG-Sak	pegulated staphylokinase
RCA	right (coronary) artery
r-PA	recombinant plasminogen activator [reteplase]
rscu-PA	recombinant single-chain urinary-type plasminogen activator
rt-PA	recombinant tissue-type plasminogen activator
r-pro-UK	recombinant glycosylated pro-urokinase
scu-PA	single-chain urinary-type plasminogen activator
tcu-PA	two-chain urinary-type plasminogen activator
SK	streptokinase
SF	soluble fibrin
sP-selectin	soluble P-selectin
Sak	recombinant staphylokinase
STEMI	ST elevation myocardial infarction
TAFI	thrombin-activatable fibrinolysis inhibitor
TAFIa	activated thrombin-activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin [complex]
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIMI	thrombolysis in myocardial infarction
TNKase	Tenecteplase
tcu-PA	two-chain urinary-type plasminogen activator
t-PA	tissue-type plasminogen activator
TSP	thrombospondin protein
T80	Tween 80
TxA_2	thromboxane A_2
UA	unstable angina
UFH	unfractionated heparin
UK	urokinase
u-PA	urinary-type plasminogen activator
vWF	von Willebrand factor

CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

CHAPTER 1: INTRODUCTION

1.1 CORONARY THROMBOSIS

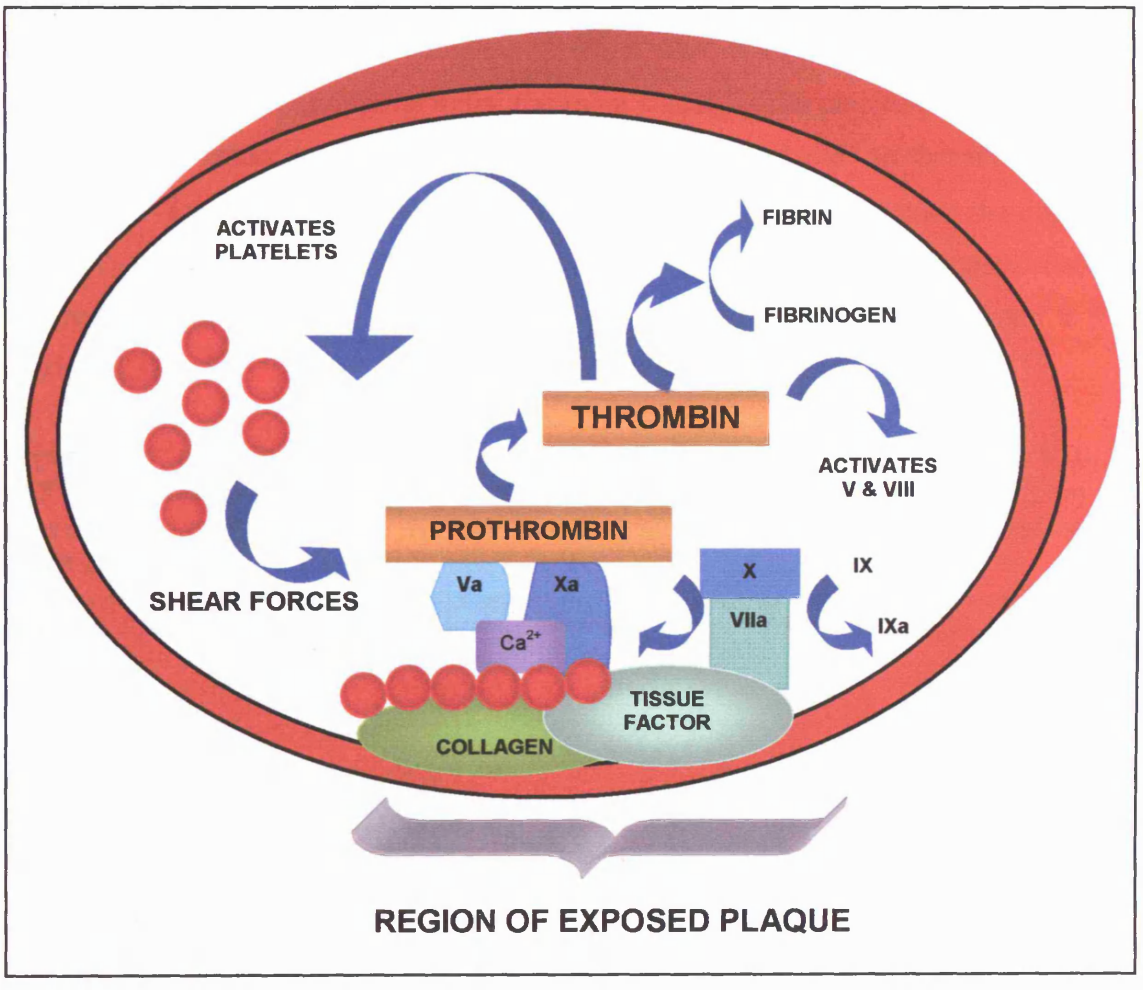
Coronary heart disease (CHD) is one of the leading causes of death in the United Kingdom. In 2002, 117,476 deaths were attributed to coronary heart disease (Office for National Statistics, 2003). This accounts for approximately one in five deaths in men, and one in six deaths in women. It is estimated that CHD currently costs the British economy at least £7,055 million per year [1]. The bulk of deaths from CHD are precipitated by the acute occlusion of a coronary artery by thrombus following plaque disruption [2]. Despite dramatic improvements in the treatment of acute ST-segment elevation myocardial infarction (STEMI), which is of primary interest in this study, approximately 1 in 10 patients still die of this disease [3].

It is advanced atheromatous lesions that usually form the substrate for coronary thrombosis. The lipid core of the atheromatous plaque contains apoptotic cells (such as dead smooth muscle cells and macrophages), mesenchymal cells, and abundant free cholesterol crystals [4]. The lipid core of these lesions is rich in tissue factor, which, upon plaque rupture and exposure to the circulating blood, initiates the coagulation cascade and thrombin generation [5]. Tissue factor is associated with and probably generated by macrophages within the plaque [6,7]. The degree of plaque disruption (erosion, fissure, or ulceration) and the amount of stenosis caused by the disrupted plaque and the subsequent mural thrombus are key factors for determining thrombogenicity at the local arterial site. When deep ulceration occurs, tissue factor from the atherosclerotic lipid core is exposed to flowing blood and released into the lumen [8,9]. As will be explained later, tissue factor is a potent initiator of coagulation which leads to conversion of prothrombin to thrombin. The high tissue factor activity contributes to the procoagulant activity of disrupted atherosclerotic lesions and the superimposed mural thrombi [8]. Disruption of advanced plaques with exposure of the highly thrombotic lipid core to the flowing blood triggers the formation of thrombi up to 6 times larger than thrombi generated by exposure of other components of the arterial wall [4].

The vulnerability of a plaque to rupture includes features that are related to size of the lipid pool, thickness of the fibrous cap, content and metabolic activity of lipids [10,11], and the activity and density of macrophages [12,13]. The external physical forces that expose the vessel wall to blood flow at different shear rates also influence the occurrence and progression of plaque disruption, thrombosis, and arteriosclerosis [14-17].

In order to fully comprehend the processes which develop following plaque rupture, it is essential to have an understanding of the haemostatic system which results in coagulation and subsequently fibrinolysis.

Figure 1-1: Overview of coagulation: activation of the coagulation cascade as a consequence of plaque rupture and exposure of tissue factor and collagen. Tissue factor forms a complex with factor VIIa that induces activation of factors IX and X. Factor Xa associates with factor Va on platelet membranes and, as part of the 'prothrombinase complex', activates prothrombin to thrombin. Platelets are activated by thrombin, exposed collagen and tissue factor, and shear forces induced by coronary stenosis.



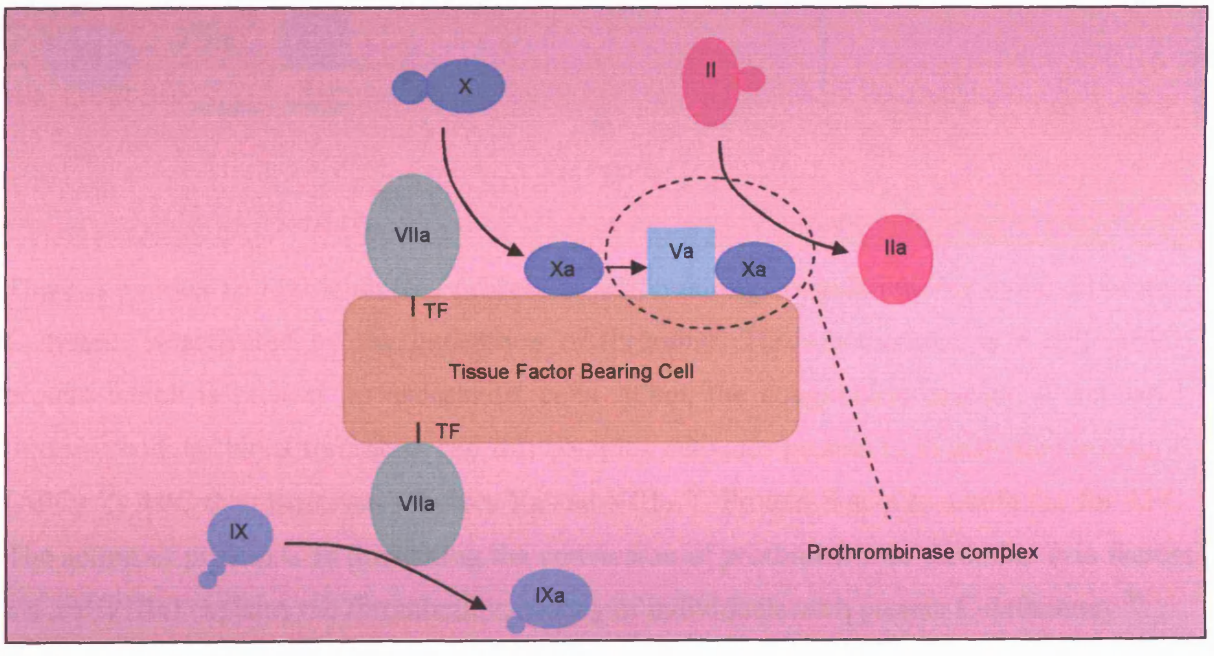
1.2 THE COAGULATION CASCADE

The ultimate consequence of coagulation is the conversion of prothrombin to thrombin and subsequently conversion of soluble fibrinogen to insoluble fibrin (**Figure 1-1**). In order to achieve this final step, a variety of complex systems interact with one another. The division of the coagulation cascade into *intrinsic* (factors XII, XI, and IX) and *extrinsic* (tissue factor mediated) pathways is artificial and simplistic, as will become clear [18,19].

Tissue factor (TF) is an integral membrane protein of many cell types, particularly in the vessel wall, and is the principal determinant of coagulation in vivo. Expressed following vessel injury, TF binds factor VII which is rapidly activated [20] to VIIa by coagulation proteases [21] and by noncoagulation proteases, depending on the cellular location of TF [22]. The factor VIIa–TF complex can activate factors X and IX. The activated forms of these two proteins play very different and distinct roles in subsequent coagulation reactions.

Factor Xa can activate plasma factor V on the TF cell [23]. The factor Xa that remains on the cell surface can then combine with factor Va to produce small amounts of thrombin (IIa) via the so-called prothrombinase complex [24]. Even this small amount of thrombin plays a significant role in amplifying the initial thrombin signal [25]. The conversion of prothrombin to thrombin is enhanced ~278,000-fold in the presence of the prothrombinase complex compared to the reaction involving factor Xa alone [26]. This helps to explain the importance of factor Va which acts as the protein cofactor in the prothrombinase complex. Factor V is released from platelets upon activation by thrombin [27]. Up to 25% of blood factor V is stored within platelets [28].

Figure 1-2: Initiation of coagulation: in the initiation of coagulation, factor VIIa bound to TF activates factor IX and also factor X. Factor Xa then activates factor V on the TF-bearing cell, complexes with factor Va (the prothrombinase complex), and converts a small amount of II to IIa.

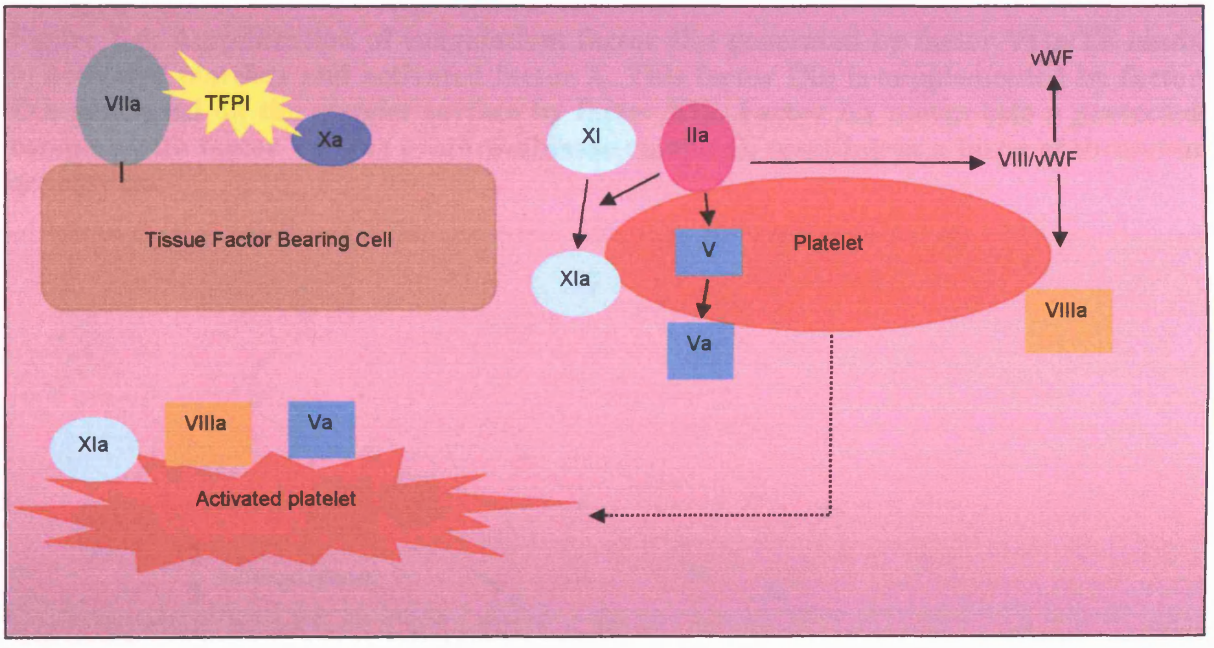


If factor Xa diffuses from the protective environment of the cell surface, it is rapidly inhibited by TF pathway inhibitor (TFPI) [29] or antithrombin III (AT-III). AT-III is normally present in plasma at over twice the concentration of any potential target coagulation enzyme generated

by the TF pathway. AT-III is an effective neutraliser of all the procoagulant serine proteases [30]. The targets of AT-III are primarily the uncomplexed enzyme products of these reactions, including thrombin.

The initial factor VIIa-TF complex is subsequently also inhibited by the action of TFPI in complex with factor Xa [31]. About 20% of TFPI circulates in plasma, and about 80% is normally bound to endothelium.

Figure 1-3: Propagation of coagulation: the small amount of initial IIa activates platelets, causing release of alpha granule contents including factor V, activates factor V, activates factor XI, and activates factor VIII by cleaving it from von Willebrand factor (vWF). Cofactors bind to the platelet surface before their respective enzymes. The factor VIIa-TF complex is shut down through the action of the TF pathway inhibitor (TFPI) in complex with factor Xa.



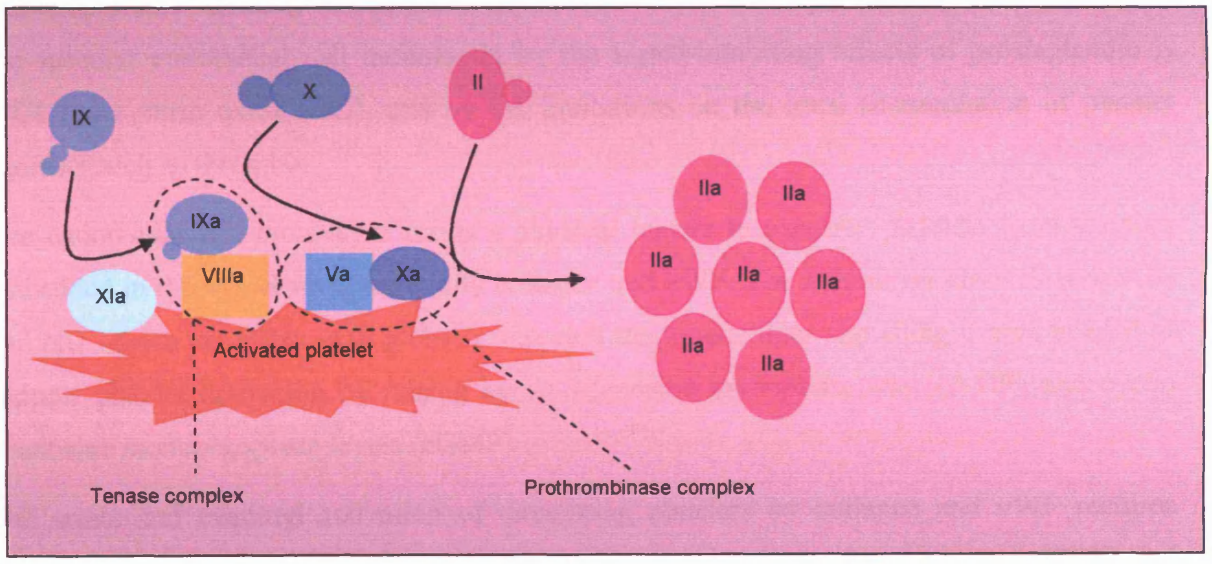
There is another natural inhibitory process in action during coagulation: the dynamic protein C system is activated by the generation of thrombin. Thrombomodulin is a cell-surface protein which is present on endothelial cells. When the coagulation cascade is activated, thrombomodulin binds thrombin, and this complex activates protein C to activated protein C (APC) [32]. APC then inactivates factors Va and VIIIa [33]. Protein S acts as a cofactor for APC. The action of protein C in preventing the conversion of prothrombin to thrombin (via factors Va and VIIIa) explains the thrombotic tendency of individuals with protein C deficiency [34].

In the initial phase of coagulation (**Figure 1-2**) the small amount of initial thrombin binds to platelets that have adhered to extravascular matrix components at the site of arterial injury, mediated in part by the binding of von Willebrand factor (vWF) to collagen [35]. vWF is a

protein synthesised by endothelial cells and is integral in the platelet/collagen and platelet/platelet interactions that occur at the site of vascular injury.

The process of binding to these matrix proteins partially activates platelets and also localises them near the site of TF exposure. Thrombin generated further activates platelets in an amplified manner when acting in synergy along with collagen [36]. Thrombin activation results in platelet granule release with production of partially active factor V from platelet α granules [36,37]. Co-stimulation of the platelet collagen receptor results in a proportion of platelets expressing high levels of factor V [36]. Thrombin then activates factor V (**Figure 1-3**) [38]. Thrombin also releases factor VIII from vWF [38], and activates factor XI bound to the platelet surface [39,40]. The result is an activated platelet which is bound to cofactors Va, VIIIa and factor XIa.

Figure 1-4: Amplification of coagulation: factor IXa generated by factor VIIa/TF binds to activated platelets and activated factor X. This factor IXa is supplemented by factor IXa generated on the platelet surface by factor XIa. Factor Xa moves into a protected complex with factor Va (the prothrombinase complex), resulting in a burst of thrombin generation.



In the final phase of coagulation, factor IXa complexes with VIIIa when the former reaches the platelet surface (**Figure 1-4**). Factor XIa bound on the platelet can provide additional factor IXa directly [39]. Factor X is recruited to the activated platelet surface and is activated by the factor VIIIa-IXa complex (the tenase complex). This allows factor Xa to move directly into a complex with factor Va [41]. In the presence of prothrombin, factor Xa is protected from inhibition by TFPI [42] and antithrombin [43]. The platelet surface prothrombinase (Xa/Va) complex generates a large amount of thrombin sufficient to convert fibrinogen into soluble

fibrin monomers. The soluble fibrin is then converted into insoluble crosslinked fibrin clot by the action of transglutamidase (factor XIIIa) under the influence of thrombin.

The activation of coagulation and generation of thrombin can be measured and monitored in a variety of ways. The conversion of prothrombin to thrombin results in the release of two fragments from the amino-terminal end of prothrombin: prothrombin fragments F_1 and F_2 [44]. The plasma levels of F_{1+2} reflect the enzymatic activity of Factor Xa on prothrombin, and thus thrombin generation. An additional method of determining thrombin generation is to measure the complex formed between thrombin and its natural inhibitor antithrombin III (Thrombin-antithrombin complex: TAT) [45]. In this study, thrombin production was reflected by the measurement of soluble fibrin levels.

1.2.1 PLATELETS

The central role of platelets in the initiation of thrombus formation following plaque rupture justifies a closer look at the processes involved in platelet activation (**Figure 1-5**).

Human platelets normally circulate in a quiescent state. They are prevented from activating by the vascular endothelial call monolayer, by the signal-inhibiting effects of prostaglandin I_2 (PGI_2) and nitric oxide (NO), and by the limitations on the local accumulation of platelet agonists such as thrombin.

The endothelial cell monolayer forms a physical barrier to separate platelets from agonists embedded in the vessel wall, especially collagen and vWF. The monolayer also releases PGI_2 and NO whose net effect is to globally suppress the intracellular signalling events needed to support platelet activation by raising cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate levels (cGMP).

The arrest and eventual activation of circulating platelets by collagen and vWF requires several receptors on the platelet surface. Some receptors bind directly to collagen (GP VI and the integrin $\alpha_2\beta_1$) and some bind to collagen indirectly via vWF (the GP Ibα/IX/V complex and the integrin $\alpha_{IIb}\beta_3$). $\alpha_{IIb}\beta_3$ is more widely known as glycoprotein IIb/IIIa (GP IIb/IIIa). The consequence of collagen binding is to initiate the release of bound calcium from the dense tubular system within the platelet [46]. The increase in free ionic calcium levels within the platelet is central to the process of platelet activation. The result is activation of protein C kinase, causing protein phosphorylation, granule secretion, and conformational change ('inside-out' signalling) in the GP IIb/IIIa receptor to the form that binds fibrinogen and, to a

lesser extent, soluble vWF [47]. The conformational change in the GP IIb/IIIa receptor allows more platelet/platelet binding via bridges of fibrinogen and vWF.

Expression of the GP IIb/IIIa receptor can be measured employing flow cytometry and monoclonal antibodies directed against the receptor. This method was used in this study in order to assess platelet activation in patients following myocardial infarction and thrombolytic therapy.

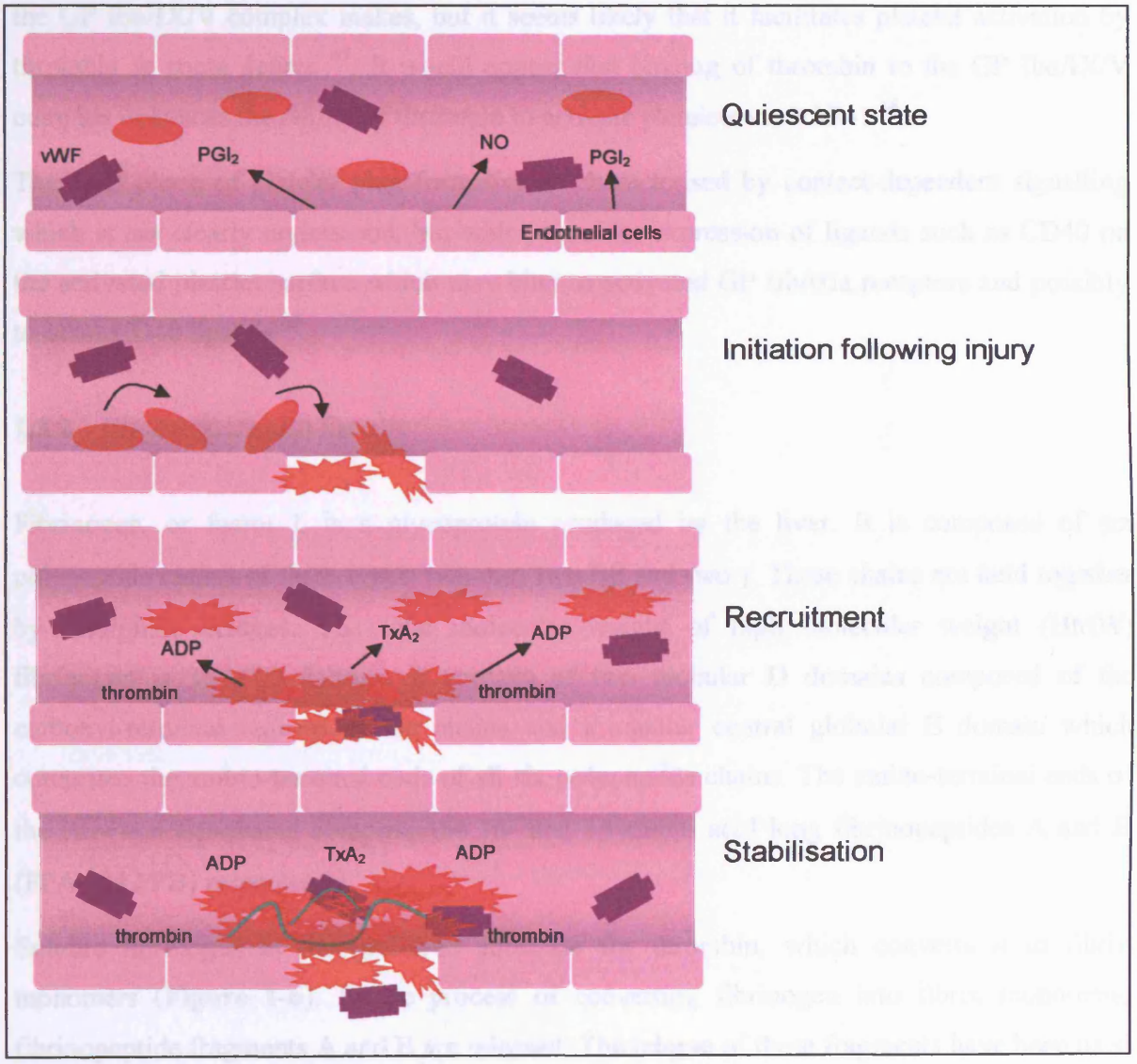
The GP Ibα/IX/V complex binds to vWF which is circulating in association with factor VIII [48]. This brings factor VIII and vWF in close proximity to thrombin on the platelet surface allowing for more efficient activation of factor VIII and release of factor VIIIa from vWF to the platelet surface.

After the initial recruitment of platelets, further activated platelets accumulate on top of the initial monolayer of platelets bound to collagen. Crucial to this accumulation is the presence of platelet surface receptors that can respond to soluble agonists such as thrombin, ADP and thromboxane A_2 (TxA_2). Further extension of the platelet plug requires the activation of the GP IIb/IIIa receptor as outlined above.

Upon their activation, platelets secrete a variety of contents from α granules and dense bodies. Dense granules contain ATP, ADP, ionised calcium (factor IV), magnesium, adrenaline, pyrophosphates and serotonin. ADP is the most important constituent as it promotes recruitment and activation of additional platelets to the site of vascular injury. α granules contain platelet-derived growth factor (PDGF), heparin neutralising factor (platelet factor-4: PF4), protein S, plasminogen activator inhibitor (PAI-1), fibronectin, β thromboglobulin, thrombospondin protein (TSP), albumin, fibrinogen (factor I), factor V, factor XIII and vWF.

Thrombospondin is a large glycoprotein released from α granules that appears to enhance platelet adherence and aggregation by attaching to corresponding platelet receptors. PF4 and β thromboglobulin prevent heparin (released by basophils and mast cells) neutralising thrombin and other blood clotting enzymes. Both PF4 [49-51] and β thromboglobulin [51-54] have been used as markers of platelet activation in the setting of coronary artery disease. Unfortunately, neither of these markers necessarily represents true platelet activation in vivo. The assays are prone to considerable error, and other disease states can cause interference [55]. β thromboglobulin is raised in hypercholesterolaemia [56], and in diabetes mellitus [56-58].

Figure 1-5: Stages in platelet plug formation. In the quiescent stage, platelets are prevented from activation by release of inhibitors including PGI_2 and NO released from endothelium, and the inability of normal plasma vWF to bind spontaneously to the platelet surface. The development of the plug can be initiated by the exposure of collagen and vWF in the vessel wall, and by the local generation of thrombin. Rolling platelets adhere and spread on the collagen matrix, forming a monolayer of activated platelets that can act as a surface for subsequent recruitment of platelets by thrombin, ADP and TxA_2.



When activated, platelet granule membrane proteins translocate to the cell surface. One such protein acts as a marker for activated platelets: P-selectin. Initially identified as GMP-140, P-selectin can be targeted by monoclonal antibodies in order to identify activated, degranulated platelets. With flow cytometry this marker can be used to differentiate resting platelets from activated platelets in whole blood [59,60]. A soluble from of P-selectin circulates in plasma [61].

The platelet receptors which respond to thrombin were first identified in 1990 as the G protein-coupled receptor (GPCR) [62,63]. Now known as protease-activated receptor 1 (PAR-1), stimulation with thrombin results in activation of the G protein: G_q [64], the raising of cytosolic Ca^{2+} and inhibition of cGMP formation. Platelets also release ADP and TxA_2 which bind in turn with their own receptors on the platelet surface. Another apparently less effective thrombin receptor (PAR-4) has also been identified [65,66]. It is not yet clear what contribution the GP Ibα/IX/V complex makes, but it seems likely that it facilitates platelet activation by thrombin to some degree [67]. It would appear that binding of thrombin to the GP Ibα/IX/V complex enhances the ability of thrombin to activate platelets via PAR-1 [68].

The final phase of platelet plug formation is characterised by contact-dependent signalling which is not clearly understood, but which involves expression of ligands such as CD40 on the activated platelet surface which may bind to activated GP IIb/IIIa receptors and possibly to other CD40 ligands [69].

1.2.2 FIBRINOGEN AND FIBRIN

Fibrinogen, or factor I, is a glycoprotein produced by the liver. It is composed of six polypeptide chains of three types: two Aα, two Bβ and two γ. These chains are held together by disulphide bridges. The total molecular weight of high molecular weight (HMW) fibrinogen is 340,000 daltons. It consists of two globular D domains composed of the carboxyl-terminal regions of the chains and a smaller central globular E domain which comprises the amino-terminal ends of all six polypeptide chains. The amino-terminal ends of the Aα- and Bβ-chains comprise the 16- and 14-amino acid long fibrinopeptides A and B (FPA and FPB) respectively.

Soluble fibrinogen is the preferred substrate for thrombin, which converts it to fibrin monomers (**Figure 1-6**). In the process of converting fibrinogen into fibrin monomers, fibrinopeptide fragments A and B are released. The release of these fragments have been used as a marker of thrombin activity in several studies of patients with coronary artery disease [52,70-74].

Removal of FPA and FPB expose polymerisation sites within the E domain such that the E domains associate tightly and non-covalently with sites on the D domains of neighbouring molecules to initiate the formation of fibrin polymers. At the same time, factor XIII (approximately half of which originates in platelets) is activated to factor XIIIa which catalyses the formation of covalent bonds between D domains of adjacent fibrin monomers [75].

The result is a fibrin mesh which resembles a three dimensional spider's web. Fibrin polymers themselves are an important cofactor in the generation of factor XIIIa [76].

Estimation of plasma fibrinogen is performed in one of three main ways. The thrombin time is a crude method but is prolonged when very low levels of fibrinogen are present. The Clauss method [77] is the most frequently used and measures the time between the addition of thrombin and clotting. It measures clottable "functional" fibrinogen but its accuracy is impaired giving spuriously low fibrinogen levels when there are elevated levels of fibrin degradation products, as is observed during thrombolysis [78]. "Intact fibrinogen" can be measured by enzyme immunoassay [79]. Elevated levels of fibrinogen have been associated with an increased risk of myocardial infarction [80,81]. Soluble fibrin, which reflects thrombin activity and fibrinogen consumption, can also be assayed employing monoclonal antibodies [82,83]. Levels of soluble fibrin and intact fibrinogen were both measured in this study employing monoclonal antibodies.

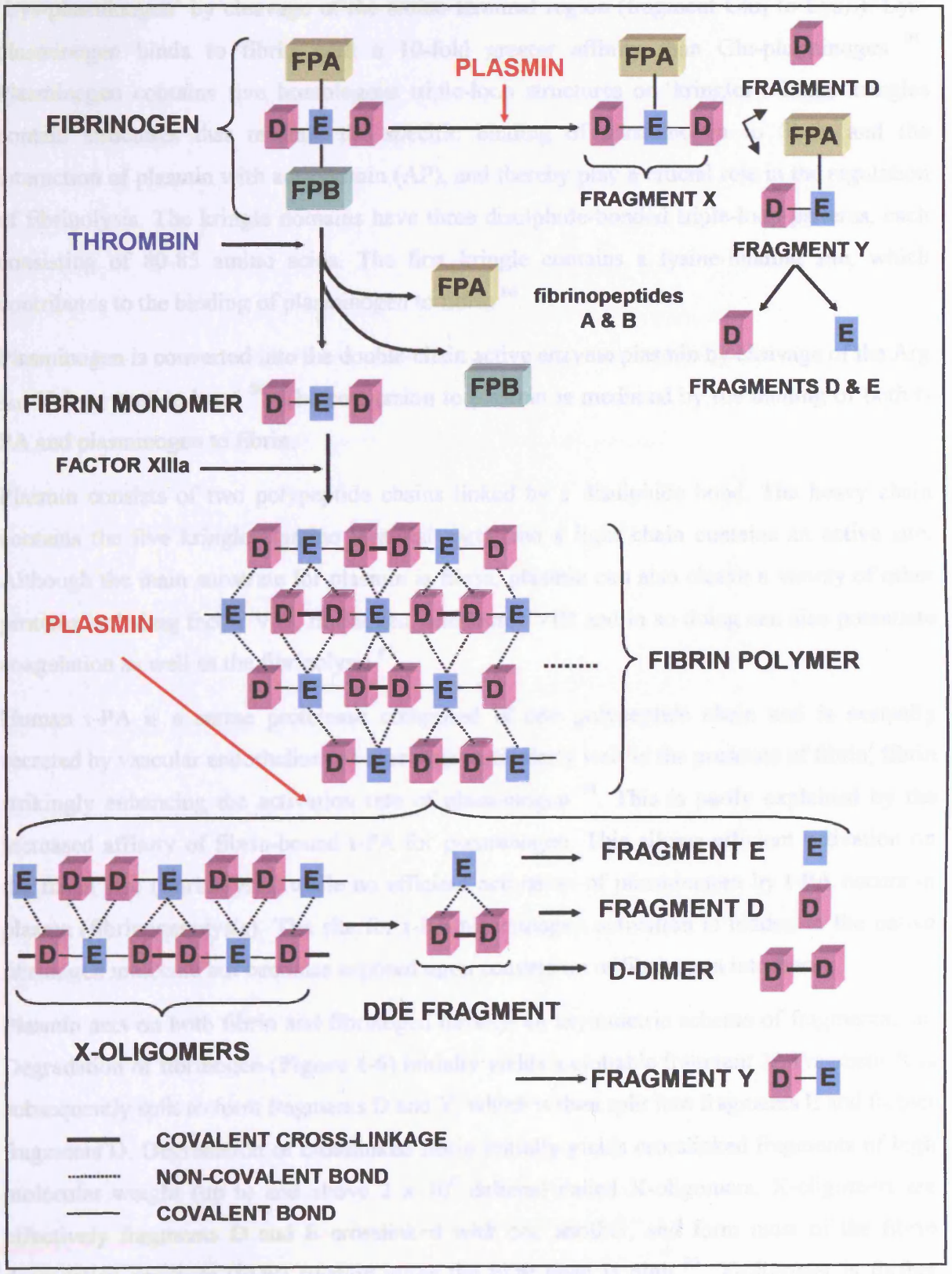
1.3 FIBRINOLYSIS

The formation of a blood clot in response to a vascular lesion is a physiological localised process limited by antithrombotic mechanisms that are simultaneously triggered and depend on a normally functioning endothelium. These mechanisms include:

- Signal transduction pathways mediated by nitric oxide and prostacyclin that produce endothelium-dependent vascular relaxation and inhibition of platelet aggregation [84].
- Inhibition of fibrin deposition by serine proteinase inhibitors of activated coagulation factors and by the protein C-protein S system that neutralise factors Va and VIIIa [32].
- The fibrinolytic system that eliminates excess fibrin [85,86].

Fibrinolysis essentially co-exists with coagulation - at the plasma fibrin or plasma membrane interface. An inactive precursor (plasminogen) is transformed into active enzyme (plasmin) by proteolytic processing mediated by serine proteinases (tissue-type plasminogen activator: t-PA, and urinary-type plasminogen activator: u-PA) [87]. The active enzyme remains surface bound via its kringle domains and free from inhibition by proteins circulating in the blood. Plasminogen and t-PA react optimally on the surface of fibrin polymers. t-PA binds strongly to fibrin [88], whereas plasminogen binds loosely, this latter binding being enhanced by partial degradation of the plasminogen [89].

Figure 1-6: Fibrin polymerisation and degradation.



Plasminogen is secreted by the liver as a single-chain glycoprotein of 791 amino acids with glutamic acid as its amino-terminal residue [90]. This 'Glu-plasminogen' circulates in plasma at

a concentration of 1·5-2·0μM (0·2-0·3g/l). It is easily converted to modified forms called 'Lys-plasminogen' by cleavage of the amino terminal region (fragment Glu_1 to Lys_{77}). Lys-plasminogen binds to fibrin with a 10-fold greater affinity than Glu-plasminogen [91]. Plasminogen contains five homologous triple-loop structures or 'kringles'. These kringles contain structures that mediate the specific binding of plasminogen to fibrin and the interaction of plasmin with antiplasmin (AP), and thereby play a crucial role in the regulation of fibrinolysis. The kringle domains have three disulphide-bonded triple-loop patterns, each consisting of 80-85 amino acids. The first kringle contains a lysine-binding site, which contributes to the binding of plasminogen to fibrin [86].

Plasminogen is converted into the double-chain active enzyme plasmin by cleavage of the Arg_{561}–Val_{562} peptide bond [86]. The conversion to plasmin is mediated by the binding of both t-PA and plasminogen to fibrin.

Plasmin consists of two polypeptide chains linked by a disulphide bond. The heavy chain contains the five kringles (amino terminal part), and a light chain contains an active site. Although the main substrate for plasmin is fibrin, plasmin can also cleave a variety of other proteins including factor V [92], fibrinogen, and factor VIII and in so doing can also potentiate coagulation as well as the fibrinolysis [93].

Human t-PA is a serine proteinase composed of one polypeptide chain and is normally secreted by vascular endothelium. It functions particularly well in the presence of fibrin, fibrin strikingly enhancing the activation rate of plasminogen [94]. This is partly explained by the increased affinity of fibrin-bound t-PA for plasminogen. This allows efficient activation on the fibrin clot (fibrinolysis) while no efficient activation of plasminogen by t-PA occurs in plasma (fibrinogenolysis). The site for t-PA plasminogen activation is hidden in the native fibrinogen molecule but becomes exposed upon conversion of fibrinogen into fibrin.

Plasmin acts on both fibrin and fibrinogen through an asymmetric scheme of fragmentation. Degradation of fibrinogen (**Figure 1-6**) initially yields a clottable fragment X. Fragment X is subsequently split to form fragments D and Y, which is then split into fragments E and further fragments D. Degradation of crosslinked fibrin initially yields crosslinked fragments of high molecular weight (up to and above 2×10^6 daltons) called X-oligomers. X-oligomers are effectively fragments D and E crosslinked with one another, and form most of the fibrin degradation products (FDP) fraction when the FDP level is high [95]. X-oligomer is further degraded to smaller intermediate fragments (DDE, YD, DXD/YY, etc.).

Finally D-dimer fragments [96] are formed along with fragment Y. D-dimer fragments are further cleaved to form individual D fragments. All of these fragments of fibrinogen and fibrin inhibit clotting to an extent in vitro.

While free D and E fragments and X and Y fragments in the plasma can be the result of plasmin degradation of fibrinogen or fibrin monomers, the presence of D-dimer or X-oligomers in the plasma signifies plasmin degradation of crosslinked fibrin. Fibrin degradation products can be assayed by immunoassay. Fragment X can be measured indirectly by the plasma concentration of the specific 42 amino acid peptide Bβ1-42 released from the amino end of the Bβ chain. The high molecular weight crosslinked FDP (X-oligomers) can be detected with the monoclonal antibody Mab 123 (NIBn123) developed in Dr Patrick Gaffney's laboratory at the NIBSC [97]. This monoclonal antibody reacts with high molecular weight crosslinked fibrin fragments but not non-crosslinked fragments. X-oligomer levels were measured in this study. D-dimer measurements are well established in clinical practice and can be achieved by ELISA or latex column methods.

1.3.1 INHIBITORS OF FIBRINOLYSIS

There are a number of inhibitors of the fibrinolytic system (**Figure 1-7**). Their interactions with fibrinolysis are illustrated below.

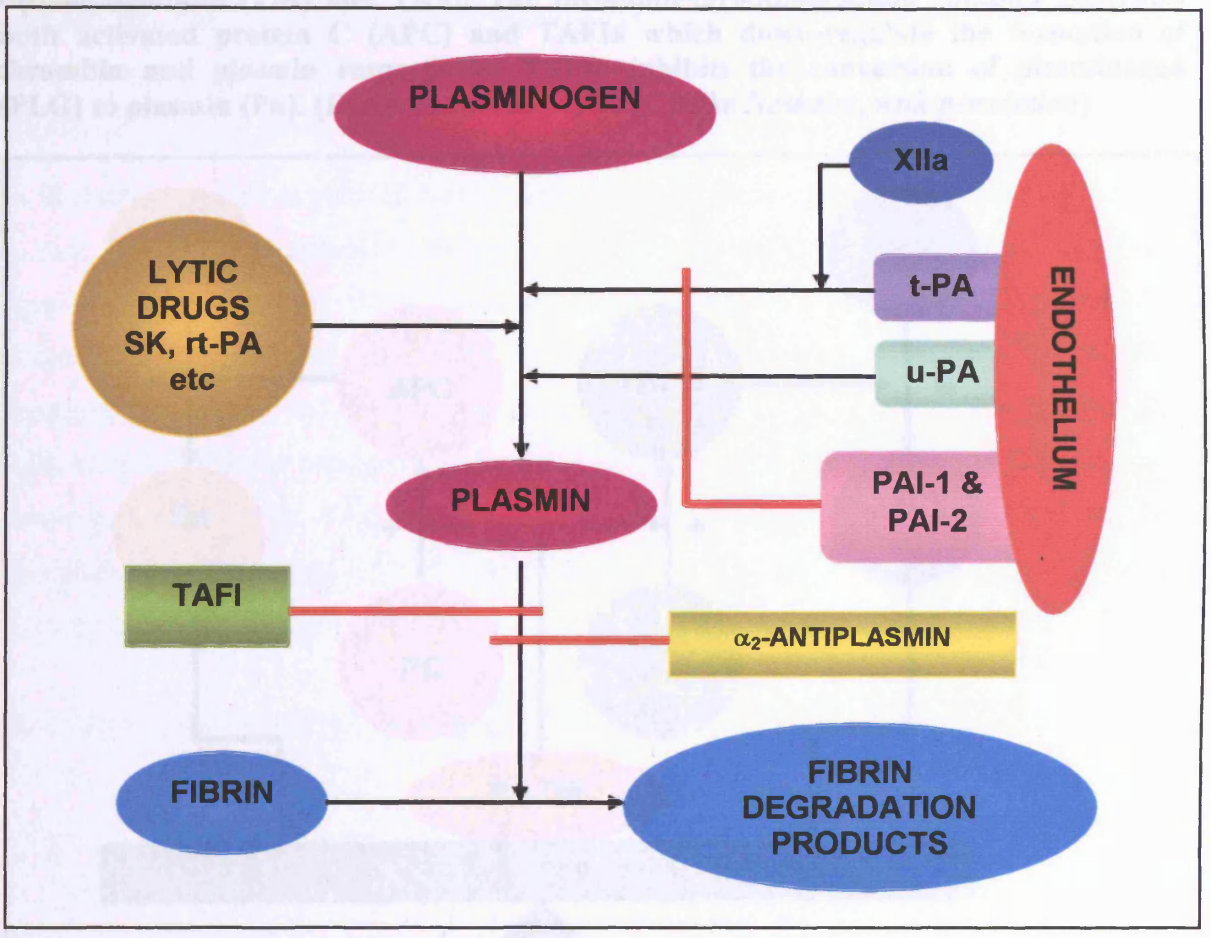
1.3.1.1 ALPHA $(\alpha)_2$-ANTIPLASMIN (AP)

There is a specific inhibitor of plasmin, α_2-antiplasmin (AP), which attaches at the lysine binding site to form a 1:1 complex with freely circulating plasmin [98]. This single-chain glycoprotein can only inhibit circulating plasmin and not fibrin-bound plasmin. It circulates in normal plasma at a concentration of about 1μM/l (70mg/l). AP belongs to the serine proteinase inhibitor protein family (serpins). This inhibitor is also crosslinked rapidly to the α-chains of fibrin by factor XIII [99], and it is argued that in this immobilised condition AP prevents the clot from degradation by plasmin [100]. α_2-macroglobulin is another plasmin inhibitor which tends to act when a large plasmin excess is formed.

1.3.1.2 Plasminogen Activator Inhibitor Type-1 (PAI-1)

Another physiological inhibitor of fibrinolysis is plasminogen activator inhibitor type-1 (PAI-1), which acts at the level of the plasminogen activators. This glycoprotein is secreted by endothelial cells and the liver. It can neutralise circulating but not fibrin-bound t-PA [101]. Platelets are another source of PAI-1 in the circulation [102,103]. It is not altogether clear whether PAI-1 plays a significant role in neutralising the activity of t-PA/plasminogen in the circulation [104]. It may be that α_2-macroglobulin protects t-PA from inhibition from PAI-1 [105].

Figure 1-7: Activators and inhibitors of the fibrinolytic system.



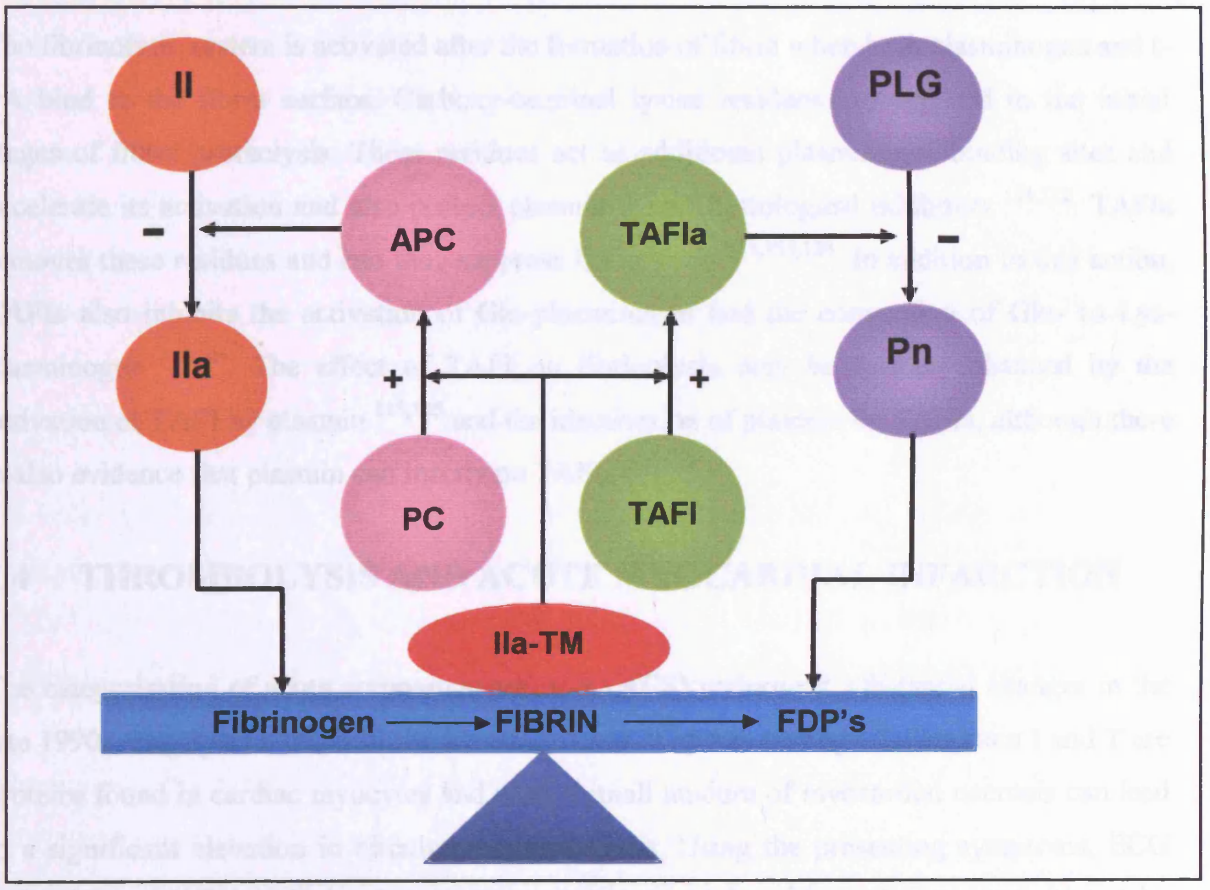
1.3.1.3 Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)

Plasma carboxypeptidase B (CPB) is an α-globulin which possesses a functional specificity similar to that of bovine pancreatic CPB. It is a proteolytic enzyme produced by the liver, capable of hydrolysing peptide bonds to release certain carboxyl-terminal basic amino acids

(Lys, Arg) from peptides and proteins [106]. It consists of a single polypeptide chain. CPB has a specific precursor, procarboxypeptidase B, from which it is formed by tryptic activation [107].

Forms of CPB probably occur in the cellular secretions of the pancreas of most vertebrates. Activity toward CPB substrates has been detected in the body fluids urine [108,109], plasma [110], lymph [108], kidney cortex [111] and spleen [112]. One form of carboxypeptidase, carboxypeptidase N (CPN) found in plasma inactivates the complement derived anaphylatoxins C3a, and C5a, as well as bradykinin and kallidin.

Figure 1-8: Regulation of coagulation and fibrinolysis: a model depicting a connection between the coagulation and fibrinolytic cascades established by thrombin (IIa), thrombomodulin (TM) and TAFI. The thrombin-thrombomodulin complex generates both activated protein C (APC) and TAFIa which down-regulate the formation of thrombin and plasmin respectively. TAFIa inhibits the conversion of plasminogen (PLG) to plasmin (Pn). (*Diagram courtesy of Prof. Mike Nesheim, with permission*)



Recently a thrombin-activatable fibrinolysis inhibitor (TAFI) has been recognised which appears to be a precursor of plasma CPB (procarboxypeptidase) and which has CPB-like activity [113]. TAFI is synthesised in the liver as a prepropeptide consisting of 432 amino acids. Its plasma concentration has been estimated at 4-15μg/ml [114,115], but figures still differ widely. It is thought that it circulates in plasma in complex with plasminogen [116], although this has not

been proven. TAFI is also found in circulating platelets, and it is thought that the different glycosylation of platelet-derived TAFI (compared with plasma-derived TAFI) suggests that platelet-derived TAFI is synthesized in the megakaryocyte [117].

The fact that TAFI has a greater affinity for Lys-plasminogen may suggest that a TAFI-plasminogen complex only forms when plasminogen is activated. The complex may thus prevent plasmin formation in the fluid phase [116].

TAFI is converted to activated TAFI (TAFIa) by proteolysis of Arg $_{92}$ - Arg $_{93}$ [113,116,118]. Conversion is best achieved by the combined action of thrombin and thrombomodulin [119]. The contribution of thrombomodulin appears to enhance the activation achieved by thrombin by a factor of 1250. Calcium also appears to be essential to successful activation of TAFI [120]. It can be seen with reference to activation of protein C, that the thrombomodulin thrombin complex seems to be central in down-regulating both coagulation and fibrinolysis (**Figure 1-8**).

The fibrinolytic system is activated after the formation of fibrin when both plasminogen and t-PA bind to the fibrin surface. Carboxy-terminal lysine residues are exposed in the initial stages of fibrin proteolysis. These residues act as additional plasminogen binding sites and accelerate its activation and also protect plasmin from physiological inhibitors [121,122]. TAFIa removes these residues and can thus suppress fibrinolysis [113,123,124]. In addition to this action, TAFIa also inhibits the activation of Glu-plasminogen and the conversion of Glu- to Lys-plasminogen [113,124]. The effect of TAFI on fibrinolysis may be further enhanced by the activation of TAFI by plasmin [118,125] and the inactivation of plasmin by TAFIa, although there is also evidence that plasmin can inactivate TAFIa [124,125].

1.4 THROMBOLYSIS AND ACUTE MYOCARDIAL INFARCTION

The categorisation of acute coronary syndromes (ACS) underwent substantial changes in the late 1990s, largely as a result of the introduction of Troponin testing [126]. Troponin I and T are proteins found in cardiac myocytes and even a small amount of myocardial necrosis can lead to a significant elevation in circulating blood levels. Using the presenting symptoms, ECG and subsequent Troponin level, patients presenting acutely with cardiac chest pain can be grouped as follows:

ST elevation MI (STEMI): Patients presenting with cardiac-sounding chest pain with persistent ST segment elevation (or new LBBB) on their ECG. Subsequent Troponin levels will be elevated along with the creatine kinase (CK).

Non-ST elevation MI (NSTEMI): Patients presenting with cardiac-sounding chest pain. ECG may show ST segment depression, T wave inversion or may be normal. Subsequent Troponin levels will be elevated. CK may be elevated.

Unstable angina (UA): Patients presenting with cardiac-sounding chest pain. ECG may show ST segment depression, T wave inversion or may be normal. Subsequent Troponin and CK levels will be normal.

The key decision to make when a patient is admitted is whether they require thrombolysis, based upon the history and the ECG. Patients presenting with new or presumed new ST-segment elevation of at least 0·1mV in at least two inferior leads (II, III, and aVF), in at least two adjacent precordial leads (V_1 to V_6), or in leads I and aVL are classified as STEMI and should be considered for thrombolysis. Patients with new or presumed new left bundle branch block are also included in this category.

The distinction between NSTEMI and UA is retrospective - it can only be made when the Troponin result is available. Patients presenting with cardiac sounding chest pain but no persistent ST elevation should be treated as UA, and can later be formally diagnosed as NSTEMI or UA once the result of the Troponin test is available. The overall approach to the management of UA and NSTEMI is the same, and neither condition is eligible for thrombolysis.

1.4.1 THROMBOLYSIS

Thrombolysis, the therapeutic method of activating the fibrinolytic system and clearing coronary artery thrombus, is currently the most widely adopted therapy for the treatment of STEMI. It is over forty years since the first clinical study, employing streptokinase (SK) for thrombolysis in myocardial infarction [127]. It has only been since the 1980's however, when the importance of thrombosis in the pathogenesis of ACS was recognised [128], that thrombolysis has become firmly established as the first-line treatment of STEMI [129]. The meta-analysis by the Fibrinolytic Therapy Trialists (FTT) Collaborative Group [130], showed that thrombolytic therapy is beneficial in all patients with suspected myocardial infarction admitted to hospital within 12 hours after the onset of symptoms and with ST elevation or new bundle branch block (but *not* ST depression or a normal ECG). Mortality rates in the thrombolysis group at 5 weeks were 10·1% versus 13·0% in the control group, an absolute difference of 2·9% (SD 0·4%; 2P < 0·00001). Only one observational study has questioned the

overall impact of thrombolytic therapy [131], citing the difference between the carefully selected patients of clinical trials and the general population of patients presenting with myocardial infarction.

The concept that the "open artery is best" forms the backbone of the management of myocardial infarction [132]. It requires that patency is achieved as early as possible and is maintained. In addition, there is evidence that outcome is improved by an open vessel independent of (and additive to) early perfusion [132]. Coronary artery patency and flow has traditionally been measured employing a method termed the TIMI flow grade (**Table 1-1**). This was a methodology first employed in 1985 [133], and has been used in all major trials of coronary flow in ACS ever since.

Table 1-1: Definition of TIMI Flow Grades.

Grade 0 (*no perfusion*): there is no antegrade flow beyond the point of occlusion
Grade 1 (*penetration without perfusion*): the contrast passes beyond the area of obstruction but 'hangs up' and fails to opacify the entire coronary bed distal to the obstruction for the duration of the cineangiographic filming sequence
Grade 2 (*partial perfusion*): contrast passes the obstruction and fills the coronary bed distally. The rate of entry into the distal bed or its clearance (or both) are slower than its entry into or clearance from comparable areas not perfused by the previously occluded vessel - e.g., the opposite coronary artery or the coronary bed proximal to the obstruction
Grade 3 (*complete perfusion*): Antegrade flow into the bed distal to the obstruction occurs as promptly as antegrade flow proximal to the obstruction, and clearance from the involved bed is as rapid as clearance from an uninvolved bed in the same vessel or the opposite artery

The higher the TIMI grade, the lower the mortality at 30 days [134], and TIMI grade 3 should be the goal. Indeed, it is increasingly suspected that even TIMI grade 2 flow arteries are effectively blocked [135], and may carry an increased risk of reocclusion [136], although this was not seen in the GUSTO study [137]. Long-term follow-up data from the European Cooperative study group suggests that the prognosis with TIMI grade 2 is as bad as that with TIMI grades 0 and 1 [138]. Similar results were found in four German multicentre studies [139].

Three major thrombolytic agents are currently in widespread use: SK – a first generation thrombolytic, recombinant tissue-type plasminogen activator (rt-PA) – a second generation thrombolytic, and Reteplase (r-PA) – a third generation thrombolytic.

1.4.1.1 STREPTOKINASE (KABIKINASE, STREPTASE)

Streptokinase (SK) is a single-chain polypeptide produced by several strains of β-haemolytic streptococci. It is an indirect activator that forms a 1:1 stoichiometric complex with native plasminogen. These complexes can then catalyse the activation/conversion of plasminogen to plasmin. The plasminogen-SK complex is subsequently converted to a plasmin-SK complex which can also convert plasminogen to plasmin. Plasmin itself is unable to convert plasminogen to plasmin. The complex activates both circulating and fibrin-bound plasminogen relatively indiscriminately and can cause extensive systemic activation of the fibrinolytic system [140]. This may result in degradation of several plasma proteins, including fibrinogen, factor V [92], and factor VIII. The plasmin(ogen)-SK complex is not inhibited by AP.

Mild allergic reactions are reported in about 4·4% of patients treated with SK versus 0·9% of controls [141]. Serum-sickness-like reactions and transient proteinuria have also been described. Hypotension requiring vasopressor support occurs in approximately 7 to 10% [141]. Unfortunately SK, with its bacterial origins, is immunogenic. Consequently, antibodies can develop against SK [142], potentially neutralising its activity on subsequent use [143]. High SK neutralisation titres persist for a long time after the use of SK as thrombolytic treatment [144,145]. It should therefore only be administered to an individual once.

1.4.1.2 RECOMBINANT TISSUE-PLASMINOGEN ACTIVATOR (RT-PA, ALTEPLASE , ACTILYSE, DUTEPLASE)

Using recombinant DNA techniques, tissue-type plasminogen activator (t-PA) has been manufactured (rt-PA) which activates plasminogen in the same way as native t-PA by cleaving the Arg $_{561}$-Val $_{562}$ peptide bond [146]. Although a 2-chain preparation was studied initially, the current clinically available agent, Alteplase, is single chain. It does not appear to stimulate the generation of antibodies and therefore can be used repeatedly in the same individual. In addition rt-PA is fibrin-specific and activates plasmin only in the presence of

fibrin. These features of rt-PA suggested that it would be the perfect thrombolytic, but this promise has not been fully realised in large clinical trials [147].

Arguments will continue as to whether SK or rt-PA is the most effective agent with two major trials [148] showing no significant difference, and one major study suggesting a possible slight advantage with front-loaded/accelerated (100mg over 90 minutes as opposed to 3 hours) rt-PA [149]. Benefit was most apparent in younger patients, those presenting early, and those with anterior STEMI [134]. There was however, a slightly higher risk of stroke with rt-PA compared with SK [148].

It has become clear that the currently available thrombolytic regimens are far from perfect, both in terms of achieving early/immediate patency and maintaining it. Analysis of major angiographic studies reveals the disappointing success rates of thrombolytic agents [150]. At 60 and 90 minutes, the rates of TIMI 3 flow are 57·1% and 63·2%, respectively, with accelerated rt-PA; 39·5% and 50·2% with standard-dose rt-PA; and 31·5% at 90 minutes with SK. It is likely that true patency rates are even less than this as the conventional 90 minute angiogram may well overestimate thrombolytic efficacy [151]. However in about 20% of patients there is a failure to reperfuse at all and, after discontinuation of the treatment, reocclusion occurs in 5% to 30% of patients [134,152], with reocclusion rates apparently being higher with rt-PA [153]. Overall, in one meta-analysis, reocclusion rates were reported as 11·8% with standard-dose rt-PA versus 6·0% for accelerated rt-PA and 4·2% for SK [150]. Reocclusion of the infarct-related artery is an important issue as it is associated with an adverse prognosis [152,154].

Overall, the second generation drugs have not shown any significant improvement in the management of STEMI. Although the systemic lytic state induced is less pronounced than that seen with SK, there still appears to be a higher risk of cerebral bleeding with rt-PA compared with SK (0·7% versus 0·55%).

Current thrombolytic therapy suffers from several major drawbacks. Because of lack of fibrin selectivity, bleeding is a significant problem with standard thrombolytic regimens, with intracranial haemorrhage being the most devastating. Overall intracranial haemorrhage resulting in death or disabling stroke occurs in 0·6% to 1·4% of patients receiving thrombolytic therapy, disproportionately affecting elderly individuals [130,155]. The search therefore continues for agents with greater fibrin selectivity so that fresh thrombus in coronary arteries is the only target of therapy.

Because of the short half-life of many agents, most require administration by infusion followed, in some cases, by antithrombotic treatment such as heparin. This introduces delays

in achieving reperfusion, and the administration of heparin and other adjunctive anticoagulant therapies complicates management and potentially increases the haemorrhagic risks. The advantages of agents with a prolonged half-life are that they can be given by bolus injection and therefore may achieve reperfusion sooner. In addition, the potential fibrinolytic effect may be longer lasting and may reduce the incidence of reocclusion.

Many agents are derived from re-engineered proteins derived from bacteria or other non-human sources. These agents have the potential to be immunogenic which may limit their initial efficacy, or at the very least may result in them being allowed to be administered to an individual only once.

1.4.1.3 RETEPLASE (r-PA, RAPILYSIN)

Recombinant plasminogen activator (Reteplase, r-PA) is a non-glycosylated truncated version of wild-type tissue plasminogen activator lacking kringle 1 and the finger domains. Unlike t-PA, Reteplase has a reduced fibrin affinity, a longer half-life, and greater thrombolytic potency [156]. The RAPID II study compared r-PA with front-loaded rt-PA and randomised 324 patients [157]. TIMI 3 flow rates at 90 minutes were 59·9% for r-PA compared to 45·2% for rt-PA. Although patency rates were clearly higher, this was not translated into survival benefit. The likely explanation is the recent recognition of microvascular obstruction.

Ito *et al* used contrast echocardiography and showed that at least one in four patients with TIMI 3 flow do not have tissue level perfusion [158]. Many groups have confirmed these findings. Porter *et al* have shown that end systolic cavity size and ejection fraction are impaired in patients with TIMI 3 flow but without tissue level perfusion [159]. Another study showed that evidence of microvascular obstruction carried a fourfold increase in adverse events including death, reinfarction, or the development of congestive heart failure [160].
The INJECT trial compared r-PA with SK in 6010 patients and revealed no significant difference between the two regimes in terms of mortality benefit, although there were significantly fewer cases of atrial fibrillation, asystole, cardiac shock, heart failure, and hypotension in the Reteplase group [161]. The GUSTO III study compared r-PA with accelerated rt-PA in 15 059 patients and found no significant difference between the two regimes [162]. It is unlikely that Reteplase offers any significant advantages over SK or rt-PA, although the bolus regimen has made Reteplase attractive for pre-hospital thrombolysis [163,164].

1.4.1.4 OTHER THROMBOLYTIC AGENTS

A number of other thrombolytic agents have been assessed or are in development. One of the first thrombolytic agents available for clinical use was ***urokinase***, otherwise called two-chain urinary-type plasminogen activator (tcu-PA). It is an effective thrombolytic agent activating plasminogen directly to plasmin. tcu-PA, like SK, is a first generation thrombolytic agent which, probably as a consequence of its cost, has not been adopted as a mainstream thrombolytic. In addition, compared to SK and rt-PA, surprisingly few studies have specifically examined its use as a thrombolytic agent in the setting of STEMI [165]. It does appear to enjoy similar efficacy to rt-PA and SK, and possibly has a lower incidence of reocclusion associated with its use [166]. TIMI 2 or 3 flow grades are quoted at about 65·8% [166].

Anisoylated plasminogen streptokinase activator complex (***APSAC***) comprises of an equimolar non-covalent complex between human plasminogen and SK. Temporary protection of the active site on the plasminogen molecule by acylation achieves a longer half-life (90 minutes compared to 20 minutes for SK, and 5 minutes for rt-PA). This allows APSAC (30U) to be given by rapid injection, and it therefore lends itself to use as a pre-hospital thrombolytic [167]. Studies suggest that APSAC possesses no particular advantages over rt-PA or SK in terms of its overall efficacy or safety profile [148]. Although there appears to be a slight increase in bleeding risk with APSAC, this has to be looked at in the context of the clinical trials which often (with the exception of ISIS-3 [148] where the safety profile was similar) employed heparin as adjunctive therapy.

The non-immunogenic recombinant unglycosylated single-chain precursor of urokinase, termed rscu-PA (***pro-urokinase***, or ***Saruplase***), unlike tcu-PA, is presumed to selectively activate fibrin-bound plasminogen. The induced clot lysis is amplified by plasmin-triggered conversion of scu-PA to tcu-PA on the surface of fibrin. Pro-urokinase has been investigated in the management of STEMI and initial results have been favourable. The PRIMI Trial compared a bolus of rscu-PA (20 mg) followed by a 60 minute infusion (60 mg) with standard SK in 401 patients [168]. It found that rscu-PA led to higher patency rate (71·2% compared to 63·9%), earlier reperfusion, less disturbance of haemostasis, and fewer bleeding complications than did intravenous SK, although there was no significant difference in left ventricular function [169]. Later evidence suggested equivalence with SK in the COMPASS study [170]. Initial studies (PASS and SESAM) comparing rscu-PA with rt-PA appeared to reveal a similar efficacy and safety profile for the two drugs [171]. Longer term data from the

PRIMI trial however suggested more adverse events with Saruplase [172], and the drug has not been widely adopted.

Novel plasminogen activator (n-PA, ***Lanoteplase***) is a deletion mutant of t-PA, but one of the kringle domains is preserved. It has a long half-life and can therefore be administered by a single bolus injection. Initial data suggested that it was as effective as rt-PA [173]. It was subsequently assessed in a large-scale multicentre trial (InTIME) which suggested possible improved patency rate at 90 minutes with Lanoteplase compared with Alteplase [174]. However, with respect to overall mortality at 30 days Lanoteplase and Alteplase were equally effective (InTIME-2 trial) [175]. The latter trial also demonstrated an increased rate of haemorrhagic stroke with Lanoteplase (1.12%) than with Alteplase (0.64%, $p = 0.004$). This increase in the most severe complication of thrombolytic therapy has stopped Lanoteplase from entering the market to date.

Recombinant ***Staphylokinase*** (Sak), derived from staphylococcus aureus is a polypeptide comprising 136 amino acids. Like SK, Staphylokinase is not an enzyme but forms a 1:1 stoichiometric complex with plasminogen. This inactive complex is then converted to the active plasmin-staphylokinase complex. Unlike the plasminogen-streptokinase complex, the plasminogen-staphylokinase complex is rapidly neutralised by antiplasmin in plasma in the absence of fibrin. Consequently, less systemic activation of plasminogen occurs. In the presence of fibrin, the complex is relatively resistant to antiplasmin neutralisation and therefore provides some fibrin specificity [176]. Staphylokinase is a potent plasminogen activator with similar efficacy to SK in terms of fibrinolytic efficacy, fibrin selectivity, and potency towards platelet-rich clots. In a dose-ranging study, also employing Aspirin and intravenous heparin, Sak displayed similar efficacy as front-loaded rt-PA with a good safety profile [177]. Data was published on 102 patients who received either front-loaded rt-PA or double bolus Sak [178]. TIMI grade 3 perfusion at 90 minutes was 68% in the STAR group and 57% in the rt-PA group. The problem however was that it suffered from the drawback of being immunogenic, which may have limited its application [176]. This has led to the development of recombinant variants such as pegulated Sak (PEG-Sak), which showed reduced immunogenicity as well as a more prolonged half-life, allowing single bolus administration [179]. PEG-Sak appeared to be as effective as rt-PA in a recent dose-ranging study [180].

Agents are being developed which are resistant to inhibitors such as PAI-1 [181]. One such re-engineered molecule is TNKase (***Tenecteplase***) , derived from t-PA which has undergone

site-specific changes with the substitution of certain amino acid sequences. It is fibrin-specific, and is largely resistant to PAI-1 [182]. Initial experience with this drug (TIMI 10A) appeared promising, with a reasonable safety profile, and 90 minute TIMI 3 flow rates in the region of 60% [183]. Its long half-life allows its administration as a single bolus injection [184]. TIMI 10B was a phase 2 trial which suggested that TNK-tPA enjoyed similar patency to accelerated rt-PA [185]. This was followed by the ASSENT-2 trial which recruited 16,949 patients and compared Tenecteplase with Alteplase, again showing equivalence and ease of administration with Tenecteplase [186].

Monteplase is a new mutant of t-PA with enhanced resistance to PAI-1. It has a prolonged half-life of over 20 minutes lending itself to bolus injection. The COMA trial has evaluated its use in patients with AMI being transferred for PCI [187]. Results in a cohort of 154 patients revealed initial patency rates of 56% compared to a spontaneous patency rate of 33% in the untreated group. No major differences were seen in clinical outcomes.

Recombinant glycosylated pro-urokinase (r-pro-UK, ***Prolyse***) is a new thrombolytic agent in development and is based on the native pro-urokinase molecule [188]. Pilot dose-ranging studies in humans have suggested that employing 60mg of r-pro-UK (preceded by 250000 IU of r-UK) achieved a best TIMI 3 flow of 57·7% [189]. At the dose regimen quoted, no severe bleeding complications were seen and in addition, reocclusion rates were remarkably low. To date, most clinical research has been directed at the treatment of acute ischaemic stroke [190].

1.4.1.5 COMBINATION THROMBOLYTIC THERAPY

No studies have convincingly shown improved thrombolytic efficacy when thrombolytic agents are combined together. One of the major concerns is that combination therapies tend to be associated with an increased risk of bleeding, lower TIMI 3 perfusion rates, and higher morbidity and mortality [149,191]. One study employing low-dose rt-PA followed by pro-urokinase (the PATENT Trial) did show patency rates similar to that seen with accelerated rt-PA [192]. The regime was well tolerated, and had a low reocclusion rate. Previous smaller studies employing a combination of rt-PA with urokinase [193] and low-dose rt-PA with pro-urokinase [194] showed similarly favourable results. Studies have also looked at the combination of low-dose rt-PA with full-dose SK [195], and although the regime was reasonably well tolerated, no significant additional benefits (except cost over rt-PA alone) were seen when one compares the results with accelerated rt-PA or SK alone.

1.4.2 ADJUNCTIVE DRUG THERAPY

1.4.2.1 ASPIRIN

The value of adjunctive drug therapy is best illustrated by aspirin [141]. Aspirin is now well established as first-line therapy for ACS. Aspirin prevents the production of thromboxane A_2 by inhibiting the enzyme cyclooxygenase. This leads to inhibition of the mechanisms of both haemostasis and thrombosis. As aspirin binds to platelet cyclooxygenase irreversibly, it inhibits the function of the platelets for the rest of their 8-10 day lifespan. Aspirin also inhibits cyclooxygenase in the endothelium of the arteries and veins, and hence blocks the production of prostacyclin, a powerful inhibitor of platelet aggregation. This aspirin-induced loss of prostacyclin production potentially reduces the overall antithrombotic action of aspirin, but the clinical significance is not known. The inhibition of prostacyclin formation is reversible, because the endothelium is capable of resynthesising cyclooxygenase.

1.4.2.2 TICLOPIDINE AND CLOPIDOGREL

Ticlopidine inhibits platelet function by inhibiting aggregation in response to ADP, adrenaline, thrombin and collagen – possibly blocking expression of the platelet fibrinogen receptor GP IIb/IIIa [196]. A major drawback is the incidence of neutropenia, about 2·4% - with a severe neutropenia in 0·85% [197]. Ticlopidine is no longer used routinely in the United Kingdom.

Clopidogrel, which exerts its antiplatelet action in a very similar manner, may be safer with respect to bone marrow toxicity. The role of the latter has been examined in a large secondary prevention trial (CAPRIE) [198]. Further subgroup analysis suggests that Clopidogrel reduces the risk of myocardial infarction by nearly 20% compared to Aspirin [199]. The bulk of the experience with Ticlopidine has been involved with the prevention of thrombosis after placement of intracoronary stents [200]. Clopidogrel has now superseded Ticlopidine as the drug of choice following stenting [201,202].

The role of both of these agents has yet to be examined with respect to STEMI and thrombolysis, although initial results in a small study suggested that Clopidogrel was superior to Aspirin [203]. An ongoing trial (CLARITY/TIMI-28) should resolve this issue. Certainly, in

patients with NSTEMI, in the CURE trial, Clopidogrel appeared to confer additional benefit to Aspirin [204].

1.4.2.3 PLATELET GLYCOPROTEIN IIB/IIIA INHIBITORS

Agents directed against the platelet fibrinogen receptor GP IIb/IIIa are now established as being beneficial when used in the context of ACS and PCI. Three agents have been extensively studied: abciximab (ReoPro), eptifibatide (Integrilin) and tirofiban (Aggrastat).

As to whether co-administration of GP IIb/IIIA inhibitors with thrombolytic therapy is beneficial in the management of myocardial infarction, a number of studies have suggested only marginal benefit. The IMPACT-AMI [205] and Platelet Aggregation Receptor Antagonist Dose Investigation and Reperfusion (PARADIGM) [206] were early and relatively small pilot trials (IMPACT-AMI: Integrilin with rt-PA; PARADIGM: Lamifiban with rt-PA or SK). These small trials showed acceptable safety profiles and modest benefits in angiographic or electrocardiographic markers of reperfusion but no differences in major clinical end points.

Other more recent trials have studied the role of abciximab combined with lower-dose thrombolytic agents. The results of TIMI 14 [207] (higher levels of TIMI 3 flow with abciximab plus half-dose rt-PA) and the Strategies for Patency Enhancement in the Emergency Department (SPEED) trial [208], using half-dose Reteplase, formed the basis for GUSTO-V, a large-scale phase 3 trial evaluating treatment with a full dose of abciximab plus a half dose of Reteplase versus treatment with a full dose of Reteplase alone. GUSTO-V revealed no benefit with use of abciximab in this setting [209].

Integrilin has also been used with SK in 171 patients with AMI [210]. In this small study, full-dose SK was employed, and bleeding risk was increased with only modest improvement in 90 minute TIMI 3 flow. Tirofiban has only been examined in relatively small pilot studies, although has been examined in patients with STEMI ineligible for thrombolysis. No benefit was seen [211].

It may be that patency can be achieved employing antiplatelet GP IIb/IIIa antibody even in the absence of a thrombolytic agent [212]. Other GP IIb/IIIa inhibitors are under investigation, including compounds which are active orally. An early study (TIMI 12) employing the orally active IIb/IIIa inhibitor Sibrafiban resulted in an increased incidence of bleeding [213]. Subsequent secondary prevention trials (SYMPHONY) of this drug suggested excessive bleeding risk, possible increased mortality and certainly no obvious clinical benefit [214,215].

Inhibition of platelet glycoprotein Ib may also be of potential use as an adjunct to thrombolysis [216].

1.4.2.4 PCI AND GP IIB/IIIA INHIBITORS

The value of abciximab in patients undergoing primary angioplasty for acute MI was prospectively evaluated in a trial of 483 patients who were randomly assigned to therapy with abciximab or placebo (RAPPORT) [217]. The 30-day composite end point of death, reinfarction, or urgent revascularization was reduced in patients treated with abciximab. In the EPILOG Trial 2,792 relatively low-risk patients were randomly assigned to therapy with Aspirin plus standard-dose weight-adjusted heparin and abciximab, or Aspirin plus low-dose weight-adjusted heparin and abciximab, or Aspirin plus weight-adjusted heparin alone [218]. The 30-day major event rate was reduced in the abciximab-treated patients. There were no differences in major bleeding rates among the three groups although minor bleeding was more frequent among patients receiving abciximab with standard-dose heparin.

Hamm *et al* [219], for the CAPTURE investigators, also reported that elevated troponin-T levels correlated with increased 6-month cardiac risk. This study indicated that serum troponin-T levels identified a subgroup of patients with refractory unstable angina suitable for coronary intervention who would particularly benefit from antiplatelet treatment with abciximab.

Eptifibatide was evaluated in the IMPACT-II trial which revealed a reduction in death, MI, unplanned surgical or repeat PCI or stent implantation for abrupt closure [220]. In patients undergoing early PCI for unstable angina, 30-day composite events occurred less often in patients receiving eptifibatide [221,222]. The ESPRIT (Enhanced Suppression of the Platelet IIb/IIIa Receptor with Integrilin Therapy) Trial evaluated the efficacy and safety of eptifibatide treatment as adjunctive therapy during non-emergency coronary stent implantation. There was a marked reduction in all composite end points [221,222].

Tirofiban employment during coronary angioplasty was evaluated in the RESTORE Trial of 2,139 patients with unstable angina or AMI [223]. The primary 30-day end point was reduced from 12·2% in the placebo group to 10.3% in the tirofiban group Patients treated with tirofiban had a 38% relative reduction in the composite end point at 48 hours, and a 27% relative reduction at 7 days. A larger clinical benefit with tirofiban was seen in patients with unstable angina undergoing coronary angioplasty in the PRISM-PLUS Study. Coronary angioplasty was performed in 30·5% of patients between 49 to 96 h after randomisation [224]. The composite end points were all significantly reduced.

1.4.2.5 HEPARIN

Heparin works as an anticoagulant by potentiating the action of antithrombin III, as it is similar to the heparan sulfate proteoglycans which are naturally present on the cell membrane of the endothelium.

Maintenance of patency is as important as the rate at which it is achieved and although the addition of heparin in the case of rt-PA appeared to be beneficial [149], a meta-analysis suggests that the routine addition of heparin does not confer additional benefit in patients who have also been administered Aspirin [225]. There is certainly no evidence to support the routine use of heparin after SK [148], although placebo controlled evidence is lacking.

Intravenous unfractionated heparin prevents clot formation at the site of arterial injury and on coronary guidewires and catheters used for PCI, and is routinely administered at the beginning of the procedure [226].

There is increasing evidence that low molecular weight heparin (LMWH) offers advantages over intravenous unfractionated heparin (UFH). Apart from enjoying convenient once- or twice-daily standardised administration without the need for activated partial thromboplastin time monitoring, there is some evidence that they may improve the efficacy of thrombolytic therapy. LMWH exert their anticoagulant effect by preferentially inhibiting activated Factor Xa and less so thrombin.

LMWH has been compared with UFH for the treatment of unstable angina and NSTEMI in several large trials [227-229] and shown a clear benefit and, in the case of enoxaparin, possible superiority over UFH [230]. The latest trial questions this superiority (SYNERGY), but certainly suggests equivalence with UFH [231]. Fewer trials have been completed evaluating LMWH with thrombolytic therapy in ST-elevation myocardial infarction. Enoxaparin has been most widely studied with SK [232], rt-PA [233] and TNK [234,235]. Overall results have been favourable, although results from ASSENT 3 PLUS suggest a possible tendency to increased bleeding risk [235].

Given the favourable results demonstrating the superiority of an invasive approach in patients with NSTEMI/UA [236,237] and STEMI [238] treated with LMWH, there are relatively few studies examining the use of LMWH specifically in elective PCI [239-242]. The data from the urgent PCI studies in high risk patients however, along with these smaller studies in elective PCI, all suggest that LMWH may well supplant UFH in these settings [243]. The problem of rebound hypercoagulability seen with discontinuation of UFH [244] however, may well be shared by LMWH [245].

1.4.2.6 WARFARIN

Warfarin is an oral anticoagulant that is an antagonist of vitamin K and acts by inhibiting the synthesis of vitamin K dependent coagulation factors. The use of Warfarin would be expected to reduce the risk of cardiac events following thrombolysis, but this has not been borne out by clinical trials [152]. Indeed, there appears to be an increased risk of haemorrhagic complications in patients formally anticoagulated compared to those receiving aspirin alone [246]. One recent study did show a tendency for Warfarin given with aspirin, or alone, to be better than aspirin; although there was again an increased risk of bleeding [247].

1.4.2.7 DIRECT THROMBIN INHIBITION

Other regimens are being examined to see if further improvement in maintaining patency can be achieved and these are currently looking at newer anticoagulant and antiplatelet drugs. These include the antithrombins hirudin [248], and argatroban [249]. Hirudin, a potent antithrombin, has been extensively investigated and although pilot studies (GUSTO IIa [248], TIMI 9A [250], HIT III [251]) were encouraging, the doses employed produced an unacceptable increase in haemorrhagic complications [248]. Although the trials were continued at lower (and apparently safer) doses (TIMI 9B [252], GUSTO IIb [253], HIT-4 [254]), it would appear that overall, the use of hirudin with thrombolytic therapy confers no significant advantage over the use of heparin alone. Only one study (HERO) has suggested that hirudin may confer benefit in this scenario [255].

Subsequent to the hirudin trials, a new synthetic analogue of hirudin has been developed and studied. Bivalirudin is a specific and reversible inhibitor of thrombin and binds directly with both fluid-phase and clot-bound thrombin. Initial results have been very favourable in the context of NSTEMI/UA [256]. In STEMI, when administered with SK, there was no major advantage over UFH, but there was a slight reduction in reinfarction [257]. Its major niche is likely to be in the context of PCI, where it may well replace the role of UFH [258].

Further studies with argatroban in the setting of STEMI also appear favourable [259,260]. There was improvement in TIMI 3 flow and no significant bleeding risk in one study (MINT) comparing argatroban with heparin following rt-PA [259].

1.5 PERCUTANEOUS CORONARY INTERVENTION (PCI) AND MYOCARDIAL INFARCTION

The problem with thrombolytic therapy is the incidence of residual stenosis and subsequent reocclusion. Normal TIMI 3 flow in the infarct-related artery 90 minutes after the start of thrombolytic therapy was found in only 29-54%, and in 51-58%, 5 to 7 days later [134,153,261]. It is now well documented that TIMI grade 2 flow has a similar outlook as TIMI grades 0 and 1 [139,262,263], and therefore TIMI 3 flow is the goal of therapy. The fact that infarct vessel reocclusion is relatively common (10-30%) and associated with an adverse prognosis [264,265] has led to investigation of methods to improve and maintain vessel patency after AMI. Other problems with thrombolysis are the potential haemorrhagic complications and the fact that there is a lag time to reperfusion (median time of about 45 minutes). Another major issue is the fact that as many as half of the patients admitted to coronary care units with STEMI are ineligible for thrombolysis anyway [266]. Reasons for lack of eligibility include late presentation, non-diagnostic ECG changes or contra-indications due to co-morbidities. Elderly patients (75 years and older), those presenting without chest pain, and those with a history of diabetes, congestive heart failure, previous myocardial infarction, or coronary bypass surgery are most likely to not receive thrombolytic therapy [267].

Because of the deficiencies of thrombolysis, the role of PCI in AMI has been examined in a number of studies.

1.5.1 PRIMARY PCI

Primary PCI is defined as balloon angioplasty undertaken as the primary reperfusion strategy for STEMI without previous or concomitant thrombolytic therapy. There have been a total of 23 randomised trials comparing primary PCI with thrombolysis. These trials differ in many respects, including patient sample size, type of thrombolytic therapy, and whether stents, with or without GP IIb/IIIa inhibitors, were used.

Table 1-2 shows a summary of the features of the 23 trials. In total, 7739 patients were assigned either PCI or thrombolytic therapy. Of the patients randomised to primary PCI, 0·6% crossed over to thrombolytic therapy, and 3% to primary PCI.

Overall it can be seen from **Table 1-3** that patients assigned to primary PCI were less likely to die, have a non-fatal reinfarction or stroke. Outcomes are also maintained in longer-term follow-up [155,238,268-287]. The only endpoint which appears to occur with greater frequency in the PCI group is major bleeding [288]. Overall it would appear that primary PCI is better than thrombolytic therapy at reducing major adverse cardiac events.

The major problem with primary PCI is the fact that it is often logistically difficult [201,289,290]. in addition, concern has been expressed that results are likely to be less impressive in centres where there is lower patient throughput [291-293]. A more attractive proposition in many countries would be PCI of the infarct-related vessel after failed thrombolysis as this should logically reduce the degree of stenosis or open an occluded artery.

Key for Table 1-2 (next page):

LBBB= left bundle branch block; MI= myocardial infarction; NA= data not available; ST↑= elevation; ST↓= depression; t-PA= tissue-type plasminogen activator; 68% of patients in the Grines study [294] and 70% in the Hochman study [268] received accelerated t-PA.

* From admission. † From randomisation. ‡ Average time. § From symptom onset to reperfusion. ¶ Median time. ‖ Time to the permitted delayed revascularisation procedure (percutaneous or surgical) in the initial medical stabilisation group. ** Both the PRAGUE [295] and the LIMI [280] trials included a third group of individuals who had thrombolytic therapy followed by transfer for subsequent PCI (n= 100 and n= 74 patients, respectively); these 174 patients are not included in the analysis.

Table 1-2: Summary of the 23 randomised trials of primary angioplasty versus thrombolytic therapy.

	Eligibility	Symptom duration (h)	Number PCI (n= 3872)	Number Lytic (n= 3837)	Stents used	GP IIb/IIIa used	Lytic Agent	Time to PCI (min)	Time to lytic (min)
Streptokinase trials (n=1837)									
Zijlstra [269]	<75, ST↑	<6	152	149	No	No	SK	62 *	30 *
Ribeiro [270]	<75, ST↑	<6	50	50	No	No	SK	238	179
Grinfeld [296]	ST↑	<12	54	58	No	No	SK	63 †	18 †
Zijlstra [271]	ST↑, low risk	<6	47	53	No	No	SK	68 *	30 *
Akhras [272]	ST↑	<12	42	45	No	No	SK	NA	NA
Widimsky [295] **	ST↑, LBBB	<6	101	99	No	No	SK	80 †	70 †
De Boer [273]	>76, ST↑	<6	46	41	No	No	SK	59 *	31 *
Widimsky [297]	ST↑	<12	429	421	Yes	Yes	SK	277 ‡ §	245 ‡ §
Fibrin-specific trials (n=5902)									
DeWood [298]	<76, ST↑	<12	46	44	No	No	Duteplase	126 *	84 *
Grines [274]	ST↑	<12	195	200	No	No	t-PA (3hr)	60 †	32 †
Gibbons [275]	<80, ST↑	<12	47	56	No	No	Duteplase	45 †	20 †
Ribichini [276,299]	<80, inferior MI, anterior ST↓	<6	55	55	No	No	Accelerated t-PA	40 †	33 †
Garcia [300]	Anterior MI		95	94	No	No	Accelerated t-PA	84	69
GUSTO IIb [155]	ST↑, LBBB	<12	565	573	No	No	Accelerated t-PA	114 †	72 †
Le May [278]	ST↑, LBBB	<12	62	61	Yes	Yes	Accelerated t-PA	77 † ¶	15 †
Bonnefoy [301]	ST↑		421	419	Yes	Yes	Accelerated t-PA	190 †	130 †
Schömig [279]	ST↑	<12	71	69	Yes	Yes	Accelerated t-PA	65 * ¶	30 * ¶
Vermeer [280] **	<80, ST↑	<6	75	75	Yes	No	Accelerated t-PA	100 †	85 †
Andersen [302]	ST↑	<12	790	782	Yes	NA	Accelerated t-PA	NA	NA
Kastrati [281]	ST↑, LBBB	<12	81	81	Yes	Yes	Accelerated t-PA	75 * ¶	35 * ¶
Aversano [282]	ST↑	<12	225	226	Yes	Yes	Accelerated t-PA	102 * ¶	46 * ¶
Grines [294]	ST↑	<12	71	66	Yes	Yes	Accelerated t-PA	155 *	51 *
Hochman [268]	Cardiogenic shock	<36	152	150	Yes	Yes	Accelerated t-PA	75 † ¶	6168 † ¶ ‖

Table 1-3: Short-term clinical outcome of the 23 randomised trials of primary angioplasty versus thrombolytic therapy.

	Death Number (%)		Non-fatal reinfarction Number (%)		Total stroke Number (%)		Haemorrhagic stroke Number (%)		Death, reinfarction, stroke Number (%)	
	PCI	Lytic	PCI	Lytic	PCI	Lytic	PCI	Lytic	PCI	Lytic
Streptokinase trials (n=1637)										
Zijlstra [269]	2/152	11/149	1/152	18/149	1/152	3/149	1/152	2/149		
	1%	7%	1%	12%	0·7%	2%	1%	1%		
Ribeiro [270]	3/50	1/50	2/50	1/50	0/50	0/50	0/50	0/50		
	6%	2%	4%	2%						
Grinfeld [295]	5/54	8/58	1/54	2/58						
	9%	14%	2%	3%						
Zijlstra [271]	1/47	1/53	0/47	7/53	1/47	2/53	0/47	0/53	2/47	10/53
	2%	2%		13%	2%	4%			4%	19%
Akhras [272]	0/42	4/45	0/42	5/45						
		9%		11%						
Widimsky [295] **	7/101	14/99	1/101	10/99	0/101	1/99			8/101	23/99
	7%	14%	0·01%	10%		1%			8%	23%
De Boer [273]	3/46	9/41	1/46	6/41	1/46	3/41			4/46	12/41
	7%	22%	2%	15%	2%	7%			9%	29%
Widimsky [297]	29/429	42/421							36/429	64/421
	7%	10%							8%	15%
TOTAL	**5%**	**10%**	**1%**	**10%**	**1%**	**2%**	**0·4%**	**0·6%**	**6%**	**16%**
Fibrin-specific trials (n=5902)										
DeWood [298]	3/46	2/44								
	7%	5%								
Grines [274]	5/195	13/200	5/195	14/200	0/195	7/200	0/195	4/200	10/195	32/200
	3%	7%	3%	7%		1%		1%	5%	16%
Gibbons [275]	2/47	2/56	1/47	3/56	0/47	0/56	0/47	0/56		
	4%	4%	2%	5%						
Ribichini [276,299]	1/55	3/55	1/55	6/55	0/55	0/55	0/55	0/55		
	2%	6%	2%	11%						
Garcia [300]	3/95	10/94	4/95	5/94						
	3%	11%	4%	5%						
GUSTO IIb [155]	32/565	40/573	25/565	37/573	6/565	11/573	0/565	8/573	54/565	78/573
	6%	7%	4%	7%	1%	2%		1%	10%	14%
Le May [278]	3/62	2/61	3/62	8/61	1/62	2/61			7/62	10/61
	5%	3%	5%	13%	2%	3%			11%	16%
Bonnefoy [301]	20/421	16/419	7/421	15/419	0/421	4/419	0/421	2/419	28/421	34/419
	5%	4%	2%	4%		1%		1%	6%	8%
Schömig [279]	3/71	5/69	2/71	4/69					5/71	9/69
	4%	7%	3%	6%					7%	13%
Vermeer [280] **	5/75	5/75	1/75	7/75	2/75	2/75	0/75	1/75	8/75	14/75
	7%	7%	1%	9%	3%	3%		1%	11%	19%
Andersen [302]	52/790	59/782	13/790	49/782	9/790	16/782			63/790	107/782
	7%	8%	2%	6%	1%	2%			8%	14%
Kastrati [281]	2/81	5/81	0/81	4/81	1/81	1/81				
	3%	6%		5%	1%	1%				
Aversano [282]	12/225	16/226	11/225	20/226	3/225	8/226			24/225	40/226
	5%	7%	5%	9%	1%	4%			11%	18%
Grines [294]	6/71	8/66	1/71	0/66	0/71	3/66	0/71	2/66	6/71	9/66
	8%	12%	1%			5%		3%	9%	14%
Hochman [268]	71/152	84/150			5/152	1/150	0/152	2/150		
	47%	56%			3%	1%		1%		
TOTAL	**8%**	**9%**	**3%**	**6%**	**1%**	**2%**	**0%**	**1%**	**8%**	**14%**

PCI following thrombolysis in AMI can be divided into various categories depending on the reason and timing for the procedure.

Rescue PCI is defined as early (within a few hours) angioplasty when thrombolysis has failed (**Table 1-4**).

Immediate PCI is described as early angioplasty when thrombolysis has been successful and there is a significant residual stenosis (**Table 1-5**).

Delayed PCI is defined as angioplasty 1 to 7 days after thrombolysis when thrombolysis may or may not have been successful (**Table 1-6**).

1.5.2 RESCUE PCI

Several studies have been performed in the last 15 years, and many of these are summarised in **Table 1-4**. Most of the studies are nonrandomised, with only five randomised studies being performed [280,295,303-305]. Most studies identified patients with TIMI 0 or 1 flow as having experienced failed thrombolysis. Those studies that included patients with higher flow, but significant residual stenoses following thrombolysis, are included, but an attempt has been made to identify only those patients with TIMI 0 or 1 flow for inclusion in the tables.

Overall it can be seen that generally the procedures are successful, but there is a significant reocclusion rate of up to 29% (average 19·4%). The mortality rates when successful are largely acceptable, and compare well with the mortality rates of patients not receiving rescue PCI (average 7·4% compared to 5·3%). When the procedure is unsuccessful however, mortality rates are very high and up to 33 to 44% (average 19%).

The latest study to be published is the MERLIN trial [306]. This study recruited 307 patients with persistent ST elevation after thrombolysis predominantly with SK. This study revealed no benefit with rescue PCI but was seriously underpowered.

It is difficult to interpret the results of these studies with any degree of certainty. The failure of rescue angioplasty may be the cause of the high mortality in these patients, but these patients may have represented a higher risk subgroup that may have died regardless of the therapeutic manoeuvres employed. Overall, the data supports the strategy of considering rescue angioplasty for patients with TIMI grade 0 or 1 flow in the infarct vessel after thrombolytic therapy. TAMI-5 and RESCUE identified patients with prior infarction and anterior AMI respectively who are most likely to benefit from this approach [303,305], and benefit was maintained up to a year [307]. Further examination of the individual studies reveals that

rescue angioplasty improves regional wall motion along with exercise LV function and may reduce the risk of congestive cardiac failure, shock or death [308]. Few studies appear to show any impact on resting left ventricular ejection fraction [309,310].

Table 1-4: Studies of rescue PCI.

			IN-HOSPITAL OUTCOME (%)					
STUDY	**n**	**Thrombolytic**	**Success**	**Reocclusion**	**Death**	**Death if failure**	**Control Group Mortality**	**Notes**
Abbottsmith et al, 1990 [263]	192	rt-PA, rt-PA + UK, rt-PA + iloprost, or UK	88	21	5·9	39·1	4.6	
Baim et al, 1988 [311]	37	rt-PA	92	26	5·4	-	7·0	
Belenkie et al, 1992 [304]	28	SK or rt-PA	81	-	0	33·3	-	No control group. Study compared immediate angioplasty for residual stenoses with angioplasty for occluded vessels (this group).
Califf et al, 1988 (TAMI-1) [312]	86	rt-PA	73	29	12	44	0	Only 10 patients in control group.
Califf et al, 1991 (TAMI-5) [303]	52	rt-PA, UK, or both	83	9	-	-		
CORAMI Study Group, 1994 [313]	72	rt-PA, SK, APSAC, or rt-PA + SK	90	7	4	14	-	61 of the 72 patients underwent check angiography
Ellis et al, 1992 [314]	173	rt-PA, rt-PA + UK, or SK	78	19	13	-		
Ellis et al, 1994 (RESCUE) [305]	151	SK, rt-PA, or UK	92	8	5·2	-	9·6	Mortality figures are for 30 days
Fung et al, 1986 [315]	13	SK	92	16	7·7	0	-	No control group. Study compared immediate angioplasty for residual stenoses with angioplasty for occluded vessels (this group).
Gibson et al, 1997 (TIMI 4) [310]	57		88	4·0	10	28·5	4·5	
Grines et al, 1989 (KAMIT) [316]	12	½ dose rt-PA + SK	100	8	-	-	2·5	
Grines et al, 1991 [195]	10	½ dose rt-PA + SK, or rt-PA	90	12	10	0	5·0	

Table 1-4 *continued*: Studies of rescue PCI.

			IN-HOSPITAL OUTCOME (%)					
STUDY	**n**	**Thrombolytic**	**Success**	**Reocclusion**	**Death**	**Death if failure**	**Control Group Mortality**	**Notes**
Hartzler et al, 1983 [317]	4	intracoronary SK	100	75	0	0	-	Not stated whether reocclusion occurred in patients treated with SK first or not.
Holmes et al, 1990 [318]	34	SK	71	-	3	0	-	No control group. Study compared immediate angioplasty for residual stenoses with angioplasty for occluded vessels (this group).
McKendall et al, 1995 (TIMI) [319]	33	rt-PA or SK	82	17	12	33	7·0	
O'Connor et al, 1989 [320]	90	SK	89	14	17	-	-	
Papapietro et al, 1985 [321]	7	intracoronary SK	57	-	0	-	-	
Rogers et al, 1990 (TIMI- IIa) [322]	29		93·1	20·8	6·9	-	8·6	Mortality figures in the invasive group are 21-day compared to 6 weeks in the conservative group.
Ross et al, 1993 (GUSTO) [323]	214	SK, rt-PA, or both	91	13	0	-		
Topol et al, 1988 (TAMI II) [193]	22	rt-PA + UK	86·4	4·3	0	-	-	
Vermeer et al, 1999 [280]	224	rt-PA	90	-	9·5	-	8	Mortality figures are at 1 year
Whitlow, 1990 (CRAFT) [324]	44	rt-PA or UK	85	27	4	-	-	Mortality figures are for 30 days. No control group.
Widimsky et al, 2000 (PRAGUE) [295]	40	SK	-	7	20	-	18	
Wnek et al, 1995 [325]	270	SK	91·5	29·8	28·6	-	3·9	Mortality for both successful AND failed PCI (intervention group). Control group are patients with TIMI-3 flow.

1.5.3 IMMEDIATE PCI

Meyer et al (1982) first described the use of PCI to dilate high-grade stenoses after successful thrombolysis [326]. Initial results were entirely favourable and led to a number of multicentre, randomised, controlled trials (meta-analysis: Michels and Yusuf [327]). These studies largely demonstrated that this approach was associated with an increased complication rate and a trend towards higher mortality (**Table 1-5**). In addition, there does not appear to be any impact on other parameters such as ejection fraction. These studies are not perfect however. Only one of the studies included routine administration of Aspirin prior to PCI, and anticoagulation was not necessarily aggressive or tightly controlled. Indications for PCI varied, but were not restricted to high-grade stenoses or vessels with TIMI 2 flow only. The incidence of other complications also appears to be higher (recurrent ischaemia, infarction, haemorrhage, etc.).

Table 1-5: Studies of immediate PCI.

		Invasive Group (%)			Conservative Group (%)		
Study	**Thrombolytic**	**n**	**Deaths at 6 weeks**	**Deaths at 1 year**	**n**	**Deaths at 6 weeks**	**Deaths at 1 year**
Belenkie et al, 1991 * [328]	SK or rt-PA	50	2·0	-	39	2·6	-
Califf et al, 1988 (TAMI I) ** [312]	rt-PA	99	3·0	6·0	189	6·0	-
Califf et al, 1991 (TAMI 5) [303]	rt-PA, UK, or both	287	5·6	8·4	288	4·5	6·9
El Deeb et al, 1990 [329]	SK	74	-	2·7	No control group		
Erbel et al, 1986 *** [330]	iv + ic SK	83	7	-	79	14	-
Erbel et al, 1989 [331]	iv + ic SK	103	11·7	17·5	103	16·5	21·4
Holmes et al, 1990 **** [318]	SK	29	0	3·0	No control group		
Papapietro et al, 1985 **** [321]	ic SK	11	9·0	-	No control group		
Rogers et al, 1990 (TIMI 2A) [322]	rt-PA	195	7·7	8·2	197	8·6	10·2
Simoons et al, 1988; *Arnold et al*, 1992 (ECSG) ***** [332,333]	rt-PA	183	6·6	9·3	184	2·7	5·4

* No conservative group. Study compares immediate with delayed PCI This is the immediate group.

** Control group consists of patients in whom thrombolysis was successful, and in whom no PCI took place.

*** In-hospital mortality.

**** No conservative group. Study compared immediate PCI for residual stenoses (this group), with PCI for occluded vessels.

***** 2 week mortality

A retrospective review of the original TAMI 1 trial data suggested that PCI in the setting of TIMI 2 flow may have a modest impact on ejection fraction, but overall there was no

significant advantage of intervention over medical therapy [334]. Routine treatment of patients with opened (TIMI 3 flow) vessels suggest actual harm by attempted intervention [332,335,336]. On the basis that there may have been some improvement in the available technology since TAMI 1 was performed, the RESCUE II study was undertaken [307].

This study recruited patients with TIMI 2 flow after thrombolysis of larger infarcts. Only 29 patients were randomised from 44 eligible. 30 day mortality in the PCI group was 7·1% (one patient) compared to 0% in the conservative group, but no significant difference at 1 year (7·1 vs 6·7%). There did appear to be an improvement in LV function in those treated with PCI. The study is far too small to draw any meaningful conclusions.

Overall the data suggest that PCI should not be employed after successful thrombolysis (TIMI-3 flow) unless there is ongoing ischaemia or haemodynamic instability.

1.5.4 DELAYED PCI

This refers to angioplasty performed electively 1 to 7 days (or longer) after thrombolysis. Several studies have examined this approach and appear to show an increased risk overall in the invasive group (see **Table 1-6**). There is again the problem of a failure to routinely administer Aspirin pre-procedure in some of the studies, and apparent poor anticoagulation protocols post-procedure. Overall, despite study shortcomings, delayed PCI cannot be recommended as a routine option. Analysis of individual studies reveals no significant impact on left ventricular function, and rates of surgical revascularisation are high. Those studies with angiographic follow-up suggest significant restenosis (51%) and reocclusion (13%) rates in this group of patients [337]. There is also a high rate of in-hospital complications [338]. One study not listed in **Table 1-6** looked at the effect of angioplasty in patients who had been treated with rt-PA or placebo [339]. It is not clear in this study, amongst the patients who underwent PCI, how many of them received rt-PA or placebo. Of the 42 patients who did undergo angioplasty however, there was an improvement in ejection fraction during exercise but not at rest.

The data suggests that routine delayed PCI in the absence of spontaneous or provoked ischaemia is not warranted.

It is not entirely clear why there is no obvious benefit from routine PCI in the setting of thrombolysis, but it appears that reocclusion (or re-reocclusion) and restenosis are largely responsible [337]. The reason why angioplasty is less successful following thrombolysis is not

fully understood. PCI is patently successful as primary treatment of myocardial infarction, and therefore the administration of thrombolytic therapy must render the environment unfavourable. Partly this may be due to the danger of early dissection of the haemorrhagic plaque [81,340]. It may also be because of reperfusion damage in a haemorrhagic infarct zone [340]. It is however more likely that the failure and/or risk of PCI is due predominantly to the relatively procoagulant milieu that exists following thrombolytic therapy. This is possibly largely as a consequence of poor fibrinolytic capacity due to low levels of plasminogen.

This environment can be partly influenced by the use of anticoagulant and antiplatelet agents, although Aspirin and heparin are already used routinely.

Table 1-6: Studies of delayed PCI.

			LVEF (%)		Mortality (%) at 6 weeks		Mortality (%) at 1 year	
Study	**n**	**Thrombolytic**	**Inv**	**Cons**	**Inv**	**Cons**	**Inv**	**Cons**
Barbash et al, 1990 * [338]	201	rt-PA	50	49	5·2	3·8	8·2	3·8
Bauters et al, 1995 ** [337]	300	rt-PA, SK or other	-	52	1·0	-	2·8	-
Belenkie et al, 1991 *** [328]	39	SK or rt-PA	55·4	-	2·6	-	-	-
Ellis et al, 1992b (TOPS) + [314]	87	rt-PA, SK or other	47	49	0	0	0	0
Özbek et al, 1990 (SIAM) [341]	324	SK	55	55	8·9	6·0	11·4	9·0
Rogers et al, 1990 (TIMI-2A) [322]	194	rt-PA	50·3	48·9	5·7	8·6	7·7	10·2
SWIFT trial study group, 1990 (SWIFT) [342]	800	APSAC	51·7	51	2·7 *	3·3 *	5·8	5·0
TIMI study group, 1989, *Williams et al*, 1992 (TIMI-2B) [343,344]	3262	rt-PA	50·5	49·5	5·2	4·7	6·9	7·4
Topol et al, 1987 (TAMI-1) ++ [335]	98	rt-PA	55	NA	1·0	6·0	-	-
Topol et al, 1992 (TAMI-6) [345]	71	late rt-PA	52	51	8·8	5·4	8·8	10·8
van den Brand et al, 1992 +++ [346]	218	rt-PA	51	50	1·8	2·9	-	-

* In-hospital mortality (not 6 week mortality).

** There is no conservative group. In-hospital mortality (not 6 week mortality). Follow-up data is for 6 months, not 1 year.

*** There is no conservative group. Study compares immediate with delayed PCI This is the delayed group.

\+ PCI 4 to 14 days post-thrombolysis.

++ Control group consists of patients in whom thrombolysis was successful, and in whom no PCI took place.

+++ 13-week mortality data

The use of platelet GP IIb/IIIa receptor antibodies seems to confer additional benefit in this scenario [347,348], and the benefit is sustained [349]. An increasing number of studies suggest that abciximab administered during rescue PCI improves outcome with only a small increase in the risk of bleeding [350].

Later restenosis is of course an issue that is not confined to PCI following thrombolysis, but is the single most troublesome problem facing interventionists today [351,352,352,353,353-355]. The mechanisms here are slightly different however and involve the interaction of thrombin, platelets and the endothelium over a longer period of time.

It is likely that stents, particularly drug-eluting stents, will have a positive impact in the setting of post-thrombolysis PCI. The major changes in interventional techniques over the past decade cast doubt over the current validity of many of the trials discussed. Although the bulk of the data now emerging for stents in AMI refer to primary angioplasty, there is accumulating data that they are beneficial in PCI after thrombolysis [356,357], and drug-eluting stents may confer additional benefit [358].

Preliminary data from the latest trial of rescue PCI, comparing intervention with repeat thrombolytic or conservative therapy (the REACT trial) was presented at the British Cardiac Society meeting in May 2004. Initial analysis certainly appears to favour intervention in the setting of clinically failed thrombolysis.

1.6 PURPOSE OF THIS STUDY

It is clear that thrombolytic therapy is far from the ideal management for STEMI due to its failure to restore and maintain TIMI 3 flow in as many as 30-40% of patients treated. In the United Kingdom however, despite the Department of Health's emerging strategy of expansion of hospital cardiac catheter laboratory facilities, it is likely that thrombolytic therapy will remain the mainstay of treatment for the majority of patients for some time. With the expansion of interventional facilities however, it is likely that more patients will be in a position to receive rescue PCI if appropriate.

Rescue PCI, by definition, happens in the immediate post-thrombolytic phase. This study sets out to determine the coagulation and fibrinolytic factors that may be important during this period. In determining the behaviour of various coagulation and fibrinolytic parameters following thrombolytic therapy, a hypothesis as to why thrombolytic therapy may fail may also be developed.

As a surrogate marker of the generation of thrombin, levels of soluble fibrin were measured in a cohort of patients undergoing thrombolytic therapy with SK for STEMI. In the same cohort, the levels of fibrinogen were also measured in order to determine the impact of lytic therapy on the substrate of fibrin.

Levels of plasminogen were measured in order to determine the impact of SK administration and also to determine the time taken for plasminogen levels to begin to return to normal. As a marker of lytic activity and plasmin generation, levels of the fibrin degradation product X-oligomer were measured. No previous studies have examined the behaviour of TAFI in this setting, and the opportunity was therefore taken to see whether there was any activity demonstrable and whether lytic therapy exerted influence.

It is also well established that platelets play a fundamental role in the acute coronary syndromes. This study is the most detailed analysis of platelet activation performed in the context of STEMI and sets out to determine when, and to what extent, platelet activation might influence events.

In a smaller substudy, the levels of soluble fibrin and X-oligomer were measured in a cohort of patients undergoing elective PCI. This was performed in order to determine whether there was any evidence for the generation of thrombin or plasmin in this setting.

CHAPTER 2: METHODS

2.1 PATIENTS

Two groups of patients were studied. A cohort of patients undergoing elective PCI, and a separate cohort of patients undergoing thrombolytic therapy for acute myocardial infarction. Written permission was gained from the ethics committees at both Glenfield Hospital and Derbyshire Royal Infirmary.

2.1.1 COLLECTION & PROCESSING OF SAMPLES FROM ELECTIVE PCI PATIENTS

Patients undergoing elective PCI were recruited from Glenfield General Hospital. Informed consent was obtained from each patient the day prior to the planned procedure. Information sheets were given to the patients and are included in the appendix. PCI was performed according to the discretion of the individual operator. All patients were given Aspirin (150 to 300mg) 1 to 3 hours before the procedure and 10,000 units of intravenous heparin after collection of the first sample (and prior to the PCI procedure itself). Patients were recruited until the target number of 20 suitable patients was reached.

Blood samples were taken from the arterial sheath (always discarding the first 10ml) immediately following arterial puncture, and before administration of heparin, and labelled time 0. Further samples were taken from the *arterial* sheath in the same manner at 15 minutes, 1 and 2 hours after the final balloon inflation. A further *venous* sample was obtained at 24 hours. This was collected without the use of a tourniquet. It was the routine practise of some operators to administer heparin intravenously at a dose of 1000 units per hour until 06·00 hours the day following the procedure. Occasionally intravenous glyceryl trinitrate was administered during the same period.

Within 2 minutes of acquisition, each sample was added to one or two polypropylene bottles containing 0·5ml of 0·129M **CITRATE** and gently mixed (total volume in each bottle being 5·0ml, resulting in a ratio of 9 parts blood: 1 part citrate). 2·5ml was then taken from each tube and added to a polypropylene tube containing 25µl of **APROTININ** (TRASYLOL®- 10,000/ml) - final concentration of aprotinin being 100 IU/ml. 5 patients had **HIRUDIN** (final concentration being 20iu/ml) added to one of the aprotinin samples. The bottles were then immediately spun down in a Labofuge 200 centrifuge for 20 minutes at 2900g (5000rpm). Plasma was removed and snap frozen in suitable aliquots. Samples were then stored at -80°C until analysis.

Details concerning the patients' clinical history and matters relating to the PCI procedure were noted on a data sheet included in the appendix. The data is displayed in **Table 3-4**.

2.1.2 COLLECTION & PROCESSING OF SAMPLES FROM PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

Patients with AMI undergoing therapy with SK were recruited at Derbyshire Royal Infirmary. AMI was suspected if patients exhibited new or presumed new ST-segment elevation of at least 0·1mV in at least two inferior leads (II, III, and aVF), in at least two adjacent precordial leads (V_1 to V_6), or in leads I and aVL. Patients with new, or presumed new, left bundle branch block were also included. Patients with cardiogenic shock or contraindications to SK (or where rt-PA was the preferred agent) were excluded. Patients were excluded from the study if subsequently AMI was not proven (i.e. ECG changes chronic, cardiac enzymes normal).

Verbal informed and witnessed consent was obtained from any patients admitted with AMI (see definition above) during recruitment periods. Information sheets were given to both the patients and their relatives (appendix). All patients had been administered Aspirin (150-300mg) prior to entering the study. All patients subsequently received a 1 hour infusion of 1·5 million IU of SK.

A large bore cannula was then placed in a suitable peripheral vein (contralateral to SK therapy). Blood, samples were drawn immediately prior to thrombolysis with SK and at 1, 2, 4, 12, 24, 36 and 48 hours post-thrombolysis. All samples were collected without the use of a tourniquet, and after discarding the first 5ml drawn. After drawing blood, the cannula was flushed with 0·9% sodium chloride. If the cannula became difficult to use or showed signs of infection, it was removed and a new cannula sited with permission from the patient.

All samples were collected and processed by the author.

Samples were then processed as with the elective PCI patients except that a plasma sample in citrate, citrate plus aprotinin *and* citrate plus aprotinin and hirudin was collected at each collection time for all patients.

At the same time as the preparation of the plasma samples, in a series of 12 patients, platelets were labelled with fluoroscein isothiocyanate (FITC) conjugated anti-P-Selectin, anti-GP IIb/IIIa and anti-GP Ib antibodies, fixed and prepared for later analysis by flow cytometry employing a technique developed during the period of this study.

Details concerning the patients' clinical history and matters relating to the AMI were noted on a data sheet (see appendix). The data is displayed in **Table 3-1**.

2.2 MEASUREMENT OF FIBRINOLYTIC AND HAEMOSTATIC VARIABLES IN PLASMA

2.2.1 SOLUBLE FIBRIN

Samples of plasma were assayed for soluble fibrin (SF) employing the monoclonal antibody (Mab) 5F3 (NIBn5F3.C4.B10). This Mab has been produced by Dr. Patrick Gaffney's laboratory at the NIBSC and was originally raised to the thrombin-treated N-terminal disulphide knot (T-NDSK) of fibrinogen [83]. Mab 5F3 has been shown to have high affinity for crosslinked fibrin via an epitope on fragment-E, which is not exposed in fibrinogen [359].

The method followed is outlined below:

The wells of a flexible microtitre plate (Dynatech) were coated with 100µL of monoclonal antibody (Mab) 5F3 in phosphate buffered saline (PBS) at a concentration of 3µg/ml overnight at 4°C. The plates were washed three times with 200µl/well of PBS containing 0·1% (v/v) Tween 80 (PBS/T80).

To block any non-specific binding sites in the wells of the plate, the plates were incubated at 37°C with 200µl/well of PBS/T80 for 30 to 60 minutes. The plates were then washed with 200µl/well with PBS/T80. Dilutions of the standard (1500u/ml soluble fibrin – NIBSC stock) were prepared by doubling serial dilutions of the standard with citrate/saline containing 1mg/ml Gly-Pro-Arg-Pro (GPAP) and 100iu/ml hirudin.

100µl of each standard solution was pipetted, in duplicate, onto the washed microtitre plate along with 100µl of each patient sample. A control sample was also pipetted into a row of wells. Blank wells were also included and contained 100µl of citrate/saline. The plate was then incubated for 1 hour at 37°C. The plate was then washed three times with PBS/T80, using 200µl per well.

100µl of Mab A11-biotin conjugate diluted 1:1000 in PBS/T80 was added to each well and the plates incubated for 20 minutes at 37°C. Following this the plates were washed three times with PBS/T80, using 200µl per well, streptavidin-HRP conjugate (diluted 1:2000 in PBS/T80) added, and the plates incubated for a further 20 minutes. The triple washing step was then repeated.

Finally 100µl of o-tolidine substrate (5ml H_2O_2 to 10ml o-tolidine) was added to each well until a blue colour developed and then the reaction was terminated by adding 50µl of 3M HCl to each well. The colour then changed from blue to yellow and the absorbance read at 450nm using an automated plate reader.

The results are expressed in units per litre (u/l). Data from significant numbers of individuals collected and analysed by the NIBSC suggest that values ***less than*** **5u/l** fall within the normal range.

In a small cohort of patients, SF levels were measured by Professor Wilhelm Nieuwenhuizen's group at the Gaubius Laboratory. They employed an in-house Mab-based enzyme immunoassay utilising an antibody (Mab anti-Fb-1/2) against an epitope in Aα which is involved in the fibrin-induced rate enhancement of plasminogen activation by t-PA. A sandwich technique using a horseradish peroxidase-labelled antibody (Mab G8 - which has its epitope in the carboxy-terminal section of the fibrin α-chains) is employed. This prevents interference from fibrin(ogen) degradation products which anti-Fb-1/2 can react with. Normal values for this assay are **42ng/ml** (95% CI 38-45 ng/ml, n = 81).

2.2.2 X-OLIGOMER/CROSSLINKED FIBRIN DEGRADATION PRODUCTS

Samples of plasma were assayed for crosslinked FDP employing the monoclonal antibody Mab 123 (NIBn123) produced in Dr Patrick Gaffney's laboratory at the NIBSC [360]. This monoclonal antibody reacts with high molecular weight crosslinked fibrin fragments but not non-crosslinked fragments.

The wells of a flexible microtitre plate (Dynatech) were coated with 100µL of monoclonal antibody (Mab) 5F3 in phosphate buffered saline (PBS) at a concentration of 3µg/ml overnight at 4°C. The plates were washed three times with 200µl/well of PBS containing 0·1% (v/v) Tween 80 (PBS/T80). To block any non-specific binding sites in the wells of the plate, the plates were incubated at 37°C with 200µl/well of PBS/T80 for 30 to 60 minutes. The plates were then washed with 200µl/well with PBS/T80.

A lyophilised preparation of X-oligomer (NIBSC reagent, code: 87/502) was used as the standard. It contains 1000ng/ml of X-oligomer and is reconstituted by addition of 1ml distilled water per ampoule. Serial doubling dilutions were made using PBS/T80 with the standard curve starting at 1000ng/ml.

100µl of each standard solution was pipetted, in duplicate, onto the washed microtitre plate along with 100µl of each patient sample. A control sample was also pipetted into a row of

wells. Blank wells were also included and contained 100µl of citrate/saline. The plate was then incubated for 1 hour at 37°C. The plate was then washed three times with PBS/T80, using 200µl per well.

100µl of a polyclonal horseradish peroxidase conjugated anti-fibrinogen diluted 1:1000 in PBS/T80 diluted 1:1000 in PBS/T80 was added to each well and the plates incubated for 20 minutes at 37°C. Following this the plates were washed three times with PBS/T80, using 200µl per well, streptavidin-HRP conjugate (diluted 1:2000 in PBS/T80) added, and the plates incubated for a further 20 minutes. The triple washing step was then repeated.

Finally 100µl of o-tolidine substrate (5ml H_2O_2 to 10ml 0-tolidine) was added to each well until a blue colour developed and then the reaction was terminated by adding 50µl of 3M HCl to each well. The colour then changed from blue to yellow and the absorbance read at 450nm using an automated plate reader.

The results are expressed in nanograms per millilitre (ng/ml). Values ***less than*** **300ng/ml** are expressed as falling within the normal range.

2.2.3 INTACT FIBRINOGEN

Intact fibrinogen was measured in Dr. Wilhelm Nieuwenhuizen's laboratory at the Gaubius Institute in Leiden, The Netherlands. Citrated plasma samples with aprotinin added were sent to the Gaubius Institute on dry ice.

Employing a unique enzyme-linked immunoassay the levels of intact fibrinogen were calculated [79]. The monoclonal antibody employed (designated as Mab G-8) recognises an epitope near the carboxyl-terminal 150 amino acid region of the fibrinogen Aα-chains. A second monoclonal antibody labelled with horseradish peroxidase (designated as Y-18) is directed against fibrinopeptide A [361], covalently bound to the Aα-chains (i.e. against the amino-terminal regions of the Aα-chains). As a result of the specificities of the two antibodies, the EIA is specific for fibrin(ogen) molecules with at least one fibrinopeptide A and one intact carboxyl-terminal end of an Aα-chain. As a result, 96% of circulating fibrinogen molecules (HMW and LMW forms) are recognised. Results are expressed in **g/l**.

2.2.4 ACTIVATABLE PLASMINOGEN

The usual method for determining plasminogen levels in the setting of thrombolysis is to adopt the method of Friberger *et al* [362]. Briefly this entails adding an excess of SK to the plasma being tested. The SK forms a complex with plasminogen present which can act upon a

chromogenic substrate designated S-2251. By reading the optical density at 405nm, and comparing the results obtained against a plasminogen standard, the amount of activatable plasminogen present can be calculated.

Although this approach has become the accepted method for plasminogen determination following thrombolysis, it has obvious shortcomings. Plasma taken from individuals following thrombolytic therapy already has SK present. In order to prevent the thrombolytic process continuing in this study, aprotinin was added to certain aliquots collected. This would have rendered the Friberger method impossible.

With these points in mind, a novel plasminogen assay has been developed for the purposes of this study in collaboration with Mr Colin Whitton in Dr Patrick Gaffney's laboratory at the NIBSC. The final method is outlined below:

Microtitre plates were coated with 100µl per well of rabbit anti-human plasminogen antibody (Dako, code: A0081) diluted 1:200 in bicarbonate buffer. Plates were left at 4°C overnight or at 37°C for 2 hours. They were then decanted and washed four times in washing buffer (PBS + 0·1% Tween 80), using 250µl per well. Non-specific binding sites were blocked by adding 250µl of the washing buffer and incubating the plate for 30-60 minutes at 37°C.

Glu-Plasminogen standard (code: 78/646) was reconstituted in 1·0ml of distilled water and then diluted 1:500 with diluting buffer (citrate/saline containing 10u/ml of aprotinin) to produce a concentration of 600ng/ml. Serial doubling dilutions were then made employing further diluting buffer. Plasma samples were then diluted 1:200 with diluting buffer.

100µl of standard or test plasma was added to appropriate wells on the microtitre plate. Wells containing diluting buffer alone acted as blanks. Both standards and duplicates were tested in duplicate.

The plates were then incubated for 1 hour at 37°C and subsequently washed four times in washing buffer. Substrate S-2403 was diluted 1:5 with citrate/saline and urokinase was added to the wells (resulting in a final concentration of 100IU/ml of urokinase).

100µl of the substrate/urokinase mixture was then added to each well and the plates incubated for three hours at 37°C. A third well for each sample tested had substrate alone added to determine the presence of native plasmin activity. For all samples tested, these wells were similar to the blank wells.

Plates were read at 405nm employing an automated plate reader. Final results are expressed in μg/ml. Normal plasma stock samples at the NIBSC gave plasminogen levels in the region of **150-200μg/ml**.

2.2.5 CARBOXYPEPTIDASE B / TAFI ACTIVITY

Plasma samples containing citrate, aprotinin and hirudin were assayed. A chromogenic assay using furylacroleyl-alanyl-arginine (***BACHEM***) as substrate was used [363]. Absorbance at 336nm was measured. The procedure followed was that adapted from Dr. Edward Plow's team at the Cleveland Clinic Foundation (*personal communication*). Two possible assay methods were considered: a spectrophotometer method, and a multiplate method. After assessment of both, the spectrophotometer method was used [123].

2.2.5.1 SPECTROPHOTOMETER METHOD

50μl of plasma was added to 550μl of 100 mM Hepes, pH 7·4 and 150mM NaCl (buffer) and mixed gently. 600μl of 0·72mM substrate was added and mixed gently. Absorbance @ 336nm was measured in a spectrophotometer at 1 minute intervals for 20 minutes. Edward Plow's team have previously shown that a 0·001 decrease in absorbance at 336nm per minute is equivalent to 1u/l of enzyme in plasma (*personal communication*). The activity measured under these conditions is the sum of CPB and CPN (the major plasma carboxypeptidase which accounts for the constitutive carboxypeptidase activity of plasma) within the sample. To obtain plasma CPB activity, the inhibitable portion of the total carboxypeptidase activity is determined. The inhibitable portion is that which is inhibited by a specific TAFIa inhibitor: potato carboxypeptidase inhibitor.

The procedure was therefore repeated as follows:

50μl aliquots of plasma was added to 550μl of buffer with 60μg of potato carboxypeptidase inhibitor (concentration 5mg in 45·8ml of buffer) ultimately producing a final concentration of potato carboxypeptidase inhibitor in the 1200μl aliquot of 50μg/ml. Samples were left for 5 minutes. 600μl of 0·72mM substrate was added and mixed gently. Absorbance @ 336nm was again measured in a spectrophotometer at 1 minute intervals for 20 minutes. In order to determine plasma CPB activity, the value was subtracted from that recorded without the inhibitor present.

Assays were performed on a Biochrom 4060 spectrophotometer employing accompanying reaction kinetics software. Final results are expressed in u/l. Dr. Edward Plow's team have identified **2-9u/l** as being in the normal range.

2.2.6 TAFI ANTIGEN LEVELS

Levels of TAFI antigen were measured by Laszlo Bajzar's team on hirudin samples sent on dry ice to the Hamilton Civic Hospitals Research Centre, Ontario, Canada. They have developed their own in-house Mab-based ELISA technique [114]. Briefly, this entailed coating wells of a 96-well microtitre plate with MoAbTAF#13 by incubating 10μg/ml MoAbTAFI#13 in 50mmol/L sodium carbonate, pH 9·6, for 2 hours at 22°C. Nonspecific sites were blocked with bovine serum albumin (BSA). Samples were diluted in 0·1% BSA in HEPES buffered saline with 0·1% Tween 20 and 100μL of each sample was applied to each well and incubated for I·5 hours at 22°C. Horseradish peroxidase-conjugated anti-TAFI was then incubated with each sample and specific binding was determined o-phenylenediamine dihydrochloride. The colour was allowed to develop for 10 minutes. The reaction was then quenched by the addition of sulphuric acid to each well. Absorbance was measured at 490nm and corrected by subtracting the absorbance at 650nm using a Thermomax microtitre plate reader. Using this ELISA, the concentration of TAFI in a plasma pool at the time of this assay was determined to be **54nM/L**.

2.2.7 SOLUBLE P-SELECTIN

Soluble P-selectin (sP-Selectin) was assayed employing a commercially available immunoassay (Parameter, Human soluble P-Selectin Immunoassay, R&D Systems Europe). This assay employs the quantitative sandwich immunoassay technique.

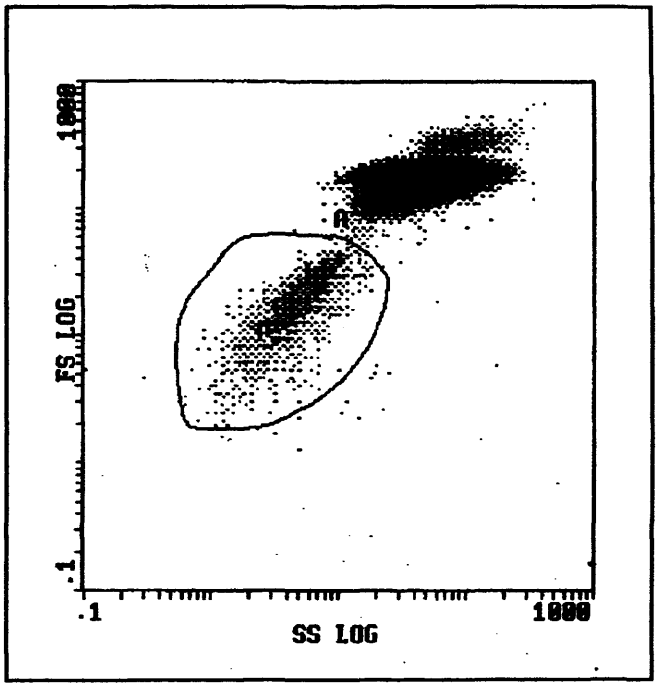
Monoclonal antibody specific for sP-Selectin is already pre-coated onto a microplate. Standards and the control were diluted with deionised water, according to instructions. Citrated plasma samples were diluted 20-fold with a supplied diluent. 100μl of the diluted standards, samples and a control solution were pipetted into the wells, together with a polyclonal antibody specific for sP-Selectin which is conjugated to horseradish peroxidase. The plate was left to stand at room temperature for 1 hour. Unbound conjugated antibody was removed by a triple washing stage employing a wash buffer which is supplied. Finally 100μl of substrate was added to each well and the plate left at room temperature for 15 minutes. 100μl of a Stop Solution was then added and the plate read on an automated plate-reader (set to 450nm with wavelength correction set to 620nm) within 30 minutes.

2.2.8 FLOW CYTOMETRY FOR THE MEASUREMENT OF PLATELET MEMBRANE P-SELECTIN, GP IB AND GP IIB/IIIA

This method can be used to quantify the amount of fluorescent antibody which is attached to individual cells, and thus determine the relative levels of antigens on each cell.

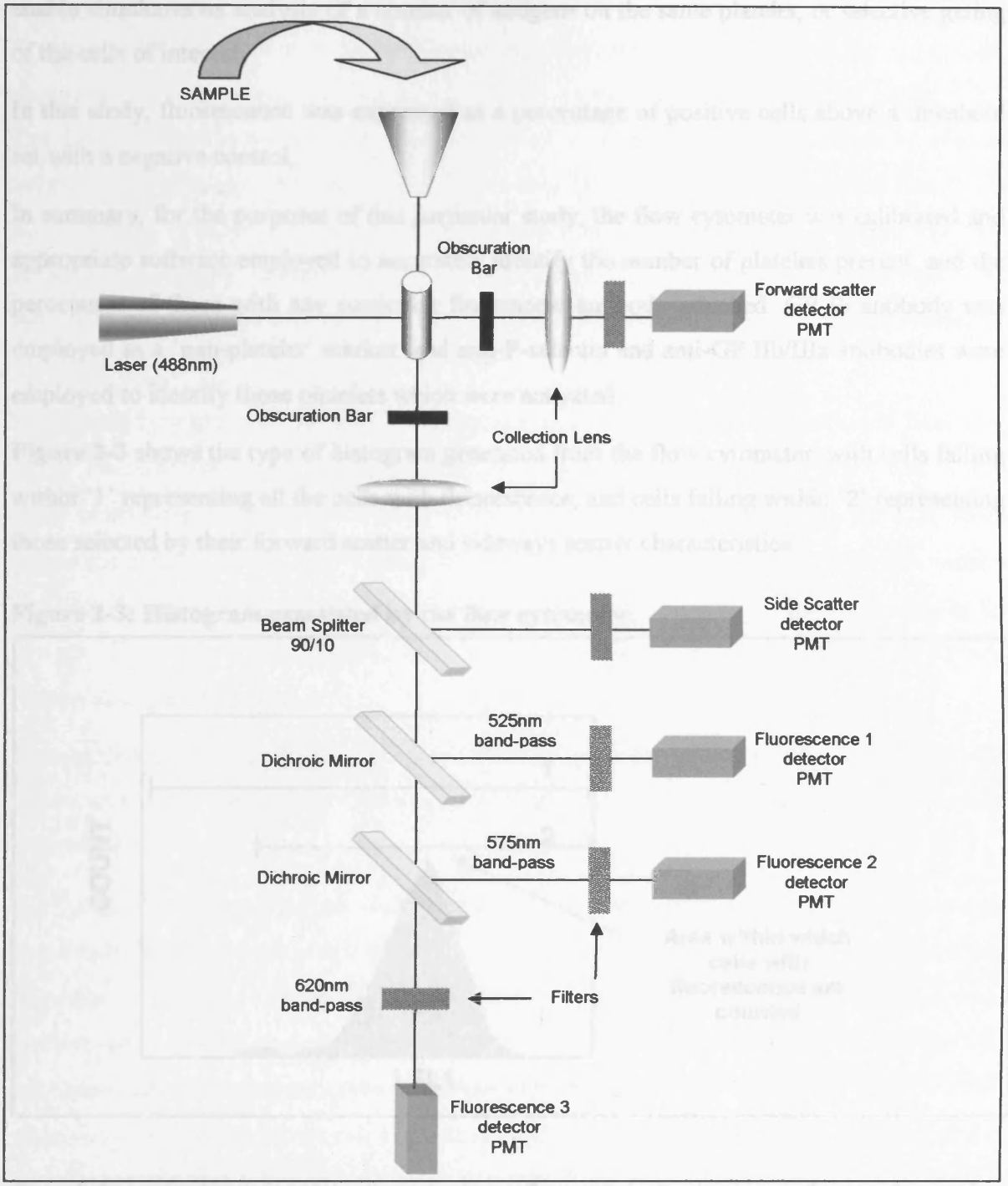
One of many advantages of flow cytometry is that it allows rapid analysis of various aspects of platelet biology on samples of whole blood. Thousands of platelets are rapidly passed through in front of a laser beam (**Figure 2-2**). Light which passes through these particles is collected by photodiodes or photomultiplier tubes (PMT) and translated into an electronic signal, which represents side scatter (SS: a measure of cell granularity) and forward scatter (FS: a measure of cell size). In this way, different cell types can be identified in a mixed sample (such as a whole blood sample) and effectively separated.

Figure 2-1: Appearance of whole blood analysis on the flow cytometer.



These can then be graphed as they are identified and different populations can be seen. **Figure 2-1** illustrates the image generated by the flow cytometer. Samples are analysed by side scatter (SS axis) and forward scatter (FS axis). Platelets are identified in electronic gate "A" and analysed for fluorescence (FL1). The large population of cells to the top right of the platelet population are red cells and those with the highest FS signal are leucocytes.

Figure 2-2: Simplified diagram of a flow cytometer.



By incubating the blood sample with fluorescently-labelled antibodies that recognise antigens on the platelet surface, the fluorophore is excited as the platelets pass through the laser and emits fluorescence at a characteristic wavelength. This is detected in the flow cytometer by photomultiplier tubes and converted into an electronic signal that is directly proportional to the number of fluorescent antibody molecules on each cell, and thus to the number of antigens on each platelet. Modern flow cytometers normally have 3 or 4 such photomultipliers, each set to detect fluorescence of different wavelengths by means of selective mirrors and filters.

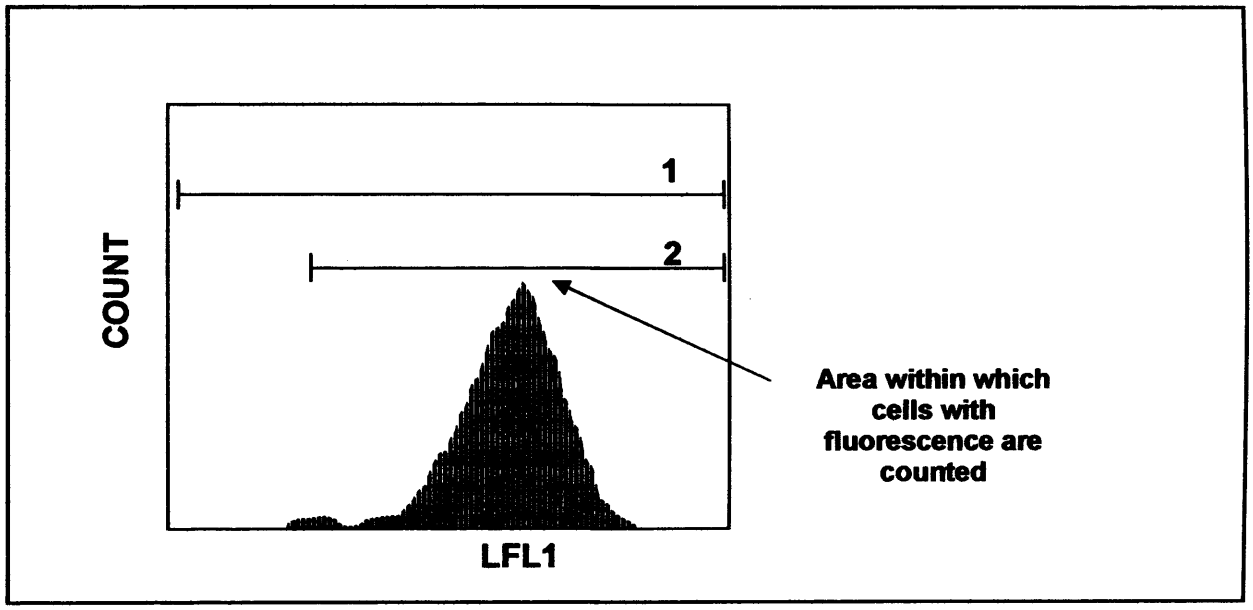
Thus combinations of antibodies coupled to fluorophores with different emission spectra enable simultaneous analysis of a number of antigens on the same platelet, or selective gating of the cells of interest.

In this study, fluorescence was expressed as a percentage of positive cells above a threshold set with a negative control.

In summary, for the purposes of this particular study, the flow cytometer was calibrated and appropriate software employed to accurately identify the number of platelets present, and the percentage of these with any particular fluorescent antibody attached. GP Ib antibody was employed as a 'pan-platelet' marker, and anti-P-selectin and anti-GP IIb/IIIa antibodies were employed to identify those platelets which were activated.

Figure 2-3 shows the type of histogram generated from the flow cytometer, with cells falling within '1' representing all the cells with fluorescence, and cells falling within '2' representing those selected by their forward scatter and sideways scatter characteristics.

Figure 2-3: Histogram generated by the flow cytometer.



One of the main disadvantages of employing a flow cytometer in this study was the need to process samples very soon after taking them. In view of the practicalities of collecting platelet samples over a 24 hour period, over 30 miles away from the flow cytometer laboratory, a method of stabilising the platelets for subsequent assessment of their activation status was required.

2.2.8.1 Developing the Method for Platelet Collection and Processing

A variety of flow cytometric methods have been employed [364-366], although the same basic approach to sample preparation can be used, based on methods developed in the 1980s [367-370]. Our flow cytometric method has been developed to measure platelet activation in clinical samples. Our laboratory had previously shown that fixation with anything greater than 0·2% formaldehyde changed the interaction of fibrinogen and the GP IIb/IIIa receptor and thus affected the assay of platelet GP IIb/IIIa expression [371]. Despite this, other groups have advocated the use of formaldehyde or paraformaldehyde fixed samples to measure P-selectin expression [372].

It was originally planned to collect and process platelets according to the method of Becker *et al* [372]. This method involves 50µl whole blood anticoagulated with EDTA being placed into tubes containing 1·0ml of 2·0% Paraformaldehyde. These are then left to stand at room temperature for 2 hours, or overnight at 4°C before being washed with Tyrode's buffer, centrifuged, washed, centrifuged and finally resuspended. Samples are then snap frozen on dry ice. The method we employed differed from Becker *et al*, only insofar as citrate was employed as the anticoagulant.

Despite repeated attempts at this method considerable problems were encountered with extensive red cell haemolysis following the washing stages. Closer scrutiny of the method was required.

A 5·0µl (as opposed to 50µl) citrated sample from a volunteer was stood at room temperature in 2·0% Paraformaldehyde for 2 hours. It was then processed according to Becker *et al* and immediately analysed on the flow cytometer after 20 minutes incubation with fluoroscein isothiocyanate (FITC) conjugated anti-P-Selectin or anti-GP Ib antibodies. The sample was <u>not</u> frozen after the washing stages. Comparison was made with an identical blood sample which had been processed according to standard local practice (which entails 5·0µl of citrated blood being added to 50µl freshly filtered Hepes buffer with either anti-P-Selectin antibody or anti-GP Ib antibody for 20 minutes and the reaction fixed with 2·0% formyl saline for 10 minutes). An experiment was run in tandem with an identical sample activated with 0.32M thrombin.

It was found that there was significant haemolysis producing difficulties in measuring any level of platelet activation after employing the 2·0% Paraformaldehyde/Tyrode's buffer method of Becker *et al*. No problems were encountered using the standard local procedure.

The experiment was repeated using freshly made solutions of Paraformaldehyde and Tyrode's buffer. Identical problems were found.

In order to determine whether the fixation method was the problem, we tried fixing the samples after incubation with the antibodies. Briefly, 5·0µl citrated blood samples from ten healthy volunteers were added to 50µl Hepes buffer with either anti-P-Selectin antibody (6µl, 1:10 dilution) or anti-GP Ib antibody (5µl) for 20 minutes. The samples were then fixed with formyl saline (500µl) or different concentrations of Paraformaldehyde (500µl of 0·5%, 1·0% and 2·0%). An experiment was run in tandem with samples activated with ADP (5µl, 10^{-3} solution) so that platelet activation could be measured and compared. In order to determine whether the samples could be left in fixative and analysed later, samples were analysed in the flow cytometer immediately and at 1, 24, and 48 hours.

The samples were stored at 4°C overnight and analysed again at 24 and 48 hours. There was an increase in P-Selectin expression over time in activated samples fixed in formyl saline. This suggests that there was either continued activation from the ADP added to the samples and/or activation from ADP released from lysed cells. Either way, it can be concluded that formyl saline does not completely fix platelets in this method. No obvious trend was seen in samples fixed in the varying concentrations of Paraformaldehyde apart from 2·0% Paraformaldehyde which did not change too much over the 48 hours (although these samples did not reveal as much activation in the first place).

There are differences at 1 hour between the different fixatives, but all are statistically insignificant when analysed employing the Mann Whitney U test ($p>0·05$).

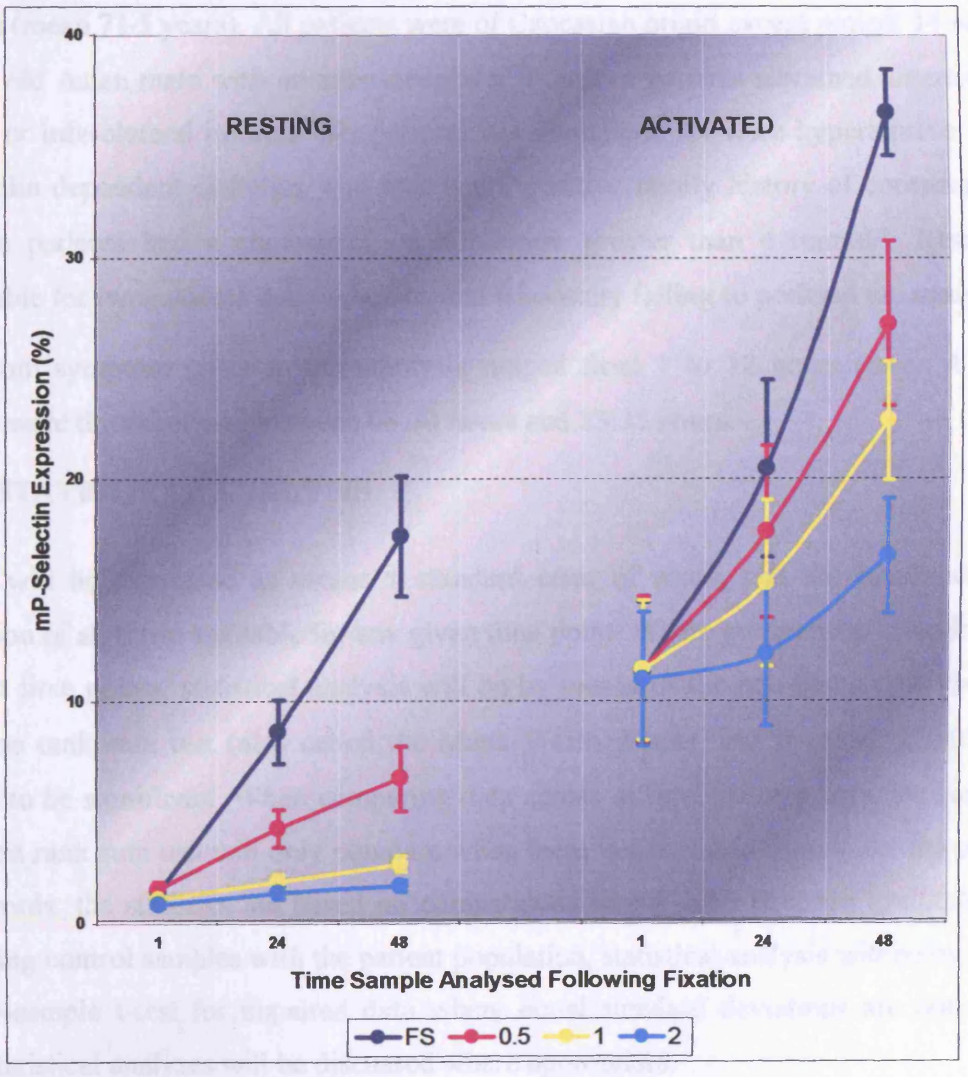
Statistical analysis employing one way ANOVA reveals a significant difference over time when using formyl saline as fixative for both resting ($p=0·0001$) and activated platelets ($p<0·0001$). 0·5% paraformaldehyde also showed a significant difference over 24 hours in resting platelets ($p=0·007$), but no significant change in activated platelets ($p=0·24$). The least significant changes over 24 hours were observed in platelets fixed with 2·0% paraformaldehyde (resting $p= 0·177$, activated $p=0·795$). In view of the fact that samples were anticipated to contain activated platelets and samples were going to be analysed on the flow cytometer within 12 hours, 0·5% paraformaldehyde was chosen as the fixative in our method. Higher concentrations clearly resulted in lower expression of mP-selectin.

It was therefore found that the fixative employed was indeed of great significance. Both resting and activated platelets revealed lower expression of mP-Selectin, the higher the concentration of fixative (with formyl saline being the weakest, and 2% Paraformaldehyde

being the strongest), and platelet expression of mP-selectin increases over time in a weaker fixative.. The results are displayed graphically in **Figure 2-4**. This has since been confirmed by other workers [373].

The final method employed in the collection and processing of citrated samples from AMI patients was to add the appropriate antibodies at the time of sample collection. Briefly, 5µl of citrated blood was added immediately after sampling to 50µl of Hepes buffer containing the appropriate antibodies (anti-mP-Selectin, anti-GP Ib and anti-GP IIb/IIIa). The samples were then incubated at room temperature for 20 minutes and finally 500µl of 0·5% paraformaldehyde added to fix the samples. All samples were then analysed within 12 hours on the flow cytometer.

Figure 2-4: The effect of different fixatives over time on expression of mP-selectin on resting and activated platelets (10 healthy volunteers). FS = formyl saline, 0.5, 1.0 and 2.0 = the % concentrations of paraformaldehyde.



CHAPTER 3: RESULTS

3.1 PATIENTS

26 patients were enrolled into the study. These patients were all treated with thrombolytic agents on the assumption they had sustained an AMI. Only 21 patients were included in the final analysis. Reasons for exclusion were as follows:

1) subsequent correction of diagnosis: suspicious ECG but normal cardiac enzyme series (n=2), normal ECG but abnormal enzyme series (n=1).

2) reinfarction during the study period and repeat administration of SK (n=1)

3) thrombolytic agent employed was rt-PA (n=1)

Patient details and characteristics are shown in **Table 3-1**. Fifteen were male, whose ages ranged from 48 to 84 years (mean 67·7 years). Six were female whose ages ranged from 68 to 79 years (mean 71·5 years). All patients were of Caucasian origin except patient 14 who was a 64 year old Asian male with an anterior infarct. Fourteen patients sustained anterior and six inferior or inferolateral infarcts. Six patients were smokers, six were hypertensive, six were non-insulin dependent diabetics, and four had a positive family history of coronary disease. Eighteen patients had a cholesterol on admission greater than 4·8mmol/l. Results were unavailable for two patients due to the hospital laboratory failing to perform the assay.

Time from symptom onset to thrombolysis ranged from 1 to 12 hours (mean 4·5 hours). Patients were thrombolysed between 08:30 hours and 23:35 hours.

3.2 STATISTICAL ANALYSIS

Results will be expressed as means ± standard error of mean, and the results will be an expression of all those available for any given time point. Where comparison is made between different time points, statistical analysis will be by means of the non-parametric two-sample Wilcoxon rank sum test (also called the Mann-Whitney test), and p values $\leq 0{\cdot}05$ will be deemed to be significant. When comparing data across different time points, the two-sample Wilcoxon rank sum test will only compare when there is a matched sample for the patient (in other words, the statistics are based on comparisons where valid data are available). When comparing control samples with the patient population, statistical analysis will be by means of the two-sample t-test for unpaired data where equal standard deviations are not assumed. Other statistical analyses will be discussed where appropriate.

Table 3-1: Myocardial infarction patient characteristics.

[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	75	M					111	3373	3421	158			4.5	2.5	22:35	ANT
[redacted]	69	F					76	4674	8562	224			5.9	1.75	8:30	INF
[redacted]	60	M	√				104	6473	3167	269			5.3	1.75	15:30	ANT
[redacted]	70	M		√			160	1051		148			5.1	1	22:30	ANT
[redacted]	48	M	√				88	946		237			7.5	2	23:35	INF
[redacted]	75	M					1219	1069		304			6.1	3	14:45	ANT
[redacted]	79	F			√		59	1452		341	330		5.7	1.5	12:00	ANT
[redacted]	67	M		√	√	√	736	622	585	194			5.8	5	14:00	INF
[redacted]	76	M					88	433		253			5.1	6.5	18:50	INF
[redacted]	64	M	√		√		277	2385	628	300	261	222	6	6	22:30	ANT
[redacted]	73	F		√	√		216		382	243		253		5	14:50	ANT
[redacted]	55	M	√			√	840	2463	1213	243		172	5	6	14:00	INF
[redacted]	67	M					332	912	1129	270	241	229	7.5	10	14:50	INF
[redacted]	64	M			√	√	353	4927	2802	214	206	206	5.8	6.5	17:25	ANT
[redacted]	68	F	√				51	2850	3109	343	266	285	4.2	1.25	14:30	INF
[redacted]	63	M		√			82	2213	1109	197	173	198	6.9	4	14:10	ANT
[redacted]	84	M			√		648	914		523	412			8	22:00	ANT
[redacted]	71	F	√	√		√	110	1350	1801	279	230	242	7.7	3	18:00	INF
[redacted]	69	F					994	1030	506	234	242	223	6.5	12	15:15	ANT
[redacted]	84	M		√			238	1253	1216	266	284	275	6.7	6	9:20	INF
[redacted]	63	M					319	2697		230	248		6.9	2	9:30	INF

Key to the table: PATIENT ID= specific patient identifier number. NIDDM= type 2 diabetes. CK= creatine kinase level (IU/l). PLAT= platelet count ($x10^3/\mu l$). CHOLESTEROL= total cholesterol level (mmol/l). ONSET= time since pain commenced to administration of SK. TIME OF THERAPY= time SK given. SITE OF INFARCT= ECG territory of infarct (ANT= anterior, INF= inferior). PRE= sample taken before thrombolytic. 12= 12 hours post-thrombolytic. 24= 24 hours post-thrombolytic.

3.3 SOLUBLE FIBRIN

Soluble fibrin (SF) levels were measured in all 21 AMI patients who were enrolled into the study.

Results for SF are displayed in **Table 5-1** to **Table 5-3** (appendix). Blank squares indicate that no result is available at that time point. The reason for this was that the assay gave a significantly spurious result, usually as a consequence of cloudy or partly coagulated plasma. Alternatively, no sample was available, or the sample was coagulated.

3.3.1 Influence of Anticoagulant Employed

Before examining the results of the SF assay in detail, it is worth noting that there was no significant difference between the three anticoagulant mixtures employed (citrate, citrate plus aprotinin, citrate plus aprotinin and hirudin). Statistical analysis of the results reveals that the results correlate very well. These are displayed in **Table 3-2**.

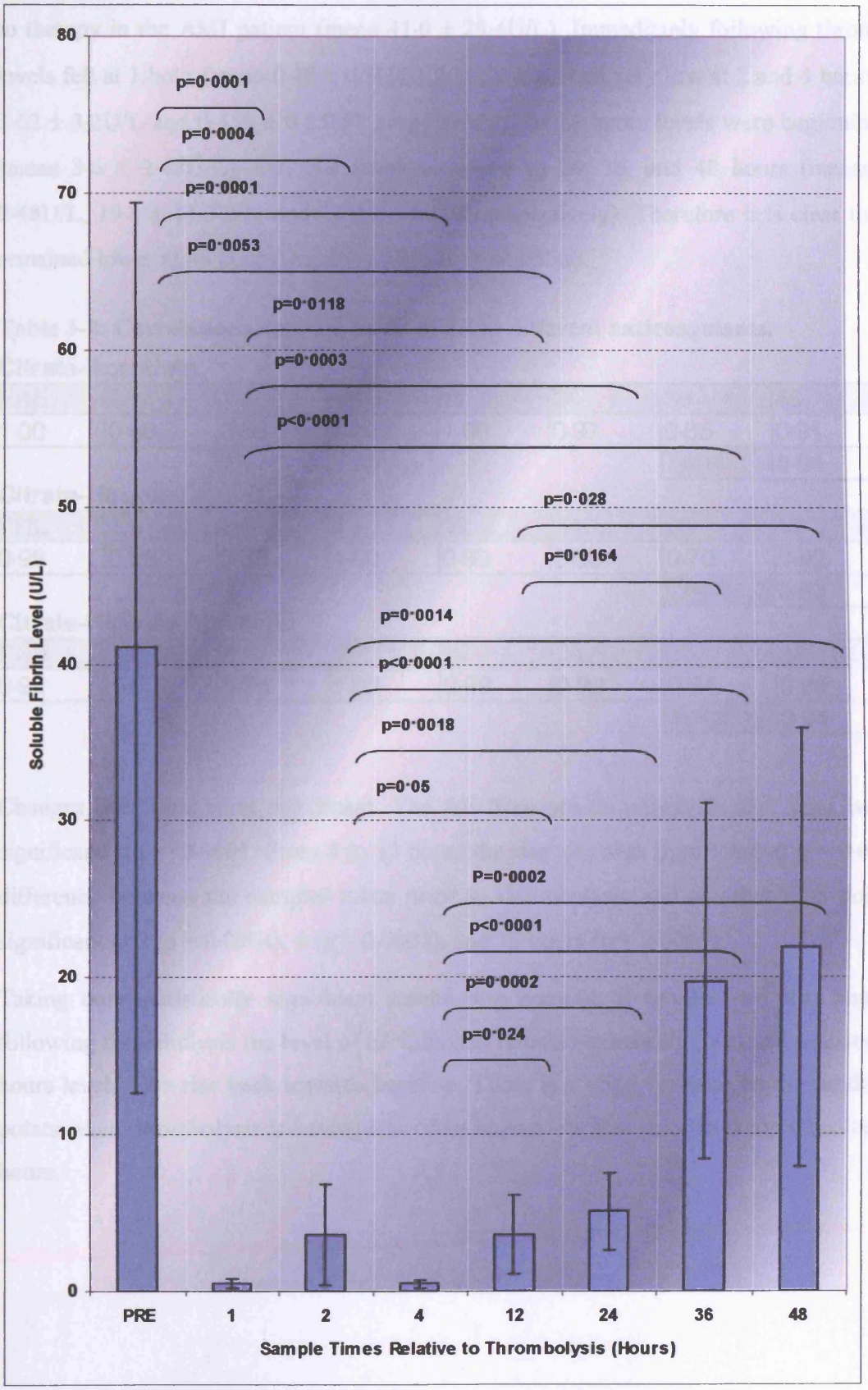
The close correlation is apparent at all time points except at 2 hours. This can be explained by examining the results more closely. This reveals the occasional very high value at this time point, whereas mostly SF is unrecordable at 2 hours.

The fact that the results correlate well suggests that there is no significant lysis occurring following sampling and, in addition, there is little further generation of thrombin (and thus fibrin).

For the purposes of further analysis, the results and statistics discussed for SF will be those with the triple anticoagulant mixture. This allows for the fact that hirudin is present, and therefore excludes any possible thrombin generation of fibrin following sampling producing the occasional spurious result.

Results for SF are displayed graphically in **Figure 3-1**.

Figure 3-1: Soluble fibrin levels following thrombolysis with SK (means ± SEM). Samples anticoagulated with citrate, aprotinin and hirudin. N=21.



Results from several hundred controls, performed at the NIBSC, suggest that SF levels are normally less than 5U/l. It can therefore be seen that SF levels are significantly elevated prior to therapy in the AMI patient (mean 41·0 ± 28·4U/L). Immediately following thrombolysis, levels fell at 1 hour (mean 0·48 ± 0·3U/L). Levels remained very low at 2 and 4 hours (means 3·62 ± 3·2U/L and 0·456 ± 0·25U/L respectively). At 12 hours levels were beginning to rise (mean 3·6 ± 2·48U/L), and this trend continued to 24, 36, and 48 hours (means 5·06 ± 2·48U/L, 19·8 ± 11·3U/L, and 21·9 ± 14·0U/L respectively). Therefore it is clear that levels remained lower at 48 hours than they were at presentation.

Table 3-2: Correlations between levels of SF in different anticoagulants.

Citrate-Aprotinin

1·00	0·98	0·58	1·00	1·00	0·97	0·85	0·91
						Total	**0·94**

Citrate-Hirudin

0·98	0·98	0·86	1·00	0·99	0·98	0·70	0·92
						Total	**0·93**

Citrate-Hirudin-Aprotinin

0·98	1·00	0·08	1·00	0·99	0·99	0·64	0·98
						Total	**0·94**

Changes over time were significant. The fall from pre-thrombolysis to 1 hour was highly significant at p = 0·0001. From 4 to 12 hours the rise was also significant at p = 0·0239. The difference between the samples taken prior to thrombolysis and at other time points were significant at 2 (p = 0·0004), 4 (p = 0·0001), and 12 hours (p = 0·0053).

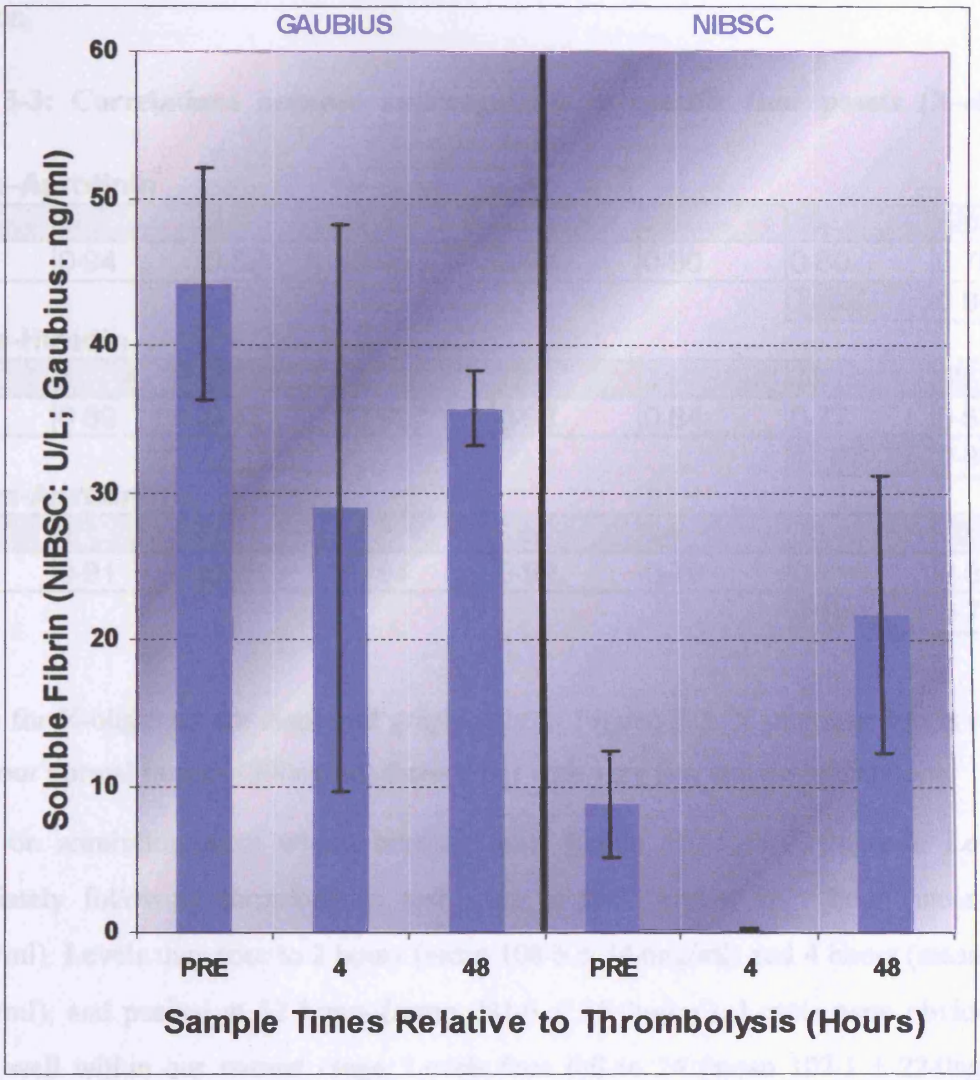
Taking only statistically significant results into account, it can be said that immediately following thrombolysis the level of SF falls and remains extremely low until 4 hours. From 4 hours levels then rise back towards baseline. There is a wide variation in the results at time points when thrombolysis is having less of an impact: such as prior to therapy and beyond 12 hours.

3.3.2 Soluble Fibrin Levels Employing A Different Monoclonal Antibody (Gaubius Laboratory)

In a small cohort of ten patients (ID: 3, 11-16, 18-21, and 26), SF levels were measured employing a fibrin-specific monoclonal antibody (Mab anti-Fb-1/2) developed at the Gaubius Laboratory by Professor Wilhelm Nieuwenhuizen's group. Samples were assayed pre-thrombolysis and at 4 and 48 hours. Assays were performed on aprotinin samples, and compared directly with paired samples employing the Mab developed at the NIBSC: Mab 5F3. Patient details can be seen in **Table 3-1**.

Results are shown in **Table 5-4**, and displayed graphically in **Figure 3-2**. The mean value of SF prior to thrombolysis was 44·19 ± 7·84ng/ml. This is only marginally above their quoted normal value of **42ng/ml**. It can be seen that the overall trend is the same however, with SF levels falling following thrombolysis, and returning towards normal at 48 hours. For both antibodies the fall to 4 hours is of significance in the Gaubius samples (Gaubius: $p = 0{\cdot}0028$; NIBSC: $p = 0{\cdot}0168$), and so is the subsequent rise to 12 hours (Gaubius: $p = 0{\cdot}0028$; NIBSC: $p = 0{\cdot}0037$).

Figure 3-2: Levels of SF following thrombolysis with SK employing two different monoclonal antibodies (means ± SEM). N=10, matched patient samples.



3.4 X-OLIGOMER

X-oligomer levels were measured in all 21 AMI patients who were enrolled into the study. Results are shown in full in **Table 5-5** to **Table 5-7** (appendix). For the purposes of further analysis, the results and statistics discussed for X-oligomer will be those employing the citrate/aprotinin anticoagulant mixture. This allows for the fact that aprotinin is present, and therefore excludes any possible fibrin degradation following sampling producing the occasional spurious result.

3.4.1 Influence Of Anticoagulant Employed

As with SF, there was no significant difference between the three anticoagulant mixtures employed. Statistical analysis of the results reveals that the results correlate very well. These

are displayed in **Table 3-3**. Correlations are not as impressive as with the SF results, and partly this can be explained by the occasional very high results skewing the standard deviation.

Table 3-3: Correlations between anticoagulants at specific time points (X-oligomer data).

Citrate-Aprotinin

0·64	0·94	0·87	0·95	0·93	0·90	0·89	0·75
							0·90

Citrate-Hirudin

0·72	0·89	0·43	0·91	0·98	0·84	0·77	0·67
							0·80

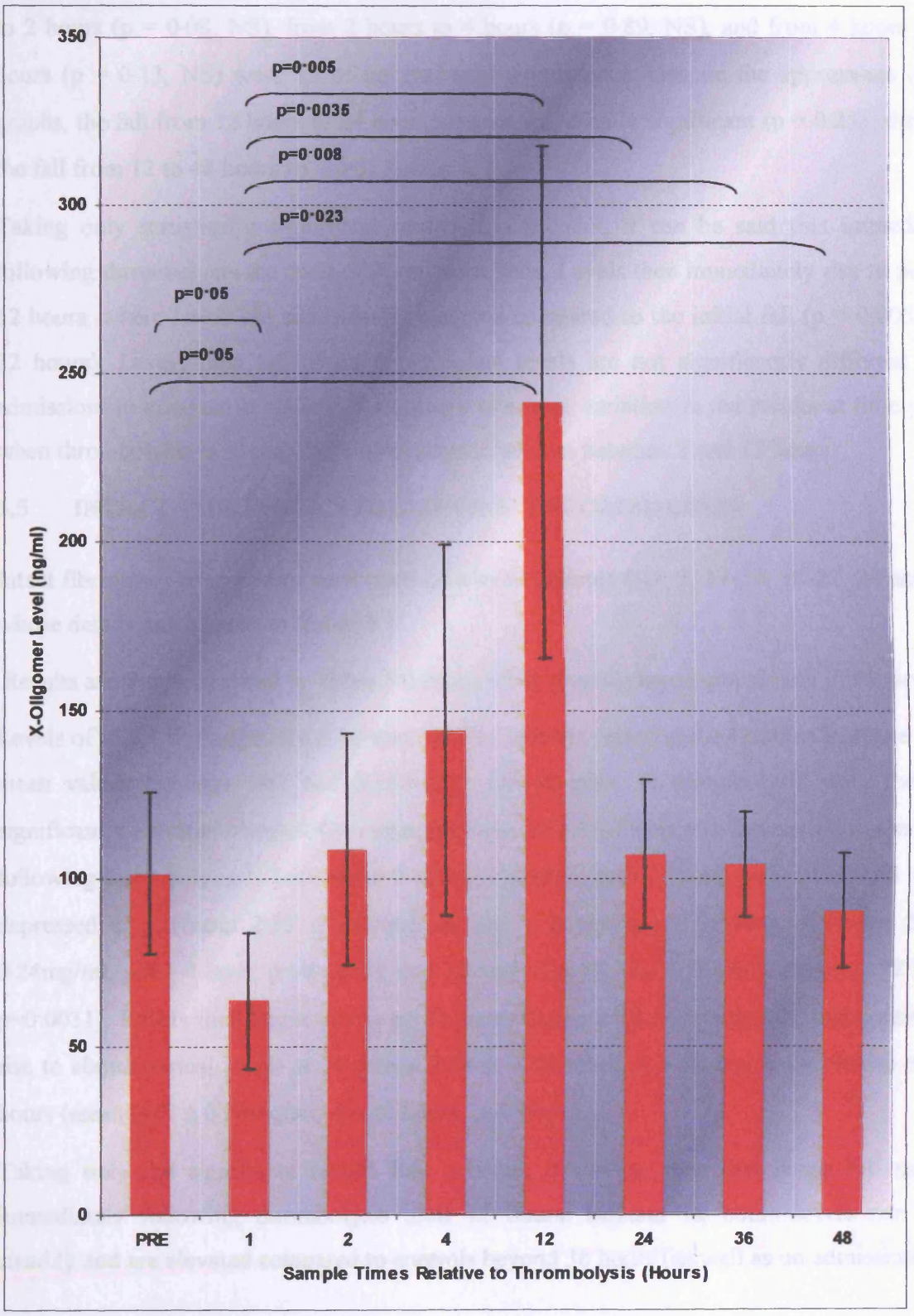
Hirudin-Aprotinin

0·76	0·91	0·26	0·94	0·92	0·79	0·71	0·84
							0·74

Results for X-oligomer are displayed graphically in **Figure 3-3**. X-oligomer levels remained within our normal range (<500ng/ml) throughout with very few isolated exceptions.

Levels on admission were within normal limits (mean 101·5 ± 23·9ng/ml). Levels fell immediately following thrombolysis and were at their lowest at 1 hour (mean 63·5 ± 20·1ng/ml). Levels then rose to 2 hours (mean 108·6 ± 34·6ng/ml) and 4 hours (mean 144·2 ± 55·1ng/ml), and peaked at 12 hours (mean 241·6 ± 75·9ng/ml). Levels were obviously still largely well within our normal range. Levels then fell to 24 (mean 107·1 ± 22·0ng/ml), 36 (mean 104·0 ± 15·6ng/ml) and 48 hours (mean 90·4 ± 17·0ng/ml).

Figure 3-3: X-oligomer levels following thrombolysis (means ± SEM). Samples anticoagulated with citrate and aprotinin. N=21.



Levels fell significantly to 1 hour (p = 0·0527). At 2 hours levels were not significantly different from those prior to therapy (p = 0·92, NS), but at 4 hours levels had risen compared to admission (p = 0·79, NS), and peaked at 12 hours (pre–12 hours, p = 0·18, NS). The reason

for the lack of significance in the results at 4 hours compared to admission is probably as a consequence of the large standard error in the results at this time point. The rises from 1 hour to 2 hours (p = 0·08, NS), from 2 hours to 4 hours (p = 0·89, NS), and from 4 hours to 12 hours (p = 0·13, NS) were all of no statistical significance. Despite the appearance of the graphs, the fall from 12 hours to 24 hours was not statistically significant (p = 0·25), although the fall from 12 to 48 hours (p = 0·013) was.

Taking only statistically significant results into account, it can be said that immediately following thrombolysis the level of X-oligomer falls. Levels then immediately rise to peak at 12 hours, where levels are significantly elevated compared to the initial fall (p = 0·005, 1 to 12 hours). Levels then fall to 48 hours, when levels are not significantly different from admission. In contrast to soluble fibrin, there is a wide variation in the results at time points when thrombolysis is having more of an impact: such as between 2 and 12 hours.

3.5 INTACT FIBRINOGEN FOLLOWING THROMBOLYSIS

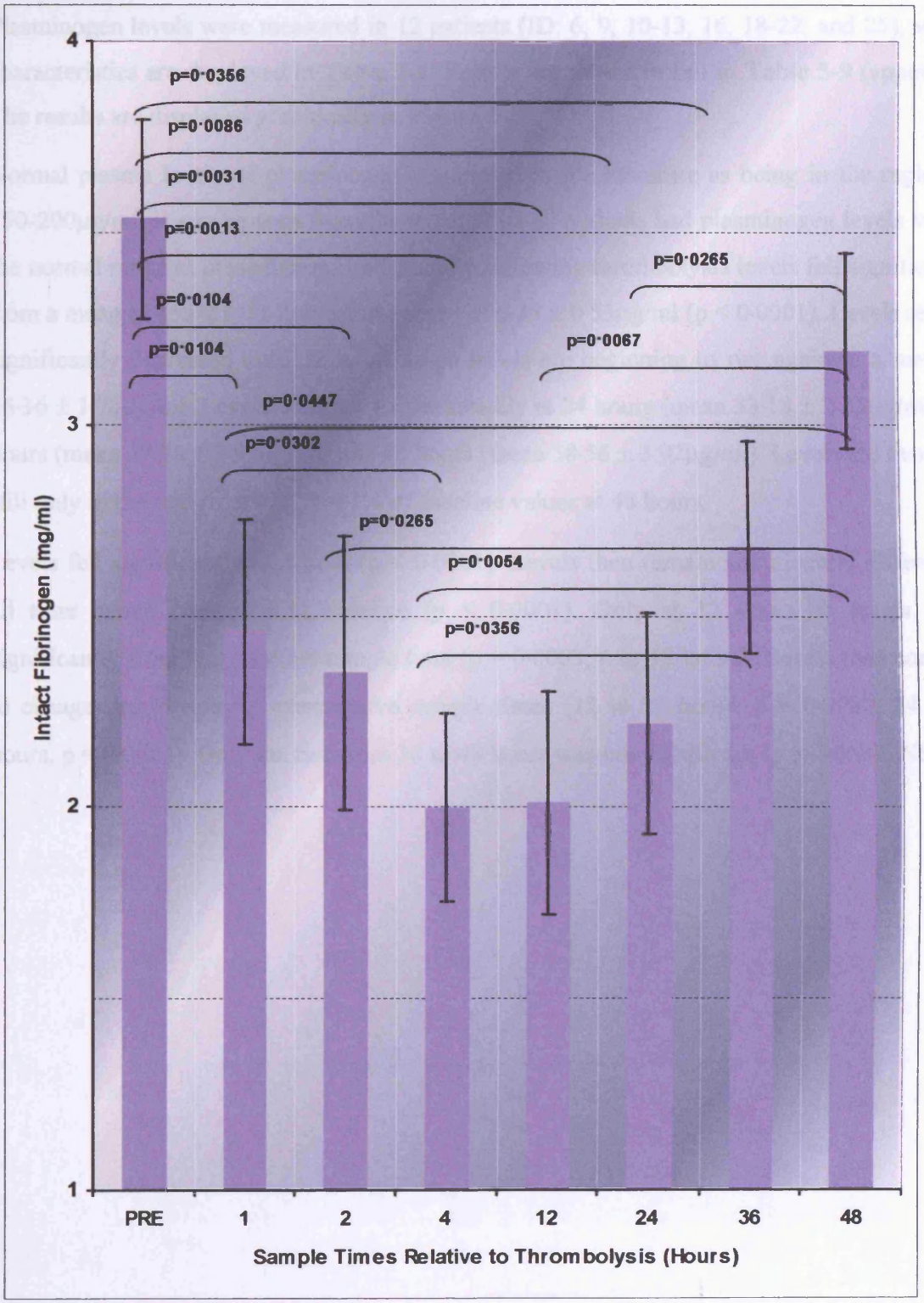
Intact fibrinogen assays were performed on eleven patients (ID: 3, 11-16, 18-21, 24 and 26), whose details can be seen in **Table 3-1**.

Results are shown in detail in **Table 5-8** (appendix), and displayed graphically in **Figure 3-4**.

Levels of intact fibrinogen from normal healthy subjects tested at the Gaubius institute reveal mean values between 1·87 and 2·63mg/ml. Levels prior to thrombolysis were therefore significantly elevated compared to controls (mean 3·54 ± 0·26mg/ml). Levels fell immediately following thrombolysis (1 hour mean 2·46 ± 0·29mg/ml, pre – 1 hour, p= 0·0104) and remain depressed at 2 (mean 2·35 ± 0·36mg/ml, pre – 2 hours, p= 0·0104), 4 (mean 2·00 ± 0·24mg/ml, pre – 4 hour, p= 0·0013), and 12 hours (mean 2·01 ± 0·29mg/ml, pre – 12 hours, p=0·0031). Levels then begin to rise at 24 hours (mean 2·22 ± 0·29mg/ml), and continue to rise to above normal levels at 36 (mean 2·68 ± 0·28mg/ml, 4 – 36 hours, p=0·0356) and 48 hours (mean 3·19 ± 0·26mg/ml, 4 – 48 hours, p=0·0054).

Taking only the significant results into account, it can be seen that levels fall steadily immediately following thrombolysis until 12 hours. Beyond 12 hours levels rise again steadily and are elevated compared to controls beyond 36 hours (as well as on admission).

Figure 3-4: Intact fibrinogen levels following thrombolysis (means ± SEM). N=11.



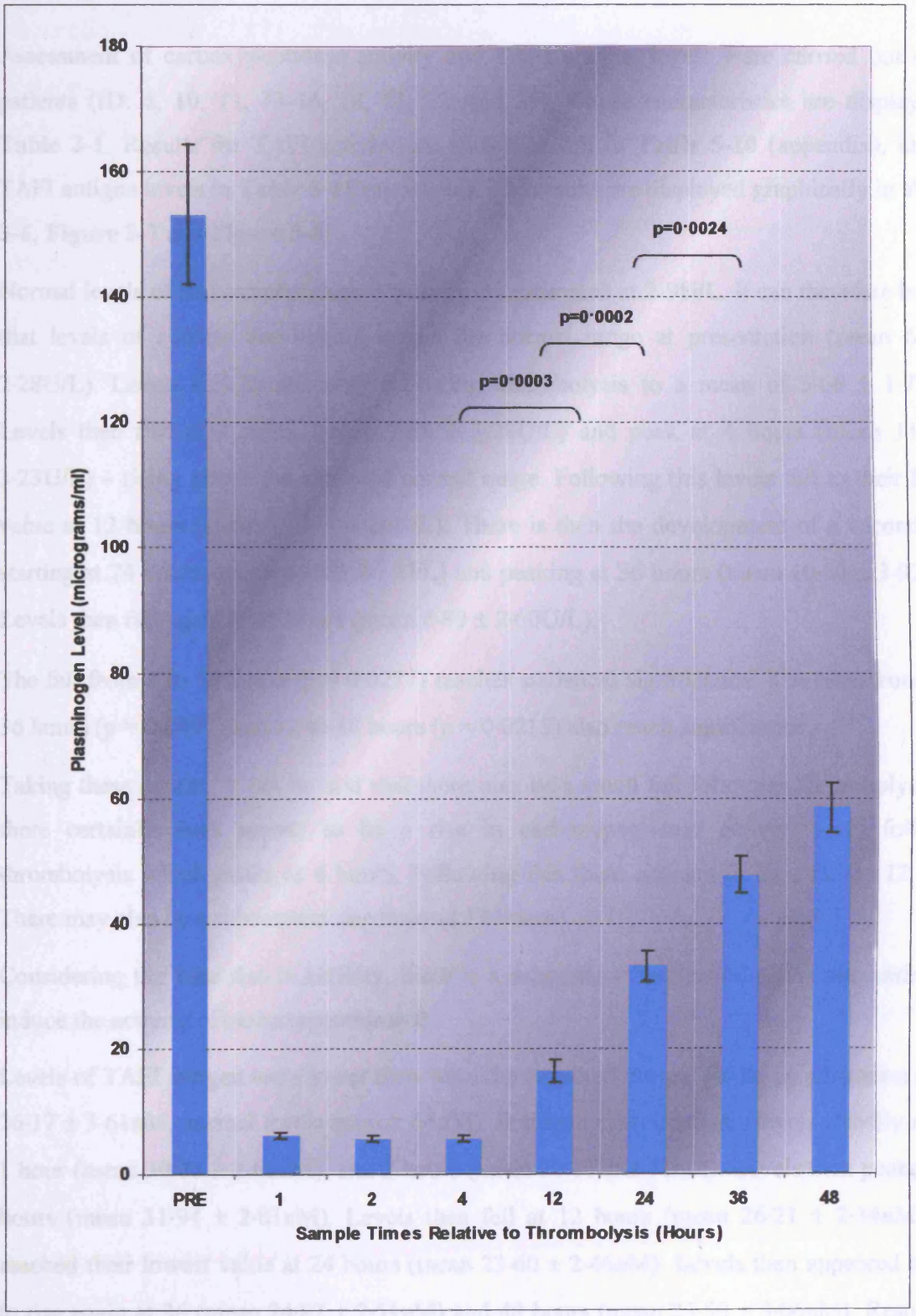
3.6 PLASMINOGEN LEVELS FOLLOWING THROMBOLYSIS

Plasminogen levels were measured in 12 patients (ID: 6, 9, 10-13, 16, 18-22, and 25), whose characteristics are displayed in **Table 3-1**. Results are shown in full in **Table 5-9** (appendix). The results are displayed graphically in **Figure 3-5**.

Normal plasma levels of plasminogen are quoted in the literature as being in the region of 150-200μg/ml. It can be seen from the results that all patients had plasminogen levels within the normal range at presentation. Immediately following thrombolysis levels fell significantly from a mean of 153·2 ± 11·2μg/ml to a mean of 6·18 ± 0·53μg/ml ($p < 0·0001$). Levels remain significantly depressed until 12 hours when levels are beginning to rise again to a mean of 16·36 ± 1·76μg/ml. Levels continue to rise steadily at 24 hours (mean 33·18 ± 2·35μg/ml), 36 hours (mean 47·75 ± 2·92μg/ml) and 48 hours (mean 58·56 ± 3·92μg/ml). Levels are therefore still only in the region of 41·2 ± 4·1% of baseline values at 48 hours.

Levels fell significantly to 1 hour ($p < 0·0001$). Levels then remain significantly different at all time points compared to baseline ($p < 0·0001$). Only at 12 hours do levels differ significantly from the previous sample time ($p = 0·0003$, 4 to 12 hours). Levels then continue to change significantly at consecutive sample times (12 to 24 hours, $p = 0·0002$; 24 to 36 hours, $p = 0·0024$). Only the rise from 36 to 48 hours was not significant ($p = 0·0689$, NS).

Figure 3-5: Plasminogen levels following thrombolysis with streptokinase (means ± SEM). N=12.



3.7 CARBOXYPEPTIDASE B (TAFI) ACTIVITY AND TAFI ANTIGEN LEVELS FOLLOWING THROMBOLYSIS

Assessment of carboxypeptidase activity and TAFI antigen levels were carried out on 10 patients (ID: 6, 10, 11, 13-16, 18, 21, 22, and 25), whose characteristics are displayed in **Table 3-1**. Results for TAFI activity are shown in full in **Table 5-10** (appendix), and for TAFI antigen levels in **Table 5-11** (appendix). The results are displayed graphically in **Figure 3-6**, **Figure 3-7** and **Figure 3-8**.

Normal levels of carboxypeptidase B activity are estimated at 2-9U/L. It can therefore be seen that levels of activity are largely within the normal range at presentation (mean 6·53 ± 2·28U/L). Levels fall immediately following thrombolysis to a mean of 3·66 ± 1·77U/L. Levels then rise at 2 hours (mean 7·55 ± 3·28U/L) and peak at 4 hours (mean 11·83 ± 3·23U/L) – rising above the expected normal range. Following this levels fall to their lowest value at 12 hours (mean 2·38 ± 1·26U/L). There is then the development of a second peak starting at 24 hours (mean 8·32 ± 3·1 U/L) and peaking at 36 hours (mean 10·50 ± 3·92U/L). Levels then fall again at 48 hours (mean 8·89 ± 2·60U/L).

The fall from 4 to 12 hours ($p = 0·0277$) reaches statistical significance. The rises from 12 to 36 hours ($p = 0·0407$) and 12 to 48 hours ($p = 0·0215$) also reach significance.

Taking these results, it can be said that there may be a small fall following thrombolysis, but there certainly does appear to be a rise in carboxypeptidase activity early following thrombolysis which peaks at 4 hours. Following this there appears to be a fall to 12 hours. There may also be a subsequent rise beyond 12 hours.

Considering the later rise in activity, there is a suggestion that thrombolysis may ultimately induce the activity of carboxypeptidase B.

Levels of TAFI antigen were lower than were the expected normal limits on admission (mean 26·17 ± 3·61nM, normal levels approx 54nM). Following thrombolysis levels steadily rose at 1 hour (mean 30·75 ± 2·62nM), and 2 hours (mean 30·75 ± 2·71nM). Levels then peaked at 4 hours (mean 31·94 ± 2·81nM). Levels then fell at 12 hours (mean 26·21 ± 2·34nM), and reached their lowest value at 24 hours (mean 23·60 ± 2·46nM). Levels then appeared to start to rise again at 36 (mean 24·82 ± 2·51nM) and 48 hours (mean 25·50 ± 3·06nM). Results are displayed graphically in **Figure 3-7**.

The changes over time were statistically significant only between 4 and 24 hours ($p = 0.0412$), when levels are seen to fall.

In summary, levels of TAFI antigen seem to rise to 4 hours and then certainly fall.

Relating TAFI levels with TAFI activity it would appear that thrombolysis promotes the release of TAFI, and this is accompanied by a rise in TAFI activity. At four hours the levels of TAFI antigen fall, but paradoxically it would appear that TAFI activity subsequently begins to increase. It would appear that there is a complex balance between production and/or release of TAFI and its ultimate clearance and/or inhibition.

Figure 3-6: Carboxypeptidase activity following thrombolysis (means ± SEM). N=10.

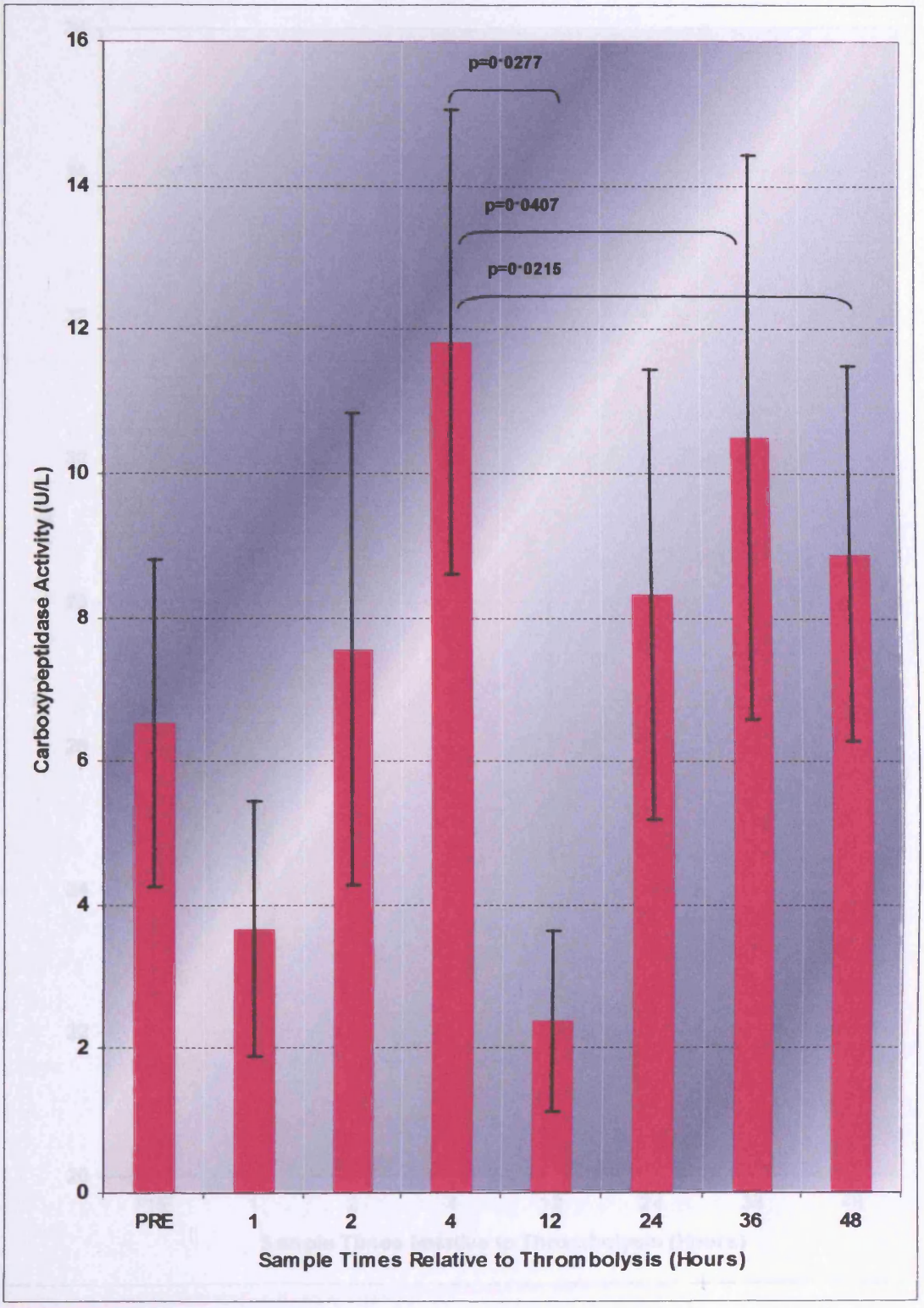


Figure 3-7: Levels of TAFI antigen following thrombolysis (means ± SEM). N=10.

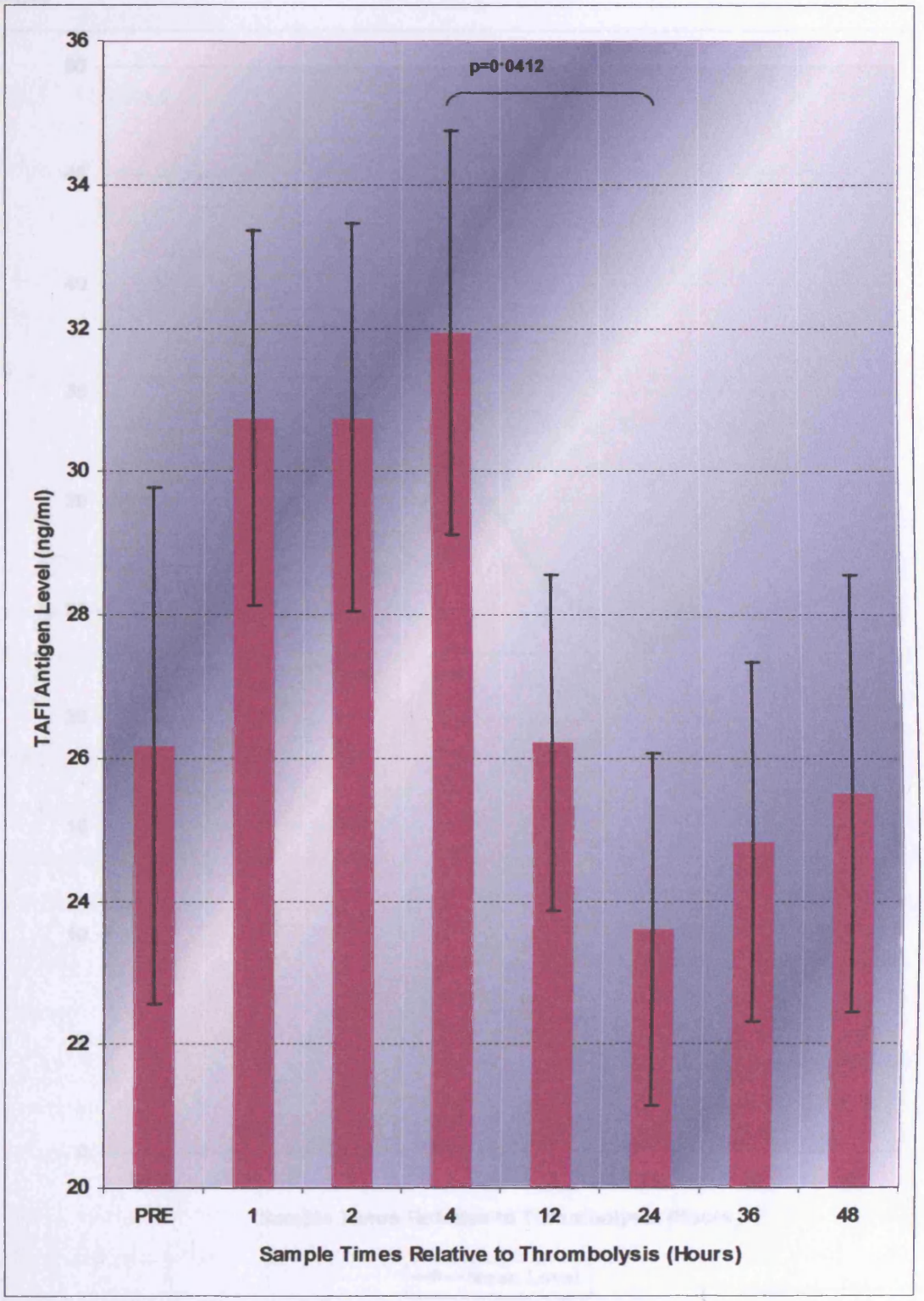
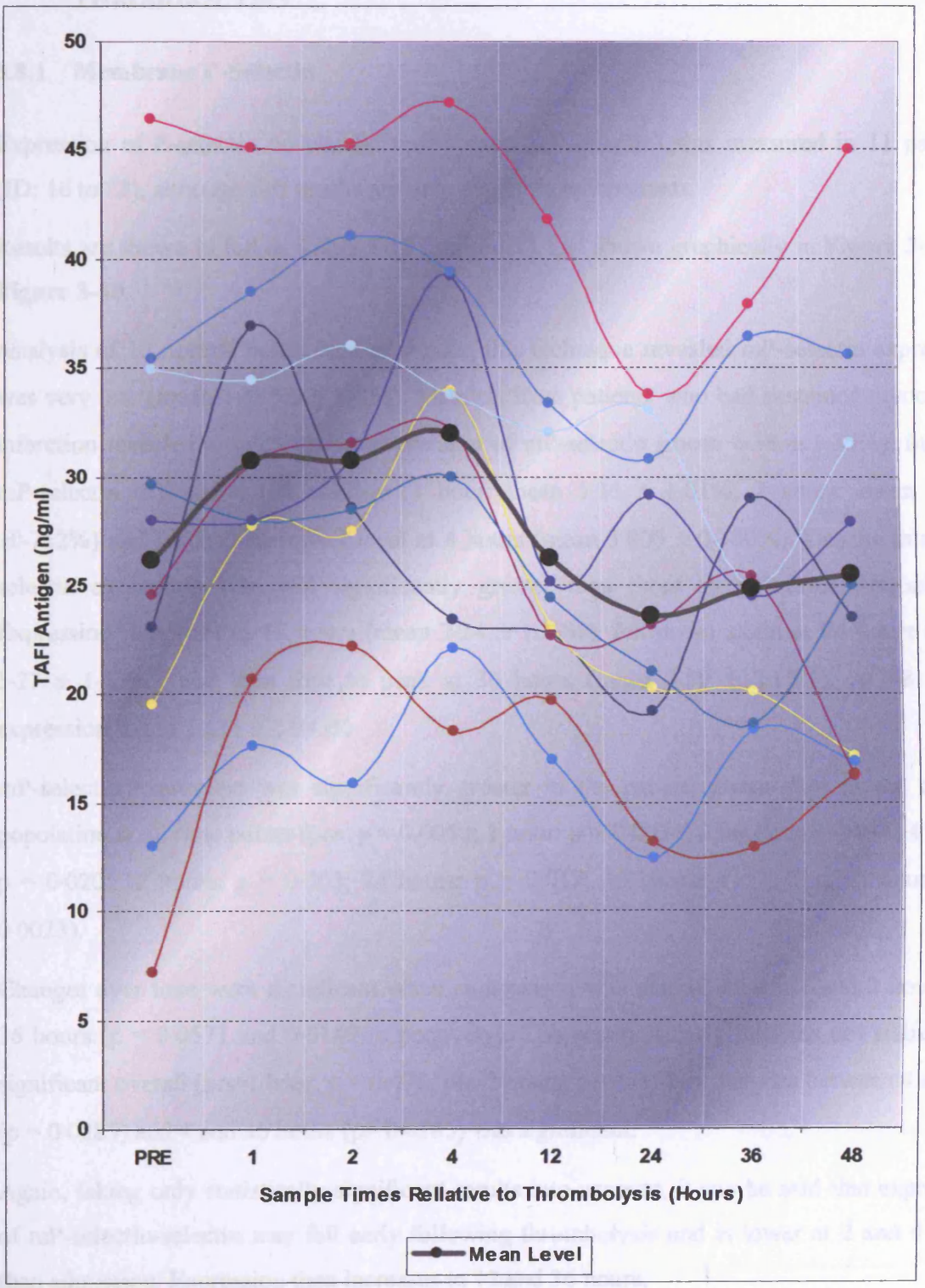


Figure 3-8: TAFI antigen following thrombolysis with streptokinase: individual patient data.



3.8 MEMBRANE MARKERS OF PLATELET ACTIVATION FOLLOWING THROMBOLYSIS

3.8.1 Membrane P-Selectin

Expression of P-selectin on platelet membranes (mP-selectin) was measured in 11 patients (ID: 16 to 28), although full results are only available in 9 patients.

Results are shown in full in **Table 5-12** (appendix), and shown graphically in **Figure 3-9** and **Figure 3-10**.

Analysis of 10 normal volunteers employing this technique revealed mP-selectin expression was very low (mean 1·535 ± 0·352%). Platelets from patients who had sustained myocardial infarction revealed a much higher expression of mP-selectin (mean 6·38 ± 1·35%). Initially mP-selectin expression fell steadily (1 hour: mean 5·35 ± 1·01%; 2 hours: mean 3·955 ±0·752%) and reached its lowest level at 4 hours (mean 3·805 ± 0·780%). Despite this, mP-selectin expression was still significantly greater than those in a 'normal' population. Expression then rose to 12 hours (mean 7·24 ± 1·37%), fell down again at 24 hours (mean 5·27 ± 1·23%), and then rose to peak at 36 hours (mean 8·59 ± 2·10%). At 48 hours, expression fell to 5·611 ± 0·943%.

mP-selectin expression was significantly greater in the patient group than in the control population at all time points (pre: $p = 0·0052$; 1 hour: $p = 0·0038$; 2 hours: $p = 0·011$; 4 hours: $p = 0·020$; 12 hours: $p = 0·002$; 24 hours: $p = 0·017$; 36 hours: $p = 0·011$; 48 hours: $p = 0·0023$).

Changes over time were significant when comparison was made between 1 and 2 hours and 36 hours ($p = 0·0571$ and 0·0149 respectively). The apparent early fall was not statistically significant overall (pre-1 hour, $p = 0·470$; pre-2 hours, $p = 0·1886$). The rise between 4 and 12 ($p = 0·0385$) and 4 and 36 hours ($p = 0·0185$) was significant.

Again, taking only statistically significant results into account, it can be said that expression of mP-selectin-selectin may fall early following thrombolysis and is lower at 2 and 4 hours than admission. Expression then increases to 12 and 36 hours.

Figure 3-9: Membrane P-selectin expression following thrombolysis (means ± SEM, n=11).

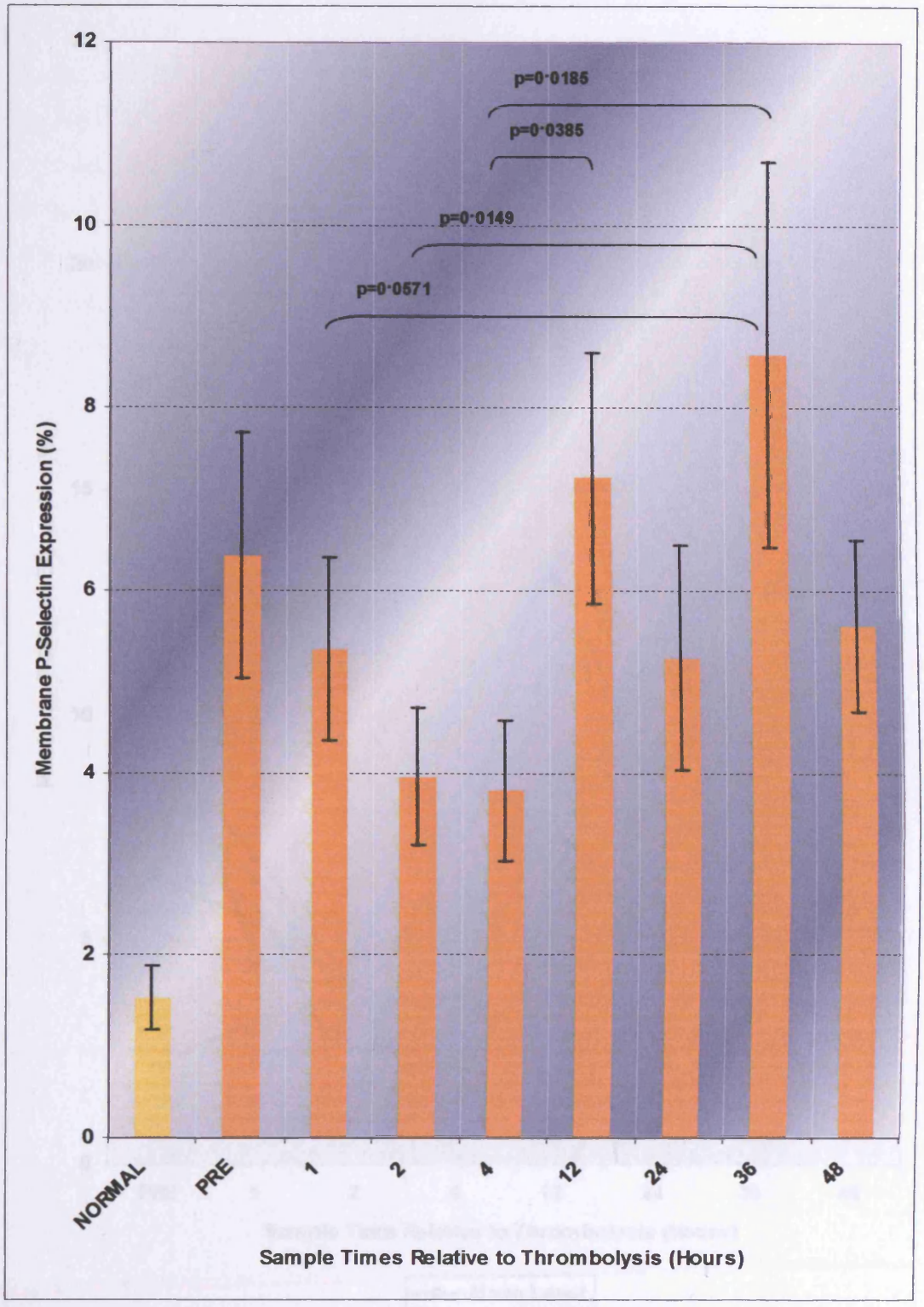
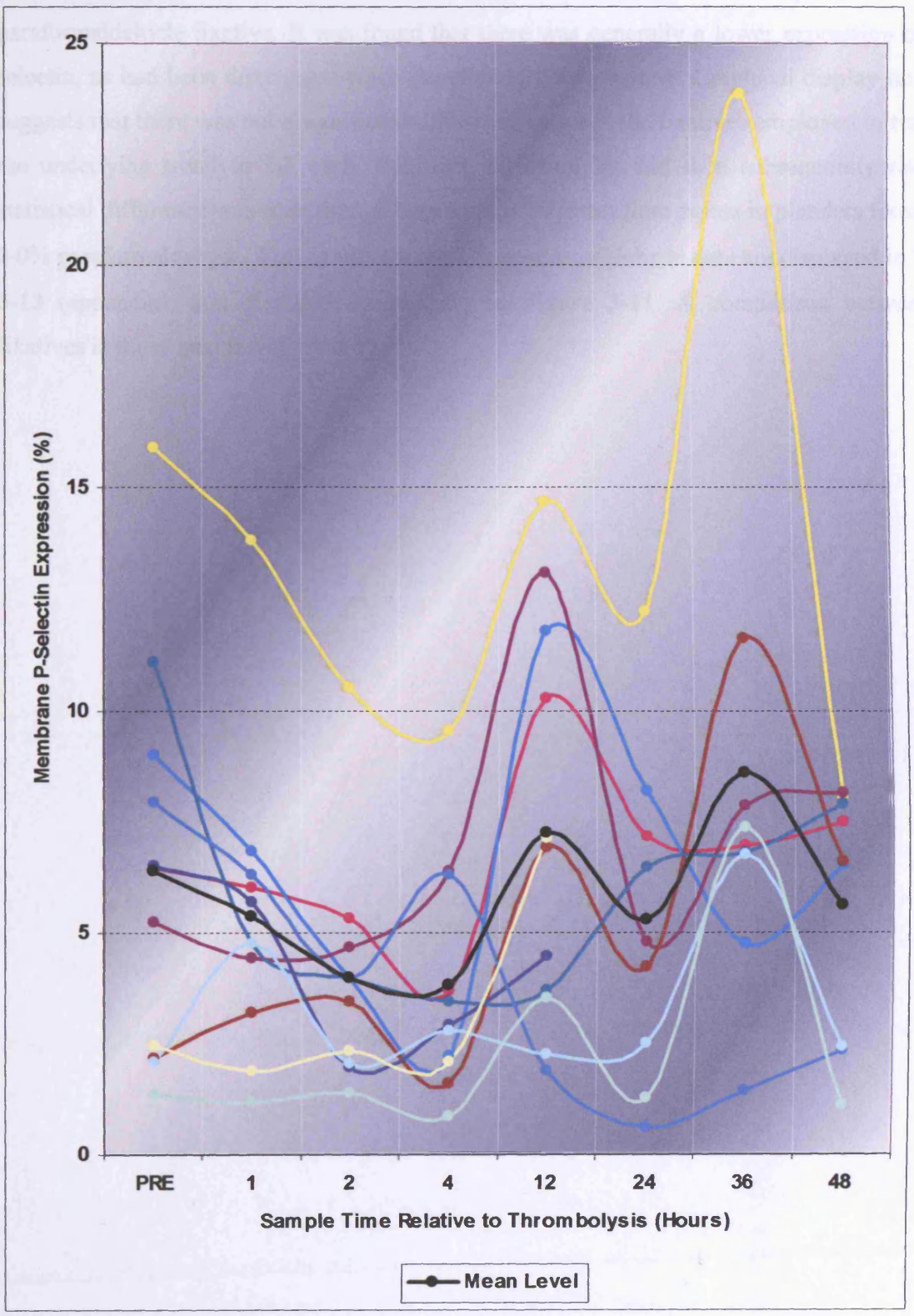


Figure 3-10: Membrane P-selectin expression following thrombolysis (individual patient data).



Samples were also analysed in a small cohort of patients (patients 23 to 28) employing 2% paraformaldehyde because of concern about the possible inadequacy of 0·5% paraformaldehyde fixative. It was found that there was generally a lower expression of mP-selectin, as had been discovered when developing the technique. Graphical display however suggests that there was not a significant difference between the fixatives employed in terms of the underlying trend to fall early following thrombolysis and then subsequently rise. No statistical difference was seen though between the different time points in platelets fixed with 2·0% paraformaldehyde. Full results for the 2% paraformaldehyde data are displayed in **Table 5-13** (appendix), and displayed graphically in **Figure 3-11**. A comparison between the fixatives is illustrated in **Figure 3-12**.

Figure 3-11: Membrane P-selectin expression following thrombolysis employing 2·0% paraformaldehyde as fixative (means ± SEM, n=5).

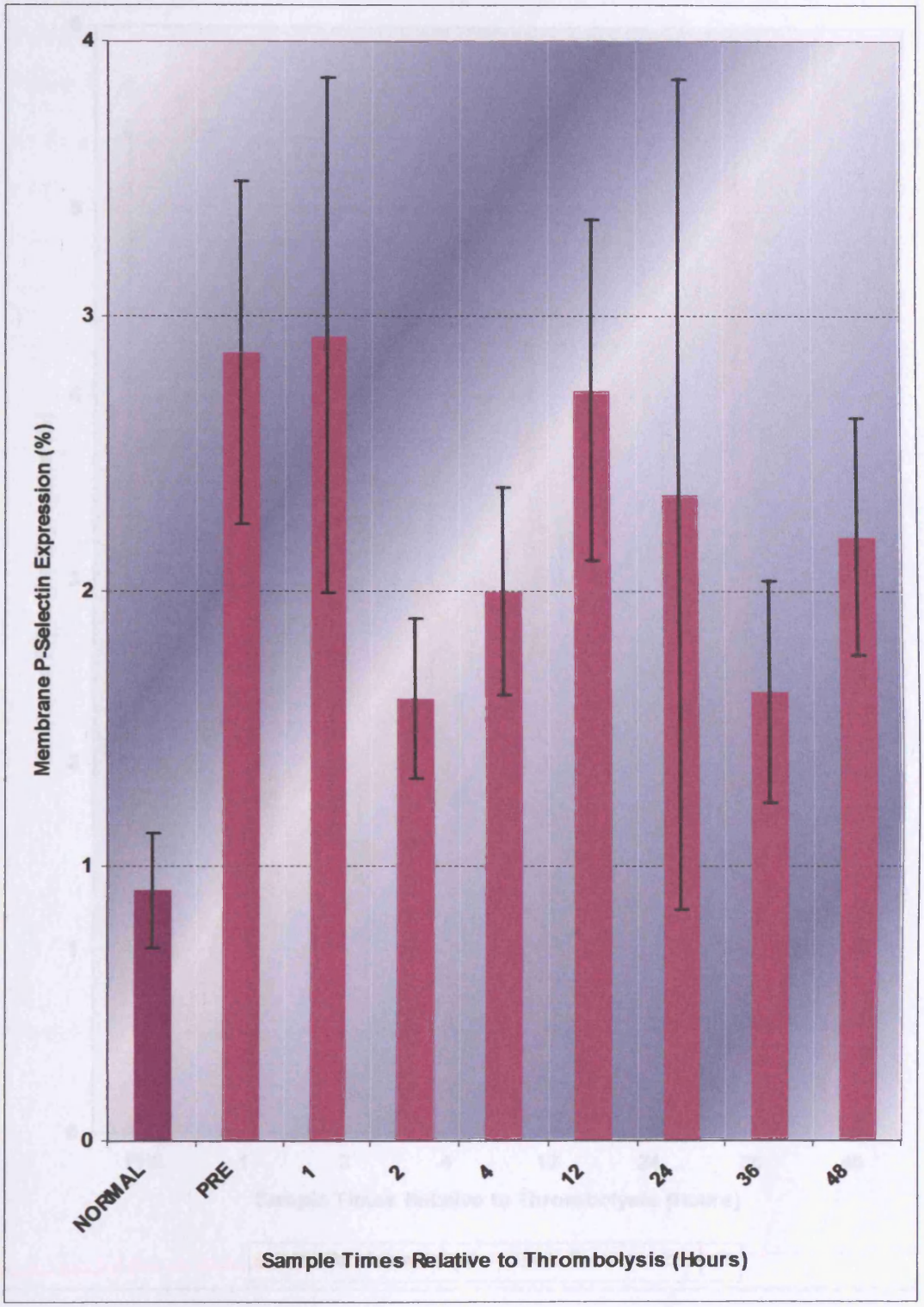
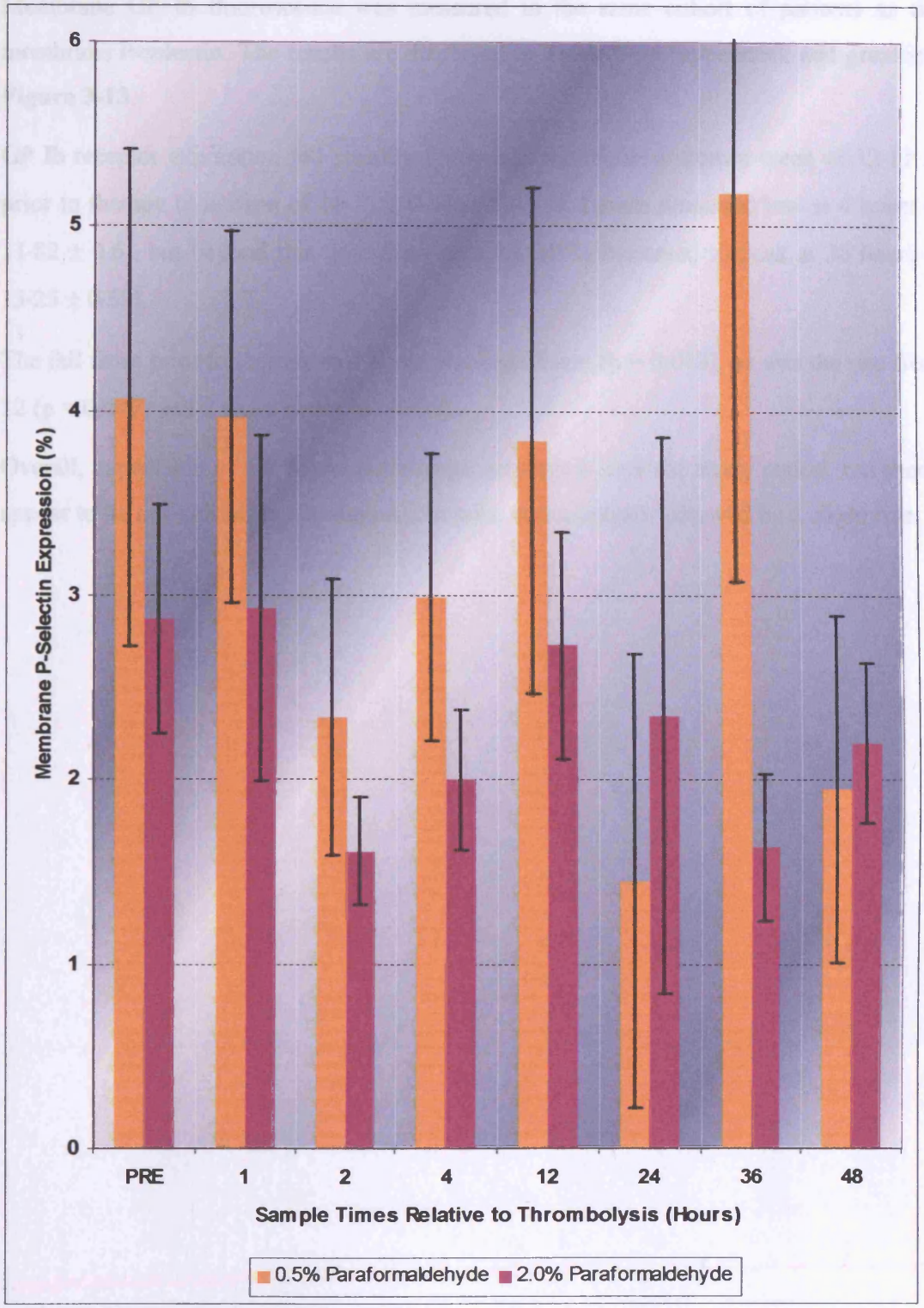


Figure 3-12: Effect of 0·5% and 2·0% paraformaldehyde fixatives on the expression of membrane P-selectin following thrombolysis (matched patient means, n=5).



3.8.2 GP Ib Fluorescence Following Thrombolysis

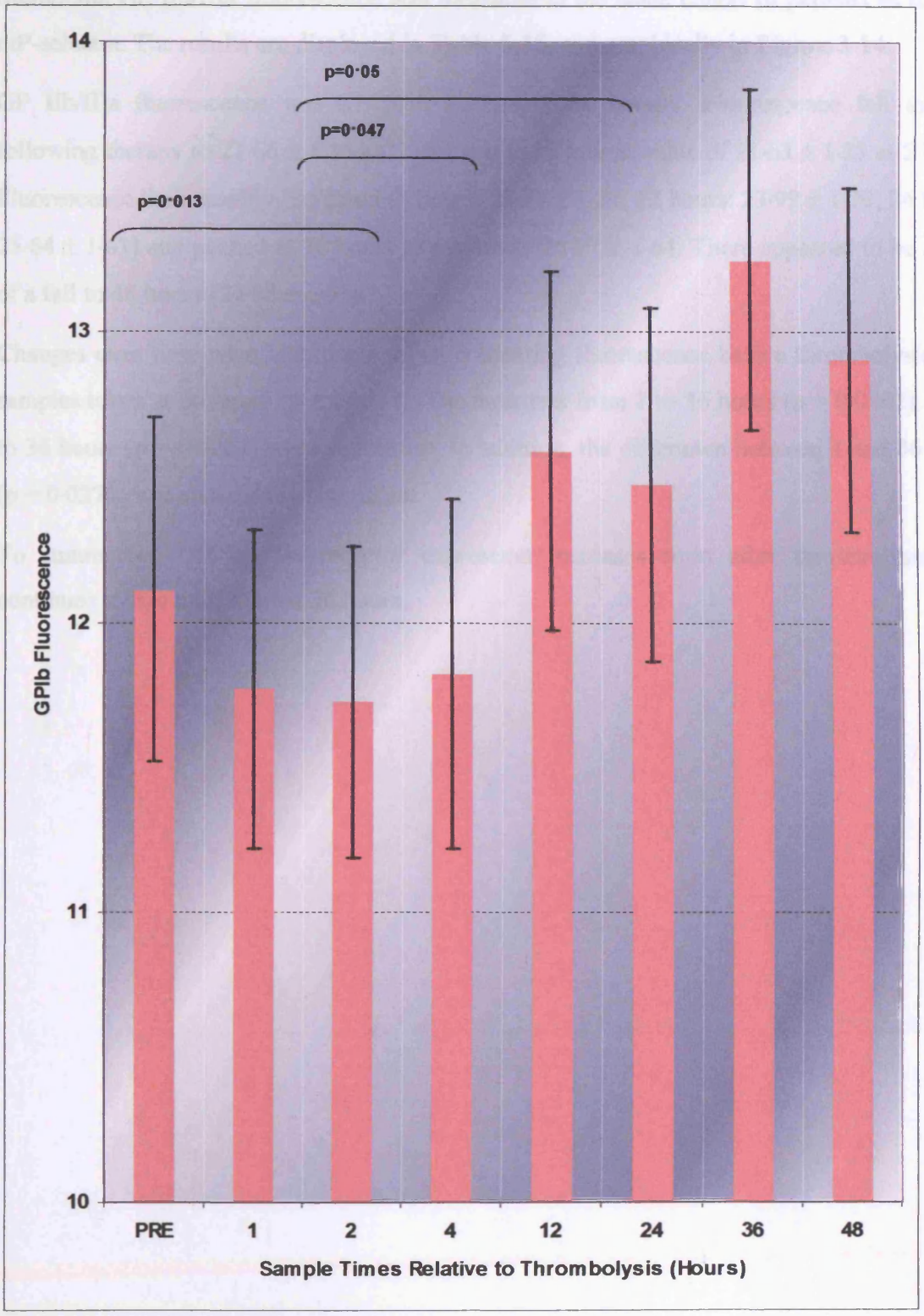
Membrane GP Ib fluorescence was measured in the same cohort of patients as that for membrane P-selectin. The results are displayed in **Table 5-14** (appendix), and graphically in **Figure 3-13**.

GP Ib receptor expression fell steadily following thrombolysis from a mean of 12·11 ± 0·59 prior to therapy to a mean of 11·73 ± 0·54 at 2 hours. Levels remained low at 4 hours (mean 11·82 ± 0·6), but beyond this time expression of GP Ib increased to peak at 36 hours (mean 13·25 ± 0·59).

The fall from prior to therapy to 4 hours was significant ($p = 0·013$), as was the rise from 2 to 12 ($p = 0·047$) and 2 to 36 hours ($p = 0·05$).

Overall, expression of GP Ib did not change very much over the study period, but there does appear to be a slight fall in expression following thrombolysis followed by a slight rise.

Figure 3-13: GP Ib fluorescence following thrombolysis with streptokinase (means ± SEM, n=11).



3.8.3 Membrane GP IIb/IIIa Fluorescence Following Thrombolysis

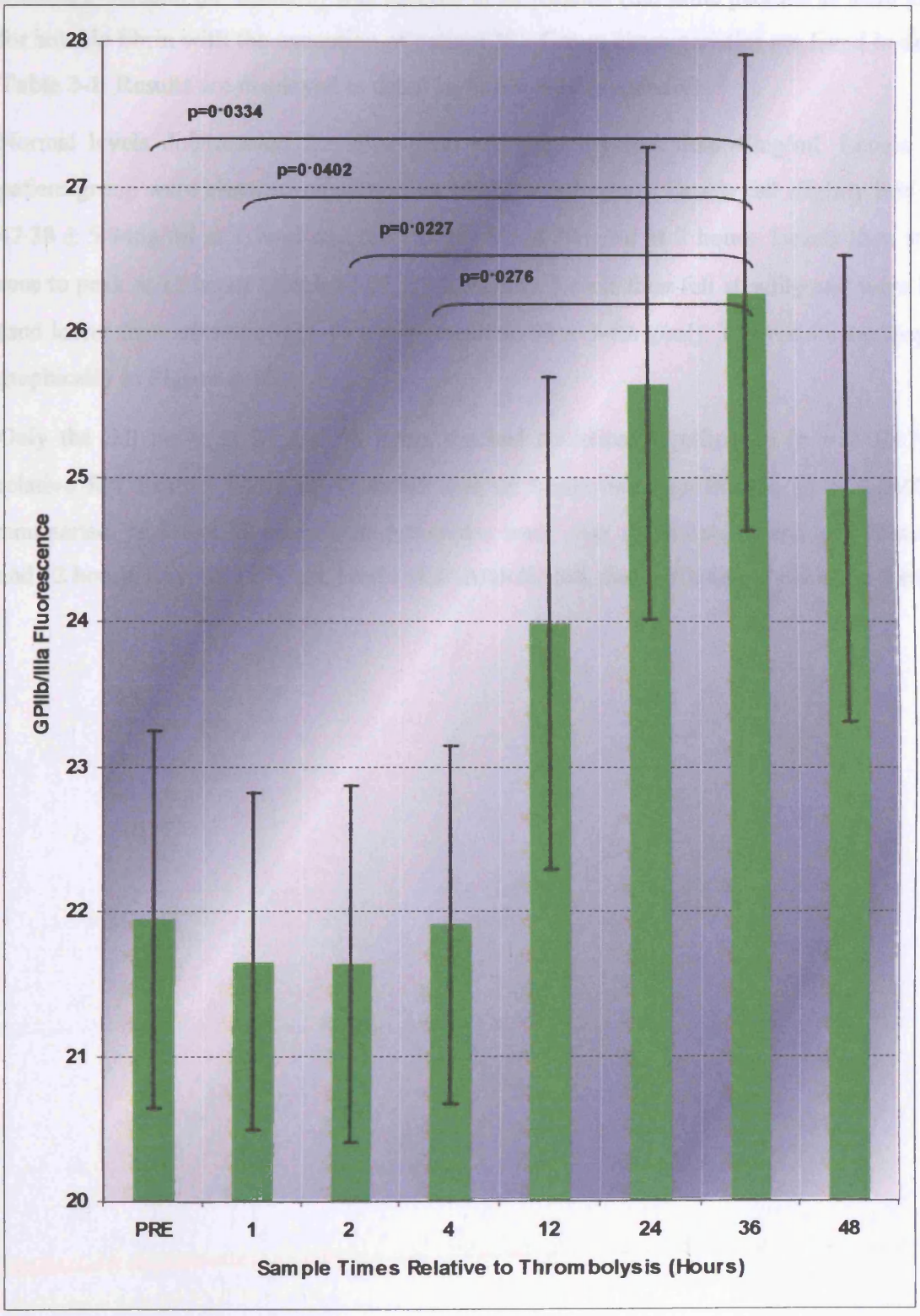
Membrane GP IIb/IIIa fluorescence was measured in the same cohort of patients as that for mP-selectin. The results are displayed in **Table 5-15**, and graphically in **Figure 3-14**.

GP IIb/IIIa fluorescence was 21·95 ± 1·30 prior to therapy. Fluorescence fell initially following therapy to 21·66 ± 1·16 at 1 hour and to its lowest value of 21·63 ± 1·23 at 2 hours. Fluorescence then steadily increased (4 hours: 21·91 ± 1·24; 12 hours: 23·99 ± 1·70; 24 hours: 25·64 ± 1·63) and peaked at 36 hours to a mean of 26·27 ± 1·64. There appeared to be a start of a fall to 48 hours (24·92 ± 1·61).

Changes over time were significant when comparing fluorescence before thrombolysis with samples taken at 36 hours ($p = 0·0334$). The increases from 1 to 36 hours ($p = 0·0402$), and 2 to 36 hours ($p = 0·0227$) were significant. In addition, the difference between 4 and 36 hours ($p = 0·0276$) was statistically significant.

To summarise, GP IIb/IIIa receptor expression increases soon after thrombolysis, and continues to rise until at least 36 hours.

Figure 3-14: Membrane GP IIb/IIIa fluorescence following thrombolysis with streptokinase (means ± SEM, n=11).



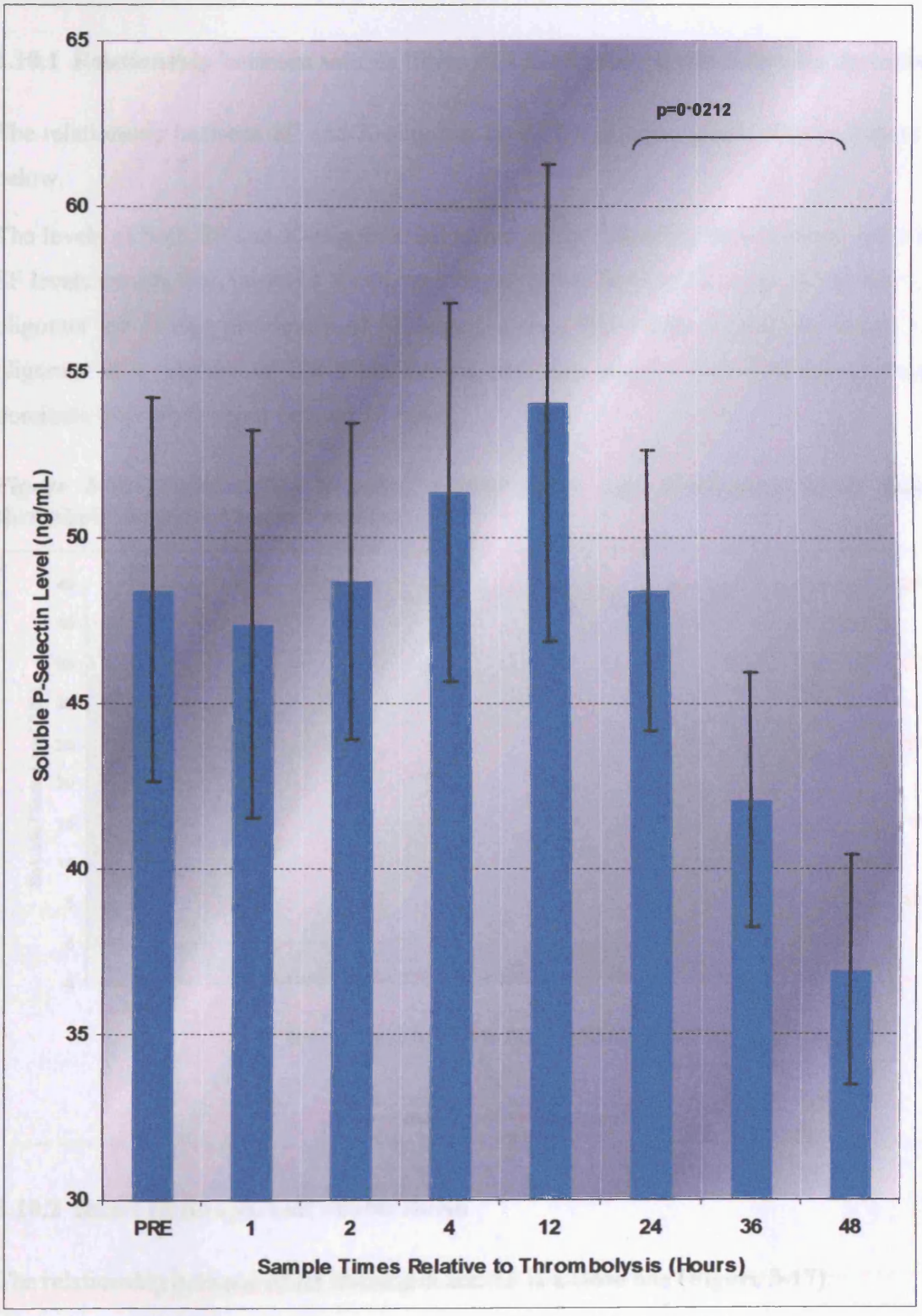
3.9 SOLUBLE P-SELECTIN LEVELS FOLLOWING THROMBOLYSIS

Soluble P-selectin (sP-selectin) was assayed in 20 patients (the same patients as were assayed for soluble fibrin with the exception of patient 28). Group characteristics are listed in detail in **Table 3-1**. Results are displayed in detail in **Table 5-16** (appendix).

Normal levels documented for sP-selectin are generally less than 40ng/ml. Levels in the patient group were elevated on admission (48·42 ± 5·8ng/ml). Levels fell slightly initially to 47·38 ± 5·84ng/ml at 1 hour and rose to 48·68 ± 4·79ng/ml at 2 hours. Levels then steadily rose to peak at 12 hours (mean 54·07 ± 7·22ng/ml). Levels then fell steadily and were lowest (and lower than admission) at 48 hours (mean 36·94 ± 3·49ng/ml). The results are displayed graphically in **Figure 3-15**.

Only the fall between 24 and 48 hours reached statistical significance ($p = 0·0213$). The relative fall from 4 hours to 48 hours was of borderline significance ($p = 0·0607$). To summarise, levels of sP-selectin may increase soon after thrombolysis and peak between 4 and 12 hours. Beyond 12 hours, levels of sP-selectin fall, and fall below those at presentation.

Figure 3-15: Soluble P-selectin levels following thrombolysis (means ± SEM, n=20).



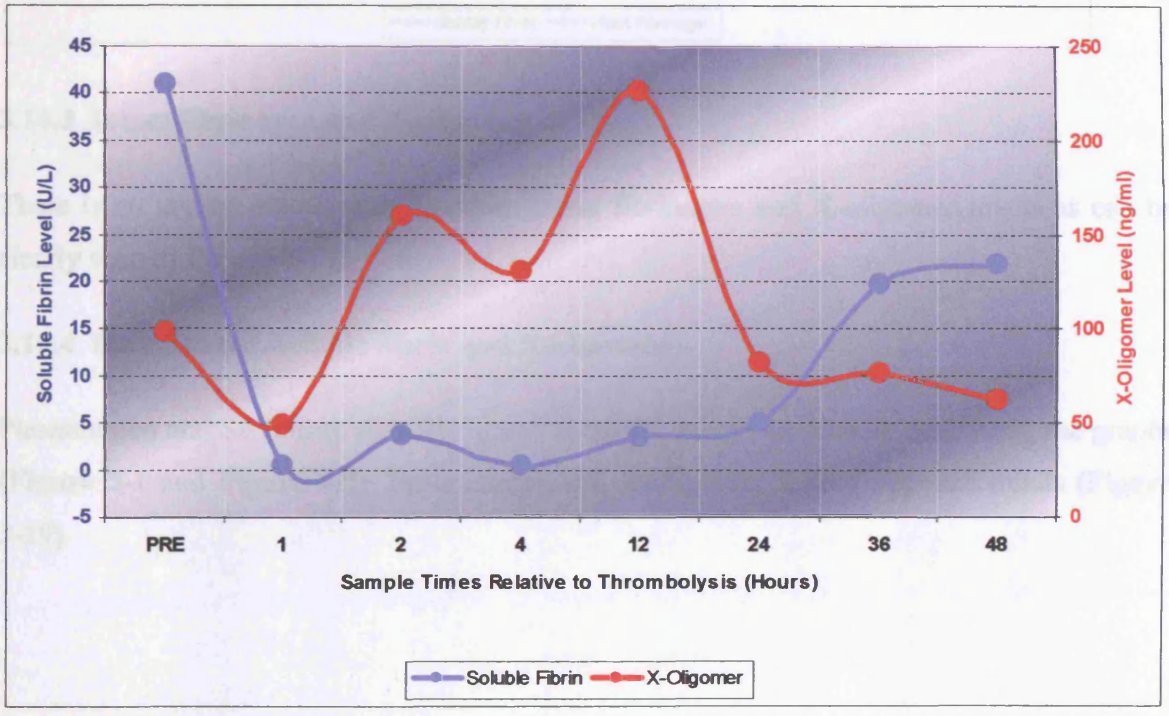
3.10 RELATIONSHIP BETWEEN THE VARIOUS MARKERS

3.10.1 Relationship between soluble fibrin and X-oligomer levels following thrombolysis

The relationship between SF and X-oligomer levels is displayed graphically in **Figure 3-16**, below.

The levels of both SF and X-oligomer fall immediately following thrombolysis, but whereas SF levels remain low, levels of X-oligomer begin to rise. Beyond 12 hours, as the levels of X-oligomer are falling, the levels of SF begin to rise. These data support the claim that X-oligomer is a product of fibrin breakdown, and also suggest that coagulation begins to dominate over fibrinolysis beyond 12 hours.

Figure 3-16: Relationship between soluble fibrin and X-oligomer levels following thrombolysis (paired patient means).

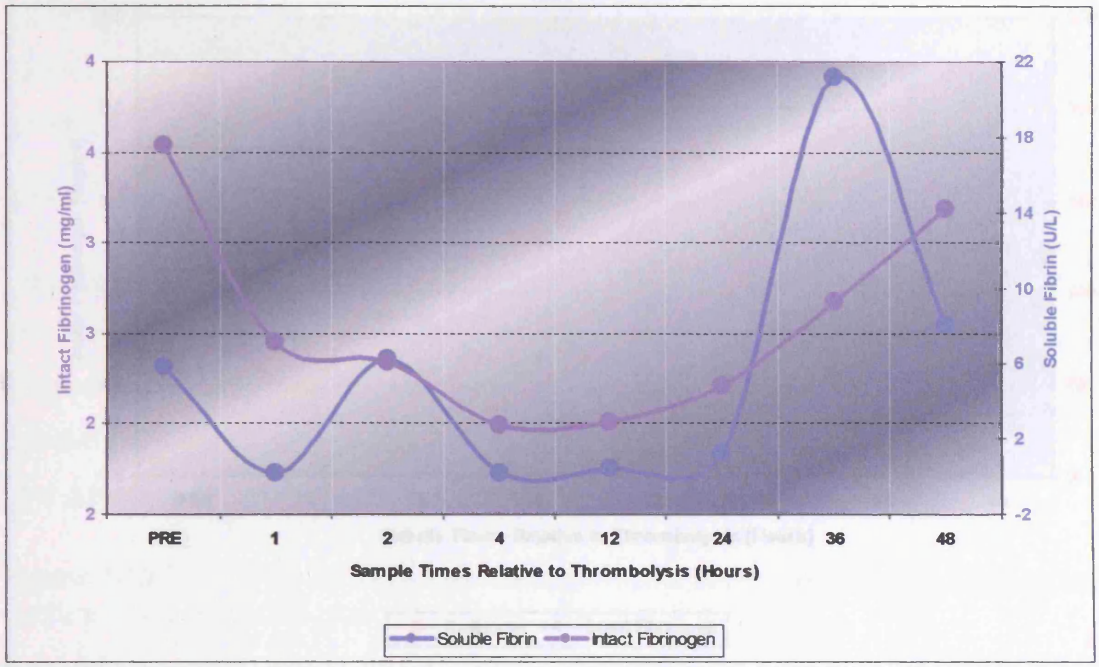


3.10.2 Intact fibrinogen and soluble fibrin

The relationship between intact fibrinogen and SF is a close one (**Figure 3-17**).

It can be seen that levels of both fibrinogen and SF fall and rise in unison. These data confirm the systemic fibrinogenolysis seen with SK therapy, but in addition, confirm that fibrinogen levels are not completely depleted as suggested by Clauss data.

Figure 3-17: Intact fibrinogen and soluble fibrin levels following thrombolysis (paired patient data).



3.10.3 Intact fibrinogen and X-oligomer

There is an inverse relationship between intact fibrinogen and X-oligomer levels as can be clearly seen in **Figure 3-18**.

3.10.4 Plasminogen, soluble fibrin and X-oligomer

Plasminogen and SF follow virtually identical trends, as can be seen by examining the graphs (**Figure 3-1** and **Figure 3-5**). Plasminogen and X-oligomer follow opposite trends **(Figure 3-19)**.

Figure 3-18: Intact fibrinogen and X-oligomer levels following thrombolysis.

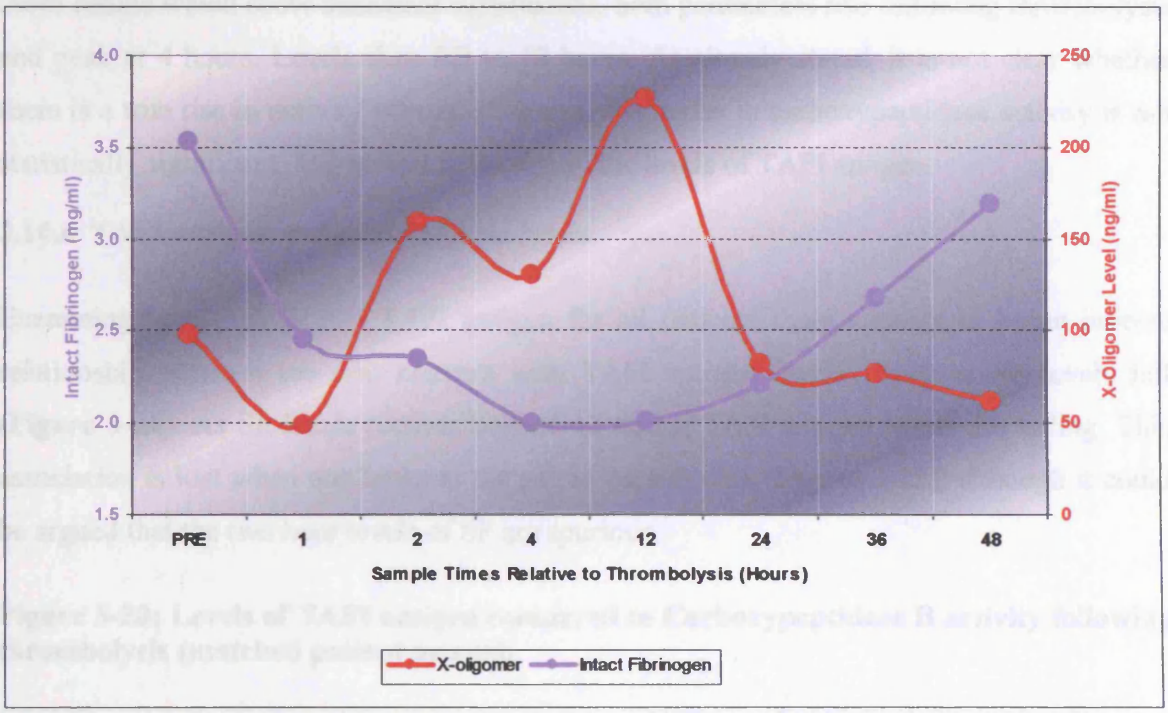


Figure 3-19: Relationship between plasminogen and X-oligomer levels following thrombolysis (matched patient means).

3.10.5 Carboxypeptidase B activity and TAFI antigen levels

The relationship between carboxypeptidase B (TAFI) activity and TAFI antigen levels are displayed graphically in **Figure 3-20**. Although not immediately apparent on the graph,

carboxypeptidase activity does appear to follow levels of TAFI antigen. Considering only those results which show statistical significance, both parameters rise following thrombolysis and peak at 4 hours. Levels then fall to 12 hours. As already stated, it is not clear whether there is a true rise in activity beyond 12 hours, as the rise in carboxypeptidase activity is not statistically significant, and neither is the rise in the levels of TAFI antigen.

3.10.6 TAFI antigen and soluble fibrin levels

Examining levels of SF and TAFI antigen for all patients there appears to be an inverse relationship between the two markers with TAFI antigen levels rising as SF levels fall (**Figure 3-21**). As SF levels recover beyond 12 hours, TAFI antigen levels are falling. This association is lost when one looks at the paired patient data (**Figure 3-22**), although it could be argued that the two hour levels of SF are spurious.

Figure 3-20: Levels of TAFI antigen compared to Carboxypeptidase B activity following thrombolysis (matched patient means).

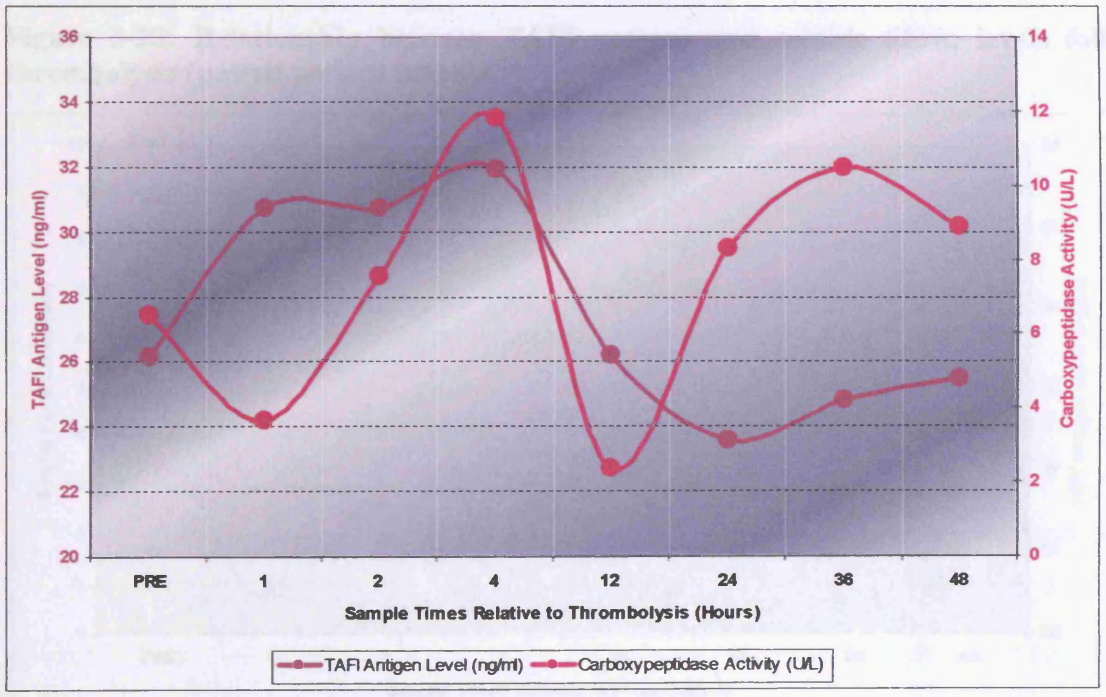


Figure 3-21: Relationship between TAFI antigen and soluble fibrin levels following thrombolysis (unmatched patient means).

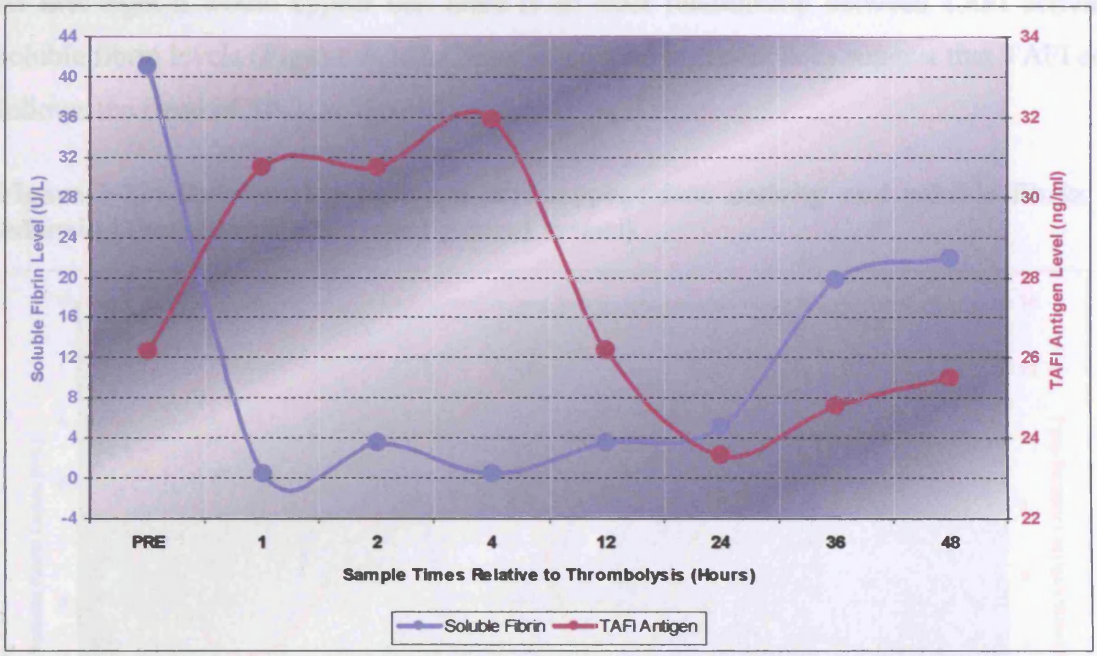
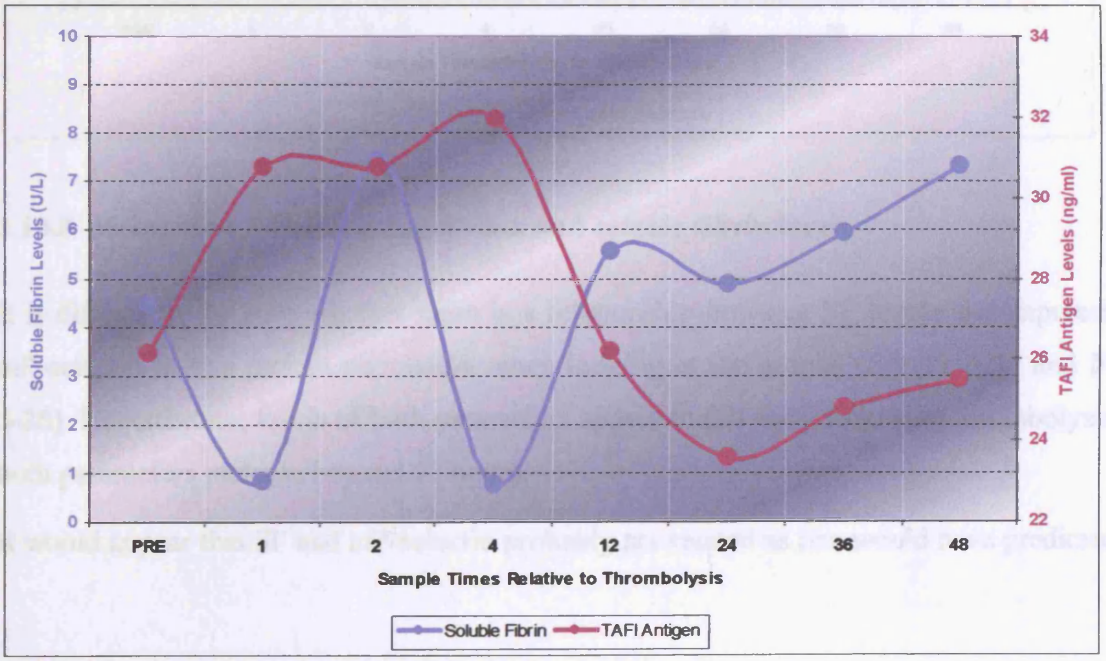


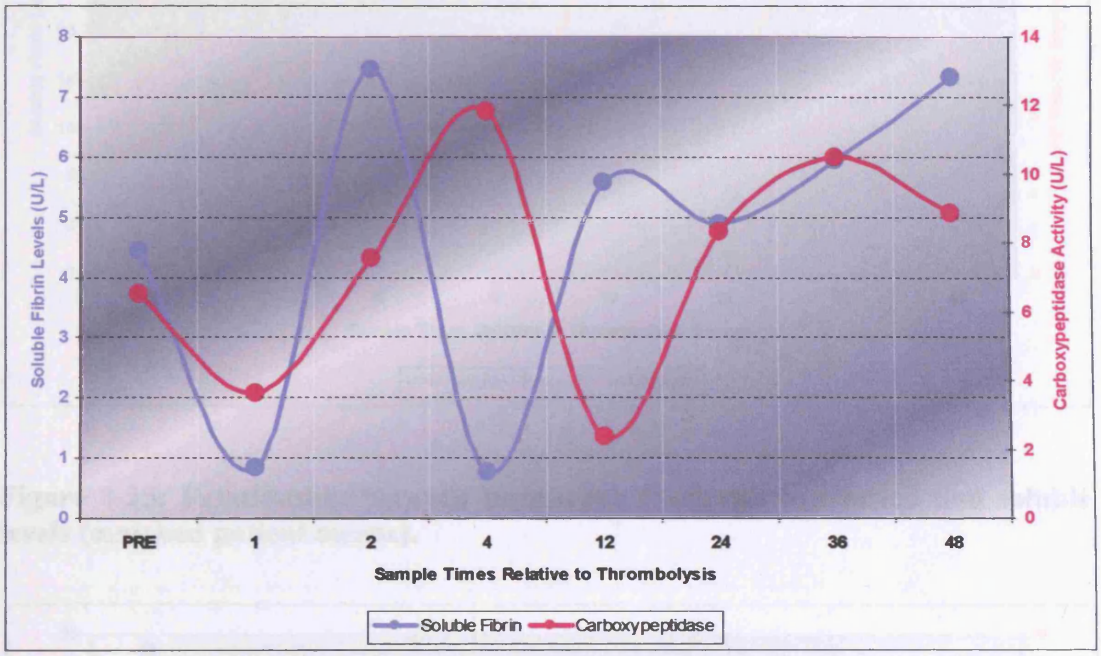
Figure 3-22: Relationship between TAFI antigen and soluble fibrin levels following thrombolysis (paired patient means).



3.10.7 Carboxypeptidase activity and soluble fibrin levels

At first sight it would appear that there is no clear relationship between TAFI activity and soluble fibrin levels (**Figure 3-23**). Closer inspection however does suggest that TAFI activity follows the trend of SF.

Figure 3-23: Relationship between carboxypeptidase activity and soluble fibrin levels following thrombolysis (matched patient means).



3.10.8 Membrane P-Selectin expression and soluble fibrin levels

It is difficult to be sure whether there is a relationship between SF levels and expression of mP-selectin in this patient population when looking at the graphs (**Figure 3-24** and **Figure 3-25**). Nevertheless, levels of both parameters appear to fall early following thrombolysis, and both parameters increase beyond 12 hours.

It would appear that SF and mP-selectin probably are related as one would have predicted.

Figure 3-24: Relationship between membrane P-selectin expression and soluble fibrin levels following thrombolysis (unmatched means).

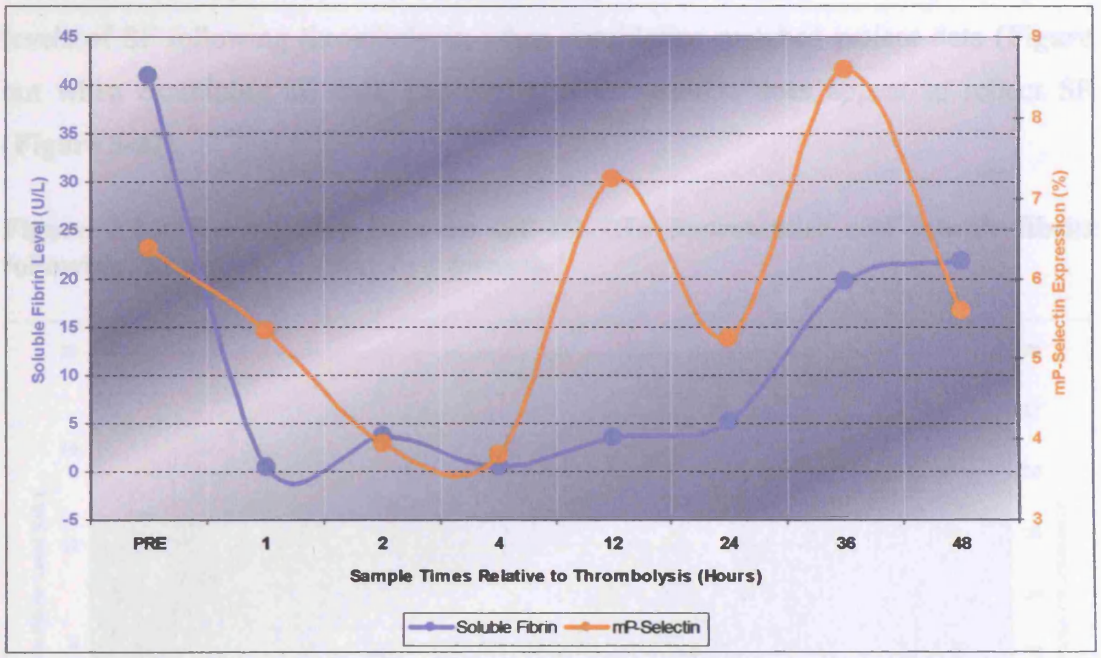
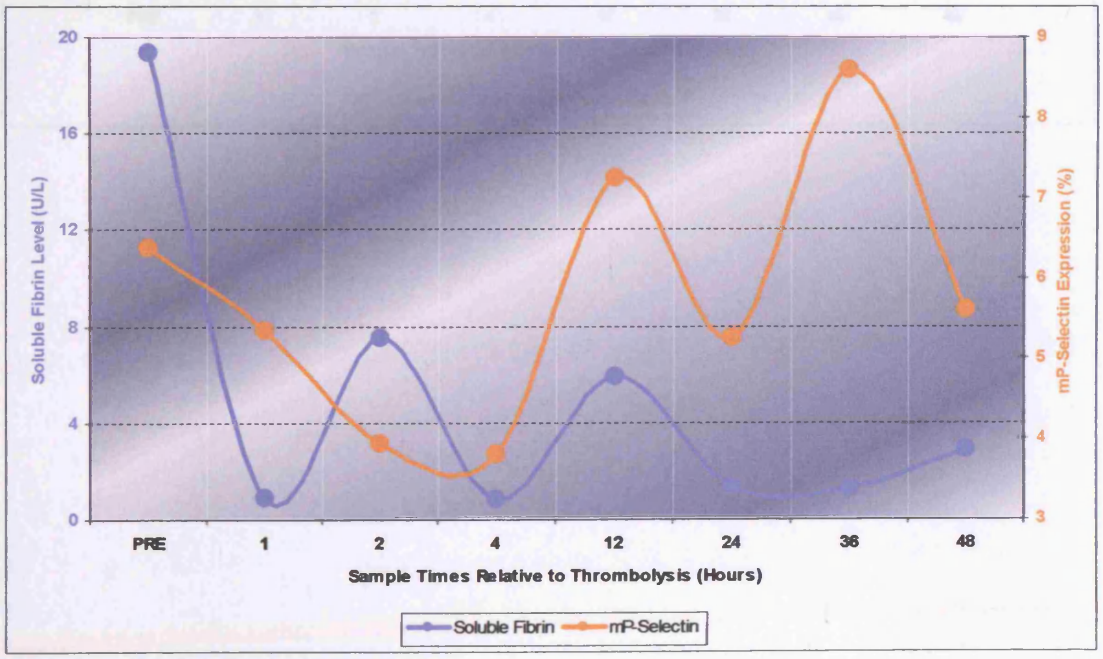


Figure 3-25: Relationship between membrane P-selectin expression and soluble fibrin levels (matched patient means).



3.10.9 GP IIb/IIIa expression and soluble fibrin levels

There does not appear to be a close relationship between GP IIb/IIIa receptor expression and levels of SF following thrombolysis, when considering matched patient data (**Figure 3-26**), but when examining all data, expression of GP IIb/IIIa does appear to reflect SF levels (**Figure 3-27**).

Figure 3-26: Relationship between GP IIb/IIIa fluorescence and soluble fibrin levels following thrombolysis (matched means).

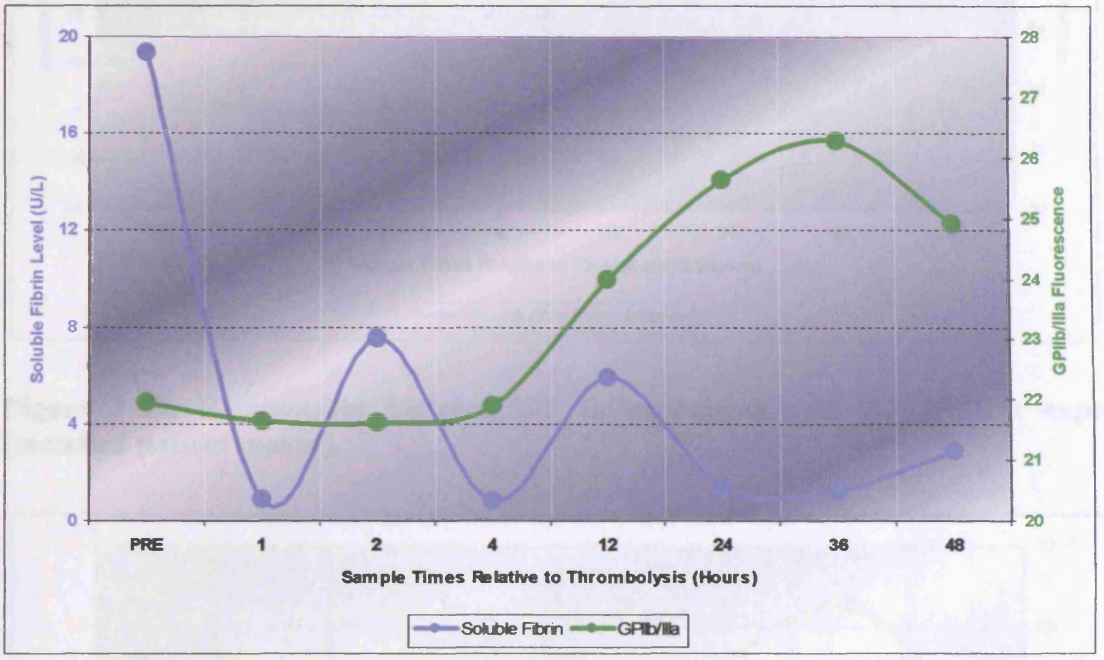


Figure 3-27: Relationship between GP IIb/IIIa fluorescence and soluble fibrin levels following thrombolysis (unmatched patient means).

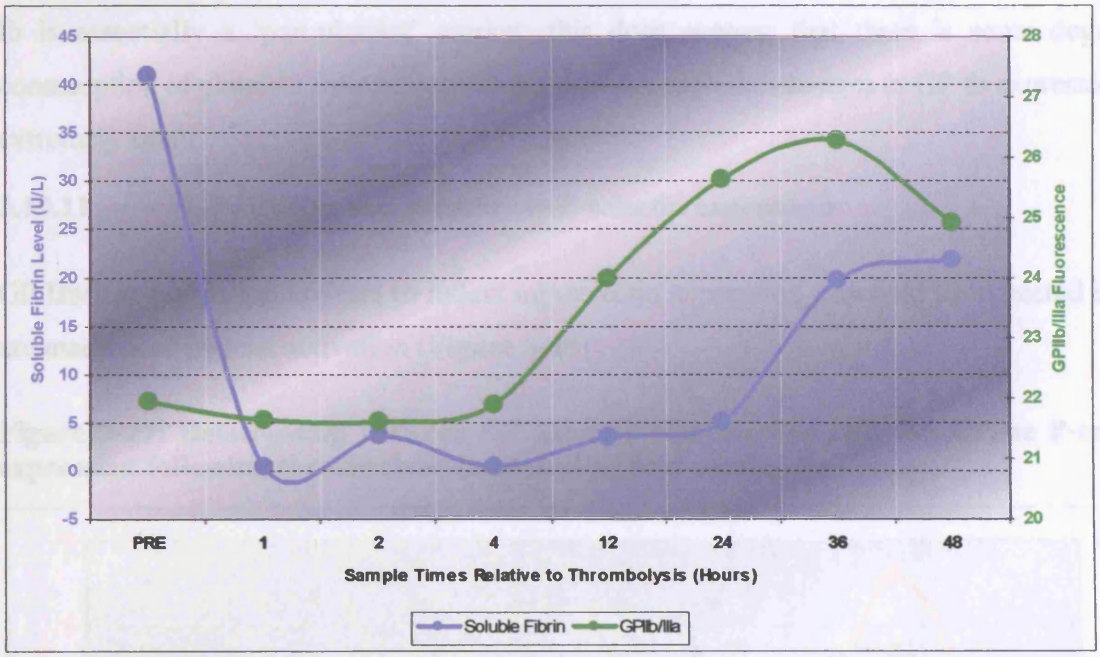
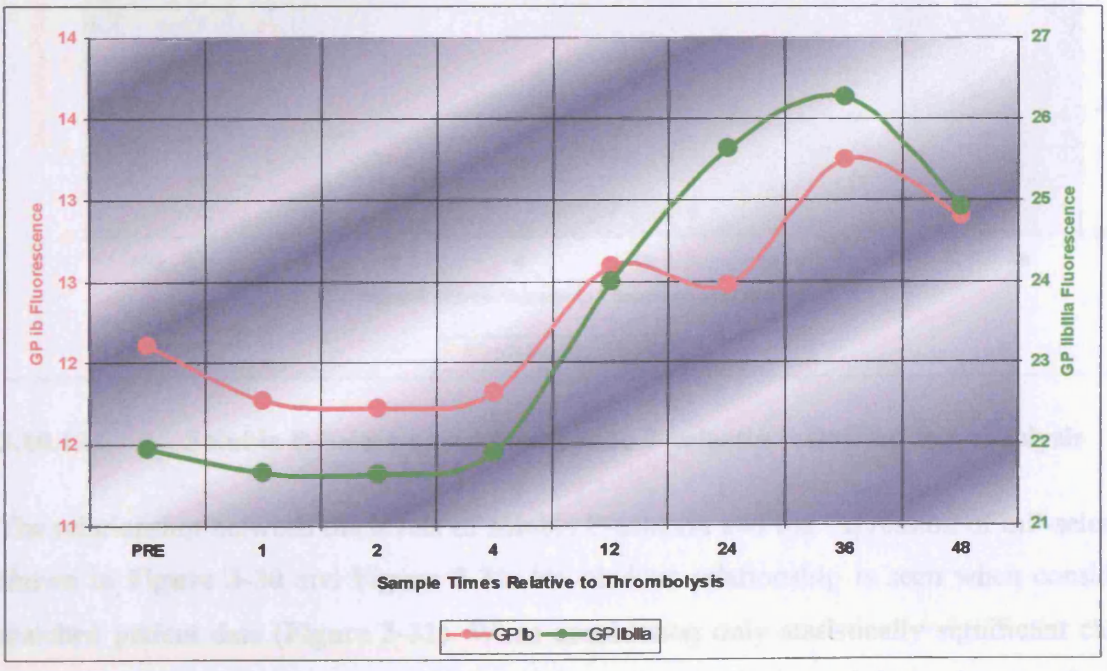


Figure 3-28: Relationship between GP Ib expression and GP IIb/IIIa expression (matched patient means).



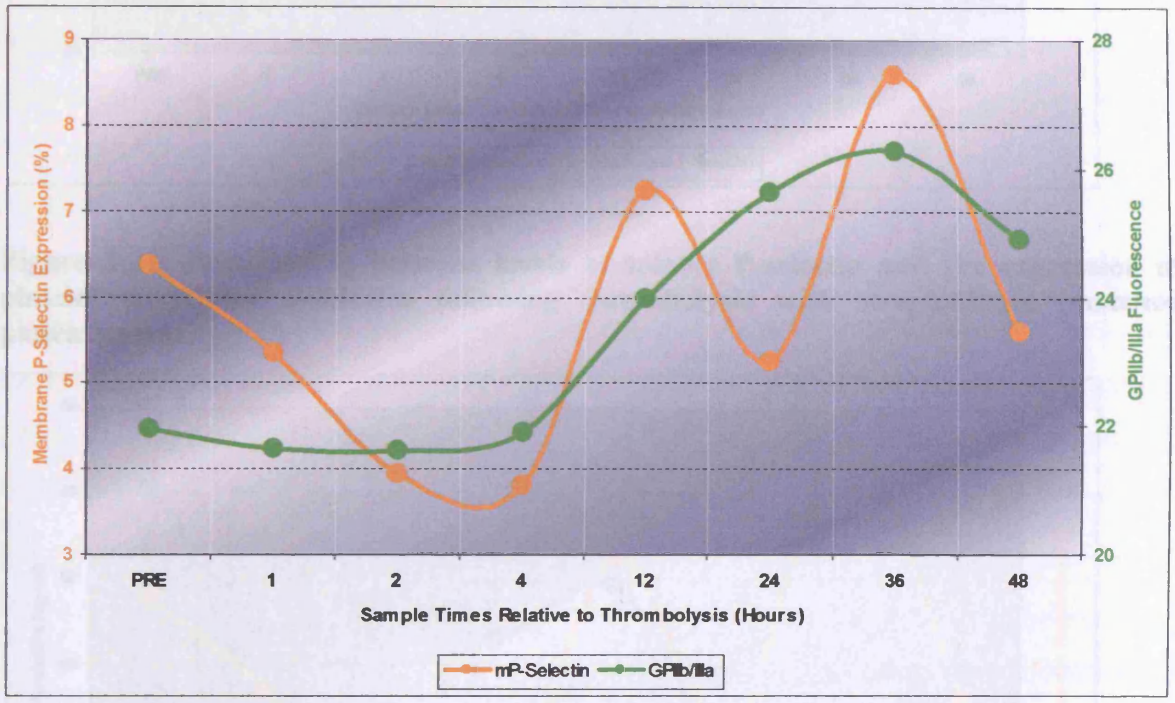
3.10.10 GP Ib and GP IIb/IIIa expression

GP Ib expression and GP IIb/IIIa expression follow very similar trends (**Figure 3-28**). As GP Ib is essentially a 'pan-platelet' marker, this does suggest that there is some degree of consumption of platelets following thrombolysis although the changes in GP Ib expression are extremely small.

3.10.11 GP IIb/IIIa and Membrane P-selectin expression

GP IIb/IIIa expression appears to reflect mP-selectin expression as would be expected if both are markers of platelet activation (**Figure 3-29**).

Figure 3-29: Relationship between GP IIb/IIIa fluorescence and membrane P-selectin expression following thrombolysis (matched patient means).



3.10.12 Soluble P-Selectin and membrane P-selectin following thrombolysis

The relationship between the levels of soluble P-selectin and the expression of mP-selectin is shown in **Figure 3-30** and **Figure 3-31**. No obvious relationship is seen when considering matched patient data (**Figure 3-31**). When considering only statistically significant changes however, levels of both parameters fall early post-thrombolysis, and then subsequently rise to peak in the region of 12 hours.

Figure 3-30: Relationship between levels of soluble P-selectin and the expression of platelet membrane P-selectin following thrombolysis with streptokinase (unmatched means).

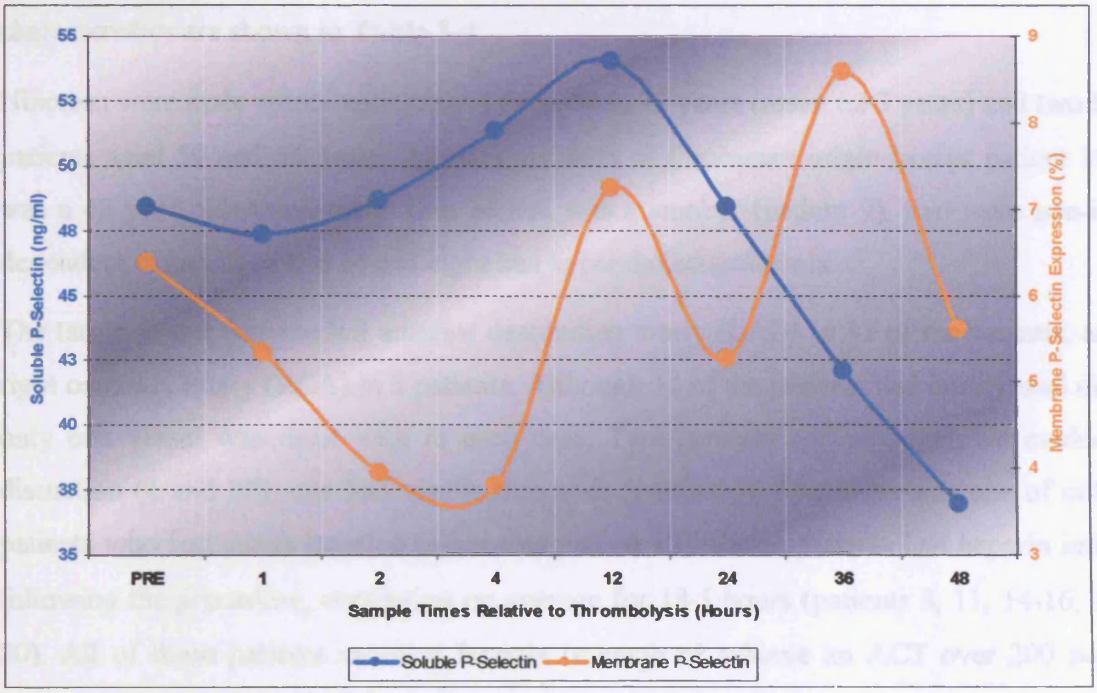
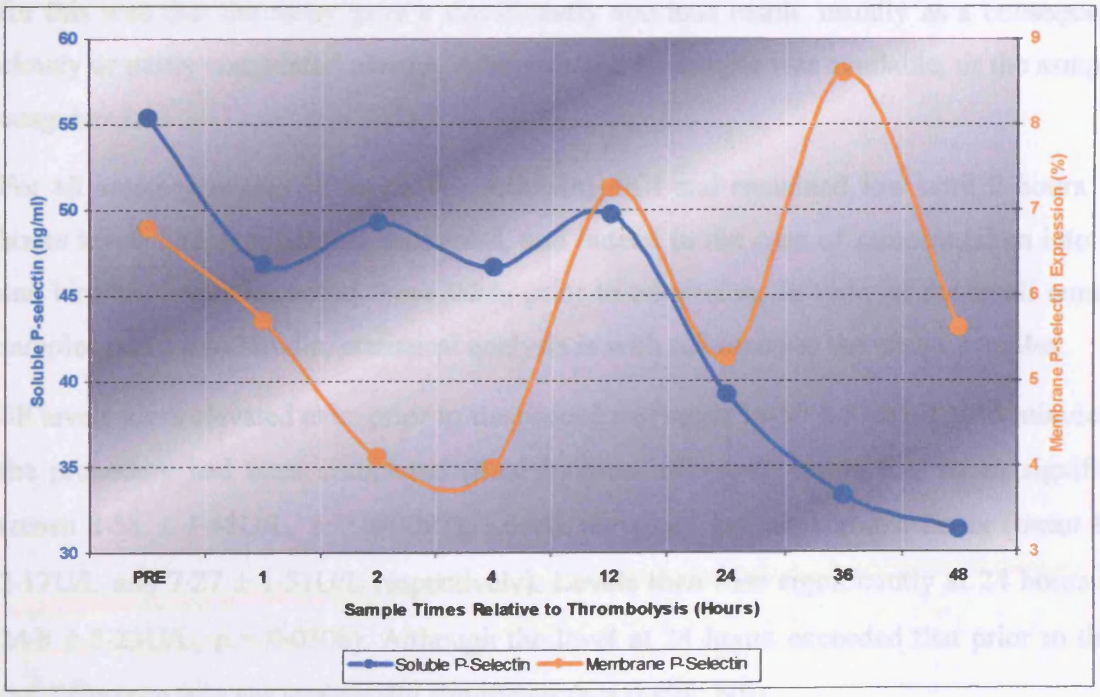


Figure 3-31: Relationship between levels of soluble P-selectin and the expression of platelet membrane P-selectin following thrombolysis with streptokinase (matched patient means).



3.11 Soluble Fibrin Following Elective PCI

Levels of soluble fibrin were measured in 21 patients undergoing elective PCI. Patient characteristics are shown in **Table 3-4**.

Nineteen were male whose ages ranged from 48 to 72 years (mean 62·3 years) and two female patients aged 59 and 66 years. All patients were of Caucasian origin except patient 18 who was a 48 year old Asian male. One patient was a smoker (patient 9), two were non-insulin dependent diabetics (3 and 6) and eight had hypercholesterolaemia.

The target vessel was the left anterior descending artery (LAD) in 11 of the patients, and the right coronary artery (RCA) in 5 patients. Although 14 of the patients had multivessel disease, only one vessel was dealt with in each case. Two patients had angiographic evidence of dissection (4 and 20), one had visible thrombus (patient 5). The latter was one of only two patients who had stents inserted (other was patient 12). Seven patients had heparin infusions following the procedure, continuing on average for 18·5 hours (patients 8, 11, 14-16, 18 and 20). All of these patients received heparin (enough to achieve an ACT over 200 seconds) because of the preference of the individual operator.

Results for SF are displayed in **Table 5-17** (appendix). Results are displayed graphically in **Figure 3-32**. Blank squares indicate that no result is available at that time point. The reason for this was that the assay gave a significantly spurious result, usually as a consequence of cloudy or partly coagulated plasma. Alternatively, no sample was available, or the sample was coagulated.

For all anticoagulants, SF levels fell following PCI and remained low until 2 hours. At 24 hours levels had significantly recovered, and indeed in the case of samples taken into citrate and hirudin, levels exceeded those taken prior to admission. In view of the small number of samples taken into hirudin, statistical analysis is with reference to the citrate samples.

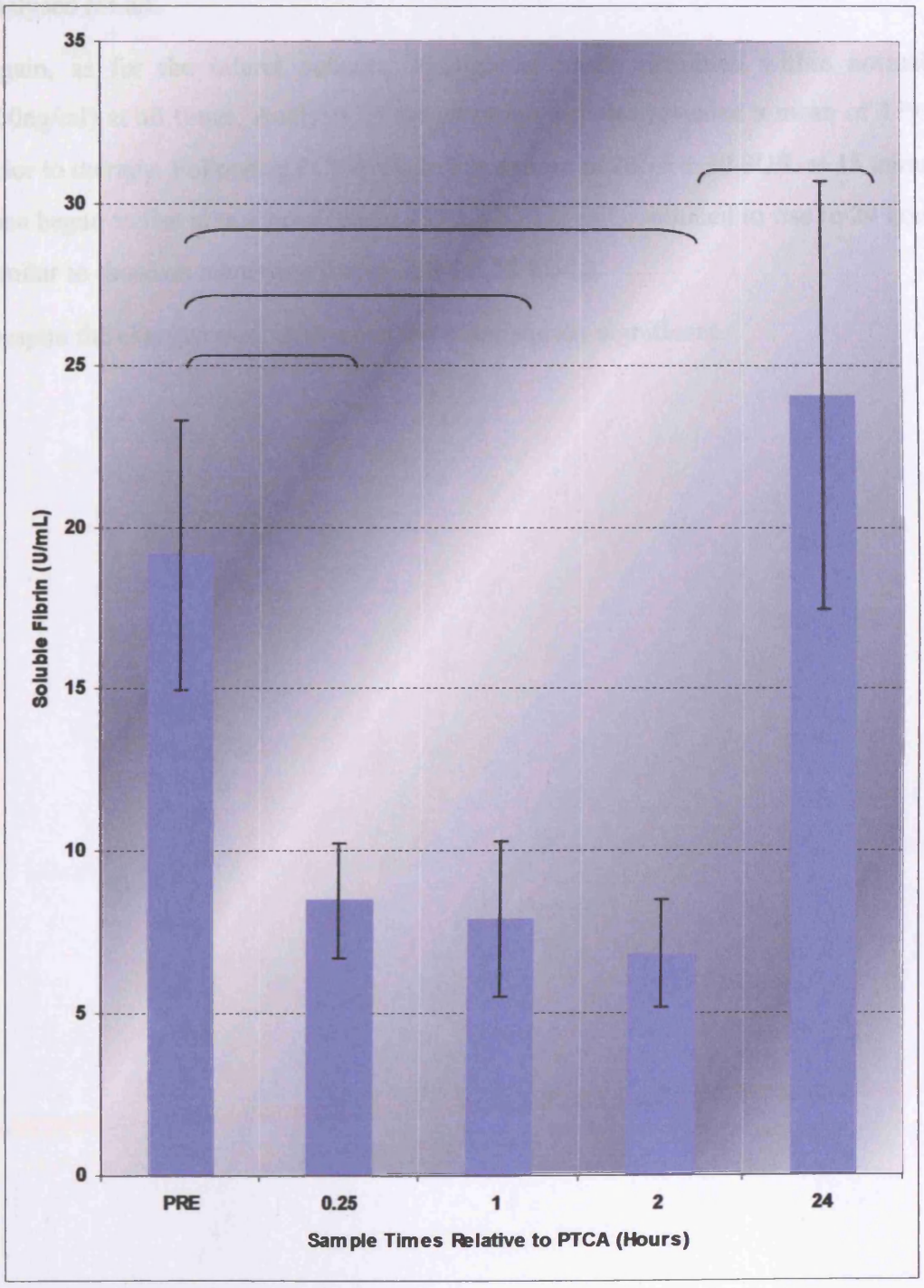
SF levels were elevated even prior to the procedure (mean 19·59 ± 3·43U/L). 15 minutes after the procedure had been completed (final balloon inflation), levels had fallen significantly (mean 8·58 ± 1·48U/L, $p = 0·0097$). Levels remained low at 1 and 2 hours (mean 8·63 ± 2·17U/L and 7·27 ± 1·51U/L respectively). Levels then rose significantly at 24 hours (mean 24·8 ± 5·23U/L, $p = 0·0308$). Although the level at 24 hours exceeded that prior to therapy, the difference was not statistically significant ($p = 0·898$, NS).

Table 3-4: Elective PCI patient characteristics.

	55	M			√						3		√	9420					
	70	M				√					2		√	2264					
	71	M		√				√			2		√	988					
	65	M				√					3		√	1418	√			√	
	56	M								√	2		√	6980		√	√		
	67	M		√	√	√					3		√	3303					
	59	F								√	3	√		1783					
	62	M						√			3		√	669					√
	60	M	√		√		√				3	√		3800					
	66	F							√		3	√		3348					
	63	M								√	1		√	3437					√
	65	M				√					3	√		1654			√		
	55	M				√					3		√	794					
	65	M								√	2	√		2952					√
	68	M			√	√					3	√		3450					√
	59	M			√	√					3		√	7856					√
	68	M			√	√					3	√		7936					
	48	M			√	√					3		√	6642					√
	65	M			√					√	2		√	1400					
	49	M				√					3		√	1008	√				√
	72	M				√					3		√	11405					

When comparison is made with the samples taken at the time of myocardial infarction, samples prior to elective PCI in patients with stable angina pectoris are surprisingly high at a mean of 19·59U/L compared to 38·1U/L (for the comparable citrate samples). When compared employing the two-sample t-test, the results are not statistically significantly different ($p = 0·46$).

Figure 3-32: Soluble fibrin levels following elective PCI (means +/- SEM, n=21).



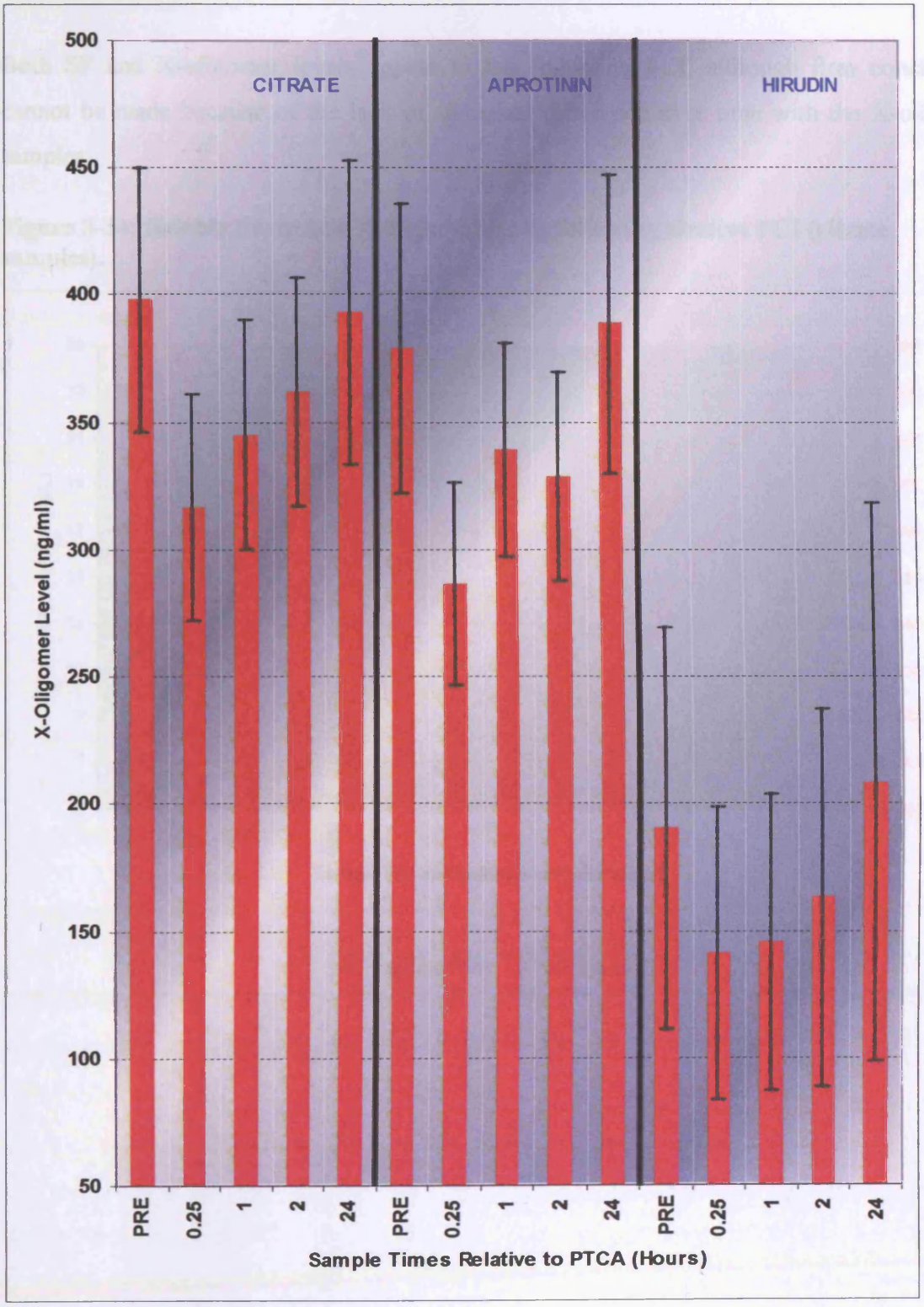
3.12 X-oligomer Following Elective PCI

X-oligomer levels were measured in the same cohort of patients as those for SF. Results are displayed in **Table 5-18** (appendix), and displayed graphically in **Figure 3-33**. Unlike SF, the anticoagulant mixture possibly does influence the results – although there are too few patients in the hirudin group to draw any firm conclusions. When comparison is made between the anticoagulant in the infarct patients no such difference was noted, and similar numbers were analysed for all.

Again, as for the infarct patients, X-oligomer levels remained within normal limits (< 500ng/ml) at all times. Analysis of the aprotinin samples revealed a mean of 379 ± 57·1U/L prior to therapy. Following PCI levels fell to a mean of 286·5 ± 39·8U/L at 15 minutes. Levels then began to rise at one hour (mean 339 ± 42U/L), and continued to rise to 24 hours to levels similar to those on admission (mean 388·8 ± 58·8U/L).

Despite the changes over time, none were statistically significant.

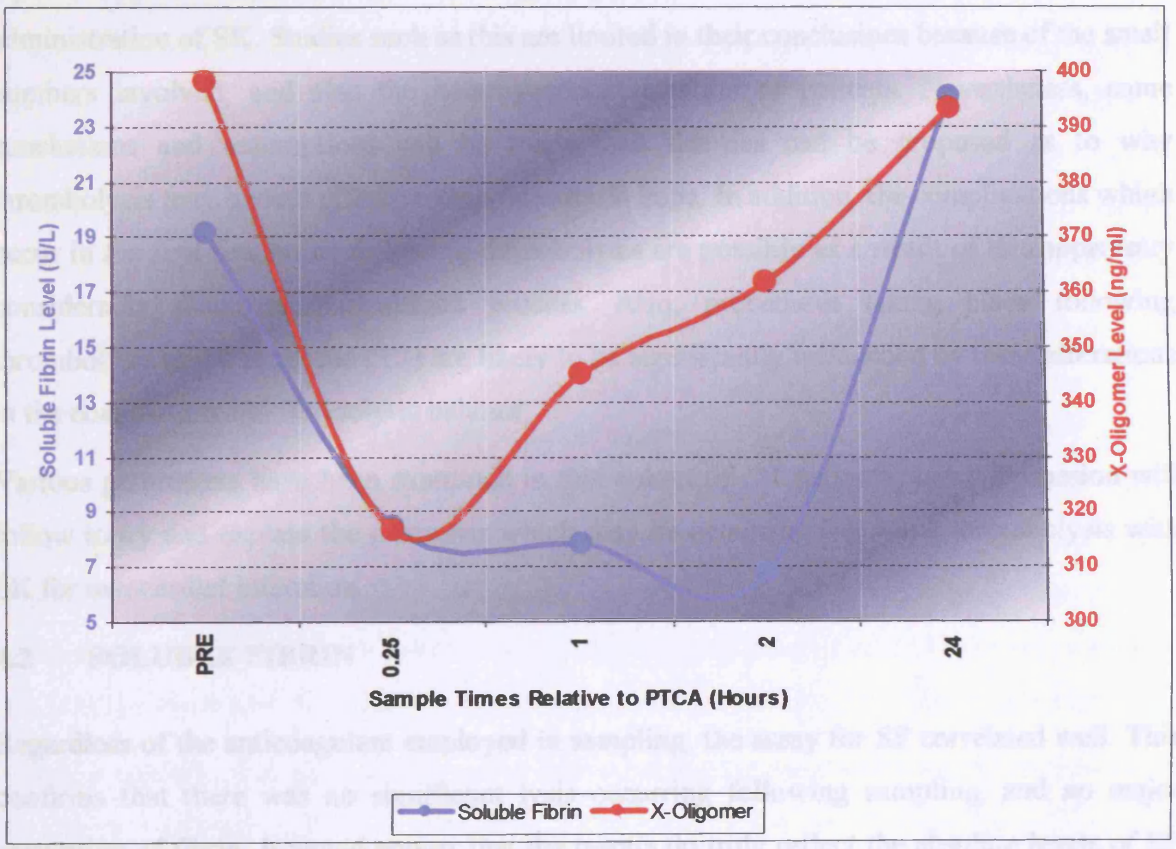
Figure 3-33: X-oligomer levels following elective PCI (means +/- SEM, n=21).



3.13 COMPARISON OF SOLUBLE FIBRIN AND X-OLIGOMER FOLLOWING PCI

Both SF and X-oligomer levels appear to fall following PCI, although firm conclusions cannot be made because of the lack of statistical difference over time with the X-oligomer samples.

Figure 3-34: Soluble fibrin and X-oligomer levels following elective PCI (citrate samples).



CHAPTER 4: DISCUSSION

4.1 THROMBOLYSIS

As has already been discussed in the introductory chapter, thrombolytic therapy fails to achieve a satisfactory outcome in a large proportion of the patients treated. There are obviously many reasons why this might be so, and this study has merely examined a few aspects of the coagulation and fibrinolytic systems which may be involved after the administration of SK. Studies such as this are limited in their conclusions because of the small numbers involved, and also the heterogeneous selection of patients. Nevertheless, some conclusions and assumptions can be made, and theories can be proposed as to why thrombolysis may be less effective than one would hope. In addition, the complications which occur in the first few hours following thrombolysis are possibly as a result of this apparently considerably disturbed haemostatic process. Also, procedures taking place following thrombolysis (such as rescue PCI) are likely to be significantly influenced by these alterations in the coagulation and fibrinolytic balance.

Various parameters have been examined in this cohort of 21 patients, and a discussion will follow to try and explain the processes which may be occurring following thrombolysis with SK for myocardial infarction.

4.2 SOLUBLE FIBRIN

Regardless of the anticoagulant employed in sampling, the assay for SF correlated well. This confirms that there was no significant lysis occurring following sampling, and no major generation of fibrin. It would appear that the results do truly reflect the absolute levels of SF present at each given sampling time, and that both thrombin generation and lytic activity have been halted.

Our data confirms the fact that SF levels are elevated at baseline in this AMI population with a mean level of 41·0u/l (hirudin samples), when normal levels are less than 5·0u/l (as established at the NIBSC). Other studies have also reported levels of SF are reported to be increased in patients with AMI and unstable angina [374-376]. The problem with these studies however is that the assays employed were not specific for SF, and fibrin degradation products may have been measured as well. Measurements of SF employing more specific monoclonal antibody techniques have only recently emerged. This is the first such detailed study of AMI patients employing such novel antibodies.

Although for the majority of patients levels of SF were high pre-thrombolysis, there were significant differences amongst patients. There was no obvious relationship between time since onset, site of infarct, age of the patient, etc (data not presented, but information accessible from the appendix). This brings into question the validity of venous levels of SF employing this particular assay, particularly if one proposes to use SF as a marker of such a localised coagulative process. It cannot be said that the assay employed by the Gaubius Laboratory is more sensitive, although the numbers are too small to be sure. Previous studies have also shown SF to be unreliable as a sensitive marker of myocardial infarction [377].

Other studies examining thrombin generation in this context have displayed heterogeneity at baseline, and some have suggested that higher levels of SF may be associated with an adverse outcome [378], although this is not supported in other studies [377]. Our study was too small to examine outcomes, although all patients survived. Angiography was not performed however, and no correlation can be made between levels of SF and TIMI flow achieved by SK therapy.

One could hypothesise that SF levels would fall immediately following SK therapy due to the direct action of plasmin, but this is contrary to other studies which have measured other markers of thrombin generation in the context of thrombolytic therapy [52,70,261,379-383]. These studies measured markers such as Fibrinopeptide A (released when fibrinogen is converted to fibrin), thrombin-antithrombin complex (TAT) and prothrombin fragments F_1 and F_2. All of these studies suggested thrombolytic therapy resulted in thrombin generation, whereas our study clearly showed SF levels were suppressed. Some of these studies have also postulated that greater degrees of thrombin generation may be associated with an adverse outcome [261].

Again, it could be that SF is simply not sensitive enough to demonstrate what is happening at the site of the plaque rupture. Alternatively, although there are increased markers of thrombin generation, they may not necessarily translate ultimately into fibrin generation. This does tend to contradict other data [261,379-383], including that established in this study from other markers, that coagulation begins to predominate early after thrombolytic therapy. It is likely that SF is simply not a reliable marker of underlying thrombin generation in this setting. Reasons could include rapid breakdown of any fibrin generated in the early phase of thrombolytic therapy.

Our data also conflicts with other studies which suggest that thrombin activity is falling back towards baseline at 12 hours [384], whereas our data suggest that recovery of SF levels is still below baseline at 48 hours.

With respect to changes over time, the NIBSC assay does appear to show that levels of SF fall rapidly and remain very low for the first 24 hours. Levels then begin to recover. It is difficult

to be sure whether levels remain low because of suppression of fibrin generation or because there is continuing lysis. It is difficult to postulate continuing lysis however, when plasminogen levels are so low and when the bulk of lysis occurs as a consequence of fibrin-bound plasminogen.

The rise in SF levels beyond 12 hours does raise an important issue regarding the possible use of heparin following SK therapy. Perhaps it could be argued that employing heparin a few hours after administration of SK, and continuing therapy for up to 48 hours, would potentially counteract any thrombin released from thrombus and any new thrombin generated. Prolonged or delayed use of unfractionated heparin has not been examined in any placebo controlled trials, but the recent AMI-SK trial did suggest potential benefit with a combination of enoxaparin and SK [232]. Even greater potential clinical benefit may be seen if more direct thrombin inhibitors such as Bivalirudin were employed shortly after administration of the thrombolytic. The only study of Bivalirudin in AMI showed no advantage over UFH, but administration was immediate following SK rather than delayed [257].

Although we have not examined it in this study, Factor V appears to play a pivotal role in the activation of coagulation following thrombolysis. The high levels of plasmin generated by thrombolysis, may directly activate Factor V as a result of enzymatic cleavage [92] and induce a procoagulant state [93,385,38672]. It achieves this because activated factor V (Va) forms part of the 'prothrombinase complex' which converts prothrombin to thrombin. However thrombin is the more recognised activator of factor V by positive feedback. Thrombin released by the thrombolytic agents can also directly activate factor V, and appears to activate the intrinsic pathway through factor XII following thrombolysis [386]. In summary, there is significant 'cross-talk' between the coagulation and fibrinolytic systems allowing therapeutic fibrinolysis (thrombolysis) to activate, or at least sensitise, the coagulation system. Factor V levels are already reduced at presentation in patients with AMI [387] suggesting a procoagulant state which is also apparent with the elevation of fibrinogen and D-dimers [387,388]. Only one group saw no significant change in factor V activity after AMI [389].

Few studies have specifically looked at factor V in the context of infarction and subsequent thrombolysis. Samama *et al* [390] looked at factor V activity after the administration of APSAC and confirmed the expected fall, whereas Topol *et al* [391] found no effect after the administration of rt-PA. Collen *et al* [392] did show a fall of about 30-45% after rt-PA, and a fall of about 15-25% following SK infusion. Tracy *et al* directly examined the effect of

thrombolysis on Factor V [393]. This clearly demonstrated extensive Factor V cleavage was plasmin-mediated rather than thrombin-mediated.

Thrombolysis also seems to generate an endogenous anticoagulant enzyme called activated protein C (APC) [394], which controls activation of coagulation through the proteolytic degradation of central components (namely factors Va and VIIIa) of the coagulation cascade (**Figure 1-8**) [33]. Elevated levels of APC appear to correlate with increased thrombin generation in patients with AMI. One study demonstrated that there was a significant relation between plasma APC level and the responsiveness to thrombolytic therapy and suggested that increased thrombin generation was the cause of thrombolytic resistance [395]. By contrast, co-administration of APC with Alteplase in another study resulted in suppression of PAI-1 activity and thus potentially could enhance lysis [396].

Individuals who are resistant to protein C as a consequence of a point mutation in coagulation factor V may be prone to produce more thrombin during thrombolytic therapy [397]. This is not of purely esoteric interest, as the prevalence of heterozygosity for the mutation in patients with ischaemic heart disease is 5-6% [398].

4.3 FIBRIN DEGRADATION PRODUCTS AND THROMBOLYSIS

D-dimer is a major structural component of all fibrin degradation products [399] and is a marker of fibrin formation in the circulation [400]. D-dimer levels are already elevated in-patients with AMI [388], suggesting that there is haemostatic activation. All studies that have measured fibrin degradation products document a further significant increase after thrombolytic therapy which parallels the fall in fibrinogen [401-408]. X-oligomer levels are said to be consistently elevated after AMI [409]. Fragment X species appear to be the predominant degradation product after SK [44,402] and rt-PA infusion [78], although significant elevations in D-dimer are also observed [405,410,411,411]. In general, the levels fall to baseline (pre-treatment) after about 48 hours.

The fact that fragment X forms the major degradation product may well have clinical significance. Fragment X is the only degradation product with the capacity to form clots in response to thrombin [412]. Although these clots have a low tensile strength and are readily susceptible to plasmin-mediated lysis, there is a period of plasminogen (and thus plasmin) depletion as already outlined above. The period of plasminogen depletion coincides with the time when high levels of fragment X are still circulating: fragment X persists in the blood for at least 24 hours after thrombolytic therapy [78].

In the context of AMI and thrombolysis, D-dimer appears to reflect fibrinolysis of the coronary thrombus rather than breakdown of circulating crosslinked fibrin [413], although this is challenged by some authors [405]. It should however be emphasised that D-dimer levels do not necessarily reflect underlying successful reperfusion [404,405,414], although some studies suggest they do [411]. The actual rate of breakdown of fibrin may be more important than the absolute level of degradation products [415].

Levels of X-oligomer as measured in this study are again remarkably constant regardless of the anticoagulant employed. This is surprising in view of the expectation that, in the absence of aprotonin, the lytic process would be continuing *in vitro* unchecked. As with the SF data, it adds considerable support to the veracity of the samples acquired at the individual time points. It is likely that the process of snap freezing the samples and analysing immediately on thawing resulted in a true reflection of the coagulation and fibrinolytic processes at the given moment.

Examination of our X-oligomer data would suggest that the lytic process is tailing off significantly beyond 12 hours. This is also reflected by the gradual recovery of activatable plasminogen. The fall off of the lytic process is also accompanied by the increase in SF levels. This change occurs sometime around 12 hours, and it could be argued that this is the time at which the patient's coagulation system is beginning to dominate. From the clinical point of view, this is of great importance. It would suggest that the patient is at greater risk at this time. It could be argued that heparin, which is not currently recommended with SK, may be of benefit if its administration is delayed until 6 to 12 hours after administration. This could be accompanied by other therapies such as antiplatelet strategies employing GP IIb/IIIa inhibitors.

Our data does differ from other published studies of FDP levels following therapy with SK. Of particular interest is the change of levels over time. Our data reveals levels peaking at 12 hours whereas other studies suggest a much earlier peak. Mentzer *et al* had a peak at 1·5 hours, with the predominant degradation product being fragment X [402]. Lurie *et al* had a peak at 6 hours [407], whilst Rao *et al* had a peak at 3 hours [404]. Of course, different methodologies and/or different monoclonal antibodies have been employed in the other studies. In other words, different breakdown species of fibrin are potentially being measured. Whilst this is a possible explanation, another important fact needs to be established. The other studies make an assumption that the lytic state peaks very soon after administration of the SK. Our study suggests that the lytic state is more prolonged – both as evidenced by the continued rise in X-

oligomer to 12 hours, and also as illustrated by the suppression of SF levels until beyond 12 hours.

As far as our X-oligomer data is concerned, it should also be noted that in the first hour levels of this fragment actually fall. This suggests that plasmin might itself degrade this protein. The subsequent increase further implies that the degradation of fibrin dominates over the degradation of X-oligomer. One might postulate that the binding site for plasminogen/plasmin in fibrin is more accommodating than the binding site on X-oligomer.

There is another important issue regarding the X-oligomer data. Despite there being a profound fibrinolytic state induced by the administration of SK, levels of X-oligomer remained within normal limits throughout, despite the changes demonstrated over time. This is very surprising and raises crucial issues. There is the possibility that previously established normal ranges employing this assay are spurious. A more likely explanation is that this X-oligomer assay is extremely specific and that the amount of cross-linked fibrin within the coronary artery is so small in the overall scheme of things, that no major rise above the normal range would be anticipated. This also assumes that there are no significant deposits of crosslinked fibrin elsewhere within the circulation which would be vulnerable to the massive amounts of plasmin generated.

4.4 FIBRINOGEN AND THROMBOLYSIS

Fibrinogen levels tend to be higher in patients with AMI [387], and indeed, hyperfibrinogenaemia appears to be an independent risk factor for ischaemic heart disease [81].

It has been consistently shown that the doses of thrombolytic agents currently in use all cause a degree of systemic fibrinogenolysis and ultimately a fall in fibrinogen levels. Whether the agent is SK [403,404,407,416], APSAC [390,403,417], or rt-PA [391,391,404,418-421]. The effects are dose-dependent.

Most studies of fibrinogen following SK reveal falls very soon after administration, which is mirrored by our own study. This could be anticipated by the knowledge that generated plasmin would break down fibrinogen. Many also suggest a marked fall in circulating fibrinogen to less than 20% of baseline [403,416]. Our data do not show such a profound fall. Levels of intact fibrinogen are at their lowest at 4 hours when they are, on average, 57% of baseline. This result is more in keeping with the findings of Lurie *et al* who showed a fall to 44% of baseline at 6 hours and Rao *et al* who had a fall to 42% of baseline at 5 hours [404,407]. Earlier studies have generally inferred a systemic fibrinolytic state as being present if

fibrinogen levels fall <70% of baseline. Employing the Gaubius assay, it is possible that more types of circulating fibrinogen molecules are being recognised compared to other laboratories. This is based on the assumption that the Gaubius assay is genuinely measuring 96% of circulating fibrinogen (HMW and LMW forms) due to its recognition of fibrinopeptide A and the carboxyl-terminal end of an Aα-chain.

Confirmation of systemic fibrinogenolysis is seen when looking at the level of fibrinogen compared to the level of SF. Essentially, fibrinogen and SF levels fall and rise in unison. The opposite is seen with fibrinogen and X-oligomer data where the levels of the degradation product reflect the opposite of what is happening to the fibrinogen.

It is difficult to establish the precise effect that thrombolysis has on the assay of fibrinogen and this has resulted in difficulty in comparing one study with another. It is clear that when there are elevated levels of degradation products, fibrinogen levels tend to be spuriously low [78,421]. Nevertheless, it is clear that functional fibrinogen levels (as measured by the Clauss assay) fall significantly within an hour of administration of the thrombolytic agent. The greatest reduction in fibrinogen occurs when the pre-treatment levels are high, and it may take up to 72 hours to return to baseline (in the case of SK).

4.5 PLASMINOGEN AND THROMBOLYSIS

The consequences of activation and depletion of circulating plasminogen has attracted interest since the earliest days of therapeutic fibrinolysis. Doses of SK used in the earliest clinical trials were calculated on the basis that a continuing plasminaemia was desirable. Subsequently, Verstraete & Vermylen [422] proposed that, since active plasmin has a short circulating half-life, it might be safer to give a large initial loading dose, activate and clear all circulating plasminogen, and subsequently to concentrate on activating clot-bound plasminogen. This has been the conceptual basis for subsequent 'short-term/high dose' thrombolytic regimens. It was appreciated that if any thrombus were to form during the period of induced aplasminogenaemia (absence of circulating plasminogen) it would be hard to lyse, and this formed the basis of combining short-term fibrinolysis with subsequent heparin therapy.

In this study we have measured plasminogen employing a novel assay. The usual method of determining plasminogen levels employing the *Friberger* method [362] could not be used in our samples because of the addition of aprotinin. The latter neutralises SK, and the *Friberger* method relies on a thrombolytic agent to convert any remaining plasminogen to plasmin, and

the resulting plasmin is assayed by means of a chromogenic substrate. This does not really take into account that there is already thrombolytic agent in the plasma (which may still be active), and does not account for the fact that plasmin formed will be complexed to a degree with any remaining α_2-antiplasmin.

Our assay confirms that plasminogen levels in our subjects were within the normal NIBSC range with a mean of 153·18μg/ml before administration of SK. As would be anticipated, levels fell immediately following the commencement of the SK infusion. That plasminogen is depleted has been well documented [423,423]. The extent of plasminogen depletion depends on the dose and type of activator used but is particularly marked with SK, which may drop plasminogen levels to less than 25% of normal [403,404,407,416]. rt-PA reduces levels by 40% to 60% [392,424,425].

Our assay revealed that at one hour plasminogen fell to 5% of baseline and remained at approximately 4% to 4 hours. At 12 hours a degree of recovery is seen, but still to only 12% of baseline. Some investigators have suggested a less pronounced fall, with Rao *et al* [404] showing a fall to 18% of baseline at 5 hours, and Lurie *et al* [407] a fall to 7% at 6 hours. The latter group also showed a rise to about 35% of baseline at 24 hours whereas our study suggests a return to only 24% at this time. They also claimed that levels return to 65% of baseline by 48 hours, whereas our results suggest levels remain more significantly suppressed at about 41%. Our results are more in keeping with other reports which did suggest that plasminogen may take greater than 48 hours to return to baseline levels [403,416,417]. By the end of the infusion of SK, Mentzer *et al* suggested ALL of the plasminogen in the circulation, except for the remaining 1% that could have been complexed with SK to form an SK-plasminogen or SK-plasmin activator complex, has been converted to plasmin [402]. They also suggested that almost all of the plasmin is neutralised at this time by the formation of complexes with circulating plasmin inhibitors.

The lack of available circulating plasminogen for so long following administration of SK does have potential implications. Current practice is to consider giving repeat thrombolytic therapy to patients who re-infarct early following therapy. The published data of so-called 'rescue' thrombolytic therapy is limited, but patency rates appear to be in the region of 54% to 73% with bleeding rates from 13% to 23% [426-428]. In addition, there is evidence that there is a beneficial effect on ejection fraction [429]. The success rates are surprisingly good, especially when one considers the lack of available circulating plasminogen at the very time that 'rescue' thrombolysis is administered. If 'rescue' thrombolysis does work, it is presumably

because of the action of clot-bound plasminogen. This is an inadequate explanation however, as one has to explain why this clot-bound plasminogen was not activated the first time round. In addition, the issue of inhibitors to thrombolysis needs to be considered. For instance, TAFI appears to inhibit thrombolysis by its action on clot-bound plasminogen.

One other issue is worth exploring in trying to explain the low levels of circulating plasminogen following thrombolysis. It may actually be a reflection of continued conversion to plasmin. Other studies measuring plasminogen are effectively measuring plasminogen indirectly – they are actually assaying plasmin. It is impossible to determine whether the plasmin being measured is purely that produced by the methodology of *Friberger* (with plasminogen being converted to plasmin by the action of SK in the assay, or whether there is *in vitro* conversion as a consequence of continued thrombolysis due to the SK administered as therapy. In all the studies previously reported in the literature, plasminogen had to be measured on citrate plasma. Our study differs because of the addition of aprotinin which truly reflects a 'snapshot' of what is happening at any given time, as aprotinin will have blocked any continued lysis.

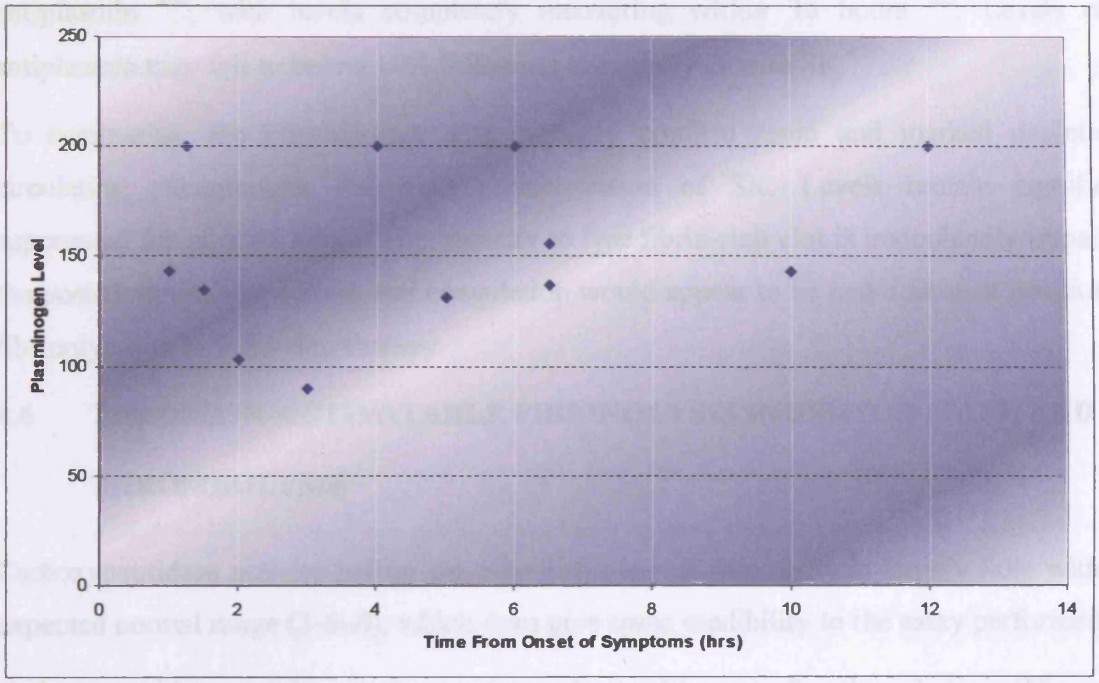
The low levels of circulating plasminogen may also have potential implications for rescue PCI. This procedure is being performed at a time when coagulation is in a position to dominate over fibrinolysis. It is well established that PCI itself results in deep arterial injury and that platelets are recruited to the site within minutes [430,431]. The subsequent activation of platelets and potential thrombin generation may be occurring at a time when available plasminogen is truly deficient, and there is a potential imbalance between the coagulation and fibrinolytic systems.

This is of course attenuated by the routine administration of drugs such as Aspirin and heparin. It would also suggest that additional efforts may be required such as routine administration of GP IIb/IIIa inhibitors. The use of these antibodies does seem to confer additional benefit in this scenario [347,348], and the benefit is sustained [349]. An increasing number of studies suggest that abciximab administered during rescue PCI improves outcome with only a small increase in the risk of bleeding [350].

There is also a strong argument to reconsider the idea of plasminogen supplementation. Previous studies have shown that giving plasminogen with the thrombolytic agent may improve its efficacy [432,433]. Gaffney *et al* [434] suggested that it was the availability of plasminogen that dictated the efficacy of clot lysis by SK. It was Sobel's group who first coined the term "plasminogen steal" when they described the attenuation of fibrinolysis

caused by plasminogen depletion during therapy [435]. They subsequently demonstrated that clot lysis by plasminogen activation is dependent on clot-associated plasminogen, which in turn depends on the concentration of plasminogen in plasma - which is reduced by thrombolytic therapy [436]. Lijnen *et al* found that thrombolytic capacity returned to normal in homozygous plasminogen-deficient mice when murine plasminogen was injected and levels restored [437]. The rapid activation and clearance of plasminogen has been invoked as a possible explanation for the disappointing rates of coronary patency in the combined SK + rt-PA arm of the GUSTO trial [149]. Other investigators have suggested that higher levels of plasminogen at baseline are associated with greater thrombolytic efficacy of rt-PA [438].

Figure 4-1: Baseline plasminogen levels according to time of onset of symptoms.



We have not been able to assess whether the patients with a higher baseline level of plasminogen enjoyed greater thrombolytic efficacy, as no patients underwent angiography. We have already seen that non-invasive methods of determining whether thrombolysis has failed are not very reliable. Whether there has been consumption of plasminogen as a consequence of endogenous activation can potentially be determined by assessing whether patients who had symptoms for longer had lower levels of plasminogen at baseline. This is not borne out by our data (**Figure 4-1**). Of course, there is almost certainly going to be variability in circulating plasminogen from individual to individual, and analysis of the data in this way, especially with such small numbers, is prone to error.

There is surprisingly little data on the levels of plasmin following thrombolysis. Part of the problem is the fact that plasmin is rapidly neutralised by circulating α_2-antiplasmin [98]. As a consequence, plasmin is usually barely recordable [402]. It would appear that plasmin activity remains below 1·0CU/ml (caseinolytic units) which is equivalent to less than 1·0μmol/l [439]. Some studies have examined the level of plasmin indirectly from the level of circulating plasmin-α_2-antiplasmin (PAP) complexes, but this assumes that all circulating plasmin is complexed which would not be true in the context of thrombolysis because of the massive amounts of plasmin produced - saturating α_2-antiplasmin. Similarly, plasmin production can be estimated from the levels of α_2-antiplasmin – which would fall as it complexes with plasmin. As one would expect, thrombolysis induces a significant fall in levels of α_2-antiplasmin [390], with levels completely recovering within 36 hours [403]. Levels of α_2-antiplasmin may fall to below 10% following thrombolysis with SK [423].

To summarise, the plasminogen data certainly confirm rapid and marked depletion of circulating plasminogen following administration of SK. Levels remain significantly suppressed for over 48 hours. The capacity to lyse fibrin-rich clot is undoubtedly impaired in the post-thrombolysis period, and coagulation would appear to be in a dominant position over fibrinolysis early following therapy.

4.6 THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) AND THROMBOLYSIS

Carboxypeptidase activity before the administration of thrombolytic largely falls within the expected normal range (2-6u/l), which does give some credibility to the assay performed.

Carboxypeptidase activity appears to rise early (to 4 hours) after thrombolysis (**Figure 3-8**) suggesting potential activation of TAFI to TAFIa by plasmin. TAFIa has previously been shown to be generated in animal models of thrombosis and thrombolysis [440]. When carboxypeptidase activity was blocked by the use of the inhibitor PCI: potato carboxypeptidase inhibitor, the time to achieve 50% clot lysis was accelerated by an average of 27%. More recent studies have shown that inhibitors of TAFI can enhance thrombolysis in various animal models [441-443].

The activation of TAFI by plasmin has been analysed and shown to be stimulated by glycosaminoglycans [125]. No information as to whether SK can directly activate TAFI is available, although urokinase and rt-PA were not capable of activating TAFI [444]. Our study

suggests that there may be activation of TAFI by SK, although the initial rise in activity was not statistically significant, and it is likely that the early rise is purely plasmin-mediated.

Beyond 4 hours there is a definite fall in carboxypeptidase (and hence TAFIa) activity. This fall could represent destruction and inactivation of TAFI and TAFIa as plasmin excesses are formed. Other investigators have described a modest reduction in TAFI antigen levels by plasmin but a significant reduction in TAFI activity [445].

Beyond 12 hours activity then begins to increase again. At this time we have already seen that levels of SF are also rising and X-oligomer levels are falling. At about 12 hours therefore, the lytic process is tailing off and coagulation predominating. On the assumption that thrombin is being generated, one would have anticipated that TAFI activity would be on the rise [119]. The graph of SF levels and carboxypeptidase activity (**Figure 3-23**) illustrates this very nicely with carboxypeptidase activity appearing to follow the production of SF. This is further evidence that coagulation is dominant so soon after thrombolytic therapy.

A potential shortcoming of the data is the relatively small numbers involved. The small fall in activity to one hour may well be spurious. The subsequent rise, although not statistically significant from 1 to 4 hours, is almost certainly real and would be expected. The statistically significant fall from 4 to 12 hours could not have been anticipated and again is likely to be real. Beyond this point there is a steady recovery of activity to nearer baseline levels.

Data from TAFI antigen levels is more difficult to interpret as the fluctuations in the level of the antigen are relatively small. There are also potential problems with the levels measured. The normal range on the assay as it was in 1997 was approximately 54nM, making our results relatively low. The same ELISA today is giving a normal range today nearer to 100nM according to Prof. Laszlo Bajzar, (*personal communication* April 2004). This may have been due to a problem with the TAFI standard being employed as a control back in 1997. As a consequence, it is fair to say that this data can only really be commented upon with respect to the pattern over time rather than the absolute values.

This particular assay is measuring all TAFI: active, inactive and certain cleavage products. The only significant change is observed between 4 and 24 hours when the levels fall. This is most likely to be a consequence of plasmin-mediated degradation and consumption, as well as potential complex formation with Lys-plasminogen. The subsequent steady recovery seen beyond 24 hours suggests replacement of TAFI by the liver and less degradation by plasmin.

It would therefore appear that TAFI is of potential clinical importance in thrombolysis. This has already been suggested by work in a canine model of thrombolysis, where TAFI was

inhibited and thrombolysis consequently enhanced [440]. The influence of TAFI may contribute further to the apparent dominance of coagulation over fibrinolysis after approximately 12 hours. It is difficult to be sure whether TAFI is exerting an influence in the very early stages of thrombolysis.

Whether TAFI has a role to play in the pathophysiology of coronary disease remains unclear. In one large cohort in France, elevated levels of TAFI antigen appeared to be a risk factor for coronary disease, whereas the same study found this was not the case in Northern Ireland [446]. Other studies have suggested that individuals with coronary disease tend to have higher levels of TAFI [447], whereas another study found levels of TAFI were lower in patients with recent myocardial infarction [448]. Part of the problem in the interpretation of the various studies is the fact that there is no recognised uniform assay procedure. In addition, TAFI levels do not necessarily correlate with TAFI activity, and individuals with higher levels of TAFI activity may be more at risk from coronary disease [449]. Elevated levels of TAFI were also found in type 2 diabetics [450].

Whether drugs that inhibit TAFIa or suppress TAFI activation could be employed as adjuncts to thrombolysis has yet to be seen. Preliminary studies in a canine model suggested that administration of the thrombin inhibitor melagatran inhibited TAFI activation during thrombolysis [451]. It is likely that inhibition by melagatran was indirect by preventing thrombin activation. Whether drugs could be employed to promote endogenous fibrinolysis and consequently offer protection from undesirable thrombotic events is worthy of investigation.

There are other inhibitors of thrombolysis which were not examined in this study.

From a purely physiological point of view, it is hard to imagine that PAI-1 can possibly play a role in the scheme of things when huge amounts of a plasminogen activator are administered as a thrombolytic agent. The levels of PAI-1 found in the normal circulation are very low (~0·1nM), and half or more of PAI-1 present in the circulation is in an inhibited complex. Nevertheless, there is a body of evidence to suggest that PAI-1 is of physiological relevance to thrombolysis.

A circadian variation in t-PA and PAI-1 levels is well recognised. A consequence is an adverse effect on the activity of the fibrinolytic system which is low in the early morning and higher later in the day [452]. PAI-1 exhibits peak activity around 4·00am to 6·00am [453-455]. Subsequent to this discovery it was found that t-PA also has a circadian pattern of efficacy with a significant morning resistance to lysis [456-458], the very time when AMI is more likely to occur [459]. A high level of PAI-1 increases the risk of AMI [460-462] and appears to be associated

with a greater likelihood that thrombolysis will fail [463]. Sinkovic *et al* demonstrated evidence that pre-treatment levels of PAI-1 could predict the outcome of thrombolysis with SK, and that high levels of PAI-1 were associated with a failure of thrombolysis [464]. Diabetics have increased levels of PAI-1 [465], and this has been implicated in their increased likelihood to experience a failure of thrombolysis [466].

Studies that have measured PAI-1 following thrombolysis for AMI found that levels of this glycoprotein fell early after thrombolysis but subsequently increase significantly [467-469]. There is evidence that the increase in PAI-1 activity is greater after SK than after rt-PA [469]. Some authors suggest that the increase in PAI-1 is a drug-independent antifibrinolytic rebound phenomenon in response to thrombolytic treatment [470].

This may be why reocclusion may occur more commonly after thrombolysis than one would expect [463]. In vitro experiments implicate thrombolysis-activated platelets with the release of functionally active PAI-1 [471,472]. In the rabbit model, activation of platelets accompanying thrombosis or thrombolysis (or both), markedly increased PAI-1 activity in plasma [473]. Evidence supporting the hypothesis that PAI-1 acts as a significant inhibitor of t-PA was provided in a canine model of coronary thrombosis [474]. In this model, administration of an antibody to PAI-1 reduced time to reperfusion and delayed the occurrence of reocclusion.

Similar, but less pronounced rises in PAI-1 levels appear to occur following direct angioplasty for AMI [468,469] suggesting that thrombolysis itself is partly the trigger for a rebound anti-fibrinolytic state following treatment.

4.7 PLATELETS AND THROMBOLYSIS

Membrane P-selectin (mP-selectin) is a useful and potentially 'dynamic' marker of platelet activation. We have demonstrated very clearly that platelets are activated at the time of AMI employing this marker, and also that they remain relatively activated following thrombolysis. Other studies have demonstrated similar findings [372,475]. We believe that our study is the first to employ flow cytometry to dynamically observe changes in mP-selectin and glycoprotein receptors in a serial manner so close to myocardial infarction, and in such detail. Other studies have looked at platelet activation in the days or weeks following the event, but these would not detect the influence of the thrombolytic agent, and the markers used were clearly not as specific [54].

In this study, the precise influence of SK however is difficult to determine. The expression of mP-selectin is seen to fall in the majority of patients early following thrombolysis, and

although not statistically significant, examination of individual patient data (**Figure 3-10**) suggests that mP-selectin expression does indeed decrease. This could be anticipated by the knowledge that some fibrinogen degradation products including fragment D inhibit platelet aggregation by binding to the GP IIb/IIIa receptor [476]. One of the few studies examining mP-selectin after myocardial infarction suggested that expression of this marker may depend on the patency of the infarct-related artery; expression fell in the first six hours if the artery was patent, and rose if the artery was occluded [477]. This may account for the different behaviour seen in some of the patients in our study. We did not have angiographic validation of the success or failure of thrombolysis. Even if one employed ECG criteria to determine successful thrombolysis, the data in this study would not be very helpful. Only three patients in the platelet substudy showed early resolution of ST changes.

The activation of platelets appears therefore to be reduced very early following thrombolysis. This may be an effect of the plasmin generated, which has been shown in vitro to inhibit platelets [478-483]. In addition however, all patients will have received Aspirin just prior to thrombolysis.

In this study, the depression of platelet activation is not hugely impressive suggesting that SK itself may not be inhibiting platelets to any great extent, despite the in vitro data suggesting otherwise. Similarly, one has to conclude the same for Aspirin in our cohort of patients. Platelet inhibition is of course achieved with Aspirin but this is not complete [484] despite its beneficial effect on mortality after AMI [141].

The subsequent increase in platelet activation seen beyond 2 to 4 hours is certainly more convincing. There is considerable evidence confirming platelet activation after SK [485,486,486] and rt-PA [487,488], and evidence to suggest that this is largely as a consequence of the thrombolytic agent and not the AMI itself [484,485]. Platelet α granule release appears to occur within the first 12 hours following therapy, and the administration of Aspirin does not appear to abolish this release [51].

The activation of platelets could be due, in large part, to the release of thrombin. Several studies have confirmed the generation of thrombin in a clinical setting by the finding of elevated levels of fibrinopeptide A [52,70,72,261,379,38372,72], prothrombin fragments 1+2 (F_{1+2}) [45,72,261,381,382], and thrombin-antithrombin III-complexes (TAT) [45,72,72,261,380,382,383] during and after the infusion of thrombolytic agents. There is a suggestion that raised levels of these markers may be predictive of an adverse outcome [72,489]. There are likely to be various sources of thrombin following thrombolytic therapy. Activated platelets may convert prothrombin to

thrombin, and there is release of clot-bound thrombin during thrombolysis [490]. There may also be exposure of the factor Xa/Va complex enabling further activation of prothrombin. Thrombin may also be formed following the activation of clotting factors as a direct consequence of the thrombolytic drug. Thrombin has a key role to play in arterial thrombosis and almost certainly is crucial to the reocclusion of successfully thrombolysed vessels.

Alternatively, platelet activation could be mediated by collagen and tissue factor from exposed and ruptured atherosclerotic plaque. In the case of SK, there has been a suggestion that platelets may be activated by the binding of anti-streptokinase antibodies to the platelet surface [486]. rt-PA may also act directly on the platelet to increase thrombin generation [491]. A further possibility is that thromboxane A_2, released in large amounts following thrombolysis [485,487,488], is responsible for the activation of platelets. If thrombolysis is successful in clearing the thrombus, there may also be changes in shear rate within the vessel as a consequence of residual stenosis resulting in platelet activation.

The major end product of thrombolysis, plasmin, appears to potentiate the platelet release reaction in response to thrombin. It probably achieves this by increasing the availability of factor V on the platelets, thus enhancing prothrombin activation [492]. Plasmin may also be able to directly activate platelets [493] and is obviously released in significant amounts during thrombolysis [385,439]. Plasmin also appears to cause the degranulation of platelets and release of ADP resulting in the induction of platelet aggregation [494].

In contrast, a few in vitro studies have suggested that plasmin has no effect on platelet function [495], and further studies have suggested that platelet function is impaired after exposure to plasmin [478-483]. Very few clinical studies have confirmed the platelet inhibition seen in vitro, although this has been inferred from the fact that bleeding time may be prolonged soon after therapy [496], and that platelet aggregation is attenuated [497]. Some investigators have suggested that there is probably both inhibition and activation occurring after thrombolytic treatment [53]. Recent studies certainly tend to support the notion of early inhibition of platelets soon after thrombolytic therapy. It would also appear that the extent of inhibition depends on the thrombolytic employed [498]. This study certainly suggests partial early inhibition followed by later activation.

It has been established that SK stimulates platelet membrane GP IIb/IIIa to form an active fibrinogen receptor and platelet degranulation, and rt-PA promotes fibrinogen binding to platelets [499]. Flow cytometry has confirmed the increased expression of the GP IIb/IIIa receptor following thrombolysis [475]. Our study has similarly revealed a steady increase in GP

IIb/IIIa expression, but this only begins beyond 4 hours. It tends to precede the increase of other markers in this study of coagulation activity following thrombolysis. This offers further support that platelets themselves have a crucial role in the early dominance of coagulation over fibrinolysis. Both SK and plasmin appear to be able to stimulate the synthesis and release of platelet activating factor (PAF) from endothelial cells, with the release beginning after approximately sixty minutes and lasting up to three hours [500].

The fact that thrombolytic agents appear in certain circumstances to activate platelets may partly explain why there is such a high incidence of reocclusion following thrombolytic therapy [499]. In addition, clots which are rich in platelets are more resistant to the action of thrombolytic agents from the outset [501]. Platelets contain factor XIII which stabilises crosslinked fibrin making it more resistant to lysis [502]. Platelets also contain PAI-1 [102,103], and AP in their granules [503]. In summary, as well as potentially being responsible for the activation of coagulation following thrombolytic therapy, platelets may also directly inhibit thrombolytic activity.

This may be as a consequence of thrombin generation, or may alternatively be the cause of the thrombin generation as the increase in platelet activity seems to precede the regeneration of SF. In vitro studies have certainly suggested that plasmin may be able to directly activate platelets[493].

The subsequent increase in platelet activation could have significant clinical consequences. The increase occurs relatively early and is marked. This suggests that attempts to provide platelet inhibition – such as with the GP IIb/IIIa inhibitors – may be more effectively given 2 to 4 hours <u>after</u> thrombolysis rather than <u>with</u> the thrombolytic agent. The implications for procedures such as rescue PCI are also important. It may well be that routine administration of IIb/IIIa inhibitors in this setting is appropriate. Initial studies have certainly suggested that employing the GP IIb/IIIa inhibitor abciximab improved outcome in rescue PCI [504].

The previous practice of employing platelet inhibitors at the same time as the thrombolytic agent would appear flawed . Not only does this increase bleeding risk a shown in the trials [505], but it does not appear entirely necessary. If the deployment of GP IIb/IIIa inhibitors were delayed, their administration would be appropriate as this is the time when platelet activity appears to be on the increase. This may subsequently attenuate the dominance of coagulation over thrombolysis that is seen so clearly beyond 12 hours.

Whether soluble P-selectin (sP-selectin) is an accurate marker of platelet activation is unclear. sP-selectin is derived from both activated platelets and endothelial cells [506]. That it is derived from platelets is suggested by the finding that loss of surface mP-selectin from platelets is accompanied by a rise in sP-selectin [507]. Other studies examining the levels of sP-selectin following myocardial infarction have also had difficulties in defining the precise contribution of endothelial cells to plasma levels of sP-selectin [508-510]. Our results correlate well with these studies in so far as levels are elevated on admission. In Shimomura's study serial changes were measured on admission and at 1, 4, 24, 48 hours and one week. Their levels peaked at 4 hours and then fell. We showed that levels actually peaked around 12 hours, and then fell. The rise in sP-selectin in our patients was less marked, but the subsequent fall was significant. Interpreting these changes is difficult. sP-selectin does not tally particularly well with mP-selectin and suggests that the former is a less satisfactory marker of platelet activation. The fact that endothelial cells are a source of sP-selectin, and that endothelial cell dysfunction is a prerequisite of myocardial infarction, suggests that there is almost certainly a contribution from a source other than platelets. As one would expect, mP-selectin appears to be a much more reliable marker of platelet activation, and certainly can detect more dynamic and subtle changes in platelet function over time.

4.8 SOLUBLE FIBRIN FOLLOWING PCI

Levels of SF in patients undergoing elective PCI were already higher than one would expect in a random population. One possible explanation for this is that SF may be a marker of vascular disease in general and a potentially pro-thrombotic state. Other studies have shown a tendency for levels of SF to be higher in patients with unstable angina, but not in patients with stable coronary disease [375]. It is difficult to provide an alternative explanation, and no direct comparison can be made with other studies because we have employed a novel assay. Although the levels of SF were higher than one would have expected, they were still well below the levels recorded in the AMI population.

The fact that levels fall immediately following the procedure almost certainly is attributable to the administration of heparin immediately before the passage of the guidewire down the diseased artery. The subsequent rise in SF levels is to be expected as both the levels of circulating heparin gradually fall, and the coronary plaque has been ruptured as part of the angioplasty procedure. Interestingly, despite plaque rupture, levels of SF were not significantly greater at 24 hours than they were at baseline. This may suggest that the PCI procedure does not cause a significant amount of coagulation activation, possibly because of

the administration of heparin. In addition, this would add support to the AMI data, that SF is insufficiently sensitive to be a marker of coagulation at the level of the coronary artery. There is certainly a difference between the stable PCI patients and the AMI patients, but this would not prove discriminatory enough to employ SF as a marker of coagulation in this population.

There have been surprisingly few studies looking at the coagulation and fibrinolytic systems at around the time of PCI, especially in the setting of AMI.

Sakata *et al* [511] revealed that there appears to be impaired fibrinolysis early after PCI in patients who restenose as reflected by raised PAI-1 levels in these patients. Other groups have reported similar findings [512,513]. This has been quoted as a potential reason why diabetic patients are more likely to restenose following PCI – because of higher basal levels of PAI-1 [514]. Contrastingly, another group found that reduced levels of PAI-1 after PCI was associated with a significantly reduced risk for restenosis [515].

Peltonen *et al* [516] found that in about a third of patients undergoing successful PCI there was systemic as well as local evidence of activation of the coagulation and fibrinolytic systems as reflected by an increase in prothrombin fragments F_{1+2} and D-dimer (despite adequate heparinisation), and a reduction in fibrinogen. As in this study, other groups have not found evidence of activation of coagulation during uncomplicated procedures, even when sampling from the coronary arteries [517]. When there is clear evidence of thrombus formation or dissection, markers of thrombin generation are increased [518].

Few studies have examined the effect of primary PCI on the coagulation and fibrinolytic systems. Interestingly, in one group of patients routinely given intravenous heparin during and then six hours after direct PCI [519], the procedure did not appear to have a significant effect on the systems. This may explain why primary PCI appears to be more successful than thrombolytic therapy, and may suggest that it is thrombolysis itself which promotes an anti-fibrinolytic/procoagulant state following treatment. Another group however showed a single peak of PAI-1 activity 16 hours after direct PCI [468]. It would appear that heparin (which is routinely given to patients during elective PCI) does not significantly affect the fibrinolytic parameters in terms of fibrinogen, plasminogen, α_2-antiplasmin, and fibrin(ogen) degradation products [417]. It is unlikely that these studies can be directly applied to the arena of rescue angioplasty as heparin does appear to attenuate the prothrombotic state induced by SK [520]. Platelet activity appears to be reduced early after direct PCI but is increased after about 24 hours [521].

4.9 X-OLIGOMER FOLLOWING PCI

The variation of X-oligomer following PCI is minimal, suggesting that there is no significant activation of the fibrinolytic system that can be measured by this assay. This provided further evidence that there is very little in the way of thrombin generation during and immediately after PCI, and thus less crosslinked fibrin to break down. In view of the low incidence of acute thrombosis following elective PCI, this is of no great surprise.

Previous studies have suggested generation and breakdown of fibrin following balloon angioplasty. Ring *et al* measured translesional levels of D-dimer in 31 patients undergoing angioplasty and showed that intracoronary degradation of fibrin can be detected after (but not before) routine coronary angioplasty despite pre-treatment with antithrombotic therapy, and concluded that this was in response to balloon-induced arterial injury and fibrin formation [522].

Some authors have suggested that an impaired fibrinolytic response may be implicated in the restenotic process [523]. Similarly, individuals who appear to generate less PAI-1 following PCI appear to have a significantly lower risk of restenosis [515].

The literature is far from clear in terms of the precise response of the coagulation and fibrinolytic systems following PCI. It would be unethical to take samples without the influence of routinely administered agents such as Aspirin, Heparin or Clopidogrel. The fact that there is activation of coagulation and fibrinolysis however is beyond any doubt. The positive impact of drugs like Aspirin, Ticlopidine and Clopidogrel on acute thrombosis following PCI is testament to this fact [200,201]. The additional benefit of GP IIb/IIIa inhibitors confirms the important role of platelet activation following PCI [348,349]. The recent successful substitution of Bivalirudin for Heparin in this setting further confirms that inhibition of thrombin is crucial for the prevention of complications [258].

4.10 CLOSING SUMMARY AND CLINICAL IMPLICATIONS

One clear limitation of this study is the relatively small number of patients studied, and in particular the fact that not all parameters were measured in all patients. This inevitably will raise doubt as to the veracity of the results, but patterns do appear to emerge. How the various parameters actually relate to each other is similarly difficult to determine because of the small numbers, but some assumptions have been made. In addition to these limitations, there is clearly no control group in this study for the myocardial infarction cohort. As a consequence, emphasis has concentrated on trends over time rather than absolute values. It is unlikely that the results were purely a consequence of the myocardial infarction rather than thrombolytic therapy, as the response of SF, X-Oligomer and plasminogen dramatically change following SK administration.

This study does appear to provide further evidence of the complexity of coagulation and fibrinolytic processes following thrombolytic therapy of AMI. Data accumulated in this study further refines the activities of various aspects of the two processes at specific times following administration of the thrombolytic agent. The dominance of coagulation over fibrinolysis at approximately 12 hours is supported by a variety of data, and especially the SF and X-oligomer data. Further support is provided by the more marked and prolonged depletion of plasminogen than has been suggested previously. This dominance of coagulation may partly explain why thrombolytic therapy, at least with SK, is not as effective as one would hope.

There are a variety of mechanisms whereby thrombolysis may fail. SK, being a single-chain polypeptide derived from β-haemolytic streptococci, is immunogenic. Consequently, antibodies can develop to SK [142], potentially neutralising its activity [143]. High SK neutralisation titres persist for a long time after the use of SK as thrombolytic treatment for AMI [144, 145]. This alone cannot explain thrombolytic failure as rt-PA does not stimulate the production of antibodies and overall enjoys a similar efficacy.

This study demonstrates quite clearly the marked and prolonged depletion of plasminogen which occurs after administration of SK. The absence of available plasminogen is clearly disadvantageous if there is residual coronary thrombus or propagating thrombus, and this has a variety of implications. It would limit the response to any repeat administration of thrombolytic agent and illustrates that rescue PCI is occurring at a time when plasminogen levels are deficient. An additional factor which needs to be considered is the fact that antibodies to prothrombin, which are known to be an independent risk factor for MI in healthy middle-aged men [524], also cross-react with plasminogen in patients who develop AMI [525].

These antibodies may interfere with the fibrinolytic function of plasminogen (whose levels are rapidly depleted and remain so) and therefore attenuate the effects of thrombolysis.

The SF data show a dramatic response to thrombolysis, but it is unclear whether SF performs as a true marker of thrombin generation in this study. SF levels do appear to rise beyond 12 hours suggesting recovery of coagulation. Antithrombotic therapy is no longer routinely employed following SK, but there is an argument that greater benefit may be seen if adjunctive therapy were delayed until coagulation activity recovers. The fall off of fibrinolytic activity (as reflected by X-oligomer levels) during a similar time frame would certainly point to coagulation becoming more dominant beyond 12 hours.

The platelet data does not appear to contradict the other results. The fluctuations in platelet activity following thrombolysis have never before been so clearly defined, and strongly support the suggestion from other investigators that platelet activation occurs relatively soon after therapy, but is not immediate.

In the clinical situation, it can be argued that strategies to inhibit platelet activity in AMI following thrombolysis should be staggered. The previous practice of employing platelet inhibitors at the same time as the thrombolytic agent would appear flawed. Not only does this increase bleeding risk, but it does not appear entirely necessary. If the deployment of GP IIb/IIIa inhibitors were delayed by approximately 4 hours, their administration would be appropriate as this is the time when platelet activity appears to be on the increase. This may subsequently attenuate the dominance of coagulation over thrombolysis that is seen so clearly beyond 12 hours.

The activation of platelets is also appearing to occur at the very time that rescue PCI would be happening. This has very real implications, and would certainly support the idea of routinely employing GP IIb/IIIa inhibitors during rescue PCI.

The fluctuations in activity of TAFI have never been recorded following thrombolysis and suggest a complex role for this substance with its pivotal relationship between the coagulation and fibrinolytic cascades. It is likely that in the early stages following thrombolytic therapy its influence is modest, but its precise role has yet to be defined. Unfortunately this study does not establish a potential role, only that it does appear to be influenced by the administration of SK.

With respect to the PCI data, SF again fails to demonstrate its usefulness as a marker of plaque rupture in the setting of PCI. This may be attributable to the administration of heparin, but it is likely that SF is simply not sensitive enough to detect changes at the level of the

coronary plaque. Similarly, it would appear that there is no significant activation of the fibrinolytic system following PCI as reflected by X-oligomer levels.

In conclusion, there does appear to be a pro-coagulant response occurring within a few hours of thrombolytic therapy. This is likely to be driven partly by SK directly, and partly indirectly by the generation of plasmin. Platelets appear to play a crucial role in this process, and the use of platelet inhibitors in the setting of thrombolysis should possibly be more appropriately timed in order to coincide with peaks of platelet activity. This would possibly enhance the efficacy of thrombolytic therapy, as well as improving the milieu in which rescue PCI is performed. In addition, the bleeding complications observed with adjunctive therapies may be reduced.

CHAPTER 5: APPENDIX

P.T.C.A. PATIENT INFORMATION SHEET

YOU ARE BEING ASKED TO PARTICIPATE IN A CLINICAL STUDY WHICH AIMS TO EXAMINE THE ROLE OF THE BLOOD CLOTTING SYSTEM AT AROUND THE TIME OF ANGIOPLASTY.

THIS STUDY IS NOT EXPERIMENTAL AND WILL NOT AFFECT WHAT TREATMENT YOU RECEIVE IN ANY WAY. THE TREATMENT FOR YOUR CORONARY DISEASE WILL BE ACCORDING TO INTERNATIONALLY AGREED GUIDELINES WHICH ARE ADHERED TO IN THIS DEPARTMENT.

IF YOU AGREE TO PARTICIPATE IN THE STUDY, A TOTAL OF FIVE BLOOD SAMPLES WILL NEED TO BE TAKEN OVER THE FIRST 24 HOURS. WE WILL DO THIS VIA THE TUBE THAT GOES IN THE ARTERY AND AVOID REPEATED NEEDLES BEING USED. ONE BLOOD SAMPLE WILL NEED TO BE TAKEN FROM A VEIN AT 24 HOURS, AS THE TUBE WILL PROBABLY NOT STILL BE IN THE ARTERY AT THAT TIME. BASICALLY, SAMPLES WILL BE TAKEN JUST AFTER THE TUBE HAS BEEN PUT IN THE LEG AND AT 15 MINUTES, 1, 2, AND 24 HOURS AFTER THE PROCEDURE.

THIS DOES MEAN THAT YOU ARE REQUIRED TO STAY IN FOR 24 HOURS AFTER THE PROCEDURE

YOU MAY WITHDRAW FROM THE STUDY AT ANY TIME AND THIS WILL NOT PREJUDICE ANY TREATMENT YOU SUBSEQUENTLY RECEIVE. BELOW IS A BRIEF EXPLANATION OF WHAT WE ARE PLANNING TO DO AND WHY.

The coronary arteries are the blood vessels which supply blood to the heart. Angina occurs when blood clots or fatty deposits partially block one or more of the coronary arteries.

Angioplasty is when a balloon is passed into the blockage and inflated to open up the artery. This treatment can lead to fresh clotting and potentially the artery may narrow back down again either early on after angioplasty or over the first few weeks.

We plan to look at the whole clotting system around the time of angioplasty, and try and find out how the body's normal clotting and repair systems become activated. In a future study we may be able to influence these systems to prevent arteries narrowing down again.

THANK YOU FOR READING THIS INFORMATION SHEET AND CONSIDERING OUR REQUEST.

MYOCARDIAL INFARCTION PATIENT INFORMATION SHEET

YOU ARE BEING ASKED TO PARTICIPATE IN A CLINICAL STUDY WHICH AIMS TO EXAMINE THE ROLE OF THE BLOOD CLOTTING SYSTEM AT AROUND THE TIME OF A HEART ATTACK.

THIS STUDY IS NOT EXPERIMENTAL AND WILL NOT AFFECT WHAT TREATMENT YOU RECEIVE IN ANY WAY. THE TREATMENT FOR YOUR HEART ATTACK WILL BE ACCORDING TO INTERNATIONALLY AGREED GUIDELINES WHICH ARE ADHERED TO IN THIS DEPARTMENT.

IF YOU AGREE TO PARTICIPATE IN THE STUDY, A TOTAL OF EIGHT BLOOD SAMPLES WILL NEED TO BE TAKEN OVER YOUR FIRST TWO DAYS IN HOSPITAL. SAMPLES WILL BE TAKEN BEFORE YOUR INITIAL TREATMENT AND 1, 2, 4, 12, 24, 36 AND 48 HOURS AFTERWARDS. WE HOPE TO DO THIS VIA A SMALL TUBE THAT STAYS IN A VEIN AND AVOID REPEATED NEEDLES BEING USED. VERY OCCASIONALLY MORE THAN ONE BLOOD SAMPLE MAY NEED TO BE TAKEN IF THE SMALL TUBE BECOMES BLOCKED.

YOU MAY WITHDRAW FROM THE STUDY AT ANY TIME AND THIS WILL NOT PREJUDICE ANY TREATMENT YOU SUBSEQUENTLY RECEIVE.

BELOW IS A BRIEF EXPLANATION OF WHAT WE ARE PLANNING TO DO AND WHY.

The coronary arteries are the blood vessels which supply blood to the heart. Heart attacks occur when blood clots block one of the coronary arteries. Thrombolytic or 'clot-busting' drugs are given in an attempt to re-open the blocked artery and prevent significant heart muscle damage occurring. The clot-busting drugs work by activating one of the body's own defence mechanisms, a substance called plasminogen which is converted to a clot dissolving enzyme, plasmin. There may be a potential for this plasminogen to be used up, and subsequently newer clots may be harder to dissolve.

This has implications for patients who have to undergo operations like angioplasty within a few days of a heart attack. Angioplasty is when a balloon is passed into the blockage and inflated to open up the artery. This treatment can lead to fresh clotting and if plasminogen has been used up there is nothing around to stop the clots from blocking the artery again.

We plan to look at the whole clotting system including plasminogen around the time of heart attacks, find out how much is used up and how quickly it is restored. In a future study we will see if we can help patients by giving extra plasminogen.

THANK YOU FOR READING THIS INFORMATION SHEET AND CONSIDERING OUR REQUEST.

P.T.C.A. PATIENT DATA SHEET

NAME						
UNIQUE IDENTIFIER NUMBER						
GROUP CATEGORY	ELECTIVE		UNSTABLE		PRIMARY	
DATE OF PROCEDURE						
DATE OF BIRTH						
AGE						
SEX	MALE			FEMALE		
ETHNIC GROUP						
ON REGULAR ASPIRIN	YES			NO		
IF YES, DOSE						
ASPIRIN ADMINISTERED TODAY	YES			NO		
TIME ADMINISTERED						
ANGINA	YES			NO		
IF YES, C.C.S. GRADE						
PREVIOUS MYOCARDIAL INFARCTION	YES			NO		
IF YES, SITE						
PREVIOUS C.A.B.G.	YES			NO		
IF YES, WHEN						
IF YES, VESSELS GRAFTED	LAD	DIAG		OM	RCA	
PREVIOUS P.T.C.A.	YES			NO		
IF YES, WHEN						
IF YES, TARGET VESSEL	LAD	DIA	CX	OM	RCA	PDA
IS THIS RESTENOSIS	YES			NO		
PREVIOUS THROMBOLYTIC	YES			NO		
IF YES, WHEN						
IF YES, AGENT USED						
SMOKER	YES	NO		AMT.	EX	
HYPERTENSION	YES			NO		
DIABETES	YES	NO		DUR^N	TYPE	
HYPERCHOLESTEROLAEMIA	YES	LEVEL		NO		
POSITIVE FAMILY HISTORY	YES			NO		

TARGET VESSEL	LAD	DIA	CX	OM	RCA	PDA
DIAMETER OF TARGET VESSEL						
PERCENTAGE STENOSIS						
TIMI GRADE OF TARGET VESSEL						
DISEASE SEVERITY	SINGLE VESSEL			MULTIVESSEL		
LV FUNCTION	>50%	40-50%		30-40%	<30%	

TIME OF FIRST INFLATION						
TIME AT END OF PROCEDURE						
FINAL BALLOON SIZE & TYPE						
INFLATIONS (PRESSURE/TIME)	/	/	/	/	/	/
	/	/	/	/	/	/
PERCENTAGE RESIDUAL STENOSIS						
TIMI GRADE POST-P.T.C.A.						
DISSECTION POST-P.T.C.A.	YES			NO		
IF YES, TYPE						
VISIBLE THROMBUS	YES			NO		
COMPLICATIONS	YES			NO		

STENT INSERTED	YES	NO
IF YES, INDICATION		
IF YES, SIZE AND TYPE OF STENT DEPLOYED		

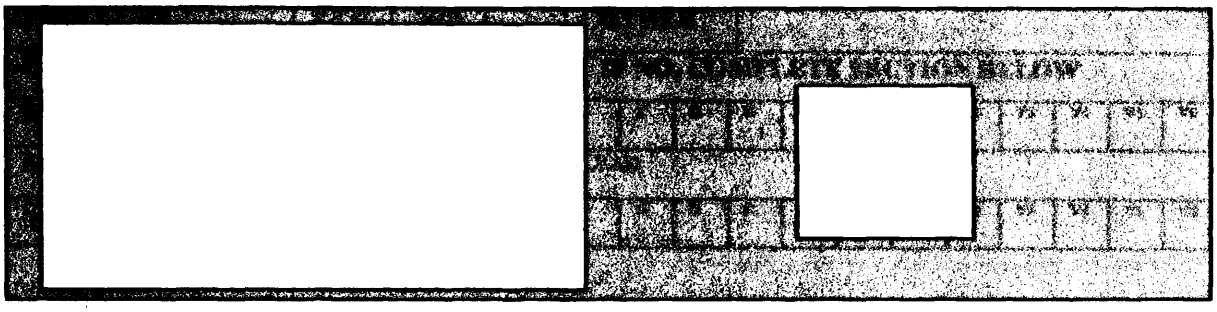
DOSE OF HEPARIN ADMINISTERED				
TIME HEPARIN ADMINISTERED				
ACT RECORD (TIME/RESULT)	/	/	/	/

TIME OF SAMPLES

IMMEDIATELY PRIOR TO P.T.C.A.	
15 MINUTES POST-P.T.C.A.	
1 HOUR POST-P.T.C.A.	
2 HOURS POST-P.T.C.A.	
24 HOURS POST-P.T.C.A.	

OTHER COMMENT

MYOCARDIAL INFARCTION PATIENT DATA SHEET



DATE OF RECRUITMENT												
AGE												
SEX	**MALE** ☐					**FEMALE** ☐						
ETHNIC GROUP	**CAUC** ☐		**ASIAN** ☐			**AFRIC** ☐		**ORIENT** ☐				
PRESENTATION	TIME OF PRESENTATION					TIME SINCE ONSET						
ON REGULAR ASPIRIN	**YES** ☐ **NO** ☐					***IF YES, DOSE***						
ASPIRIN ADMINISTERED TODAY	**YES** ☐ **NO** ☐					***IF YES, DOSE***						
TIME ADMINISTERED												
TIME OF THROMBOLYSIS (TIME 0)												
SITE OF ST ELEVATION (LEADS)	I	II	II	R	L	F	V1	V2	V3	V4	V5	V6
MAXIMAL ST ELEVATION	LEAD						MM					
PREVIOUS MYOCARDIAL INFARCTION	**YES** ☐ **NO** ☐					***IF YES, SITE***						
SMOKER	**YES** ☐ **NO** ☐					AMT.			EX			
HYPERTENSION	**YES** ☐ **NO** ☐					***DURATION***						
DIABETES	**YES** ☐ **NO** ☐					***TIME***			NIDDM ☐ IDDM ☐			
HYPERCHOLESTEROLAEMIA	**YES** ☐ **NO** ☐					***RESULT***						
POSITIVE FAMILY HISTORY	**YES** ☐ **NO** ☐					FATHER		MOTHER		BROTH		SISTER

CLINICAL FEATURES ON ADMISSION

PULSE		**RHYTHM**		**B.P.**		**J.V.P.**	
L.V.F.		**YES** ☐ **NO** ☐		**OTHER**			

BLOOD RESULTS AT PRESENTATION

Hb		**WCC**		**PLATELETS**			
SODIUM		**POT**		**UREA**		**CREAT**	
GLUCOSE		**CK MB**		**CHOL**			

CLINICAL FEATURES AT 12 HOURS

PULSE		RHYTHM		B.P.		J.V.P.							
L.V.F.		YES☐ NO☐		OTHER									
E.C.G. RESOLVED		YES☐ NO☐		IF NO, COMPLETE SECTION BELOW									
SITE OF ST ELEVATION (LEADS)				I	II	II	R	L	F	V1	V2	V3	V4	V5	V6
MAXIMAL ST ELEVATION				LEAD						MM					
SITE OF Q WAVES (LEADS)				I	II	II	R	L	F	V1	V2	V3	V4	V5	V6

BLOOD RESULTS AT 12 HOURS

Hb		WCC		PLATELETS	
SODIUM		POT		UREA		CREAT	
GLUCOSE		CK MB	

CLINICAL FEATURES AT 24 HOURS

PULSE		RHYTHM		B.P.		J.V.P.							
L.V.F.		YES☐ NO☐		OTHER									
E.C.G. RESOLVED		YES☐ NO☐		IF NO, COMPLETE SECTION BELOW									
SITE OF ST ELEVATION (LEADS)				I	II	II	R	L	F	V1	V2	V3	V4	V5	V6
MAXIMAL ST ELEVATION				LEAD						MM					
SITE OF Q WAVES (LEADS)				I	II	II	R	L	F	V1	V2	V3	V4	V5	V6

BLOOD RESULTS AT 24 HOURS

Hb		WCC		PLATELETS	
SODIUM		POT		UREA		CREAT	
GLUCOSE		CK MB	

COMPLICATIONS DURING ADMISSION

V.T./V.F.	YES☐ NO☐	D.C. CARDIOVERSION	YES☐ NO☐
TEMPORARY PACING	YES☐ NO☐	HYPOTENSION	YES☐ NO☐
INOTROPIC SUPPORT	YES☐ NO☐	REINFARCTION	YES☐ NO☐
P.T.C.A.	YES☐ NO☐	A.F.	YES☐ NO☐

USE OF HEPARIN

WAS HEPARIN USED YES☐ NO☐	IF YES, REASON		
APTT RESULTS (TIME/RESULT)	/	/	/
	/	/	/

MUGA SCAN RESULTS

COMMENTS

THE METHOD FOR 0·129 ^{M} CITRATE PRODUCTION (pH 5·5)

In order to produce 0·129^{M} citrate the following measures are required:

CITRIC ACID

M.W.=	210·1g
1·0^{M}=	210·1g/l
	21·01g/100ml
0·1^{M} =	2·101g/100ml
0·129^{M} =	**2.710g/100ml**

SODIUM CITRATE

M.W.=	294·1g
1·0^{M} =	294·1g/l
	29·41g/100ml
0·1^{M} =	2·941g/100ml
0·129^{M} =	**3·794g/100ml**

IT IS APPROPRIATE TO START WITH A LARGER VOLUME OF SODIUM CITRATE AND ADD ALIQUOTS OF CITRIC ACID UNTIL THE pH FALLS TO 5·5. APPROXIMATE RATIO IS 60:20

THE METHOD FOR THE PRODUCTION OF 1000 ML OF TYRODES'S BUFFER

NaCl	8·00g
KCl	0·20g
NaH_2PO_4	0·07g
MgCl	0·18g
$NaHCO_3$	1·00g
Dextrose	1·00g
Na_2EDTA	3·36g

Filter the solution through a 0·22μm filter and store at 4°C.

THE METHOD FOR THE PRODUCTION OF 1000 ML OF PHOSPHATE BUFFERED SALINE (PBS)

NaCl	8·00g
KCl	0·20g
NaH_2PO_4	1·44g
KH_2PO_4	0·24g

pH to 7·4 using 1 M HCl. Alternatively, PBS tablets can be used. Stored at 4°C.

METHOD FOR THE PRODUCTION OF 200 ML 10% PARAFORMALDEHYDE

20·0g of paraformaldehyde in 200ml of PBS

Heat and stir the solution in a fume cupboard until the temperature is maintained at approximately 60°C for about 2 hours.

Filter the final solution of 10% paraformaldehyde and dilute according to requirements. Store frozen until required.

METHOD FOR THE PRODUCTION OF 500 ML OF HEPES BUFFERED SALINE (HBS) – PLATELET FLOW CYTOMETRY

NaCl	4·383g
KCl	0.186
$MgSO_4$	0.123g
Hepes (Sodium salt)	1.302g

pH to 7·4

Filter the solution through a 0·22μm filter and store at 4°C.

METHOD FOR THE PRODUCTION OF 100 ML OF HEPES BUFFERED SALINE (HBS) – CARBOXYPEPTIDASE ASSAY

For the production of 100mM Hepes and 150mM Sodium Chloride

Hepes (Sodium Salt)	2·603g
NaCl	0·876g

pH to 7·4

METHOD FOR THE PRODUCTION OF POTATO CARBOXYPEPTIDASE INHIBITOR

For a final concentration of 50μg/ml (in a 1200μl solution)

5mg in 48·83ml in Hepes buffered saline

METHOD FOR THE PRODUCTION OF CARBOXYPEPTIDASE SUBSTRATE (FA-ALA-ARG-OH)

For a final solution of 0·72M

10mg in 1ml methanol
Add the methanol-dissolved substrate to 38ml Hepes buffered saline

Table 5-1: Soluble fibrin levels following thrombolysis (citrate samples). U/L

PATIENT	PRE	1	2	4	12	24	36	48
1	7·239	0	0	0	1·315	4·674	3·757	1·17
2	559·4		0·083	0·105	2·992	123·4	112	163·8
3	1·434	0·065	0·063	0·091	0·699	3·028	42·25	64·42
6	6·699	0	0	0	0·001	76·04	2·609	15·88
9	2·788	0·354	0·352	0·363	1·76	0·815	1·491	2·218
10	3·206	0·358	0·352	0·374	0·565	2·169	1·126	27·46
11	0	0	0	0	0	0	0	0
12	35	0·324	0·405	0·253	0·281	7·682		9·687
13	14·47	0·061	0·064	0·074	0·505	4·646	1·19	0·949
14	20·46	0·006	0·01	0·01	0·086	0·652	1·341	3·181
16	1·584	0·427	0·427	0·427	0·519	3·558	6·636	46·64
17	1·439	0·451	0·443	0·468	0·551	4·492	2·648	1·453
18	9·389	0·018	6·194	0·023	0·043	0·92	1·107	2·233
19	1·46	0·426	0·427	0·444	1·173	1·203	1·222	2·65
21	4·072	0·774	0·727	0·762	1·3	1·439	2·346	65·51
22		2·693	4·075	4·704	32·56			
23	1·443			0·048	1·884			
24	0	0	0	0	0	0	19·47	4·656
25	0	0	0	0	0	0	0	0
26							1·282	
28	53	0	0	0	0·127			

Median	**3·21**	**0·06**	**0·08**	**0·08**	**0·54**	**2·17**	**1·49**	**3·18**
Mean	**38.06**	**0.33**	**0.72**	**0.41**	**2.32**	**13.81**	**11.79**	**24.23**
SEM	**29·13**	**0·15**	**0·37**	**0·23**	**1·60**	**8·12**	**6·76**	**10·26**
SD	**127·00**	**0·63**	**1·61**	**1·03**	**7·16**	**33·47**	**27·88**	**42·29**

Table 5-2: Soluble fibrin levels following thrombolysis (aprotinin samples). U/L

PATIENT	PRE	1	2	4	12	24	36	48
1	5·325	0	0	0	2·602	2·554	2	8·589
2	729·2	0·087	0·081	0·095	3·347	49·21	278·7	335·8
3	3·426	0·074	0·069	0·091	0·423	1·691	71·54	144·8
6	8·191	0	0	0	0·001	42·16	7·003	12·57
9	1·76	0·349	0·358	0·365	1·968	1·968	1·284	2·066
10	2·087	0·352	0·354	0·374	0·584	1·332	1·227	102·7
11	0	0	0	0	0	0	155·8	0
12	35·97	0·29	0·285	0·245	0·328	2·431	0	7·458
13	6·268	0·067	0·065	0·134	0·503	12·35	3·347	2·035
14	18·3	0·007	0·008	0·016	0·079	0·376	1·26	2·102
16	2·334	0·426	0·426	0·426	0·529	2·543	5·107	10·08
17	1·654	0·458	0·466	0·463	0·511	2·774	1·365	1·275
18	14·68	0·026	0·442	0·024	0·046	1·552	0·892	2·2
19	1·64	0·43	0·431	0·444	1·329	0·92	1·013	1·854
21	4·513	0·705	0·746	0·782	1·385	1·474	1·963	17·89
22		5·859	3·991	5·099	36·07			
23	1·754	0·029	0·002	0	3·231			
24	0	0	0	0	0	0	25·95	5·561
25	0	0	0	0	0	0	0	0
26							2·847	
28	64·23	0	0	0	0·236			

Median	**3·43**	**0·07**	**0·08**	**0·09**	**0·51**	**1·69**	**1·98**	**5·56**
Mean	**47.44**	**0.46**	**0.39**	**0.43**	**2.66**	**7.26**	**31.18**	**38.65**
SEM	**38·05**	**0·29**	**0·20**	**0·25**	**1·77**	**3·59**	**17·18**	**20·97**
SD	**165·85**	**1·29**	**0·88**	**1·12**	**7·94**	**14·78**	**72·90**	**86·48**

Table 5-3: Soluble fibrin levels following thrombolysis (hirudin samples). U/L

PATIENT	PRE	1	2	4	12	24	36	48
1	25·7	0	0	0	9·228	2·928	5·562	1·599
2	506·7	0·081	0·08	0·105	2·668	33·69	95·1	223·1
3	1·384	0·068	0·069	0·087	0·487	0·871	162·4	57·76
6	6·6	0	0	0	0·042	30·11	0·49	6·55
9	1·588	0·359	0·356	0·361	1·471	0·778	0·882	1·525
10	1·668	0·352	0·352	0·372	0·541	1·105	1·167	35·87
11	0	0	0	0	0	0	39·35	0
12	28·09	0·079	0·138	0·217	0·265	1·075		4·835
13	8·936	0·069	0·067	0·075	0·427	5·612	0·906	0·885
14	2·475	0·017	0·013	0·014	0·082	0·468	1·353	1·16
16	1·808	0·433	0·43	0·433	0·492	1·469	1·791	1·589
17	1·518	0·479	0·468	0·471	0·539	1·872	0·981	1·167
18	10·69	0·035	61·07	0·448	0·046	1·526	0·77	1·226
19	1·968	0·44	0·445	0·452	1·488	0·875	1·137	1·987
21	3·477	0·77	0·712	0·724	1·232	1·202	1·702	11·43
22		5·859	4·609	4·898	47·34			
23	1·24	0·027	0·002	0	1·84			
24	0	0	0	0	0	0	0·755	0
25						2·401		
26							1·935	
28	134·2	0	0·001	0	0·194			

Median	**2·22**	**0·07**	**0·08**	**0·11**	**0·49**	**1·20**	**1·26**	**1·59**
Mean	**41.00**	**0.48**	**3.62**	**0.46**	**3.60**	**5.06**	**19.77**	**21.92**
SEM	**28·37**	**0·30**	**3·20**	**0·25**	**2·48**	**2·48**	**11·32**	**13·98**
SD	**120·35**	**1·32**	**13·95**	**1·10**	**10·80**	**10·21**	**45·29**	**55·93**

Table 5-4: Soluble fibrin levels employing different monoclonal antibodies.

	GAUBIUS ng/ml			NIBSC U/L		
PATIENT	PRE	4	48	PRE	4	48
3	39·6	12·8	49·2	1·43	0·09	64·42
11	28·0	7·6	35·1	0	0	0
12	63·5	11·6	31·8	35	0·25	9·69
13	23·9	11·6	23·2	14·47	0·07	0·95
14	37·0	10·1	38·1	20·46	0·01	3·18
16	33·5	6·9	28·6	1·58	0·43	46·64
18	53·8	9·4	31·6	9·39	0·02	2·23
19	30·6	9·8	35·9	1·46	0·44	2·65
21	26·8	7·4	49·5	4·07	0·76	65·51
26	105·2	202·6	34·3	0	0	
Median	**35·25**	**9·95**	**34·70**	**2·83**	**0·08**	**3·18**
Mean	**44.19**	**28.98**	**35.73**	**8.79**	**0.21**	**21.70**
SEM	**7·84**	**19·30**	**2·62**	**3·64**	**0·09**	**9·50**
SD	**24·80**	**61·04**	**8·30**	**11·51**	**0·26**	**28·50**

Table 5-5: X-oligomer levels following thrombolysis (citrate samples). ng/ml

PATIENT	PRE	1	2	4	12	24	36	48
1	1·22	9·85	4·85	3·43	2·01	3·18	9·55	1·15
2	10·20		22·84	50·09	133·80	99·19	120·50	114·20
3	10·38	26·96	42·36	110·30	169·20	61·24	48·49	62·06
6	103·90	40·83	98·83	168·10	118·50	214·60	107·40	134·60
9	54·81	22·44	40·84	42·52	59·33	209·10	50·76	60·62
10	109·60	25·11	43·78	85·37	64·53	50·55	114·60	98·40
11	141·20	101·30	376·40	389·80	537·00	404·80		112·90
12	40·80	32·26	96·91	88·57	65·49	322·50	143·40	74·89
13	56·14	65·16	80·99	72·05	106·60	124·50	54·11	74·17
14	95·58	22·70	17·52	30·77	123·30	194·90	184·60	154·90
16	26·42	12·48	10·10	26·12	39·56	139·40	117·50	25·23
17	76·91	44·10	48·07	87·01	170·40	104·80	121·00	72·19
18	107·00	53·47	155·90	77·25	181·70	172·00	142·40	82·95
19	35·36	15·15	14·26	51·67	97·96	87·93	66·76	51·98
21	56·63	31·32	58·52	95·27	149·60	52·61	53·68	57·44
22	222·10	76·09	199·70	174·90	433·10	213·20	207·50	245·50
23	177·90	211·10	513·70	701·70	1330·00			
24	220·90	61·59	170·30	192·40	309·00	284·30	254·40	227·60
25	75·10	41·60	159·30	90·05	136·00	106·50	118·00	57·02
26	52·85	39·74	41·60	42·34	23·56	15·23	29·77	19·20
28	155·90	65·99	151·50	105·30	309·50			
Median	**75·10**	**40·29**	**58·52**	**87·01**	**133·80**	**124·50**	**116·05**	**74·17**
Mean	**87·19**	**49·96**	**111·82**	**127·86**	**217·15**	**150·55**	**108·02**	**90·89**
SEM	**14·25**	**9·97**	**27·78**	**33·86**	**62·86**	**24·48**	**15·17**	**14·68**
SD	**65·31**	**44·57**	**127·32**	**155·18**	**288·07**	**106·71**	**64·34**	**63·97**

Table 5-6: X-oligomer levels following thrombolysis (aprotinin samples). ng/ml

PATIENT	PRE	1	2	4	12	24	36	48
1	1·04	7·38	1·25	4·25	2·40	1·60	1·01	1·01
2	44·92	11·28	10·74	10·92	36·90	15·27	159·70	132·60
3	45·91	20·97	34·59	70·37	57·56	65·16	67·03	65·99
6	105·90	55·30	115·40	173·30	118·00	163·50	144·20	138·10
9	38·34	12·26	47·79	46·73	96·55	184·70	59·33	104·40
10	39·37	6·97	25·11	79·30	140·50	93·56	127·40	97·01
11	160·50	233·40	149·60	373·20	676·80	396·90	147·60	96·47
12	32·26	31·87	98·25	37·14	100·60	109·00	92·24	46·89
13	38·07	32·86	68·49	35·94	90·24	109·10	58·37	55·53
14	422·40	20·84	13·48	81·31	79·68	90·41	157·30	286·20
16	35·66	28·79	55·65	30·28	15·45	48·02	61·49	35·36
17	47·28	38·59	53·95	47·28	150·90	51·98	59·37	35·72
18	25·23	33·46	61·18	33·46	268·60	134·60	118·00	126·00
19	44·69	16·33	32·37	18·70	24·34	4·11	19·89	35·36
21	18·08	6·66	29·01	30·81	227·40	60·15	54·48	65·87
22	206·90	90·03	214·20	420·60	293·90	226·30	209·50	251·40
23	299·00	396·20	746·20	1071·00	1445·00			
24	250·50	51·34	148·40	181·10	297·90	197·00	261·40	90·45
25	125·10	103·70	121·80	117·20	94·04	54·36	150·20	24·66
26	39·74	41·22	47·95	21·74	22·83	29·77	27·58	29·04
28	111·20	94·47	205·40		834·60			

Median	**44·92**	**32·86**	**55·65**	**47·01**	**100·60**	**90·41**	**92·24**	**65·99**
Mean	**101·53**	**63·52**	**108·61**	**144·23**	**241·63**	**107·13**	**104·00**	**90·42**
SEM	**23·88**	**20·06**	**34·59**	**55·07**	**75·92**	**22·04**	**15·63**	**17·04**
SD	**109·45**	**91·92**	**158·50**	**246·30**	**347·91**	**96·07**	**68·15**	**74·27**

Table 5-7: X-oligomer levels following thrombolysis (hirudin samples). ng/ml

PATIENT	PRE	1	2	4	12	24	36	48
1	49·00	7·70	1·10	3·81	1·72	4·61	1·62	9·75
2	56·95	12·54	10·38	18·39	41·19	61·24	55·32	29·80
3	28·67	30·57	26·21	90·11	187·90	42·36	50·49	45·71
6	91·26	53·72	91·76	152·80	113·90	129·50	105·40	102·90
9	49·70	17·55	47·37	44·20	34·18	108·40	22·85	83·34
10	59·33	20·20	10·43	42·10	73·30	57·39	42·10	30·87
11	85·90	191·90	343·50	351·40	519·40	161·00	157·00	82·06
12	27·59	20·17	40·44	39·71	43·33	74·56	72·21	68·52
13	59·60	43·74	71·00	84·87	122·30	84·01	71·84	76·71
14	170·60	19·01	19·92	29·45	54·60	120·20	110·10	141·90
16	26·42	12·78	37·76	56·26	76·79	95·36	106·50	51·67
17	80·82	43·31	49·14	80·26	61·78	39·37	56·90	13·57
18	71·23	62·22	1477·00	126·30	139·10	64·93	74·83	87·91
19	59·33	20·48	15·15	39·26	67·70	34·76	46·81	30·28
21	47·81	41·47	56·90	109·50	139·10	36·76	43·05	30·81
22	246·00	27·68	226·30	407·60	395·50	221·50	193·10	213·70
23	402·20	214·70	479·70	588·50	1935·00			
24	138·60	73·15	136·50	208·10	276·10	171·60	110·20	40·48
25	126·30	29·41	41·22	76·66	58·16	55·50	96·85	18·84
26	44·58	48·33	34·56	35·66	34·56	9·47	41·60	23·56
28	140·60	41·29	142·10	167·90	379·80			

Median	**59·60**	**30·57**	**47·37**	**80·26**	**76·79**	**64·93**	**71·84**	**45·71**
Mean	**98·21**	**49·14**	**159·93**	**131·09**	**226·45**	**82·76**	**76·78**	**62·23**
SEM	**19·29**	**11·82**	**70·90**	**32·29**	**90·61**	**13·17**	**10·67**	**11·61**
SD	**88·41**	**54·19**	**324·92**	**147·99**	**415·21**	**57·41**	**46·50**	**50·60**

Table 5-8: Intact fibrinogen levels following thrombolysis (mg/ml).

PATIENT	PRE	1	2	4	12	24	36	48
3	3·64	1·86	1·77	1·61	1·43	1·81	2·82	2·86
11	3·07	1·99	1·61	1·69	1·48	2·52	3·39	3·85
12	5·28	4·16	4·34	3·05	3·4	3·9	3·33	3·92
13	2·47	1·79	1·48	1·43	1·36	1·42	1·63	1·92
14	3·83	2·46	2·27	1·81	1·89	2·06	2·68	3·43
16	3·07	2·02	1·84	1·62	1·55	1·24	1·8	2·22
18	4·66	2·65	2·49	2·19	2·41	2·14	2·6	2·97
19	3·43	1·72	1·91	1·64	1·83	2·38	2·92	3·43
21	2·39	1·93	1·52	1·26	1·38	1·36	1·77	2·75
24	3·25	1·94	1·64	1·7	1·2	1·54	1·85	
26	3·81	4·52	4·96	3·98	4·2	4·03	4·69	4·56
Median	**3·43**	**1·99**	**1·84**	**1·69**	**1·55**	**2·06**	**2·68**	**3·20**
Mean	**3·54**	**2·46**	**2·35**	**2·00**	**2·01**	**2·22**	**2·68**	**3·19**
SEM	**0·26**	**0·29**	**0·36**	**0·25**	**0·29**	**0·29**	**0·28**	**0·26**
SD	**0·86**	**0·97**	**1·19**	**0·81**	**0·96**	**0·96**	**0·92**	**0·81**

Table 5-9: Plasminogen levels following thrombolysis with streptokinase (µg/mL).

PATIENT	PRE	1	2	4	12	24	36	48
6	143·00	4·07	3·59	4·60	14·38	30·46	54·15	70·73
9	103·00	6·68	5·42	4·70	18·54	34·35	46·10	49·82
10	90·20	6·72	6·68	6·78	17·87	34·43	44·09	49·61
11	135·00	6·34	4·95	4·75	13·06	33·18	47·33	58·28
12	131·00	5·78	5·42	7·75	18·00	39·38	55·55	73·45
13	156·00	7·44	7·04	6·56	24·38	40·72	55·98	72·75
16	200·00	3·70	3·62	3·51	4·49	18·01	33·43	45·56
18	143·00	10·24	9·77	7·94	19·14	43·89	60·33	76·51
19	137·00	8·07	7·01	7·59	27·23	44·27	63·01	71·76
21	200·00	4·42	3·96	3·58	9·24	23·41	36·98	49·61
22	200·00	5·09	4·23	4·30	14·52	25·89	33·77	38·62
25	200·00	5·62	4·85	4·96	15·44	30·20	42·25	46·07
Median	**143·00**	**6·06**	**5·19**	**4·86**	**16·65**	**33·77**	**46·72**	**54·05**
Mean	**153·18**	**6·18**	**5·55**	**5·58**	**16·36**	**33·18**	**47·75**	**58·56**
SEM	**11·20**	**0·53**	**0·52**	**0·47**	**1·76**	**2·35**	**2·92**	**3·92**
SD	**38·79**	**1·85**	**1·81**	**1·64**	**6·11**	**8·15**	**10·11**	**13·58**

Table 5-10: Results of carboxypeptidase activity following thrombolysis (U/L).

PATIENT	PRE	1	2	4	12	24	36	48
6	1·82	18·2	33·42	19·75	0	29·91	14·91	5·65
10	14·39	3·13	9·04	16·01	0	11·53	41·27	17·26
11	17·42	0	0	24·64	0·55	20·4	16·75	26·21
13	0	0	0	0	0	0	7·29	12·76
14	9·47	5·56	8·12	26·93	11·8	3·49	4·58	10·1
16	0·97	0	0	13·49	0	6·66	4·57	0
18	2·18	0·31	0·04	2·76	3·91	0	2·34	1·47
21	2·3	5·96	7·97	0	6·63	7·93	13·25	4·93
22	16·77	0·89	14·27	12·85	0	0	0	9·45
25	0	2·59	2·68	1·87	0·9	3·23	0	1·03
Median	**2·18**	**0·31**	**0·04**	**2·76**	**0·55**	**3·23**	**4·57**	**4·93**
Mean	**6·53**	**3·66**	**7·55**	**11·83**	**2·38**	**8·32**	**10·50**	**8·89**
SEM	**2·28**	**1·77**	**3·28**	**3·23**	**1·26**	**3·13**	**3·92**	**2·60**
SD	**7·22**	**5·59**	**10·37**	**10·21**	**3·99**	**9·90**	**12·38**	**8·22**

Table 5-11: Levels of TAFI antigen following thrombolysis (nM).

PATIENT	PRE	1	2	4	12	24	36	48
6	23·1	36·9	28·6	23·4	22·8	19·2	29	23·5
10	46·4	45·1	45·3	47·2	41·8	33·8	37·9	45
11	19·5	27·7	27·5	33·9	23·1	20·3	20·1	17·1
13	13	17·6	15·9	22·1	17	12·4	18·4	16·9
14	24·6	30·6	31·5	32·1	22·7	23·2	25·4	16·2
16	7·2	20·7	22·2	18·3	19·7	13·2	12·9	16·3
18	29·6	28	28·5	30	24·4	21	18·6	25
21	28	28	30·9	39·2	25·2	29·1	24·5	27·9
22	35·4	38·5	41·1	39·4	33·4	30·8	36·4	35·6
25	34·9	34·4	36	33·8	32	33	25	31·5
Median	**26·30**	**29·30**	**29·75**	**32·95**	**23·75**	**22·10**	**24·75**	**24·25**
Mean	**26·17**	**30·75**	**30·75**	**31·94**	**26·21**	**23·60**	**24·82**	**25·50**
SEM	**3·61**	**2·62**	**2·71**	**2·81**	**2·34**	**2·46**	**2·51**	**3·06**
SD	**11·43**	**8·27**	**8·57**	**8·87**	**7·40**	**7·78**	**7·94**	**9·68**

Table 5-12: Membrane P-selectin expression following thrombolysis (%).

PATIENT	NORM	PRE	1	2	4	12	24	36	48
16	2·75	6·45	6·00	5·30	3·70	10·25	7·15	6·95	7·50
17	1·25	15·90	13·85	10·50	9·55	14·70	12·25	23·85	8·00
18	1·00	9·00	6·85	3·95	2·20	11·80	8·20	4·75	6·50
19	1·25	5·25	4·40	4·65	6·40	13·10	4·80	7·85	8·15
21	0·10	2·15	3·20	3·45	1·60	6·90	4·20	11·60	6·60
22	0·15	11·10	4·75	3·95	3·45	3·70	6·45	6·80	7·90
23	2·25	6·50	5·70	1·95	2·90	4·45			
24	2·20	7·95	6·30	3·95	6·30	1·90	0·60	1·45	2·35
25	3·50	2·10	4·75	2·05	2·80	2·25	2·50	6·75	2·40
26		1·35	1·2	1·4	0·85	3·5	1·25	7·35	1·1
28		2·45	1·90	2·35	2·10	7·10			

Median	1·25	6·45	4·75	3·95	2·90	6·90	4·80	6·95	6·60
Mean	1·54	6·38	5·35	3·95	3·80	7·24	5·27	8·59	5·61
SEM	0·35	1·35	1·01	0·75	0·78	1·37	1·23	2·10	0·94
SD	1·11	4·48	3·34	2·49	2·59	4·56	3·70	6·31	2·83

Table 5-13: Membrane P-Selectin expression employing 2% paraformaldehyde as fixative.

PATIENT	NORM	PRE	1	2	4	12	24	36	48
23	1·05	1·65	6·10	1·45	1·95	3·90			
24	0·85	4·20	3·95	2·65	3·45	4·40	1·15	0·85	1·65
25	1·75	2·90	1·35	1·60	1·55	2·25	5·35	2·20	3·05
26	0·55	4·3	2·2	0·85	1·3	1·05	0·55	1·85	1·9
28	0·10	1·3	1·05	1·5	1·75	2·05			
	0·05								
	1·75								
	1·25								
	0·85								

Median	0·85	2·90	2·20	1·50	1·75	2·25	1·15	1·85	1·90
Mean	0·91	2·87	2·93	1·61	2·00	2·73	2·35	1·63	2·20
SEM	0·21	0·62	0·94	0·29	0·38	0·62	1·51	0·40	0·43
SD	0·62	1·39	2·10	0·65	0·85	1·39	2·62	0·70	0·75

Table 5-14: GP Ib fluorescence following thrombolysis (%).

PATIENT	PRE	1	2	4	12	24	36	48
16	15·90	15·05	15·61	15·50	16·58	14·69	15·84	15·49
17	15·55	14·70	13·53	15·12	13·47	15·67	14·24	14·45
18	12·46	12·08	11·78	12·40	12·60	12·05	13·58	13·19
19	11·04	9·85	10·68	10·86	10·51	11·46	11·96	11·65
21	10·54	9·75	9·56	9·38	12·55	11·39	13·64	14·34
22	12·69	13·13	12·83	12·61	15·04	13·86	15·17	14·08
23	10·99	10·71	10·12	10·68	11·00			
24	11·89	11·34	12·27	11·59	11·96	10·74	10·71	11·02
25	10·03	10·11	10·03	9·55	11·05	12·01	13·12	11·34
26	10·54	11·32	11·03	10·79	11·15	10·43	11·00	10·61
28	11·63	11·44	11·57	11·59				

Median	**11·63**	**11·34**	**11·57**	**11·59**	**12·25**	**12·01**	**13·58**	**13·19**
Mean	**12·11**	**11·77**	**11·73**	**11·82**	**12·59**	**12·47**	**13·25**	**12·90**
Max	**15·90**	**15·05**	**15·61**	**15·50**	**16·58**	**15·67**	**15·84**	**15·49**
SEM	**0·59**	**0·55**	**0·54**	**0·60**	**0·62**	**0·61**	**0·59**	**0·59**
SD	**1·96**	**1·83**	**1·78**	**2·00**	**1·96**	**1·83**	**1·76**	**1·78**

Table 5-15: Expression of GP IIb/IIIa following thrombolysis (%).

PATIENT	PRE	1	2	4	12	24	36	48
16	29·42	29·35	28·93	29·64	32·19	31·99	32·29	30·02
17	27·95	24·23	27·34	27·93	30·36	30·22	29·11	29·66
18	19·53	20·68	19·24	19·31	19·97	22·76	22·93	22·50
19	23·06	21·60	21·89	21·64	24·42	25·45	24·84	24·17
21	22·01	21·95	21·69	21·47	25·27	25·35	31·01	
22	26·86	27·05	26·55	26·36	30·30	32·12	32·19	30·76
23	19·63	20·59	18·86	19·13	19·52			
24	18·47	18·36	17·77	19·32	17·44	21·02	20·65	21·21
25	17·57	17·39	18·46	17·79	21·59	23·58	23·43	21·94
26	19·62	19·57	19·34	19·52	18·87	18·28	19·97	19·14
28	17·31	17·44	17·90	18·92				

Median	**19·63**	**20·68**	**19·34**	**19·52**	**23·01**	**25·35**	**24·84**	**23·33**
Mean	**21·95**	**21·65**	**21·63**	**21·91**	**23·99**	**25·64**	**26·27**	**24·92**
Max	**29·42**	**29·35**	**28·93**	**29·64**	**32·19**	**32·12**	**32·29**	**30·76**
SEM	**1·30**	**1·16**	**1·23**	**1·24**	**1·70**	**1·63**	**1·64**	**1·61**
SD	**4·32**	**3·85**	**4·09**	**4·11**	**5·38**	**4·89**	**4·93**	**4·55**

Table 5-16: Soluble P-selectin levels following thrombolysis (ng/ml).

PATIENT	PRE	1	2	4	12	24	36	48
1	48·60	61·40	77·40	101·40	80·60	69·40	58·20	43·80
2	26·20	37·40	43·80	47·00	106·20	95·00	83·80	75·80
3	53·40	59·80	71·00	67·80	67·80	66·20	48·60	43·80
6	37·40	42·20	45·40	47·00	47·00	48·60	39·00	29·40
9	27·80	34·20	32·60	34·20	34·20	34·20	34·20	21·40
10	39·02		39·00	35·80	31·00	39·00	39·00	
11	39·00	55·00	71·00	77·40	75·80	63·00	64·60	39·00
12			24·60					
13	23·00	31·00	37·40	37·40	35·80	47·00	37·40	35·80
14	79·00	63·00	39·00	61·40	51·80	47·00	35·80	40·60
16				21·40	26·20	32·60	26·20	31·00
17	31·00	23·00	32·60	31·00	21·40	23·00		
18	56·60	31·00	58·20	31·00	45·40	50·20	35·80	29·40
19	43·80	40·60	34·20	47·00	56·60	50·20	39·00	26·20
21	31·00	39·00	43·80	47·00	39·00	32·60	24·60	
22	51·80	32·60	32·60	31·00	27·80	32·60	26·20	27·80
23	128·60	125·40	107·80	109·40	146·20			
24	50·20	51·80	61·40	67·80	56·60	43·80	43·80	35·80
25	40·60	21·40	31·00	21·40	21·40			
26	64·60	56·60	42·20	59·80	56·60	48·60	37·40	37·40

Median	**42·20**	**39·80**	**39·00**	**47·00**	**45·40**	**47·00**	**36·60**	**35·80**
Mean	**48·42**	**47·38**	**48·68**	**51·38**	**54·07**	**48·41**	**42·10**	**36·94**
SEM	**5·80**	**5·84**	**4·79**	**5·70**	**7·22**	**4·23**	**3·86**	**3·49**
SD	**24·60**	**24·09**	**20·90**	**24·84**	**31·45**	**17·43**	**15·44**	**13·05**

Table 5-17: Soluble fibrin levels following elective PCI (U/L).

	CITRATE					APROTININ					HIRUDIN				
PATIENT	PRE	0-25	1	2	24	PRE	0-25	1	2	24	PRE	0-25	1	2	24
1	8·0	6·5	5·4	5·8		5·9	4·3	5·8	6·5						
2	3·7	6·2	5·7	5·0		10·7	5·8	5·1	1·2						
3	23·7	8·8	2·7	4·7	3·9	35·0	17·4	15·6	7·8	2·4					
4		3·4					4·3								
5	5·2	6·6	3·6	4·1	11·5	9·3	4·7	5·9	4·5	36·3					
6	29·4	25·2	44·0	31·5	59·2	54·3	32·8	24·7	22·2	25·2					
7	34·6	8·6	4·6	3·9	2·4	19·2	9·6	7·2	5·5	4·9					
8	3·0	2·5	1·3	0·2	1·3	1·2	2·5	1·0	0·1	1·0					
9	17·1	3·9	3·0	4·4		7·8	2·9	2·7	2·0						
10	0·9	0·4	0·1	0·1	1·4	0·5	0·5	0·7	0·3	3·9					
11			5·3	5·8	18·4			20·7	7·2	29·8					
12	5·4	5·7	3·8	2·6	65·8	17·6	4·4	3·1	2·1	32·0					
13	8·8	7·5	4·2	1·8	5·6	4·8	3·5	2·2	1·4	3·8					
14	61·4	17·0	11·1	12·4	43·2	128·3	32·7	26·7	8·1	19·3					
15	9·3	3·7	7·3	7·0	37·8	9·9	5·7	9·0	7·8	55·9					
16	20·2	2·5	0·4	1·1	40·7	18·1	2·9	2·7	4·5	95·2	6·4	0·6	1·4	0·4	63·4
17	11·3	18·6	20·2	13·8		10·3	4·8	4·3	1·0		4·3	11·1	14·6	7·5	
18	55·9	3·9		1·0	3·6	22·8	0·1		0·0	4·2	27·2	1·4		1·5	9·9
19	15·5		3·4	10·6	8·9	59·0		6·5	1·6	8·1	6·1		1·0	0·9	2·0
20		27·3	20·2	17·9	79·8		36·4	33·7	21·6	184·8		19·5	19·5	12·6	42·8
22	30·9	2·5	3·3	2·9	1·8	45·7	4·5	4·8	3·9	4·0					

Median	13·40	6·22	4·16	4·53	10·18	14·14	4·53	5·76	4·17	13·71	6·28	6·25	8·01	1·48	26·33
Mean	19·14	8·47	7·88	6·83	24·08	25·57	9·47	9·59	5·45	31·92	11·03	8·16	9·12	4·57	29·51
SEM	3·43	1·48	2·17	1·51	5·23	6·53	2·16	1·87	1·19	10·40	2·46	2·06	2·15	1·25	6·62
SD	17·59	7·79	10·41	7·50	26·45	31·14	11·51	9·78	6·26	47·91	10·84	8·95	9·37	5·32	28·65

Table 5-18: X-oligomer levels following elective PCI (ng/ml).

	CITRATE					APROTININ					HIRUDIN				
PATIENT	PRE	0-25	1	2	24	PRE	0-25	1	2	24	PRE	0-25	1	2	24
1	255-5	216-9	234-0	276-2		197-0	217-9	288-7	278-2						
2	378-5	276-7	281-0	317-6		284-9	225-7	297-7	287-1						
3	288-4	324-2	328-0	403-6	340-0	285-4	328-9	372-1	388-9	362-6					
4		503-7					430-1								
5	132-4	97-5	119-3	52-6	134-3	135-3	118-4	121-2	136-2	140-9					
6	660-7	525-9	621-1	633-4	643-8	711-1	561-7	694-4	775-0	763-3					
7	419-7	400-2	437-0	507-4	495-2	466-4	423-1	489-2	502-5	430-1					
8	137-4	116-7	128-9	114-5	128-4	119-4	96-7	115-8	123-5	137-4					
9	783-1	691-2	683-0	684-4		821-3	394-6	517-2	365-4						
10	260.3	142-0	162-6	154-9	301-2	230-5	153-2	175-9	168-3	295-9					
11			478-4	618-7	422-0			335-8	388-9	359-9					
12	822.8	764-3	699-5	654-6	970-6	730-2	497-7	607-0	658-6	888-1					
13	187.1	238-5	260-7	280-6	412-4	181-9	244-2	284-5	322-8	466-1					
14	587.9	445-6	643-4	592-8	817-0	736-7	701-6	717-1	589-3	697-8					
15	270.2	148-8	110-4	205-1	290-0	221-8	168-7	181-0	177-2	238-0					
16	329.3	268-5	253-7	258-1	302-8	316-3	208-9	229-9	253-7	323-1	87-4	70-1	59-5	61-5	66-3
17	640.5	345-8	302-8	362-0		438-0	240-9	248-9	274-6		500-4	363-1	365-4	453-0	644-8
18	170.7	144-5		138-6	142-8	49-7	23-1		61-0	54-1	165-9	147-8	161-0	157-3	136-9
19	525.8		401-9	526-4	395-4	597-7		330-4	332-1	555-1	105-4	78-6	91-1	86-5	133-8
20		169-7	185-3	186-9	231-5		196-5	216-4	241-2	258-1	91-1	46-2	49-7	56-1	59-0
22	316.2	193-5	224-9	270-8	267-4	297-7	211-7	218-5	247-5	250-0					

Median	**322-8**	**268-5**	**281-0**	**299-1**	**321-4**	**291-6**	**225-7**	**288-7**	**282-7**	**341-5**	**105-4**	**78-6**	**91-1**	**86-5**	**133-8**
Mean	**398-1**	**316-5**	**345-0**	**362-0**	**393-4**	**379-0**	**286-5**	**339-0**	**328-6**	**388-8**	**190-1**	**141-2**	**145-3**	**162-9**	**208-2**
SEM	**51-9**	**44-5**	**45-1**	**45-1**	**59-8**	**57-1**	**39-8**	**42-0**	**41-1**	**58-8**	**78-9**	**58-0**	**58-4**	**74-7**	**110-0**
SD	**220-1**	**193-9**	**196-4**	**201-6**	**239-4**	**242-3**	**173-6**	**182-9**	**183-7**	**235-3**	**176-3**	**129-7**	**130-5**	**167-1**	**246-8**

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Hurrah!