HAEMODILUTION AND COAGULATION

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PhD THESIS TITLE:

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- Assistance with statistical analysis by Professor James

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I am now presenting the thesis for re-examination for the degree of PhD.

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LIST OF ABREVIATIONS

α-angle	alpha angle
ACT	activated clotting time
ACT-C	activated coagulation time with celite
ACT-K	activated coagulation time with kaolin
ADP	adenosine diphosphate
ANOVA	analysis of variance
aPTT	activated partial thromboplastin time
ATIII	antithrombin III (Heparin Cofactor I)
BT	bleeding time
Ca^2	ionised calcium
CLIA	American Clinical Laboratory Improvement Act of 1988
COP	colloid oncotic pressure
CSF	cerebrospinal fluid
DIC	disseminated intravascular coagulopathy
DVT	deep vein thrombosis
ECF	extra-cellular fluid
FBC	full blood count
FFP	fresh frozen plasma
FIB	fibrinogen
GP Ib/IX/V	glycoprotein Ib/IX/V
GP IIb/IIIa	glycoprotein IIb/IIIa
Hb	haemoglobin
HCT	haematocrit
HES	hydroxy-ethyl starch
HMW	high molecular weight
INR	international normalized ratio
ISI	international sensitivity index
K time	coagulation time
LMW	low molecular weight
LSD	Fisher least significant difference
MA	maximum amplitude
Max	maximum
Min	minimum
mmHg	millimetres of mercury
MMW	medium molecular weight

MODS	multiple organ dysfunction syndrome
NS	Normal Saline
PAI-1	plasminogen activator inhibitor-1
PE	pulmonary embolism
pH	hydrogen ion concentration – acid base status
PLTS	platelet count
PPP	platelet poor plasma
PRP	platelet rich plasma
PT	prothrombin time
PT-FIB	PT-Fibrinogen recombinant
PTR	prothrombin time ratio
QXDP	Quantitative D-Dimer test
r time	reaction time
RBC	red blood cell count
RCT / RCB	routine coagulation tests / routine coagulation battery
RL	Ringer's Lactate
SCT	Sonoclot analysis
SD	standard deviation
SEM	standard error of mean
SERPINS	serine protease inhibitors
TAT	thrombin antithrombin complex
TEG	thrombelastograph / thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TF / VIIa	tissue factor/factor VIIa
TT	thrombin time
vWF	von Willebrand factor
WPBTS	Western Province Blood Transfusion Service
XDP	D-Dimers

ABSTRACT

HAEMODILUTION AND COAGULATION

Studies on the effect of haemodilution on coagulation have previously concentrated primarily on differences between crystalloids and colloids, often using crystalloids as a control, with conflicting results. However, it was previously demonstrated that the onset of coagulation is actually *enhanced* after rapid haemodilution with crystalloid. The reason for this enhanced coagulation and the mechanism by which it occurs have remained unclear.

This thesis establishes that the effect is reproducible with haemodilution in healthy volunteers with no confounding variables. It investigates the effect of various diluents on the enhancement of coagulation and the range of dilution over which the effect occurs. The focus then moves on to the mechanisms of enhanced coagulation and attempts to define and explain these. The role of platelet activation by the standard cyclo-oxigenase pathway is investigated through the administration of aspirin. It is demonstrated that the enhancement of coagulation occurs on the basis of haemodilution lowering the levels of anti-coagulants and thereby the threshold for positive feedback of thrombin, while not diminishing the spontaneous activation of thrombin, thus resulting in an imbalance. This imbalance leads to increased thrombin formation, which results in activation of platelet aggregation, which further enhances activation of thrombin. Two clinical studies set out to identify whether the effect could be demonstrated in a clinical setting.

Coagulation studies were primarily based on the thrombelastogram, which provides a dynamic measure of whole blood coagulation, rather than standard tests measuring endpoints in plasma with excesses of coagulation factors added, as the latter may obscure the finer coagulation interactions determining the state of onset of coagulation.

This thesis demonstrates the effect of enhanced coagulation following haemodilution and the cause of this enhancement of coagulation is now established. While it is still difficult to prove a clinical difference in outcome between diluted and undiluted groups, the effect may pose clinical questions regarding patients receiving large volumes of intravenous crystalloid. This is an area that will need further research in time to come. The length of time this effect persists should also be investigated.

PREFACE

My interest in investigating this subject using the thrombelastograph (TEG) began when I was a Registrar. I never realised that it would one day include writing a PhD thesis on the subject.

However, following the original study and a literature review, it became evident that the concept of haemodilution affecting coagulation was not as simple as it seemed. From this my research developed, demonstrating that moderate haemodilution actually increases the onset of coagulability of blood. Even though this was later borne out by further work and other authors, the cause and extent of this effect have never been described. It was with this in mind, that I set out to do the work incorporated in this thesis. This was done under the guidance of Professor James, who was always there to provide the support and assistance, but allowed me to develop the process in my own mind.

The TEG was used as a measuring tool, because of its established role in assessing whole blood coagulation and because it is a dynamic measure of interactions in the balance of coagulation and anti-coagulants, rather than an endpoint measure. The Department of Anaesthesia at Groote Schuur Hospital initially had one TEG, which increased to 3 machines that were made available for use in this project.

The Medical Research Council and the University funded the disposables and laboratory investigations. Fresenius AG supplied the pentastarch for the original *in vivo* dilution study and funding towards that and the clinical (Chapter 10) trial. Althea Fordyce of Sanofi Omnimed donated the ATIII.

Manta Medical Systems loaned a TEG® machine, and Dr Eli Cohen, Chairman of HAEMOSCOPE Inc. donated the three TEG machines to the Department, as well as giving background information on the TEG and allowing me to reproduce the pictures of the TEG.

The University of Cape Town Research and Ethics Committee approved all of the experiments performed for this thesis. All the blood samples were taken from consenting volunteers in the Department of Anaesthesia at Groote Schuur Hospital and Western Cape Blood Transfusion Services; or in the clinical trials, from consenting patients undergoing surgery. They were only enrolled after consent was taken. Disposal of contaminated waste was in accordance with accepted hospital norms.

Many of the volunteers were colleagues in the Department, and I would like to thank them for donating their blood so unflinchingly; in addition Ms Gail Neil and Ms Pat Ellis of our research laboratory for their help in running the TEG. Sister Rencia Gillespie, our clinical research assistant also helped out with the TEG. Thanks go to Dr Arthur Bird and Sister Heather Money of the Western Province Blood Transfusion Services for their assistance and to Professor James for his help in the statistical analysis of the raw data.

CHAPTER ONE
INTRODUCTION

For many years the assumption has been made that most intravenous colloid solutions cause a decreased coagulation of blood, while with moderate crystalloid dilution (20-40%), coagulation remains inert. This was based on the fact that haemodilution decreases the concentration of clotting factors in the blood and thus theoretically results in a decreased ability to form a clot. Indeed, this assumption was borne out in various studies comparing the effect of colloid haemodilution with the crystalloid diluted "control group"[95,100,130,132], or not making allowance for any crystalloid the patient was given in addition to the colloid[101,126]. Crystalloids such as normal saline (NS) and Ringer's lactate (RL) are distributed through the interstitial space. Although effective, crystalloids tend to require larger volumes for infusion and oedema remains a problem[57] as these solutions pass freely across the capillary membrane[113] and decrease oncotic pressure[92]. Less than 25 percent of infused saline is retained in intravascular fluid compartments after 1 hour[94]. As a result, the volume of fluids required with crystalloids is two to four times that of colloids[3].

On the colloid side, plasma, albumin and synthetic colloids may all be used for volume expansion. There are three classes of synthetic colloid; dextrans, gelatins and hydroxyethyl starches (HES)[182]. The colloid oncotic pressure (COP) of the plasma proteins, which is about 25 mmHg, is the main factor for the retention of intravascular volume and the prevention of interstitial oedema. Solutions with high COP increase the intravascular volume due to resorption of interstitial fluid[1]. Dextran, gelatin, and HES can all induce a specific decrease of von Willebrand factor and factor VIII:c. Blood coagulation is most

impaired by dextran and high molecular weight HES. The effects of HES on blood coagulation have been shown to depend on its molecular weight and rate of elimination[24].

The induction of some degree of impairment of coagulation is certainly the case once haemodilution in excess of 60% has occurred. It was noted, however, that patients receiving crystalloids demonstrated an increase in coagulability as measured by the thrombelastograph (TEG), but this was not attributed directly to the crystalloid infusions[213,219], even though it might have implied that moderate haemodilution could cause blood to clot more readily. In fact, in recent practice, the management of stroke is based on hypervolaemic haemodilution to improve perfusion of relatively ischaemic areas of brain[59].

On the other hand, while colloids provide highly effective volume expansion, there is a question against all of the synthetic colloid solutions regarding their effects on haemostasis. Common practice is to limit their use to 2 units (1000 ml) in the average 70 kg adult[100]. Mardel *et al.* have suggested that gelatin-based products may become incorporated into developing clots and reduce the function of fibronectin in forming covalent cross-linkages and normal covalent associations with fibrin, thus interfering with polymerisation of fibrin monomers[111]. The dextrans are thought to pose a greater risk than either modified gelatins or hydroxyethyl starch[122].

HES solutions have varying physicochemical properties with regard to molecular weight and degree of hydroxylation, which influences elimination kinetics, as well as coagulation[91]. Various clinical studies have been conducted on the effect of the hydroxyethyl starches on coagulation, with conflicting results. There appears to be an increased risk of bleeding,

including small, but significant, prolongation in partial thromboplastin, prothrombin and bleeding times, decreases in fibrinogen, antithrombin III and factor VIII concentrations and an increase in clot lysis[201,202], but these tests do not reflect any potential effect on the dynamic enhancement of the onset of coagulation. It must also be noted that many of these studies have used a crystalloid dilution as control, rather than taking the undiluted base sample as the control. For example, Kuitunen *et al.* demonstrated both types of hydroxyethyl starch (HES) solutions (Hetastarch: HMW - 450,000 daltons; and Pentastarch: MMW - 264,000 daltons) to have adverse effects on laboratory indices of haemostasis compared with Ringer's acetate when used as priming solutions for cardiopulmonary bypass[95], while replacement of blood loss using HES was reported to have no adverse effects on coagulation when compared with the use of 5% albumin in gynaecological and neuro-surgery[20]. Pentastarches have been found, by Strauss *et al*[200] to exert significantly lesser effects on standard tests of blood coagulation than HES despite a greater effect on haemodilution. Penner *et a*[147] looked at specific dilutions (20%) of a 10% hydroxyethyl starch and its effect on haemostasis and found no apparent clinical effects. In a recent study, the influence of moderate and profound *in vitro* hemodilution with different HES solutions (70.000/0.5, 130.000/0.4, 200.000/0.5) on blood coagulation was investigated. HES 130.000 had some advantages over other hydroxyethyl starches solutions (70.000, 200.000)[91]. There appears to be no consensus in the literature as to the effect of various starch solutions on haemostasis. In addition, many of these studies were limited as they were performed in conjunction with surgery where multiple factors such as surgical trauma, heparinisation, protamine

reversal, stress response, endothelial exposure, etc. could all play a role in altered haemostasis.

From reviewing the available literature and previous work[178], it has become apparent that a crystalloid, for example 'normal', 0.9% saline, is not inert as an intravenous fluid, and that it has an effect on enhancing coagulation. In 1951, Tocantins *et al.* suggested that the effect was present with whole blood dilutions of up to 40%[206], while, in 1959, Monkhouse[123] reported that moderate haemodilution with crystalloids could provoke an increase in whole blood coagulation. He speculated on an imbalance between pro- and anti-coagulants. However, quite why haemodilution should produce such a procoagulant state was not explained. This effect was later borne out by Janvrin[71] in a clinical trial that investigated the incidence of postoperative deep vein thrombosis related to intra-operative fluid administration and correlating with laboratory findings confirming dilution induced enhanced coagulability using the Biobridge® measuring impedance clotting time.

My own findings demonstrated that the onset of coagulation is enhanced after rapid haemodilution with both crystalloid (normal saline) and colloid (gelatin) haemodilution, with the final absolute clot strength (increased maximum amplitude (MA) on the TEG) only being increased with crystalloid haemodilution and remaining unchanged with colloid haemodilution[178].

This effect has been identified[173]; however, neither the causes of this enhancement of coagulation following haemodilution, nor the mechanisms have been established, with only a single study measuring the effect and clinical response thereof[71], going as far as stating that "drips should be added to the list of risk factors for patients awaiting surgery with fractured necks of

femur" and referring to the "myth that dehydration and haemo-concentration are risk factors in DVT."[72]

The hypothesis for this thesis is that: 'Rapid haemodilution enhances the onset of coagulation through an imbalance between coagulation factors and their inhibitors'. This thesis sets out to primarily determine whether this effect is reproducible with in vivo haemodilution in a controlled state. Once this has been established, the effect of the types of fluid and the range of dilution over which this occurs needs to be determined. Following on from this, the final step will be to identify and explain the mechanism and the potential clinical effect of the enhanced coagulability of blood induced by haemodilution. The University of Cape Town Human Research Ethics committee approved all the studies, apart from the study in blood donors (Chapter 9) which was approved by the Research Ethics Committee of the Western Province Blood Transfusion Service (WPBTS). Informed consent was obtained from each volunteer prior to them entering into a study.

The Thrombelastograph was the major tool for measuring coagulation in this thesis and these tests, as well as the obtaining of all blood samples, were run or supervised by myself. The standard haematological tests as well as the more specialised tests like the AT III, TAT and platelet aggregation were performed by the Department of Haematology.

The work is represented in the following chapters:

2.) Mechanisms of coagulation.

3.) Methods of coagulation testing

4.) A study in normal volunteers, utilising a 20% *in vivo* haemodilution achieved by the infusion of 1000 ml of either crystalloid or colloid, and the TEG for *ex vivo* pre- and post-dilution coagulation studies.

5.) An *in vitro* study using warmed Plasmalyte B (buffered / warmed to 37°C / normal constituents of plasma) or a protein-poor body fluid (cerebrospinal fluid (CSF) – taken at the time of dural puncture in patients undergoing spinal anaesthesia) repeating the methodology.

6.) An *in vitro* study looking at the effect of different percentage dilutions on coagulation.

7.) An *in vitro* study, using the methodology as before, in normal volunteers who take aspirin for 3 days before being tested again, to determine whether the effect is present when an antiplatelet agent is used.

8.) An *in vitro* study repeating the methodology as before, using a plasma-like fluid that has normal levels of antithrombin III (Plasmalyte B with additional ATIII) to determine whether the effect is attenuated or inhibited in the face of haemodilution with normal levels of ATIII.

9.) An *in vivo* study in volunteers donating a unit of blood, to measure their coagulation response as they auto-infuse their intravascular volume, thus causing haemodilution.

10.) A clinical study using patients undergoing peripheral vascular surgery under regional anaesthesia, to determine whether *in vivo* dilution causes a reproducible and/or clinical effect on coagulation.

11.) Conclusion

CHAPTER TWO
PLASMA COAGULATION FACTORS

OVERVIEW

The integrity of the circulation is maintained through the provision of a rapid, potent, but tightly localised coagulation response to vascular damage. There is, however, one extraordinary problem in the regulation of haemostasis - blood flows. Procoagulant products, whether clotting factors or platelets, must therefore be spatially localised to the site of damage, and/or kinetically controlled so that they are inactive when distant from the damaged vessel. The blood coagulation cascade is usually initiated when sub-endothelial tissue factor is exposed/expressed to the blood flow following either the damage or activation of the endothelium[16,222,226]. As a result, the haemostatic mechanism is invoked through a complex series of regulated events, involving the interactions of the components of blood and tissue proteins, resulting in a spectrum from haemorrhage, through controlled haemostasis, to thrombosis.

This response has been characterised as a *cascade* or *waterfall* of enzymatic reactions, which result in α-thrombin converting soluble plasma fibrinogen to the insoluble fibrin polymer[103,104]. This led to observation that the coagulation process is a series of reactions in which the sequentially derived products have the capacity to amplify small stimuli to result in the rapid generation of large amounts of α-thrombin[160]. Tissue factor, factor Xa, and thrombin are pivotal; together with physiological controls (positive and negative feedback loops, and natural anticoagulants) that first enhance thrombin generation but

then preserve vessel patency by limiting haemostatic plug formation to areas of injury. Abnormalities in these mechanisms can increase thrombosis risk[46].

THE PATHWAYS OF BLOOD COAGULATION

EXTRINSIC PATHWAY

The extrinsic pathway is the route of thrombin formation, through which the physiological haemostatic response occurs primarily. It occurs through contact with a non-blood protein source. The physiological initiator of coagulation is *tissue factor,* which comes into contact with the blood at the site of vascular injury and combines with plasma serine protease, factor VII[32,124]. Being a trans-membrane protein, it is *ipso facto* localised[10]. The tissue factor/factor VIIa (TF/VIIa) complex activates factor X to factor Xa. The limited amounts of the serine protease factor Xa produced generate minute concentrations of thrombin, which partially activates platelets and cleaves the pro-cofactors factor V and factor VIII generating the active cofactors--factor Va and factor VIIIa, respectively[14,155]. It can, however, also activate factor IX to IXa[98,145]. Thus the extrinsic pathway catalyst could be responsible for factor X activation through both pathways[80,228]. Furthermore, the efficiency of factor X activation by the factor VIIIa/IXa complex is significantly greater than the efficiency of the TF/VIIa complex. It is relevant that the factor IX-dependent route, in contrast to the direct activation of factor X by TF/VIIa, involves two enzyme-catalysed reactions in sequence and therefore provides extra amplification over the direct activation of factor X, but will take longer[161]. The reactions occur on the platelet surface, but only on those platelets that are activated and involved at the site of vascular injury, thus the response is localised to the site of vascular damage. Multiple control mechanisms guard

against procoagulant materials entering the general circulation and causing disseminated intravascular coagulation[73]. Obviously, since the latter condition exists, these safeguards can on occasion be overrun.

INTRINSIC PATHWAY

The intrinsic or contact pathway of coagulation consists of a group of plasma proteins that are activated by interaction with exogenous negatively charged surfaces such as glass or kaolin. This pathway starts with the formation of factor XIIa[151], which can cleave prekallikrein to provide kallikrein[107], which in turn reciprocally activates factor XII[162].

Factor XIIa, in the presence of high-molecular-weight kininogen, converts factor XI to factor XIa, which in turn converts factor IX to factor IXa[43]. Factor IXa binds with its cofactor protein factor VIIIa, in the presence of calcium and the appropriate membrane surface, and activates factor X to factor Xa[67].

Factor VIIIa forms the *intrinsic factor Xase* complex with the serine protease, factor IXa, on a membrane surface provided by platelets, microparticles, and endothelial and other cells[204] and activates factor X at a 50-100-fold higher rate than the factor VIIa-tissue factor complex[98]

Factor VIII circulates in association with von Willebrand factor in blood and is released from von Willebrand factor during activation of the factor VIII molecule by thrombin. Factor Xa binds to its cofactor protein factor Va, in the presence of calcium and the appropriate membrane surface, and activates factor II (prothrombin) to IIa (α-thrombin)[69].

The two soluble plasma proteins, factor V and factor VIII, participate in the formation of vitamin K-dependent protein complexes. Once formed, they are unstable in solution, being inactivated by protein C.

COMMON PATHWAY

The common pathway involves the conversion of prothrombin to α-thrombin and is accomplished via a catalytic subunit, factor Xa in the presence of calcium ions, and a regulatory subunit, factor Va[81,135]. Human factor X circulates in plasma, and is converted to its active form, factor Xa, by either the extrinsic pathway (factor VIIa in the presence of tissue factor) or by the intrinsic pathway (factor IXa and its cofactor, factor VIIIa).

Factor V circulates in the blood, both in platelet (~20%), and as the soluble plasma protein (~80%). Since a deficiency of platelet factor V is associated with decreased factor Xa binding and a bleeding diathesis[208], it is clear that the platelet pool of this protein plays a major physiological role. The factor Xa/Va complex activates prothrombin on an anionic phospholipid membrane: the platelet surface.

Thrombin generation is the central biochemical reaction in both normal haemostasis and thrombosis. The thrombin produced further amplifies its own generation through other important coagulation events like activating factor XI[45], completing platelet aggregation[220], plus factor V and VIII activation[14]. It also causes the transformation of soluble fibrinogen into insoluble fibrin[26,125], and the activation of factor XIII to factor XIIIa[133], which stabilises the blood clot[12,115]. Thrombin also participates in damping of its own production by complexing with thrombomodulin,[37] which is released by endothelial cells.

These also participate in the activation of proteins C and S, which result in the inactivation of factors Va and VIIIa[38]. Residual serine proteases are inhibited by antithrombin III[11,28,35,65,142,159].

At initiation of coagulation thrombin is generated at a relatively low rate, with partial activation of platelets and proteolysis of factor V, factor VIII, factor VIIa, factor IXa, factor Xa, and factor XIa are also generated[14,97,155].

Subsequently, in the presence of all procoagulant proteins at their mean plasma concentrations, the propagation phase of thrombin generation occurs, with the clot occurring at the inception of this phase. The characteristic features of the propagation phase are a prothrombin conversion at a high rate, completion of platelet activation, and solid clot formation[12,14,18,97,155].

PLATELETS

Platelets are essential for the maintenance of haemostasis and, on the other hand, play a pivotal role in the formation of a thrombus with platelet membrane glycoproteins mediating binding to subendothelial tissue and aggregation into haemostatic plugs[49]. Reduced platelet activity will result in a bleeding tendency, while stimulation of platelets can lead to thrombosis[25]. Platelets are an essential component of the blood coagulation process *in vivo*. Aggregated, activated platelets provide procoagulant phospholipid-equivalent surfaces upon which the complex-dependent reactions of the blood coagulation cascade are localised[168,221].

Platelets are small (2-μm-diameter), non-nucleated blood cells, which are activated rapidly after blood vessel injury or blood exposure to artificial

surfaces. They are a crucial component of the primary clotting response, providing haemostasis by 4 interconnected mechanisms: (1) adhering to sites of vascular injury or artificial surfaces, (2) releasing compounds from their granules, (3) aggregating together to form a haemostatic platelet plug, and (4) providing a procoagulant surface for activated coagulation protein complexes on their phospholipid membranes[93].

The adhesion to the subendothelium is the initial step in platelet activation[110], which is extremely rapid and is facilitated by vWF binding to platelet surface glycoprotein Ib/IX/V[117,193,210]. Platelets can also adhere to vascular wall associated fibrin or fibrinogen through interaction with platelet surface glycoprotein IIb/IIIa[171,188]. After adhering to the subendothelium, platelets undergo activation leading to a shape change with development of pseudopods. Intracellular signalling processes lead to increased cytoplasmic calcium and initiation of a secretory release reaction, whereby products are released into the surrounding milieu[44]

Platelets have numerous intrinsic glycoproteins embedded in the outer surface of their plasma membrane that are receptors for agonists, such as adrenaline, thrombin, collagen, and platelet-activating factor, which can initiate platelet aggregation[146].

Activated platelets also play a vital procoagulant role, linking platelet function and coagulation activation. Platelet membrane phospholipids undergo a rearrangement during activation, providing a binding site for phospholipid-dependent coagulation complexes that activate both factor X and prothrombin[231], accelerating coagulation through the assembly of multi-enzyme complexes with great catalytic efficiency. The platelet surface

membrane also interacts with factors Xa and Va to form the prothrombinase complex, as well as promoting the assembly of the factor IXa-VIIIa complex required for factor Xa activation[230]. These interactions should not be overlooked, since they dramatically enhance enzymatic formation of both thrombin and factor Xa[229] and in the absence of membrane the formation of the complex is either abolished or impaired[109,209]. Calcium is also an essential component for efficient complex formation[134].

Consequently, the proposed basic mechanism of platelet reaction is: 1.) Tissues and blood vessels are injured. Vascular subendothelium and collagen fibrils are extended; 2.) platelets adhere to the subendothelium and collagen through vWF; 3.) adenosine diphosphate (ADP), adrenaline and thromboxane A_2 are released from adhering platelets; 4.) platelets are aggregated by released ADP, adrenaline and thromboxane A_2, and collagen through fibrinogen; 5.) platelets are further irreversibly aggregated by small amounts of thrombin generated from extrinsic blood coagulation; 6.) fibrin thrombi resulting from blood coagulation are formed over the platelet thrombi[203].

FIBRIN FORMATION

Activated α-thrombin rapidly converts soluble plasma fibrinogen to an insoluble fibrin polymer by limited proteolysis, thus consolidating the haemostatic platelet plug. Fibrinogen is synthesised in the liver and is present in plasma and platelet α-granules. It is an acute-phase reactant: with maximal stimulation, fibrinogen synthesis can be increased 20-fold. Thrombin also activates the plasma transglutaminase, factor XIII[56], which covalently cross-links the fibrin polymers both longitudinally and

transversely[99]. Calcium enhances lateral fibre growth. This is then relatively resistant to proteolytic attack.

INITIATION OF BLOOD COAGULATION

Tissue factor

Coagulation *in vivo* is initiated by tissue factor, the intrinsic membrane protein, which is present on the surface of many cell types that are not normally in contact with the circulation. However, it is exposed to blood when vascular damage occurs. Thus, vascular damage may itself lead to increasing levels of tissue factor at the site of injury. In association with the serine protease factor VIIa, it initiates the blood coagulation cascade. The interaction of tissue factor and factor VIIa requires no calcium[10,32,124].

Factor VII

Factor VII has the shortest half-life of all the clotting factors (~ 4 to 6 h), making it difficult to sustain normal plasma levels with replacement therapy. It is converted to VIIa by several serine proteases, including thrombin, factor IXa, factor Xa, factor VIIa and factor XIIa. It is poorly neutralised in blood, and small amounts of the activated protein are thought to circulate. Factor VIIa activates factor X, at extremely low rates, in the absence of tissue factor[158,194].

Substrates of the TF/VIIa complex

Tissue factor forms a complex with factor VII - TF/VIIa[157]. Anionic membranes significantly enhance the proteolysis of factor X and factor IX by this complex[79]. The relevance to haemostasis of the tissue factor-dependent activation of factor IX was questioned because the direct activation of factor X by TF/VIIa is faster than the activation of the intrinsic pathway through factor

IX[80,228]. However, the direct factor IX activation, while slower, forms exponentially increasing amounts of thrombin.

Tissue factor and platelets

Tissue factor is not released into the circulation in a soluble form during normal haemostasis. If there is a major injury, there is a potential for it to be swept into the circulation, which may lead to disseminated intravascular coagulation (DIC)[68]. However, at the site of damage, platelets interact with components of the vessel wall at the same time that plasma factor VII comes into contact with tissue factor. The platelet plug thus prevents the unchecked release of the TF/VIIa complex.

ALTERNATIVE PATHWAYS

Factor XI activation

The alternative route of activation of factor XI via thrombin[131] is a positive feedback system, which explains both the requirement for factor XI and the lack of a requirement for factor XII. Thus, a reasonable scenario is that tissue factor initiates coagulation, leading to fast thrombin production. Thrombin then feeds back, producing small amounts of factor XIa. The XIa in itself may then have a positive feedback on itself, thus leading to an increase in the rate of factor XI activation.

It has been shown that the absence of, or deficiency in, factor XI has almost no effect on thrombin generation, platelet activation, and clot formation at a relatively high tissue factor concentration[14,18]. This is due to slow factor XI activation by thrombin and, as a consequence, a relatively late accumulation of factor XIa. However, when the initiation phase of thrombin generation is

prolonged either due to low tissue factor concentration or caused by inhibitory effects of elevated concentrations of some proteins involved in blood coagulation, factor XI plays an important role[18].

Factor IX activation by TF/VIIa

Although factor XIa is an activator of factor IX in one of the simplest reactions of coagulation, requiring only calcium as a cofactor, the factor VIIa-tissue factor complex activates factor IX as well as activating factor X[89,98,145]. This links the extrinsic pathway directly into the intrinsic one.

Factor VIII activation by Thrombin and Factor Xa

Factor VIII circulates as a complex with von Willebrand factor, and normally exists in an almost inactive state, requiring activation to factor VIIIa. Activation is due to the feedback action of either factor Xa or thrombin. Upon factor VIII activation by thrombin or factor Xa, it is released from von Willebrand factor, which enables binding of the activated cofactor.

POSITIVE FEEDBACKS

Positive feedback in coagulation plays a major role in the architecture of the system: there are at least six feedback loops. In a positive feedback an enzyme formed later in the pathway acts to provide something that accelerates its own formation. The known major feedback loops of clotting, all originating via either factor Xa or thrombin, result in the formation of both enzymes and activated cofactors. Thus, the production of thrombin is rapid (≤ 1 minute) and self-reinforcing because of its feedback activation of numerous coagulation proteins (factor V, VIII, XI, and VII)[28,45,108,154] that contribute to thrombin production. However, while the existence and effects of

the feedback loops are clear, their benefit has not been documented. Nevertheless, it may be speculated that this provides the threshold preventing uncontrolled exponential clot formation. As it is, the requirement for feedback activation of the TF/VIIa complex produces a significant delay, similar points applying to factors VIII and V.

Platelet activation

Platelets have numerous receptors for ligands ranging from fibrinogen, collagen, thrombin, and thrombospondin to von Willebrand factor (vWF) and fibronectin[146]. Of note is that most of the platelet activators exert their action by binding to specific receptors on the platelet surface membrane. A possible exception to this is activation by thrombin, one of the most potent platelet activators known[229]. Minor quantities of thrombin formed early in haemostasis, prior to coagulation[192], bind to platelet receptors, to elicit release and aggregation. Platelet aggregation itself also involves positive feedbacks through the release from platelets of platelet agonists such as adenosine diphosphate (ADP) and thromboxane A_2.

Factor VII activation

The major activator of factor VII is factor Xa, which generates factor VIIa. Although anionic phospholipid alone does support this reaction, it is much faster in the presence of tissue factor. Tissue factor therefore has two distinct cofactor roles in coagulation: enhancing the activity of factor VIIa on factors IX and X, and accelerating the feedback activation of factor VII by factor Xa. Factor VII can also be feedback-activated, but at lower rates, by the TF/VIIa complex and by thrombin and factor IXa[13].

Cofactor activation

Both major plasma cofactors, factors VIII and V, are activated in feedback loops. The principal activator of both is thrombin[136], but both are also activated by factor Xa in the presence of anionic phospholipid[5,51]. Factor VIII activation by factor Xa constitutes a feedback, but activation of factor V does not. It is possible that factor Xa action plays a significant role in the early phases of coagulation before significant thrombin generation.

POSITIVE FEEDBACKS IN THE PRESENCE OF INHIBITORS

The enzymes catalysing positive feedbacks, thrombin and factor Xa, are also the major targets of plasma inhibitors, chiefly antithrombin III (ATIII). Inhibition may therefore be expected to play a role in the regulation of the feedbacks. Positive feedback loops, when subject to control by inhibitors such as ATIII, confer threshold properties on the system[78]. Below the threshold no response will occur, while above the threshold the response should be at or near maximum.

NEGATIVE FEEDBACKS

Control of coagulation reactions

Several natural anticoagulant mechanisms are able to exert damping effects upon each step of the coagulation cascade[164]. Two types of inhibition exist in the regulation of thrombin production. The first depends on the existence in plasma of permanent and ample inhibitory capacity in the form of protease inhibitors, the major example being ATIII[167], with possibly α_2-macroglobulin as a minor player. In addition to this, protein C anticoagulant mechanisms, will regulate activated cofactors[36]. However,

Antithrombin III does appear to be the most quantitatively important. It is able to effectively neutralise all serine proteases produced during the blood coagulation process[142].

The second type of inhibition, which is so far unique, is the tissue factor pathway inhibitor (TFPI), involving a negative feedback in which an inhibitory species is formed only when the system is activated. It can bind and inactivate both factor Xa and factor VIIa simultaneously[159]. While TFPI is also an important regulator of the serine proteases, its principle targets being factor Xa and the factor VIIa-TF / factor Xa complex[11], this is not as clearly related to injury free plasma dilution and may therefore not be as crucial in this thesis.

The dynamic inhibitory system is generated when thrombin binds to its cofactor thrombomodulin and activates protein C[37]. Activated protein C attenuates the coagulation reaction of factor V/Va and factor VIII/VIIIa[28,65].

Protein C system and antithrombin III have prominent effects on the propagation phase of thrombin generation[216,217]. In the presence of both, TFPI and antithrombin III, the concentration of factor Xa (the limiting reactant of thrombin generation) is decreased. Additionally, antithrombin III also inhibits other serine proteases produced *in situ* during the reaction[142]. As a consequence of synergistic effects, thrombin generation becomes a threshold-limited process, influenced by relatively small changes in the concentrations of procoagulants and their inhibitors[13].

Therefore, a number of plasma proteins are able to inhibit serine proteases involved in coagulation, fibrinolysis, and kinin formation. These

include ATIII, heparin cofactor II, α_2-macroglobulin, α_1-antitrypsin, tissue factor pathway inhibitor, protein C inhibitor, α_2-antiplasmin, and plasminogen activator inhibitor-1 (PAI-1). The similarity in structure and mechanism of action of these proteins (with the exception of α_2-macroglobulin) has led to the general term *serpins* for these serine protease inhibitors:[212]

Antithrombin III, which is also known as antithrombin or heparin cofactor I, inhibits its target enzymes by forming 1:1 enzyme: inhibitor complexes in which the active site of the enzyme is blocked[166]. Factors IXa, Xa, XIa and XIIa are inactivated by ATIII in a manner similar to that outlined for thrombin[96,165,197]. Although the interaction is reversible[74], dissociation is probably too slow to be significant. Since the plasma ATIII concentration is in significant excess of the sum of the concentrations of all the coagulants, there is ample mass of ATIII available. However, despite the level being adequate in mass terms, the second-order kinetics of its action mean that the rates of inhibition of target enzymes are a function of its concentration, both in pure systems and plasma[27,76,77].

Heparin cofactor II (HCII) is a serine protease inhibitor (serpin) that is synthesised by the liver and circulates in plasma. About 40% of the protein equilibrates with an extravascular compartment and it has been detected in the intima of normal human arteries. It inhibits thrombin, but has no activity against other proteases involved in coagulation or fibrinolysis[207].

α_2-Macroglobulin can inhibit numerous serine proteases, including thrombin, factor Xa, plasmin and kallikrein, albeit at a relatively modest rate compared with other inhibitors[36].

Tissue factor pathway inhibitor is a serum component that has been shown to form a quaternary complex with factor Xa, factor VIIa, and tissue factor in the presence of calcium and thereby suppresses the activity of the extrinsic cascade[156,187].

Protein C - a blood coagulation inhibitor, autoprothrombin IIa, is generated when thrombin is converted to prothrombin. The vitamin K-dependent protein, which was termed protein C was subsequently found to be the same protein as autoprothrombin IIa[189].

Plasminogen activator inhibitor-1 plays the major physiological part in suppressing the function of tissue plasminogen activator as well as urokinase, thus preventing the activation of the fibrinolytic pathway. In contrast to most other serine protease inhibitors in plasma, the normal level of PAI-1 is quite low. PAI-1 can also inhibit other coagulation system proteases including plasmin, activated protein C, and thrombin[30,31].

The coagulation cascades are represented in Appendix 1.

CHAPTER THREE
MONITORING OF COAGULATION ABNORMALITIES

INTRODUCTION

As discussed in the previous chapter, normal haemostasis is the ability of the haemostatic system to control activation of clot formation and clot lysis in order to prevent haemorrhage without causing thrombosis. It involves vasoconstriction, platelet adhesion and aggregation at the site of injury, leading to a plug formation. This is followed by fibrin formation consolidating the plug and rendering it stable. All of this occurs with no clotting elsewhere. This basic definition of normal haemostasis, depending on several finely balanced mechanisms, shows that:

1. There are three systems involved simultaneously in haemostasis: the formation of clots, involving platelets and the coagulation pathways; this is opposed by the anti-coagulant system, and thirdly, the fibrinolysis of clots once they have been formed.

2. The absence or presence of haemorrhage or thrombosis depends on a delicate balance between the procoagulant and anticoagulant systems, while the clot maturity, or early breakdown once formed, will depend on the fibrinolytic system. In other words, an excess of *activated* pro-coagulants will primarily result in thrombosis. On the other hand, an increased anticoagulant potential (antithrombin, protein C, etc.), or decrease in activation of procoagulant factors will result in haemorrhage. In addition, enhanced fibrinolysis will also lead to bleeding.

3. Given the intricate interactions between the three systems, any instrument measuring coagulation endpoints may only be able to identify single points, rather than the interaction between systems. This is because coagulation is a dynamic process dependent on the interaction of an array of factors rather than one determined by actual values of individual agents. It is important to note that some of the end-point measurements may also provide artifactual data, i.e. prolonged bleeding time, where inappropriate wiping could stop the formation of a platelet plug, rather than a clotting abnormality.

MONITORING OF COAGULATION

As the understanding of the normal mechanisms of coagulation grows, so does the ability to monitor haemostasis. Evaluation of haemostasis will become more specific and accurate and instrumentation techniques will become simpler and more efficient as technology progresses, facilitating the monitoring of haemostasis in the clinical setting[223]. The haemostatic process of coagulation is extremely complex, with multiple interactions between factors, including the coagulation and fibrinolytic proteins, platelets, activators and inhibitors. This is in order to avoid uncontrolled action in either direction, and is accomplished by numerous positive and negative inhibiting and activating feedback mechanisms. As a result, the actual *quantity or level* of an individual factor does not necessarily reflect its functional status. With this complicated and interactive dynamic system, the measurement of static endpoints will not reflect the dynamics of the system.

Any medical coagulopathy, as opposed to a surgical bleed, needs the actual underlying cause determined in order to direct the therapy appropriately.

Traditionally, haematological tests like prothrombin time (PT) and its derived value, the international normalised ratio (INR), activated partial thromboplastin time (aPTT), bleeding time and activated clotting time (ACT) (see below) have been done. However, while they are useful endpoint markers to identify the lack of a given factor, they do not measure the dynamic interaction between the different systems, as blood coagulation occurs efficiently on cell surfaces such as activated platelets and monocytes, and fibroblasts. Both prothrombin time (PT) and activated partial thromboplastin time (APTT) are highly artificial in vitro systems with major limitations[4].

STANDARD TESTS OF COAGULATION

Most of the routine tests of coagulation are performed on blood that has had the coagulation cascade suspended through the addition of sodium citrate 3.8% which removes the calcium from the blood. The sample is then spun down leaving only plasma, with almost no platelets. In order to measure the various tests of coagulation endpoints, calcium is added in abundance, as are the various factors used to initiate coagulation. As a result, these tests are generally useful for measuring absolute values, or endpoints. However, they will not measure the interaction between pro- and anticoagulant factors, because of the addition of excess initiating factors.

Prothrombin time (PT)[121]

This test measures the overall efficiency of the extrinsic system. It was introduced as an assay of prothrombin in plasma before many of the factors participating in the coagulation reactions were identified. It is now used as a screening tool of the activities of fibrinogen, prothrombin and factors V, VII,

and X. It is also used to monitor the oral anticoagulants, since these lower the levels of prothrombin, VII and X.

The test is initiated by recalcifying citrated plasma in the presence of thromboplastin. Thromboplastin is added far in excess of normal levels and is a source of tissue factor in a suspension of phospholipids. The time that elapses until the plasma clots is the PT and is predominantly determined by: i) factor VIIa/tissue factor in the presence of phospholipids and calcium activating factor X; ii) factor Xa/Va in the presence of phospholipids and calcium activating prothrombin; iii) thrombin cleaving fibrinogen; and iv) fibrin monomers polymerising.

The test has to be standardised and consists of taking blood in a ratio of 9 parts blood to 1 part 3.8% citrated blood. Inter-assay variability is increased by the variability of methods used to detect clot formation.

In order to interpret this, the prothrombin time ratio (PTR) needs to be calculated, i.e. the patient's result compared with the mean reference range. It then needs to be corrected for the slope of response of the assay to oral anti-coagulants. This is the international sensitivity index (ISI). The International Normalised Ratio (INR) is then calculated as $INR = PTR^{ISI}$. "INR shorter than the reference range are uncommon and have not been consistently correlated with any clinical situation.[1]" Calculation of the INR improves standardisation, but is still limited by variations in reagents and methods. As a measure of anticoagulation, the INR only has meaning for patients on a stable dose of anticoagulants; therefore this cannot be used in patients who have not been taking their anti-coagulants for at least a week or in patients who have abnormal prothrombin times for reasons such as liver disease. However, in

practice, the INR is used to test for intrinsic liver function in hepatitis, as factor VII is produced in the liver.

The ACL 200 analyser simultaneously determines the prothrombin time and fibrinogen using the PT-Fibrinogen Recombinant (PT-FIB) reagent. The normal INR range is: 1,0 to 1,3.

Activated partial thromboplastin time (aPTT)[87,119]

This is usually used as a screening test to identify acquired or inherited deficiencies or inhibitors of factors VIII, IX, and XI. Reduced activation of fibrinogen, prothrombin, or factors V and X can prolong the aPTT. It is also used to monitor heparin therapy.

Again the test is initiated with citrated platelet-poor plasma being incubated and recalcified after the addition of a source of platelets or platelet substitute together with a contact activator and phospholipid to "activate" coagulation. The time that elapses until the plasma clots is the aPTT. While the normal has been difficult to establish as there is great variability, and hence the "controlled activation" of citrated blood to give an endpoint, it is again important to note that the variability of coagulation results from subtle changes occurring in the dynamic coagulation system and that these are lost in the aPTT.

The aPTT is sensitive to heparin and is usually used to monitor anticoagulant therapy. It is noted that some patients with disseminated intravascular disease (DIC) or malignancies may have a shortened aPTT reflecting the *in vivo* state, however, a shortened aPTT or PT rarely point to significant findings of enhanced coagulation.

The ACL 200 analyser determines the activated partial thromboplastin time using the APTT reagent and calcium, giving the printout aPTT-result in seconds, with the normal range being: 26 to 40 seconds.

Red Blood Cell count (RBC) / Platelet count[8]

Using fresh EDTA anticoagulated blood, both red blood cell and platelet counts are analysed by a single optical cytometer after appropriate dilution of the blood sample with ADVIA 120 RBC/PLT reagent. The red blood cells are isovolumetrically sphered and lightly fixed with glutaraldehyde to preserve the spherical shape. Red cells and platelets are counted from the signals from a common detector with 2 different gain settings, with the platelet signals being amplified considerably more than the RBC signals. The Haemoglobin Concentration (HCT) value is calculated from the RBC count and the MCV.

The test is performed on the **ADVIA® 120**

Bleeding time (BT)[120]

This is a screening test for inherited or acquired disorders of platelet function and for von Willebrand's disease. It is measured as the time required for a standardised skin wound to stop bleeding. It is thought to be determined by the rate of platelet plug formation. It should only be performed by experienced operators and each centre should have its own reference range (~ 2.5-10 min.). A lower platelet count can prolong the bleeding time and it may be affected by multiple other factors. It is not possible to reliably demonstrate abnormal bleeding in anyone with an acquired platelet dysfunction. The test has a poor sensitivity and specificity. The major conclusions of a position article on bleeding time are: (1) In the absence of a history of a bleeding disorder, the bleeding time is not a useful predictor of the risk of haemorrhage

associated with surgical procedures. (2) A normal bleeding time does not exclude the possibility of excessive haemorrhage associated with invasive procedures. (3) The bleeding time cannot be used to reliably identify patients who may have recently ingested aspirin or non-steroidal anti-inflammatory agents[148].

Activated clotting time (ACT)[118]

This is mostly used to monitor heparin anticoagulation. The result is available within minutes of starting the test, and is therefore often used in a clinical situation. It is conceptually similar to the aPTT, except that fresh whole blood is tested rather than citrated plasma. Whole blood is added into a tube containing an activator, and the time that elapses until clot is formed is the ACT. This assay thus depends on the same reactions as the aPTT *plus* the effects of platelets and any other cells that can influence coagulation. In this effect it reflects *in vivo* conditions much better. However, it only measures an endpoint and does not identify where the source of a coagulation abnormality rests.

Platelet Aggregation[64]

Platelet rich plasma (PRP) is stirred and a suspension of collagen is added to it. As a result some of the platelets adhere to the collagen coating the fibres. After the adherence has occurred, the free platelets begin to swell and stick together, with the reaction accelerating until large platelet masses have formed. This is the process of *platelet aggregation*; it is distinct from the adherence to collagen although initiated by it. Platelet aggregation occurs when aggregation agents are added to platelet-rich plasma (PRP), which is continually being stirred. As aggregation proceeds, platelet clumping occurs.

In the plasma, this reduces the optical density of the platelet-rich plasma (PRP) allowing more light (infra red) to pass through. If all the platelets clumped, the resultant optical density would be equivalent to that of the platelet-poor plasma (PPP). The light transmittance through the platelet-poor plasma represents 100% and that of the platelet-rich plasma (PRP) 0% aggregation. The aggregometer develops a voltage proportional to the transmittance of light through the plasma. This voltage is recorded on a strip chart recorder as a function of time. Since the optical channel is automated, a voltage representing the difference in transmittance between the platelet-rich plasma (PRP) and platelet-poor plasma (PPP) cuvettes is automatically developed and this voltage is registered on the strip chart recorder.

Tests on the Whole Blood Aggregometer Optical Channel - This detects platelet aggregation in a 0.45 ml sample of platelet rich plasma (PRP) by measuring infrared light transmission through the sample.

Calculations - Provided that the recorder is run long enough to permit a plateau or a reversal of the responses to occur, a plot of the maximum aggregation achieved during the time verses the logarithm of the agonist concentration gives a characteristic sigmoidal curve.

Normal Range - The platelets of normal subjects usually produce a single reversible primary wave with 1 µmol/L ADP or less, a biphasic aggregation with 2,5 µmol/L, and a single irreversible wave at 5 to 10 µmol/L. A single phase response is observed after a lag phase lasting not more than 1 min with 1 and 4 µg/ml of collagen. A single phase or biphasic response is seen with 1,2 mg/ml of ristocetin and biphasic aggregation is observed with 2-10 µmol/L of adrenaline.

Interpretation of results - The length of time between the addition of the aggregating agent and the onset of the recorders pen deflection provides a clue as to how the aggregation agent works; whether directly aggregating platelets or through stimulating the release of ADP. The slope of the curve allows estimation of the speed of aggregation while the course, maximum height and return to baseline provides information on the responsiveness of the platelets, from different sources, to the same stimulus.

ADP – Low concentrations of ADP (adenosine diphosphate) (0,5 TO 2,5 µmol/L) cause primary or reversible aggregation. At very low concentrations of ADP, platelets may disaggregate after the first phase. In the presence of higher concentrations of ADP an irreversible secondary wave of aggregation is associated with the release of dense and α-granules due to activation of the arachidonic acid pathway. Failure to aggregate with ADP is caused by the following: aspirin consumption, storage pool disease (no α or dense granules released), thrombasthenia (due to a missing or defective platelet membrane glycoprotein).

Collagen – The aggregation response to collagen is preceded by a short "lag" phase lasting 10-60 seconds. The duration of the lag phase is inversely proportional to the concentration of the collagen used and to the responsiveness of the platelets tested. This phase is succeeded by a single wave of aggregation due to activation of the arachidonic pathway. Higher doses of collagen can cause sudden increase in intra-platelet calcium concentration, thus bypassing the prostaglandin pathway. The following causes defective or no response to collagen aggregating agents: aspirin

consumption, thrombasthenia thromboxane synthetase deficiency or cyclo-oxygenase deficiency.

Ristocetin – Ristocetin reacts with von Willebrands Factor (vWF) and the membrane receptor to induce platelets to clump together (agglutination). It does not activate any of the three aggregation pathways, nor cause the initial granule release. Defective aggregation is caused by a glycoprotein deficiency or defect and is associated with disease.

Adrenaline – No shape change precedes aggregation, but the response thereafter resembles the ADP response. Such a response is usually obtained with concentrations of 2-10 µmol/ml. Some clinically normal people will fail to aggregate with adrenaline.

Antithrombin III (AT III)[62]

Antithrombin III or Heparin Cofactor I is the major inhibitor of blood coagulation and as such, a deficiency is associated with a high risk of thromboembolic disorders. It is also essential for effective heparin therapy. By inhibiting the coagulation proteases, especially thrombin, Factors Xa and IXa, AT III prevents uncontrolled coagulation and thrombosis. The assay is based on a chromogenic substrate and on Factor Xa inactivation, with the method being consequence specific and not influenced by Heparin Cofactor II.

Antithrombin levels in patient plasma are measured automatically on the **ACL Futura** in two stages:

- Incubation of the plasma with Factor Xa reagent in the presence of an excess of heparin.

- Quantification of the residual Factor Xa activity with a synthetic chromogenic substrate.

The paranitroaniline released, monitored at 405 nm, is inversely proportional to the Antithrombin III level in the test sample. The result is reported in percentage activity and is automatically calculated, with the method being linear from 10% - 150%. The Normal Range is: 85% - 120%.

Thrombin Antithrombin complex (TAT)[22]

This is an enzyme immunoassay for the determination of human thrombin / antithrombin III complex. **Enzygnost® TAT micro** is an enzyme immunoassay for the quantitative determination of thromblin/antithromblin III complex in human plasma and is used for the diagnosis of disturbances in blood coagulation, which are associated with changes in the activity of the coagulation system. Persons predisposed to thrombosis and DIC are found to have elevated concentrations of TAT.

D-Dimer (XDP)[63]

D-Dimer containing moieties are formed by plasmin degradation of factor XIIIa cross-linked fibrin, with elevated levels being found in clinical conditions such as deep vein thrombosis (DVT), pulmonary embolism (PE) and disseminated intravascular coagulation (DIC).

For quantitative analysis using the **ACL Futura** the degree of agglutination is directly proportional to the concentration of D-dimer in the sample and is determined by measuring the decrease of the transmitted light at 405 nm caused by the aggregates (turbidimetric immunoassay).

Quantitative Test - The QXDP Test is used and results are reported in ng/ml, with the interpretation of results being that agglutination will occur within 180 seconds for samples containing more than 250 ng/ml D-dimer. The mean level of D-dimer (XDP) in the healthy population is between 8 and 135 ng/ml and neat plasma from normal healthy individuals should not agglutinate. If no agglutination is observed a thrombotic condition is unlikely. The circulatory half-life of D-dimers is about 12 hours and therefore elevated levels can persist for some time after the active process has ceased.

Fibrinogen

This is measured using an **IL Test™ PT-Fibrinogen Recombinant** kit. The test is based on a combined assessment of the prothrombin time and fibrinogen level by the addition of recombinant rabbit thromboplastin to the plasma. The fibrinogen is quantified by relating the absorbance or light scatter during clotting to a calibrator

Catecholamines

Heparinised samples, immediately centrifuged at 4000rpm for 10 minutes, plasma is frozen at –70°C for batching. Plasma catecholamine concentrations are measured by electrochemical detection after separation with reverse phase high-pressure liquid chromatography (using dihydrobenzylamine as internal standard). The coefficient of variation is 7.9% for noradrenaline and 8.7% for adrenaline; the limit of sensitivity is 20pg.ml^{-1}.

WHOLE BLOOD DYNAMIC TESTS OF COAGULATION

There are two tests using whole blood to measure clot formation rather than measuring end-points as is done in the standard tests.

Sonoclot

Sonoclot (**Sonoclot R Coagulation Analyser, Sienco, Inc**) analyses provide a simple test of cellular and plasmatic coagulation properties[90]. It and the thrombelastograph (TEG)(see below) assess the coagulation process in whole blood and may therefore be physiologically more relevant than assays of isolated haemostatic components. Hypercoagulability is detected in a high proportion by both techniques, with the clotting rates of the Thrombelastograph and Sonoclot being significantly correlated[42,198]. In addition, Sonoclot coagulation analyses correlate better with blood loss than aPTT and platelet count in the routine coagulation analyses. [33].

Thromboelastography (TEG)

The Thrombelastograph Haemostasis Analyser is a non-invasive diagnostic instrument designed to analyse the coagulation state of a blood sample in order to assess haemostasis conditions from haemorrhagic through thrombotic. It will be discussed in more detail, as the TEG is the main tool used to evaluate coagulation in this thesis.

TEG DESIGN AND OPERATING PRINCIPLES (Data from Haemoscope Corp.)

The TEG analyser's approach to the monitoring of patient haemostasis is based on these two facts:

- *The end result of the haemostasis process is a single product—the clot.*
- *The clot's physical properties (rate, strength, and stability) will determine whether the patient will have normal haemostasis, will haemorrhage or will develop thrombosis.*

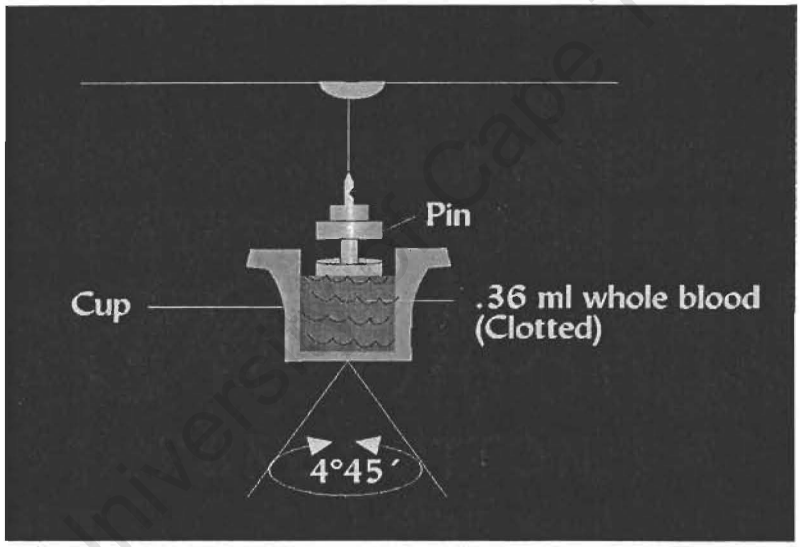


Fig 3.1. TEG sample cup design (Figure supplied from Haemoscope Corp.)

The TEG analyser involves two mechanical parts to measure the physical properties of the clot, a cup and a pin. The special stationary cylindrical cup holds the blood (0.36 ml) and is oscillated through an angle of 4°45′ at 37°C (Figure 3.1).

Each rotation cycle lasts 10 seconds. The pin is suspended in the blood sample by a torsion wire and is monitored for motion, which is either transduced mechanically to a heated stylus, moving across heat-sensitive

graph paper on a chart recorder, or in the newer models, it is converted by a mechanical-electrical transducer to an electrical signal, which can be monitored by a computer. When no clot exists, the motion of the cuvette does not affect the pin and a straight line is recorded. The torque of the rotating cup is only transmitted to the immersed pin after fibrin-platelet bonding has linked the cup and pin together. The strength of these fibrin-platelet bonds affects the magnitude of the pin motion, such that strong clots move the pin directly in phase with the cup motion. Thus, the magnitude of the output is directly related to the strength of the formed clot. As the clot retracts or lyses, these bonds are broken and the transfer of cup motion is diminished.

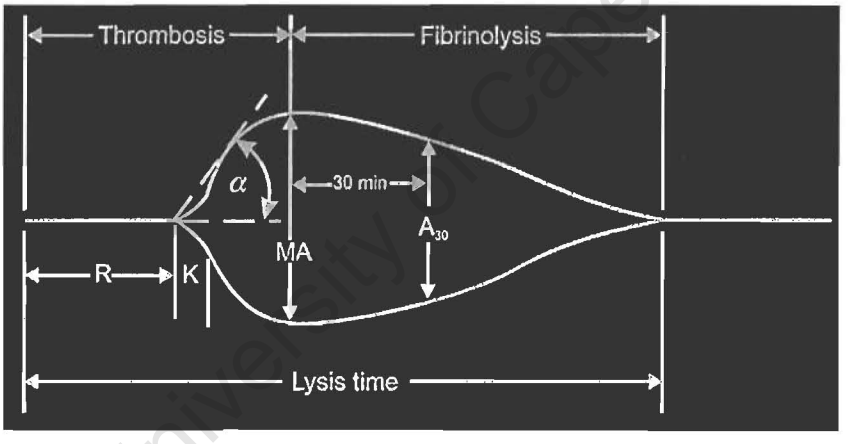


Fig 3.2. TEG tracing parameters (Figure supplied from Haemoscope Corp.)

The resulting haemostasis profile is a measure of the time it takes for the first fibrin strand to be formed, the kinetics of clot formation, the strength of the clot and dissolution of clot (Figure 3.2).

TEG ASSESSMENT (Data from Haemoscope Corp.)

The coagulation profile evaluated by the TEG is a graphic method of displaying a measure of the kinetics of clot formation and dissolution as well as of clot quality. It monitors shear elasticity, a physical property of a blood

clot, and is, therefore, sensitive to all the interacting cellular and plasmatic components in the blood that affect the rate or structure of a clotting sample and its breakdown.

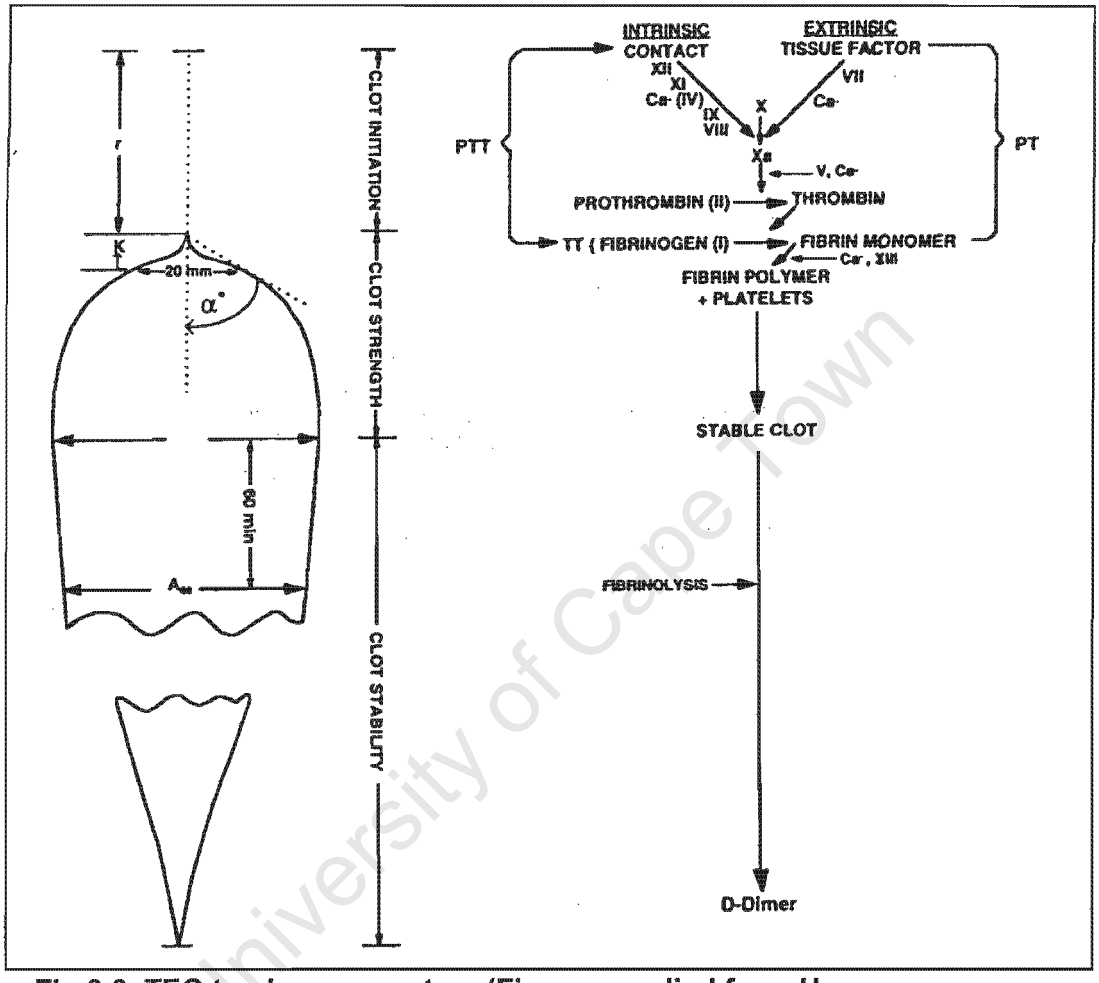


Fig 3.3. TEG tracing parameters (Figure supplied from Haemoscope Corp.)

The clot's ability to perform useful mechanical work (the work of haemostasis) is a function of the net result of the interactive coagulation proteins and cellular elements involved in the process of haemostasis. In essence, the TEG analyser measures the ability of the clot to perform mechanical work throughout its structural development (Figure 3.3).

A recent advance is computerisation of the TEG, which essentially gives greater accuracy and consistency than manual measurements by removing

the human error from the measurements. The TEG pattern is divided into component variables. (Table 3.1)

r	r-time is the period of time of latency from the time that the blood was placed in the TEG analyser until the initial fibrin formation.
k	k-time is a measure of the speed to reach a certain level of clot strength
α	Alpha angle measures the rapidity of fibrin build-up and cross-linking (clot strengthening)
MA	MA, or Maximum Amplitude, is a direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.
LY30	LY30 measures the rate of amplitude reduction 30 minutes after MA.

Table 3.1. TEG tracing parameters (Table supplied from Haemoscope Corp.)

Reaction Time. The time from the start of a sample run until the first significant levels of detectable clot formation (amplitude = 2mm in the TEG tracing). This is the point at which most traditional coagulation assays reach their end-points. r-time is prolonged by anticoagulants and factor deficiencies and shortened by hypercoagulable conditions.

Achievement of a certain clot firmness. The time from the measurement of r (beginning of clot formation) until a fixed level of clot firmness is reached (amplitude = 20mm). Therefore, k-time is a measure of the speed or clot kinetics to reach a certain level of clot strength. k is shortened by increased fibrinogen level and, to a lesser extent, by platelet function, and is prolonged by anticoagulants that affect both.

The kinetics of clot development. The α- angle is closely related to k-time, since they both are a function of the rate of polymerisation. The angle is more

comprehensive than k-time, since there are hypocoagulable conditions in which the final level of clot firmness does not reach an amplitude of 20 mm (in which case k is undefined). Similar to k, α is larger by increased fibrinogen levels and, to a lesser extent, by platelet function, and is decreased by anticoagulants that affect both.

Maximum Amplitude. This is the measurement of maximum strength of stiffness (maximum shear modulus) of the developed clot. Clot strength is the result of two components — the modest contribution of fibrin to clot strength and the much more significant contribution of the platelets.

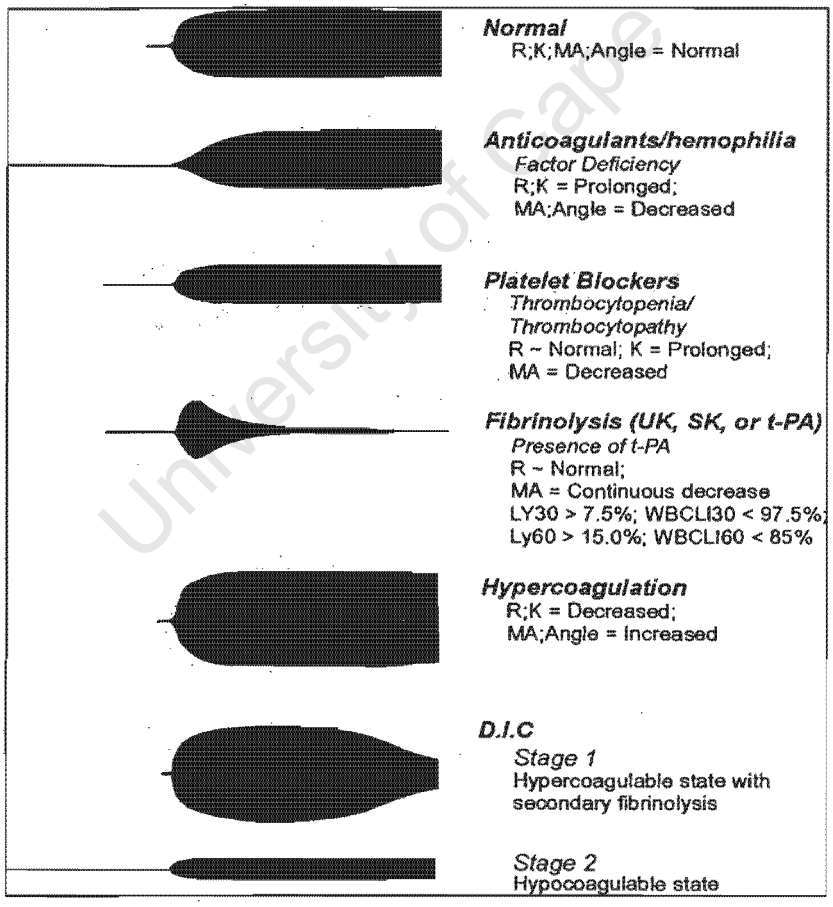


Fig 3.4. Qualitative Analysis (Figure supplied from Haemoscope Corp.)

The basic TEG measurements of kinetics, strength and stability of a clot can be determined by using a native whole blood sample. This has provided a sensitive method for monitoring hypercoagulation, with the patterns being

interpreted without measurement to determine conditions of hyper-, hypo-, normal coagulation, and fibrinolysis (Figure 4). However, by using the measurements and established normal ranges and indices, the patterns can be quantified as to the degree of abnormality.

QUALITY ASSURANCE -

The methods providing comprehensive quality assurance for the TEG® analyser are based on the recommendation of the American Clinical Laboratory Improvement Act of 1988 (CLIA)

Laboratory Techniques and Precautions

Standardised methods for the collection and handling of specimens were used. Everyone using the TEG® analyser was familiar with standard laboratory procedures, techniques, and precautions. The absolute values are all within the instrument accuracy of measurement, which is+/- 1 unit. Of importance is:

- Using the two-syringe technique to eliminate tissue factors or contamination from catheter lines – 3 to 5 ml of blood drawn in a separate syringe and discarded before the sample is taken.
- Avoiding heparin contamination.
- Avoiding clot activation in the drawn sample by exposure to glass.
- Adhering to the constant time intervals established for native whole blood analysis, which state that blood samples should be placed on the TEG analyser within 4 minutes of being drawn. (Because coagulation begins as soon as blood is drawn.)
- Avoiding touching of the working surfaces of the disposable cups and pins.
- Operating temperature 15 to 30°C

General Quality Control

- Maintenance and function checks.
- Calibration and calibration verification
- Control procedures

These are performed semi-annually by trained TEG® users or Haemoscope technicians to the check the baseline.

Local normal ranges

	R (min)	K (min)	α-Angle (deg)	MA (mm)
Native	15.5-23.0	5.5-10.5	22.0-38.0	47.0-58.0

Table 3.2. Standard Parameter Measurement (n=132)(Haemoscope Corp)

Haemoscope provides reference normal ranges for the TEG (Table 3.2).

	r-time (min)	k-time (min)	alpha-alpha	MA
Number	**163**	**163**	**156**	**163**
Mean	13.8	6.7	37.5	52.7
95% CI	13.1	6.3	35.7	51
	14.5	7.1	39.3	54.3
C'tile 25	11	4.5	29	46
C'tile 75	16	8	44	58
Min	4.5	1.5	19.5	19
Max	34	22	68	79
Quartile Range	5	3.5	15	12
SD	4.4	2.6	11.4	10.7
SEM	0.3	0.2	0.9	0.8

Table 3.3. UCT Parameter Measurements (Calculated by MFM James)

Different population composition and other factors may affect the measurement capability, performance, and sensitivity of the analyser and may produce a different set of values for normal ranges. For this reason, Haemoscope suggest that centres should set up a local normal reference range from "normal control individuals". These individuals should be healthy and not taking any medications which affect haemostasis. This was done for the population in which investigations of this thesis were performed (Table 3.3).

THROMBOELASTOGRAPHY HISTORY AND EVALUATION

The TEG provides a quantitative and qualitative indication of the coagulation state of a blood sample by measuring coagulation parameter and recording the kinetic changes in a sample of whole blood, plasma or platelet rich-plasma as it clots, retracts, and breaks apart (lysis). As a dynamic measurement of coagulation, it reflects the interaction between cell surfaces and coagulation factors (pro- and anti-) involved in the localisation, amplification, and modulation of coagulation functions. It is a technique first developed by Hartert in 1948[60]. It had little clinical use for many years until it was re-evaluated in the surgical setting in 1974[66]. The main reason for the current interest in the device is the fact that it assesses whole blood coagulation rather than individual components of the coagulation pathway. However, the global non-specific nature of the TEG measurement may be both its greatest weakness and strength[224]. Although standard laboratory tests are poor predictors of bleeding, transfusion medicine specialists have been reluctant to accept whole-blood assays. Their justification has been: (1) the non-specific

nature of the TEG measurement; (2) lack of quality-assurance; and (3) insufficient scientific evidence or appropriately powered outcome studies[48].

Having said that, it is important to understand that the TEG measures haemostasis by analysing the functional aspects of the coagulation cascade, rather than endpoints, in its use to evaluate blood coagulation, with analysis of native blood yielding the most reliable TEG results[15]. It has been indicated that TEG is a global test of coagulation evaluating the elastic properties of whole blood, particularly in the detection of hypercoagulable states with excellent reproducibility for all parameters[17,191,218], providing a method for evaluation of the coagulation system from initial clot formation to clot retraction or dissolution. It is thus a reliable method of evaluating hypercoagulability[52]. The TEG is a 'near patient' coagulation test, which poses many theoretical advantages to standard tests, not least of which is the yield of information relating to cumulative effects of several components of coagulation at a given instant [183,213].

Although the TEG was developed so long ago, it has only recently acquired widespread acceptance. Historically, the thrombelastograph has been run with poor standardisation causing a negative bias towards research carried out using it. There was also limited published data on performance with regard to assay precision or inter-assay variability that led to scepticism. There have, however, been fundamental changes to the instrument that have allowed standardisation of results. The traces have been improved and the TEG cups and pins, initially reusable stainless steel causing a potential for a variation in the assay result, are now made of disposable plastic, thus increasing the standardisation[218].

As a result, the TEG has emerged as a useful method for evaluating haemostasis in several clinical settings. More recently, the contribution of platelets and soluble clotting components to clot strength has been the focus of clinical studies using thromboelastography[139]. While the dilutional changes in TEG are influenced by both coagulation factors and platelets, thus causing the evaluation complex, two variables of TEG, MA and k, were linearly related with platelet count[144]. It can also potentially correlate with the degree of anticoagulation produced by low molecular weight heparin, with the r-time from the thrombelastogram correlating with serum anti-Xa concentration[88]. Analysis of the TEG tracing reveals information about the rapidity of clot formation, the strength of the formed clot, the presence of fibrinolysis, and platelet dysfunction, which may not be evident in terms of abnormal PT and aPTT values until they are more than 50% reduced[211,223].

In summary, of importance for this thesis is that it is very sensitive in the identification and measurement of hypercoagulability, which is not detected by routine laboratory tests[2], unless the platelet count or fibrinogen concentration is markedly increased[106]. TEG variables indicative of hypercoagulability are a shortening of the r and k times, and an increase in MA and α-angle. It can provide rapid information on coagulation status and specific treatment options within 30 min, specifically in the evaluation of the hypercoagulable states[183]. It is, in fact, the leading point-of-care monitor for hypercoagulability [50].

COMPARISON BETWEEN THE TEG AND STANDARD TESTS

The TEG has been widely used as a measure of coagulation in clinical anaesthesia[106], but there have been conflicting opinions on its validity as a stand-alone test.

In an editorial in Anesthesia & Analgesia, Samama casts a critical light on thromboelastography[185]. He observes that amongst other things, thromboelastography is the leading point-of-care monitor for hypercoagulability[42,50] and has become so popular that many groups consider it a self-sufficient coagulation test. However he goes on to state "that it is not a conventional hemostasis test, thus remaining a second-level test and therefore not reaching the standards acceptable of most academic hemostasis societies". His opinion is that these major drawbacks have to be taken into account when thromboelastography is proposed as the only available haemostasis test in a study, going on to state that "thromboelastography *is not* validated, as far as international standards are concerned, and *it has never been standardised*". He further states that "postoperative hypercoagulability exploration requires the use of such specific hemostasis tools as platelet aggregation variables, platelet activation measurements (flow cytometry variables), thrombin-antithrombin complexes, prothrombin fragment 1 + 2, type 1 plasminogen activator inhibitor, or plasmin-antiplasmin complexes[186]", although that reference makes no comment on the TEG. In the opinion of Samama "even modified-thromboelastography, should not be used alone to express a hypercoagulable state." In closing, the editorial agrees with Whitten[224], "that if thromboelastography is to be considered a promising hemostasis tool, its global management must be improved as it deserves a new evaluation leading to a final validation or it will remain an expensive, non-validated point-of-care monitor[185]."

Conversely, thromboelastography has been quoted as a proven useful research tool and compared to six common tests (the haematocrit, platelet

count, fibrinogen, prothrombin time, activated thromboplastin time and fibrin split products) by Zuckerma[227]. The results indicated that, although there is a strong relationship between the thromboelastographic variables and these common laboratory tests, thromboelastographic variables contain additional information on the haemostatic process. The TEG has been used to confirm normal coagulation in the face of an abnormal prothrombin time[150]. Kang *et al*[83] found the relationship between TEG and standard laboratory tests of coagulation to be similar to that of other researchers[66,227], with the greatest correlation occurring between *r*-time and aPTT, while McNicol *et al* found the relationship between standard tests of coagulation and the TEG to be an inconsistent correlate[116]. However, the authors comment that this is not surprising given that the TEG variables are interdependent, measuring the interaction of the coagulation cascade and platelets in whole blood rather than specific endpoints in centrifuged plasma samples. The dynamic data provided by the TEG allows far more appropriate replacement therapy[19].

The TEG is a real time monitor of global haemostasis, which provides readily available analysis of coagulation in a shorter period of time than laboratory coagulation tests would take under ideal circumstances, thus proving useful and prompt identification of coagulation disorders[149]. It allows rapid intra-operative diagnosis and specific management[213], covering the entire field of haemostasis in the peri-operative setting, from platelet function, to fibrinolysis. On the other hand are the findings of Gravlee *et al* prospectively evaluating numerous tests of clotting function, including activated clotting time, activated partial thromboplastin time, prothrombin time, thrombin time, fibrinogen, fibrin/fibrinogen degradation products, platelet count, and Duke's earlobe

bleeding time, in 897 consecutive adult cardiac surgical patients. Statistically significant correlations were found between these tests and mediastinal drainage. However, scatter plots indicated that these relationships, while statistically significant, had little predictive value, with the best multivariate model constructed explaining only 12% of the observed variation in postoperative blood loss[55].Furthermore, Davis *et al* found in their study that of various assays tested, only abnormal TEG values: alpha angle ($P < 0.01$) and k-time ($P < 0.04$) were associated with an increased risk of bleeding. Bleeding times were not predictive of an increased risk of post-biopsy bleeding. All prothrombin time, partial thromboplastin time, and platelet count abnormalities were mild and none of these assays predicted bleeding. It was concluded that the TEG was the best assay for detecting mild coagulation abnormalities associated with an increased risk of bleeding[23]. With the advent of computerisation in the last decade, TEG has evolved from a research laboratory tool into a compact processor, providing global information on the entire coagulation process. The battery of traditional coagulation tests, including bleeding time, prothrombin time, partial thromboplastin time, thrombin time, fibrinogen and factor assays, and platelet function studies, are based on the isolated, static end points of standard laboratory tests and as a result, they do not take into account the interaction of clotting cascade and platelets in the whole blood. Haemostasis is an integrated, interactive, dynamic, and extremely complex process involving coagulation proteins, activators, cellular elements, and inhibitors. It is important to realise that the TEG measures this interactive dynamic coagulation[196], while standard tests of coagulation stop measuring at the first onset of clot formation; in other words,

at static endpoints of blood coagulation, and so provide no information on the dynamics of clot formation, strength and stability. As a result, it is difficult to assess what the exact dynamic process for inhibition of clotting is.

In the clinical setting, the TEG is used to rapidly assess coagulation abnormalities. Kaufmann investigated its use in the initial assessment of trauma patient coagulation, comparing it to platelet count, prothrombin time/partial thromboplastin time. Only TEG ($p < 0.05$) was predictive of early transfusion, leading to the conclusion that the TEG is a rapid, simple test that can broadly determine coagulation abnormalities. In response to this, Dr. Robert C. Lim (San Francisco, California) agreed that: "thrombelastogram is a test that has been used primarily in research in coagulation. It corresponds to the different steps in haemostasis and so gives a global picture of the haemostatic status. It has been confirmed that the thrombelastogram correlates with standard tests, such as PTT, PT and platelet count"[85].

Clinical tests, such as the TEG and Sonoclot analysis (SCT) are measures of viscoelastic properties of coagulating blood, which allow rapid intraoperative evaluation of coagulation factor and platelet activity as well as overall clot integrity from a single blood sample. Routine coagulation tests (RCT) include activated clotting time, prothrombin time, partial thromboplastin time, fibrinogen level, and platelet count. Tuman *et al* found that after cardio-pulmonary bypass, mean values for RCT were normal, but there were abnormalities in TEG and SCT parameters reflecting platelet-fibrin interaction. Both viscoelastic determinants of clot strength, TEG and SCT were 100% accurate in predicting bleeding and significantly better predictors of postoperative haemorrhage than RCT[214]. Essell *et al* tested the utility of TEG

versus platelet studies (bleeding time, platelet count, mean platelet volume) and standard coagulation tests (prothrombin time, activated partial thromboplastin time, fibrinogen). The sensitivities of the bleeding time (71.4%) and platelet count (100%) were similar to the TEG (71.4%), but the specificity (89.3%) of the TEG in predicting postoperative bleeding was greater than that of the bleeding time (78.5%) and platelet count (53.6%), suggesting that patients with a normal TEG who have a post-bypass bleed should not be given blood products empirically, but that a surgical cause should rather be excluded[39]. Kang *et al*[83] found thromboelastography to be a reliable and rapid monitoring system during liver transplantation and Martin *et al* validate the role of the TEG during paediatric liver surgery, finding a 100% sensitivity (8/8) and 73% specificity (8/11) in predicting increased postoperative bleeding[112]. In a study comparing the sensitivity of the ACT, aPTT and TEG, the ACT was less sensitive to residual heparin anticoagulation than aPTT or TEG[127]. In addition, the TEG, using anticoagulated blood during cardiac surgery is useful to guide treatment with haemostatic components. Twelve-hour chest tube losses were not different between groups despite the threefold reduction in the use of haemostatic products, showing that intra-operative monitoring of coagulation in the anticoagulated patient can be used to guide treatment[170]. The thrombelastograph, by providing information on the interaction of all the coagulation precursors, gives more clinically useful information on coagulation than that available from the coagulation profile or the activated clotting time alone. Thromboelastography was a significantly better predictor (87% accuracy) of postoperative haemorrhage and need for re-operation than was the activated clotting time (30%) or coagulation profile (51%)[195], with the TEG

providing diagnostic data within 30 minutes of blood sampling. Few studies have evaluated the comparative reproducibility of the TEG, of the Sonoclot and of the activated coagulation time with celite (ACT-C) or kaolin (ACT-K), measured with the Hemochron, in clinical on-site monitoring of haemostasis. Forestier *et al* determined the reproducibility of those measurements, and evaluated the ability of various devices to substitute for the ACT-C. In the clinical conditions of use, on-site haemostasis monitoring devices providing the most reproducible measurements are, in decreasing order, the TEG, the Hemochron and the SCT[40].

While the TEG is known to be a dynamic measure of coagulation, its role in determining platelet function has not been clarified. In a study by Wong, the TEG is compared to a routine coagulation battery (RCB) consisting of prothrombin time, partial thromboplastin time, platelet count, and bleeding time, which is commonly performed to assess coagulation status. The TEG was found to allow evaluation of overall coagulation activity, with an abnormal TEG maximum amplitude value always correlating with a prolonged bleeding time, making the TEG useful in assessing platelet function in the presence of thrombocytopenia[225]. In another study by Orlikowski, the platelet count, bleeding time TEG variables and the correlation between these variables were measured in 49 pregnant patients presenting with pre-eclampsia or eclampsia. The TEG variables, k time and maximum amplitude had a strong correlation with platelet count. There was no correlation between bleeding time and thrombocytopenia (platelet count < or = 150 x 10^9) or between bleeding time and any measured TEG variable. An MA of 53 mm, which is the lower limit for normal pregnancy, correlated with a 54 x 10^9 platelet count[143].

In screening for hypercoagulability, standard laboratory tests that may indicate hypercoagulability include a high platelet count, high levels of coagulation factors VII, VIII, IX, X, and XI or fibrinogen, low levels of protein S, protein C, antithrombin, and the presence of a lupus anticoagulant, while the TEG indicators of hypercoagulability are a short *r* time, a large MA or an alpha angle that is large and rapidly increasing. This has been noted with increased frequency in a variety of patient groups who are considered to be at increased risk of thrombosis, with the predictive value of the TEG being higher than other systems.

Of importance to this thesis is the role of the TEG in evaluating enhanced coagulation.Preliminary data suggests that the TEG may be of value in screening patients for a suspected prothombotic state[183]. The TEG has been established as a sensitive test for the global assessment of haemostatic function in several clinical settings, specifically for monitoring hypercoagulability. Of importance is that this hypercoagulability may not be reflected by standard coagulation monitoring and may be predominantly influenced by increased platelet reactivity[105], which needs to be measured as an dynamic test and not as an end-point.

CHAPTER FOUR
THE EFFECTS OF *IN VIVO* HAEMODILUTION WITH PENTASTARCH AND NORMAL SALINE ON COAGULATION

RATIONALE

The interest in the possibility of haemodilution enhancing the onset of coagulation first began during studies performed toward my MMed(Anaes) thesis[173]. The findings were confirmed in an *in vitro* study that formed the basis of that thesis and was subsequently published[178]. As discussed in Chapter 1, this phenomenon was first described as early as the 1950s when Tocantins *et al*[206] and Monkhouse[123] reported that moderate haemodilution with crystalloids could induce a hypercoagulable state. This was later borne out by Janvrin *et al*[71] in a clinical study. This chapter sets out to investigate whether these findings can be reproduced by *ex vivo* testing in volunteers receiving *in vivo* haemodilution without any surgical stress.

Many of the studies which comment on the effects of colloids on coagulation have used a crystalloid control[100,132,95,130] or have not made allowance for any crystalloid the patient was given in addition to the colloid, for example in cardiopulmonary bypass pump prime[126,101]. Popov-Cenic *et al*[152] refer to the effect of surgery on enhancing coagulation and the modification thereof by hydroxyethyl starch. Vinnazzer and Bergmann[219] in 1975, in a double-blind study, compared standard tests of coagulation as well as thromboelastography pre- and postoperatively in two groups, viz. one treated with hydroxyethyl starch and the other with isotonic saline peri-operatively. Their findings show a postoperative hypercoagulable state in the control

(saline) group, and an insignificant change in the test (hydroxyethyl starch) group. As their study was performed postoperatively, it does not show whether these changes of coagulability were already present after infusion of the intravenous fluid, before any surgical stress response occurred which might further have influenced coagulation parameters.

Thromboelastography, as discussed earlier (Chap 2), is essentially a graphic method of displaying the stages in the formation of a whole blood clot (thrombosis) and provides a visual pattern of functional clotting and clot breakdown status. An ACT only measures the end-point of coagulation, whereas the TEG provides a continuous measurement of the coagulation process. Of relevance to this and later studies is the fact that the TEG is an established monitor for detecting enhancement of coagulation (Chapter 3). If the correct methods of blood sampling (two-syringe technique), as described in Chapter 3, are used, the results are reproducible in samples of blood taken from an indwelling cannula or from another puncture site.

This chapter is therefore a study in which commonly used tests of coagulation as well as the thrombelastograph (TEG) were employed to determine the effect that *in vivo* haemodilution with commonly used intravenous fluids might have on coagulation. Whilst the dilution was performed *in vivo*, the coagulation studies were performed *ex vivo*, as is the case for all measures of coagulation, except for the bleeding time.

METHODS

This investigation was performed to measure the effects of the rapid intra-venous infusion of intra-vascular volume on the coagulability of whole blood. This was accomplished using either 1000 cc of crystalloid (0.9% saline) or

colloid (200/0.5 pentastarch, HAES-Steril®) in fit, healthy, volunteers with a range of body weight between 55 kg and 85 kg (expected dilution of ~20%).

Using the standard technique, blood was taken for haematological tests - haematocrit (HCT), platelet count (plts), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (fib), antithrombin III (ATIII), platelet aggregometry and thromboelastography (TEG). The subjects were then randomised to receive either crystalloid 1000 ml, or colloid 1000 ml, via the cannula. This was infused over a period of 30 minutes. Provision was made to stop the infusion immediately and exclude any subject who experienced any ill effects. At the end of the infusion, blood was again sampled through the cannula, using the standard technique and the coagulation studies and TEG were repeated.

STATISTICAL ANALYSIS:

Data were collected using Microsoft Excel 2000 and statistical analysis performed using Statistica Version 6 statistical package using a two-way ANOVA for repeated measures with the group and pre-and post-fluid as the independent variables (StatSoft, Inc, Tulsa, OK, USA. Version 6, 2001). The Fisher least significant difference (LSD) post-hoc test was used to identify individual within and between group differences. The changes from the pre-dilutional control found following fluid infusion were calculated as a difference from pre-infusion values.

RESULTS

The infusion of the crystalloid produced the anticipated reduction of HCT of approximately 10%, and the colloid infusion reduced HCT by approximately

19% (Table 4.1.). Since the red cells were not taken out of the circulation, or damaged, this drop of HCT could be attributed to an *increase* in plasma volume of ~ twice the decrease in HCT. Similar reductions in platelet counts were observed in both groups.

Statistically different increases in PT and aPTT were seen in both groups, but this was expected following the plasma dilution as they measure coagulation cascade end points in plasma samples, which have coagulation initiated through the addition of a coagulation stimulant. However, they do not measure the balance between pro- and anti-coagulation factors within the coagulation framework.

Fibrinogen and ATIII concentrations were decreased in both groups. However, when comparing their decrease to plasma dilution, the results were interesting. The decrease of AT III in the crystalloid group was greater than could be explained by plasma dilution alone, while the change in AT III in the colloid group was similar to the plasma dilution.

	Saline			HES		
	Baseline (N=20)	Post-dilution (N=20)	Change from control (%)	Baseline (N=20)	Post-dilution (N=19)	Change from control (%)
Hct(%)	43.3	39.0*	-4.1 (9.9)	42.5	34.5*†	-7.9 (18.8)
SD	4.7	3.9	1.8	3.2	3.3	2.1
Plts(10^9/L)	208.0	188.8	-19.3 (9.2)	215.9	171.7*	-44.3 (20.5)
SD	36.5	36.4	11.4	45.9	33.5	22.1
PT	13.2	13.9*	0.7 (5.3)	13.1	14.6*	1.5 (11.2)
SD	1.1	1.1	0.5	0.8	1.0	0.70
aPTT	33.9	34.7	0.7 (2.4)	33.2	36.6*	3.4 (10.2)
SD	2.7	2.9	1.4	2.9	3.6	2.1
Fib (g/L)	2.7	2.2*	-0.5 (19.2)	2.7	1.9*	-0.8 (29.1)
SD	0.8	0.6	0.3	0.7	0.5	0.3
ATIII(%)	116.1	80.1*	-37.7 (32.5)	108.8	62.8*	-45.9 (42.1)
SD	33.7	15.9	43.4	30.2	16.0	36.2

PT - prothrombin time (sec), aPTT - activated partial thromboplastin time (sec), ATIII - antithrombin III as percentage of laboratory normalised value.

* $P < 0.001$ for within group differences from baseline.

† $P < 0.05$ for between group differences .

Table 4.1. Haematological data before and after haemodilution with saline or hydroxyethyl starch (HES) (mean (SD)).

Therefore, the percentage decrease in ATIII after haemodilution was different between the groups with a three-fold decrease in the crystalloid group, which could not be explained on the basis of *plasma* dilution alone. The change in fibrinogen did not appear different in the two groups compared to control. (Table 4.1.)

	Saline			HES		
	Baseline (N=20)	Post-dilution (N=20)	Change from control (%)	Baseline (N=20)	Post-dilution (N=19)	Change from control (%)
Bl. Time (min)	3.4	3.5	0.1 (3.0)	3.3	4.4*	1.1 (32.3)
SD	0.9	0.9	0.7	0.8	1.0	0.5
ADP 100μmol	76.6	79.8†	3.3 (4.2)	72.2	72.3	0.1 (0.1)
SD	11.1	9.8	6.1	7.4	10.8	7.1
ADP 10μmol	69.3	72.0†	2.5 (3.9)	63.4	62.9	0.6 (-0.8)
SD	12.8	13.5	8.0	9.9	14.1	7.8
ADP 5μmol	58.5	63.0	4.5 (7.7)	54.0	52.0	2.0 (-3.7)
SD	17.9	18.5	11.2	13.9	17.3	9.4
Adrenaline	53.7	56.0	2.2 (4.3)	58.2	38.4*	19.8 (-34.0)
SD	27.8	31.4	23.9	25.0	29.7	23.0
Ristocetin	83.4	88.2†	4.7 (5.8)	80.6	82.0	1.5 (1.7)
SD	8.9	9.9	9.9	7.2	10.0	8.0

* $P < 0.001$ for within group differences from baseline

† $P < 0.05$ for between group differences

Table 4.2. Measures of platelet aggregation (% of light transmission) before and after haemodilution with saline or hydroxyethyl starch (HES) (mean (SD)).

Platelet aggregation as measured by adenosine diphosphate (ADP) 100 μm, ADP 10 μm and ristocetin stimulation was marginally raised following dilution with crystalloid, whereas colloid dilution resulted in either no change or a small reduction in platelet aggregation. However, platelet aggregation following crystalloid dilution was significantly greater than that following colloid dilution using these three stimulants. Adrenaline-stimulated platelet aggregability was significantly decreased in the colloid group (Table 4.2.).

The TEG results showed significant reduction in *r*- time and k-time, and increased alpha-angle and MA in the crystalloid group. The only change in the colloid group was a significant reduction in MA (Table 4.3.).

	Saline			HES		
	Baseline (N=20)	Post-dilution (N=20)	Change from control (%)	Baseline (N=19)	Post-dilution (N=18)	Change from control (%)
***r*-time (min.)**	13.4	10.2*	-3.2 (23.9)	13.1	11.7	-1.4 (10.7)
SD	3.5	3.0	1.2	3.6	3.6	4.7
***k*-time (min.)**	7.1	5.3*	-1.8 (25.4)	7.1	6.6†	-0.5 (7.0)
SD	1.8	1.6	1.2	2.0	2.2	2.4
alpha-angle(°)	37.0	45.7*	8.7 (23.5)	37.8†	38.1†	0.3 (0.8)
SD	7.4	9.4	5.5	7.6	9.4	8.6
MA (mm)	50.0	53.2	3.2 (6.4)	50.8	45.0*	-5.8 (11.4)
SD	5.4	5.4	2.4	8.6	6.6	6.5

*$P < 0.01$ for within group differences from baseline.

† $p < 0.05$ for between group differences after dilution.

Table 4.3. Thrombelastograph data before and after haemodilution with saline or hydroxyethyl starch (HES).

DISCUSSION

These results demonstrate a predictable drop (10% and 19%) in HCT and platelet count due to haemodilution in the two groups, the greater drop being evident in the colloid group, where presumably, less immediate extravasation of the fluid occurs. Standard tests of coagulation such as PT, aPTT and bleeding time are generally more sensitive in

hypocoagulable states than they are in hypercoagulable states, and as such were not of great help in this study as anticipated (see Chapter 3).

However, the level of ATIII dropped significantly in both groups. In the crystalloid group this was 3 times greater than the drop in HCT (33% vs. 10%), which is greater than the plasma dilution, whereas in the colloid group, the change was approximately twice that of the drop in HCT (42% vs. 19%) which is in keeping with the plasma dilution.

This drop of ATIII in the crystalloid group cannot be explained by dilutional factors alone, especially when considered in conjunction with the TEG results, which demonstrate a significantly shortened r and κ time in the saline group. A non-dilutional decrease in ATIII is usually due to its binding to activated thrombin, thus the 3-fold reducing of ATIII levels, greater than can be explained by pure haemodilution, is most likely related to binding with thrombin. These findings are in keeping with the assumption of intra-venous infusion of crystalloid, but not colloid, enhancing the formation of thrombin via the coagulation pathway as measured by the TEG.

Monkhouse[123] originally postulated an imbalance between the formation of thrombin and the activity of ATIII as being responsible for the enhanced clotting demonstrated after haemodilution. The findings of this study suggest that haemodilution *per se* induces the formation of thrombin, which is a powerful mechanism of positive feedback (Chap 2), as well as one of the most potent platelet activators known. Even minor quantities of thrombin formed early in haemostasis bind to platelet receptors to elicit aggregation[229]. Platelets play a role in accelerating the intrinsic pathway reactions[49], with the interaction between platelet surface membrane and

various plasma proteins playing an essential role in several reactions, thus enhancing enzymatic activity and resulting in increased complex formation. This, in turn, ensures rapid generation of factor Xa and thrombin[229]. Therefore, coagulation is accelerated by virtue of binding of clotting factors to the platelet plasma membranes, resulting in the assembly of multi-enzyme complexes with great catalytic efficiency. With haemodilution, a decrease in heparin-dependent *serpin* activity coupled with unchanged VIII:C reactivity results in hypercoagulability detected by thromboelastography[114]. However, HES molecules may coat the platelets, leading to limited access of ligands to their binding sites on the platelet surface, thus limiting the access of coagulation factors to the phospholipid surface of the platelet, and thereby limiting platelet reactivity[47] (see chapter 2). The anti-platelet effects of the starch have previously been described[199].

These TEG results show the shortened r and k times, and an increased α-angle, as well as an increased MA in the crystalloid group, which are in keeping with the enhanced coagulability described in previous *in vitro* work. In the starch group the TEG variables are not significantly changed, other than a smaller MA. This is in keeping with decreased platelet aggregation.

These results of *in vivo* dilution confirm the previous findings following *in vitro* haemodilution, that haemodilution with crystalloid solutions enhances the coagulability of blood, as well as the *in vitro* suggestion that colloid solutions may have a lesser effect, possably mediated through an anti-platelet action. The post-dilutional results fell outside the 95% confidence interval (Chapter

3). It is of interest that a consumption of ATIII occurs in the crystalloid group. Further investigation is needed to clarify possible mechanisms for this finding.

This study suggests that the infusion of intravenous crystalloid (saline), but not colloid (starch) could enhance the formation of thrombin via the activation of the coagulation pathway and/or platelets. If clinical implications of this finding could be deduced, they could be potentially important. If crystalloid haemodilution can enhance the onset of coagulation, this may increase the risk factors of thrombogenic complications.

In order to determine whether this effect is due to the type of crystalloid used, or to the haemodilution itself, this study must be repeated using warmed, isotonic, solutions at a pH of 7.4. This will establish which attributes of the diluent provide the cause for the enhancement of coagulation following haemodilution. In addition it is important to test for coagulation at varying degrees of dilution in order to determine the range of haemodilution that enhances coagulation, before the dilutional effect becomes too great to sustain hypercoagulability. This will be described in the following chapters.

CHAPTER FIVE

THE EFFECT OF 20% *IN VITRO* HAEMODILUTION WITH WARMED BUFFERED SALT SOLUTION AND CEREBROSPINAL FLUID ON COAGULATION

RATIONALE

As discussed in the previous chapter, haemodilution will decrease the concentration of clotting factors in the blood, and intuitively, should induce some degree of impairment of coagulation, but the opposite has been demonstrated, with the cause thereof needing to be identified. As previously shown, both *in vitro*[178] and *in vivo*[176] haemodilution with 0.9% saline solution causes an increase in the coagulability of whole blood as measured by the thrombelastograph (TEG) (Chapter 3). Part of the enhanced coagulability could have been due to the infusion of a cold, non-physiological, acidic solution such as 0.9% saline. It is worth noting that the colloid is also suspended in saline. On the basis of this, this chapter focuses on an *in vitro* study in which haemodilution was performed with a physiologically balanced salt solution containing normal constituents of the blood with the exception of calcium and buffered to a normal pH (Plasmalyte B) and warmed to 37°C, or with a protein-poor body fluid, cerebrospinal fluid (CSF).

This chapter investigates, using the TEG, whether *in vitro* haemodilution with warmed physiological crystalloid or CSF will demonstrate similar enhancement of coagulation.

METHODS

The investigation was conducted in two parts. In the first part, blood samples were obtained from twenty healthy, conscious volunteers using the standard technique. In a 10 ml syringe 8ml of blood were taken and separated into 2 aliquots of 4 ml each in polypropylene plastic tubes. A physiological balanced salt solution containing normal constituents of the blood with the exception of calcium and buffered to a normal pH (Plasmalyte B) was kept warmed at 37°C in a fluid warming cupboard, and 1 ml was added to one sample while the other sample was left undiluted and served as a control. Specimen tubes containing each sample were inverted several times to ensure thorough and similar mixing of the blood in each tube. The control samples were inverted in a similar fashion to those of the diluted group to ensure that, as far as possible, each sample was treated similarly. Specimens from each sample tube (0.36 ml) were then pipetted into a TEG cuvette and simultaneous traces recorded of the normal blood sample and of the diluted sample. The TEG trace was recorded for one hour and the reaction (r) and coagulation (K) times, alpha-angle (α), and maximum amplitude (MA) were measured for each sample.

In the second limb of the study the method described above was repeated, except that the study consisted of blood taken from parturient patients undergoing spinal anaesthesia for caesarean section. At the time of the dural puncture, 1 ml of CSF was taken from the spinal needle, and this served as diluent in the study sample, while an undiluted blood specimen served as the control. Macroscopic appearance of a bloody tap served as an exclusion criterion.

STATISTICAL ANALYSIS:

Data from each diluent group was compared with that obtained from the matched controls in each limb of the experiment and any differences noted were analysed for statistical significance using a paired Student's t-test.

RESULTS

In the first limb of the experiment, there were statistically significant differences between the warmed Plasmalyte-B-treated samples and their untreated controls in all the parameters measured by the TEG, except the MA (Table 5.1.).

	Control	Plas B	% change
r time(min)	13.4	11.0 *	-17.9
SD	2.7	2.3	
κ time(min)	5.9	3.9 §	-33.3
SD	1.2	1.2	
α-angle	43.2	52.8 §	22.2
SD	7.4	7.6	
MA(mm)	56.5	56.8	0.8
SD	4.1	4.6	

* $P < 0.01$

§ $P < 0.001$

Table 5.1. TEG values in control and warmed Plasmalyte-B-diluted samples.

In the second part of the study, there were statistically significant differences between the CSF-treated samples and the untreated controls in all the parameters measured by the thrombelastograph (Table 5.2).

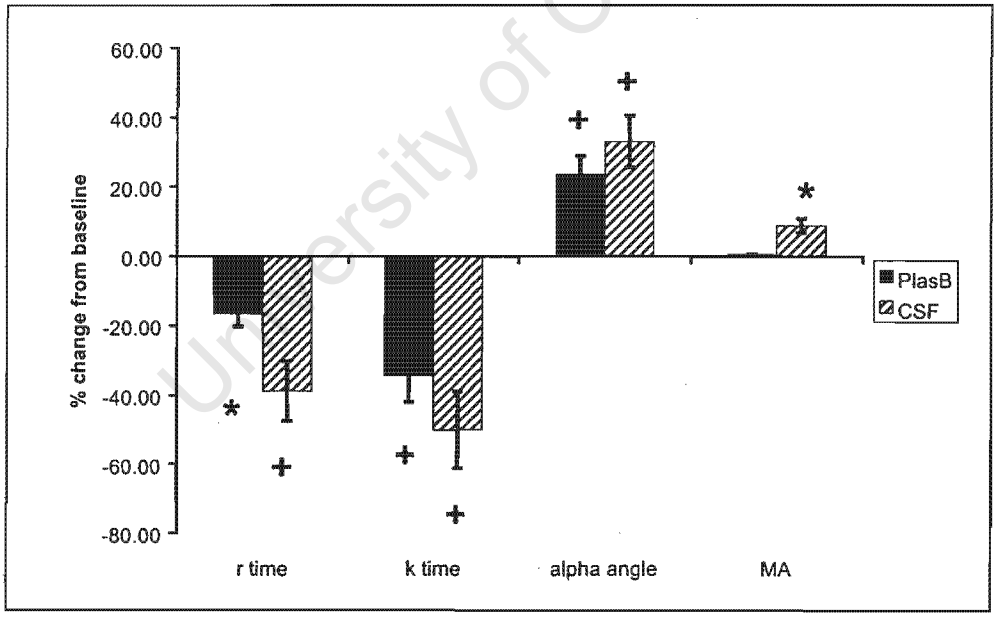
The difference compared with the control is best shown as a percentage change from baseline, and these are depicted in Figure 5.1.

	Control	CSF	% change
r time (min) SD	10.5 1.8	6.3 §) 2.2	-39.6
κ time (min) SD	4.0 1.2	1.9 § 0.5	-50
α-angle (°) SD	54.5 8.5	71.0 § 4.8	30.3
MA (mm) SD	65.7 7.5	71.0 * 4.4	8.1

* $P < 0.01$

§ $P < 0.001$

Table 5.2. TEG values in control and CSF-diluted samples.(r-and k-time are in minutes, α-angle in degrees, MA in mm)



* $P < 0.05$ for differences from baseline,

+ $P < 0.05$ for differences from baseline.

Fig. 5.1. Differences from undiluted control samples following *in vitro* haemodilution with either Plasmalyte B or CSF (mean ± SEM).

DISCUSSION

These findings confirm that the results obtained previously with *in vitro*[178] and *in vivo* (Chapter 4) saline haemodilution were reproducible *in vitro* when the diluent solution is a buffered salt solution maintained at normal body temperatures throughout the experiment. This is an important finding, as the previous findings could be questioned as to whether the cause of the enhanced onset of coagulation was related to the physical attributes of the diluent. This study was performed investigating a 20% haemodilution, as this is in keeping with the previous percentage of dilution used to demonstrate the effect. Of note is that, as the study occurred in controlled surroundings, there were no other factors playing a role in the demonstrated effect of enhanced clotting. These findings are similar to those by Egli *et al*[29], who artificially corrected for calcium deficits and hydrogen ion changes in their samples and, again, were able to demonstrate an increase in coagulability at haemodilution of 30%.

This investigation suggests that the electrolyte and acid base balance of the diluent fluid plays little, if any, role on the observation that crystalloid haemodilution produces hypercoagulability. However, direct comparisons between 0.9% saline and Plasmalyte B were not made, so it is difficult to state with absolute certainty that the magnitude of the effect of the two solutions is similar. However, it demonstrates clearly that the trigger for this process cannot be any biochemical abnormalities of the crystalloid diluent fluid, but rather some aspect related to haemodilution itself.

The haemodilution with CSF produced an interesting result. Pregnant patients are expected to demonstrate hypercoagulability, as this is an established

physiological change during pregnancy. This was recently reconfirmed by Sharma *et al*[190]. It is of interest that the pre-dilution TEG values obtained in the pregnant patients were almost identical to those found in normal subjects after haemodilution with crystalloid. Since pregnant patients have an increased risk of thrombotic events, this suggests that the magnitude of these coagulation changes post dilution in the non-pregnant patients may be sufficient to exert clinically relevant changes in coagulation.

The dramatic increase in coagulability produced by the addition of CSF, however, cannot be explained simply haemodilutional terms and this suggests that the original hypothesis, that CSF is a physiologically neutral body fluid without coagulation effects, is incorrect. It must be assumed, on the basis of this study, that CSF markedly enhances the coagulation of whole blood, although the mechanism by which it might do this is currently not fully explained. This was described by Graeber and Stuart[54] in 1978 in a study which showed that CSF has procoagulant activity. The effect that CSF has on initiation of coagulation was investigated by Nagda[129] who also described a method of isolating a procoagulant substance from human CSF[128].

In conclusion, this chapter has addressed the constituents of the diluent as a possible cause of the enhancement of coagulability of whole blood following haemodilution and has proven this effect to be unrelated to the constituents, pH, or temperature of the dilution fluid. The next step is to identify the range of dilution over which the effect can be shown to occur.

CHAPTER SIX
A DOSE-RESPONSE STUDY OF THE EFFECTS OF HAEMODILUTION ON COAGULATION

RATIONALE

It has become evident from *in vitro*[178,179] and *in vivo*[176] dilution of whole blood at a dilution of 20%, that haemodilution causes enhanced coagulation as measured by the thrombelastograph (TEG)(Chapters 4 & 5). Other authors have confirmed this[50,70,138]. The observations suggest that it is the haemodilution *per se,* rather than the nature of the diluents used that enhances the coagulability of whole blood. While it has been previously described that there is a serial effect of haemodilution with crystalloid on coagulation, which is greater than the enhanced coagulation found with colloids, with saline 0.9% increasing the alpha angle significantly up to 50% haemodilution[205], no clear dose-response curve for dilution has been defined. In order to define the effect, it is important to identify the range of dilution over which this occurs. This has been done by means of an *in vitro* study measuring the effect of crystalloid haemodilution on coagulation at an increasing percentage of dilution.

METHODS

This study sets out to determine the effect of different percentages of dilution on coagulation. Blood samples were obtained from twenty healthy, conscious volunteers using the standard technique.

As only 4 TEG channels were available at any time, the study was performed in two steps. Repeated blood samples were taken using a venepuncture site

that was more distal than the previous one, or on the other arm (to avoid contamination). Previous work in this laboratory has shown that reliable coagulation tests can be performed on blood obtained by these means.

Each sample was split into 4 aliquots, of which three were then diluted in random order by pre-calculated amounts of saline to achieve a calculated dilution of 10%, 20%, 30%, 40%, 50% or 60%. Randomisation was achieved by drawing labelled slips of paper from an envelope. These diluted samples were placed randomly in three of the four TEG channels, with the fourth sample serving as control. A repeat of this using a second blood sample some time later, completed the normal saline series of increasing dilutions.

All samples were placed in the TEG curvets within 4 minutes of sampling. The TEG reaction (*r*) time, coagulation (*K*) time, alpha angles and maximum amplitude (MA) were compared with the control to assess the effect of dilution on coagulation at different concentrations.

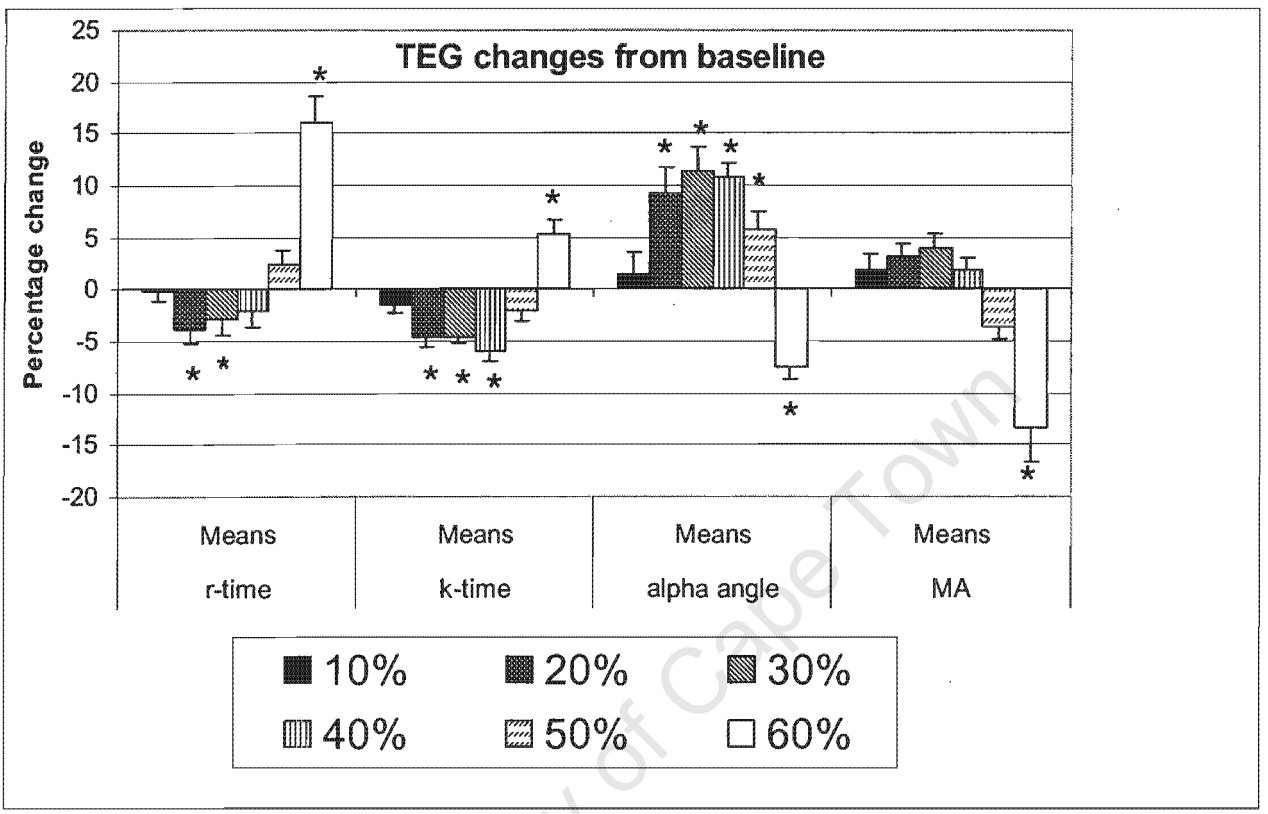
STATISTICAL ANALYSIS:

Analysis of variance (ANOVA) for repeated measures was used to analyse the various coagulation parameters with the Fisher LSD post-hoc test to define significantly different samples.

RESULTS

There was shortening of the *r* time (implying more rapid onset of coagulation) in all the samples up to a dilution of 40%, after which the *r* time was prolonged at 50% and 60%. The change was statistically significant at 20%, 30% and 60% haemodilution. There was shortening of the *K* time (speed of polymerisation of fibrin once coagulation has started) in all percentages up to

50%, after which the coagulation was decreased at 60%. Of these differences, 20-40% and 60% were statistically significant.



* statistically significant– $p<0.05$

Fig. 6.1. Percentage Changes from baseline at increasing percentage of dilution as measured by the TEG

The alpha-angle was increased significantly in samples 20-50%, and decreased significantly in the 60% dilution. Clot strength (MA) was increased but not significantly in dilutions of 10-40%, and decreased after 50% dilution. Dilution at 60% significantly decreased clot strength (Figure 6.1.).

DISCUSSION

Enhanced coagulation, following haemodilution has previously been reported by several authors[50,70,138,176,178,179]. However, while it has been shown that moderate haemodilution enhances coagulation, the effect of increasing degrees of haemodilution on coagulation is not clearly quantified. The previous chapters have focussed on a fixed 20% dilution to investigate the

effect of enhanced coagulation following dilution. By evaluating the effect of increasing levels of crystalloid dilution on coagulation the dilutional effect is now more clearly documented, with a trend towards enhanced coagulation shown at 10% dilution and a statistically significant change ($P < 0.05$) occurring at 20% to 30% dilution in both the onset of coagulation, and the rate of fibrin polymerisation once clotting was initiated. The latter is still significantly increased at 40% dilution (The alpha angle still significant at 50%). Haemodilution of 60% impaired all aspects of coagulation including the speed of onset of coagulation, the rate of clot propagation and clot strength. These findings are in keeping with other authors[34,163], who have described a biphasic response of coagulation to dilution, with an initial increase in coagulability, followed by a decrease at extreme dilutions.

In an *in vitro* study such as this, other aspects of dilution must be considered, notably the effects on calcium. Ionised calcium (Ca^{2+}) was not measured in this study, but it can reasonably be assumed that Ca^{2+} decreased in proportion to the degree of dilution, as did the concentration of clotting factors. It may be due to these reductions that coagulation was impaired at extreme dilutions. Subsequent studies in this laboratory have shown that 60% dilution is associated with a reduction in ionised calcium to <0.5 mmol/l, unless the diluent fluid contains calcium. The critical level below which ionised calcium affects coagulation is 0.56 mmol/l (James & Roche, ASA 2001).

Therefore rapid, large-volume haemodilution *per se* initially results in the enhanced onset of coagulation, the greatest effect occurring at 20-30%. This effect is already trending towards significance at lower dilutions and is

maintained until about 50% dilution, at which time the coagulation effects are reduced, with hypocoagulability occurring at 60% dilution.

In Chapter 5, it was shown that the mechanism is not based on the type of crystalloid used. It is now apparent that haemodilution *per se* enhances coagulation.

Based on the discussion in Chapter 4, even minor quantities of thrombin formed early in haemostasis bind to platelet receptors to elicit platelet aggregation[229], while platelets play a role in accelerating the intrinsic pathway reactions[49], with the interaction between platelet surface membrane and various plasma proteins leading to rapid generation of factor Xa and thrombin[229]. The question then arises whether haemodilution has a primary effect on platelet clumping and so leading to thrombin generation, or whether it causes increased thrombin formation and so activating platelets. The question must be: "What comes first after haemodilution, platelet activation, or thrombin generation?"

This will be investigated in two steps. The first step is to take away normal platelet aggregability (but not activation by thrombin), through the administration of aspirin, to investigate whether this would prevent the enhanced onset of coagulation following haemodilution. The second step is to investigate whether keeping antithrombin III levels constant throughout dilution, to prevent an increase in thrombin formation from baseline values, would prevent the enhancement of coagulation.

CHAPTER SEVEN

THE PROCOAGULANT EFFECT OF *IN VITRO* HAEMODILUTION IS NOT INHIBITED BY ASPIRIN

RATIONALE

As demonstrated in Chapter Four, haemodilution with 0.9% saline solution causes enhanced onset of coagulation of whole blood, associated with a significant decrease in antithrombin III (ATIII) concentrations. Chapter Five demonstrated that the pH, temperature and components of the crystalloid where not the cause for this to occur. There is a range of dilutions over which this effect occurs as demonstrated in Chapter six. These observations suggest that crystalloid haemodilution enhances the onset of coagulation *per se*, possibly either through an increase of the aggregability of platelets or the increased formation of thrombin.

One possibility is that addition of crystalloid solutions to whole blood activates platelets. The interaction between the platelet surface membrane and various plasma proteins plays an essential role in several reactions, which are necessary for normal haemostasis, including interactions with factors Xa and Va to form the prothrombinase complex, as well as promoting the factor IXa-VIIIa complex required for factor X activation. These interactions dramatically enhance both thrombin and factor Xa formation[230].

This chapter investigates whether platelets play a primary role. This investigation into the role of platelets, using the TEG, was conducted in normal volunteers to investigate whether this effect of haemodilution on coagulation could be reproduced after the administration of an anti-platelet

agent (Aspirin). Platelet adhesion, activation, and aggregation are key processes in the pathogenesis of coagulation. Aspirin use has been associated with reduced platelet aggregation response to adrenaline (P=0.01)[82] and aspirin mono-therapy started within 48 hours of stroke onset may result in a modest clinical improvement[9]. It has been available as anti-platelet therapy for over 100 years, and it improves outcome in a wide range of clinical situations. However, aspirin only inhibits platelet activation mediated by thromboxane A_2[102], and thromboxane-stimulated release (and aggregation) can be by-passed, since activation of platelets by thrombin or higher concentrations of collagen is not inhibited by aspirin[84]. Aspirin has previously been shown to have no effect of itself on the TEG. Thus any observed effect of aspirin on previously established haemodilution-enhanced coagulation would demonstrate an involvement of normal platelet aggregability playing a role in this effect. No change in the enhanced onset of coagulation after haemodilution following the administration of aspirin would suggest that the effect might be *primarily* mediated through other forms of coagulation activation such as an increase in activated thrombin levels.

METHOD

Blood samples were obtained using the standard technique, from twenty healthy, conscious volunteers, none of whom had taken aspirin or any non-steroidal analgesics during the previous 7 days. In a 10cc syringe, 8 ml of blood were taken and separated into 2 aliquots of 4 ml each in polypropylene plastic tubes. Normal saline, 1 ml, was added to one sample while the other sample was left undiluted and served as a control. Specimen tubes containing each sample were inverted several times to ensure thorough and

similar mixing of the blood in each tube. The control samples were inverted in a similar fashion to those of the diluted group to ensure that, as far as possible, each sample was treated in a similar fashion. Specimens from each sample tube (0.36 ml) were then added to the TEG and simultaneous traces recorded of the normal blood sample and of the diluted sample. The TEG trace was recorded for 1 hour and the reaction (r) time, coagulation (K) time, alpha angle (α), and maximum amplitude (MA) were measured for each sample. Following this test, all volunteers then took 375mg of soluble aspirin daily for 3 days, after which the procedure described above was repeated.

Data obtained after aspirin had been taken were compared with those obtained from the same volunteer before the administration of aspirin. All volunteers confirmed that they had taken the aspirin as directed.

A small pilot study (6 subjects) was conducted, in which the experimental protocol was repeated exactly, except for the administration of aspirin, to rule out the possibility that the mere act of prior venesection might have altered the coagulability of blood significantly at the time of taking the second sample. No effect was observed.

STATISTICAL ANALYSIS:

Statistical analysis was performed using multifactorial ANOVA with aspirin treatment (yes or no) and dilution (yes or no) as the predictor variables. Post hoc analysis was performed using the LSD test.

RESULTS

There were no differences in the pre-dilutional TEG values before and after the administration of aspirin, neither was there any difference in the degree of

hypercoagulability induced by haemodilution after the administration of aspirin for 3 days. The MA was unchanged from pre-dilutional values after haemodilution in the two groups. The results are tabulated in Table 7.1.

	Pre-Aspirin (SD)		Post-Aspirin (SD)		Pre vs. Post (p)
No Dilution	*r*	12.5 (2.3)	*r*	12.5 (4.5)	1
	K	6.6 (1.5)	*K*	6.3 (2.4)	0.54
	α	39.5 (6.6)	α	42.9 (14.0)	0.28
	MA	52.4 (3.9)	MA	54.1 (6.2)	0.13
Post-Dilution	*r*	10.1 (1.6)	*r*	10.4 (3.2)	0.67
	K	4.4 (1.0)	*K*	4.0 (1.2)	0.36
	α	49.6 (7.9)	α	52.2 (8.7)	0.32
	MA	54.4 (4.8)	MA	54.3 (6.2)	0.95
Post-dilution change	*r*	2.4 (0.8)†	*r*	2.1 (1.4)*	0.62
	K	2.2 (0.55)†	*K*	2.3 (0.9)†	1
	α	-10.1 (7.4)†	α	-9.4 (11.1)†	0.8
	MA	-2.0 (2.6)	MA	-0.1 (5.4)	0.18

* $P < 0.01$

† $P < 0.001$

Table 7.1. TEG values before and after saline haemodilution, and before and after administration of 375 mg of soluble aspirin daily for 3 days, as well as changes of TEG parameters from undiluted values following haemodilution in both groups. All values are mean (SD) with r and k values in minutes, MA in millimetres and α-angles in degrees)

DISCUSSION

After exposure to multiple agonists, platelets may undergo a multitude of morphological and biochemical changes resulting in adherence to areas of damaged endothelium[103]. This primary haemostasis results in the formation of a platelet plug without the generation of fibrin. Aspirin interferes with this process through deactivation of platelet cyclo-oxygenase. This action is

almost instantaneous and irreversible at doses in excess of 100 mg/d[169]. Once aspirin has been taken for 3 days, thromboxane-mediated platelet aggregation is completely inhibited.

Clotting occurs when the platelet plug is then stabilised as circulating inactive forms of haemostatic proteins are activated on the platelet surface, thus generating thrombin at the site of injury. Thrombin, a serine protease, cleaves soluble fibrinogen to form insoluble fibrin strands that cross-link and form the basis of the final stable clot. In addition, the activated thrombin is integrally involved in positive feedback, via the intrinsic pathway, in platelet aggregation, as well as in the stimulation of the fibrinolytic pathway. (Chap 2)

Platelets play a role in accelerating the intrinsic pathway reactions[49], with coagulation being accelerated by virtue of binding of clotting factors to the platelet plasma membranes, resulting in the assembly of multi-enzyme complexes with great catalytic efficiency. This, in turn, ensures rapid generation of factor Xa and thrombin[229]. Therefore, coagulation is accelerated by virtue of binding of clotting factors to the plasma membranes of a platelet plug.

This study has confirmed that the enhanced coagulation, demonstrated with *in vitro* and *in vivo* haemodilution with saline, was reproducible. Furthermore, it has shown that this effect is not attenuated when an anti-platelet agent, stopping primary platelet clumping, is administered. The presence of aspirin has no effect on the observation that crystalloid haemodilution produces enhanced onset of coagulation, and that this effect is not primarily mediated via platelet-endothelial interaction. It is known that thrombin activates platelets during TEG measurements, and unlike conventional coagulation tests (like

prothrombin time and partial thromboplastin time) that are based only on the time to the onset of clot formation, TEG allows acquisition of quantitative information beyond the gel point, allowing measurement of the absolute strength attained by clots[86], thus showing that this was not affected by aspirin.

Haemodilution would thus seem to enhance clot formation through a primary effect on the coagulation cascade. This may in turn lead to increased thrombin generation, resulting in increasing positive feedback loops that further stimulate coagulation via the intrinsic pathway, as well as stimulating platelet aggregation directly (Chap2). Platelet activation by thrombin, one of the most potent activators known, is suggested to be an exception to receptor-binding on the platelet surface membrane. As a result aspirin does not inhibit platelet activation through thrombin. Platelets stimulated by the combined action of thrombin and collagen promote a ten-fold increase in prothrombin activation and a twenty-fold increase in Factor X activation. Even minor quantities of thrombin formed early in haemostasis bind to platelet receptors to elicit platelet aggregation[229].

The trigger for the enhanced coagulability must be some aspect related to haemodilution itself, possibly resulting in an increase of activated thrombin, which then triggers an exponential increase in coagulation.

CHAPTER EIGHT
HAEMODILUTION-INDUCED ENHANCEMENT OF COAGULATION IS ATTENUATED *IN VITRO* BY MAINTAINING ANTITHROMBIN III AT PRE-DILUTION CONCENTRATIONS

RATIONALE

In the previous chapters it was demonstrated that crystalloids enhance clot formation, with the constituents, temperature and pH being ruled out as being the primary cause. In addition, while platelet activation is of great importance in clot formation, it is a secondary event. The most likely route would then be through a primary effect of dilution on the coagulation cascade. This, in turn, leads to increased thrombin generation. If the trigger for the enhanced coagulability is related to haemodilution itself, it may possibly result from an increase of activated thrombin, which triggers coagulation[176]. Even minor quantities of thrombin formed early in haemostasis bind to platelet receptors to elicit aggregation[229]. As proven in Chapter 7 the effect persists, even after taking aspirin to impair platelet clumping, which is in line with the thinking that thrombin may be the central modulator of coagulation.

Normal plasma contains idling levels of thrombin[140,141], which are continuously being formed, but have no effect. As a result inhibition must play a role in controlling a positive feedback of thrombin into the coagulation cascades[78], conferring threshold properties on the system[77]. A decrease of the anti-coagulants may possibly lower the threshold, thus allowing positive feedback to occur earlier. ATIII is the prime factor (~50%) regulating the activation of

factor X and thrombin[176]. It has second-order kinetics, which means that the rates of inhibition of its target enzymes are a function of its concentration[27,77,78]. Clinical evidence has shown that a decrease in concentration of ATIII, to ~70% of normal, is linked to an increased risk of thrombosis, particularly venous thrombosis[71]. Since, at this level there is still a mass excess of ATIII relative to thrombin, partial inhibitor deficiency is likely to be a kinetic rather than a capacity defect.

This *in vitro* study, using the TEG, was conducted to determine whether the enhancement of coagulation following haemodilution can be prevented, or at least moderated, when antithrombin III (ATIII) levels are kept at a pre-dilutional constant level throughout haemodilution.

METHOD

Blood samples were obtained using the standard technique, from twenty healthy, conscious volunteers. These were separated into 4 aliquots of 4 ml each in polypropylene plastic tubes. One sample was left undiluted and served as a control (C). Another was diluted by 20% with the addition of 1 ml of 0.9% saline to demonstrate the previously documented enhanced coagulation (S). The third sample was diluted with 1 ml of 0.9% saline to which 2 units of ATIII had been added to achieve a calculated level within the normal range (70-130%) of ATIII in the diluent (SA). The fourth remained undiluted, but had 2 units of ATIII (in a volume of 40 μL) added to it (CA). (ATIII produced by Immuno: Heat treated concentrate, containing 50u AT III and protein 20-50 mg / ml.) Specimen tubes, containing each of the four samples, were inverted several times to ensure thorough and similar mixing of the blood in each tube. As far as possible, each sample was treated in a

similar fashion. Specimens from each sample tube (0.36 ml) were then placed in the TEG cuvette within 4 minutes of venesection and simultaneous traces recorded of the four samples. The TEG trace was recorded for 1 hour and the reaction (r) time, coagulation (K) time, alpha-angle (α), and maximum amplitude (MA) were measured for each sample.

In order to validate the calculation of the ATIII content in the diluted samples, a further experiment was conducted. In 5 subjects, ATIII concentrations were compared between control and 20% saline dilution, and between control and 20% saline plus ATIII dilution.

STATISTICAL ANALYSIS:

Statistical analysis was performed using the STATISTICA package running under Windows 98 on an IBM-compatible computer. Data obtained from each group were compared using analysis of variance with the LSD test used for post-hoc identification of significantly different groups. Power analysis using anticipated differences and standard deviations obtained from previous work, indicated that sample size of 20 would have 90% power to detect 20% difference in TEG variables. Data were further analysed by calculating the variation of each group from its control value, and differences were again tested using analysis of variance (ANOVA) for repeated measures and Fisher's least significant difference (LSD) test.

RESULTS

The 20% dilution of whole blood with saline alone (group S) resulted in a significant enhancement of coagulation as measured by the TEG, in line with previous observations.

The dilution of whole blood by 20% with saline containing 2 units of ATIII (group SA) resulted in an *r* time that was not significantly different from that of the undiluted control (group C), or from that of the undiluted sample to which 2 units of ATIII had been added (group CA); however it was significantly different from group S (Table 8.1.).

	r time (min)	*K* time (min)	Alpha (°)	MA (mm)
Control (group C)	17.5	8.3	27.3	52.2
SD	4.1	2.4	8.5	6.1
Saline diluted (group S)	13.6 †*	5.1 ‡†	37.9 ‡†	55.4
SD	3.5	1.4	8.5	5.4
ATIII/saline (group SA)	17.3	6.7 §	32.8	53.0
SD	6.1	2.7	10.5	7.4
ATIII (group CA)	17.9	8.7	27.0	52.4
SD	5.4	2.8	9.4	6.1

No significant differences from control induced by addition of antithrombin III alone in any of the variables (group C vs. CA).

* $P < 0.05$ for group S compared to the other three groups

† $P < 0.01$ for differences between group S and group CA.

‡ $P < 0.01$ for difference from control sample

§ $P < 0.05$ difference between ATIII-containing diluted & undiluted samples (SA vs. CA)

Table 8.1. TEG variables in native whole blood (control)(C), diluted whole blood (saline diluted)(S), diluted whole blood plus antithrombin III (saline/ATIII)(SA) and whole blood plus antithrombin III (ATIII)(CA)(n = 20).

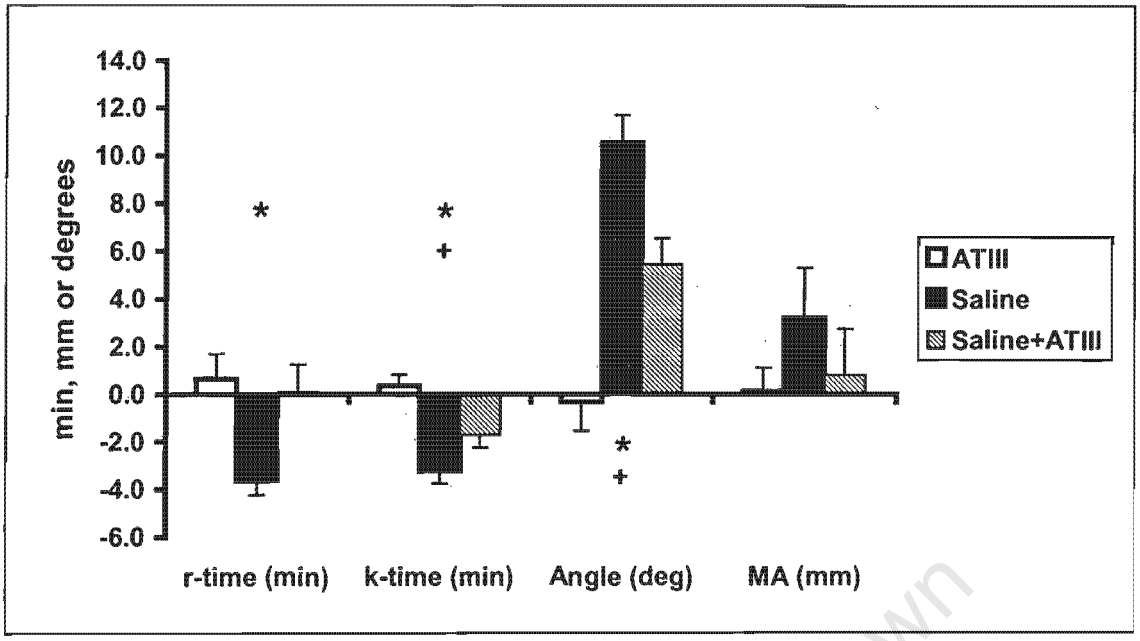
The *K* time obtained from group SA was significantly shorter (showing increased rate of clot formation) than that obtained from group CA. There was no statistical difference in *K* time between the saline plus ATIII group (group SA) compared with the pure saline group (group S) ($P = 0.051$).

Therefore, rate of clot formation in group SA, while still accelerated, was slightly slower than in group S, but this did not achieve statistical significance.

There was a significant increase in alpha-angle in group S as compared with groups C and CA (P <0.001). There was no increased alpha-angle in group SA compared with group C (P =0.06) and group CA (P = 0.052). There was no significant difference in alpha-angle between group S and group SA. The MA was not changed significantly in any of the groups.

The addition of 2 units of ATIII to an otherwise undiluted sample of whole blood had no effect on coagulation variables compared with those obtained from the untreated control sample, i.e. group CA was not different from group C in any respect.

All changes from control values are shown in Figure 8.1. Thus the change in r time produced by saline was significantly different from that produced by saline plus ATIII, as well as significantly different from both ATIII alone or zero. The changes in K time produced by both saline and saline plus ATIII were different from zero and from that produced by ATIII alone; they also approached significant differences from each other. Saline produced a change in alpha-angle that was significantly different from zero (C) and ATIII (CA), and also greater than saline plus ATIII (SA); group SA produced a change that was significantly different from that in group CA, but not different from zero.



* P < 0.05 for differences between the saline and ATIII and saline plus ATIII samples

\+ P < 0.05 for differences between the ATIII and saline plus ATIII samples

Fig. 8.1. Changes in TEG variables from the control (undiluted) sample by addition of ATIII or dilution with saline or dilution with saline plus ATIII (SEM)(n = 20).

The differences from control in ATIII concentrations after 20% saline dilution, as well as saline plus ATIII dilution, are reflected in Table 8.2. There was an expected drop in ATIII concentration after pure saline dilution. The ATIII levels were maintained at slightly above the control level in the group where dilution with saline plus ATIII was performed.

	Control	20% saline dilution	20% Saline+ATIII dilution
Mean	91	68.2*	100.1
SD	12.2	3.6	4.9

p< 0.01 for differences between saline diluted samples, control samples and samples diluted with saline + ATIII.

Table 8.2. ATIII activity (expressed as percentage of standardised normal activity) in the control samples, samples diluted 20% with plain saline and samples diluted 20% with saline plus ATIII (n = 5)

DISCUSSION

The entire clotting process, from its initiation through to fibrin production, is spatially restricted in the vasculature. *Thrombin generation* is the central biochemical reaction in normal haemostasis and thrombosis, with positive feedback playing a major role in the architecture of the system. Thrombin also activates platelet aggregation and stimulates the fibrinolytic pathway[58] (Chapter 4). The system has idling, non-zero levels of activated clotting enzymes, without generating a response. This implies that blood is protected against sub-threshold thrombin levels through a balance between intra-vascular coagulants and anti-coagulants.

Once thrombin has overcome the anticoagulant-induced threshold, it results in an exponential increase in platelet aggregation (Chap 2 & 7) and further thrombin formation, thus ensuring that a clot is formed.

The findings in this chapter can be summarised as follows: (a) 20% haemodilution with saline causes an enhancement of coagulation; (b) the addition of small amounts ATIII to normal blood has no effect on coagulation; (c) addition of ATIII to the diluent (saline), to maintain post-dilutional blood concentrations at pre-dilution levels initially prevents the enhancement of coagulation normally produced by dilution, but once begun, the rate of coagulation again increased.

While prothrombin is continuously being spontaneously activated, the actual quantity of *activated thrombin* makes up only a minute fraction of total prothrombin[140]. However, it is eliminated in a one to one, enzyme to inhibitor, reaction of thrombin with the AT III[166]. Therefore, even a doubling of the activated thrombin levels will not result in a significant decrease in the

absolute level of AT III. On the other hand, a moderate decrease in the AT III concentration, resulting from dilution, will lower the threshold and allow positive feedback of thrombin into the Intrinsic Pathway, since the second order kinetics of anti-thrombin III (ATIII) mean that rates of inhibition of its target enzymes are a function of its concentration[27,77,78].

In other words, haemodilution decreased the levels of ATIII[176] (Chap 4) and possibly other inhibitors of coagulation. As the thrombin : AT III ratio is altered the threshold for positive feedback is lowered. (There is a depletion of *anticoagulants* after dilution while spontaneous activation of coagulants remains unaffected). Unchecked positive feedback then occurs and accelerated formation of thrombin takes over once the threshold is breached.

This study shows that when the concentration of ATIII is maintained within the normal range throughout dilution, the effect of enhanced onset of coagulation can be attenuated, but not inhibited completely. It must, however, be taken into account that ATIII is only one of several inhibitors of positive feedback[75] acting on the coagulation cascade, accounting for between 40% and 50% of the effect. It is therefore possible to speculate that one could produce greater attenuation of this enhanced coagulation by keeping the levels of the other naturally occurring inhibitors of coagulation constant as well.

In summary, this study demonstrates that enhanced coagulation following haemodilution is likely to be on the basis of decreased concentrations of the coagulation inhibitors, while having no effect on the spontaneous formation of activated coagulants. The decrease in the concentration of the inhibitors reduces the threshold at which positive feedback occurs along the coagulation pathway, thus leading to the unopposed exponential propagation of thrombin.

The threshold properties of the system imply that if the level of *activated* thrombin is below threshold, there is no positive feedback, while when the level of anti-coagulants drops allowing the thrombin level to rise above the threshold; the positive feedback is at, or near, maximum.

CHAPTER NINE
THE EFFECTS OF A ONE UNIT BLOOD DONATION ON AUTO-HAEMODILUTION AND COAGULATION

RATIONALE

The previous chapters have attempted to define the mechanism of the enhanced onset of coagulation following both *in vitro*[175,178-180] and *in vivo*[29,176] haemodilution, using the thrombelastograph (TEG) and other tests. Specifically, the interactions between a significant thrombin formation and an increase in platelet aggregability, as well as the dilution induced decrease in anti-coagulant levels/concentration have been addressed.

In a recent clinical study Ng *et al* have demonstrated enhanced coagulation after haemodilution when compared to no dilution, in patients having been anaesthetised, but before the commencement of surgery[137].

As discussed in Chapter 8, with haemodilution, the naturally occurring *activated* circulating anticoagulants, like antithrombin III (ATIII), are diluted, while the haemodilution has no effect on the spontaneous activation of thrombin (even though the prothrombin is diluted equivalently) as activated thrombin makes up only a minute fraction of the total prothrombin[180]. This imbalance between coagulants and anti-coagulants results in enhanced onset of coagulation through positive thrombin feedback into the Intrinsic Pathway (Chapter 8) and activation of platelets (Chapter 7), which further enhance thrombin formation. This suggests a dynamic "activation" of the coagulation cascade after haemodilution.

This study aims to investigate the rate of autodilution, as well as the effect this has on coagulation, following the removal of approximately 10% (~500 ml) of whole blood from the circulation. This is an *in vivo* study measuring the rate of haemodilution with interstitial space fluid after a unit of blood is taken from volunteers and, using *ex vivo* tests, whether the pro-coagulant effect of haemodilution can be demonstrated.

It has previously been found that during blood donation some subjects experience stress reactions. However, the frequency of these reactions is higher in first-time donors than in experienced ones, with increased cortisol levels before the first donation, but not observed in experienced donors. Blood donation *per se* is not a stressful event and moderate stress, as suggested by increased cortisol levels and heart rate at the first donation, is secondary to emotional rather than to physical stimuli[7]. In determining the relative roles of autonomic regulation and hormones during blood donation, Fortrat *et al*[41] found that blood donation led to increases in the plasma catecholamines and an *increase* in systolic blood pressure. Heart rate, cardiovascular variability and the spontaneous baroreflex sensitivity did not change. Catecholamine concentrations, particularly noradrenaline, reflect sympathetic activation, rather than a hormonal response.

METHODS

Ten healthy, conscious volunteers, all regular blood donors with a body weight of 55-85 kg (~4000 – 6000 ml of circulating blood volume), were enrolled to participate. Exclusion was based on the local blood transfusion service donation guidelines, and the taking of any anticoagulant, aspirin, anti-inflammatory or procoagulant drugs. Informed written consent was obtained

from all volunteers prior to participation. The volunteers were admitted unstarved to the Western Province Blood Transfusion Services (WPBTS) in the morning and asked to continue taking oral fluid to prevent any stress associated with fluid deprivation - they were requested to drink 200ml fluid prior to commencement of the study, followed by 500ml in the first hour after donation, 200ml in the second hour, and 100ml per hour thereafter until 6 hours had elapsed. As this was taken enterally, it would not have a direct effect on the intra-vascular compartment.

The drop in haematocrit measured the amount of auto-dilution that occurred following a 10% blood loss, while the TEG was used to measure the effect on coagulability.

The baseline blood samples were obtained using the standard technique, prior to the blood donation being commenced, for TEG, ATIII and fibrinogen levels, catecholamine assay and haematocrit. Thereafter, a standard unit of blood (approx. 500ml (~8.3 – 12.5% of total blood volume)) was taken according to WPBTS guidelines. All ten samples were taken within 15 minutes, and a second blood sample was then taken immediately post donation, and the same tests were performed. These were repeated at 1, 2, 4 and 6 hours post blood donation. Each sample was taken from a site more distal to any previous venous puncture site.

STATISTICAL ANALYSIS:

Analysis of variance for repeated measures, with post hoc LSD testing was performed on the data. The study had an estimated power of 90% based on an anticipated haemodilution of 10% and previous results of changes in TEG

variables at this dilution, as obtained in chapter 6. Noradrenaline values were found to be non-significantly distributed, and changes were analysed using Kruskal-Wallis, non-parametric ANOVA.

RESULTS

There was a significant decrease in r-time in the sample taken immediately post blood donation compared to the control sample. The other TEG measures in the post donation sample, shortened k-time, increased α-angle and Maximum Amplitude, did not reach significance. The values returned towards baseline at 1-hour post donation and remained there. There were no significant changes in any other TEG variables (Table 9.1.).

	r-times (SD)	K-times (SD)	α angles (SD)	MA (SD)
Control	15.2 (4.9)	6.2 (2.4)	36.9 (11.5)	54.7 (7.5)
Immed Post	11.7 (5.2)*	5.2 (2.3)	41.7 (12.4)	56.7 (7.3)
1h	17.2 (2.7)	6.7 (2.0)	34.2 (8.1)	54.0 (8.6)
2h	15.9 (4.3)	6.4 (2.0)	35.4 (7.0)	53.5 (6.1)
4h	15.2 (2.7)	5.8 (2.5)	38.4 (10.4)	55.1 (7.6)
6h	16.0 (3.2)	6.2 (2.6)	36.8 (11.3)	54.7 (6.8)

* $P < 0.01$

Table 9.1. Thromboelastography results – r- and k-times in minutes, alpha angle in degrees and maximum amplitude in mm.

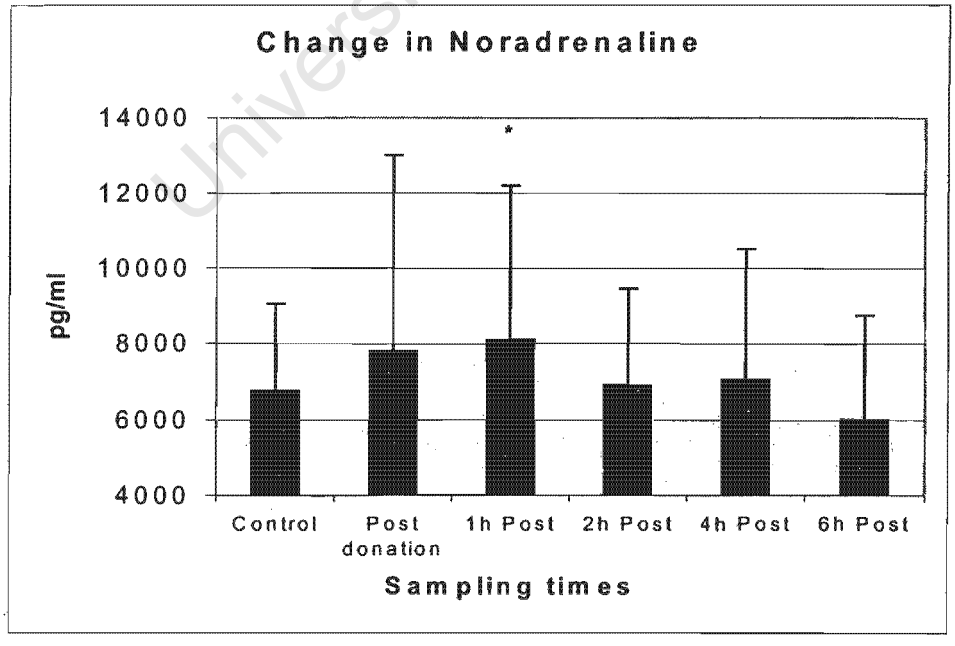
There was a marked decrease in Antithrombin III (ATIII) between the Control and the immediate post-dilution sample, but this did not reach significance (p=0.17). The other AT III and fibrinogen levels did not show any difference from control (Table 9.2.).

	ATIII Mean	(SD)	Fibg. Mean	(SD)
Control	110.7	(16.1)	2.6	(0.5)
Immed Post	100.3	(12.7)	2.9	(1.0)
1h Post	110.0	(17.2)	2.7	(0.7)
2h Post	112.3	(15.6)	2.8	(0.9)
4h Post	102.2	(17.7)	2.8	(1.0)
6h Post	116.4	(16.4)	2.9	(0.7)

No statistical difference

Table 9.2. Antithrombin III and Fibrinogen Levels

Fractional plasma noradrenaline changes were significantly raised at 1-hour post donation ($p<0.05$), but returned to baseline within 2 hours (Figure 9.1.).

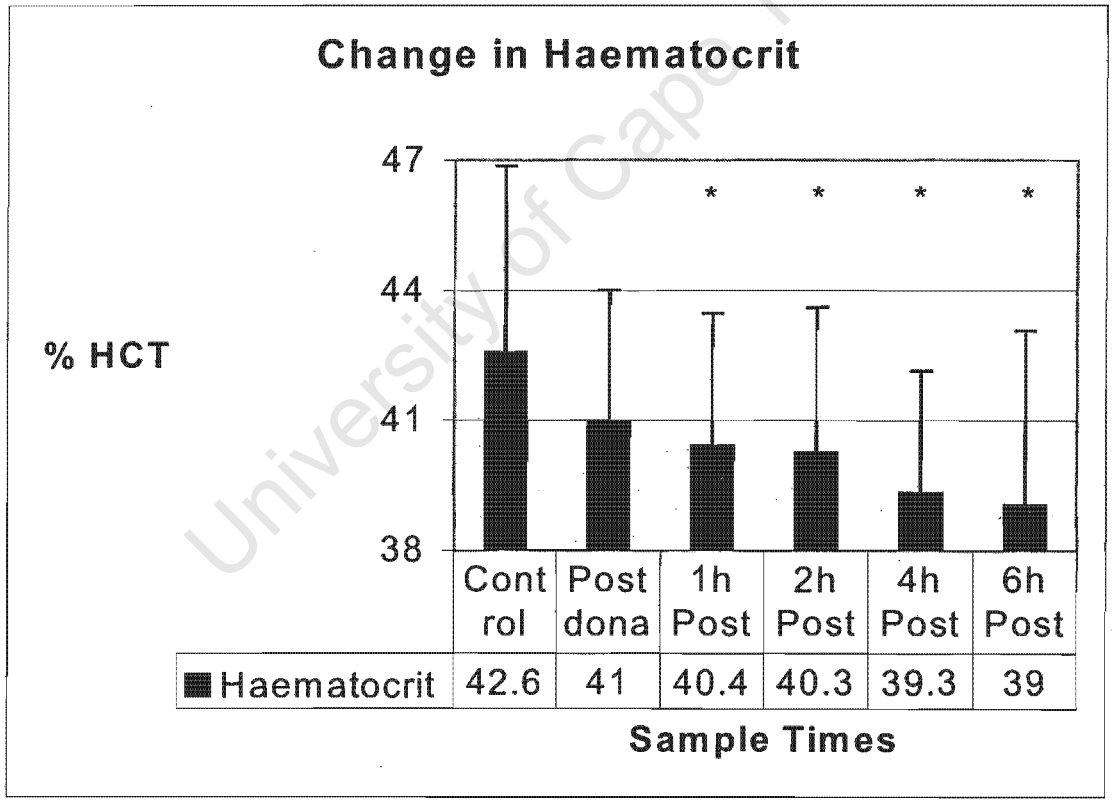


* $P < 0.01$

Fig. 9.1. Noradrenaline levels (pg/ml) – Samples taken represented on the x-axis as control, post donation, 1 hour post, 2 hours post, 4 hours post and 6 hours post donation of blood. (SD in bars)

Adrenaline concentrations were below the lower level of sensitivity of the method (20 pg/ml) throughout the study.

The haematocrit showed a rapid (~4%) drop during donation, followed by a slow, but progressive decrease over 6 hours, falling by a mean of 8.3% from pre-donation values. This rapid initial reduction in haematocrit from baseline, failed to achieve significance in the immediate post donation samples (p=0.053). The difference was significant from one hour post donation (p≤0.05) (Figure 9.2.).



* $p < 0.05$

Fig. 9.2. Haematocrit – changes following blood donation

DISCUSSION

A significant shortening of r-time compared to control occurred in the post donation sample, with the other TEG variables, the shortened k-time, increased alpha angle and maximum amplitude, not achieving significance.

The finding reflects an enhancement of the onset of coagulation and a trend towards hypercoagulability. All TEG results returned to baseline from 1 hour post donation. There was no significant hypercoagulability after the initial rapid blood dilution (Hb drop of 4%). The shortened r-time was not related to how the sample was taken. The coagulation effects of an indwelling cannula, *per se,* have been documented by Gorton *et al*[53].

While the faster onset of coagulation occurred immediately after donation of the blood, returning to normal by one hour, the noradrenaline levels were only significantly raised by 1 hour, and remained raised until 2 hours post donation, with plasma adrenaline remaining unmeasurable throughout. It is of interest to note that a 10% loss of blood volume in a healthy donor is easily compensated for by the sympathetic response, through an increase in the tone of blood vessels as is evidenced by the level of noradrenaline rising, while the adrenaline remains unchanged. This is reflected by the findings in this study, rather than a pure sympathetic stress response and this, together with the change r-time not being related to the rise in noradrenaline, argues against the catecholamines making a major contribution to the observed enhancement of coagulation after haemodilution.

It is important to note that the rate of decrease in Haematocrit is greatest in the ~15 minutes during which the blood is being lost, especially when compared to the drop over the first hour post donation, and later over the 2-hour periods (Fig.2). A drop of the haematocrit of 4% (42.6-41.0% drop in HCT) implies a plasma dilution of ~10% in the 15 minutes of blood donation. The enhanced coagulation, similar to that of 10% dilution in Chap 6, can thus be attributed to the immediate rapid auto-dilution with interstitial fluid. As the

vascular tone increases, dilution slows and coagulation returns to normal. It must be noted, that while a lowering of haematocrit has been described as decreasing coagulation on the basis of decreased blood viscosity leading to a faster arterial blood flow[153], this does not have a major effect in the micro-vascular circulation, especially the venous side, where flow is slow and not as dependent on the viscosity of the blood.

The likelyhood that the phenomenon of haemodilution-induced enhanced coagulability may be the consequence of introduction of foreign material to the blood is thus unlikely, since the enhanced coagulation has been seen to occur with an auto-dilution. It is likely that after the rapid initial dilution (Drop of Haematocrit mean from 42.6 to 41 ~ 4%, plasma dilution ~10%, in 15 minutes), the continuing rate of dilution may be too slow and too small to create the necessary dynamic imbalance. The AT III had a 10% drop (although no statistical difference, p=0.17) during the 15 minutes between samples 1 and 2, before returning to baseline, or even higher (Table 9.2). This is in line with the previously observed finding of the relationship between AT III and thrombin. The fibrinogen levels did not show a statistically significant increase.

It would thus appear that autodilution of blood to replace a suden 10% loss does not induce major hypercoagulability after the effect demonstrated following the initial *rapid* dilution. This can be explained, as the total drop in Haematocrit is only 8.3%, occurring over 6 hours. This means that after the initial dilution (4% drop in HCT in 15 minutes) the next 4.3% drop occurred over 6 hours, which will not precipitate the imbalance between anti-coagulants and spontaneously activated coagulants, which is required for the

enhancement of coagulation, as is the case following a rapid, large volume (20-30%) haemodilution.

Seen in the light of the previous work on enhanced coagulation after haemodilution[29,50,70,173,175,176,178-180,213] on one hand and the minimal autonomic and coagulation changes between 1 hour and 6 hours after donation in healthy blood donors, it could be postulated, given the findings in Chapter 8, that the speed of dilution creates a change in the ratio between AT III and thrombin, which will cause the enhanced onset of coagulation. The next step is to measure the effect on coagulation of crystalloids vs colloids in a clinical setting.

CHAPTER TEN
THE EFFECTS OF INTRAVENOUS COLLOID OR CRYSTALLOID ON COAGULATION IN PATIENTS UNDERGOING PERIPHERAL VASCULAR SURGERY

RATIONALE

The previous chapters have validated that haemodilution enhances coagulation, both *in vitro* and *in vivo*, and have demonstrated the probable cause of this effect. In this chapter the aim is to determine the effect in a clinical setting. The study sets out to investigate the effect of haemodilution on coagulation in patients undergoing vascular surgery. As previously discussed, Popov-Cenic*et et al*[152] have referred to the effect of surgery on enhancing coagulation and the modification thereof by hydroxyethyl starch. In 1975 Vinnazzer and Bergmann[219] demonstrated a post-operative hypercoagulable state in the control (saline) group, and an insignificant change in the test (hydroxyethyl starch) group. As their coagulation testing was only performed post-operatively, it is difficult to determine if the enhanced coagulation was due to the saline therapy or the surgery; similarly, the apparently normal coagulation profile in the starch group could be interpreted as either a negligible effect of the starch on coagulation, or as an impairment of surgery-induced enhanced coagulation. A review by Colombo and Tuman suggested that the decreased incidence of thrombotic events in patients undergoing peripheral vascular surgery under epidural anaesthesia and analgesia may be due to either attenuation of hypercoagulation, or other mechanisms such as reduced vasoconstriction or improved blood flow[21], but did not comment on

the role of intravenous fluids or the influence of surgical stress response on coagulation.

While this is of great interest, it has not yet been demonstrated whether coagulation enhancement induced by rapid fluid infusion is relevant in a clinical setting in patients undergoing surgery, and whether any effect occurs on the basis of a stress response or on the basis of the haemodilution induced enhancement of coagulation.

This chapter is a controlled study investigating effects of intravenous fluid on coagulation in patients undergoing peripheral vascular surgery under regional anaesthesia, comparing the coagulation effects of fluid administration and surgical stimulus with the baseline coagulation state, as well as investigating the different effects of crystalloid and colloid.

Therefore, this study was designed to investigate if the now established and well-described effect of haemodilution on enhancing coagulation can still be demonstrated under regional anaesthesia; if so, whether it is demonstrable prior to surgical stimulus, or only after the surgery has begun; and whether the nature of the fluid used had an influence on the effect.

METHODS

Patients considered for inclusion in the study were those presenting for peripheral vascular surgery on the lower limb who were suitable for regional anaesthesia and who gave informed consent for both the regional anaesthetic and the study. Patients were excluded from the study if they were already receiving intravenous fluids at the time of arrival in theatre, if they had received aspirin or any non-steroidal anti-inflammatory drug during the

previous 7 days or if they were receiving any anticoagulant medication. Once informed consent had been obtained, patients were randomised to either a colloid or crystalloid group using a sequence determined from tables of random numbers. This table was kept by a research assistant and the group revealed to the anaesthetist performing the case only once the patient had been included in the study.

All subjects received a premedication of temazepam 10 or 20 mg orally 1 hour prior to surgery. When the patient arrived in the operating theatre an intravenous cannula was inserted into a suitable forearm vein. Blood samples were obtained using the standard technique from the opposite forearm. An intravenous co-load infusion of either 1000 ml of crystalloid (Plasmalyte B or modified Ringer's lactate) or 500 ml of hydroxyethyl starch (HAES-Steril) was commenced. The choice of crystalloid was made on the basis of whether patients were diabetic or not, with Plasmalyte B being administered to diabetic patients and modified Ringer's lactate (from which calcium had been excluded) to the remainder. All patients received an anaesthetic consisting of epidural bupivacaine 0.5% administered through an epidural catheter inserted at a suitable lumbar disc space. No additional sedative agents were administered, and if the regional analgesic technique was inadequate, the patient was excluded from the study. The volume of bupivacaine used initially was 10-12 ml, with subsequent top-up doses administered as necessary to achieve and maintain a sensory level at T10 or above. Approximately half the volume of the fluid load was administered prior to the insertion of the epidural catheter and the remainder during the procedure.

Hypotension requiring the administration of vasopressor agents was a post-hoc exclusion criterion. Once a satisfactory block was established, the patients were transferred to the operating room and surgery was commenced. During surgery, patients received only crystalloid or colloid depending on their group, but the volume of fluid infused was judged against clinical criteria of fluid requirement. Blood samples obtained prior to the administration of any intravenous fluid (Sample 1), were repeated after completion of both the regional anaesthetic and the initial fluid co-load (Sample 2), during surgery immediately prior to heparin administration and arterial clamping (Sample 3) and a final sample 24 hours after the administration of the regional block (Sample 4). The following tests were performed: TEG, HCT, INR, aPTT, D-Dimers and TAT complexes.

STATISTICAL ANALYSIS:

Power analysis for the study was performed using the assumptions that intraoperative haemodilution would be approximately 15%, and that the changes in TEG variables would be similar to those found in previous studies. On this basis, a sample size of 20 subjects per group was sufficient to demonstrate significant changes in coagulation variables. Statistical analysis was performed using Statistica Version 6. A multifactorial ANOVA was used with sample time and diluent as the categorical variables. Post hoc LSD testing was performed to identify individually significantly different groups. In order to control for different initial baselines, the change within each group from baseline was also calculated and tested in the same way.

RESULTS

The two groups were similarly matched in terms of age and gender, and the time taken between samples 1 and 3 was the same. The volume of crystalloid used intra-operatively prior to cross-clamp was greater in the crystalloid group than in the colloid group (Table 10.1.).

	Crystalloid	Colloid
Age (years)	63.0 (± 8.6)	58.1 (±13.6)
Gender (m:f)	16:4	15:5
Epidural IV fluid co-load (l): (Given between sample 1-2)	1.0	0.5
Intra-op IV fluid (l): (Given between sample 2-3)	1.6 (± 0.8)	1.2 (± 0.4)
Total fluid (l): (Given between sample 1-3)	2.6 (± 0.8) *	1.7 (± 0.4) *
Operating time (min): (Between sample 2-3)	92.6 (± 38.5)	88.9 (± 33.4)

* $P < 0.03$

Table 10.1. Group Analysis – Age and gender of patients, fluid volume given during epidural and during surgical time, prior to heparinisation, and operating time from start to heparin dose.

Comparable haemodilution was achieved in both groups as demonstrated by an equivalent decrease in haematocrit of approximately 10% in both groups. In the crystalloid group, a faster onset of coagulation, an increase in the rate of clot formation and an increase in clot strength was demonstrated after the initial fluid load (Sample 2) as well as just prior to administration of heparin (Sample 3), using the TEG (reaction time (*r*) and coagulation time (*K*) shortened, maximum amplitude (MA) increased). No enhancement of coagulation was observed in the colloid group (Table 10.2.). There were no changes from control evident after 24 hours (Sample 4) in either group.

▪ Crystalloid										
	r-time (min)		k-time (min)		Alpha angle (°)		MA (mm)		HCT (%)	
	Mean (SD)	*P**	Mean (SD)	*P* *	Mean (SD)	*P* *	Mean (SD)	*P* *	Mean (SD)	*P* *
1	14,0 (5,8)		5,7 (2,6)		43,6 (15,1)		54,6 (14,5)		42,4 (6,2)	
2	10,1 (4,9)	0,024	3,5 (1,7)	0,006	54,9 (13,9)	0,009	62,6 (13,3)	NS	38,2 (7,6)	0,039
3	8,8 (3,9)	0,007	4,0 (3,1)	0,012	54,9 (12,6)	0,012	64,2 (11,1)	NS	36,8 (6,3)	0,023
4	13,6 (7,6)	NS	5,7 (3,3)	NS	46,3 (14,9)	NS	55,0 (16,4)	NS	35,6 (6,0)	0,002
▪ Colloid										
	Mean (SD)	*P*	Mean (SD)	*P*	Mean (SD)	*P*	Mean (SD)	*P*	Mean (SD)	*P* *
1	11,3 (4,3)		5,4 (2,5)		46,2 (12,1)		51,9 (15,1)		42,5 (5,8)	
2	9,6 (3,7)	NS	5,1 (2,6)	NS	46,7 (11,3)	NS	52,0 (15,2)	NS	37,9 (7,7)	0,034
3	11,5 (4,0)	NS	5,1 (2,5)	NS	44,5 (12,4)	NS	51,3 (13,0)	NS	34,4 (6,7)	0,002
4	15,1 (11,1)	NS	5,4 (2,8)	NS	43,6 (13,2)	NS	54,7 (15,1)	NS	34,6 (8,7)	0,002

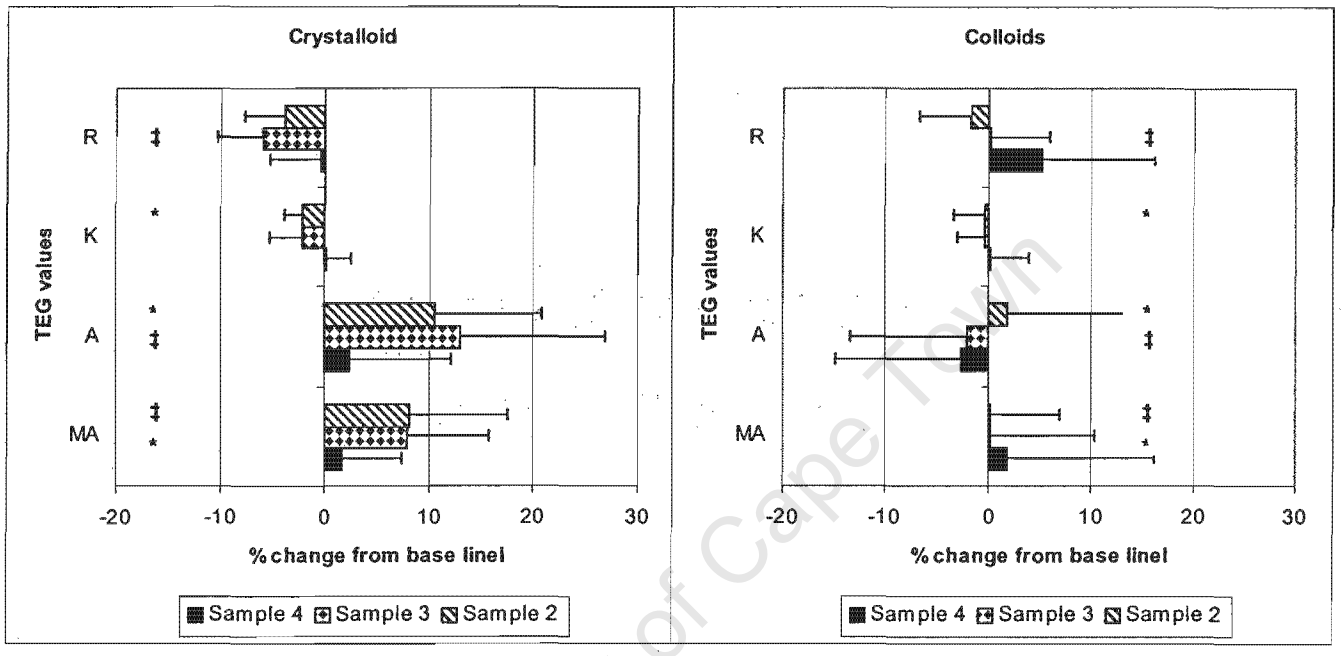
* $P < 0.05$ compared to sample 1

NS – Not significant

Table 10.2. Within group analysis of changes in thromboelastography from baseline

The between group difference in TEG values compared with control shows a statistically significant *r* time difference at the time of sample 3 ($p < 0.05$). The *K* time between group differences from control were statistically significant at sample 2 ($p < 0.05$). There was a significant between group difference in change from control in alpha angle at sample 2 ($p < 0.03$) and a greater

difference in sample 3 (P <0.002). The MA changes from control demonstrate a significant between group difference from control in sample 2 (P <0.01) and sample 3 (P <0.02). There were no between group differences in change from control in sample 4 at 24 hours after surgery (Figure 10.1.).



* - Significant between group difference in change from baseline sample 1 (P<0.03)

‡ - Significant between group difference in change from base line sample 1 (P<0.005)

Fig. 10.1. Between group comparison of TEG value changes from sample 1 (baseline) (r- and k-time in minutes, α-angle in degrees and MA in mm)

There was an increase in the absolute value of the TAT and D-Dimers in both groups, but this did not achieve statistical significance (Figure 10. 2.).

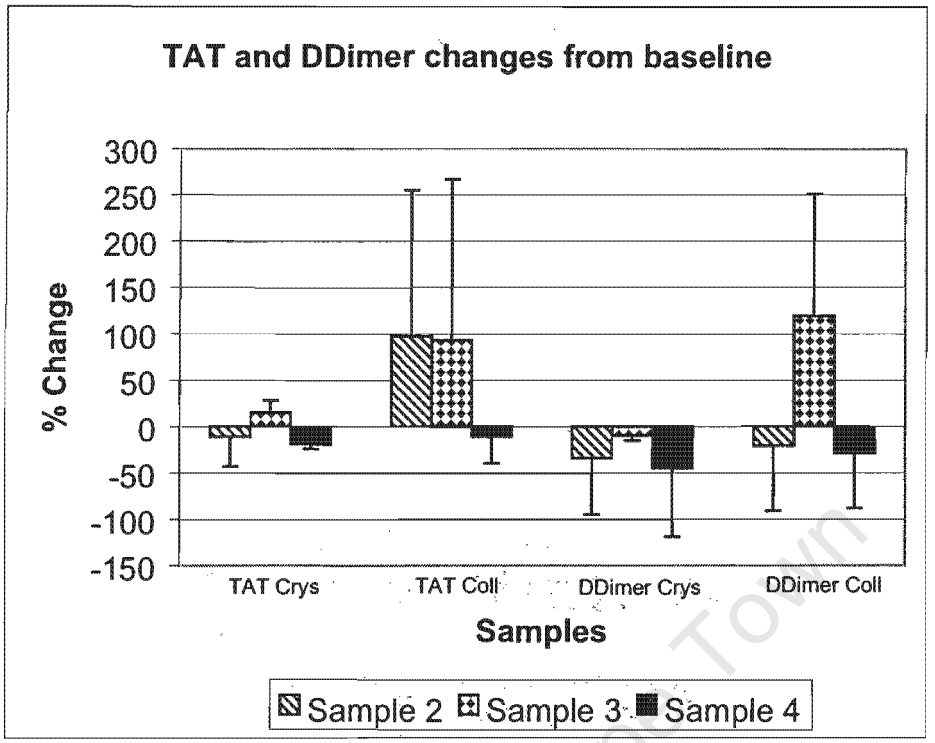


Fig. 10.2. Difference from baseline in TAT and D-dimer values No difference between groups. (Standard deviation indicated by bars)

There were no significant within group changes in INR or aPTT. The INR had a significant between group difference in sample 3 and there were no between group differences in aPTT at any time (table 10.3.).

Measure	Sample	Crystalloid	Colloid	p
INR	Sample 1	1.2 ± 0.2	1.3 ± 0.3	ns
	Sample 2	1.2 ± 0.3	1.4 ± 0.4	ns
	Sample 3	1.2 ± 0.2	1.5 ± 0.4	0.025
	Sample 4	1.3 ± 0.3	1.5 ± 0.8	ns
aPTT	Sample 1	31.9± 1.3	31.7± 2.0	ns
	Sample 2	32.0 ± 1.3	31.7 ± 2.0	ns
	Sample 3	31.8 ± 0.9	31.8 ± 1.1	ns
	Sample 4	31.7 ± 1.4	31.4 ± 1.5	ns

p - indicates between group differences. There were no within-group differences.

Table 10.3. International Normalised Ratio (INR), and activated Partial Thromboplastin Time (aPTT) at various sample times

DISCUSSION

This study demonstrated that the previously described enhancement of coagulation by crystalloid fluid haemodilution is present under regional anaesthesia and that it occurs prior to commencement of surgery. It was also shown that the effect persisted well into the operative phase, and was demonstrable up to the time of administration of heparin.

Regional anaesthetic is highly effective at blocking the surgical stimulation, which might normally have an effect on the enhancement of coagulation. It is noted that while the major part of the enhanced coagulation observed in this study was due to the crystalloid fluid administered, it cannot be totally excluded that the surgical trauma possibility resulted in the release of procoagulant factors into the systemic circulation, which may have contributed to the enhanced coagulation seen in the crystalloid group. Had the stress of surgery produced a discernable effect on coagulation, some change in the TEG would have been expected in the groups after the infusion of fluid and before the administration of heparin (between Samples 2 and 3). However, this was not demonstrable. The TEG measures were virtually unchanged by the time of heparin administration. Indeed the colloid studied, hydroxyethyl starch 200/0.5, had almost no effect on coagulation either at the time of acute haemodilution, or during surgery. While it is possible that the colloid impaired a purely surgically induced enhancement of coagulation, this is unlikely, and it appears that the major part of the alteration in coagulation seen in the crystalloid group was attributable to the fluid administered, rather than the surgical stimulus as there is no statistical within group difference between samples 2 + 3.

It is clear that enhanced coagulation, in the crystalloid group, occurred before commencement of surgery, and continued during the surgical stimulus. In the colloid group, enhancement of coagulation may have been inhibited by the anti-platelet activation effect of the hydroxyethyl starch preventing the platelet clumping usually brought on by minor quantities of activated thrombin binding to platelet receptors to elicit aggregation[229] early in haemostasis.

As discussed previously, platelets play a role in accelerating the intrinsic pathway reactions and thrombin activation[49]. This effect may result in the findings of non-altered coagulation following dilution in the starch group as opposed to the crystalloid group. The coagulation enhancement appears to be modulated by the use of colloids, as has also been previously demonstrated, even though there is a rise in the amount of TAT complexes formed with haemodilution (Figure 2). Of interest is that the initial increase in TAT is greater in the colloid group, in line with the greater plasma dilution achieved by the starch. However, the ultimate coagulation was not enhanced, probably because of the coating of platelets by the starches, which prevents a 10-fold exponential increase in thrombin formation (Chap 2 & 4). Therefore starches preventing platelet activation[114], result in the findings of non-altered coagulation in the starch group compared with the crystalloid group.

It has been demonstrated that the probable cause for the hypercoagulability is an imbalance between the naturally occurring anticoagulants and activated pro-coagulants, with a reduction in antithrombin III probably being the most important (Chapters 8 & 9). This effect results in lowering the threshold above which positive feedback into the intrinsic pathway occurs, thus leading to the enhanced coagulation[180,181]. The positive feedback of thrombin into the

intrinsic pathway is not dependent on absolute levels of Anti-thrombin and other naturally occurring anticoagulants, as even a relatively small decrease in anticoagulants (~20-30%) can result in this imbalance[77,78]. It is dependent, rather, on a relationship, or ratio, between the anticoagulants, and spontaneously activated coagulants, so that even a limited degree of reduction of anti-coagulants can lower the threshold above which the feedback is uninhibited (Chap 8).

Janvrin *et* al[71] have previously shown an increase in coagulation and incidence of deep vein thrombosis in patients receiving crystalloid fluid peri-operatively. This has now been taken one step further, in demonstrating that in a clinical setting the enhanced coagulability is induced by the *rapid* infusion of fluid, which alters the balance between anti-coagulants and the activated fraction of procoagulants, which by definition results in an enhancement of clot formation, thus defining the onset of enhanced coagulation, related to haemodilution *per se*. This effect is independent of the response to surgery. Of note was that both groups were outside the normal range of coagulation for the Cape Town population following the dilution. This is not surprising in a group of vascular disease patients.

Neither group demonstrated enhanced coagulability at 24 hours after surgery. This is in line with the observations that regional anaesthesia may be associated with a lower incidence of deep vein thrombosis[184,215]. The lack of difference either within, or between groups, at 24 hours (Sample 4) was probably attributable to the fact that there were many variables in both groups, like the receipt of anticoagulants during the post-operative period to minimise

the risk of graft thrombosis; however, this study was not powered to look at outcome variables.

There were no changes in the INR and aPTT tests, other than a between group difference of the INR at sample 3, but this was expected as they measure coagulation cascade end points in plasma samples, which have coagulation initiated through the addition of a coagulation stimulant. However, as discussed in Chapter 3 & 4, they do not measure the balance between pro- and anti-coagulation factors within the coagulation framework.

While it is tempting to speculate that the increase in coagulation during the pre-heparin phase in the crystalloid group may lead to an increased risk of occlusive vascular events, such a conclusion cannot be drawn on the basis of these results. However, the findings in this chapter confirm that the mechanism of enhanced coagulation is related to dilution itself, and thus an imbalance between coagulants and anti-coagulants and that this is triggered by the *rapid* crystalloid haemodilution. Given that vascular surgical patients may be at an increased risk of coagulation, especially peri-operative cases, more work must be done to establish the clinical relevance of this effect.

CHAPTER ELEVEN
DISCUSSION

In analysing the effect of enhanced coagulation occurring with haemodilution, the clotting cascade and its interaction with platelet activation has been explored in order to define exactly at which point, and through which mechanism the effect occurs. There may well be many factors that have an impact on the fine balance of coagulation enhancement and inhibition. With this complicated and interactive dynamic system, it has been important to define all aspects in the process that may contribute to the increase in coagulability with haemodilution. The TEG measures the complete dynamic coagulation process in whole blood, rather than testing the activity of coagulant factors in anticoagulated plasma, as is commonly done in conventional analysis of coagulation and this has made the TEG the primary tool in the work performed for this thesis[174].

In setting out to perform the work it was therefore crucial to confirm the validity of the TEG results. It was important to establish that the effect of enhanced onset of coagulation following haemodilution was not an artefact due to the technique of the TEG. The thrombelastograph is a valid tool for measuring coagulation, especially hypercoagulability and this has been established in Chapter 3. However, to take all doubt out of the potential effect that the TEG mode of measuring coagulation might have on the effect, every sample of blood measured for enhancement of coagulation following dilution, had its own undiluted control. As a result the enhanced coagulation is on the basis of dilution, since this was the only variable.

Having established the effect of *in vitro* dilution[173], the first investigation in this thesis was an *in vivo* dilution to confirm that this would show the same result (Chapter 4). The rapid administration of crystalloid resulted in the onset of enhanced coagulation, while this did not occur in the colloid (hydroxy-ethyl starch) group[176]. This confirmed that the effect can be reproduced with crystalloid dilution in human subjects and is not only a laboratory artefact. There were no other confounding variables like surgery, stress, disease, etc. playing a role. Of interest was that the antithrombin III levels sank lower than anticipated from dilution alone. In addition, in Chapter 9, where blood is taken from volunteers and dilution occurs through fluid being drawn from the interstitium with no other factors to encourage coagulation, the onset of coagulation was still enhanced by the initial dilution, further supporting the findings of the effect occurring with *in vivo* dilution.

It is also important to clarify that this thesis is examining and identifying the effect of coagulation enhancement and not defining hypercoagulability, although in Chapter 5 the findings after dilution in group one were very similar to the undiluted controls in group two – pregnant mothers undergoing spinal anaesthesia for Caesarean Section. It is known that pregnancy poses an increased risk of pathologically enhanced coagulation. Therefore, it may well be possible to speculate that there may be a clinical effect associated with dilution of blood. However, while this is an interesting observation, that is not what this thesis has set out to achieve.

From previous work it was established that there is an enhancement of clot formation following haemodilution with 0.9% saline and other crystalloid solutions, but the mechanism had remained incompletely explained. Since the

effect was first described by Monkhouse[123] and Tocantins *et al*[206] in the 1950s, it has been addressed by several authors[50,70,138,152,173,213,219]. This thesis confirms the speculation of Monkhouse that dilution produces an imbalance between procoagulant and anticoagulant factors. As discussed below, the mechanism is probably explained by the fact that the procoagulant factors are present in the blood as inactive precursors in vast excess. Haemodilution, although reducing the concentration of the precursors, has almost no effect on the availability of active factors until dilution reaches extreme levels (Chapter 6). However, coagulation inhibitors including antithrombin III, α_2-macroglobulin, protein C and protein S are all present in their active form and their effectiveness is therefore susceptible to a reduction in their concentration by dilution.

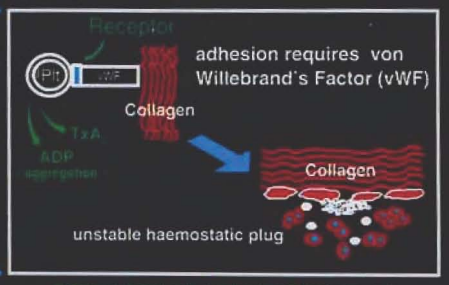
While a lowering of haematocrit has been described as decreasing coagulation on the basis of decreased blood viscosity leading to a faster arterial blood flow[153], this does not reflect the process of coagulation itself, especially in the microvascular circulation, where flow does not impact on the intrinsic ability of blood to clot. It is rather the activation of coagulation factors and platelets as well as vascular damage (2 factors of Virchow's triad) that will have a greater effect on the coagulation at this level.

Having established the effect to occur following *in vivo* dilution, the variables of the diluent, such as pH, temperature, ions and osmolality, were ruled out as factors resulting in the enhancement of coagulation (Chapter 5)[179], followed by an investigation into the degree of dilution at which this enhancement of coagulation occurs. The effect first becomes evident at a low percentage (10%) dilution, is most marked at a moderate (20 – 30%) dilution, starting to

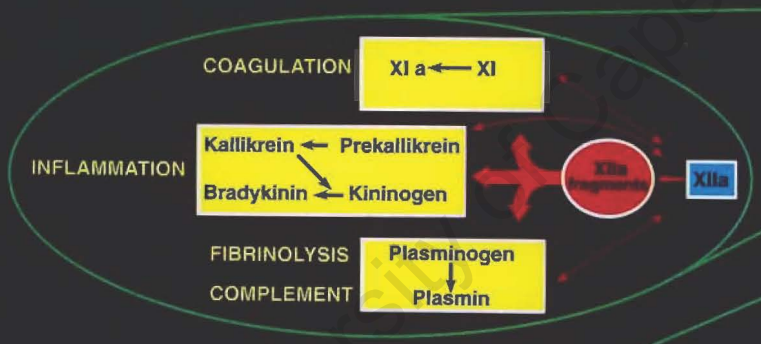
THE COAGULA

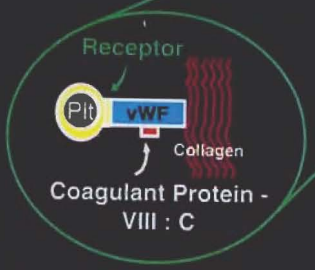
NORMAL HAEMOSTATIC MECHANISM :

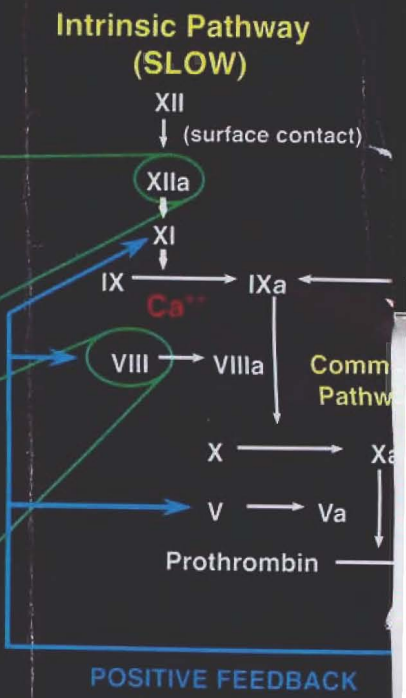
- Vessel severed
- Vascular Constriction -transient. lasting less than 60 secs -via Thromboxane A2
- Platelet Adhesion
- Platelet Aggregation
- Blood Coagulation -Aggregated platelets supply a surface on which blood coagulation occurs
  -Fibrin consolidates the plug rendering it stable
  -The fibrin component of the plug gradually increases in amount as platelets lyse
  -After 24 hours the haemostatic plug is completely transformed to fibrin
  -Fibrin is then digested by the plasma fibrinolytic system and leucocytes
  -The defect in the vessel wall is recovered with endothelium

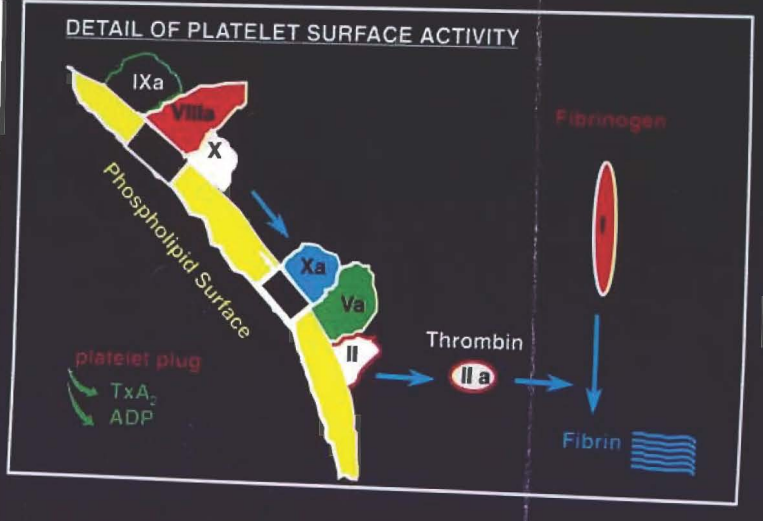


FORMATION OF HAEMOSTATIC PLUG









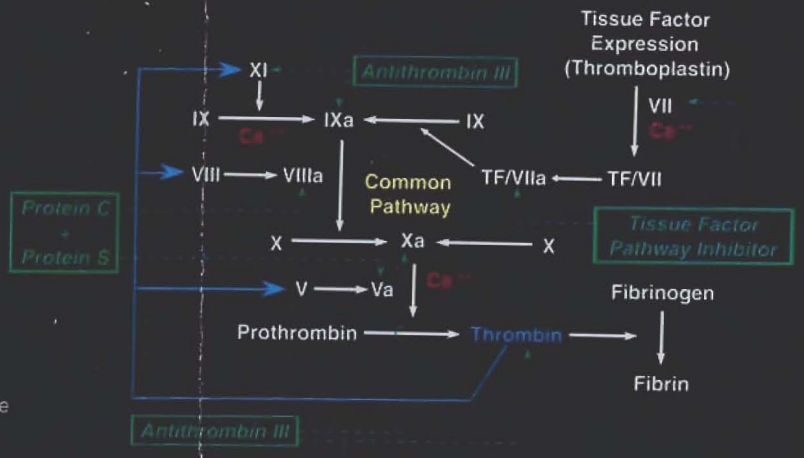
ON CASCADE

ROLE OF INHIBITORS

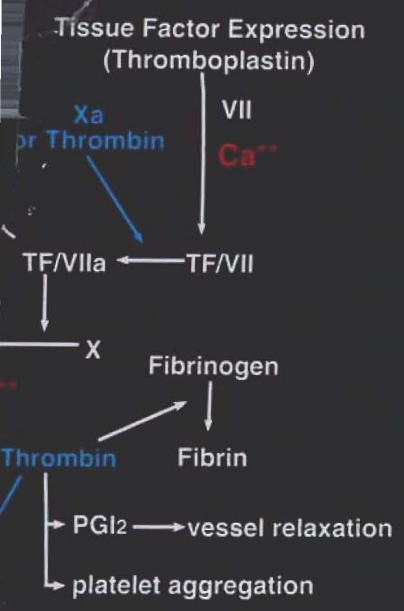
• Thrombin acts on FACTORS XI, VIII AND V in a self-propagating positive feedback cycle in order to produce more Thrombin.

• The Extrinsic and Intrinsic pathways do not function independently, but rather initial Thrombin production via the faster Extrinsic pathway is amplified by the slower Intrinsic pathway.

• The role of Inhibitors (Antithrombin III, Protein C and S, and Tissue Factor Pathway Inhibitor) is therefore important in control and cessation of this cascade, thereby preventing intravascular thrombosis.

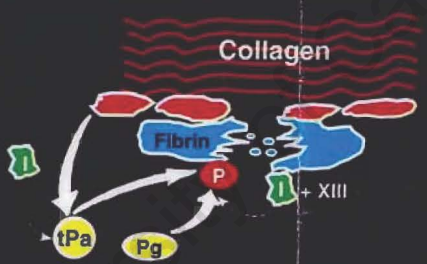


Extrinsic Pathway (FAST)



FIBRINOLYTIC SYSTEM

• Plasma - hydrolysis of Fibrin via Plasmin



• Tissue plasminogen activator (tPa) is released from endothelial cells to complex with circulating plasminogen (Pg) to form plasmin (P) bound to the fibrin clot.

• Factor XIII is responsible for Fibrin cross-linkage and clot stabilisation.

• Plasmin hydrolyses Fibrin, Fibrinogen and other coagulation proteins.

• Both tPa and P are modulated by Inhibitors ( ).

• Cellular - proteolytic enzymes released from Leucocytes

DEFECTS IN HAEMOSTASIS

Bleeding Disorders

- vascular defects
- thrombocytopaenia
- platelet function defect
- coagulation defects
- excessive fibrinolysis

Thrombosis

Thrombogenic Stimuli

- vascular damage
- stimulation of platelet aggregation
- activation of coagulation
- stasis (*Virchow*)

Protective Mechanisms

- inactivation by inhibitors
- clearance by liver or RE cells
- dissolution by fibrinolysis

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decrease from its peak thereafter (40%) and a reduction below control levels only becoming apparent with crystalloid dilution of 60% (Chapter 6).

There is, therefore, no doubt that haemodilution itself enhances coagulation. It appears that all crystalloid solutions produce similar effects. The conclusions regarding crystalloid haemodilution seem clear. Rapid intravenous administration of crystalloid solutions will enhance coagulation up to a haemodilution of 40%. Beyond this, further haemodilution with crystalloids will begin to impair coagulation.

If it is *haemodilution* creating an imbalance between procoagulant and anticoagulant factors, the colloid solutions ought to exert a similar or greater procoagulant effect to that seen with crystalloids, however the influence of various colloids on the coagulation process itself, specifically platelet activation, offsets the effects of dilution of anticoagulant factors as demonstrated in Chapters 4 and 10. The fact that coagulation is maintained near normal led to the investigation into the effect of haemodilution on platelets.

With haemodilution itself causing the enhancement of coagulation, the mechanism of the whole process needed to be clarified. It was demonstrated that following haemodilution, coagulation is not mediated via cyclo-oxigenase induced platelet-endothelial interaction, as the effect still persists after the administration of aspirin (Chapter 7)[175]. While this ruled out the role of haemodilution causing direct platelet activation through this pathway, it did not address the direct activation of platelets by other factors such as thrombin. However, with the limitation of the enhanced onset of coagulation in the starch group in Chapter 4, it can be deduced that platelets do play a role in the

process through direct activation and that this is impaired through the starches coating the platelet surface[172]. Further studies of haemodilution with both crystalloids and colloids, using the platelet ADP inhibitors (e.g. clopidogrel) or GpIIb/IIIa inhibitors (such as abciximab) may throw a greater light on this aspect.

Haemostasis involves a very complex interaction between initial thrombin production via the faster extrinsic pathway and its amplification by the slower intrinsic pathway, occurring through both the direct activation of factor IX by tissue factor/ factor VIIa complex (linking the extrinsic to the intrinsic pathways) and via the positive thrombin feedback into the intrinsic pathway as well as direct platelet activation (Chapter 2). Activated platelets play a vital procoagulant role, linking platelet function and coagulation activation. Platelet membrane phospholipids undergo a rearrangement during activation, providing a binding site for phospholipid-dependent coagulation complexes that activate both factor X and prothrombin[231], accelerating coagulation through the assembly of multi-enzyme complexes with great catalytic efficiency. The platelet surface membrane also interacts with factors Xa and Va to form the prothrombinase complex, as well as promoting the assembly of the factor IXa-VIIIa complex required for factor Xa activation[230]. These interactions should not to be overlooked, since they dramatically enhance enzymatic formation of both thrombin and factor Xa[229] and in the absence of the platelet membrane the formation of the complex is either abolished or impaired[109,209]. Calcium is also an essential component for efficient complex formation[134].

However, it must be emphasised that thrombin generation is the central biochemical reaction in both normal haemostasis and pathological thrombosis. The production of thrombin is self-reinforcing because of its feedback activation of numerous coagulation proteins (factor V, VIII, XI, and VII) that contribute to thrombin production. Positive feedback in coagulation plays a major role in the architecture of the system[78]. As discussed above and in Chapter 2, thrombin triggers both platelet aggregation and fibrin formation[58]. The findings in Chapter 8 suggest that the cause for the enhanced coagulation occurring after haemodilution may well be attributed to a decrease in the concentration of the anti-coagulants, lowering the threshold at which positive feedback could occur into the coagulation pathway and thus removing the "break" of the positive feedback.

What does this mean? As discussed in Chapter 8, Nossel and co-workers have demonstrated that normal plasma contains idling levels of *activated* thrombin[140,141], while Jesty *et al* established that inhibition of the positive feedback loop, through the effect of anticoagulants, plays a role in modulating the exponential enhancement of the coagulation cascades, conferring threshold properties on the system. In other words, if the level of activated thrombin is below the threshold, no response will occur, while if it rises above the threshold, the response should be at, or near, maximum. As a result, once the thrombin level has overcome the anticoagulant-induced threshold, it results in an exponential increase of further thrombin formation, and so ensures that a clot is formed.

In a study by Butenas and Mann the concentrations of blood coagulation proteins and their inhibitors were varied from 50 to 150% of their mean

plasma values[13], i.e., in the range that is generally considered as being "normal". The difference in thrombin generation for these two extremes reaches almost 30-fold. The dominant contributors to these extremes are prothrombin and antithrombin III. They demonstrated a marked influence of the ratio between the two.

Therefore, the ability of the entire system to cope with idling, non-zero levels of activated clotting enzymes, without generating a response, implies that the balance of intravascular coagulation is protected against sub-threshold stimuli. The role of inhibitors (ATIII, proteins C and S, and tissue factor inhibitor *inter alia*) is therefore critically important in control and cessation of this cascade, thereby preventing inappropriate activation of coagulation beyond areas of injury or dissemination of intravascular thrombosis. It was possible to demonstrate that it is the imbalance that is brought about by haemodilution that leads to an enhancement of coagulation (Chapter 8), as demonstrated through modulating this by maintaining the AT III levels at a *constant* value in spite of dilution, which prevented the enhanced onset of clotting brought on by dilution[180]. Clinical evidence has shown that a decrease in concentration of ATIII, to below 70% of normal, is linked to an increased risk of thrombosis on the basis of its second-order kinetics[27,75-77]. Moreover, the actual quantity, or level of an individual procoagulant factor, therefore does not necessarily reflect their functional status, as the percentage of activated thrombin formation is so minute.

Therefore, when examining the interaction of ATIII with thrombin, it is important to clarify that the actual *activated* thrombin makes up this minute fraction of the total amount of prothrombin present. Dilution thus leads to a

decrease in ATIII and *prothrombin*, but does not effect the spontaneous thrombin generation. There is a one-to-one, enzyme-to-inhibitor reaction of thrombin with the ATIII[166], thus even a *doubling* of the activated thrombin levels will not necessarily result in a significant decrease in the absolute level of ATIII, while breaching the threshold of positive feedback of thrombin Chapter 8).

Having demonstrated the effect as occurring with both *in vitro* and *in vivo* dilution, the response to autodilution following a 10% loss of blood was examined in Chapter 9. The findings confirmed that a rapid dilution of intra-vascular blood occurs with blood loss and that this is associated with a faster onset of coagulation. This coagulation state returned to normal once the dilution followed a slower process as the vasoconstriction brought about by noradrenaline came into play.

Given the identification of the mechanism of this effect it may be speculated that the evolution of this system may have survival advantages, as it would not be beneficial if an injured animal, suffering blood loss, lost the ability to form clot following intra-vascular auto-dilution in an attempt to maintain adequate circulating blood volume. As demonstrated in Chapter 9, the initial phase of this auto-dilution is rapid even though complete restoration of blood volume took several hours. Logically, the fact that dilution enhances coagulation is therefore not as counter-intuitive as might first appear to be the case.

Does this have a clinical effect? Given the findings of an enhancement of coagulation following haemodilution, it is easy to extend this into a clinical setting where an individual has a significant loss of blood with rapid

restoration of circulating blood volume externally from intra-venous fluid administration or internally from the interstitial fluid, leading to an enhancement of the onset of coagulation. This was demonstrated in chapter 10 which investigated the comparison of haemodilution with volume expansion for a vaso-dilation brought on by a regional anaesthetic in vascular surgical cases, comparing the coagulation effects of fluid administration and surgical stimulus with the baseline coagulation state, as well as investigating the different effects of crystalloid and colloid[177]. Of interest is that in Chapter 10, the TEG r-time values of both groups fell to outside the 25%tile range of the UCT normal value, thus theoretically putting them outside the normal range. This was also the case for the k- α- and MA values in the crystalloid group, which is in keeping with the findings of enhanced coagulation resulting from haemodilution. While a criticism might be that the UCT standard values may be based on too small a baseline, it is interesting to note that the crystalloid group post-dilution values were similar to the pregnant patients pre-dilution values in Chapter 5 and as discussed earlier, pregnancy is well known to be a cause for pathological hypercoagulability. However, it is beyond the scope of this thesis to draw any clinical conclusions from these findings.

As mentioned above, it is interesting to observe that when internal haemodilution occurs after blood loss, enhanced coagulability may prevent further haemorrhage. However, a point may be reached where bleeding occurs, as the clotting factors are used up through enhanced clot formation at a capillary level. This in turn leads to a marked increase in bleeding, as an imbalance between procoagulant factor activation and anticoagulants or enhanced fibrinolysis, occurs - the syndrome of disseminated intravascular

coagulopathy (DIC). In the natural state this may well result in the speedy demise of the individual. Indeed, decreased plasma concentration of ATIII may be indicative of the role of DIC in the pathogenesis of multiple organ dysfunction syndrome (MODS)[6], its baseline value correlating with mortality, and its plasma concentration being decreased in DIC, particularly in sepsis and shock.

Clinical work has suggested that the procoagulant effect of crystalloid infusions may be of relevance during surgery, with Ng and Lo[138] demonstrating that during surgical blood loss with crystalloid volume replacement, a hypercoagulable state developed which was related to the degree of haemodilution and Heather *et al* reported the "saline predictor test" - the probability of patients developing a postoperative DVT[61], which could be used as a predictor of the risk of formation of DVT formation, confirming the suggestion by Janvrin *et al*[71]. While the potential clinical implications are interesting and have been speculated on[173], for example the potential adverse effects of excessive coagulation, particularly in the postoperative patient with risks of deep vein thrombosis, coronary artery thrombosis and stroke[59], this speculation falls outside the scope of this thesis.

However, this thesis has set out to identify the cause and mechanism of the process and that it is not just a laboratory observation, which it has achieved. There is no doubt that it is reproducible with *in vivo* dilution, even auto-dilution, and that this effect is most likely due to the lowering of the anti-coagulant threshold thus allowing thrombin induced positive feedback into the intrinsic pathway and platelet activation, which leads to an exponential increase in the formation of a clot. The TEG, as the main tool for identifying

enhanced coagulation has been defended as an accurate tool in both the literature review and the reproducibility of findings in this laboratory.

Arising out of this thesis, further work should be done to establish the duration of the enhanced coagulation once the dilution resolves, whether the effect is maintained with ongoing dilution and whether the effect is also brought on by a slower infusion of a similar volume.

APPENDIX 1 – COAGULATION CASCADE

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APPENDIX 2 - CHAPTER 4

SALINE

	HCT (%)		PLATELETS		FIB		ANTI TH.III (%)	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST
1	43	39	261	233	3.4	2.4	85	83
2	44	42	225	203	2	1.8	100	86
3	47	41	157	143	2.6	2.2	92	83
4	42	4	195	193	1.7	1.5	91	85
5		36		157	2.4	2.1	85	83
6	39	35	199	178	2.8	2.6	80	86
7	46	44	191	205	3.3	2.6	117	78
8	53	44	170	150	2.8	1.9	90	92
9	38	33	176	141	4.3	3.4	108	114
10	46	42	173	148	2.6	2.2	180	41
11	46	43	282	269	2	1.7	86	97
12	45	4	250	227	2.8	2.2	101	64
13	39	37	212	192	2.2	1.8	75	65
14	4	37	256	221	2.8	2.4	150	69
15	4	37	213	204	1.7	1.7	158	98
16	38	34	251	240	3.4	2.6	137	65
17	47	41	191	170	2.6	2.2	167	77
18	5	45	201	183	1.7	1.3	156	67
19	45	39	180	171	1.8	1.2	156	75
20	34	31	170	149	4	3	107	94
	43.3	39.0	208.0	188.8	2.7	2.2	116.1	80.1
	4.7	3.9	36.5	36.4	0.8	0.6	33.7	15.9

HAES-STERIL

	HCT (%)		PLATELETS		FIB		ANTI TH.III (%)	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST
1	43	30	228	166	3	1.7	92	49
2	42	32	248	183	2.2	1.7	86	68
3	43	34	203	182	2.4	2	82	68
4	43	36	194	164	2	1.4	94	69
5	43	36	162	135	2.4	1.5	95	50
6	41	33	188	154	3.6	2.6	95	107
7	37	32	187	160	3.4	2.3	94	54
8	48	38	172	134	3	2.2	90	58
9	37	31	175	129	4	3	78	61
10	44	38	159	130	2	1.5	87	67
11	43	37	272	234	2.2	1.5	86	67
12	47	38	288	235	2	1.5	69	55
13	41	35	274	228	2.6	2	138	65
14	41	31	309	195	2.8	1.7	165	49
15	41	31	211	153	2.4	1.5	147	64
16	38	29	258	193	3	2.2	139	60
17	45	38	216	180	3	2.2	147	90
18	48	40	182	154	3.4	2.4	131	63
19	42	37	177	153	1.5	1.2	152	30
	42.5	34.5	215.9	171.7	2.7	1.9	108.8	62.8
	3.2	3.3	45.9	33.5	0.7	0.5	30.2	16.0

SALINE

	PT (min)		aPTT (sec)		BL. TIME (min)	
	PRE	POST	PRE	POST	PRE	POST
1	12.5	13.5	38	37	4	4
2	14.4	15.3	34	37	2.5	5
3	12.5	13.5	34	34	3	3
4	13.5	14.4	37	37	3.5	3
5	13.5	14.4	30	31	2.5	3.5
6	12.5	13.5	36	35	3.5	3.5
7	15.3	16.2	36	34	6	6
8	12.5	13.5	31	32	3	3
9	13.5	14.4	36	39	3	3
10	13.5	13.5	34	36	3	3
11	13.5	13.5	32	32	2.5	3
12	13.5	14.4	38	38	3	3
13	12.5	12.5	30	30	3	2.5
14	11.6	12.5	35	37	4	4
15	11.6	13.5	33	34	3	3
16	12.5	13.5	34	35	2.5	2.5
17	13.5	13.5	31	31	3	3
18	15.3	15.3	32	34	4	3
19	13.5	14.4	38	40	3	3
20	11.6	11.6	30	31	5	5
	13.2	13.9	33.9	34.7	3.4	3.5
	1.1	1.1	2.7	2.9	0.9	0.9

HAES-STERIL

	PT (min)		aPTT (sec)		BL. TIME (min)	
	PRE	POST	PRE	POST	PRE	POST
1	12.5	15.3	36	46	3	4.5
2	13.5	14.4	35	38	4	5
3	12.5	13.5	31	35	4.5	6
4	13.5	15.3	36	38	4.5	6
5	13.5	14.4	30	33	4.5	6
6	11.6	12.5	39	39	3.5	5
7	14.4	17.1	35	39	2	3.5
8	12.5	14.4	32	34	4.5	5.5
9	13.5	14.4	33	34	2	2.5
10	13.5	14.4	35	39	3	4.5
11	13.5	14.4	32	34	3	4
12	14.4	16.2	34	37	2.5	3.5
13	12.5	14.4	33	38	3	4
14	12.5	14.4	28	31	2.5	3.5
15	11.6	13.5	31	36	3.5	4
16	12.5	14.4	29	33	3.5	5
17	13.5	14.4	30	32	3.5	4
18	13.5	13.5	36	41	3	4
19	13.5	15.3	36	39	3.5	3.5
	13.1	14.6	33.2	36.6	3.3	4.4
	0.8	1.0	2.9	3.6	0.8	1.0

SALINE

	ADRENALIN		COLLAGEN		RISTOCETIN	
	PRE	POST	PRE	POST	PRE	POST
1	19	15	58	56	63	88
2	21	66	66	69	85	83
3	18	24	70	70	88	85
4	16	11	69	79	88	93
5	35	25	66	21	80	85
6	78	85	80	80	80	80
7	79	13	81	80	74	88
8	89	80	90	91	91	88
9	25	81	81	85	86	94
10	63	80	94	75	94	90
11	23	23	90	65	79	95
12	81	83	69	81	80	97
13	78	85	70	70	86	100
14	30	35	75	86	81	85
15	63	16	83	83	76	88
16	46	58	88	100	100	100
17	75	84	69	71	83	86
18	68	83	75	64	82	83
19	100	100	100	100	100	100
20	68	73	60	63	73	55
	53.7	56.0	76.7	74.5	83.4	88.2
	27.8	31.4	11.6	17.2	8.9	9.9

HAES-STERIL

	ADRENALIN		COLLAGEN		RISTOCETIN	
	PRE	POST	PRE	POST	PRE	POST
1	75	10	85	63	86	71
2	68	52	69	10	69	65
3	69	28	63	75	76	75
4	19	11	69	69	69	63
5	20	16	44	38	76	88
6	68	80	73	75	86	86
7	68	8	69	64	86	80
8	89	89	65	83	80	90
9	70	14	75	81	84	81
10	63	40	68	74	84	91
11	24	23	86	60	68	65
12	80	56	63	70	84	83
13	93	83	80	94	97	88
14	33	16	69	86	86	89
15	20	15	68	63	81	94
16	60	14	60	70	80	83
17	75	75	70	70	84	98
18	31	18	64	69	75	84
19	80	81	75	81	80	85
	58.2	38.4	69.2	68.2	80.6	82.1
	25.0	29.7	9.4	18.4	7.2	10.0

SALINE

	100um ADP		10um ADP		5um ADP	
	PRE	POST	PRE	POST	PRE	POST
1	65	75	40	50	46	49
2	65	76	59	68	44	66
3	63	63	54	58	40	54
4	65	68	71	61	58	45
5	85	85	60	65	42	45
6	86	84	81	88	71	83
7	65	65	74	60	31	33
8	88	90	78	85	80	78
9	88	86	80	88	85	91
10	84	74	75	71	73	59
11	73	78	55	60	41	44
12	81	91	81	75	60	66
13	76	88	75	84	45	80
14	68	76	68	75	64	70
15	73	70	59	50	41	35
16	94	94	70	68	50	52
17	78	79	69	75	70	78
18	65	78	70	73	65	64
19	100	100	100	100	100	100
20	70	75	68	85	65	68
	76.6	79.8	69.3	72.0	58.6	63.0
	11.1	9.9	12.8	13.5	17.9	18.5

HAES-STERIL

	100um ADP		10um ADP		5um ADP	
	PRE	POST	PRE	POST	PRE	POST
1	69	63	63	48	40	31
2	64	54	61	52	54	33
3	63	68	68	63	55	39
4	66	56	51	45	48	39
5	69	71	56	58	42	40
6	83	93	78	90	81	86
7	73	69	71	58	56	45
8	85	81	71	78	73	68
9	75	69	75	70	69	64
10	75	81	63	69	55	58
11	64	63	55	52	42	41
12	75	86	65	76	52	59
13	81	75	68	63	46	44
14	81	81	71	81	61	63
15	60	58	36	31	20	25
16	68	73	60	59	55	59
17	69	85	68	69	69	65
18	71	69	54	58	45	44
19	81	79	71	74	63	85
	72.2	72.3	63.4	62.9	54.0	52.0
	7.4	10.8	9.9	14.1	13.9	17.3

SALINE

	Cont-r (mm)	Cont-k (mm)	Cont-α (°)	Cont-MA (mm)	Sal-r (mm)	Sal-k (mm)	Sal-α (°)	Sal-MA (mm)
1	12	7	57	60	9	4	68	64
2	15	11	41	54	9	11	44	59
3	28	15	36	49	24	14	36	49
4	22	12	42	52	18	10	45	53
5	22	12	40	45	16	6	61	53
6	32	15	36	47	26	11	46	51
7	24	11	44	59	18	7	55	59
8	31	17	31	45	16	12	41	51
9	24	11	43	52	19	9	49	54
10	32	16	32	47	31	15	32	49
11	21	12	41	55	13	8	52	57
12	29	17	30	50	24	15	35	49
13	31	15	33	44	23	13	41	50
14	25	13	38	55	19	8	52	58
15	37	16	32	45	26	12	41	51
16	23	15	37	58	18	7	55	64
17	27	15	35	49	22	11	45	51
18	34	18	27	47	27	14	35	50
19	40	24	23	43	28	14	36	45
20	27	12	42	44	24	10	45	46
	26.8	14.2	37.0	50.0	20.4	10.6	45.7	53.2
	6.9	3.6	7.4	5.4	6.0	3.2	9.4	5.4

HAES-STERIL

	Cont-r (mm)	Cont-k (mm)	Cont-α (°)	Cont-MA (mm)	Haes-r (mm)	Haes-k (mm)	Haes-α (°)	Haes-MA (mm)
1								
2	22.0	13.0	39.0	47.0	18.0	9.0	46.0	49.0
3	38.0	23.0	24.0	44.0	22.0	13.0	37.0	43.0
4	20.0	13.0	39.0	51.0	14.0	15.0	33.0	50.0
5	29.0	15.0	32.0	45.0	25.0	16.0	32.0	38.0
6	29.0	14.0	38.0	47.0	27.0	11.0	41.0	46.0
7	14.0	8.0	51.0	77.0	21.0	9.0	50.0	55.0
8	23.0	12.0	42.0	49.0	31.0	17.0	32.0	37.0
9	22.0	12.0	43.0	60.0	5.0	5.0	63.0	53.0
10	37.0	17.0	33.0	49.0	29.0	15.0	34.0	44.0
11	29.0	12.0	40.0	50.0	21.0	12.0	39.0	48.0
12	28.0	13.0	39.0	45.0	29.0	13.0	38.0	48.0
13	23.0	13.0	41.0	50.0	25.0	12.0	39.0	42.0
14	22.0	9.0	49.0	62.0	18.0	9.0	51.0	52.0
15	32.0	17.0	32.0	46.0	29.0	16.0	33.0	38.0
16	28.0	14.0	35.0	54.0	16.0	7.0	35.0	54.0
17	34.0	17.0	30.0	46.0	30.0	16.0	32.0	43.0
18	11.0	9.0	48.0	54.0	29.0	21.0	26.0	37.0
19	30.0	22.0	25.0	39.0	31.0	21.0	25.0	33.0
	26.2	14.2	37. 8	50.8	23.4	13.2	38.1	45.0
	7.2	4.0	7.6	8.6	7.2	4.4	9.4	6.6

APPENDIX 3 - CHAPTER 5

Control v Plas B dilution (r- and k-times in mm, α-angle in ° and MA in mm)

	cont-r	contr-k	cont-α	cont-MA	plas-r	plas-k	plas-α	plas-MA
1	26	11	45	56	18	8	56	54
2	20	10	50	54	22	9	49	56
3	25	13	38	54	24	9	50	57
4	32	14	36	53	18	8	51	56
5	24	12	43	51	20	4	48	48
6	14	5	67	62	13	4	67	66
7	30	13	39	59	22	8	45	58
8	19	10	49	55	17	8	53	53
9	29	11	45	57	19	6	60	58
10	27	11	42	61	24	6	57	59
11	26	13	41	56	24	11	43	59
12	31	13	39	58	26	7	58	60
13	28	12	42	55	25	7	54	57
14	18	14	39	53	19	7	58	56
15	28	13	42	52	24	12	41	49
16	27	9	51	64	17	5	65	66
17	28	10	46	66	19	6	60	62
18	31	15	34	52	26	12	42	53
19	34	13	41	54	32	7	55	55
20	35	15	34	57	28	11	43	54
Means	26.8	11.8	43.2	56.5	22.0	7.8	52.8	56.8
	5.4	2.4	7.4	4.1	4.6	2.4	7.6	4.6

Control v CSF dilution (r- and k-times in mm, α-angle in ° and MA in mm)

	cont-r	cont-k	cont-α	cont-MA	csf-r	csf-k	csf-α	csf-MA
1	13	3	61	67	6	4	70	70
2	18	3	68	69	12	3	70	71
3	22	5	45	62	12	4	72	71
4	20	4	50	65	14	5	64	65
5	17	4	49	65	8	2	78	73
6	23	5	45	57	14	4	71	70
7	22	5.5	50	75	7	3	76	78
8	18	3	60	66	12	3	77	71
9	22	2	71	72	16	3	73	76
10	24	4.5	52	71	16	4	68	78
11	34	6.5	39	50	18	4	67	65
12	33	4	52	66	14	3	73	72
13	7	3	58	56	6	5	64	63
14	7	2.5	60	70	4	3	73	69
15	24	4	54	62	16	6	64	69
16	28	4	53	85	17	3	75	76
17	21	3.5	57	65	14	3	74	73
18	20	5	51	59	15	5	62	67
19	28	5	46	64	19	4	72	76
20	20	2.5	69	68	11	3	76	67
Means	21	8.0	54.5	65.7	12.6	3.8	71.0	71.0
Std Dev	3.6	2.4	8.5	7.5	4.4	1.0	4.8	4.4

APPENDIX 4 - CHAPTER 6

Volunteer	Code	Con No.	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
1	1	1	10%	20	13	34.5	52
	2	2	20%	24.5	11	36.5	51.5
	3	1	30%	15	11	36	51
	4	2	40%	19	7	24.5	51
	5	1	50%	21	16	45	39
	6	2	60%	35	29	16	29
	Control		con 1	22	15.5	27	51.5
	Control		con 2	32	20.5	21.5	46.5
2	1	1	10%	22	8	42	57
	2	2	20%	17	6	52	58
	3	1	30%	21	7.5	52	56
	4	2	40%	22.5	5	55	58.5
	5	1	50%	33	19	23.5	48
	6	2	60%	38	21	22	42.5
	Control		con 1	23	14	30.5	53
	Control		con 2	21	9	38	57
3	1	2	10%	24	9	38	54
	2	1	20%	25	8	43	53
	3	2	30%	20.5	7.5	48	55.5
	4	1	40%	34.5	12	29.5	49.5
	5	2	50%	27	12	32.5	47
	6	1	60%	45	24	19.5	37.5
	Control		con 1	23	13	32	53
	Control		con 2	21	11.1	36.5	58
4	1	1	10%	26.5	14.5	28.5	54
	2	2	20%	21	7	45.5	55
	3	1	30%	25	11	32	48
	4	2	40%	26.5	13	30.5	49
	5	1	50%	23	9	39	50
	6	2	60%	51	18	19.5	38
	Control		con 1	35	20.3	21.5	45
	Control		con 2	23	9	36	54
5	1	1	10%	29	9	44.5	66.5
	2	2	20%	27.5	6.5	54	69.5
	3	1	30%	27	6.5	51	67
	4	2	40%	31	7	49.5	64
	5	1	50%	24	10	38.5	56
	6	2	60%	53	13	28.5	53
	Control		con 1	32	13	30.5	56
	Control		con 2	33.5	13	35	64
6	1	1	10%	19.5	8	45	60.5
	2	1	20%	18.5	7	48	60
	3	1	30%	13	5.5	56	66
	4	2	40%	34	7.5	45.5	54.5
	5	2	50%	35	15	34	50
	6	2	60%	50	14	28	49
	Control		con 1	13	4.5	60	63
	Control		con 2	37.5	12	34	56.5

Volunteer	Code	Con No.	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
7	1	1	10%	26.5	11	35.5	54.5
	2	1	20%	26	11.5	37.5	53
	3	1	30%	21.5	7	45	56
	4	2	40%	17	8.5	38	52
	5	2	50%	15	9	42.5	51
	6	2	60%	28	19	23	41.5
	Control		con 1	19.5	8	41.5	60
	Control		con 2	17.5	13.5	31	53.5
8	1	1	10%	23.5	11.5	34.5	55.5
	2	1	20%	12.5	4.5	59	62
	3	1	30%	19.5	6.5	52	59
	4	2	40%	23.5	10	41	56
	5	2	50%	33	8	32	51.5
	6	2	60%	44	22.5	18	42
	Control		con 1	18	8	41	60
	Control		con 2	31.5	18	24	48
9	1	1	10%	30.5	8.5	42.5	62
	2	1	20%	23	6	52	61.5
	3	1	30%	16	6.5	46	63.5
	4	2	40%	20	7	45	58
	5	2	50%	26.5	10	42	54
	6	2	60%	53	25	16	44.5
	Control		con 1	36.5	14	27.5	54.5
	Control		con 2	20	11	30	59
10	1	1	10%	25	16	28	50
	2	1	20%	30	10	35.5	51
	3	1	30%	31	9.5	38	51.5
	4	2	40%	40	9.5	37	48
	5	2	50%	54.8	16	44	25
	6	2	60%	67.5	27	13.5	38
	Control		con 1	28	16	22	49
	Control		con 2	30.5	23	22	43
11	1	2	10%	35.5	13	31.5	54
	2	1	20%	29.5	10	39.5	54
	3	2	30%	36.5	8.5	46	55.5
	4	1	40%	21	5.5	50	57
	5	2	50%	32.5	12	33	50
	6	1	60%	44	15	27	42
	Control		con 1	30.5	18	28	50.5
	Control		con 2	29	18	22	52.5
12	1	2	10%	23.5	10.5	33.8	50.5
	2	2	20%	19	7	45	56
	3	2	30%	19.5	8	43	56
	4	1	40%	27.5	10	37	54
	5	1	50%	25	9	36	51
	6	1	60%	44	16	25.8	41.5
	Control		con 1	23	12.5	32	52
	Control		con 2	27.5	16	27.5	46

Volunteer	Code	Con No.	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
13	1	2	10%	22	11.5	32.5	56
	2	2	20%	16.5	6.5	45	55
	3	2	30%	14	7	50.5	55.5
	4	1	40%	20	9	40	53
	5	1	50%	34.5	13	29.5	49
	6	1	60%	49	19	20.5	43
			con 1	28.5	12	32.8	48
			con 2	26.5	11.5	37.5	53
14	1	1	10%	24	11	33	54
	2	2	20%	24	11	38	54
	3	1	30%	22	11	38.5	53.5
	4	2	40%	21	7.5	44	54
	5	1	50%	26	7	41.5	50
	6	2	60%	24	12	29	44
			con 1	26	15.5	27.5	47
			con 2	26	15.5	27.5	47
15	1	1	10%	28.5	14.5	28	51
	2	1	20%	24	11	30	55
	3	1	30%	28	10.5	30	50
	4	2	40%	26	16	30	47
	5	2	50%	24	12	31	44
	6	2	60%	25	22.5	17	37
			con 1	36.5	19	22	49.5
			con 2	26	17	21.5	51
16	1	1	10%	31.5	10	38	59.5
	2	1	20%	20	7	44	61
	3	1	30%	29.5	5.5	48.5	61.5
	4	2	40%	30	7	45.5	50
	5	2	50%	31	11	37	53.5
	6	2	60%	30.5	29	26	46.5
			con 1	22	12	33	61
			con 2	28.5	13	32	56
17	1	2	10%	25	13	32	58
	2	2	20%	24	14	32.5	50
	3	2	30%	24.5	12.5	34	54
	4	1	40%	24.5	9	31.5	54
	5	1	50%	35.5	20.5	20	48
	6	1	60%	70	32.5	13	40
			con 1	32.5	22	19	43.5
18	1	1	10%	10.5	6	31.5	43.5
	2	1	20%	12	4	43.5	48
	3	1	30%	11.5	3	54	58
	4	1	40%	11.5	4	46	53.5
	5	1	50%	13.5	5	37.5	49.5
	6	1	60%	21.5	9	23.5	41
			cont 1	15.5	6.5	34	49.5

Volunteer	Code	Con No.	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
19	1	1	10%	16	7.5	24.5	40.5
	2	1	20%	13.5	6	36	49
	3	1	30%	14	4	46	51
	4	1	40%	14.5	6	32.5	47.5
	5	1	50%	18	9	23	40
	6	1	60%	38.5	12	18	-29.5
			cont 1	16.5	8.5	25	43
20	1	1	10%	17	8.5	25	41
	2	1	20%	16.5	8.5	25.5	42.5
	3	1	30%	14.5	5.5	35	47.5
	4	1	40%	18	6	32.5	45
	5	1	50%	20	10	21.5	38.5
	6	1	60%	34	13.5	14	30.5
			cont 1	19	9	24.5	46.5

APPENDIX 5 - CHAPTER 7

Number	Date	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
1	27.1.98	1	27	11	47	60
		2	20	8	54	63
	29.1.98	11	12	5	68	67
		12	9	3	74	71
2	26.1.98	1	27	15	35	44
		2	25	10	44	46
	28.1.98	11	23	10	50	53
		12	23	8	50	49
3	3.3.98	1	23	16	33	53
		2	20	9	50	54
	6.3.98	11	10	4	71	65
		12	7	6	57	51
4	17.3.98	1	30	11	45	58
		2	21	6	62	66
	19.3.98	11	32	14	39	60
		12	25	5	65	67
5	9.3.98	1	31	17	31	52
		2	20	11	45	53
	11.3.98	11	30	14	36	52
		12	15	12	41	56
6	16.2.98	1	32	16	35	49
		2	21	8	52	56
	18.2.98	11	26	15	36	50
		12	19	8	51	59
7	20.1.98	1	18	10	44	54
		2	14	6	59	57
	22.1.98	11	14	6	60	60
		12	15	5	65	59
8	3.2.98	1	25	12	43	48
		2	20	10	47	48
	5.2.98	11	24	13	38	49
		12	20	8	51	51
9	24.2.98	1	17	9	51	57
		2	16	8	54	54
	26.2.98	11	25	14	37	50
		12	21	8	55	55
10	2.3.98	1	24	15	35	52
		2	17	11	44	52
	4.3.98	11	28	15	37	53
		12	22	8	54	55
11	23.3.98	1	24	12	44	52
		2	20	7	54	50
	25.3.98	11	26	13	40	50
		12	21	9	48	50

Number	Date	Sample	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
12	10.2.98	1	22	13	39	50
		2	21	9	48	51
	12.2.98	11	31	15	35	46
		12	24	12	38	46
13	9.2.98	1	21	10	46	56
		2	15	5	64	58
	11.2.98	11	5	3	73	66
		12	19	7	53	55
14	19.1.98	1	20	11	44	52
		2	17	8	33	53
	21.1.98	11	19	10	44	53
		12	15	6	60	57
15	23.3.98	1	28	18	30	51
		2	24	12	41	51
	25.3.98	11	38	18	32	49
		12	32	10	45	50
16	24.3.98	1	33	16	32	48
		2	26	11	42	51
	26.3.98	11	30	17	31	51
		12	26	11	43	48
17	7.4.98	1	28	20	28	48
		2	22	10	48	53
	9.4.98	11	31	18	30	49
		12	27	9	47	48
18	6.4.98	1	26	12	42	55
		2	23	10	45	57
	8.4.98	11	28	15	34	55
		12	22	9	49	54
19	20.4.98	1	24	12	42	54
		2	22	10	45	57
	22.4.98	11	39	20	27	50
		12	29	9	49	52
20	21.4.98	1	20	10	44	55
		2	20	6	61	58
	23.4.98	11	29	14	39	55
		12	27	9	50	53

Sample number:
1.) First sample prior to dilution.
2.) First sample after dilution.
11.) Sample prior to dilution after aspirin.
12.) Sample after dilution and after aspirin.

APPENDIX 6 - CHAPTER 8

Number	SAMPLE	r-time (mm)	AT III maintenance k-time (mm)	α-angle (°)	MA (mm)
1	1	29	11	32	55
	2	27	8	43	59
	3	32.5	12.5	34.5	57.5
	4	22	5	55.5	61
2	1	34	21	19.5	48.5
	2	24	11	35	54
	3	24	16	27	53
	4	22	11	33	53
3	1	22	18	24	47
	2	23	11	34	48
	3	29	19	23	50.5
	4	23	14.5	27	48
4	1	35	16	28	62
	2	29	6	55	63
	3	31	15	29	63.5
	4	34	6.5	53.5	67.5
5	1	15	5.5	55.5	58.5
	2	6.5	6.5	54	55
	3	12	5	54	57
	4	14	6	52	52
6	1	41	21	18	44
	2	32	8	42	51
	3	47.5	24	19.5	42.5
	4	49.5	21.5	20	41.5
7	1	40	13	34	59
	2	32	10	40	62
	3	49	17	25	56.5
	4	54	18.5	28.5	68
8	1	33	23.5	20.5	53
	2	19.5	13	33.5	63
	3	31	21	21	49
	4	24	13	32	49
9	1	42	13	28	54
	2	27	8	41	61
	3	43	18.5	28	55.5
	4	37.5	12	33.5	61
10	1	35	18	22	47
	2	26	10	35	49
	3	40.5	23.5	18.5	41
	4	43	13.5	29.5	47
11	1	39	13	32	62
	2	31	11	28	55
	3	58.5	21.5	18	53.5
	4	33.5	12	29.5	58

AT III maintenance

Number	SAMPLE	r-time (mm)	k-time (mm)	α-angle (°)	MA (mm)
12	1	29	14	33	62
	2	23	9	45	60.5
	3	28	10	38	60
	4	29	10	36	59
13	1	38	18	24	51
	2	32	8	43	56
	3	44	21	21	53
	4	37.5	14.5	28.5	57
14	1	51.5	26.5	18	47.5
	2	41	11.5	32	50.5
	3	40	25	17	47
	4	46	18	22	46
15	1	36	22	22	48
	2	27	7	44	56
	3	45.5	20	22	57
	4	61.5	28	15.5	45
16	1	28.5	14.5	30.5	56
	2	23.5	9.5	40	55.5
	3	25	7	42	55
	4	32	17	25	49
17	1	36	20	22	44
	2	26	13	32	60
	3	36	17	25.5	51.5
	4	41.5	13.5	31	48.5
18	1	45.5	12.5	33.5	52
	2	31.5	13.5	31	55
	3	24	13	34	53
	4	24	8	38	49
19	1	26	18	25	46
	2	36	17	21	43
	3	42.5	21	22.5	40
	4	37.5	11.5	31.5	48.5
20	1	34	16	25	48
	2	27	14	29	52
	3	32	21.5	20.5	51
	4	28	11.5	34	52.5

AT III – test group

Number	Control	20% Dil.	20% Dil./AT
1	76	64	95
2	84	72	108
3	102	69	107
4	105	71	91
5	88	65	100

APPENDIX 7 - CHAPTER 9

Number	r 1 (mm)	r 2 (mm)	r 3 (mm)	r 4 (mm)	r 5 (mm)	r 6 (mm)
1	31.5	26.5	37	25	34	39
2	17	6.5	23	25.5	34.5	38.5
3	31.5	5	42	35.5	36	31
4	23.5	28.5	29	22.5	28	24
5	31.5	21.5	33	49.5	28.5	28.5
6	25	26.5	29.5	25.5	28	23
7	36.5	30	36.5	39	21.5	39.5
8	25	36.5	36.5	38	34.5	36.5
9	53	20	31	29	22.5	26.5
10	28.5	32	35	28.5	35.5	33.5
Mean	30.4	23.4	34.4	31.8	30.4	32.0
SD	9.8	10.4	5.4	8.6	5.4	6.4

Number	k 1 (mm)	k 2 (mm)	k 3 (mm)	k 4 (mm)	k 5 (mm)	k 6 (mm)
1	14	11	14.5	12.5	13.5	16
2	7	5	16	14.5	10	15
3	14.5	6.5	12	10	9.5	10
4	15.5	12	16.5	8	12	13.5
5	7	5.5	10	10	7	5
6	6.5	10	11	11.5	9.5	11
7	16.5	20.5	18	20	21	17.5
8	10	12.5	8	13	7.5	8
9	21	9.5	18.5	18	18.5	21
10	11	11	8.5	9	6.5	7
Mean	12.4	10.4	13.4	12.8	11.6	12.4
SD	4.8	4.6	4.0	4.0	5.0	5.2

Number	α 1 (°)	α 2 (°)	α 3 (°)	α 4 (°)	α 5 (°)	α 6 (°)
1	31	39	30	37.5	33.5	28
2	51.5	61	30	30	39.5	29
3	33.5	52	33.5	42.5	42.5	39
4	28	34	28	43.5	36	31
5	52	58.5	40	43	49.5	58.5
6	50.5	39.5	38	33	39.5	36.5
7	24.5	20	24.5	23.5	21.5	25.5
8	40	33.5	47	34.5	47.5	45.5
9	20.5	40	25.5	27	22.5	25
10	37	39	45.5	39.5	52	49.5
Mean	36.9	41.7	34.2	35.4	38.4	36.8
SD	11.5	12.4	8.1	7.0	10.4	11.3

Number	MA 1 (mm)	MA 2 (mm)	MA 3 (mm)	MA 4 (mm)	MA 5 (mm)	MA 6 (mm)
1	56	59	59.5	57.5	55.5	58.5
2	63.5	66.5	59.5	53	44.5	59
3	58.5	51	55.5	61	61	52.5
4	46.5	54	39.5	52	52.5	49
5	64.5	64.5	63.5	57.5	62	61.5
6	53	52	44.5	42	45	42
7	49.5	41.5	45	45.5	49.5	48.5
8	57	58.5	63	56	63	62
9	40.5	58.5	49.5	50.5	52	53
10	58	61.5	60	59.5	66	61
Mean	54.7	56.7	54.0	53.5	55.1	54.7
SD	7.5	7.3	8.6	6.1	7.6	6.8

Number	Hct 1 (%)	Hct 2 (%)	Hct 3 (%)	Hct 4 (%)	Hct 5 (%)	Hct 6 (%)
1	50.7	46.5	46.4	46.3	44.5	48.1
2	41.1	39.8	39.7	40.2	38.6	37.7
3	39.4	38.3	37.3	37	36.4	35
4	40.1	40.1	37.8	39.3	37	36.1
5	38.4	38.7	39.1	37.5	38.2	37
6	39.2	38.9	38.2	38.5	38.5	37.5
7	46.5	44.9	43.6	43	42.7	42.7
8	38.7	38.8	39.7	37.8	37.2	37
9	47	44.3	43.7	45.1	42.5	42.1
10	44.5	39	38.6	37.8	37.5	37.2
Mean	42.6	41	40.4	40.3	39.3	39.0
SD	4.3	3.1	3.1	3.4	2.8	4.0

Number	ATIII 1 (%)	ATIII 2 (%)	ATIII 3 (%)	ATIII 4 (%)	ATIII 5 (%)	ATIII 6 (%)
1	104	86	89	104	97	103
2	104	91	101	106	93	
3	97		98	99	109	115
4	92	100	84	92	104	99
5	124	123	130	135	76	114
6	128	107	112	118	107	136
7	94	110	120	127	129	
8	117	109	116	136	131	98
9	136	88	138	101	87	141
10		89	112	105	89	125
Mean	110.7	100.3	110.0	112.3	102.2	116.4
SD	16.1	12.7	17.2	15.6	17.7	16.4

Number	Fibri 1	Fibri 2	Fibri 3	Fibri 4	Fibri 5	Fibri 6
1	2.3	2.2	2.5	1.9	2.6	2.8
2	2.7	1.6	2.2	2.4	2.2	
3	2.6		2.8	2.6	2.8	2.7
4	1.8	1.7	1.6	1.3	1.4	1.4
5	3.8	3.5	3.4	4.2	5	3.8
6	2.4	2.9	2.9	2.6	3	2.6
7	2.6	2.3	1.9	2.4	2.3	
8	2.9	3.5	3.3	3.3	2.1	3.6
9	2.5	4.1	2.9	2.9	3	2.8
10	4.7	4.5	3.9	3.9	3.4	3.3
Mean	2.6	2.9	2.7	2.8	2.8	2.9
SD	0.5	1.0	0.7	0.9	1.0	0.7

Number	N'adren 1	N'adren 2	N'adren 3	N'adren 4	N'adren 5	N'adren 6
1	5550	6225	7617	7783	9009	9407
2	4169	4368	5252	4368	4015	4368
3	5683	8379	7683	5650	4777	5009
4	4258	4059	5285	3871	5893	3849
5		4468	5120	4103	5341	3683
6	8236	10004	12103	7451	7860	6004
7	7164	6655	8423	7131	10655	9263
8	9816	8446	9760	12357	13208	9794
9	10357	21716	17628	9418	6269	4843
10	3661	3329	3020	5097	3539	4368
Mean	6543.7	7764.8	8189.2	6722.9	7056.6	6058.8
SD	2484.9	5373.9	4225.8	2690.8	3108.9	2455.2

APPENDIX 8 - CHAPTER 10

Crystalloids (TEG data: **r- and k-times in mm,** α-angle in ° and MA in mm)

		TEG values				TEG diff from control			
Number	**Sample**	**r time**	**k time**	**α-angle**	**MA**	**r time**	**k time**	**α-angle**	**MA**
1	1	20	6	66	43				
	2	14	5	70	50	6	1	-4	-7
	3	3	3	74	66	17	3	-8	-23
	4	24	8	63	41	-4	-2	3	2
2	1	23	9	65	37				
	2	10	4	77	65	13	5	-12	-28
	3	18	6	73	51.5	5	3	-8	-14.5
	4	24	7	71	44	-1	2	-6	-7
3	1	13	6	63	55				
	2	11	5	63	58	2	1	0	-3
	3					0	0		
	4	22	5	61	52	-9	1	2	3
4	1	30	19	48	26				
	2	28	13	54	28	2	6	-6	-2
	3	24	28	47	21	6	-9	1	5
	4	40	19	49	19	-10	0	-1	7
5	1	40	11	56	30				
	2	18	7	71	46	22	4	-15	-16
	3	28	7	64	46	12	4	-8	-16
	4	20	18	55	32	20	-7	1	-2
6	1	22	7	66	41				
	2	11	3	78	73	11	4	-8	-32
	3								
	4	15	5	71	56	7	2	-15	-15
7	1	21	7	44	61	0	0		
	2	14	4	63	65	7	3	-19	-4
	3								
	4	15	10	40	58	6	-3	4	3
8	1	27	12	32	50				
	2	8	4	63	64	19	8	-31	-14
	3	13	5	55	62	14	7	-23	-12
	4	26	9	35	50	1	3	-3	0
9	1	32	16	27	52				
	2	16	6	53	64	16	10	-26	-12
	3	18	8	44	59	14	8	-17	-7
	4	16	7	49	62	16	9	-22	-10
10	1	35	19	22	50				
	2	16	9	40	58	19	10	-18	-8
	3	18	11	35	53	17	8	-13	-3
	4	19	13	30	51	16	6	-8	-1
11	1	23	11	36	54				
	2								
	3	23	12	37	57	0	-1	-1	-3
	4	16	9	34	53	7	2	2	1

Number	Sample	r time	k time	α-angle	MA	r time	k time	α-angle	MA
12	1	30	8	47	77				
	2	22	5	60	80	8	3	-13	-3
	3	14	3	72	81	16	5	-25	-4
	4	25	7	53	84	5	1	-6	-7
13	1	34	18	31	76				
	2	24	11	48	82	10	7	-17	-6
	3	15	9	49	85	19	9	-18	-9
	4	40	15	29	80	-6	3	2	-4
14	1	28	8	49	77				
	2	23	5	52	82	5	3	-3	-5
	3	24	8	50	77	24	0	-1	0
	4	40	6	55	81	-12	2	-6	-4
15	1	30	15	33	57				
	2	15	6	54	73	15	9	-19	-17
	3								
	4								
16	1	25	14	27	56				
	2	27	13	30	53	-2	1	-3	3
	3	8	3	68	67	17	11	-41	-13
	4	34	28	30	56	-9	-14	-3	0
17	1	13	3	62	69				
	2	17	6	46	63	-4	-3	16	6
	3	23	7	49	66	-10	-4	13	3
	4	25	10	36	62	-12	-7	26	7
18	1	21	7	40	58				
	2	21	7	50	63	0	0	-10	-5
	3	13	5	55	67	10	2	-15	-9
	4	20	7	50	63	1	0	-10	-5
19	1	37	7	49	70				
	2	38	6	55	72	-1	1	-6	-2
	3								
	4								
20	1	21	13	33	61				
	2	19	7	45	65	2	6	-12	-4
	3								
	4								
21	1	27	22	20	46				
	2	26	15	26	48	1	7	-6	-2
	3	8	6	52	61	19	16	-32	-15
	4	30	22	22	46	-3	0	-2	0

Colloids (TEG data: r- and k-times in mm, α-angle in ° and MA in mm)

		TEG values				TEG diff from control			
Number	**Sample**	**r time**	**k time**	**α-angle**	**MA**	**r time**	**k time**	**α-alpha**	**MA**
1	1	21	8	58	38				
	2	16	9	58	37	5	-1	0	1
	3	17	7	56	44	4	1	2	-6
	4	20	4	56	56	1	4	2	-18
2	1	21	15	51	36				
	2	17	8	71	42	4	7	-20	-6
	3	36	15	60	36	-13	0	-9	0
	4	25	11	56	44	-4	4	-5	-8
3	1	23	10	53	32				
	2	25	9	53	37	-2	1	0	-5
	3	19	9	48	39	4	1	5	-7
	4	16	5	60	57	7	5	-7	-25
4	1	40	13	52	27				
	2	23	11	50	33.5	17	2	2	-7.5
	3	14	7	44	49	26	6	8	-22
	4	0	0						
5	1	39	26	43	19				
	2	28	19	45	20	11	7	-2	-1
	3	28	18	43	25	11	8	0	-6
	4	29	8	52	42	10	18	-9	-23
6	1	25	10	68	39				
	2	28	9	69	33	-3	1	-1	6
	3	7	4	64	56	18	6	4	-17
	4	100	20	49	9	-75	-10	19	30
7	1								
	2								
	3								
	4	6	6	52	50				
8	1	18	7	58	45				
	2	14	10	51	37	4	-3	7	8
	3	20	13	44	29	-2	-6	14	16
	4								
9	1	17	8	42	61				
	2	8	6	55	65	9	2	-13	-4
	3								
	4	28	10	36	63	-11	-2	6	-2
10	1	24	11	35	62				
	2	11	13	33	61	13	-2	2	1
	3	29	10	35	51	-5	1	0	11
	4	28	9	36	51	-4	2	-1	11
11	1	12	6	45	64				
	2	10	5	51	70	2	1	-6	-6
	3	15	7	49	62	-3	-1	-4	2
	4	21	7	47	65	-9	-1	-2	-1

Number	Sample	r time	k time	α-angle	MA	r time	k time	α-alpha	MA
12	1	19	7	50	63				
	2	20	6	50	61	-1	1	0	2
	3	21	5	58	66	-2	2	-8	-3
	4	30	10	41	64	-11	-3	9	-1
13	1	9	4	60	65				
	2	39	20	21	51	-30	-16	39	14
	3	38	21	19	50	-29	-17	41	15
	4	40	16	26	52	-31	-12	34	13
14	1	27	13	30	61				
	2	26	10	37	61	1	3	-7	0
	3	30	9	36	61	-3	4	-6	0
	4	4	5	51	64	23	8	-21	-3
15	1	21	9	40	52				
	2	14	5	58	66	7	4	-18	-14
	3	19	6	45	56	1	3	-5	-4
	4	23	7	45	61	-2	2	-5	-9
16	1	25	12	34	55				
	2	18	24	34	50	7	-12	0	5
	3	23	8	41	55	2	4	-7	0
	4								
17	1	4	4	65	58				
	2	13	13	34	45	-9	-9	31	13
	3	14	5	58	67	-10	-1	7	-9
	4	11	4	63	76	-7	0	2	-18
18	1	20	8	49	79				
	2	20	6	56	77	0	2	-7	2
	3	22	6	55	76	-2	0	-6	3
	4	35	18	48	78	-15	-10	1	1
19	1	30	12	39	57				
	2	24	9	44	60	6	3	-5	-3
	3								
	4								
20	1	25	11	38	68				
	2	18	7	47	70	7	4	-9	-2
	3	25	15	37	56	0	-4	1	12
	4	31	16	25	55	-6	-7	13	13
21	1	29	14	30	55				
	2	12	6	51	61	17	8	-21	-6
	3	28	16	25	47	1	-2	5	8
	4	32	20	25	50	-3	-6	5	5
22	1	27	17	27	53				
	2	21	10	36	55	6	7	-9	-2
	3	32	13	29	49	-5	4	-2	4
	4	66	18	22	48	-39	-1	5	5

Crystalloids

Number	Sample	HCT (°)	INR	Plts	TAT	D-Dimers
1	1	35	1.4	29	7.2	756
	2	33	1.5	28	16.3	993.5
	3	30	1.4	23	238.8	978
	4	30	1.6	33	3.2	635.8
2	1	39	1.4	27	22.1	260.4
	2	36	1.7	25	40.4	551.8
	3	33	1.1	22	80.1	586.6
	4	35	1.5	28	21.5	390.1
3	1	44	1.4	27	3.2	226.7
	2	38	1.5	31	12.2	886
	3	33	1.5	28	3.1	207.5
	4	36	1.8	120	17.8	561.9
4	1	52	1.3	27	5.1	264.4
	2	49	1.3	28	11	443.4
	3	44	1.3	27	24.6	421.2
	4	42	1.4	31	0.9	328.2
5	1	42	1.2	30	0	196.5
	2	39	1.2	25	88.5	2479.5
	3	35	1.3	30	3.9	948.1
	4	31	1.6	30		
6	1	38	1	26	5.5	249.9
	2	37	1.1	24	14.2	498.6
	3	36	1	20	14.2	299.8
	4	32	1.1	27	33.1	722.4
7	1	48	0.8	32	291	92.38
	2	43	0.9	32	101.2	247.5
	3				680.3	1158
	4	39	0.9	33		
8	1	45	1.2	24		
	2	40	1.1	24		
	3	41	1	24		
	4	40	1	30		
9	1	44	1.1	38	22.1	1067
	2	41	1.1	31	62.5	996.2
	3	36	1.3	34	47.3	555.8
	4	35	1.1	40	20.5	1058
10	1	48	1.1	32	120.1	3032.5
	2	43	1.1	33	23.8	573.2
	3	43	1.2	31	33.1	613.5
	4	44	1.2	35	68	635.5
11	1	52	1.3	28	15.5	580.2
	2	46	1.4	28	9.5	545
	3	44	1.5	27	23.1	558.6
	4	43				

Number	Sample	HCT (°)	INR	Plts	TAT	D-Dimers
12	1	37	1.8	35	5.8	234.3
	2	36	1.3	41	98.1	497.8
	3	26	1.5	36	20.9	222.1
	4	25	1.5	33	2.5	215.8
13	1	36	1.3	32	15.5	380.6
	2	29	1.3	32	61.9	1028
	3	26	1.3	32	1.7	724.4
	4	27	1.3	32		
14	1	37	1.2	29	5.9	733.1
	2	17	2.3	41		
	3	39	1.5	31	4.2	661.9
	4	31	1.2	26	2	940
15	1	48	1.4	29	7.6	217.8
	2	49	1.4	26	23.2	367.4
	3					
	4					
16	1	47	0.8	37	181	1082
	2	42	0.8	38	122.5	850.3
	3	42	0.8	38	40.4	272.1
	4	40	0.9	35		
17	1	27	1.1	42	193.5	1184
	2	27	0.8	34	191.4	1132
	3					
	4	30	1.3	49		
18	1	43	1.7	39		
	2	41	0.8	33	32	1024
	3		1.1	32	242.2	1067
	4		0.9	34	263.5	1220
19	1	39	1.1	29	3.9	241.2
	2	35	1.1	29	66.6	295
	3					
	4					
20	1	42	1.1	22		
	2	35	1.1	22		
	3					
	4	36	1.1	43		
21	1	48	1.2	32	4.2	238.2
	2	47	1.2	32	3	187.6
	3	44	1.1	25		
	4	45	1.2	32	4.6	342.2

Colloids

Number	Sample	HCT (°)	INR	Plts	TAT	D-Dimers
1	1	37	1.2	24	16.6	103.8
	2	33	1.2	22	115	750.2
	3	29	1.4	26	17.2	110.7
	4	20	1.6	30	61.1	4.66
2	1	40	1	27	5.1	266.5
	2	35	1.1	27	40.4	244.1
	3	26	1.3	33	3.8	135.3
	4	25	1.3	35	1.9	167.3
3	1	43				
	2	36				
	3	34				
	4	25	1.3	33		
4	1	47	1.5	31	1.9	213.2
	2	40	1.6	32	2.2	172.2
	3	36	1.7	29	78.4	5615
	4					
5	1	50	1.4	29	2.1	79.94
	2	47	1.6	29	9.4	138.4
	3	44	1.6	29	5.4	125.5
	4	47	1.4	30	15.7	889.6
6	1		1.7	34	0.6	287.5
	2	37	1.8	36	32	437
	3	32	1.6	23	89.7	11080
	4				1.9	317.5
7	1	43	1.2	29	5.9	849.5
	2	38	1.3	29	4.4	720.5
	3	30	1.5	35	41.7	4645
	4	28	1.4	24	20.3	453.9
8	1					
	2					
	3					
	4					
9	1	47	0.8	31	99.4	95.45
	2	42	0.9	27	134.9	1272.5
	3					
	4	39	0.9	30		
10	1	43	0.8	30	36.9	1074
	2	38	0.8	31		
	3	34	1	37	57.9	205.8
	4	33	0.9	36	41.9	174.6
11	1	43	1	29	101.2	119.5
	2	39	1	29	1017.3	136.9
	3	35	2.3	31		
	4	37	0.8	33	81	275.8

Number	Sample	HCT (°)	INR	Plts	TAT	D-Dimers
12	1	35	0.9	36		1205
	2	33	0.9	35	433.5	1270
	3	28	0.9	36	680.3	1117
	4	33	0.9	28	117.8	762.2
13	1		0.9	35		
	2	50	0.9	39	72.5	774.2
	3	48	1.1	41	60.3	427
	4	38	0.9	40	9.9	208
14	1	46	1.5	32	58.7	184
	2	41	1.4	31	162.5	280
	3	38	1.4	34	68.4	283
	4	44	2.9	47	56.3	1202
15	1	40	1.2	33	112.2	990
	2	37	1.2	32	105.8	770
	3	36	1.3	35	79.3	342.6
	4	35	1.2	35		
16	1	42	1	26	5.1	266.5
	2	36	1.1	29	40.4	244.1
	3	35	1.1	28	3.8	135.3
	4		1.1	22	1.9	167.3
17	1	40	1	23	80.5	707.4
	2		2.2	41	19.1	785
	3	27	1.5	26	35.5	782.7
	4	34	1.3	23		
18	1	38	1.8	30	2.2	129.7
	2	36	1.9	31	3.7	142.2
	3	29	2.1	34	2	140.9
	4	33	2	31		
19	1	37				
	2	35	1.5	29	5	198
	3					
	4					
20	1	41	1.6	30	2.4	292.2
	2	36	1.6	29	46.3	372.8
	3	33	1.5	29	6.2	156.9
	4	32	3.7	40	71.7	2145
21	1	56	1.9	46	3.8	187.1
	2	55	2	43	47.1	2704
	3	49	2.1	52	4.6	160.9
	4	49	1.7	43	3.8	222.6
22	1	50	1.4	33	35.4	323.3
	2	45	1.4	34	24.6	307.9
	3	39	1.5	35		
	4	48	1.4	34		

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