The Control Of Thrombin Generation In Haemoglobinopathies and Other Haemolytic Red Cell Disorders In Children

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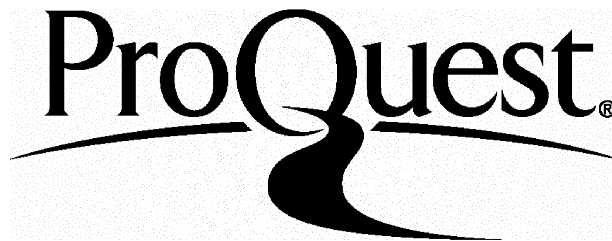

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Abstract

Vascular occlusion is a complex process that has a central role in the pathophysiology of sickle cell disease (SCD) and it is known that thrombosis contributes to large vessel and possibly also to small vessel occlusion. In childhood the most serious complication of SCD is stroke secondary to cerebrovascular disease (CVD) which is symptomatic in 7-10% of children and a further 14% have asymptomatic disease on imaging.

The role of haemostatic abnormalities in the development of vascular occlusion in children with SCD, in particular those with CVD, has not been comprehensively studied before. The principle objectives of this thesis were to perform a prothrombotic profile, including coagulation inhibitors and markers of thrombin generation, in children with SCD and to compare results between different groups; untransfused and transfused; those with CVD and those without; those with haemoglobin SS (HbSS) and those with haemoglobin SC (HbSC), thalassaemia major, other haemolytic red cell disorders and normal controls.

I have found significant reductions in the majority of the natural anticoagulants in children with HbSS compared to sibling controls and also substantial elevation in markers of thrombin generation in the steady-state and evidence of acquired activated protein C resistance. These findings were only partially reversed by transfusion and were not as marked in the group with HbSC though thalassaemics also had reduced levels of most inhibitors. There was no evidence that antiphospholipid antibodies were involved in any group. In the groups with or without CVD there were no differences for any of the parameters measured except for lower haemoglobin levels and higher white cell counts in those with asymptomatic CVD so the abnormalities found in the HbSS children do not appear to play a primary role in the development of CVD.

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Abbreviations

ABS	adult bovine serum
ACL	anticardiolipin antibodies
ACS	acute chest syndrome
Alb	albumin
APA	antiphospholipid antibodies
APC	activated protein C
APTT	activated partial thromboplastin time
AST	aspartate transaminase
β_2-GP1	β_2-glycoprotein 1
Bili	bilirubin
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
C4b-BP	C4b binding protein
CRP	C-reactive protein
CSSCD	Co-operative study of sickle cell disease
CVA	infarctive stroke
CVD	cerebrovascular disease
FBC	full blood count
Fib	fibrinogen
FII	prothrombin
FIX	factor IX
FPA	fibrinopeptide A
FPS	free protein S
FV	factor V
FVII	factor VII
FVIII	factor VIII
FVR506Q	factor V Leiden mutation
FX	factor X
FXI	factor XI
FXII	factor XII
G6PD	glucose-6-phosphate dehydrogenase
Hb	haemoglobin
HbA	haemoglobin A
HbAC	haemoglobin C-trait
HbAS	sickle cell trait
HbF	fetal haemoglobin
HbH	haemoglobin H
HbS	sickle haemoglobin
HbSβ^+thal	haemoglobin Sβ^+ thalassaemia
HbSβ^0thal	haemoglobin Sβ^0 thalassaemia
HbSC	heterozygous haemoglobin S and haemoglobin C
HbSS	homozygous haemoglobin S disease
HCII	heparin cofactor II
HMWK	high molecular weight kininogen

HRG	histidine-rich glycoprotein
HRP	horseradish peroxidase
HS	hereditary spherocytosis
KK	kallikrein
MCV	mean corpuscular volume
MRA	magnetic resonance angiography
MRI	magnetic resonance imaging
MTHFR-T	methyltetrahydrofolate reductase - thermolabile variant
NC	normal controls
OPD	orthophenylenediamine
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PAI-3	plasminogen activator inhibitor-3
PBS	phosphate buffered saline
PC	protein C
PCI-I	protein C inhibitor-I
PEG	polyethylene glycol
PF1+2	prothrombin fragment F1+2
PK	pyruvate kinase
PKK	prekallikrein
Plts	platelet count
PS	protein S
PT	prothrombin time
PTS	phosphatidylserine
RBC/rbc	red blood cell
Retics	reticulocytes
SCD	sickle cell disease
Sec	second
SSRBC	sickle erythrocytes
TATs	thrombin-antithrombin complexes
TCD	transcranial doppler ultrasound
TF	tissue factor
TPA	tissue plasminogen activator
TPS	total protein S
TT	thrombin time
UPA	urokinase-type plasminogen activator
VTE	venous thromboembolism
VWF	von Willebrand factor
VWF:RAg	von Willebrand factor antigen
WBC	white blood cell count

Acknowledgments and declaration

This thesis describes work carried out over a 3-4 year period whilst I was employed as a Senior Registrar rotating between Great Ormond Street Hospital for Children NHS Trust and University College Hospitals NHS Trust. I am very grateful to my supervisor Professor Samuel J Machin and to Dr Ian Mackie, Senior Lecturer at the Haemostasis Research Unit for their guidance and help. The collection and processing of all samples and data and the majority of the laboratory work was performed by myself. However I am very grateful to Janet Cookson for her teaching and supervision of the factor and inhibitor assays and to Natalie Sturt who performed the PCR for Factor V Leiden. I am also grateful to Sally MacDonald, Andrew Chitolie and Siobhan Donohoe, staff of the Haemostasis Research Unit at University College London, for their help with the assays for antiphospholipid antibodies and the markers of thrombin generation.

Chapter 1

Introduction

1.0 *The Haemostatic System*

1.0.1 *Introduction*

It is necessary to give an overview of the haemostatic system and how abnormalities within it can cause an increased tendency to fibrin formation and thrombosis before considering what pathophysiological mechanisms are important in sickle cell disease (SCD) and what evidence there is that haemostatic abnormalities may contribute towards the complications of sickle cell disease.

In response to damage to the vessel wall a haemostatic plug is formed following a complex interplay between platelets, plasma proteins and the damaged vessel. This response must be effective within minutes and yet any inappropriate clot formation can be disastrous, so it is not surprising that the control of the haemostatic system is extremely elaborate, involving both positive and negative feedback loops and mechanisms to localise coagulation to the required site. Once a haemostatic plug has served its purpose and the vessel has been repaired it needs to be removed by the fibrinolytic system, which is also responsible for lysing intravascular fibrin formed inappropriately.

1.0.2 *Primary haemostasis*

Blood platelets play a critical role in the process of haemostasis. Originating as anucleate fragments of megakaryocyte cytoplasm, enveloped by a highly organised unit membrane, they circulate in the form of smooth discs. When the endothelial lining of the vessel wall is breached, bringing platelets into contact with subendothelium or deeper layers of vessel wall, within seconds the platelets rapidly adhere and spread over the surface of these tissues. They then rapidly undergo the process of activation, which is marked by morphologic changes with the formation of pseudopodia, the generation of biologically active mediators and

degranulation. These processes facilitate the linkage of platelets together to form a haemostatic plug, the final and most visible step in primary haemostasis, platelet aggregation. This primary haemostatic plug is unstable until reinforced by fibrin strands generated by plasma coagulation reactions (see section 1.0.3). Platelet adhesion to subendothelium and their subsequent aggregation require platelets to interact with components of the vessel wall, as well as with adhesive proteins present in blood plasma. In addition, for successful primary haemostasis, a proper complement of platelet membrane receptors, an intact platelet cytoskeletal system, and the necessary biochemical machinery for transducing intracellular signals must be present for appropriate platelet activation.

As well as the functions of the platelet detailed above, it is now recognised that the influence of the platelet extends further to other aspects of haemostasis. The platelet provides a phospholipid surface for many of the coagulation reactions (see section 1.0.3) and it generates biologically active mediators, such as thromboxane A_2, that modulate vascular tone and the degree of platelet activation. It also secretes a broad array of granule constituents that stimulate vessel repair, induce platelet aggregation, inhibit the heparin-antithrombin interaction, or limit the degree of fibrinolysis.

The same processes that ordinarily lead to platelet plug formation can also produce arterial thrombosis which, though usually important in atherosclerotic vessels in adults, is seen in childhood in some conditions, for example, SCD, stroke, Kawasaki disease and haemolytic uraemic syndrome. However there are no well-characterised abnormalities in the platelet that predispose to arterial thrombosis but it is thought that inappropriate platelet activation, possibly triggered by dysfunctional endothelium, may be important.

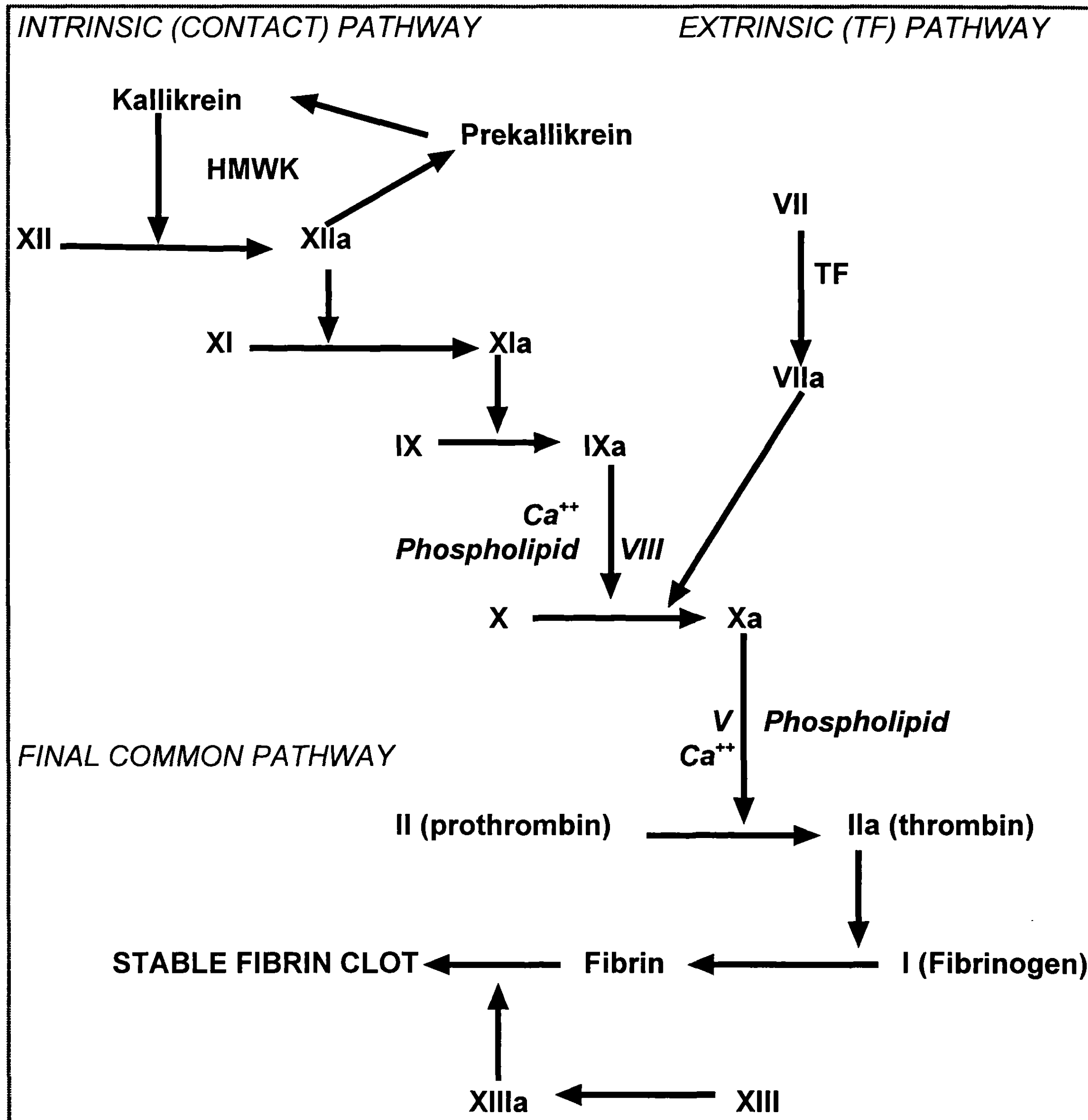
1.0.3 The coagulation pathway

Blood coagulation involves a biological amplification system in which relatively few precursor substances sequentially activate by proteolysis a cascade of circulating precursor proteins (the coagulation factor enzymes) which culminates in the generation of thrombin. It is now understood that *in vivo* the contact (intrinsic) and the tissue factor (extrinsic) pathways of coagulation are intimately interrelated with each other, though in the past they were thought to separately be capable of activating FX to bring about prothrombin activation and thrombin generation - the 'classical' coagulation pathway - see figure 1. However for *in vitro* purposes of diagnosing patients with haemostatic abnormalities and for monitoring patients receiving anticoagulation therapy the use of the prothrombin time (PT) to monitor the integrity of the tissue factor pathway and the activated partial thromboplastin time (APTT) to measure the contact pathway have proven enormously useful.

The contact pathway is initiated by the exposure of negatively charged phospholipid containing surfaces, usually by disruption or damage of the vessel wall endothelium. Factor XII (FXII) binds strongly to such surfaces, and prekallikrein (PKK) and factor XI (FXI) bind to a cofactor, high molecular weight kininogen (HMWK), which links them to the surface. It has been proposed that surface bound FXII makes the first proteolytic cleavage that activates PKK to kallikrein (KK). KK then activates FXII to FXIIa and this then converts more PKK to KK as well as activating FXI. The above steps are known as contact activation. FXI consists of 2 identical subunits and upon activation these are separated to give 2 molecules of FXIa which activate factor IX (FIX) to FIXa. In the presence of calcium FIXa complexes with activated factor VIII (FVIIIa) and factor X (FX) on a

Figure 1 The 'classical' coagulation pathway.

The intrinsic system assumes an exposure to an activating surface 'contact activation'. The extrinsic system assumes release of TF from blood vessel damage.



phospholipid surface. The FX in this 'tenase' complex is cleaved to the active form, FXa. This is the classical 'intrinsic' pathway or contact pathway and coagulation through this pathway must be important because FVIII and FIX deficiencies cause significant bleeding disorders, namely haemophilia A and B. However if the only mechanism for FIX activation was through the intrinsic pathway then severe deficiencies of the proteases required for this activation (HMWK, FXII and FXI)

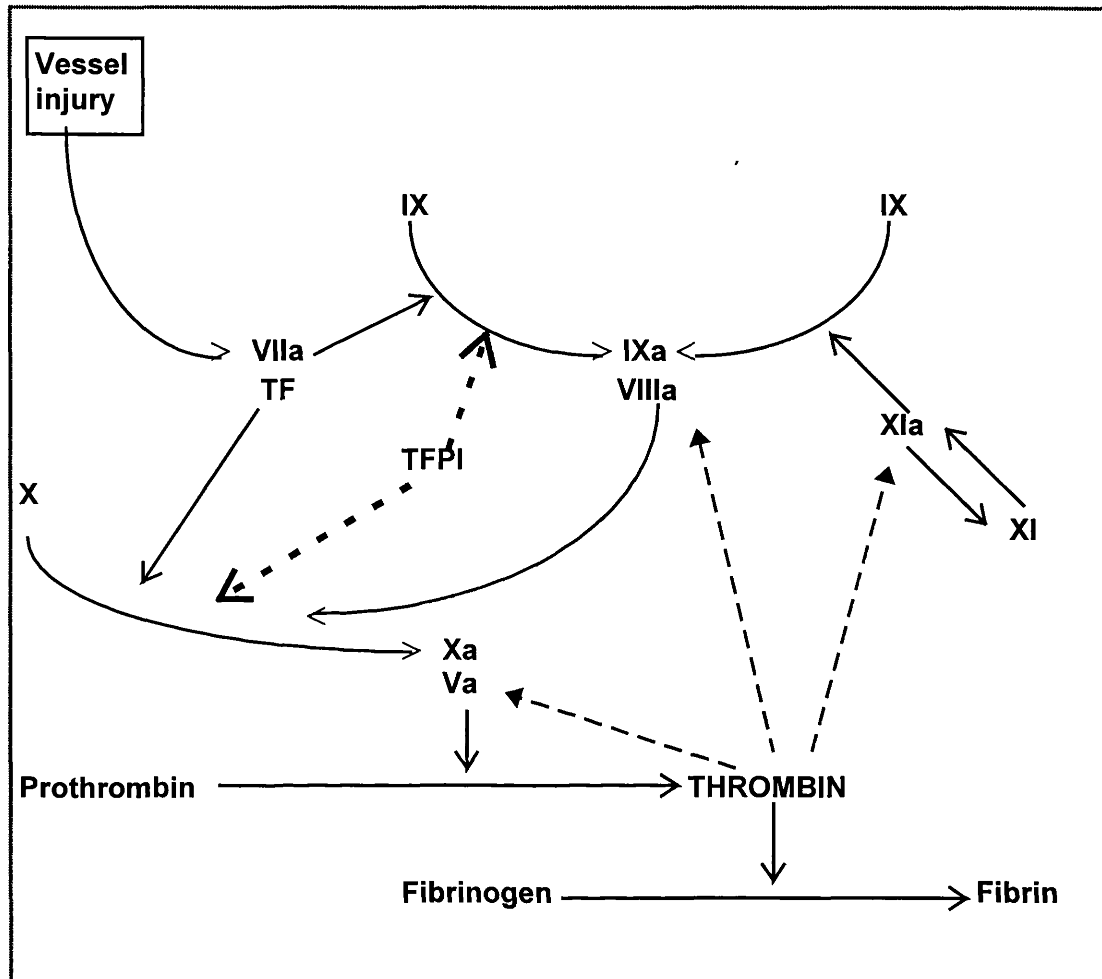
would result in a clinical picture similar to haemophilia. This, in fact, is not what is observed. Congenital FXI deficiency results in a relatively mild bleeding diathesis whereas deficiencies of the contact activation factors (FXII, PKK, HMWK) do not predispose to bleeding. However as factor VII (FVII) is essential for tissue factor initiated coagulation a deficiency state can confer a significant haemorrhagic tendency. This indicates that coagulation is probably initiated predominantly through the extrinsic pathway and that mechanisms other than the intrinsic pathway exist for FIX activation.

The 'extrinsic' or tissue factor pathway is triggered when tissue damage or cell perturbation exposes a lipoprotein known as tissue factor (TF). TF is not normally expressed by cell types in direct contact with blood such as vascular endothelial cells but is expressed constitutively by fibroblasts and pericytes which underlie the vascular endothelium and also by activated monocytes under certain conditions. When expressed, TF binds to and activates FVII and this TF/VIIa complex is a potent activator of FX and also directly activates FIX (Osterud and Rapaport, 1977). FVII is also itself activated by FXa and FIXa forming a short positive feedback loop, and it is also activated by thrombin and by FXIIa. FVIIa has no activity but remains relatively stable in the absence of TF. The physiological inhibitor of TF initiated coagulation, tissue factor pathway inhibitor (TFPI), appears to target mainly the TF/VIIa complex rather than unbound FVIIa and inhibition of TF/VIIa by TFPI is dependent on initial binding to FXa.

To summarise, the current revised hypothesis of coagulation (see figure 2) supposes that coagulation is initiated when FVII or FVIIa in blood gains access to TF at the site of a blood vessel wound. The resulting TF/VIIa complex would then activate some FX to FXa and FIX to FIXa. With the initial generation of FXa, however, the inhibitory properties of TFPI become manifest and inactivate TF/VIIa and also feedback to inhibit FXa (Galiani and Broze, 1997). Any additional FXa

Figure 2 The revised hypothesis of coagulation.

All procoagulants are integrated into single pathway. Feedback inhibition by TFPI is indicated by heavy short-dashed arrows. Thrombin activation is indicated by long-dashed arrows with filled arrowheads.



required for haemostasis must then be provided through the activity of FIXa in the presence of its cofactor, FVIIIa, and additional FIXa through the activity of FXIa. The FXa dependent inhibition of TF/VIIa by TFPI explains the need for intact extrinsic (FVII) as well as intrinsic (FIX and FXI) pathways for proper clot formation. In this revised model the bleeding in haemophilic patients is likely to be due to the lack of a mechanism for sustaining coagulation after the initiation complex, TF/FVIIa, has been inhibited by TFPI. This does not explain why FXI deficient patients can have a bleeding diathesis but it may be that during severe haemostatic

challenges the FIXa, activated initially by the TF/FVIIa complex, may be insufficient for proper clot formation and in these situations supplemental FIXa provided through the activity of FXIa is required and it also appears that both thrombin and FXIa can feedback to activate FXI (Galiani and Broze, 1991; Naito and Fujikawa, 1991).

In the final common path, FXa forms a complex with activated factor V (FVa) and prothrombin on a phospholipid surface in the presence of calcium. The prothrombin in this 'prothrombinase' complex is cleaved to form thrombin. Thrombin converts fibrinogen to fibrin and activates FXIII which then cross-links the fibrin strands. The cofactors V and VIII are activated by thrombin and less efficiently by factor Xa. Calcium ions are required for many of the reactions including all the phospholipid dependent steps. Thrombin is also a potent platelet activator which propagates further platelet aggregation, but conversely trace amounts also play a critical role in the anticoagulant pathway (see section 1.0.4). Thrombin therefore plays a critical central role in the control of coagulation reactions and it is possible to detect 'hyperactivity' and overproduction of thrombin through the coagulation pathway by measuring such parameters as the complexes formed between thrombin and antithrombin (TAT complexes) and also prothrombin fragment F 1+2 (PF1+2) which is produced when prothrombin is cleaved to form thrombin. These provide sensitive markers on the extent of *in vivo* activation of coagulation.

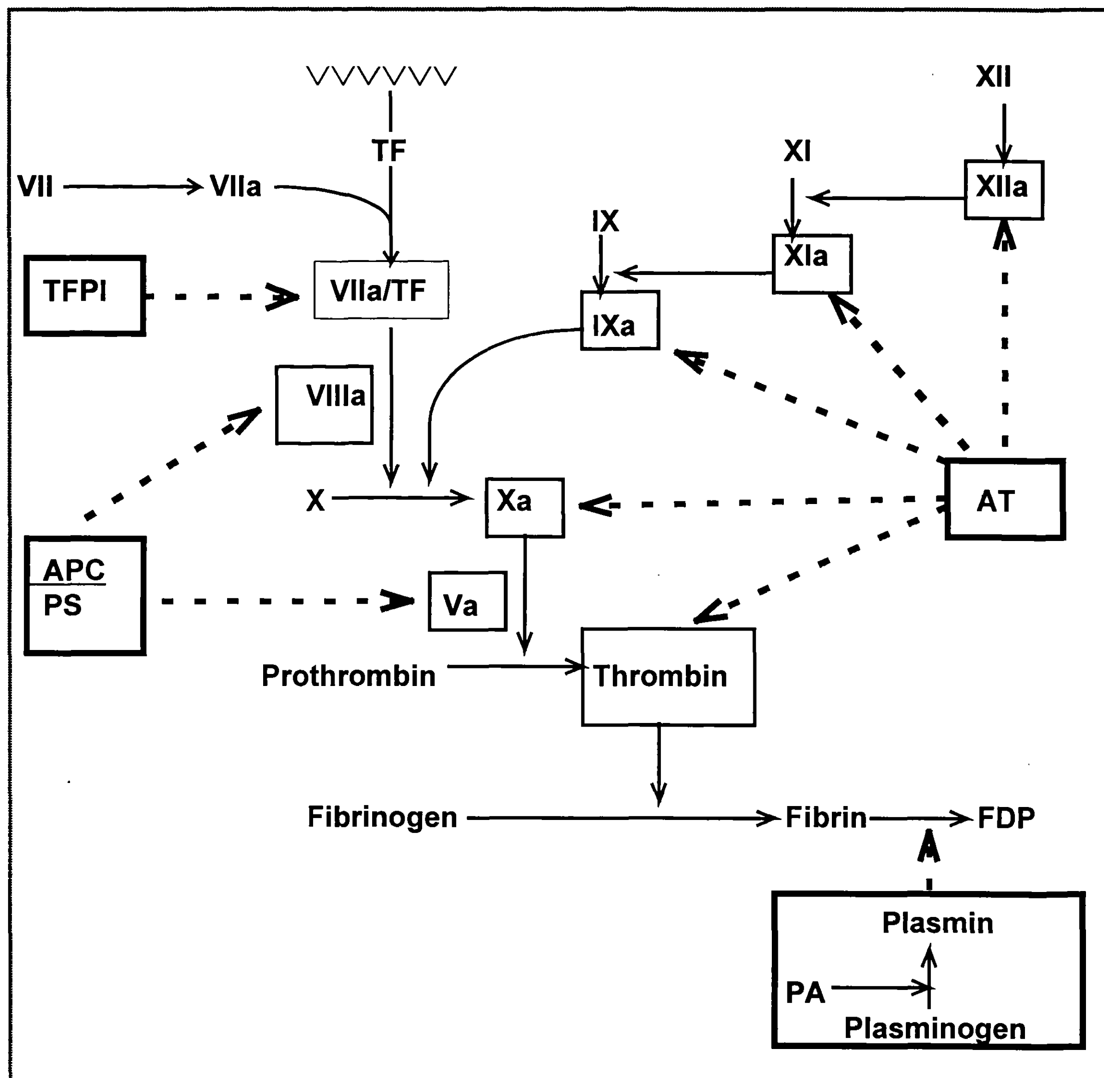
1.0.4 Inhibitors of coagulation

The coagulation system with its many positive feedback loops must be carefully controlled by natural inhibitors to provide the delicate balance that is required to achieve adequate coagulation when necessary without excess fibrin

formation and thrombosis. These natural anticoagulants preserve systemic flow under normal circumstances and participate in limiting blood clots to sites of vascular injury. There are four main antithrombotic pathways - antithrombin, protein C / protein S, the fibrinolytic system and tissue factor pathway inhibitor as shown in figure 3. As the figure shows these proteins essentially blanket the entire coagulation system, acting at various strategic points to quench the cascade and reduce fibrin accumulation in the circulation.

Figure 3 Sites of the four major physiological antithrombotic pathways

The antithrombotic pathways are indicated by heavy borders and sites of inactivation by heavy dashed arrows.



Antithrombin (AT)

The first of these pathways involves antithrombin (AT), which is a *ser*ine *p*rotease *in*hibitor, a member of the 'serpin' superfamily of plasma proteins. It is considered to be the main physiological inhibitor of thrombin and FXa and *in vitro* it also neutralises other serine proteases - FXIIa, FXIa and FIXa but the contribution to their inactivation *in vivo* is not fully determined. Its activity is greatly enhanced in the presence of heparin and natural heparin-like glycosaminoglycans found on the blood vessel endothelial surface. Heparin promotes the formation of a complex with AT and proteases, in which the active site of the protease is brought into close contact with the reactive site of AT. This reduces the half-time of thrombin inhibition in plasma from approximately 40 seconds to 10 milliseconds (Bjork et al. 1989). Its role as the major inhibitor of thrombin is illustrated by the significant (mainly venous) thromboembolic complications found in patients with inherited deficiency of AT (Egeberg, 1965). This is an autosomally dominant inherited condition occurring in 0.2 - 0.4% of the general population (Tait et al. 1990) and can be due to either a quantitative deficiency of the protein or qualitative defect in the molecule. Therefore a functional assay is preferable to an immunological assay to screen for AT deficiency. In unselected series of patients with a history of VTE the frequency of AT deficiency is approximately 1.1% (Heijboer et al. 1990).

Heparin cofactor II (HCII) is a similar protein to AT. It specifically inhibits thrombin but not FXa and its activity is only moderately enhanced by heparin but strongly by another glycosaminoglycan, dermatan sulphate. Familial HCII deficiency has been suggested as a risk factor for venous thrombosis (Tran et al. 1985; Sie et al. 1985).

The protein C / protein S pathway

Protein C (PC) and protein S (PS), like FII, FVII, FIX and FX are vitamin K dependent proteins synthesised in the liver. PC is cleaved and converted to activated PC (APC) by thrombin in the presence of an endothelial receptor, thrombomodulin (see figure 3). This is a phospholipid dependent reaction and the high affinity binding of thrombin to thrombomodulin produces a 20,000-fold increase in the rate of PC activation (Dahlback, 1995). Once thrombin binds to thrombomodulin it has greatly reduced activity towards fibrinogen and no longer activates platelets. APC binds to its cofactor PS and the principal function of the PC/PS complex is then to inactivate FVa and FVIIIa, also on a phospholipid surface. When APC inactivates membrane-bound FVa it does so through an initial cleavage at Arg506, which is required for optimal exposure of the other cleavage sites at Arg306 and Arg679 (Kalafatis et al. 1994).

PS acts by enhancing the affinity of APC for negatively charged phospholipids, forming a membrane-bound APC-PS complex that renders FVa and FVIIIa more easily accessible to APC-mediated cleavage. Approximately two-thirds of circulating PS is bound to C4b-binding protein (C4b-BP) of the complement system and as such has no APC-cofactor activity. Only the free form is thought to be active as a cofactor for APC. More recently it has been reported that PS itself also has anticoagulant activity in that it may inhibit (in an APC-independent way) the activity of both the tenase and the prothrombinase complexes, some reactions being independent of the presence of C4b-binding protein (Heeb et al. 1993; Heeb et al. 1994; Koppelman et al. 1995). Whether these actions are of physiological importance is not known.

PC and PS deficiency are also inherited autosomally and the prevalence of heterozygous PC deficiency is 0.1-0.5% in the general population (Miletich et al. 1987) but that of PS deficiency is not known. Deficiency of either PC or PS

predisposes to thrombotic disease and in families with a history of symptomatic thrombosis 50% of heterozygotes with PC deficiency can be expected to develop venous thromboembolism (VTE) (Allaart et al. 1993; Comp and Esmon, 1984; Griffin et al. 1981). As with AT deficiency both quantitative and qualitative defects of PC and PS have been identified and functional tests are more sensitive. The frequency of PC deficiency is approximately 3.2% and of PS deficiency 2.2% in unselected patients with VTE (Heijboer et al. 1990).

In 1993 it was observed that plasma samples from some patients with familial thrombosis were resistant to the action of APC in that there was impairment of the ability of added APC to prolong the APTT of these thrombophilic plasmas (Dahlback et al. 1993). In 1994 Bertina *et al* (Bertina et al. 1994) found that APC resistance is associated with a point mutation in the FV gene that causes significant reduction in the rate of inactivation of FVa by APC. The mutation involves a G to A transition of nucleotide 1691 in exon 10 which leads to the substitution of Arg506 by Gln and this removes the initial cleavage site for the action of APC and hence delays the inactivation of FVa. APC resistance is the most frequent cause of inherited thrombophilia, accounting for 20-50% of cases (Griffin et al. 1993; Svensson and Dahlback, 1994; Koster et al. 1993) and over 95% of cases are due to this mutation - the 'factor V Leiden' mutation (FVR506Q) (Bertina et al. 1994; Zoller and Dahlback, 1994) though other much rarer mutations in the FV gene have also been described (Chan et al. 1998; Williamson et al. 1998). The FVR506Q mutation has a relatively high frequency in Caucasian populations (up to 6%) but a lower frequency in Africa and Asia than in European populations (Rees et al. 1995). APC resistance in individuals lacking mutant FV can be explained in part by the poor specificity of the functional assay used to detect APC resistance though there is ongoing interest in the role of 'acquired' APC resistance as a risk factor for thrombosis. The specificity of the APC resistance assay for mutant FV can be

improved greatly by using the 'modified' assay where diluted FV is added to the test plasma.

APC is subject to some inhibitory control; a heparin dependent APC inhibitor, protein C inhibitor-I (PCI-I), has been identified and is identical to plasminogen activator inhibitor-3 (PAI-3) (see section 1.0.5). A heparin independent APC inhibitor, protein C inhibitor-II, appears to be α_1-antitrypsin (van der Meer et al. 1989) and the existence of another heparin independent APC inhibitor, protein C inhibitor-III, has also been postulated. APC, in addition to acting as an anticoagulant, also stimulates fibrinolysis by inhibiting plasminogen activator inhibitor-1 (PAI-1), again with PS as a cofactor (D'Angelo et al. 1987). These profibrinolytic properties of APC have been related to activation of a recently described protein, thrombin-activatable fibrinolysis inhibitor (TAFI) by APC (Bajzar et al 1995).

Tissue factor pathway inhibitor.

The importance of TFPI in the control of the coagulation network is outlined above in section 1.0.3 and indicated in figure 2. Thrombophilia caused by abnormalities of the TFPI system has not yet been documented.

1.0.5 Fibrinolysis

Fibrin is broken down by the serine protease plasmin which exists in plasma in a precursor form, the zymogen plasminogen (figure 4). Plasminogen contains an important high affinity lysine binding site through which it binds to fibrin or histidine rich glycoprotein (HRG) in the plasma which modulates the level of free plasminogen available for binding to fibrin. It is activated to plasmin by at least two different plasminogen activators, tissue type plasminogen activator (tPA) and

urokinase-type plasminogen activator (uPA) and can also be activated by a contact system dependent process which depends on KK but the physiological role of this is unclear. TPA is the physiologically important activator and is secreted from the vascular endothelial cell. In the absence of fibrin it is a poor activator of plasminogen but when both tPA and plasminogen are bound to fibrin to form a ternary complex there is efficient plasminogen activation. UPA does not bind to fibrin and the main physiological role of the uPA system may be in tissue remodeling rather than in the lysis of intravascular fibrin.

There are a number of inhibitors which control fibrinolysis. The principle inhibitor of plasmin is the serpin α_2-antiplasmin which reacts rapidly with plasmin in the circulation but reacts very slowly with fibrin-bound plasmin. The main plasminogen activator inhibitor - PAI-1 - is secreted from vascular endothelial cells and hepatocytes and inhibits both tPA and uPA. Secreted PAI-1 loses its activity unless protected by the plasma protein vitronectin. This inactive or latent PAI-1 can be reactivated *in vitro* by denaturants and by phosphatidylserine (PTS). Platelets also contain PAI-1, the role of which is unclear as when platelets release it *in vitro* it is mainly as the inactive form. Other inhibitors have been described but PAI-1 is of overriding physiological importance. Plasminogen activator inhibitor-2 (PAI-2) is secreted by the placenta and may play a local role in pregnancy. The main role of PAI-3 is as the heparin dependent APC inhibitor -PCI-I.

A variety of fibrinolytic defects have been implicated in the pathogenesis of thrombosis. These include hypo- and dys-plasminogenaemia, dys-fibrinogenaemia, decreased release of tPA, increased PAI-1 levels and increased HRG levels. However on the whole primary hypercoaguable states due to defects of fibrinolysis have not been as well characterised as those due to deficiencies of AT, PC, and PS.

1.0.6 Other causes of thrombophilia

There are a number of other inherited genetic mutations and acquired abnormalities of proteins involved in the coagulation pathway that are recognised causes of an increased tendency to arterial and/or venous thrombosis.

Antiphospholipid antibodies (APA) are a heterogeneous group of antibodies that react to proteins bound to phospholipids. Patients with APA can be free of symptoms or can have thrombocytopenia or arterial or venous thrombotic complications including stroke and fetal loss and in patients with systemic lupus erythematosus, APA are usually the cause of the thromboembolic events that are associated with this condition. The mechanisms by which the APA cause these clinical problems are not fully explained. The most important of the phospholipid-bound proteins are probably prothrombin and β_2-glycoprotein 1 (β_2GP1) and antibodies to these can be demonstrated in some patients. However for the purposes of diagnosis of the 'antiphospholipid syndrome' 2 or more assays are recommended because no single test identifies all APA owing to the heterogeneity of the antibodies. Other assays include the demonstration of a 'lupus anticoagulant', which is characterised by prolongation of phospholipid-dependent coagulation assays, and anticardiolipin antibodies (ACL) (Exner et al. 1991).

Mild ***hyperhomocysteinaemia*** has been recognised as a risk factor for arterial disease for over a decade but has more recently been found in 19% of patients with juvenile venous thrombosis and family studies showed that in most cases this phenotype was inherited (Falcon et al. 1994). The homozygous inheritance of a thermolabile variant of the enzyme methyltetrahydrofolate reductase (MTHFR-T) is a common, significant cause of mild hyperhomocysteinaemia, present in 5-15% of Caucasian populations (Frosst et al. 1995; Arruda et al. 1997). Another highly prevalent mutation was first reported in

1996 (Poort et al. 1996); this involves the prothrombin gene - ***prothrombin 20210 allele*** - and is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. It is present in approximately 2-3% of Caucasian populations.

One further genetic defect that has been discussed with respect to its possibility as a risk factor for thrombosis is partial ***factor XII deficiency***. Some studies have suggested a high frequency of heterozygotes for FXII deficiency in cohorts of thrombophilic patients (Halbmayer et al. 1992) but another study has not been able to confirm these findings (Koster et al. 1994) .

Finally, there has been recent interest in ***high factor VIII levels*** as a risk factor for venous thrombosis in large patient-control studies (O'Donnell et al. 1997; Koster et al. 1995). The hereditability of this phenotype and the eventual underlying molecular defects have not yet been reported.

1.0.7 Haemostatic risk factors for arterial disease.

The thrombophilic conditions that have been discussed above are known to contribute towards the development of venous, and sometimes arterial, thrombosis but there are also a number of other changes in the haemostatic system which are thought to predispose towards arterial but not venous disease (Lowe, 1997). As I will discuss in section 1.2, patients with SCD can develop significant arterial occlusion, particularly in the cerebrovascular system.

Elevated fibrinogen level is the best of the haemostatic variables to accurately predict arterial thrombotic events (both ischaemic heart disease and stroke), and there have been many large prospective studies to show that this is the case. The first of these was the Northwick Park Heart Study which reported that plasma fibrinogen was predictive of cardiovascular death (Meade et al. 1980).

A recent meta-analysis of the seven largest studies examining the relationship between plasma fibrinogen and cardiovascular events has collectively included over 15,000 individuals followed for a median time of 6.4 years and has confirmed a clinically significant increase in cardiovascular risk in the third of the population with the highest fibrinogen levels, which resulted in a relative risk of 2.45 (95% confidence intervals 2.05-2.93) (Resch and Ernst, 1995). It has also been shown that the plasma fibrinogen level is a strong, consistent risk factor for arterial events in persons with established arterial disease, including stroke (Ernst et al. 1991).

Elevated FVII:C activity also proved to be important in the prediction of ischaemic heart events in the Northwick Park Heart Study (Meade et al. 1980) and also in the parallel "PROCAM" study from Munster (Heinrich et al. 1994). Factor VII activity is related to dietary fat intake, blood lipids and obesity which may provide a link between these risk factors and thrombotic disease.

There have also been a number of investigations as to whether ***high FVIII levels,*** with or without elevated vWF:RAg, play a causative role in arterial disease and, although the evidence is not as convincing as it is for fibrinogen and FVII, current beliefs are that there may well be an effect of these factors on the development of clinical events (Lowe, 1997).

The role of inappropriate ***platelet activation*** in arterial thrombosis is discussed briefly in section 1.0.1 and the biological importance of platelets in this situation is highlighted by convincing meta-analyses that show beneficial effects of the anti-platelet agent, aspirin, in ischaemic heart disease and cerebrovascular disease in adults.

1.1. *Background to Sickle Cell Disease*

1.1.0 Basic pathophysiology

The sickle mutation results from a single nucleotide change (GAT → GTT) in the sixth codon of exon 1 of the gene for the β-globin chain of the haemoglobin molecule. As a result the normal glutamic acid at position 6 of the β-globin chain is replaced by valine, thus leading to the formation of sickle haemoglobin (HbS). This single base substitution in the 3 billion base pairs of the human genome leads to a complex disease process that involves multiple organs. It also produces the different electrical charge that is used in the detection of HbS by electrophoresis and it changes the behaviour of Hb molecules so that when deoxygenated there is a hydrophobic interaction between HbS molecules which tend to aggregate into large polymers. This polymerisation or 'gelation' of deoxygenated HbS results in distortion of the shape of the red cells and a marked decrease in their deformability causing rigid, deformed cells. HbS also has adverse effects on the red cell membrane that cause oxidative damage, cellular dehydration, abnormal phospholipid asymmetry and increased adherence to endothelial cells (Hebbel, 1991). The net result of these cellular abnormalities is a shortened red cell lifespan and intermittent episodes of vascular occlusion that cause tissue ischaemia and acute and chronic organ dysfunction.

1.1.1 Genetics

Sickle cell anaemia (HbSS) is the homozygous state in which the sickle gene is inherited from both parents and this is the most common form of SCD. Electrophoresis over the age of 1 year shows that patients with HbSS will normally have 80-95% HbS and although the majority of subjects with HbSS will have levels of fetal haemoglobin (HbF) of between 0.5% and 10% there are some with HbF

levels up to 30%. Co-inheritance of HbS with other abnormal Hb molecules can give rise to other forms of SCD and next in frequency among people of West African ancestry is sickle cell/haemoglobin C (HbSC) disease, resulting from the inheritance of one HbS gene with one gene for HbC which gives approximately 50% HbS and 50% HbC on electrophoresis. Inheritance of the HbS gene with a gene for β-thalassaemia may cause either sickle cell/β^+-thalassaemia (HbSβ^+thal) with a mild clinical picture and 5-30% HbA, 65-90% HbS, 3.5-6.0% HbA_2 and 0.5-10% HbF or sickle cell/β^0-thalassaemia (HbSβ^0thal) where there is no HbA, 80-92% HbS, 3.5-7.0% HbA_2 and 0.5-15% HbF and more severe disease. However although HbSS and HbSβ^0thal tend to have the more severe clinical manifestations there is a great deal of individual heterogeneity in the severity of the vasoocclusive complications of each of these disorders. The heterozygote forms of sickle cell trait (HbAS) and Hb C trait (HbAC) are benign and important principally for their genetic counselling implications.

1.2 Clinical manifestations of sickle cell disease

The important clinical manifestations of SCD are listed in table 1, divided into 2 sections on the basis of which underlying process is most likely to contribute to causation.

Vascular occlusion and tissue ischaemia can result in acute and chronic injury to virtually any organ of the body but injury to three major organs, the spleen, the lungs and the brain, are directly or indirectly responsible for much of the morbidity and most of the mortality seen in childhood (Gill et al. 1995; Thomas et al. 1982; Platt et al. 1994). Although historically microvascular occlusion by poorly deformable sickle erythrocytes (SSRBC) was thought to be the central

Table 1 Clinical manifestations of sickle cell disease

Clinical sequelae due primarily to vascular occlusion
• Stroke • Acute chest syndrome • Acute painful crisis (eg musculoskeletal, dactylitis, abdominal) • Splenic sequestration • Functional asplenia • Hyposthenuria and enuresis • Papillary necrosis • Chronic nephropathy and renal failure • Priapism • Avascular necrosis of bone • Proliferative retinopathy • Leg ulcers
Clinical sequelae due primarily to haemolysis
• Chronic anaemia • Jaundice • Aplastic crises • Cholelithiasis • Delayed growth and maturation

pathophysiological event, the actual mechanisms of vascular obstruction are now recognised to be very complex and the contribution of other vasocclusive processes such as vascular intimal hyperplasia, thrombosis and vasospasm are not clearly defined. In fact, it is entirely possible that the genesis of vaso-occlusion varies among patients and among different vascular regions.

Although there is much overlap between the clinical consequences of the two most common forms of SCD, namely HbSS and HbSC, there are also some important differences; HbSS is characterised by a higher mortality, a higher risk of stroke, chest syndrome and leg ulcers but a lower risk of retinopathy compared to HbSC (Platt et al. 1994; Thomas et al. 1982; Serjeant, 1985; Vichinsky et al. 1997). There is also a belief that aseptic necrosis of bone is more common in HbSC but this may be because patients with HbSC tend to live longer and therefore reach the age groups at which this complication is most likely to occur (Serjeant, 1985). The

reasons for these clinical differences between HbSS and HbSC are not entirely understood but one factor which may contribute to the different spectrum of complications in HbSC may be the less severe anaemia and higher blood viscosity in HbSC.

There are also a variety of genetic and acquired risk factors that are known to modify the phenotypic expression of SCD. Some of these exert predictable effects on the sickling risk. Examples are HbF which dilutes and inhibits polymerisation of HbS as the level of HbF rises and α-globin gene number - any α-chain deletion will lower the mean cell Hb concentration, also inhibiting polymerisation of HbS molecules. There are many factors which affect the phenotype less predictably and include environmental influences. For example, high socioeconomic status can be protective (Farber et al. 1985) and there are also many pathophysiological variables which can be important (see section 1.3).

The clinical manifestations of SCD that are due to vascular occlusion will be discussed in the following paragraphs. Those of most relevance to this thesis - cerebrovascular disease, stroke and the acute chest syndrome - will be considered in most detail and pathophysiological aspects will discussed more fully in section 1.3.

1.2.1 Stroke

Infarctive stroke (CVA) is probably the most severe of the complications of SCD in childhood in terms of long-term morbidity and is also an important cause of mortality (Thomas et al. 1982; Balkaran et al. 1992; Powars et al. 1978; Leikin et al. 1989). It occurs at a median age of 6-8 years and affects at least 8% of children with HbSS by the age of 14 years (Balkaran et al. 1992; Powars et al. 1978; Ohene Frempong, 1991). A recent longitudinal study of over 4000 patients from the

Cooperative Study of Sickle Cell Disease (CSSCD) (Ohene Frempong et al. 1998) found that CVA was infrequent in HbSC, in HbSβ^+thal, below the age of 2 yrs and in adulthood when haemorrhagic strokes are more common. Haemorrhagic strokes have also been reported in children with SCD but occur less frequently than CVA (Van Hoff et al. 1985).

The risk of developing a CVA is greatest in those with a high white cell count (WBC) and lowest Hb - often acutely lowered - (Balkaran et al. 1992; Ohene Frempong, 1991). There also seems to be a temporal association between episodes of acute chest syndrome (ACS) and CVA (Ohene Frempong et al. 1998), though CVA can also occur spontaneously or in the setting of infection, painful crisis or dehydration (Russell et al. 1984). Although high HbF levels are known to generally ameliorate the severity of SCD (Powars et al. 1984), there has been no convincing evidence that higher levels prevent the development of CVA (Balkaran et al. 1992; Powars et al. 1978; Ohene Frempong et al. 1998). Another factor suggested to have a possible triggering role for CVA is recurrent pneumococcal tonsillitis and obstructive sleep apnoea (Davies et al. 1989; Madderin et al. 1989; Robertson et al. 1988; Ajulo, 1994), but prospective studies are needed as this was not addressed in the CSSCD study.

Occlusion of large cerebral vessels is the cause of the majority of CVA in SCD (see section 1.3) and hemiplegia is the commonest presenting symptom and residual deficit (Balkaran et al. 1992; Powars et al. 1978; Ohene Frempong, 1991; Portnoy and Herion, 1972) with a 50-70% chance of recurrence within 3 years of the first event (Balkaran et al. 1992; Powars et al. 1978).

For the last 2-3 decades, the management of CVA in SCD has been directed towards the prevention of recurrence by chronic transfusion regimes which maintain the %HbS level at below 30% (Russell et al. 1984; Pegelow et al. 1995; Russell et al. 1976; Wilimas et al. 1980; Buchanan et al. 1983; Wang et al. 1991;

Sarnaik and Lusher, 1982). However recurrences have occurred at %HbS levels of lower than 30% (Wilimas et al. 1980; Buchanan et al. 1983) and therefore many now advocate a %HbS level of <20% as a more protective cut-off level. Attempts to stop such programmes after periods up to 12 years have then been followed by recurrence rates as high as 70% within 3 months (Wilimas et al. 1980; Wang et al. 1991) though other groups have disputed this (Rana et al. 1997).

The continuation of a regular transfusion regime in the long-term carries risks of red cell alloimmunisation, transfusion reactions, transfusion-acquired infections, iron overload, and venous access difficulties. Because of these problems and the associated morbidity, recent interest in this field has concentrated on the identification of individuals at risk of stroke prior to the first episode. The development of newer non-invasive methods for imaging and detecting cerebrovascular disease (CVD) have been instrumental in this. Eight to ten years ago it was shown that transcranial Doppler ultrasound (TCD) is sensitive and specific for the detection of the arterial vasculopathy of SCD and correlates well with cerebral angiography which had hitherto been the 'gold standard' investigation but is more invasive (Adams et al. 1990; Adams et al. 1988a; Adams et al. 1992). Larger studies then showed that TCD can identify large vessel disease in 14% of an asymptomatic group of children with SCD and a finding of a mean arterial velocity of greater than 170 cm/sec in the middle cerebral artery identifies those children at highest risk of CVA (Adams et al. 1992; Adams et al. 1997). TCD is therefore a useful screening tool to aid in the implementation of primary prevention of stroke.

Magnetic resonance imaging (MRI) and angiography (MRA) have also been proven as a useful diagnostic modalities. Vascular lesions found in large arteries on MRA correlate well with cerebral angiography and TCD (Seibert et al. 1993; Verlhac et al. 1995; Wiznitzer et al. 1990; Kandeel et al. 1996) and MRI

demonstrates cerebral infarcts, the majority of which are consistent with major vessel occlusion involving anterior, middle and posterior cerebral arteries (el Gammal et al. 1986; Adams et al. 1988b; Pavlakis et al. 1988). Several studies have again identified a number of asymptomatic children with subclinical CVD; a report from the CSSCD found that 13% of 312 children had infarction or ischaemic changes on MRI in the absence of a recognised cerebrovascular accident (Moser et al. 1996) and other smaller studies have agreed with these figures (Seibert et al. 1993; Glauser et al. 1995). A further report from the CSSCD has shown that these children with 'silent infarcts' perform significantly worse in neuropsychological tests than those with no MRI abnormality though those with a CVA performed the poorest (Armstrong et al. 1996).

The correct management of asymptomatic children with abnormal vessels or silent infarcts has until very recently been unclear. However a recent multicentre randomized trial in the USA - the 'STOP' (stroke prevention in sickle cell anaemia) trial has shown conclusively that a chronic transfusion programme prevents more first strokes in children with abnormal TCD than no transfusion (Adams et al. 1998). The authors accept that this benefit has to be weighed against the risks of long term transfusion programmes and ideally future work must be directed at identifying which of the children with TCD abnormalities are the subgroup who are going to stroke. There is little experience with the use of antiplatelet agents, anticoagulation, hydroxyurea (which raises %HbF level) or surgery in either the primary or secondary prevention of stroke but future therapeutic studies need to address these options.

1.2.2 The chest syndrome

One of the other most serious complications of SCD is the acute chest syndrome (ACS), a condition in which infiltrates on the chest X-ray are associated with respiratory symptoms including progressive hypoxia and chest pain (Haupt et al. 1982). There is often confusion and overlap with pulmonary infection. CSSCD data found a mortality rate from ACS in adults of 18 deaths in 419 events (4.3%) and in children 14 in 1322 events (1.1%) (Vichinsky et al. 1997). The incidence is age-dependent; 24.5 events per 100 patient years in <10 yr age group falling to 8.8 events in older adults and it occurs most frequently in the HbSS and HbSβ^0thal genotypes (Vichinsky et al. 1997; Castro et al. 1994). Studies suggest that over 50% of paediatric patients with HbSS will suffer from one or more episodes of ACS and it is the second leading reason for hospitalisation (Gill et al. 1995; Castro et al. 1994). HbF levels and the degree of anaemia are inversely proportional to the rate of ACS and the WBC is directly proportional to the rate of ACS (Castro et al. 1994).

Episodes of ACS are probably caused by a spectrum of pathology (Vichinsky et al. 1997; Poncz et al. 1985; Sprinkle et al. 1986; Vichinsky et al. 1994) with infection, infarction, pulmonary sequestration and fat embolism all potentially contributing. Coexistent viral or bacterial infections and seasonal epidemics of these are known to be important (Vichinsky et al. 1997; Poncz et al. 1985) and the risk is increased if there has been a previous pulmonary event, during pregnancy, post-operatively and after anaemic events (Vichinsky et al. 1997). There is also a striking association with painful crises in that >30% of ACS events are immediately preceded by infarction in a long bone or particularly rib or sternal infarctions (Rucknagel et al. 1991) when hypoventilation and narcosis also play a contributory part (Vichinsky et al. 1994). Recurrent attacks of ACS predispose adolescents and adults to the development of chronic restrictive sickle lung disease with pulmonary

fibrosis, pulmonary hypertension and cor pulmonale (Collins and Orringer, 1982; Powars et al. 1988).

1.2.3 The painful crisis

This is the commonest clinical problem in patients with SCD and over 90% of hospital admissions for SCD are for the management of acute painful crises (Platt et al. 1991). The report from the CSSCD found that the 'pain rate' (episodes per year) is a measure of clinical severity and correlates with early death in adults with SCD (Platt et al. 1991). The pain rate increased as patients grew older from 0 to 30 years and declined thereafter. The important risk factors for pain are higher haematocrit and Hb and lower %HbF (Powars et al. 1984; Platt et al. 1991; Baum et al. 1987).

Inducing higher levels of Hb F is a logical therapeutic objective for recurrent painful crises and the recently completed trial of hydroxyurea in the USA (Charache et al. 1995) showed significant reduction in painful crises and in transfusion requirements in selected severely affected adults. However hydroxyurea does have potential dangers in reducing WBC and platelet counts, it must be carefully monitored and may have long-term effects so the selection of patients suitable for this intervention must be carefully considered. Its use in children is still limited (de Montalembert et al. 1997; Ferster et al. 1996).

1.2.4 Functional asplenia and splenic sequestration

The spleen is uniquely susceptible to damage by SSRBC because its slow tortuous microcirculation provides an environment conducive to the polymerisation of HbS. Splenic injury in HbSS begins as early as 3-6 months and is often asymptomatic but progressive, resulting in 'autosplenectomy' in the majority of

children by the age of 5 yrs. The importance of this is underscored by the repeated observation that bacterial infection, particularly with encapsulated organisms, is the leading cause of death worldwide in children with SCD (Thomas et al. 1982). This mortality can be significantly reduced by prophylactic penicillin (Gaston et al. 1986).

In splenic sequestration the spleen rapidly enlarges and there is an acute fall in Hb which can be fatal if appropriate urgent resuscitation is not given (Gill et al. 1995; Emond et al. 1985). The mechanisms for triggering these episodes are not clear but splenic sequestration often accompanies viral illness and can occur in combination with other complications of SCD.

1.2.5 Renal disease

The microvasculature of the kidney is especially sensitive to hypoxia induced by vaso-occlusion and problems can arise from the length of the nephron from the glomerulus to the papillary tip. Renal insufficiency occurs in 4 to 18% of SCD patients, depending on their genotype - commonest in HbSS (Thomas et al. 1982; Powars et al. 1991). It is associated with proteinuria and advancing age and can progress rapidly once it begins (Powars et al. 1991). It is one of the leading causes of death in older sickle cell patients (Platt et al. 1994; Thomas et al. 1982).

1.2.6 Priapism

This is a distressing complication of SCD that is commonest in males with HbSS, a low %HbF, a low reticulocyte count and a high platelet count (Fowler et al. 1991; Edmond et al. 1980; Hasen and Raines, 1962). There is a bimodal distribution in the age at onset with peaks at 5 to 13 years (Miller et al. 1995) and at 21 to 29 years. It requires urgent aggressive treatment to avoid long-term erectile dysfunction.

1.2.7 Avascular necrosis of bone

Bone and joint lesions are a frequent complication of patients with SCD. Blockage of the microcirculation causes infarction of bone marrow and adjacent bony structures. Localized infarcts can produce swelling with tenderness; the earliest example of this is dactylitis in early childhood and 25-45% of infants and young children will experience at least one episode (Gill et al. 1995). Osteonecrosis of the femoral and humeral heads are important causes of morbidity in adolescents and adults with SCD. It occurs in all genotypes of SCD and although historically it was thought to be more common in HbSC disease, this is probably only because patients with HbSC have a longer life expectancy and are therefore more likely to develop it in their lifetime (Serjeant, 1985; Milner et al. 1993). Neither high levels of HbF or α-thalassaemia protect from this complication (Hawker et al. 1982; Milner et al. 1993; Milner et al. 1991). The onset is often in late childhood or early adulthood and can cause considerable chronic pain. Progressive cases usually require joint replacement (Clarke et al. 1989).

1.2.8 Retinal problems

Small vessel occlusion in the peripheral retinal vasculature causes ischaemia and can culminate in the development of proliferative sickle retinopathy which may cause vitreous haemorrhage with transient visual loss or retinal detachment with permanent blindness. This complication is more prevalent in the milder forms of SCD, namely HbSC and HbSβ^+thal. It also occurs in older patients with HbSS (14% in > 40 yr age group), particularly males with a high Hb and a low %HbF (Hayes et al. 1981).

1.2.9 Leg ulcers

These are the commonest cutaneous manifestation of SCD and also occur in other haemolytic anaemias. They account for significant morbidity in patients with HbSS or HbSβ^0thal but are extremely rare in other forms of SCD (Koshy et al. 1989). The underlying pathophysiology is not fully understood but histology of these lesions suggest that capillary and arteriolar damage and occlusion by red cells is contributory. The incidence is rare in <10 yrs age group but leg ulcers were present in 2.5% of over 2000 subjects >10 yrs enrolled in the CSSCD study in the USA and the overall incidence of ulcers was 25% based on patient history or occurrence during the study period (Koshy et al. 1989). Prevalence rates in other geographical areas can vary widely as phenotypic, social and economic factors influence the development and persistence of leg ulcers.

1.3. Pathophysiology Of Vaso-Occlusion In SCD

It is generally agreed that vascular occlusion plays a pivotal role in the clinical course of SCD (Hebbel, 1991; Francis, Jr. and Johnson, 1991) and that this is fundamentally related to the abnormal properties of the sickle erythrocyte. However the mechanism by which the abnormal red cell causes occlusion is likely to be a very complex, multifactorial process and although many factors appear to play a role in the pathogenesis of the occlusion our understanding of how these interact in any given clinical situation is usually unclear. Vaso-occlusion can involve both the microcirculation, which is believed to have a major contributory role in acute painful sickle crises, and the macrocirculation which is more associated with cerebrovascular disease and organ failure though there is undoubtedly some overlap between these even though direct evidence for this overlap is as yet scanty

(Cheung et al. 1997). The factors that may contribute to vascular occlusion are listed in Table 2 and the evidence for these is discussed in the following sections. The majority of the investigations into the pathophysiology of vascular occlusion have focused on HbSS and therefore the relevance to HbSC is inferred rather than demonstrated in the following sections.

Table 2 Vascular occlusion in SCD - possible contributory factors

1. **Red cell factors**
    - polymerisation of Hb S
    - rheological properties of SS rbc
        - cellular dehydration
        - rbc deformability
        - blood viscosity
        - dense cells
        - membrane phospholipid asymmetry
2. **White cell factors**
    - neutrophil activation
    - neutrophil dysfunction
    - increased neutrophil adhesiveness
3. **Endothelial factors**
    - deformable cells
    - membrane abnormalities
    - plasma factors
    - activated endothelial cells
4. **Vascular factors**
    - dynamics of microvasculature
    - vascular intimal hyperplasia
5. **Coagulation abnormalities and thrombogenic mechanisms**
    - thromboembolism
    - platelet activation and dysfunction
    - procoagulant changes
    - changes in plasma inhibitors
    - increased thrombin generation

1.3.1 Red cell factors

The ***polymerisation and gelation of HbS*** upon deoxygenation is believed to play a central role in microvascular occlusion. The rate and extent of this is dependent on a number of physiological factors such as the intracellular concentration and composition of Hb, the percentage of oxygen saturation, temperature, pH and 2,3-DPG. Occlusion is likely to occur whenever intra- and extracellular conditions promote the generation of sufficient numbers of rigid sickle cells with polymerised Hb capable of blocking capillaries and initially it was postulated that this occurred when the transit time through the microcirculation was delayed beyond the lag time required for initiation of HbS polymerisation. However it is now evident that some SSRBCs, especially 'dense cells' (see below), contain polymerised HbS even at arterial oxygen saturation thus reducing the lag time to gelation during microvascular transit (Eaton and Hofrichter, 1987; Noguchi and Schechter, 1981). There is also evidence that rapid deoxygenation can occur, resulting in the formation of extensive HbS polymers without classic morphological sickling but resulting in selective trapping of dense cells (Kaul et al. 1989). Situations that slow microvascular transit time such as altered vascular reactivity and increased adhesion of SSRBC to the endothelium may be as important as HbS gelation in modulating microvascular occlusion *in vivo* (see below) (Eaton and Hofrichter, 1987).

Despite *in vitro* evidence linking HbS gelation to microvascular occlusion direct *in vivo* evidence is lacking but there is indirect evidence to suggest it is important in clinical situations. There is an inverse correlation between the mean gelation time and average clinical severity of HbSC and HbSS disease (Eaton and Hofrichter, 1987) and some evidence of an inverse correlation between %HbF (which inhibits HbS gelation) and many of the vaso-occlusive complications of SCD

such as chest syndrome, stroke, retinopathy, aseptic necrosis of the femoral head and leg ulcers (Powars et al. 1984; Hawker et al. 1982; Hayes et al. 1981; Koshy et al. 1989). There is also trial data accumulating on the use of hydroxyurea, which increases HbF (Charache et al. 1992) and reduces sickling, which has found that the frequency of painful crises and possibly the degree of microvascular occlusion can be reduced by its use (Charache et al. 1995; Ferster et al. 1996).

The ***rheological properties of SSRBC*** also play a critical role in the pathogenesis of vaso-occlusion but again these are influenced by a number of interrelated factors such as plasma viscosity, haematocrit, cell haemoglobin concentration, membrane mechanical properties and surface area to volume ratio of red cells. Thus once a sickle RBC is dehydrated as a consequence of the intracellular polymerisation of HbS, the cell density increases, the cellular deformability decreases (Nash et al. 1984) and this in turn increases blood viscosity. This fraction of rigid 'dense cells' appears largely responsible for the abnormal rheology of the blood but to what extent the dense cells contribute to the primary process in microvascular occlusion and the painful crisis is not clear (Akinola et al. 1992; Ballas et al. 1988; Billett et al. 1986; Stuart and Johnson, 1987). However the one *in vivo* study that has been published has shown that these less deformable cells have a reduced rate of entry into capillaries and increased transit time through the microcirculation where they can become trapped (Lipowsky et al. 1987) and *in vitro* studies confirm this (Kaul et al. 1986). Deoxygenation worsens the deformability and accentuates their deleterious effect on the dynamics of the microcirculation leading to blockage of blood flow, tissue hypoxia and damage.

The clinical relevance of elevated blood viscosity is that it can have detrimental effects on the cardiovascular system and tissue oxygenation and there are recent studies showing a direct relationship between increased frequency of

painful crises and a higher haematocrit value (Platt et al. 1991; Baum et al. 1987). HbSS patients with coexistent α-thalassaemia and those with a higher Hb have a higher blood viscosity and this results in a higher incidence of retinopathy and avascular necrosis than do patients with HbSS and no α-gene deletion (Hawker et al. 1982; Milner et al. 1993; Hayes et al. 1981; Fox et al. 1993).

A further effect of dehydration and deoxygenation on the SSRBC is to alter the membrane phospholipids. In normal RBC the membrane phospholipids are asymmetrically distributed in the red cell membrane. The choline-containing phospholipids (phosphatidylcholine and sphingomyelin) are essentially located in the outer leaflet of the cell membrane whereas the aminophospholipids (phosphatidylethanolamine [PE] and phosphatidylserine [PTS]) are found in the inner leaflet. Sickling of the cells significantly disturbs this asymmetry (Hebbel, 1991; Middelkoop et al. 1988) resulting in a greater exposure of PE and PTS on the outer surface compared to normal RBCs and to non-sickled SSRBCs (Lubin et al. 1981; Wood et al. 1996). This abnormality could contribute to the pathophysiology of SCD in several ways; firstly exposure of PTS has also been shown to activate prothrombin and promote blood coagulation *in vitro* and may do so *in vivo* (Chiu et al. 1981; Helley et al. 1996), secondly it could contribute to abnormal adhesion of SSRBC to the endothelium (Hebbel et al. 1982), and lastly it may be partially responsible for the decreased red cell survival characteristic of this disorder because exposure of PTS promotes macrophage recognition and splenic clearance of SSRBCs (Schwartz et al. 1985).

1.3.2 White blood cell factors

The contributory role of WBC in mediating vaso-occlusion of the microcirculation is poorly understood as it has received little investigative attention

until very recently. However there is good circumstantial evidence that WBC may play a co-pathogenic role;

- high WBC counts in SCD patients are associated with a higher mortality (Platt et al. 1994).
- many patients have a moderately elevated WBC in the steady-state and functional asplenia and autosplenectomy may be partially responsible.
- there is an association between sickle cell crises and infection (Serjeant, 1985) and theoretically this could cause enhancement of WBC-endothelial cell interaction which may be sufficiently obstructive to allow sickling or adhesion of RBCs in areas proximal to retarded flow.
- acute painful crises are often accompanied by a marked leucocytosis (Serjeant, 1985) and there is evidence for associated neutrophil dysfunction and increased 'stickiness' during crisis (Lachant and Oseas, 1987).
- reports from cohorts of children with HbSS have shown that higher baseline white cell counts are associated with a relatively higher risk for stroke (Balkaran et al. 1992; Ohene Frempong, 1991) but there is no evidence as to whether this is cause or effect.
- the beneficial effects of hydroxyurea are coincidental with reduction of the WBC (Charache et al. 1995; Charache et al. 1992)

More recent work has concentrated on neutrophil activation in SCD. The high affinity Fc receptor 'CD64' is a marker of neutrophil activation and CD64+ neutrophils have been found in increased numbers in SCD patients compared to controls and were increased further in patients during crisis (Fadlon et al. 1998). This study also suggested that the neutrophils expressing CD64 show increased adherence to vascular endothelium which lends support to a mechanism by which aberrant cellular-endothelial interactions may contribute towards vascular occlusion

in SCD. One further area that is currently being investigated is the expression of TF by circulating monocytes. It is postulated that increased TF expression may well have a crucial role in the initiation of the 'hypercoaguable state' that is present in steady-state SCD - see section 1.3.7.

1.3.3 Endothelial factors

SSRBC are known to be abnormally adherent to vascular endothelium and this is thought to be an important contributory factor to small vessel vaso-occlusion (Kaul et al. 1989; Mohandas and Evans, 1984; Hebbel et al. 1980). There is an inverse relationship between RBC density and adhesiveness and current thinking favours a model in which the young, less dense, more deformable cells adhere better than dense, irreversibly sickled cells (Barabino et al. 1987). This may be mediated to some extent via the $\alpha 4\beta_1$ receptor on the sickle reticulocyte which binds to the endothelial receptor VCAM-1 (Gee and Platt, 1995). There is also some evidence that painful crises are more frequent in patients with a greater percentage of these less dense, more deformable cells (Ballas et al. 1988; Lande et al. 1988) and during crises the level of dense red cells falls (Billett et al. 1986). It is postulated that the reason for this is that the deformable cells initiate vascular obstruction by adhering to endothelial cells and the poorly deformable dense cells subsequently logjam behind these adherent cells and propagate the obstruction (Kaul et al. 1989; Kaul et al. 1994). However this theory is not supported by a recent study in which the dense, irreversibly sickled cells were more adherent than the less dense cells to endothelium treated by the inflammatory cytokine tumour necrosis factor (TNF) (Vordermeier et al. 1992). The role of cytokines in the promotion of red cell-endothelial attachment needs further investigation.

As well as cell density other properties of the red cell may also influence cell adhesivity; intrinsic membrane abnormalities of SS RBC that have been implicated as causes of increased adhesiveness include sialic acid abnormalities, oxidative damage and loss of phospholipid asymmetry (Hebbel, 1991; Schwartz et al. 1985; Hebbel et al. 1989). Adhesion is also thought to be enhanced by a number of plasma factors including fibrinogen, FVIII, von Willebrand factor (vWF) as well as platelet microparticles and thrombospondin from activated platelets (Mohandas and Evans, 1984; Wick et al. 1987; Brittain et al. 1993; Sugihara et al. 1992).

Further evidence supporting a role for the endothelium suggests that perturbation of vascular wall endothelium itself by SSRBC may contribute. Findings include increased numbers of circulating activated endothelial cells particularly during crisis (Sowemimo Coker et al. 1989; Solovey et al. 1997) and the presence of elevated levels of endothelial-cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin in the blood (Blei et al. 1994). Whereas normal endothelium can regulate vasoreactivity by producing endothelium-derived relaxing factor (EDRF, thought to be identical to nitric oxide) in response to agonists such as acetylcholine, SSRBC appear to inhibit this process, possibly contributing to the development of vaso-occlusive episodes (Mosseri et al. 1993; French II et al. 1997). The possible role of platelets and abnormal platelet release in the process of endothelial injury is largely unexplored.

1.3.4 Vascular factors

Changes in ***vascular tone, blood flow*** *or* ***microvascular dynamics*** are likely to be important in vaso-occlusion but this is technically difficult to assess and little work has looked at this to date in the microvasculature. The only study to have examined the dynamic behaviour of SSRBC in the human looked at blood

flow in nailfold capillary loops using intravital microscopy and the HbSS subjects were found to exhibit increased frequency of intermittent flow and flow stasis compared to HbAA controls (Lipowsky et al. 1987).

However ***vascular intimal hyperplasia*** with or without superimposed thrombosis (see section 1.3.5) is thought to play an important role in large vessel occlusion in SCD. Current evidence indicates that it is the major cause of stroke (Merkel et al. 1978; Stockman et al. 1972; Rothman et al. 1986) and may occur in other vascular beds such as pulmonary (Oppenheimer and Esterly, 1971), splenic, penile and renal but the frequency and significance of intimal hyperplasia in these extracerebral vascular beds is largely not explored.

Large vessel cerebral arterial disease in sickle cell subjects was first convincingly demonstrated over 25 years ago when 6 of 7 patients with neurological deficits were found to have partial or complete occlusion of one or more large cerebral artery on cerebral angiography (Stockman et al. 1972) and the 'moyamoya' pattern of basal occlusive disease with associated collateral proliferation was noted in 2 subjects. Other studies have confirmed these findings (Russell et al. 1984; Russell et al. 1976; Merkel et al. 1978; Rothman et al. 1986; Boros et al. 1976; Koshy et al. 1990). Histologically there is segmental thickening of the vessel walls, intimal hyperplasia with proliferation of fibroblasts and smooth muscle cells, splitting and clumping of the internal elastic lamina and focal fibrosis of the media. Occlusion results from intimal hyperplasia alone, superimposed thrombosis (see section 1.3.5) and/or distal thromboembolisation (Merkel et al. 1978; Rothman et al. 1986; Boros et al. 1976; Koshy et al. 1990). In some patients small cortical vessel occlusion may be important and this may be detected particularly in asymptomatic patients and may precede symptomatic stroke (Pavlakis et al. 1988; Moser et al. 1996).

The underlying causes of cerebral vascular intimal hyperplasia in SCD are not fully appreciated but the apparent predilection for sites of arterial bifurcation (Russell et al. 1984; Russell et al. 1976; Merkel et al. 1978; Rothman et al. 1986; Boros et al. 1976) and the efficacy of transfusion therapy in decreasing intimal irregularities and halting the progression of the stenosis (Russell et al. 1984; Russell et al. 1976) suggest that endothelial injury at sites of high flow turbulence may be a key factor. *In vitro* studies indicate that SSRBC are most likely to adhere to the endothelium at sites of vascular tortuosity, where flow is most turbulent (Burns et al. 1985). However direct observations of SSRBC-endothelial adhesion have thus far been confined to the microvasculature (Kaul et al. 1989; Lipowsky et al. 1987) and there is at present no direct evidence that SSRBC adhere abnormally to the intima of large arteries *in vivo.*

1.3.5 The role of thrombosis

There is little evidence that thrombosis alone plays a primary role in the pathogenesis of most of the vaso-occlusive complications of SCD but there is considerable evidence to show an increased thrombotic risk in these patients and evidence that thrombosis complicates other pathophysiological mechanisms operating in some of the complications of SCD. There is also evidence that coagulation is abnormally activated in SCD and a 'hypercoaguable state' exists with increased thrombin generation even in the non-crisis, steady state situation (see section 1.3.7).

Cerebrovascular thrombosis.

Thrombosis at sites of intimal hyperplasia in large cerebral vessels as a cause for CVA was recognised first almost 60 years ago and has been confirmed

on post-mortem studies since (Merkel et al. 1978; Rothman et al. 1986). In the study by Rothman and colleagues (Rothman et al. 1986), 12 of 24 autopsied SCD patients had infarcts that were associated with organising and recannalising thrombi involving the proximal divisions of the internal carotid artery system, especially the anterior-middle cerebral artery border zone. Once it was possible to investigate CVA patients with angiography these pathological findings were confirmed, and endothelial hyperplasia, stenosis and occlusion were found in 60-95% of patients commonly in the intracranial internal carotid, middle cerebral and anterior cerebral arteries (Russell et al. 1984; Stockman et al. 1972). It is thought that the irregular, damaged endothelial surface at sites of intimal hyperplasia may be thrombogenic, analogous to a ruptured atherosclerotic plaque. Remarkably, there have been no studies of antiplatelet or anticoagulant agents in the primary or secondary prevention of CVA in SCD, the one vasocclusive complication in which thrombosis is clearly implicated as an important contributory factor.

Pulmonary thrombosis and thromboembolism.

Pulmonary infarction secondary to obstruction or thrombosis of small or medium-sized vessels is important in the pathogenesis of the ACS. In the CSSCD review on the ACS, 9 of the 16 post-mortems performed on the patients who died of the ACS showed evidence of pulmonary embolus (Vichinsky et al. 1997). Studies of causes of death in SCD (Thomas et al. 1982; Haupt et al. 1982) have histologically documented pulmonary embolism in 8% and 25% of SCD subjects who died after the age of 10. There have also been other autopsy reports of extensive thrombotic occlusion of medium to small pulmonary arteries in sickle subjects with acute and/or chronic lung disease (Collins and Orringer, 1982; Oppenheimer and Esterly, 1971; Durant and Cortes, 1966). Abnormal ventilation/perfusion lung scans and pulmonary angiographic findings consistent

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with pulmonary thrombosis/embolism have been reported (Walker et al. 1979; Maggi and Nussbaum, 1987; Babiker et al. 1985) and there is evidence from high resolution CT scanning of microvascular occlusion due to microthrombi during episodes of the ACS (Bhalla et al. 1993). Pulmonary thromboembolism does appear to be uncommon as a direct cause of acute pulmonary disease (Haupt et al. 1982) but it may be that the 'hypercoaguable state' (see section 1.3.7) acts more indirectly by activating the inflammatory system and stimulating cellular activation and adhesion in the lung (Fabry and Kaul, 1991; Francis, Jr. 1989; Francis, Jr. 1991). Recent dynamic studies have suggested that occlusion in pulmonary vessels is precipitated by the adhesion of RBC to endothelial cells at these sites (Brittain et al. 1992).

It is not known whether pulmonary arterial thrombi in SCD form *in situ* or embolise from abdominal, pelvic, and/or lower extremity veins but fat embolism from necrotic bone marrow can occur - it has been detected on post-mortem (Haupt et al. 1982) and also has been found a cause of the ACS (Vichinsky et al. 1994). Fat globules in the coronary, cerebral and renal microvasculature has also been found in patients with extensive marrow infarction (Charache and Page, 1967).

Thrombosis during pregnancy.

In a recent large prospective trial of the efficacy of transfusion therapy in 189 pregnant subjects with SCD, thrombosis of the maternal sinuses was noted in over half of the 170 placentas examined independent of the transfusion status of the mother (Koshy et al. 1988). Acute chest syndrome occurred in 12, including 2 transfused and pulmonary embolism occurred in 3 patients. The 'hypercoaguability' of pregnancy may interact with hypercoaguable changes in SCD (see section 1.3.7) to cause placental thrombosis and miscarriage.

Thrombosis in other complications of SCD.

There is at present little evidence that thrombosis plays a significant role in other vasocclusive complications of SCD but thrombosis of small vessels is very difficult to detect clinically and angiographic studies to detect the frequency and significance of acute thrombosis in the bone marrow, bone, retinal, penile and other vasculature in subjects at the onset of the clinical symptoms of crisis, aseptic necrosis, retinopathy, priapism etc have never been done. Since the factors that predispose to vessel occlusion in the cerebral and pulmonary circulations are probably operative throughout the vasculature it can be hypothesised that arterial and/or venous occlusion may contribute to many of the other vaso-occlusive complications of SCD. There are case reports of massive venous thrombosis in unusual sites such as portal vein and cerebral venous sinuses (Arnold et al. 1993; Oguz et al. 1994).

1.3.6 Platelet abnormalities

Moderate thrombocytosis is commonly seen in patients with SCD in the steady state with increased numbers of circulating platelet aggregates and megathrombocytes (Freedman and Karpatkin, 1975; Mehta and Mehta, 1979; Haut et al. 1973). These findings are usually attributed to functional asplenia and/or autosplenectomy, a consequence of repeated splenic infarction (Freedman and Karpatkin, 1975; Haut et al. 1973; Kenny et al. 1980), though increased thrombopoiesis has not been excluded as a cause. If functional asplenia is the major cause then these abnormalities should not be present in younger children with better preserved splenic function or in adults with HbSC who have better preserved splenic function but these detailed studies have not been reported. It is also not clear whether platelet survival is altered in that some workers report

shortened (Semple et al. 1984) and some prolonged (Haut et al. 1973) platelet survival following radiolabelled studies.

In vitro platelet aggregability appears to be increased in adults with steady-state SCD (Haut et al. 1973; Kenny et al. 1980; Westwick et al. 1983) but the limited studies done in children suggest normal or reduced aggregability (Mehta and Mehta, 1980; Mehta and Albiol, 1982; Stuart et al. 1974), again possibly related to better preserved splenic function.

Other studies on platelet function in SCD have found increased plasma levels of the platelet α-granule protein, β-thromboglobulin, and increased platelet factor 4 (PF4) levels and thromboxane B2 production in the steady-state (Westwick et al. 1983; Semple et al. 1984; Buchanan and Holtkamp, 1983; Mehta, 1980; Foulon et al. 1993; Longenecker et al. 1992; Kurantsin Mills et al. 1994; Papadimitiriou et al. 1993), though results have been discrepant as to whether there is further increase during crisis. One study showed platelet ADP levels were significantly reduced during painful crisis suggesting that there is further platelet activation in crisis compared to steady-state (Beurling Harbury and Schade, 1989). There is also disagreement as to whether these abnormalities are consistent with increased *in vivo* platelet activation and secretion or whether they are caused by *in vitro* release but the most recent studies using monoclonal antibodies and flow cytometry strongly suggest that there is *in vivo* platelet activation (Wun et al. 1998). There has to date been no investigation of platelet function in any of the other non-crisis vasocclusive complications such as stroke. In one study levels of β-thromboglobulin and PF4 were reported to be normal in a small number of subjects with stroke (Buchanan and Holtkamp, 1983) but most were investigated during hypertransfusion therapy or at a time remote from their acute event.

1.3.7 Coagulation abnormalities

Procoagulant changes

Factor VIII/vWF is significantly increased in the steady state (Semple et al. 1984; Leslie et al. 1975; Famodu, 1987; Mackie et al. 1980; Hagger et al. 1995). The mechanisms by which this occurs are not clear but FVIII/vWF is an acute phase reactant and may be released in response to humoral mediators of the acute phase reaction such as tumour necrosis factor, interleukin-1 or interleukin-6 (Bauer et al. 1989; Bevilacqua et al. 1986). There is also evidence that other acute phase proteins such as C-reactive protein, serum amyloid A and transferrin are raised in the steady-state (Hedo et al. 1993; Singhal et al. 1993). It also may be that the increased thrombin generated in SCD stimulates endothelial release of vWF. There is no consistent finding as to whether FVIII levels are further increased during crisis with 2 studies showing no differences between steady-state and crisis (Mackie et al. 1980; Richardson et al. 1979) and one reporting significantly higher levels during crisis (Famodu, 1987).

Fibrinogen levels have been normal (Leslie et al. 1975; Mackie et al. 1980; Hagger et al. 1995; Richardson et al. 1979) or increased in the studies performed (Famodu, 1987; Green and Scott, 1986) leaving discrepancies as to whether or not levels are affected and similarly discrepant results have been found when investigators have looked to see if there is any change in levels during crisis (Famodu, 1987; Richardson et al. 1979; Green and Scott, 1986). As it is a major determinant of whole blood viscosity and it enhances RBC adhesion to the endothelium (Wautier et al. 1983), its contribution could be important.

Contact system factors. Significant reductions in contact system factors have been reported in steady-state SCD. One paediatric study (Gordon et al. 1985) reported significant reductions in FXII activity, FXII antigen, PKK activity and

HMWK activity in 12 children with SCD compared to controls and another found reduced FXI and FXII (Abildgaard et al. 1967). Reductions in these factors have been attributed to increased consumption as a result of abnormal coagulation activation though this has not been proven to be the mechanism. During crisis FXII activity fell further though FXII antigen levels were unchanged (Famodu, 1987; Green and Scott, 1986; Gordon et al. 1985).

Studies of *other coagulation factors* have showed reduced levels of FV (Leslie et al. 1975; Famodu, 1987; Richardson et al. 1979) in the steady-state which may well reflect increased thrombin generation as it is proteolysed by thrombin, and reduced FVII and FVII zymogen levels, consistent with increased FVII turnover and TF expression (Kurantsin Mills et al. 1992).

Changes in plasma inhibitors

There is no large comprehensive evaluation of the changes in plasma inhibitors and the control of thrombin generation in either adults or children with SCD. The published studies that have been done have several problems; they have only measured one or two of the inhibitors; many have used unreliable outdated methods; many have findings that conflict with other studies and many have small sample numbers which may well be partially responsible for the discrepancies found between studies. Further difficulties have arisen as the majority of the studies on PC and TPS/FPS have combined data on patients with different genotypes of SCD and have not attempted to separate HbSS and HbSC patients into different groups even though the spectrum of clinical complications varies. Also, none of the published work has included significant numbers of HbSC patients.

Protein C levels are known to be reduced in normal children compared to adult values so that children have a normal but physiological deficiency that

persists until early adulthood (Andrew et al. 1992). This highlights the importance of age-matched controlled studies in children. PC activity has been reported to be reduced in steady state SCD in a number of controlled but small studies in both children (Peters et al. 1994) and young adults with HbSS using a variety of assay methods (Hagger et al. 1995; Green and Scott, 1986; el Hazmi et al. 1993; Wright et al. 1997). Three studies have also reported low PC antigen (Karayalcin and Lanzkowsky, 1989; Peters et al. 1994; Wright et al. 1997). However normal PC activity has also been reported in 14 adult SCD patients (Francis, Jr. 1988b). Results have also been inconsistent in crisis with some studies reporting no differences between crisis and steady state (Hagger et al. 1995; Green and Scott, 1986; el Hazmi et al. 1993; Francis, Jr. 1988b) and another showing significantly reduced levels in crisis (Karayalcin and Lanzkowsky, 1989).

Results from studies on *protein S* levels have also been inconsistent. A recent study in 16 children with HbSS found reduced levels of both mean FPS and TPS antigen in steady state compared to controls (Peters et al. 1994) and adult studies had similar results (Wright et al. 1997; Francis, Jr. 1988b) with levels unchanged during crisis. C4b-BP levels were normal in crisis and steady-state, suggesting that the low FPS levels were not caused by increased levels of C4b-BP and another mechanism(s) is likely to be responsible for the reduction. A further study on 30 adults, mainly with HbSS, found normal levels of both TPS and FPS in steady state but reduced FPS in crisis (Hagger et al. 1995).

The causes of the reduction of PC, TPS and FPS levels in SCD may well be multi-factorial but several possible mechanisms have been proposed; Lane (Lane et al. 1994; Lane et al. 1990) has presented evidence to suggest that reduced FPS in SCD might be due to binding of FPS to the anionic phospholipids abnormally exteriorised in the SSRBC membrane by showing that radiolabelled FPS binds to spectrin-depleted membrane vesicles from normal RBC as well as those shed in

the formation of irreversibly sickled RBC (Lubin et al. 1981; Chiu et al. 1981; Allan et al. 1982) and this binding was calcium-dependent. Whether this process occurs with PC or other vitamin K-dependent factors has not been confirmed. A more recent study has suggested that hepatocellular dysfunction rather than coagulation activation or haemolysis is responsible for the low levels of FPS and PC (Wright et al. 1997) and other workers have also supported this (Karayalcin and Lanzkowsky, 1989).

Antithrombin activity has also been investigated by a number of studies but again results have been discrepant. The earliest controlled studies on HbSS patients in the steady-state found levels to be normal (Leslie et al. 1975) or reduced (Onyemelukwe and Mba, 1984; Onyemelukwe and Jibril, 1992) using an immunoassay or significantly elevated using an outdated activity assay (Richardson et al. 1979). One later study in children found normal antigen levels but reduced activity (Karayalcin et al. 1984). Further studies using chromogenic assays showed normal AT levels in 16 children compared to 16 age-matched controls (Peters et al. 1994) and in 30 adults with HbSS (Hagger et al. 1995). Two studies reporting on AT levels in the crisis situation both agree that AT activity is lower in early crisis compared with the steady-state (Richardson et al. 1979; Karayalcin et al. 1984).

Heparin cofactor II levels have been found to be reduced in one study on adult patients with SCD and other conditions characterised by intravascular haemolysis (Porter et al. 1993). Levels increased towards normal during crises and with regular blood transfusion.

Activated protein C resistance

The published literature on APCR in SCD is scanty. The reasons for this are probably two-fold; first of all it is a recently described cause of thrombophilia (Dahlback et al. 1993) and secondly the FVR506Q mutation is uncommon in Afro-

Caribbean populations (Rees et al. 1995). One study in 50 young adults with SCD found that there was a significant reduction in APCR ratio by the 'standard' test compared to controls (Wright et al. 1997). Only one had the FVR506Q mutation and another one had a lupus anticoagulant. This study did not address the problems with this assay in patients with a prolonged baseline APTT (see chapter 3) but suggested that high FVIII levels may have contributed to increased resistance to APC.

Antiphospholipid antibodies

The possible role of APA in the complications of SCD has not been fully explored though there are 2 published studies with divergent findings. One study found that 17 of 25 SCD patients (68%) had increased levels of APA (Kucuk et al. 1993) but the second study found slightly elevated levels of IgG APA in only 9 of 108 (8%) unselected patients and these did not appear to have any clinical relevance (De Ceulaer et al. 1992). However one case report has reported the association of APA with a right atrial thrombus in a patient with SCD (Yeghen et al. 1995).

Hyperhomocysteinaemia

This has recently been identified as a risk factor for stroke and other vascular diseases and as the pathogenesis of vasculopathy in hyperhomocysteinaemia is presumed to be due to endothelial injury, co-inheritance of MTHFR-T and investigation of homocysteine levels in patients with SCD may reveal important findings and longitudinal prospective studies are needed. The prevalence of homozygosity for this mutation in Afro-Caribbean's is thought to be around 1.5%. One recent uncontrolled study examined serum homocysteine levels in 100 SCD patients including 16 who had had a stroke and found levels were

significantly higher in the stroke group compared to those without stroke and values correlated inversely with folate levels (Houston et al. 1997).

Fibrinolysis

There has been very little work on what role defective fibrinolysis may play in the pathogenesis of complicated SCD and what has been done has looked at clot lysis times which are not specific for any particular component of the fibrinolytic system and are subject to interference by alterations in fibrinogen levels (Famodu, 1988). Investigators that have looked at tPA activity and antigen levels after venous occlusion have had discrepant results (Hagger et al. 1995; Francis, Jr. 1988a; Gordon et al. 1974; Phillips et al. 1990; Phillips et al. 1988) but on balance it seems unlikely that fibrinolysis is significantly impaired in most subjects with SCD but this does warrant further investigation, particularly in those with severe microvascular disease as the small numbers studied to date appear to have lower levels of tPA antigen and activity (Francis, Jr. 1988a).

Increased thrombin generation - SCD as a hypercoaguable state.

There is now some good evidence that a hypercoaguable state exists in SCD so that the alterations in the blood tip the haemostatic balance towards inappropriate fibrin deposition with a consequent increased tendency to thrombosis. The direct evidence for increased thrombin generation and fibrin formation in steady-state SCD includes studies on fibrin D-dimer fragment, a specific marker of cross-linked fibrin formation and dissolution. Levels were elevated in two controlled studies on steady-state adult SCD patients (Francis, Jr. 1989; Kurantsin Mills et al. 1992). As might be expected there is a rise in the D-dimer levels during vaso-occlusive crisis and in patients with other chronic complications of SCD (Devine et al. 1986). Increased levels of fibrinopeptide A (FPA), which reflect thrombin

proteolysis of fibrinogen, have also been found in the steady-state (Green and Scott, 1986; Kurantsin Mills et al. 1992; Billett et al. 1988) but the elevation of FPA levels appear unrelated to the progression of crises (Green and Scott, 1986; Billett et al. 1988).

More recently the more sensitive assays for detecting coagulation activation that have been developed, namely assays for TATs and PF1+2, have been used to look for enhanced thrombin generation. Peters *et al* have reported on 16 children who have significantly increased plasma concentrations of both parameters compared to age-matched children (Peters et al. 1994) and 45 adults have had similar results in the TATs assay (Kurantsin Mills et al. 1992).

Other more indirect evidence includes the findings of reduced plasma levels of factors proteolysed or consumed by thrombin such as FV, PC and PS and increased FVII turnover indicating increased TF expression. The mechanism for this is not clear but suggestions are that various procoagulant modifiers, such as TNF and IL1, may promote TF expression in endothelial cells and/or monocytes (Bauer et al. 1989; Bevilacqua et al. 1986). A further hypothesis is that C-reactive protein could induce monocytic TF synthesis (Cermak et al. 1993).

Coagulation abnormalities in non-crisis vasocclusive complications of SCD.

Very few studies of coagulation abnormalities in vaso-occlusive complications other than crisis have been reported, even in thrombotic complications such as stroke. Devine *et al* (Devine et al. 1986) have reported that fibrin D-dimer fragment was significantly elevated in 86% of samples from subjects with complications other than crisis (leg ulcers, aseptic necrosis of bone, joint pain, stroke, cholecystitis and infection). Cacciola *et al* (Cacciola et al. 1989) reported that AT activity was significantly reduced in subjects with HbSβthal and leg ulcers and that infusion of AT concentrate improved ulcer healing. A small pilot study has

examined the levels of protein C and protein S in SCD children with stroke and found significantly reduced levels compared to those who had not had clinical events but this finding needs confirmation in a bigger controlled study (Tam, 1997). Apart from these latter studies there has been no other investigation of any changes that may occur in plasma inhibitors in relation to any of the other non-crisis vasocclusive complications. The possible role of hyperhomocysteinaemia has been suggested but requires more study.

1.4 Beta - thalassaemia

1.4.1 Background

The thalassaemias are a group of disorders that cause moderate to severe haemolytic anaemia, acute and chronic tissue damage, organ failure and premature death. They are caused by mutations or deletions in or around the globin gene DNA that alter the rate of synthesis of globin, leading to deficient globin chain production and imbalanced synthesis of the globin moiety of haemoglobin. In β-thalassaemia, depending on the genetic defect, either no β chains (β^0) or small amounts are synthesised (β^+). Excess α chains precipitate in erythroblasts and in mature red cells causing severe ineffective erythropoiesis and haemolysis. Individuals who have β^0-thalassaemia (β-thalassaemia major) are transfusion dependent whereas some with β^+-thalassaemia can survive with moderate chronic anaemia but without regular transfusion (β-thalassaemia intermedia).

1.4.2 The role of thrombosis, platelet and coagulation abnormalities in β-thalassaemia.

Thromboembolic events are thought to be an important cause of morbidity and mortality in β-thalassaemia (Michaeli J et al. 1992), although it is difficult to determine the true incidence of these events in the light of recent advances in the management which result in longer life expectancy. However there are reports of strokes and transient ischaemic attacks (Wong et al. 1990; Logothetis J et al. 1972), pulmonary embolism (Sonakul and Fucharoen, 1992), renal vein thrombosis and deep vein thrombosis (Michaeli J et al. 1992).

Studies looking for mechanisms to explain this thrombotic tendency have been scanty, particularly in children, and a comprehensive study of haemostasis in this condition is lacking. Those that have been published have included investigation of platelet and coagulation abnormalities in thalassaemia and overall the evidence that exists is similar to that in SCD in that there are both platelet and coagulation anomalies and there is a chronic hypercoaguable state. There is reduced platelet survival and in vivo platelet activation, detected both by increased excretion of platelet thromboxane urinary metabolites and by measuring GMP-140 expression, an activation dependent marker (Eldor et al. 1989; Eldor et al. 1991; del Principe et al. 1993). An inverse relationship has been found between GMP-140 expression and high density lipoprotein cholesterol (del Principe et al. 1993) and there is a suggestion that the dyslipidaemia may play a central role in the activation of blood platelets and in the early development of atherosclerosis (Goldfarb et al. 1991).

A study on 30 children with homozygous beta-thalassaemia found reduced levels of the majority of procoagulants with only fibrinogen, FV and FVIII being normal (Caocci et al. 1978). The vitamin-K dependent factors, II, VII, IX and X

were all slightly reduced whereas FXI and FXII were markedly reduced, suggesting that the chronic haemolysis causes ongoing activation of the contact pathway.

Levels of coagulation inhibitors have been found to be reduced in 2 studies on mainly adult thalassaemics (Shirahata et al. 1992; Musumeci et al. 1987). Protein C values were reduced in the majority and AT was reduced in some of the patients in the first study (Musumeci et al. 1987) whereas the second of these found low PC and TPS levels which were significantly lower than the concentrations of other liver-dependent factors (Shirahata et al. 1992). However this latter study measured only TPS but not FPS and used a method that is known to be unreliable due to poor precision. As in SCD, heparin cofactor II levels appear to be related to red cell turnover, being low in untransfused thalassaemia intermedia and approaching normal levels in patients on transfusion regimes (O'Driscoll et al. 1995). It has also been found that thalassaemic red cells, like SS RBC's seem to have undergone lipid rearrangements that result in excess PS exposure and this may contribute towards the hypercoaguable, thrombogenic state that has been associated with thalassaemia (Helley et al. 1996; Borenstein et al. 1993). A recent study in adults has found correlation between the red cell anionic phospholipid exposure in thalassaemia major red cells and markers of platelet activation, suggesting that the procoagulant surface of the red cells accelerates thrombin generation *in vivo* which, in turn, triggers platelet activation (Ruf et al. 1997). There were no differences in the degree of red cell and platelet abnormalities between the splenectomised and non-splenectomised patients. This is of particular interest as there have been recent observations that patients with hereditary haemolytic anaemias, including thalassaemia major, have an increased risk of thrombo-embolic disease post-splenectomy (Kemahli et al. 1997; Winichagoon et al. 1981; Visudhiphan et al. 1994).

1.5 Summary

Vascular occlusion has a central role in the pathogenesis of the complications that can arise in sickle cell disease but the sequence of events that occurs to result in this occlusion is likely to be complex and is still largely ill-understood. The role of thrombosis in the vaso-occlusion has been the subject of some debate and although it plays a more proven role in large vessel events, such as CVD, there is little evidence so far that thrombosis *alone* is responsible for micro-vascular occlusion.

As a preface to this thesis I have introduced the haemostatic system and given an overview of the clinical sequelae and what is known about the underlying processes in the pathophysiology of this disease. I have also shown that it is still not clear what abnormalities are present in the haemostatic system as no large comprehensive study of the control of thrombin generation has previously been performed and there has been very little consideration of how this may relate to different vaso-occlusive complications. This thesis has been designed to address these issues.

Chapter 2

Outline and aims of study

2.0 Aims of this thesis

As outlined in detail in Chapter 1 many of the previous studies that have been performed on adults and children with SCD to look at the causes of the increased thrombotic risk in these patients have had a number of weaknesses;

- conflicting findings.
- outdated methods.
- small numbers.
- many were uncontrolled and as normal ranges for procoagulant and inhibitor proteins can vary with age, sex, between laboratories and possibly between ethnic groups it is important to compare to ethnically matched controls.
- different genotypes were analysed together in some studies and very small numbers of patients with Hb SC have been tested.
- very few studies relate the findings to non-crisis vaso-occlusive complications.

Therefore the principal objective of this thesis was to perform a comprehensive thrombophilia screen in a large cohort of children with SCD to provide definitive data as to whether there are important consistent abnormalities that contribute to the thrombotic potential in this disease. Results were compared to a control group.

There were also a number of other questions which I proposed to examine with the data collected;

- was there any difference between the different genotypes of SCD ?
- was there any difference between the children who have had a clinical cerebral thrombotic event, those who have asymptomatic cerebrovascular disease and those who have no detectable abnormality of cerebral vasculature? As I have outlined in chapter 1 the CVD that develops in SCD usually involves large arteries and although abnormalities in the parameters measured in this study (see section 2.1) are associated with an increase in the risk of *venous* thromboembolic

disease, the possible contribution towards the development of cerebral arterial disease is still unclear, especially in the setting of abnormal red blood cells in SCD.

- Do these investigations give any further clues as to the important processes underlying the development of CVD and, if so, are there any therapeutic possibilities that may be beneficial ?
- what were the differences between chronically transfused children and non-transfused children - i.e. which parameters are reversed by transfusion?
- was there any relationship between any of the parameters measured and the severity of SCD as measured by crisis rate and number of chest crises?
- was there any relationship between baseline haematological parameters such as Hb, WBC, %HbF and any of the thrombotic proteins measured?
- was there any influence of splenectomy on the thrombotic proteins measured?
- how did the results for children with sickle cell disease compare to those with other disorders such as thalassaemia and other chronic haemolytic conditions? What were the findings that were unique to SCD and which could be contributed to increased red cell turnover?
- what were the findings in thalassaemic children, who have been poorly studied but also *may* have an increased thrombotic risk?

2.1 Outline of the study

The thrombophilia screen was designed to be as comprehensive as possible. All patients and controls had baseline haematology, biochemistry and coagulation screening. Fibrinogen was measured as very high levels are associated with an increased risk of thrombosis and it is also an 'acute phase' protein. CRP was measured as a second 'acute phase' protein. The inhibitor proteins measured were PC, TPS and FPS, AT and HCII. In order to assess whether deficiencies of PC and PS may be due to a general deficiency of vitamin K

dependent proteins or liver disease rather than excess depletion of coagulation inhibitors two vitamin K dependent procoagulants, FVII and FX were measured and one non-vitamin K dependent procoagulant, FXI was also measured. FVII levels were also important as high levels are a risk factor for arterial disease. FXII was done as part of the prothrombotic profile. APC resistance ratios were performed and when appropriate, the factor V Leiden genotype was determined. TAT complexes and PF1+2 were done as measures of thrombin generation. A comprehensive antiphospholipid screen included ACL IgG and IgM, anti-β_2GP1 antibodies and a textarin time. An ecarin time was set up for the abnormal/borderline textarin times.

Chapter 3

Methods

3.0 Study population

The study population consisted of a total of 144 children with known inherited haemolytic red cell disorders. They were enrolled from a weekly haemoglobinopathy clinic at Queen Elizabeth Hospital for Children, London E2. The diagnosis had been made previously in all cases by family studies and cellulose acetate haemoglobin electrophoresis at pH 8.6 and/or high performance liquid chromatography in the children with SCD and thalassaemia. The children with other miscellaneous red cell disorders had had the diagnosis made by standard laboratory techniques.

One hundred and twenty children had SCD; 91 were homozygous for haemoglobin S (HbSS), 5 had haemoglobin Sβ^0thalassaemia (HbSβ^0thal) and 24 had haemoglobin SC (HbSC). Since HbSβ^0thal has a similar phenotype to HbSS these two groups were combined for analysis (see chapters 4 and 5). At the time of sampling (see below) these patients were in the steady non-crisis state, were free from infection and were not on any regular medication except for prophylactic antibiotics and folic acid. Thirteen children with HbSS were on a regular transfusion programme to keep the %HbS below 20% at all times and minimise the risk of further sickling, and all had been transfused 3-4 weeks prior to study. In 10 of the 13 the indication for a transfusion regime was clinical symptomatic stroke. The remaining 107 children with SCD had not had a blood transfusion in the 12 week period prior to testing.

The other 24 study patients did not have SCD; 13 had β-thalassaemia major and were all on a regular transfusion programme (every 3-4 weeks) and the other 11 were a heterogeneous group of red cell disorders - hereditary spherocytosis (HS), pyruvate kinase (PK) deficiency, haemoglobin H (HbH) disease and glucose 6-phosphate dehydrogenase (G6PD) deficiency.

3.1 *Normal controls*

In order to establish a normal range for the assays performed in this study ethnically matched healthy children were required. All parents were therefore asked at the outpatient visit whether they would be willing to bring any healthy unaffected siblings in at their next attendance to be venesected and act as normal controls. A total of 30 controls was the original aim but it proved difficult to recruit and a total of 21 were enrolled by the end of the study. However one was found to have FXI deficiency and a further two were found to be G6PD deficient and were excluded leaving a control group of 18 siblings.

3.2 *Ethical approval*

This study was submitted to and approved by the ethics committee at Great Ormond Street Hospital for Children as at that stage Queen Elizabeth Hospital for Children was administratively part of Great Ormond Street.

When families attended the out patient clinic all received an information sheet and informed consent was obtained from at least one parent before the blood samples were taken in all cases. There was a different information sheet for controls in which issues particularly relevant to venesection in healthy siblings were covered.

3.3 *Screening for cerebrovascular disease*

Of the 96 children with HbSS/HbSβ^0thal, a total of 64 had had investigations for CVD. Ten of these had had symptomatic stroke confirmed on CT and/or MRI and were on regular transfusions. The other 54 asymptomatic children had all had

transcranial doppler (TCD) studies performed - the majority had been part of a previous study looking at TCD in the assessment of CVD in this clinic population (Hewes et al. 1994). All abnormal TCD studies had been repeated on at least one occasion to confirm high (>170 cm/sec) middle cerebral artery velocities (see section 1.2.1). The majority of the children with abnormal TCD had also had MRI performed (see chapter 4 for results).

3.4 Sample collection

Clinical data was collected and blood samples were taken at times of routine outpatient attendance at the haemoglobinopathy clinic at Queen Elizabeth Hospital for Children, London E2.

Venous blood samples (10-15ml) were drawn into plastic syringes and then transferred immediately into plastic bottles containing 0.106 M tri-sodium citrate as anticoagulant in a ratio of nine parts blood to one part citrate. EDTA and lithium heparin samples were also taken to perform baseline haematological and biochemical investigations.

3.5 Sample processing and storage

Platelet poor plasma was prepared within an hour of collection by centrifugation twice at 2000 *g* for 15 minutes at room temperature. This method was validated at the start of the study by checking that the platelet count was unrecordable in both patient and control plasmas. The plasma aliquots were stored at -40 to -80°C until use.

3.6 Parameters measured

The following tests were done on each sample;

•FBC including MCV, WBC and differential and platelet count

•Reticulocyte count

•Hb electrophoresis to measure the %HbF

•Biochemical screen - liver function, renal function tests and bone profile.

•CRP was measured as a marker of 'acute phase reaction'.

•Coagulation screen - PT, APTT and TT.

•Fibrinogen

•Factor VII

•Factor X

•Factor XI

•Factor XII

•Protein C

•Total Protein S

•Free Protein S

•Antithrombin

•Heparin cofactor II

•Activated protein C resistance

•Factor V genotype

•Thrombin-antithrombin complexes

•Prothrombin fragment F1+2

•Anticardiolipin IgG and Ig M

•Anti-β_2GP1 antibodies

•Clotting time using Pseudonaja textilis venom

•Clotting time using Echis carinatus venom (in a small number of samples)

3.7 Methods

3.7.1 Baseline Investigations

The FBC, biochemical and liver function tests and CRP were performed by standard automated laboratory procedures. The reticulocyte count was done by a manual counting method using a supravital stain - new methylene blue. Hb electrophoresis was performed to measure %HbF by high purity liquid chromatography.

Additional tests included standard automated coagulation screening tests performed on an ACL 3000+ coagulometer (Instrumentation Laboratories Ltd., Warrington, Cheshire). The PT was done using 0.1 or 0.2 ml samples of test plasma and normal control plasma (Instrumentation Laboratories Ltd.) and a lyophilised thromboplastin (PT-Fib HS PLUS, Instrumentation Laboratories Ltd.). The APTT was performed with the same volumes of test and control plasma and APTT lyophilised silica (Instrumentation Laboratories Ltd.). The normal ranges for children > 1 year in this laboratory are PT 12.0-15.0 seconds, APTT 27.0-40.0 seconds.

The thrombin time (TT) was done by manual techniques incorporating Seegers titration mixture containing calcium chloride to overcome the effect of the high levels of sialic acid residues found in fetal fibrinogen in infants. 0.1 ml aliquots of normal control (Instrumentation Laboratories Ltd.) and test plasmas were warmed in a waterbath and 0.3 ml of Seegers titration mixture and 0.1 ml of bovine thrombin (Armour Pharmaceuticals, Illinois, U.S.A., 10 U/ml diluted with Owren's buffered saline) were added and the time to clot formation was measured following tube tilting. The normal range for children > 1 year in this laboratory is 9.0-11.0 seconds.

3.7.2 *Fibrinogen*

Fibrinogen was measured by a Clauss clotting assay (von Clauss, 1957). Dilutions (1/10, 1/15, 1/20, 1/25, 1/30) of Reference Plasma 100% (Immuno, Vienna, Austria) and test plasma (1/10, 1/20) were made in imidazole buffer. 0.2 ml aliquots were clotted with 0.1 ml bovine thrombin (100u/ml, Organon Teknika Ltd, Cambridge, U.K.). Test results were read from the standard curve (log/log plot). A control plasma of known potency (Instrumentation Laboratories Ltd.) was run with each test to ensure continued precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range is 2.0-4.0 g/l.

3.7.3 *Factor VII*

This was performed with a one-stage PT-based assay on the ACL 3000+ coagulometer. FVII deficient plasma was added to dilutions of the patient and reference plasma of known FVII content. A PT was then performed on these dilutions and the ability of the test plasma to correct the deficient plasma was compared to that of the reference and this is used in the standard curve to give a value for the test sample.

The standard curve was prepared from 1/5, 1/10 and 1/20 dilutions of 100% Reference Plasma (Immuno) in factor assay diluent (Instrumentation Laboratories Ltd.). 1/5 and 1/10 dilutions of test plasma were also prepared in the same way and all dilutions, the FVII deficient plasma (Diagnostic Reagents Ltd, Thame, Oxon, U.K.) and the thromboplastin (PT-FIB, Instrumentation Laboratories Ltd.) were loaded appropriately into the coagulometer. The FVII value of the reference plasma was entered and the ACL 3000+ was programmed to perform a single factor assay according to manufacturers instructions. From the PT results a direct

reading of the FVII value from each test plasma was obtained from the 1/5 dilution and the result from the 1/10 dilution was doubled and the average of the 2 results was reported. A control plasma of known potency (Immuno) was run with each test to ensure between batch precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range for children > 1 year is 50-150 iu/dl.

3.7.4 Factor X

This was performed as for the FVII assay but the coagulometer was programmed for a FX assay and FX deficient plasma (Instrumentation Laboratories Ltd.) was used. The laboratory reference range for children >1 year is 50-150 iu/dl.

3.7.5 Factor XI

FXI was measured by a one-stage APTT-based assay on the ACL 3000+ coagulometer. The principle of the assay, the dilutions for the standard curve and test plasmas and the reagents were all the same as for the FVII assay except that an APTT was performed on all dilutions using an APTT reagent (Instrumentation Laboratories Ltd.) and FXI deficient plasma (Instrumentation Laboratories Ltd.). . The laboratory reference range for children > 1 year is 50-150 iu/dl.

3.7.6 Factor XII

FXII was measured by an amidolytic assay modified for microtitre plates. The FXII present is converted to FXIIa by an ellagic acid-containing-activator preparation. Any kallikrein generated by the action of FXIIa on prekallikrein is blocked with a kallikrein inhibitor reagent. FXIIa then cleaves a chromogenic

peptide substrate and liberates p-nitroaniline pNA which can be measured photometrically.

The standard (Immuno Reference Plasma 100%) and test plasmas were initially treated with acetone in a ratio of 3:1 plasma to acetone. The mixture was left for 15 minutes at 4°C and then spun in a microcentrifuge at 10 000g for 4 minutes and then placed on ice. The test plasma and the 100% standard plasma dilution were prepared by diluting 100μl of the acetone treated plasma with 300 μl of Tris buffer. Further standard dilutions of 125%, 75%, 50%, 25%, 0% were prepared in the same way. Duplicate 25 μl of standard or test plasma dilutions were then placed in the wells of a microtitre plate and 25 μl of FXII activator (Unicorn Diagnostics Ltd, London, U.K.) was added to each well, mixed and incubated at 37°C for 10 minutes. 75 μl of kallikrein inhibitor (Unicorn Diagnostics Ltd) diluted 1/50 with Tris buffer and warmed to 37°C was added and incubated for exactly 1 minute. Then 50 μl of FXII chromogenic substrate (2 AcOH.H-D-CHT-Gly-Arg-pNA, Unitrate FXII, Unicorn Diagnostics Ltd.) was added and again mixed and incubated at 37°C for 10 minutes. The reaction was stopped with 50 μl citric acid (1M) and absorbance was read at 405 nm. Absorbance was plotted against %FXII concentration for the standard plasma dilutions and the values for the test plasma were abstracted from the standard curve and corrected according to the potency of the standard. A control plasma of known potency was run with each batch of tests to ensure between-batch precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range is 73-145 iu/dl.

3.7.7 Protein C

PC activity was measured by an amidolytic assay on the ACL 3000R coagulometer using Copperhead snake (Agkistrodon contortrix) venom (Protac C,

Unicorn Diagnostics Ltd.) to activate PC. The level of APC was measured by detection of released nitroaniline following its cleavage of a chromogenic substrate.

A standard curve was prepared by diluting 100% Reference Plasma (Immuno) with tris/imidazole buffer pH 8.4 to give the following dilutions; 150%, 100%, 75%, 50%, 25% and 0%. The test plasma was diluted 1/2 in the same buffer. The standard curve dilutions and the test plasma dilution were all placed into cups in the autosampler tray and Protac C and a tripeptide chromogenic substrate (2 AcOH.H-D-Lys(Cbo)-Pro-Arg-pNA, Unitrate PC, Unicorn Diagnostics Ltd.) were placed in the reagent reservoirs. The coagulometer was set to a reaction time of 300 seconds and absorbance was measured at 405 nm. The assay was repeated substituting diluted buffer for Protac to obtain background values and these were subtracted from each test absorbance which was then plotted against concentration. A control plasma of known potency was run with each batch of tests to ensure between-batch precision. All abnormal results were repeated with a second assay for confirmation. The coagulometer was primed between assays to prevent carry-over. The reference range used for children in this laboratory was 40-92 iu/dl at age 1-6 years, 45-93 iu/dl at age 6-11 years, 55-111 at age 11-16 years and 70-130 iu/dl for those >16 years (adapted from Andrew (Andrew et al. 1992) and established on normal adults).

3.7.8 Total and free Protein S antigen

TPS and FPS antigen were measured by ELISA using the method of Woodhams (Woodhams, 1988) with some modifications. PS exists in plasma both free and bound to C4b-BP. The bound PS can be precipitated with polyethylene glycol (PEG) so that FPS can be measured.

Duplicate microtitre plates were coated with antisera against PS, one was used to measure FPS and the other TPS. They were coated overnight at 4°C with 100 μl of 1/1000 rabbit anti-human PS (Dako Ltd, High Wycombe, Bucks U.K.) diluted in phosphate buffered saline (PBS). To prepare test and standard (100% Reference Plasma, Immuno) plasmas for FPS measurement, 200 μl aliquots were placed in microfuge tubes and warmed to 37°C. To each tube 50 μl of 18.75% PEG 8000 (Sigma® Chemical Company, St Louis, U.S.A.) was added (final concentration 3.75%) and, after mixing, the tubes were placed on melting ice for 30 minutes. They were then centrifuged at 3000 RPM for 20 minutes and the supernatant was pipetted into clean tubes and used for the FPS assay.

The standard curve for the FPS assay was prepared by diluting 100 μl standard plasma in 3.9 ml PBS tween plus 3% PEG 8000 to make a 1/40 (125%) dilution and from this 1/50 (100%), 1/75, 1/100, 1/200, 1/400 and 1/800 dilutions were prepared. The test plasma was diluted in the same way to make 1/50 and 1/75 dilutions. The standard curve for the TPS assay was made by diluting 50 μl of reference plasma with 8.0 mls of PEG buffer to make a 1/160 dilution (125%) and from this 1/200 (100%), 1/240, 1/400, 1/800, 1/1600 and 1/3200 dilutions were prepared. The test plasma for the TPS assay was diluted 1/200 and 1/300. The plates were washed with PBS tween and 100 μl of the standard and sample dilutions added in duplicate. After a 3 hour incubation at room temperature the plates were washed as before and then 100 μl of 1/1000 rabbit anti-human PS conjugated to horseradish peroxidase (HRP) (Dako Ltd.) diluted in PBS tween plus 3% PEG 8000 were added to each well. After a further 90 minute incubation at room temperature plates were washed as before and 100 μl of freshly made up orthophenylenediamine (OPD) substrate were added to each well. This substrate was made up by adding 10 mg 1,2-phenylenediamine dihydrochloride

(orthophenylenediamine, Sigma®) and 7 μl 3% hydrogen peroxide to 15 ml citrate phosphate buffer pH 5.0. The plates were then incubated in the dark until optimum colour development had occurred (after about 3 to 6 mins) and the reaction was then stopped with 100 μl sulphuric acid (2M). Absorbance was measured at 492 nm on a microtitre plate reader. For each plate log concentration was plotted against absorbance and test samples read from the respective standard curve. Each test sample is converted for dilution factor and only those dilutions giving results within the linear region of the standard curve were accepted. The results were corrected as below to account for the plasma dilutions;

TOTAL:	1/200 - direct reading	FREE:	1/50 - direct reading
	1/300 - multiply by 1.5		1/75 - multiply by 1.5

A control plasma of known TPS and FPS was run with each test to ensure continued precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range for children >1 year was 66-120 iu/dl for TPS and 70-125 iu/dl for FPS (adapted from Andrew (Andrew et al. 1992) and established on normal adults).

3.7.9 Antithrombin

AT was measured by an amidolytic assay modified for microtitre plates. Normal or test plasma is incubated with heparin, which acts to potentiate the action of AT, and excess bovine thrombin. A proportion of the thrombin is inhibited by the AT in the plasma and the residual thrombin is able to cleave a peptide chromogenic substrate for thrombin, liberating p-nitroaniline pNA, which can be measured photometrically. The amount of colour is inversely proportional to the AT

concentration. As HCII is a poor inhibitor of thrombin this system primarily measures AT.

The test plasma and the standard (100% Reference Plasma, Immuno) dilutions were prepared by adding 25 μl of the plasma to 1500 μl of buffer (tris/EDTA/saline buffer pH 8.4 with unfractionated sodium heparin [C.P. Pharmaceuticals Ltd, Wrexham, Clywd, U.K] at 3 iu/ml) and then 200 μl of this dilution was added to a further 600 μl of buffer to give the 100% dilution. Further standard dilutions of 125%, 75%, 50%, 25%, 0% were prepared. Duplicate 100 μl of standard or test plasma dilutions were placed in the wells of a microtitre plate. 25 μl of bovine thrombin (5 iu/ml in tris/EDTA/saline buffer pH 8.4 with bovine serum albumin [BSA, Sigma®] at 10g/l) was added to each well and after incubation at 37°C for 60 seconds, 50 μl of thrombin substrate (1 mM 2AcOH.H-D-CHG-Gly-Arg-pNA, Unitrate TH, Unicorn Diagnostics Ltd) was added. After a further incubation of exactly 120 seconds the reaction was stopped with 75 μl citric acid (1M). Absorbance was read at 405 nm and was plotted against concentration and the results were read off the standard curve. These values were corrected according to the potency of the standard as follows:-

$$\text{AT (iu/dl)} = \frac{\text{\% activity x standard potency (iu/dl)}}{100}$$

A control plasma of known potency was run with each batch of tests to ensure between-batch precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range for > 1 year is 80-120 iu/dl.

3.7.10 Heparin cofactor II

HCII was measured with a similar assay to AT but using dermatan sulphate instead of heparin and human rather than bovine thrombin. Dermatan sulphate potentiates the action of HCII but not of AT. Test samples and the 100% standard were prepared by diluting 25 μl with 2000 μl buffer (tris/EDTA/saline polybrene, pH 8.2). Further 150%, 75%, 50%, 25% and 0% standards were prepared. Duplicate 50 μl amounts of standard or test plasma dilutions were placed in the wells of a microtitre plate. 50 μl dermatan sulphate (0.3 μg/ml, Unicorn Diagnostics Ltd) was added and the mixture incubated at 37°C for 2 minutes. Then 50 μl human thrombin (Unicorn Diagnostics Ltd, 1.2u/ml in 1% BSA) were added for a further 5 minute incubation period. 50 μl of thrombin substrate (1 mM 2AcOH.H-D-CHG-Gly-Arg-pNA, [Unitrate TH, Unicorn Diagnostics Ltd]) was then added for a final 5 minute incubation. The reaction was stopped by adding 50 μl of citric acid (1M). Absorbance was measured at 405 nm and was plotted and calculated as for the AT assay. A control plasma of known potency was run with each batch of tests to ensure between-batch precision. All abnormal results were repeated with a second assay for confirmation. The laboratory reference range was 65-148 iu/dl.

3.7.11 Activated protein C resistance

APC resistance was assessed by measuring the anticoagulant response of test plasma to the addition of APC using the ACL 3000R coagulometer and kits supplied by Chromogenix (Coatest, Chromogenix, Mölndal, Sweden). Aliquots of 250 μl of test plasma were used and an APTT was performed using APTT reagent and calcium chloride ($CaCl_2$) supplied in the kit. An APTT was also done following the addition of APC to the test plasma and the ratio of APTT with APC / APTT with $CaCl_2$ gave the APC resistance ratio. In test samples with a long baseline APTT

the standard assay is inaccurate and therefore a 'modified' APC resistance assay was performed by adding factor V deficient plasma (V-DEF plasma, Chromogenix) to sample plasma in a ratio of 4 to 1 and then measuring the anticoagulant response on addition of APC. All assays were controlled using internal controls supplied in the kit. All abnormal results were repeated with a second assay for confirmation. Following each assay the coagulometer was primed to prevent carry-over. The laboratory reference range is 2.8-5.5 for the standard APC resistance ratio and 2.3-4.0 for the 'modified' APC resistance ratio.

3.7.12 Factor V genotype

The presence of the factor V Leiden mutation (FVR506Q) was determined by preparation of genomic DNA from EDTA peripheral blood leucocytes and then a modification (Beauchamp et al. 1994) of the original polymerase chain reaction method (Bertina et al. 1994) was used. The region of intron 10 of the factor V gene surrounding the Arg506Gln mutation was amplified using the primers as follows;

Fv3 5'- CATGAGAGACATCGCCTCTG
Fv5 5'- GACCTAACATGTTCTAGCCAGAAG

The reaction times were 95°C for 7 minutes then 35 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute. This was followed by 10 minutes at 72°C and soak at 4°C. The 147 bp fragment produced was digested with the restriction enzyme *Mnl* 1 during incubation for 3 hours at 37°C. The G1691A mutation removes a restriction site yielding fragments of 25 and 122 bp as opposed to 25, 37 and 85 bp in the wild type. The digested products were run on a 3% Metaphor XR agarose gel stained with ethidium bromide and were compared to known controls homozygote, heterozygote and negative for FVR506Q.

3.7.13 Thrombin-antithrombin complexes

Evidence of activation of coagulation was assessed by measuring TAT complexes using a commercial ELISA (Enzygnost TAT micro, Behring, Marburg, Germany). The conversion of prothrombin into active thrombin is pivotal in the coagulation pathway. Thrombin is inhibited by AT and the inactive proteinase/inhibitor complex can be measured and the amount of complex present reflects the degree of activation of coagulation. Microtitre plates are supplied that are coated with rabbit antibodies against human thrombin. 50 μl aliquots of 4 standard dilutions supplied in the kit, a control plasma with a known assigned value and the test plasma are put into the wells in duplicate with 50 μl of sample buffer (Tris buffer solution 100 mmol/l, Tween 10 ml/l and EDTA 37 g/l). The plate is incubated for 15 minutes at 37°C and was then washed 3 times to remove unbound constituents with the supplied washing solution (phosphate buffer solution 90 mmol/l containing tween 18 g/l). In the second reaction 100 μl of rabbit anti-human AT peroxidase conjugated antibody diluted 1/50 with conjugate buffer (Tris buffer 50 mmol/l with BSA) was added and again incubated for 15 minutes at 37°C and washed 3 times. The substrate solution used was OPD dihydrochloride in citrate buffer with hydrogen peroxide. 100 μl volumes were added and the plate was incubated in the dark for 30 minutes and the reaction then stopped with sulphuric acid (0.5 M). The resulting colour intensity was proportional to the concentration of TAT. The absorbance was read at 492 nm and the 4 standard dilutions were plotted on log/log paper. The TAT concentrations of test plasma were read directly from the reference curve via their respective absorbance values. The reference range quoted by the manufacturer was 1.0-4.1 μg/l. Ex-vivo activation was minimized by avoiding the use of samples which had previously been thawed and re-frozen and care was taken with sample handling. The same operator performed

all the assays to maximize reproducibility and avoid inter-assay variation and a control plasma of known potency was also run with each batch to ensure between-batch precision.

3.7.14 Prothrombin fragment F 1+2

Further evidence of activation of coagulation was assessed by measuring PF1+2 using a commercial ELISA (Enzygnost F1+2 micro, Behring, Marburg, Germany). When prothrombin is converted to active thrombin, PF1+2 is generated and it is therefore possible to quantify exactly the amount of thrombin generated. The method is similar to that for TATs and uses the same volumes but the microtitre plates supplied are coated with rabbit antibodies against human F1+2. The first incubation step is for 30 minutes and the first washing step has 2 cycles. In the second reaction rabbit anti-human prothrombin peroxidase conjugated antibody binds to the free F1+2 determinants. The substrate solution and stopping solution used was as in the TAT complex method and the absorbance was read at 492 nm and the 4 standard dilutions were plotted on log/log paper. The PF1+2 concentrations of test plasma were read directly from the reference curve via their respective absorbance values. The reference range quoted by the manufacturer was 0.4-1.1 nmol/l. Ex-vivo activation was minimized by avoiding the use of samples which had previously been thawed and re-frozen and care was taken with sample handling. The same operator performed all the assays to maximize reproducibility and avoid inter-assay variation and a control plasma of known potency was also run with each batch to ensure between-batch precision.

3.7.15 Anticardiolipin antibodies

ACA IgG and IgM were measured by ELISA (Loizou et al. 1985) and standardised using sera calibrated against International Reference samples in GPL and MPL units (Harris et al. 1987), the GPL and MPL being arbitrary units which were derived from the activity of affinity purified sera. Half of each microtitre plate was coated with 30 μl per well of 50 mg/l cardiolipin (Sigma®) and the other half was coated with ethanol alone to act as blanks to account for non-specific binding. The plates are incubated overnight to allow the ethanol to evaporate and then are washed with PBS. To block non-specific sites 75 μl of adult bovine serum (ABS, Sigma®) diluted 10% in PBS was added to each well and incubated at room temperature for 1 hour and then washed again with PBS. 50 μl aliquots of doubling dilutions of reference plasma (1/50 - 1/1600) and 1/50 dilutions of test samples in ABS buffer were added in duplicate to both halves of the plate which was then incubated at room temperature for 3 hours. After washing with PBS, 50 μl aliquots of alkaline phosphatase conjugated goat anti-human IgG or IgM (Sigma®, diluted 1 in 1000 in ABS buffer) were added to each well and the plates incubated for 90 minutes at room temperature. After further washing with PBS, 50 μl of 1g/l substrate (p-nitrophenyl phosphate disodium hexahydrate [phosphatase tablets] Sigma®) in diethanolamine buffer was added to each well, and the plate incubated in the dark at 37°C until suitable colour development (approximately 45 minutes). The reaction was stopped with 50 μl of sodium hydroxide (3M). The absorbance was read at 405 nm using 490 nm as a reference wavelength. The average blank reading was subtracted from the average test reading and a standard curve obtained by plotting log absorbance against log concentration. The test results were calculated as a percentage of the reference plasma. A control plasma of known potency was run with each batch of tests to ensure between-batch

precision. All abnormal results were repeated with a second assay for confirmation. A positive ACA result was considered to be IgG >5 GPL or IgM >3 MPL (Harris et al. 1987).

3.7.16 Anti-β2GPI antibodies

Anti-β2GPI antibodies were detected by ELISA following purification of β2GPI (McNally et al. 1995) and results were expressed as the percentage binding of a well-characterized positive serum from a patient with the antiphospholipid syndrome.

Half of each microtitre plate was coated with 100 μl per well of β2GPI (1mg/ml) diluted 1/100 with PBS and the other half was coated with 100 μl of 10 μg/ml BSA (Merck Ltd) in PBS to act as blanks. The plates were incubated overnight at 4°C and unbound protein was removed by washing the plate 3 times with 150 μl volumes of PBS tween. To block non-specific sites 125 μl of 1% w/v BSA in PBS was added to each well and incubated at room temperature for 1 hour and then washed again with PBS tween. Test and standard plasmas were diluted in PBS tween containing 1% w/v of BSA. A standard curve was prepared from a reference plasma with a known high anti-β2GPI at 6 doubling dilutions from 1:100 to 1:3200, which has been calibrated against affinity purified anti-β2GPI. Tests were diluted 1/100. 100 μl aliquots of tests and standards were added to duplicate test and blank wells and incubated at room temperature for 2 hours. Unbound protein was removed by washing 4 times with PBS tween. 100 μl volumes of peroxidase conjugated goat anti-human IgG (Sigma®, diluted 1 in 2000 in PBS tween containing 1%w/v BSA) were added to the wells for 2 hours at room temperature. After 3 further washes with PBS tween, 100 μl aliquots of 3,3',5,5'-tetramethylbenzidine (Sigma®, 0.1mg/ml in phosphate citrate buffer) was added to

each well and following colour development the reaction was stopped with 2M H_2SO_4 and absorbance read at 450 nm. The anti-β2GPI level of test plasma was derived from a log/log standard curve. A plasma with a known anti-β2GPI concentration was assayed in each batch to ensure between-batch reliability. All abnormal results were repeated with a second assay for confirmation.

3.7.17 Clotting times using Pseudonaja textilis and Echis carinatus venoms

To avoid problems due to abnormal levels of clotting factors in the patients serum the presence of a lupus anticoagulant (LA) was tested for using a phospholipid dependent prothrombin activating protease purified from the venom of *Pseudonaja textilis* (Sigma Aldrich Chemical Co., Poole, Dorset) using a method modified from that of Triplett *et al* 1993 (Triplett et al. 1993). The protease was diluted in PBS and mixed with 'Bell & Alton' phospholipid (Diagnostic Reagents Ltd) to give a clotting time of approximately 20 seconds. Clotting times were performed on a Sysmex CA-6000™ (Sysmex UK Ltd). 50μl aliquots of test plasma were added to 50μl of diluted venom and incubated at 37°C for 4 minutes. 50μl of warm $CaCl_2$ was added and the clotting time was measured from the addition of $CaCl_2$. Samples giving results >2 SD above the mean normal time were suspected to be positive but confirmation of the presence of a lupus anticoagulant was performed by then doing a clotting time with *Echis carinatus* venom. This assay is based on the ability of a protein fraction of the *Echis carinatus* venom to activate prothrombin in a phospholipid *independent* manner. 50μl aliquots of test plasma were incubated at 37°C for 3 minutes and added to 100μl of warm *Echis carinatus* venom (50 Ecarin units/vial) which had been reconstituted according to the manufacturers instructions (Unicorn Diagnostics Ltd). The clotting time was measured on a

Sysmex CA-6000™ (Sysmex UK Ltd). All abnormal results were repeated with a second assay for confirmation.

3.8 Statistics

The majority of the data did not conform to a normal distribution so results are expressed as a median with the interquartile range and total range. I used the Mann-Whitney U test for non-parametric unpaired comparisons between groups and Spearman's correlation test was used to determine correlation between different measurements. The statistics were performed using the 'Astute' statistics add-in for Microsoft Excel 5.0.

Chapter 4

Results

4.0 Patient characteristics

The 144 patients were divided into 7 groups on the basis of their disease, the presence or absence of CVD and transfusion status to facilitate analysis and comparison to 18 normal sibling controls. As HbSβ^0thal has a similar phenotype to HbSS these two groups were combined for analysis.

The characteristics of the NC and these groups are shown in table 3 and summarised as follows;

- Group NC = normal sibling controls.
- Group A = HbSS - untransfused with CVD - abnormally high cerebral artery velocities on TCD - but asymptomatic.
- Group B = HbSS or HbSβ^0thal with no evidence of CVD on TCD.
- Group C = HbSS or HbSβ^0thal who had not had TCD.
- Group D = HbSS or HbSβ^0thal on transfusion programme, in 10 cases for symptomatic CVD [group D(s)].
- Group E = HbSC disease.
- Group F = Thalassaemia major on transfusion programme.
- Group G = Heterogeneous group of other haemolytic disorders.

The results of each of the controls and 7 patient groups will be presented separately in the following sections. The results of groups A, B, and C will also be presented together in a further section to allow comparison of data between all 83 untransfused children with HbSS or HbSβ^0thal with the 13 in the transfused group D as well as those with HbSC, the thalassaemics and those with other red cell disorders. Section 4.5 will include data on Group D - the transfused group - but the data on the 10 stroke patients [group D(s)] will also be presented separately to facilitate a comparison between these symptomatic patients with CVD, the 17 untransfused asymptomatic children with CVD (group A) and the 37 who are known to have normal cerebral vessels (group B).

Table 3. Characteristics of control group (NC) and patient groups A-G.
Ages are given as median and interquartile range.

Group	NC	A	B	C	D	E	F	G
Number of subjects	18	17	37	29	13	24	13	11
Age in years	8.1 (5.5 - 12.1)	8.8 (6.2 - 11.5)	8.9 (7.4 - 10.9)	4.6 (3.2 - 6.6)	11.4 (8.1 - 12.2)	7.6 (4.8 - 10.4)	12.7 (12.3 - 15.3)	7.2 (4.0 - 13.4)
Male / female	12/6	11/6	20/17	19/10	6/7	14/10	5/8	8/3
Haemoglobin electrophoresis or diagnosis	6 HbAA 9 HbAS 3 HbAC	17 HbSS	34 HbSS 3 HbSβ^0 thal	28 HbSS 1 HbSβ^0 thal	12 HbSS 1 HbSβ^0 thal	24 Hb SC	13 Thal major	5 HS 2 PK 1 HbH 3 G6PD
Cerebrovascular disease	Not tested	Yes	No	Not tested	Yes (10/13)	Not tested	Not tested	Not tested
On transfusion programme	No	No	No	No	Yes	No	Yes	No
Splenectomy	0/18	1/17	4/37	1/29	4/13	0/24	4/13	1/11

4.1 Group NC - normal controls

4.1.1 Clinical data including cerebrovascular studies

This group of 18 sibling controls were healthy with no chronic disease and did not have any apparent intercurrent illnesses at the time of venesection. They had not had cerebrovascular studies performed.

4.1.2 Investigations

Baseline investigations are shown in table 4. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 5, inhibitors and APC resistance ratios in table 6 and activation markers and results of the antiphospholipid screen in table 7.

Table 4 - Baseline investigations in group NC - 18 normal controls.

Parameter	median	interquartile range	total range
Hb (g/dl)	12.0	11.5 - 13.7	10.7 - 15.3
WBC (10^9/l)	5.5	4.1 - 6.8	3.1 - 8.1
Plts (10^9/l)	263	207 - 306	161 - 416
MCV (fl)	81	76 - 83	70 - 100
Retics (%)	1.0	0.7 - 1.4	0.43 - 2.1
Hb F (%)	0.5	0.4 - 0.7	0.1 - 1.3
AST (u/l)	43	40 - 47	33 - 73
Alb (g/l)	41	40 - 43	39 - 45
Bili (mmol/l)	10	8 - 11	6 - 18
CRP (mg/l)	1.2	0.7 - 1.8	0.3 - 2.3

Table 5 - Coagulation screen and procoagulant factors in group NC.

Parameter	median	interquartile range	total range
PT (seconds)	15.0	14.0 - 15.0	13.0 - 18.0
APTT (seconds)	36.0	35.0 - 37.0	30.5 - 40.0
TT (seconds)	10.5	10.0 - 11.0	9.0 - 12.0
Fib (g/l)	2.6	2.3 - 2.7	1.8 - 4.2
FVII (iu/dl)	88	77 - 97	70 - 150
FX (iu/dl)	87	84 - 98	66 - 104
FXI (iu/dl)	89	81 - 94	72 - 105
FXII (iu/dl)	102	84 - 118	47 - 170

Table 6 - Inhibitors and APC ratios in group NC.

Parameter	median	interquartile range	total range
PC (iu/dl)	72	66 - 81	56 - 107
TPS (iu/dl)	82	75 - 99	64 - 101
FPS (iu/dl)	96	83 - 110	55 - 143
AT (iu/dl)	102	92 - 110	62 - 136
HCII (iu/dl)	105	94 - 118	73 - 134
PC / FVII ratio	0.82	0.75 - 0.93	0.54 - 1.04
FPS / FX ratio	1.11	0.95 - 1.32	0.57 - 1.81
APCR ratio*			
standard (18/18)	3.5	3.2 - 4.0	2.5 - 5.0
modified (3/18)			*2.6, 2.6, 2.6*

* The modified APCR ratio was only done in 3 subjects and individual results are given. 16/18 had PCR for FVR506Q mutation - all were negative.

Table 7 - Activation markers and antiphospholipid screen in group NC.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	2.28	1.91 - 2.58	1.07 - 6.83
PF1+2 (nmol/l)	0.39	0.29 - 0.46	0.18 - 0.83
ACL IgG (GPL)	3	1 - 4	0 - 8
ACL IgM (MPL)	0	0 - 0	0 - 2
Anti β2GP1 (%)	0.29	0.23 - 0.35	0.08 - 1.17
TXT (sec)	21.5	20.5 - 21.9	19.8 - 22.6

4.2 Group A

4.2.1 Clinical data including cerebrovascular studies

This group of 17 patients all had evidence of CVD on TCD or MRI but had normal neurological examination and were asymptomatic at the time of testing. Results of TCD screening and other clinical data on this group is shown in table 8.

Table 8 - Clinical complications in Group A - 17 subjects.

Average number of admissions per year for all crises median (range)	0.77 (0 - 4.17)
Chest crises:	
Number who have had a chest crisis	5 (29%)
Number with recurrent (>1) chest crisis	2 (11%)
Cerebrovascular disease:	
TCD abnormal MRI normal	1
TCD abnormal MRI abnormal	9
TCD abnormal MRI not done	7

4.2.2 Investigations

Baseline investigations are shown in table 9. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and factors are shown in table 10, inhibitors and APC resistance ratios in table 11 and activation markers and results of the antiphospholipid screen in table 12.

Table 9 - Baseline investigations in group A.

Parameter	median	interquartile range	total range
Hb (g/dl)	7.5	6.9 - 8.4	6.6 - 9.1
WBC (10^9/l)	14.3	11.8 - 16.7	9.8 - 22.1
Plts (10^9/l)	392	344 - 442	236 - 604
MCV (fl)	90	85 - 95	70 - 106
Retics (%)	15.0	10.7 - 18.0	3.6 - 27.0
Hb F (%)	6.3	2.2 - 7.9	0.2 - 10.4
AST (u/l)	82	75 - 98	69 - 125
Alb (g/l)	43	41 - 44	39 - 50
Bili (mmol/l)	57	42 - 87	29 - 92
CRP (mg/l)	0.6	0.3 - 1.4	0.2 - 4.5

Table 10 - Coagulation screen and procoagulant factors in group A.

Parameter	median	interquartile range	total range
PT (seconds)	17.0	16.5 - 17.0	15.0 - 18.5
APTT (seconds)	38.0	35.5 - 39.5	32.0 - 48.0
TT (seconds)	10.0	10.0 - 10.5	9.0 - 11.5
Fib (g/l)	2.3	2.1 - 2.4	1.5 - 3.6
FVII (iu/dl)	72	68 - 79	56 - 106
FX (iu/dl)	67	62 - 83	54 - 104
FXI (iu/dl)	72	58 - 80	49 - 94
FXII (iu/dl)	81	63 - 94	44 - 150

Table 11 - Inhibitors and APC ratios in group A.

Parameter	median	interquartile range	total range
PC (iu/dl)	62	57 - 68	47 - 79
TPS (iu/dl)	68	62 - 74	50 - 99
FPS (iu/dl)	65	55 - 74	44 - 91
AT (iu/dl)	88	82 - 100	69 - 113
HCII (iu/dl)	70	62 - 75	55 - 91
PC / FVII ratio	0.85	0.72 - 0.96	0.60 - 1.05
FPS / FX ratio	0.93	0.82 - 1.07	0.51 - 1.31
APCR ratio;*			
standard (16/17)	3.0	2.8 - 3.3	2.6 - 3.8
modified (4/17)			*2.4, 2.5, 2.7, 2.4*

* The APCR ratios were not done in all patients - the number tested is in parentheses and individual results are given for the modified test. All patients had either the standard or the modified test. The 5 with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 12 - Activation markers and antiphospholipid screen in group A.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	5.08	4.46 - 6.94	3.38 - 11.12
PF1+2 (nmol/l)	1.26	0.95 - 1.89	0.48 - 2.60
ACL IgG (GPL)	1	0 - 1	0 - 11
ACL IgM (MPL)	0	0 - 0	0 - 1
Anti β2GP1 (%)	0.3	0.16 - 0.50	0.12 - 0.99
TXT (sec)	21.0	20.1 - 21.2	18.5 - 24.0

4.3 Group B

4.3.1 Clinical data including cerebrovascular studies

This group of 37 patients had all been screened for CVD with TCD and all were found to have normal cerebral vasculature. They had normal neurological examination and were asymptomatic at the time of testing. Clinical data on this group is summarised in table 13.

Table 13 - Clinical complications in Group B - 37 subjects.

Average number of admissions per year for all crises median (range)	0.86 (0 - 3.53)
Chest crises: Number who have had a chest crisis Number with recurrent (>1) chest crisis	 9 (24%) 7 (18%)
Cerebrovascular disease: TCD normal	 37

4.3.2 Investigations

Baseline investigations are shown in table 14. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 15, inhibitors and APC resistance ratios in table 16 and activation markers and results of the antiphospholipid screen in table 17.

Table 14 - Baseline investigations in group B.

Parameter	median	interquartile range	total range
Hb (g/dl)	8.1	7.3 - 9.0	6.8 - 12.5
WBC (10^9/l)	11.8	8.6 - 13.7	6.3 - 16.6
Plts (10^9/l)	357	286 - 440	214 - 592
MCV (fl)	87	82 - 92	66 - 103
Retics (%)	13.1	8.1 - 17.7	2.3 - 26.2
Hb F (%)	5.3	2.5 - 9.5	0.3 - 25.0
AST (u/l)	73	65 - 88	44 - 123
Alb (g/l)	43	42 - 45	37 - 49
Bili (mmol/l)	49	37 - 84	14 - 157
CRP (mg/l)	0.3	0.3 - 0.9	0.3 - 1.8

Table 15 - Coagulation screen and procoagulant factors in group B.

Parameter	median	interquartile range	total range
PT (seconds)	16.5	16.0 - 17.5	14.5 - 20.5
APTT (seconds)	37.0	33.5 - 41.0	30.0 - 48.5
TT (seconds)	10.5	10.0 - 11.0	9.0 - 11.5
Fib (g/l)	2.2	2.0 - 2.5	1.7 - 3.2
FVII (iu/dl)	75	64 - 86	50 - 104
FX (iu/dl)	75	69 - 81	40 - 114
FXI (iu/dl)	80	64 - 94	49 - 121
FXII (iu/dl)	69	51 - 89	34 - 143

Table 16 - Inhibitors and APC ratios in group B.

Parameter	median	interquartile range	total range
PC (iu/dl)	64	55 - 70	43 - 82
TPS (iu/dl)	66	60 - 77	45 - 99
FPS (iu/dl)	69	53 - 81	31 - 122
AT (iu/dl)	93	84 - 99	73 - 116
HCII (iu/dl)	76	65 - 82	47 - 89
PC / FVII ratio	0.82	0.71 - 0.96	0.53 - 1.40
FPS / FX ratio	0.90	0.75 - 1.07	0.27 - 1.95
APCR ratio;*			
standard (30/37)	2.9	2.7 - 3.2	2.1 - 3.9
modified (13/37)	*2.5*	*2.3 - 2.6*	*2.3 - 2.8*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test. 10 patients with low APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 17 - Activation markers and antiphospholipid screen in group B.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	5.50	2.43 - 6.90	1.12 - 28.76
PF1+2 (nmol/l)	1.29	0.78 - 1.75	0.40 - 3.67
ACL IgG (GPL)	0	0 - 1	0 - 4
ACL IgM (MPL)	0	0 - 0	0 - 3
Anti β2GP1 (%)	0.27	0.16 - 0.43	0.01 - 0.79
TXT (sec)	20.5	19.7 - 21.7	18.5 - 23.3

4.4 Group C

4.4.1 Clinical data including cerebrovascular studies

This group of 29 patients comprises many of the youngest patients tested and had not been screened for CVD with TCD but were asymptomatic at the time of testing. Other clinical data on this group is summarised in table 18.

Table 18 - Clinical complications in Group C - 29 subjects.

Average number of admissions per year for all crises median (range)	0.37 (0 - 4.87)
Chest crises:	
Number who have had a chest crisis	5 (17%)
Number with recurrent (>1) chest crisis	1 (3%)

4.4.2 Investigations

Baseline investigations are shown in table 19. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 20, inhibitors and APC resistance ratios in table 21 and activation markers and results of the antiphospholipid screen in table 22.

Table 19 - Baseline investigations in group C.

Parameter	median	interquartile range	total range
Hb (g/dl)	8.0	7.5 - 9.1	6.3 - 11.1
WBC (10^9/l)	11.1	9.8 - 15.2	7.1 - 23.1
Plts (10^9/l)	338	271 - 437	206 - 534
MCV (fl)	80	77 - 88	63 - 107
Retics (%)	9.4	7.6 - 10.4	2.5 - 27.0
Hb F (%)	11.6	4.8 - 14.3	0.3 - 24.3
AST (u/l)	74	66 - 92	38 - 115
Alb (g/l)	43	41 - 45	38 - 48
Bili (mmol/l)	38	25 - 51	10 - 81
CRP (mg/l)	0.3	0.3 - 1.1	0.3 - 2.5

Table 20 - Coagulation screen and procoagulant factors in group C.

Parameter	median	interquartile range	total range
PT (seconds)	15.5	15.0 - 16.5	13.0 - 18.0
APTT (seconds)	36.0	34.0 - 37.0	30.5 - 49.0
TT (seconds)	10.0	10.0 - 10.5	9.0 - 12.0
Fib (g/l)	2.4	2.2 - 3.0	1.8 - 4.4
FVII (iu/dl)	82	74 - 91	55 - 124
FX (iu/dl)	87	75 - 95	54 - 143
FXI (iu/dl)	82	72 - 97	58 - 140
FXII (iu/dl)	78	60 - 102	39 - 157

Table 21 - Inhibitors and APC ratios in group C.

Parameter	median	interquartile range	total range
PC (iu/dl)	62	57 - 67	46 - 82
TPS (iu/dl)	69	63 - 81	43 - 108
FPS (iu/dl)	72	56 - 84	31 - 127
AT (iu/dl)	103	93 - 117	82 - 146
HCII (iu/dl)	78	72 - 87	57 - 99
PC / FVII ratio	0.76	0.67 - 0.84	0.52 - 1.02
FPS / FX ratio	0.87	0.70 - 1.01	0.31 - 1.34
APCR ratio;*			
standard (26/29)	3.4	3.0 - 3.6	2.5 - 4.1
modified (5/29)			*2.4, 2.6, 2.7, 2.2, 2.5*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test and individual results are given for the modified test. 7 patients with low APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 22 - Activation markers and antiphospholipid screen in group C.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	6.32	4.22 - 7.86	1.81 - 19.28
PF1+2 (nmol/l)	1.15	0.75 - 1.64	0.49 - 3.79
ACL IgG (GPL)	1	0 - 4	0 - 11
ACL IgM (MPL)	0	0 - 0	0 - 11
Anti β2GP1 (%)	0.22	0.14 - 0.35	0 - 7.43
TXT (sec)	21.2	20.4 - 21.6	18.6 - 23.4

4.5 Group D and D(s)

4.5.1 Clinical data including cerebrovascular studies

This group of 13 children comprises those who are on a regular transfusion programme. In 10 cases the indication for transfusion was clinical stroke (Group D[s]). Other clinical data on this group is summarised in table 23.

Table 23 - Clinical complications in Group D - 13 subjects.

Average number of admissions per year for all crises median (range)	1.40 (0 - 4.90)
Chest crises:	
Number who have had a chest crisis	6 (46%)
Number with recurrent (>1) chest crisis	3 (23%)
Clinical symptomatic stroke	10 (77%)

4.5.2 Investigations

Baseline investigations are shown in table 24. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 25, inhibitors and APC resistance ratios in 26 and 27 and activation markers and results of the antiphospholipid screen in 28.

Table 24 - Baseline investigations in group D and D(s).

Parameter	Results group D			Results group D(s)		
	median	interquartile range	total range	median	interquartile range	total range
Hb (g/dl)	10.2	9.9 - 10.8	8.9 - 12.7	10.0	9.9 - 11.9	8.9 - 12.7
WBC (10^9/l)	10.6	8.6 - 19.3	7.1 - 24.8	13.9	8.6 - 20.1	7.1 - 24.8
Plts (10^9/l)	421	307 - 464	222 - 742	365	287 - 459	222 - 742
MCV (fl)	88	85 - 91	81 - 94	87	85 - 90	81 - 94
Retics (%)	7.3	2.9 - 10.7	0.7 - 16.1	7.0	2.9 - 10.7	0.7 - 16.1
Hb F (%)	1.0	0.6 - 1.4	0.2 - 4.0	0.9	0.5 - 1.4	0.2 - 2.5
Hb S (%)	14.0	7.5 - 28.0	1.0 - 32.0	12.0	6.0 - 21.0	1.0 - 29.0
AST (u/l)	58	54 - 86	37 - 118	60	54 - 89	48 - 118
Alb (g/l)	42	41 - 44	40 - 48	43	41 - 45	40 - 48
Bili (mmol/l)	38	25 - 60	16 - 79	35	20 - 42	16 - 79
CRP (mg/l)	0.9	0.3 - 1.7	0.3 - 1.8	1.0	0.3 - 1.7	0.3 - 1.8

Table 25 - Coagulation screen and procoagulant factors in groups D and D(s).

Parameter	Results group D			Results group D(s)		
	median	interquartile range	total range	median	interquartile range	total range
PT (sec)	16.5	16.0 - 17.5	14.0 - 19.5	16.5	16.0 - 17.5	14.0 - 19.5
APTT (sec)	40.0	36.0 - 42.5	32.0 - 51.5	40.0	35.0 - 42.0	32.0 - 47.0
TT (sec)	11.0	10.0 - 11.0	10.0 - 12.0	11.0	10.0 - 11.0	10.0 - 12.0
Fib (g/l)	2.4	2.0 - 2.7	1.5 - 2.9	2.6	2.2 - 2.8	1.5 - 2.9
FVII (iu/dl)	84	62 - 93	56 - 113	76	61 - 93	56 - 113
FX (iu/dl)	72	64 - 75	55 - 118	70	64 - 75	55 - 118
FXI (iu/dl)	58	55 - 64	41 - 81	59	55 - 70	47 - 81
FXII (iu/dl)	91	46 - 107	36 - 134	84	46 - 112	36 - 134

Table 26 - Inhibitors and APC ratios in group D.

Parameter	Results group D		
	median	interquartile range	total range
PC (iu/dl)	61	55 - 75	47 - 83
TPS (iu/dl)	70	65 - 79	55 - 90
FPS (iu/dl)	69	54 - 76	36 - 132
AT (iu/dl)	99	89 - 104	83 - 127
HCII (iu/dl)	76	69 - 82	60 - 95
PC / FVII ratio	0.81	0.71 - 0.93	0.61 - 0.98
FPS / FX ratio	0.99	0.79 - 1.04	0.60 - 1.33
APCR ratio;*			
standard (8/13)	3.8	3.7 - 3.9	3.6 - 4.0
modified (5/13)			*2.7, 2.5, 2.7, 2.6, 2.7*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test and individual results are given for the modified test. 8 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 27 - Inhibitors and APC ratios in group D(s).

Parameter	Results group D(s)		
	median	interquartile range	total range
PC (iu/dl)	59	52 - 75	47 - 83
TPS (iu/dl)	70	65 - 79	55 - 85
FPS (iu/dl)	61	54 - 73	36 - 132
AT (iu/dl)	96	87 - 103	83 - 111
HCII (iu/dl)	76	69 - 81	60 - 95
PC / FVII ratio	0.81	0.71 - 0.93	0.61 - 0.98
FPS / FX ratio	0.95	0.72 - 1.03	0.60 - 1.12
APCR ratio;*			
standard (7/10)	3.8	3.7 - 3.9	3.6 - 4.0
modified (4/10)			*2.5, 2.7, 2.6, 2.7*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test and individual results are given for the modified test. 7 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 28 - Activation markers and antiphospholipid screen in groups D and D(s).

Parameter	Results group D			Results group D(s)		
	median	interquartile range	total range	median	interquartile range	total range
TAT complexes (μg/l)	3.57	2.26 - 5.66	1.30 - 9.04	3.60	2.19 - 5.70	1.30 - 9.04
PF1+2 (nmol/l)	1.15	0.81 - 1.55	0.42 - 2.16	0.98	0.72 - 1.38	0.42 - 1.63
ACL IgG (GPL)	0	0 - 1	0 - 5	1	0 - 3	0 - 5
ACL IgM (MPL)	0	0 - 0	0 - 3	0	0 - 2	0 - 3
Anti β2GP1 (%)	0.23	0.17 - 0.38	0.12 - 3.68	0.26	0.21 - 0.42	0.12 - 3.68
TXT (sec)	20.5	20.1 - 21.5	19.1 - 22.7	20.6	20.4 - 21.5	19.2 - 22.7

4.6 Group E

4.6.1 Clinical data including cerebrovascular studies

This group of 24 children with Hb SC have not had TCD screening as cerebrovascular complications are uncommon in this genotype. Clinical data on this group is summarised in table 29.

Table 29 - Clinical complications in Group E - 24 subjects.

Average number of admissions per year for all crises median (range)	0.25 (0 - 1.54)
Chest crises:	
Number who have had a chest crisis	1 (4%)
Number with recurrent (>1) chest crisis	0 (0%)

4.6.2 Investigations

Baseline investigations are shown in table 30. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 31, inhibitors and APC resistance ratios in table 32 and activation markers and results of the antiphospholipid screen in table 33.

Table 30 - Baseline investigations in group E.

Parameter	median	interquartile range	total range
Hb (g/dl)	10.7	10.3 - 11.9	8.0 - 14.7
WBC (10^9/l)	7.7	6.3 - 10.0	5.1 - 13.2
Plts (10^9/l)	261	197 - 337	146 - 447
MCV (fl)	78	75 - 81	81 - 94
Retics (%)	3.0	2.5 - 5.1	0.6 - 12.0
Hb F (%)	1.8	0.7 - 3.6	0.2 - 14.0
AST (u/l)	55	50 - 69	19 - 166
Alb (g/l)	44	42 - 45	38 - 46
Bili (mmol/l)	25	19 -34	12 - 55
CRP (mg/l)	0.3	0.3 - 0.8	0.3 - 1.7

Table 31 - Coagulation screen and procoagulant factors in group E.

Parameter	median	interquartile range	total range
PT (seconds)	15.5	15.0 - 16.5	12.5 - 17.5
APTT (seconds)	39.0	34.0 - 40.5	30.5 - 51.0
TT (seconds)	10.0	10.0 - 11.0	9.5 - 12.0
Fib (g/l)	2.4	2.3 - 2.8	1.8 - 3.6
FVII (iu/dl)	88	79 - 97	58 - 137
FX (iu/dl)	85	82 - 99	71 - 138
FXI (iu/dl)	83	64 - 103	38 - 124
FXII (iu/dl)	77	62 - 96	44 - 131

Table 32 - Inhibitors and APC ratios in group E.

Parameter	median	interquartile range	total range
PC (iu/dl)	73	62 - 81	47 - 90
TPS (iu/dl)	75	70 - 83	49 - 103
FPS (iu/dl)	78	68 - 95	33 - 125
AT (iu/dl)	103	97 - 108	66 - 143
HCII (iu/dl)	78	69 - 97	57 - 130
PC / FVII ratio	0.80	0.70 - 0.93	0.54 - 1.28
FPS / FX ratio	0.90	0.78 - 1.10	0.42 - 1.27
APCR ratio;*			
standard (21/24)	3.4	3.3 - 3.7	2.7 - 4.0
modified (3/24)			*2.6, 2.6, 2.5*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test and individual results are given for the modified test. 8 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 33 - Activation markers and antiphospholipid screen in group E.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	2.78	2.12 - 3.89	1.64 - 17.55
PF1+2 (nmol/l)	1.08	0.83 - 1.44	0.40 - 3.75
ACL IgG (GPL)	0	0 - 3	0 - 4
ACL IgM (MPL)	0	0 - 0	0 - 2
Anti β2GP1 (%)	0.30	0.20 - 0.39	0.05 - 0.79
TXT (sec)	21.1	20.3 - 21.9	19.7 - 26.1*

* One patient in this group had a TXT that was >2 SD above the mean normal time - an Ecarin time was performed which was also marginally elevated (26 seconds)

4.7 Group F

4.7.1 Clinical data

The 13 children in this group have β-thalassaemia major and are all maintained on regular transfusion programmes.

4.7.2 Investigations

Baseline investigations are shown in table 34. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 35, inhibitors and APC resistance ratios in table 36 and activation markers and results of the antiphospholipid screen in table 37.

Table 34 - Baseline investigations in group F - 13 subjects.

Parameter	median	interquartile range	total range
Hb (g/dl)	9.6	9.1 - 10.2	8.1 - 12.5
WBC (10^9/l)	9.1	6.5 - 10.6	4.2 - 16.1
Plts (10^9/l)	394	313 - 515	181 - 712
MCV (fl)	86	85 - 87	84 - 89
Retics (%)	0.3	0 - 0.6	0 - 8.4
Hb F (%)	0.7	0.4 - 1.1	0.2 - 1.8
AST (u/l)	40	31 - 56	23 - 82
Alb (g/l)	40	39 - 43	36 - 44
Bili (mmol/l)	24	19 - 28	10 - 39
CRP (mg/l)	0.4	0.3 - 0.8	0.3 - 1.8

Table 35 - Coagulation screen and procoagulant factors in group F.

Parameter	median	interquartile range	total range
PT (seconds)	15.5	15.5 - 17.5	14.5 - 20.0
APTT (seconds)	43.5	41.0 - 47.0	38.5 - 54.5
TT (seconds)	10.0	10.0 - 11.0	9.5 - 11.5
Fib (g/l)	2.3	2.0- 2.5	1.3 - 3.9
FVII (iu/dl)	89	71 - 104	40 - 125
FX (iu/dl)	77	72 - 82	47 - 93
FXI (iu/dl)	49	46 - 66	30 - 79
FXII (iu/dl)	90	81 - 97	42 - 115

Table 36 - Inhibitors and APC ratios in group F.

Parameter	median	interquartile range	total range
PC (iu/dl)	59	49 - 69	45 - 78
TPS (iu/dl)	72	62 - 75	44 - 95
FPS (iu/dl)	80	74 - 90	61 - 110
AT (iu/dl)	88	80 - 104	64 - 111
HCII (iu/dl)	91	88 - 93	64 - 105
PC / FVII ratio	0.64	0.56 - 0.74	0.47 - 1.95
FPS / FX ratio	1.08	1.01 - 1.17	0.85 - 1.70
APCR ratio;*			
standard (7/13)	3.6	3.4 - 4.3	2.7 - 4.4
modified (8/13)	*2.7*	*2.4 - 2.7*	*2.3 - 2.9*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test. 10 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 37 - Activation markers and antiphospholipid screen in group F.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	1.08	0.99 - 1.67	0.24 - 4.00
PF1+2 (nmol/l)	0.64	0.59 - 0.89	0.38 - 1.09
ACL IgG (GPL)	0	0 - 2	0 - 4
ACL IgM (MPL)	0	0 - 0	0 - 7
Anti β2GP1 (%)	0.26	0.16 - 0.40	0.08 - 4.28
TXT (sec)	19.9	19.3 - 20.6	19.0 - 22.5

4.8 Group G

4.8.1 Clinical data

The 11 children in this group have a heterogeneous group of red cell disorders and the results are therefore presented separately as well as a group.

4.8.2 Investigations

Baseline investigations are shown in table 38. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are shown in table 39, inhibitors and APC resistance ratios in table 40 and activation markers and results of the antiphospholipid screen in table 41.

Table 38 - Baseline investigations in group G - 11 subjects.

Parameter	HS n = 5	PK n = 2	HbH n = 1	G6PD deficiency n = 3	Results for whole group		
					median	interquartile range	total range
Hb (g/dl)	9.1, 15.6, 12.6, 11.9, 9.3	8.6, 5.6	10.9	11.5, 11.6, 11.3	11.3	9.1 - 11.9	5.6 - 15.6
WBC (10^9/l)	11.8, 8.2, 8.1, 5.2, 6.6	4.7, 5.5	9.8	6.1, 5.9, 9.6	6.6	5.5 - 9.6	4.7 - 11.8
Plts (10^9/l)	419, 631, 324, 258, 292	202, 275	338	359, 284, 275	292	275 - 359	202 - 631
MCV (fl)	78, 78, 71, 85, 88	83, 102	67	68, 84, 81	81	71 - 85	67 - 102
Retics (%)	18.1, 4.2, 3.6, 4.3, 6.0	8.5, 25.1	4.7	1.5, 16.3, 20.5	6.0	4.2 - 18.1	1.5 - 25.1
Hb F (%)	0.5, 0.2, 0.9, 0.9, 1.0	0.1, 1.0	0.2	0.7, 0.5, 0.5	0.5	0.2 - 0.9	0.1 - 1.0
AST (u/l)	50, 44, 36, 36, 35	36, 69	87	39, 56, 47	44	36 - 56	35 - 87
Alb (g/l)	43, 40, 46, 47, 47	41, 42	43	46, 47, 45	45	42 - 47	40 - 47
Bili (mmol/l)	50, 50, 23, 37, 18	39, 69	70	12, 21, 8	37	18 - 50	8 - 70
CRP (mg/l)	0.9, 0.8, 1.0, 0.3, 0.5	0.6, 0.3	0.8	1.2, 1.6, 1.2	0.8	0.5 - 1.2	0.3 - 1.6

Table 39 - Coagulation screen and procoagulant factors in group G.

Parameter	HS n = 5	PK n = 2	Hb H n = 1	G6PD deficiency n = 3	Results for whole group		
					median	interquartile range	total range
PT (sec)	17.0, 17.5, 15.0, 17.0, 14.5	16.0, 16.5	18.0	13.0, 15.0, 16.0	16.0	15.0 - 17.0	13.0 - 18.0
APTT (sec)	40.5, 43.0, 42.0, 35.5, 37.0	35.0, 35.5	43.0	40.0, 43.0, 46.0	40.5	35.5 - 43.0	35.0 - 46.0
TT (sec)	10.5, 10.0, 10.0, 14.0, 12.0	10.0, 10.5	10.5	10.0, 10.0, 12.5	10.5	10.0 - 12.0	10.0 - 14.0
Fib (g/l)	1.7, 2.3, 1.9, 2.2, 3.1	2.3, 2.4	2.0	2.6, 2.8, 2.5	2.3	2.0 - 2.6	1.7 - 3.1
FVII (iu/dl)	60, 60, 80, 73, 110	85, 62	71	117, 84, 78	78	62 - 85	60 - 117
FX (iu/dl)	80, 76, 71, 60, 88	75, 73	66	128, 95, 110	76	71 - 95	60 - 128
FXI (iu/dl)	50, 56, 60, 78, 83	78, 64	62	67, 55, 62	62	56 - 78	50 - 83
FXII (iu/dl)	98, 46, 104, 108, 90	96, 128	57	99, 54, 52	96	54 - 104	46 - 128

Table 40 - Inhibitors and APC ratios in group G.

Parameter	HS n = 5	PK n = 2	Hb H n = 1	G6PD deficiency n = 3	Results for whole group		
					median	interquartile range	total range
PC (iu/dl)	49, 71, 59, 70, 70	64, 61	60	83, 77, 67	67	60 - 71	49 - 83
TPS (iu/dl)	65, 98, 70, 56, 62	65, 60	45	94, 63, 81	65	60 - 81	45 - 98
FPS (iu/dl)	80, 127, 93, 58, 80	77, 88	42	123, 60, 110	80	60 - 110	42 - 127
AT (iu/dl)	73, 90, 101, 109, 70	117, 99	92	114, 110, 119	101	90 - 114	70 - 119
HCII (iu/dl)	77, 61, 73, 80, 89	81, 77	57	126, 71, 93	77	71 - 89	57 - 126
PC / FVII ratio	0.82, 1.18, 0.74, 0.96, 0.64	0.75, 0.98	0.85	0.71, 0.92, 0.86	0.85	0.74 - 0.96	0.64 - 1.18
FPS / FX ratio	1.00, 1.67, 1.31, 0.97, 0.91	1.03, 1.21	0.64	0.96, 0.63, 1.00	1.00	0.91 - 1.21	0.63 - 1.67
APCR ratio;*							
standard (7/11)	2.8, 2.9, 3.3	3.3, 3.2	3.8	3.6	3.3	2.9 - 3.6	2.8 - 3.8
modified (4/11)	*2.6, 2.5*			*2.5, 2.5*			

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test.
5 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 41 - Activation markers and antiphospholipid screen in group G.

Parameter	HS n = 5	PK n = 2	Hb H n = 1	G6PD deficiency n = 3	Results for whole group		
					median	interquartile range	total range
TAT complexes (μg/l)	4.13, 1.17, 1.33, 1.08, 1.33	1.30, 2.32	1.57	2.87, 2.30, 2.16	1.57	1.30 - 2.32	1.08 - 4.13
PF1+2 (nmol/l)	0.69, 0.42, 0.66, 0.50, 0.87	1.27, 1.18	0.50	0.55, 0.37, 0.45	0.55	0.45 - 0.87	0.37 - 1.27
ACL IgG (GPL)	0, 0, 0, 1, 2	0, 0	4	3, 0, 2	0	0 - 2	0 - 4
ACL IgM (MPL)	0, 0, 0, 0, 0	0, 0	0	0, 0	0	0 - 0	0 - 0
Anti β2GP1 (%)	0.03, 0.26, 0.44, 0.28, 0.63	0.19, 0.08	0.28	0.31, 0.29, 0.23	0.28	0.14 - 0.44	0.03 - 0.63
TXT (sec)	20.7, 19.8, 20.4, 20.9, 21.6	20.1, 19.3	20.9	25.4*, 19.6, 18.9	20.4	19.6 - 20.9	18.9 - 25.4

* One patient in this group had a TXT that was >2 SD above the mean normal time - an Ecarin time was performed which was not elevated (23 seconds)

4.9 Group ABC

4.9.1 Clinical data

In this section the results of all 83 untransfused children with Hb SS or Hb $S\beta^0$ thal. are presented to facilitate comparison between untransfused and transfused children and the other groups tested. A summary of the clinical data is presented in table 42.

Table 42 - Clinical complications in Group ABC - 83 subjects.

Average number of admissions per year for all crises median (range)	0.57 (0 - 4.86)
Chest crises:	
Number who have had a chest crisis	19 (23%)
Number with recurrent (>1) chest crisis	12 (14%)
Cerebrovascular disease:	
TCD abnormal MRI normal	1
TCD abnormal MRI abnormal	9
TCD abnormal MRI not done	7
TCD normal	37
TCD not done	29

4.9.2 Investigations

Baseline investigations are shown in table 43. Renal function, calcium and phosphate levels were normal in all subjects. Coagulation screen and procoagulant factors are in table 44, inhibitors and APC resistance ratios in table 45 and activation markers and results of the antiphospholipid screen in table 46.

Table 43 - Baseline investigations in group ABC.

Parameter	median	interquartile range	total range
Hb (g/dl)	8.1	7.1 - 8.9	6.3 - 12.5
WBC (10^9/l)	12.1	9.9 - 14.5	6.3 - 23.1
Plts (10^9/l)	357	289 - 441	206 - 604
MCV (fl)	85	78 - 92	63 - 107
Retics (%)	10.7	8.0 - 17.7	2.3 - 27.1
Hb F (%)	6.4	3.0 - 11.6	0.2 - 25.0
AST (u/l)	78	67 - 91	38 - 125
Alb (g/l)	43	42 - 45	37 - 50
Bili (mmol/l)	47	34 - 80	10 - 157
CRP (mg/l)	0.8	0.3 - 1.2	0.2 - 4.5

Table 44 - Coagulation screen and procoagulant factors in group ABC.

Parameter	median	interquartile range	total range
PT (seconds)	16.5	15.5 - 17.0	13.0 - 20.5
APTT (seconds)	37.0	33.5 - 39.0	30.0 - 49.0
TT (seconds)	10.0	10.0 - 11.0	9.0 - 12.0
Fib (g/l)	2.3	2.2 - 2.7	1.5 - 4.4
FVII (iu/dl)	76	66 - 86	50 - 124
FX (iu/dl)	76	68 - 88	40 - 143
FXI (iu/dl)	79	64 - 91	49 - 140
FXII (iu/dl)	76	54 - 94	34 - 157

Table 45 - Inhibitors and APC ratios in group ABC.

Parameter	median	interquartile range	total range
PC (iu/dl)	62	55 - 69	43 - 82
TPS (iu/dl)	68	61 - 78	43 - 108
FPS (iu/dl)	69	55 - 81	31 - 127
AT (iu/dl)	95	85 - 105	69 - 146
HCII (iu/dl)	76	65 - 83	47 - 99
PC / FVII ratio	0.78	0.70 - 0.93	0.52 - 1.40
FPS / FX ratio	0.89	0.72 - 1.07	0.27 - 1.95
APCR ratio			
standard (71/83)	3.1	2.8 - 3.4	2.1 - 4.1
modified (22/83)	*2.5*	*2.4 - 2.6*	*2.2 - 2.8*

* The APCR ratios were not done in all patients - the number tested is in parentheses. All patients had either the standard or the modified test. 22 patients including those with lowest APCR ratios had PCR for FVR506Q mutation - all were negative.

Table 46 - Activation markers and antiphospholipid screen in group ABC.

Parameter	median	interquartile range	total range
TAT complexes (μg/l)	5.50	3.96 - 7.14	1.12 - 28.76
PF1+2 (nmol/l)	1.26	0.77 - 1.77	0.40 - 3.79
ACL IgG (GPL)	0	0 - 2	0 - 11
ACL IgM (MPL)	0	0 - 0	0 - 11
Anti β2GP1 (%)	0.24	0.14 - 0.44	0 - 7.43
TXT (sec)	20.8	20.1 - 21.6	18.5 - 24.0

Chapter 5

Comparisons of data

5.0 Introduction

In this chapter I will present the results of statistical comparisons between groups for the different parameters measured. The *p* values for MWU tests are shown in tables 47, 48 and 49 and all significant correlation coefficients between parameters in table 50 and 51. Box-Whisker plots for a number of the parameters measured are shown in Figs 3 and 4 with the relevant *p* values in table 47 and 49. Findings are summarised in the following sections.

5.1 The non-transfused Hb SS children - groups A, B and C and group ABC v normal controls (group NC).

5.1.1 Clinical data

Age. Only group C had significantly lower age range compared to NC. Groups A and B and the group as a whole, group ABC, did not differ significantly compared to NC.

Total crisis rate. There was no significant difference in total or chest crisis rate between any of the 3 non- transfused Hb SS groups, A,B, or C and though group C had a lower average annual crisis rate than groups A and B this was not significant and may reflect the younger age of group C as many sickle children are crisis-free for the first 6 - 18 months of life.

Table 47 - *P* values for MWU comparisons between all patient groups and NC.

	A v NC	B v NC	C v NC	D v NC	E v NC	F v NC
Age	NS	NS	0.0023	NS	NS	0.0093
Hb	<0.0001	<0.0001	<0.0001	0.0005	0.0016	<0.0001
WBC	<0.0001	0.0016	<0.0001	<0.0001	0.0005	0.0023
Plts	0.0002	0.0002	0.0069	0.0008	NS	0.0065
Retics	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0008
Hb F	<0.0001	<0.0001	<0.0001	0.0215	0.0003	NS
AST	<0.0001	<0.0001	<0.0001	0.0037	0.0291	NS
Alb	0.040	0.0030	0.0200	0.030	0.0300	NS
CRP	NS	0.0017	NS	NS	0.0016	0.0370
Fib	NS	0.0134	NS	NS	NS	NS
FVII	0.0011	0.0014	NS	NS	NS	NS
FX	0.0017	0.0004	NS	0.0028	NS	0.0092
FXI	0.0014	NS	NS	<0.0001	NS	<0.0001
FXII	0.0476	0.0027	0.0407	NS	0.0420	NS
PC	0.0086	0.0095	0.0012	NS	NS	0.0123
TPS	0.0008	<0.0001	0.0073	0.0130	NS	0.0068
FPS	<0.0001	<0.0001	0.0005	0.0022	0.0214	0.0290
AT	NS	NS	NS	NS	NS	NS
HCII	<0.0001	<0.0001	<0.0001	<0.0001	0.0006	0.0073
APCR	0.0028	0.0001	NS	NS	NS	NS
PC/FVII	NS	NS	NS	NS	NS	0.0200
FPS/FX	0.0175	0.0188	0.0014	0.0374	0.0095	NS
TATs	<0.0001	0.0015	<0.0001	0.0392	NS	0.0011
PF12	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
ACLIgG	0.0120	0.0004	NS	0.0252	0.0283	0.0323
ACLIgM	NS	NS	NS	NS	NS	NS
Antiβ2GP1	NS	NS	NS	NS	NS	NS
TXT	NS	NS	NS	NS	NS	0.0280

Table 48 - *P* values for MWU comparisons between groups ABC and NC, D, E, F and G

	ABC v NC	ABC v D	ABC v E	ABC v F	ABC v G
Age	NS	0.0134	NS	0.0004	NS
Hb	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
WBC	<0.0001	NS	<0.0001	0.0028	<0.0001
Plts	<0.0001	NS	0.0001	NS	NS
Retics	<0.0001	0.0055	<0.0001	<0.0001	NS
Hb F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
AST	<0.0001	NS	<0.0001	<0.0001	<0.0001
Alb	0.0033	NS	NS	0.0019	NS
CRP	0.0070	NS	NS	NS	NS
Fib	NS	NS	NS	NS	NS
FVII	0.0024	NS	0.0027	NS	NS
FX	<0.0001	NS	0.0005	NS	NS
FXI	NS	0.0002	NS	<0.0001	0.0088
FXII	0.0044	NS	NS	NS	NS
PC	0.0010	NS	0.0005	NS	NS
TPS	<0.0001	NS	0.0142	NS	NS
FPS	<0.0001	NS	0.0236	0.0146	NS
AT	NS	NS	0.0117	NS	NS
HCII	<0.0001	NS	NS	<0.0001	NS
APCR	0.0014	0.0001	0.0035	0.0122	NS
PC/FVII	NS	NS	NS	0.0124	NS
FPS/FX	0.0020	NS	NS	0.0054	NS
TATs	<0.0001	0.0270	0.0007	<0.0001	<0.0001
PF12	<0.0001	NS	NS	0.0006	0.0003
ACLIgG	0.0047	NS	NS	NS	NS
ACLIgM	NS	NS	NS	NS	NS
Antiβ2GP1	NS	NS	NS	NS	NS
TXT	NS	NS	NS	NS	NS

Table 49 - *P* values for MWU comparisons between groups A and B, and A and B and D(s).

	A v B	A v D(s)	B v D(s)
Age	NS	NS	NS
Hb	0.0271	<0.0001	<0.0001
WBC	0.0086	NS	NS
Plts	NS	NS	NS
Retlcs	NS	0.0067	0.0087
Hb F	NS	0.0024	<0.0001
AST	NS	NS	NS
Alb	NS	NS	NS
CRP	NS	NS	NS
Fib	NS	NS	NS
FVII	NS	NS	NS
FX	NS	NS	NS
FXI	NS	NS	0.0050
FXII	NS	NS	NS
PC	NS	NS	NS
TPS	NS	NS	NS
FPS	NS	NS	NS
AT	NS	NS	NS
HCII	NS	NS	NS
APCR	NS	0.0007	0.0002
PC/FVII	NS	NS	NS
FPS/FX	NS	NS	NS
TATs	NS	NS	NS
PF12	NS	NS	NS
ACLIgG	NS	NS	NS
ACLIgM	NS	NS	NS
Antiβ2GP1	NS	NS	NS
TXT	NS	NS	NS

Table 50 - Spearman correlation coefficients between procoagulants, coagulation inhibitors, markers of thrombin inhibition and AST values.

* = *p*<0.05, ** = *p*<0.01 refer to the significance of the r values between the 2 parameters compared. Non-significant r values are not quoted.

		Fib	FVII	FX	FXI	FXII	PC	FPS	FPS/X	PC/VII	PF12
FVII	A	0.55*									
	C	0.45*									
	ABC	0.39**									
	G	0.61*									
FX	NC	-	0.82**								
	B	-	0.38*								
	C	0.65**	0.75**								
	ABC	0.45**	0.60**								
	E	-	0.57**								
	G	0.66*	-								
FXI	B	-	-	0.54**							
	C	0.57**	0.43*	0.69**							
	ABC	0.34**	0.32**	0.62**							
	D	0.58*	-	-							
	E	0.44*	-	0.54**							
	F	0.59*	-	-							
FXII	A	-	0.55*	-	-						
	C	-	-	0.39*	-						
	ABC	-	-	0.28*	0.30**						
	D	-	-	0.85**	-						
PC	NC	-	0.48*	0.69**	-	-					
	B	0.44**	-	0.34*	-	-					
	C	0.44*	0.70**	0.64**	0.49**	-					
	ABC	0.35**	0.32**	0.36**	0.26*	-					
	D	-	0.69**	-	-	-					
	E	-	-	0.47*	-	-					
	F	0.61*	-	-	0.65*	0.70**					
	G	0.75**	-	-	-	-					
FPS	NC	0.58*	-	-	-	-	-				
	B	-	-	-	-	0.37*	-				
	ABC	-	-	-	-	0.23*	-				
	D	-	0.72**	0.65*	-	-	0.67*				
	E	-	-	-	0.41*	-	-				
	F	-	-	0.72**	-	-	-				
TPS	NC	0.52*	0.52*	-	-	-	-	0.62**			
	A	-	0.77**	0.57*	-	-	-	-			
	B	-	-	0.57**	-	-	-	0.46**			
	C	0.62**	0.47**	-	-	-	0.38*	0.48**			
	ABC	0.40**	0.37**	0.58**	0.32**	-	0.33**	0.45**			
	D	-	-	0.73**	-	-	-	-			
	E	-	-	0.59**	0.64**	-	-	0.52**			
	F	-	-	0.75**	-	-	-	0.60**			
	G	-	-	-	-	-	-	0.86**			
AST	B	-	-	-	-	-	-	-0.33*	-	-	
	C	-	-	-	-	-	-	-0.38*	-	-	
	ABC	-	-	-	-	-	-	-0.32**	-0.29**	-	
	D	-	0.61*	-	-	-	-	-	-	-	
	F	-	-	-	-	-	-	-	-	-0.61*	
TAT	B										0.66**
	C										0.67**
	ABC										0.61**
	E										0.56**

Table 51 - Spearman correlation coefficients between baseline haematological parameters, the coagulation inhibitors and TATs.

There were no significant findings between PF1+2 and all other values.
Other results for AST are in table 50. There were no correlations in the NC group.
*= $p<0.05$, ** = $p<0.01$ refer to the significance of the r values between the 2 parameters compared. Non-significant r values are not quoted.

		Hb	WBC	Plats	%HbF	AST	AT	HCII
Fib	C	-	-	-	-		0.55**	0.58**
	E	-	-	-	-		-	0.57**
	F	-	-	-	-		-	0.60*
	ABC	-	-	-	-		0.45**	0.37**
PC	ABC	-	-	-	-		-	0.27*
FPS	B	-	-	-	-		-	0.38*
	C	0.49**	-	-	-		-	-
	E	0.52**	-	-	-		-	-
	ABC	0.29**	-	-	-		-	0.33**
TPS	A	-	-	-	-		-	0.66**
	C	0.42*	-	-	-		0.49**	0.48**
	E	-	-	-	-		-	0.53**
	F	-	-	-	-		-	0.62*
	ABC	0.26*	-	-	-		0.36**	0.41**
APCR	B	-	−0.55**	-	-	-	-	-
	ABC	-	−0.40**	-	-	-	-	-
C/VII	C	-	0.39*	-	-	-	-	-
S/X	E	0.56**	-	-	-	-	-	-
	ABC	0.22*	-	-	-	-	-	-
TATs	D	−0.61*	0.60*	-	-	-	-	-
	ABC	-	0.22*	-	-	-	-	-
AT	A	-	-	-	-	-		0.73**
	B	0.55**	-	-	-	-		0.49**
	C	-	-	-	0.62**	-		0.54**
	D	-	-	-	-	-		0.58*
	E	-	-	-	-	-		0.59**
	ABC	0.33**	-	-	0.44**	-		0.61**
HCII	ABC	-	-	-	-	−0.28*		

Figure 4. PC, FPS and HCII levels in all groups.

All levels are in iu/dl.

P values between groups are shown in tables 47 and 49.

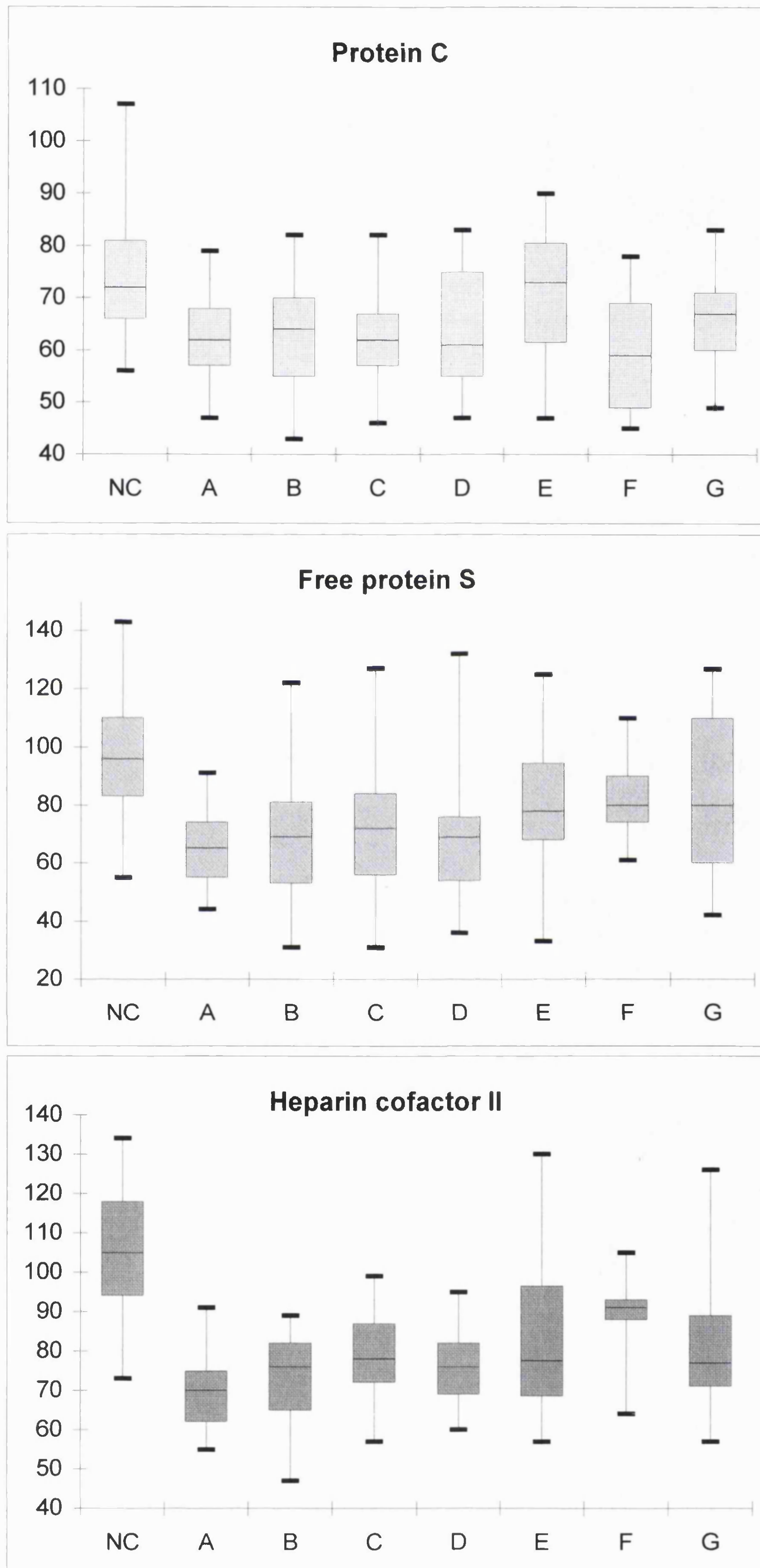
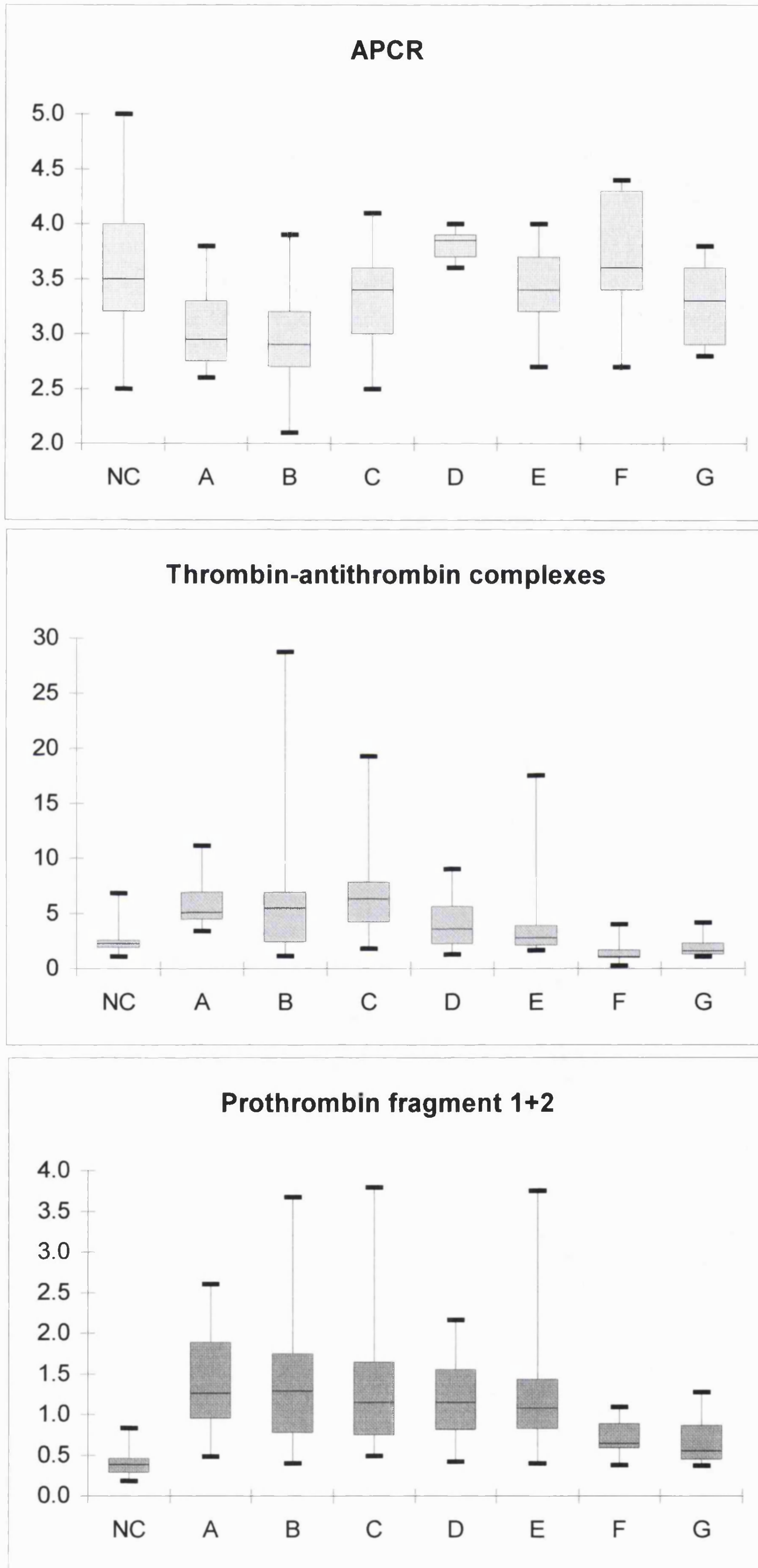


Figure 5. APCR ratios, TATs (in micrg/l) and PF12 levels (in nmol/l) in all groups.
P values between groups are shown in tables 47 and 49.



5.1.2 Laboratory findings

The patient groups A, B and C and group ABC had significant differences compared to the control group as follows. *P* values are shown in table 47 and 48.

Baseline data:
↓ Hb
↑ WBC
↑ platelets
↑ reticulocytes
↑ %HbF
↑ AST
↑ albumin
↓ CRP (groups B & ABC)

Procoagulant factors:
↓ fibrinogen (group B only)
↓ FVII (not group C)
↓ FX (not group C)
↓ FXI (group A only)
↓ FXII (all groups)

Inhibitors and APC ratios;
↓ PC
↓ TPS
↓ FPS
↓ HCII
↓ APCR (not group C)
↓ FPS / FX

Activation markers and antiphospholipid screen;
↑ TATs
↑ PF12
↓ ACL IgG

5.1.3 Summary of results

Results of between-group comparisons: The untransfused children with HbSS, groups A, B and C, had lower Hb values with increased reticulocytes and %HbF, and higher WBC and platelets compared to controls. They had evidence of liver dysfunction with elevated levels of AST though albumin levels were actually increased rather than decreased as would be expected with significant liver dysfunction. There was no evidence of an acute phase response in that the CRP values were actually lower than controls. Findings for procoagulant factors were variable but the group as a whole (ABC) had reduced FVII, FX and FXII compared to controls. All inhibitors, PC, TPS, FPS and HCII, were lower and there was evidence of excess depletion of FPS compared to FX. PC values are known to vary with age with lowest levels at the youngest ages (Andrew et al. 1992) but as there was no significant difference in age between groups A, B, ABC and controls these comparisons are likely to be valid. For group C the age range was lower

than controls so the comparison must be considered with some caution but the *p* value was highly significant at 0.0012 and whereas all the controls were within published normal ranges, 5 of the 29 children in group C had values below the lower limit of the age-adjusted range.

The patient groups also showed evidence of activated protein C resistance compared to NC but all those tested were negative for FVR506Q. Markers of increased thrombin generation were considerably elevated but there was no evidence of antiphospholipid antibodies.

Significant correlations between procoagulants and anticoagulants; There were many cross-correlations in all sickle groups between the natural anticoagulants. In the controls the significant correlations were that FVII, FX and PC all correlated with each other; fibrinogen with both FPS and TPS; TPS also with FPS and FVII. In group A FVII correlated with fibrinogen, FXII, and TPS and TPS also correlated with FX. HCII correlated with both TPS and AT. In group B FX correlated with FVII, FXI and TPS; PC correlated with fibrinogen and FX; FPS with FXII, TPS and HCII; AT with HCII; TATs with PF12 and there was negative correlation between AST and FPS. In group C fibrinogen correlated with FVII and both of these factors correlated with FX, FXI, PC and TPS. Fibrinogen also correlated with AT and HCII. FX correlated with FXI, FXII and PC; FXI with PC; TPS with PC, FPS, AT and HCII; TATs with PF12 and AT with HCII. There was negative correlation between AST and FPS and FPS/FX ratio. In group ABC fibrinogen, FVII, FX, FXI, PC and TPS all correlated with each other and TPS, FPS, AT and HCII also all correlated with each other. Fibrinogen and PC both correlated with AT and HCII; FXII with FX, FXI and FPS; TATs with PF12 and AST correlated negatively with FPS.

Significant correlations between baseline parameters and inhibitors. There were few correlations between baseline haematological values and the inhibitors

measured. In the control group and group A there were no correlations between Hb, WBC, plats and %HbF and any inhibitors. In group B and ABC Hb correlated with AT and WBC correlated negatively with APCR. In group C and ABC Hb correlated with FPS and TPS and %HbF with AT. Also in group ABC WBC correlated weakly with TATs and in group E and ABC Hb correlated with S/X ratios.

Clinical correlates; In order to establish whether there was a relationship between any of the parameters measured and overall severity of disease I examined the correlation coefficients between average annual crisis rate and the following parameters; Hb, WBC, plats, %Hb F, AST fibrinogen, FVII, FX, FXI, FXII, PC, TPS, FPS, AT, HCII, APCR ratio, TATs and PF1+2. The only significant correlations were between crisis rate and %Hb F ($r = -0.35$, $p = 0.0012$), which is an expected finding, and between crisis rate and AT levels ($r = -0.24$, $p = 0.03$), which is an unexpected weak negative correlation. To see if this was related to AST values I looked at correlation between crisis rate and AT and AST but there was no correlation between AST and either measure.

I also separated the patients in group ABC into 2 cohorts; those with and without chest crises but there were no significant differences between the 2 groups for any parameters measured.

Influence of splenectomy; As there were only 6 children enrolled in this study who had had operative splenectomy the number was probably too small to make any meaningful comparisons for any parameters between those with and those without splenectomy.

5.2 The transfused Hb SS children - group D v normal controls (group NC).

5.2.1 Clinical data

Age. Group D did not have significantly different age range compared to NC.

5.2.2 Laboratory findings

Compared to controls group D had significant findings as follows. *P* values are shown in table 47.

Baseline data;	↓ Hb ↑ WBC ↑ platelets ↑ reticulocytes ↑ %Hb F ↑ AST ↑ albumin	**Procoagulant factors;**	↓ FX ↓ FXI
Inhibitors and APC ratios;	↓ TPS ↓ FPS ↓ HCII ↓ FPS / FX	**Activation markers and antiphospholipid screen;**	↑ TATs ↑ PF12 ↓ ACL IgG

5.2.3 Summary of results

Despite a regular transfusion programme this group of children with HbSS had the same pattern of significant findings in baseline data compared to controls as the non-transfused HbSS groups though the level of significance was less for the majority of the parameters. There was evidence of reduction in FX levels as with group ABC but there were also reduced levels of FXI, a non-vitamin K dependent factor. Significant reductions in TPS, FPS and HCII were present though the degree of significance was less for the first 2 of these compared to group ABC v NC. PC values were similar in group D to those in group ABC but those in group D v NC did not achieve significance probably because the number of

children in this group is smaller. The transfusion programme also appears to have reversed the reduction in APCR ratios found in the non-transfused group and all those tested were negative for FVR506Q. Evidence of increased thrombin generation is present but the *p* value for the TAT's was only just significant in this transfused group. There was again no evidence of antiphospholipid antibodies.

The only significant correlations in group D were between fibrinogen and FXI; FVII with PC, FPS and AST; FX with FXII, FPS and TPS; PC with FPS; AT with HCII. There was also negative correlation between TATs and Hb and positive between TATs and WBC.

5.3 The transfused Hb SS (group D) v the non-transfused Hb SS (group ABC).

5.3.1 Clinical data

Age. Group ABC had a significantly lower age range compared to group D.

Total crisis rate. There was no significant difference in total or chest crisis rate between group ABC or group D.

5.3.2 Laboratory findings

Compared to group ABC, group D had significant findings as follows. *P* values are shown in table 48.

Baseline data;	↑ Hb ↓ reticulocytes ↓ %Hb F	**Procoagulant factors;**	↓ FXI
Inhibitors and APC ratios;	↑ APCR	**Activation markers and antiphospholipid screen;**	↓ TATs

5.3.3 Summary of results

The comparison between these transfused and non-transfused groups shows few significant findings. The expected changes in red cell parameters are increased Hb, reduced reticulocytes and %HbF. The reduction in APCR ratios in the non-transfused groups was not present in the transfused group and TATs were lower in group D than ABC but were still raised compared to controls. FXI alone was significantly lower in the transfused patients compared to the non-transfused.

5.4 The Hb SC group - group E v normal controls (group NC).

5.4.1 Clinical data

Age. Group E did not have significantly different age range compared to NC.

5.4.2 Laboratory findings

The *p* values from comparisons between groups E and NC are shown in Table 47. Compared to NC group E had significant findings as follows:

Baseline data	↓ Hb ↑ WBC ↑ reticulocytes ↑ %Hb F ↑ AST ↑ albumin ↓ CRP	**Procoagulant factors**	↓ FXII
Inhibitors and APC ratios	↓ FPS ↓ HCII ↓ FPS / FX	**Activation markers and antiphospholipid screen**	↑ PF12 ↓ ACL IgG

5.4.3 *Summary of results*

The children with Hb SC had the same pattern of significant findings in baseline data compared to controls as the non-transfused group except that the level of significance in group E was less for the majority of the parameters and there was no difference in platelet count between groups E and NC. Procoagulant factors were unchanged from control values except for FXII and only FPS, HCII and FPS/FX were reduced compared to NC. One of the activation markers was increased - PF12 but not TATs and as with other groups there was no evidence of antiphospholipid antibodies.

Significant correlations in group E were fibrinogen with FXI, AT and HCII; FX with FVII, FXI, PC and TPS; FXI with FPS and TPS; FPS and HCII with TPS; Hb with FPS and S/X ratio.

5.5 *The Hb SC group (group E) v the non-transfused Hb SS (group ABC).*

5.5.1 *Clinical data*

Age. Group E did not have significantly different age range compared to group ABC.

Total crisis rate. The HbSC children in Group E had a significantly lower average annual crisis rate compared to group ABC ($p = 0.0052$) and fewer chest crises ($p = 0.0389$).

5.5.2 *Laboratory findings*

Compared to group ABC, group E had significant findings as follows. *P* values are shown in table 48.

Baseline data	↑ Hb ↓ WBC ↓ platelets ↓ reticulocytes ↓ %Hb F ↓ AST	**Procoagulant factors**	↑ FVII ↑ FX
Inhibitors and APC ratios	↑ PC ↑ TPS ↑ FPS ↑ ATIII ↑ APCR	**Activation markers and antiphospholipid screen**	↓ TATs

5.5.3 Summary of results

The HbSC children had higher Hb values and less evidence of chronic haemolysis with lower reticulocyte and lower %HbF values than the HbSS children in group ABC. The WBC, platelet count and AST were also all lower and nearer normal values in group E. The reductions in FVII, FX, PC, TPS and APCR ratios found in group ABC were not present in group E and though FPS was reduced in group E compared to NC the reduction was not as severe and the levels in group E were still significantly higher than in group ABC. The difference between PC values in the 2 groups are likely to be valid as there was no difference in age ranges between the 2 groups. TATs were not raised in group E and were therefore lower than in group ABC.

5.6 The thalassaemic group - group F v normal controls (group NC).

5.6.1 Clinical data

Age. Group F had a significantly higher age range compared to NC.

5.6.2 Laboratory findings

Compared to NC group F had significant findings as follows. *P* values are shown in table 47.

Baseline data	↓ Hb ↑ WBC ↑ platelets ↑ reticulocytes ↓ CRP	**Procoagulant factors**	↓ FX ↓ FXI
Inhibitors and APC ratios	↓ PC ↓ TPS ↓ FPS ↓ HCII ↓ PC / FVII	**Activation markers and antiphospholipid screen**	↓ TATs ↑ PF12 ↓ ACL IgG ↓ TXT

5.6.3 Summary of results

Despite a regular transfusion programme these thalassaemic children had lower Hb and higher reticulocyte values than controls but this is expected as the blood samples were taken just before the next transfusion was due. The WBC and platelet count were also higher than NC. However 4 of the 13 in the group had had splenectomies and this may account for the higher values as the sub-group within this group who had had splenectomy had significantly higher WBC (p=0.008) and platelets (p=0.03) than the sub-group who had not.

The 2 procoagulant factors reduced in this group were FX and FXI and PC, FPS, TPS, HCII and PC/VII ratios were also all reduced compared to controls. As this group and controls were not well matched for age the comparison for PC may not be entirely valid but in fact the higher age range of the group F patients means that the lower PC values were likely to be even more important. There was discrepancy in the markers of thrombin generation with elevated PF12 but lower TATs in group F compared to controls but there was no indication of antiphospholipid antibodies in this patient group.

The only correlations found in this group were between fibrinogen and FVII and HCII; PC and fibrinogen, FXI and FXII. FPS, TPS and FX all correlated with each other and AST with PC/VII ratio. TPS also correlated weakly with HCII.

5.7 The thalassaemic group (group F) v the non-transfused HbSS (group ABC).

5.7.1 Clinical data

Age. Group F had a significantly higher age range compared to group ABC.

5.7.2 Laboratory findings

Compared to group ABC, group F had significant findings as follows. *P* values are shown in table 48.

Baseline data	↑ Hb ↓ WBC ↓ reticulocytes ↓ %Hb F ↓ AST ↓ albumin	**Procoagulant factors**	↓ FXI
Inhibitors and APC ratios	↑ FPS ↑ HCII ↑ APCR ↓ PC / VII ↑ FPS / FX	**Activation markers and antiphospholipid screen**	↓ TATs ↓ PF12

5.7.3 Summary of results

Compared to non-transfused HbSS children the thalassaemic children in this study had higher baseline Hb but reduced WBC, reticulocytes and %HbF. Their liver function was better preserved with lower AST values despite high ferritin levels resulting from iron overload (median 1524, range 740 - 7116). The only

procoagulant factor reduced in the thalassaemics compared to sicklers was FXI and some inhibitor levels, namely FPS and HCII were increased towards 'normal' values. APCR and FPS/FX ratios were also increased but PC/VII ratios were less than in group ABC, possibly because PC levels in group F were reduced compared to NC but FVII levels were normal. Group F had less evidence of increased thrombin generation than group ABC though levels of TATs and PF12 were between those of NC and group ABC.

5.8 Other haemolytic red cell disorders (group G) v the non-transfused Hb SS (group ABC).

5.8.1 Clinical data

Age. Group G did not have significantly different age range compared to group ABC.

5.8.2 Laboratory findings

Compared to group ABC, group G had significant findings as follows. *P* values are shown in table 48.

Baseline data	↑ Hb ↓ WBC	**Procoagulant factors**	↓ FXI
Inhibitors and APC ratios	No differences found	**Activation markers and antiphospholipid screen**	↓ TATs ↓ PF12

5.8.3 Summary of results

The main differences between this heterogeneous group of haemolytic disorders and group ABC were that they had higher Hb values, lower WBC and

lower FXI levels compared to group ABC. They also had TAT and PF12 values that approached normal.

Correlations found in group G were between fibrinogen and FVII, FX and PC and between TPS and FPS.

5.9 *The non-transfused Hb SS children with asymptomatic CVD (group A) v the group without CVD (group B).*

5.9.1 Clinical data

Age. There was no significant difference in age range between groups A and B.

Total crisis rate. There was no significant difference in total or chest crisis rate between groups A and B.

5.9.2 Laboratory findings

Compared to group B, group A had significant findings as follows. *P* values are shown in table 49.

Baseline data	↓ Hb ↑ WBC	**Procoagulant factors**	No differences found
Inhibitors and APC ratios	No differences found	**Activation markers and antiphospholipid screen**	No differences found

5.9.3 Summary

The only differences found between the non-transfused children with documented but asymptomatic cerebrovascular disease and those without were a

lower Hb and higher WBC in the former group. There were no other differences in any of the other parameters measured.

5.10 The non-transfused Hb SS children with asymptomatic CVD (group A) v the transfused group with stroke (group D[s]).

5.10.1 Clinical data

Age. There was no significant difference in age range between groups A and D(s).

Total crisis rate. There was no significant difference in total or chest crisis rate between groups A and D(s).

5.10.2 Laboratory findings

Compared to group D(s), group A had significant findings as follows. *P* values are shown in table 49.

Baseline data	↓ Hb ↑ reticulocytes ↑ %Hb F	**Procoagulant factors**	No differences found
Inhibitors and APC ratios	↓ APCR	**Activation markers and antiphospholipid screen**	No differences found

5.10.3 Summary of results

The baseline findings are as expected for an untransfused compared to a transfused group of children with sickle cell disease. The only other finding was that the untransfused children had evidence of APC resistance compared to the transfused stroke cohort.

5.11 *The non-transfused HbSS children without CVD (group B) v the transfused group with stroke (group D[s]).*

5.11.1 Clinical data

Age. There was no significant difference in age range between groups A and D(s).

Total crisis rate. There was no significant difference in total or chest crisis rate between groups B and D(s).

5.11.2 Laboratory findings

Compared to group D(s), group B had significant findings as follows. *P* values are shown in table 49.

Baseline data	↓ Hb ↑ reticulocytes ↑ %Hb F	**Procoagulant factors**	↑ FXI
Inhibitors and APC ratios	↓ APCR	**Activation markers and antiphospholipid screen**	No differences found

5.11.3 Summary

The results of group B v D(s) were similar to the previous section - group A v D(s) - except that group B also had higher FXI levels than group D(s) as these were significantly reduced in group D as a whole compared to the non-transfused HbSS.

Chapter 6

Discussion of results and conclusions

6.0 The HbSS children

6.0.1 The natural anticoagulants

In this study I have shown significant reductions in the majority of the naturally occurring anticoagulants in a large number of children with steady-state HbSS or HbSβ^0 thalassaemia compared to healthy sibling controls. I will discuss these findings in detail in the following paragraphs.

Protein C

There was a striking decrease in the PC levels in the 3 non-transfused groups (A, B and C) compared to controls. As outlined in section 5.1.3, the age range of group C was lower than NC and because of age-related physiological variations in PC levels (Andrew et al. 1992) the comparison between group C and NC must be considered with caution but group C did contain 5 apparently 'deficient' children. When all the untransfused HbSS children were taken as a whole - group ABC - and compared to controls there was no difference in age but PC levels were significantly lower. The only other study in children with HbSS in which PC activity was measured by an established chromogenic method was from Peters *et al* (Peters et al. 1994), published just prior to the start of my study. They found that 16 children had lower PC levels than age-matched but not race-matched controls. My study adds a further 83 children to support this finding.

As so little data has been previously published on the clinical correlates of haemostatic abnormalities in this disease, I looked at how measures of clinical severity such as annual crisis rate or incidence of chest crisis may correlate with parameters measured. In fact, there was no evidence from my data that PC levels, or indeed levels of any of the anticoagulants correlated significantly with these

measures of clinical severity. I will discuss cerebrovascular disease in section 6.0.8.

Protein S

Levels of TPS and FPS were also very low for groups A, B and C compared to NC and as physiological levels in children are the same as adult ranges from over 1 yr of age (Andrew et al. 1992) the comparisons should be valid for all 3 groups. Once again my study has confirmed with much larger numbers of children the findings of Peters *et al* (Peters et al. 1994) which is the only other study looking at TPS and FPS in the paediatric population.

Antithrombin

AT was the only one of the 4 natural anticoagulants measured to have levels that were no different in any of the 3 HbSS groups from NC. The other 2 paediatric studies previously published were Peters *et al* (Peters et al. 1994) and an abstract from Karayalcin *et al* (Karayalcin et al. 1984). The results of the former once again concurred with this study but the latter found reduced AT activity in 75 steady state children but normal AT antigen suggesting a functional deficiency of AT. Unfortunately this was only published in abstract form and the methods were not outlined. All the most recent observations measuring AT activity by chromogenic methods in adults and children have found that levels are not affected in steady state SCD and my study supports the validity of these other studies.

Heparin cofactor II

This is the first study looking at HCII levels in children with Hb SS and it confirms that levels of this anticoagulant are also reduced. A previous study

published in adults (Porter et al. 1993) using the same method also found low levels.

6.0.2 Activated protein C resistance ratios

APCR ratios measured by the 'standard' assay were significantly reduced in groups A, B and ABC as a whole compared to NC. There were no FVR506Q positive patients and as this mutation is rare in this ethnic group (Rees et al. 1995) this is not unexpected. These findings are similar to the one published study which is in adults with SCD (Wright et al. 1997). Therefore this relative, and probably acquired, resistance to APC is present in childhood in SCD and may contribute towards thrombotic risk. It may well be that high FVIII levels, which have been reported in other series (Leslie et al. 1975; Famodu, 1987), are contributing towards the functional APC resistance demonstrated in these children. Unfortunately there was insufficient plasma left for most of the subjects in this study to examine the FVIII levels and see if there was a relationship between FVIII and APCR ratios.

6.0.3 Markers of coagulation activation

TAT complexes and PF1+2 were markedly increased in groups A, B and C compared to NC with highly significant *p* values, confirming previous adult work (Francis, Jr. 1989; Kurantsin Mills et al. 1992; Devine et al. 1986) though only Peters *et al* has studied a small group of children (Peters et al. 1994). This indicates that even at a young age SCD is associated with increased thrombin generation and a hypercoaguable state.

6.0.4 Antiphospholipid antibodies

A comprehensive screen for the presence of APA in this cohort of children with HbSS has found no real evidence of an antiphospholipid syndrome in any of our patients though a stronger association between APA and SCD, but not between any specific clinical correlate, has been reported previously in one adult series (Kucuk et al. 1993) but disputed in a larger series (De Ceulaer et al. 1992). I did find very weakly positive ACA in 4 SCD children and also in 3 NC but these were likely to have been transient and possibly related to recent viral infection though all children were clinically well at the time of testing. As these subjects were only studied on one occasion this could not be confirmed.

6.0.5 The effect of red cell transfusion programmes

The current accepted management of symptomatic stroke is a transfusion programme that replaces Hb S with Hb A and keeps the %HbS below 20%. This is discussed in detail in section 1.2.1. There is also recent evidence that similar regimes can prevent stroke in previously asymptomatic children with CVD (Adams et al. 1998), though exactly what the mechanism as to why this is the case is not clear. In order to examine how regular transfusion alters haemostatic abnormalities and to get an idea as to whether reversing these abnormalities may contribute towards the benefit gained by transfusions, I compared group D to NC and also to group ABC.

The significant differences between groups D and NC for the anticoagulants measured were essentially the same as between groups A, B and C and NC and there were no differences between ABC and D. TPS and FPS were significantly lower in group D than NC but the *p* values were less significant than for groups A, B or C. As the ranges of values were similar to those in the untransfused groups, the

reason for this is probably because the numbers in group D were smaller. HCII values were also much lower with highly significant *p* values but AT values were normal. PC measurements, however, were not statistically different to controls even though the range of PC values in group D was similar to group ABC. Again, this is likely to be an effect of smaller numbers in group D.

There is no evidence, therefore, from my data that transfusion corrects the anticoagulant abnormalities present in untransfused sickle children. The only other published investigation of what difference transfusion makes to these abnormalities is the adult study on HCII levels (Porter et al. 1993) which found that levels *were* normalised by regular transfusion. The reasons for this discrepancy is not clear; the timings between transfusion and sampling were more uniform in my study in that all children were sampled 3-4 weeks after transfusion and just before the next transfusion was due. In the other study (Porter et al. 1993) samples were taken between 2 and 90 days after transfusion and it may be that those with the highest HCII levels were those who had been transfused most recently but by the time 3 weeks had passed the levels had fallen again.

One parameter that was reversed by transfusion was the APCR ratios which were significantly higher in group D than ABC and those in group D were also no different from controls. This is an interesting finding, especially in the light of the fact that anticoagulant levels are not altered by transfusion, as outlined above. Therefore there must be other factors affecting this ratio which have been changed by transfusion. This study did not measure FVIII or FIX levels which may be relevant, but FXI, another APTT based factor, was significantly lower in group D than controls and ABC and this may have contributed. All the other procoagulants measured were unchanged between group ABC and D. The cause of the lowered FXI levels in group D is unexplained but it is intriguing that the thalassaemics in group F, who are also transfused, also had very low levels of FXI.

The chronic increase in thrombin generation was not completely reversed by transfusion in that group D also had significantly elevated TAT and PF12 levels compared to controls. However the *p* value for the TATs was only just significant and there was significance between groups ABC and D showing that TATs were reduced to a certain extent by transfusion but PF12 levels were not.

6.0.6 The pathogenesis of the haemostatic abnormalities in HbSS.

The role of the liver and liver dysfunction

There has been recent interest in how and to what extent impaired liver hypofunction and vitamin K deficiency may contribute to reduced protein synthesis in HbSS. A recent adult study performed on Jamaican patients proposes that reduced levels of PC and FPS are due to impaired hepatocellular function and chronic intrahepatic sickling (Wright et al. 1997). To examine this further I included measurements of 2 vitamin K dependent procoagulants, factors VII and X, and one non-vitamin K dependent procoagulant, factor XI and ratios and correlations between these procoagulants and anticoagulants were calculated. A number of other parameters measured are also synthesised in the liver or reflect liver function, namely fibrinogen, AT, HCII, albumin and AST.

There was definite evidence of a degree of hepatocellular dysfunction with elevated AST levels in all HbSS groups compared to controls but in all patients the elevation was mild with most values between 50 and 100 and the highest was 125 u/l. Other markers for liver damage were not affected; albumin levels were actually higher in the HbSS rather than lower; AT levels were unaffected as discussed

above and fibrinogen levels were also unchanged. It therefore seems unlikely that the liver was severely impaired in these groups of patients.

In groups A, B and ABC both FVII and FX were reduced compared to NC and in groups C and ABC FVII correlated with PC suggesting a possible common cause for their depletion though FX did not correlate with FPS but did with TPS in groups A, B and ABC. However there was no significant inverse correlation between AST values and PC, FPS, FVII or FX levels for any group except for a weak correlation between FPS and AST in groups B and C but not when larger numbers were examined in group ABC. PC/FVII ratios were not different in patient groups compared to NC and again did not correlate with AST. However the FPS/FX ratios were significantly lower in A, B, C and ABC than NC and there was negative correlation with AST values for ABC only. This suggests that FPS levels are depleted in excess of FX, which has a similar half-life, and liver dysfunction may play a contributory role. In group D only FX, but not FVII, was reduced compared to NC but PC was not lowered either. Neither the PC/VII nor FPS/FX ratios were different to NC and the only correlation with AST was between AST and FVII.

The depletion in procoagulant factors was not confined to the vitamin-K dependent factors as FXI levels were depleted in groups A and D but not in B, C or ABC. FXII levels were also low in all non-transfused groups, in agreement with previous studies on reduction of contact factors in SCD (Gordon et al. 1985; Abildgaard et al. 1967). There was no correlation between FXI or FXII and AST. HCII, a non-vitamin K dependent anticoagulant, correlated weakly and negatively with AST in group ABC only but it did correlate strongly in all HbSS groups with AT, which in turn was unrelated to AST, though both HCII and AT correlated with fibrinogen and TPS in group C and ABC, and HCII only with FPS in group B and ABC.

It is likely that the correlations I have demonstrated reflect the common synthetic property of the liver, but as there is no evidence of a direct relationship they do not lend support to the hypothesis that liver dysfunction has a major contribution in depleting plasma levels of procoagulants and anticoagulants. However this data does suggest that liver dysfunction is one of several components in a complex, multi-factorial process.

The role of the red cell membrane and chronic haemolysis

It is likely that haemostatic proteins binding to the surface of damaged red cells is an important mechanism contributing to plasma depletion of coagulation inhibitors and enhanced thrombin generation in the sickle cell syndromes. Although this study was not specifically designed to address the question as to what extent this binding contributes towards low levels, some of the relationships between groups of data collected may be informative as I did measure a number of parameters that indicate the degree of on-going haemolysis. Other studies have shown that the damaged, deoxygenated and irreversibly sickled red cell membrane, and also circulating spectrin-free membrane vesicles shed during sickling, have abnormally exteriorised procoagulant anionic phospholipids (Chiu et al. 1981; Helley et al. 1996; Allan et al. 1982), which are capable of binding, activating, and increasing consumption of coagulation factors and inhibitors. The two inhibitors for which there is currently good evidence for red cell binding in SCD are FPS and HCII (Lane et al. 1994; Chitolie et al. 1996).

The indices I measured which reflect the degree of ongoing haemolysis best were retics, Hb and %HbF. In fact, the retic count did not correlate with any of the procoagulants and anticoagulants measured though, as expected, it did correlate negatively with Hb in all HbSS groups. Hb correlated with both TPS and FPS in groups C and ABC, AT in group B and ABC, and FXI in group ABC. It did not

correlate with PC unlike in the recent adult Jamaican study (Wright et al. 1997). The %HbF levels were also unrelated to most parameters and only correlated with AT in groups C and ABC. There is therefore no convincing demonstration of a relationship between markers of haemolysis and procoagulants and inhibitors measured. It is interesting however that the 2 inhibitors for which there is good evidence for red cell binding, namely FPS and HCII, do correlate positively with each other in groups B and ABC. TPS and HCII also correlate in groups A, C and ABC. This raises a suggestion that the significant depletion in the HbSS groups in these anticoagulants may well arise by a common process and as they are known to bind to sickled red cell membranes this process is likely to be contributing. It can be postulated that PC may also bind to the red cell surface, even though this has not been demonstrated, but that the reason that AT is not affected is that it does not bind in the same way as the other inhibitors. As far as I am aware there is no evidence supporting or refuting this theory.

The other haemolytic disorders investigated in this thesis were thalassaemia (group F) and small numbers of children with HS, PK, HbH and G6PD deficiency (group G). G6PD deficiency is associated more with acute haemolytic crises whereas the others are chronic haemolytic states. As can be seen from section 4.8 many of the children in group G also had reduced levels of PC, TPS, FPS and HCII. As a result, when groups ABC and G were compared there were no significant differences for any of these parameters between the 2 groups, suggesting that chronic red cell breakdown, whatever the mechanism, plays an important part in the reduction of these anticoagulants. However the numbers studied in group G was small and therefore these results must be treated with some caution though this area does warrant further study in larger numbers. It would also be interesting to look at the role of splenectomy on these parameters in a longitudinal study on patients with these chronic haemolytic states but as the

numbers post-splenectomy were so small in this study (group ABC 6 patients, group G 1 patient) no conclusions can be made. There is some evidence from the literature that patients with hereditary haemolytic anaemias, including thalassaemia major and HS, have an increased risk of thrombo-embolic disease post-splenectomy (Kemahli et al. 1997; Winichagoon et al. 1981; Visudhiphan et al. 1994) but no studies on how any possible abnormalities in anticoagulant levels might contribute have been done to date. Potentially the consumption of PC, FPS and HCII could increase post-splenectomy as the more damaged red cells will have an increased circulation time when the spleen has been removed.

The thalassaemic children (group F) also had reduced levels of the four anticoagulants PC, TPS, FPS and HCII compared to controls but the reductions were not as significant as for the untransfused HbSS children v controls. For FPS and HCII comparisons between groups ABC and F were still significant even though the levels in group F were approximately mid-way between those in group ABC and the controls. As the thalassaemics are all on regular transfusion this may explain 'partial' reversal of some of these abnormalities whereas none of the children in group G had been transfused in the preceding months and showed greatly reduced levels.

Results for procoagulants in groups F and G were not significantly different to group ABC except that both had very low levels of FXI compared to the untransfused HbSS children, as did the transfused children in group D. Therefore transfused HbSS, transfused thalassaemics and other haemolytic disorders all have apparent FXI deficiency. The mechanism for this is not clear and is not mirrored by any of the other factors measured.

Neither group F or G had as striking evidence of increased thrombin generation as the untransfused HbSS group and in both groups levels of TATs and PF12 were lower than group ABC though there was some elevation above normal

control values for PF12 only. This suggests that the mechanisms resulting in depletion of inhibitors and those causing increased thrombin generation may not be closely linked and may be separate processes even though some evidence exists for an association between inherited inhibitor deficiencies and enhanced thrombin production (Mannucci et al. 1992; Conard et al. 1993) (see below).

6.0.7 Markers of thrombin generation

The findings of increased levels of TAT complexes and PF1+2 strongly suggests that thrombin generation is increased above normal levels in the steady-state in HbSS children. This adds further evidence to existing adult data that a 'hypercoaguable' state exists even in young children with HbSS, characterised by acceleration of coagulation reactions *in vivo* and resulting in increased thrombin generation. This is consistent with reports that SSRBC accelerate prothrombinase activity *in vitro* due to a loss of membrane phospholipid symmetry (Chiu et al. 1981; Helley et al. 1996). Whether other phospholipid-dependent coagulation reactions (activation of factors VII, IX and X) are similarly accelerated in steady-state SCD is not known but an unpublished observation from this study is that a small number of samples (3 from NC, 8 from group A and 7 from group B) were processed for FVIIa levels and there was no evidence of elevation in levels in the HbSS samples from this very small pilot analysis. Obviously conclusions cannot be drawn from this but depletion in levels of the procoagulants measured in this study do suggest that there may be acceleration of these other reactions as well.

Another possible source of acceleration of coagulation activation is increased TF expression by endothelial cells and monocytes via increased TNF levels, which have been shown to induce procoagulant activity (Bauer et al. 1989; Bevilacqua et al. 1986) but confirmatory data is lacking. However other

suggestions that raised CRP levels may be responsible for inducing monocytic TF expression and coagulation activation (Cermak et al. 1993) are refuted by my findings of completely normal CRP levels in all the steady-state HbSS children.

One further mechanism that may contribute towards thrombin formation was mentioned in the last section; namely low levels of PC. In asymptomatic non-SCD patients with PC deficiency markers of thrombin production were also elevated, suggestive of a prothrombotic state (Mannucci et al. 1992). Moreover, in heterozygous PC deficiency the level of thrombin formation could be normalised by infusing PC concentrate (Conard et al. 1993). These data confirm a direct association of inhibitor deficiencies and enhanced thrombin production and a similar association may play a role in the observed prothrombotic state in SCD.

Lastly, it must be considered whether the elevated markers of thrombin generation are due to impaired clearance in patients with SCD. TAT complexes are cleared by the Kupffer cells of the liver and are known to have a very short plasma half-life of 3 minutes. As well as being elevated in sepsis they are known to be elevated in severe liver impairment and chronic renal failure. It seems unlikely that the very mild degree of liver impairment demonstrated in the patients in this study and discussed in full in section 6.0.6 will have contributed towards poor clearance of these enzyme-inhibitor complexes. Correlation analyses did not demonstrate any relationship between TATs and PF1+2 and AST or any of the other parameters that reflect liver function such as albumin and fibrinogen.

6.0.8 Is there any evidence that haemostatic abnormalities contribute to the development of cerebrovascular disease?

There has been very little work published on coagulation abnormalities in vaso-occlusive complications of SCD other than painful crisis and, in particular,

there has been no comprehensive study of how levels of procoagulants and anticoagulants may contribute towards the development of CVD in SCD. The only study in the literature was published while my thesis was underway (Tam, 1997) and was a small pilot study on 9 older children with HbSS (mean age 12.6 years) with a past history of stroke but no recent transfusion. This patient group had significantly lower PC and PS activity levels than a control group of 4 HbSS children without clinical or radiological evidence of stroke. This study is obviously too small to draw any real conclusions from though the authors do suggest that there is a relationship between PC and PS deficiencies and an increased risk of stroke and that it would be of benefit to screen children for deficiency and start them on a transfusion programme before a clinical event occurs.

One of the main aims of this study was to establish whether or not children with known CVD had any significant differences in any of the factors controlling thrombin generation or inhibition compared to those who had normal vasculature. The screening programme to detect asymptomatic CVD with TCD +/- MRI/MRA, which was in place in this clinic, has allowed me to look at this in untransfused patients as all those with symptomatic CVD remained on long-term regular transfusion which potentially could reverse abnormalities present that may have been important in the pathogenesis of the original cerebral event. To analyse my data I divided the non-transfused HbSS children into 3 groups; group A were those who had proven CVD on at least 2 TCD studies with or without MRI/MRA abnormalities but were asymptomatic; group B were those who had been found to have normal cerebral vasculature on such studies and group C were those who had not been screened. I compared groups A and B to each other and to the subgroup of group D - group D(s) who were the 10 transfused children with stroke (table 49).

My results have found no excess risk for thrombosis or stroke as measured by levels of natural anticoagulants, response to APC, and the presence of an antiphospholipid antibody in the children with abnormal cerebral vessels in group A comparing them to group B, suggesting that none of these are important in the pathophysiology of CVD in this disease. Similarly, there were no differences in these parameters between group D(s) and groups A or B except that both A and B had significantly lowered APC resistance ratios compared to group D(s). Therefore the process that occurs to prevent further cerebral events when the %HbS is lowered to less than 20 - 30% during a transfusion programme does not appear to involve reversal of deficiencies in any of the natural anticoagulants but somehow APC resistance, which is probably acquired, reverses. As the 'standard' APC resistance ratio is derived from a APTT based assay with and without the addition of APC then any of the parameters that affect the APTT or the action of APC in inhibiting activated FV or activated FVIII can affect this ratio. As I stated in section 6.0.3. I was unable to examine a possible relationship between FVIII levels and APC resistance ratios. FXI and FXII were the only APTT-based factors that were studied and group D(s) did have reduced levels compared to group B but not A and there were no differences for FXII between these 3 groups. More detailed coagulation assays would be required to examine what is responsible for the normalisation of the functional APC resistance in group D(s) compared to groups A and B.

The lack of an association between the development of CVD and deficiency of the natural anticoagulants, response to APC, or the presence of an antiphospholipid antibody is an important negative finding of my study. First of all it refutes completely the results of the small pilot study already mentioned (Tam, 1997) which did find lower levels of PC and PS in children who had had a stroke. Secondly, there is still considerable dispute in the literature as to whether these

thrombophilia conditions are important in the pathogenesis of cerebral arterial thrombosis in children without SCD. All published studies are small and include case reports, which have to be regarded as anecdotal (Nowak Gottl et al. 1996a; Nuss et al. 1995; Gurgey et al. 1996; Olson et al. 1994; Devilat et al. 1993; Nowak Gottl et al. 1996b). There are no large controlled studies published to date and the data from this study does not support suggestions that these conditions cause childhood arterial thrombosis. The role of a patent foramen ovale, which has recently been recognised as an important cause of stroke in young adults, deserves evaluation in children with stroke with and without SCD.

The *only* significant differences between groups A and B in this study were that the children with asymptomatic CVD had lower Hb and higher WBC values than those with normal vasculature. Both of these are known to signify more clinically severe SCD and be related to higher mortality rates and also a higher risk of stroke in other published series (Platt et al. 1994; Balkaran et al. 1992; Ohene Frempong, 1991). It is not clear whether or not a high WBC *per se* or aberrant WBC-endothelial interactions are important in the initiation of CVD or, conversely, whether a higher WBC is reactive, secondary to intimal hyperplasia and endothelial damage in the cerebral vessels or to sites of chronic infection. One common site for chronic infection in children, particularly those with SCD, is the hypertrophied tonsillar bed and as a causative relationship between obstructive sleep apnoea and stroke has been proposed (Davies et al. 1989; Madderin et al. 1989; Robertson et al. 1988), one hypothesis for the association between a reactive elevation in WBC and the development of CVD is that chronic tonsillar hypertrophy could play an important central role (Ajulo, 1994). One interesting study looking at neutrophils in SCD patients has found increased numbers of circulating activated neutrophils which show increased adherence to vascular endothelium (Fadlon et al. 1998). Further work is obviously required in this field to determine exactly what is the role

and importance of WBC in this disease and whether interactions between WBC and endothelial cells or between WBC and platelets such as WBC-platelet complexes play a role. The importance of tonsillar hypertrophy in the pathogenesis of CVD also needs to be examined as this was not addressed in the recent CSSCD study (Ohene Frempong et al. 1998). However a further finding in my study suggests that even though the group with CVD have a higher WBC than those without, this is not reversed by transfusion as there was no difference in WBC between groups A and D(s). As transfusion is known to prevent clinical recurrence but does not correct the high WBC this is evidence against a prominent role for WBC in CVD.

6.1 Therapeutic implications

6.1.1 Introduction

Prevention or amelioration of vascular occlusion via pharmacological alteration of haemostasis would be the strongest possible evidence that the haemostatic system contributes towards the pathogenesis of vascular occlusion in SCD. Unfortunately the therapeutic potential of anticoagulant, antiplatelet, and thrombolytic agents in SCD have not been adequately tested. All of the studies so far conducted have been relatively small, and concerned with prevention or amelioration of painful crisis. There have been no studies of these agents in the primary or secondary prevention of stroke, the vaso-occlusive complication most clearly linked to thrombosis (Russell et al. 1984; Stockman et al. 1972; Rothman et al. 1986). The 'standard' treatment for strokes remains transfusion but hydroxyurea is being used increasingly in this disease (see section 6.1.3) and if the risks of this agent are deemed acceptable this *may* provide a possible alternative to

transfusion in the future but obviously it requires a randomised controlled trial to fully assess this. There has also been much recent attention on the use of allogeneic bone marrow transplantation from unaffected siblings as a curative procedure for this disease and it has been performed in a few children with CVD. However because of the lack of donors and the high risk of this procedure it is only suitable or acceptable at present to a small minority of children.

6.1.2 Chronic transfusion regimes

The current standard therapeutic approach for children with strokes is a chronic transfusion regime but because of associated risks of transfusion programmes clinicians are reluctant to embark on this when the child remains asymptomatic even when screening has identified those at highest risk of clinical stroke. However the recently published 'STOP' (stroke prevention in sickle cell anaemia) trial (Adams et al. 1998) has shown that a chronic transfusion programme does prevent more first strokes in asymptomatic children with abnormal TCD. The authors accept that this benefit has to be weighed against the considerable risks of long term transfusion programmes and ideally future work must be directed at identifying additional risk factors which label the subgroup of children with TCD abnormalities who are going to stroke in order to limit the number requiring transfusion programmes while other therapeutic possibilities are being evaluated.

6.1.3 Hydroxyurea

The possible contributory role of elevated WBC levels in the pathogenesis of vascular occlusion in SCD is again raised by the use of hydroxyurea (HU) in SCD. This agent has been used primarily because it raises %HbF levels and HbF

inhibits sickling, though the molecular mechanism through which it does this is not known. It also reduces the WBC and platelet count and has been shown to be of benefit in reducing the incidence of painful crisis and transfusion requirements in severely affected SCD adults (Charache et al. 1995; Charache et al. 1992). Clinicians have been reluctant to use this agent in children because of concerns about its long-term effects, particularly its leukaemogenic potential. Therefore its use in children has still to be fully evaluated even though 2 early pilot reports describe its use in a total of 57 children (de Montalembert et al. 1997; Ferster et al. 1996) and find reductions in painful events and in hospitalisation days. There have been no studies to date examining whether or not it is of benefit in preventing a first cerebral event or recurrent events in either adults or children with SCD.

It has been generally assumed that the benefit of treatment with HU in patients with SCD is due to the induction of HbF but a multivariable analysis of data from the national multicentre clinical trial (Charache et al. 1995) showed that the percentage of F cells was inversely correlated with the rate of painful crises only during the first three months of therapy but in contrast there was a strong correlation between the neutrophil count and the rate of painful crises throughout the two-year study and this effect may therefore contribute to the drug's efficacy.

Data on the use of other drugs that stimulate %HbF production such as butyrate and other short-chain fatty acids is limited at present but are of interest as they are thought to be non-mutagenic.

6.1.4 Anticoagulants

Other therapeutic possibilities for the primary or secondary prophylaxis of stroke in SCD include anticoagulant agents. Low dose warfarin has been shown to normalise the steady-state 'hypercoaguability' in 7 adult patients (Wolters et al.

1995) as measured by PF1+2 levels but the use of warfarin has not been studied in larger numbers. Its use may well warrant therapeutic trials in children with asymptomatic CVD and there are proposals for multicentre studies in the U.K. in such children. However, there are 2 reasons for caution when considering these trials; one is that a small but documented number of children have haemorrhagic strokes, or have haemorrhage associated with an infarctive stroke and if the child was a 'treatment failure' on anticoagulation this could worsen the degree of damage. What is not clear is whether the pre-stroke TCD findings are different for infarctive and haemorrhagic strokes, though the fact that one of the children in the 'STOP' trial randomised not to receive transfusions developed a haemorrhagic stroke suggests that it may not be possible to predict which sort of stroke these children are at risk from. The other note of caution is that many SCD children, as confirmed in this study, have very low baseline levels of both PC and FPS. Starting anticoagulants will drop these levels further and would introduce a short-term risk of an increase in hypercoaguability and further thrombotic problems.

6.1.5 Antiplatelet agents

There is also little experience with the use of antiplatelet agents in SCD even though evidence is accumulating for *in vivo* platelet activation (Wun et al. 1998). Antiplatelet agents could be useful in preventing stroke in SCD, but whether currently available antiplatelet agents are capable of preventing platelet aggregation and secretion at sites of vascular intimal injury is not known. My study did not address the role of platelets in CVD but future proposals on this population of children do include studying platelet activation, platelet-endothelial and platelet-leucocyte interactions in the pathogenesis of CVD, which are to date largely unexplored. Clearly it is vitally important that future therapeutic trials then

incorporate specific measurements of platelet activity to determine whether adequate inhibition of platelet function by anti-platelet agents is attained and using modern flow techniques it is likely that this can be achieved.

6.2 The HbSC children

Most previous studies on levels of coagulation inhibitors in SCD have concentrated on HbSS but it is clear that there are important clinical differences in HbSC disease; CVD is rare but other complications in which microvascular thrombosis may be important such as proliferative retinopathy and aseptic necrosis of the femoral head and are as common or more common in Hb SC than in Hb SS (Serjeant, 1985). There have been no studies published on haemostatic changes in a selected population of Hb SC children or adults. Several studies looking at levels of coagulation inhibitors that have included small numbers of HbSC patients have analysed patients with HbSC together with Hb SS (Hagger et al. 1995; Francis, Jr. 1988b; Karayalcin et al. 1984). Porter *et al* (Porter et al. 1993) studied AT and HCII levels in 10 HbSC adults and found reduced levels of HCII but normal levels of AT, similar to the findings in their HbSS population. There have been no publications looking at APC resistance but 2 studies on markers of thrombin generation have included HbSC patients; one measured fragment D-dimer levels in SCD and included 10 HbSC patients but these were analysed together with the HbSS patients (Devine et al. 1986). The other more recent study included 11 HbSC adults with a larger number of HbSS and measured PF1+2 and TAT complexes (Helley et al. 1997). Results did not reach significance compared to controls for either parameter despite elevated levels in 3 or 4 subjects for each measurement.

Because of the clinical differences between HbSS and HbSC and the paucity of data on HbSC I included 24 children with HbSC disease (group E) in this study. The main differences between group E and NC were a significant reduction in FPS, HCII and FXII and increased PF1+2. PC and TPS values, which were reduced in all Hb SS groups were 'normal' in the Hb SC children and this group did not show resistance to APC. Markers of thrombin generation were discrepant in that there were significantly higher PF1+2 values compared to controls but TAT complex levels were not significantly different as the majority of the children in the group had levels similar to NC. However there were 5 children in the group with TAT values above 5 μg/l and all of these also had high PF1+2 over 1.0 nmol/l. None of them appeared to have more severe clinical complications than the remainder of the group in terms of painful crisis rate, chest crisis or symptomatic cerebral events. Similarly, none of the children in this group had suffered from symptomatic avascular necrosis and due to their young age they had not yet been screened for proliferative retinopathy and so it is not possible to relate my findings to any clinical correlates.

The results in this group suggest that the degree of increased thrombotic risk and increased thrombin generation in the steady-state suffered by subjects with SC disease is less than in Hb SS but there are still distinct abnormalities present which may be of pathogenetic significance but I have not been able to confirm this. It is interesting that the 2 inhibitors that are lowered in Hb SC are the 2 for which there is good evidence for binding to the sickle red cell surface and consequent plasma depletion of levels of the inhibitor (Lane et al. 1994; Chitolie et al. 1996), again suggesting that the consumption may arise by a common process and binding to the membrane of the Hb S- and C-containing red cell is likely to be contributing. Why PC levels are not reduced in Hb SC is not clear but does infer that the causative process is different and does not occur in Hb SC disease.

6.3 *The thalassaemic group*

Much of the findings in this group have been discussed in section 6.0.6 when comparing findings between the different red cell haemolytic disorders. However as I outlined in section 1.4 thalassaemic patients probably do have an increased thrombotic risk and so I will summarise my findings on group F in this section.

Levels of the anticoagulants PC, TPS, FPS and HCII were all reduced compared to controls but generally the reductions were not as significant as for the untransfused HbSS groups. Coagulation inhibitors have not been measured in a study on a paediatric population before though in one of the 2 published adult studies (Shirahata et al. 1992; Musumeci et al. 1987) 16 children had PC and TPS levels measured but the findings were analysed with the adult results and only adult controls were used which will have exaggerated the differences between the patient groups and controls for PC values (Shirahata et al. 1992). The consensus from these previous studies is that thalassaemics have low PC levels which my findings agree with. However the comparison between group F and controls for PC is complicated by the poor age-matching in my study in that the controls were significantly younger than the thalassaemic group. However this patient group had *lower* PC values than controls so it is likely that if these patients were age-matched with controls there would be an even greater difference in PC levels with a more significant *p* value.

There have been no previous studies measuring FPS values but the previous study on TPS agreed that levels were also reduced (Shirahata et al. 1992). As with SCD studies there is dispute in the scanty published literature as to whether AT levels are affected but the most recent study in adults (O'Driscoll et al.

1995) and my findings agree that there is no reduction in AT levels compared to controls but there is reduction in HCII.

The mechanism(s) for the reductions in anticoagulants found is no more apparent than it is for SCD, although it is unlikely that liver dysfunction contributes in any way as both the AST and albumin levels were no different to controls and so liver function appeared normal in these children. There is evidence of some red cell membrane lipid rearrangement and excess PS exposure (Helley et al. 1996; Borenstein et al. 1993) so it seems more likely that this is contributory. There is at present no evidence that this phospholipid exposure plays a role in the dramatic reductions in FXI found in my thalassaemic group and one other study (Caocci et al. 1978) but I would expect that this apparent FXI deficiency balances out to some extent the prothrombotic tendency conferred by the low levels of PC, FPS and HCII.

The results for the 2 markers of thrombin generation measured in this study were conflicting in this group in that the TAT complexes were actually lower than controls but the PF1+2 were higher so the evidence for increased thrombin generation and a chronic hypercoaguable state is not conclusive. There was also no indication that either APC resistance or antiphospholipid antibodies contribute towards thrombogenic potential in thalassaemics. The significantly different results for ACLIgG and TXT compared to controls were because these were actually *lower* than controls rather than higher, confirming that there is no evidence of either anticardiolipin antibodies or a lupus anticoagulant.

6.4 Conclusions

This study has provided conclusive evidence for an increased thrombotic risk in children with HbSS, demonstrated by significant reduction in the majority of the naturally occurring anticoagulants and evidence of increased thrombin generation in the steady state and the majority of these abnormalities are not reversed by a chronic transfusion regime. However I was not able to relate these findings to the most serious of the vascular occlusion syndromes in childhood SCD, namely cerebrovascular disease. I have shown that the spectrum of abnormalities is more limited in HbSC, which *may* either reflect or contribute towards the more benign course of HbSC disease. Thalassaemics also have reduced levels of natural anticoagulants, excepting AT, as did the few children tested with other haemolytic disorders, suggesting that red cell turnover may be important in the pathogenesis of the inhibitor deficiencies.

Future research into the pathogenesis of CVD and other thrombotic complications in children with SCD will need to concentrate on cellular mechanisms and the contribution from adhesion molecules in the vaso-occlusive process. The role of platelet activation using modern flow techniques has not been examined previously and whether platelet-endothelial and/or red cell-endothelial interactions are contributory in the development of vascular intimal hyperplasia has also not been established but warrants further study.

Appendices

Appendix 1

Papers and abstracts published from the work in this thesis

Liesner R, Mackie I, Cookson J, McDonald S, Chitolie A, Donohoe S, Evans J, Hann I, Machin S. (1998) Prothrombotic changes in children with sickle cell disease; relationship to cerebrovascular disease and transfusion.
British Journal of Haematology **103** 1037-1044.

Liesner R, Cookson J, Mackie I, Chitolie A, McDonald S, Machin S, Hann I. (1996) Prothrombotic changes in children with sickle cell disease and thalassaemia (abstract). Abstracts from the 26th Congress of the International Society of Haematology.

Liesner R, Mackie I, Cookson J, Chitolie A, McDonald S, Hann I, Machin S. (1996) Prothrombotic changes in children with sickle cell disease and other red cell disorders (abstract).
British Journal of Haematology **93** Suppl. 1.

Appendix 2

Assay buffers

Citrate phosphate buffer

0.05 M citrate, 0.1 M phosphate, pH 5.0

Citric acid	10.3 g/l	0.049M
Na_2HPO_4.	36.5 g/l	0.102M

Diethanolamine buffer

Diethanolamine	97 g/l	0.92M
$MgCl_2.6H_2O$	0.1 g/l	0.5mM
NaN_3	0.2 g/l	3mM

Adjust pH to 9.8 with HCl

Imidazole buffer

0.05M pH 7.3

Imidazole	3.4 g/l	0.05M
NaCl	5.84 g/l	0.1M

Owrens buffer

Na barbitone	5.875 g/l
NaCl	7.335 g/l

Adjust pH to 7.35 with HCl

Phosphate buffered saline (PBS)

0.01 M phosphate, 0.145 M saline, pH 7.2

$NaH_2PO_4.2H_2O$	0.39 g/l	0.0025M
$Na_2HPO_4.12H_2O$	2.69 g/l	0.0075M
NaCl	8.47 g/l	0.145M

PBS tween

0.01 M phosphate, 0.5 M saline, 0.1% (v/v) tween 20, pH 7.2

$NaH_2PO_4.2H_2O$	0.39 g/l	0.0025M
$Na_2HPO_4.12H_2O$	2.69 g/l	0.0075M
NaCl	29.22 g/l	0.5M
Tween 20	1ml/l	

Seegers titration mixture

NaCl 0.85%	167.5 mls
$CaCl_2$ 1.0%	33.0 mls
Imidazole buffer pH 7.3	333.0 mls
15% acacia (in Na Cl)	66.5 mls

Tris buffer

pH 7.9		
Tris	8.959 g/l	0.05M
Methylamine	8.102 g/l	0.12M
EDTA	3.36g/l	
Adjust pH to 7.9 with HCl		

Tris/EDTA/saline buffer

pH 8.4		
NaCl	10.2 g/l	0.175M
Tris	6.1 g/l	0.05M
Disodium EDTA	2.8 g/l	7.5mM
Adjust pH to 8.4 with HCl		

Tris/EDTA/saline/polybrene buffer

pH 8.2		
NaCl	8.76 g/l	0.15M
Tris	6.057 g/l	0.05M
Disodium EDTA	2.8 g/l	7.5mM
Polybrene	2 mg/l	
Adjust pH to 8.2 with HCl		

Tris Imidazole buffer

pH 8.4		
Tris	3.5 g/l	0.029M
Imidazole	1.98 g/l	0.029M
NaCl	12.8 g/l	0.22M
Adjust pH to 8.4 with HCl		

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