AN INVESTIGATION INTO THE
REGULATION OF HAEMOSTASIS IN
SICKLE CELL DISEASE
AND
β THALASSAEMIA

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B. App. Sci (M.L.S.)

2002

A thesis submitted to the
University of London
for the degree of
Master of Philosophy

University College London
United Kingdom
Abstract

Sickle cell disease (SCD) and β thalassaemia (β thal) are congenital blood disorders caused by abnormal or inadequate haemoglobin synthesis respectively. These pathological conditions are characterised by the production of abnormal red blood cells (RBCs) that have a shortened life-span as well as abnormal biological, biochemical and adhesive properties. Chronic RBC haemolysis and anaemia, as well as repetitive episodes of RBC sickling and vasocclusion in SCD, may result in delayed growth and development, susceptibility to infection and progressive organ failure. Blood coagulation properties also appear to be affected as some patients experience thrombotic complications, which can be associated with significant morbidity and/or mortality. I embarked on this study because a well controlled, comprehensive investigation of the haemostatic (blood clotting) system had not been undertaken in these patients. I examined aspects of haemostasis in SCD and β thal, paying particular attention to abnormalities that predispose individuals to a heightened risk of venous thrombosis while addressing issues that had complicated previously published work: my subjects were free of acute clinical complications; they were compared to control groups of similar ethnicity; their genotype and transfusion status was taken into account and liver function was assessed.

Haemostatic variables of interest were initially examined in healthy Black (n = 28) and Caucasian (n = 33) subjects with HbAA genotypes. Levels of each variable were compared and reference ranges were independently established within the two ethnic groups. Mild variation was noted for some variables; significant differences included higher prothrombin fragment 1+2 levels in Caucasians, but longer APTTs and higher thrombin:antithrombin complexes, prothrombin and soluble E-selectin levels in Blacks.

44 SCD (HbSC or HbSS) and 41 β thal (intermedia or major) patients were investigated and compared to the Black and Caucasian control groups respectively. Haemostasis was altered in SCD: thrombin generation was increased, plasma levels of clotting factors, physiological inhibitors and other proteins were reduced and fibrin deposition was increased. Certain haemostatic variables were more significantly altered in HbSS compared to HbSC, which may reflect greater preservation of hepatic function and less RBC damage in HbSC, however the extent of abnormal thrombin generation was similar in the two genotypes. Mild activation of the endothelium was evident in untransfused HbSS patients and was strongly associated with inflammation. Patients with β thal intermedia were untransfused and showed broadly similar abnormalities to
untransfused HbSS patients, including excessive thrombin generation and endothelial cell activation; abnormalities tended to be milder in β thal major (Thal Maj) patients, who were transfusion-dependent.

Altered plasma levels of coagulation system proteins in SCD and β thal represent a chronic, consumptive coagulopathy that is probably exacerbated by mildly impaired hepatic synthesis. There was no evidence of gross liver disease in this cohort, although some hepatic dysfunction could not be entirely excluded, particularly in patients with substantial iron overload. There was no relationship between the extent of thrombin generation and the degree of anaemia, HbS or HbF levels, nor was there evidence of other inherited or acquired pathology that was likely to contribute to hypercoagulability. β2GPI-independent antibodies to phospholipid were detected in some patients and attributed to prior infection or chronic RBC damage.

An important observation arising from this work was that relative reductions in plasma levels of physiological inhibitors of coagulation is not associated with the extent of thrombin generation, perhaps because clotting factors are concomitantly depleted. It is, perhaps, surprising that relatively few individuals experience thrombotic complications as hypercoagulability (i.e. increased thrombin generation and/or fibrin deposition), with or without endothelial cell activation is quite common amongst these patients, despite an absence of symptoms. It is likely that hypercoagulability is propagated by vascular damage that results from abnormal mechanical and biological interactions between the endothelium and sickled or thalassaemic RBCs; this may be exacerbated by their exposure of negatively charged phospholipids, which provide a procoagulant surface for the assembly of clotting factor complexes. However, while the coagulation system is clearly activated in these patients, an adjusted equilibrium appears to be maintained in the absence of further prothrombotic stimuli.

Excessive thrombin generation persists despite regular exchange transfusions in HbSS and β thal patients, however fibrin deposition is considerably decreased and activation of the endothelium is almost entirely abated in both disorders. This goes some way to explaining the dramatic reduction in recurrent thrombotic events afforded by long-term transfusion therapy in SCD and β thal.
Table of Contents

Abstract ....................................................................................................................................1
Table of Contents ....................................................................................................................3
List of Tables ...........................................................................................................................5
List of Figures .........................................................................................................................6
Abbreviations ..........................................................................................................................8
Acknowledgements ...............................................................................................................10
Chapter 1 Introduction .....................................................................................................11
  1.1 General introduction ..................................................................................................11
  1.2 Normal haemostasis ...............................................................................................12
      1.2.1 Platelet activation .......................................................................................13
      1.2.2 Blood coagulation ......................................................................................14
      1.2.3 Physiological inhibition of coagulation ....................................................20
      1.2.4 Fibrinolysis ..................................................................................................22
      1.2.5 The vasculature ..........................................................................................22
  1.3 Thrombosis and thrombophilia ................................................................................24
      1.3.1 Congenital thrombophilia ...........................................................................27
      1.3.2 Acquired risk factors for thrombosis .........................................................29
  1.4 Haemoglobin ..............................................................................................................31
  1.5 Sickle cell disease ......................................................................................................32
  1.6 ß thalassaemia ............................................................................................................39
  1.7 Red blood cells ..........................................................................................................43
      1.7.1 Normal red blood cell membrane ..............................................................43
      1.7.2 Red blood cells in sickle cell disease ........................................................44
      1.7.3 Red blood cells in ß thalassaemia .............................................................46
  1.8 Thrombophilia in sickle cell disease ........................................................................47
  1.9 Thrombophilia in ß thalassaemia .............................................................................51
  1.10 Aims of the thesis ....................................................................................................55
Chapter 2 Methods ..........................................................................................................57
  2.1 Blood samples: collection and processing ..............................................................57
  2.2 Clotting tests .............................................................................................................58
      2.2.1 Prothrombin time (PT) ...............................................................................58
      2.2.2 Activated Partial Thromboplastin Time (APTT) .......................................59
      2.2.3 Thrombin Clotting Time (TT) ....................................................................59
      2.2.4 Fibrinogen (Fg) ..........................................................................................59
      2.2.5 Factor II (FII) .............................................................................................60
      2.2.6 Activated FVII (FVIIa) ...............................................................................61
      2.2.7 Modified activated protein C resistance (APCR) .....................................61
      2.2.8 Dilute Russell’s Viper Venom Time (DRVVT) .......................................63
  2.3 Amidolytic substrate assays .......................................................................................64
      2.3.1 Antithrombin III (ATIII) .............................................................................64
      2.3.2 Heparin cofactor II (HCII) .........................................................................66
      2.3.3 Protein C (PC) .............................................................................................67
      2.3.4 Alpha-2-antiplasmin (α2AP) ....................................................................68
      2.3.5 Alpha-1-antitrypsin (α1AT) .......................................................................69
      2.3.6 Factor VII (FVII) .........................................................................................70
      2.3.7 Factor X (FX) ...............................................................................................71
      2.3.8 Plasminogen (Plg) .........................................................................................72
2.4 Enzyme-linked immunosorbent assays (ELISAs) .................................................. 73
2.4.1 Prothrombin fragment 1+2 (F1+2) ........................................................... 73
2.4.2 Thrombin:antithrombin complex (TAT) .................................................. 74
2.4.3 Free protein S (PSf) .................................................................................... 74
2.4.4 D-Dimer (D-D) ........................................................................................... 76
2.4.5 Cardiolipin antibodies (aCL) ..................................................................... 76
2.4.6 β2-glycoprotein I antibodies ...................................................................... 78
2.4.7 Soluble E-selectin (sE-s) ........................................................................... 79
2.4.8 Soluble thrombomodulin (sTM) ............................................................... 80

2.5 DNA analysis .............................................................................................................. 80

2.6 Haematological and biochemical analyses .............................................................. 83
2.6.1 Total Cholesterol (CHOL) and Triglyceride (TRIG) .............................. 84
2.6.2 Lactate dehydrogenase (LDH) ................................................................... 84
2.6.3 C-reactive protein (CRP) ............................................................................ 85

2.7 Data presentation and statistical analysis ............................................................... 85

Chapter 3 Haemostatic variables in healthy Caucasian and Black subjects.............. 86
3.1 Introduction .................................................................................................... 86
3.2 Methods ........................................................................................................... 87
3.3 Results ............................................................................................................. 88
3.4 Discussion ..................................................................................................... 102

Chapter 4 The haemostatic system in sickle cell disease .......................................... 104
4.1 Introduction ................................................................................................... 104
4.2 Methods ......................................................................................................... 106
4.3 Results ............................................................................................................ 106
4.4 Discussion ..................................................................................................... 143

Chapter 5 The haemostatic system in β thalassaemia .............................................. 156
5.1 Introduction ................................................................................................... 156
5.2 Methods ......................................................................................................... 158
5.3 Results ............................................................................................................ 159
5.4 Discussion ..................................................................................................... 188

Chapter 6 Summary & conclusions ............................................................................. 198

Bibliography ...................................................................................................................... 208

Appendices ......................................................................................................................... 228

Appendix I. Manufacturers and suppliers of equipment and reagents. ......................... 228
Appendix II. Reference ranges for haematological and biochemical variables. ................. 230
Appendix III. Haematological variables in clinical sub-groups. .................................... 231
Appendix IV. Thrombin generation markers and physiological inhibitor levels in patients with sickle cell disease ............................................................. 232
Appendix V. Sickle cell disease sub-groups: comparative analysis. ................................. 233
Appendix VI. Thrombin generation markers and physiological inhibitor levels in patients with β thalassaemia ................................................................. 234
Appendix VII. Comparison between (a) D-Dimer, (b) thrombin:antithrombin and (c) soluble E-selectin levels in sickle cell disease and β thalassaemia clinical groups ................................................................. 235

Publications pertaining to material presented in this thesis ............................................. 237
List of Tables

Table 1.1 Risk factors for venous thrombosis ...............................................................27
Table 1.2 The prevalence and relative risk associated with congenital risk factors for venous thromboembolic disease .................................................................29
Table 1.3 Pathological features of sickle cell disease....................................................37
Table 1.4 Pathological features of β thalassaemia .........................................................42
Table 3.1 Profiles of Caucasian and Black control groups ...........................................88
Table 3.2 Clotting screens in control groups ...............................................................89
Table 3.3 Clotting factors in control groups ...............................................................90
Table 3.4 Markers of coagulation activation in control groups ....................................91
Table 3.5 Coagulation inhibitors in control groups .......................................................94
Table 3.6 Screening tests for activated protein C resistance in control groups .....96
Table 3.7 Components of fibrinolysis in control groups .............................................97
Table 3.8 Screening tests for phospholipid-dependent antibodies in control groups 98
Table 3.9 Markers of endothelial cell activation in control groups ..............................99
Table 3.10 Reference ranges for haemostatic variables .................................................101
Table 4.1 Characteristics of patient groups (sickle cell disorders) ..............................108
Table 4.2 Clotting factors in sickle cell disorders ........................................................111
Table 4.3 Markers of thrombin generation and tissue factor-mediated activation in sickle cell disorders ............................................................................113
Table 4.4 Tests for phospholipid-dependent antibodies in sickle cell disorders ...117
Table 4.5 Physiological inhibitors of coagulation in sickle cell disorders ...............118
Table 4.6 Serum lipids in sickle cell disorders ............................................................122
Table 4.7 Components of fibrinolysis in sickle cell disorders ...................................123
Table 4.8 Markers of endothelial cell activation in sickle cell disorders .................126
Table 4.9 Evidence of acute phase reactivity in a subgroup of 'steady-state' HbSS patients ........................................................................................................127
Table 4.10 Biochemical tests of liver function, red blood cell parameters and ferritin levels in untransfused patients with sickle cell disease .............................................131
Table 4.11 Characteristics of regularly transfused HbSS patients ................................133
Table 4.12 Characteristics of patients in sickle crisis .................................................141
Table 5.1 Characteristics of control and patient groups (β thalassaemic disorders) 160
Table 5.2 Clotting factors in β thalassaemic disorders ...............................................162
Table 5.3 Biochemical tests of liver function, red blood cell parameters and ferritin levels in patients with β thalassaemia ............................................................167
Table 5.4 Markers of coagulation system activation in β thalassaemic disorders ....170
Table 5.5 Physiological inhibitors of coagulation in β thalassaemic disorders ......172
Table 5.6 Serum lipids in β thalassaemic disorders ....................................................176
Table 5.7 Components of fibrinolysis in β thalassaemic disorders .........................178
Table 5.8 Tests for phospholipid-dependent antibodies in β thalassaemic disorders 181
Table 5.9 Endothelial cell activation in β thalassaemia ..............................................182
List of Figures

Figure 1.1 A conceptual representation of haemostatic mechanisms ..........................17
Figure 1.2 Development of a deep vein thrombosis ......................................................26
Figure 1.3 Red blood cell morphology ...........................................................................36
Figure 1.4 The pathophysiology of vaso-occlusion in sickle cell disease .................37
Figure 1.5 Schematic representation of a normal red blood cell membrane in cross-section .............................................................................................................44
Figure 3.1 Activated partial thromboplastin times in control subjects ........................89
Figure 3.2 Factor II in control subjects ...........................................................................91
Figure 3.3 Prothrombin fragment 1+2 levels in control subjects .................................92
Figure 3.4 Thrombin:antithrombin complexes in control subjects ............................92
Figure 3.5 Activated FVII levels in control subjects .....................................................93
Figure 3.6 Alpha-1-antitrypsin levels in control subjects .............................................95
Figure 3.7 Free protein S levels in Caucasian control subjects ..................................95
Figure 3.8 Free protein S levels in Black control subjects ..........................................96
Figure 3.9 Activated protein C resistance screening tests in control subjects ..........97
Figure 3.10 Soluble E-selectin in control subjects .....................................................99
Figure 4.1 Fibrinogen levels in sickle cell disorders ...................................................111
Figure 4.2 Factor II levels in sickle cell disorders .....................................................112
Figure 4.3 Factor VII levels in sickle cell disorders ....................................................112
Figure 4.4 Factor X levels in sickle cell disorders .....................................................113
Figure 4.5 Prothrombin fragment 1+2 levels in sickle cell disorders ........................114
Figure 4.6 Thrombin:antithrombin levels in sickle cell disorders ............................115
Figure 4.7 The relationship between prothrombin fragment 1+2 and thrombin:antithrombin complex levels in patients with sickle cell disease ..........115
Figure 4.8 Activated factor FVII levels in sickle cell disorders ................................116
Figure 4.9 Antithrombin III levels in sickle cell disorders ........................................119
Figure 4.10 Heparin cofactor II levels in sickle cell disorders ...................................119
Figure 4.11 Protein C levels in sickle cell disorders ....................................................120
Figure 4.12 Free protein S levels in sickle cell disorders ...........................................121
Figure 4.13 Alpha-1-antitrypsin levels in sickle cell disorders ..................................121
Figure 4.14 Cholesterol levels in sickle cell disorders ...............................................122
Figure 4.15 D-Dimer levels in sickle cell disorders .....................................................124
Figure 4.16 Plasminogen levels in sickle cell disorders .............................................124
Figure 4.17 Alpha-2-antiplasmin levels in sickle cell disorders ................................125
Figure 4.18 Soluble E-selectin levels in sickle cell disorders .....................................126
Figure 4.19 The impact of regular exchange transfusions on soluble E-selectin levels in patients with sickle cell disease .........................................................135
Figure 4.20 The impact of regular exchange transfusions on total leucocyte counts in patients with sickle cell disease .................................................................136
Figure 4.21 The relationship between total leucocyte counts and soluble E-selectin levels in untransfused HbSS patients .................................................................136
Figure 4.22 The impact of regular exchange transfusions on thrombin:antithrombin levels in patients with sickle cell disease .........................................................137
Figure 4.23 The impact of regular exchange transfusions on heparin cofactor II levels in patients with sickle cell disease .................................................................137
Figure 4.24 The impact of regular exchange transfusions on protein C levels in patients with sickle cell disease .................................................................138
Figure 4.25 The impact of regular exchange transfusions on D-Dimer levels in patients with sickle cell disease .................................................................138
Figure 4.26  Thrombin:antithrombin levels in paired samples from transfused patients
with sickle cell disease..........................................................................................140

Figure 4.27  Altered levels of thrombin generation markers and physiological inhibitors
in 9 SCD patients during crisis episodes............................................................142

Figure 5.1  Fibrinogen levels in β thalassaemic disorders.........................................163
Figure 5.2  Factor II levels in β thalassaemic disorders.............................................163
Figure 5.3  Factor VII levels in β thalassaemic disorders...........................................164
Figure 5.4  Factor X levels in β thalassaemic disorders.............................................164
Figure 5.5  Prothrombin fragment 1+2 levels in β thalassaemic disorders..............170
Figure 5.6  Thrombin:antithrombin complex levels in β thalassaemic disorders......171
Figure 5.7  Antithrombin III levels in β thalassaemic disorders...............................174
Figure 5.8  Heparin cofactor II levels in β thalassaemic disorders............................174
Figure 5.9  Protein C levels in β thalassaemic disorders............................................175
Figure 5.10 Free protein S levels in β thalassaemic disorders...................................175
Figure 5.11 Alpha-1-antitrypsin levels in β thalassaemic disorders..........................176
Figure 5.12 Cholesterol levels in β thalassaemic disorders......................................177
Figure 5.13 D-Dimer levels in β thalassaemic disorders...........................................179
Figure 5.14 Plasminogen levels in β thalassaemic disorders.....................................180
Figure 5.15 Alpha-2-antiplasmin levels in β thalassaemic disorders.......................180
Figure 5.16 Soluble E-selectin levels in β thalassaemia..........................................182
Figure 5.17 Serial observations in a Thal Int patient commencing regular exchange
transfusions........................................................................................................186
Abbreviations

\(\alpha_1\) AT  Alpha-1-antitrypsin
\(\alpha_2\) AP  Alpha-2-antiplasmin
\(\alpha_2\) M  Alpha-2-macroglobulin
aCL  Cardiolipin antibodies
Alk Phos  Alkaline phosphatase
APAs  Phospholipid-dependent antibodies
APC  Activated protein C
APCR  Activated protein C resistance
APTT  Activated partial thromboplastin time
AST  Aspartate transaminase
ATIII  Antithrombin III
\(\beta_2\) GPI  \(\beta_2\) Glycoprotein I
\(\beta\) thal  \(\beta\) thalassaemia (intermedia and/or major)
CHOL  Cholesterol
CRP  C-reactive protein
D-D  D-Dimers
DNA  Deoxyribonucleic acid
DRVVT  Dilute Russell’s viper venom time
EDTA  Ethylenediamine tetra-acetic acid
ELISA  Enzyme-linked immunosorbent assay
F1+2  Prothrombin fragment 1+2
Fg  Fibrinogen
FPA  Fibrinopeptide A
FVIIa  Activated FVII
FVL  Factor V Leiden
Hb  Haemoglobin
HbS  Haemoglobin S
HbSC  Sickle cell-haemoglobin C disease
HbSS  Homozygous sickle cell disease
HbSS(tx)  Regularly transfused HbSS patients
HbSS(untx)  Untransfused HbSS patients
HCII  Heparin cofactor II
HCT  Haematocrit
HMWK  High molecular weight kininogen
HPLC  High performance liquid chromatography
IgG aCL  IgG specific cardiolipin antibodies
IgM aCL  IgM specific cardiolipin antibodies
ISCs  Irreversibly sickled cells
KK  Kallikrein
LA  Lupus anticoagulant
LDH  Lactate dehydrogenase
MCH  Mean cell haemoglobin
MCHC  Mean cell haemoglobin concentration
MCV  Mean cell volume
NS  Not significant (p > 0.05)
OC  Estrogen-based oral contraceptive
PAI  Plasminogen activator inhibitor
PBS  Phosphate buffered saline
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<tr>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>P-e</td>
<td>Phosphatidylethanolamine</td>
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<td>PGM</td>
<td>Prothrombin G20210A</td>
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<tr>
<td>PKK</td>
<td>Prekallikrein</td>
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<tr>
<td>PL</td>
<td>Anionic phospholipid</td>
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<td>Plg</td>
<td>Plasminogen</td>
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<td>pNA</td>
<td>p-nitroaniline</td>
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<td>PS</td>
<td>Protein S</td>
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<td>Retic</td>
<td>Reticulocyte</td>
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<td>RSC</td>
<td>Reversibly sickled cells</td>
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<tr>
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<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<td>β thalassaemia intermedia</td>
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<tr>
<td>Thal Maj</td>
<td>β thalassaemia major</td>
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<td>TM</td>
<td>Thrombomodulin</td>
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<td>Triglyceride</td>
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<td>UCLH</td>
<td>University College London Hospitals NHS Trust</td>
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<td>WBC</td>
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Acknowledgements

I greatly appreciate the following people’s support of this study: Dr. I.J. Mackie for supervision and constructive criticism; Professor S.J. Machin for providing the opportunity to undertake the project; Mr. A.S. Lawrie for technical support and helpful advice; Mr. G. Purdy for logistical assistance in performing several of the automated assays; Professor J.B. Porter for referring patients to take part in the investigation and a cohort of very amenable patients and volunteers.
CHAPTER 1

INTRODUCTION

1.1 General introduction

Sickle cell disease (SCD) and β thalassaemia (β thal) are congenital blood disorders caused by abnormal or inadequate haemoglobin (Hb) production respectively. Hb is the protein that facilitates oxygen and carbon dioxide transport between the lungs and tissues by red blood cells (RBCs). Pathological or inadequate Hb production gives rise to RBCs that display abnormal rheological and biochemical properties and SCD and β thal share several characteristics: both are the consequence of β globin chain abnormalities; both are characterised by chronic haemolysis, anaemia, progressive organ damage and susceptibility to infection and some patients are transfusion-dependent. Both disorders may be attended by thrombotic complications, whereby pathological blood clots (thromboses) develop, suggesting that the coagulation (blood clotting) system is abnormally activated in these patients.

140-175 infants with SCD and 10-25 with β thal are born in the United Kingdom each year (Hickman et al, 1999). It is estimated that 1-5 % of β thal patients experience thrombotic complications during their lifetime (Borgna-Pignatti et al, 1998a), however the prevalence of thrombosis has not been established in SCD: much of the supporting data for a heightened thrombotic risk in these patients is based on case reports, anecdotal observations and laboratory evidence of abnormal coagulation. Large vessel arterial disease (intimal hyperplasia with superimposed thrombosis) is the most important cause of cerebrovascular accidents (strokes) in SCD and thrombosis has been implicated in pulmonary infarction and hypertension (Francis, 1991; Fauroux et al, 1998). Thromboses in SCD and β thal often occur at unusual sites, are frequently recurrent and may be associated with significant morbidity or mortality. Long-term transfusion therapy reduces the likelihood of further thrombotic events and eliminates the recurrence of strokes in most patients, however it is desirable to identify patients most at risk of thrombotic complications with a view to prophylaxis.
Haemostatic abnormalities had been reported in association with SCD and β thal prior to my commencing this investigation. The interpretations of some studies were limited, however, because they were not well controlled, few variables were studied, little attention was paid to the patients' transfusion status or they employed methodologies that are now outdated. I aimed to verify and extend these findings by examining variables that provide information regarding thrombin generation and regulation, congenital and acquired risk factors for venous thrombosis, clotting factor synthesis, fibrinolysis and vascular damage, as well as assessing their relationship to haematological and biochemical variables and each other. I limited my investigation to patients who did not have overt clinical complications at the time of testing as some coagulation proteins are acute phase reactants and plasma levels may be altered as a consequence of inflammatory processes.

Before the factors potentially contributing to hypercoagulability in SCD and β thal can be appreciated, an understanding of (i) physiological haemostatic mechanisms, (ii) abnormalities predisposing to increased thrombin generation, fibrin formation and thrombosis and (iii) the consequences of abnormal or insufficient Hb production on the morphological and functional characteristics of RBCs is required.

1.2 Normal haemostasis

Healthy endothelium lines the internal aspect of blood vessel walls and is in direct contact with circulating cells and proteins, providing a metabolically active interface between blood and extravascular tissues. Blood is normally isolated from substances that promote clotting and products of endothelial cells actively maintain the haemostatic equilibrium in favour of anticoagulation (section 1.2.5). Haemostasis is the physiological response to a breach of the vasculature, whereby haemorrhage is arrested and vascular integrity and blood flow are subsequently restored. This process involves complex interactions between endothelial cells, cellular components of blood (RBCs, leucocytes and platelets) and plasma proteins: localised reflex vasoconstriction limits blood loss and exposure of the subendothelium at the site of the breach initiates local activation of platelets; these adhere to the wound site and each other, forming an unstable plug that is consolidated when successive activation of plasma clotting factor proteins culminate in the conversion of soluble fibrinogen (Fg) to insoluble fibrin. A stable blood clot is formed when fibrin strands create an interlocking meshwork through the platelets, as well as RBCs and leucocytes. Wound healing, clot dissolution,
restoration of vascular patency and blood flow are ultimately achieved by the combined actions of the fibrinolytic system and cellular products (Hutton, 1989).

Several mechanisms regulate the extent to which activation of the plasmatic coagulation system occurs: efficient activation of clotting factors require a negatively charged surface on which coagulation complexes are assembled, which is provided by sub-endothelial substances and the membrane of activated or damaged endothelial cells, local leucocytes and platelets, but not by healthy, non-activated cells, so that coagulation progresses efficiently in the region of vascular disturbance only; activation of several of the clotting factors is calcium-dependent; positive and negative feedback mechanisms influence the rate of various reactions; physiological inhibitors and inactivators target specific products of the coagulation process. Competent haemostasis requires a rapid pro-coagulant response as well as regulation and localisation of clot formation. Failure of pro-coagulant mechanisms to remain confined to sites of vascular injury may lead to systemic activation of coagulation or under some circumstances, culminate in thromboses that are associated with significant morbidity.

My study is primarily concerned with plasmatic coagulation. Discussion of the role of activated endothelial cells, leucocytes and platelets as well as the involvement of other systems (e.g. inflammation and complement activation) will therefore be limited, although these are inherently involved in haemostasis.

1.2.1 Platelet activation

Platelet activation and the formation of a primary platelet plug is crucial to haemostasis: activated platelets adhere to the damaged vessel wall, express anionic (i.e. negatively charged) phospholipids (PLs) on their membranes and release constituents that enhance their aggregation and influence the function of other cells that promote inflammation and tissue repair. Platelets do not adhere to the endothelium under physiological conditions because: (i) sufficient production of prostacyclin by intact endothelial cells reduces the availability of free calcium ions via increased intracellular adenyl cyclase and cyclic AMP levels; (ii) procoagulant molecules contained within platelets are not released until the platelet is activated by an external stimulus such as collagen or thrombin and (iii) integrin receptors that bind elements of the subendothelial matrix are not fully functional in quiescent cells (Miller, 1994; Triplett, 2000).

A breach of the endothelium exposes platelets to sub-endothelial collagen microfibrils and to von Willebrand factor, which is bound to the underlying connective
tissue matrix and secreted by damaged endothelial cells: GPIb:von Willebrand factor-mediated adhesion of the platelet to the sub-endothelium initiates platelet activation. The cell surface area of activated platelets is increased by the formation of pseudopodia and bulky surface protrusions, facilitating interaction between membrane glycoprotein receptors and the endothelium, adjacent platelets, plasma clotting factors and agents that modulate platelet function. Substances that promote and regulate platelet adhesion and aggregation are released from platelet granules and mobilisation of PLs, particularly phosphatidylserine (P-s), to the outer membrane of activated platelets provide a suitable surface for the assembly of clotting factor complexes (Mann, 1999). Activated platelets also express specific binding sites for such complexes (e.g. for FIXa:FVIIIa and FXa:FV a) (Tracey et al, 1985; Nesheim et al, 1988).

Platelet aggregation continues over the damaged area until the wound is sealed by a loosely bonded plug, while production of anticoagulant molecules by the surrounding, intact endothelial cells help restrict the haemostatic response to the site of injury. Successive activation of plasma clotting factors result in the formation of solid fibrin strands throughout the plug that stabilise the clot and trap additional blood cells, with eventual consolidation.

### 1.2.2 Blood coagulation

Activation of the plasmatic coagulation system triggers a series of proteolytic reactions that culminate in the production of insoluble fibrin (Hutton, 1989; Mann, 1999). Successive complexes are formed between an enzyme (an activated clotting factor protein), its substrate (an inactive clotting factor pro-enzyme) and a catalytic co-factor on the PL surfaces found in the subendothelium and on activated platelet and endothelial cell membranes. The reactions proceed inefficiently in the absence of PL and some are calcium dependent. A conceptual representation of the activation and regulatory mechanisms of haemostasis is presented in Figure 1.1. Clotting factors are identified by an assigned Roman numeral and ‘a’ distinguishes the active form of the protein (e.g. FVII/FVIIa).

Clotting factors are primarily synthesised in hepatocytes (liver cells), although small amounts of some factors are also produced in platelets, endothelial cells and the spleen. Most of the proteins circulate as inactive pro-enzymes; their catalytic region is exposed following proteolytic cleavage and subsequent release of one or more ‘activation peptide’ fragments. Factors IIa (thrombin), VIIa, IXa, Xa, XIa, XIIa and
kallikrein (KK) are serine proteases (the active site of their catalytic regions contain a serine residue). Factors II, VII, IX and X require post-translational carboxylation of specific glutamic acid (\textit{gla}) residues for enzymatic activity, a process that is vitamin K dependent. Substrate binding sites and \textit{gla} residues are found on the heavy chain of activated proteins: \textit{gla} residues enable calcium binding to the clotting factors, effecting a conformational change that in turn, facilitates binding to PL molecules. Inadequate functional protein synthesis (secondary to congenital abnormalities or impaired liver function), malabsorption of vitamin K or ingestion of vitamin K antagonistic drugs (e.g. warfarin), is reflected by reduced clotting factor activity in the circulation.

FVa and FVIIIa are co-factors that bind negatively charged surfaces via specific PL receptors. They accelerate the rate of enzymatic activity of the complex toward its relevant substrate by altering the spatial orientation of the molecules on the surface to which the complex is bound. Formation of an enzyme:co-factor complex increases the catalytic activity of the serine proteases $10^5 - 10^6$-fold (Mann, 1999).

Disruption of the vasculature exposes blood components to tissue factor (TF) in the subendothelial matrix; coagulation is initiated when FVII or FVIIa binds TF (Mann, 1999). TF is a cell surface glycoprotein whose transmembrane domain anchors the complex to the cell surface and a requirement for PL limits its co-factor activity to the site of vascular injury; TF has no inherent enzymatic activity and does not function in the absence of the PL that is expressed on activated platelets and endothelial cells and to a lesser extent on leucocytes (Bach \textit{et al}, 1981). TF remains isolated from blood components under normal conditions, but is abundantly available in the event of haemorrhage as cells in the subendothelium of blood vessels and organ capsules, as well as cells of epithelial surfaces and the nervous system express TF (Drake \textit{et al}, 1989). TF expression can also be induced in endothelial cells and monocytes by endotoxin, interleukin-1 and tumour necrosis factor \textit{in vitro} (Osterud \textit{et al}, 1995; Wada \textit{et al}, 1995), which affords an explanation as to why thrombotic complications are frequently encountered in association with sepsis and malignancy (Semeraro & Colucci, 1997).

TF catalyses the action of FVII or FVIIa on FX. A minute amount of FVIIa (approximately 1% of FVII) is present in the circulation under physiological conditions, however its activity is not efficiently expressed unless bound to TF, a property that protects it from inactivation by antithrombin III (ATIII) (Lawson \textit{et al}, 1992; Morrissey \textit{et al}, 1993; Mann, 1999). In addition, and uniquely among the clotting factors, the FVII zymogen expresses trace enzymatic activity (1-2% of that of FVIIa) (O'Brien, 1989).
These attributes of FVII/FVIIa provide a ‘priming’ mechanism that enables a rapid procoagulant response at sites of vascular perturbation or injury: albeit inefficiently, FXa generated by the proteolytic activity of TF:FVII/FVIIa on FX is capable of converting prothrombin to thrombin, which subsequently activates the co-factors FV and FVIII that in turn, form complexes with FXa and FIXa respectively. Formation of these complexes is critical, as FX activation by FIXa-FVIIIa is 50 times more efficient than by TF:FVIIa and the ‘prothrombinase’ complex (FXa:FVa:TF/PL:Ca^{2+}) dramatically accelerates the conversion of prothrombin to thrombin (Mann, 1999). FXa generation is rapidly augmented via reciprocal FXa-mediated activation of TF:FVII, TF:FVIIa-mediated activation of FIX and thrombin-mediated activation of FXI in the presence of endothelial cell-derived glycosaminoglycans (Osterud & Rapaport, 1977; Gailani & Broze, 1991; Mann, 1999).

FXa generation is moderated by tissue factor pathway inhibitor (TFPI), which is present on surrounding, intact vascular cells as well as in low concentrations in plasma: TFPI binds the FXa-TF:FVIIa complex with high specificity and limits FXa and FIXa production by this pathway so that FXa is subsequently generated via FIXa:FVIIIa only (Mann, 1999). Therefore, where the endothelium is only slightly perturbed, coagulation will be controlled by TFPI, but in the event of significant haemorrhage and sufficient TF exposure, this initial regulatory mechanism will be overridden and thrombin will augment its own generation by positive feedback mechanisms.
Figure 1.1  A conceptual representation of haemostatic mechanisms. Key: aqua: inactive precursors; blue: active enzymes; green: cofactors; purple: physiological inhibitors; pink: activation peptides/complexes/products. Abbreviations as per text.
Another series of reactions between the so called 'contact' factors provide an auxiliary coagulation mechanism. FXII undergoes limited proteolysis by cellular or complement proteases or plasmin, or after binding to negatively charged surfaces, generating FXII', an intermediate capable of the slow activation of prekallikrein (PKK) to kallikrein (KK). Reciprocal catalytic action between KK and FXII in the presence of high molecular weight kininogen (HMWK) enhances the generation of FXIIa. When FXII is fully activated, it converts FXI to FXIa, which acts on its substrate, FIX and FIXa activates FX. Further proteolysis of FXIIa produces a βXIIa fragment that retains activity when associated with PKK but is unable to cleave FXI, providing a means of limiting the reaction (Mackie & Bull, 1989). The concentration of the electronegative surface regulates generation and release of FXIIa and KK, which are both inactivated by C₁-esterase inhibitor (C₁-INH) (Mitropoulos, 1999). Generation of FXa by means of FXIIa, KK, FXIa and FIXa is considerably slower than direct activation by FVIIa, taking minutes rather than seconds because more factors are involved, proteolytic cleavage of factors XII and XI is slow and sufficient platelet PL may not be immediately available to accelerate the reactions (Mackie & Bull, 1989).

Irrespective of its mechanism of generation, the crucial effect of FXa is its activation of prothrombin to thrombin. Prothrombin consists of three principle regions: fragment 1 contains the gla residues required for calcium-mediated binding to PL; fragment 2 contains binding sites for a co-factor (FVa); the remainder is the thrombin forming region. Progressive cleavage steps by the 'prothrombinase' complex generates a series of derivatives (including meizothrombin, α-, β- and γ-thrombin) that are biologically active but do not all play a significant role in haemostatic mechanisms (DeLa Cadena et al, 1994). The release of thrombin into the circulation is accompanied by generation of prothrombin fragment 1+2 (F1+2), an activation peptide whose levels in plasma provide an objective means of quantifying in vivo activation of the coagulation system, however smaller fragments may not be detected by the monoclonal antibody employed in the ELISA (Pelzer et al, 1991; Mann, 1994b). The relatively short half-life of F1+2 in plasma (3 minutes) results from its susceptibility to further proteolysis by thrombin (Ofosu, 1995) while the complex formed between thrombin and its primary inhibitor, ATIII (i.e. the thrombin:antithrombin complex (TAT)) persists for longer (its half life is 90 minutes) and is therefore considered a more reliable indicator of ongoing thrombin generation.
The generation of thrombin is a key event in the haemostatic process as this auto-regulator is a powerful potentiator of mechanisms that either augment or down-regulate further thrombin generation via a series of positive and negative feedback mechanisms, several of which are mediated by endothelial cells (Mann, 1994a). Critical procoagulant functions of thrombin include activation of co-factors FV and FVIII, conversion of Fg to fibrin and activation of FXIII. Fg is a hetero-dimer consisting of paired polypeptide chains linked by disulphide bonds; thrombin’s enzymatic activity on Fg releases monomers that polymerise to form insoluble polymers that are susceptible to degradation by plasmin and remain haemostatically incompetent until cross-linked by FXIIIa. A large part of thrombin’s anticoagulant activity occurs via activation of PC and subsequent inhibition of FVa (section 1.2.3). Thrombin also influences the activity of elements involved in platelet and leucocyte activation, cellular migration and growth, fibrinolysis and tissue repair (Miller, 1994).

FXa production continues until sufficient thrombin binds the thrombomodulin (TM) that is constitutively expressed by endothelial cells: TM catalyses thrombin-mediated activation of protein C (PC), which in turn inactivates the 'prothrombinase' complex by inhibiting the activity of its co-factor FVa, so that thrombin’s enzymatic activity is self-regulating (section 1.2.3). Residual thrombin and activated clotting factors are inactivated by the physiological anticoagulants ATIII and heparin cofactor II (HCII), whose activities are potentiated by glycosaminoglycans on the surrounding, intact vascular wall. ATIII has a broad spectrum of target substrates while HCII specifically inhibits thrombin (section 1.2.3).

Reciprocal amplification mechanisms are inherent in the haemostatic response to vascular damage, enabling trace concentrations of proteins involved in the initial reactions to rapidly generate high concentrations of subsequent proteins, but it is obviously undesirable for this process to proceed unchecked, as blood clot formation would extend inappropriately. Several mechanisms help limit propagation of the clot: coagulation is stringently regulated by the requirement for a PL surface on which the assembly of clotting factor complexes are assembled; blood flow disperses coagulation enzymes and platelet-activating substances from the site of injury to be inactivated in the systemic circulation and the liver; negative feed-back mechanisms that involve direct proteolytic activity or activation of inhibitory systems limit the extent to which activated clotting factors are generated and physiological inhibitors are directed at specific proteases.
1.2.3 Physiological inhibition of coagulation

Tissue factor pathway inhibitor

Tissue factor-mediated coagulation is regulated by TFPI, whose three serial kunitz domains bind FVIIa and FXa as well as heparin (Girard et al, 1989). The majority of TFPI is bound to the endothelium via glycosaminoglycans, however small quantities can be found in association with plasma lipoproteins and lesser amounts in platelets or circulating freely in plasma (Novotony, 1994). TFPI initially forms a reversible complex with FXa, inhibiting its activity by binding its catalytic region before forming an inactive quaternary complex with TF:FVIIa. The action of the antithrombotic agent heparin is effected by instigating a rapid 2-10 fold increase in plasma TFPI levels (Sandset et al, 1988). Reduced plasma levels of TFPI are not risk factors for thrombosis, however congenital TFPI deficiency appears to be incompatible with life (Novotony et al, 1991).

Serine protease inhibitors (SERPINS)

Serine proteases are regulated by members of the family of serine protease inhibitors that includes ATIII, HCII, alpha-2-macroglobulin ($\alpha_2$M), alpha-2-antiplasmin ($\alpha_2$AP), alpha-1-antitrypsin ($\alpha_1$AT), plasminogen activator inhibitor (PAI) and C1-esterase inhibitor (C1-INH) (Hutton, 1989). Serine protease inhibitors inactivate the proteolytic capability of target enzymes by binding their active serine centre, forming an irreversible 1:1 complex that is subsequently eliminated by the liver, with the exception of $\alpha_2$M, where inhibition occurs via steric hindrance.

ATIII is the principal inhibitor of thrombin in vivo. The primary substrates of ATIII are thrombin and FXa, although it does have some inhibitory effect on all the clotting factor proteases including TF:FVIIa (Lawson et al, 1993). Inactivation of thrombin by ATIII is accelerated 1000-fold by heparin, while heparan sulphate, a glycosaminoglycan located on the surface of endothelial cells, potentiates the activity of ATIII in vivo (Rosenberg & Bauer, 1994).

The anticoagulant activity of HCII has no inhibitory effect on FXa and is specifically directed at thrombin. HCII is less responsive to heparin derivatives than is ATIII, but is more capable of inhibiting meizothrombin, the first intermediate formed during the conversion of prothrombin to $\alpha$-thrombin (Han & Tollefsen, 1997). The fact that HCII activity is accelerated by its co-factor (dermatan sulphate) in the presence of fibroblasts and vascular smooth muscle cells but not endothelial cells in vitro suggests a
role for HCII in the regulation of thrombin activity at extravascular sites (Tollefsen et al., 1983; Tollefsen, 1995). HCII also regulates fibroblast proliferation, monocyte chemotaxis, adhesion of neutrophils to endothelial cells, production of prostacyclin by endothelial cells and neurite outgrowth (Rossi et al., 1999).

\( \alpha_1 \text{AT} \) activity has little direct effect on thrombin, but accounts for 70% of FXIa and 50% of FXa neutralising activity in plasma (Hutton, 1989). C1-INH is the major inhibitor of \( \alpha_- \) and \( \beta \) FXIIa and neutralises some KK, FXIa and plasmin activity, although the activated form of the first component of the complement system is its primary target (Mackie & Bull, 1989).

Plasmin inhibition is principally achieved by \( \alpha_2 \text{AP} \), which also possesses weak activity against several of the contact factors (Hutton, 1989). PAI-1 and PAI-2 regulate other components of fibrinolysis; endothelial cells produce PAI-1 and a small amount is contained within platelets. PAI-1 inhibits tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).

\( \alpha_2 \text{M} \) has a broad spectrum of activity that includes thrombin, activated protein C, plasmin and KK. Its alternative mechanism of inhibition results in the retention of some amidolytic activity on the small peptide substrates that are employed in certain laboratory assays (Mackie et al., 1992). \( \alpha_2 \text{M} \) is an acute phase reactant and it is likely that its activity becomes more significant under conditions of stress or when thrombin and plasmin inhibition is compromised.

**The protein C system**

The key protein of this regulatory system is activated protein C (APC), a vitamin K-dependent serine protease that degrades the potent co-factor activity of PL-bound FVa and FVIIa, with protein S (PS) acting as a co-factor, thereby limiting the assembly of 'prothrombinase' and 'ten-ase' complexes. 60% of PS circulates in the plasma bound to one of the regulatory proteins of the complement system, C4b-binding protein (C4bp); only the remaining free PS (PSf) retains functional activity (Dahlback, 1991). C4bp is an acute phase protein, but while total C4bp levels increase during inflammatory processes, differential regulation of the alpha- and beta-chain isoforms of C4bp maintain stable plasma levels of PSf (Garcia de Frutos et al., 1994).

APC is a critical regulator of coagulation as thrombin generation is autoregulated by activating PC. This process is significantly accelerated when thrombin is bound to endothelial cell-bound TM, an endothelial cell surface glycoprotein with a high-affinity receptor for thrombin. Thrombomodulin forms a surface-bound, 1:1 complex with
thrombin, inhibiting its activity on platelets and potentiating the activation of PC 1000-fold, altering thrombin’s substrate specificity from Fg to PC, and consequently its function from a procoagulant protease to a potent anticoagulant (Esmon, 1989). In the presence of cytokines, activated neutrophils and macrophages, TM is enzymatically cleaved from the endothelial cell surface, releasing soluble fragments that lack the transmembrane and cytoplasmic domains of tissue TM and are ultimately excreted in urine (Boffa & Karmochkine, 1998; Nakano et al, 1998). Increased plasma levels of soluble thrombomodulin (sTM) provide a marker of endothelial cell injury and increased levels have been reported in pathological conditions associated with diffuse vascular damage (Wada et al, 1993; Nyberg et al, 1997; Reverter et al, 1997).

1.2.4 Fibrinolysis

The dissolution of a formed blood clot is primarily regulated by plasmin, which is generated from plasminogen (Pig) by tPA in the presence of fibrin, urokinase, neutrophil elastase or by contact factor dependent mechanisms (Hutton, 1989; Lijnen & Collen, 1995). Plasmin splits fibrin into progressively smaller fragments by proteolysing both fibrin and cross-linked fibrin to yield cleavage products that include D-Dimer (D-D), a plasmin-resistant degradation product that is generated exclusively from the degradation of cross-linked fibrin (i.e. not Fg). Plasma D-D levels reflect the extent of fibrin deposition that has occurred in vivo. Regulation of fibrinolytic activity may be effected by specific inhibitors directed at tPA in the case of plasminogen activator inhibitors, or at plasmin, primarily by α₂AP.

1.2.5 The vasculature

Endothelial cells produce substances that regulate vessel tone and growth, platelet and leucocyte interactions, and coagulation and fibrinolytic activity in response to various stimuli. Intact endothelial cells do not express surface molecules capable of initiating a procoagulant response and produce substances (i.e. prostacyclin and nitric oxide) that inhibit coagulation and potentiate each other’s activity (Pearson, 1993). Prostacyclin is derived from arachadonic acid following its liberation from membrane PLs on activated cells. Basal levels of prostacyclin are insufficient to influence platelet function but the hormone is rapidly upregulated by thrombin and ATP. Similar agonists augment the synthesis of nitric oxide, which also inhibits platelet adhesion to the subendothelial basement membrane but can be rapidly inactivated by free Hb. As
discussed earlier, thrombin bound to endothelial cell surface-expressed TM has a reduced affinity for Fg and accelerates PC-mediated inactivation of FVa and FVIIIa. Endothelial cell-associated glycosaminoglycans (heparan sulphate and dermatan sulphate) potentiate the activity of the thrombin inhibitors ATIII and HCII.

Activated or damaged endothelial cells promote clot formation at sites of vascular perturbation by facilitating platelet aggregation, exposing clotting factor binding sites and expressing negatively charged PL molecules, providing a suitable surface for the assembly of coagulation factor complexes. von Willebrand factor is stored by endothelial cells and its secretion is up-regulated by neurohumoural factors (Pearson, 1993). Endothelial cell surface-associated secretion of platelet-activating factor stimulates both platelets and leucocytes while platelet aggregation and binding to exposed collagen is mediated by von Willebrand factor, fibronectin, Fg and thrombospondin. Activated or damaged endothelial cells express TF, initiating coagulation in the presence of PL molecules by complexing FVII/FVIIa. Endothelial cells synthesise and express clotting factor V and possess binding sites for factors IX, IXa, Va and Fg, enabling the rapid acceleration of coagulation enzyme complex formation on the luminal surface (Mann, 1999). In turn, procoagulant mechanisms of the endothelium are regulated by other endothelial-cell derived substances; platelet-derived ADP and serotonin are degraded by secretion of ectonucleotidases; TF:FVIIa complex activity is inhibited by TFPI and tPA and PAI regulate fibrinolytic activity (Pearson, 1993).

Endothelial cells also express a variety of adhesion molecules (including ICAM-1, VCAM-1 and IL-6) in response to pathogenic or inflammatory stimuli, which facilitate endothelial cell-leucocyte interactions (Pearson, 1993). E-selectin is an inducible, cell-surface adhesion molecule, specifically derived from endothelial cells, which primarily mediates neutrophil (but also monocyte and memory T-cell) adhesion (Boehme et al, 2000). Its expression is highly regulated and degradation of cell-surface E-selectin takes place by endocytosis and proteolysis in lysosomes. Expression of E-selectin by cultured endothelial cells is up-regulated by a variety of inflammatory stimuli, including interleukin-1, tissue necrosis factor-alpha and lipopolysaccharide/endotoxin (Sengoelge et al, 1998). A soluble form (sE-s) is released into plasma following the proteolytic cleavage of the surface-expressed molecule (Nurden & Nurden, 1993); by contrast, sTM is not released until interaction between cytokine-activated endothelial cells and neutrophils has occurred (Boehme et al, 2000).
Soluble adhesion molecules in plasma provide a means of quantifying active vascular disease: sE-s is found in the plasma of healthy subjects and because expression of E-selectin is confined to endothelial cells, raised sE-s in plasma may be considered a specific indicator of endothelial cell activation; elevated levels are seen in patients with substantial inflammation of the endothelium and vascular disease (Newman \textit{et al}, 1993; Ghaisas \textit{et al}, 1997; Okajima \textit{et al}, 1997; Musolino \textit{et al}, 1998).

Disturbance of the finely balanced equilibrium that exists between procoagulant and regulatory mechanisms creates a tendency toward either a thrombotic or haemorrhagic diathesis. If further triggers are acquired, the imbalance may reach a critical threshold, culminating in thrombosis or haemorrhage. This thesis considers coagulation abnormalities that are associated with hypercoagulability and venous thrombosis.

1.3 Thrombosis and thrombophilia

The haemostatic equilibrium is normally balanced in favour of anticoagulation so that blood flow is unimpeded throughout the vasculature. However, a breakdown in the balance between thrombogenic factors and regulatory mechanisms may result in the inappropriate formation of a pathological blood clot, i.e. ‘thrombosis’. Thrombosis is the product of a multifactorial disease process that involves interaction between congenital abnormalities which confer a life-long increased risk of thrombosis and/or acquired pathological and lifestyle factors. It should be kept in mind that our current understanding of the elements involved in propagating thrombosis is incomplete, as no known risk factors can be identified in many afflicted patients. Thromboses develop on the inner aspect of the vessel wall, particularly at sites of vascular perturbation, relatively static blood flow or hypoxia. Thrombi may occur at any site within the circulation, i.e. in arteries, veins, capillaries or within the chambers of the heart. The haemodynamics of blood flow, the cellular and protein constituents of the blood and the condition of the vessel wall determine the site and extent of thrombosis formation.

The term ‘thrombophilia’ describes an increased potential to develop thrombosis in association with genetic and/or acquired abnormalities (The British Committee for Standards in Haematology, 1990). This ‘pre-thrombotic’ or ‘hypercoagulable’ state is identified when enhanced activation of the coagulation system can be objectively demonstrated. Thrombophilia is associated with a variety of abnormalities including enhanced activation of platelets or plasmatic clotting proteins, inadequate regulation by
physiological inhibitors, incompetent fibrinolysis, the acquisition of antibodies to PL and damage to the endothelium. While patients in whom the haemostatic equilibrium is altered in favour of clot formation are clearly at a heightened thrombotic risk, hypercoagulability does not inevitably lead to thrombosis and many such patients remain asymptomatic. Additional prothrombotic stimuli, e.g. trauma/surgery, infection, pregnancy or long periods of immobility often instigate thrombosis in hypercoagulable patients.

Thrombi that arise within the arterial or venous circulation display inherently different characteristics (Thomas, 1981; Hirsh et al, 1994). The vessel wall is invariably abnormal at sites of arterial thrombus formation, however arterial blood flow is relatively undisturbed; arterial thrombi therefore consist primarily of platelet aggregates and a little fibrin and tend to adhere to one side of the vessel wall, so that blood flow may continue beyond the periphery of the thrombus. Venous thrombi can materialise on patent vessel walls, although perturbation of the endothelium will promote thrombus formation by exposing TF and disrupt the haemodynamics of blood flow.

The majority of venous thrombi occur in the deep veins of the lower limbs due to the characteristics of blood flow required for adequate venous return; thrombi materialise at sites where local venous stasis/arrest, eddy formation or perturbation of the endothelium in venules or larger veins allows the accumulation of activated clotting factors (Figure 1.2). Critical levels of thrombin may be produced locally and if not readily inactivated, promote platelet aggregation and fibrin deposition. Coagulation can proceed to a greater extent in a region of stasis so these thrombi are rich in fibrin and RBCs and may extend to completely occlude the vessel. Rapid blood flow through the vessel is often sufficient to disband the initial platelet-fibrin deposit and disperse products of coagulation activation (i.e. FXa and thrombin) into the circulation to be rapidly inactivated by ATIII. Such asymptomatic thrombi remain localised and eventually undergo lysis, organisation or re-canalisation. Failure to resolve the developing thrombus may result in its propagation to larger veins, culminating in symptomatic venous obstruction or pulmonary embolism, which may be fatal. Venous thromboses at other sites are considerably less common, but include the mesenteric, sinus and cerebral veins.
Relative stasis in venous valve pockets allows localised thrombin generation. The arrow indicates the direction of blood flow.

Trace amounts of thrombin promote platelet aggregation and fibrin formation.

The platelet-fibrin nidus propagates in a region of stasis.

Blood flow is obstructed, resulting in retrograde extension of the thrombus and partial or complete occlusion of the vein.

**Figure 1.2** Development of a deep vein thrombosis.

Venous thromboembolic events occur in approximately 1 in 1000 individuals per year in the general population of so-called 'developed' countries but are considerably less common elsewhere (Rain, 1988; Adoh et al, 1992a; Nordstrom et al, 1992). Certain congenital, physiological and acquired conditions have been identified in association with the development of thromboses and are considered risk factors (Table 1.1).
Table 1.1 Risk factors for venous thrombosis.

### Congenital

<table>
<thead>
<tr>
<th>Congenital</th>
<th>Acquired</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III deficiency</td>
<td>Increasing age</td>
<td>Obesity</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>Prolonged immobilisation</td>
<td>Previous thrombosis</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>Trauma or major surgery</td>
<td></td>
</tr>
<tr>
<td>Factor V Leiden (FV R506Q)</td>
<td>Malignancy</td>
<td></td>
</tr>
<tr>
<td>Factor II 20210A</td>
<td>Pregnancy and puerperium</td>
<td></td>
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<tr>
<td>Dysfibrinogenaemia</td>
<td>Antiphospholipid syndrome</td>
<td></td>
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<tr>
<td>Abnormalities of fibrinolysis</td>
<td>Oral contraceptive use</td>
<td></td>
</tr>
<tr>
<td>Hyperhomocystinaemia</td>
<td>Hormonal replacement therapy</td>
<td></td>
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<tr>
<td>Deficiency of physiological coagulation inhibitors</td>
<td>Malignancy</td>
<td></td>
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<tr>
<td>Enhanced platelet activation</td>
<td>Myeloproliferative disorders</td>
<td></td>
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<tr>
<td>Inadequate fibrinolysis</td>
<td>Polycythaemia</td>
<td></td>
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<tr>
<td>Hyperlipidaemia</td>
<td>Deficiency of physiological coagulation inhibitors</td>
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<tr>
<td>Hyperviscosity</td>
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<tr>
<td>Vasculitis and endothelial cell activation/damage</td>
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<tr>
<td>Increased levels of clotting factors (e.g. Fg, FVIII)</td>
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#### 1.3.1 Congenital thrombophilia

The prevalence of congenital thrombophilia is three times greater than that of inherited haemorrhagic disorders, with an incidence of 1/7500 in the general population of 'developed' countries (Mannucci & Tripodi, 1988). The most commonly encountered genetic abnormalities produce: (i) an abnormal FV molecule that is resistant to inactivation by APC; (ii) a relative increase in plasma concentration of prothrombin, or (iii) quantitative or functional defects of coagulation inhibitors. The relative frequencies of these abnormalities in general and thrombophilic populations are compared in Table 1.2. Venous thromboembolic disease is a multicausal process and interaction between two or more co-existing congenital abnormalities often results in a relative risk that is greater than their additive effect (Rosendaal, 1999).

**Factor V Leiden**

Factor V Leiden (FVL) is produced by a single point mutation in the FV gene, where Arg506 is substituted by Gln (Bertina et al, 1994), with the subsequent loss of one of three APC-cleavage sites: the mutant FV therefore possesses normal procoagulant co-factor activity but is resistant to proteolytic cleavage by APC, giving rise to biological ‘activated protein C resistance’ (APCR) and a life-long increased risk of thrombosis (Dahlback, 1997a). FVL is the most common heritable thrombophilic
abnormality detected in general Caucasian populations, although its prevalence shows distinct geographical variability; it is most common in northern Europe and the Mediterranean and may be present in up to 60% of patients with venous thromboembolic disease. FVL is extremely rare in non-Caucasians as the mutation originated after the genetic divergence of Africans from non-Africans and Caucasians from Asians. Heterozygosity and homozygosity for FVL are associated with a 5-10-fold and a 50-100-fold life-long increased risk of thrombosis respectively (Dahlback, 1995).

**Prothrombin gene mutation**

A mutation in the prothrombin gene was described after I began work on this study. Prothrombin G20210A (PGM) confers hypercoagulability by giving rise to plasma prothrombin (FII) levels that tend to lie in the upper quartile of those found in the general population (Poort et al, 1996). In population-based control studies, the overall prevalence of PGM is approximately 2%, although there are significant geographical variations, with a higher frequency in southern compared to northern Europe and a very low prevalence in African populations (Rosendaal et al, 1998). Heterozygosity for this mutation is associated with a 3-fold increased risk of venous thrombosis (Zoller et al, 1999).

**Congenital abnormalities of physiological anticoagulants**

The importance of the anticoagulant properties of ATIII, PC and PS are illustrated by the high incidence of thromboembolic events in individuals with congenital deficiencies (Egeberg, 1965; Griffin et al, 1981; Comp et al, 1984). Quantitative deficiencies are more common than qualitative abnormalities. Their prevalence in general and thrombotic populations are shown in Table 1.2.

Congenital HCII deficiency has occasionally been reported in thrombotic patients (Sie et al, 1985; Tran et al, 1985; Levy et al, 1994), although the association is not convincing as it appears to be equally prevalent in healthy populations (Bertina et al, 1987).

Qualitative and quantitative Plg defects have been detected in 2-3% of young patients with thromboses, although family studies have failed to establish a definitive association with thrombosis (Sartori et al, 1994). Congenital dysfibrinogenaemia accounts for approximately 1% of venous thromboses in young adults, and arterial thrombosis may occur within the same kindred (Mannucci & Tripodi, 1988).
Table 1.2 The prevalence and relative risk associated with congenital risk factors for venous thromboembolic disease. Compiled from (Rosendaal, 1997; Alhenc-Gelas et al, 1999; Gaustadnes et al, 1999; Rosendaal, 1999). * clinically recessive PC deficiency, rarely associated with thrombosis.

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<thead>
<tr>
<th>Risk factor</th>
<th>Prevalence (heterozygous)</th>
<th>Relative risk for venous thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>General populations</td>
<td>Thrombophilic Populations</td>
</tr>
<tr>
<td>FV Leiden (R506Q)</td>
<td>3 - 7 %</td>
<td>20 - 60 %</td>
</tr>
<tr>
<td>Prothrombin 20210A</td>
<td>1 - 4 %</td>
<td>6 - 8 %</td>
</tr>
<tr>
<td>ATIII deficiency</td>
<td>0.02 %</td>
<td>1 - 4 %</td>
</tr>
<tr>
<td>PC deficiency</td>
<td>&lt;0.01%</td>
<td>5 %</td>
</tr>
<tr>
<td>PS deficiency</td>
<td>0.3 %*</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

1.3.2 Acquired risk factors for thrombosis

Acquired risk factors that predispose to thrombosis were outlined in Table 1.1 and may be encountered in any individual, however long periods of immobility, hyperviscosity, acquired deficiencies of coagulation inhibitors, impaired fibrinolytic activity, the acquisition of antibodies to PL and vascular damage may be particularly relevant to patients with SCD or β thal. Factors that increase the likelihood of arterial disease (culminating in myocardial infarction or stroke) include smoking, hypertension and hyperlipidaemia, however these do not appear to increase the risk of venous thrombosis.

Inadequate protein synthesis

The liver is the principal site of synthesis of the coagulation system proteins, including the physiological anticoagulants. Therefore, reductions in plasma levels of any of these proteins may occur secondarily to impaired protein synthesis when significant liver disease is present and concentrations are usually commensurate with the extent of hepatocellular damage. Low ATIII levels due to liver disease are not usually associated with thrombosis but the relationship between PC, PS and the occurrence of thrombotic events is less clear (Jespersen et al, 1999): when reductions in inhibitor levels are related to hepatic insufficiency, procoagulant proteins are often comparably reduced, so that the equilibrium between pro- and anticoagulant mechanisms is largely maintained. Accelerated utilisation of these proteins may be inadequately compensated by the liver
when consumption exceeds the maximal rate of synthesis (e.g. in severe liver disease or disseminated intravascular coagulation).

Phospholipid-dependent antibodies (APAs)

Phospholipid-dependent antibodies (APAs) are a heterogeneous group of pathological immunoglobulins that prolong PL-dependent clotting tests in vitro and are implicated in recurrent miscarriage, venous thrombosis and autoimmune disease (Bick & Baker, 1994; Petri, 1997; Roubey & Hoffman, 1997; Triplett, 1999). Target antigens for APAs include cardiolipin, or in the case of the lupus anticoagulant (LA), a variety of proteins that have an affinity for PLs, including β2-glycoprotein I (β2GPI), prothrombin and PC (Triplett, 1992; Boutiere et al, 1994).

β2GPI is an essential co-factor in the pathogeneity of cardiolipin antibodies (aCL): it inhibits the contact phase of blood coagulation and the 'prothrombinase' activity of dormant platelets in vivo, however damaged endothelial cells and activated platelets that express P-s provide an appropriate PL surface to which β2GPI is able to bind (Esmon et al, 1997; Thiagarajan & Shapiro, 1998). β2GPI-mediated binding of aCLs to endothelial cell PL disrupts normal cell function and promotes endothelial cell procoagulant activity via several candidate mechanisms: induced expression of adhesion molecules; inhibition of prostacyclin synthesis; interference in PC, PS and TM interactions and induction of TF synthesis on the laminar surface of endothelial cells; or interference with fibrinolytic processes (Triplett, 1992).

Transient, alloimmune aCLs may be detected during the convalescence period of an infectious illness, but are not β2GPI-dependent and are not considered pathological (Triplett, 1995). Autoimmune APAs tend to be persistent, IgG specific and are associated with a variety of pathological manifestations: arterial and venous thromboses, recurrent spontaneous abortion, obstetric complications, thrombocytopenia and neurological disorders. APAs are detected in 3-5% of the general population, however additional prothrombotic stimuli are usually required before thrombosis develops. Thromboses occur in approximately 30% of patients with APAs and approximately two thirds of these are venous (Gastineau et al, 1985; Lechner & Pabinger-Fasching, 1985). Elevated F1+2 and fibrinopeptide A (FPA) confirm hypercoagulability in patients with APAs (Ginsberg et al, 1993), which appear to have a causative role in thrombosis: a prospective study in healthy male physicians revealed higher aCL titres in those who went on to experience thromboses compared to a well matched control group (Ginsberg et al, 1992). The latter study ascertained a relative risk for deep vein thromboses similar
to that associated with resistance to APC. LA positivity is a greater risk factor for thrombosis than isolated aCL (Derksen et al, 1988).

Identification of pathological APAs require the demonstration of a PL-dependent antibody and/or elevated titres of \( \beta_2 \)GPI-dependent IgG specific antibodies to cardiolipin (IgG aCL) that persist for at least six weeks (Greaves et al, 2000); both abnormalities are present in approximately 60% of affected patients (Triplett, 1995).

1.4 Haemoglobin

Normal Hb has a tetrameric structure made up of two alpha-like (\( \alpha \) or \( \zeta \)) or beta-like (\( \beta, \epsilon, \gamma \) or \( \delta \)) globin chains (Serjeant, 1992a). Each chain is covalently linked to a haem molecule that consists of ferrous iron surrounded by a porphyrin ring. Oxygen molecules bind reversibly to each iron atom, with high affinity in the lungs and low affinity in tissues: weak binding of an oxygen molecule alters the structure of deoxygenated Hb so that subsequent oxygen molecules are bound with increasing affinity; 2,3-diphosphoglycerate and carbon dioxide promote the release of oxygen from oxygenated Hb in the tissues by preferentially binding Hb. The equilibrium between deoxygenated Hb and fully saturated oxyhaemoglobin is shifted toward deoxygenated Hb in acidic environments because of its higher affinity for protons (the Bohr effect).

The genetic ‘blue-print’ for Hb protein synthesis is contained within the double-stranded helical deoxyribonucleic acid (DNA) molecule of the RBC nucleus. DNA is composed of alternating phosphate and deoxyribose molecules that provide a backbone for the attachment of four major mononucleotide bases. Amino acid (and therefore protein) synthesis is determined by the sequence of these nucleotides, where a codon, consisting of three bases, represents the genetic information for synthesis of a single amino acid. Non-coding sequences (introns or intervening sequences) within the gene have a regulatory role but are not represented in the final protein product. When a termination, or ‘stop’ codon is reached, the completed protein chain is released into the cell cytoplasm.

Hbs vary throughout normal stages of human development: coordinated switching of Hb synthesis determines the site of erythropoiesis, the polypeptide chains produced and therefore the Hb synthesised. Embryonic Hbs are expressed during the first eight weeks of gestation, before a switch to foetal haemoglobin F (HbF) occurs. HbF (\( \alpha_2 \gamma_2 \)) comprises 85% of Hb from six weeks gestation to term and 5-10% HbA is synthesised from at least eight weeks. HbF persists until approximately three months of
age, after which time the principle adult haemoglobin, HbA (α2β2) predominates: HbA comprises 97% of normal adult Hb and is accompanied by < 3% HbA2 (α2δ2: a minor Hb produced in association with HbA) and < 1% HbF.

The 'haemoglobinopathies' are a family of congenital blood disorders caused by the production of structural Hb variants or by inadequate globin-chain synthesis. Abnormal Hb synthesis is most commonly the result of a single point mutation and the subsequent substitution of a single amino acid, although not all single-base substitutions result in clinically significant abnormalities. Single point mutations that affect the STOP codon of messenger RNA produce elongated, abnormal Hb chains and a thalassaemic syndrome. Frame-shift changes result from the deletion or insertion of nucleotides close to the end of the chain so that subsequent amino acids are read abnormally.

Sickle cell syndromes are characterised by the presence of an abnormal haemoglobin (haemoglobin S (HbS)). Thalassaemic disorders result from a globin-chain imbalance due to reduced or absent globin chain synthesis and are classified according to the globin chain affected (i.e. α or β thalassaemia).

1.5 Sickle cell disease

'Sickle cell disease' describes the pathological conditions attributable to the homozygous or compound heterozygous inheritance of HbS: heterozygous inheritance of HbS with normal HbA is a carrier state with no significant pathology and is specifically excluded from this definition (Serjeant, 1992a). HbS is produced following a single nucleotide substitution in codon 6 of the β globin gene whereby the normal codon for glutamine (GAG) is replaced by that for valine (GUG). Compound heterozygosity occurs when βS is inherited in conjunction with another abnormal Hb that co-polymerises with HbS (e.g. HbC, HbD Punjab or HbO Arab), so that two abnormal β globin chains are present and no normal HbA is synthesised. Other abnormal Hbs have no impact on the expression of HbS, so their phenotypes resemble that of HbAS. The substitution of lysine (AAG) for glutamine at the same site in the β chain as HbS produces haemoglobin C (HbC). Sickle cell-β thalassaemia (HbS/β thal) is another form of SCD that arises from co-inheritance of βS and a gene for β thal. Clinical manifestations depend on the amount of HbA produced by the β thal gene as the phenotypic expression of βS is ameliorated by higher concentrations of HbA: HbS/βthal mimics that of homozygous SCD (HbSS) because no HbA is produced,
whereas HbS/β^+ thal types I – III are associated with increasing amounts of HbA and less severe clinical disorders.

Abnormal Hbs are differentiated on the basis of their electrophoretic mobility, net electrical charge, ionic strength and retention properties \textit{in vitro}. The presence of HbS is confirmed in tests that exploit its reduced solubility under deoxygenated conditions. HbS/β^0 thal is differentiated by an elevated percentage of HbA2 in association with HbS. HbS and HbC are present in approximately equal amounts in sickle cell-haemoglobin C (HbSC) disease. HbF concentrations vary within genotypes.

\textbf{Pathophysiology and clinical features of homozygous sickle cell disease}

The classic feature of HbS is its tendency to undergo intracellular polymerisation under deoxygenated conditions, unlike normal HbA, which remains in solution. Slow or partial deoxygenation initially causes the formation of a single aggregate of deoxyHbS tetramers: the rate of assembly of these tetramers is dependent on the intracellular concentration of deoxyHbS, temperature, pH and cell age and may involve a time delay of approximately three minutes, which is usually sufficient for HbS-containing cells to traverse the low-oxygen environment of the microcirculation without forming deoxygenated Hb polymers (Serjeant, 1992a). When a critical nucleus of tetramers form, however, the reaction is thermodynamically shifted in favour of rapid polymer formation. Rapid deoxygenation produces multiple, independent polymers that do not immediately alter the shape of the cell, but extend and align in parallel to give rise to the classic ‘sickle’ shaped cell following prolonged deoxygenation (Figure 1.3). As well as distorting the cell, polymer formation adversely affects the cell membrane, causing oxidative damage, dehydration, destabilisation of PL asymmetry and altered adhesive properties (Figure 1.4). Rigid and adhesive ‘sickled’ cells may become trapped in the microvasculature, occluding the affected blood vessel and impeding the blood supply to the target tissue: the splenic, pulmonary and central nervous systems are particularly vulnerable. Repetitive de- and re-oxygenation cycles cause a progressive loss of membrane flexibility and the cells become increasingly more likely to undergo further sickling. Eventually, the cells lose the capacity to return to a normal shape even when adequately oxygenated; these irreversibly sickled cells (ISCs) are very dense, have deformed membranes and probably represent the final stage of the cell’s life as they are rapidly sequestered by the spleen (Francis & Johnson, 1991).
The net result of these abnormalities is a shortened RBC lifespan and intermittent episodes of vascular occlusion that cause acute and chronic tissue ischaemia and subsequent organ dysfunction (Table 1.3). SCD is characterised by a chronic haemolytic anaemia punctuated by occasional or frequent episodes of acute, debilitating illness known as 'crises', although many patients may remain virtually asymptomatic (in a so-called 'steady-state') for extended periods. Crises may be vaso-occlusive, aplastic (occurring secondarily to a viral infection), haemolytic or manifest as acute splenic sequestration. Vaso-occlusive, also called 'painful', episodes are the most common and while they are not usually accompanied by a fall in Hb, the debilitating, dull, intense throbbing pain may persist for extended periods; a patient enrolled in my study likened it to that felt on impact of a hammer blow, without relief. Precipitating events include exposure to cold, hypoxia, exertion and infection, however the trigger frequently remains elusive.

SCD is associated with a reduced life expectancy: even in countries with advanced medical support, the median age at death is 42 years in males and 48 years in females (Platt et al, 1994). Clinical expression is highly heterogeneous between patients and may vary within individuals over time. The general pathology is attributable to the abnormal molecular behaviour of mutant HbS, however the severity of the disease is influenced by the effect of genes that interact with $\beta^S$, as well as lifestyle factors: the HbS mutation is known to have arisen on three separate occasions in Africa as well as independently in the Middle East and the four distinct $\beta^S$ gene clusters (haplotypes) have some bearing on the phenotype; co-inheritance of alpha thalassaemia ameliorates the clinical course of SCD by reducing the globin chain imbalance within the cell and persistence of high levels of HbF is associated with milder clinical and haematological features (Serjeant, 1993; Serjeant, 1997).

Peripheral blood

The peripheral blood of 'steady-state' SCD patients reflects their haemolytic anaemia: RBC fragments, dense cells, moderate poikilocytosis, occasional ISCs, target cells, nucleated RBCs, a degree of hypochromia and a mild reticulocytosis may be observed and the relative proportion of each cell type vary according to clinical status (Serjeant, 1992c). A mild leucocytosis is common even in the absence of infection. Anaemia is usually more pronounced in HbSS (Hb 6-9 g/dL) than in HbSC patients and the presence of Howell-Jolly bodies signify compromised splenic function.
Alterations in the peripheral blood that occur during some crisis episodes reflect the pathophysiology involved: the red blood cell count (RCC) and haematocrit (HCT) may fall and the reticulocyte (retic) count increase during a haemolytic crisis, with a rapid increase in the number of sickled red blood cells (SRBCs); aplastic crises are characterised by a rapid reduction in all cell lines in the peripheral blood, which return to 'steady-state' levels when bone marrow activity is restored. Perhaps surprisingly, significant alterations in the peripheral blood cells are not evident during the majority of vaso-occlusive crises.

**Therapy**

Patients are encouraged to avoid conditions known to instigate or accelerate the sickling process, i.e. dehydration, cold, high altitude, infection, physical/emotional stress and long periods of inertia. Immunisation against common viruses and penicillin are administered to compensate for diminished splenic function. Dietary folate supplements are prescribed to expiate erythroid hyperplasia. In the event of an acute episode, supportive measures aimed at pain relief, infection control and restoration of body temperature, haematological and biochemical equilibrium are instituted as appropriate. Transfusion of red blood cell concentrates (RBCCs) may be required to reduce the percentage of circulating HbS or to rectify symptomatic anaemia.

The standard form of therapy instituted following a cerebrovascular accident is regular exchange transfusions of RBCC (section 4.3.1). Therapy is directed toward maintaining the HbS level below 30%, or even 20% in some patients and reduces the risk of recurrent stroke from 90 to 10% (Russell et al, 1984). Other indications for a hypertransfusion regime include frequent, painful crises that cause significant morbidity, or ulceration that is unresponsive to standard therapy. Many of the severe complications of SCD, including retardation of growth and development, delayed onset of puberty and pulmonary involvement (particularly the acute chest syndrome) can also be ameliorated by regular exchange transfusions.

Hydroxyurea, a myelosuppressive agent, has recently been shown to afford a marked clinical improvement by improving HbF levels and reducing absolute neutrophil counts (Charache et al, 1996). Gene-replacement therapy is a focus of research, but bone marrow transplantation is currently the only cure for SCD, which carries its own profound risks of fatal infection and graft rejection; this procedure is therefore undertaken only in carefully selected patients (Apperley, 1993).
(a) normal (HbAA)

(b) homozygous sickle cell disease

(c) sickled red blood cells viewed by scanning electron microscopy (× 7100).

(d) β thalassaemia intermedia

Figure 1.3 Red blood cell morphology. Micrographs (a), (b) and (d) reproduced from Department library. Micrograph (c) from (Barnhart et al., 1976).
Sickle haemoglobin (HbS) 

\[ \text{Deoxygenation} \]

- Instability
- Reduced solubility
- oxidant generation
- iron decompartmentalisation
- Hb/membrane interaction
- deoxyHbS polymerisation
- RBC dehydration
- abnormal microrheology
- phospholipid destabilisation
- phagocytosis
- endothelial adhesivity
- vasocclusion and haemolysis

**Figure 1.4** The pathophysiology of vaso-occlusion in sickle cell disease (Hebbel, 1991).

<table>
<thead>
<tr>
<th>Clinical manifestations primarily attributable to vaso-occlusion</th>
<th>Clinical manifestations primarily attributable to haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrovascular accident (stroke)</td>
<td>Chronic anaemia</td>
</tr>
<tr>
<td>Acute chest syndrome</td>
<td>Jaundice</td>
</tr>
<tr>
<td>Acute painful crisis</td>
<td>Aplastic crisis</td>
</tr>
<tr>
<td>Splenic sequestration / functional asplenia</td>
<td>Cholelithiasis</td>
</tr>
<tr>
<td>Hyposthenuria and enuresis</td>
<td>Delayed growth and maturation</td>
</tr>
<tr>
<td>Papillary necrosis</td>
<td>Congestive heart failure secondary to anaemia</td>
</tr>
<tr>
<td>Chronic nephropathy and renal failure</td>
<td></td>
</tr>
<tr>
<td>Priapism</td>
<td></td>
</tr>
<tr>
<td>Avascular necrosis of bone</td>
<td></td>
</tr>
<tr>
<td>Chronic pulmonary failure (sickle cell lung disease)</td>
<td></td>
</tr>
<tr>
<td>Proliferative retinopathy</td>
<td></td>
</tr>
<tr>
<td>Slow healing ulceration of lower limbs</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3** Pathological features of sickle cell disease.
Haemoglobin SC (HbSC) disease

HbC is the second most common Hb variant; consequently, HbSC disease is the most common form of SCD after HbSS (Serjeant, 1992b). HbSC RBCs contain approximately equal amounts of HbS and HbC (i.e. half the concentration of HbS than HbSS cells), so lower oxygen tensions are required to induce sickling: the spectrum of pathology is similar to that described in homozygous patients, however the frequency and severity of complications is usually less. HbSC disease is characterised by a later onset of symptoms and patients are usually less anaemic than those with HbSS, despite an commensurate degree of haemolysis; the oxygen affinity of the cells is only slightly decreased and well compensated by a mild reticulocytosis, so that Hb is maintained at 10–12 g/dL. The peripheral blood is characterised by many target cells, frequent dense cells and occasional ISCs. The RBC lifespan is modestly reduced (to 20–60 days compared to 120 days in normal cells), but is considerably longer than in HbSS (8–12 days). A relatively low plasma volume in HbSC disease helps moderate the effect of increased viscosity which may otherwise contribute to vaso-occlusive episodes.

Some patients remain virtually asymptomatic into adulthood, though severely affected patients resemble those with HbSS. HbSC patients are prone to acute pulmonary episodes: typically, pulmonary embolism from necrotic bone marrow following bone infarction gives rise to the acute chest syndrome. Splenomegaly is more frequently encountered in HbSC than HbSS because the infarctive splenic atrophy that occurs in older HbSS individuals does not usually manifest in HbSC disease. Episodes of acute splenic sequestration are usually associated with an identifiable precipitating event, often in association with hypoxia. Proliferative sickle retinopathy is more common in HbSC patients (Condon & Sergeant, 1980), but rates of growth and sexual development are usually normal. Infarctive episodes involving the heads of long bones and avascular necrosis of the femoral head is encountered in approximately 12% of HbSC patients, however early reports of this complication being more prevalent in HbSC than HbSS patients do not appear to be founded (Sebes, 1989).

Sickle cell trait

In sickle cell trait (HbAS), the percentage of HbA predominates that of HbS, distinguishing the carrier state from Hbs/β^thal (Serjeant, 1992d). RBCs contain 20–45% HbS and intracellular polymerisation does not occur at physiological oxygen tensions. RBC survival is normal in HbAS subjects and sickle cell trait is not usually
associated with significant pathology, however the complications described in SCD may be encountered under conditions of extreme hypoxia, acidosis, fever or dehydration. Renal abnormalities may be caused by sickling in the hypoxic, acidic and hypertonic conditions of the renal medulla and manifest as hypostenuria, haematuria, asymptomatic urinary tract infections or pyelonephritis in pregnancy. There are occasional case studies of thrombotic events in HbAS subjects in the literature, but these do not appear to be more common than in the general population and may reflect a selective bias due to their carrier status: superior sagittal sinus thrombosis, recurrent DVT as well as thromboses in association with pregnancy and estrogen-based oral contraceptive (OC) use have been reported (Feldenzer et al, 1987; Humphries & Wheby, 1992; Serjeant, 1992d).

1.6 β thalassaemia

β thalassaemia (β thal) is caused by disturbed control mechanisms of protein synthesis that affect the β globin chain and give rise to reduced or absent chain synthesis and a subsequent globin chain imbalance within the RBC. Single amino acid substitutions, duplications, deletions, inversions, faulty chromatid pairing and translocation abnormalities have been described as the molecular basis for many of the well characterised abnormalities (Modell & Berdoukas, 1984b; Serjeant, 1992a).

β thalassaemia (intermedia and major)

Reduced or absent β-globin chain production in β thal manifests as reduced or absent production of normal HbA. The lack of adequate β-globin chain synthesis creates a globin chain imbalance and early precipitation of unpaired α-chains within immature erythroblasts. These are subsequently catabolized by lysosomal enzymes of reticuloendothelial cells (in the bone marrow following extraction from retics or in the spleen, following removal from RBC circulating in the peripheral blood) so that the majority of cells are prematurely destroyed: the half-life of a β thal erythrocyte is reduced from 120 to approximately 17 days (Modell & Berdoukas, 1984a). Cells in which non-α-globin synthesis approximates 40-50% of α-globin synthesis survive this process, as is apparent in the typical RBCs of 'carrier' β thal trait subjects. Early polychromatic erythroblasts undergo maturation arrest when the rate of intracellular Hb synthesis is maximal, but prior to the accumulation of significant amounts of Hb. Even at this early stage in the life-cycle of the cell, cellular damage associated with abnormal protein
precipitates is evident: the membrane is visibly abnormal, excessively sensitive to oxidation and hyper-permeable to Na\textsuperscript{+}. RBCs appear in the circulation following a selective process in the bone marrow that favours HbF-containing cells. Therefore the majority of the Hb contained in circulating RBCs in β thal is HbF, although the concentration varies considerably between cells.

The principle feature of β thal is ineffective erythropoiesis, reflected by the abnormally rapid uptake and premature release of iron and accelerated turnover of haem pigments. The patient remains anaemic, despite gross extramedullary expansion of the bone marrow that when untreated, results in progressive distortion and fragility of the bones. The physiological response to anaemia in HbAA subjects is an increase in RBC 2,3-diphosphoglycerate, which reduces the affinity of HbA for oxygen and promotes the release of oxygen to the tissues. HbF however, has a high affinity for oxygen and responds poorly to alterations in concentrations of 2-3-DPG, so that oxygen delivery to the tissues remains poor, although production of erythropoietin continues and the bone marrow remains maximally stimulated. In contrast, HbS, which has a low affinity for oxygen, responds well to raised levels of 2-3 DPG and oxygen delivery to the tissues in 'steady-state' SCD patients at rest approximates normal.

The chronic anaemia in β thal persists, despite an increased blood volume that retards maturation and growth (which may in turn precipitate high output cardiac failure). Abnormal processing of iron causes its excessive gastro-intestinal absorption and favours movement of iron from the gut directly to tissue stores, resulting in toxic iron-overload. This is exacerbated by the preferential incorporation of the iron contained in phagocytosed α-chains into tissue stores; in contrast, iron in normal (and SCD) bone marrow, where Hb is not precipitated, is immediately available for incorporation into newly developing cells. Congestion of the reticulo-endothelial system by abnormal RBCs leads to hypersplenism, which in turn worsens the anaemia, further stimulating the bone marrow and exacerbating the existing pathology. The pathological impact of Thai Maj is outlined in Table 1.4.

The peripheral blood of β thal patients shows severe anisocytosis, poikilocytosis and hypochromia, with target cells, fragmented RBCs, microcytes, tear drop cells and nucleated RBCs (Figure 1.3). Splenectomised patients present with even more bizarre RBC morphology as grossly abnormal cells, nucleated RBCs and α-chain precipitates are no longer removed but remain in the circulation.
The terms β thalassaemia ‘intermedia’ or ‘major’ are designations that reflect the severity of clinical manifestations. A patient's phenotype is influenced by a variety of factors, including the causative genetic abnormality, hereditary persistence of foetal Hb and coincidental inheritance of one or more α-thalassaemia genes that lessen the degree of globin chain imbalance within the developing RBC and ameliorate the associated pathology. Symptoms in patients with β thal intermedia (Thai Int) vary widely; patients may be only mildly affected or suffer a chronic, debilitating illness with occasional requirements for transfusion. Patients with β thal major (Thai Maj) typically experience a severe clinical course and are transfusion-dependent: RBCC transfusion is undertaken to reduce or correct symptomatic anaemia and to diminish the erythropoietic stimulus to the bone marrow.

**Therapy**

Patients are prescribed penicillin and are immunised against common viruses to help compensate for the splenic dysfunction that makes them susceptible to infection. Folate is prescribed to satisfy the hyperactive bone marrow’s increased requirement for folic acid. Regularly transfused patients are prescribed an iron chelating agent to help reduce iron toxicity: desferrioxamine may be delivered subcutaneously, or intravenously in cases of severe iron overload. Patients with gross hypersplenism or a high transfusion requirement (indicating a large blood volume is being sequestered within the spleen) may be candidates for splenectomy, however each individual must be carefully assessed with respect to likely post-splenectomy transfusion requirements. The two most common post-splenectomy complications are infection and thrombosis; the latter has been attributed to the presence of platelet aggregates and the marked or persistent thrombocytosis sometimes observed during this period (Winichagoon *et al*, 1981; Moratelli *et al*, 1998).

**β thalassaemia trait**

The carrier state for β thal results from the inheritance of an abnormal β gene from one parent and normal HbA from the other, so that β chain synthesis is reduced by approximately 50% in each RBC. This imbalance gives rise to excess, unstable α-chains that are rapidly catabolized by proteases in erythrocyte precursors. Slower removal of unpaired α-chains in more mature cells forms precipitates that resemble those detected in β thal, however they are competently removed by the spleen in β thal trait and RBC survival is not significantly altered (Modell & Berdoukas, 1984a). Morphologically, the
RBCs are microcytic and hypochromic, however the mean cell haemoglobin (MCH), intracellular oncotic pressure and intracellular fluid volume are also reduced, so that the mean cell haemoglobin concentration (MCHC) is adequately maintained. Compensatory Hb synthesis, possibly due to extended survival of globin messenger RNA, causes a relative and absolute increase in HbA2 which identifies this condition.

Overt pathology is rare in β thal trait. A degree of ineffective erythropoiesis is evident in only a minority of patients, characterised by a mild anaemia and associated weakness/fatigue, however severe anaemia may be encountered in pregnancy.

<table>
<thead>
<tr>
<th>Affected organ / process</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>Cortical thinning, fine or coarse cystic space formation, 'hair on end' appearance of the skull caused by florid proliferation of the bone marrow and erosion of the cortex in extreme cases, disturbance of normal epiphyseal function by hyperactive bone marrow, pathological fractures. Extramedullary expansion of the bone marrow producing characteristic facies.</td>
</tr>
<tr>
<td>Blood</td>
<td>Gross plasma volume expansion secondary to shunting of blood to hypertrophied marrow may result in terminal high-output cardiac failure. A dilutional effect caused by bone marrow expansion worsens anaemia.</td>
</tr>
<tr>
<td>Gastro-intestinal iron absorption</td>
<td>Iron recovered from phagocytosed erythroblasts and α-chain inclusions is directed to tissue stores. Hepatic and reticulo-endothelial cells store iron more rapidly than it can be released. Remainder of high iron requirement (20-30 times normal) for incorporation into new RBCs is obtained by increased absorption from the gut.</td>
</tr>
<tr>
<td>Growth</td>
<td>Early (&lt; 10 years) growth failure secondary to grossly overactive bone marrow, hypotransfusion or compensatory bone marrow expansion caused by hypersplenism. Poor growth characterised by lack of muscular tissue, low exercise tolerance and weakness.</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Splenomegaly caused by reticulo-endothelial hyperplasia. Membrane rigidity and inclusions impede RBCs traversing splenic sinusoids. RBC inclusions are trapped between endothelial cells, retained and phagocytosed - the 'pitted' cell re-enters the circulation. Trapping of large numbers of abnormal cells causes the spleen to become engorged and enlarged. Similarly, lymphadenopathy affects lymph nodes, tonsils and adenoids.</td>
</tr>
<tr>
<td>Extramedullary erythropoiesis</td>
<td>Erythropoiesis in the liver, spleen, abdominal lymph nodes. Occasionally, paravertebral or mediastinal erythropoietic masses develop in untransfused patients.</td>
</tr>
<tr>
<td>Hepatic / renal</td>
<td>Increased urinary uric acid secretion, uric acid nephropathy, uric acid crystalluria (occasionally). Non-pathological enlargement of the kidneys. Hyperbilirubinaemia with development of pigment gallstones in Thai Int.</td>
</tr>
</tbody>
</table>

Table 1.4 Pathological features of β thalassaemia (Modell & Berdoukas, 1984c).
1.7 Red blood cells

1.7.1 Normal red blood cell membrane

The RBC membrane is made up of 50% protein, 40% lipids (60% PL: 30% cholesterol and other neutral lipids: 10% glycolipids) and 10% carbohydrate (Contreras & Lubenko, 1989). Its general organisation is typical of other biological membranes, consisting of a lipid bilayer with which a number of cytoskeletal and integral membrane proteins are associated (Figure 1.5). This bilayer is arranged asymmetrically so that 75-80% of the PLs containing choline (i.e. phosphatidylcholine (P-c) and sphingomyelin) are located in the outer monolayer, while the inner monolayer retains most of the aminophospholipid (i.e. 100% of P-s and 80% of the phosphatidylethanolamine (P-e)). Maintenance of this asymmetrical conformation is by active ATP-dependent PL translocating activity as well as the passive interaction of P-s with spectrin and band 4.1, although it is possible for PLs to migrate between the monolayers (Pradhan et al., 1991; Hebbel, 1991).

Specific features of the RBC membrane confer a biconcave disc shape to the cell and provide it with sufficient elasticity/flexibility to enable passage through narrow capillaries: it has an excessive surface area (approximately 150% relative to the cell volume), the arrangement of the membrane components maintain the interior of the membrane in a semi-fluid state and the membrane is extensively and tightly anchored to the cytoskeleton (a complex network of cytoplasmic protein filaments and tubules) (Contreras & Lubenko, 1989). Maintenance of RBC shape is primarily attributed to spectrin, a contractile protein located in the cytoskeleton (Figure 1.5).

Natural senescence is associated with a loss of membrane integrity and function, causing a loss of potassium, lipids and protein from the membrane, an increase in intracellular potassium and reduced activity of RBC enzymes (Luzzatto, 1989). Gradual dehydration increases cell density and osmotic fragility, so that the characteristic discoid shape and associated deformability is lost. The cells become more spherical, with abnormal distribution and function of membranous proteins and increased binding of IgG molecules. Destabilisation of the PL arrangement culminates in exteriorisation of P-s on the external surface of senescent cell membranes, enabling recognition by reticulo-endothelial macrophages that subsequently remove them from the circulation (Schroit et al., 1985; Connor & Pak, 1994).
1.7.2 Red blood cells in sickle cell disease

RBC containing HbS are strikingly heterogeneous compared to normal cells: they are morphologically abnormal and exhibit markedly disordered cation regulation, membrane iron deposition, rearrangement of membrane lipids and altered adhesive properties. Studies employing continuous density gradients have identified distinct subpopulations of low-density reties, normal density discocytes, dense discocytes and ISCs (Kaul et al, 1983). SRBC are inherently, though variably more adhesive than normal cells and the adhesive properties of each subpopulation increase with dehydration and in association with vasoocclusive episodes (Hebbel et al, 1989). Adhesive properties are inversely related to cell density, whereby younger, low density reties are more strongly adhesive than very dense discocytes (Gee & Platt, 1995). Mechanical interaction with circulating rigid dense SRBCs, which may form up to 50% of the circulating population (Kaul et al, 1983), impart a persistent, self-propagating insult to the vascular endothelium, as do repetitive episodes of SRBC adhesion and detachment. Raised levels of soluble adhesion molecules and activated endothelial cells in the plasma of SCD patients suggest that in vivo interactions between SRBC and the endothelium are abnormal (Duits et al, 1996; Solovey et al, 1997; Saleh et al, 1998).
Normal, intact RBCs have a limited effect on haemostasis because P-s is confined to the cytoplasmic surface of the membrane and is inaccessible to platelets and plasma coagulation proteins. In contrast, the outer membrane leaflet of deoxygenated, SRBC is enriched with P-s and P-e, as evidenced by enhanced availability to digestion by external phospholipases (Lubin et al, 1981). Deoxygenation does not alter the PL arrangement of normal cells, but deoxygenation-induced polymerisation of HbS destabilises the lipid bilayer of the SRBC membrane, accelerating the translayer mobility of P-c by approximately 50% (Mohandas et al, 1985): oxidative damage to the membrane, dissociation of lipids and proteins that accompany the sickling process and enhanced transbilayer movement of P-c culminate in simultaneous translocation of P-c from the external to the internal surface of the lipid bilayer and movement of P-s and P-e to the outer layer in a so-called 'flip-flop' mechanism (Franck et al, 1985). Sensitive flow cytometric analyses employing annexin V (AV), a calcium-dependent antibody with a high affinity and specificity for anionic PLs (Lubin et al, 1981; Tait & Gibson, 1994) can identify minute subpopulations of AV positive cells (that have lost PL asymmetry); these are 8-10-fold higher in SCD than in normal subjects (Kuypers et al, 1996). Normal RBCs possess approximately 275 AV binding-sites per cell, which increase to more than 850 on ageing, whereas SRBC possess approximately 12,400 sites per cell (Tait & Gibson, 1994). P-s exteriorisation is virtually a universal feature of SCD; increased AV binding has been reported in 96% of samples collected during 'steady-state' (Wood et al, 1996).

The exteriorisation of P-s on the membrane is critical because RBCs that express increased P-s are capable of accelerating blood clotting reactions by activating platelets and providing a suitable catalytic surface for the assembly of coagulation factor complexes, in a manner akin to the physiological response to localised vessel injury by activated platelets (Zwaal et al, 1989). P-s is unusual in this respect, as only P-s, in combination with appropriate amounts of P-c, P-e or sphingomyelin appears to significantly accelerate this process (Chiu et al, 1981). Exteriorisation of P-s is confined to the spicules of RSC and ISCs, suggesting that deoxygenation does not destabilise the PL arrangement if significant membrane deformation can be prevented; isolated spicules released from RSC following repeated sickling and re-oxygenation cycles markedly enhance the rate of thrombin formation in the presence of purified 'prothrombinase' and prothrombin, whereas remnant despiculated cells do not (Westerman et al, 1984; Franck et al, 1985; Choe et al, 1986). The procoagulant activity of RSC and ISCs is directly
attributable to the abnormal PL organisation as these results can be duplicated using liposomes with an identical PL composition to the outer lipid leaflet of reversibly sickled or ISCs in the absence of protein (Chiu et al, 1981; Helley et al, 1996). P-s is also capable of binding plasma PS (Lane et al, 1994). Exteriorisation of P-s also contributes to the pathophysiology of SCD by mediating SRBC adhesion to the endothelium, facilitating recognition of SRBCs by macrophages and therefore accelerating their destruction and activating the alternate complement pathway via C3b-binding (Schwartz et al, 1985; Wang et al, 1993; Chudwin et al, 1994; Setty et al, 1996).

Transmobility of P-c, subsequent exteriorisation of P-s and associated procoagulant activity are properties of deoxygenated, but reversibly sickled cells (RSC) as well as fully oxygenated or deoxygenated ISCs (Chiu et al, 1981; Schwartz et al, 1985; Blumenfeld et al, 1991). Notably, PL asymmetry is unaltered in normal and HbAS RBCs and oxygenated SRBC (Zachowski et al, 1985). PL destabilisation and its subsequent effects are reversible with reoxygenation of RSC but remain permanent in ISCs (Lubin et al, 1981; Blumenfeld et al, 1991).

1.7.3 Red blood cells in β thalassaemia

The pathophysiology of β thal is attributable to ineffective erythropoiesis by abnormal and highly heterogeneous thalassaemic RBCs (section 1.6). When my study was undertaken, P-s exteriorisation on thalassaemic RBCs had not been investigated extensively. P-s in the outer leaflet of the membrane was capable of accelerating in vitro clotting reactions (Borenstain-Ben et al, 1993) and was attributed to passive diffusion movements, rather than altered aminophospholipid translocase activity that was evident in murine studies (Muller et al, 1993). Aberrant P-s expression in β thalassaemic RBCs and its relationship to hypercoagulability has subsequently been confirmed and will be discussed further in Chapter 5.

Similarly, there was limited understanding of interactions between thalassaemic cells and the endothelium. In vitro studies demonstrated increased binding between RBCs from β thal patients that was not apparent with cells from normal or α-thalassaemic subjects (Butthep et al, 1992). Endothelial damage, particularly in patients who had not been splenectomised or who had leg ulcers, was indicated by immature platelets in the circulation and elevated levels of sTM (Butthep et al, 1995). Thalassaemic RBC do not adhere to intact endothelium (Smith & La Celle, 1987).
1.8  Thrombophilia in sickle cell disease

Thrombosis can be a devastating complication of SCD: arterial thromboses typically present as cerebrovascular accidents; venous thrombosis may manifest in the deep veins, as pulmonary emboli, or at other, more unusual sites. There is some evidence that thrombosis also contributes to other complications, including osteonecrosis (Glueck et al, 1997). Conversely, SCD is a recognised risk factor for thrombosis (Coull & Clark, 1993).

Arterial thrombosis

Thrombosis is clearly implicated in the evolution of cerebrovascular accidents in SCD (Thomas et al, 1982). Angiography has revealed endothelial hyperplasia, stenosis and occlusion in the cerebral arteries of 60-95% of patients (Rothman et al, 1986). Further, thromboses at sites of intimal hyperplasia in large cerebral vessels have been confirmed in post-mortem studies, where approximately 50% of patients had infarcts associated with organised and recanalised thrombi in the internal carotid arteries (Rothman et al, 1986). The irregular, damaged endothelial surface at sites of intimal hyperplasia are thrombogenic, analogous to the surface of a ruptured atherosclerotic plaque. Other case reports include thromboses within the brachial artery (Adoh et al, 1992b), internal carotid artery (Tuohy et al, 1997) and the middle cerebral arteries (Verlhac et al, 1995).

Venous thromboses

Thromboses of small or medium-sized pulmonary vessels are important contributors to the acute chest syndrome and have been implicated in both acute and chronic lung disease (Walker et al, 1979; Haupt et al, 1982; Thomas et al, 1982; Maggi & Nussbaum, 1987). Occlusion is precipitated by the adhesion of HbS-containing RBCs to endothelial cells in the pulmonary vasculature (Brittain et al, 1992). The incidence of pulmonary embolism in SCD increases with age (Haupt et al, 1982) and has been documented histologically as the cause of death in 8% of SCD subjects who died after the age of 10 years (Thomas et al, 1982), however another autopsy study found that the incidence of pulmonary emboli in adult SCD patients is similar to controls (Francis & Johnson, 1991).

There are case reports of massive venous thromboses occurring in unusual sites, including the portal and mesenteric veins (Arnold et al, 1993); cerebral venous sinuses (Oguz et al, 1994); the inferior vena cava (Berzine et al, 1979) and cerebral and renal
veins (Magid et al, 1987; Di Roio et al, 1997). Necrosis of the terminal ileum and complete bowel obstruction may be caused by microvascular thrombosis and congestion of SRBCs (Engelhardt et al, 1989). Reports of an increased incidence of thromboembolic complications associated with central venous access in SCD appear to be unfounded (Abdul-Rauf et al, 1995). Pregnancy carries its own thrombotic risk, however thromboses within the maternal sinuses have been reported in more than 50% of placentas from mothers with SCD, who appear to be particularly at risk during the post-partum period (Anionwu et al, 1981; Koshy et al, 1988).

**Hypercoagulability in sickle cell disease**

The association between SCD and venous thrombosis appears to be largely anecdotal or based on case reports in the literature, however the relationship is supported by laboratory-based evidence of hypercoagulability in SCD; coagulation appears to be abnormally activated and excessive thrombin generation is attended by an array of coagulation abnormalities involving platelet activation, procoagulant changes, altered plasma levels of coagulation inhibitors and increased fibrinolysis. These changes may be exacerbated during episodes of crisis, but are often present in 'steady-state'.

**Platelet activation**

Platelets are activated in SCD, irrespective of the functional status of the spleen. Constituents released by activated platelets are detectable in plasma and urine and increased levels of β-thromboglobulin, platelet factor 4 and platelet-specific thromboxane B2 derivatives indicate enhanced platelet activation and shortened survival (Billett et al, 1988; Adamides et al, 1990; Kurantsin-Mills et al, 1994). Flow-cytometric detection of activation-dependent platelet antigens and increased numbers of platelet microparticles in plasma relative to race-matched controls support these observations (Wun et al, 1998), however reduced thromboxane release from platelets in vitro suggest a degree of desensitisation and 'exhaustion' (Longenecker et al, 1992). Circulating platelet aggregates have been detected, particularly during vaso-occlusive crises (Mehta & Mehta, 1979) and alterations in platelet ADP levels and their response to agonists in vitro reflect an up-regulation of platelet activation at this time (Kenny et al, 1980; Westwick et al, 1983; Beurling-Harbury & Schade, 1989). It is probable, however, that this accompanies, rather than precipitates the crisis and the degree of exacerbation reflects the extent of local stasis and inflammation (Babiker et al, 1987). Platelet activation in HbSC subjects and children with SCD is less pronounced than in adult
HbSS patients, which may be partially attributable to better preserved splenic function in those groups (Buchanan & Holtkamp, 1983; Browne et al, 1996).

**Thrombin generation**

Elevated D-D levels in plasma testify to excessive fibrin formation and subsequent degradation of cross-linked fibrin. Elevated D-D levels have been reported in 'steady-state' SCD patients with further increases during vaso-occlusive episodes (Francis, 1989; Kurantsin-Mills et al, 1992; Hagger et al, 1995). One study concluded that elevated D-D in the absence of painful crises signified the presence of other complications, e.g. ulceration, chronic cholecystitis, aseptic necrosis or infection (Devine et al, 1986). Others, however, have reported elevated D-D in the absence of these complications, indicating that increased thrombin activity and fibrin deposition are features of SCD. Increased FPA levels in 'steady-state' SCD also indicate accelerated thrombin activity and fibrin formation (Green & Scott, 1986; Billett et al, 1988), although this may not be so pronounced when compared to ethnically matched controls (Hagger et al, 1995).

The recent availability of sensitive assays for coagulation activation peptides and complexes (F1+2 and TAT) has provided a more direct means of quantifying the degree of thrombin generation *in vivo*. Prior to my commencing this study, three studies measured these variables in SCD and reported excessive thrombin generation in homozygous adults and children (Kurantsin-Mills et al, 1992; Peters et al, 1994; Hagger et al, 1995).

**Clotting factors**

Fg, FVIII and vWF are acute phase reactants; plasma levels vary in SCD and may be elevated during crisis episodes (Famodu & Reid, 1987; Akinola et al, 1992; Hagger et al, 1995). Accelerated FVII turnover has been confirmed (Kurantsin-Mills et al, 1992). Examination of the contact factors in SCD has been limited, however a paediatric study reported reduced levels of FXII, PKK and HMWK activity as well as FXII antigen in steady state and a further decrease in crisis that was attributed to increased consumption (Gordon et al, 1985). Inadequate hepatic synthesis, accelerated consumption by up-regulated coagulation or both, may contribute to reduced levels of any of the clotting proteins.
Inflammation

Thrombosis may complicate severe inflammatory disorders and the interaction between inflammation, endothelial cell activation and thrombotic mechanisms are being elucidated (Nachman & Silverstein, 1993; Dosquet et al, 1995; DeGraba, 1997). A degree of inflammation is common in 'steady-state' SCD and accounts for the elevated levels of acute phase reactive proteins that are sometimes encountered: biochemical and rheological fluctuation occurs even in the absence of clinical symptoms and are consistent with minor episodes of microvascular occlusion (Akinola et al, 1992; Singhal et al, 1993). This is often insufficient to produce overt tissue dysfunction or painful crisis, but may generate a covert inflammatory response that is mediated by the up-regulation of inflammatory cytokines, including tumour necrosis factor and interleukins 1 and 6 (Malave et al, 1993; Taylor et al, 1995; Bourantas et al, 1998). These humoral mediators stimulate the production of a variety of proteins and elevated levels of these acute phase reactants denote a response that is relative to the degree of stimulation: many of these proteins are involved in haemostasis, e.g. Fg, FVIII, HCII, α1AT and α2AP. Levels of a rapidly responding acute-phase protein, C-reactive protein (CRP), correlate well with clinical severity in SCD and provide a useful means of monitoring acute phase reactivity (Monnet et al, 1993; Stuart et al, 1994).

Physiological inhibitors of coagulation

Prior to this investigation, studies of the plasma proteins that regulate thrombin generation were limited in SCD: the majority did not measure all of the inhibitors, some used methods that have subsequently been superseded, many of the investigations focused on children or co-investigated patients in 'steady-state' and crisis and transfusion status was not generally taken into account. Despite this, some trends were apparent.

PC and its co-factor PSf have been investigated most extensively. Reduced PC activity has been reported by a variety of assay methods, although levels are variable and frequently normal, with no significant alteration during crisis episodes (Green & Scott, 1986; el-Hazmi et al, 1993; Marfaing-Koka et al, 1993; Hagger et al, 1995). Studies in children concur, although levels may be reduced in crisis (Karayalcin & Lanzkowsky, 1989; Peters et al, 1994). A mild reduction in total PS and a more substantial reduction in free (PSf) has been reported in both adults and children (Francis, 1988a; Marfaing-Koka et al, 1993; Peters et al, 1994). Crossed immunoelectrophoresis reveals a marked reduction in PSf, with normal levels of PS bound to C4bp. Further, PS activity relative to the PSf antigen level is reduced. None of these studies identified a significant
alteration in PSf during crisis episodes and this has been confirmed in a larger, well controlled investigation that reported consistent PSf levels within individual patients, with lower levels encountered in those with more severe disease and frequent episodes of crisis (el-Hazmi et al, 1993). Another study of 30 adults observed a fall in PSf during crisis, but normal levels of both PSf and total PS in 'steady-state', compared to race-matched controls (Hagger et al, 1995).

Reports of ATIII levels in SCD are variable: one study reported low ATIII levels in 7/49 children using an immunogenic assay (Onyemelukwe & Jibril, 1992), but this has not been supported elsewhere. The majority of investigations have yielded normal ATIII levels with no significant alteration during episodes of vaso-occlusive crisis in adults or children (Babiker et al, 1987; Porter et al, 1993; Peters et al, 1994; Hagger et al, 1995; Nsiri et al, 1996).

Colleagues have previously reported conspicuously low HCII antigen and activity levels in SCD, which prompted my more extensive investigation (Porter et al, 1993). Increased HCII activity was detected during crisis, probably due to acute phase reactivity, as well as in patients using OC.

_Phospholipid-dependent antibodies_

The potential contribution of APAs to hypercoagulability in SCD was unclear when I undertook this investigation. In one article, weak IgG aCL were detected in 8% of unselected patients but were not related to clinical complications (De Ceulaer et al, 1992). A subsequent, smaller study found 65% of patients to be positive for IgG specific antibodies to a variety of PLs, including P-e and P-s, that are thought to be raised in response to membrane structural abnormalities (Kucuk et al, 1993).

1.9 _Thrombophilia in β thalassaemia_

Thromboses may occur in children or adults with β thal, at sites that are unusual in other thrombophilic populations and they are frequently recurrent. Their reported incidence is variable, which probably reflects the heterogeneity of clinical presentations as well as variable standards of medical support available world-wide. Up to 10% of Thal Int patients may experience thromboembolic complications and the incidence ranges between 1-4% in Thal Maj: the most recent and largest study, involving more than 1100 Thal Maj patients, reported thromboses in 1.1% over a 10-18 year period. (Michaeli et al, 1992; Borgna-Pignatti et al, 1998a; Moratelli et al, 1998).
The majority of thromboses develop within the venous system; sites include the pulmonary, portal, mesenteric and splenic veins, while deep vein thromboses may be localised within the upper or lower limbs (Girot et al, 1984; Borgna-Pignatti et al, 1998a). Arterial thromboses are less common but have occurred within the cardiac and renal arteries, causing infarction (Landing et al, 1989; Tamary et al, 1994). Pulmonary microthromboembolism with associated hypoxaemia is a major determinant of chronic lung disease, subsequent cardiac failure and cor pulmonale, which in turn, are major causes of death in adult patients (Sonakul & Fucharoen, 1992; Chuansumrit et al, 1993; Eldor et al, 1993).

Patients may be more vulnerable when exposed to secondary prothrombotic risk factors such as pregnancy or post-splenectomy thrombocytosis (van Teunenbroek et al, 1989; Bianconcini et al, 1993; Moratelli et al, 1998) but are especially at risk when transfusion induces a rapid, marked increase in HCT (Michaeli et al, 1992; Giordano et al, 1998a). Pathology that frequently accompanies β thal, including diabetes, complex cardiopulmonary abnormalities and hypothyroidism and liver function anomalies poses an additional risk (Borgna-Pignatti et al, 1998a).

That thrombotic disease is implicated in the clinical complications of β thal is even more striking at autopsy: post-mortem examinations frequently reveal resolved as well as recent pulmonary and renal infarcts, multiple platelet microthrombi, premature atherosclerotic change and arterial thrombosis (Landing et al, 1989; Sumiyoshi et al, 1992; Eldor et al, 1993). These findings are corroborated in patients with phenotypically similar HbE/β thal; organised pulmonary thrombi with a thickened arterial wall were found in 24/58 patients in one study, 12 of whom were splenectomised, and the incidence increased with age, although this is considerably lower in β thal (Sonakul & Fucharoen, 1992; Visudhiphan et al, 1994).

**Hypercoagulability in β thalassaemia**

Laboratory investigations of hypercoagulability in β thal have been relatively limited compared to those in SCD. Several of the studies co-investigated Thal Int and Thal Maj and did not necessarily consider transfusion status, however there is general evidence that coagulation is up-regulated in these patients.

**Platelet activation**

Post-splenectomy thrombocytosis poses a theoretical risk of thrombosis, although relatively few patients actually experience problems during this period. Platelet
activation is a common feature of $\beta$ thal, however, irrespective of splenic status: increased numbers of circulating platelet aggregates, increased in vitro aggregation, a reduced platelet life-span and enhanced excretion of urinary metabolites of thromboxane $A_2$ and prostacyclin by activated platelets denote platelet activation in vivo; (Winichagoon et al, 1981; Eldor et al, 1989; Eldor et al, 1991) and a relationship between platelet activation and dyslipidaemia in these individuals has been proposed (Del Principe et al, 1993). Platelet hyperactivity is also evident in children (Isarangkura et al, 1987; Schettini et al, 1987). Perpetual platelet activation may contribute to venous thromboembolic disease by enhancing thrombin generation and is implicated in cerebrovascular disease (Akopov et al, 1996), although chronic platelet ‘exhaustion’ may eventually manifest as a bleeding tendency (Triadou et al, 1990).

**Thrombin generation**

Surprisingly, there were no reports of the definitive markers of thrombin generation being measured in $\beta$ thal, despite the thromboembolic complications associated with this disorder. Mine is the first study to measure F1+2, TAT and D-D in both Thal Int and Thal Maj.

**Inflammation**

A low-grade up-regulation of many inflammatory cytokines occurs in $\beta$ thal in the absence of any significant clinical pathology. Abnormal levels of tumour necrosis factor-alpha, IL-1 beta and interferon-gamma are particularly evident and raised further following splenectomy, predictably (Meliconi et al, 1992; Lombardi et al, 1994; Chuncharunee et al, 1997; Salsaa & Zoumbos, 1997). Increased levels of these inflammatory cytokines have a negative effect on erythropoiesis and may contribute to the worsening anaemia observed during acute infections (Salsaa & Zoumbos, 1997), as well as contributing to hypercoagulability.

**Clotting factors and physiological inhibitors of coagulation**

Limited investigations have been made of clotting factors and the inhibitors of coagulation in $\beta$ thal. Mild alterations in basic screening tests reflect altered levels of clotting factors; vitamin K dependent factors II, VII, IX and X are most commonly affected (Caocci et al, 1978; Musumeci et al, 1987): there was clear biochemical evidence of liver disease in many of the 30 children involved in the second study, however a disproportionate reduction in FIX, FXII and PKK observed in Thal Maj (Caocci et al, 1978; Schettini et al, 1987) has been attributed to contact activation
following intravascular haemolysis and multiple blood transfusions. The only other study measuring factors II, VII and X in β thal showed no significant alteration in levels (Shirahata et al, 1992).

Examination of coagulation inhibitors reveal abnormal PC levels, variable PS levels and normal levels of ATIII in the majority of patients (Musumeci et al, 1987; Schettini et al, 1987; Chuansumrit et al, 1993; O'Driscoll et al, 1995). Functional PC and PS may be reduced in both children and adults, with PC levels being more abnormal than PS, particularly in older, splenectomised patients or those with chronic hepatitis (Musumeci et al, 1987; Schettini et al, 1987; Shirahata et al, 1992). In the second study (of 70 Thai Maj patients), PC deficiency was attributed to chronic hepatitis as PC levels correlated with albumin, the prothrombin time (PT), Fg and serum transaminase, but there was no association with thrombotic complications (Musumeci et al, 1987).

Mild reductions in ATIII have been reported in Thal Maj, again in association with splenectomy (Musumeci et al, 1987). Another report, which incorporated data from 30 adults and 18 children with HbE/β thal, Thal Maj and HbE disease found no alteration in ATIII levels (Shirahata et al, 1992). This was confirmed by colleagues at University College London, who also demonstrated low levels of HCII activity and antigen in Thal Int (O'Driscoll et al, 1995). HCII is only a secondary risk factor for thrombosis and levels appeared to be related to RBC turnover. Interestingly, HCII levels improved when transfusions were commenced.

The contribution of FVL, PGM and PL-dependent antibodies to hypercoagulability in β thal had not been investigated when I commenced my study.
1.10 Aims of the thesis

This investigation was initiated when colleagues reported HCII deficiency in SCD and β thal patients, which was attributed to intravascular RBC haemolysis and accelerated turnover and appeared to be ameliorated by transfusion in β thal, but not in SCD. I set out to verify and extend these findings in a well controlled study that examined this, as well as other determinants of plasmatic coagulation. The haemostatic system in adult SCD and β thal patients had not been methodically assessed when I commenced this investigation, despite a heightened risk of thrombosis being well accepted in connection with these disorders. There were limitations associated with some of the previous studies: most examined only a limited number of variables; several observations were made in children, did not employ ethnically-matched controls or involved small patient groups; patients with different genotypes were frequently co-investigated, despite phenotypic expression being broadly dissimilar; some earlier studies employed unreliable or outdated methodologies and no substantial investigations had been performed in HbSC patients. Many of the observations regarding SCD were made during crisis episodes and did not necessarily take the impact of therapeutic regimes, especially transfusion, into account.

The remit of my study was to examine aspects of haemostasis in SCD and β thal, with an emphasis on risk factors that may contribute to an increased risk of venous thrombosis. I selected asymptomatic adult HbSC, HbSS, Thal Int and Thal Maj patients for investigation and whereas several previous studies had focused on relatively few variables, I set out to assess an extensive array of haemostatic, haematological and biochemical variables and their relationships to each other. Clinical groups were compared to healthy HbAA subjects of similar ethnicity and I paid particular attention to the patients' genotype and transfusion status.

Variables were selected to assess:

- thrombin generation
- congenital mutations (FVL, PGM)
- clotting factor synthesis
- physiological inhibition of coagulation
- aspects of fibrinolysis
- phospholipid-dependent antibodies and
- abnormalities of the vasculature
I specifically aimed to address the following issues:

- Do levels of haemostatic variables differ between healthy HbAA subjects of Caucasian and Black ethnicity?

- How does the haemostatic system in SCD and β thal patients compare to that of ethnically similar control groups?

- Do haemostatic abnormalities vary according to genotype (i.e. HbSC vs. HbSS and Thal Int vs. Thal Maj)?

- Is altered haemostasis associated with haematological or biochemical abnormalities, haemolysis, hepatic insufficiency or organ dysfunction?

- What is the effect of regular exchange transfusion therapy on haemostatic variables?

- What is the effect of therapeutic interventions such as splenectomy and hydroxyurea on hypercoagulability?
CHAPTER 2

METHODS

Manufacturers and suppliers of the equipment and reagents utilised during this investigation are cited in Appendix I. Reagents were prepared as directed by the manufacturer. Chemicals were from Merck Eurolab Ltd. unless otherwise stated.

Quality control material was included in all assays and results were accepted when its potency fell within defined limits: these were supplied in commercial assay kits or alternatively, 'in-house' material was prepared using pooled plasma from more than 20 healthy subjects and target ranges were defined as the mean ± 2SD of assays performed on at least 20 separate occasions.

2.1 Blood samples: collection and processing

Sample collection

Blood samples were collected from adult patients attending University College London Hospitals NHS Trust (UCLH) as well as healthy adult volunteers. All samples were obtained during a single phlebotomy: patient samples were collected during phlebotomy for routine tests; samples from regularly transfused patients were collected immediately prior to transfusion.

Venous blood was withdrawn by clean venepuncture and with minimal stasis using a 21-gauge needle and an evacuated sample tube system (Vacutainer®: Becton Dickinson Ltd.): all tubes were made from siliconised glass and contained ethylenediamine tetra-acetic acid tripotassium salt (EDTA), 0.105M aqueous tri-sodium citrate dihydrate, 167 IU lithium heparin or no anticoagulant. Blood was collected into sodium citrate (ratio 9:1) for coagulation studies, into EDTA for full blood and retic counts, Hb variant identification and quantification and buffy coat preparation, into lithium heparin for biochemical analyses and into a non-anticoagulated tube for the preparation of serum.
Sample processing

Platelet poor plasma was prepared from citrated blood within an hour of sample collection: blood was centrifuged at 2000 × g for 15 minutes at ambient temperature; plasma was removed and subjected to a second, identical centrifugation. Samples collected into non-anticoagulated tubes were allowed to clot for a minimum of two hours at room temperature prior to centrifugation at 2000 × g for 15 minutes. Aliquots of platelet poor plasma and serum were stored at -70°C in polypropylene microtubes (Sarstedt Ltd.) and thawed to 37°C immediately prior to processing.

EDTA-anticoagulated blood was centrifuged at 2000 × g for 10 minutes at ambient temperature. Buffy coats were removed, stored in polypropylene microtubes (Sarstedt Ltd.) at -20°C and thawed at ambient temperature prior to processing.

2.2 Clotting tests

Clotting tests were performed using automated coagulometers: an ACL300R (Instrumentation Laboratory (UK) Ltd.) was used for activated factor VII and plasminogen assays and a CA-6000™ (Sysmex UK Ltd.) was used for the remaining tests. Reagents were from Dade Behring unless otherwise stated. Standards and quality control material were tested in duplicate; tests were highly reproducible, so test samples were assayed singly to conserve sample volume. Grossly abnormal results were verified on a second aliquot.

2.2.1 Prothrombin time (PT)

Reagent

- Innovin®: containing recombinant human tissue factor and calcium.

Method

- 50μl plasma was incubated at 37°C for three minutes.
- 100μl Innovin® was added and the time for clot formation to occur was measured.
2.2.2 Activated Partial Thromboplastin Time (APTT)

Reagents

- DiaCel-L APTT reagent (Diamed AG.): containing cephaloplastin and complexed kaolin.
- Calcium chloride (CaCl₂), 0.02 M.

Method

- 50μl plasma was incubated at 37°C for one minute.
- 50μl APTT reagent was added and incubated at 37°C for three minutes.
- 50μl CaCl₂ was added and the time for clot formation to occur was measured.

2.2.3 Thrombin Clotting Time (TT)

Reagent

Thromboclotin®: containing bovine thrombin (reconstituted to 5.0 NIH U/ml).

Method

- 100μl plasma was incubated at 37°C for two minutes.
- 50μl Thromboclotin® was added and the time for clot formation to occur was measured.

2.2.4 Fibrinogen (Fg)

Reagents

- Bovine thrombin, 90 NIH U/ml.
- Buffer: Owren's Veronal Buffer: pH 7.35.
- Immuno Coagulation Reference Plasma (Baxter AG).

Method

A standard curve ranging from 1 to 5 g/L Fg was generated using reference plasma.

- 10μl plasma was diluted in 90μl buffer and incubated at 37°C for three minutes.
- 50μl thrombin was added and the time for clot formation to occur was measured.

Fg concentrations were derived from a log/log standard curve where each dilution of the reference material was plotted against its respective clotting time. Samples with Fg concentrations outside the linear range of the standard curve were automatically re-tested at a higher or lower dilution as appropriate.
2.2.5 Factor II (FII)

Dilute plasma was mixed with a substrate plasma that was completely deficient in FII. A modified PT was then performed and the ability of the test plasma to correct the prolonged PT of the substrate plasma was compared to that of a standard of known potency. Pre-dilution of test plasma in FII deficient plasma normalised levels of clotting factors or inhibitors that may otherwise affect the test so that the clotting time was dependent on the FII activity of the test plasma.

Reagents

- Buffer: Owren’s Veronal Buffer: pH 7.35.
- FII deficient plasma (Instrumentation Laboratory (UK) Ltd.).
- Innovin®.
- Immuno Coagulation Reference Plasma (Baxter AG).

Method

A six point standard curve ranging from 0 to 1.50 IU/ml FII was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.

- 5μl plasma was diluted in 45μl buffer.
- 50μl FII deficient plasma was added and incubated at 37°C for three minutes.
- 100μl Innovin® was added and the time for clot formation to occur was measured.

FII levels were derived from a log-log standard curve where the FII concentration at each dilution of the reference material was plotted against its respective clotting time.

2.2.6 Activated FVII (FVIIa)

This assay was based on the method of Morrissey et al (1993) and utilised a mutant, truncated form of TF that lacks transmembrane and cytoplasmic domains: this soluble TF retains co-factor function for the FVIIa-catalysed activation of FX but is unable to promote autoactivation of FVII by FVIIa or to enhance the FXa-catalysed activation of FVII, rendering the assay insensitive to FVII levels. Test plasma was incubated with soluble TF and PL prior to initiating coagulation with CaCl₂. Pre-dilution of test plasma in FVII deficient plasma increased the specificity of the test.

Reagents

- Assay buffer (TBSA): 100mM NaCl, 50mM Tris, pH 7.4, containing 1% bovine serum albumin (BSA) fraction V, A-7030 (Sigma-Aldrich Company Ltd.).
- Soluble tissue factor (TF219): 47μg/ml tissue factor mutant-219 was a gift from Professor E Tuddenham (RPMS Hammersmith). Stored at −70°C.
● PL: Bell and Alton Platelet Substitute (Diagnostic Reagents Ltd.).
● Working reagent: PL was mixed with TBSA and TF-219 at a ratio of 5:9:1 and maintained on ice until required.
● FVII deficient plasma (Diagnostic Reagents Ltd.).
● CaCl₂: 25mM (Instrumentation Laboratory (UK) Ltd.).
● Reference preparation: 1.2 mg/vial recombinant Factor VIIa (Novo Nordisk A/S: prepared for therapeutic use, expired). Stock solution: 6ug/ml in TBSA, stored at -70°C.
● In-house quality control preparation: prepared by cold-activation of normal citrated plasma for 18 hours at 4°C.

**Method**

Plasma samples were maintained at ambient temperature prior to testing to avoid artificially raising FVIIa levels by cold activation. Immediately prior to assay, stock recombinant FVIIa was diluted in TBSA to yield a 10 ng/ml solution that was serially diluted 1/1.66 to yield a five point standard curve ranging from 1.3 to 10 ng/ml FVIIa.

- 5μl test plasma was diluted in 45μl FVII deficient plasma and incubated at 37°C for two minutes.
- 75μl of the ‘working reagent’ was added and incubated at 37°C for one minute.
- 50μl CaCl₂ was added and the time for clot formation to occur was measured.

FVIIa levels were derived from a log/linear standard curve where the FVIIa concentration at each dilution of the reference material was plotted against its respective clotting time.

**2.2.7 Modified activated protein C resistance (APCR) test**

The ratio of clotting times in the presence and absence of APC is calculated in this APTT-based test. APC markedly prolongs the clotting time of normal plasma by inactivating FVa and FVIIIa; resistance to inactivation of FVa by APC in patients with FVL results in a lesser prolongation of the clotting time and therefore a reduced ratio. Specificity of the test is increased by pre-diluting test samples in FV-depleted plasma as abnormal protein levels (particularly FVIII), may otherwise affect clotting times. OC use or the presence of a strong PL-dependent antibody occasionally yield spuriously low ratios.
Reagents
Tests were performed using a commercial kit (COATEST® APC™ Resistance - C) and chemically depleted FV-deficient plasma from Chromogenix AB. The APTT reagent was purified PL containing colloidal silica as a contact activator. Human APC was co-lyophilised with CaCl₂.

Method
Test plasma was diluted 1/5 in FV-deficient plasma prior to assay.

Test in the presence of APC
- 50μl diluted plasma was incubated at 37°C for one minute.
- 50μl warm APTT reagent was added and the mixture incubated at 37°C for three minutes.
- 50μl warm APC/CaCl₂ reagent was added and the time for clot formation to occur was measured.

Test in the absence of APC
- 50μl diluted plasma was incubated at 37°C for 60 s.
- 50μl warm APTT reagent was added and the mixture incubated at 37°C for three minutes.
- 50μl warm CaCl₂ solution was added and the time for clot formation to occur was measured.

The modified APCR test is a specific screening test for FVL and a study undertaken in our department established that abnormal samples are well differentiated using this method (Chitolie et al, 2001). Samples yielding low ratios were subject to confirmatory FV genotyping using a polymerase chain reaction technique (section 2.5).
2.2.8 Dilute Russell’s Viper Venom Time (DRVVT)

This test for the lupus anticoagulant incorporated a platelet neutralising procedure according to current guidelines (Greaves et al, 2000). Russell’s Viper venom directly activates FX in the presence of dilute PL and coagulation is initiated by addition of CaCl₂. The LA is capable of prolonging the clotting time in the presence of dilute PL, but its activity is reduced or neutralised by the high concentration of PL in lyophilised washed normal platelets. ‘False positive’ results may be obtained from plasma containing heparin, warfarin, or marked FII, FV, FX or Fg deficiencies. Tests were performed using a commercial kit (Lupus Anticoagulant Kit) from Unicorn Diagnostics Ltd. that contained Phospholipid Reagent (lyophilised phospholipid extract), Platelet Neutralising Reagent (lyophilised washed human platelets), lyophilised Russells’s Viper venom and CaCl₂.

Method

Detection procedure

- 40μl plasma was warmed for 24 s.
- 40μl Phospholipid Reagent was added and incubated at 37°C for one minute.
- 40μl Russell’s Viper venom was added and incubated at 37°C for 30 s.
- The clotting time was measured from the addition of 40μl CaCl₂ (0.025M).

Confirmation procedure

- 40μl plasma was warmed for 24 s.
- 40μl Platelet Neutralising Reagent was added and incubated at 37°C for one minute.
- 40μl Russell’s Viper Venom was added and incubated at 37°C for 30 s.
- The clotting time was measured from the addition of 40μl CaCl₂ (0.025M).

Interpretation

The presence of a PL-dependent antibody is indicated when the ratio of test to control plasma is greater than the upper limit of the reference range in the ‘detection procedure’, accompanied by a correction to normal or a reduction of at least 10% in the ‘confirmation procedure’ (Greaves et al, 2000).
2.3 Amidolytic substrate assays

Methods for the determination of ATIII, HCII, α₂AP and α₁AT levels were adapted for use on a Sysmex CA-6000™ automated coagulation analyser from microtitre plate techniques previously used in our department. Reaction conditions were optimised for each assay: the relative potency of test and control samples were derived from standard curves where the functional activity at each dilution of the reference material was plotted against a relative change in absorbance (ΔAabs) following the addition of a peptide substrate; linear colour development was verified at each concentration of the standard to ensure that substrate depletion did not occur. Accuracy of the results was verified using a range of commercial preparations with known potencies.

Standards and quality control material were tested in duplicate for all assays; tests were highly reproducible, so test samples were assayed singly to conserve sample volume. Grossly abnormal results were verified on a second aliquot.

Standardisation

Amidolytic substrate assays were standardised against a single batch of a commercial, lyophilised preparation obtained from a large pool of donors. A potency had been determined by the manufacturer for many of the variables assayed, but where these were unavailable, the material was assigned an arbitrary potency of 1.00 U/ml and checked for quality against a pool of plasma from 20 healthy subjects.

2.3.1 Antithrombin III (ATIII)

Plasma was diluted in a heparinised buffer and incubated with excess bovine thrombin, which was neutralised by (heparin-potentiated) ATIII activity in the sample. Residual thrombin activity cleaved p-nitroaniline (pNA) from a chromogenic substrate and the reaction was measured kinetically. The rate of change in absorbance was inversely proportional to the concentration of functional ATIII. The use of bovine thrombin rendered the assay insensitive to HCII activity while incorporation of methylamine into the buffer blocked inhibition by α₂M.
**Reagents**

- Unfractionated heparin: 1000U/ml (Monoparin®: CP Pharmaceuticals).
- Assay buffer: 175mM NaCl (NaCl), 50mM Tris (hydroxymethyl) methylamine, 7.5mM disodium EDTA and 0.15M methylamine, pH 8.4. 3 IU/ml heparin was added to the assay buffer before use.
- Bovine thrombin: 6 IU/ml (Unicom Diagnostics Ltd.).
- Unitrate™ THR thrombin substrate [2AcOH,H-D-CHG-Gly-Arg-pNA]: 1mM (Unicom Diagnostics Ltd.).
- Immuno Coagulation Reference Plasma (Baxter AG).

**Method**

A six point standard curve ranging from 0 to 1.50 IU/ml ATIII activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.

- 5μl plasma was diluted in 115μl assay buffer.
- 10μl dilute plasma was further diluted in 90μl assay buffer and incubated at 37°C for two minutes.
- 25μl thrombin was added and incubated at 37°C for one minute.
- 50μl warm thrombin substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

ATIII activity was derived from a linear standard curve.
2.3.2 Heparin cofactor II (HCII)

A mixture of dilute plasma and dermatan sulphate was incubated with excess human thrombin, which was neutralised by the (dermatan sulphate-potentiated) activity of HCII in the sample. Residual thrombin activity cleaved pNA from a chromogenic peptide substrate and the reaction was measured kinetically. The rate of change in absorbance was inversely proportional to the concentration of functional HCII. The use of polybrene® in the buffer renders the assay insensitive to ATIII by neutralising any heparin present and methylamine blocks the effect of plasma α2M.

Reagents

- Assay buffer: 0.05M Tris (hydroxymethyl) methylamine, 0.15M NaCl, 0.008M disodium EDTA, 0.002g polybrene®, pH 8.2.
- Dermatan sulphate: 0.3mg/ml (Unicom Diagnostics Ltd.).
- Human thrombin: 2.8 IU/ml (Unicom Diagnostics Ltd.).
- Unitrate™ THR thrombin substrate [2AcOH.H-D-CHG-Gly-Arg-pNA]: 1mM (Unicorn Diagnostics Ltd.).
- Immuno Coagulation Reference Plasma (Baxter AG).

Method

A six point standard curve ranging from 0 to 1.00 U/ml HCII. Samples were assayed at the same dilution as the 1.00 U/ml standard.

- 10μl plasma was diluted in 70μl assay buffer.
- 5μl of dilute plasma was further diluted in 45μl assay buffer and incubated at 37°C for 12 s.
- 50μl dermatan sulphate was added and incubated at 37°C for two minutes.
- 50μl human thrombin was added and incubated at 37°C for five minutes.
- 50μl warm thrombin substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

HCII activity was derived from a linear standard curve.
2.3.3 Protein C (PC)

In this assay, PC in dilute plasma was activated to APC, which cleaved pNA from a synthetic peptide substrate. The reaction was monitored kinetically and the rate of change in absorbance was proportional to the concentration of functional PC in the sample. The assay was also performed in the absence of activator to test for non-specific protease activity (see below).

**Reagents**

- PC levels were determined using a commercial kit (Coamatic® Protein C) from Chromogenix AB. The enzymatic activator was extracted from venom of the Southern Copperhead Snake and reconstituted to 0.27 U/ml.
- Immuno Coagulation Reference Plasma (Baxter AG).

**Method**

A six point standard curve ranging from 0 to 1.50 IU/ml PC activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.

**Protein C assay**

- 12μl of plasma was diluted in 48μl deionised water.
- 60μl activator was added and incubated at 37°C for five minutes.
- 60μl substrate S-2366 was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

**Test for non-specific protease activity**

- 12μl of plasma was diluted in 48μl deionised water.
- 60μl deionised water was added and incubated at 37°C for five minutes.
- 60μl substrate S-2366 was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

PC activity was derived from a linear standard curve.

*Non-specific protease activity*

Amidolytic substrate assays for PC are performed with relatively little dilution of the sample and the substrate is susceptible to cleavage by other proteases as well as α2M:protease complexes (Mackie *et al*, 1992). This may be a particular problem in patients with enhanced haemostatic activation and can give rise to inaccurate results.
Therefore, plasma from each control subject and patient was assessed for non-specific protease activity. Every sample yielded 0.00 IU/ml PC under these conditions, confirming the absence of non-specific protease activity in the plasma samples and validating the results obtained by chromogenic assays in this study.

2.3.4 Alpha-2-antiplasmin (α₂AP)

Excess plasmin was incubated with dilute plasma, forming plasmin:α₂AP complexes while plasmin inhibition by other protease inhibitors was limited by short incubation times. Methylamine was incorporated into the buffer to block α₂M activity. Residual free plasmin activity cleaved pNA from a chromogenic peptide substrate and the reaction was measured kinetically. The log rate of change in absorbance was inversely proportional to the concentration of α₂AP activity in the sample.

**Reagents**
- Assay buffer: 0.05M Tris-HCl, 0.1M NaCl, 0.15M methylamine, pH 7.4.
- Plasmin solvent: 50% glycerol (Sigma-Aldrich Company Ltd.) in 2mM HCl containing 5 g/l Carbowax 6000 (Sigma-Aldrich Company Ltd.).
- Human plasmin, 29 CU/ml (Unicom Diagnostics Ltd.). Reconstituted in plasmin solvent to yield a 0.25 CU/ml solution.
- Plasmin substrate: S2251™, 1mM (Chromogenix AB).
- Immuno Coagulation Reference Plasma (Baxter AG).

**Method**
A six point standard curve ranging from 0 to 1.50 IU/ml α₂AP activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.
- 25μl plasma was diluted in 75μl assay buffer.
- 30μl dilute plasma was further diluted in 30μl assay buffer and incubated at 37°C for three minutes.
- 50μl of plasmin was added and incubated at 37°C for 30 s.
- 40μl warm plasmin substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.
α₂AP activity was derived from a log/linear standard curve.
2.3.5 Alpha-1-antitrypsin ($\alpha_1$AT)

Excess trypsin was incubated with dilute plasma, forming trypsin:$\alpha_1$-AT complexes. Incorporation of methylamine in the buffer inhibited complexing of trypsin by $\alpha_2$M. Residual free trypsin cleaved pNA from a synthetic chromogenic peptide substrate and colour generated by the liberation of pNA following addition of the substrate was measured kinetically. The log rate of change in absorbance was inversely proportional to the concentration of $\alpha_1$AT activity in the sample.

Reagents

- Assay buffer: 0.05M Tris, 0.15M methylamine, pH 8.0.
- Porcine trypsin (Unicom Diagnostics Ltd.). Reconstituted in 10 ml of 1 mmol/L HCl then diluted 1/40 in 1 mmol/L HCl before use.
- Unitrate™ TRY trypsin substrate [Bz-Val-Lys-Arg-pNA] (Unicom Diagnostics Ltd.): 10μmol/vial reconstituted in sterile water to 1 mmol/L.
- Immuno Coagulation Reference Plasma (Baxter AG).

Method

Reference plasma and samples were diluted 1/10 in assay buffer prior to assay. A six point standard curve ranging from 0 to 1.50 U/ml $\alpha_1$AT activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 U/ml standard.

- 10μl dilute plasma was diluted in 60μl assay buffer.
- 5μl very dilute plasma was diluted further in 45μl assay buffer and incubated at 37°C for two minutes.
- 50μl trypsin was added and incubated at 37°C for five minutes.
- 50μl warm trypsin substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance ($\Delta$Abs/min) following substrate addition.

$\alpha_1$AT activity was derived from a log/linear standard curve.
2.3.6 Factor VII (FVII)

Dilute plasma was incubated with thromboplastin in the presence of calcium and FX, so that FVII in the sample was converted to FVIIa, which subsequently activated FX. FXa activity was then measured by cleavage of pNA from a chromogenic peptide substrate and the reaction was monitored kinetically. The rate of change in absorbance was directly proportional to the FVII activity of the sample. The complete conversion of FVII to FVIIa in the first stage avoids spuriously elevated FVII concentrations derived from plasma activated in vitro, which may occur in a one-stage clotting assay.

Reagents

- Buffer: Owren's Veronal Buffer: pH 7.35.
- CaCl₂: 25mM.
- Dilute Innovin®. Reconstituted as instructed by the manufacturer then diluted 1/5 in 25mM CaCl₂.
- Human FX [10U] (Sigma-Aldrich Company Ltd.). Reconstituted with 1.0 ml sterile water and diluted to 1.0 U/ml in buffer.
- FX substrate S2765™ [Z-D-Arg-Gly-Arg-pNA·2 HCl]: 25mg (Chromogenix AB). Reconstituted to 2mM in sterile water.
- Immuno Coagulation Reference Plasma (Baxter AG).

Method

A six point standard curve ranging from 0 to 1.50 IU/ml FVII activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.

- 10μl plasma was diluted in 90μl buffer.
- 10μl dilute plasma was further diluted in 30μl buffer.
- 40μl FX was added and incubated at 37°C for 3 minutes.
- 40μl dilute Innovin® was added and incubated at 37°C for one minute.
- 40μl warm substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

FVII levels were derived from a log/log standard curve.
2.3.7 Factor X (FX)

The reactions on which this assay is based occurred in two stages: FX was activated by Russell’s Viper venom in the presence of calcium; proteolytic cleavage of pNA from a chromogenic substrate by FXa was then monitored kinetically. The rate of change in absorbance was proportional to the FX activity in the sample.

Reagents
- Assay buffer: 0.05M Tris and 227mM NaCl, pH 8.3
- Russell’s Viper venom (RVV): 0.2 mg/ml (Diagnostic Reagents Ltd.).
- CaCl₂: 0.40M.
- RVV/CaCl₂: two volumes of 0.2mg/ml RVV were mixed with one volume of 0.40M CaCl₂.
- Factor Xa substrate S2765™ [Z-D-Arg-Gly-Arg-pNA·2 HCl]: 25mg (Chromogenix AB). Reconstituted to 2mM in sterile water.
- Immuno Coagulation Reference Plasma (Baxter AG).

Method
A six point standard curve ranging from 0.06 to 1.50 IU/ml FX activity was generated using reference plasma. Samples were assayed at the same dilution as the 1.00 IU/ml standard.
- 20µl plasma was diluted in 80µl assay buffer
- 12µl dilute plasma was further diluted in 48µl assay buffer and incubated at 37°C for three minutes.
- 15µl RVV/CaCl₂ was added and incubated at 37°C for two minutes.
- 75µl warm substrate was added.
- The reaction was monitored kinetically at 405nm for one minute and reported as the change in absorbance (ΔAbs/min) following substrate addition.

FX levels were derived from a linear standard curve.
2.3.8 Plasminogen (Plg)

Dilute plasma was incubated with excess streptokinase, which formed a complex with free Plg. On formation of the complex, the active serine site of Plg was exposed, cleaving pNA from a chromogenic peptide substrate. Colour generated by the liberation of pNA was inversely proportional to the concentration of functional Plg in the sample and was measured photometrically. The use of excess streptokinase resulted in all free Plg being converted to plasmin, rendering the assay insensitive to plasma protease inhibitors.

**Reagents**

- Assay buffer: 0.05M Tris and 0.1M sodium chloride, pH 7.4.
- Streptokinase (Unicom Diagnostics Ltd.): reconstituted in sterile water then diluted to 8000 IU/ml in normal saline.
- Plasminogen substrate: [2AcOH • HD-Ala-CHT-Lys-pNA] (Unicom Diagnostics Ltd, London UK), 10 μmol/vial. Reconstituted in sterile water then diluted to 1mM in assay buffer.
- Immuno Coagulation Reference Plasma (Baxter AG).

**Method**

Plg assays were performed using the research mode and chromogenic cycle of an ACL300R coagulometer. A six point standard curve ranging from 0 to 1.50 IU/ml Plg activity was prepared using reference plasma and plasma was diluted in assay buffer: samples were assayed at the same dilution as the 1.00 IU/ml standard. Loading, incubation and acquisition conditions were programmed:

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample volume</td>
<td>60 μl</td>
</tr>
<tr>
<td>Reagent Volume Position 2 (streptokinase)</td>
<td>30 μl</td>
</tr>
<tr>
<td>Reagent Volume Position 3 (substrate)</td>
<td>60 μl</td>
</tr>
<tr>
<td>Reaction Time</td>
<td>600 s</td>
</tr>
<tr>
<td>Inter Ramp Interval</td>
<td>5 s</td>
</tr>
<tr>
<td>Delay Time</td>
<td>0 s</td>
</tr>
<tr>
<td>Acquisition Time</td>
<td>300 s</td>
</tr>
<tr>
<td>Speed</td>
<td>1200 s</td>
</tr>
</tbody>
</table>

The change in absorbance following substrate addition was reported. Plg activity was derived from a linear standard curve.
2.4 Enzyme-linked immunosorbent assays (ELISAs)

*Standardisation and equipment*

Assays using kit-form ELISAs were standardised against reference preparations supplied by the manufacturer. The remaining assays were standardised against material with a known potency. All tests were performed in duplicate and accepted when the CV between duplicates was less than 10%. Substrate mixtures and stopping agents were added at timed intervals to ensure uniformity of colour development: substrate colour development was allowed to progress until optimum gradation of the standard curve was achieved. Relative potencies of test and control samples were derived from standard curves where the concentration of each dilution of the reference material was plotted against its respective absorbance (see specific methods). Grossly abnormal results were verified on a second aliquot.

Absorbances were measured using a scanning microtitre plate reader (Anthos 2001 Reader from Labtec International). Sample potencies were calculated using ANELISA™ software for Microsoft® Windows™ (Epsilon Technology Ltd.).

2.4.1 Prothrombin fragment 1+2 (F1+2)

F1+2 levels were determined using a commercial kit (Enzygnost® F1+2 micro) from Dade Behring, which utilised microtitre plates coated with rabbit antibodies against human F1+2 and peroxidase-conjugated rabbit anti-human prothrombin.

*Meth od*

- 50μl sample buffer was added to each well of the test plate.
- 50μl volumes of standard, control and test plasma were loaded into duplicate wells and incubated at 37°C for 30 minutes.
- The wells were washed three times in 250μl/well volumes of wash buffer.
- 100μl immunoconjugate was added to each well and incubated at 37°C for 15 minutes.
- The wells were washed three times in 250μl/well volumes of wash buffer.
- 100μl active substrate (o-phenylenediamine dihydrochloride/H₂O₂) was added to each well and incubated in the dark at ambient temperature for 15 minutes.
- 100μl sulphuric acid was added to each well to stop colour development.
- The optical density of each well was measured at 492nm.

F1+2 levels were derived from a linear interpolation curve fit applied to a log/log standard curve.
2.4.2 Thrombin:antithrombin complex (TAT)

TAT levels were determined using a commercial kit (Enzygnost® TAT micro) from Dade Behring, which utilised microtitre plates coated with rabbit antibodies directed against human thrombin and peroxidase-conjugated rabbit anti-human ATIII.

Method

- 50μl sample buffer was added to each well of the test plate.
- 50μl volumes standard, control and test plasma were loaded into duplicate wells and incubated at 37°C for 15 minutes.
- The plate was washed three times in 250μl/well volumes of wash buffer.
- 100μl immunoconjugate was added to each well and incubated at 37°C for 15 minutes.
- The plate was washed three times in 250μl/well volumes of wash buffer.
- 100μl active substrate (o-phenylenediamine dihydrochloride/H₂O₂) was added to each well and incubated in the dark at ambient temperature for 15 minutes.
- 100μl sulphuric acid was added to each well to stop colour development.
- The optical density of each well was measured at 492nm.

TAT levels were derived from a log/log standard curve.

2.4.3 Free protein S (PSf)

Free Protein S (PSf) antigen levels were determined according to the method of (Gardiner et al, 1998). This ELISA utilised two monoclonal antibodies that react with distinct epitopes of PSf and are devoid of reactivity with PS:C₄BP complexes: one binds the PS domain masked by C4bp binding, rendering the assay specific for PSf. This method obviates the need to separate C4bp-complexed PS and PSf before testing, avoiding the cumbersome sample preparation of the polyethylene glycol (PEG) precipitation technique, but correlates well with that assay.

Reagents

Reagents were from Sigma-Aldrich Company Ltd. unless otherwise stated.

- Phosphate buffered saline (PBS): tablets dissolved in deionised water to produce a buffer containing 0.01M phosphate, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4.
- Wash buffer: PBS containing 1ml Tween 20 (polyoxyethylene-sorbitan monolaurate), pH 7.4.
Sample (and immunoconjugate) buffer: 3% PEG 6000 dissolved in PBS containing 1ml Tween 20.

Monoclonal anti-human free Protein S 15C₄ (Kordia Laboratory Supplies).

Monoclonal anti-human free Protein S 34G₂ (Kordia Laboratory Supplies).

Immunoconjugate: horseradish peroxidase (HRP)-conjugated rabbit anti-human protein S (Dako Ltd.).

Substrate buffer: phosphate-citrate buffer with urea hydrogen peroxide: 0.05M phosphate-citrate buffer containing 0.014% H₂O₂, pH 5.0. One tablet dissolved in 100ml deionised water.

Substrate: o-phenylenediamine dihydrochloride. Two 15mg tablets dissolved in 24ml substrate buffer.

2M sulphuric acid (Merck Eurolab Ltd.).

Reference Plasma: 7ᵗʰ British Standard for coagulation factors (National Institute for Biological Standards and Control).

Method

A mixture containing 1/500 dilutions of both monoclonal antibodies (15C₄ and 34G₂) was prepared in PBS.

100µl volumes of the dilute primary antibodies were added to each well of a 96 well gamma-irradiated microtitre plate.

The plate was washed three times in 250µl/well volumes of wash buffer.

Serial dilutions of plasma were prepared in sample buffer: reference plasma was assayed at seven doubling dilutions from 1/100 to 1/6400. Test and control plasmas were assayed at three doubling dilutions from 1/200 to 1/800.

100µl volumes of reference, control and test plasma were loaded onto the plate, sealed and incubated at ambient temperature for two hours.

The plate was washed three times in 250µl/well volumes of wash buffer.

100µl of a 1/1000 dilution of immunoconjugate in sample buffer was added to each well, the plate sealed and incubated at ambient temperature for two hours.

The plate was washed three times in 250µl/well volumes of wash buffer.

100µl of fresh substrate was added to each well and incubated at ambient temperature.

100µl of sulphuric acid was added to each well to stop colour development.

The absorbance of each well was measured at 492nm.

PSf levels were derived from a log/linear standard curve.
2.4.4 D-Dimer (D-D)

D-D levels were determined using a commercial kit (Dimertest® Gold EIA Kit: Agen Biomedical Limited.) that utilised microtitre plates coated with a monoclonal antibody (DD-3B6) directed against D-D and utilised a peroxidase-conjugated mouse monoclonal antibody to fibrin degradation products.

Method

- The reference preparation was diluted to create an eight point standard curve ranging from 0 to 2000 ng/ml D-D.
- The microtitre plate was washed three times with 250μl/well volumes of assay buffer prior to assay to remove preservative (sodium azide).
- 100μl assay buffer was added to each well.
- 25μl volumes of plasma were added to duplicate wells, before the plate was sealed and incubated at ambient temperature for 15 minutes with continual mixing.
- The plate was washed three times with 250μl/well volumes of assay buffer.
- 50μl/well immunoconjugate was added to each well before the plate was sealed and incubated at ambient temperature for 15 minutes with continual mixing.
- The plate was washed three times with 250μl/well volumes of assay buffer.
- 100μl active substrate (2,2'-Azino-bis (3-ethylbenzthiazoline Sulfonic Acid)) was added to each well before the plate was sealed and incubated at ambient temperature for 15 minutes with continual mixing.
- 50μl Stopping Reagent was added to each well.
- The optical density of each well was measured at 405nm.

D-D levels were derived from a linear standard curve. The assay was not linear below 32 ng/ml; samples yielding D-D levels less than 32 ng/ml were therefore assigned a level of 16 ng/ml to facilitate statistical analysis.

2.4.5 Cardiolipin antibodies (aCL)

aCL were measured according to a modification of the method of Loizou et al (1985) by McNally et al (1995b). The assays were standardised using sera calibrated against appropriate International Reference Samples (Harris & Hughes, 1987). Titres were expressed as IgG or IgM specific PL-antibody (GPL and MPL) units respectively.

Reagents

Reagents were from Sigma-Aldrich Company Ltd. unless otherwise stated.
- Ethanol (Merck Eurolab Ltd.).
Cardiolipin from bovine heart (ethanol solution) diluted to 50µg/ml in ethanol.

PBS: tablets dissolved in deionised water to produce a buffer containing 0.01M phosphate, 0.0027M potassium chloride and 0.137M NaCl, pH 7.4.

10% ABS buffer: 10% (v/v) adult BSA in PBS.

Immunoconjugate: alkaline phosphatase-conjugated goat anti-human IgG or IgM diluted 1/1000 in 10% ABS buffer prior to use.

0.92M diethanolamine buffer, pH 9.8, containing 0.5mM magnesium chloride hexahydrate (Merck Eurolab Ltd.).

Substrate: 1 mg/ml p-nitrophenyl phosphate disodium hexahydrate phosphatase in diethanolamine buffer.

3M sodium hydroxide (Merck Eurolab Ltd.).

Reference sera for IgG aCL and IgM aCL (calibrated locally).

**Method**

- 30µl cardiolipin was added to four (test) rows of a gamma-irradiated microtitre plate (Nunc Polysorp: Nunc A/S). 30µl ethanol was added to the remaining four (blank) rows. The plate was incubated overnight at 4°C to allow evaporation of the ethanol.

- The plate was washed three times in 250µl/well volumes of PBS.

- 75µl of 10% ABS buffer was added to each well (blocking step).

- The plate was sealed and incubated at ambient temperature for one hour.

- The plate was washed once in 250µl/well volumes of PBS.

- A six point standard curve was prepared by diluting reference sera 1:50 to 1:1600 in 10% ABS buffer. Control and test sera were diluted 1:50 in 10% ABS buffer.

- 50µl volumes of dilute sera were added to duplicate test and blank wells, then sealed and incubated at ambient temperature for three hours.

- The plate was washed three times in 250µl/well volumes of PBS.

- 50µl volumes of IgG or IgM immunoconjugate were added to each well and incubated at ambient temperature for 90 minutes.

- The plate was washed three times in 250µl/well volumes of PBS.

- 50µl/well active substrate was added and incubated in the dark at 37°C to allow colour development.

- 50µl/well sodium hydroxide was added to stop colour development.

- The absorbance of each well was measured at 405nm.

The mean absorbance of the blank wells was subtracted from that of the test wells. IgG and IgM aCL antibody levels were derived from the relevant log/log standard curve.
2.4.6 β₂-glycoprotein I antibodies

Antibodies directed against β₂GPI were measured according to a modification of the method of Matsuura et al (1994) by McNally et al (1995b). This ELISA employed highly purified human β₂GPI antigen and a peroxidase-conjugated monoclonal antibody to human IgG. The assay was standardised using pooled normal serum calibrated against purified β₂GPI.

Reagents
- PBS: 0.01M phosphate, 0.145 M NaCl, pH 7.2.
- Wash buffer: PBS containing 0.1% (v/v) Tween 20.
- Assay buffer: PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) BSA.
- BSA: 10 mg/ml BSA in PBS.
- Blocking solution: 1% w/v BSA in PBS.
- β₂GPI: 1 mg/ml in PBS. Purified according to the method of McNally et al, 1995b.
- Immunoconjugate: peroxidase conjugated goat anti-human IgG (Sigma-Aldrich Company Ltd.): diluted 1/2000 in PBS.
- Substrate buffer: 0.05M phosphate citrate buffer with urea hydrogen peroxide (Sigma-Aldrich Company Ltd.).
- Substrate: 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich Company Ltd.): 0.1 mg/ml in substrate buffer.
- 2M sulphuric acid.
- Reference serum: serum with a known high concentration of antibodies directed against β₂GPI and calibrated against affinity purified anti-β₂GPI.

Method
- Purified β₂GPI was diluted 1/100 in PBS.
- 100μl dilute β₂GPI was added to the wells of a microtitre plate (Nunc Polysorp: Nunc A/S). The plate was sealed and incubated at 4°C overnight before washing three times in 150μl/well volumes of wash buffer to remove unbound protein.
- 125μl blocking solution was added to all wells and incubated at ambient temperature for one hour.
- The plate was washed once with 150μl/well volumes of wash buffer.
- A six point standard curve was prepared by diluting reference serum 1:100 to 1:3200 in assay buffer.
- Control and test serum was diluted 1:100 in assay buffer.
- 100μl volumes of dilute reference and test sera was added to duplicate test and blank wells and incubated at ambient temperature for two hours.
- The plate was washed 4 times with 150μl/well volumes of wash buffer.
- 100μl immunoconjugate was added to each well and incubated at ambient temperature for two hours.
- The plate was washed three times with 150μl/well volumes of wash buffer.
- 100μl active substrate was added to each well and incubated at ambient temperature to allow colour development.
- 100μl sulphuric acid was added to each well to stop colour development.
- The optical density of each well was measured at 450nm.

β2GPI antibody levels were derived from a log/log standard curve.

2.4.7 Soluble E-selectin (sE-s)

Soluble E-selectin levels were determined using a commercial kit (Human Soluble E-selectin) from R & D Systems Europe, utilising a microtitre plate coated with murine antibody to human E-selectin and peroxidase-conjugated anti-E-selectin.

**Method**

- Serum samples were diluted 1/20 in sample diluent.
- 100μl conjugate was added to each well of the microtitre plate.
- 100μl volumes of reference material or dilute sera were loaded onto the test plate, agitated to mix, sealed and incubated at ambient temperature for 90 minutes.
- The plate was washed six times in 300μl/well volumes of wash buffer.
- 100μl substrate was added to each well and incubated in the dark at ambient temperature for 30 minutes.
- 100μl acid solution was added to each well to stop colour development.
- The absorbance of each well was measured at 450nm using a correction wavelength of 620nm.

sE-s levels were derived from a linear standard curve.
2.4.8 Soluble thrombomodulin (sTM)

Soluble thrombomodulin levels were determined using a commercial kit (Asserachrom Thrombomodulin) from Diagnostica Stago, which employed a microtitre plate coated with F(ab')2 fragments of a monoclonal antibody to TM and peroxidase conjugated anti-TM.

Method

- Serial dilutions of the Reference Plasma were prepared to yield five assay calibrators ranging from 6.25 to 100 ng/ml thrombomodulin.
- Test plasma was diluted 1/5 in dilution buffer.
- 200μl volumes of calibrator or dilute test plasma were loaded onto the test plate and incubated at ambient temperature for two hours.
- The plate was washed five times in 300μl/well volumes of wash buffer.
- 200μl immunoconjugate was added to each well and incubated at ambient temperature for two hours.
- The plate was washed five times in 300μl/well volumes of wash buffer.
- 200μl o-phenylenediamine dihydrochloride/urea H2O2 substrate was added to each well and incubated in the dark at ambient temperature for eight minutes.
- 50μl sulphuric acid was added to each well to stop colour development.
- The absorbance of each well was measured at 492nm.

sTM levels were derived from a log/log standard curve and adjusted for the dilution factor (i.e. 1/5).

2.5 DNA analysis

DNA was extracted from buffy coat preparations and analysed using polymerase chain reaction (PCR) techniques described by Bertina et al (1994) and Poort et al (1996). The PCR is a method for the enzymatic synthesis of specific DNA sequences: double-stranded DNA is denatured to form two single strands; complementary oligonucleotide primers that flank the region of interest in the target DNA are annealed onto each single-strand; polymerase enzyme activity catalyses the addition of further nucleotides and therefore extends the primers, using the original DNA as a template so that two copies of the original target strand are produced. Repetitive PCR cycles result in the exponential accumulation of the specific fragment whose termini are defined by the 5’ ends of the primers. The amplified DNA is enzymatically digested at the site of
interest, producing fragments whose size is altered by single point mutations in the DNA. The fragments are visualised using ethidium bromide-stained agarose gel electrophoresis.

**Reagents**

- 0.17M ammonium chloride (NH$_4$Cl).
- 0.05M sodium hydroxide (NaOH).
- Tris ([hydroxymethyl] amino-methane) buffer, 1M, pH 7.7.
- PCR buffer [100mM Tris-HCl, 500mM KCl, 15mM MgCl$_2$, 0.01% gelatin, pH 8.3], conc: ×10 (Sigma-Aldrich Company Ltd.).
- Nucleotide product preparation (NTPs): containing 2mM ATP, CTPc, TTP and GTP (Labtech International).
- Oligonucleotide primers (Genosys Biotechnologies (Europe) Ltd.): reconstituted to 1µg/µl in sterile water.
  - **FVL:** 5'-TGCCCAGTGCTTAACAAGACC (95.4nmol) and 5'-TGTTATCACACTGGTGCTAA (68.7nmol),
  - **PGM:** 5'-TCTAGAAACAGTTGCCTGGC (159.7 nmol) and 5'-ATAGCACTGGGAGCATTGAAGC (135.9 nmol).
- Red Hot DNA Polymerase: 5u/µl (Advanced Biotechnologies Ltd.).
- Mineral oil (Sigma-Aldrich Company Ltd.).
- Restriction enzymes [FVL: Mnl I (10u/µl), buffered with Buffer G+ [10nM Tris-HCl, 10mM MgCl$_2$, 50mM NaCl, 0.1 mg/ml BSA, pH 7.5], conc ×10 (Biolabs),
  - **PGM:** Hind III (80u/µl), buffered with Buffer E [60mM Tris-HCl, 1M NaCl, 60mM MgCl$_2$ and 10mM DTT, pH 7.5] (Promega UK Ltd.).
- Electrophoresis buffer: Tris Borate EDTA (TBE) buffer (Severn Biotech Ltd.) 10× solution diluted 1/10 in deionised water for use. 0.5g/l ethidium bromide (Sigma-Aldrich Company Ltd.) was added
- SeaKem® LE Agarose (FMC Bio Products).
- Loading dye: 13.7% sucrose in 1×TBE buffer, containing xylene cyanole FF (for FVL) or bromophenol blue (for PGM).

Heating and amplification cycles were performed using a DNA Thermal cycler (Perkin Elmer Ltd.). Gels were visualised and photographed using AlphalImager™ 1200I v4.03/AlphaEase™ Version 4.0 camera and software (Flowgen Instruments Ltd.).
Method

DNA extraction

- 800µl NH₄Cl was added to 100µl frozen-thawed buffy coat sample and mixed by inversion for one hour.
- The sample was centrifuged at high speed for two minutes and the supernatant discarded. The pellet was washed in 1ml sterile saline.
- 200µl NaOH was added to the pellet and vortexed to mix: the re-suspended pellet was heated to 99°C for 10 minutes.
- The DNA was neutralised with 40µl Tris buffer and stored at −20°C until analysed.

DNA amplification (i.e. polymerase chain reaction)

- The ‘reaction mixture’ incorporated 7.5µl sterile water, 2.5µl PCR buffer, 2.5µl NTPs, 2.5µl FVL or PGM primers and 0.5µl polymerase per sample.
- 15µl ‘reaction mixture’, then 10µl DNA was pipetted into a 0.2ml thin-walled reaction tube (Alpha Laboratories Ltd.) and mixed.
- Two drops of mineral oil were layered above the mixture before the DNA was amplified according to the relevant protocol:
  
  **FVL**
  
  Denaturation: 94°C for two minutes.
  Annealing: 94°C for 40 s followed by 60°C for 40 s: 36 cycles.
  Extension: 72°C for five minutes.
  
  **PGM**
  
  Denaturation: 94°C for five minutes.
  Annealing: 94°C for 45 s followed by 58°C for 45 s, followed by 72°C for 45 s: 35 cycles.
  Extension: 72°C for 10 minutes.

- The amplified PCR product remained at 6°C until digested.

PCR Product Digestion

- The ‘digest mixture’ incorporated 9µl sterile water, 1µl buffer [FVL: buffer G+, PGM: buffer E] and 0.5µl restriction enzyme [FVL: MnlI, PGM: Hind III] per sample.
- 10µl of PCR product was mixed with 10µl of the ‘digest mixture’ and incubated at 37°C overnight.
Electrophoresis

- An agarose gel [FVL: 2%, PGM: 3%] was prepared in electrophoresis buffer.
- 10μl digested PCR product was added to 3μl loading dye for each sample: 12μl of the mixture was electrophoresed in a positive direction at 100V and 50mA, [FVL: one hour, PGM: 2.5 hours].

Relevant electrophoretic bands were examined under ultra violet light. Control and ‘blank’ samples were included in each gel: the ‘blank’ contained sterile water in place of DNA to ensure no erroneous PCR product was present due to contamination of the reaction mixtures by DNA or DNA-ase.

2.6 Haematological and biochemical analyses

Haematological analyses and biochemical tests of liver function (LFTs) comprised part of the study protocol. In addition, renal and thyroid function tests, glucose measurements and ferrokinetic studies were performed as required for the patients’ clinical management. Analyses were performed in the Departments of Haematology and Biochemistry at UCLH and their reference ranges are cited in Appendix II. Full blood counts were performed on a Coulter® STKS automated cell counter (Beckman Coulter UK Ltd.). Manual leucocyte differentials were performed on peripheral blood films stained by modified Wright’s method and total leucocyte counts (WBCs) were corrected for the presence of nucleated RBCs when necessary. Retics were counted by flow-cytometric analysis using an SE-9500™ automated cell counter with RAM-1™ (Retic analysis module) (Sysmex UK Ltd.). Hb variants were identified and quantified using a high performance liquid chromatography (HPLC) system (Variant™: Bio-Rad Laboratories Ltd.). LFTs, comprising total bilirubin (TBIL), alkaline phosphatase (Alk Phos), aspartate transaminase (AST) and albumin determinations were performed using an ISP-1000™ automated analyser (Synermed Europe Ltd.).

I performed total cholesterol (CHOL), triglyceride (TRIG), lactate dehydrogenase (LDH) and CRP determinations using a Cobas Mira automated biochemical analyser (ABX UK). Protocols, reagents, calibrators and quality control material were supplied by kit manufacturers (see sections 2.6.1-3).
2.6.1 Total Cholesterol (CHOL) and Triglyceride (TRIG)

Standard methods were used to measure CHOL and TRIG, whereby the rate of colour (quinoneimine dye) production was directly proportional to the total CHOL or TRIG concentration and was measured photometrically. CHOL levels represent the total cholesterol found in low density (where it is the major component), very low density and high density lipoprotein fractions.

**Method**

Tests were performed using commercial kits (CHOLESTERIN 20 and TRIGLYCERIDE [GPO-TRINDER] 10) from Sigma-Aldrich Company Ltd.

- 3μl of cholesterol (200mg/dL) or triglyceride (250 mg/dL) calibrator as appropriate, or test serum was diluted in 10μl distilled water.
- 300μl working reagent (for cholesterol or triglyceride) was added to the dilute sample.
- The rate of change in absorbance at 500nm was monitored between 25 and 325 seconds following the addition of the relevant working reagent.

A supplied calibration factor was used to generate CHOL or TRIG levels in mmol/L.

2.6.2 Lactate dehydrogenase (LDH)

Lactate dehydrogenase catalyses the interconversion of lactate and pyruvate (in erythrocytes) and elevated serum levels provide a sensitive indicator of intravascular haemolysis (Myhre & Rasmussen, 1970). Catalytic LDH activity was determined in accordance with the recommendations of the Société Française de Biologie Clinique (SFBC) (1982). The rate of colour production due to oxidisation of NADH is directly proportional to LDH activity and is measured photometrically.

**Method**

Tests were performed using a commercial kit (Unimate 3 LDH SFBC) containing (i) 0.20 mmol/L NADH and (ii) TRIS buffer with 1.6 mmol/L pyruvate and 200 mmol/L sodium chloride, pH 7.2: NADH was reconstituted with buffer to produce a working reagent, according to instructions of the manufacturer (ABX UK).

- 4μl serum was diluted in 20μl distilled water.
- 225μl working reagent was added to the dilute plasma.
- The rate of change in absorbance at 340nm was monitored between 75 and 275 seconds following the addition of working reagent.

A calibration factor was supplied to generate LDH activity in U/L.
2.6.3 C-reactive protein (CRP)

CRP in serum was specifically bound to goat anti-human CRP, yielding an insoluble aggregate that determined the turbidity of a reaction mixture. The degree of turbidity, reflected by a change in absorbance of the assay mixture, was proportional to the amount of CRP present and was measured photometrically.

**Method**

Tests were performed using a commercial kit (C-REACTIVE PROTEIN (CRP)) from Sigma-Aldrich Company Ltd. containing goat anti-human CRP antibody solution (0.9 mg/ml, pH 7.0); assay buffer containing 120 mmol/l phosphate and reference serum: human serum containing a known level of CRP calibrated against the IFCC Standard (85/506).

- 14μl serum was diluted with 10μl distilled water.
- 330μl assay buffer was added to the dilute plasma.
- 50μl antibody solution and 10μl distilled water were added to the assay mixture.
- The rate of change in absorbance at 340nm was monitored between 275 and 600 seconds following the addition of antibody solution with distilled water.

A calibration factor was supplied to generate CRP levels in mg/dL.

2.7 Data presentation and statistical analysis

Data was non-Gaussian for some variables, therefore the median and interquartile range were reported and non-parametric tests were used for statistical analysis. The number of samples analysed is indicated as occasional results were unobtainable due to inadequate sample volume. Statistical analyses were undertaken using ANALYSE-IT® add-in to Microsoft® Excel® (Analyse-It, Software Ltd.): variation between groups was assessed using the Wilcoxon-Mann-Whitney test for non-parametric data; correlation between variables was assessed using Spearman rank correlation analysis for non-parametric data, where the degree of association between two variables is reported as the Spearman rank correlation coefficient ($r_s$). Data is represented graphically when the difference between variables in clinical and control groups was statistically significant; the median value of each data series is indicated by a horizontal bar and results of significance testing between groups is shown. Statistical analysis was considered significant at the 5% level for all tests, i.e. when ‘$p’ < 0.05. (NS: not significant).
CHAPTER 3

HAEMOSTATIC VARIABLES IN HEALTHY
CAUCASIAN AND BLACK SUBJECTS

3.1 Introduction

The effect of a pathological condition on normal physiological processes cannot be determined before acceptable limits of variation have been defined within healthy, but otherwise analogous subjects. As far as possible, the control group should be comparable to the age, sex, genetic background, medication, lifestyle and dietary habits of the subjects under investigation, although this may be impractical in some instances. Comparative analysis is valid only when samples from control subjects and those under investigation are collected, processed and assayed in the same manner.

Plasma levels of haemostatic proteins may be altered as a result of the interaction between inflammatory, immunological and other regulatory mechanisms and there is also normal physiological variation between healthy individuals. Some variation may be attributable to age, sex, diet, lifestyle (e.g. stress, labour/exercise, obesity, smoking and alcohol consumption), medication (e.g. aspirin, barbiturates, anticonvulsants and hormonal steroids), environmental factors (e.g. altitude, endemic infection) or ethnicity. Of particular relevance to this study is the impact of ethnicity on haemostatic variables as patients from two distinct ethnic backgrounds were to be investigated: the majority of the SCD cohort are Black, while the majority of the β thal patients are Caucasian.

Physiological differences in some haematological and haemostatic variables have been reported between healthy Black and Caucasian subjects. Lower Hb, mean cell volumes (MCVs) and WBCs have been reported in healthy Black subjects and the leucocyte differential is reversed due to a relative neutropaenia (Williams, 1981; Zezulka et al, 1987). Blood group B, which is associated with 15% higher FVIII activity than group O, is encountered more commonly in Black populations than in Caucasians and is an independent risk factor for venous thromboembolism (Issitt, 1994; O'Donnell et al, 1997); certain antigens (such as Rh, Duffy, Kell and Kidd) are more frequently encountered in Caucasians than in Blacks (Sosler et al, 1993). Variation in components of coagulation and fibrinolysis that are associated with ethnicity may contribute to the
considerably lower incidence of thrombosis generally observed in Black populations relative to Caucasians (Gelfand & Ross, 1976; Rain, 1988). Of course, differences attributed to ethnicity may incorporate dietary, lifestyle, medicinal and environmental factors.

Some recent studies involving SCD patients have recruited a race-matched control group, but prior to my commencing work for this thesis, only one comparative study of haemostatic variables in healthy Caucasian and Black adults had been undertaken (Hagger et al, 1995). That investigation revealed significant differences in Fg, FPA, beta-thromboglobulin, D-D, PAI and ATIII. Previous reports of so-called ‘abnormal’ platelet aggregation responses to low concentrations of ristocetin in Black subjects were subsequently found to be physiological variations and attributed to the presence of a plasma inhibitor (Buchanan et al, 1981). For the purposes of my study, it was important to establish levels of the haemostatic parameters of interest in healthy Caucasian and Black adults with HbAA genotypes and no history of thrombotic disease. Reference ranges established by UCLH were not utilised as several of the assays I planned to perform embraced mild variations in reagents and instruments or were not in routine use within the hospital. In any case, ethnic-based reference ranges are not established independently at UCLH, despite the diverse ethnic background of attending patients. This situation is typical of most European laboratories and while minor variations are usually not of clinical significance, awareness of a patient’s ethnic origin may assist interpretation of routine tests.

In this chapter, I determined the levels of haemostatic variables in two healthy control groups of different ethnicity. The ‘Caucasian’ control subjects were primarily European and did not include any subjects of African ethnicity; ‘Black’ subjects were of African and Caribbean origin. Results obtained in the two groups were compared and independent reference ranges were established for each variable.

3.2 Methods

Patients, blood samples and assays

Blood samples were collected from healthy, non-pregnant adult subjects as described in section 2.1. Each subject answered a questionnaire that confirmed date of birth and racial background, no history of thrombosis and no recent drug ingestion. OC use was noted when applicable. Assays were performed as described in Chapter 2.
Control groups

The control subjects were divided into two groups according to ethnic background. The ‘Caucasian’ group incorporated subjects from the United Kingdom (n=24), Australia (n=3), New Zealand (n=2), the Republic of Ireland (n=1), Spain (n=1), India (n=1) and Lebanon (n=1). The ‘Black’ group was comprised of subjects from Jamaica (n=9), the West Indies (n=5), Ghana (n=4), Nigeria (n=2), Mauritius (n=2), Barbados (n=1) and St Vincent (n=1). The HbAA genotype of each control subject was confirmed by HPLC.

Exclusions

Fourteen healthy volunteers were excluded from the study due to abnormal genotype (HbAC: n=2; slow chain Hb variant: n=1 and six in whom alpha-thalassaemia could not be excluded without globin chain analysis), mild anaemia (n=2), mild macrocytosis (n=1) or heterozygosity for FVL (n=2).

3.3 Results

Each subject enrolled in a control group was well at the time of testing and had no history of thrombosis. Haematological profiles were normal. The number of males and females in each group was approximately equal and although the subjects were not matched for age, there was no significant difference in the age distribution between the two ethnic groups as assessed by Kruskal-Wallis one way analysis of variance. Eight women were using OCs. The profiles of both control groups are shown in Table 3.1.

<table>
<thead>
<tr>
<th></th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n)</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>17 (3)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>19 - 48</td>
<td>20 - 46</td>
</tr>
<tr>
<td>Median age</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>Genotype</td>
<td>HbAA</td>
<td>HbAA</td>
</tr>
</tbody>
</table>

Table 3.1 Profiles of Caucasian and Black control groups. The number of females using estrogen-based oral contraceptives are shown in parentheses.
3.3.1 Routine clotting profiles

Routine clotting profiles were performed and summary data is shown in Table 3.2. The PTs and TTs in the two control groups were not significantly different, however APTTs were slightly longer in the Black controls (Figure 3.1), which may reflect altered concentrations of some clotting factors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>10.3 (10.2 – 10.7)</td>
<td>10.4 (10.0 – 10.6)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 26</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>30.5 (29.1 – 32.2)</td>
<td>32.7 (31.0 – 34.7)</td>
</tr>
<tr>
<td></td>
<td>n = 32</td>
<td>n = 27</td>
</tr>
<tr>
<td>TT (s)</td>
<td>14.9 (13.8 – 15.4)</td>
<td>14.2 (13.5 – 15.2)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 27</td>
</tr>
</tbody>
</table>

Table 3.2 Clotting screens in control groups. Median results are reported (with the interquartile range in parentheses).

Figure 3.1 Activated partial thromboplastin times in control subjects.
3.3.2 Clotting factors

Fg and clotting factors II, VII and X were measured and summary data is shown in Table 3.3. Fg, FVII and FX levels were comparable, whereas FII levels were significantly higher in the Black subjects (p < 0.05, Figure 3.2). Clotting factor assays were performed on a single aliquot on the same day, so inter-batch variation does not account for this observation. The range of FII levels determined within each group were approximately equal (Caucasians: 0.92 – 1.38 and Blacks: 0.96 – 1.41 IU/ml).

Factors II and X were correlated in the Caucasian controls (r_s = 0.72, p < 0.0005), but neither were correlated with FVII. Statistical associations were demonstrable between all three factors in the Black control subjects, but they were not strong (FII and FVII: r_s = 0.49, p < 0.02; FII and FX: r_s = 0.43, p < 0.05; FVII and FX: r_s = 0.48, p < 0.02).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fg</td>
<td>2.3 (1.9 – 2.6)</td>
<td>2.2 (1.9 – 2.6)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 24</td>
</tr>
<tr>
<td>FII</td>
<td>1.05 (0.98 – 1.22)</td>
<td>1.18 (1.08 – 1.25)</td>
</tr>
<tr>
<td></td>
<td>n = 20</td>
<td>n = 24</td>
</tr>
<tr>
<td>FVII</td>
<td>1.07 (0.96 – 1.24)</td>
<td>1.01 (0.93 – 1.19)</td>
</tr>
<tr>
<td></td>
<td>n = 29</td>
<td>n = 26</td>
</tr>
<tr>
<td>FX</td>
<td>0.95 (0.89 – 1.07)</td>
<td>1.11 (0.90 – 1.28)</td>
</tr>
<tr>
<td></td>
<td>n = 21</td>
<td>n = 26</td>
</tr>
</tbody>
</table>

Table 3.3 Clotting factors in control groups. Median results are reported (with the interquartile range in parentheses).
3.3.3 Markers of coagulation system activation

Summary data for F1+2, TAT and FVIIa is shown in Table 3.4.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1+2</td>
<td>0.8 (0.7 – 1.0)</td>
<td>0.6 (0.5 – 0.8)</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
<tr>
<td>TAT</td>
<td>2.3 (1.3 – 2.6)</td>
<td>2.8 (2.1 – 4.3)</td>
</tr>
<tr>
<td>(μg/L)</td>
<td>n = 32</td>
<td>n = 28</td>
</tr>
<tr>
<td>FVIIa</td>
<td>0.99 (0.76 – 1.45)</td>
<td>1.64 (1.08 – 2.23)</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>n = 18</td>
<td>n = 24</td>
</tr>
</tbody>
</table>

Table 3.4 Markers of coagulation activation in control groups. Median results are reported (with the interquartile range in parentheses).

Markers of thrombin generation

Statistically significant differences were noted between the levels of F1+2 and TAT in the two control groups (Figures 3.3 and 3.4). F1+2 levels were higher in the Caucasians (p < 0.02), however Black controls demonstrated slightly higher TAT levels (p < 0.05). F1+2 and TAT were not correlated within either group.
Figure 3.3 Prothrombin fragment 1+2 levels in control subjects.

Figure 3.4 Thrombin:antithrombin complexes in control subjects.
**Activated factor VII (FVIIa)**

FVIIa levels were significantly higher in the Black controls (p < 0.02, Figure 3.5) and were associated with FVII within both control groups (Caucasian: \( r_s = 0.61, p < 0.01 \); Black: \( r_s = 0.55, p < 0.05 \)).

![Activated FVII levels in control subjects.](image)

**3.3.4 Physiological inhibitors of coagulation**

Levels of physiological inhibitors of coagulation (ATIII, HCII, PC, PSf and \( \alpha_1 \text{AT} \)) were measured. Summary data is shown in Table 3.5. There were no statistically significant differences detected in ATIII, HCII, PC or PSf levels between the two groups, however \( \alpha_1 \text{AT} \) levels were slightly higher in the Black controls (p < 0.02, Figure 3.6).

A tendency toward higher median HCII levels in the Black controls were associated with greater variability and it was possible that this reflected proportionally greater OC use compared to Caucasians (Mackie *et al*, 1990). This was not the case, though, as median levels were not significantly altered when OC users were excluded from the analysis. It was likely that OC use contributed to the difference in \( \alpha_1 \text{AT} \) levels however, as the higher \( \alpha_1 \text{AT} \) levels were observed in OC users and there was no
difference between the two groups when these were excluded (1.03 vs. 1.13 U/ml, p = 0.12). An increase in $\alpha_1$AT is known to be associated with OC use (Campbell et al, 1993).

PSf levels tend to be lower in females than males and lowest in females using OC (Tait et al, 1997). Therefore, I assessed levels in males and females within each ethnic group separately and the reference ranges subsequently established in females exclude OC users: PSf was consistently higher in males (Figures 3.7 & 3.8), but there was no significant difference in the levels found in males or females between ethnic groups. A weak negative association between PSf and age was demonstrated in Black males ($r_s = -0.60, p < 0.05$) and in Caucasian females, irrespective of OC use (female (all): $r_s = -0.51, p < 0.05$ and female (-OC): $r_s = -0.64, p < 0.02$).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIII (IU/ml)</td>
<td>1.09 (0.98 - 1.14)</td>
<td>1.05 (1.00 - 1.10)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
<tr>
<td>HClI (U/ml)</td>
<td>1.03 (0.96 - 1.12)</td>
<td>1.24 (0.98 - 1.37)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
<tr>
<td>PC (IU/ml)</td>
<td>1.02 (0.91 - 1.13)</td>
<td>0.98 (0.89 - 1.18)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
<tr>
<td>PSf (IU/ml)</td>
<td>0.84 (0.74 - 0.96)</td>
<td>0.84 (0.71 - 0.97)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
<tr>
<td>$\alpha_1$AT (U/ml)</td>
<td>1.04 (0.90 - 1.24)</td>
<td>1.14 (1.07 - 1.37)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 28</td>
</tr>
</tbody>
</table>

Table 3.5 Coagulation inhibitors in control groups. Median results are reported (with the interquartile range in parentheses).
Figure 3.6  Alpha-1-antitrypsin levels in control subjects.

Figure 3.7  Free protein S levels in Caucasian control subjects. M: male, F(+OC): female (including OC users), F (-OC): female (excluding OC users).  • OC users.
3.3.5 Activated Protein C Resistance (APCR)

Summary data for APCR screening tests is shown in Table 3.6. APTTs were significantly longer in the Black controls in both the presence (p < 0.002) and absence (p < 0.0001) of APC (Figure 3.9), consistent with the differences noted in the standard APTT using the kaolin based reagent in section 3.3.1. This was not attributable to the notably shorter clotting times of one subject in each ethnic group. Despite this, APCR ratios in the two groups were not significantly different.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT:APC (s)</td>
<td>107.6 (103.4)</td>
<td>112.0 (107.6 – 114.7)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 28</td>
<td></td>
</tr>
<tr>
<td>APTT:nAPC (s)</td>
<td>40.0 (39.1 – 41.1)</td>
<td>43.6 (42.5 – 44.6)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 28</td>
<td></td>
</tr>
<tr>
<td>APCRm (ratio)</td>
<td>2.64 (2.59 – 2.72)</td>
<td>2.57 (2.49 – 2.66)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 28</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6 Screening tests for activated protein C resistance in control groups. Median results are reported (with the interquartile range in parentheses). APTT:APC: test in the presence of activated protein C; APTT:nAPC: test in the absence of activated protein C; APCRm: modified activated protein C resistance ratio.
3.3.6 Components of fibrinolysis

D-D, Plg and α2AP were measured and summary data is shown in Table 3.7.

These variables were similar in the two ethnic groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-D (ng/ml)</td>
<td>16 (16 – 33)</td>
<td>18 (16 – 31)</td>
</tr>
<tr>
<td>n = 26</td>
<td>n = 28</td>
<td></td>
</tr>
<tr>
<td>Plg (IU/ml)</td>
<td>1.13 (1.02 – 1.26)</td>
<td>1.13 (1.03 – 1.24)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 26</td>
<td></td>
</tr>
<tr>
<td>α2AP (IU/ml)</td>
<td>1.04 (0.99 – 1.11)</td>
<td>1.05 (1.04 – 1.12)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 28</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7 Components of fibrinolysis in control groups. Median results are reported (with the interquartile range in parentheses).
3.3.7 Phospholipid-dependent antibodies

Tests for PL-dependent antibodies were undertaken: clotting times were determined using Dilute Russell’s Viper Venom and titres of IgG and IgM aCL were measured. Summary data is shown in Table 3.8. Interpretation of the DRVVT was described in section 2.2.8: DRVVTs did not differ significantly between ethnic groups and the ratios calculated to discriminate LA positive plasmas were the same (i.e. 1.06).

Weak IgG aCL were detected in two Caucasian subjects (titres: 6 and 14 GPL units), however the demonstration of a ‘true’ PL-dependent antibody requires two samples to test positive at least six weeks apart, as weak IgG aCL may occur in association with infection or certain drug therapies, but these tend to be transient in nature (Greaves et al, 2000). The confines of this study did not permit re-testing on subsequent occasions, but neither subject yielded an abnormal APTT or DRVVT and antibodies to β2GPI, the co-factor for PL antibody binding, were not detected in either subject. The slightly higher incidence of weak IgG aCL positivity in the Caucasians was not statistically significant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRVVT</td>
<td>0.95</td>
<td>0.93</td>
</tr>
<tr>
<td>(PL ratio)</td>
<td>(0.93 – 0.98)</td>
<td>(0.90 – 0.98)</td>
</tr>
<tr>
<td>n = 29</td>
<td>n = 27</td>
<td></td>
</tr>
<tr>
<td>IgG aCL</td>
<td>2 / 33</td>
<td>1 / 26</td>
</tr>
<tr>
<td>(%)</td>
<td>6.1 %</td>
<td>3.9 %</td>
</tr>
<tr>
<td>IgM aCL</td>
<td>1 / 33</td>
<td>1 / 26</td>
</tr>
<tr>
<td>(%)</td>
<td>3.0 %</td>
<td>3.9 %</td>
</tr>
</tbody>
</table>

Table 3.8 Screening tests for phospholipid-dependent antibodies in control groups. Median PL ratios are reported (with the interquartile range in parentheses). aCL reported as the number abnormal / tested (see text).
3.3.8 Markers of endothelial cell activation

Plasma levels of sE-s and sTM were measured to assess the condition of the endothelium. Summary data is shown in Table 3.9. sE-s levels were significantly higher in the Black controls (p < 0.02, Figure 3.10). sTM levels were comparable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HbAA (Caucasian)</th>
<th>HbAA (Black)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sE-s (ng/ml)</td>
<td>33.3 (26.9 – 44.4)</td>
<td>45.7 (35.1 – 57.5)</td>
</tr>
<tr>
<td></td>
<td>n = 31</td>
<td>n = 26</td>
</tr>
<tr>
<td>sTM (ng/ml)</td>
<td>51.5 (37.2 – 58.9)</td>
<td>37.2 (26.9 – 51.8)</td>
</tr>
<tr>
<td></td>
<td>n = 15</td>
<td>n = 15</td>
</tr>
</tbody>
</table>

Table 3.9 Markers of endothelial cell activation in control groups. Median results are reported (with the interquartile range in parentheses).

Figure 3.10 Soluble E-selectin levels in control subjects.
A reference range defines the limits of expected results in a specified population and I established reference ranges for haemostatic variables independently in the Caucasian and Black HbAA control groups (Table 3.10). The limits were defined as two standard deviations (2 S.D.) above and below the mean of log transformed data for each variable. Log transformation was performed to compensate for the effects of skewed data when the data distribution was non-Gaussian. By definition, the reference range would be expected to exclude 2.5% of ‘normal’ subjects at the upper and lower limit of the range where the distribution is normal.

OCs affect levels of some haemostatic proteins including Fg, FVII, FX, PS and HCII. Within this study, variables in OC users did not significantly affect the profile of the remainder of their respective groups, but for consistency, data from females using OCs was excluded from the calculation of all reference ranges. FX and PSf levels were higher in Black males than in females (FX: 1.14 vs. 0.94 IU/ml, p < 0.02 and PSf: 0.89 vs. 0.72 IU/ml, p < 0.005), so sex-dependent ranges were calculated for these variables. Independent ranges were also calculated for males and females with respect to $\alpha_1$AT and PSf in the Caucasian group, as sex-related differences were evident ($\alpha_1$AT: 0.92 vs. 1.22 U/ml, p < 0.05 and PSf: 0.94 vs. 0.79 IU/ml, p < 0.01).

The reference ranges established in both ethnic groups compared favourably to those cited by the Department of Haematology at UCLH, or the manufacturers of assay kits where applicable, including those that incorporated small numbers because of sex-dependence (i.e. $\alpha_1$AT and PSf in Caucasians and FX and PSf in Blacks). The reference ranges established for D-D in Caucasians and Blacks (<67 and <40 ng/ml respectively) lay well within that provided by the manufacturer of the assay kit (<120 ng/ml): the considerably lower levels may be due to the smaller number of controls used to establish the reference range in my study, or to different sample populations. The manufacturer’s range, established in 100 healthy subjects, was employed in this instance so that the number of patients with abnormal D-D levels was not overestimated.

Reference ranges established in the two ethnic control groups were compared (Table 3.10). Limits of the ranges defined for each variable differed slightly between groups and significant differences were observed with respect to APTT, F1+2, sE-s and $\alpha_1$AT in males. TAT and sTM levels were more variable in the Black controls, but this was not statistically significant.
### Table 3.10 Reference ranges for haemostatic variables.

Reference ranges were derived using data from males and females not using estrogen-based oral contraceptives. Significant sex-related differences were evident in α1AT and PSf in Caucasians and FX and PSf in Blacks, therefore sex dependent reference ranges were established. Reference ranges that differed significantly between the control groups are indicated in bold type in the ‘significance’ column.

* Reference ranges provided by the manufacturer were utilised for D-D.

** Reference values for aCL were established in our laboratory using a group of healthy normal subjects of mixed ethnicity.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Abbreviation</th>
<th>Reference range</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time (s)</td>
<td>PT</td>
<td>9.5 - 11.6</td>
<td>NS</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (s)</td>
<td>APTT</td>
<td>24.1 - 39.3</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Thrombin clotting time (s)</td>
<td>TT</td>
<td>12.7 - 16.9</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>Fg</td>
<td>1.5 - 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Factor II (IU/ml)</td>
<td>FII</td>
<td>0.86 - 1.36</td>
<td></td>
</tr>
<tr>
<td>Factor VII (IU/ml)</td>
<td>FVII</td>
<td>0.75 - 1.46</td>
<td></td>
</tr>
<tr>
<td>Factor X (IU/ml)</td>
<td>FX</td>
<td>0.54 - 1.61</td>
<td>NS</td>
</tr>
<tr>
<td>Factor X (IU/ml)</td>
<td>Female</td>
<td>0.65 - 1.37</td>
<td>NS</td>
</tr>
<tr>
<td>Prothrombin fragment 1+2 (nmol/L)</td>
<td>F1+2</td>
<td>0.3 - 1.8</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Thrombin:antithrombin complex (μg/L)</td>
<td>TAT</td>
<td>0.8 - 5.3</td>
<td>NS</td>
</tr>
<tr>
<td>Activated Factor VII (ng/ml)</td>
<td>FVIIa</td>
<td>0.39 - 3.07</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Antithrombin III (IU/ml)</td>
<td>ATIII</td>
<td>0.84 - 1.37</td>
<td>NS</td>
</tr>
<tr>
<td>Heparin cofactor II (U/ml)</td>
<td>HCH</td>
<td>0.73 - 1.53</td>
<td>NS</td>
</tr>
<tr>
<td>Protein C (IU/ml)</td>
<td>PC</td>
<td>0.69 - 1.42</td>
<td>NS</td>
</tr>
<tr>
<td>Free protein S (IU/ml)</td>
<td>PSf</td>
<td>0.62 - 1.37</td>
<td>NS</td>
</tr>
<tr>
<td>Free protein S (IU/ml)</td>
<td>Female</td>
<td>0.58 - 1.03</td>
<td>NS</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin (U/ml)</td>
<td>α1AT</td>
<td>0.63 - 1.37</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Activated protein C resistance (ratio)</td>
<td>ACPR</td>
<td>2.35 - 3.01</td>
<td>NS</td>
</tr>
<tr>
<td>D-Dimer (ng/ml)</td>
<td>D-D</td>
<td>&lt;120 *</td>
<td>NS</td>
</tr>
<tr>
<td>Plasminogen (IU/ml)</td>
<td>Plg</td>
<td>0.80 - 1.59</td>
<td>NS</td>
</tr>
<tr>
<td>Alpha-2-antiplasmin (IU/ml)</td>
<td>α2AP</td>
<td>0.88 - 1.22</td>
<td>NS</td>
</tr>
<tr>
<td>Dilute Russell’s viper venom time (ratio)</td>
<td>DRVVT</td>
<td>1.06</td>
<td>NS</td>
</tr>
<tr>
<td>IgG specific anticardiolipin antibodies (GPL units)</td>
<td>IgG aCL</td>
<td>&lt;5 **</td>
<td></td>
</tr>
<tr>
<td>IgM specific anticardiolipin antibodies (GPL units)</td>
<td>IgM aCL</td>
<td>&lt;11 **</td>
<td></td>
</tr>
<tr>
<td>Soluble E-selectin (ng/ml)</td>
<td>sE-s</td>
<td>18.8 - 67.5</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Soluble thrombomodulin (ng/ml)</td>
<td>sTM</td>
<td>30.0 - 81.7</td>
<td>NS</td>
</tr>
</tbody>
</table>
3.4 Discussion

The broad aim of this thesis is to examine elements of the haemostatic system in SCD and β thal: all of the SCD patients enrolled in this study are Black, whereas most of the β thal patients are Caucasian. Levels of some haematological and haemostatic variables vary with ethnicity (Hagger et al, 1995), so it was important to establish 'normal' levels of the variables of interest in healthy HbAA individuals who were of a similar ethnic background to the prospective patients. In this chapter therefore, I assessed haemostatic variables in healthy adults of Black African or Caucasian ethnicity: a normal Hb genotype was confirmed in each subject and the data obtained within the two ethnic groups was compared; the control groups were of a similar age and sex distribution to each other and comparable to the respective SCD and β thal patient cohorts. This data was also used to define reference ranges for each variable within the two independent control groups: the data obtained for some variables had a non-Gaussian distribution, so it was normalised by log transformation. Non-parametric tests were used for statistical analysis.

A statistically significant difference was noted between the two ethnic groups for some variables: APTTs were slightly longer and FII, α₁AT and sE-s levels were higher in Blacks. The difference in APTTs may be attributable to variations in plasma levels of FVIII, FIX, FXI or FXII, although I did not measure these; the LA, which may prolong the APTT if present, was not detected in any subject. It was not possible to test the control subjects for PGM at the time of performing this work, however it was unlikely that higher prothrombin levels were associated with inheritance of PGM, as the mutation is now known to be rarely encountered in Black individuals of African ethnicity (Rosendaal et al, 1998). FVIIa levels were significantly higher in the Black subjects and were associated with FVII within both control groups. F1+2 levels tended to be higher in Caucasians, however TAT levels were significantly higher and more variable in Black subjects. F1+2 and TAT were not correlated within either control group, which was consistent with the narrow range of data expected in normal subjects: high levels (that reflect increased thrombin generation) are not encountered in the quiescent state. Also, F1+2 and TAT are generated at different stages of the coagulation process and the activation peptide (F1+2) and enzyme:inhibitor complex (TAT) have different half-lives in plasma (90 vs. 3 minutes respectively). F1+2 may also undergo further proteolysis by thrombin, liberating separate prothrombin fragments 1 and 2, which are not efficiently detected by the F1+2 assay method (Cardigan, 1998).
Plasma levels of Fg, FVII and FX, proteins that regulate coagulation (i.e. ATIII, HCII, PC and PSf) as well as components of fibrinolysis (D-D, α2AP and Plg) were comparable between the two ethnic groups, as were modified APCR ratios, IgG aCL titres, DRVVT ratios and sTM levels. α2AP have been reported to be higher in Caucasians than Africans, however this was not evident in my cohort.

Reference ranges established in appropriate control populations provide a means of identifying anomalies that may be significant in patients with disease. ‘Normal’ levels of the haemostatic variables examined in this chapter were broadly similar in the two ethnic groups, however this chapter confirms the importance of utilising an appropriate group of control subjects for comparative analysis as limits of the references ranges differed slightly between the two control groups and this was statistically significant for some variables. Values at the limits of a given reference range may be incorrectly classified as ‘normal’ or ‘abnormal’ when an inappropriate reference range is applied.
CHAPTER 4

THE HAEMOSTATIC SYSTEM IN
SICKLE CELL DISEASE

4.1 Introduction

The pathophysiology of SCD is attributable to the abnormal behaviour of deoxygenated HbS, whereby chronic haemolysis and recurrent episodes of RBC sickling and microvascular occlusion culminate in pain, anaemia and progressive organ damage. RBC abnormalities include dehydration, membrane instability, PL destabilisation and enhanced adhesiveness. Repetitive cycles of RBC sickling and 'de-sickling' cause progressive membrane loss and increasing rigidity of the cell.

SCD is associated with a heightened risk of thromboembolic complications that may occur within the venous or arterial vasculature. Thromboses do not appear to play a primary role in the pathogenesis of vaso-occlusive complications of SCD, but are associated with significant morbidity and mortality: large vessel arterial disease is the most important cause of stroke in children with SCD and pulmonary arterial thrombosis/embolism contributes to pulmonary disease and hypertension; thromboses within the deep veins may occur at sites that are unusual in other thrombophilic populations and are frequently recurrent. Long-term transfusion therapy diminishes the recurrence of strokes as well as thromboses in most patients, however it is desirable to identify those most at risk of thrombotic complications with a view to prophylaxis.

Thrombophilia is a multicausal process and patients with SCD are susceptible to a variety of pathological factors that independently enhance hypercoagulability and may culminate in thrombosis: perpetual aggravation of the endothelium by sickled cells create localised areas of haemodynamic disturbance and regions of vascular perturbation that propagate the development of thromboses and stimulate inflammation; inflammatory responses increase blood viscosity by up-regulating plasma levels of acute-phase proteins; protracted periods of clinical immobility as well as hypoxia and microvascular occlusion by SRBC give rise to pockets of venous stasis; repetitive
episodes of sickling within the hepatic sinusoids may cause liver cell dysfunction with inadequate synthesis of coagulation proteins.

At the time of planning this work, reports of low plasma levels of physiological coagulation inhibitors as well as indirect evidence of excessive thrombin generation and fibrin formation indicated that haemostasis was disturbed in SCD, although the interpretations of some of these studies were limited (section 1.10). In this chapter, I undertook an extensive evaluation of the plasmatic haemostatic system in a population of adult SCD patients in 'steady-state' using a variety of functional, immunological, biochemical and molecular techniques. I sought to identify the extent of potential abnormalities that may contribute to an increased risk of venous thrombosis as well as their possible relationships to Hb genotype, haemolysis, inflammation, liver function, splenic function, hydroxyurea therapy and transfusion.

Material presented in Chapter 3 affirmed the importance of establishing ‘normal’ levels of the variables of interest in an appropriate control group; SCD patients were therefore compared to the Black HbAA control subjects as well as to each other. Patients were examined in the absence of clinical complications to avoid the impact of inflammatory processes on plasma levels of certain haemostatic proteins that are acute phase reactants or may otherwise be affected during acute illness. I aimed to address the following questions:

■ Are plasma levels of the haemostatic variables of interest significantly altered in HbSC and/or HbSS patients when compared to healthy, race-matched controls of HbAA genotype?

■ Are the haemostatic profiles of HbSC and HbSS patients significantly different?

■ Is hypercoagulability a feature of SCD in the absence of acute clinical complications?

■ Is a potential hypercoagulable state attributable to poor regulation of haemostasis by physiological inhibitors in these patients?

■ Are potential haemostatic abnormalities significantly associated with compromised liver function, endothelial cell damage, haemolysis or inflammation?

■ Is there evidence of underlying pathology that contributes to hypercoagulability?

■ What are the effects of therapeutic interventions (i.e. regular exchange transfusion, splenectomy and hydroxyurea) on haemostasis in SCD?
4.2 Methods

Patients

44 adult SCD patients (HbSC: n = 13; HbSS: n = 31) were enrolled in this study and a group of eight healthy heterozygous (HbAS) subjects were investigated concurrently. No patient was pregnant and all patients were in ‘steady-state’ at the time of testing, i.e. were free of clinical complications and had not recently experienced sickle-related pain. No intervention was made in the clinical management of any patient. Each subject answered a questionnaire that confirmed date of birth, ethnic background and prescribed medication; OC use was noted when applicable and any history of surgical splenectomy, thrombotic complications or stroke was recorded. Transfusion status was confirmed by blood transfusion records. Hb genotypes were confirmed by HPLC and subjects were divided into three groups on this basis (i.e. HbAS, HbSC or HbSS). Two patients with HbS/βthal were co-investigated with HbSS patients as neither genotype produces HbA and the two are phenotypically similar.

Exclusions

Volunteers who presented with current/resolving vasocclusive crises, thrombosis or other complications at the time of investigation were excluded from the study. A further six patients were excluded on the basis of age (<12 years, n=4), pregnancy (n=1) or a co-existing myeloproliferative disorder (n=1).

Control subjects

Patients cited African or West Indian/Caribbean ethnicity with the exception of two Caucasian Cypriots and one patient from the United Arab Emirates. It was therefore considered most appropriate to compare them to the healthy Black HbAA control group described in Chapter 3.

Sample collection and processing

Blood samples were collected and processed as described in section 2.1. Assays were performed as described in Chapter 2.

4.3 Results

4.3.1 Characteristics of subjects under investigation

The clinical groups investigated in this chapter are characterised in Table 4.1. The age and sex distributions of each group were not significantly different as assessed by Kruskal-Wallis one-way analysis of variance. Five patients had undergone surgical
splenectomy and five had previously experienced thrombotic complications (right axial
vein thrombosis, left leg DVT, portal vein thrombosis or stroke). Three sets of siblings
HbSS (each with an HbSS genotype) were enrolled.

Data is presented with respect to the following patient categories: HbSC: all
HbSC patients; HbSC(untx): untransfused HbSC patients; HbSS: all HbSS patients;
HbSS(untx): untransfused HbSS patients; HbSS(tx): regularly transfused HbSS
patients.

Transfusion

In this study, ‘transfusion’ refers to an exchange transfusion of plasma reduced,
irradiated and leucodepleted RBCCs: one unit (approximately 350ml) of physiological
sodium chloride (saline) is transfused to the patient while a unit of whole blood is
simultaneously withdrawn; subsequent units are then transfused while a unit of whole
blood is correspondingly withdrawn so that for a transfusion of three RBCC, four units
of whole blood are withdrawn. In this manner, blood volume is maintained while blood
viscosity and the percentage of circulating HbS is reduced and iron overload is limited
as far as possible.

Eight HbSS patients were regularly transfused (2-4 RBCC units, 3-4 weekly);
samples were collected between nine and 48 days following the most recent transfusion
(median 21 days). Another four HbSS patients were not transfused regularly, but had
received RBCC between 43 and 90 days prior to sample collection. Only one HbSC
patient had been transfused (22% HbA, 1% HbF, 38% HbS, 39% HbC). The remaining
12 HbSC and 19 HbSS untransfused patients had not received blood products for at
least 120 days prior to sample collection.

Medication

Patients were prescribed folic acid and penicillin. Antithrombotic drugs were not
prescribed for any patient and none admitted recent aspirin ingestion. Five patients were
treated with hydroxyurea. Regularly transfused patients were prescribed an iron-
chelating agent (desferrioxamine mesylate), with varying degrees of compliance.

Oral contraceptive use

One HbAS and one HbSS patient used oestrogen-based OCs, however their
inclusion did not alter the distribution of haemostatic profiles of their respective groups.
Another HbSS patient was prescribed Depo-provera for contraception; this compound
contains progesterone only and is not associated with thrombotic complications.
Table 4.1 Characteristics of patient groups (sickle cell disorders). * The HbSS group included 2 HbS/βthal patients (1 male and 1 female). Females using estrogen-based oral contraceptives are indicated in parentheses. For details of transfusion status, see text.

Sickle cell trait

HbAS subjects are generally healthy and no significant haemostatic abnormalities were detected in this cohort. Quantitative Hb analysis revealed 67 (61 – 67) % HbA; 33 (33 – 39) % HbS. Plasma levels of clotting factors (Fg, II, VII and X), inhibitors (ATIII, HCII, PC, PSF and α1AT), lipids (CHOL and TRIG), markers of coagulation system activation (F1+2, TAT and FVIIa) and endothelial cell activation (sE-s and TM) as well as components of fibrinolysis (D-D, Plg and α2AP) were comparable to those observed in the Black control subjects. PL-dependent antibodies were not detected in any subject.

Sickle cell disease

Haematological profiles

Haematological profiles reflected the broad clinical differences between the patient groups and were therefore more severely altered in HbSS than in HbSC and improved in transfused patients (Appendix III). Quantitative Hb analysis was performed and median levels and the interquartile range in each group were determined: untransfused HbSC [HbA: 0%, HbF: 2 (1 – 4) %, HbS: 49 (47 – 50) %, HbC: 50 (49 – 51) %]; untransfused HbSS [HbA: 0, HbF: 9 (4 – 15) %, HbS: 91 (85 – 96) %]; regularly transfused HbSS [HbA: 58 (48 – 69) %, HbF: 2 (2 – 4) %, HbS: 37 (30 – 50)
WBCs were normal in HbSC and mildly elevated in HbSS, irrespective of transfusion status, although the counts were more variable in untransfused HbSS patients (ranges: 4.1 – 21.0 vs. 9.0 – 13.3 × 10^9/L, p < 0.01). Abnormal RBC indices were as follows: untransfused HbSS patients [RCC: 2.5 (2.1 – 2.9) × 10^12/L; Hb: 7.9 (7.3 – 9.2) g/dL; HCT: 0.24 (0.21 – 0.27) l/l]; regularly transfused HbSS [RCC: 3.6 (3.2 – 3.7) × 10^12/L; Hb: 10.5 (9.3 – 10.8) g/dL; HCT: 0.32 (0.28 – 0.32) l/l]; untransfused HbSC [RCC: 4.1 (3.7 – 4.7) × 10^12/L; Hb: 11.6 (10.5 – 11.8) g/dL; HCT: 0.35 (0.32 – 0.36) l/l]. The remaining RBC indices were unremarkable. Platelet counts tended to be highest in untransfused HbSS patients, although this should be interpreted with caution as microcytic RBCs and RBC fragments may be erroneously categorised as platelets by some automated cell counters (including the Coulter® STKS) and a manual platelet count was not routinely performed in the Haematology laboratory at UCH.

4.3.2 Routine clotting profiles

Routine clotting profiles (PT, APTT and TT) were undertaken as a basic haemostatic screen.

Prothrombin time (PT)

Eight (26%) HbSS patients had slightly abnormal PTs, although the degree of prolongation (within 1 second of the reference range) was not considered clinically pertinent. These patients had normal Fg levels and no evidence of LA (section 4.3.6). Marginal prolongation of the PT in this subgroup was attributed to deficiencies of single or multiple clotting factors: each of the patients had low FII levels; six had low FVn levels; five were deficient in FX and four had multiple deficiencies (section 4.3.3). The PT is a relatively sensitive indicator of the synthetic capability of the liver; the clotting factor deficiencies and subsequent PT anomalies observed may therefore reflect hepatic dysfunction. Liver function is investigated in section 4.3.13.

Activated partial thromboplastin time (APTT)

One HbSC and two HbSS subjects had mildly prolonged APTTs (44.0, 41.6 and 46.5s respectively). Each sample corrected when mixed with an equal volume of normal plasma, suggestive of clotting factor deficiencies and samples from all patients were negative for LA (section 4.3.6).
**Thrombin clotting time (TT)**

TTs were normal with the exception of one HbSC patient, whose TT (24.2s) did not correct with 1% protamine sulphate, confirming the absence of heparin: a partial correction to 17s with toluidine blue accompanied by a normal Fg concentration was suggestive of dysfibrinogenemia and attributable to hepatic insufficiency following a portal vein thrombosis three and a half months previously. Persistent hepatic dysfunction was confirmed by hypoalbuminaemia (25g/L; R.R. 35 - 50g/L) and a mild prolongation in TT persisted for approximately one year. This patient had also experienced acute renal failure, although renal function tests were normal when samples were collected for this study. There was evidence of residual acute phase reactivity (CRP was slightly increased at 1.43 mg/dL) and TAT was mildly elevated (16.0 μg/L), although inclusion of this patient in the study did not significantly alter the profile of the HbSC group.

### 4.3.3 Clotting factors

Levels of Fg, FII, FVII and FX were measured and summary data is shown in Table 4.2. Six HbSS patients had raised Fg levels, but three of these were marginal and Fg in the HbSS and HbSC groups did not differ substantially from the controls, however Fg in HbSS were significantly higher than in HbSC (p < 0.05, Figure 4.1). CRP levels in patients with 4.3, 4.5 and 5.8 g/L Fg were 1.4, 5.1 and 8.2 mg/dL respectively (R.R.: ≤ 0.50 mg/dL). Fg and CRP are acute phase reactants; it is likely that raised levels were associated with sub-clinical inflammatory processes in these patients, the third of which also had marginally raised α1AT (1.66 U/ml), another acute phase protein.

Factors II, VII and X were relatively reduced in HbSS (p < 0.0001) and HbSC, however a tendency toward lower FVII levels in HbSC did not reach statistical significance (Figures 4.2 to 4.4). Factors II and X correlated well in HbSS patients (r_s = 0.84, p < 0.0001); weaker associations were observed between FII and FVII (r_s = 0.56, p = 0.002) and between FVII and FX (r_s = 0.46, p < 0.02). The shorter half-life of FVII (4-6 hours) compared to FII (72 hours) and FX (10-20 hours) may account for the weaker associations involving FVII. The strong relationship between FII and FX levels observed in HbSS contrasted with that in HbSC, where no association was observed at all. FII and FVII levels were positively, but weakly associated in HbSC (r_s = 0.69, p < 0.02), as were FVII and FX (r_s = 0.68, p < 0.05).
<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
<th>HbAS</th>
<th>HbSC</th>
<th>HbSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fg (g/L)</td>
<td>2.2</td>
<td>2.2</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>(1.9 - 2.6)</td>
<td>(2.1 - 2.6)</td>
<td>(2.0 - 2.3)</td>
<td>(2.1 - 3.3)</td>
</tr>
<tr>
<td>n</td>
<td>27</td>
<td>8</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>FII (IU/ml)</td>
<td>1.18</td>
<td>1.06</td>
<td>0.98</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>(1.08 - 1.25)</td>
<td>(1.08 - 1.25)</td>
<td>(0.87 - 1.15)</td>
<td>(0.80 - 1.03)</td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>8</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>FVII (IU/ml)</td>
<td>1.01</td>
<td>0.92</td>
<td>0.83</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>(0.93 - 1.19)</td>
<td>(0.85 - 1.10)</td>
<td>(0.72 - 1.13)</td>
<td>(0.54 - 0.78)</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>8</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>FX (IU/ml)</td>
<td>1.11</td>
<td>0.96</td>
<td>0.81</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>(0.90 - 1.28)</td>
<td>(0.92 - 1.15)</td>
<td>(0.66 - 1.05)</td>
<td>(0.68 - 0.92)</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>8</td>
<td>11</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 4.2 Clotting factors in sickle cell disorders. Median results are reported (with interquartile range in parentheses). Reference ranges: Fg: 1.4 - 3.3 g/L; FII: 0.92 - 1.43 IU/ml, FVII: 0.70 - 1.45 IU/ml, FX: 0.82 - 1.56 IU/ml (M), 0.57 - 1.34 IU/ml (F).

Figure 4.1 Fibrinogen levels in sickle cell disorders. Dotted lines indicate the limits of the reference range.
Figure 4.2  Factor II levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.

Figure 4.3  Factor VII levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.
4.3.4 Markers of coagulation system activation

Plasma levels of F1+2 and TAT were measured to assess the degree of in vivo thrombin generation. FVIIa was measured to assess the extent of TF-mediated activation. Summary data is shown in Table 4.3.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
<th>HbAS</th>
<th>HbSC</th>
<th>HbSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1+2 (nmol/L)</td>
<td>0.6 (0.5 - 0.8)</td>
<td>0.6 (0.4 - 0.6)</td>
<td>0.9 (0.6 - 1.2)</td>
<td>1.3 (0.7 - 1.7)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 7</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>TAT (μg/L)</td>
<td>2.8 (2.1 - 4.3)</td>
<td>2.2 (1.1 - 2.9)</td>
<td>6.0 (4.2 - 10.7)</td>
<td>10.6 (5.6 - 13.7)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 7</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>FVIIa (ng/ml)</td>
<td>1.64 (1.08 - 2.23)</td>
<td>1.40 (0.28 - 2.94)</td>
<td>1.45 (0.84 - 2.00)</td>
<td>0.90 (0.66 - 1.30)</td>
</tr>
<tr>
<td>n = 24</td>
<td>n = 6</td>
<td>n = 12</td>
<td>n = 31</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Markers of thrombin generation and tissue factor-mediated activation in sickle cell disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: F1+2: 0.2 - 1.5 nmol/L, TAT: 0.8 - 10.1 μg/L, FVIIa: 0.41 - 4.14 ng/ml.
Increased thrombin generation was demonstrated by significantly elevated F1+2 and TAT levels in both HbSC (p < 0.02) and HbSS (p < 0.0001) (Figures 4.5 and 4.6). Levels of both variables in HbSC and HbSS were not substantially different, nor was the proportion of patients with abnormal levels within the two groups: elevated levels of either or both markers of thrombin generation were found in 5/13 patients with HbSC and 17/31 (55%) patients with HbSS. F1+2 and TAT levels were weakly correlated in HbSC (rₚ = 0.66, p < 0.02), but not in HbSS (Figure 4.7) and the relationship between F1+2 and TAT levels in HbSC was more apparent when atypical results from one patient (F1+2: 2.7 nmol/L; TAT: 4.4 μg/L) were excluded (rₚ = 0.82, p < 0.002).

There were no statistically significant associations between the clotting factor levels assessed and F1+2 or TAT in HbSC or HbSS. Neither was there an apparent association between elevated F1+2 or TAT levels and any clinical variable (i.e. splenectomy, history of thrombosis, exchange transfusion, hydroxyurea therapy or OC use), although the numbers in each of these groups were quite small.

![Figure 4.5](image.png)
Figure 4.6  Thrombin:antithrombin levels in sickle cell disorders. The dotted line indicates the upper limit of the reference range.

Figure 4.7  The relationship between prothrombin fragment 1+2 and thrombin:antithrombin complex levels in patients with sickle cell disease (see text). Dotted lines indicate the upper limits of the respective reference ranges.
Tissue factor-mediated activation in sickle cell disorders

FVIIa levels were significantly reduced in HbSS patients (p = 0.001) but not in HbSC and no subject had elevated levels (Figure 4.8). FVIIa and FVII levels were strongly correlated in HbSC (r_s = 0.88, p = 0.0001) but not in HbSS.

4.3.5 Genetic mutations associated with an increased risk of venous thrombosis

The prevalence of FVL and PGM is now known to be very low in Black populations of African descent although this had not been established when I undertook my study. However, the presence of either mutation cannot be arbitrarily excluded in individual patients and both were sought in this section.

Activated protein C resistance

Mildly abnormal modified APCR ratios in two HbSC and five HbSS subjects (ratios: 2.20 - 2.34) were unlikely to be due to the FVL mutation (see section 2.2.7) but were subjected to PCR analysis for confirmation; all were normal. A ratio of 1.98 in one HbSS patient was confirmed in a second sample collected after a six week interval and may have been due to her OC use. A strong association between low APCR ratios and TAT levels (r_s = -0.91, p = 0.002; n = 8) was not evident when ratios were normal.
Clotting times in HbSC and HbSS were shorter than the control group in both the presence and absence of APC in this APTT-based test (99.8 and 98.8 vs. 112.0 s, p < 0.0001 respectively; 39.5 and 39.3 vs. 43.6 s, p < 0.001 and 0.0001 respectively). This contrasted with the APTT measurements in section 4.3.2 and may be attributable to the different activators (i.e. kaolin and silica) and PL formulations employed in the two methods.

Prothrombin G20210A
26 / 44 SCD patients were tested for PGM: all were normal.

4.3.6 Phospholipid-dependent antibodies

The LA as well as aCL and β2GPI antibodies were sought in this section and results are summarised in Table 4.4. LA was not detected in any patient: none yielded an abnormal DRVVT ratio using dilute PL. It was not possible to perform DRVVTs for three HbSS patients due to insufficient plasma volume, however each had a normal APTT (LA will often prolong the APTT).

Moderately elevated IgG aCL (15 - 20 GPL units) were detected in four HbSS patients; weakly abnormal levels were detected in a further five (5 - 6 GPL units), as well as two HbSC patients (7 & 10 GPL units). The higher incidence of IgG aCL positive patients in HbSS relative to HbSC was not statistically significant. IgM aCL were detected in one HbAA and HbSC subject only (14 and 20 MPL units respectively) and no patient had increased titres of both IgG and IgM aCL. It was possible to repeat measurements in five of the IgG aCL positive patients: each were negative at the next presentation.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
<th>HbAS</th>
<th>HbSC</th>
<th>HbSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRVVT (Positive / Tested)</td>
<td>0 / 27</td>
<td>0 / 8</td>
<td>0 / 13</td>
<td>0 / 28</td>
</tr>
<tr>
<td>IgG aCL (Positive / Tested)</td>
<td>1 / 26</td>
<td>0 / 6</td>
<td>2 / 12</td>
<td>9 / 31</td>
</tr>
<tr>
<td></td>
<td>(3.9 %)</td>
<td>(0 %)</td>
<td>(16.7 %)</td>
<td>(29.0 %)</td>
</tr>
<tr>
<td>IgM aCL (Positive / Tested)</td>
<td>1 / 26</td>
<td>0 / 6</td>
<td>1 / 12</td>
<td>0 / 31</td>
</tr>
<tr>
<td></td>
<td>(3.9 %)</td>
<td>(0 %)</td>
<td>(8.3 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td>Anti β2GPI antibodies</td>
<td>None detected</td>
<td>Not tested</td>
<td>None detected</td>
<td>None detected</td>
</tr>
</tbody>
</table>

Table 4.4 Tests for phospholipid-dependent antibodies in sickle cell disorders. Median PL ratios are reported (with the interquartile range in parentheses). aCL reported as the number abnormal / tested (see text).
4.3.7 Physiological inhibitors of coagulation

Physiological inhibitors (ATIII, HCII, PC, its co-factor PSf and \(\alpha_1\)AT) were measured. Summary data is shown in Table 4.5. Low levels of at least one inhibitor were detected in 7/13 HbSC and 25/31 HbSS patients: there was no consistent pattern of deficiencies amongst either group, although HCII deficiency was easily the most common (Appendix IV). There was no significant association between reduced levels of any inhibitor and elevated F1+2 or TAT levels in HbSC or HbSS.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
<th>HbAS</th>
<th>HbSC</th>
<th>HbSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIII (IU/ml)</td>
<td>1.05 (1.00 - 1.10)</td>
<td>1.01 (0.99 - 1.13)</td>
<td>1.01 (0.87 - 1.10)</td>
<td>0.90 (0.81 - 1.04)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 8</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>HCII (U/ml)</td>
<td>1.24 (0.98 - 1.37)</td>
<td>1.18 (1.09 - 1.20)</td>
<td>0.68 (0.60 - 0.76)</td>
<td>0.66 (0.57 - 0.72)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 8</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>PC (IU/ml)</td>
<td>0.98 (0.89 - 1.18)</td>
<td>0.98 (0.92 - 1.11)</td>
<td>0.91 (0.71 - 1.00)</td>
<td>0.78 (0.68 - 0.91)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 8</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>PSf (IU/ml)</td>
<td>0.84 (0.71 - 0.97)</td>
<td>0.81 (0.67 - 0.89)</td>
<td>0.88 (0.71 - 0.96)</td>
<td>0.77 (0.55 - 1.01)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 8</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>(\alpha_1)AT (U/ml)</td>
<td>1.14 (1.07 - 1.37)</td>
<td>1.13 (1.07 - 1.32)</td>
<td>1.00 (0.91 - 1.01)</td>
<td>1.21 (1.00 - 1.31)</td>
</tr>
<tr>
<td>n = 28</td>
<td>n = 8</td>
<td>n = 13</td>
<td>n = 31</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Physiological inhibitors of coagulation in sickle cell disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: ATIII: 0.80 - 1.25 IU/ml, HCII: 0.71 - 1.79 U/ml, PC: 0.59 - 1.59 IU/ml, PSf: 0.60 - 1.35 (M), 0.44 - 1.04 IU/ml (F), \(\alpha_1\)AT: 0.78 - 1.62 U/ml.

Antithrombin III (ATIII)

Low ATIII levels were detected in 4/13 HbSC and 8/31 (26 %) HbSS patients, but overall, levels were significantly altered in HbSS only (\(p < 0.001\), Figure 4.9).

Heparin cofactor II (HCII)

Low HCII was a common finding in SCD; 7/13 (54%) HbSC and 23/31 (74%) HbSS patients had reduced levels. The contrast between HCII levels in HbSC and HbSS relative to the control group was striking and highly significant in both groups and levels were similar in HbSC and HbSS patients (Figure 4.10). ATIII and HCII were correlated in both HbSC and HbSS (HbSC: \(r_s = 0.56\), \(p < 0.05\), HbSS: \(r_s = 0.57\), \(p < 0.001\)). The HbSS patient with > 1.2 U/ml HCII was a 52 year old man who, ironically,
experienced thrombotic strokes at 26 (when he was a heavy smoker) and at 51 years of age (after he had stopped smoking).

**Figure 4.9** Antithrombin III levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.

**Figure 4.10** Heparin cofactor II levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.
Protein C and free protein S

There was a clear tendency toward lower PC levels in the HbSS patients relative to the control group although only 3/31 HbSS patients were overtly deficient (Figure 4.11). Reduced PC levels were also detected in 2/13 HbSC patients although levels in HbSC were comparable to the controls overall. Two HbSC patients (one male and one female) and five male HbSS patients yielded low PSf, however levels in the clinical groups did not differ significantly from the controls, nor was there a significant difference in PSf levels between HbSC and HbSS (Figure 4.12). ATIII and PC were correlated in HbSC and HbSS (HbSC: $r_s = 0.62$, $p < 0.05$, HbSS: $r_s = 0.42$, $p < 0.02$). There was a positive association between HCII and PC ($r_s = 0.70$, $p < 0.01$) and between PC and PSf ($r_s = 0.65$, $p < 0.02$) in HbSC, but not in HbSS. Conversely, ATIII and PSf were positively associated in HbSS only ($r_s = 0.52$, $p < 0.005$).

Alpha-1-antitrypsin

$\alpha_1$AT levels in the HbSC group were significantly lower than both the controls and HbSS patients ($p < 0.01$), although only one patient was actually deficient (Figure 4.13). $\alpha_1$AT correlated with HCII in HbSS only ($r_s = 0.44$, $p < 0.02$).

![Figure 4.11 Protein C levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.](image-url)
Figure 4.12 Free Protein S levels in sickle cell disorders. The broken and dotted lines indicate the lower limit of the reference range for males and females respectively. * indicate abnormal values in male patients.

Figure 4.13 Alpha-1-antitrypsin levels in sickle cell disorders. The dotted lines indicate the limits of the reference range.
4.3.8 Serum lipids

CHOL was moderately reduced in SCD (Table 4.6) and the difference between levels in both genotypes compared to controls was highly significant (p = 0.0002 and p < 0.0001 in HbSC and HbSS respectively, Figure 4.14). CHOL tended to be lower in HbSS than HbSC whereas TRIG levels were not significantly altered. CHOL and TRIG were well correlated in HbSC (r_s = 0.75, p < 0.01) but not in HbSS and there was a positive association between CHOL and PC within both groups (r_s = 0.66, p < 0.02; r_s = 0.52, p < 0.005 respectively). TRIG was weakly correlated with PSf in HbSC only (r_s = 0.59, p < 0.05).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
<th>HbAS</th>
<th>HbSC</th>
<th>HbSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOL (mmol/L)</td>
<td>5.0 (4.4 - 5.2)</td>
<td>4.8 (4.3 - 5.2)</td>
<td>3.8 (3.6 - 4.1)</td>
<td>3.1 (2.7 - 3.5)</td>
</tr>
<tr>
<td>n = 25</td>
<td>n = 6</td>
<td>n = 12</td>
<td>n = 29</td>
<td></td>
</tr>
<tr>
<td>TRIG (mmol/L)</td>
<td>0.8 (0.6 - 1.4)</td>
<td>0.8 (0.7 - 1.2)</td>
<td>0.7 (0.5 - 0.8)</td>
<td>0.7 (0.4 - 1.1)</td>
</tr>
<tr>
<td>n = 25</td>
<td>n = 6</td>
<td>n = 12</td>
<td>n = 29</td>
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</tbody>
</table>

Table 4.6 Serum lipids in sickle cell disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: CHOL: 3.6 - 6.5 mmol/L, TRIG: 0.5 - 1.8 mmol/L.

Figure 4.14 Cholesterol levels in sickle cell disorders. Dotted lines indicate the limits of the reference range.
4.3.9 Components of fibrinolysis

D-D, Plg and α₂AP were measured and summary data is shown in Table 4.7.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Black)</th>
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</thead>
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<tr>
<td>D-Dimer</td>
<td>18</td>
<td>16</td>
<td>69</td>
<td>201</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(16 - 31)</td>
<td>(16 - 39)</td>
<td>(31 - 99)</td>
<td>(96 - 426)</td>
</tr>
<tr>
<td>n</td>
<td>28</td>
<td>7</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>Plg</td>
<td>1.13</td>
<td>1.21</td>
<td>1.08</td>
<td>0.97</td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(1.03 - 1.24)</td>
<td>(1.08 - 1.40)</td>
<td>(0.91 - 1.13)</td>
<td>(0.86 - 1.05)</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>8</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>α₂AP</td>
<td>1.05</td>
<td>1.06</td>
<td>0.98</td>
<td>0.94</td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(1.04 - 1.12)</td>
<td>(0.99 - 1.20)</td>
<td>(0.91 - 1.08)</td>
<td>(0.84 - 1.06)</td>
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<tr>
<td>n</td>
<td>28</td>
<td>8</td>
<td>13</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 4.7 Components of fibrinolysis in sickle cell disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: D-Dimer: < 120 ng/ml, Plg: 0.90 - 1.31 IU/ml, α₂AP: 0.89 - 1.26 IU/ml.

D-Dimers

Two HbSC patients had abnormal D-D levels (Figure 4.15). In contrast, D-D were considerably increased in HbSS, with 20/31 patients yielding elevated levels and the difference compared to the control group was highly significant (p < 0.0001). D-D levels correlated with F1+2 in HbSS (rₛ = 0.46, p < 0.01), but no other associations with F1+2 or TAT levels in HbSC or HbSS were observed.

Plasminogen and alpha-2-antiplasmin

Median Plg levels were significantly lower than controls in HbSS, but not in HbSC patients however the range of levels within the two genotypes was similar (Figure 4.16). α₂AP followed a similar pattern to Plg; α₂AP levels in HbSC and HbAA were not significantly different, whereas they tended to be lower in HbSS (p < 0.0005, Figure 4.17). α₂AP were correlated with ATIII and HCII levels in both HbSC (rₛ = 0.76, p < 0.0005 and rₛ = 0.86, p = 0.0001 respectively) and HbSS (rₛ = 0.72, p < 0.0001; rₛ = 0.64, p < 0.0001).
**Figure 4.15** D-Dimer levels in sickle cell disorders. The dotted line indicates the upper limit of the reference range.

**Figure 4.16** Plasminogen levels in sickle cell disorders. The dotted line indicates the lower limit of the reference range.
4.3.10 Markers of endothelial cell activation

Plasma levels of sE-s and sTM were measured to assess endothelial cell activation and damage. Summary data is shown in Table 4.8. sE-s levels were significantly elevated in HbSS (p < 0.001) but not in HbSC (Figure 4.18): 11/31 (36%) HbSS patients yielded abnormal levels (range: 112 - 214 ng/ml). The variability of sE-s levels in HbSS patients negated a statistically significant difference between HbSS and HbSC, but the fact that abnormal levels were observed in HbSS patients only is likely to be important.

Abnormal sE-s levels in HbSS were associated with elevated WBCs (14.0 vs. 9.2 x 10^9/L, p = 0.0005) as well as slightly higher α1AT (1.29 vs. 1.16 U/ml, p < 0.05) and platelets (457 vs. 291 x 10^9/L, p < 0.002). Interestingly, there was a weak association between sE-s and age, with higher levels observed in patients ≤ 30 years (119 vs. 59 ng/ml, p < 0.05). This paralleled a similar association between the WBC and age (13.1 vs. 9.2 x 10^9/L, p < 0.005), although sE-s and WBCs were not correlated within the two age brackets. sTM levels in HbSC and HbSS were not significantly altered from those of the control group and no patient yielded abnormal levels.
Table 4.8 Markers of endothelial cell activation in sickle cell disorders. Median results are reported (with interquartile range in parentheses). Reference ranges: sE-s: 24.5 - 93.5 ng/ml, sTM: 15.3 - 93.9 ng/ml.

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<th>HbSC</th>
<th>HbSS</th>
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<td>sE-s (ng/ml)</td>
<td>45.7 (35.1 - 57.5)</td>
<td>43.5 (32.6 - 51.5)</td>
<td>67.4 (57.7 - 83.6)</td>
<td>73.3 (55.5 - 123.3)</td>
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<td>n = 26</td>
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<td>n = 12</td>
<td>n = 31</td>
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<tr>
<td>sTM (ng/ml)</td>
<td>37.2 (26.9 - 51.8)</td>
<td>Not Tested</td>
<td>57.6 (49.0 - 64.9)</td>
<td>51.6 (41.5 - 72.0)</td>
</tr>
<tr>
<td>n = 15</td>
<td>n = 13</td>
<td>n = 29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.18 Soluble E-selectin levels in sickle cell disorders. The dotted line indicates the upper limit of the reference range.
4.3.11 Acute phase reactivity

I sought evidence of acute phase reactivity in my patients by measuring levels of the acute phase protein CRP. To confirm that this marker was sufficiently sensitive, I tested 12 samples from nine HbSS patients who were not enrolled in my study and were not in 'steady-state': samples were collected during the recovery period following priapism, cardiac arrest, portal vein thrombosis or crisis episodes and were expected to show raised CRP levels consistent with inflammation: CRP levels in healthy subjects are ≤ 0.50 mg/dL; levels above 1.0 mg/dL are consistent with acute phase reactivity (Singhal et al, 1993). CRP levels ranged between 4.50 and 12.7 mg/dL in each ‘recovery’ sample, demonstrating that acute phase reactivity was easily detectable using this marker.

CRP is a rapidly responding acute phase protein whereas Fg responds more slowly. CRP was increased in one HbSC patient discussed earlier (CRP: 1.43 mg/dL), which was consistent with his relatively recent history of portal vein thrombosis (section 4.3.2). Elevated CRP reflected acute phase reactivity in 8/29 (28%) of the HbSS patients tested (Table 4.9); three of these also had elevated Fg, suggesting a prolonged episode. A tendency toward higher levels of other acute phase reactants (HCII, α1AT and α2AP) was not statistically significant in these patients and were not correlated with CRP or Fg. CRP was not correlated with F1+2, TAT, D-D or sE-s levels.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>CRP (mg/dL)</th>
<th>Fg (g/L)</th>
<th>WBC (×10⁹/L)</th>
<th>Transfused</th>
</tr>
</thead>
<tbody>
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<td>Y</td>
</tr>
<tr>
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<td>M</td>
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<td>1.9</td>
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<tr>
<td>DOS</td>
<td>M</td>
<td>5.09</td>
<td>4.5</td>
<td>9.3</td>
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<td>KAC</td>
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<td>3.3</td>
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<td>12.0</td>
<td>N</td>
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</table>

Table 4.9 Evidence of acute phase reactivity in a subgroup of 'steady-state' HbSS patients. Reference ranges: CRP: ≤ 0.50 mg/dL, acute phase reactivity: > 1.0 mg/dL; Fg: 1.4 – 3.3 g/L; WBC: 3.0 – 10.0 × 10⁹/L.
4.3.12 Underlying pathology that may contribute to hypercoagulability

Diabetes, thyroid dysfunction and cardiac anomalies may be encountered in association with SCD and each are recognised risk factors for thrombosis. Biochemical investigations to assess pancreatic, thyroid and cardiac function (requested by the clinician) were available for many of the SCD patients. Tests were not necessarily performed at the same time as the samples for my study, however successive measurements were reasonably consistent within individuals. Thyroid stimulating hormone and free thyroxin were measured to assess thyroid function; fasting glucose was determined. AST was used to screen for abnormal cardiac function and creatinine kinase measurement was available when cardiac dysfunction was suspected clinically, however these enzymes are derived from both cardiac and hepatic tissue and raised levels may reflect dysfunction of either organ.

Diabetes

Mild hyperglycaemia (glucose: 5.7 – 7.0 mmol/L) was detected in 4/8 HbSC and 9/21 HbSS patients tested. An additional HbSC patient showed evidence of poorly controlled diabetes, with 18.5 mmol/L glucose and elevated fructosamine (495 μmol/L: Reference range: 215 – 264 μmol/L). Normal sodium and potassium levels indicated no patient was dehydrated and glucose levels were not associated with age.

Levels of clotting factors and inhibitors tended to be higher in hyperglycaemic HbSS patients, however there was no difference in the amount of thrombin generated as indicated by F1+2, TAT and D-D levels.

Thyroid function

Thyroid function tests were performed for five HbSC and 16 HbSS patients. Adequately compensated hypothyroidism was detected in one HbSS patient (free thyroid stimulating hormone: 5.5 μU/ml, reference range: 0.5 – 5.0 μU/ml; free thyroxin: 11.8 pmol/L, reference range: 9.1 – 23.8 pmol/L), free thyroxin was mildly reduced in another (8.8 pmol/L) and the remainder were normal.

Cardiac abnormalities

AST levels were abnormal in 14 HbSS patients and the single HbSC patient with a history of liver pathology; in the absence of any symptomatic cardiac dysfunction, these were thought to be hepatocellular derived and creatinine kinase measurement had not been indicated for any patient.
4.3.13 Biochemical tests of liver function (LFTs)

In this section, I aimed to assess the extent of any hepatic insufficiency in my SCD patients and to assess its likely contribution to the altered plasma levels of haemostatic proteins observed earlier in the chapter. The LFT panel performed routinely at UCLH measured plasma levels of hepatocellular-derived TBIL, Alk phos, AST and albumin (section 2.6): reduced albumin concentrations attest to significantly impaired synthetic capability while abnormal levels of the remaining variables may occur as a consequence of various hepatocellular pathologies; the character and progression of disease determines which LFTs are abnormal and to what extent. In addition, I measured serum LDH, a hepatocellular enzyme that is released into the circulation following cell injury; LDH is also present in RBCs and is released in vast quantities by haemolysed RBCs, so haematological variables that reflected the degree of in vivo haemolysis (i.e. reties, Hb and RCC) were simultaneously evaluated to help gauge the impact of haemolysis on abnormal TBIL and LDH levels. This analysis was limited to untransfused patients, however the biochemical profiles in transfused patients were entirely consistent with haemolysis (i.e. they had no evidence of hepatic insufficiency). The HbSC patient with clinical liver pathology discussed in section 4.3.2 was omitted from this assessment.

Results

LFTs in the untransfused HbSC (n = 11) and HbSS (n = 19) patients are shown in Table 4.10. Broadly, TBIL levels were appreciably higher in HbSS than HbSC: 45 (32–52) vs. 22 (20–25) μmol/L, p < 0.0001; Alk phos was normal in the majority of patients in both groups; AST was considerably higher in HbSS, with mildly abnormal levels in approximately half the patients: 58 (48 – 64) vs. 25 (23 – 31) IU/L, p < 0.0005; albumin concentrations were normal in all. LDH levels were considerably higher in HbSS: 891 (619 – 1043) vs. 396 (354 – 542) BB U/ml, p < 0.0005.

Haemolysis

TBIL was mildly raised in all HbSC patients and several also yielded elevated LDH. LDH was strongly correlated with RCC (r_s = -0.76, p < 0.01) and retics (r_s = 0.71, p < 0.02) in HbSC, suggesting that increased LDH levels were primarily due to haemolysis in this group; raised TBIL levels were therefore considered to be essentially attributable to haemolysis in HbSC patients. TBIL and LDH were elevated in all HbSS patients, however LDH was not correlated with TBIL, Retics or RCC.
LFTs were assessed in collaboration with a clinician: AST levels more than twice the upper limit of the reference range (i.e. > 110 IU/ml) were broadly designated criteria for significant hepatocellular damage within this clinical population. AST levels were normal in all HbSC patients and no HbSS patient yielded > 110 IU/ml AST.

Elevated TBIL levels in 8/11 HbSC and 8/19 HbSS patients in whom the remaining LFTs were normal, were entirely consistent with haemolysis. Mildly elevated Alk phos observed in two HbSC and four HbSS patients aged between 12 and 19 years was attributed to adolescent bony growth. Mildly raised Alk phos and normal AST in a 59 year old HbSC patient was consistent with chronic cholecystitis (confirmed in medical records). 10 of the remaining 11 HbSS patients yielded only mildly raised AST: one (ID: OLD) also had mildly elevated Alk phos, suggestive of cholestasis. Serum ferritin levels above 3000μg/L in two patients (ID: ADA & OLD) confirmed significant iron overload. Normal albumin concentrations in the 30 patients assessed in this section (as well as in the transfused patients) indicated that any compromise in liver function was not severe as levels are affected only when liver dysfunction is pronounced.

AST was mildly elevated in only two of the four HbSS patients in whom PTs were marginally abnormal (section 4.3.2). Examination of clotting factor levels did not aid the interpretation of LFTs in this cohort as levels were similar in patients with normal and abnormal AST and there was no relationship between abnormal AST levels and any of the clotting factors, nor the physiological inhibitors, F1+2, TAT or D-D levels. Plg, α2AP and HCII may be reduced in association with liver disease and these variables were significantly altered in HbSS but they bore no relationship to AST levels.

Summary

Biochemical evidence of liver significant hepatocellular damage was clearly excluded in 11/11 HbSC and 12/19 (63%) HbSS patients. Mildly raised AST and/or Alk phos levels in seven HbSS patients denoted a degree of mild hepatocellular damage that was not considered alarming within the context of SCD. Substantial iron overload meant that iron toxicity was likely in two patients, one of whom had cholestasis.
<table>
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<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>TBil (µmol/L)</th>
<th>Alk Phos (IU/L)</th>
<th>AST (IU/L)</th>
<th>Albumin (g/L)</th>
<th>LDH (BB U/ml)</th>
<th>Ferr (µg/L)</th>
<th>Code</th>
</tr>
</thead>
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</table>

Table 4.10  Biochemical tests of liver function, red blood cell parameters and ferritin levels in untransfused patients with sickle cell disease. Reference ranges: TBIL: 3-17 µmol/L; Alk Phos: 100-280 IU/L; AST: 11-55 IU/ml; Albumin: 35-53 g/L; LDH: 231-381 BB U/ml. Ferr: 22-275(M); 7-204(F) µg/L. * normal at contiguous presentations. Code: H: haemolysis; B: adolescent bony growth; Cy: cholecystitis; Ch: cholestasis; I: substantial iron overload; LD: hepatocellular damage. Interpretation as per text.
4.3.14 The effect of transfusion on haemostatic variables in HbSS patients

It was possible that transfusion-induced haemodynamic fluctuations affect plasma levels of clotting proteins and consequently influence thrombin generation. Ideally, the effect of transfusion would be assessed in longitudinal studies, monitoring patients prior to and following the commencement of transfusion, but longitudinal data was available on only one patient, who was lost to follow-up after only three transfusions when his family emigrated. In that patient, Hb rose from 6.1 to 9.3 g/dL, HbA rose from 0 to 86%; HbS fell from 98 to 13% and HbF remained essentially unchanged after three exchange transfusions on days 0, +16 and +35. Even after adjustment for haemodynamic changes relative to the haematocrit on day 0, levels of all the haemostatic parameters tested had fallen to abnormal levels by +35 days; this was likely to be an effect of haemodilution and statistical analysis showed a negative association between many of the variables and the HCT, however the data points were too small to be reliable. However, the magnitude of reductions in F1+2 (0.70 to 0.27 nmol/L), TAT (11.1 to 1.4 μg/L), D-D (155 to 28 ng/ml) and sE-s (130 to 63 ng/ml) were greater than the increase in HCT and the variables were not correlated; transfusion appeared to ameliorate thrombin generation and endothelial cell activation in this patient.

I was obliged to assess the impact of transfusion in my HbSS cohort indirectly, comparing variables in transfused and untransfused patients. The effect of transfusion was not assessed in HbSC as only one patient from that group was transfused.

Patients

This analysis considered the regularly transfused HbSS patients, who underwent exchange transfusions of 2-4 units of RBCC every 4-6 weeks (see Table 4.11). Four ‘recently’ transfused patients were specifically excluded from this assessment. The transfused (HbSS(tx)) and untransfused (HbSS(untx)) groups were assessed in relation to the Black HbAA controls and to each other.
Table 4.11 Characteristics of regularly transfused HbSS patients.

### Results

**Untransfused HbSS patients**

In general, the differences noted between the HbSS and HbAA control groups earlier in the chapter were evident in HbSS(untx), with similar degrees of significance (Appendix V). There was evidence of enhanced thrombin generation and endothelial cell activation (raised F1+2, TAT, D-D and sE-s levels); levels of clotting factors (II, VII, X and VIIa) and inhibitors (ATIII, HCII and PC) were reduced; Plg and α2AP were lower than the controls, but not overtly abnormal. Fg levels were an exception, however: Fg was slightly, yet significantly higher in HbSS(untx) compared to the controls (2.9 vs. 2.2 g/L, p < 0.05), but similar to HbSS(tx). Plg, another acute phase protein, showed a similar pattern and was weakly associated with Fg in HbSS(untx) (r_s = 0.57, p < 0.02).

Low PC levels correlated strongly with FII (r_s = 0.79, p < 0.0001) and FX (r_s = 0.64, p < 0.005) and less well with FVII (r_s = 0.48, p < 0.05), suggesting a common mechanism of deficiency. There was no association with AST levels.

sE-s and WBC (Figures 4.19 & 4.20) were strongly associated in HbSS(untx) (Figure 4.21); sE-s also correlated strongly with the absolute neutrophil count (r_s = 0.70, p < 0.001) and platelets (r_s = 0.64, p < 0.005). In turn, WBCs and platelets were positively associated (r_s = 0.71, p < 0.001). A weaker association between sE-s and the absolute eosinophil count was also evident (r_s = 0.57, p < 0.02). Of the 19 untransfused
HbSS patients, 11 had elevated WBCs and 9 of these also had elevated sE-s (Figure 4.21): the eight remaining patients had normal levels of both variables. Collectively, these associations provide convincing evidence of a relationship between endothelial cell activation and inflammation in HbSS(untx) that was not observed in HbSS(tx), nor in the untransfused HbSC patients (data not shown).

There was no association between elevated sE-s and any other haemostatic, haematological or biochemical variable in HbSS(untx). Specifically, raised sE-s did not correlate with increased F1+2 or TAT. 8/9 HbSS patients with raised sE-s had increased D-D, however fibrin deposition had also occurred in 7/10 patients with no evidence of endothelial cell activation and there was no relationship between the variables.

**Transfused HbSS patients**

Predictably, RBC parameters were improved in transfused patients compared to HbSS(untx): Hb: 10.5 (9.3 – 10.8) vs. 7.9 (7.3 – 9.2) g/dL; RCC: 3.55 (3.17 – 3.70) vs. 2.50 (2.10 – 2.85) x 10^9/L; HCT: 0.32 (0.28 – 0.32) vs. 0.24 (0.21 – 0.27) l/l. No other significant haematological differences were noted. Median HbS was 36.5% compared to 91.5% in HbSS(untx) patients and HbF was significantly lower in HbSS(tx) (2.1 vs. 8.5%, p < 0.02).

Raised F1+2, TAT and D-D as well as a relative reduction in FVIIa, FII, FVII, FX, HCII and α2AP levels persisted in HbSS(tx). Conversely, ATIII, PC, Plg, Fg, and sE-s (which were altered in untransfused patients) approximated levels observed in the control group (Appendix V). Associations between PC, FII, FX, FVII and Fg observed in HbSS(untx) subjects were not evident in this smaller transfused group. However, there was a tendency toward lower AST levels in HbSS(tx); also, where 10/19 untransfused patients had mildly raised levels, AST was overtly abnormal in only one transfused HbSS patient. The number of transfused patients in this evaluation is quite small, but this data suggests that regular exchange transfusions may lessen insult to the liver in HbSS patients.

**Comparison between untransfused and transfused HbSS patients**

Haemostatic variables in HbSS(untx) and HbSS(tx) were compared (Appendix V). Notably, TAT and HCII levels were not significantly different (Figures 4.22 and 4.23). In fact, PC, D-D and sE-s were the only variables that showed substantial improvement in transfused patients: PC levels were normal in HbSS(tx) and not significantly different from the control group (Figure 4.24); D-D were significantly higher in both HbSS(tx) and HbSS(untx) compared to the control group (140 and 340 vs. 18 ng/ml, p < 0.0001.
respectively): the highest levels were found in untransfused patients and the difference between the two HbSS groups was significant ($p < 0.05$, Figure 4.25). Interestingly, the moderately raised sE-s levels seen in HbSS(untx) were not evident in transfused patients (Figure 4.19); only one transfused patient showed evidence of mild endothelial cell activation compared to 50% of untransfused patients. The highest WBCs were observed in untransfused patients, although this was not statistically significant due to variable counts within that group (Figure 4.20).

**Figure 4.19** The impact of regular exchange transfusions on soluble E-selectin levels in patients with sickle cell disease. The dotted line indicates the upper limit of the reference range.
Figure 4.20  The impact of regular exchange transfusions on total leucocyte counts in patients with sickle cell disease. The dotted line indicates the upper limit of the reference range.

Figure 4.21 The relationship between total leucocyte counts and soluble E-selectin levels in untransfused HbSS patients. Dotted lines indicate the upper limits of the respective reference ranges.
Figure 4.22  The impact of regular exchange transfusions on thrombin:antithrombin levels in patients with sickle cell disease. The dotted line indicates the upper limit of the reference range.

Figure 4.23  The impact of regular exchange transfusions on heparin cofactor II levels in patients with sickle cell disease. The dotted line indicates the lower limit of the reference range.
Figure 4.24 The impact of regular exchange transfusions on protein C levels in patients with sickle cell disease. The dotted line indicates the lower limit of the reference range.

Figure 4.25 The impact of regular exchange transfusions on D-Dimer levels in patients with sickle cell disease. The dotted line indicates the upper limit of the reference range.
The acute effect of transfusion on haemostatic variables in sickle cell disease

The median time since the most recent transfusion in this HbSS cohort was 21 (range 9 to 48) days and it is possible that some short-term haemostatic benefits of transfusion were lost once the percentage of HbS returned to a critical level. The opportunity arose to examine acute changes in haemostatic variables in samples collected shortly following transfusion. Variables were measured in 13 samples from 10 SCD patients (nine HbSS and one HbSC) who were transfused 2 - 4 units of RBCC. Four were in 'steady-state'; the others presented with vasocclusive crisis, pregnancy, thrombosis or myelodysplastic disease.

Results

As expected, each patient showed an increase in HbA: 44.8 (37.6 – 55.5) vs. 61.2 (56.6 – 67.7) %, with a corresponding fall in HbS: 47.0 (36.5 – 53.5) vs. 33.5 (27.4 – 39.4) % following transfusion. Haemostatic variables in post transfusion samples were adjusted for small changes in HCT. Unexpectedly, TAT showed a dramatic increase of up to 20 times those of pre-transfusion levels in all but one sample (Figure 4.26). The patient in whom post-transfusion TAT was 258 µg/L was hospitalised with a portal vein thrombosis and interestingly, the only (HbSC) patient in whom TAT levels showed no real change following the transfusion was pregnant and anticoagulated with fragmin. These two patients were clearly atypical of the remainder of the group and when they were excluded from statistical analysis, there was a strong association between pre-transfusion TAT levels and the magnitude of their increase (rs = -0.82, p < 0.005) but not the number of units transfused: TAT levels rose from 9.5 (4.3 – 11.0) to 41.6 (30.0 – 54.5) µg/L, p < 0.0001, accompanied by a similar degree of change in F1+2: 0.7 (0.6 – 1.7) vs. 1.5 (1.0 – 2.3) nmol/L, which was not statistically significant, but the percentage increase in the two variables correlated well (rs = 0.91, p < 0.0001). Three of the patients were evaluated on two separate occasions (6, 9 and 85 days apart); TAT levels had returned to ‘baseline’ levels prior to the second transfusion in each.

ATIII, HCII, PC, α1AT, Plg, α2AP and Fg showed a persistent, though less dramatic fall immediately following exchange transfusion; this was statistically significant for ATIII: 0.94 (0.76 – 1.01) vs. 0.81 (0.63 – 0.87) IU/ml; Plg: 0.97 (0.80 – 1.12) IU/ml and α2AP: 0.93 (0.79 – 1.03) vs. 0.80 (0.65 – 0.92) IU/ml, p < 0.05 respectively, however none correlated with the increase in F1+2 or TAT.
Figure 4.26 Thrombin:antithrombin levels in paired samples from transfused patients with sickle cell disease. Samples were collected from 9 HbSS and 1 HbSC patients: TAT levels in samples collected 30 minutes post-transfusion were corrected for haemodynamic changes. The dotted line indicates the upper limit of the reference range. The arrow indicates the only anticoagulated (HbSC) patient. The p value was derived using the Wilcoxon matched-pairs signed rank test, excluding data from the two patients denoted by * as per text.

4.3.15 Thromboembolic history and sickle cell crisis

Thromboembolic history

It was not possible to evaluate potential relationships between abnormal plasma levels of haemostatic variables and a history of thromboembolic manifestations as only five of the patients studied (two with HbSC, three with HbSS) had previously experienced such complications (venous thromboembolism: n = 3; stroke: n = 2). These groups were too small to enable convincing conclusions to be drawn, and variation in the clinical presentations would further complicate any interpretation.

Sickle cell crisis

The aim of this study was to examine haemostasis in 'steady-state' SCD, however the opportunity arose to assess nine of the HbSS patients who were hospitalised during crisis episodes on separate occasions (Table 4.12). Small, yet
statistically significant changes in RBC parameters, neutrophil count, CRP levels and α₁AT were consistent with haemolysis and inflammation. Six of the patients had been transfused within 120 days of collecting the ‘crisis’ sample and because PC, D-D and sE-s levels were significantly altered by transfusion (section 4.3.14), these variables were not assessed here. Other variables (F1+2, TAT, ATIII, HCII, PSf, α₁AT, Plg, α₂AP and Fg) were compared to levels measured in the same patients during 'steady-state'. Collectively, these variables were not significantly altered in association with crisis episodes, which may be due to considerable variability between individual patients and a lack of consistent trends as shown in Figure 4.27, reinforcing the strict criteria applied to the selection of 'steady-state' patients for this study.

<table>
<thead>
<tr>
<th>I.D.</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Sex</th>
<th>HbS (%)</th>
<th>No. days post last transfusion</th>
<th>Crisis</th>
<th>Comment</th>
</tr>
</thead>
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<tr>
<td>ALO</td>
<td>HbS/β⁺thal</td>
<td>51</td>
<td>M</td>
<td>14</td>
<td>76</td>
<td>Resolving painful crisis</td>
<td></td>
</tr>
<tr>
<td>ANI</td>
<td>HbSS</td>
<td>24</td>
<td>M</td>
<td>29</td>
<td>30</td>
<td>Priapism</td>
<td>Regular transfusions</td>
</tr>
<tr>
<td>BOL</td>
<td>HbSS</td>
<td>32</td>
<td>M</td>
<td>0</td>
<td>111</td>
<td>Painful crisis – knees</td>
<td></td>
</tr>
<tr>
<td>KAC</td>
<td>HbSS</td>
<td>41</td>
<td>F</td>
<td>0</td>
<td>&gt; 6 years</td>
<td>Mild painful crisis</td>
<td></td>
</tr>
<tr>
<td>LUJ</td>
<td>HbSS</td>
<td>41</td>
<td>F</td>
<td>0</td>
<td>&gt; 120</td>
<td>Painful crisis</td>
<td></td>
</tr>
<tr>
<td>NUS</td>
<td>HbSS</td>
<td>21</td>
<td>M</td>
<td>0</td>
<td>106</td>
<td>Mild painful crisis</td>
<td></td>
</tr>
<tr>
<td>OKE</td>
<td>HbSS</td>
<td>29</td>
<td>F</td>
<td>0</td>
<td>135</td>
<td>Mild painful crisis</td>
<td></td>
</tr>
<tr>
<td>SMI</td>
<td>HbSS</td>
<td>18</td>
<td>F</td>
<td>12</td>
<td>108 days</td>
<td>Mild painful crisis</td>
<td></td>
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<tr>
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<td>31</td>
<td>M</td>
<td>69</td>
<td>2 days</td>
<td>Painful crisis</td>
<td>Regular transfusions</td>
</tr>
</tbody>
</table>

*Table 4.12  Characteristics of patients examined during sickle crisis.*
Figure 4.27  Altered levels of thrombin generation markers and physiological inhibitors during crisis episodes in nine patients with SCD.
4.4 Discussion

Patients studied in this chapter had a diagnosis of SCD (HbSC or HbSS) confirmed by HPLC and a subgroup of HbSS patients were regularly exchange transfused. The patients were examined while in 'steady-state' (with the exception of ancillary evaluations in sections 4.3.14 & 4.3.15, where patients were re-assessed when they were hospitalised on another occasion) and none were treated with antithrombotic drugs. Haemostatic variables in HbSC and HbSS patients were assessed and compared to those measured in a healthy HbAA control group of similar ethnicity as well as to each other. SCD is an inherently dynamic disease and the plasmatic environment is subject to physiological, pathological and therapy-related fluctuations; samples for all tests were therefore collected during a single venepuncture to help identify potential relationships between haemostatic, haematological and biochemical variables.

Acute phase reactivity

Patients were investigated in the absence of vasocclusive or other crises, ulceration, infection or other inflammatory conditions, as some haemostatic proteins are acute phase reactants and may respond to inflammatory complications of SCD. Despite this careful patient selection, however, CRP levels above 1.0mg/dL demonstrated acute phase reactivity in 28% of HbSS patients. A degree of inflammation is a familiar finding in asymptomatic SCD (Akinola et al, 1992; Singhal et al, 1993); subclinical vasocclusion is thought to promote a covert inflammatory response that is mediated by cytokines (Bourantas et al, 1998). There was no significant impact on haemostatic protein levels in these patients however, with the exception of Fg, another acute phase reactant, which was slightly higher in HbSS than HbSC. Interestingly, there was no evidence of acute phase reactivity in the HbSC cohort, with the exception of a single patient with a known cause (section 4.3.2).

It was possible to examine nine of the HbSS patients on separate occasions when they were hospitalised during crisis episodes. Changes in plasma levels of thrombin generation markers and physiological inhibitors were not uniform and reinforced the need to assess these patients’ haemostatic systems in the absence of acute clinical complications.
Activation of the coagulation system

An interesting observation made in the previous chapter was that the upper limit of the TAT reference range established in Black HbAA controls was approximately double that of the Caucasians: abnormal thrombin generation may therefore be overestimated in SCD patients when appropriate ethnic controls have not been utilised for comparative analysis. Taking this into account however, raised plasma levels of either or both markers of thrombin generation provided direct evidence of enhanced thrombin generation in 39% of the HbSC and 55% of the HbSS patients. TAT complex levels were considered more reliable indicators of ongoing thrombin generation than F1+2 (section 1.2.2 ) and were were abnormal in 31% HbSC and 40% HbSS patients. Both F1+2 and TAT tended to be higher in HbSS than HbSC but this did not reach statistical significance as occasional HbSC patients showed marked derangement. Increased thrombin generation persisted in regularly transfused patients.

D-D are generated following the degradation of cross-linked fibrin by plasmin; raised plasma levels therefore testify to increased fibrin deposition and fibrinolytic activity and may represent a more sensitive indicator of enhanced activation of the coagulation system than F1+2 or TAT (Francis, 1989). D-D were normal in the majority of HbSC patients but overtly abnormal in 65% of those with HbSS. Raised D-D levels are frequently encountered in association with inflammatory complications of SCD such as chronic cholecystisits, aseptic necrosis and ulceration (Devine et al, 1986), but were not related to inflammatory markers (i.e. CRP, WBC or sE-s) in my asymptomatic cohort.

My study confirms indirect reports of hypercoagulability in ‘steady-state’ HbSS by other authors, providing direct evidence of increased thrombin generation and fibrin deposition in HbSS and is the first to show direct evidence of excessive thrombin generation in ‘steady-state’ HbSC patients. In an independent study of children with SCD, I demonstrated increased thrombin generation in infants as young as twelve months old (Liesner et al, 1998). It is apparent that hypercoagulability is a common feature of ‘steady-state’ SCD, irrespective of genotype or transfusion status and is present in both children and adults.

Having established that thrombin generation was increased in my adult patients, I examined a series of potential risk factors that may have contributed. Repetitive or severe sickling episodes may cause small vessel infarction leading to progressive organ dysfunction: diabetes, abnormal thyroid function and cardiac anomalies are frequently
encountered in SCD and independently heighten the risk of thrombosis. However, there was no common underlying pathology here (section 4.3.12); mild hyperglycaemia was common in both HbSC and HbSS, but haemostatic profiles did not differ significantly between normo- and hyperglycaemic patients. Hypercoagulability was not a function of age or OC use in this cohort.

**Congenital thrombophilic mutations**

No SCD patient had FVL: the extremely low frequency of this mutation in Black populations of African ethnicity has subsequently been established and the findings of my study are confirmed in more recent studies of SCD patients; (Kahn *et al*, 1997; Andrade *et al*, 1998). APCR in the absence of FVL has been reported in SCD (Wright *et al*, 1997), however the authors had not pre-diluted samples in FV deficient plasma (a process that renders the test system highly specific for FVL) and it is possible that abnormal FVIII concentrations, which are frequently reported in SCD, affected clotting times.

PGM was described after I commenced this investigation so it was possible to test only 26 of the 44 patients enrolled. None were abnormal and this mutation has since been found to be extremely rare in Black populations of African descent (Rosendaal *et al*, 1998; Zivelin *et al*, 1998).

**Phospholipid-dependent antibodies**

Pathological antibodies to PL, including LA and β2GPI-dependent aCL, are quite common in other thrombophilic populations but they did not contribute to hypercoagulability in this SCD cohort. APAs were sought using clotting tests and immunological techniques according to recommended guidelines (Greaves *et al*, 2000): a normal DRVVT in each patient excluded the presence of LA; IgG aCL in 17% HbSC and 29% HbSS patients were not β2GPI-dependent and since β2GPI antibodies show a better association and greater specificity for thrombosis than aCL (McNally *et al*, 1995a), I concluded that these antibodies were not pathogenic for thrombosis and were likely to be mounted in response to infection or chronic P-s exposure by damaged RBC (McNally *et al*, 1995b). IgG aCL in association with SCD has subsequently been confirmed, however there is no apparent relationship between their incidence or titres and the major complications of SCD (Nsiri *et al*, 1998). Antibodies directed against other PLs (i.e. P-e, P-s, phosphatidylinositol and antiphosphatidic acid) have also been
identified in SCD patients and it has been proposed that they are associated with structural changes in the SRBC membrane (Kucuk et al, 1993).

Clotting factors and inhibitors

The shift in haemostatic equilibrium to a hypercoagulable state represents poor control of thrombin generation; a possible cause was inadequate regulation by the physiological inhibitors of coagulation. Variable plasma levels of these inhibitors (ATIII, PC and its co-factor PS) in SCD patients have been reported by other authors, but most found a reduction in PC and no significant change in ATIII. In my cohort, ATIII and PC levels were substantially reduced in HbSS compared to controls, but few patients had overtly abnormal levels and no consistent patterns were apparent. PSf was not significantly altered, which contrasted with the findings in some previous studies that used alternative methodologies and did not necessarily employ an ethnically similar control group for comparison. All the HbSS patients in my study that had evidence of acute phase reactivity had normal PSf levels despite C4bp (the protein to which PS is bound in plasma) being an acute phase reactant. I did not measure C4bp, however normal levels have been reported elsewhere (Francis, 1988a). In any case, differential regulation of C4bp α- and β-chain expression, as well as the concentration of PSf being a consequence of the molar excess of protein S over C4bp β+, has since been shown to stabilise plasma levels of functional PSf during acute phase processes (Criado-Garcia et al, 1997).

Decreased plasma levels of ATIII that occur secondarily to hepatic failure are not usually associated with thrombotic disorders, although the relationship between PC, PS and thrombosis is less clear (Jespersen et al, 1999) When reductions in inhibitor levels are a consequence of hepatic insufficiency, procoagulant proteins may be simultaneously reduced, so that the equilibrium between pro- and anticoagulant mechanisms is largely maintained. This was apparent in my patients, in whom clotting factor levels were concomitantly reduced, but not correlated.

HCII was by far the most common inhibitor deficiency in my cohort, with overtly reduced levels in 54% HbSC and 74% HbSS patients and the levels were comparable between the two genotypes. This may of course reflect inadequate hepatic synthesis, but it is likely that an alternative mechanism contributes as HCII levels were disproportionately reduced relative to the other inhibitors. HCII may provide a 'back-up' means of regulating thrombin generation that is depleted before levels of the principle
inhibitor ATIII are significantly altered. Alternatively, HCII may be actively depleted from plasma by binding abnormal RBC membranes (Chitolic et al., 1996) in a similar manner to that which involves aberrantly expressed P-s on SRBC binding PSf (Lane et al., 1994) and perhaps FVIIa (see below).

$\alpha_1$AT was the only inhibitor in which levels differed substantially between my HbSC and HbSS patients. Slightly higher levels in HbSS approximated those in HbAA subjects and may have reflected the mild sub-clinical inflammation not evident in HbSC patients, as $\alpha_1$AT is an acute phase reactant. That CRP levels were mildly increased in HbSS but not HbSC and HCII and $\alpha_1$AT, both acute phase proteins, were correlated within the HbSS group only, supports this notion.

An important finding in this study was that there was no apparent relationship between deficiencies of any of the coagulation inhibitors and the extent of abnormal thrombin generation. This may be due to concurrent depletion of clotting factors (at least FII, FVII and FX were altered here). FVII were lowest in HbSS and while FVIIa were proportional to FVII levels in HbSC, FVIIa was disproportionately reduced in HbSS; therefore, FVIIa levels do not merely reflect low levels of FVII in HbSS patients, suggesting that FVIIa may be preferentially removed from plasma, perhaps by binding the abnormal SRBC membrane. This was a somewhat unexpected finding, as I had examined FVIIa levels as a means of quantifying the extent of TF-mediated coagulation activation (since TF is the principal ligand for FVIIa) and anticipated increased FVIIa levels in vivo. This phenomenon is echoed in other disorders, however (i.e. disseminated intravascular coagulation and sepsis), in which there may be enhanced activation of TF-mediated coagulation (Cardigan, 1998). Reduced FVIIa levels in SCD, that are not mediated by increased TFPI activity, have subsequently been confirmed (Key et al., 1998). It has been proposed that FVIIa binds to the TF expressed on circulating endothelial cells, which is increased 6-8-fold in SCD (Solovey et al., 1998).

**Plasma lipids**

MacCallum et al. (1998) described a strong association between PC antigen/activity and both serum CHOL and TRIG levels, whereby PC levels increase by approximately 0.25 U/ml when the lipid concentrations increase from the 5th to 95th percentile (MacCallum et al., 1998). A similar association was reported for PS. This report prompted me to examine the relationship between these variables in my patients as PC was clearly lower in HbSC and HbSS compared to controls and anomalies have
been reported with respect to lipids in SCD. CHOL was moderately reduced in both SCD genotypes, which concurred with findings by Sasaki et al (1983) and el-Hazmi et al (1987) and correlated positively with PC, suggesting that PC deficiency in SCD may be partially a consequence of lipid disturbances associated with abnormal morphology of the SRBC. CHOL levels in HbSS(tx) were improved, but not normalised, although each patient had a normal PC concentration, irrespective of whether they were transfused on an occasional or a regular basis.

The distribution of CHOL in biological cell membranes is asymmetrical and there is limited exchange with plasma lipids across the membrane (Schroeder et al, 1995). It has been proposed that spicule formation in SRBC physically alters the structural domains of membrane CHOL, so that it becomes readily exchangeable with plasma CHOL (Kavecansky et al, 1995): plasma CHOL becomes depleted, while SRBC lipids contain high concentrations that are associated with decreased electrolytic and enzymatic activity (Sasaki et al, 1983).

Fibrinolysis

Elevated D-D demonstrated that increased fibrin deposition and enhanced degradation of cross-linked fibrin by plasmin commonly occurs in HbSS patients. This was consistent with the excessive thrombin generation described in association with this genotype and was accompanied by mild reductions in Plg and α₂AP. Plg levels did not approach those seen in congenital deficiencies and the relative reduction of these two variables in plasma may be related to liver disease/insufficiency (see below). I did not undertake a comprehensive evaluation of the fibrinolytic system because of logistical constraints, however the assessment of various components of fibrinolysis by other authors corroborate mildly disordered fibrinolytic activity in SCD: elevated D-D and PAI-1 levels have been reported while FPA levels are variable (Kurantsin-Mills et al, 1992; Hagger et al, 1995; Nsiri et al, 1996). The capacity of the endothelium to synthesise and release tPA does not appear to be impaired, even during crisis (Francis, 1988b; Nsiri et al, 1996). Deranged fibrinolysis has recently been implicated in the development of osteonecrotic lesions that are common in SCD (Glueck et al, 1997). Enhanced fibrinolytic activity was not evident in my HbSC patients, as D-D levels were normal in most and Plg and α₂AP were not significantly altered.
Liver function

Liver pathology is a common feature of SCD (Charache, 1983; Schubert, 1986). Perpetual SRBC sequestration and sludging within the hepatic sinusoids presents a chronic insult to hepatocytes that may progressively compromise liver function. Transfusion presents a further insult: frequently transfused patients are subject to iron overload, haemosiderosis, cholelithiasis and cirrhosis (Mills et al, 1988) in addition to the risk of transfusion-acquired infection, although this is minimal in the UK due to stringent screening procedures. Gall stones are common (Schubert, 1986; Bond et al, 1987) and autopsy studies reveal a spectrum of abnormalities: distention and/or obstruction of hepatic sinusoids, Kupffer cells containing phagocytosed SRBC, focal parenchymal necrosis, portal fibrosis, regenerative change, haemochromatosis and cirrhosis (Bauer et al, 1980). Low plasma levels of any of the proteins involved in haemostasis may well be a consequence of inadequate hepatocellular synthesis, although few studies have actually examined liver function in association with haemostatic anomalies.

Examination of biopsied liver tissue is required for true assessment of hepatic pathology, however this was beyond the scope of my study. I therefore assessed biochemical tests of liver function. Because RBCC transfusions will impact on some of the biochemical variables in question (see below), I restricted this aspect of the study to the untransfused patients.

The interpretation of (biochemical) LFTs is complicated in SCD patients: TBIL levels are increased in some forms of liver disease and as a consequence of RBC haemolysis; Alk phos comprises a family of isoenzymes that are derived from a variety of tissues and is therefore non-specific; albumin is only reduced in severe hepatic dysfunction. Although LDH is abundantly present in hepatocytes, its widespread distribution in other tissues (particularly kidney, skeletal muscle and myocardium) renders it non-specific and RBC haemolysis releases massive amounts into plasma. Another enzyme, alanine transaminase, is specifically derived from hepatocytes and raised plasma levels indicate hepatocellular damage, but unfortunately, this enzyme was substituted for AST in the routine LFT panel at UCLH only after completion of my study. Plasma AST is not derived specifically from hepatic cells (i.e. it may also be released from degenerate cardiac muscle), but in the absence of cardiac abnormalities in this cohort, it was considered the best available indicator of hepatic function. Trends and alterations in biochemical tests of liver function provide significant information
regarding the functional state of the liver, however repeated measurements were not possible within the time constraints of this study and a limitation of this assessment is that LFTs were measured on blood samples collected on a single occasion (however the results obtained were typical of those obtained over a period of several months, according to hospital records).

There was no biochemical evidence of liver impairment in the HbSC patients at all: abnormal TBIL levels were entirely consistent with haemolysis. Mild hepatic insufficiency could not be excluded in approximately half of the HbSS patients however, (who had mildly raised AST or evidence of iron overload) although AST levels were not considered clinically significant by a treating physician and albumin synthesis was adequate in all. Plasma concentrations of haemostatic proteins are more sensitive to liver dysfunction than biochemical indices and provide a more accurate reflection of the synthetic capability of hepatocytes: mild reductions in plasma levels of haemostatic proteins (FII, FVII, FX, ATIII, HCII, PC, α2AP and Plg) showed similar patterns relative to the control group that were loosely associated with each other, suggesting that a common mechanism contributed to their decline. Clotting factor levels were not sufficiently abnormal to significantly affect PTs, which provide a sensitive indicator of hepatic function and normal albumin concentrations indicated that any compromise in the synthetic capability of the liver was not severe.

I examined the ratio of PC to FVII, in an attempt to discriminate between hepatic insufficiency and accelerated turnover as a primary contributor to its altered levels in plasma: PC and FVII have similar plasma half-lives, a property that has been utilised to confirm PC deficiency in warfarin-treated patients: functional protein synthesis is reduced to a similar extent by warfarin, resulting in PC:FVII ratios akin to those of normal subjects, whereas the ratio falls below 0.76 in truly PC deficient individuals (Jones et al, 1991). A disproportionate reduction in PC indicates specific consumption of the protein, whereas a parallel reduction in both proteins (and therefore a normal ratio) would be expected when inadequate synthesis occurs secondarily to liver disease. PC:FVII ratios were reduced in only three HbSC and three HbSS patients.

I propose that mild hepatic insufficiency is likely to have contributed to the abnormal plasma levels of haemostatic proteins observed in the patients studied here, particularly in those who have received multiple transfusions and are heavily iron overloaded, however the extent of any liver disease was not morbid. Altered plasma levels of the principle inhibitors of coagulation reflect an uncompensated coagulopathy,
but do not appear to be the primary cause of excessive thrombin generation as several patients were hypercoagulable despite normal protein levels; other mechanisms evidently contribute to enhanced thrombin generation in these patients. It is likely, however, that SCD patients are less able to mount a competent response to further procoagulant stimuli.

**The endothelium**

My study demonstrated endothelial cell activation in HbSS patients in the absence of overt clinical manifestations. Recent reports of circulating endothelial cells and raised plasma levels of endothelin-1 in 'steady-state' SCD patients corroborate this finding (Solovey et al, 1997; Werdehoff et al, 1998). Endothelial cell damage is frequently implicated in the complications of SCD and adhesion of SRBC to the endothelium is thought to initiate vaso-occlusive episodes (Hebbel et al, 1980). SRBC are abnormally adherent and their adhesive properties are closely related to cell density: retics and younger cells are more adhesive than dense ISCs which, because of their permanently altered shape are minimally adherent, yet play a distinct role in postcapillary obstruction (Kaul et al, 1994). The weak association that I observed between sE-s and age has subsequently been reported in healthy subjects (Sack et al, 1998).

Raised sE-s accompanied by normal sTM levels in HbSS indicated that endothelial cell activation had occurred without overt endothelial cell damage. Endothelial cell activation was not directly related to hypercoagulability in this cohort (there was no relationship between sE-s or sTM and F1+2, TAT or D-D levels), but was strongly associated with inflammation. Slightly higher $\alpha_1$AT and platelets in patients with evidence of endothelial cell activation supports this. Endothelial cell activation was not a feature of HbSC disease.

An important finding was that all but one of the regularly exchanged HbSS patients showed entirely normal sE-s levels, suggesting an ameliorating effect of transfusion on endothelial cell activation. This may well account for the efficacy of transfusion in preventing recurrent stroke in patients with SCD (Russell et al, 1984; Pegelow et al, 1995); perhaps maintenance of HbS below 30% improves blood rheology sufficiently to avert endothelial cell activation and thrombogenesis. This concept was supported anecdotally when an immediate and sustained fall in sE-s (from 150 to 60 ng/ml, reference range: 24.5 – 93.5 ng/ml) accompanied a decrease in HbS from 98 to...
13% in a HbSS patient who commenced regular exchange transfusions during the course of the study (data not shown).

**Transfusion**

I anticipated that thrombin generation may have been suppressed in the altered plasmatic environment afforded by regular exchange transfusions. This was not borne out, however, as F1+2 and/or TAT levels were abnormal in the majority of my regularly transfused HbSS patients and there was no association between either variable and the length of time following the most recent transfusion. HbS levels in these subjects were significantly lower than those of untransfused patients, but remained considerably higher than those recommended following a stroke. It was possible that the beneficial effect of transfusion is not afforded when HbS levels remain relatively high, yet this did not appear to be the case: the extent of thrombin generation was not merely a function of the concentration of HbS present, as there was no relationship between HbS or HbA concentrations and F1+2 or TAT levels in this cohort. Further, the HbAS subjects, who had approximately 67% HbS (albeit with minimal sickling), displayed no evidence of excessive thrombin generation. Regular exchange transfusions did seem to ameliorate fibrin deposition, however.

Transfusion had no appreciable effect on inflammation or on plasma levels of most of the haemostatic proteins, however there was a marked improvement in PC levels and on endothelial cell activation, which was abnormal in only one transfused patient (in contrast to half those who were untransfused); PC levels were entirely normal in all, and significantly higher than in untransfused patients. This may be related to an improvement in RBC rheology as well as plasma CHOL levels. Incidentally, PC levels in the ‘occasionally’ transfused patients were also normal (data not shown).

It is possible that the results were biased by the assignment of patients to a chronic transfusion regime. Patients with severe clinical disease (i.e. those who have experienced a cerebrovascular accident or frequent, severe crises, or who have significantly impaired organ function) may be expected to have more gross haemostatic derangement, but as these patients are more likely to be transfused, severe abnormalities which may otherwise be evident may actually be masked.

An interesting observation was that even after taking dilutional effects into account, a rapid and marked increase in thrombin generation occurred immediately following transfusion. It was not clear whether this was preventable with anticoagulation, as the only anticoagulated patient was also the only one with an HbSC
genotype. Patients may be at a heightened thrombophilic risk in the period shortly following an exchange transfusion of RBCC, although anecdotal observations do not associate this period with the onset of thrombotic complications. The magnitude of the increase in TAT was strongly associated with pre-transfusion TAT levels, but not with the number of units transfused.

The RBCC that are transfused to patients with SCD are plasma reduced and any residual plasma would be expected to be rapidly and massively diluted in the circulation. In any case, it was unlikely that the marked increase in TAT levels was due to thrombin generation within stored blood packs: given that the total plasma volume in a regularly transfused HbSS patient approximates 3400ml (assuming a 70kg weight and a HCT of 30% and that each of an average of three RBCC contain approximately 30ml of residual plasma), TAT levels in the region of 350µg/L per pack would have to be generated and transfused into the patient to achieve the four-fold increase in TAT that was observed in vivo. In addition, RBCC are anticoagulated with citrate phosphate dextrose adenine (CPDA), which should inhibit thrombin generation during storage. To verify this, I measured F1+2 and TAT levels in residual plasma samples from six RBCC units: F1+2 levels ranged between 0.2 and 0.5 nmol/L; TAT levels between 0.5 and 0.9 µg/L. These very low levels demonstrate that thrombin generation is adequately suppressed within the blood packs but it is possible that the mechanical or storage induced stresses on the RBCs leads to perturbation of the cell membrane and once transfused, the exposed procoagulant surface is capable of binding coagulation factors in the presence of physiological concentrations of plasma calcium, so that thrombin is generated in vivo. This hypothesis is supported by the observation that the patient receiving fragmin showed no such increase in TAT or F1+2, because any FXa formed would have been rapidly inhibited so that thrombin generation could not follow, although it should be noted that she was the only patient with the milder HbSC genotype. The return of TAT to ‘baseline’ levels before a subsequent transfusion in three patients is consistent with the 90 minute half-life of TAT and with recovery of the transfused normal RBCs in the circulation. The fact that the elevation in F1+2 was not as dramatic as that of TAT would be in keeping with the mechanism of thrombin generation, since F1+2 remains bound to the cell surface when thrombin is formed, so that the released TAT complex might be expected to appear in plasma before F1+2 (Mann, 1994a).
**HbSC disease**

The clinical course of SCD tends to be milder in patients with the HbSC genotype and it is reasonable to expect that any alterations in the haemostatic system would be less severe than observed in HbSS. Thrombin generation and fibrin deposition were increased relative to the HbAA cohort and there was a trend toward lower FII, FVII, FX and Plg levels, although this was statistically significant for FII and FX only. Pronounced abnormalities were observed with respect to HCl and CHOL and the disproportionately low levels of these variables may again reflect abnormal binding to and interactions with SRBC (discussed earlier with respect to HbSS patients): HCII was reduced to the same extent as in homozygous patients, although CHOL were significantly less abnormal. No patients presented with congenital thrombophilic abnormalities or pathological APAs and the remaining variables were unremarkable.

When the untransfused HbSC patients were compared to the HbSS(untx) cohort, several variables appeared relatively less altered but this was significant for ATIII, Fg, FII, FVII, α1 AT and Plg only, however thrombin generation was increased to a similar extent in both genotypes. The most pertinent clinical observation with respect to the HbSC group was that very few patients had abnormal fibrin deposition and they displayed no evidence of inflammatory activation of the endothelium at all, in contrast to untransfused HbSS patients.

**Sickle cell trait**

There were no significant haemostatic abnormalities amongst the group of healthy HbAS subjects. Specifically, there was no evidence of hypercoagulability as thrombin generation and fibrinolyis were normal, levels of clotting factors and inhibitors resembled those of the control group and there was no evidence of abnormal activation of the endothelium. This is consistent with the general well-being of HbAS subjects, who only experience sickle-related complications under conditions of extreme duress (Serjeant, 1992d).
Conclusion

The haemostatic system is abnormally activated in so-called 'steady-state' SCD and is a feature of HbSC and HbSS. The extent of thrombin generation associated with the two genotypes is similar and occurs against a heterogeneous background of altered plasma levels of haemostatic proteins. Hypercoagulability (increased thrombin generation and/or fibrin deposition) is uniformly demonstrable in HbSS and in approximately half of HbSC patients, despite an absence of clinical complications. An important observation was that increased fibrin deposition occurred in 94% of individuals with HbSS but was relatively uncommon in HbSC. Hypercoagulability was not attributable to congenital thrombophilic abnormalities (FVL or PGM), nor to the presence of pathological PL-antibodies and is not merely attributable to liver disease.

The shift in the haemostatic equilibrium toward coagulation may be partially compensated for by a simultaneous reduction in clotting factor levels. Also, enhanced fibrinolytic activity may temper increased thrombin generation, which may go some way to explaining why more patients do not experience thrombotic complications.

In the main, this haemostatic derangement is independent of transfusion status in HbSS. The exception is the profound effect of regular transfusions on PC levels and endothelial cell activation: there is a strong relationship between endothelial cell activation and inflammation in untransfused HbSS patients, which is dramatically abated by regular exchange transfusions. PC levels appeared to be completely restored by transfusion but there was no apparent relationship between PC and inflammation or endothelial cell activation and this may have been attributable to improved RBC rheology and restoration of plasma CHOL. Fibrin deposition was considerably reduced in transfused patients, despite persistent thrombin generation.

Thromboembolic complications occur in association with other congenital haemolytic anaemias (Barker & Wandersee, 1999). I questioned whether the haemostatic abnormalities observed in my cohort of SCD patients were peculiar to SCD, or features of other disorders that involve similar abnormalities of the RBC membrane. In order to address this issue, I undertook a similar investigation in a group of β thal patients in the next chapter.
CHAPTER 5

THE HAEMOSTATIC SYSTEM IN

β THALASSAEMIA

5.1 Introduction

In this study, I investigated a group of patients with β thal using a similar structure as the SCD patients in the previous chapter. β thal shares several characteristics with SCD: it too is a congenital β globin chain disorder that gives rise to a chronic haemolytic anaemia, multi-organ pathology, susceptibility to infection and many patients are transfusion-dependent. The availability of safe blood products, iron chelation therapy and antibiotics in developed countries have improved the prognosis for patients with β thal, however a heightened risk of thrombosis remains associated with this disorder (section 1.9). Until relatively recently, reports of thrombotic complications were based on case studies and anecdotal accounts in the literature, but large multi-centre studies now estimate the prevalence of thromboembolic complications in β thal to be 1-5%, or as high as 9.6% when Thal Int patients are considered separately (Borgna-Pignatti et al, 1998a; Borgna-Pignatti et al, 1998b; Moratelli et al, 1998). The disparity between centres probably reflects differences in patient classification and therapeutic regimes, as well as genetic factors.

Aspects of blood coagulation had been investigated in β thal by other authors prior to my commencing this study: abnormal platelet activation and chronic platelet 'exhaustion' were typical features; mild alterations in basic screening tests were caused by abnormal levels of clotting factors; a pronounced reduction in levels of contact factors in Thal Maj has been attributed to contact activation following intravascular haemolysis and multiple blood transfusions (Caocci et al, 1978; Schettini et al, 1987); examination of coagulation inhibitors reveal abnormal PC levels (particularly in older, splenectomised patients and those with chronic hepatitis), variable PS levels and normal levels of ATIII in the majority of patients (Musumeci et al, 1987; Schettini et al, 1987; Chuansumrit et al, 1993; O'Driscoll et al, 1995).
The relevance of some of these earlier studies was limited because many examined Thal Maj patients only, or co-investigated Thal Int and Thal Maj patients and therefore failed to consider their transfusion status. Several of the observations were made in association with HbE/β thal, which is more common in geographical regions that do not have access to the high standard of health facilities that are available in Europe. Therefore, dietary and environmental factors as well as the reduced availability of antibiotics and adequate and safe transfusion support will have had a detrimental impact on the clinical status of those patients. Several of the earlier studies were also performed in children.

In my study, thrombin generation, plasma levels of clotting factors and physiological inhibitors, congenital and acquired abnormalities, fibrinolytic capacity, endothelial cell activation, inflammation and liver function are assessed in adult β thal patients who presented without overt clinical complications. I aimed to establish whether the haemostatic abnormalities identified in SCD patients extended to other disorders characterised by similar RBC abnormalities (i.e. dehydration, membrane instability, shortened survival, phospholipid destabilisation and enhanced adhesiveness). Patients with β thal are almost invariably iron overloaded as a consequence of RBC haemolysis and/or multiple blood transfusions and it has been assumed that low plasma levels of clotting factors and inhibitors are secondary to impaired liver function and inadequate synthesis, although this had not been convincingly demonstrated. I therefore aimed to assess the synthetic capability of the liver in my patients. Patients with Thal Int were untransfused and those with Thal Maj were transfusion-dependent: the two cohorts were investigated independently, avoiding physiological and haemodynamic alterations that may result from transfusion.
5.2 Methods

Patients

41 patients with β thal were enrolled; Hb genotypes were confirmed by HPLC and the patients were divided into two groups (i.e. Thal Int or Thal Maj) by a clinician on the basis of clinical status and transfusion-dependence. No patient was pregnant, each patient was free of acute clinical complications at the time of testing and no intervention was made in the clinical management of any patient. Each subject answered a questionnaire that confirmed date of birth, ethnic background and prescribed medication; OC use was noted when applicable and any history of surgical splenectomy, thrombotic complications or stroke was recorded. Transfusion records confirmed that the Thal Maj patients were transfusion-dependent and all Thal Int patients were untransfused. Five β thal minor (Thal Min) subjects who were identified during screening of potential Caucasian control subjects were included for anecdotal comparison. The three clinical groups were compared to the Caucasian HbAA control group described in Chapter 3.

Exclusions

Two referred patients were omitted from the study on the basis of age (<12 years, n=2). Two patients anticoagulated with warfarin following previous thrombotic complications (DVT with PE and frequent, rapid clotting of Port-A-Cath lines) were not enrolled as warfarin reduces plasma levels of several variables under investigation. Two Thal Int patients who had been transfused within 120 days of sample collection were also excluded to avoid difficulties in the interpretation of results due to transfusion-related haemodynamic disturbances and altered plasmatic environment.

Control subjects

The majority of thalassaemic patients were Caucasians of Mediterranean origin, however a small number of patients cited Chinese, Asian or Middle Eastern ethnicity. It was considered most appropriate to compare them to the Caucasian group of healthy adult control subjects described in Chapter 3.

Sample collection and processing

Blood samples were collected and processed as described in section 2.1. Assays were performed as described in Chapter 2.
Transfusion

'Transfusion' refers to an exchange transfusion as described in section 4.3.1. Thal Maj patients were transfused 2-4 units of RBCC on a 3-5 weekly basis, however some required additional transfusions during the interval: samples were collected during a single phlebotomy immediately prior to a transfusion. The majority of samples were collected between 7-35 days (median 19 days) following the most recent transfusion; samples from four patients were collected within four days of transfusion. Thal Int patients had not been transfused for at least 120 days prior to sample collection.

5.3 Results

5.3.1 Characteristics of subjects under investigation

Characteristics of the clinical groups investigated in this chapter are summarised in Table 5.1. Sex distributions did not differ significantly between groups as assessed by Kruskal-Wallis one-way analysis of variance, however the Thal Maj patients were significantly younger than the controls (23 vs. 29 years, $p < 0.0001$): all subjects enrolled in the study were of an age where haemostatic variables had reached adult levels and the age difference between the two groups was not considered a complicating factor. 22 patients had undergone surgical splenectomy and another had experienced a PE three years prior to this investigation, but was no longer anticoagulated. Three sets of siblings were enrolled: two were brothers (Thai Int/Thai Int; Thai Int/Thai Maj), the third were brother and sister (Thai Int/Thai Maj).

Medication

Patients were prescribed folic acid and penicillin. Antithrombotic drugs were not prescribed for any patient and none admitted recent aspirin ingestion. All Thal Maj and some Thal Int patients were prescribed desferrioxamine mesylate, with varying levels of compliance.

Oral contraceptive use

Two Thal Maj patients were prescribed estrogen-based hormone replacement therapy (Prempak-C) to assist growth and endocrine development; one patient had increased thrombin generation (TAT: 11.3 μg/L), however levels of up to 19.3 μg/L were observed in other females; her inclusion did not alter the haemostatic profile of the group and the remaining haemostatic variables were unremarkable in both young
women. One patient used progesterone-based Depo-provera for contraception, which is not associated with thrombotic complications.

<table>
<thead>
<tr>
<th></th>
<th>HbAA (Caucasian)</th>
<th>β thalassaemia minor</th>
<th>β thalassaemia intermedia</th>
<th>β thalassaemia major</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n)</td>
<td>33</td>
<td>5</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>2</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>17 (3)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>14 (2)</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>19 - 48</td>
<td>21 - 47</td>
<td>12 - 43</td>
<td>14 - 35</td>
</tr>
<tr>
<td>Surgical splenectomy</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Transfused</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HbAA, HbA₂</td>
<td>HbA and mildly</td>
<td>≥ 60% HbF, variable</td>
<td>&gt; 79% HbA, 1-4% HbA₂ and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>elevated HbA₂</td>
<td>HbA and HbF (untransfused)</td>
<td>variable % Hbf (transfused)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Characteristics of control and patient groups (β thalassaemic disorders). Females using estrogen-based oral contraception or hormone replacement therapy are indicated in parentheses.

**β thalassaemia minor**

Quantitative Hb analysis of the Thal Min group revealed 92 (89 - 95)% HbA, 6 (3 - 6)% HbA₂ and 2 (2 - 6)% HbF. This small, healthy group of individuals displayed no significant abnormalities with respect to plasma levels of clotting factors (Fg, II, VII and X), inhibitors (ATIII, HCII, PC, PSf and α₁AT), lipids (CHOL and TRIG), markers of coagulation system activation (F1+2, TAT, FVIIa) or elements of fibrinolysis (D-D, Plg and α₂AP) and PL-dependent antibodies were not detected in any subject. Endothelial cell activation was not assessed.

**β thalassaemia intermedia and β thalassaemia major**

Haematological profiles

HPLC revealed medians of 14.0% HbA and 80.5% HbF in the Thal Int group and 96.3% HbA and 1.0% HbF in Thal Maj. RBC indices were more abnormal in Thal Int, reflecting the microcytic hypochromic anaemia and haemolysis characteristic of β thal, whereas this was masked to a great extent by transfusion in Thal Maj patients: Hb: 9.2 vs. 11.0 g/dL, p < 0.0005; HCT: 0.294 vs. 0.335 l/l, p < 0.02; MCV: 81.2 vs. 87.1 fl, p < 0.01 and MCH: 25.6 vs. 29.2 pg, MCHC: 31.1 vs. 33.8 g/dL and retics: 5.7 vs. 0.3
% p < 0.0001 respectively. RCCs were analogous: 3.61 and 3.79 x 10^12/L. WBCs were variable, ranging between 5.3 - 19.1 x 10^9/L in Thal Int and between 3.1 - 24.2 x 10^9/L in Thal Maj, but were generally higher in Thal Int. Platelets appeared to be higher in Thal Int (664 vs. 328 x 10^9/L, p < 0.0001), however this should be interpreted with caution (see section 4.3.1).

5.3.2 Routine clotting profiles

Routine clotting profiles (PT, APTT and TT) were undertaken as a basic haemostatic screen. Mildly prolonged PTs were observed in one Thal Int and four Thal Maj patients (11.8, 11.8, 11.8, 12.5 and 14.0s). Two Thal Int and 10 Thal Maj patients yielded mildly prolonged APTTs that were not due to reduced Fg levels, nor to interference by heparin or the presence of fibrin degradation products (there was no corresponding prolongation of the TT and D-D levels were normal). Clotting times in each of the samples corrected when mixed with an equal volume of normal plasma, which was suggestive of factor deficiencies and the presence of LA was definitively excluded in section 5.3.10. TTs were significantly shorter in Thal Int and Thal Maj compared to the control subjects (p < 0.0001 respectively), with five Thal Int and 10 Thal Maj patients having clotting times between 11.1 and 12.6s (Reference range: 12.7 – 16.9s). Increased plasma viscosity may affect TTs performed using a mechanical clot detection system, however an optical clot detection system was used here. Other possible explanations for the shortened TTs include an increased fibrin monomer polymerisation rate (possibly due to elevated amounts of partially proteolysed Fg molecules).

Of the five patients in whom the PT was slightly prolonged, FVII was reduced all; three also had reduced levels of factors II and X. The PT is a very sensitive indicator of liver function and the PTs in this sub-group may be attributable to mildly impaired, subclinical hepatic function and a consequential reduction in clotting factor synthesis; this was supported by prolonged APTTs in three of the five, although plasma levels of the hepatic enzyme AST, which are raised when significant liver cell damage or death has occurred, was abnormal in only one patient. The patient with the longest PT also had a chronic, mild prolongation of the APTT and he was hospitalised with jaundice shortly after the sample was collected for this study.
5.3.3 Clotting factors

Fg and factors II, VII and X were assayed: summary data is shown in Table 5.2. Fg levels were significantly reduced in Thal Int (p < 0.005) but not in Thal Maj (Figure 5.1). The remaining factors were substantially lower in both Thal Int and Thal Maj compared to controls (Figures 5.2 to 5.4). FII and FX levels were correlated in Thal Maj (r_s = 0.56, p < 0.005) but not in Thal Int and no other relationships were demonstrable within either group. The PT was a function of FVII levels in Thal Maj only (r_s = -0.65, p = 0.0002).

Despite levels of FII, FVII and FX being lower in Thal Int and Thal Maj than the HbAA controls, the PT was affected in only five patients, and not to an extent to be considered clinically pertinent in four of them. Clotting factor deficiencies in both Thal Int and Thal Maj may occur as a consequence of depletion from plasma or inadequate hepatic synthesis. Liver function is assessed in section 5.3.4.

<table>
<thead>
<tr>
<th></th>
<th>HbAA (Caucasian)</th>
<th>Thalassaemia Minor</th>
<th>Thalassaemia Intermedia</th>
<th>Thalassaemia Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fg (g/L)</td>
<td>2.3 (1.9 - 2.6)</td>
<td>1.9 (1.7 - 2.4)</td>
<td>1.6 (1.2 - 2.2)</td>
<td>2.0 (1.6 - 2.4)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
</tr>
<tr>
<td>FII (IU/ml)</td>
<td>1.05 (0.98 - 1.22)</td>
<td>1.17 (1.09 - 1.28)</td>
<td>0.74 (0.73 - 0.83)</td>
<td>0.92 (0.80 - 1.02)</td>
</tr>
<tr>
<td></td>
<td>n = 20</td>
<td>n = 3</td>
<td>n = 11</td>
<td>n = 27</td>
</tr>
<tr>
<td>FVII (IU/ml)</td>
<td>1.07 (0.96 - 1.24)</td>
<td>0.98 (0.88 - 1.07)</td>
<td>0.79 (0.68 - 0.89)</td>
<td>0.92 (0.78 - 1.03)</td>
</tr>
<tr>
<td></td>
<td>n = 29</td>
<td>n = 4</td>
<td>n = 11</td>
<td>n = 28</td>
</tr>
<tr>
<td>FX (IU/ml)</td>
<td>0.95 (0.89 - 1.07)</td>
<td>0.98 (0.80 - 1.01)</td>
<td>0.59 (0.52 - 0.75)</td>
<td>0.85 (0.62 - 0.94)</td>
</tr>
<tr>
<td></td>
<td>n = 21</td>
<td>n = 3</td>
<td>n = 11</td>
<td>n = 27</td>
</tr>
</tbody>
</table>

Table 5.2 Clotting factors in β thalassaemic disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: Fg: 1.5 - 3.2 g/L; FII: 0.86 - 1.36 IU/ml, FVII: 0.75 - 1.46 IU/ml, FX: 0.61 - 1.46 IU/ml.
Figure 5.1  Fibrinogen levels in β thalassaemic disorders. The dotted lines indicate the limits of the reference range.

Figure 5.2  Factor II levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.
Figure 5.3  Factor VII levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.

Figure 5.4  Factor X levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.
Biochemical tests of liver function

Biochemical tests of liver function and variables that reflect the degree of RBC haemolysis in vivo were assessed as described with respect to the SCD patients in section 4.3.13. Data is shown in Table 5.3. Broadly, TBIL, AST and LDH levels were notably higher in Thai Int than in Thai Maj (TBIL: 53 vs. 26 μmol/L, p < 0.005; AST: 60 vs. 33 IU/ml, p < 0.01; LDH: 612 vs. 285 BB U/ml, p = 0.0005), however Alk phos levels were similar (252 vs. 216 IU/L). AST was elevated in 6/9 Thai Int patients compared to only 4/28 with Thai Maj and albumin was marginally reduced in one Thai Int patient only. Iron toxicity as a consequence of chronic haemolysis and multiple transfusions is a potential cause of hepatic dysfunction in β thal patients; serum ferritin levels were used to assess iron stores where available.

Iron overload

β thal patients are considered to be grossly iron overloaded when serum ferritin concentrations exceed 3000 μg/L. Iron deposition was considerably greater in the Thal Maj cohort (Ferritin: 1877 vs. 467 μg/L, p < 0.005): levels in 8/10 Thal Int patients were less than 670 μg/L and none exceeded 3000 μg/L (Table 5.3). Gross iron overload was evident in approximately one third of the Thal Maj patients; ferritin is also an acute phase reactant and two other acute phase proteins (α1AT and CRP) were slightly, but significantly higher in this sub-group, although levels were not abnormal (α1AT: 0.99 vs. 0.94 U/ml; CRP: 0.33 vs. 0.15 mg/dL, p < 0.01 respectively). AST levels were also significantly higher in these patients than those with < 3000μg/L ferritin (47.5 vs. 29.5 units, p < 0.02), although the variables were not convincingly associated. These results suggest a greater degree of hepatic cell death and associated inflammation in Thal Maj patients with gross iron overload, however the haemostatic and biochemical variables that I went on to examine did not differ significantly between patients with and without severe iron overload.

Biochemical tests of liver function

LFTs were assessed in collaboration with a clinician. AST levels greater than twice the upper limit of the reference range (i.e. > 110 IU/L) were broadly designated criteria for clinically significant hepatocellular damage within this patient population. This was evident in only two patients, who also yielded elevated Alk Phos, although accelerated RBC destruction in the bone marrow may have contributed to this: both
patients were grossly iron overloaded, however, so it is likely that their liver function was in fact compromised, despite adequate albumin synthesis. As mentioned earlier, another patient (ID: TSA) presented with clinical jaundice shortly following the collection of the sample for my study: despite being severely iron overloaded, TBIL was the only LFT that proved abnormal, yet plasma levels of several haemostatic proteins were reduced (FX: 0.64, FVII: 0.61, FX: 0.52, PC: 0.32, PSf: 0.48, $\alpha_2$AP: 0.61 and Plg: 0.51 IU/ml respectively), with normal thrombin generation (F1+2: 0.4 nmol/L; TAT 2.0 μg/L; D-D: 16ng/ml). Albumin was marginally reduced in only one patient and levels of most of his haemostatic proteins were also mildly decreased, probably due to inadequate synthesis in this case (FII: 0.59, FVII: 0.63, FX: 0.43, ATIII: 0.80, HCII: 0.52, PC: 0.32, PSf: 0.49, $\alpha_2$AP: 0.73 and Plg: 0.59 IU/ml respectively).

Raised Alk phos in the presence of normal (n = 9) or only mildly elevated (n = 3) AST was likely to be related to accelerated RBC destruction in the bone marrow rather than hepatocellular damage. One Thal Int and 11 Thal Maj patients had elevated TBIL accompanied by normal LDH levels; mild hyperbilirubinaemia was the only abnormality in six of these. In the absence of significant RBC haemolysis, raised TBIL may indicate an element of liver disease, or merely reflect accelerated processing of Hb from haemolysed RBC. There was a trend toward lower levels of some haemostatic proteins in association with raised TBIL, however the relationships were not striking.
## Table 5.3 Biochemical tests of liver function, red blood cell parameters and ferritin levels in patients with β thalassaemia.

Reference ranges: TBIL: 3-17 μmol/L; Alk Phos: 100-280 IU/L; AST: 11-55 IU/ml; Albumin: 35-53 g/L; LDH: 231-381 BB U/ml. Ferr: 22-275(M); 7-204(F) μg/L. Interpretation as per text. I: gross iron overload; LD: hepatocellular damage; BMH: accelerated RBC haemolysis within bone marrow.
The interpretation of routine LFTs were complicated in \( \beta \) thal patients for similar reasons to those discussed in relation to SCD (section 4.3.13). In this study, AST was considered the best available indicator of hepatocellular damage as ALT was not measured, TBIL and LDH levels are profoundly affected by haemolysis, Alk Phos is not specifically hepatocellular-derived and plasma albumin levels are generally maintained until liver function is severely compromised.

Biochemical evidence of substantial hepatic dysfunction did not appear to be common amongst my Thai Int and Thai Maj patients. In fact, significantly raised AST was identified in only two, both of whom were severely iron overloaded. The higher AST levels observed in Thai Int was interesting, given that transfusion-related iron overload was apparently more severe in Thai Maj; it is possible that early instigation of transfusion therapy in these patients reduces the long-term impact of their disease on the liver. Adequate albumin concentrations in all but one patient provided further evidence that any compromise in the synthetic capability of the liver was not pronounced in this cohort. Iron accumulation was well controlled in the Thai Int patients and was not of clinical concern in the majority of Thai Maj patients; in any case, the iron status of this cohort of Thai Maj patients had no gross impact on plasma levels of the coagulation proteins I tested. Clotting tests (PT and APTT) were mildly abnormal in a minority of patients and consistent with factor deficiencies.

The extent of liver damage in \( \beta \) thal patients is not always evident from biochemical tests (Masera et al, 1980): measurement of plasma levels of coagulation proteins provide a more sensitive indication of the liver's synthetic capability. Reduced levels of haemostatic proteins in my patients were consistent with some hepatic insufficiency, however a precise appraisal of toxic iron deposition and likely hepatic dysfunction would require the histological examination of biopsied tissue, which was beyond the scope of my investigation.

**Conclusion**

Patients with \( \beta \) thal are susceptible to liver damage resulting from chronic haemolysis, iron toxicity and inflammatory or infectious hepatitis. Despite this, moderately raised AST levels indicated substantial hepatocellular injury in only two (heavily iron overloaded) patients. Gross iron overload was indicated in nine Thai Maj patients and albumin synthesis was inadequate in one with Thai Int, however the biochemical abnormalities observed in these and the remaining subjects were considered mild within the milieu of their disease and may be partially attributable to chronic
haemolysis and accelerated RBC destruction within the bone marrow. Normal PTs and albumin concentrations testify to adequate synthetic function in the majority of patients in this cohort. There was no evidence of gross derangement in the majority of these patients, however I cannot definitively exclude hepatic dysfunction as contributing to the haemostatic abnormalities I went on to observe in this study as it is reasonable to expect some compromise of liver function in adult β thal patients (see Discussion).

5.3.5 Coagulation system activation

Thrombin generation

Summary data for plasma F1+2 and TAT levels is shown in Table 5.4. Lower F1+2 in Thai Maj than the Thai Int and control groups may reflect suppression by transfusion, but as levels were not actually abnormal in Thai Int, this was unlikely to be significant (Figure 5.5). In contrast, TAT levels were raised in 8/11 Thai Int and 10/30 Thai Maj patients (Figure 5.6), providing direct evidence of enhanced in vivo thrombin generation in both clinical groups. The disparity between TAT levels in Thai Maj and the controls was not attributable to the high TAT (44.5 μg/L) level detected in one patient; this result was clearly atypical of the remainder of the group and was detected in a 29 year old male patient (ID: COG) who died as a result of heart failure and gross iron overload (due to very poor compliance with chelation therapy); this patient had experienced a pulmonary embolism some years prior to my study. It was possible that transfusion suppressed thrombin generation in some Thai Maj patients although there was no relationship between TAT levels and the length of time following the most recent transfusion: a proportionally lower incidence of abnormal TAT levels were observed in Thal Maj (33 vs. 73%, p < 0.02) and median TAT levels were significantly lower in Thal Maj compared to Thal Int (p < 0.05), despite abnormal levels in a third of Thal Maj patients (Figure 5.6). There was no convincing association between TAT and any of the clinical, haematological, biochemical or haemostatic variables assessed in this study.

Activated factor VII

FVIIa levels were measured in 11 Thai Int patients: none had elevated levels and they were actually reduced relative to the controls (0.86 vs. 1.27, p < 0.05). FVIIa and FVII levels were not correlated.
Table 5.4 Markers of coagulation system activation in \( \beta \) thalassaemic disorders.

Median results are reported (with the interquartile range in parentheses).

Reference ranges: \( F1+2: 0.3 - 1.8 \text{ nmol/L} \), \( TAT: 0.8 - 5.3 \text{ pg/L} \), \( FVIIa: 0.39 - 3.07 \text{ ng/ml} \).

<table>
<thead>
<tr>
<th></th>
<th>( \text{HbAA} ) (Caucasian)</th>
<th>( \beta ) Thalassaemia Minor</th>
<th>( \beta ) Thalassaemia Intermedia</th>
<th>( \beta ) Thalassaemia Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F1+2 ) (nmol/L)</td>
<td>0.8 (0.7 - 1.0)</td>
<td>0.5 (0.5 - 0.6)</td>
<td>1.0 (0.7 - 1.2)</td>
<td>0.6 (0.4 - 0.7)</td>
</tr>
<tr>
<td></td>
<td>( n = 33 )</td>
<td>( n = 5 )</td>
<td>( n = 11 )</td>
<td>( n = 30 )</td>
</tr>
<tr>
<td>( TAT ) (( \mu \text{g/L} ))</td>
<td>2.3 (1.3 - 2.6)</td>
<td>2.8 (2.0 - 3.2)</td>
<td>8.5 (3.6 - 12.7)</td>
<td>3.4 (2.5 - 6.9)</td>
</tr>
<tr>
<td></td>
<td>( n = 32 )</td>
<td>( n = 5 )</td>
<td>( n = 11 )</td>
<td>( n = 30 )</td>
</tr>
<tr>
<td>( FVIIa ) (ng/ml)</td>
<td>0.99 (0.76 - 1.45)</td>
<td>Not tested</td>
<td>0.77 (0.32 - 1.46)</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>( n = 18 )</td>
<td>( n = 7 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.5 Prothrombin fragment 1+2 levels in \( \beta \) thalassaemic disorders. The dotted line indicates the upper limit of the reference range.
5.3.6 Physiological inhibitors of coagulation in β thalassaemia

Summary data for levels of the physiological inhibitors of coagulation is shown in Table 5.5. All of the Thal Int and 12/30 Thal Maj patients showed deficiencies of more than one inhibitor and concurrent HCII and PC deficiency was most commonly encountered, however there was no consistent pattern of abnormalities in Thal Int or Thal Maj (Appendix VI). Elevated TAT were detected in the presence of normal levels of each inhibitor in some individuals and in contrast, normal markers of thrombin generation were detected against a background of reduced inhibitor levels in other patients. Consequently, it was not surprising that there was no statistically significant associations between levels of any of the inhibitors and F1+2 or TAT.
### Table 5.5 Physiological inhibitors of coagulation in β thalassaemic disorders.

Median results are reported (with the interquartile range in parentheses). Reference ranges: ATIII: 0.84 - 1.37 IU/ml, HCII: 0.73 - 1.52 U/ml, PC: 0.69 - 1.42 IU/ml, PSf: 0.62 - 1.37 IU/ml (M), 0.58 - 1.03 IU/ml (F), α₂AT: 0.63 - 1.67 U/ml (M), 0.73 - 1.83 U/ml (F).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>ATIII (IU/ml)</th>
<th>HCII (U/ml)</th>
<th>PC (IU/ml)</th>
<th>PSf (IU/ml)</th>
<th>α₂AT (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Caucasian)</td>
<td>Minor</td>
<td>Intermedia</td>
<td>Major</td>
<td></td>
</tr>
<tr>
<td>ATIII</td>
<td>1.09</td>
<td>0.96</td>
<td>0.89</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(0.98 - 1.14)</td>
<td>(0.95 - 1.00)</td>
<td>(0.79 - 0.99)</td>
<td>(0.91 - 1.08)</td>
<td></td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCII</td>
<td>1.03</td>
<td>0.90</td>
<td>0.61</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>(U/ml)</td>
<td>(0.96 - 1.12)</td>
<td>(0.78 - 1.06)</td>
<td>(0.55 - 0.68)</td>
<td>(0.65 - 0.88)</td>
<td></td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>1.02</td>
<td>1.00</td>
<td>0.49</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(0.91 - 1.13)</td>
<td>(0.87 - 1.01)</td>
<td>(0.44 - 0.66)</td>
<td>(0.62 - 0.84)</td>
<td></td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSf</td>
<td>0.84</td>
<td>0.87</td>
<td>0.59</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>(IU/ml)</td>
<td>(0.74 - 0.96)</td>
<td>(0.85 - 0.89)</td>
<td>(0.48 - 0.75)</td>
<td>(0.64 - 0.92)</td>
<td></td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₂AT</td>
<td>1.04</td>
<td>1.06</td>
<td>1.15</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>(U/ml)</td>
<td>(0.90 - 1.24)</td>
<td>(1.05 - 1.10)</td>
<td>(1.03 - 1.20)</td>
<td>(0.82 - 0.97)</td>
<td></td>
</tr>
<tr>
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<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antithrombin III**

Significantly lower ATIII levels were seen in Thai Int and Thai Maj compared to controls (p < 0.002 respectively, Figure 5.7): 5/11 Thai Int and 3/30 Thai Maj patients had overtly reduced levels and plasma concentrations in the two groups were similar.

**Heparin cofactor II**

HCII levels in both Thai Int and Thai Maj were significantly reduced (p < 0.0001 respectively) (Figure 5.8): levels were abnormal in 10/11 of the Thai Int patients and borderline in the remaining subject. Results were more variable in Thai Maj, with overtly reduced levels in 16/30 patients. The patient in whom HCII was markedly reduced (0.22 U/ml) had normal levels of all the other haemostatic variables tested but was unavailable for follow-up. HCII levels were significantly lower in Thai Int than Thai Maj (p < 0.02).

**Protein C**

There was a substantial reduction in PC in Thai Int, with only one patient having a clearly normal level (Figure 5.9). PC levels in Thai Maj were also reduced relative to the controls, although the median was significantly higher than in Thai Int (0.73 vs. 0.49, p < 0.01). PC was overtly reduced in 11/30 Thai Maj patients. There were weak,
but statistically significant associations between PC and HCII levels in both Thal Int ($r_s = 0.66, p < 0.05$) and Thal Maj ($r_s = 0.60, p < 0.001$), suggesting a common mechanism of depletion of HCII and PC in $\beta$ thal: 10/11 Thal Maj patients with overtly reduced PC also had low HCII and 13/19 patients with normal PC had normal HCII. PC was weakly associated with ATIII in Thal Maj only ($r_s = 0.49, p < 0.01$). There was a disproportionate reduction in PC relative to FVII (which has a similar half-life in plasma) in 8/10 Thal Int patients and at least 8/11 Thal Maj patients with PC deficiency (FVII levels were unavailable in two cases), indicating that specific consumption of the inhibitor contributed to low levels (see Chapter 4, Discussion), however there was no relationship to F1+2, TAT or D-D.

*Free protein S*

PSf levels were abnormal in six Thal Int and four Thal Maj patients, although levels were significantly altered in the Thal Int cohort only, relative to the controls ($p = 0.002$, Figure 5.10). PSf and ATIII were weakly, but significantly associated in Thal Int ($r_s = 0.66, p < 0.05$) and Thal Maj ($r_s = 0.43, p < 0.02$).

*Alpha-1-antitrypsin*

$\alpha_1$AT was slightly lower in Thal Maj compared to the controls, but was unlikely to be clinically relevant as levels were normal in 27/30 patients (Figure 5.11). Higher $\alpha_1$AT levels in Thal Int may reflect a degree of inflammation that is absent or suppressed by transfusion in Thal Maj patients.
Figure 5.7  Antithrombin III levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.

Figure 5.8  Heparin cofactor II levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.
Figure 5.9 *Protein C levels in β thalassaemic disorders.* The dotted line indicates the lower limit of the reference range.

Figure 5.10 *Free Protein S levels in β thalassaemic disorders.* The broken and dotted lines indicate the lower limit of the male and female reference ranges respectively. *abnormal male.*
5.3.7 Serum lipids

Summary data for CHOL and TRIG measurements is shown in Table 5.6. CHOL was reduced in Thal Maj and further in Thal Int (Figure 5.12) and the difference in levels between the two groups was significant ($p < 0.005$). TRIG levels in the patients and control subjects were comparable. CHOL and TRIG were not correlated with each other, nor with PC or PSf levels in Thal Int or Thal Maj.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Caucasian)</th>
<th>β thalassaemia minor</th>
<th>β thalassaemia intermedia</th>
<th>β thalassaemia major</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOL (mmol/L)</td>
<td>5.2 (4.3 – 6.0)</td>
<td>3.8 (3.7 – 4.6)</td>
<td>2.3 (2.1 – 2.8)</td>
<td>3.3 (2.9 – 4.1)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 10</td>
<td>n = 24</td>
<td></td>
</tr>
<tr>
<td>TRIG (mmol/L)</td>
<td>1.2 (0.7 – 1.7)</td>
<td>0.6 (0.5 – 0.8)</td>
<td>1.1 (0.9 – 2.2)</td>
<td>1.2 (0.8 – 1.6)</td>
</tr>
<tr>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 24</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6 Serum lipids in β thalassaemic disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: CHOL: 3.6 – 6.5 mmol/L, TRIG: 0.5 – 1.8 mmol/L.
5.3.8 Factor V Leiden and prothrombin G20210A

Activated protein C resistance

Modified APCR ratios were abnormal in 15 and 16 year old Turkish brothers with Thal Int (ratios: 1.63 and 1.60) and one 23 year old with Thal Maj (ratio: 1.72): heterozygosity for FVL was confirmed by PCR in each subject. TAT (but not F1+2) was mildly elevated in the siblings (8.5 and 8.3 μg/L), although levels up to 15.3 μg/L were observed in patients with normal FV and these two patients were not atypical of the remainder of their clinical group. Haemostatic variables were normal in the Thal Maj patient with the exception of a short TT and slightly elevated sTM (88.4 ng/ml). Two FVL heterozygotes had been excluded from the Caucasian control group, so the incidence of FVL in the patient and control cohorts were similar (4.9 vs. 5.7 %, p = NS).

Prothrombin G20210A

27/41 β thal patients were tested for PGM; all were normal.
5.3.9 Components of fibrinolysis

Summary data for fibrinolytic variables is shown in Table 5.7. Relationships between levels of fibrinolytic variables and markers of thrombin generation were sought: there was no significant association between D-D, Plg or $\alpha_2$AP and F1+2 or TAT levels in either group.

<table>
<thead>
<tr>
<th>Analysis (IU/ml)</th>
<th>HbAA (Caucasian)</th>
<th>$\beta$ thalassaemia minor</th>
<th>$\beta$ thalassaemia intermedia</th>
<th>$\beta$ thalassaemia major</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Dimer (ng/ml)</td>
<td>16 (16 - 33)</td>
<td>16 (16 - 16)</td>
<td>57 (16 - 111)</td>
<td>16 (16 - 16)</td>
</tr>
<tr>
<td></td>
<td>n = 26</td>
<td>n = 3</td>
<td>n = 11</td>
<td>n = 26</td>
</tr>
<tr>
<td>Plg (IU/ml)</td>
<td>1.13 (1.02 - 1.26)</td>
<td>0.98 (0.79 - 1.18)</td>
<td>0.76 (0.63 - 0.89)</td>
<td>0.85 (0.73 - 0.98)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
</tr>
<tr>
<td>$\alpha_2$AP (IU/ml)</td>
<td>1.04 (0.99 - 1.11)</td>
<td>0.94 (0.92 - 1.16)</td>
<td>0.95 (0.85 - 1.01)</td>
<td>0.92 (0.87 - 1.01)</td>
</tr>
<tr>
<td></td>
<td>n = 33</td>
<td>n = 5</td>
<td>n = 11</td>
<td>n = 30</td>
</tr>
</tbody>
</table>

Table 5.7 Components of fibrinolysis in $\beta$ thalassaemic disorders. Median results are reported (with the interquartile range in parentheses). Reference ranges: D-D: < 120 ng/ml, Plg: 0.79 – 1.59 IU/ml, $\alpha_2$AP: 0.88 – 1.22 IU/ml.

D-Dimers

Abnormal D-D levels were seen in only two Thal Int patients (Figure 5.13). 507 ng/ml D-D observed in one Thal Maj patient was clearly not representative of the remainder of the group and the cause of such an atypical result remains unexplained; TAT was only slightly increased in this subject (6.0 µg/L).

Plasminogen

Plg was significantly reduced in both Thal Int and Thal Maj compared to the controls (p < 0.0001 respectively). 6/11 Thal Int and 13/30 (43%) Thal Maj patients had mildly reduced levels (ranging between 0.51 – 0.77 IU/ml). Levels in the larger Thal Maj group appeared to be more variable, but were not substantially altered from those with Thal Int (Figure 5.14).

Alpha-2-antiplasmin

$\alpha_2$AP levels were significantly lower in Thal Int and Thal Maj than the controls (p < 0.002 and p < 0.0001 respectively, Figure 5.15). The lower limit of the reference range established in Chapter 3 (0.88 IU/ml) was somewhat higher than those employed in diagnostic laboratories, so I interpreted levels above 0.70 IU/ml as being equivocal in
this study and certainly not clinically problematic. A distinctly low level (0.61 IU/ml) was observed in only one patient. \( \alpha_2 \)AP levels in Thal Int and Thal Maj were not significantly different.

**Figure 5.13**  
D-Dimer levels in \( \beta \) thalassaemic disorders. The dotted line indicates the upper limit of the reference range.
Figure 5.14 Plasminogen levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.

Figure 5.15 Alpha-2-antiplasmin levels in β thalassaemic disorders. The dotted line indicates the lower limit of the reference range.
5.3.10 Phospholipid-dependent antibodies

The results of PL-dependent antibody screening are given in Table 5.8. LA was not detected in any patient. The proportion of subjects in whom IgG aCL were detected appeared to be higher in Thal Int and Thal Maj compared to the controls, but this was not statistically significant and titres in Thal Int (11 and 23 GPL units) and Thal Maj (6, 7 and 28 GPL units) were similar. It was possible to re-test only two patients: weak antibodies persisted for over a period of more than 10 weeks, however antibodies directed against \( \beta_2 \)GPI were not detected in any patient.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HbAA (Caucasian)</th>
<th>( \beta ) thalassaemia minor</th>
<th>( \beta ) thalassaemia intermedia</th>
<th>( \beta ) thalassaemia major</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRVVT</td>
<td>0/27</td>
<td>0/5</td>
<td>0/11</td>
<td>0/29</td>
</tr>
<tr>
<td>IgG aCL</td>
<td>2/33 (6.1%)</td>
<td>0/5</td>
<td>2/11</td>
<td>3/26 (11.5%)</td>
</tr>
<tr>
<td>IgM aCL</td>
<td>1/33 (3.0%)</td>
<td>0/5</td>
<td>0/11</td>
<td>0/26</td>
</tr>
<tr>
<td>Anti ( \beta_2 )GPI antibodies</td>
<td>None detected</td>
<td>Not tested</td>
<td>None detected</td>
<td>None detected</td>
</tr>
</tbody>
</table>

Table 5.8 Tests for phospholipid-dependent antibodies in \( \beta \) thalassaemic disorders.

5.3.11 Endothelial cell activation

Summary data for sE-s levels are shown in Table 5.9. sE-s was significantly elevated in Thal Int (p < 0.0001), whereas levels in Thal Maj resembled those of the control group (Figure 5.16). 8/11 (73%) Thal Int patients showed increased levels compared to only one Thal Maj patient, in whom the level was only marginally elevated and the difference in levels between the two groups was highly significant (p < 0.0001). sE-s was not correlated with WBCs in Thal Int or Thal Maj, but had a weak, yet statistically significant relationship with PC in Thal Int (\( r_s = -0.62 \), p < 0.05). It was possible to determine soluble sTM levels in only six Thal Int patients: four were normal, one was equivocal, one was mildly elevated and there was no association with sE-s.
### Table 5.9 Endothelial cell activation in β thalassaemia. Median results are reported (with the interquartile range in parentheses). Reference range: sE-s: 18.8 – 67.5 ng/ml.

<table>
<thead>
<tr>
<th>Analysis</th>
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<th>β thalassaemia intermedia</th>
<th>β thalassaemia major</th>
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</thead>
<tbody>
<tr>
<td>sE-s</td>
<td>33.3 (26.9 – 44.4)</td>
<td>Not tested</td>
<td>92.9 (67.6 – 133.1)</td>
<td>39.9 (29.0 – 47.4)</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td>n = 31</td>
<td></td>
<td>n = 11</td>
<td>n = 26</td>
</tr>
</tbody>
</table>

**Figure 5.16 Soluble E-selectin levels in β thalassaemia.** The dotted line indicates the upper limit of the reference range.

#### 5.3.12 Underlying pathology that may contribute to hypercoagulability

Pathology that involves the pancreas, thyroid glands and heart may complicate β thal as a consequence of oxygen deprivation or iron overload and may be independently associated with an increased risk of thrombosis. The function of these organs had been assessed in the majority of my β thal patients as described with respect to SCD patients in section 4.3.12. Samples for these tests were not necessarily collected at the same time as those for haemostatic investigations, however successive measurements were reasonably consistent within individuals.
Diabetes

Mild hyperglycaemia (glucose: 5.7 – 7.5 mmol/L) was detected in 2/7 Thai Int and 7/22 (31.8%) Thai Maj patients in whom glucose results were available. One additional Thai Maj patient showed evidence of poorly controlled diabetes, with 13.1 mmol/L glucose and elevated fructosamine (410 μmol/L; reference range: 215–264 μmol/L). Glucose levels were not correlated with age and no patient was dehydrated as indicated by sodium and potassium levels.

The effect of hyperglycaemia on haemostatic profiles in Thai Int patients was not evaluated due to insufficient numbers. Haemostatic variables in mildly hyperglycaemic Thai Maj patients were not substantially altered when compared to normoglycaemic patients, with the exception of α1AT, which was slightly higher, though not abnormal (0.97 vs. 0.85 U/ml, p < 0.001). This may indicate a generally more severe course in these patients, with increased inflammatory processes and worse tissue (i.e. pancreatic, in this case) damage. Specifically, there was no difference in the amount of thrombin generated between hyper- and normo-glycaemic patients.

Thyroid function

Thyroid stimulating hormone and free thyroxine levels were available for all Thai Int and 29/30 Thai Maj patients. Hypothyroidism in one Thai Maj patient was evident by markedly reduced free thyroxine and an inadequate compensatory response by thyroid stimulating hormone (free thyroxine: < 6.4 pmol/L, reference range: 9.1 – 23.8 pmol/L; thyroid stimulating hormone: 51.6 μU/ml, reference range: 0.5 – 5.0 μU/ml). A further three Thai Maj patients showed evidence of adequately compensated thyroid dysfunction, with mildly raised thyroid stimulating hormone levels only (5.2, 5.8 and 10.4 μU/ml).

Cardiac abnormalities

No patient displayed symptoms of cardiac dysfunction, nor had creatinine kinase measurement been indicated in any patient. Levels of AST were elevated in 6/9 Thai Int and 4/28 Thai Maj patients. As discussed previously, creatinine kinase may be derived from cardiac and/or hepatic tissue and may therefore reflect dysfunction of either organ. Hepatic or cardiac abnormalities in this cohort may have occurred in association with iron overload and/or inflammation, as AST was weakly but significantly correlated with ferritin levels in Thai Maj (r_s = 0.40, p < 0.05).
Splenectomy

One of the aims of this study was to examine haemostasis in β thal patients with respect to surgical splenectomy. Ideally, the impact of splenectomy on haemostatic variables should be assessed longitudinally in untransfused patients, but only three Thal Int patients had not undergone surgical splenectomy, so statistical analysis was not possible.

14 Thal Maj patients had been splenectomised. Significant differences between splenectomised and non-splenectomised patients were observed with respect to FX: 0.91 vs. 0.71 IU/ml, p < 0.05, HClI: 0.79 vs. 0.66 U/ml; Plg: 0.92 vs. 0.74 IU/ml and Fg: 2.31 vs. 1.65 g/L, p < 0.01 respectively. Marginally longer PTs noted in splenectomised patients correlated negatively with FVII levels (r_s = -0.60, p < 0.02). A trend toward higher PC levels in splenectomised patients did not reach statistical significance (0.77 vs. 0.69, p = 0.06) and the extent of thrombin generation and endothelial cell activation in the two groups was similar (F1+2: 0.6 vs. 0.7 nmol/L; TAT: 3.9 vs. 2.7 μg/L; sE-s: 38.5 vs. 41.5 ng/ml). A trend toward higher platelet counts in splenectomised patients did not reach statistical significance (345 vs. 281, p = 0.07), which was not unexpected as the patients were no longer in an acute post-operative period.

Conclusion

Hyperglycaemia was detected in a significant proportion of patients in my study, although diabetes was diagnosed in only one patient. Thrombin generation did not appear to be affected by hyperglycaemia in Thal Maj but it was not possible to assess this in Thal Int due to insufficient data points. There was no evidence of common underlying pathology that contributed to the hypercoagulable state in this cohort. Improved FX, HClI, Plg and Fg levels were noted in splenectomised Thal Maj patients, however the clinical significance of this is unclear.

Acute phase proteins

Evidence of an acute phase reactivity was sought by measuring serum CRP levels. Slightly raised CRP was detected in only two patients: one with Thal Int (0.88 mg/dL) and the other with Thal Maj (0.92 mg/dL), but these were not sufficiently abnormal to suggest acute phase reactivity. Also, Fg was mildly elevated in only one Thal Maj patient, who had normal CRP; median α1AT levels were higher, but not abnormal, in Thal Int and levels were not correlated with other haemostatic variables. Acute phase reactivity was not a significant feature in this cohort.
5.3.13 Serial observations in a patient commencing regular exchange transfusions

During the course of the study, one Thai Int patient ('GEB') commenced regular exchange transfusions in an attempt to stabilise progressive retinopathy. She began a regime of two unit exchanges on a three weekly basis and samples were collected immediately prior to each transfusion. Two samples were collected prior to the first exchange (samples 1 & 2) and eight samples were subsequently collected over an 11 month period (samples 3 - 10). A full blood count was performed prior to each exchange with the exception of sample 5, which was collected one week later. Levels of each haemostatic variable were corrected for minor haemodynamic alterations and compared to the baseline sample, obtained immediately prior to the first transfusion (Figure 5.17).

The percentage of HbA rose from a pre-transfusion level of 20% to a consistent level of approximately 75% over the period of observation. This was accompanied by a corresponding decrease in HbF from 76% to approximately 21%. TAT levels were variable: TAT was slightly elevated in sample 1 but had normalised by the time the baseline sample was collected; there was an initial decrease following the first four transfusions; samples collected at +15 and +42 weeks yielded moderately elevated levels (13.0 and 18.3 μg/L), which normalised by collection of the last sample at +46 weeks. There was no apparent clinical impact of these fluctuating TAT levels and they did not correspond to inhibitor levels, which showed no consistent pattern. F1+2 levels decreased slightly, but were not abnormal at the outset. HClI levels fluctuated a little, but showed a gradual improvement from an abnormal pre-transfusion level of 0.66 IU/ml to a maximum, normalised level of 0.84 IU/ml at seven months.

There was a moderate and consistent reduction in sE-s following transfusion, however sTM levels were unremarkable. This supports the suggestion, from data presented earlier in the chapter, that an increase in HbA-containing cells (and a corresponding decrease in lysis and damage caused by thalassaemic cells) reduces activation of the endothelium and in fact, sE-s were weakly associated with HbA levels ($r_s = -0.68, p < 0.05$) in this instance.
Figure 5.17  Serial observations in a Thal Int patient commencing regular exchange transfusions. (a) quantitative haemoglobin, (b) markers of thrombin generation and (c) markers of endothelial cell activation. The first transfusion is indicated by ▽.
Comparison between haemostatic profiles in SCD and β thal patients.

I made an arbitrary comparison between levels of each variable in the Thal Int group with those in the untransfused HbSS patients described in the previous chapter. Plasma levels of ATIII, α₂AP, F1+2, TM, sE-s and FII were similar in HbSS(untx) and Thal Int, however D-D levels were notably higher in HbSS(untx). PSf levels tended to be lower in Thal Int but this was not statistically significant. TAT levels were comparable, but because the upper limit of the reference range is higher in Black subjects, a greater proportion of Thal Int patients had abnormal levels (73 vs. 37%). α₁AT, Plg, Fg and FX tended to be lower in Thal Int and may reflect a relative reduction in protein synthesis in those patients, who were likely to have greater iron overload. A comparison between untransfused HbSC and Thal Int patients yielded a similar pattern with the exception of D-D levels, which approximated those seen in Thal Int. It is possible that lower FVII in HbSS(untx) reflects greater binding to damaged RBC than occurs in β thal, a notion supported by greater RBC haemolysis in HbSS(untx) (LDH: 891 vs. 612 BB U/ml, p < 0.05), although HCII and FVIIa levels were reduced to a similar extent. An important observation was that fibrin deposition was greater in HbSS(untx) than Thal Int, despite similar degrees of thrombin generation and endothelial cell activation (Appendix VII).
5.4 Discussion

In this chapter, I examined patients with Thal Int or Thal Maj who presented without acute clinical complications and compared them to the healthy Caucasian HbAA control group described in Chapter 3. The Thal Int patients were untransfused and those with Thal Maj were transfusion-dependent. Hypothyroidism was detected in one patient and another had cardiac disease secondary to substantial iron overload. One patient had poorly controlled diabetes and although mild hyperglycaemia was not uncommon in this cohort, there was no evidence of common underlying pathology that contributed to hypercoagulability.

Thrombin generation

When I commenced this investigation, reports of enhanced platelet activation, reduced levels of physiological anticoagulants and impaired fibrinolysis indicated a hypercoagulable state in β thal. Elevated TAT levels in my Thal Int and Thal Maj cohorts confirmed this, providing novel, direct evidence of excessive thrombin generation. There was no relationship between the extent of thrombin generation and the length of time following the most recent transfusion in Thal Maj. Median TAT levels in Thal Maj were notably lower than those observed in Thal Int, although levels were more variable in Thal Maj; abnormal levels were more frequently encountered in Thal Int than Thal Maj, suggesting a role for transfusion in the amelioration of thrombin generation, despite persistent, increased thrombin generation in a third of Thal Maj patients. It may be that transfusion suppresses thrombin generation in select β thal patients only, or that the beneficial effect of transfusion can be overcome by sufficient stimulation of the coagulation system in markedly hypercoagulable individuals.

F1+2 and TAT levels were not correlated in Thal Int or Thal Maj and possible reasons for this were discussed in reference to SCD patients (section 3.4). The relatively higher TAT levels compared to controls as well as overtly abnormal levels in a significant proportion (44%) of patients provided evidence of ongoing thrombin generation in β thal that was not associated with a history of splenectomy, transfusion status, HbF concentration or any of the haemostatic variables tested. To date, only one other study has examined markers of thrombin generation in Thal Maj and confirmed elevated levels in adults as well as children (Eldor et al, 1999).
Liver function

It was reasonable to anticipate that altered liver function may be sufficient to interfere with synthetic capacity in my thalassaemic cohorts. The primary site of synthesis of most of the coagulation proteins is the liver and liver disease/insufficiency is a common cause of morbidity and mortality in β thal (Zurlo et al, 1989). The liver is at risk of toxic damage because excessive amounts of iron released from haemolysed RBCs are deposited in this, as well as other organs. Furthermore, Thai Int patients are particularly prone to excessive gastro-intestinal iron absorption and Thai Maj patients are subject to gross iron overload following frequent transfusions. Hepatomegaly is frequently encountered; marked haemosiderosis is a universal finding, which may be accompanied by various degrees of fibrosis and inflammatory hepatitis and most regularly transfused adult thalassaemic subjects show features of post-necrotic cirrhosis (Jean et al, 1984). Multiple transfusions also increase the risk of exposure to viral hepatitis. Indeed, grossly elevated serum ferritin levels in at least a third of the Thai Maj patients indicated significant iron overload, so it was important to try and establish the impact of any liver dysfunction on plasma levels of haemostatic proteins.

Only four of my patients presented with definitive biochemical evidence of significantly altered liver function. Of the remaining individuals, altered TBIL and LDH levels were attributed to accelerated intravascular or bone-marrow associated RBC haemolysis. AST levels were normal or only slightly elevated and albumin concentrations were adequate in all patients, so any impairment of synthetic function could only be considered mild (criteria for significant LDI was outlined in section 4.3.13). Chronic liver disease is thought to play an important role in the deterioration of glucose tolerance (De Sanctis et al, 1986), which may explain the mild hyperglycaemia evident in at least a third of my Thal Maj cohort, however there was no convincing association with AST levels in this sub-group and their haemostatic profiles were not significantly altered from the remaining patients.

It was difficult to assess the extent of likely hepatic insufficiency in these patients. Biochemical indicators do not always reflect the state of the liver in β thal (Masera et al, 1980; Buonanno et al, 1984). Chronic hepatitis may be demonstrable by liver biopsy in 30% of patients with normal transaminase levels and 57% of patients with transaminase levels that would be considered only mildly elevated (Angelucci et al, 1995). In contrast, normal clotting factor levels may be maintained in patients with markedly elevated AST (Rapaport SI, 2000). Accurate assessment of liver disease (i.e.
due to iron overload, siderosis, hepatitis or cirrhosis) requires examination of biopsied
tissue and this was beyond the scope of my study. However, most β thal patients show
features of cirrhosis by the age of 15-16 years (Jean et al, 1984), so it is reasonable to
expect that a some deterioration of synthetic function has occurred in my (adult)
patients.

I surmise that the relative reductions in plasma levels of haemostatic proteins I
observed here were consistent with mildly impaired hepatocellular synthesis: decreased
levels of physiological inhibitors and clotting factors were concomitant; the reductions
were usually mild and although protein concentrations were not correlated, they were
generally reduced to a similar extent. The exceptions were concentrations of HCII and
PC, which were disproportionately reduced (see below). There was no evidence of gross
hepatic derangement in this cohort, despite substantial iron overload in a third of the
Thal Maj patients.

Clotting factors and physiological inhibitors of coagulation

Clotting factors (II, VII, X and Fg) and inhibitors (ATIII, HCII, PC, and PSf)
were measured. With the exception of PSf and Fg in Thal Maj, each variable was
significantly reduced in Thal Int and Thal Maj relative to the controls, a significant
proportion of the patients in each group had overtly abnormal levels and each tended to
be lower in the untransfused Thal Int patients. Reduced clotting factor levels impacted
on PTs and APTTs in both Thal Int and Thal Maj, although very few patients had
overtly abnormal clotting times and these were not likely to be associated with clinical
problems.

The functional activities of FII, FVII, FX, PC and PSf, are vitamin K dependent,
however simultaneous reductions in the activities of proteins that do not require
carboxylation for activity (i.e. ATIII, HCII, Fg, α₂AP and α₁AT) indicated that
mechanisms other than malabsorption or antagonism of vitamin K were operating here.

Acquired ATIII deficiency is thought not to be a strong risk factor for thrombosis
(Jespersen et al, 1999) and only slight reductions were seen here: a relative reduction in
ATIII was observed in both Thal and Thal Maj, but few patients were actually deficient.
PC levels were abnormally low in nearly all the Thal Int patients and a third of the Thal
Maj group. Free PS was reduced in Thal Int, with overtly abnormal levels in six patients
as well as four with Thal Maj. These findings confirm reports of altered levels of the
physiological anticoagulants in β thal, where pronounced ATIII and PC deficiency has
been linked to increasing age and splenectomy (Musumeci et al, 1987; Shirahata et al, 1992) although this was not apparent in my cohort: PC levels tended to be higher in my splenectomised patients, but this did not reach statistical significance and there was no association with age. 10/11 of my Thai Maj patients with abnormal PC had normal ATIII levels, a pattern that has previously been associated with chronic hepatitis (Musumeci et al, 1987), however I was unable to confirm or refute this in my cohort without histological examination of the liver. There was no relationship between low PC and iron overload, hepatocellular damage or abnormal levels of any other haemostatic variable. PC was correlated with HCII in both Thal Int and Thal Maj, but not in the controls, suggesting a common mechanism of depletion in the patients.

Again, acquired deficiencies of coagulation proteins may be a consequence of hepatic insufficiency, or accelerated consumption by an abnormally activated coagulation system. I was unable to detect convincing biochemical evidence of gross hepatic disease in the vast majority of these patients, but plasma levels of haemostatic proteins provide a more sensitive indication of the synthetic capability of the liver than do biochemical tests, and although a recent paper reported normal levels in the presence of markedly abnormal LFTs (Rapaport SI, 2000), it was likely that the concomitant reduction in haemostatic protein levels reflected this. To investigate further, the ratio of PC to FVII was again examined in patients with absolute PC deficiencies (Chapter 4, Discussion). The disproportionate reduction in PC compared to FVII in the majority of cases suggests that PC deficiency in these individuals was primarily due to accelerated consumption of the inhibitor, rather than inadequate synthesis secondary to liver disease.

Low levels of HCII have occasionally been reported in thrombotic patients, however HCII deficiency is not a likely candidate for thrombosis as discussed previously, but HCII levels were borderline or abnormal in all the Thal Int and in half the Thal Maj patients. I did not measure antigen levels in this study, but they have previously been shown to mirror functional levels in β thal (O'Driscoll et al, 1995). HCII may be markedly reduced in liver disease (Rak, 1988; Velasco et al, 1992) and was correlated with AST in my Thal Int patients, but not in the Thal Maj cohort, in whom AST levels were generally normal. This is consistent with a contribution of hepatic insufficiency to abnormal HCII, although levels were disproportionately reduced compared to the other inhibitors and it is likely that other mechanism(s) play a role.

The significantly higher HCII levels in Thal Maj compared to Thal Int suggest an ameliorating role for transfusion. Prior to my study, colleagues suggested that low levels
are related to the accelerated RBC turnover that characterises Thal Int, which is normalised once the haemolysis has been suppressed by hypertransfusion (O'Driscol et al, 1995). That senescent and abnormal RBCs are capable of adsorbing HCII, depleting it from plasma, has subsequently been confirmed (Chitolie et al, 1996) and recent reports that a subpopulation of thalassaemic RBCs express increased amounts of P-s, identifies a mechanism by which platelet activation, coagulation activation, thrombin generation and endothelial cell activation may be stimulated (Borenstein-Ben et al, 1993; Ruf et al, 1997; Kuypers et al, 1998).

If the disproportionate depletion of HCII is indeed a function of RBC turnover, regular transfusion of these patients would be expected to increase HCII levels (by improving the RBC's rheology, biochemical properties and interactions with the endothelium). This indeed occurred in the Thal Int patient who commenced regular transfusions during the course of my study; her HCII levels fluctuated a little, but gradually normalised, rising from 0.61 to 0.84 U/ml over the course of seven months of monitored therapy and the improvement was independent of AST levels or any alteration in PT. FVII, which may also bind the P-s expressed on the thalassaemic RBC membranes, displayed a similar pattern (increasing from 0.71 - 0.86 IU/ml).

Although liver pathology and impaired protein synthesis probably contributes to altered plasma levels of haemostatic proteins in these patients, it seems likely that HCII is actively depleted from plasma in association with chronic haemolysis and adsorption of HCII to abnormal RBCs. Transfusion may ameliorate HCII levels by improving RBC rheology, reducing P-s expression, lessening the chronic haemolytic insult on the liver and suppressing RBC death in the bone marrow. In any case, the fact that HCII deficiency was almost a universal finding in Thal Int as well as being common in Thal Maj, but that only one Thal Maj patient (with a history of other problems) had experienced a thromboembolic event suggests that HCII deficiency is not a significant contributor to the development of thromboses in these patients. This was further supported by the lack of any relationship between HCII levels and the markers of thrombin generation or fibrin deposition.
Factor V Leiden and prothrombin G20210A

The prevalence of FVL in my β thal cohorts approximated that of the control group and was comparable to that reported in populations of similar ethnicity (Dahlback, 1997b). FVL is an important risk factor for venous thrombosis and may have contributed to mildly increased thrombin generation in three of my patients who were heterozygous for this mutation; none had experienced a thrombotic event, but they were all quite young at the time of investigation. It was possible to test two thirds of the patients for PGM and all were normal.

At the time I undertook this investigation, no other assessment had been made as to the contribution of these thrombophilic mutations to thrombotic risk in β thal. Heterozygosity for FVL has since been reported in a child who experienced a stroke following transfusion, however a rapid and pronounced increase in HCT was likely to have been the precipitating factor in that case (Giordano et al, 1997). My study indicated a low clinical impact of FVL and PGM in β thal, which has subsequently been confirmed in a larger cohort (Giordano et al, 1998a), although it should be noted that the prevalence of these mutations is relatively high in some ethnic groups in whom β thal is also common and their coincidental inheritance with β thal will compound the thrombotic risk in individual patients.

Phospholipid-dependent antibodies

IgG aCL were detected in a few of my patients, but not more frequently than observed in the control group. Another study recently reported a 34% incidence of IgG aCL in β thal, which they associated with previous hepatitis C infection (Giordano et al, 1998b). Serological testing for hepatitis and other infections had not been performed in the majority of my patients, so I was unable to confirm their relevance here, but the absence of β2GPI antibodies in my patients made it unlikely that the aCL antibodies were pathogenic. They may be associated with past infection or be stimulated in response to chronic RBC damage (McNally et al, 1995b).

Endothelial cell activation

Interactions between thalassaemic RBC and the endothelium had not been widely investigated when I began this study. Reports of RBC adherence to cultured endothelial cells were contradictory, being normal under low shear conditions (Smith & La Celle, 1987) or increased, particularly in the presence of autologous platelet rich plasma (Butthep et al, 1992). Raised sTM levels have been observed in β thal/HbE
patients, with the greatest increases in patients with leg ulcers, consistent with extensive vascular damage (Butthep et al., 1995).

Raised sE-s levels in the plasma of my Thal Int patients testified to endothelial cell activation in vivo. Unlike the SCD patients though, this did not appear to be linked with inflammation, as sE-s was not accompanied by increased WBC, α1AT, Fg or CRP. One patient (in whom sE-s was 77.2 ng/ml) had chronic, mild ulceration of the foot, however other patients had considerably higher sE-s levels without symptoms caused by localised breakdown of the vasculature. There was a trend toward lower PC levels in association with raised sE-s in this relatively small group, however the significance of this is unclear and there was no relationship between fibrin deposition or activation of the endothelium with thrombin generation or any of the other variables tested.

These observations have been confirmed in the only other study of endothelial cell activation in β thal, which found increased levels of sE-s as well as other soluble intercellular and vascular adhesion molecules and von Willebrand factor in the plasma of HbE/β thal patients (Butthep et al., 1997). The authors reported higher sE-s levels in splenectomised patients, whereas the reverse was the case in my cohort, although the numbers were too small to enable meaningful analysis. It also appears that thalassaemic sera contains one or more factors that influence growth and proliferation of the endothelium, as serum from thalassaemic patients can inhibit mitosis and induce apoptotic changes in cultured endothelial cells (Banjerdpongchai et al., 1997; Butthep et al., 1997).

The stark contrast between sE-s levels in Thal Int and Thal Maj in my study indicate that endothelial cell activation can be entirely suppressed by transfusion in β thal and levels in Thal Maj patients actually resemble those of HbAA subjects (despite persistent thrombin generation and an elevated WBC in more than half the patients). Further, there was an immediate and sustained normalisation of sE-s levels in the Thal Int patient who began regular exchange transfusions during the course of my investigation.

Transfusion

The global impact of regular exchange transfusions on the plasmatic environment in β thal can be indirectly assessed by comparing variables in the untransfused Thal Int group with the regularly transfused Thal Maj patients. Statistically significant differences were identified between the Thal Int and Thal Maj cohorts with respect to thrombin generation (F1+2 and TAT), fibrinolytic activity (D-D), clotting
factors (II and X) and inhibitors (HCII and PC). In each case, levels of the variables tended to be improved in the Thal Maj patients, suggesting an ameliorating role for transfusion that was statistically significant for FII, FX, HCII, PC, and PSf, although levels were still abnormal in some patients. Thrombin generation and fibrin deposition was curbed: F1+2 and D-D levels, while not being overtly abnormal, were significantly lower in Thal Maj than Thal Int and the difference in TAT levels between the two groups was statistically significant. As discussed earlier, TAT levels in Thal Maj were highly variable, remaining elevated in one third of the patients and raised sE-s levels in Thal Int reflected a degree of endothelial cell activation that was not evident in Thal Maj. Slightly higher α1AT levels (an acute phase protein) in Thal Int may be associated with inflammatory processes that are also curbed by transfusion in Thal Maj.

Hepatocellular damage as a result of iron toxicity is a more significant factor in β thal than in SCD, which is reflected in the more generalised, albeit mild reductions in plasma levels of haemostatic proteins, but this bore no apparent relationship to the extent of thrombin generation. Having established that hypercoagulability in β thal is not associated with underlying complications of the disease (i.e. cardiac, thyroid or pancreatic pathology), congenital defects (i.e. FVL or PGM) or the acquisition of PL-dependent antibodies (i.e. LA or aCL), the question arises as to what is activating coagulation in these patients? The possibility that the thalassaemic RBC themselves possess procoagulant properties (akin to that proposed in SCD) is being investigated by other authors.

In vitro studies have demonstrated the enhanced thrombogenic activity of thalassaemic RBCs: prothrombin activation by FXa in a purified system is accelerated three-fold by normal (i.e. HbAA) RBCs but β thalassaemic cells reduce the Km of the reaction 17-fold, which is comparable to the acceleration induced by SRBC (Borenstain-Ben et al, 1993; Helley et al, 1996). Sensitive flow cytometric assays using AV have fully ascribed this capacity to abnormal P-s expression on the outer surface of the membrane of a subpopulation of thalassaemic RBCs (Helley et al, 1996; Srinivasan & Basu, 1996). This in turn, has been accounted for by oxidative damage that interferes with aminophospholipid translocase activity and calcium-induced randomisation of aminophospholipids (de Jong et al, 1997) as well as accelerated repair of oxidatively damaged PLs (Kuypers et al, 1998). In murine studies, abnormal P-s expression been attributed to alterations that occur as a result of passive diffusion movement (i.e. not to
alterations in amino-phospholipid translocase activity (Muller et al, 1993). Ruf et al have identified a positive relationship between the procoagulant activity of RBCs, Annexin V expression (i.e. P-s exposure) and platelet activation in Thal Maj patients (Ruf et al, 1997). Abnormal P-s expression on the surface of thalassaemic RBCs is thought to provide a procoagulant surface capable of accelerating thrombin generation in vivo, which may in turn, trigger platelet activation and stimulate coagulation.

**β-thalassaemia trait**

There were no significant haemostatic abnormalities amongst the very small group of healthy Thal Min subjects co-investigated in this chapter. Specifically, there was no evidence of hypercoagulability as thrombin generation and fibrinolytic activity was normal, levels of clotting factors and inhibitors resembled those of the control subjects and there was no evidence of abnormal activation of the endothelium. This is consistent with the usual (asymptomatic) clinical course associated with Thal Min (Modell & Berdoukas, 1984b).

**Conclusion**

My study confirms a hypercoagulable state in β-thal, even in the absence of significant clinical complications. Congenital thrombopholic abnormalities (i.e. FVL and PGM) and acquired PL-dependent antibodies do not contribute to this hypercoagulability. Enhanced thrombin generation is accompanied by altered plasma levels of clotting factors, physiological inhibitors and components of fibrinolysis that are likely to be attributable to hepatocellular damage and reduced protein synthesis. This does not exclude a consumptive coagulopathy (and PC appears to be selectively depleted) however there was no apparent relationship between plasma levels of the physiological inhibitors of coagulation and the extent of thrombin generation. Coagulation is clearly activated, but as discussed in relation to SCD patients in the previous chapter, the concomitant reduction in coagulation inhibitors and clotting factors may allow a modified haemostatic equilibrium to be maintained in the absence of further prothrombotic challenge. In the main, plasma levels of haemostatic proteins were improved in Thal Maj relative to Thal Int cohort; this may be due to better preserved hepatocellular function in the former group (as AST levels were significantly higher in Thal Int despite lower iron stores) or associated with an altered plasmatic environment afforded by regular exchange transfusions, or both.
A disproportionate reduction in HCII is consistent with its active depletion from plasma as HCII is known to bind abnormal thalassaemic RBC membranes. Abnormal P-s expression on the membrane of a subpopulation of cells may provide a chronic, prothrombotic stimulus that results in up-regulated coagulation and demonstrable hypercoagulability in some patients.

The most clinically pertinent observation made here was that regular exchange transfusions entirely suppress endothelial cell activation in β thal. Normalisation of sE-s and PC points toward an improved condition of the endothelium in transfused patients. This goes some way to explaining the very low recurrence rate of thrombotic complications (including strokes) in hypertransfused patients despite ongoing excessive thrombin generation and inflammation.

Despite hypercoagulability being demonstrable in the majority of Thal Int patients and a significant proportion of those with Thal Maj, relatively few experience clinical thrombotic events. While coagulation is clearly activated and the haemostatic equilibrium is shifted toward coagulation, the concomitant reduction in both clotting factors and inhibitors of coagulation appears to result in a fragile equilibrium being maintained unless the patient is challenged with a further prothrombotic stimulus, such as immobility, acute inflammation, infection, pregnancy or a marked haemodynamic change. It would, of course, be interesting to monitor patients on a regular basis over an interval of years, to determine whether a thromboembolic event can be predicted by a rapid or even gradual change in plasma concentrations of thrombin generation markers, endothelial cell activation markers or other indicators within individual patients, however this was beyond the scope of my study.
CHAPTER 6

SUMMARY & CONCLUSIONS

The remit of my study was to examine the haemostatic system in SCD and β thalassaemia, two congenital disorders characterised by chronic haemolytic anaemia, progressive organ damage and susceptibility to infection. These disorders produce RBCs with abnormal morphological and biochemical properties, increased adhesitivity and aberrant phospholipid exteriorisation. Sickled RBC may physically occlude regions within the microvasculature. I selected adult patients with SCD (HbSC or HbSS) or β thal (intermedia or major) for parallel investigations: in general, HbSC patients experience a milder clinical course than those with homozygous SCD and Thai Int patients are less severely affected than those with Thal Maj, who require transfusion support from infancy so that gross manifestations of the disorder are considerably abated. Particular attention was paid to mechanisms that may propagate excessive thrombin generation and predispose affected individuals to venous thrombosis, as reports of thromboembolic complications in association with SCD and β thal suggested that thrombin generation was poorly regulated in these patients.

Prior to my commencing this research, colleagues had reported low plasma levels of a physiological thrombin inhibitor (HCII) in SCD and β thal patients, however this was unlikely to be the root of their thrombotic problems as isolated HCII deficiency may be encountered in healthy individuals and is not a convincing risk factor for thrombosis. Other authors described additional haemostatic abnormalities, although no comprehensive evaluation of the haemostatic system had been performed in adult patients at the time of planning this work. When designing my study, I addressed several issues that clouded the interpretation of some previously published data: I (i) measured an extensive array of haemostatic, haematological and biochemical variables in clinical groups classified by Hb genotype; (ii) used ethnically relevant control subjects with normal Hb (HbAA) for comparative analysis; (iii) studied patients in the absence of significant clinical complications; (iv) limited the investigation to adults and (v) took into account the transfusion status of the patients. Variables that provided information regarding thrombin generation and regulation, clotting protein synthesis, congenital and
acquired thrombophilic abnormalities, fibrinolytic activity and the condition of the vasculature were examined.

All of the SCD patients in my study were Black, while most of the β thal patients were Caucasian and physiological levels of some biological variables vary with ethnicity. Therefore, I determined plasma levels of the haemostatic variables of interest in healthy Caucasian and Black control subjects prior to evaluating the patients (Chapter 3). Reference ranges were independently established using data from these two control groups and minor differences were noted for several variables, confirming the importance of utilising an appropriate group of control subjects for comparative analysis: longer APTTs, higher α₁AT and sE-s as well as lower F1+2 levels in the Black controls were statistically significant. SCD and β thal patient cohorts were then compared to the Black and Caucasian control groups respectively: a quarter of the HbSS patients and all the Thai Maj patients were regularly exchanged transfused; the majority of the remaining patients had not been transfused for at least three months.

It was important to determine the contribution of any underlying disease to hypercoagulability in my patients. The chronic haemolysis, iron overload, inflammation and repeated sickling episodes that accompany SCD and β thal may cause progressive pancreatic, thyroid or cardiac pathology that independently poses a thrombotic risk to affected individuals: mild hyperglycaemia was common amongst my cohort and laboratory records confirmed diabetes in one HbSC and one Thai Maj patient, however there was no apparent impact on hypercoagulability. The haemostatic profiles of splenectomised Thai Maj patients were not significantly altered from those with intact (but possibly dysfunctional) spleens and the acute haemostatic changes sometimes encountered following splenectomy were not observed here, as surgery had been performed years earlier. Despite the patients being asymptomatic at the time of investigation, it was clear from mild to moderately elevated WBCs that underlying inflammation persisted in 61% of the HbSS cohort and CRP levels were consistent with acute phase reactivity in 37% of these, although there was no significant impact on levels of the haemostatic proteins that are acute phase reactants within this group. Mild inflammation was also evident in the majority of the Thal Int patients and half the Thal Maj group, without evidence of acute phase reactivity.

Coagulation screening tests detected marginal and clinically insignificant prolongations of the PT or APTT in a minority of patients. These were attributed to mild deficiencies of clotting factors that were subsequently found to be a common
phenomenon in each clinical group. These global tests of haemostasis were not expected to detect a hypercoagulable state, but were performed to identify any gross haemostatic abnormalities and provide background information about the general haemostatic status of the patients.

Patients with SCD and β thal were investigated independently, however there were some general findings that were common to both disorders. Crucially, hypercoagulability was demonstrable in each clinical group, with approximately half the patients yielding direct evidence of excessive thrombin generation (i.e. raised F1+2 and/or TAT). This tended to be more pronounced in HbSS than HbSC, but the difference did not reach statistical significance and was independent of transfusion status. The extent of thrombin generation in untransfused HbSS and Thal Int patients was similar, although this represented a greater proportion of hypercoagulable Thal Int patients. Median TAT levels were significantly lower in Thal Maj than Thal Int, however levels remained high in a third of patients, suggesting only a partial ameliorating effect of transfusion; a similar phenomenon occurred in the HbSS group.

Increased fibrin deposition occurred in HbSS only: D-Ds were commonly elevated in HbSS patients (providing further evidence of coagulation system activation) but not in HbSC, Thal Int or Thal Maj. F1+2, TAT and D-D levels were not correlated within clinical groups, possibly because each have different half-lives in plasma and are derived at different stages of the dynamic process that involves thrombin generation and dissolution of fibrin. Also, I performed discrete observations only; serial observations may have provided more information as to whether thrombin generation was increasing or subsiding in individual patients.

Hypercoagulable individuals are, by definition, at a heightened risk of thrombosis and several mechanisms may contribute to this increased potential for thrombus formation. I went on to test for the more common haemostatic abnormalities known to contribute to such an altered equilibrium in other thrombophilic populations. My study illustrated the low clinical impact of FVL and PGM in patients with SCD and β thal: their prevalence in my clinical groups mirrored that of healthy populations of similar ethnicity, which has subsequently been confirmed by other authors. PL-dependent antibodies were sought using clotting tests and immunological techniques in accordance with national guidelines. LA was not detected in any patient, however 25% SCD and 14% β thal patients had increased titres of IgG aCL. Because these antibodies were not β2GPI-dependent, they may be associated with recent infection or chronic cell
damage, rather than being directly pathogenic. This notion is supported by clinical studies that have found no relationship between the incidence or titres of aCL and the major complications of SCD or β thal.

In my study, ATIII and PC levels were significantly altered in HbSS (but not HbSC) as well as both β thal groups, however overtly abnormal ATIII levels were uncommon. An interesting observation was that overt PC deficiency was almost uniformly encountered in Thal Int, but only a third of the transfused Thal Maj patients. A similar phenomenon occurred in HbSS, whereby transfused patients showed entirely normal PC levels that were significantly higher than those in untransfused patients. There was also a positive association between CHOL and PC in HbSC and HbSS, but not β thal: CHOL contained within the SRBC membrane is readily exchangeable with plasma CHOL: SRBC lipids therefore contain high concentrations while plasma levels become depleted. Improved RBC rheology afforded by transfusion is likely to account for improved levels of CHOL and PC in transfused patients. I examined the ratio of PC:FVII in those patients with overtly reduced PC levels as the two proteins have similar half-lives in plasma: PC was disproportionately reduced in the vast majority of both Thal Int and Thal Maj with overtly reduced levels, suggesting that this inhibitor was being specifically depleted as a parallel reduction in FVII would be expected if low levels were merely attributable to reduced synthesis. In contrast, few SCD patients were actually PC deficient and only one HbSC and one HbSS patient yielded a relative reduction in PC.

PSf deficiency was not common in my patients and Thal Int was the only clinical group in whom levels were significantly altered overall. This contrasted with reports by other authors and the reason for this is unclear. Not all studies have utilised ethnically similar control groups and the lower limit of the reference range for healthy Black females in my study was lower than that for Caucasians; perhaps true PSf deficiency has been overestimated in SCD. Extensive liver disease in other cohorts may have contributed to their decreased levels, although the only (HbSC) patient with a clinical history of hepatic complications in my study had entirely normal PSf. Another explanation may be that alternative methodologies measure slightly different aspects of PSf: I utilised a relatively new ELISA that employed two monoclonal antibodies (section 2.4.3), although this assay has been shown to have good correlation with functional assays. PSf levels were loosely correlated with reductions in other clotting
factors and inhibitors in my HbSS group, but as levels were normal in 26/31 patients, this does not appear to be clinically relevant.

Having demonstrated that hypercoagulability occurs against a mildly disordered background of plasma levels of thrombin inhibitors in both SCD and β thal, I endeavoured to determine whether this typically occurred as a result of hepatic dysfunction and reduced protein synthesis or reflected an uncompensated, consumptive coagulopathy. The liver is particularly susceptible to pathology in patients with these haemoglobinopathies: chronic haemolysis and iron toxicity in both disorders as well as recurring episodes of RBC sickling in SCD potentially compromise hepatocellular function; multiple transfusions propagate iron overload and carry a risk of exposure to viral hepatitis. However, a chronic pro-coagulant challenge mounted by abnormal RBC interactions with the endothelium, vaso-occlusion of the microvasculature and aberrant P-s exposure by sickled and thalassaemic RBC may perpetually activate coagulation and result in a chronic depletion of coagulation proteins. Determining the impact of these potential mechanisms and their relationship to the extent of thrombin generation turned out to be rather a complicated issue.

The extent of hepatocellular damage was difficult to assess in SCD and β thal as some tests of liver function are affected by the haemolysis that characterises both disorders. True assessment of liver pathology requires the examination of biopsied tissue, which was beyond the scope of my study, although variable degrees of iron deposition, fibrotic and inflammatory change are known to occur in both disorders (and were evident in patients for whom histological reports were available). I therefore assessed biochemical tests of liver function in collaboration with a clinician: with the exception of one patient who had a congenital hepato-biliary deformity, abnormalities observed in the HbSC patients were entirely attributable to haemolysis and there was no biochemical evidence of significant hepatocellular injury amongst those with HbSS, Thal Int or Thal Maj.

Measurement of plasmatic haemostatic proteins provide a more sensitive indication of the synthetic capability of the liver. Clotting factor deficiencies did not generally affect the PT, which is considered a sensitive indicator of hepatic function, but disordered levels of the plasma inhibitors in HbSS, Thal Int and Thal Maj were often accompanied by mild, relative alterations in FII, FVII, FX, α1AT, Plg and α2AP, suggesting a degree of hepatic insufficiency. However, as AST levels were not considered significantly elevated within the context of these disorders and albumin
levels were maintained, this could not be considered severe. Still, increased thrombin
generation with or without increased fibrin deposition evidently occurs in these patients,
so accelerated consumption in a systemic coagulopathy cannot be excluded either. An
alternative mechanism that may explain the disproportionately low levels of HClII that
were common to each clinical group is discussed below.

When I began this investigation, I anticipated a demonstrable relationship
between plasma levels of thrombin inhibitors and markers of thrombin generation in
SCD and β thal, as reduced levels of the proteins coincided with indirect evidence of
hypercoagulability in the literature. This was not the case, however: irrespective of the
exact mechanisms that culminate in altered plasma concentrations of haemostatic
proteins, an important observation was that there was no consistent trend amongst the
levels of coagulation inhibitors within or between patient groups, nor was there a
perceptible relationship between their levels and the extent of thrombin generation or
fibrin deposition. Patients in whom levels of one or more of the inhibitors was relatively
or absolutely reduced often yielded entirely normal F1+2 and TAT levels and
conversely, elevated F1+2 and/or TAT were observed in patients with normal inhibitor
levels. The same was true with respect to D-Ds.

The period following an exchange transfusion may represent a time of heightened
thrombotic risk as in vivo thrombin generation markedly increases as a function of pre-
transfusion TAT levels during this period (section 4.3.14). I verified that thrombin is not
generated during storage of the RBCC; it is possible that storage-induced stresses
sufficiently alter the RBC membrane so that it provides a suitable surface for binding
coaulation factors in the presence of physiological concentrations of calcium, before
regaining normal rheology in the circulation. Patients with multiple thrombotic risk
factors may benefit from prophylactic anticoagulation during this period, which is
limited in any case, as TAT rapidly returns to baseline levels.

One of the objectives of my investigation was to ascertain whether haemostatic
profiles varied significantly between different SCD and β thal genotypes. Some
variables appeared to be less severely altered in untransfused HbSC patients relative to
untransfused HbSS patients, but this did not always reach statistical significance. The
most significant observation with respect to HbSC patients was that they displayed no
evidence of abnormal endothelial cell activation or fibrin deposition, in contrast to
untransfused HbSS patients, despite equivalent degrees of abnormal thrombin
generation. There was a trend toward more abnormal levels of most of the haemostatic proteins in Thal Int compared to Thal Maj, though not always to a degree that was statistically significant: the extent of thrombin generation in the two groups was comparable, however endothelial cell activation and mildly abnormal fibrin deposition were features of Thal Int only.

*My data advocates that:*

- it is important to establish 'normal' levels of biological variables of interest in appropriate control subjects. Some physiological variation may be encountered between different ethnic groups and if this is statistically significant, patients may be incorrectly categorised as 'normal' or 'abnormal' when an inappropriate reference range is applied. For example, the number of my SCD patients who are hypercoagulable may otherwise have been overestimated as the reference range for TAT is significantly higher in healthy Black HbAA subjects compared to Caucasians;

- a lack of acute symptoms in SCD and \(\beta\) thal does not necessarily represent an absence of pathology. The haemostatic system in these patients is clearly not normal, whether compared to healthy controls or heterozygous 'carrier' subjects: features include increased thrombin generation, reduced levels of clotting factors, physiological anticoagulants and other haemostatic proteins as well as increased fibrin deposition and endothelial cell activation;

- hypercoagulability is a common feature of SCD and \(\beta\) thal despite an absence of clinical complications; excessive thrombin generation and/or fibrin deposition is a consistent finding in HbSS patients as well as a significant proportion of those with HbSC, Thal Int or Thal Maj. The extent of thrombin generation amongst untransfused HbSC, HbSS and Thal Int patients is similar, although significantly increased fibrin deposition occurs in HbSS only. Fibrin deposition, but not thrombin generation, is appreciably assuaged in regularly transfused HbSS and \(\beta\) thal patients;

- hypercoagulability in SCD and \(\beta\) thal is not attributable to the abnormalities most commonly encountered in other thrombophilic populations, i.e. FVL, PGM, acquired PL-dependent antibodies or underlying disease;
• mild or moderate HCII deficiency is a consistent finding in SCD and β thal and is often the only abnormality detected amongst the physiological inhibitors of coagulation in SCD. Further, HCII is often disproportionately reduced relative to the other inhibitors, suggesting an independent mechanism of depletion. HCII is known to bind PL abnormally expressed on the membrane of sickled or thalassaemic RBC with subsequent removal from plasma, which probably accounts for such altered levels. This was corroborated when clearly normal HCII were only detectable in (some) HbSC, transfused HbSS and Thal Maj patients, who would be expected to have improved RBC rheology relative to untransfused HbSS and Thal Int patients. I was able to confirm improved HCII levels in transfused β thal (but not HbSS) patients that were originally reported by my colleagues: greater P-s exposure may persist in HbSS. Anecdotally, HCII (as well as FVII and PSf, which may also bind abnormal RBC membranes) gradually normalised in a Thal Int patient who's percentage of abnormal circulating RBC decreased when she commenced regular exchange transfusions.

• hypercoagulability in SCD and β thal appears to be a consequence of the RBC pathology that characterises these disorders: a commensurate degree of thrombin generation is evident in untransfused HbSC, HbSS, Thal Int and Thal Maj patients and abnormal thrombin generation is demonstrable in SCD children as young as 12 months old, who have better preserved hepatic function (Liesner et al, 1998).

• hypercoagulability does not predict imminent thrombotic complications in these patients. Abnormally activated coagulation was common to each of the clinical groups I investigated, yet thromboses manifest in relatively few individuals with SCD or β thal; persistent hypercoagulability may be tempered by chronic anaemia and additional prothrombotic stimuli must coincide before a critical threshold is breached;

One of the most interesting observations to have arisen from this study was the clear evidence that in vivo activation of the endothelium occurs in untransfused HbSS and Thal Int patients, but not in HbSC, transfused HbSS or Thal Maj. The degree of endothelial cell activation was strongly associated with systemic inflammation in HbSS only, but bore no relationship to the extent of thrombin generation in either group. Regular exchange transfusions appear to suppress activation of the endothelium (there is no evidence of abnormal endothelial cell activation in transfused HbSS or Thal Maj
patients) and reduce the deposition of fibrin, despite having no significant impact on thrombin generation in SCD or β thal.

These findings are consistent with the improvement in vascular patency and the dramatic reduction in recurrence of arterial and venous thromboses observed in hypertransfused patients and suggest that vascular perturbation and fibrin deposition play a greater role in the development of thrombotic complications than does systemic thrombin generation. A reduction in the number of abnormal RBCs in the circulation following transfusion is likely to decrease the mechanical and pathological insult to the endothelium as well as improve the haemodynamics of blood flow, inhibiting the development of thromboses despite ongoing thrombin generation. This is supported anecdotally in two HbSS patients whose F1+2, TAT and sE-s levels gradually improved following the instigation of regular exchange transfusions (data not shown).

Conclusion

I conclude that abnormal activation of the coagulation system is a feature of SCD and β thal: hypercoagulability is common in HbSC, HbSS, Thal Int and Thal Maj patients, irrespective of transfusion status. Abnormal exteriorisation of P-s may contribute to chronic activation of the coagulation system and a consumptive coagulopathy, but this is likely to be propagated by pathological interactions between abnormal RBCs and the endothelium, with associated inflammation and fibrin deposition. A progressive decline in the synthetic capability of the liver may render it incapable of normalising plasma levels of haemostatic proteins, exacerbating the pathology. Relative reductions in levels of the physiological inhibitors of coagulation do not appear to govern the extent of thrombin generation. Disproportionately low levels of specific proteins (HCII, PSf, FVIIa, as well as CHOL) appear to be a consequence of aberrant binding to abnormal RBC membranes with active depletion from plasma. Concomitant reductions in plasma levels of clotting factors and coagulation inhibitors may contribute to a haemostatic equilibrium being maintained in the absence of further prothrombotic insult, despite being altered in favour of coagulation. The patient's capacity to cope with an aggressive prothrombotic challenge is probably compromised, however and when a critical threshold is breached, thromboses develop. The dramatic fall in the incidence of recurrent thromboses that is associated with regular exchange transfusions is likely to be afforded by curbing endothelial cell activation and fibrin
deposition, rather than by creating an environment in which thrombin generation is competently regulated.

Hypercoagulability is only one of several thrombotic risk factors potentially affecting patients with SCD and β thal: hyperviscosity, extended periods of immobility, inflammation, aberrant P-s expression by abnormal RBCs and perpetual insult to the endothelium each heighten the risk of thrombosis. It is important to differentiate between three distinct, but interactive processes that were evident here: (i) hypercoagulability (increased thrombin generation and/or fibrin deposition), (ii) a relative reduction in plasma levels of haemostatic proteins that remains uncompensated, probably as a consequence of mildly impaired hepatic function in HbSS and β thal at least, and (iii) inflammatory activation of the endothelium. The extent to which each of these predominates in an individual patient will determine the likelihood of his experiencing thrombotic complications. My study was limited to an examination of the plasmatic environment: while hepatic insufficiency may account for mildly altered plasma levels of haemostatic proteins, this did not account for the extent of thrombin generation encountered in each of the clinical groups investigated. The question remains as to what instigates activation of the coagulation system in these patients. Apparently, hypercoagulability is not merely a function of abnormal interactions between the endothelium and abnormal sickled and thalassaemic RBCs that express adhesion molecules and P-s: endothelial cell activation was entirely abated in transfused patients, yet significant hypercoagulability persisted. Perhaps P-s expression by these RBC, as well as circulating spicules and endothelial cells in SCD (that have been shown by other authors to have significant procoagulant characteristics) is sufficient to provide a chronic prothrombotic stimulus. In any case, the recurrence of thrombotic complications are dramatically reduced by long-term, regular exchange transfusion therapy, so it appears that the propagation of thromboses in these patients are primarily attributable to abnormal endothelial cell interactions rather than excessive thrombin generation per se. Perhaps prophylactic therapeutic measures directed at regulating inflammatory activation of the endothelium (in contrast to anticoagulation) could provide a means of reducing the occurrence of thromboses in SCD and β thal. Of course, it is possible that abnormal interactions between RBCs and the endothelium extend to involve leucocytes and/or platelets also, which remains a focus of future work.
Bibliography


Osterud, B., Bajaj, M.S. & Bajaj, S.P. (1995) Sites of tissue factor pathway inhibitor (TFPI) and tissue factor expression under physiologic and pathologic conditions On behalf of the Subcommittee on tissue factor Pathway Inhibitor (TFPI) of the Scientific and Standardization Committee of the ISTH. Thromb Haemost, 73: 873-875.


## Appendices

### Appendix I. Manufacturers and suppliers of equipment and reagents.

<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
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<tr>
<td>ABX UK</td>
<td>Warren Court Chicksands, Shefford, Bedfordshire SG17 5QB</td>
</tr>
<tr>
<td>Advanced Biotechnologies Ltd.</td>
<td>Unit 7, Mole Business Park, Randalls Road, Leatherhead, Surrey KT22 7BA.</td>
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<tr>
<td>AGEN Biomedical Ltd.</td>
<td>Distributed in the UK by Quadratech Ltd.</td>
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<tr>
<td>Alpha Laboratories Ltd.</td>
<td>40 Parham Drive, Eastleigh, Hants. S05 4NU</td>
</tr>
<tr>
<td>Analyse-It, Software Ltd.</td>
<td>P.O. Box 77, Leeds, LS12 5XA</td>
</tr>
<tr>
<td>Axis-Shield plc</td>
<td>Luna Place, The Technology Park, Dundee, DD2 1XA Scotland</td>
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<tr>
<td>Baxter AG</td>
<td>Distributed in the UK by Technoclone Ltd. Eclipse House, 7 Curtis Road, Dorking, Surrey RH4 1EJ</td>
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<td>Beckman Coulter UK Ltd.</td>
<td>High Wycombe Bucks HP11 1JU</td>
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<tr>
<td>Becton Dickinson UK Ltd.</td>
<td>Between Towns Road, Cowley, Oxford, OX4 3LY</td>
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<tr>
<td>Bio-Rad Laboratories Ltd.</td>
<td>Maylands Avenue, Hemel Hempstead, Herts. HP2 7TD</td>
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<td>Biotechnologies Ltd.</td>
<td>16A Cambridge Science Park, Milton Road, Cambridge CB4 4GH.</td>
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<td>Chromogenix AB</td>
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<tr>
<td>CP Pharmaceuticals Ltd.</td>
<td>Ash Road North, Wrexham Industrial Estate, Wrexham, Clwyd. LL13 9UF</td>
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<td>Dade Behring</td>
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<td>Dako Ltd.</td>
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<tr>
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<td>Epsilon Technology Ltd.</td>
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<td>Flowgen Instruments Ltd.</td>
<td>Broadoak Enterprise Village, Broadoak Road, Sittingbourne, Kent ME9 8AQ.</td>
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<tr>
<td>Instrumentation Laboratory (UK) Ltd.</td>
<td>Kelvin Close, Birchwood, Warrington, Cheshire WA3 7PB</td>
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<td>Kordia Laboratory Supplies</td>
<td>Oude Singel 160, 2312 RG Leiden, The Netherlands.</td>
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<td>Labtec International</td>
<td>Woodside, Easons Green, Uckfield, East Sussex TN22 5RE</td>
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<td>Life Technologies Ltd.</td>
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<td>Merck Eurolab Ltd.</td>
<td>Hunter Boulevard, Magna Park, Lutterworth, Leicester LE17 4XN</td>
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<tr>
<td>National Institute for Biological Standards and Control (NIBSC)</td>
<td>Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG.</td>
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<td>Novo Nordisk A/S</td>
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Quadratex Ltd.  
R & D Systems Europe Ltd.  
Roche Diagnostic Ltd.  
Sarstedt Ltd.  
Severn Biotech Ltd.  
Sigma-Aldrich Company Ltd.  
Synermed Europe Ltd.  
Sysmex UK Ltd.  
Unicorn Diagnostics Ltd.  

Delta House, Chilworth Research Centre, Southampton SO16 7NS  
P.O. Box 167 Epsom, Surrey KT18 7YL  
4-10 The Quadrant, Barton Lane, Abingdon, OX14 3YS  
Bell Lane, Lewes., East Sussex BN7 1LG.  
68 Boston Road, Beaumont Leys, Leicester LE4 1AW  
Unit 2 Park Lane, Kidderminster, Worcestershire, D411 6TJ.  
Fancy Road, Poole, Dorset BH17 7NH  
67 Victoria Road, Burgess Hill, West Sussex RH15 9YL  
Sunrise Parkway, Linford Wood (East), Milton Keynes, Bucks. NK14 6QF  
‘Sandettie House’ Hawkesdown Estate, Walmer, Kent CT14 7PH
Appendix II. Reference ranges for haematological and biochemical variables.

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Reference ranges were supplied by the Departments of Haematology and Biochemistry at UCLH, or the reagent manufacturer where indicated by *.
## Appendix III. Haematological variables in clinical sub-groups.

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*Abbreviations as per text. Results are reported as the median (in bold type) and interquartile range.*
Appendix IV. Thrombin generation markers and physiological inhibitor levels in patients with sickle cell disease.

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<th>HCII (U/ml)</th>
<th>PC (IU/ml)</th>
<th>PSF (IU/ml)</th>
<th>α1AT (U/ml)</th>
<th>F1+2 (nmol/L)</th>
<th>TAT (µg/L)</th>
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Shading indicates abnormal levels (reference ranges: Table 3.10). tx: regularly transfused, tx*: recently transfused.
## Appendix V. Sickle cell disease sub-groups: comparative analysis.

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<td>0.58 - 0.69</td>
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<td>0.60 - 0.90</td>
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<td>(IU/ml)</td>
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<tr>
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<td>1.9 - 2.4</td>
<td>2.4 - 3.3</td>
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Abbreviations as per text. Results are reported as the median and interquartile range.
### Appendix VI. Thrombin generation markers and physiological inhibitor levels in patients with β thalassaemia

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<th>Patient ID</th>
<th>Diagnosis</th>
<th>ATIII (IU/ml)</th>
<th>HCII (U/ml)</th>
<th>PC (IU/ml)</th>
<th>PSF (IU/ml)</th>
<th>α2AT (U/ml)</th>
<th>F1+2 (nmol/L)</th>
<th>TAT (μg/L)</th>
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*Shading indicates abnormal levels. Reference ranges: Table 3.10.*
Appendix VII. Comparison between (a) D-Dimer, (b) thrombin:antithrombin and (c) soluble E-selectin levels in sickle cell disease and β thalassaemia clinical groups. Dotted lines indicate the upper limit of the respective reference ranges.
Appendix VII (continued).

(c) $p = \text{NS}$

- $p < 0.005$
- $p < 0.001$
- $p < 0.01$

(d) $p = \text{NS}$

- $p = \text{NS}$
- $p = \text{NS}$
- $p < 0.02$
- $p < 0.05$
- $p < 0.01$
Publications pertaining to material presented in this thesis.

