DEVELOPMENT OF THE ROUTINE LABORATORY DIAGNOSIS OF ACTIVATED PROTEIN C RESISTANCE AND ITS EVALUATION IN A POPULATION OF PREGNANT WOMEN

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Ethics approval was obtained from the
Committee for Research on Human Subjects,
University of the Witwatersrand.
Clearance Certificate Numbers
M951117 and M970336.

DECLARATION

I declare that this Research Report is my own, unaided work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) to the University of theWitwatersrand. It has not been submitted before for any degree or examination at any other University.

Marion Münster
October 1997

ABSTRACT

Venous thromboembolic disease is a common health problem. It contributes considerably to morbidity as well as to mortality. Thrombosis usually occurs due to an underlying risk factor which may be environmental or genetic in origin. The recently described activated Protein C (APC) resistance is the commonest cause of familial thrombophilia documented to date. The molecular lesion is a single point mutation in the factor V (FV) gene which abolishes a cleavage site whereby it is normally inactivated by APC. This defect, termed the FV Leiden mutation, is highly prevalent in normal Caucasian populations. Although it would appear to have arisen due to a founder effect, there is a paucity of data concerning non-Caucasian populations.

As part of this study a polymerase chain reaction (PCR) based assay to detect this defect was established at the Johannesburg Hospital. Analysis of the FV genotype of 130 healthy Black blood donors did not reveal a single FV Leiden positive individual. This together with the lack of detection of this mutation in any of the Black patients investigated for thromboses at the Johannesburg Hospital since the PCR based assay became available, suggests that the FV Leiden mutation is absent in the South African Black population.

For purposes of population screening, the feasibility of pooling deoxyribonucleic acid (DNA) samples for FV Leiden genotyping was investigated. The results indicate that analysis of DNA pools from 5 separate individuals could reliably detect a single FV Leiden heterozygote. When the pool size was increased to 10 individuals, the detection rate was 80%. One should however be able to circumvent this shortcoming by including relevant control samples. This strategy would reduce the cost of the test should routine screening be implemented for populations known to harbour the mutation.

Plasma samples from the same Black blood donor study group were subjected to functional APC resistance testing by means of a commercial activated partial

thromboplastin time (APTT)-based assay. The mean activated Protein C sensitivity ratio (APC-SR) of the 82 samples suitable for analysis was found to be 2.18 (2SD 0.72) with no significant difference between males and females. The lower limit of normal for APC-SRs for this study population is therefore 1.46 (mean - 2SD). This value is much lower than others quoted in the literature. This finding is interesting particularly as the Black population seems to lack the FV Leiden mutation. Factor V and factor VIII (FVIII) functional activities were determined on a subset of plasmas but this was not helpful in determining the reason for the low APC-SRs. As specimen handling is critically important, the effect of temperature of storage and time delay prior to plasma separation from whole blood was explored in an attempt to explain the low APC-SRs. The results of these investigations were however uninformative in this regard. Furthermore, definite recommendations concerning handling of blood specimens for APC resistance testing cannot be made but it would appear reasonable to recommend that if a time delay is unavoidable the blood should be kept at room temperature for not more than 24 hours.

The development of acquired functional APC resistance in the absence of the FV Leiden mutation is well described, particularly in association with pregnancy. Pregnancy is not only an independent risk factor for thrombosis but is also associated with changes in the functional activities of some of the naturally occurring anticoagulant proteins. The above two findings make the interpretation of laboratory tests performed as part of routine investigation of venous thromboembolic disease in pregnant women extremely difficult. In order to facilitate such interpretation and to alert clinicians to this effect the second part of this study was concerned with the establishment of pregnancy-specific reference ranges for Protein C, Protein S, Antithrombin and the APC resistance functional assay.

A total of 47 pregnant women were studied. The original control group comprised 25 healthy female South African Institute for Medical Research (SAIMR) laboratory staff members. As they were predominantly Caucasian and the pregnant subjects were predominantly Black, an additional 20 female control subjects were recruited from the blood donor group. This was done as it was not known whether racial differences exist for the parameters being studied. The findings of low APC-SRs and low Protein S levels

in pregnancy are in accordance with reports in the literature. Although less pronounced and probably clinically insignificant, Protein C levels tended to be higher and Antithrombin levels tended to be lower in pregnancy.

Significant differences in the Protein S and APC-SRs were obtained for the Black control group compared with the predominantly Caucasian group. This will necessitate a re-evaluation of the locally used normal laboratory reference ranges with the establishment of race and sex-specific normal values as necessary.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC	activated Protein C
APC-SR	activated Protein C sensitivity ratio
nAPC-SR	normalized activated Protein C sensitivity ratio
APTT	activated partial thromboplastin time
Arg	arginine
Gln	glutamine
OD	optical density
UV	ultra violet
dNTPs	deoxyribose nucleotide triphosphates
DNA	deoxyribonucleic acid
bp	base pair
K_3EDTA	tripotassium ethylenediamine tetra-acetate
$CaCl_2$	calcium chloride
$MgCl_2$	magnesium chloride
*Mnl*1	*Moraxella nonliquefaciens* (restriction endonuclease)
DVT	deep vein thrombosis
PE	pulmonary embolus
PCR	polymerase chain reaction
PCR-SSP	PCR using sequence specific primers
FV	coagulation factor V
FVa	activated coagulation factor V
FVIII	coagulation factor VIII
FVIIIa	activated coagulation factor VIII
FXa	activated coagulation factor X
SAIMR	South African Institute for Medical Research
CV	coefficient of variation
SD	standard deviation

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DVT	deep vein thrombosis
PE	pulmonary embolus
PCR	polymerase chain reaction
PCR-SSP	PCR using sequence specific primers
FV	coagulation factor V
FVa	activated coagulation factor V
FVIII	coagulation factor VIII
FVIIIa	activated coagulation factor VIII
FXa	activated coagulation factor X
SAIMR	South African Institute for Medical Research
CV	coefficient of variation
SD	standard deviation

1 INTRODUCTION

Venous thromboembolic disease is a common health problem affecting about 1 per 1000 people annually. It therefore contributes considerably to morbidity and mortality in the general population (Goldhaber, 1994; Thomas, 1994; Allaart and Briët, 1994). Venous thrombosis is a multifactorial disease usually occurring due to an underlying risk factor which may be environmental or genetic in origin, or both. The acquired risk factors include immobilization, surgery, trauma, oral contraceptive use, pregnancy, the puerperium, obesity, malignancy as well as cardiac disease resulting in sluggish venous return. The known genetic predispositions towards thrombosis until recently, were primarily deficiencies of Protein C, Protein S and Antithrombin and rarely dysfibrinogenaemia, though together they accounted for only 5% to 10% of cases (Dahlbäck, 1995a).

1.1 ACTIVATED PROTEIN C RESISTANCE

The yield of laboratory investigations in patients with thrombosis has improved dramatically since the discovery in 1993 of a new mechanism of familial thrombophilia, namely the phenomenon of resistance to the anticoagulant action of APC (Dahlbäck *et al*, 1993).

1.1.1 The Protein C - Protein S anticoagulant system

Protein C is a vitamin K dependent naturally occurring anticoagulant protein. Its function, together with its cofactor Protein S, is to counterbalance procoagulant forces generated at sites of vascular injury so that haemostasis remains localized. Thrombin generated at sites of vascular injury exerts a positive feedback signal on coagulation factors, aggregates platelets and converts fibrinogen to fibrin. In contrast, thrombin manifests potent anticoagulant properties when exposed to intact endothelial cells by complexing with thrombomodulin, a surface membrane protein (Dahlbäck and Stenflo, 1994; Esmon, 1989). This thrombin-thrombomodulin complex activates Protein C. Activated Protein C is a serine protease with potent anticoagulant properties which together with Protein S, limits thrombus formation during normal haemostasis by proteolytic inactivation of activated factors V (FVa) and VIII (FVIIIa) (Dahlbäck, 1995a) (Figure 1).

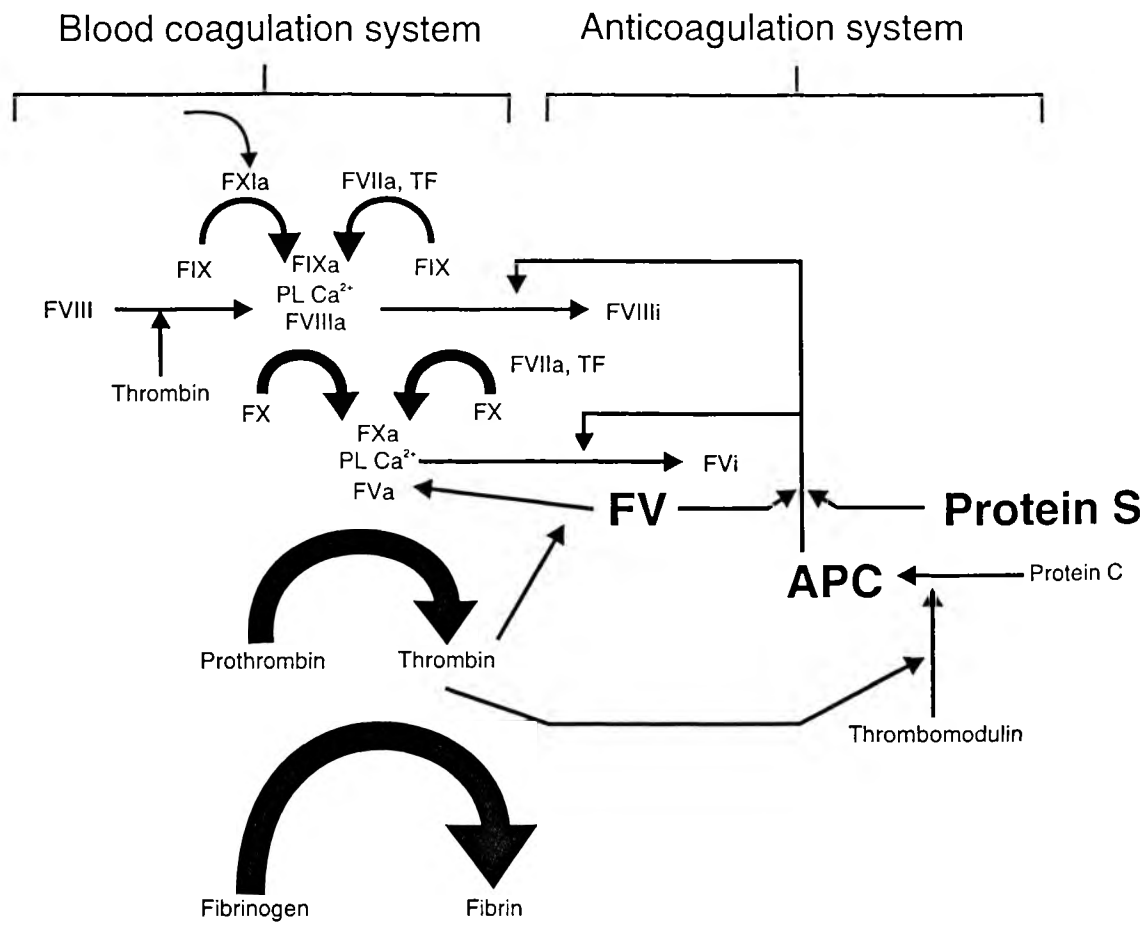

Figure 1 Simplified scheme of blood coagulation and the Protein C anticoagulant system

Thrombin bound to thrombomodulin activates Protein C. APC cleaves and inhibits membrane-bound factors Va and VIIIa. Protein S and FV function as synergistic cofactors to APC. Thrombin activation of FV is association with loss of anticoagulant activity and gain of procoagulant functions. TF denotes tissue factor, PL denotes phospholipid (Adapted from Dahlbäck 1995b).

1.1.2 Origins of the Activated Protein C Resistance functional assay

The physiological importance of the Protein C-Protein S anticoagulant system is borne out by the well documented risk of venous thrombosis in Protein C (Koster *et al*, 1995; Horellou *et al*, 1984; Broekmans *et al*, 1983) or Protein S deficient individuals (Broekmans *et al*, 1985; Comp and Esmon, 1984). As these deficiencies are however only documented in a minority of individuals with venous thromboembolic disease and a family history of thrombosis (Malm *et al*, 1992), Dahlbäck *et al* (1993) postulated that a poor response to APC may be responsible for those previously unidentified families with thrombophilia. Based on this hypothesis they developed a new coagulation assay to measure the anticoagulant effects of exogenous APC in plasma. This consisted of a simple APTT-based method. This was performed in duplicate, once as a conventional APTT and once with the addition of a known quantity of APC. Addition of APC results in degradation of the activated forms of both FVIII and FV which delays thrombin generation and hence formation of the fibrin clot. In view of this the expected effect of the addition of APC to plasma in normal individuals was prolongation of the clotting time whilst failure to do so was considered as resistance to APC. If APC resistance is present, this results in a shorter clotting time as FVa and FVIIIa remain active and continue to generate thrombin. This phenomenon was clearly demonstrated in the proband case, who had a history of multiple episodes of deep vein thrombosis, as well as amongst his relatives who also had a high incidence of thrombosis.

1.1.3 Molecular basis of APC Resistance

Discovery of the phenotype of APC resistance prompted a search for a molecular explanation. Dahlbäck *et al* (1993) originally postulated that APC resistance was probably due to an inherited deficiency of a previously unrecognized cofactor to APC. Dahlbäck and his team subsequently did further work to try to isolate and characterize this cofactor from the plasma of an individual with pronounced inherited APC resistance. They devised a purification procedure that revealed that the putative APC cofactor was identical to FV (Dahlbäck and Hildebrand, 1994). They showed that APC resistant plasma contained normal FV procoagulant activity. Their results indicated that although FV procoagulant activity was unaffected, FV plays an important role in the anticoagulant system as a cofactor to APC and that a selective defect in this anticoagulant property was responsible for APC resistance.

Dahlbäck *et al* (1993) originally thought of the possibility of a mutation in the FV or FVIII gene in or close to the APC cleavage site but these ideas were dismissed; the FVIII mutation on the basis of DNA linkage analysis and a FV mutation on the basis that the poor APC response was less pronounced in a factor Xa (FXa)-based assay (which reflected only FVa degradation) than in the APTT-based assay and a factor IXa-based assay which are sensitive to both FVa and FVIIIa inactivation.

Bertina *et al* (1994) subsequently defined the molecular basis for the APC resistance phenotype by showing that it was associated with a single point mutation within exon 10

of the FV gene at nucleotide position 1691 involving substitution of guanine by adenine. This base change results in a codon change and hence the synthesis of an abnormal FV protein where arginine (Arg - codon CgA) at position 506 has been replaced by glutamine (Gln - codon CAA). This abnormal FV has been termed FV Leiden (Bertina *et al*, 1994). The pathophysiological consequence of this amino acid substitution is the abolition of an APC cleavage site within the FV molecule. Activated Protein C cleaves the human FVa protein at a number of sites including Arg^{506} thereby resulting in its inactivation (Kalafatis *et al*, 1994). The presence of the FV Leiden mutation prevents an initial cleavage which occurs in normal FVa at Arg^{506}. As cleavage at Arg^{506} normally facilitates subsequent cleavage at Arg^{306} and Arg^{679} (Griffin *et al*, 1995), the presence of the mutation renders the FV protein relatively resistant to inactivation by APC (Dahlbäck, 1995a; Bertina *et al*, 1994; Dahlbäck, 1994; Heeb *et al*, 1995) (Figure 2).

Recent data support the hypothesis that the cleavage at Arg^{306} by APC in Gln^{506} FVa is sufficient for inactivation although this cleavage is about 10 times slower for the variant than for normal FVa (Griffin *et al*, 1995).

Besides the removal of an APC cleavage site, the FV Leiden mutation appeared to have further indirect effects on the impairment of the anticoagulant Protein C - Protein S pathway. *In vitro* experiments have shown that intact FV was required as a cofactor for APC for efficient inactivation of FVIIIa and that it is synergistic with the action of Protein S (Shen and Dahlbäck, 1994). Impaired cleavage of the FV Leiden molecule results in ongoing thrombin generation and hence positive feedback activation of the

coagulation cascade including that of FV. This ongoing activation results in a reduction in the pool of intact FV. As FV, upon activation, loses its ability to enhance FVIIIa inactivation by APC (Shen and Dahlbäck, 1994), there is a reduction in the availability of an important APC cofactor to degrade FVIIIa. The FV Leiden mutation thereby indirectly diminishes the degradation of normal FVIIIa.

The APC cofactor function of FV also appeared to be directly affected by the mutation. Whilst normal FV, in the absence of free Protein S, was able to inactivate FVIIIa, the mutant FV demonstrated no cofactor activity. The addition of free Protein S was able to enhance the effect of FV Leiden on FVIIIa inactivation although the quantities required were 10-fold higher than normal FV to achieve similar effects (Váradi *et al*, 1996).

In vitro work by Rosing *et al* (1995) showed that the presence of Protein S and FXa almost completely abolished differences in the rates of APC catalyzed inactivation of FV Leiden and normal FV. This suggests that the thrombotic tendency associated with FV Leiden is not primarily due to abnormal regulation of FVa activity by APC *in vivo* but rather due to loss of the cofactor activity of FV in APC mediated inactivation of FVIIIa.

As there is data to support both views it is likely that the physiological consequences of the $Arg^{506} \rightarrow$Gln mutation are multiple. There is however intense controversy in the literature concerning the role of FV as a cofactor to APC in FVIIIa inactivation.

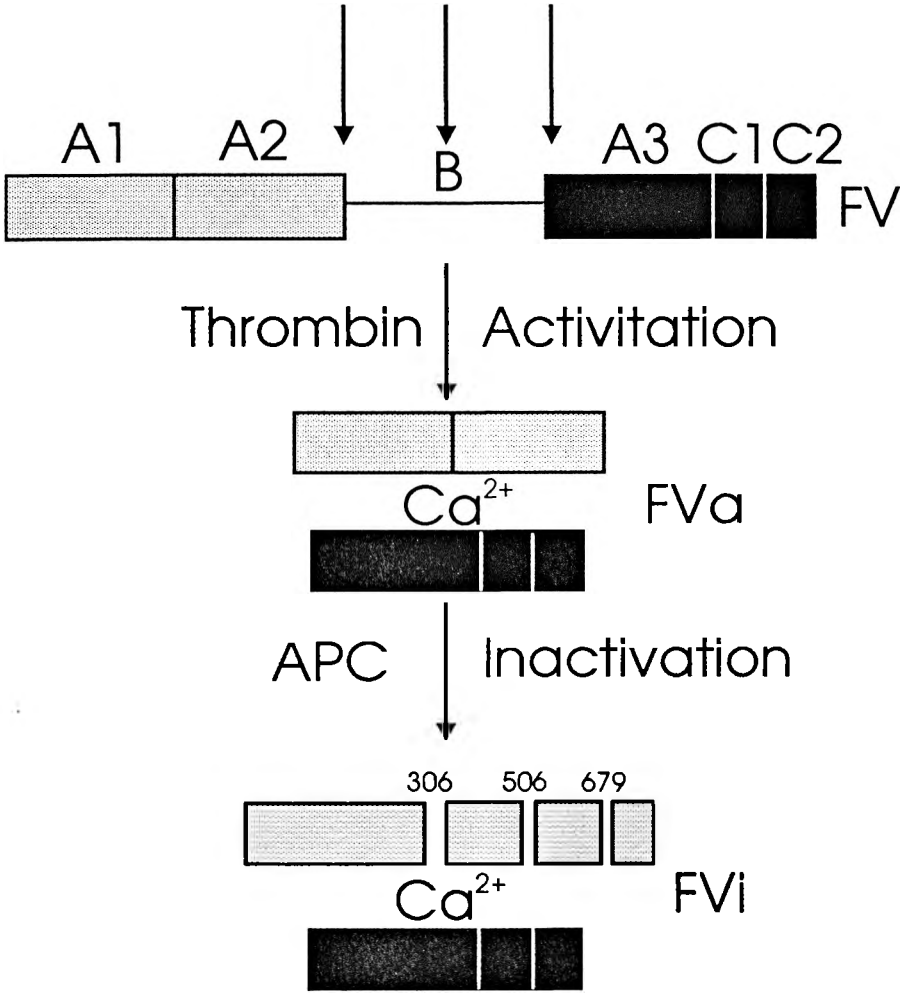

Figure 2 Schematic model of activation and inactivation of factor V

Factor V is composed of three homologous A repeats, one B region and two homologous C repeats. Thrombin activation of factor V occurs as a result of cleavage of 3 peptide bonds as indicated by the arrows. In factor Va, the A_1-A_2 containing heavy chain and the A_3-C_1-C_2 containing light chain form a calcium-dependent complex. During inactivation several peptide bonds are cleaved as shown (from Dahlbäck, 1995a).

Inactivation kinetics of FVIIIa (Pötzsch *et al*, 1995) as well as various APC resistance assays using purified Gln^{506} FVa and normal FVa (Griffin *et al*, 1995) find no support for the hypothesis that FV is a significant cofactor to APC. Although the findings of Lu *et al* (1996) would support a cofactor role for FV in the presence of Protein S in the inactivation of FVIII, it would appear to be only the B-domain fragments generated by APC or α-thrombin cleavage of membrane bound FV that is responsible, rather than intact single chain FV. They postulate that the FV fragments facilitate interaction of APC and Protein S thereby enhancing APC activity.

Likewise, there is no consensus as to the preferred substrate for APC (Shen and Dahlbäck, 1994; Lu *et al*, 1996). The observation that FVIIIa appears to be the preferred substrate for APC was made based on a FVIIIa degradation experiment using a mixture of APC, Protein S and FV whereby inclusion of increasing concentrations of FVa appeared to have no influence on the inhibitory effect on FVIIIa (Shen and Dahlbäck, 1994). As the plasma concentration of FV is at least 100-fold higher than FVIII, the preference of FVIIIa as a substrate for APC would appear to be necessary (Shen and Dahlbäck, 1994). If this were not so, the relative excess of FVa after activation of the coagulation cascade would prevent FVIIIa degradation which, in view of its lesser quantity would appear to present an easier target for control of excess coagulation. Although Shen and Dahlbäck (1994) interpreted these findings based on the understanding that the primary pathophysiological consequence of the FV Leiden mutation is as a direct result of ineffective FVa degradation, and hence an imbalance between procoagulant and anticoagulant forces, their findings are not entirely

incongruous with those of Váradi *et al* (1996) who emphasize the importance of the cofactor function.

In contrast to the findings of Shen and Dahlbäck (1994), more recent data indicate that FV/FVa rather than FVIII/FVIIIa are the physiologically significant substrates for APC (Lu *et al*, 1996). This conclusion was based on observations that large amounts of APC and approximately 4 hours were required for complete inactivation of membrane bound recombinant FVIIIa whereas FV and FVa could be rapidly inactivated by catalytic amounts of APC (Lu *et al*, 1996; Kalafatis *et al*, 1994). In contrast to FVa, the inactivation of FVIIIa was largely spontaneous with 60% to 80% being lost as a result of dissociation of the A_2 domain subsequent to α-thrombin cleavage. This accounts for the very short half life of FVIIIa (~2 minutes) (Curtis *et al*, 1994). The remaining activity is slowly inhibited by APC at Arg^{336}. Thus, α-thrombin cleavage at Arg^{372} initiating activation of FVIII would appear to play the same role in its inactivation as does APC cleavage of FVa at Arg^{506} (Lu *et* al, 1996; Kalafatis *et al*, 1994). Both events are preliminary in that they render the respective molecules more susceptible to the inactivating cleavage at Arg^{336} (FVIIIa) and Arg^{306} (FVa). Cleavage at Arg^{306} only occurs efficiently when FVa is membrane bound reducing its activity by 80%. The remaining activity is lost following membrane-independent cleavage at Arg^{679}. In addition, FV activation can be prevented by APC proteolysis at Arg^{306}, Arg^{506}, Arg^{679} and Lys^{994}. Cleavage at Arg^{306} occurs first, unlike in FVa, and prevents activation of FV in the first instance (Kalafatis *et al*, 1994).

Although obviously of extreme importance by virtue of its strong association with thrombophilia (Broekmans *et al*, 1985; Comp and Esmon, 1984), the exact role of Protein S is also not clear. Earlier data showed an important role for Protein S in abrogating the ability of FXa to protect FVa from APC within the prothrombinase complex (Nesheim *et al*, 1982; Solymoss *et al*, 1988). More recently Protein S has been shown to have a greater enhancing effect on the inactivation of FVIII, increasing the response by 6.4-fold compared with only a 2-fold increase for FVa, whereas membrane-bound FV showed no Protein S dependency (Lu *et al*, 1996). The authors do however point out that their experiments were conducted in the absence of von Willebrand Factor, the carrier protein for FVIII, which has been shown to protect FVIII from APC inactivation (Rick *et al*, 1990). Therefore the physiological importance of *in vitro* experiments determining the relative roles of Protein S and FV in FVIII inactivation remain to be established.

A third prothrombotic effect associated with FV Leiden has been described. When excess thrombin is generated this may lead to clot stabilization because of inhibition of fibrinolysis through the activation of **T**hrombin **A**ctivatable **F**ibrinolysis **I**nhibitor (TAFI), also known as plasma procarboxypeptidase β (Bajzar *et al*, 1995; Eaton *et al*, 1991; Bajzar *et al*, 1996b). Normally APC would promote fibrinolysis by attenuating thrombin generation and hence TAFI would not be activated.

Bajzar *et al* (1996a) have demonstrated clearly that the profibrinolytic effect of APC is markedly attenuated in the presence of FV Leiden compared with normal FV. Thus an

impaired TAFI-dependent profibrinolytic response to APC in individuals with APC resistance would appear to contribute to the overall hypercoagulability of FV Leiden by enhancing stability of the thrombus formed as a result of excess thrombin generation.

Some older studies suggest that the profibrinolytic activity of APC *in vitro* is due, at least partly, to APC-Plasminogen Activator Inhibitor 1 (PAI 1) complex formation but the reported results are conflicting (Dahlbäck and Stenflo, 1994).

Whilst there may not be consensus on the exact sequence and physiological importance of events involved in the normal Protein C-Protein S anticoagulant mechanism, the hypercoagulability associated with the phenomenon of APC resistance is almost certainly due to multiple pathophysiological effects resultant from the mutant FV molecule.

In families with familial APC resistance the APC resistance phenotype has been associated with the FV Leiden mutation in 94% of cases (Zöller *et al*, 1994). In a limited number of individuals with inherited APC resistance, the FV gene mutation was not found suggesting that other as yet unknown genetic defects may also cause APC resistance. In view of this the possibility of APC cleavage site point mutations in the FVIII molecule analogous to the $Arg^{506} \rightarrow$Gln mutation in FV Leiden has been postulated as an explanation for some of these unexplained cases of inherited APC resistance. A recent study undertaken to investigate this possibility did however not identify any such mutations in the FVIII molecule (Roelse *et al*, 1996).

1.1.4 LABORATORY DIAGNOSIS OF APC RESISTANCE

The laboratory diagnosis of functional APC resistance generally utilizes a modified APTT assay that measures the degree of prolongation of plasma clotting time consequent to the addition of APC (Dahlbäck *et al*, 1993). Results are expressed as an APC-SR which is the ratio of clotting times of plasma in the presence of APC to that of clotting times in its absence. Although the principle of the assay is sound, interpretation is complex.

The manufacturers of the commercial kit most widely used in studies reported in the literature (COATEST®, Chromogenix, Sweden) first recommended that an APC-SR of < 2.00 should be considered to be abnormal. Chromogenix has subsequently recommended that each laboratory should determine its own cut-off point (mean -2SD) obtained by analysis of a normal control group. This would appear to have been an important amendment as a number of studies now indicate cut-off points below 2.00 (De Stefano *et al*, 1995; Hakala *et al*, 1995).

Although the use of a group of healthy individuals is a well established procedure in establishing normal reference ranges for laboratory tests, with regards to the APC-SR there is a lack of uniformity as to what constitutes a normal control. Firstly, the number of individuals in a control group from which cut-off points discriminating between normal and abnormal are derived, have varied from 21 to 120 in the literature (Rintelen *et al*, 1996; Hakala *et al*, 1995). Secondly, separate reference values for men and women are

generally not quoted despite documentation that females in the absence of FV Leiden have significantly lower APC-SRs than men (Henkens *et al*, 1995a; Wagner *et al*, 1995; Hakala *et al*, 1995). Exogenous oestrogen intake further suppresses the anticoagulant response to APC thereby reducing the specificity of the clotting assay in predicting an underlying FV Leiden mutation (Henkens *et al*, 1995a; Olivieri *et al*, 1995). Thirdly, there is no mention as to whether a family history of thrombosis was considered an exclusion to entry into a control group for the purposes of establishing a reference value. Fourthly, the ethnic background of members of the control group is not usually stated although one might expect this to reflect that of the general population which a particular laboratory serves. This would be important as APC resistance has been shown to be highly prevalent in some populations and absent in others.

In addition to the need to standardize criteria of control groups for the determination of normal references, de Ronde and Bertina (1994) have pointed out that the actual prolongation of the APTT (in seconds) is dependent on multiple variables. These would include sample handling, citrate, calcium chloride and APC concentrations. They advocate the use of a normalized APC-SR (nAPC-SR) whereby the APC-SR of the patient is divided by that obtained for pooled normal plasma in the same test run. They determined the lower limit of normal to be 0.84 for the nAPC-SR by measuring the plasma of 100 anonymous healthy volunteers. All 4 individuals who had values less than 0.84 in their control group were later shown to be heterozygous for the FV Leiden mutation by DNA analysis.

Comparison with DNA analysis has revealed that there is significant overlap between the APC-SRs for normal individuals and FV Leiden heterozygotes whereas prediction of homozygosity appears to be accurate (Stirling *et al*, 1995; Zehnder and Benson, 1996). Whilst the screening test is able to detect the small number of FV Leiden homozygotes, it does not clearly discriminate between normal individuals and FV Leiden heterozygotes. As molecular diagnosis would be required for confirmation, the APTT-based assay in its original state does not appear to be a good screening test for heterozygotes (Zehnder and Benson, 1996).

A further problem of the functional clotting assay is that it is unsuitable for patients on anticoagulant therapy where the baseline APTT is prolonged (de Ronde and Bertina, 1994). Although heparin adsorption prior to testing may be feasible, the current assay cannot be applied to warfarinized patients as the test is exquisitely sensitive to low levels of factors II and X (de Ronde and Bertina, 1994).

In order to overcome the problem of screening for APC resistance in warfarinized patients a modified test has been devised (Jorquera *et al*, 1994). This involves a 1 in 5 dilution of plasma samples in FV-deficient plasma and has been shown to have increased the sensitivity of the APTT-based test. This modification promises to be very helpful as many patients being investigated for thrombosis are also anticoagulated. The reliability of the modified test in accurately discriminating between normal and the presence of the FV Leiden mutation in patients taking oral anticoagulants has been confirmed (Trossaërt *et al*, 1994). Engel *et al* (1996) utilising a 1:10 dilution in FV-deficient plasma

demonstrated a remarkable 100% concordance with DNA analysis whilst the unmodified test results were unsatisfactory.

The inclusion of FV-deficient plasma in the APC resistance assay provides optimal results regardless of oral anticoagulation. However, Jones *et al* (1997) found that test results are greatly influenced by the specific lot of FV-deficient plasma that is used. They suggest pooling of FV-deficient plasma from multiple donors to allow for standardization and optimal patient testing. In view of the limitation of the original coagulation based assays for APC resistance, several alternative tests have since been devised exhibiting excellent correlation with DNA analysis. A chromogenic assay based on the capacity of APC to limit the generation of FXa by inactivation of FVIIIa which accurately detected 23 out of 24 FV Leiden carriers has been reported (Váradi *et al*, 1995). The response to APC is determined by the ratio of FXa amidolytic activity without APC to that with the addition of APC to the test plasma. Liebman *et al* (1996) evaluated a newly described one-stage tissue factor dependent FV coagulation assay comparing it to PCR-based DNA analysis for the FV Leiden mutation in control subjects as well as patients with thrombosis, many of whom were anticoagulated or had a lupus anticoagulant which is known to give false positive results (Aznar *et al*, 1997). The results of their trial were excellent.

Although modifications of the original APTT-based assays and the more recently described non-clotting based assays have significantly improved the specificity of screening for the FV Leiden mutation, discrimination between normal and abnormal

relies on statistical and therefore somewhat arbitrary criteria. In view of this, if only phenotypic analysis is performed the potential for false positive and false negative results remains a constant threat, the consequences of which may be significant for the patient concerned.

The gold standard for the detection of the FV Leiden mutation remains direct DNA analysis. This involves PCR amplification of a portion of the FV gene spanning the mutation at nucleotide position 1691. In the original method of Bertina *et al* (1994), the PCR product was subjected to *Moraxella nonliquefaciens* (*Mnl*1) restriction endonuclease digestion. The guanine to adenine missense mutation abolishes a restriction site and hence a different restriction fragment length pattern is obtained for the normal and abnormal alleles with clear distinction between heterozygotes and homozygotes.

1.1.5 Activated Protein C Resistance and Venous Thromboembolic Disease

Activated Protein C resistance is the commonest laboratory abnormality found among individuals with venous thrombosis. Its prevalence has been reported to be between 21% and 64% in cohorts of patients with deep venous thrombosis (Koster *et al*, 1993; Griffin *et al*, 1993; Voorberg *et al*, 1994; Svensson and Dahlbäck, 1994). Differences in selection procedures of studies of patients with venous thromboembolic disease may partly explain this wide variation in results obtained. Patients with a positive family history of thrombosis tend to have higher prevalence rates than series of consecutive patients.

Contrary to expectation the prevalence of the FV Leiden mutation has been reported to be lower in patients presenting with pulmonary embolism (PE) compared to those with deep vein thromboses (DVTs) with an estimated three-fold increased risk for PE in comparison with a seven-fold increased risk for DVT (Manten *et al*, 1996). The findings of Manten *et al* (1996) support the hypothesis that the structure of a thrombus is different depending on its aetiology and that the FV Leiden mutation leads to thrombi with smaller embolic risk. The findings of a prevalence of 5.5% of resistance to APC of unselected patients with PE compared with 4% in patients in whom PE was excluded suggest an even weaker association between PE and the FV Leiden mutation (Desmarais *et al*, 1996). The difficulty in accurately diagnosing PE may also account for this discrepancy.

There appears to be great heterogeneity in the clinical expression of the FV Leiden mutation. Although some thromboses occur spontaneously, in the majority of both heterozygous and homozygous cases, thrombosis is associated with circumstantial risk factors, the most common being pregnancy (Hirsch *et al*, 1996; Bokarewa *et al*, 1996), oral contraceptive usage (Vandenbroucke *et al*, 1994; Samama *et al*, 1995) as well as trauma and surgery (Zöller *et al*, 1994). These findings suggest that a combination of genetic and environmental factors determine the risk of thrombosis. The risk of thrombosis in heterozygotes has been estimated to be increased five to ten-fold (Bertina *et al*, 1994; Ridker *et al*, 1995), whilst homozygotes have a 50 to 100-fold increased risk (Rosendaal *et al*, 1995). Despite this, individuals may lead healthy lives without experiencing thrombosis (Zöller *et al*, 1994) even though they have encountered high risk situations (Samama *et al*, 1995).

The risk of recurrence of DVTs in individuals homozygous for the mutation has been reported as 9.5% per year compared with 4.8% for heterozygotes and 5% for individuals with thrombosis with no identifiable inherited risk factor (Rintelen *et al*, 1996). These findings of a lack of increased risk of recurrence of venous thromboembolic disease amongst FV Leiden heterozygotes are supported by those of Eichinger *et al* (1997) but not by Simioni *et al* (1997). Whilst there may be a lack of consensus with regards to the risk of recurrence, there seems to be general agreement that prolongation of anticoagulation beyond the currently recommended three to six month period is not generally indicated (Simioni *et al*, 1997; Eichinger *et al*, 1997; Rintelen *et al*, 1996). Individual circumstances such as near-fatal thrombotic episode, strong family history or personal history of recurrence must obviously be carefully considered when making decisions regarding the management of symptomatic patients (Dahlbäck, 1997). Whilst the currently available literature on venous thromboembolic disease in FV Leiden homozygotes indicates that the risk of thrombosis and recurrence of thrombosis is greater than in heterozygotes (Rintelen *et al*, 1996) the thrombotic tendency is mild compared to deficiencies of anticoagulant proteins (Emmerich *et al*, 1997). Firm guidelines regarding the management of homozygous FV Leiden positive patients have not yet been established and must await larger prospective studies. Although limited data are available it would appear that, as most thromboses in homozygotes are not spontaneous (Emmerich *et al*, 1997), long-term oral anticoagulation is not generally recommended after a single venous thromboembolic episode (Rintelen *et al*, 1996; Samama *et al*, 1995; Rosendaal *et al*, 1995). Long-term oral anticoagulation after a recurrence would however seem to be warranted. In all cases, the benefits of anticoagulation must be weighed against the risk of

bleeding complications, particularly if long-term use is contemplated. In contrast to the presence of FV Leiden, treatment for longer than six months may be warranted in Protein C, Protein S and Antithrombin deficiency states due to the increased risk of recurrence (Thaler and Lechner, 1981; Engesser *et al*, 1987; Bovill *et al*, 1989). Compound heterozygosity for FV Leiden and deficiency of either Protein C, Protein S or Antithrombin warrants long-term treatment with oral anticoagulants (Dahlbäck, 1997).

An emerging concept from epidemiological studies of the inherited thrombotic disorders is that many individuals with recurrent thrombosis have more than one genetic risk factor. The observation of both clinically dominant and recessive Protein C deficiency led to investigations in search of a second hereditary risk factor as a possible explanation for this observed difference in thrombotic risk between families. This was subsequently confirmed by documenting that the prevalence of the FV Leiden mutation was high (19%) among symptomatic Protein C deficient probands (Koeleman *et al*, 1994; Gandrille *et al*, 1995). Likewise the co-existence of the FV Leiden mutation in Protein S (Koeleman *et al*, 1995; Zöller *et al*, 1995) and Antithrombin deficient individuals increases the thrombotic risk with those affected becoming symptomatic at a younger age (van Boven *et al*, 1996). Patients with concurrent homocystinuria and FV Leiden have also been shown to have an increased risk of thrombosis (Mandel *et al*, 1996). Venous thrombosis is not only multifactorial in origin due to its strong association with circumstantial risk factors, but can also be considered to be a polygenetic disease in certain families.

1.1.6 Activated Protein C Resistance and Arterial Thrombosis

The Physicians Health Study, a very large population based study, showed no difference in the FV Leiden mutation amongst disease-free individuals compared with those who had suffered myocardial infarction or stroke (Ridker *et al*, 1995). Likewise Emmerich *et al* (1995), van Bockxmeer *et al* (1995) and van der Bom *et al* (1995) conclude that the FV Leiden mutation does not appear to play an important role in the pathogenesis of coronary artery disease. In contrast , März *et al* (1995) did show an increased prevalence of FV Leiden in myocardial infarction compared with control subjects. The findings of Nowak-Göttl *et al* (1995) also challenge the notion that there is no association between arterial disease and the FV Leiden mutation, at least in the paediatric age group. They detected 38% of paediatric patients with arterial thromboembolism to be heterozygous for the FV Leiden mutation compared with 52% with venous thrombosis. As the vast majority of arterial thromboses occurred in neonates in their study, 22% of whom had either another exogenous or inherited prothrombotic risk factor, it is difficult to draw conclusions with regards to the general lack of association between FV Leiden and arterial thrombosis in adults.

Thus in contrast to venous thromboembolic disease, there appears to be no strong association between FV Leiden and arterial thrombosis at least in the adult population.

1.1.7 Population Genetics of Factor V Leiden

An explanation for the wide variation in results observed for the occurrence of FV Leiden in cohorts of patients with DVTs may be partly explained by differences in the selection procedure of subjects for inclusion into studies (patients with a positive family history tend to have higher prevalence rates than series of consecutive patients). Other factors such as ethnicity and geographic variation, need to be borne in mind as the prevalence of APC resistance may be markedly different in various population groups (Hillarp *et al*, 1995). The reported prevalence of APC resistance or heterozygosity for the FV Leiden mutation in the general population varies between 2% and 10% (Bertina *et al*, 1994; Svensson and Dahlbäck, 1994; Beauchamp *et al*, 1994; Ridker *et al*, 1995; Lee *et al*, 1996). These studies indicating a relatively high prevalence rate of the FV Leiden mutation have almost all been conducted in Caucasian populations. There is a paucity of data from other population groups. A reported prevalence of 6.6% in Egyptian individuals (Hammoud *et al*, 1996) is in conflict with two other studies that did not detect the FV Leiden mutation in any of the African, Middle Eastern, Australasian and Far East Asian non-Caucasian populations studied (Rees *et al*, 1995; Helley *et al*, 1996).

In view of the low prevalence of the mutation outside of Europe (or people of European descent), it has been suggested that the FV Leiden mutation occurred as a single event in the European population. A study looking at different haplotypes within exon 13 of the FV gene suggests that the $Arg^{506} \rightarrow$Gln mutation occurred as a single event, giving support to the hypothesis that it has arisen due to a founder effect (Cox *et al*, 1996).

The proposed single genetic origin rather than the occurrence of frequent mutations of FV Leiden in Caucasians has been independently confirmed by Zivelen *et al* (1997) who speculate that the mutation originated in a single Caucasoid individual approximately 21000 to 34000 years ago.

This has been disputed by an American Study that looked at the prevalence of the FV Leiden mutation in unselected Black and White hospital patients and found 1.4% and 1.6% heterozygotes respectively (Pottinger *et al*, 1996). As ethnic background was determined from hospital records it is possible that there may have been individuals of mixed race and thus the possibility of the introduction of an ancestral Caucasian gene into the Black population should be considered. A further report showed the prevalence of APC resistance to be 2% and 4.5% in Black and White United States Air Force recruits respectively although genotyping was not performed (Eby *et al*, 1996). The lower figure for Black subjects would not be incongruous with the suggestion of introduction of FV Leiden into the Black population by racial mixing.

Despite this, there appears to be general consensus that the FV Leiden mutation is common in Caucasian populations and that it is the commonest inherited risk factor for venous thrombosis documented to date. What remains to be ascertained is whether this mutation is also present in non-European people. This would aid explaining the relatively lower occurrence of DVTs in Africa and Asia (Rees *et al*, 1995; Lane *et al*, 1996).

As the issue of the lack of information concerning the prevalence of the FV Leiden mutation in developing countries has been repeatedly raised (Hillarp *et al*, 1995, Lane *et al*, 1996), this research report will address this problem in the South African context by screening healthy Black blood donors for the FV Leiden mutation.

1.2 THROMBOPHILIC PROFILES IN NORMAL PREGNANCIES

A second issue that will be addressed is the establishment of normal reference values for the laboratory diagnosis of underlying prothrombotic risk factors during pregnancy. Pregnancy in normal healthy women is a well recognized risk factor for venous thromboembolic disease, the risk being five times higher in a pregnant than in a non-pregnant woman of similar age (National Institutes of Health Consensus Development Conference, 1986). Pulmonary embolism, although relatively uncommon is currently recognized as the leading cause of maternal death (Toglia and Weg, 1996). Furthermore, pregnancy commonly exposes previously undiagnosed inherited prothrombotic conditions such as Protein C, Protein S and Antithrombin deficiency and more recently APC resistance (Bokarewa *et al*, 1996; Bauer, 1995). Once a thrombotic event has been objectively diagnosed, investigation for a possible underlying predisposition should be undertaken. Besides acquired abnormalities such as antiphospholipid antibodies, the routine laboratory investigation should include screening for Protein C deficiency, Protein S deficiency, Antithrombin deficiency as well as APC resistance.

1.2.1 Physiological Changes of the Natural Anticoagulant proteins during Pregnancy

It is well documented that there are numerous physiological changes in pregnancy, including alterations in certain proteins of the coagulation and fibrinolytic systems (Greer; 1994). In general there seems to be an imbalance between anticoagulant and procoagulant factors which is likely to contribute to the hypercoagulable state associated with pregnancy. The most well documented and consistent observation is the marked reduction in both functional and immunological Protein S levels (Faught *et al*, 1995; Bremme *et al*, 1992; Comp *et al*, 1986; Fernández *et al*, 1989; Boerger *et al*, 1987). The decrease occurs early in pregnancy, returning to normal a few days after delivery (Malm *et al*, 1988). Whilst the exact mechanism of the change is not known, it appears to be a hormonal rather than a dilutional effect (Alving and Comp, 1992). The changes appear to be due to an absolute reduction in total Protein S (Fernández *et al*, 1989) as well as a progressive decrease in its availability due to an increase of C4b binding protein which appears to be maximally increased at the time of delivery (Malm *et al*, 1988).

Unlike Protein S, changes in Protein C and Antithrombin levels are not as well documented and appear to be inconsistent. Whilst some studies have shown a mild to moderate increase in Protein C activity which appears to be maximal in the second trimester (Malm *et al*, 1988; Pinto *et al*, 1988), others have shown no change in comparison with normal controls (Lao *et al*, 1989; Manucci and Vigano, 1982).

Antithrombin levels have been reported to have remained unchanged throughout normal pregnancy (Gilabert *et al*, 1988; Pekonen *et al*, 1986; Weenink *et al*, 1982) or to be slightly reduced (Pinto *et al*, 1988).

A similar but less pronounced alteration in Protein S levels was observed in women using oral contraceptives (Malm *et al*, 1988; Alving and Comp, 1992) with varying reports on the effect on Protein C levels (Malm *et al*, 1988; Gilabert *et al*, 1988). This suggests that the Protein C/S anticoagulant pathway is at least partially hormone dependent (Wagner *et al*, 1995). Whether or not concurrent Protein S deficiency would further reduce the Protein S level in pregnancy or whether it would fall within the "normal pregnancy range" remains to be seen. Likewise the potential exists that Protein C deficiency may be masked by pregnancy.

1.2.2 Diagnosis of true deficiency states of the Natural Anticoagulants in Pregnancy

Whilst changes of the above factors are well documented , very little attention has been focused on the diagnosis of true deficiency states in pregnancy. In this context, it would be important to establish pregnancy specific reference ranges for these factor levels in our laboratory. These ranges would alert any clinicians who may be unaware of these changes so that Protein S deficiency is not inappropriately diagnosed during pregnancy and that Protein C deficiency is not inadvertently missed. Ideally all women presenting with

venous thromboembolic disease during pregnancy should be re-evaluated at a later stage to overcome the limitations of diagnosing Protein S and Protein C deficiency during pregnancy. Where a life-threatening event has occurred, the accurate diagnosis of such inhibitor deficiencies would be further hampered by prolonged or even lifelong anticoagulant therapy. As both Protein C and Protein S are vitamin K dependent factors, warfarin therapy would result in decreased functional activity of these proteins thereby not allowing accurate diagnosis of a true deficiency state (Dahlbäck and Stenflo, 1994). In these instances family studies would be required. In view of the multitude of mutations that have been documented in these conditions, in the absence of knowledge of the specific mutation affecting that family, direct analysis of the genetic defect would not be viable for routine diagnosis.

1.2.3 Acquired Activated Protein C Resistance

In contrast to Protein C and Protein S deficiency, there is a lot of recent literature concerning the diagnosis of APC resistance in pregnancy. It has been reported that pregnancy induces a state of acquired APC resistance, as determined by the functional assay, in the absence of the FV Leiden mutation (Bokarewa *et al*, 1996; Mathonnet *et al*, 1996a; Cumming *et al*, 1995). This acquired defect reverts to normal post-partum (Cumming *et al*, 1995). The cause of this change in functional APC activity has not been fully elucidated. There are however a number of pregnancy related factors which may influence APC anticoagulant activity. The reduction of Protein S activity, usually to about 40% to 50% of normal, may be expected to manifest in functional APC resistance

as Protein S is an important co-factor for APC (Cumming *et al*, 1995). In terms of the APC resistance functional assay, as Protein S has been shown to be of significance only when its functional activity falls below 20%, it is unlikely that changes in Protein S levels are responsible for the pregnancy associated resistance to APC (de Ronde and Bertina, 1994). Similarly as FVIII is also a substrate for APC and FVIII levels go up in pregnancy, this has been proposed as an alternative explanation (Mathonnet *et al*, 1996a; Cumming *et al*, 1995). Further support for the role of elevated FVIII levels in the development of acquired APC resistance stems from a study of patients with inflammatory disease who did not harbour the mutation (Mathonnet *et al*, 1996b).

Regardless of the underlying mechanism of the development of APC resistance in pregnancy, the APTT-based functional APC resistance assay would clearly not be suitable to accurately predict the presence of the FV Leiden mutation in pregnant women. As FV Leiden is strongly associated with venous thromboembolic disease in pregnancy (Bokarewa *et al*, 1996) it would nevertheless be important to identify those women at risk, particularly if they have a personal history of venous thromboembolic disease, as in these cases prophylactic anticoagulant therapy throughout pregnancy has been recommended (Bauer, 1995). Anticoagulation does however have certain associated risks and therefore any decision to commence such therapy could be aided in some cases by a laboratory diagnosis. The definitive diagnosis of the FV Leiden mutation in pregnancy should therefore be based on direct DNA analysis.

Whether or not routine screening for the APC resistance genotype in all pregnant women would be beneficial has not yet been established and would require large prospective studies. If consensus is reached that routine screening during pregnancy or prior to the commencement of oral contraceptives would be beneficial to the individual, geographic and ethnic variation would need to be considered so that populations who are known not to have the mutation are not screened unnecessarily. If such a programme were to be implemented nationally, in order for it to be sustained, the expenditure would need to be balanced by the anticipated cost-saving by reducing the incidence of venous thromboembolic disease. In an effort to reduce costs, the feasibility of pooling patient DNA samples for FV genotyping needs to be evaluated. If a pooled sample reveals the mutation, then all samples in that batch will need to be individually re-evaluated. The optimal batch size will depend on the anticipated or known frequency of occurrence of the mutation in the group being screened as well as the sensitivity of the technique. If the FV Leiden mutation is shown to be absent in the South African Black population, which is not unlikely in view of the proposed European founder effect, then not only would routine screening be of no value, but it would also obviate the need to look for inherited APC resistance in Black patients presenting with thrombosis.

As PCR technology is not available in many laboratories, diagnosis of the FV Leiden mutation in pregnancy may not always be possible. If patient specimens cannot be referred to a central laboratory then an alternative would be to use a modification of the original APTT-based APC resistance assay. This was originally devised so that the APC resistance phenotype could be accurately detected in patients with venous

thromboembolic disease who had already commenced anticoagulant therapy and therefore had prolonged baseline clotting times. This involves predilution of patient plasma with FV-deficient plasma prior to testing as per the original assay (Jorquera *et al*, 1994). Predilution with FV-deficient plasma was shown to abrogate the APC resistance phenotype associated with pregnancy but still allowed detection of the FV Leiden mutation (Cumming *et al*, 1996).

Whether acquired activated protein C resistance is merely an interesting physiological change in pregnancy or whether it contributes significantly to hypercoagulability leading to thrombosis is an intriguing question but is beyond the scope of this report.

2 STUDY OBJECTIVES

- To introduce a PCR assay for routine diagnostic purposes to detect the FV Leiden gene defect responsible for the activated Protein C resistance phenotype.

- To establish the prevalence of the FV Leiden gene defect in the South African Black population.

- To establish the feasibility of pooling samples for purposes of screening for the FV Leiden mutation to reduce the cost of the test.

- To compare the DNA - based assay with the functional APC resistance assay.

- To establish pregnancy specific reference ranges for Protein S, Protein C, Antithrombin and the APC resistance functional assay to facilitate interpretation of these laboratory tests when thrombotic screens are performed for venous thromboembolic events in pregnant women.

3 MATERIALS AND METHODS

3.1 ESTABLISHING THE TECHNIQUE OF POLYMERASE CHAIN REACTION FOR DETECTION OF THE FACTOR V LEIDEN MUTATION

3.1.1 SAMPLES

K_3EDTA (Tripotassium ethylenediamine tetra-acetate) anticoagulated blood samples submitted from the Johannesburg Hospital on patients who were being investigated for thrombosis by the SAIMR Haematology Laboratory were used. These specimens were submitted for full blood count analysis and would otherwise have been discarded after testing. Thirty samples were utilized in the development phase of this assay. The buffy layer was separated from whole blood after centrifugation at 2000g for 15 minutes and was stored in 5 ml plastic tubes at -70 °C until further analysis.

3.1.2 DNA EXTRACTION

DNA extraction was performed in accordance with the technique that is currently used routinely for the molecular confirmation of B and T cell tumour monoclonality. This rapid DNA extraction protocol was originally described by Talmud *et al* (1991). Specimens were frozen and then thawed to facilitate lysis of contaminating red cells prior to DNA extraction.

Four hundred microlitres of freshly prepared 0.17 M ammonium chloride was added to 100 µl of thawed buffy coat specimen. This was mixed well by inversion and then left at room temperature for 20 minutes before being spun down in a microcentrifuge for 30 seconds. The supernatant was then discarded, the pellet resuspended in 0.9% sodium chloride by vortexing and then centrifuged again. This washing process was repeated three times. After the third wash, the pellet was resuspended in 200 µl 0.05 M sodium hydroxide. This was boiled for 10 minutes and then neutralized by the addition of 25 µl 1 M Tris hydrochloric acid (pH 8.0). The extracted DNA was quantitated by spectrophotometric means and stored at -20 °C until further analysis.

3.1.3 DNA Quantitation

To determine the concentration of the DNA in solution, an aliquot of 20 µl of the post-extraction sample was diluted to a final volume of 1 ml by the addition of 980 µl H_2O. The samples were inserted into the spectrophotometer individually and the optical density (OD) at 260 nm was read. An OD_{260} of 1 corresponds to approximately 50 µg/ml of double-stranded DNA.

The concentration of DNA in µg/ml of the original sample was then calculated according to the following formula:

$$\text{DNA concentration } (\mu g/\mu l) = \frac{OD_{260} \text{ x } 50 \text{ x dilution factor } (50)}{1000}$$

$$= OD_{260} \text{ x } 2.5$$

3.1.4 Polymerase Chain Reaction

For PCR amplification the set of primers described in the original method of Bertina *et al* (1994) were used (Figure 3). These primers were obtained from Genosys.

As all current routine and most research PCR assays performed in the Haematology Department utilize a commercial PCR kit (PCR Core Kit, Boehringer Mannheim) this rather than the complex reaction buffer described in the original method of Bertina *et al* (1994) was used. The kit supplies reaction buffer with or without magnesium chloride ($MgCl_2$), additional $MgCl_2$, nucleotide mix in the form of deoxyribose nucleotide triphosphates (dNTPs) and the enzyme *Taq* polymerase.

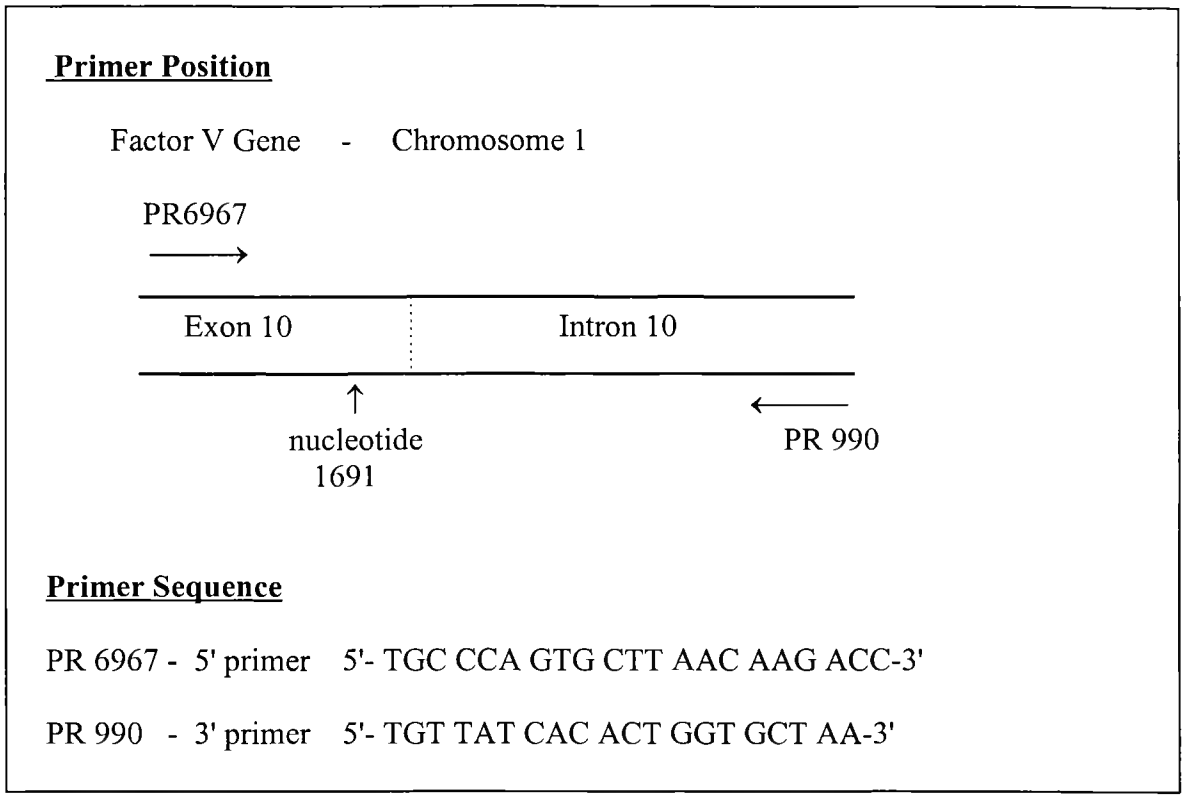

Figure 3. Sequence and Position of Primers used for the amplification of the segment of the Factor V Gene on Chromosome 1 spanning the site of occurrence of the Factor V Leiden Mutation.

A reaction volume of 50 µl was used. The reaction mixture comprised buffer, $MgCl_2$, nucleotides, enzyme, both primers, genomic DNA and distilled sterile water, the concentrations of which were adjusted accordingly until the conditions were considered to be optimal.

Initially, in accordance with the original method, a high $MgCl_2$ concentration was used (5.5 mM) although the optimal concentration was found to be 1.5 mM. As this is the

concentration of $MgCl_2$ in the buffer already containing $MgCl_2$, this premixed buffer was subsequently used obviating the need for addition of extra $MgCl_2$ to the reaction mixture.

Initially 2.5 µl of a 10 µM solution of each primer was added. This was subsequently adjusted to 1.25 µl. For each 50 µl reaction volume, both 0.25 and 0.5 µg of genomic DNA were tried. However, as 0.5 µg was the standard concentration used for other well established routine PCR techniques in the Haematology Laboratory, this concentration was adopted as there was no appreciable difference between the two. In order for a standard volume of 5 µl to be used for each reaction, an aliquot of extracted DNA (X µl) was diluted to a final concentration of 0.1 µg / µl in a final volume of 20 µl according to the following formula:

X = sample volume of parent DNA solution to be added to H_2O to a final volume of 20 µl.

$$X = \frac{2}{\text{DNA concentration after extraction}}$$

$$= \frac{2}{(OD_{260} \times 2.5)} \quad \mu l$$

Volume of H_2O = 20 - X

Of this 20 µl of DNA working solution (0.1 µg/µl), 5 µl was used for each reaction.

If the parent DNA solution concentration was less than or equal to 0.1 μg/μl, no further dilution was performed.

Taq polymerase and nucleotide concentrations were used in accordance with the kit instructions, namely 0.25 μl and 1 μl per 50 μl reaction volume respectively.

After experimentation, the final reaction conditions that were adopted for this technique are shown in Table 1.

Table 1 Optimal reaction mixture for a 50 μl PCR reaction volume

Reagent	**Volume (μl)**	
Buffer + $MgCl_2$	5	
dNTPs	1	
Primer PR6967	1.25	Master
Primer PR990	1.25	Mix
Taq polymerase	0.25	
H_2O	36.25	
Sample DNA	5	
Total	**50**	

The original description of 36 cycles at 91 °C for 40 seconds (denaturation), 55 °C for 40 seconds (annealing) and 71 °C for 2 minutes (elongation) was modified according to standard DNA PCR programmes in use in the Haematology Laboratory. Subsequently various changes were made including a reduction to 30 cycles; an increase of denaturation time to 1 minute; an increase of denaturation temperature to 94 °C; an increase in the annealing temperature up to 60 °C; prolongation of annealing time to 1 minute and an increase of elongation temperature to 72 °C.

The thermocycling conditions that were considered to be optimal were an initial 7 minutes denaturation at 94 °C followed by 30 cycles at 94 °C for 1 minute (denaturation), 58 °C for 1 minute (annealing) and 72 °C for 2 minutes (elongation).

The amplifications were carried out with a Perkin Elmer Gene Amp PCR System 2400 thermal cycler.

The use of the two primers PR6967 and PR990 results in the amplification of a 267 base pair (bp) fragment spanning the base at position 1691 in the FV gene. The DNA of every individual, whether they carry the normal FV gene (1691G) or the FV Leiden mutation (1691A) or both, should yield a 267 bp fragment when subjected to this PCR assay. As failure to do so implies failed PCR, this serves as an internal control.

3.1.5 Visualization of PCR Product

A 2 % agarose (w/v) gel of 5 to 7 mm thickness was used for electrophoretic separation of the amplified PCR products using TRIS Acetate EDTA (TAE) as buffer. A 50x stock solution of TAE consists of 242g TRIS, 100 ml 0.5 M EDTA pH 8.0 and 57.1 ml glacial acetic acid. The working solution of TAE buffer was made up of 20 ml of the above plus 980 ml distilled water. Forty microlitres of concentrated ethidium bromide was added to this to facilitate visualization of the size-separated PCR products under ultraviolet (UV) light.

Twenty microlitres of each PCR product mixed with a small quantity (±2 µl) of blue dye (Bromophenol Blue and Xylene Cyanol) was added to a well within the agarose gel and then subjected to electrophoresis. The blue dye is required to determine the point at which separation of product according to size has been adequate. This is in order to prevent excess exposure of the gel to UV light and hence fading of the product bands. A 100 bp molecular weight ladder (Boehringer Mannheim) was used as a reference to determine the size of the PCR products.

3.1.6 CONTROLS

A negative control (blank) sample containing no DNA was included in each run. If any amplification was detected in this sample, contamination was deemed to have occurred and the entire batch would be rerun. As DNA of every single individual, regardless of FV genotype is expected to yield a 267 bp fragment, this inbuilt control precludes the need for positive controls to be run with each batch. If a 267 bp band was not detected, the PCR reaction was considered to have failed and was subsequently rerun.

3.1.7 RESTRICTION DIGESTION USING *MNL*1 ENDONUCLEASE

This step is necessary for the actual detection of the factor V Leiden mutation. The enzyme *Mnl*1 is a restriction endonuclease sourced from the bacterium *Moraxella nonliquefaciens* which recognizes and cuts a specific DNA sequence. This specific sequence is represented twice within the 267 bp fragment of the normal FV allele. One of these sites involves the guanine at position 1691 so that when the guanine is replaced by adenine, as in the FV Leiden mutation, this restriction site is abolished. Different restriction patterns are therefore obtained for the FV Leiden positive and negative individuals.

The enzyme, obtained from Amersham Life Sciences, was stored at -20 °C in its original storage buffer containing 50% glycerol until required.

The original method by Bertina *et al* (1994) used 0.4 U *Mnl*1 to digest 7 to 10 µl of the 267 bp PCR product. As *Mnl*1(Amersham Life Sciences) contains 1.5 U/µl, it was empirically decided to use 1 µl per 20 µl PCR product. As specific details were not provided in the original method, various combinations of volumes of PCR product, PCR buffer (including $MgCl_2$) and assay buffer (supplied with the enzyme) were tried in the development phase of the *Mnl*1 restriction digestion (Table 2).

Table 2 Restriction mixtures used in the development phase of *Mnl*1 PCR product digestion (volumes given in µl)

	1	2	3
PCR buffer (+$MgCl_2$)	-	0.5	-
Enzyme buffer	-	-	2.5
H_2O	-	3.5	1.5
Enzyme (*Mnl*1)	1	1	1
PCR product	20	20	20
Total	21	25	25

After the addition of *Mnl*1 to 20 µl of PCR product in an Eppendorf tube, the mixture was incubated in a water bath at 37 °C overnight followed by electrophoretic separation as previously described using a 50 bp molecular weight ladder as a size reference.

The addition of 1µl of *Mnl*1 enzyme to 20 µl of PCR product was found to be effective, and as it was the simplest procedure, this was adopted for routine use. As *Mnl*1 is relatively expensive and double the recommended quantity of enzyme was being used, restriction digests using 0.3 µl (0.45 units), 0.7 µl (1.05 units) and 1 µl (1.5 units) of enzyme were compared.

The normal allele should yield 37, 67 and 163 bp fragments whilst the abnormal allele has lost a restriction site yielding only the 67 bp and a 200 bp (163 + 37) band. A normal individual will therefore have 37, 67 and 163 bp bands, a heterozygote for FV Leiden will have 37, 67, 163 and 200 bp bands whereas a homozygote will have only the 67 and 200 bp bands (Figure 4). DNA samples from a homozygous and heterozygous FV Leiden individual that were kindly supplied by Professor Bertina were used as controls in the development phase.

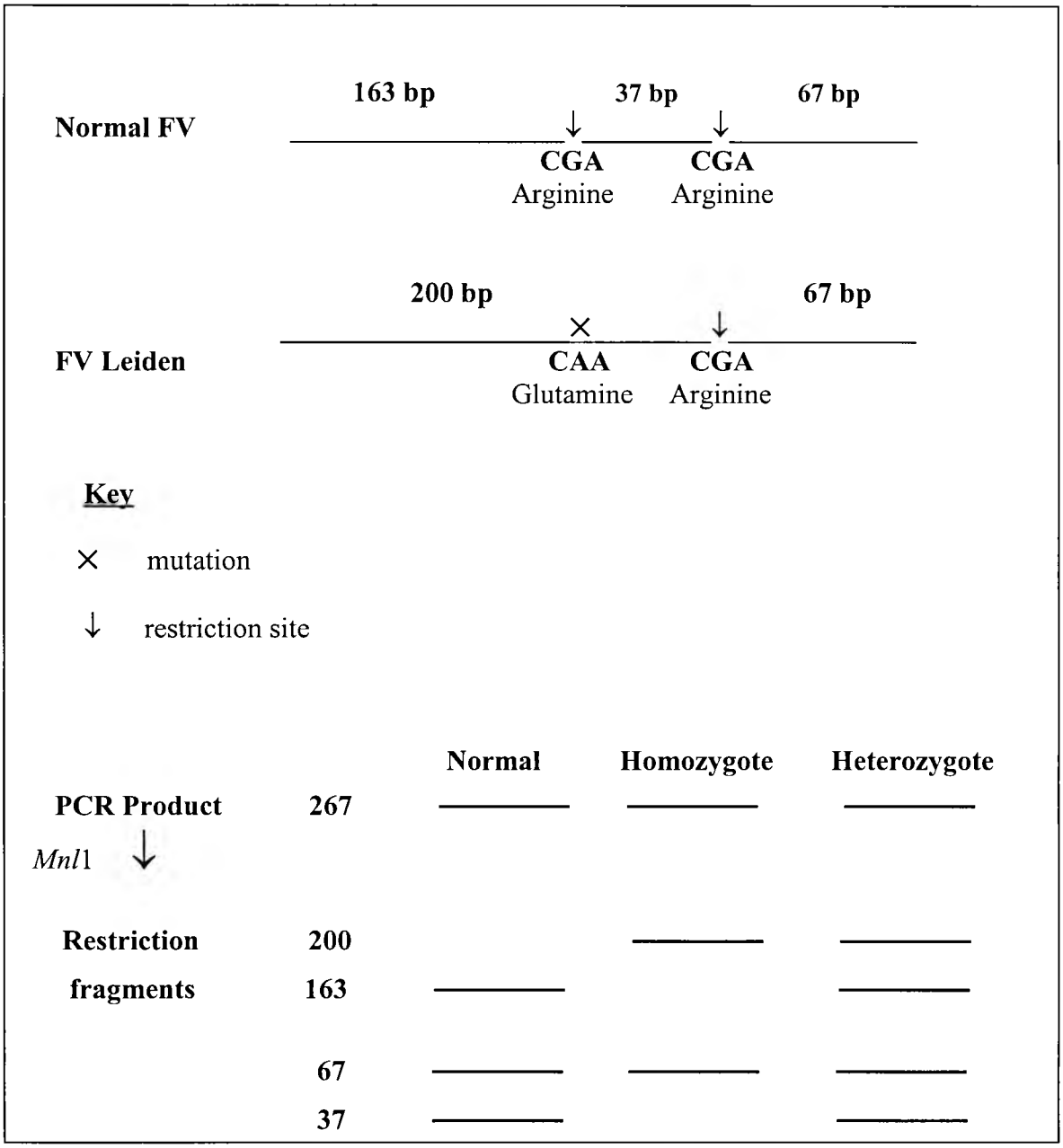

Figure 4 Schematic representation of influence of factor V Leiden mutation (1691 G→A) on restriction digestion pattern obtained with *Mnl*1 after PCR amplification of FV gene spanning mutation.

3.2 DETERMINING THE PREVALENCE OF THE FACTOR V LEIDEN MUTATION IN THE SOUTH AFRICAN BLACK POPULATION

3.2.1 STUDY POPULATION

Black blood donors presenting to the South African Blood Transfusion Services donor clinic at Baragwanath Hospital and its associated mobile blood collection clinics were studied. The first 130 volunteers constituted the study sample. Verbal consent was obtained by the attendant nursing sister.

3.2.2 SAMPLE COLLECTION

Two 4.5 ml venous blood specimens were collected at the time of blood donation. After the unit of blood had been collected, the tubing was tied, clamped and cut. The specimens were then collected by unclamping the tubing still connected to the vein in the antecubital fossa of the donor thereby allowing free flow of blood into the specimen tube. Additional venepuncture was therefore not required. One specimen was collected in K_3EDTA the other in 0.105 M trisodium citrate (9 volumes blood:1 volume citrate).

3.2.3 SAMPLE ANALYSIS

3.2.3.1 Activated Protein C Resistance - Factor V Genotype analysis

The K_3 EDTA specimen was processed as previously described to ascertain the FV genotype. All 130 specimens were analysed individually.

3.2.3.2 DNA Pooling for FV Leiden Population Screening

Pools of DNA from FV Leiden negative individuals were "spiked" with FV Leiden positive DNA to determine whether routine FV Leiden genotyping by means of PCR amplification and *Mnl*1 restriction was significantly sensitive to detect the abnormal genotype.

Equal quantities of DNA (10 μl of a ~0.1 μg/μl solution) from 9 individuals who had been shown to have a normal FV genotype were pooled. A 10 μl aliquot from a ~0.1 μg/μl solution of DNA from a known FV Leiden heterozygous individual was then added to this pooled mixture. The 1691A FV allele frequency was thus artificially created to be ~1 in 20.

A second pool comprising DNA from 4 normal individuals and one FV Leiden heterozygous individual was set up creating a 1691A allele frequency of ~1 in 10.

Each DNA pool was then subjected to routine PCR amplification and *Mnl*1 restriction digestion together with repeat analysis of the individual constituents of each pool within the same PCR run. The above analysis was performed five times to ensure repeatability of the screening process.

If the technique is sufficiently sensitive to reliably detect a single heterozygous individual in a pool of ten, then for population screening, batches of ten could be processed followed by individual analysis should a pool be shown to be positive.

As this is potentially labour intensive an alternative approach may be feasible. Knowledge of the identity of the FV Leiden positive sample/s is not necessary when an analysis is performed solely to determine the population prevalence of this defect. In view of the observation of an apparently constant relationship between the intensity of the 200 bp and 163 bp restriction fragments as visualized on ethidium bromide stained agarose gels after electrophoretic separation, it was postulated that it may be possible to predict the allele frequency in a pooled DNA sample. This would depend on an altered ratio of the two bands which are used to discriminate between normal (200 bp absent) and abnormal (200 bp present). To test this hypothesis a series of "spiked" DNA pools with diminishing FV Leiden positive DNA content was set up and analysed [i.e. 1 in 2 (neat heterozygous positive DNA); 1 in 4 (1 heterozygote, 1 normal); 1 in 6 (1 heterozygote, 2 normal); 1 in 8 etc. up to 1 in 20].

DNA samples from six different FV Leiden positive heterozygotes were processed to see whether the visually observed impression of a constant relationship between the 200 bp and 163 bp bands with regards to their intensity when visualized / photographed on an ethidium bromide stained 2% agarose gel under UV light could be documented objectively. This was performed by taking a negative image of the agarose gel under UV light followed by densitometry scanning. The sequential pooled samples were also subjected to densitometry scanning.

3.2.3.3 Activated Protein C Resistance - Functional Assay

This was performed using the COATEST® APC ™ Resistance commercial kit from Chromogenix, Sweden. This is based on an APTT assay for semi-quantitative determination of the response towards human APC.

In an APTT reaction, the addition of APC results in degradation of both FVIIIa and FVa which delays thrombin generation and hence formation of the fibrin clot. The FV $Arg^{506} \rightarrow$ Gln mutation is associated with partial resistance to degradation of mutated FVa by APC, but the mutated FVa expresses normal procoagulant properties (Dahlbäck, 1994; Heeb *et al*, 1995). Consequently thrombin generation is not properly impaired in the presence of APC which results in less prolongation of the clotting time.

For this test, the citrated blood specimens were centrifuged at 2000g for 20 minutes. The plasma was then removed avoiding the platelet-rich buffy layer region, and transferred into a 5 ml plastic tube with screw cap. These samples were snap frozen and stored at -20 °C. This took place at the Baragwanath Hospital SAIMR Coagulation Laboratory. Specimens were collected weekly, transported on dry ice to avoid thawing and placed in a -70 °C freezer in the Johannesburg Hospital SAIMR Coagulation Laboratory where analysis took place. At the time of analysis, plasma samples were rapidly thawed in a 37 °C waterbath and processed within one hour.

The reagents supplied with the kit are calcium chloride, APTT reagent and premixed APC/$CaCl_2$. The assay was performed manually. A sufficient volume of APC/$CaCl_2$ was prewarmed at 37 °C. One hundred microlitres of plasma was then added to a test tube followed by 100 µl of APTT reagent. This was then incubated at 37 °C for 5 minutes. One hundred microlitres of $CaCl_2$ was then added whilst timing of clot formation was begun simultaneously. The time taken for clot formation was recorded. A second analysis was then performed on the plasma exchanging $CaCl_2$ with APC/ $CaCl_2$.

The result was then recorded as an APC-SR which was calculated as follows:

$$\text{APC-SR} = \frac{\text{Clot Time (APC/CaCl}_2\text{)}}{\text{Clot Time (CaCl}_2\text{)}}$$

The current laboratory interpretation of this test is that < 2.00 is considered to be abnormal.

Quality control plasmas available from Chromogenix, Control Level 1 (normal) and Control Level 2 (abnormal) were run with each batch.

3.2.3.3.1 Factor V and Factor VIII Assays

As the results of the above assay were lower than expected with 25% of the females and 28% of the males having APC-SRs of less than 2.00, plasma samples were divided into two groups for further analysis.

Group I comprised all plasma samples with an APC-SR $\geq$ 2.00 and Group II all those plasma samples with an APC-SR of < 2.00. Five plasma samples with the highest recorded APC-SRs and five plasma samples with the lowest APC-SRs were selected to represent Group I and Group II respectively. This was performed prior to the completion of the study therefore the samples did not necessarily have the lowest and highest APC-SRs of the total study population. As the APTT with APC values were shown to be responsible for the observed differences in APC-SRs between the two groups, FV and FVIII levels were determined for these selected plasma samples as these are the known substrates of APC.

The FVIII assays were performed on an ACL 300R Coagulometer (Instrumentation Laboratories, Milan, Italy) using FVIII-deficient plasma from Baxter and automated APTT reagent from Organon-Teknika using the single factor assay (FVIII) programme. The FV assays were performed on an ACL 3000 Coagulometer (Instrumentation Laboratories, Milan, Italy) using FV-deficient plasma from Stago and the recombinant

thromboplastin Innovin (Dade). The assay was performed using the single factor assay programme (FV) in accordance with the instrument manual instructions. Results were recorded as percentage values.

3.2.3.3.2 Further Testing of Samples with Prolonged Baseline APTTs

Prior to the completion of functional APC resistance testing on all samples, several plasma samples were shown to have markedly prolonged baseline APTTs. As these were all derived from healthy individuals these abnormal findings were considered to be due to specimen handling. As FV and FVIII activity is lost with "aging" of plasma, this was postulated to be the likely explanation. An APTT correction assay was performed on 8 samples with prolonged baseline APTTs. This involves mixing an equal volume of sample plasma with normal control plasma followed by routine APTT testing using the ACL 300R coagulometer and automated APTT reagent from Organon-Teknika. Factor V and FVIII functional assays were subsequently performed on these 8 samples as previously described.

3.2.3.3.3 Effect of Temperature of Storage of Whole Blood and Time Delay prior to Plasma Separation

Prior to completion of the APC resistance functional testing on all plasma samples, it was noted that several were either activated, had prolonged baseline APTTs or very low APC-SRs. As specimen handling was considered to be primarily responsible for these changes,

the effect of temperature at which whole blood specimens were kept as well as the effect of the length of time prior to centrifugation, plasma separation and freezing was determined.

Twenty eight 4.5 ml citrated tubes of peripheral blood were obtained from one individual. Venepuncture was performed using a 23 gauge butterfly needle. Blood was collected directly into Becton Dickinson vacutainer tubes.

The first four tubes were immediately centrifuged at 2000g for 20 minutes, the plasma separated, snap frozen and stored at -70 °C. Two of these tubes constituted the baseline (time 0) specimen against which all others were compared. Plasma obtained from the other two tubes was divided into 10 aliquots for the assessment of APC resistance functional assay interbatch variation. One aliquot of plasma was included whenever a new batch of reagent was used for a run of APC resistance functional assays.

The remaining 24 tubes were processed as follows: 2 tubes each were kept at room temperature and at 4 °C for 1 hour, 2 hours, 4 hours, 6 hours, 12 hours and 24 hours respectively prior to plasma separation and storage as described above. Each set of plasmas was then processed for APC-SR determination as previously described. Each plasma set was analysed 10 times to determine the intrabatch variation.

3.3 THROMBOPHILIC PROFILES IN NORMAL PREGNANCIES

3.3.1 STUDY POPULATION

Healthy females with normal uncomplicated pregnancies attending the Johannesburg Hospital antenatal clinic were asked to volunteer for the study. The first 20 volunteers in each of the first, second and third trimesters of pregnancy were meant to constitute the study sample. As patients at this clinic generally attend only once in a fairly advanced stage of pregnancy, only two subjects in the first trimester of pregnancy were available to participate in this study. In an attempt to overcome this shortcoming, obstetricians in the private sector were approached to recruit further first trimester pregnant women into the study. Despite this, only five further patient samples could be obtained. The total study sample thus comprised 7 women in the first trimester, 20 in the second trimester and 20 in the third trimester of pregnancy.

3.3.2 CONTROL SUBJECTS

The control group was comprised of 25 healthy female volunteers who were recruited from the SAIMR Laboratory staff at the Johannesburg Hospital, most of whom were Caucasian. As it is not known whether there are differences in normal values for the parameters under study (Protein C, Protein S, Antithrombin and APC resistance functional assay) amongst Blacks and Whites, the first 20 female volunteers aged

between 16 and 40 years of the Black blood donor group were additionally recruited as the pregnant study group comprised both White and Black patients.

3.3.3 SAMPLES

One 4.5 ml peripheral blood specimen in K_3EDTA and one 4.5 ml specimen in 0.105 M trisodium citrate was obtained from each volunteer. The K_3EDTA specimen was used to determine the FV genotype as previously described. Platelet poor plasma derived from the citrated sample was stored at -70 °C as previously described. The plasma sample was then assayed for APC resistance, Protein C, Protein S and Antithrombin levels by means of functional assays.

3.3.4 SAMPLE ANALYSIS

3.3.4.1 Activated Protein C Resistance

This was performed as previously described.

3.3.4.2 Protein C

This assay was performed according to the routine laboratory technique using the COAMATIC® Protein C diagnostic kit from Chromogenix, Sweden which is a chromogenic-based assay. The Protein C in plasma is activated by a specific enzyme

from Southern Copperhead snake venom. The amount of activated Protein C is then determined by the rate of hydrolysis of a synthetic substrate S-2366. The paranitroaniline release measured at 405 nm is proportional to the Protein C level in the range from 0 to 120% of normal plasma.

The test is automated and was performed using the ACL 300R coagulometer. Patient values were calculated automatically by comparison with a standard curve and were expressed as a percentage value.

3.3.4.3 Protein S

This assay was performed according to routine laboratory techniques using a commercial Protein S kit from Instrumentation Laboratories. The functional activity of Protein S, co-factor for Protein C, is proportional to the prolongation of the prothrombin time of Protein S-deficient plasma to which diluted sample has been added. Activated Protein C is generated by activation of Protein S-deficient plasma with Protac. This mixture was kept at 37 °C for 20 minutes before use. Both Protac and Protein S-deficient plasma are supplied with the kit.

Protein S levels in plasma were measured automatically on the ACL 3000 Plus coagulometer, using bovine thromboplastin as the reagent to initiate clotting. The Protein S level was recorded as a percentage value by comparison to a standard curve generated from a number of dilutions of Protein S control plasma.

3.3.4.4 Antithrombin

The assay was performed according to the routine technique currently in use in the Coagulation Laboratory at the Johannesburg Hospital.

This utilises the COAMATIC® Antithrombin kit and Chromtrol (control plasma) from Chromogenix, Sweden. Antithrombin has a powerful and immediate antithrombin action in the presence of heparin. The assay was performed using the ACL 300R Coagulometer and was carried out in 2 steps:

- incubation of the sample with excess FXa (which generates excess thrombin) in the presence of heparin
- detection of the residual thrombin activity on a synthetic chromogenic substrate S-2765.

The paranitroaniline release monitored at 405 nm is inversely proportional to the Antithrombin level and was reported as a percentage value in comparison with the standard control curve.

3.4 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was performed to determine whether there are differences between data obtained for the first, second and third trimester pregnant women and normal controls for Protein C, Protein S, Antithrombin and APC resistance functional assays. The students t-test was used for all other comparative analyses. A significance level of 5% was used. Where applicable, the co-efficient of correlation (r-value) was calculated.

4 RESULTS

4.1 FACTOR V LEIDEN GENOTYPING

4.1.1 POLYMERASE CHAIN REACTION AMPLIFICATION OF FV GENE SPANNING NUCLEOTIDE 1691

Amplification of genomic DNA using the primers PR6967 and PR990 results in a 267 bp product in all individuals, irrespective of the presence or absence of the mutation, when optimal amplification conditions prevail. Figure 5 shows the results of agarose gel electrophoresis of the PCR products obtained from genomic DNA from 10 different individuals.

The most critical factor in determining successful amplification was found to be the $MgCl_2$ concentration. Although the original description of PCR amplification for FV Leiden indicated a high $MgCl_2$ concentration, this was not successful when adapted for use in conjunction with the Boehringer Mannheim PCR core kit (Figure 6a).

As the general recommendation of $MgCl_2$ concentration for use with the PCR core kit is 1.5 mM, as well as it being the concentration of $MgCl_2$ in the standard "pre-mixed" buffer, this was tried next with success under otherwise identical conditions (Figure 6b).

A reduction in primer concentration from 2.5 µl of a 10 µM stock solution to 1.25 µl per 50 µl reaction volume resulted in slightly less primer-dimer formation (Figure 7) with no obvious loss of intensity of the 267 bp band. Although the presence of primer-dimer bands does not interfere with interpretation of the PCR product restriction digestion pattern, the lower primer concentration was selected for its resultant clearer picture as well as for cost-saving purposes.

Alterations of annealing temperature, DNA concentration and the number of cycles were found to have only a minor effect on amplification (not shown). The PCR amplification conditions that were considered to be optimal and thus selected for routine diagnostic purposes are set out in the materials and methods section.

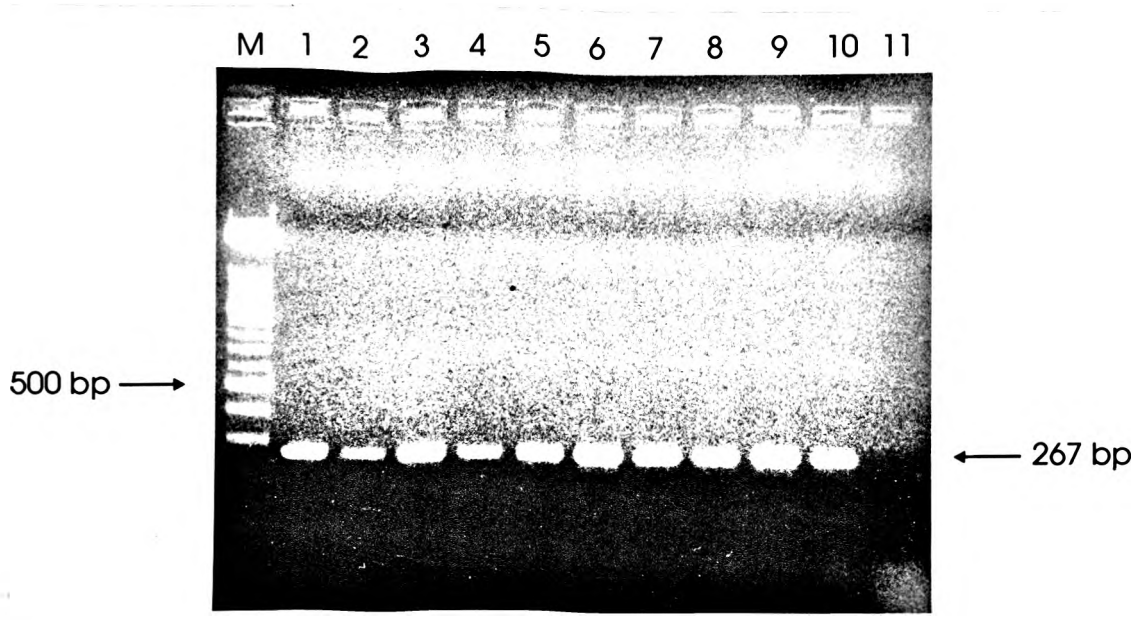

Figure 5 PCR Amplification of portion of Factor V Gene spanning Nucleotide 1691

Ethidium bromide stained 2% agarose gel showing PCR amplification of genomic DNA from 10 individuals (lanes 1-10). Lane 11 is a negative control sample containing no DNA. M is a 100 bp molecular weight ladder (Boehringer Mannheim).
Reaction conditions: $MgCl_2$ 1.5mM, 30 cycles at 94 °C (1 min), 58 °C (1 min), 72 °C (2 min).

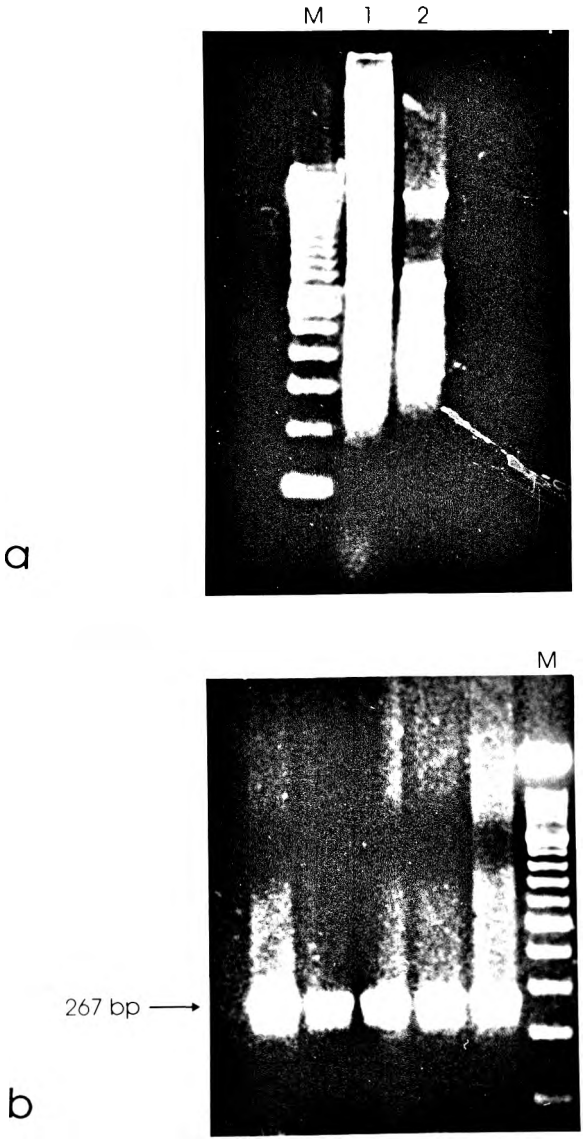

Figure 6 Effect of $MgCl_2$ concentration on PCR Amplification

a) Ethidium bromide stained 2% agarose gel comparing PCR products obtained under reaction conditions with varying $MgCl_2$ concentrations, namely 5 mM (lane 1) and 5.5 mM (lane 2) respectively. Both concentrations resulted in smudging and the production of multiple bands. Although a faint band just smaller than the 300 bp marker was present in both lanes, this was insufficiently clear to be representative of the desired 267 bp product.
b) PCR reaction with $MgCl_2$ concentration of 1.5 mM.
M = 100 bp molecular weight marker.

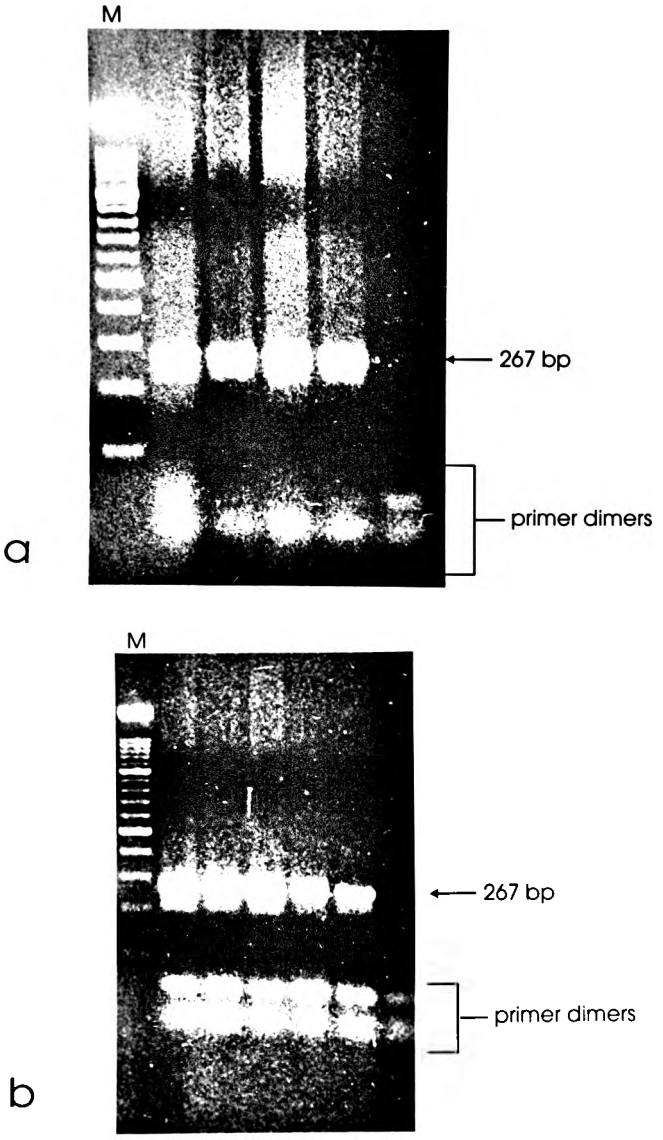

Figure 7 Effect of Primer Concentration on PCR Amplification

Ethidium bromide stained 2% agarose gel showing less primer-dimer formation after amplification of part of the FV gene using 1.25 µl (a) of a 10 µM stock solution of each primer rather than 2.5 µl (b) per 50 µl reaction volume. M is a 100 bp molecular weight ladder (Boerhinger Mannheim).

4.1.2 RESTRICTION DIGESTION USING *MNL*1

The varying restriction fragment length patterns obtained after subjecting the PCR products to *Mnl*1 digestion at 37 °C overnight are shown in Figure 8.

The restriction patterns obtained allow clear distinction between individuals who are homozygous for the FV Leiden mutation (200 bp, 67 bp), heterozygotes (200 bp, 163 bp, 67 bp, 37 bp) as well as those with a normal FV genotype (163 bp, 67 bp, 37 bp).

In the development phase of the *Mnl*1 digestion component of FV genotyping for the detection of FV Leiden, various combinations of enzyme buffer, PCR buffer and *Mnl*1 enzyme were tested the results of which are shown in Figures 9 and 10.

Suboptimal digestion was obtained when the enzyme-specific buffer was added to the restriction mixture. This is clearly indicated by the presence of a prominent residual 267 bp band in lane 3 of Figure 10 although some digestion did occur. As no obvious difference was noted whether PCR buffer was added or not, this was omitted from the final enzyme restriction method adopted for routine use.

As the *Mnl*1 enzyme is very expensive (~R2200/250 units) restriction digests using varying amounts of enzyme were compared. The results indicate that digestion using the

lowest quantity (0.45 units/20 μl PCR product) is as good as 1.05 units and 1.5 units of enzyme (Figure 10).

The final procedure adopted for restriction digestion was the addition of *Mnl*1 restriction endonuclease directly to the unmodified PCR product solution. If only a single sample was being analysed, 1 μl (1.5units) of enzyme was added as accuracy of pipetting diminished when smaller volumes were used. For larger sample batches an enzyme "master mix" was created by diluting enzyme with distilled sterile water so that 2 μl of this solution contained 0.45 units of *Mnl*1. Two microlitres of this "master mix" were then added to each post amplification 20 μl sample aliquot.

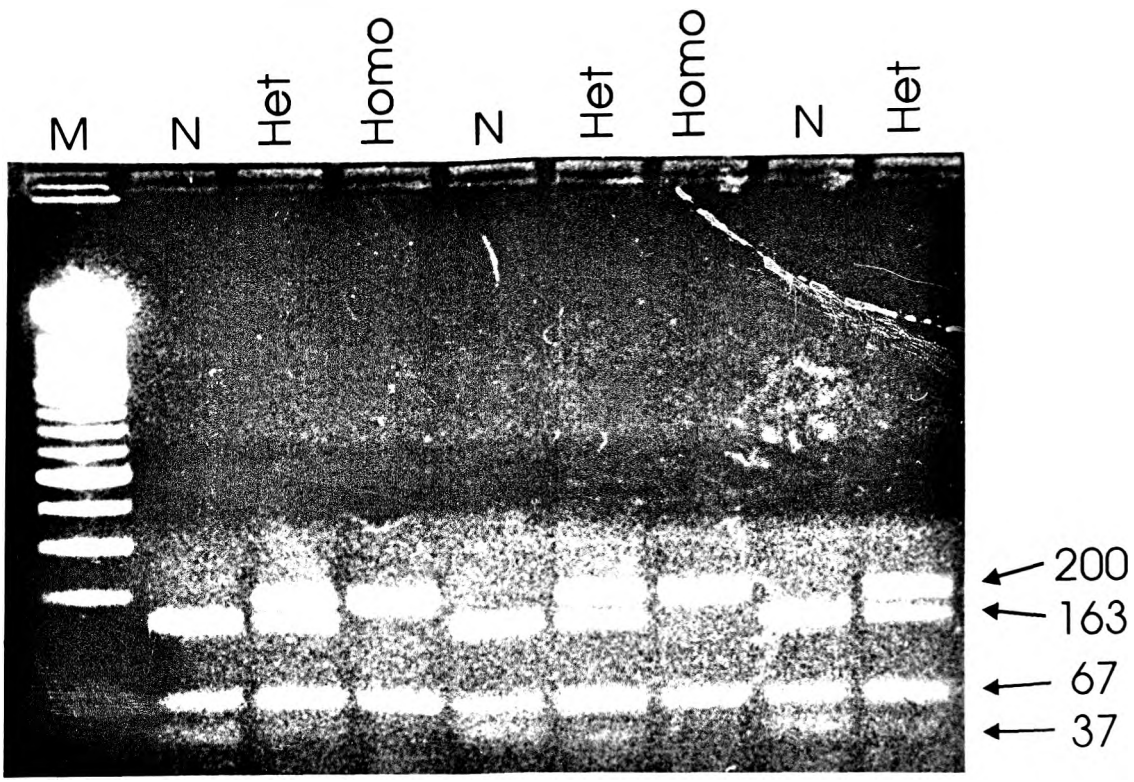

Figure 8 ***Mnl*1 Restriction Digestion of PCR Products**

Ethidium bromide stained 2% agarose gel showing the varying restriction fragment length patterns for normal individuals (N), FV Leiden heterozygotes (Hetero) and FV Leiden homozygotes (Homo). M is a 100 bp molecular weight ladder.

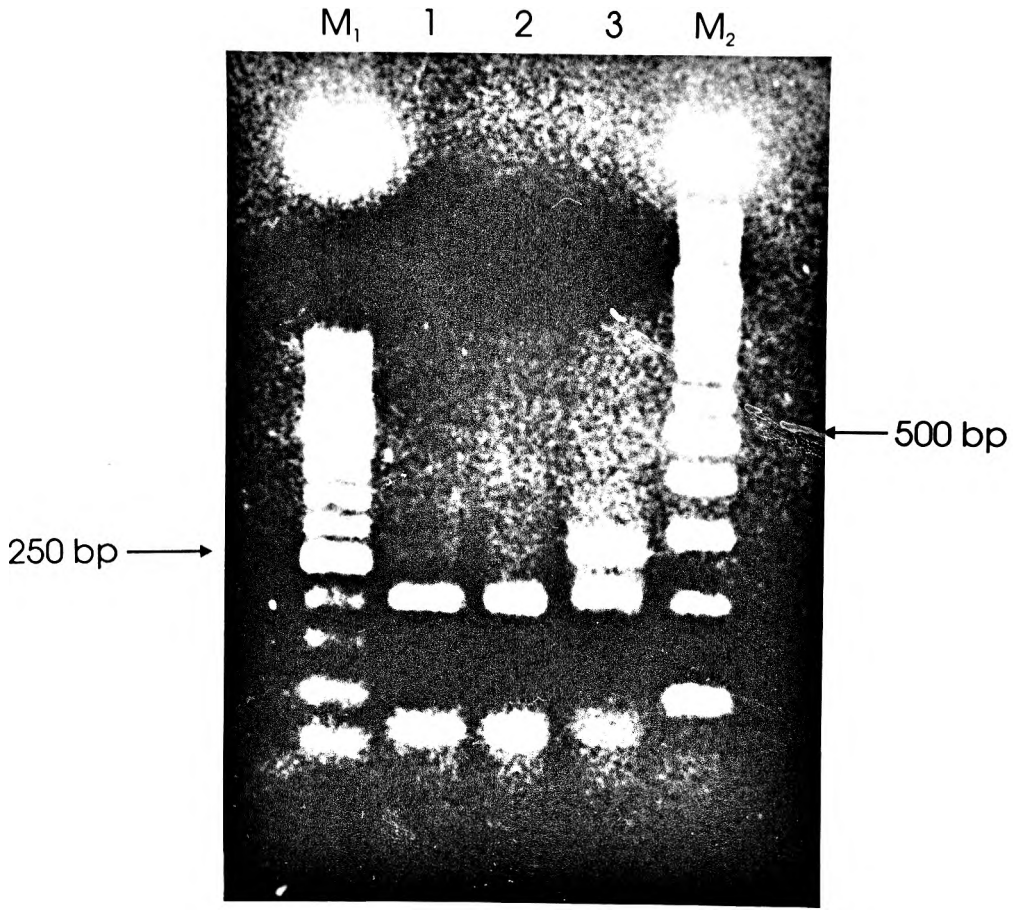

Figure 9 Effect of various Buffers on *Mnl*1 Restriction Digestion

Ethidium bromide stained 2% agarose gel showing the digestion fragments obtained after subjecting aliquots of the same 267 bp PCR products from a FV Leiden heterozygote to the restriction enzyme *Mnl*1 at 37 °C for 16 hours under varying conditions as indicated: **lane 1** - 1 µl enzyme, 20 µl PCR product; **lane 2** - 1 µl enzyme, 20 µl PCR product, 0.5 µl PCR buffer ($MgCl_2$ 1.5 mM), 3.5 µl H_2O; **lane 3** - 1 µl enzyme, 20 µl PCR product, 2.5 µl *Mnl*1 enzyme buffer, 1.5 µl H_2O.
The presence of both a 200 bp and 163 bp band is clearly seen in lanes 1 and 2.
M_1 is a 50 bp and **M_2** a 100 bp molecular weight marker.

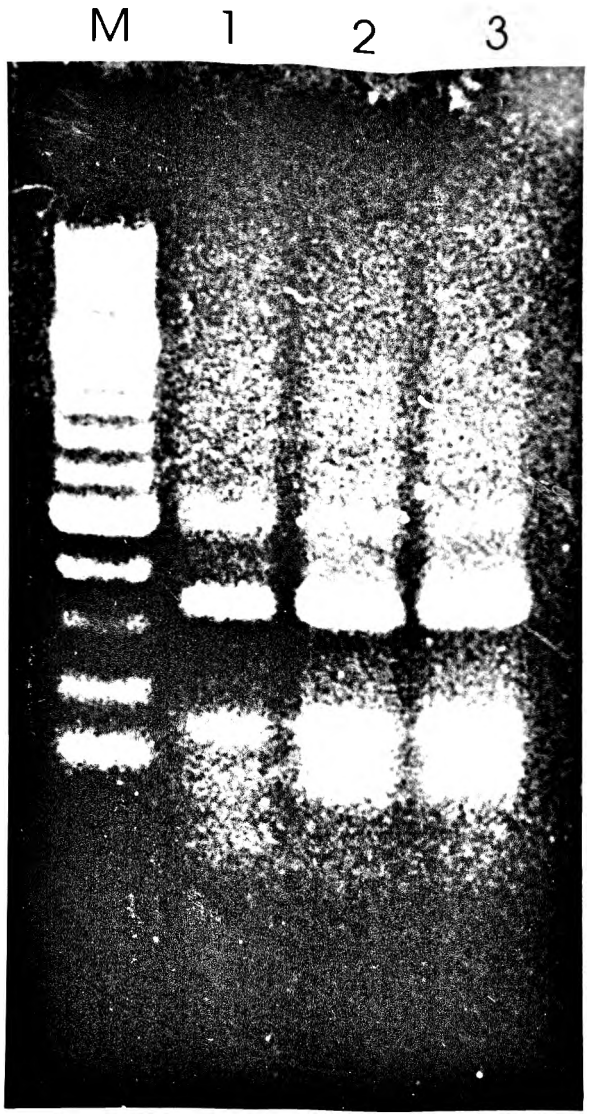

Figure 10 Effect of *Mnl*1 Enzyme Concentration on restriction digestion

Ethidium bromide stained 2% agarose gel showing the effect of progressive reduction of *Mnl*1 enzyme concentration on the restriction digestion of aliquots of PCR products obtained from a single individual. The quantities used were 1.5 units (lane 1), 1.05 units (lane 2) and 0.45 units (lane 3) per 20 µl of PCR product. M is a 50 bp molecular weight ladder.

4.2 PREVALENCE OF FACTOR V LEIDEN MUTATION IN SOUTH AFRICAN BLACK POPULATION

4.2.1 STUDY POPULATION

One hundred and thirty healthy Black blood donors were studied. This group comprised 73 males, 44 females and 13 individuals in whom sex was not stated. The mean ages were males 30.6 years (range 16 - 57), females 27.1 years (range 16 - 58) and in the unspecified group 31.5 years (range 22 - 40).

4.2.2 GENOTYPIC ANALYSIS

Polymerase chain reaction amplification of DNA followed by *Mnl*1 restriction digestion did not reveal a single FV Leiden positive individual (Figure 11).

4.2.3 POOLING OF DNA SAMPLES FOR FV LEIDEN POPULATION SCREENING

Analysis of the pooled DNA samples detected the FV1691A allele on 4 out of 5 occasions when using the 1 in 20 mix (1 heterozygote, 9 normals) and on all occasions (5/5) using the 1 in 10 mix (1 heterozygote, 4 normals). This is shown in Figure 12.

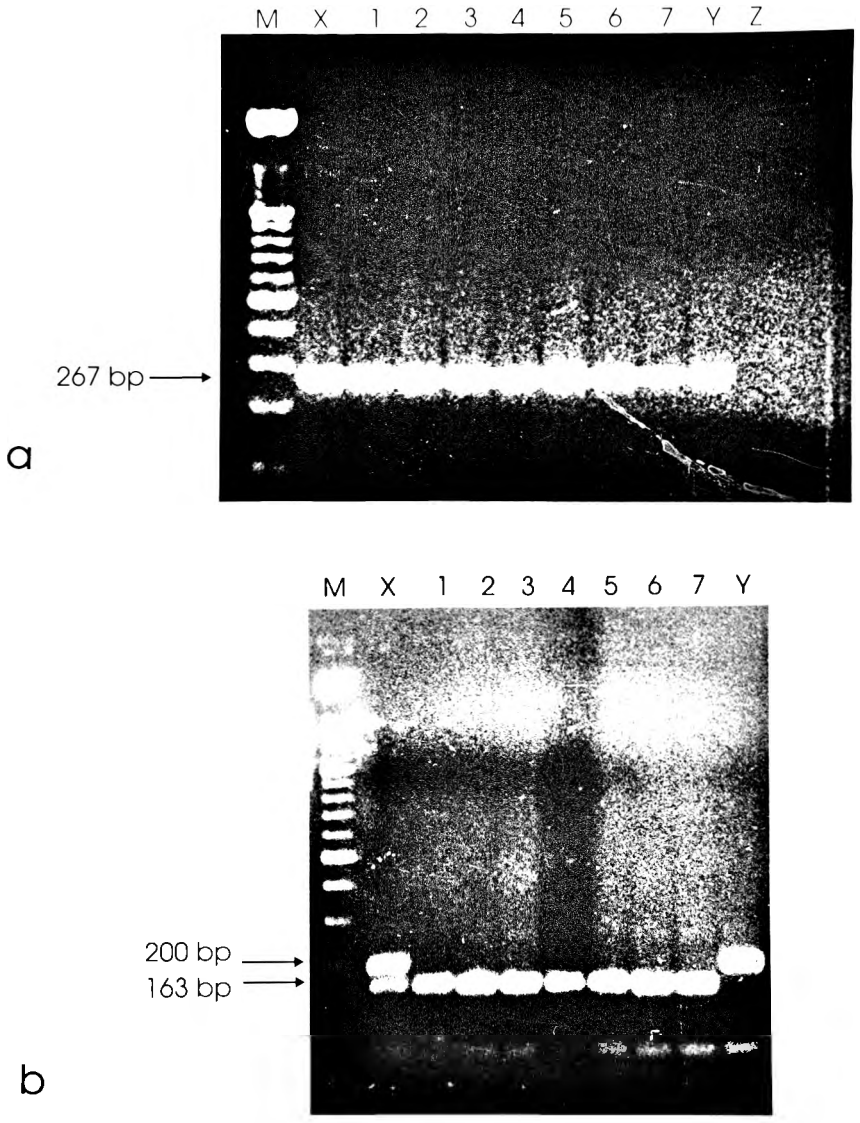

Figure 11 FV Leiden Genotyping of Black Blood Donors

a) Ethidium bromide stained 2% agarose gel showing 267 bp PCR product for all individuals analysed (lanes 1 - 7). Lane X = positive control (FV Leiden heterozygote); Lane Y = positive control (FV Leiden homozygote); Lane Z = negative control (blank). M = 100 bp molecular weight marker.
b) Agarose gel showing normal *Mnl*1 restriction pattern (163, 67, 37 bp bands) in all individuals analysed (lanes 1 - 7). Lane X = positive control exhibiting expected pattern for a FV Leiden heterozygote (200, 163, 67, 37 bp bands). Lane Y = positive control exhibiting expected pattern for a FV Leiden homozygote (200, 67 bp bands).

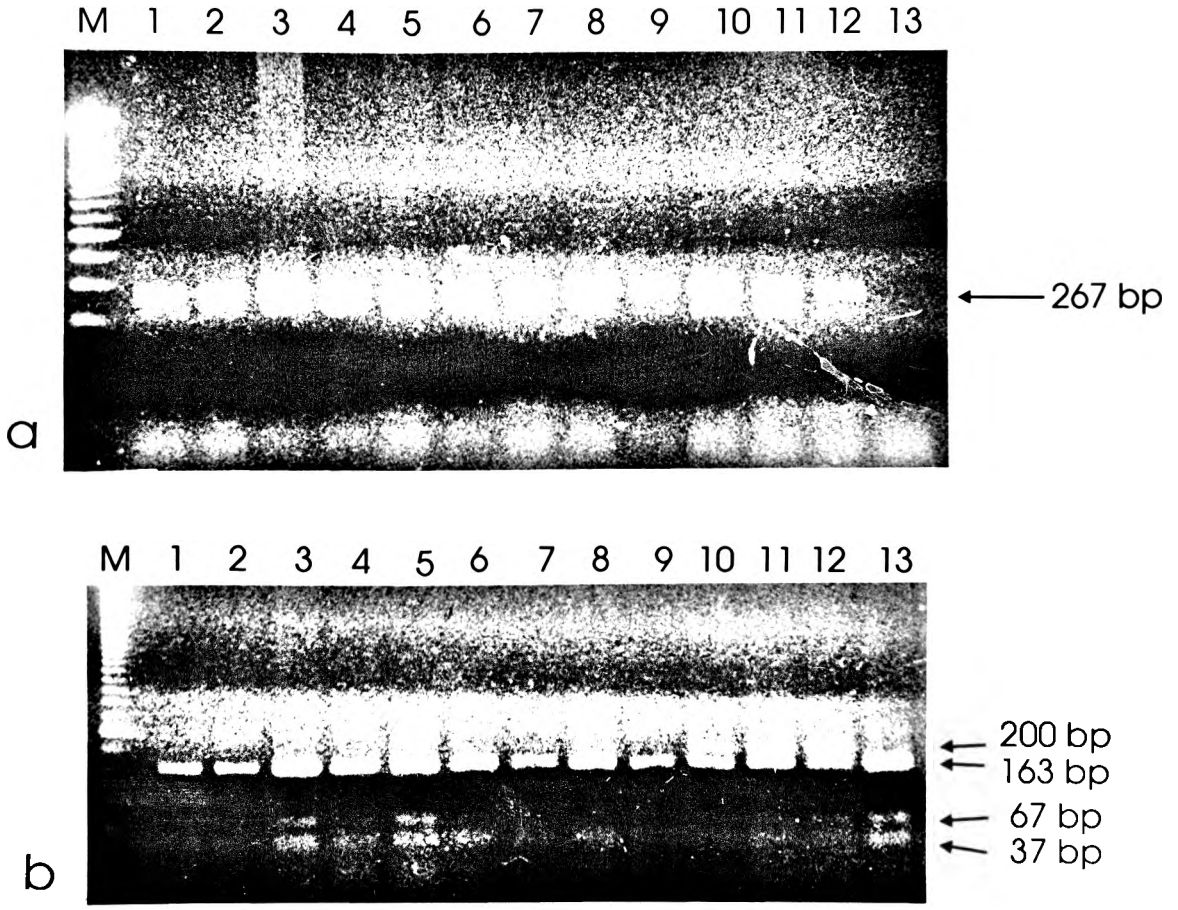

Figure 12 Detection of FV1691A Allele in Pooled DNA Samples

a) Ethidium bromide stained 2% agarose gel showing products of PCR amplification of 9 normal individuals (**lanes 1 to 9**), pooled DNA - FV1691A allele frequency 1 in 10 (**lane 10**), pooled DNA - FV1691A allele frequency 1 in 20 (**lane 11**), FV Leiden heterozygote control (**lane 12**) and a blank control (**lane 13**). **M** is a 100 bp molecular weight ladder.
b) Agarose gel showing *Mnl*1 restriction digestion patterns of individually analysed normal DNA samples [1691G/1691G] (**lanes 1 to 9**) and pooled DNA samples that were "spiked" with an equal quantity of DNA from a heterozygous FV Leiden positive individual [1691A/1691G]. **Lane 10** represents the pooled DNA (FV1691A frequency 1 in 10) of individuals in **lanes 1 to 4** and that of the positive control DNA (FV1691G/FV1691A) which is shown in **lane 12. Lane 11** represents the pooled DNA of the individuals in **lanes 1 to 9** and that of the positive control in **lane 12** (FV1691A allele frequency 1 in 20). **Lane 13** is a normal control. **M** is a 50 bp molecular weight marker.

Analysis of the series of DNA pools with a stepwise reduction in FV Leiden 1691A allele content is shown in Figure 13. Although there is an obvious difference in the intensity of the 200 bp and 163 bp bands between the neat FV Leiden heterozygote (1 in 2 FV1691A allele frequency) and the 1 in 20 DNA pool on visual inspection, the differences between the various mixes are too subtle to allow for any reliable assessment of FV1691A allele frequency within the DNA pool.

Densitometry scanning of the 200 bp and 163 bp bands obtained from 6 individual FV Leiden heterozygotes was performed. The result, shown in Table 3, did not yield a constant ratio between the two bands contrary to what was expected from simple visual observation. Likewise, similar analysis of the same series of DNA pools as shown in Figure 13 could not predict the FV1691A allele frequency within the DNA pools (Table 4). An example of a densitometry scan is shown in Figure 14.

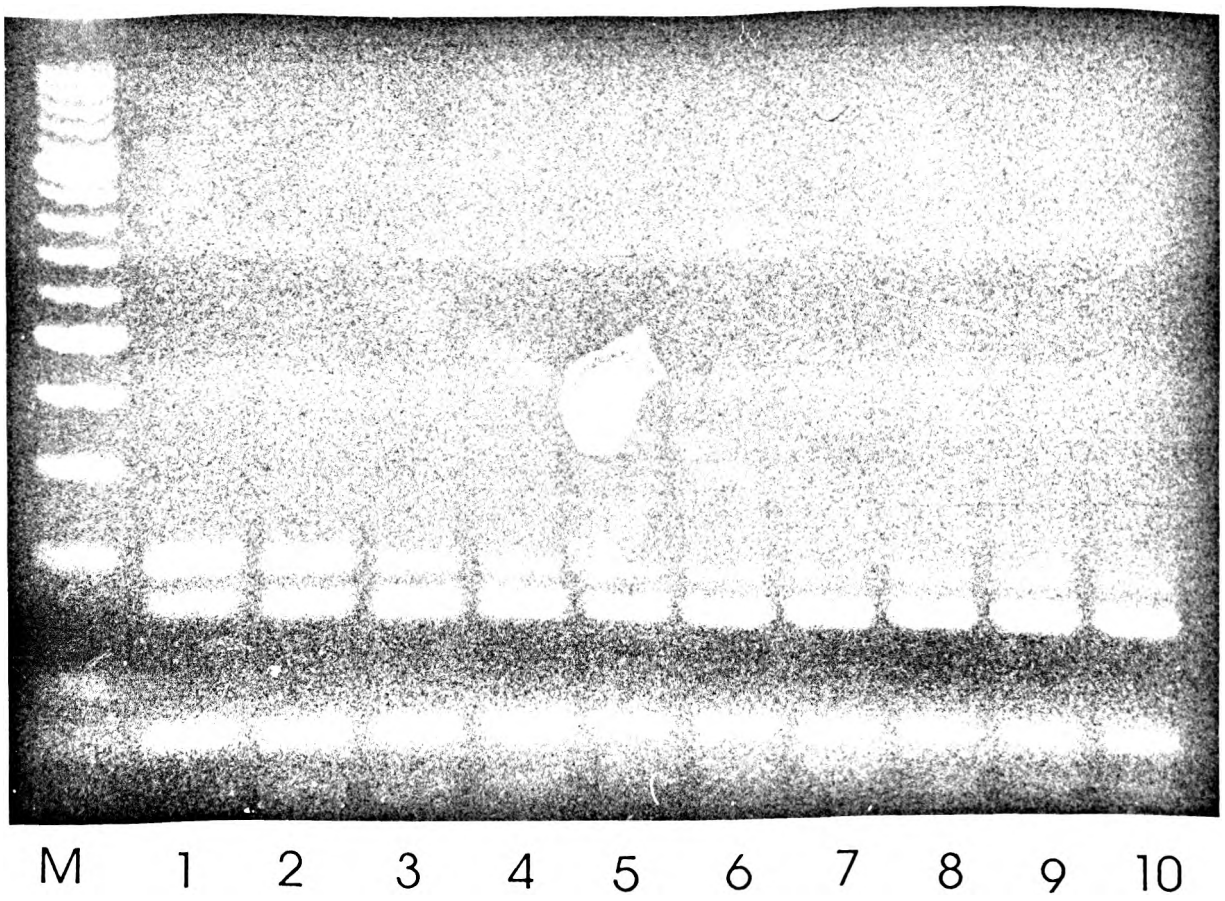

Figure 13 Detection of FV1691A in a series of Pooled DNA samples

Ethidium bromide stained 2% agarose gel showing *Mnl*1 restriction digestion patterns of DNA samples with diminishing FV1691A allele frequency. **Lane 1**- 1 in 2 (neat Het); **Lane 2** - 1 in 4 (1 Het, 1 N); **Lane 3** - 1 in 6 (1 Het, 2 N); **Lane 4** - 1 in 8 (1 Het, 3 N); **Lane 5** - 1 in 10 (1 Het, 4 N); **Lane 6** - 1 in 12 (1 Het, 5 N); **Lane 7** - 1 in 14 (1 Het, 6 N); **Lane 8** - 1 in 16 (1 Het, 7 N); **Lane 9** -1 in 18 (1 Het, 8 N); **Lane 10** - 1 in 20 (1 Het, 9 N). **M** = 100 bp molecular weight ladder.
Het = heterozygote; N = normal.

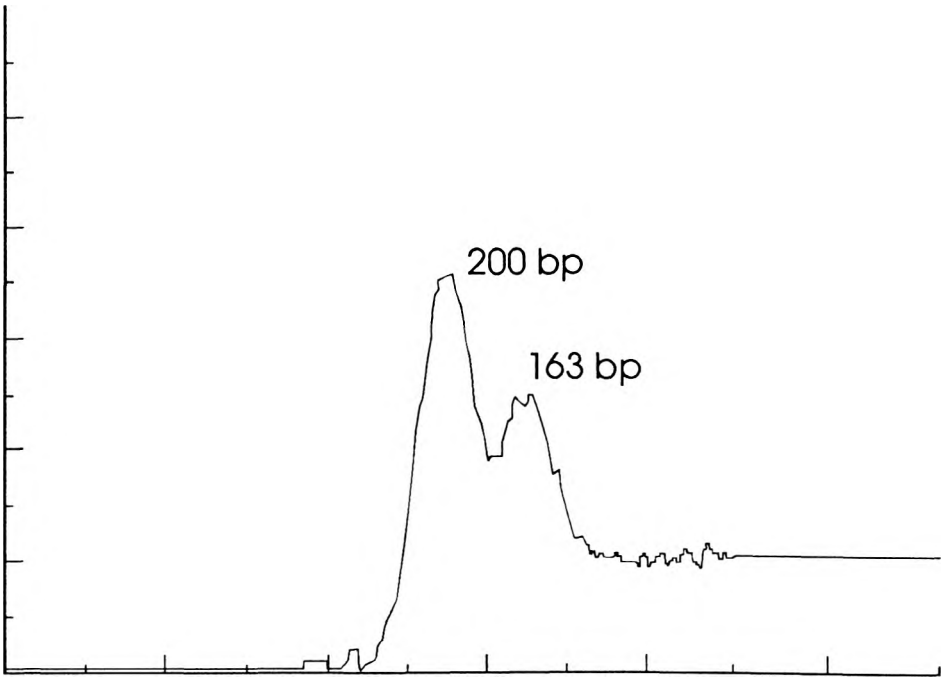

Figure 14 Example of a Densitometry Scan

Example of a densitometry scan performed on a negative image of an ethidium bromide stained 2% agarose gel showing electrophoretically separated *Mnl*1 restricted PCR products using genomic DNA from an individual who is heterozygous for the FV Leiden mutation. The 200 bp and 163 bp peaks corresponding to the bands on the gel are shown. Based on the measured areas under the curve for each peak, the calculated 200:163 ratio is 1.24.

Table 3 Densitometry scanning of the 200 bp and 163 bp *Mnl*1 restriction fragments of PCR products obtained from individual DNA samples from FV Leiden Heterozygotes

	200 bp	163 bp	200 bp:163 bp ratio
1	11.5	9.9	1.16
2	9.1	10.5	0.87
3	11.4	10.3	1.11
4	16.4	13.2	1.24
5	10.2	10.3	0.99
6	35.1	17.1	2.05

Table 4 Densitometry Scanning of the 200 bp and 163 bp *Mnl*1 restriction fragments of PCR products obtained from a series of DNA Pools with diminishing FV1691A allele content

FV1691A allele frequency *	200 bp	163 bp	200 bp:163 bp ratio
1 in 2 (neat Het)	35.1	17.1	2.05
1 in 4 (1 Het, 1N)	34.6	21.9	1.58
1 in 6 (1 Het, 2N)	17.3	24	0.72
1 in 8 (1 Het, 3N)	13.2	17.3	0.76
1 in 10 (1 Het, 4N)	19.1	16.0	1.19
1 in 12 (1 Het, 5N)	14.4	20.6	0.70
1 in 14 (1 Het, 6N)	12.2	17.6	0.69
1 in 16 (1 Het, 7N)	20.1	22.3	0.90
1 in 18 (1 Het, 8N)	14.5	21.0	0.69
1 in 20 (1 Het, 9N)	16.7	31.9	0.52

* Het = FV Leiden heterozygote (FV1691A/FV1691G)
N = normal FV genotype (FV1691G/FV1691G)

4.2.4 Activated Protein C Resistance Functional Assay

Of the original 130 plasma samples, 48 were not included in the analysis. Of these discarded samples 33 were activated (visible fibrin strands), 2 were haemolysed, 9 had a markedly prolonged baseline APTT and 4 could not be found after storage. Thus a total of 82 samples were analysed. The demographic details of this study population are given in Table 5.

Table 5 Blood Donor Study Population

Sex	Number	Age in years		
		mean	SD	range
Male	47	31.6	11.7	16 - 57
Female	24	28.3	12.6	16 - 58
Unknown	11	32.9 *	6.4	22 - 40
Total	82	30.7 †	11.6 †	16 - 58 †

* Age was only known in 7 of the 11 individuals in this category
† n = 78

The results obtained for the APC resistance functional test are shown in Table 6.

Table 6 APC Resistance Functional Assay Results

Sex	n	APC-SR		APTT (seconds) $APC/CaCl_2$		APTT (seconds) $CaCl_2$	
		mean	2 SD	mean	2 SD	mean	2 SD
Males	47	2.24	0.79	98.8	57.1	43.8	16.1
Females	24	2.10	0.65	84.7	34.9	40.5	13.0
Unknown	11	2.07	0.45	87.1	37.2	41.9	14.0
Total	82	2.18	0.72	93.1	50.4	42.6	15.1

According to these results, the lower limit of normal for the APC-SR for this study population is therefore 1.46 (mean - 2SD). The cut-off obtained is 1.45 for both males and females when analysed separately. There was no significant difference between values obtained for males and females.

4.2.4.1 Comparison of Group I (APC-SR ≥2.00) and Group II (APC-SR<2.00)

As 1.46 is well below 2.00, the first described and most commonly quoted point used to discriminate between normal and abnormal, the results were subdivided into two groups, namely those with APC-SRs ≥ 2.00 (group I) and those with APC-SR < 2.00 (group II) for further analysis. There was no difference in the proportion of males (28%) and females (25%) who had an APC-SR of < 2.00. The results are shown in Table 7.

Table 7 Comparison of groups with APC-SR ≥ 2.00 vs APC-SR < 2.00

Group	Sex	n	APC-SR		APTT (seconds) APC/$CaCl_2$		APTT (seconds) $CaCl_2$	
			mean	SD	mean	SD	mean	SD
I	Total	59	2.34	0.28	100.9	25.0	43.0	8.4
APC- SR	Males	31	2.43	0.29	107.6	28.2	44.1	8.9
≥ 2.00	Females	18	2.24	0.24	90.6	15.7	40.7	7.2
	Unknown	7	2.19	0.14	94.5	19.0	43.1	8.4
II	Total	23	1.76	0.14	73.2	11.0	41.5	4.8
APC-SR	Males	13	1.76	0.13	75.9	12.2	42.9	1.5
<2.00	Females	6	1.69	0.12	66.9	7.7	39.7	3.8
	Unknown	4	1.86	0.18	74.0	8.7	39.9	3.6

Statistical analysis by means of the students t-test has shown that the APTT in the presence of APC is highly significantly different between the two groups ($p<0.0000$) whereas the standard APTT in the absence of APC is similar in both groups. This indicates that the APC-SR is determined primarily by the APTT in the presence of APC. Correlation analysis has confirmed this association as the Pearson correlation co-efficient (r value) is 0.74 for APTT with APC compared with 0.16 for the APTT without APC.

The mean age of the group with APC-SR ≥ 2.00 is 29.3 years (SD 10.8, $n = 56$) whilst that of the group with APC-SR< 2.00 is 34.1 years (SD 13.2, $n = 21$). No significant difference in age was detected between the two groups ($p > 0.05$).

Based on the analysis of all APC-SRs ≥ 2.00, the mean value obtained for males was significantly higher than for the females ($p = 0.0323$). However, no sex difference could be demonstrated in the group with APC-SRs < 2.00.

Functional assays for FVIII and FV were performed on a subset of plasma samples ($n = 5$) from each of the two groups. The plasma samples with the 5 lowest and 5 highest APC-SRs that were available were chosen. This was performed prior to the completion of all 130 samples therefore these are not the highest and lowest within the total study

group. This analysis was however not helpful in determining the reason for the low APC-SRs (Table 8). The students t-test could not demonstrate any difference between FV and FVIII levels in these two groups.

Table 8 Analysis of a subset of Groups I and II

	APC-SR	FV (%)	FVIII (%)
Group I	2.61	118.6	143.2
Group II	1.58	90.6	135.0

(mean values are given)

4.2.4.2 Analysis of Plasma Samples with Prolonged Baseline APTT values

Eight of the nine plasma samples that were shown to have a prolonged baseline APTT were investigated further to determine the cause of this unexpected finding in healthy individuals. An APTT correction, FV and FVIII functional assays were performed on each specimen. The results are shown in Table 9.

Table 9 Analysis of Plasma Samples with Prolonged Baseline APTTs

Specimen	Baseline APTT (seconds)	APTT correction (seconds)	FV (%)	FVIII (%)
1	> 200	56.7	0	4
2	> 200	31.2	6	11
3	> 200	29.4	10	28
4	197	46.4	0	6
5	183	47.9	2	5
6	138	47.9	6	5
7	70	42.2	53	33
8	56	39.7	41	75

These findings indicate that the prolonged baseline APTT observed during the APC resistance functional assay were due to loss of FV and FVIII activity. Although the handling specifications of these specimens could not be individually traced, the findings are almost certainly due to "aging" of the plasma due to a delay in plasma separation and freezing.

4.2.4.3 Investigation of the Effect of Temperature and Time Delay prior to Separation of Plasma from Whole Blood on the APC Resistance Functional Assay

Only sixty three percent of plasma samples were suitable for analysis of functional APC resistance. This was considered to be suboptimal. Parameters of specimen handling that could have affected the quality of the plasma were therefore analysed. The effects of temperature (4 °C or room temperature) and time delay prior to plasma separation on the APC resistance functional assay are indicated in Tables 9 and 10. The results shown are the mean values of 10 replicate measurements performed on plasma from a single individual.

Table 10 Effect of Time Delay prior to Plasma Separation on APC-SR when Whole Blood Samples are left at Room Temperature (± 24 °C)

Time	APC-SR			APTT (APC) *			APTT *		
	mean	SD	CV	mean	SD	CV	mean	SD	CV
0	2.65	0.13	4.8	104.4	7.68	7.4	39.5	3.14	8.0
1 hour	2.44	0.30	12.5	97.7	9.88	10.1	40.4	3.52	8.7
2 hours	2.25	0.24	10.7	85.4	4.14	4.8	38.3	2.83	7.4
4 hours	2.47	0.34	13.8	97.8	7.63	7.8	40.0	4.14	10.4
6 hours	2.24	0.21	9.4	84.4	6.93	8.2	37.7	2.30	6.1
12 hours	2.28	0.31	13.6	91.1	8.81	9.7	40.4	4.11	10.2
24 hours	2.67	0.28	10.5	105.4	12.2	11.6	39.5	2.32	5.9

* measured in seconds
CV reported as a percentage value

Table 11 Effect of Time Delay prior to Plasma Separation on APC-SR when Whole Blood Samples are left at 4 °C

Time	APC-SR			APTT (APC) *			APTT *		
	mean	SD	CV	mean	SD	CV	mean	SD	CV
0	2.65	0.13	4.8	104.4	7.68	7.4	39.5	3.14	8.0
1 hour	2.45	0.23	9.4	91.6	8.64	9.4	37.4	1.95	5.2
2 hours	2.55	0.43	16.8	97.4	8.32	8.5	38.8	3.85	9.9
4 hours	2.44	0.43	17.7	93.7	16.40	17.5	38.5	1.81	4.7
6 hours	2.55	0.29	11.3	99.5	11.43	11.5	39.2	3.72	9.5
12 hours	2.97	0.44	14.7	113.0	17.87	15.8	38.0	1.78	4.7
24 hours	2.32	0.15	6.3	86.6	3.32	3.8	37.6	3.02	8.0

* measured in seconds
CV reported as a percentage value

There was a general trend for APC-SRs to be less than those observed at time 0 when whole blood samples were kept at room temperature and plasma separation was delayed (Figure 15). It could however not be demonstrated that the degree of reduction in APC-SR was time-dependent. An interesting finding in this regard was the 24 hour APC-SR which at 2.67 was almost identical to that at baseline (2.65) although the CV was greater (10.5% vs 4.8%). Statistical analysis by means of the students t-test indicated that only the 1 hour ($p = 0.0217$), 2 hour ($p = 0.0156$), 6 hour ($p = 0.0039$) and 12 hour ($p = 0.0125$) plasma samples were significantly less than those noted at baseline. In all cases the reduction was due to shorter APTTs in the presence of APC although only the 6 hour ($p = 0.002$) and 12 hour ($p = 0.0218$) values achieved statistical significance.

For plasma samples that were stored at 4 °C prior to plasma separation the APC-SRs, with the exception of that obtained at 12 hours, were lower than those observed at baseline (Figure 16) with the 1 hour ($p = 0.0469$) and 24 hour ($p = 0.002$) values reaching statistical significance. The 12 hour sample had an APC-SR that was significantly greater than the baseline value ($p = 0.0365$). Again the changes in APC-SR were due to changes in the APTT with APC rather than the APTT without added APC with the 1 hour ($p = 0.0059$), 2 hour ($p = 0.0283$) and 24 hour ($p = 0.002$) values reaching statistical significance. As for the samples stored at room temperature, the degree of reduction in APC-SR could not be demonstrated to be time-dependent in the samples stored at 4 °C.

Comparison of plasma stored at room temperature with plasma stored at 4 °C prior to separation showed that where significant differences did occur, the APC-SRs were generally lower in the room temperature group (2 hours - $p < 0.05$; 6 hours - p , 0.01; 12 hours - $p < 0.005$). Only the 24 hour sample had a significantly greater APC-SR than the 4 °C sample ($p < 0.005$).

The interbatch variation of APC-SR was shown to be 16% compared with 4.8% intrabatch variation (time 0) although the mean values were essentially the same (2.61 vs 2.67).

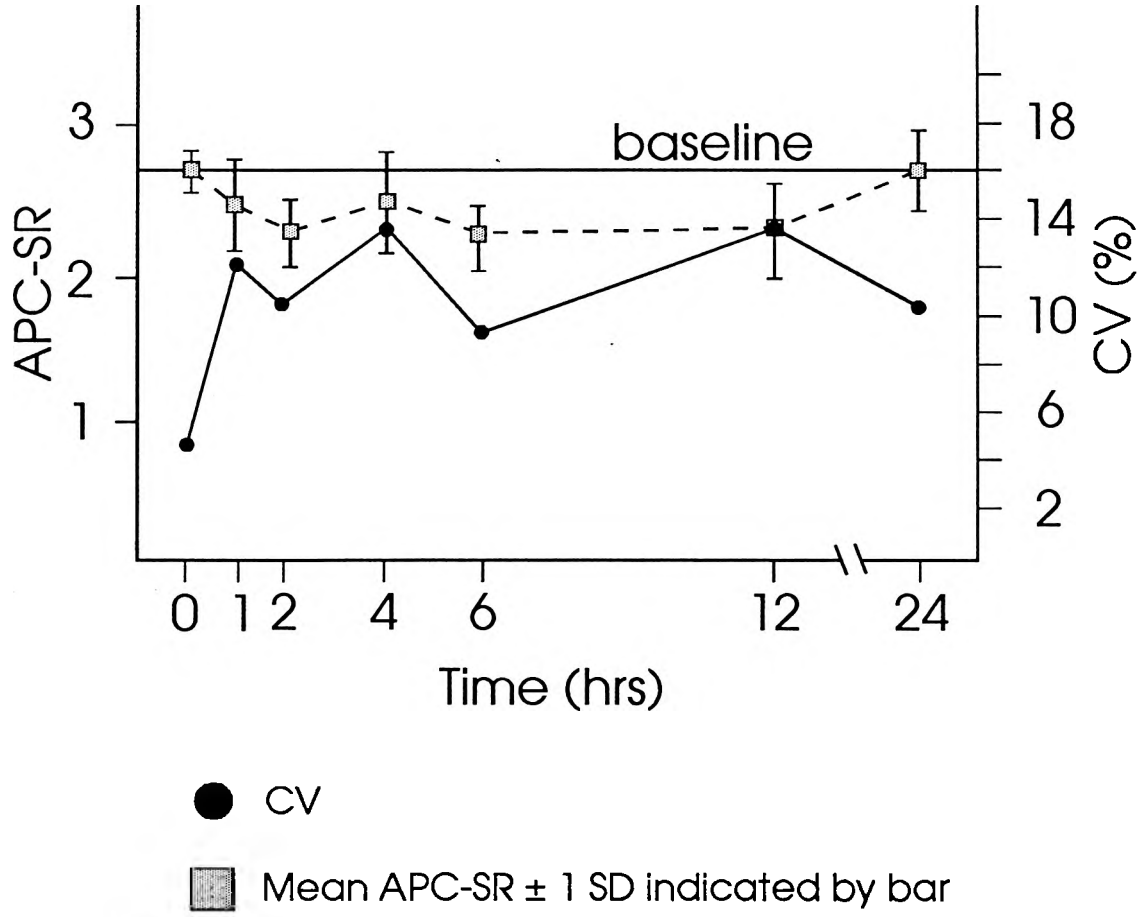

Figure 15 APC-SRs in relation to Storage of Whole Blood at Room Temperature and Time Delay prior to Plasma Separation

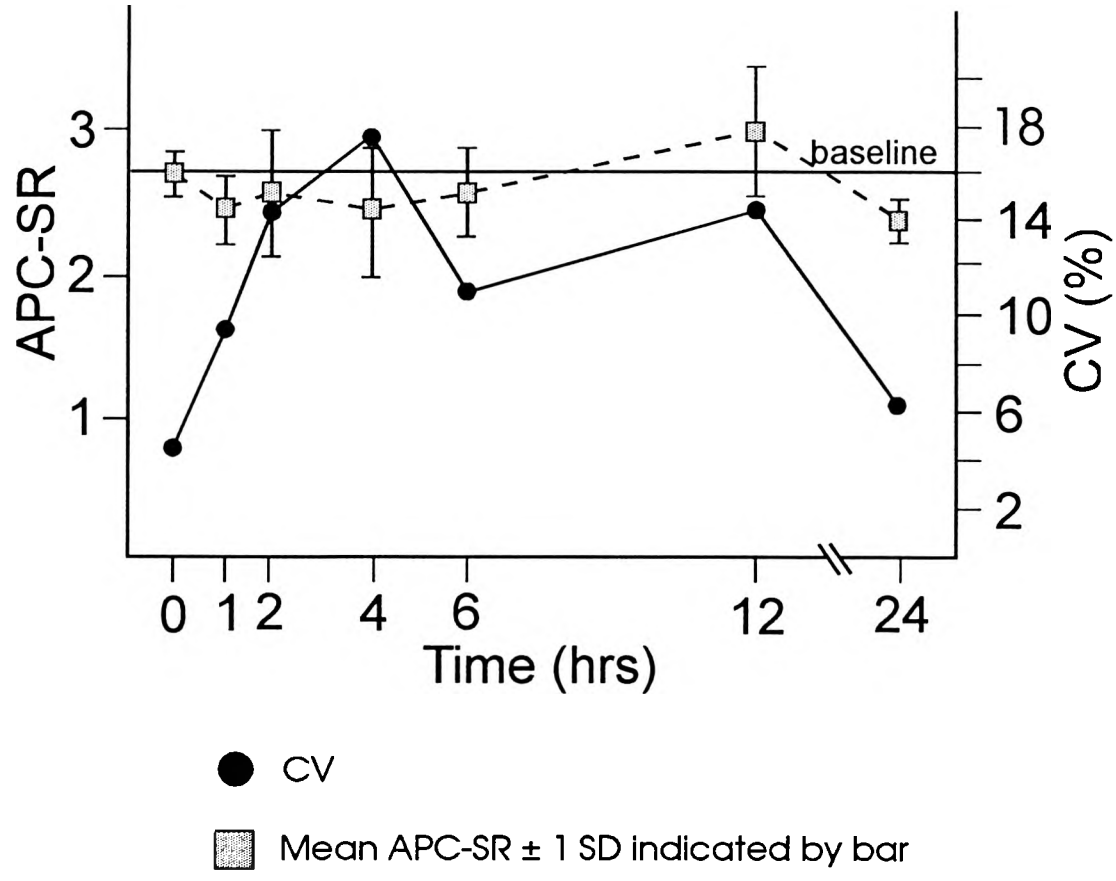

Figure 16 **APC-SRs in relation to Storage of Whole Blood at 4 °C and Time Delay prior to Plasma Separation**

4.3 THROMBOPHILIC PROFILES IN NORMAL PREGNANCIES

4.3.1 STUDY POPULATION

A total of 47 pregnant women were studied; 7 in the first trimester and 20 each in the second and third trimesters of pregnancy. The mean age was 27.8 years (n=36, range 21 - 41 years). Most of the pregnant women were Black (70%).

The control group comprised 25 female SAIMR Laboratory staff members, the majority of whom were Caucasian (23/25), as well as the first 20 Black female blood donors, aged between 16 and 40 years (mean =24.9 years). As the SAIMR-based control subjects were anonymous volunteers, their exact ages are not known but all were within the range of 20 to 40 years inclusive.

4.3.2 ACTIVATED PROTEIN C RESISTANCE

4.3.2.1 Factor V Leiden Mutation Analysis

Deoxyribonucleic acid was only available for 41 of the 47 pregnant patients included in this study. Of these 41, only one, a Caucasian women, was shown to be heterozygous for the FV Leiden mutation while all others had a normal FV genotype. Of all the pregnant subjects, 14 were known to be Caucasian, therefore the prevalence of FV Leiden in this

group is 7.1%. In comparison 2 of the control subjects, both of whom are Caucasian, were positive for the mutation in the heterozygous state. The laboratory control group of whom 23 are Caucasian, therefore has a FV Leiden prevalence rate of 8.7%.

4.3.2.2 Activated Protein C Resistance Functional Assay

The mean APC-SRs for the first, second and third trimester groups were 2.43 (SD 0.88), 1.78 (SD 0.31) and 1.89 (SD 0.29) respectively. In contrast the control group APC-SRs were 2.69 (SD 0.47) for the laboratory staff, 2.25 (SD 0.31) for the blood donors and 2.49 (SD 0.46) for the combined group. The APC-SRs belonging to the 3 FV Leiden carriers were excluded from this analysis as the physiological effects of pregnancy on APC resistance functional testing were being evaluated. It is interesting to note that the APC-SR of the pregnant FV Leiden carrier (1.92) was greater than 1 of the 2 non-pregnant control subject FV Leiden carriers (1.74 and 1.98).

Analysis of variance (ANOVA) showed that there were significant differences between the groups under study ($p = 0.0001$). The second and third trimester group showed significantly more functional APC resistance when compared with the normal controls. Interestingly the control groups, when analysed separately, revealed the predominantly Caucasian laboratory staff group to have significantly higher APC-SRs ($p = 0.0052$) compared with the Black blood donor group. When combined as a single control group, the second and third trimester group APC-SRs remain significantly lower (Table 12). The first trimester group APC-SRs were not significantly different from the control group.

Although the study sample is very small, the results are nevertheless interesting. The very large SD obtained for this group in comparison with the other pregnancy groups suggests that this is not a homogeneous group. The two samples obtained from the Johannesburg Hospital antenatal clinic were both from late first trimester patients and both had low APC-SRs, namely 1.26 and 1.62. In contrast, the five samples obtained from the private sector all had APC-SRs above 2.00 (mean 2.82) and all were from very early pregnancies (< 7 weeks).

4.3.2.3 Protein C

The mean Protein C functional activity values were 88.1% (SD 13.3), 99.6% (SD 16.3) and 100.7% (SD 24.5) for the first, second and third trimester groups respectively. Protein C values obtained for the control groups were 87.2% (SD 14.1) and 90.5% (SD 24.3) for the laboratory staff and blood donors respectively. No significant difference could be detected between the two control groups, the combined mean of whom was 88.6% (SD 19.0).

Although there was a trend for Protein C levels to increase with the progression of pregnancy, ANOVA just failed to reach significance ($p = 0.0541$). The second and third trimester groups did however have significantly elevated Protein C levels when compared with the laboratory control group ($p = 0.0377$ and $p = 0.0240$) and the combined control group ($p = 0.04$ and $p = 0.0242$) but not the blood donor control group (Table 12).

4.3.2.4 Protein S

The mean functional Protein S activity obtained for the first, second and third trimester groups were 72.3% (SD 11.3), 44.6% (SD 8.5) and 42.2% (SD 11.5) respectively. In contrast the control values were 107.9% (SD 12.0) for the laboratory staff and 89.1% (SD 20.6) for the blood donor group. The combined control group mean was 100.0% (SD 18.5).

Analysis of variance of all the groups showed them to be statistically significantly different from each other ($p = 0.0001$). Again a difference was detected between the Protein S values obtained for the two control groups with the laboratory staff having a significantly higher average Protein S activity level. Whether analysed as separate or combined control groups, all three pregnancy groups had significantly lower Protein S levels with $p = 0.0001$ for all except for the first trimester group vs blood donor control group ($p = 0.0062$). The second and third trimester group Protein S levels in turn were significantly lower than the first trimester value ($p = 0.0001$ for both) although no difference was detected between the second and third trimester groups (Table 12).

4.3.2.5 Antithrombin

The mean values for Antithrombin activity for the first, second and third trimester patients were 107.9% (SD 10.7), 99.2% (SD 8.7) and 98.3% (SD 11.5) respectively. The

laboratory staff control group had a mean Antithrombin level of 114.8% (SD 9.0) and that of the blood donor group was 109.5% (SD 19.3). The control groups were not significantly different from each other and when combined had a mean value of 112.4% (SD 14.5).

Analysis of variance indicated that both the second and third trimester groups had significantly lower Antithrombin levels than the control group with p values of 0.0002 and 0.0001 respectively. They were not significantly different from each other. Although the first trimester Antithrombin level was not significantly lower than the control group, it did follow the trend of lower Antithrombin values in pregnancy as established by the results for the second and third trimester groups (Table 12).

Table 12 Thrombophilic Profiles in Normal Pregnancies

		First Trimester	Second Trimester	Third Trimester	Control (Lab)	Control (BD)	Control (All)
APCR (ratio)	**Mean**	2.43	1.78 ↓	1.89 ↓ *	2.69 *	2.25 ⇓	2.49
	SD	0.88	0.31	0.29	0.47	0.31	0.46
	CV (%)	36.3	17.5	15.0	17.5	14.0	18.5
Protein C (%)	**Mean**	88.1	99.6 ↑	100.7 ↑	87.2	90.5	88.6
	SD	13.3	16.3	24.5	14.1	24.3	19.0
	CV (%)	15.0	16.4	24.4	16.1	26.9	21.4
Protein S (%)	**Mean**	72.3 ↓	44.6 ↓	42.2 ↓	107.9	89.1 ⇓	100.0
	SD	11.3	8.5	11.5	12.0	20.6	18.5
	CV (%)	15.7	18.9	27.3	11.1	23.1	18.5
Anti-thrombin (%)	**Mean**	107.9	99.2 ↓	98.3 ↓	114.8	109.5	112.4
	SD	10.7	8.7	11.5	9.0	19.3	14.5
	CV (%)	9.9	8.8	11.7	7.9	17.6	12.9

* FV Leiden positive individuals excluded from analysis
Lab = Laboratory staff; BD = Blood donors; All = combined control group
↓ = significantly less than combined control group
⇓ = BD control significantly less than Lab control
↑ = significantly greater than combined controls

4.3.2.6 "Normal Values"

By convention, normal reference ranges for many laboratory tests are established by taking the mean ± 2SD of a normal population sample. If this conventional rule were to be applied using the control group data the reference ranges obtained would be different to the current normal values in use in the SAIMR Coagulation Laboratory at the Johannesburg Hospital (Table 13).

Table 13 Normal Reference Ranges

Test	"New Normal Reference Range"			Established normal values
	Lab Staff	Blood Donors	Combined	
APC-SR	≥ 1.75	≥ 1.63	≥ 1.57	≥ 2.00
PC (%)	59 - 115	42 - 139	51 - 127	70 - 160
PS (%)	84 - 132	48 - 131	63 - 137	60 - 140
AT (%)	97 - 133	71 - 148	83 - 141	87 - 125

When comparing the results obtained for the pregnant women with the "new reference range", Protein S clearly is the most dramatically altered parameter with 89% of observations falling below 63% (lower cut-off limit). Twenty one percent of the APC-SR measurements and 11% of the Antithrombin measurements are below the control values whilst 4% of Protein C levels are elevated (Figure 17). Pregnancy-specific reference ranges obtained from these data are given in Table 14.

Table 14 Normal Pregnancy-specific "Reference Ranges"

Test	Range
Protein C	58 - 138 %
Protein S	10 - 81 %
Antithrombin	79 - 121 %
APC-SR	≥ 1.10

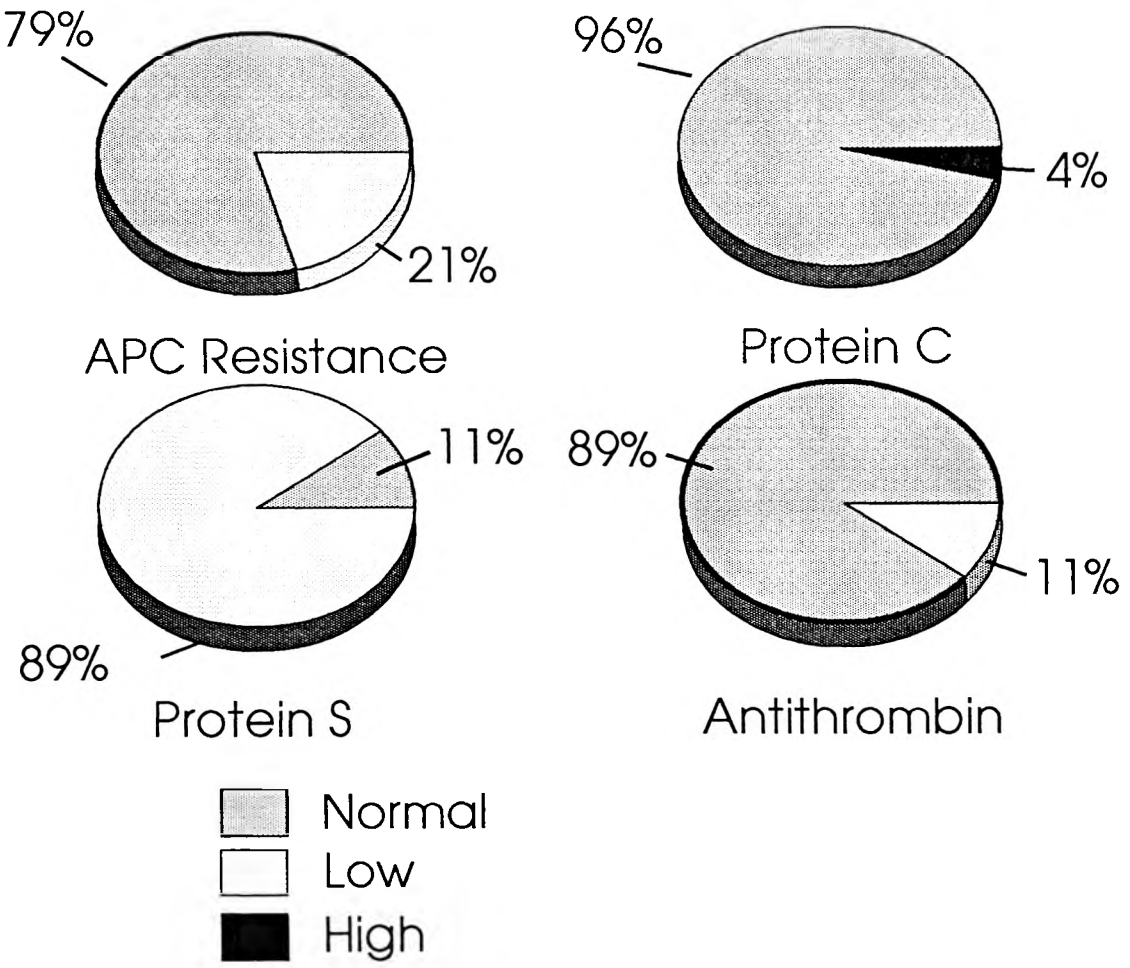

Figure 17 **Results of Functional Assays for Protein C, Protein S, Antithrombin and Activated Protein C Resistance in Pregnancy compared with Normal Controls**

5 DISCUSSION

5.1 FACTOR V LEIDEN MUTATION DETECTION

The prevalence of the FV Leiden mutation associated with activated APC resistance and its importance as a genetic risk factor for venous thrombosis have necessitated the establishment of an accurate assay for its detection.

Polymerase chain reaction amplification of part of the FV gene spanning nucleotide 1691 followed by *Mnl*1 restriction endonuclease digestion has been shown to be a robust and reliable technique for FV Leiden genotyping. The technique has a number of inbuilt controls which is what makes it extremely reliable for routine diagnostic purposes. Firstly, in contrast to the use of PCR for the detection of clonal immunoglobulin gene rearrangements, failure of amplification always implies a technical problem. DNA from each and every individual should yield a 267 bp fragment using the original primers as described by Bertina *et al* (1994). Secondly, the 267 bp PCR product from all individuals should restrict in at least one site to yield the 67 bp fragment. Failure to do so implies a failed restriction irrespective of the individual's genotype with regards to the FV Leiden mutation. Thirdly, clear distinction between normal (163 bp, 67 bp, 37 bp), heterozygotes (200 bp, 163 bp, 67 bp, 37 bp) and homozygotes (200 bp, 67 bp) is possible. Whilst all bands can be seen, genotyping essentially relies on identification of the 200 bp and

163 bp bands only (see Figure 8). This technique has now been established as a routine test in our laboratory with approximately 60 samples being processed each month.

As the *Mnl*1 enzyme is expensive (± R2200 / 250 units) Rabès *et al* (1995) devised a primer which by means of a mismatched nucleotide (T rather than the native G) at the 3' penultimate position would create a *Taq*1 endonuclease (low-priced) cleavage site in the normal but not the abnormal allele. Distinction between normal and abnormal would still rely on the detection of different restriction fragment length patterns. Although this method is quicker and cheaper, a disadvantage is the potential problem of failed digestion which would result in the incorrect diagnosis of a FV Leiden homozygote.

There is another potential shortcoming in the use of restriction fragment length polymorphism analysis for the assignment of FV genotype. A substitution in any one of the 4 basepairs that constitute the *Mnl*1 recognition site (GAGG) would prevent restriction and consequently give the same result as for the FV Leiden mutation. A silent mutation in the CGA codon for arginine (CGC or CGG) would prevent restriction, but at a protein level the APC cleavage site is present and the FV molecule does not exhibit APC resistance. Liebman *et al* (1996) report the finding of a false positive diagnosis of heterozygosity for FV Leiden using PCR and *Mnl*1 digestion. This patient had inflammatory bowel disease with no personal or family history of thrombosis and a normal coagulation assay for APC resistance but appeared to be a heterozygote for FV Leiden by PCR analysis. Sequencing of the *Mnl*1 restriction site of his aberrant FV gene revealed a silent A to C transition at position 1692.

Although screening for FV Leiden using PCR-based restriction endonuclease assays may overestimate its incidence, the findings of Cumming *et al* (1997) suggest that the silent base change at position 1692 does not appear to be common.

A technique that avoids the post amplification restriction step is the use of sequence specific primers for PCR amplification (PCR-SSP), also known as Amplification Refractory Mutation System (ARMS). For this technique a sense primer complementary to both FV alleles (1691A and 1691G) is coupled with either of two antisense allele specific primers, one complementary to the FV Leiden allele and one to the normal FV allele. The FV genotype is assigned based on whether or not each allele specific primer set produces an amplified product. Assignment of genotype using PCR-SSP has been shown to correlate 100% with those determined by PCR amplification followed by *Mnl*1 digestion (Kirschbaum and Foster, 1995).

The PCR-SSP technique for FV Leiden detection described by Kirschbaum and Foster (1995) requires two PCR reaction tubes per patient analysed. Failed amplification, unrelated to primer complementarity, could potentially occur in one of the two reaction tubes (one for each allele specific primer) resulting in false genotype interpretation.

The technique described by Radtke and Scharrer (1996) overcomes both the shortcomings of the two tube reaction and the reliance on restriction endonuclease digestion. This involves the generation of a PCR product using two allele specific 3' primers labelled with fluorescent markers, FITC (normal allele - 1691G) and rhodamine

(mutant allele - 1691A) respectively. The 5' primer, common to both alleles, is biotinylated. Polymerase chain reaction products from the normal allele are therefore FITC-labelled whereas those from the mutant allele are rhodamine-labelled. All products are biotinylated. For the detection of the FV genotype, PCR products are transferred to a streptavidin coated microtiter plate and incubated for 15 minutes. The plates are then washed and analysed in a fluorescent plate reader. This assay appears to be fast and simple and has the advantage of processing large numbers of samples at a time.

Other techniques that have been utilized for the detection of the FV Leiden mutation include the use of standard PCR followed by single strand conformation polymorphism (SSCP) (Larsen *et al*, 1997; Kouidou *et al*, 1997) as well as the ligase chain reaction (Kvasov *et al*, 1997).

Irrespective of the specific technique used, FV genotype analysis for the detection of the FV Leiden mutation has become an integral part of the laboratory investigation of patients presenting with thromboembolic disease. Since the PCR-based assay was included in thrombotic screens at the Johannesburg Hospital, the yield in terms of detecting underlying prothrombotic conditions has improved dramatically (data not shown). It is hoped that with the planned addition of similar assays for the detection of the recently described Prothrombin 20210A point mutation (Poort *et al*, 1996) and the thermolabile variant of the Methylenetetrahydrofolate Reductase enzyme (Frosst *et al*, 1995) that screening of thrombotic patients will become even more informative.

5.2 LOCAL PREVALENCE OF THE FACTOR V LEIDEN DEFECT

Polymerase chain reaction analysis did not reveal a single FV Leiden positive individual amongst the 130 Black blood donors studied. This would support the previous findings of Helley *et al* (1996) and Rees *et al* (1995). It is theoretically possible that the use of a healthy blood donor group would have excluded individuals with thrombosis and may therefore be biased against the detection of the FV Leiden mutation. However, in view of the highly variable thrombotic tendency, one would expect a proportion of assymptomatic heterozygotes (and homozygotes) to be present in this donor group (Zöller *et al*, 1994; Greengard *et al*, 1994). These findings would suggest that the FV Leiden mutation is absent in Sub-Saharan Africans. In support of the absence of the FV Leiden mutation in South African Blacks is the lack of detection of FV Leiden positivity in any of the approximately 200 symptomatic Black patients screened to date at the Johannesburg Hospital Coagulation Laboratory (data not shown). As no studies in North Africans have been reported it is not possible to conclude that the FV Leiden mutation would be absent in all Africans as North Africans are generally genetically distinct from the Sub-Saharan Africans (Rees, 1996).

In contrast to the Black population, the FV Leiden defect is highly prevalent in people of European descent. Although the apparent prevalence within Europe varies widely from country to country, with a peak carrier frequency of 15% found in Greeks (Rees *et al*, 1995), the average FV Leiden allele frequency is 2.7% (Rees, 1996). The finding of 8.7% (2/23) FV Leiden heterozygotes amongst the Caucasian female laboratory staff indicates

that this mutation is common locally. Although the group is small, there is no reason to suspect that the sample was biased in anyway. Individuals from the laboratory who provided blood samples for the pregnancy trial control group were all healthy and had no history of previous thromboses. As participation was anonymous it was not possible to identify the individuals concerned to establish whether or not they had a positive family history of thrombosis. The absence of males from this study should not alter the observed FV Leiden prevalence because the FV Leiden mutation is inherited in an autosomal dominant fashion.

A recent study performed in the Free State area of South Africa indicated that APC resistance was detected in only 5% of the 104 thrombophilic patients investigated (Alexander, 1997). This figure is lower than that of the Caucasian control subjects in this study and much lower than figures of 21% to 64% published in the literature (Koster *et al*, 1993; Griffin *et al*, 1993; Voorberg *et al*, 1994; Svensson and Dahlbäck, 1994). A possible explanation for this discrepancy which would likewise support the findings of this study, would be that the study group was comprised largely of Black patients although this data was not supplied.

5.3 ACTIVATED PROTEIN C RESISTANCE FUNCTIONAL ASSAY

5.3.1 PRE-ANALYTICAL INFLUENCES

The fact that only 63% of the 130 collected plasma samples from the blood donor group could be utilized for the determination of APC-SRs was considered to be suboptimal. This illustrates the critical importance of correct specimen handling. These blood specimens were all collected from mobile blood donor clinics and only brought back to the Baragwanath Hospital blood bank in the late afternoon. From there they were taken to the Baragwanath Hospital Haematology Laboratory where the blood specimens were cenrifuged, the plasma separated and stored at -20 °C. As a result, at least some of the specimens may have been left for up to 8 hours prior to separation. In view of this, a controlled experiment was performed to determine the effect of temperature and time delay prior to plasma separation from whole blood. No specimens kept at either room temperature or 4 °C up to 24 hours prior to plasma separation resulted in a prolonged baseline APTT, were activated or haemolysed. These findings are supported by de Ronde and Bertina (1994) who showed that samples stored as whole blood for 24 hours prior to plasma separation had the same APC-SRs as those of samples processed immediately. Therefore, although this data was not provided in their study, the baseline APTT s could not have been markedly prolonged. Likewise Luddington *et al* (1996) showed that storage of whole blood at either room temperature or at 4 °C had no significant effect on the APC-SR. However, following 24 hour storage a rapid fall in APC-SR was observed due to a shortening of the APTT in the presence of APC. Delay in plasma separation, for

samples processed the same day as was prescribed in the instructions given to the blood bank staff, was therefore not considered to be a satisfactory explanation.

The plasma samples with the prolonged baseline APTTs were not all from a single donor clinic therefore this finding cannot be attributed to an entire batch of specimens being erroneously processed a few days after collection. These samples were all shown to have low FV and FVIII levels in proportion with the degree of APTT prolongation. Combined FV and FVIII deficiency is relatively rare (Rizza, 1994) and these were otherwise healthy blood donors, therefore this finding must be due to incorrect specimen handling. Factor V and FVIII are fairly labile factors with activity diminishing as plasma is "aged" therefore it is highly probable that there was a substantial delay in processing although the handling of individual samples could not be traced.

A satisfactory reason for the large number (25.4%) of activated specimens could not be obtained. A potential cause could be traumatic venepuncture. Whilst this was considered it was thought to be unlikely for a number of reasons: Firstly, blood transfusion staff are highly skilled in phlebotomy; secondly, a wide bore needle was used and thirdly, the samples were obtained after the unit of blood had been collected using the same tubing without repeat venepuncture. What must be considered though is the potential influence of time delay between clamping, tying and cutting the collection tubing prior to obtaining the blood sample. Although this was not reported by the blood transfusion services staff, it is nevertheless possible that some difficulties may have been experienced as this procedure is not part of their standard operating practices. It is also not known to what

extent prolonged pressure from the cuff applied to the upper arm of the donor, which in some instances may be up to 15 to 20 minutes, may be contributing to sample activation. A separate study would be required to explore both these variables. A further potential problem is that specimen tubes may have become contaminated, and hence activated, at the time of cap removal and recapping as the blood was collected directly from the tubing into the specimen tube.

Whilst direct venepuncture specific for the collection of blood samples for this study using the vacutainer system would have circumvented many of these problems, the management of the South African Blood Transfusion Services understandably did not wish to inconvenience their donors in any way as they are heavily reliant on their continued support.

Centrifugation of the blood donor specimens and initial storage took place at the Baragawanath Hospital SAIMR Haematology Laboratory. These specimens were collected weekly. They were transported on dry-ice in a cooler box, the transit time from freezer to freezer being less than one hour. No visible thawing appeared to have occurred during this time. Even if it did, this should not have influenced the results to any great extent (de Ronde and Bertina, 1994).

5.3.2 APC-SRs

The mean APC-SR of 2.18 obtained for the Black blood donor group is lower than other reported normal references (Table 15) utilizing the unmodified APTT-based assay (COATEST®, Chromogenix).

Table 15 Examples of Normal Values for APC-SR quoted in the literature using the Unmodified COATEST® from Chromogenix

subjects	Mean APC-SR	SD	Normal Value	Country	Reference
30	2.66	0.30	≥2.15	Canada	Jobin *et al*, 1995
43	2.38	0.14	≥2.11	Italy	De Stefano *et al*, 1995
21	2.92	0.43	≥2.06	England (F)	Cumming *et al*, 1995
49	3.10	0.60	≥1.90	Netherlands	Roelse *et al*, 1996
53	2.75	0.34	≥2.08	Spain	Aznar *et al*, 1995
40	3.28	0.44	≥2.40	Argentina	Varela *et al*, 1997
40	3.48	0.65	≥2.18	England	Luddington *et al*, 1996
85	2.85	0.55	≥2.30	Netherlands	Henkens *et al*, 1995a
60	N/A	N/A	≥1.90	Finland (M)	Hakala *et al*, 1995
60	N/A	N/A	≥1.80	Finland (F)	Hakala *et al*, 1995

F = Female subjects only
M = Male subjects only
N/A = not available

Using the conventional method of determining the cut-off value (mean -2SD) used to discriminate between normal and abnormal, an abnormal APC-SR as determined from the Black blood donor group would be < 1.46. This is much lower than would be expected from the literature (Table 15). Values obtained for homozygous FV Leiden positive individuals by others using the same commercial kit (COATEST®) would not have been detected by this cut-off (Stirling *et al*, 1995).

The overall plasma sample quality was poor with only 63% of samples generating an APC-SR. All factors with a possible impact on lowering the APC-SR were thus considered. In support of this Van Acker *et al* (1997) observed that markedly lower APC-SRs were obtained in the absence of FV Leiden for plasma samples received for analysis from outside their hospital. This resulted in poor sensitivity of the functional test for predicting FV Leiden status. They postulate that these differences were due to pre-analytical handling, in particular centrifugation and problems with plasma stability.

Whilst centrifugation was performed in accordance with recommendations for sample preparation in the COATEST® package insert, it cannot be ascertained whether physical plasma separation by means of pipetting was carried out correctly on all occasions. If too much plasma was removed, platelet contamination may have occurred.

Lower sensitivity ratios have been noted to occur in freeze-thawed plasma samples by some (Girolami *et al*, 1994; Trossaërt *et al*, 1995) but not by others (de Ronde and Bertina, 1994). This phenomenon has been attributed to platelet contamination, the

platelets being disrupted upon freeze-thawing (Luddington *et al*, 1996). Luddington *et al* (1996) propose that the most likely explanation is exposure of anionic-phospholipid which would normally only occur after platelet activation at sites of endothelial damage. The rate of inactivation of FVa is accelerated on anionic membranes (Bakker *et al*, 1992) but the nett effect is an overall enhancement of thrombin generation (Lee and Baglin, 1995). The results of Luddington *et al* (1996) indicate that it is shortening of the APTT in the presence of APC that is responsible for lower APC-SRs in platelet contaminated plasma samples. This effect could be abrogated by filtration of freeze-thawed plasma through a 0.2 μm syringe filter prior to analysis to remove platelet particles or by filtering fresh plasma to remove intact platelets. They also showed that storage of whole blood at both room temperature and 4 °C for longer than 24 hours, resulted in a rapid fall in APC-SRs despite double centrifugation for plasma preparation. Again, the lower APC-SRs were due to shortening of the APTT in the presence of APC. This suggests that platelets become disrupted upon prolonged storage as whole blood with the particles being inaccessible to removal by centrifugation.

Although APC-SRs may be lower for freeze-thawed plasma samples compared with fresh plasma, as long as the control group from whom the normal range is determined is treated in the same way, this should not pose a problem. de Ronde and Bertina (1994) showed that up to 10 freeze-thawing cycles had a negligible effect on APC-SR (↑4%) in comparison with a single cycle. Whilst these results cannot be compared with those of Luddington *et al* (1996) who looked at fresh vs freeze-thawed plasmas, their findings are nevertheless interesting. With increasing numbers of freeze-thawing cycles, de Ronde and

Bertina (1994) showed an increase (19%) in the APTT in the presence of APC rather than a decrease. Likewise, the baseline APTT became longer (15%) with a nett effect of minimal change to the APC-SR, albeit an increase.

Analysis of these results indicates that whilst more than one cycle of freeze-thawing may not have a significant effect on plasma quality, valid conclusions cannot be drawn when comparing fresh with frozen plasma samples. As all the plasma samples in this study were stored at -70 °C prior to analysis, this is not an explanation for the low APC-SRs obtained for the blood donor group.

The results of the investigations looking at the effect of temperature and time delay prior to separation of plasma from whole blood on the APC resistance functional assay were not conclusive in ascertaining possible reasons for the low APC-SRs in the blood donor group. For the specimens kept at room temperature, although there was a trend for the mean APC-SR to decrease and the CV to increase, the 24 hour sample mean was essentially unchanged from the baseline value, although the SD was double (0.28 vs 0.13). This is similar to the findings of others (de Ronde and Bertina, 1994) indicating that results for 24 hour old specimens are less precise. The SD of 0.37 obtained for the blood donor group is however well within the range of other published reports as shown in Table 15 (range 0.135 - 0.65). The results obtained for the samples kept at 4 °C generally showed larger SDs (mean SD 0.33 vs 0.28) than those kept at room temperature. The refrigerated specimens also had larger CVs (mean CV 12.7% vs 11.75%) although this comparison did not reach statistical significance. From these data

it cannot be concluded what the recommended maximum time delay should be from venepuncture to plasma separation. Ideally, samples should be processed immediately but this is not always feasible. In the absence of more conclusive data it would appear reasonable to recommend that if a time delay is unavoidable, the blood should be kept at room temperature for not more than 24 hours in accordance with the findings of others (de Ronde and Bertina, 1994; Luddington *et al*, 1996).

As the evaluable blood donor group comprised predominantly males with a male to female ratio of about 2:1, and the sex-specific mean APC-SRs were not significantly different, the lower APC-SRs are not due to oral contraceptive use which has been associated with acquired APC resistance (Wagner *et al*, 1995; Henkens *et al*, 1995; Olivieri *et al*, 1995).

Bearing in mind all the potential extrinsic influences on the blood donor group APC-SRs, the possibility of genuinely lower values than previously reported would nevertheless need to be considered. None of the studies referred to in Table 15 state the ethnic origin of the control group used to define normality. In addition, most were conducted in Western Europe, with one Canadian and one Argentinean study. Although some of these control groups may have included non-Caucasian individuals, there appears to have been no previous report referring to APC-SRs exclusively for healthy Black individuals. This is an important issue which is worthy of re-evaluation under conditions of strict control over all potential confounding pre-analytical variables.

This finding of lower APC-SRs in Blacks is extremely interesting in view of the apparent absence of the FV Leiden mutation in this population group. These findings emphasize the need for laboratory reference ranges to reflect the patient population they serve, and where differences are significant, requires the establishment of race-specific reference ranges to prevent inappropriate interpretation of seemingly "abnormal" results.

The assessment of APC resistance in this study was performed using the unmodified APTT-based assay (COATEST®) as this is in routine use in the Coagulation Laboratory. Since it was first described it has become clear that a number of variables influence the result of the APTT-based test. These include choice of reagents, instrument and pre-analytical conditions all of which may be dependent on other factors (Bertina, 1997). In addition, APTT reagent choice might influence results due to particular sensitivity to platelet contamination (Sidelmann *et al*, 1995), elevated FVIII levels (Henkens *et al*, 1995b), lupus anticoagulants (Villa *et al*, 1995; Martorell *et al*, 1995) or oral contraceptive use (Henkens *et al*, 1995a). A further severe limitation of the original assay is that it cannot be used to test patients receiving anticoagulant therapy, either heparin or warfarin (Bertina, 1997).

These limitations resulted in modification of the original assay by predilution of sample plasma in FV-deficient plasma (Jorquera *et al*, 1994). The effect of this is two-fold: firstly, this will normalize other factors that might affect the APC-SR, in particular FVIII and Protein S levels and secondly, if the dilution factor is sufficient, this may prevent interference by inhibitors such as heparin or lupus anticoagulants (Bertina, 1997). This

modified test has now been fairly extensively evaluated and appears to be highly sensitive and specific as a predictor of an underlying FV Leiden mutation (Borg *et al*, 1997; Vu *et al*, 1997; Burstein *et al*, 1997).

Whilst other non-APTT based APC resistance tests have been developed (Table 16), they have not yet been extensively evaluated. Preliminary studies however, show that the Russel Viper Venom Time-based assay, using plasma prediluted with FV-deficient plasma was superior to the APTT-based method in the separation of normals from FV Leiden carriers (Aboud and Ma, 1997). In their study, when plasma was prediluted with FV-deficient plasma, both assays had 100% sensitivity and specificity.

Table 16 Examples of Non-APTT-based APC Resistance Tests

Test	Reference
Chromogenic APC response assay (factor Xa based)	Váradi *et al*, 1995
Tissue-factor dependent FV assay (PT-based)	Le *et al*, 1995
Factor Xa clotting time	Tripodi *et al*, 1997
Russel Viper Venom Time	Tripodi et al, 1997 Ibrahim *et al*, 1997
Textarin Time Assay	Hoagland *et al*, 1996

As DNA-based tests are relatively easy and relatively inexpensive to perform, it may be questioned as to why functional tests are still used. Continuation of the use of functional APC resistance tests can however be justified. Not all laboratories are equipped for DNA-based diagnosis. A functional test that is easy to perform and has close to 100% sensitivity such as the modified APTT-based APC resistance assay (Johnston *et al*, 1997; Vu *et al*, 1997) would therefore be desirable. This would facilitate selection of individuals with low APC-SRs for confirmation of the FV Leiden mutation by DNA-based techniques. The 100% sensitivity would ensure that no cases would be missed. Confirmation of FV Leiden status by genotypic analysis would permit identification of those individuals with apparent inherited thrombophilia with the phenotype of APC resistance but absence of the FV Leiden mutation (Zöller *et al*, 1994).

As decisions regarding long-term oral anticoagulation may be based on these tests, irrespective of vastly improved sensitivities and specificities with modified APC resistance assays, the functional assays should continue to be regarded as a screening test.

5.4 Acquired Activated Protein C Resistance

An emerging concept in the literature is that of acquired APC resistance. Besides identifiable variables that interfere with the APC resistance APTT-based assays, such as elevated FVIII levels (Mathonnet *et al*, 1996b) and the presence of a lupus anticoagulant (Aznar *et al*, 1997), other conditions have been associated with phenotypic APC

resistance. A study of patients with advanced gastrointestinal cancer revealed 18% to have functional APC resistance (unmodified APTT assay) of which only 1/25 carried the FV Leiden defect (De Lucia *et al*, 1997). Of the patients with a low APC-SR, 84% had suffered a DVT within the previous two years compared with 34% of those with a normal APC-SR. Further investigations of these patients indicated that acquired APC resistance phenotype is strongly associated with increased thrombin generation. These findings suggest that in this group of patients acquired APC resistance may be associated with increased thrombotic risk. A similar study by Green *et al* (1997), although the type of cancer was not specified, confirmed the development of APC resistance in association with advanced malignancies (13%) but could not conclude that this phenotype was predictive of thrombosis.

A well documented cause of acquired APC resistance is oral contraceptive usage (Henkens *et al*, 1995a; Olivieri *et al*, 1995). Oral contraceptives have a multitude of effects on haemostasis variables (Kluft and Lansink, 1997). The combination of these altered parameters results in phenotypic APC resistance in the absence of the FV Leiden mutation, whereas predilution with FV-deficient plasma abrogates this effect (Toulon *et al*, 1996). As different hormones have different effects, it is not surprising that different sensitivities to APC were observed in women using second and third generation oral contraceptives (Rosing *et al*, 1997). They showed that women who used third generation oral contraceptives were significantly less sensitive than those who used second generation oral contraceptives, the ratios falling in the range as that observed for FV Leiden heterozygotes. A combination of oral contraceptive use and heterozygosity for

FV Leiden revealed APC-SRs that would normally predict homozygosity. This finding of APC resistance may well explain the increased risk of venous thrombosis in oral contraceptive users, especially in women using third generation oral contraceptives (WHO Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception, 1995).

It remains to be proven whether or not phenotypic APC resistance as determined by the unmodified assay is in itself a risk factor for thombosis (Bertina, 1997). Until further light is shed on this issue, the unmodified test is unlikely to be widely maintained as a baseline screening test for patients presenting with thrombosis, particularly in laboratories that do not perform FV genotypic analysis on all samples. With this changeover, sensitivity and specificity of screening for the FV Leiden mutation may be enhanced, but at the risk of not detecting other causes of APC resistance.

Whilst changing from the unmodified to the modified APTT assay for APC resistance was considered, it was decided not to introduce the modified test locally. The reasons for this are twofold. Firstly, FV genotyping by PCR amplification followed by *Mnl*1 restriction is well established and performed routinely on all patients being investigated for thrombosis at the Johannesburg Hospital SAIMR Coagulation Laboratory. This test is robust, easy to perform and relatively inexpensive. It also serves to collect DNA on all thrombotic patients for storage in a DNA bank. This may be extremely valuable for future studies, particularly in the light of future discoveries of new prothrombotic genetic defects. The second reason for maintaining the original APC resistance assay is for the

detection of cases of acquired APC resistance which would be missed by the modified test. Elevated FVIII levels have been shown to be an independent risk factor for thrombosis by the Leiden Thrombophilia Study (Felix *et al*, 1997). Detection of functional APC resistance due to the influence of elevated FVIII levels on the assay may therefore be potentially useful. Accurate documentation and follow-up of all cases of acquired APC resistance could contribute towards clarifying whether or not this, in its own right, should be regarded as a risk factor for thrombosis. In this regard it would be of particular importance to verify whether true differences exist between Blacks and Whites as suggested by this study. In order to do so, sex and race-specific control samples will need to be rigorously evaluated and new reference ranges established as necessary.

In view of the apparent lack of the FV Leiden mutation in the local Black population, the policy of performing FV genotyping on all patients with thromboses may need to be revised. Costs saved by not performing PCR on these samples should ideally be invested in introducing other diagnostic tests that could provide further insight into the aetiology of thrombosis in the local community.

5.5 THROMBOPHILIC PROFILES IN NORMAL PREGNANCIES

Pregnancy is a commonly cited risk factor for venous thromboembolic disease in healthy normal individuals as well as those with underlying genetic prothrombotic risk factors (Bokarewa *et al*, 1996; Bauer, 1995). The risk of venous thromboembolic disease in

pregnancy has been reported to be 5 times higher than in non-pregnant females of similar age (National Institutes of Health Consensus Development Conference, 1986). In a referral teaching hospital one would therefore expect to encounter cases of pregnancy-associated venous thromboembolic disease from time to time. Once a thrombotic event has been objectively diagnosed, investigation for a possible underlying predisposition to thrombosis should be undertaken. In the Johannesburg Hospital Coagulation Laboratory investigation of thrombotic patients includes screening for APC resistance (both functional and genotypic analysis), lupus anticoagulant, anticardiolipin antibodies, Protein C, Protein S and Antithrombin functional deficiencies.

As normal pregnancy is associated with major changes in coagulation and fibrinolytic variables (Greer, 1994), it was decided to document the magnitude of these changes in those variables included in the thrombotic screen performed in our laboratory and to establish normal pregnancy-specific reference values. This was considered important as blood specimens for thrombotic screens frequently arrive in the laboratory with no accompanying clinical history. Furthermore, a particular concern is that clinicians who may be unaware of the pregnancy-associated coagulation changes may inadvertently interpret physiological changes as pathological conditions. This study was therefore undertaken to establish such reference values and to issue these with each report.

The results of this study support the previously documented findings that pregnancy is associated with acquired APC resistance (Cumming *et al*, 1995). Although the first trimester study sample was too small for meaningful comparison, the findings

nevertheless showed an interesting trend suggesting that the APC resistance phenotype becomes established after 7 weeks gestation. The second and third trimester group mean APC-SRs were significantly lower than both control groups. Furthermore, the single FV Leiden heterozygote detected in the pregnancy study group had an APC-SR (1.92) that was greater than the group mean (1.89). As reduced sensitivity to APC is a physiological consequence of pregnancy, the unmodified APC resistance APTT-based assay can clearly not be used as a screening test for the detection of FV Leiden in pregnancy.

Predilution of test plasma with FV-deficient plasma abolishes the pregnancy-associated effect on the APC-SR (Cumming *et al*, 1996). This modification should enable good discrimination for the detection of the FV Leiden mutation in pregnant women being investigated for thrombosis. Whether or not the degree of acquired resistance to APC is itself predictive of thrombotic risk in the absence of FV Leiden remains to be determined.

The finding of very low functional Protein S levels, particularly in the second and third trimester, is in accordance with several previous reports (Lao *et al*, 1989; Fernández *et al*, 1989; Comp *et al*, 1986; Malm *et al*, 1988; Gilabert *et al*, 1988). The reduction in Protein S activity in pregnancy falls within the range of heterozygous Protein S deficiency. In view of this, it is particularly important to inform clinicians that these changes are physiological so that genetic Protein S deficiency is not inadvertently diagnosed as this could result in inappropriate lifelong anticoagulation.

The pregnancy-associated change in Protein C level is far less pronounced than that for Protein S. There was nevertheless a definite trend for levels to increase with the progression of pregnancy, with the second and third trimester group values being significantly elevated when compared with the combined control group but not the Black control group. The pregnancy group was comprised predominantly of Black patients whilst the combined control group had more or less equal numbers of Black and White subjects (20 vs 23). In view of this it is not possible to conclude whether this difference is due to the pregnant state or due to genuine racial differences.

Reports in the literature regarding the effect of normal Protein C levels are variable, either stating that levels remain unchanged (Gilabert *et al*, 1988; Lao *et al*, 1989) or that levels increase (Malm *et al*, 1988). One can conclude from this that if changes do occur, they are probably minor and are unlikely to mask the presence of true Protein C deficiency. In this regard it is important to note that a significant decrease in Protein C has been observed in pre-eclamptic states, particularly as this group of patients are prone to thrombosis (Gilabert *et al*, 1988).

Although Antithrombin levels have generally been reported to remain unchanged during normal pregnancy (Pekonen *et al*, 1986; Weenink *et al*, 1982; Gilabert *et al*, 1988), the results of this study are in agreement with Pinto *et al* (1988) in showing that a mild drop occurs. Even though this change was shown to be statistically significant it is unlikely to be clinically relevant. Most of the work on Antithrombin in pregnancy has focused on

pre-eclampsia. Whilst there is agreement that Antithrombin levels drop significantly in pre-eclampsia (Weenink *et al*, 1985; Saleh *et al*, 1988; Aghai *et al*, 1990) there is no consensus with regards to the predictive value of this drop.

The findings of this study together with those of the literature indicate that pregnant status is an important cause of deranged values. Knowledge of these physiological changes is imperative to prevent misinterpretation of results obtained for laboratory investigations of pregnant women with thromboses. The particular danger is misdiagnosis of inherited Protein S deficiency. As the current practice is to perform PCR routinely in our laboratory, the low APC-SRs are not problematic. Pregnancy-specific reference values (Table 14) for thrombotic screens have now been established for the Johannesburg Hospital Coagulation Laboratory. These will be issued with reports that lack adequate clinical information. This is solely to alert clinicians and is no way meant to be used to diagnose deficiency states if laboratory values fall below the pregnancy-specific values. It is hoped that by supplying these values that incorrect diagnoses of inherited thrombophilic states may be prevented.

5.6 Normal Values

The finding of significant differences in the Protein S and APC-SR values obtained for the two control groups compared with each other as well with the current laboratory reference ranges will necessitate a re-evaluation of these reference ranges. Oral contraceptive use was not established for either group. As oral contraceptive usage has

been shown to result in both lower APC-SRs (Henkens *et* al, 1995a; Olivieri *et* al, 1995) as well as lower Protein S levels (Boerger *et al*, 1987; Lowe *et al*, 1997) this is therefore a potential confounding variable. Although there is no obvious reason why there should be any difference between the two groups with regard to oral contraceptive usage, this is in itself an important point to consider when determining normal values.

Whilst the Protein C and Antithrombin values of the two control groups were not significantly different, the values obtained for the Black group followed the trend observed in pregnancy i.e. Protein C levels were higher and Antithrombin levels lower.

5.7 Screening for Factor V Leiden

Knowledge of race and sex-specific differences are not only important for the interpretation of laboratory reports but would also facilitate rational decision-making with regards to screening for abnormalities in patients with thrombosis. Documentation of the local population genetics of FV Leiden would be necessary to facilitate the formulation of rational screening programmes. If FV Leiden is truly absent in the South African Black population as it appears to be, then continued screening for this mutation in Black patients presenting with a thrombotic episode would be inappropriate.

From the female laboratory control group it is evident that the FV Leiden mutation is common in Caucasians in South Africa (8.7%). Review of routine thrombotic screens

confirms that the FV Leiden mutation is the commonest cause of an inherited predisposition to thrombosis at the Johannesburg Hospital (data not shown).

It would be of value to determine whether the FV Leiden mutation is present in other population groups that are served by the Johannesburg Hospital.

As the performance of individual PCR analyses would be costly for population studies and the expected positivity rate would be low, the feasibility of pooling DNA samples for analysis was investigated. Analysis of pooled DNA samples that were "spiked" with DNA from a FV Leiden carrier detected the FV1691A allele in 4 out of 5 runs when using the 1 in 20 mix (1 heterozygote, 9 normals) and 5 out of 5 times using the 1 in 10 mix (1 heterozygote, 4 normals). Pools of DNA from 5 rather than 10 individuals should therefore be used for population screening. An alternative strategy would be to analyse pools of DNA from 10 individuals as long as a positive control (1 in 20 mix) is included in each run. If a positive signal (i.e. 200 bp fragment) is detected after *Mnl*1 restriction of the PCR product, then DNA from the individuals would need to be processed separately to determine the number of positive cases in the pool of 10.

The identity of the individual with the FV Leiden mutation is not necessary when determining the population prevalence rate. In view of this an alternative strategy was investigated. During the establishment of the PCR-based assay for FV Leiden genotyping, it was observed that on visual inspection there was a constant relationship between the intensity of the 200 bp and 163 bp restriction fragments when the ethidium

bromide stained gels were visualized under UV light. Visual inspection of the restriction digestion patterns of a series of DNA pools with diminishing FV1691A allele content could however not be relied upon to predict the ratio of normal to mutated alleles present in the mix. An attempt was then made to determine the intensity of the 200 bp and 163 bp bands more objectively by densitometry scanning. This failed to show a constant ratio between the bands and did not permit accurate prediction of FV1691A allele frequency in the DNA pool.

Whilst the technique has theoretical potential, it would require refinement in order for it to be sufficiently accurate to replace individual analysis for population screening to determine the prevalence of genetic mutations.

In contrast to population surveys, general screening of populations or select groups for FV Leiden for the "benefit" of the individual is currently a widely debated but unresolved issue. However, new clinical data are continuously being reported on APC resistance and consensus regarding screening for this mutation will hopefully soon emerge.

There are obvious potential benefits of general screening for APC resistance, particularly in individuals who are exposed to circumstantial risk factors such as surgery, oral contraceptive use and pregnancy. If an individual with no family history of thrombosis is identified as a FV Leiden carrier, then short term prophylactic anticoagulation would be recommended in high risk situations, but it remains to be established whether this would be applicable in all situations associated with an increased risk of thrombosis (Dahlbäck,

1977). In contrast homozygous individuals and heterozygotes who have an additional deficiency of one of the anticoagulant proteins, should probably receive prophylaxis in all risk situations regardless of personal or family history of thrombosis (Dahlbäck, 1997).

Carriership of FV Leiden increases the risk of deep vein thrombosis about 8-fold. When combined with oral contraceptive usage this risk increases to about 30-fold (Vandenbroucke *et al*, 1994). As these risk factors are synergistic, thrombosis will occur in a substantial number of women who would not have experienced thrombosis if they had only one of the two risk factors. In view of this FV Leiden status should be considered when prescribing oral contraceptives to young women with a family history of thrombosis.

Whether all young women should be screened for the FV Leiden mutation prior to taking oral contraceptives has not been resolved. Such a policy, if implemented, might deny effective and acceptable contraception to about 5% of Caucasian women, while preventing only a small number of deaths due to pulmonary emboli (Helmerhorst *et al*, 1997). It would be beneficial to take a careful personal and family history of venous thromboembolic disease in order to detect individuals at risk. Such individuals would probably benefit from FV Leiden screening. The FV Leiden mutation is however not considered to be an absolute contraindication for oral contraceptive use by all, but decisions regarding oral contraceptive use should always be subjected to the careful assessment of an individual's risk (Helmerhorst *et al*, 1997).

6 CONCLUSIONS

The establishment of the PCR-based assay for the detection of the FV Leiden mutation has made a significant impact on the yield of routine laboratory investigation of patients with thrombosis. In its unmodified form the APTT-based APC resistance functional assay is not a good predictor of FV Leiden status but does allow for the detection of acquired APC resistance when used in conjunction with FV genotyping. With the excellent reported sensitivities and specificities of the modified APTT assay, whereby plasma samples are prediluted with FV-deficient plasma, most laboratories use this as a screening test for FV Leiden. Those samples with a low APC-SR are then subjected to genotyping for confirmation.

Whilst this might seem to be an appropriate change to institute in our laboratory this has not been implemented. If the modified assay were to replace the currently used unmodified assay, the infrastructure for FV genotyping would still need to be available. As FV genotyping is now well established as a routine test in the coagulation laboratory this will be maintained as a baseline test. Under these circumstances there would be no added value in changing to the modified functional assay as this is specifically designed to detect the FV Leiden mutation exclusively. The significance of functional APC resistance, in the absence of the FV Leiden mutation, has not been established. In an attempt to resolve this issue it was decided to continue to perform the unmodified APC resistance functional assay in parallel with the PCR assay.

In view of the apparent absence of the FV Leiden mutation in the local Black population which is in accordance with the findings of others, the policy of screening for this mutation in all patients with thrombosis will need to be reconsidered. Costs saved from such a policy change should ideally be channeled into the development of other assays which may further decrease the number of "unexplained" thromboses. Knowledge of the genetics of the FV Leiden mutation in all the population groups served by the Johannesburg Hospital would be important for long-term policy decisions regarding the rational investigation of patients with thrombosis. Whether or not general population screening would be beneficial must await the accumulation of more prospective data and cost-benefit analyses before any general recommendations can be made.

Normal reference values for APC resistance functional testing as well for the anticoagulant proteins should be reviewed because of the potential racial differences as well as the known gender differences and influences of oral contraceptives. As pregnancy is a common condition and venous thromboembolic disease is commonly associated with pregnancy, it is particularly important to alert clinicians with regards to the physiological changes of haemostasis variables to prevent misdiagnosis of inherited thrombophilic states with its attendant consequences.

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