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Activated Protein C Resistance in Pregnancy

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A thesis for the degree of Doctor of Medicine submitted to the University of Glasgow in June 1999

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Factor V Leiden (FVL), the principal inherited cause of activated protein C resistance (APCR), has been linked to the failure of pregnancy with an increased risk of venous thrombosis, fetal loss and pre-eclampsia. Both venous thrombosis and pre-eclampsia are associated with inflammation. In particular pre-eclampsia is associated with a monocyte/macrophage infiltration of the placental bed. That APCR, without FVL, (acquired APCR) may be a marker of thrombotic potential out-with pregnancy has been suggested by the association of venous thrombosis with the combined oral contraceptive pill and more recently by the association of venous thrombosis with a low activated protein C sensitivity ratio (the APC:SR).

Changes in sensitivity to APC (assessed by the APC:SR) occur in normal pregnancy. The aetiology and significance of this phenomenon has not been fully characterised. In this thesis, the pattern of change in APC:SR in pregnancy was detailed in FVL negative subjects. The relationship of the APC:SR to other coagulation factors and thrombin generation was investigated. The significance of the APC:SR to the mother and the fetus was also examined. To investigate if a link (via thrombin) exists between the APC:SR and the inflammatory response, the inflammatory marker ICAM-1 was examined on the monocyte. The influence of thrombin on the expression of this marker was examined in vitro and the relationship between the APC:SR in pregnancy and ICAM-1 was examined ex vivo.

In a prospective study of 1046 pregnancies, the APC:SR at 7-15 weeks gestation showed a significant relationship with blood group and smoking, with higher APC:SRs observed in non-smokers and blood group O subjects. No significant relationship of APC:SR with body mass index, waist circumference, age, alcohol consumption, or family history of venous thrombosis or pregnancy-induced hypertension in a first degree relative was observed. In ~75% of pregnancies, a fall in APC:SR with increasing gestation was seen. Those who showed a fall in APC:SR (i.e. an increase in APCR) with increasing gestation had higher absolute APC:SR values at 7-15 weeks gestation than those who did not show a fall. APC:SR values less than the reference range observed in non-pregnant females were seen in 38% of subjects in the third trimester of pregnancy (28 to 40 weeks gestation) in the absence of FVL or elevated ACAs. In subjects without elevated ACAs or FVL, the APC:SR showed a significant negative relationship with factor VIIIc, factor Vc, and a positive relationship with free protein S. The relationship to factor VIIIc is compatible with the association of APCR and inflammatory disorders and with the association of non-blood group O, higher factor VIIIc levels and venous thrombosis. The relationship with factor Vc is consistent with in vitro evidence that the APC:SR may be affected by factor V.

The fall in APC:SR with gestation paralleled the fall observed in the red cell count, haemoglobin and mean red cell volume. This appeared to be due to the effect of gestation on both, rather than an effect of the

APC:SR on the blood count. It has been suggested that heterozygous carriage of FVL results in a reduction in blood loss at delivery. In FVL negative subjects, no relationship between pre-natal, peri-natal, or post-natal blood loss and APC:SR was observed. Similarly, in the small group of FVL positive subjects (n=37) observed no difference in pre-natal, peri-natal, or post-natal blood loss was observed when compared with FVL negative subjects.

No significant relationship between the APC:SR and placental weight was observed, although in subjects with an APC:SR less than the reference range for non-pregnant females, smaller birth weights were noted. When birth weights were corrected by adjustment for parity, gestation at delivery and fetal sex and the influence of gestation on the APC:SR was minimised, no relationship between the APC:SR and birth weight was found. However significantly smaller birth weight centiles were observed in subjects in whom a fall in APC:SR with gestation occurred.

Significantly lower APC:SRs at 7-15 weeks were observed in subjects, normotensive at recruitment and with no past history of hypertension, who subsequently developed pregnancy-induced hypertension (PIH) or pre-eclampsia (PET) in the current pregnancy. This difference persisted for PIH when the analysis was restricted to primigravid subjects. No relationship between the APC:SR at 27-34 weeks gestation and the occurrence of PIH or PET was noted. A 2 fold ($C.I._{95}$ 1.1-3.6) increased risk of PIH and 3 fold increased risk of PET

($C.I._{95}$ 1.2-8.0) in the current pregnancy was observed in those subjects in whom no decrease in APC:SR was seen when compared with those in whom the APC:SR did decrease from 7-15 to 27-34 weeks. These results suggest that, in the absence of FVL, subjects with a lower APC:SR early in pregnancy do not increase resistance to APC with increasing gestation and are at a higher risk of development of PIH and PET. They also appear to have larger babies than those in whom an increase in APCR occurs. As lower APC:SRs are associated with venous thrombosis, and as a decrease in APC:SR occurs in the majority of pregnancies, this suggests that a higher APC:SR early in pregnancy and a fall in APC:SR with increasing gestation is a physiological response which mirrors fetal development. Individuals with a lower APC:SR early in pregnancy, do not become more resistant with increasing gestation and the (physiological) change in APC:SR does not occur. The response to APC appears to be set early in pregnancy and the sensitivity to APC may influence, or be influenced by, fetal development. The APC:SR, in the absence of FVL, does not, however, appear to predict fetal loss or neonatal death.

A significant inverse relationship between F1+2 levels and APC:SR was observed, although this may reflect the influence of gestation on both variables. When the effect of increasing gestation was excluded, a significant inverse relationship between APC:SR and TAT concentration was observed at 28-32 weeks, suggesting that the APC:SR may reflect thrombin generation in vivo.

Monocyte ICAM-1 expression, as assessed by flow cytometry, was influenced in a specific dose-dependent fashion by human alpha thrombin and may therefore be a useful indicator of the link between the coagulation cascade, thrombin and the inflammatory response of this cell. In pregnancy, a significantly higher level of monocyte ICAM-1 was observed when compared with non-COCP using female subjects. No significant relationship between ICAM-1 and gestation, parity or smoking was observed. In pregnant subjects, a significant positive relationship between monocyte ICAM-1 and plasma ICAM-1 was observed. This suggests that the monocyte contributes to plasma ICAM-1, which has been used widely as a marker of inflammation. A small but significant inverse relationship of both monocyte ICAM-1 and plasma ICAM-1 with APC:SR at 28-32 weeks gestation was noted. This suggests that in individuals with a normal pregnancy outcome the thrombin generation associated with the APC:SR at 28-32 weeks gestation is mirrored by the inflammatory response of the monocyte. However, as the monocyte has a significant, independent role in the activation of coagulation, it is conceivable that activated monocytes could also influence the sensitivity to APC in plasma by modulating the generation of factor VIIIa and factor Va.

A change in the sensitivity to APC appears to be a physiological response in pregnancy which mirrors fetal development. Whether acquired APCR has an independent role or simply reflects the sum total of contributing coagulation factors is unknown. However, the APC:SR

reflects overall thrombin generation and may have a useful role in the prediction of disease states in pregnancy.

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Chapter 1

Introduction

1.1 In recent years there has been a substantial increase in knowledge linking pro-thrombotic tendencies with several adverse outcomes of pregnancy. This has been paralleled by an increased awareness of the central co-ordinating role of vascular endothelium in inflammation, atherosclerosis and haemostasis. The exact mechanism whereby pro-thrombotic (or 'thrombophilic') tendencies lead to pregnancy failure is unknown. Thrombophilic abnormalities are associated with an increase in thrombin generation[1, 2]. As thrombin is both procoagulant and pro-inflammatory, this may result in stimulation of an inflammatory response from the endothelium and thrombosis of the placental vessels. Stimulation of an inflammatory response in the endothelium also results in secretion of pro-adhesive molecules and cytokines and alteration of the anticoagulant protein C system on the endothelial surface[3-5].

1.2 Thrombin and the protein C/protein S system

1.2.1 The cascade of zymogen activation in the coagulation cascade leads to the formation of thrombin (Figure 1.1). Thrombin is a serine protease which is responsible for fibrin formation, platelet activation, activation of factor XIII (with cross-linking of the fibrin network) and the feedback activation of clotting factors Vc and VIIIc.

1.2.2 Thrombin has a direct action on the endothelium via a transmembrane thrombin receptor[6] (Figure 1.2). Via this interaction thrombin participates in tissue remodelling, wound repair, leukocyte chemotaxis, leukocyte

adhesion, vascular contraction and changes in vascular permeability (reviewed in[7]). This receptor is present on a variety of cells including platelets and monocytes[8]. On endothelial cells proteolytic cleavage of this receptor results in a wide variety of cellular responses including expression of leucocyte adhesive proteins such as P-selectin[9, 10] and intercellular adhesion molecule-1 (ICAM-1), the ligand for lymphocyte function antigen-1[11], on the endothelial surface. This response results in leukocyte adherence to the endothelium and further enhancement of the inflammatory response. Thus thrombin can participate in leukocyte chemotaxis and adherence (reviewed in[7]). Thrombin may also affect immunological surveillance by stimulating T-cell activation and cytokine production[12]. The importance of the role of thrombin in the inflammatory response is confirmed by the consistent finding of thrombin generation accompanying leukocyte accumulation in several forms of inflammation[13, 14] and by the association of an inflammatory response with venous thrombosis[15, 16].

1.2.3 Thrombin also functions as an activator of the anticoagulant protein C pathway (Figures 1.3 and 1.4). Surface components and intracellular products of the endothelium play a significant role in the control of coagulation. In particular, the cell surface possesses a thrombin binding glycoprotein, thrombomodulin (TM)[17]. TM forms a high affinity (Kd=0.5nM) 1:1 complex with thrombin. Although thrombin may activate protein C directly, the complex formation accelerates the activation 20,000 fold[17]. Formation of this complex directly inhibits the capacity of thrombin to cleave fibrinogen and activate platelets[17-20]. The proteolytic cleavage

of protein C by thrombin results in the generation of the anticoagulant, activated protein C (APC). The complex of APC and plasma protein S assembles on membrane surfaces and inactivates activated coagulation factors, V and VIII (Figure 1.4). These interactions require the presence of Ca^{2+}. It is also likely that the inactivate factor Vc acts as a cofactor in the inactivation of factor VIIIa[21].

1.2.4 Thrombin's effect on protein C is, however, complex. Acting via the endothelial thrombin receptor, thrombin produces an increase in TM RNA in the endothelium. However this does not result in an increase in TM expression on the endothelial cell surface[22, 23]. Recently, another membrane-linked protein C receptor on the endothelial surface has been described. This endothelial protein C receptor (EPCR) also has a protein C activating function but has been found to bind protein C as well as APC with similar affinity[24]. The significance of this receptor is unknown, although interestingly, it may also have a role in the inactivation of APC[25]. It has been shown to have differing distribution in endothelial cell types, with a higher density found in large vessels rather than capillaries[26].

1.2.5 The protein C pathway appears to be critical to the control of the coagulation pathway as it is known that homozygous deficiency of protein C or protein S is associated with lethal thrombosis in neonates[27, 28] and gene deletion of TM is associated with embryonic lethality in mice[29]. Furthermore, inherited resistance of activated factor V to the effects of APC (Factor V Leiden-FVL) is the most frequently identified risk factor for a heritable thrombotic tendency[30]. The recent increase in understanding of the mechanism

whereby acquired disorders, such as antiphospholipid syndrome[31, 32], hyperhomocysteinaemia[33] and dyslipidaemia[34, 35] interfere with the protein C/protein S system reinforces the central role of the protein C pathway in the control of haemostasis.

1.2.6 A strong link exists between the protein C pathway and inflammation. This is evident in disseminated intravascular coagulation. In primates, APC infusion prevents septic shock and death from otherwise lethal doses of bacteria[36] and inhibition of the action of the protein C pathway enhances the coagulant and inflammatory response to the bacteria[36, 37]. The mechanism whereby the protein C pathway influences the inflammatory process is unknown, although APC infusion in rodents prevents the septic shock response to endotoxin and diminishes cytokine elaboration, an action which may be mediated by an effect on monocytes[38, 39].

1.3 The function of ICAM-1

ICAM-1 results in leucoyte adherence via LFA-1. This will result in leukocyte recruitment to the inflammatory response. A soluble form of ICAM-1 is found in plasma. The physiological function of this soluble form is unknown, although it retains its ability to bind lymphocyte function antigen-1 (LFA-1)[40] and may have an immuno-modulatory role. Soluble ICAM-1 has been used widely as a marker of endothelial activation in atherosclerosis[41-43], inflammation[44] and pregnancy disorders characterised by thrombosis and inflammation such as pre-eclampsia[45]. There is, however, little direct

evidence confirming the relationship between soluble ICAM-1 and cellular expression[46]. ICAM-1 is expressed on a variety of cells in addition to endothelial cells[47-49] and, in-vitro, studies have shown that induction of membrane-bound ICAM-1 can be accompanied by an increase in shedding of soluble ICAM-1 from the surface of these cells[40, 50-52]. In the vascular system, the contribution that individual cell types make to the soluble plasma pool of ICAM-1, measured, in-vivo, is unknown.

1.4 The endothelium and coagulation

Aside from synthesis and expression of TM, the endothelium participates in the regulation of haemostasis and thrombosis by a number of other mechanisms (Figure 1.5). By synthesis of von-Willebrand factor and tissue factor the endothelium promotes platelet adhesion and triggers the coagulation cascade. The endothelium also regulates fibrinolysis by the synthesis and secretion of tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI)(reviewed in[53]). The extracellular matrix around the endothelium, which contains heparans, enhances the activity of antithrombin[54]. Heparans also release tissue factor pathway inhibitor from its endothelial cell stores[55]. Thus the endothelium is pivotal to the regulation of coagulation, fibrinolysis and the endogenous anticoagulant system. Furthermore, the endothelium provides a link between the coagulation system and the inflammatory response. Clinical and experimental studies have shown that inflammation/infection is associated

with enhanced production of inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα). Infusion of TNFα to normal humans induces a sustained activation of coagulation with increased turnover of thrombin evident for 6-12 hours after infusion[56]. In cell culture and animal experiments, TNFα elicits tissue factor expression on endothelial cells[57] and results in a significant increase in plasma TM despite an inhibitory effect on TM RNA transcription[4], translation[58] and expression on endothelial cells[59, 60]. Endotoxin, which has been utilised to model the inflammatory response in-vitro, also reduces TM expression and enhances tissue factor release from the endothelial surface[60]. In addition thrombin will promote expression of cell adhesion molecules which control leukocyte recruitment and activation at sites of inflammation.

1.5 The monocyte, inflammation and coagulation.

1.5.1 Monocytes have a central function in the inflammatory response and express a variety of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1)[8, 61]. Like endothelial cells, monocytes possess thrombin receptors[62] and stimulation of monocytes with thrombin receptor agonist peptides has been shown to result in an increase in intracellular calcium[63]. Thrombin interacts with monocytes to enhance secretion of TNFα, interleukin-1[64] and plasminogen activator inhibitor-2[65]. Although

thrombin stimulates adhesion molecules on the endothelium, it is not known whether thrombin influences ICAM-1 expression on the monocyte.

1.5.2 Monocytes participate in the coagulation cascade by a variety of means including production of tissue factor[66], activation of coagulation factor X[67] and participation in the control of fibrinolysis[68]. Monocytes also possess TM[69] and a specific receptor for APC has been reported to occur on the surface of the cell[70]. Inflammatory mediators, such as TNFα, increase the expression of TM on the monocyte surface[38], but decrease its expression on the endothelial surface[60]. The increase in monocyte TM expression is accompanied by an increase in ICAM-1 expression on the cell surface[38].

1.6 Activated protein C resistance

Resistance to the anticoagulant effect of APC added in-vitro to plasma was first recognised as an important cause of familial thrombosis in 1993[71]. Depending on selection criteria, it is present in 20% to 70% of those individuals who have had a venous thrombotic event[72, 73]. In the vast majority of individuals with familial APC resistance the resistance is due to the presence of a point mutation Glycine→Arginine at position 1691 (G→A 1691) in the gene coding for coagulation factor V (Factor V Leiden - FVL)[74]. This mutation is highly prevalent in European populations (2-7%)[75]. Screening for APC resistance (APCR) is commonly based on an activated partial thromboplastin test (APTT). In this test a ratio of the APTT

in the presence and absence of added APC is obtained (i.e. APTT plus added APC/APTT minus added APC). This is designated as an 'APC sensitivity ratio' (APC:SR). A low APC:SR implies APCR. The significance of APCR occurring in the absence of the FVL mutation is unknown. Low ratios have been described in the latter stages of pregnancy[76], in subjects on oestrogen therapy[77] and with other variations in the factor V gene[78, 79]. Furthermore, the standard APTT-based assay is unreliable in the presence of antiphospholipid antibodies[80] or anticoagulant therapy[81]. Pre-dilution of patient plasma with factor V depleted plasma prior to APTT testing renders the test insensitive to anticoagulants or gestation and more specific for the FVL mutation[81, 82].

1.7 Acquired Activated Protein C Resistance

1.7.1 Although the importance of APCR in the absence of the FVL mutation is poorly understood, a number of published observations would suggest that APCR may be a potential marker of a prothrombotic tendency. A negative correlation between FVIIIc and APC:SR (a positive correlation between FVIIIc and APCR) was reported in 1996[83, 84]. Elevated FVIIIc levels, a common manifestation of inflammation, have been shown to be an independent risk factor for venous thrombosis[85]. This association of FVIIIc and thrombosis may be linked to blood group[85-87] in that non-blood group O subjects have higher FVIIIc levels and a higher incidence of venous thrombosis.

1.7.2 Both elevated anticardiolipin antibodies and the presence of a lupus inhibitor may be associated with a reduction in the APC:SR as measured by the classical APTT-based test[88, 89]. Individuals with an APCR phenotype show a strong relationship between APCR and FVL carriage in those with 'weak' lupus inhibitor activity[88]. In contrast, those with strong lupus inhibitor activity show a weak association between APCR and FVL. The observation of an influence of anti B_2 glycoprotein-1 antibodies on the APC:SR of normal plasmas suggest that antiphospholipid antibodies may have a direct effect on the inactivation of FVa[90] and result in APCR. Interestingly, in a subsequent small study, Aznar et al suggested that the degree of APCR associated with antiphospholipids may correlate with thrombotic risk[89].

1.7.3 In 1994 Vandenbrouke et al reported that subjects who are heterozygous for FVL and who use a combined oral contraceptive (COCP) have a 30 fold higher risk of development of thrombosis than non FVL, non-COCP users[91]. In keeping with a previous study[92], they also noted an approximately 4 fold relative risk of venous thrombosis and use of COCPs in the absence of FVL. In 1994, Osterud et al noted that COCP use was associated with resistance to APC when assessed by an APTT-based method[93]. This finding was confirmed by Henkens et al[94] and Olivieri et al[77], who also reported that cessation of the COCP in 2 subjects, without FVL, resulted in normalisation of the APC:SR. These findings were not supported by Mathonnet et al[95] who found no difference in APC:SR between COCP and non-COCP users. In this study however, a significantly lower APTT (without APC) was observed in COCP users and one individual (1/34) of the control

group was found to carry the FVL mutation. This would result in a significant reduction in the APC:SR "normal" range obtained and exclusion of FVL in all subjects has been recommended in the construction of "normal" pool material for analysis of the APC:SR[96].

1.7.4 Resistance to the effects of APC added in-vitro to plasma has also been assessed by the pattern of thrombin generation which occurs when plasma is stimulated by tissue factor (i.e. by activation of the extrinsic coagulation cascade)[97]. Using this technique Rosing et al have reported similar APCR in non FVL, COCP users to that observed in non-COCP users who are heterozygous for FVL. They suggest that acquired APCR may explain the 4 fold increased risk of venous thrombosis observed in users of COCPs[98].

1.7.5 In a study of 20 pregnancies, a reduction in APC:SR with increasing gestation was reported in 1995[76]. However, only at 14-20 weeks gestation did the APC:SR correlate with the plasma level of FVIIIc. A reduction of APC:SR in pregnancy was confirmed by Mathonnet et al[95], who observed a lower APC:SR in 32 pregnant subjects at delivery, in comparison to control non-pregnant, non-COCP using subjects and COCP users. The delivery APC:SR correlated with delivery levels of FVIIIc. Although a higher level of FVc was observed in comparison to control non-COCP and COCP-using subjects, no correlation with FVc and APC:SR was observed. Confirmation of a relationship between FVIIIc and APC:SR in early pregnancy was reported by Bokarewa et al in 1997[99]. No information was available regarding FVc. Interestingly, the standard deviation of the mean APC:SR in the third trimester was less than that observed post-partum and subjects with the

lowest post-partum APC:SR values showed the least change during pregnancy. These observations would be consistent with tight control of the response to APC in pregnancy. As 57-76% of women develop an APC:SR less than non-pregnant reference ranges during pregnancy[76, 99], this is clearly a physiological adaptation. The significance of this adaptation is, however, unknown.

1.8 Heritable abnormalities of the protein C/protein S system

A number of heritable abnormalities of elements of the protein C pathway have been described.

1.8.1 Protein C deficiency

Heritable deficiency of protein C has been estimated to occur in 1 in 300 to 500 of the population[100, 101]. In studies investigating blood donor populations, the majority of individuals identified biochemically or genetically are symptom free[100, 101]. Protein C deficiency has been classified into two types: Type 1, a quantitative reduction in functionally normal protein C and Type 2, a higher level of protein C antigen than activity. Type 2 deficiency results from production of a functionally abnormal protein C molecule.

1.8.2 Protein S deficiency

The prevalence of protein S deficiency in the general population has not been established in a large scale study of healthy individuals. Protein S is a vitamin K-dependent single chain glycoprotein. Plasma levels of protein S are dependent on age, sex[102], lipid levels, oestrogen[103] or oral anticoagulant usage. In plasma, approximately 60% of circulating protein S is bound to C4b binding protein. Only free protein S is able to function as a cofactor for protein C. Heterozygous protein S deficiency is a significant risk factor for thrombosis[104]. At present there is no unified classification of protein S deficiency. Three classes of protein S deficiency have been proposed: Type 1, a reduction in total and free protein S; Type 2, a reduction is protein S activity with normal free and bound antigen levels and Type 3, a reduction in free antigen and activity with a normal total protein S. In 1993 Faioni reported[105] that some protein S functional assays give falsely low protein S activity assessments in the presence of FVL. This finding has subsequently been confirmed in other studies[106, 107]. There is currently, therefore, no universally accepted functional protein S assay.

1.8.3 Factor V Leiden

The presence of FVL can be detected directly by mutation analysis of DNA for the G→A mutation at nucleotide position 1691 of the factor V gene. Recently a factor V gene polymorphism (the HR2 haplotype)[78] and another gene

mutation (Arg306→Thr, Factor V Cambridge) in factor V have been described[79]. These also result in an APCR phenotype, although the prevalence of these genetic variations is unknown.

1.8.4 Thrombomodulin

The glycoprotein TM is synthesised within endothelial cells and expressed on the endothelial surface. The gene encoding TM is found on chromosome 20[108, 109]. Although little evidence links genetic mutations of the TM gene with clinical thrombosis, in animal studies congenital absence of TM results in lethality in-utero[29]. An amino acid dimorphism in the TM gene at position 1418, which predicts an alanine or valine at position 455 in the TM molecule, has been associated with premature myocardial infarction[110], but has been reported to be neutral as regards the occurrence of venous thrombosis[111]. The mechanism whereby this contributes to abnormal TM function or alters TM thrombin affinity is unknown and as yet, there is no information regarding the influence of this genetic variation on pregnancy outcome. TM expression is down-regulated by a number of influences on the endothelium including cytokines, endotoxin and hypoxia[112, 113]. Oxidant injury also results in a decrease in TM activity[114]. TM expression on endothelial cells is influenced by a number of inflammatory mediators including TNFα[4, 58-60] and endotoxin[60]. TM can also be shed from the endothelial surface by neutrophil elastase[115] and soluble TM (sTM) has

been used as a marker of the effect of inflammation on the endothelium[116-118].

1.9 Haemostasis and endothelial function in normal pregnancy

1.9.1 Coagulation and the protein C/protein S system

Normal pregnancy is associated with changes in many coagulant and anticoagulant factors. By the end of the normal gestation there is a mean rise in the vitamin K dependent factors IIc, VIIc and Xc[119]. In addition, a small increase in factor XIc and factor Vc has been reported[119], although this was not been confirmed in a subsequent study[120]. Protein C antigen and activity levels are not progressively affected by gestation[121, 122], although an increase in protein C activity has been reported at the beginning of the puerperium[123]. Normal pregnancy is associated with a progressive fall in free and total protein S antigen[119]. APC:SR, when assessed by the classical APTT-based test has been shown to fall with increasing gestation[76]. Modification of the test, by pre-dilution of patient plasma with coagulation factor V depleted plasma, results in an insensitivity of the test to the effects of pregnancy[124]. Antithrombin activity has consistently been reported to be unaffected by normal gestation[125-127].

1.9.2 Thrombin generation

Enhanced thrombin generation may result from an increase in procoagulant factors[1], a decrease in circulating natural anticoagulants[1], from inflammation[128, 129] and from thrombosis itself. Thrombin formation has been assessed, ex-vivo, by the circulating levels of a number of marker proteins formed as a consequence of thrombin formation or inactivation. As prothrombin is cleaved by factor Xa to form thrombin, a fragment is released. This fragment is called prothrombin fragment 1+2 (F1+2) and can be measured in plasma by an enzyme linked immunoabsorbent assay (ELISA)[130]. Thrombin formation can also be assessed by the circulating level of the complex that it forms with its principle inhibitor, antithrombin (the thrombin-antithrombin or TAT complex)[130].

An increase in TAT complex formation occurs in normal pregnancy[127, 131, 132]. TAT complex levels higher than non-pregnant control values have been reported to be present in 50% of women during the first trimester with all subjects showing elevated levels in the second and third trimesters[127]. A significant positive correlation between gestational stage and elevated F1+2 has also been shown[133, 134], with levels elevated beyond non-pregnant subjects seen early in pregnancy[134]. These results show that activation of coagulation occurs in normal pregnancy. Delorme et al[135] have shown that pregnant plasma in in-vitro experiments is capable of more rapid and elevated generation of thrombin than non-pregnant plasma. This result, in combination with evidence of an increase in prothrombin activation

and TAT complex formation, suggests that heightened thrombin generation may be a feature of normal pregnancy.

Fibrin formation is known to be essential for normal placental implantation[136, 137]. It is therefore plausible that the enhanced thrombin generation of pregnancy is stimulated by the placenta[138]. As thrombin has both procoagulant and anticoagulant functions it is not clear whether the enhanced thrombin generation of pregnancy contributes to a pro-thrombotic state.

1.9.3 Protein C activation

As described above, protein C is activated by thrombin in the presence of TM. APC is known to be regulated by several inhibitors: protein C inhibitor-1[139], α1-antitrypsin (also known as α1-antiproteinase)[140], α2-antiplasmin, C1 esterase inhibitor[141] and α2-macroglobulin[142]. The recently described EPCR[5] may also have an inhibitory function on APC, although its role in protein C homeostasis is, as yet, unknown. It is possible to assess the activity of thrombin on protein C activation by measuring APC[143], by measuring the peptide released from protein C on activation[144] or by measuring complexes of APC with its inhibitor α1-antitrypsin[140]. An increase in APC/α1-antitrypsin in the third trimester to double the level observed in the first trimester has been reported to occur in normal pregnancy[145]. In this study APC/α1-antitrypsin levels did not correlate

with α1-antitrypsin. This would be consistent with an increase in the activation of protein C, and therefore thrombin activity, with gestation.

1.9.4 Thrombomodulin

The influence of normal pregnancy on endothelial expression of TM is not known. Thrombin, via its endothelial receptor, is capable of down regulating TM expression on the endothelial surface[22, 23]. As with other indices of endothelial activation, plasma levels of sTM have been shown to increase progressively with gestation in normal pregnancies[146].

1.10 APC resistance and thrombin generation

A number of studies have shown that carriage of FVL is associated with an increase in the markers of thrombin generation[1, 2] similar to that seen in individuals with a past history of thrombosis. The effect of acquired APCR on thrombin generation has not been assessed. However, in the diagnosis of acquired APCR associated with COCP use Rosing et al have shown that plasma from 3rd generation COCP users has greater potential to generate thrombin than non-COCP and 2nd generation COCP users[98]. The relationship between this increase in thrombin potential and thrombin generated in-vivo is not known.

1.11 The protein C/protein S system and pregnancy failure

1.11.1There is now a considerable body of evidence suggesting that inherited disorders such as FVL[147-149], protein C or protein S deficiency[148, 150-152] can lead to pregnancy failure and pregnancy-related thrombosis. In particular, recent investigations have concentrated on the potential role of these pro-thrombotic abnormalities in the occurrence of pre-eclampsia, fetal loss and the protection of the blood count in pregnancy.

1.11.2Pre-eclampsia

Pre-eclampsia is a multi-system disorder which presents with proteinuria and hypertension. It is characterised by widespread vascular endothelial damage and dysfunction which is associated with failure of trophoblastic invasion of the spiral arteries of the placenta[153]. A number of the spiral arteries of the developing placenta are occluded by fibrin deposits and surrounded by foam cell infiltration[154, 155], a feature similar to atherosclerosis and termed 'acute atherosis'.

Inflammation

Endothelial dysfunction is common to all the pathological features of pre-eclampsia[156]. It is unclear, however, what triggers this endothelial disturbance, although abnormalities of platelets[157, 158], endothelial

function[159-161], and neutrophil activation have all been implicated in the pathophysiology[162, 163]. Several studies have shown that the serum of patients with pre-eclampsia have increased levels of circulating VCAM-1 (vascular cell adhesion molecule-1)[45, 156] and ICAM-1 (intercellular adhesion molecule-1)[45]. These integrins are involved in the adherence of leucocytes to the endothelium and an increase in the expression of ICAM-1 and V-CAM-1 may result in an increase in leucocyte adherence[164, 165]. The cause of the increase in expression and shedding of CAMs is not known, but various cytokines including interleukins[166] and TNFα[167] upregulate the expression of cellular adhesion molecules on endothelial cells. Elevated concentrations of both TNFα[168] and interleukin-6[169] have been reported in the maternal plasma and amniotic fluid of women with severe pre-eclampsia. An increase in mRNA expression for TNFα has been found in pre-eclamptic placentas[170]. Although the source of its production is unknown, TNFα may be produced by macrophages present in the placental stroma. In addition to the upregulation of CAM expression on endothelial cells[167], TNFα has been shown, in-vitro, to directly impair vascular endothelial function by activating neutrophils directly to adhere to endothelial cells[171]. Thus the monocyte/macrophage system appears to be integral to the necrotising arteriopathy of pre-eclampsia which is characterised by accumulation of lipid-laden macrophages and mononuclear perivascular infiltration[155].

Coagulation

It has been known for many years that pre-eclampsia is associated with activation of the coagulation and fibrinolytic systems. In those subjects with hypertensive disorders of pregnancy, a number of changes in the coagulation system similar to those described in chronic disseminated intravascular coagulation have been reported. These include, a reduction in antithrombin activity, plasminogen level and platelet count and an increase in fibrin degradation products[172, 173]. Both Reinthaller et al[132] and Halligan et al[174] have shown that high TAT complex levels (higher than those found in normal pregnancies) are seen in individuals with pre-eclampsia. As antithrombin levels appear unaffected by gestation and as antithrombin may even be consumed in pregnancy-induced hypertension[175], these results suggest that exaggerated thrombin generation is a feature of gestational hypertension. The overall thrombotic potential of this increase in thrombin activity in pre-eclampsia is not clear, as an increase in protein C activation has also been reported[145]. Coagulation markers of endothelial activation, such as von-Willebrand factor[176], fibronectin[174] and plasma TM[177-179] are elevated in pre-eclampsia and, along with antithrombin activity[180], may have a role as predictive markers for the disease. In two studies pre-eclampsia has been associated with an elevation of plasminogen activator inhibitor-1 (PAI-1) and a reduction in plasminogen activator inhibitor-2 (PAI-2) levels when compared with normotensive pregnancies[174, 181]. The mechanism and significance of these

observations remains speculative at present. It is likely that these changes in coagulation activation and fibrinolysis are, however, a secondary phenomenon rather than the primary initiators of hypertension in pregnancy.

The protein C pathway

APCR and FVL

Studies of the impact of APCR on the incidence of pre-eclampsia have not, in the main, distinguished between FVL and other causes of APCR. Two case reports have linked the heterozygous inheritance of the FVL gene with both pre-eclampsia[182] and the HELLP (haemolysis, elevated liver enzymes and low platelet count) syndrome[183]. In subjects with a history of pre-eclampsia, a 2 fold higher carrier rate for FVL has been described[184] and a higher rate of occurrence of APCR in pre-eclamptic women is also reported by Dekker et al[152]. In this study no assessment of FVL status was made and although patients were assessed for the presence of a lupus inhibitor and anticardiolipin antibodies, it is not clear if subjects positive for these abnormalities (29.4%) were excluded from the APC:SR assessment. Lindoff also describes an odds ratio of 2.5 ($C.I._{95}$ 0.9-9) for prevalence of FVL in 50 subjects with pre-eclampsia versus controls sampled more than 6 months after delivery[185]. As with the paper by Dekker no distinction is made between APCR and the presence of the FVL mutation. A 5 fold relative risk ($C.I._{95}$ 1.3-18.3) of inheritance of FVL is also reported in a larger case control

study of pre-eclampsia and FVL[186]. In a more recent study, APC:SR and FVL were assessed together in 279 subjects with a history of pre-eclampsia, eclampsia or HELLP syndrome. All subjects were sampled at least 10 weeks after pregnancy. Interestingly, an APC:SR lower than local reference range was found in 11.1% of subjects with these disorders but in none of the controls. From this study it appears that only 6% of patients of subjects with these disorders were positive for FVL. The aetiology of the APCR in the remainder is not stated. At 10 weeks post-partum the acquired APCR associated with normal pregnancy[76] is likely to have resolved and whether these results point to an independent role for acquired APCR in the development of pre-eclampsia remains to be confirmed.

Protein C and protein S deficiency

In 1987 a case study suggested an association between inherited protein C deficiency and the development of pre-eclampsia[187]. In a subsequent study by Dekker, 24% of subjects with severe early-onset pre-eclampsia had evidence of protein S deficiency[152]. However only 1 of the 85 patients tested showed evidence of protein C deficiency.

1.11.3 Fetal loss

A single spontaneous fetal loss occurs in 14-19% of clinically recognised pregnancies[188], whereas three or more consecutive pregnancies losses are

uncommon and occur in only 0.4-0.8% of women[189]. Early pregnancy losses are much more common than late pregnancy loss (6.1/1000 total births) and a variety of other endocrine, infectious and mechanical variables should be considered before a link between thrombophilia and fetal loss can be made. In the study of fetal loss and thrombophilia, two approaches predominate: case/control studies where subjects with fetal loss represent the cases, and case/control studies where subjects with inherited thrombophilia represent cases. The results of these two approaches may not be directly comparable. Furthermore it is likely that different thrombophilic factors may not have an identical influence on the gestation at which fetal loss occurs. Fetal loss may be associated with placental insufficiency and infarction[190] and placental infarction has been reported in association with abnormalities of the protein C/protein S system[191], abnormalities of homocysteine metabolism[192], with the presence of lupus inhibitors[193] and, in animal studies, with the absence of TM[29].

FVL and APCR

A number of case reports have suggested that maternal carriage of FVL or APCR is associated with fetal loss[182, 194, 195]. That APCR influences recurrent later rather than early pregnancy loss was suggested by Rai et al[196, 197] and subsequent studies have confirmed that there is no significant excess of inheritance of FVL in women with recurrent 1st trimester miscarriages[198-200] or fetal loss before 28 weeks gestation[148]. A

relative risk of stillbirth with FVL of 2 ($C.I._{95}$ 0.5-7.7)[148] and a relative risk of FVL inheritance and recurrent late pregnancy loss of 4.39 ($C.I._{95}$ 1.3-14.7)[201] have been reported. Interestingly, one study has also suggested that fetal carriage of FVL may influence fetal loss and placental infarction[191], but as yet, there is little evidence that paternal FVL influences fetal outcome.

Protein C and Protein S

Two case reports in 1987 reported extensive placental infarction in association with third trimester intrauterine death and protein C deficiency[187, 202]. Subsequently conflicting family based studies have shown no[150], or a 2.5 relative risk[151] of fetal loss in subjects with protein C deficiency. Results from a large cohort of patients with hereditary thrombophilia (EPCOT) observed odds ratios of 1.4 and 2.3 for miscarriage (fetal loss less than 28 weeks) and stillbirth (fetal loss greater than 28 weeks) respectively in subjects with protein C deficiency[148]. The overall results of this study also suggest that heritable thrombophilia may have a greater impact in later than early pregnancy loss. Like protein C deficiency, protein S deficiency was also associated with a higher relative risk of stillbirth than miscarriage (O.R. 3.3 vs 1.2). This is in keeping with the positive risk of fetal loss with protein S deficiency previously reported (relative risk of fetal loss 1.5)[151] and in keeping with the absence of protein C or S deficiency in 522 subjects with more than three 1st trimester miscarriages[200].

1.12 Blood loss in pregnancy and FVL

As the FVL mutation has a restricted distribution to individuals of European descent it has been suggested that the mutation has a single origin[203] and it has been suggested that the mutation was present in the first Europeans around 30-40,000 years ago[204]. This persistence in the population at a relatively high frequency may relate to an absence of effect on reproductive fitness despite its possible association with an increased relative risk of fetal loss. It has also been speculated that the persistence of the mutation may indicate a beneficial effect to the carrier. The most obvious potential advantage of an increase in thrombotic potential would be in the reduction of blood loss and the protection of red cell and haemoglobin levels. This hypothesis has been examined in pregnancy in a single study[205] which showed a significantly reduced risk of intrapartum bleeding episodes and better preservation of the post-partum haemoglobin levels in FVL heterozygote subjects when compared to the non-FVL pregnant subjects. The estimation of blood loss in this study was highly subjective and it is likely that the changes in blood volume and differences in the type of delivery have a greater effect on post-partum haemoglobin measurements than FVL.

1.13 Summary of proposed investigation

1.13.1As yet, acquired APCR of pregnancy has only been examined in small studies and there is no consistent information regarding the aetiology or prevalence

of this phenomenon. Furthermore the clinical significance of this physiological adaptation as regards the outcome of pregnancy for the mother and the fetus has not been investigated. Thrombin generation is the presumed mechanism whereby heritable thrombophilia results in failure of pregnancy and thrombosis and in a variety of clinical circumstances thrombin generation is closely linked to the inflammatory response. Modulation of the inflammatory response occurs in normal pregnancy[146, 206] and a heightened inflammatory response is observed in pregnancy disorders such as pre-eclampsia. Pre-eclampsia is characterised by acute atherosis which associated with monocyte/macrophage infiltration of the placenta (reviewed in[207]). The relationship of acquired APCR in pregnancy with thrombin generation and inflammation is unknown.

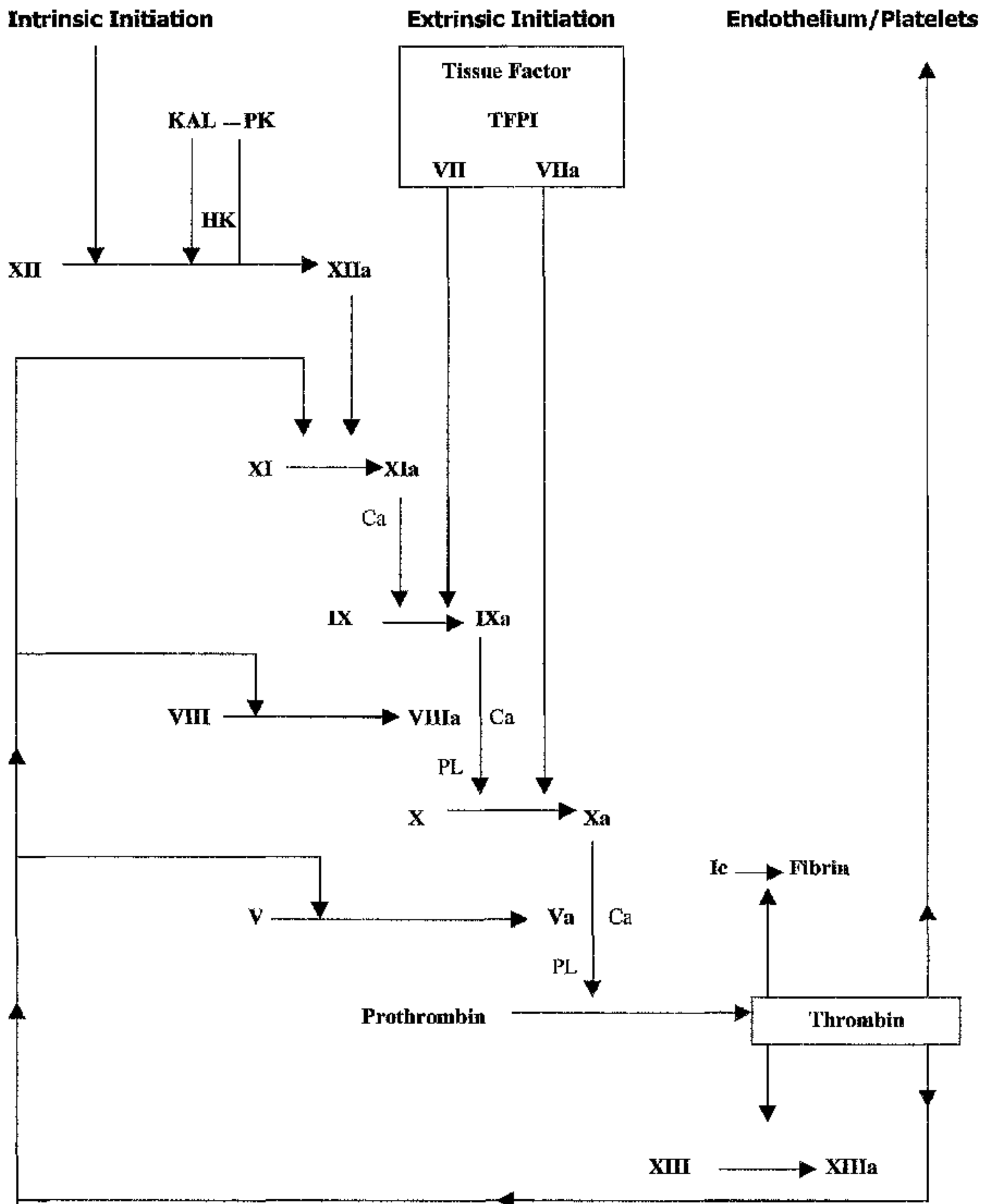
1.13.2It is proposed to examine

- the change in APC:SR in pregnancy and to determine the influence of other coagulation factors on the APC response.
- the relationship between acquired APCR and markers of thrombin generation.
- the significance of the APC response, in the absence of FVL, to fetal growth.
- the relationship of acquired APCR with blood loss, changes in the blood count and occurrence of maternal hypertension.

To further investigate the link between thrombin, acquired APCR and inflammation it is proposed

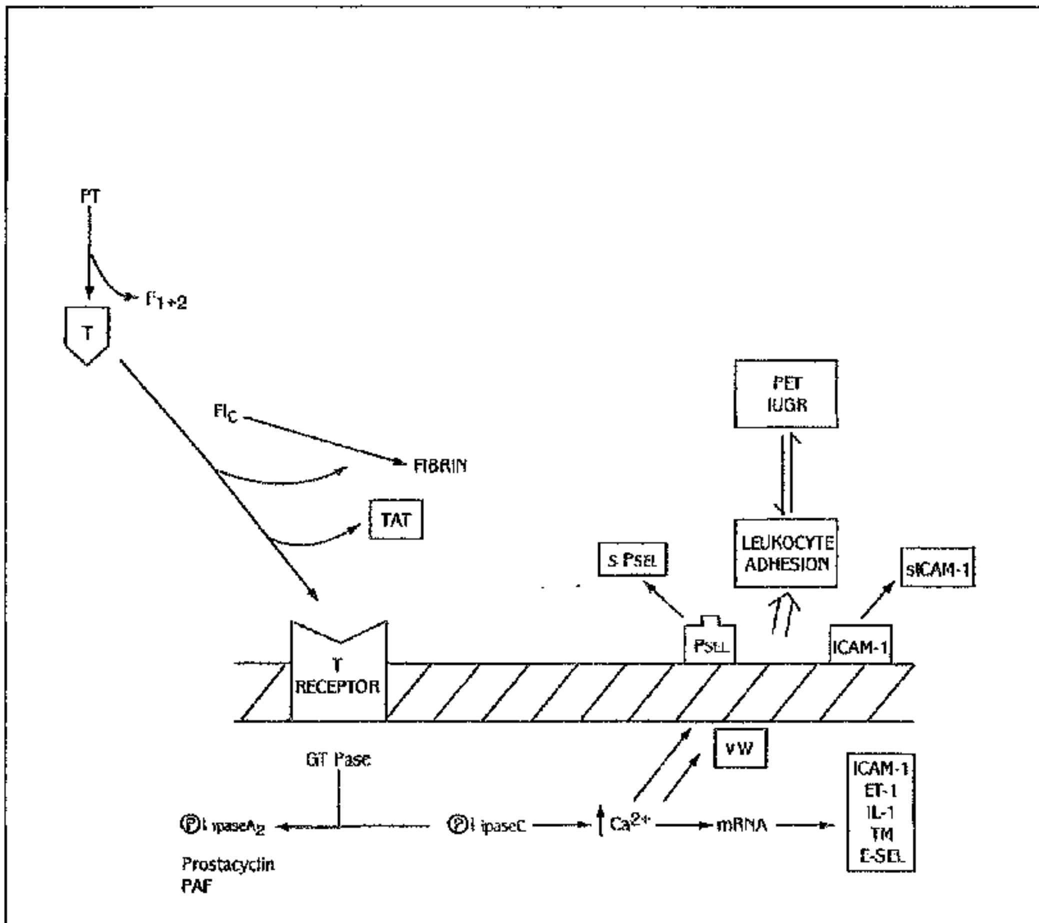
- to examine the response of an inflammatory marker (the adhesion molecule ICAM-1) on the monocyte to thrombin in-vitro to determine if this has potential as a marker of thrombin's influence on monocyte function.
- to determine the relationship between monocyte ICAM-1 and plasma ICAM-1 in pregnancy.
- to determine if the APCR of pregnancy is related to monocyte ICAM-1 and plasma ICAM-1 in vivo.

Figure 1.1 The Coagulation Cascade



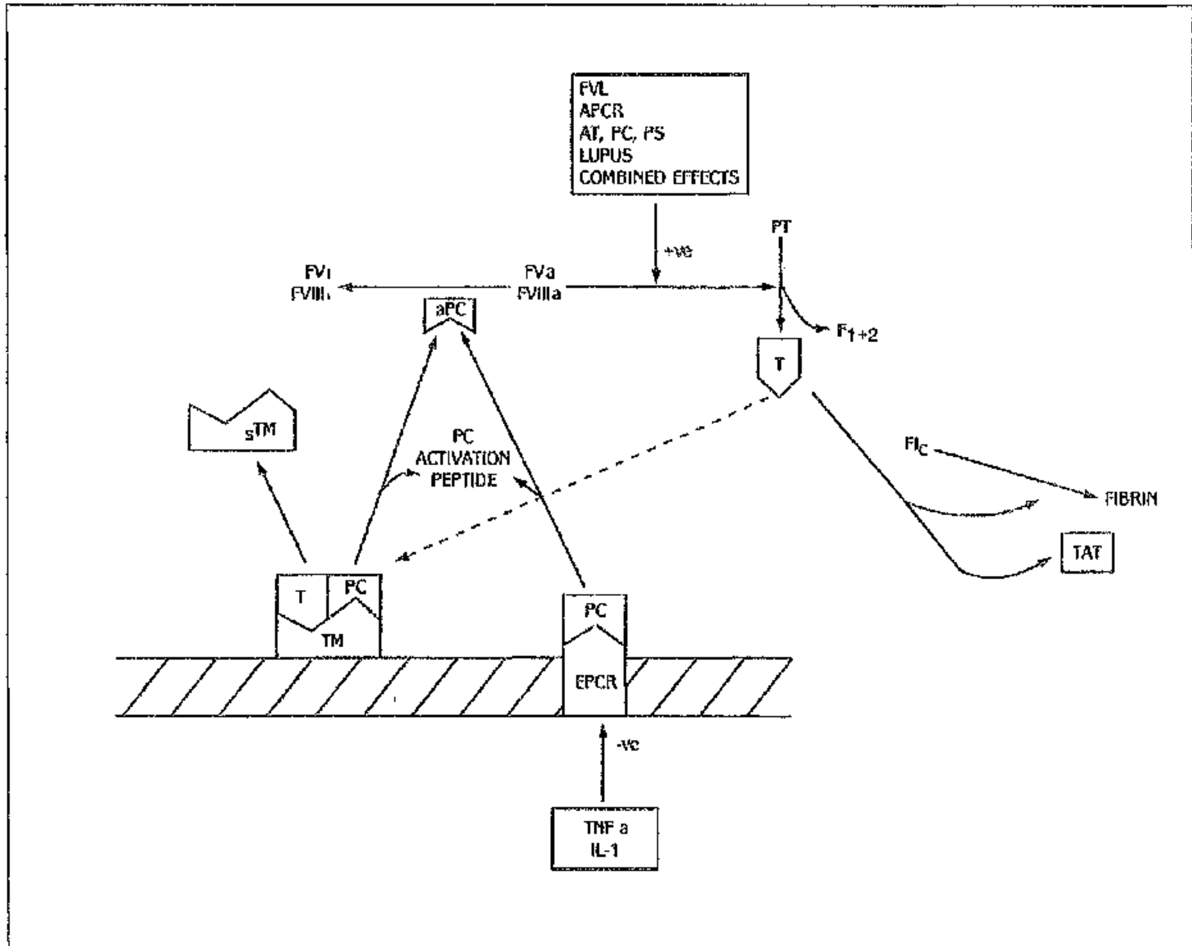
Legend The activation of the coagulation factors with cofactors phospholipid (PL) and Ca++ (Ca). Pre-kallikrein (PK) and high molecular weight kininogen (HK) is shown. The result of this cascade is the generation of thrombin, which is pivotal in fibrin formation, platelet/endothelial activation, fibrin stabilisation as well as the anticoagulant functions outlined in Figure 1.4.

Figure 1.2 The interaction of thrombin with the endothelium



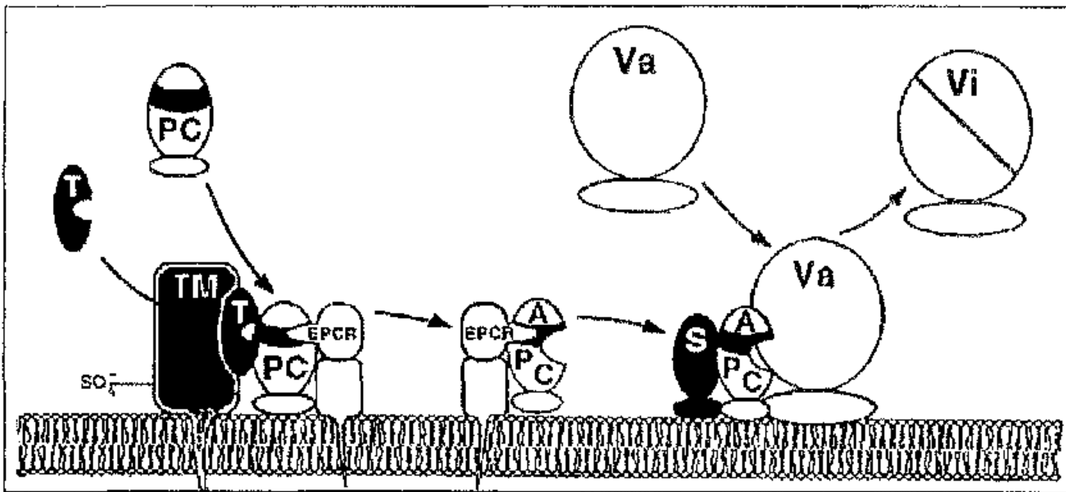
Legend Thrombin (T) from the coagulation cascade interacts with a specific transmembrane receptor (T RECEPTOR). This results in a rise in intracellular Ca $^{2+}$ and an increase in ICAM-1, endothelin-1 (ET-1), interleukin-1 (IL-1), thrombomodulin (TM) and E-selectin (E-SEL) mRNA. This is accompanied by an increase in the release of P selectin (PSEL) and von-Willebrand factor (vW) from endothelial stores and an increase in ICAM-1 expression on the endothelial surface. This results in leukocyte adherence to the endothelium. The resultant inflammatory response may be associated with pre-eclampsia (PET) and fetal growth retardation (IUGR).

Figure 1.3 The interaction of the protein C/protein S system with the endothelium



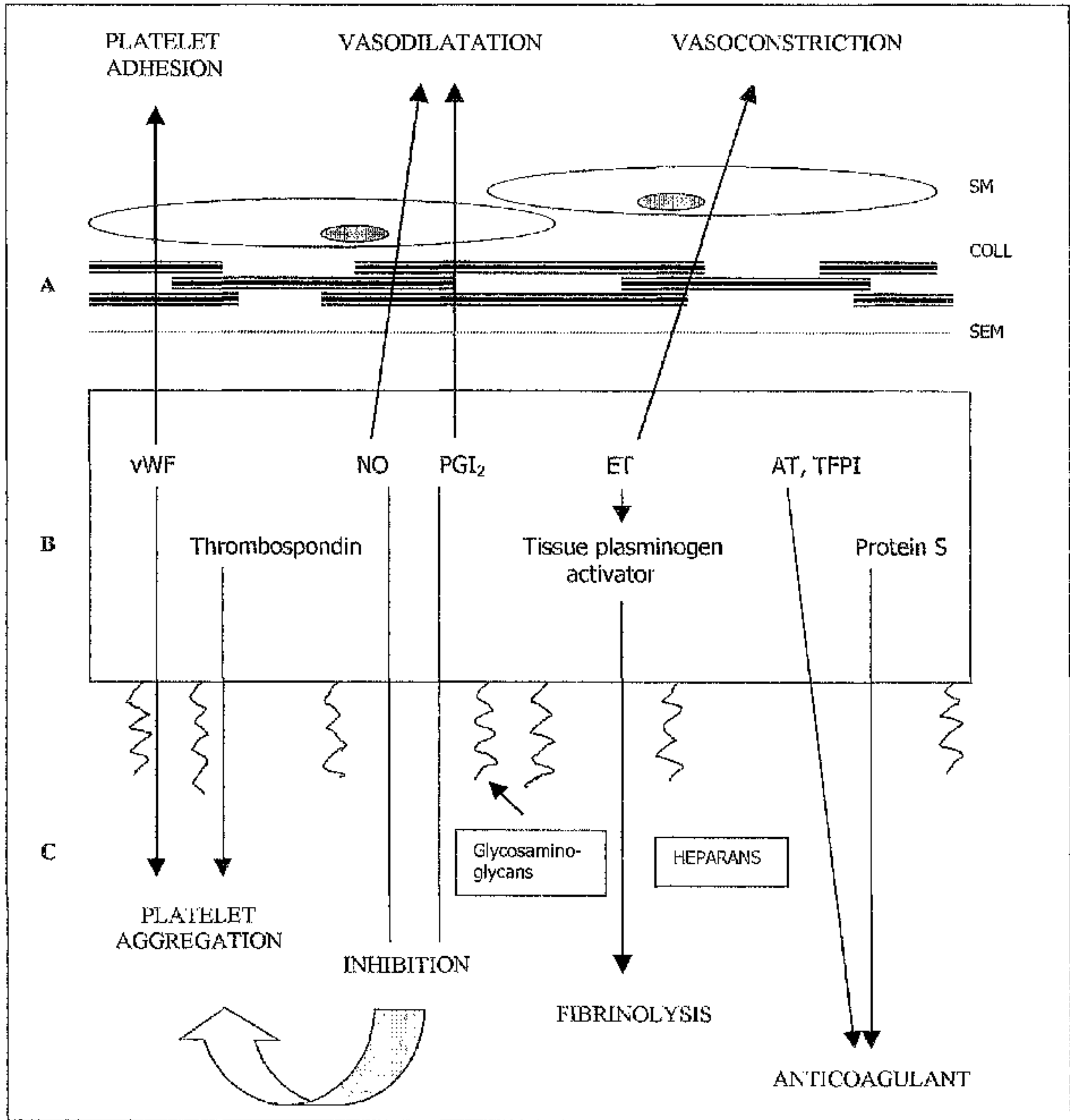
Legend The generation of thrombin (T) from the coagulation cascade is shown. T generation depends on the availability of activated factors V (Va) and VIII (VIIIa). FVL, APCR, AT protein C and S and lupus inhibitors have a positive effect on T generation. T interacts with protein C (PC) and thrombomodulin (TM) to activate PC to activated PC (aPC). T also activates fibrinogen (F1c) to fibrin. The source of prothrombin (PT) fragment 1+2 (F1+2) and thrombin-antithrombin (TAT) complexes is shown. The reported influence of inflammation via TNF alpha (TNF a) and interleukin-1 (IL-1) on the system is also shown. Inflammation may also release TM from the endothelium (sTM).

Figure 1.4 The protein C/protein S system



Legend Thrombin (T) interacts with thrombomodulin (TM) on the endothelial surface. This alters the specificity of thrombin so that it activates PC to activated PC (APC). PC and APC interact with the endothelial surface via phospholipid and may also bind to the endothelial PC receptor (EPCR). The cofactor protein S (S), along with APC inactivates activated factor V (Va) to inactivated factor V (Vi). By a similar mechanism APC also inactivates factor VIIIa.

Figure 1.5 The endothelium and coagulation



Legend A = the vessel wall, B = the endothelium and C = the vessel lumen. SM = smooth muscle, COLL = collagen and SEM = subendothelial microfibrils. The endothelium contributes to platelet aggregation by synthesis and release of von Willebrand Factor (vWF) and thrombospondin. This is inhibited by nitric oxide (NO) and prostacyclin (PGI_2). The endothelium participates in fibrinolysis via endothelin (ET), tissue plasminogen activator and plasminogen activator inhibitors. Via antithrombin (AT), tissue factor pathway inhibitor (TFPI), protein S, and heparans as well as protein C activation, the endothelium provides anticoagulant functions. The effect of these substances on platelet adhesion, vasodilatation and vasoconstriction is also shown.

Chapter 2

Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy

2.1 Pregnancy is associated with significant changes in haemostasis. Over the last decade a number of studies have implicated the haemostatic mechanism with the occurrence of maternal and fetal disorders such as thrombosis[150, 208], pre-eclampsia[133, 152, 183], fetal loss[148] and recurrent miscarriage[197]. As outlined in Chapter 1, recent interest has focused on inherited resistance to the anticoagulant effect of APC added in-vitro to plasma[71]. Two small studies have suggested that APC resistance, occurring in the absence of FVL, may be a feature of normal pregnancy [76,95]. However the prevalence, aetiology and significance of activated protein C resistance occurring in the absence of the FVL mutation remains unclear.

In this chapter, systematic information on a range of haemostatic parameters in normal pregnancy and the early puerperium is presented. A study detailing the effect of gestation on APC response assessed by the original unmodified APTT-based assay and an APTT-based assay modified by pre-dilution of subject samples with factor V depleted plasma[209] was carried out. The frequency of acquired APCR in normal pregnancy was determined and the changes in components of the protein C/protein S system which may contribute to alterations in APC response were investigated.

2.2 Materials and methods

2.2.1 Subjects

A prospective, cross-sectional study of women during normal pregnancy was carried out. 239 healthy women attending a routine antenatal clinic at Glasgow Royal Maternity Hospital were entered into the study. Subjects had no previous history of hypertension or recurrent miscarriage, no significant medical disorder and no personal or family history of thrombosis. The study was approved by the hospital ethical committee and written consent was obtained from all participating subjects. Each subject was sampled on a single occasion. Samples were obtained at all stages of gestation and at three days post-partum (3PN) by venepuncture from the antecubital vein and anticoagulated in 1/10 volume of 0.105M sodium citrate. Following delivery, patient case records were re-examined to confirm that all pregnancies remained uncomplicated. Pregnancy duration, blood pressure, urinalysis results, blood loss at delivery, placental and fetal weight were recorded.

2.2.2 Assay Methodology

Immediately after collection, samples were stored at 4°C for less than one hour, prior to double centrifugation at 3000G for 12 minutes. The resultant platelet poor plasma was stored at -70°C until analysis.

The APCR was assessed using the Coatest APC resistance kit (Chromogenix AB, Sweden), using the automated coagulometer ACL 3000 and expressed as an APC sensitivity ratio (APC:SR). The Coatest APC resistance kit is an Activated Partial Thromboplastin (APTT) based test which provides a semi-quantitative determination of the human response to APC. Subject plasma is incubated with the APTT reagent (a mixture of purified phospholipids with colloidal silica as a contact activator) at 37°C. Coagulation is triggered by the addition of calcium chloride ($CaCl_2$) in the absence and presence of APC and the time for clot (fibrin) formation is recorded. A ratio of clot time with APC to clot time without APC is calculated. This is the APC:SR.

The ACL 3000 uses an optical detection method to detect fibrin formation. The light source is a light emitting diode (wavelength 660 nm). The light ray is directed to the measuring cuvette by means of an optic fibre system. The scattered light is read at 90° with respect to the light source by a solid state detector. Fibrin formation is detected by the change in light scatter associated with the formation of fibrin clot.

The Modified APC:SR (mAPC:SR) was measured using Coatest APC resistance kit (Chromogenix AB, Sweden). In this test the subject plasma is pre-incubated with factor V depleted plasma in a dilution of 1 part subject plasma to 4 parts factor V depleted plasma (Chromogenix AB, Sweden). The APC response of the mixture is obtained as above. References ranges for APC:SR and mAPC:SR were obtained from the analysis of plasma obtained from 25 healthy, non-pregnant, non-COCP using females.

Protein C (PC) activity was assessed using an automated amidolytic assay using Immunochrom Protac Activation kit (Immuno, Austria). The test is based on the chromogenic substrate PCa-1 for the enzyme activated PC. PC in the test sample is activated with Protac®, an extract of the venom of the snake Agkistrodon contortix. The resultant activated PC then reacts directly with the substrate PCa-1 to release the chromogen p-nitroaniline. The level of p-nitroaniline is directly proportional to the level of PC.

Total protein S (PST) and free protein S (PSF) antigen were measured using an enzyme linked immunoassay (ELISA, Dako Ltd, UK). In plasma, approximately 60% of circulating protein S is bound to C4b binding protein. Only free protein S is able to function as a cofactor for protein C. Polyethylene glycol 8000 (PEG 8000) is used to precipitate the C4b-binding protein-protein S complex from the plasma leaving free protein S in the supernatant. The method allows the simultaneous measurement of PSF and PST by ELISA using anti-protein S IgG antibody as a capture antibody and the same antibody, linked to horse radish peroxidase, as the detection antibody.

IgG and IgM anticardiolipin antibody (ACA) assays were performed using sandwich ELISAs (Cambridge Life Sciences, UK). These employ antigen (cardiolipin) coated micropins. The pins are immersed into wells containing subject or reference plasma. Any antibody present will bind to the pin surface. The pins are then dipped into wells containing anti-IgG-peroxidase or anti-IgM-peroxidase conjugate which will bind to any captured antibody. Unbound antibody is removed by a washing stage. The pins are then dipped

into wells containing a colourless substrate. The intensity of yellow colour formed from the reaction is proportional to the concentration of anticardiolipin antibody bound in the first incubation.

F1+2 was assessed by ELISA ('Enzygnost F1+2 micro', Behring, Germany)[210]. This ELISA is based on a sandwich principle. During the first incubation, F1+2 antigen in the sample binds to anti-F1+2 antibodies fixed to the surface of the micro-titration plate. The plate is then washed. Peroxidase-conjugated antibodies are added and, in the second incubation, bind to F1+2. A subsequent wash step removes free peroxidase-conjugated antibodies. Quantification of bound antibody is determined photometrically from the reaction with hydrogen peroxide.

Antithrombin activity (AT) was measured using a fully automated amidolytic method ('Antithrombin III kit', Boehringer-Mannheim, Germany). The test principle is that antithrombin in test plasma is exposed to heparin in the test kit. An excess of thrombin is added. Available thrombin in the resultant solution converts the substrate Tos-Gly-Pro-Arg-pNA to Tos-Gly-Pro-Arg-OH and p-nitroanaline. The level of p-nitroaniline derived from the subject plasma is subtracted from that derived from a mixture of excess thrombin and Tos-Gly-Pro-Arg-pNA alone. This figure is converted to IU/ml of antithrombin.

FVL is identified in DNA isolated from peripheral blood leucocytes. The G→A 1691 mutation disrupts a restriction enzyme site on the DNA. The polymerase chain reaction is used to amplify the DNA spanning the nucleotide change (147 base pairs) and the amplified product is cut using the

enzyme Mnl 1. The products of the reaction are electrophoresed and visualised on 2.5% agarose gel.

Fibrinogen activity level (FIc) was determined by Clauss assay (Organon Teknika Corporation, UK). Prothrombin, factor V, and factor X coagulant activities (c) were determined by automated prothrombin time (PT) clotting assays using the relevant deficient-plasma substrates (Instrumentation Laboratory, UK). Factor VIII, IX XI, and XII coagulant activities (c) were assessed by automated activated partial thromboplastin time (APTT) assays using the relevant deficient-plasma substrates (Diagnostic Reagents Ltd [VIII, IX]; Instrumentation Laboratory, UK [XI, XII]) on an ACL 3000 coagulometer. IL-Test™ calibration plasma, (calibrated against the 1st International Standard Plasma-84/665, Instrument Laboratory, UK) was used as reference plasma in the prothrombin, FIXc and FXc assays and Diagen Normal Reference Control Plasma (calibrated against the 3rd International standard plasma-91/666, Diagnostics Reagents Ltd, UK) as reference plasma for the FVIIIc assay. Von-Willebrand factor Antigen (vWF Ag) level and Von-Willebrand Ristocetin Cofactor Antigen (vWF Ricof) levels were determined by ELISA using double-antibody 'sandwich' techniques (Dako Ltd, UK and Shield Diagnostics, UK).

2.2.3 Statistical Analysis

Results during pregnancy were divided into gestational age periods of 6-11, 12-16, 17-23, 24-28, 29-35, 36-40 weeks and three days post-natal (3PN).

Mean and 2 standard deviation (2 SD) normal ranges were produced for each period. For non-parametric data, data were logarithmically transformed prior to statistical analyses. Normal ranges were derived for APC:SR and mAPC:SR after the exclusion of subjects with elevated anticardiolipin activity or the G→A 1691 mutation. Where the data had been transformed, the normal ranges for the transformed data were transformed back in order to provide normal ranges for the original (untransformed) data. In those subjects with no evidence of increased anticardiolipin activity or the G→A 1691 mutation, linear regression analyses were carried out to compare changes in APC:SR, factor Vc, factor VIIIc, free protein S antigen and haematocrit with gestation. Linear regression analyses were also used to compare APC:SR with factor Vc, factor VIIIc and free protein S antigen levels during pregnancy and the puerperium. Analysis of variance was used to compare parameters at different gestations. Statistical analyses were carried out using Minitab® for Windows® Release 11 statistical software (Minitab Inc, USA).

2.3 Results

2.3.1 The change in coagulation parameters with gestation

Increasing concentrations of factor VIIIc (from 107iu/dl, SD 79 at 6-11 weeks to 192iu/dl, SD 138 at 3 days post-natal) were seen as pregnancy advanced, with levels remaining elevated at 3 days post-natal (Table 2.1 and Figure. 2.1). Factor Vc rose after 16 weeks gestation (from 101u/dl, SD 61

at 12-16 weeks to 141u/dl, SD 70 at 3 days post-natal) and elevated levels persisted to 3 days post-natal (Table 2.1 and Figure 2.1). AT (Table 2.1) and PC (Table 2.1 and Figure 2.1) levels showed no relationship to increasing gestation, although an increase in protein C activity was seen at 3PN (Table 2.1: analysis of variance of 3PN and 36-40 week values, $p=0.0001$). PST and PSF fell progressively through pregnancy (Table 2.1 and Figure 2.1). A fall in APC:SR was observed with increasing gestation (Figure 2.2, Linear regression $R^2=13\%$, $t=-4.00$, $p<0.001$). mAPC:SR showed no significant change with increasing gestation (Table 2.1 and Figure 2.2). A significant rise in F1+2 was noted when 12-16 week and 24-28 week values were compared (Table 2.1: analysis of variance of 12-16 week and 24-28 week values, $p=0.006$). F 1+2 levels remained elevated at 3PN (Table 2.1). Summary data for other factors are shown in Table 2.1.

2.3.2 FVL and anticardiolipin antibody positive subjects

Six individuals of 239 were heterozygous for the FVL mutation. All six subjects had a normal gestation and uncomplicated delivery at term. In these subjects there was no observed difference in F1+2 in comparison with subjects without the FVL mutation. A further seven individuals of 239 (2.9%) showed levels of ACAs beyond 2 SD above the mean for the gestational period of the sample. Only one subject with elevated ACAs had an APC:SR less than 2.6 (APC:SR=2.1). All seven subjects had a normal gestation and

uncomplicated delivery at term. In these subjects there was no observed difference in F 1+2 in comparison with subjects without elevated ACAs.

2.3.3 Relationship of FVIIIc, FVc, protein S with acquired APCR

APC:SRs of less than 2.6 (local non-pregnant female reference range: 2.6-4.5) were seen in 38% (22/58) of subjects in the third trimester of pregnancy (28 to 40 weeks gestation) in the absence of the FVL mutation or elevated ACAs. These women were defined as having acquired APCR.

In those subjects without elevated ACAs or FVL (n=226), regression analysis revealed a significant negative relationship between factor VIIIc (linear regression R^2=3.7%, t=-2.81, p=0.005), factor Vc (linear regression R^2=5.6%, t=-3.45, p=0.001), and a positive relationship of PSF (linear regression R^2=6.8%, t=4.09, p=0.001) with APC:SR. No correlation of APC:SR with haematocrit was observed (data not shown, p>0.05). Previous studies have demonstrated that APC:SR in non-pregnant subjects is unaffected by factor Vc at levels of 12.5% to 100%[211, 212] but elevation of factor Vc beyond 100% of 'normal' is associated with a significant reduction in APC:SR[213]. To minimise the effect of factor Vc on the observed relationship between FVIIIc and APC:SR, samples with factor Vc values greater than/or equal to 120 u/dl were excluded from the analysis. The mean factor Vc level for the whole period of gestation was 120 u/dl and as factor V rose throughout gestation, this value was chosen to facilitate comparison with previous studies in non-pregnant subjects[211-213]. A

significant relationship was observed between factor VIIIc and APC:SR in the 106 subjects who had a factor Vc less than 120u/dl (Figure 2.3: linear regression R^2=11%, t=-3.58, p=0.001).

This procedure was repeated to determine if the relationship between FVc and APC:SR persisted when high values of factor VIIIc were excluded from the analysis. 57 subjects had factor VIIIc levels less than 120 iu/dl. In this group a significant correlation between factor Vc and APC:SR was observed (Figure 2.4: linear regression R^2=10.5%, t=-2.52, p= 0.01).

Free protein S levels also showed a significant correlation with APC:SR in the 37 individuals with levels of both factor Vc and factor VIIIc less than 120u/dl (linear regression R^2=12.7%, t=2.26, p=0.03).

2.3.4 Relationship between APC:SR, placental weight, fetal weight and F1+2

No significant difference in placental weight was observed between those with acquired APCR and those with a normal APC:SR (acquired APCR mean placental weight 631g vs 614g, two sample t test p>0.05). In subjects with acquired APCR smaller birth weights were observed (mean weight 3240g vs 3566g, difference 320g, two sample t test p=0.03).

For all FVL negative subjects, a significant inverse relationship between F1+2 levels and APC:SR (linear regression R^2=16.7%, t=-4.08, p=0.001) was observed. In subjects sampled in the third trimester a similar relationship was observed (Figure 2.5, linear regression R^2=21.4%, t=-3.30, p=0.001),

although a stronger positive relationship between F1+2 and gestation (linear regression R^2=30.7%, t=5.65, p=0.0001) was noted.

2.4 Discussion

2.4.1 This study provides data on APC response, protein C, protein S and other haemostatic variables in 239 women during pregnancy. A 29% rise in factor Vc was observed from 6-11 weeks to 36-40 weeks gestation (mean, SD, p=0.01). In previous studies no consistent elevation of factor Vc has been reported[119, 120] although in the study by Stirling et al[119], an elevation of FVc was observed early in pregnancy and immediately after delivery. Higher levels of FVc have also been reported to occur in normal pregnant subjects at delivery[95]. Elevation of factor VIIIc was seen throughout pregnancy and is consistent with previous studies[119, 126]. It is likely that these changes are multifactorial, but relate in part to changes in hormones during gestation. These elevations persisted into the early puerperium. As with many previous studies no effect of gestation on AT activity was seen[125, 126]. Consistent with previous investigations PC activity showed no consistent trend with gestation[121, 122] and the protein C levels observed during pregnancy in this study are within the local reference range for protein C activity in equivalent age non-contraceptive using females[102]. A rise in PC activity at three days post-partum was observed. This is consistent with the rise in PC antigen levels in the immediate post-partum period previously reported[121]. The mechanism of the rise in PC levels in

the puerperium is unclear, as PC is not considered to be an acute phase reactant[214]. Rising PC levels are seen in a number of clinical circumstances including oral anticoagulant withdrawal[215, 216], anabolic steroid usage[217] and in the late normalisation of reduced levels occurring after major surgery[218, 219]. In population-based studies a strong positive correlation between PC levels and lipids has been reported[220, 221]. An elevation in blood lipids has been reported to occur at the end of pregnancy[222] and, as no reduction in PC values is seen in normal pregnancy this elevation of lipids may contribute to the rise in PC observed in the early puerperium.

2.4.2 The observed change in PSF level with increasing gestation is consistent with the recent study by Faught et al[223]. However, in the current study a progressive fall in PST with increasing gestation was observed. This was not observed by Faught et al, but has been previously reported[120, 123, 224]. The normal ranges for both PST and PSF at 6-11 weeks gestation are below the local normal ranges for non-oral contraceptive using females (PST: 64-154 iu/dl, PSF:54-154iu/dl) and suggest that a fall in protein S occurs during the first weeks of pregnancy. These changes present significant difficulty in the diagnosis of inherited protein S deficiency in pregnancy.

2.4.3 The mechanism of the physiological change in APC:SR has been attributed to elevation of factor VIIIc. Similar acquired APC resistance has been found in association with inflammatory disorders[83] and use of the combined oral contraceptive pill[94]. In this study, a fall in APC:SR was observed with increasing gestation in the absence of elevated anticardiolipin antibodies, a

prolonged APTT or the factor V Leiden mutation. This fall correlated with changes in factor VIIIc, factor Vc, and Protein S, but not with changes in haematocrit. This correlation between APC:SR and factor VIIIc persisted when subjects with factor Vc of >/= to 120u/dl were excluded from the analysis. The correlation between APC:SR and factor Vc also persisted when subjects with a factor VIIIc of >/=120iu/dl were excluded from the analysis. In the small number of subjects in the study with both factor Vc and factor VIIIc <120u/dl it was possible to find a correlation between PSF and APC:SR. These results show a relationship between factor VIIIc and APC:SR independent of factor Vc, but also suggest a potential role for factor Vc in APC resistance. Although APC:SR has been shown to be insensitive to Factor Vc levels at normal concentrations[212], the results suggest that high factor Vc levels may influence APC:SR and are in keeping with one other published study which has suggested that APC:SR may be affected by elevated factor Vc levels[213]. It has been suggested that protein S only influences APC:SR when protein S levels fall below 20% of normal[211]. It is possible in pregnancy that APC:SR may be affected by the extreme reduction in protein S values seen in some individuals in the latter stages of normal gestation.

2.4.4 The correlation of factor VIIIc with APC:SR is consistent with previous studies of APC:SR determinants[95, 124] and is in keeping with a previous longitudinal study of APC:SR in normal pregnancy[225]. Although that study utilised different sample timing it reported similar changes in factor VIIIc and APC:SR between 28 and 37 weeks gestation as seen in this study. Modification of the APC:SR assay by pre-dilution of patient plasma with factor

V depleted plasma abolished the pregnancy-associated change in APC:SR. This is presumably by normalisation of coagulation factors (except factor FVc) by dilution and is in keeping with the high specificity of the assay for the factor V Leiden mutation in a variety of clinical circumstances[81]. The normal range for mAPC:SR for the whole of gestation (2.0-2.76) is comparable with the local laboratory non-pregnant female reference range for mAPC:SR (2.2-2.6). The observed rise in F1+2 between 12-17 weeks and 24-28 weeks gestation is consistent with recent other studies utilising the same assay[134, 226] and indicates that a degree of activation of coagulation occurs at a relatively early stage in normal pregnancy.

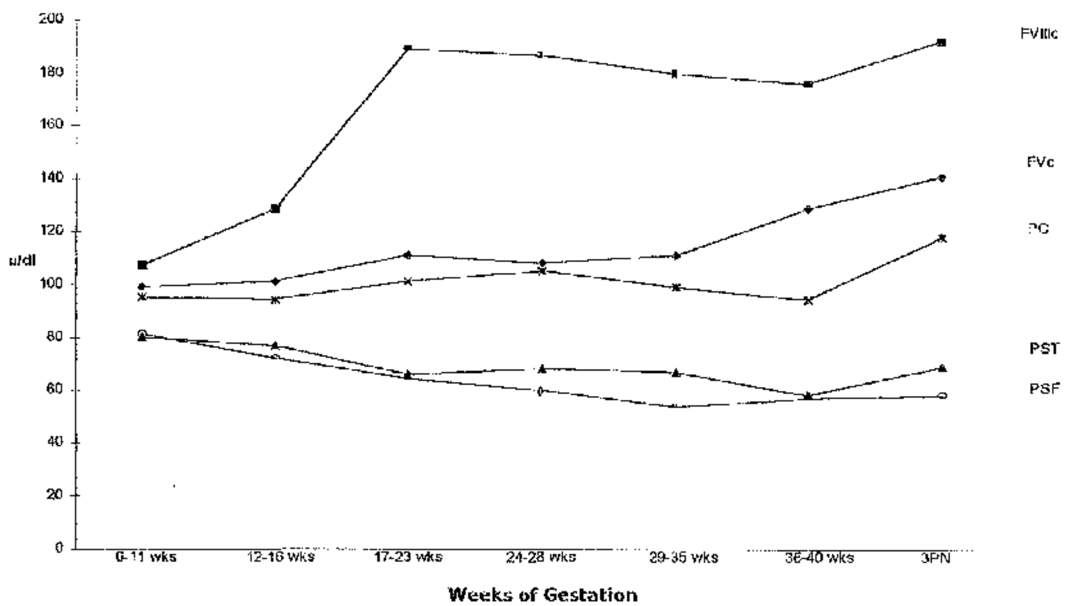
2.4.5 A significant inverse relationship between the marker of thrombin generation F1+2 and APC:SR was observed in this study. This may indicate that the APC:SR is related to thrombin generation. However further study is required to determine if this relationship is independent of gestation. If acquired APCR results in an increase in thrombin generation, this may influence a number of outcomes of pregnancy which have been linked to thrombosis and heritable APCR, such as venous thrombosis and pre-eclampsia. Thrombin generation may also influence, and be influenced by, placental function and fetal development. In this study a small, but significant difference in birth weights was observed in comparison of those subjects with an APC:SR less than 2.6 and those with an APC:SR greater than 2.6 in the third trimester of pregnancy. This would be consistent with a relationship between the sensitivity to APC in pregnancy and fetal development.

Table 2.1 Mean and 2SD reference ranges for haemostatic variables in pregnancy

	6-11WKS N=41		12-16WKS N=28		17-23WKS N=10		24-28WKS N=19		29-35WKS N-35		36-40WKS N-23		3PN N=87	
	MEAN	RR	MEAN	RR	MEAN	RR	MEAN	RR	MEAN	RR	MEAN	RR	MEAN	RR
PST (u/dl)	80	34-126	77	45-109	66	40-92	68	38-98	67	27-106	58	27-90	69	37-85
PSF (u/dl)	81	47-115	72	44-101	64	38-90	60	34-86	54	32-76	57	15-95	58	29-87
PC (u/dl)	95	65-125	94	62-125	101	63-139	105	73-137	99	60-137	94	52-136	118	78-157
AT (iu/dl)	96	70-122	100	72-128	100	74-126	104	70-138	104	68-140	102	70-133	108	77-137
APC:SR	3.12	1.94-4.30	2.82	1.70-3.94	2.82	2.22-3.42	2.62	2.08-3.16	2.64	2.00-3.28	2.63	1.77-3.49	2.76	2.14-3.38
mAPC:SR	2.39	1.91-2.87	2.25	1.65-2.85	2.44	2.08-2.80	2.31	1.85-2.77	2.39	1.89-2.89	2.24	1.68-2.80	2.39	1.99-2.79
F 1+2(nmol/l)	1.1	<2.9	1.1	<1.5	1.3	<2.1	1.8	<3.4	2.0	<3.9	1.9	<3.5	2.2	<4.9
D-dimers(ug/ml)	-	<0.25	-	<0.25	-	<0.25	-	<0.25	-	<0.25	-	<0.25	-	<0.25
ACA IgG (u/ml)	3.1	<8.9	2.9	<8.9	2.5	<8.9	2.5	<8.9	1.8	<8.9	3.4	<8.9	2.4	<6.0
ACA IgM (u/ml)	2.6	<6.6	2.6	<6.6	1.0	<6.6	1.0	<6.6	2.0	<6.6	3.1	<6.6	2.0	<6.6
F1c (g/l)	3.6	2.5-4.8	3.8	2.5-5.1	3.62	2.56-4.68	4.4	2.9-5.9	4.1	2.5-5.8	4.2	3.2-5.3	4.5	3.1-5.8
FIIc (iu/dl)	153	107-200	160	111-209	153	41-265	172	92-252	153	100-211	162	107-217	169	8-231
FVc (u/dl)	99	39-159	101	39-162	111	47-175	108	50-166	111	43-179	129	65-194	141	71-211
FVIIc (iu/dl)	94	48-180	106	51-161	150	64-236	156	60-252	145	65-226	164	96-270	238	90-398
FVIIIc (iu/dl)	107	62-220	129	82-178	189	59-159	187	71-341	180	31-328	176	50-302	192	54-331
FIXc (iu/dl)	100	49-151	106	82-130	96	74-118	121	59-183	109	65-154	114	79-150	136	65-207
FXc (iu/dl)	125	88-162	129	78-180	128	50-206	159	52-263	146	81-212	152	113-191	162	69-254
FXIc (u/dl)	102	50-154	103	58-147	86	58-114	102	45-162	100	31-169	92	36-181	96	46-146
FXIIc (u/dl)	137	70-204	160	52-268	186	64-247	170	54-286	178	78-278	179	62-296	174	86-262
VWAg (iu/dl)	118	52-248	141	30-273	123	37-209	185	72-341	147	88-415	221	98-464	226	102-545
RICOF (iu/dl)	117	47-258	132	55-298	128	50-206	204	68-360	169	86-466	240	100-544	247	97-630

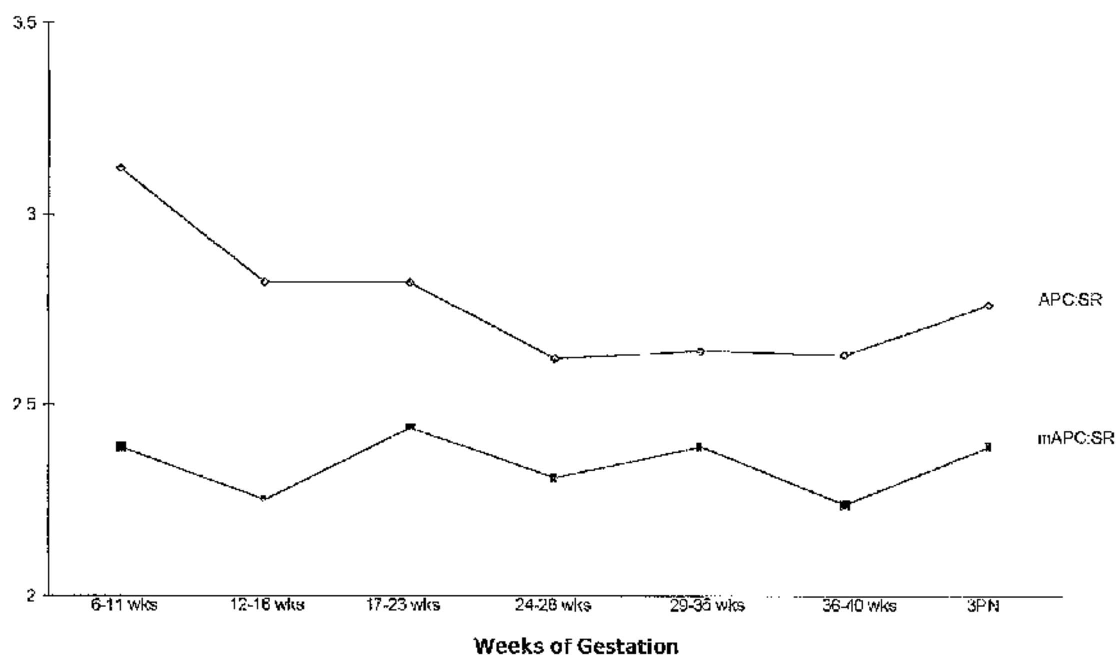
Legend Abbreviations: N = number of subjects examined; RR = 2 S.D. reference range; F = factor; c = coagulant activity. Abbreviations for each factor are explained in Materials and Methods. * Mean results derived after log transformation of original data. Due to the nature of distribution of F1+2, and ACAs, an upper limit of normal is shown.

Figure 2.1 Changes in factors VIIIc, Vc, protein S and protein C with gestation



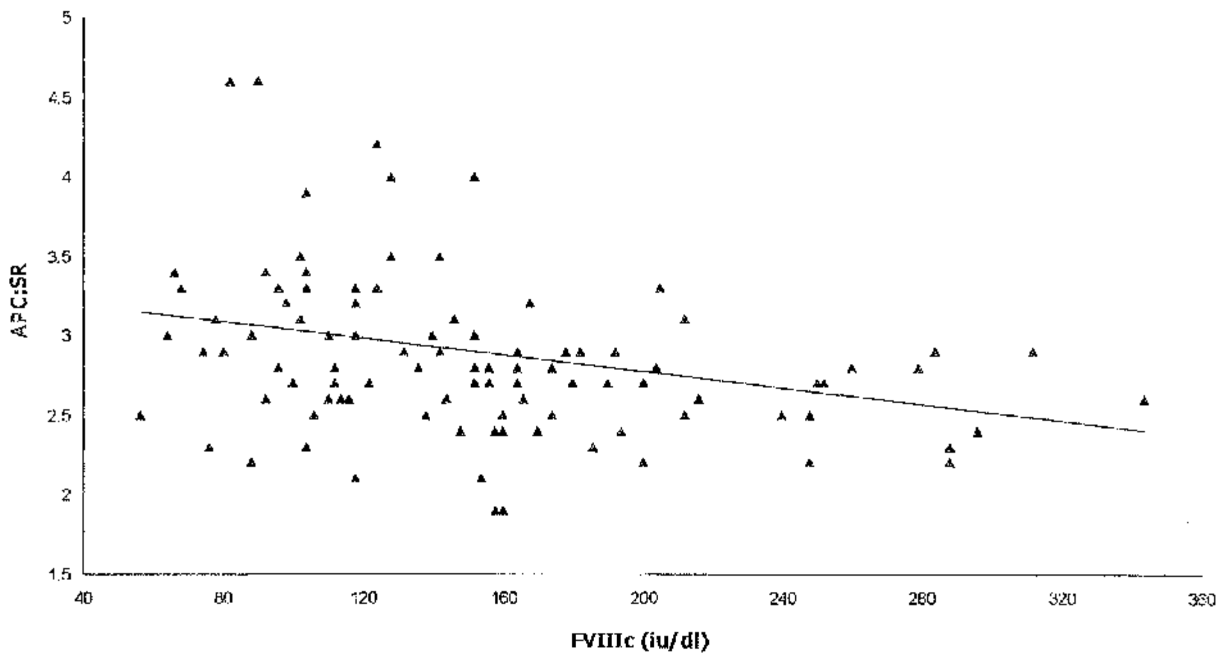
Legend Mean values for each variable against periods of pregnancy (weeks) and at 3 days post-natal (3PN) are shown. Abbreviations for each factor are explained in Materials and Methods. FVc (diamond), FVIIIc (square), PST (triangle), PSF (open circle), PC (*).

Figure 2.2 Changes in APC:SR and mAPC:SR with gestation



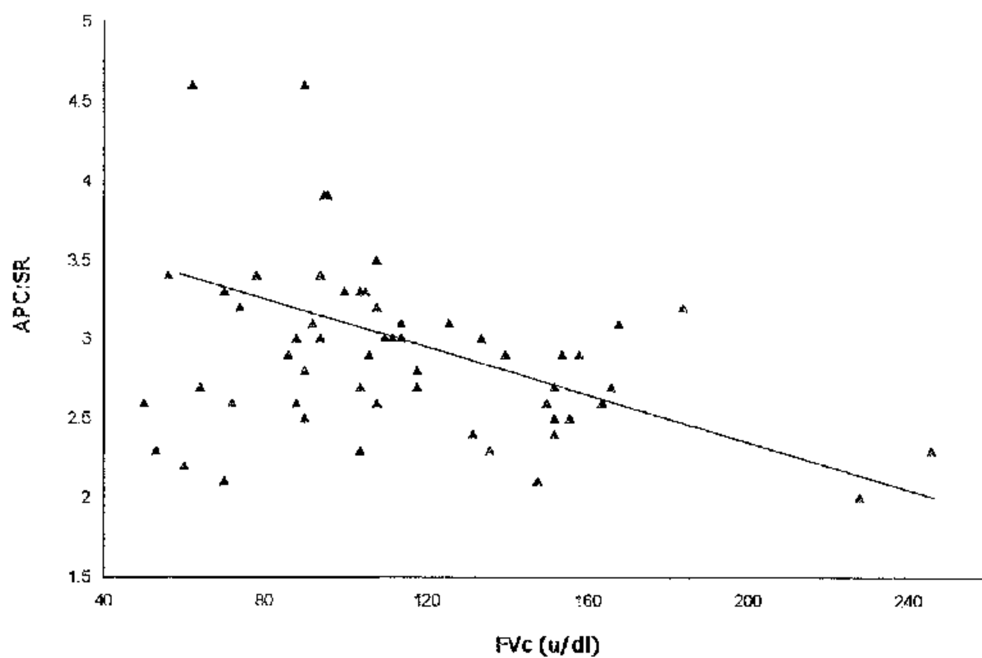
Legend Mean values for each variable against periods of pregnancy (weeks) and at 3 days post-natal (3PN) are shown. Abbreviations for each factor are explained in Materials and Methods. APC:SR (square), mAPC:SR (diamond).

Figure 2.3 APC:SR vs FVIIIc when FVc <120u/dl



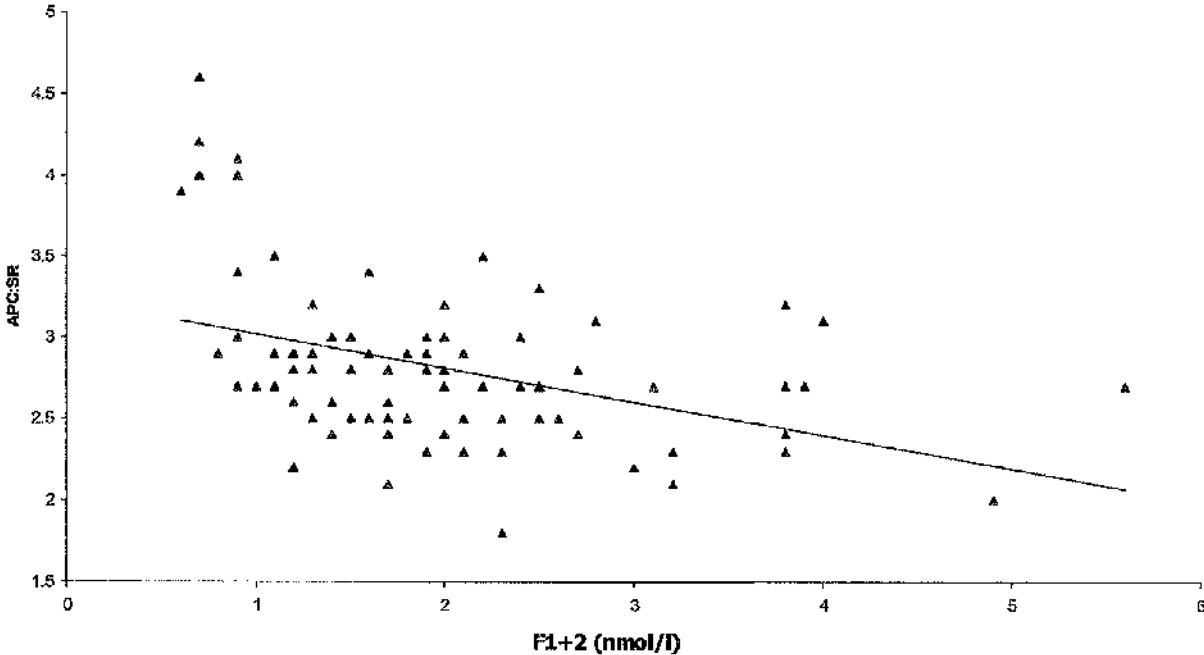
Legend Correlation of APC:SR and FVIIIc in subjects with a FVc level <120u/dl. For guidance a linear regression line is shown.

Figure 2.4 APC:SR vs FVc when FVIIIc <120iu/dl



Legend Correlation of APC:SR and FVc in subjects with a FVIIIc level <120iu/dl. For guidance a linear regression line is shown.

Figure 2.5 Prothrombin fragment 1+2 (F1+2) with APC:SR



Legend Correlation of APC:SR and prothrombin fragment 1+2 at 28-40 weeks gestation. For guidance a linear regression line is shown.

Chapter 3

Acquired activated protein C resistance and markers of thrombin generation

3.1 APCR associated with FVL has been shown to be associated with higher plasma levels of two markers of thrombin generation; F1+2 and thrombin-antithrombin (TAT) complexes[227, 228]. TAT complexes are formed as thrombin binds to its principal inhibitor antithrombin. In view of this and the association between F1+2 and APC:SR observed in Chapter 2, a further study of the relationship between thrombin generation and acquired APCR was carried out. To exclude any influence of FVL, all individuals were screened to exclude this mutation. To exclude an effect of gestation, all subjects were sampled at presentation for antenatal care and at 28-32 weeks gestation. Subjects with at least one successful pregnancy were chosen as these would constitute a homogenous low risk pregnancy group. Individuals with a history of fetal loss have been reported to have a higher prevalence of heritable thrombophilia[148] and subjects with a history of recurrent fetal loss have been shown to have higher levels of TAT (when assessed out with pregnancy)[229]. Consequently this study was designed to examine the relationship between APC:SR and the change in APC:SR with gestation with markers of thrombin generation and fetal weight. To refine the examination of fetal weight, birth weights were normalised for gestational age, sex and maternal parity.

3.2 Materials and Methods

3.2.1 Subjects

A prospective longitudinal study of 134 pregnant women (median age 30, range 25-35 years) was carried out. Consecutive parous subjects attending a routine antenatal clinic at Glasgow Royal Maternity Hospital were entered into the study. Subjects with at least one successful pregnancy were chosen as these would constitute a homogenous low risk pregnancy group. The study was approved by the hospital ethical committee and written consent was obtained from all participating subjects. To determine the significance of physiological changes in APC:SR, women with a personal history of VTE (n=1) or FVL (n=2) were excluded from the study. In a further 3 subjects, APC:SRs at presentation were not available.

3.2.2 Assay methodology

Blood sampling, plasma processing, APC:SR, F1+2 and FVL analyses were carried out as detailed in Chapter 2. APC:SRs were assessed at presentation for antenatal care (median gestation 12 weeks, range 7-15) and at 28-32 weeks gestation. F1+2 and TAT concentrations were measured at 28-32 weeks gestation. TAT concentrations were measured by ELISA (Enygnost; Behring, Marburg, Germany)[230]. Body mass index (BMI) for each subject was calculated at the time of the initial blood sample (kg/m^2). All subjects

had uncomplicated pregnancies. Case records were retrieved at six weeks after delivery and birth weight centiles were calculated by use of the Medical Research Council normogram[231].

3.2.3 Statistical analysis

As there is no published information on the significance of the change in APC:SR in pregnancy, simple subtraction was used to calculate the difference in booking and 28-32 weeks gestation APC:SRs and subjects were grouped into those with an increase (Group A, n=90, mean increase 0.43, SD 0.32) or no increase (Group B, n=38) in resistance with increasing gestation. Comparison of groups was carried out by Student t test analysis for parametric and Mann-Whitney U test for non-parametric data where appropriate. The relationships of APC:SR with age, height, weight, BMI, TAT, F1+2 and birth weight centile were examined by linear regression using Minitab®for Windows® Release 11 statistical software (Minitab Inc, USA).

3.3 Results

3.3.1 No significant relationship between APC:SR and gestation between 7 and 15 weeks was observed (mean APC:SR 2.76, SD=0.38.) At 7-15 weeks no relationship of APC:SR with blood group, smoking history (Table 3.1), age, height, weight or BMI (Table 3.2) was observed.

3.3.2 A fall in APC:SR (mean fall 0.43, SD=0.32) was observed in 90 subjects. This group (group A) was defined as showing an increase in APCR with gestation. No difference in TAT or F1+2 values was observed in comparison with those individuals in which no increase in APCR (n=38) was observed (group B). The median birthweight centile in the offspring of women of group A (median centile 49, I.Q.R. 18-75) was significantly lower (Mann-Whitney U test p=0.02) than those in group B (median centile 67, I.Q.R 41-82). These results are shown in Figure 3.1.

3.3.3 No relationship of APC:SR with gestation between 28 and 32 weeks was observed (mean APC:SR 2.50, SD=0.37). At 28-32 weeks gestation a significant inverse relationship between APC:SR and TAT concentration (mean 7.25µg/l, SD=2.6) was observed (Figure 3.2: linear regression R^2=3.8%, t=-2.11, p=0.03). No significant relationship between smoking, blood group (Table 3.1), age, booking weight, height, booking BMI (Table 3.2), F1+2 (mean 2.95 nmol/l, SD=0.90) or birthweight centile and 28-32 week APC:SR was noted.

3.4 Discussion

3.4.1 Unlike non-pregnant subjects[232] no relationship between APC:SR and BMI at booking was observed. No relationship between cigarette smoking and APC:SR was found in this study. This may be due to the sample size of the study as the larger study detailed in Chapter 4 detected a significant positive relationship between APC:SR and smoking.

3.4.2 Normal gestation is associated with a progressive increase in thrombin generation[233]. Additionally, increased markers of thrombin generation, in non-pregnant women, have been associated with a history of recurrent miscarriage[229]. An increase in thrombin generation has also been reported in APCR associated with FVL[228]. In this study a significant inverse relationship between the APC:SR at 28-32 weeks gestation and TAT complex levels in plasma was observed. This relationship appeared to be independent of gestation and suggests that the level of APCR in plasma during pregnancy may reflect thrombin generation. This would be consistent with the association of elevated FVIIIc[85] and protein S deficiency[208] with venous thrombosis.

3.4.3 Although an inverse relationship between APC:SR and F1+2 was observed in Chapter 2 this association was not confirmed in this study. This apparent disparity cannot be explained by the different case-mix of the two studies and comparable F1+2 levels were observed in both. That the relationship between F1+2 and APC:SR was not confirmed when the confounder of gestation was removed suggests that the influence of gestation on both APC:SR and F1+2 resulted in the correlation of APC:SR and F1+2 observed in Chapter 2. It is conceivable that TAT complex levels more sensitively represent thrombin generation as F1+2 has been reported to be less sensitive than TAT to changes in thrombin generation during warfarin therapy[234]. However both assays do appear to have similar profiles in subjects with FVL[227].

3.4.4 In 70.3% of pregnant subjects an increase in APCR with gestation was observed in the absence of the FVL mutation. The significance of this physiological adaptation has not previously been studied. In subjects demonstrating an increase in ACPR with gestation, no significant difference in markers of thrombin generation was observed in comparison to subjects in whom no increase in APCR was noted. However significantly smaller birth weight centiles were observed in those demonstrating an increase in ACPR. The mechanism of this association is unknown. However as with the study described in Chapter 4, it is of interest that those subjects who do not show an increase in APCR with gestation have lower APC:SR values at 7-15 weeks gestation (mean 2.50 vs 2.84, t=4.99, p=0.00001).

Table 3.1 APC:SR at 7-15 weeks and 28-32 weeks gestation compared with blood group and smoking

	APC:SR 7-15 weeks		T Test p	APC:SR 28-32 weeks		T Test P
	mean	SD		mean	SD	
Blood group O	2.78	0.37	>0.05	2.56	0.43	>0.05
Non-group O	2.70	0.42		2.42	0.32	
Smoker	2.76	0.38	>0.05	2.50	0.40	>0.05
Non-smoker	2.73	0.41		2.47	0.36	

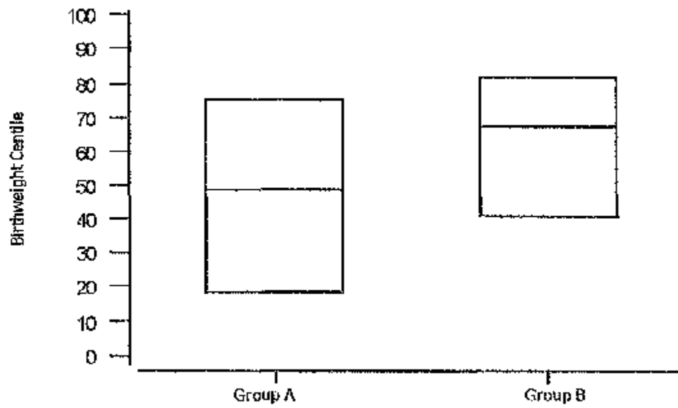
Legend APC:SR values (mean and SD) for blood group O, non-blood group O, smokers and non-smokers are shown at 7-15 and 28-32 weeks gestation. The p values of the t test comparisons of APC:SR for blood group O vs non-blood group O and for smokers vs non-smokers at both 7-15 and 28-32 weeks gestation are shown.

Table 3.2 The relationship of APC:SR with age, height, weight and BMI

	At 7-15 weeks		APC:SR 7-15 weeks			APC:SR 28-32 weeks		
	mean	SD	R^2	T	p	R^2	T	p
Age (yrs)	30	3	0.0%	0.24	0.8	0.5%	-0.78	0.4
Height (m)	1.63	0.06	0.0%	0.03	0.9	0.5%	0.79	0.4
Weight (kg)	68.5	14.2	0.0%	-0.1	0.9	0.6%	0.91	0.4
BMI (kg/m^2)	25.7	5.0	0.0%	-0.16	0.9	0.3%	0.61	0.5

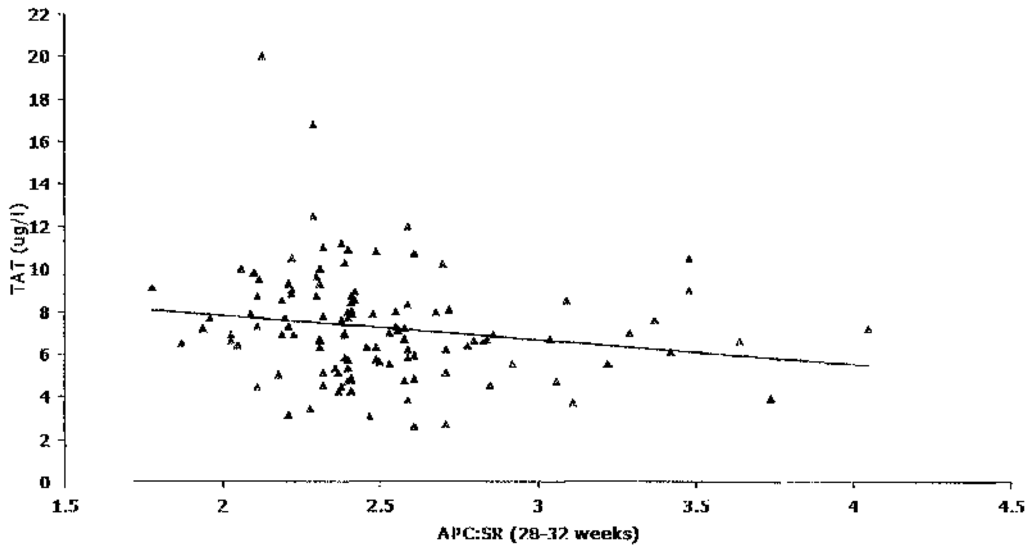
Legend The mean and SD of age, weight, height and body mass index (BMI) at 7-15 weeks gestation is shown. The results of linear regression analysis of each parameter with APC:SR at 7-15 and 28-32 weeks gestation is shown.

Figure 3.1 Comparison of birthweight centile in Group A vs Group B



Legend Median (line), upper and lower limits of interquartile range (upper and lower box limits) are shown for those subjects with an increase (Group A) or no increase (Group B) in APCR with gestation.

Figure 3.2 TAT concentration vs APC:SR at 28-32 weeks gestation



Legend The relationship between APC:SR and TAT (ug/l) at 28-32 weeks gestation is shown. A linear regression line is shown.

Chapter 4

The relationship between APCR, pregnancy outcome, the full blood count and bleeding in pregnancy

4.1.1 In keeping with its prothrombotic potential, a number of studies have suggested that heterozygous carriage of FVL confers at least a 2 fold risk of both pregnancy-induced hypertension[152, 184, 185] and late fetal loss[148, 201]. However, as it seems likely that the FVL mutation was present in the first Europeans around 30-40,000 years ago (reviewed in[204]) and in view of its relatively high prevalence in European subjects[75], it has been speculated that FVL may confer a beneficial effect on the carrier. The most obvious potential advantage of an increase in thrombotic potential would be the reduction of blood loss and the protection of red cell levels. This hypothesis has been examined in pregnancy in a single study[205], which showed a significantly reduced risk of intrapartum bleeding and better preservation of the post-partum haemoglobin levels in FVL heterozygote subjects when compared to the non FVL pregnant subjects.

4.1.2 Changes in APCR, occurring in the absence of FVL, are seen in the majority of normal pregnancies (see Chapters 2 and 3). The influence of this physiological adaptation on the outcome of pregnancy is unknown. In family and case-control studies, individuals with thrombosis (without FVL) have lower APC:SR values than non-thrombotic individuals[235, 236]. Low APC:SRs have also been associated with thrombosis in Japanese subjects who are unlikely to have FVL[237]. More recently de Visser et al have shown a 2.5 relative risk ($C.I._{95}$ 1.5-4.2) of venous thrombosis associated with the lowest quartile of APC:SR in FVL negative subjects[238]. Whether the APC:SR (in the absence of FVL) influences the occurrence of thrombosis-

linked complications of pregnancy such as fetal loss and pre-eclampsia is unknown.

4.1.3 To examine the significance of physiological changes in APCR during pregnancy, the APC:SR was examined, in a prospective longitudinal study of 1046 subjects, at two time points during pregnancy. The APC:SR was analysed in relation to the demographics, personal and family history of the study population, as well as pregnancy outcome measures of maternal blood loss, changes in maternal blood count and the occurrence of fetal loss and maternal hypertension.

4.2 Materials and methods

4.2.1 Subjects

1046 subjects (median age 29 years, interquartile range 25-32) who consecutively attended for routine antenatal care at the Glasgow Royal Maternity Hospital were recruited. Ethical approval was obtained from the local ethical committee at the Glasgow Royal Infirmary NHS Trust and written consent was obtained from all participating subjects. The study was blinded to the subjects and their clinicians until 6 weeks after delivery of the current pregnancy. Past obstetric and medical history was collected by questionnaire at entry to the study and the data on the outcome of the current pregnancy was obtained by examination of case records six weeks after delivery.

4.2.2 Laboratory Analysis

Blood samples were collected from the antecubital vein at 7-15 weeks gestation and between 27 and 40 weeks gestation in all subjects. Plasma samples were anticoagulated in 1/10 volume of 0.105 M sodium citrate. Sample processing, APC:SR, mAPC:SR and mutation analysis for the G→A 1691 mutation in the gene coding for coagulation factor V were assessed as detailed in Chapter 2. Samples for full blood count analysis were anticoagulated in K_3 EDTA. Analysis of full blood count parameters (white blood cell count (WBC), red cell count (RBC), haemoglobin (Hb), haematocrit (HCT), mean red cell volume (MCV), mean cell Hb concentration (MCHC), red cell distribution width (RDW) and platelet count) was carried out using the Coulter 'GenS' System (Coulter Electronics, USA).

4.2.3 FVL classification

The APC:SR and the modified APC:SR were measured in all subjects between 7-15 weeks gestation. The mAPC:SR has been shown to be highly specific for the FVL mutation in a variety of circumstances[209, 239] and is unaffected by gestation (Chapter 2). Svensson et al [239] have shown that in normal, non pregnant subjects (and in those presenting with VTE) the mAPC:SR is 98.8% specific and 100% sensitive for FVL at greater than/equal to 1.7 SD below the mean mAPC:SR for normal control subjects. To ensure exclusion of FVL in this study a more stringent cut off of >1.3 SD below the

mean was employed. Subjects with an mAPC:SR >1.3 SD below the mean (n=100) underwent direct mutation analysis for the G→A 1691 mutation. 37 Subjects were found to be heterozygous for the FVL mutation. All FVL positive subjects lay beyond 2 SD below the mean (Figure 4.1 mAPC:SR range 1.37-1.72). 1009 subjects were classified as FVL negative (mAPC:SR 1.49-4.0). In 583 non-FVL subjects the APC:SR was also measured at 27-34 weeks gestation. In the remainder the second APC:SR assessment was carried out beyond or before this gestation and to exclude an effect of gestation on the APC:SR these subjects were not included in the analysis.

4.2.4 Definition of pregnancy-induced hypertension (PIH) and pre-eclampsia (PET)

PIH was defined as diastolic BP >90 mmHg on two or more occasions 4 hours apart, in subjects normotensive at recruitment, with no history of hypertension. PET was defined as PIH with >2+ proteinuria.

4.2.5 Statistical Analysis

Relationships between FBC parameters and APC:SR were assessed by linear regression analysis. Comparison of blood count parameters between FVL positive and negative subjects was carried out using the two sample 't' test. Comparison of non-parametric data was achieved by Mann-Whitney U test (MW) analysis. Comparison of continuous variables was achieved by linear regression. These analyses were completed on Minitab®for Windows®

Release 11 statistical software (Minitab Inc, USA). Comparison of categorical data was achieved by Mantel and Haentzel Odds Ratio analysis (OR_{MH}).

4.3 Results

4.3.1 APC:SR and mAPC:SR values

The mean value of APC:SR at 7-15 weeks for all subjects was 2.72 (SD 0.56). The mean value for mAPC:SR for all subjects at 7-15 weeks was 2.28 (SD 0.25). In the FVL negative group no correlation with gestation and APC:SR was observed between 7 and 15 weeks. Similarly there was no correlation of APC:SR with gestation between 27 and 34 weeks. In the current pregnancy, 83 (of 1046) subjects developed PIH and 22 PET. PIH occurred in 11.9% and PET in 2.5% of primigravid women.

4.3.2 Comparison of FVL negative with FVL positive subjects

The results are summarised in Table 4.1. In first degree relatives of the whole study group a family history of venous thromboembolism (VTE) was noted in 11.7%, and of PIH in 10.6%. There was no significant difference in the occurrence of a positive family history of VTE, PIH, cerebrovascular disease (CVD), or ischaemic heart disease (IHD) in the FVL positive subjects when compared with the FVL negative subjects. No significant difference in the occurrence of PIH in previous pregnancies or of personal history of VTE

was observed between the two groups. In the index pregnancy no significant difference in the occurrence of hypertensive disorders was observed between FVL positive and negative subjects.

Blood count and Blood Loss

No significant difference in any FBC parameter at either 7-15 weeks or 27-34 weeks was observed between FVL positive and negative subjects (Table 4.2). Furthermore, no difference in the change of FBC parameters during pregnancy between the groups was observed. In the comparison of FVL positive and negative subjects (Table 4.3) there was no significant difference in the occurrence of excess blood loss at delivery (estimated by eye at >500mls) or blood loss at delivery requiring additional syntometrine therapy, transfusion or surgery. No difference was found when analysis was restricted to those subjects who had had a spontaneous vertex delivery (SVD) which did not require instrumentation. No significant difference in the occurrence of post-partum haemorrhage (PPH) or PPH requiring readmission, syntometrine or surgery was observed between the two groups. A similar result was obtained when the analysis was restricted to SVD subjects. Anaemia of less than 10g/dl at any gestation was observed in 187 of 1046 subjects. No significant difference in the occurrence of anaemia was observed when FVL positive and negative subjects were compared. Per-vaginal blood loss was reported in 145 subjects prior to 24 weeks gestation and in 102 subjects at or after 24 weeks gestation (72 subjects required

admission, although none required blood transfusion). No difference in the occurrence of blood loss was observed in comparison of FVL positive and negative subjects.

Pregnancy Outcome

11 subjects (of 1046) had a miscarriage between booking for antenatal care and 24 weeks gestation. 12 subjects suffered fetal loss at or after 24 weeks gestation. No miscarriages or fetal losses were noted in FVL positive subjects. There were 3 neonatal deaths. Two occurred in the FVL positive group (OR_{MH} 57.6, $C.I._{95}$ 5.1-647). One of these was found to have suffered neonatal asphyxia and the other to have multiple developmental anomalies.

4.3.3 The APC:SR in FVL negative subjects

No significant difference in APC:SR at 7-15 weeks was observed when those subjects with and without a family history of VT, IHD, PIH, or CVD in a first degree relative were compared. In those subjects with a history of PIH in a previous pregnancy there was no difference in the APC:SR at 7-15 weeks or 27-34 weeks. No relationship between the APC:SR at 7-15 weeks and waist circumference, weight, body mass index, age, alcohol consumption or diastolic blood pressure at recruitment was observed (Table 4.4). Lower APC:SRs were noted in non-blood group O individuals at both 7-15 weeks (mean 2.67, SD 0.45 vs 2.81, SD 0.42: $t=4.61$, $p<0.00001$) (Figure 4.2) and

27-34 weeks gestation (mean 2.36, SD 0.31 vs 2.49, SD 0.38: $t=4.68$, $p<0.00001$). Lower APC:SRs were also observed in non-smokers (Figure 4.2) at both 7-15 weeks (mean 2.72, S.D. 0.45 vs 2.79, S.D. 0.43: $t=-2.29$, $p<0.02$) and 27-34 weeks (mean 2.40, S.D. 0.34 vs 2.49, S.D. 0.36: $t=-3.12$, $p<0.0002$).

Blood count and Blood Loss

No significant relationship between WBC, RBC or platelet count at 7-15 weeks and the APC:SR at 7-15 weeks was observed, although a positive relationship between the APC:SR at 7-15 weeks and the 27-34 week Hb (linear regression R^2=0.7%, p=0.02), HCT (R^2=0.6%, p=0.04), MCV (R^2=1.2%, p=0.004) and MCH (R^2=1.2%, p=0.003) was noted. At 27-34 weeks a similar slight, but significant, positive relationship between the RBC (R^2=0.7%, p=0.04), Hb (R^2=2.7%, p=0.0001), MCH (R^2=1.0%, p=0.02) and the APC:SR at 27-34 weeks was observed. However a much greater negative relationship of gestation with APC:SR (R^2=13.3%, p=0.0001), Hb (R^2=39.3%, p=0.0001) and RBC (R^2=37.3%, p=0.0001) was observed. No significant difference in APC:SR was observed in comparison of subjects who experienced per-vaginal blood loss during pregnancy or subjects who experienced excess loss of blood during delivery or in the early puerperium.

Pregnancy Outcome

A significantly lower APC:SR at 7-15 weeks was observed in subjects, normotensive at recruitment and with no past history of hypertension, who subsequently developed PIH in the current pregnancy (mean APC:SR 2.64, SD 0.34 vs 2.76, SD 0.45, t=-2.89, p=0.004, Figure 4.3). A more marked difference was observed in subjects who developed PET (mean APC:SR 2.50, SD 0.26 vs 2.75, SD 0.44, t=-4.26, p=0.0003, Figure 4.3). A significant difference for PIH persisted when the analysis was restricted to primigravid subjects (mean APC:SR 2.64, SD 0.31 vs 2.76, SD 0.41, t=-2.23, p=0.03) and although a lower mean APC:SR was noted in primigravid subjects who subsequently developed PET (mean APC:SR 2.52, SD 0.316 vs 2.76, SD 0.41), this did not reach statistical significance (p=0.08). There was no relationship between the APC:SR and the occurrence of PIH or PET when the APC:SR was measured at 27-34 weeks gestation.

4.3.4 Changes in APC:SR with gestation

Of 594 subjects, in whom an APC:SR was available at both 7-15 and 27-34 weeks, 467 subjects showed an increase in APCR with increasing gestation (mean increase 0.50, SD 0.39). In the remainder no increase in resistance was observed. FBC parameters for each group are shown in Table 4.5. No significant difference in any FBC parameter at 7-15 weeks or 27-34 weeks gestation was observed. The changes in the red cell parameters between 7-

15 and 27-34 weeks gestation for those subjects with an increase in APCR with gestation are shown in Figure 4.4.

A significantly increased relative risk of PIH (OR_{MH}=2.0, $C.I._{95}$ 1.1-3.6) and PET (OR_{MH}=3.1, $C.I._{95}$ 1.2-8.0) in the current pregnancy was observed in those subjects in whom no increase in APCR was seen in comparison with the group in which APCR increased from 7-15 to 27-34 weeks. This unexpected relationship may be explained by the observation that significantly lower APC:SRs at 7-15 weeks (mean APC:SR 2.41, SD 0.27 vs 2.84, SD 0.45, t=-13.18, p=0.00001) and at 27-34 weeks (mean APC:SR 2.74, SD 0.49 vs 2.50, SD 0.26, t=8.54, p=0.00001) were observed in subjects in whom no increase in resistance with gestation was observed.

In those subjects who suffered fetal loss or neonatal death there was no difference in the APC:SR at either 7-15 weeks or 27-34 weeks when compared with those with a live birth.

4.4 Discussion

4.4.1 As shown in Chapter 2, acquired APCR in pregnancy relates, in part, to FVIIIc levels. An association between blood group and FVIIIc levels has previously been documented[87], with higher FVIIIc levels noted in non-blood group O subjects. The finding of higher APC:SR values in blood group O individuals when compared with non-blood group O is consistent with an influence of blood group on APCR in studies of non-pregnant subjects[232]. In contrast to a previous study[240], no relationship between blood group and PIH or

PET was observed. Body mass index (BMI) in early pregnancy is known to be important in the subsequent development of PET. Although this was confirmed in this study, no relationship between booking BMI and APC:SR at any gestation was observed. Waist circumference has been suggested as an independent predictor of the development of ischaemic heart disease in the general population[241]. In this study a significant relationship between waist circumference and the development of PIH (median 81.0 cm vs 77.5 cm, p=0.00001) and PET (median 80.5 cm vs 78.0 cm, p=0.02) was observed. However no relationship between waist circumference and APC:SR was noted.

4.4.2 The finding of higher APC:SRs in smokers is compatible with the reduced risk of PIH[240] and reduced thrombin generation[226] associated with smoking in pregnancy. The association of lower APC:SR levels early in pregnancy and subsequent PIH suggests that the control of APCR may be important in the development of this condition. As APCR, due to FVL, has been implicated in the development of PET, this study parallels the recently observed association between APCR (occurring in the absence of the FVL mutation) and venous thrombosis[238]. Both these observations would be consistent with the evidence of thrombosis in pre-eclamptic placentas[155]. That the balance of coagulation early in pregnancy reflects the occurrence of clinical PET later in pregnancy is consistent with the observation that the failure of normal placental development which characterises PET is found early in pregnancies which subsequently develop the condition[153]. That there is no relationship between acquired APCR, measured at 27-34 weeks, and PIH may

be due, in part, to the occurrence of clinically evident PIH or PET prior to 35 weeks gestation. 31% of PIH cases and 41% of PET cases occurred prior to this time. A lack of sensitivity of the APC:SR at this gestation may relate to the consumption of FVIIIc which has been reported to occur during clinically evident PET[242]. A reduction of available FVIIIc in subjects during PET would result in a rise in APC:SR.

4.4.3 A small, but significant, relationship between APC:SR and FBC parameters was observed. However, no difference in the FBC at 7-15 or 27-34 weeks gestation was noted when subjects showing an increase in APCR were compared to those with no increase. In addition, a much stronger relationship between gestation itself and FBC was observed. This suggests that changes in APCR are not the main influence on the blood count in pregnancy.

4.4.4 The observation of a significant association between the carriage of FVL and neonatal death is unlikely to be of biological significance given the small number of cases observed and the presence of significant alternative aetiologies of neonatal death in each case.

Table 4.1 Family, past medical and current pregnancy history in FVL positive and negative subjects

	FH				*PMH*			*CURRENT PREGNANCY*				
	VTE	*PIH*	*CVD*	*IHD*	*HBP*	*PIH*	*VTE*	*PIH*	*PET*	*MISC*	*SB*	*NND*
FVL +ve	4	1	9	17	1	1	0	3	1	0	0	2*
(SD)	(10.8)	(2.7)	(24.3)	(47.2)	(2.7)	(2.7)[#]	(0)	(8.1)	(4.2)	(0)	(0)	(5.5)
FVL –ve	105	120	260	524	18	70	6	89	21	11	13	1
(SD)	(10.4)	(11.8)	(25.7)	(51.8)	(1.7)	(10.3)-	(0.6)	(8.8)	(2.0)	(1.1)	(1.3)	(0.1)

Legend Frequency of a positive family history (FH), in a 1st degree relative of venous thrombosis (VTE). Pregnancy-induced hypertension (PIH), cerebrovascular disease (CVD), and ischaemic heart disease (IHD)in subjects positive (+ve, n=37) and negative (-ve, n=1009) for FVL. Past medical history (PMH) of high blood pressure (HBP), PIH in parous subjects or VTE. Occurrence in the current pregnancy of PIH, pre-eclampsia (PET), fetal loss less than 24 weeks gestation (MISC), fetal loss after 24 weeks (SB) and neonatal death (NND): * (OR_{MH} 57.6, C.I. 5.1-647).

Table 4.2 FBC during pregnancy in FVL positive and negative subjects

	WBC	RBC	Hb	HCT	MCV	MCH	RDW	PLAT	WBC	RBC	Hb	HCT	MCV	MCH	RDW	PLAT
	7-15	7-15	7-15	7-15	7-15	7-15	7-15	7-15	27-34	27-34	27-34	27-34	27-34	27-34	27-34	27-34
FVL +ve	9.2	4.1	12.6	0.37	91	31	12.6	232	10.5	3.6	11.2	0.33	90	31	12.9	245
(SD)	(1.8)	(0.3)	(1.1)	(0.03)	(3.7)	(1.2)	(0.9)	(51)	(2.3)	(0.3)	(0.8)	(0.02)	(3.4)	(1.4)	(0.8)	(62)
FVL –ve	8.8	4.1	12.6	0.37	92	31	12.7	238	10.4	3.6	11.1	0.32	90.1	31	12.8	243
(SD)	(2.1)	(0.3)	(0.9)	(0.03)	(4.3)	(1.6)	(1.0)	(49)	(2.5)	(0.3)	(0.9)	(0.03)	(5.4)	(2.1)	(1.4)	(58)

Legend FBC parameters at 7-15 and 27-34 weeks gestation in subjects positive (+ve) and negative (-ve) for FVL. Mean and standard deviation (SD) values for each parameter are shown. Values for WBC ($x10^9$/l), RBC ($x10^{12}$/l), Hb (g/dl), HCT (l/l), MCV (fl), MCH (pg), MCHC (g/dl), RDW (%), PLAT ($x10^9$/l) are shown.

Table 4.3 Blood loss during pregnancy in FVL positive and negative subjects

Blood Loss	<24	<24 A	>24	>24 A	>24 D	IPH	IPH S	IPH OP	PPH	PPH A	PPH S	PPH OP	Hb <10
FVL +ve	4	0	4	3	1	2	1	1	1	0	0	0	3
(%)	(10.8)	(0)	(10.8)	(8.1)	(2.7)	(11.7)	(5.8)	(5.8)	(5.8)	(0)	(0)	(0)	(8.1)
FVL –ve	141	20	98	70	11	22	13	1	21	5	16	4	184
(%)	(13.9)	(1.9)	(9.7)	(6.9)	(1.1)	(3.4)	(2.0)	(0.2)	(3.3)	(0.8)	(2.5)	(0.6)	(18.7)

Legend Number of subjects with at least one episode of blood loss at: less than 24 weeks gestation (<24); less than 24 weeks gestation requiring admission to hospital (<24 A); greater than 24 weeks gestation (>24); at greater than 24 weeks requiring delivery (>24 D). Number of subjects with blood loss greater than 500 mls during a spontaneous vertex delivery (IPH); IPH requiring an additional dose of syntometrine (IPH S); surgical intervention (IPH OP). Number of subjects with post-partum haemorrhage from a spontaneous vertex delivery (PPH); PPH requiring admission to hospital (PPH A); PPH requiring an additional dose of syntometrine (PPH S); PPH requiring surgical intervention (PPH OP). Subjects with anaemia of <10g/dl at any gestation before delivery (Hb<10). Actual number of cases and percentage of FVL positive or negative subjects (in parenthesis) is shown.

TABLE 4.4 Presentation characteristics and relationship with APC:SR at 7-15 weeks gestation of non-FVL subjects

			APC:SR (7-15)	
	mean	**SD**	**R^2**	**P**
Waist (cm)	78.5	13.5	0.0	0.77
Age (yrs)	29	6	0.2	0.20
Height (m)	1.63	0.1	0.4	0.05*
Weight (kg)	65	13.2	0.1	0.30
BMI (wt/Ht^2)	24.6	4.7	0.0	0.71
SBP	110	11	0.5	0.03*
DBP	66	8	0.2	0.14

Legend Mean values and standard deviation (SD) for each parameter: waist, age, height, weight, BMI, systolic and diastolic blood pressure-SBP and DBP.

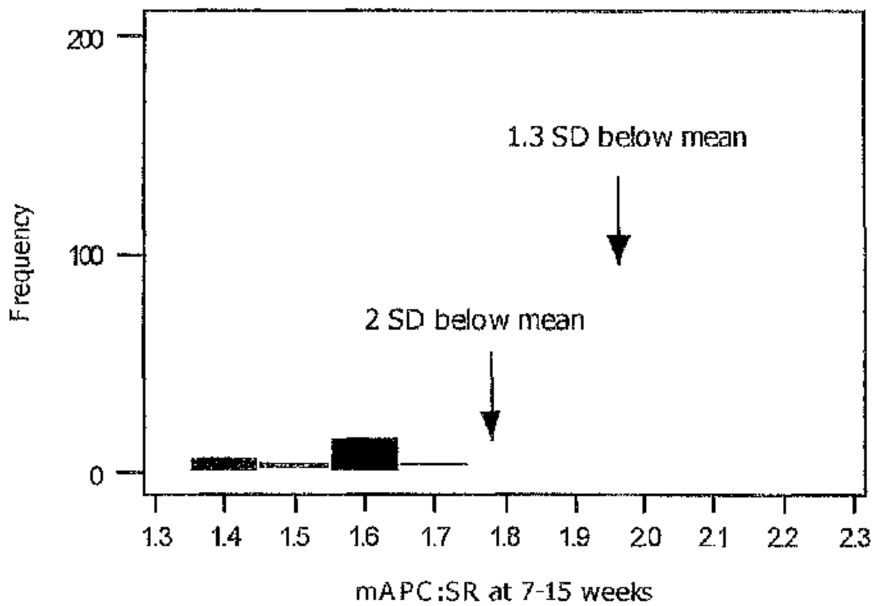
R^2 (%) and p values (p) for linear regression analysis of each parameter with APC:SR at 7-15 weeks gestation is shown.

Table 4.5 FBC parameters in subjects with an increase or no increase in APCR with gestation

	Increase in APCR						No increase in APCR					
	median	Q1	Q3	Median	Q1	Q3	median	Q1	Q3	median	Q1	Q3
	7-15 wks			27-34 wks			7-15 wks			27-34 wks		
WBC (x10^9/l)	8.6	7.5	10.1	10.2	8.6	11.9	8.4	7	10	9.9	8.1	11.7
RBC (x10^{12}/l)	4.1	3.9	4.3	3.6	3.4	3.8	4.09	3.9	4.3	3.62	3.4	3.8
Hb (g/dl)	12.5	12.0	13.1	11.2	10.5	11.8	12.6	12	13.2	11.3	10.7	11.8
HCT (l/l)	0.4	0.4	0.4	0.33	0.31	0.35	0.375	0.36	0.39	0.331	0.32	0.35
MCV (fl)	92	89	94	92	89	94	92.3	89	95	91.1	88	95
MCH (pg)	31	30	32	31	30	32	30.9	30	32	31.2	30	32
MCHC (g/dl)	34	33	34	34	34	34	34	33	34	34	34	34
RDW (%)	12.6	12.1	13.1	12.6	12.1	13.2	12.5	12.1	12.9	12.4	12.0	12.9
PLAT (x10^9/l)	234	205	264	240	206	276	226	201	251	230	195	261

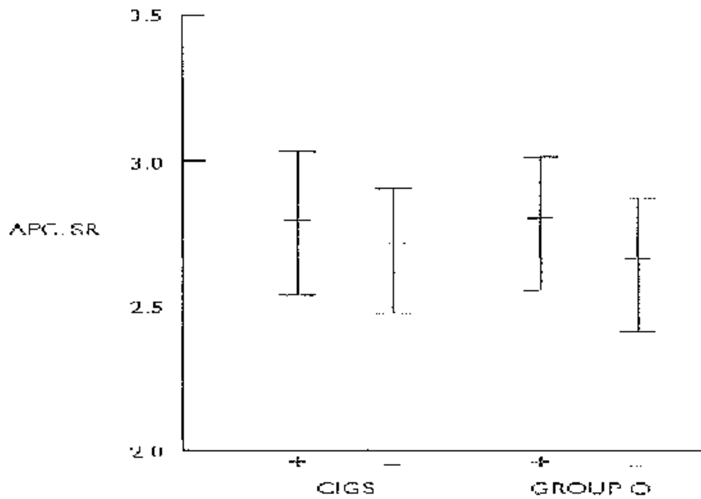
Legend Median, upper and lower interquartile ranges for FBC parameters at 7-15 weeks (wks) and 27-34 wks gestation for subjects showing an increase (n=378) or no increase in APCR (n=112) with gestation.

Figure 4.1 The mAPC:SR and Factor V Leiden



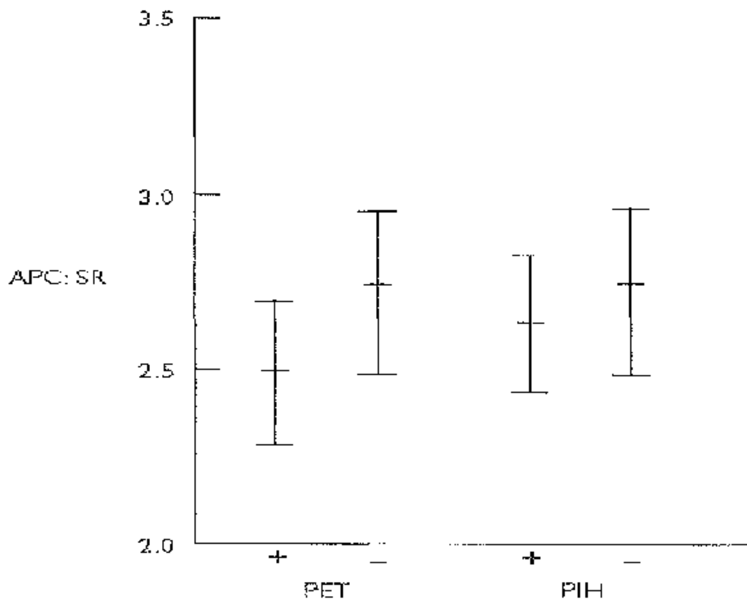
Legend A histogram of the mAPC:SR at 7-15 weeks gestation is shown. Subjects positive for FVL are shown in blue. Subjects negative for FVL are shown in yellow. The arrows indicate 1.3 SD and 2 SD below the mean value of mAPC:SR for all subjects at 7-15 weeks gestation.

Figure 4.2 APC:SR at 7-15 weeks with smoking and blood group



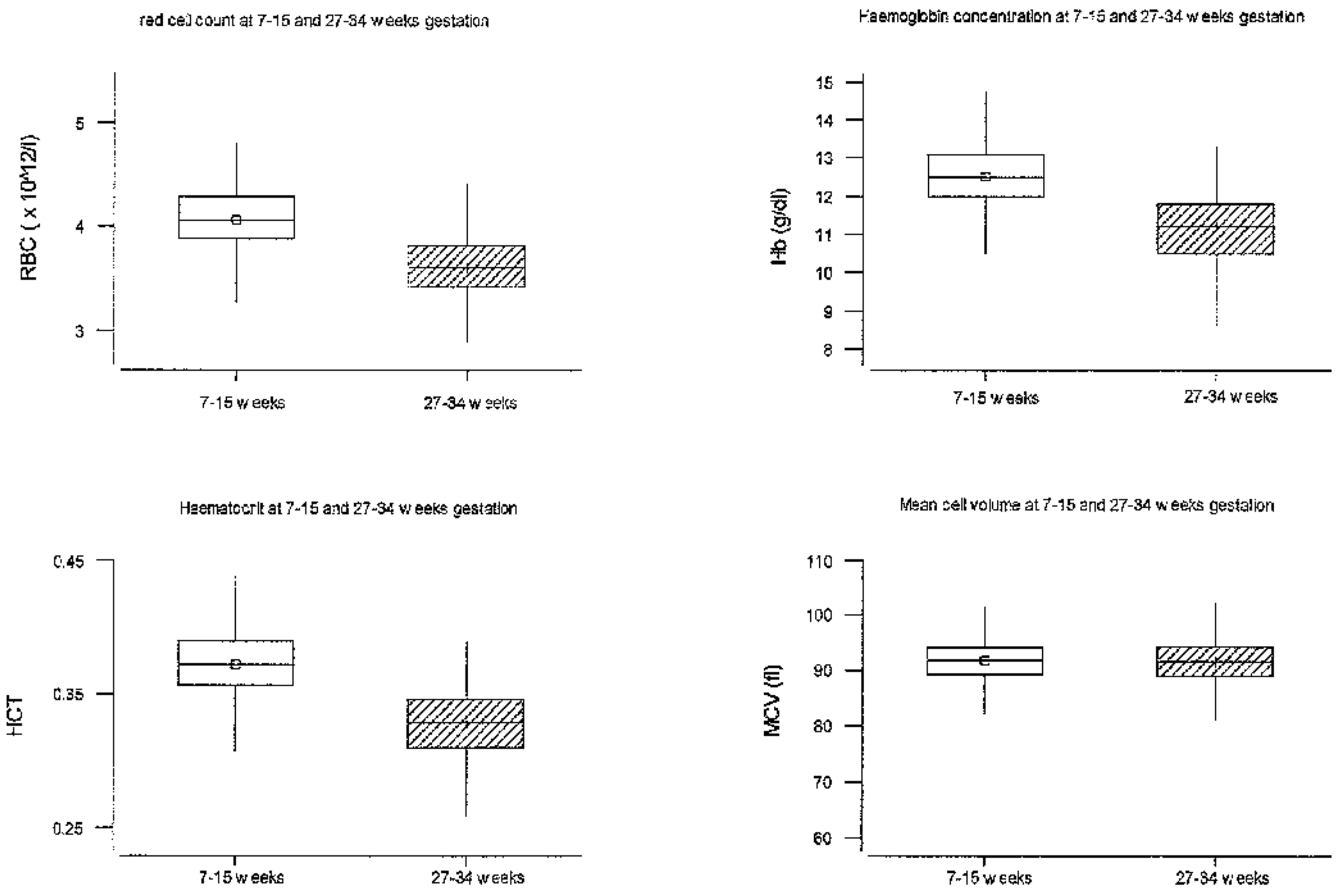
Legend Mean and interquartile ranges for APC:SR are shown for smokers (CIGS +) vs non-smokers (CIGS -) and blood group O + (GOUP O +) vs non blood group O subjects (GROUP 0 -).

Figure 4.3 APC:SR at 7-15 weeks and occurrence of PIH or PET in the current pregnancy



Legend Mean and interquartile ranges are shown for APC:SR for subjects with pre-eclampsia (PET +) vs no PET (PET -) and pregnancy-induced hypertension (PIH +) vs no PIH (PIH -).

Figure 4.4 Changes in FBC with gestation in subjects with an increase in APCR



Legend Median (central symbol and line), upper and lower limits of the interquartile ranges (upper and lower box limits) for each parameter are shown.

Chapter 5

Thrombin induction of intercellular adhesion molecule-1 (ICAM-1) expression on human monocytes

5.1.1 From the studies detailed in Chapters 3 and 4, acquired APCR is associated with an increase in thrombin generation and appears linked to the occurrence of pre-eclampsia (a condition which is associated with both heritable thrombophilic disorders, infiltration of the placenta by monocyte/macrophages and an increase in the inflammatory response – Chapter 1). In Chapter 5 and 6 the link between acquired APCR and inflammation was investigated. This investigation centred on the expression of adhesion molecule ICAM-1 (CD 54) on the human monocyte. An increase in ICAM-1 expression is likely to increase the recruitment of leukocytes to the inflammatory response. As detailed in Chapter 1, thrombin can increase the expression of ICAM-1 on the endothelium. It is not known whether thrombin can also modulate the expression of ICAM-1 on the monocyte. Such an interaction would link thrombin to the pro-inflammatory response of the monocyte.

5.1.2 In this chapter the influence of thrombin on monocyte ICAM-1 expression in vitro was investigated. The effect of human alpha thrombin on monocytes from normal human donors was examined to determine if a specific dose-response relationship between monocyte ICAM-1 and thrombin could be observed. The response of monocyte ICAM-1 to thrombin was also examined on the monocyte cell line THP-1. To prove the specificity of the response the effect of the thrombin receptor agonist peptide (TRAP-6) and a pre-incubated mixture of thrombin with the direct thrombin inhibitor hirudin on ICAM-1 expression was also investigated.

5.1.3 ICAM-1 expression was measured by means of flow cytometry to allow the quantifiable assessment of a dose-response. Flow cytometric measurement would also allow examination of expression with minimal sample manipulation and facilitate more direct comparison with the investigation of in vivo monocyte ICAM-1 expression and soluble ICAM-1 levels detailed in Chapter 6.

5.2 Materials and Methods

5.2.1 Subjects

Monocytes were obtained from whole blood from 8 healthy volunteers (7 female, 1 male, age range 25-40 years). The response of these cells to incubation, human alpha thrombin (Alpha Laboratories, UK), the thrombin receptor agonist peptide SFLLRN (TRAP-6, Bachem, UK) and a pre-incubated mixture of thrombin and a direct thrombin inhibitor hirudin (Alpha Laboratories, UK) was examined.

5.2.2 Human monocyte cell line THP-1 cells

The human monocyte cell line THP-1, a cell line derived from acute monocytic leukaemia cells (European Collection of Animal Cell Cultures, Salisbury, UK), was cultured in liquid phase to allow flow cytometric analysis of ICAM-1 expression. Cells were cultured in standard culture media: RPMI-

1640 (Sigma, UK) supplemented with 10% fetal calf serum (FCS, ICAN Flow, UK), 2mmol/l L-glutamine, 100 iu/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, UK). The concentration of cells in the final reaction mixture was ~500,000/300μl. Cells were incubated with human alpha thrombin TRAP-6 and thrombin, pre-incubated with hirudin. THP-1 cell viability, after 24 hour incubation was >90% determined by trypan blue exclusion.

5.2.3 Human peripheral blood monocyte preparation

Human peripheral blood monocytes were prepared from whole blood anticoagulated in 1/10 volume of 0.105 M sodium citrate by density gradient centrifugation. For each experiment 40 ml of blood was centrifuged against Lymphoprep (Nycomed, UK) at 300g for 15 minutes. The resultant cells were resuspended in RPMI 1640 (Sigma, UK) supplemented with 10% fetal calf serum (FCS, ICAN Flow, UK), 2mmol/l glutamine, 100iu/ml penicillin and 100μg/ml streptomycin (Gibco BRL, UK). The final concentration of monocytes was 2.16 x 10^9/l. Cell viability for each procedure, after the 24 hour incubation, was >95%, as determined by trypan blue exclusion.

5.2.4 Thrombin, TRAP-6 and thrombin-hirudin stimulation of monocytes

Human alpha thrombin (Alpha Laboratories, UK), diluted in 0.5% bovine serum albumin, ('BSA', Sigma, UK) was added to human monocyte and THP-1 cell suspensions in a final reaction volume of 300μl. Thrombin at 0.75 iu,

3.1 iu, 6.25 iu, 12.5 iu, 18.75 iu and 25 iu/300μl reaction mix was added to cell suspensions for 24 hours at 37°C in a 5% CO_2/95% air humidified atmosphere. Cells were also stimulated with TRAP-6 for 24 hours at a final concentration of 2μg/ml, 5 μg/ml and 10μg/ml. Cell suspensions were also incubated with a mixture of 1.5 iu of thrombin, pre-incubated for one hour at 37°C with 15 antithrombin units of hirudin (Alpha Laboratories, UK).

5.2.5 Flow cytometric analysis of thrombin/TRAP and thrombin-hirudin stimulation of ICAM-1 expression

Flow Cytometry principles

The Facscan Flow Cytometer (Becton Dickinson Ltd, UK) analyses the optical properties of single cells moving in a fluid stream through a focused argon laser beam. The forward scatter of incident light on the cell indicates the relative size of the cell and the side scatter indicates relative granularity. The flow cytometer can also measure the fluorescence emitted by various dyes or fluorescent-labelled antibodies. The fluorochromes absorb energy from the laser and release the absorbed energy by vibration, heat and by emission of photons of a longer wavelength. The fluorochrome Fluorescein Isothiocyanate (FITC) emits at ~515nm and is detected on channel FL-1 and Phycoerythrin (PE) emits at ~580nm and is detected on channel FL-2 of the flow cytometer. The amount of fluorescent antibody a cell will bind (the

fluorescence intensity) is proportional to the number of antibody binding sites on the cell.

Sample Preparation

Monocytes were identified by antiCD14. CD14, a receptor which binds the complex lipopolysaccharide-binding proteins, is expressed on 90-95% of monocytes[243] and is considered to be a specific monocyte marker for use in identification of monocytes in flow cytometric analysis. Monoclonal antibodies against CD54 (ICAM-1[244]) were used to quantify ICAM-1 on the monocyte surface. Mononuclear cell preparations and THP-1 cells were stained using anti-CD14 PE (Becton Dickinson Ltd, UK) and anti-CD54 FITC (Dako Ltd, UK) allowing simultaneous identification of monocytes and quantification of antibody binding to CD54. Cell counts per tube were adjusted to 1×10^6 cells in phosphate-buffered saline (PBS) azide containing 2% blood group AB serum (PBS-AB) to a final volume of 100μl. The appropriate volumes of anti-CD14 and anti-CD54 were added and incubated at room temperature for 15 minutes. 2 ml of FACS lysing solution (Becton Dickinson Ltd, UK) was added and incubated for 10 minutes at room temperature prior to centrifugation. After the supernatant was discarded, the resultant preparation was washed once in PBS-AB. The cells were then re-suspended in 0.5ml PBS-azide. Isotype and fluorochrome matched mouse immunoglobulin was used as a negative control.

Analysis and quantification

Samples were acquired on a Facscan Flow Cytometer (Becton Dickinson Ltd, UK) using Lysis II software with a logarithmic amplification of the FL1 and FL2 signals. List mode data were collected from a minimum of 5000 events for each tube by 'live gating' on the CD14 positive monocyte population. The monocytes were then analysed to determine the mean fluorescence intensity (MFI) of CD54 on the monocyte population (a sample output showing the MFI is shown in Figure 5.1). The CD54 MFI was then converted into antibody binding capacity (ABC), using the Quantum Simply Cellular Microbeads Kit (Sigma Ltd, UK). The ABC is a measure of the number of antibodies bound per cell[245, 246]. This kit contains a set of standards to calibrate the MFI into equivalent units of Antibody Binding Capacity (ABC) for CD54. This standardisation facilitates the assessment of a dose-response relationship between a stimulus and the MFI of antibody binding as well as inter-laboratory comparison of results.

5.3 Results

5.3.1 In vitro monocyte stimulation with thrombin and TRAP-6: preliminary experiments to determine optimal incubation and dose

ICAM-1 expression was measured after 0, 4, 24 and 48 hrs incubation at 37° C with 0, 6.25 and 25 IU/300µl of human alpha thrombin. The rise in ICAM-1 expression in the absence of thrombin with time is shown as MFI (Figure 5.2) and ABC (Figure 5.3). Results of incubation with 6.25 and 25 IU/300µl alpha thrombin are also shown in Figure 5.3. At 48 hours, a fall in total ICAM-1 expression in control and thrombin treated cells at all concentrations, when compared with 24 hour values, was observed. This pattern is consistent with a previous study of isolated monocyte ICAM-1 response to incubation[247] and is consistent with the maximal up-regulation of monocyte ICAM-1 at 24 hours noted previously[248]. All subsequent experiments were conducted at 24 hours.

5.3.2 The dose response of human monocyte ICAM-1 to thrombin and TRAP-6

A dose-response relationship between thrombin and human monocyte ICAM-1 expression at 24 hours was observed (Figure 5.4). At 24 hours incubation with 25IU thrombin, a mean rise in ICAM-1 expression of 54278 ABC (184%) over 24 hour control values was observed (Table 5.1). This rise is equivalent to the rise in mean fluorescence intensity of CD54 observed on monocytes

exposed to glutamine[249]. A rise of 143-147% (mean 25,080 ABC) in ICAM-1 expression was observed at 24 hours on incubation with 2µg/ml to 10µg/ml of TRAP-6. In a separate experiment, incubation of human monocytes with 1.5 IU/300µl of thrombin resulted in an increase (over control) of 46,709 ABC at 24 hours. Repeating the experiment with pre-incubation of thrombin with 30IU of the thrombin inhibitor hirudin resulted in inhibition of the ICAM-1 response (maximal rise </= 3,633 ABC).

5.3.3 The dose response of THP-1 ICAM-1 to thrombin and TRAP-6

Incubation of THP-1 cells at 37°C for 24 hours resulted in little increase in baseline ICAM-1 ABC (mean rise 560). Incubation with 25 IU/300µl of thrombin for 24 hours resulted in a mean rise of 24,222 ABC over 24 hour controls (Table 5.1). As with human cells, pre-incubation of thrombin with hirudin resulted in a failure of elevation of ICAM-1 expression. In a separate experiment incubation of THP-1 cells with TRAP-6 (at 5µg/ml) for 24 hours resulted in an increase of 22,084 ABC compared with 24 hour control values (Table 5.1).

5.4 Discussion

5.4.1 In addition to a central role in coagulation, thrombin has been shown to have an important role in the co-ordination of the inflammatory response. Thrombin can induce selectin[9, 10] and adhesion molecule expression[11]

on the endothelial surface and thereby induce leukocyte adhesion and subsequent activation. Thrombin, in-vitro, has also been shown to be capable of influencing the inflammatory and coagulation repertoire of the monocyte by stimulation of interleukin-1, TNFα[64] and plasminogen activator inhibitor-2[65] expression. Monocytes also function as antigen presenting cells in the immune system, a function which is influenced by the expression of the LFA-1 ligand ICAM-1 (CD54) on the cell surface. Thrombin is also capable of activating T cells[12], which express LFA-1, the ligand for ICAM-1.

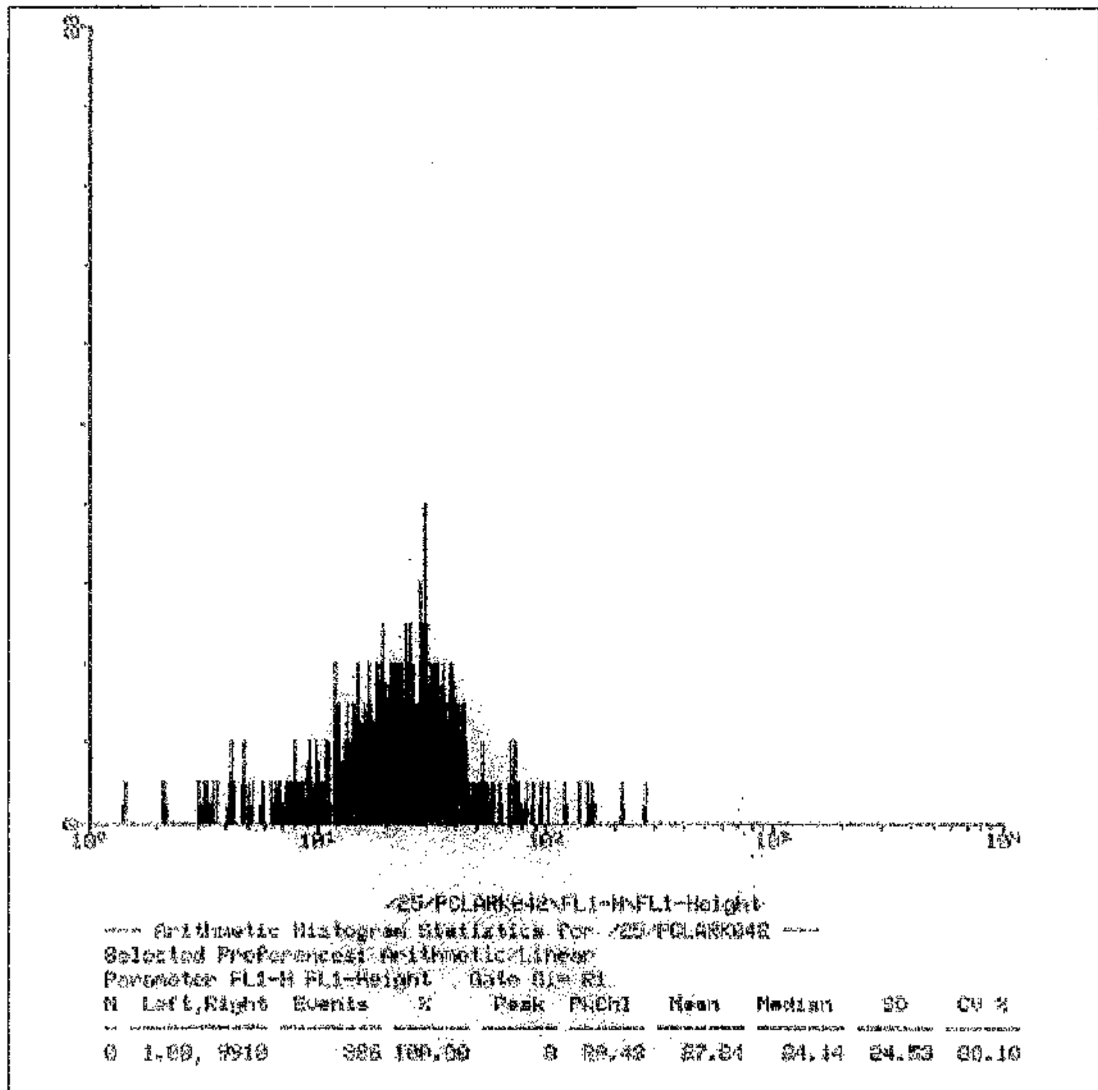
5.4.2 The present findings show that thrombin also has a direct effect on monocyte ICAM-1 expression. Thus activation of the coagulation system may directly influence the monocyte inflammatory response. This may be important in vascular injury and disorders such as atherosclerosis and pre-eclampsia, where vascular damage is characterised by deposition of lipid-laden macrophages[154, 155]. A similar, in-vitro, induction of monocyte ICAM-1 with the hexapeptide SFLLRN (TRAP 6) was observed. SFLLRN has been shown to be a specific thrombin receptor activator[250, 251], suggesting that thrombin induction of monocyte ICAM-1 is due to an interaction of thrombin with its specific receptor[6]. Furthermore the thrombin action appears specific as it was inhibited by hirudin, a specific thrombin inhibitor and was also demonstrated on the monocyte cell line THP-1.

Table 5.1 The response of human monocytes and THP-1 cells to human alpha thrombin and SFLLRN (TRAP-6)

	Human Mean ICAM-1 rise over 24hr control (ABC)	Mean ICAM-1 rise over 24hr control (%)	**THP-1** Mean ICAM-1 rise over 24hr control (ABC)	Mean ICAM-1 rise over 24hr control (%)
24 hr + Thrombin (25IU/300ul)	54278	184%	24,222	235%
24 hr + TRAP (5ug/ml)	24,827	145%	22,084	222%

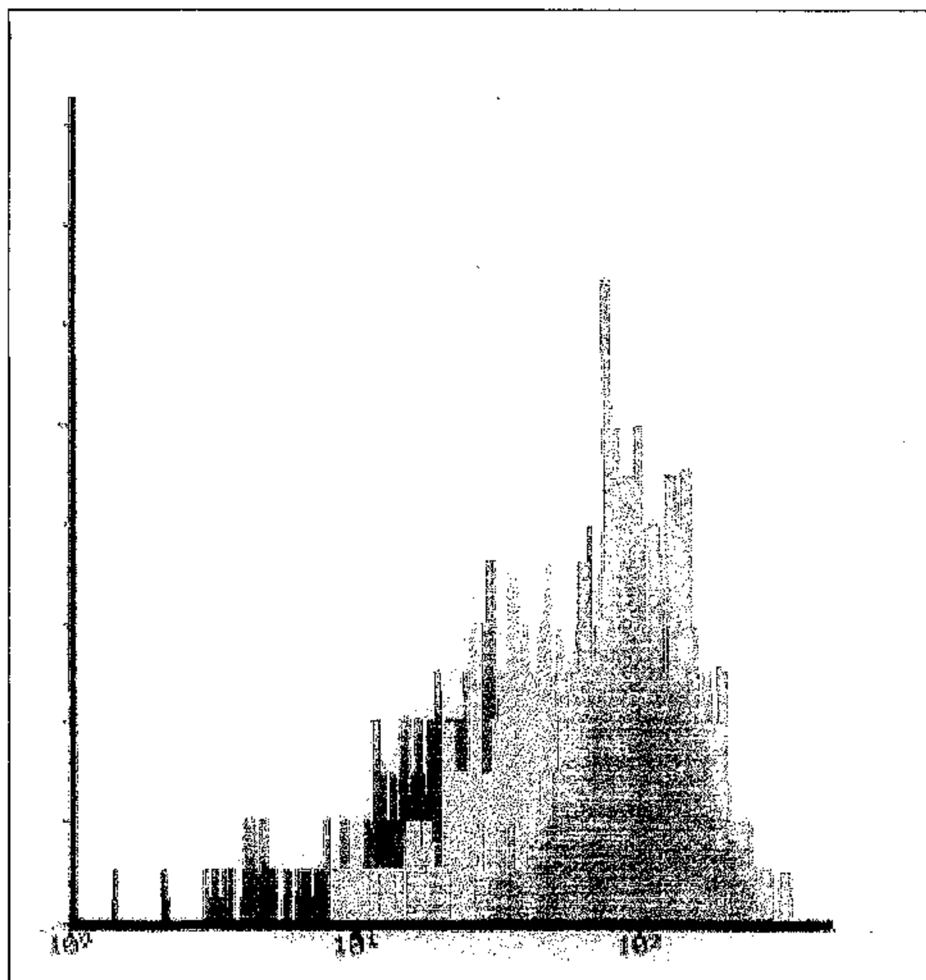
Legend The mean rise (and percentage rise) in monocyte ICAM-1 in response to the maximal dose of thrombin (25iu/300ul) and TRAP-6 (TRAP 5ug/ml) at 24 hours incubation in human monocytes and THP-1 cells is shown.

Figure 5.1 ICAM-1 (CD54) expression on human monocytes



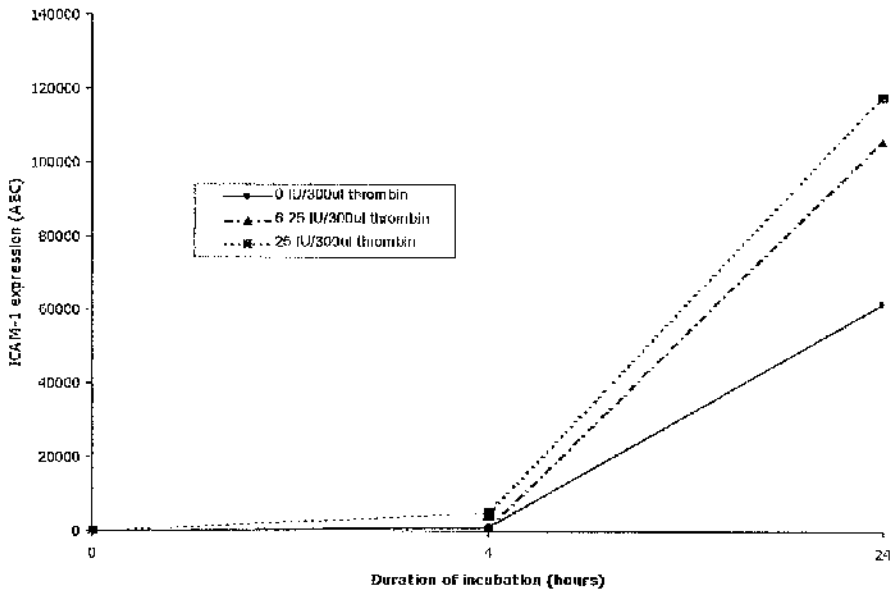
Legend ICAM-1 expression (CD54) on isolated monocytes at 0 hours incubation in the absence of thrombin.A mean fluorescence of 27.84 is shown.

Figure 5.2 The effect of incubation on ICAM-1 (CD54) expression on human monocytes



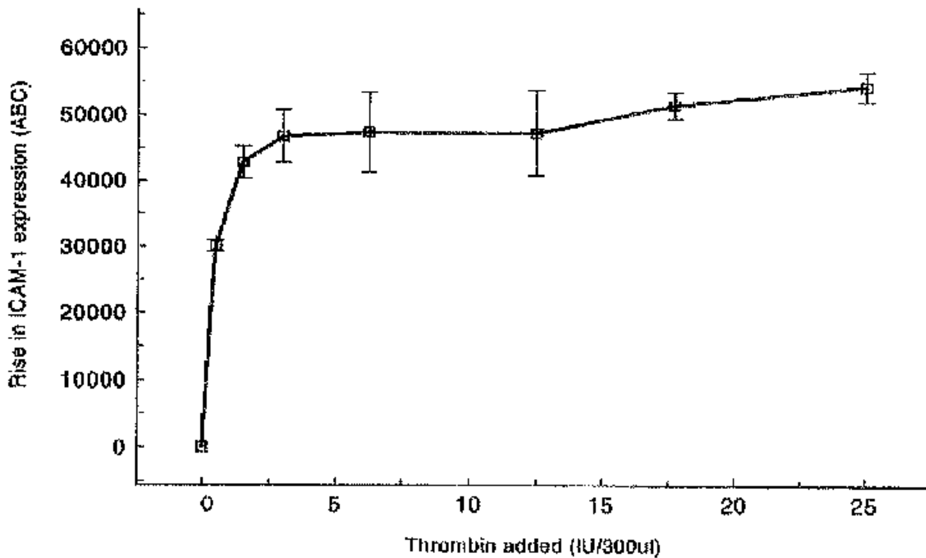
Legend ICAM-1 expression (CD54) on isolated monocytes at 0 hours (blue), 4hours (yellow) and 24 hours (red) incubation in the absence of thrombin. Increasing mean fluorescence intensity is shown on the x axis.

Figure 5.3 Monocyte ICAM-1 expression: effect of incubation with human alpha thrombin



Legend The change in ICAM-1 expression (antibody binding capacity-ABC) with time in the absence of thrombin (solid line), with 6.25 iu/300ul human alpha thrombin (dot and dash line) and 25 iu/300ul of thrombin (dotted line) is shown.

Figure 5.4 The dose response relationship between thrombin and monocyte ICAM-1



Legend The change in ICAM-1 expression (ABC) with increasing concentrations of human alpha thrombin with mean values and interquartile ranges at each concentration is shown.

Chapter 6

The relationship of the APC:SR in pregnancy with monocyte ICAM-1 expression and plasma soluble ICAM-1

6.1 Both pregnancy[127, 134, 233] and the APCR of pregnancy (Chapter 3) are associated with an increase in thrombin generation. Furthermore oestrogen may modify cellular ICAM-1 expression[252]. In this study the relationship of monocyte ICAM-1 to pregnancy and to pregnancy-associated APCR was examined ex vivo. A relationship between APC:SR and monocyte ICAM-1 would be compatible with the link between monocyte ICAM-1 expression and thrombin generation in vivo and a link between the APCR of pregnancy and adhesion molecule expression/inflammation. Furthermore, as in vitro studies suggest that an increase in cellular ICAM-1 expression is associated with an increase in shedding of ICAM-1 from the cell surface[40, 50-52], the study also determined whether there is a relationship between monocyte ICAM-1 expression and plasma soluble ICAM-1 ex vivo.

6.2 Materials and Methods

6.2.1 Subjects

In a prospective study, 149 healthy pregnant subjects (age: mean 30 years, range 20-40 years) were recruited. All subjects had uncomplicated pregnancies. 60 were primigravid and 48 were smokers. Subjects were sampled at 8-15 weeks (n=27), 16-28 weeks (n=58), 29-34 weeks (n=47) and 35-40 weeks gestation (n=21). In addition 18 healthy, non-smoking, non-oral contraceptive using, female subjects (mean age 30 years, range 25-39 years) and 10 healthy non-smoking male subjects (mean age 30 years,

range 25-39 years) were recruited. Institutional ethical approval was obtained and each individual was sampled on a single occasion.

6.2.2 Sample preparation

Samples were obtained by venepuncture from the antecubital vein and anticoagulated in 1/10 volume of 0.105 M sodium citrate. Samples for ex vivo assessment of monocyte ICAM-1 were stored at 4°C for less than 24 hours before flow cytometric analysis. Samples for soluble plasma ICAM-1 and APC:SR assessment were stored at ambient temperature for less than one hour, prior to double centrifugation at 3000G for 12 minutes. The resultant platelet poor plasma was stored at −70°C until analysis. APC:SR was assessed as detailed in Chapter 2. Plasma soluble ICAM-1 levels were determined by ELISA (R+D Systems Ltd, UK).

6.2.3 Flow cytometric analysis for ex-vivo ICAM-1 expression

Sample Preparation

Monocytes in whole blood were identified by antiCD14 and monoclonal antibodies against CD54 (ICAM-1[244]) were used to quantify ICAM-1 on the monocyte surface. Whole blood preparations were stained using anti-CD14 PE (Becton Dickinson Ltd, UK) and anti-CD54 FITC (Dako Ltd, UK). The

subsequent sample preparation was as described in Chapter 5 aside from substitution of PBS-AB with PBS alone.

Analysis and quantification

Samples were acquired on a Facscan Flow Cytometer (Becton Dickinson Ltd, UK) using Lysis II software with a logarithmic amplification of the FL1 and FL2 signals as used in Chapter 5. The monocytes were then analysed to determine the mean fluorescence intensity (MFI) of CD54 on the monocyte population. The CD54 MFI was then converted into antibody binding capacity (ABC) as before.

6.2.4 Statistical Analysis

ANOVA of groups, linear regression analysis of monocyte ICAM-1 with plasma ICAM-1, APC:SR and gestation and two sample t test comparisons of smoking and parity were carried out using Minitab®for Windows® Release 11 statistical software (Minitab Inc, USA).

6.3 Results

6.3.1 No significant difference in monocyte ICAM-1 ABC between males (mean 14,979, SD 2049) and non-oral contraceptive (COCP) using female (mean 15,554 ABC, SD 1652, F=0.62, p=0.44) was observed (Figure 6.1). A

significantly higher level of monocyte ICAM-1 ABC was observed in pregnant subjects (mean 18,381, SD 4,499) in comparison with both male and non-COCP female subjects (F=5.91, p=0.003, Figure 6.1 and Table 6.1). This difference in expression is equivalent to the difference in mean fluorescence intensity of ICAM-1 which is observed when patients with a myocardial infarction are compared with non-myocardial infarction controls[253]. No significant relationship between ICAM-1 ABC and weeks of gestation (R^2=0.0%, p>0.05) was observed. No significant difference in monocyte ICAM-1 ABC in comparison of primigravid vs parous subjects (t=-0.20, p>0.05) or smoking vs non-smoking subjects (t=0.90, p>0.05) was observed (Table 6.1).

6.3.2 No significant difference in plasma ICAM-1 was observed between male (mean 10.9 μg/ml, SD 2.9), non-OCP females (mean 9.2 μg/ml, SD 2.1) and pregnant subjects (mean 10.6 μg/ml, SD 3.2, F=1.61, p=0.2, Table 6.1). No significant relationship between gestation (linear regression R^2=0.0%, p>0.05) or parity (t=1.97, p>0.05) and plasma ICAM-1 was observed. In pregnancy, a significantly higher level of soluble ICAM-1 was observed in smokers (mean 12.2μg/ml, SD 3.95) than non-smokers (mean 9.74μg/ml, SD 2.46, t=3.31, p=0.002, Table 6.1).

6.3.3 In pregnant subjects linear regression analysis revealed a significant positive relationship between monocyte ICAM-1 ABC and plasma ICAM-1 (Figure 6.2, R^2 12.1%, p <0.0001). This relationship persisted when smokers were removed from the analysis (R^2 12.0%, p=0.002). No significant relationship was demonstrated in non-pregnant women or men. A small but significant

inverse relationship between monocyte ICAM-1 (R^2 3.9%, p=0.05) and plasma ICAM-1(R^2 4.4%, p=0.03) with APC:SR at 28-32 weeks gestation was observed (Figures 6.3 and 6.4).

6.4 Discussion

6.4.1 In pregnancy, a condition characterised by a physiological increase in thrombin generation[127, 134, 233], an increase in the expression of monocyte ICAM-1 and a relationship between expression and the plasma level of its soluble form was observed. This suggests that soluble ICAM-1 levels in pregnancy reflect monocyte expression and is consistent with the in-vitro experiments, which show that up-regulation of ICAM-1 is accompanied by an increase in shedding of soluble ICAM-1 from stimulated cells[40, 50-52]. Smoking in pregnant subjects appears to contribute to higher plasma levels of ICAM-1, but is not associated with higher monocyte ICAM-1 levels. Smoking may increase plasma ICAM-1 by cleavage of ICAM-1 from the monocyte surface, or by stimulation of expression of ICAM-1 on cells other than monocytes. The lack of an effect on monocyte ICAM-1 expression would be consistent with the published evidence on the protective effect of smoking on thrombin generation and pre-eclampsia detailed in Chapter 4.

6.4.2 No correlation of monocyte ICAM-1 expression with gestation after 7 weeks was observed. Although other studies[133, 134] have reported a correlation between F1+2 and gestation, elevated levels of F1+2 are a common feature of early normal pregnancy[134, 233]. In addition, TAT complex levels are

reported to be elevated in the first trimester of 50% of normal pregnancies[127]. Thus the results presented here may be consistent with an increased sensitivity of monocytes to thrombin or to a combined up-regulating effect of increasing thrombin, cytokines and oestrogen[252] on monocyte ICAM-1 expression in pregnancy.

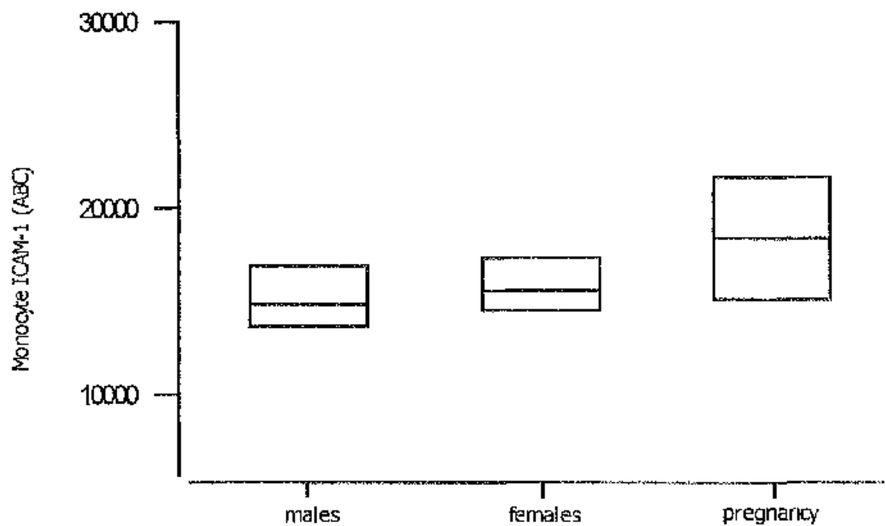
6.4.3 A significant negative relationship was observed between APC:SR at 28-32 weeks gestation and both monocyte and soluble ICAM-1 at that gestation. This would be compatible with the negative correlation between APC:SR and thrombin generation observed in Chapter 3 and would suggest that ICAM-1 expression in pregnancy is influenced by thrombin generation in vivo. Furthermore the relationship of APC:SR with monocyte ICAM-1 suggests that a lower APC:SR in pregnancy occurs along with an upregulation of the antigen presenting function of the monocyte.

Table 6.1 The relationship between monocyte and plasma ICAM-1 with sex, pregnancy and smoking

	Monocyte ICAM-1 (ABC)			Plasma ICAM-1 (ug/ml)		
	Mean	SD	p	Mean	SD	p
Males	14,979	2,049	0.42	10.9	2.9	0.3
Females	15,554	1,652		9.2	2.1	
Pregnancy	18,381	4,499	0.003*	10.6	3.2	0.2^{∞}
Primigravid	18,423	4,279	0.68	9.7	2.6	0.09
Parous	18,104	4,555		10.7	3.1	
Smoking	18,691	3,756	0.37	12.2	3.9	$0.002^{\varnothing}$
Non-smoking	18,013	4,270		9.7	2.5	

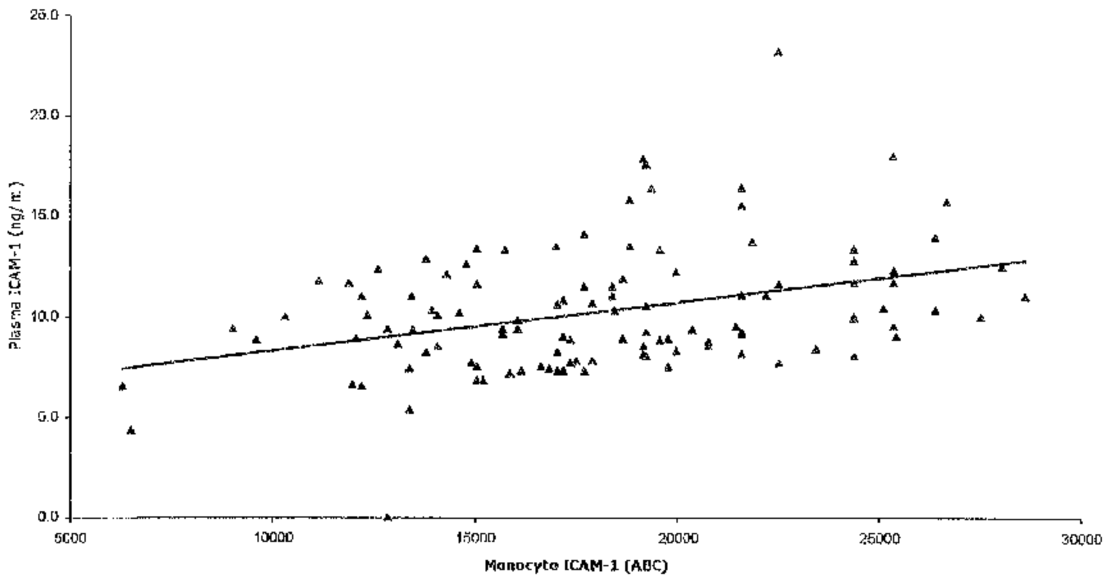
Legend The mean and SD monocyte ICAM-1 (ABC) and plasma ICAM-1 (ug/ml) for males, females, all pregnant subjects (pregnancy), primigravid pregnant subjects, parous pregnant subjects, smoking and non-smoking pregnant subjects is shown. P values for ANOVA and t test for comparisons of males and females, pregnant vs non-pregnant females (*p significant at 0.003), primigravid vs parous women and pregnant smokers vs non-smokers ($^{\varnothing}$ p significant at 0.002) is shown.

Figure 6.1 Monocyte ICAM-1 (ABC) in males, females and in pregnancy



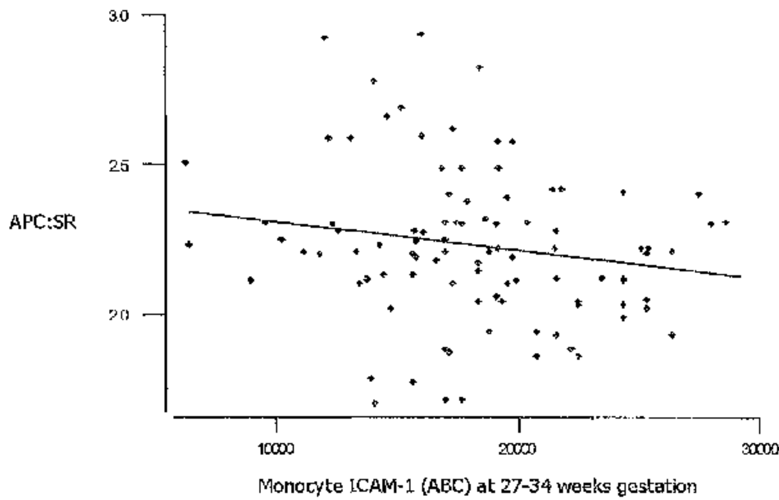
Legend The median and upper and lower interquartile range of ICAM-1(ABC) for males, females and pregnant subjects is shown.

Figure 6.2 Monocyte ICAM-1 vs plasma ICAM-1 in pregnancy



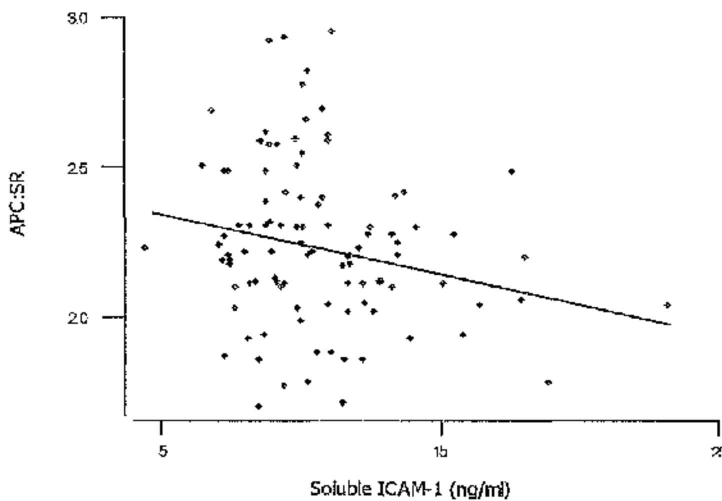
Legend Monocyte ICAM-1 expression (ABC) vs plasma ICAM-1 levels (ng/ml) in pregnancy. For guidance a linear regression line is shown.

Figure 6.3 APC:SR at 28-32 weeks gestation vs monocyte ICAM-1



Legend Monocyte ICAM-1 expression (ABC) vs APC:SR at 28-32 weeks gestation. For guidance a linear regression line is shown.

Figure 6.4 APC:SR at 28-32 weeks gestation vs plasma ICAM-1



Legend Plasma ICAM-1 levels (ng/ml) vs APC:SR at 28-32 weeks gestation. For guidance a linear regression line is shown.

Chapter 7

Conclusions

7.1 FVL, the principal inherited cause of APCR, has been linked to the failure of pregnancy with an increased risk of venous thrombosis[254], fetal loss[148] and pre-eclampsia[186]. Both venous thrombosis and pre-eclampsia are associated with inflammation. In particular pre-eclampsia is associated with a monocyte/macrophage infiltration of the placental bed[154, 155]. That APCR, without FVL, (acquired APCR) may be a marker of thrombotic potential out-with pregnancy has been suggested by Rosing et al[98] and more recently by de Visser et al[238].

7.2 Changes in sensitivity to APC (assessed by the APC:SR) occur in normal pregnancy[76]. The aetiology and significance of this phenomenon has not been fully characterised. In this thesis, the pattern of change in APC:SR in pregnancy was detailed in FVL negative subjects. The relationship of the APC:SR to other coagulation factors and thrombin generation was investigated. The significance of the APC:SR to the mother and the fetus was also examined. To investigate if a link (via thrombin) exists between the APC:SR and the inflammatory response, the inflammatory marker ICAM-1 was examined on the monocyte. The influence of thrombin on the expression of this marker was examined in vitro and the relationship between the APC:SR in pregnancy and ICAM-1 was examined ex vivo.

7.3 In a large prospective study, the APC:SR at 7-15 weeks gestation showed a significant relationship with blood group and smoking, with higher APC:SRs observed in non-smokers and blood group O subjects. No significant relationship of APC:SR with body mass index, waist circumference, age, alcohol consumption, or family history of venous thrombosis or pregnancy-

induced hypertension in a first degree relative was observed. In ~75% of pregnancies, a fall in APC:SR with increasing gestation was seen. Those who showed a fall in APC:SR (i.e. an increase in APCR) with increasing gestation had higher absolute APC:SR values at 7-15 weeks gestation than those who did not show a fall. APC:SR values less than the reference range observed in non-pregnant females were seen in 38% of subjects in the third trimester of pregnancy (28 to 40 weeks gestation) in the absence of FVL or elevated ACAs. In subjects without elevated ACAs or FVL, the APC:SR showed a significant negative relationship with factor VIIIc, factor Vc, and a positive relationship with free protein S. The relationship to factor VIIIc is compatible with the association of APCR and inflammatory disorders[83] and with the association of non-blood group O, higher factor VIIIc levels and venous thrombosis[85-87]. The relationship with factor Vc is consistent with in vitro evidence that the APC:SR may be affected by factor Vc[213]. A significant contribution of protein S alone to the APC:SR may also occur.

7.4 The fall in APC:SR with gestation paralleled the fall observed in the red cell count, haemoglobin and mean red cell volume. This appeared to be due to the effect of gestation on both, rather than an effect of the APC:SR on the blood count. In the study by Lindqvist et al[205] it was suggested that heterozygous carriage of FVL results in a reduction in blood loss at delivery. In that study the assessment of blood loss was highly subjective. In a small group of FVL positive subjects (n=37) (Chapter 4) no difference in pre-natal, peri-natal, or post-natal blood loss was observed when compared with FVL negative subjects. Similarly, in FVL negative subjects, no relationship

between pre-natal, peri-natal, or post-natal blood loss and APC:SR was observed.

7.5 No significant relationship between the APC:SR and placental weight was observed, although in subjects with an APC:SR less than the reference range for non-pregnant females, smaller birth weights were noted. When birth weights were corrected by adjustment for parity, gestation at delivery and fetal sex[231], and the influence of gestation on the APC:SR was minimised, no relationship between the APC:SR and birth weight was found. However significantly smaller birth weight centiles were observed in subjects in whom a fall in APC:SR with gestation occurred.

7.6 Significantly lower APC:SRs at 7-15 weeks were observed in subjects, normotensive at recruitment and with no past history of hypertension, who subsequently developed PIH or PET in the current pregnancy. This difference persisted for PIH when the analysis was restricted to primigravid subjects. No relationship between the APC:SR at 27-34 weeks gestation and the occurrence of PIH or PET was noted. A 2 fold ($C.I._{95}$ 1.1-3.6) increased risk of PIH and 3 fold increased risk of PET ($C.I._{95}$ 1.2-8.0) in the current pregnancy was observed in those subjects in whom no decrease in APC:SR was seen when compared with those in whom the APC:SR did decrease from 7-15 to 27-34 weeks. These results suggest that, in the absence of FVL, subjects with a lower APC:SR early in pregnancy do not increase resistance to APC with increasing gestation and are at a higher risk of development of PIH and PET. They also appear to have larger babies than those in whom an increase in APCR occurs. As lower APC:SRs are associated with venous

thrombosis[238], and as a decrease in the APC:SR occurs in the majority of pregnancies, this suggests that a higher APC:SR early in pregnancy and a fall in APC:SR with increasing gestation is a physiological response which mirrors fetal development. Individuals with a lower APC:SR early in pregnancy, do not become more resistant with increasing gestation and the (physiological) change in APC:SR does not occur. The response to APC appears to be set early in pregnancy and the sensitivity to APC may influence, or be influenced by, fetal development. The APC:SR, in the absence of FVL, does not, however, appear to predict fetal loss or neonatal death.

7.7 A significant inverse relationship between F1+2 levels and APC:SR was observed, although this may reflect the influence of gestation on both variables. When the effect of increasing gestation was excluded, a significant inverse relationship between APC:SR and TAT concentration was observed at 28-32 weeks, suggesting that the APC:SR may reflect thrombin generation in vivo.

7.8 Monocyte ICAM-1 expression, as assessed by flow cytometry, was influenced in a specific dose-dependent fashion by human alpha thrombin and may therefore be a useful indicator of the link between the coagulation cascade, thrombin and the inflammatory response of this cell. In pregnancy, a significantly higher level of monocyte ICAM-1 was observed when compared with non-COCP using female subjects. No significant relationship between ICAM-1 and gestation, parity or smoking was observed. In pregnant subjects, a significant positive relationship between monocyte ICAM-1 and plasma ICAM-1 was observed. This relationship was independent of

smoking. A small but significant inverse relationship of both monocyte ICAM-1 and plasma ICAM-1 with APC:SR at 28-32 weeks gestation was noted. This suggests that in individuals with a normal pregnancy outcome the thrombin generation associated with the APC:SR at 28-32 weeks gestation is mirrored by the inflammatory response of the monocyte. However, as the monocyte has a significant, independent role in the activation of coagulation, it is conceivable that activated monocytes could also influence the sensitivity to APC in plasma by modulating the generation of factor VIIIa and factor Va.

7.9 Although the APC:SR early in pregnancy predicts the occurrence of PIH and PET, the APC:SR assessed at 27-34 weeks appears insensitive to these disorders. Further study would be required to show if clinically evident PIH or PET is associated with an alteration of monocyte ICAM-1 expression. Furthermore, assessment of the relationship of monocyte ICAM-1 with markers of thrombin generation such as TAT would allow the link between the APC:SR and monocyte ICAM-1 to be confirmed as being due to thrombin generation in vivo.

7.10 Alteration of the balance of coagulation factors and inhibitors is a physiological response to pregnancy. The principal outcomes of the coagulation cascade include thrombin generation and fibrin formation. As shown here, increasing gestation is associated with an increase in thrombin formation. Although thrombin can have an anticoagulant effect via protein C activation and is rapidly inhibited by $alpha_2$ macroglobulin[135], pregnancy is associated with an increased risk of ante-partum deep venous thrombosis[254]. This increased risk suggests that the thrombin generation

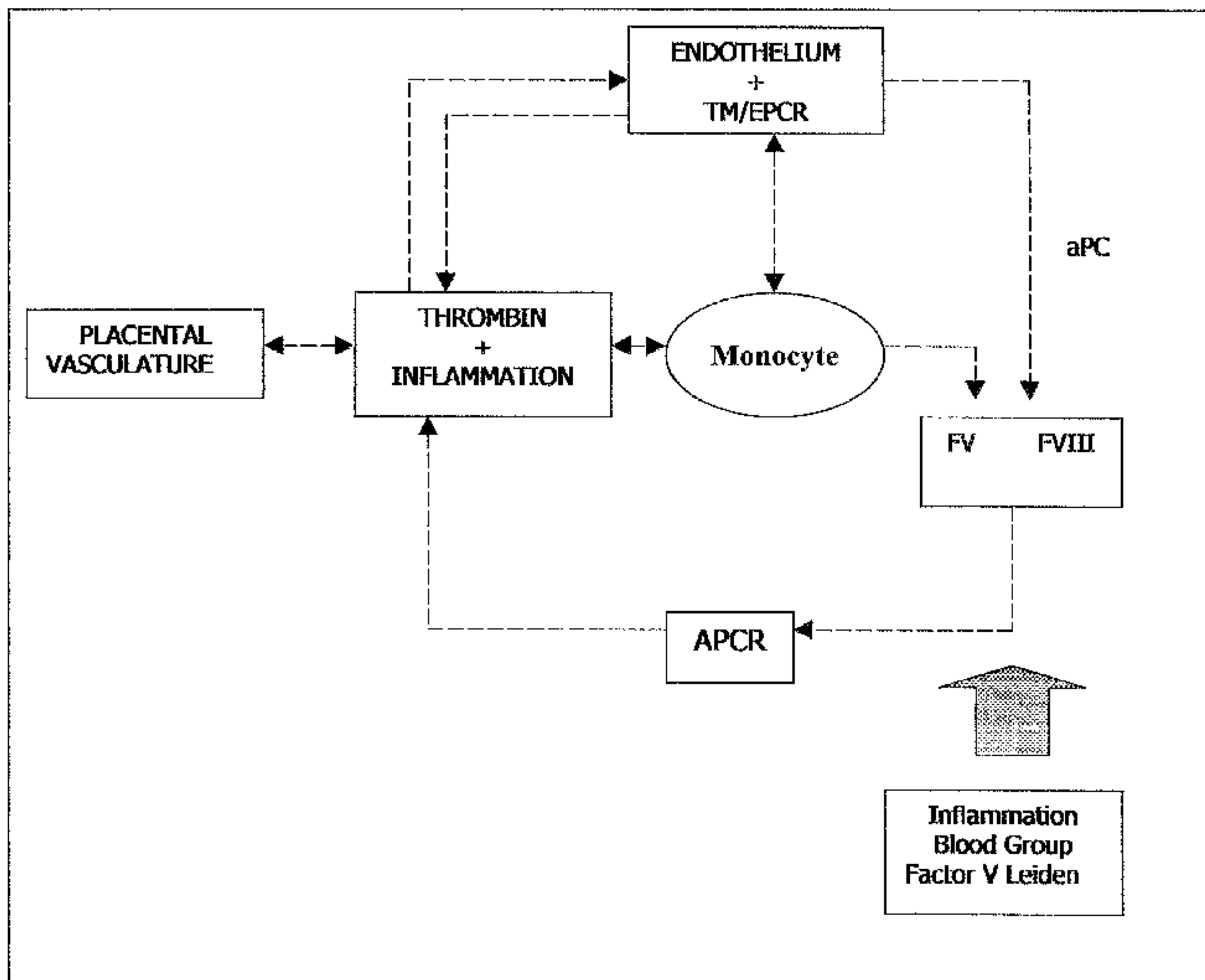
of pregnancy is associated with a hypercoagulable state which is compensated in the majority of subjects. In those subjects with heritable thrombophilia (such as FVL), this compensation may not occur and venous thrombosis[255], PET[186] and fetal loss[148] will result. An increase in thrombin generation, due to an increase in coagulation factors (including FVIIIc and FVc) occurs with increasing gestation and is likely to be a preparation for the haemostatic challenge of delivery. This is mirrored by the increase in ACPR with gestation which occurs in ~75% of pregnancies.

In addition to thrombin, fibrin formation appears to be integral to successful placental development[137] and has been shown to be important in chorionic villous repair[256]. The placenta is a significant source of tissue factor[257] and can also modulate the coagulation cascade via thrombomodulin[258]. It is likely then that the placenta is the driving force behind the increased thrombin generation observed in normal pregnancy. The aetiology of pre-eclampsia is unknown, but is assumed to be due to an immunological response to the developing pregnancy. This results in abnormal trophoblast invasion of the developing placental bed. A process which is normally complete by 20 weeks gestation (reviewed in[259]). In pre-eclampsia it is conceivable that the reported alterations in maternal lipids, maternal coagulation (including an increase in tissue factor[260]) and the increase in inflammation, are a response to the pathological process in the developing placenta.

In normal pregnancy a relationship between the APC:SR, thrombin generation and the inflammatory response of the monocyte is seen. Whether

pregnancies complicated by pre-eclampsia show an exaggerated monocyte ICAM-1 response requires further investigation. The potential interactions between these factors and the placenta are shown in Figure 7.1. In 25% of pregnancies the physiological increase in ACPR with gestation does not occur. In these individuals a lower APC:SR is observed between 7 and 15 weeks gestation. Thus a relationship between the APC:SR (assessed at 7-15 weeks gestation) and the occurrence of pre-eclampsia exists before pre-eclampsia would be evident pathologically in the placenta. This indicates that the APC:SR is either a sensitive measure of the maternal response to early pre-eclampsia, or that the APC:SR represents a phenotype (a combination of FVIIIc, FVc, PS, blood group, smoking and inflammation) which, like FVL, increases the susceptibility to the condition. This would be consistent with the observation that a low APC:SR, in the absence of FVL, is associated with venous thrombosis[238] and would also be consistent with the increased risk of VTE observed when FVL positive subjects are exposed to acquired APCR via the combined oral contraceptive[91] or pregnancy[254]. Peri-conceptual studies would be required to show how early in pregnancy the response to APC is set and would assist in refining the understanding of the maternal and fetal contribution to PET.

Figure 7.1 The interaction between thrombin, the monocyte and APCR



Legend The interaction of the placenta with thrombin and maternal coagulation is shown. The function of TM and EPCR is altered by inflammation. TM = thrombomodulin, EPCR is the endothelial protein C receptor.

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