**Shendi University**

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# Role of Activated Protein C Resistant and Anticoagulant Deficiency in Vascular Complication of Pregnancy in Shendi locality, Sudan.

*A Thesis Submitted in Fulfillment for the Requirements of the PhD Degree in Medical Laboratory Sciences (Haematology)*

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# الآيـة

( قُلْ مَنْ رَبُّ السَّمَاوَاتِ وَالْأَرْضِ قُلِ اللَّهُ قُلْ أَفَاتَّخَذْتُمْ مِنْ دُونِهِ أَوْلِيَاءَ لَا يَمْلِكُونَ لِأَنْفُسِهِمْ نَفْعًا وَلَا ضَرًّا قُلْ هَلْ يَسْتَوِي الْأَعْمَىٰ وَالْبَصِيرُ أَمْ هَلْ تَسْتَوِي الظُّلُمَاتُ وَالنُّور أَمْ جَعَلُوا لِلَّهِ شُرَكَاءَ خَلَقُوا كَخَلْقِهِ فَتَشَابَهَ الْخَلْقُ عَلَيْهِمْ قُلِ اللَّهُ خَالِقُ كُلِّ شَيْءٍ وَهُوَ الْوَاحِدُ الْقَهَّار )

(16). سورة الرعد

## Dedication

To the soul of my father. May ALLAH reward him in the paradise

To my mother for her endless patience and constant support.

To my brothers, sister and family members who have encouraged me in my education journey all through the time and for their persistent assistance.

To my wonderful lovely wife, and beautiful daughters and sons. Thank you for your support, patience, and humor throughout this process. I love you!

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## List of abbreviation

| | |
|---|---|
| APC | Activated protein C |
| APC-R | Resistance to activated protein C |
| APTT | Activated partial thromboplastin time |
| Arg | Arginine |
| BMI | Body mass index |
| C4BP | C4b binding protein |
| CI | Confidence interval |
| COC | Combined oral contraceptive |
| DNA | Deoxyribonucleic Acid |
| DVT | Deep venous thrombosis |
| EDTA | Ethylene Diamine Tetra Acetate |
| ELISA | Enzyme immunosorbant linkage assay |
| EPCR | Endothelial protein C receptor |
| F II | Prothrombin |
| F IIa | Thrombin |
| F IX | Coagulation factor IX |
| F IXa | Activated factor IX |
| F V | Coagulation factor V |
| F Va | Activated factor V |
| F VII | Coagulation factor VII |
| F VIIa | Activated factor VII |
| F VIII | Coagulation factor VIII |
| F VIIIa | Activated factor VIII |
| F X | Coagulation factor X |
| F Xa | Activated factor X |
| F XI | Coagulation factor XI |
| F XIa | Activated factor XI |
| F XIII | Coagulation factor XIII |
| F XIIIa | Activated factor XIII |
| FVL | Factor V Leiden |
| G20210A | Mutation at 20210 in the prothrombin gene |
| Gln | Glutamine |
| IUGR | Intrauterine growth restriction |
| LETS | Leiden Thrombophilia Study |
| LMWH | Low molecular weight heparin |
| MTHFR | Methylene tetra hydrofolate reductase |

| | |
|---|---|
| OAC | Oral anticoagulant |
| OR | Odds ratio |
| PAI-1 | Plasminogen Activator Inhibitor Type I |
| PAI-2 | plasminogen activator inhibitor 2 |
| PAR | population attributable risk |
| PAR% | population attributable risk proportion |
| PAR-1 | protease activated receptors |
| PC | protein C |
| PCCs | Prothrombin Complex Concentrates |
| PCI | Protein C Inhibitor |
| PCR | Polymerase Chain Reaction |
| PPROM | Preterm premature rupture of membranes |
| PS | Protein S |
| PTA | Prothrombin Activator |
| P-value | Probability value |
| RVV-V | Russell’s viper venom-Factor V |
| SD | Standard deviation |
| TAFI | Thrombin-activatable fibrinolysis inhibitor |
| TF | Tissue factor |
| TFPI | Tissue factor pathway inhibitor |
| TM | Thrombomodulin |
| UFH | Unfractionated heparin |
| VLBW | Very low birth weight |
| VTE | Venous thromboembolism |
| vWF | Von Willebrand factor |

## ملخص البحث


**المقدمة:-** القابلية لحدوث التخثر تترافق مع حالات مرضية كثيرة و تشمل النوع الوراثى مثل الحالات التى تحدث فيها طفرات جينية فى الجين المورث لمضادات التجلط الطبيعية مثل بروتين سى ، برتين اس و مضاد الثرومبين او عوامل التجلط مثل العامل الخامس و العامل الثانى.

تعتبر القابلية للتجلط من مضاعفات الخطرة ليست لمضاعفات الحمل فقط و انما لفقدان الجنين و مضاعفات الاوعية الدموية.

**الهدف :-** تهدف الدراسة لمعرفة العلاقة بين القابلية لحدوث الجلطات الوراثى مع مضاعفات الحمل المشيمى.

**المنهجية :-** أجريت هذه الدراسة المقطعية التحليلية بمدينة شندى فى الفترة من 2013 الى 2018م و شملت الدراسة اربعة و خمسون من النساء السودانيات. 20 منهن سودانيات ذوات حمل طبيعى كعينة ضابطة و 34 من النساء ذوات الحمل المتعثر كعينة اختبارو اللاتى تتراوح اعمارهن من 18 الى 50 سنة بمتوسط عمرى 30.7 سنة.

حللت النتائج بواسطة برنامج الحزم الاحصائية الاجتماعية مشتملة على المتوسط و الانحراف المعيارى و اختبار تى المستقل و اختبار كاى

اختبرت الفحوصات المعملية عاى النحو التالى: عامل التجلط الخامس المقاوم لبوتين سى النشط ، مستوى بروتين س و مستوى بروتين اس باستخدام جهاز قياس التجلط بينما اختبر مستوى بروتين سى غير المرتبط بطريقة الانزيمات المرتبطة اما مستوى مضاد الثرومبين فقد قيس بجهاز قياس الطيف الضوئى

**النتائج :-** اظهرت النتائج ان نسبة المتجانسة لمقاومة بروتين س النشط كانت منعدمة تماما، بينما غيرالمتشابهة منه كانت 47.1%

متوسط مستوى بروتين س فى عينة الاختبار كانت 43.7% مقارنة بنسبة 81.4% للعينة الضابطة مع قيمة احتمالية 0.001 و كذلك متوسط برتين اس فى عينة الاختبار يلغت 76.2% مقارنة 86.6% التى سجلت فى العينة الضابطة و كانت نسبة غير المرتبط منه 97.9% فى عينة الاختبار مقارنة مع 95.8% فى العينة الضابطة بقيمة احتمالية 72.0

كذلك اظهرت نتائج الدراسة ان متوسط الثرومبين فى العينة الضابطة 127.3 بينمافى عينة الاختبار 122.9 مع قيمة احتمالية 0.766

ايضا اوضحت الدراسة ان تكرار عوز بروتين س ، بروتين اس ، مضاد الثرومبين وبروتين سي المقاوم كان ( 91.2%، 12% ، 13%، 0%) على التوالي

**الخلاصة :-** خلصت الدراسة الى ان عوز بروتين س يعتبر من عوامل مضاعفة الخطر لحدوث الحمل المتعثر، بينما برتين اس و مضاد الثرمبين هما اقل ارتباطا بتعثر الحمل و المقاومة ليروتين س النشط ليس لهم علاقة مع الحمل المتعثر.

## Abstract

**Background**: Thrombophilia can be defined as a predisposition to thrombosis. Abnormalities in haemostasis that are associated with clinical thrombophilia include heritable defects, such as mutations in the genes encoding the natural anticoagulants antithrombin, protein C, and protein S, or clotting factors prothrombin and factor V. Women with thrombophilic defects have been shown to be at increased risk, not only of pregnancy associated thromboembolism, but also of other vascular complications of pregnancy, including pre-eclampsia and fetal loss.

**Objective:** To determine the association of thrombophilia with placental complicated pregnancy.

**Methods:** In this cross sectional analytical study, a total of 54 Sudanese subjects were enrolled in the study, from July 2014 till July 2018 in Shendi town. The details of the subject go as follows: 20 normal pregnant Sudanese women as control group and the rest 34 Sudanese women with complicated as study group, with age range was between 18 and 50, with the mean of 30.7 years. The data were analyzed by using Statistical Package for Social Sciences (SPSS); the mean, STD, independent t test and cross tabulate were used.

A novel prothrombin-based activated protein C resistance, protein C (PC) activities and protein S (PS) activities were measured bycoagulometer usedACTICLOT reagents.Free protein S (PS) was measured by (ELISA) used IMUCLONE free protein S ELISA. Antithrombin III (ATIII) activities were measured byspectrophotometer used, all the applied reagent were supplied from (Sekisui Diagnostics GmbH,Pfungstadt, Germany).

**Results:** The findings of this study showed that APC-R homozygous was (0%), heterozygous was (47.1%) and wild type was (52.9%).Also the mean of PC in

study group was (43.7%), while in control group (81.4%), with P. Values of (0.001).the mean of protein S in study group was (76.3%), while in control group was (86.6%), with P. Value of (0.073). The mean of FPS in study group was (97.9%), while in study group was (95.80 %) with P. Value of (0.720). The mean anti-thrombin level in study group was (122.9%), while in control group was (127.30%), with P value of (0.766).

Also the study demonstrated the frequency of PC, PS, AT III deficiency and APC-R were (91.2 12%, 13% and 0%) respectively.

**Conclusion:** Protein C, deficiency were found to be the most frequent risk factors in women with complicated pregnancy, while PS , AT III deficiency were found less frequent and APC-R were not associated with placental complicated pregnancy .

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## 1.1. Introduction

Hereditary deficiencies of antithrombin *(AT)*, protein C *(PC)* and protein S *(PS)* and activated protein C resistance *(APC-R)* are strong risk factors for venous thromboembolism *(VTE)*.[1-3]Women with these deficiencies are at higher risk of VTE during pregnancy and the puerperium, because of the acquired hypercoagulable state associated with this condition.[4]It is likely that not only maternal veins but also maternal–placental vessels are more prone to the development of thrombosis, as has been demonstrated in women with mild thrombophilic defects.[4] Consequently, women with strong thrombophilic defects, i.e. deficiencies of *(AT)*, *(PC)*, *(PS)* or *(APC-R)*, may be at higher risk of fetal loss, because of placental insufficiency as a result of placental infarction. Thus far, only a few family studies addressed fetal loss in women with these rare deficiencies.[4]Although the reported risk of fetal loss was increased compared with controls, this result was not consistent in meta-analyses.[5] It was demonstrated that the concomitance of other thrombophilic defects increases the risk of *(VTE)* in subjects with hereditary deficiencies of *(AT)*, *(PC)* or *(PS)*. Moreover, concomitance was frequently observed in families with these deficiencies.[6] Similarly, concomitance might also increase the risk of fetal loss.

The incidence of all types of thrombophilia in women with obstetrical complications is not so rare and every specialist should think about these conditions and has to investigate and to suspect them in their patients with recurrent fetal loss or severe complication of the pregnancy. Approximately 1 to 5 percent of pregnant women have serious complications of pregnancy, such as severe preeclampsia, abruptio placentae, intrauterine fetal death, or severe fetal growth retardation.[7]

For diagnosis of inherited thrombophilias should be investigated some labs and genetic factors -Leiden factor V mutation (R560Q) (DNA test by PCR),protein C levels, protein S levels, activated protein C activity and PAI-1 gene mutation.[7]

We performed our study to assess thrombophilia (protein C, protein S, antithrombin III levels & activated protein C resistance) as risk factors for placental complicated pregnancy.

### 1.2. Justification

Pregnancy is associated with an increased risk of venous thromboembolism (VTE), and this condition remains an important cause of maternal morbidity and mortality.

Approximately 50% of gestational VTE are associated with thrombophilia. Recent studies suggest that there is also a link between thrombophilia and pregnancy loss, as well as other gestational vascular complications. Heritable thrombophilia is becoming more frequent.

This study for the prevalence of heritable thrombophilia during pregnancy, in an area that remains particularly challenging because of the potential for anticoagulant-related fetal as well as maternal complications and the paucity of good-quality data upon which to base clinical decisions.

## 1.3. Objectives:-

### 1.3.1. General Objective:-

Assessment of protein C, protein S, antithrombin III levels & activated protein C resistance; as risk factors for placental complicated pregnancy.

### 1.3.2. The specific objectives:

1.3.2.1. To assess PC, PS and ATIII deficiency among case and control.

1.3.2.2. To determine the APC-R frequency among placental complicated pregnancy.

1.3.2.3. To correlate the APC-R with types of placental complicated pregnancy.

# Literature review

## 2.1. Hemostasis:-

The main factors maintaining the balance between bleeding and thrombosis are the vessel wall, platelets, coagulation system, and fibrinolytic system. At the site of a vessel wall injury, platelets serve as the first hemostatic plug by adhering to exposed collagen directly and through von Willebrand factor. Aggregated and activated platelets support local coagulation by providing a negatively charged phospholipid surface for the coagulation cascade, which eventually forms a stable fibrin clot. Coagulation is regulated by natural anticoagulant mechanisms, to limit the process at the site of injury. Finally, the clot is dissolved by the fibrinolytic system.(8)

### 2.1.1. Primary haemostasis:-

Is a series of events including vasoconstriction and platelet activation, vasoconstriction diverts blood from the site of the injury and protects it from exposure to sub-endothelial structures that activate blood coagulation. Platelets adhere to the injured vessel wall, a process mediated by glycoprotein *(Ib)* which binds to *(vWF)* on the endothelial cells. In addition, activation of platelets leads to a change in shape, leading to exposure of new receptors on the platelet membrane which makes the platelets able to form aggregates, mediated by the bridging of glycoprotein *(GIIb, GIIIa)* to glycoprotein *(GIIb, GIIIa)* on another platelet by fibrinogen. This primary platelet plug is very fragile and must, in order to avoid detachment and subsequent resumed bleeding, be stabilized and more firmly anchored to the vessel wall; this is achieved via fibrin formation by the coagulation pathway also called secondary haemostasis.(8)

### 2.1.2. Secondary haemostasis:

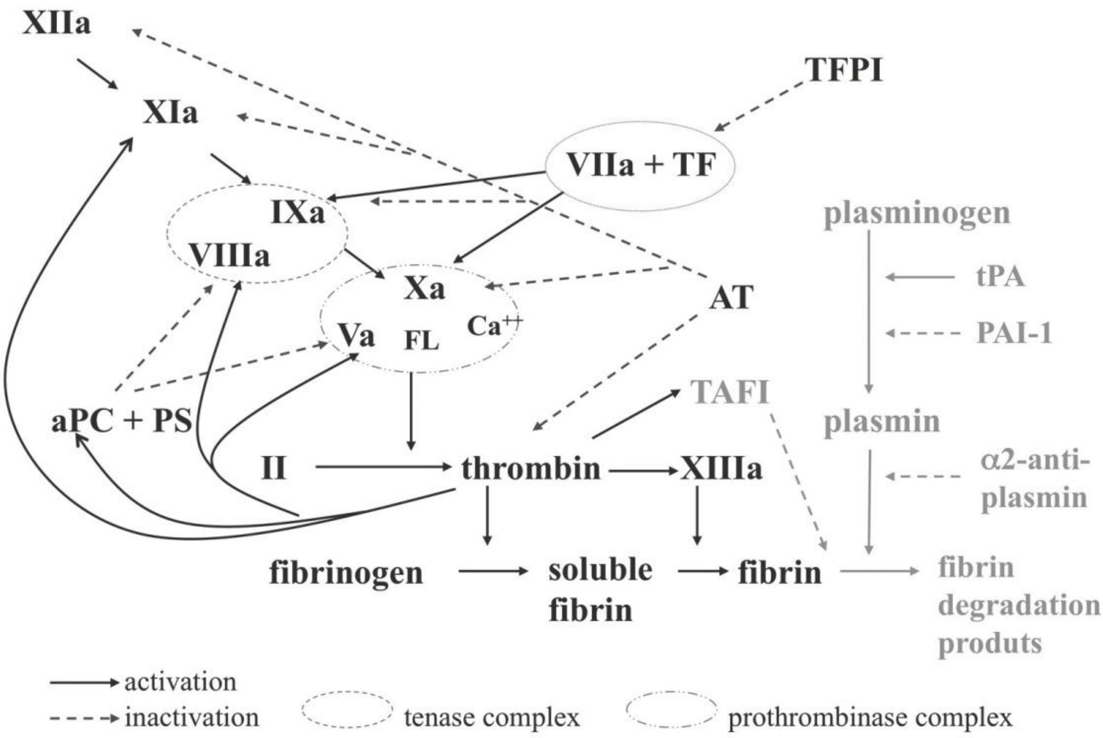



**Figure (2-1): sketch of the coagulation cascade.**[9]

The procoagulant coagulation cascade is composed of serine protease enzymes and their cofactors. The end point of this cascade is the formation of active thrombin. The coagulation cascade occurs on a phospholipid surface, mainly on the activated platelets or the injured endothelium, in the presence of *(Ca++)*. The coagulation process begins when tissue factor *(TF)* is exposed to blood and binds with *(FVIIa)*, which pre-exists in trace amounts in the blood. *(F VIIa)* needs to be bound to TF to gain proteolytic activity. *(TF - F VIIa)* complex activates *(F IX)* and more efficiently *(F X)*. [9] The first small amounts of *(F Xa)*

activate *(F V)*, and together they form a prothrombinase complex to activate prothrombin to thrombin.[10]

After this initiation phase, the newly formed thrombin activates *(FV, F VIII)* and *(F XI)* thereby accelerating its own activation and leading to a very efficient propagation phase of coagulation. *(F IXa)* with its now activated cofactor *(F VIIIa)* (tenase complex), activates efficiently *(F X)*, and then *(F Xa)*, with its cofactor *(FVa)* (prothrombinase complex), activates prothrombin to thrombin. *(F XIa)* serves as another activator for *(F IX)* to ensure the efficiency of the thrombin formation process.[9] Thrombin converts the soluble fibrinogen into insoluble fibrin, which forms a network in and around the platelet plug. Thrombin also activates *(F XIII)*, which cross-links fibrin molecules to form a stable clot.[8] In addition, thrombin further activates platelets,[9]ensuring excellent conditions for coagulation to proceed on the phospholipid surface. As a link between coagulation and inflammation, thrombin can activate endothelial cells, mononuclear cells, platelets, fibroblasts, and smooth muscle cells through *(PAR-1, PAR-3)*, and *(PAR-4)* (protease activated receptors) on their surface, leading to the production of several cytokines and growth factors.[11]

Anticoagulant mechanisms regulate the coagulation cascade rigorously to limit thrombosis at the site of vessel wall trauma. Limiting factors include several phenomena: adhered, activated platelets remain at the site of injury, serine proteases involved in the process need to be proteolytically activated, and physiologic anticoagulants - tissue factor pathway inhibitor *(TFPI)*, antithrombin, and the protein C system - control critical points of the coagulation cascade.[9]

Platelet factor 4 released from platelets increases protein C activation rate and this may also limit thrombus formation outside the site of injury.[12]

*(TFPI)* neutralizes stoichiometrically the *(TF - F VII)* complex.[9] Antithrombin can neutralize all the procoagulant serine proteases by binding to them,[9] the primary targets being thrombin, *(F Xa)*, and*(F IXa)*.[13] The protein C system regulates the coagulation process dynamicall by responding to the presence of thrombin. [12] This anticoagulant system is described in detail in the next section.

**2.1.3. Protein C:-**

Protein C is a 62-kD glycoprotein, synthesized in the liver as a zymogen, which circulates in the blood at a concentration of (4µg/mL). Activation of protein C requires conversion of the single-chain zymogen into a 2-chain serine –protease-like enzyme required for catalytic activity and a key component of the natural anticoagulant pathway.[14]

Protein C circulates as single chain protein is converted by thrombin into its active form *(APC)* which, with its cofactor protein S, degrades factor *(Va)* and factor *(VIIIa)*. The activation of *(PC)* to *(APC)* occurs relatively slowly in the presence of thrombin alone but the rate of activation is increased significantly when thrombin is bound to the transmembrane protein thrombomodulin *(Tm)*.[15]

Thrombin bound to *(Tm)* has no procoagulant activity but significant anticoagulant activity through the protein C-protein S pathway.In addition to its anti-coagulant role, activated protein C exhibits anti-inflammatory and anti-apoptotic activities. *(APC)* also binds to *(PAI-1)* (Plasminogen Activator Inhibitor Type I) and inhibits its activity, so preventing inhibition of *(T-PA)* and thereby enhancing fibrinolysis.[15]

Activated protein C is inhibited by protein C inhibitor *(PCI)* - historically known as *(PAI-3)* - a member of the serpin family of serine protease inhibitors.[15]

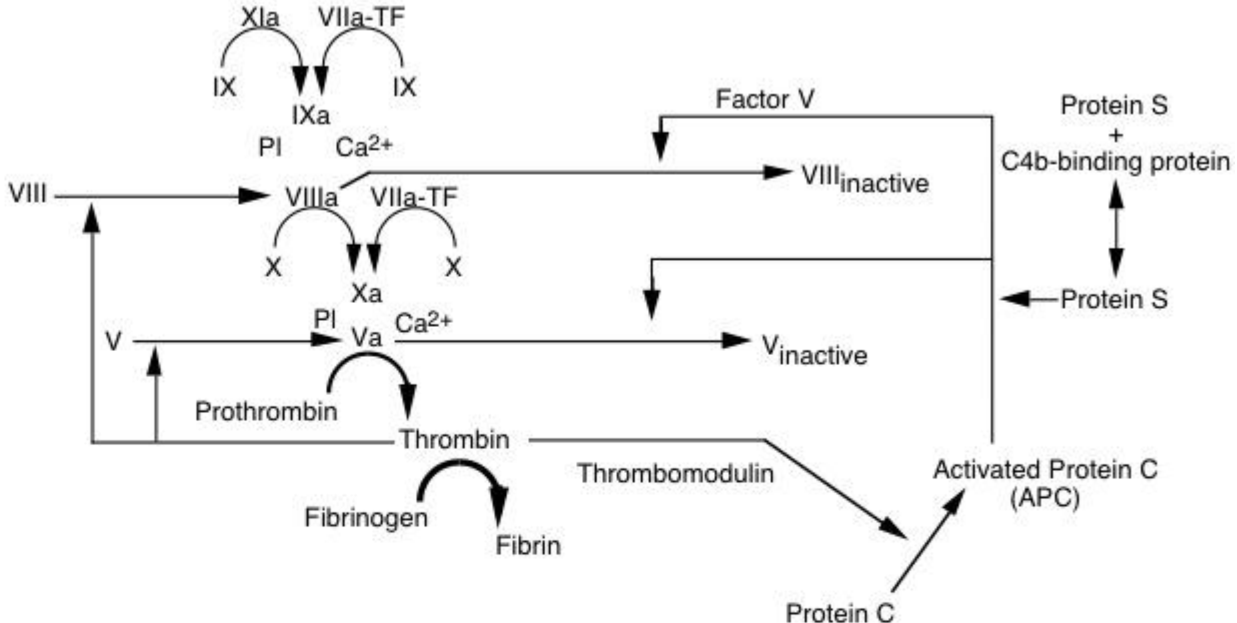



**Figure (2-2):Protein C pathway**[15]

Protein C deficiency is inherited in an autosomal dominant manner and increases the risk of *(VTE)* to a magnitude depending on whether it is homozygous or heterozygous.[15]

Homozygous protein C deficiency is rare and presents in newborns with purpura fulminans (a form of disseminated intravascular coagulation characterized by extensive cutaneous haemorrhage and necrosis) which is rapidly fatal unless treated with protein C replacement.[15]

In individuals with heterozygous protein C deficiency, warfarin may produce a similar phenomenon early in its use by reducing the level of protein C which has a short half-life approximately 6 hours; before significant falls in the other vitamin K dependent procoagulants occurs *(FII)* is slower 4 to 5 days. This, despite warfarin's role as an anticoagulant, without therapeutic heparin, produces a procoagulant state characterized by thrombus formation in the small dermal vessels and extensive skin necrosis. It is a rare phenomenon.[15] Activated protein C resistance is the inability of protein C to cleave factors *(Va)* and/or *(VIIIa)*.

This may be hereditary or acquired but is usually due to abnormalities in the targets of activated protein C activity rather than to abnormalities of protein C itself. The most common example of activated protein C resistance is due to the Factor *(FVL)* mutation.[15]

**2.1.4. Protein S:-**

Protein S is a vitamin K-dependent glycoprotein and is a key component of the protein C-protein S natural anticoagulant pathway.[14]

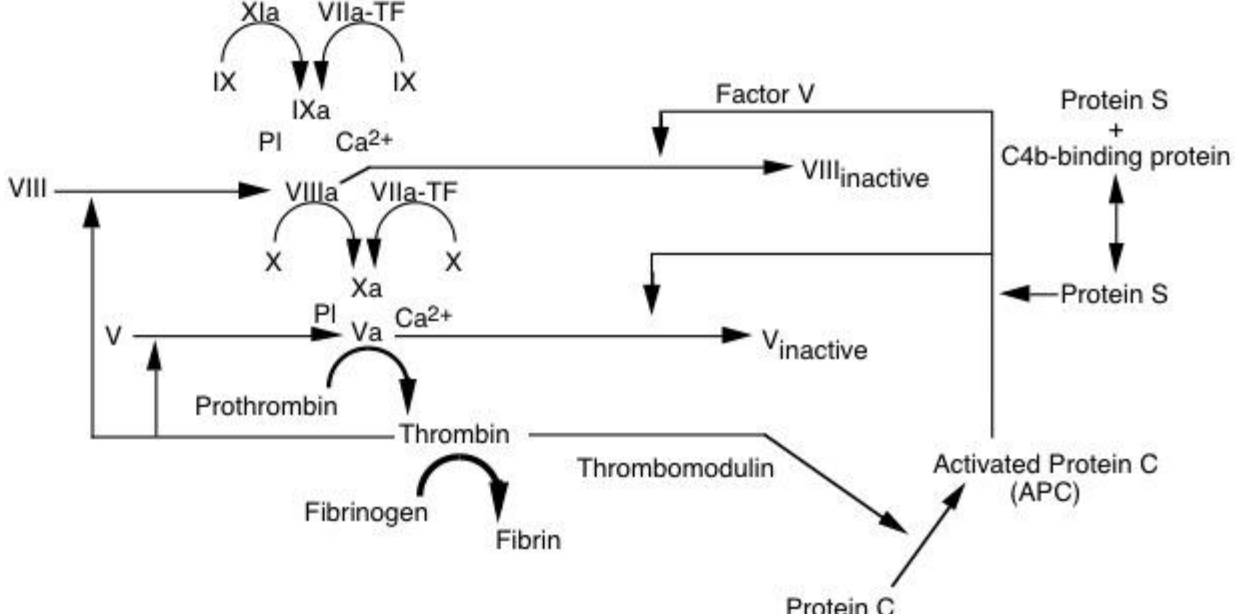



**Figure (2-3):Protein S pathway.**[15]

Protein S acts as a cofactor for the serine protease *(APC)* in the inactivation of factors *(Va)* and *(VIIIa)*. Protein S also exerts activated protein C-independent anticoagulant activity through direct binding to *(FVa)*, *(FXa)* and *(FVIII)*. In addition to its anti-coagulant role activated protein S plays a role in enhancing the phagocytosis of apoptotic cells.[16]

Protein S exists in 2 forms – bound and free. About 65% is in the bound form, complexed to C4b-binding protein *(C4bBP)*, and is inactive. Only the free form has activity. The proportion of bound and unbound forms is regulated by the

availability of *(C4bBP)*. Protein S is synthesized in the liver, endothelial cells and megakaryocytes and has a T½ of 42 hours.[14]

Protein S deficiency is inherited in an autosomal dominant manner and increases the risk of *(VTE)*. Homozygous protein S deficiency may present in newborns with purpura fulminans (a form of disseminated intravascular coagulation characterized by extensive cutaneous haemorrhage and necrosis) which is rapidly fatal unless treated with protein S replacement, usually *(FFP)* as no protein S concentrate exists although prothrombin complex concentrates *(PCCs)* contain in addition to factors*( II, VII, IX and X )* protein C and S.[15]

There are three types of hereditary protein S deficiency:

**Table (2-1): Show types of hereditary protein S deficiency**[15]

| Type | Free protein S | Bound protein S | Total protein S | Protein S function |
|---|---|---|---|---|
| I | ↓ | ↓ | ↓ | Normal |
| II | Normal | Normal | Normal | ↓ |
| III | ↓ | ↑ | Normal | Normal |

However, it is probable that type I and III are phenotypic variants of the same genetic mutation and the sub-division into types I and III at least in some families may be inappropriate. Protein S levels increase with age and this may in part explain this phenotypic variation.[15]

Many cases of type II deficiency have on re-investigation been found to be due to the presence of the *(FV L)*mutation.[15]

### 2.1.5 Protein. C anticoagulant pathway:-

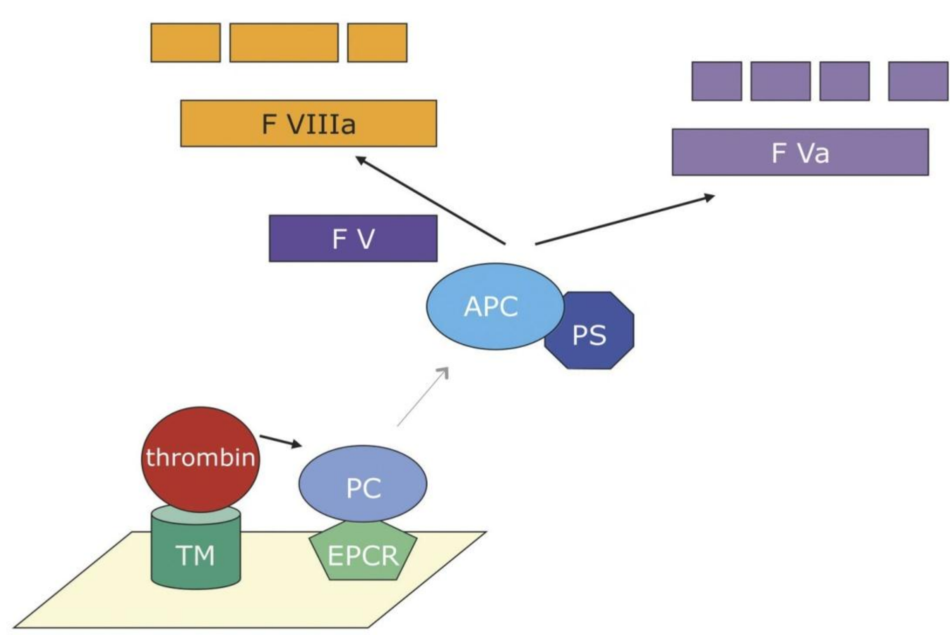



**Figure (2-4): Protein C anticoagulant pathway [9]**

After thrombin is formed, it down-regulates its own formation through the thrombin-thrombomodulin-protein C system.[9] When thrombin binds to thrombomodulin present on the surface of the intact endothelium, it loses its procoagulant activity. Thrombomodulin-bound thrombin is not only efficiently inactivated by antithrombin and other inhibitors, but it also activates protein C to *(APC)*.[12] Endothelial cell protein C receptor *(EPCR)*, also present on the endothelium, presents protein C to the thrombin-thrombomodulin complex enhancing protein C activation.[12]

*(APC)* with its cofactor protein S, inactivates *(FVa)* and *(FV IIIa)* by cleaving certain peptide bonds in them. *(F Va)* is cleaved at least at the sites *(R306, R506)*and *(R679)* and *(F VIIIa)* at the sites *(R336)* and *(R562)*.[17]This inactivation

of central factors in the propagation phase of the coagulation cascade efficiently reduces the formation of thrombin and eventually also the formation of*(APC)*.*(APC)* is slowly inactivated by protein C inhibitor and alfa-1 antitrypsin.[17]

The thrombin-thrombomodulin complex efficiently activates also thrombin activatable fibrinolysis inhibitor *(TAFI)*, which renders fibrin clot more resistant to lysis.[18] The protein C pathway is also involved in limiting inflammatory responses.[11, 12]

Factor V *(FV)*, which was discovered in 1943,[19]has proved to be an important regulator of the hemostatic balance with both procoagulant and anticoagulant properties.[17] The gene of (*F V)* is on the chromosome 1 (1q23), and this single-chained glycoprotein of (2,196 amino acids) is synthesized in the liver. Of the total *(F V)*, (20%) is stored in platelet α-granules; the rest circulates in plasma.[10]The *(F V)* in platelets is of plasma origin, but it is already modified in platelets by partial proteolysis, giving it considerable *(FXa)*-cofactor activity.[10] This seems to be an efficient way to ensure that this important factor is immediately present at the site of vessel wall injury and ready to function. *(F V)* is activated by *(F Xa)* or thrombin to *(FVa)* by the cleavage of three peptide bonds (Arg709, Arg1018, Arg1545) .[10] The inactivation of *(FVa)* is mediated through (*APC*), which cleaves the *(FVa)* at the sites (Arg506, Arg306, and Arg679), usually in this order.[17] The (Arg506) is the preferred site for proteolysis, but protected by *(FXa)* in prothrombinase complex when coagulation is in process.[17]

However, protein S accelerates the slower proteolysis at the site (Arg306) [18]and helps APC to reach the Arg506 site.[13]After cleavage at the site (Arg506), *(FVa)* still has partial procoagulant activity, which is abolished when the (Arg306) and

(Arg679) peptide bonds are cleaved.[20] *(F V)* has procoagulant as well as anticoagulant properties. In its activated form, *(FVa)* serves as an essential cofactor for *(FXa)* (the prothrombinase complex) in the formation of thrombin.[10] On the other hand, the intact *(FV)*acts as a cofactor in the protein C system by stimulating the cofactor activity of protein S in the inactivation of *(FVIIIa)* by *(APC)*.[21]This anticoagulant activity appears after the cleavage of a peptide bond at the (Arg506) by *(APC)*.[17] Mutations in the *(FV)* gene may lead to hemorrhagic and thrombotic tendencies.[17]

**2.1.6. Antithrombin:-**

Antithrombin *(AT)* is a natural anticoagulant that plays a pivotal role in hemostasis by inhibiting the serine proteases thrombin *(IIa)*, *(FXa)*,*(FXa)* and to a lesser extent *(FXIa)*, and *(FIXa)*.[14]

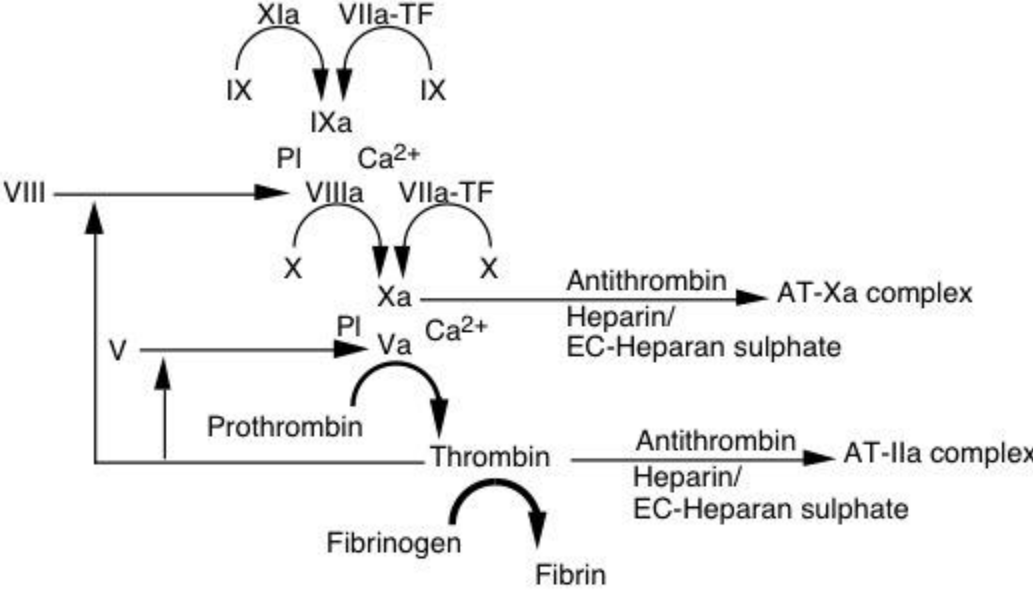



**Figure (2-5): Antithrombin pathway**[15]

Inherited deficiencies of antithrombin are associated with an increased risk of venous thromboembolic disease *(VTE)* and can be detected in (1-2%) of patients with *(VTE)* as compared to (0.02-0.2%) of the normal population. Inherited antithrombin deficiency has a prevalence; in the general population between (0.2/1000 and 0.5/1000). Some patients with *(AT)*deficiency may be relatively heparin-resistant as defined by an apparently sub-therapeutic *(APTT)* despite high doses of *(UFH)*.[22]

Antithrombin is synthesized primarily in the liver and circulates in plasma as single chain protein of (432 amino acids) with a molecular weight of (58,200 Daltons). The normal plasma level is (150 µg/mL) and the plasma half-life is approximately 3 days.[14]

The gene encoding *(AT)* is located on chromosome 1 and a wide variety of mutations have been identified in individuals with antithrombin deficiency and venous thrombosis. Plasma *(AT)* can be measured both by immunological (quantitative) or functional (qualitative) assays and it is on the basis of these assays that antithrombin deficiency is classified:[22]

**Table (2-2): Show classification of Antithrombin Deficiency**[22]

| Type | Interpretation |
|---|---|
| Type I | Quantitative variants - Type I deficiency is associated with a parallel reduction in functional and immunological antithrombin to approximately 50% of normal. |
| Type II | Qualitative variants - Type 2 deficiency is associated with a greater reduction in the functional assay in comparison to the immunological assay |
| Type II HBS: Mutations affecting the heparin binding domain of antithrombin | |
| Type II RS: Mutations that affect the reactive site of antithrombin | |
| Type II PL: Mutations that affect both the heparin binding domain and the reactive site of the molecule. | |

## 2.2. Factor V Leiden:

In 1993, a new phenomenon was described i.e., poor anticoagulant response to activated protein C, in a family with a history of venous thrombosis. The phenomenon was thought to be due to the deficiency of a new protein C cofactor and the laboratory phenomenon was named *(APC)* resistance.[23]

In May 1993, a commercial *(APC)* resistance test became available, *(APC)* resistance was quickly demonstrated to be a common risk factor for venous thrombosis. In the Leiden thrombophilia study *(LETS)*, *(APC)* resistance was present in (21%) of venous thrombosis patients and in (3%) of controls.[24] In a Swedish material, about (40%) of (104) consecutive venous thrombosis patients had *(APC)* resistance compared with (7%) of controls.[25]*(APC)* resistance was also shown to be present in over (50%) of previously unexplained thrombophilic

patients and concomitantly in a few with a previous diagnosis of protein C or protein S deficiency.[26]

In early 1994, there was a report that the cause of *(APC)* resistance to be a property of factor V.[27] At the same time, in Leiden, The Netherlands also concluded that *(FV)* was involved. In June 1994, they published a paper showing that a single *(G)* to *(A)* substitution at the nucleotide position (1691) in the *(FV)* gene was associated with *(APCR)*.[28] The point mutation causes the replacement of amino acid *(Arg)* to *(Gln)* at the site (506) in *(FV)* resulting in the inadequate inactivation of mutated *(FVa)*. The mutation was named as *(FV)* Leiden.[28] As the site (Arg506) is a cleaving site for *(APC)*, it was now easy to understand why the disappearance of this site can cause resistance to *(APC)*. The workers initial idea of the lack of a new protein C cofactor behind *(APC)* resistance has also proved to be partially true as the *(FV)* Leiden mutation abolishes the cofactor activity of *(FV)* in the in activation of *(FVIIIa)*.[17]And also found*(FV)* Leiden mutation in almost all of their fifty Swedish *(APC)* resistant families proving this mutation to be the most prevalent cause for*(APC)*resistance.[29]

**2.2.1. APC resistance and the FVL mutation:-**

*(APC)* resistance was first described in 1993. A new *(APTT)*-based coagulation assay was tested with a fixed amount of added *(APC)*. When this test was run on plasma from a man suffering from recurrent episodes of venous thrombosis, prolongation of the clotting time was much shorter than expected. Several of the proband’s relatives demonstrated a similar poor in vitro response to *(APC)* and family studies suggested that the disorder was inherited as an autosomal dominant trait with decreased penetrance. The term used for describing this condition was *(APC)* resistance, and the quotient between the two clotting times was called the *(APC)* ratio. The molecular basis of this phenotype was identified

as the *(FVL)* mutation,[28] a single mutation *(G)* to *(A)* in position (1691) that converts arginine 506 of *(FV)* to glutamine *(FV:Q)*. *(FVa:Q506, 306)* and Arg is (10-fold) less sensitive to *(APC)*-mediated degradation. However, it is slowly cleaved at Arg506679,[30]which explains the partial *(APC)* sensitivity of individuals with *(FVa:Q)*. Thrombi formed in a *(FVL)* carrier are more resistant to fibrinolysis due to sustained activation of *(TAFI)* by *(APC)* and subsequent inhibition of fibrinolysis,[31]another way the mutation may contribute to thrombosis. More than (90%)of patients with *(APC)* resistance are carriers of the *(FVL)*mutation.[29] It is the most common thrombophilia among Caucasians and the prevalence of heterozygosity in caucasian populations is reported at (2-15 %). The highest prevalences are found in northern europe and the middle east. Haplotype analyses support a single origin for *(FVL)* and the mutation is estimated to have arisen between (17 000 and 29 000) years ago, i.e. after the evolutionary divergence of Africans from non-Africans and of Caucasoid from Mongoloid subpopulations towards the end of the last glacial period. It has been suggested that the founders of both this and the prothrombin gene mutations lived in the middle east because the current prevalences of both mutations are higher in ancient middle eastern populations than in other white populations.[32] The *(FVL)* mutation's widespread presence among Caucasians suggests that it may be a balanced polymorphism with some advantages conferred upon heterozygotes, especially in pre-modern times when death from bleeding associated with childbirth, trauma, or warfare was a significant risk and when scurvy was a common condition. The increase in risk of a first *(VTE)* was found to be (7.9) (95 % confidence interval (CI) 4.1-13) in heterozygotes and (91) (95 % CI 26-322) in homozygotes.[33] The presence of other thrombophilic mutations multiplies the risk of *(VTE)*.[33]

Deficiency of protein Z leads to a bleeding tendency in non-carriers but raises the risk of *(VTE)* in*(FVL)* carriers.[34] Since 1994, a few other rare *(FV)* mutations leading to *(APC)* resistance have been found: Arg to glycine *(FV)* Hong-Kong or threonine *(FV)* Cambridge and mutations leading to low *(FV)* levels (FV R2 haplotype). Concomitant heterozygous *(FVL)* mutation in one gene and mutation in the other gene leading to *(FV)* deficiency is called pseudo-homozygous *(APC)* resistance and results in an *(APC)* ratio in the reference range for homozygosity of the *(FVL)* mutation. This is explained by the fact that *(FV)* is not only a procoagulant but also an anticoagulant, as it is a cofactor, together with protein S, in the degradation of *(FVIIIa)*.[34]

*(APC)* resistance can be evaluated in plasma using different commercially available methods, many of which are derived from the original coagulation test *(APC)* resistance test method, available since 1993. These tests, classic *(APC)* resistance assays, measure the classic *(APC)* ratio as clotting time in the presence of *(APC)*, divided by clotting time in the absence of *(APC)*. These assays have been shown to have some shortcomings; many haemostatic variables can interfere because the test is based on the *(APTT)*. The assays are susceptible to platelet contamination and to clinical conditions such as age; gender; body mass index *(BMI)*; smoking; blood group; *(APTT)*; fibrinogen, homocysteine, triglyceride and total cholesterol levels; all conditions giving rise to high levels of plasma *(FVIII)*. Low classic *(APC)* ratios have been found among individuals with the metabolic syndrome,[35]during oral contraceptive use and pregnancy [36],[37],[38] and in individuals with lupus anticoagulants *(LA)*.[38] In one study a correlation was found between the classic *(APC)* ratio and the protein C level.[39] The *(APC)* ratio is not useful during treatment with heparin or oral anticoagulants *(OAC)*. It has also been shown that the *(APC)* ratio increases in plasma samples

with only slightly reduced levels of *(FII)*, *(FVIII)* and *(FX).*[40] The condition with a classic *(APC)* ratio below the lower limit of the reference interval in the absence of a (*FV)* gene mutation is called acquired (*APC)* resistance. Studies have shown that there is a correlation between this condition, blood coagulation activation and subsequent incidences of *(VTE).*[41] The results from studies assessing the relationship between acquired *(APC)* resistance and the incidence of placenta-mediated complications are conflicting[34, 42, 43]but a relationship between a low ratio and thrombotic lesions in the placenta has been found.[44] Modified *(APC)* resistance tests are based on pre-dilution (1:4) of the sample plasma with *(FV)*-deficient plasma prior to analysis, which is then performed according to routine. This procedure makes the method much more specific for alterations in the (*FV)* molecule, resulting in a very high discrimination of the FV:R 506Q mutation as well as the ability to analyze plasma from *(OAC)*- and heparin-treated individuals and pregnant women.[45] The modified *(APC)* resistance test used in the research underlying this thesis has been reported to have (100%) sensitivity and (100%) specificity for the *(FVL)* mutation and distinguishes homozygous from heterozygous individuals.[46-50]It cannot, however, be ruled out that analysis of plasma from patients with high *(LA)* titres and from patients with *(FV)* deficiency may yield misleading results.[50]

**2.2.2. Prothrombotic mutation: gain of function - loss of function:-**

FV Leiden is a prothrombotic mutation. It is at the same time a gain of function mutation and a loss of function mutation.[51]

First, due to disappearance of the cleavage site at the Arg506, APC is unable to inactivate *(FVa)* optimally leading to increased thrombin formation.[20]

Second, due to the disappearance of that cleavage site, *(APC)* is unable to cleave intact *(FV)* so that *(FV)* could function as cofactor for *(PC-PS)* complex in the

inactivation of F *(VIIIa)*. This loss of anticoagulant function leads, again, to increased thrombin formation.[17]

However, the risk for venous thrombosis caused by *(FV)* Leiden is relatively low. This may be explained by the fact that although cleavage at the site (Arg506) accelerates inactivation of *(FVa)* remarkably by exposing the cleavage sites (Arg306 and Arg679) to *(APC)*, cleavage at the site Arg506 is not absolutely necessary for the inactivation of *(FVa)*.[20] In addition, in the prothrombinase complex, the capability of *(APC)* to inactivate *(FVa)* is similar for the wild type *(FVa)* and *(FVa)* Leiden, because the (Arg506) cleavage site of the wild type *(FVa)* is protected by *(FXa)*, and in *(FVa)* Leiden this cleavage site does not exist.[19, 20] Mechanisms that reduce the effect of this potentially injurious mutation include the acceleration of the cleavage of *(F Va)* by protein S at the site (Arg306).[19]

### 2.2.3. Epidemiology and evolutionary advantage:-

According to haplotype analyses, *(FV)* Leiden is a founder mutation, which occurred about (21,000 years) ago.[33, 52, 53] The mutation is present at a variable frequency (mean 5%) in Caucasians, but absent or nearly absent in other races.[54]-[55] This indicates that *(FV)* Leiden most likely occurred in a Caucasoid subpopulation after the separation of non-Africans from Africans, and Caucasoid populations from Mongoloid populations.[53, 56]

The high prevalence of *(FV)* Leiden in Caucasians suggests an association with evolutionary advantage and many findings support a favourable selection pressure.[56] Data exist indicating that *(FV)* Leiden might protect against peripartal bleeding[57-59] and heavy menstrual blood loss.[60] This could have provided considerable advantage by reducing iron depletion and by protecting against life-threatening post-partum hemorrhage. However, conflicting

observations about pregnancy-related blood loss exist.[61] Similarly to protecting against excessive bleeding in association with surgery,[62]*(FV)* Leiden may have protected against excessive bleeding in association with trauma in the past.Some evidence, although partly conflicting, exists that simultaneous carriage of *(FV)* Leiden might attenuate bleeding symptoms also in hemophiliacs.[63] Other possible selective advantages include a more favourable embryo implantation in carriers of *(FV)* Leiden,[64, 65] and an increased fecundity (shorter time to pregnancy) in the male carriers of *(FV)* Leiden.[66] This is supported by an observation of a slightly increased sperm count in the male carriers of *(FV)* Leiden.[67]

**2.3. F II G20210A:-**

In 1996 a point mutation was reported in the coagulation *(FII)*(prothrombin) gene.[68] The mutation causes a G to A substitution at the nucleotide position of (20210) in the 3'-untranslated region of the gene. The point mutation is associated with elevated prothrombin levels and is therefore a gain of function mutation. FII G20210A allele is associated with about a 2-fold increased risk for venous thrombosis.[68] The mutation is of single origin and is mainly found in the Caucasian population.[33]

**2.4. Pregnancy and haemostasis:-**

Pregnancy is a period of reproduction during which a woman carries one or more live offspring from implantation of a fertilized zygote in the uterus throughout gestation. There are several physiological changes that occur in pregnancy. Physiology of a normal pregnancy involves major changes in both the coagulation system and hematological parameters. These changes appear to be related to the development of the utero placental circulation and provide a protective mechanism during delivery. In the blood, the most important

alterations during normal pregnancy are increased plasma volume, physiologic decrease of hemoglobin, occasional mild thrombocytopenia, neutrophilia, increases in many procoagulant factors, and attenuated fibrinolysis.[69]

Increases in many coagulation factor levels, decrease of anticoagulant activity, and diminished fibrinolysis lead to a hypercoagulable state protecting from excessive bleeding during delivery. The most prominent changes are a decrease in protein S activity (due to the increase of C4BP); acquired protein C resistance; increased levels of von Willebrand factor, *(FVIII)*, and *(FVII)*; increased fibrinogen; and increased activity of fibrinolytic inhibitors *(TAFI, PAI-1, PAI-2)*. Usually the levels of *(F II, F V, F IX)*, and *(FX)* increase slightly and the level of *(F XI)* decreases slightly.[69-72] *(F XIII)* level is increases early in the pregnancy but decreases thereafter .[70] Coagulation parameters usually reach their baseline levels by eight weeks postpartum.[69]

**2.5. Venous thromboembolism:-**

Venous thrombosis can be seen as a classic example of complex common disease which is caused by interaction of acquired and inherited risk factors.[73]Thrombosis occurs when many risk factors are simultaneously present. Each risk factor increases the thrombotic potential and eventually a trigger point for thrombosis is exceeded. The risk of thrombosis increases with age. Therefore, in young adults more risk factors are needed for thrombosis to occur than in old age. Among women of fertile-age the incidence of thrombosis is about (1:10,000) women years.[74]

According to Virchow's triad from the 1856, the emergence of thrombosis is due to changes in the vessel wall, in blood, and in the velocity of blood flow[17]. Venous and arterial thrombosis only partly shares the same risk factors and both also have their own risk factors.[75]

Thrombophilia can be inherited or acquired. Defects of natural inhibitors of coagulation or gain of function of coagulation factors can disturb the strictly regulated balance to favour thrombus extension. Antithrombin deficiency, protein C deficiency, and protein S deficiency are well known, although rare, inherited risk factors for venous thrombosis. They are strong risk factors for venous thrombosis, the estimated increase of risk being about 10-fold.(76) Gain-of-function mutations, *(FV)* Leiden and prothrombin (G20210A)*(FII G20210A)*, are moderate risk factors for venous thrombosis, increasing the risk (5-fold and 2- to 3-fold), respectively.(76)They do not have a major impact on arterial thrombosis, although in special subgroups of young patients they may be involved to some extent.(77)

For acquired thrombophilia, antiphopholipid antibodies are of great importance. Antiphopholipid antibodies are risk factors for venous and arterial thrombosis as well as for pregnancy complications.(75)

Non-O blood group is associated with a (2 to 4-fold) increased risk for venous thrombosis compared with blood group O.(76-78) This is probably due to the higher levels of von Willebrand factor *(vWF)* and *(FVIII)* in individuals with these blood groups. The lower level of *(vWF)* in individuals with blood group O may be due to more efficient clearance of *(vWF)*, which may be determined by ABH antigens on *(vWF)*.(79)

The increasing thrombosis risk associated with increasing age may be due to the progressive increase of many coagulation factors, impaired function of fibrinolytic system, and age-related structural and functional changes in vessel walls.(75)Other recognized acquired risk factors for venous thrombosis include obesity, previous venous thrombosis, surgery, trauma, immobilization, cancer, oral contraceptives, hormone replacement therapy, and pregnancy.(75)

## 2.6. Venous thromboembolism in pregnancy:-

Pregnancy-associated venous thromboembolism is a rare cause of maternal morbidity occurring in less than (1 in 1,000) pregnancies in western countries.[80-84]In these countries, it is, however, a major cause of maternal mortality.[80, 85-87] In Finland also, thromboembolism is the main cause of maternal deaths.[88, 89] Pregnancy increases the risk for venous thrombosis (4- to 10-fold). Besides being a hypercoagulable state, pregnancy causes venous stasis in lower extremities due to the enlarged uterus, and during labour the endothelium of pelvic vessels may be damaged. Thrombosis in the veins of the left lower extremity is overrepresented compared with thrombosis occurring in non-pregnant state. This may be due to the pronounced compression of the left iliac vein by the right iliac artery. Most pregnancy-related venous thrombosis occur during pregnancy, but the risk for venous thrombosis is higher in postpartum period.[80, 85-90]

## 2.7. Inherited predisposition to VTE:-

The most important inherited biochemical disorders that are associated with VTE result from :[91]

- Defects in the naturally occurring inhibitors of coagulation: deficiencies of antithrombin, protein C, or protein S: and
- Resistance to activated protein C, which is caused by the *(FVL)* mutation the majority of cases.

The first of these disorders are rare in the general population (combined prevalence of <1%), have a combined prevalence of approximately 5% in patients with a first episode of *(VTE)*, and are associated with a 10- to 40-fold increase in the risk of *(VTE)*.[91]

The *(FVL)*mutation is common, occurring in approximately 5% of Caucasians and approximately (20%) of patients with a first episode of *(VTE)* (i.e. an approximate 4-fold increase in *(VTE)* risk).[91]

A mutation in the3'untranslated region of the prothrombin gene *(G20210A)*, which is associated with an approximately (25%) increase in prothrombin levels, occurs in about (2%) of Caucasians and approximately (5%) of the those with a with a first episode of *(VTE)* (i.e. an approximate (2.5-fold increase in risk).[91]

Elevated levels of a number of coagulation factors *(I, II, VIII, IX, XI)* are associated with thrombosis in a "dose-dependent" manner. It is probable that such elevations are often inherited, with strong evidence for this in the case of Factor *(VIII)*.[91]

**2.8. Prevention of gestational VTE:-**

Using an assessment tool based on the known risk factors for gestational *(VTE)*, all pregnant women should be assessed for thrombotic risk at the time of booking, at each antenatal visit, on admission for delivery, and following delivery.[28]

Routine screening of all women for thrombophilic defects is not justifiable, but screening of women who have a history of previous *(VTE)* is frequently recommended, and many clinicians would also offer thrombophilia screening to women who give a family history of proven *(VTE)*.[92]

All women assessed to be at increased risk of gestational *(VTE)* should be encouraged to wear graduated compression stockings throughout their pregnancy and puerperium. Given the evidence that the risk of *(VTE)* is greatest following delivery, many obstetricians would also offer women assessed to be at increased risk of pregnancy-associated *(VTE)*, who have no contraindication to anticoagulation or antithrombotics, pharmacological thrombophylaxis following

delivery usually daily prophylactic doses of *(LMWH)*, self-administered subcutaneously, for 6 weeks following delivery.[93]

Consideration may be given to offering pharmacological thrombophylaxis during pregnancy to women perceived to be at relatively higher risk of gestational *(VTE)*. [92, 93] This group includes:

- Any woman who has a history of spontaneous (idiopathic) *(VTE)*.
- Women who have hand a thrombotic event in relation to a previous pregnancy or while using a combined oral contraceptive *(COC)*.
- Any woman who has been found to have a thrombophilic defect because she has been investigated following a previous thrombotic event.
- Women who have no personal history of thrombosis, but who have been investigated because of a family history of *(VTE)* and have been found to have thrombophilic defect associated with a relatively risk of gestational *(VTE)* (e.g. type1antithrombin deficiency, homozygosity for factor *(V)* Leiden or prothrombin (G20210A), or double heterozygosity for factor *(V)* Leiden and prothrombin (G20210A).

Daily self – administered LMWH in prophylactic doses throughout pregnancy and for 6 weeks following delivery is usually considered adequate for most of these women at higher risk, but in some cases, the daily dose of *(LMWH)* may be increased to a level intermediate between that which is usually used for prophylaxis and the dose usually used for treatment of acute *(VTE)*.[92, 93]

The incidental finding of antiphospholipids in pregnancy should trigger increased clinical surveillance, but pharmacological intervention should be reserved for these women with antiphospholipids who are symptomatic. Women with antiphospholipids and a past history of *(VTE)* may usually be considered to be at highly increased risk of recurrent *(VTE)* associated with pregnancy and offered

pharmacological thrombo-prophylaxis, using intermediate dose of *(LMWH)* as described above during pregnancy and the puerperium.[94]

**2.9.FV Leiden and venous thromboembolism in pregnancy:-**

Studies that assess the risk associated with *(FVL)* for pregnancy-associated venous thromboembolism (VTE) varies in many respects. Study designs, selection of cases and controls, reporting of ethnicity, definition of puerperium (from 3 weeks to 3 months postpartum), inclusion of recurrent VTE events, and inclusion of homozygotes in analyses differ.[95]

In case-control studies, the odds ratio of pregnancy-associated venous thromboembolism for FV Leiden varies from (2.8 to 18.3), a pooled OR of six case-control studies was 8.6 (95% CI 5.9-12.6), although these studies were found to be heterogeneous.[92]

In cohort studies, the odds ratio of pregnancy-associated venous thromboembolism for mainly heterozygous FV Leiden varies from (3.7 to 8.3).[40, 82, 86]. For homozygous FV Leiden the OR has been (41.3).

In the meta-analysis, a pooled OR of cohort studies was 4.5 (95% CI 1.8-10.9).[92] This meta-analysis included cohorts fromthrombophilic families[96-98]as well as prospective cohorts of pregnant women.[58, 99]Prospective population-based cohort studies would give the best estimationof the risk associated with *(FV)* Leiden in general population. However, the three prospective studies available [58, 100] consist of less than (4,700) mainly White women, of whom only (383) are carriers of FVL. Numbers are too small to give a definite estimate of risk given that pregnancy-associated venous thrombosis is so rare, usually less than (1/1,000) pregnancies.[100]

In a systematic review and meta-analysis, heterozygous and homozygous carriers of *(FVL)* were analyzed separately for the risk of pregnancy-associated venous

thrombosis. The pooled OR was 8.3 (95% CI 5.4-12.7) for heterozygotes, and 34.4 (95% CI 9.9-120) for homozygotes.[101]

There were no signs of heterogeneity although the eight studies included case-control and cohort studies, as well as family studies. However, population-based studies are still needed to assess the risk in carriers of FV Leiden from the general population.[101]

**2.10. Pre-eclampsia:-**

Pre-eclampsia is an important cause of maternal and fetal morbidity complicating (2-7%) of pregnancies.[102] Pre-eclampsia is one of the leading causes of maternal mortality.[86] In Finland, pre-eclampsia and eclampsia cause about (12%) of maternal deaths.[89] Pre-eclampsia is defined as high blood pressure after (20 weeks) of gestation in a previously normotensive woman plus new-onset proteinuria. Definitions vary slightly among studies, but usually the *(ACOG)* criteria[103] are applied. Pre-eclampsia may be mild, just fulfilling the definition, or severe, including symptoms and findings like thrombocytopenia, elevated liver enzymes, epigastric or right upper-quadrant pain with nausea or vomiting, oliguria, cerebral symptoms, pulmonary edema, and seizures.[102] Pre-eclampsia is ultimately cured only by delivery, therefore often leading to preterm birth. Prematurity and fetal growth restriction, which is often, associated with pre-eclampsia, affect the health of the newborn.[102]

The etiology of this heterogeneous disease entity is still unknown.[102, 104] Pre-eclampsia can be divided to placental pre-eclampsia originating from abnormal placental perfusion, and maternal pre-eclampsia originating from pre-existing problems in mother.[105]However, in an individual, pre-eclampsia may be caused by variable interaction of placental fetal and maternal factors.[104] Factors that have been associated with an increased risk for pre-eclampsia

include primigravidity, multifetal gestation, previous pre-eclampsia, obesity, pregestational diabetes, chronic hypertension or renal disease, family history of pre-eclampsia, and controversially thrombophilia.[102] Endothelial dysfunction is considered to be an important factor in its development.[105, 106] Endothelial cell injury can lead to the activation of coagulation, vasoconstriction, reduced plasma volume due to "leaking endothelium", and glomerular capillary protein leak.[106]

**2.11. FV Leiden and pre-eclampsia:-**

Numerous studies with different designs have assessed association between pre-eclampsia and *(FV)* Leiden and many meta-analyses have tried to determine the true association. In a meta-analysis,[42] the OR for association of *(FV)* Leiden with pre-eclampsia varied from (0.2-12.9) in (24) case-control studies. Studies were so heterogeneous that pooled OR was not calculated. In seven studies specifying severe pre-eclampsia, pooled OR was 3.0 (95% CI 2.0-4.7). These studies included (753 cases) and (1,120) controls of women with reported ethnicity of Caucasian or Israeli. Heterozygous and homozygous carriers of FV Leiden were pooled.[107]

In a meta-analysis, the combined OR for FV Leiden in (12) case-control studies assessing all pre-eclampsia was 1.8 (95% CI 1.1-2.9). Heterozygous and homozygous carriers were pooled. Statistical test for heterogeneity was significant (p=0.04) and a funnel plot analysis suggested publication bias (small negative studies missing). The studies included (1,798) cases and (1,471) controls of mostly Caucasian origin; in one study the participants were Japanese and in one Australian study only (83%) were Caucasian.[107]In their meta-analysis of (11) case-control studies assessing severe pre-eclampsia, the pooled OR for FV Leiden was 2.2 (95% CI 1.3-3.9). Statistical test for heterogeneity was significant (p=0.009), but there were no suggestion of

publication bias. These studies included (1,135) cases and (1,471) controls of mostly Caucasian origin; in three studies, (90-95%) of women were Caucasian and in one study only (40%) were Caucasian. As *(FV)* Leiden is mostly limited to the Caucasian population, inclusion of other ethnicities may influence the results.[107]

In a systematic review and meta-analysis., heterozygous and homozygous carriers of FV Leiden were analyzed separately for the risk of pre-eclampsia. Fourteen studies assessing heterozygous *(FV)* Leiden had pooled OR of 2.2 (95% CI 1.5-3.3) with signs of heterogeneity (p=0.04). The studies included both mild and severe pre-eclampsia and study designs varied from retrospective case-control and cohort studies to one prospective cohort study. Studies included (1,951) cases and (1,971) controls, ethnicity was not specified.[103]

When five studies of severe pre-eclampsia were analyzed separately, the pooled OR for heterozygous FV Leiden was 2.0 (95% CI 1.2-3.4) without signs of heterogeneity. Five studies assessing homozygous FV Leiden had pooled OR of 1.9 (95% CI 0.4-7.9) without signs of heterogeneity. These studies included 612 cases and 536 controls, ethnicity was not specified.[108]

In a systematic review and meta-analysis of nine prospective cohort studies assessing the association between FV Leiden and pre-eclampsia (106),the pooled OR was 1.23 (95% CI 0.89-1.70) indicating that FV Leiden is not associated with an increased risk for pre-eclampsia. There were no signs of heterogeneity. The meta-analysis comprised (21,833) unselected prospectively enrolled women with a spontaneous singleton pregnancy from Ireland, Israel, the United States, the United Kingdom, Australia, Greece, Sweden, and Canada. FV Leiden carriers had a (3.8%) absolute risk for pre-eclampsia whereas in FV Leiden non-carriers

the absolute risk was (3.2%). The prevalence of FV Leiden in these populations varied from (2.7% to 10.9%), ethnicity of participants was not specified.[109]

Only two population-based studies of at least predominantly Caucasian study populations assess FV Leiden as a risk factor for pre-eclampsia. Both are retrospective registry-based cohort studies of geographically well-defined area, one from Scotland,[110] and one from Norway.[111]Both studies pooled heterozygotes and homozygotes in their analyses. The study from Scotland analyzed (494) pre-eclampsia cases and (163) controls, ethnicity was not reported. In this study, FV Leiden was not associated with an increased risk for pre-eclampsia (OR 0.9, 95% CI 0.4-2.1).[109]

The study from Norway analyzed (14,393) pregnancies of (5,874) women, ethnicity was not reported. In this study, FV Leiden was associated with an increased risk for pre-eclampsia (OR 1.6, 95% CI 1.2-2.3). As the study analyzed pregnancies, not women, several pregnancies for each woman were included.[109]

In another study , FV Leiden was not significantly associated with an increased risk for verified pre-eclampsia in a cohort of pregnant women *(FVL)* in (17/243) cases and (204/4,206) controls, OR 1.19, 95% CI 0.64-2.23).[109]

However, when they combined their study with five other cohort studies in meta-analysis to increase power, association between FV Leiden and pre-eclampsia became significant (pooled OR 1.49, 95% CI 1.13-1.96). The pooled analysis included (860) cases and (18,340) controls from the United Kingdom, Sweden, the United States (two studies), Norway, and Ireland. Meta-analyses described above include partly the same studies.[112]

This nested case-control study within a prospective cohort of pregnant women consisted of (13) pre-eclampsia cases and (443) controls. The study included different ethnicities, which were not specified. FV Leiden was not associated

with an increased risk for pre-eclampsia (OR 1.1, 95% CI 0.4-2.7). The study showed that histopathologic features consistent with placental under perfusion were more common in cases than in controls. However, FV Leiden was not associated with these features.[112]

**2.12. Stillbirth:-**

Although stillbirth is a rare pregnancy complication in developed countries, it causes strong emotional burden for the particular family. The stillbirth rate has been estimated to be (4.2-6.8/1,000) deliveries in developed countries.[113] In Finland, like in other Nordic countries, the stillbirth rate is even less and among the smallest in the world. However, due to different definitions the stillbirth rate is difficult to compare between countries.[114]

The precise definition of stillbirth varies in different countries and in different studies. The definition is based on gestational age of the fetus at the time of stillbirth (usually ≥20-24 weeks) or on the fetal weight (usually ≥500g).[114-116] In Finland, stillbirth is defined as stillbirth at or after (22) weeks of gestation, or fetal weight (≥ 500 g).[114] Stillbirths are sub classified as early stillbirths at or before 28 weeks of gestation and late stillbirths after 28 weeks of gestation.[116]

Risk factors for stillbirth include multiple pregnancy, nulliparity, advanced maternal age, pre-pregnant obesity, smoking, maternal diseases, previous stillbirth, and low socio-economic status.[116, 117]The causes of stillbirth include maternal infections, placental lesions like abruptio placentae, or major infarction of the placenta, umbilical cord complications like prolapse, strangulation, or knot, and congenital anomalies. However, (25-60%) of stillbirths remain unexplained. Thrombophilia has been hypothesized as one possible risk factor for stillbirth.[116, 117]

### 2.13. FV Leiden and stillbirth:-

Studies assessing the association between FV Leiden and stillbirths vary in many respects. Study designs, selection of cases and controls, definition of stillbirth, reporting of ethnicity, inclusion of women with previous thromboembolism, and inclusion of homozygotes in analyses differ.[5]

In case-control studies, the odds ratio for association between FV Leiden and stillbirth varied from 0.7 to 9.2. In six of the nine studies, the association was statistically significant. Only two of the studies assessing unexplained stillbirth reported the study population to be Caucasian.[5, 118].

In retrospective cohort studies, the odds ratio varied from (1.3 to 4.4). In only one of these studies, the association was statistically significant (*OR* 2.2, 95% CI 1.5-3.4), but there the studied unit was not a woman but pregnancy.[111]In the only prospective cohort study to this date, FV Leiden was associated with almost a (9-fold) risk for stillbirth (OR 8.85, 95% CI 1.6-48.9). Cohort consisted of (1,707) nulliparous healthy women with a singleton pregnancy and heterogeneous ethnic background. However, there were only six stillbirths in the cohort.[119]

In a meta-analysis, the pooled OR for association between FV Leiden and fetal loss after 19 weeks of gestation was 3.3 (95% CI 1.8-5.8). Analysis included six retrospective studies with no signs of heterogeneity (372 cases, 1,888 controls).[5]

In a meta-analysis, the pooled OR for *(FVL)* was 2.8 (95% CI 1.3-6.2) when assessing only isolated third trimester fetal losses. There were no signs of heterogeneity in this post hoc sub analysis of five studies. In a systematic review and meta-analysis, the pooled OR for association between heterozygous FV Leiden and late fetal loss (third trimester) was 2.06 (95% CI 1.1-3.9) with no signs of heterogeneity. Analysis included six retrospective case-control and

cohort studies with (151) cases and (1,503) controls, ethnicities were not reported.[120]

In a recent review, a meta- analysis of eleven heterogeneous studies yielded a pooled OR of 3.6 (95% CI 2.1-6.2). The above four meta-analyses included partly same studies.[5]

Taken together, the results of individual studies are partly conflicting, perhaps resulting from heterogeneity of the studies. In meta-analyses, FV Leiden has been associated with quite a constant 2-fold to 3-fold increased risk. Population- based studies are few.[54]

**2.14. Preterm birth:-**

Preterm birth (birth before 37 completed weeks of gestation), occurring in (5-13%) of deliveries in developed countries, is a major cause of neonatal morbidity and mortality.[121]. In Finland, preterm delivery occurs in about (5%) of deliveries. Preterm birth is a heterogeneous clinical entity. Many pregnancy complications may lead to it, but in about half of the cases the cause of preterm birth remains unknown. Preterm birth can be categorized to 1) spontaneous preterm birth due to a) onset of preterm labour or b) preterm premature rupture of membranes *(PPROM)*, and to 2) indicated preterm birth including a) induced labour and b) cesarean section performed for maternal or fetal reasons.[121]

Preterm birth can also be categorized by gestational age to extremely preterm (<28 gestational weeks), severely preterm (28-31 gestational weeks), and late preterm birth (32-36 gestational weeks). Preterm births at 32-36 gestational weeks have also been sub-classified to moderate prematurity (32-33 gestational weeks), and to near term (34-36 gestational weeks).[121]

However, as infants born at (34-36) gestational weeks are immature and have a greater risk of morbidity and mortality than infants born at term, it is

recommended to refer them as late-preterm infants.[122] As a result, late preterm birth has different definitions in the literature. The etiological causes of preterm birth are partly different during different phases of pregnancy. Inflammatory mechanisms have a key role in the initiation of normal labour .[123]

Mechanisms that are thought to be involved in the initiation of preterm labour include infection or inflammation, utero-placental ischemia or hemorrhage, uterine over distention, stress, and other immunologically mediated processes.[121] In addition, genetic factors and race contribute to preterm birth.[121] Factors that are associated with an increased risk for preterm birth include multiple gestation, uterine anomalies, maternal diseases, extremes of maternal age, low pre-pregnancy BMI, smoking, and low socioeconomic and educational status.[124]

Theoretically, thrombophilia could influence the initiation of preterm labour via thrombosis in the placenta causing utero-placental ischemia or oxidative stress, or by activating inflammatory mechanisms. Polymorphisms in genes involved in coagulation and inflammation, including factor V, have been associated with preterm birth.[125, 126]

**2.15. FV Leiden and preterm birth:-**

Only three heterogeneous reports have shown positive association between FV Leiden and preterm birth. One study compared (205) very low birth weight *(VLBW)* preterm infants with (205) term infants (OR for FV Leiden 2.1, 95% CI 1.01-4.4).[127] One study observed increased prevalence of *(FVL)* in (50) women with preterm birth compared with the population prevalence (18% vs. 6.3%).[128] FV Leiden has also been associated with preterm birth with evidence of placental hemorrhage (OR 4.8, 95% CI 1.6-14.2.[129] Other current studies have not found a significant association between *(FVL)* and preterm birth.[91, 98, 130-132]Many of the studies are small or have other limitations. Study design, selection of cases and

controls, ethnicity, exclusion criteria, and even definition of preterm birth differ.[132]

Taken together, only a limited number of heterogeneous studies have assessed the association between FV Leiden and preterm birth, and with conflicting results.[132]

Cervical insufficiency is one cause of preterm birth. In one study, *(FVL)* was associated with a (4-fold) risk for cervical insufficiency and subsequent preterm birth (OR 4.2, 95% CI 1.5-13.6).[133] The authors speculated that increased thrombin production caused by FV Leiden could intensify activation of inflammatory processes in the cervix leading to cervical insufficiency.[133]

### 2.16. Screening of inherited thrombophilia in pregnancy complications:-

Screening for thrombophilia has been under debate since the first findings of association between thrombophilia and placenta-mediated pregnancy complications. However, screening for a risk factor is indicated only if the result influences the treatment of the patient.[134] LMWH is increasingly used in women at increased risk for these complications.[133] However, with the current knowledge, prophylaxis with low molecular weight heparin *(LMWH)* is not routinely recommended in women with a prior placenta-mediated pregnancy complication (pregnancy loss, pre-eclampsia, *(IUGR)*, placental abruption), whether they have inherited thrombophilia or are unselected.[133, 134] Further randomized controlled trials are urgently needed. In the light of this, in current guidelines, screening for inherited thrombophilia, or *(FVL)*, is not recommended in unselected women with placenta-mediated pregnancy complications. However, screening for thrombophilia in women with personal or family history of venous thrombosis is considered reasonable as it may influence

the timing and intensity of venous thrombosis prophylaxis during pregnancy and puerperium.[134]

## 2.17. Assessment of risk associated with a genetic risk factor:-

Several different study designs can be used to assess the association between a specific genetic risk factor and a disease entity. Each design has its advantages and limitations.[54]

In case-control studies, cases are selected on the basis of developing a specific disease (outcome). The disease entity should be as homogeneous as possible to minimize the risk of any true association remaining unobserved. Controls should be from the same population as cases, i.e., if a person without the disease had developed the disease, she/he would have been selected as a case. The case-control design is particularly suitable for rare diseases and it allows many risk factors to be evaluated simultaneously. Being less expensive and time-consuming than cohort studies, case-control studies are often more feasible. However, they are susceptible to selection bias (inclusion of cases or controls is somehow dependent of the studied risk factor) and information bias (knowledge of disease status, recall bias, reporting bias, research bias, misclassification). Therefore, studies should be carefully planned to avoid these biases. Well-planned and conducted case-control studies can provide valuable information on the association between a risk factor and disease and they can be reliably used to test epidemiologic hypothesis.[133]

In case of genetic risk factors, case-control studies are efficient and reliable in estimating risks if their sizes are in accordance with the prevalence of the studied mutation, i.e., if they have enough statistical power. However, false positive and false negative associations are possible if the studied population includes genetically heterogeneous subgroups. Genetic association studies cannot prove

causality as the studied genetic marker may only be linked to the causative genetic factor.[134]

In cohort studies, individuals are selected on the basis of having or not having an exposure or risk factor. Exposed and unexposed individuals are then followed to assess the risk for an endpoint or disease. The exposed and unexposed should be as similar as possible except for the studied risk factor. The cohort design is particularly suitable when the risk factor is rare. Also, it allows assessment of many endpoints for a single exposure and direct calculation of endpoint incidence rates in the exposed and unexposed.[10]

The best way to establish whether and how much a single mutation alters the risk for a specific disease, is to study the absolute risk of the disease in carriers and non-carriers of the mutation in a fixed population-based cohort over a defined time. Prospective cohort studies may have the lowest risk for selection bias as the cohort has been identified before the development of the disease. However, these studies are seldom feasible as large cohort studies needed for rare diseases can be extremely expensive and time-consuming.[10]A more feasible variation of a cohort study is a nested case-control study in which only cases and a sample of controls in a fixed cohort are assessed in detail.[10] In genetic association studies with this design, only cases and controls are genotyped for the studied mutation. In this setting, it is possible to study relative risks and their ratios and even population parameters that are readily generalizable to the known reference population if the sampling is unbiased.[54]

Sometimes the term retrospective study is used as a synonym for a case-control study, because in this design researchers have first an outcome for which they aim to ascertain a cause. Analogously the term prospective study is sometimes used as a synonym for a cohort study, because in this design researches have first

a suspected risk factor and they follow up a cohort for an outcome. However, the terms retrospective and prospective are often used to define whether the outcome has occurred before or after the study started. Therefore, case-control and cohort studies can be either retrospective or prospective, although this distinction is usually used only for cohort studies.[66]

In all epidemiological studies, it is vital that information has been gathered identically from all study subjects. Information about exposure and outcome should be accurate and complete.[133]When the information is gathered retrospectively, adequate records should be available, and sometimes several sources may have to be used. Whether the risk has been assessed in family studies, hospital-based studies, registry-based studies, or population-based studies, the populations the results can be generalized to must be carefully considered.[133]

In case of thrombophilia, cohorts of carriers (exposed) and non-carriers (unexposed) of a mutation are most readily available from thrombophilic families. However, population-based studies give more generalizable results.[133]

In hospital-based studies, cases often represent the most severe cases of the specific disease, which may distort the results leading to an overestimation ofthe risk associated with the mutation. Register-based studies are also used in genetic association studies. They are feasible but only as accurate and reliable as the information in the registers. Therefore, the validity of diagnoses in the register is of great importance. Registers can be used to identify cohorts or cases and controls, which then are recruited for the study to give samples for DNA. Register-based studies become laborious, but also more accurate, when diagnoses and clinical data are checked from the medical records.[135]

As in all research, possible publication-bias should be kept in mind when reviewing the literature about genetic risk factors. Publication bias exists when researchers, reviewers, or editors submit or accept papers for publication depending on the direction or strength of the results.(136)

**2.18.(APC-R) test:-**

Activated protein C *(APC)* degrades factor Va*(FVa)* and factor VIIIa *(FVIIIa)* by proteolytic cleavage at specific arginine residues.(137)In factor V Leiden *(FVL)*, a single point mutation in position (506) of the factor V *(FV)* gene replaces an arginine to a glutamine residue. This results in a (10-fold) decrease in the rate of *(FVa)* inactivation and in an *(APC)* resistance *(APC-R)* phenotype.(147)The increase in *(FVa)* half-life results in increased clotting. More than (95%) of *(APC-R)* cases are due to the (*FVL*) mutation.(138)

This mutation leads to the most common inherited form of a primarily venous thrombophilia. Heterozygosity for *(FVL)* occurs in (3% to 8%) of the general US and European populations. The frequency of homozygosity for the (*FVL)* mutation is approximately (1 in 5,000). The risk for venous thrombosis is approximately (3- 10-fold) in individuals who are heterozygous for the (*FVL)* mutation. Homozygous individuals have been reported to have an approximately (80-fold) risk over baseline for thrombosis.(139)

The original test for *(APC-R)* used in clinical laboratories is an activated partial thromboplastin time *(aPTT)* performed in the presence and absence of exogenously supplied *(APC)*.(140)In healthy patients, the *(APC)* degrades the patient's *(FVa)* and *(FVIIIa)* and, on that basis, prolongs the *(aPTT)*. In patients with an (*FVL)* mutation, the degradation of *(FVa)* does not occur to the same extent, and, therefore, the *(aPTT)* does not become as prolonged. The ratio of the *(aPTT)* with *(APC)* vs the *(aPTT)* without *(APC)* is calculated. Healthy

individuals typically have a ratio of (2.0) or greater, and individuals with *(FVL)* typically have a ratio less than (2.0). However, there is considerable overlap between healthy subjects and heterozygotes.[141]

In a modified *(APC-R)* assay, the patient plasma sample first is diluted (1:5) with *(FV)*-deficient plasma before the analysis and then assayed.[142]

The presence of the deficient plasma provides all factors except *(FV)* to offset any *(aPTT)*-related factor deficiencies and to minimize the effect of an elevation of FVIII, which would shorten the *(aPTT)*. The modified assay also contains polybrene, which neutralizes unfractionated heparin and low-molecular-weight heparin in the specimen. Sensitivity and specificity values reported for the modified *(aPTT)*-based *(APC-R)* assay are almost (100%).[143]

In smaller evaluations, sensitivities and specificities of (100%) have been reported.[144]

The discrimination gap between wild-type *(FV)* and *(FVL)* is narrow.One potential major interference of the *(aPTT)*-based *(APC-R)* assays is the presence of lupus anticoagulants in the sample. Because the tests are phospholipid-based, the lupus anticoagulant is capable of producing interference in the assay. Thus, patients with a lupus anticoagulant should be evaluated directly with a genetic assay to determine whether the (*FVL)* mutation is present.[145]

Other functional *(APC-R)* assays have been developed[159]; however, the *(aPTT)*-based method is still the most commonly used functional *(APC-R)* detection method.[145]

The "gold standard" *(DNA)* assay for the (*FVL)* mutation involves the use of the polymerase chain reaction *(PCR)* method.[146] The testing by *(PCR)* allows specific identification of patients with *(FV)* wild-type, (*FVL)* heterozygosity, and (*FVL)* homozygosity. The genetic tests are much more expensive and labor-

intensive than the clot-based screening assay. Owing to the need for special instrumentation, genetic testing usually also has significantly longer turnaround times than functional *(APC-R)* testing.[145]

A new prothrombin-based activated protein C resistance *(APC-R)* test is described. In this method, the patient sample is prediluted in a plasma depleted of factor V *(FV)*. A reagent containing *(APC)* and a specific activator of *(FV)* is added. After an incubation period, clotting is initiated by the addition of the *(FV)*-dependent prothrombin activator Noscarin. They analyzed (703) samples from patients undergoing thrombophilia screening. By using a predefined cutoff ratio of (2.5, 100%) sensitivity and specificity for the detection of a factor V Leiden (*FVL*) mutation was found. With a cutoff ratio of (1.2), a complete but narrow distinction of *(FVL)* heterozygous (n = 192) and *(FVL)* homozygous samples (n = 27) was determined. No interference by the international normalized ratio, activated partial thromboplastin time *(aPTT)*, protein S activity, fibrinogen and factor VIII *(FVIII)* levels, or lupus anticoagulant ratio was detected. The new prothrombin-based *(APC-R)* assay provides improved distinction of (*FV)* wild-type and (*FVL)* carriers compared with the *(aPTT)*-based method. By the use of an *(FV)*-dependent prothrombin activator, the assay is not influenced by *(FVIII)* concentration or lupus anticoagulants.[146]

## Material and Methods

### 3.1. Study design:-

This is a case control study conducted at Shendi Teaching Hospital & Elmek Nemir University Hospital in the period from  to 2013 to 2018

### 3.2. Study area:-

Shendi locality, River Nile State, Sudan, Which is located at the north of Khartoum with about 176 km. The total areas of the Shendi locality are about 1496 km2. Shendi population about 245000 persons male 48.7% female 51.3% (, 2008), most of them are farmers.

### 3.3. Study populations:-

The study populations were comprised of pregnant women with past history or at high risk to develop pregnancy complications as study group. Controls were comprised of pregnant women with no history of pregnancy loss and who have delivered at least one term infant without any complications were enrolled into the study. Besides that, their complicated pregnancy confirmed and diagnosed on the basis of clinical criteria by obstetrian at Shendi outpatient clinic.

### 3.4. Inclusion criteria:-

Any pregnant women, with past history of thrombophilia or at high risk to develop pregnancy complications in Shendi locality

### 3.5. Exclusion criteria:-

- Acquired thrombophilia disorders
- Any pregnancy complication reasons rather than primary coagulability

### 3.6. Sample size:-

The sample size is calculated according to the known formula, which is used to reach a certain desired margin of error in the results. The sample size in this study is calculated for each category (on average) to give a maximum of error (0.02) with a probability of ($\alpha = 0.05$). Plus the 3% of non-responsive the sample size is as

Follows:-

$$n = \frac{z^2 .p .q}{d^2}$$

$$= \frac{(1.96)^2 \times (0.03) \times (0.9)}{(0.05)^2} = 41.5$$

z = the value in normal curve corresponding to level of confidence 95% = 1.96

P = probability prevalence in the community is 3% or 0.03

- q = (1-p) = 1-0.1= 0.97
- d = margin of error = 0.05

According to the above formula; sample size should be enrolled in this study is (41) and the actual collected samples size (34) Sudanese ladies with placental complicated pregnant women attended to the obese and gynecological clinic as study group) the reduction in the sample size based on the number of test that done by the one kit and also the price of the kit was expensive.

### 3.7. Data collection tools:-

The study group data collected using structure questionnaire to collect information about age, parity, medical and obstetric history, family medical and obstetric history.

## 3.8. Specimen Collection and sample preparation:-

The participants were instructed to get rest for 10 min prior sampling.

Then 4.5 ml of maternal blood were collected in 0.5 ml of 0.1 M trisodium citrate. The blood samples were centrifuged at 3,500 x g for 15 minutes. The citrated plasma was stored at -20 for 1 month and the frozen plasma was thawed once at 37°C, 30 minutes before use.[147]

## 3.9. Laboratory studies:-

Quantitative functional clotting assay for protein C, protein S and activated protein C resistance (APC-R) for factor V Leiden were determined, antithrombin III activity was measured by chromogenic assay.

### 3.9.1. Protein C assays:-

#### 3.9.1.1. Principle:-

Protac is a rapid protein C activator derived from the venom of the viper Agkistrodon contortrix. Under the conditions described below, protac converts human protein C to the active protease within 5 minutes. In this assay protac is co-lyophilized with an APTT reagent to form a reagent that activates both protein C and the contact factors of the intrinsic pathway. With this reagent, the clotting time of normal plasma is very long (>100 seconds) while that of protein C deficient plasma is essentially the same as the APTT (approx. 30-40 seconds). When patient plasma is mixed with protein C deficient plasma the prolongation of the clotting time is proportional to the amount of protein C in the patient plasma.[148]

**3.9.1.2. Reagents:** (Appendix 1)

1. Acticlot activator: 3 vials each containing 1.5 units protac co-lyophilized with APTT reagent (rabbit brain cephalin and colloidal silica activator).
2. Protein C deficient plasma: 3 vials each containing 1.5 mL of freeze-dried human plasma substrate that has been artificially depleted of protein C by adsorption on an immobilized immunospecific goat polyclonal antibody to human Protein C.
3. Protein C control plasma: 3 vials each containing 0.5 mL of freeze-dried normal human plasma that has been assayed for protein C antigen and activity against the 1st International Standard for protein C.
4. Dilution buffer: 3 vials each containing 5 mL of a 10-fold concentrate. After dilution, the buffer contains 0.12 M NaCl, 0.03M imidazole pH 7.35. The buffer also contains protamine sulfate to neutralize up to 1 USP unit/ml heparin in the plasma sample.

**3.9.1.3. Result reading and interpretation:-**

The results were obtained from the calibration curve construct protein C control and the values less than 70% were to be deficient (normal 87.0 ± 17.0)according to the manufacture protocol.

**3.9.1.4. Assay procedure:-** (Appendix 1)

Reagents were reconstituted as described by manufacture.

Acticlot activator and calcium chloride were transferred to 37˚C, Prepare dilutions as described by manufacture.

**3.9.1.5.Calibration standards curve preparation:** 0.1 mL protein C deficient plasma + 0.1 mL standard dilution were incubated for 2 minutes at 37˚C , 0.1 mL acticlot activator was added , then incubated for 5 minutes at 37˚C , 0.1 mL

calcium chloride (0.025 M) was added , then the clot timer was started and clotting time was noted , the above mentioned steps were repeated to get the mean of reading .

**3.9.1.6.Test reading**: 0.1 mL protein C deficient Plasma + 0.1 mL of the test citrate plasma were incubated for 2 minutes at 37˚C , 0.1 mL acticlot activator was added , then incubated for 5 minutes at 37˚C , 0.1 mL calcium chloride (0.025 M) was added , then the clot timer was started and clotting time was noted , the above mentioned steps were repeated to get the mean duplicate reading .

**3.9.1.7. Functional activity reading**: Protein C percentage activity of calibration standards were plotted on the x-axis vs. mean clotting time on the y-axis. The participant's samples were read from the above created curve

**3.9.2. Protein S assays:-**

**3.9.2.1. Principle:-**

The ACTICLOT protein S assay is a clotting-based plasma assay. In the assay, dilutions of patient plasma are mixed with protein S depleted plasma. A reagent that contains factor Xa, activated Protein C and phospholipids is added to the mixture. Following a 5-minute incubation period, calcium chloride is added to initiate clot formation. Under these conditions, the prolongation of the clotting time is directly proportional to the concentration of protein S in the patient plasma.

**3.9.2.2. Reagents:-**

The contents of the reagents are as follows:

- R1 4 vials ACTICLOT Activator Reagent (each vial contains 1 mL lyophilised material containing human activated Protein C, bovine factor Xa, and rabbit brain phospholipid).

- R2 4 vials protein S depleted plasma (each vial contains 1 mL of lyophilised human plasma that has been depleted of protein S by immunoadsorption on a column of immobilized goat antibody to human Protein S).
- R3 2 vials sample dilution buffer, 10X concentrate (2.5 mL concentrate, which upon dilution contain 0.2 M NaCl, 0.03 M HEPES and a sufficient quantity of polybrene to neutralize up to 1.2 U/mL heparin in a plasma sample).
- R4 2 vials protein S Control plasma (each vial contains 0.5 mL of citrated lyophilised normal human plasma whose protein S clotting activity level has been determined and is printed on the vial label).

**3.9.2.2. Result reading and interpretation:-**

The results were obtained from the calibration curve construct protein S control and the values less than 55% were to be deficient (normal 55- 160)according to the manufacture protocol.

**3.9.2.3. Calibration standards curve preparation:-**

A new standard curve must be constructed each time the assay is performed. Pooled normal plasma (PNP) from at least 20 normal donors, which has been collected in the same manner as the plasma to be tested, should be used for the preparation of the assay standards. Commercially prepared reference plasma in which the Protein S level has been determined may also be used. Prepare the protein S standards, control and patient plasma samples just before testing, as follows:

#### 3.9.2.4. Assay procedure:-

- ACTICLOT activator reagent and calcium chloride was transferred to the 37°C reagent wells of a clot timer instrument. Incubate for two minutes.
- 0.1 mL protein S depleted plasma and 0.1 mL test plasma dilution to a coagulation cuvette were added ad incubated for 2 minutes at 37°C.
- 0.1 mL prewarmed ACTICLOT activator Reagent to the cuvette was added incubated for exactly 5 minutes at 37°C.
- 0.1 mL prewarmed Calcium Chloride was added.
- The timer was started and the clotting time was recorded.

#### 3.9.2.5. Test reading:-

**Functional activity reading**: Protein S percentage activity of calibration standards were plotted on the x-axis vs. mean clotting time on the y-axis. The participant's samples were read from the above created curve

### 3.9.3. Resistance to activated protein C Assays:-

Acticlot protein C resistance is a phenotypic assay with the sensitivity to distinguish between homozygous and heterozygous carriers of Factor V Leiden, without using molecular (PCR) methods[(146)]

#### 3.9.3.1. Principle of the method:-

The acticlot protein C resistance assay is a plasma-based functional clotting assay and differs from other functional *(APC)* resistance tests by acting specifically on the prothrombinase complex level. It is based on a FV-dependent prothrombin activator isolated from snake venom. Robustness and specificity of the assay is enhanced by elimination of possible disturbing influences by factors upstream the coagulation cascade and independency from calcium. Interference from *(UFH)*, *(LMWH)* and pentasaccharide in the blood sample is precluded by a heparin inhibitor added to reagents 1 and 2. Sample plasma is pre-diluted with reagent 4

(dilution plasma) and incubated at 37 °C with *(FV)* activator from snake venom (*RVV-V* from Daboia russelli). Coagulation is triggered by the addition of a *(FV)* dependent prothrombin activator from snake venom from Notechis scutatus in the absence of calcium. The clotting times are recorded and the ratios (clotting time in the presence of APC/clotting time in the absence of *(APC)* are calculated.[146]

**3.9.3.2. Reagents :-**( Appendix 2)

The contents of the reagents are as follows:

**1.** R1 APC / RVV-V (+APC) Reagent

(APC, RVV-V, Polybrene, Hepes, BSA)

3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)

**2.** R2 RVV-V (-APC) Reagent

(RVV-V, Polybrene, Hepes, BSA)

3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)

**3.** R3 PTA Reagent

(Prothrombin Activator, EDTA, Hepes, BSA)

3 vials (lyophilisate, to be reconstituted in 4.0 ml of deionized water per vial)

**4.** R4 Dilution Plasma

(Processed Human Plasma)

3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)

-Incubate reconstituted solutions R1-R4 in closed vials for 30’ at room temperature and swirl gently before use.

### 3.9.3.3. Result reading and interpretation of APCR test:-

Differentiation of homozygous, heterozygous inherited APCR resistance was based on the typical ratio ranges calculated from results obtained from patients' plasma. The ratio range as follows:

– Negative APCR ≥3.

– Heterozygous hereditary APCR 1.3-1.9.

–Homozygous hereditary APCR 0.9-1.1.

All quality control procedure were adopted to get the validation of the assay, each test was done parallel with negative and positive controls; the acticlot protein C resistance control plasmas (REF 840C) as a control reference was done, the negative control.

### 3.9.3.4. Procedure:-

Thawed samples were mixed for homogenization. The clotting time was determined in the presence of activated protein C and also in the absence of activated protein C and the ratio was calculated according to the following scheme: (Appendix 2)

**Table (3.1): Show procedure of APC-R estimation:-**

| | | + APC | - APC |
|---|---|---|---|
| | **Sample or control plasma** | **30 µL** | **30 µL** |
| **R4** | **Dilution Plasma** | **20 µL** | **20 µL** |
| | | **mix prior to use** | **mix prior to use** |
| **R1** | **APC/RVV-V (+APC) Reagent** | **50 µL** | **-** |
| **R2** | **RVV-V (-APC) Reagent** | **-** | **50 µL** |
| | **Incubation** | **3 min, 37°C** | **3 min, 37°C** |
| **R3** | **PTA Reagent** | **50 µL** | **50 µL** |
| | | **Determine clotting time** | **Determine clotting time** |
| | **Ratio calculation =** | **Clotting time + APC/Clotting time – APC** | |

**3.9.3.5. Clotting time for samples and controls in the presence of APC: -** Determination of a clot in the in the presence of APC, 30 µL of plasma or control was add to 20 µL of the diluent, these reagents were mixed well, 50 µL of RVV-V (+APC) was added to the above mixture, the reactant was incubated for 3 min at 37°C, 50 µL of clot initiating reagent (PTA) was added and the clotting time was determined. (Appendix 2)

**3.9.3.6. Clotting time for samples and controls in the absence of APC:-** Determination of a clot in the in the presence of APC, 30 µL of plasma was add to 20 µL of the diluent, these reagents were mixed well, 50 µL of RVV-V (-APC) was added to the above mixture, the reactant was incubated for 3 min at 37°C, 50

μL of clot initiating reagent (PTA) was added and the clotting time was determined. (Appendix 2)

**3.9.3.7. APC-R calculation:-**

APC-R was calculated as a ratio between clotting time with APC/Clotting time without APC (Appendix 2)

**3.9.4. Free protein S assays: -** (Appendix 3)

**3.9.4.1. Assay principle:-**

A calcium-dependent monoclonal antibody specific for Free Protein S coupled to horse radish peroxidase (HRP) is added to a microwell coated with another calcium-dependent monoclonal antibody specific for free protein S. Next, a diluted plasma sample or biological fluid is immediately added to the microwell and the immunological reaction begins. If present, Free Protein S binds onto the monoclonal antibody coated solid phase via one epitope and binds to the second monoclonal antibody coupled to HRP via a second epitope. Following a wash step, the peroxidase substrate, 3, 3', 5, 5'–Tetramethylbenzidine (TMB), in the presence of hydrogen peroxide ($H_2O_2$), is added to the microwell and the subsequent enzymatic reaction yields a blue colored solution. The addition of sulphuric acid stops the reaction and turns the solution color to yellow. The amount of color is directly proportional to the concentration of human Free Protein S in the tested sample. (Appendix 3)

**3.9.4.2. Result reading and interpretation:-**

The results were obtained from the calibration curve construct with FPS calibrator and the normal free protein S concentration in normal human plasma is usually in the range (60–150%), therefore the abnormal range is (< 60%)according to the manufacture protocol.

**3.9.4.3. Reagents: -** (Appendix 3)

The contents of the reagents are as follows:

1. 12 strips of 8 antibody coated microwells (total of 96 wells) in frame holder.
2. 2 vials of protein S sample diluent (contains calcium), ready to use (50 mL).
3. 3 vials of plasma protein S calibrator, 1:50 prediluted (lyophilized).
4. 1 vial of protein S control plasma I, high (lyophilized).
5. 1 vial of protein S control plasma II, low (lyophilized).
6. 3 vials of Anti-human free protein S-HRP immunoconjugate (lyophilized).
7. 1 vial of Protein S Conjugate Diluent, ready to use (15 mL).
8. 1 vial of Wash Solution, 20 fold concentrate (50 mL).
9. 1 vial of TMB Substrate, ready to use (25 mL).
10. 1 vial of Stop Solution, 0.45M $H_2SO_4$ (6 mL).

**3.9.4.4. Procedure:-**

ELISA-based assays were used in which 100 µL of anti-human free protein S-HRP conjugate was added to each microwell, 100 µL of free protein S calibrator concentrations or diluted controls or diluted sample was immediately added to the appropriate microwell, then incubated for 1 hour at room temperature (18°-25°C) with manually gentle shaking. the wells was washed (5 times) with (300 µL) of protein S wash Solution , then 200 µL TMB Substrate solution was added to each microwell , then the micro plates were Incubated for exactly 5 minutes at room temperature (18-25°C). Following exactly the same time intervals used for adding the substrate, 50 µL of stop Solution (0.45 M

$H_2SO_4$) was added to stop the reaction, the reactance tubes were waited for 10 minutes in order to allow the color to stabilize, then absorbance was measured at (450 nm). Subtract the blank value from the measurements. (Appendix 3)

**3.9.5. Antithrombin assays: -**

Actichrome AT III is intended for the quantitative determination of antithrombin III in human plasma by chromogenic assay.[149]

**3.9.5.1. Principle:-**

Antithrombin III is an inhibitor of plasma serine proteases. An important function of antithrombin III is the inhibition of thrombin activity. Normally the rate of thrombin inhibition by antithrombin III is slow (progressive antithrombin activity). However, the rate of inhibition can be enhanced several thousand- fold in the presence of heparin (heparin cofactor activity). In the present two-stage method, [48] thrombin is added to a plasma dilution containing antithrombin III in the presence of excess heparin. After an initial incubation (stage 1) residual thrombin is determined with a thrombin-specific chromogenic substrate (stage 2). The residual thrombin activity is inversely proportional to the antithrombin III concentration.

Tolefsen and blank have reported another rapid heparin-dependent thrombin inhibitor, heparin cofactor II, in human plasma. [49] This protein can interfere with antithrombin III determinations especially at high (2 USP units/mL) heparin concentrations. In order to confer specificity to antithrombin III the present assay system uses a lower (1.0 USP units/mL) final heparin concentration where heparin-enhanced inactivation of thrombin by heparin cofactor II is negligible. In addition, human heparin cofactor II reacts more readily with human thrombin than with bovine thrombin.[55] Thus, further specificity for antithrombin III is imparted in the present assay system by the u se of bovine thrombin.[149]

**3.9.5.2. Result reading and interpretation:-**

The results were obtained from the calibration curve construct antithrombin III control and the normal range of ATIII in plasma is (75%-125%). Activity levels of (30-60%) observed in patients with hereditary ATIII deficiency, according to the manufacture protocol.

**3.9.5.3. Reagents: -** (Appendix 4)

1. R1 Bovine Thrombin: 6 vials (lyophilized).
2. R2 Spectrozyme TH: 6 vials each containing 1.8 µmoles thrombin substrate (lyophilized).
3. R3 Assay buffer: 6 vials each containing 5 mL of buffer, 10-fold concentrate. Working strength buffer contains 50 mM Tris-HCI, 175 mMNaCI, 7.5 mMNaEDTA and 1.0 USP units/mL sodium heparin, pH 8.4.

**3.9.5.4. Assay procedure – endpoint method: -** (Appendix 4)

200 µL of standard and unknown plasma was added to a plastic tube, incubated for 3min at 37ºC, 200 µL of bovine thrombin was added to the tube , then incubated for 1minute at 37ºC , 200 µL of spectrozyme was added , then incubated for 1minute at 37ºC , 200 µL of 50% glacial acetic acid was added . The absorbance was read at 405 nm in a 1 cm semi-micro cuvette (Bio system 3030) against a blank prepared in the following order:

200 µL acetic acid

200 µL standard dilution

200 µL bovine thrombin

200 µL spectrozyme

#### 3.9.5.5. Representative Standard Curve:-

The absorbance obtained for each antithrombin III standard was plotted against the percent of antithrombin III on linear graph paper. Interpolate the antithrombin III level of the unknown plasma sample was interpolated from the calibration curve. (Appendix 4)

#### 3.9.5.6. Quality control:-

Commercial antithrombin III control plasma (e.g. Hemostasis Reference Plasma, American diagnostica catalog may be used for quality control of the assay. If commercial antithrombin III control plasma has been used to construct the calibration curve for the assay, then a different lot of control plasma should be used for quality control. (Appendix 4)

### 3.10. Ethical consideration:-

Ethical clearance was obtained from the scientific committee of postgraduate college, Shendi University.

Volunteers were informed about the study purposes and consent was taken verbally by the obstetrician and they were asked to fill a highly confidential questionnaire. Neither the participant's name nor her institution was used in any of study materials and each participant was assigned unique identification number.

### 3.11. Data processing:-

All categorical variables were analyzed by descriptive statistics. The numerical variables were presented as Mean ± SD. Independent sample T- test was used to compare between coagulation tests results of patients and controls groups. Pearson chi square test (cross tabulate) was used for association between coagulation test results in studied group and some associated clinical history and diagnosis. All statistical analyses were carried by Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 16.0.P value ≤0.05 was considered statistically Significant.

### 3.12. Data presentations:-

The obtained results were presented in in the form of tables and figures

# Results

This chapter describes characteristics of the sample and addressed the research questions. The Statistical Package for the Social Sciences (SPSS) 16.0 was used to analyze the collected data.

The data were collected from 34 females with documented placental complicated pregnancy

## 4.1. Demographical data:-

This is a case control study was conducted at Shendi during the period from August 2013 to February 2018 to detect association between hereditary thrombophilia and placental complicated pregnancy, our study population include many races as revealed in table (4.2) and figure (4.1) and with age of (18 to 50 years) with average of (30.7 years) as mentioned in and table (4.2).

**Table (4.1): Show frequency of race among study population:-**

| Race | Frequency | Percent |
|---|---|---|
| Galieen | 16 | 47.1 |
| Unkown | 8 | 23.5 |
| Shaigia | 5 | 14.7 |
| Ababda | 1 | 2.9 |
| Hasania | 1 | 2.9 |
| Nuba | 1 | 2.9 |
| Falata | 1 | 2.9 |
| Robatab | 1 | 2.9 |
| Total | 34 | 100.0 |

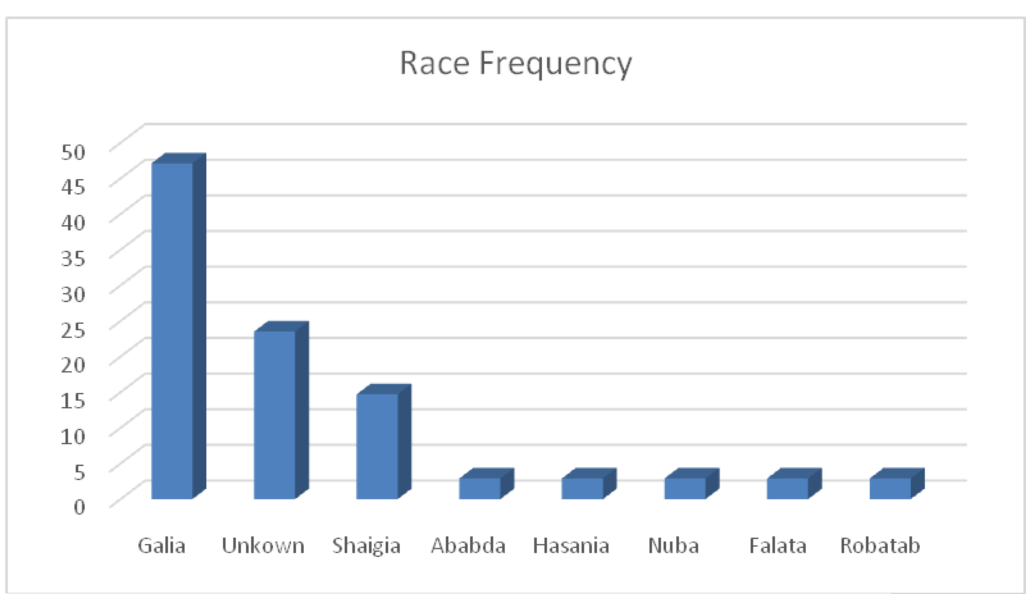



**Figure (4.1): Show frequency of races among study population:-**

**Table (4.2): Show age distribution among study population:-**

| | N | Minimum | Maximum | Mean | Std.deviation |
|---|---|---|---|---|---|
| Age | 34 | 18.0 | 50.0 | 30.7059 | 8.09987 |

## 4.2. Clinical Data:-

### 4.2.1: Frequency types of complicated pregnancy:-

The frequency of diagnosis of under study group revealed that the participant with still birth represent(8.8%),pre-term labour (8.8%), pre- eclampsia (2.9 %), miscarriage (61.8), still birth with pre-eclampsia (2.9%), pre-term with miscarriage (8.8%), still birth with miscarriage (2.9%), still birth with

miscarriage and pre eclampsia (2.9%) as demonstrated in table (4.3) and figure(4.2).

**Table (4.3): Show frequency of types of complicated pregnancy with in study group:-**

| **Diagnosis** | Frequency | Percent |
|---|---|---|
| Miscarriage | 21 | 61.8 |
| Stillbirth | 3 | 8.8 |
| PTL | 3 | 8.8 |
| Pre with miscarriage | 3 | 8.8 |
| Pre-eclampsia | 1 | 2.9 |
| Stillbrith+pre-eclampsia | 1 | 2.9 |
| Stillbirth+ miscarriage | 1 | 2.9 |
| Stillbirth+pre-eclampsia+miscarrige | 1 | 2.9 |
| Total | 34 | 100.0 |

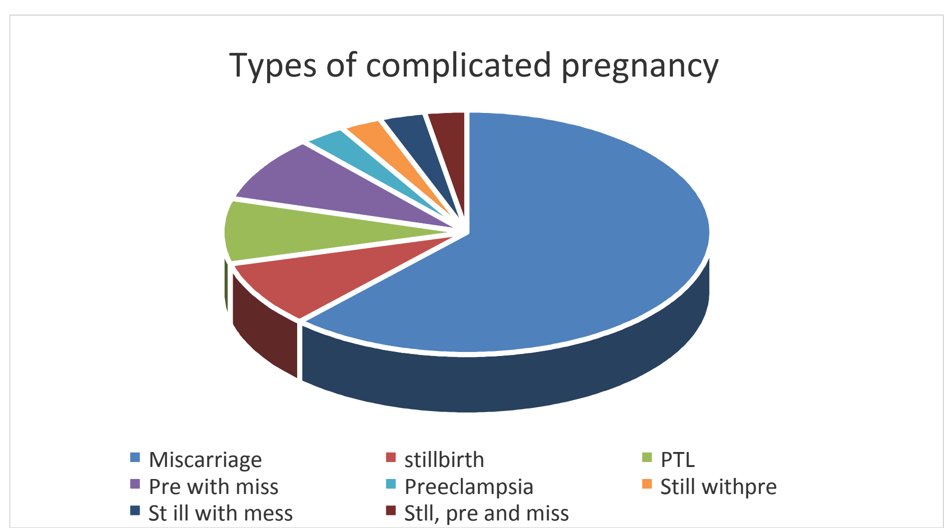



**Figure (4.2): Show frequency of types of complicated pregnancy with in study group:-**

**4.2.2: Past family history:-**

The participants with past family history thrombosis was represent (61.8%), while those with no family history was (38.2%) as noted in table (4.4) and figure (4.3).

**Table (4.4): Show past family history of thrombosis in study population:-**

| Past family history | Frequency | Percent |
|---|---|---|
| Yes | 21 | 61.8 |
| No | 13 | 38.2 |
| Total | 34 | 100 |

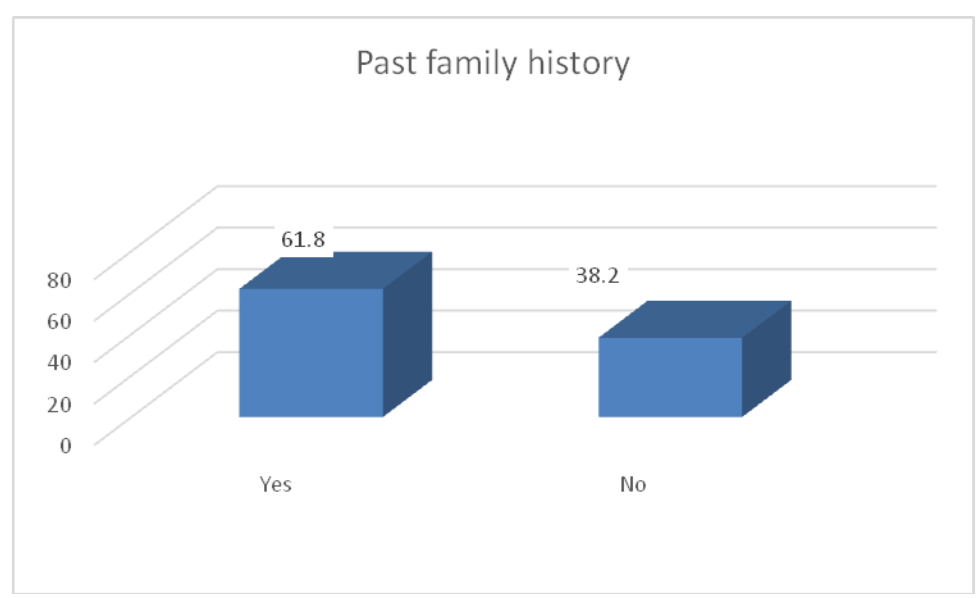



**Figure (4.3): Show past family history of thrombosis in study population:-**

### 4.2.3: Chronic disease:-

Other diseases were distributed among study population as; thrombosis (5.9%), DM (2.9%), hypertension (2.9 %), no other disease (88.2%) as noted in table (4.5) and figure (4.4).

**Table (4.5): Show frequency of chronic diseases among study population:-**

| **Chronic disease** | Frequency | Percent |
|---|---|---|
| Healthy | 30 | 88.2 |
| Thrombosis | 2 | 5.9 |
| D.M | 1 | 2.9 |
| Hypertension | 1 | 2.9 |
| Total | 34 | 100.0 |

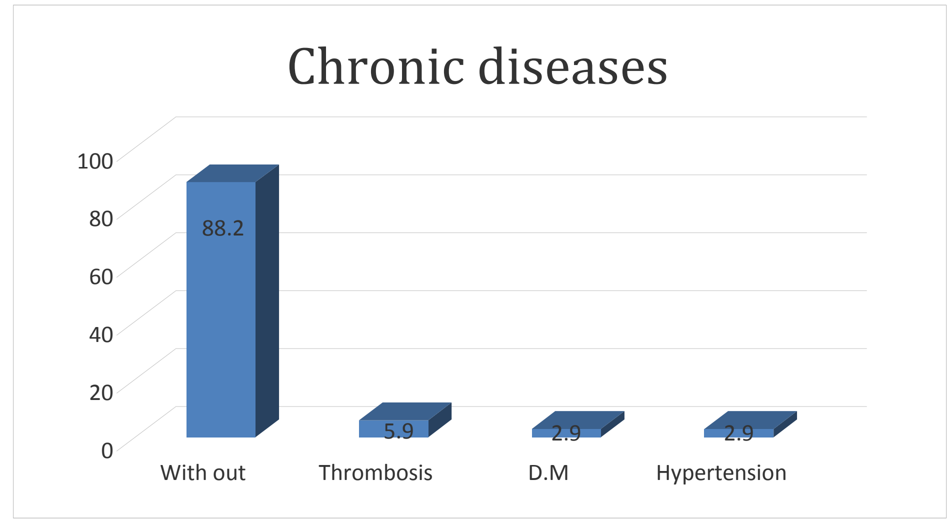



**Figure (4.4): Show frequency of other diseases among study population:-**

### 4.3. Laboratory Results:-

### 4.3.1Frequencies of APC-R:-

The results of APC-R revealed that there was no homozygous for FVL, while those with heterozygous was (47.1%) and those wild type was (52.9%) as shown in table (4.6) and figure (4.5).

**Table (4.6): Show frequency of APC-R among study population:-**

| APC-R | Frequency | Percent |
|---|---|---|
| Negative | 18 | 52.9 |
| Heterozygous | 16 | 47.1 |
| Homozygous | 00 | 00.0 |
| Total | 34 | 100.0 |

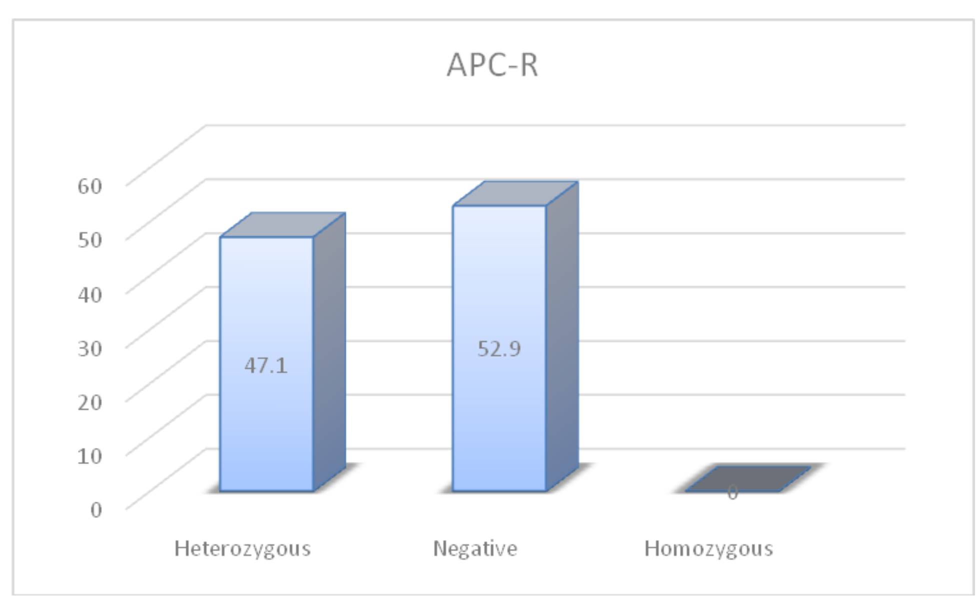



**Figure (4.5): Show frequency of APC-R among study population:-**

### 4.3.2 Comparison of coagulation tests between cases and control groups:-

#### 4.3.2.1 PC:

In comparison of coagulation tests results between study and control group, in which the mean of PC in study group was (43.7%), while in control group (81.4%) as shown in figure (4.6),independent sample test was applied and results revealed statistically significant differences with P. Values of(0.001) as mentioned in table (4.7). The frequency of protein C deficiency among study group was (91.2%), compared with (40%) among control group as demonstrated in table (4.8).

**Table (4.7) shows the mean of PC in study and control group:-**

| Study group | Number | Mean of protein C | P.value |
|---|---|---|---|
| Case | 34 | 43.7 | 0.001 |
| Control | 20 | 81.4 | |

Independent sample test was applied (*p.value 0.001*).

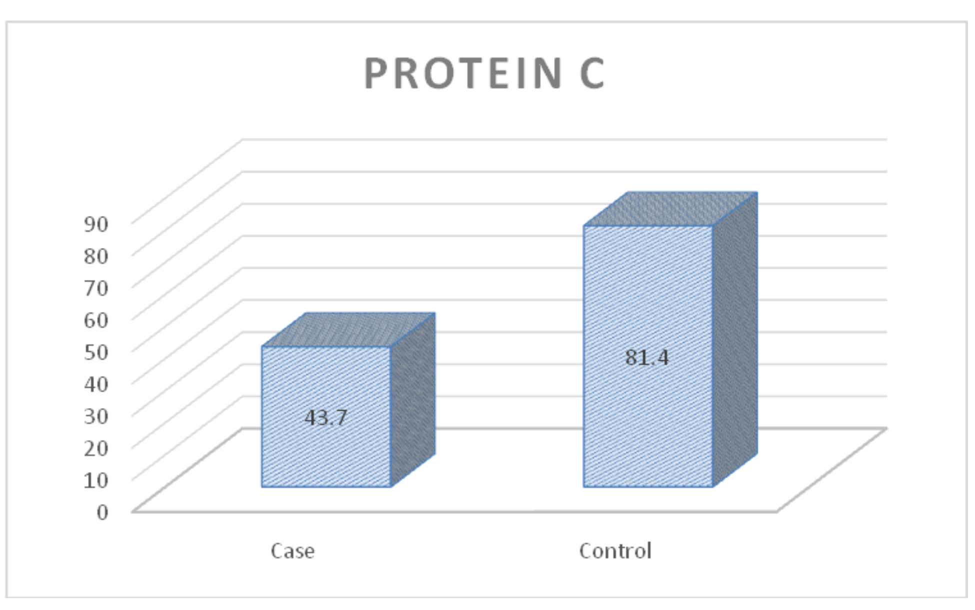



**Figure (4.6) shows the mean of PC in study and control group:-**

**Table (4.8) shows frequency of PC defiency in study and control group:-**

| Study group | Frequency | Percent |
|---|---|---|
| Case | 31/34 | 91.2 |
| Control | 8/20 | 40.0 |

### 4.3.2.2Protein S:-

Regarding to protein S; the mean of study group was (76.32%), while in control group was (86.55%) as noted in figure (4.7). Statistically insignificant differences were observed with P. Value of(0.073) when compare two mean by using independent sample test as shown in table (4.9).The frequency of protein S deficiency among study group was (12.0%), compared with (0%) among control group as demonstrated in table (4.10).

**Table (4.9) shows the mean of protein S in study and control group:-**

| Study group | Number | Mean of protein S | P.value |
|---|---|---|---|
| Case | 34 | 76.32 | 0.073 |
| Control | 20 | 86.55 | |

Independent sample test was applied (*p.value0.073*).

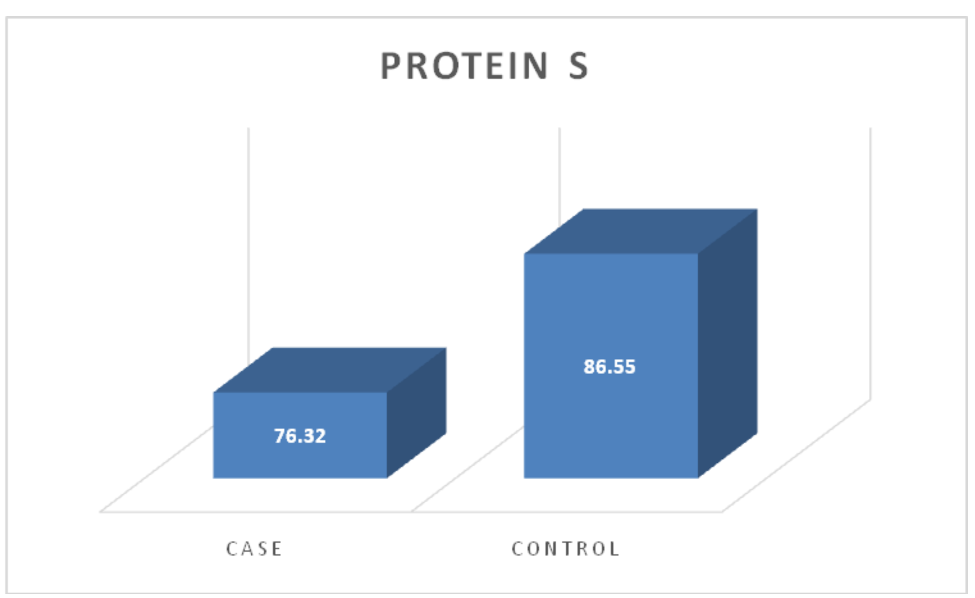



**Figure (4.7): Shows the mean of protein S in study and control group:-**

**Table (4-10): Shows frequency of PS deficiency in study and control group:**

| Study group | Frequency | Percent |
|---|---|---|
| Case | 4/34 | 12.0 |
| Control | 0/20 | 00 |

### 4.3.2.3 FPS:-

The compression of free protein S results in study and control group revealed that there was no statistically differences with P. Value of (0.720) as noted in table (4.11).This based on the results gives by independent sample test in which the mean of FPS in study group was (97.92%), while in study group was (95.80) as noted in figure (4.8).

**Table (4.11) shows the mean of free protein S in study and control group:-**

| Study group | Number | Mean of FPS | P.value |
|---|---|---|---|
| Case | 34 | 97.92 | 0.638 |
| Control | 20 | 95.80 | |

Independent sample test was applied (*p value 0.638*).

**Figure (4.8) shows the mean of free protein S in study and control group:-**

#### 4.3.2.4 ATIII:-

The results of anti-thrombin level among study group reveal also there was no statistically significant differences when compared with the means of controls group with P value of (0.766) as shown in in table (4-12).The mean anti-thrombin level in study group was (122.88%), while in control group was (127.30%) as demonstrated in figure (4.9).The frequency of anti-thrombin deficiency among study group was (13.0%), compared with (0%) among control group as demonstrated in table (4.13).

**Table (4.12) shows the mean of anti-thrombin level in study and control group:-**

| Study group | Number | Mean of ATIII | P.value |
|---|---|---|---|
| Case | 34 | 122.88 | 0.720 |
| Control | 20 | 127.30 | |

Independent sample test was applied (*p.value 0.72*).

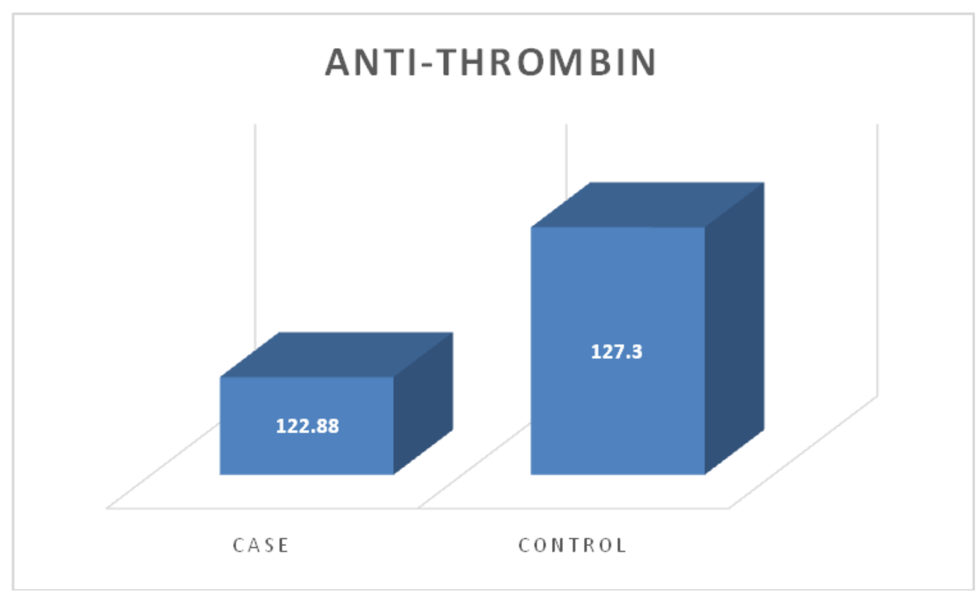



**Figure (4.9): Show the mean of anti-thrombin level in study and control group**

**Table (4-13): Show frequency of anti-thrombin deficiency in study and control group**

| Study group | Frequency | Percent |
|---|---|---|
| Case | 5/34 | 13 |
| Control | 0/20 | 0.0 |

**4.3.3Relationship of APC-R results with clinical data:-**

**4.3.3.1Relationship of APC-R results with types of complicated pregnancy:-**

Regarding the relation between APC-R results and diagnosis; statistical analysis revealed that miscarriage with frequency of (61.8%) (21/34), (38.2%) of them was heterozygous (13/21), and wild type (negative) was (23.5%) (8/21) as noted in table (4.14).

**Table (4.14): Show relationship of APC-R results with types of complicated pregnancy**

| Types of complicated pregnancy | | APC-R | | Total |
|---|---|---|---|---|
| | | Heterozygous | Negative | |
| Still birth | Number | 1 | 2 | 3 |
| | % of total | 2.9 | 5.9 | 8.8 |
| PTL | Number | 1 | 2 | 3 |
| | % of total | 2.9 | 5.9 | 8.8 |
| Preeclampsia | Number | 0 | 1 | 1 |
| | % of total | 0 | 2.9 | 2.9 |
| Miscarriage | Number | 13 | 8 | 21 |
| | % of total | 38.2 | 23.6 | 61.8 |
| Preeclampsia +still birth | Number | 0 | 1 | 1 |
| | % of total | 0 | 2.9 | 2.9 |
| Miscarriage +preeclampsia | Number | 0 | 3 | 3 |
| | % of total | 0 | 8.8 | 8.8 |
| Miscarriage +still birth | Number | 1 | 0 | 1 |
| | % of total | 2.9 | 0 | 2.9 |
| Still+preeclampsia + Miscarriage | Number | 0 | 1 | 1 |
| | % of total | 0 | 2.9 | 2.9 |
| Total | Number | 16 | 18 | 34 |
| | % of total | 47.1% | 52.9% | 100% |
| **Chi-Square Tests** | | | | |
| Pearson Chi-Square | Value | df | Asymp. Sig. (2-sided) | |
| | 8.770 | 7 | .270 | |

### 4.3.4 Relationship between chronic disease and diagnosis:-

The association between other disease and diagnosis, reflect that (88.2%) with No other disease, (52.9%) of them with miscarriage as noted in table (4.16).

**Table (4-15): Relationship between chronic disease and types of complicated pregnancy:-**

| Other disease | | Diagnosis | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Still birth | PTL | preeclampsia | Miscarriage | Stil+Pre | Pre+miss | Stil+Mess | Stil+Pre+Mess | |
| Thrombosis | Number | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| | % | 0 | 0 | 0 | 2.9 | 0 | 0 | 2.9 | 0 | 5.9 |
| DM | Number | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| | % | 0 | 0 | 0 | 2.9 | 0 | 0 | 0 | 0 | 2.9 |
| Hypertension | Number | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| | % | 0 | 0 | 0 | 2.9 | 0 | 0 | 0 | 0 | 2.9 |
| No | Number | 3 | 3 | 1 | 18 | 1 | 3 | 0 | 1 | 30 |
| | % | 8.8 | 8.8 | 2.9 | 52.9 | 2.9 | 8.8 | 0 | 2.9 | 88.2 |
| Total | Number | 3 | 3 | 1 | 21 | 1 | 3 | 1 | 1 | 34 |
| | % | 8.8 | 8,8 | 2.9 | 61.8 | 2.9 | 8.8 | 2.9 | 2.9 | 100 |

**Chi-Square Tests**

| Pearson Chi-Square | Value | df | Asymp. Sig. (2-sided) |
|---|---|---|---|
| | 18.133 | 21 | .641 |

## 5. 1: Discussion

The aim of this study was to determine the role of APC-R and deficiencies in coagulation inhibitors (antithrombin, protein C and protein S) among Sudanese ladies with placental complicated pregnancy. Although in Sudan numbers of studies were conducted in thrombophilia. But as far as we know, this is the first study extended to screen for both common thrombotic (FVL 1691G>A, and coagulation inhibitors deficiencies in Sudanese ladies with placental complicated pregnancy.

The current study give that homozygous APC-R was 0%, the finding consist with the study conducted by Montagnana and his colleges; they reported that the frequency of *FVL 1691G>A* varies from (0% to 15%) according to ethnicity and geographic distribution worldwide.[150] However *FVL* mutation is higher in Caucasian than non-Caucasian. Low frequencies were reported in African, Asian and South European populations (0%–3%). It is relatively high in North America (5%) and very high in Mediterranean populations (13.6% in Syria, 12.3 % in Jordan and Lebanon 39.7%).[151]However our findings were in agreement with a Sudanese study conducted by Yousif and his team; they get (0 %) frequency of FVL.[152]But a findings regarding the frequency of APC-R were reported by Safia Khalil and his colleges among Sudanese; they get (33.3%) were positive for APCR.[153]

Our finding of the heterozygous APC-R was 47.1; while The Heterozygous alleles G/A in factor V gene was 8.0% in all cases related with recurrent miscarriage.[154]

The present study demonstrated that protein C level was significantly decrease among study group compared with control group, with P .value (0.001). The frequency of protein C, protein S and anti -thrombin III deficiency among study

group was (91.2%), (12%) and (13%) respectively.However several published studies analyze the relationship between thrombophilic conditions with types of complicated pregnancy and gives a variant results;the findings of Payandeh et al, who reported PC deficiency as the most frequent hereditary risk factors in VTE patients (28%). (155)PC defiency found in patients with adverse pregnancy outcome (45%).(156)in the study of Hansda cooperated with Roy Chowdhury, they concluded that: protein C deficiency was present in 15.09 % of patients with RPL. Protein S deficiency was present in 50.94 % of patients with RPL (p = 0.000) (157), Marietta et al.(158), in their study, also observed (5 and 11.25 %) patients with recurrent abortion had protein C and protein S deficiency, respectively. Nazli H et al.(156)observed that (45%) of patients were found deficient for protein C and S in their case series, The Findings of of Suehisa et al. who reported PS deficiency (17.7%), PC deficiency (7.9%), AT (1.77%) and combined deficiency of PC and PS (0.88%) in Japanese patients with VTE.(159) Moreover, in study conducted in Kuwait (16.8%) of VTE patients had PS deficiency, (13.1%) had PC deficiency and (8.3%) of patients had AT deficiency.(160)There are contradicting results in Kuwait study that indicate higher frequency of PS deficiency in VTE patients than PC and AT deficiency. This variation could be explained by ethnic and geographical differences in addition to variations of patient's demographics. In addition to some limitation of technique because not all commercial kits or laboratory developed tests measure the plasma protein in the same manner. Also PS has limitation influenced by different biological and pre analytical variables

Regarding the association of APC-R result and ethnicity, our finding illustrated that most APC-R were heterogeneous, the incidence of homogenous does not exist, the same findings were reported by Hoff brand and Vizcanio in Africans

and blacks [165,166].This results can be explained by consanguineous marriages in the studied group.

## 5.2: CONCLUSION:-

- Protein C is the most frequent risk factors in Sudanese ladies with placental complicated pregnancy.
- Protein S and ant thrombin are less frequent risk factors in Sudanese ladies with placental complicated pregnancy.
- The FV Leiden *(APC-R)* represents non-significant risk factors for the occurrence of placental complicated pregnancy in this study population.

### 5.3: Recommendations:-

- A case control study should be carried out since the results have highlighted the significance of this problem
- All patients diagnosed with Obstetrical complications must be screened for thrombophilia and dealt with accordingly, to reduce the possibility of a new attack.
- Pregnancies which are complicated by severe pre eclampsia, abruption or unexplained fetal growth restriction, and in women whose pregnancies end up in unexplained still birth should be tested for genetic or acquired markers for thrombophilia
- Large studies are required to determine pregnancy associated complications the most important underlying prothrombophilic gene mutations which lead to in Sudanese population.
- Factor V Leiden DNA test should be performed only to abnormal activated protein C resistance.

## References

1. Sanson B-J, Simioni P, Tormene D, Moia M, Friederich PW, Huisman MV, et al. The incidence of venous thromboembolism in asymptomatic carriers of a deficiency of antithrombin, protein C, or protein S: a prospective cohort study. Blood. 1999;94(11):3702-6.
2. Brouwer JLP, Veeger NJ, Schaaf Wvd, Kluin-Nelemans HC, Meer Jvd Difference in absolute risk of venous and arterial thrombosis between familial protein S deficiency type I and type III. Results from a family cohort study to assess the clinical impact of a laboratory test-based classification. British journal of haematology. 2005;128(5):703-10.
3. Brouwer J-LP, Veeger NJ, Kluin-Nelemans HC, van der Meer J. The pathogenesis of venous thromboembolism: evidence for multiple interrelated causes. Annals of internal medicine. 2006;145(11):807-15.
4. Folkeringa N, Brouwer JLP, Korteweg FJ, Veeger NJ, Erwich JJH, Van Der, Meer J. High risk of prenancy-related venous thromboembolosim in women with multiple thrombophilic defects. British journal of haematology. 2007;138(1):110-6.
5. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. The Lancet. 2003;361(9361):901-8.
6. Zoller B, Berntsdotter A, De Frutos PG, Dahlback B. Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S. Blood. 1995;85(12):3518-23.
7. Pavlova E, Chemev T, Chemev A, Karagiozova Z. Pregnancy and issues with inherited and acquired thrombophilia. Journal of IMAB-Annula Proceeding. 2008:21-2.
8. Goodnight S. In Goodnight SH, Hathaway WE (eds): Disorders of Hemostasis and Thrombosis: A Clinical Guide. New York, McGraw-Hill; 2001.
9. Mann KG. Thrombin formation. CHEST Journal. 2003;124(3_suppl):4S-10S.
10. Duckers C, Simioni P, Rosing J, Castoldi E. Advances in understanding the bleeding diathesis in factor V deficiency. British journal of haematology. 2009;146(1):17-26.
11. Yeh Y-C, Wang M-J, Lin C-P, Fan S-Z, Tsai J-C, Sun W-Z, et al. Enoxaparin sodium prevents intestinal microcirculatory dysfunction in endotoxemic rats. Critical care. 2012;16(2):R59.

12. Esmon CT. The protein C pathway. CHEST Journal. 2003;124(3_suppl):26S-32S.
13. Lippi G, Favaloro EJ, Franchini M, Guidi GC, editors. Milestones and perspectives in coagulation and hemostasis. Seminars in thrombosis and hemostasis; 2009: © Thieme Medical Publishers.
14. Kumar M, Ananthakrishnan N. When and How to Investigate? Roshan Lall Gupta's Recent Advances in Surgery-13. 2013:176.
15. Perry D, Todd T. Practical-haemostasis. com: a web-based resource for laboratory haemostasis. Journal of Thrombosis and Haemostasis. 2009;7:860.
16. Cronin J, Sivarajaratnam M. Poor efficacy of haemodiafiltration to treat dabigatran-associated coagulopathy. Journal of the Intensive Care Society. 2014;15(2):154-7.
17. Dahlbäck B. Advances in understanding pathogenic mechanisms of thrombophilic disorders. Blood. 2008;112(1):19-27.
18. Rosing J, Hoekema L, Nicolaes GA, Thomassen MCL, Hemker HC, Varadi K, et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. Journal of Biological Chemistry. 1995;270(46):27852-8.
19. Stormorken H. The discovery of factor V: a tricky clotting factor. Journal of Thrombosis and Haemostasis. 2003;1(2):206-13.
20. Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the Molecular Defect in Factor VR506A. Journal of Biological Chemistry. 1995;270(8):4053-7.
21. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. Journal of Biological Chemistry. 1994;269(29):18735-8.
22. Godfrey E, Godfrey A, Perry D, Shaw A. Don't be a clot: A radiologist's guide to haemostasis including novel antiplatelet and anticoagulant therapies. Clinical radiology. 2011;66(8):693-700.
23. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proceedings of the National Academy of Sciences. 1993;90(3):1004-8.

24. Koster T, Vandenbroucke J, Rosendaal FR, de Ronde H, Briët E, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. The Lancet. 1993;342(8886-8887):1503-6.
25.Svensson PJ, Dahlback B. Resistance to activated protein C as a basis for venous thrombosis. New England Journal of Medicine. 1994;330(8):517-22.
26.Griffin JH, Evatt B, Wideman C, Fernandez J. Anticoagulant protein C pathway defective in majority of thrombophilic patients [see comments]. Blood. 1993;82(7):1989-93.
27. Dahlbäck B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proceedings of the National Academy of Sciences. 1994;91(4):1396-400.
28. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369(6475):64-7.
29. Zöller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. The Journal of clinical investigation. 1994;94(6):2521-4.
30. Kalafatis M, Haley P, Lu D, Bertina R, Long G, Mann K. Proteolytic events that regulate factor V activity in whole plasma from normal and activated protein C (APC)-resistant individuals during clotting: an insight into the APC-resistance assay. Blood. 1996;87(11):4695-707.
31. Bajzar L, Kalafatis M, Simioni P, Tracy PB. An antifibrinolytic mechanism describing the prothrombotic effect associated with factor VLeiden. Journal of Biological Chemistry. 1996;271(38):22949-52.
32. Zivelin A, Mor-Cohen R, Kovalsky V, Kornbrot N, Conard J, Peyvandi F, et al. Prothrombin 20210G> A is an ancestral prothrombotic mutation that occurred in whites approximately 24 000 years ago. Blood. 2006;107(12):4666-8.
33. Bertina RM. Factor V Leiden and other coagulation factor mutations affecting thrombotic risk. Clinical chemistry. 1997;43(9):1678-83.
34. Kjellberg U. Factor V Leiden mutation and pregnancy.
35. Tosetto A, Missiaglia E, Gatto E, Rodeghiero F. The VITA project: phenotypic resistance to activated protein C and FV Leiden mutation in the general population. Vicenza Thrombophilia and Atherosclerosis. Thrombosis and haemostasis. 1997;78(2):859-63.

36. Cumming AM, Campbell Tait R, Fildes S, Hay CR. Diagnosis of APC resistance during pregnancy. British journal of haematology. 1996;92(4):1026-7.
37. Vasse M, Leduc O, Borg J-Y, Chrétien MH, Monconduit M. Resistance to activated protein C: evaluation of three functional assays. Thrombosis research. 1994;76(1):47-59.
38. Nojima J, Iwatani Y, Ichihara K, Tsuneoka H, Ishikawa T, Yanagihara M, et al. Acquired activated protein C resistance is associated with IgG antibodies to protein S in patients with systemic lupus erythematosus. Thrombosis research. 2009;124(1):127-31.
39. Mahieu B, Jacobs N, Mahieu S, Naelaerts K, Vertessen F, Weyler J, et al. Haemostatic changes and acquired activated protein C resistance in normal pregnancy. Blood Coagulation & Fibrinolysis. 2007;18(7):685-8.
40. De Ronde H, Bertina R. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thrombosis and haemostasis. 1994;72(6):880-6.
41. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood. 1999;93(4):1271-6.
42. Morrison ER, Miedzybrodzka ZH, Campbell DM, Haites NE, Wilson B, Watson M, et al. Prothrombotic genotypes are not associated with pre-eclampsia and gestational hypertension: results from a large population-based study and systematic review. Thrombosis and haemostasis. 2002;87(05):779-85.
43. Gardiner C, Cohen H, Austin S, Machin S, Mackie I. Pregnancy loss, tissue factor pathway inhibitor deficiency and resistance to activated protein C. Journal of Thrombosis and Haemostasis. 2006;4(12):2724-6.
44. Sedano S, Gaffney G, Mortimer G, Lyons M, Cleary B, Murray M, et al. Activated protein C resistance (APCR) and placental fibrin deposition. Placenta. 2008;29(9):833-7.
45. Jorquera JI, Aznar J, Fernández MA, Montoro J, Curats R, Casaña P. A modification of the APC resistance test and its application to the study of patients on coumarin therapy. Thrombosis research. 1996;82(3):217-24.
46. Freyburger G, Javorschi S, Labrouche S, Bernard P. Proposal for objective evaluation of the performance of various functional APC-resistance tests in genotyped patients. Thrombosis and haemostasis. 1997;78(5):1360-5.

47. Gouault-Heilmann M, Leroy-Matheron C. Factor V Leiden-dependent APC resistance: improved sensitivity and specificity of the APC resistance test by plasma dilution in factor V-depleted plasma. Thrombosis research. 1996;82(3):281-3.
48. Hall C, Andersson N-E, Andras M, Zetterberg U, Rosén S. Evaluation of a modified APTT-based method for determination of APC resistance in plasma from patients on heparin or oral anticoagulant therapy. Thrombosis research. 1998;89(5):203-9.
49. Van Oerle R, Van Pampus L, Tans G, Rosing J, Hamulyák K. The Clinical Application of a New Specific Functional Assay To Detect the Factor VLeiden Mutation Associated With Activated Protein C Resistance. American journal of clinical pathology. 1997;107(5):521-6.
50. Kjellberg U. Factor V Leiden mutation and pregnancy. Haemostasis during pregnancy in non-carriers and carriers of factor V Leiden mutation, with special emphasis on placenta-mediated and venous thromboembolic complications and on blood coagulation and fibrinolysis markers for prediction of complications: Institute of Clincial Sciences. Department of Obstetrics and Gynecology; 2009.
51. Castoldi E, Brugge JM, Nicolaes GA, Girelli D, Tans G, Rosing J. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. Blood. 2004;103(11):4173-9.
52. Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, et al. A single genetic origin for a common Caucasian risk factor for venous thrombosis. Blood. 1997;89(2):397-402.
53. Rees DC. The population genetics of factor V Leiden (Arg506Gln). British journal of haematology. 1996;95(4):579-86.
54. Hiltunen L, Rautanen A, Rasi V, Kaaja R, Kere J, Krusius T, Vahtera E, Paunio M. An unfavourable combination of factor V Leiden with age, weight, and blood group causes high risk of pregnancy-associated venous thrombosis – a population-based nested case-control study. Thromb Res 2007;119:423-32.
55. Lindqvist PG, Svensson PJ, Dahlbäck B, Marsùál K. Factor V Q506 mutation (activated protein C resistance) associated with reduced intrapartum blood loss–a possible evolutionary selection mechanism. Thrombosis and haemostasis. 1998;80(01):69-73.

56. Rees DC, Chapman NH, Webster MT, Guerreiro JF, Rochette J, Clegg JB. Born to clot: the European burden. British journal of haematology. 1999;105(2):564-6.
57. Lindqvist PG, Svensson PJ, Maršál K, Grennert L, Luterkort M, Dahlbäck B. Activated protein C resistance (FV: Q506) and pregnancy. Thrombosis and haemostasis. 1999;81(04):532-7.
58. Kjellberg U, van Rooijen M, Bremme K, Hellgren M. Factor V Leiden mutation and pregnancy-related complications. American journal of obstetrics and gynecology. 2010;203(5):469. e1-. e8.
59. Lindqvist PG, Zöller B, Dahlbäck B. Improved hemoglobin status and reduced menstrual blood loss among female carriers of factor V Leiden–an evolutionary advantage? Thrombosis and haemostasis. 2001;86(04):1122-3.
60. Clark P, Walker ID, Govan L, Wu O, Greer IA. The GOAL study: a prospective examination of the impact of factor V Leiden and ABO (H) blood groups on haemorrhagic and thrombotic pregnancy outcomes. British journal of haematology. 2008;140(2):236-40.
61. Donahue BS, Gailani D, Higgins MS, Drinkwater DC, George AL. Factor V Leiden protects against blood loss and transfusion after cardiac surgery. Circulation. 2003;107(7):1003-8.
62. Franchini M, Lippi G. Factor V Leiden and hemophilia. Thrombosis research. 2010;125(2):119-23.
63. Göpel W, Ludwig M, Junge AK, Kohlmann T, Diedrich K, Möller J. Selection pressure for the factor-V-Leiden mutation and embryo implantation. The Lancet. 2001;358(9289):1238-9.
64. Van Dunne F, Doggen CJM, Heemskerk M, Rosendaal F, Helmerhorst F. Factor V Leiden mutation in relation to fecundity and miscarriage in women with venous thrombosis. Human reproduction. 2005;20(3):802-6.
65. Van Dunne F, De Craen A, Heijmans B, Helmerhorst F, Westendorp R. Gender-specific association of the factor V Leiden mutation with fertility and fecundity in a historic cohort. The Leiden 85-Plus Study. Human reproduction. 2006;21(4):967-71.
66. Cohn D, Repping S, Büller H, Meijers J, Middeldorp S. Increased sperm count may account for high population frequency of factor V Leiden. Journal of Thrombosis and Haemostasis. 2010;8(3):513-6.

67. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 39-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88(10):3698-703.
68. Sheskey AM. Evaluating the need for anticoagulant prophylaxis during pregnancy in asymptomatic heterozygous carriers of factor V Leiden. 2012.
69. Bremme KA. Haemostatic changes in pregnancy. Best practice & research Clinical haematology. 2003;16(2):153-68.
70. Szecsi PB, Jørgensen M, Klajnbard A, Andersen MR, Colov NP, Stender S. Haemostatic reference intervals in pregnancy. Thrombosis & Haemostasis. 2010;103(4):718.
71. Kjellberg U, Andersson N-E, Rosen S, Tengborn L, Hellgren M. APC resistance and other haemostatic variables during pregnancy and puerperium. Thrombosis and haemostasis. 1999;81(04):527-31.
72. Reitsma P, Rosendaal F. Past and future of genetic research in thrombosis. Journal of Thrombosis and Haemostasis. 2007;5(s1):264-9.
73. Rosendaal FR. Venous thrombosis: a multicausal disease. The Lancet. 1999;353(9159):1167-73.
74. Martinelli I, Bucciarelli P, Mannucci PM. Thrombotic risk factors: basic pathophysiology. Critical care medicine. 2010;38:S3-S9.
75. Rosendaal F, Reitsma P. Genetics of venous thrombosis. Journal of Thrombosis and Haemostasis. 2009;7(s1):301-4.
76. Reiner AR, Siscovick DS, Rosendaal FR. Hemostdtic Rssk Factors and Arterlal TTuombotic Disease. Thromb Haemost. 2001;85:584-95.
77. Jick H, Westerholm B, Vessey M, Lewis G, Slone D, Inman WW, et al. Venous thromboembolic disease and ABO blood type: a cooperative study. The Lancet. 1969;293(7594):539-42.
78. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? Transfusion. 2006;46(10):1836-44.
79. Walker ID, editor Venous and arterial thrombosis during pregnancy: epidemiology. Seminars in vascular medicine; 2003: Copyright© 2003 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel.:+ 1 (212) 584-4662.

80. McColl M, Ramsay J, Tait R, Walker I, McCall F, Conkie J, et al. Risk factors for pregnancy associated venous thromboembolism. Thrombosis and haemostasis. 1997;78(4):1183-8.
81. Andersen B, Steffensen F, Sørensen H, Nielsen G, Olsen J. The cumulative incidence of venous thromboembolism during pregnancy and puerperium. Acta obstetricia et gynecologica Scandinavica. 1998;77(2):170-3.
82. Simpson E, Lawrenson R, Nightingale A, Farmer R. Venous thromboembolism in pregnancy and the puerperium: incidence and additional risk factors from a London perinatal database. BJOG: An International Journal of Obstetrics & Gynaecology. 2001;108(1):56-60.
83. Gherman RB, Goodwin TM, Leung B, Byrne JD, Hethumumi R, Montoro M. Incidence, clinical characteristics, and timing of objectively diagnosed venous thromboembolism during pregnancy. Obstetrics & Gynecology. 1999;94(5):730-4.
84. Schutte J, Steegers E, Schuitemaker N, Santema J, de Boer K, Pel M, et al. Rise in maternal mortality in the Netherlands. BJOG: An International Journal of Obstetrics & Gynaecology. 2010;117(4):399-406.
85. Erkkola R, editor Maternal mortality in Finland 1970-89. Annales chirurgiae et gynaecologiae Supplementum; 1994.
86. Tikkanen M, Gissler M, Metsäranta M, Luukkaala T, Hiilesmaa V, Andersson S, et al. Maternal deaths in Finland: focus on placental abruption. Acta obstetricia et gynecologica Scandinavica. 2009;88(10):1124-7.
87. Farquharson D, Dale S, McMaster R, Miyazaki L, Pacheco T. Maternal mortality in British Columbia. 2008.
88.Kearon C, Crowther M, Hirsh J. Management of patients with hereditary hypercoagulable disorders. Annual review of medicine. 2000;51(1):169-85.
89. Key N MM, O'Shaughnessy, Lillicrap D. Practical hemostasis & thrombosis Isobel D.walker; 2009.
90. Walker ID, Greaves M, Preston F. Investigation and management of heritable thrombophilia. British journal of haematology. 2001;114(3):512-28.
91. Bates SM, Greer IA, Pabinger I, Sofaer S, Hirsh J. Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians evidence-based clinical practice guidelines. CHEST Journal. 2008;133(6_suppl):844S-86S.

92. Biron-Andreani C, Schved J-F, Daures J-P. Factor V Leiden mutation and pregnancy-related venous thromboembolism: What is the exact risk? Thrombosis and haemostasis. 2006;96(01):14-8.
93. Simioni P, Sanson B-J, Prandoni P, Tormene D, Friederich PW, Girolami B, et al. Incidence of venous thromboembolism in families with inherited thrombophilia. Thrombosis and haemostasis. 1999;82(02):198-202.
94. Lensen R, Rosendaal F, Vandenbroucke And J, Bertina R. Factor V Leiden: the venous thrombotic risk in thrombophilic families. British journal of haematology. 2000;110(4):939-45.
95. Tormene D, Simioni P, Prandoni P, Luni S, Zerbinati P, Sartor D, et al. Factor V Leiden mutation and the risk of venous thromboembolism in pregnant women. Haematologica. 2001;86(12):1305-9.
96. Dizon-Townson D, Miller C, Sibai B, Spong CY, Thom E, Wendel Jr G, et al. The relationship of the factor V Leiden mutation and pregnancy outcomes for mother and fetus. Obstetrics & Gynecology. 2005;106(3):517-24.
97. Murphy RP, Donoghue C, Nallen RJ, D'mello M, Regan C, Whitehead AS, et al. Prospective evaluation of the risk conferred by factor V Leiden and thermolabile methylenetetrahydrofolate reductase polymorphisms in pregnancy. Arteriosclerosis, thrombosis, and vascular biology. 2000;20(1):266-70.
98. Baglin T, Gray E, Greaves M, Hunt BJ, Keeling D, Machin S, et al. Clinical guidelines for testing for heritable thrombophilia. British journal of haematology. 2010;149(2):209-20.
99. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. The Lancet. 2005;365(9461):785-99.
100. Practice ACoO. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2002;77(1):67.
101. Roberts JM, Hubel CA. The two stage model of preeclampsia: variations on the theme. Placenta. 2009;30:32-7.
102. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. American journal of obstetrics and gynecology. 1996;175(5):1365-70.

103. Roberts J, Taylor R, Musci T, Rodgers G, Hubel C, McLaughlin M. Preeclampsia: An endothelial cell disorder. International Journal of Gynecology & Obstetrics. 1990;32(3):299-.

104. Brenner B. COMMENTARY: Thrombophilia and pregnancy loss in first intended pregnancy. Journal of Thrombosis and Haemostasis. 2005;3(10):2176-7.

105. Lin J, August P. Genetic thrombophilias and preeclampsia: a meta-analysis. Obstetrics & Gynecology. 2005;105(1):182-92.

106. Rodger MA, Betancourt MT, Clark P, Lindqvist PG, Dizon-Townson D, Said J, et al. The association of factor V leiden and prothrombin gene mutation and placenta-mediated pregnancy complications: a systematic review and meta-analysis of prospective cohort studies. Plos medicine. 2010;7(6):e1000292.

107. Nurk E, Tell G, Refsum H, Ueland P, Vollset S. Factor V Leiden, pregnancy complications and adverse outcomes: the Hordaland Homocysteine Study. Journal of the Association of Physicians. 2006;99(5):289-98.

108. Nurk E, Refsum H, Tell G, Ueland P. Factor V Leiden, pregnancy complications and adverse outcomes. Qjm. 2006;99(9):639-40.

109. Dudding T, Heron J, Thakkinstian A, Nurk E, Golding J, Pembrey M, et al. Factor V Leiden is associated with pre-echlampsia but not with fetal growth restriction: agenetic association study and meta-analysis. Journal of Thrombosis and Haemostasis. 2008;6(11):1868-75.

110. Kahn SR, Platt R, McNamara H, Rozen R, Chen MF, Genest J, et al. Inherited thrombophilia and preeclampsia within a multicenter cohort: the Montreal Preeclampsia Study. American Journal of Obstetrics and Gynecology. 2009;200(2):151. e1-. e9.

111. Stanton C, Lawn JE, Rahman H, Wilczynska-Ketende K, Hill K. Stillbirth rates: delivering estimates in 190 countries. The Lancet. 2006;367(9521):1487-94.

112. Kim YM, Chaemsaithong P, Romero R, Shaman M, Kim CJ, Kim J-S, et al. Placental lesions associated with acute atherosis. The Journal of Maternal-Fetal & Neonatal Medicine. 2015;28(13):1554-62.

113. Pasupathy D, Wood AM, Pell JP, Fleming M, Smith GC. Time of birth and risk of neonatal death at term: retrospective cohort study. Bmj. 2010;341:c3498.

114. Silver RM, Varner MW, Reddy U, Goldenberg R, Pinar H, Conway D, et al. Work-up of stillbirth: a review of the evidence. American journal of obstetrics and gynecology. 2007;196(5):433-44.
115. Martinelli I, Taioli E, Cetin I, Marinoni A, Gerosa S, Villa MV, et al. Mutations in coagulation factors in women with unexplained late fetal loss. New England Journal of Medicine. 2000;343(14):1015-8.
116. Sottilotta G, Oriana V, Latella C, Luise F, Piromalli A, Ramirez F, et al. Genetic prothrombotic risk factors in women with unexplained pregnancy loss. Thrombosis research. 2006;117(6):681-4.
117. Said JM, Higgins JR, Moses EK, Walker SP, Borg AJ, Monagle PT, et al. Inherited thrombophilia polymorphisms and pregnancy outcomes in nulliparous women. Obstetrics & Gynecology. 2010;115(1):5-13.
118. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. The lancet. 2008;371(9606):75-84.
119. Muglia LJ, Katz M. The enigma of spontaneous preterm birth. New England Journal of Medicine. 2010;362(6):529-35.
120. Wu O, Robertson L, Twaddle S, Lowe G, Clark P, Greaves M, et al. Screening for thrombophilia in high-risk situations: systematic review and cost-effectiveness analysis. The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) study. 2006.
121. Engle WA, Tomashek KM, Wallman C. “Late-preterm” infants: a population at risk. Pediatrics. 2007;120(6):1390-401.
122. Velez DR, Fortunato SJ, Thorsen P, Lombardi SJ, Williams SM, Menon R. Preterm birth in Caucasians is associated with coagulation and inflammation pathway gene variants. PloS one. 2008;3(9):e3283.
123.Göpel W, Kim D, Gortner L. Prothrombotic mutations as a risk factor for preterm birth. The Lancet. 1999;353(9162):1411-2.
124. Cedergren MI. Maternal morbid obesity and the risk of adverse pregnancy outcome. Obstetrics & Gynecology. 2004;103(2):219-24.
125. Erhardt E, Stankovics J, Molnár D, Adamovich K, Melegh B. High prevalence of factor V Leiden mutation in mothers of premature neonates. Neonatology. 2000;78(2):145-6.
126. Gargano JW, Holzman CB, Senagore PK, Reuss ML, Pathak DR, Friderici KH, et al. Polymorphisms in thrombophilia and renin-angiotensin system

pathways, preterm delivery, and evidence of placental hemorrhage. American journal of obstetrics and gynecology. 2009;201(3):317. e1-. e9.
127. Kocher O, Cirovic C, Malynn E, Rowland CM, Bare LA, Young BA, et al. Obstetric complications in patients with hereditary thrombophilia identified using the LCx microparticle enzyme immunoassay: a controlled study of 5,000 patients. American journal of clinical pathology. 2007;127(1):68-75.
128. Valdez LL, Quintero A, Garcia E, Olivares N, Celis A, Rivas F. Thrombophilic polymorphisms in preterm delivery. Blood Cells, Molecules, and Diseases. 2004;33(1):51-6.
129. Resch B, Gallistl S, Kutschera J, Mannhalter C, Muntean W, Mueller WD. Thrombophilic polymorphisms–factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations–and preterm birth. Wiener Klinische Wochenschrift. 2004;116(17-18):622-6.
130. Uvuz F, Kilic S, Yilmaz N, Tuncay G, Cakar E, Yuksel B, et al. Relationship between preterm labor and thrombophilic gene polymorphism: A prospective sequential cohort study. Gynecologic and obstetric investigation. 2009;68(4):234-8.
131.Kramer MS, Kahn SR, Rozen R, Evans R, Platt RW, Chen MF, et al. Vasculopathic and thrombophilic risk factors for spontaneous preterm birth. International journal of epidemiology. 2009;38(3):715-23.
132.Ulander V-M, Wartiovaara U, Hiltunen L, Rautanen A, Kaaja R. Thrombophilia: A new potential risk factor for cervical insufficiency. Thrombosis research. 2006;118(6):705-8.
133.Hennekens CH, Buring JE, Mayrent SL. Epidemiology in medicine: Boston: Little Brown and Company, 1987; 1987.
134. Ziv E, Burchard EG. Human population structure and genetic association studies. Pharmacogenomics. 2003;4(4):431-41.
135. Haukka J. Finnish health and social welfare registers in epidemiological research. Norsk epidemiologi. 2009;14(1).
136. Parekh-Bhurke S, Kwok CS, Pang C, Hooper L, Loke YK, Ryder JJ, et al. Uptake of methods to deal with publication bias in systematic reviews has increased over time, but there is still much scope for improvement. Journal of clinical epidemiology. 2011;64(4):349-57.

137. Greengard J, Sun X, Xu X, Fernandez J, Griffin J, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. The Lancet. 1994;343(8909):1361-2.
138. Zehnder JL, Benson RC. Sensitivity and Specificity ofthe APC Resistance Assay in Detection of Individuals With Factor V Leiden. American journal of clinical pathology. 1996;106(1):107-11.
139. Jorquera J, Montoro J, Fernández MA, Aznar J, Aznar J. Modified test for activated protein C resistance. The Lancet. 1994;344(8930):1162-3.
140. Trossaert M, Conard J, Horellou M, Elalamy I, Samama M. The modified APC resistance test in the presence of factor V deficient plasma can be used in patients without oral anticoagulant. Thrombosis and haemostasis. 1996;75(3):521-2.
141.Denson K, Haddon M, Reed S, Davidson S, Littlewood T. A more discriminating test for APC resistance and a possible screening test to include protein C and protein S. Thrombosis research. 1996;81(1):151-6.
142. Gardiner C, Cooper P, Makris M, Mackie I, Malia R, Machin S. An evaluation of screening tests for defects in the protein C pathway: commercial kits lack sensitivity and specificity. Blood coagulation & fibrinolysis. 2002;13(2):155-63.
143. Van Cott EM, Soderberg BL, Laposata M. Activated protein C resistance, the factor V Leiden mutation, and a laboratory testing algorithm. Archives of pathology & laboratory medicine. 2002;126(5):577-82.
144. Dahlback B. Resistance to activated protein C, the Arg506 to Gln mutation in the factor V gene, and venous thrombosis. Functional tests and DNA-based assays. Pros and Cons Thromb Haemost. 1995;73:739-42.
145. Voelkerding KV, Wu L, Williams EC, Hoffman SM, Sabatini LM, Borcherding WR, et al. Factor V R506Q gene mutation analysis by PCR-RFLP: optimization, comparison with functional testing for resistance to activated protein C, and establishment of cell line controls. American journal of clinical pathology. 1996;106(1):100-6.
146.Wilmer M, Stocker C, Bühler B, Conell B, Calatzis A. Improved distinction of factor V wild-type and factor V Leiden using a novel prothrombin-based activated protein C resistance assay. American journal of clinical pathology. 2015;122(6):836-42.

147.Walker I. Blood collection and sample preparation: pre-analytical variation. Laboratory Techniques in Thrombosis—a Manual: Springer; 1999. p. 21-8.
148. Stocker K, Fischera H, Meier J, Brogli M, Svendsen L. Characterization of the protein C activator Protac® from the venom of the southern copperhead (Agkistrodon contortrix) snake. Toxicon. 1987;25(3):239-52.
149. Ødegård O, Lie M, Abildgaard U. Heparin cofactor activity measured with an amidolytic method. Thrombosis research. 1975;6(4):287-94.
150. Montagnana M, Favaloro EJ, Franchini M, Guidi GC, Lippi G. The role of ethnicity, age and gender in venous thromboembolism. Journal of thrombosis and thrombolysis. 2010;29(4):489-96.
151. Geerts W, Cook D, Selby R, Etchells E. Venous thromboembolism and its prevention in critical care. Journal of critical care. 2002;17(2):95-104.
152. Yousif A, Muddathir A, Elamin E, Alhadi A. The Role of Factor V Leiden 1691G> A and Prothrombin Gene 20210G> A Mutations in Hypercoagulable State Associated with Venous Thromboembolism among Sudanese Patients. J Blood Disord Transfus. 2017;8(386):2.
153. Taha SKA, Kordofani AAY. Activated Protein C Resistance in Adult Sudanese Patients diagnosed with Deep Vein Thrombosis. Science Journal of Medicine and Clinical Trials. 2014;2014.
154. Babker AMAAA, Gameel FEMH. The frequency of factor V leiden mutation among Sudanese pregnant women with recurrent miscarriage. Journal of American Science. 2014;10(9).
155. Payandeh M, Zare ME, Kansestani AN, Mansouri K, Rahimi Z, Hashemian AH, et al. Protein C and S deficiency in deep vein thrombosis patients referred to Iranian blood transfusion organization, Kermanshah. International Journal of Hematology-Oncology and Stem Cell Research. 2011;5(2):5-8.
156. Hossain N, Shamsi T, Soomro N. Frequency of thrombophilia in patients with adverse pregnancy outcome. JOURNAL-PAKISTAN MEDICAL ASSOCIATION. 2005;55(6):245.
157. Hansda J, Roychowdhury J. Study of thrombophilia in recurrent pregnancy loss. The Journal of Obstetrics and Gynecology of India. 2012;62(5):536-40.
158. Marietta M, Facchinetti F, Sgarbi L, Simoni L, Bertesi M, Torelli G, et al. Elevated plasma levels of factor VIII in women with early recurrent miscarriage. Journal of Thrombosis and Haemostasis. 2003;1(12):2536-9.

159. Suehisa E, Nomura T, Kawasaki T, Kanakura Y. Frequency of natural coagulation inhibitor (antithrombin III, protein C and protein S) deficiencies in Japanese patients with spontaneous deep vein thrombosis. Blood coagulation & fibrinolysis. 2001;12(2):95-9.
160. Marouf R, Mojiminiyi O, Qurtom M, Abdella N, Al Wazzan H, Al Humood S, et al. Plasma homocysteine and hematological factors in patients with venous thromboembolic diseases in Kuwait. Acta haematologica. 2007;117(2):98-105.
161. Hoffbrand A, Petit J, Moss P. Essential Hematology,4th ed, oxford black well.2003;273-275.
162. Vizcaino G, Torres E, Quintero J, Herrmann F, Grimm R, Diez-Ewald M, et al. Prevalence of the activated protein C resistance in indigenous and Black populations of the western Venezuela. Investigacion clinica. 2000;41(1):29-36.

# *Questionnaire*

**University of Shendi**
**Faculty of Post-Graduate studies**

# Role of APC-R and hereditary anticoagulant deficiency in vascular complication of pregnancy

S. Number ….............................................................................
Hospital……………………………..…………………………………

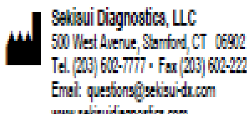
- Lab no: ……………………….………………………………….
- Age:…………………………………………………………
- Number of previous pregnant………..……..………….
- Pregnancy stage……………………………………….
- Race or tribe
- Region
- Family history of pregnancy complication ......................…………
    - Relation degree
    - Diagnosis:-
        - Stillbirth
        - Pre-eclampsia
        - IUGR
        - Placental abruption
        - Chorionamnionitis
- **History of other disease**:
- Thrombosis………………………..…………..…..…
- Abortion……………………………………………………..
- Bleeding……………………..……………………………….
- DM ……………………...………….…………………………
- Hypertension…………………………………………………
- Any drugs intake……………………………………..…..…
- Others……...…………………………………………………...

**(8) Results**:

- APC-R……………………………………..……
- ATIII ……………………………………..………
- PC………………………………………..………….
- PS……………………………..…………….
- Free PS………………………………………….

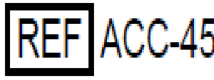
*Date*……………..…………

Appendix 1


Sekisui Diagnostics, LLC
500 West Avenue, Stamford, CT 06902
Tel. (203) 602-7777 • Fax (203) 602-2221
Email: questions@sekisui-dx.com
www.sekisuidiagnostics.com

# ACTICLOT® C

REF ACC-45



## INTENDED USE

ACTICLOT® C is intended for the measurement of Protein C activity in human plasma via an end-point clotting assay. The assay is for in vitro diagnostic use.

## EXPLANATION OF THE TEST

Protein C is a vitamin K-dependent anticoagulant protein that normally circulates as an inactive zymogen. After activation, Protein C inactivates factors V and VIII thus prolonging the clotting time. While Protein C can be activated by thrombin, the rate of activation in vitro is slow. Under such conditions Protein C inhibitor protein inactivates Protein C as fast as it is activated.

## PRINCIPLE OF THE METHOD

The venom of the copperhead snake Agkistrodon contortrix is a rapid activator of Protein C.[1] Under the assay conditions of ACTICLOT C, the ACTICLOT Activator, formulated with the venom from Agkistrodon contortrix, converts human Protein C to its active protease within 5 minutes.[2] The ACTICLOT Activator reagent is formulated to activate both Protein C and the contact factors of the intrinsic pathway. With this reagent, the clotting time of normal plasma is very long, greater than 100 seconds, while the clotting time of a Protein C deficient plasma is essentially the same as the clotting time of an APTT test, approximately 30-40 seconds. When an unknown test plasma is mixed with Protein C deficient plasma, the Protein C level is proportional to the prolongation of the clotting time.

## REAGENTS

1. **ACTICLOT Activator:** 3 vials each containing 1.5 units of *Agkistrodon contortrix* venom lyophilized with rabbit brain cephalin and colloidal silica activator.

2. **Protein C Deficient Plasma:** 3 vials each containing 1.5 mL of lyophilized human plasma depleted of Protein C by immunoadsorption on a column of immobilized antibody immunospecific to human Protein C.

3. **Protein C Control Plasma:** 3 vials each containing 0.5 mL of lyophilized normal human plasma.

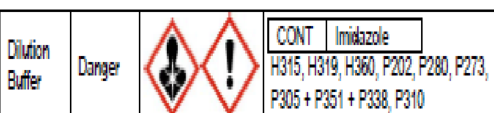
4. **Dilution Buffer:** 3 vials each containing 5 mL of a 10-fold concentrate. At working strength, the buffer contains 0.12 M NaCl, 0.03M Imidazole, pH 7.35.

## WARNINGS AND PRECAUTIONS

This product contains *human* source material that has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using registered methods. As no known test method can provide complete assurance that products derived from human specimens will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, this product should be handled as recommended for any potentially infectious human specimen.

This product contains *animal* source material. As no known test method can provide complete assurance that products derived from animal specimens will not transmit blood-borne pathogens, this reagent should be handled as recommended for any potentially infectious specimen.

The Dilution Buffer contains sodium azide that may react with lead or copper plumbing to form highly explosive metal azides. Materials discarded into a sink should be flushed with a large volume of water to prevent azide build-up.



| Dilution Buffer | Danger | | CONT Imidazole<br>H315, H319, H360, P202, P280, P273, P305 + P351 + P338, P310 |
|---|---|---|---|

**Hazard Statements:**
H315 Causes skin irritation.
H319 Causes serious eye irritation.
H360 May damage fertility or the unborn child.

**Precautionary Statements:**
P202 Do not handle until all safety precautions have been read and understood.
P264 Wash thoroughly after handling.
P280 Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352 IF ON SKIN: Wash with plenty of water.
P332 + P313 If skin irritation occurs: Get medical advice/attention.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical advice/attention.



## REAGENT PREPARATION AND STORAGE

Unreconstituted reagents are stable until the expiration date indicated on the label when stored at 2° - 8°C.

1. **ACTICLOT Activator**: Reconstitute with 1.5 mL purified water.
   Stability at -20°C: 3 months
   at 2°- 8°C: 48 hours
   at 37°C: 4 hours

   Some automated equipment may require a larger Activator volume in order to adequately fill the reagent reservoir and pump tubing. In this case, reconstitute all three vials of Activator provided and pool to obtain a reagent volume of 4.5 mL after use. The remaining contents of the reservoir and pump tubing may be returned to the vial, capped, frozen at -20°C and reused. Activator can be frozen and thawed virtually without loss of activity.

2. **Protein C Deficient Plasma**: Reconstitute with 1.5 mL purified water. Let stand at room temperature for 20 minutes for complete dissolution then swirl gently. Use immediately or store on melting ice until use.

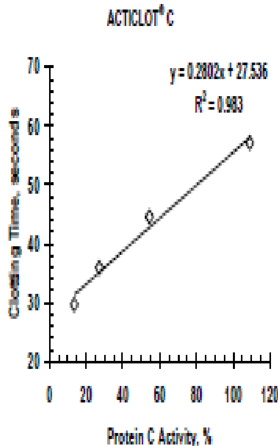
3. **Protein C Control Plasma**: Reconstitute with 0.5 mL purified water. Let stand at room temperature for 20 minute for complete dissolution then swirl gently. Use immediately or store on melting ice until use. Use as a quality control reagent when performing the assay.

4. **Dilution Buffer**: Dilute to 50 mL with purified water.
   Stability at room temperature: 1 week
   at 2 - 8°C: 1 month

## SPECIMEN COLLECTION AND PREPARATION

See "Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays; Approved Guidelines-Fifth Edition", CLSI Document H21-A5, Vol. 28, No. 5, 2008.[3]

Nine volumes of blood are collected in 1 volume of 0.1M trisodium citrate and centrifuged at 3000 x g for 10 minutes. Plasma should be stored at 2°- 8°C and assayed within 2 hours. Alternatively, plasma may be stored at -20°C for 1 month and thawed once at 37°C, 30 minutes before use.




## CALCULATION OF RESULTS

Determine the % Protein C in the test sample by interpolating from the standard curve and multiplying the result by two to correct for dilution. In the case of patients with lupus anticoagulants or abnormally high Protein C activity, where multiple patient dilutions were assayed, correct the Protein C level for the dilution. Corrected Protein C levels from at least two dilutions must agree.

## QUALITY CONTROL

Use Protein C Control Plasma provided in the kit for quality control of the assay.

## PERFORMANCE CHARACTERISTICS

In a clinical study comparing ACTICLOT C to a Protein C ELISA, the following results were obtained:

Plasma Protein C Concentration
(% of pooled normal plasma, mean + s.d.)

| Population | n | ACTICLOT C | ELISA |
|---|---|---|---|
| Normal | 40 | 89.0 ± 17.0 | 94.0 ± 16.0 |
| DIC | 10 | 29.4 ± 11.9 | 34.2 ± 13.0 |
| Liver Disease | 10 | 18.6 ± 9.6 | 20.1 ± 14.6 |
| Congenital Def. | 10 | 37.9 ± 7.1 | 45.0 ± 8.1 |
| Heparin | 10 | 93.7 ± 16.1 | 93.5 ± 14.2 |
| Coumadin | 20 | 23.2 ± 9.0 | 57.7 ± 15.7 |
| *Sick Neonates | 12 | 21.9 ± 5.3 | 19.3 ± 8.0 |

* e.g. respiratory distress syndrome, sepsis, thrombosis, renal failure



Correlation between ACTICLOT C and ELISA (coumadin patients on coumadin not included):

| Regression Line | Correlation Coefficient | Standard Error of Estimate |
|---|---|---|
| y = 0.93x + 0.0014 | 0.952 | 0.086 |

The coefficient of variation of the assay has been determined using plasma samples prepared by mixing plasma that has been totally immunodepleted of Protein C with normal pooled plasma to obtain Protein C levels of 10%, 50% and 100%.

| Protein C Level | Intra-Assay C.V. | Inter-Assay C.V. |
|---|---|---|
| 100% | 5.9% | 2.4% |
| 50% | 4.7% | 3.9% |
| 10% | 9.1% | 9.3% |

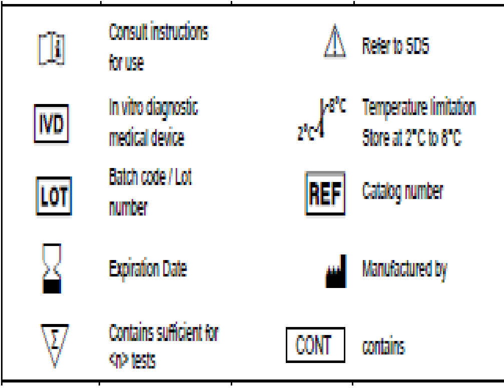
## REFERENCES

1. Stocker, K., Fischer, H., Mejer, J., Brogil, M. and Svendsen, L: Characterization of the protein C activator Protac from the venom of the Southern Copperhead (Agkistrodon contortrix) snake. *Toxicol.* 1987, 25: 239-252.

2. Martinoli, J. L. and Stocker, K. Fast functional protein C assay using Protac, a novel protein C activator. *Thromb. Res.* 1986, 43: 253- 264.

3. Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays and Molecular Hemostasis Assays; Approved Guidelines-Fifth Edition", CLSI Document H21-A5, Vol. 28, No. 5, 2008.

## DEFINITIONS OF SYMBOLS






ACC-45_C08D20151223

Appedex 2

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# ACTICLOT® Protein C Resistance

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*ENGLISH*

## INTENDED USE

The ACTICLOT® Protein C Resistance assay is a plasma based functional assay for the determination of resistance to activated protein C caused by the factor V Leiden mutation (FV:Q506). For *in-vitro* diagnostic use.

## INTRODUCTION

Activated protein C (APC) resistance is the most frequent hereditary defect associated with deep vein thrombosis. Over 95% of the APC resistance phenotype can be explained by the Factor V Leiden mutation [1,2,3,4,5,6]. This defect is caused by point mutation in the factor V gene resulting in a replacement of the amino acid Arg 506 by a Gln residue [2,3,7]. The heterozygous (het) defect is associated with a 5 to 10 fold, the homozygous (hom) defect with a 50 to 100 fold increased thrombosis risk [5,8,9].

There are two possibilities of detecting factor V (FV) Leiden. Plasma based functional assays identifying the phenotype expression of the defect [1] or genotype determination which can be done by PCR technology [10].

## PRINCIPLE OF THE METHOD

The ACTICLOT® Protein C Resistance assay is a plasma-based functional clotting assay and differs from other functional APC resistance tests by acting specifically on the prothrombinase complex level. It is based on a FV-dependent prothrombin activator isolated from snake venom. Robustness and specificity of the assay is enhanced by elimination of possible disturbing influences by factors upstream the coagulation cascade and independency from calcium. Interference from UFH, LMWH and Pentasaccharide in the blood sample is precluded by a heparin inhibitor added to reagents 1 and 2.

Sample plasma is pre-diluted with reagent 4 (dilution plasma) and incubated at 37 °C with FV activator from snake venom (RVV-V from *Daboia russelli*). Coagulation is triggered by the addition of a FV dependent prothrombin activator from snake venom from *Notechis scutatus* in the absence of calcium. The clotting times are recorded and the ratios (clotting time in the presence of APC/clotting time in the absence of APC) are calculated.

## REAGENTS

| Reagent | Reagent content |
|---|---|
| R1 | **APC / RVV-V (+APC) Reagent**<br>(APC, RVV-V, Polybren, Hepes, BSA)<br>3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial) |
| R2 | **RVV-V (-APC) Reagent**<br>(RVV-V, Polybren, Hepes, BSA)<br>3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial) |
| R3 | **PTA Reagent**<br>(Prothrombin Activator, EDTA, Hepes, BSA)<br>3 vials (lyophilisate, to be reconstituted in 4.0 ml of deionized water per vial) |
| R4 | **Dilution Plasma**<br>(processed Human Plasma)<br>3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial) |

Incubate reconstituted solutions R1-R4 in closed vials for 30' at room temperature and swirl gently before use.

Attention: Extended incubation of reagent R4 may – due to its high protein content – cause a phase separation characterized by a clear solution with a fine, whitish layer on its surface. This may be erroneously interpreted as coagulation. Therefore, the reagent must absolutely be brought in its initial homogeneous and slightly cloudy form just before use.

## MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized water
- Calibrated pipettes (1000–5000 µl)
- Automated or semi-automated coagulation instruments using mechanical or optical detection methods

Note: When using automated or semi-automated coagulation analyzers refer always to manufacturer's operator manual or ask for a detailed adaptation protocol.

## STORAGE AND STABILITY

The test kit may be used up to the expiry date given on the label when stored unopened at 2–8 °C.

Stability of the reagents after reconstitution:

| Reagent | Stability | |
|---|---|---|
| R1 | -20°C<br>2 - 8°C<br>15 - 25°C | 6 months<br>14 days<br>24 hours (on-board) |
| R2 | -20°C<br>2 - 8°C<br>15 - 25°C | 6 months<br>14 days<br>24 hours (on-board) |
| R3 | -20°C<br>2 - 8°C<br>15 - 25°C | 6 months<br>14 days<br>24 hours (on-board) |
| R4 | -20°C<br>2 - 8°C<br>15 - 25°C | 6 months<br>14 days<br>24 hours (on-board) |

Frozen reagents should be thawed at 37 °C and gently mixed before use. Freeze only once.

## QUALITY CONTROLS

Use the ACTICLOT® Protein C Resistance Control Plasmas (REF ADG840C) as a control reference for the validation of the assay. Negative control or wild-type (neg) shows normal responce to APC whereas heterozygous control (het) shows responce to the presence of the heterozygous type of FV: Q506 mutation. A control run should be made with each test series.
For preparation, use and interpretation of the controls, refer to the instructions and certified ranges mentioned in the package insert of the corresponding control kit.
Different clotting times will be obtained with different types of instruments depending on the clot detection principle. If values outside the certified range (ratio) are obtained, a complete check of reagents should be made and the analysis should be repeated. If the problem persists, a complete instrument check should be made and the analysis should be repeated.

## BLOOD COLLECTION AND SAMPLE PREPARATION

The patient should be at rest for 10 min prior sampling. Collect venous blood carefully in either 104 mM or 129 mM sodium citrate (volume ratio 9+1). Mix gently blood and anticoagulant directly after sampling, avoid foam formation. Centrifuge immediately at no less than 2000x g for at least 20 min at room temperature. Take care to avoid contaminations from the platelet layer into plasma when the plasma is separated from the cells. As a general rule hemolytic plasma samples should not be used.
For storage freeze undiluted plasma rapidly at −70 °C in aliquots. Freeze only once. Avoid repeated freezing and thawing cycles. To ensure negligible loss of activity of labile coagulation factors and absence of cryoprecipitate, thawing should be done rapidly (within 5 min) in a water bath at 37 °C. For more information see NCCLS document H21-A2 [11].

Stability of undiluted samples (plasma):

| | |
|---|---|
| −80 °C | at least 1 year |
| −20 °C | 2 months |
| 2–8 °C | 24 hours |
| 15–25 °C | 4 hours |

## PROCEDURE

Prepare reagents and samples as described above. Mix gently thawed sample for homogenization, avoid foam formation. Determine +APC clotting time (clotting time in the presence of Activated Protein C), −APC clotting time (clotting time in the absence of Activated Protein C) and calculate the ratio according to the following scheme:

| | | + APC | - APC |
|---|---|---|---|
| | Sample or control plasma | 30 µL | 30 µL |
| R4 | Dilution Plasma | 20 µL | 20 µL |
| | | mix prior to use | mix prior to use |
| R1 | APC/RVV-V (+APC) Reagent | 50 µL | - |
| R2 | RVV-V (-APC) Reagent | - | 50 µL |
| | Incubation | 3 min, 37°C | 3 min, 37°C |
| R3 | PTA Reagent | 50 µL | 50 µL |
| | | Determine clotting time | Determine clotting time |
| | Ratio calculation = | Clotting time + APC<br>Clotting time - APC | |

2

## INTERPRETATION OF THE TEST RESULTS

Differentiation of homozygous, heterozygous and negative samples is based on the typical ratio ranges measured with genotyped patient plasma samples (see tables below). These ratios may vary depending on laboratory, instrument and lot. Therefore, it is recommended to establish individual ranges and cut-offs for each laboratory and each instrument (if necessary also for each lot) by testing series of known genotyped patient plasmas.

## EXPECTED VALUES

Typical ratio ranges for PCR-genotyped patient plasmas on different devices are shown in the table below.

| KC-4/-10 A™ micro | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 99 | ≥ 3.0 |
| heterozygous | 166 | 1.3–1.9 |
| homozygous | 25 | 0.9–1.1 |

| BCS® (Siemens Coagulation System) | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 143 | ≥ 3.0 |
| heterozygous | 170 | 1.4–2.2 |
| homozygous | 27 | 0.9–1.1 |

| CA-1500 / CA-7000 | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 235 | ≥ 3.0 |
| heterozygous | 56 | 1.5–1.8 |
| homozygous | 2 | 1.0–1.1 |

| ACL 9000™ | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 127 | ≥ 3.0 |
| heterozygous | 119 | 1.4–2.1 |
| homozygous | 24 | 0.9–1.2 |

| STA® C | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 134 | ≥ 2.9 |
| heterozygous | 83 | 1.3–1.8 |
| homozygous | 27 | 0.9–1.1 |

| AMAX CS-190 | | |
|---|---|---|
| Genotype FV:Q506 | n | Ratio range (min/max) |
| negative | 74 | ≥ 3.0 |
| heterozygous | 62 | 1.2–2.3 |
| homozygous | 10 | 0.9–1.1 |

When using these tables, following restrictions should be considered:

1. These are examples and no reference ranges or cut-offs guaranteed by the manufacturer.
2. Certain interference factors (refer to "Limitations and Interferences") may cause ratio values which cannot clearly be attributed to a particular genotype, or may lead to clotting times that exceed the maximum admitted detection time of the instrument. In these cases, further investigation by PCR and the determination of individual factors are absolutely essential.

## SENSITIVITY AND SPECIFICITY

With the samples tested so far ACTICLOT® Protein C Resistance assay provided 100% sensitivity and 100% specificity for carriers of heterozygous and homozygous FV:Q506 mutation as determined by BCS® (n=340), KC-4/-10 A™ micro (n=290), CA-1500/CA-7000 (n=293), ACL 9000™ (n=270), STA® C (n=244) and AMAX CS-190 (n=146).
Due to the functional detection technique the assay is supposed to detect other FV mutations leading to APC-R phenotype as well. However their prevalence is very low compared to the FV Leiden mutation.

## ACCURACY AND REPRODUCIBILITY

With 2 genotyped plasma samples (neg/het) a series of 25 measurements were taken on the same day on 2 different fully automated analytical systems (BCS®, CA-500). Correlation of variance (CV) was determined based on the ratio. For both instruments and plasma genotypes the CV was below 5%.

3

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## IMUCLONE® Free Protein S ELISA

**REF 842**

**For Research Use Only**

### INTENDED USE

The IMUCLONE® Free Protein S is a an enzyme-linked immunosorbent assay for measuring human Free Protein S (the activated Protein C cofactor) in plasma or any biological fluid where Free Protein S may be present. The ELISA is intended For Research Use Only.

### EXPLANATION OF THE TEST

Protein S is an 80,000 D molecular ratio, vitamin K dependent glycoprotein synthesized in the liver. The concentration of Protein S in normal human plasma is approximately 25 µg/mL[1] and is found in two forms: Free Protein S comprises approximately 40% (10 µg/mL) of the total amount while approximately 60% (15 µg/mL) circulates in blood as a non-covalent complex with C4b Binding Protein (C4b-BP). Only the Free Protein S possesses anticoagulant activity as the cofactor of Activated Protein C. The balance between the Free and C4b-BP complexed forms of Protein S plays an important role as only the Free Protein S is active.

### PRINCIPLE OF THE METHOD

A calcium-dependent monoclonal antibody specific for Free Protein S coupled to horse radish peroxidase (HRP) is added to a microwell coated with another calcium-dependent monoclonal antibody specific for Free Protein S. Next, a diluted plasma sample or biological fluid is immediately added to the microwell and the immunological reaction begins. If present, Free Protein S binds onto the monoclonal antibody coated solid phase via one epitope and binds to the second monoclonal antibody coupled to HRP via a second epitope. Following a wash step, the peroxidase substrate, 3,3',5,5'–Tetramethylbenzidine (TMB), in the presence of hydrogen peroxide ($H_2O_2$), is added to the microwell and the subsequent enzymatic reaction yields a blue colored solution. The addition of sulfuric acid stops the reaction and turns the solution color to yellow. The amount of color is directly proportional to the concentration of human Free Protein S in the tested sample.

### REAGENTS

96 Microwell Plate pre-coated with anti-(h) Free Protein S; 12 strips, 8 wells/strip in a frame holder plus storage bag with desiccant
2 vials of Protein S Sample Diluent (contains calcium), ready to use (50 mL)
3 vials of Plasma Protein S Calibrator, 1:50 prediluted (lyophilized)
1 vial of Protein S Control I, High (lyophilized)
1 vial of Protein S Control II, Low (lyophilized)
3 vials of Anti-(h)-Free PS-HRP Conjugate (lyophilized)
1 vial of Protein S Conjugate Diluent, ready to use (15 mL)
1 vial of Protein S Wash Solution, 20 fold concentrate (50 mL)
1 vial of TMB Substrate (Peroxidase Substrate), ready to use (25 mL)
1 vial of Stop Solution, 0.45 M $H_2SO_4$ (6 mL)

### WARNINGS AND PRECAUTIONS

Source material for some of the reagents in this kit is of human origin. This material has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2). As no known test method provides complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, reagents should be handled as recommended for any potentially infectious human specimen. Discard all waste associated with test specimens and human source reagents in a biohazard waste container.

Limited for research use only in the United States. For *in vitro* use only. Not for internal use in humans or animals. Do not use the kit components beyond the stated expiration date. Do not mix reagents from different kits. Avoid microbial contamination of the reagents. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled. Do not pipette reagents by mouth. Wear laboratory coat and disposable gloves throughout the test procedure and wash hands thoroughly afterwards. Avoid splashing or aerosol formation.

| Stop Solution | Warning | CONT: Sulfuric acid<br>H315, H319, P264, P280, P302 + P352, P305 + P351 + P338, P332 + P313, P337 + P313 |
|---|---|---|

**Hazard Statements:**
H315 Causes skin irritation.
H319 Causes serious eye irritation

**Precautionary Statements**
P264 Wash thoroughly after handling.
P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
P302 + P352 IF ON SKIN: Wash with plenty of water.
P305 + P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P332 + P313 If skin irritation persists: Get medical advice/attention.
P337 + P313 If eye irritation persists: Get medical advice/attention.

### REAGENT PREPARATION AND STORAGE

Unopened and lyophilized reagents are stable until the expiration date printed on the box when properly stored at 2°-8°C. Allow the reagents to warm to room temperature for 30 minutes before use.

1. **Microwell Strips pre-coated with anti-(h) Free Protein S:** Once removed from the aluminium pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present, protected from any moisture, and stored in the provided storage bag.
2. **Protein S Sample Diluent:** Supplied ready to use, once opened, the diluent may be used for up to 4 weeks when stored at 2°-8°C. The Diluent contains 0.05% Kathon CG.
3. **Plasma Protein S Calibrator:** Reconstitute each vial with 2.0 mL of Protein S Sample Diluent. This calibrator is equivalent to normal plasma containing at a 1:50 dilution. Reconstituted calibrator is stable for at least 8 hours at room temperature (18°-25°C).
4. **Protein S Control I:** Reconstitute this vial 0.5 mL of distilled water. This control is a high plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at –20°C providing bacterial contamination is avoided.
5. **Protein S Control II:** Reconstitute this vial 0.5 mL of distilled water. This control is a low plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at –20°C providing bacterial contamination is avoided.
6. **Anti-(h)-Free PS-HRP Conjugate:** Reconstitute each vial with 4.0 mL of Protein S Conjugate Diluent. Shake the vial gently to homogenize the content. Reconstituted immunoconjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2°-8°C.
7. **Protein S Conjugate Diluent:** Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C. This conjugate diluent contains 0.05% Kathon CG.
8. **Protein S Wash Solution:** If solids are present, incubate the vial for 15-30 minutes in a 37°C water bath. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL is sufficient to prepare 1 Liter of Wash Solution). The Wash Solution may be used for up to 4 weeks after opening when stored at 2°-8°C in its original vial. Diluted Wash Solution may be used for up to 7 days when stored at 2°-8°C. This wash solution contains 0.05% Kathon CG and calcium and must be used for this Free Protein S ELISA.
9. **TMB Substrate (Peroxidase Substrate):** Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C.
10. **Stop Solution (0.45 M $H_2SO_4$):** Supplied ready to use. *Caution:* Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

### SPECIMEN COLLECTION AND PREPARATION

Citrate collected platelet poor plasma or serum may be used for this assay. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 2,500 x g for 20 minutes.

(...over)

3. Plasma should be stored at 2°-8°C and assayed within 4 hours. Alternatively, plasma may be stored at –20°C for up to 6 months.
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at 2°-8°C and assayed within 4 hours.

Test samples and the Protein S Controls must be diluted 1:50 in the Protein S Sample Diluent. For expected Protein S levels >100%, samples must be assayed at higher dilutions, 1:100 or greater. For Protein S <10%, the sample may be assayed at a lower dilution, less than 1:50.

## PROCEDURE

**Materials Provided** – See Reagents

**Materials Required But Not Provided**

Distilled water
50-300 μL eight channel multi-pipette
0-200 μL, 200-1000 μL single pipettes
Microwell plate reader for reading absorbance at 450 nm
Microwell plate shaker, Microwell plate washer (optional)

**Preparation of the Free Protein S Concentration Levels**

Free Protein S concentrations are expressed as a % of pooled normal plasma. The 100% concentration corresponds to a pooled normal human plasma diluted 1:50, the standard assay dilution. Using the Plasma Protein S Calibrator provided, with a Free Protein S concentration "C" as indicated on the flyer provided in the kit, prepare the following calibrator concentrations.

| Free Protein S Concentration (%) | C | C/2 | C/4 | C/10 | C/20 | 0 |
|---|---|---|---|---|---|---|
| Vol. of Plasma Protein S Calibrator | 1.0 mL | 0.5 mL | 0.25 mL | 0.10 mL | 0.05 mL | 0 mL |
| Vol. of Protein S Sample Diluent | 0 mL | 0.5 mL | 0.75 mL | 0.90 mL | 0.95 mL | 1.0 mL |

Mix gently for a complete homogenization. The dilutions of calibrator are stable for at least 4 hours at room temperature (18°-25°C).

**Assay Procedure**

Remove the required number of microwell strips from the aluminium pouch sufficient for the number of assays to be performed. Place the strips in the frame provided. To the appropriate microwells, add the reagents and perform the various assay steps as indicated on the following table:

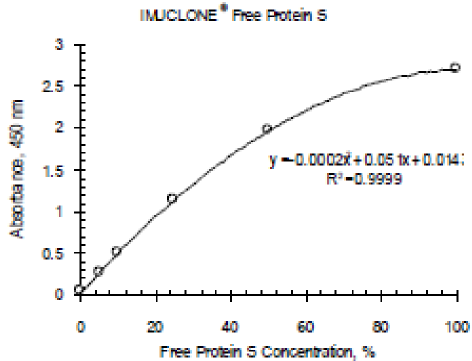
| Reagent | Volume | Procedure |
|---|---|---|
| Anti-(h)-Free PS-HRP Conjugate | 100 µL | Add the anti-human Free Protein S-HRP Conjugate to each microwell. |
| Free Protein S concentrations, diluted Controls, diluted sample or Protein S Sample Diluent (blank) | 100 µL | Immediately add the Free Protein S calibrator concentrations or diluted controls or diluted sample to the appropriate microwell. |
| Incubate for 1 hour at room temperature (18°-25°C) while gently shaking either manually or using an orbital microwell plate shaker. | | |
| Protein S Wash Solution | 300 µL | Wash the wells 5 times. |
| TMB Substrate | 200 µL | Add the substrate to each microwell immediately after the wash step. |
| Incubate for exactly 5 minutes at room temperature (18-25°C). | | |
| Stop Solution (0.45 M $H_2SO_4$) | 50 µL | Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 0.45 M $H_2SO_4$ |
| Wait for 10 minutes in order to allow the color to stabilize and measure the absorbance at 450 nm. Subtract the blank value from the measurements. | | |

**Notes:**

1. Avoid letting the plate in the bright sunlight during incubations and particularly during color development.
2. Do not allow the microwells to dry out between the addition of reagents or following a washing step. Add the next reagent within 3 minutes in order to prevent the microwells from drying, which could damage the immobilized components. If necessary, fill the microwells with prepared Wash Solution and empty it just before the introduction of the next reagent.
3. When adding the TMB Substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

## RESULTS

Construct a calibration curve by plotting the mean absorbance value for each Free Protein S concentration (ordinate) versus its corresponding concentration in % (abscissa). A calibration curve should be generated each time the assay is performed. The following calibration curve is for demonstration purposes only.




## CALCULATIONS

From the calibration curve generated, directly deduce the Free Protein S concentration in assayed samples at the standard 1:50 dilution. If higher dilutions are used, the Free Protein S concentration must be multiplied by the complementary dilution factor D (i.e. multiply the concentration by 2 for a 1:100 sample dilution or by 4 for a 1:200 sample dilution). If lower dilutions are used, the concentration obtained must be divided by 50:D. Alternatively, an ELISA software (i.e. Dynex, etc.) can be used for the calculation of concentrations.

## LIMITATIONS OF THE PROCEDURE

As the monoclonal antibodies used in the ELISA are calcium dependent, only the wash buffer supplied, which contains calcium, may be used in the assay. If the wash steps are not correctly performed, samples can produce a high absorbance value. In order to avoid non-specific color development, check that the wash steps are performed efficiently.

## EXPECTED VALUES

The Free Protein S concentration in normal human plasma is usually in the range 60–150%, therefore the abnormal range is < 60%. The concentration is higher in males than in females and tends to increase with age and blood lipid concentration.

## PERFORMANCE CHARACTERISTICS

The IMUCLONE® Free Protein S ELISA is specific for the native and functional forms of Free Protein S. The ELISA is not reactive with Protein S-C4b-BP complexes.

## REFERENCES


1. Henkens, C. A. A., *et al.* Plasma Levels of Protein S, Protein C, and Factor X: Effects of Sex, Hormonal State and Age. *Thromb. Haemost.* 1995, **74**: 1271-1275.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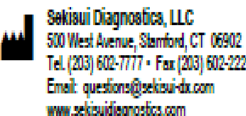
Appendex 4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# ACTICHROME® AT III

REF 838

*Chromogenic assay for measuring anti-thrombin III activity in human plasma*

## INTENDED USE

ACTICHROME® AT III is intended for the quantitative determination of antithrombin III in human plasma by chromogenic assay. The assay is intended for in vitro diagnostic use.

## EXPLANATION OF THE TEST

Antithrombin III is an inhibitor of plasma serine proteases. An important function of antithrombin III is the inhibition of thrombin activity. Normally the rate of thrombin inhibition by antithrombin III is slow (progressive antithrombin activity). However, the rate of inhibition can be enhanced several thousand- fold in the presence of heparin (heparin cofactor activity).

Tolefsen and Blank have reported another rapid heparin-dependent thrombin inhibitor, Heparin Cofactor II, in human plasma. This protein can interfere with antithrombin III determinations especially at high (2 USP units/mL) heparin concentrations. In order to confer specificity to antithrombin III the present assay system uses a lower (1.0 USP units/mL) final heparin concentration where heparin-enhanced inactivation of thrombin by heparin cofactor II is negligible. In addition, human heparin cofactor II reacts more readily with human thrombin than with bovine thrombin (Friberger et al.). Thus, further specificity for antithrombin III is imparted in the present assay system by the u se of bovine thrombin.

## PRINCIPLE OF THE METHOD

In the present two-stage method (Odegard, et al.), thrombin is added to a plasma dilution containing antithrombin III in the presence of excess heparin. After an initial incubation (stage 1) residual thrombin is determined with a thrombin-specific chromogenic substrate (stage 2). The residual thrombin activity is inversely proportional to the antithrombin III concentration of the plasma.

## REAGENTS

The kit contains sufficient reagents to perform 60 tests using semi-micro methodology.

R1 **Bovine Thrombin:** 6 vials (lyophilized).

R2 **SPECTROZYME® TH:** 6 vials each containing 1.8 µmoles thrombin substrate (lyophilized).

R3 **Assay Buffer:** 6 vials each containing 5 mL of buffer, 10-fold concentrate. Working strength buffer contains 50 mM Tris-HCl, 175 mM NaCl, 7.5 mM Na-EDTA and 1.0 USP units/mL sodium heparin, pH 8.4.

## WARNINGS AND PRECAUTIONS

Do not use kit components beyond the expiration date. Do not mix reagents from different kit lots. Avoid microbial contamination of the kit components. Do not mouth pipette or ingest reagents.

| | | | |
|---|---|---|---|
| Bovine Thrombin | Warning | (!) | H315, H319; P264, P280, P302 + P352, P305 + P351 + P338, P337 + P313 |
| SPECTROZYME® TH | Warning | (!) | CONT: H-D-cyclohexylalanyl-alanyl-arginine-para-nitroanilide diacetate salt<br>H315, H319, H335, P261, P264, P280, P302 + P352, P305 + P351 + P338, P337 + P313 |
| Assay Buffer | - | - | Observe good laboratory hygiene practices. |

**Hazard Statements:**
H315 Causes skin irritation.
H319 Causes serious eye irritation.
H335 May cause respiratory irritation.

**Precautionary Statements:**
P261 Avoid breathing dust.
P264 Wash thoroughly after handling.
P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
P302 + P352 IF ON SKIN: Wash with plenty of water.
P305 + P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical advice/attention.

## REAGENT PREPARATION AND STORAGE

Intact vials of reagents are stable until the label expiry date when stored at 2° - 8°C.

R1 **Bovine Thrombin:** Reconstitute with 2 mL of filtered deionized water. Reconstituted reagent is stable for 1 week at 2-8°C and for up to one month at -20°C

R2 **SPECTROZYME TH:** Reconstitute with 2 mL of purified water filtered deionized water. Reconstituted reagent is stable for 1 week at room temperature, 2 months at 2° - 8°C and 6 months at -20°C (aliquot and freeze).

R3 **Assay Buffer:** Dilute to 50 mL with filtered deionized water purified water. Working strength buffer is stable for 1 week at room temperature and for 1 month at 2° - 8°C.

## SPECIMEN COLLECTION AND PREPARATION

Citrate collected platelet poor plasma may be used for this assay. See "Collection, Transport and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays-Approved Guideline", CLSI Document H21-A5, Vol. 28, No. 5, January 2008. Plasma collection should be performed as follows:



1. Collect 9 parts of blood into 1 part of 3.2% (0.109 M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 1,500 x g for 15 minutes.
3. Plasma should be stored at 2° - 8°C and assayed within 2 hours. Alternatively, plasma may be stored at –20°C for up to 1 month.
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at 2° - 8°C and assayed within 24 hours.

## PROCEDURE

**Materials Provided – See Reagents**

**Materials Required But Not Provided**

filtered deionized $H_2O$
0-200 µL, 200-1000 µL single pipettes
Plastic test tubes
Laboratory timer
37°C wet or dry bath
50% glacial acetic acid
Spectrophotometer operable at 405 nm
Pooled normal plasma or commercial reference plasma

**Assay Calibration**

Pooled normal human plasma (at least 10 normal donors), which has been collected in the same way as plasmas to be tested, may be used for preparation of the antithrombin III standards. Since oral contraceptives and other estrogen/progesterone preparations may affect antithrombin III levels, plasma from users of such preparations should be excluded from the pool. Commercially prepared plasma standard in which antithrombin III has been determined may also be used.

Prepare plasma antithrombin III standards and unknown plasma samples as follows:

| Standard | Volume of Pooled Normal Plasma | Volume of Assay Buffer |
|---|---|---|
| 100% | 25 µL | 1000 µL |
| 50% | 500 µL of 100% Standard | 500 µL |
| 0% | 0 µL | 1000 µL |
| Unknowns | 25 µL Plasma Specimen | 1000 µL |

### Assay Procedure – Endpoint Method

#### Endpoint Method

1. Add 200 µL of standard or unknown plasma to a plastic tube.
2. Incubate at 37ºC for 2-4 minutes.
3. Add 200 µL of Bovine Thrombin.
4. Mix and incubate at 37ºC for 1 minute.
5. Add 200 µL of SPECTROZYME TH.
6. Mix and incubate at 37ºC for 1 minute.
7. Add 200 µL of 50% glacial acetic acid.
8. Mix
9. Add 200 µL of water* (optional).

Read the absorbance at 405 nm in a 1 cm semi-microcuvette against a blank prepared in the following order:

200 µL acetic acid
200 µL standard dilution
200 µL Bovine Thrombin
200 µL SPECTROZYME TH
200 µL water* (optional)

(*Some spectrophotometers require a minimum of 1 mL volume in the cuvette.)

Multiple Simultaneous Determinations - As many as ten determinations can be performed simultaneously with the same stopwatch by staggering pipetting steps at five second intervals.

### Assay Procedure - Kinetic Method

A kinetic analyzer may be used to measure the initial rate of hydrolysis of the chromogenic substrate. The procedure to be used is as follows:

1. Add 5 µL of standard or unknown plasma to 200 µL of Dilution Buffer.
2. Incubate at 37ºC for 2-4 minutes.
3. Add 200 µL of Bovine Thrombin.
4. Mix and incubate at 37 ºC for 1 minute.
5. Add 200 µL of SPECTROZYME TH.
6. Measure rate of change of absorbance at 405 nm.

## RESULTS

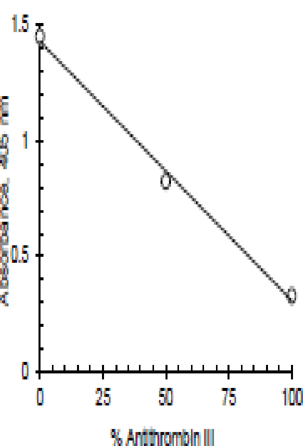
### Representative Standard Curve

Plot the absorbance obtained for each antithrombin III standard against the percent of antithrombin III on linear graph paper. Interpolate the antithrombin III level of the unknown plasma sample from the calibration curve. If a commercial antithrombin III reference standard was used, adjust the antithrombin III value determined for the unknown plasma sample as follows:

% AT III (adjusted) = % AT III (unknown plasma) x % AT III (reference)/100



The calibration curve shown below is for example only. A new calibration curve must be constructed each time the assay is performed.




## QUALITY CONTROL

Commercial antithrombin III reference plasma may be used for quality control of the assay. If commercial antithrombin III control plasma has been used to construct the calibration curve for the assay, then a different lot of control plasma should be used for quality control.

## LIMITATIONS OF THE PROCEDURE

Icteric, lipemic and hemolyzed samples may interfere with the assay. If the unknown plasma is very icteric, a second blank containing the unknown plasma dilution instead of the standard dilution should be prepared and its absorbance subtracted from the absorbance obtained for the unknown antithrombin III determination

## EXPECTED VALUES

The normal range of ATIII in plasma is 75%-125%. Activity levels of 30-60% may be observed in patients with hereditary ATIII deficiency. Several clinical conditions associated with acquired ATIII deficiency include liver disease, DIC, nephrotic syndrome, pulmonary embolism, stroke and thrombophlebitis. In addition, oral contraceptive use may reduce ATIII levels.

## PERFORMANCE CHARACTERISTICS

### Accuracy

In clinical studies comparing ACTICHROME ATIII to several other commercially available chromogenic antithrombin III kits the following correlation was observed:

% ATIII (other assays) = 0.93 % ATIII (ACTICHROME) + 5.9 (n=53, r = 0.80)