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College of Graduate Studies

Effects of Oral Contraceptive Pills Intake on Factor V Leiden Gene Mutation among Sudanese Women

تأثير تناول حبوب منع الحمل الفموية على الطفرة الجينية لعامل التجلط الخامس (ليدن) لدى النساء السودانيات

Dissertation Submitted in Partial Fulfillment of Requirments for the award of the M.Sc. Degree in Hematology and Immune hematology

Submitted by:

Hiba Abdelmalik Eldow MohamedAhmed

(B.Sc.in H. Hematology and Immunohematology, SUST, 2015)

Supervisor:

Dr. Kawthar Abdelgaleil Mohammed Salih Ibrahim

Assoc.prof

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Approval Page

(To be completed after the college council approval)

Name of Candidate: Hiba | Abdelmalik | ELdaw | Mohammed Ahmed

Thesis title: Effect of Oral Contraceptive pills Intake on Factor V Leiden Gene Mutation among Sudanese women

Degree Examined for: Master

Approved by:

1. External Examiner

Name: Mubarak Sayeed ElKarsani

Signature: ..... Date: 10-03-2019

2. Internal Examiner

Name: Mansour Mohammed Mansour

Signature: ..... Date: 10-03-2019

3. Supervisor

Name: Kawthar Abdelgaleil Mohammedsaleh

Signature: ..... Date: 10-03-2019

بسم الله الرحمن الرحيم

الايـــــــــة

قالَ تَعَالَى :

(قُلْ لَوْ كَانَ الْبَحْرُ مِدَادًا لِكَلِمَاتِ رَبِّي لَنَفِدَ الْبَحْرُ قَبْلَ أَنْ تَنْفَدَ كَلِمَاتُ رَبِّي وَلَوْ جِئْنَا بِمِثْلِهِ مَدَدًا)

صدق الله العظيم

سوره الكهف (الايه رقم109)

Dedication

To…my lovely mother and father

To …my dears sisters

To…my darling Entasar and nora

To… all my family

To…my special friends, teacher and all colleagues who support

Me… idedicated this work….

Hiba…

Acknowledgments

Great thanks firstly and finally to Allah who gave me willing to accomplish this work.

I would like to thank my wonderful teacher Dr.Kawthar Abdelgaleil for her advice and encouragement to conduct this study.

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Great thank for everyone who help me to complet this study.

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Abstract

This case control study was conducted in Khartoum state – Sudan during the period from April to November 2018. The study aimed to study effect of oral contraceptive pill on factor V leiden.

The Study included 50 women who use oral contraceptive as cases and 50 women don't use oral contraceptive pill as control, all subject were informed verbally about the study and approved for participation. From each participant 3 ml of venous blood collected in EDTA container then sample prepared for PCR (Eppendorf, master cycler) to analyze factor V Leiden gene mutation in exon 10 by using specific primer, the wild-type primer is 5' GGACAAAATACCT GTATTCCTC'3 the mutant primer is 5' GGACAAAATAC CTGTATTCCTT'3 and the common primer is 5'CTTTCA GGCAGGAACAACACC'3.

Result analyzed by SPSS (version 16) computerized program.

The result obtained from cases show that mean of age is30±5.5and it divided into three group less than 20 with least frequency 4%(2/50), (20-35) was most frequancy80%(40/50) and more than 35 years has frequancy16%(8/50).

Most case use oral contraceptive pill for more than 1years with frequency 60%(30/50) with mean 2±0.8.

The most frequent oral contraceptive pill use was levonorgestrel 88%(44/50) followed by desogestrel12%(6/50).

The result show no gene mutation with no significant different between user and non-user oral contraceptive pill.

Also there no significant association was found between mutation and demographic data as well as mean of age between user and non-user oral contraceptive pill *P*.value (*P*=0.52).

مستخلص البحث

اجريت هذه الدراسه بطريقه حاله المقارنه في لايه الخرطوم خلا الفتره الزمنيه من ابريل الي نوفمبر 2018

هدفت هذه الدراسه الي دراسه تاثير تعاطي حبوب منع الحمل علي العامل الخامس من عوامل التجلط شملت الدراسه 50 امراه تستخدم حبوب منع الحمل و50 منهن لا يستخدمن حبوب منع الحمل اخطر كل المشاركين في الدراسه شفاهه عن مفهومها واخذت موافقتهم علي المشاركه. تم جمع 3مل من الدم الوريدي عامل التجلط (ثنائى امين الايثيلين رباعي حمض الاسيتيك) من كل امراه في الدراسه ثم تم تحضيره واستخدم جهاز البولييميز المتسلسل لتحليل الطفره الجينيه للعامل التجلط الخامس (لبدن) في اكسون 10 باستخدام الاشعال المحددة التمهيدي الطبيعي 5' GGACAAAATACCT GTATTCCTC'3التمهيدي المتحول5'CTTTCAGGCAGGAACAACA'3والتمهيدي المشترك 5'GGACAAAATACCTGTATTCCTT'3 . CC'3

حللت النتائج باستخدام برنامج الحزم الاحصائيه للعلوم الاجتماعيه اصدار16

وان متوسط الاعمار هو5.5±30تم الاعمار الي ثلاث فئات اقل من 20،20-35،اكثر من 35سنه. وان الفئه 20-35 هي الاكثر ترددا80% (40\50) بينما الفئه اقل من 20 هي اقل ترددا 4%(2\50).

وان معظم الحالات تستخدم حبوب منع الحمل لاكثر من سنه بتردد 60%(30\50)ومتوسط2±0.8.

وان اكثر حبوب منع الحمل استخداما هي الليفونورغيستريل بتردد 88%(44\50) بينما ديزوغيسترل هي الاقل استخداما بتردد 12%(6\50).

اشارت كل النتائج علي عدم وجود طفره جينيه ولايوجد فرق ذو دلاله احصائيه بين مستخدمين وغير مستخدمين حبوب منع الحمل.

ولايوجد علاقة بين الطفره جينيه و البيانات السكانية مع العلم ان متوسط الاعمار بين مستخدمين وغير مستخدمين حبوب منع الحمل(القيمه الاحتماليه=0.52).

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List of abbreviations

Abbreviation	Full name
ADP	Adenosine Di Phosphate
ARMS	Amplification Refractory Mutation System
AT	Anti Thrombin
COCs	Combined Oral Contraceptive pills
DVT	Deep Vein Thrombosis
DNTPs	Deoxy nucleotide tri phosphate
FSAP	Factor VII-Activating Protein
GP	G-Protein
OCs	Oral Contraceptive
PAs	Plasminogen Activators
PAI-I	Plasminogen Activator Inhibitor -1
POPs	Progestin Only Contraceptive Pills
PCR	Polymerase Chain Reaction
PT	Pro thrombin
P2Y1	Pyrogenic receptor 2band cytosolic Ca
P2Y12	Pyrogenic receptor 2band cytosolic Ca12
RCLB	Red Cell Lysis Buffer
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TFPI	Tissue factor pathway inhibitor

TF	Tissue Factor
TXA2	Thromboxane A2
UPA	Urokinase type Plasminogen Activator
VTE	Venous ThromboEmbolism
VWF	Von Will Brand
WCLB	White Cell Lysis Buffer

Chapter One

Introduction and Literature Review

Chapter One
Introduction and literature review

1.1Introduction:

Oral contraceptive (OCs) also known as the pill is most popular method of contraception. The primary mechanism action is inhibition of ovulation in addition oral contraceptive produces endometrium that isn't receptive to ovum implantation and cervical mucus that became thick and hostile to sperm transport.(Hall *et al*.,2011)

There are different type of oral contraceptive:

- Combined oral contraceptive (COCs) which contain estrogen and progestin.
- Progestin only contraceptive(POPs) which contain progestin but no estrogen this pill referred as mini pill (Adamopoulou and Vgeneopoulou,2015).

Estrogen containing oral contraceptive increase plasma concentration of clotting factors II, VII, X,XII,VIII, fibrinogen and thrombin activatable fibrinolysis inhibitor by affect the gene transcription of various protein, Estrogen crosses the cell membrane for particular target tissue, inside cytoplasm bind to nuclear recrptors then Estrogen –nuclear receptor complex then travel into nucleus where it recognize and bind to specific recognition sites this binding turn on gene transcription by allowing RNA Polymerase IIto transcribe the protein in that region of DNA in this case new protein are the clotting factors (Trenor *et al*.,2011).

The Factor V is one of several substances that help blood clot which cause increase in blood clotting, the clotting action of factor V is controlled by anther protein called activated protein C dosenot work well on abnormal factor V leiden factor V and increase chance of developing blood clot in deep vein (DVT) in leg or in lung (pulmonary embolus) (Gruber and Bull,2012).

1.2 Literature review:

1.2.1Hemostasis:

Hemostasis is a process to prevent hemorrhage by arresting and keeping the blood within the damaged vessel walls. It is a complex process that is contingent on the complex interaction of platelets, plasma coagulation cascades, fibrinolytic proteins, blood vasculatures and cytokine mediators. Upon tissue injury, the hemostatic mechanism employs a plethora of vascular and extravascular receptors, in accordance with the blood components, to seal off the impairments to the vasculature and closing it off from the encircling tissues.(Vandy and Wakefield,2009)

1-2-2Type of hemostasis:

1-2-2-1Primary hemostasis:

Mechanim is vasoconstriction and platelet plug formation upon platelet adhesion and aggregation.

Vasoconstriction:

Vascular spasm occurs whenever there is an injury or damage to the blood vessels. This will trigger a vasoconstriction, which could eventually stop the blood flow. This reaction can be responded within 30 minutes, and is localized to the injured area. At this stage, exposed collagen fibers will release ATP and other inflammatory mediators to recruit macrophages. In addition, the ECM becomes highly thrombogenicity, promoting platelet adhesion and aggregation. (Garmo and Burns,2018).

Platelet plug formation:

Following vasoconstriction, exposed collagen from the damaged surface will encourage platelets to adhere, activate and aggregate to form a platelet plug, sealing off the injured area (Storti *et al*.,2014)

Platelet adhesion:

platelet adhesion mechanism is generally supported by the particular interactions between the membrane receptors and absorbed plasma proteins. platelet membrane receptors are enriched with Gp receptors embedded in the phospholipid bilayer, including tyrosine kinase receptors, integrins, leucine rich receptors; G-protein coupled transmembrane receptors, selectins and immunoglobulin domain receptors. These are the important proteins involved to facilitate

hemostatic function by mediating the interactions within cell-platelet and platelet substrates. The initial event that occurs upon hemostasis is the rolling and adherence of the platelets to the exposed subendothelium. Platelet adhesion is mediated by von Willebrand Factor (vWF) that binds to Gp Ib-IX in the platelet membrane. vWF is a blood Gp that serves as an adhesive protein, which could bind to other proteins, especially factor VIII at the wound sites.(Ruggeri and Mendolicchio,2007)

Platelet activation:

A variety of stimuli can activate platelets. Platelet cells can also be activated through biomaterial surface stimulation. Adhered platelets undergo degranulation and release cytoplasmic granules that contain serotonin, platelet activating factors and ADP. ADP is an important physiological agonist stored in the dense bodies of platelets that play an essential function in normal hemostasis and thrombosis. Platelets are activated to change shapes into a pseudopodal form upon the adhesion to the injured area which will activate the collagen receptors on their surface membrane, named GpIIbIIIa, to undergo release reactions. The GpIIbIIIa complex, organized through calcium-dependent association of GpIIb and GpIIIa that is a necessary step in platelet aggregation and endothelial adherence. At the same time, platelets tend to synthesize and discharge thromboxane A2 (TXA2) increase vasoconstriction and platelet aggregation. In addition, GpIIbIIIa integrins and P-selectin move from the α-granule membrane to the platelet membrane to support platelet aggregation Additionally, these are the receptors that could act as the catalytic surface and facilitate the hemostasis process(Yun *et al*.,2016).

Platelet aggregation:

Platelet aggregation begins once platelets become activated, triggering the GpIIbIIIa receptor (50100/platelets), which attach to vWF or Fibrinogen. Each activated platelet extends pseudopods, clumping and becoming aggregated. These activations are further heightened by the generation of thrombin via the hemostasis mechanism. the aggregation promotes a primary platelet plug. while ADP receptor interconnects with a family of ADP receptors (P2Y1 and P2Y12), which could be detected on platelets as helping with aggregation. P2Y1 receptors assist in stimulating the initial platelet shape changes and platelet aggregation. At the same time, P2Y12 is an important mediator for blood clotting. It increases significantly, responding

to ADP to complete the aggregation process. Eventually, the formed platelet plug stabilized by the formation of fibrin(Rumbaut andThiagarajan ,2010).

1-2-2-2Secondary hemostasis:

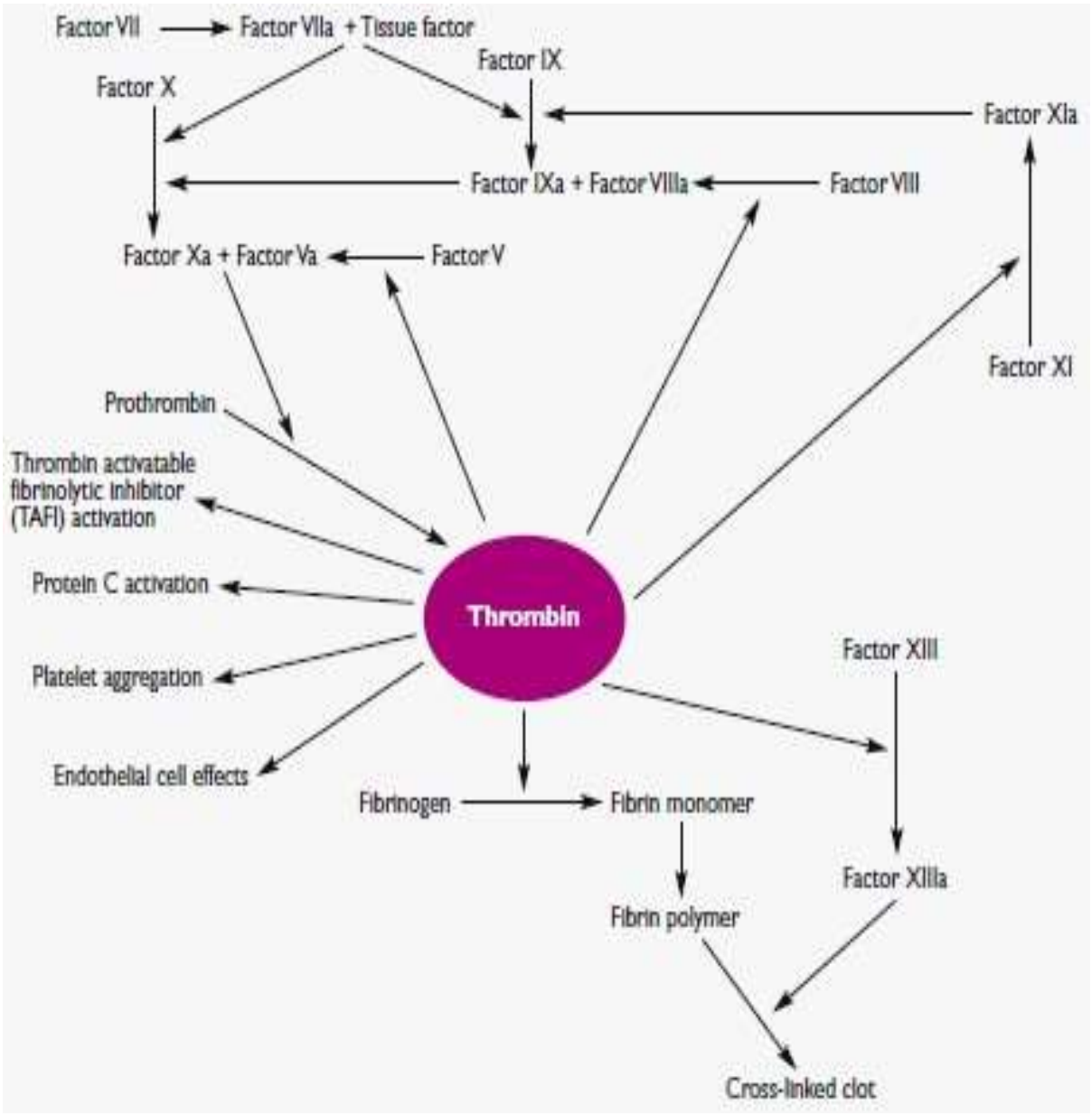

Figure A: Coagulation mechanism (Halim *et al.*,2016)

1.2.2.2.1 Intrinsic Pathway

This pathway is the longer pathway of secondary hemostasis, It begins with the activation of Factor XII (a zymogen, inactivated serine protease) which becomes Factor XIIA (activated serine protease) after exposure to endothelial collagen, Endothelial collagen is only exposed when endothelial damage occurs. Factor XIIA acts as a catalyst to activate factor XI to Factor XIAthen goes on to activate factor IX to factor IXA goes on to serve as a catalyst for turning factor X into factor Xa This is known as a cascade. When each factor is activated, it goes on to activate many more factors in the next steps When factor II is activated by either intrinsic or extrinsic pathway, it can reinforce the intrinsic pathway by giving positive feedback to factors V, VII, VIII, XI, XIII.(Chaudhry and Babiker,2017).

1.2.2.2.2Extrinsic Pathway

is the shorter pathway of secondary hemostasis Once the damage to the vessel is done, the endothelial cells release tissue factor which goes on to activate factor VII to factor VIIa then goes on to activate factor X into factor Xa This is the point where both extrinsic and intrinsic pathways become one. (Barmore and Burns , 2018).

1.2.2.2.3Common Pathway

This pathway begins at factor X which is activated to factor Xa The process of activating factor Xa is a complicated reaction Tenase is the complex that cleaves factor X into factor Xa, Tenase has two forms: extrinsic, consisting of factor VII, factor III (tissue factor) and Ca2+, or intrinsic, made up of cofactor factor VIII, factor IXA, a phospholipid, and Ca2+,Once activated to factor Xa, it goes on to activate factor II (prothrombin) into factor IIa (thrombin) Also, factor Xa requires factor V as a cofactor to cleave prothrombin into thrombin. Factor IIa (thrombin) goes on to activate fibrinogen into fibrin, Thrombin also goes on to activate other factors in the intrinsic pathway (factor XI) as well as cofactors V and VIII and factor XIII then Fibrin subunits come together to form fibrin strands, and factor XIII acts on fibrin strands to form a fibrin mesh This mesh helps to stabilize the platelet plug. (Palta *et al*.,2014).

1.2.2.3. fibrinolytic system:

functions to remove the clot after the vasculature is repaired, as well as to degrade clots that form in the bloodstream, The final step in this pathway is the plasmin-mediated cleavage of fibrin, creating fibrin degradation products, Plasmin is produced from plasminogen by plasminogen activators (PAs): urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), The latter is produced primarily in endothelial cells and secreted in response to a variety of stimuli, including molecules such as thrombin and histamine, as well as shear stress and venous occlusion, tPA is considered the major intravascular activator of plasminogen, Plasmin cleaves both PAs from single-chain to two-chain polypeptides, which increases their activity. Fibrin has a self-limiting property of localizing plasminogen and tPA on its surface, which markedly increases the ability of tPA to convert plasminogen to plasmin(Rogers *et al.*,2018). Cleavage of fibrin exposes lysine residues to which plasminogen and tPA can also bind and The fibrinolytic system is also regulated by inhibition of PAs by plasminogen activator inhibitors (most commonly PAI-1), produced by a variety of tissues, and direct inhibition of plasmin by serpins, such as α_2-plasmin inhibitor, which is present in platelet α-granules (Fedan,2007).

1.2.2.4Phase of hemostasis:

1-2-2-4-1Initiation:

It occurs by expression of TF in damaged vessel which binds factor VIIa to activate factor IX and factor X. This activation of factor IX by TF-VIIa complex serves as the bridge between classical extrinsic and intrinsic pathways. Factor Xa then binds to factor II to form thrombin (factor IIa). Thrombin generation through this reaction is not robust and can be effectively terminated by TF pathway inhibitor (Thomas *et al*.,2016).

1-2-2-4-2Amplification:

Since the amount of thrombin generated is not sufficient, therefore numerous positive feedback loops are present that bind thrombin with platelets. Thrombin that is generated in the initiation phase further activates factor V and factor VIII, which serves as a cofactor in prothrombinase complex and accelerates the activation of Factor II by FXa and of FXa by FIXa, when enzyme complexes accumulated (tenase complex and prothrombinase complex) on platelet surface support robust amounts of thrombin generation and platelet activation This ensures continuous generation of thrombin and subsequently fibrin to form a sufficiently large clot this called propagation phase (Versteeg *et al*.,2013).

1-2-2-4-3Stabilization:

Thrombin generation leads to activation of factor XIII (fibrin stabilizing factor) which covalently links fibrin polymers and provides strength and stability to fibrin incorporated in platelet plug. In addition, thrombin activates fibrinolysis inhibitor(TAFI)that protects the clot from fibrinolysis (Versteeg *et al*.,2013).

1.2.3Factor V:

Factor V (F V), which was discovered by Paul Owren in 1943, has proved to be an important regulator of the hemostatic balance with both procoagulant and anticoagulant properties, it is located on 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 mF V is activated by F Xa or thrombin to FVa by the cleavage of three peptide bonds (Arg709, Arg1018, Arg1545). The inactivation of F Va is mediated through APC, which cleaves the FVa at the sites Arg506, Arg306, and Arg679(Hiltunen and Rautanen,2011).

1-2-4FactorV leiden:

The factor V Leiden R506Q mutation (G1691A) occurs in 8% of the population with a specific G→A substitution at nucleotide 1691 in the gene for factor V. The defective protein product is cleaved less efficiently (10%) by activated protein C, resulting in deep vein thrombosis (DVT), recurrent miscarriages, portal vein thrombosis in cirrhotic patients, early kidney transplant loss, and other forms of venous thromboembolism (VTE) and there dramatic increase in the incidence of thrombosis is seen in women who are taking oral contraceptives, Both prothrombin G20210 and factor V Leiden mutation in the presence of major risk factors may contribute to atherothrombosis (a thrombus that forms due to rupture of an atherosclerotic plaque) Antithrombin drugs play a crucial role in the management of these thrombotic disorders(Fareed and Iqbal ,2016).

This alteration result in an inability of activated protein C to cleave factor Va promoting coagulation via continued thrombin production (Tranijr and Lawson ,2007).

factor V leiden gene present as result of gene inherited from one parent from one this is heterozoygous type body have 50% factor leiden and 50% normal factor V, if inherited 2 factor V leiden genes have the homozygous type of factor V leiden body have have 100% of factor V leiden and no normal factor V Serious illness, srgery and chronic condition such as diabetes have greater chance of developing clot also Women have greater chance of developing a blood clot when use hormones and birth control pill that increase risk of blood clots due to acquired activated protein C resistance (Gruber and Bull,2012).

1.2.5Oralcontraception pills:

Oral contraception (OCs) also known as the pill. Primary mechanism action is inhibition of ovulation in addition oral contraceptive produce an endometrium that is not receptive to ovum implantion and cervical mucus that becomes thick and hostile to sperm transport. Tubal and end endometrial motility are slowed.(Hall *et al*.,2011)

1.2.5.1Types of oral contraception pills:

There are two types of oral contraceptives:

1.2.5.1.1combined oral contraceptive pills (cocs):

which contain an estrogen and progestin and it available in 2 basic formulation Monophasic formulation in which eachactive pill contain the same dose of estrogen and progestin or multiphasic formulation can have varying amount of estrogen and /or progestin in the active pill and their Multiple different patterns of combined oral contraceptive pill use 28day cycling-most pill have 21 active hormone pill and 7 in active pill or shortened pill- free interval starting the new pack of pillon the first day of menstration usually decrease the pill –free interval thus allowing less time for new follicle to develop pill free interval should not more than 7 days or extended regimens there no biological reason to have monthly with drawal bleeding on oral contraceptive. (Szabo and Schaff ,2013).There are multiple extended regimens and there some pills that are formulated and packaged specifically for this type of extended regimen use for women who need to control the timming of their bleeding or have sever symptoms when bleeding .must be counseled as such bicycling placebo pill skipping at the end of every other pack of pill yield one period after 6 week of active pill or tri cycling- skipping the placebo pill at the end of 2out of every 3 pack of pill yield one period after 9 weeks of active pill and other extended regimens (e.g seasonale) COCs may be packed by manufactures as extended regimens. Seasonale for example has 84 active pill followed by 7inactive pill but contious the client takes only active pill daily contiously break through bleeding will occur (Nappi *et al*.,2016)

2-progestin only contraceptives (pop) which contain progestin but no estrogen this pill is often referred to as the mini pill, Its taken every day without interruption. (Adamopoulou and Vgeneopoulou,2015).

1.2.6Relation between oral contraceptive pills and factor V leiden:

Estrogen containing oral contraceptives increase the plasma concentrations of clotting factors II, VII, X, XII, factor VIII, fibrinogen, and thrombin activatable fibrinolysis inhibitor (TAFI) However, not all of the increases in clotting factors are of the same magnitude (Afsar *et al*.,2008). As it relates to the magnitude of increase in factor VII concentrations, it appears that desogestrel containing oral contraceptives confer the greatest impact when compared to a second generation oral contraceptive containing levonorgestrel(Vinogradova *et al*.,2014). There is a small decrease in factor V, which is necessary for the activation of prothrombin (II) to thrombin (IIa). While a decrease in factor V may appear to be beneficial, factor V actually works synergistically with protein S to inhibit factor VIII ,Estrogen, like many lipophilic hormones, affects the gene transcription of various proteins itis increases plasma concentrations by crosses the cell membrane for a particular target tissue, which there are many that estrogen influence, and once inside the cytoplasm binds to nuclear receptors , The estrogen/nuclear receptor complex then travels into the nucleus where it recognizes and binds to specific recognition sites, called hormone response elements or in this case, "estrogen response elements". This binding then turns on gene transcription by allowing RNA polymerase II to transcribe the protein in that region of the DNA, these new proteins are the clotting factors and proteins. (Vrtacnik *et al* .,2014).

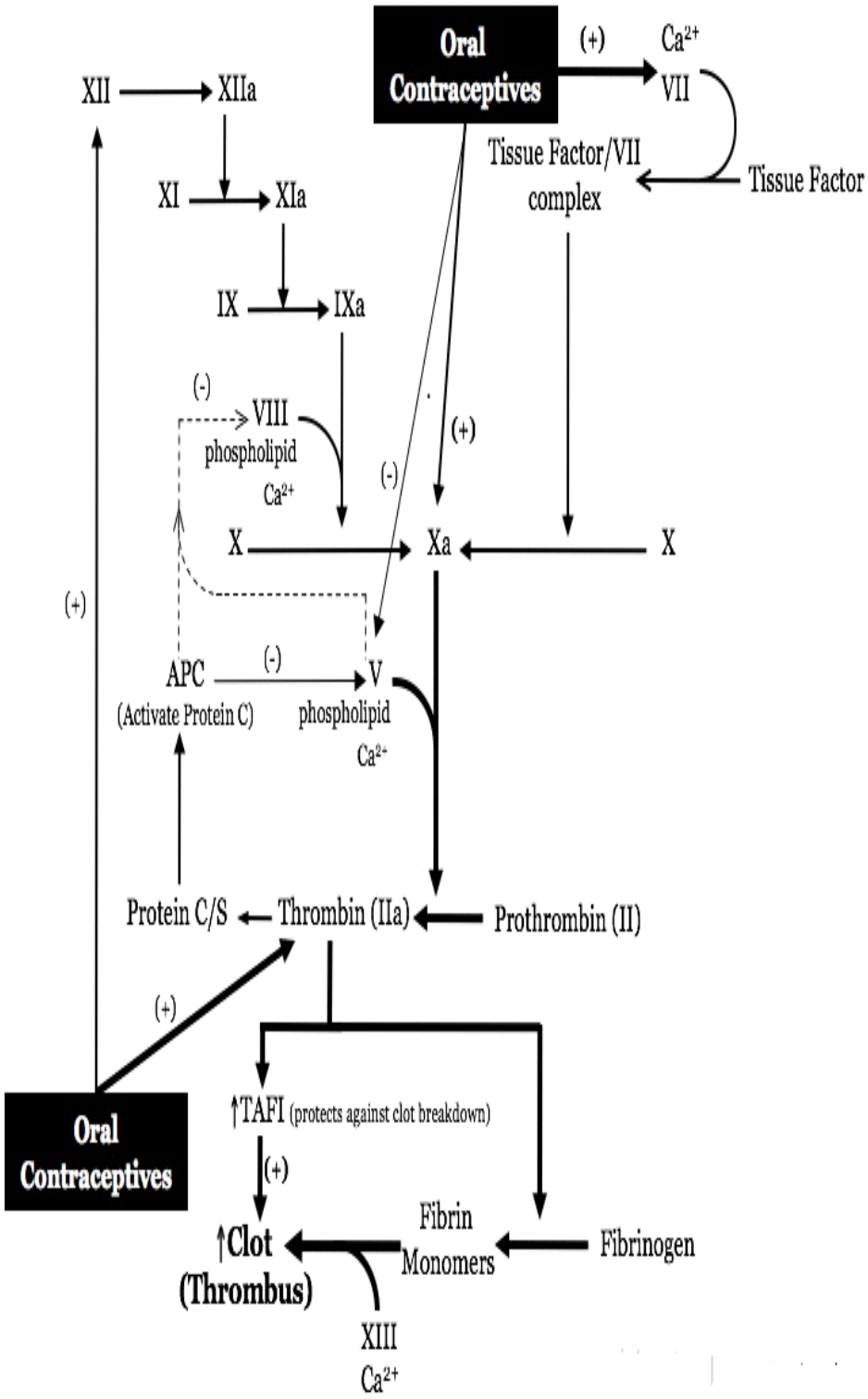

Figure B: Mechanism of oral contraceptive induce clot or thrombus formation (Evidance Base Medcine,2015)

1.3Previous studies:

In 2018 Ibrahim and other study risk factor for deep vein thrombosis of lower extremities in Sudanese women by detection of factor V leiden mutation by using multiplex polymerase and found there no mutation in all subject and risk factor are most significant affect patient were oral contraceptive. (Ibrahim *et al*., 2018)

In 2015 Zia and other study hypercoagulability in aldoscent girls on oral contraception global coagulation profile and estrogen receptor and found that oral contraceptive induced change on coagulation are complex with high inter individual variabity .striking finding was elevated FVIII level, FV was absent in all.(Zia *et al*.,2015)

In 2014 Bergendal and other study association venous thromboembolism with hormonal contraception and thrombophilic genotype and found thrombophilic genotype such factor V leiden increase risk of venous thromboembolism in user of combined hormonal contraception.(Bergendal *et al*.,2014)

In 2014 Raps and other study thyroid function, activated protein C resistance and the risk of venous thrombosis in users of hormonal contraceptive and found that use of combined hormonal contraceptive is associated with three to eight fold increase risk of venous thrombosis compare with nonuse because oral contraceptive lead to resistance to activated protein C which may be serve as marker for the risk of venous thrombosis (Raps *et al*.,2014).

Also in 2014 Tchaikovsky and other study change in hemostatic prameters during oral contraceptive and found the risk of venous thromboemblolism that depend on the oral contraceptive formulation and explained by impaired functions of protein C system and TFPI system and their increase in PAC resist (2-4)fold and decrease In plasma concentration In FV.(Tchaikovsk *et al.,* 2014)

In 2013 Kim and Kim study pulmonary embolism and deep vein thrombosis related to oral contraceptive use and found all coagulation profile are normal and FV leiden are negative and CT show pulmonary embolism and discus it by venous thromboembolism arise from acquired condition and oral contraceptive is one of acquired risk factors. (Kim and Kim , 2013)

In 2011 Piparva and Buch study deep vein thrombosis in women taking oral combined contraceptive pills and found in every 100000 women aged 15-44 years approximately 5-10 are likely to develop blood clot in year and risk increase 3-4 time in those using second generation and 6-8times in those using third generation. (Piparva and Buch , 2011)

In2010 Tchaikovsk and other study mechanism of estrogen induced venous thromboemblolism and found plasma level of coagulation factors change during oral contraceptive use. (Tchaikovsk *et al*., 2010)

1.4Rationale:-

In 2018 Ibrahim and other study risk factor for deep vein thrombosis of lower extremities in Sudanese women by detection of factor V leiden mutation by using multiplex polymerase and found there no mutation in all subject and risk factor are most significant affect patient were oral contraceptive. (Ibrahim *et al*., 2018). Venous thromboembolism poses public health threat with increased incidence it arise from acquired condition, inherited disorder, or both Acquired risk factors include obesity, cigarette smoking, hypertension, surgery, oral contraceptive (Kaunitz *et al*.,2013).Major problem cause by oral contraceptive is thrombosis and form in deep veins (leg) DVT this may lead pulmonary embolism or cerebral embolism in last may lead to death, while Factor V leiden mutation is important and most common risk factor for pulmonary embolism or DVT during oral contraceptive (Kim and Kim ,2013).

So this research is done to study effect of oral contraceptive on factor V gene to evaluate problem more objectively and implement appropriate result.

1.5Objective:

1.5.1General objective:

To study effect of oral contraceptive pills intake on factor V gene mutation.

1.5.2 Specific objectives:

To detect mutation in factor V among subjects using oral contraceptive pills by using PCR technique.

To determine possible association between thrombosis and oral contraceptive pills.

To find correlation with mutation and other risk factors (age, gravidity).

Chapter Two

Materials and methods

Chapter two

Materials and Methods

2-1 Study Design:

This was prospective case control study.

2-2 Study area and Duration:

This study was conducted at women use oral contraceptive during period from April to November 2018.

2-3Study Population:

women use oral contraceptive was taken as cases and matched group of apparently healthy women did not use contraceptive as controls after their verbal consent.

2-4 Inclusion Criteria:

Case women use oral contraceptive.

Control group only apparently Healthy women did not use any contraception

2-5 Exclusion Criteria:

Women have coagulation problem or last time exposure to bleeding or thrombosis or take vitamin affect on coagulation system**.**

2-6 Sample Size:

This study included 50 cases and 50 controls individuals.

2-7 Sampling:

Three mls of venous blood was collected from each participant (women use oral contraceptive) control using disposable sterile syringe after dis- infection the collection site with70% alcohol then the blood is dispensed in a sterile EDTA blood container.

2-8 Data Collection:

Data was be obtained by direct interviewing structured questionnaire.

2-9 Principle and Procedures:

2-9-1 Principle:

PCR involve the primer mediated enzymatic amplification of DNA. it is based on using the ability of DNA polymerase to synthesize new strand of complementary to offered tamplets and primer is needed DNA polymerase can add nucleotide only on to a preexisting 3-OH group to add the first nucleotide, DNA polymerase then elongate its 3 end by adding more nucleotides to generate an extended region of double stranded DNA. (Joshi and Deshpande,2010).

2-9-2 Test Procedure:

2-9-2-1DNAextraction:

2-9-2-1-1 Procedure:

DNA was extracted from whole blood using Guanidine hydrochloride protocol:

Four mls of red blood lysis buffer (RCLB) was de added to each sample then sample centrifuged for 5 min at 6000 rpm, the above step was repeated 2 times until a clear bellet of white blood cell appear, supernatant was be discard then 2 ml of white blood lysis buffer (WCLB), 1ml of guanidine hydrochloride, 300µ of NH_4 acetate and 10µ of proteinase K was added, Sample incubated overnight at 37C^o after overnight incubation the sample will be cooled at room temperature, and then 2ml of pre chilled chloroform will be added, after that samples will be centrifuge for 5 min at 6000rpm , upper layer will be collected to new falcon tube, 10 ml of cold absolute ethanol is added then kept at -20C^o over night , aFter overnight incubation the sample will be centrifuge for 10 min at 6000rpm, the supernant will be drained, bellet will be washed

with 4ml 70% ethanol, then will centrifuge for 10 min at 6000 rpm and supernant will be poured off and pellet will be allowed to dry pellet will be dissolved in 100µlof dd H_2O then incubated at 4Cº. (Chomczynski and sacchi, 2006).

2-9-2-2PCR reaction:

Each PCR reaction was performed in a final volume of 20 contain2.5Mm dNTPs , 2 target DNA.1(10pmol) of each primer(C, N, M) and 2.5U (5U/) Taq polymerase then samples ran in thermal cycler (appendorf master cycler).For Factor V Leiden the PCR reaction were carried out by ARMS, the wild-type primer is 5, GGACAAAATACCT GTATTCCTC3, the mutant primer is 5, GGACAAAATAC CTGTATTCCTT3, and the common primer is 5CTTTCA GGCAGGAACAACACC3. The thermal cycling conditions consisting of 5 min denaturation at 95 followed by 35 cycles of denaturation at 95 C for 30 s, annealing at 60 C for 30 s, extension at 68 C for 60 s, then the final extension step at 72 C for 5 min, kept at 4 C until use. PCR reaction was run on 2 % agarose gel for 45 min at 150 V and stained with ethidium bromide. (Dajani *et al.*,2013)

2-10 Ethical considerations:

Participants were informed verbally in their simple language about the research, its benefits and method of sample collection, then their approval taken.

2-11 Statistical analysis:

The statistical analysis of result was performed by using the Statistical Package for Social Sciences (SPSS) version 16 for interpretation of results significant difference will be determined using Chi square test less than 0.05 Mean ±STD, Median and Frequency.

Chapter Three

Results

Chapter three

3. Results

This study was carried out in Khartoum state during period from April to November 2018.

Fifty volunteers using oral contraceptive pill were enrolled in this study and matched group of apparently healthy women not use oral contraceptive or other type of contraceptive pill with mean age ± StD.

All volunteers not take any durg affect on hemostatic parameters and not have previous thrombosis or family history of bleeding or thrombosis.

Paraticipated were classified according to age in three group less than 20, (20-35) and more than 35 years. The age group of (20-35) years are most frequent, while the least one was less than 20 years (table3.1)

Table 3.1: Distribution of Age group among study subjects:

Age group	**Frequency / percent in case**	**Frequency / percent in control**
Less than20	2 /50,4%	6 /50,12%
20-35	40 /50,80%	31 /50,62%
More than 35	8 /50,16%	13 /50,26%
Total	50 /50,100%	50 /50,100%

Table3.2: Distribution of demographic data among subjects:

Variable	Frequency / percent in case	Frequency /Percent in control
Gravity		
Primy (one child)	3 /50,6%	4 /50,8%
Secondy (two child)	14 / 50,28%	19 /50,38%
Multi (three child)	14 /50,28%	19 /50,38%
Grand multi (more than three child)	19 /50,38%	8 /50,16%
Type of oral contraceptive		
Levonorgestrel	44 /50,88%	-
Desogestrel	6 /50,12%	-

Table 3.5: Mean ± StD and *p*.value of age and duration among subjects group:

Variable	Mean± StD	*p*.value
Age case	30± 5.5	0.52
Control	29±8.0	

Table 3.6: Mean ± StD Frequency and percent of duration among subjects group:

Variable	Mean± StD	Frequency / percent
Duration	2±0.8	
Less than 1	-	20 /50,40%
More than 1	-	30 /50,60%

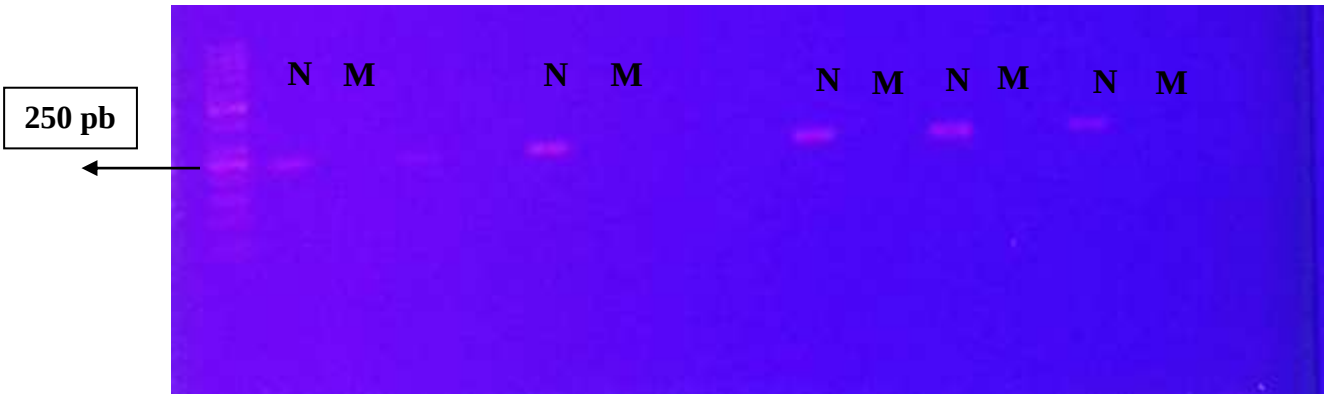

Figure 2 ARMS- PCR for detecting Factor V Leiden mutation in DNA isolated from whole blood samples of preeclamptic Sudanese women. Photograph shows ethidium bromide –stained 2% agarose gel , 50bp DNA ladder . 250 bp factor V PCR product.

N : normal ,M : mutant . the result show all sample are normal.

Chapter Four

Disscussion, Conclusion and Recommendations

Chapter Four

4.1 Discussion

The study performed to find out subsequent effect of oral contraceptive on factor V gene it is include 50 women using oral contraceptive age of participated patient with mean 30.

The factor V gene is located on chromosome 1q23 and contain 25 exons.the factor V gene defect occur in exon 10 where there is a G-A substitution at nucleotide 1691 in the present study showed that there no significant change on factor V this agree with study of Ibrahim and his worker 136 women was enrolled in the study including 75 DVT patient and 61 healthy control and found there no mutation in FV measuring by using multiplex polymerase in all subject but disagree in that most significant affect patient in age 18-45 is oral contraceptive and in this study there no significant affect in age *P.Value*=0.5243 and there no correlation with mutation and other risk factors (age , gravity).(Ibrahim *et al*.,2018).Also agree with Piparva when study deep vein thrombosis in women taking oral contraceptive and notice it in woman taking oral combined contraceptive for 3.5 months, developed DVT thrombosis of left leg, herediatory and acquired cause of DVT were excluded (there no mutation in factor V) and believe that risk of blood clot due to dose of estrogen (Piparva and Buch, 2011).

In present study duration of use oral contraceptive found most cases use oral contraceptive for more than one year this mean use of oral contraceptive for long time act as protection by decrease risk of thrombosis this agree with study of Martinelli that divide case use into 3 group short less than year, long (1-5) year, very long more than 5years and found the risk of VTE in oral contraceptive user decrease over time. (Martinelli *et al*.,2016).

The Recent study most of cases use levonorgestrel that mean this type of oral contraceptive not cause or have lower effect this agree with Vinogradova study risk of venous thromboembolism and exposure to combined oral contraceptive and found that type of progestogen hormone (drospirenone, desogestrel, gestodene and cyproterone) are associate with increased risk of venous thromboembolism when compare to pill containing older progestogen (levonorgestrel and norethisterone).(Vinogradova *et al.*, 2014).

Effect of oral contraceptive increase probality of VTE development depend on dose in medication and type of contraceptive use.(Wolski, 2014).

4.2Conclusion

In study found that oral contraceptive pills that not had effect on factor V.

There no correlation between mutation and other risk factor (age, gravity).

4.3Recommendation

Increase sample size and using advance technique

Study effect of different type oral contraceptive pills.

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Appendices

Appendix I

Questionnaire

Sudan university of science & technology

College of Graduate studies

Department of hematology

Effects of Oral Contraceptive Pills Intake on Factor V Leiden Gene Mutation in Sudanese Women

تاثير تناول حبوب منع الحمل الفمويه علي الطفره الجينيه للعامل التجلط الخامس (لبدن) في النساء السودانيات

patient ID number :……

Age…… year.

History of chronic disease?

Yes ☐ No ☐

Do you have previous bleeding or thrombosis?

Yes ☐ No ☐

If your family have history of bleeding or thrombosis?

Yes ☐ No ☐

The number of gravidity ?.....

Type of contraceptive ……..

Duration……

Appendix II

Sudan university of science & technology

College of Graduate studies

Department of hematology

Effects of Oral Contraceptive Pills Intake on Factor V Leiden Gene Mutation in Sudanese Women

تاثير تناول حبوب منع الحمل الفمويه علي الطفره الجينيه للعامل التجلط الخامس (لبدن) في النساء السودانيات

Consent form

نموذج الموافقه

تعتبر حبوب منع الحمل من اكثر الوسائل استخدامالتنظيم النسل وتاخير الحمل الا ان الدراسات اظهرت ان حبوب منع الحمل قد تضاعف خطر اصابه المرأة بالجلطات و اثبت الدراسات ان وجود طفرة جينيه في عامل التجلط الخامس يزيد من خطر الاصابه بالجلطه وهذه الدراسه تهدف لمعرفه تاثير حبوب منع الحمل علي الطفره الجينيه لعامل الخامس وهذا البحث يتطلب عينات دم بغرض اجراء بعض التحاليل التي تخص البحث اعلاه لذا نرجو كريم فضلكم بالمشاركه في هذا البحث بالموافقه على اخذ العينات بغرض البحث واتعهد بأن استخدم كل النتائج في الأغراض البحثيه فقط.

اسم المتبرع :..... الامضاء :.....

اسم الباحث :..... ت :.....

Appendix III

Material and Equipment

A. Reagents:

- DNA extraction:

Solutions:

1- Red cell lysis buffer:

NH_4CL 8.3g

$KHCO_3$ 1.0g

EDTA 5% 1.8g

Compelet to 1000 ml D.W.

2- White cell lysis buffer :

Tris HCL 7.88g.

EDTA 7.44g

Sodium choloride 1.45g

SDS 1.0g

Dissolved in 500ml D.W.

3- Guanidine hypochloride 57.2g/100ml D.W.

4- NH_4acetate 57.81g/100ml D.W

5- proteinase K

6- Chloroform -20C

7- Absolute ethanol -20C.

- Master Mix

- Ladder

- Primers

- Agarose gel

B. Equipments:

- Sterile disposable plastic syrings.
- Cotton
- Ethanol tabs.
- Gloves
- Blood containers
- Falcon tube
- Eppendorf tube
- White tips
- Yellow tips
- Blue tips
- Automatic pipettes
- Pasteur pipettes
- Centrifuge
- Master cycler (eppendorf)
- Electrophoresis
- UV Light
- Sensitive balance
- Flask
- Graduated cylinders
-Incubater