بسم الله الرحمن الرحيم

Sudan University of Science and Technology

Faculty of Graduate Studies

Detection of Methylenetetrafolate Reductase Polymorphisms (MTHFR C677T) in pregnant women with Unexplained Recurrent Pregnancy Loss at Elgezira State in Sudan

كشف الصور المتعددة لجين **MTHFR C677T** كعامل لحدوث اسقاط الحمل المتكرر مجهول السبب فى ولاية الجزيرة بالسودان

A thesis Submitted for the Partial Fulfillment of the Requirements of MSc degree in Medical Laboratory Sciences (Haematology and immunohematology)

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بسم الله الرحمن الرحيم

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ﴿1﴾ خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ ﴿2﴾ اقْرَأْ وَرَبُّكَ الْأَكْرَمُ ﴿3﴾ الَّذِي عَلَّمَ بِالْقَلَمِ ﴿4﴾ عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ ﴿5﴾

صدق الله العظيم

سورة العلق الآيات (1-5)

Dedication

This research is dedicated to the soul of my father who taught

Me the meaning of life.

To my mother for their endless Love, Support & Encouragement.

To My beloved brothers and sisters, who gave

Me their confidence.

To my doctors, teachers, friends &Colleagues who

Supported alot.

Acknowledgement

Firstly I would like to express my thanks and gratitude to supervisor Dr.Munsoor Mohammed Munsoor for his supervision ,endless help and continuous follow up ,and his precise observations throughout this study.My appreciation also goes to the member of Alhassahessa (obstetrics and gynecology ward) teaching hospital for their assistance.Lastly whole heartily thanks extend to everyone help me directly or indirectlyto complete this study.

ABSTRACT

This was retrospective analytical case control study conducted at Elgezira state during period of April to December 2018 ,aimed to detection of Methylentetrahydrofolate reductase polymorphism (MTHFR C677T) in Pregnant Women with Unexplained Recurrent Pregnancy Loss .

Total of 80 women were involved in this study, 40 of them with recurrent pregnancy loss (cases), and 40 women with no history of recurrent pregnancy loss (controls).

2 ml of venous blood samples were collected from each subject in EDITA blood container for PCR techniques. Data were analysis by SPSS version 16.

Methylenetetrahydrofolate reductase (MTHFR C677Tpolymorphism) plays a key role in folate metabolism, MTHFR C677T poly morphism reduce enzyme activity lead to decrease conversation of homocystine to methionine subsequently result in homocystine accumulation in the blood (Hyperhomocysteinaemia), hyperhomocysteinaemia is linked to atherosclerosis resulting in arterial and venues thromboembolism.

The study population were distributed according to mutation to mutant type (MTHFRC677T polymorphism) represent 37.5% among cases and 0% among controls, and wide type were 2.5% in cases and 41.3% in controls. while the heterozygous were 10% among cases, and 8.8% among controls . This subjects were distributed according to number of abortion to two times, three times, four times ,five times, and six times .The most frequent number of mutation two times and least number of mutation six times. The result show an association between number of abortion and mutation in

MTHFR C677T gene polymorphism .The age groups are from 20-36 years. There is no association between age and mutation of MTHFR C677T gene polymorphism. The mutation distributed between cases and controls.

In conclusion this study conducted that Methylentetrahydrofolate reductase MTHFR C677T polymorphism consider as risk factor for unexplained recurrent pregnancy loss URPL at Elgezira state in sudan.

المستخلص

اجريت هذه الدراسه في في ولايه الجزيره وتمت المقارنه بين المرضي (اسقاط الحمل المتكرر مجهول السبب) والاصحاء, في الفتره من شهر ابريل وحتي ديسمبر 2018 بغرض كشف الصور المتدده لجين (MTHFR C677T) كعامل لحدوث اسقاط الحمل التكرر مجهول السبب .

اجريت الدراسه علي 80 امراه,40 يعانون من اسقاط الحمل المتكرر مجهول الاسباب , و40 امراه ليس لديهم تاريخ مرضي باسقاط الحمل المتكرر. تم اخذ 2 مل من الدم واجري عليها فحص الجينات PCR للجين MTHFR C677T.

لهذا الجين دور اساسي في استقلاب الفوليت, الطفره في هذا الجين تؤدي الي نقص نشاط الانزيم الذي يحول الهوموسستسن الي ميثونين,مما يؤدي الي تجمع الهوموسستين في الدم , والذي يؤدي بدوره الي حدوث الجلطات في الاورده والشراين.

قسمت هذه المجموعه علي حسب الطفره للجين MTHFR C677T polymorphism الي متماثل الطفره الذي يمثل 37.5% في المرضي و 5% في الاصحاء ,طبيعي (ليس به طفره) الذي يمثل 2.5% من المرضي و 41.3% من الاصحاء , وايضا متعدد الطفره الذي يمثل 10% من المرضي و 8.8% من الاصحاء. وايضا تم تقسيم هذه المجموعه علي حسب عدد مررات الاجهاض التي تتراوح بين مرتين ,ثلاثه مرات , اربعه مرات , خمسه مرات , وسته مرات. اكثر التكرارات الموجوده هو مرتين واقلها سته مرات في النساء الائي يعانين من الاجهاض المتكرر. اظهرت الدراسه ان هناك علاقه بين عدد مرات الاجهاض والطفره الجينيه تراوحت الفئات العمريه في هذه الدراسه من 20–36 سنه. اظهرت الدراسه انه ليس هناك علاقه بين العمر والطفره الجينه للجين MTHFR C677T . تتوزع الطفره الجينيه بين النساء المصابات باسقاط الحمل المتكرر غير معروف الاسباب والنساء الاصحاء.

تخلص هذه الدراسه الي ان الطفره الجينه للجين MTHFR C677T polymorphism تعتبر كعامل لحدوث اسقاط الحمل المتكرر مجهول الاسباب للنساء في ولايه الجزيره بالسودان.

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List of Abbreviation and Symbols

MTHFR	Methylen tetra hydrofolate reductase
PCR	Polymerase chain reaction
DNA	Deoxy ribonucleic acid
URPL	Unexplained recurrent pregnancy bloss
D.W	Distilled water
RCLP	Red blood cells lysis buffer
WCLB	White blood cells lysis buffer
RPM	Round per minute
EDTA	Ethylene diamine tetra acetic acid
AT	Anti thrombin
APC	Activated partial protein C
FVL	Factor V linden
SDS	Sodium dodecyl sulfate
TBE	Tris borate EDTA
TE	Tris EDTA

Chapter One

Introduction

1. Introduction and literature review

1.1 Introduction:

Pregnancy itself may be considered a hypercoagulable state wherein changes occur in the blood coagulation system in favor of the procoagulant branch with decreased levels of anticoagulant factors and increased levels of procoagulant factors (RuchaPatil*et al*.,2013). Recurrent pregnancy loss (RPL) is defined as two or more failed pregnancies, wherein the pregnancy is defined as a clinical pregnancy documented by ultrasonography or histopathological test(Farghali*et al.*, 2015). Recurrent Pregnancy loss (RPL) affects approximately 1–2%of healthy females, and poses a significantphysical and emotional burden on women experiencingthe incidence and their family members (Jianting*et al*.,2017).One miscarriage increases the risk of miscarriage in future pregnancy to 24%; this risk increases to 26% with two previous miscarriages and reaches 32% with three previous miscarriages (Farghali*et al*.,2015).If a woman has not previously given birth, the loss of the fetus is referred to as primary recurrent miscarriage ; if a woman has had a previous live birth, the pregnancy loss is referred to as secondary recurrent miscarriage (Tothet*etal* .,2013). The aim of this study was to investigate whether the MTHFR C677T polymorphism is associated with increased risk for recurrent pregnancy loss in Sudanese population .

Thrombosis is the pathological process whereby platelets and fibrin interact with vessel wall to fold form a haemostatic plug to cause vascular obstruction(Mehta and Hoffbrand, 2000).The term thrombophilia is used to describe inherited or acquired disorders of the haemostatic mechanism that

predisposes thrombosis (Hoffbrand and Moss, 2016).Thrombophilia may be inherited or acquired, and the hypercoagulabilitystate may arise from an excess or hyperfunction of a procoagulant or a deficiency of an anticoagulant moiety (Wahed and Disgupta , 2015). Suggestions of an inherited thrombophilia include thrombosis without any predisposing condition(no surgery, injury, prolonged inactivity), thrombosis at a young age(less than about 40 to 45),thrombosis in unusual site(upper extremities, mesenteric vessels, hepatic or portal veins, cerebral veins), and a family history of thrombosis(Kern, 2002). Several studies have described correlations between recurrent pregnancy loss(RPL) and thrombophilia which comprises of hereditary conditions linked to deficiencies in natural coagulation inhibitors (antithrombin III, proteins S and C) or methylenetetrahydrofolate reductase (*MTHFR*) polymorphism , prothrombin (mutation G20210A) or factor V Leiden genes; and acquired conditions such as antiphospholipid antibody syndrome and patients that produce lupus anticoagulants.[Mervie*et al*.,2017).The C667T mutation in MTHFR gene results in a higher level of homocysteine which is essential for metabolizing vitamin B12 and folate (Elisabeth M *et al.,* 2013). Folic acid is an essential factor for the growing placental tissue and it acts as a substrate for the metabolism of several amino acids and is involved in the transmethylation pathway. MTHFR also maintains the methyl pool for the control of gene expression by DNA methylation during implantation and invasion of the embryo in the first trimester of pregnancy.(NanneCroles*et al*., 2017)..

1.2: Rationale:

Thrombophilia(hypercoagulability or prothrombotic state) is an abnormality of blood coagulation that increases the risk of thrombus formation. Established genetic factors associated with thrombophilia include factor V Leiden, prothrombin gene mutation, protein C or S deficiency, and antithrombin III (AT III) deficiency, or methylenetetrahydrofolate reductase (*MTHFR)*genepolymorhism which is considered as cause of recurrent pregnancy loss (RPL). Approximately half of recurrent pregnancy losses have unexplained etiology.In Sudan studies concerning Methylenetetrahydrofolate reductaseMTFR C677T polymorphism detection are limited; this study is conducted to provide guideline in diagnosis of unexplained recurrent pregnancy loss(URPL)patients in Gezira state and to compare it with other studies carried at other parts of the world. The results of this study may addsnew records of unexplained recurrent pregnancy loss (URPL) and its diagnosis among Sudanese females.

1.3 Objectives:

1.3.1General objectives:

- Evaluation of polymorphisms of methylenetetrafolate reductase (MTHFR C677T) as a risk factor for unexplained recurrent pregnancy loss at Gezira State in Sudan.

1.3.2 Specific objectives:

1. To detect MTHFR C677T polymorphism in unexplained recurrent pregnancy loss (URPL) and healthy control group using allele specific PCR.
2. To compare the PCR results of the two groups and get prevalence of mutation for each group.
3. To corelate between MTHFR C667T polymorphism frequency inUnexplained recurrent pregnancy loss (URPL) and previous number of abortions.

Chapter Two

Literature review

2. Literature review

2.1: Pregnancy:

Pregnancy is a normal physiological state that predisposes to thrombosis, a hypercoagulability (thrombophilia), determined by changes in the body, arising from the special hormonal constellation. Such changes occur in blood flow (venous stasis), in the vascular wall (hypotonia, endothelial lesion) and in coagulation factors (increased levels of factor VII, factor VIII, factor X, von Willebrand factor) and decreased activity levels of natural anticoagulants (protein C, protein S). All these changes persist for another 6 weeks post partum (Coriu et *al*., 2014)

2.1.1: Pathophysiologic changes during pregnancy:

The physiological changes that occur during pregnancy are mainly responsible for the increased thrombogenicity of the peripartum period. A number of clotting factors including factor VII, factor VIII, Factor X, von Willebrand factor, and fibrinogen are elevated as a result of hormonal changes. At the same time, resistance to activated protein C increases in the second and third trimesters and the activity of protein S is decreased due to changes in the total protein S antigen level .There is also an increase in a number of inhibitors of the fibrinolytic pathway such as activatable fibrinolytic inhibitor (TAFI) and plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2).In addition, the physical changes of pregnancy result in an increased thrombotic state. Increased pressure on the pelvic veins from the gravid uterus and decreased flow in the lower extremities result in increased stasis. Relative compression of the left iliac vein by the right iliac artery as it courses across the vessel leads to an increase of clots in the left iliac vein .Although stasis increases throughout the course of pregnancy and

leg pain and swelling are more frequent during the third trimester, incidence of DVT is distributed relatively equally across trimesters.(Elisabeth*et al.*, 2013).

2.2:Recurrent Pregnancy Loss:

Recurrent pregnancy loss is defined by American Society of Reproductive Medicine (ASRM) as two or more failed pregnancies

(Ocak*et al., 2013)*, wherein the pregnancy is defined as a clinical pregnancy documented by ultrasonography or histopathological test(Farghali*et al.*, 2015). *Spontaneous pregnancy loss is a surprisingly common occurrence, withapproximately 15% of all clinically recognized pregnancies resulting inpregnancy failure (*Holly *et al* .,2009).

One miscarriage increases the risk of miscarriage in future pregnancy to 24%; this risk increases to 26% with two previous miscarriages and reaches 32% with three previous miscarriages; thus, women with two or more consecutive miscarriages merit meticulous study to detect the definite cause and possible treatment(Farghali*et al.*, 2015). The risk of recurrent spontaneous miscarriage is much higher in patients with previous losses. The risk gets worse with increasing maternal age .(Jeve and Davies ,2014). There is a strong belief that RPL is a multifactorial condition that many factors affect such as chromosomal abnormalities, uterine anatomic malformation, endocrine dysfunction, immunologic factors, infections, and environmental factors. However, the etiology of RPL remains unknown in ~50 % of cases (Zonouzi*et al*., 2013). If a woman has not previously given birth, the loss of the fetus is referred to as primary RM; if a woman has had a previous live birth, the pregnancy loss is referred to as secondary RM (Tothet*etal*., 2013).When investigating patients with RPL, it is very important to exclude

other possible causes of the losses, such as uterine malformation; diabetes mellitus; connective tissue diseases such as systemic *lupus erythematosus*(SLE); chromosomal abnormalities, and thyroid dysfunction (Abu-Heija 2014.

2.2.1: Etiology of recurrent pregnancy loss:

Various factors are implicated in the pathophysiology of repeated miscarriage. Fetal causes such as single gene or genomic imprinting defects account for 3.5–5% of the cases of repeated miscarriage; other fetal defects include fetal infections and developmental abnormalities. Maternal causes of repeated miscarriage include immunological causes, accounting for 30% of the cases, with anti-phospholipid antibody syndrome being the most common autoimmune cause .Endocrine dysfunction accounts for 48.71% of the cases, while other maternal factors, including anatomical detects and sub-clinical endometrial infection, account for a minimal number of cases . Approximately 50% of repeated miscarriages are unexplained, with no definitive etiology. Several authors suggest the cause to be alloimmune rejection of the fetus (Farghali*et al*., 2015).

2.3: Thrombosis:

Thrombi are solid masses or plugs formed in the circulation from blood constituents. Platelets and fibrin form the basic structure. There clinical significances result from ischemia from local vascular obstruction or distant embolization . Thrombi are involved in the pathogenesis of myocardial infarction , cerebrovascular disease deep vein thrombosis(DVT) and pulmonary emboli (PE). Thrombosis both arterial and venous is more common as age increases and is frequently associated with risk factors (eg: surgery or pregnancy) (Hoffbrand and Moss, 2010).

Thrombosis is multifaceted disorder resulting from abnormalities in blood flow, coagulation system, platelet function, leukocyte activation

molecules, and the blood vessels; It is the inappropriate formation of platelets or fibrin clot that obstruct blood vessels(Keohane*et al*., 2016).

2.3.1: Arterial thrombosis:

This occurs in relation to damaged endothelium, exposed collagen and released tissue factor cause platelet aggregation and fibrin formation(Mehta and Hoffbrand, 2000).

2.3.2:Venous thrombosis:

The term venous thrombosis describes the clinical situation—more common during pregnancy, after surgery, or serious illness—in which a blood clot lodges in a vein. One specific type, which is more serious, involves the clot forming in a major vein in the lower leg and thigh and is termed a deep venous thrombosis. The clot can block blood flow and cause swelling and pain, but more seriously, can break off and move through the bloodstream, causing an embolism. An embolism can get stuck in the brain (and cause a stroke), lungs (and cause a pulmonary embolism), heart (to cause a heart attack), and/or other areas of the body, leading to severe damage(Flinterman *et al*.,2012).

Pregnancy increases the risk of venousthromboembolism VTE in women fivefold to sixfoldcompared with age matched controls. A positive family history for VTE further increases the risk of pregnancy associated VTE 3.7-fold to 8.5-fold.(Croles*et al*.,2017)

2.4:Thrombophelia:

The term thrombophiliais used to describe inherited or acquired disorders of the haemostatic mechanism that predispose to thrombosis(Hoffbrand and Moss, 2016).Thrombophilia may be inherited or acquired, and the hypercoagulability. State may arise from an excess or hyperfunction of a procoagulant or a deficiency of an anticoagulant moiety(Wahed and Disgupta, 2015).Thrombophilia is heritable or acquired disorder of haemostatic mechanism predisposing to thrombosis, typically venous, Arterial thrombosis is usually the result of atherosclerosis not blood hypercoagulability (Provan*et al*., 2004).

2.4.1:**Acquired causes of thrombophilia:**

Acquired causes of thrombophilia are more common that inherited causes;Trauma, surgery, immobilization, pregnancy, hormone replacement therapy, Use of oral contraceptives, paroxysmal nocturnal hemoglobinuria, etc, However, lupus anticoagulant (lupus anti-body) and anticardiolipin antibodies are commonly encountered in patients with a higher risk of thrombotic event, the use of oral contraceptives increases the risk of venous thromboembolism as well as arterial thrombosis .

(Wahed and Disgupta, 2015).

2.4.2:Inherited causes of thrombophilia:

genetic factors associated with thrombophilia include factor V Leiden, prothrombin gene mutation, protein C or S defi-ciency, and antithrombin III (AT III) deficiency, whereas rare genetic defects such as hyperhomocysteinemia and dysfibrinogenemia are also established

causes of thrombophilia, Intermediate genetic factors related to thrombophilia include elevated coagulation factors such as elevated factor VIII activity.Elevated activities of factors IX and XI may also be associated with thrombophilia (Wahed and Disgupta, 2015).An increased level of homocysteine in plasma (hyperhomocysteinemia) also leads to throthrombotic events and is related to the presence of C677T (rs1801133) and A1298C (rs1801131) mutations. (Vivian Dionisio Tavares Niewiadonski*et al*., 2015)

Inherited thrombophilias such as factor V Leiden mutation, prothrombin gene mutation (PT 20210A), and deficiencies of natural anticoagulants protein C, protein S, and antithrombin are associated with recurrent miscarriage. (Jeve and Davies ,2014)

2.4.2.1: Factor v Leiden gene mutation (Activated protein C resistance):

There is failure of activated protein C (APC) when added to plasma to prolong the activated partial thromboplastin time (APPT) test. Protein C when activated break down activated factor V so APC should slow the clotting reaction and prolong the APTT . APC resistance is cause by a genetic polymorphism in the factor v gene (replacement of arginine at position 506 with glutamine Arg 506 Gln) which make factor V less susceptible to cleavage by APC .this is called factor V Leiden mutation.

(Hoffbrand and Moss, 2010).

2.4.2.2: Prothrombin gene mutation:

The G20210A mutation is a genetic variation of the prothrombin (Factor II) gene consisting of a single nucleotide change (guanine to adenine) at position 20 210 of the 3'-untranslated region. The G20210A mutation is the

second most frequent genetic prothrombotic mutation after FVL. lead to greater prothrombin plasma levels , and increases the risk of venous thrombosis about threefold (Georgia Tsiolakidou*et al., 2008)*

2.4.2.3: Antithrombin deficiency:

Antithrombin (AT) deficiency is a heterogeneous disorder. AT It is usually inherited in an autosomal dominant fashion, thereby affecting both sexes equally. There are different AT deficiencies based on the subtypes, as follows:

Type I – The type I deficiency state results from reduced synthesis of biologically normal protease inhibitor molecules. In these cases both the antigenic and functional activity of AT in the blood are reduced. The values are reduced by approximately 50 percent in the heterozygote.

Type II – Type II AT deficiency is produced by a discrete molecular defect within the protein. While the AT immunologic activity is normal in this deficiency, plasma AT functional activity is markedly reduced leading to risk of thrombosis.

Type III – This type is characterized by normal functional and antigenic antithrombin levels but impaired interaction between AT and heparin .(*Khan et al* 2006).

2.4.2.4: Protein C :

Protein C deficiency is inherited in an autosomal dominant manner and is associated with familial venous thrombosis. The gene for protein C is located on chromosome 2 (2q13–14) and appears to be closely related to the gene for factor IX . The primary effect of activated protein C (APC) is to inactivate coagulation factors Va and VIIIa, which are necessary for efficient

thrombin generation and factor X activation .The inhibitory effect of APC is markedly enhanced by protein S, another vitamin K-dependent protein.

(*Khan et al* 2006)

2.4.2.5: Protein S :

Protein S serves as a cofactor for activated protein C , Three phenotypes of protein S deficiency have been defined on the basis of total protein S antigen concentrations, free protein S concentrations, and protein S functional activity.Type I – The classic type of protein S deficiency is associated with a decreased level of total S antigen (approximately 50% of normal), and marked reductions in free protein S antigen and protein S functional activity.Type II – This type of protein S deficiency is characterized by normal total and free protein S levels, but diminished protein S functional activity.Type III – Also known as type IIa, this is characterized by total protein S antigen measurements in the normal range and selectively reduced levels of free protein S and protein S functional activity to less than approximately 40 percent of normal.(*Khan et al* 2006)

2.4.2.6: Dysfibrinogenemia :

The abnormal production of fibrinogen can result in dysfibrinogenemia. The abnormal fibrinogen usually exhibits an abnormal thrombin-mediated conversion to fibrin. While most patients with dysfibrinogenemia are clinically asymptomatic, some present with a bleeding diathesis, others with thrombophilia, and occasionally with both, bleeding and thromboembolism .(*Khan et al* 2006)

2.4.2.7: Hyperhomocysteinemia :

Homocysteine is a non-protein-building sulfhydryl amino acid resulting from the intracellular demethylation of methionine . (NadjaBogdanova *et al.*, 2010*)*. Hyperhomocysteinemia may be both a genetic and acquired abnormality. Homocystinuria and hyperhomocysteinemiacan be caused by rare inborn errors of metabolism that result in marked elevations of plasma and urine homocysteine concentrations.Hyperhomocysteinemia occurs when increased amounts of the amino acid accumulate in the blood due to of impaired intracellular metabolism of homocysteine. Homocysteine is metabolized by the body in two possible pathways: transsulfuration, and remethylation. The transsulfuration of homocysteine produces cysteine and the reaction is catalyzed by cystathionine-β-synthase. This process requires pyridoxal phosphate (Vitamin B) as a cofactor. Remethylation of homocysteine produces methionine. This reaction is catalyzed either by methionine synthase or by betaine-homocysteine methyltransferase. Vitamin B12 (cobalamin) is the precursor of methylcobalamin, which is the cofactor for methionine synthase. Elevations in the plasma homocysteine concentration can occur due to genetic defects in the enzymes involved in homocysteine metabolism as well as due to nutritional deficiencies in vitamin cofactors, or to other factors including some chronic medical conditions and drugs.The most common form of genetic hyperhomocysteinemia results from production of a thermolabile variant of methylene tetrahydrofolate reductase (MTHFR) with reduced enzymatic activity (T mutation) . The gene encoding for this variant contains an alanine-to-valine substitution at amino acid 677 (C677T) .(*Khan et al* 2006).

2.5: MTHFR:

The *MTHFR* gene is localized on chromosome 1 at 1p36.6 . The C677T polymorphism is a C to T transition at base pair 677 resulting an alanine to valine substitution(Boyi Yang *et al*.,2014).Methylenetetrahydrofolate reductase (MTHFR) plays a key role in folate metabolism by irreversibly catalyzing the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate, which serves as both a cofactor and substrate for the regeneration of methionine. (Xia *et a*l., 2014). MTHFR is a key enzyme that plays an important role in the metabolism of folate in the human body, by maintaining the normal metabolism of folate, which can regulate intracellular DNA.It also plays a part in maintaining the stability of single- and double-strand DNA, DNA methylation, and chromosomal integrity. The variation in the fourth gene exon consists of a cytosine (C) mutation to thymine (T) that results in an alanine-encoding amino acid substitution. The variation causes the thermal stability of MTHFR to become reduced, thereby reducing the enzyme activity. It has been reported that the MTHFR enzyme activity of the individual carrying homozygous mutant genotype is only 30% of that among the wild-type carriers.The C677T (rs1801133) and A1298C (rs1801131) polymorphisms are the most common SNPs in the *MTHFR*gene. (Tong*et al2018).* A reduction in the enzyme activity decreases the conversion of homocysteine into methionine, subsequently resulting in homocysteine (Hcy) accumulation in the blood.Hyperhomocysteinaemia(HHcy) has been shown to be caused by both enzyme deficiencies (MTHFR) and environmental factors related to the lack of cofactors in methionine metabolism (vitamins B6 and B12 and folic acid).(Mervie*l et*

al.,2016).However, in the presence of MTHFR gene C677T polymorphism, MTHFR activity slows down even with normal folic acid levels in bodyThe resulting hyperhomocysteinemia causes severe vascular endothelial cell injury. The damaged lumen of blood vessels acts as a precursor for thrombotic events(Nema*et al*., 2018). Folic acid is an essential factor for the growing placental tissue and it acts as a substrate for the metabolism of several amino acids and is involved in the transmethylation pathway ,MTHFR also maintains the methyl pool for the control of gene expression by DNA methylation during implantation and invasion of the embryo in the first trimester of pregnancy (Mervie*et al*.,2016).

2.6: Treatment:

Treatment with low-dose aspirin, enoxaparin and folic acid was the most effective therapy in women with RM who carried a C 6 7 7 T *MTHFR* polymorphism (Mervie*et al*., 2017).

2.7: Previous studies.

A study done in North India (Saraswathy*et al* ., 2018) found that the distribution pattern of methylated allele between RPL cases and controls was compared with respect to the MTHFR C677T genotypic status , there seems to be a significant difference between RPL cases and controls, wherein the frequency of methylated allele was found to be significantly higher among RPL cases carrying CC and CT genotypes of MTHFR C677T polymorphism as compared to the controls.A study done in China (Yunli Cao *et al*., 2012) showed that a significant association between MTHFR C677T and URPL in the East Asian subgroup and mixed subgroup. Another study done in Syria (Al-Achkar*et al*., 2017) .In RPL group the genotype frequencies of MTHFR C677T Polymorphism were CC (41%) ,(CT (41%) , and TT(18%) , and in the control group the frequency were CC (62.2%) , CT (36.7%) , and TT (1%) .Statistical analysis show homozygous TT genotype at T allele were significantly different. Study done in Egypt (Settin*et al*., 2011) show that mutation related MTHFR gene polymorphism are increase in case than control group but not statistically significant in Egyptian women with Unexplained Recurrent Pregnancy Loss (URPL). Ahmed PoursadeghZonouzi et al in 2012 found that there is no significant difference in the prevelance of MTHFR C677T genotype among women with RPL and healthy control.

Study done in Tunisia (Mtiraoui*et al*., 2006) found that the homozyggosity of MTHFR C677T is risk factor factor for recurrent pregnancy loss.

Chapter Three

Materials and Methods

3- Materials and Methods

3.1:Study design:

- This study is descriptive, cross sectional case control study.

3.2: Study area and duration:

- This study carried out in period from April to December 2018 to detect MTHFR C667T mutation among. Unexplained recurrent pregnancy loss Sudanese female patients in Elgazira state (Alhasahesa hospital)

3.3: Study population:

- Sudanese female with recurrent pregnancy loss in Aljazeera state Alhasahesa hospital. The study carried out in 80 females. 40 females diagnose for URPL (cases) ,and 40 females with no history of URPL (controls).

3.4: Inclusion criteria:

- Female with recurrent pregnancy loss

3.5: Exclusion criteria:

- Female with one time abortion, uterine malformation; diabetes mellitus; connective tissue diseases such as systemic *lupus erythematosus*(SLE) and thyroid dysfunction .

3.6:Ethical consideration:

- Sample will be collected after approval of patients.
- Data will be kept confidentially.
- 3.7: Sample Size:

- 80 samples were collected 40 with Unexplained Recurrent Pregnancy loss URPL (case), and 40 with no history of Unexplained Recurrent Pregnancy Loss URPL (control)

3.8: Data collection:

- Data was collected using questionnaire which specifically designed to obtain information about demographic and clinical data that helped in either including or excluding from the study.

3.9:Sampling Technique:

- Two ml of blood were collected into EDTA containers for DNA extraction using salting out technique and subsequent PCR.

3.10:Data analysis:

- Data will be analyzed by statistical package for social sciences (SPSS) version 16.
- 3.11: Methodology:

3.11.1:DNA extraction by salting out method:

- 300ul of blood was placed in 1.5 ml eppendorf tube, 1000ul of RCLB was added to the blood , mixed well and centrifuged at 2500 rpm for 10 minutes, supernatant was discarded and the pellet (WBCs) washed again with 1000 ul of RCLB many times until clear white pellet obtained .300 ul of SDS and WCLB plus 100 ul of 6M NaCL was added to the pellet and incubated at 56 c for 30 minutes After incubation 200 ul of cold chloroform was added to the mixer and centrifuged at full speed (14000 rpm) for 5 minutes . the aqueous layer was transferred to clean Eppendorf tube and added a double

volume of cold absolute ethanol, centerfugde at 14000 rpm for 5 minutes, then washed with 600 ul 70% ethanol by centrifugation at 5000 rpm for 5 minutes ,then left the tube to dry , the pellet resuspended in 50 ul TE buffer and preserved at -20 c until PCR performed.

3.11.2:Detection of MTHFR C677T Polymorphism:

All DNA samples were examined for the C677T polymorphism using allele specific PCR.

The primer sequences used were as follow:

Table 3.1:

Primers	**Sequences**	**CC**	**TT**
Wild type R	TGC GTG ATG ATG AAA TCC G	**124bp**	
Mutant type R	TGG GTC ATG ATG AAA TCC A-		**50bp**
Common F	TCT CCT GAC TGT CAT CCC TA		

3.11.3:PCR Program:

- **Initial denaturation**: 95cfor 5 minutes.
- **Second denaturation** :95c for 30 seconds
- Annealing 51.8c for 30 seconds and 35 cycles (for normal). For the mutant, annealing was 50.7 for 30 second and for 35 cycles.
- **Extension**: done in 72c for 30 seconds.
- **Last extension**: 72c for 5 minutes.

3.11.4:Master mix tube preparation for detection of wild allele:

- **Table 3.2:**

Reagent	volume
D.W	8 ul
Wild R	0.5 ul
Common F	0.5 ul
Master Mix	6 ul
Template DNA	5 ul
Total reaction tube	20 l

3.11.5 :Master Mix tube preparation for detection of mutant allele:

Table 3.3:

Reagents	Volume
D.W	8 ul
Mutant R	0.5 ul
Common F	0.5 ul
Master Mix	6 ul
Template DNA	5 ul
Total reaction tube	20 ul

Chapter four

Results

4. Results

4.1. Characteristics of study population.

A total of 80 women were participated in this study, 40 women with recurrent pregnancy loss (cases) and 40 women with no history with recurrent pregnancy loss (controls). The age range from 20 to 36 years .and divided into 3 age groups (20-24),(25-30), (>30). (table 4.1,figure 1)

Table (4.1). Frequency distribution of age group of cases:

Age group	Frequency	Percent
20-24	4	10
25-30	25	62.5
>30	11	27.5
Total	40	100

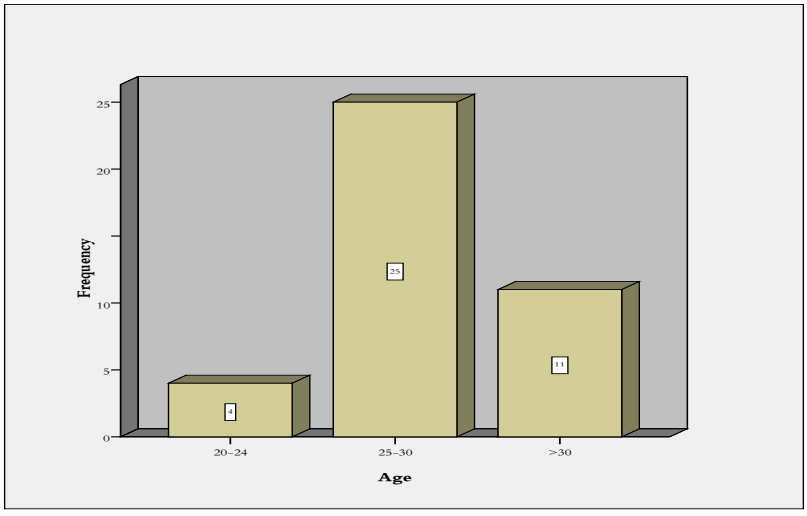

Fig. 1. Frequency distribution of age group of cases

4.2:Distribution of number of Abortion:

These subjects were distributed according to number of abortions into 5 groups (2 ,3,4,5,6 times) (table4. 2, figure2).

Table (4.2). Frequency distribution of number of abortion among the cases

Number of abortion	Frequency	Percent
2	20	50
3	13	32.5
4	3	7.5
5	3	7.5
6	1	2.5
Total	40	100

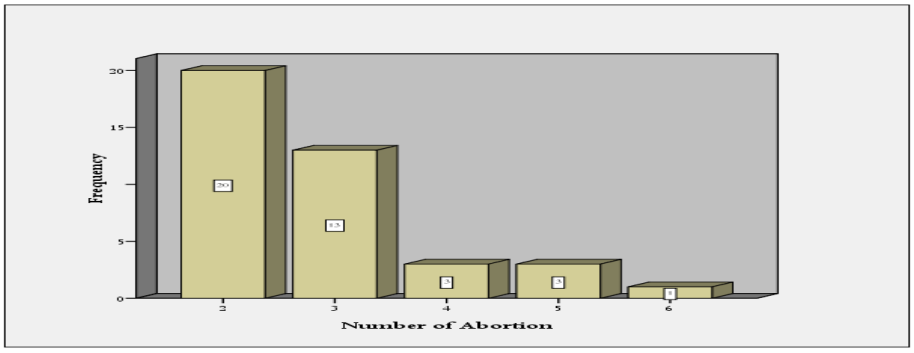

Fig. 2. Frequency distribution of number of abortion among cases

4.3: Distribution of mutation:

The study populations were distributed according to mutation into (homozygous with frequency 30 times), (heterozygous with frequency 8 times), (normal with frequency 2 times) (table4.3 and figure 3).

Table no. (4.3.): Frequency distribution of Mutation

Mutation	Frequency	Percent
Homozygous	**30**	**75**
Normal	**2**	**5**
Heterozygous	**8**	**20**
Total	**40**	**100**

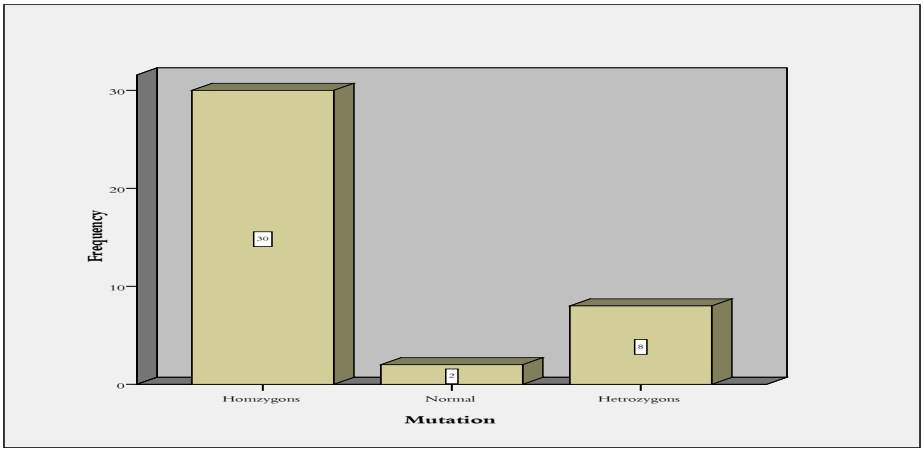

Fig. (3): Frequency distribution of Mutation

4.4:Relation between number of abortion and mutation

These subjects were distributed according to number of abortions into 5 groups (2 , 3, 4,5,6 times) (table4.2 figure 2). The most frequent was females with 2 time (20 females represent 50% of population and the least frequent was 6 times one female represent 2.5 of population)(table4. 4).

Table 4 .4: Relation between number of abortion and mutation:

Number of abortion	Homozygous	Normal	Heterozygous	Total
2	10 25%	2 5%	8 20%	20 50%
3	13 32.5%	0 0%	0 0%	13 32.5%
4	3 7.5%	0 0%	0 0%	3 7.5%
5	3 7.5%	0 0%	0 0%	3 7.5%
6	1 2.5%	0 0%	0 0%	1 2.5%
Total	30 75%	2 5%	8 20%	40 100%

4.5:Relation between age groups and mutation:

The results show no relation between age groups and mutation (P =0.335)(table 4.5).

Table 4. 5. Relation between age groups and mutation:

Age group	Homozygous	Normal	Heterozygous	Total
20-24	4 10%	0 0%	0 0%	4 10%
25-30	16 40%	2 5%	7 17.5%	25 62.5%
>30	10 25%	0 0%	1 2.5%	11 27.5%
Total	30 75%	2 5%	8 20%	40 100%

4. 6:Frequency of mutation between cases and controls:

The study results indicate a relation between mutation on one hand and studied population on the other with a P value of 0.000 (table 4.6). The homozygous distribution were 37.5% among cases and 0% among controls. The normal were 2.5% in cases and 41.3% in controls. While the heterozygous were 10% among cases and 8.8% among controls (table4. 6).

Table 4.6. Frequency of mutation between cases and controls

Mutation	Case	Control	Total
Homozygous	30 37.5%	0 0%	30 37.5%
Normal	2 2.5%	33 41.3%	35 43.8%
Heterozygous	8 10%	7 8.8%	15 18.8%
Total	40 50%	40 50%	80 100%

Chapter five

Discussion, conclusion and recommendations

5 .Discussion, conclusion and recommendations

5.1. Discussion:

In this study the frequency of mutation of MTHFR C677T polymorphism among cases 95% and 8.8% in control ,this indicate the distribution of MTHFR C677T polymorphism among cases and controls in this respect this result agreed with result reported by Sarawathy *et al* .,2018 .according to this study the mutation of MTHFR C677T polymorphism report as main cause o unexplained recurrent pregnancy loss among cases.

The results show that the mutation MTHFR C677T polymorphism are significantly increased in cases than control. This result contradict a result reported in study in Egypt by Sittin and his coworkers in 2011 where they found that no statistical difference in mutation of MTHFR C677T polymorphism on cases compared to control.

In this study the result indicated the frequency of homozygous mutation of MTHFR C677T polymorphism was 75% and heterozygous 20% .this indicated that homozygosity of MTHFR C677T gene polymorphism is risk factor for unexplained recurrent pregnancy loss .This result agree with result reported in Tunis by Metraoui *et al* 2006.

This study observed that an association between number of abortion on one hand and mutation on other hand, Cases with two times of abortion represented highest number of mutation among cases the less frequent was six times abortion as observation.

5.2: Conclosions:

- MTHFR C677T gene polymorphism among the present study represent a risk factor for unexplained recurrent pregnancy loss.
- The MTHFR C677T gene polymorphism is distributed among case and control.
- There is association between number of abortion and mutation of MTHFR C677T .

5.3: Recommendation:

-Early diagnosis is useful to avoid unexplained recurrent pregnancy loss.

- Further studies should assess polymorphism of MTHFRC677T gene among Sudanese women with unexplained recurrent pregnancy loss , with larg sample size.

- Mutation of MTHFR C677T gene must be considered in diagnosis of unexplained recurrent pregnancy loss.

Chapter Six

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Appendix

Appendix:

Molecular reagents:

-RBCs lysis buffer consist of 8.3 g NH4CL, 1.19 g of NaHCO3 or KHCO3, 1.8 g of 5% EDTA dissolved in 1 litter of D.W.

-WBCs lysis buffer contain 7.88 g of Tris HCL, 5.44 g of EDTA, 0.146 g of NaCL and 10g of SDS dissolved in 500 ml of D.W.

-Agarose gel 1% prepared by dissolving 0.5 g of agarose powder in 50 ml TBE buffer 1x and this solution must be heated in microwave for 3 minutes until the powder dissolve completely.

-1x TBE buffer prepared by dissolving 27 g of Tris base powder ,14 g of boric acid and 1.9 g of EDTA in 2500 ml of D.W.

-Master mix was premixed , ready to use solution containing 0.25U/ul Taq DNA polymerase, 0.4 Mm dNTPs, 3.2 mM Mg+2, 0.02% bromophenol blue and 2x reaction buffer at optimal concentrations for efficient amplification of DNA template by PCR.

Questionnaire

Sudan University of science and technology

Faculty of graduate studies

Hematology department

Detection of Methylentetrafolate Reductase Polymorphism MTHFR C677T in pregnant women with Unexplained Recurrent Pregnancy Loss at Elgezira state in Sudan

Hospital Name…….

Date……

Patient ID…….

Patient serial No……..

Age……

Cause of recurrent pregnancy loss……..

No of recurrent pregnancy loss ……..

Other disease…….

If yes what disease…….