SUDAN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF GRADUATE STUDIES

Association of Mythylenetetrahydrofolate Reductase C677T Polymorphism and Myocardial Infarction Risk among Sudanese patients in Khartoum State

العلاقة بين تعدد الأشكال الجيني لإنزيم ميثيلين تتراهيدروفوليت C677T المختزل وخطر الاصابة باحتشاء عضلة القلب لدي المرضي السودانيين بولاية الخرطوم

A thesis submitted for Partial Fulfillment of the Requirements for M.Sc. Degree in Medical Laboratory Science (Hematology and Immunohematology)

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قال تعالى : (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 – الأيات (1-5)

DEDICATION

All thanks for Allah for the guidance, strength, power of mind, protection and skills and for giving me a healthy life.

I dedicate this study to my beloved parents, who have been my source of inspiration and who continually provide their moral, spiritual, emotional, and financial support.

To my son, brother, sisters, relatives, friends, and colleagues who shared their words of advice and encouragement to finish this study.

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ABSTRACT

This was an analytical case-control study that has been carried out in Khartoum state from October 2018 to June 2019 aimed to investigate the association between Methylenetetrahydrofolate C677T polymorphism and myocardial infarction risk. Eighty subjects were recruited for this study, forty patients with a history of myocardial infarction aged between 27 to 80 years and forty healthy volunteers as a control group. 13 of patients were suffering from diabetes mellitus, 14 had hypertension, 3 of them had the two diseases and ten of them did not have any risk factor. Data wer collected from participants (cases and controls) directly by questionnaire, included age, gender, other diseases and family history for cases and age and gender for control subjects.

2.5 ml intravenous blood samples were collected from all participants in EDTA anticoagulant containers, genomic DNA was extracted by salting out method and the methylenetetrahydrofolate C677T polymorphism was analyzed using allele-specific polymerase chain reaction. Amplified fragments separated on 2% agarose gel stained with ethidium bromide and demonstrated by gel documentation system, which produced two single bands one at 124 bp represented C wild allele and the other one at 50 bp represented T the mutant allele.

The data analyzed by computer program statistical package for social sciences SPSS, version 16. The results showed that the frequencies of CC, CT and TT genotypes within case group were 21 (52.5%), 17 (42.5 %) and 2 (5%) respectively, while this frequencies within control group were 25 (62%), 15 (37%) and 0 respectively. The relative risk for methylenetetrahydrofolate C677T polymorphism was calculated (0.9) by SPSS program (95%CI: 0.885 to 1.020), it was shown that there was no significant association between having this mutation and risk of MI occurrence.

Methylenetetrahydrofolate C677T genotypes distribution showed no significant difference between the case group and the control group P. value (0.290). Also, there was no significant relationship between risk factors which patients suffer from in this study and methylenetetrahydrofolate C677T polymorphism.

In conclusion, there was no significant association between Methylenetetrahydrofolate C677T polymorphism and myocardial infarction risk among Sudanese patients in Khartoum state.

المستخلص

كانت هذه دراسة تحليلية للحالات والمجموعة الضابطة) تم اجراؤها في ولاية الخرطوم في الفترة مابين اكتوبر2018 الي يونيو 2019. هدفت الدراسة لاستقصاء العلاقة بين تعدد الاشكال الجيني لانزيم تتراهيدروفوليت C677T وخطر الاصابة باحتشاء عضلة القلب . تم تعيين مجموعة من 80 شخص لهذه الدراسة ، 40 مريض تم تشخيصهم مسبقا بمرض احتشاء القلب تتراوح اعمارعم بين 27 الي 80 سنة و40 متطوعين أصحاء كمجموعة ضابطة.

ثلاثة عشر من المرضي كانو يعانون من مرض السكري،اربعة عشر يعانون من ارتفاع ضغط الدم وثلاثة مرضي يعانون من كلا المرضين وعشرة مرضي لم نسجل لهم اي من هذه الامراض. تم جمع بيانات المشاركين مباشرة بواسطة الاستبيان.

2.5 مل من الدم تم جمعها من جميع المشاركين في حاوياتEDTA المضادة للتخثر. تم فصل الجزء المستهدف الحمض النووي بواسطة طريقة التمليح ، ثم تحليل تعدد الأشكال الجيني لإنزيم تتراهيدروفوليت المختزل بتفاعل البلمرة المتسلسلة ، الأجزاء المتضخمة فرقت بواسطة 2% من الجل المصبوغ بالإثيديوم بروماید وأظهرت النتائج بنظام توثيق الجل التي انتجت جزء واحد عند 124 مثل الأليل العادي C وجزء واحد آخر عند 50 مثل الأليل المتحور T. تم تحليل البيانات بواسطة برنامج الحزم الإحصائية للعلوم الإجتماعية الإصدار 16.إختلافات ذات دلالة معنوية ($P<0.05$).

أظهرت النتائج أن نسب الاشكال المتعددة للجين CC، CT وTT هي 52.5%،42.5%و5% علي التوالي بين فئة الحالات بينما هذه النسب في فئة المجموعة الضابطة كانت 62%،37% وصفر% علي التوالي، حيث مثل النمط الوراثي CC من تعدد الأشكال الجيني للإنزيم النمط المتماثل للجين العادي، وCT النمط المتخالف الجينات بينما مثل النمط TT النمط الجيني المتماثل للجين المتحور. ولايوجد اختلاف هام بين توزيع نسب تعدد اللأشكال الجيني للإنزيم بين المجموعتين حيث كانت قيمة P هي (0.9).

ايضا تم احتساب المخاطر النسبية لتعدد الأشكال الجيني لهذا الإنزيم بواسطة برنامج SPSS(0.9) (95% CI: 0.020-0.885) ، وقد تبين أنه لا يوجد أي ارتباط كبير بين وجود هذا التحور وخطر حدوث إحتشاء عضلة القلب، كما لا توجد علاقة هامة بين عوامل الخطورة التي يعاني منها المرضي وبين وجود هذه الطفرة في حدوث المرض.

في الخلاصة، أوضحت الدراسة عدم وجود علاقة ذات دلالة إحصائية بين تعدد الأشكال الجيني لإنزيم ميثيين تتراهيدروفوليت المختزل C677T وخطر الإصابة بمرض إحتشاء عضلة القلب بين المرضي السودانيين في ولاية الخرطوم.

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List of abbreviations:

MI	Myocardial Infarction
MTHFR	Methylenetetrahydrofolate
VTE	Venous Thromboembolism
FVL	Factor V Leiden Mutation
Pm	Prothrombin (FII) Mutation
AT	Anti-Thrombin
Thcy	Total Homocysteine
SAM	S-Adenosylmethionine
SAH	S-Adenosylhomocysteine
Met	Methionine
MS	Methionine Synthase
HPLC	High Performance Liquid Chromatography
CAD	Coronary Artery Disease
CVD	Cardio Vascular Disease
Hhcy	Hyper Homocysteinemia
THF	Tetrahydrofolate
CTN	Cardiac Troponin
STEMI	ST-Segment Elevation Myocardial Infarction
NSTEMI	Non ST- Segment Elevation Myocardial Infarction
ECG	Electrocardiography
FT	Fibrinolytic Therapy
P-PCI	Primary- Percutaneous Coronary Intervention
RCLB	Red Blood Cells Lysis Buffer
WCLB	White Blood Cells Lysis Buffer
TBE	Tris/ Borate/ EDTA Buffer
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
LOX-1	Lecithin-Like Oxidized Low-Density Lipoprotein Receptor-1
ICAM	Intracellular Adhesion Molecule Type 1
VCAM	Vascular Adhesion Molecule
PECAM1	Platelet Endothelial Cell Adhesion Molecule Type 1
MMP	Matrix Metalloprotein

Chapter I

INTRODUCTION

CHAPTER ONE

1. INTRODUCTION

1.1 Introduction

Although lifestyle and environmental factors influence the prevalence of myocardial infarction, genetic epidemiological studies have suggested that several genetic variants increase the risk for this condition (Yamada, 2006).

Ischemia occurs when blood flow to the myocardium is reduced. Ischemia of prolonged duration induces myocardial infarction (MI), and MI is a common cause of heart failure (Tanai and Frantz, 2015).

Adjustable risk factors for ischemic heart disease consist of elevated arterial blood pressure, hyperlipidemia, chronic tobacco use, increased body weight, sedentary life style, diabetes mellitus, and anxiety. Other aspects, such as gender and age are unchangeable cardiovascular risk factors. If a patient admitted to the hospital for typical symptoms of an acute thrombotic event (acute myocardial infarction), without any significant cardiovascular risk factors, the clinician is required to exclude genetic disorders as etiology for the acute diagnosis (Jaco and Siko, 2017).

Genetic causes that have been shown to be independent risk factors for acute thrombotic events include the mutation of several genes that encode the coagulation factors, such as antithrombin, C and S protein, fibrinogen, prothrombin, and factor V from the clotting cascade. The genetic mutation of the methylenetetrahydrofolate reductase (MTHFR) gene, which causes a transitional shift in cytosine to thymidine on the 677 nucleotide, lead to low enzymatic activity and a modified folic acid state with increased folate necessity. The C677T mutation of the gene that encodes the MTHFR causes C/C, C/T and T/T genotypes, leading to an elevated serum level of homocysteine lead to early atherosclerotic lesion formation and venous thromboembolism (Tempelhoff *et al*., 2016).

Myocardial infarction is the most acute form of coronary artery disease. Apart from the traditional risk factors of myocardial infarction, recently many reports have suggested that hyperhomocysteinemia plays an important role in myocardial infarction (Angeline *et al*., 2005).

The role of hyper homocysteinemia in vascular and thromboembolic disease has been extensively studied. Previous studies reported significant vascular disease in patients with markedly elevated total homocysteine (tHcy) level (Brevik *et al.,* 2005).

Basic studies demonstrated that homocysteine can induce vascular damage by promoting platelet activation, oxidative stress, endothelial dysfunction, hypercoagulability, vascular smooth muscle cell proliferation, and endoplasmic reticulum stress (De Bree *et al*., 2002).

1.2 Rationale

Myocardial infarction is one of the most common causes of death in the world and now genetic investigations play an important role in the detection and prediction of many diseases. Many studies have found that genetic in addition to known risk factors (including age, gender, arterial hypertension, smoking, diabetes, and dyslipidemia) are the common causes of MI.

One of the genetic causes have been reported to be associated with MI is (MTHFR C677T) polymorphism Which results in increased serum homocysteine leading to early atherosclerotic lesion formation and venous thromboembolism, but results from previous studies are conflicting.

In Sudan, I did not find studies that done to find out the association between MTHFR C677T polymorphism and myocardial infarction but there was many studies conducted to associate this polymorphism and many diseases such as deep venous thrombosis, leukemia and many types of cancer. This study aimed to explore whether this polymorphism plays a role in the genetic susceptibility to MI.

1.3 Objectives:

1.3.1 General objective:

To study the association of MTHFR polymorphism with myocardial infarction risk among Sudanese patients.

1.3.2 Specific objectives

1. To detect MTHFR C677T polymorphism in patients with myocardial infarction.

2. To determine the frequency of MTHFR C-T genotyping variants among patients of MI.

3. To study the association between MTHFR C-T polymorphism and risk of MI.

4. To associate MTHFR polymorphism with patient gender.

5. To study the interaction between MTHFR polymorphism and other known risk factors of MI such as diabetes mellitus and arterial blood pressure.

Chapter II

LITERATURE REVIEW

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Homocysteine and Hyperhomocysteinemia:

Elevated plasma homocysteine (tHcy) and the MTHFR 677C > T variant have been postulated to increase the risk of venous thromboembolism (VTE) (Joachim, 2013).

Hcy is thought to increase thrombotic risk by inducing endothelial injury in venous and arterial vasculature; however, the precise prothrombotic mechanisms are poorly understood (Undas *et al.*, 2005).

It has been reported that elevation of Hcy in the blood is associated with an increased risk for arteriosclerosis, myocardial infarction, venous thrombosis, stroke, and neural tube defects (Toyoda *et al*., 2014).

The elevation of homocysteine may result in the dysfunction of endothelial and smooth muscle cells in the vascular wall. Endothelial injuries such as the inhibition of cellular binding sites for tissue plasminogen activator, decreasing expression of thrombomodulin , and production of endoplasmic reticulum stress and growth arrest are observed in hyperhomocysteinemic animals. The effect of hemostasis, induced by homocysteine, promotes blood clotting and reduces fibrinolysis (Perła *et al*., 2007).

The effects of hyperhomocysteinemia also include: inhibition of prostacyclin synthesis, activation of factor V, inhibition of protein C activation, down regulation of thrombomodulin expression and blocking of binding of t-PA (but not plasminogen) to endothelial cells. The toxic effect of homocysteine also results in increased ptatelet adhesion, impaired regulation of endothelium-derived relaxing factor and related nitrogen oxides, induction of tissue factor, suppression of heparin sulfate expression, stimulation of smooth muscle cell proliferation and oxidation of low-density lipoprotein (Mayer *et al*., 1996).

Homocysteine, a sulfhydryl-containing amino acid, is an intermediate product in the normal biosynthesis of the amino acids methionine and cysteine (Faeh *et al.*, 2006).

It is a key determinant of the methylation cycle, it methylated to methionine, which undergoes S-adenosylation and forms S-adenosylmethionine (SAM). S-adenosylmethionine is the principal methyl donor for all methylation reactions in cells (Loscalo and Handy, 2014).

The loss of the methyl group can result in the transfer of SAM to S-adenosylhomocysteine (SAH), and the hydrolysis of SAH catalyzed by SAH hydrolases yields homocysteine (Zhang *et al*., 2013).

Homocysteine is metabolized through two biochemical pathways: remethylation and transsulfuration. In remethylation, homocysteine can be remethylated into Met via methionine synthase (MS) or betaine-homocysteine methyltransferase to begin another methyl group transfer cycle. In contrast, in transsulfuration, homocysteine combines with serine and is irreversibly converted to cystathionine via Vitamin B6-dependent enzyme: cystathionine β-synthase. Subsequently, cystathionine is hydrolyzed to yield cysteine, a precursor to the antioxidant glutathione. Deficiencies of folic acid, Vitamin B6, and Vitamin B12, as well as abnormal methylenetetrahydrofolate reductase (MTHFR), cystathionine β-synthase, and MS, can lead to metabolic disturbances of homocysteine. Plasma total homocysteine consists of free homocysteine, protein-bound homocysteine (S-linked and N-linked), oxidized forms, and homocysteine thiolactone (Manolescu *et al*., 2010).

The normal range of plasma homocysteine is 5–15 μmol/L when assessed by high performance liquid chromatography or 5.0–12.0 μmol/L when immunoassay methods used) (Moll and Varga, 2015).

Hyperhomocysteinemia is defined as a medical condition characterized by an abnormally high level (above 15 μmol/L) of homocysteine in the blood (Guo, 2009).

It is classified into mild, moderate, and severe forms. A concentration between 15 μmol/L and 30 μmol/L is considered mildly elevated, between 30 μmol/L and 60 μmol/L is considered moderately elevated, and above 60 μmol/L is considered severely elevated (Moll and Varga, 2015).

2.1.1 Causes of hyperhomocysteinemia:

Hyperhomocysteinemia may arise from genetic defects of enzymes involved in homocysteine metabolism. The enzymes involved can be 5, 10-methylene tetrahydrofolate reductase, methionine synthase, and cystathionine-β-synthase. The most common one that is detected worldwide and has a high incidence in different populations, is single nucleotide polymorphisms of 5,10-methylenetetrahydrofolate reductase which has been associated with mild (13–24 μM) and moderate (25–60 μM) hyperhomocysteinemia.

Hyperhomocysteinemia can also arise from nutritional deficiencies of folate, vitamin B6, and vitamin B12. Blood levels of folate, vitamin B12 and to a lesser extent, vitamin B6 are related inversely to total homocysteine; therefore a person with a nutritional deficiency that leads to low blood concentrations of the aforementioned is at increased risk of hyperhomocysteinemia (Currò, 2014).

Increasing age, male sex, smoking, coffee consumption, high blood pressure, unfavorable lipid profile, high creatinine, and faulty diet are some of the factors associated with increased homocysteine levels. On the other hand, physical activity, moderate alcohol consumption, good folate and vitamin B12 status are associated with lower homocysteine levels. Vegetarians may be at a higher risk of hyperhomocysteinemia due to low plasma B12 levels but the difference is likely to be insignificant (Shenoy *et al.*, 2014).

It was reported that hyperhomocysteinemia is related to the occurrence and development of many diseases, for example, CAD, pregnancy complications, autism, osteoporosis, multiple sclerosis, psoriasis, and Alzheimer's disease (Bebchuk *et al.*, 2015).

2.1.2 Hyper homocysteinemia and cardiovascular disease:

There has been an indication towards a significant correlation between hyperhomocysteinemia and cardiovascular disease and its complications such as heart attacks and strokes it is believed that hyperhomocysteinemia leads to endothelial cell damage, reduction in the flexibility of vessels, and alters the process of hemostasis. Hyperhomocysteinemia may lead to an enhancement of the adverse effects of risk factors like hypertension, smoking, lipid and lipoprotein metabolism, as well as the promotion of the development of inflammation (Baszczuk and Kopczynski, 2014).

2.2 MTHFR and myocardial infarction:

The relationship between homocysteine, MTHFR 677C>T polymorphism, and CVD is equally controversial and may involve differential associations with ischemic stroke and myocardial infarction (MI) (Spence, 2007).

Some data suggest that MTHFR TT genotype and elevated homocysteine levels increase the risk for incident ischemic stroke and MI (Yamada, 2006). According to the World Health Organization, every year ~50 million people succumb due to ischemic heart diseases, particularly myocardial infarction (MI), which is a worldwide leading cause of fatality. In general, MI is a result of myocardium necrosis, which occurs when the latter does not receive enough oxygen, due to a sudden occlusive thrombosis of the coronary artery that irrigates this section of myocardium (Hmimech, 2016).

Several studies have identified that genetic in addition to known risk factors (including age, gender, arterial hypertension, smoking, diabetes and dyslipidemia) strongly increases the risk of MI occurrence. Among the numerous genes that were previously found to be in association with MI susceptibility, 5,10-methylenetetrahydrofolate reductase (MTHFR) and factor II prothrombin (FII) genes are the most widely reported (Sakowicz, 2010).

The 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is located on chromosome 1 at 1p36.3. MTHFR is the key metabolic enzyme of homocysteine (Hcy). It catalyses 5, 10-methylenetetrahydrofolate reduction to 5-methyltetrahydrofolate, which as a methyl donor induces Hcy remethylation to methionine (Biselli *et al.*, 2010).

The abnormity of MTHFR structure or function can take part in the occurrence of Hyperhomocysteinemia (Moll and Varga, 2015).

Two polymorphic variants, including C677T (rs1801133) and A1298C (rs1801131), have been identified in MTHFR gene (Xia *et al*, 2014).

MTHFR C677T represents an alanine-to-valine substitution at nucleotide position 677 in exon 4 resulting in thermolability and concurrent decreased activity of the enzyme (Christ-Crain, 2003).

Variants of C677T in methylenetetrahydrofolate reductase (MTHFR), were shown to be associated with increased circulating Hcy, since the catabolic activity of the enzyme was reduced to 65% and 30% in CT and TT carriers as compared with that in wild type CC (Liew and Gupta, 2015).

Homozygous mutation MTHFR enzyme lead to homocystinuria characterized by very high levels of plasma hcy, severe mental and skeletal abnormalities, premature thromboembolism, and lens dislocation (Mudd, 2001).

Heterozygous mutations in these enzymes or nutritional deficiency of cofactors can lead to moderate increase in plasma hcy known as hyperhomocysteinemia (Hhcy). Hyperhomocysteinemia is an independent risk factor for cardiovascular diseases and associated with an increased risk of coronary artery disease and myocardial infarction (Wierzbicki, 2007).

2.2.1 MTHFR enzyme and methylation:

Into the one-carbon pathway enter glucose and amino acids, and out of it one expects a wealth of components used for processes encompassing all aspects of life: biosynthesis, genome integrity and epigenetics. The folate cycle begins with folic acid, which, upon cell entry, is reduced to tetrahydrofolate (THF). THF, in turn, is modified to 5,10-methylene-THF (me-THF). This is when methylenetetrahydrofolate reductase (MTHFR) comes into play, and reduces me-THF to 5-methyltetrahydrofolate (mTHF). It is exactly mTHF that is at the hub of the folate cycle and the methionine cycle. MTHF is demethylated to release a carbon, which is taken up by homocysteine and thus generating methionine. Methionine, then, is adenylated to make S-adenosylmethionine (SAM), which is the ultimate methyl donor for methylation reactions in the cell. Thus, carbon units are transferred between the folate cycle and the methionine cycle. Its innate cyclic nature makes it ideal to be an integrator of nutrient status, cell growth and human diseases (Locasale, 2013).

2.3 Thrombophilia :

Thrombophilia is a disorder of hemostasis in which there is a tendency for the occurrence of thrombosis. This tendency can be inherited or acquired.

2.3.1 Acquired thrombophilia:

Venous thrombosis and arterial cardiovascular disease have been traditionally regarded as separate diseases with distinct causes and treatment. However, several studies in the past decade have shown that patients with venous thrombosis (i.e., deep vein thrombosis or pulmonary embolism) have an increased risk of subsequent arterial disease (Lijfering *et al*., 2011). In many cases of venous thromboembolism (VTE), there is an obvious precipitating factor, such as the postoperative state. In a minority, VTE is apparently unprovoked or spontaneous. Acquired thrombophilic conditions should be considered in all cases of apparently spontaneous VTE. Identifying such a condition may have important management implications for the patient, as anticoagulant treatment may minimize the risk of thrombosis recurrence. If the precipitating factor is not reversible then long-term anticoagulation may be indicated. Other risk factors, such as obesity, immobility, diabetes mellitus, arterial blood pressure, hyper lipidemia, inflammatory bowel disease and overt malignancy, may be easily identified (Merriman and Greaves, 2006).

2.3.2 Inherited thrombophilia:

Inherited thrombophilia constitutes a group of abnormalities of blood coagulation, including the factor V Leiden mutation (FVL) (homozygous or heterozygous), the prothrombin (FII) G20210A mutation (Pm) (homozygous or heterozygous), and deficiencies of the endogenous anticoagulants, antithrombin (AT), protein C, and protein S. Among these conditions, FVL and Pm are relatively common, while the others are rare. FVL is a point mutation (G1691A), resulting in an altered factor resistant to inactivation by protein C. The Pm leads to a 20% to 50% increase in plasma prothrombin levels (Tempelhoff *et al.*, 2016).

Also elevation of procoagulant factors such as factor VIII, von Willebrand factor, and factors V, VII, IX and XI. These appear to be weaker risk factors for venous thromboembolism in general (Cushman, 2007).

2.4 Myocardial infarction (MI):

Myocardial infarction represents death of myocard cells due to irreversible ischemia progressing to necrosis (Thygesen, 2007). According to the World Health Organization's estimates, every year approximately 6 million people around the world experience a myocardial infarction, and the lethal outcome occurs in over 25% of cases (WHO, 2015).

Mortality rates for myocardial infarction are dropping in North America and many North and West European countries, while in Central and East Europe these rates are increasing (WHO, 2015).

Around two thirds of the myocardial infarction mortality rates declines in developed countries are due to reduced exposure to risk factors, while the last third is the result of adequate treatment and improved survival (WHO, 2011).

2.4.1 Etiology and risk factors of MI:

Etiology of myocardial infarction is complex and still not completely elucidated (Mahmood, 2014).

Age, smoking, hypertension, high cholesterol levels, diabetes mellitus and male sex are recognized as major risk factors for myocardial infarction. Other risk factors

include obesity, physical inactivity, dietary factors, positive family history and psychosocial factors (Milena *et al.*, 2018).

2.4.2 Pathophysiology of MI:

Approximately 90% of all cases of myocardial infarction are the result of acute thrombus, causing obstruction of an artery vessel in places of atherosclerosis plaque rupture. The etiopathogenesis of atherosclerosis plaque is still not clear. Several theories or hypotheses about atherogenesis have been proposed. The most probable is the unified theory in which atherosclerosis is caused by complicated interactions among cells of the endothelium, blood cells, and lipoprotein. Damage to the endothelium leads to its dysfunction and activation (Heusch and Gersh, 2017).

This activation is related to the increased expression of chemoattractant cytokines, lecithin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and adhesion molecules such as selectin E, intracellular adhesion molecule type 1 (ICAM1), vascular adhesion molecule (VCAM), and platelet endothelial cell adhesion molecule type 1 (PECAM1). These molecules help leukocytes adhere to the endothelium and migrate into the intima layer of the artery. This process increases inflammation in the vessel. Lymphocytes, macrophages, and other cells (e.g., smooth muscle cells) secrete many different cytokines, metalloproteinases, and factors involved in the progression and disruption of the lesion. The matrix metalloproteins MMP1, MMP2, MMP3, and MMP9 are involved in degradation of the extracellular matrix, weakening the fibrous cap and subsequently destabilizing atherosclerotic lesions. The fibrous cap overlies the athermanous lipid core. The disruption of the atherosclerotic plaque exposes the procoagulants (e.g., phospholipids, tissue factor or platelet-adhesive matrix molecules) that accumulate in the lipid core to blood, which triggers thrombosis. It has been hypothesized that impaired fibrinolytic function may also be a risk factor for ischemic events (Sakowicz, 2013).

2.4.3 Classification of myocardial infarction:

Myocardial infarction is generally classified into two types according to ST-segment elevation on ECG:

ST-segment elevation myocardial infarction (STEMI) and Non ST-segment elevation myocardial infarction (NSTEMI). The ST segment is that portion of the ECG cycle from the end of the QRS complex to the beginning of the T wave (Fig. 1). It

represents the earliest phase of ventricular repolarization. The normal ST segment is usually isoelectric (i.e., flat on the baseline, neither positive nor negative), but it may be slightly elevated or depressed normally (usually by less than 1 mm). Pathologic conditions, such as myocardial infarction (MI), that produce characteristic abnormal deviations of the ST segment are a major focus of clinical ECG diagnosis (Ary *et al.*, 2018).

Myocardial infarction was classified into 5 types in 2007 and this classification introduced as an important component of the universal definition. It suggests that a classification for patients with myocardial infarction based on cause to provide more sensitive markers of myocardial necrosis (Thygesen *et al*., 2012).
This classification differentiates between type 1 myocardial infarction, due to thrombosis of an atherosclerotic plaque, and type 2 myocardial infarction, due to an imbalance of myocardial blood supply and demand that may arise in many acute medical and surgical conditions. Patients with myocardial necrosis, but no symptoms or signs of myocardial ischemia are classified as acute or chronic myocardial injury. The other types include Myocardial infarctions presenting as sudden death (type 3), or after percutaneous coronary intervention (type 4) and coronary artery bypass grafting (type 5) are also defined (Chapman *et al*., 2017).

2.4.4 Clinical features of MI:

People who have heart attacks do not experience the same symptoms or the same severity of symptoms, it can be quite varied.
The most common symptoms of a heart attack include: pressure or tightness in the chest, pain in the chest, back, jaw, and other areas of the upper body that lasts more than a few minutes or that goes away and comes back, shortness of breath, sweating, nausea, vomiting, anxiety, a cough, dizziness and a fast heart rate. Chest pain is the most commonly reported symptom among both women and men. However, women are more likely than men to have: shortness of breath, jaw pain, upper back pain, lightheadedness, nausea and vomiting (Debra *et al*., 2005).

2.4.5 Diagnosis of MI:

The definition of MI requires cardiac myocyte necrosis with an increase and/or a decrease in plasma of cardiac troponin (cTn). At least one cTn measurement should be greater than the 99th percentile normal reference limit during: (1) symptoms of myocardial ischemia; (2) new (or presumably new) significant ECG ST-segment/T-wave changes or left bundle branch block; (3) the development of pathological electrocardiographic (ECG) Q waves; (4) new loss of viable myocardium or regional wall motion abnormality identified by an imaging procedure; or (5) identification of intracoronary thrombus by angiography or autopsy. Cardiac troponin (I or T) has high myocardial tissue specificity as well as high clinical sensitivity because cTn T and I are essential contractile components of myocardial cells and are expressed almost exclusively in the myocardium. Release of cardiac troponin from the myocardium can result from normal turnover of myocardial cells, myocyte apoptosis, myocyte release of troponin degradation products, increased myocyte wall permeability and bleb formation, or myocyte necrosis (Thygesen *et al*.,2012).

Troponin, expressed in ng/L or pg/mL and the blood sample for the measurement of cTn should be drawn during the initial patient assessment and repeated 3-6 h later (Taylor J, 2012).

The electrocardiogram (ECG) is a cornerstone in the diagnosis of MI and should be acquired and interpreted within 10 min after patient presentation (thygesen, 2007).

It also should be acquired at 15-30 min intervals, especially if the initial ECG is unclear. Wide spread and profound ST-T changes are associated with greater degrees of myocardial ischemia. The extent and severity of coronary stenosis, collateral coronary circulation and prior myocardial necrosis impact on the ECG manifestations of myocardial ischemia (Zimetbaum and Josephson, 2003).

2.4.6 Treatment of MI:

The patient must be admitted quickly to the hospital to receive the treatment that aims to unblock blood vessels, reduce blot clot enlargement, reduce ischemia, and modify risk factors with the aim of preventing future MIs. Reperfusion therapy which include fibrinolytic therapy (FT) and Primary Percutaneous Coronary Intervention (P-PCI) are the main treatment for MI especially STEMI. P-PCI, when performed is superior to FT. However, P-PCI is not universally available. When P-PCI is not available, FT is

an alternative. It reduced mortality and morbidity when carefully administered within 12 h of symptom onset. (Antman, 2008).

Fibrin specific agents such as tenecteplase, retaplase and alteplase are preferred and tenecteplase is the most fibrin specific. These fibrin specific agents are not antigenic (Reddy *et al*., 2015).

Aspirin and Clopidogrel which are an antiplatelet anticoagulant are given as a loading dose with the goal of inhibition the platelet aggregation caused by adenosine diphosphate, thus decreasing ischemic events (Hai-Rong *et al*., 2018).

Nitroglycerin remains a first-line treatment for acute myocardial infarction. It achieves its benefit by giving rise to nitric oxide, which causes vasodilation and increases blood flow to the myocardium (Ferreira and Mochly-Rosen, 2012).

B blockers which are a group of medications that block the adrenergic β-receptors that found on cells of the heart muscles, have been used for decades in the treatment of cardiovascular disease protect the heart from a second heart attack (MI) after a first heart attack (András and Szentes, 2017).

2.5 Previous studies:

A study was done by Hmimech *et al* (2016) among 100 moroccan MI patients and 182 apparently healthy controls investigate the potential association of C677T 5, 10-methylenetetrahydrofolate reductase (MTHFR) and G20210A factor II prothrombin (FII) polymorphisms with the susceptibility of MI. Following extraction by the standard salting-out procedure, DNA samples were genotyped by polymerase chain reaction-restriction fragment length polymorphism and the result show No significant association was observed between the C677T MTHFR and MI occurrence, and there was more heterozygote CT in the patient group compared to the controls.

Another study conducted by Alizadeh *et al* (2016) which performed a MEDLINE search to identify published case-control studies correlating (MTHFR C677) polymorphisms and MI risk. A total of 47 studies were finally included in this meta-analysis and the results showed no statistically significant association between C667T polymorphisms and MI risk. However, in subgroup analysis by ethnicity, the T allele of C677T polymorphism was associated with a 63% increased risk of MI compared

with the C allele (T vs. C, OR = 1. 63, 95%CI = 1.15–2.10, fixed effects) in African populations.

Nasiri *et al* (2014) conducted a case-control study in north of fars province consists of 54 patients with a history of MI and 54 genders matched control to investigate whether genetic polymorphism of MTHFR(C677T and A1298C) contributed to the development of MI. The result showed there was a strong positive relationship between the TT genotype and the risk of MI supported with a significant p-value 0.001(OR=11.87, 95%CI: 4.7-29.9).

A study done by Ucar and Erkut (2011) in the eastern black region of turkey concluded that the MTHFR C677T mutant genotype is a risk factor for patients of MI in this region.

Also another case-control study done by Majluf and Isordia (2010) included 167 of unrelated mexican patients under the age 45 with a diagnosis of STEMI and 167 unrelated controls subjects matched by age and gender and their results showed no significant difference in the genotype distribution between groups (p: 0.69) and the mutation is not a risk factor for MI.

Chapter III

MATERIALS AND METHODS

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Study design:

This study designed as an analytical case-control study.

3.2 Study area and duration:

This study achieved in the period from October 2018 to April 2019 at Alshaab hospital in Khartoum state.

3.3 Study population:

Sudanese Patients diagnosed with myocardial infarction at Alshaab hospital as case group and apparently healthy individuals as a control group.

3.4 Inclusion criteria:

All patients diagnosed with myocardial infarction by ECG and increased serum enzymes

All ages will be included.

3.5 Exclusion criteria:

Patients with other cardiac diseases.

3.6 Ethical consideration:

Approval was taken from Khartoum state ministry of health research department, Alshaab hospital and all participants.

3.7 Sampling:

2.5 ml of venous blood was collected in EDTA containing containers.

3.8 Sample size:

The sample was collected from 40 MI patients as (case) group and 40 healthy individuals as (control) group.

3.9 Data collection:

Data was collected from participants by using questionnaire.

3.10 Methodology:

3.10.1 DNA extraction by salting out method:

300µl of blood sample was placed in 1.5 ml Eppendorf tube,1000µl of RCLB(Appendix 1) was added to the blood, mixed well and centrifuged at 2500 rpm for 10 min, supernatant was discarded and the pellet (WBCs) washed again with 1000µl of RCLB many times until clear white pellet obtained. 300µl of SDS and WCLB (Appendix 2) plus 100 µl of 6M NaCl was added to the pellet and incubated at 56 c for 30 min. After incubation 200µl of cold chloroform was added to the mixture and centrifuged at full speed (14000 rpm) for 5 min. The aqueous layer was transferred to a clean Eppendorf tube and added a double volume of cold absolute ethanol, centrifuged at 14000 rpm for 5 min. The supernatant was poured without disrupting the precipitate and then washed with 600 µl 70% ethanol by centrifugation at 5000 rpm for 5 min, ethanol discharged an left the tube to dry, the pellet resuspended in 50µl TE buffer (Shokrzadeh and Mohammadpour, 2018).

3.10.2 Determination of DNA quality:

The quality and purity of DNA determined by gel electrophoresis (Appendix 7) by using a mixture of loading dye and part of the DNA solution.

3.10.3 DNA storage:

DNA preserved at – 20 c until PCR is performed.

3.10.4 Molecular analysis:

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

3.10.4.1The primers sequence used were as follow:

Reverse normal: TGCGTGATGATGAAATCCG
Reverse mutant: TGCGTGATGATGAAATCCA
Forward common: TCTCCTGACTGTCATCCCTA (Nasab *et al*., 2016).

3.10.4.2 Primer preparation:

1. Centrifuge the primer tubes, 8000 rpm for 30 seconds to get all the lyophilized DNA to the bottom of the tube.

2. Dissolving:

The primers that we ordered for our lab came as lyophilized materials in small tubes, and we re-suspend them with a specific amount of water to get a concentration 100 uM. The information regarding the specific amount of water that needed to add for each primer in an attachment information sheet which must comes along with the primers that ordered. For doing the dilution after finishing re-suspending the lyophilized primers, we prepared 1/10 dilution from the re-suspending primers to get 10 uM concentration and we stored them at -20C until PCR performed.

3.10.4.3 Master Mix preparation:

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

Table 3.1 Primers sequence

Primers	Sequence	CC	TT
Wild type R	TGCGTGATGATGAAATCCG	124bp	
Mutant type R	TGCGTGATGATGAAATCCA		50bp
Common F	TCTCCTGACTGTCATCCCTA		

Table 3.2 PCR protocol

Profile	Temperature	Time duration	Number of cycles
Initial Denaturation	95 c	5 min	1
Denaturation	95 c	30 sec	35
Annealing	51.8 for wild allele 50.7 for mutant allele	30 sec	
Extention	72	30 sec	
Final Extention	72	5 min	1

Table 3.3 Master Mix tube preparation for detection of wild allele:

Reagents	Volume
D.W	8 µl
Wild R	0.5 µl
Common F	o.5 µl
Master mix	6 µl
Template DNA	5 µl
Total reaction tube	20 µl

Table 3.4 Master Mix tube preparation for detection of Mutant allele:

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

3.10.5 Demonstration of PCR product:

Fourµl of PCR product was loaded in 1% agarose gel (Appendix 3) which stained with 1µl ethidium bromide for electrophoresis performance, 1X TBE buffer (Appendix 4) was used as running buffer. The voltage applied to the gel was 100 volt with time duration of 30 min. 25 bp DNA ladder was used as a molecular weight marker with each batch of samples. Finally, the PCR product was demonstrated by gel demonstration system.

3.11 Data analysis:

The data were analyzed by using Statistical Package for Social Sciences (SPSS) version 16 and the P-value will be considered statistically significant at 0.05 by using chi-square test which determines if the MTHFR mutation is a risk of MI.

Chapter IV

RESULTS

CHAPTER FOUR

4. RESULTS

4.1 Demographic data:

This study aimed to investigate the association between the MTHFR C677T polymorphism and myocardial infarction risk. A total of forty Sudanese patients with a history of MI and forty healthy subjects were enrolled in this study. Their age, gender was taken by questionnaire in addition to patient's family history and other risk factors (diabetes mellitus, arterial hypertension, smoking and hyperlipidemia).

4.2 Genotyping:

The distribution of MTHFR genotypes within case and control groups were shown in table (3.1). C allele represent the normal gene while T allele represent the mutant allele. The frequencies of CC, CT and TT genotypes within case group was 21 (52.5%), 17 (42.5 %) and 2 (5%) respectively, while this frequencies within control group was 25 (62%), 15 (37%) and 0% respectively.

The relative risk (RR) for MTHFR mutation was calculated (0.9) by SPSS program (95%CI: o.885 to 1.020). It was shown that there was no significant association between having this mutation and risk of MI occurrence.

There was no statistically significant association between MTHFR polymorphism and patient gender as shown in table (4.2). Also, there was no significant relationship between risk factors which patients suffer from in this study and MTHFR polymorphism according to table (4.3).

MTHFR genotypes distribution showed no significant difference between the case group and control group P. value (0.290) as shown in table (4.1).

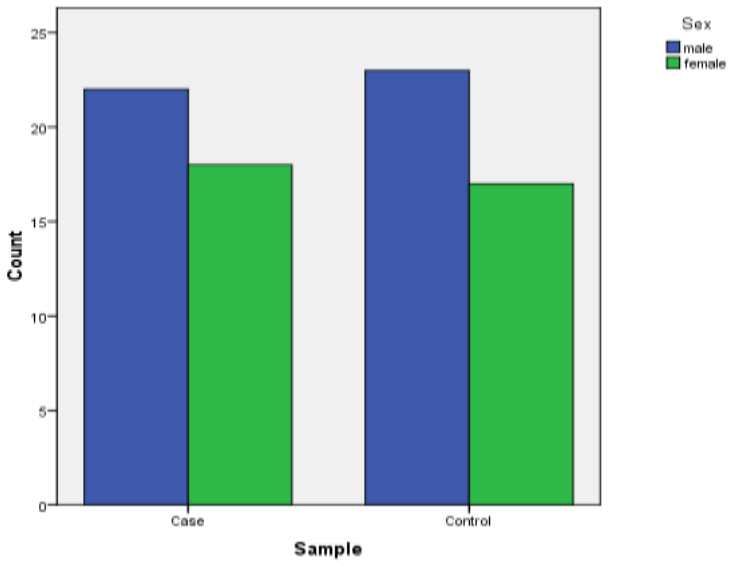

Figure (4.1) represents the Distribution of study group according to sex group.

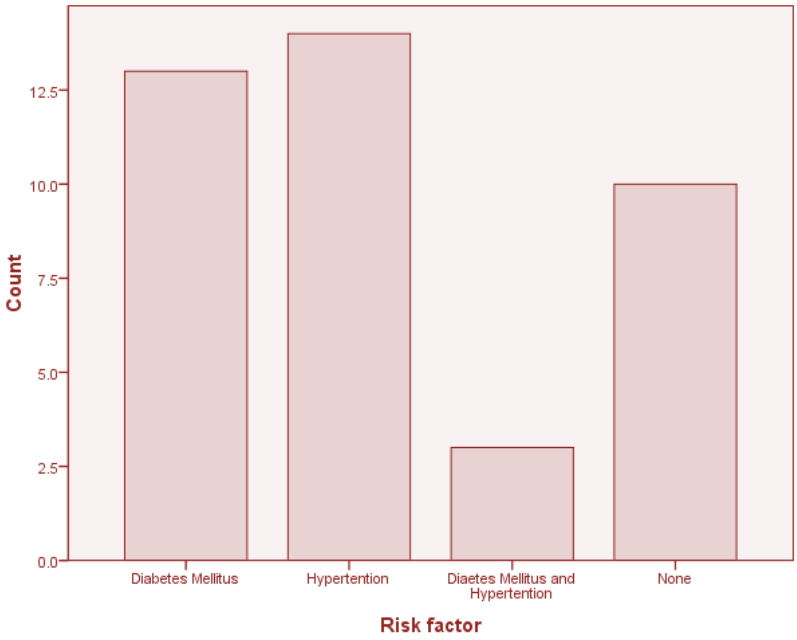

Figure (4.2) Distribution of case group according to risk factor group

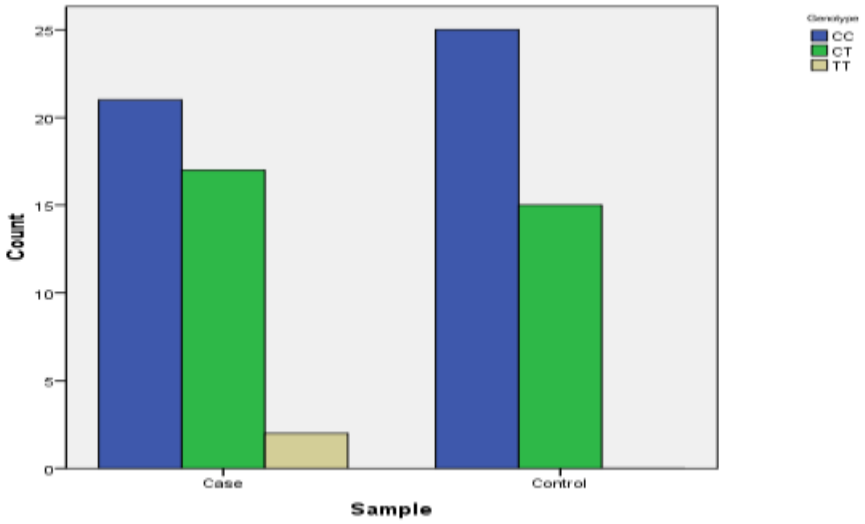

Figure (4.3) Distribution of study group according to genotype group

Table 4.1 Frequencies of MTHFR genotypes and RR:

Genotype	Case	Control	RR (95%CI)	P. value
CC	21 (52.5%)	25 (62%)	0.9	0.290
CT	17 (42.5%)	15 (37%)		
TT	2 (5%)	0 (0%)		

Table 4.2 Association between MTHFR genotypes and patient gender:

Genotype	Male	Female	Total	Pearson Chi-Square P. value
CC	13 (32.5%)	8 (20%)	21 (52.5%)	0.187
CT	7 (17.5%)	10 (25.5%)	17 (42.5%)	
TT	2 (5%)	0 (0%)	2 (5%)	
Total	22	18	40	
% of total	55.0%	45.0%	100%	

Table 4.3 Interaction between MTHFR genotypes and risk factors of MI:

	Diabetes Mellitus	Hypertension	Diabetes Mellitus and Hypertension	None	Total	P. value
Genotype CC	6	9	1	5	21	0.823
CT TT	6 1	4 1	2 0	5 0	17 2	
Total	13	14	3	10	40	

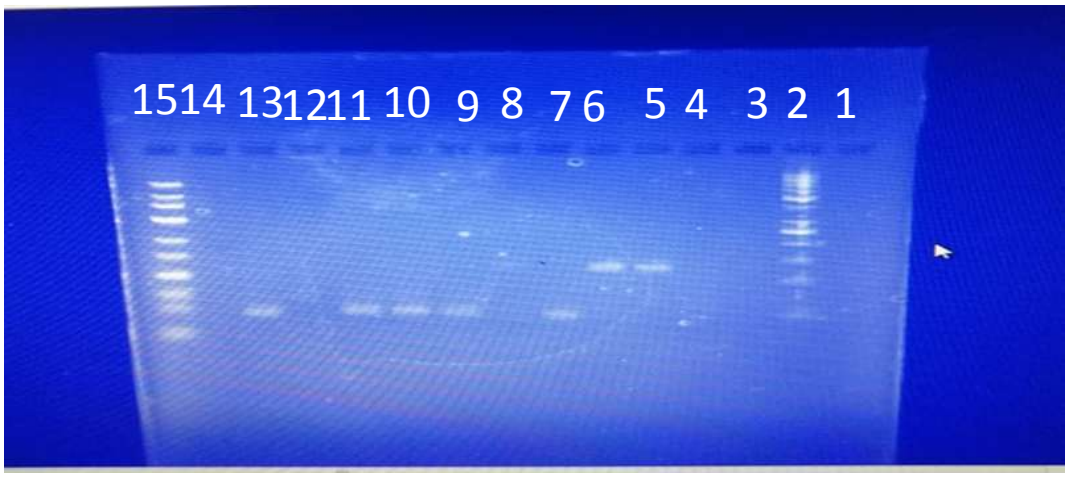

Figure (4.4) DNA fragment of MTHFR gene in gel electrophoresis in which lane 2 represents the 50 bp DNA ladder, lane 5 and 6 represent the wild allele (C) of 124 bp and lanes 7,9,10,11 and 13 represent the mutant allele (T) of 50 bp. The last lane repre

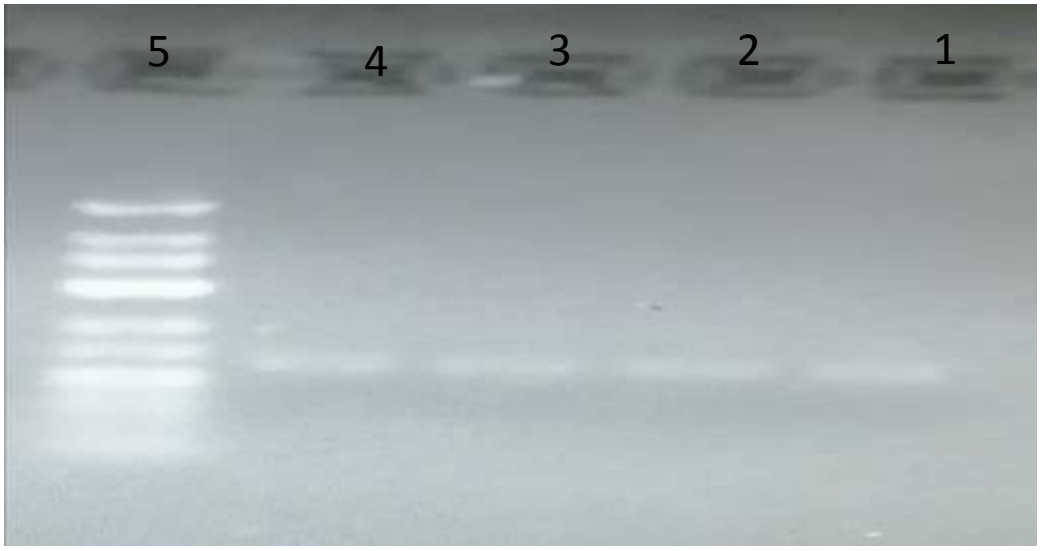

Figure (4.5) Show DNA fragments of MTHFR gene, the lane labeled 5 represent the 25 bp DNA ladder and the other lanes represent the wild allele fragmet oof 124 bp in length.

Chapter V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion:

Myocardial infarction (MI) was regarded as a polygenic disease with a high mortality rate that results from the mutual action of environmental and genetic factors. Several previous genome association and gene studies have been performed to identify MI susceptible genes and help susceptible individuals to prevent MI development. Different studies conducted to identify the role of MTHFR C677T polymorphism in MI risk and different conclusions have been reported.

This present study aimed to identify the role of MTHFR C677T polymorphism in MI risk in Khartoum state, it found that there was no significant association between MTHFR C677T polymorphism and the risk of MI, and the frequency of the mutant allele TT was few with 5% within the patients and there was more heterozygote CT (42.5%) within the patient group compared to the controls (37%). These results agreed with that reported by Hmimech *et al., (*2016) concluded that there was no significant association observed between the C677T MTHFR and MI occurrence, and there was more heterozygote CT in the patient group compared to the controls.

A study done by Ucar and Erkut, (2011) in the eastern black region of turkey concluded that the MTHFR C677T mutant genotype is a risk factor for patients of MI in this region and disagreed with our results. Also, Nasiri *et al*., (2014) conducted a case-control study disagreed with reported results and found that there was a significant strong positive relationship between the TT genotype and the risk of MI. A meta-analysis study done by Alizadeh *et al.*, (2016), the results showed no statistically significant association between C667T polymorphisms and MI risk agreed with reported results but, in subgroup analysis by ethnicity, the T allele of C677T polymorphism was associated with a 63% increased risk of MI compared with the C allele in African populations.

5.2 Conclusion:

On the bases of outcomes concluded that there was no significant association observed between the C677T MTHFR and MI occurrence, and there was more heterozygote CT in the patient group compared to the controls.

There was no significant variation between the C677T MTHFR genotypes distribution within case and control groups.

5.3 Recommendations:

On the bases of this study recommended that other studies should be conducted to identify the risk of MTHFR C677T mutation in the occurrence of MI and preferably accompanied by estimation of coagulation tests, homocysteine and B12 levels. Also, the number of samples should be increased.

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Appendices

Appendices

Appendix (1):

Molecular reagents:

RBCs lysis buffer consist of 8.3 g of NH4Cl, 1.19 g of NaHCo3 or KHCo3, 1.8 g of 5% EDTA dissolved in 1 liter of D.W.

WBCs lysis buffer contain of 7.88 g of Tris HCl, 5.44g of EDTA, 0.146g of NaCl and 10g of SDS dissolved in 500 ml of distilled water.

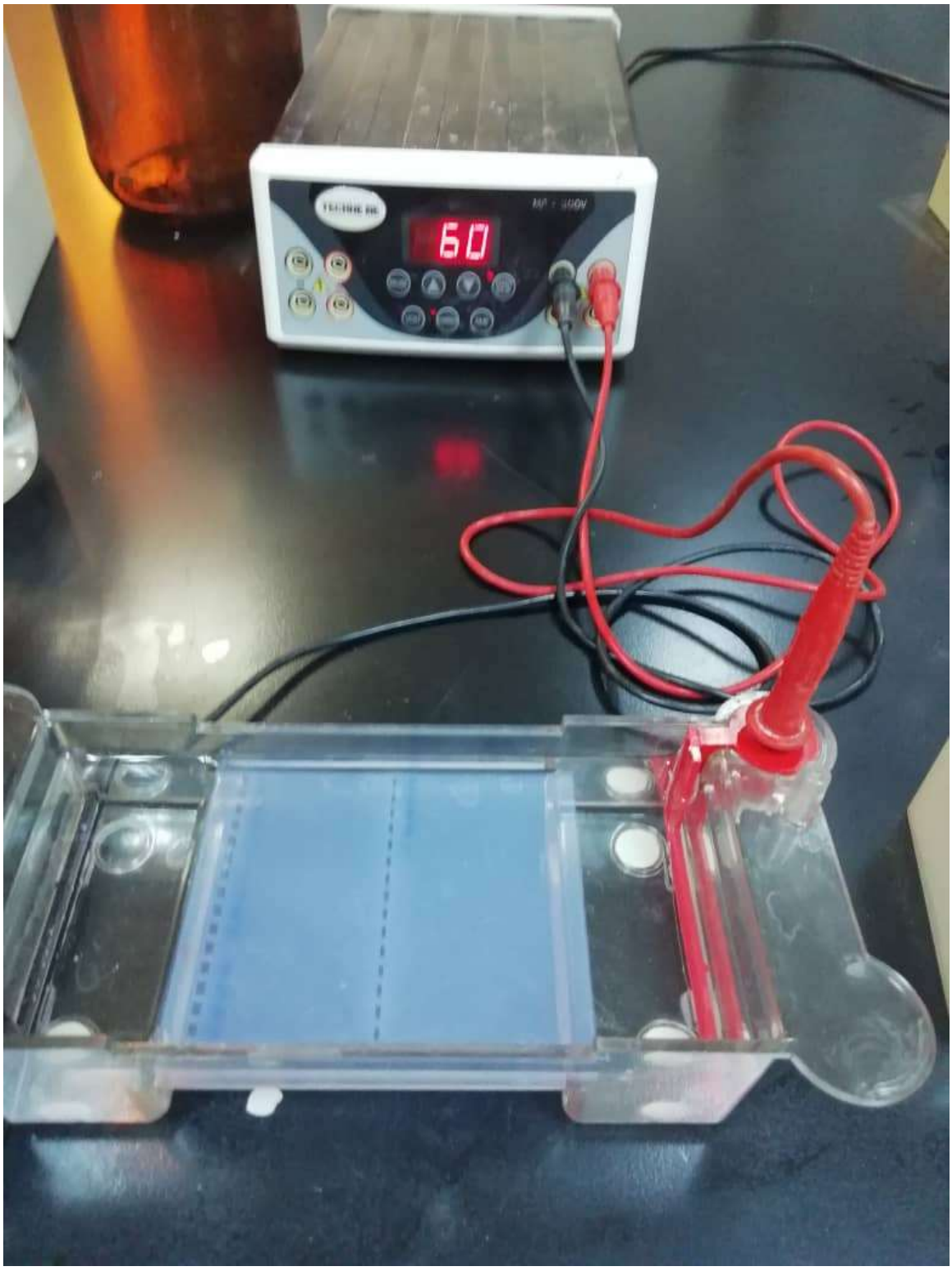
Agarose gel 1% prepared by dissolving 0.5 g of agarose powder in 50 ml of TBE buffer 1x and this solution must be heated in microwave for 3 min until the powder dissolve completely.

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

Appendix (2)

PCR machine

Appendix (3) Gel electrophoresis machine



الموافقة المستنيرة

.الاسم : ابتهال عثمان ابوزيد عثمان طالبة ماجستير بجامعة السودان للعلوم والتكنولوجيا

عنوان البحث : العلاقة بين الطفرة الجينية المتعلقة بانزيم ميثيلين تتراهيدروفوليت وخطر الزبحات القلبية.

الهدف من الدراسة : معرفة مدي اثر هذه الطفرة في حدوث الزبحة القلبية وتحديد نسبة وجودها في المرضي السودانيين .

الاجراءات : ساقوم بأخذ متبقي عينة الدم المتواجدة بمعمل المستشفى وسأجري عليها التحاليل المخبرية لمعرفة وجود الطفرة الجينية ومعرفة تأثيرها على حدوث الزبحة أو زيادة حدوثها ، ولا توجد أي مخاطر متوقعة على المشارك. كما سأجري البحث بسرية تامة من حيث المعلومات والوثائق الخاصة بالمريض.

سأقوم بأخذ المعلومات المتعلقة بالدراسة من ملفات المرضى الموجودة بالمستشفى بقسم الاحصاء وذلك بعد أخذ الموافقة من جامعة السودان للعلوم والتكنولوجيا ووزارة الصحة ولاية الخرطوم ومستشفى الشعب التعليمي وسوف أقوم بأخذ المعلومات في أوقات لا تؤثر على سير العمل وسوف اقوم بالمحافظة على الملفات وارجاعها لمكانها بعد الانتهاء من أخذ المعلومات.

سأقوم بتقديم النصح والتوعية بخطر اهمال معالجة الامراض وأخذ الدواء التي تعتبر سبب في حدوث الزبحات كمرض السكري وضغط الدم وزيادة الكولسترول والسمنة.

ستكون مشاركتك في البحث طوعية ورفضك المشاركة في البحث لا تفقدك الحق في أية فوائد من البحث أو العلاج .

من حقك الانسحاب من البحث في أي وقت والتوقيع على طلب الانسحاب.

يمكنني في ظروف معينة ايقافك من البحث دون أخذ موافقتك.

العدد التقريبي للمشاركين في البحث هو 50 مشارك.

لأي استفسار يمكنك الاتصال بي علي الرقم:

0927261920

Sudan University of science and technology

College of graduate studies

Association of Mythylenetetrahydrofolate Reductase C677T polymorphism and Myocardial Infarction Risk among Sudanese patients in Khartoum State

Code number:

Doctor unit:

Age:

Gender:

Male □

Female □

Diagnosis of MI:

STEMI □

Not STEMI □

Medical history:

Arterial blood pressure □

Diabetes Mellitus □

High cholesterol □