An-Najah National University

Faculty of Graduate studies

Association between Genetic Thrombophilia Mutations and Homocysteine Level on In Vitro Fertilization Outcomes Effects on Positive Pregnancy Results.

By

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Dedication

For all those who encouraged me to fly toward dreams.

My man Luai

For his advice, his patience, his bearing, and his support.

My father and mother,

Whose affection, love, encouragement and prays of day and night, make me able to get such success and honor.

My kids Tia and Jad,

Without whom this work would have been completed two years earlier.

I Dedicate My Work

Acknowledgment

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الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Association between Genetic Thrombophilia Mutations and Homocysteine Level on In Vitro Fertilization Outcomes Effects on Positive Pregnancy Results.

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
APC	Activated protein C
APS	Antiphospholipid syndrome
ARMS-PCR	Amplification Refractory Mutation System
ART	Assisted Reproductive Techniques
AZF	Azoospermia factor region
CAVD	Congenital absence of the Vas Deference
CBS	Cystathionine B-synthase
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CGS	Cysteinylglycine
DNA	Deoxyribonucleic Acid
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
FII	Prothrombin
FVL	Factor V Leiden
GnRH	Gonadotropin releasing hormone
HCG	Human chorionic gonadotropin
Hcy	Homocysteine
ICSI	Intracytoplasmic sperm injection
IUFD	Intrauterine fetal death
IUGR	Intrauterine growth restriction
IUI	Intra Uterine Insemination
IVF	In Vitro Fertilization
LMWH	Low molecular weight heparin
MAT	Methionine Adenosyltransferase
MTHFR	Methylenetetrahydrofolate Reductase

NK	Natural killer
NTD	Neural tube defect
OAC	Oral anticoagulant
PCOS	Polycystic ovarian syndrome
PCR	Polymerase Chain Reaction
RPL	Recurrent pregnant loss
SAHH	Adenosylhomocysteine hydrolase
THF	Tetrahydrofolate
UFS	Unexplained sterility patient
VTE	Venous thromboembolism
WHO	World health organization

Association between Genetic Thrombophilia Mutations and Homocysteine Level on *In Vitro Fertilization* Outcomes Effects on Positive Pregnancy Results.

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Abstract

Fertility is the normal capacity to produce offspring from two male and female cells. However, the lake of the ability to reproduce takes a place in the fertility problems, which needs an assisted reproductive technique called in vitro fertilization. That involves the injection of the male sperm in the female oocyte in embryological laboratory after treatment, to increase the chance of pregnancy. Many factors arises the need to these techniques, whether it was male causes; such as the number, motility, shape of sperm cells or a female causes; as low of fertility because of, endometriosis, ovulation disorders, mutated genes which leads to IVF failure.

Thrombophilia term means the accumulation of abnormal blood clots in the blood vessels, this clots may pass through the blood stream and cause (venous thromboembolism), or may reach the lungs causing (pulmonary embolism) according to inherited or acquired causes.

Homocysteine is an amino acid produced by the body, it is safe if founded in the normal range, but the elevated level meaning a many problems in the

body included the narrowing of heart vessels. In this study blood samples collected from 20 women failed in IVF treatment and tested for the presence of the four mutations, then DNA extracted and certain regions of the genes were amplified using ARMS-PCR technique.

The results shows that the patients with these mutations (FVL, FII, MTHFR 677, and MTHFR 1298), have also an increase in the homocysteine level because of the decrease in the enzyme, which breaks down the Hcy so that will arise the pregnancy complications. Such patients need a folate and many B vitamins to pass their genetic problems.

The aim of this study is to develop a rapid and cost effective method to screen inherited thrombophilia mutations in Factor V Leiden G1691A/R506Q (FVL), prothrombin G20210A (FII) and methylenetetrahydrofolate reductase (MTHFR) C677T/ C1298A. to check the prevalence screening of genetic thrombophilia mutations among a selected samples of Palestinian women's in IVF cycle. Then, to correlate the level of homocysteine in patients with or without the inherited thrombophilia mutations and the effect on IVF success (positive pregnancy after treatment).

Keywords: Homocysteine, Prothrombin, ARMS-PCR, mutations, IVF.

Chapter One
Introduction

1.1 Infertility

In general Infertility, term defined as the inability to get pregnancy in a normal state with unprotected intercourse, affects about 15-20% of the couples trying for pregnancy. Which is mean after 12 months of regular and undefended ejaculation in a woman under age 35 years, because of the male or female causes. some studies show that the male causes of infertility estimate about 20-30%. However, female causes are 20-35%, and 25-40% due to complex problems in both of couples. The male causes appear in the problems of semen itself and the channels that carry it, whereas the female causes are lot according to her biological clock and physiological state (1). Etiological factors acting at pre-testicular, testicular, or post-testicular level that is alter sperm production and function (15).

In addition, folate metabolic pathway plays an important role in the physiology of the cell as in nucleotide synthesis, repair and methylation of DNA, and stability of the genome. However, any defect in such pathways due to the insufficient folate intake, or gene mutations causing infertility complications (6).

Treatment methods are varies according to the infertility case, either male or female structural problems, functional or genetic disorders, hormonal dysfunction, or immunological disorders. The most common causes of infertility are located in three main factors: the first factor is male agents, in

either sperm production or quality. The second factor is ovulation or hormonal production, the last one is the joining and the transportation of sperm and egg into the uterus (13).

However, if there are no problems in the male and female reproductive system anatomy, but there are mistakes in the fertilization itself, the medical treatment will be the best solution. The physician will suggest an assisted reproductive technique's (ART); either intrauterine insemination (IUI), (which includes the semen transferring immediately to the uterus by long catheter with or without fertility drugs). Alternatively, in vitro fertilization (IVF) which means that eggs from womancollected and fertilized with the man sperms in the laboratory tubes.Thenreturned to the uterus. In addition, other techniques like Intracytoplasmic Sperm Injection (ICSI) (16).

Generally, ART techniques involved many steps, which starts with the stimulation of ovaries by drugs (to increase the number of matured eggs) that includes gonadotropin releasing hormone agonists (GnRH), pergonal, clomid, or human chorionic gonadotropin (HCG).Then the physician extracted the eggs and fertilized them in the laboratory, and after few days of being a good embryo, the fertilized eggs retrieved to the uterus, in a process called embryo transfer.However, there are factors that affected on the success or failure of IVF process will discussed below.(16)

1.1.1 Male and Female Infertility

The infertility prevalence estimated as 15% of couples. The male causes have many reasons but the genetic abnormalities accounts for 15%-30%, including the chromosomal abnormalities, Y chromosome micro deletions, autosomal gene mutations, polymorphisms, and the epigenetic errors (2).

The chromosomal abnormality accounts for 5% of male infertility. It is include the incorrect chromosome number, either Aneuploidy (the high incidence of aneuploidy appears in men with nonobstructive azoospermia), Or Klinefelter syndrome in its two forms: no mosaic, 47, XXY and mosaic, 47, XXY/ 46, X. which is considered as the most common chromosomal abnormality that has a prevalence of 5% in men with severe oligospermia and 10% in azoospermic men. Moreover, the chromosomal translocations which is another source of aneuploidy that ends with the loss of genetic material (2).

The Y chromosome microdeletionof some genes that located on the long arm of Y chromosome in the euchromatic region, contain three major sub-regions called azoospermia factor region (AZFa, AZFb. AZFc AZFd). A deletion in these regions will effect on the male infertility because they play an essential role in spermatogenesis. Since, it is a major cause of azoospermia caused by the congenital absence of the vas deference (CAVD) which responsible for transporting sperms from the epididymis to the ejaculatory ducts (1). A deletion in the genes located in AZFa region and called (USP9Y, DBY) causes a Sertoli cell-only syndrome

characterized by the appearance of only Sertoli cells in the testes with a loss of spermatozoa in the ejaculate. However, a deletion in the genes (RBMY, PRY) which is located in AZFb region causes arrest of spermatogenesis at the primary spermatocyte stage. Since, it codes for an RNA binding proteins (2).

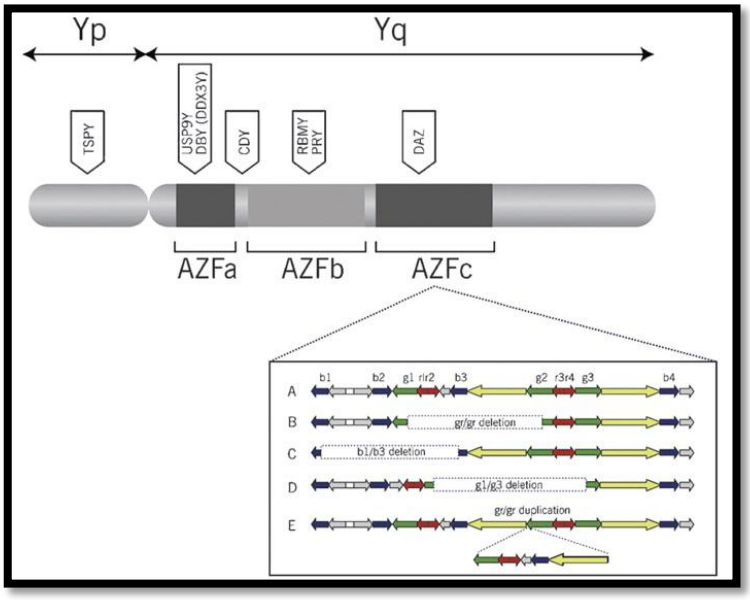

Fig.(1): schematic illustration of the Y chromosome and the AZF sub-regions: The Y chromosome micro deletion of some genes that located on the long arm of Y chromosome in the euchromatic region, contain three major sub-regions called azoospermia factor region (AZFa, AZFb. AZFc AZFd) (1).

A deletion in genes that located in AZFc region causes a reduction in spermatogenesis associates with the low sperm concentration. The intensity of deletion drives the spectrum of phenotypes ranging from oligospermia to azoospermia. A complete deletion in AZFc region may occur in order to a previous deletion in this region, or spontaneous deletion. Other genes in this region regulates translation, control of meiosis, and repairing the primordial germ cell population (2).

In summary, AZFb deletion regions cause severe defects in spermatogenesis associated with high diffusion of sertoli cell-only syndrome. Nevertheless, the deletions in AZFc region in men and their mate together are candidate them for intracytoplasmic sperm injection (1).

The epigenetic causes of male infertility refers to the modifications in the genetic code that does not effects on the basic DNA sequence. Such as the methylation, acetylation, ubiqitination of DNA which modify the regulation of transcription and then gene expression. Imprinting, the methylation of DNA is critical for normal embryo development, because it determining which genes from parental or maternal genomes expressed in the embryo. Every reproductive cycle the imprinted regions are reset. In order to allow the remaining of just one copy of a gene, a differential marking of DNA regions with histone modifications, methylation or both, in order to achieve the imprinting process (2).

Many Autosomal gene mutations and polymorphisms are also effects on male infertility. The Cystic fibrosis transmembrane conductance regulator gene (CFTR), a gene provides instructions for proteins that act as a channel across the membrane of cells that make mucus, sweat, saliva, and tears (1). Which is located on chromosome 7 (2), is an example of those gene mutations, if it is mutated it may cause an abnormal secretion of electrolytes and fluid across the epithelial membranes of most exocrine organs, diabetes mellitus, pancreatic insufficiency, and elevated sweat electrolytes (2).

Homocysteine is a thiol containing amino acid (11), called sulfhydryl amino acid that produced from the breakdown of methionine as it metabolized to cysteine (14); methionine is an essential amino acid and the only source of Hcy (7), that found in our foods like, meat, dairy food, seafood, and eggs. It can produced in the large amount even if the person is vegetarian, because the vegan proteins have a little amount of methionine, and vegetarian diets have less quantity of vitamin B12 which is important in a methylation reaction in reconverting the methionine back from homocysteine (3). As shown in this figure.

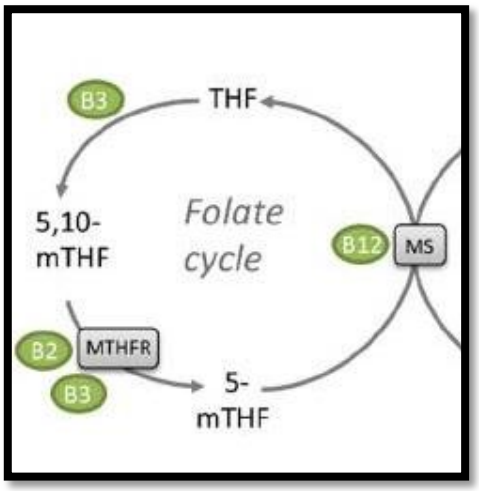

Fig.(2): Folate cycle: a folic acid will reduced to tetrahydrofolate (THF) after be imported to the cell. Then converted immediately to 5, 10-methylene THF through a serine hydroxymethyltransferase (SHMT). An enzyme that depends on vitamin B6 and uses serine as a one-carbon donor. (3)

The low level of B6 and B2 also affected on rising the homocysteine level according to trans-sulphation reaction (3).

Major cause of homocysteinemia is according to the imbalance between folate, cobalamine, pyridoxine, and methionine or variations in the genetic material. The rates of implantation and pregnancy success in women with low follicular Hcy level are more than in women with high follicular Hcy. Moreover, it is available in pregnant women lower than in non-pregnant (34). A follicular Hcy levels reflects by the systemic levels. Since high concentration of Hcy in the follicular fluid (>9 µmol/L) decreases the quality of embryo by causing decreased division and increased fragmentation of cells. Good embryo quality does not always mean pregnancy because the implantation may not be accomplished (7).

Methionine is essential for fetal development; since, the embryo quality appears clearly when comparing the mean Hcy in embryos of grade 3 (bad quality embryo), which is higher than mean Hcy of embryos with grade 1-2 (good quality embryo). Because such increase, have a toxic effect on gonadal cells due to the high production of oxygen radicals. (37) However, low Hcy level associated with mature oocyte count if compared withnormal level.Suitable amount of oxidative stress is essential for achieving pregnancy (7).

Folate is a co-enzyme that is belong to the water-soluble vitamin B group (B9), B vitamins play important role in homocysteine metabolism and keep it in normal range. Since, the lower concentration attributed to folic acid supplement use, decreased albumin concentrations, hemodilution, and an increase in glomerular filtration rate (35). Moreover, these important

nutrients help the body convert food into energy, in addition to perform other important functions. Generally, B1 and B2 are important for healthy functioning of the muscles, nerves and heart. B3 helps regulate the nervous and digestive system (36). B5 and B12 are required for normal growth and development (36). B6 supports the immune system and aids the body in breaking down protein (36). B7 is involved in the production of hormones. B9 help cells make and maintain DNA (36).

It is vital for pregnant women to receive adequate nutrition, and B-complex vitamins, in particular, are important for the proper growth and development of unborn babies (36).

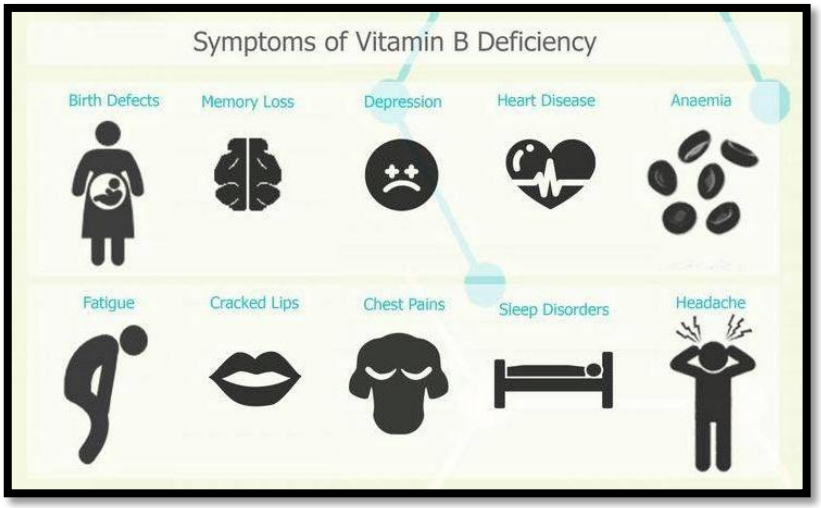

Fig.(3): symptoms of Vitamin B Deficiency: a defect in vitamin B may affect on the pregnant women and cause heart disease, then problems to the fetus, other symptoms like headache, fatigue, chest pains, anemia, depression and memory loss (36).

To date, a large number of studies have reported inverse associations between B vitamin status and circulating homocysteine concentrations (39). Folic acid supplementation consistently shown to lower homocysteine and

additional roles of vitamin B-12, riboflavin, and vitamin B-6 have identified (40).

In addition to their function in homocysteine metabolism, folate and choline are essential nutrients for fetal development. Folate is crucial for DNA and RNA biosynthesis (42), whereas choline has a number of key metabolic roles in lipid metabolism, cell membrane formation, and neurotransmission in the form of acetylcholine (43). Pregnant women, therefore, expected to have higher requirements for both folate and choline, thereby leading to lower status and higher circulating total homocysteine (tHcy) concentrations (43).

The B vitamins are involved in amino acid metabolism as purine and pyrimidine synthesis, methylation of nucleic acids, proteins and lipids. Therefore, it is necessary in production during a rapid cell growth periods and proliferation of the follicular and embryonic cells. On the other hand, the insufficient intake of the folate causes spontaneous abortions in early stages, birth defects and negative pregnancy outcomes (10).

Folate deficiency leads to increase deoxyuridine monophosphate misincorporation to DNA, disrupts the integrity of DNA, slows the replication of DNA and causes apoptosis and necrosis to the target cells. In summary, low dietary intake causes a defection in the DNA methylation, which altered the gene expression and causes the fragility of chromosome (10).

Such deficiency may result in an elevation in serum homocysteine concentration (Hcy) that called hyperhomocysteinemia. That is associated with several pathologies including pregnancy complications since Hcy is a mediator between the negative effect of insufficient folate level and the polymorphisms in genes offolate metabolism pathway (10).

1.1.2 Metabolism of Homocysteine

Methylation and demethylation on the CpG loci in DNA may affected on gene silencing or gene activation, respectively.In order to regulate mammalian gene expression and cellular differentiation. On the other hand, methylation of ribosomal RNAs interfere in mRNA function and integrity (12). In the beginning of metabolism pathway, amethionine with ATP catalyzed by Methionine adenosyltransferase (MAT) to make of S-adenosylmethionine (AdoMet) (17). The (AdoMet) donate a methyl group to DNA, RNA, Protein, and neurotransmitters for example. In order to produce S-adenosylhomocysteine (AdoHcy) which suppress the methyltransferase enzyme, but Adenosylhomocysteine hydrolase (SAHH) may hydrolyzes AdoHcy to make adenosine and homocysteine. Both of them metabolized of transported out of the cell to stop (AdoHcy) aggregation (12).

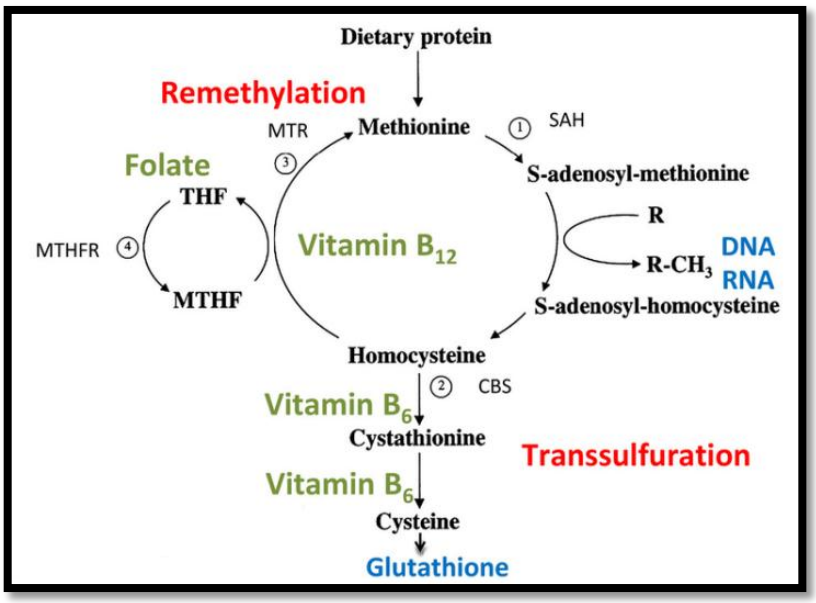

Fig. (4): Methionine-Homocysteine cycle: A re-methylation pathway defined as a conversion of homocysteine to methionine that catalyzed by methionine synthase (MTR) enzyme, a transsulfuration pathway involves a degradation of homocysteine to cysteine. Through two different enzymes, which is, depends on vitamin B6: cystathionine B-synthase (CBS) that catalyzes the condensation of homocysteine and serine to cystathionine (12).

If the AdoMet level is very low, the methylation activity will reduced. In addition, if women decreased her methionine intake, the risk of having neural tube defects (NTD) will increase. Due to the unfavorable (low) ratio between AdoMet and AdoHcy. Nevertheless, this ratio may restored if the methionine intake increased. The high level ofAdoHcy give a negative feedback on the Transmethylation reaction. Since homocysteine accumulation, may impair the methylation activity (12).

In the folate cycle, a folic acid will reduced to tetrahydrofolate (THF) after be imported to the cell. Then converted immediately to 5, 10-methylene THF through a serine hydroxymethyltransferase (SHMT). An enzyme that depends on vitamin B6 and uses serine as a one-carbon donor. However,

Methylene THF is then reduced by methylenetetrahydrofolate reductase (MTHFR) enzyme to 5-methylenetetrahydrofolate (mTHF). On the other hand, turned into 10-formyltetrahydrofolate (F-THF). Now, with a methionine synthase enzyme and its cofactor vitamin B12, the methylation of homocysteine will take place through a demethylation of mTHF to complete folate cycle and the carbon donated into the methionine cycle (3).

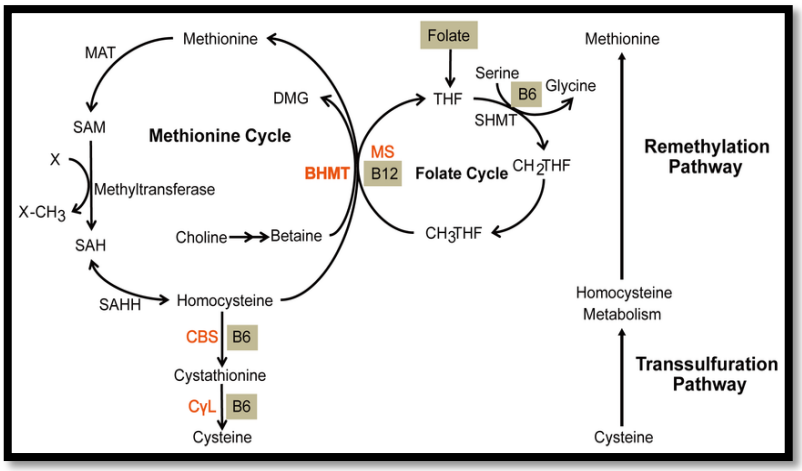

Fig. (5): Methionine-Folate cycle: the methylation of homocysteine will take place through a demethylation of mTHF to complete folate cycle and the carbon donated into the methionine cycle (18).

The metabolism pathway of homocysteine is complicated according to the enzyme participations in human body. It is included two different pathways, the first one is re-methylation but the other is trans-sulfuration (18). A re-methylation pathway defined as a conversion of homocysteine to methionine that catalyzed by methionine synthase (MTR) enzyme, which can binds the folate cycle with homocysteine metabolism. However, the enzyme needs cobalamin (Cbl) as a cofactor to form Cbl (I) MTR complex. Which links the methyl group of 5-methylTHF to compose methylcbl (III) MTR. Nevertheless, Cbl (I) MTR will reformed when the methyl group

transferred to homocysteine, and it can take another methyl group from 5-methyltettrahydrofolate (5-methylTHF) (3).

However, a transsulfuration pathway involves a degradation of homocysteine to cysteine. Through two different enzymes, which is, depends on vitamin B6: cystathionine B-synthase (CBS) that catalyzes the condensation of homocysteine and serine to cystathionine (19). Moreover, cystathionine v-lyase that catalysis the hydrolysis of cystathionine to cysteine and a-ketobutyrate (3).

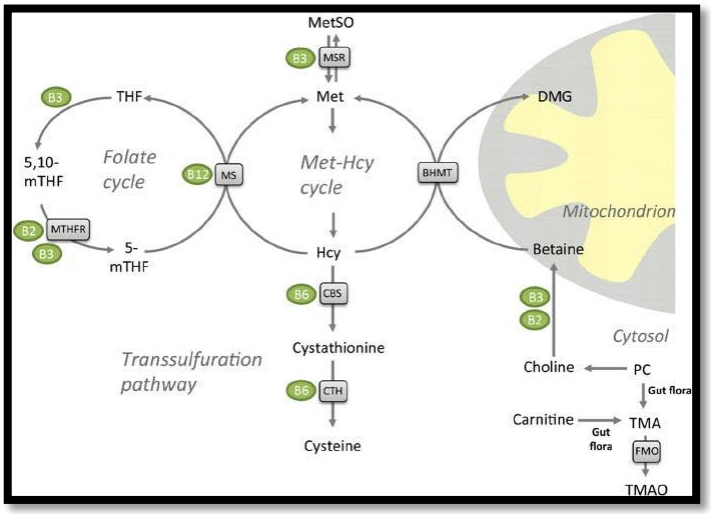

Fig. (6): Transsulfuration pathwaya transsulfuration pathway involves a degradation of homocysteine to cysteine. Through two different enzymes, which is, depends on vitamin B6: cystathionine B-synthase (CBS) that catalyzes the condensation of homocysteine and serine to cystathionine (24).

1.1.3 The Defects in Homocysteine Pathways

Any deficiency in these two pathways will causes an accumulation of homocysteine, which called hyperhomocysteinemia (21). The hereditary error of cystathione B-synthase enzyme is an autosomal recessive disease

includes a high concentration of homocysteine in plasma and urine (22). Effected patient with this type suffering from mental retardation and atherosclerosis ends with death. A woman who have this problem were exposed to lose her fetal about 50% (23). Nevertheless, the mild forms of hyperhomocysteinemia appears related to about 50% deficiency in the enzymatic activity of methylenetetrahydrofolate reductase (MTHFR) gene. Because of the homozygosity for the nucleotide 677 of C-to-T mutation, which causes thermolability of MTHFR and hyperhomocysteinemia. The frequency of this mutation is about 5-15% in the general population (23).

Nutrient related factors as; a deficiency of folate, vitamin B12, or vitamin B6 are another reasons for mild hyperhomocysteinemia. Because these factors are very important in the homocysteine pathways, and any deficiency in them causes an accumulation of homocysteine in the body. Since remethylation reaction, requires folate and vitamin B12, and transsulfuration requires vitamin B6 (24).

1.1.4 Hyperhomocysteinemia as a risk factor for many diseases

There are several causes of hyperhomocysteinemia, either insufficient acquired of the B vitamins, folic acid (Folate) and vitamin B12 or inherited defects in homocysteine metabolisms, or the polymorphisms in MTHFR gene. However, the insufficient intake of folate supplementation not only combined with the insufficient dietary intake but also the variations in maternal methylenetetrahydrofolate reductase was the major genetic determinant of hyperhomocysteinemia (9).

Some disorders, which combined with immune system activation, as cardiovascular, neurodegenerative and autoimmune diseases accompanied with hyperhomocysteinemia.Women with recurrent pregnancy loss may get a favorable pregnancy outcome when the Natural killer (NK) cells regulated down. Sincewomen with RPL get a positive result, when they had low the NK cells if compared with women whose suffer from subsequent pregnancies miscarried again (9).

The immune activation marker (Th1) and Neopterin (which is a disease marker that increase the production of reactive oxygen species) correlated well with homocysteine and reversely with folate concentration in case of coronary artery disease. Since homocysteine activates the immune system and promote the inflammatory process by stimulating Th1 cytokines, IL-6, IL-12, and IL-18. However, homocysteine promotes cytokine production in synoviocytes (cells that lines the inner surface of tendon sheaths and joints) as in rheumatoid arthritis (9).

A high concentration of homocysteine may affect the body in several ways like a cardiovascular disease and thrombosis (27). and also associated with microalbuminuria that is a strong indicator for the future risk of renal dysfunction (28). and cardiovascular disorders. Homocysteine prevents and declines the function of three main components of arteries: collagen, elastin, and proteoglycans. However, it affected on the structure and function of proteins as a cysteine disulfide bridges and lysine amino acid residues (29).

Furthermore, elevated homocysteine level linked with Alzheimer's disease (30). Since a high level of homocysteine and low level of vitamin B6 and B12 are risks for mild cognitive impairment and dementia (31). Inducing an oxidative stress causing schizophrenia (32). Moreover, it has also linked to increased fractures in elderly persons. Because it is an auto-oxidizes factor and interact with reactive oxygen intermediates (33). In addition, more diseases as atherosclerosis, thromboembolic, and neurodegenerative disorders. Other symptoms related to a number of developmental abnormalities, neural tube defects, and late pregnancy complications as pre-eclampsia, abruption placentae, intrauterine growth retardation, and intrauterine fetal death (3).

Moreover, the pathophysiological effect of hyperhomocysteinemia such as gene polymorphism of methylenetetrahydrofolate reductase (MTHFR), folate or Vitamins B6/B12 deficiencies due to low dietary intake or dysregulation in their metabolism. In addition, there are a strong relation between the recurrent pregnancy loss and deficiency of protein C, protein S or antithrombin. Alternatively, with other gene variants linked with thrombophilia as factor V Leiden and/or prothrombin A20210G. Finally, an antiphospholipid syndrome, which is an acquired condition, or clotting factor VIII that is increased plasma level, all of them are associated with recurrent pregnancy loss (RPL) (5).

Low folate associated with the binding of uracil with DNA instead of thymine, and the normal process of removing the misincorporated uracil

fails. That is lead to disturb the chromosome stability then promotes apoptosis (12). Since the folate deficiency, has a direct effect on the nucleic acid synthesis. The multiplication of rapidly dividing cells as precursors of male germ cell. The indirect effect being in accumulation of homocysteine that run in the male reproductive tract. Then, it will effect an inflammatory cytokines and altered the nitric oxide (NO) bioavailability, which combined with male infertility and poor sperm parameters. Some studies insights for normal sperm function and sperm-oocyte fusion a small amount of reactive oxygen species (ROS) needed but the excessive amount make an opposite effect in the fertilization and early embryo development (3).

1.1.5 The effect of hyperhomocysteinemia

The first harmful effect of hyperhomocysteinemia is free radicals produced from oxidation of Hcy that is toxic to the vascular endothelium. Furthermore, the Hcy interrupts the coagulation cascade and endothelium, becomes more thrombotic (25). The second effect is the decreasing synthesis and bioavailability of nitric oxide (NO) in the vascular endothelium. While NO participatory in all steps of female reproduction as; ovulation, early embryonic cleavage, implantation, organization of arterial pressure, uterine constriction and expansion (26).

1.1.6 Folates and homocysteine on female fertility

A concentration of serum methionine, vitamin B6, B12 is higher from their concentration in a follicular fluid. Because of the passive diffusion of blood plasma between theca interna and granulosa layer through the basement membrane, In addition to the active secretion by granulosa cells. The oocyte and granulosa cells depends on the theca capillary network to diffuse the blood plasma nutrient prior to ovulation (3).

A polish study demonstrated that the homocysteine concentration in the follicular fluid is lower in patients whom take a folate supplements and higher in women with endometriosis. In addition, there is a negative relation between the homocysteine concentration and the level of maturity of the retrieved oocytes. As well as a seminal homocysteine concentration, there is a negative relation with embryo quality after IVF on day three (20).

The correlation between folic acid and ovarian function demonstrated in 1960s. When the ovarian biopsy was taken from monkeys fed with prevented folate diets, it shows that a declination of graafian follicles, with an increase in cystic follicles and atretic, in addition to a depletion of granulosa cells, and a decrease or absence of the corpora lutea (3).

Most of studies shows that there is an inhibitory effect of estrogen on homocysteine production, after notice that the homocysteine level was higher in a bilateral oophorectomy patients, but lower in such patients in order to undergoing to the hormonal replacement therapy (HRT), but other studies did not found any significant effect. Moreover, there is a relation

between polycystic ovary syndrome (POS) and an increase in the cardiovascular problems, which assisted to the insulin resistance, then hyperandrogenism or hyperhomocysteinemia. The insulin sensitizers such as metformin can improve the ovulation induction, which is cause a further increase of serum homocysteine in such patients in spite of the decrease in insulin resistance (3).

Since a folate supplementations needs to increase the ovulation induction treatments for IVF. A patients with MTHFR 677 genotype polymorphism either CT type or TT type, required a higher doses of FSH stimulation, nevertheless, a number of collected oocytes and a concentration of serum estradiol was lower in such patients than in homozygous wild-type patients. Patients with MTHFR 677 CT genotype has a slightly but increased chance of getting a viable pregnancy than TT genotype. While MTHFR 1298 polymorphism with AA genotype combined with a better IVF outcome. Finally, according to recent study by Dobson in 2007 that demonstrated the importance of taking folate supplementation previously to perform the IVF trial in order to masked these polymorphisms (20).

Such mutations has another effect on pregnancy. Even on follicular growth or maturation, when researchers (54) found a lower prevalence of these mutations in women that became pregnant with a dichorionic twins. Then, a reduction in MTHFR activity associated with hyperhomocysteinemia, which in turn could inhibit polyovulation that responsible for a spontaneous dichorionic pregnancy. Furthermore, a poor level of folate even because a

low dietary intake or genetically polymorphism, lead to a miscarriage of dichorionic twins. Therefore, there is an opposite relation between a prevalence of MTHFR polymorphism and the rate of twin births. Such relation take a place when we describe the embryo availability (3).

1.1.7 Folate metabolism and early pregnancy loss (RPL)

Recurrent pregnancy loss is a heterogeneous condition, with many etiological factors as prothrombolic states, anomalies in uterine structure, chromosomal abnormalities, endocrinological defects (44), immunological problems as antiphospholipid syndrome, and thrombophilia (49). It observed in 5% of pregnant women. 50% with identified causes, and 50% with unexplained cause, which is, involves immunologic, thrombophilic, environmental factors and maternal hyperhomocysteinemia (9).

Because cystathione β-synthase CBS-deficiency associated with homocystinuria causing a severe hyperhomocysteinemia so presenting a spontaneous abortion with a rate of 50%. Moreover, moderate hyperhomocysteinemia (HHCY) considered as a risk factor for recurrent early pregnancy loss (RPL) that defined as a spontaneous abortion two or more times before 16 weeks of menstrual age (3).

Elevated levels of homocysteine is found more commonly in women with pregnancy complication such as: preeclampsia "pre-eclampsia: A complication of pregnancy that affect blood pressure and other organ system, diagnosed when high blood pressure and proteinuria , it is found in pregnant women who is beyond 20 week gestation" , placental abruption,

miscarriage and small, low birth weight babies. When the homocysteine level gets too higher especially during pregnancy it can cause a condition called hypercoagulability which mean that the blood clots much more easily than it should, not only put the pregnant women at a higher risk for a heart attacked and stroke, but it can put your baby in a danger as well. As small blood clots begin to develop in the uterus, the placenta can be cut off leaving the fetus void of the oxygen and nutrient supply it need to survive, this can induce a spontaneous abortion (38).

A karyotyping study must performed in order to check the reason of RPL or female sterility. Such as anatomic evaluation of the uterine cavity by transvaginal ultrasound, endocrinological assessment as screening for polycystic ovarian syndrome (PCOS), chronic inflammations to check an immunological diseases as rheumatoid arthritis, and alterations of homeostasis especially in thrombophilia cases as testing protein C, protein S, and antithrombin deficiency (5).

Briefly, homocysteine level and the availability of MTHFR C677T homozygosity were higher in unexplained sterility patients (UFS) and recurrent pregnancy loss (RPL) patients than control subjects were. Serum folic acid levels and vitamin B12 were lower in (UFS), (RPL) patients than control subjects (5).

1.1.8 Pathophysiology of folate deficiency-induced female infertility

A folate deficiency and homocysteine accumulation have a deleterious effect on female and male fertility, in reducing cell division either in oogonia during oogenesis or granulosa cells during folliculogenesis, a production of inflammatory cytokine, metabolism of altered NO, oxidative stress, already been demonstrated in other tissue as the endothelium and trophoblast (3).

Endogenous thiols affect fertility in sub-fertile couple. Since, the prevalence of subfertility is approximate 10% between couples; there are some factors that responsible for endogenous antioxidant that is involved in maintaining the balance between the pro-oxidant-antioxidant statuses in human tissues. A thiol glutathione (GSH) defined as such factors. However, cysteine (Cys), homocysteine (Hcy), and cysteinylglycine (CGS) are endogenous thiols. Since Cys is, a precursor amino acid of GSH and both acquired by food, or created as a metabolic product of Hcy. Cysteine and Glycine is a building block of cysteinylglycine, which is a main intermediate in the transport or synthesis of GSH. GSH is very important factor that is eliminate reactive oxygen species (ROS) (4).

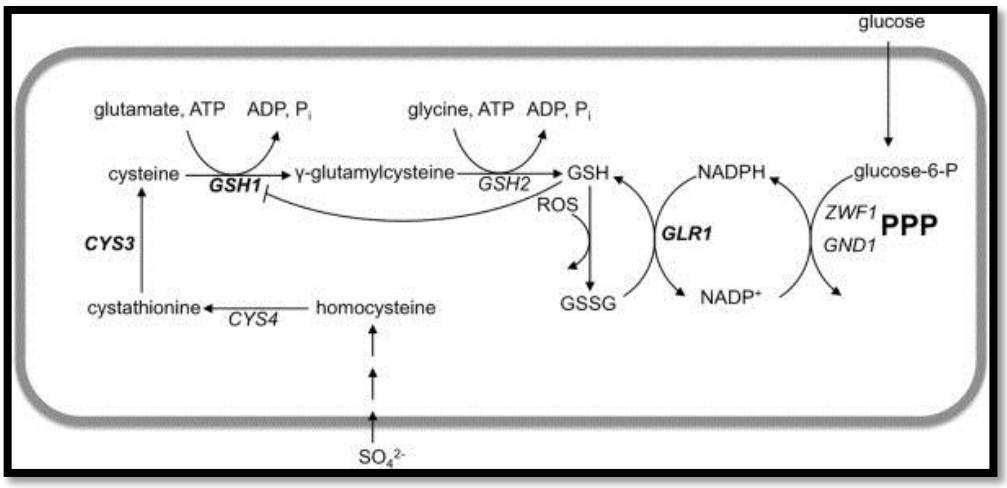

Fig. (7): Schematic summary of glutathione metabolism: Cysteine and Glycine is a building block of cysteinylglycine, which is a main intermediate in the transport or synthesis of GSH. GSH is very important factor that is eliminate reactive oxygen species (ROS) (4).

Thiols and reactive oxygen species (ROS) are important in human reproduction. Since spermatozoa give ROS that is, affect hyperactivation of spermatozoa, and the connection of spermatozoa with oocyte to be fertilized in the acrosomic reaction. On the other hand, the oxidative thiols influence the tail structure stabilization, motility of sperm, and protection of sperm DNA from physical or chemical damage. Although ROS is a very important factor, the excessive amount may lead to damage the DNA and plasma membrane (3).

Since in the final steps of spermatozoa production when spermatozoa lose its cytoplasm, therefore the defensive enzymes will be lost also, and these cells susceptible to ROS. When the peroxidation of lipids increased, the membrane fluidity will also altered. That causes a dysfunctional sperms through impaired metabolism, so acrosome reaction and the ability of sperm to combine with oocyte breaks up. Finally, as a result,

concentrations of sperm, its motility and abnormal morphology leading to loss fertility (4).

On the other hand, thiols and ROS are necessary in female reproduction. Since it involves in maturation of oocyte, luteolysis, production of progesterone by corpus luteum, and atretic regression of the group of newly grown follicles to prepare only one follicle for ovulation. In addition, excessive ROS amount leads to damage the plasma membrane of oocyte. Although the follicular fluid contains a free radical scavengers, which maintain the level of ROS in balance, and protect the embryo and oocyte (3).

1.2 Thrombophilia

Defined as an increasing tendency of blood for clotting in order to inherited causes (family history) which represented by genetic mutations in factor V Leiden, prothrombin 20210, MTHFR polymorphisms, Proteins C, S deficiencies, and antithrombin deficiency. On the other hand, The acquired causes (52).

A strong relationship between pregnancy complications such as severe preeclampsia intrauterine growth retardation, abruption placentae and stillbirth, recurrent miscarriage, and thromboembolism. That is associated with thrombophilia term since pregnancy is a hypercoagulable state, and women with thrombophilia exposed to thrombosis during pregnancy and adverse maternal and fetal symptoms. Meaning a rapidly abnormal tendency toward blood clotting or thrombosis (44).

Thrombophilias may be inherited or acquired conditions. Inherited type included three important subtypes. A mutation in Factor V Leiden called Adenine 506 Guanine (A506G) mutation combined with increased risk for venous thromboembolism, the heterozygosity of FVL mutation is responsible of 20-30% of venous thromboembolism events appear in 5% of the population. On the other hand, prothrombin mutation Guanine 20210 Adenine that linked with higher plasma prothrombin concentrations, increased risk for thromboembolism and cerebral-vein thrombosis. In addition, Homozygosity of methylenetetrahydrofolate reductase gene (C677T) mutation Cytosine 677 Thymine associated with increasing in plasma homocysteine concentrations that linked with thrombosis, which found in 5-15% of populations (44).

Venous thromboembolism (VTE) is a disease that includes deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE which is linked with inherited or acquired thrombophilia may amplified by a genetic factors as a homozygosity of mutation, presence of multigenic defects or thrombophilic abnormalities and other risk factors as post-surgical state and immobilization. Defects in placental hemostasis and vasculature are associated with preeclampsia, abruption placentae, intrauterine growth restriction (IUGR), intrauterine fetal death (IUFD) influence the morbidity and mortality of fetus or mom (49).

Endothelial dysfunction, vasoconstriction, placental ischemia (represented by the failure to deliver nutrients and oxygen to the placenta). Problems

that amplify the abnormal placental development, which combined with preeclampsia. The subsequent vasculopathy, secondary thrombosis from hypercoagulation and abnormal interaction between mother and fetus, caused by the abnormal invasion of trophoblast of the spinal arteries. Result in making small narrowed vessels, which act as a muscular wall for blocking the blood flow to the placenta and abnormal pregnancies because of that abnormality. Instead of the normal invasion of the trophoblast that is responsible for allowing such blood flow. These events result in inadequate perfusion of the intervillous space, preeclampsia, placental infracts, IUGR, IUFD and placental abruption (49).

To emphasize the role of thrombophilia in recurrent miscarriage (RM) there are a direct and indirect evidence. Since indirect evidence, women with history of RM noticed that they has a higher levels of thrombin antithrombin (TAT) complex at first and second trimester fetal losses, and a chronic state of endothelial stimulation that linked with the activation of coagulation system. In addition, they have an excess production of thromboxane at weeks 4-7 of gestation, meaning higher blood clotting and constriction of blood vessels. Moreover, deficiency in prostacyclin, which is a powerful vasodilator, at week 8-11 of gestation (52).

Direct evidence, 50-70% of women with first trimester recurrent miscarriage have an increased prevalence of antiphospholipid antibodies (APA), especially at the early period of pregnancy after the activation of fetal heart. Therefore, to improve the live birth rate, treatment with

antithrombotic therapy as, Aspirin and Heparin or low molecular weight Heparin should started in the first trimester of pregnancy (44).

1.2.1 Inherited mutations

Inherited thrombophilia associated with significant conditions as factor V Leiden, prothrombin mutations, Protein S, C deficiencies, MTHFR mutations, and anti-thrombin III deficiency (49).

1.2.1.1 The MTHFR polymorphisms

The methylenetetrahydrofolate reductase gene (MTHFR) is such a gene that located on the short arm of chromosome 1. This catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyl tetrahydrofolate. It is an important enzyme that essential in folate metabolic pathway, which plays important roles in DNA methylation and repair, synthesis of nucleotides, spermatogenetic process, and stability of the genome. Therefore, the deficiency in such enzyme exhibit hypomethylation of global DNA (since all of genes that interfere in spermatogenesis regulated by DNA methylation, and any alteration in methylation model resulting in global hypomethylation of a genome. That is demonstrate the relation between MTHFR gene and infertility). Hyperhomocysteinemia, increased S-Adenosyl homocysteine level (SAH), and compromised spermatogenesis (6).

In male fertility, The MTHFR enzyme altered methylation of sperm DNA in infertile individual, and epigenetic regulation of genes included in spermatogenesis. Such decreasing in MTHFR activity due to inadequate intake of folate and vitamin B12, or due to genetic modifications, which lead to hyperhomocysteinemia that might cause auto-oxidation resulting in oxidative stress (6). However, some studies did not found any relation between homocysteine and any type of MTHFR polymorphisms (11).

The intensive activity of MTHFR enzyme resulting in the excessive amount in homocysteine level. Since hyperhomocysteinemia associated with many pathological effects, as atherosclerosis, vascular disorders, defects in neural tube, Parkinson disease, pregnancy complications, polycystic ovarian syndrome, and male infertility. There are two common polymorphisms in such enzyme, c.677 C>T and c.1298 A0>C that is decreased the catalytic activity of the MTHFR enzyme and increased its thermolability (6).

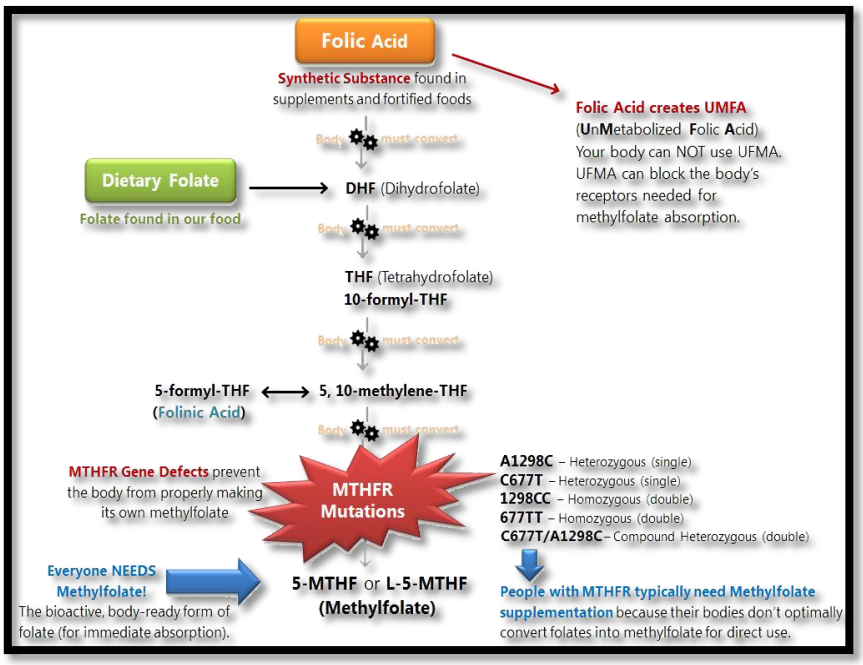

Fig. (8): The MTHFR enzyme altered methylation of sperm DNA in infertile individual, and epigenetic regulation of genes included in spermatogenesis. Such decreasing in MTHFR activity due to inadequate intake of folate and vitamin B12, or due to genetic modifications, which lead to hyperhomocysteinemia that might cause auto-oxidation resulting in oxidative stress (6).

The first polymorphism c.677 C>T resulting from the substitution of Alanine by Valine, the presence of T at 677 locusresponsible for increase the risk of infertility. The residuals activity of the enzyme is 30% in homozygous condition and 70% residual activity in heterozygous condition (6). Individual with TT variant is associated with neural tube defects (NTDs) because of this variant decrease the enzyme activity so they had higher level of Hcy than those with CC, CT variants did. Notably, they have a normal or high folate content but their methyl-THF level is low (12). Since the wild type CC genotype is the most beneficial genotype for health that is associated with lowest serum Hcy and highest enzyme activity and concentration (10).

However, maternal heterozygous CT genotype is beneficial compared with homozygous CC and TT genotypes with a greater proportion of good quality embryos, and a proportion of CD3-/CD56+ bpNK cells. Since the increased level of homocysteine tended to decrease the proportion of CD3-/CD56+ pbNK cells (9). On the other hand, CT genotype combined with an increased chance for positive HCG test and clinical pregnancy (10).

Fetuses with heterozygous genotype CT are more viable than fetuses with homozygous genotypes. The wild type CC genotype is associated with good cellular methylation reactions, nevertheless, the TT genotype is associated with folate cofactor balance that better supports DNA biosynthesis and decreases deoxyuridine monophosphate misincorporation into DNA (10).

Decreased embryo viability with the MTHFR 677 CC genotype maycaused by the increased hypermethylation of DNA linked with the more active form of the wild type MTHFR enzyme showing that the elevated concentration of methionine may have more effect on embryo survival than high homocysteine concentration (11).

Table 1: MTHFR 677 polymorphisms and their effect on the body (6).

MTHFR 677 polymorphisms	The effect on the body
Wild type CC genotype	Enzyme activity ⬆ Folate concentration ⬆ Efficient cellular methylation ⬆
CT genotype (heterozygous)	Positive HCG and get pregnancy ⬆ Viable fetus, embryo quality ⬆ Blood folate ⬇ More CD3-/CD56+pbNK
TT genotype (homozygous) (more severe= 2 mutations)	Folate cofactor balance to support DNA biosynthesis⬆ Deoxyuridine monophosphate misincorporation into DNA⬇ Homocysteine concentration ⬆ Folate concentration ⬇

However, the second polymorphism is c.1298 A >C that resulting from the substitution of Glutamic acid by Alanine which is reducing the enzymatic activity but lesser than the first polymorphism. Even though, A to C conversion at this locus does not affect infertility risk in Indian men. The same in Moroccan and French populations which found a weak relation between c.1298 A >C and male infertility. Nevertheless, Moroccan population found that the frequency of CC in infertile individuals was higher than fertile (6).

The variant C allele is associated with higher essential FSH concentrations and minimized response to ovarian stimulation. As CC genotype tends to decrease ovarian responsiveness to FSH stimulation compared with AA and AC genotypes (10).

Table 2: MTHFR 1298 polymorphisms and their effect on the body in destroying the folate metabolism, and arises homocysteine level. (6)

MTHFR 1298 polymorphisms	**Effect on the body**
Wild type AA genotype	Beneficial genotype for health
CC genotype (homozygous) (More severe=2 mutations)	Folate cofactoravailability that affecting DNA biosynthesis ⬇ Apoptosis ⬆ Ovarian responsiveness to FSH ⬇ FSH requirement ⬆

MTHFR polymorphisms can destroy folate metabolism, and arises homocysteine level. Infertile women that have polycystic ovarian syndrome PCOS are at a critical state of having higher homocysteine level at the same time diagnosed as repeated miscarriage compared with others. Some women have a higher level of homocysteine compared with another woman, which affected on the fertility and IVF success, and it considered as a hidden factor of infertility that can destroy the pregnancy success, because it is rarely tested before the fertility treatment (6).

1.2.1.2 Factor V Leiden mutation

Factor V Leiden is a point mutation caused by a substitution of arginine for glutamine at position 24.2 of chromosome 1 in the factor V gene. Meaning the site in which activated protein C cleaves factor Va. Which is disturb the procoagulant-anticoagulant balance. FVL is an important factor predispose its carrier to venous thromboembolism, if linked with a hypercoagulable state like pregnancy. Since it is the most common cause of primary and recurrent venous thromboembolism in pregnancy. Hence, its carrier

complain from early onset gestational hypertension and HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) (47).

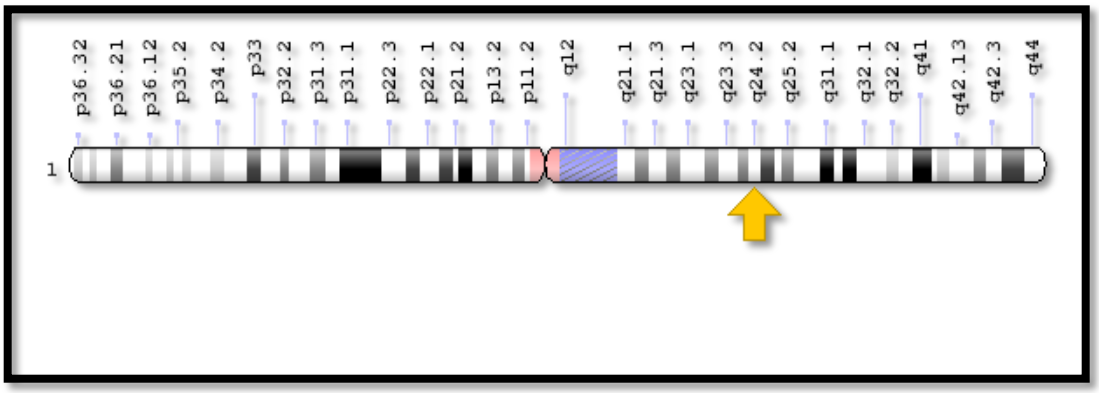

Fig. (9): Cytogenetic location of Factor V Leiden which is a point mutation caused by a substitution of arginine for glutamine at position 24.2 of chromosome 1 in the factor V gene (47)

The factor V Leiden is responsible for 95% of the activated protein C (APC) resistance. However, activated protein C is essential in maintaining balance between procoagulant and anticoagulant mechanisms. By binding the thrombin to thrombomodulin of endothelial cell surfaces. It can selectively degrades the coagulation factors Va and VIIIa, if protein S is present which is, another anticoagulant and cofactor to protein C (56).

Heterozygosity for the FVL is associated with 5-10-fold increase in the risk of thrombosis, while homozygosity increase the risk to 50-100-fold (47). Homozygous FVL have a long probability to cause high rates of morbidity and mortality (48).

Screening for factor V Leiden recommended under the following circumstances: personal or family history of venous thromboembolism, onset or recurrent pre-eclampsia, fetal growth resistance, fetal loss or

stillbirth and placental abruption without any symptoms. However, the mutation detected by isolation the patient DNA and amplified the exon 10 of the factor V gene by using an adequate primers, this 267 base pair gene segment contains 1691 nucleotides, a position where such mutation occur. Then, the amplified segment fractionated by size on the gel electrophoresis apparatus (47).

1.2.1.3. Prothrombin mutation

Prothrombin is a blood protein called factor II that responsible for blood clotting. A substitution of Guanine to Adenine at locus 20210 of a prothrombin gene (F2 gene) in a cytogenetic location p11.2 of chromosome 11. Causes a problem in the blood clotting. Prothrombin mutation (F2) is an inherited disorderrepresented by accumulationof blood clots in the blood vessels, impedes the flow in the deep veins of the legs, which defined as (deep venous thrombosis). These clots may pass through the blood stream and reach the lungs as (pulmonary embolism) (58).

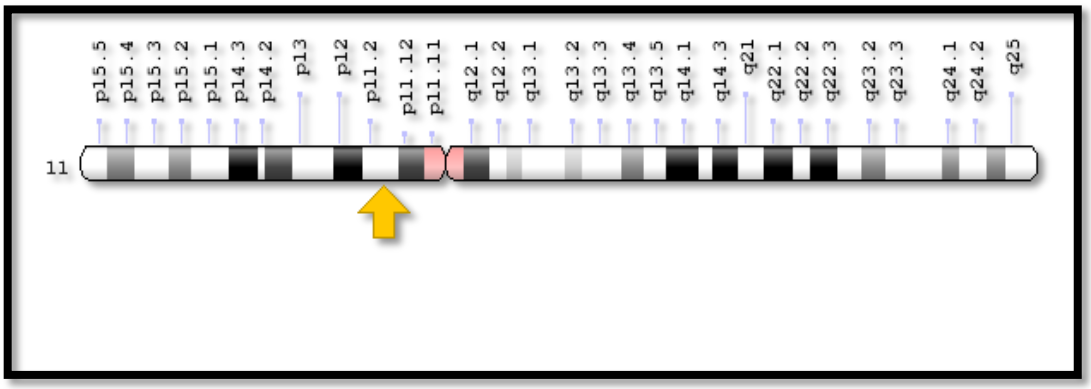

Fig. (10): Prothrombin mutation, substitution of Guanine to Adenine at locus 20210 of a prothrombin gene (F2 gene) in a cytogenetic location p11.2 of chromosome 11 (58).

The severity of homozygous polymorphism is higher than heterozygous. As mentioned above, the first symptoms of prothrombin-related thrombophilia is a venous thrombosis and pulmonary embolism (PE). In addition, the PE in patients with F2 heterozygous mutation is in a higher rate than FVL heterozygous mutation, because of the formation of isolated pulmonary emboli, and increasing the develop of VTE at a younger age. The heterozygosity of this mutation linked with the thrombosis of the upper part of the body such as thrombosis of the cerebral vein, portal vein, hepatic thrombosis, and the unusual locations as retinal vein thrombosis (57).

On the other hand, the heterozygosity is also associated with pregnancy complications in the second and third trimester as preeclampsia, restriction of fetal growth and abruption of placenta. Therefore, patients may predict to thrombosis from other symptoms by checking the numbers of F2 gene alleles, some co-existing genetic abnormalities like FVL mutation, F8 mutation, deficiencies in anticoagulant proteins like protein S, protein C, and family history (58).

1.2.2 Acquired thrombophilia

An acquired autoimmune disorder called antiphospholipid syndrome (APS) that characterized by elevated levels of antibodies that is act persistently against membrane anionic phospholipids (45). Such as lupus anticoagulant (LAC) and/or anticardiolipin antibodies (aCL) that is associated with (adverse pregnancy outcomes) represented by recurrent miscarriage,

thrombosis, preeclampsia, IUGR, and placental abruption. On the other hand, antiphospholipid antibodies (APA) linked with placental vascular thrombosis, decidual vasculopathy, intervillous fibrin deposition, and placental infraction. Such changes resulting in pregnancy complications as miscarriage, IUGR, stillbirth, and early severe preeclampsia (44).

1.2.2.1. Antiphospholipid syndrome (APS)

This syndrome is include deep vein thrombosis (DVT) and pulmonary embolism (PE), coronaryor peripheral artery thrombosis, cerebrovascular or retinal vessel thrombosis and pregnancy morbidity. Women with such syndrome, who have had prior thrombosis, must receive thromboprophylactic therapy during pregnancy and the post-partum period. Either Heparin (5000 IU) or LMWH and low dose Aspirin (0.1 g/day) is the recommended therapy. In high risk patients and DVT patients treated with LMWH (e.g.: Enoxaparin 1 mg/kg), but in post-partum period, LMWH replaced by Warfarin (44).

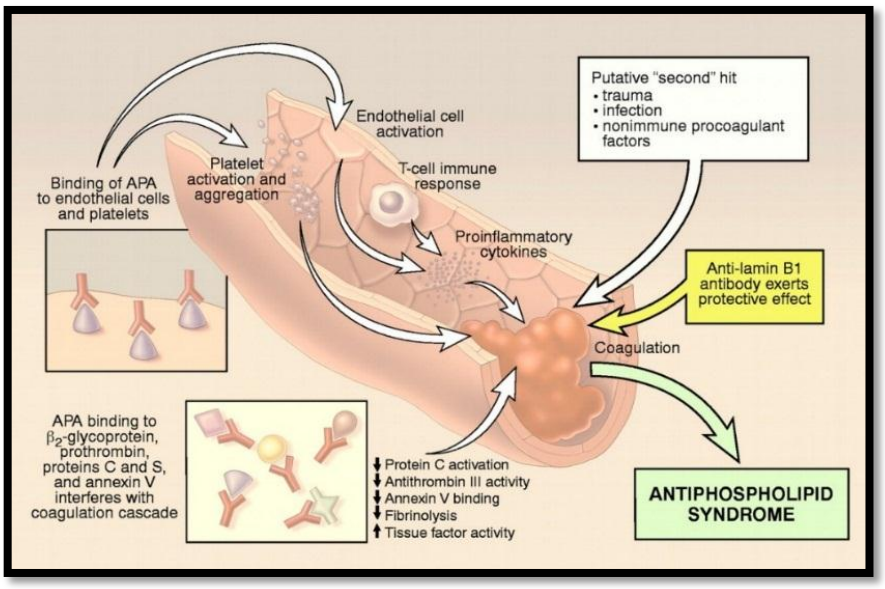

Fig.(11): Antiphospholipid syndrome is an autoimmune condition. This means the immune system, which usually protects the body from infection and illness, attacks healthy tissue by mistake. (44)

However, an acute venous thrombosis in an APS cases treated by higher doses of LMWH depending on the thrombotic event severity and history. During pregnancy, LMWH and UFH are given, and then they must stopped before delivery.After that,heparin therapy is reinstated with (oral anticoagulants) OAC. Low dose Aspirin (0.1g/day) recommended in women with APS in addition for prevention of arterial thrombosis (cerebral events). Whereas high dose of aspirin (0.3-0.5g/day) and full therapeutic doses of UFH or LMWH recommended for, women with APS associated with recurrent arterial or venous thrombotic events, and women who develop stroke or transient focal neurologic deficits during pregnancy (44).

UFH mechanism of action is differ from LMWH. Since, UFH is inhibits a blood protein called factor Xa and thrombin equally while, LMWH has a

higher effect against factor Xa only. Since, factor Xa is a blood protein that plays a role in blood clotting to stop bleeding (46).

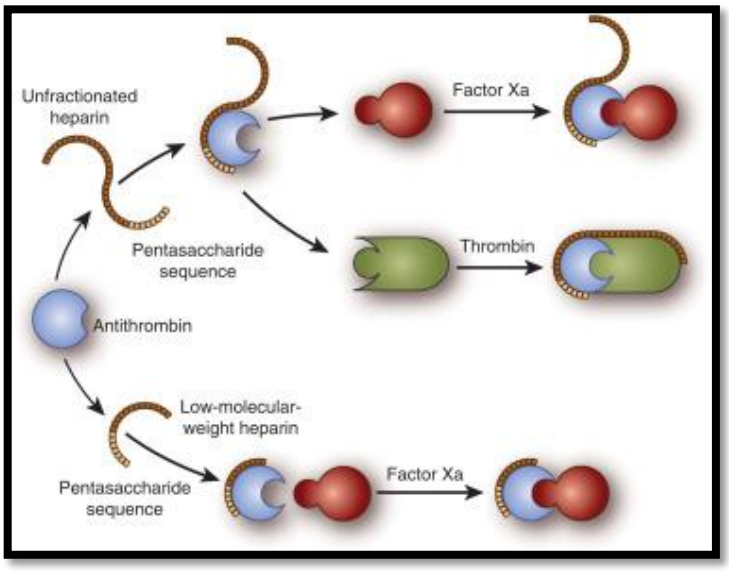

Fig.(12): Mechanism of action of unfractionated and low-molecular-weight heparin UFH is inhibits a blood protein called factor Xa and thrombin equally while, LMWH has a higher effect against factor Xa only. Since, factor Xa is a blood protein that plays a role in blood clotting to stop bleeding (46).

There is a relation between severe preeclampsia, which is a multigenetic disease, and thrombophilia. Since in a maternal with hypercoagulable state, the low-pressure villous blood flow may trigger placental fibrin precipitation to make a placental plug, which may stimulate development of early severe disease but not mild. Because a severe preeclampsia linked with FVL mutation, hyperhomocysteinemia, and deficiencies of protein S, C and antithrombin III (44).

Factor V Leiden mutation, activated protein C resistance (APCR); that acquired during the second and third trimester of normal pregnancy because of increases in FV and factor VIII and decreased protein S levels.

Moreover, Prothrombin G20210A mutation, and protein S deficiency are associated with early and/or late fetal loss, recurrent or not recurrent. In addition, women with both FVL and Prothrombin gene mutations are at a higher risk of getting venous thromboembolism (VTE) in pregnancy or puerperium more than women get only one mutation. Nevertheless, methylenetetrahydrofolate reductase mutations, protein C and antithrombin III deficiencies were not significantly associated with fetal loss (44).

1.2.2.2. Venous thromboembolism in pregnancy (VTE)

Venous thromboembolism is the existence of blood clot in the vein. It is included two types: Deep vein thrombosis (DVT) appears commonly in the left leg, and pulmonary embolism (PE) occurs if the vein blood clots breaks free and travels to the lungs, especially during the third trimester or the post-partum period. Pregnant women are at a risk of VTE six times more than non-pregnant. Hence, VTE is a major cause of death in pregnancy or puerperium (44).

Major factors, which is increase the risk of VTE in pregnant women, is obstruction of venous return by the enlarging uterus, venous atonia. In addition, the acquired prothrombotic changes include the rising of fibrinogen and FVIII, and functional resistance to activated protein C, then a decrease in protein S, and increases in plasminogen activator inhibitors 1 and 2, which decrease fibrinolysis and the activation of platelet. Other maternal factors as age, obesity, smoking habits, ethnicity and immobilization, contribute to the hypercoagulable state (44).

DVT range percent is 0.08-1.2 in vaginal delivery whereas 2.2-3.0 in cesarean section. So, women need for surveillance in the puerperium, because of the increasing proportion of both poet-partum DVT, and pulmonary embolism after delivery from hospital (44).

Table 3: Management of fetal loss associated with thrombophilia (44)

Category	Recommendation
Successful pregnancy with Prem Delivery	Prophylactic heparin 5000 IU. Or LMW I And aspirin 0.1 gm per day
Solitary thrombophilia	Enoxaparin 40 mg/day
Combined thrombophilia	Enoxaparin 80 mg/day
APS	Aspirin

Table 4: Management of adverse pregnancy outcome associated with thrombophilia (44)

Category	Recommendation
History of severe preeclampsi Abruptio placentae IUGR	Enoxaparin 40 mg/day And aspirin 100 mg/day (from 8-12 weeks ge

1.3 Management of women with VTE during Pregnancy

To manage the thrombophilia during pregnancy there are two main procedures. The first one is, primary prophylaxis in asymptomatic women, the other is, secondary prophylaxis of recurrences in women who have previously developed thrombosis, and the treatment of acute thrombotic cases. The diagnosis of VTE cases is a crucial point, since the implications not only influence the immediate pregnancy, but also for the future pregnancies. In addition, Heparin is a good choice for preventing and treating of VTE during pregnancy.Hence, it gradually replaced by low-molecular-weight heparins (LMWH). That is present a number of

advantages over unfractionated heparin (UFH). Since it improved bioavailability, longer half-life, facility of administration, no monitoring requirement and lesser side effects. On the other hand, LMWHs are not teratogenic or fetotoxic and do not cross the placenta (44).

Tables below demonstrate the recommended doses of VTE cases according to the severity state. The types of LMWH and how to use it.

Table 5: the treatment of VTE according to the severity of injury (44).

Category	Patients	Recommendations
Very high risk for VTE	Previous VTE on anticoagulant VTE in current pregnancy Antithrombin deficiency	LMWH (Enoxaparin twice day) mg/kg Or Heparin adjusted dose with confirmation of pregnancy
High risk for VTE	Previous VTE Protein C, S deficiency + familyHistory of VTE Homozygote FV or prothrombin mutation Combined thrombophilia	LMW Heparin (Enoxaparin) 40 mg/day until 6-12 weeks Postpartum or fixed dose Heparin
Moderate risk for VTE	Heterozygote FV or prothrombin mutation PS deficiency Family history of VTE	Postpartum anticoagulation LMWH (Enoxaparin) 40mg/day
Relatively low risk for VTE	Heterozygote FV or prothrombin mutation No personal or family history VTE	Monitor for additional risk for VTE

Since Aspirin treatment alone is not effective for patients whom suffering from recurrent miscarriage, and sole protein S deficiency together. LMWH defined as a beneficial choice to increase the livebirth rate in such case. Nevertheless, this treatment is failed in preventing other pregnancy complications as placental insufficiency, and intrauterine growth

restriction. Therefore, this table demonstrate the LMWH types and the recommended doses to get good results (49).

Table 6: Types of low molecular weigh Heparin, their commercial name, and its recommended doses (49).

LMWH	Commercial name	Recommended dose
Dalteparin	Fragmin	200 anti-factor Xa units/kg once a day
Enoxaparin	Clexane, Lovenox	1.0 mg/kg twice a day or 1.5 mg/kg once a day
Nadroparin	Fraxiparin	200 anti-factor Xa units/kg once a day
Tinzaparin	Innohep	175 anti-factor Xa units/kg once a day

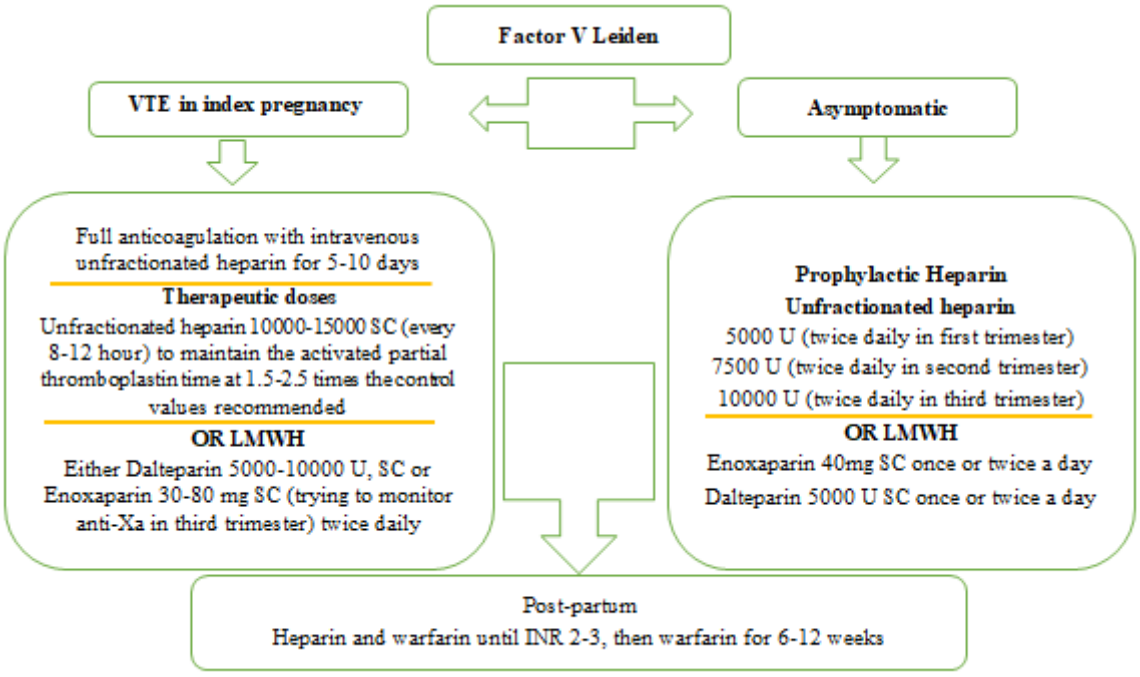

Fig. (13): Treatment of venous thromboembolism (VTE) in two types of paitents, with or without previous symptoms and family history, showing the perfect doses for each case (49).

1.4 Prophylaxis of thrombosis

Primary prophylaxis recommended for women without previous symptoms, but checked for the presence of FVL or prothrombin mutations, and deficiencies of protein C, S. these cases must take a prophylaxis doses at the last weeks of pregnancy and 2-6 weeks in the puerperium. A subcutaneous heparin (5000 IU twice a day) or LMWH (enoxaparin 40mg/day, or dalteparin 5000 IU/day). A monitoring of women with homozygote for FVL mutation be throughout pregnancy and post-partum, but women with both FVL and FII monitored in post-partum period (44).

Secondary prophylaxis recommended for women with previous thrombosisand other additional risk factors as hyperemesis, obesity, and immobilization, surgery, having preeclampsia, nephrotic syndrome, inflammatory bowel disease or infection. Such cases must receive thromboprophylaxis during pregnancy and puerperium, with heparin or LMWH (44).

1.5 Management of Hyperhomocysteinemia

Homocysteine level must be in the normal range in order to promote the cardiovascular health and supporting the neurotransmitter synthesis (59). Folate supplement may keep this favorable range and it is considered as a first treatment way. Especially in the patients whom has an insuffient amount of the enzyme, that converts folate to its active form 5-methylene tetrahydrofolate reductase. Therefore, Optimized Folate (L-Methylfolate) 1000 mcg, 100 vegetarian tablets must be taken to maintain a healthy homocysteine level. Each tablet contains

microcrystalline cellulose, dicalcium phosphate, croscarmellose sodium, silica, vegetable stearate and stearic acid (60).

The second treatment is the three B's injection, divided into two ampoules. One ampoule of 2 ml contains 100 mg Thiamine HCL (vitamin B1), and 200 mg Pyridoxine HCL (vitamin B6). Other ampoule of 1 ml contain 1000 mcg Cyanocobalamine (vitamin B12). The complementary action of these therapeutic vitamins limited to pain relief within the limits of hematological indications and metabolic disorders (61).

Chapter Two
Literature review

2.1 The relation between elevated homocysteine level and vascular diseases

The elevated homocysteine level is associated with atherosclerosis and venous thrombosis as discussed previously. Some studies illustrate the vascular toxicity of homocysteine and its mechanism, as the smooth muscles stimulated by homocysteine to proliferate and to build a collagen, the atherogenesis hallmarks. On the other hand, homocysteine affects on the anticoagulant system and the vascular endothelium in the negative form. The accurate effect of the homocysteine summarized in blocking the endothelial cell-surfacethrombomodulin expression and activity, blocking the binding activity of endothelial heparan sulfate proteoglycan of the antithrombin III. Hence, inhibiting the AT III anticoagulant effect. Blocking the endothelial surface fibrinolytic properties, by inhibiting the tissue plasminogen activator binding to cell-surface receptor (62).

Homocysteine stimulates the activity of Human umbilical vein endothelial cells (HUVECs) which is derived from the endothelium veins of the umbilical cord.that is increase the affinity between Lipoprotein and fibrin, making a link between lipoprotein, atherosclerosis and elevated homocysteine level (62).

Many studies demonstrate the relation between elevated homocysteine level and vascular disease. Some of these studies worked on elderly men at age (68-84 years old) that founded a strong relation between high level of homocysteine and cerebrovascular and coronary heart disease. Other studies included a young and middle-aged population, founded that there is a strong relation between higher level of homocysteine and myocardial infarction and stroke (63).

A study by Coen (55) done on 878 men within (64-84 years old) by checking their systolic and diastolic blood pressure according to the specific criteria included: the severe chest pain, changes on electrocardiography, elevation of specific enzymes. After that, a venous blood samples centrifuged for 60 minutes, then the serum homocysteine level was measured and given as whole numbers. Finally, they follow-up the patients status during 10 years until to find out the causes of death.

In the same study by Coen, 30% of patients had a high level of homocysteine which is mean, more than 17µmol/L (optimal range 11.4-15.8 µmol/L). other studies finding a strong relation between that high level and myocardial infarction and stroke. (63)

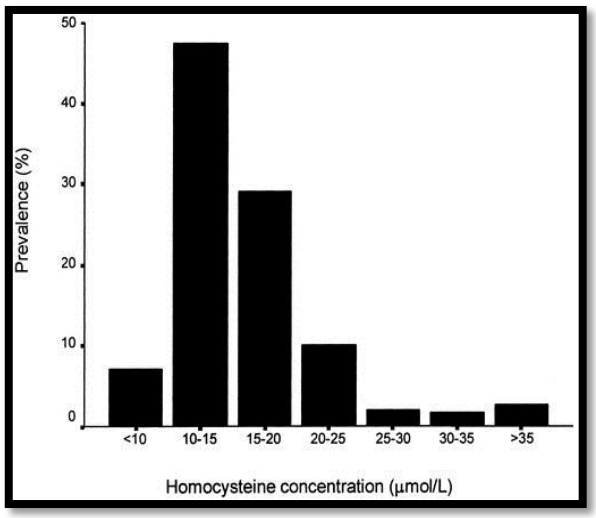

Fig. (14): The distribution of Homocysteine level in 878 elderly men (63).

To summarize the factors that affects on the homocysteine level, they starts with smokers people, black people more than white, hypertension, non-insulin-dependent diabetes mellitus and age. But a vitamin B complex and Folate treatment may decrease this high level.

Chapter Three
Material and Methods

3.1 Study population

The study population included fifteen women subjects (between 25-35 years old). experienced two or more previously failed IVF-embryo transfer cycles and tested for the presence of inherited (factor V Leiden (FVL) mutation, prothrombin G20210A mutation (FII), and methylenetetrahydrofolate reductase (MTHFR) C677T / C1298A mutation.

* Samples collected from patients in Al-Shunnar Center for Infertility Treatment and In Vitro Fertilization.

3.2 Blood samples collection

Peripheral blood sample (5 ml) collected in EDTA tubes, from patients who have undergone the IVF cycles and get a negative results. The blood samples collected from fifteen women prepared for IVF cycles at Al shunner Infertility and Genetics center.

3.3 Total DNA Extraction

The whole blood samples collected in an anticoagulant (EDTA) tube according to GenElute Blood Genomic DNA Kit (sigma-Aldrich Co.) and equilibrated to room temperature before beginning preparation.

3.4 Blood lysate preparation

A 20 µL of the proteinase K solution placed into 1.5 mL microcentrifuge (Eppendorf) tube and 200µL of the whole blood sample added to the tube and was vortex for 5-6 seconds to ensure thorough mixing of the enzyme. In addition, 20µL of RNase a solution added to catalyze the degradation of un-necessary RNA into smaller components. Then incubated for 2 minutes at room temperature. A 200 µl of lysis solution C added to the mixture and mixed by vortex carefully 15 seconds then incubated at 55ºC for 10 minutes. In addition, 200 µl of absolute (100 %) ethanol added to the lysate and mixed by vortex carefully for 5-6 seconds.

3.4.1 DNA Binding

A 500 µl of the column preparation solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at maximum speed 12,000 x g for 1 minute. Then, the flow-through liquid discarded. The lysate transferred into the treated column and centrifuged at 6500 x g for 1 minute, then the spin column was placed in a new collection tube and the old one was discarded.

3.4.2 DNA Washing

A 500 µl of pre-wash solution was added to the column and centrifuged at 6500 x g for 1 minute then placed in two new ml collection tube and the old tubes was discarded. The second wash was completed by adding 500 µl of the wash solution to the column then centrifuged 3 minutes at maximum

speed (12.000 x g). The old tubes was discarded but the flow-through liquid added to a new 2 ml collection tubes.

3.4.3 DNA Elution

A 200 µl of the elution solution was added to the center of the column, and incubated for 5 minutes at room temperature.Then centrifuged for 1 minute at 6500-x g to elute the DNA. A second elutionwas done by repeating the last step, with adding another 200 µl of the elution solution.Then incubated for 5 minutes at room temperature and centrifuged for 1 minute at 6500-x g in the same collecting tubes. The elute contains pure genomic DNA was stored at 2-8 ºC.

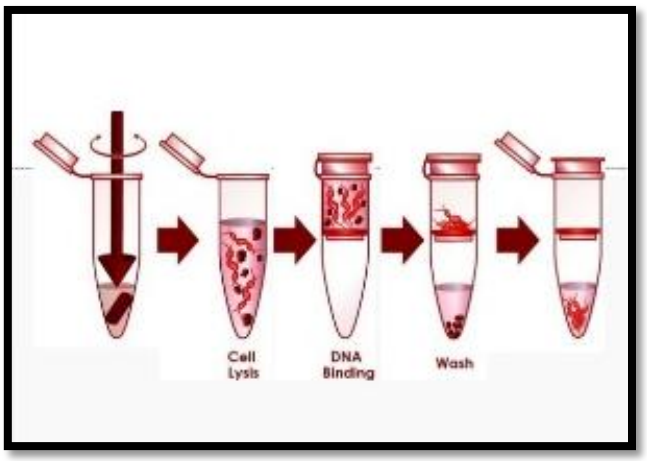

Fig (15): DNA extraction involves separating the nucleic acids in a cell away from proteins and other cellular materials.

3.5 Evaluation of the Extracted DNA

3.5.1 Spectrophotometric Analysis of the Extracted DNA

The concentration of the DNA was determined using Nano-dropper spectrophotometer machine from JENWAY Genova Nano at 260 nm.

3.6 Polymerase Chain Reaction (PCR)

PCR is a technique, depends on the using of a single copy or few copies of a DNA segment, and amplified to make a thousand to millions of a particular DNA sequences.

In our study, we chose Tetra-primers ARMS-PCR technique; it is a combined method that, collect the Tetra primer PCR (uses four allele-specific primers and the mismatch located in the middle of them). On the other hand, ARMS PCR technique (Tetra primer amplification refractory mutation system-polymerase chain. That is use five allele-specific primers, and the mismatches are lies in the 3' terminus of them). (51)

Therefore, Tetra-primers ARMS-PCR depends on using four primers (two forward and two reverse) and differentiated by its higher-specificity. Since the mismatch does not occur only on the 3' terminus, but also at the position-2 of the same allele-specific primer from the same terminus.

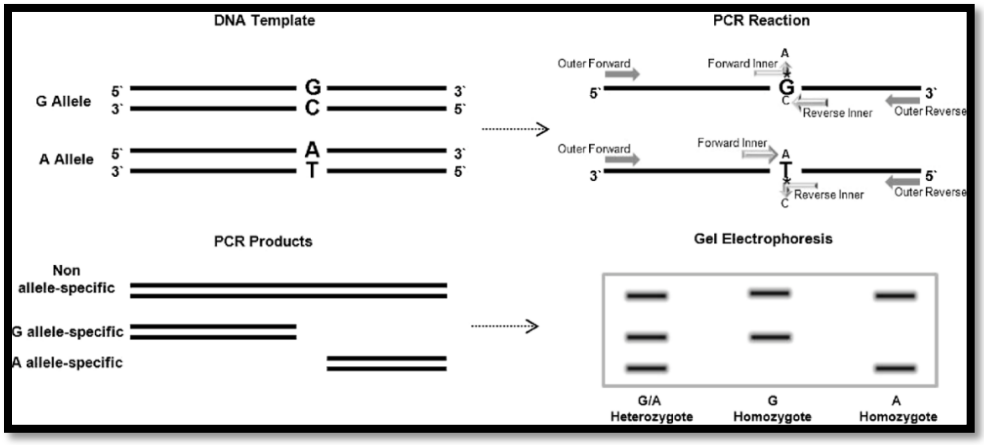

Fig.(16): Illustration of Tetra-Primers ARMS-PCR mechanism of action: starting with the specific DNA fragment that contain the mutation, then the binding of the four primers in their locations, after that, the building of the complementary strands and showing the results on the gel electrophoresis (51).

Note: a primer design found at the computer software: http://primer1.soton.ac.uk/primer1.html. (51) The following steps will describe the PCR amplification used in this study.

3.6.1 PCR Primers for PCR Reactions

Table 7: The tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) that includes four primers in a single PCR reaction. And here are primers concentration used for each mutation of the four-thrombophilia mutations that used in our study (51).

Primers	Conc. From stock	Water conc.
FVL-common-Forward	1.75	98
FVL-common-reverse	3.6	96
FVL-G	2.4	97
FVL-A	2.19	98
Prot-common-forward	2.15	98
Prot-common-reverse	2.09	98
Prot-A	3.44	96

Prot-G	1.49	98
MTHFR-677-common-F	1.6	98
MTHFR-677-common-R	1.7	98
MTHFR-677-C	2.3	97
MTHFR-677-T	1.99	98
MTHFR-1298-common-F	2.2	97
MTHFR-1298-common-R	3.5	96
MTHFR-1298-A	2.04	98
MTHFR-1298-C	1.32	98

Table 8: Primers for all the triplex tetra-primer ARMS PCR methods, including their final reaction concentrations (Conc.), optimal annealing temperatures (Ta) and expected product sizes in base pairs (bp). the sequences of the forward and reverse primers. Which produce amplicons contain the mutation, with representation the size of each product (50).

Mutation	**Primer sequence (5'-3')**	**Vol. (µl)**	**Product size (bp)**
FVl c.1691G>A	FVL-Common F: CAGGAACAACACCATGATCAGAGC	0.5	501
	FVL-Common R: TAATCAACTTGCTCAACACATCCGA	0.6	
	FVL-G: AAGAGCAGATCCCTGGACAGACG	0.8	319
	FVL-A: CAAGGACAAAATACCTGTATTCGTT	1.6	228
FII g.20210G>A	FII-Common F: GCCTGAAGAAGTGGATACAGAAGGTCAT	0.4	245
	FII-Common R: CACCAGGTGGTGGATTCTTAAGTCTTCT	0.5	
	FII-A: TGGTTCCCAATAAAAGTGACTCTCATCA	1.2	169
	FII-G: GAATAGCACTGGGAGCATTGAGGATC	0.8	130
MTHFR c.677C>T	677-Common F: CCCAGCCACTCACTGTTTTAGTTCAGGC	0.9	407
	677-Common R: GGTGAGAGTGGGGTGGAGGGAGCTTAT	1.0	
	677-C: CAAAGAAAAGCTGCGTGATGATGAAATAGG	0.8	273
	677-T: TTGAAGGAGAAGGTGTCTGCGGGCGT	0.8	190
MTHFR c.1298A>C	1298-Common F: GAAGAAGTTTGCATGCTTGTGGTTG	1.2	593
	1298-Common R: CAGGCAAGTCACCTGGGAGAGA	1.3	
	1298-A: GGCAAAGAACGAAGACTTCAAAGACACATT	2.0	281
	1298-C: GAGGAGCTGACCAGTGATGC	0.2	361

3.6.2 PCR Master Mix

Manually PCR Mixture prepared by using 1µl from template DNA that extracted from patients and stored in the refrigerator at 2^{o}C. It was added to 1.2 µl $MgCl_2$that is act as a cofactor to the Taq enzyme. Then 2µl dNTPs (building blocks for PCR)was added to the mixture. Hence, nucleotides can built by using 0.1 µl Taq polymerase enzyme. Then, 2.5µl PCR buffer added to resisting the PH alterations after that, primers was added according to the previous table, which is, demonstrate the accurate primers concentration and volume. Finally, sterile distilled water was added up to 25µl total volume.

3.6.3 PCR Conditions for DNA Amplification

The PCR reaction mix shown in the bottom tables. Now, The PCR components mixed in the Eppendorf tubes after preparing the DNA sample. Then, the thermocycler adjusted according to the protocol as shown in the table below.

Table 9: PCR reaction mix used in the study for the four point mutations, starting from DNA template, then MgCl2 to increase the higher productivity of Taq Polymerase enzyme, PCR buffer to create optimal conditions for the enzyme, dNTPs to supply the 'bricks', Primers to determine the DNA fragment to be amplified.

Reagent	Volume
Template DNA	1µl
$MgCl_2$	1.2µl
Taq Polymerase	0.1µl
PCR buffer	2.5µl
dNTPs	2.0µl
Tetra Primers	˜5µl
Distilled Water	To 25 µL
Total volume	25 µL

Table 10: PCR conditions for amplification reaction in the thermocycler, explaining the temperature degree, the time, and the number of cycles. That is suitable for each PCR step.This type of PCR is used to get a specific product, for strength of binding, to prevent the chance of primers binding to another place although they are specific.

Step	Temperature ºC	Time	Number of cycles
Initial denaturation	94	5 min	1
Denaturation	94	25 sec	
Annealing	60.5-61.8	30 sec	32
Extension	72	25 sec	
Final extension	72	10 min	1

3.7 Agarose Gel Electrophoresis of PCR Products

Agarose gel of 1.5 % (w/v) was prepared by weighting1.5 g of agarose powder and added to 100 ml of 1X TAE buffer in the Erlenmeyer flask. Then heated 2 minutes in the microwave and poured on the gel tray of the electrophoresis apparatus that provided with comb, which make wells in the gel to put the PCR solution. Moreover, when the PCR reaction finished, 5µl of 100bp DNA ladder and 10 µl of each sample was loaded in the well of the ultrapure agarose gel directly. Then it was electrophoresed for one hour at 100 voltage.

In addition, few drops of Ethidium Bromide was diluted in can filled of water, which placed in a sink in order to dye the gel. Then the results was visualized by using a MultiDoc-IT^{TM} 120 Imaging system with LM-20 Transilluminator at 302 nm.

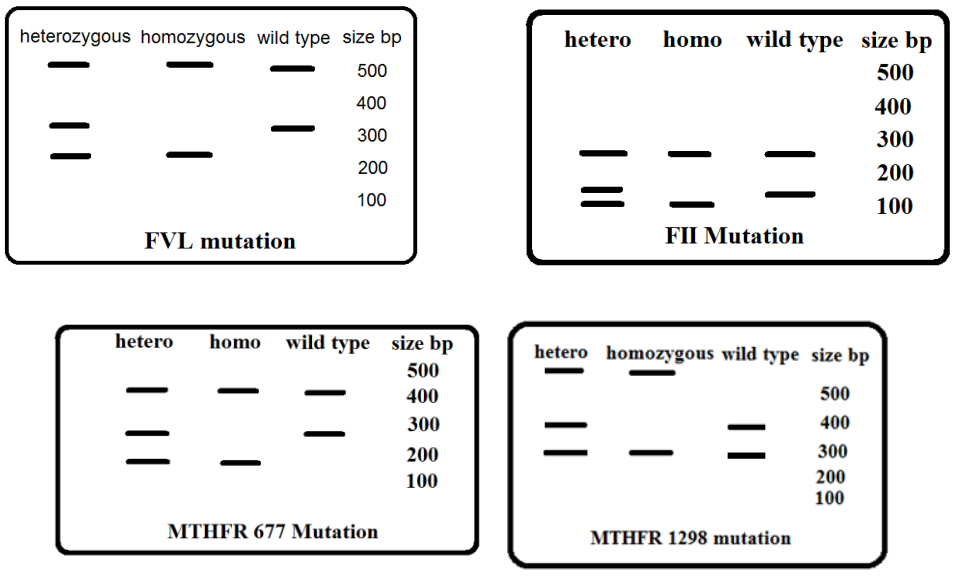

Fig.(17): Schematic illustration of bands on the gel electrophoresis, according the base sizes and the homo or heterozygosity status for our four mutations of thrombophilia.

3.8 Homocysteine Analysis

The second part of our study is includes the measuring of homocysteine level and its effect on the thrombophilia and so the pregnancy status.

3.8.1 Specimen

Homocysteine level was analyzed by using Human (Homocysteine liquiUV) Kit. Twenty samples was collected from patients undergoing IVF treatment many times, in Al-Shunnar Center for Fertility. To avoid the increasing concentration of homocysteine level the samples was placed on ice in a Heparin tubes after collection. The samples kept on ice prior to centrifugation and the separation of the Plasma. Then stored at -20^{o}C to check all samples in the same time, it can be stored up to 8 months.

3.8.2 Reaction Principle

Tris (2-carboxyethyl) phosphine (TCEP) reduced the oxidized form of Homocysteine. Therefore, the reduced form of Hcy reacts with serine then catalyzed by cystathionine β-synthase (CBS) to form cystathionine. Cystathionine β-lyase (CBL) breaks down the cystathionine to homocysteine, Pyruvate, and ammonia. However, lactate dehydrogenase (LDH) in turn can convert pyruvate to lactate with NADH.

3.8.3 Analysis Procedure

A substrate reagent (NADH, LDH, Serine, Trizma base 1, Trizma hydrochloride 1, Sodium azide and TCEP) was added to the cuvettes about 1000 µl (all reagents are ready to use). Then 70µl from Calibrator 1 (an aqueous homocysteine blank), and Calibrator 2 (an Aqueous homocysteine solution) added to the same cuvette, and after mixing, it was incubate for 5 minutes at 37^{o}C. Then 100µl from the enzyme reagent (cystathionine β-synthase, cystathionine β-lyase, and sodium azide) and incubated for 1 minute. Moreover, the initial absorbance recorded at the start point and after 3 minutes exactly. Finally, the average between two-measure absorbance were calculated.

Chapter Four
Results and Discussion

4.1 PCR and Gel Electrophoresis

According to the previous data related to the PCR reaction, the sample criteria, and the Gel features. The resulting information is shown in the following figures.

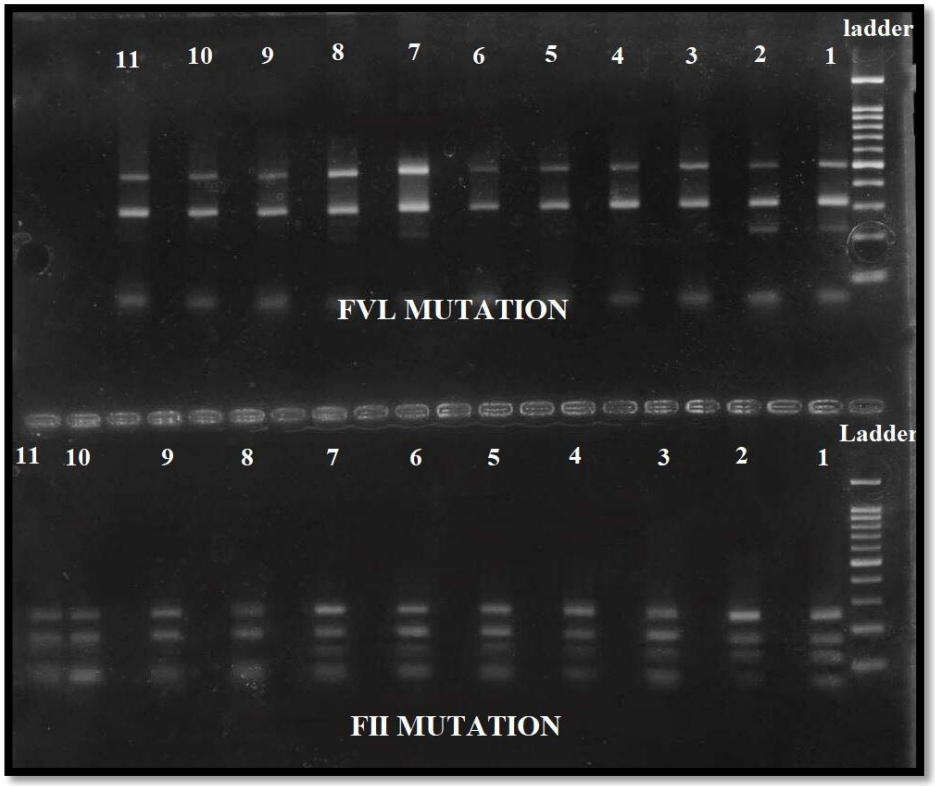

Fig. (18): Gel electophoresis image of the PCR Products of FVL, FII genes

In the FVL mutation image, the sample 1, 2, and 7 considered as a heterozygous patients, but the rest samples are wild type forms meaning that does not carry this mutation. While the second mutation (FII mutation), the samples 1 to 7 are heterozygous patients, the rest are in the wild type.

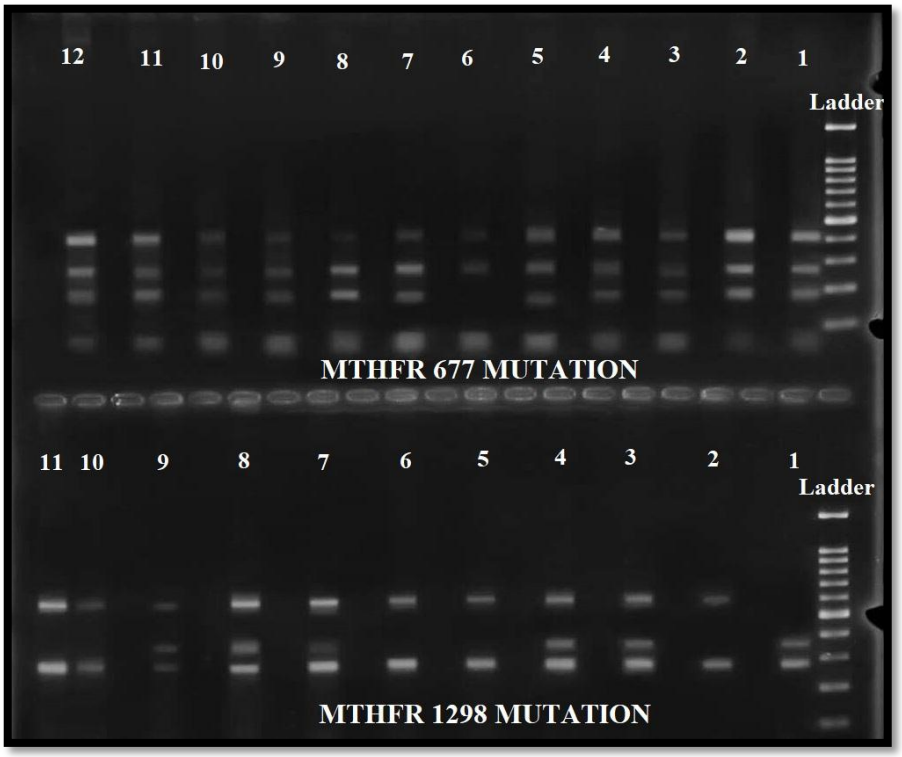

Fig. (19): Gel electrophoresis of MTHFR 677, MTHFR 1298 mutations

The first form of the MTHFR mutation gel illustrates that the sample 6 considered as a wild type form, but the rest are carries the mutation in the heterozygous form. The second mutation gel demonstrate that 3, 4, 7, 8, 9 patients are carries the gene in its wild form, but sample 2 is in homozygous form, and sample 1 in the wild form.

Table 11: DNA concentration per ml and the absorbance spectroscopy for each sample

Sample ID	Ng/ml at 260 nm	Absorbance spectroscopy (ABS)
P1	84.3	0.084
P2	38.8	0.039
P3	38	0.038
P4	55	0.055
P5	50.5	0.051
P6	68	0.068
P7	106	0.106
P8	71	0.071
P9	69	0.069
P10	61	0.061
P11	68	0.068
P12	53	0.053
P13	44	0.044
P14	77	0.077
P15	76	0.076

Table 12: Homocysteine level before and after B-Complex treatment: since, before treatment the level was high, and after first and second doses a data decreased, which is mean a good choice, that affect a positive pregnancy, but in some result a pregnancy status was negative because of other factors that affects the status.

Patient number	Hcy level before B-complex	Hcy after B-complex treatment (within 10 days)	Pregnancy status
Patient 1	7.99	7.37	+ve
Patient 2	11.57	7.46	+ve
Patient3	10.71	9.14	-ve
Patient 4	12.51	9.04	-ve
Patient 5	17.59	9.4	+ve
Patient 6	14.71	8.53	-ve
Patient 7	14.94	6.7	+ve
Patient 8	9.28	8.74	+ve
Patient 9	9.36	6.3	+ve
Patient 10	19.51	11.8	-ve

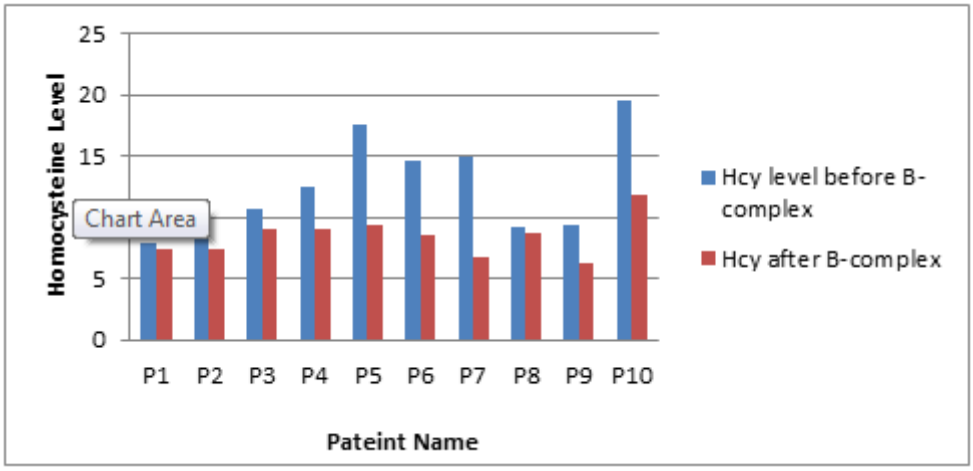

Fig. (20): Homocysteine level before and after B-complex treatment

Table 13: Homocysteine levels before and after Folate treatment: since, before treatment the level was high, and after first and second doses a data decreased, which is mean a good choice, that affect a positive pregnancy, but in some result a pregnancy status was negative because of other factors that affects the status.

Patient number	Hcy level before folate treatment	Hcy level after folate treatment within 5 days	Hcy level after 10 days of folate treatment	Pregnancy status
Patient 1	32.62	22.03	11.15	+ve
Patient 2	11.36	8.48	7.67	+ve
Patient 3	10.24	7.59	-----	-ve
Patient 4	11.99	5.02	-----	+ve
Patient 5	14.34	8.55	8.59	+ve
Patient 6	33.36	9.28	-----	+ve
Patient 7	8	13.47	-----	-ve
Patient 8	16.55	13.95	11.47	+ve
Patient 9	11.86	14.6	19.86	-ve
Patient 10	19.01	12.11	9.3	+ve
Patient 11	9.24	5.25	-----	-ve
Patient 12	13.59	12.84	-----	-ve
Patient 13	14.8	10.15	-----	+ve
Patient 14	11.46	9.89	13.27	+ve
Patient 15	11.78	13.01	-----	-ve
Patient 16	20.97	9.73	-----	+ve
Patient 17	9.5	8.09	-----	-ve
Patient 18	11.92	11.57	-----	+ve
Patient 19	44.1	28.52	-----	-ve

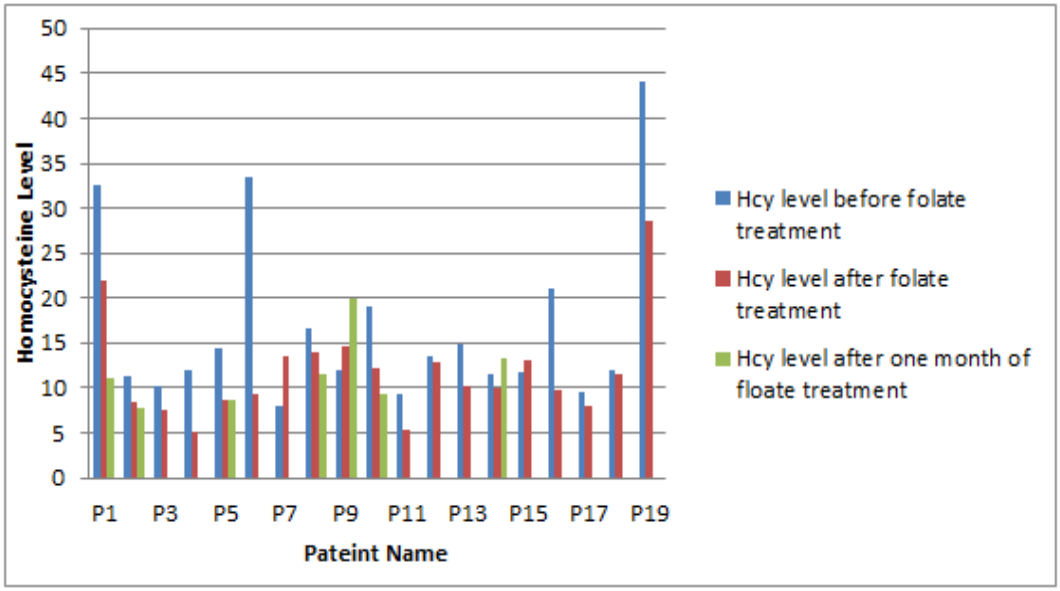

Fig. (21): Homocysteine level before and after Folate treatment

Tetra primers ARMS PCR was a rapid and cost effective method in checking the mutations, after optimized the annealing temperature, primers concentration, and pcr thermo cycler program. We get a clear results. On the other hand, the homocysteine level was calculated after using homocysteine liquiUV kit and huma star 200 device. The data show that the level was decreased remarkably until reach the normal range, after using the folate or B complex treatment within 10 days of starting the IVF protocol. Since, folate metabolic pathway plays an important role in the physiology of the cell as in nucleotide synthesis, repair and methylation of DNA, and stability of the genome. However, any defect in such pathways due to the insufficient folate intake, or gene mutations causing infertility complications.

a folic acid will reduced to tetrahydrofolate (THF) after be imported to the cell. Then converted immediately to 5, 10-methylene THF through a serine hydroxymethyltransferase (SHMT). An enzyme that depends on vitamin B6 and uses serine as a one-carbon donor. So the homocysteine level will reduced also.

Chapter Five
Conclusion and Recommendations

5.1 Conclusion and recommendations

Tetra-Primers ARMS PCR is a very good choice to check single nucleotide polymorphism (SNP) in point mutation status, since it is differ from other procedures in allowing the evaluation of the wild and mutant forms of mutation in the same tube and determining the specific gene sequence. Since it being a fast and economical method (doesn't cost more than 3.1$), the reaction finished in 3-4 hours (45-60 min in DNA extraction, 90-100 min in PCR reaction, and one hour to get results), On the other hand, it is more suitable than other methods by cancelling the need to use restriction enzymes hence, less time wasting for incubation. ARMS-PCR give adequate and reliable results even if the amount of DNA do not pass 1-5 ng (50).

Patients with these mutations (FVL, FII, MTHFR 677, and MTHFR 1298) suffering from many problems in their body. Since, some mutations in MTHFR gene called MTHFR 677, MTHFR 1298 causes an increase in the homocysteine level because of the decrease in the enzyme, which breaks down the Hcy, so that will arise the pregnancy complications, neurodegenerative disorders and renal problems. According to the dietary deficiency of the vitamins, smoking, low thyroid hormones and advanced age. In addition, the treatment should be by taking a folate supplementations or B vitamins.

Other mutations that cause a harmful effect on the body are thrombophilia mutations (FVL, FII). Which caused by mutations in gene five and two, respectively. Represented by developing a blood clots because of the resistance of the activated protein C in the patients with factor V Leiden. Alternatively, the preventing the prothrombin protein in patents with FII mutation. Even if the infection appear in the legs (deep venous thrombosis) or in lungs (pulmonary embolism). The treatment doses are varies according to the cause and severity of the mutation by using anticoagulants like (Heparin, Warfarin)

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جامعة النجاح الوطنية

كلية الدراسات العليا

فحص طفرات الثرومبوفيليا (FVL, FII, MTHFR 677, MTHFR 1298) بطريقة ARMS-PCR وإيجاد العلاقة بين وجود هذه الطفرات ومستوى الهموسستين بالدم

اعداد

ولاء ياسر محمود زيد

اشراف

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين

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الملخص

الخصوبة هي مفهوم يطلق على حدوث الحمل بالصورة الطبيعية عند اندماج خلية ذكرية تناسلية مع خلية انثوية، وأي خلل في هذه العملية يؤدي الى اضطرابات ومشاكل مما تقتضي اللجوء الى وسائل حمل مساعدة مثل عملية الحقن المجهري داخل الرحم. التي تتضمن حقن الحيوان المنوي داخل البويضة الناضجة في مختبرات الاخصاب، وهناك العديد من الأسباب التي تستدعي اللجوء لمثل هذه الطرق وهي أسباب ذكرية: أي مشاكل في عدد وحركة وشكل الحيوانات المنوية. وأسباب أخرى انثوية: مثل مشاكل التبويض وبطانة الرحم المهاجرة وطفرات جينية تؤدي الى فشل عملية التلقيح.

تخثر الدم (الثرومبوفيليا) هو مرض ناتج عن تجمع خثرات الدم بالشكل غير الطبيعي في الاوعية الدموية، هذه الخثرات ممكن ان تنتقل عن طريق تيار الدم حتى تصل الى الشعيرات الصغيرة في الارجل، وممكن ان تصل الى الرئتين مسببة ما يعرف ب (الانسداد الرئوي) وذلك نتيجة أسباب وراثية جينية او مكتسبة.

الهموسستين، هو عبارة حمض اميني يتم انتاجه في الجسم وهو أمن عند تواجده بالمعدل الطبيعي، اما إذا زادت نسبته عن الحد الطبيعي فانه يؤدي الى مشاكل عديدة في الجسم منها تراكمه داخل الاوعية الدموية. وفي هذه الدراسة تم جمع عينات دم من 20 مريضة خضعوا لبرنامج الحقن المجهري وفشلت الزراعة لديهم عدة مرات في مركز الشنار لعلاج العقم وأطفال الانابيب، وتم

فحص طفرات الثرومبوفيليا الأربعة لديهم عن طريق استخلاص الدي ان ئي من عينات الدم وتكثيف بعض مناطق من جيناتهم وهي المسؤولة عن تخثر الدم باستخدام تقنية .ARMS-PCR

النتائج كانت تشير الى وجود طفرات التخثر في اجسام المرضى الذين عندهم ارتفاع حقيقي في مستوى الهموسستين، وذلك بسبب نقص الانزيم المسؤول عن تحويل الهموسستين الى المثيونين السليم، حيث نتج عن ذلك حدوث مشاكل في الحمل إضافة الى مشاكل أخرى، ولتجاوز مثل هذه المشاكل يتم اعطاءهم جرعات مناسبة من حبوب الفوليت، او حقن فيتامينات بي بتراكيز مناسبة.

هدف الدراسة هو إيجاد طريقة فعالة وامنة وغير مكلفة لفحص طفرات تخثر الدم الأربعة (Factor V Leiden G1691A/R506Q (FVL), prothrombin G20210A (FII) and methylenetetrahydrofolate reductase (MTHFR) C677T/ C1298A.)، ومدى انتشار هذه الطفرات بين السيدات اللواتي خضعن لعملة الحقن المجهري عدة مرات، وإيجاد علاقة بين وجود مثل هذه الطفرات ومستوى الهموسستين في الدم ونجاح عملية الحقن المجهري أي (الحمل الإيجابي).