CHAPTER ONE

Introduction and literature review:

1.1 Introduction:

Spontaneous abortion and miscarriage are synonymous terms. In the medical literature, spontaneous abortion is most often used, while in clinical practice and among the general population miscarriage is the preferred term. Spontaneous abortion or miscarriage is defined as the involuntary end of pregnancy before 20 weeks of gestation. Recurrent pregnancy loss (RPL), also known as recurrent miscarriages, is defined by the consecutive loss of two or more pregnancies with the same partner, and having no more than one living child (Aruna and Reddy, 2006).

Also Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses before twenty week of gestation, which affects 1-3% of couples (Calder and Greer, 2005). The American Society for Reproductive Medicine defines the numbers of previous miscarriages in RM as two or more whereas Europe Society of Reproduction and Embryology defines it as three or more (Asrm, 2008, Eshre, 2006). The exact frequency of miscarriages is, however, unknown as miscarriages frequently occur before the woman is aware of her pregnancy.

There are numerous factors that may cause (RPL), but the underlying problem often remains undetected. Although much work has been done to identify the underlying mechanisms, the cause of miscarriage can be identified in only ~50% of cases. The known causes of RSA include chromosomal and metabolic abnormalities, uterine anomalies, and immunologic factors. RPL is estimated to occur in 2%–4% of reproductive-agecouples (Stephenson and Kutteh, 2007). Recurrent pregnancy loss (RPL) is one of the most frustrating and difficult areas in reproductive medicine because the etiology is often unknown and there are few evidence-based diagnostic and treatment strategies. Studies on the etiology, evaluation, and management of RPL are often flawed. Women with RPL at first trimester were classified into three separate groups, as primary, secondary and tertiary aborters. Primary aborters are women with no previous

live birth, secondary aborters if there was a live birth followed by pregnancy losses, and tertiary aborters are women who had pregnancy losses followed by a live birth. Common methodologic weaknesses include failure to adhere to generally accepted criteria for RPL, ascertainment bias, improper selection of controls, uneven monitoring of cohorts, no exclusion of aneuploid fetuses, lack of stratification for important factors such as number of previous losses, premature termination of study after interim analysis, and excessive post randomization patient withdrawal (Christiansenm, *et.al*., 2005). The pregnancy associated with hypercoagulability sets a foundation for hemostatic abnormalities during pregnancy and may be associated with pregnancy complications (Calder and Greer, 2005). Thrombophilia is considered still a debated problem that may be common in women with unexplained recurrent pregnancy loss, with prevalence as high as 65% in selected populations (Blickstein, 2006). The thrombophilias are a number of prothrombotic factors, which can either be inherited or acquired. The influence of thrombophilia in pregnancy is a popular research topic in recurrent miscarriage. The inherited thrombophilias include activated protein C resistance 95% due to factor V Leiden (FVL) mutation], protein S deficiency, protein C deficiency, antithrombin III deficiency, FII (prothrombin) mutation and hyper homocysteinaemia (Doyle, 2004). Factor V Leiden (FVL) and prothrombin (G20210A) mutations are the most common causes have been implicated as risk factors of hereditary thrombophilias which in turn can result in placentation (Doyle, 2004).

Epidemiologic data have suggested that the patients with a poor obstetrical history are at significantly increased risk of recurrence. Inherited thrombophilic conditions are increasingly being implicated in these pregnancy outcomes, yet paradoxically, the majority of patients harboring the most common mutations, such as Factor V Leiden, methylene tetrahydrofolate reductase (MTHFR) gene and prothrombin gene mutation G20210A are asymptomatic (Ehrenforth, *et.al*., 2004). Acquired or hereditary thrombophilia have been related to adverse pregnancy outcome and a higher incidence of early recurrent abortion was associated with factor V Leiden, the methyleneterhydrofolate reductase (MTHFR) C677T and to the prothrombin G20210A variant (Vossen, *et.al*., 2004).

1.2 Literature Review:

1.2.1 Pregnancy:

Pregnancy is period of reproduction during which a women carries one or more live offspring from implantation of a fertilized zygote in the uterus throughout gestation. Childbirth usually occurs about 38 weeks after conception; in women who have a menstrual cycle length of four weeks, this is approximately 40 weeks from the start of the last normal menstrual period (WHO, 2006). The normal menstrual cycle is 28 days long; with ovulation usually occurring on day fourteen Implantation of the fertilized zygote occurs 7 days after conception, which is day 21 of the cycle. A normal pregnancy is 40 weeks long (plus or minus two weeks), counted from the date of the last menstruation, which is two weeks longer than the age of the fetus. About once every 28 days, in the middle of a woman's menstrual cycle, an ovum bursts from one of her ovaries, and is drawn into one of two fallopian tubes that lead to the hollow uterus. While the ovum is traveling, the spot on the ovary from which it was released, now called the corpus luteum, secretes hormones that prepare the lining of the uterus to receive a fertilized ovum. If pregnancy does not occur, the corpus luteum shrinks, and the lining of the uterus is discarded two weeks later with menstruation (Berk, 2011). After sexual intercourse, sperms are transported upward from the vagina and through the uterus and fallopian tube, where fertilization usually takes place. One spermatozoon out of hundreds of millions ejaculated by the man may penetrate the outside layer of the ovum and fertilize it. Through fertilization, the egg is activated to begin its developmental process, and the haploid nuclei of the two gametes come together to form the genome of a new diploid organism. The fertilized egg, known as a zygote, then moves toward the uterus, a journey that can take up to a week to complete. Cell division begins approximately 24 to 36 hours after the male and female cells unite. Cell division continues at a rapid rate and the cells then develop into what is known as a blastocyst which arrives at the uterus and attaches to the uterine wall, a process known as implantation. The mass of cells is now known as an embryo (Guyton and Hall, 2011).

Pregnancy is typically divided into three periods, or trimesters, each of about three months. In medicine, pregnancy is often defined as beginning when the developing

embryo becomes implanted in the endometrial lining of a woman's uterus. The first 12 weeks of pregnancy are considered to make up the first trimester. According to the American Pregnancy Association, by the end of the first trimester, the fetus will be about 3 inches (76 mm) long and will weigh approximately 1 ounce (28 gm) (American Pregnancy Association, 2010). By the end of the second trimester, the expanding uterus has created a visible "baby bump". The third trimester of pregnancy spans from week 28 to the birth. The woman's belly will transform in shape as the belly drops due to the fetus turning in a downward position ready for birth. The fetus has a good chance of survival if born during this time. Size increases. Lungs mature. Rapid brain development causes sensory and behavioral capacities to expand. In the middle of this period, a layer of fat is added under the skin. Antibodies are transmitted from mother to fetus to protect against disease. Finally fetus becomes 50 cm length and 3.4 kg in weight. Most fetuses rotate into an upside-down position in preparation for birth (Berk, 2011). Successful outcome of pregnancy requires frequent monitoring of biochemical and hematological parameters to avoid complications throughout the trimesters of pregnancy.

1.2.2 Physiological and hemostatic changes during pregnancy:

Pregnancy is associated with normal physiological changes that assist the nurturing and survival of the fetus. Biochemical parameters reflect these adaptive changes in most organ system and are clearly distinct from the non-pregnant state (Tran, 2005). The physiology of normal pregnancy involves major changes in the coagulation system. These changes appear to be related to the development of the uteroplacental circulation and provide a protective mechanism during delivery (Brenner, 2004).

Hemostasis is the complex process that maintains the balance between clotting and bleeding. It is composed of several tightly coupled biochemical reactions involving the dynamic interaction between circulating coagulation factors, the anticoagulation and fibrinolytic systems, vascular endothelium, platelets, and blood flow (Furie and Furie, 2008). Physiological changes in pregnancy affect the coagulation and fibrinolytic systems. Many of the clotting factors increase and anticoagulation factors decrease causing augmented coagulation and decreased fibrinolysis. Pre existing coagulopathy may affect the course of pregnancy and nature of coagulopathy may also be modified by

pregnancy. Changes in coagulation affect the mode of delivery and the approach to analgesia and anesthesia in patients with hypocoagulable disorders. Different physiological changes occur during the process of pregnancy, which affect all of the woman systems; these include metabolic adaptations and hormonal changes. These changes that affect the coagulation factors. It is due to secondary increase in the concentrations of pre-coagulant factor, a reduction of the naturally occurring anticoagulant proteins and increase in fibrinogen, which characterized pregnancy with hypercoagulability (Martinelli, *et.al.*, 2000). Normal pregnancy is associated with increased concentrations of most clotting factors, decreased or unchanged concentrations of natural anticoagulants and reduced fibrinolysis. These changes are interpreted as mainly being due to increased estrogen levels (Bremme, 2003). During the course of normal pregnancy dramatic changes occur in the haemostatic system. Coagulation factors increase physiologically in pregnancy and this is thought to be an evolutionary mechanism to prevent excessive blood loss at childbirth These physiological changes in the coagulation system may increase the risk of pregnancy failure if are associated with thrombophilia (Namee, *et.al.*, 2012).

1.2.1.2 Abnormal Pregnancy:

Sometimes a pregnancy ends unhappily, but it is not technically a miscarriage. There are four main types of abnormal pregnancies. These include an early pregnancy failure, an ectopic pregnancy, a blighted ovum, and a molar pregnancy. It is important to know the signs and symptoms of abnormal pregnancies, so that you can seek our medical attention, if you believe you are at risk

1.2.1.2 .1 Ectopic pregnancy:

Is a normal fertilized egg that gets stuck in the fallopian tube or fall into the abdominal cavity and implants there. This type of pregnancy cannot survive to term and increases risk for severe hemorrhage and possibly even death to pregnant women. When the ectopic is discovered, it is essential to surgically and immediately remove the baby. Symptoms associated with this situation include: sharp, intense pain in abdomen or possibly in shoulders; a pregnancy test that is positive, then turns negative a few days

later; and spotty red bleeding that continues. With rare exceptions, ectopic pregnancies are not viable. Furthermore, they are dangerous for the mother, since internal hemorrhage is a life-threatening complication. Most ectopic pregnancies (93-97%) occur in the distal Fallopian tube (so-called tubal pregnancies), but implantation can also occur in the cervix, ovaries, and abdomen. (Crochet, *et.al.*, 2013)

Ectopic are usually caused by scar tissue in the fallopian tubes that could have been caused by previous surgery in the pelvic region, uterus, tubes; a pelvic infection such as Chlamydia or pelvic inflammatory disease; or endometriosis that blocks the entrance to the tubes

1.2.1.2.2 A Molar Pregnancy:

A Molar Pregnancy is a very rare type of pregnancy, is an abnormal form of pregnancy in which a non-viable fertilized egg implants in the uterus and will fail to come to term. A molar pregnancy is a gestational trophoblast disease (American Cancer Society, 2014).

The baby usually does not form, but the uterus is filled with big bubble clusters. A molar pregnancy is caused when a sperm fertilizes an empty egg (called a complete molar pregnancy) and no baby grows, or when two sperm fertilize an egg and both the baby grows a little as well as an abnormal placenta (called a partial molar.) Even if a baby does grow, it cannot survive. The longest documented molar pregnancy which has seen was a 24-week stillbirth. Molar pregnancies usually present with painless vaginal bleeding in the fourth to fifth month of pregnancy. The uterus may be larger than expected, or the ovaries may be enlarged. The most common symptom is vaginal bleeding, especially between the 6th and 16th weeks of pregnancy. Another symptom is bleeding that continues for a long time after delivery. Small amounts of bleeding can show up as a watery brown discharge from the vagina, there may also be more vomiting than would be expected (hyperemesis). Sometimes there is an increase in blood pressure along with protein in the urine. Blood tests will show very high levels of human chorionic gonadotropin (hCG) (Ganong, *et.al.*, 2005).

1.2.1.2.3 A stillbirth:

According the National Stillbirth Society, stillbirth is defined as the intrauterine death and subsequent delivery of a developing infant that occurs beyond 20 completed weeks of gestation. A stillbirth is technically any pregnancy that ends after the 20th week and the baby does not survive. Some babies die in utero and are discovered when the heartbeat is not found. A stillbirth occurs when a fetus dies in the uterus. A wide variety of definitions exist (Nguyen and Wilcox, 2005) .The most common causes of this are uterine abnormalities, a knot or other umbilical cord accident, infections of the lining of the gestational sac or cord, and placental abruptions that cause the placenta to pull away from the uterine wall. These babies are usually born through the induction of labor. Other babies are lost through early labor. The causes of early labor are premature rupture of membranes, uterine abnormalities that make the uterus too small to hold the baby, and an incompetent cervix, which opens up and lets the baby out. It is unknown how much time is needed for a fetus to die. Behavior is consistent and a change in the fetus' movements or sleep wake cycles can indicate fetal distress (Jason, *et.al.*, 2009).

1.2.1.2.4 An an embryonic gestation:

Early pregnancy failure (also known as blighted ovum or an embryonic gestation) is a common cause of miscarriage. An an embryonic gestation (also known as a blighted ovum) is a pregnancy in which the very early pregnancy appears normal on an ultrasound scan, but as the pregnancy progresses a visible embryo never develops or develops and is resorbed (Kim, 2013). A blighted ovum causes about one out of two miscarriages in the first trimester of pregnancy. A miscarriage is when a pregnancy ends on its own within the first 20 weeks. The bleeding, if that happens before the blighted ovum is found via ultrasound, is slow and brown. Symptoms will seem to go away. A blighted ovum is believed to be caused by an egg or sperm with poor genetic material. When the egg is fertilized, instead of creating both a sac and a baby, the part that should be a baby never grows. Some women do experience more than one blighted ovum, but most women go on to later have a baby (Kim, 2013).

The criteria depend on the type of ultrasound examination performed. A pregnancy is an embryonic if a transvaginal ultrasound reveals a sac with a mean gestational sac diameter (MGD) greater than 25 mm and no yolk sac, or an MGD >25 mm with no embryo. Tranabdominal imaging without transvaginal scanning may be sufficient for diagnosing early pregnancy failure when an embryo whose crown–rump length is 15 mm or more has no visible cardiac activity. (Campion, *et,al*., 2013).

1.2.2 Recurrent Spontaneous abortion:

Recurrent spontaneous abortion (RSA), which is also referred to as repeated pregnancy loss (RPL) and habitual abortion, is defined as three or more consecutive spontaneous miscarriages (Eshre, 2006).

The experience of repeated pregnancy loss is physically and emotionally traumatic to women who are trying to have children. The overall frequency of RM was estimated from 1% to 3% (Christiansen, *et .al*., 2008). The exact prevalence of RM depends on its definition. To date there is no consensus on the definition of RM with regard to the numbers of previous miscarriages and the gestational age of RM. The American Society for Reproductive Medicine defines the numbers of previous miscarriages in RM as two or more whereas Europe Society of Reproduction and Embryology defines it as three or more (Eshre, 2006).

One of the greatest challenges in obstetrics is the high fetal loss which occurs in the first and second trimesters. Spontaneous pregnancy loss is a surprisingly common occurrence, with approximately 15% of all clinically recognized pregnancies resulting in pregnancy failure. Recurrent pregnancy loss (RPL) has been inconsistently defined. When defined as 3 consecutive pregnancy losses prior to 20 weeks from the last menstrual period, it affects approximately 1% to 2% of women. This review highlights the current understanding of the various etiologies implicated in RPL, including factors known to be causative, as well as those implicated as possible causative agents. The appropriate diagnostic evaluation, therapy, and prognosis are also addressed. (Ford, *et.al.*, 2009).

Historically, recurrent abortion was defined as three or more clinically recognized pregnancy losses before 20 weeks of gestation. Using this definition, RPL occurs in approximately 1 in 300 pregnancies. However, many recommend that clinical investigation and intervention be initiated after two consecutive spontaneous abortions, especially if any of the following are coexistent: fetal heart activity was identified before any of the pregnancy losses, fetal karyotyping of pregnancy tissues revealed normal chromosome content, the woman older than 35 years of age, or the couple also shows sub fertility. (Walter, *et.al.,* 2013).

Also Recurrent spontaneous abortion (RSA), defined as three consecutive pregnancy failures, is estimated to affect ~1% of all couples trying to conceive. There are numerous factors that may cause RSA, but the underlying problem often remains undetected. Although much work has been done to identify the underlying mechanisms, the cause of miscarriage can be identified in only ~50% of cases the known causes of RSA include chromosomal and metabolic abnormalities, uterine anomalies, and immunologic factors. Even though RSA is a heterogeneous condition and the progress in identifying causative factors has been slow, the repetitive pregnancy losses in some couples and the high percentage of unexplained RSA indicate that there are specific underlying causes yet to be found (Rai and Regan, 2006).

1.2.2.1 Stages and Types of Spontaneous Abortions:

There are various stages and types of spontaneous abortions (threatened, inevitable, incomplete and complete abortions, missed abortion, and fetal/embryonic demise). These types are clearly defined as follow:

- Spontaneous abortion/miscarriage: A pregnancy that ends spontaneously before the fetus has reached a viable gestational age. The World Health Organization defines it as expulsion or extraction of an embryo or fetus weighing 500 g (typically corresponds to a gestational age of 22 weeks).

-Threatened abortion: Threatened abortions may progress to inevitable, spontaneous, incomplete, or complete abortions. With good medical management, most of these cases can reach full term and normal pregnancy. Bleeding through a closed cervical during the

first half of pregnancy. The bleeding is often painless, although it may be accompanied by mild suprapubic pain. On examination, the uterine size is appropriate for gestational age, and the cervix is long and closed. Fetal cardiac activity can be detect-able if the gestation is sufficiently advanced.

- **Inevitable abortion:** When abortion is pending, there may be increased bleeding, intensely painful uterine cramps, and a dilated cervix. The gestational tissue can often be felt or visualized through the internal cervical.

- **Incomplete abortion**: When the fetus is passed, but significant amounts of placental tissue may be retained, also called an abortion with retained products of conception (RPOC) (commonly occurs after 12 weeks' gestation). On examinations, the cervical is open, gestational tissue may be observed in the vagina/cervix, and the uterus is smaller than expected for gestational age but not well contracted. The amount of bleeding varies but can be severe enough to cause hypovolemic shock. Painful cramps are often present.

- **Complete abortion:** When an abortion occurs (usually before 12 weeks of gestation) and the entire contents of the uterus are expelled. More than one-third of all cases are complete abortions. If a complete abortion has occurred, the uterus is small and well contracted with a closed cervix; slight vaginal bleeding and mild cramping can be present.

- **Missed abortion:** Refers to in utero death of the embryo or fetus prior to the 20th week of gestation, with prolonged retention of the pregnancy (4–8 weeks). Vaginal bleeding may occur, and the cervix is usually closed.

- **Septic abortion:** An abortion accompanied by fever, chills, malaise, abdominal pain, vaginal bleeding, and frequently purulent discharge. Physical examination may reveal tachycardia, tachypnea, lower abdominal tenderness, and a tender uterus with dilated cervix. Infection is usually due to Staphylococcus aureus, Gram-negative bacilli, or some Gram-positive cocci. Mixed infections (anaerobic organisms and fungi) can also be encountered. The infection may spread, leading to salpingitis, generalized peritonitis, and septicemia. (Adi, *et. al.*, 2011).

1.2.2.2 Incidence of recurrent Spontaneous abortion:

Spontaneous abortion is the most common complication of early pregnancy (Regan and Rai , 2000) the frequency decreases with increasing gestational age. Eight to 20 percent of clinically recognized pregnancies at less than 20 weeks of gestation will undergo spontaneous abortion; 80 percent of these occur in the first 12 weeks of gestation (Wang, *et.al*., 2003). The overall risk of spontaneous abortion after 15 weeks is low (about 0.6 percent) for chromosomally and structurally normal fetuses, but varies according to maternal age and ethnicity (Wyatt, *et.al*., 2005).

Loss of unrecognized or subclinical pregnancies is even higher, occurring in 13 to 26 percent of all pregnancies (Lohstroh, *et.al*., 2005). Early pregnancy losses are unlikely to be recognized unless daily pregnancy tests are performed. A study that compared women's bleeding following a pregnancy loss before 6 weeks of gestation with their typical menstruation found that mean bleeding length following a pregnancy loss was 0.4 days longer than the woman's average menses and the amount of bleeding was light (Promislow , *et.al*., 2007).

1.2.2.3 Epidemiology of recurrent Spontaneous Abortion:

The World Health Organization (World Health Organization.) has defined spontaneous abortion as “the expulsion or extraction from its mother of an embryo or fetus weighing 500 g or less (Adolfsson, 2006). Early spontaneous abortions are defined as those that occur before the 12th week of gestation, with late spontaneous abortions being those that occur from 12-20 weeks of pregnancy, and 500g or less (Kallen 1988; Statistics Canada 2004). Women’s experience of miscarriage is obvious and distressing, both psychologically and physiologically. The reported ratio of the number of clinically recognizable miscarriages to the number of known pregnancies in general population studies varies between 12 and 15 %. The reported ratio of the number of clinically recognizable miscarriages to the number of known pregnancies in general population studies varies between 12 and 15 %(Regan and Rai, 2000).

Epidemiological studies have suggested that the condition might be multifactorial with a possible genetic predisposition and environmental factors in its pathogenesis (Cramer

and Wise, 2000). A better understanding of the role of various gene and gene environment interactions will enable identification of high risk individuals and propose a genetic mechanism to explain the unknown etiology of RPL. With the completion of human genome project it is imperative to understand the genetic basis of diseases and to identify the population and race polymorphism. Since pregnancy is a complex process and in about 50% of the RPL cases the cause is unidentified, it is essential to explore the contribution of the genetic variations in RPL (Cramer and Wise, 2000) .

The prevalence of these mutations varies among different populations and ethnic groups. Factor V Leiden mutation is rare in Asian and African populations and is higher in European populations with the highest frequency reported in the Eastern Mediterranean region, notably Lebanon. The frequency of the prothrombin mutation varies between worldwide and affects few of healthy subjects in our population (Taher, *et.al.,* 2003). The frequency of the T/T genotype of the MTHFR mutation also varies geographically. It is low in Asia and Africa, with higher frequencies noted in Europe and is estimated in many of healthy Lebanese subjects in the homozygous and heterozygous states, respectively (Almawi, *et.al.,* 2004).

Another case control study conducted by Laila et al. With an objective to determine Prevalence of factor V Leiden, prothrombin and methylene tetrahydrofolate reductase mutations in women with adverse pregnancy outcomes in Lebanon The prevalence of factor V Leiden it highest found fallow by prothrombin and methylene tetrahydrofolate reductase gene mutations was not significantly different between study subjects and control subjects.(Laila, *et .al.,* 2006).

Pihusch, et al. Found a higher frequency of factor II mutations which was more prominent than other studied gene polymorphisms in their case series among German populations. This effect was significant in a subgroup with abortions exclusively in the first trimester. (Pihusch, *et.al.,* 2002). Also, Krause and his college observed among German women that cases with early fetal loss had FVL and FII mutations were detected, while cases with late fetal loss had low respectively. They concluded that FVL appeared more in early pregnancy loss Krause and his college, Grandone et al. among Italian women found a statistically significant difference between women with only

early fetal loss vs. those with late events was observed. They demonstrated a strong association between FV Leiden and fetal loss. Furthermore, they indicate that late events are more common in these patients. (Grandone, *et. al.*, 1998).

Also Another case control study conducted by Gihan., etal about the Molecular Characterization of Factor V Leiden G1691A and Prothrombin G20210A Mutations in Saudi Females with Recurrent Pregnancy Loss The results showed that Factor V (Leiden) carries genotypes (AA& GA) mutations frequency was higher among cases with low age group <30 years than those with high age group >30 .However there no significant association between age of the cases as risk factor and presence of factor V (Leiden) mutation . Also, Factor II (Prothrombin) carries genotypes (AA& GA) mutations frequency was significantly higher among studied cases with passive smoking compared with negative cases. (Gihan, *et.al.*, 20013).

Altintas et al. observed that FVL or FII mutation among Turkish women were nonsignificant compared to controls. (Altintas, *et. al.*, 2007).

Factor V Leiden mutation is prevalent among the Caucasian people, with low incidence rate. Heterozygous genotype is found also in Caucasian population and fold increased of the relative thromboembolic risk (Semsettin, *et.al.*, 2012).

Several studies on the relationship between factor V Leiden mutation and poor pregnancy outcomes were reported. Conducted a case control study in order to estimate the prevalence of Factor V Leiden mutation among women with at least one poor pregnancy outcome, Factor V Leiden mutation was confirmed in significantly higher prevalence among cases than that among controls. Their study concluded that pregnant women with serious complications have an increased incidence of factor V Leiden mutation, predisposing them to a higher risk of developing thrombosis compared to the control group (Kupferminc, *et.al.*, 1996).

Another case control study conducted by Finan et al. With an objective to determine the prevalence of factor V Leiden mutation and other thrombophilic mutations among Lebanese women with recurrent idiopathic abortions. In this study Leiden mutation was found highest among cases than controls group. (Finan, *et.al.*, 2002).

In Sudan published data regarding no any study about the Prevalence of Factor V Leiden, Prothrombin and methylene gene mutation among recurrent spontaneous abortion women report.

1.2.2.4 Etiology of Recurrent spotonus abortion:

Historically, recurrent miscarriage has been attributed to either genetic, structural, infective, endocrine, immune, or unexplained causes. Thrombophilic disorders are thought to play a part in the cause of recurrent pregnancy loss, which widens the scope of investigations and management options for recurrent miscarriage .Many syndromes associated with recurrent fetal loss include anatomic anomalies, endocrine/hormonal abnormalities, genetic, chromosomal abnormalities, and blood coagulation protein/platelet defects (Bick, *et. al*., 1998).

The etiology of recurrent pregnancy loss (RPL) remains unclear, but it may be related to a possible genetic predisposition together with involvement of environmental factors. Etiology of recurrent pregnancy loss is among the most studied, yet unresolved issue in modern gynecology. Among the various proposed etiological factors, abnormal parental karyotype, antiphospholipid syndrome and uterine anatomic abnormalities were reported in about 50% of the patients; however, in remaining 50%, the cause is unknown (Cramer and Wise, 2000).

RSA is a heterogeneous condition and it is unlikely that only a single pathological factor is attributed to RM. Current literature suggests that the cause of RM is only identifiable in up to 40%-50% of cases. The remaining RM cases are classified as idiopathic. Hence, this merits further research to seek other possible underlying causes of RM. To date the identifiable causes of RM have been categorized as parental, fetal, environmental and psychological factors. The etiologies of spontaneous miscarriage, as well as of recurrent miscarriage are to some degree the same and to some degree different. Some of the medical causes have a higher incidence in cases of recurrent miscarriage (Jablonowska, 2003)

1.2.2.4 .1 Parental Factors:

1.2.2.4.1.1 Chromosomal Abnormality:

Parental balanced structural chromosomal rearrangement accounts for 2%-4% of RM (Ford and Schust, 2009). There are many factors that come in to play when the egg and sperm unite and form that first cell. Even if both the egg and sperm come with perfect chromosomes, the first few cell divisions can see an abnormality crop up that would certainly be devastating. The main chromosomal abnormalities are autosomal trisomies, polyploidy, and monosomy X. Most trisomies show a maternal age effect, with chromosomes 16 and 22 most commonly involved, triploidy and tetraploidy accounts for 30% of chromosomal abnormal abortions. Chromosomal abnormalities are less likely to occur in spontaneous abortions for women younger than age 36 with a history of recurrent abortion (Stephenson, *et.al*., 2002).The most common chromosomal rearrangement is balanced reciprocal or Robertsonian translocation which may lead to unbalanced gene translocations in the fetus, resulting in miscarriage (Fortuny, *et. al*., 1988, Suzumori and Ogasawara,2010). Other chromosomal anomalies associated with RM include chromosomal inversion, insertions and mosaicism (De la Fuente, *et. al*., 2009).

1.2.2.4 .2 Maternal Factors:

1.2.2.4 .2 .1 Ages:

Paternal age also plays a part. It is well recognized that female fertility declines with advancing age, which manifests in increases in miscarriage and trisomy 21 and monosomy X of the fetus. Frequency of chromosomal anomalies in sperm appears to increase with age. Independent of maternal age, paternal age of more than 40 years carries 1:6 odds of miscarriage compared with paternal age of 25 to 29 years. It is well recognized that female fertility declines with advancing age, which manifests in increases in miscarriage and trisomy 21 and monosomy X of the fetus. RM as part of a range of reproductive failures shares common risk factors. Studies have shown that in women with RM maternal age is positively associated with the numbers of repeated miscarriages (Garrisi , *et.al*., 2009) and also is an important factor predicting the

occurrence of miscarriage (Metwally , *et.al*., 2010). In IVF treatment the pregnancy rate of women with RM declined with advancing maternal age (Zhang, *et. al*., 2008). The value of preimplantation genetic screening (PGS) on the reduction of pregnancy loss in RM women with advanced age is not yet clear. Some studies have shown that PGS significantly reduces the rate of pregnancy loss following IVF treatment in RM patients older than 35 years (Munne, *et.al*., 2005). Adoption or the use of donor gametes may be recommended to the older couples if IVF has failed.

1.2.2.4 .2 .2 Endocrinological Factors:

Both estrogen and progesterone play essential roles in pregnancy. During the menstrual cycle the first half is estrogen-dominated while the second half is progesterone dominated .Estrogen and progesterone initially prepare the Endometrium for implantation by initiating a cascade of local morphological and physiological events via their respective receptors (Daniel, 2010). Progesterone acts on the reproductive tract in preparation for the initiation and maintenance of pregnancy by inhibiting contraction of the uterus and the development of new follicles (Niswender, *et. al*., 2000). Following fertilization of the oocyte, the developing embryo secretes human chorionic gonadotropin (HCG) which sustains progesterone levels. During pregnancy, fetoplacental estrogens, progestogens and adrenocorticoids are secreted into both fetal and maternal circulation (Gabbe, *et. al*., 2002). Estrogen production is mainly under the control of the fetus and is the primary signaling method by which the fetus directs essential physiologic processes that affect fetal well-being. By the 20th week of pregnancy, approximately 90% of maternal estriol excretion can be accounted for by dehydro epiandrosterone sulfate (DHEA-S) production by the fetal adrenal gland. Estrogens affect progesterone production, uterine blood flow, mammary gland development and fetal adrenal gland function. Many endocrine disturbances have been assumed to be responsible for RSA. Higher rates of spontaneous abortions are observed among women with polycystic ovary syndrome (PCOS). This may be due to hyper androgenemia, hyper secretion of LH, or insulin resistance. High levels of androgens have been shown to interfere with normal endometrial development. They alter the production of certain growth factors and may be responsible for pregnancy failure. LH

stimulates ovarian androgen synthesis; therefore, LH hyper secretion is likely to interfere with early pregnancy via hyperandrogenism. (Toner, 2003).

1.2.2.4 .2 .3 Anatomic Factors:

Anatomic abnormalities account for 16 - 18% of RM cases (Jaslow, *et.al*., 2009). The common anatomic abnormalities include congenital uterine anomalies, uterine adhesions, uterine fibroids and polyps. These abnormalities may cause inadequate vascularity of the Endometrium where the embryos implant, resulting in placental abruption and consequently miscarriage (Propst and Hill, 2000).

Among these anatomic abnormalities, congenital uterine abnormalities such as arcuate, septate or bicornuate uterus may be associated with second trimester miscarriages more than early pregnancy losses (Saravelos, *et.al*., 2010). Women with anatomic anomalies may benefit from uterine sonography and HSG in the initial diagnosis. A definitive diagnosis can be obtained by using combined laparoscopy and hysteroscopy as well as 3D sonography (Saravelos, *et.al*., 2008). Surgical resection of the uterine septum and adhesions, removal of submuscous fibroids and polyps may improve subsequent pregnancy outcomes in these women (Propst and Hill, 2000).

1.2.2.4 .2 .4 Immunological Factors:

The implanting embryo inherits its antigens from both the mother and the father. The paternal antigens are identified as foreign by the maternal immune system. In order to prevent the rejection of the pregnancy, this immune response needs to be modulated. It has been proposed that in otherwise unexplained pregnancy losses, dysregulation of the immune system could be responsible for the failure .The immunological interaction between the mother and the fetus remains a scientific enigma. In normal pregnancies, the maternal immune system does not react to spermatozoa or the embryo, even though they express antigens that are exogenous to the maternal system. Maternal-fetal tolerance has been compared to that of a semi-allogenic fetal “graft”, and may be the result of a complex array of mechanisms (including HLA-G expression of trophoblast; the leukemia inhibitory factor and its receptor, indoleamine 2,3-dioxygenase; the Th1/Th2 balance; suppressor macrophages; and hormones such as progesterone, or the placental

growth hormone, CD95, and its ligand and annexin II) that may be pregnancy-specific and interconnected (Thellin , *et.al.*, 2000). Immunological mechanisms are involved in successful implantation. Maternal adaptation of immunological responses to the implanting embryo is a key process in the establishment of the feto-placental unit. Miscarriage may therefore be a consequence of inappropriate humoral or cellular immunological responses towards the embryo APS belongs to the well-known risk factors of RM and has been reported in 15% of RM patients. Antibodies against anionic phospholipids such as cardiolipins, phosphatidylserine as well as cofactors such as 2-glycoproteins can be found disproportionately more of tenin RM patients as compared to healthy controls. Also functional tests for lupus anticoagulants frequently show haemostatic changes in APS patients. The diagnosis of APS requires fulfillment of the criteria defined in the international consensus statement (Miyakis, *et.al*., 2006). There is evidence that aspirin combined with LMWH significantly increases live birth rate in RM patients with APS.

1.2.2.4 .3 Infections:

Infective causes of recurrent miscarriage remain speculative. For any infective agent to be implicated, it must be capable of persisting in the genital tract undetected and must cause few maternal symptoms. The pathogenetic mechanisms of these infections are unique. Because of their relatively low virulence, the organisms involved seldom lead to fetal death beyond the earliest stages of embryogenesis. Since the fetus is essentially a graft of foreign tissue in the uterus, the placenta constitutes a protective immunologic barrier that shields the fetus from the mother's humoral and cell-mediated immune responses. This makes the fetus especially susceptible to infection during the first trimester. (Mims, *et.al.*, 2001).

A number of micro organisms have been suggested to be associated with spontaneous miscarriage, including Chlamydia trachomatis, Listeria monocytogenes, Toxoplasma gondii, rubella, herpes simplex virus (HSV). Bacterial vaginosis (BV) seems to be associated with premature rupture of membranes resulting in mid trimester loss and preterm labor more than early pregnancy losses (Nelson, *et.al.*, 2007). Repeated second-trimester fetal losses following cervical dilatation or rupture of membranes can be

attributed in many cases to bacterial infections, as well as early preterm delivery. These patients should be screened for bacterial vaginal infections and treated if treatment is carried out before 20 weeks of gestation, it succeeds in preventing preterm delivery (Pildne and Schneider, 2009).The most common infection among pregnant women is Toxoplasmosis and Cytomegalovirus

1.2.2.4 .2.1Toxoplasmosis:

Toxoplasmosis caused by a protozoan parasite called Toxoplasma gondii with long-term living in the humans and animal body. One third of the general population is approximately infected by the Parasite (Sensini, 2006). The seroprevalence studies indicate that toxoplasmosis is one of the most common human infections in many parts of the world (Elsheikha, 2008).

Three different ways of Toxoplasma infection induction are: eating the cysts in not fully cooked contaminated meats, using water or food contaminated with oocytes excreted from the feces of cats and transmission from mother, who has been contaminated by the previous ways, to fetus Although toxoplasmosis is often benign in the women, disease transmission through the placenta can lead to serious consequences such as abortion, still birth, different degrees of mental or physical retardation, hydrocephaly and blindness. Elsheikha, (2008) reported that the seroprevalence of Toxoplasma gondii antibodies in pregnant women varies from the 6.1 to 75.2 percent based on the geographical region.

1.2.2.4 .2.2 Cytomegalovirus (CMV):

The human cytomegalovirus (CMV) or human herpes virus 5 is one of the major causes of congenital infections. Its clinical manifestations range from asymptomatic forms (90% of cases) to severe fetal damage and, in rare cases, death due to abortion. Furthermore, 10%–15% of the children who are asymptomatic at birth may develop late sequelae, especially hearing defects, after a period of months or even years Latency following a primary infection (first contact with the virus) may be punctuated by periodic reactivations that give rise to recurrent infections, and in utero transmission may occur during either primary or recurrent infections. Actually recurrent infections may be due to reinjection with a new strain or to reactivation, but it is likely that most recurrent

infections are due to reinjection. The risk of congenital infection is much higher during primary infection. (Boppana, *et. al.*, 2001).

1.2.2.4 .2.3 Rubella:

Rubella is a common, normally mild disease that mainly affects children aged 2–12 years. Rubella in pregnancy may cause abortion, stillbirth and congenital anomalies, or congenital rubella syndrome (CRS. Prior to the introduction of rubella vaccine in 1969, the disease was distributed evenly throughout the world. In temperate regions, the incidence was usually highest in late winter and early spring. Minor epidemics occurred every 6–9 years, with major epidemics occurring at intervals ranging from 10 to 30 years (World Health Organization, 2000).

1.2.2.4 .4 Thrombophilias:

Thrombophilia can be defined as a predisposition to form clots inappropriately. Thrombotic events are increasingly recognized as a significant source of mortality and morbidity The predisposition to form clots can arise from genetic factors, acquired changes in the clotting mechanism, or, more commonly, an interaction between genetic and acquired factors (Salwa and Joseph, 2006).A successful pregnancy requires a well developed placenta and sufficient placental function to sustain an adequate fetomaternal microcirculation and the normal coagulation pathway is pivotal for the pregnancy outcomes. Also any kind of disorder in coagulation pathway may cause thrombophilia that may be the reason of placental insufficiency and PL. Recently; it has become clear that prothrombotic changes are associated with a substantial proportion of these fetal losses. Therefore, the role of thrombophilias in RPL has generated a great deal of interest. This heterogeneous group of disorders results in increased venous and arterial thrombosis. Although some thrombophilic states in RPL may be acquired such as antiphospholipid antibody syndrome (APAS) (Reznikoff, *et.al.*, 2001). Pregnancy may be compromised by prothrombotic disorders leading to subsequent miscarriages .The term thrombophilia refers to inherited or acquired conditions that predispose individuals to thromboembolic events.

Thrombophilias have been implicated in a variety of obstetrical complications including preeclampsia (PE), intrauterine growth restriction (IUGR), placental abruption, and fetal loss (Benedetto, *et.al.*, 2010) The thrombophilia represent a spectrum of coagulation disorders associated with a predisposition for thrombotic events (deep vein thrombosis (DVT) and pulmonary embolism (PE) (Kaandorp, *et.al.*, 2009). Pregnancy itself initiates a hypercoagulable state and involves a balance between procoagulants and anticoagulant pathways (Kujovich 2004). Thrombophilias can be both inherited and acquired. There is link between acquired antiphospholipid antibodies and recurrent pregnancy loss (Vinatier, *et.al.*, 2001) and a variety of therapies and combination therapies that include heparin and aspirin have been recommended to support the maintenance of pregnancy until birth (Empson, *et.al.*,2002). In the antiphospholipid syndrome (APS), antiphospholipid antibodies occur in association with venous thrombosis, arterial thrombosis, pregnancy loss or thrombocytopenia. However, the exact mechanism by which antiphospholipid antibodies lead to thrombosis is unknown. More recently, several other inherited and acquired thrombophilic disorders have been linked with recurrent pregnancy loss including: Factor V Leiden, deficiencies of natural anticoagulant proteins C, S and antithrombin, hyperhomocystinemia, prothrombin gene mutation, and homozygous point mutation (C677T) in the methyleneterhydrofolate reductase (MTHFR) gene (Kujovich, 2004). Thrombophilic disorders have generated considerable interest in the field of RPL. Thrombophilia is an important predisposition to thrombosis due to a procoagulants state. Treatment for thrombophilias remains controversial, but may include heparin and aspirin. Recently, Cochrane Database review concluded that women with recurrent miscarriage and thrombophilia do not benefit from aspirin or heparin therapy (Kaandorp, *et.al.,* 2009).

1.2.2.4 .4 .1 Inherited thrombophilia:

Inherited thrombophilia are the leading cause of maternal Thromboembolism and are associated with an increased risk of certain adverse recurrent miscarriage including second- and third-trimester fetal loss, abruptions, and severe intrauterine growth restriction, and early onset, severe preeclampsia. Inherited thrombophilia are the leading cause of maternal thrombo embolism and are associated with an increased risk of certain

adverse recurrent miscarriage including second and third trimester fetal loss, abruptions, severe intrauterine growth restriction, and early-onset, severe preeclampsia. Current information suggests that all patients with a history of prior venous thrombotic events and those with these characteristic adverse pregnancy events should be evaluated for thrombophilia Current information suggests that all patients with a history of prior venous thrombotic events and those with these characteristic adverse pregnancy events should be evaluated for thrombophilia (Lockwood, 2002).The most common inherited thrombophilic disorders are deficiencies of antithrombin III, protein C and protein S, Factor V Leiden mutation, methylene tetrahydrofolate reductase (MTHFR) and prothrombin gene mutation (G20210A). The most common cause of hyper homocysteimia rarer thrombophilias includes autosomal-dominant deficiencies of antithrombin, protein C (PC), and protein S (PS).

1.2.2.4 .4 .1.1 Prothrombin gene mutation (G20210 mutation):

Prothrombin is a protein in the blood that is required for the blood to clot. It is also called factor II. It is a vitamin K-dependent protein which is synthesized in the liver and circulates with a half-life of approximately three to five days. Vitamin K acts as a cofactor for posttranslational gamma-carboxylation of prothrombin which is required for functional activity. Blood clots are composed of a combination of blood platelets and a meshwork of the blood clotting protein fibrin. Prothrombin is a blood clotting protein that is needed to form fibrin. If somebody has too little prothrombin, he or she has a bleeding tendency (Elizabeth, *et.al.*, 2013).

Prothrombin gene (G20210A) mutation is associated with an increased risk of thrombosis and it is the most identifiable risk factor for venous thrombosis and is in fact the second most common genetic defect for inherited thrombosis, with Factor V Leiden being the most common. It is an autosomal dominant disorder, with Heterozygotes being at a 3- to 11-fold greater risk for thrombosis in both men and women and for all age groups. Although homozygosity is rare, inheritance of two 20210A alleles would increase the risk for developing thrombosis (Salwa and Joseph, 2006).

The mutation leads to an increased amount of thrombin circulating in the person's blood stream. The exact mechanism by which the prothrombin gene mutation results in a thrombophilic state is unclear. It is thought that the increased amount of circulating prothrombin provides a springboard upon which the clotting cascade can get started and that, in some circumstances, it may run out of control because of that springboard potential. The prothrombin gene mutation (PT) is signaled by a defect in clotting factor II at position G20210A and the human prothrombin gene spans 21 kb on chromosome 11p11-q12 and consists of 14 exons and 13 introns, which account for 90 percent of the sequence. This mutation occurs as a result of the G to A transition at nucleotide 20210 in the prothrombin gene. The reported prevalence in Europe is around 2 %to 6% and the risk of venous thrombosis to heterozygous carriers is three times the normal population (Poort, *et.al.*, 1996). More recent studies have shown that G20210A mutation is associated with RM (Yenicesu, *et.al.*, 2010). However several studies found that the incidence of G20210A mutation was rare in women with RM (Agorastos, *et.al.*, 2002, Altintas , *et.al.*, 2007, Behjati , *et.al.*, 2006). Therefore, the role of G20210A mutation in RM still needs to be elucidated.

1.2.2.4 .4 .1.2 Factor V Leiden mutation:

Factor V is one of the essential clotting factors in the coagulation cascade. Its active form, factor Va, acts as a cofactor allowing factor X to stimulate the conversion of prothrombin to thrombin. Thrombin is then able to cleave fibrinogen to fibrin and a fibrin clot is formed. Activated protein C is a natural anticoagulant it limits the extent of clotting by destroying factor V and reducing further thrombin formation. Factor V Leiden (FVL) mutation (named after the Dutch university where it was discovered) is a point mutation in the gene for clotting factor V (Van and Levi, 2013). It has autosomal dominant inheritance and is the most common cause of inherited thrombophilia the mutation of Factor V Leiden causes acquired protein C resistance, resulting in thrombophilia both in veins and spiral arteries of the placenta. This may lead to placenta abruption and consequently results in miscarriage heterozygote’s have a three to five times increased risk of thrombosis. Women with this mutation are two to three times more likely to have multiple (recurrent) miscarriages or a pregnancy loss during the

second or third trimester. Some research suggests that the factor V Leiden mutation may also increase the risk of other complications during pregnancy, including pregnancy-induced high blood pressure (preeclampsia), slow fetal growth, and early separation of the placenta from the uterine wall (placental abruption). However, the association between the factor V Leiden mutation and these complications has not been confirmed. Most women with factor V Leiden thrombophilia have normal pregnancies and Homozygotes are much less common but have a much higher thrombotic risk, around eight times increased risk (van Vlijmen, *et. al.*, 2011). The association between the FVL mutation and RPL seems stronger for non-recurrent second-trimester pregnancy loss compared with recurrent early pregnancy loss (Robertson, *et.al.*, 2006). Factor V Leiden mutation is the most common hypercoagulable disorder occurring in 5% of the white population. This mutation leads to a form of factor V that when activated to factor Va is resistant to degradation by activated protein C. There is increased procoagulants activity and therefore increased risk of Thromboembolism (Kujovich, 2011). FVL according to epidemiology is present in around 5% of Caucasians and It is rare or absent in people of black African, Far East Asian, native Australian and native American origin and The chance of developing an abnormal blood clot depends on whether a person has one or two copies of the factor V Leiden mutation in each cell. People who inherit two copies of the mutation, one from each parent, have a higher risk of developing a clot than people who inherit one copy of the mutation (van and Levi, 2013).

1.2.2.4 .4 .1.3 Methylene tetrahydrofolate reductase deficiencies:

Methylene tetrahydrofolate reductase (MTHFR) is one of the main regulatory enzymes in the metabolism of homocysteine that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Forges , *et.al.*, 2007).

Methylene tetrahydrofolate reductase (MTHFR) is a rare genetic defect that leads to complications in pregnancy (Ivy, *et.al.*, 2007). MTHFR gene produces an enzyme called methylene tetrahydrofolate reductase and mutation in the gene inhibits the production of this enzyme, result in hyperhomocystinemia, which is an elevated level of an enzyme homocysteine found in blood plasma. When the body is deficient in methylene trahydrofolate reductase, its ability to absorb folate, such as folic acid, is inhibited. Folic

acid and B9 are both essential to the development and health of the fetus. Because of a mother with MTHFR ’inability to efficiently metabolize folic acid and vitamin B9, the disorder has been linked to a variety of pregnancy complications such as congenital malformations. Elevated levels of homocysteine have been associated with placental disease, preeclampsia and RPL (Foka, *et. al*., 2000).

Deficiency in the homocysteine metabolism pathway resulting in an elevation of homocysteine level in plasma (hyperhomocystinemia) has been regarded as a cause of Thrombophilia (Michael, 2003). Mutations in MTHFR gene lead to decreased activity of enzyme and hyperhomocystinemia, which induces platelet aggregation through promotion of endothelial oxidative damage. Although several mutations within the MTHFR gene but C677T and A1298C mutations are the two most common mutations (Mtiraoui, *et.al*., 2006). Single-nucleotide polymorphisms (SNPs) in metabolic pathways, which regulate enzymes such as MTHFR, are considered to be risk factors for thrombophilia. MTHFR is the key enzyme in folate, methionine, and homocysteine metabolism. The disturbances in MTHFR activity could be the cause of increased serum level of homocysteine. Hyperhomocystinemia is a risk factor for changes in coagulation cascade through direct Cytotoxic influence on endothelium, atherogenesis, activation of coagulation factor V and VII, increased level of thrombin, platelet aggregation, and a tendency toward venous thrombosis. (Spiroski, *et.al.*, 2008).

The common C677T missense mutation in the MTHFR gene, which converts an alanine to a valineresidue, decreases the enzymatic activity and leads to high homocysteine and low folate levels in plasma (Botto and yang ,2000). Homocysteine is metabolised by either the transsulfaration pathway (excess homocysteine is converted to methionine) or the remethylation pathway (recycling of homocysteine to form methionine). Increased homocysteine is an independent risk factor for venous thrombo-embolism (Perry, 1999). The 667 C to T MTHFR mutation results in a thermo labile enzyme with reduced activity for the remethylation of homocysteine. The homozygous form of the mutation induces a state of hyperhomocystinemia (Kujovic, 2004).

1.2.2.4 .4 .1.4 Factor XII:

Factor XII (Hageman factor) is an important protease that plays a major role in the initiation of the intrinsic pathway of blood coagulation and fibrinolysis and kinin formation. Although congenital factor XII deficiency (up to 50% of normal) is not associated with a clinical bleeding tendency, it can be identified on a routine coagulation test, such as a prolonged activated partial thromboplastin time (Sotiriadis, *et. al*., 2007). This deficiency is a rare autosomal recessive disorder. It is still unclear whether factor XII deficiency causes any disorders during pregnancy. Disruption of this system may be a risk factor for early pregnancy losses and recurrent miscarriages and placental abruption were reported in cases with factor XII deficiency (Pauer, *et. al*., 2003 ; Jones *et. al*., 2006). It is well known that congenital thrombophilia is associated with fetal loss and to cause significant maternal complications, and possibly has an adverse effect on normal fetal development (Inomo, *et. al*., 2008). Thus, factor XII deficiency and hypo fibrinolysis (mainly high plasminogen activator inhibitor activity) are the most frequent hemostasis-related abnormalities found in unexplained primary recurrent aborters. In patients with antiphospholipid antibodies or hypo fibrinolysis, there is a non-inflammatory ongoing chronic elevation of markers of endothelial stimulation associated with coagulation activation

1.2.2.4 .4 .1.5 Protein C and Protein S deficiencies:

Protein C inactivates factor Va and VIIIa involved in the anticoagulant process and this function is enhanced in the presence of protein S. Protein C deficiency results from a decrease in protein C antigen or the activity of protein C also Protein C is a 62-kD, vitamin K-dependent glycoprotein synthesized in the liver. It circulates in the blood as an inactive zymogene at a concentration of 4 μg/ml. Its activation into the serine-protease like enzyme, activated protein C (aPC), is catalyzed by thrombin when it is bound to the endothelial proteoglycan thrombomodulin (Dahlback , 2008) Protein S is a vitamin K-dependent, single-chain glycoprotein, which is synthesized in the liver and vascular endothelium, and acts mainly as a cofactor to aPC in the inactivation of FVIIa and FVa. Protein S is the principle cofactor of activated protein C , and deficiency states mimic protein C deficiency with increased fibrin formation Protein . Bind directly to

inhibit factors Va, VIIIa, and Xa. Proteins exists in two distinct forms in plasma the free form accounts for 35 to 40% of total protein S , whereas the remainder is found in a form bound to C4b binding protein. Only the free protein S can serve as a cofactor for protein. The plasma level of protein S depends upon age, sex, lipid levels, estrogen, oral anticoagulant usage and the presence of acute thrombosis. In the plasma, around 60% of circulating protein S is bound to C4b binding protein, and only free protein S can function as a cofactor to aPC. Heritable protein S deficiency is transmitted as an autosomal trait. Those with heterozygous deficiency are at increased risk of venous thrombotic events (VTE), as well as warfarin-induced skin necrosis (Clark and Greer, 2006). Proteins S deficiency results from a decrease in the concentration or the function of protein S (Ten and Van, 2008). Protein S plays a role in inhibition of the clotting cascade. Protein S and C inactivate factors VIIIa and Va, required cofactors for factors IXa and Xa. This is important because the most important natural inhibitor of clotting, the tissue factor pathway inhibitor, can be short circuited by factor IXa; so inhibition of the clotting cascade requires inhibition of factors IXa and Xa. This is achieved with the complex of activated protein C and protein S (Mary and Peter, 2005).

Sixty percent of protein S circulates in a protein bound form, and only the remaining 40% free form is biologically active. Certain conditions, such as pregnancy, inflammation, and surgical stress, lead to increased levels of the complement 4b-binding protein, which binds to protein S, and thereby decrease protein S activity. In addition, pregnancy is a thrombogenic state because of other alterations in the coagulation pathway. There is a 20% to 200% increase in levels of fibrinogen and some clotting factors. At the same time, the tissue factor pathway inhibitor increases only minimally, whereas antithrombin and protein C levels remain constant. Also, the level of plasminogen activator inhibitor, the main inhibitor of fibrinolysis, increases 3-fold during pregnancy Protein S deficiency is an autosomal dominant mutation that confers a modest risk of thrombo embolism 5% to 20% risk during pregnancy and the postpartum period. The risk of miscarriage does not seem to be increased with a deficiency of protein S or protein C. However, there may be an increased risk of fetal loss later in pregnancy, severe preeclampsia, abruption placenta, and fetal growth restriction with

protein C or S deficiency. There are no randomized prospective trials to show the efficacy of different anticoagulation regimens in affected patients. (Namee, *et.al*., 2012).

Protein C and S deficiencies have been linked to an increased risk of miscarriage. For example, a study examined the effect of thrombo prophylaxis on the reduction of pregnancy losses. They found that women who received low-molecular-weight heparin treatment for deficiency of Protein C and S had a significantly lower miscarriage rate than those who did not receive the treatment (Folkeringa, *et.al*., 2007), so the best recommendations are based on expert opinion suggesting that Protein C and Protein S deficiencies may be a contributory factor of miscarriage. All women with a history of thrombo embolism who are planning pregnancy should be tested for inherited thrombophilias. In addition, women with a history of fetal loss, abruption, severe preeclampsia, and severe intrauterine growth restriction should also be tested. It is not yet clear whether patients with a history of recurrent early pregnancy losses at <10 weeks' gestation should be tested.

1.2.2.4 .4 .1.6 Antithrombin III deficiencies:

Antithrombin is a potent inhibitor of the reactions of the coagulation cascade. Although the name, antithrombin, implies that it works only on thrombin, it actually serves to inhibit virtually all of the coagulation enzymes to at least some extent. The primary enzymes it inhibits are factor Xa, factor IXa and thrombin (factor IIa). It also has inhibitory actions on factor XIIa, factor XIa and the complex of factor VIIa and tissue factor. Its ability to limit coagulation through multiple interactions makes it one of the primary natural anticoagulant proteins. Its numerous interactions are depicted on the above figure.(Hyers, 2001). Antithrombin acts as a relatively inefficient inhibitor on its own. However, when it is able to bind with heparin, the speed with which the reaction that causes inhibition occurs is greatly accelerated; this makes the antithrombin-heparin complex a vital component of coagulation. This interaction is also the basis for the use of heparin and low-molecular-weight heparins as medications to produce anticoagulation. There are two primary types of antithrombin deficiency: type I and type II. Type I antithrombin deficiency is characterized by an inadequate amount of normal antithrombin present. In this case, there is simply not enough antithrombin present to

inactivate the coagulation factors. In type II antithrombin deficiency, the amount of antithrombin present is normal, but it does not function properly and is thus unable to carry out its normal functions. In many cases, the antithrombin in type I deficiencies has a problem binding to heparin, although there have been multiple other changes to the antithrombin molecule described. (Brenner, *et. al.*, 2000).

The clinical relevance of a distinction between antithrombin I and antithrombin II deficiency lies in the higher risk of thrombosis associated with the type I variety. Antithrombin III is the most important inhibitor of thrombin, factor Xa, IXa and XII a. Antithrombin III deficiency results from the decrease in the concentration or the function of antithrombin III (Patnaik and Moll, 2008).

1.2.2.4 .4 .1.7 Plasminogen Activator Inhibitor 1 (PAI1):

Plasminogen activator inhibitor-1 is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the activators of plasminogen and hence fibrinolysis. Plasminogen activator inhibitor 1 (PAI-1) inhibits plasminogen activators (u-PA and t-PA) by forming stable complexes endocytosed via a low-density lipoprotein receptor super family member-dependent mechanism. PAI-1 circulates actively in plasma and latently in platelets but is also secreted and deposited into the matrix by several cells, where it participates in tissue repair processes. Endothelial PAI-1 expression is modulated by a 4G/5G polymorphism in the PAI-1 promoter, which is 675 bp upstream from the start site of transcription. Angiotensin II plasma levels also influence PAI-1 expression. Homozygosity for the 4G allele of the PAI-1 gene increases the risk for pregnancies, predisposing to prematurity, intrauterine growth retardation, miscarriage and stillbirth (Buchholz, *et.al*., 2003)

1.2.2.4 .4 .2 Acquired thrombophilia:

Acquired thrombophilias are hypercoagulable states secondary to various aetiologies. In particular, during pregnancy the risks are exaggerated due to the underlying physiological changes. The most common acquired thrombophilia associated with RM is the antiphospholipid syndrome (APS). Antiphospholipid antibodies are auto antibodies

against negatively charged phospholipids. APS is categorized as primary (where it occurs in isolation) and secondary (George and Erkan , 2009).

1.2.2.4 .4 .2.1 Acquired hyperhomocystinemia:

Hyperhomocystinemia has been underlined as an emerging risk factor for several diseases such as arterial and/or venous thrombosis Hyperhomocystinemia may be acquired secondary to dietary and lifestyle factors such as a reduced intake of folate, vitamin B6 or vitamin B12, excessive caffeine consumption and excessive coffee intake. The acquired form of hyperhomocystinemia may also result from certain medical conditions such as hypothyroidism or renal impairment. Inherited and acquired conditions have been involved to explain pathophysilogy as gene polymorphism .The Homocysteine Lowering Trial Collaboration (Clark, *et.al*., 2007) has suggested that endothelial dysfunction, alteration of platelet reactivity and disruption of prostacyclin pathways, may be some of the mechanisms responsible for the reported venous thrombosis risk as well as the theoretical risk of pregnancy loss. A meta-analysis of ten studies concluded that acquired hyperhomocystinemia is a risk factor for recurrent pregnancy loss (Nelen, *et.al.*, 2000).

1.2.2.4 .4 .2.2.2 Acquired activated protein C resistance:

APCR is the most prevalent risk factor for thrombosis. The presence of the factor V Leiden mutation produces a protein that is intrinsically resistant to activated protein C, causing the pathological phenotype .The pathophysilogy underlying APCR not caused by the FVL mutation is still not completely understood. In different studies, it has been suggested that acquired factors might be the cause of APCR in the absence of FV Leiden .A number of coagulation factors can affect the activated partial thromboplastin time (aPTT). Previous literature suggested a possible positive correlation between levels of factors V, VIII and IX and acquired APCR. Protein S and protein C, levels can (or may) affect acquired APCR. (Sara, *et.al*., 2011).

1.2.2.4 .4 .2.2.3 Antiphospholipid syndromes:

Antiphospholipid syndrome is the most important treatable cause of recurrent miscarriage. Anti phospholipid antibodies are a family of about 20 antibodies that are

directed against phospholipid binding plasma proteins. Evidence for pregnancy loss having a thrombotic basis is based mostly in the association between anti-phospholipid (aPL) antibodies and RPL (Carp, 2006).

They include lupus anticoagulant and anti cardiolipin antibodies. Antiphospholipid syndrome was originally defined as the association between antiphospholipid antibodies and recurrent miscarriage, thrombosis, or thrombocytopenia ,anti-phospholipid antibody syndrome is characterized by the presence of aPL, anti-lupus coagulant, anti-cardiolipin, and/or anti-beta-2-glycoprotein I antibodies that bind to negatively charged phospholipids on the membranes of endothelial cells, monocytes, and platelets (Brenne, 2010).

1.2.2.4 .4 .2.4 Disseminated intravascular coagulation (DIC):

Disseminated intravascular coagulation is a pathological disruption of haemostasis characterized by a systemic activation of coagulation leading to widespread fibrin deposition (stage I), subsequent depletion of platelets and coagulation factors that culminates in severe bleeding (stageIII) . (Thachil and Toh , 2009). The most commonly described obstetric causes of DIC include amniotic fluid embolism, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, pre-eclampsia/eclampsia, placental abruption, and septic abortion. The pathophysiological mechanisms that lead to DIC are unknown, but in many cases placental insufficiency and utero-placental hypo-perfusion are thought to play a causal role. The classical findings associated with DIC include prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT), low platelet counts, elevated products of fibrin breakdown (e.g. D-dimer), and low plasma levels of coagulation inhibitors (ATIII) (Ferasatkish , *et.al.,* 2007).

Although these features together with clinical evidence of simultaneous bleeding and micro thrombi can aid diagnosis, they are not without limitations. The current tools used to diagnose DIC pose problems since a correct diagnosis can be masked by haemostatic changes that occur during pregnancy. For instance, the levels of D-dimmers are inherently high in normal pregnancy and cannot simply be attributed to DIC.

1.2.2.4 .5 Fetal Factors:

Chromosomal anomaly is the commonest fetal cause of RM. Aneuploidy is the most prevalent chromosomal abnormalities of abortuses in RM (Carp, *et.al.*, 2001). Evidence from preimplantation genetic diagnosis (PGD) has shown that women with RM had a higher incidence of chromosomally abnormal embryos after Aneuploidy screening than those without RM (Rubio, *et.al.*, 2005, Rubio , *et.al.*, 2003).

1.2.2.4 .5.1 Fetal-blocking antibodies:

Fetal blocking antibodies work to protect the baby from the mother's immune system, which will recognize the father's genetic material as foreign to her body and attack it. When the sperm penetrate the egg, it provides foreign material, but it also contains histo compatibility locus antigens (HLA). The sperm's HLA will "talk" to the mother's HLA, which would normally attack the baby, and stimulate the mother's body to protect the baby. In some cases, however, the father's genetic material is too similar to the mother's In that case; the mother's .response is weak and insufficient to prevent her white blood cells from attacking the new cells. This type of problem usually causes an early miscarriage, well before 12 weeks, and is often suspected when several miscarriages have occurred at the exact same time in the pregnancy (Aldrich and Karrison, 2001).

1.2.2.4 .5.2 Umbilical cord abnormalities:

The umbilical cord is a narrow, tube-like structure that connects the developing fetus to the placenta. The umbilical cord begins to form about five weeks after conception. There are three blood vessels inside the umbilical cord—two arteries and one vein. The vein carries oxygen-rich blood and nutrients from the placenta to the baby, while the two arteries transport waste from the baby back to the placenta where waste is transferred to the mother’s blood and disposed of by her kidneys. A number of abnormalities can affect the umbilical cord. Sometimes the cord is too long, too short, connects improperly to the placenta or becomes knotted or compressed. Cord abnormalities can lead to problems during pregnancy or during labor and delivery. In some cases, cord problems can affect mother and baby. The followings are some of the most frequent umbilical cord problems and how they can affect mother and baby (Cunningham, *et. al*., 2001).

1.2.2.4 .6 Environmental Factors:

Environmental factors; Heavy metals, organic solvents, and ionizing radiation are confirmed teratogens, and exposure to these can contribute to pregnancy loss. Alcohol and cocaine are also confirmed teratogens, while caffeine and smoking are suspected teratogens but their teratogenic impact is still controversial .Caffeine consumption and smoking has been implicated in increasing the risk of miscarriage (Kaare, 2009). Recent studies have shown that women who are homozygous for CYP1A2 -1F alleles (an enzyme responsible for caffeine metabolism) had a high risk of RM with a dose dependent effect of daily caffeine intake (Sata, *et.al*., 2005). Other studies have shown that women exposed to environmental tobacco smoke had a high risk of spontaneous miscarriage in combination with caffeine and alcohol consumption (Windham, *et.al*., 1999).

1.2.2.4 .7 Stress factor:

Pregnancy is a special time for a woman and her family. It is a time of many changes in a pregnant woman's body, in her emotions and in the life of her family. Stress stimulation can trigger a series of physiological adaptive responses. These changes often add new stresses to the lives of busy pregnant women who already face many demands at home and at work. However, when physical or emotional stress builds up to uncomfortable levels, it can be harmful for pregnant women. The systems that respond to stress are the hypothalamo-pituitary-adrenal (HPA) axis and the sympathoadrenal system. Stress can affect the secretion of the parvocellular neurons (PVN) in the hypothalamus and the release of neuropeptides, corticotrophin releasing hormone (CRH) and arginine vasopression (AVP)Recently, the impact of psychological stress on RM has received more attention. Studies have suggested that stress may play a role in RM through maternal neuro-endocrine-immune network response (Andalib, *et.al*., 2006). Stress, namely pressure or tension, has been defined as a form of psychological, physiological and behavioural transaction between people and environment, i.e. Person-environment fit. In this definition the external environmental stimulus is regarded as a stressor and the psychological response to the external stimulus is termed as stress (Ogden, 2007). The effect of stress may be beneficial to enable fight or flight adaptive

response to escape from the harmful situation and the homeostasis may still be resumed. However, effect of stress may be detrimental by mediating a series of physiological responses resulting in adverse somatic consequences, including impaired cognition, abnormal metabolism, immune function and impaired reproduction. Two types of stress are differentiated including acute stress and chronic stress. Acute stress is a response to an immediate threat, such as an exam or a public presentation.

1.2.2.4 .8 Alcohol and smoking:

Drinking alcohol during pregnancy can cause physical and mental birth defects. According to the Centers for Disease Control and Prevention each year between 1,300 and 8,000 babies in the United States are born with fetal alcohol syndrome (FAS), a combination of physical and mental birth defects. Consuming alcohol during pregnancy increases the risk of miscarriage, low birth weight and stillbirth. Heavy drinkers are two to four times more likely to have a miscarriage between the fourth and sixth months of pregnancy than are nondrinkers (Kesmodel, *et. al*., 2002). This is a major public health problem because not only can smoking harm a woman's health, but smoking during pregnancy can lead to serious health problems in newborns (Samet and Yoon, 2001). Smoking has been associated with a number of pregnancy complications. Early in pregnancy smoking appears to increase a woman's risk of having an ectopic pregnancy, placental complications, thus increasing the risk of miscarriage (Wang , *et. al*., 2002).

1.2.2.5 Signs and Symptoms of spontaneous abortion:

1.2.2.5.1 The followings are considered the main signs:

- Strong cramps that cause breathe in a huffy way followed by quick bleeding.

- Heavy bleeding that soaks a pad in a few hours or less.

- Passage of tissue, resembling large thick blood clots in the earliest weeks up to pinkish/grayish material, with or without cramps or pain

1.2.2.5.2 Other possible signs include:

- Vaginal bleeding: The bleeding associated with spontaneous abortion ranges from scant brown spotting to heavy vaginal bleeding. The volume or pattern of bleeding does not predict a spontaneous abortion. Vaginal bleeding is common in the first trimester, occurring in 20 to 40 percent of pregnant women even heavy, prolonged bleeding can be associated with a normal outcome. As an example, in a prospective study of over 4000 pregnant women, 12 percent of women with first trimester vaginal bleeding had a miscarriage, but miscarriage also occurred in 13 percent of women without bleeding (Hasan, *et.al*., 2010).

Pelvic pain: The pain that accompanies a spontaneous abortion is typically crampy or dull in character and may be constant or intermittent.

- **Cramping:** Is usually frequent and random during the whole pregnancy and it becomes of concern if breathing becomes in a labor-like huff, or if it is associated with bleeding.

- Loss of pregnancy symptoms: While the complete and sudden loss of pregnancy symptoms can signal a pending miscarriage, usually it is not the first sign. Women at this stage may not feel pregnant for many days when nausea abates for a day or two and the breasts are less sore. Around weeks 10 to 14, this is completely normal, as hormone levels even out and the placenta take over. The loss of pregnancy symptoms during a miscarriage is usually something you see in hindsight, not ahead of time.

- **Incidental finding on ultrasound**: Spontaneous abortion may be detected due to the absence of fetal cardiac activity on a hand-held Doppler or pelvic ultrasound examination.

- If products of conception remain in the uterus after spontaneous abortion, vaginal bleeding may occur, sometimes after a delay of hours to days. Infection may also develop, causing fever, pain, and sometimes sepsis.

- A pregnancy test that is positive, then negative: this is a classic sign of an Ectopic and often associated with spotting.

1.3 Rationale:

Miscarriage is a common occurrence in the life cycle of the woman. Exactly how common this experience is not known exactly. Pregnancy is a complicated physiological process that might lead to negative outcomes and could threaten the women`s life or the fetus. Current literature suggests that the cause of RM is only identifiable in up to 40%-50% of cases. Women with Factor V Leiden, prothrombin and methylene tetrahydrofolate reductase mutation have been link to an increased risk of early, late and recurrent pregnancy loss.

The Study of these mutations among Sudanese women was not done previously in Sudan and there are no clear data bases on the genetic which can cause recurrent spontaneous abortion among Sudanese women. This study will be a base line to determine where the cause of thrombophilia in recurrent Spontaneous Abortion is the mutation of one of above gene or to others causes among Sudanese pregnant women with recurrent Spontaneous Abortion, or women who had Deep Vien Thrombosis (DVT) and Thrombooembolic (TE) after delivery. This study may be useful in the improvement of gynecological care of women with recurrent pregnancy loss and accurate knowledge of all significant complications in these women regarding thrombophilia and formulate a plan to diagnosis and development of new treatment strategies is essential.

Also the clinical importance of identifying of these genetic defects in women with spontaneous recurrent abortion can rests with the possibility of counseling them for primary prophylaxis or supports in situations.

1.4 Hypothesis:

-There will be a significant association between the Factor V, Prothrombin and methylene tetrahydrofolate reductase gene mutation with the causes of recurrent spontaneous abortion.

- There will be a significant association between the recurrent spontaneous abortion and the selected demographic variables.

1.5 Objectives:

1.5.1 General objective:

To study the Thrombophilic Mutation of Factor V G1691A, Prothrombin G20210A and methylene tetrahydrofolate reductase C677T gene mutation among recurrent spontaneous abortion Sudanese Women

1.5.2 Specific objectives:

1- To measure the coagulation (PT and PTT) among recurrent spontaneous abortion women.

2- To detect the incidence of Factor V G1691A mutation among recurrent abortion Sudanese women.

3- To detect the incidence of Prothrombin G20210A mutation among recurrent abortion Sudanese Women.

4- To detect the incidence of methylene tetrahydrofolate reductase C677T mutation among recurrent abortion Sudanese Women.

5- To determine the Relationship between maternal age and recurrent pregnancy loss.

CHAPTER TWO

Materials and Methods:

2.1 Materials:

2.1.1 Study area:

Omdurman is the largest city in Sudan and Khartoum State, lying on the western banks of the River Nile, opposite the capital, Khartoum. Omdurman has a population of 2,577,780 (2010) and is the national centre of commerce. With Khartoum and Khartoum North or Bahri, it forms the cultural and industrial heart of the nation. Omdurman is located at 15.6445 latitude and 32.4777 longitudes at an elevation/altitude of meters. The average elevation of Omdurman, Sudan is 391 meters.

Wikimedia Commons, the free media repository

Figure: 1.2 Khartoum state maps

2.1.2 Study Setting:

Omdurman Maternity Hospital (Sudan).

2.1.3 Study design:

A prospective analytical case control study

2.1.4 Study population:

Sudanese women who experienced three or more of the adverse pregnancy Outcomes during their reproductive age attended Omdurman Maternity Hospital.

2.1.5 Controls:

The control group included ninety four healthy women who attended the same medical facilities (mean age was 30 ± 4. years) with at least more than 2 normal pregnancies and without any history of adverse pregnancy outcome or recurrent miscarriages.

2.1.6 Inclusion Criteria:

Each woman who has at least three or more consecutive RPL outcomes with unknown caused.

2.1.7 Exclusion criteria:

Woman who had any of the following criteria were excluded from the study :

- A history of vascular thrombotic disease
- Fetal congenital anomalies
- Fetal chromosomal anomalies
- Uterine abnormalities
- A known causes of the abortion.

2.1.8 Sample size:

One hundred women selected by conventional random sampling because limited resources and there were no any statistical data available about the number of women with RPL. But the represents samples should be selected according to the fallowing equation:

$n= N /1 +N (e)^2$

n= sample

N= population size

e= precision

2.1.9 Data Collection

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

2.2 Method:

2.2.1 Sample collection:

Five ml venous blood was collected from each participant into EDTA and tri-sodium citrate container after interviewed verbal and written consent was obtained from each participant blood Specimens were labeled with patient name, medical record number, date and time of collection.

2.2.2 Hemostatic analysis:

2.2.2.1 Preparation of platelets poor plasma (PPP):

Within 3 hours of blood collected, centrifuged capped citrate tube for 10 minutes at an RCF (relative centrifugal force) of 2000g . Used a plastic transfer pipet, removed the top 3/4 of plasma was placed it in a plastic centrifuge tube with cap. Centrifuged the plasma (in the plastic centrifuge tube) for another 10 minutes at 2000g. Using a plastic transfer pipet, removed the top ¾ of plasma into a plastic tube. Without disturbing the plasma in the bottom of the spun tube, where any residual platelets will be. (Marco, *et.al*., 2007)

2.2.2.2 Determination of Prothrombin Time:

PT an aliquot of test platelet-poor plasma was incubated at 37C° with a reagent containing a tissue factor, phospholipid (thromboplastin), and $CaCl_2$. (Jackson, *et.al.*, 2003). The time required for clot formation was measured by coagulometer (MSLBA13) the results was reported in seconds (prothrombin time), or as a ratio compared to the laboratory mean normal control (prothrombin ratio, PTR).

2.2.2.3 Determination of Partial Prothrombin Time:

The aPTT an aliquot of undiluted, Platelet poor plasma [PPP] was incubated at 37°C then phospholipid (cephalin) and a contact activator (e.g. Kaolin, micronized silica or ellagic acid) were added followed by calcium (all pre-warmed to 37°C). Addition of calcium initiates clotting and timing begins. The aPTT result was reported as the time required for clot formation after the addition of $CaCl_2$ and then measured by coagulometer (MSLBA13). (Jackson, *et.al.*, 2003)

2.2.3 Molecular analysis:

The detection of MTHFR, Prothrombin and factor V leiden gene were analyzed by Polymerase chain reaction (PCR) method.

2.2.3.1 Blood collection and DNA extraction:

Five ml venous blood was collected from each participant into EDTA tubes after consent obtained from each participant.

DNA was extracted from the blood samples using Master pure DNA purification kit for blood GF-1 BLOOD DNA EXTRACTION KIT, 100 PREPS (cat. No. GF-BD-050, Vivantis Technologies Sdn. Bhd., MALAYSIA).

2.2.3.1.1 GF-1 Blood DNA Extraction Kit:

The GF-1 Blood DNA Extraction Kit is designed for rapid and efficient purification of genomic DNA from up to 400µl whole blood. This kit used a specially treated glass filter membrane fixed into a column to efficiently bind DNA in the presence of high salt. This kit applies the principle of a mini-column spin technology and the use of optimized buffers to ensure that only DNA is isolated while cellular proteins, metabolites, salts and other low molecular weight impurities were removed during the subsequent washing steps. High-purity genomic DNA is then eluted in water or low salt buffers and has an A260/280 ratio between 1.7 and 1.9 made it ready to use in many routine molecular biology applications such as restriction enzyme digestion, PCR, Southern blotting, DNA fingerprinting, and other manipulations. The procedure is based on using Buffy coat, according to the following steps;

2.2.3.1.2 Blood Lysis:

Buffer BB 200 µl was added into 200 µl of blood in centerfuge tube. Then was mixed by pulsed vortexing then add 20 µl proteinase K. was added and mixed homogeneously by pulsed vortexing and then incubate at 65°Cfor 10 min.

2.2.3.1.3 Removal of RNA

RNA-free DNA is required, 20µl of RNase A was added (DNase-Free, 20mg/ml). Mix and incubate at 37°C for 10 min.

2.2.3.1.4 Addition of ethanol:

About 200µl of absolute ethanol was added, mixed immediately and thoroughly to obtain a homogeneous solution. (Mix immediately to prevent any uneven precipitation of nucleic acids due to high local ethanol concentrations.)

2.2.3.1.5 Loading to column:

The sample was transferred into a column assembled in a clean collection tube (provided). Centrifuge at 5,000 x g for 1 min. flow through was discarded.

2.2.3.1.6 Column washing 1:

The column washed with 500µl Wash Buffer 1 and centrifuged at 5,000 x g for 1 min, the flow through was discarded.

2.2.3.1.7 Column washing 2:

Washed the column with 500µl Wash Buffer 2 and centrifuged at 5,000 x g for 1 min. discarded flow through. Wash column again with 500µl Wash Buffer 2 and centrifuged at maximum speed for 3 minutes

2.2.3.1.8 DNA elution:

Place the column into a clean micro centrifuge tube. Add 100µl of preheated Elution Buffer, TE buffer or sterile water directly in to column membrane and stranded for 2 min. Centrifuged at 5,000 x g for 1 min to elute DNA.

DNA was used immediately for PCR analysis or stored at -20°C.

2.2.3.1.9 DNA Quantification:

Extracted Genomic DNA was quantified spectrophotometrically by measuring the absorbance at 260 nm spectrophotometer on Gene Quant (Amersham bioscence.UK).

2.2.3.2 Polymerase chain reaction (PCR):

PCR is a fast and inexpensive technique used to amplify small and targeted segments of DNA to produce million of copies, sometimes called "molecular photocopying" of a specific gene fragment.

2.2.3.2.1 PCR Components:

A basic PCR technique requires certain components and reagents that include:

1. Two primers (Forward and Reverse): short pieces of artificially prepared DNA that will target the gene fragment of interest in the entire genome. They are complementary to the

3’ ends of each strand of the double stranded target gene i.e. DNA.

2. Taq polymerase (DNA polymerase): It’s an enzyme whose function is to extend the new DNA strand. Taq polymerase attaches near the end of the primer and start added nucleotides. It requires double stranded DNA to become functional.

The DNA polymerase in our bodies broke down at temperatures below 95 °C - the temperature necessary to separate two complementary strands of DNA in a test tube. The DNA polymerase (Taq polymerase) that's used in PCR comes from a strain of bacteria called thermus aquaticus that live in the hot springs. It can survive near boiling temperatures and worked well at 72 °C.

3. Deoxynucleotide triphosphate (dNTP's): They were the building blocks from which the DNA polymerase synthesized a new DNA strand. Taq polymerase grabs nucleotides that were floated in the liquid around it and attached them to the end of a primer.

4. Buffer solution: provided a suitable chemical environment for optimum activity and stability of the DNA polymerase.

5. MgCl2: acts as a cofactor for the polymerase enzyme.

6. Extracted DNA sample: containing the target region to be amplified

2.2.3.2.2 PCR Steps:

PCR was a three-step process which is repeated in several cycles. The three steps are:

1- Denaturation step: This step consists of heating the reaction to 94 °C. (For 1min)

It causes DNA separated by disrupted the hydrogen bonds between complementary bases, yield single strands of DNA (Aleman, *et. al.,* 2013),

2- Annealing step: The reaction temperature is lowered according to the gene protocol for factor V (55°C for 30 seconds), Prothrombin and methylene (58°C for 1min seconds) allowing hybridization of the primers to the single-stranded DNA template.

3. Extension/Elongation step: at 72°C for 1 min at this step, the Taq polymerase synthesized a new DNA strand complementary to the DNA template stranded by adding dNTPs that are complementary to the template in 5' to 3' direction.

This process is repeated as many as 30 times, leading to more than one billion exact copies of the original DNA segment. The entire cycling process of PCR was automated and can be completed in just a few hours used a machine called a thermal cycler.

2.2.4 Detection of prothrombin gene mutation (G20210) gene:

For detection of G20210 prothrombin gene mutation, we used a PCR method that has been described previously by (Aleman, *et. al.,* 2013), a 345-bp genomic DNA fragment encompassing a part of the prothrombin gene that contains the mutation was amplified

by PCR using specific primers Forward (5'TCT AGA AAC AGT TGC CTG GC-3') and Reverse primer (5'ATA GCA CTG GGA GCA TTG AAG C-3)

2.2.5.1 Agarose gel electrophoresis:

The working solution of 1X TBE was prepared from the stock solution (1 L) which contains the following: 89 mM Tris base (108 gm), 89 mM boric acid (55 gm)
40 ml of 0.5M EDTA, adjust pH to 8.0 .
1.5% agarose was prepared from 1x TBE, and 5µl PCR products was loaded by mixed PCR products with 1µl loading dye, run on the gel for 30 mins and visualized on UV transellimantor

2.2.4.2 Digestion:

The PCR product (10 µL) was digested with 20 U of Hind III, at 37°C for 16 h, and loaded into 2% low melting point agarose gel, eletropherosed at 90 volts for 60 mins

2.2.4.3 Detection of fragments:

The digested products was visualized by UV transellimantor and photographed.

2.2.4.4 Result and interpretation:

the wild-type DNA yields a solitary 345-bp band, heterozygous yields two bands of 345 and 322 bp, respectively, and homozygous mutation only one band of 322 bp.

2.2.5 Detection of Factor V Leiden gene mutation (G1691A) gene:

Extracted genomic DNA was tested for the presence of FVL mutation used polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). 267-basepair (bp) segment of the factor V gene was amplified used specific forward primer (5'TCA GGC AGG AAC AAC ACC AT-3') and reverse primer 5'GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT 3

2.2.5.1 Agarose gel electrophoresis:

The working solution of 1X TBE was prepared from the stock solution (1 L) which contains the following: 89 mM Tris base (108 gm), 89 mM boric acid (55 gm)

40 ml of 0.5M EDTA, adjust pH to 8.0(It need not be autoclave)

1.5% agarose was prepared from 1x TBE, and 5µl PCR products was loaded by mixing PCR products with 1µl loading dye, run on the gel for 30 mins and visualized on UV transellimantor

2.2.5.2 Digestion:

Digested with 10 µl of DNA restriction enzyme MnI1 at 37°Cfor 18 h, subjected to 2% low melting point agarose.

2.2.5.3 Detection of fragments:

Low melting point agarose gel 2% electrophoresis and viewed under ultraviolet light after staining the gel with ethidium bromide.

2.2.5.4 Result interpretation:

The wild-type DNA yields a solitary gave 3 bands of 163, 67 and 37 bp bands, heterozygous yields two bands of 241 and 209 bp, respectively, and homozygous mutation yield tow band of 209/32 bp . (Ulehlova, *et.al*., 2014).

2.2.6 Methylene tetra hydrofolate reductase (MTHFR) Gene Mutations:

PCR will be carried out to made millions of copies of a specific DNA fragment consisting of a known functional mutation in the MTHFR gene by used site specific primers Forward (5' TGA AGG AGA AGG TGT CTG CGG GA-3') and

Reverse primers: 5'AGG ACG GTG CGG TGA GAG AGT G -3'.

2.2.6.1 Method of PCR:

A master mix was prepared by adding Nuclease free water,10x buffer,dNTP,tow primers,$Mgcl_{2,}$Taq DNA polymerase and DNA, the mixture was loaded into thermocycler according to the specific Temperature profile .

2.2.6.2Agarose gel electrophoresis:

The working solution of 1X TBE is prepared from the stock solution (1 L) which contains the following: 89 mM Tris base (108 gm), 89 mM boric acid (55 gm)

40 ml of 0.5M EDTA, adjust pH to 8.0(It need not be autoclave)

1.5% agarose was prepared from 1x TBE, and 5µl PCR products was loaded by mixing PCR products with 1µl loading dye, run on the gel for 30 mins and visualized on UV transellimantor.

2.2.6.3 MTHFR digestion:

The master mix reagents was added into the 0.5 ml PCR tube started with water, and the enzyme (Hindfl) at the latest stage, mixed well by pipptting up and dawn, preferably quick spin for seconds to collect the reagents to the bottom, to insure complete digestion and proper invermoments for setting the mixture working all the time at ice , by Added 10 µl mixtures to the 10 µl MTHFR products, a quick spinning is needed, 5- Incubated at 37 ºC 18 hours, and the reaction was Stopped with 4 µl prom phenol blue dye, then 18 µl digested products was loaded into 2% agarose .

2.2.6.4 Results interpretation:

The wild-type DNA yields a solitary gave (198 bp) bands and heterozygous yields three bands (198, 175, 23 bp) respectively, and homozygous mutation yield tow band of (175, 23 bp). (Shazia, *et.al*., 2008)

2.3. Data analysis:

Data were entered and analyzed by SPSS prograrmme (version: 17.0). All demographic data of the study population were presented as mean ± SD in the text and Odds Ratio was used for detecting the power of relationship between the determinant and the outcome and 95% confidence interval was calculated Data were analyzed using the Chi-square test for comparison the prevalence of MTHFR , Prothrombin gene and FVL mutation between patients and controls (The test considered significant when P.value <0.05)

CHAPTER THREE

RESULTS:

The participants included 194 women subjects. Out of them, 100 had a history of 3 or more events of recurrent fetal loss (abortion, miscarriage or still birth). Their mean age± SD was 25 ± 4. And 94 women were healthy the mean age of was 30 ± 4. (Table 3.1) The prothrombin time PT (p=0.93) and PTT (p=0.69) were normal among all women with RM and controls (100%) had normal PT and PTT. As shown in flowing table. There were no significant correlation between the PT, PTT and recurrent miscarriage (Table 3.2).

Factor V Leiden mutation distribution showed higher prevalence among cases group than controls group. The mutation was detected in 8 out of cases (8.0%) and in 6 out of 94 controls (6.4%) P- Value =0.66, Odds Ratio=1.28, 95% CI (0.42 to 3.84) The prevalence of heterozygous FVL mutation in RM Women was found to be 8 % but in control was found to be 6.4%. Normal homozygous (G/G) among cases show 92% but in controls show 93.6%. Alleles G allele occurred with a frequency of 96. % among cases and 96.8% in controls while mutant allele (A) was seen only in 4 % of the cases. Frequency of mutant allele (A) was 3.2 % and G allele occurred with a frequency of 96.8 % among controls. According to this result there is statistically insignificant between the cases and controls group (Table 3.3).

Prevalence of the Prothrombin gene was 3% among cases with P- Value =0.091.but no any mutant gene detected among control group. According to the genotyping in cases showed (Heterozygotes, 3.0%; Homozygotes, 97.0%), Alleles G (98.5%) and Alleles A (1.5%) while in controls group show Normal homozygous G/G (100%) and Alleles G (Alleles G) . No significant association between cases carriage any of this mutation and risk with recurrent pregnancy miscarriage (Table 3.4).

Frequency of Heterozygous C/T MTHFR gene was 3.0% in cases with P- Value =0.091, there was no mutant gene was detected among the controls group. Normal homozygous gene was 97.0% in cases and 100% show in controls .The frequency of Alleles C was 98.5% in cases and 100% in controls while Alleles T was 1.5%. There was no

significant association between cases carriage any of this mutation and risk of recurrent miscarriage (Table 3.5).

The cases group in our study was divided into subgroups based on time of recurrent abortion from second to eight times of repeated miscarriage. Our data indicates that factor V was most frequent with recurrent miscarriage, repeated in women with three time of repeated miscarriage by (37.65) fallow by women with four time of repeated miscarriage by(50%) and found in women with five time of repeated miscarriage by(12.5%) Prothrombin was found only among those women with three time of recurrent miscarriage with 100% and MTHFR present in three, four and five times of recurrent miscarriage women with equal percentage 33.3% foe each (Table 3.6).

Table 3.1 Distribution of Study Subjects According to Demographic Characteristics

characteristics		Patients N (%)	Controls N (%)
Age group	17-24	10(10.1)	13(13.8)
	25-29	29(29.3)	28(29.8)
	30-34	27(27.3)	36(38.3)
	35-39	21(21.2)	8(8.5)
	≥40	12(12.1)	9(9.6)
Area of resident	Khartoum	8(8.1)	85(90.4)
	Omdurman	88(88.9)	5(5.3)
	Bahri	3(3.0)	4(4.2)
	AB	6(6.1)	11(11.8)

Table 3.2 Average distribution of PT and PTT in patient and control groups

Risk factor	Patients Mean±SD	Controls Mean±SD	P-value
PT	13.82±1.13	13.84±1.17	0.93
PTT	32.47±3.26	32.67±3.54	0.69

Table 3.3 Frequency of factor V (Leiden) mutation among cases of recurrent pregnancy loss compared to controls:

Genotype	Patients N(%)	Controls N(%)	P-value	OR (95%CI)
Heterozygous G/A	8(8.0)	6(6.4)	0.66	1.28(0.42 to 3.84)
Normal homozygous G/G	92(92.0)	88(93.6)		
Alleles G	192(96.0)	182(96.8)	0.67	0.76(0.27 to 2.33)
Alleles A	8(4.0)	6(3.2)		

Table 3.4 Frequency of Prothrombin mutation among cases of recurrent pregnancy loss compared to controls:

Genotype	Patients N(%)	Controls N(%)	P-value	OR (95%CI)
Heterozygous G/A	3(3.0)	0	0.091	0
Normal homozygous G/G	97(97.0)	94(100)		
Alleles G	194(98.5)	188(100)	0.089	0
Alleles A	3(1.5)	0		

Table 3.5 Frequency of MTHFR mutation among cases of recurrent pregnancy loss compared to controls

Genotype	Patients N(%)	Controls N(%)	P-value	OR (95%CI)
Heterozygous C/T	3(3.0)	0	0.091	0
Normal homozygous C/C	97(97.0)	94(100)		
Alleles T	3(1.5)	0	0.089	0
Alleles C	194(98.5)	188(100)		

Table 3.6 Frequency of factor V (Leiden), Prothrombin and MTHFR related to times of recurrent pregnancy loss

Times of recurrent abortion	Factor V		Prothrombin		MTHFR	
	Positive	Negative	Positive	Negative	Positive	Negative
Twice	0	8(8.8)	0	8(8.3)	0	8(8.3)
Three times	3(37.5)	57(62.6)	3(100)	57(59.4)	1(33.3)	59(61.5)
Four times	4(50.0)	16(17.6)	0	20(20.8)	1(33.3)	19(19.8)
Five times	1(12.5)	6(6.6)	0	7(7.3)	1(33.3)	6(6.2)
Six times	0	1(1.1)	0	1(1.0)	0	1(1.0)
Seven times	0	1(1.1)	0	1(1.0)	0	1(1.0)
Eight times	0	2(2.2)	0	2(2.1)	0	2(2.1)

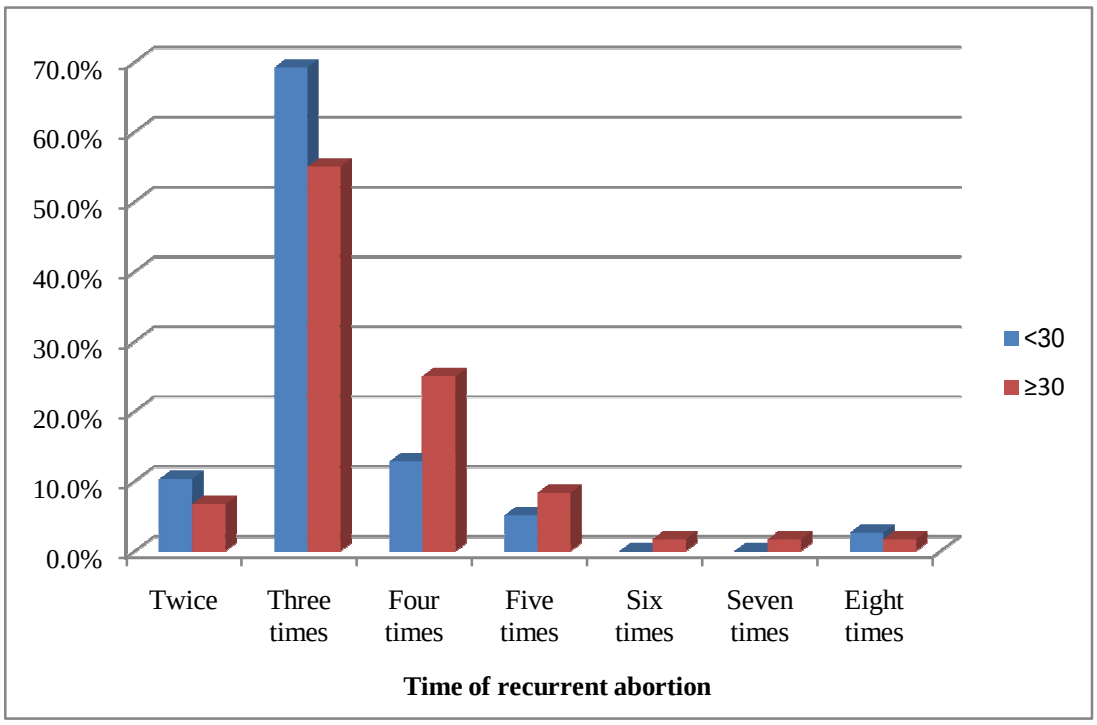
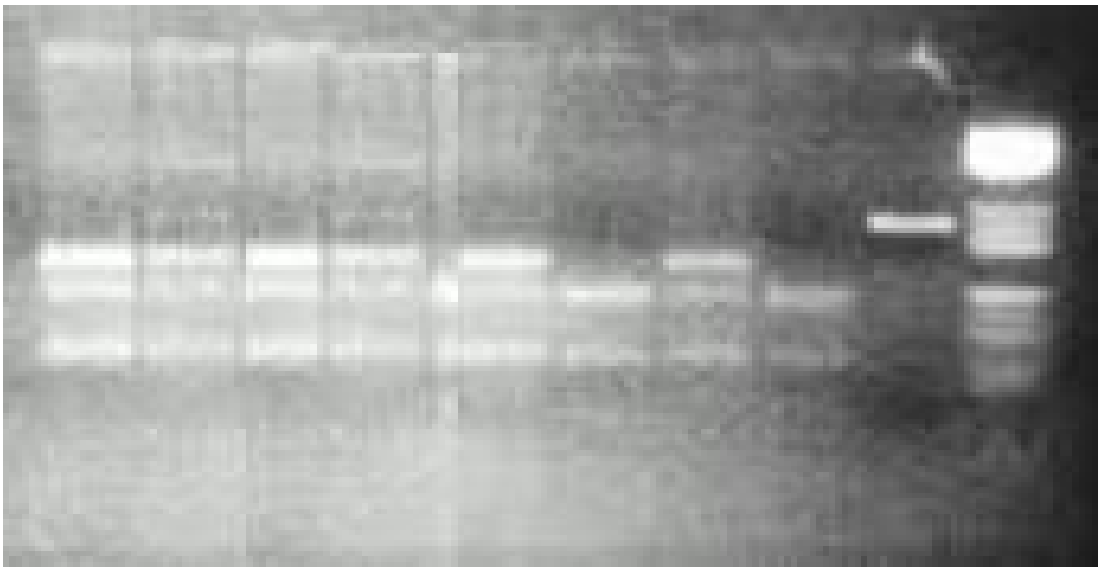


Figure 3.1 Distribution of recurrent misscreage cases according to age groups :

Maternal age was divided into 5 major groups (17-24,25-29,30-34,35-39 and ≥ 40). Abortions rates among these groups were represented by 38(18.8),26(12.9%), 39(19.2%), 40(19.8%) and 59(29.2%), respectively. Differences on prevalence of current abortion cases and maternal age were



10 9 8 7 6 5 4 3 2 1

Fig 3.2: PCR amplification of FVL gene mutation:

Digestion of factor v gene with MnI1 enzyme on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide , Lane 1 molecular weight marker 50 bp, lane2 undigested PCR products lane 3 and 5 were hetrozygous mutant (AG), Lane 4,6,7 and 8 ,9 and 10 were Wild typ (AA), The 267 bp DNA products digested with MnI1.

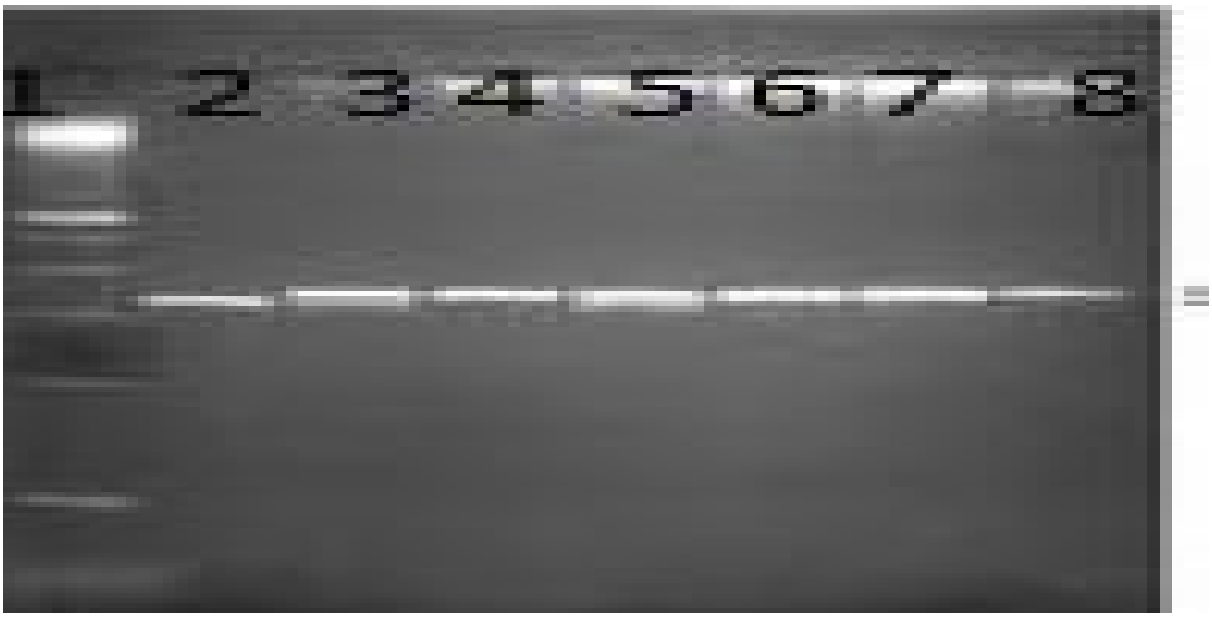


Fig 3.3 PCR amplification of Prothrombin gene mutation

Digestion of prothrombin gene with Hind III on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide , Lane 1 molecular weight marker 100 bp, lane 2 (322 bp) , mutant(AA), control ,lane 3 and 5 were hetrozygous mutant (GA), Lane 4,6 and 7 were Wild type (GG), lane 8 undigested(345 bp)

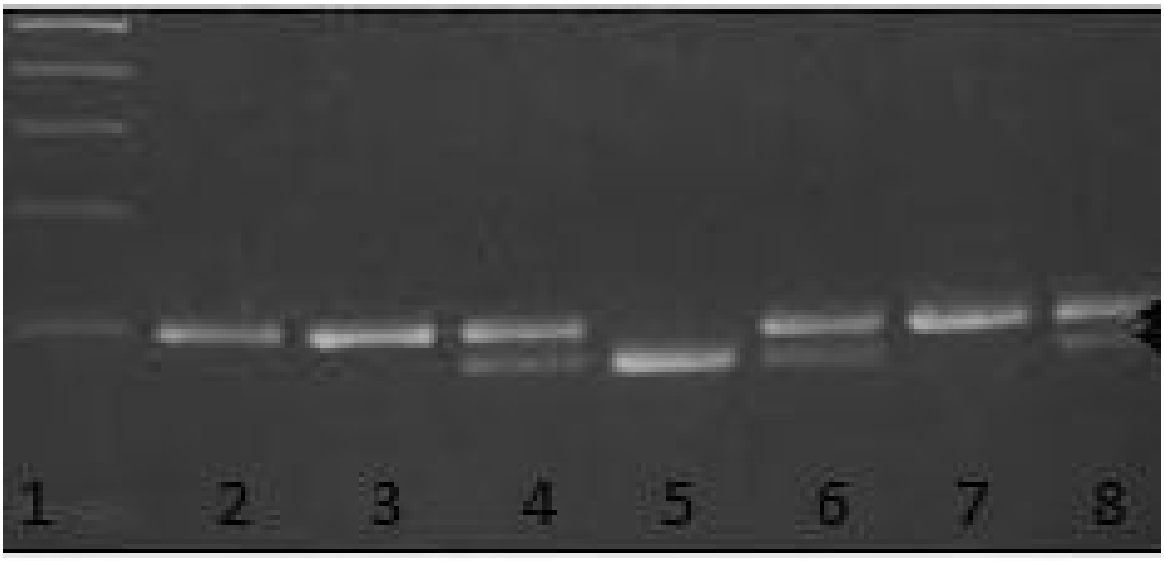


Fig 3.4 PCR amplification of MTHFR gene mutation

Digestion of MTHFR gene with Hindf1 on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide , Lane 1 molecular weight marker 100 bp, lane 2 undigested(198 bp) , lane 3 wild type (CC), Lane 4,6 and 8 were hetrozygous mutant(CT), lane 5 was Control homozygous mutant(TT)

CHAPTER FOUR

Discussion, Conclusion and Recommendation:

4.1 Discussion:

Recurrent spontaneous abortion is the most common complication of pregnancy, is the spontaneous loss of a pregnancy before the fetus has reached viability. Improvement of pregnancy outcome is considered as an important area of action for those concerned with the improvement of women's health and pregnancy outcome. Exploring the relation between Factor V Leiden. Prothrombin and methylene gene mutation with recurrent miscarriages is a challenge. This is due to the fact that recurrent miscarriages are with multiple etiologies, where genetic factors are considered one of those etiologies. Advances technology in molecular genetics provides an accurate and reliable tool to precisely study the genetic abnormalities associated with many diseases. Several studies identified thrombophilia as the principal cause of recurrent pregnancy loss. However, reported studies often do not evaluate other causes of miscarriages in their inclusion and exclusion criteria. So the aim of our study was to investigate the role of these genes in women with RPL.

Brenner, *et.al.*, (2000), identified thrombophilia as a principal cause in more than 40% of women affected by RPL. Other studies underlined a pathogenetic role of inherited thrombophilia in women affected by RFL. Sanson, *et .al.*, (1996), reported an increased frequency of antithrombin III, protein C, and protein S deficiency in women with RPL, while several studies underlined the role of inherited thrombophilia (in particular related to factor V Leiden gene polymorphism and prothrombin A20210G gene polymorphism) in the pathophysilogy of recurrent pregnancy loss. Yet increasing evidence is available on the relationship between hyperhomocystinemia and methylene-tetra-hydrofolate reductase (MTHFR) C677T gene polymorphism and unexplained recurrent pregnancy loss. Several reports have described an association between early recurrent pregnancy loss and hyperhomocystinemia and/or MTHFR C677T gene polymorphism (Fatini, *et .al.*, 2000).

The prevalence of Factor V Leiden mutation was tested and calculated in both case and control groups. The presence of Factor V Leiden mutation was slightly higher among cases group compared to the controls group. The prevalence of the mutation among cases group was 8% while it was found to be 6% among controls group. Factor V Leiden mutation, involved in the etiology of poor pregnancy outcomes, and has been proposed as one of the leading factors that is associated with poor pregnancy outcomes. We did not found a strong association with factor V Leiden gene polymorphism and recurrent spontaneous abortion.

Result of the present was in agreement with findings from the several large meta-analysis studies that have explored to determine any significant association between the factor V Leiden mutation and the presence of recurrent miscarriage during pregnancy. This result is agreement with study performed by Samieh, *et.al.,* (2010), among Iranian women in which the frequency of factor V Leiden gene mutation in total, (8.6%) of cases and 2(1%) of controls showed the factor V Leiden mutation and the incidence of factor V Leiden was typically higher in preeclamptic women than control group, concluded that pregnant women with factor V Leiden mutation were prone for preeclampsia syndrome during pregnancy, but this risk factor was not correlated to pregnancy complications in the studied women. Also, Altintas, *et. al.*, (2007), observed that FVL or FII mutation among Turkish women were nonsignificant compared to controls (7.9% vs. 7%, P=0.780) and (1.7 vs. 1.6%, P=0.931) respectively, while the presence both FVL and FII mutations among cases compared to controls (9.6% vs. 8.6%, P=0.756). The result obtained was comparable with the reported prevalence of 6% (2/33), 8.9% (4/21) and 10% (4/40) in Davalos, *et.al.*,(2005), Dizon,et.al.,(1999), and Prasmusinto, *et.al.*, (2004), studies, respectively In this study, there was statistically no significant correlation between the factor V Leiden mutation and pregnancy complication. Also the result was in agreement with large study performed by Jaslow, *et.al,* (2010), when diagnosed factors identified in 1020 women with two versus three or more recurrent pregnancy losses were found that the frequency of factor v was 6.8% among cases and 6.8% among control group and there were no any clinically significant between these result and recurrent pregnancy loss. Also, in contrast Preston, *et. al.*, (1996), failed to establish a significant association between Leiden mutation and

recurrent pregnancy losses in European women participating in the European Prospective Cohort on Thrombophilia.

Some studies reported the prevalence of factor V Leiden mutation varies among a nation to other nation and it's observed the lowest prevalence of the mutation among Asian nations especially in Indonesian and Japanese population (Bauduer,*et.al.*,2005), (Limdi,*et.al.*,2006)and (Kobashi,*et.al.*,1999).

On the whole, our data has indicated no statistically significant difference in the prevalence of FVL mutation between the two groups. These data which was observed in our study was in contrast with a previous report on the literature. One of these studies reported by Gihan and Osama, (2013) were found that FV (27% vs. 25% , P>0.0001) compared to control group and concluded that recurrent pregnancy loss among Saudi pregnant women was strongly associated with thrombophilic mutations related to both FVL . Also another studies done by Wolf, *et.al.*,(2003), among German women confirmed that FVL was significantly more common in cases with RPL compared to controls (10% vs. 2%, p=0.02), while FII was not associated with RPL. Also our study disagreement with small retrospective study done by, Kovacheva, *et.al.,* (2007), found that among Bulgarian women that Factor V Leiden was more common in the group of women with fetal loss in second and third trimester (OR-6.25; P<0.001) and significant protection for RFL in first trimester (OR-0.16; P<0.001). Mutation of prothrombin was more common in group of women with fetal loss in first trimester compared to the controls (28.3% vs. 11.2% respectively=0.009; OR-3.11). His conclusion was genetic thrombophilic defects were common in women with RFL and were associated with late fetal loss.

Extended to our study, the prothrombin G20210A mutation our result revealed that the mutation not common among recurrent spontaneous aborted Sudanese women they were found in 3 out of 100 women with frequency 3% and did not found any mutated gene among control group. We found there was no significant association between the prothrombin G20210A mutation and repeated of spontaneous abortion among Sudanese women. . This finding was agree with study performed by Robert, *et.al.,* (2010), were found there was a total of 157 (3.8%) women had the prothrombin gene mutation and

concluded that there was no association between the prothrombin G20210A mutation and pregnancy loss. Also the result is complete agreement with the one performed by Reaza , *et.al.,* (2006), where were found no significant difference was observed in the frequencies of FII mutations between the patients and controls , were found that the frequency of prothrombin was 3.2% in cases and 0.0%, in the control group . In another study done by Majid, *et.al.,* (2013), among 80 Iranian women with recurrent pregnancy loss no factor II mutation in cases and controls was found and concluded these data did not confirm that factor II prothrombin gene G20210 mutation might play a role in recurrent pregnancy loss in Iranian women. This result was disagreement with findings from the several large meta- analysis studies that have explored to determine any significant association between the prothrombin gene G20210 and the presence of recurrent pregnancy loss. The result of this study was different from ones reported by Sehirali, *et. al.,* (2005), observed that FII mutation was significantly higher in Turkish women with RM compared to controls (10.9% vs. 2.04%, $P<0.05$). Also Gihan and Osama, (2013), were found that FII was [50.1% vs. 38%, $P<0.0001$] , compared to control group and concluded that recurrent pregnancy loss among Saudi pregnant women was strongly associated with thrombophilic mutations related to FII. Characteristically, these cases showed a high frequency of factor II mutation. On the whole, our data has indicated no statistically significant difference in the prevalence of mutation between the two groups. This difference may be explained by differences in the populations or by using low numbers of samples. This difference in our result and other which found significant association may be explained by differences in the populations' ethnicity or by using low numbers of samples. Because some of literature done by Chang, *et.al.,* (2009), reports related to FII mutation could be due the ethnic difference of the studied groups. Some studies found that F2 variant was rare in African and Asian population (0.6% in African American).

In addition, we extended our study to the Methylene tetrahydrofolate reductase C677T gene mutation which suspected have role and associated with inherited thrombophilias among Sudanese women with recurrent miscarriage. The prevalence of MTHFR C677T variant among women with recurrent miscarriage is still a matter of controversy. In the our study we found that they did not found significant between this gene and recurrent

pregnancy loss among those women and the frequency of Heterozygous C/T MTHFR gene was 3.0% in cases with P- Value 0.091and there was no mutant gene was detected among the controls group. Our finding was agreement with several studies reported that there were no any statistical significant between MTHFR C677T and recurrent pregnancy loss. One of these studies done by Hasan and Sara, (2014), among iraqian women with Recurrent Abortion and reported that there was no significant difference in the prevalence of C677T genotype among women with RSA and healthy controls (P = 0.37). Also no statistically significant difference in the frequency of A1298C MTHFR gene mutation was detected between the two groups (P=0.23). In a similar study done by Ahmed, *et. al.*,(2012), in Women with Recurrent Spontaneous Abortions in the Northwest of Iran The frequencies of MTHFR 677T and MTHFR 1298C alleles were (23.4%, 34.8%) in patients and (24%, 40%) and concluded showed no significant variations in MTHFR C677T and A1298C genotype distribution among patients who suffered from Recurrent Spontaneous Abortions and controls. Also this result was in accordance with earlier investigations (Goodman *et, al.*, 2006; Yenicesu *et.al.*, 2010), which reported no association between MTHFR and RSA.

The present study was disagreed with findings from the several large studies that determine the significant association between the MTHFR C677T mutation and the presence of recurrent miscarriage during pregnancy. Behjati, *et.al.*, (2006); Jeddi, *et. al.*, (2011); they found that there were significant association between this gene mutation and recurrent pregnancy loss.Also our study disagreement with another study done by Mtiraoui, *et.al.*, (2006), among 200 Tunisian women with more than three consecutive RPLs and 200 age-matched parous control women, were found frequency of MTHFR 677T/T (30.0 vs 7.0%) and 1298C/C (13.5 vs 4.0%) genotypes was significantly higher in patients and concluded that homozygosity for MTHFR C677T were risk factor for RPL.

Noteworthy those not all retrospective studies showed a relationship between the prothrombin G20210A, MTHFR C677T and Factor V gene mutation and obstetric complications. Several case control studies failed to show an association between this mutation and abruption. Explanations for differences in results among studies may

include different ethnic populations, different definitions for adverse outcomes, combining thrombophilias or adverse outcomes or both into summary statistics, incomplete data regarding the gestational age of lost pregnancies. In addition, the difference in sample size number between the various studies may be a good determinant in observed opposite conclusions.

There are many pieces of literature same like our studies have discussed the matter that MTHFR, factor V Leiden and prothrombin as group of gene mutations might be a risk factor for recurrent spontaneous abortions mutation and the presence of preeclampsia during pregnancy our result was agreement with findings from the several large meta-analysis studies . Someone done by Dalmaz, *et .al.,* (2006), in Brazilian population, they considered the correlation between methyleneterhydrofolate reductase (MTHFR), Prothrombin Mutation (FII), Plasminogen activator inhibitor (PAI-1) and also factor V Leiden concluded that In conclusion, in the population analyzed, the presence of the genotype risk factors alone does not seem to be associated with the development of preeclampsia even in the severe presentation form. However, an interaction among the MTHFR, FII, FV and PAI-1 gene polymorphisms on the development of the preeclampsia was indicated.

The result of our study were agreement with another studies done by Thiago, *et.al.*, (2014), where investigated hereditary factors FV Leiden, F II 20210A mutation and the polymorphism C677T of the MTHFR, as singly and as in association, in a group of women from Ceará state-Northeast Brazil with severe preeclampsia. Were we found that FV Leiden, FII G20210A mutation and MTHFR C677T didn't increase risk for preeclampsia development. FV Leiden and FII G20210A mutations had low frequency in the population studied, which may justify the absence of association. Also, the polymorphism of C677T was not associated to preeclampsia. Other genetic and environmental risk factors should contribute to the development of preeclampsia in the population studied.

Another study was done by Laila, *et.al.*, (2006), to determine the prevalence of factor V Leiden, prothrombin, and methylene tetrahydrofolate reductase gene mutations among Lebanon women with adverse pregnancy outcome compared with women who had

uneventful pregnancies and concluded In this study, the frequencies of factor V, prothrombin ,and MTHFR gene mutations were not found to be significantly different in a group of patients with adverse obstetric outcomes compared with patients who had uneventful pregnancies and the case-control study confirms the high prevalence of mutations in the genes that encode factor V, prothrombin, and MTHFR but fails to establish an association between these mutations and adverse obstetric outcome in otherwise healthy women. Hence, in our population, screening for these thrombophilias cannot be advocated on the basis of our results.

Also our study was disagreement with study done by Foka , *et.al.*, (2002), the study was to investigate the relationship between recurrent miscarriages and factor V Leiden, prothrombin G20210A and C677T methylene tetrahydrofolate reductase (MTHFR) mutations was determined in a consecutive series of 80 recurrent miscarriage patients and 100 controls and suggest that that the presence of factor V Leiden and prothrombin G20210A polymorphism, but not MTHFR C677T homozygosity, could be additional risk factors for recurrent miscarriages. Furthermore, it was suggested that the prevalence of factor V Leiden and prothrombin G20210A mutations is more prominent in second trimester, primary fetal losses and it is independent of the existence of additional pathology predisposing to recurrent fetal losses.

They concluded from our study the presence of these mutations alone is not able to predispose pregnant women for recurrent pregnancy loss and we found that FV Leiden, FII G20210A mutation and MTHFR C677T didn't increase risk for pregnancy loss. MTHFR and FII G20210A mutations had low frequency in the population studied, which may justify the absence of association. Also, the polymorphism of FVL was not associated to pregnancy loss. Other genetic and environmental risk factors should contribute to the development of pregnancy disorder in the population studied.

Prothrombin time (PT) and partial thromboplastin time (PTT) in women with recurrent miscarriage in this study were not affected significantly (P=0. 93 and P=0.69) respectively this is similar to the normal results reported by Ghulam, *et.al.*, (2014) among Sixty three pakistanian women with history of three spontaneous abortions in their first three months of pregnancy .

Also our result with normal range agree with result reported by Salamat, et. *al*, (2000). Another reported by Shahida, *et.al.*, (2011), in 245 women with recurrent abortions showed that PT and PTT were normal.

4.2 Conclusion:

The studies included in this thesis explored the association between inherited thrombophilic gene mutation (Factor V Leiden, prothrombin and MTHFR) among women with recurrent spontaneous abortion and we also investigated differential risk factor for pregnancy related to obstetric disorder such as (age, ABO, ethnic, smoking and families history) and our data strengthen the hypothesis of differential pathophysilogy between pregnancy and recurrent spotonus abortion and our conclusions were as follows:

- The result of Factor V Leiden mutation showed no significant variations among women with RSA case group (8.0%) compared to controls group (6.4%) and didn't increase risk for recurrent spotonus abortion development.

- No significant variations in MTHFR C677T genotype distribution among women who suffered from RSA (3.0%), and there was no mutant gene was detected among the controls group.

- FII G20210A mutation (3.0%), and there was no mutant gene was detected among the controls group and there was no significant association between cases carriage any of this mutation and risk with recurrent pregnancy miscarriage.

- PT and APTT result showed within normal range among all recurrent abortion women.

4.3 Recommendations:

1- Further studies on large Sudanese women population with recurrent spontaneous abortion are needed to classify all DNA thrombophilic mutations by using more sensitive and accurate molecular methods in studying potential affected DNA Mutations regions such as real time PCR technique

2- As findings of the current study seems to indicate limited importance of genetic factors and a pronounced special concern should be paid for couples with recurrent miscarriage should be tested for immunologic, infections and other physical abnormalities in women reproductive system to find more risk factor among these women.

3- More prospective studies are required to explain the relationship between thrombophilia especially factor XIII, XI and recurrent spontaneous abortion because it showed more frequency among these women.

4- Conduct studies about the possible prophylactic management of thrombophilias in risky women especially during pregnancy.

5- Increase the awareness of the medical personnel with regard to this result to seek to different causes beside thrombophilias.

6- Increase the awareness of the women about the other risk factors that may increase the risk of spontaneous recurrent abortion.

REFERENCES:

- Adi Y, Weintraub, Eyal Sheiner (2011) . Early Pregnancy Loss. New York, Springer. 25–27.

- Adolfsson AS.(2006). MiscarriageWomen's Experience and its Cumulative Incidence. (Doctoral dissertation). Institutionen för molekylär och klinisk medicin.

- AgorastosT, Karavida A , Lambropoulos A , ConstantinidisT, Tzitzimikas S, Chrisafi, Set al .(2002) Factor V Leiden and prothrombin G20210A mutations in pregnancies with adverse outcome. J Matern Fetal Neonatal Med.12:267-273.

- Ahmad P Z, Nader C, Mehrdad A E, Mahzad M S, Laya F, Alieh G, et al. (2012) Methylenetetrahydrofolate Reductase C677T and A1298C Mutations in Women with Recurrent Spontaneous Abortions in the Northwest of Iran. ISRN Obstet Gynecol. Epub Nov 14.

- Aldrich C L, Stephenson , Karrison T. HLA-G (2001). genotypes and pregnancy outcome in couples with unexplained recurrent miscarriage. MolHum. Reprod . 12:1167-1172.

- Aleman MM, Walton BL, Byrnes JR, Wang JG, Heisler MJ, Machlus KR, et al. Elevated prothrombin promotes venous, but not arterial, thrombosis in mice. Arterioscler Thromb Vasc Biol 2013;33:1829–36.

- Almawi WY, Finan RR, Tamim H, Daccache JL, Irani-HakimeN.(2004). Differences in the frequency of the C677T mutation in the meth-ylenetetrahydrofolate reductase (MTHFR) gene among the Leba-nese population. Am J Hematol .76:85-7

- Altintas A, Pasa S, Akdeniz N, CilT, Yurt M, Ayyildiz O, et al . (2007) Factor V Leiden and G20210A prothrombin mutations in patients with recurrent pregnancy loss: data from the southeast of Turkey. Ann Hematol. 86: 727-731.

- American Pregnancy Association (2010). Pregnancy Week by week Symptoms". http://www.pregnancybegins.com/week-by-week.php.

- American Cancer Society.(2014). Gestational Trophoblastic Disease Last Medical Review: 2014. www.cancer.org.html.

- Andalib A, Rezaie A, Oreizy F, Shafiei K and Baluchi S.(2006). A study on stress, depression and NK cytotoxic potential in women with recurrent spontaneous abortion . Iran J Allergy Asthma Immunol, 5, 9-16.

- Aruna M, Reddy BM (2006). Recurrent spontaneous abortions: An overview of the Genetic and Non-Genetic backgrounds. Int J Hum Genet. . 6: 109-17

- Asrm.(2008). Definitions of infertility and recurrent pregnancy loss. Fertil Steril, 90, S60.

- Bauduer F, Lacombe D.(2005).Factor V Leiden, prothrombin 20210a, methylenetetrahydrofolate reductase 677t, and population genetics. Mol Genet Metab. 86:91–99.

- Baumann K, Beuter-Winkler P, Hackethal A, Strowitzki T, TothB, Bohlmann MK (2013). Maternal factor V leiden and prothrombin mutations do not seem to contribute to the occurrence of two or more than two consecutive miscarriages in Caucasian patients. Am J Reprod Immunol. ;70:518–21

- Behjati R, Modarressi MH, Jeddi-tehraniM, Dokoohaki P, Ghasemi J, et al. (2006). Thrombophilic mutations in Iranian patients with infertility and recurrent spontaneous abortion. Ann Hematol, 85, 268-71.

- Benedetto C, Marozio L, Tavella AM, Salton L, Grivon S, Di Giampaolo F.(2010). Coagulation disorders in pregnancy: acquired and inherited thrombophilias. Ann N Y Acad Sci.1205:106-17.

- Berk, L.E. (2011).Infants, children, and adolescents, Sevth Edition, USA, Pearson, p 96.

- Bick, R.L, Madden, J, Heller, K.B. and Toofanian A (1998) . 12. Medscape Women's Health Impact and Implications of Chromosomal Abnormalities. 3:2-4

- Blickstein I (2006) .Thrombophilia and women's health: An overview. Obstet Gynecol Clin North Am 33: 347-356.

- Boppana SB , Rivera S B, Fowler KB , Mach M. and Britt W (2001).Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. Engl. J. Med. vol. 344. no. 18. pp. 1366–1371.

- Botto LD and Yang Q (2000). 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am. J. Epidemiol. 151:862-877.

-Bremme KA (2003). Haemostatic changes in pregnancy. Best.Pract.Res.Clin.Haematol. 16:153-168.

- Brenner B, HoffmanR, BlumenfeldZ,Weiner Z, Younis JS (2000). Gestational outcome in thrombophilic women with recurrent pregnancy loss treated by enoxaparin. Thromb Haemost.83(5): p. 693-7

- Brenner B (2004). Haemostatic changes in pregnancy.Thromb Res . 114:409–414.

- Brenner B (2010). Hypercoagulability and recurrent miscarriages. Clin Adv Hematol Oncol. 8(7):467-9.

- Buchholz T, Lohse P, Rogenhofer N, Kosian E, Pihusch R, Thaler CJ. (2003). Polymorphisms in the ACE and PAI-1 genes are associated with recurrent spontaneous miscarriages Hum Reprod . ;18:2473-2477.

- Calderwood CJ, Greer IA .(2005).The role of factor V Leiden in maternal health and the outcome of pregnancy. Curr Drug Targets. 6(5):567-76.

- Campion, Edward W, Doubilet, Peter M, Benson, Carol B, et al (2013). "Diagnostic Criteria for Nonviable Pregnancy Early in the First Trimester". New England Journal of Medicine 369 (15): 1443–1451

- Carolyn R, Jaslow, Judi L, Carney, band William, Kutteh (2010). Diagnostic factors in 1020 women with RPL: fertility and Sterility: Vol. 93, No. 4.

- Carp H, Toder V, Aviram A, Daniely M, Mashiach S and Barkai,G.(2001). Karyotype of the abortus in recurrent miscarriage. Fertil Steril, 75, 678-82.

- Carp HJ.(2006). Thrombophilia and Recurrent Pregnancy Loss. Obstet Gynecol Clin North Am.; 33(3):429-42.

- Chang MH, Lindegren ML, Butler MA,. (2009).Prevalence in the United States of selected candidate gene variants: Third National Health and Nutrition Examination Survey. Am J Epidemio;169:54–66.

- Christiansen OB, Nybo AM, Bosch E, Daya S, Delves PJ, Hviid, TVet al (2005).based investigations and treatments of recurrent pregnancy loss.;83(4):821

- Christiansen O B, Steffensen R, Nielsen H S and Varming K. (2008).Multifactorial etiology of recurrent miscarriage and its scientific and clinical implications. Gynecol. Obstet. Invest. 66. 257-267.

- Clark R, Armitage J, Lewington S, Collins R. (2007). B-vitamin treatment trialists' collaboration of homocysteine-lowering trilas for prevention of vascular disease: protocol for a collaborative meta-analysis. Clin Chem lab Med; 45 (12): 1575-81.

- Clark, P. and GreerA . (2006). Hematology measurements in pregnancythrombophilia and pregnancy outcome (Chapter 7), antithrombotic therapy in pregnancy (Chapter 5) and management of thrombotic disorders (Chapter 6). In:"Practical Obstetric Hematology", 1st edition, Taylor and Francis.

- Cramer DW and Wise LA (2000). The epidemiology of recurrent pregnancy loss. Semin Reprod Med . 18:331-339.

- Critchley HO (1999). Factors of importance for implantation and problems after treatment for childhood cancer. Med Pediatr Oncol 33:9-14.

- Crochet JR, Bastian LA, Chireau MV. (2013). "Does this woman have an ectopic pregnancy?: the rational clinical examination systematic review". JAMA **309** (16): 1722–9

- Cunningham FG (2001). Abnormalities of the umbilical cord, Williams Obstetrics, 21st edition, New York, McGraw-Hill MedicalPublishing Division, 831-835.

- Dahlback B (2008). Advances in understanding pathogenic mechanisms of thrombophilic disorders. Blood. Jul 1.112(1):19-27

- Dalmáz CA, Santos KG, Botton MR, Tedoldi CL, Roisenberg I.(2006). Relationship between polymorphisms in thrombophilic genes and preeclampsia in a brazilian population. Blood Cells Mol Dis. ;37:107–110.

- Daniel M, Green Jane M, Lange, Eve M, Peabody, Natalia N , et al. (2010). Pregnancy outcome after treatment for wilms tumor: A report from the national wilmstumor long-term follow-up studyJournal of Clinical Oncology. 28(17):2824-2830.

- Dávalos IP, Moran MC, Martínez-Abundis E, González-Ortiz M, Flores-Martínez SE, Machorro V, et al.(2005). Methylenetetrahydrofolate reductase c677t polymorphism and factor V Leiden variant in mexican women with preeclampsia/eclampsia. Blood Cells Mol Dis.35:66–69.

- De la Fuente, Cerda F, Davila R, Garcia V, De la R A, Cortes G E .(2009) . Chromosomal abnormalities and polymorphic variants in couples with repeatedmiscarriage in Mexico. Reprod Biomed Online, 18, 543-8.

- Derksen, RH, Pg, DE, Koike, T, et al. (2006). "International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS)." J Thromb Haemost 4(2): 295-306.

- Dizon DS, Nelson LM, Easton K, Ward K. (1996).The factor V Leiden mutation may predispose women to severe preeclampsia. Am J Obstet Gynecol. 175:902–905.

- Doyle NM, Monga M. (2004). Thromboembolic disease in pregnancy. Obstet Gynecol Clin North Am 31: 319-344, vi.

- E.Trabetti . (2008).“Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk,” Journal of Applied Genetics, vol. 49, no. 3, pp. 267–282, .

- Elizabeth A. Varga, MS and Stephan Moll.(2013). Prothrombin 20210 Mutation. American heart association. 110: e15-e18.

- Elsheikha, H.M.. (2008). Congenital toxoplasmosis: priorities for further health promotion action. Public Health. 122(4): 335-353

- Ehrenforth S, Nemes L, Mannhalter C, et al; (2004). Impact of environmental and hereditary risk factors on the clinical manifestation of thrombophilia in 15 homozygous carriers of factor V:G1691A. J Thromb Haemost. Mar;2(3):430-6.

- Empson M, Lassere M, Craig JC, Scott JR. (2002). Recurrent pregnancy loss with antiphospholipid antibody: a systematic review of therapeutic trials. Obstet Gynecol 99:135-144. Epidemiology of Human Reproduction. Boca Raton, Florida: CRC Press Inc.

- Eshre .(2006). Evidence-based guidelines for the investigation and medical treatmentof recurrent miscarriage. Hum. Reprod. 21. 2216-2222.

- Fatini C, Gensini F, Battaglini B, Prisco D, Cellai AP and Fedi S. (2000). Angiotensin convertine enzyme DD genotype, angiotensin receptor CC genotype, and hyperhomocysteinemia increase first-trimester fetal-loss susceptibility. Blood Coagul Fibrinolysis. 11:657–62.

- Ferasatkish R, Naddafnia H, Alavi SM, Naseri MH.(2007). Diagnosis and treatment of disseminated intravascular coagulation: a case report. Arch Iran Med. 10(3):4048.

- Finan R, Tamim H, Ameen G, et al (2002). Prevalence of Factor V G1691A(Factor V Leiden) and Prothrombin gene mutations in a recurrent miscarriage population .American Journal of Hematology . 71:300-305.

- Foka ZJ, Lambropoulos AF, Saravelos H, Karas GB, and Karavida A.(2000) .Factor V Leiden and prothrombin G20210A mutations, but not methylenetetrahydrofolate reductase C677T, are associated with recurrent miscarriages. Hum.Reprod. 15(2): 458-462.

- Folkeringa, N, Brouwer, J L, Korteweg, F J, Veeger ,et al .(2007). Reduction of high fetal loss rate by anticoagulant treatment during pregnancy in antithrombin, protein C or protein S deficient women. Br J Haematol, 136, 656-61.

- Ford HB and Schust DJ. (2009). Recurrent pregnancy loss: etiology, diagnosis, and therapy. Rev Obstet Gynecol, 2, 76-83.

- Fortuny A, Carrio A, Soler A, Caraach J, Fuster J and Salamic C. (1988). Detection of balanced chromosome rearrangements in 445 couples with repeated.

- Forges T, Pellanda H, Diligent C, Monnier P, Guéant JL. (2007).Impact of folate and homocysteine metabolism on human reproductive health," Human Reproduction Update, vol. 13, no. 3, pp. 225–238.

- Furie B, Furie BC.(2008).Mechanisms of thrombus formation. N Engl J Med. 28;359(9):938-49.

- Gabbe SG, Niebyl JR, Simpson JL.(2002).Obsetrics: Normal and Problem Pregnancies. Churchill Livingstone. 729–753.

- Ganong WF, McPhee SJ, Lingappa VR (2005). Pathophysiology of Disease: An Introduction to Clinical Medicine (Lange). McGraw-Hill Medical. p. 639.

- Garrisi J G, Colls P, Ferry KM, Zheng X, Garrisi MG and Munne S. (2009). Effect of infertility, maternal age, and number of previous miscarriages on the outcome of preimplantation genetic diagnosis for idiopathic recurrent pregnancy loss. Fertil Steril, 92, 288-95.

- George D and Erkan D. (2009). Antiphospholipid syndrome. Prog Cardiovasc Dis, 52, 115-25.Gestational Trophoblastic Disease at American Cancer Society. Last Medical Review: 04/14/2011.

- Ghulam S N , Rashid A M , Asghar kh , Saeed SS , Ikram UU.(2014). Analysis of anti phospholipid antibodiesin women with recurrent spontaneous abortion. Isra medical journal | volume 6 - issue 2 | apr - jun .

- Giacomucci E, Bulletti C, Polli V, Prefetto RA, Flamigni C. (1994). Immunologically mediated abortion (IMA). J Steroid Biochem Mol Biol 49:107-121.

- Gihan EH and Osama AK.(2013). Molecular Characterization of Factor V Leiden G1691A and Prothrombin G20210A Mutations in Saudi Females with Recurrent Pregnancy Loss: J Blood Disorders Transf, 4:6 .

- Goodman CB, Coulam R.S, Jeyendran VA, Acosta, and Roussev, (2006).Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss?" American Journal of Reproductive Immunology.56, pp. 230–236.

- Grandone E, Margaglione M, Colaizzo D, Andrea G, Cappucci G.(1998) Genetic susceptibility to pregnancy-related venous thromboembolism: roles of factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations. Am J Obstet Gynecol 179:1324-1328.

- Guyton, A.C. and Hall, J.E. (2011): Text Book of Medical Physiology, Twelve

Edition, Elsevier Saunders, Philadelphia, Pennsylvania, pp 1027-1041.

- Hasan R, Baird DD, Herring AH, et al.(2010). Patterns and predictors of vaginal bleeding in the first trimester of pregnancy. Ann Epidemiol 20:524.

- Hemminki E, Forssas E. (1999).Epidemiology of miscarriage and its relation to other repro-ductive events in Finland. Am J Obstet Gynecol . 181: 396-401.

- Hyers TM.,(2001). Antithrombotic therapy for venous thromboembolic disease. Chest, **119**(1 Suppl): p. 176S-193S.

- Inomo A S and Fujita Y. (2008). The Antigenic Binding Sites ofAutoantibodies to Factor XII in Patients with Recurrent Pregnancy Losses Thrombosis and Haemo. Stasis. Vol. 99, No. 2. pp. 316-323.

- IvyA, A A and Louis MA. (2007). The 5, 10 methylenetetrahydrofolatereductase C677T mutation and risk of fetal loss: a case series and review of theliterature. Thromb. J. 5: 17.

- Jablonowska B. (2003). Recurrent spontaneous abortion: a clinical, immunological and ge-netic study, Linköping University, Linköping, , 2003. 88 p.

- Jackson CM, Esnouf MP, Lindahl TL. (2003). A criti-cal evaluation of the prothrombin time for moni-toring oral anticoagulant therapy. Pathophysiol Haemost Thromb 33(1):43-5- Kitchens CS. 2005. To bleed or not to bleed? Is that.

- Jaslow C , Carny J L and K utteh W H. (2009). Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril, 1234-43.

- Jason H, Charles L, Collins, Candace C. (2009).Silent Risk: Issues About the Human Umbilical Cord, retrieved 2009-03-17.

- Jeddi TR, Torabi A.and Zarnani H.(2011). Analysis of plasminogen activator inhibitor-1, in tegrin beta3, beta fibrinogen, and Methylenetetrahydrofolate reductase polymorphisms in Iranian women with recurrent pregnancy loss. Am J Reprod Immunol **66**(2): 149-156.

- Jones DW, Gallimore MJ, Winter M and More M.(2006). Pathogenic Antibodies to Coagulation Factors. Part II: Fibrinogen, Prothrombin, Thrombin, Facto.

- Kaandorp S, Di Nisio M, Goddijn M, Middeldorp S. (2009). Aspirin or anticoagulants for treating recurrent miscarriage in women without antiphospholipid syndrome (Review). The Cochrane Library Issue 1.

- Kaare, M. (2009). Genetic studies on recurrent miscarriage, university of Helsinki, unpublished data.

- Källén B. (2004) . Statistics Canada. Vital - Statistics - Stillbirth Database.

- Kesmodel U,Wisborg K., Olsen S. F., Henriksen TB, Secher N.J. (2002). Moderate alcohol intake during pregnancy and the risk of stillbirth and death in the first year of life. American Journal of.Epidemiology, volume 155, 305-312.

- Kim Mackenzie-Morris. "What is a blighted ovum.(2013). Fallow at Babycentre.co.uk. Retrieved 19 December 2013.

- Kobashi G, Yamada H, Asano T, Nagano S, Hata A, Kishi R, et al.(1999) The factor V Leiden mutation is not a common cause of pregnancy-induced hypertension in japan. Semin Thromb Hemost. Semin Thromb Hemost. 25(5):487-9.

- Kovacheva K, Ivanov P, Konova E, Simeonova M, Komsa-Penkova R (2007).Genetic thrombophilic defects (Factor V Leiden, prothrombin G20210A, MTHFR C677T) in women with recurrent fetal loss]. Akush Ginekol (Sofiia) 46:10-16.

- Kujovich JL.(2004).Thrombophilia and pregnancy complications. American Journal of Obstetrics and Gynecology 191:412-424.

- Kujovich JL (2011). Factor V Leiden thrombophilia. Genet Med. Jan;13(1):1-16.

- Kujovic JL. (2004) Thrombophilia and pregnancy complications. Am J Obst Gyne.; 191:412-424.

- Kupferminc MJ, Eldor A, Steinman N, et al.(1999). Increased frequency of genetic thrombophilia in women with complications of pregnancy. N. Engl J. Med. 340:9-13.

- Kurzawińska G, Seremak MA, Drews K, Barlik M, Mrozikiewicz PM. (2009).Genetic conditioned changes in activity of 5,10-methylenetetrahydro-folate reductase (MTHFR) and recurrent miscarriages. Ginekol Pol. 80(10):762-7.

- Laila F, Zahed, Roni F. Rayes ,Rami A. Mahfouz, *et. al* .(2006). Prevalence of factor V Leiden, prothrombin andmethylene tetrahydrofolate reductase mutations in women with adverse pregnancy outcomes in Lebanon. American Journal of Obstetrics and Gynecology. 195, 1114–8.

- Limdi NA, Beasley TM, Allison DB, Rivers CA, Acton RT.(2006). Racial differences in the prevalence of factor V Leiden mutation among patients on chronic warfarin therapy. Blood Cells Mol Dis. 37:100–106.

- Li TC, Tuckerman EM, Laird SM (2002a). Endometrial factors in recurrent miscarriage. Hum Reprod Update. 8:43-52.

- Lockwood and K. A. Bauer (2008) .Inherited thrombophilias in pregnancy," UpToDate,

- Lockwood C.J. (2002). Inherited thrombophilias in pregnant patients. Prenat.

Neonat.Med.6:3–14.

- Lohstroh PN, Overstreet JW, Stewart DR, *et.al*.(2005). Secretion and excretion of human chorionic gonadotropin during early pregnancy. Fertil Steril . 83:1000.

- Majid A, Hossein N, Abbas A , Nasrin Ghasemi and Mohammad S.(2013). Case control study of the factor V Leiden and factor II G20210A mutation frequency in women with recurrent pregnancy loss: Iran J Reprod Med. Jan . 11(1): 61–64.

- Martinelli I, Emanuela T, Cetin I, et al. (2000). Mutations in coagulation factors in women with unexplained late fetal loss. N Engl J Med . 343:1015-8.

- Mary K and Peter S B Disclosures. (2005).Recurrent Pregnancy Loss and Thrombophilia.Avilable from : www.medscape.com/viewarticle/516199.

- Marco Cattaneo, Anna Lecchi, Maddalena Loredana Zighetti, Federico Lussana.(2007) "Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count". Haematologica,92(05) the question for the PTT? J Thromb Haemost 3(12):2607-2611.

- MetwallyM, SaravelosS., LedgeWL and LI (2010). Body mass index and risk of miscarriage in women with recurrent miscarriage. Fertil Steril, 94, 290-5.

- Michael JK.(2003) .“Thrombophilia and pregnancy,” Reproductive Biology and Endocrinology, vol. 111, no. 1.

- Mims C A , Nash, Stephen. (2001). Mims' Pathogenesis of Infectious disease.5ed.

.Academic press. London .

- Miyakis S, Lockshi , Atsumi T, Branch DW, Brey RL, Cervera R, et al . (2012) Evaluation of Female Infertility: J MEDICINE 2012; 13 : 200-209

- MtiraouiW, Zammiti L, and Ghazouani G. (2006). Methylenetetrahydrofolate reductase C677T and A1298C polymorphism and changes in homocysteine. Reproduction. 131(2):395-401

- Munne S, C hen S, Fischer J, Colls P, Zheng X, Stevens J, et al. (2005). Preimplantation genetic diagnosis reduces pregnancy loss in women aged 35 years and older with a history of recurrent miscarriages. Fertil Steril, 84, 331-5.

- Namee K, Dawood F, Farquharson R (2012).Recurrent miscarriage and thrombophilia: an update. Curr Opin Obstet Gynecol.24(4):229-34.

- Nelen WL, Blom HJ, Steegers EA, den Heijer M, Eskers TK. (2000) Hyperhomocysteinaemia and recurrent early pregnancy loss: a meta-analysis. Fertil Steril; 74(6): 1196-9.

- Nelson DB, Bellamy S, Nachamkin I, Ness R B, Macones G A and AllenT .(2007). First trimester bacterial vaginosis, individual microorganism levels, and risk of second trimester pregnancy loss among urban women. Fertil Steril, 88, 1396-403.

- Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. (2000). Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 80:1-29.

-Nguyen RH and Wilcox AJ (2005). "Terms in reproductive and perinatal epidemiology: 2. Perinatal terms". J Epidemiol Community Health **59** (12): 1019–21.

- Nybo AAM, Wohlfahrt J, Christens P, Olsen J, Melbye M. (2000). Maternal age and fetal loss: population based register linkage study. BMJ 320: 1708-1712.

- Ogden J.(2007). Health psychology : a textbook Open University Press. OLIVE, D. L. 1991. The prevalence and epidemiology of luteal-phase deficiency in normal and infertile women. Clin Obstet Gynecol, 34, 157-66.

- Patnaik MM and Moll S. (2008). Inherited antithrombin deficiency: a review. Haemophilia, 14, 1229-39.

- Pauer HU, Burfeind P, Kostering H, Emons G, Hinney B.(2003).Factor XII Deficiency Is Strongly Associated with Primary Recurrent Abortions.Fertility and Sterility, Vol. 80. No. 3. Pp: 590-594.

- Perry DJ.(1999). Hyperhomocystenaemia. Baillieres Best Practice Res Clin Haematol . 12:451-477.

- Pihusch R, Buchholz T, Lohse P, Rübsamen H, Rogenhofer N, et al. (2001) Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. Am J Reprod Immunol 46:124-13

- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. (1996) A common genetic variation in the 3' untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood; 88:3698-3703.

- Prasmusinto D, Skrablin S, Fimmers R, van der Ven K. (2004).Ethnic differences in the association of factor V Leiden mutation and the c677t methylenetetrahydrofolate reductase gene polymorphism with preeclampsia. Eur J Obstet Gynecol Reprod Biol. 112:162–169.

- Preston FE, Rosendal FR, Walker ID et al.(1996). Increased fetal loss in women with heritable thrombophilia .Lancet. 348:913-916.

- Promislow JH, Baird DD, Wilcox AJ, Weinberg CR.(2007). Bleeding following pregnancy loss before 6 weeks' gestation. Hum Reprod . 22:853.

- Propst AM and Hill J A. (2000). Anatomic factors associated with recurrent pregnancy loss. Semin Reprod Med, 18, 341-50.

- Rai R, Regan L (2006). Recurrent miscarriage. Lancet. 368:601-611.

- Regan L and Rai R (2000) Epidemiology and the medical causes of miscarriage. Baillieres Best Pract Res Clin Obstet Gynaecol 14,839–854.

- Reza B, Mohammad HM, Mahmood JT , Pouneh D, Jamileh G, Amir H Z . et al .(2006) Thrombophilic mutations in Iranian patients with infertility and recurrent spontaneous abortion. Volume 85, Issue 4, pp 268-271.

- Reznikoff Etievan. *et. al*. Factor V. (2001).Leiden G20210A prothrombin mutations are risk factors for very early recurrent miscarriage. BJOG.108 12 1251 1254

-Robertson L, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GD, et,al.(2006) .Thrombophilia in pregnancy: a systematic review. Br J Haematol 132:171-196.

- Robert M, Silver, Yuan Z .(2010). Prothrombin Gene G20210A Mutation and Obstetric Complications: O bstet Gynecol. 115(1): 14–20.

- Rubio C, Simon C, Vidal F, Rodrigo L, Pehlivan T, Remohi J. (2003). Chromosomal abnormalities and embryo development in recurrent miscarriage couples. Hum Reprod, 18, 182-8.

- Rubio C, Pehlivan T, RodrigoL, Simon, C, Remohi J. and Pellicer A.(2005). Embryo aneuploidy screening for unexplained recurrent miscarriage: a minireview. Am J Reprod Immunol, 53, 159-65.

-Salamat N, Saleem M, Ahmed T.(2000). Lupus coagulant and anticardiolipin antibodies in patients with recurrent fetal loss: A case control Study. Ann Saudi Med. 20(5-6):450-3.

- Salwa K and Joseph D D.(2006).Hereditary thrombophilia.Thromb J. 4: 15.

- Samieh K, Majid Y, Azadeh A, Minoo R, Maryam A. (2010).Evaluation the frequency of factor V Leiden mutation in pregnant women with preeclampsia syndrome in an Iranian population. Iranian Journal of Reproductive Medicine.(1): 59-66.

- Sanson BJ, Fierich PW, Simioni P, et al. (1996).The risk of abortion and stillbirth in antithrombin-, protein C, and protein S deficient women. Thromb Haemost.75:387–8.

- Sara S, Mark L, Brendan C, Margaret, Geraldine G, and Majella M.(2011). “Acquired Activated Protein C Resistance, Thrombophilia and Adverse Pregnancy Outcomes: A Study Performed in an Irish Cohort of Pregnant Women,” Journal of

Pregnancy, vol. 2011, Article ID 232840, 9 pages, 2011. doi:10.1155/2011/232840.

- Saravelos S H, Cocksedge K A,Li TC.(2010). The pattern of pregnancy loss in women with congenital uterine anomalies and recurrent miscarriage. Reprod Biomed Online, 20, 416-22.
- Saravelos S H, Cocksedge K A,Li TC.(2008). Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal. Hum Reprod Update, 14, 415-29.
- Sata F, Yamada H, Suzuki K, SaijoY, Kato EH, Morikawa M , et al. (2005). Caffeine intake, CYP1A2 polymorphism and the risk of recurrent pregnancy loss. Mol Hum Reprod, 11, 357-60.
- Sehirali S, Inal MM, Yildirim Y, Balim Z, Kosova B, et al. (2005) Prothrombin G20210A mutation in cases with recurrent miscarriage: a study of the mediterranean population. Arch Gynecol Obstet 273: 170-173.
- Semsettin S, Ismail B, Leyla. (2012).Distribution of prothrombin G20210A, factor V Leiden, and MTHFR C677T mutations in the middle Black Sea area (Tokat) of Turkey. Turk J Med Sci 42 (6): 1093-1097.
- Sensini A. (2006). Toxoplasma gondii infection in pregnancy: opportunities and pitfalls of serological diagnosis. Clin. Microbiol. Infect. 12(6): 504-512
- Shahida M, Amber I, Lubna RD ,Ghulam R,Shabbir HM, Ikram U.(2011). Levels of Anti Phospholipid antibodies in females with recurret abortions. Ann. Pak. Inst. Med. Sci. 2011; 7(3): 156-159.
- Shazia Michael, Raheel Qamar, Farah Akhtar, Wajid Ali Khan, and Asifa Ahmed.(2008). C677T polymorphism in the methylenetetrahydrofolate reductase gene is associated with primary closed angle glaucoma. Mol Vis. 14: 661–665.
- Sotiriadis A. Makrigiannakis A.Stefos T, Paraskevaidis E ,Kalantaridou SN. (2008). Occasional antiphospholipid antibody positive patients with recurrent pregnancy

loss also merit aspirin therapy: a retrospective cohort-control study. Am J Reprod Immunol, 59, 235-41.

- Sotiriadis A, Makrigiannakis , Stefos T, Para S E. and Kalantaridou S N. (2007). Fibrinolytic Defects and Recurrent Miscarriage: ASystematic Review and Meta-Analysis. Obstet. Gynecol. Vol. 109 No. 5. P: 1146-1155.

- Speroff L, Glass RH, Kase NG. (1999). Clinical Gynecologic Endocrinology and Infertility. Lippincott Williams & Wilkins.

- Spiroski I, Kedev S, Antov S, Arsov T, Krstevska M, Dzhekova S S, et al. (2008). Methylenetetrahydro-folate reductase (MTHFR-677 and MTHFR-1298) genotypes and haplotypes and plasma homocysteine levels in patients with occlusive artery disease and deep venous thrombosis. Acta Biochim Pol. 55(3):587-94.

- Stephenson M, Kutteh WH. (2007). Evaluation and management of recurrentearly pregnancy loss. Clin Obstet Gynecol .50:132–45.

- Stephenson , Awartani K A, Robinson W P. (2002). Cytogenetic - analysis of miscarriages from couples with recurrent. Hum Reprod. 17(2):446-51.

- Suzumori N. & Ogasa Wara M. 2010. Genetic factors as a cause ofmiscarriage. Curr Med Chem, 17, 3431-7.

- Taher A, Khalil I, Abou-Merhi R, Shamseddine A, Bazarbachi A.(2003).High prevalence of prothrombin G20210A mutation among pa-tients with deep venous thrombosis in Lebanon. Thromb Haemost89:945-6.

-Ten k, M. K. & Van Der M J.(2008). Protein S deficiency: a clinical perspective. Haemophilia, 14, 1222-8.

-Thachil J, Toh CH.(2009). Disseminated intravascular coagulation in obstetric disorders and its acute haematological management. Blood Rev. 23(4):167-76.

-Thellin O, Coumans B, Zorzi W, Igout A, Heinen E. (2000). Tolerance to the foeto-placental 'graft': ten ways to support a child for nine months. Curr Opin Immunol 12:731-737.

-Thiago FV, Freire, Gervina BM, Holanda, Debora M, Zuleika S ,te al.(2014). Rabenhorst. Relationship between Methylenetetrahydrofolate Reductase (C677T), Factor V Leiden (G1691A), Prothrombin Mutation (G20210A) and Severe Preeclampsia in a Brazilian Population. Open Journal of Obstetrics and Gynecology, 4, 628-635 34.

- Toner J. (2003). Age=egg quality, FSH level=egg quantity. Fertil Steril.;79:491.

- Tran HA, (2005): "Biochemical tests in pregnancy". Australian Prescriber(28): 98-101.

-Van Mens TE, Levi M, Middeldorp S. (2013). Evolution of Factor V Leiden. Thromb Haemost. 2013 Jul;110(1):23-30.

- Ulehlova Jana, Slavik Ludek, Kucerova Jana, Krcova Vera, Vaclavik Jan, and Indrak Karel .(2014).Genetic Testing and Molecular Biomarkers. Vol. 18, No. 9: 599-604.

-Van Vlijmen EF, Veeger NJ, Middeldorp S, et al, (2011). Thrombotic risk during oral contraceptive use and pregnancy in women with factor V Leiden or prothrombin mutation: a rational approach to contraception. Blood. 25;118(8):2055-61.

-Vinatier D, Dufour P, Cosson M, Houpeau JL. (2001). Antiphospholipid syndrome and recurrent miscarriages. European Journal of Obstetrics and Gynecology and Reproductive Biology 93:37-50.

- Von PS and Schneider KTM, .(2009).“Recurrent spontaneous abortions-an update on diagnosis and management,” Journal of Reproductive Medicine and Endocrinology, vol. 6, no. 1, pp. 11–16.

- Vossen CY, Preston FE, Conard J, et al.(2004). Hereditary thrombophilia and fetal loss:a prospective follow-up study. Thromb Haemost. 592–6.

- Walter V, Keiko K, Kyoko H, Junko Y, Akihiko S, etal .(2013).Placental expression of microRNA-17 and -19b is down-regulated in early pregnancy loss Volume 169, Issue 1 , Pages 28-32 ,

- Wang X, Chen C, Wang L ,Chen D, Guang W, French J.(2003). Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. Fertil Steril 79:577.

- Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer J P, Canfield R. E.et al.(1988) Incidence of early loss of pregnancy. N Engl J Med 1988; 319:189.

-Windham GC, Von B J, W aller AK. , Fenster L. (1999). Exposure to environmental and mainstream tobacco smoke and risk of spontaneous abortion. Am J Epidemiol, 149, 243-7.

- Wolf CE, Haubelt H, Pauer HU, Hinney B, Krome-Caser C, et al. (2003).Recurrent pregnancy loss and its relation to FV Leiden, FII G20210A and polymorphisms of plasminogen activator and plasminogen activator inhibitor.Pathophysiology of Haemostasis Thrombosis. 33:134-13.

- World Health Organization (2006): International Statistical Classification of Diseases and Related Health Problems, 10th revision. Geneva (CH).

- Wyatt PR, Owolabi T, Meier C, Huang T. (2005). Age-specific risk of fetal loss observed in a second trimester serum screening population. Am J Obstet Gynecol . 192:240.

- Yenicesu G I, Cetin M, Ozdemir O, CetinA, Ozen F, Yenicesu C ,et al.(2010). A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss. Am J Reprod Immunol 63, 126-36.

- Z hang XY, Sona WY, Holzer H , Tana S L, Aoa A and Group M P. (2008). Effect of maternal age on the pregnancy rate of patients with repeated implantation failure and recurrent miscarriage following PGS Abstracts of the Scientific Oral & Poster

Sessions Program Supplement, American Society for Reproductive Medicine 64th Annual Meeting Fertility and Sterility.

APPENDICS

Appendix I

Questionnaire for Recurrent Aborters Women

Sudan University of Science and Technology

College of Graduate studies and scientific research

Thrombophilic Mutation of Factor V G1691A, Prothrombin G20210A

And Methylene tetra hydrofolate reductase C677T Gene mutations among Recurrent Spontaneous Abortion Sudanese Women

Health Center:……..……

Date :……. ……..…….…….……

Serial No:……..……

Personal data:

Name:……

Age (Date of birth):……..……..…

Residence…….…….…..

Tribe:…….……...……

Phone No……

Blood group……

Have anyone in your family had recurrent spontaneous abortion?

Yes…….No……. If yes who……..……...…..

Number of miscarriages……

Gestational age before miscarriage……

Having a live birth……..

Number of a live birth before RPL…….

Time after last miscarriage (months)……..

Have you got any history of thrombotic disease.....

No……. yes…… what……

……..…

Diabetes……

Hypertensive…….

Other cases of abortion ……

.if present specify……

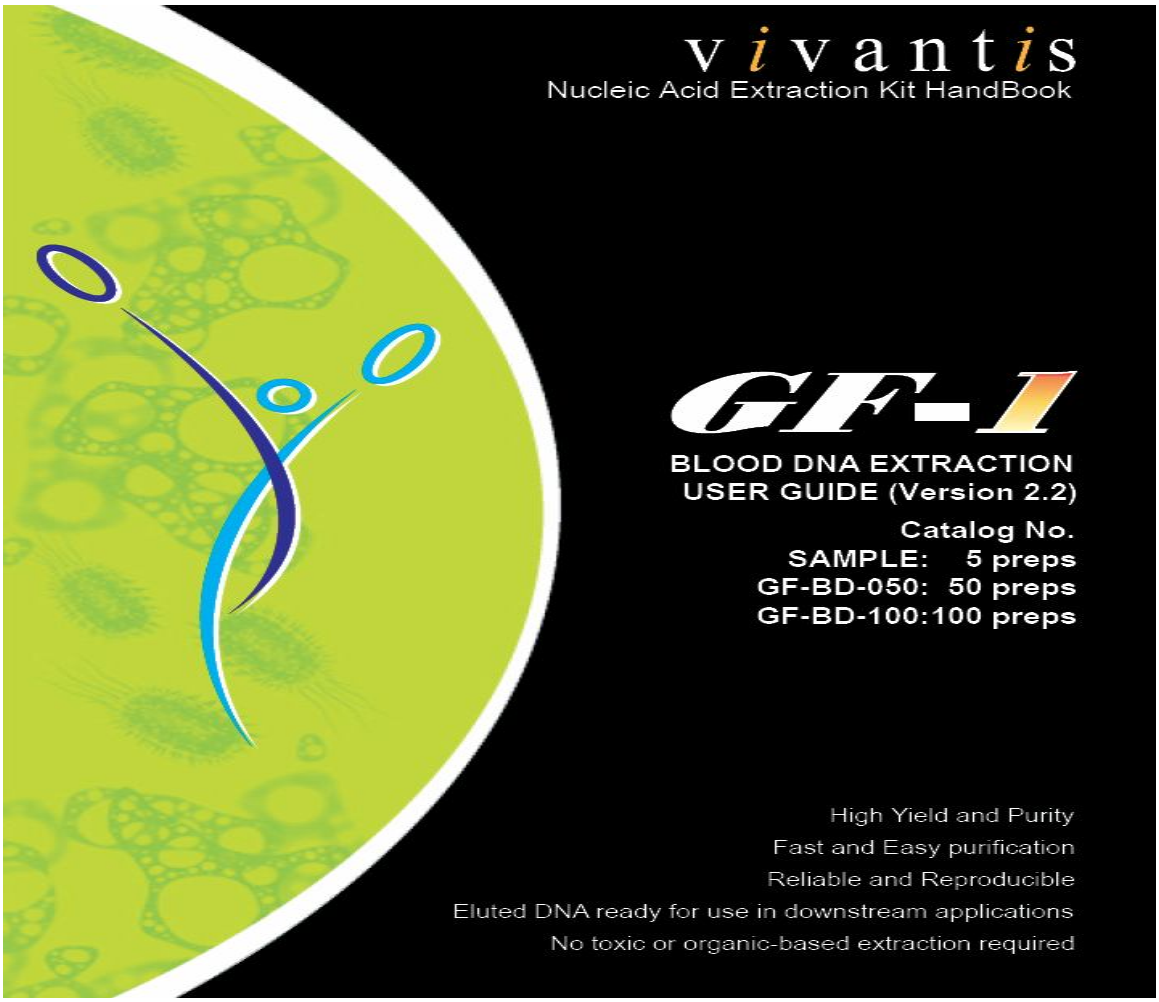
Receiving any medication.....

If yes,…….

Thank you

Appendix II

GF-1 Blood DNA Extraction Kit



GF-1 Blood DNA Extraction Kit:

The GF-1 Blood DNA Extraction Kit is designed for rapid and efficient purification of genomic DNA from up to 400µl whole blood. This kit uses a specially treated glass filter membrane fixed into a column to efficiently bind DNA in the presence of high salt. This kit applies the principle of a mini-column spin technology and the use of optimized buffers to ensure that only DNA is isolated while cellular proteins, metabolites, salts and other low molecular weight impurities are removed during the subsequent washing steps.

GF-BD-100 Kit component:

NO	Components	100 Preps GF-BD-100
1	GF-1 columns	100
2	Collection tubes	100
3	Blood Lysis Buffer (Buffer BB)	24 ml
4	Wash buffer 1 (Concentrate)	30 ml
5	Wash buffer 2 (Concentrate)	34 ml
6	Elution Buffer	20ml
7	Proteinase K	2x 1.05 ml

Preparation of GF-BD-100 (100 preps):

Add 30ml of absolute ethanol into the bottle labeled Wash Buffer 1.

Add 80ml of absolute ethanol into the bottle labeled Wash Buffer 2.

Store Wash Buffer at room temperature with bottle capped tight after use.

Storage and Stability:

• Store all solutions at 20°C - 30°C.

• Store Proteinase K at -20°C.

Procedures of DNA extraction:

Reminder

• All steps are to be carried out at room temperature unless stated otherwise.

1. Blood Lysis:

Add 200µl of Buffer BB into a 200µl blood sample in a micro centrifuge tube. Mix thoroughly by pulsed-vortexing. Add 20µl of Proteinase K and mix immediately. Incubate at 65°C for 10 min.

Optional: Removal of RNA:

If RNA-free DNA is required, add 20µl of RNase A (DNase-Free, 20mg/ml). Mix and Incubate at 37°C for 10 min.

2. Addition of ethanol:

Add 200µl of absolute ethanol. Mix immediately and thoroughly to obtain a Homogeneous solution.

3. Loading to column:

Transfer the sample into a column assembled in a clean collection tube (provided). Centrifuge at 5,000 x g for 1 min. Discard flow through.

4. Column washing 1:

Wash the column with 500µl Wash Buffer 1 and centrifuge at 5,000 x g for 1 min. Discard flow through.

5. Column washing 2:

Wash the column with 500µl Wash Buffer 2 and centrifuge at 5,000 x g for 1 min. Discard flow through. Wash column again with 500µl Wash Buffer 2 and centrifuge at Maximum speed for 3 minutes.

6. DNA elution

Place the column into a clean micro centrifuge tube. Add 100µl of preheated Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. Centrifuge at 5,000 x g for 1 min to elute DNA. Store DNA at 4°C or -20°C.

Appendix III

Master Mix preparation

10X TBE Tris-borate-EDTA (TBE) buffer preparation:

The working solution of 1X TBE is prepared from the stock solution (1 L) which contains the following:

89 mM Tris base(108 gm)

89 mM boric acid (55 gm)

40 ml of 0.5M EDTA, adjust pH to 8.0(It need not be autoclave)

Primer preparation:

Each of the upstream and downstream primers were prepared as follows: 10µl of each stock primer (100 µM) were added to 90µl PCR water and aliquoted in 0.5 ml PCR polypropylene tube to yield a concentration of 10µM, and the solution was mixed carefully using sterile tips to ensure the homogeneity.

Dexonucleotides (dNTPs) preparation:

All four dexonuclotides with 100 mM concentration were prepared by adding 10µl of each nucleotides (total volume 40), in 60 µl of sterile PCR water to a final concentration 10 mM in a PCR tube, vortexed to collect any dNTPs from the tube surface in the button of the tube.

Master Mix for FV Leiden mutation recipe:

	Conic	**Volume**	**Final Conic**
H20		23.50 µl	
PCR buffer	10x	03.00 µl	1x
$Mgcl_2$	50	00.90 µl	1.5 mM
dNNTPs	10 mM	00.90 µl	300µM
Primer1	10 mM	00.75 µl	250 pM
Primer2	10 mM	00.75 µl	250 pM
Taq DNA	5 unit	00.20 µl	1 unit

Master mix for prothrombin gene mutation recipe:

	Conic	Volume	Final Conic
H20		18.80µl	
PCR buffer	10x	03.00µl	1x
Mgcl	50 mM	03.00µl	5 mM
dNNTPs	10 mM	02.40µl	800µM
Primer1	100 mM	01.20µl	400 pM
Primer2	100 mM	01.20µl	400 pM
Taq DNA	5 unit	00.4 µl	2.0 unit

Master Mix for MTHR:

	Conic	Volume	Final Conic
H20		24.40 µl	
PCR buffer	10x	03.00 µl	1x
dNNTPs	10 mM	00.90 µl	300µM
Primer1	100 mM	00.75 µl	250 pM
Primer2	100 mM	00.75 µl	250 pM
Taq DNA	5 unit	00.20 µl	1 unit