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Estimation of Cyclophilins A and Gene Expression of miRNA in Women with Miscarriage Associated Cytomegalovirus Infection.

A Thesis

Submitted to the Council of College of Medicine-University of Babylon in Partial Fulfillment for the Requirements for the Doctor of Philosophy in Science / Medical Microbiology

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بسم الله الرحمن الرحيم

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﴿قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ﴾

صدق الله العلي العظيم

سورة البقرة – الآية(٣٢)

Supervision Certificate

I We certify that this thesis was prepared under our supervision at the Department of Medical Microbiology, College of Medicine, University of Babylon, in a partial fulfillment for the requirements of the degree of Doctorate of Philosophy in Medical Microbiology.

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Dedication

To my family

for their deep love and Endless Support

Hawraa \ 2023

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Summary:

A total of 250 women were included in this study, 50 women of them were represented the control group (healthy women who were not pregnant and had no previous miscarriages) and 200 had a history of miscarriage (case group). In this study out of 200 blood samples, only 60 cases were positive for CMV, which also further categorized into six with IgM only, 14 positive for IgM and IgG, and 40 positive for IgG only. In samples that tested positive for CMV, Cyclophilin A was estimated, The results showed that these samples had significantly higher levels of Cyclophilin A. The mean value of Cyclophilin A in the group that had CMV infection and experienced miscarriage was 34.337. The results showed that CMV IgM antibodies alone significantly increased Cyclophilin A concentration (mean=64±4.76). However, in patients with CMV IgM and IgG, the Cyclophilin A level was lower (mean=48.15±16.72). For women patients with CMV IgG, the Cyclophilin A level decreased even further (mean±SD=25.004±2.34).

Single nucleotide polymorphisms were done to show the effect of nucleotide variants on the *PPIA* gene which has a relationship with cyclophilin A levels in the blood of women patients with CMV infections at two reference loci.

The results revealed that *PPIA* polymorphism at a reference rs4720485 was found in the TA genotype and was higher than other genotypes TT and AA. The study showed that cyclophilin A levels are higher among women with allele type TT and to a lesser extent in patients with TA genotype.

On the other hand, SNPs were also studied for the *PPIA* gene at a reference sequence rs8177826, It was found their different genotypes in this locus CC, CG, and GG genotypes. cyclophilin A levels were found high in women patients with the CC genotype and to a lesser degree among women with the CG genotype.

Regarding microRNA levels, miR-US25-5p, and miR-UL112_5p genes were upregulated in women who had undergone miscarriage with CMV infection compared to healthy married women. The expression of CMV-miR-UL112-5p in the patient group was compared to the control group, with a mean fold change of 12.47 in women patients and 1.077 in the control group. Additionally, miR-US25-5p was also compared, with a mean fold change of 14.96 in women patients and 1.02 in the control group . This means that miR-US25-5p and miR-UL112-5p are highly increased in miscarriage women infected with Cytomegalovirus. The presence of high rates of fold change of two types of microRNA will give good biomarkers for CMV infections.

Concerning the study that compared serum cortisol and plasma ACTH levels between patients and a control group, the results showed that the patient group had significantly lower levels cortisol of (mean 42.1158) than the control group's average of (114.2062). However, the patient group had higher levels of Plasma Adrenocorticotropic hormone (ACTH) compared to the control group, with a mean value (78.1468, *P* 0.001) compared to the control group's 45.18. However, these hormones are understudying and cannot be used as biomarkers for CMV infection.

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List of Abbreviations

Abbreviation	Meaning
ACTH	Adrenocorticotropic hormone
CMV	Cytomegalovirus
CRH	Corticotropin-releasing hormone
CypA	Cyclophilin A
EDTA	Ethylene diamine tetra acetic acid
ELFA	Enzyme-linked fluorescent assay
ELISA	Enzyme-linked immunosorbent assay
HPA	Hypothalamic –pituitary- adrenal
IE	Immediate –early
LncRNA	Long non-coding RNA
miRNA	microRNA
Ml	Milliliter
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PPIA	PeptidylProylIsomerase A
siRNA	Small-interfering RNA
SNPs	Single nucleotide polymorphisms

TBE	Tries base boric acid
TNF	Tumor necrosis factor

CHAPER ONE

INTRODUCTION

AND

LITERATURE

REVIEW

1.1 Introduction:

Cytomegalovirus (CMV) infection is a significant concern during pregnancy, particularly in pregnant women who acquire the infection for the first time. CMV is a member of the herpesvirus family and is highly prevalent in the general population. Although CMV can cause symptoms in healthy adults, it is true that many primary CMV infections in pregnant women go unnoticed because they often present with mild or no symptoms. In some cases, the symptoms may resemble those of a common cold or flu, making diagnosis difficult without specific testing (Shoham *et al.*, 2023).

CMV can establish latent infections in the body, which means that the virus can remain dormant and asymptomatic for long periods. In some cases, women previously infected with CMV before pregnancy may reactivate the virus during pregnancy, potentially posing a risk to the developing fetus. The main concern regarding primary CMV infection during pregnancy is the risk of transmitting the virus to the fetus, which can lead to congenital CMV infection (Al-Mukhtar,2020).

Congenital CMV infection can cause a range of birth defects and developmental issues, including hearing loss, intellectual disabilities, and vision problems. Preventing primary CMV infection during pregnancy can be challenging because the virus is widespread in the population. Pregnant women can reduce their risks by practicing good hygiene, such as frequent hand washing, avoiding close contact with young children's bodily fluids, and refraining from sharing eating utensils or cups with them. Routine prenatal care may include CMV testing; however, no vaccine is currently available for CMV (Adane and Getawa, 2021) .

Congenital CMV transmission can occur at any stage of pregnancy, but it is generally considered more severe when it occurs during the first trimester. Infections during this period may increase the risk of miscarriage or birth defects. Miscarriage refers to the loss of a pregnancy before the 20th week. It is a relatively common occurrence, with estimates suggesting that about 10-20% of known pregnancies end in miscarriage. The majority of miscarriages occur during the first trimester, which spans from conception to the 12th week of pregnancy (Giakoumelou *et al* .,2016).

During the first trimester, which spans from conception to 12 weeks of gestation, miscarriages are most commonly caused by chromosomal abnormalities. However, infections such as CMV can also contribute to first-trimester miscarriages. CMV is transmitted through bodily fluids, including saliva, urine, blood, and breast milk. CMV infection during early pregnancy can increase the risk of miscarriage by crossing the placenta and infecting the developing fetus. However, not all CMV infections during pregnancy result in miscarriage, as the risk varies depending on factors such as the timing of infection and the immune response of the mother (Hardy *et al* .,2018). If a pregnant woman becomes infected with CMV for the first time or experiences a reactivation of a latent CMV infection during pregnancy, there is a risk of the virus crossing the placenta and infecting the developing fetus. This can lead to congenital CMV infection, which can cause a range of health problems in the baby (Al-Mukhtar, 2020).

In the second trimester, spanning from 13 to 27 weeks of gestation, miscarriages are less common but can still occur due to various factors, including infections like CMV. CMV infection during the second trimester can lead to severe complications such as fetal growth restriction, central nervous system abnormalities, and even fetal death. These complications may increase the risk of miscarriage during this stage of pregnancy (Magnus *et al* .,2019).

CMV, like many other viruses, has a lytic cycle during which it actively replicates and produces new virus particles to infect other cells. This cycle is regulated by specific genes known as lytic genes. Cyclophilin A is a cellular protein that interacts with viral proteins during CMV's lytic cycle. It plays a role in facilitating the production of new virus particles. It can interact with various viral proteins, including viral kinases, and modulate their functions to support viral replication.CMV can also establish latent infections in host cells ,where Interestingly, Cyclophilin A has been implicated in the establishment of CMV latency and the maintenance of the latent viral genome. During this phase, Cyclophilin A may play a role in helping the virus remain dormant and persist in the host cell without active replication. The virus becomes dormant and doesn't actively produce new virus particles (Abdullah *et al.*,2018; Zhao *et al.*,2021).

Cyclophilin A (CypA) is a potential biomarker for CMV infections and its levels may show some correlation with the presence of IgM and IgG antibodies, it's important to note that CypA is not a widely established or widely used biomarker for CMV infections in clinical practice (Suga *et al.*, 2023).

MiRNAs are short non-coding RNAs that can regulate gene expression by binding to messenger RNAs (mRNAs) and inhibiting their translation or promoting their degradation. During CMV infection, both viral and host-encoded miRNAs can come into play (Zhang *et al* .,2020).

CMV has its own set of miRNAs, which can regulate both viral and host genes. These viral miRNAs may help the virus evade the host immune response and maintain latency. Host-encoded miRNAs can also target CMV genes, limiting viral replication and spread. Some miRNAs might directly target CMV mRNAs, hindering viral protein production. LncRNAs are a diverse group of non-coding RNAs that are longer than miRNAs. They can exert regulatory effects through

various mechanisms, including chromatin remodeling and interacting with other RNA molecules. LncRNAs have also been implicated in CMV infection, Some host lncRNAs can modulate the host immune response to CMV infection by regulating the expression of genes involved in immune signaling pathways. They may act as positive or negative regulators of the antiviral immune response. CMV produces its own lncRNAs, which can play roles in viral gene regulation and the establishment of latency. (Janković *et al.*, 2022).

miR-UL112 This specific miRNA appears to have a role in CMV immunoevasion and immune modulation. It's important to note that miR-UL112 is a viral miRNA encoded by CMV itself. Viral miRNAs like miR-UL112 can target host genes and modulate host immune responses. In the context of CMV, it may inhibit certain host immune factors and promote viral persistence. miR-US25-1: This miRNA seems to play a role in preventing viral replication and direct viral damage. Again, it's important to clarify whether this miRNA is a host-encoded or viral miRNA. Host-encoded miRNAs can target viral genes and inhibit viral replication. In doing so, they contribute to the host's antiviral defenses. (Picarda and Benedict, 2018; Yu M *et al.*,2022).

The potential impact of cytomegalovirus (CMV) infection on hormone levels and hormone disturbances, particularly in the context of women experiencing miscarriage due to CMV infections, is an area of ongoing research.

CMV is known for its ability to establish latent infections and can cause a wide range of health issues, including congenital infections, but its direct influence on hormonal regulation is not fully understood.

Aim of study:

The aim of this study is investigation CMV infection in women with miscarriage and detection the association between Cyclophilin A, nucleotide polymorphism *PPIA*, and gene expression microRNA.

The objective of the study was:

1) Screening of women with history miscarriage screen CMV infections by detecting IgG and IgM.
2) Detection of Cyclophilin A levels and study of some loci for Cyclophilin A gene polymorphisms.
3) Detection of some miRNA associated with CMV infection (miR-UL112 and miR-US25) .
4) Detection of two hormones Cortisol and ACTH to show their levels in those women.

1.2 Literature review

1.2.1 Cytomegalovirus

Cytomegalovirus (CMV) belonging to the family Herpesviridae, specifically the subfamily Betaherpesvirinae. The family Herpesviridae consists of a large group of DNA viruses that infect various animal species, including humans. This family is further divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae.(Auriti, *et al.*, 2021)

Cytomegalovirus is classified within the Betaherpesvirinae subfamily, which also includes human herpesviruses 6 and 7 (HHV-6 and HHV-7). These viruses share similar characteristics and mechanisms of infection. CMV is referred to as Human Herpesvirus 5 (HHV-5) within the herpesvirus family (Biswas and Kansal, 2023).

The Betaherpesvirinae subfamily is distinct from the other two subfamilies in terms of their biological properties, clinical manifestations, and latency patterns. Betaherpesviruses have a relatively long replication cycle and tend to establish persistent infections, often causing chronic and recurrent diseases. (Abiri *et al.*, 2021).

Virtual three-dimensional model of HCMV showing various components of the virus as shown in figure (1-1).

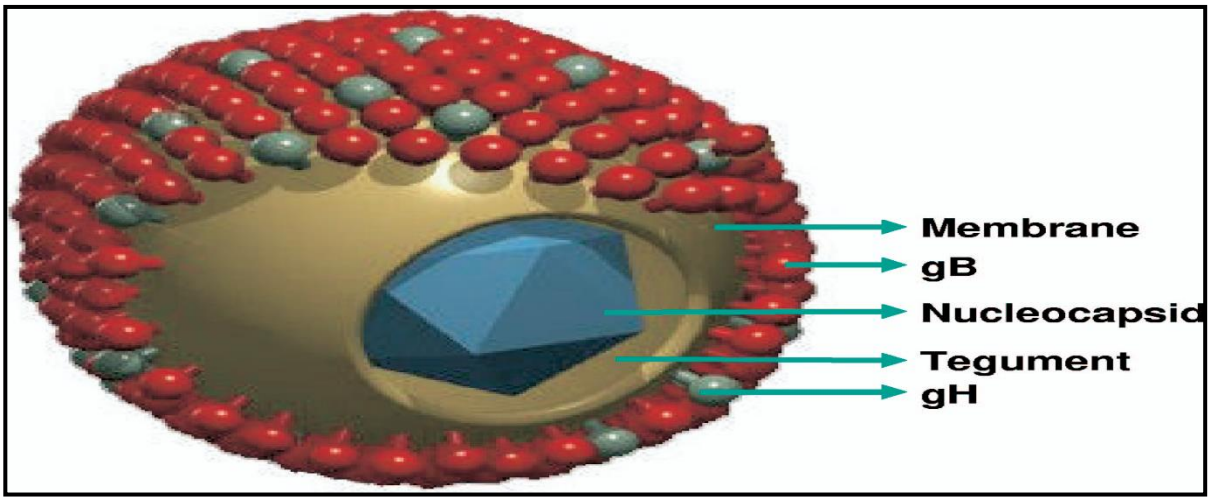


Figure (1-1):Virtual three-dimensional model of CMV showing various components of the virus (Crough and Khanna, 2009)

1.2.2 Cytomegalovirus (CMV) infection.

Cytomegalovirus has ability to cause disease infection , which belongs to the herpes virus family. CMV is a ubiquitous virus, meaning it is found in people all over the world and can infect individuals of all ages. Most people who become infected with CMV do not experience any symptoms or only have mild symptoms. However, it can be a serious concern for certain groups of people, especially those with weakened immune systems (Godsell *et al*.,2021).

When CMV infection occurs in association with humans, particularly in individuals with weakened immune systems or during specific circumstances, it can cause several diseases and complications (Shi *et al.*, 2023) such as Congenital CMV infection which can be transmitted from an infected mother to her fetus during pregnancy. Congenital CMV infection can lead to various health issues in newborns, including hearing loss, developmental delays, intellectual disability, vision problems, and potentially life-threatening conditions.

CMV mononucleosis which is similar to infectious mononucleosis caused by Epstein-Barr virus (EBV), CMV infection can cause a mononucleosis-like illness in some individuals. Symptoms may include fever, sore throat, fatigue, swollen lymph nodes, and general malaise. Also this virus causes CMV-related organ diseases which affecting specific organs in individuals with weakened immune systems, such as transplant recipients or those with HIV/AIDS. These diseases can include CMV pneumonia, CMV hepatitis, and gastrointestinal CMV infection. Moreover, CMV infection can affect the retina of the eye, leading to a condition known as CMV retinitis. This primarily occurs in individuals with severely compromised immune systems, such as those with advanced HIV/AIDS. If left untreated, CMV retinitis can cause vision loss or blindness (Godsell *et al.*, 2021; Jassim *et al.*, 2021).

After the primary infection with cytomegalovirus (CMV), the virus can indeed establish a lifelong latent infection in the body. During latency, CMV remains in a dormant or inactive state within certain cells, particularly white blood cells (specifically, monocytes and T cells). The virus's ability to remain latent in the host's cells is a characteristic of herpes viruses, to which CMV belongs. However, the virus can reactivate under various circumstances, which can lead to symptomatic infections, particularly in individuals with weakened or compromised immune systems. Reactivation of CMV can occur in People with weakened immune systems, such as those with HIV/AIDS, individuals receiving immunosuppressive medications after organ transplantation, or individuals undergoing chemotherapy, are at a higher risk of CMV reactivation. The virus can become active when the immune system's ability to control it is compromised, Stress, illness, and other factors that weaken the immune system's function can trigger CMV reactivation (Mabilangan *et al.*, 2020 ;Gupta *et al.*, 2021).

Also, Pregnant women, particularly those who have not been previously exposed to CMV, can experience primary infection during pregnancy. In some cases, the virus can reactivate, leading to complications for both the mother and the developing fetus. CMV reactivation can become more common as people age and their immune systems weaken. When CMV reactivates, it can cause symptoms similar to those of the primary infection, including fever, fatigue, and other flu-like symptoms. In individuals with compromised immune systems, CMV reactivation can lead to more severe and potentially life-threatening complications, such as pneumonia, hepatitis, and retinitis(Griffiths and Reeves, 2021; Almishaal, 2022).

Most healthy individuals with CMV infection do not require specific treatment, as the infection often resolves on its own, However, antiviral medications may be prescribed for severe or symptomatic CMV infections, especially in individuals with weakened immune systems.(Liu *et al.*, 2022) . Life cycle of CMV in a human cell as shown in figure (1-2)

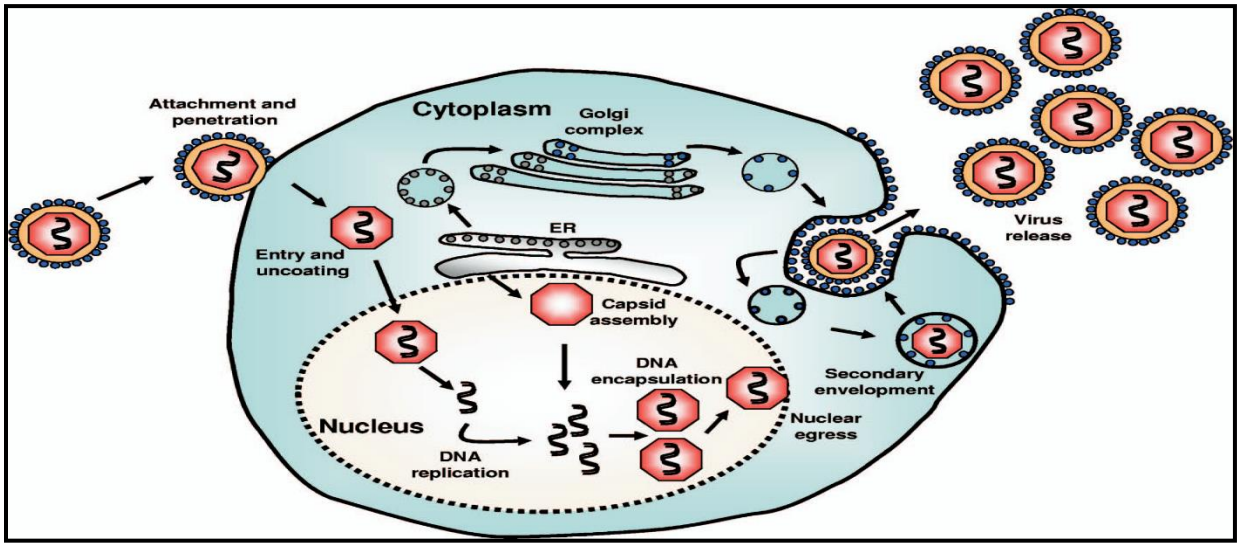


Figure (1-2):Life cycle of CMV in a human cell(Crough and Khanna, 2009).

1.2.3 Spread and Transmission of Cytomegalovirus (CMV)

Cytomegalovirus (CMV) has a wide distribution and can be found worldwide. It is estimated that a large percentage of the population has been infected with CMV at some point in their lives. the spread and transmission of CMV can occurs in several ways such as Person-to-person transmission through close contact with infected individuals. This can occur through direct contact with bodily fluids such as saliva, urine, blood, semen, vaginal secretions, and breast milk. Common modes of transmission include kissing, sexual contact, sharing utensils or toothbrushes, and breastfeeding(Fellah *et al.*, 2020). CMV can be transmitted from an infected mother to her unborn baby during pregnancy. This is known as congenital CMV infection. The virus can cross the placenta and infect the fetus, potentially leading to various complications and long-term health effects (Linthorst *et al.*, 2023). CMV can be transmitted through organ transplantation or blood transfusion from an infected donor. This is more common in cases where the donor or recipient is CMV-positive. also , the healthcare workers, daycare workers, and individuals working in close contact with children are at higher risk of CMV infection due to potential exposure to bodily fluids. CMV can be transmitted through sexual activity, including vaginal, anal. It is important to note that the risk of sexual transmission is generally lower compared to other sexually transmitted infections.(de Vries *et al.*, 2021).

1.2.4 The symptoms and diagnosis Cytomegalovirus (CMV):

1.2.4.1 Symptoms:

Many people infected with CMV may not experience any symptoms or may only have mild symptoms similar to a flu-like illness. This is known as an asymptomatic or subclinical infection(Leruez-Ville *et al.*, 2020). In

immunocompromised patients, such as those with HIV/AIDS or undergoing organ transplantation, CMV can cause severe complications and symptoms such as pneumonia, retinitis (inflammation of the retina), hepatitis, and gastrointestinal issues(Safdar and Armstrong, 2019). while in congenital CMV infection, symptoms may vary but can include hearing loss, developmental delays, intellectual disabilities, vision problems, and, in severe cases, organ damage(Leruez-Ville *et al.*, 2020).

1.2.4.2 Diagnosis:

There are two primary types of blood tests used for CMV diagnosis the serologic Tests which determine the presence of CMV-specific antibodies in the blood. The body produces antibodies in response to CMV infection. Different types of CMV antibodies are produced at different stages of infection. Serologic tests can help identify the stage of infection, whether it's a primary infection or a reactivation, and Polymerase Chain Reaction (PCR) which is a highly sensitive method that detects the presence of CMV DNA in the blood. This test can identify even low levels of the virus. PCR testing is particularly useful for diagnosing active infections and monitoring the viral load in individuals, especially those with compromised immune systems. (Rosenheck *et al.*, 2021).

1.2.4 Cytomegalovirus and miscarriage women

Cytomegalovirus (CMV) infection during pregnancy can potentially be associated with complications, including the risk of transmission of the virus to the developing fetus. However, it's important to emphasize that most pregnant women who acquire CMV infection for the first time during pregnancy will still have uncomplicated pregnancies and give birth to healthy babies. The risk of transmission

to the fetus and the severity of potential complications depend on several factors, the gestational age at which a pregnant woman is first infected with CMV is a crucial factor. Generally, the risk of transmission to the fetus is higher if the infection occurs during the first trimester of pregnancy. The mother's immune response plays a role in determining the risk of transmission and the severity of fetal complications. Women with a strong immune response may be less likely to transmit the virus to the fetus or may transmit it with fewer severe consequences. Some strains of CMV may carry a higher risk of fetal transmission and complications than others. The Complications associated with congenital CMV infection in the fetus can includes Hearing impairment is one of the most common complications of congenital CMV infection. CMV can affect the central nervous system, leading to developmental delays, intellectual disabilities, and other neurological problems. It**'s** can cause vision impairments and even blindness in some cases. Fetal growth restriction and low birth weight can also occur in cases of congenital CMV infection. (Uchida *et al.*, 2020 ; Auriti *et al.*, 2021; Pesch *et al.*, 2021).

Congenital CMV infection occurs when a pregnant woman acquires a primary CMV infection during pregnancy or experiences reactivation of a latent infection. The virus can be transmitted to the fetus through the placenta, leading to an intrauterine infection(Auriti *et al.*, 2021).

The transmission rate of cytomegalovirus (CMV) from a pregnant woman to her fetus does indeed vary, but it is generally estimated to be in the range of 30% to 40% in cases of primary CMV infection during pregnancy. However, it's important to note that the majority of infants born to CMV-infected mothers do not have significant health problems, even if they acquire the infection during pregnancy. While a substantial proportion of infants born to mothers with primary CMV infection may become infected with the virus, the outcomes can vary widely. Many

of these infants will not experience any immediate or long-term health issues. Only a smaller percentage of these infants will develop symptoms or complications related to congenital CMV infection. The severity of congenital CMV infection in affected infants can range from mild to severe, with some infants being asymptomatic (showing no symptoms at birth) and others experiencing more significant health challenges (Walsh *et al.*, 2021).

According to the Centers for Disease Control and Prevention (CDC), it was seen that approximately 1 in 200 infants are born with congenital CMV infection. This makes it more common than other well-known congenital infections, such as Down syndrome or spina bifida(Demmler-Harrison *et al.*, 2020). The prevalence of congenital CMV infection varies across populations and regions, but it is generally considered a significant public health concern. The exact incidence of congenital CMV infection varies depending on factors such as geographical location, socioeconomic status, and population studied(Mussi-Pinhata and Yamamoto, 2020).

Early detection, proper prenatal care, and appropriate management strategies can help in the prevention, identification, and management of congenital CMV infection. Pregnant women who have concerns or questions about CMV should consult with their healthcare providers for guidance and recommendations based on their individual circumstances. (Angueyra *et al.*, 2020; Prosser *et al.*, 2021).

1.3 Cyclophilin A

Cyclophilins are part of a larger family of proteins known as immunophilins, which also includes FK-506-binding proteins (FKBPs) and parvulins. These proteins are collectively referred to as immunophilins because they are often targeted by immunosuppressive drugs, such as cyclosporine and FK-506, which have therapeutic applications in transplantation and autoimmune diseases(Singh *et al*

.,2021). They are a proteins known for their peptidyl-prolyl cis-trans isomerase (PPIAse) activity. Its catalyze the cis-trans isomerization of peptide bonds involving proline residues. This enzymatic activity is important in the folding and conformational changes of proteins. By facilitating the interconversion between cis and trans forms of proline bonds, cyclophilins play a role in protein folding, stability, and function. Moreover, Cyclophilins are found in various organisms, including prokaryotes (bacteria) and eukaryotes (including humans). The presence of cyclophilins in such a wide range of organisms suggests their evolutionary significance and conserved roles in cellular processes. Humans have a total of 16 known cyclophilin proteins, each with distinct cellular functions. One of the most well-known human cyclophilins is cyclophilin A (CypA), which has been extensively studied for its interactions with the HIV virus and its potential as a therapeutic target.They can interact with a wide range of target proteins and influence their stability and activity(Singh *et al.*, 2021; Harikishore and Sup Yoon, 2016).

There are seven key cyclophilins, including Cyclophilin A (CypA) Also known as hCyp-18a, it is one of the most well-studied and abundant cyclophilins in humans. It has a molecular mass of around 18 kDa. CypA is found in the cytoplasm and is involved in various cellular processes, including protein folding and the immune response. It is also known for its interaction with the HIV-1 virus. Cyclophilin B (CypB), Also known as hCyp-22/p, it has a molecular mass of around 22 kDa. It is another important member of the cyclophilin family and is found both inside and outside of cells. Its plays a role in protein folding, cellular trafficking, and other processes. Cyclophilin C (CypC) is another member of the cyclophilin family but is less studied compared to CypA and CypB. It is involved in protein folding and may have specific roles in mitochondrial processes. Cyclophilin D (CypD)is a

mitochondrial cyclophilin. It plays a role in mitochondrial permeability transition, which is important in cell death pathways. CypD is often associated with mitochondrial dysfunction in various pathological conditions. Cyclophilin E (CypE) is less well-characterized compared to some other cyclophilins. It is involved in protein folding and may have specific roles in cellular processes. Cyclophilin 40 (Cyp40) has a molecular mass of around 40 kDa and is known for its larger size compared to other cyclophilins. It is involved in protein folding and is often found in complex with the heat shock protein Hsp90. Cyclophilin NK (CypNK) was first discovered in humans and is involved in protein folding and other cellular processes. It may have specific functions in immune responses (Wang and Heitman,2005; Molenberghs *et al.*, 2020) .

Cyclophilin A (CyPA) has been found to play a role in human cytomegalovirus (HCMV) infection. and it can establish both lytic and latent infections in host cells(Liao *et al.*, 2021). Studies have shown that CyPA is required for various stages of the HCMV life cycle, including lytic infection, latency establishment, and reactivation. its interacts with HCMV proteins and contributes to the successful replication and persistence of the virus within host cells (Yu *et al.*, 2022). During lytic infection, CyPA is involved in the efficient production of infectious virus particles. It interacts with viral proteins, such as the viral kinase pUL97, and modulates their functions, leading to enhanced viral replication and release of mature virions(Molenberghs *et al.*, 2020). In addition to lytic infection, HCMV can establish a latent infection in certain cell types, particularly hematopoietic cells. During latency, the virus enters a dormant state, and viral gene expression is limited. Studies have shown that CyPA is required for the establishment of HCMV latency. It interacts with viral proteins involved in the

regulation of latency and contributes to the maintenance of the latent viral genome(Yang *et al.*, 2023).

Reactivation of latent HCMV can occur under certain conditions, such as immune suppression or other immune challenges. CyPA has also been implicated in the reactivation process. It is involved in the regulation of viral gene expression and the transition from latency to the lytic phase, leading to the production of infectious virus particles(Balasubramaniam *et al.*, 2019).

Cyclophilin A (CyPA) has been implicated in regulating immediate-early (IE) protein and lytic gene expressions during the replication cycle of human cytomegalovirus (HCMV). Understanding the interactions between host cell factors like CyPA and viral replication processes is critical for unraveling the mechanisms of viral infections.

Immediate-early (IE) proteins are among the first viral proteins expressed upon infection and play a crucial role in initiating the replication cycle of HCMV. These proteins are involved in modulating host cell functions to facilitate viral replication. Cyclophilins, as peptidyl-prolyl cis-trans isomerases, can influence protein folding and conformational changes. Therefore, their involvement in regulating viral gene expression is not surprising, as it can affect the folding and activity of viral proteins crucial for replication. (Vincenzi and Leone, 2021; Adamson and Nevels, 2020).

CyPA has been found to interact with and regulate the expression of HCMV IE proteins. Specifically, it has been shown to enhance the transcriptional activity of IE gene promoters, thereby promoting the expression of IE proteins. This regulation of IE protein expression by CyPA can impact subsequent stages of the HCMV replication cycle(Monk and Zwezdaryk, 2020).

Furthermore, CyPA can influence the expression of lytic genes during HCMV replication. Lytic genes are expressed during the later stages of the viral replication cycle and are responsible for viral assembly, maturation, and release. CyPA has been reported to modulate the expression of specific lytic genes, potentially influencing viral replication and the production of infectious virions (Pavitrakar *et al.*, 2021).

Cyclophilin A (CypA) is a cellular protein that has been implicated in various physiological and pathological processes, including viral infections. While the specific role of Cyclophilin A in miscarriage is not well-characterized, it has been associated with immune dysregulation and inflammatory responses, which can potentially contribute to pregnancy complications, including miscarriage. Cyclophilin A has been shown to modulate immune responses by interacting with immune cells and influencing their functions (Yang, *et al.*, 2023).

It can act as a chaperone protein and participate in the folding and trafficking of immune-related proteins. In the context of pregnancy, immune tolerance and proper immune modulation are crucial for maintaining a successful pregnancy. Dysregulation of immune responses, including aberrant activation or inadequate suppression, can lead to pregnancy complications, including miscarriage. Also it can act as a pro-inflammatory molecule by stimulating the production of inflammatory cytokines and chemokines. It can activate immune cells and promote the recruitment of inflammatory cells to the site of infection or inflammation. Excessive inflammation within the placenta can disrupt normal placental function, compromise fetal development, and increase the risk of miscarriage. Certain viral infections, including CMV, have been associated with miscarriage. Cyclophilin A has been implicated in the life cycle of CMV, where it plays a role in viral replication, as well as establishment and maintenance of latency. CMV infection during pregnancy can lead to immune dysregulation, inflammation, and placental dysfunction, which can

contribute to pregnancy complications, including miscarriage. (Abdullah *et al* ., 2018; Geldenhuys *et al* .,2019; Njue,2020).

1.3.1 The Cyclophilin A gene

The Cyclophilin A gene, also known as *PPIA* (Peptidylprolyl Isomerase A), encodes the Cyclophilin A protein. The Cyclophilin A gene is located on human chromosome 7 (7p13) and consists of several exons and introns. It spans approximately 7 kilobases in length(Hadpech and Thongboonkerd, 2022). The protein encoded by the Cyclophilin A gene, Cyclophilin A, is a highly conserved protein that is expressed in a wide range of tissues and cell types. It is known to interact with numerous cellular proteins, including those involved in immune responses, inflammation, and viral infections(Zhou *et al.*, 2022).

1.3.2 Cyclophilin A with CMV infection

Cyclophilin A has been shown to interact with specific CMV proteins and promote viral replication. For example, CyPA interacts with the CMV DNA polymerase processivity factor UL44, UL44 is a CMV protein that acts as an accessory factor for the viral DNA polymerase. It helps the DNA polymerase remain attached to the DNA template during replication, thus increasing the efficiency of viral DNA synthesis. also known as the viral DNA polymerase accessory protein, and enhances its activity. This interaction facilitates efficient viral DNA replication and overall CMV replication(Wu *et al.*, 2022).

CMV has developed various mechanisms to evade the host immune response. Studies have suggested that CyPA may play a role in CMV immune evasion. It interacts with the CMV-encoded immunomodulatory protein, viral inhibitor of caspase-8 activation (vICA), and contributes to the inhibition of host immune signaling pathways, including the tumor necrosis factor (TNF) signaling

pathway(Cheng, 2019). It has been implicated in the late stages of CMV replication, specifically viral assembly and maturation. It interacts with the CMV capsid protein, contributing to proper capsid formation and stabilization. This interaction is important for the assembly of infectious CMV particles(Rossi *et al.*, 2021). Given its involvement in CMV replication, CyPA has been explored as a potential therapeutic target against CMV infection. Inhibiting CyPA's interaction with CMV proteins or its enzymatic activity has been investigated as a strategy to disrupt CMV replication and reduce viral load.(Wu *et al.*, 2022).

1.4 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play a crucial role in regulating gene expression in various organisms, including eukaryotes and certain viruses, such as herpesviruses. These molecules have emerged as important regulators of gene expression at the post-transcriptional level(Kamalidehghan *et al.*, 2020).

MiRNAs were first discovered in the early 1990s, and since then, extensive research has shed light on their diverse functions and significance in biological processes(Peng *et al.*, 2023). They are typically around 21-23 nucleotides in length and are transcribed from specific genes. They function by binding to target messenger RNA (mRNA) molecules, leading to their degradation or inhibition of translation(Kryovrysanaki *et al.*, 2021).

The regulation of gene expression by miRNAs is a complex and highly orchestrated process. Initially, miRNA genes are transcribed into primary miRNA transcripts (pri-miRNAs), which undergo processing steps to generate precursor miRNAs (pre-miRNAs)(Chen *et al.*, 2020).

Pre-miRNAs are then further processed into mature miRNAs, which are incorporated into a protein complex known as the RNA-induced silencing complex (RISC). Within the RISC, the mature miRNAs guide the complex to complementary sequences in target mRNAs, resulting in the repression of gene expression.(Ergin and Çetinkaya, 2022)

In eukaryotic organisms, miRNAs are involved in numerous biological processes, including development, cellular differentiation, proliferation, and apoptosis. They have been implicated in various diseases, such as cancer, cardiovascular disorders, neurodegenerative diseases, and viral infections.

In the case of viral infections, miRNAs play a dual role by regulating both host and viral gene expression(Jiao *et al.*, 2021).

Herpesviruses, a family of DNA viruses, have been shown to encode their own miRNAs. These viral miRNAs can target both viral and host mRNAs, influencing viral replication, immune evasion, and the establishment of viral latency. By manipulating host gene expression, viral miRNAs can create an environment favorable for viral survival and persistence within the host(Zhang *et al.*, 2020; Letafati *et al.*, 2022). They can target host cell mRNAs, leading to the repression of specific genes involved in antiviral defense mechanisms or immune responses. By downregulating host genes that are critical for mounting an effective immune response, also they can help the virus to establish and maintain infection within the host. This allows the virus to evade immune detection and clearance, promoting its survival and replication(Dass *et al.*, 2023). Additionally, viral miRNAs can also target viral mRNAs, affecting viral gene expression and replication. By regulating the expression of viral genes, these miRNAs can modulate the production of viral proteins involved in various stages of the viral life cycle. This enables the virus to control its own replication, promote the establishment of latency, or enhance the

production of infectious viral particles.(Abu-Izneid *et al.*, 2021; Diggins and Hancock, 2023).

Understanding the functions and mechanisms of miRNAs has opened up new avenues for research and potential therapeutic interventions. The ability of miRNAs to regulate gene expression with high specificity makes them attractive targets for developing diagnostic tools and therapeutic strategies for various diseases, including viral infections(Abdel Halim *et al.*, 2023).

In the context of cytomegalovirus (CMV) infection, miRNAs expressed by the virus can be functionally relevant during the relatively brief lytic replication cycle by exerting specific regulatory effects(Luan *et al.*, 2022). The virus induces the expression of numerous genes and triggers various cellular responses. To be functionally relevant within this short timeframe, CMV-encoded miRNAs are believed to primarily target specific genes induced upon infection or repress the synthesis of short-lived proteins.(Afshari *et al.*, 2022)

By targeting and inhibiting the expression of specific host genes induced by CMV infection, viral miRNAs can interfere with host cellular processes and immune responses. This allows the virus to manipulate the cellular environment to create a more favorable setting for viral replication. The targeting induced host genes also assists CMV to counteract host antiviral defenses and evade immune system.(Qin *et al.*, 2023)

In addition, viral miRNAs can also repress the synthesis of short-lived proteins. This mechanism enables the rapid attenuation of specific cellular effects induced by CMV infection. Thus viral miRNAs can modulate specific cellular pathways and prevent the production of proteins that might interfere with viral replication or immune evasion(Diggins *et al.*, 2021).

The regulatory effects of CMV miRNAs help the virus to finely tune host gene expression and cellular responses to its advantage. By modulating gene expression, CMV can manipulate the cellular environment, counteract host immune defenses, and promote its own replication.(El Baba and Herbein, 2021)

miRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level. Growing evidence suggests that aberrant expression of miRNAs can contribute to the pathogenesis of various diseases, including miscarriage ,explore the involvement of miRNAs in different aspects of miscarriage, including implantation failure, pregnancy complications, and fetal abnormalities, the potential diagnostic and therapeutic applications of miRNAs in miscarriage management. (Karami *et al.*, 2018).

The expression of cytomegalovirus (CMV) microRNAs in placental tissues of women with spontaneous miscarriage is an interesting area of research in the context of viral infections during pregnancy. CMV is known to be a potential cause of congenital infections when a pregnant woman is exposed to the virus. Congenital CMV infection can lead to a range of birth defects and complications, including spontaneous miscarriage (miscarriage). CMV encodes its own set of microRNAs, including miR-UL112-5p and others, as mentioned earlier. These viral microRNAs can influence gene expression in host cells, including placental cells. Some studies have reported the detection of CMV microRNAs in placental tissues of women who experienced spontaneous miscarriage, suggesting that CMV infection may have played a role in the pregnancy loss. The exact mechanisms by which CMV microRNAs may contribute to spontaneous miscarriage are not fully understood. However, it is hypothesized that CMV infection in the placenta could disrupt normal placental development, cause inflammation, and potentially lead to fetal

complications and miscarriage.(Smith *et al.*,2015; Wang *et al.*,2021; Patel *et al.*,2020).

The mechanisms by which cytomegalovirus (CMV) microRNAs (miRNAs) promote immune evasion in the placenta and potentially lead to an inflammatory environment, impairing fetal development and increasing the risk of pregnancy loss, are complex and not yet fully understood. CMV miRNAs may target host immune response genes in placental cells. By binding to specific messenger RNA (mRNA) molecules of host immune genes, these miRNAs can inhibit the translation of these mRNAs into functional proteins. Suppression of key immune signaling pathways, such as interferon (IFN) signaling, can dampen the host's antiviral immune response. This allows the virus to replicate and persist in placental tissues. CMV infection in the placenta can trigger inflammatory responses as part of the host's attempt to control the infection. CMV miRNAs may modulate the balance of pro-inflammatory and anti-inflammatory cytokines in placental cells. Imbalances in these cytokines can lead to an excessive inflammatory response. Chronic inflammation in the placenta can disrupt normal fetal development and contribute to pregnancy complications, including pregnancy loss. CMV miRNAs may influence the recruitment and activation of immune cells in the placenta. Some miRNAs can target chemokines and cytokines involved in immune cell trafficking. Altering the composition and activation status of immune cells in the placenta can contribute to inflammation and tissue damage. CMV may employ immune tolerance mechanisms to evade the host's immune response. This can involve inhibiting the activation of immune cells that could target infected placental cells. CMV miRNAs may play a role in these tolerance mechanisms by suppressing immune responses against infected placental cells while promoting inflammation. CMV may employ immune tolerance mechanisms to evade the host's immune response. This can involve

inhibiting the activation of immune cells that could target infected placental cells. CMV miRNAs may play a role in these tolerance mechanisms by suppressing immune responses against infected placental cells while promoting inflammation. The combined effects of CMV infection, inflammation, and immune evasion mechanisms in the placenta can increase the risk of pregnancy complications, including miscarriage or stillbirth.(Letafati *et al.*, 2022; Zhao *et al*.,2021).

1.4.1 miR-US25-1

miR-US25-1 is a microRNA (miRNA) encoded by human cytomegalovirus (HCMV). It is a viral miRNA that has been identified and characterized in HCMV-infected cells. It is transcribed by HCMV during the lytic replication cycle and has been found to play various roles in the viral infection, it's have the ability to targets specific host cellular mRNAs to regulate gene expression and modulate cellular processes. Several target genes of miR-US25-1 have been identified, including genes involved in immune responses and cellular signaling pathways. By modulating the expression of these target genes, miR-US25-1 can influence host-virus interactions and promote viral replication(Dass *et al.*, 2023). miR-US25-1 can target the major histocompatibility complex class I-related chain B (MICB), which is involved in immune recognition and natural killer (NK) cell activation. By downregulating MICB expression, it can potentially inhibit immune responses and promote viral immune evasion(Yu *et al.*, 2022).

Furthermore, miR-US25-1 has also been shown to target other host genes involved in cellular signaling pathways, such as the protein phosphatase PPM1A and the transcription factor NFATC3. Modulation of these target genes by miR-US25-1 can impact cellular processes and create a favorable environment for viral

replication(Diggins *et al.*, 2021). The functional significance and precise mechanisms of miR-US25-1 in HCMV infection are still an active area of research. Further studies are needed to fully elucidate its role and the implications for host-virus interactions and viral pathogenesis.

1.4.2 miR-UL112-5p

miR-UL112-5p is indeed a microRNA (miRNA) encoded by the human cytomegalovirus (HCMV), which is a member of the Herpesviridae family. miRNAs are small non-coding RNA molecules that play important roles in the regulation of gene expression. In the case of HCMV, viral miRNAs like miR-UL112-5p are transcribed and expressed during the lytic replication cycle of the virus. miR-UL112-5p is primarily expressed and active during the lytic replication cycle of HCMV. This is the phase of the viral life cycle when the virus replicates and spreads within the host cell. Like other miRNAs, miR-UL112-5p is involved in regulating gene expression. It does this by binding to specific messenger RNA (mRNA) molecules and inhibiting their translation into proteins. By doing so, the miRNA can influence the host cell's machinery and create a more favorable environment for viral replication. Some studies suggest that miR-UL112-5p may be involved in immune evasion strategies employed by HCMV. By modulating host cell gene expression, it can potentially interfere with the host's immune response, allowing the virus to persist and replicate more effectively(Zhang *et al.*, 2020; Diggins *et al.*, 2021; Yu *et al.*, 2022).

One of the notable targets of miR-UL112-5p is the major histocompatibility complex class I-related chain B (MICB). By targeting MICB, miR-UL112-5p can downregulate its expression and potentially interfere with the immune recognition

of infected cells by natural killer (NK) cells and other immune effector cells. This targeting of MICB by miR-UL112-5p may aid in the immune evasion strategies employed by HCMV(Zhang *et al.*, 2020). Also, miR-UL112-5p may have other targets involved in immune responses, viral replication, and cellular signaling pathways.

Further research is needed to fully elucidate the specific targets and functions of miR-UL112-5p and their implications in HCMV infection(Yu *et al.*, 2022).

Understanding the role of viral miRNAs, such as miR-UL112-5p, in HCMV infection can provide insights into the intricate interactions between the virus and the host immune system. It may also have implications for the development of antiviral strategies targeting viral miRNAs as a potential therapeutic approach against HCMV infection(El Baba and Herbein, 2021).

1.5 Cortisol and ACTH in CMV infection

Cytomegalovirus (CMV) is a common viral infection that can affect various organs and systems in the body. One area of interest in CMV research is its potential impact on hormone levels, particularly cortisol and ACTH. Cortisol is a steroid hormone produced by the adrenal glands, which are located on top of the kidneys. It is involved in a wide range of physiological processes, including metabolism, immune response, and stress regulation. ACTH, on the other hand, is a hormone produced by the pituitary gland in the brain, and it stimulates the production and release of cortisol from the adrenal glands(Onpoaree *et al.*, 2022).

However, it is important to note that cortisol levels can fluctuate throughout the day, with higher levels in the morning and lower levels in the evening. Therefore, the timing of the blood test may influence the results, and multiple samples may be

necessary to get a comprehensive understanding of cortisol pattern(Russell and Lightman, 2019) . In addition to cortisol, ACTH levels can also provide valuable information about the functioning of the adrenal glands. High levels of ACTH may suggest an overactive adrenal gland, while low levels may indicate an underactive gland.

The relationship between CMV infection and ACTH levels is still being studied, and further research is needed to fully understand the mechanisms involved(Mooney, 2020).

The relationship between cytomegalovirus (CMV) infection and cortisol and ACTH levels involves a complex mechanism. CMV infection can stimulate the release of pro-inflammatory cytokines, such as interleukin-6 (IL-6), which can then affect the hypothalamic-pituitary-adrenal (HPA) axis, the regulatory system that controls cortisol production. When CMV infects the body, it triggers an immune response characterized by the release of various inflammatory molecules. This immune response can lead to the activation of the HPA axis. The hypothalamus releases corticotropin-releasing hormone (CRH), which then stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH, in turn, stimulates the adrenal glands to produce and release cortisol(Mahmood and Al-Ghurabi, 2020).

The increased production of cortisol serves as an adaptive response to the infection. Cortisol plays a crucial role in regulating the immune system and dampening excessive inflammation. It helps control inflammation and modulates the immune response, preventing it from becoming overly aggressive. Cortisol also supports the body's stress response and helps maintain homeostasis during times of infection or other physiological challenges.(Quatrini and Ugolini, 2021)

In some cases, CMV infection can lead to prolonged activation of the HPA axis, resulting in chronically elevated cortisol levels. This prolonged exposure to high cortisol levels can have negative effects on the immune system and overall health. It may impair immune function, increase susceptibility to other infections, and contribute to the development of certain health conditions, such as metabolic disorders and mood disturbances(Capellino *et al.*, 2020).

On the other hand, in severe or advanced stages of CMV infection, particularly in immunocompromised individuals, the adrenal glands may become compromised, leading to reduced cortisol production. This can result in low cortisol levels and a weakened stress response, which can further exacerbate the impact of the infection on the body(Teles *et al.*, 2022).

1.6 The Mechanisms of Cytomegalovirus (CMV) in Fetal Miscarriage.

The Cytomegalovirus CMV can modulate the maternal immune response, leading to an imbalance in immune tolerance and potential immune-mediated fetal damage(McElwain *et al.*, 2021). Moreover the CMV can infect placental cells, disrupting their normal function and compromising the exchange of nutrients and oxygen between the mother and fetus(Ortega *et al.*, 2022).In cases where CMV crosses the placenta and infects fetal tissues, it can cause direct damage to fetal organs, leading to miscarriage (Abas *et al.*, 2020) . also the CMV infection can trigger an inflammatory response in the mother, releasing cytokines and immune mediators that may adversely affect the developing fetus(Shuid *et al.*, 2021).

1.7 Mechanisms of CMV-Induced Miscarriage

Cytomegalovirus (CMV) infection can lead to miscarriage through various mechanisms involving both direct and indirect effects on the developing fetus and the maternal immune response. CMV can infect placental cells, particularly the syncytiotrophoblasts, leading to placental dysfunction. This can result in reduced nutrient and oxygen supply to the developing fetus, compromising its growth and development. Placental dysfunction may also disrupt hormonal balance, leading to hormonal insufficiency and abnormal placental function, which can contribute to miscarriage(Zhao *et al.*, 2021).

Also, CMV infection can impair trophoblast invasion and spiral artery remodeling, which are crucial processes for establishing adequate blood supply to the developing fetus. Impaired invasion and inadequate remodeling can lead to inadequate blood flow to the placenta, compromising fetal development and increasing the risk of miscarriage(Weckman *et al.*, 2019).

CMV infection can dysregulate the maternal immune response, leading to an imbalance between pro-inflammatory and anti-inflammatory factors. This immune dysregulation can result in an exaggerated inflammatory response, which may have detrimental effects on placental function and fetal development. Excessive inflammation in the placenta can lead to tissue damage and impair nutrient and oxygen exchange, contributing to miscarriage(Woods *et al.*, 2023).

The CMV can cross the placenta and directly infect the developing fetus, resulting in congenital CMV infection. The virus can target various fetal tissues, including the central nervous system, liver, spleen, and lungs. CMV-induced fetal infection can disrupt normal fetal development and cause severe organ damage, which may lead to miscarriage or fetal demise. In addition to Their ability to trigger

an immune response in the placenta, leading to immune-mediated damage (Zhao *et al* .,2021).

Inflammatory cells, including natural killer (NK) cells and cytotoxic T cells, can infiltrate the placenta in response to CMV infection. The release of pro-inflammatory cytokines and cytotoxic molecules by these immune cells can cause placental tissue damage and compromise fetal well-being, potentially resulting in miscarriage, moreover CMV infection can promote a pro-thrombotic state, leading to the formation of blood clots in placental blood vessels. Thrombosis within the placental vasculature can impair blood flow and nutrient delivery to the fetus, contributing to fetal growth restriction and increasing the risk of miscarriage. It is important to note that the exact mechanisms underlying CMV-induced miscarriage may vary depending on the timing of infection during pregnancy, the immune status of the mother, and the virulence of the CMV strain. The interplay between direct viral effects, placental dysfunction, immune dysregulation, and fetal damage contributes to the complex pathogenesis of CMV-associated miscarriage. (Njue *et al.*, 2020).

1.8 Molecular Mechanisms Underlying CMV-Induced Miscarriage

The molecular mechanisms underlying CMV-induced miscarriage are complex and multifactorial. Here, we will discuss some of the key molecular processes involved(Krstanović *et al.*, 2021):

A. Viral Replication and Spread:

Upon CMV infection, the virus replicates in various cell types, including trophoblasts and placental cells. CMV employs multiple strategies to promote its own replication and spread within the host. Viral proteins and miRNAs can target host factors involved in cell cycle regulation, apoptosis, and immune response,

facilitating viral replication and evading host immune defenses. The robust viral replication within placental cells can directly cause cellular damage and compromise placental function, contributing to miscarriage(Lee *et al.*, 2021)

B. Trophoblast Dysfunction:

CMV infection can disrupt the normal functioning of trophoblast cells, which are crucial for successful implantation and placental development. The virus can interfere with trophoblast proliferation, migration, invasion, and differentiation, impairing the establishment of a functional placenta. CMV proteins and miRNAs can target host factors involved in trophoblast function, leading to abnormal placental development and potential pregnancy complications, including miscarriage(van Dijk and Oudejans, 2011).

C. Immune Evasion:

CMV has developed sophisticated mechanisms to evade the host immune response, allowing it to establish persistent infection. The virus encodes various proteins that interfere with immune recognition and response. CMV proteins can inhibit major histocompatibility complex (MHC) class I expression, impairing antigen presentation and reducing the effectiveness of cytotoxic T cell responses. Additionally, CMV miRNAs can modulate the expression of host immune-related genes, dampening immune activation. Immune evasion strategies employed by CMV contribute to its ability to persist and cause prolonged damage, including miscarriage(Manandhar *et al.*, 2019).

D. Inflammatory Responses:

CMV infection triggers an immune response characterized by the production of pro-inflammatory cytokines and chemokines. The release of these inflammatory mediators is necessary to control viral replication and initiate an antiviral response. However, excessive or dysregulated inflammation can have detrimental effects on pregnancy. Chronic inflammation in the placenta can lead to tissue damage, impaired nutrient and oxygen exchange, and increased risk of miscarriage(Chudnovets *et al.*, 2020)

E. Angiogenesis and Vascular Dysfunction:

Proper placental development relies on the process of angiogenesis, which involves the formation of new blood vessels. CMV infection can disrupt angiogenesis by altering the balance of pro-angiogenic and anti-angiogenic factors. This disruption can lead to abnormal placental vascularization, compromising fetal blood supply and increasing the risk of miscarriage. CMV-induced vascular dysfunction may also result in thrombosis within placental blood vessels, further impairing blood flow and contributing to fetal demise(Huang *et al.*, 2021).

F. Hormonal Imbalance:

CMV infection can interfere with hormonal balance, particularly affecting hormones essential for maintaining pregnancy, such as progesterone and estrogen. The virus can disrupt the production, regulation, and responsiveness of these hormones, leading to hormonal imbalances that can contribute to miscarriage. Hormonal insufficiency caused by CMV infection can result in inadequate uterine support for the developing fetus(Littauer and Skountzou, 2018).

It is important to note that these molecular mechanisms are interconnected and can act in a synergistic manner, exacerbating the detrimental effects of CMV infection on pregnancy. Further research is needed to elucidate the specific molecular interactions and signaling pathways involved in CMV-induced miscarriage, which could potentially unveil targets for therapeutic interventions aimed at preventing or mitigating the adverse effects of CMV infection on pregnancy outcomes(Njue *et al.*, 2020).

CHAPTER TWO

MATERIALS

AND

METHODS

2 Materials and methods:

2.1 Materials

2.1.1 Laboratory Equipment and Instrument

Table (2-1) lists the laboratory equipment used in this study.

Table (2-1): Laboratory apparatus and Equipment.

No.	Item	Company	Country
1	Autoclave	Hirayama	Japan
2	Biological safety cabinet	Thermo Scientific	Germany
3	Centrifuge	Hettich	Germany
4	Digital camera	Nikon	USA
5	ELISA reader	Biotec	USA
6	ELISA shaker	Pasture	France
7	ELISA washer	Biotec	USA
8	Horizontal gel electrophoresis	Biorad	Japan
9	Hot plate	Medico	USA
10	Incubator	Incucell	Germany
11	Micropipettes,(2- 20µl), (5-50µl) (20-200µl) , (100-1000µl)	Slamed	Germany
12	Multichannel pipette	Slamed	Germany
13	PCR thermal cycler	Biometra	Germany
14	Printer	Epson	Indonesia
15	Refrigerated centrifuge	Hettich	Germany
16	Refrigerator	Concord	Italy
17	Sensitive digital balance	Sartorios	Germany

18	UV- transilluminator	Cleaver	England
29	Vortex shaker	Stuart	Germany
20	Water bath	Grant	England
21	Water distiller	GFL	Germany
22	VIDAS	Biomereux	France
23	Real-time PCR	Bioneer	Korea

2.1.2 Laboratory Instruments

The study required many glassware and disposable materials listed in the following table (2-2).

Table (2-2): Glassware and plastic equipment

No.	Equipment	Company	Origin
1	Disposable Sterile Syringes,5 ml	Dolphin	Syria
2	EDTA-tubes	Afco	Jordan
3	Ependrof Tubes 1.5 ml	Biobasic	Canada
4	Gel Tube10 ml	AL- Jawaher	Jordan
5	Test tube rack	Himedia	India
6	Tips 200,500,1000µl	BAG Health Care	Germany

2.1.3 Commercial Kits and chemical materials

2.1.3.1 Commercial kits

The commercial kits used in the study are listed in Table (2-3).

Table (2-3): Commercial kits used in the study

No.	Kits	Company	Origin
1	CMV IgM VIDAS test kit	Biomereux	France
2	CMV IgG VIDAS test kit	Biomeriex	France
3	Genomic DNA extraction kit	Geneaid	UK
4	Human Cyclophilin A ELISA Kit	BT LAB	China
5	Cortisol Kit	Biomeriex	France
6	PCR master mix	Promega	USA
7	Adrenocorticotropic Kit	Biomeriex	France
8	AccuZoL™	Bioneer	Korea
9	AccuPower® Green Star ™qpcr PreMix	Bioneer	Korea
10	AccuPower® RocketScript™ RT-PCR PreMix	Bioneer	Korea

Table (2-4): DNA extraction kit (Geneaid/UK).

Materials
GB buffer
W1 buffer
Wash buffer
Elution buffer
Proteinase K.
A bsolute ethanol

Table (2-5): AccuZoL™(BIONEER/Korea).

Materials
AccuZoL™ Reagent
Chloroform
Isopropyl alcohol
Ethanol
RNase -Free Water

Table (2-6):AccuPower ^{r} RocketScript ^{TM} RT-PCR PreMix (Bioneer/Korea).

Materials
RocketScript^{TM} Reverase Transcriptase
Reaction buffer
DTT
dNTPs(dATP,dCTP,dGTP,dTTP)
RNase inhibitor
TopDNA polymerase
Stabilizer and tracking dye

Table (2-7): AccuPower ^{r} Green Star qPCR PreMix (Bioneer/Korea).

Materials
Top DNA Polymerase
Hot Start buffer with 1.5mM $MgCL_2$
Intercalating dye
dNTps(dATP,dCTP,dGTP,dTTP)

Table (2-8):Human Cyclophilin A ,CYPA ELISA kit (E0672Hu).

Materials
Stander solution
Pre-coated ELISA plate
Standard dilution
Streptavidin-HRP
Stop solution
Substrate solution A
Substrate solution B
Wash buffer concertation
Biotinylated Human PPIA antibody

2.1.3.2 Chemical Materials

The chemical materials used in this study were listed in table (2-9).

Table (2-9): The Chemical Materials

No.	Material	Company	Origin
1	Absolute Ethanol	Scharlau	Spain
2	Agarose	Pronadisa	Spain
3	Ethidium Bromide	BioBasic	Canada

4	Free nuclease water	Cyntol	Russia
5	Ladder 50bp and 100bp	Promega	USA
6	TBE buffer 10 X(Tris base ,boric acid and EDTA)	Thomas Baker	Inda
7	Primers for polymorphism	Macrogen	Korea
8	DNA loading dye	Promega	USA
9	Primer for microRNA	Bioneer	Korea

2.2 Methods

2.2.1 Solutions

2.2.1.1 Ethidium Bromide Solution

It was prepared by dissolving 0.05gm of ethidium bromide in 10 ml distilled water and stored in a dark reagent bottle (Sambrook and Rusell, 2001).

2.2.1.2 Agarose Gel Preparation

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 2gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60^{o}C and mixed with 0.5µg/ml ethidium bromide.

2.2.1.3 1x TBE from 10XTBE:

TBE buffer: 27g of Tris base 14 g Boric acid and 1.86 g of EDTA (pH 8) dissolved in 500ml distal water

2.2.2 Collection of Clinical Specimens

2.2.2.1 Study Design

A case-control study was designed to include 200 samples obtained from patient's women with miscarriages who attended Babylon Teaching Hospital for Maternity and Children (outpatient clinic of obstetric and Gynecologic Department) as well as a private clinic, during a period of six months (from February 2022 to August 2022), Additionally, a control group of 50 women with no history of miscarriage was included.

2.2.2.2 Patients and Specimens Collection

A total of 250 women have been included in this study, 50 women of them were represent control group (a healthy women who were not pregnant, had no previous miscarriages) and 200 had a history of miscarriage (case group). The study was conducted at Babylon Teaching Hospital for Maternity and Children (outpatient clinic of the obstetric and Gynecologic Department) as well as a private clinic, during a period of six months (February 2022 to August 2022). Blood specimens were collected from participants who had provided informed consent, and these samples were then analyzed in a laboratory.

2.2.2.2.1 Inclusion Criteria for Patients

The inclusion criteria for the patient group consist of women within the reproductive age group of 16-40 years who have a history of miscarriages time.

2.2.2.2.2 Exclusion Criteria for The Patients

Exclusion criteria involve patients with chronic diseases, pregnant women, obese women, women with corticosteroid therapy, and adrenal disorders.

2.2.2.3 Blood specimens

Five ml of freshly venous blood were collected from each participant in the morning. 2ml of which was kept in an EDTA tube and use for extraction of human DNA and plasma ACTH after centrifugation. The other 3ml of blood kept in the gel tube without anticoagulant until be clotted. It was undergone centrifugation at 2500 rpm for 15 minutes then the serum was collected and preserved at -20°C until being used for Anti-Cytomegalovirus IgM & IgG, and hormonal Cortisol, which were measured using VIDAS, while human Cyclophilin A was measured using ELISA technique. Finally, measurement of gene expression for microRNA.

2.2.2.4 Ethical approval

Verbal consent is taken from each patient before sampling. This study was approved by the Committee of publication ethics at the College of Medicine, University of Babylon, Iraq.

2.3 Estimation of Cytomegalovirus(IgM, IgG), Cortisol Hormone, and Adrenocorticotrophic Hormone(ACTH) by VIDAS.

VIDAS (Vitek Immuno Diagnostic Assay System) is an automated immunoassay system developed by bioMérieux, a leading global in vitro diagnostics company. The VIDAS system is designed to rapidly and precisely detect various infectious diseases, including viruses, bacteria, parasites, and markers of various health conditions.

The principle of VIDAS is based on the enzyme-linked fluorescent assay (ELFA) technology, which is a varied from enzyme-linked immunosorbent assay (ELISA). The process odf VIDAS system involved:

1- Sample Preparation and incubation: The first step involves preparing the patient's sample, which could be blood, serum, plasma, urine, or other body fluids. This sample is collected and appropriately handled to ensure accurate test results, The prepared sample is introduced into the VIDAS instrument, where it undergoes an incubation step. During incubation, the target analyte (e.g., antigen or antibody) in the sample binds to specific antibodies that are immobilized onto solid-phase micro or beads

2- Washing Step: After the incubation, unbound substances are removed by washing the solid-phase micro particles, leaving only the specific antigen-antibody complexes attached to the micro particles.

3- Reaction with Enzyme-Labeled Antibodies: The VIDAS system then introduces enzyme-labeled antibodies that can recognize and bind to the target analyze. These enzyme-labeled antibodies also react with the antigen-antibody complexes immobilized on the micro particles.

4- Washing Step: After a brief incubation period, unbound enzyme-labeled antibodies are washed away to remove any non-specific binding.

5- Addition of Substrate: A substrate specific to the enzyme is added to the system. The enzyme on the micro particles catalyzes a reaction with the substrate, resulting in the production of a fluorescent signal.

6- Signal Detection and Quantification: The VIDAS instrument detects and measures the fluorescence the enzymatic reaction emits. The amount of fluorescence is directly proportional to the concentration of the target analyte in the patient's sample.

7- Interpretation of Results: Based on the fluorescence signal obtained, the VIDAS system interprets the results and provides a quantitative or qualitative result for

the target analyte. The results are displayed on the instrument's interface and are typically available quickly. Interpretation of results for CMV IgM and IgG as well as serum cortisol and plasma ACTH obtained through the VIDAS assay can differ significantly due to the distinct nature of these tests and the hormones or antibodies they measure. Here's a general overview of the interpretation for each:

A- The CMV IgG antibody test using VIDAS measures the presence and concentration of IgG antibodies against Cytomegalovirus in the patient's blood sample. The interpretation of the results of the CMV IgG antibody test is based on the fluorescence signal obtained from the sample. The test result can be categorized into three main interpretations: negative, equivocal, and positive. were as follows:

- Negative: If the test result shows a fluorescence signal below the established cutoff value (usually < 4 AU/mL), it indicates no detectable IgG antibodies against CMV. This suggests either no previous exposure to CMV or an insufficient immune response to produce detectable levels of IgG antibodies.
- Equivocal: If the test result falls within a specific range (typically ≥ 4 to < 6 AU/mL), it is considered equivocal. This means the fluorescence signal is close to the cutoff value, and the presence of CMV IgG antibodies is uncertain. In such cases, additional or repeat testing might be recommended to confirm the status.
- Positive: If the test result shows a fluorescence signal equal to or above the established cutoff value (usually ≥ 6 AU/mL), it indicates the presence of CMV IgG antibodies in the patient's blood. A positive result suggests that the individual has been exposed to CMV in the past, and their immune system has developed specific IgG antibodies against the virus

B- The CMV IgM antibody test is another important serological test used to diagnose Cytomegalovirus (CMV) infections, The interpretation of the results is as follows:

- Negative: If the test result is less than 0.70, it is considered negative, indicating no detectable CMV IgM antibodies in the patient's blood. This suggests that the individual has not recently acquired a primary CMV infection.
- Equivocal: If the test result falls within the range of ≥ 0.70 to < 0.90,

  it is considered equivocal. This means that the CMV IgM antibody levels are in a gray area, and it is not definitive whether the infection is present or not. Additional testing or repeat testing may be required to confirm the result.
- Positive: If the test result is 0.90 or higher, it is considered positive, indicating the presence of CMV IgM antibodies in the patient's blood. This suggests that the individual has a recent or active CMV infection.

C- The interpretation of serum cortisol results obtained through the VIDAS Cortisol assay is based on the reference ranges or cutoff values established by the laboratory or the manufacturer of the assay. The reference ranges may vary depending on factors such as the time of day when the sample was collected, and the population being studied

- For the morning cortisol levels, the expected range is 54.94 ng/mL to 287.56 ng/mL. Most healthy individuals' morning cortisol levels will be within this range.
- For the afternoon cortisol levels, the expected range is 24.61 ng/mL to 171.52 ng/mL, reflecting the range where most healthy individuals' afternoon cortisol levels are expected to fall.

D- The results for ACTH using the VIDAS assay. Based on the reference range for ACTH levels using the VIDAS method is: Reference Range: 6.0 - 76.0 pg/mL

This means that in a healthy population, the typical ACTH levels fall within this range. Any ACTH results below 6.0 pg/mL or above 76.0 pg/mL would be considered outside the normal range.

So, The VIDAS system's automation, speed, and precision make it a valuable tool in clinical laboratories for diagnosing infectious diseases and monitoring patient health. It is widely used worldwide and has contributed significantly to the field of in vitro diagnostics.

2.4 Estimation of Cyclophilin A by ELISA technique:

2.4.1-A Principle

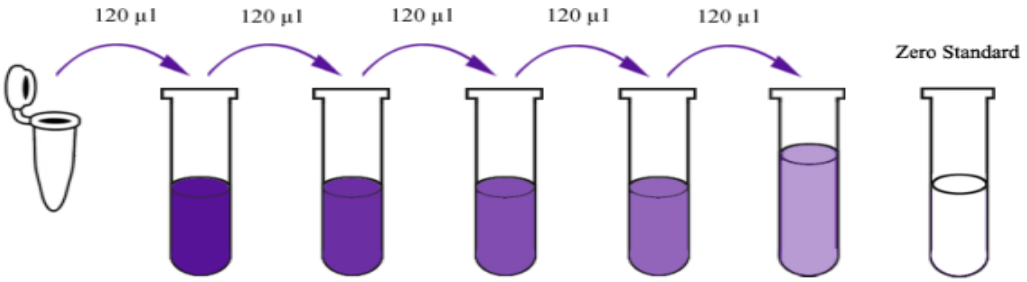
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human Cyclophilin A (CypA) antibody. CypA in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human CypA Antibody is added and binds to CypA in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated CypA antibody. After incubation, unbound Streptavidin- HRP is washed away during a washing step. Substrate solution is added and color develops proportionately to the amount of Human CypA. The reaction is terminated by adding an acidic stop solution, and absorbance is measured at 450 nm.

2.4.1-B Reagent Preparation

1. All reagents should be brought to room temperature before use.
2. Standard Reconstitute the 120µl of the standard (240ng/L) with 120µl of standard diluent to generate a 120ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate

standard points by serially diluting the standard stock solution (120ng/L) 1:2 with standard diluent to produce 60ng/L, 30ng/L, 15ng/L, and 7.5ng/L solutions. Standard diluent serves as the zero standard (0 ng/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

120ng/ml	Standard No.5	120ul Original standard + 120ul Standard diluent
60ng/ml	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
30ng/ml	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
15ng/ml	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
7.5ng/ml	Standard No.1	120ul Standard No.2 + 120ul Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
240ng/ml	120ng/ml	60ng/ml	30ng/ml	15ng/ml	7.5ng/ml

Figure (2-1) The Diluting of Stock Solution of CypA.

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionizedor distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.4.1-C Assay Procedure

1. All reagents, standard solutions, and samples are prepared as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.

2. The number of strips required for the assay was determined. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.

3. Fifty µl of standard added to the standard well. Note: Didn't add a biotinylated antibody to the standard well because the standard solution contained a biotinylated antibody.

4. Forty µl of sample to sample added wells and then added 10µl CypA antibody to sample wells, and 50µl streptavidin-HRP tosample wells and standard wells (Not blank control well). Mixed well. Covered the plate with a sealer. Incubated for 60 minutes at 37°C.

5. The sealer was removed, and washed the plate 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted each well and washed it 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.

6. Fifty µl of substrate solution A was added to each well, and then added 50µl substrate solution B to each well. The incubated plate was covered with a new sealer for 10 minutes at 37°C in the dark.

7. 50µl of Stop Solution was added to each well, and the blue color changed into yellow immediately.

8. The optical density (OD value) was determined for each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.4.1-D Standard Curve

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best-fit curve was drawn through the points on the graph as shown in figure (2-2).

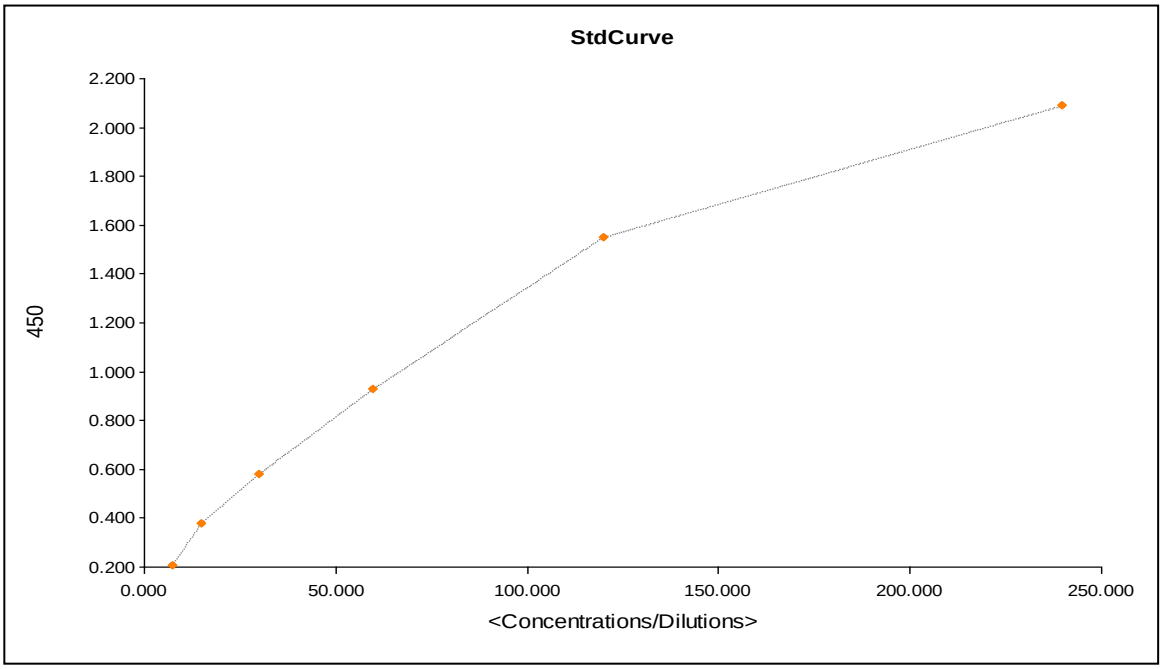


Figure (2-2) Standard curve for serum CypA contrition

2.5 Extraction of Genomic DNA from Blood Sample

The process utilized the Genomic DNA Purification Kit provided by the manufacturer, Bioneer from Korea. Genomic DNA was extracted from whole blood using the Geneaid Genomic DNA Purification Kit from the UK and followed the instructions provided by the company.

Step1:

The first step involves gently mixing the blood sample collected in an EDTA tube to prevent clotting. This is important as clotting can interfere with subsequent steps of the DNA extraction process. Once the blood sample is thoroughly mixed, 300 µl of blood is transferred to a 1.5 ml micro centrifuge tube.

Step2:

In this step, 20 µl of Proteinase K is added to the micro centrifuge tube containing the blood sample. It is crucial to ensure that ddH_2O (double-distilled water) is added to Proteinase K before mixing. After adding Proteinase K, the tube is vortexed for 10 seconds to ensure proper mixing. The tube is then incubated at 60ºC for at least 10 minutes. During the incubation, it is recommended to invert the tube every 3 minutes. Proteinase K helps in breaking down proteins present in the blood sample, facilitating DNA extraction.

Step 3:

In this step, 200 µl of GB Buffer is added to the micro centrifuge tube containing the blood sample and Proteinase K mixture. Similar to previous steps, vortexing for 10 seconds ensures proper mixing of GB Buffer with the sample. The tube is then incubated at 70ºC for at least 10 minutes. Inverting the tube every 3 minutes during incubation helps maintain a clear sample lysate. It is also mentioned that at this time, Elution Buffer (200 µl per sample) should be pre-heated to 70ºC for later use in DNA elution ,for using step (7) DNA Elution.

Step 4:

Two hundred microliter of absolute ethanol was added and mixed immediately by shaking vigorously. If a precipitate appears, break it up as much as possible with a pipette then I placed the GD Column in a 2 ml Collection tube and

transferred the mixture (including any insoluble precipitate) to the GD Column and centrifuge at 14-16,000 x g for 2 min. Discarded the 2 ml Collection tube containing the flow-through and then placed the GD Column in a new 2 ml .

Step 5:

Four hundred microliter of W1 Buffer was added to the GD Column and Centrifuge at 14-16,000 x g for 30 seconds; discarded the flow-through then placed the GD Column back in the 2 ml Collection tube.

Step 6:

Six hundred microliter of Wash Buffer (with ethanol) was added to the GD Column and then centrifuged at 14-16,000 x g for 30 seconds, discarded the flow-through and placed in the GD Column was back in the 2 ml Collection tube, and centrifuged again for 3 min. at 14-16,000 x g to dry the column matrix.

Step 7:

The dried GD Column was transferred to a clean 1.5 ml microcentrifuge tube, and added 100 µl of pre-heated Elution Buffer was placed into the center of the column matrix then I waited to stand for at least 3 min. to allow Elution Buffer to be completely absorbed and centrifuged at 14-16,000 x g for 30 seconds to elute the purified DNA.

Step 8

The DNA was kept frozen at -20°C (Hatada *et al*., 1991).

2.6 Genotyping assay

2.6.1*PPIA*-SNP selection

The studied *PPIA*-Single Nucleotide Polymorphisms (SNPs) were chosen according to *PPIA*-SNPedia as the highest frequency Two SNPs in the *PPIA gene* (*PPIA* Allele*Frequencies in World Populations Database search). Table (2-10) illustrates the *PPIA*-SNPs chosen.

Table (2-10): *PPIA*- Single-Nucleotide Polymorphisms (SNPs) chosen

Site of SNP	SNP	Frequency
chr7:44795554	rs4720485	0.357
chr7:44796669	rs8177826	0.020

2.6.2 Restriction enzyme selection

To restrict the rs4720485 SNP, the TaqI enzyme can be used. TaqI is a type II restriction enzyme that recognizes and cleaves DNA at specific sequences. It is commonly used in molecular biology research and genetic analysis. The properties of the accepted primer pair for the rs4720485 SNP can be found in Table (2-12). These properties include the sequence of the forward and reverse primers, their melting temperatures, lengths, and any additional modifications or considerations.

The details for the TaqI enzyme can be found in Table (2-11). This table provides information about the recognition sequence of TaqI, which is 5'-TCGA-3'. It also includes details about the optimal reaction conditions for using TaqI, such as the recommended buffer, temperature, and incubation time

Table (2-11) details for restriction enzyme TaqI

Restriction enzyme	**TaqI**
Recognition sequence	5..T^CGA..3 3..AGC^T..5
Source	Thermus aquatics
Optimal assay temperature	65
Incubation period	Overnight

Table (2-12): The accepted primer pair for rs4720485 and their properties.

Primer	Sequence(5'->3')	Template strand	Length	Tm	GC%	Self com ple men tarity	Self 3' comp lemen tarity	Reference
Forward Primer	CGGCTGGAATGCAGTGAT	Plus	18	57.77	55.56	5.00	3.00	An *etal*., 2007
Reverse Primer	ACGCTTCATCTTAGGAGTACGAC	Minus	23	59.93	47.83	4.00	2.00	
Product Length	**199**							

The amplicon was cut with **TaqI** restriction enzyme to produce 41bp,55bp, and 103 bp fragments for the T allele and 41 and 158 bp fragments for the A allele, as shown in appendix 1.

The enzyme HaeIII is commonly used for the restriction of DNA sequences, including single nucleotide polymorphisms (SNPs). In the case of the rs8177826 SNP, the properties of the accepted primer pair and details for the restriction enzyme HaeIII are provided in Table (2-13) and Table (2-14) respectively

Table (2-13): The accepted primer pair for rs8177826 and their properties

Primer	Sequence(5'->3')	Templatestrand	Length	Tm	GC%	Self complementarity	3' complementarity	Reference
Forward primer	AAGTCGCAGACCCGATTG	Plus	18	57.40	55.56	4.00	2.00	An *et al*., 2007)
Reverse primer	ACTTTCTGGGCCCCATTC	Minus	18	56.80	55.56	7.00	1.00	
Product length	**250**							

Table(2-14) details for restriction enzyme HaeIII

Restriction enzyme	HaeIII
Recognition sequence	5..GG^CC..3 3..CC^GG..5
Source	Haemophilus aegyptius
Optimal assay temperature	37
Incubation period	Two hours

The amplicon was cut with **HaeIII** restriction enzyme to produce 36bp, 47bp,19bp,28bp, and110 bp fragments for the C allele and 36bp,47bp,19bp, and 138 bp fragments for the G allele as shown in appendix 2

2.6.3 Reconstituting and dilution of primers

The nuclease-free H_2O was added to each primer to obtain a master stock that would be used again to obtain a working stock. The following steps were followed for reconstituting and diluting the primers :

1- The tubes were spin down before opening the caps.

2- The desired amount of free nuclease water was added according to the guidelines of manufacturer to obtain a 100 Pico moles/µl (Master Stock).

3- The tubes was vortex properly for re-suspend the primers evenly.

4- A volume of 10µl of the each primers stock was transferred to a 1.5 ml Eppendorf tube that contains 180µl of sterile, nuclease-free water (Working Stock).

5- The master stock was stored at-20

6- The working stock was store at-20

7- The working stock was thawed on ice and vortexed before using in PCR and then stored at -20 °C

2.7 Convential Polymerase chain reaction (PCR)

The PCR amplicon that's required for rs4720485 and rs8177826 in the current study was amplified by PCR amplification performed in a programmable thermal cycler gradient PCR system. The amplification of each target region was first optimized by gradient PCR and the best efficient and specific annealing temperature that produce the most efficient, specific product was chosen for further PCR amplification procedure, the latter was performed according to the addition of the components for amplification of each SNP as in table (2-15)

Table (2-15): The starting PCR reaction ingredients concentration for each optimization process.

No	Ingredient	Concentration	Added volume
1	Master mix	2.5 x	8 µl
2	DNA	20 ng / µl	2 µl
3	Primers	10 pmol / µl	1 µl for each
4	Molecular grad water		7.5 µl
Total volume			**20** µl

2.7.1 Optimization of PCR Conditions

Optimization of PCR was done by several attempts to detect the best annealing temperature and the best number of amplification cycles.

The components of PCR for all the amplified fragments and the optimized PCR programs are stated in Table (2-16),(2-17) and Table (2-18) respectively.

Table (2-16): Optimized thermo cycle PCR Condition for genotyping of rs4720485 , and rs8177826

No	Stage	Temperature	Time	Number of cycles
1	Initial denaturation	94 ºC	5 min.	1
2	Denaturation	94 ºC	30 sec.	35
3	Annealing	Gradient ± 6ºC of the lowest primer Tm	30 sec	
4	Elongation	72 ºC	30 sec	
5	Final elongation	72 ºC	5 min.	1

After optimization, the best thermo-cycling conditions for rs4720485 and rs8177826 SNPs were listed in Table (2-17) and Table (2-18) these conditions produce the most specific and sufficient PCR product. Then the amplified products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide, as shown in (appendix 3) and (appendix 4) .

Table (2-17): Optimized thermo-cycling final condition for rs4720485 PCR-RFLP genotyping.

No	Stage	Temperature	Time	Number of cycles
1	Initial denaturation	94 ºC	5 min.	1
2	Denaturation	94 ºC	30 sec.	35
3	Annealing	66ºC	30 sec	
4	Elongation	72 ºC	30 sec	
5	Final elongation	72ºC	5min.	1

Table (2-18): Optimized thermo-cycling final condition for rs8177826 PCR-RFLP genotyping.

No	Stage	Temperature	Time	Number of cycles
1	Initial denaturation	94 ºC	5 min.	1
2	Denaturation	94 ºC	30 sec.	35
3	Annealing	63ºC	30 sec	
4	Elongation	72 ºC	30 sec	
5	Final elongation	72ºC	min.	1

2.8 Agarose Gel Electrophoresis

This process was carried out according to (Lee *and* Bahaman, 2012).

Principle:

Agarose gel electrophoresis is a method of gel electrophoresis used in many scientific fields to separate macromolecules such as DNA fragments in a matrix of 1-2% dissolved agarose in TBE buffer. The DNA fragments such as PCR products were separated by applying an electric field to move the negatively charged DNA molecules through an agarose matrix from negative pole (cathode) to the positive pole (anode) and the DNA fragments were separated by size in the agarose gel matrix. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease.

Methods:

1. Agarose gel was prepared by dissolving 2 gm of agarose powder in 100 ml of (1X) TBE buffer in microwave or water bath.
2. Allowed the agarose to cool to 50°C then ethidium bromide (EtBr) was added to a concentration of 0.5 µg/ml and mixed, be careful to add EtBr in over than 50°C because the evaporation of EtBr may be carcinogenic when inhalation.
3. The comb was fixed at one end of the tray for making wells that were used for loading DNA sample then the agarose was powered gently into the tray, and allowed to solidify at room temperature. The comb was then removed gently from the tray.
4. The tray was fixed in an electrophoresis chamber which was filled with (1X) TBE buffer covering the surface of the gel, 5µl of DNA sample of PCR products was transferred gently by micropipette into the signed wells in agarose gel.
5. A volume of 5µl DNA ladder (50-1500 bp) was added to one well in order to estimated measure the size of DNA bands of PCR products.
6. The electric current was applied at 80-100 volt for 20-35 min.
7. The band was observed by a UV light box (UV transilluminator) at a wavelength of 320 nanometers and photographed with an electronic camera system.
8. The PCR product of study samples that give the same band (according to base pair size) that required in the present study was sent for detect DNA sequence.

2.9 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Technique

2.9.1 Restriction Digestion PCR-RFLP for TaqI

Restriction digestion for PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) using TaqI is commonly used in molecular biology to analyze genetic variations. This method involves the use of a restriction enzyme, TaqI, to cleave DNA at specific recognition sites, resulting in different fragment lengths that can be visualized and analyzed.

To perform the restriction digestion for PCR-RFLP using TaqI, the following steps are followed:

1. Prepare the reaction mixture:

- Take four microliters (ul) of the PCR product that needs to be digested.

- Add 1.5 ul of the selected restriction enzyme, in this case, TaqI.

- Add 1.5 ul of the appropriate restriction buffer for TaqI, supplied by the manufacturer.

- Include 0.15 ul of bovine serum albumin (BSA). BSA helps to stabilize the reaction and enhance enzyme activity.

- Complete the reaction mixture to a final volume of 15 ul by adding molecular-grade water.

2.Preventev aporation:

- To prevent evaporation during incubation, add 0.1 ul of mineral oil on top of the reaction mixture.

3.Incubation:

- Place the reaction mixture in a 65°C water bath for overnight incubation. The incubation time may vary depending on the specific protocol or experiment requirements.

During the incubation period, TaqI recognizes and binds to its specific recognition sequence in the DNA molecule. It then cleaves the DNA at these sites, generating fragments with different lengths based on the presence or absence of TaqI recognition sites within the target DNA sequence. After completing the restriction digestion, the resulting DNA fragments can be separated and visualized using gel electrophoresis. The fragments will migrate through an agarose gel based on their size, allowing for the identification of different genotypes or genetic variations

2.9.2 Restriction Digestion for PCR-RFLP for HaeIII

PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) is a technique used to detect genetic variations by digesting PCR products with specific restriction enzymes, which recognize and cleave specific DNA sequences. The resulting fragment lengths after digestion can be used to identify genetic variations.

In this case, the PCR-RFLP experiment uses the restriction enzyme HaeIII to digest the PCR product. Here's a breakdown of the steps:

Mix the following components in a tube:

5 µl of the PCR product (amplified DNA obtained through PCR).

0.5 µl of the restriction enzyme HaeIII.

1.5 µl of the restriction buffer (specific to the HaeIII enzyme, provided by the manufacturer).

Complete the reaction mixture to a total volume of 15 µl by adding about 8 µl of molecular-grade water. This step is necessary to ensure that the final reaction volume is 15 µl, as the total volume of the previous components was 7 µl (5 µl + 0.5 µl + 1.5 µl).

Incubate the reaction mixture in a 37°C water bath for two hours. During this incubation period, the restriction enzyme HaeIII will cleave the DNA at its specific recognition sites.

After the two-hour incubation, the reaction should result in the DNA being cut into fragments of various lengths, depending on the presence or absence of specific HaeIII recognition sites in the PCR product. These fragment patterns can be visualized through gel electrophoresis, allowing to identify genetic variations based on the unique fragment lengths produced by different genotypes.

2.9.3 Agarose Gel Electrophoresis for PCR-RFLP

This process was carried out according to (Lee *and* Bahaman, 2012)

Methods:

1- Agarose gel was prepared by dissolving 2 gm of agarose powder in 30 ml of (0.5X) TBE buffer in microwave or water bath.

2-Allowed the agarose to cool to 50°C then ethidium bromide (EtBr) was added to a concentration of 0.5 µg/ml and mixed, be careful to add EtBr in over than 50°C because the evaporation of EtBr may be carcinogenic when inhalation.

3-The comb was fixed at one end of the tray for making wells that were used for loading samples then the agarose was powered gently into the tray, and allowed to solidify at room temperature. The comb was then removed gently from the tray.

4-The tray was fixed in an electrophoresis chamber which was filled with (0.5X) TBE buffer covering the surface of the gel, 10µl from PCR product (after final incubation samples in restriction enzyme) were transferred gently by micropipette into the signed wells in agarose gel.

5-A volume of 5µl DNA ladder was added to one well to estimate and measure the size of DNA bands of PCR products.

The electric current was applied at 80-100 volt for 20-35 min. The band was observed by a UV light box (UV transilluminator) at a wavelength of 320 nanometers and photographed with an electronic camera system, allowing to identify genetic variations based on the unique fragment lengths produced by different genotypes

2.10 Molecular study

2.10.1 RNA Isolation

RNA purification is a crucial step in molecular biology research and various techniques have been developed to isolate high-quality RNA from biological samples. One commonly used method is the Accuzol Reagent protocol, which involves several steps to extract RNA from a sample.

First step (Sample Lysis)

The first step in the Accuzol Reagent protocol is sample lysis. This step adds a volume of AccuZolTM reagent to the sample suspension. The ratio of AccuZolTM reagent to sample is 3:1 (750 µl of AccuZolTM for every 250 µl of sample). The cells in the sample are then lysed by passing the suspension several times through a pipette or vortexing. This lysis step disrupts the cell membranes and releases the cellular components, including RNA.

The second step (Three phases of separation)

After sample lysis , the next step is three-phase separation. The steps involved in this process are as follows:

1. Add 200ul of chloroform per 1ml of AccuZolTM: In this step, chloroform is added to the AccuZolTM reagent in a specific ratio. The purpose of chloroform is to facilitate the separation of different phases during centrifugation.

2. Shake vigorously for 15 seconds: After adding chloroform, the mixture is shaken vigorously for a short duration. This shaking helps in achieving a thorough mixing of the components.

3. Incubate the mixture on ice for 5 minutes: Following the shaking step, the mixture is incubated on ice for a specific period. Cooling the mixture helps in stabilizing the RNA and preventing its degradation.

4. Centrifuge at 12,000 rpm for 15 minutes at 4℃: After the incubation step, the mixture is subjected to centrifugation at specific conditions. Centrifugation separates the components based on their density, with heavier components settling at the bottom.

5. Separation into lower organic phase, interphase, and upper aqueous phase: As a result of centrifugation, the mixture separates into three distinct phases. The lower phase, often green in color due to the presence of chloroform, contains organic compounds such as proteins and lipids. The interphase lies between the lower organic phase and upper aqueous phase and contains some residual contaminants. The upper aqueous phase is colorless and contains RNA.

6. Transfer the aqueous phase to a new 1.5ml tube: In this final step, the RNA-containing aqueous phase is carefully transferred to a new tube while avoiding any contamination from the other phases. This isolated aqueous phase can then be further processed for RNA analysis or storage.

The third step (RNA precipitation)

- RNA precipitation is the addition of an equal volume of isopropyl alcohol to the RNA sample. Isopropyl alcohol is commonly used in this process because it causes the RNA molecules to precipitate out of the solution. The addition of isopropyl alcohol disrupts the hydrogen bonding between water molecules and RNA, forming a precipitate.
- After adding isopropyl alcohol, the tube is inverted 4-5 times to ensure thorough mixing. This step helps to ensure that all RNA molecules come into contact with the isopropyl alcohol, promoting efficient precipitation.

- Following mixing, the tube is incubated at -20°C for 10 minutes. Incubation at this low temperature helps to stabilize the RNA molecules and facilitates their precipitation.
- After incubation, the tube is centrifuged at high speed (12,000 rpm) for 10 minutes at 4°C. Centrifugation causes the heavier RNA molecules to sediment at the bottom of the tube, forming a pellet. The supernatant, which contains other biomolecules and impurities, can be carefully removed without disturbing the pellet.

fourth Step (RNA washing)

- The next step in RNA precipitation involves washing the RNA pellet with ethanol. Ethanol is commonly used as a washing agent because it helps to remove residual contaminants and impurities. To wash the pellet, 1 ml of 80% ethanol is added to the tube and the mixture is mixed well by inverting or vortexing.
- The tube is centrifuged at 12,000 rpm for 5 minutes at 4°C. Centrifugation helps to remove the ethanol and any remaining impurities from the RNA pellet.
- After centrifugation, the supernatant is carefully removed, leaving behind the RNA pellet. The pellet is then air-dried to remove any traces of ethanol. Air-drying can be achieved by leaving the tube open in a laminar flow hood or using a vacuum concentrator. It is important to ensure complete drying of the pellet to prevent contamination and degradation of the RNA.

Fifth step (RNA solubility)

- The final step in RNA precipitation is the solubilization of the dried RNA pellet. Two common methods are dissolving RNA in RNase-free water or passing the solution through a pipette tip.

- The first method dissolves the RNA pellet in 50 µl of RNase-free water. The water should be free from any ribonucleases (enzymes that degrade RNA) to prevent degradation of the isolated RNA. Alternatively, passing the solution through a pipette tip multiple times can help break up the pellet and facilitate its dissolution.
- After dissolution, incubating the solution at 55 to 60℃ for 10 minutes can further aid in solubilization.

2.10.2 cDNA synthesis

cDNA (complementary DNA) synthesis is a crucial step in molecular biology research that involves the conversion of RNA (ribonucleic acid) into cDNA using reverse transcription the sequence primer is used in this experiment as shown in the table**(**2-19). This process allows for amplifying and analyzing specific RNA molecules, providing valuable insights into gene expression and regulation. The protocol you provided outlines the steps involved in performing cDNA synthesis.

1. First, 18 ul of RNA is added to 1 ul of RT primer in a tube containing the Rocket Script premix Eppendorf from Bioneer. The RT primer is a short oligonucleotide that anneals to the RNA template and serves as a starting point for reverse transcription.

2. The mixture is then mixed using an exispin or vortex to ensure proper mixing of the components. After mixing, the tube is placed in a PCR machine for thermal cycling.

The thermal cycling program consists of three temperature steps:
a. 37°C for 5 minutes: This step allows for the denaturation of secondary structures in the RNA and promotes the binding of the RT primer to the RNA template.

b. 42°C for 1 hour: This step facilitates the reverse transcription reaction, where the enzyme reverse transcriptase synthesizes cDNA using the RNA template and RT primer as a starting point. The extended incubation time at this temperature ensures efficient conversion of RNA into cDNA.

c. 95°C for 5 minutes: This final step inactivates the reverse transcriptase enzyme and denatures any remaining RNA molecules, ensuring that only cDNA remains for downstream applications.

2.10.3 The qPCR (quantitative polymerase chain reaction)

The protocol provided involves amplifying and quantifying a specific DNA sequence using a fluorescent dye called Sybr Green. The protocol consists of several steps: primer addition, cDNA addition, mixing, and running the qPCR program.

1.PrimerAddition:

In the first step, one µl of forward primer and one µl of universal reverse primer are added to each well of the qPCR plate. Primers are short DNA sequences complementary to the target DNA region and essential for initiating DNA amplification during qPCR.

2.cDNA Addition

Next, five µl of cDNA (complementary DNA) is added to each well of the qPCR plate containing Sybr Green. cDNA is synthesized from RNA using reverse transcription and serves as the template for DNA amplification in qPCR.

3. Mixing and Loading:

After adding the primers and cDNA, the contents of each well are mixed using a vortex or similar method to ensure proper dispersion. Once mixed, the plate is loaded into the qPCR device.

4. qPCR Program:

The qPCR program has several temperature steps that facilitate

DNA denaturation, primer annealing, and DNA synthesis. The program outlined in the provided protocol is as follows:

a. Initial Denaturation:

The samples are incubated at 95°C for 5 minutes to denature the double-stranded DNA into single strands, allowing subsequent amplification.

b. Denaturation:

The temperature is raised to 95°C for a short duration (typically 5 seconds) to separate the DNA strands.

c. Annealing:

The temperature is lowered to 55°C for a short duration (typically 5 seconds) to allow the primers to bind specifically to their complementary sequences on the single-stranded DNA template.

d. Extension and Fluorescence Detection:

The temperature is raised to a level suitable for DNA synthesis (usually 72°C) to allow the DNA polymerase enzyme to extend the primers and synthesize new DNA strands. During this step, the Sybr Green dye intercalates into the newly synthesized DNA, resulting in fluorescence.

e. Cycling:

Steps b-d are repeated for a specific number of cycles (40 cycles in this protocol) to amplify the target DNA exponentially. Each cycle doubles the amount of DNA present, resulting in a significant increase in fluorescence.

f. Final Extension:

After the cycling, a final extension step is performed at 72°C for 1 minute to ensure that any remaining incomplete DNA strands are fully extended.

5. Cooling:

Finally, the temperature is lowered to 25°C for 1 minute to allow the samples to cool down before further analysis or storage.

Table (2-19) the primer sequence in real-time -PCR

Primer Name	Seq.	**Reference**
mir-US25-1-5p-RT	5`- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGGTCCG-3`	Designed by this study
mir-US25-1-5p-F2	5`- GGTTTTTTGCCTCCGGATCACATGGT-3`	
GAPDH RT	5`-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAATCAG-3`	
GAPDH F	5`-GTGAACTTATTGACGGGCG-3`	
Universal Reverse	5`-GTGCAGGGTCCGAGGT-3`	
miR-UL112-5p-RT	5`- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTGAGTA-3`	
miR-UL112-5p-F2	5`- GGTTTTTTGCCTCCGGATCACATGGT-3`	

2.11 Statistical Analysis

All statistical calculations were performed using SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010). Microsoft Corp. USA).

A $P < 0.05$ was considered statistically significant. Chi-square test to assess the categorially association variables and genetic association, according to (Solé *et al*., 2006).

Allele frequencies of genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium

was estimated using the H-W calculator for two alleles, which is available free online at:

http://www.had2know.com/academics/hardy-weinbergequilibriumcalculator-3-ale;es.html.

Hardy-Weinberg equilibrium is the expected frequencies of genotypesif mating is non –assortative and there are no mutations from one allele to another. When there are two alleles for a particular gene; A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB, BB are p2, 2pq and q2, respectively. Significant differences between the observed and expected frequencies are assessed by Pearson‟s Chi-square test it (Graffelman *et al*., 2017).

Expression (ΔCT) ΔCT represents the difference in the threshold cycle (CT) value of the target gene (miR-US25-5p and miR-UL112-5p) and the reference gene (housekeeping gene). It measures the initial level of gene expression in each group. ΔCT=Ct(target) – Ct(housekeeping)

ΔΔCT is the difference in the ΔCT values between the Control and Patient groups. It indicates the change in gene expression between the two groups, relative to the reference gene. ΔΔCT= ΔCT - mean ΔCT(control)

Fold change represents the ratio of gene expression between the Patients group and the Control group. It indicates how many times the gene expression is increased (fold change > 1) or decreased (fold change < 1) in the Patients group compared to the Control group. fold change=$2^{-\Delta\Delta CT}$

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 The prevalence of CMV among women with miscarriage.

During the study period, 200 blood samples were collected from women who had miscarriages, the mean age (28) years ranging from (16-40) years old, and attended the Maternity and Children s Hospital in Hilla governorate over six months (from February 2022 to August 2022). This study revealed that out of 200 patients, 60 were seropositive for CMV, while 140 had were seronegative for CMV. As shown in Figure (3-1).

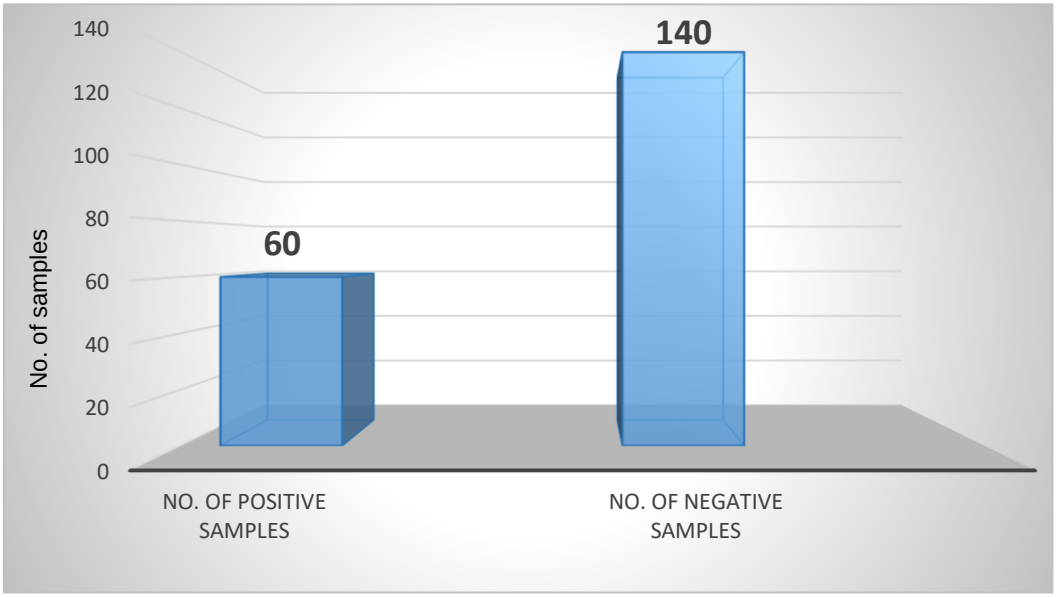


Figure (3-1): The prevalence of CMV among miscarriage women patients

In this study, the prevalence of Human Cytomegalovirus (HCMV) infection was positive in 60 (30 %), This result is different to that reported in other studies, such as those observed by Khudhair and Al-Azzawi,(2018) reported that the prevalence rate of CMV infection was (32.78%) among study population, also two other studies performed in Al- Iraq were revealed high rate of CMV infection among women reach to (60.63% & 56%) respectively (Yasir *et al.*,2020; shaker *et al* ., 2023).

While Al-Dorri,(2018) analyzed miscarriage women who visited Tikrit Teaching Hospital in Salah Al-Deen governorate, the author discovered that 16.40% of miscarriage women presented with seropositive CMV, which is lower than that reported in this study. A study performed in other developing countries reported a higher rate of CMV infection in comparison to this study, such as in Australia, the prevalence rate was (56.9%)and in France (46.8%) (Picone O *et al.*,2009).

The serological diagnosis for CMV seropositive patients found that 6 (10%) samples were seropositive to IgM, 14 (23.3%) samples were seropositive for both IgM & IgG, and 40 (66.7%) samples were seropositive for IgG, as shown in figure (3-2).

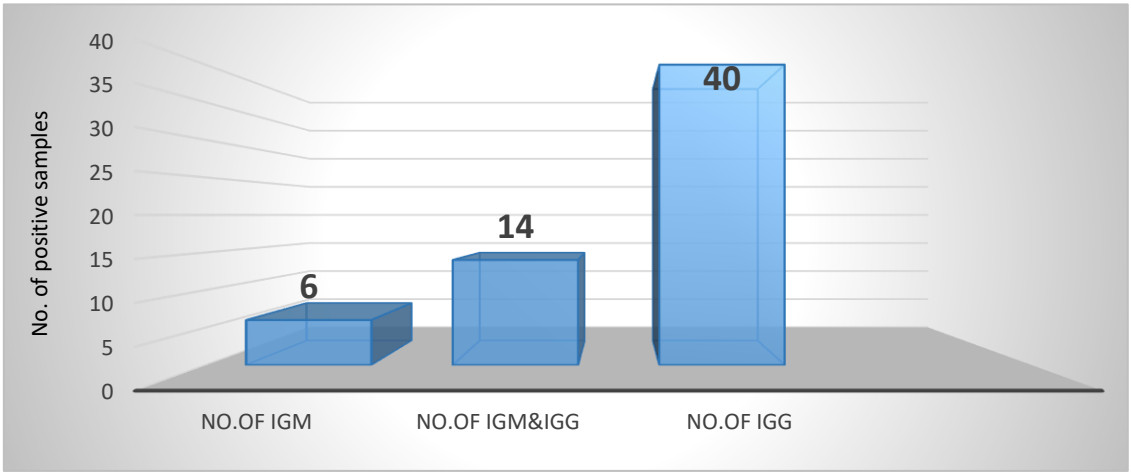


Figure (3-2): serological analysis of seropositive patients with CMV.

Several studies conducted in different governorates about CMV infection among miscarriage women patients, Al-Baiati *et al.,(*2014) revealed that the rate of IgM was (10%) and IgG was (85%) among 152 miscarriage women with CMV infection at the Al-Yarmouk Teaching Hospital for infertility. Naame *et al.*, (2021) found that the rate of IgM was (2.5%) and IgG was (30.8%) among miscarriage women attending the Basra Hospital in Basra City. Saeed *et al.,(*2022) reported that among one hundred pregnant women knowing with a

history of miscarriage, IgM was (32.6%) and IgG was (61.3%) for CMV infection, At the same time, they found that some patients have both IgM and IgG for CMV (19.3%).

Cytomegalovirus can be transmitted through bodily fluids, transplacental transmission during pregnancy, or sexually from an infected partner to a pregnant woman. Direct contact with infected fluids and exposure to young children or healthcare settings increase transmission risk (Voordouw *et al.*,(2019). The Human Cytomegalovirus is a significant cause of miscarriage, stillbirth, premature delivery, and congenital malformation. The severity of fetal infection is particularly high during the first and second trimesters. Congenital defects are less likely to occur if the infection occurs after the 20th week of gestation. This may be because the first trimester is a critical period for fetal development, and any risk factors, such as the reactivation of latent infections like CMV, can threaten the fetus. (Pereira *et al.*, 2017;Uenaka *et al.*, 2019).

The variation in the concertation of immunoglobulins for the CMV virus in the sample of patients under study may be due to the difference in the stage of infection, as the CMV IgM antibody titers become apparent between 0 and 3 weeks . This suggests that the timing of CMV infection may play a role in the development of immunity to the virus.

According to a report by Plotogea M *et al.*, (2022), acute infections are more likely to be passed on to the fetus and can result in more severe harm than recurrent infections, This is because there is a latency period after the initial infection, and the virus can reactivate, causing recurring symptoms and transmission of CMV in women.

The severity of virus infections varies across regions due to various factors, including healthy habits, cultural differences in dietary habits, levels of

education, primary healthcare programs, and early detection of infections (Kakayi *et al.*, 2021).

Some Gynaecologists do not depend on the presence of high levels of CMV IgG without high levels of IgM and may present cross-reaction with other diseases when IgG levels are high.

3.2 Cyclophilin A levels in miscarriage women with CMV infection

The level of Cyclophilin A has been quantifying using ELISA method for women who experienced miscarriages and CMV infection, the result revealed that mean value for Cyclophilin A in the group with CMV infection and miscarriage experience was 34.337, indicating an elevated protein level. On the other hand, the control group had lower levels of Cyclophilin A than women patient at mean value 16.33. As shown in Figure (3-3).

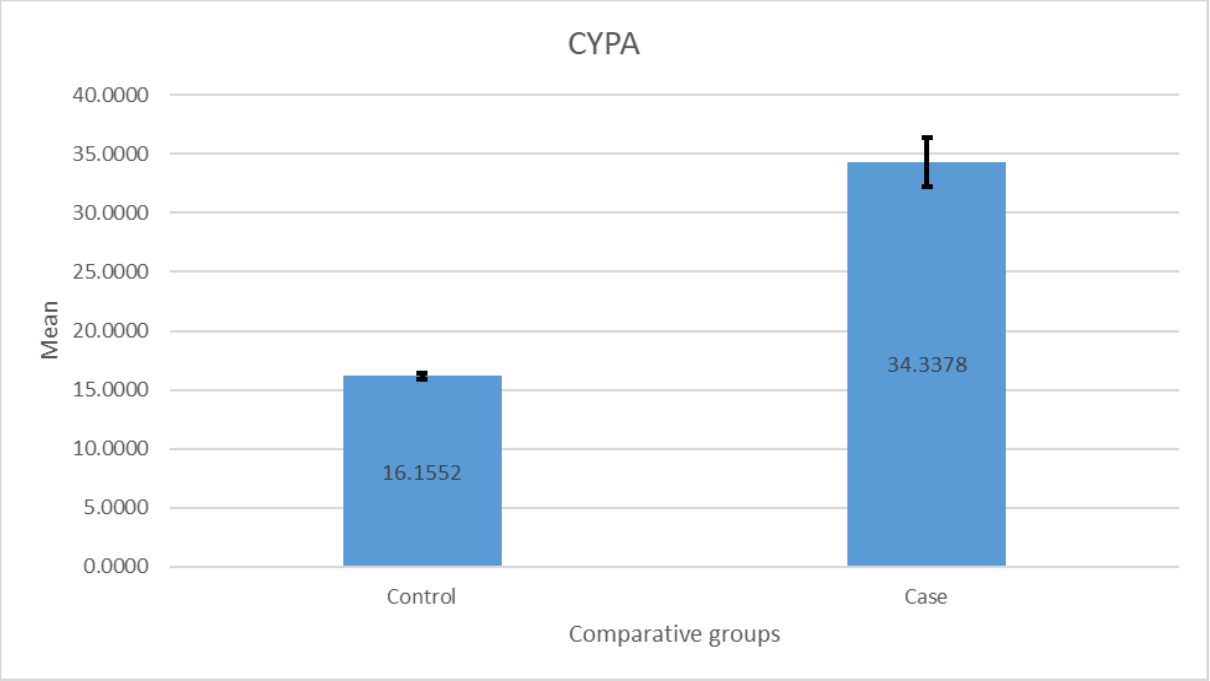


Figure (3-3) mean value of Cyclophilin A in miscarriage women patients with CMV and control group.

Numerous studies have explored the connection between CMV infection and CypA. Among these studies Keyes *et al*.,2012 and Xiao *et al.*,2016 identified a potential role for CyPA in the life cycle of HCMV. The study has shown that inhibiting or depleting CyPA can compromise HCMV replication. CypA is crucial for the efficient replication of CMV. Reducing CypA levels can delay or even inhibit the reactivation process. Using siRNA to silence CypA has significantly reduced viral production by delaying the expression of IE proteins and lowering viral DNA loads and titers. CypA primarily expresses IE proteins and facilitates virus reactivation, making it a promising target for antiviral therapies against HCMV.

Elevated CypA levels in these cases could indicate a potential CMV infection and an increased risk of associated complications like miscarriage, this could be especially valuable for predicting CMV infections in cases where the infections might otherwise go unnoticed due to the absence of symptoms. increased CypA levels could provide a signal for further investigation and preventive measure.

The study done by (Abdulla *et al* .,2018) show that CMV infection induces oxidative stress, leading to increased CyPA production then contributes to the activation of the some pathways. These pathways are linked to viral replication, particularly through the expression of IE genes. The involvement of CMV's UL4 promoter in pathway activation further highlights the virus's manipulation of host cell processes. Additionally, the roles of IE1 and IE2 proteins in controlling gene activation factors underscore their significance in promoting CMV's replication cycle and its responses to antiviral treatments. This insight provides a glimpse into the complex network of interactions that CMV exploits to ensure its successful replication within host cells

Also Kalinina *et al.,(*2023) investigate the Cyclophilin A is identified as an adverse pro-inflammatory factor that negatively influences fetal development and is associated with complications during pregnancy, Overexpression of CypA in

fetal tissues results in the death of all transgenic fetuses and complete miscarriage. This underscores the critical role of CypA in determining the viability of developing fetuses. The research suggests using secreted CypA as a novel marker for identifying complicated pregnancies. Furthermore, secreted CypA could be a potential therapeutic target for addressing pregnancy complications associated with its effects.

3.3 The relationship between the level of cyclophilin A and CMV immunoglobins levels(IgM&IgG)

In this study, the patients have been categorized into three groups according to their CMV immunoglobulin results and compared the level of cyclophilin A among these groups, the results reveal that patients with a high titer of CMV IgM antibodies and no titer of CMV IgG antibodies exhibited the highest mean level of Cyclophilin A. This suggests that the presence of CMV IgM antibodies alone is associated with a significant increase in Cyclophilin A concentration (mean±SD=64±4.76), women patients had high levels of both CMV IgM and IgG antibodies. The mean level of Cyclophilin A was lower than the IgM-only group but higher than the IgG-only group. This indicates that the presence of both CMV IgM and IgG antibodies still leads to an elevation in Cyclophilin A concentration, although it might be lower compared to the IgM-only group(mean±SD=48.15±16.72), women patients who had high levels of CMV IgG antibodies but lacked significant levels of CMV IgM antibodies, displayed the lowest mean level of Cyclophilin A. This suggests that the absence of CMV IgM antibodies is associated with decreased levels of Cyclophilin A(mean±SD=25.004±2.34). As shown in the table (3-1)

Table (3-1) Cyclophilin A levels according to the type of immunoglobulin's

Immunoglobulin Types	Patients(60)	No.
	Cyclophillin A levels (mean±SD)	
IgM	64 ± 4.76	**6**
IgG	25.004 ± 2.34	**40**
IgM + IgG	48.15 ± 16.72	**14**

These findings suggest that Cyclophilin A levels may be influenced by the presence or absence of CMV IgM antibodies. The increase in CypA concentration in the presence of CMV IgM could indicate a potential role of CypA in the early immune response to CMV infection or an active ongoing infection. Conversely, the lower CypA levels without CMV IgM might reflect a different stage of infection or a reduced immune response.

So, The level of CypA can be influenced by CMV infection. However, it is important to note that the exact impact of CMV on CypA levels may vary depending on several factors, including the stage of infection, the individual's immune status, and the specific experimental conditions.(Liao *et al* .,2021)

Cyclophilin A is known to play roles in both immune regulation and inflammatory processes. Its increased production could be a part of the immune system's attempt to control the infection, modulate immune responses, or even influence viral replication. Therefore, the presence of CMV IgM antibodies, which indicates an ongoing immune reaction, could contribute to the elevated

levels of Cyclophilin A as a component of the broader immune and inflammatory response against CMV.(Bulak *et al*.,2020)

3.4. Genotyping result

3.4.1. *PPIA* gene polymorphism at locus rs4720485 T/A

By using PCR and gel electrophoresis the result of rs4720485 T/A polymorphism in the Alu repeat region of the *PPIA* gene was showed that the gene product was located in 199 bp as show in figure(3-4). After that the PCR product was represented to RFLP technique and the product was digested with TaqI enzyme.

Depending on the genotype of the individual, the restriction enzyme will cut the DNA at specific sites, producing fragments of varying lengths that can be visualized on a gel. Homozygous genotype (TT) enzyme cuts at both TCGA sites, producing three fragments: (41bp, 55bp, and 103bp) The 55bp and 41bp fragments might not be visible due to their small size. For the homozygous genotype (AA) The mutation disrupts the TCGA site at one location, preventing the enzyme from cutting there. As a result, two fragments are observed: (41bp and 158bp), whereas the heterozygous genotype (TA) This genotype contains four bands at 41bp, 55bp, 103bp, and 158bp. as show in figure (3-5).

This result showed that T allele frequency was (57%) in patient group and (56%) in control group whereas A allele frequency was (43%) in the patient group and (44%) in the control group as show in table (3-2).

Also this table showed that the (TT) genotype have frequency (20%) in patient and (16%) in control group, whereas the heterozygous (TA) was found to be (73.3%) in patient group and (80%) in control but the genotype (AA) form was found to be (6.7%) in patient and (4%) in control.

This result was showing that most patient and control have the T allele. However, there were no statistic significant differences in the genotype and allele frequencies between patients and controls (p value=0.54) and allele frequencies between patients and controls (p value=0.94)

Table (3-2) Comparison of the allele and genotype frequencies between the miscarriage patient and control groups.

SNPs	Genotype / Allele	patient group (number 60, %)	Control group (number 50 ,%)	P value
rs4720485	**Genotype**			
	TT	12 (20)	8 (16)	
	TA	44 (73.3)	40 (80)	0.54
	AA	4 (6.7)	2 (4)	0.77
	Allele Frequency			
	T	34 (57)	28 (56)	
	A	26 (43)	22(44)	0.94

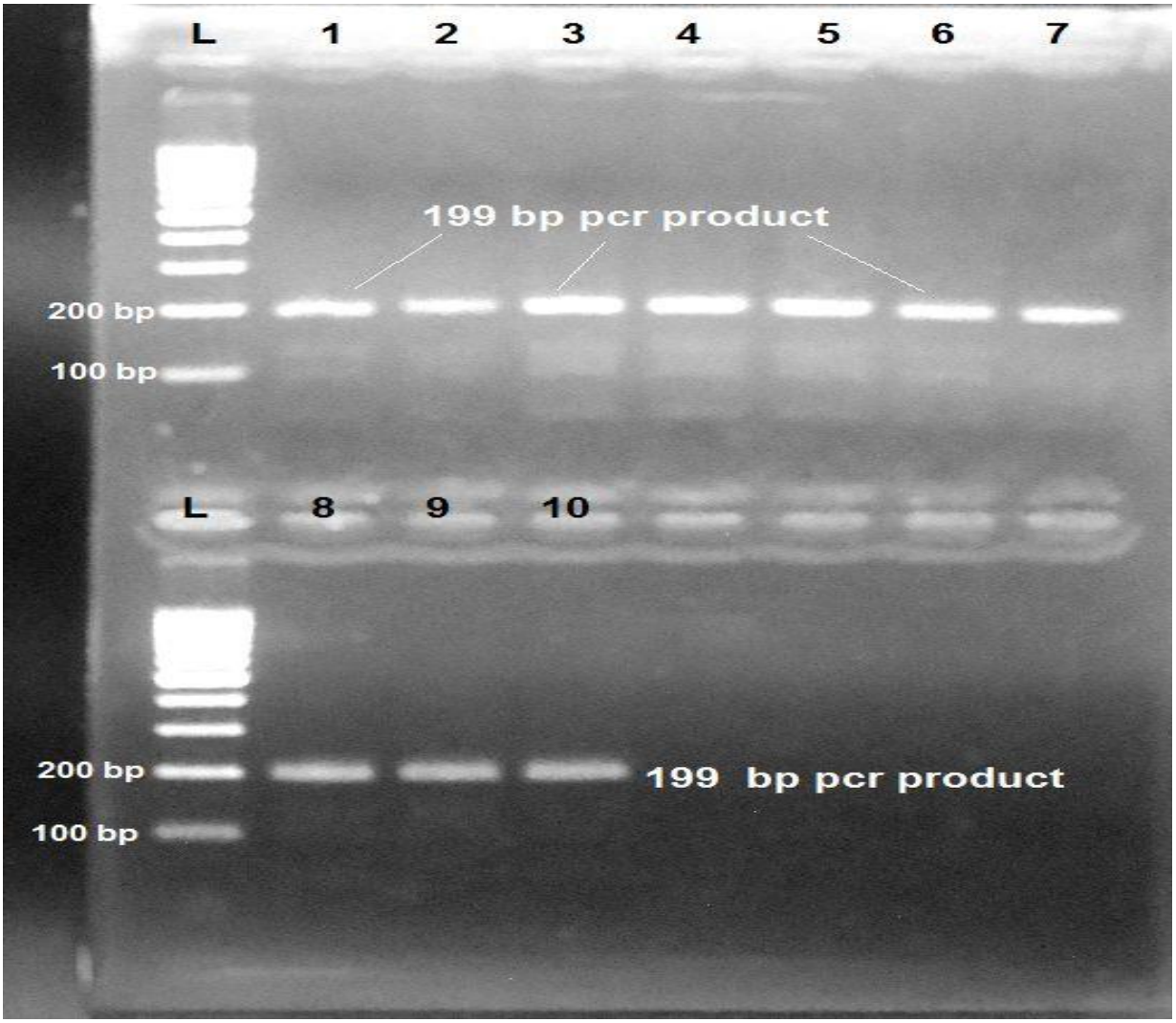


Figure (3-4): Gel electrophoresis of 2% agarose for PPIA PCR products visualized under U.V light after staining with ethidium bromide. The size of product is 199bp.

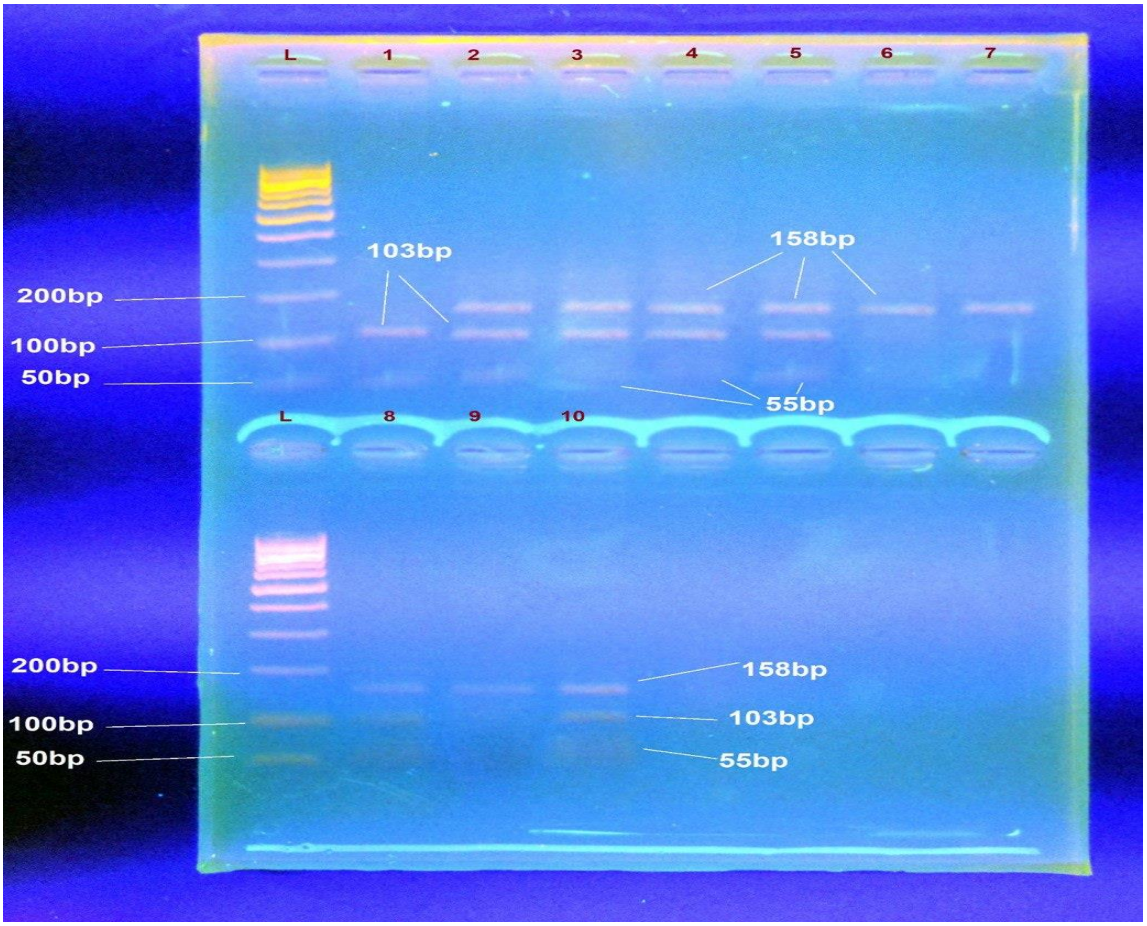


Figure (3-5) Genotyping of rs4720485 by PCR-RFLP on a grose 70volts for 20-35, PCR amplicon 199bp was digested by TaqI restriction enzyme, which produces 41+55+103 fragments when the T allele is present and produces 158 and 41 when A allele is present. lane L= DNA ladder; lanes 5,7 and 9 = AA genotype;lanes2,3,4,5,8 and 10 TA genotype; lanes 1= TT genotype.

These genotype are not naturally arising in the human genome but due to mutation particularly point mutation these single nucleotide polymorphism appear in three types AA,AT ,and TT. The allele frequency of T allele is higher than A allele in women patients and control group.

The locus SNPs in this studies consider is Non-coding SNPs in *PPIA*, that mean variants are genetic variations that occur in regions of the genome that do not code for protein sequences. These regions can still have important regulatory roles in gene expression and cellular processes. This variant rs4720485 involve the substitution of a single nucleotide T at a specific position in the DNA sequence, can be mutated to A .

Based on the information provided by the von Hahn and Ciesek , (2015) it appears that rs4720485 is a single nucleotide polymorphism located within the Alu repeat region located on chromosome 7 at position 44795554 in the human genome of the *PPIA* gene. This gene encodes the peptidyl -prolyl-isomerase A protein.

Shi *et al*., (2020) mentation the study observed polymorphism of rs4720485 (T/A) within the *PPIA* gene that appear A allele in Kawasaki Disease (KD) about 98% while in control 96.7%, were not significantly associated with the risk of Kawasaki Disease development. this implies that these specific variations in the gene do not appear to contribute significantly to the development of KD of the study population.

Palacin *et al.*, (2008) highlights that while no additional studies had been published at that time concerning non-coding variants in the *PPIA* gene and their association with viral diseases, there were reports discussing the relationship between *PPIA* variants and non-infectious diseases. This indicates that variations in the *PPIA* gene might have broader implications for various health conditions beyond viral infections. In particular, the mentioned examples of non-infectious

diseases associated with *PPIA* variants are nephrotoxicity and myocardial infarction.

In this study did not find a significant difference in the occurrence of the rs4720485 SNP between patients and controls, it implies that the presence or absence of this SNP in the *PPIA* gene's promoter region was not associated with the miscarriage women with CMV that was investigated in this study. So, the variations in this particular SNP may not play a significant role in influencing susceptibility to or development of the condition in the studied population.

3.4.2 *PPIA*gene polymorphism at a locus rs8177826 C/G

The result of rs8177826 promoter polymorphism was present in 250bp fragment. After PCR-based analysis figure (3-6), and followed by Restriction fragment length polymorphism (RFLP) analysis. The PCR products of promoter polymorphism were digested with HaeIII restriction enzyme. Figure (3-7).

This was a C/G transition and the homozygous genotype (CC) have frequency (88.3%) and (82%) for patient and control respectively, while the heterozygous (CG) have frequency (3.3%),(12%) for patient and control respectively and but the frequency of the homozygous genotype (GG) were (8.4%) and(6%) for each patient and control as shown in table(3-3) and in the same table show the C allele frequency for patient was (90%) and (88%) for control while G allele frequency was (10%) and (12%) for patient and control respectively, RFLP technique reveals that the CC genotype represented by (36bp,47bp,19bp,28bp,and 110bp)while GG genotype by (36bp,47bp,19bp,and 138bp).the fragments (28bp,36bp,19bp,and 47bp) was not visible due to its small size and CG genotype by (110bp and 138bp) fragments.

Most of the patients and controls were homozygous to C allele. The genotype and allele frequencies showed a no Significant difference when the patients were compared to the controls.

Table (3-3) Comparison of the allele and genotype frequencies between the women miscarriage with CMV group and control group.

SNPs	Genotype / Allele	patient group (number60, %)	Control group (number 50,%)	P value
rs8177826	**Genotype**			
	CC	53 (88.3)	41 (82)	
	CG	2 (3.3)	6 (12)	0.2
	GG	5 (8.4)	3 (6)	0.2
	Allele Frequency			
	C	54 (90)	44 (88)	
	G	6 (10)	6(12)	0.73

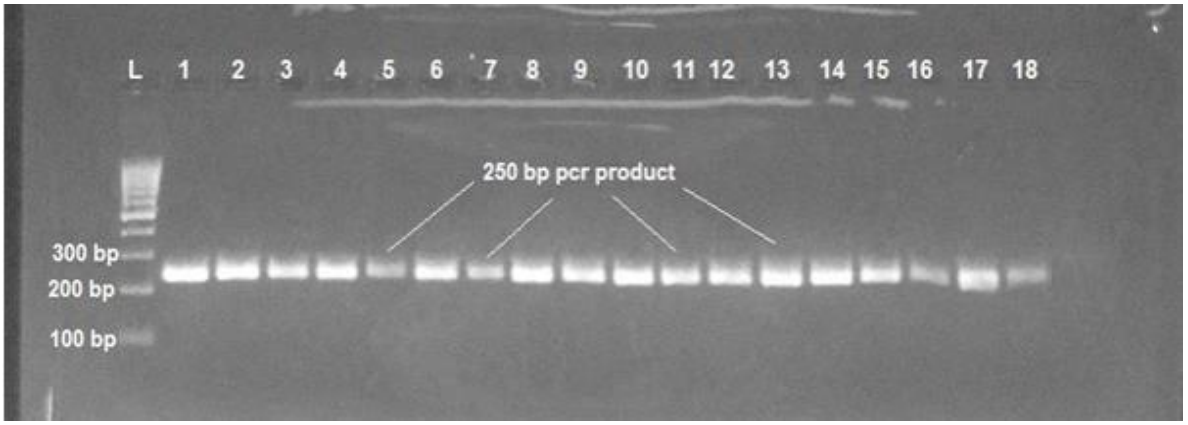


Figure (3-6): Gel electrophoresis of 2% agarose for *PPIA* PCR products visualized under U.V light after staining with ethidium bromide. The size of product is 250bp.

In this study, the genotypes have emerged at the rs8177826 locus, resulting in the presence of GG, CC, and CG genotypes due to point mutations. So, the mutations occurring at this locus are not influencing the expression of the associated gene .

There is a genetic variation in the locus rs8177826, a single nucleotide polymorphism (SNPs) located at position 44796669 on chromosome 7 in the human genome. This variation can have possible alleles: C>G, this means that there is alternative allele at this position in the genome: where the original C base has changed to a G.

The study indicates that, due to the limited sample size, no significant differences were observed between *PPIA* gene polymorphisms, including rs8177826, and CMV-related miscarriage in women patients.

The study conducted by Palacin *et al.*, (2008) investigated the association between a specific genetic variation, rs8177826, in the *PPIA* gene and the risk of developing myocardial infarction (MI) in patients with coronary artery disease. This research found no significant risk of developing myocardial infarction associated with the genetic variation rs8177826 in the *PPIA* gene among patients with coronary artery disease.

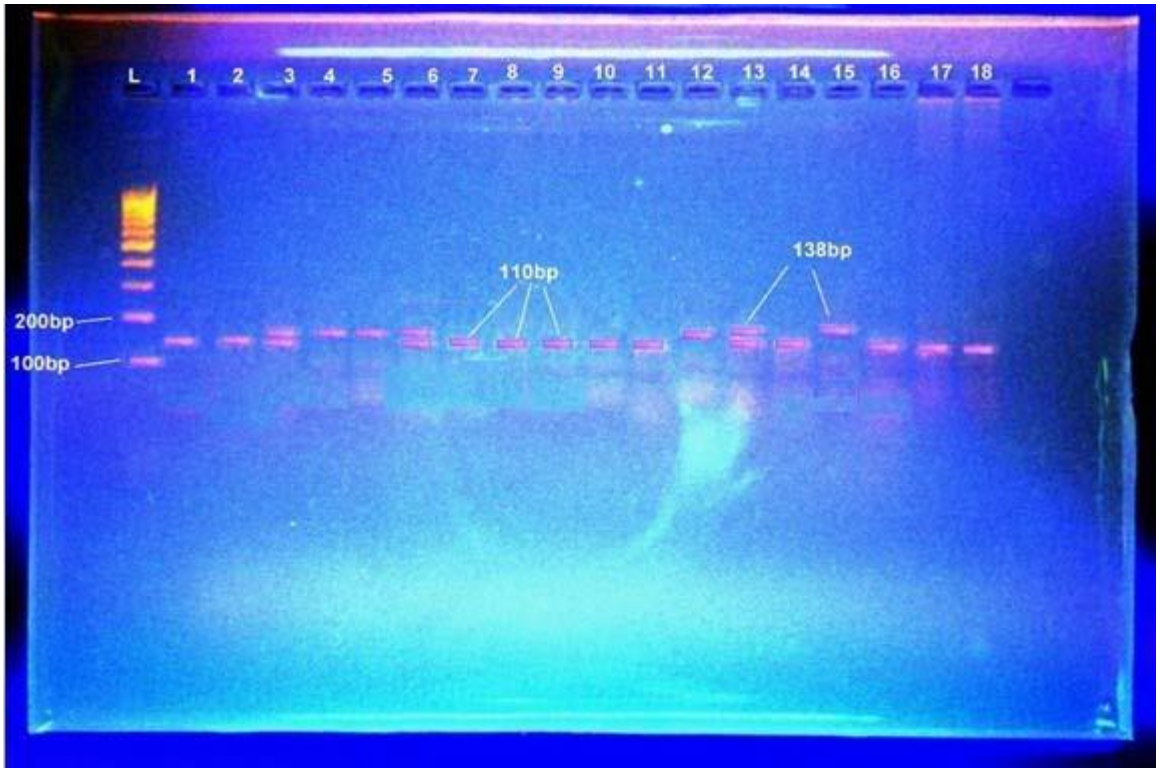
Bigham *et al.(*2014) In their study, they found that individuals with the rs8177826 variant had a significantly decreased risk of HIV-1 acquisition. This suggests that the presence of this genetic variant might confer a protective effect against HIV-1 acquisition, reducing the risk of becoming infected with the virus.

The study conducted by An *et al.(*2007) illustrated found that the SNP rs8177826, were associated with a faster progression from HIV infection to AIDS in European Americans infected with HIV-1.This implies that individuals who carried these specific genetic variants were more likely to experience a more rapid development of AIDS once they were infected with HIV.

However, this locus may associated in many other infection disease but in CMV infections, the effect of allele variants are not significant ,where the transcription factor has not a site to bind at G allele or C allele but there may be other transcription factors for other SNPs are present.

If a specific SNP doesn't directly affect a transcription factor binding site, it doesn't mean that it would not impact gene expression. It might still be involved in altering the binding affinity of other regulatory proteins or influencing the interactions between enhancers, silencers, and the gene's promoter. These interactions can be quite intricate and can result in subtle changes in gene expression levels (Degtyareva *et al.*,2021).

The CG genotype refers to a specific combination of C and G alleles at a particular locus. However, variations within this genotype can occur due to point mutations, where C and G alleles are substituted for one another. Whether these variations are considered within the normal range or not depends on the specific context and the functional consequences of these mutations.



Figure(3-7)genotyping of rs8177826 by PCR-RFLP on agrose 70volts for 20-35, PCR amplicon 250 bp was digested by *HaeIII* restriction enzyme which produce 36+47+19+28+110 fragments when C allele present and produce 36+47+19+138 when G allele present, lane L= DNA ladder; lanes 1,2,7,8,9,10,11,14,16,17, and 18 CC genotype; lanes 3,6 and 13 CG genotype; lanes 4, 5,12 and 15 GG genotype.

3.5 Cyclophilin A association with *PPIA* gene polymorphism

3.5.1 Cyclophilin A levels and *PPIA* gene polymorphism at reference sequence rs4720485

The analysis of gene polymorphisms of the *PPIA* gene at the reference sequence (rs4720485) and Cyclophilin A level revealed interesting findings. Three distinct genotypes were observed from 60 samples obtained from miscarriage women patients with CMV. Specifically, 20% had the TT genotype, 73.3% had the TA genotype, and only 6.7% had the AA genotype. This result is shown in Table (3-4).

Table(3-4)Relationship between genotype of the locus rs4720485 and CyclophilinA

Genotype	CypA concentration mean ±std	*P*-value
TT(20%)	23.53±12.48	.000
TA(73.3)	7.6±2.52	.000
AA(6.7%)	3.2±0.78	.000

*. The mean difference is significant at the 0.05 level.

Although the TA genotype is normal, the TT and AA genotypes are SNPs due to silent mutations. A study conducted found that Cyclophilin A levels are high among women patients with the genotype TT, with a mean value of 23.53±12.48, and the lowest mean value of Cyclophilin A was observed in individuals with the genotype AA, with a mean value of 3.2±0.78.

The study suggests that the reference locus, specifically the TT genotype, may increase the levels of Cyclophilin A, a protein involved in various cellular processes, including immune response and inflammation regulation. About the TA genotype, the mean value of Cyclophilin A in patients with Cytomegalovirus (CMV) infection may be close to the AA genotype mean value, with a mean value of 7.6±2.52. This indicates that most transcription factors related to Cyclophilin A can bind only with the allele T but not A, leading to increased levels of Cyclophilin A in women patients with this genotype. Overall, the study shows that Cyclophilin A levels are high in women patients with the TT genotype and low in those with the AA genotype, and the reference locus may play a role in increasing Cyclophilin A levels in individuals with the TT genotype. Additionally, the TA genotype may have intermediate levels of Cyclophilin A, closer to the AA genotype mean value.

The study found that individuals with the TT genotype for the rs4720485 SNP in the *PPIA* gene could be more susceptible to severe issues related to cytomegalovirus (CMV) infection, particularly in the context of miscarriage pregnancies. This susceptibility might be attributed to the higher levels of Cyclophilin A (CypA) associated with the TT genotype.

In the control group of 50 samples, patients with no history of CMV infection had low Cyclophilin A levels and were therefore excluded from the experiment.

There are some research suggesting a connection between certain genetic variations in *PPIA* and the levels of CypA in the body. Sun *et al.*(2019) conducted study found the CypA serum level significant with CypA genetic polymorphism in women with sever preeclampsia. Another study by Vinitha *et al.*,(2015) also points indicate that the SNPs rs6850 is associated with increased risk for elevated levels cyclophilin A and coronary artery disease in patients.

3.5.2 Cyclophilin A levels and *PPIA* SNPs polymorphism at reference sequence rs8177826

Based on the gene polymorphism results of *PPIA* at rs8177826, there are three distinct genotypes: CC, CG, and GG. The correlation between the level of Cyclophilin A and genotypes rs8177826 of patients groups have been investigated the results reveal there are no correlation have been found. The mean values of Cyclophilin A were 19.35±10.556 for CC, 8.5±1.63 for CG, and 6.48±2.76 for GG. As shown in Table (3-5).

Table(3-5) Relationship between genotype of the locus rs8177826 and CyclophilinA

genotype	CypA concentration mean ±std	*P*. value
CC(88.3%)	(19.35±10.556)	.063
CG(3.3%)	(8.5±1.63)	.328
GG(8.4%)	(6.48±2.76)	.512

The level of serum CypA levels in different genotypes of rs8177826, specifically the CC, CG, and GG genotypes, were found to have no significant difference. This suggests the differences of alleles at this locus do not indicate a relationship between these single nucleotide polymorphisms (SNPs) and Cyclophilin A levels. Furthermore, it implies that no specific transcription factors can bind with the C or G allele to modulate the synthesis of Cyclophilin A at the RNA level. This could imply that these alleles don't directly impact the regulatory processes controlling CypA production

This lack of significant difference implies that the presence of different alleles at this SNP locus does not appear to directly influence the levels of CypA protein in the serum. Additionally, that there may not be specific transcription factors that bind preferentially to the C or G allele of this SNP to regulate the synthesis of Cyclophilin A at the RNA level. In other words, this particular SNP may not play a direct and significant role in modulating the regulatory processes that control the production of CypA ,So genetic regulation of protein levels can be complex, and the absence of a significant difference in CypA levels among genotypes at this specific SNP does not rule out the possibility of other genetic or environmental factors influencing CypA production or function.

3.6 MicroRNA Expression Profiling in Women with Miscarriage Pregnancies and its Association with Cytomegalovirus (CMV) Infection.

This study investigated two CMV-encoded miRNAs genes in the test sample as well as in control samples normalized with housekeeping genes. The data showed that all patients group had a specific level of CMV-encoded miRNAs. In particular, cmv-miRUS25-1-5p and cmv-miR-UL112-5p were expressed at higher levels in women with miscarriage and CMV compared to normal controls ($P<.001$). These findings suggest that altered HCMV-encoded miRNAs may be specific to women who have experienced miscarriage and are infected with CMV.

This study observed the expression of CMV-miR-UL112-5p patients group compared control group, the mean fold in women patients is 12.47 -fold change while in the control group is 1.077.

A fold change of 12.47 means that the expression level of CMV-miR-UL112-5p is approximately 12.47 times higher in the miscarriage women patients with CMV than in the control group. this means that the gene is upregulated in

patients group compared to the control group, which means that the expression level of this specific microRNA is higher in women patients. The up regulation of miR-UL112-5p is associated with CMV infection and its potential role in pregnancy loss. as shown in Table (3-6).

The presence of miRNA in the serum of women with CMV infection may prevent the virus to establish itself in human tissues through its ability to make base paring with virus RNAs and hence degrade all viral RNA and prevent translation into proteins.

Table (3-6) The expression of the miR-UL112-5p gene among patient group and control versus the reference gene (GAPDH)

UL112	ΔCT (mean ±SD)	ΔΔCT (mean ±SD)	Fold Change (mean)
Control(50)	-1.224± 0.577	1.42E-16 ± 0.577	1.0775
Patients(60)	-4.896 ± 1.771	-3.672 ± 1.771	12.74
P-value	**0.000**	**0.000**	**0.000**

Another microRNA investigated in this study is miR-US25-5p, the level expression in the patient's group compared to the control group, the mean fold in women patients is 14.96-fold change while in the control group is 1.02.

The fold change of 14.96 indicates that the expression level of CMV-miR-US25-5p is approximately 14.96 times higher in the patient's group compared to the control group. This suggests that the gene is unregulated in miscarriage women patients with CMV, potentially indicating its involvement in the pathogenesis of CMV infection during pregnancy. The result is shown in Table (3-7) and appendix 6.

Table (3-7) The expression of the miR-US25-5p gene among patient group and control versus the reference gene (GAPDH)

US-25	ΔCT (mean ±SD)	ΔΔCT (mean ±SD)	Fold Change (mean)
Control	0.473 ± 0.306	-3.55E-17 ± 0.043	1.0213
Patients	-3.43 ± 1.194	-3.09 ± 0.22	14.965
P-value	**0.000**	**0.000**	**0.000**

The results of the study suggest that both miR-US-25p and miR-UL112_5p genes are up- regulated in women who had undergone miscarriages with CMV infection compared to healthy married women. This upregulation was found to be statistically significant, indicating that these genes may play a role in the context of miscarriages and CMV infection.

One possible interpretation of these findings is that the upregulation of these microRNAs could be a response to CMV infection in women who had

undergone miscarriages Njue *et al.*, (2020). MicroRNAs are small RNA molecules that can regulate gene expression and are known to be involved in various biological processes, including immune responses to viral infections (Letafati *et al* .,2022).

In general, microRNAs are small non-coding RNA molecules that play a crucial role in post-transcriptional regulation of gene expression(Abu-Izneid et al .,2021). They can bind to specific messenger RNA (mRNA) molecules, leading to either degradation of the mRNA or inhibition of its translation into a protein(Correia et al ., 2019). MicroRNAs are involved in various physiological and pathological processes, including immune responses to viral infections(Zhou et al .,2023).

Regarding cytomegalovirus (CMV) infection, studies have identified several microRNAs that can impact the virus-host interaction. Some microRNAs have been shown to target viral genes, thereby affecting viral replication and the establishment of a latent infection(Diggins&Hancock,2023). Conversely, CMV infection can also alter the expression of cellular microRNAs, enabling the virus to manipulate host immune responses and promote viral survival and dissemination (Dass *et al* .,2023)

Numerous studies have demonstrated the significance of microRNAs in Cytomegalovirus. Gao *et al.,* (2021) found that hcmv-miR-US25-1-5p (with a fold change of 1.75) could serve as an indicator of adverse pregnancy outcomes (APO) in pregnant women who have contracted CMV. Moreover, the authors propose that cmv-miR-US25-1-5p can be transmitted more easily to the fetus through the placenta than CMV, potentially affecting fetal development.

A study conducted by Afshari *et al.,(*2022) discovered a significant link between miR-UL112-5p and BUN levels in kidney transplant patients currently battling CMV infection. The study also revealed that only miR-UL112-5p and miR-US25-1-5p have a statistically significant association with Cr levels in those

who have undergone kidney transplants and have latent CMV infection.

Zhang *et al* .,(2020) conducted a study investigating the connection between the expression of hcmv-miRNAs in extracellular vesicles and liver damage in newborns with HCMV infection. They compared the expression levels of these miRNAs in HCMV-infected infants to those of healthy infants. The results revealed that the levels of miRNAs were significantly higher (with a 4-fold increase) in HCMV-infected infants than in non-infected infants. A study conducted by Kawano *et al*., (2016) analyzed the association between hcmv-miRNAs and clinical features in patients with congenital HCMV infection. The study focused on the expression level of hcmv-miR-US25-1-5p in infants under six months of age with congenital HCMV infection. The researchers specifically looked at clinical features such as hearing disorders, intrauterine growth restriction, developmental retardation, and abnormalities in brain imaging. The study found that some newborns with abnormal brain imaging had higher levels of miR-US25-1-5p and miR-US25-2-5p than those with normal brain imaging.

Human Cytomegalovirus has adopted multiple strategies to manipulate the host's immune responses. Among them, the expression of viral microRNAs (miRNAs) is one of the most intriguing. HCMV microRNAs, miR-UL112-5p, were found to contribute to immune evasion by directly targeting the endoplasmic reticulum (ER) aminopeptidase 1 (ERAP1), a key component of the antigen processing that generates antigenic peptides for presentation to cytotoxic CD8+ T lymphocytes and NK cells, The reduced trimming due to HCMV-specific action led to the reduction of HCMV-specific CD8+ T cell responses .as demonstrated by research conducted by Romania *et al.*, (2017); Kim *et al* .,*(*2011).

Hong YM *et al* .,(2022) menation that miR-UL112 is unique because it has the ability to regulate the expression of IE72. Specifically, miR-UL112 can bind to the mRNA of IE72, leading to the degradation of IE72 mRNA or

the inhibition of its translation. This regulatory interaction results in the suppression of IE72 protein levels within infected cells. The suppression of IE72 expression by miR-UL112 is thought to be one of the strategies employed by HCMV to establish and maintain viral latency. By inhibiting the expression of IE72, miR-UL112 may contribute to the maintenance of latency by preventing the activation of early viral genes that are required for productive viral replication.

Jiang *et al.*,(2015) conducted a study that revealed the high expression rate of hcmv-miR-US25-1-5p during both lytic and latent human cytomegalovirus infections (HCMV). This microRNA effectively inhibited viral replication, suggesting its potential as a therapeutic target for HCMV infections. Furthermore, miR-US25-1-5p was implicated in the later stages of viral lytic phase activation, where it aids in achieving a stable latent condition by blocking cell cycle progression through the modulation of various molecules, including cyclins, as described by Kim *et al.*,*(*2015).

(Qi *et al.*, 2013) mention that the expression of miR-US25-1-5p caused a significant decrease in viral DNA replication. This is because miR-US25-1-5p can repress the expression of multiple host genes, which all play essential roles in cellular bioactivity and can affect viral genome replication.

According to Zhou *et al*., (2020), certain types of miRNA tend to increase during the later stages or reactivation of infection. This study confirms earlier research by Stern-Ginossar *et al.* ,*(*2007), which found that levels of miR-UL112 go up during CMV infections. In addition, Chen *et al*. (2017) conducted research that indicated HCMV-encoded miRNAs, specifically hcmv-miR-US25-1-5p,increase significantly during the reactivation phase of lytic infection.

According to Gao *et al*. (2021), pregnant women with APO exhibit a distinct set of HCMV-encoded miRNAs. The levels of hcmv-miR-US25-1-5p

and hcmv-miR-US5-1 in their plasma are associated with APO. Furthermore, hcmv-miR-US25-1 could be a non-invasive biomarker for detecting APO in pregnant women during HCMV infection.

Studies investigating the specific functions of miRNAs in different stages of pregnancy and their involvement in various pregnancy complications are ongoing. As miRNAs are potential targets for therapeutic interventions, understanding their roles in miscarriage may lead to new diagnostic and treatment approaches in reproductive medicine (Singh *et al* .,2023).

Further research is warranted to elucidate the specific roles of miR-US-25p and miR-UL112_5p in CMV-associated miscarriages Toksvang *et al* .,*(*2022). Mechanistic studies could help uncover the underlying pathways and interactions that lead to the observed upregulation of these microRNAs (Hill et *al* .,2021).

The study provides valuable insights into the differential expression miR-US-25p and miR-UL112_5p were upregulated in CMV-related miscarriages compared to healthy married women, indicating their potential involvement in pregnancy complications. They could serve as diagnostic markers and therapeutic targets for managing CMV-related pregnancy issues. Further research is needed to understand their mechanisms in miscarriage and reproductive health.

3.7 Cortisol and Adrenocorticotropic Levels in Women Patients with CMV Infection

A study included serum cortisol and plasma ACTH levels estimation between patients and a control group. The results showed that the patient group had significantly lower cortisol levels, at mean value 42.1158, compared to the control group average of 114.2062. On the other hand, women who had miscarriages had higher levels of Plasma Adrenocorticotropic hormone (ACTH) compared to the control group, with a difference of (78.1468, *P* 0.001) compared to the control group's 45.18. These findings are illustrated in Figures (3-8) and (3-9)

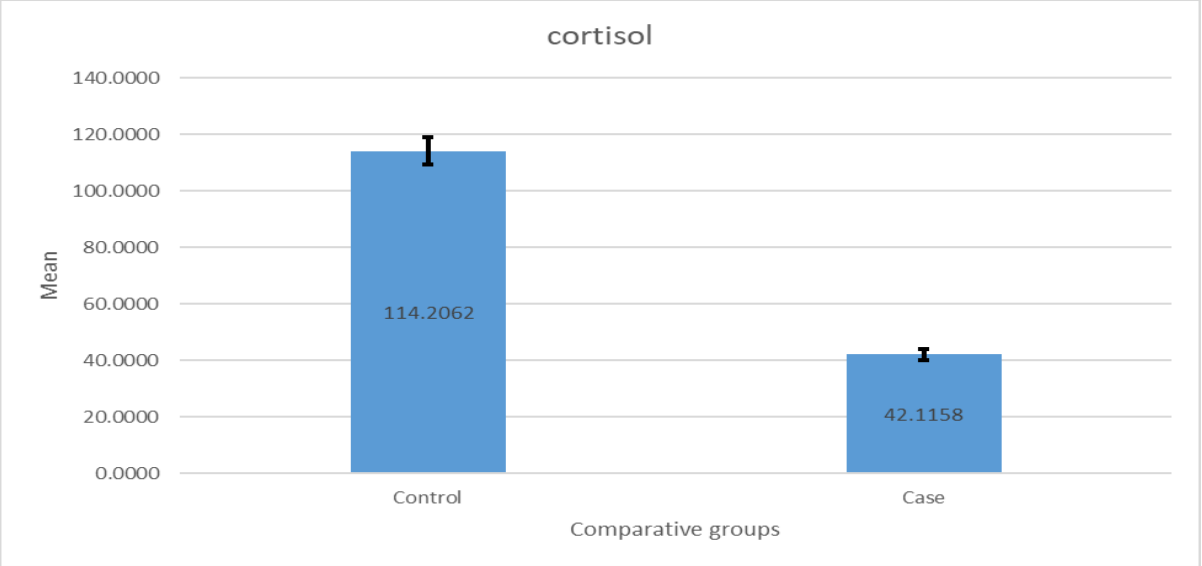


Figure (3-8) mean value of Cortisol in miscarriage women patients with CMV and control

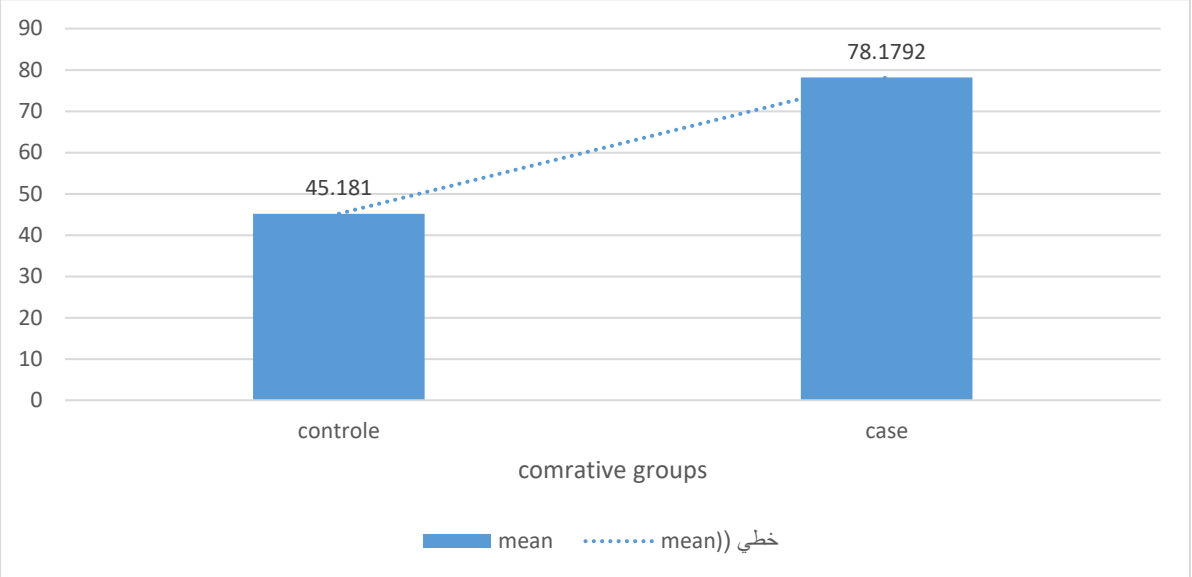


Figure (3-9) mean value of ACTH in miscarriage women patients with CMV and control

These hormones are not previously studied to show their role in CMV infections as biomarker but they need ongoing studies to show their role at genetic levels.

The relationship between CMV and cortisol/ACTH is complex and needs to be fully understood. A study by Bissinger *et al.*, (2002) found that. the lungs, pancreas, kidneys, and liver are the organs most affected by CMV, also, the adrenal glands were common organ affected by CMV.

The virus can directly infect and damage the adrenal glands, impairing cortisol synthesis. Additionally, CMV infection can also suppress the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for regulating cortisol production. The exact mechanisms by which CMV affects the HPA axis are not fully understood but may involve viral proteins interfering with signaling pathways in cortisol synthesis (Rector *et al* .,2014).

It is important to note that while CMV infection can affect cortisol synthesis, not all individuals infected with CMV will experience these effects.

The impact of CMV on cortisol production may vary depending on factors such as the individual's overall health, immune system function, and viral load (Corinna and Don ,2012).

It is unclear how CMV affects the adrenal gland, but some believe it might be an infection source. The virus seems to deplete the gland's resources and increase the likelihood of adrenal insufficiency, but symptoms only arise when over 90% of the gland has been damaged. As a result, functional issues with the gland can go unnoticed until complete adrenal insufficiency sets in, which can be life-threatening. (Pintaldi *et al* .,2019; Barthel *et al*., (2019).

If a person experiences chronic or persistent CMV infection, it may cause adrenal insufficiency. This is a condition where the body does not produce enough cortisol. The virus can damage the adrenal glands or trigger autoimmune destruction of the adrenal tissue, leading to this condition. Even if the person is stimulated by high levels of ACTH, the adrenal glands may still be unable to produce enough cortisol. (Paolo and Nosanchuk , 2006).

Although , this study included two hormones to show their relationships to CMV infection and miscarriage ,it is still requiring further investigation to show whether cortisol levels are affected due to CMV infections or there is variation in this value due to the time of samples obtaining.

CONCLUSION AND RECOMMENDATIONS

Conclusions

1- Cytomegalovirus is found in some women with miscarriage when IgM or IgG and IgM are high.
2- Cyclophilin A was found to be increased with CMV infection when the level of IgM plus IgG or IgM alone are high.
3- Cyclophilin A gene polymorphism revealed a relationship between genotyping of SNPs and Cyclophilin A levels.
4- Three genotypes are observed where SNPs are investigated at cyclohilin A gene.
5- The presence of high levels of ACTH in the blood of women with CMV.
6- There is decrease level Cortisol in women miscarriage with CMV infection which is statically significant.
7- miR-US25-5p and miR-UL112_5p were upregulated in women with miscarriage with CMV infection which may be involved in pregnancy complications.

Recommendations

1- Investigation of new methods to determine CMV infection by using MLST and RED-PCR .
2- Investigation of new microRNA in women's blood to shows their relationship to CMV infection and miscarriage.
3- Analysis of some genes related to the human reproductive system and its relation to genetic polymorphism .

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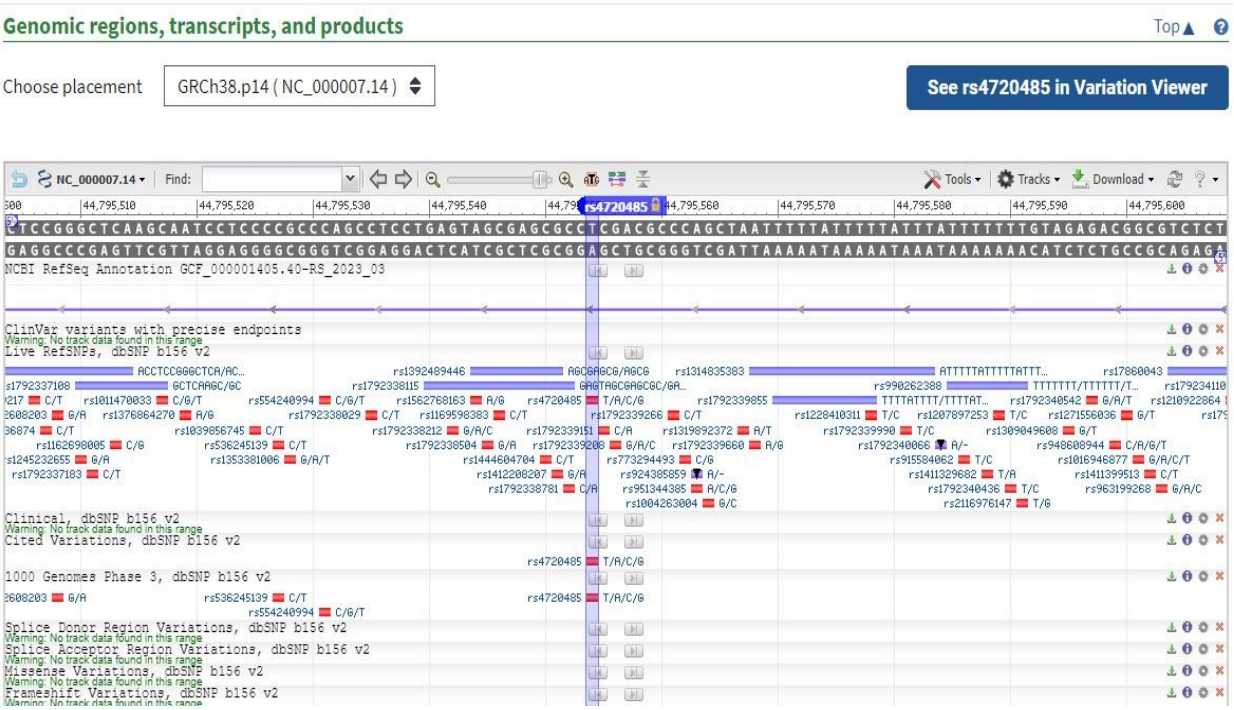
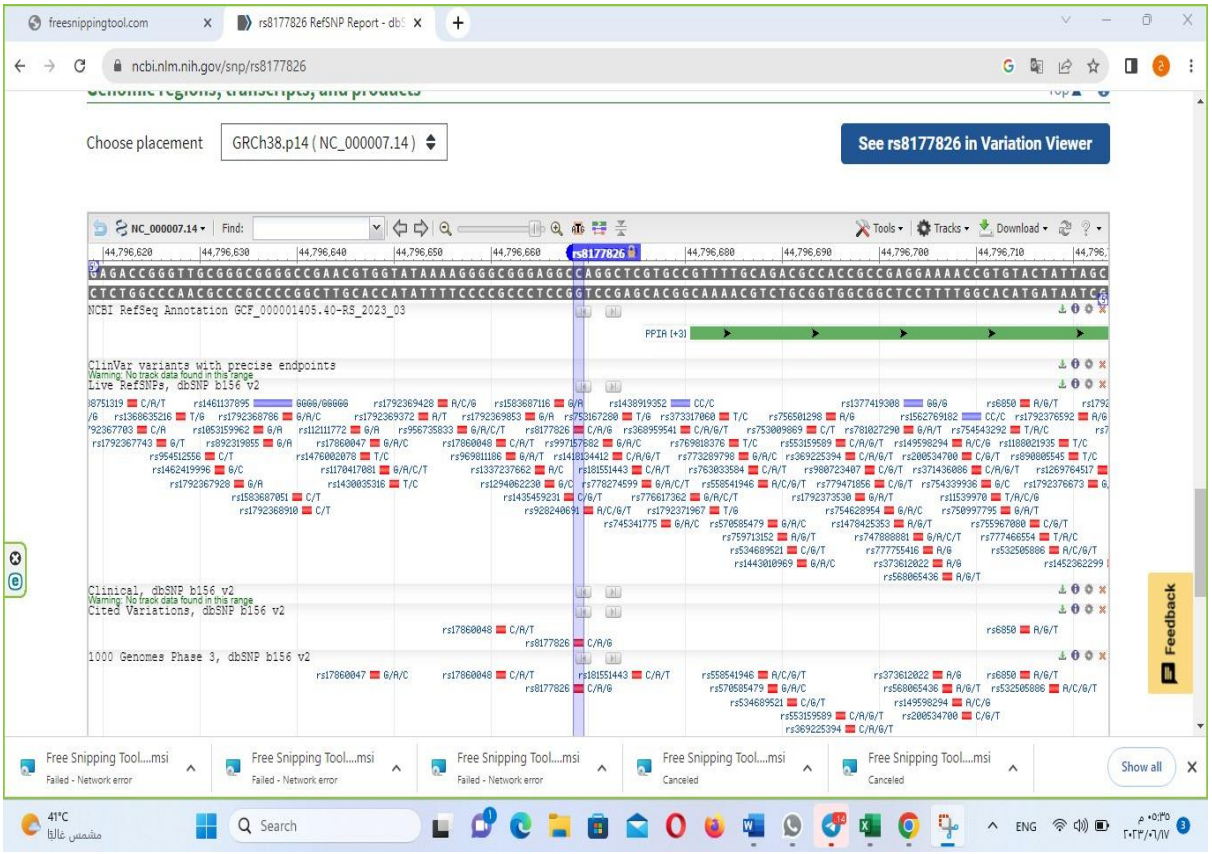
Appendix 1



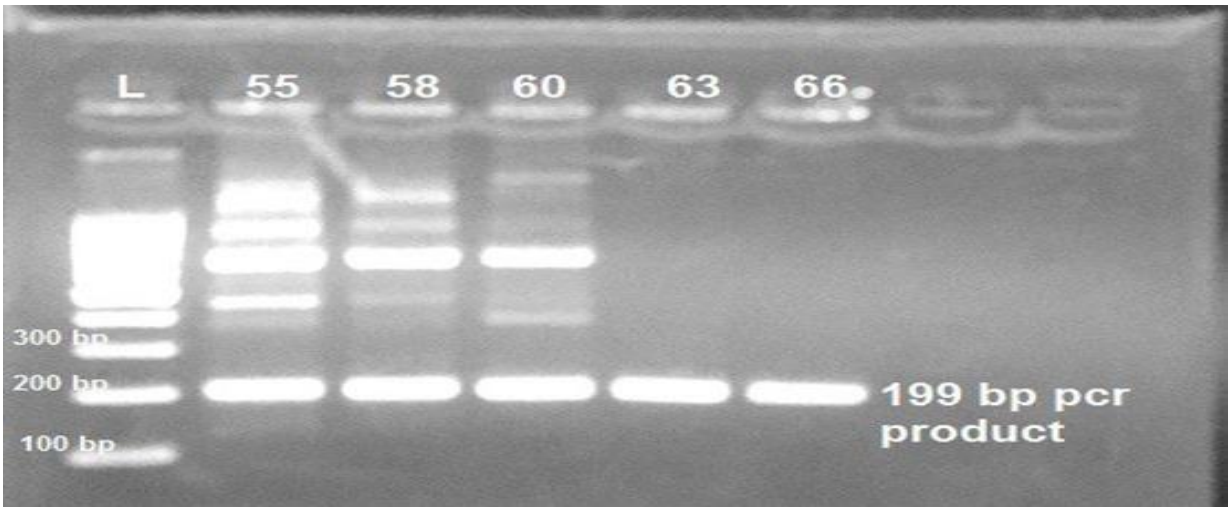
The exact position of rs4720485 and primers pair on the human genome (NCBI sequence browser).

Appendix 2



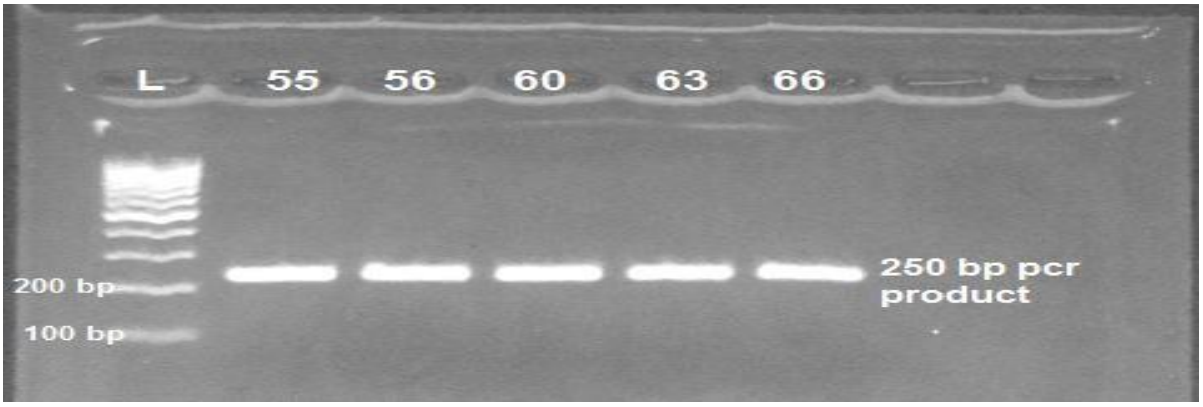
The exact position of rs4720485 and primers pair on the human genome (NCBI sequence browser)

Appendix 3



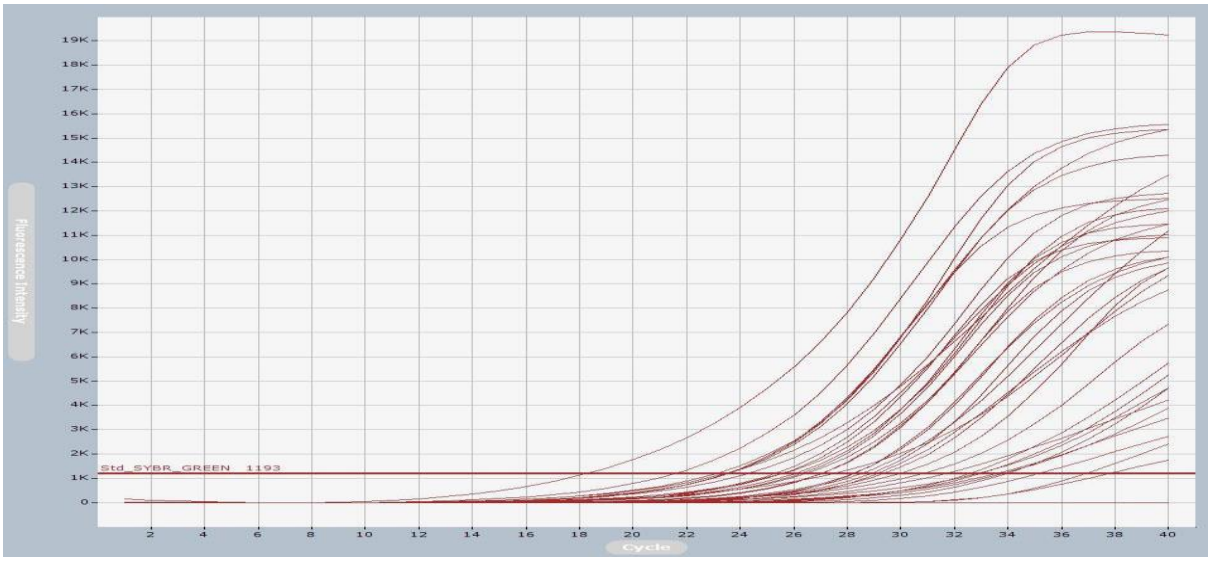
Gel electrophoresis of gradient PCR product (rs4720485) , lane L:DNA ladder, other lane represent different annealing temperatures as indicated on each lane.

Appendix 4



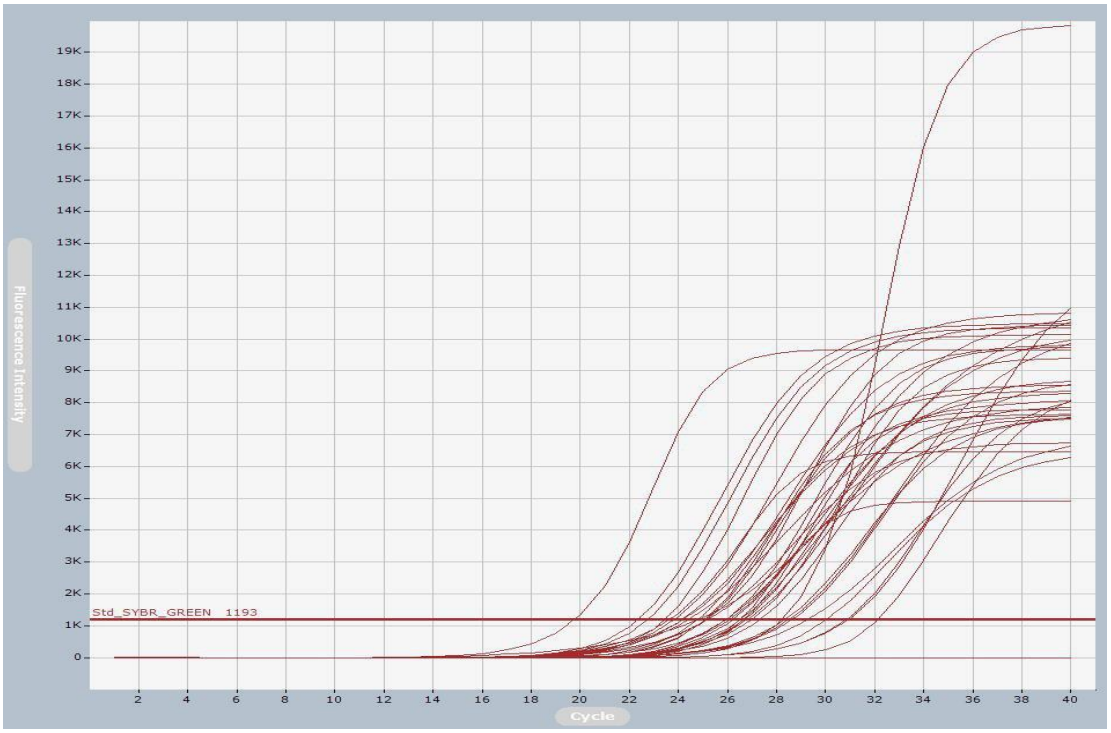
Gel electrophoresis of gradient PCR product (rs8177826) , lane L:DNA ladder, other lane represent different annealing temperatures as indicated on each lane.

Appendix 5



Gene expression for housekeeping level (GAPDH) .

Appendix 6



miRNA-US25 gene expression level.

تم اختيار ٢٥٠ امرأة في هذه الدراسة، ٥٠ امرأة منهن مثلت كونترول (نساء أصحاء غير حوامل ولم يكن لديهن أي إجهاض سابق) و٢٠٠ لديهن تاريخ إجهاض. في هذه الدراسة من بين ٢٠٠ عينة دم، كانت ٦٠ فقط، و١٤ حالة إيجابية لـ IgM، والتي تم تصنيفها أيضًا إلى ست حالات مع CMVحالة فقط إيجابية لـ CMV ، فقط. في العينات التي كانت نتيجة اختبارها إيجابية لـ IgG، و٤٠ حالة إيجابية لـ IgG وIgM ، وأظهرت النتائج أن هذه العينات تحتوي على مستويات أعلى بكثير من Cyclophilin A تم تقدير CMV في المجموعة التي أصيبت بعدوى Cyclophilin A. وكان متوسط قيمة Cyclophilin A وحدها زادت بشكل CMV IgM وتعرضت للإجهاض ٣٤,٣٣٧. أظهرت النتائج أن الأجسام المضادة (المتوسط = ٦٤ ± ٤,٧٦). ومع ذلك، في المرضى الذين يعانون من Aملحوظ تركيز السيكلوفيلين أقل (يعني = ٤٨,١٥ ± ١٦,٧٢). بالنسبة للنساء Cyclophilin A، كان مستوى IgG وIgM CMV ± SD = 25.004 بشكل أكبر (يعني ± A، انخفض مستوى السيكلوفيلين CMV IgG المصابات بـ 2.34.)

تم إجراء تعدد أشكال النوكليوتيدات المنفردة لإظهار تأثير متغيرات النوكليوتيدات على جين *PPIA* الذي له علاقة بمستويات السيكلوفيلين A في دم النساء المصابات بعدوى الفيروس المضخم للخلايا في موقعين مرجعيين

كشفت النتائج أن تعدد الأشكال *PPIA* عند مرجع rs4720485 وجد أن النمط الوراثي TA كان أعلى من النمط الوراثي الآخر TT و AA. أظهرت الدراسة أن مستويات السيكلوفيلين A أعلى بين النساء ذوات الأليل من النوع TT وبدرجة أقل في المرضى الذين يعانون من النمط الجيني AT.

من ناحية أخرى، تمت دراسة SNPs أيضًا لجين *PPIA* عند تسلسل مرجعي rs8177826. وقد وجد هناك أنماط وراثية مختلفة في الأنماط الجينية CC وGC وGG. تم العثور على مستويات السيكلوفيلين A مرتفعة في النساء المصابات بالنمط الوراثي CC وبدرجة أقل بين النساء ذوات النمط الجيني GC.

فيما يتعلق بمستويات microRNA، تم تنظيم جينات miR-US25-5p وmiR-UL112_5p لدى النساء اللاتي خضعن لعمليات الإجهاض بعدوى CMV مقارنة بالنساء المتزوجات الأصحاء. وتمت مقارنة التعبير عن CMV-miR-UL112-5p في مجموعة المرضى بالمجموعة الاصحاء، مع متوسط تغير قدره ١٢,٤٧ في المرضى النساء و١,٠٧٧ في المجموعة الاصحاء. بالإضافة إلى ذلك، تمت مقارنة miR-US25-5p أيضًا، مع متوسط تغير قدره ١٤,٩٦ في المرضى من النساء و١,٠٢ في المجموعة الاصحاء. وهذا يعني أن miR-US25-5p وmiR-UL112-5p يزدادان بشكل كبير في النساء

المجهضات المصابات بالعدوى. مع الفيروس المضخم للخلايا. إن وجود معدلات عالية لتغير لنوعين من الحمض النووي الريبي الميكروي سيعطي مؤشرات حيوية جيدة لعدوى الفايروس المضخم للخلايا

فيما يتعلق بالدراسة التي قارنت مستويات الكورتيزول في الدم ومستويات ACTH في البلازما بين المرضى ومجموعة التحكم ، أظهرت النتائج أن مجموعة المرضى كانت لديهم مستويات من الكورتيزول أقل بشكل ملحوظ من (متوسط ٤٢,١١٥٨) من متوسط المجموعة الضابطة (١١٤,٢٠٦٢). ومع ذلك ، كان لدى مجموعة المرضى مستويات أعلى من هرمون قشر الكظر البلازمي (ACTH) مقارنة بالمجموعة الضابطة ، بمتوسط قيمة (٧٨,١٤٦٨ ، P 0.001) مقارنة بالمجموعة الاصحاء ٤٥,١٨ ، ومع ذلك ، فإن هذه الهرمونات غير مدروسة ولا يمكن استخدامها كأساس. العلامات الحيوية لعدوى الفيروس المضخم للخلايا.

وزارة التعليم العالي والبحث العلمي

جامعة بابل كلية الطب

تقدير السايكلوفيلينِ A والتعبير الجيني لل miRNA لدى النساء المجهضات المصابات بفايروس المضخم للخلايا.

رسالة

مقدمة الى مجلس كليه الطب / جامعة بابل

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في

العلوم/الأحياء المجهرية الطبية

من قبل

حوراء احمد علي جبر

بكالوريوس في الاحياء المجهرية /كلية العلوم /جامعة بابل/٢٠١١

ماجستير في الاحياء المجهرية الطبية/كلية الطب/جامعة بابل/٢٠١٦

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