Republic of Iraq
Ministry of Higher Education
& Scientific Research
University of Babylon
College of Science for Women

The Impact of HLA-DR and HLA-G with Some microRNA Expression in Thalassemia Major Patients Associated with Hepatitis C virus Infection

A thesis
Submitted to the Council of the College of Science for Women of Babylon University as Partial Fulfillment of the Requirements for the Degree of Master in Biology

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2022 AD **1444 AH**

بِسْمِ اللّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي
وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا)

صدق الله العلي العظيم

سورة الآسراء: الآية 85

Dedication

To the sake of Allah, my Creator and my Master..

To my great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life..

To my homeland Iraq..

To my great parents .. which have been my strength , my confidence and my constant support....To all my family, my friends and all people whom I love..

To everyone who helped me in every possible way to make this work see the light .. I dedicate this work ..

ESTABRAQ

Acknowledgment

First of all, thanks to Allah the most gracious and the most merciful, who gave me the ability and desire to achieve this study.

I would like to express my deepest appreciation and very honest gratitude to my supervisors, Dr. Abd Alnabi J AL-Mammory. and Dr.Naeem R AL-Jubore for their guidance, support, interest and their encouragement .

My sincere thanks to all staff of Center for Genetic Blood Diseases at Babil Teaching Hospital for Maternity and Children for presenting all the facilities to finish this work .

My special and sincere thanks to Thalassemia the patients from whom I collected samples, and may Allah heal them and may Allah heal them and have mercy on those who died because of this disease..

ESTABRAQ

Certification of the Supervision

I certify that this dissertation entitled(***The impact of HLA_DR and HLA_G with some microRNA expression in thalassemia major patients associated with hepatitis C virus infection***) " was prepared by **Estabraq Hasan Alwan AL-watifi**, under our supervision at the Department of Biology College of Science for Women, University of Babylon , as a partial fulfillment of the requirements for Degree of Master in Science of Biology

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Data: / / 2022

In the view of the available recommendation, I forward this thesis for debate by the examining committee

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Summary:

Hepatitis C virus (HCV) is a virus enveloped in the positive-sense RNA genome in the genus Hepatitis C viruses related to the family Flaviviridae. Each virus particle has a size of about (55 nm–65 nm). Virus-associated transmission electron microscopy (EM) was hampered by the lack of a cell culture system that produced a sufficient amount of two viruses for visualization. This study was conducted on thalassemia patients with hepatitis C at the Center for Genetic Blood Diseases at Babylon Hospital for Women and Children. thalassemia is a heterogeneous group of hemoglobin synthesis and genetic disorders resulting from a decrease in the rate of production of one or more hemoglobin chains .

This study was conducted on 100 sample divided into three groups. Thalassemia patients infected with hepatitis C virus (60) patients, and the control groups of thalassemia patients without hepatitis C were (20) patients, and the healthy control (20) individual. The study aimed to identify the hepatitis C virus among thalassemia patients, resulting from blood transfusions contaminated with this virus, and to determine some immunological parameters affected by the virus (HLA-DR & HLA.G), The results of ELISA on patients sera showed high percentages of (HLA-DR and G) in patients compared to the positive and negative control, where the percentage of HLA-DR (8.92 ± 11.31) in patients compared to the control group (Positive and Negative Control) (4.92± 2.70 and 4.34 ± 2.16)respectively with a non-significant difference P-0.007. As for the percentage of HLA-G(811.5 ± 640.1)in patients compared to the control group (Positive and Negative Control)(220.5± 952.91 and 561.8 ± 394.1) respectively . with a significant difference.

Ribonucleic acid was extracted from blood samples and RT qPCR technology was used to determine and evaluate the gene expression of (miRNA-122 , miRNA-21) and compare them with the housekeeping genes that are considered as control genes to HCV patients. The inverse relationship of gene miR-122 was(17.67 ± 5.2) with H.K. genes(24.67 ± 4.2) P value 0.000, showed an increase in gene expression and a strong association with hepatitis C virus, As for miR-21(29.15 ± 2.3)compare with the housekeeping genes , they had low gene expression in HCV patients .This study also included the comparison of mean values of liver function test of patients hepatitis C virus with thalassemia (GOT 36.86 ± 30.50 ,GPT:30.02 ± 20.34) , The increase was clear in patients compared to the control of thalassemia at the level of (GOT :25.73± 15.28 ,GPT:26.26 ± 16.32). which was also increased compared to the control healthy level (GOT 19.80 ± 5.98, GPT 26.85 ± 5.55) This result show no significant differences in GOT in P. Value:0.05 , as well as the levels of (ferritin, Hb , PT& PTT and PCV) were measured.

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List of Abbreviations

NO.	Abbreviations	Terms
4	**Ab**	antibodies
1	**ALT**	Alanine aminotransferase
5	**APCs**	antigen-presenting cells
3	**aPTT**	activated Partial Thromboplastin Time
2	**AST**	Aspartate aminotransferase
8	**CBC**	Complete Blood Count
10	**CD 8**	Clusters of differentiation 8
9	**cDNA**	Complimentary Deoxyribonucleic Acid
6	**CHC**	Chronic hepatitis C
7	**CHV**	canine Hepacivirus
11	**CT**	Cycle thereshold
12	**EDTA**	Ethylenediamine tetraacetic acid
13	**ELISA**	Enzyme-linked Immunosorbent assay
14	**EM**	Electron microscopy
22	**H.K. gene**	Housekeeping gene
16	**HAV**	Hepatitis A Virus
15	**Hb**	hemoglobin
54	**Hb A**	Hemoglobin adult
53	**Hb F**	Hemoglobin fetal
17	**HBV**	Hepatitis B Virus
19	**HCC**	hepatocellular carcinoma
18	**HCV**	Hepatitis C Virus
20	**HDV**	Hepatitis D Virus
21	**HEV**	Hepatitis E Virus

23	**HLA**	Human leukocyte antigen
24	**IRES**	Internal ribosome entry site
25	**IUs**	International Units
56	**JFH**	Japanese fulminant hepatitis
26	**LC**	liver cirrhosis
29	**MG**	mixed globulinemia
32	**MHC**	Major Histocompatibility Complex
27	**miRNA**	MicroRNAs
28	**mRNA**	Messenger RNA
31	**MTCT**	mother-to-child transmission
35	**NK**	Natural Killer
37	**NPHV**	non-primate Hepacivirus
33	**NS-4B**	Non- structural protein 4B
34	**NS-5B**	Non- structural protein 5B
36	**NTDT**	nontransfusion-dependent thalassemia
38	**OD**	optical density
39	**PBMC**	peripheral blood mononuclear cells
40	**PCR**	Polymerase chain reaction
55	**PCV**	Packed cell volume
42	**POI**	Polymerase enzyme
41	**PT**	prothrombin time
43	**RBC**	Red blood cell
44	**RdRp**	RNA-dependent RNA polymerase
46	**RNAi**	RNA interference
45	**RT-qPCR**	Reverse Transcriptase Quantitative Polymerase Chain Reaction
47	**SD**	Standard Deviation
48	**siRNAs**	small interfering RNAs
30	**SMADH7**	mothers against decapentaplegic homolog 7
49	**SPSS**	Statistical Package for social sciences
50	**TCR**	T-cell receptor
51	**UTR**	untranslated regions
52	**WBC**	White blood cell

CHAPTER ONE

INTRODUCTION

AND

AIM OF STUDY

1.1.Introduction:

The virus of Hepatitis C (HCV) is responsible for more than 85% of the transfusion-related cases of hepatitis. The possibility of the progression of the infection into chronic case becomes over 50%, which will result in hepatocellular carcinoma or cirrhosis in 20%. Almost all world countries It has been projected that over 172 million persons have got the infection all over the worldwide. In the , the prevalence of the Hepatitis C virus (HCV) is had established their own data base regarding HCV (Gupta *et al*, 2014).HCV can be considered as the most widespread post- transfusion hepatitis (PTH) cause as well as end-stage of liver disease in numerous regions. Regular transfusions of blood in the patients that have the hereditary a, especially the thalassemia, enhanced their general rate of the survival, however, it includes certain risks of acquiring blood borne virus infections particularly the viral hepatitis(B&C) (Alberti and Benvegnu, 2003 ; Alavian *et al*., 2005).In addition to that, regarding the marked overload of the liver iron, typically unavoidable in the patients on the usual processes of the blood transfusion frequent , showed the infections of Hepatitis C virus having potentiating impact on the hepatic fibrogenesis in the Thalassimic patients (Ardalan *et al*., 2004).

Thalassemia is inherited blood disorders; it has spread in the (Mediterranean region). Middle Eastern, African and Southeast Asian and people have the ability to carrying thalassemia genes (Bhandari *et al*, 2018). It causes varying degrees of anemia by the genetic defect, it may be a mutation or deletion. Beta-thalassemia involves 3 basic types, Thalassemia minor which is often termed as (BTT)or beta-thalassemia carrier, (BTI) and (BTM) is known as Mediterranean Anemia (Al-Mosawy .,2017) Thalassemia syndromes in Mediterranean countries are the main cause of iron overload is a blood transfusions (Papanikolaou *et* *al*, 2005). Beta-thalassemia major patients usually present with sharp anemia that requires transfusions of blood frequent compared to Beta-Thalassemia intermedia (Bajwa and Basit., 2019). This causes them to suffer multiple diseases, including hepatitis.

Human leukocyte antigen (HLA) have been shown to associate with Hepatitis, none of the associations has been proven to be conclusive. The mechanism of susceptibility to chronic persistent HCV infection is not well clarified. Since the outcome of HCV infection mainly depends on the host immune response, and HLA, an integral component of the immune response, plays an important role in immunological reaction to HCV infection (Godkin *et al*., 2005).The differ types and highly polymorphic HLA has been considered as an appropriated biological candidate susceptibility that is associated with the development and the progression of chronic HBV & HCV infection. Indeed, previous studies have highlighted that HLA-DR polymorphisms influence individual immune responses, thus affecting the outcome of diseases especially the hepatitis, and that many different HLA alleles play a role in Hepatitis infection (C&B) (Singh *et al*., 2007) this meta-analysis, the identification of common HLA-DR and HLA-G causing Hepatitis susceptibility was examined through a systematic review of the literature followed by a ELISA of all case-control studies. ELISA is a powerful method for quantitatively summarizing the results from different studies. One of the advantages is to enhance the statistical power of outcomes in ethnically, and ancestrally populations and to enlarge the sample sizes, which may reduce the possibility of producing false-positive or false-negative association by random error(Blettner *et al*.,1999). There have also been many genetic studies represented microRNA

MicroRNAs are endogenous, small noncoding RNAs containing an average of 22 nucleotides that mainly regulate gene expression at the post-transcription levels through sequence-specific binding to the 3'-untranslated region (UTR), coding region or 5'-UTR of the target mRNA(Qureshi *et al*.,2014). As most miRNAs guide the recognition of imperfect matches of target mRNAs, each miRNA can have tens to hundreds of different mRNAs targets and further one mRNA can be simultaneously regulated by multiple miRNAs(Bartel ,2009). Due to their fundamental role in biological process, aberrant expression of miRNAs in the liver may be involved in pathological processes of various liver diseases. In fact, the specific deregulation of certain miRNAs, along with the roles of the miRNAs, was observed in liver diseases such as HBV/HCV infection, cirrhosis and HCC (Fromm *et al*.,2020).

1.2.The Aim of Study:

The main aim of this study ,Detection to some immunological (HLA) and molecular parameters (miRNA) of Hepatitis C virus amongst thalassemia patients

Objectives were suggested

- Detection of some immune changes represented by HLA- (DR & G) in hepatitis C patients by using ELISA technique.
- Detection of some miRNA genes. Genes (miR-122, miR-21) were used to Detected the change in gene expression that was affected by hepatitis C virus by using RT-PCR technique.
- Detection of the level of liver enzymes (GPT, GOT) and other test,(PT,PTT) PCV and ferritin changes from the normal level with changes in the immunological and molecular parameters.

CHAPTER TWO

LITERATURE REVIEW

2. Literature Review:

2.1.Thalassemia:

Thalassemia can be defined as a group of heterogeneous genetic disorders (Al-Barazanchi *et al.*, 2019).where the rate of hemoglobin production is partly or entirely blocked as a result of the reduction in the synthesis rate of the α or β- chains, (Hussain and Jaber, 2018, Schmuckler *et al.*, 2020). In thalassemia, an imbalance in globin chain synthesis leads to red cell damage. This in turn leads to the destruction of those cells in the bone marrow (i.e., inactive erythropoiesis) and the peripheral circulation (i.e., hemolysis) (Dalvand *et al.*, 2019; Demosthenos *et al.*, 2019, Tariq *et al.*, 2019).

2.2. Historic Background of Thalassemia:

In 1925, Cooley and Lee described the form of acute anemia with bone changes and an enlarged spleen. Initially referred to as thalassemia anemia, it was shortened to thalassemia from θαλασσα, "sea" by Whipple & Bradford in 1932. In 1925, Rietti described a simple type of hemolytic jaundice in which red cells showed an increase in osmotic resistance. Similar observations were later published by other researchers from Italy (Weatherall,2010). The first genetic evidence of Cooley's anemia was provided by Caminopetros in 1938 and Neel in 1950 who hinted that this is a homozygous state of the recessive trait that leads to reduced intracellular content of hemoglobin (i.e. hypochromia) and red blood cells of small sizes (i.e. microcytosis). Hereditary hemoglobin disorders are the most common monogenic condition (Li *et al.*, 2018).

2.3. Types of Thalassemia:

β- Thalassemia:

This type results from a deficiency or absence of β-globin chain synthesis, which leads to an increase in α chains. In general, β-thalassemia results in the form of acute disease, since it has caused severe anemia (Borgna et al., 2005). **β-thalassemia minor** can be defined as the carrier state of thalassemia, which is clinically asymptomatic and can be characterized by certain hematological characteristics (Lahiry *et al*., 2008). **β-thalassemia intermediate** This type has been defined as a highly heterogeneous thalassemia-like disorder group, which ranges in severity from asymptomatic carrier status to the severe transfusion-dependent type (Cao and Galanello, 2010). **β-thalassemia major**, which is also referred to as Cooley or Mediterranean anemia, can be defined as acute type of anemia dependent on blood transfusion. It is a compound heterozygous or homozygous condition of the recessive Elian disorder (Madigan and Malik, 2006, Origa *et al*., 2015). At birth, patients with β-thalassemia major have an almost normal blood ally, due to the fact that the structure of the β-globin chain is normal and hemoglobin production is sufficient. In this type, newborns need to replace fetal RBCs (ie, from fetal to adult hemoglobin exchange) with cells containing primarily HbA (β2 β2), the defect in β-globin synthesis becomes noticeable. Due to the fact that the main shift from HbF to HbA occurred throughout the first year of life, severity of β-thalassemia occurs during the first year of the neonate's life (Olivieri, 1999, Sankaran *et al*., 2010, and Sankaran *et al*., 2010).

2.4.Management of Thalassemia:

2.4.1. Transfusions:

The goals of transfusion therapy are the main methods of treatment for patients with severe beta-thalassemia in order to correct anemia, inhibit erythropoiesis and inhibit gastrointestinal iron absorption, which occur in un transfused patients due to ineffective increase. Erythropoiesis (Porter and Shah, 2010). The choice to initiate transfusion in patients confirmed to have thalassemia should be based on the presence of severe anemia (hemoglobin less than 7 g/dL for more than 2 weeks, excluding other contributing causes, such as infections) (Cappellini *et al*., 2007). However, in patients with hemoglobin levels greater than 7 g/dL, other factors should be considered, including poor growth, facial changes, evidence of bony expansion, and increased splenomegaly. Hemoglobin level after transfusion from 9 g/L to 10 g/dL - 13 g/L to 14 g/dL does not allow for impaired growth, bone abnormalities and organ damage, allowing for a normal quality of life and activities (Sharma and Pancholi, 2010). The blood transfusion frequency is usually every 2.4 weeks. Short periods can also reduce general blood requirements, however, they are not compatible with adequate quality of life (Wood, 2008).

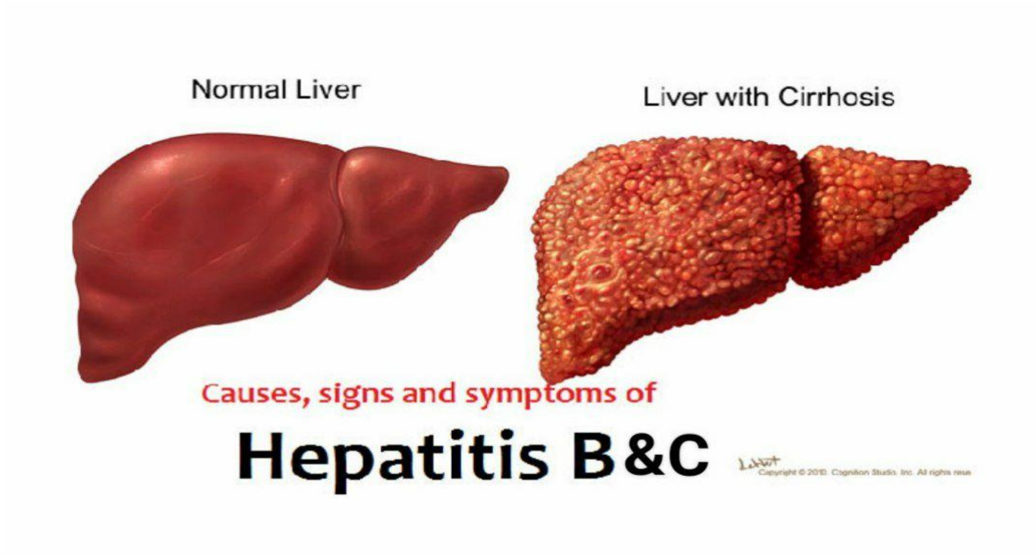
2.5. Hepatitis Virus in Thalassemia patient:

Hepatitis infection is a global public health problem (Al-Kahtani *et al*., 2019). HCV and HBV were first characterized (Choo *et al* , 1989, Hassan *et al*., 2018 ; Alter, 2019). Hepatitis C virus (HCV) can be considered the most common liver disease and post-transfusion hepatitis (PTH) caused by thalassemia patients. Regular blood transfusions in patients with hereditary hemolytic anemia, especially thalassemia, have enhanced their overall survival, however, it has been found to carry a certain risk of blood-borne viral infection, particularly viral hepatitis Type C (coppola *et al.*, 2019; Dalvand *et al.*, 2019). In addition, regarding the observed iron overload in the liver, which is usually unavoidable in patients undergoing regular blood transfusions, hepatitis C virus (HCV) infection has been shown to have a potential effect on hepatic fibrogenesis in thalassemia patients (El-Shabrawy and Kamal, 2019).

Transmission of hepatitis C and HBV interferes with direct exposure to contaminated blood, tattoos, iatrogenic exposure, body piercing, often via vertical transmission and high-risk sexual behaviors (Le Ngoc *et al.*, 2019). The diagnosis of chronic hepatitis C infection was based on the presence of both hepatitis C virus antibodies, which are detected by enzyme immunoassays, and hepatitis C virus RNA, which is detected by molecular assays and is important for the management of viral hepatitis (Coffin *et al.*,2019). Patients without non-transfusion-dependent thalassemia (NTDT) receiving blood transfusion should have annual serological monitoring for hepatitis B&C infection. In patients who have evidence of hepatitis B or C infection on serological testing, confirmatory tests using PCR should be performed, and infection monitored every 6 months (Luo *et al.*, 2019, Ngim *et al.*, 2019). Chronic hepatitis C (CHC) in the majority of affected patients leads to cirrhosis, portal hypertension, liver fibrosis and hepatocellular carcinoma. For this reason, anyone with positive tests for the hepatitis C antibody should undergo additional tests for the presence of actual hepatitis C using polymerase chain reaction (Mainar *et al.*, 2019). The combination of interferon alpha and ribavirin with its significant problems and complications delayed standard treatment of pediatric HBV and HCV infection until early 2017 (El-Shabrawy and Hassanein, 2019).

2.6.Hepatitis:

Hepatitis can be defined as inflammation of the liver. (Myers *et al*., 2014) It is also a global health problem that causes liver dysfunction (Al-Sadiq *et al*., 2017). This inflammation may be due to many causes, of which viral infection was the main cause, and also leads to significant mortality and morbidity. Viral hepatitis is caused by infection with 1 out of 5 recognized viruses, which primarily affect the liver, hepatitis A, B, C and D, as well as E viruses (HAV, HBV, HCV, HDV, as well as HEV) (Sayed and Mullman, 2019). Many people with hepatitis have no symptoms, while others have yellow skin discoloration, diarrhea, fatigue, poor appetite, abdominal pain, as well as vomiting (Kanawade *et al*., 2019; Sayed and Meuleman, 2019).). Also, hepatitis is considered acute if cured within 6 months, while it is defined as chronic if it persists for more than 6 months (Shahzad *et al*., 2018; Keykhosravi *et al*., 2019). Moreover, the pathway by which hepatitis viruses cause viral hepatitis has been excellently identified in relation to hepatitis C and B (Longo *et al*., 2013). Nor do viruses directly trigger apoptosis (cell death) (Nakamoto and Kaneko, 2003; Longo *et al*., 2013). However, hepatitis activates innate and adaptive arms in relation to the immune system resulting in an inflammatory response that leads to death and cellular damage (Nakamoto and Kaneko., 2003 ; Longo *et al*., 2013). The Figure (2.1) shows the difference between a healthy liver and an infected liver



Figure(2.1):A comparison between a normal liver and a liver after hepatitis (Malone, 2016).

2.7. Classification of viral hepatitis:

2.7.1.Hepatitis A virus (HAV):

This type may cause acute disease that will not develop into chronic liver disease. Thus, the main role with respect to screening is to assess the immune status of individuals at high risk of contracting the virus, as well as individuals with liver disease due to this type of infection that may lead to liver failure (Prevention, 2005., Norsina, 2019). People in these groups who aren't already immune may receive a vaccine against the hepatitis A virus.

2.7.2.Hepatitis B virus (HBV):

This species was partially a double-stranded DNA virus belonging to the family Hepadnaviridae, in the genus Osteovirus (Al-Sadiq *et al*., 2019) and could be considered as a causative agent of HBV, causing acute and chronic infections of hepatitis. Chronic infection with hepatitis B virus may progress to hepatocellular carcinoma (HCC) as well as cirrhosis, resulting in death. Thus, it has been identified as a life-threatening virus in the world, causing significant morbidity (Li *et al*., 2019). For this type of hepatitis, there is a three-dose vaccine that can protect against the risk of infection

2.7.3.Hepatitis D virus (HDV):

This type is one of the smallest virus-infected individuals, and hepatitis delta virus has been the cause with respect to fulminant hepatitis which is a rapid progression of liver disease in settings related to chronic hepatitis infection (Harichandran *et al.,* 2019). HDV can be considered as a defective satellite RNA virus that requires an auxiliary function associated with HBV for replication as well as assembly of new virions (Rizzetto., 2019).

2.7.4.Hepatitis E virus (HEV):

This type of hepatitis E is a causative agent and was initially identified in 1983 using electron microscopy as a viral spheroid with a size of (27-30). Also, the virus originated from the feces of a volunteer who had oral infection from the faces of non-HAV as well as non-HBV suspected cases (Balayan *et al*., 1983; Meister *et al*., 2019).

2.7.5.Hepatitis c virus (HCV):

2.7.5.1.Hepatitis C Virus epidemiology:

Based on (WHO 2013), around 150 million people are chronically infected with HCV worldwide. figure (2.2) shows the distribution of hepatitis virus spread around the world ,and there are approximately (3 to 4) new cases each year. Also, Seroprevalence varies with geographic regions, as the last four evaluations predicted that about 4% of populations in the Middle East, North Africa, South, East, Central, and Southeast Asia were infected, while 2% of the North American and Central and Western

European populations became infected. (Mohd Hanafiah *et al*., 2013, Sharma & Feld, 2014). The virus is also transmitted directly through blood or its derivatives and is sometimes related to parenteral therapy in addition to the use of intravenous drugs (Chesari, 2005).

However, another less specific route is mother-to-child transmission (MTCT), which occurred in about 5% of cases, and was also the main route with respect to hepatitis C infection in children (Yeung and Roberts, 2010, Abd al-Hadi *et al*., 2011) were asymptomatic for several years until songs related to liver damage appeared (Sharma and Field., 2014). Of infected individuals, approximately 70% will present with chronic infections, despite the presence of humeral and cellular immune responses against the virus (Bowen and Walker., 2005). Once chronic infection is detected, about 20% will develop cirrhosis as well as 2.5% will develop hepatocellular carcinoma (HCC). However, both conditions have been associated with end-stage liver disease, and these patients may also need liver transplants (Hoofnagle, 2002; Afdhal, 2004 ; Sharma and Feld, 2014).

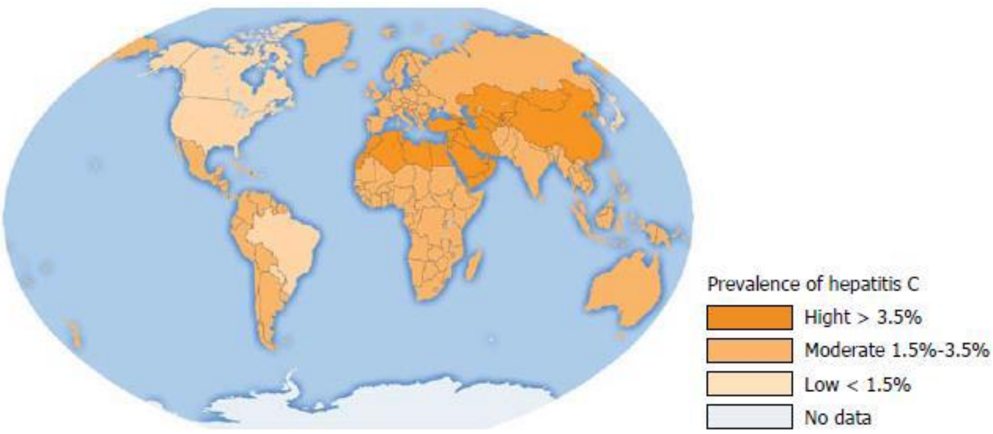


Figure (2.2): Hepatitis C virus infection in the World (Hanafiah, 2013).

2.7.5.2.The structure of HCV:

2.7.5.2.1.Virus particles:

Hepatitis C virus (HCV) is a virus enveloped in the positive-sense RNA genome in the genus Hepatitis C viruses related to the family Flaviviridae. Each virus particle has a size of about (55 nm–65 nm) (Kaito *et al*., 1994; Shimizu *et al*., 1996). Virus-associated transmission electron microscopy (EM) was hampered by the lack of a cell culture system that produced a sufficient amount of two viruses for visualization. However, recent work using the JFH1 cell culture system has enabled more virus-related characterizations (Wakkita *et al*., 2005). By studying other viruses related to the family Flaviviridae, it was indicated that HCV has decahedral arrangements in which structural glycoproteins E-1 as well as E-2 are incorporated into a bilayer lipid envelope derived from host cells. Also, the core protein forms a virus-related nuclear envelope that surrounds the RNA genome (Ishida *et al*., 2001). Moreover, there were 3 isoforms associated with this virus in serum with respect to infected individuals that include free virus and immunoglobulin-related insights as well as insights into low-density lipoproteins as well as extremely low-density (Bradley *et al*., 1991; .Thomssen *et al*., 1993)

2.7.5.2.2.Positive sense RNA genome:

HCV contains a positive-sense single-stranded RNA genome, approximately 9.50 kb. Also, the genome includes a single open reading frame flanked by 5' plus 3' untranslated regions (UTR) which are important for genome replication. The highly conserved 5' UTR is 341 nucleotides long, and also contains an internal ribosomal entry site including 4 major RNA domains along with extensive secondary structures. Moreover, the entire 5 UTR was important for IRES activity and HCV translation (Jubin *et al*., 2000, Lukavsky *et al*., 2000, Beales *et al*., 2001). Also, UTR 3 was important for the initiation of viral complications. They include poly(U)/polypyrimidine, a variable sequence of 40 nucleotides, and also a highly conserved sequence of 98 nucleotides with invariant secondary structures (Kolekhalov *et al*, 1996; Tanaka *et al*., 2000).

2.8 .Viral replication:

The process of HCV replication occurs in sponge-like inclusions attached to the ER membrane . Formation associated with such a membrane network was initiated by viral NS4B proteins, which may be of high importance for the assembly of non-structural proteins together at the replication site, including RNA-dependent RNA polymerase (RdRp) (Elazzar *et al*, 2004). Also, HCV replication proceeds via the synthesis of NS-5B complementary negative RNA from the positive-stranded genome. Also, viral (RdRp) based on an intermediate RNA template to generate a positive strand genome (Moradpor Penin and Rice, 2007;Suzuki *et al*., 2007; Kim and Chang, 2013;and Gu and Rice, 2013). Moreover, NS5B would initiate synthesis with respect to the genomes of positive and negative strands in the primer-dependent approach, in which the structures in the 3'UTR were '42-loop back transcripts. Also, the (NS-5B RdRp) was very error prone and would lead to multiple errors during the replication process (Tuplin *et al*., 2012).

Errors incorporated as viral genome-associated mutations as well as positive-stranded replicons were sometimes 100% complementary to the template (Behrens *et al*., 1996; Elazzar *et al*., 2004; Friebe *et al*., 2005). Remarkably, NS5B-related polymerase activity has the ability to transcribe heterologous and homologous RNA templates in a primer-dependent manner (Behrens *et al*., 1996). However, it is not understood how specificity of the HCV template can be achieved. It has been suggested that the regulation related to NS-5B domains may be important (Gu and Rice, 2013).

2.9.Liver function tests:

2.9.1. Oxaloacetic Transaminase (GOT):

Aside from being present in high concentrations in the liver, ALT is found in the kidneys, heart, and muscles. It catalyzes the conversion reaction, and is found only in cytoplasmic form. Any type of liver injury such as hepatitis can cause elevated ALT (Kasper *et al*., 2018). Elevation up to 300 IU/L is not specific to the liver, but could be due to damage to other organs such as the kidneys or muscles.. It can be due to hepatitis, ischemic liver injury, liver cancer, cirrhosis, and toxins that cause liver damage. (Shivaraj *et al*., 2009).

2.9.2. Glutamic-pyruvic transaminase (GPT):

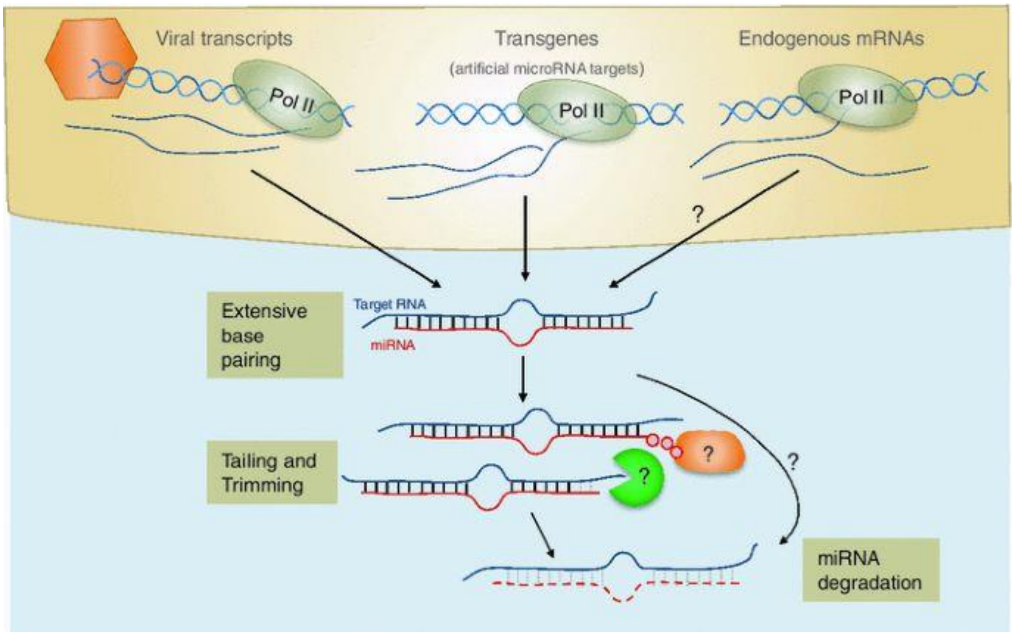
Aspartate transaminase is present in two isoenzymes, the mitochondrial form and the cytoplasmic form. It is found in highest concentration in the liver, followed by the heart, muscles, kidneys, brain, pancreas, and lungs. Compared to ALT. A significant increase in AST in the blood mitochondria indicates chronic liver disease. The mitochondrial form of balanced enzymes contributes to more than 80% of the activity of AST in the liver. AST is elevated particularly markedly in those with cirrhosis and hepatitis C & B especially (Shivaraj *et al*., 2009).

2.10.The Role of microRNA on Hepatitis Virus genome (RNA virus):

2.10.1.MicroRNAs (miRNAs):

MicroRNA (abbreviated miRNA) is a small, single-stranded, non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals, and some viruses, and functions in RNA silencing and post-transcriptional regulation of gene expression. (Qureshi *et al*., 2014; Bartle, 2018). miRNAs work by base pairing with complementary sequences within mRNA molecules. (Bartel, 2009). The Figure(2.3): show Regulation of microRNA.

As a consequence, these mRNA molecules are silenced, by one or more of the following processes: mRNA strand splitting into two pieces, mRNA destabilization through shortening of its multiple tail (A), and less efficient translation of mRNA into proteins by ribosomes (Bartel., 2009; Fabian *et al*., 2010). miRNAs are similar to small interfering RNAs (siRNAs) of the RNA interference (RNAi) pathway, except for miRNAs derived from regions of RNA transcription that fold back on themselves to form short hairpins, whereas siRNAs are derived from longer regions of double-stranded RNA. (Bartel, 2004). The human genome may encode more than 1900 miRNAs, (Bartel, 2009) although more recent analysis suggests the number is closer to 2300 (Alles *et al*., 2019), however, only about 500 human microRNAs represent the honest mRNA in Manual coordination. MirGeneDB Gene Database (Froome *et al*., 2020). As for the relationship between miRNA and hepatitis, several studies have shown that aberrant expression of specific miRNA can be detected in serum and plasma of hepatitis patients or hepatocellular carcinoma cells and tissues (Kumar, 2016).



Figure(2.3): Regulation of microRNA(Krüger & Rehmsmeier, 2006).

2.10.2. miRNA with hepatitis:

miRNAs are oligonucleotides of a small non-coding RNA structure consisting of 18–24 nucleotides (22 nt average) transcribed from highly conserved DNA regions but not translated into protein. miRNAs play an important role in the processing, regulation, and similar post-transcriptional levels of genetic information within cells in all multicellular eukaryotic organisms (Lee *et al* .,1993; Bartel, 2009, Kozomara, Griffiths-Jones. 2014). miRNAs are involved in many pathways that are critical to the cell and therefore, when they fail to function, may lead to disease susceptibility, particularly cancer (Lee *et al*., 2003). There has been a recent increase in the number of studies investigating the role of miRNAs in regulating various cellular processes such as energy production, protein synthesis, proliferation, differentiation and apoptosis (Bartel, 2009).

In the onset and progression of cancer, macromolecules (miRNA) act as tumor suppressors or oncogenes depending on the characteristics of the target genes (Bartel, 2009). Disruption of normal miRNA expression patterns has been reported in various liver diseases ranging from chronic hepatitis B (CHB) to cirrhosis (Ambros., 2004; Huntzinger; Izaurralde, 2011, Fabian and Sonenberg. 2012).

Diagnosing people with hepatitis at an early stage before clinical signs and symptoms appear is an urgent need to improve prognosis (Lewis *et al*., 2005). And was to determine gene-miRNA-21 expression, and gene-miRNA-122 expression levels in HCV cases. and associated with hepatitis B virus and HCV and their ability to become biomarkers. The miRNAs included in the study were identified by a literature review. , while other microparticles were selected from the microparticles, as studies on this topic are just beginning. (Bartel., 2009).

Discuss the applications of miRNAs as biomarkers for hepatitis diagnosis and prediction, the relationship between miRNA polymorphisms and hepatocellular carcinoma risk as well as prognosis A large number of reports have shown deregulation of miRNAs in human hepatitis, and these miRNAs can be used as new potential biomarkers for hepatitis diagnosis and prediction. Avene diagram demonstrated the role of miRNAs in hepatitis diagnosis, prognosis, or interference. Much effort has been made to develop non-invasive circulatory biomarkers for the diagnosis of hepatitis (Sonenberg., 2012 .).

2.10.3.miRNA-122 and effect of hepatitis patients.

MiR-122 is a conserved miRNA among vertebrate species. There is no miR-122 in invertebrates, and no close analogues of miR-122 have been detected (Kozomara *et al*., 2018) miR-122 is highly expressed in the liver, where it has been considered a regulator of fatty acid metabolism in rat studies. Low miR-122 levels are associated with hepatocellular carcinoma. miR-122 also plays an important positive role in regulating hepatitis C virus replication (Lagos-Quintana *et al*., 2002). miR-122 expression increases during embryonic development until it constitutes 72% of the total miRNA in the adult human liver, making it one of the most highly expressed miRNAs of any tissue (Wienholds *et al*., 2005).

Hepatitis C virus (HCV) affects more than 71 million individuals worldwide and is a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Ono *et al*., 2020). One of the most important host factors for hepatitis C virus infection is the liver-specific microRNA (miRNA), miR-122 (Zukerkandel and Pauling, 1965). On the other hand, chronic HCV infection is often associated with additional hepatic manifestations such as mixed serum globulin, B-cell lymphoma, thyroiditis, and diabetes mellitus (Galosi *et al*., 2007).

Supported by clinical observations, low levels of HCV-RNA replication have been detected in PBMCs and neural tissues in chronic hepatitis C patients (Castillo *et al*., 2005, Wilkinson ,2009; Radkowski, 2009) and in patients with chronic HCV that has progressed. B-cell lymphoma with direct-acting antivirals for HCV and lymphoma (Maciocia *et al*., 2016). These observations indicate that HCV replication can be established in non-hepatic cells in the state of miR-122 deficiency. In general, miRNAs negatively regulate translation of the target mRNA by interacting with the 3'UTR in a sequence-specific manner. In this way, miR-122 regulates gene expression involved in the maintenance of liver homeostasis, including lipid metabolism, iron metabolism, and carcinogenesis, (Hsu *et al*., 2012, Tsai *et al*., 2012).

In contrast, miR-122 has also been shown to stabilize HCV-RNA (Shimakami et al., 2012) and promote translation mediated by the internal ribosomal entry site (IRES) (Zuckerkandl and Pauling, 1965; Henke *et al*

2008, Roberts *et al*., 2011) and replicate (Masaki *et al*., 2015) for HCV-RNA by direct interaction with the 5'UTR of HCV (Jopling *et al*., 2008; Machlin and Sarnow, 2011). Recently, it has been reported that miR-122 has a function similar to that of RNA that stimulates HCV IRES folding so that it can readily associate with 80S ribosomes for efficient translation (Canizares *et al*., 2018; Schult *et al*., 2018).

2.10.4. miRNA-21 effect of hepatitis patients:

MicroRNA 21 also known as hsa-mir-21 or miRNA21 is a mammalian microRNA that is encoded by the MIR21 gene. (Lagos-Quintana *et al*., 2001). miRNA-21 was one of the first mammalian microRNAs identified. The mature miR-21 sequence is highly conserved throughout the evolution period. The human microRNA-21 gene is located on the strand of chromosome 17q23.2 (55273409-555273480) plus within the gene encoding TMEM49 (also called vacuolar membrane protein). Although it is located in the intronic regions of the gene coding downstream of transcription, it has its own promoter regions and forms a long primary miR-21 (known as pri-miR-21) transcript that is independently transcribed (Lagos-Quintana *et al*., 2001). Several have demonstrated potential value as biomarkers for detection and prediction of liver disease progression in viral hepatitis infection (Motawe *et al*., 2015). MiRNA-21 is one of the most important miRNAs that are highly expressed in HCV-infected patients. Recent studies have also revealed that in liver needle biopsies from HCV-infected patients, miRNA-21 level correlates with positive fibrosis stage and can eventually lead to increased fibrosis by targeting SMAD7 in transforming growth factor, in contrast to all other malignancies, Studies of the prognostic effects of miRNA 21 expression in hepatitis patients have been debated. Multiple studies revealed that higher levels of miRNA-21 were associated with improved prognosis in hepatitis (Lawrie *et al*., 2007; Lawrie *et al*., 2008; Chen *et al*., 2014), while others suggested a weak prognostic effect of miRNA-21. (Li *et al*., 2015).

2.11. Expression of miRNA in HCV-induced liver diseases:

Hepatitis C virus infection causes liver-specific injury, including progressive cirrhosis, cirrhosis, and hepatocellular carcinoma. These diseases result from both the direct modulation of cellular metabolism by viral proteins and the indirect consequence of the host's response to HCV infection. Cellular miRNAs are important factors in the development of hepatitis C-induced liver disease. Accumulating evidence shows that changes in RNA caused by HCV infection subsequently influence the development of cirrhosis. After comparing chronic hepatitis C patients with normal controls (Ramachandran *et al*., 2013).

Recent studies have also revealed that in liver needle biopsies from HCV-infected patients, the level of miRNA-21 is positively correlated with the stage of fibrosis and can eventually lead to increased fibrosis by targeting SMAD7 in transforming growth factor (Sarma *et al*., 2012). TGF-β signaling pathway, while miR-122 level negatively correlates with the fibrotic stage (Marques *et al*., 2010), miRNAs are directly involved in hepatocyte carcinogenesis in hepatitis C virus-infected patients (Negrini *et al*, 2011). In 2006, after analyzing microRNA expression profiles in 25 pairs of hepatocellular carcinomas and adjacent non-neoplastic tissues and nine additional samples of chronic hepatitis using a human miRNA microarray, it was found (Murakami *et al*., 2006).

That eight miRNAs have significantly different expression patterns. Among them, miR-92, miR-20, miR-18 and precursor miR-18 had a marked negative correlation with HCC differentiation, while the expression of miR-99a was consistent with the degree of tumor differentiation. Similarly, after validating the expression of 2,226 miRNAs in HCC patients infected with HCV, 2 (Diaz *et al*., 2013) identified 18 miRNAs exclusively for HCC, including three novel HCC-associated miRNAs, miR-497, 1269 and miR-424-3pexpression are several critical RNA molecules that highlight their clinical implications in liver disease progression. Analysis of changes in miRNAs and host mRNAs However, only some abnormal expression of miRNAs was directly induced by HCV, although altered expression of miRNAs in liver disease has been frequently reported on several critical miRNAs highlighting their effects, Clinical progression of liver disease, Analysis of changes in host mRNAs and miRNAs (Negrini *et al*., 2011).

2.12. Immunological Test:

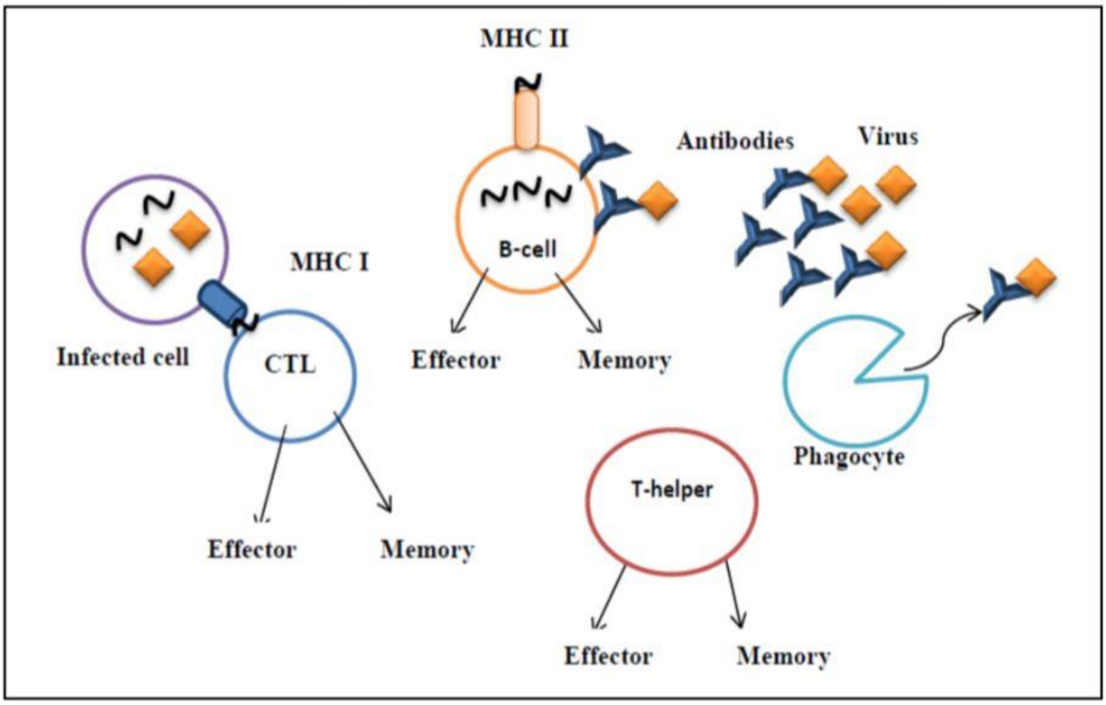
2.12.1. Human leukocyte antigen (HLA):

Human leukocyte antigen (HLA) is the name of the human Major Histocompatibility Complex MHC complex (Matsumura *et al*., 1992 ; Choo., 2007 ; Nakamura *et al*., 2019) The genetic locus of these antigens contains a number of genes associated with the immune system. These genes are located on chromosome 6 and most genes carry the genetic code for cell surface antigen presentation proteins. These proteins have been called antigens because of their historical relationship to organ transplantation. These antigens play a major role in the immune system, with each type having its own function specific HLAs corresponding to **MHC class I** (A, B, C, and G), and each HLA Class1 group, presenting peptides from within the cell (Nakamura *et al*., 2019). For example, if a cell is infected with a virus, the HLA system brings virus fragments to the cell surface so that the cell can be destroyed by the immune system by CD8

Tcell. These peptides are produced from digested proteins which are degraded into proteasomes. In general, these particular peptides are small polymers, with a length of about 8-10 amino acids. (Matsumura *et al* .,1992; Bouzid *et al*., 2021).

MHC class II proteins bind to β2-microglobulin, which in contrast to HLA proteins is encoded by a gene located on chromosome 15 HLAs corresponding to MHC class II (DP, DM, DO, DQ, and DR) displaying extracellular antigens to T lymphocytes. These specific antigens stimulate the proliferation of helper T cells (also called CD4 positive T cells), and the self-antigens are suppressed by regulatory T cells. It is difficult to predict which (parts) of the antigens will be presented to the immune system by a particular type of human leukocyte antigen, but the technology involved is improving(Taylor *et al*., 2011).

The antigen-presenting cells (APCs) engulf the pathogen through a process called phagocytosis. Proteins from the pathogen are digested into small pieces (peptides) and loaded onto human leukocyte antigens (specifically, MHC II), and then presented by antigen-presenting cells to helper T cells, (Taylor *et al*., 2011). In process, proteins (both local and foreign) produced within most cells are presented to human leukocyte antigens (specifically, MHC class I) at the cell surface. Infected cells can be recognized and destroyed by T cells (Agawal *et al*., 2017) Any cell expressing another type of HLA is 'non-self' and is viewed by the body's immune system as an invader (Korko *et al*., 2013; Jokiniemi *et al.*, 2020).



Figure(2.4): The immune adaptive responses (MHC1&MHC2) (Hadi, 2017)

2.12.2. HLA with Hepatitis infection:

Hepatitis C virus is estimated to cause cirrhosis, and a subset of these individuals eventually develop liver failure or hepatocellular carcinoma (Lai *et al*., 2003). A complex of environmental, pathogen and host genetic factors play a role in determining susceptibility to hepatitis B and HCV and the course of infection (Segal and Hill., 2003). Several epidemiological factors, such as age at infection, sex, chronic alcohol use, and co-infection with other hepatitis viruses, are suspected to influence virus persistence. In addition, host immune factors and genetic background have been considered to influence susceptibility to persistent hepatitis and its outcome (Frodsham, 2005). Family studies provide some evidence that host genes influence virus persistence, as concordance rates for HBeAg in HBV persistence were higher in identical twins than in non-identical twins (Lin *et al*., 1989). Several human leukocyte antigens (HLAs) (Kamatani *et al*., 2009; Guo *et al*., 2011) have been associated with hepatitis, and none of these has been shown to be conclusive. The mechanism of susceptibility to chronic HCV infection is not well elucidated. As the outcome of hepatitis C virus infection depends primarily on the host immune response, and HLA, an integral component of the immune response, plays an important role in the immune response to hepatitis C infection (Godkin *et al*., 2005).

The HLA gene is polymorphic and the significant polymorphism has been considered a suitable biological gene for a candidate allergy associated with the development and progression of chronic hepatitis C infection. Indeed, previous studies have shown that HLA-DR polymorphisms influence individual immune responses, and thus influence disease outcome. Antigens play a role in immune surveillance and the immune response, and in many of the studies that have been performed, conclusive evidence for the association between polymorphisms and outcome of hepatitis-infection is still lacking (Saito *et al*., 2008); The relationship between them is not universal for all populations examined. In this meta-analysis, the identification of co-occurring HLA-DR and HLA-G was examined by a systematic review of the literature followed by a meta-analysis of all case-control studies. Meta-analysis is a powerful method for quantitatively summarizing the results of different studies (Khurami *et al*., 2015).

2.12.3. Human leukocyte antigen (HLA-G):

Human leukocyte antigen (HLA-G) is a non-classical human MHC class I cell surface receptor , presenting a tissue-restricted expression pattern and encoding molecules with immunomodulation properties. HLA-G, which was first described by Geraghty and Koller in 1987 (Geraghty and Koller, 1987), presents a genetic structure similar to other classical HLA class I genes. However, in contrast to what was observed in the classical class I genes (HLA-A, -B and -C), the HLA-G is completely conserved between different populations and within the same population, (Donadi *et al.*, 2011) .

Providing only a few of non-synonymous mutations and several sites of variation characterized as synonymous modifications, interionic variations, or altered sites in regulatory regions. HLA-G does not appear to initiate immune responses like its conventional counterparts. Alternatively, HLA-G is associated with the induction of inhibitory stimuli in T and B lymphocytes (Nagy *et al.*, 2014), natural killer (NK) cells (Rouas-Freiss *et al.*, 1997), and antigen-presenting cells (APC) (Horuzsko *et al.*, 2001) HLA-G may directly interact with multiple inhibitory receptors, HLA-G was first detected in the trophoblast at the maternal-fetal interface, and HLA-G was detected in few normal tissues, including the cornea (Le Discorde *et al.*, 2003).

Much effort has been devoted to assessing HLA-G . Gene regulation after transcription. Given that the structure of HLA-G molecules has been conserved throughout evolution, the amount of molecules produced may depend primarily on factors that modulate gene expression by transcriptional and post-transcriptional mechanisms, and hepatic HLA-G expression has been associated with hepatitis virus-infected liver samples. Epidemiological has milder stages of fibrosis (Crispim *et al.*, 2012). Hemosiderin deposits (Menier.et al., 2004) Besides hepatocytes, HLA-G expression was observed on mast cells located in areas of cirrhosis (Amiot *et al*, 2014). Increased plasma HLA-G levels have been associated with chronic hepatitis C virus infection (Weng *et a.*, 2011), since mast cell treatment with IL-10 and class I interferon stimulates HLA-G expression (Amiot et al., 2014), an important role in Maintaining chronic infection and inducing chronic complications. One study linked increased HLA-G expression in hepatocytes with HBV viral load (Souto *et al*., 2011). Various studies have associated increased serum/plasma HLA-G levels with hepatitis B and C virus infection (Shi *et al*., 2011; Park *et al* ., 2012; Han *et al*., 2014; Bertol *et al*., 2020).

2.12.4. Human leukocyte antigen (HLA-DR):

Human leukocyte antigen (HLA-DR) is a class II MHC cell surface receptor encoded by the human leukocyte antigen complex on chromosome 6 region 6p21.31. The HLA-DR (human leukocyte antigen homotype-DR) complex and peptide, generally between 9 and 30 amino acids in length, form a T-cell receptor (TCR) binding (Solomon *et al*., 2015). HLA-DR is also involved in many autoimmune diseases, disease susceptibility and disease resistance. It is also closely related to HLA-DQ and this association often makes it difficult to resolve the more etiological factor (Klitz *et al*., 2003).

HLA-DR molecules are upregulated in response to signals. In the event of infection, the primary function of HLA-DR is to present peptide antigens, potentially foreign in origin, to the immune system for the purpose of eliciting or suppressing (helper) T-cell responses that ultimately lead to the production of antibodies against the same antigen. peptide; Antigen-presenting cells (macrophages, B cells, and dendritic cells) are the cells in which DR is typically present (Solomon *et al*., 2015).. Increased abundance of DR antigen at the cell surface is often in response to stimulation, and thus, DR is also a marker of immune stimulation. Although hepatitis C virus elicits innate immune responses early after infection, the virus can overcome immunity and persist. Viral clearance occurs only in the presence of antiviral CD4+ and CD8+ cell responses (Hwang *et al*., 2006) Human leukocyte class I and class II antigens (HLA) are ideal candidate genes for studying associations between HCV infection and outcome. It has been shown that specific HLA alleles influence the outcome of HCV infection (Urabe *et al*., 2013).

The second category consists of three major genes DR, DQ and DP. HLA-DR consists of multiple genes and DR is the most numerous and important which gives variability in the immune response. HCV infection has been associated with a risk allele: (El-Bendary *et al.*, 2016) Several studies have reported that HLA-DRB1 alleles may play an important role in susceptibility to hepatitis C virus infection or clearance and may also determine response to antiviral drugs. . (Thio *et al.,* 2001 Tripathy *et al* 2009; Liu *et al*., 2019) The responsibility of HLA-DR alleles for susceptibility to hepatitis C virus is highly variable from one group to another. (Theo *et al*., 2001).

2.13. HCV Complication:

2.13.1. Hepatitis, fibrosis, steatosis, and cirrhosis:

In general, hepatitis C virus is not considered as a cytopathic virus. It is assumed that the damage to the liver is primarily caused by the mechanism of immune pathogens. Nothing less, hepatitis C virus was associated with direct effects of cytopathic on the liver such as apoptosis, progression of steatosis, activation of stellate cells, and insulin resistance(Iwaisako *et al*., 2012). Liver damage is monitored clinically by increased levels of ALT. In cases of hepatitis C, liver biopsies are usually taken to assess the stage of liver disease. Hepatitis C occurs after inflammatory cells infiltrate the liver and damage to hepatocytes leads to cirrhosis and steatosis. Hepatic steatosis refers to the accumulation of fat in the liver cells which may eventually lead to the development of fibrosis. Cirrhosis is recognized by accumulations of IF matrix proteins, such as collagen, that are generated from different types of hepatocytes after damage. Cirrhosis can be reversed (Iwaisako *et al*., 2012). Cirrhosis can be considered as a final stage of liver disease, in which liver tissue is replaced by scar tissue and nodules that reduce liver function. Cirrhosis is not reversible and patients who are in advanced stages of cirrhosis will need a liver transplant (Mengshol *et al.*, 2007).

2.13.2. Lympho-proliferative disorders:

One of the optimal non-hepatic disorders identified associated with HCV infection is mixed globulinemia (MC) (Lunel., 1994), which results from the development of hepatic globulin and is composed of polyclonal IgG and monoclonal IgM or polyclonal IgM with rheumatoid factor that It is deposited in the blood at a temperature of less than 37 °C (Zignego, 1997) and signs include arthralgia, weakness and palpable purpura of the lower extremities and complications may include kidney damage and possibly liver damage as well (Ferry 1992; Kayali, 2002; and Saadoun 2006). About 5%-10% of patients infected with HCV MC (Lunel, 1994) successfully treated hepatitis C virus produces mixed cryoglobulinemia in the majority of patients (Zignego, 2007). HCV is also associated with malignant lymphomas that can be associated with mixed cryoglobulinemia. B-cell-derived non-Hodgkin's lymphoma may be considered the most common lymphoma associated with HCV infection (Devita ,1997).

2.13.3.Hepatocellular carcinoma (HCC):

Hepatitis C infection is a major risk factor for developing hepatocellular carcinoma (Hassan *et al*., 2002). The approach by which hepatitis C virus is produced in hepatocellular carcinoma is not yet known, however, a number of studies have indicated the effect of the core protein, which includes the induction of oxidative stress and steatosis. Cell signaling pathways are also altered by the core protein, as previously explained. The development of cirrhosis and cirrhosis of the liver increases the risk of developing liver cancer as well. About 1%-3% of hepatitis C patients develop hepatocellular carcinoma 3 decades after chronic infection (Hassan *et al*., 2002). Age, obesity, alcohol consumption, genotype, diabetes, and co-infection with HIV or hepatitis B virus may increase the risk of HCC in HCV-infected patients (Hassan *et al*., 2002).

CHAPTER THREE

MATERIALS

AND

METHODS

3.Materials and Methods:

3.1.Materials:

3.1.1. Study Subjects:

The presented work has been conducted on the basis of case-control study design .This study included three groups of subjects:

A- A total 60 specimens of blood samples have been collected from thalassemia subjects who have been admitted in Thalassemia hematology center in Babylon women &children Hospital in Babylon province / Iraq, during the period from September 2021 to February 2022. The patient's age were ranged from 3 to ≥ 40 years. The patient was diagnosed at the thalassemia syndrome patient was regularly attending the hematology clinic in Thalassemia hematology center in Babylon women &children Hospital ,either for transfusion and chelation and follow up of Hb level and iron status. Also, the diagnosis that is related to β-thalassemia has been on the basis of hemoglobin electrophoresis, hematological indices, clinical presentation, as well as the iron overload. An ethical verbal consent were taken from patients. **B-** 20 specimens were collected from apparently stable control group, who had no history of thalassemia. **C**-20 specimens were collected from apparently stable control group, Patients with Thalassemia, but not recorded with Hepatitis.

3.1.2.Equipment and Apparatus.

Equipment's and apparatus with their company names and origins used in the presented work have been shown in the table below. Equipment and apparatus

Table (3-1): The Equipment's and Apparatus which were used in the study

No	Item	Company	Country
1	Syringe	SUPER	China
2	Cotton	Kardelen	China
3	Sterile material (Ethanol)	Aljoud	Iraq
4	Gel tube	Afco	USA
5	EDTA tube	Memmert	Germany
6	Cold Centrifuge	FAORGEN	Taiwan
7	Deep freezer(-40°C)	Thermo-Fisher	Japan
8	Micropipette from100-1000 . micro letter Micropipette from 0.1-3 micro letter Micropipette from 10-100 micro letter Micropipette from 0.5-10 micro letter	Dragon lab.	USA
9	Vortex mixer	Dragon lab.	USA
10	Nanodrope .	Dragon lab.	USA
11	PCR tube rack	Watson Bio Lab	Turkey
12	Pipette tips (blue)	Applied Biosysyem	USA
13	Pipette tips (yellow)	Applied Biosysyem	USA
13	Tips (various volumes)	Applied Biosysyem	USA
14	Cool box	Tank	India
15	Cloves	Latex	China
16	PCR tube	Bionear	Japan

17	Collection tube	Biocomma limited	Spain
18	Eppendorf tubes	Awareness technology	USA
19	RT PCR system	Qtower3G, Analyica	German
20	FARB Mini column	FAORGEN	China
21	Laboratory Fume Hood	GFL	Germany
22	Incubator	DIALAB	Austria
23	Absorbent paper	Nano Entek	Korea
24	Clean tube	Applied Biosysyem	USA
25	Microplate reader	Applied Biosysyem	USA
26	Precision pipettes and disposable pipette tips sodium citrate tube	Latex	China
27	Precision pipettes and disposable pipette tips	Tank	China

3.1.3.Kits and their contents:

The kits that have been used in the present study are shown in table(3-2).

Table (3-2): Kits and contents which were used in the study

NO.	Kits	Company	Country
1	Total RNA Mini Kit	FAVORGEN	USA
2	GoTaq® 1-Step RT-qPCR System	Promega	USA
3	Human Leukocyte Antigen – DR ELISA Kit (HLA-DR)	BT LAB	China
4	Human Leukocyte Antigen –G ELISA Kit (HLA-G)	BT LAB	China

3.1.4.Chemical and Biological Materials:

The Chemical and biological materials that have been used in the present study shown in table

Table (3-3): Chemical and Biological Materials used in this study

NO.	Materials	The industrial company
1	ß-Mercaptoethanol	INTRON BIOTECHNOLOGY
2	RNase-free ethanol	Promega
3	Ethanol	Biotech

3.1.5.Molecular Materials:

3.1.5.1.Primers:

The primers that used in the present study shown in table (3-4).

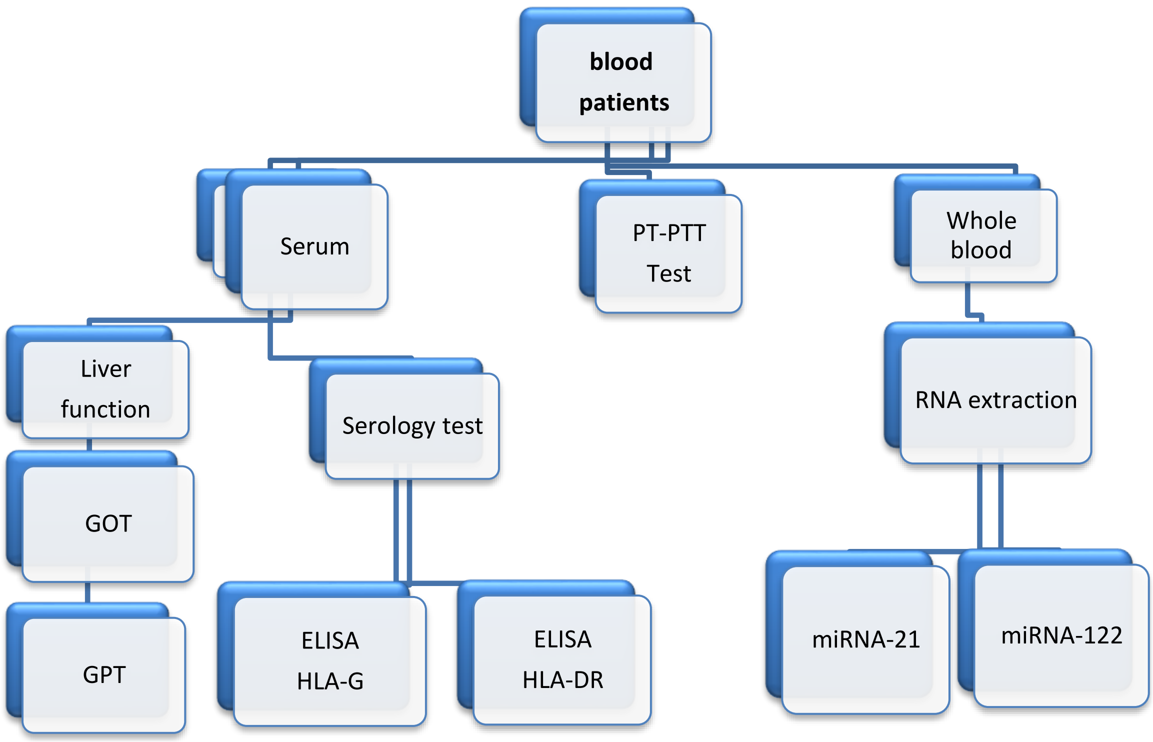
Table (3-4): Primers which were used in the study.

Gene of miRNA	Sequence	References
MiRNA-122	F: 5'-GCGAAA GCATTTGCCAAGAA-3	(Dai *et al*., 2019)
	R: 5'-CATCACAGACCT GTTATTGC-3'	
MiRNA-21	F: 5'-GCCGCTAGCTTATCAGACTGATGT-3'	(Zhuang *et al*., 2016)
	R: 5'-GTGCAGGGTCCGAGGT-3'	
H.K.gene U6	F: 5'-GTTTTGTAGTTTTGGAGTTAGTGTTGTGT-3'	(Zhuang *et al*., 2016)
	R: 5'-CTGAACCTACAATCAAAAACAACACAAACA -3'	

3.2.Methods:

3.2.1. Blood Collection:

Six milliliter of the venous blood acquired through vein puncture from each one of the subjected (controls and cases) included in this work, the blood has been almost equally divided into (2ml) EDTA tubes, also (3ml) in the gel tubes (the samples of blood have been collected into EDTA tubes and gel tubes were prepared and labeled). (2ml) blood sample obtained from each one was clot at room temperature for one hour, then the centrifugation has been conducted for a period of five minutes, at (4000) rpm for the serum separation. The sera was transferred by micropipette, used to determine the following biochemical and immunological parameters (GOT, GPT, Ferritin , HLA-DR , HLA-G), detection HCV antibodies. The sera were stored at -40 °C until the assay was done. Blood in EDTA tubes has been utilized for performing, for the extraction and purification of RNA for the purpose of carrying out the molecular diagnosis(miR-122 , miR-21), and 1ml in sodium citrate tube use to (PT , PTT) and other PCV, WBC from whole blood as can be seen in figure (3-1).



Figure(3-1): the steps of study.

3.2.2.Laboratory Investigations:

3.2.2.1 Hematological Analysis:

Packed cell volume, complete system related to reagents of control as well as to measure the proportion of blood calibrator, has been utilized for determining PCV related to Thalassemia hematology center in babylon women and children Hospital

3.2.2.2.Partial Thromboplastin Time(PTT):

The blood was drawn and was mixed immediately with anticoagulant (9:1). The mixture was centrifuged for 15 minutes at 2500. The test was done within two hours at room temperature. Plasma samples were stored frozen at -20℃ for up to two weeks, (NCCLS,Wayne, 1998).

Procedure: The sample mixture was placed in plastic or siliconized glass cuvette. 100µl plasma citrate (supernatant) was added. The 100µl of reagent (prewarmed to 37℃) was added. It was mixed and incubated for 3 – 5 minutes at 37℃. 100µl of prewarmed calcium chloride at 37℃, was added. The time for clot formation was recorded by the coaglomete

3.2.2.3.Prothrombin Time Test(PT):

Procedure : 100µl of the sample was put in plastic or siliconized glass cuvette. It was incubated for about 2 minutes at 37℃. 200µl of reagent (initially prewarmed at 37℃ was added. It was tested in duplicate

3.2.3.Biochemical analysis:

The Reflotron reagent strips of liver enzymes quantitative determination in human serum were used by Reflotron plus system device, which is an in vitro device using Reflotron test strip to diagnose the biochemistry parameters with insures reliable and rapid results. This test was used to determination of liver enzymes as following: Glutamic-Oxaloacetic Transaminase (GOT) and Glutamic-pyruvic transaminase (GPT).

-Procedure:

The device was switched on

Reflotron pipette was use to add (30) µl of sample to the center of the strip particularly on the application reading zone, with avoid warping the strip. ; The slide cover or flap was opened to place the strip in the designated area within 15 seconds only, then the slide was closed when finished. ; Each of GPT and GOT were automatically calculated by magnetic strips through 2minutes at 37℃ .The reading was recorded after measuring the depth of color at 567 nm.

3.2.4.Serological analyses:

3.2.4.1.ELISA for Human Leukocyte Antigen –DR to HCV (HLA-DR):

3.2.4.2.Intended Use :

This sandwich kit was uesd for the accurate quantitative detection of Human Leukocyte Antigen-DR , G (also known as HLA-DR) in serum, plasma, cell culture supernates, Ascites, tissue homogenates or other biological fluids.

3.2.4.3.Assay Principle:

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

3.2.4.4.Contents:

Standard Solution ; Pre-coated ELISA Plate ; Standard Diluent Streptavidin-HRP ; Stop Solution ; Substrate Solution A ; Substrate Solution ; Wash Buffer Concentrate (25x) ; Biotinylated Human HLA-DR Antibody ; User Instruction ;Plate Sealer ;Zipper bag

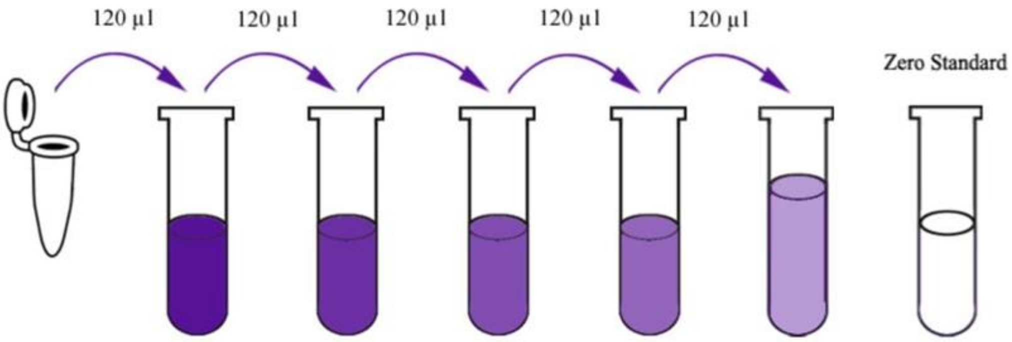
3.2.4.5.Reagent Preparation:

Before used all reagents were left to reach room temperature

Standard: Reconstitute the 120µl of the standard (40ng/ml) with 120µl of standard diluent to generate a 20ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. We Prepare duplicate standard points by serially diluting the standard stock solution (20ng/ml) 1:2 with standard diluent to produce10ng/ml, 5ng/ml, 2.5ng/ml and1.25ng/ml solutions. Standard diluent serves as the zero standard(0 ng/ml). Table (3-5): show Dilution of Standard Solutions for HLA-DR and HLA-G.

Table (3-5): Dilution of Standard Solutions for HLA-DR

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
4800U/ml	2400U/ml	1200U/ml	600U/ml	300U/ml	150U/m1



- **Wash Buffer** Dilute 20ml of Wash Buffer Concentrate 25x into deionized or 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

3.2.4.6.Assay Procedure:

1. Prepared all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature

2 . the number of strips required for the assay were Determine . The unuseded strips should be stored at 2-8°C. the strips were Insert in the frames for use. The unused strips should be stored at 2-8°C .

3 . Added 50µl standard to standard well. Note: Don't added biotinylated antibody to standard well because the standard solution contains biotinylated antibody

4 . Added 40µl sample to sample wells and then adding10µl anti-HLA-DR and G antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well)

5. Removed the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirating or decant each well and wash 5

times with wash buffer. Blot the plate onto paper towels or other absorbent material .

6. Added 50µl substrate solution A to each well and then adding 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark

7 . Added 50µl Stop Solution to each well, the blue color will change into yellow immediately

8. Determined the optical density (OD value) of each well immediately used a Microplate reader set to 450 nm within 10 minutes after adding the stop solution

3.2.4.7.Calculation of Result:

Drawn a best fit curve across the points on the graph by estimating the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis.

The measurements were better done using computer-based curve-fitting applications, also the best fit line could be found using regression analysis, as seen in figure (3-2, 3-3).

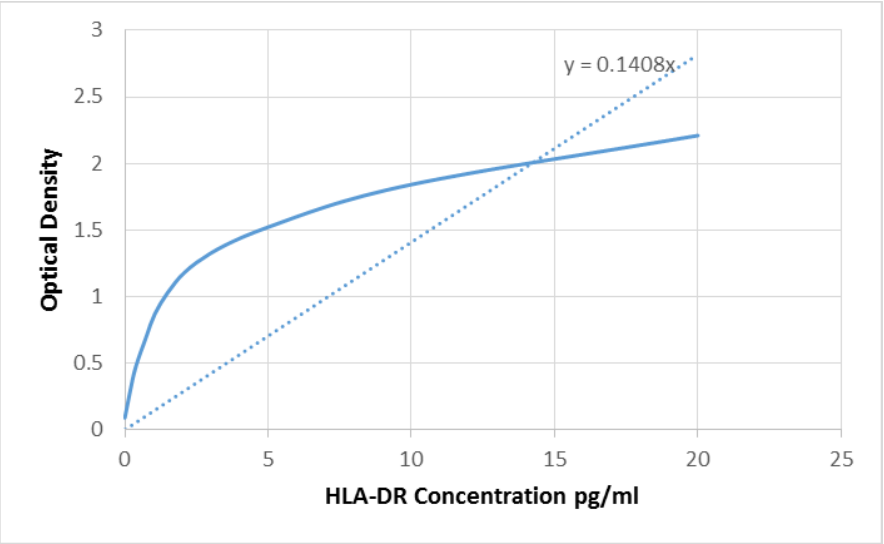


Figure (3-2): Curve the average of optical density of standard (450 nm) with the HLA-DR concentration U/ml

3.2.5.ELISA for Human Leukocyte Antigen –G to HCV (HLA-G)

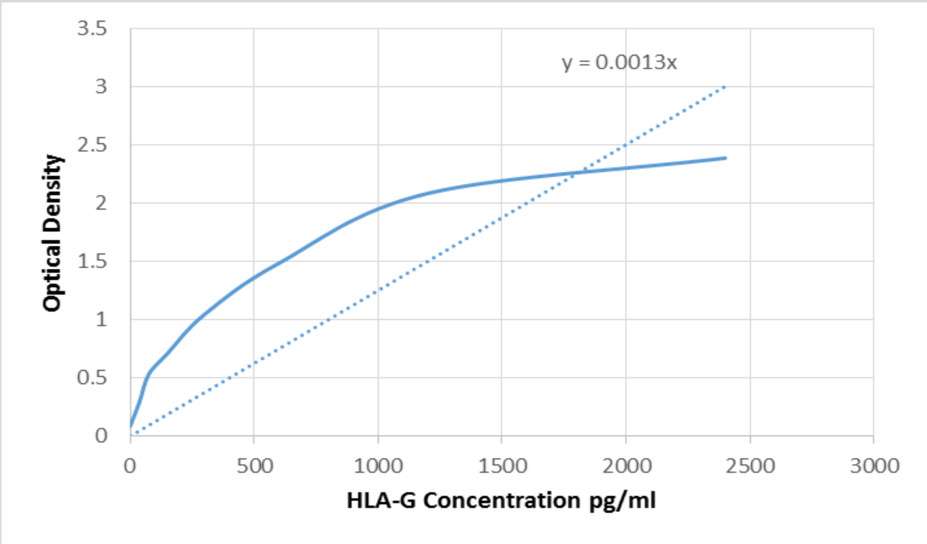


Figure (3-3): Curve the average of optical density of standard (450 nm) with the HLA-G concentration U/ml.

3.2.6.Molecular analyses:

3.2.6.1. Human RNA Extraction Kit:

Kit Contents: RL Buffer ,FARB Buffer, Wash Buffer , Wash Buffer 2 , RNase-free Water , FARB Mini Column , Filter Column , Collection Tube , Elution Tube , User Manual.

3.2.6.2.Protocol: Isolation of Total RNA from Human Whole Blood:

1.Red blood cells lysis

1-1 . Added 200 ~ 300 µl of anticoagulant-preserved fresh human whole blood to a microcentrifuge tube(1.5 ml or 2.0 ml tube) (not provided). If the sample volume is more than 200 ul, use a 2.0 ml tube as the sample container..

2-1. Mixed 5 volume of RL Buffer with 1 volume of the sample and mix well by inversion.

3-1 Incubated on ice for 10 min. Vortex briefly 2 times during incubation.

4-1. For 1 min at 4,500 rpm Centrifuge to form a cell pellet and discard the supernatant completely.

5-1. Added 600 µl of RL Buffer to resuspend the cell pellet by briefly vortexed.

6-1. For 1min at 4,500 rpm Centrifuge to form a cell pellet again and discard the supernatant completely.

2. Added 350 µl of FARB Buffer and 3.5 µl of ß-Mercaptoethanol to the cell pellet. Vortex vigorously for 1 min to resuspend the cells completely.

3. Placed a Filter Column to a Collection Tube and transfer the sample mixture to the Filter Column. Centrifuge at full speed (~ 18,000 x g) for 2 min.

4. Transferred the clarified supernatant from the Collection Tube to a new microcentrifuge tube , and measure the volume of the supernatant.

5. Added 1 volume of 70 % RNase-free ethanol and mix well by vortexes.

6. Placed a FARB Mini Column to a Collection Tube and transfer the ethanol added sample mixture (including any precipitate) to the FARB Mini Column. Centrifuge at full speed for 1 min, discard the flow-through and return the FARB Mini Column back to the Collection Tube.

7. Added 500 µl of Wash Buffer 1 to the FARB Mini Column, centrifugeat at full speed for 1 min.Discard the flow-through and return the FARB Mini Column back to the Collection Tube**.**

8. Adding 750 µl of Wash Buffer 2 to the FARB Mini Column, centrifuge at full speed for 1 min.Discard the flow-through and return the FARB Mini Column back to the Collection Tube.

9. Repeated step 9 for one more washing.

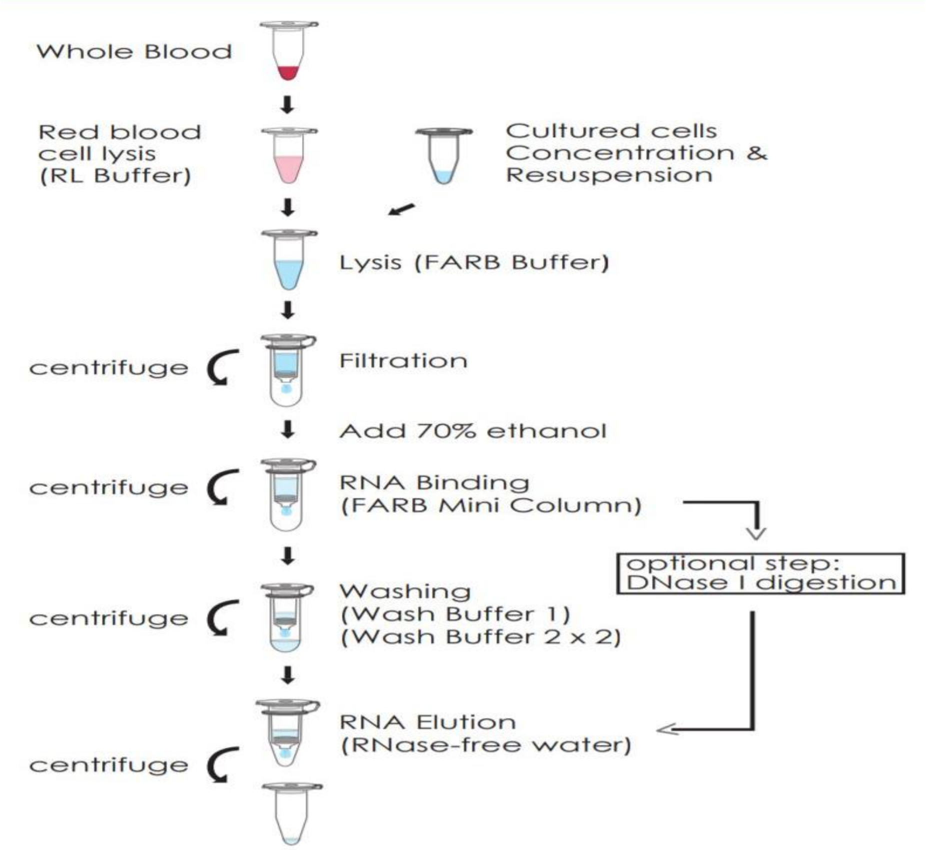
10. were Centrifuge the FARB Mini Column at full speed for an additional 3 min to dry the FARB Mini Column.This step were will avoid the residual liquid to inhibit subsequent enzymatic reaction-.

11. Placed the FARB Mini Column to a Elution Tube (1.5 ml microcentrifuge tube).

12. Added 40 ~ 100 µl of RNase-free ddH2O to the membrane center of the FARB Mini Column Stand the FARB Mini Column for 1 min

13. Centrifuge the FARB Mini Column at full speed for 1 min to elute RNA.

14. RNA at -70C were Store. The figure below represents the method of added the solutions.



Figure(3.4):Prief procedure

3.2.6.3. GoTaq 1-Step RT-qPCR System Kit.

- **Product Components**

Includes:

• 5 × 1ml GoTaq qPCR Master Mix, 2X
• 225µl GoScript™ RT Mix for 1-Step RT-qPCR
• 200µl CXR Reference Dye, 30µM
• 750µl MgCl2, 25mM
• 2 × 13ml Nuclease-Free Water

3.2.6.4.General Considerations

Preventing Contamination , qPCR Primers , RNA Template , BRYT Green® Dye**,** CXR Reference Dye and Instrument Considerations.

3.2.6.5.GoTaq1-Step RT-qPCR Protocol

A- CXR Reference Dye to the GoTaq® qPCR Master Mix (Optional)Some real-time PCR instruments require higher levels of CXR Reference Dye; For high reference dye instruments, add CXR Reference Dye to achieve a high dye concentration (500nM), as follows:

1. Thaw were the GoTaqR qPCR Master Mix.
2. Vortex the GoTaqR qPCR Master Mix for 3–5 seconds to mix.
3. When using an instrument designated as a high reference dye instrument, 0.33µl add were per 20µl reaction for a final concentration of 500nM.
4. Vortex for 3–5 seconds to mix.

3.2.6.6.Assembling the GoTaq 1-Step RT-qPCR Reaction Mix

The final reaction volume in this protocol is 20µl. The volumes given here may be scaled for larger or smaller reaction volumes.

1. the GoTaqR qPCR Master Mix and Nuclease-Free Water were Thaw.
2. Vortex the GoTaqR qPCR Master Mix for 3–5 seconds to mix. Vortex at low speed to avoid aeration.
3. the number were Determine of reactions to be set up, including negative control reactions. 1 or 2 add were reactions to this number to compensate
4. the reaction were Prepare mix (minus RNA template) by combining the GoTaq® qPCR Master Mix, GoScript™ RT Mix, PCR primers and Nuclease-Free Water as .
5. the appropriate volume add were of reaction mix to each PCR tube or well of an optical-grade PCR plate.
6. the RNA template add were (or water for the no-template control reactions) to the appropriate wells of the reaction plate.
7. the tubes or optical plate were seal, and centrifuge briefly to collect the contents of the wells at the bottom. Protect from extended light exposure or elevated temperatures. The samples are ready for thermal cycling.

Table (3-6). GoTaq1-Step RT-qPCR Reaction Mix.

Component	Volume per 20µl Reaction	Volume per 50µl Reaction	Final Concentration in Reaction
GoTaq® qPCR Master Mix, 2X	10µl	25µl	1X
Forward Primer, 10X	2µl	5µl	50–300nM
Reverse Primer, 10X	2µl	5µl	50–300nM
GoScript™ RT Mix for 1-Step RT-qPCR, 50X or Nuclease-Free Water for Minus-RT Control	0.4µl	1.0µl	1X
RNA Template (500fg–100ng) or Nuclease-Free Water for No-Template Control	4µl	10µl	variable
Optional: MgCl2, 25mM*	__µl	-µl	≥2mM
Optional: CXR Reference Dye, 30µM**	__µl	--µl	≥33mM
Nuclease-Free Water	to 20µl	to 50µl	---

3.2.6.7.Thermal Cycling

The cycling parameters below are offered as a guideline and may be modified as necessary for optimal results.

Table (3-7). General Thermo cycler Program of qPCR

Step	Cycles	Temperature	Time
Reverse transcription	1	≥37°C	15 minutes
Reverse transcriptase inactivation and GoTaqR DNA Polymerase activation	1	95°C	10 minutes
Denaturation	40	95°C	10 seconds
Annealing and data collection		60°C	30 seconds
Extension		72°C	30 seconds

Table(3-8) . summarized flaorophones , Dyes , Emission and excitation parameters used for multiplex RT PCR

Modules

Pos.	Quencher	Excitation	Detection	Dye	Gain	Measure ment	Pass. Ref.
1	Blue	470	520	FAM	5	Yes	No
2	Green	515	545	JOE	5	No	No
3	Orange	565	605	ROX	5	Yes	No
4	Red	630	670	Cy5	5	No	No

3.2.7.Calculating Gene Expression (Fold change)

There are two strategies for analyzing qPCR data are absolute and relative quantification. Absolute quantification identifies the input gene amount based on a standard curve which

In contrast, relative quantification determines changes in gene expression relative to a reference genes sample which accomplished by Errors caused by standard dilutions when creating a standard curve can also be avoided.

groups is of more interest than exact DNA/RNA molecular numbers. Therefore, relative quantification is widely performed.

Gene expression or gene fold or RQ (Relative quantification) value calculated.

RQ = 2-(ΔΔCT)

Gene fold or RQ calculated firstly by collecting CT (CT - cycle threshold or CQ - cycle quantification) average value from real time PCR device for each triplicated

sample then calculate ΔCT value for both treated and untreated samples as follow:

Δ CT = CT (gene of interest) – CT (reference gene)

To calculate ΔΔCT value which found as follow:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control)

After calculating ΔΔ CT for all samples then take final equation to calculate

gene expression or RQ as follow:

Fold change = 2-(ΔΔCT).

3.2.8.Statistical analysis

The SPSS statistical package for the Social Sciences was used to analyze the results (version 20.0 for windows, SPSS, Chicago, IL, USA), (Iuliano & Franzese,2018).

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results and Discussion:

4.1. Demographic Distribution of all studied patients:

4.1.1. Gender distribution :

The Figure (4 - 1) summarized the results of impact of β-thalassemia patients with HCV in male have (26 : 52%) in comparison with female (24 : 48 %), this result show that no significant difference at P. Value >0.05 between male and female of hepatitis C infection .

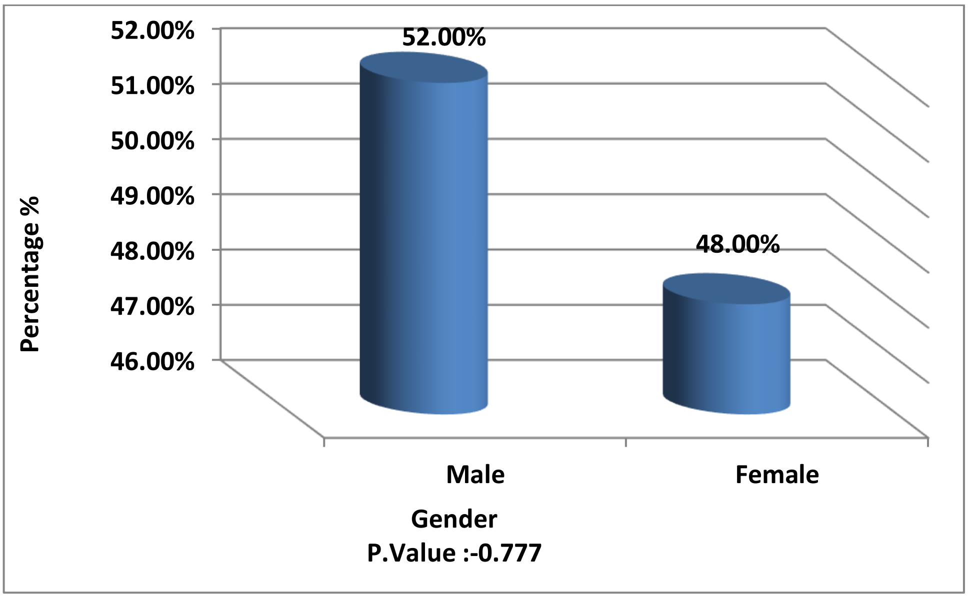


Figure (4 -1): Gender distribution of studied patients infected with HCV .

This result relatively agreement with other study , such as This result indicated that this disease affects men more than women and this may due to the women have slower rates of liver disease progression than men if they become chronic infected and women also more likely to clear the virus spontaneously because of their hormones nature (Baden *et al.*, 2014). The current study was agreed with study of (Fedeli *et al.*, 2019) from Italy, who found the high distribution of HCV infection was in male 44% than in females 38%. (Youssef *et al.*, 2017) , also reported in their study the rate of HCV infection was higher in males than in females among B-Thalassemia patients (14%) and (6%) respectively. This also corresponds to the studies of(Mukharmash *et al.*, 2017 ; Matham,2020), Province. While current result disagreed with some studies in Iraq; (Muslim, 2014; Jamil and Ahmad, 2015) Province showed a higher percentage of HCV infection among females than in males.

4.1.2. Age groups distribution:

Distribution of HCV patients show that the age range 20 -29 years have higher percentage (26 : 52%) , followed by 10 – 19 years (15 : 30 %) , while the adult age group > 40 years have (6 : 12%) , and children 1- 9 years (3 : 6 %) (Figure (4-2)) . The result might be refer to that children less exposure to infected with hepatitis –C virus that young and adult ages , in which that have lower percentage than other.

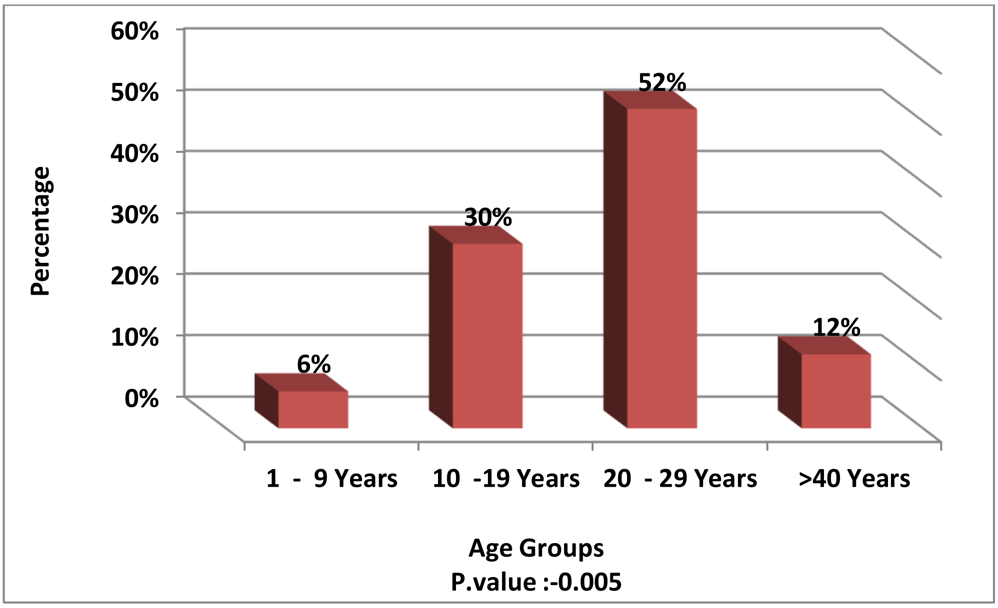


Figure (4 - 2) : Age groups distribution of studied patients HCV.

(Alao *et al.*, 2014) showed the age group of 20-29 years had the highest HCV Seroprevalence in 52 %, followed by age groups of 10- 19, 1-9 and 31-40 years with a prevalence of respectively, while the age groups above 40 years old showed no evidence of HCV antibodies. In the study of (Buseri *et al*., 2009) the highest rates of HCV Seroprevalence were found among the 18-35 years of age of B-Thalassemia in association with hepatitis C . However, a lot of studies reported that the HCV can infect people at any age but appears in adult, especially the young above 25 years, (Niu *et al*., 2016) which demonstrated that the highest prevalence of HCV infection was at the age group 25–45 years. The similar findings were reported by (Janahi *et al*., 2015; Umutesi *et al*., 2019)

4.1.3 Distribution patients based on vaccination:

The result in Figure(4.3) shown that vaccinated patients have (11 : 22%) less than the non-vaccinated that show higher percentage (28 : 56%) ,and the patients take 1 dose show (7 : 14%), but the 2 dose vaccinated patient shown (4 : 8%) in which that have lower percentage than other, this result show that no significant difference at P. Value >0.05 between number of vaccinated of B-Thalassemia in association with hepatitis C infection;

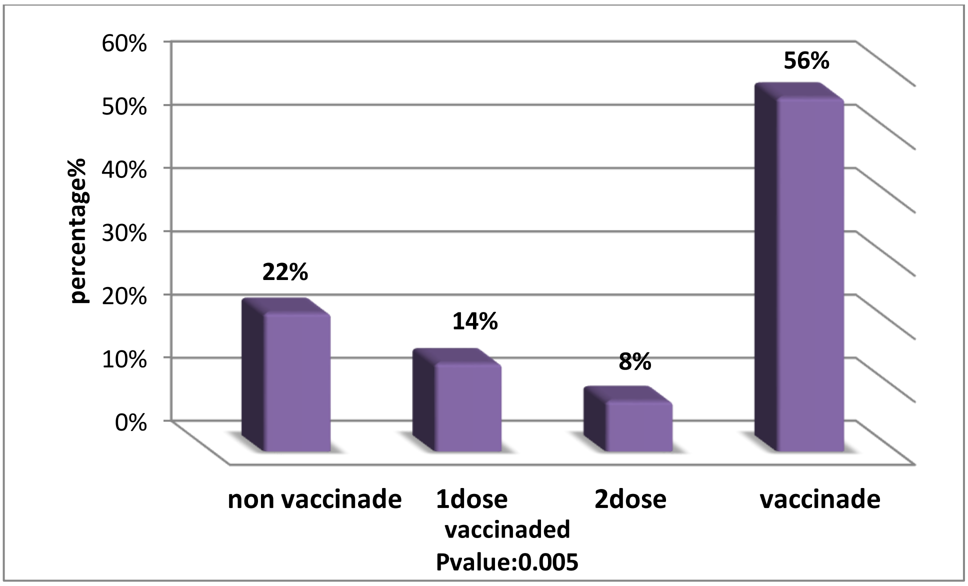


Figure (4- 3) : Distribution of patients HCV according for vaccination doses.

This result relatively agreement with other study, Hepatitis B vaccine is usually given as 2, 3, shots. should getβ- thalassemia patients dose of hepatitis B vaccine at birth and will usually complete the series at 6–18 months of age (Feuerstadt *et al*., 2010). The birth dose of hepatitis B vaccine is an important part of preventing long- term illness in infants and the spread of hepatitis Overall, only a small minority of patients with chronic hepatitis C can currently be cured in most real-world settings with interferon-based treatments (Feuerstadt *et al*., 2010).An effective preventive vaccine would considerably reduce the number of new infections and thereby reduce the burden on health care systems. However, there are many impediments the development of vaccine for HCV including the existence of multiple HCV genotypes repeatedly exposed to HCV, such asgetβ- thalassemia patients,, raises concerns that the development of long-term protective immunity for HCV may not be possible (van de Laar *et al*., 2009). The development of an effective vaccine for HCV should be achievable, as supported by vaccination studies (Lauer *et a.*,, 2004).

4.1.4. Blood group distribution:

The Figure (4 -4) summarized the result of impact blood it shown no significant differences P. Value >0.05 between test blood groups ABO blood groups have shown some association with β- thalassemia patients with hepatitis Seroprevalence of HCV Ab were found to be higher in patients who has blood group O (20 : 40%) and lowest in patients who has blood group A (5 : 10%.), The level of HCV with blood group B was (12 :24%) and in AB (8 : 16%),and loss than in A&B-ve (1 : 2%.)and in O-ve (3 : 6%.).

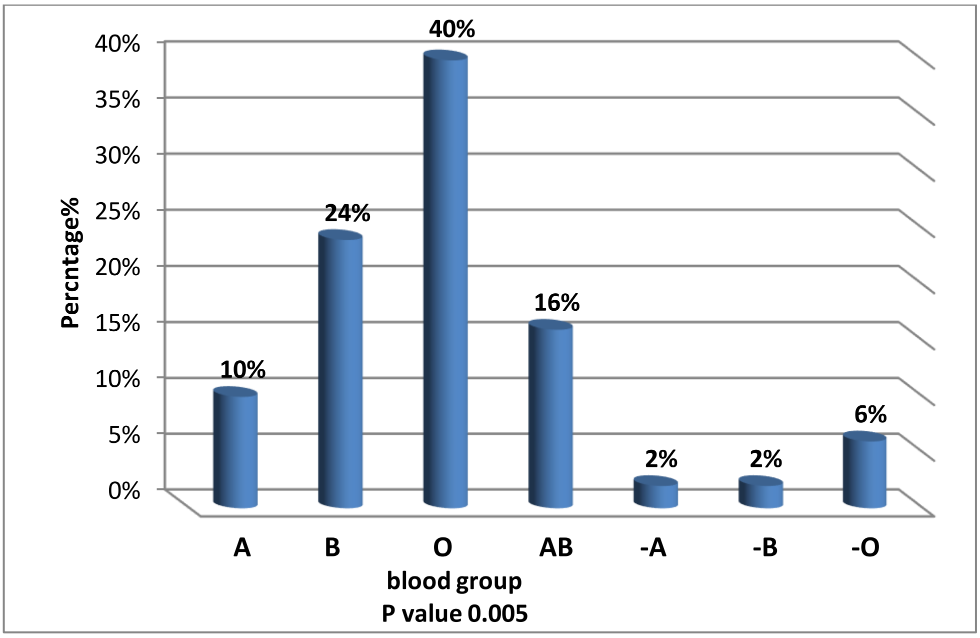


Figure (4- 4) Blood group distribution of studied patients HCV.

The results pointed that patients with blood group O were at higher risk of HCV infection than other blood groups subjects in thalassemia patients with HCV, which was consistent with some previous studies by (Lao *et al.*, 2012, Zhou *et al* .,2013; Liu *et al.*, 2013 and Abate *et al*, 2016). That means more investigation should be taken to ensure the safety of people with blood group O the precise role that ABO blood groups play in host susceptibility and Hepatitis infection has yet to be clarified, (Liu *et a.,*, 2013) Related have been observed that are most likely related to the altered immune response and systemic inflammatory response, (Zhou *et al* .,2013) which are associated with different blood group phenotypes , Further studies are warranted to elucidate the association between blood groups and Hepatitis infection, and the way the blood type influences the process of Hepatitis HCV infection. Meanwhile, several limitations need to be considered. First, although we performed subgroup analyses, analyses of previous studies have revealed that the heterogeneity cannot be ignored. Second, the analysed studies lacked basic information on the ethnicity data and the prevalence of different Hepatitis genotypes (Abate *et al.*, 2016 ; Jing *et al.*, 2020).

4.2. Hematological Test:

4.2.1. results of PT & PTT test for the patients with hepatitis and control:

As indicated in table (4.1) about HCV-infection in thalassemia patients, it was observed that the mean values of the coagulation factors partial prothrombin time (PTT),prothrombin time (PT) , showed no significant differences P. Value >0.05 The mean value in for PT female patents show (16.7 ± 2.9) increased than the male (15.7 ± 2.7) and is lower compared with the controls positive and negative (16.60 ± 3.03) , but the result in PTT patients is higher in male (45.3 ± 24.7) than that in female (38.5± 6.60) and , significantly increased of than Positive Control(Thalassimic) in PTT test (39.2 ± 9.4), and negative control(healthy) show low (34.8 ± 3.6). that mean thalassemia deases main reason for the effect PT&PTT test.

Table(4.1): Relationship of gender and (PT,&PTT) for HCV patients.

Gender and parameters		N	Mean± Std.Deviation	P.Value
PT	Male	31	15.7692 ± 2.00614	.652
	Female	29	16.0000 ± 2.87417	
	Positive Control(Thalassimic)	20	16.6000 ± 3.03315	
	Negative Control (healthy)	20	16.6000 ± 3.03315	
PTT	Male	31	45.3846 ± 24.16125	.092
	Female	29	38.5000 ± 6.60698	
	Positive Control(Thalassimic)	20	39.2000 ±9.45126	
	Negative Control (healthy)	20	34.8000 ±3.65052	

The liver plays a major role in hemostasis as most of the coagulation factors, anticoagulant proteins and components of the fibrinolysis system are synthesized by the hepatic parenchymal cells. But when the liver is diseased, these functions are impaired. The study on the effects of hepatitis C virus with β- thalassemia patients on some coagulation factors was designed to assess the changes associated with some coagulation factors following viral hepatitis (HCV). In the study, it was observed that in hepatitis C virus infection, there was an observed statistical change in all the coagulation factors analyzed. and according (Li *et al*., 2008), infection of the liver by virus causes virus-induced tumor necrosis factor production which mediates a significant liver pathology. These changes can therefore be explained based on the state of the diseased liver which is saddled with the responsibility of clotting factors synthesis (William, 2005) . In hepatitis C virus infection, a prolonged PTT, PT was observed. HCV infected people have been suggested by researchers that they fail to clear the virus during the acute phase of the disease and as result become chronic carriers. (Sy and Jamah, 2006)

4.3. Liver functions test:

As indicated in Table (4.2) about HCV-infection in thalassemia patients, it was observed that the mean values of the liver enzyme Glutamic-Oxaloacetic Transaminase (GOT) and Glutamic-pyruvic transaminase (GPT) , the comparison of mean values of liver function test of patients hepatitis C virus with thalassemia (GOT 36.86 ± 30.50 ,GPT:30.02 ± 20.34) , The increase was clear in patients compared to the control of thalassemia at the level of (GOT :25.73± 15.28 ,GPT:26.26 ± 16.32). which was also increased compared to the control healthy level (GOT 19.80 ± 5.98, GPT 26.85 ± 5.55) This result show no significant differences in GOT in P. Value:0.01 .the ages 1-9 years(96.6 ± 25.5 GOT ; 61.33 ± 22.03 GPT) were the most increased compared to the age level 10 - 19 Years (GOT:37.6 ± 21.1 ; GPT: 30.7 ± 14.80) , 20 - 29 Years (GOT: 35.1 ± 34.7 ; GPT:27.33 ± 20.1) and >40 Years(GOT:26.1 ± 27.8 ; GOT: 22.1 ± 22.03), while the value in Positive Control(Thalassimic) and Control (healthy) were (GOT:25.18 ± 14.8 ; 19.9 ± 6.1 ; GPT :26.1 ± 15.9 ; 26.7 ± 5.6) respectively. The concentration of GOT & GPT In thalassemia patients with HCV were determined for duration of diseases (HCV) in patient the concentrations of an increase in level 1-5 Year were observed (GPT :34.5 ± 20.2 ; GOT: 40.03 ± 30.7) than that in <1 years (GOT: 37.1 ± 29.8 ;GPT: 7.1 ± 0.3) and in 6 – 10 years(GPT 30.5 ± 32.2 ; GPT: 22.03 ± 21.7) the normal range: GOT=0-40 u/l, GPT=0-41 u/l.. As shown in the Table(4.2).

Table(4.2): Relationship of (GOT&GPT) and other parameter for HCV patients.

(GOT, GPT)and parameters		N	Mean ±Std. Deviation	LSD Value
GOT of patient HCV	Patients Positive Control(Thalassimic Negative Control (healthy	60 20 20	36.86 ± 30.50 25.73± 15.28 19.80 ± 5.98	0.018
GOT & age	1 - 9 Years 10 -19 Years 20 - 29 Years >40 Years	7 19 26 8	69.66 ± 25.54 37.60 ± 21.19 35.11± 34.72 26.16 ± 27.85	0.087
GOT duration of disease **(HCV)**	< 1 Year 1 - 5 Years 6 – 10 Years	12 32 16	37.00± 29.75 40.033 ± 30.77 30.00 ± 32.48	0.064
GPT & patient HCV	Patients Positive Control(Thalassimic Negative Control (healthy	60 20 20	30.02 ± 20.34 26.26 ± 16.32 12.85 ± 5.55	0.638
GPT & age	1 - 9 Years 10 -19 Years 20 - 29 Years >40 Years	7 19 26 8	61.333 ± 22.03 30.73 ± 14.80 27.80 ± 20.17 22.16 ± 22.85	0.022
GPT duration of disease **(HCV)**	< 1 Year 1- 5 Years 6 – 10 Years	12 32 16	25.55 ±17.74 34.50 ±20.32 22.80 ±21.33	0.218

In the current study the mean values of liver enzymes(GPT & GOT) in both groups were significantly higher than the normal range similarly to the other workers(Shfik *et al.*, 2011; Sikorska *et al.*, 2011) which is due to iron overload that accumulated in organs such as liver and this result from excessive hemolysis and ineffective erythropoiesis as well as increased iron absorption and use of chronic blood transfusion therapy programs in thalassemia. (Hoffbrand *et al*., 2011 ; AL-Saedi *et al*., 2019) On the other hand the mean values of GOT and GPT in thalassemia control were higher than that in healthy control which is attributed to viral hepatitis infection causing destruction of hepatocytes and release of liver enzymes . Liver dysfunction was one of complications iron over load and chelation therapy (Ferrara *et al*., 2004).

Since the liver is the first organ which is affected via iron overload in thalassemia, higher level of serum GOT and GPT in β-thalassemia patients indicate an abnormal liver function in this study showed that the iron over load affects liver function in thalassemia patients. These findings are in agreement with the finding of (Salih and AL-Mosawy, 2016) who reported Iron overload with an average ferritin level in the thalassemia patients who received multiple transfusions of blood. Comparably, it has been indicated that there are increased GOT levels , therefore, this study's results have been in accordance to other works.(Cheema and Khan, 2018). Furthermore, there are greater GOT levels in the thalassemia patients (Cheema and Khan, 2018).

In this study observed the high concentrations of GOT. Therefore, the findings of this research coincide with the outcomes of other research (Cheema and Khan, 2018). The elevation concentration of serum hepatic enzymes (GPT, GOT) might be related to increased serum ferritin levels, that is on the basis of iron overload in the thalassemia patients since due to many blood transfusions. . (Cappellini *et al*., 2014 ; Najaf *et al*.,2019).

4.4.Immunological study:

4.4.1.Relationship HLA-DR in HCV patients and other parameter:

Concentration of HLA-DR in patients HCV were determined for male and female, in female HLA-DR increase in level were observed (12.15 ± 15.4) , than that in male (8.9 ± 3.2), while the value in Positive Control(Thalassimic) and Control (healthy) were (5.9 ± 3.2 ; 4.0 ± 1.7) respectively.as for concentration in the ages of HCV in thalassemia patients , the ages 10-19 years(12.4. ± 19.6) were the most increased compared to the age 20 - 29 Years (9. 8± 5.0), 1 - 9 Years (6.1 ± 0.6) and >40 Years(7.06 ± 4.9), while the value in Positive Control(Thalassimic) and Control (healthy) were (5.18 ± 2.8 ; 4.05 ± 1.7) respectively. The concentration of HLA-DR in patients HCV were determined for duration of diseases (HCV) in patient the concentrations of HLA-DR an increase in level < 1 Year were observed (16.3. ± 24.6) than that in 1 - 5 years (8.8. ± 4.8) and in 6 – 10 years(5.9. ± 3.2) .. As shown in the Table(4.3).

Table(4.3): Relationship HLA-DR in HCV patients and other parameter

HLA-DR and parameters		**N**	**Mean ± Std. Deviation**	**LSD Value**
HLA-DR & gender	Male	26	8.94 ±4.281	0.007
	Female	24	10.15 ±15.48	
	Positive Control(β -T)	20	5.18 ± 2.88	
	Negative Control (healthy)	20	4.05 ± 1.73	
HLA-DR & age	1- 9 Years	3	6.10 ± 0.69	0.087
	10 -19 Years	15	12.47 ± 19.29	
	20 - 29 Years	26	9.86 ± 5.002	
	>40 Years	6	8.06 ± 4.99	
HLA-DR duration of disease **(HCV)**	< 1 Year	9	16.3 ± 24.57	0.200
	1 - 5 Years	30	8.9 ± 4.8	
	6 – 10 Years	11	5.96 ± 3.25	

In this study, the investigated the regulation of HLA-DR antigen in serum HCV in β-thalassemia patients with HCV been extensively used for studies of infections viral . the observations are in agreement with earlier reports suggesting that MHC class II from chronically HCV-infected patients do not respond to maturation stimulus antigens (Bain ,*et al,2001*; Pereira *et al.*,2019). Together, the last results that the negative effects of HCV on APC function could lead to reduced immunogenicity in vivo .A maturation defect in MHC generated from chronic HCV-infected (Auffermann-Gretzinger *et al.*,2001; Reith *et al.*, 2005).

On the other hand, HCV has been shown to infect not only liver cells but also affects the immune to present the antigen , This would explain how patients with chronic hepatitis C exhibit a selective deficit of anti-HCV immunity with preservation of normal immune response to unrelated antigens .Cellular immune responses are critical for the clearance of HCV (Fowler *et al*., 2005and Rosário *et al*.,2020). Failure to mount a potent and broad repertoire response results in persistent HCV infection. in previous studies It has been suggested that HCV subverts cellular immunity by inhibits or loss MHC II expression in patients inducing ,which in turn the activation of HLA-DR and development (Brady *et al*.,2003). , this studies also provided evidence that HCV in thalassemia patients can interfere with antigen presentation and increased concentration of HLA-DR, which could be a critical mechanism for HCV persistence. HCV has developed multiple strategies to escape immunity and persist within the host., Cellular immune responses appear to be mainly responsible for viral clearance following HCV infection (Schulze zur Wiesch *et al*., 2005; Komatsu *et al*.,2006).

The importance to the host immune response is the presentation of HCV antigen in the context of HLA molecules. Therefore, diversity in antigen presentation by different HLA may contribute to the effectiveness of the host immune response to HCV and consequently in outcome of infection. This the report on the correlation of HLA class II with clearance or persistence of HCV in patients . The last studies shows that while HLA- DR has a significant correlation . Studies on various Age ,gender, Vaccinated dose and duration of disease variability in the frequency of HLA among them (Thursz *et al*.,1999; Thio *et al*., 2001; Azocar *et al*., 2003; Mckiernan *et al.,* 2004; Singh *et al*., 2007 ; Pereira *et al*.,2019 ; Rosário *et al*.,2020).

Another possible explanation could be a difference in the route of HCV transmission. Several previous studies have suggested that the route of contamination may influence the relationship between some HLA and viral clearance, and that there might be an additional factor responsible for the large variation of the results in studies of HLA and HCV clearance (Harris *et al*., 2008; de Almedia *et al*., 2011). The thalassemia patients whom participated in the present study had received a high infectious dose of virus during blood transfusion. A significant correlation of the DR with chronic infection have been reported in some studies published previously (Thursz *et al*., 1999; Mckiernan *et al*., 2004; Wang *et al.*, 2009; Chan *et al*,2018). Similar observation was made by (Yenigum and Durupinar 2005, Nguyen *et al*,2014;Rosário *et al*,2020). The finding suggests that HLA-DR might play role in HCV infection among of the thalassemia patients.

4.4.2.Relationship HLA-G in HCV patients and other parameter:

According to the serum patients results to(HLA-G), Concentration of HLA-G patients HCV were determined for male and female, in female HLA-G an increase in level were observed(907.15 ± 706.4) , than that in male (723.2 ± 571.2), while the value in Positive Control(Thalassimic) (220.81 ± 952.9) this concentrations show higher compared to Control (healthy) were (561.0 ± 394.7) .as for concentration in the ages of HCV in thalassemia patients , the ages 10-19 years(888.4. ± 697.6) were the most increased compared to the age level 20 - 29 Years (840. 8± 670.0),1 - 9 Years (483.06 ± 81.6) , and >40 Years(658.3 ± 536.9), and Concentration of HLA-G in thalassemia patients with HCV were determined for duration of diseases (HCV) in patient the concentrations of HLA-G an increase in level 1 - 5 Years were observed (880.08. ± 650.4) than that in < 1 Year (107. 90 ± 785.4) and in 6 – 10 Years(673.8. ± 417.02) . As shown in the Table(4.4).

Table(4.4). Relationship HLA-G in HCV patients and other parameter.

HLA-G and parameters		N	Mean ±Std. Deviation	LSD Value
HLA-G & gender	Male Female Positive Control (β -T) Negative Control (healthy)	26 24 20 20	723.22 ± 571.81 907.15 ± 706.60 220.81± 952.91 561.73 ± 394.01	0. .323
HLA-G & age	2 - 9 Years 10 -19 Years 20 - 29 Years >40 Years	3 15 26 6	583.33 ± 81.916 888.41 ± 697.656 840.36 ± 670.420 658.33 ± 536.958	0. 632
HLA-G duration of disease **(HCV)**	< 1 Year 1 - 5 Years 6 – 10 Years	9 30 11	107.90 ± 785.2 880.08 ± 650.47 673.84 ± 417.02	0. 632

The main feature of HCV is its ability to persist in the majority of infected subjects. In this study serum examined of 50 patients hepatitis with thalassemia developed chronic infection ,the increase of HLA-G concentration was clear and striking in comparison with studied parameters and the remaining 20 HCV- non infected subjects non virus The factors that determine clearance or persistence of virus in HCV-infected patients have not yet been identified. and Other factors influence clinical expression of HLA-G and viral persistence (Thomas *et al*,. 2000; Zhou *et al*.,2022), including route of infection (transfusion in thalassemia patients), size of inoculum, duration of injury and viral genotype.

The route of infection could be an important factor especial in thalassemia patients in chronic infection because of frequent blood transfusions . in this study suggested the significant increase in plasma

HLA-G expression might play a role in to chronic infection . Increased HLA-G expression in HCV-infected has been associated with milder stages of fibrosis and hemosiderin deposit (Crispim *et al*., 2011). Besides this , HLA-G expression was observed on mast cells present in areas of liver fibrosis (Amiot *et al*. , 2014). Increased plasma HLA-G levels were associated with chronic HCV infection with genetic changes in thalassemia patients and with increased Ferritin levels (Weng *et al* , 2011, Catamo *et al*.,2017).Since the treatment of mast cells with induces HLA-G expression (Amiot *et al* ., 2014). One study has associated increased HLA-G expression in hepatocytes with the HBV&HCV viral load (Souto *et al.*, 2011).

Different studies associated the increased serum/plasma HLA-G levels with hepatitis virus infection (Shi *et al*., 2011, Park *et al*., 2012 ; Han *et al* .,2014 , Zhou *et al*.,2022), which were associated with increased percentage of CD4 regulatory and HLA-G monocytes cells in patients exhibiting acute or chronic hepatitis (Shi *et al*., 2011), active hepatitis B, C virus infection (Park *et al*., 2012). Considering the tolerogenic properties of HLA-G and considering the aphorism that the induced expression of HLA-G may be detrimental in chronic HCV viral infection, It will be important that future studies do not neglect the interaction between genetic markers from both pathogen and host, giving special attention to the host ethnic background. Accumulating evidence suggests that during HCV infection, the inactivation of immune effector cells by HLA-G may lead to inability to clear the virus. As HLA-G are crucial for restricting viral replication, yet the magnitude of in chronic HCV decreases dramatically compared with acute HCV (Chisari ,1997; Weng *et al*., 2011; Khorrami *et al*.,2015).

The tolerizing functions of HLA-G seem to be playing a significant role in viral clearance Patients who are able to clear HCV infection express only low levels of HLA-G, while non-responders express drastically higher levels of HLA-G (Catamo *et al*.,2017). In addition, high levels of HLA-G in HCV and hepatocellular carcinoma are associated with concentration of HLA-G (Bochud *et al*.,2009; Weng *et al*., 2011). Therefore, it is likely that HLA-G plays a role in virus-associated malignant cell transformation , the levels of HLA-G activation positively correlate with the amount of mast cell activation and liver fibrosis (Amiot *et al*,2014). however, several studies observed a neo-expression of HLA-G linked to several pathological conditions, including HCV infection.(Baricordi *et al*,2008; **.** Kochan *et al*,2013; Amiot *et al*.,2014; , Zhou *et al*.,2022).

4.4.3.Effect the HCV on some hematological parameters of the patient:

Concentration of some parameters in HCV patients were determined for male and female, The effect of HCV was clear, as were the genetic disorders caused by thalassemia ,the increased value of ferritin in male and female were (3661.85 ± 2831.2 ; 3191.56 ± 2434.4) respectively ,than the Positive Control(Thalassimic) (2920.61 ± 1896.2) compared to Control (healthy) (282.15 ±150.2). but the concentration decreased in Hb in both sexes patients it was clear (male & female) were(6.746 ± 1.33; 7.158 ± 0.38) respectively and in Positive Control (6.55 ±1.49), compared to Control (healthy) (12.42 ±1.10). and the concentration decreased in PCV in both sexes patients (male & female) were(20.846 ± 2.52; 22.5 ± 7.25) respectively, and in Positive Control were (21.60 ±4.03), compared to Control (healthy) (35.800 ±3.13). It was found that there was an increase in the number of WBC in patients PCV (male: 8.73 ± 8.12; female: 9.12 ± 4.16) respectively compared to the control groups (6.91 ± 3.3) this result showed no significant differences P. Value >0.05, As shown in the Table(4.5).

Table(4.5). Effect of the HCV on some hematological parameters

Gender and parameters		N	Mean±Std. Deviation	P.Value
Ferritin	Male Female Positive Control(Thalassimic) Negative Control (healthy)	26 24 20 20	3661.85 ± 2831.367 3191.65 ± 2434.471 2920.61 ± 1869.932 282.15 ± 152.528	.000
Hb	Male Female Positive Control(Thalassimic) Negative Control (healthy)	26 24 20 20	6.74 ± 1.33 7.15 ± 0.83 6.55 ± 1.49 12.24 ± 1.10	.000
WBC	Male Female Positive Control(Thalassimic) Negative Control (healthy)	26 24 20 20	8.7319 ± 3.12 9.1250 ± 4.16 6.9180 ± 3.32 6.9180 ± 3.32	.582
PCV	Male Female Positive Control(Thalassimic) Negative Control (healthy)	26 24 20 20	20.8462 ± 2.52 22.5000 ± 7.25 21.6000 ± 4.03 35.8000 ± 3.13	.000

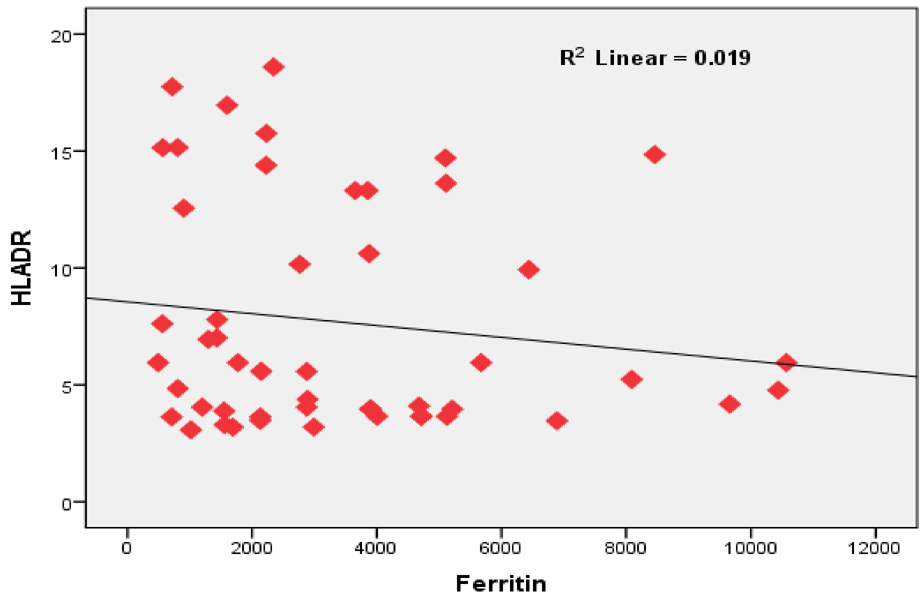
Serum ferritin in this study was significantly increase in thalassemia with HCV than in those with control. Iron's excess or overload ,This is the result of repeated blood transfusions that reach once or twice during one week. there will be increase in the ferritin's iron composition (Al-

Hakeim and Al-Hakany, 2013) and this may be the most important cause for the elevation of serum ferritin. These results indicate iron overload in the patient's group because serum ferritin is used to indicate iron overload illnesses such Plasma ferritin is considered the best single indicator of total body iron (Kalender *et al.*, 2002).In the current study, serum ferritin has been considerably high in HCV patients in comparison to control group. This is in agreement with the findings of (Fujita *et al*., 2008 ; El Lehleh *et al*., 2017).

The hematological characteristics of to study groups appeared, all the Hb and PCV parameters as well as the indices evaluated in this study indicated significant difference (P less than 0.000) between controls and β-thalassemia patients with hepatitis C virus. Extreme anemic presentations have been specified in the β-thalassemia patients. Low levels of (Hb and PCV) were significant decreased ($P < 0.001$) in thalassemia patients because β-thalassemia is an inherited Hb synthesis disorder resulting in severe anemia (Nienhuis and Nathan, 2012). This outcome was similar to other research (Sadoon and Bashir, 2010). There was a decrease in hemoglobin levels of patients comparing to the recorded level in controls as the patients and controls examined in this study shared the same genetic background.

4.4.5.Relationship of HLA-DR and Ferritin for HCV patients:

In the figure (4 - 5) . The logistic regression between HLA-DR and ferritin in hepatitis C virus patients genetically infected with Thalassemia ,show that direct relationship or Negative correlation between them , this result might be show that increased HLA-DR lead to increase in expression of ferritin , although the increased of ferritin resulting from repeated blood transfusions to patients (Lehleh *et al.*, 2017), while HLA-DR was associated with causes serious immune problems in the host's defenses against the virus.



Figure(4.5):.Relationship of HLA-DR and Ferritin for HCV patients .

4.4.6.Relationship of HLA-DR and Hb & PCV HCV patients:

The Figure (4 - 6) shown that negative correlation between PCV ; Hb and HLA-DR in hepatitis C virus patients genetically infected with Thalassemia , it was show that increased concentration of HLA-DR lead to decreased in concentration of Hb and PCV .The hematological characteristics of to study groups a There was a decrease in hemoglobin levels of patients comparing to the recorded level in controls As the patients and controls examined in this study shared the same genetic background ,these results are inversely proportional compared to the increase in the proportion of HLA-DR. the observed different frequencies of HLA-DR are likely to reflect different host’s genetic makeup and its capability to influence the outcome of HCV infection.

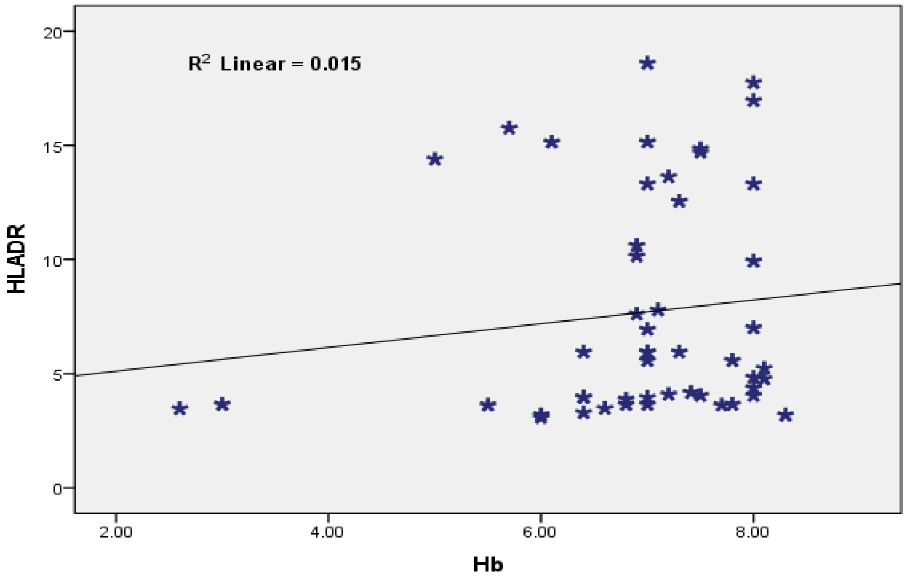


Figure (4- 6): Relationship of HLA-DR and Hb for HCV patients

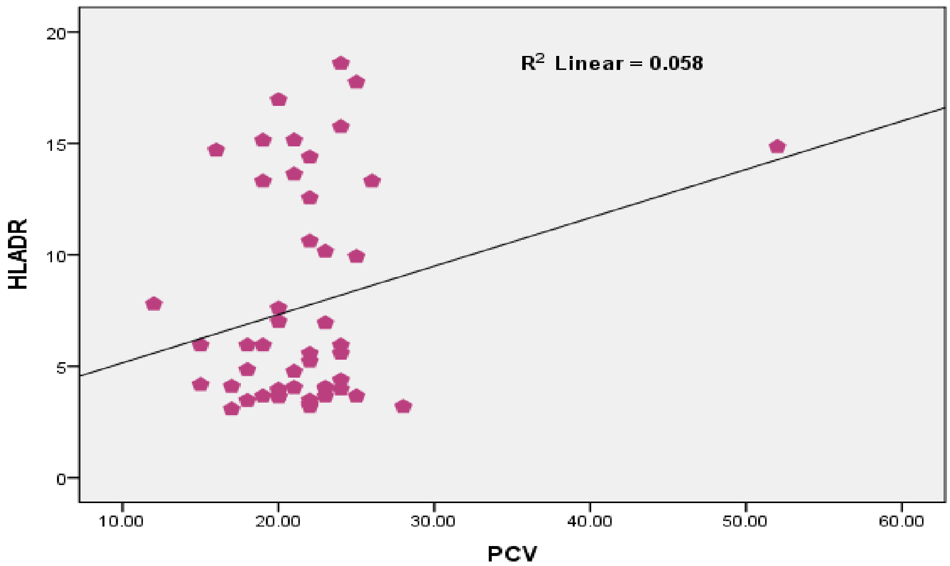
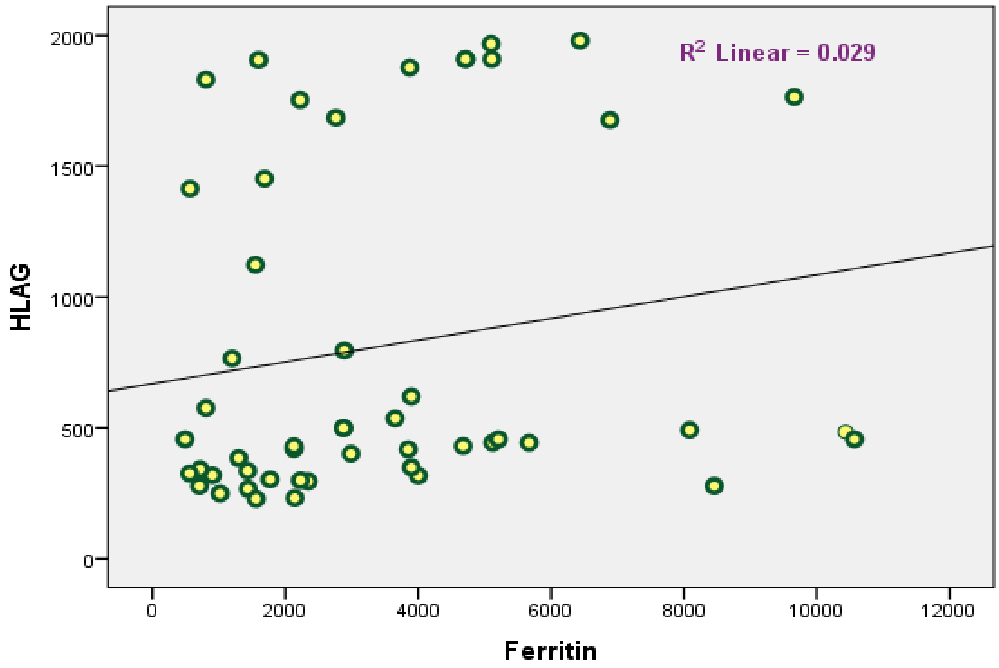


Figure (4 - 7): Relationship of HLA-DR and PCV for HCV patients .

4.4.7.Relationship of HLA-G and Ferritin for HCV patients:

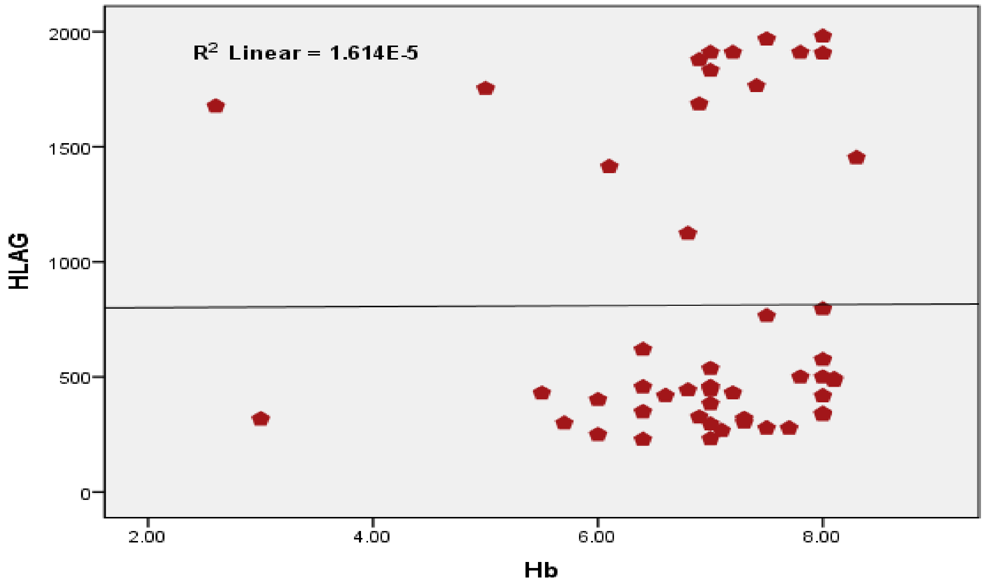
In the Figure (4 - 8) shown the regression between HLA-G and ferritin in hepatitis C virus patients genetically infected with Thalassemia ,show that direct relationship or positive correlation between them , this result might be show that increased HLA-G lead to increase in expression of ferritin , although the increased of ferritin resulting from repeated blood transfusions to patients (Lehleh *et al.*, 2017), while HLA-G was associated with causes serious immune problems in the host's defenses against the virus (Amiot *et al.*, 2014).



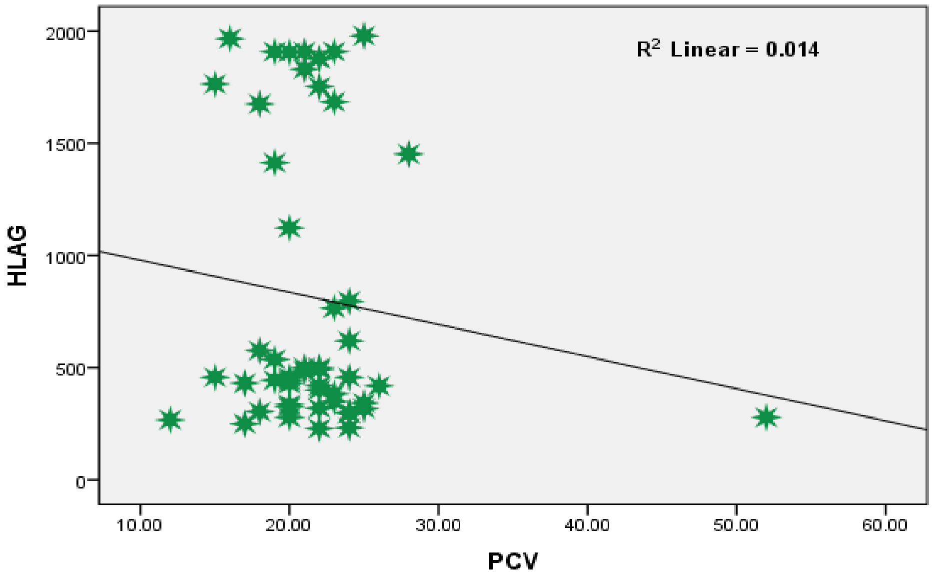
Figure(4.8):Relationship of HLA-G and Ferritin for HCV patients

3.4.8.Relationship of HLA-G and Hb & PCV for HCV patients:

The figure (4 - 9,10) show that negative correlation between PCV ; Hb and HLA-G in hepatitis C virus patients genetically infected with Thalassemia , it was show that increased concentration of HLA-G lead to decreased in concentration of Hb and PCV .The hematological characteristics of to study groups a There was a decrease in hemoglobin levels of patients comparing to the recorded level in controls As the patients and controls examined in this study shared the same genetic background ,these results are inversely proportional compared to the increase in the proportion of HLA-G.



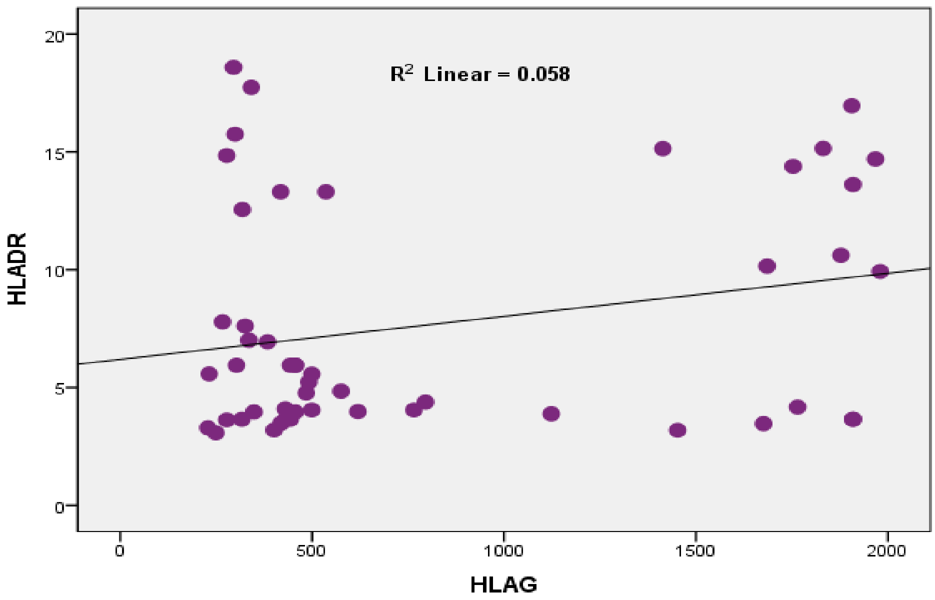
Figure(4.9): Relationship of HLA-G and Hb for HCV patients .



Figure(4.10) :Relationship of HLA-G and PCV for HCV patients.

4.4.9.Relationship of HLA-G and HLA-DR for HCV patients:

In the Figure (4 - 11) . The logistic regression between HLA-DR and HLA-G show that direct relationship or positive correlation between them , this result might be show that increased HLA-DR lead to increase in level of HLA-G , although the expression of HLA-DR might be associated with acute hepatitis infection are significantly impaired in their ability to present antigen(Pereira *et al*.,2019), while HLA-G was associated with HCV and hepatocellular carcinoma.(Weng *et al*.,2011).

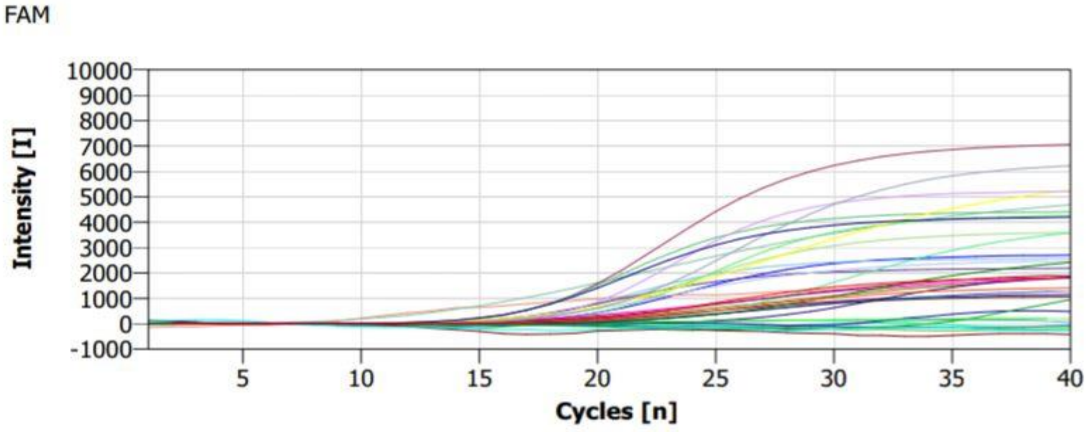


Figure(4.11):Relationship of HLA-G and HLA-DR for HCV patients .

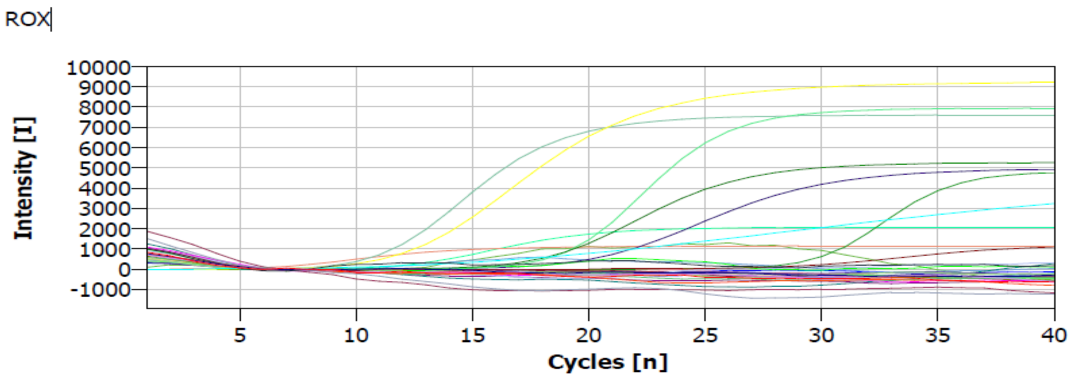
4.5.Molecular Test:

4.5.1. Quantitative RT-PCR to measurement of MIRNA in HCV patients samples:

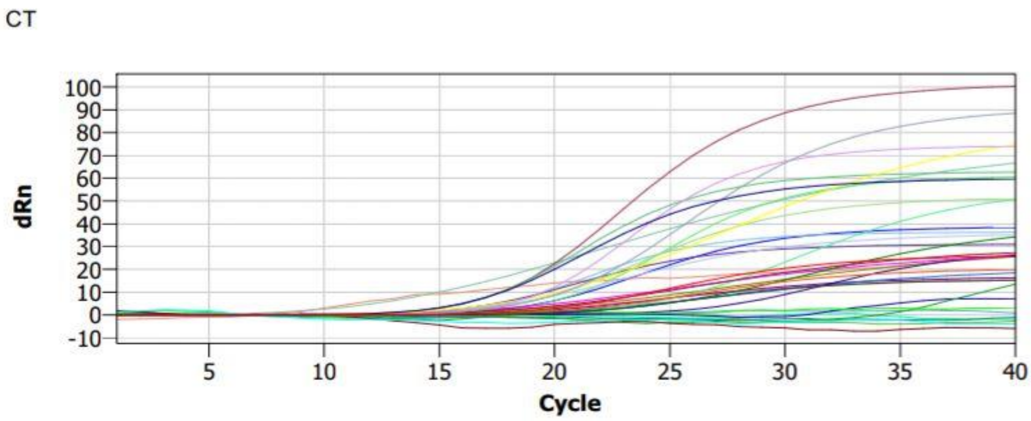
By using Quantitative RT- PCR , the assessment of MiRNA were done in a group of infected with hepatitis C virus in Thalassimic patients the result show that the FAM CT value indicated of positive miRNA , in comparison with ROX for Housekeeping gene as a control miRNA and , at high to moderate fluorescent intensity . Figure (4- 12) .



A : Positive of Target MiRNA .



B : Positive of H.K. gene as a control .



C:Positive CT value of patients.

Figure (4 - 12) : Quantitative RT-PCR of MiRNA in HCV patients.

4.5 .2. The RT-PCR result of miRNA (122 and 21) genes:

The overall results of studied genes , show that the MiRNA 122 have 100% positive RT-PCR result in comparison with 0 % in which have negative result or no ΔCT value was appear , as well while the other gene MiRNA 21 have 100 % positive result as well as housekeeping genes , as shown in table (4 - 6) . H. K. gene or control gene , which are often referred to as H.K. genes , are frequently used to normalize mRNA levels between different samples or different genes , this genes may change under certain conditions . This result might be refer to that the miRNA 122 was increased expression in patients prognosis to HCV , and decreased expression in miRNA-21 compared with H.K gene to hepatitis infection among the Thalassimic patients .It was also demonstrated that miRNA122 significantly differential expression and the survival chronic for HCV.

Table (4 - 6): The RT-PCR result of miRNA genes (122 and 21)

Gene types	RT –PCR	No.	Percentage
MiRNA 122	Positive	26	100 %
	Negative	0	0 %
MiRNA 21	Positive	26	100 %
	Negative	0	0 %
H.K. Gene Control	Positive	18	100 %
	Negative	0	0 %

4.5.3. The ΔCT value of MiRNA 122 , 21 and House Keeping gene:

The result of table show that the high level of MIRNA -122 in comparison with MiRNA 21 as well as H.K. gene , the low CT value of

MiRNA -122 (17.87) refer to high level of such parameter among HCV patients , increased ΔCT value in MiRNA 21 (29.15) indicated of lower level of expression, because that inverse relationship between CT value number and MiRNA level . While the ΔCT value of H.K. gene revealed that (24.39) , the optimum control (H.K. gene) CT value should be in between 20 - 30 ΔCT value as constant index measure . Table (4 - 7) shown the ΔCT value of the studied MiRNA .

Table (4 -7): Quantitative ΔCT value measurement of MiRNA in HCV patients

MiRNA	N	Mean ±SD	P. Value
MiRNA-122	26	17.8767 ±5.969	0.005
MiRNA-21	26	29.1596 ± 2.837	
House Keeping Gene	18	24.3900 ± 4.166	

Hepatitis C is one of the most common diseases worldwide Thalassemia patients are frequently affected because blood transfusions and high mortality rate. Currently, the diagnosis of HCV relies on imaging characteristics obtained using computed tomography (ΔCT) .However, early detection and diagnosis of small viron are relatively difficult and inaccurate (Lee *et al*.,2008; Gibriel *et al*.,2020). Many cases are unclear when diagnosed. Therefore, there is an urgent need for new and more sensitive biomarkers for early detection of Hepatitis.

The finding that miRNAs can be detected in various bio fluids including serum and plasma has opened up new opportunities in the search for biomarkers in hepatitis. High plasma miRNA levels acute infection of virus.. Hence, it used miR-21 and miR-122 as an example to examine miRNA expression in whole plasma in HCV patients and healthy volunteers from thalassemia . The results showed an increase in gene expression in miRNA-122 and a decrease expression in miRNA-21 patients in comparison with H. K gene which serves as a genetic control when detecting miRNA and the whole blood whose concentration is inversely proportional to the studied genes, may be useful for the prediction of HCV risk.

Consistent with the results published by (Tomimaru *et al*.,2012). Compared to the H.K gene miR-21 has been shown to be consistently low in hepatitis infection, including HCV and HCC (Volinia *et al*.,2006; Varnholt,2008; Bihrer *et al*.,2011). Importantly, this miRNA is not only up regulated in association with oncogenes is and viral infection, but can act as oncogenic miRNA (Iliopoulos *et al*.,2010; Gholami *et al*,2016). the hepatic level of miR-21 appears to correlate with the stage of liver fibrosis and chronic hepatitis (Marquez *et al*.,2010; Gibriel *et al*., 2020).

microRNA-21 is strongly expressed in HCV, . In the present study the miR-21 concentration in plasma showed a positive correlation with the H.K gene score, Similar to the data of the present study, it has recently been reported that sera from HCV-induced chronic hepatitis contain elevated levels of miR-21 in comparison to housekeeping genes controls it means a lower level of gene expression .However, in another study with patients suffering from HCV induced chronic hepatitis, no elevation of plasma miR-21 levels scores in these patients (Zhang *et al*.,2020), leaving the role of plasma miR-21 in HCV induced chronic hepatitis unclear. and The reasons for the different findings between the studies remain elusive . recent data show that serum levels of the liver-specific miR-122 also correlate with the H.K gene levels

However, miR-21 and miR-122 differ in that miR-21, but not miR-122,miR-21 was a low expression of either 122 was high expression in patients, both of which genes comparison to the housekeeping gene. Obviously, the serum levels of miR-21 and miR-122 overlapped, current evidence indicates that viruses use miRNAs to manipulate both viral and host gene expression (Gholami *et al*,2016; Bharali *et al*,2018). In general, Perhaps the role of miR-122 in controlling hepatitis C virus (HCV) infection is the most interesting example of the control of viruses through host miRNAs(Ono *et al*.,2020).This might be related to the different expression pattern of miR-21 and miR-122, miR-122 being highly selective for the liver(He *et al*,2021). Thus, release of miR-21 from several different cell types may contribute to the elevation of the serum miR-21 level in patients with chronic hepatitis. A drawback of the broader expression pattern of miR-21 compared to miR-122 is that comorbidities are more likely to reduce the diagnostic power of the serum miR-21 level than that of miR-122. (Xu *et al*.,2010).

4.5.4. The ΔCT value of MiRNA 122 , 21 in HCV patients and control:

The result of table (4 - 8) show that increased in miRNA - 122 expression among patients with thalassemia after infected with hepatitis C virus at ΔCT value 17.25 , after comparison with Thalassimic patients without HCV infection , while the miRNA – 21 have higher expression at Thalassimic patients (27.46) rather than thalassemia with HCV infection (29.15). This result might be refer to that the infection with HCV induced high gene expression in Thalassimic patients , the housekeeping gene was used as a control gene for only the patients samples.

Table (4 - 8) :The ΔCT value of MiRNA 122 , 21 in patients and control .

RT-PCR result		N	Mean ±SD	P. Value
MiRNA 122	Patients HCV	18	17.8767 ±5.969	0.390
	Control (Thalassemia)	8	20.736 ±8.189	
MiRNA21	Patients HCV	18	29.15 ± 2.83	
	Control (Thalassemia)	8	27.46 ± 1.21	

The role of MicroRNAs are ascertain to be vital in virus–host interactions, pathogenesis and host resistance via regulation of post-transcriptional or translational modification. The gene expression levels of two miRNAs, miR-21 and miR-122, were analysed in this study of β-thalassemia patients with HCV, Control(Thalassimic)patients ,and show abnormalities or changes have in thalassemia patients most of the studies and the relationship between gradual accumulation of molecular alterations and stepwise Hepatitis progression have been established by the earlier studies.(Midorikawa *et al*.,2009, Um *et al*.,2011) MicroRNAs were considered as regulator of this gene expression in human body. Previous reports showed that the deregulation of miRNAs might play important and different roles in HCV development and progression by various kinds of unknown mechanisms.(Murakami *et al*,2006; Zhang *et al*,2010)

In current study the miR-21 low expression and miR-122 were expression highly in blood serum of thalassemia patients with hepatitis compared to H.K. gene controls .and miR-122 was the most expressive and means the most effective in patients.MiR-21 gene expression was significantly more down regulated in the Hepatitis, than Control(Thalassimic)patients with non- hepatitis , these results were in agreement with (Abdelkhalek *et al*, 2021)suggesting a possible role of this miRNA in the pathogenesis of HCC (Harfoush *et al*,2016; Abdelkhalek *et al*, 2021). However, (Alnoanmany *et al*., 2015; Elghoroury *et al*., 2017) asserted that miR-21 is significantly higher in an Hepatitis group than in an positive control group (Alnoanmany *et al.*, 2015). . In this study, miR-122 higher expression and miR-21 less expressive was found to in HCV with thalassemia compared to controls without Hepatitis. A line significant difference of elevated serum miR-122 compared to miR-21 in hepatitis patients than compared without infection has been reported in earlier study by (Qi *et al*., 2011) ,and suggested the expression level of miR-122 in the serum of patients may reflect liver injury(Li *et al*,2013; Dai *et al*.,2019).

The abundance of hepatic microRNA-122 has made it the most commonly targeted biomarker of liver disease including HCV infection. A non-invasive technique to recognize the severity of liver disease by circulating serum miR-122 assessment essentially can become a preferable choice of diagnosis(Yang *et al*.,2015; . Ferracin *et al* .,2016;. Goldvaser *et al*.,2017; Ma *et al*.,2018). MiR-122 expression levels of HCV patients are deregulated and distinct from healthy controls, thereby making miR-122 a possible biomarker (Gao *et al*.,2009; Caviglia *et al*,2017, Gibriel *et al*.,2020) The serum miR-122 has also been previously reported as a potent diagnostic marker in determining liver injury and distinguishing viral and non-viral etiology. (sraelow *et al*.,2014; Kishta *et al*.,2020). Although research regarding the expression levels and associated functions of miRNA‑122 in thalassemia patients with HCV, and other liver diseases have been widely carried out (Bihrer *et al*.,2011, Pelizzaro *et al*.,2021), the plasma expression levels of miRNA‑122 increased significantly in hepatitis C patients and other liver injury diseases compared with the healthy controls (He *et al*.,2021). The differentiating power of miR-21 and miR-122 in Hepatitis patients and controls thalassemia without Hepatitis in current study showed that serum miR-21 and miR-122 may be used in the diagnosis of HCV

This study results showon that the expression of miR-21 and miR-122 was Variable in hepatitis patients with thalassemia, where the expression of miR-122 due to infection with the virus was higher than it is in miR-21, meaning that the effect of HCV the virus increases the expression miR-122 in patients, and this is important results in diagnosing HCV. in other studies It described that some serum/plasma miRNAs such as miR-21, miR-122, mi-125a/b, miR-199a/b, miR-221, miR-222, miR-223, miR-224 might serve as biomarkers for early diagnosis-prognosis of Hepatitis C virus.(Fiorino *et al*.,2016) Therefore validation of the potential applicability of miRNAs in the diagnosis of hepatitis HCV is very important and so more rigorous studies are necessary to confirm the same.

4.5.5 Pearson Correlation of MiRNA and HLA typing:

By using a Pearson correlation model in SPSS statistical program , the result show that there is a direct correlation between both HLA-DR , HLA-G and MiRNA 122 , and MiRNA 21 at P . Value (0.676 , 0.443) respectively , in comparison with negative correlation with H. K. gene . this result were refer to that increased HLA –DR , HLA-G level well lead to increase in MIRNA (122) gene expression . H.K. gene reduced expression in relation to HLA – DR and G , although it used as a control MiRNA in the present study . This results were listed in table (4 - 9).

Table(4-9): Pearson Correlation of MiRNA(122 , 21) gene and HLA(DR , G) typing

Correlations		HLA-DR	HLA-G	MiRNA-122	MiRNA-21	H.K. gene
HLA-DR	Pearson Correlation	1				
	Sig. (2-tailed)					
HLA-G	Pearson Correlation	.034	1			
	Sig. (2-tailed)	.753				
MiRNA-122	Pearson Correlation	-.164-	-.090	1		
	Sig. (2-tailed)	.443	.676			
MiRNA-21	Pearson Correlation	-.209-	.251	-.340	1	
	Sig. (2-tailed)	.305	.216	.104		
H.K. gene	Pearson Correlation	-.659-**	-.013-	-.061-	.224	1
	Sig. (2-tailed)	.003	.958	.809	.372	
**. Correlation is significant at the 0.01 level (2-tailed).						

HLA-DR belong MHC-II and HLA-G belongs to a non-classic MHC-I family and was considered to be an immune inhibitory molecule, which could bind to immunosuppressive receptors and induce immunosuppressive signaling (Amiot *et al*.,1998 ; Rosário *et al*.,2020). Preliminary studies found that HLA-G, DR was highly expressed in various kinds of diseases, such as HCC ,hepatitis and lung cancer cells, which lead to tumor immune escape. (Manaster *et al*.,2012)And it was shown that HLA gives high expression in viral infections compared to uninfected controls with an increase in microRNA expression of the studied genes.

An increasing number of evidence has showed that viral genes played critical roles in the chronic injuries and process of tumor formation, but the concrete mechanisms involved are still poorly understood; we need to do more to explore these. miRNAs are an endogenous, small, non-coding RNAs which can bind to specific target complementary mRNA and biological processes, and also involve in various diseases such as cancer and viral infections.(Fromm *et al*,2020). So far, , many evidence have indicated that miRNA-122 and miR21 had important roles in HBV and HCV infection and associated HCC, HCV infection altered the expression profiles of human cellular miRNAs directly or indirectly, to facilitate its replication and transcription (Thirion and Ochiya ,2013 ; Clément *et al*., 2019). And in studies, some miRNAs, such as miR-27 (Singaravelu *et al*.,2014) and miR-93-5p (He *et al*., 2018), directly targeted HCV transcripts and change its life cycle and its effect on the host.

The results of miRNA showed increased expression in hepatitis patients pre-existing with thalassemia compared with expression of H. K gene which is considered an unaffected control of the disease., targeted host genes that regulated HCV infection to participate in Hepatitis pathogenesis indirectly. Taken together, miRNAs are novel angle of view to understand the interaction between Hepatitis and human hepatocytes (Sonenberg ,2012) . What deserves to be mentioned particularly is miRNAs are also involved in anti-HCV immune response and HCV-induced immune evasion(Castelli *et al*.,2009). miR-203a (Liu *et al*.,2015,),miR-196a (Xu *et al*.,2016),miR-122(Yokoyama*et al*., 2019), miR-21(Elghoroury *et al*.,2017) and miR-192 (Kim, *et al*.,2016) have been proven to affect immune genes to support viral evasion or promote viral clearance in HCV infection .

In this study, focused on a known immune inhibitory molecular, HLA-G, which could present immuno modulatory functions in many ways. In the context of hepatitis , chronic virus injuries and hepatocellular carcinoma (Amiot *et al.*,2015),The results showed that HLA(DR ,G) increases expression in the serum of hepatitis C virus patients and this is proportional to the increase of gene expression in microRNA. Even HLA-G levels in serum could be used as a useful diagnostic marker for HCV-chronic injuries (Da Silva *et al.*,2014 ; Rosário *et al.*, 2020). But whether and how HBV and HCV regulate HLA-G and HLA-DR in hepatocytes and the detailed role of HLA-G in Hepatitis infection and prognosis is still largely unknown(Catamo *et al.*,2014).

Also, HLA-G was one target of these miRNAs, which could be down regulated by miRNAs ,So targeting miRNA might reverse highly expressed HLA-G and recover normal response during HBV infection and HCV+ HCC (Bian *et al.*,2015). Since virus, not limited to HCV, may establish chronic infection by alter cellular miRNAs pattern, certain miRNAs which bind to immune genes may be promising targets for treating viral diseases, including related Hepatitis in thalassemia patients . Taken together, this understanding between miRNAs and HCV interaction need further investigation, and HLA and miRNAs might be new potentially biomarkers for diagnosis and therapy of HCV infection. In conclusion, the clinical significance of miRNA‑122 , miR-21 and HLA-DR , HLA-G was systematically analyzed from multiple perspectives in this study. The diagnostic value of miRNA‑122 and miR-21between the thalassemia patients and hepatitis via serology was further demonstrated, and the differential diagnostic value in patients with Hepatitis compared with in thalassemia patients.

4.5.6. Correlation between miRNA -122 and miRNA-21 gene in HCV patients:

In the Figure (4 - 13) . The logistic regression between miRNA -122 and miRNA -21 show that indirect relationship or nigtive correlation between them , this result might be show that increased miRNA-122 lead to increase in expression of miRNA -21 , the expression of miRNA 122 might be associated with liver damage due to tumor invasion mostly at malignant tumor , while miRNA was associated with lung tissue or certain soft tissue in other part of human body as well as might be used as predictive marker after treatment with chemotherapy (Amaral *et al*,2014).

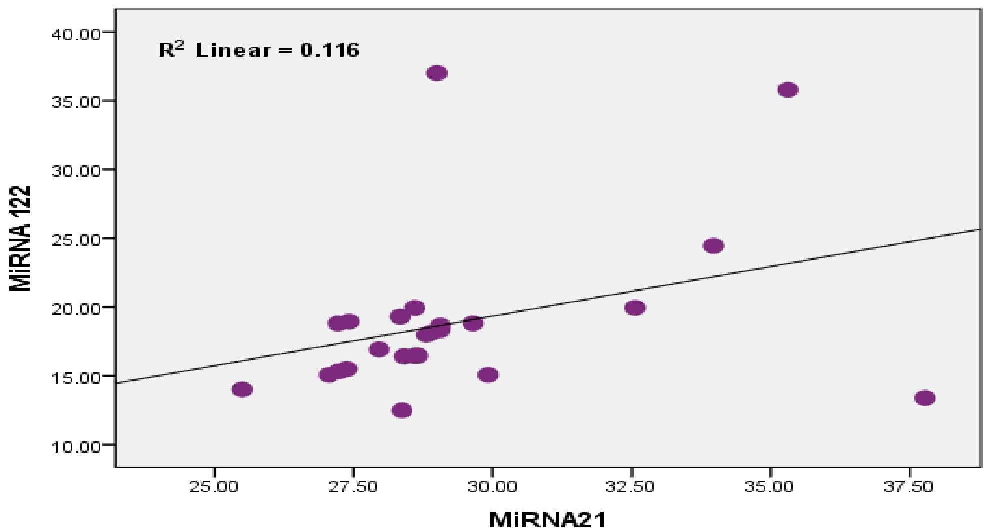


Figure (4 - 13) : Logistic regression model of miRNA- 122 and miRNA-21 in HCV patients.

4.5.7.Correlation between miRNA -122 and H. K. gene in HCV patients:

The Figure (4 -14) shown that negative correlation between miRNA-122 and H.K. gene , it was show that increased expression of miRNA -122 lead to decreased in expression of H. K. gene or control gene , which are often referred to as housekeeping genes . This result might be refer to that increased in miRNA – 122 exhibited with the other normal gene and reduced its expression such as control gene or H.K. gene.

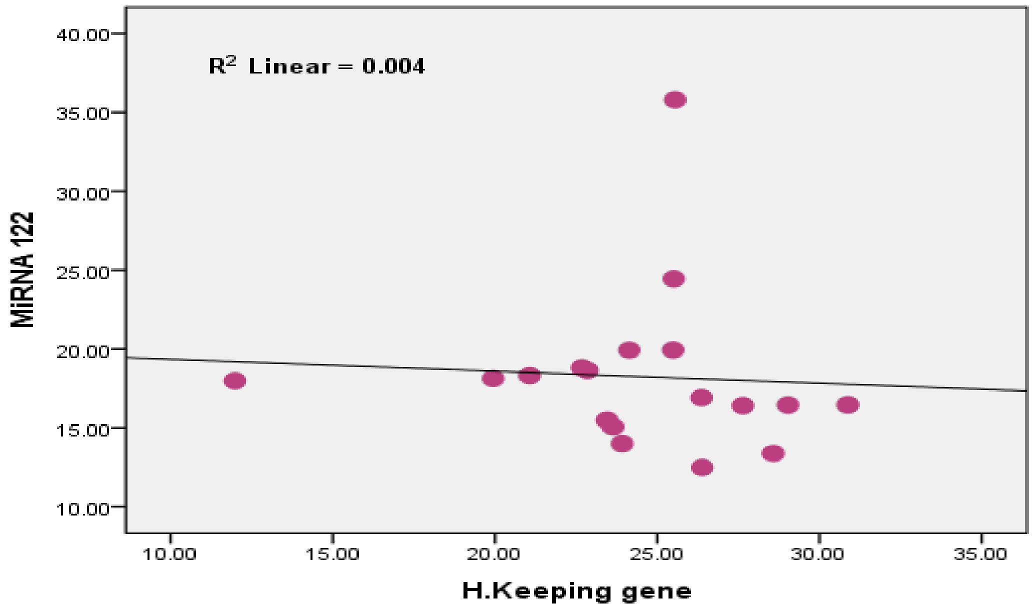
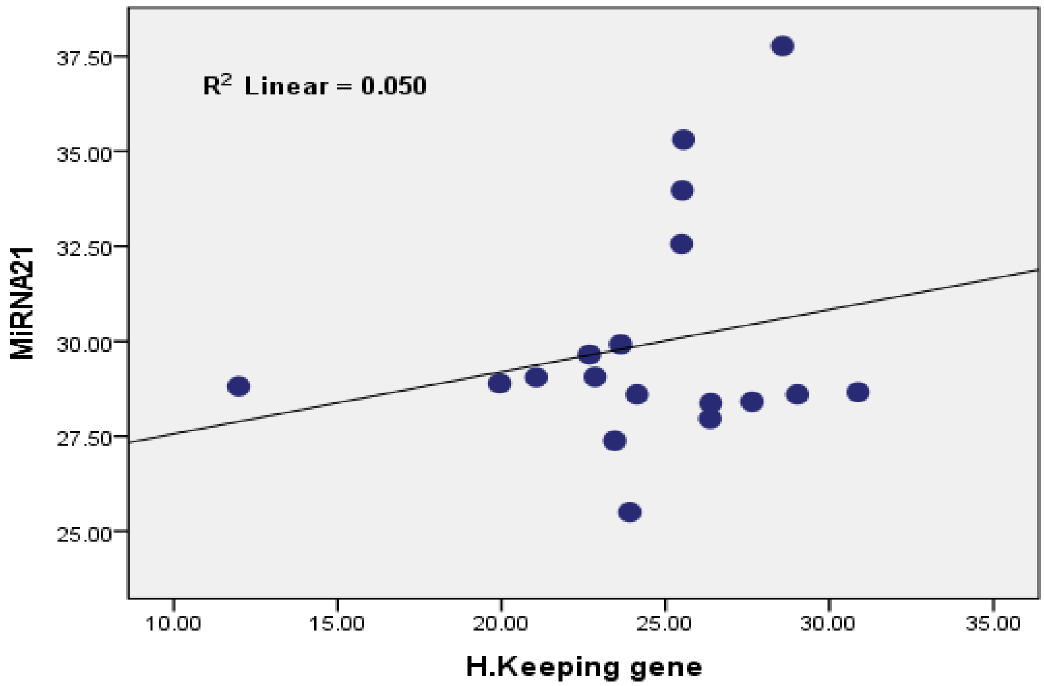


Figure (4 - 14): Correlation between miRNA -122 in HCV patients .

4.5.8. Correlation between miRNA -21 and H. K. gene in HCV patients:

The Figure (3 - 15) show that positive correlation between miRNA-21 and H.K. gene , it was show that increased expression of miRNA -21 lead to increase in expression of H. K. gene or control gene , which are often referred to as housekeeping genes . This result might be refer to that increased in miRNA – 21 exhibited with the other normal gene and elevated its expression such as control gene or H.K. gene among a group of Thalassimic patients infected with HCV infection.



Figure(4.15): Correlation between miRNA -21 and H. K. gene in HCV patients.

CONCLUSION

AND

RECOMMENDATION

Conclusions:

1.. Increased the HLA-DR level and HLA-G , might be show that the disease activity enhancement of Class 11 HLA –typing by which T-Cells activation and antibody production , and cytotoxic T-cells activation or HLA-Class 1 type especially in females patients than males.

2. The young ages between (10-19years) are the most influential The duration of disease also had a significant immune effect. The higher the age of the infection, the greater the effect of the virus on the HLA immune factor.

3. The gene expression of (miR-122, miR-21) genes, revealed clear increase in gene expression for miR-122 gene , encounter by poor expression of miR-21 . This is evidence of the strong association between gene miRNA-122 and infection and might be refer to prognosis of hepatitis infection .

4. The more susceptible blood grouping type , is the (O +ve blood group) in thalassemia patients in which that more infected with hepatitis C virus rather than other blood group.

5. Excessively high level of liver enzymes (GPT, GOT) in patients with thalassemia in which associated with hepatitis C virus

6. The association between HLA and miRNA, have a positive correlation in gene expression of miRNA-122 and HLA. In contrast to miR-21, which gave low gene expression, and these are important results for hepatitis patients with pre-existing genetic disorders caused by thalassemia.

Recommendation:

1. The use of a large number of HLA alleles and the expansion of the study of immune factors, such as monitoring the effect of the virus on the different types of HLA, which can give an idea of strengthening the immune system to overcome the virus.

2. Greatly shedding light on the study of gene expression of miRNA genes at different types , which could lead to developments in the treatment of hepatitis patients or monitor the disease prognosis.

3. Determining the relationship between the genetic and immune forms of thalassemia patients with hepatitis, and the necessity of periodic examinations for them to monitor the progression of the disease to a chronic condition.

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الخلاصة

فيروس التهاب الكبد الوبائي سي (HCV) هو فيروس يلفه جينوم الحمض النووي الريبي الموجب المعنى في فيروسات التهاب الكبد الوبائي سي المرتبطة بعائلة فلافيفيروس. يبلغ حجم كل جسيم فيروسي حوالي (55 نانومتر - 65 نانومتر). تم إعاقة الفحص المجهري الإلكتروني المرتبط بالفيروس (EM) بسبب عدم وجود نظام ثقافة خلوية أنتج كمية كافية من فيروسين للتصور. أجريت هذه الدراسة على مرضى الثلاسيميا المصابين بالتهاب الكبد سي في مركز أمراض الدم الوراثية في مستشفى بابل للنساء والأطفال. الثلاسيميا هي مجموعة غير متجانسة من تخليق الهيموجلوبين والاضطرابات الوراثية الناتجة عن انخفاض معدل إنتاج سلسلة أو أكثر من سلاسل الهيموجلوبين.

أجريت هذه الدراسة على 100 عينة مقسمة إلى ثلاث مجموعات. مرضى الثلاسيميا المصابون بفيروس التهاب الكبد سي (60) مريضا ، ومجموعات مرضى الثلاسيميا غير المصابين بالتهاب الكبد سي بلغ (20) مريضا ، والمجموعة الضابطة (20) فرد. هدفت الدراسة إلى التعرف على فيروس التهاب الكبد الوبائي سي لدى مرضى الثلاسيميا الناتج عن عمليات نقل الدم الملوثة بهذا الفيروس ، وتحديد بعض المؤشرات المناعية المتأثرة بالفيروس (HLA-DR & HLA.G)، وأظهرت نتائج اختبار ELISA على المرضى المصابين بالفيروس. نسب عالية من (HLA-DR,G) في المرضى مقارنة بالسيطرة الإيجابية والسلبية ، حيث كانت نسبة HLA-DR (8.92 ± 11.31)في المرضى مقارنة بالمجموعة الضابطة (4.92 ± 2.70 و 4.34 ± 2.16) على التوالي مع فرق غير معنويه P-0.007. أما نسبة HLA-G

(640.1 ± 811.5)في المرضى مقارنة بالمجموعة الضابطة (التحكم الإيجابي والسلبي) (232.5 ± 977.5 و 552.8 ± 386.1) على التوالي. ، كان الدليل على تأثير الفيروس على عامل المناعة HLA-DR أكبر في المرضى.

تم استخلاص الحمض النووي الريبوزي من عينات الدم واستخدمت تقنية RT qPCR لتحديد وتقييم التعبير الجيني لـ(miRNA-122, miRNA-21) ومقارنتها بجينات التدبير المنزلي التي تعتبر جينات تحكم لمرضى التهاب الكبد .C كانت العلاقة العكسية للجين miR-122 (17.67±5. 2)مع .H.K الجينات (24.76 ± 4.2) قيمةP 0.000 ، أظهرت زيادة في التعبير الجيني وارتباطًا قويًا بفيروس التهاب الكبد C ، أما بالنسبة miR-21 (29.15± 2.3)مقارنة بجينات التدبير المنزلي ، فقد كان لديها تعبير جيني منخفض في مرضى التهاب الكبد الوبائي. تضمنت هذه الدراسة أيضا مقارنة القيم المتوسطة لاختبار وظائف الكبد لمرضى التهاب الكبد C المصابين بالثلاسيميا(GOT 36.86 ± 30.50) ، (GPT 30.02 ± 20.34)، وكانت الزيادة واضحة في المرضى مقارنة مع السيطرة على الثلاسيميا على مستوى (GOT). 25.73 ± 15.28) ، .(GPT(26.26 ± 16.32التي تبين زيادتها أيضًا مقارنةً بالمستوى الصحي الضبط(GOT 19.80 ± 5.98) ، GPT (26.85 ± 5.55)هذه النتيجة لا تظهر فروقًا ذات دلالة إحصائية .

وكذلك تم قياس مستويات (الفيريتين ، الهيموغلوبين ، PT & PTT، PCV).وقد تأثرت جميع الأصناف بفيروس التهاب الكبد سي من جهة ، والاضطرابات الوراثية للمرضى من جهة أخرى.

وزارة التعليم العالي
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قسم علوم الحياة

دراسة تأثير HLA-G , HLA-DR وبعض عوامل التعبير الجيني الرايبوزي للمايكرو RNA لمرضى الثلاسيميا المصابين بالتهاب الكبد الفيروسي نوع C

رسالة مقدمة الى
مجلس كلية العلوم للبنات – جامعة بابل
كجزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

استبرق حسن علوان الوطيفي

بإشراف

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2022 م　　　　1444ه