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Association of Cytokines Gene Polymorphism in Patients With HCV in AL- Diwaniyah Province

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(وَكَايِّنَ مِنْ دَابَّةٍ لَا تَحْمِلُ رِزْقَهَا اللَّهُ يَرْزُقُهَا وَإِيَّاكُمْ وَهُوَ السَّمِيعُ الْعَلِيمُ)

صدق الله العلي العظيم

سورة العنكبوت
(الآية 60)

Supervisor's certification

We certify that this study entitled "**Association of Cytokine Gene Polymorphisms With Hepatitis C Virus Infection in AL- Diwaniyah Province Population**" has been conducted under our supervision at the department of Biology, college of sciences - University of Al-Qadisiyah, as a partial requirement for Degree of Doctor of philosophy in sciences\ Biology.

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In view of the available recommendations ,I forward this study for debate by the examining committee.

Professor.Dr.Laith Sareea Mutar

Date: / /2025

Dedication

To every patient who contributed to my scientific study project (you are not just numbers and people, but you were a great reason for the completion of my thesis and succeeding).

To the support in my life, the amazing man, and first supporter my husband .

To Father and Mother are who gives me strength and determination and always trusted my success, to those who sacrificed for us, to those who illuminate my life, my dear mother and my dear father.

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List of Abbreviations

Abbreviation	FullName
AP-1	Activator protein-1
CH25 H	Cholesterol 25- hydroxylase
COX-2	Cyclooxygenase -2
CTLA-4	Cytotoxic-lymphocyte-Associated protein4
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IFITM	Interferon - induced transmembrane protein
IRF3	Interferon Regulatory factor 3
ISG15	Interferon stimulated gene 15
KIR2DL3	Killer cell Immunoglobulin- lik receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LGP2	Laboratory of Genetics and physiology 2
MAPK	Mitogen – Activated protein
MAVS	Mitochondrial Antiviral-signaling protein
MDA5	Melanoma differentiation -Associated protein 5
NF-K β	Nuclear factor kappa B
NK	Natural killer cell
NLRP3	Nod-like receptor family pyrin domain containing3
P53	Tumor Suppressor protein
PD-1	Programed cell death
PKR	Protein kinase R
RDRP	RNA dependent RNA polymerase
RIGI	Retinoic acid inducible gene 1
ROS	Reactive oxygen species
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription3
SVR	Sustained virologic response
TCF1	T-cell factor1
TFH cells	T follicle helper cell
TGF- β	TransformingGrowthFactor- β
TIGIT	T cell immune receptor with Ig and ITIM domains
TIM-3	T cell Immunoglobulin and Musin domain-3
TRAF1	Tumor necrosis factor receptor-associated factor 1
Trege	RegulatoryT-cells

Summary

Summary

Hepatitis C virus (HCV) infection is a large health issue in developing countries. In Iraq, various studies have been performed to assess the genotypes prevalence related the virus (HCV), but, there is little data about such prevalence in Diwaniyah Province. The aim of this study was to find the prevalence of different HCV genotypes and to assess for correlation among susceptibility to infection of HCV and gene polymorphism related to *TNF α* -308G>A and IL-28B CT (rs12979860). The aim of this study was to assess for correlation across susceptibility to HCV infection and gene polymorphism related to IL-28B CT (rs12979860).

Enrolled patients were selected from the group of patients visiting the dialysis unit in Al-Diwaniyah Teaching Hospital, Al-Diwaniyah city, Iraq. Equal number of apparently healthy individuals was enrolled in order to serve as control group. The group of patients included 100 patients who were diagnosed to have HCV infection based on positivity to HCV RNA test in blood in addition to abnormal liver enzymes profiles. Five ml venous blood from antecubital vein was obtained. DNA sequencing was performed.

Rate of genetic subtypes of HCV in patients' group were as following: 11 cases (34.4 %) were genotype 4 subtypes, 10 cases (31.3 %) were genotype 1a subtypes, 7 cases (21.9 %) were genotype 1b subtype and the rest of 4 cases (12.5 %) were genotype 3b subtype.

Comparison of gene polymorphism of *TNF α* -308G>A SNP between patients group and control group, GA genotype was a significant risk factor to disease with an odds ratio in the range of 2.67 to 3.11. AA genotype was not significantly associated with the disease ($p > 0.05$).

Summary

GG genotype was significant protecting factor against the disease with an odds ratio of 0.40. In addition, allele A was significant risk factor (odds ratio = 1.94) and allele G was significant protecting factor (odds ratio = 0.52). Gene polymorphism of *TNFA* -308G>A is associated with significant variation in serum TNF-alpha level in patients with HCV infection (G/A and AA genotypes were associated with low serum level in comparison to GG genotype).

Comparison of gene polymorphism of IL-28B CT (rs12979860) SNP between patients group and control group revealed that none of genotypes or alleles was significantly associated with the disease ($p > 0.05$). Comparison of mean serum IL-28B based on IL-28B CT (rs12979860) genotypes results were as following: CT genotype showed significantly lower level of serum IL-28B in comparison with CC genotype ($p < 0.001$) and TT genotype (0.016). Comparison of IL-28 levels between patients group and control group, Mean serum IL-28 was higher significantly in patients group compared to control group, 340.86 ± 61.61 ng/ml versus 275.18 ± 91.68 ng/ml, respectively ($p < 0.001$). Mean serum TNF-alpha was higher significantly in patients group compared to control group, 93.32 ± 14.69 ng/ml versus 62.24 ± 22.62 ng/ml, respectively ($p < 0.001$). The current study lack of significant impact of virus genotypes and or sub-genotypes on serum values of IL-28B. No significant association was found when genotypes for interleukines were compared to virus genotypes /subgenotypes.

Chapter One

Introduction

1. Introduction

Hepatitis C virus (HCV) genotypes are divided into eight categories according to the phylogenetic and sequence analyses of HCV genomes, while each genotype is further subdivided into 86 subtypes. These subtypes are represented by lowercase letters such as 1a, 1b, 2a, and so on. Despite the extensive sequence diversity observed in HCV, all genotypes share inside the open reading frame (ORF) a common set of genes of co-linear kind that are of similar size. Additionally, the known variants exhibit significant sequence similarity across the entire genome. This characteristic enables their classification depending on sequences that are partial obtained from particular sites, such as NS5B or core/E1, inside the genome (Hedskog *et al.*, 2019).

While there are several techniques available for genotyping of HCV, the gold standard for precise classification still involves entire sequencing of analysis of the genome, along with subjecting the conserved and informative regions to phylogenetic analysis, such as NS5B or core/E1 (Virtanen *et al.*, 2018; Spitz *et al.*, 2019; Irekeola *et al.*, 2021).

Genetic variations among genotypes range from approximately 25% to 35% at the nucleotide level. These genotypes can be classified into subtypes, which exhibit differences of around 15% to 25% at the nucleotide level. Viral quasispecies refer to closely-related mutant spectra, such as escape mutants observed in one patient due to immune response (Sardanyés *et al.*, 2024; Tsukiyama-Kohara and Kohara, 2017).

Regarding particular groups of populations and even worldwide, the subtypes 1 (a and b), 2 (a and b), 3 (c and q), 4 (a and d) 5 (a) and 6 (a) are well recognized (Simmonds, 2013; Li *et al.*, 2014; Lu *et al.*, 2015). It is estimated that both

genotypes 1 and 3 are responsible for approximately 30 to 40 % of cases worldwide. The genotype 2 is found in 9% of cases, genotype 4 is recognized in 8 % of cases, genotype 6 is seen in 6 % of cases and genotype 5 is observed in 1 % of cases (Messina *et al.*, 2015). In Europe, it has been shown that more than 90% of cases are attributed to genotypes 1, 2 and 3 (Simão and Gonçalves, 2024).

In the United States, Europe, Japan and Australia, genotype 1 is of the highest frequency. Across Italian community, genotype 2 is widespread. Genotype 3 shows significant dominance in various countries, with Pakistan having a 22% prevalence. Genotype 1b is prevalent in Southern Europe, while genotype 1a is commonly seen in Northern Europe. With respect to genotype 2, the 2b subtype is common in Northern Europe and the 2c subtype is predominant in Southern Europe. Subtype 2c was diagnosed for the first time in the Sardinia region of Italy (Tariq *et al.*, 2022).

Genotype 3 is predominantly characterized by subtype 3a. The distribution of genotypes 4 and 5 has expanded due to emigration from Africa and the Middle East, particularly among intravenous drug addicts. In Europe, genotypes 3a and 4d are increasingly dominant among intravenous drug users. As a result, genotype 3a is responsible for approximately half of all infections in several Northern European countries. Occasionally, genotype 6 can be found among emigrants from Southeast Asia (Sistayanarain and Chaiwong, 2017).

Tumor necrosis factor alpha (TNF- α) constitutes a cytokine that is pro-inflammatory and functions both within the cellular type immune reaction and as a mediator of innate immune response (Santiago *et al.*, 2021). Aberrant levels of TNF- α have been correlated with chronic hepatitis C virus (HCV) infection (Pompili *et al.*, 2013, Talaat *et al.*, 2012). Some TNF α gene polymorphisms correspond to transcription factor binding domains and have been connected to the regulation of cytokine production (Santiago *et al.*, 2021). The TNF α -

308G>Apolymorphism has been linked to a number of infectious diseases (Santiago *et al.*, 2021, Eskandari-Nasab *et al.*, 2018; Pabalan *et al.*, 2017) and shows greater activity of transcription than the allele that is wild (Santiago *et al.*, 2021).

Naturally, human body is equipped with interferons which play an essential role in immunity against viral infection as they act by triggering some crucial pathways. One of important interferons in this regard is the interferon lambda (IFN- λ), a member of type III IFN family, which is also known as interleukin-28B (IL-28B) and its gene is located on chromosome 19 (Hajizadeh *et al.*, 2022). The stability of mRNA related to IL-28B can be affected by a number of single nucleotide polymorphisms (SNPs), thus, the durability and the function of the gene is going to be disturbed. Therefore, body immune response against HCV can be negatively affected leading to complications including persistent chronic state of infection, liver cirrhosis and even carcinoma (Al-Qahtani *et al.*, 2015).

Aim of The Study:

The aim of the present study is to assess the association of some cytokine gene polymorphisms with hepatitis C virus infection.

To achieve this aim, the following objectives were applied:

1. To find the prevalence of different HCV genotypes in a sample of Iraqi patients in Diwaniyah province.
2. To assess for correlation across susceptibility to infection-HCV and gene polymorphism related to IL-28B CT (rs12979860).
3. To assess for correlation across susceptibility to infection-HCV and gene polymorphism related to *TNF α* -308G>A.
4. To compare of IL-28 levels between patients group and control group.
5. To compare of TNF-alpha levels between patients group and control group.
6. To assess for the association of HCV genotypes to TNF-alpha and IL-28 gene polymorphism.

Chapter Two

Literatures Review

2. Literature Review

2.1. Historical Background

In the mid-1970s, Harvey James Alter, chief of the infectious diseases section of the Division of Transfusion Medicine at the National Institutes of Health, and his research team showed that most cases of post-transfusion hepatitis were not due to hepatitis A or B virus. Despite this finding, international efforts to identify the virus failed within a decade, and it was referred to as non-A, non-B hepatitis (NANBH)(Campollo *et al.*, 2022). In 1989, Michael Houghton, Guilin Zhu, Jinhong Kuo, and Dr. Daniel Bradley of Chiron worked with the Centers for Disease Control and Prevention to use a new molecular selection technique to identify unknown organisms and develop diagnostic tests(Boyer, 2001; Manns and Maasoumy, 2022).

In 1988, Alter confirmed the presence of the virus in samples of non-A, non-B hepatitis, confirming the presence of hepatitis C virus. Two articles on the discovery of a vaccine for hepatitis C were published in the April 1989 issue of Science(Choo *et al.*, 1989). This discovery greatly improved the diagnosis and antiviral treatment of hepatitis C. Alter and Bradley received the Lasker Award in 2000 "for their groundbreaking work leading to the discovery of the virus that causes hepatitis C and the development of screening methods that have reduced transfusion-associated hepatitis in the United States from 30% in 1970 to virtually zero by 2000 (Thomas *et al.*, 2015).

In 1991, the International Committee on Taxonomy of Viruses classified HCV as a member of the genus Hepatitis C virus in the family Flaviviridae (van Regenmortel and Mahy, 2004). In recognition of their

contributions to the discovery of the hepatitis C virus, the Nobel Foundation announced that the 2020 Nobel Prize in Physiology or Medicine would be awarded jointly to Harvey Alter , Michael Houghton , and Charles M. Rice , with each receiving one-third(Ghany *et al.*, 2021).

2.2.Taxonomy

Hepatitis C (English: Hepatitis C), translated as C-type hepatitis in Taiwan, often referred to as hepatitis C, is a humoral infectious disease caused by the hepatitis C virus, which mainly affects the liver(Hu et al., 2020; Dajti *et al.*, 2021).Hepatitis C virus (HCV) is an enveloped, single-stranded, positive-sense Rnavirus (Rosen, 2011), classified in the genus Hepacivirus of the family Flaviviridae (Ray and Thomas, 2009). Its peers include viruses of the genus Pestivirus, such as yellow fever virus, Borna virus, and bovine diarrhea virus.Based on the diversity of HCV sequences, the international classification view mainly adopts the Simmonds system (Zein and Persing, 1996). Using the sequence analysis of 5UTR, core, and NS5B, it is divided into 6 major types and about 12 subtypes (Shier *et al.*, 2017).

2.3. Etiology of HCV

HCV is a spherical particle with a diameter of 50 to 60 nm. The outermost layer is the envelope glycoprotein, and the inner layer is the nucleocapsid. The viral genome is wrapped by the nucleocapsid to form a core particle with a diameter of 30 to 35 nm, which is wrapped by the envelope to form a complete HCV particle with a sedimentation coefficient of 140 to 159S. According to sucrose density gradient analysis, there are two different density gradients of HCV particles in serum. One is high density (1.186~1.213kg/L), which may be free or immunoglobulin-bound HCV

particles; the other is low density (1.099~1.127kg/L), which may be HCV bound to low-density lipoprotein(Zein and Persing, 1996).

2.4. HCV Genome and Encoded Proteins

The HCV genome is a single-stranded positive-strand RNA with a total length of about 9500nt. Because the length of the polyadenylic acid tail (polyA) after 9400nt varies, the length reported by different sources varies (9400-9600nt). The genome consists of a 5'-noncoding region (5'-NCR, about 341nt long; also known as the 5'-untranslated region, 5'-UTR), an open reading frame (ORF, 9033-9099nt long) and a 3'-noncoding region (3'-NCR, also known as the 3'-untranslated region, 3'-UTR) as shown in figure (2.1) (Ashfaq *et al.*, 2011; Pirakitikulr *et al.*, 2016; Tong *et al.*, 2017; Wan *et al.*, 2022).

The 5'-NCR sequence is the most conserved in the genome sequence and contains an internal ribosome entry site (IRES), which can regulate the expression of the viral genome. From the 5' end to the 3' end, ORF is C region (encoding nucleocapsid protein), E1 and E2 regions (encoding envelope protein), P7 region (encoding cell outer membrane pore protein or ion channel protein), NS2 region (non-structural protein 2 region, encoding viral autoprotease), NS3 region (encoding viral helicase and serine protease), NS4A (encoding NS3 protease auxiliary factor), NS4B (encoding replication complex and membrane network combination factor), NS5A (encoding viral replication and assembly regulatory factor), NS5B (encoding HCVRNA-guided RNA polymerase) as shown in table (2.1). ORF first guides the synthesis of a precursor that is polyprotein comprising 3010-3033 amino-acids in length, and then, under the action of viral protease and host signal peptidase, it is cut into viral structural proteins (core protein and envelope

protein) and non-structural proteins (NS1 to NS5 proteins). The E2 region is actually what was previously called the NS1 region, so this region is sometimes called the E2/NS1 region. The NS3 protein also has strong immunogenicity and can stimulate the body to produce antibodies, which is of great value in clinical diagnosis. Non-structural proteins are mainly functional enzymes and their auxiliary factors involved in HCV replication (Ashfaq *et al.*, 2011; Pirakitikulr *et al.*, 2016; Tong *et al.*, 2017; Wan *et al.*, 2022).

The genome consist of 5' and 3' non-translated sequences and an open reading frame. It is worth mentioning that since the genome does not have a cap structure, there is an internal ribosome entry site (IRES) consisting of four collar loop domains at the 5' end. Immediately adjacent to the 5' end are the core protein and envelope proteins 1 and 2, which are structural proteins. Non-structural proteins include (P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) and 3'UTR (Cuypers *et al.*, 2015). HCV infection and proliferation is a rapid process, The number of viral replications will reach a peak about 2 months after infecting humans or chimpanzees (Scott *et al.*, 2023). However, this high replication efficiency, due to the lack of an effective proofreading mechanism for RNA dependent RNA polymerase (RdRp), results in a high mutation rate in the HCV genome, which poses a severe challenge to the research and development of antiviral drugs for HCV.

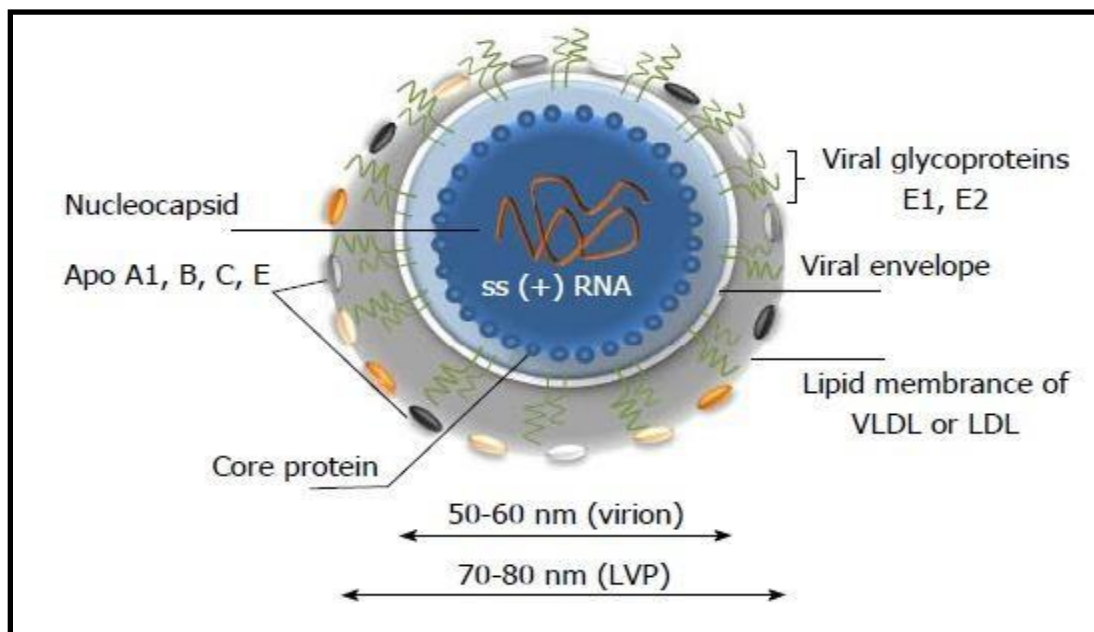


Figure 2.1: The structure of hepatitis C virus (HCV) (Vercauteren *et al.*, 2014).

Table 2.1: Summarization of proteins sizes related to the virus hepatitis C virus

Molecular weight of protein, kDa	Amino acid position	Number of amino acids	Protein
4	1658-1711	54	NS4A
7	747-809	63	p7
21	1-174	174	Core mature
21	810-1026	217	NS2
23	1-191	191	Core immature
27	1712-1972	261	NS4B
35	192-383	192	E1
56	1973-2420	448	NS5A
66	2421-3011	591	NS5B
70	384-746	363	E2
70	1027-1657	631	NS3
Approximately	16-17	126-161	F protein of ARF protein

2.5. HCV Genotypes

HCV has high genetic heterogeneity. HCV can be divided into different genotypes and subtypes based on the differences in nucleotide sequences. When the difference in the whole gene sequence of HCV is more than 30%, it can be distinguished into different genotypes; when the difference in the whole gene sequence of HCV of the same genotype is more than 20%, it can be distinguished into different subtypes. In the past, HCV was divided into types I to IV according to the Okamoto method, but it is now abandoned. According to the Simmonds method, HCV is mainly divided into 7 genotypes, and each genotype can be divided into different subtypes (a, b, c, etc.)(Zein, 2020; Kumar *et al.*, 2018; Keikha *et al.*, 2020; Castro *et al.*, 2020; Virtanen *et al.*, 2018).

There are 6 HCV genotypes that have been studied more thoroughly, divided into 11 subtypes: 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a, 5a, and 6a; type 1 is the most common, accounting for 40% to 80%. The distribution of HCV genotypes in different countries and regions varies greatly. Type 1 is the most common in China, Japan, and the United States. Type 3 is common in India, Pakistan, Australia, Scotland, etc. Type 4 is common in the Middle East and Africa. Type 5 is common in South Africa (Zein, 2020; Kumar *et al.*, 2018; Keikha *et al.*, 2020; Castro *et al.*, 2020; Virtanen *et al.*, 2018).

Type 6 is common in Hong Kong, Macau, Guangdong, and Chongqing, China. Type 7 was isolated from a small number of patients in Central Africa. Its clinical importance is still unclear. Type 1b HCV has a high RNA load and severe pathological changes in the liver, which can easily lead to cirrhosis and liver cancer. However, some scholars believe that there is no obvious correlation between genotype and disease severity. HCV genotype is

also closely related to the efficacy of IFN. When developing HCV vaccines, it is also necessary to design them for different HCV genotypes(Zein, 2020; Kumar *et al.*, 2018; Keikha *et al.*, 2020; Castro *et al.*, 2020; Virtanen *et al.*, 2018).

2.6. Pathogenesis of HCV

HCV invasion of host cells is a complex process mediated by multiple receptors. The proposed HCV receptors mainly include CD81, low density lipoprotein receptor (LDLR), scavenger receptor class B type I (SR-BI), tight junction protein family, epidermal growth factor receptor (EGFR), tyrosine kinase EphA2 receptor (ephrin receptor A2, EphA2), NPC1L1 receptor (niemann-Pick C1-like1cholesterol absorption receptor), etc. The mechanism of HCV infection of hepatocytes may be achieved through the binding of its envelope protein E2 to the corresponding receptor CD81 molecule on the surface of hepatocytes. In the past, it was believed that the pathogenesis of hepatitis C was the direct damage of HCV to hepatocytes, but now it is believed that this is only a secondary mechanism. The following points suggest that the pathogenesis of hepatitis C may be involved in immune mechanisms: ① The number of hepatocytes infected by HCV is small, while the inflammatory response of liver tissue is obvious, which forms a contrast. ②Immunohistochemistry proved that the necrotic area of liver parenchyma in hepatitis C was mainly infiltrated by CD8⁺ lymphocytes, and immunoelectron microscopy observed direct contact between CD8⁺ cells and hepatocytes. ③HCV-specific T cell clones were isolated from the liver of hepatitis C patients. ④IFN- α treatment can reduce the number of CD8⁺ cells in the liver. HLA molecules and ICAM-1 molecules are expressed on the surface of hepatocytes in hepatitis C patients. These findings are similar to hepatitis B(Irshad *et al.*, 2013; Antonelli *et al.*, 2003; Kohla and Bonacini, 2006; Rios

et al., 2021; Elgretli *et al.*, 2023).

Although HCV infection induces specific CTL response, due to the variability of the highly variable region of HCV RNA, a series of quasi-species and other variants are formed. Specific CTL cannot recognize its epitope, making antiviral immunity ineffective. This is the fundamental reason why HCV infection is very easy to become chronic. Its chronic mechanism also includes the following points: ① The level of HCV in the blood is very low, which can easily induce immune tolerance. ② HCV is pantropic and difficult to eliminate. ③ Immune cells can be infected by HCV, resulting in immune disorder. In addition, some scholars have proposed that type 1b HCV infection is more prone to chronicity, and the reason remains to be studied. Autoimmune response may also be involved in the onset of hepatitis C (Irshad *et al.*, 2013; Antonelli *et al.*, 2003; Kohla and Bonacini, 2006; Rios *et al.*, 2021; Elgretli *et al.*, 2023).

The reason why Asian (yellow) chronic hepatitis C (CHC) patients have a higher response to IFN- α than Caucasians (whites) is still unclear. Host genetic changes are associated with IFN- α response, spontaneous viral clearance, and ribavirin (RBV)-induced anemia in CHC patients. Two groups of reports have studied the single nucleotide polymorphism (SNP) rs12979860 3kb upstream of the IL-28B gene encoding type III IFN- α . The results showed that the T/T and T/C sites were not only associated with a significant decrease in the response to IFN- α relative to C/C (the difference in response was 2-fold), but also confirmed that the IL-28B RNA level in the blood of patients with T/T and T/C IFN- α response failure was lower. However, the reason why the IFN- α efficacy in the Chinese population is generally higher does not seem to be completely explained by the above findings, and it is speculated that it is also related to viral factors. (Irshad *et al.*, 2013; Antonelli *et al.*, 2003; Kohla and Bonacini, 2006; Rios *et al.*, 2021; Elgretli *et al.*, 2023).

2.7. Replicative Cycle of HCV

The replicative cycle of the hepatitis C virus (HCV) initiates with the attachment of a virion to specific entry factors or receptors on hepatocytes. Once attached, the virion enters the cytoplasm and releases its genomic RNA. Subsequently, the HCV genomic RNA serves the purpose of both polyprotein translation and viral replication. The replication of HCV occurs within the membranous web (MW) located in the endoplasmic reticulum (ER). Finally, HCV utilizes the biosynthetic pathway of very-low-density lipoprotein (VLDL) to assemble and release viral particles from cells (Li *et al.*, 2021).

The alterations in lipid metabolism induced by HCV play a crucial role in its replication and particle assembly. HCV can modify the host's serum lipid profile, leading to steatosis. This condition is more prevalent and severe in HCV genotype 3 patients, and its severity correlates with higher HCV RNA levels. Steatosis serves as both a marker of liver disease progression and an indicator of reduced therapy response in HCV-infected individuals. However, effective antiviral therapy can mitigate non-metabolic or alcoholic steatosis. The presence of HCV genomes in extrahepatic sites, such as leukocytes, suggests a potential reservoir of the virus even after treatment. The diversity of HCV quasi-species in different cell types, like plasma and peripheral blood monocytes, indicates independent viral spread. The infection of B-cells, as demonstrated in non-Hodgkin's lymphoma, raises questions about the significance of these extrahepatic reservoirs and their potential impact on immune manipulation and virus persistence (ModaresiEsfeh and Ansari-Gilani, 2016).

Replicative cycle of HCV is shown in figure 2.2. Hepatitis C virus entry is facilitated by a variety of receptors and signaling pathways. Upon viral entry, the positive sense RNA genome is released into the cytoplasm from endosomal acidification. The viral RNA undergoes replication and translation at the rough

endoplasmic reticulum to produce a single polyprotein chain at the endoplasmic reticulum membrane that is cleaved by viral and host proteases into 10 different viral proteins (structural and non-structural). The virus particles are assembled on lipid droplets and associate with very-low-density lipoproteins which mature at the Golgi apparatus and are released *via* the secretory pathway. HSPG: Heparin sulfate proteoglycan receptor; LD: Lipid droplets; VLDL: Very-low-density lipoproteins (D'souza *et al.*, 2020). An infected person produces approximately 10^{12} viruses per day (Lerat H, Hollinger, 2004). Viral replication is not limited to the liver, but also occurs in white blood cells (Zignego *et al.*, 2012).

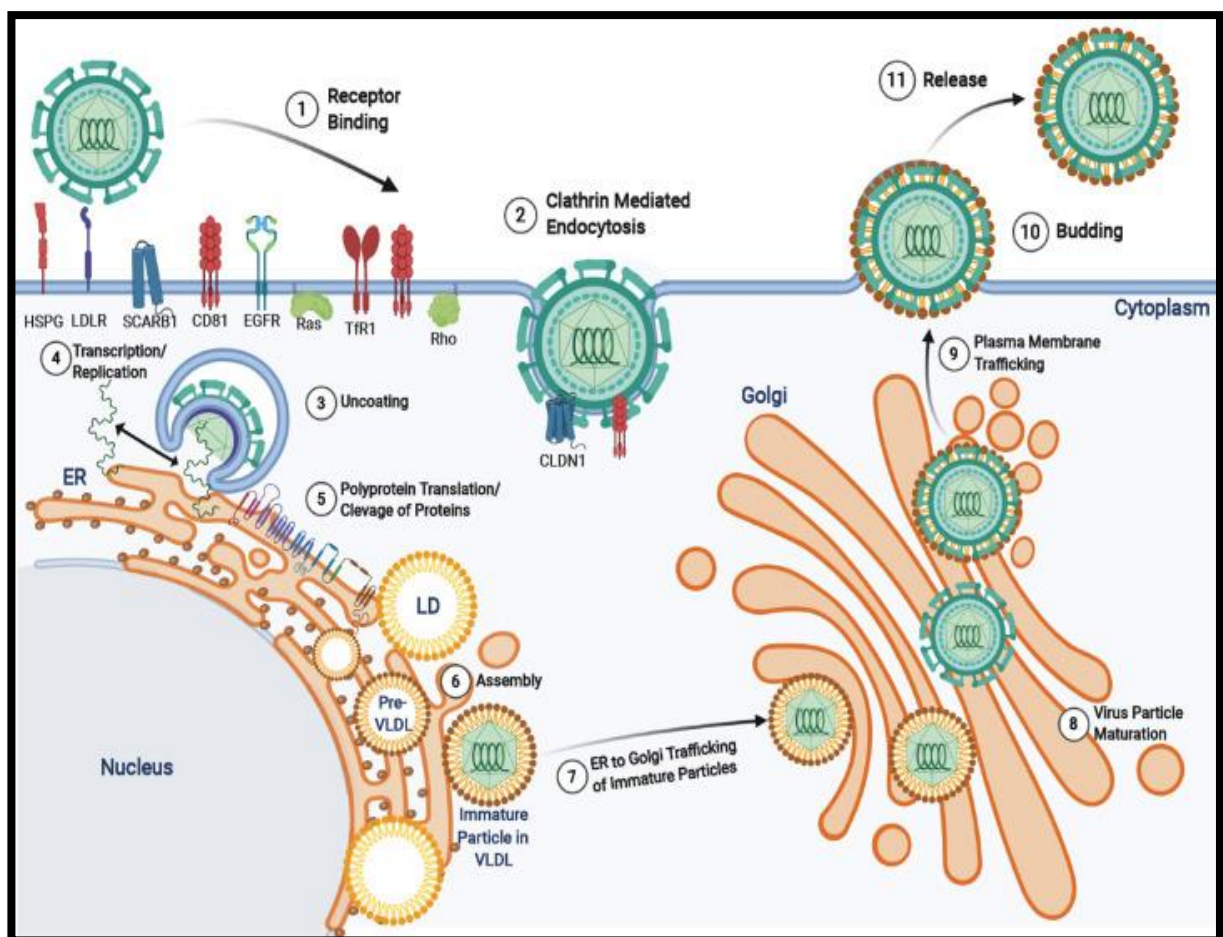


Figure 2-2:Hepatitis C virus replicative cycle . (D'souza *et al.*, 2020)

2.8 HCV Genotyping and Vaccine Development

Due to the existence of various HCV genotypes, subtypes, and quasispecies mutations and immune escape, the virus is able to escape the host immune system and cause persistent viral infection. Studies on chimpanzees have shown that the humoral immune response of HCV has a high degree of virus strain variability. Due to the high frequency of HCV mutation, mutants that are tolerant to HCV antibodies can escape the body's humoral immune response and maintain HCV infection (Stuart *et al.*, 2021). There are different mechanisms for the chronicity of HCV infection, such as the downregulation of HCV-specific T cell immune responses by regulatory T cells (Treg) (Fouad *et al.*, 2016) or programmed death receptor 1 (PD-1) (Ma *et al.*, 2011).

That is, in chronic HCV infection, the frequency of Treg cells is significantly higher than that in acute HCV infection. In addition, studies have also shown that CTL cells in chronic hepatitis C patients can specifically recognize HCV. The antigenic sites encoded by the C, E1, E2/NS-1, NS-3, and NS-5 regions, especially the C and NS-5 regions, can kill target cells expressing the above antigens. However, HCV-infected people still develop chronic disease, which may be due to the highly mutated viral genes that cause immune escape, or the low viral titer that causes the body's CTL cell response to be too weak. The existence of various HCV genotypes, subtypes, and quasi-species mutations has brought great difficulties to the development of hepatitis C vaccines. Ezelle *et al.* (2002) used a recombinant vesicular stomatitis virus (VSV) vector that can express HCV structural region proteins (C, E1, E2) to immunize animals. The results showed that the immunized mice produced strong cellular and humoral immunity against all HCV structural region proteins, especially humoral immunity against HCV-E2. Therefore, it was concluded that the VSV recombinant virus vaccine expressing HCV structural region proteins can be used as a method for developing anti-HCV infection and therapeutic vaccines (Baryluk *et al.*, 2005).

The glycoproteins E1 and E2 were used for treatment in human volunteers, and the E1/E2 protein recombinant showed moderate immunogenicity (Frey *et al.*, 2010). Currently, French scientists (Garrone *et al.*, 2011) have developed a hepatitis C vaccine using virus-like particles. After being injected into mice and macaques, this vaccine will induce high titers of anti-E2 and/or anti-E1 antibodies and neutralizing antibodies (Nabs) against HCV with pseudovirus particles of E2 and/or E1 HCV envelope glycoproteins. These antibodies can neutralize the infectivity of various HCV variants, thereby playing a preventive role. This vaccine is effective against five HCV variants, and further experiments are needed to clarify the effect of this vaccine on humans.

HCV has an exceptional ability to evade clearance by host immune response to develop its chronicity, with 60%–85% of infected patients develop into chronic infections no known other flaviviruses have this high chronicity rate in humans. The persistent liver infection can lead to chronic hepatitis, liver cirrhosis, and in some cases, hepatocellular carcinoma (HCC). HCV is an enveloped RNA virus containing a positive-sense, single-stranded genomic RNA of approximately 9600 nucleotides.. Sandwiched by 5'- and 3'-untranslated regions, the open reading frame of genomic RNA encodes a polyprotein with approximately 3000 amino acids. The polyprotein is cleaved post-translationally by virus and host proteases into structural proteins and non-structural proteins (Mani *et al.*, 2021).

The structural proteins, including core, E1, and E2, are the building blocks assembling into virus particles. The non-structural proteins, including ion channel protein p7 (NS1), NS2, NS3, NS4A, NS4B, NS5A, and NS5B, are responsible for forming virus replication systems and virus productions. The best-known primary function of HCV core protein is to assemble into nucleocapsid and encapsulate the virus genomic RNA. However, additional properties of the HCV core protein for modulating host cell transcription, inhibition or stimulation of apoptosis, and suppression of host immunity have been documented. These functions and properties

will be discussed and summarized in the following sections(Feng *et al.*, 2015; Shen *et al.*, 2018).

HCV core protein, derived from N-terminal 191 amino acids of the polyprotein, is the only HCV protein to directly interact with and encapsulate HCV genomic RNA and form nucleocapsids. The HCV core protein consists of three domains: the highly cationic domain 1, with approximately 117 amino acids, is the major domain to associate with HCV genomic RNA and to form nucleocapsids; the hydrophobic domain 2 is responsible for association with lipid droplets crucial for infectious virus particle productions; the domain 3, also hydrophobic, is the endoplasmic reticulum (ER) anchoring domain. The majority of HCV core protein is found to locate at the ER, mitochondria, and lipid droplets in cytoplasm. The interaction between HCV core protein and mitochondria reduces electron transfer complex I activity and increases reactive oxygen species (ROS) productions. On the other hand, nuclear localization of the core protein has been detected and was found to be mediated by nuclear localization signal-like sequences within protein domain 1. HCV core protein can be secreted from HCV-infected hepatocytes and detected in the circulating bloodstream (Mani *et al.*, 2021).

The roles played by the core protein in the modulations of host transcription and lipid metabolism, stimulation or inhibition of apoptosis, alterations of immune cell differentiations, and pro- and anti-inflammatory cytokine releases have been investigated and the ability of HCV core protein to trigger hepatic angiogenesis was also discovered(Tu *et al.*, 2012; Feng *et al.*, 2015). The core protein-induced dysfunction of components of several important pathways, including p53, AP-1, MAPK , transforming growth factor β , vascular endothelial growth factor, Wnt/ β -catenin, cyclooxygenase 2 (COX-2), are suggested to be involved in the development of HCC. Immune suppression and liver damage induced by HCV core protein were also demonstrated. The effects caused by the HCV core protein on host immune and inflammatory response are discussed in the following sections(Zahra *et al.*, 2018;

Mahmoudvand *et al.*, 2019).

The inflammation regulations by HCV core protein are very complicated and still not fully understood. Both pro- and anti-inflammatory roles have been suggested by different studies. The induction of oxidative stress in hepatocyte by the HCV core protein was observed. Microarray analysis on immortalized human hepatocytes suggested that the HCV core protein induces inflammatory cytokines through the STAT3 signaling pathway and extracellular HCV core protein activations of STAT3 in human monocytes, macrophages, and dendritic cells (DCs) were observed to related to an interleukin (IL)-6 pathway (Tacke *et al.*, 2011) (Liu *et al.*, 2020). On the other hand, the anti-inflammatory effects by the HCV core protein were also evidenced. Multiple studies have identified that the HCV core protein suppressed IL-12 and no production in activated macrophages and human blood monocytes, thus inhibiting inflammatory responses. IL-10 is considered as an anti-inflammatory cytokine, which played a central role in the production of other cytokines (Pang *et al.*, 2016).

While a study claimed that the lipopolysaccharide-induced level of IL-10 was not upregulated by HCV core protein other studies observed elevated level of IL-10 produced from HCV core protein-stimulated monocytes, cultured DCs and HCV patients' DCs. Cellular inflammatory response can also be inhibited by the core protein in cells via inhibition of COX-2 expressions. Acute inflammation is a mean for the host to defend against virus infection. Host inflammation can indeed be triggered by HCV core protein. On the other hand, the core protein is found to be able to downregulate the host inflammation, contributing the development of persistent virus infection (Pang *et al.*, 2016; Mani *et al.*, 2021).

2.9. Epidemiology of HCV

Chronic HCV affects more than 71 million people annually and causes nearly 700,000 deaths globally. There are regional and intra-population variations in HCV prevalence (Tariq *et al.*, 2022). It is estimated that between 123 and 180 million people, or 2% to 3% of the world's population, are either carriers or infected with HCV (Kretzer *et al.*, 2014).

The subtypes 1a, 1b, 2a, 2b, 3c, 3q, 4a, 4d, 5a, and 6a are well explained both globally and within specific demographic groups (Simmonds, 2013; Li *et al.*, 2014; Lu *et al.*, 2015). Nearly 30% to 40% of all infections are caused by genotypes 1 and 3. 9%, 8%, 1%, and 6% of infections are caused by genotypes 2, 4, 5, and 6, respectively (Messina *et al.*, 2015). 90% of all infections in Europe are caused by genotypes 1, 2, and 3 (Esteban *et al.*, 2008).

In United States, Europe, Japan and Australia, genotype 1 is the most common. In Italy, genotype 2 is widespread. Genotype 3 shows significant dominance in various countries, with Pakistan having a 22% prevalence. Within genotype 1, subtype 1a is predominant in Northern Europe, while 1b is more common in Southern Europe. Among genotype 2, subtype 2b is dominant in Northern Europe, while 2c is the prevailing subtype in Southern Europe. In Italy, a patient from Sardinia was diagnosed with subtype 2c for the first time (Bukh *et al.*, 1993).

Genotype 3 is predominantly characterized by subtype 3a. The distribution of genotypes 4 and 5 has expanded due to emigration from Africa and the Middle East, particularly among intravenous drug addicts. In Europe, genotypes 3a and 4d are increasingly dominant among intravenous drug users. As a result, genotype 3a is responsible for approximately half of all infections in several Northern European countries. Occasionally, genotype 6 can be found among emigrants from Southeast Asia (Corbet *et al.*, 2003).

Moreover, type 4 genetic variant is predominantly found in Middle East and Africa, although the 4d subkind is initially identified in a patient from Denmark (Bukh *et al.*, 1993; Bukh *et al.*, 1994). In Asian country of (Thailand), genotypes of less severity such as 7b and 7a is prevalent. The impact of HCV genotypes on chronic HCV infections is minimal. Consequently, severe infections of hepatic tissues is correlated to genotypes as a whole, while genotype 3 is the principal cause of steatosis (Negro, 2006). It is recognized that genetic kinds play a role in the reaction to therapies that are based on interferon (INF). Genetic types 4 and 1 exhibit poor response to the therapy, unlike genotypes 2 and 3 (Pawlotsky *et al.*, 2015). Genotype 3 is the most challenging to treat with novel-IFN free DAA-based therapies (Goossens and Negro, 2014; Pawlotsky, 2014). The HCV prevalence rate in Iraq stands at 3.9% according to Merzah *et al.* (2019). While there is no national study in Iraq estimating the prevalence rates of different HCV genotypes, a report by Ghaderi-Zefrehi *et al.* (2016) indicated that genotype 4 has the highest prevalence at 60%, followed by genotype 1 at 26%.

It is estimated that between 150 and 200 million people are infected with hepatitis C worldwide, with most infections occurring in Africa, Central Asia, and East Asia (Hashmi *et al.*, 2010). In 2013, hepatitis C caused more than 343,000 deaths from liver cancer and more than 358,000 deaths from cirrhosis of the liver Ghaderi-Zefrehi *et al.* (2016). Hepatitis C was first thought to be a non-A or non-B form of hepatitis, first conceived in the 1970s and confirmed in 1989 (Manns and Maasoumy, 2022). Hepatitis C infects only humans and chimpanzees (Ploss and Kapoor, 2020).

In the United States, 70% of cases are caused by genotype 1, 20% of cases are caused by genotype 2, and the other genotypes only cause about 1% of hepatitis C (Wilkins *et al.*, 2010). Genotype 1 is also common in South America and Europe (Rosen, 2011). Japan is also mainly dominated by type 1, type 2 is more common in Japan and China, type 3 is more common in Thailand, Singapore, and parts of India, type 4 is mainly in Central Africa, Middle East and Egypt, and types 6

and 5 are more common in Southeast Asia and South-Africa (Ploss and Kapoor, 2020).

2.10. Mode of Transmission

The disease is transmitted through shared needles, inadequately sterilized medical equipment, needlestick injuries during health care work, and blood transfusions. With the advent of blood screening, infection through blood transfusions is less than one in two million. Another route of transmission is from mother to baby during childbirth. It is not contagious through daily contact (Woyesa, S. B., & Amente *et al.*, 2023).

2.10.1. Transmission Through Blood

It can be transmitted through transfusion of infected or contaminated whole blood and blood products, but this route has been controlled since my country screened blood donors for anti-HCV. Another route of transmission through blood is through broken skin and mucous membranes, which is currently the most important mode of transmission. Intravenous drug injection, use of non-disposable syringes and needles, dental instruments that are not strictly sterilized, endoscopes, invasive operations and acupuncture are important routes of transmission through skin and mucous membranes. Sharing razors, toothbrushes, tattoos and earring holes are also potential ways of transmission through blood (Zeuzem *et al.*, 1996).

2.10.2. Sexual Transmission

People who have sexual intercourse with HCV infected people or engage in promiscuous sexual behavior have a higher risk of contracting HCV (Nijmeijer *et al.*, 2019).

2.10.3. Mother-To-Child Vertical Transmission

Mothers who are anti-HCV positive or HCV RNA positive are at risk of transmitting HCV to their newborns, and the higher the HCV viral load, the higher the risk of transmission. Generally, kissing, hugging, sneezing, coughing, food, drinking, sharing tableware and cups, as long as there is no skin damage or other blood exposure, generally will not transmit HCV(Tovo *et al.*, 2016).

So far, there is no vaccine to prevent hepatitis C (Cox, 2020). According to the above known transmission routes, it is very important to take corresponding preventive measures, so as to avoid HCV infection as much as possible. For example: strictly implement the "Blood Donation Law of the People's Republic of China", strictly screen blood donors by testing serum anti-HCV and alanine aminotransferase (ALT) to ensure that infected or contaminated blood sources are excluded.

The Hepatitis C virus can be transmitted through various means, including blood transfusion, injections, sexual contact, surgery, and tattooing. Research by Keikha *et al.* (2020) indicates that most patients contract HCV through blood transfusion, with HCV-RNA typically being detected 7-21 days post-transfusion (Grebely *et al.*, 2012). During the acute phase, many infected patients are asymptomatic and unaware of their condition (Loomba *et al.*, 2011). It is worth noting that spontaneous clearance of the virus occurs in 20% to 30% of infected individuals (Micallef *et al.*, 2006).

Patients with disorders such as thalassemia, hemophilia, chronic renal failure requiring hemodialysis, and various cancers greatly rely on blood transfusion for their continuous treatment (O'Brien *et al.*, 2007). Another significant route of HCVtransmission is through unsafe injection practices, particularly the reuse of syringes and needles (Kermode,2004).

Intravenous drug use is a significant means of transmitting HCV among

individuals. The transmission of the HCV virus from a mother to her fetus is restricted to a maximum of 5% (Benova *et al.*, 2014). Needle stick injuries have been identified as a significant source of HCV infection among healthcare professionals (Kiyosawa *et al.*, 1991).

Hepatitis C virus (HCV) infection can be transmitted through sexual contact and is more common among individuals with multiple sexual partners. The risk of HCV transmission is further heightened when there is co-infection with HIV (Terrault, 2002). In addition to sexual transmission, nosocomial transmission of HCV has been observed due to the use of unsterile medical devices such as endoscopy, angiography, and surgical instruments. Furthermore, certain practices like tattooing, piercing, and cupping have also been identified as potential modes of HCV transmission. Various medical procedures including gynecology and cardiology operations, angiography, endoscopy, and colonoscopy can contribute to an increased rate of HCV infection when unsterile devices are utilized (Zamani *et al.*, 2013).

2.11. Clinical Features

Acute HCV infection typically does not exhibit noticeable symptoms, but it can lead to malaise, nausea, and pain in the upper right quadrant of the abdomen. Subsequently, dark urine and jaundice may occur, which are indistinguishable from symptoms of other acute viral hepatitis cases. Individuals who are persistently infected with HCV often do not experience symptoms. The symptoms that do arise are nonspecific and include fatigue, intermittent pain in the upper right quadrant of the abdomen, joint pain, and an overall feeling of being unwell, resulting in a reduced quality of life. It is difficult to attribute these symptoms solely to HCV, as there may be a psychological component due to the awareness of having an underlying chronic disease (Basit *et al.*, 2023).

Between 10% and 20% of individuals infected with HCV and suffering from

cirrhosis will experience clinical decompensation within a five-year period. This is characterized by the emergence of conditions such as portal hypertension, esophageal varices, ascites, coagulopathy, encephalopathy, or hepatocellular carcinoma. During this phase, they may exhibit physical symptoms that indicate chronic liver disease, including caput-medusae, spider angiomas, palmar erythema, asterixis, anasarca, and fluid thrill. Additionally, they might display signs and symptoms of various extrahepatic manifestations such as mixed cryoglobulinemia, membranoproliferative glomerulonephritis, porphyria cutanea tarda, lichen planus, neurocognitive changes, insulin resistance, and B cell lymphoproliferative disorders (Basit *et al.*, 2023).

The initial symptoms are mild or even absent. Occasionally, there will be symptoms such as fever, yellow and dark urine, abdominal pain and jaundice. About 75% to 85% of the first-time patients will have the virus persist in the liver. Early chronic hepatitis C generally has no symptoms, but after many years it will become liver disease and occasionally cirrhosis. Some patients with cirrhosis will have other complications, such as liver failure, hepatocellular carcinoma, esophageal varices or gastric varices (Hu *et al.*, 2020; Dajti *et al.*, 2021). Only 25% of adults can clear up hepatitis C infection on their own (Grebely *et al.*, 2012). The half-life of virus particles in plasma ranges from 45 minutes to 3 hours (Pockros, 2011).

2.12. Diagnosis of HCV Infection

There are two types of tests used to diagnose and manage HCV infection: serologic tests that detect antibodies to the virus and molecular tests that detect viral genetic material. However, these tests do not provide information on the severity of the disease or prognosis (Li and Lo, 2015). The initial step in diagnosing HCV involves conducting a serum antibody test. If the test yields a non-reactive result, it indicates that there has been no prior exposure to HCV. However, a reactive antibody test cannot differentiate between a current or past infection. It is

worth noting that around 15-25% of individuals who come into contact with HCV naturally eliminate the virus from their system without any treatment and do not experience chronic infection. Despite not having an active infection, these patients will still test positive for HCV antibodies (Guss *et al.*, 2018; Abu-Freha *et al.*, 2022).

Patients who have been previously treated and cured will still exhibit a positive result on an antibody test. For patients who have a reactive or indeterminate/equivocal antibody test, it is recommended to conduct a qualitative or quantitative HCV- RNA test to determine if there is an active infection. If HCV RNA is detected, it confirms the diagnosis of HCV infection. On the other hand, the absence of HCV RNA indicates a past HCV infection that has been cleared or a false-positive result. Patients who have a reactive antibody test and a HCV RNA viral load number higher than the detection level have an active HCV infection and are eligible for treatment (AASLD/IDSA HCV Guidance Panel, 2015). The diagnosis of acute or chronic HCV infection usually involves testing serum for both anti-HCV antibodies and HCV RNA. It is recommended to use a sensitive quantitative HCV RNA assay for diagnosis as it also provides information on the virus level, which is helpful for management. The difference between acute and chronic HCV infection is based on clinical symptoms, jaundice, and previous ALT elevation. HCV RNA can be detected in serum before antibodies, typically 2 weeks after exposure, while anti-HCV is usually detectable after 8-12 weeks. The interpretation of HCV assays depends on the presence or absence of anti-HCV and HCV RNA, and careful analysis is needed to understand the results (Ghany *et al.*, 2009) (Table 2.2).

Table 2.2: Interpretation of HCV Assays (Ghany *et al.*, 2009)

Anti-HCV	HCV RNA	Interpretation
Positive	Positive	Acute or chronic HCV depending on the clinical context
Positive	Negative	Resolution of HCV; Acute HCV during period of low-level viremia.
Negative	Positive	Early acute HCV infection; chronic HCV in setting of immunosuppressed state; false positive HCV RNA test
Negative	Negative	Absence of HCV infection

Acute hepatitis C often presents asymptotically in 90% of cases, with asthenia, fever, and muscle/joint ache occurring in a few cases, Jaundice symptoms are infrequent. Elevated serum transaminases characterize the condition, with viral RNA detectable 1-2 weeks post-exposure and anti-HCV IgG appearing 7-8 weeks later. Spontaneous resolution occurs in 20% through innate and adaptive immunity, clearing viral RNA within 3-4 months. Factors like host immune response and interleukin (IL) 28B gene polymorphism influence viral clearance. Hepatitis is classified as chronic when viral replication continues for more than six months following an initial infection. The majority of people with chronic hepatitis are asymptomatic and may not exhibit any general symptoms like fatigue, arthralgia, or myalgia. The factors that accelerate the disease progression are the following: acquisition of more than 40 years, male gender, co-infection by HIV, higher body mass index, fatty liver and alcohol consumption.

After 10 to 30 years, about 20 to 30% of patients develop a cirrhosis. The cirrhosis may be associated with a liver failure. In cirrhotic patients, the risk of death from complications is 4% per year, and their risk of developing an hepatocellular carcinoma (HCC) is 1 to 5% per year. Thirty-three percent of patients with HCC die within one year after diagnosis (Morozov and Lagaye, 2018). Diagnosis is by testing the blood for serum antibodies or viral RNA. Screening is recommended for anyone

who has been exposed to risk factors (Gupta *et al.*, 2014) (Figure 2.3).

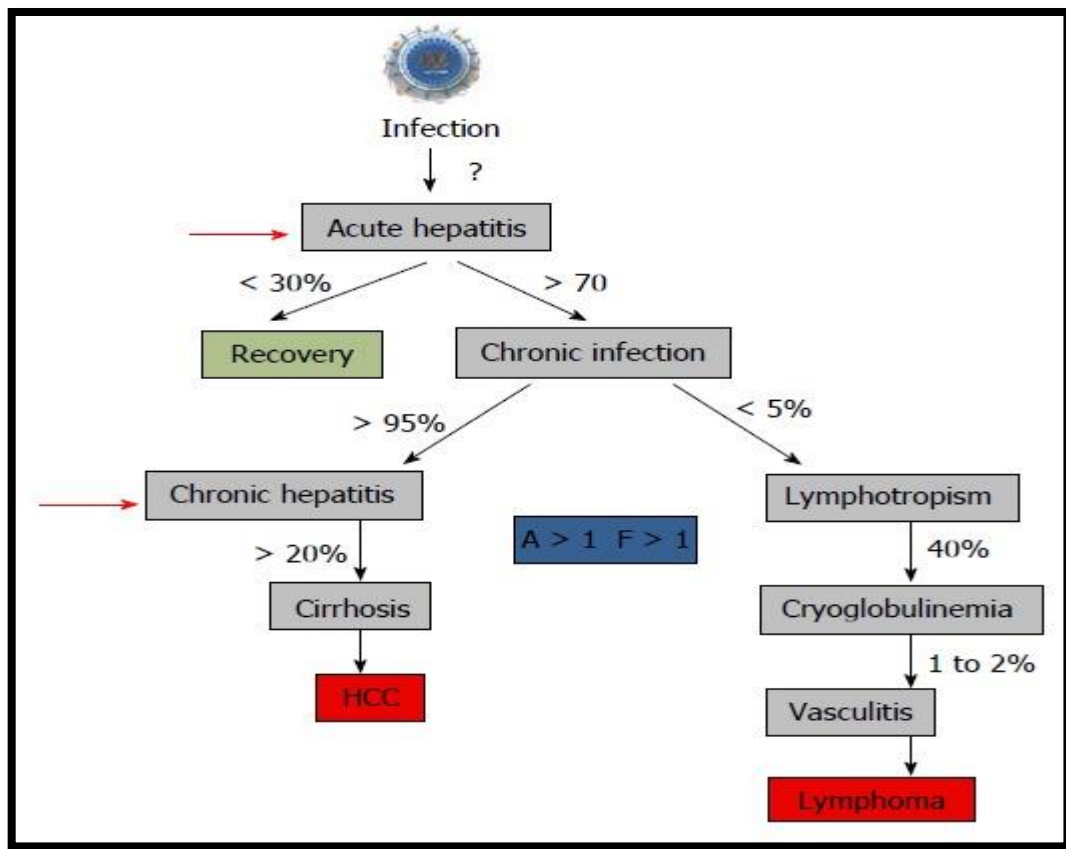


Figure 2.3: Natural history of hepatitis C virus infection (Morozov and Lagaye, 2018).

2.13. Precaution

There is currently no effective vaccine for hepatitis C (Echeverría *et al.*, 2021). Prevention methods include harm reduction programs for long-term intravenous drug use and testing of blood donated by donors (Grebely and Dore, 2011; Cappy *et al.*, 2022).

2.13.1. Primary prevention

Unlike hepatitis A and B, there is currently no vaccine to prevent hepatitis C virus infection. You can reduce your risk of infection by avoiding the following: unsafe and not necessary forms of injectable materials; products of

blood that are not safe; collections of sharp tools that are disposable and not safe; making equipment of injection common and using illegal therapies; Unprotected sexual contact with someone infected with HCV; sharing personal tools that are sharp and carry infected blood; Using contaminated tools for tattooing, piercing and acupuncture (Alter and Moyer, 1998).

2.13.2. Secondary and Tertiary Prevention

People infected with HCV should: receive education and counseling regarding health care and treatment options; Get vaccinated against hepatitis A and B to prevent co-infection with the hepatitis A and B viruses and protect their livers; Receiving appropriate medical management early, including appropriate antiviral treatment; Get regular checkups to detect liver disease as early as possible of (Zhang *et al.*, 2021).

The European Association for the Study of the Liver (EASL), the American Association for the Study of the Liver Disease (AASLD), and various local national associations have recently revised their guidelines on HCV treatment. The updated recommendations aim to streamline the diagnosis, pre-treatment assessments, and treatment process. Studies have shown a high success rate of around 95–100%, with factors such as genotype, fibrosis grade, and patient history influencing the outcome (Abu-Freha *et al.*, 2022).

Different medications can be used for treatment, including antiviral agents like Sofosbuvir, velpatasvir, Glecaprevir, pibrentasvir, and elbasvir. The primary goal of antiviral treatment using direct acting antivirals (DAA) is to achieve a sustained virological response (SVR), which is defined as having undetectable HCV RNA 12 weeks after completing the antiviral treatment (Di Marco *et al.*, 2022).

2.14. Treatment

Chronic infection is cured in about 90% of patients with medications including sofosbuvir (brand name Sovaldi) or simeprevir (brand name Olysio) (Sclair *et al.*, 2016). Earlier treatments included combination therapy with interferon Pegasys (Peginterferon alfa-2a) and ribavirin, which has a cure rate of only about 50% and has strong side effects (Rong and Perelson, 2010). Patients who already have cirrhosis or liver cancer may need to consider liver transplantation. Hepatitis C is the leading cause of liver transplantation, but many patients still have a recurrence of the virus after liver transplantation (Martini, 2018). At the same time, effective interferon treatment for type 1 is not as effective as that for types 2 and 3.

2.15. Immunological Aspects of Innate Immune Response**2.15.1. RNA pathogen recognition receptors**

The outcome of HCV infection, whether it leads to viral clearance or chronic infection, is determined by the interplay across HCV and cellular elements of innate immune system. When HCV infects hepatocytes, it triggers the activation of various intracellular pathogen recognition receptors such as toll-like receptor 3 (TLR-3), (MDA-5), (DHX-58 or LGP-2) the DExH-box helicase 58, (DDX-58 or RIGI) the DExD/H-box helicase 58 and (PKR) the eukaryotic translation initiation factor 2 alpha kinase 2, (figure 2.4).

(Brisse and Ly, 2019; Suzuki, *et al.*, 2019). HCV RNA within hepatocytes can exist in two forms: an RNA that is single-stranded with end of a free 5' triphosphate and tract of 3' poly-U:UC and an RNA that is a double-stranded (ds-RNA) of intermediate replication (Du *et al.*, 2017). The 3' poly-U motif structure and 5'-triphosphate of RNA of HCV are recognized by RIGI, which then recruits

mitochondrial antiviral signaling protein (MAVS). This recruitment leads to the activation after being phosphorylated of factors of transcription NF- κ b and IRF3, ultimately leading the expressing of genes of interferon (IFN)-encoding (Brisse and Ly, 2019). RIGI can also be activated through alternative pathways such as signaling that is Riple, hat is a target of N-S3-4-A (Vazquez *et al.*, 2019). MDA5 is triggered by the long noncoding RNA induced by virus (lncITPRIP-1 or CFAP58-DT) and intermediates of replication of HCV dsRNA to stimulate interferons, through a route depending on MAVS (Du *et al.*, 2017; Xie *et al.*, 2018). LGP2 acts upstream of MDA5 and plays a positive role in regulating IFN signaling that is induced by the virus induced inside liver cells that are infected (Hei and Zhong, 2017). The precise activation and control of mechanisms of TLR3 and PKR at time of infection by HCV remain uncertain. While certain studies have indicated that activation of PKR and subsequent shutoff of translation in liver carcinoma cells can impede HCV replication, others have found that activation has no impact on the expression of genetic materials of HCV or the virus amplification. Involvement, in infection with HCV, of PKR is intricate due to the varied effects of various domains of IRES related to HCV on PKR activation and inhibition. Furthermore, the HCV proteins NS5A and NS5B interact with PKR during the later replacave stages to modulate its related functionality (Suzuki *et al.*, 2019).

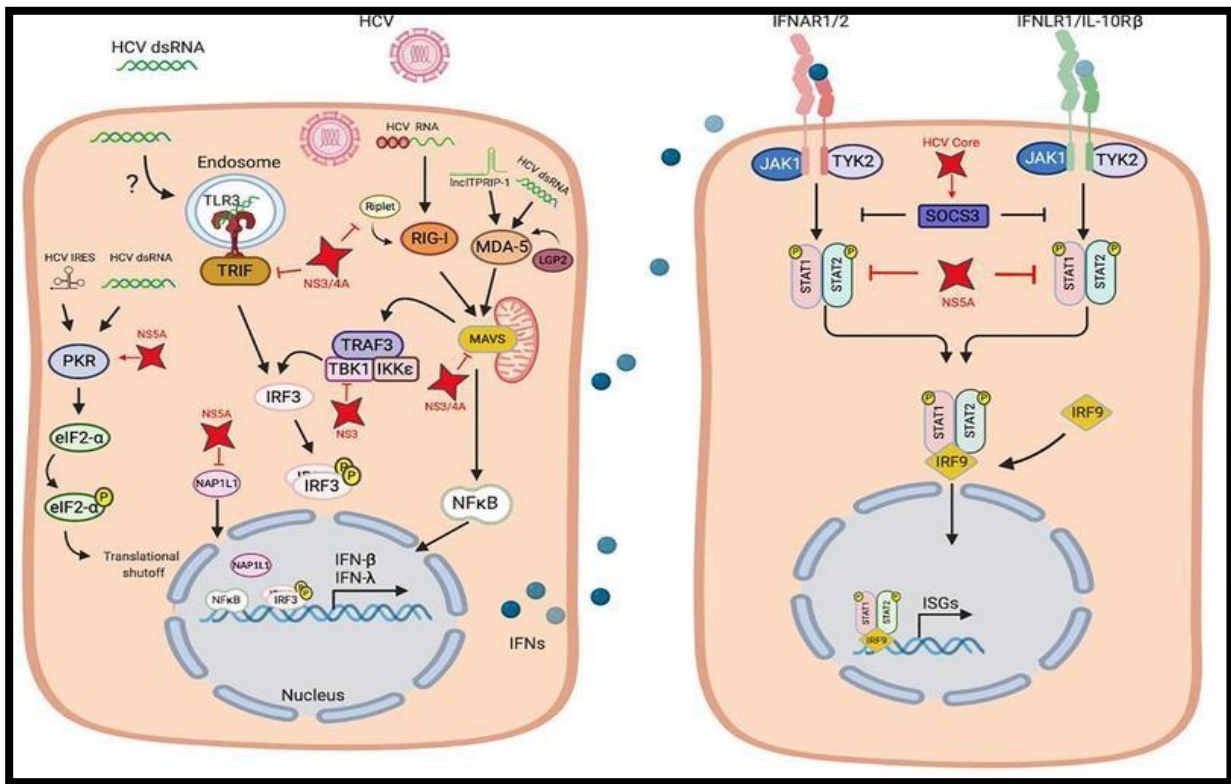


Figure 2.4: Evasion and Induction of Responses that are innate against the virus HCV

Additional investigation is necessary to gain a comprehensive understanding of how the interplay between NS-5A and PK-R influences the control of replication of HCV. TLR-3 serves as a detector for virus and transmits signaling through complex of adaption, which consists of TRAF the (TNF receptor-associated factor) and (TRIF or TICAM1) the toll-like receptor adaptor molecule 1. This signaling cascade leads to the activation of IRF3 and NF-κb. The intriguing aspect of HCV dsRNA being localized in the cytosol is its potential interaction with endosomal TLR3, suggesting a potential way for transporting RNA of HCV to vesicle of endocytosis .Further in vestigation is imperative to comprehend the impact of HCV-produced activation of TLR-3 on replication of virus within lier cell containing the infection (Stuart *et al.*, 2021).

2.16. New Roles for IFNs

The production of IFNs plays a crucial role in the innate immune response to HCV infection. Type I IFNs (IFN-A or IFN-B), which are expressed throughout the body, and type III IFNs (IFNL1–IFNL4), which are produced by various immune cells and epithelial cells , are responsible for initiating antiviral responses (Wong and Chen, 2016). Type 1 IFNs (IFNB) secretion induces IFNA production, which was utilized in the treatment of chronic HCV infection prior to the advent of direct-acting antiviral (DAA) agents (Padmanabhan *et al.*, 2014). According to a study, the F protein, which is the frameshift product of HCV core protein, has been found to stimulate the expression of IFNs in a manner that is dependent on the genotype of HCV (Lai *et al.*, 2020).

Type III IFNs have been linked to the natural elimination of HCV and the eradication of infection after receiving ribavirin treatment and pegylated IFN-A. Recent studies about genome-wide has pinpointed specific SNPs in genes of the IFNL-4 and IFNL-3 that are connected to HCV clearance (Rosenberg *et al.*, 2018). Nevertheless, conducting more in-depth analyses on the relationship among SNPs in IFNL-4 and IFNL-3 and the associated effect on expression of protein and activity can be a valuable area for further investigation (Stuart *et al.*, 2021).

2.16.1. IFN-Stimulated Genes

The expression and secretion of IFNs are induced by pathogen recognition receptors, leading to the detection of HCV. These IFNs then bind to interferon-receptors on hepatocytes and immune cells, activating signaling pathways that result in the expression of interferon-stimulated genes (ISGs). In HCV-infected tissues of liver, there is an upregulation of ISGs such as CH25H, USP18, ISG15, IFITM, IFIT, IRF7, IRF1, ISG20, PKR, ADAR, DDX60, RIGI, ribonuclease L, and 2'–5'-oligoadenylate synthetase 1 (OAS1)(Wong *et al.*, 2016). Patients with

chronic HCV infection exhibit the expression of ISGs in their liver tissues, but there is a negative correlation between the low amounts of expression of ISG and response to combination therapy ribavirin and pegylated IFNA (Dustin *et al.*, 2014).

This phenomenon is most likely caused by the continuous synthesis of cytokines and IFNs by liver cells that contains the virus and cells of innate immunity, which are mediated by ISGs. As a result, it leads to profound inflammatory response and hepatic damage. Humans as well as animals who have a great ISGs expression in the hepatic tissue prior to undergoing treatment have a lower chance of clearing HCV infection. Additionally, they tend to have unfavorable outcomes when treated with pegylated IFNA and ribavirin (Ramamurthy *et al.*, 2018; Sung and Shin, 2020). Furthermore, patients with SNPs in IFNL3, which are associated with HCV clearance, exhibit lower levels of ISGs in their liver tissues compared to patients without these genetic variations (Świątek-Kościelna *et al.*, 2017). Liver with high levels of ISG expression decreases the likelihood of achieving a lasting response to anti-HCV treatment. Increased resistance to ISG, like IFITMs, strengthens the production of neutralizing antibodies and facilitates the escape of HCV variants under selection pressures (Wrensch *et al.*, 2019). The evolution of hepatic inflammatory reaction at time of infection with the virus involves the interaction between cells of innate immunity and liver cells, such as macrophages of liver known as Kupffer cells, dendritic cells and cells of natural killing (NK).

The expansion and differentiation of NK cells during persistent HCV infection can vary among patients, as observed in a study by Strunz *et al.* in 2018. Furthermore, the identification of phenotype of NK cell and expression of receptor showed changes in NK cell function during both acute and chronic HCV infection, as highlighted by Njiomegne *et al.* in 2020.

Plasmacytoid dendritic cells (pDCs) are known for their ability to produce high levels of IFNA, which in turn enhances the innate immune response. On the

other hand, myeloid dendritic cells, including classical BDCA1⁺ and cross-presenting BDCA3⁺ cells, produce IFNL to trigger the adaptive immune response. In patients with chronic HCV infection, the frequency of circulating pDCs is decreased. However, even in these patients, pDCs can still respond to HCV when stimulated with TLR7 and TLR8 agonists, leading to the production of significant amounts of cytokines and chemokines (Dolganiuc *et al.*, 2006; Doyle *et al.*, 2019).

The frequencies of plasmacytoid dendritic cells (pDC) from individuals with chronic HCV infection who are undergoing liver transplantation are comparable between the liver and blood. Nevertheless, the frequencies of BDCA1⁺ myeloid DCs are elevated in the blood, whereas the frequencies of BDCA3⁺ myeloid DCs are increased in the liver. These findings indicate that the function of DCs during end-stage liver disease plays a role in the inflammation linked to persistent HCV infection (Yoshio *et al.*, 2013).

HCV components or progeny virions may undergo phagocytosis by Kupffer cells, leading to the production of IL1B and IL18 (Li *et al.*, 2017). IL1B serves as an inflammation marker in individuals with chronic hepatitis. Patients with cirrhosis exhibit elevated levels of IL18 in their bloodstream and increased levels of IL1B in the liver (Estevez *et al.*, 2017). Specifically, HCV proteins such as core and p7 trigger the NLR family pyrin domain containing 3 (NLRP3) inflammasome, resulting in the release of IL1B from Kupffer cells, primary human macrophages, and transformed macrophages (Farag *et al.*, 2017; Negash *et al.*, 2019).

The results of a meta-analysis examining genetic variations in IL18 and CTL4 have shown that certain SNPs, including CTLA4 rs231775, CTLA4 rs5742909, IL18 rs1946518, and IL18 rs187238, are associated with an increased risk of HCV infection. Further investigations are needed to understand how these SNPs affect gene expression and function, as well as to explore the involvement of Kupffer cells in HCV infection. This area of research holds promise for future studies (Yu *et al.*, 2019).

2.17. Adaptive Immune Response

2.17.1. CD8+ Tcells

CD8+ T lymphocytes play a crucial role in eliminating acute HCV infection. The spontaneous resolution of initial acute infection is linked to widespread, HCV-specific responses of CD8+ T cells, which emerge 8–12 weeks post-infection in the bloodstream and liver of both humans and chimpanzees (Sung *et al.*, 2014). CD8+ T cells specific to HCV in individuals who naturally clear the infection express CD127 and PD-1 surface markers, secrete high levels of IFNG, TNFA, and perforin, and recognize various HCV epitopes. Conversely, CD8+ T cells in circulation of individuals with persistent infection exhibit reduced proliferation and cytotoxic activities and recognize fewer HCV epitopes, potentially favoring the proliferation of mutant viruses that evade T cell recognition (Seigel *et al.*, 2013; Walker *et al.*, 2015).

Additionally, a majority of these cells exhibit functional exhaustion, as indicated by their elevated levels of inhibitory markers such as PD-1, 2B4, CD160, TIM3, and KLRG1 (Aregay *et al.*, 2019; Chen *et al.*, 2020). The exhaustion of CD8+ T cells in the context of HCV infection could potentially stem from changes in the expression of nucleosome genes, specifically histone proteins, that play a role in regulating T cell differentiation (Wolski *et al.*, 2017).

CD8+ T lymphocytes play a crucial role in providing lasting immunity against HCV reinfection. After the clearance of HCV, CD8+ T cells that are specific to HCV differentiate into either T-effector (TEM) or T central memory (TCM) cells. The long-lasting memory CD8+ cells exhibit high levels of CD127. When chimpanzees are reinfected with the same strain of HCV, there is a rapid infiltration of memory CD8+ T cells expressing IFNG into the liver (Stuart *et al.*, 2021).

Improved polyfunctionality and increased specificity of the CD8⁺ T-cell receptor are associated with the effective treatment of secondary HCV infection. On the other hand, HCV-specific CD8⁺ T cells are less polyfunctional and have less-specific TCR with chronic infection. They also display a terminally exhausted (PD1^{hi}Eomes^{hi}TCF1[–]CD127[–]) (Stuart *et al.*, 2021).

Thymocyte selection-associated high mobility group box (TOX) expression may epigenetically preserve the tired HCV-specific CD8⁺ T lymphocytes, which prevents them from producing potent memory responses when they are reexposed to the antigen. Interferon-free treatments do not stop intrahepatic CD8⁺ T cell fatigue or viral escape, but they do partially restore CD8⁺ T cell proliferation during chronic HCV infection (Stuart *et al.*, 2021).

2.17.2. CD4⁺Tcells

CD4⁺ T lymphocytes play a crucial role in eliminating primary HCV infection. The presence of robust, long-lasting, HCV-specific CD4⁺ T cells is linked to the spontaneous resolution of HCV. Conversely, individuals with chronic HCV infection tend to have deficient or short-lived HCV-specific CD4⁺ T cells. The majority of HCV-specific CD4⁺ T cells in individuals who naturally clear the virus are T-helper 1 (Th1) cells expressing IFNG, IL2, and TNF, along with Th17 cells expressing IL17A and IL21 (Chen *et al.*, 2020). Th1 cells proliferate post-HCV vaccination and during direct-acting antiviral therapy, coinciding with decreased viremia levels (Burchill *et al.*, 2015).

Although chronically infected HCV persons have less HCV-specific CD4⁺ T cells than spontaneous resolvers, both groups of patients have comparable CD4⁺ T cell frequencies and specificities during the early acute phase of infection. Several studies have demonstrated that CD4⁺ T cells are unable to regulate HCV

in individuals who experience persistent infection. The decline of HCV-specific CD4⁺ T cells in chronically infected patients may be related to T-cell exhaustion, which is characterized by elevated expression of PD-1, CTLA-4, and TIM-3 among other inhibitory markers (Chen *et al.*, 2020). However, since PD-1 and other inhibitory molecules can be blocked to restore activity and proliferation, some of these worn-out cells may still have effector function. These findings suggest that medications that target T-cell inhibitory molecules, such as PD-1, may be created to treat chronic HCV infection (Kared *et al.*, 2013).

CD4⁺ T cells, like CD8⁺ T cells, play a crucial role in protecting against HCV reinfection. Individuals who have successfully resolved HCV infections have a higher frequency of HCV-specific CD4⁺ memory T cells, which exhibit decreased expression of inhibitory surface molecules. Additionally, these cells demonstrate enhanced proliferative capacity and cytokine production compared to cells from individuals who develop a persistent secondary infection after resolving the primary infection (Abdel-Hakeem *et al.*, 2014).

Prior to the commencement of DAA or interferon-based therapy, the presence of heightened proliferation of memory Th1 cells in individuals with chronic HCV infection is positively associated with a sustained virologic response (SVR). Additionally, the ability of CD4⁺ T cells to specifically target evolving HCV antigens is crucial in providing protection against reinfection (Puig *et al.*, 2006).

Tfh cells play a crucial role in the spontaneous clearance of acute HCV infection by mediating germinal center reactions that enhance the affinity maturation of antigen-specific B cells. In individuals who resolve the infection spontaneously, both circulating and intrahepatic HCV-specific Tfh cells expand during the early stages of infection, correlating with the presence of HCV-specific neutralizing antibodies. Conversely, patients with chronic infections do not experience this expansion of Tfh cells. Following DAA treatment, individuals who

achieve SVR show a rapid increase in HCV-specific Tfh cells as viremia decreases, while HCV-specific Th1 cells decrease. Given the significance of Tfh cells in clearing HCV infection, future vaccine strategies should focus on inducing strong HCV-specific Tfh-cell responses alongside neutralizing antibodies and other immune responses (Raziorrouh *et al.*, 2016; Zhang *et al.*, 2019; Smits *et al.*, 2020).

While Th1 and Tfh cells play a crucial role in promoting HCV clearance, various CD4⁺ T cell subsets have been shown to impede the effective control of HCV infection. Patients with chronic HCV infection exhibit high levels of T-regulatory cells (Treg cells, CD4⁺CD25⁺Foxp3⁺) in both peripheral blood and liver, which in turn suppress HCV-specific CD8⁺ and CD4⁺ T cells. Consequently, the presence of Tregs post successful DAA and/or interferon therapy could potentially contribute to secondary HCV infection upon re-exposure (Barjon *et al.*, 2015; Langhans *et al.*, 2017).

2.17.3. B Cells

There has been a debate regarding the involvement of B cells in the elimination of acute HCV infection. At first, it was believed that B cells played a minor role in HCV clearance, given that humans and chimpanzees could recover from acute HCV infection without generating significant amounts of neutralizing antibodies (nAbs) against HCV. Nevertheless, in recent years, several studies have suggested a potential link between the early presence of nAbs during acute infection and the spontaneous resolution of the virus (Kinchin *et al.*, 2018; Walker *et al.*, 2019).

Indeed, the rapid production of neutralizing antibodies (nAbs) may promote the elimination of primary acute HCV infection more effectively than increased nAb cross-reactivity. This is because individuals with chronic HCV infection also generate nAbs against various HCV genotypes throughout the persistent infection.

Nevertheless, the processes involved in the early production of nAbs in individuals who successfully clear acute HCV infection remain unclear (Swann *et al.*, 2016; Rosenberg *et al.*, 2018).

Furthermore, a higher quantity of Tfh cells is associated with the rapid generation of antibodies in individuals who naturally clear their infections, indicating that the early emergence of Tfh cells facilitates a quicker proliferation of HCV-specific B cells that generate neutralizing antibodies. Nevertheless, further investigation is required to as certain if these Tfh cells directly engage with HCV-specific B cells to facilitate the spontaneous resolution of infection (Stuart *et al.*, 2021).

NAbs could potentially provide protection against HCV reinfection. Chimpanzees and mice with humanized livers were shielded from HCV challenge through the passive transfer of nAbs. Moreover, immunizing chimpanzees with recombinant HCV E1E2 glycoproteins resulted in the generation of nAbs and robust CD4⁺ T cell responses, ultimately enhancing the clearance of infection following HCV challenge (Meunier *et al.*, 2011; Bukh *et al.*, 2015). Furthermore, a portion of injection drug users who successfully cleared more than one HCV infection maintained neutralizing antibodies to conserved regions on the E2 glycoprotein (Merat *et al.*, 2019). Nevertheless, not all instances of spontaneous HCV clearance are linked to long-lasting neutralizing antibodies. Paradoxically, levels of HCV-specific antibodies decline over time in the blood of certain individuals who naturally resolve acute HCV infection, as well as in patients who achieve sustained virological response (SVR) through treatment with interferon or DAAs (Kee *et al.*, 2012). Sustaining antibodies against HCV may, therefore, necessitate ongoing activation of antigen-specific B cells (Boisvert *et al.*, 2016).

2.18. Role of Cytokines

Hepatitis C virus (HCV)-specific CD8 + T cells are progressively exhausted during HCV persistent infection (PI). This process may involve positive immune checkpoints 4-1BB/4-1BBL due to loss of their signal transducer TRAF1. To address this question, peripheral HCV-specific CD8 + T cells from patients with PI and resolved infection (RI) after treatment were studied. The duration of HCV infection and the rate of liver fibrosis progression were inversely related to the possibility of detecting peripheral CD8 + cells. In PI, CD8 + cells were impaired in antigen-specific reactivity. *Short/* mid-term PI was characterized by detectable peripheral PD-1 + CD127^{low}TRAF1^{low} cells. After T cell receptor (TCR) triggering, TRAF1 levels were positively correlated with CD127, Mcl-1, and CD107a expression and proliferation intensity, and negatively correlated with PD-1 expression, thus correlating TRAF1- low expression with fatigue. *In vitro* interleukin 7 (IL-7) treatment upregulated TRAF1 expression, whereas transforming growth factor- β 1 (TGF- β 1) treatment did the opposite, suggesting that in addition to TCR stimulation, the IL-7/TGF- β 1 balance may also be involved in TRAF1 regulation. Indeed, serum TGF- β 1 concentrations were higher in PI patients than in RI patients and negatively correlated with TRAF1 expression. Consistent with IL-7 increasing TRAF1 expression levels, IL-7 plus 4-1BBL treatment *in vitro* T cell reactivity was enhanced in patients with short/intermediate infection. However, for patients with long-acting PIs, anti-PD-L1 had no effect in patients with slow progression of fibrosis (slow fibroblasts) but not in rapid fibroblasts, except for the combination of IL-7 and 4-1BBL. In conclusion, peripheral blood hyporeactive TRAF1- low HCV-specific CD8 + T cell responses, which could be restored by treatment with 4-1BBL plus IL-7, are characteristic of short/intermediate PIs. In long-term disease, HCV-specific CD8 + T cells were rarely detected *ex vivo* , but therapy with 4-1BBL, IL-7, and anti-PD-L1 restored their reactivity *in vitro* in slow fibroblasts (Peña-Asensio *et*

al., 2021).

Globally, 71 million people are infected with the hepatitis C virus (HCV). Chronic illness, which can result in cirrhosis and hepatocellular cancer, affects two-thirds of people. Even after using direct-acting antiviral medications, some individuals continue to recur. Additional immunotherapy may be very important in these situations. CD8 + T cells specific to the virus are essential for an effective anti-HCV immune response. These cells' ability to operate is compromised after a prolonged infection, most likely as a result of a costimulation failure. Low expression levels of TRAF1, a signal transducer of the positive costimulatory pathway 4-1BB/4-1BBL, are indicative of tired specific T lymphocytes, as shown in this work. In patients with short/moderate disease, IL-7 upregulation the TRAF1 expressing and enhanced T cell responsiveness, whereas blockade of the negative PD-1/PD-L1 checkpoint was also necessary for patients with long-term infection. If the findings are collectively analysed, this research makes supportto novel approach to restore specific CD8 ⁺ T cell responses, elucidating the importance of TRAF1 signaling. This may be a promising target for future immunotherapies (Peña-Asensio *et al.*, 2021).

There have been more than 100 cytokines identified, each categorized based on their main function. Cytokines can also be grouped into subcategories based on their specific roles (Fallahi *et al.*, 2012):

- pro-inflammatory cytokines (interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-alpha);
- T-helper (Th)1 cytokines, which are produced by Th1-activatedlymphocytes (interferon (IFN)-gamma, IL-12, IL-18);
- Th2-type cytokines have a crucial function in suppressing the production

of cytokines from Th1 cells. This, in turn, leads to the suppression of Th1 immune responses by downregulating the antigen-presenting capacity of macrophages. Additionally, Th2-type cytokines promote the proliferation of B-cells, resulting in increased antibody production. Notable examples of Th2-type cytokines include IL-10, IL-4, IL-5, and IL-13.

- Th17 cytokines play a crucial role in the development of Th17 lymphocytes. The combination of IL-23, IL-6, and transforming growth factor (TGF)- β triggers the transformation of Th0 cells into Th17 cells, responsible for producing IL-17A, IL-17F, TNF- α , and IL-1, ultimately resulting in a proinflammatory response.

Cytokines play a crucial role in regulating viral control and liver damage caused by hepatitis C virus (HCV) infection. The intricate network of cytokines during the initial stages of infection enables a coordinated and effective immune response, involving both innate and adaptive immunity. However, HCV disrupts the cytokine system at various levels and evades immune detection by promoting a T-helper (Th)2/T cytotoxic 2 cytokine profile. Failure to control the infection leads to the recruitment of inflammatory cells into the liver tissue through interferon (IFN)- γ -inducible CXC chemokine ligands (CXCL)-9, -10, and -11 chemokines. This persistent inflammation ultimately results in liver damage and the development of liver cirrhosis (Fallahi *et al.*, 2012).

2.19. Polymorphism

Single base pair substitutions that take place both inside and outside of genes are known as single-nucleotide polymorphisms, or SNPs. Numerous well-characterized human characteristics, like as medication response and disease susceptibility and resistance, are explained by SNPs. Consequently, over the last two years, SNP finding efforts have significantly increased. SNP frequencies

differ between coding and noncoding sequences as well as between genomic regions. The amount of diversity of nucleotide is in the range of 0.0003 to 0.005; in other words, there are from 3 to 50 SNPs per 10 kilobases when two chromosomes are compared (Shen *et al.*, 1999).

Two or more allelic variants of mRNA can arise from genes with one or more SNPs. The interactions of mRNAs with cellular components involved in mRNA synthesis, maturation, transport, translation, or destruction can differ depending on the bases present at SNP sites. Numerous single base-pair alterations have been shown to change or add crucial sequence components for human mRNA splicing, processing, or translation. These SNPs are linked to changes in the steady-state level and/or length of cytoplasmic mRNA. However, SNPs that have no effect on protein sequences or RNA consensus have not been thoroughly examined. Such SNPs might potentially have phenotypic impacts as well, most likely via mechanisms that are not consensus-dependent (Shen *et al.*, 1999).

2.19.1. Tumor Necrosis Factor -308 G/A Association With Hepatitis C Virus Infection

Genetic alterations can lead to abnormal cytokine production and impact the development of chronic HCV infection, as the equilibrium between pro- and anti-inflammatory cytokines plays a crucial role in determining the infection's outcome (Santiago *et al.*, 2021). Tumor necrosis factor- α (TNF- α) is a powerful antiviral cytokine that exhibits a wide array of proinflammatory effects. It plays a crucial role in the host immune response to HCV infection as TNF- α is secreted by cytotoxic T lymphocytes (CTLs) in the liver. The level of circulating TNF- α rises during HCV infection, and the hepatitis viral infection triggers TNF- α production in human hepatocytes. An increased TNF- α level is associated with the severity of hepatic inflammation, fibrosis, and tissue damage. Prolonged immune-mediated

hepatic injury can set off the progression of fibrosis, cirrhosis, and, ultimately, HCC (Talaat *et al.*, 2012).

Certain variations in the TNF- α gene have been linked to the control of cytokine production and align with the regions where transcription factors bind. The TNF- α -308G>A polymorphism exhibits greater transcriptional activity compared to the normal allele and has been connected to various infectious diseases (Santiago *et al.*, 2021). TNF- α expression is rigorously regulated both at the transcriptional and post-transcriptional levels. There have been reports of six diallelic polymorphisms in the TNF- α promoter, located at positions -1031, -863, -857, -376, -308, and -238, which are believed to impact TNF- α production. Specifically, the polymorphism at -308 in the human TNF- α promoter involves the substitution of guanine with adenosine in the less common alleles. Various infectious diseases and inflammatory disorders have been linked to TNF- α -308 alleles. The potential role of TNF- α polymorphisms in the development of HCV infection has been explored, although conflicting results have been observed. These inconsistencies could be attributed to variations in ethnicity among the studied populations, resulting in a distinct distribution of cytokine gene polymorphisms (Talaat *et al.*, 2012).

2.19.2. IL-28B rs 12979860 C/T role in Hepatitis C virus infection

The eradication of HCV infection is influenced by genetic factors within the host of infected individuals. Among these factors, interferon lambda 3 (IFN- λ 3) is one of the most extensively studied. IFN- λ 3, located on chromosome 19 of the IL-28B gene, is believed to play a crucial role in mounting a robust response against HCV infection (Mahboobi *et al.*, 2011; Bruno *et al.*, 2015). This cytokine regulates Treg cells and enhances adaptive cellular immunity. Through the pathway of JAK-

STAT IL-28B induces the expressing of interferon-enhanced genetic materials, thereby exerting its antiviral effects (Safarnezhad *et al.*, 2016).

Multiple research studies have demonstrated the significance of the single nucleotide polymorphism (SNP) on specific regions of the IL-28B gene (on its genetic variant) in relation to the function of IFN- λ 3 and its response to PEG-IFN treatment (Suppiah *et al.*, 2009; Tanaka *et al.*, 2009; Mahboobi *et al.*, 2011; Aziz *et al.*, 2015; Taheri *et al.*, 2015). Suppiah *et al.* (2009) and Tanaka *et al.* (2009) emphasized the importance of SNP rs8099917 and sustained virological response (SVR), where the T allele was linked to a higher SVR (78% for genotype TT). The SNP rs12979860 also exhibits a similar association with **SVR** (Aziz *et al.*, 2015; Taheri *et al.*, 2015). Generally, individuals of African descent tend to have a lower response to therapy compared to Europeans, with SVR rates in CC genotypes being 2-3 times higher than in TT genotypes (Taheri *et al.*, 2015).

The efficacy of treatment in CHC may be influenced by the allelic variants of the interleukin-28B (IL28B) rs12979860 polymorphisms, as stated by Hodo *et al.* in 2013. Additionally, Lau *et al.* found that the IL-18-137G/C (rs187238) SNP could potentially increase the risk of HCC. Numerous studies have examined the relationship between the IL-28B rs12979860 C/T SNP and the development of liver cirrhosis and HCC in different populations. However, the results have been inconclusive and inconsistent, as mentioned by Suo *et al.* in 2013. For instance, Fabris *et al.* reported that the T allele of IL-28B rs12979860 is more common in patients with HCV-related liver cirrhosis and may enhance the risk of HCC development.

Nevertheless, some studies failed to establish a link between this SNP and the risk of HCC (Agúndez *et al.*, 2012). It has been documented that possessing the IL-28B T allele can exacerbate the severity of liver cirrhosis; on the other hand, the presence of the C allele alongside the T allele can mitigate cirrhosis severity

(Attallah *et al.*,2018).We postulated a correlation between the distribution of IL-28B alleles and the clinicopathological characteristics of viral infection in this research. To accomplish this, we will examine the influence of the IL-28B rs12979860 C/T polymorphism, situated on chromosome 19 of the IL-28B gene, on the advancement of HCV infection in Iraqi patients through the utilization the amplification-refractory mutation system polymerase chain reaction (ARMS-PCR). The relationship between the distribution of this polymorphism and its effect on cytokine levels in the serum will be assessed.

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Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Equipment and Instruments used in this study are demonstrated in table 3.1.

Table 3.1: Equipment and Instruments used in the present study

Rank	Equipment & instrument	Company
1	Beakers	Eppendorf /Germany
2	Blood collection tubes	Concord /Lebanon
3	Digital camera	Samsung/ china
4	Exispin vortex centrifuge	Bioneer/ Korea
5	Flasks	CYAN/ Belgium
6	Gel electrophoresis	Bioneer/ Korea
7	High Speed Cold centrifuge	Eppendorf /Germany
8	Incubator	Memmert/Germany
9	Microcentrifuge tubes	Biobasic/ Canada
10	Micropipettes 5-50, 0.5-10,100-1000µl	CYAN/ Belgium
11	Microwave	Argose/Germany
12	Oven	Memmert/Germany
13	PCR tubes	Biobasic/ Canada
14	Refrigerator	Concord /Lebanon
15	Sensitive Balance	Sartorius/Germany
16	Thermocycler PCR	BioRad/ USA
17	UV Transilluminator	ATTA/ Korea
18	Vortex	CYAN/ Belgium
19	Water Bath	Memmert/Germany

3.1.2 Kits

Table 3.2: Kits used in this study

Rank	Kit	Company	Country
1	gSYAN DNA Extraction Kit	Geneaid	Taiwan
	GST buffer		
	GSB buffer		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
	Proteinase K 10mg/ml		
2	GoTaq® G2 Green Master Mix kit	Promega	Korea
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris.HCl pH 9.0		
	KCl		
	MgCl ₂		
	Loading dye		
3	Viral RNA extraction kit	Bioneer	Korea
	AccuZol™ reagent 100ml		
4	One-Step RT-PCR Premix Kit for cDNA synthesis and PCR	iNtRON	Korea
	OptiScript™ RT System		
	RT-PCR buffer (10×)		
	dNTPs		
	i-StarTaq™ DNA polymerase		
	Stabilizing buffer		

3.1.3 Primers

Table 3.3: Primers for HCV

Primer	Sequence 5'-3'	
HCV-5' UTR - F	ACGCAGAAAGCGTCTAGCCATGGCGTTAGT	256bp
HCV-5' UTR - R	TCCCGGGGGCACTCGCAAGCACCCCTATCAGG	

The Tetra-ARMS-PCR Primers for TNF- α -308 G>A (rs1800629) are shown in table 3.4.

Table 3.4: The Tetra-ARMS-PCR Primers for TNF- α -308 G>A (rs1800629) gene polymorphism with their sequence and amplicon size:

T-ARMS-PCR Primer	Sequence (5'-3')	Product size
Mutant type Forward inner primer (A allele):	GCAATAGGTTTTGAGGGGCAG GA	203bp
Wild type Reverse inner primer (G allele)	CTGGAGGCTGAACCCCGTACC	273bp
Forward outer primer	CCTTTCCTGAGGCCTCAAGCC T	432bp
Reverse outer primer	CCCTGCACCTTCTGTCTCGGTT T	

The Tetra-ARMS-PCR Primers for IL-28B C>T(rs12979860) gene polymorphism are shown in table 3.5.

Table 3.5: The Tetra-ARMS-PCR Primers for IL-28B C>T(rs12979860) gene polymorphism with their sequence and amplicon size:

T-ARMS-PCR Primer	Sequence (5'-3')	Product size
Mutant type Forward inner primer (T allele):	CAGGGAGCTCCCCGAAGGAGT	191bp
Wild type Reverse inner primer (C allele)	AGTGCAATTCAACCCTGGTGCG	167bp
Forward outer primer	GACAAGCGGCGCTTATCGCATA	315bp
Reverse outer primer	CTATGTCAGCGCCCACAATTCCC	

3.1.4 Chemicals that used in this study are shown in table 3.6.

Table 3.6: the chemicals that used in this study with their company and country of origin:

Rank	Chemical	Company and Origin
1	Absolute ethanol	BDH (England)
2	Chloroform	BDH (England)
3	Isopropanol	BDH (England)
4	Agarose	iNtRON (Korea)
5	10x TBE buffer	iNtRON (Korea)
6	Ethidium Bromide 10mg/ml	BioBasic (Canada)
7	Nuclease free water	BioLabs/ UK
8	DNA Marker ladder (100bp)	iNtRON (Korea)

3.2. Method**3.2.1. Study Group and Sample Collection**

This study case control study included 100 patients with HCV infection diagnosed by specialist physician in medicine according to serological and molecular tests. The age range of those patients were between (29-73) and they included (64) males and (36) females. In addition the study included 100 control subjects with comparable age range and sex proportions. The patients included in the study were recruited from dialysis unit, in Al-Diwaniyah Teaching Hopsital, AL-Diwaniyah Province, Iraq. The study period for sample collection extended from January 2024 to August 2024; whereas, the period of accomplishing the study extended to June 2025 .

After proper sterilization, 5 ml venous blood from antecubital vein was obtained from 100 patients. Those patients were recruited from renal dialysis unit and blood transfusion unit in Adewaniyah Teaching Hospital. Blood samples were also obtained from 100 control subjects of comparable age range. Blood samples were transferred into EDTA tube for purpose of DNA techniques later on. Another 2 ml blood sample was obtained from all participants and transferred into test tube and was allowed for 2 hours at room temperature and was then centrifuged and the supernatant was transferred to clean tube and kept at -20 C to be used then for ELISA.

The flow chart of study design is shown in figure (3.1) .

3.2.2. Inclusion criteria

- Any patients with chronic HCV with an adult age more than or equal 18 years of either sex.
- Any patient, whether on treatment or not was included in the study.
- Any patients with comorbidities shuch as essential hypertension or

diabetes mellitus was included.

- Any patients with complication of disease such as liver fibrosis or cirrhosis.

3.2.3.Exclusion criteria

- Any patient with mixed HCV and HBV infection was excluded.
- Any pregnant woman was excluded.
- Any patient with hepatocellular carcinoma was excluded.
- Any patient who refused to be enrolled in this study.

3.2.4.Ethical Approval

The study was conducted in accordance with the ethical principles that have their origin in the declaration of Helsinki .It was carried out with patients verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 8 (including the number and the data on June 4, 2024) to get this approval.

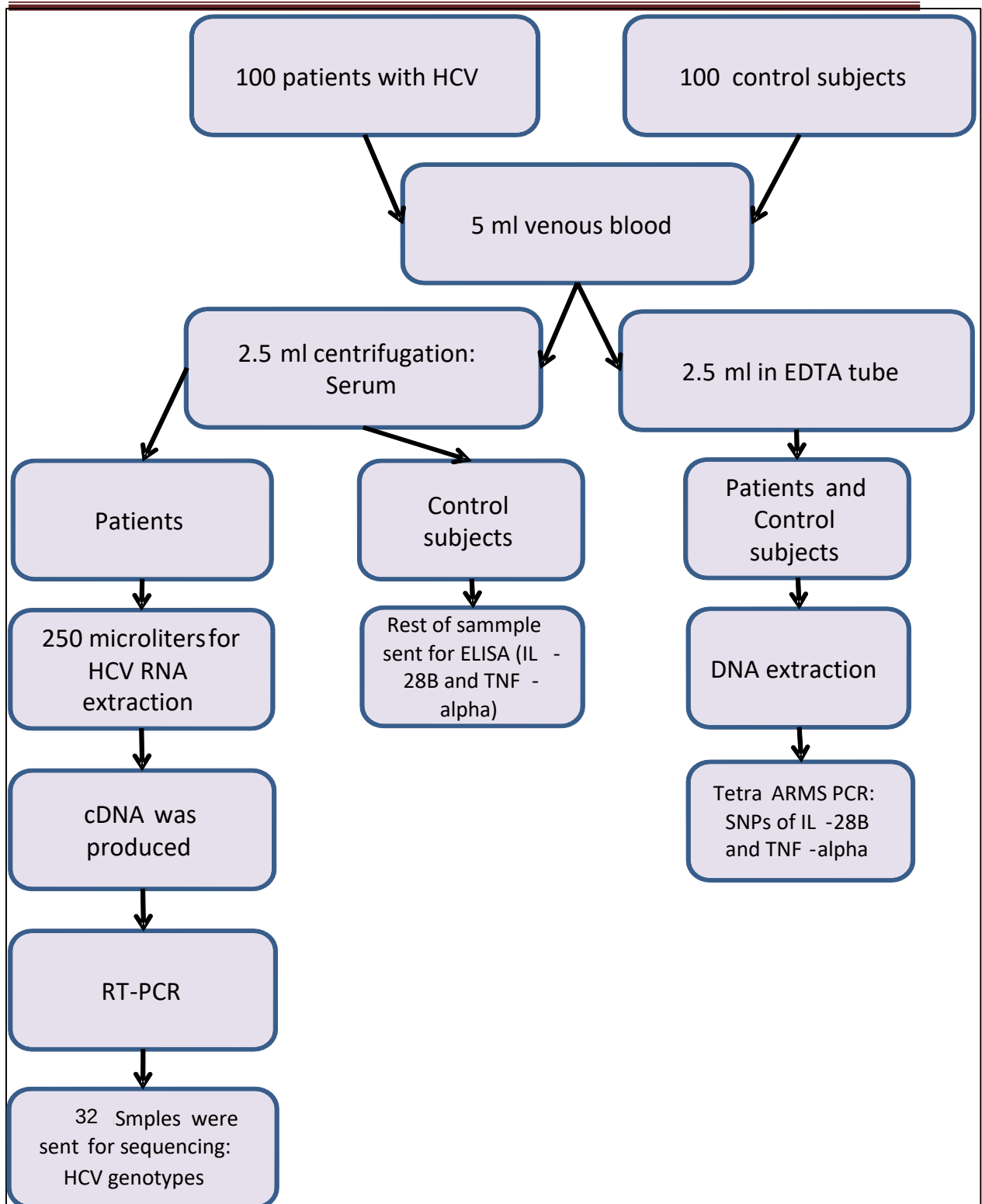


Figure (3.1): Flow chart of study design showing steps of the analysis

3.2.5. RT-PCR

The RT-PCR method was performed for detection Hepatitis C Virus based on 5' UTR region of HCV RNA genome. This method was done according to (Saha et al., 2014) as following steps:

3.2.5.1. Viral RNA Extraction

Viral RNA was extracted from serum samples by using AccuZol™ Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as following steps:

1. A 250µl serum samples was transferred by pipette into sterile and clean 1.5ml Eppendorf tube, then 1ml AccuZol reagent an mixed by vortex.
2. Chloroform (200 µl) was added to each Eppendorf tube and mixed vigorously for 30 seconds.
3. The mixture was incubated on ice for 5 minutes.
4. After that, the mixture was centrifuged at 12,000 rpm, 4C°, for 15 minutes.
5. Supernatant was transferred to a new Eppendorf tube, and 500µl isopropanol was added.
6. The mixture Mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes.
7. The mixture was centrifuged at 12,000 rpm, 4C°, for 10 minutes.
8. The supernatant was discarded.
9. 80% Ethanol was added into each tube and mixed by vortex, then centrifuged at 12,000 rpm, 4C° for 5 minutes.
10. The supernatant was discarded and the RNA pellet left to dry at room air for 5 minutes.
11. After that, DEPC water (50µl) was added to RNA pellet tubes and mixed by vortex to dissolved the RNA pellet.

12. The extracted RNA sample was kept at -80 deep freezers.

3.2.5.2. Estimation of Extracted RNA

The extracted RNA were estimated by using Nanodrop spectrophotometer that used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio (1.8-2.0) as pure RNA, and done as following steps:

- 1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
- 2- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2 μ L of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.
- 3- After that, the pedestals are cleaned and pipette 1 μ L of total RNA sample for measurement.

3.2.5.3. RT-PCR Master Mix Preparation:

RT-PCR master mix was prepared by using (**One-Step RT-PCR Premix Kit**) and done according to the company instructions in two step the following **Table 3.7**:

RT-PCR master mix components:

PCR master mix	Volume
RNA template 5-100ng	5 μ L
HCV-5' UTR forward primer (10pmol)	1 μ L
reverse primer (10pmol)	1 μ L
HCV-5' UTR RT-PCR premix	8 μ L
Nuclease free water	5 μ L
Total	20 μ L

These RT-PCR reaction mixes were placed in sterile 0.2ml PCR tubes and mixed and centrifuged for 3000rpm for 3 minutes by Exispin centrifuge, after that transferred into PCR thermocycler.

3.2.5.4. RT PCR Thermocycler Conditions:

RT PCR thermocycler conditions was according to primer annealing temperature as in the following **Tables 3.8:**

Step	Condition	Cycle
cDNA synthesis	42 °C 30 min.	1
Pre-Denaturation	95 °C 5 min.	1
Denaturation	95 °C 30 sec.	35
Annealing	55 °C 30 sec.	
Extension	72 °C 1min.	
Final extension	72 °C 5 min.	1
Hold	4 °C	Forever

3.2.5.5. RT PCR Product Analysis

The RTPCR products was analysed by agarose gel electrophoresis as shown in figure (3.2) following steps:

- 1- A 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.
- 2- Then 3µl of ethidium bromide stain were added into agarose gel solution.
- 3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.
- 4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.
- 5- A 10µl of RT PCR product were loaded in to each comb well and 5µl of (100bp Ladder) in first well.
- 6- Then electric current was performed at 90 volt and 80 mA for 1.5hour.
- 7- RT-PCR products were visualized by using UV ttransilluminator.

3.2.6. DNA Sequence Method

The DNA sequencing method was performed for genotyping analysis based on based 5' UTR region of HCV RNA genome. The RT PCR products were sent to Macrogen Company in Korea and the DNA sequencing by Applied Biosystems DNA sequencing system.

The DNA sequencing analysis was conducted by using Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis of the partialpolyprotein gene based ClustalW alignment analysis and The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method. The homology sequence identity analysis by using NCBI BLAST analysis.

3.2.7 Genotypeing of Hepatitis C virus:

The DNA sequence analysis of local Hepatitis C virus (HCV) isolates were to determine the genotypes and phylogenetic relationships of Hepatitis C virus (HCV) isolates. The analysis begins with a multiple sequence alignment of the 5' untranslated region (5'UTR) of the HCV polyprotein gene. The alignment shows clear differences in nucleotide sequences between the local HCV isolates and reference sequences from the NCBI GenBank database, representing different HCV genotypes. The phylogenetic tree analysis elucidates the relationships between the local HCV isolates and the reference genotypes at total genetic change (0.300-0.050%). The local isolates cluster into four distinct clades, corresponding to HCV genotypes G4, G1a, G1b, and G3b. The majority of the 11 local isolates are closely related to the genotype G4 reference sequence (AM885125.1), while 10 isolates are grouped with the genotype G1a reference (EU781783.1), 7 isolates are

associated with the genotype G1b reference (MZ702786.1), and only 4 isolates are associated with the genotype G3b reference (KC844044.1).

The NCBI BLAST homology analysis corroborates these findings, showing that the local HCV isolates have (99.10-99.85%) sequence identity with the corresponding reference genotypes. This high level of sequence similarity suggests that the local strains are closely related to the well-characterized HCV genotypes G4, G1a, G1b, and G3b, which are prevalent in various regions around the world.

The distribution of HCV genotypes within the local population was show that Genotype G4 is the most common form globally, responsible for approximately (34%) of all HCV infections, followed by genotypes G1a (31%), G1b (21%), and G3b (14%).

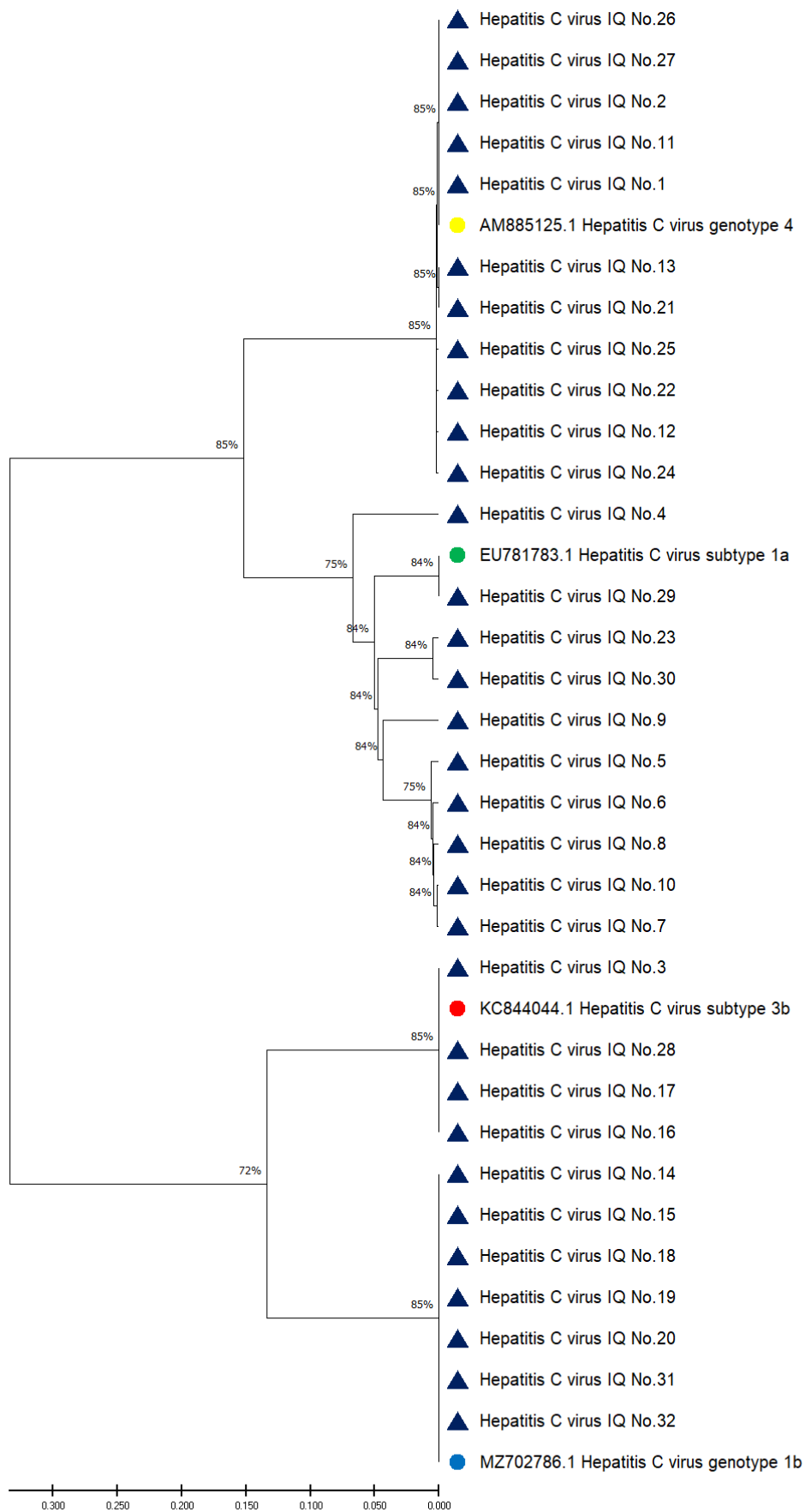


Figure (3.2): Phylogenetic tree analysis based on the Hepatitis C virus 5'UTR polyprotein gene partial sequence that used for Genotyping analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Hepatitis C virus isolates (No.1-No.32) were show closed related to NCBI-Blast Hepatitis C virus genoptype G4, G1a, G1b and G3b at total genetic change (0.300-0.050%).

Table (3.9): NCBI BLAST Homology sequence identity between local Hepatitis C virus isolates and closed related to NCBI-Blast Hepatitis C virus genotypes

Local HCV isolate	NCBI BLAST Homology sequence identity		
	Identical Genotypes	NCBI Accession number	Identity %
Hepatitis C virus No.1	G4	AM885125.1	99.44%
Hepatitis C virus No.2	G4	AM885125.1	99.41%
Hepatitis C virus No.3	G3b	KC844044.1	99.85%
Hepatitis C virus No.4	G1a	EU781783.1	99.10%
Hepatitis C virus No.5	G1a	EU781783.1	99.24%
Hepatitis C virus No.6	G1a	EU781783.1	99.34%
Hepatitis C virus No.7	G1a	EU781783.1	99.15%
Hepatitis C virus No.8	G1a	EU781783.1	99.16%
Hepatitis C virus No.9	G1a	EU781783.1	99.56%
Hepatitis C virus No.10	G1a	EU781783.1	99.14%
Hepatitis C virus No.11	G4	AM885125.1	99.56%
Hepatitis C virus No.12	G4	AM885125.1	99.34%
Hepatitis C virus No.13	G4	AM885125.1	99.23%
Hepatitis C virus No.14	G1b	MZ702786.1	99.55%
Hepatitis C virus No.15	G1b	MZ702786.1	99.55%
Hepatitis C virus No.16	G3b	KC844044.1	99.45%
Hepatitis C virus No.17	G3b	KC844044.1	99.40%
Hepatitis C virus No.18	G1b	MZ702786.1	99.34%
Hepatitis C virus No.19	G1b	MZ702786.1	99.34%
Hepatitis C virus No.20	G1b	MZ702786.1	99.45%
Hepatitis C virus No.21	G4	AM885125.1	99.56%
Hepatitis C virus No.22	G4	AM885125.1	99.56%
Hepatitis C virus No.23	G1a	EU781783.1	99.40%
Hepatitis C virus No.24	G4	AM885125.1	99.34%
Hepatitis C virus No.25	G4	AM885125.1	99.45%
Hepatitis C virus No.26	G4	AM885125.1	99.34%
Hepatitis C virus No.27	G4	AM885125.1	99.45%
Hepatitis C virus No.28	G3b	KC844044.1	99.56%
Hepatitis C virus No.29	G1a	EU781783.1	99.23%
Hepatitis C virus No.30	G1a	EU781783.1	99.23%
Hepatitis C virus No.31	G1b	MZ702786.1	99.55%
Hepatitis C virus No.32	G1b	MZ702786.1	99.55%

3.2.8. Tetra- ARMS-PCR Method

T-ARMS-PCR method was performed for detection and genotyping of TNF- α -308 G>A (rs1800629) and IL-28B rs12979860 C>T gene polymorphism in patients and healthy control samples. This method was carried out according to described by (.....REF.....). as following steps:

3.2.8.1. Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA kit extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions as following steps:

1. A 200 μ l of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 30 μ l of proteinase K and mixed by vortex. And incubated at 60°C for 5 minutes.
2. After that, 200 μ l of lysis buffer GSB was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70°C for 10 minutes, and inverted every 3 minutes through incubation periods.
3. 200 μ l absolute ethanol were added to lysate and immediately mixed by shaking vigorously.
4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow through were discarded and placed the column in a new 2 ml collection tube.

5. 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.
6. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.
7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.
8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated elution buffer were added to the center of the column matrix.
9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3.2.8.2. Genomic DNA Estimation

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.

3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

3.2.8.3.T-ARMS-PCR Master Mix Preparation

T-ARMS-PCR master mix was prepared by using (**GoTaq® G2 Green Master Mix kit**) and this master mix done two reactions for each samples according to company instructions as following **Tables 3.10**:

Standard T-ARMS-PCR reaction Mix:

T-ARMS-PCR Master mix	Volume
DNA template	5µl
Forward inner primer (10pmol)	1µl
Reverse inner primer (10pmol)	1µl
Forward outer primer (10pmol)	1µl
Reverse outer primer (10pmol)	1µl
G2 Green Master Mix	12.5µl
Nuclease free water	3.5µl
Total volume	25µl

After that, these PCR master mix component that mentioned in table above were transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (BioRad. USA).

3.2.8.4. PCR Thermocycler Conditions

All PCR thermocycler conditions were done for each gene independent as following **Tables 3.11**:

1- TNF- α -308 G>A (rs1800629)

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	35cycle
Annealing	60°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

2- IL-28B C>T (rs12979860)

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	35cycle
Annealing	62°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

3.2.8.5. T-ARMS-PCR Product Analysis

The T-ARMS-PCR products were analyzed by agarose gel electrophoresis following steps as in figures (3.3)and (3.4):

1- 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

1- Then 3 μ L of ethidium bromide stain were added into agarose gel solution.

- 2- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.
- 3- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.
- 4- 10µl PCR product were added in to each comb well and 3µl of (100bp Ladder) in First well.
- 5- The electric current was performed at 100 volt and 80 mA for 1hour.
- 6- The T-ARMS-PCR products were visualized by using UV transilluminator.

3.2.9.ELISA Method

Elisa method was used for quantitative determination of Human Interleukin 28 (IL-28)and Tumor Necrosis Factor Alphain patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

Table 3.12:A. ELISA components:

Reagent	Quantity
Standard Solution	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash BufferConcentrate (25x)	20ml x1
BiotinylatedHuman Antibody	1ml x1

B. Assay Procedure:

1. All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.
2. A 50µl standard serial dilution was added into to standard well.
Without added antibody to standard well because the standard solution contains biotinylated antibody.
3. A 40µl sample was added to sample wells and then add 10µl anti-antibody to sample wells, then 50µl streptavidin-HRP added in to sample wells and standard wells (Not blank control well).
4. The ELISA mixed well and covered the plate witha sealer. Then incubated at 60 minutes at 37°C.
5. The sealer was removed and wash the plate 5 times with wash buffer. wells soaked
6. with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For
7. A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
8. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
9. A 50µl Stop Solution was added to each well, then the blue colour will change into yellow immediately.
10. The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

C. Calculation of Results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was

plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph as in figures (3.3) and (3.4).

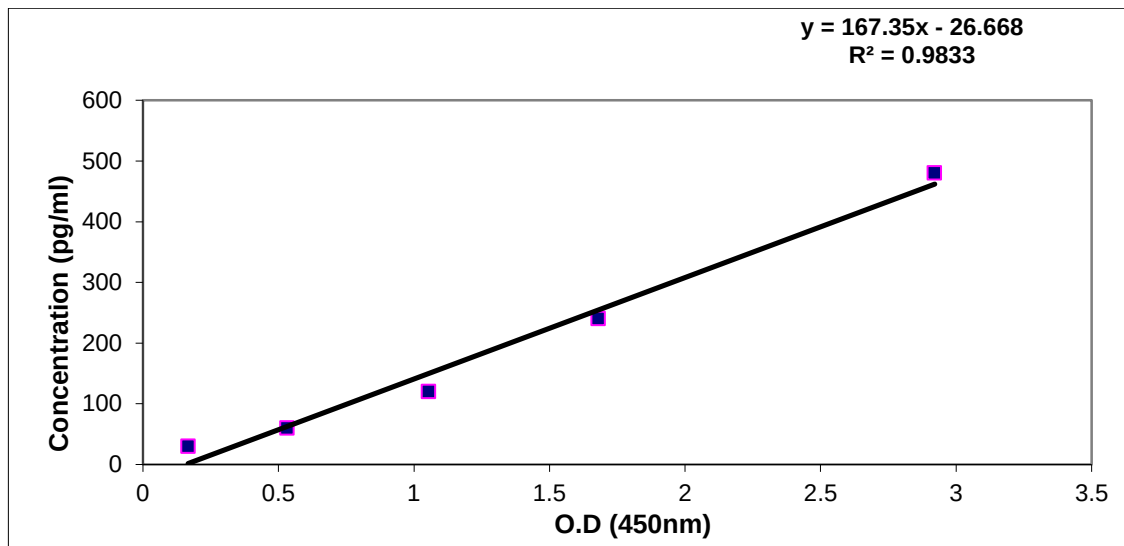


Figure (3.3) Human TNF standard curve

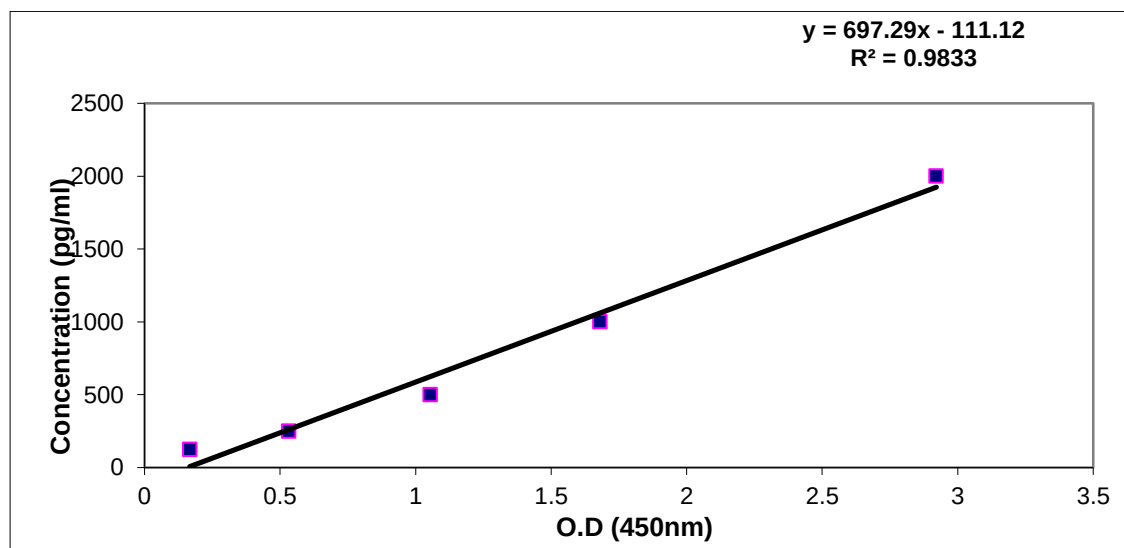


Figure (3.4) Human IL-28 standard curve

3.2.10. Statistical Analysis

Microsoft Office Excel 2010 and the statistical program for social sciences (SPSS) version 23 were used to gather, compile, analyze, and present the data. In contrast to quantitative (numeric) variables, which were first assessed for normality distribution using the Kolmogorov-Smirnov test, qualitative (categorical) variables were expressed as numbers and percentages. Consequently, normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion), while those that were not normally distributed were expressed as median (an index of central tendency) and inter-quartile range (an index of dispersion). The following statistical tests were used:

1. **Independent Samples Student T-Test** was used to evaluate difference in mean of numeric variables between two groups provided that these numeric variables were normally distributed.
2. **Chi-square test** was use to evaluate association between any two categorical variables provided that less than 20 % of cells have expected count of less than 5.
3. **Receiver operating characteristic** curve analysis was done to find cutoff values with corresponding sensitivity and specificity..
4. Risk was estimated using **odds ratio** with corresponding 95 % confidence interval.

The level of significance was considered at *P*-value of equal or less than 0.05.

Chapter Four

Results and Discussion

4. Results and Discussion

4.1. Demographic characteristics compared between patients group and control group

Demographic characteristics compared between patients group and control group are shown in table 4.1. The mean age of patients group was 49.08 ± 11.96 years and that of control group was 48.99 ± 6.04 years. The age was ranging between 29 -73 years in patients group and between 25 -61 years in control group. Statistical comparison of mean age revealed no significant difference between patients group and control group ($p = 0.947$).

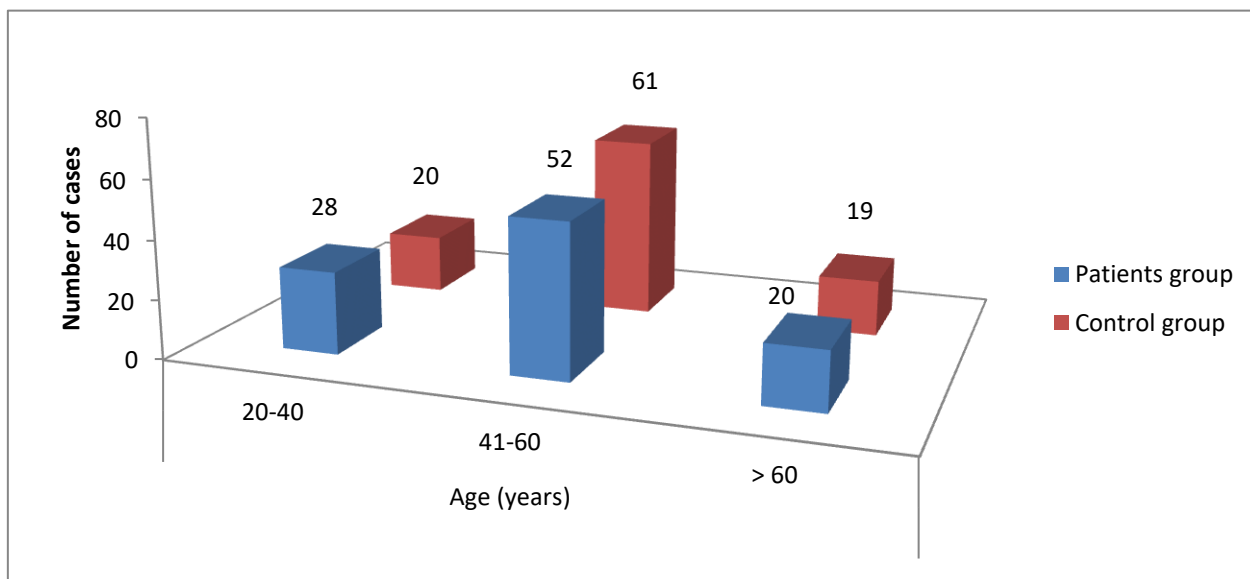
The distribution of patients according to age was as following: 28 (28.0 %) between 20-40 years, 52 (52.0 %) between 41-60 years and 20 (20.0 %) > 60 years. The distribution of control subjects according to age was as following: 20 (20.0 %) between 20-40 years, 61 (61.0 %) between 41-60 years and 19 (19.0 %) > 60 years. Statistical companions also revealed no significant variation in the distribution ($p = 0.354$), as shown in figure 4.1.

With respect to sex proportions, the group of patients enrolled 64 (64.0 %) males and 36 (36.0 %) females and the male to female ratio was 1.78:1. The group of control subjects included 65 (65.0 %) males and 35 (35.0 %) females. Statistical comparison of sex proportions between patients group and control group revealed no significant difference ($p = 0.883$), as shown in figure 4.2.

Table 4.1: Demographic characteristics compared between patients group and control group

Characteristic	Patients group <i>n</i> = 100	Control group <i>n</i> = 100	<i>p</i>
Age (years)			
Mean ±SD	49.08±11.96	48.99±6.04	0.947 I
Range	29 -73	25 -61	NS
20-40 years, <i>n</i> (%)	28(28.0%)	20(20.0%)	0.354 C NS
41-60 years, <i>n</i> (%)	52(52.0%)	61(61.0%)	
> 60 years, <i>n</i> (%)	20(20.0%)	19(19.0%)	
Sex			
Male, <i>n</i> (%)	64(64.0%)	65(65.0%)	0.883 C
Female, <i>n</i> (%)	36(36.0%)	35(35.0%)	NS

n: number of cases; *SD*: standard deviation; *I*: independent samples student *t*-test; *C*: chi-square test; *NS*: not significant

**Figure (4.1) :** Three dimensional column graph showing the distribution of frequency of control subjects and patients based on age

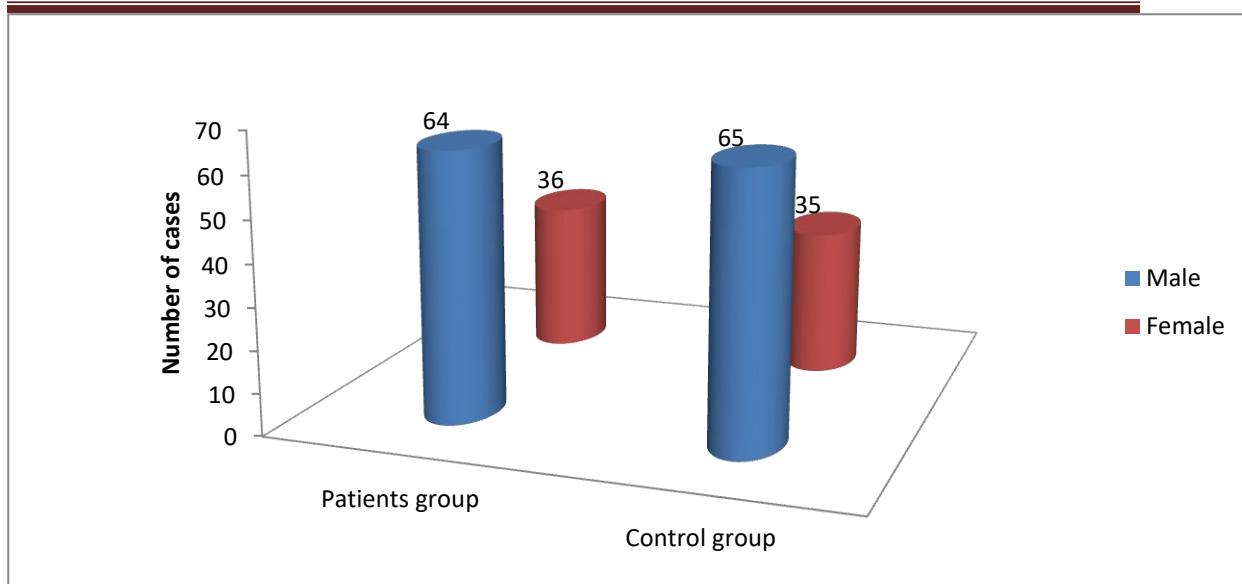


Figure (4.2): Three dimensional cylinder graph showing the frequency distribution of patients and control subjects according to sex

Comparison of males and females proportion and mean age according to sex in both patients group and control group is shown in table 4.2. The frequency distribution of male patients according to age groups was as following: 8 (12.5 %) in 20-40 years interval, 44 (68.8 %) in 41-60 age interval and 12 (18.8 %) in > 60 years age interval. The frequency distribution of female patients according to age groups was as following: 20 (55.6 %) in 20-40 years interval, 8 (22.2 %) in 41-60 age interval and 8 (22.2 %) in > 60 years age interval. Statistical wise, comparison of this frequency distribution between male patients and female patients revealed no significant variation ($p = 0.875$).

The frequency distribution of male control subjects according to age groups was as following: 13 (20.0 %) in 20-40 years interval, 41 (63.1 %) in 41-60 age interval and 11 (16.9 %) in > 60 years age interval.

The frequency distribution of female patients according to age groups was as following: 7 (20.0 %) in 20-40 years interval, 20 (57.1 %) in 41-60 age interval and 8 (22.9 %) in > 60 years age interval. Statistical wise, comparison of this frequency distribution between male control subjects and female control subjects revealed no

significant variation ($p = 0.758$).

Moreover, comparison of mean age between male patients and female patients was not significant ($p = 0.381$), 48.60 ± 6.66 years versus 49.71 ± 4.66 years, respectively, as shown in figure 4.3. In addition, comparison of mean age between male control subjects and female control subjects was not significant ($p = 0.053$), 50.81 ± 9.53 years versus 46.00 ± 15.03 years, respectively, as shown in figure 4.3.

Table 4.2: Comparison of males and females proportion and mean age according to sex in both patients group and control group

Age (years)	Patients group $n = 100$		Control group $n = 100$	
	Sex		Sex	
	Male $n = 64$	Female $n = 36$	Male $n = 65$	Female $n = 35$
20-40	8 (12.5 %)	20 (55.6 %)	13 (20.0 %)	7 (20.0 %)
41-60	44 (68.8 %)	8 (22.2 %)	41 (63.1 %)	20 (57.1 %)
> 60	12 (18.8 %)	8 (22.2 %)	11 (16.9 %)	8 (22.9 %)
p	0.875 C NS		0.758 C NS	
Mean $\pm$ SD	48.60 $\pm$ 6.66	49.71 $\pm$ 4.66	50.81 $\pm$ 9.53	46.00 $\pm$ 15.03
Range	25 -61	44 -59	37 -70	29 -73
p	0.381 I NS		0.053 I NS	

n : number of cases; SD: standard deviation; I: independent samples student t -test; C: chi-square test; NS: not significant; ***: significant at $p \leq 0.001$

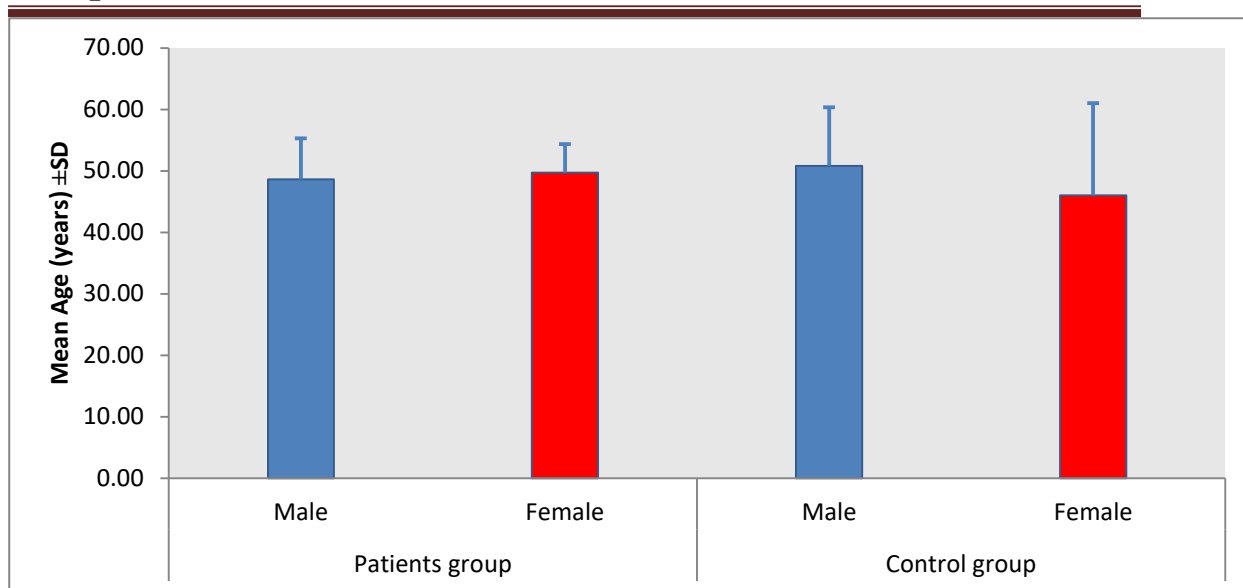


Figure (4.3): Bar chart showing comparison of mean age between male and female in patients group and in control group

Pyenson et al in 2018 found that the mean age of patients with HCV was 49.5 years which is nearly the same as that reported in current study (48.99 years). In addition, Pyenson *et al* (2018) reported low incidence rate of HCV in patients younger than 40 years which is also in support for our findings as we found that only 28 % of cases were younger than 40 years and that most cases were between 41 and 60 years of age. Moreover, Naser et al. in 2025 found that 60 % of cases of HCV were more than 40 years of age and this is also in accordance with our findings. Moreover, Siaw *et al.* (2024) found that the mean age of patients with HCV was 45.4 (± 16.3) years which slightly lower than the mean age in our study. Niu *et al.* (2016) found Prior to age 60, the prevalence of HCV infection gradually increased. After age 60, the prevalence of HCV infection decreased, possibly as a result of a drop in economic strength and physical mobility, which lowers the number of older examinations.

In their study, Butsashvili *et al.* (2025) found that age > 40 was an independent risk factor for HCV infection. The most acceptable explanation for high incidence rate of HCV in age > 40 years can be due to the high rate of

chronicity of HCV, thus, most patients older than 40 will be discovered after seroconversion and that younger patients may be asymptomatic and the disease passed unnoticed until 40s of age. Another explanation is the presence of chronic comorbidities such as diabetes mellitus and hypertension which may interfere with immune responses leading to exacerbation of infection with HCV.

According to Hussein S. A, and Al-Azzawi (2020), ninety eight patients with Hepatitis C virus (HCV), 52 without treatment with average age (45.26 ± 2.97) years and 46 received treatment their average age (39.30 ± 3.90) years.

Increased susceptibility to environmental factors like oxidative stress as one ages, decreased hepatic flow rate, diminished mitochondrial capacity, weakened immunity, and heightened carcinogenic potential due to a diminished capacity to repair DNA are some of the aging-related mechanisms that have been hypothesized to increase the risk of liver disease outcomes in the context of HCV infection.(Horiike et al., 1995; Poynard *et al.*, 2001).

Additionally, there is little evidence linking HCV infection to elevated immune-senescence markers, which have been demonstrated to occur in the context of HIV infection and are believed to contribute to the early onset of aging-related comorbidities in HIV infection. The loss of early-differentiated T cells and the subsequent buildup of chronically-activated, late-differentiated senescent T cells may be linked to HCV infection itself. According to a single, short study (Barathan *et al.*, 2015) that compared HCV-infected people with healthy controls, all of whom were under 54, the CD4 and CD8 T cells from HCV-infected people exhibited a marked increase in the T cell immunosenescent phenotype, which is more frequently linked to aging. It is unclear if this rise is linked to the early beginning of both liver and non-liver clinical consequences associated with aging in those infected with HCV(Chou and Effros, 2013).

With respect to sex, in our study, most of cases were males and this observation is consistent with previous observations of several authors (Abdel-

Gawad *et al.*, 2023; Butsashvili *et al.*, 2025). Female sex is associated with greater likelihood of spontaneous clearance of HCV infection, potentially a function of sex differences in immunological functioning and inflammatory responses (Levinsson *et al.*, 2024). Irish and German women who received HCV-contaminated Rh immune globulin in late pregnancy or early postpartum had the best clearance rates (45%), according to two studies (Kenny-Walsh, 1999; Wiese *et al.*, 2000). It is widely accepted that women clear HCV well, probably better than men, based on the findings of these two investigations.

Furthermore, the findings of three of the biggest population-based studies—the Trent Study in the UK, the NHANES in the USA, and Dionysos in Italy—were contentious. Only the non-Hispanic Black subgroup in the NHANES sample showed a sex difference in HCV clearance, whereas the non-Hispanic White and Mexican American groups showed no sex difference (Alter *et al.*, 1999). No sex difference in HCV clearance was observed in the Trent and Dionysos investigations (Bellentani *et al.*, 1999; Mohsen *et al.*, 2001; Hoofnagle, 2002). In contrast to our findings, Hussein S. A. and Al-Azzawi (2020) discovered that women were more likely than men to be infected with HCV.

There is currently no solution to the gender disparity in HCV clearance rates. Under the standard assumptions ($\alpha = 0.05$; two-sided tests), a prospective study comparing the HCV clearance rate between males and females following acute HCV infection would need 376 patients of each sex to have 80% power to detect an absolute 10% difference (40% v 30%) in clearance rate. Even with the best design, such a study is nearly impossible to conduct, and the only way to compare HCV clearance rates by sex is to use retrospective studies. This study's estimated female HCV clearance rate was 44.6%, which is nearly the same as the rates for East German (45.5%) and Irish (44.6%) women infected with HCV-contaminated anti-D immune globulin (Kenny-Walsh, 1999). According to Wiese *et al.* (2000), it was greater than that of men (44.6% vs. 33.7%; $p = 0.001$).

In the case of HCV the steroid hormones have been suggested to be possible players of the reported sex differences. It has been shown that treatment with E2 of HCV infected cell cultures reduced the production of HCV virions, probably inhibiting virus assembly /secretion, whereas E2 did not affect HCV RNA replication nor virus protein synthesis. However, in the post-menopausal women, when estrogen levels decrease, these sex differences are mitigated and partially reversed. In addition, sex hormones not only directly affect viral replication but, also, exert potent immunomodulating effects. Estrogens potentiate immune response, both humoral and innate, whereas androgens are mainly immunosuppressive (Ruggieri and Malorni, 2015).

Indeed, the liver is a highly sexual dimorphic organ, accounting for at least 72% of sexually differentiated genes. This sex difference is reflected in the different susceptibility, progression, and outcomes of many acute and chronic liver diseases in females and males (Sayaf *et al.*, 2022).

The lifestyle of males makes them more exposed to various risk factors for HCV transmission (El-Adly, and Wardany, 2017).

4.2. Chronic Medical Disorders Reported in Patients Group and in Control Group

Chronic medical disorders reported in patients group and in control group is shown in table 4.3. Diabetes has been reported in 17 % and hypertension was seen in 12 %.

Table 4.3: Chronic medical disorders reported in patients group and in control group

Characteristic	Patients group <i>n</i> = 100	Control group <i>n</i> = 100	<i>p</i>
Diabetes			
Positive, <i>n</i> (%)	17(17.0%)	12(12.0%)	0.315 C NS
Negative, <i>n</i> (%)	83(83.0%)	88(88.0%)	
Hypertension			
Positive, <i>n</i> (%)	12(12.0%)	13(13.0%)	0.831 C NS
Negative, <i>n</i> (%)	88(88.0%)	87(87.0%)	

n: number of cases; C: chi-square test; NS: not significant

However, despite the fact that many of these studies were not prospective, had small sample sizes, or included cases of liver cirrhosis, which can raise the risk of diabetes on its own, the presence of HCV antibodies (HCV Ab) appears to increase the risk of both incident and prevalent diabetes (Hong *et al.* 2017). The pooled OR with diabetes was 1.68 in a meta-analysis of 17 studies that compared patients with and without HCV infection (Naing *et al.*, 2012).

Because the liver is essential for the metabolism of glucose, severe hepatic conditions including cirrhosis and hepatocellular carcinoma cause dysregulation in glucose homeostasis. However, it is unclear what mechanisms underlie the disturbance in glucose metabolism in viral hepatitis without cirrhosis (Naing *et al.*,

2012). In one investigation, there were no differences between HBV and HCV infection, but there was a high correlation between diabetes and the degree of fibrosis in liver biopsies in individuals with either chronic HCV infection or increased liver enzymes (Papatheodoridis *et al.*, 2006). In patients with persistent HCV infection, an Italian study also discovered that the only two characteristics that were independently linked to the presence of diabetes were age and cirrhosis (Mangia *et al.*, 1998).

Other explanations for the altered glucose metabolism in HCV infection have been proposed. According to experimental research, HCV can directly disrupt the insulin signaling cascade in infected hepatocytes, which may be influenced by pathways specific to a person's genotype (Bugianesi *et al.*, 2012; Qadri *et al.*, 2004; Sheikh *et al.*, 2008). By producing peripheral insulin resistance in the muscle tissue of chronic HCV patients, HCV may also indirectly disrupt glucose metabolism (Vanni *et al.*, 2007).

According to Drazilova *et al.* in 2018, because the hepatitis C virus disrupts the hepatocyte insulin signaling pathway in several ways, such as (i) increased production of tumor necrosis factor- α , (ii) phosphorylation of the insulin receptors, (iii) overexpression of the suppressor of cytokines (SOCS-3), and (iv) induction of SOCS-7, a chronic HCV infection can result in increased insulin resistance. Insulin sensitivity is compromised in both the liver and the entire body, even in HCV-infected patients who do not have metabolic syndrome. Also mediators produced by an HCV-infected liver lead to elevated insulin resistance at extrahepatic locations, namely in skeletal muscles.

Recent research has demonstrated that hepatitis C infection also has a role in the development of metabolic abnormalities, which are important risk factors for hypertension and cardiovascular disease. Because of HCV's discernible impact on the development of obesity, insulin resistance, diabetes, lipid abnormalities, and

hepatic steatosis, some authors have dubbed metabolic abnormalities during the infection "metabolic and virus syndrome," while hepatic steatosis is referred to as an organ form of a metabolic syndrome (Rajewski *et al.*, 2022).

4.3. Duration of Disease and Proportion of Patients Receiving Treatment

Minimum duration of disease was 3 months and maximum duration of disease was 8 years and the mean duration ($\pm$ standard deviation) was 3.49 ± 2.21 years. The frequency distribution of patients according to duration of disease is shown in figure 4.4. Sixteen patients were less than 1 year; 8 patients were 1 year; 12 patients were 2 years; 16 patients were 3 years; 16 patients were 4 years; 12 patients were 5 years; 12 patients were 6 years and 8 patients were 8 years.

The proportion of patients receiving treatment is shown in figure 4.5. Fifty six patients were receiving treatment.

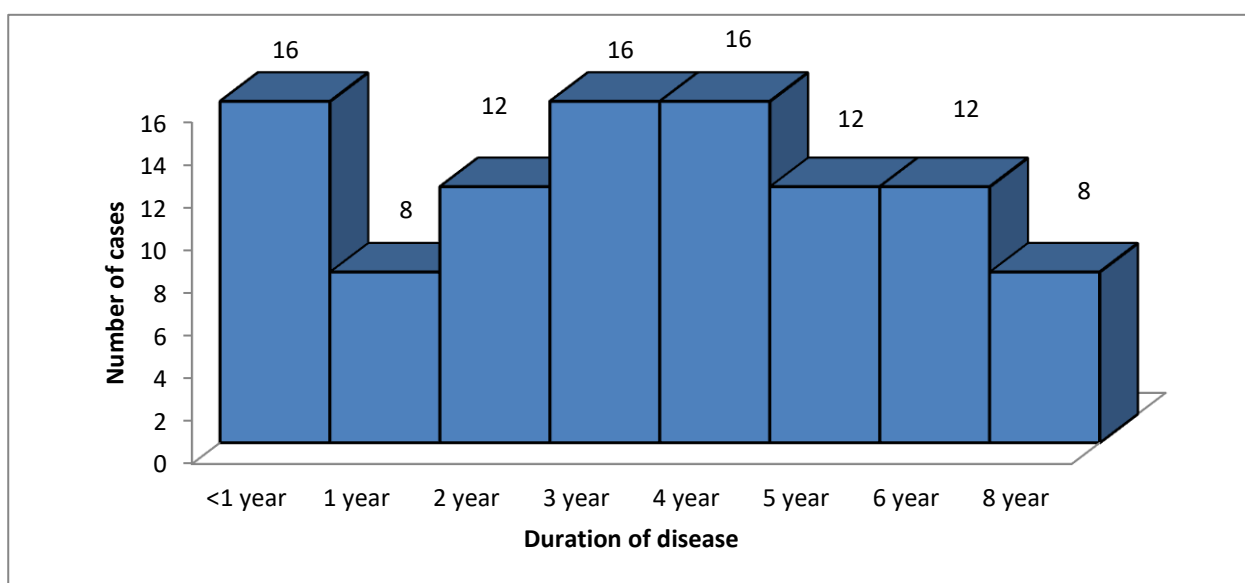


Figure (4.4): Three dimensional clustered column showing the number of patients according to duration of disease .

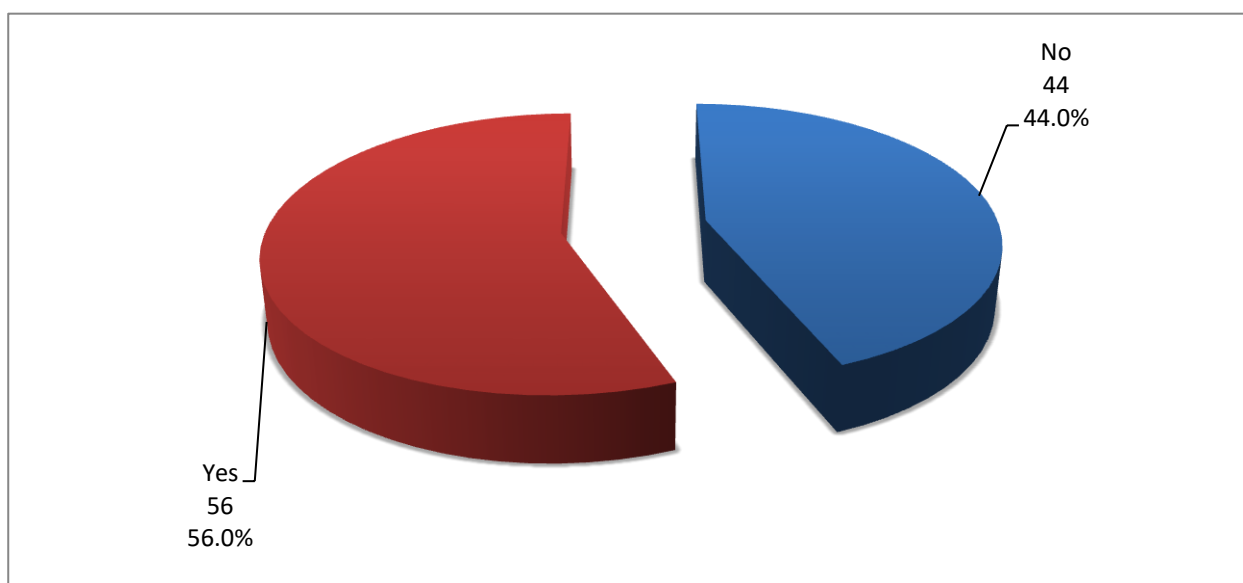


Figure (4.5): the proportion of patients receiving treatment

Direct-acting antiviral (DAA) treatments are currently the gold standard for treating both acute and chronic hepatitis C, replacing IFN-based approaches. Based on their targets in HCV proteins, DAAs can be divided into three main groups. Protease inhibitors (PIs) that block the processing of HCV polyproteins fall into the first type. These include non-structural protein 3/4A (NS3/4A). NS5A inhibitors, which can prevent viral proliferation and assembly, are within the second category (Fasano *et al.*, 2024).

NS5B polymerase inhibitors, which can prevent HCV RNA replication, make up the third group. The potential "efficacy advantage" of treatment in the early stages of HCV infection has been lessened by the great results obtained with DAA treatment in a chronic HCV infection. On the other hand, identifying and treating a recent HCV infection should make it easier to receive multidisciplinary care, stop chronic liver disease from developing and becoming worse, and lessen the risk of transmission in important populations. Thus, in the DAA era, treating an infection early rather than waiting until it becomes chronic is still a viable strategy that is backed by cost-effectiveness analysis (Bethea *et al.*, 2018).

4.4. Comparison of Cytokines Levels Between Patients Group and Control Group

Comparison of cytokines levels between patients group and control group is shown in table 4.4. Mean serum IL-28 was higher significantly in patients group compared to control group, 340.86 $\pm$ 61.61 ng/ml versus 275.18 $\pm$ 91.68 ng/ml, respectively ($p < 0.001$), as shown in figure 4.6.

Mean serum TNF-alpha was higher significantly in patients group compared to control group, 93.32 $\pm$ 14.69 ng/ml versus 62.24 $\pm$ 22.62 ng/ml, respectively ($p < 0.001$), as shown in figure 4.7.

Table 4.4: Comparison of cytokines levels between patients group and control group

Characteristic	Patients group <i>n</i> = 100	Control group <i>n</i> = 100	<i>p</i>
IL-28 ng/ml			
Mean ±SD	340.86±61.61	275.18±91.68	<0.001 I ***
Range	199.87 -421.61	145.48 -450.2	
TNF ng/ml			
Mean ±SD	93.32±14.69	62.24±22.62	<0.001 I ***
Range	70.54 -116.23	31.55 -111.04	

n: number of cases; *SD*: standard deviation; *I*: independent samples student *t*-test; **IL-28**: interleukin-28; **TNF- α** : tumor necrosis factor-alpha; ***: significant at $p \leq 0.001$

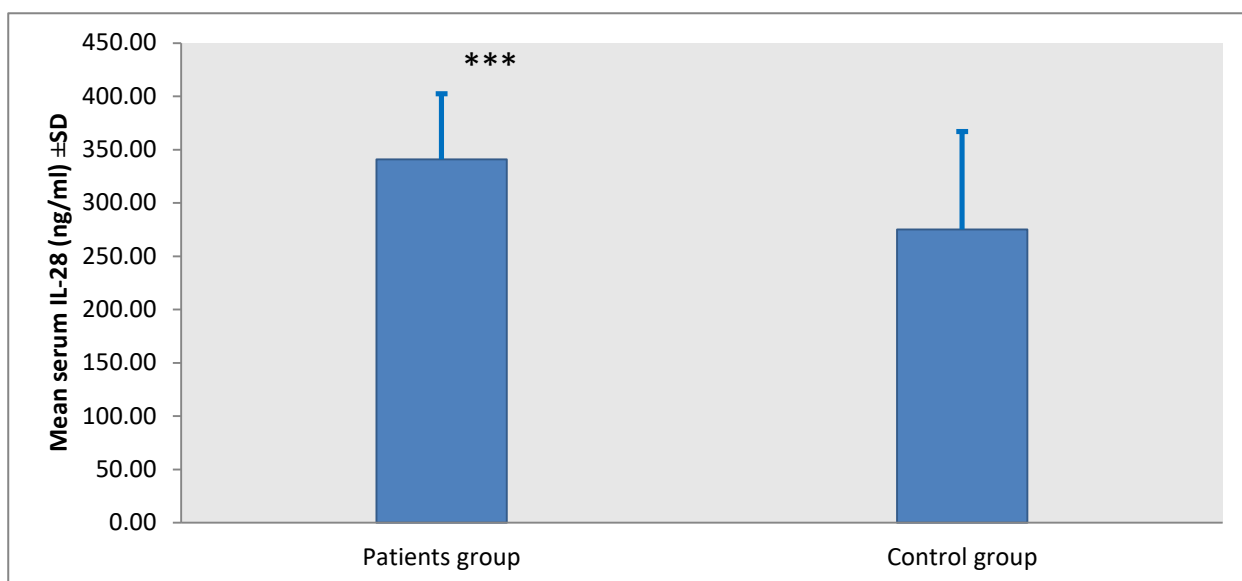


Figure (4.6): Bar chart showing comparison of IL-28 levels between patients group and control group

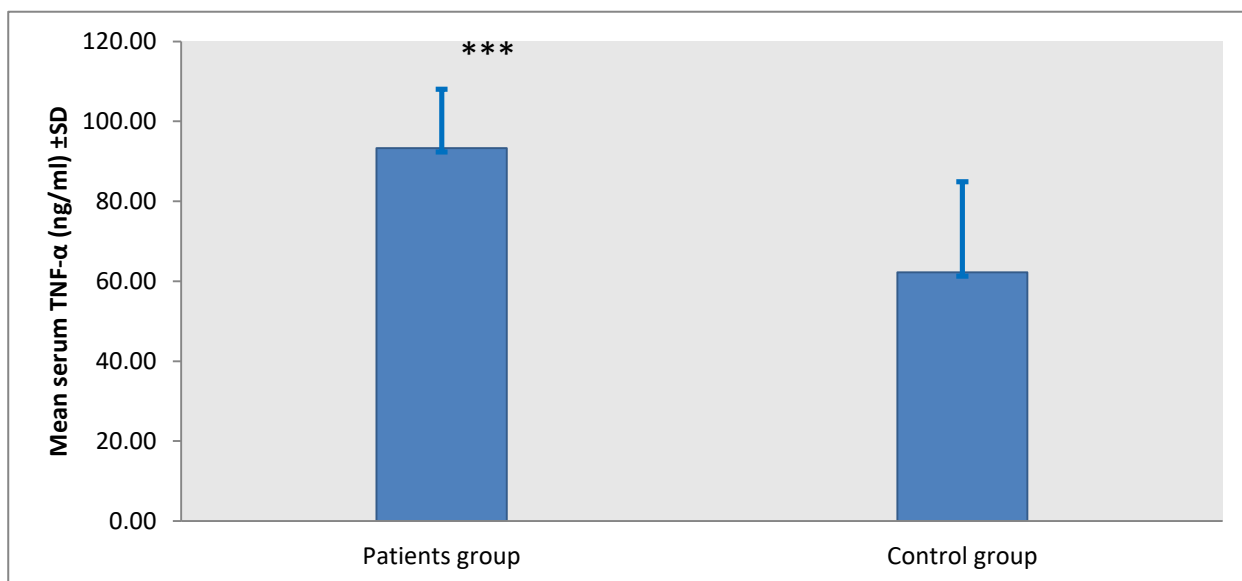


Figure (4.7): Bar chart showing comparison of TNF-alpha levels between patients group and control group

The significant differences in mean serum IL-28 and TNF-alpha are suggestive of possible diagnostic potential of both serum markers with respect to HCV infection; therefore, receiver operating characteristic (ROC) curve analysis was carried out and the results were outlined in figures 4.8 and 4.9 and tables 4.5,

4.6 and 4.7. With respect to serum IL-28, the cutoff value based on Youden Index was > 227 ng/ml with an are under the curve (AUC) of 0.712 making it valid test because the area in more than 0.700 and this is was further documented by significant p-value (<0.001). The sensitivity was 96.0 %, the specificity level was 45.0 %, the positive predictive value (PPV) was 61.5 %, the negative predictive value (NPV) was 90.9 % and the accuracy level was 68.0 %.

With respect to serum TNF-alpha, the cutoff value based on Youden Index was > 63.18 ng/ml with an are under the curve (AUC) of 0.700 making it valid test because the area in equal to 0.700 and this is was further documented by significant p-value (<0.001). The sensitivity was 100.0 %, the specificity level was 70.0 %, the positive predictive value (PPV) was 76.9 %, the negative predictive value (NPV) was 100.0 % and the accuracy level was 85.0 %.

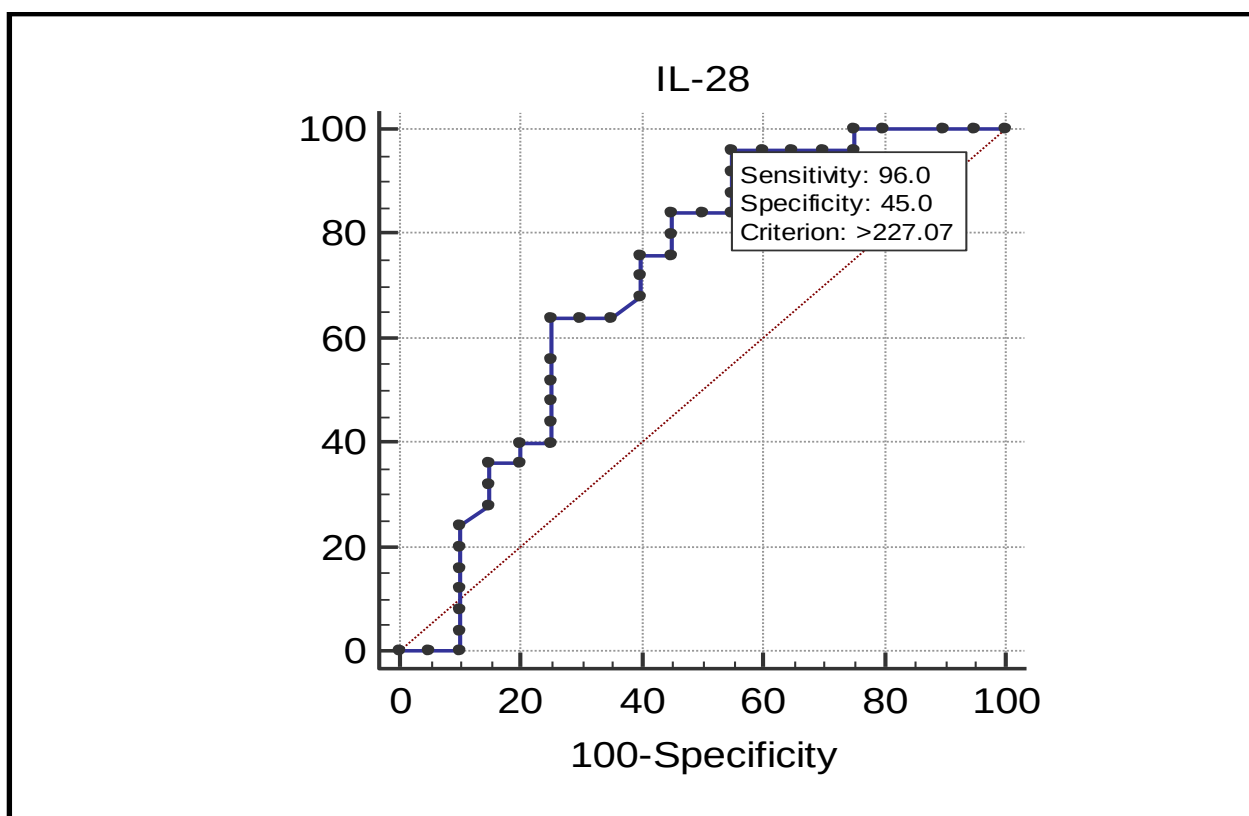


Figure (4.8): Receiver operating characteristic (ROC) curve analysis to find the cutoff value at which serum IL-28 is used to predict diagnosis of HCV infection

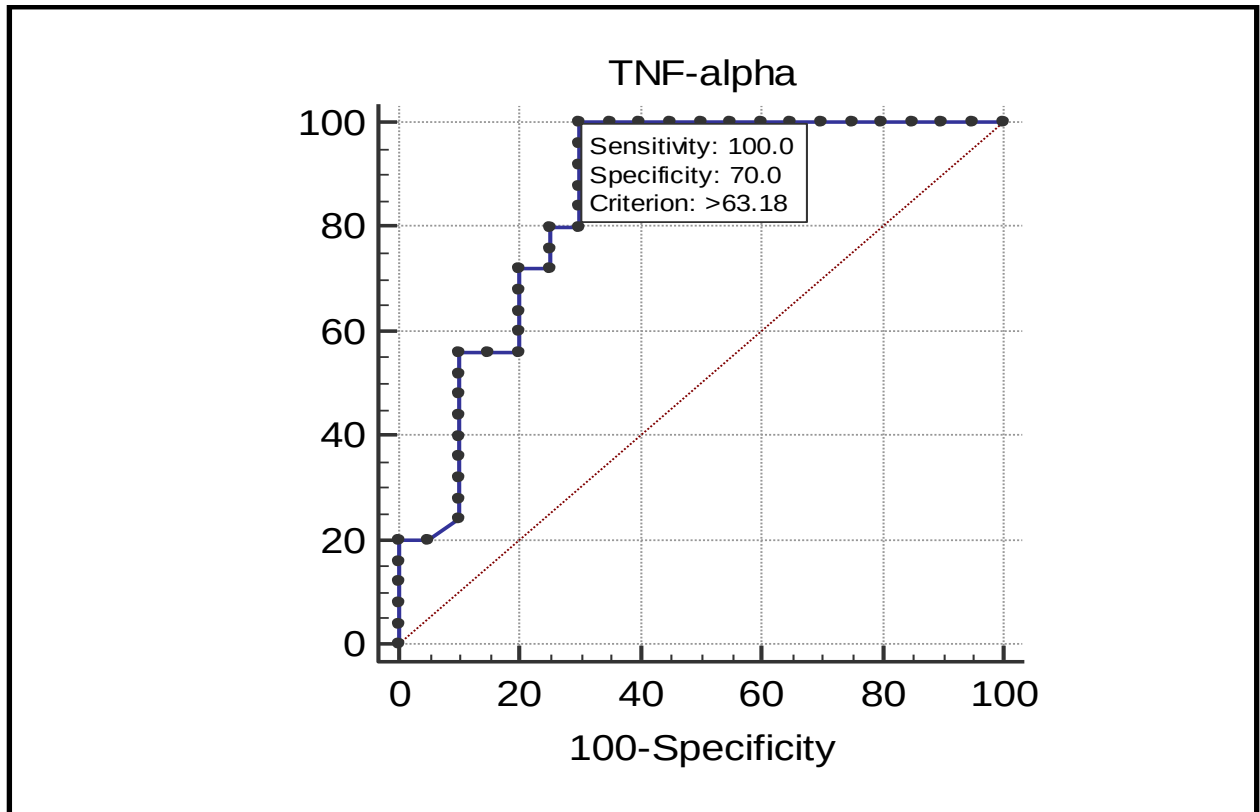


Figure (4.9): Receiver operating characteristic (ROC) curve analysis to find the cutoff value at which serum TNF-alpha is used to predict diagnosis of HCV infection

Table 4.5: The statistical parameters obtained from ROC curve analysis of IL-28 and TNF-alpha

Characteristic	IL-28	TNF-alpha
Cutoff value	> 227.07 ng/ml	>63.18
Youden index J	0.410	0.700
AUC	0.712	0.853
95% CI	0.644 to 0.774	0.796 to 0.899
<i>p</i>	<0.001***	<0.001***
Sensitivity %	96.0	100.0
Specificity %	45.0	70.0
PPV %	61.5	76.9
NPV %	90.9	100.0
Accuracy %	68.0	85.0

AUC: area under curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value

The distribution of patients and control subjects based on the diagnostic potential of serum IL-28 is shown in table 4.6. Accordingly, 96 patients (48 %) were true positive, 40 control (20 %) were true negative, 4 patients (2 %) were false negative and 60 control (30 %) were false positive.

Table 4.6: The distribution of patients and control subjects based on the diagnostic potential of serum IL-28

IL-28	Patients group	Control Group	Total
Positive (> 227 ng/ml)	96/TP	60/FP	156
Negative ($\leq$ 227 ng/ml)	4/FN	40/TN	44
Total	100	100	200

TP: true positive; FP: false positive; FN: false negative; TN: true negative

The distribution of patients and control subjects based on the diagnostic potential of serum TNF-alpha is shown in table 4.7. Accordingly, 100 patients (50 %) were true positive, 70 control (35 %) were true negative, 0 patients (0 %) were false negative and 30 control (15 %) were false positive.

Table 4.7: The distribution of patients and control subjects based on the diagnostic potential of serum TNF-alpha

TNF-alpha	Patients group	Control Group	Total
Positive (> 63.18 ng/ml)	100/TP	30/FP	130
Negative ($\leq$ 63.18 ng/ml)	0/FN	70/TN	70
Total	100	100	200

TP: true positive; FP: false positive; FN: false negative; TN: true negative

Correlations of serum IL-28 and TNF-alpha levels to patients' characteristics are shown in table 4.8. There no significant correlations ($p > 0.05$).

Table 4.8: Correlations of serum IL-28 and TNF-alpha levels to patients' characteristics

Characteristic	IL-28 ng/ml		TNF ng/ml	
	<i>r</i>	<i>p</i>	<i>R</i>	<i>p</i>
Age (years)	0.067	0.509	-0.044	0.666
Sex (1 = male; 2 = female)	-0.030	0.701	0.083	0.314
DM (1= positive; 0 = negative)	0.100	0.325	-0.099	0.325
Hypertension (1 = positive; 0 = negative)	0.093	0.358	-0.024	0.810
Duration (years)	0.161	0.109	0.036	0.725
Treatment (1= yes; 0 = no)	-0.077	0.448	0.124	0.221

In 2002, two separate groups identified type III IFNs, sometimes known as IFN- λ s, of which IL-28B is a member. In terms of their signaling and biological activity, IFN- λ s and Type-1 IFNs share a similar mechanism, despite their structural differences. IFN- λ s have recently attracted a lot of attention due to their association with the spontaneous resolution and effective treatment of HCV infection, even though both types of IFNs are important for antiviral activity. Zhang *et al.* (2011). Our findings with respect to IL-28 are in accordance with findings of Dolganiuc *et al.* (2012). In addition, Diegelmann *et al.*, in 2010 also found that serum IL-28 is significantly higher in association with HCV infection. Thus, the high level of IL-28 linked to the body's immuneresponse to the virus, especially the production of (IFN- λ) which is produced by IL-28 gene, the increased levels of IL-28B and IFN- λ in HCV patient, particularly those with chronic infection suggest its role in the body's try to control or eliminates the virus, the high level of IL-28 association with HCV can be used as an aid in diagnosis of suspected cases and can be used to evaluate response to treatment.

In the contrary to our results, Hussein S. A, and Al-Azzawi (2020) found no significant difference in mean serum level of IL-28 between patients group and control group ($p > 0.05$). Some of local prior outcomes similar to the present study such as Yosur, (2018), Alazzawy, (2018), Al-Qahtani *et al.* (2015), and other universal studies like Lee *et al.* (2014), Dolganiuc *et al.* (2012) and Diegelmann *et al.* (2010).

According to a previous Arabic study by Al-Qahtani *et al.* (2015), serum levels of IL28B have a significant association with the various outcomes of HCV infection, especially among patients with cirrhosis and HCC, where serum levels of IL28 appear to increase with disease progression. However, the recent study showed a non-significant increase of IL-28 in patient groups, which may be due to the small

sample size. Additionally, Alazzawy (2018) found a highly significant correlation between IL-28_α and HCV clearance and that IL-28_α levels were elevated in PCR-negative Hepatitis C Virus patients. Furthermore, earlier studies in Iraqi patients by Yosur (2018) showed that patients with HCV infection had higher serum IL28_α levels (0.14 ± 0.005 pg/ml) than the control group (0.06 ± 0.002 pg/ml). According to research by Diegelmann et al. (2010) and Dolganiuc *et al.*, (2012), patients with chronic HCV infection had higher levels of IL-28 than did volunteers who appeared to be in good condition and patients who did not have viral liver disease. According to Lee *et al.*, (2014), HCV patients had higher levels of IL-28 transcription than controls.

In contrast, a previous study conducted in Iraq by Sara (2017) found that HCV patients' levels of IL-28_α were not substantially lower (145.578 ± 235.520 pg/ml) than those of the control group (235.418 ± 173.181 pg/ml). However, Alborzi *et al.* (2017) found that the amount of IL-28_α in HCV patients' serum is substantially lower than that in quality control. When comparing untreated HCV patients, recovered individuals, and healthy controls, another study found that the untreated group's levels of IL-28 and mRNA transcription were significantly lower than those of the clearance and healthy control groups (Shi *et al.*, 2012). In line with our results, Hussein S. A, and Al-Azzawi (2020) found no significant association between age or sex and serum IL-28.

With respect to TNF- α , Despite advances in medicine and technology, morbidity and mortality rates of CHC are high, especially in developing countries (Bilgiç *et al.*, 2015). In recent studies, it has been reported that the increase in proinflammatory cytokines such as TNF- α may be effective in the development of tumors and chronic inflammatory diseases (Zimmermann *et al.*, 2011). In chronic viral hepatitis cases, increased cytokine levels cause inflammation in the liver (Borekci *et al.*, 2014). Therefore, measuring cytokine levels provides useful information about

the activity and prognosis of the disease (Conde *et al.*, 2013). Tumor necrosis factor (TNF)- α is a pro-inflammatory and antiviral cytokine secreted by macrophages and cytotoxic T lymphocytes in the liver, regulating immune reaction, cell growth and apoptosis. Therefore, TNF- α expression is considered to be an important molecular link between liver inflammation, steatosis, and fibrosis (Bader *et al.*, 2016). The role of TNF- α in chronic HCV infection is not well understood. Serum levels of TNF- α is significantly higher in HCV-infected patients than in healthy subjects. Serum levels of TNF- α correlate with serum transaminase levels, histological activity and fibrosis (Pompili *et al.*, 2013).

Larrea *et al.*, in 1996 found that serum TNF- α levels were elevated in all chronic hepatitis C patients including those cases presenting sustained biochemical remission of the disease after interferon therapy. In contrast to our study, there was no significant difference in the mean serum level of TNF- α in HCV compared to control group (Che Noh *et al.*, 2022). TNF- α is an immune system modulator with a strong pro-inflammatory peripheral effect. It plays a crucial role in cell proliferation, differentiation, and apoptosis (Zelová & Hošek, 2013). TNF- α has been implicated in the pathogenesis of viral hepatitis and is associated with inflammation and fibrotic changes in hepatocytes (Moura *et al.*, 2009). A study carried out on 53 patients with chronic HCV infection in Florida found significantly higher serum levels of TNF- α than those of healthy controls (Nelson *et al.*, 1997). Also, our study was able to obtain a similar statistically significant result. In 1993, Zhang *et al.* found that serum TNF- α was higher in HCV in comparison with control group. Further discussion of IL-28 and TNF- α will be presented in the section of gene polymorphism.

4.5. Genetic Study

4.5.1. Diagnosis and Identification of Genetic Subtypes

Based on RT-PCR results, 100 patients were proved to be positive cases. Rate of genetic subtypes of HCV in patients' group were as following: 11 cases (34.4 %) were genotype 4 subtypes, 10 cases (31.3 %) were genotype 1a subtypes, 7 cases (21.9 %) were genotype 1b subtype and the rest of 4 cases (12.5 %) were genotype 3b subtype, as shown in figure (4.10) (4.11).

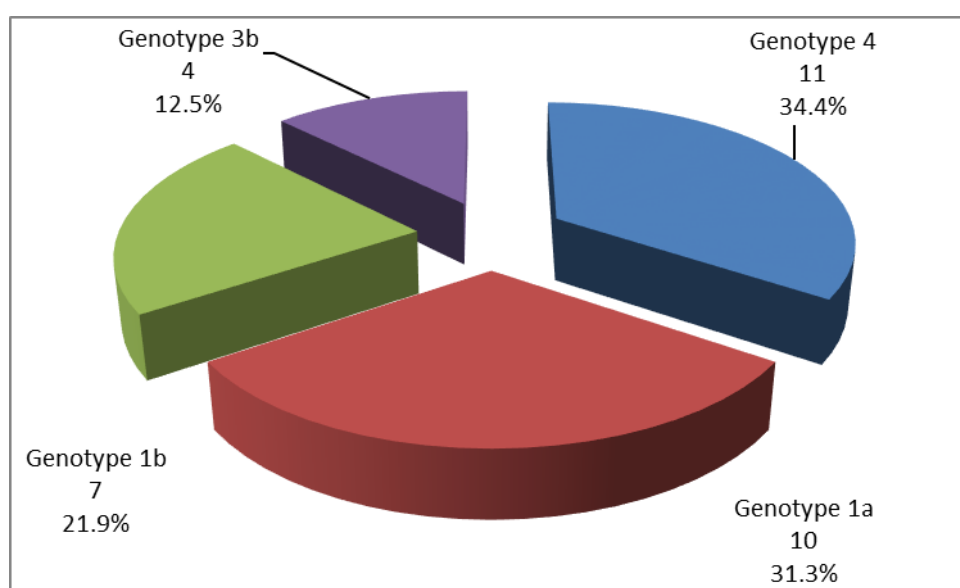


Figure (4.10): Rate of genetic subtypes of HCV in patients' group

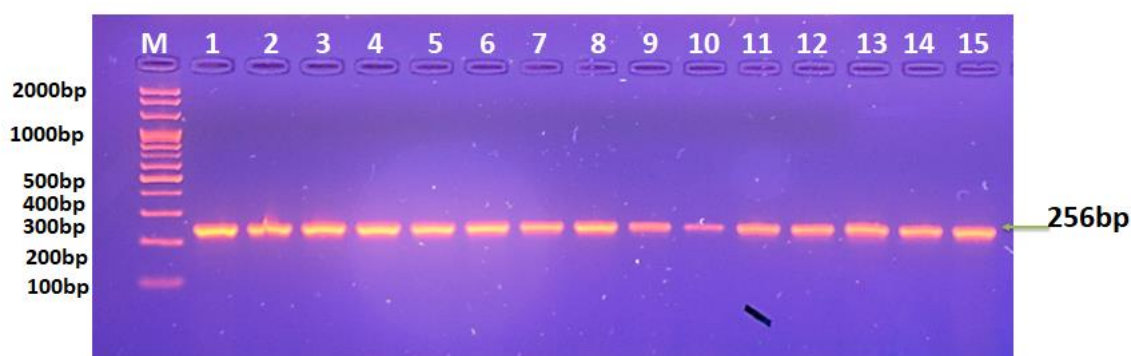


Figure (4.11): Agarose gel electrophoresis image that showed RT-PCR product analysis of 5' UTR region of HCV RNA genome from hepatitis infection patient. M (Marker ladder 2000-100bp). Lane (1-15) Showed only positive RT-PCR at (256bp) PCR product size.

When evaluating therapy choices and strategies, genotypes are an important consideration. It has been demonstrated that genotypes can affect the severity, prognosis, and response to treatment of a disease (Antaki et al. 2013; Wyles and Gutierrez 2014). The results of current included , rate of genetic subtypes of HCV in patients' group were as following: 11 cases (34.4 %) were genotype 4 subtypes, 10 cases (31.3 %) were genotype 1a subtypes, 7 cases (21.9 %) were genotype 1b subtype and the rest of 4 cases (12.5 %) were genotype 3b subtype. According to Ghaderi-Zefrehi et al. in 2016, in Iraq, the genotype 4 was the mostly detected one followed by genotype 1, 60% rate versus 26 % rate, respectively. Based on the study of Hussein et al., in 2021, the most common genotype in Iraqi individuals was 4 when contrasted other genotypes of HCV, as the genotype 4 was recorded in 76.92% (40/52).

According to Abdullah et al. in 2012, the following was obtained: genotype 1a was seen in 34.48%, genotype 1b was observed in 13.79%, genotype 3a was detected in 3.45%, while genotype 4 was detected in 41.38%; added to that, mixed infection was recognized in 2 patients having both genotypes 4 and 1b. Based on another Iraqi report from Duhok city, genotype 4 was recognized in 50 % of cases,

genotype 1 was seen in 43.3% and genotype 3 was seen in 6.7 % (Hussein et al., 2017). The study of Othman *et al.* (2014), revealed that the mostly recognized genotype was genotype 4 followed by 3a, 1b and 1a, 52.9%, 17.1%, 12.9%, and 1.4%, respectively, and that 7.1 % of cases had mixed infection with genotypes 4, 3a and 1b. The study of Khalid and Abdullah in 2012 showed that genotype 4 was isolated from 94 % of thalassemia patients, 85 % of chronic liver disease patients and from 80 % of blood donors individuals who are carriers of HCV; they also detected genotype 1b in 5%, 10 % and 10 % of same categories of patients and mixed infection by genotype 4 and 1b in 1%, and 5% from thalassemia category and chronic liver disease category, respectively; they also stated that 20 % of blood donor group had the subtype 1b. Furthermore, Al-Mola et al. in 2013 stated that genotype 4 was the most commonly recognized subtype (89.4%) followed by other subtypes including 1b, 2b, 3a and 6a. Al-Kubaisy et al. in 2006 reported that the most frequently detected subtypes were as following: 1b, 1a, 4 and 4 mixed with 3a. In the study of Kareem and Salih in 2014, genotype 1 was the most common (87.5%) followed by genotype 2 (8.3%) and lastly by genotype 4 (4.2%) in a sample of patients recruited from Sulaimani governorate. In the study of Niu et al ., 2016 , They discovered that, with 71.98 percent of the individuals affected, genotype 1 was the most common, followed by genotypes 2 (17.14 percent), 3, (5.25 percent), and 6 (3.22 percent). These patients did not have genotypes 4 or 5. According to two studies, genotype 4 was the most prevalent in Saudi Arabia, followed by genotypes 1b and 1a, while genotype 3a (40.96%), 3b, and 1a were the most common in Pakistan (Al Ashgar *et al.* 2013; Idrees et al. 2009).

4.5.2. Gene Polymorphism

Comparison of gene polymorphism of TNF- α -308 GA (rs1800629) SNP between patients group and control group is shown in table 4.9 and figure (4.12). GA genotype was a significant risk factor to disease with an odds ratio in the range of 2.67 to 3.11. AA genotype was not significantly associated with the disease ($p > 0.05$). GG genotype was significant protecting factor against the disease with an odds ratio of 0.40. In addition, allele A was significant risk factor (odds ratio = 1.94) and allele G was significant protecting factor (odds ratio = 0.52).

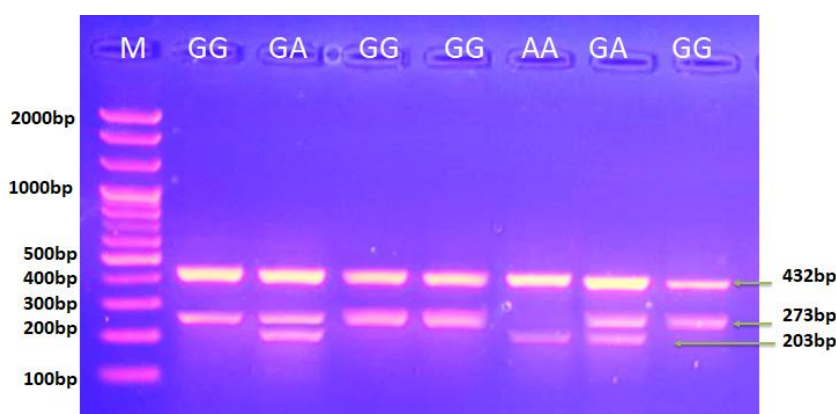


Figure (4.12): Agarose gel electrophoresis image that showed the T-ARMS-PCR product analysis of for TNF- α -308 G>A (rs1800629) gene polymorphism. Where M: marker (2000-100bp). The lane (GG) wild type homozygote were showed only G allele at 273bp T-ARMS-PCR product. The lane (AA) mutant type homozygote were showed only A allele at 203bp T-ARMS-PCR product, whereas the (GA) heterozygote were showed as both G and A allele at 273bp and 203bp T-ARMS-PCR product. The outer internal control were observed at 432bp T-ARMS-PCR product.

Table 4.9: Comparison of gene polymorphism of TNF-a-308 GA (rs1800629) SNP between patients group and control group

Mode	Genotypes or alleles	Patients Group Genotypes (n = 100) Alleles (n = 200)	Control Group Genotypes (n = 100) Alleles (n = 200)	<i>p</i>	OR	95% CI
Co-dominant	GG	48	70	Reference	Reference	Reference
	G/A	32	15	0.001 C ***	3.11	1.52 -6.36
	AA	20	15	0.085 C NS	1.94	0.91 -4.17
Dominant mode	GG	48	70	0.002 C **	0.40	0.22 -0.71
	G/A+AA	52	30	Reference	Reference	Reference
Recessive mode	AA	20	15	0.352 C NS	1.42	0.68 -2.96
	G/A+GG	80	85	Reference	Reference	Reference
Hyper-dominant mode	G/A	32	15	0.005 C **	2.67	1.34 -5.32
	GG+AA	68	85	Reference	Reference	Reference
Alleles	G	128	155	0.003 C **	0.52	0.33 -0.80
	A	72	45		1.94	1.25 -3.01

C: chi-square test; OR: odds ratio; CI: confidence intervals; NS: not significant; **: significant at $p \leq 0.01$; ***: significant at $p \leq 0.001$

Comparison of gene polymorphism of IL-28B CT (rs12979860) SNP between patients group and control group is shown in table 4.10 and figure (4.13). None of genotypes or alleles was significantly associated with the disease ($p > 0.05$).

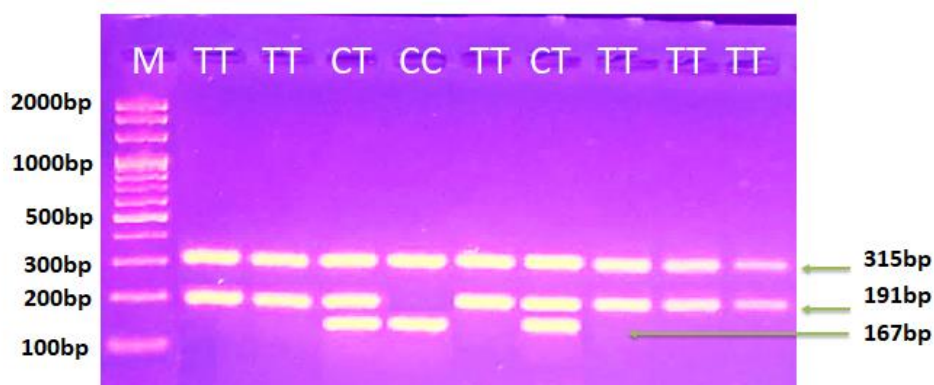


Figure (4.13): Agarose gel electrophoresis image that showed the T-ARMS-PCR product analysis of for IL-28B C>T (rs12979860) gene polymorphism. Where M: marker (2000-100bp). The lane (C) wild type homozygote were showed only C allele at 167bp T-ARMS-PCR product. The lane (TT) mutant type homozygote were showed only T allele at 191bp T-ARMS-PCR product , whereas the (CT) heterozygote were showed as both C and T allele at 167bp and 191bp T-ARMS-PCR product. The outer internal control were observed at 315bp T-ARMS-PCR product.

Table 4.10: Comparison of gene polymorphism of IL-28B CT (rs12979860) SNP between patients group and control group

Mode	Genotypes or alleles	Patients Group Genotypes (n = 100) Alleles (n = 200)	Control Group Genotypes (n = 100) Alleles (n = 200)	<i>p</i>	OR	95% CI
Co-dominant	CC	56	55	Reference	Reference	Reference
	C/T	36	30	0.598 C NS	1.18	0.64 -2.17
	TT	8	15	0.171 C NS	0.52	0.21 -1.33
Dominant mode	CC	56	55	0.887 C NS	1.04	0.60-1.82
	C/T+TT	44	45	Reference	Reference	Reference
Recessive mode	TT	8	15	0.121 C NS	0.49	0.20 -1.22
	C/T+CC	92	85	Reference	Reference	Reference
Hyper-dominant mode	C/T	36	30	0.367 C NS	1.31	0.73 -2.37
	CC+TT	64	70	Reference	Reference	Reference
Alleles	C	148	140	0.373 C NS	1.22	0.79 -1.89
	T	52	60		0.82	0.53 -1.27

C: chi-square test; OR: odds ratio; CI: confidence intervals; NS: not significant

Comparison of mean serum TNF-alpha based on TNF-a-308 GA (rs1800629) genotypes is shown in figure (4.14), comparison of mean serum IL-28 based IL-28B CT (rs12979860) genotypes is shown in figure (4.15).

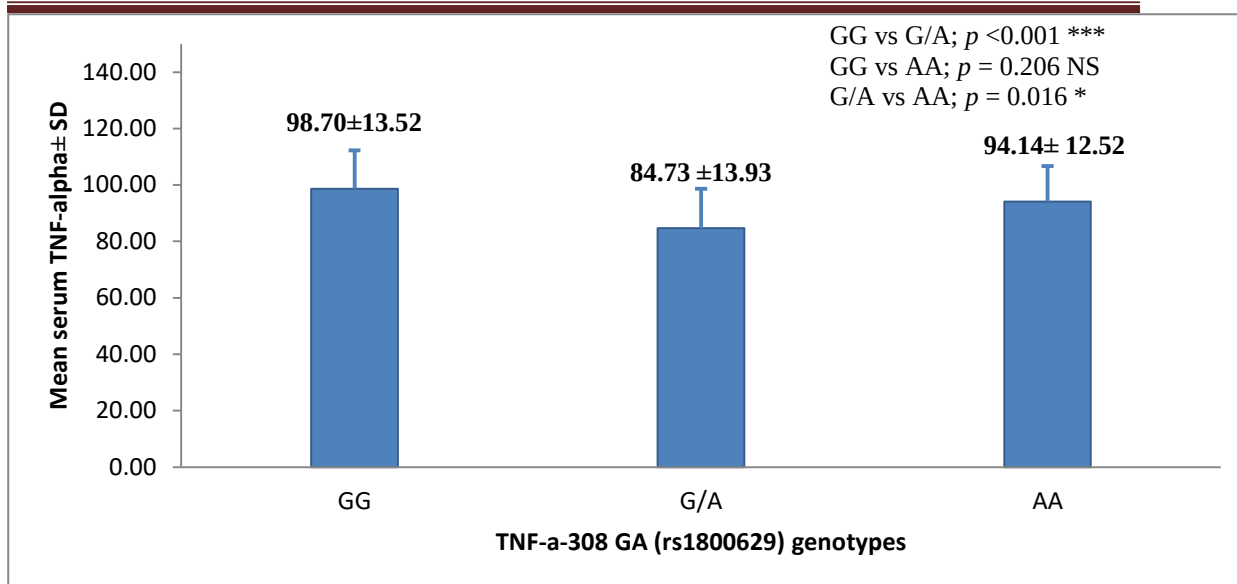


Figure (4.14): comparison of mean serum TNF-alpha based on TNF-a-308 GA (rs1800629) genotypes

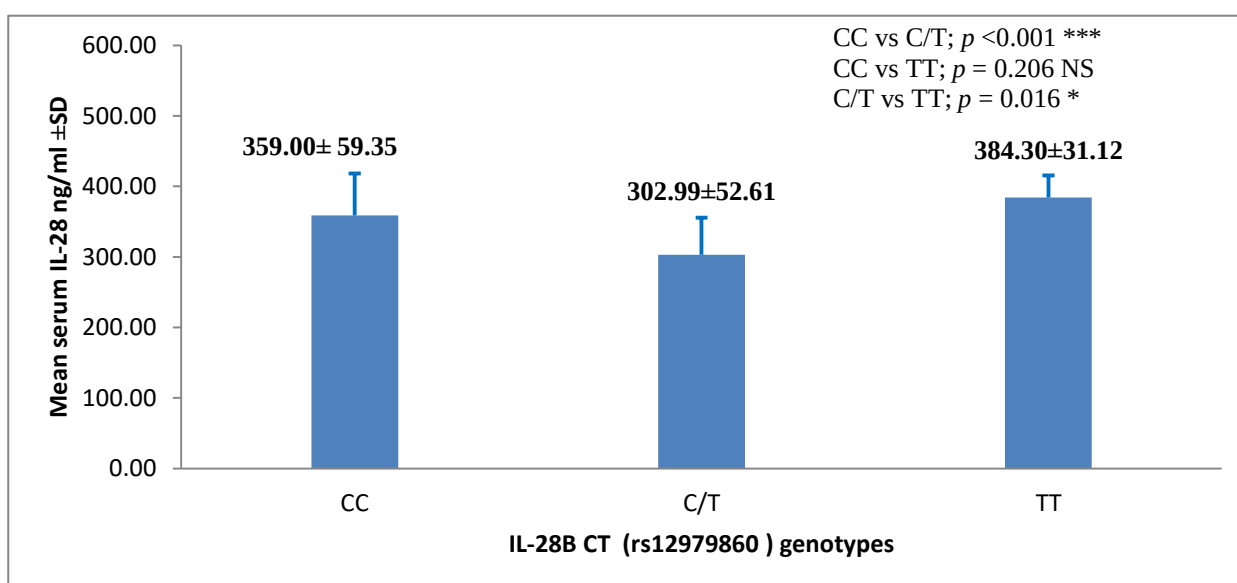


Figure (4.15): comparison of mean serum IL-28 based IL-28B CT (rs12979860) genotypes

In this study, it has been shown that polymorphism in genotypes for the *TNF* α -308G>A is correlated to susceptibility to infection with HCV in such a way that AA genotype and A allele are risk factors for development of disease and that allele G is protective against HCV infection. In addition, Heterozygous

combination of G/A was also a risk factor for HCV infection. Moreover, it has been shown that gene polymorphism of *TNFA* -308G>A is associated with significant variation in serum TNF-alpha level in those having with HCV (G/A and AA genotypes were associated with low serum level in comparison to GG genotype).

Up to 85% of cases of HCV, a hepatotropic virus, evade the immune system and cause acute and chronic necroinflammatory illness (Fallahi *et al.*, 2012). Numerous cytokines have two functions in viral infection: they cause tissue damage and virus removal (Guidotti *et al.*, 2001). In the immunological response, TNF- α is a crucial cytokine that mediates the process of inflammation through pathways of innate immune response and activates the response of cells, causing necrosis or programmed cell death (Holbrook *et al.*, 2019). Therefore, the development of HCV infection may be aided by genetic variants in the gene of TNF-alpha that change the amounts of production of cytokine.

Serum levels of the cytokine were shown to correlate with the genotype that is polymorphic for TNF α -308G>A variant in the current investigation; these results are consistent with earlier observations (Kroeger *et al.*, 1997). Current hypothesize that the homozygous variant allele (GG) may help improve immune regulation and stop the spread of HCV infection. Additionally, elevated TNF-alpha stimulates endothelial cells and macrophages and increases the production of vascular endothelial adhesion molecules, potentially improving infection clearance (Guidotti *et al.*, 2001).

Allele (A) is more frequent in patients with HCV suggesting a role in getting higher risk of acquiring this particular viral infection in addition o higher probability of having chronic long term inflammatory course of disease. These suggestion can be explained by the fact that under production of TNF- α by dendritic cells may

enhance the transformation of CD4⁺ T cells into non-IFN- γ -producing and IL-10-producing cells.

Performance of studies, in other ethnic categories, on polymorphism of the *TNFA* -308G>A revealed conflicting results (Santiago *et al.*, 2021; Bouzgarrou *et al.*, 2010, Dogra *et al.*, 2011; Santos *et al.*, 2010). Some authors denied existence of correlation between HCV infection suitability and *TNFA* -308G>A polymorphism (Santiago *et al.*, 2021; Bouzgarrou *et al.*, 2010). Other authors proved such association (Dogra *et al.*, 2011; Santos *et al.*, 2010). Thus, it seems that consensus about the effect of polymorphism of *TNFA* -308G>A in contribution to HCV infection susceptibility is need for future further research work. Additionally, the methods employed to genotype the polymorphisms may have varied, which could account for the discrepancies in the results. Neither the fibrosis score nor varying degrees of necroinflammatory activity were linked to the *TNFA*-308G>A SNPs. Numerous investigations have also failed to find any connection between these TNF gene polymorphisms and the various phases of the disease (Talaat *et al.*, 2012; Gao *et al.*, 2009; Bouzgarrou *et al.*, 2010; He *et al.*, 2011, Ghoneim, 2019). This is likely because the host genetic profile is not the sole factor contributing to the disease's progression.

The importance of evaluating the association between *TNFA* -308G>A polymorphism and degree of hepatic involvement in those afflicted by HCV relies on presence of a substantial proportion of subjects with HCV infection are going to develop hepatocellular carcinoma in the future, thus identifying patients with such gene polymorphism may lead to more close follow up, early detection of malignant transformation and early intervention which may reduce risk of mortality in such cohort of patients (Aroucha *et al.*, 2016). Aroucha *et al.*, (2016) conducted a study to assess for correlation across risk of developing liver malignancy in patients with HCV and the gene polymorphism of *TNFA* -308G>A; they concluded significant

association between risk of cancer development and such gene polymorphism. The primary cause of Liver malignancy (HCC) is the HCV. The more severe the hepatic fibrosis and liver inflammation, the higher the chance of developing HCC. From clinical perspective, HCV disease course and the likelihood of developing HCC are thought to be determined by the equilibrium across the production of anti- and pro-inflammatory classes of cytokines (Aroucha *et al.*, 2016).

Nevertheless, several previous reports have been published dealing with lack of association between severity of inflammation and fibrosis of hepatic tissue in patients with HCV infection and the TNFA -308G>A (Bouzgarrou *et al.*, 2010, Larijani *et al.*, 2016).

In this study, it has been shown that polymorphic genotype for the IL-28B CT (rs12979860) is not associated with susceptibility to HCV infection. However, we were able to show that such gene polymorphism has significant impact on serum level of IL-28B CT in such a way that heterozygous pattern is associated with the least production of this IFN. In a previous Iraqi report concerning the association between IL-28B CT (rs12979860) SNP and HCV infection, 67 patients with HCV and 53 control subjects were evaluated and the results showed that genotype CC was less frequently detected in patients group compared to control group. Indeed, our results revealed no such an association despite enrolling larger sample size of 100 patients and 100 control subjects. Another study shown that genotype TT is the more frequent genotype in patients with HCV (EL-Awady *et al.*, 2012) and this finding is contrary with our observation since we reported TT genotype in 8 patients only.

Additionally, the distribution of CT and CC genotypes in IL-28B rs12979860 SNPs was higher than other types in patients with hepatitis C and healthy individuals, respectively, according to reports of IL-28B rs12979860 genotypes and

alleles in Iranian patients. There were no statistically significant variations in the distribution of IL-28B genotypes between rs12979860-related hepatitis C patients and healthy persons. In IL-28B rs12979860 SNPs, the C allele was more widely distributed than the T allele in hepatitis C patients and healthy people, respectively. There were statistically significant differences in the distribution of IL-28B rs12979860 alleles between hepatitis C patients and healthy people (Mousavi Nasab *et al.*, 2015). Thus, current results are in agreement with the latter Iranian study with respect to lack of significant association between genotypes of IL-28B rs12979860 and susceptibility to infection with HCV.

The finding of the present study that genotype variation of IL-28B rs12979860 has an impact on serum level of IL-28B is an important finding that may explain partly the variation in immune response against HCV and viral clearance in various patients. It can be suggested that patients with CT genotype may be more liable for persistent infection and development of HCV complications at a higher rate in comparison with patients having other genotypes probably because of under production of IL-28B. (Jiménez *et al.*, 2013) found that rs12979860 C/C genotype is associated with SVR in Caucasian, Asian, European, Hispanic and African-American populations.

Up to 85% of cases of HCV, a hepatotropic virus, evade the immune system and cause acute and chronic necroinflammatory illness (Lampertico *et al.*, 2013). Numerous cytokines have two functions in viral infection: they cause tissue damage and virus removal (Rauch *et al.*, 2010).

4.5.3. The Correlation of HCV Genotypes to other Characteristics of Enrolled Patients

The association of HCV genotypes to age groups and to sex proportions of patients is shown in table 4.11. There was significant association with age ($p = 0.003$) in such a way that age interval 41-60 was mainly involved with genotype 1a,

3a and 4 (64.5 %, 50 % and 61.8 %) and age interval 20-40 was mainly involved by genotype 1b (57.1 %). There was also significant association with sex ($p = 0.006$); males were mainly affected by genotypes 1a, 3a and 4; whereas, females mainly have genotype 1b.

Table 4.11: The association of HCV genotypes to age groups and to sex proportions of patients

Characteristic	Genotype 1a <i>n</i> = 31	Genotype 1b <i>n</i> = 21	Genotype 3a <i>n</i> = 14	Genotype 4 <i>n</i> = 34	<i>p</i>
Age (years)					
20-40, n (%)	3 (9.7 %)	12 (57.1 %)	6 (42.9 %)	7 (20.6 %)	0.003 C **
41-60, n (%)	20 (64.5 %)	4 (19.0 %)	7 (50.0 %)	21 (61.8 %)	
> 60, n (%)	8 (25.8 %)	5 (23.8 %)	1 (7.1 %)	6 (17.6 %)	
Sex					
Male, n (%)	23 (74.2 %)	7 (33.3 %)	8 (57.1 %)	26 (76.5 %)	0.006 C **
Female, n (%)	8 (25.8 %)	14 (66.7 %)	6 (42.9 %)	8 (23.5 %)	

C: chi-square test; **: significant at $p \leq 0.01$

The variation in age prevalence of various genotypes may be due to variation to immune response in relation to particular HCV genotype. The association of HCV genotypes to diabetes mellitus and to hypertension is shown in table 4.12. Statistical analysis revealed significant association with DM in such a way that genotype 4 has the highest rate (29.4 %) followed by genotype 1a (19.4 %) and then by genotype 1b (4.8 %) and lastly by genotype 3a; however, there was no significant association with hypertension ($p = 0.172$).

HCV genotype 1 was the most common (73.39%) in the study by Niu et al. (2016), followed by genotypes 2 (17.14%), 3 (5.25%), and 6 (3.22%). These patients did not have genotypes 4 or 5. Subtype 1b was the most common (71.98%), followed by genotypes 2a (17.14%). Five patients had a combination of HCV subtype infections. In both male (73.27%) and female (73.47%) patients, genotype 1 was the most prevalent of all the genotypes, followed by genotype 2.

Male patients were more likely than female patients to have genotype 1 (male: 29.84 % of 496, vs female: 43.55 % of 496, $\chi^2 = 20.07$, $P < 0.0001$), genotype 2 (male: 6.25 % of 496, vs female: 10.89 % of 496, $\chi^2 = 6.81$, $P = 0.009$), and 6 (male: 1.41 % of 496, vs female: 1.81 % of 496, $\chi^2 = 0.626$, $P = 0.401$).

In the 50–59 age range Male patients were most likely to have genotype 1 (29.05 percent of 148), followed by those aged 20 to 29 years (23.65 percent of 148); those aged 60 to 69 years (12 cases of 31); those aged 50 to 59 years (4 cases of 13); and those aged 30 to 39 years (4 cases of 7) were most likely to have genotype 6. Before the age of 60, female patients had a considerably greater frequency of HCV prevalence than male patients; however, after that age, the opposite effect was seen. Subtype 1b was the most prevalent HCV genotype in WuHan, followed by subtype 2a, and it was more prevalent in female patients than male patients.

While HCV genotype 4 has been commonly detected in females, the frequency of HCV genotype 1 was found to be strongly related with males (Brahim *et al.*, 2012). According to Rouabhia *et al.* (2013), there is no difference between HCV genotypes and gender because the ratio of each HCV genotype in males and females is the same. Female gender was with negative association with type 3 genotype and female with relative risk to infection with type 4 genotype 11 times to infection with type 3 genotype (Koura, 2022). According to Bakir *et al.* in 2023, genotype distribution was not significantly different among various age groups ($P = 0.067$).

Based on observation of (Martinez-Gonzalez *et al.*, 2022)HCV genotype distribution differed significantly ($p < 0.001$) between genders, with genotype 1 being more frequent in females, whereas genotype 3 was more common in males. The highest percentage of HCV infections was at the age-group 40-59 (44.8% of 6,097) and the lowest one at the age-group < 18 (0.6% of 6,097). Genotype 3 was highly represented in younger patients (19-39 age- group), while genotype 1 was

more common in older ones (> 60 age-group).

Table 4.12: The association of HCV genotypes to diabetes mellitus and to hypertension

Characteristic	Genotype 1a <i>n</i> = 31	Genotype 1b <i>n</i> = 21	Genotype 3a <i>n</i> = 14	Genotype 4 <i>n</i> = 34	<i>p</i>
DM					
Positive, <i>n</i> (%)	6 (19.4 %)	1 (4.8 %)	0 (0.0 %)	10 (29.4 %)	0.030 C *
Negative, <i>n</i> (%)	25 (80.6 %)	20 (95.2 %)	14 (100.0 %)	24 (70.6 %)	
HT					
Positive, <i>n</i> (%)	6 (19.4 %)	0 (0.0 %)	1 (7.1 %)	5 (15.6 %)	0.172 C NS
Negative, <i>n</i> (%)	25 (80.6 %)	21 (100.0 %)	13 (92.9 %)	29 (84.4 %)	

DM: diabetes mellitus; **HT:** hypertension; **C:** chi-square test; **NS:** not significant; *: significant at $p \leq 0.05$

The association between DM and various genotypes may reflect affection of immune system in diabetes mellitus in such a way that certain HCV genotype may have higher opportunity to infect diabetic patients than others. The association of HCV genotypes to treatment and to duration of disease is shown in table 4.13 and statistical analysis revealed no significant association ($p = 0.791$ and 0.054 , respectively).

According to (Farshadpour et al., 2018) of the 11 HCV-seropositive diabetic patients, 10 (91%) had HCV viremia with genotype 3a. In addition, the effect of some specific HCV genotypes on the development of type 2 DM should also be considered. Some previous studies have reported high frequencies of genotypes 1 and 4 in diabetic patients (Moucari et al., 2008; Chehadeh et al., 2011).

According to Rajewski et al., (2022), patients infected by HCV genotype 1 or 4 suffered from hypertension more often than patients with HCV genotype 3. They stated that the relevance between HCV genotype and the incidence of hypertension and cardiovascular diseases has not been proved. Low number of patients with an HCV genotype other than type 1 participating in the study, resulting from the population genotype distribution, may have contributed to the above mentioned

fact.

Table 4.13: The association of HCV genotypes to treatment and to duration of disease

Characteristic	Genotype 1a <i>n</i> = 31	Genotype 1b <i>n</i> = 21	Genotype 3a <i>n</i> = 14	Genotype 4 <i>n</i> = 34	<i>p</i>
Treatment					
Yes, <i>n</i> (%)	18 (58.1 %)	10 (47.6 %)	9 (64.3 %)	19 (55.9 %)	0.791 C
No, <i>n</i> (%)	13 (41.9 %)	11 (52.4 %)	5 (35.7 %)	15 (44.1 %)	NS
Duration (years)					
Median (IQR)	5 (3)	4 (3.83)	2.5 (3.75)	3 (2.25 %)	0.054 K NS

C: chi-square test; K: Kruskal Wallis test; NS: not significant

Different hepatitis C genotypes respond differently to treatment, according to numerous research. It has been proposed that individuals with HCV genotypes 1b are less likely than those with genotypes 2 or 3 to respond favorably to interferon therapy. After six months of interferon treatment, Zein et al. (1996) found that 60 to 70% of patients with HCV genotype 2 and 10 to 15% of those with genotype 1 had a full biochemical response.

Both the pretreatment HCV RNA levels and liver histologic characteristics had no bearing on this differential, which was also seen for sustained response. Given that HCV genotype 2 is more common in Europe than in the US or Japan, this could help to explain some of the greater rates of long-term response to interferon treatment that have been documented there. Although there was a difference, the non-1 HCV genotype had a modest predictive value for response to therapy (58% for response at the end of treatment and 55% for sustained response), according to a recent meta-analysis of the most pertinent trials (Davis and Lau, 1997). The relevance of prognostic indicators for response to therapy and the

differential response of HCV genotypes may become less evident with the development of more potent medications, such as the combination of interferon and ribavirin (Davis and Lau, 1997).

Patients with HCV genotypes other than 1 had higher rates of sustained response to therapy in more recent treatment trials employing interferon plus ribavirin in patients with chronic HCV who were interferon-naïve (McHutchison *et al.*, 1998) or in patients whose prior interferon treatment had failed (Davis *et al.*, 1998). According to McHutchison *et al.* (1998), patients with HCV genotype 1 needed 48 weeks of treatment to get a response comparable to those of patients infected with other genotypes who received 24 weeks of treatment.

According to a different study, genotypes 1a and 1b tended to have more severe liver disease and a weaker response to interferon therapy (Wyles and Gutierrez 2014). Patients with genotype 1 infection benefit greatly from an IFN plus ribavirin combination therapy (SVR more than 90%). However, according to Zuberi *et al.*, genotypes 2 and 3 respond poorly to SVR therapy, with corresponding responses of 27.8% and 58.8% (Robinson *et al.*, 2015). Other research revealed that whereas genotypes 2 and 3 exhibited mixed responses to the combined therapy of IFN with Ribavirin, genotypes 1 and 4 reacted favorably. Additionally, during 48 weeks, genotypes 5 and 6 respond appropriately to ribavirin and peg-IFN (Cartwright *et al.*, 2013).

Table 4.12 displays the correlation between HCV genotypes and TNF-alpha and IL-28 gene polymorphisms. Statistical analysis showed no significant correlation ($p = 0.198$ and 0.198 , respectively). The most promising host-related predictors of treatment-induced clearance in HCV genotype-1 patients, according to recent studies, are SNPs within or adjacent to IL28B (Asselah *et al.*, 2010; Rauch *et al.*, 2010). In contrast, the relationship between IL28B genotype and outcome is less clear in studies of people infected with genotypes 2 and 3, including rs12979860 with treatment-induced clearance (Moghaddam *et al.*, 2011; Mangia *et al.*, 2010).

There is currently little evidence of how genetic IL28B variability affects viral removal during treatment in G4. As with HCV genotype-1, we showed in this study that SVR in patients with HCV G4 was strongly influenced by CC genotypes of rs12979860. Furthermore, sensitivity to PEG-IFN/ribavirin therapy was three times higher in hosts with at least one copy of the C allele than in hosts with rs12979860-negative status. The host innate immune response may be the mechanism and reason for the correlation between spontaneous clearance and genetic differences in the IL28B gene. IFN- λ 3, which is involved in viral control, particularly HCV, is encoded by IL28B. IFN- α stimulates the production of IFN- λ genes in vitro, which prevents HCV from replicating (Marcello *et al.*, 2006).

Regarding the relationship between genetic polymorphisms and treatment response, Asselah *et al.* (2012) found that SNP rs12979860 was highly correlated with SVR in 164 HCV-G4 patients from various ethnic backgrounds.

Table 4.14: The Association of HCV Genotypes to TNF-alpha and IL-28 gene Polymorphism

Characteristic	Genotype 1a <i>n</i> = 31	Genotype 1b <i>n</i> = 21	Genotype 3a <i>n</i> = 14	Genotype 4 <i>n</i> = 34	<i>P</i>
TNF-a-308 GA (rs1800629)					
GG	13 (41.9 %)	12 (57.1 %)	7 (50.0 %)	16 (47.1 %)	0.198 C NS
G/A	11 (35.5 %)	2 (9.5 %)	6 (42.9 %)	13 (38.2 %)	
AA	7 (22.6 %)	7 (33.3 %)	1 (7.1 %)	5 (14.7 %)	
IL-28B CT (rs12979860)					
CC	14 (45.2 %)	9 (42.9 %)	11 (78.6 %)	22 (64.7 %)	0.198 C NS
C/T	15 (48.4 %)	9 (42.9 %)	3 (21.4 %)	9 (26.5 %)	
TT	2 (6.5 %)	3 (14.3 %)	0 (0.0 %)	3 (8.8 %)	

C: chi-square test; NS: Not significant

During our current study, we did not find a significant relationship between genotypes for virus and genotypes for interleukins. While other studies have been conducted by (Pineda *et al.*, 2010) (Pedergnana *et al.*, 2012) are found HCV-positive patients with genotype 1 and 4 revealed that the virus spontaneous clearance was higher in patients with CC genotype of rs12979860 than other rs12979860 genotypes. Also (Nasab *et al.*, 2015) rs12979860CC is associated with clearance of HCV genotype 3a. The frequency of rs12979860 CC in patients who were infected with HCV genotypes 3a and those infected with HCV genotypes 1a is equal and higher than other genotypes. This finding does not support the previous researches demonstrating the higher frequency of CC genotype in patients infected with HCV genotypes 3a than genotypes 1 (Nasab *et al.*, 2015) (McCarthy *et al.*, 2010). Also (Aalaei-Andabili, 2014) found, there were significant linkages between HCV genotypes and IL28B genotypes/alleles. A study conducted by (

Santos *et al.*, 2015), In this study it was not possible to observe an association between IL-28B genotype and HCV geno- types. However, the frequency of IL-28B genotypes according to the HCV genotypes is not in agreement with different reports.

The association of HCV genotypes to IL-28 and TNF-alpha levels is shown in table 4.15 and statistical analysis revealed no significant association ($p = 0.137$ and 0.496 , respectively).

Table 4.15: The Association of HCV Genotypes to IL-28 and TNF-Alpha Levels

Characteristic	Genotype 1a <i>n</i> = 31	Genotype 1b <i>n</i> = 21	Genotype 3a <i>n</i> = 14	Genotype 4 <i>n</i> = 34	<i>P</i>
IL-28 ng/ml					
Mean $\pm$ SD	347.77 $\pm$ 56.82	321.73 $\pm$ 49.89	321.15 $\pm$ 78.43	354.51 $\pm$ 62.34	0.137 O NS
TNF ng/ml					
Mean $\pm$ SD	93.39 $\pm$ 14.98	97.33 $\pm$ 14.05	90.36 $\pm$ 15.56	91.98 $\pm$ 14.57	0.496 O NS

SD: standard deviation; O: one way ANOVA; NS: not significant

Current results show no association between HCV genotypes or subgenotypes to IL-28 and TNF-alpha levels. This is in line with recent research that looked at the relationship between HCV genotypes and serum cytokine levels (Akbar *et al.*, 2011; Zhang *et al.*, 2011; Al-Qahtani *et al.*, 2015), These investigations showed no association between serum levels of cytokines and HCV genotypes.

Conclusions and Recommendation

Conclusions and Recommendation

Conclusions

1. The HCV genotype with highest frequency in Diwaniyah province is genotype-4 followed by genotype 1a, followed by genotypes 1b and lastly by genotype 3b.
2. TNF- α) -308G>A polymorphism: the AA genotype and A allele are risk factors for development of disease and that allele G is protective against HCV infection.
3. Gene polymorphism of IL-28B CT (rs12979860) has no significant impact on susceptibility of infection with HCV; however, heterozygous pattern was associated with reduced serum level of IL-28B
4. Mean serum TNF-alpha was higher significantly in patients group compared to controlgroup
5. Mean serum TNF-alpha was higher significantly in patients group compared to controlgroup
6. Lack of significant impact of virus genotypes and or sub-genotypes on serum values of IL-28B. No significant association was found when genotypes for interleukines were compared to virus genotypes/subgenotypes.

Conclusions and Recommendation

Recommendation

1. Evaluation of correlations of HCV genotypes to degree of liver disease, stage of fibrosis and cirrhosis.
2. Study of contribution of HCV genotypes to response to therapy against HCV in pediatric and in adult age groups.
3. Assessment of IL-28B and TNF-alpha gene polymorphism in association with degree of liver fibrosis, ultrasound changes, cirrhosis and carcinogenic pathology.
4. Contamination should be avoided during blood collection, transportation, and preparation of blood products; psychological counseling and safety education should be provided to intravenous drug users to persuade them to quit drugs; disposable syringes and needles should be used; dental instruments, endoscopes and other medical instruments should be strictly disinfected.
5. Correlation between HCV infections and SNPs of the IL-28B gene variants such as rs8099917, it can play a significant role in HCV treatment.

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A

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الخلاصة

الخلاصة

هدفت هذه الدراسة إلى تحديد انتشار الأنماط الجينية المختلفة لفيروس التهاب الكبد الوبائي سي، وتقييم العلاقة بين قابلية الإصابة بعدوى فيروس التهاب الكبد الوبائي سي وتعدد أشكال الجينات المرتبط بعامل نخر الورم ألفا-308. G>A. كما هدفت إلى تقييم العلاقة بين قابلية الإصابة بعدوى فيروس التهاب الكبد الوبائي سي وتعدد أشكال الجينات المرتبط بصبغة IL-28B CT (rs12979860).

اختير المرضى المسجلون من مجموعة المرضى الذين يزورون وحدة أمراض الجهاز الهضمي ووحدة غسيل الكلى في مستشفى الديوانية التعليمي، مدينة الديوانية، العراق. تم تسجيل عدد متساوٍ من الأفراد الأصحاء ظاهرياً، من أجل تحديد شدة حالتهم كمجموعة ضابطة. ضمت مجموعة المرضى 100 مريض تم تشخيص إصابتهم بعدوى فيروس التهاب الكبد الوبائي سي بناءً على إيجابية اختبار الحمض النووي الريبوزي لفيروس التهاب الكبد الوبائي سي في الدم، بالإضافة إلى اختلالات في إنزيمات الكبد. تم الحصول على خمسة مل من الدم الوريدي من الوريد المرفقي، وتم إجراء تسلسل الحمض النووي. كانت نسبة الأنواع الفرعية الجينية لفيروس التهاب الكبد الوبائي سي في مجموعة المرضى كما يلي: 11 حالة (34.4%) كانت من النمط الجيني 4، و 10 حالات (31.3%) كانت من النمط الجيني 1، و 7 حالات (21.9%) كانت من النمط الجيني 1ب، وبقية الحالات الأربع (12.5%) كانت من النمط الجيني 3ب. كان النمط الجيني GA عامل خطر مهم للإصابة بالمرض بنسبة احتمالات تتراوح بين 2.67 و 3.11. لم يكن النمط الجيني AA مرتبطاً بشكل كبير بالمرض. ($p > 0.05$) كان النمط الجيني GG عامل حماية مهم ضد المرض بنسبة احتمالات بلغت 0.40. بالإضافة إلى ذلك، كان الأليل A عامل خطر مهم (نسبة الأرجحية = 1.94) والأليل G عامل حماية مهم (نسبة الأرجحية = 0.52). يرتبط تعدد أشكال جين TNFA-308G>A بتباين كبير في مستوى TNF-alpha في مصل المرضى المصابين بعدوى فيروس التهاب الكبد C ارتبطت الأنماط الجينية G/A و AA بانخفاض مستوى المصل مقارنةً بالنمط الجيني GG. أظهرت مقارنة تعدد أشكال جين SNP لـ IL-28B CT (rs12979860) بين مجموعتي المرضى والمجموعة الضابطة عدم وجود ارتباط كبير بين أي من الأنماط الجينية أو الأليلات والمرض. ($p > 0.05$) كانت نتائج مقارنة متوسط مصل IL-28B على أساس النمط الجيني (rs12979860) IL-28B CT على النحو التالي: أظهر النمط الجيني CT مستوى أقل بكثير من IL-28B في المصل بالمقارنة مع النمط الجيني CC ($p < 0.001$) والنمط الجيني TT. مقارنة مستويات IL-28 بين مجموعتي المرضى والمجموعة الضابطة، كان متوسط IL-28 في المصل أعلى بشكل ملحوظ في مجموعة المرضى مقارنةً بالمجموعة الضابطة، حيث بلغ 61.61 ± 340.86 نانوغرام/مل مقابل 91.68 ± 275.18 نانوغرام/مل، على التوالي ($p < 0.001$). كان متوسط TNF-alpha في المصل أعلى بشكل ملحوظ في مجموعة المرضى مقارنةً بالمجموعة الضابطة، حيث بلغ 14.69 ± 93.32 نانوغرام/مل مقابل 22.62 ± 62.24 نانوغرام/مل، على التوالي ($p < 0.001$). وقد أشرنا إلى عدم وجود تأثير كبير للأنماط الجينية للفيروس أو الأنماط الجينية الفرعية على قيم IL-28B في المصل. ولم يُعثر على أي ارتباط ذي دلالة إحصائية عند مقارنة الأنماط الجينية للإنترلوكينات بالأنماط الجينية/الأنماط الجينية الفرعية للفيروس.

الاستنتاج: الأنماط الجينية لفيروس التهاب الكبد الوبائي سي هي: 4، 1أ، 1ب، و3ب. تعدد الأشكال: النمط الجيني AA والأليل A

الخلاصة

لـ $TNF-\alpha-308G>A$ هما عاملان خطر، بينما الأليل G وقائي. تعدد أشكال جين $IL-28B$ CT (rs12979860) ليس له تأثير يُذكر.



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
كلية العلوم قسم علوم الحياة

علاقة تعدد أشكال جينات السيتوكينات لدى مرضى التهاب الكبد الفيروسي C في محافظة الديوانية

اطروحة مقدمة إلى

مجلس كلية العلوم – جامعة القادسية
وهي جزء من متطلبات نيل شهادة الدكتوراه في علوم الحياة

من قبل

نجوان عبد الكريم حبيب

بكالوريوس علوم في علوم الحياة 2008
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