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# Investigating circulatory microRNA expression profiles in Egyptian patients with HCV induced hepatocellular carcinoma

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THE AMERICAN UNIVERSITY IN CAIRO
الجامعة الأمريكية بالقاهرة

School of Sciences and Engineering

# Investigating circulatory microRNA expression profiles in Egyptian patients with HCV induced hepatocellular carcinoma

A Thesis Submitted to
The Chemistry Master's Program
In partial fulfillment of the requirements for
The degree of Master of Science
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# ABSTRACT

**The American University in Cairo**

## Investigating circulatory microRNA expression profiles for Egyptian patients affected with HCV mediated hepatocellular carcinoma.

By: **Amany Mohamed Mohamed Mahmoud Al-Anany**

Under the supervision of: **Prof. Dr. Hassan M.E. Azzazy**

Hepatitis C virus (HCV) infection is a serious health challenge affecting over 185 million individuals globally. Egypt has the highest rate for chronic HCV infection worldwide. Recent studies have shown that from 10%-30% of HCV infected individuals would progress to more deteriorating conditions such as cirrhosis and/or hepatocellular carcinoma (HCC). Current biomarkers for HCC diagnosis are lacking both sensitivity and specificity. As a result there is demand to develop reliable biomarkers that are sensitive, specific and non-invasive for early diagnosis and rapid intervention of HCC to increase the survival rate of patients. Recently, circulatory mi-RNAs have drawn great attention as promising non-invasive biomarkers for various diseases. They are highly stable in blood and their expression profiles reflect disease progression and/or drug response status. Besides, a number of miRNAs were found to be commonly dysregulated in HCC.

In this study, we investigated mi-RNA expression profile from the plasma of Egyptian patients with chronic hepatitis C, cirrhosis and HCV associated HCC compared with healthy control. We aimed to assess the diagnostic potential of the selected miRNAs to differentiate between healthy, HCV and HCV associated disorders (cirrhosis and HCC). In addition to studying the expression pattern in the different studied stages.

Using real time PCR, we compared the levels of circulating mir-122, miR-21 and miR-155 in plasma from healthy control (n=40), cirrhosis (n=39) and HCC (n=40) to CHC (n=37). Synthetic miR-39 was spiked in the samples to be used as a normalizing control for the samples. Plasma miR-122 was significantly up-regulated

in HCC than in the three other categories (P<0.001). The plasma level of miR-122 in HCC patients was significantly higher than healthy control and cirrhotic patients (P = 0.002 and P<0.001, respectively). The plasma level of miR-21 was significantly up-regulated in HCC compared to cirrhotic patients (P=0.03). Although Control and HCC in mir-21 increased by 3.52 and 6 log2 scale respectively, they were in marginal insignificance (*p*=0.093) that might be due to limited number of samples used in this study. There had been no significant dys-regulation in the expression levels of miR-155 between the 4 categories.

Receiver operating characteristic curves' analysis revealed that miR-122 differentiated HCC patients from healthy control with a specificity and sensitivity 57.50% and 65.52% respectively (at a cutoff >1.86). To discriminate cirrhotic patients from HCC subjects, a specificity and sensitivity of 61.54% and 1.43% (at a cutoff> 0.43) were determined. Additionally, analyzing ROC curve indicated that miR-21 differentiated HCC patients from cirrhotic patients with best sensitivity and specificity 72.97% and 50% (at a cutoff >-0.43).

In conclusion, plasma miR-122 and miR-21 may be further investigated as potential markers for HCV associated HCC.

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| | |
|---|---|
| **AFP** | Alfa feto protein |
| **ALP** | Alkaline phosphatase |
| **ALT** | Alanine amino transferase |
| **APC** | Antigen presenting cells |
| **Apo** | Apolipoprotein |
| **AST** | Aspartate amino transferase |
| **CD81** | Cluster of differentiation 81 |
| **CHC** | Chronic hepatitis C |
| **CLDN1** | Claudin 1 |
| **ECM** | Extracellular matrix |
| **ER** | Endoplasmic reticulum |
| **HCC** | Hepatocellular carcinoma |
| **HCV** | Hepatitis C virus |
| **HIV** | Human Immunodeficiency virus |
| **IFN** | Interferon |
| **IRES** | Internal ribosomal entry site |
| **LDLR** | lipoprotein receptor |
| **LDs** | Lipid droplets |
| **mi-RNA** | MicroRNAs |
| **MVBs** | Multivesicular bodies |
| **PBMC** | Peripheral blood mono-nuclear cells |
| **PCR** | Polymerase chain reaction |
| **ROC** | Receiver operator characteristic |
| **SVR** | Sustained virologic response |

# Chapter 1 INTRODUCTION

## 1.1 Overview

Hepatitis C virus (HCV) infection is a serious health challenge affecting over 185 million individuals globally. Egypt has the highest rate for chronic HCV infection globally. Unresolved chronic HCV infection could progress to more deteriorating conditions such as cirrhosis and/or hepatocellular carcinoma (HCC). The latter is estimated to be the second leading cause for cancer related mortalities and the seventh most common cancer globally. Current conventional HCC biomarkers lack both sensitivity and specificity. Discovering novel and reliable biomarkers that are sensitive, specific and non-invasive is very crucial for early diagnosis and rapid intervention for HCV related liver diseases. Recently, circulatory mi-RNAs have drawn great attention as promising non-invasive biomarkers for various diseases. They are highly stable in blood and their expression profiles reflect disease progression and/or drug response status. The aim of this study is to explore expression profiles for some circulatory mi-RNAs such as miR-122, miR-155 and miR-21 in healthy controls, cirrhotic and HCC Egyptian patients against chronic HCV samples. Comparative mi-RNAs profiling results could be utilized as novel biomarkers for early diagnosis and prognosis of HCV related liver diseases.

## 1.2 Research Problem and objectives

One of the major global health issues is hepatitis C virus infection (HCV). Two types of assays are available for determining the occurrence of HCV infection. The first test is a serological immunoassay that detects antibody raised against HCV infection following week 10. The second one is a polymerase chain reaction (PCR) based molecular assay that detects presence of HCV RNA within few days following infection. Although serological immunoassay is efficient, it can't discriminate between active and resolved HCV infection. The only disadvantage concerning PCR assay is its extreme expensive costs compared to immune-assay [1].

The progression of HCC is mainly due to the delay in diagnosis and treatment [2]. Traditional biomarkers show poor performance in the surveillance, diagnosis and prognosis of HCC [3]. Recent studies have indicated that AFP and des γ-carboxy

prothrombin (DCP) tests lack adequate specificity and sensitivity for effective surveillance and diagnosis [2]. AFP in particular is currently not considered as an ideal test for HCC diagnosis by the American association for the study of liver diseases. This necessitates the search for novel and potential biomarkers that could scan the progression of liver deterioration across various stages to enable early diagnosis and intervention [3].

The overall goal of this thesis is to investigate if the selected mi-RNAs could be used as novel biomarkers for early diagnosis and prognosis of several stages of HCV-associated liver diseases as an alternative to existing biomarkers. The specific objectives are: (1) collect clinical specimens from healthy control, patients infected with HCV at different stages and subjects diagnosed with HCC. (2) Develop real-time PCR for detection of mi-RNAs in spiked plasma. (3) Explore the expression profiles for circulatory mi-RNAs (miR-122, miR-155 and miR-21), as non-invasive method, in Egyptian patients affected with various degrees of HCV infection stages. (4) Elucidate the correlation between one or panel of mi-RNAs and HCC. (5) Study the correlation between some clinical data and mi-RNAs expression.

## 1.3 Methodology

To achieve the study objectives listed above, 156 Egyptian individuals were enrolled in the study and divided into groups as follows; (37) chronic HCV infected patients, (39) HCV mediated cirrhotic patients, (40) HCV mediated HCC infected patients and (40) healthy controls group. All samples were collected from the National Hepatology and Tropical Medicine Research Institute (NHTMRI) in Cairo under thorough supervision of Prof. Dr. Mohamed Ezz El Arrab the head of the HCC and intervention unit at the NHTMRI in Cairo.

Total RNA and mi-RNA were extracted from patients' and healthy controls' plasma. RNA purity and concentration were evaluated by Nano-Drop. Extracted RNA was poly adenylated, reverse transcribed. This step was followed by quantitative RT-PCR for the synthesized cDNA. Finally statistical analysis of the data was achieved using SPSS software.

## 1.4 Organization of the thesis

This thesis is divided into five chapters. The first chapter is an introduction that involves an overview, the research problem, the study objectives and finally the

methodology. Chapter 2 displays a literature review of hepatitis C virus and mi-RNA. Chapter 3 presents a discussion of the criteria of subjects enrolled in the study and the methods utilized. The results and statistical analysis are discussed in Chapter 4. A discussion of the results was presented in Chapter5. Finally, a summary of the conclusions drawn from this study and future perspectives are presented in chapter 6.

# Chapter 2 LITERATURE REVIEW

## 2.1 History of hepatitis C virus

In 1989 Michael Houghton and his colleagues at the Chiron Corporation in Emeryville, CA, together with another team in the Centers for Disease Control in Atlanta, GA were able to discover Hepatitis C virus (HCV). HCV infection was known before that time as post-transfusion non-A, non-B hepatitis NANBH [4]. The NANBH was postulated to be caused by a small enveloped RNA virus which is readily communicable to Chimpanzees [5]. At that time, HCV infection was first thought to be of minimal importance, affecting a number of drug users and populations experiencing blood transfusion in developed countries [6].

Hepatitis C virus discovery launched a new research era, given that it was the primary virus which was detected only by a molecular approach that was based on cloning of complementary DNA (cDNA) isolated from infected plasma. The detection process was achieved with no tissue culture, electron microscopy or serology approaches but with a direct molecular approach based on viral genome cloning. Since HCV discovery, it has been considered as one of the major causes for chronic liver diseases, cirrhosis, hepatocellular carcinoma HCC and different types of hepatitis including post-transfusion and intravenous drug injection mediated hepatitis [7].

## 2.2 Epidemiology of hepatitis C virus infection

More than quarter a century after HCV discovery, HCV is currently recognized as a global health issue [6]. In 2015, the estimated mortality caused by viral hepatitis was 1.34 million deaths which was higher than that caused by HIV and comparable to that caused by tuberculosis. While the incidence rate of tuberculosis and HIV deaths is apparently declining over time, deaths from viral hepatitis is rising (**Figure 1**) [8].

Hepatitis C universal prevalence estimates, built upon the presence of anti-HCV antibodies has been approximated at 1.6% (range: 1.3–2.1%), which is equivalent to 115 million (range: 92–149 million) patients. Yet, these individuals are not all currently HCV-infected; certain population have cleared the viral infection

either spontaneously or as a result of therapy. Accordingly, the viraemic prevalence which is based on being positive for HCV RNA is dropped to 1% (range: 0.8–1.14%) or 71 million (range: 62–79 million) individuals with HCV infection (**Figure 2**) [9].

Over the last 15 years, estimates have shown that HCV infection has increased to 2.8% of the whole population and this means that more than 185 million individuals are affected with HCV. This increase in the disease prevalence is mainly due to multiple drug injections and hemodialysis [10]. According to the 2017 World Health Organization (WHO) global hepatitis report, 399 thousand lose their lives each year to HCV. The majority of these losses are due to cirrhosis and hepatocellular carcinoma. Worldwide, in 2015 the estimated figure for chronically affected HCV patients was 71 million. The Eastern Mediterranean Region has the highest prevalence (2.3%) of the world population followed by the Western pacific and the European Region (1.5%) (**Figure 3**) [8]. Cameroon, Egypt, Gabon, Georgia, Mongolia, Nigeria and Uzbekistan all have more than 5% prevalence of anti-HCV antibody between adult population [9, 11]. Of the total global HCV infections, Genotype 4 (GT 4) comprises 12%-15% of the cases, and is the predominant HCV epidemic in Egypt, particularly subtype 4a. Approximately, 2-4% of HCV-infected individuals can eventually progress to irreversible cirrhosis and/or hepatocellular carcinoma (HCC) on a yearly basis [1]. In Egypt the starting cause for HCV infection spread was the intravenous treatment for schistosomiasis (bilharzia) in the 1960–1970s [9, 11].

A primary cause for hepatic related mortality, cirrhosis and HCC in the USA is HCV infection, which makes it a serious public health problem [12]. Hepatitis C is the main signal for liver transplantation in many segments of the world [13]. There is still new incidences of HCV infection each year which are estimated to be up to 4 million new infections. This is due to the sustained use of improperly screened blood transfusions and blood products, the use of inadequately sterilized medical tools, and the growth in injectable drug handling in formerly unaffected regions. The majority of HCV infected patients' progress from acute to chronic infection without being aware of the disease or its progression [14].

## 2.3 HCV virology

A massive analysis of the virus' genome after its discovery in 1989 has elucidated its structure. It is well known as an enveloped virus, of diameter ranging from 50-80nm and a member of the family Flaviviridae that comprises foremost human and animal pathogens. The viral particle is made up of a viral envelop which is made up of a lipid bi-layer and on the envelope there are surface proteins. A protein capsule known as nucleocapsid is then found inside the envelop that protects the viral genetic material (**Figure 4**) [15, 16].

HCV genome consists of a positive-sensed or a plus stranded RNA molecule of about 9.6 kilo-bases. Plus stranded RNA is running from the 5'-prime end to the 3'-prime end and could be easily translated to a large poly-protein (around 3000 amino acids). This large poly-protein is a precursor to at least 10 proteins: the core, envelope 1 (El), E2, p7, nonstructural (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B (**Figure 5**). The viral structural proteins are the core protein (C) and the envelope glycoproteins E1 and E2. Then comes a small membrane bound protein p7 which acts as an ion channel. The remaining proteins play a key role in the intracellular life cycle of the virus. The ten former proteins are readily released from the cleavage of the precursor by proteinase enzymes of the host and the virus itself. These HCV proteins play a very important role not only in viral replication but also in multiple cellular functions [15, 17].

An important feature of HCV infection is the heterogeneity of the virus density circulating in blood. It varies from very low to low, intermediate and high density fractions. Very low to low density HCV fractions are called Lipo-Viro-Particle (LVP) in which the virus is linked to triglyceride-rich lipoproteins (TRL) these include apo-lipoproteins B, C1, C2, C3 and E. High density virions are mainly composed of naked nucleocapsids; which is the viral genome interacting with the core protein, while those of intermediate density appear in an enveloped viral form which is known as "canonical" form [18].

## 2.4 HCV Life cycle

### 2.4.1 HCV entry and un-coating

Hepatitis C viral infection is well known only in humans and chimpanzees. It is mainly transmitted through blood including wrong blood transfusion from HCV infected subjects or contaminated blood products, insecure medical treatment, dialysis and medical operation  In Egypt, the main reason for HCV epidemic is the intravenous injection therapy for anti-schistosomiasis. Besides the lack of disposable syringes and the improper use of health care facilities made the prevalence of the infection became faster [19].

Hepatitis C virus reaches the liver through the bloodstream. Even though HCV infection was reported in other cells than hepatocytes such as B cells, dendritic cells and others, productive infection of HCV occurs only within hepatocytes (**Figure 6**). In other words, hepatocytes are the only human cells that support HCV replication which makes this viral infection unique [18, 20]. The virus comes in contact with hepatocytes through the passage across liver sinusoids which are types of blood vessels with fenestrated, discontinuous endothelium [21].

Upon reaching the hepatocyte surface, the viral particle interacts first with the surface receptors and attachment factors. This step is mediated by Low density lipoprotein receptor (LDLR), glycosaminoglycans (GAGs) and heparan sulfate proteoglycan syndecan-1 or syndecan-4 or by the scavenger receptor class B type1 (SRB1). The viral interaction with these receptors, based on the viral particles' density, is initiated by binding to Apo-lipoprotein E (APoE) which resides on LDLR [20]. This is then followed by binding to more specific receptors, including the scavenger receptor B1 and the tetraspanin protein (CD81). The new complex of the viral particle with these entry factors reaches tight junction and participates in future interactions with claudin-1 (CLDN1) and occlud (OCLN) [21, 22, 23]. The lateral membrane transfusion is actin-mediated and depends on the activation of many transduction pathways [22].

The viral particle consecutively goes into the cell via receptor-and clathrin mediated endocytosis. After reaching the cytosol, the clathrin-coated vesicle is transported along microtubules to reach Ras-related protein **Rab-5A (**RAB5A) containing the endoplasmic reticulum area where HCV fusion occurs. At low PHs, the

endosome lumen provokes conformational modifications of the viral envelop glycoproteins, that successively triggers a fusion process of viral and endosomal membranes. Following membrane fusion a process of nucleocapsid un-coating is achieved leading to the release of the Viral RNA genome into the cytosol [22, 23].

### 2.4.2 HCV RNA translation

Binding and gathering of ribosome subunit on the viral RNA is the initial unit of hepatitis C virus polyprotein translation which would subsequently initiate viral replication. A signal sequence sited at the viral 5′ non coding region (NCR) allows the ribosome to be targeted to the translocation channel on the endoplasmic reticulum (ER) membrane. The 5′-NCR comprises four highly well-organized domains running from I to IV. Domain I and II play a crucial role in HCV RNA replication and domain I is not involved in internal ribosomal entry site (IRES) activity. The IRES is made up of 5'-NCR domains II, III and IV in addition to the 20-40 nucleotides at the beginning of the core coding sequence of the virus genome [9, 20].

The initial step in HCV translation is the development of a complex between the IRES and the 40S ribosomal subunit. Subsequently an assembly of eukaryotic translation initiation factor 3 (eIF3) and ternary complex (eIF2•Met-tRNAi •GTP) occurs. This in turn leads to the association of a 48S-like complex at the AUG initiation codon to start translation. The rate limiting step for the translation process is the GTP-dependent assembly of the 60S subunit to form an 80S complex [20]. Translation can thus be carried on further producing a poly-protein that is processed by cellular-endoplasmic reticulum- signal peptidases and by viral proteases into 10 mature structural and non-structural proteins [9, 20]. Endoplasmic reticulum peptidase processes the structural proteins and P7 polypeptide while the two viral proteases, the NS2-3 protease and the NS3-4A serine protease process the non-structural proteins. The released proteins are either used for translation and replication which is the case for non-structural proteins, or packaged into new viral particles.The non-structural protein NS2–3 and NS3/4A are having a catalytic protease activity. P7 is an amino acid protein that has an ion channel activity. NS4B induces the development of the membranous web which is essential for HCV viral replication. NS5B catalyze the formation of a complementary negative-strand RNA and the consequent synthesis of genomic positive-strand RNA from this negative-strand RNA template [20].

### 2.4.3 HCV RNA replication

HCV non-structural proteins such as NS3/4A, NS4B, NS5A, and NS5B in concert with host factors constitute the replication complex. NS4B integral membrane protein would first induce changes in the ER membrane, leading to the development of double-membrane vesicles. These vesicles cluster to develop the membranous web, which represents the site for HCV viral replication [9]. RNA dependent RNA polymerase activity of NS5B, which acts together with other viral non-structural proteins, along with several host factors would catalyze the synthesis of a viral RNA positive strand. The synthesis of a negative RNA strand intermediate is then completed using the positive-strand genome RNA as a template. Multiple positive-strand RNAs are generated from the negative RNA strand intermediate. These multiple positive-strand RNAs are consequently utilized either for translation, the preparation of new intermediates for viral replication or packaging into nucleocapsid [23].

### 2.4.4 Packaging, assembly and particle release

Viral particle assembly relies mainly on the presence of lipid droplets (LDs) which keep triacylglycerides and cholesteryl esters surrounded by a phospholipid monolayer incorporating numerous proteins. It is assumed that core proteins interact with LDs to trigger the assembly of other viral components involved in viral particle synthesis [21]. LDs act as transporters conveying core protein from RNA translation and replication sites to assembly sites. NS5A then carries the viral RNA to core proteins for assembly into nucleocapsids. Nucleocapsids are formed at the ER and ER derived membranes, where E1 and E2 are delivered associated with p7, NS2 and host factors including apolipoprotein E. The viral envelop glycoproteins are developed by budding, a process which is common in members of the *Flaviviridae* [24]. Newly synthesized virus particles are thought to be transported to Golgi apparatus where they would be packed up and placed in endosomes that acts as export vesicles which via the cellular secretory pathway would release the new virus particles to the cell surface. Finally, by exocytosis they are discharged from the cell to reach the blood circulation. It has been reported that HCV can directly infect neighboring cells with no need for HCV entry factors [25].

## 2.5 HCV genotypes, subtypes and quasi-species

Remarkable genetic heterogeneity is a distinguishing feature of HCV genome. Genetic diversity exist at several levels including six major genotypes, with various subtypes (over 80) and minimal variants formed during evolution called "quasispecies". HCV genotypes are created as a result of accidental nucleotide errors that are often introduced by RNA-dependent RNA polymerase during replication of HCV genome and due to the lack of associated repair mechanism [26, 27]. Studies have revealed that there only six major genotypes each with multiple subtypes and that newly proposed genotypes such as 7,8 and 9 are only subtypes of genotype 6 and genotype 10 is a subtype of genotype 3B [27, 28].

Worldwide distribution of HCV genotypes is extensive, which reflects dissimilarities in epidemiology, including pathogenesis, diagnostic implications, response to therapy and racial variability. The sequence differences among these genotypes are momentous which make it rational to believe that viruses compromising these genotypes have different traits. For instance, certain HCV genotypes seem to have higher response to interferon therapy than others [29]. HCV genotypes 1, 2 and 3 have broad geographical distribution, whereas HCV genotypes 4, 5 and 6 are restricted to definite areas (**Figure 7**). The most predominant genotype in Africa and the Middle East is genotype 4 with highest prevalence in Egypt. A proposed study in 2012 reported the prevalence of HCV in Egypt about 7 per 1,000 person-years, which is equivalent to 500,000 HCV infections annually [26], with 85% of genotype 4 [30]. HCV genotype1 is mostly prevalent in Argentina while genotype 2 is mostly prevalent in Taiwan and Republic of Korea. Pakistan, India and Thailand are having the highest prevalence of genotype3. Genotype5 main prevalence is in South Africa, yet genotype6 is mainly prevalent in Vietnam [26].

The main difference between genotypes and quasispecies that the later genetic heterogeneity is observed within a single infected individual while genotypes heterogeneity is detected among diverse HCV isolates [29]. Quasispecies are genetically related variants with only minimal sequence differentiations (i.e., less than 5% of the viral genome), these variants' genomes are concentrated around one master sequence [27, 29]. The Quasispecies nature of HCV infection can be a reason for

persistent infection, altered response to interferon therapy and finally resistance to host immune response [29].

## 2.6 HCV and immune response

In the first weeks of infection, the host evokes innate immune responses, then on later weeks, an adaptive immune responses are elicited to clear invading virus C [31]. The minority of HCV infected patients that have the ability to clear the viral infection is mainly due to a rapid induction of innate immune response. On the other hand, most patients are unable to clear the virus and develops viral persistence challenging continuing immune responses. This due to the virus multiple strategies that are elaborated to avoid these immune responses [32].

### 2.6.1 Innate immune response

Innate immune response is considered to be a rapid and specific immune response, besides being the first line of defense against virus C infection. This kind of immune response involves the participation of both natural killer cells (NK) and dendritic cells. Natural killer cells would attack the infected hepatocytes causing their lyses in addition to its role in shaping the downstream adaptive immune response. Dendritic cells (**DCs**) are professional antigen presenting cells (APC) that has an important function in immune surveillance, antigen (Ag) capture, and Ag presentation, which in turn provides a bridge between innate and adaptive immune responses [31].

Upon infection, the virus is recognized by several pathways these include; the toll-like receptor (TLR) dependent pathway and the cytosolic pathway. These pathways are elicited by interaction between the viral RNA, the RNA helicases retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation antigen 5 (Mda5). Both (RIG-1) and (Mda5) act as pattern recognition receptors that sense virus C infection. The key transcription factors NF-jB as well as the interferon regulatory factors (IRF) 3 and 7 are activated as the toll-like receptor (TLR) and the cytosolic pathway join. Activated IRF3 and NF-jB would finally lead to NK cells activation which in turn stimulates the release of cytokines (gamma interferon (IFN-**γ)**, tumor necrosis factor-α [**TNF-α**]) and chemokines (macrophage inflammatory factor [**MIP-1**] α/β and interferon **γ** inducible protein 10 [**IP-10**]), providing an important front line of defense against hepatitis C viral infection [31].

All interferons are capable of eliciting an antiviral response upon transcriptional activation of hundreds of genes [33]. Type I IFNs (including several IFN-α and one IFN-β) and type III IFNs (IFNk1,-k2, and -k3; also termed IL29, IL28A, and IL28B) are produced by the infected cells and by key cells of the innate immune system: macrophages and dendritic cells [32].

### 2.6.2 Adaptive immune response

Humoral antibody and T cells are two arms of the adaptive immune system that play an important role in hepatitis C viral clearance. After 6-8 weeks of acute infection, the former system activity could be detected via producing antibodies against epitopes associated with both viral structural and non-structural proteins. Studies have shown that only small portion of the released anti bodies, known as neutralizing antibodies, is having anti-viral activity by inhibiting virus binding, entry or un-coating. $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells are very important cells that are directed against multiple epitopes within the different HCV proteins [32]. Interestingly, another study have suggested a delayed development of neutralizing antibodies and T-cells in patients with chronic infection, but an early time development in people with resolving infection. $CD4^+$ cells are in charge of stimulation and conservation of successful $CD8^+$ T-cell responses. $CD4^+$ cells migrate to the hepatocytes where APCs stimulate $CD4^+$ activity and release of cytokines. This in turn causes the maturation of $CD8^+$ which are key modulators in regulating viral infection through cytolysis (by perforin cytolytic protein release) and secretion of anti-viral cytokines such as IFN-γ (**Figure 8**) [34,35].

### 2.6.3 Viral escape from immune response

Studies have proven that HCV has established multiple strategies to not only escape but also work against host immune defenses (**Figure 9**). These are probably the main causes that contribute to the persistence of HCV infection. First, the association of virus C particles with host lipid proteins such as lipoproteins that resembles VLDL particles enables the virus to be masked from recognition by the host immune system. Second, the fact that the virus replicates its RNAs within re-arranged cytoplasmic membrane vesicles which are highly compartmentalized microenvironment may protect HCV replication complexes from attacks by cellular nucleases and proteinases [33]. Third, Studies have reported that HCV evokes autophagy, stimulating the cells to recycle cytoplasmic constituents via lysosomal

pathways which eventually enable the virus to escape innate immunity antiviral activity [33, 36]. Fourth, miR-122, a liver specific mi-RNA, is known to be hijacked by hepatitis C virus to promote and facilitate the replication of its genome [33]. miR-122 3'end would bind the viral RNA 5'end, leading to viral RNA protection from the 5' to 3' exonuclease activity and host innate immunity [37]. Fifth, the presence of hepatitis C in the form of viral variants named quasispecies allow the virus to escape cytotoxic T cells and neutralizing antibodies [33]. Besides, in case of the absence of CD4+ T cell help or the effect of regulatory T cells or cytokines may lead to virus-specific CD8+ T cell exhaustion, enabling HCV escape from the adaptive immune response [34].

## 2.7 HCV and liver diseases

### 2.7.1 Chronic HCV infection

Hepatitis C viral infection remains asymptomatic for decades in its acute infection stage in the majority of cases before its detection in late chronic stages, when noticeable symptoms will be recognized. Acute infection may develop with symptoms such as jaundice, fatigue, right upper abdominal pain, discomfort or joint pain in minor cases [9]. Up to 85% of HCV Egyptian patients develop chronic hepatitis while others experience spontaneous clearance of the virus. On long term chronic infection, in which the patient harbor the disease for an average between 20–40 years, HCV may complicate to liver fibrosis, cirrhosis, and eventually, HCC [30, 38]. Liver fibrosis is distinguished by excessive deposition of extracellular matrix proteins (ECM- proteins) such as collagen which would subsequently alter the liver structure by developing a fibrous scar, and then forming nodules of regenerating hepatocytes named cirrhosis. Fibrosis is thought to be a model of wound-healing response to chronic hepatic damage which is reversible in early stages however if it reached a cirrhosis stage it would be irreversible [39]. Cofactors, for instance; patient age, male sex, alcohol, HIV infection and schistosomiasis can make the progression towards end-stage liver disease goes faster in other words to be having synergistic effect [8, 9, 30].

Cirrhosis may progress to some complications including hepatic decompensation which is distinguished by hepatic failure, ascites, upper gastrointestinal bleeding, and liver encephalopathy. The later complications, cirrhosis and HCC are mostly the causes for mortalities between HCV patients [30]. Patients

may suffer from symptoms such as fatigue, weight loss, muscle and joint pain, or right upper abdominal discomfort, pain or itching prior to developing decompensation symptoms [8, 9, 30].

### 2.7.2 HCV induced HCC

Nowadays HCC is estimated to be the second leading cause for cancer related mortalities and the seventh most common cancer globally [40]. HCC is a highly lethal cancer as the majority of the cases are detected at a late stage in patients with underlying hepatic dysfunction. There are different etiologies for HCC among these is hepatitis B, hepatitis C, alcoholic liver diseases and non-alcoholic fatty liver diseases. HCV has been a primary cause of HCC in developed nations and is the first warning for hepatic transplantation for patients with HCC in the United States [40, 41].

Pathogenesis of HCV-induced HCC involves multiple steps: establishment of acute HCV infection, prognosis to chronic infection, chronic hepatitis, gradual liver fibrosis, appearance of neoplastic clones associated with permanent somatic genetic/epigenetic changes, and finally progression of the malignant clones in an oncogenic tissue microenvironment which is usually referred to as "field cancerization" (**Figure 10** ). This cirrhotic oncogenic microenvironment accelerates genetic abnormalities and cellular transformation which in turn allows development and promotion of carcinogenic clones. HCV chronic infection, leads to hepatic damage which is manifested in the release of inflammatory mediators for instance: reactive oxygen species (ROS), cell death signals, nucleotides and hedgehog ligands; proteins that regulate cell growth and its fate. A complex series of events then occurs that leads to hepatic stellate cell activation. These events include the activation of intracellular inflammation factors, a family of transcription factors and other transcriptional events. Hepatic stellate cell activation stimulates liver scarring as a result of proliferation, fibro-genesis, matrix degradation and inflammatory signaling [40].

## 2.8 Diagnosis of HCV and consequent liver diseases

### 2.8.1 Serological assay (Immunoassay)

A number of virological tools can be utilized for diagnosis and monitoring of hepatitis C infection. The frequently used screening tool is immunoassay which includes enzyme immunoassay (EIA), micro-particle EIA, chemi-luminescence immunoassay (CIA). These assays are based on the ability to detect total anti-HCV

antibodies (IgG and IgM) in serum, plasma , whole blood or cervicular oral fluid [9]. They are specific, inexpensive and have easy automation. A limitation of these assays that detection cannot distinguish between acute and chronic HCV infection [42]. Additionally, testing only for anti-HCV antibodies might miss early infection when the amount of antibodies released are non-detectable [9]. The fact that anti-HCV antibodies persist for years or even decades in the body ever after the clearance of the viral infection make it impossible to discriminate between recovered patients and concurrently diagnosed leading to false positive results. To aid in solving false-positive EIA test problem new supplemental tests (recombinant immune-blot assay (RIBA)) were developed. These include second-generation RIBA (RIBA-2) and third-generation RIBA (RIBA-3) EIAs [43]. Currently, the third-generation EIAs are used to detect antibodies directed against several HCV epitopes in plasma or serum. These epitopes include recombinant antigens from core and non-structural proteins 3, 4 and 5 [42].

### 2.8.2 Molecular assay

#### 2.8.2.1 Polymerase chain reaction

Polymerase chain reaction is a molecular assay that is characterized by high sensitivity and specificity. It's not only a qualitative assay but a quantitative one as well. PCR assay has the ability to discriminate between actively infected and recovered patients. The essay is based on quantifying the viral RNA which is known as viral load and the results of the assay are expressed in IU per ml [9]. This assay is used in monitoring both viral replication and viral clearance by the infected host. This in turn is used in diagnosis and assessment of anti-viral therapy efficiency. There are several reasons contributing to PCR assay variability for instance: sample handling and storage settings, precise design of amplification primers, inconsistent biochemical reactions and efficiency of post-amplification detection systems. This means that highly controlled quality control procedures should be utilized when performing this assay [43].

#### 2.8.2.2 Branched DNA assays

Branched DNA assay is another quantitative assay based on signal amplification rather than target amplification; which is the case in PCR assay. This technology is based on capturing HCV RNA in a micro-well by hybridization, and then measuring the quantity of captured RNA via signal amplification of chemi-

luminescent produced in response to RNA hybridization. Comparing branched DNA assay to PCR, the later has been found to be much more sensitive ruling out false negative results in cases of low viremia load. On the other hand results from branched DNA assay have been found to be more standardized and reproducible [44].

### 2.8.3 HCV genotype testing

HCV genotype testing is essential to guide treatment algorithm which involves the course of therapy, the period of treatment and the addition of interferon regimen. The reference methods fall into two main categories either analyzing points of alteration within HCV genome through screening tests or sequence analysis of larger segments of HCV genome as in confirmatory testing [43].

### 2.8.4 Assessment of liver disease severity

#### 2.8.4.1 Invasive technique (Liver biopsy)

Recent studies have proposed that proper liver disease staging has a major therapeutic impact, knowing that liver fibrosis is reversible, in other words it can be modified by treatment. Histo-pathological assessment using a biopsy or a surgical sample is considered as the "gold standard" or a reference test for the evaluation of liver fibrosis .Despite being a gold standard, it is associated with several limitations. For instance: being painful, sampling error that leads to improper-estimation of the feature being assessed, inter- and intra-observer variation in pathology reporting and finally significant mortality and morbidity owing to the invasive nature of the technique. With these limitations, noninvasive methods of liver fibrosis assessment have been developed [45, 46].

#### 2.8.4.2 Non-Invasive techniques

Alternative to invasive biopsy, non-invasive techniques are frequently utilized for liver fibrosis and cirrhosis staging besides clinical findings. These techniques include transient elastography (TE) and biomarkers' detection, that are classified to direct and routine serum-based laboratory tests known as indirect biomarkers. Direct and indirect biomarkers are listed in (**Tables 1)**. Despite the fact that the level of indirect biomarkers is altered in hepatitis, they are not specific. On the other hand, direct biomarkers include soluble or secreted proteins that are produced by hepatic stellate cells during liver fibrosis. In addition to biomarkers, several scoring algorithms are utilized in liver disease staging. These include: FibroTest, Fibrosis-4 (FEB-4) and the AST to PLT ratio index (APRI). Despite their ability to diagnose

advanced fibrosis (Stage-2), many of these non-invasive techniques cannot distinguish between early stages of liver fibrosis [47].

### 2.8.4.3 HCC assessment

Over four decades ago, alfa feto protein (AFP) which is a serum glycoprotein, has been identified as a marker for HCC. Not showing highly elevated levels in all HCC cases have made AFP of less accuracy and sensitivity. Another frequently utilized tumor marker is Des-gamma-carboxy prothombin (DCP), also known as PIVKA II (protein induced by vitamin K absence). On one hand some studies have shown 95% sensitivity of DCP, on the other hand other studies have demonstrated its poor sensitivity in tumors of size less than 3cm [48].

Diagnostic imaging technology is another aspect that plays a crucial role in HCC diagnosis. This imaging technology include ultrasonography, computed tomography scan (CT scan) and magnetic resonance imaging (MRI). Ultrasonography has been widely replaced by CT scans and MRI owing to its low sensitivity. In spite of being sensitive, CT scanning is considered to be an invasive technique because of the requirement for contrast agent infusion for imaging technique. Magnetic resonance imaging have recently became the diagnostic imaging model for HCC in different institutions all over the world despite the fact that it might develop some allergic reactions in some patients because of the contrast agent. Even with the high sensitivity of these imaging techniques, they are considered to be costly. Nowadays, research studies is focus on developing ways to improve diagnostic techniques and approaches with the intention of identifying earlier stages of HCV mediated HCC [48].

## 2.9 Treatment of HCV

### 2.9.1 Interferon therapy

The goal of HCV therapy is to reach a sustained virologic response (SVR). SVR is unnoticeable serum HCV RNA 6 months after completing the course of therapy. Studies have shown that patients who attain SVR with treatment have less than 5% chance of relapse of hepatitis C infection [49]. In early 1960s, steroids were firstly used for hepatitis management through regulating ALT levels. On one hand this improved the patients' health, on the other hand later studies have shown that this is detrimental and accordingly they were no longer used [7]. In 1991 the US Food and

Drug administration (FDA) approved the use of interferon α-2b (IFN- α-2b) for chronic hepatitis C management. The mechanism of action of interferon involves 2 phases; a rapid dose dependent phase followed by a slower one. The first phase involves direct inhibition of the viral genome replication while the second phase is characterized by an indirect antiviral activity through the stimulation of host mediated immune response [50]. Studies have shown that a 24-week course of interferon therapy was effective in management of many cases of hepatitis C, even though many patients relapsed after treatment because of low SVR rates [51].

### 2.9.2 Ribavirin

Ribavirin is a potential antiviral agent, having broad spectrum of activity and acting against both DNA and RNA viruses. It has an inhibitory effect on HCV RNA dependent RNA polymerase besides modifying the expression of interferon stimulating genes. Antiviral therapy for hepatitis c had been then improved by adding ribavirin-based regimens with interferon; SVR rates increased to 38-42% with conventional IFN and ribavirin (RBV). Knowing that SVR with interferon (IFN) monotherapy was around 6-2% [52].

### 2.9.3 Pegylated IFN-α

Aiming to improve the pharmacokinetics and dosing regimen of interferon, it had been covalently attached to polyethylene glycol (PEG) moiety. This in turn led to long-lasting antiviral activity and as a result, patients were given a weekly dose of interferon rather than three doses in the same week [52]. Besides on using pegylated IFN (peg-IFN) and RBV together the SVR had been elevated to 63% [49].

### 2.9.4 Direct acting antiviral agents

The launch of direct-acting antiviral agents (DAA) era was in 2011 when the two NS3/4A protease inhibitors were introduced and used in combination with interferon-based regimens. This era started after the major advances in understanding the viral genome and accordingly formulating antiviral agents that targeted the viral non-structural proteins and in turn terminating viral replication. The main advantages of this new DAA regimens that unlike interferon, they are all administered orally and not injections. Besides being highly effective and well tolerated. On average, DAA requires only 8-12 weeks of therapy for the majority of patients. Current DAA includes NS3-4A protease inhibitors, Nucleotide analogues, Non-nucleoside

inhibitors and NS5A inhibitors (**Figure 11**). [52,53,54] NS-4A protease inhibitors include simeprevir, paritaprevir and asunaprevir. On the other hand, examples of NS5A inhibitors are daclatasvir, ledipasvir and omitasvir. Finally, examples of NS5B inhibitors are sofosbuvir and dasabuvir and beclabuvir [54].

## 2.10 MicroRNAs biogenesis and regulation

After their discovery in nematodes "Caenorhabditis elegans" in 1993, microRNAs (mi-RNAs) have been found to play an important role in many biological processes in plants and animals. MicroRNAs are a family of short non coding RNAs that are around 22 nucleotides in length. These non-coding RNAs are too short to code for proteins and are thought to control specific genes' expression via base-pairing to target messenger RNAs [55]. The first discovered mi-RNA was ln 4-RNA that was noticed at the time of its discovery to play an important role in the regulation of ln 14-mRNA, causing less expression of proteins from that messenger RNA. A huge number of mi-RNAs have been discovered in worms, flies, fish, frogs, mammals and plants using different molecular biology approaches such as molecular cloning and bioinformatics [56].

In humans, mi-RNAs biogenesis starts with a precursor (pri-mi-RNA) that folds into a double stranded (dsRNA)-like hairpin structure (**Figure 12**). Pri-mi-RNA originates either from mi-RNA genes' transcription by RNA polymerase II or found as parts of introns of protein-coding RNA polymerase II transcripts. RNase III type endonucleases Drosha (RN3) and Dicer consecutively undergo two step catalysis after the hairpin structure formation. The two endonucleases Drosha and Dicer always perform their role in association with proteins containing dsRNA-binding domains (dsRBDs). The Drosha will partner with DiGeorge syndrome critical region gene 8 (DGCR8) in mammals to form Drosha–DGCR8 complex which will then convert the pri-mi-RNA to pre-mi-RNA which is a hairpin of about 70-nucleotides. The formed Pre-mi-RNAs are subsequently transported from the nucleus to the cytoplasm by exportin5, where they encounter Dicer-_TRBP complex that lops off the loop of the hair pin structure to yield ~20-bp mi-RNA duplexes. The Dicer-_TRBP complex is formed from the binding of Dicer with TAR RNA binding protein (TRBP) in mammals. Following the formation of mi-RNA duplex, one strand of the complex formed by the activity of helicase enzyme would be loaded to the Argonaute protein

(AGO) to form RNA induced silencing complex (RISC). Some mi-RNA might by pass Drosha processing during their formation an example of this case is when the primary transcript is an intron that can fold directly to a hair pen structure known as Mirtron that would be directly recognized by Dicer [57].

The formed mi-RNA within the AGO protein will be directed to specific mRNA regulating their expression. Mi-RNAs play an important role in post-transcription gene silencing. They might base pair with specific messenger RNAs leading to mRNA decay and translation repression. Other mi-RNAs are key regulators in mammalian cells development. Mi-RNAs expression is tightly controlled, each mi-RNA is having its unique expression pattern. Despite the fact that the same mi-RNA might be expressed in several cell types, some mi-RNA are exclusively expressed in certain cells [57].

## 2.11 Detection of mi-RNAs

Nowadays mi-RNAs have become significant targets in several research fields, because of their potential to be utilized as noninvasive biomarkers. Accordingly great efforts have been made to develop efficient and sensitive techniques for their detection. Conventional methods include northern blotting, real time RT-PCR (qRT-PCR), microarrays and others. Innovative techniques include the use of nanoparticle-derived probes, isothermal amplification, electro-chemical methods and others. The main difference between the traditional techniques and the innovative ones that the latter have higher efficiency of detection by combining a multistep signal amplification with some sensitive signal output unit [58].

### 2.11.1 Northern blotting for mi-RNA detection

The expression of both the mature and precursor mi-RNAs have been frequently studied using northern blot analysis. Besides enabling quantitation of the expression levels of detected mi-RNAs, northern blot analysis allows mi-RNA size determination. The technique involves four main steps: First, fractionating RNA molecules; a step which is catalyzed using polyacrylamide gel electrophoresis. Second, passing on small RNA molecules from the gel onto a membrane. Third, Using crosslinking method to fix these small RNA molecules onto the membrane. Finally, utilizing radiolabeled oligonucleotide probes for membrane hybridization [59]. The main limitation of using northern blotting is the need for radiolabeling

which might lead to a contamination, in addition to being complex technique that has low sensitivity. The low sensitivity of this technique is attributed to the need of a great amount of mi-RNA per sample to be adequately analyzed, which is not the case all the time especially when the available sample is cell or tissue [58, 60].

### 2.11.2 qRT-PCR for mi-RNA detection

PCR product real time detection is the main idea of qpcr technique, which is considered to be the gold standard for mi-RNA detection. In this approach, all mi-RNA molecules are firstly reverse transcribed then elongated by an identical tail usually a poly A tail used to prime the reverse transcription into a complementary DNA (cDNA) [61]. There are several tactics for qpcr normalization, either using a normalization gene or what so called a housekeeping gene or adding a synthetic spike-in. The most well know housekeeping gene in most of mi-RNA studies is RNU6B (U6). However, studies have revealed that the expression of such housekeeping genes is not stable among different individuals with different disorders. The other approach of using a synthetic spike in which is a synthetic mi-RNA from another organism such as Caenorhabditis elegans have shown better normalization results [62]. A specific mi-RNA forward and reverse primers are used to form multiple copies of the targeted mi-RNA sequence making this method highly sensitive and specific for mi-RNA detection [63].

### 2.11.3 Microarray for mi-RNAs detection

Microarray is a technology based on hybridizing the target mi-RNA sequence with a complementary probe. The targeted mi-RNA is usually labelled with a fluorescent dye and upon binding with the complementary probe it would result in fluorescence emission. Recent studies have shown the high biocompatibility of nanomaterials toward nucleic acid probes; hence, the use of these materials has shown highly improved efficiency and sensitivity. Despite the enhanced efficiency of using nanomaterials in mi-RNA detection, the existence of a specialized nanotechnology platform is essential for this technique [59, 61].

## 2.12 mi-RNAs and HCV infection.

MicroRNAs have been acknowledged as very important in hepatitis C virus infection induced hepatic complications; the alteration in the expression of mi-RNA have been reported to be involved in the changes involved with the viral replication

[64, 65], translation [66], gene expression [67, 68], and in controlling response to interferon (IFN) therapy [69].

On one hand some mi-RNAs would regulate HCV translation and replication stimulating both processes which is the case in miR-122. On the other hand, other mi-RNAs would inhibit the viral genome production such as miR-199a* and those regulated by interferon beta (IFN-β). This in turn would moderate IFN-mediated antiviral response. The expression of miR-155, which enhances the proliferation and carcinogenesis of liver cells, was noticed to be induced after the infection with hepatitis C virus [70]. Specific mi-RNAs for liver, such as miR-122, miR-155, miR-125b, miR-16 and miR-34a, are experiencing different expression in the serum of HCV infected patients [71, 72].

## 2.13 Circulatory mi-RNAs as biomarkers in HCV infection

Mi-RNAs are essential in screening and diagnosing of hepatitis C infection. These small RNA molecules could be either detected within body cells and tissues or circulating as extracellular mi-RNA. On one hand, extracellular mi-RNAs are either circulating in membrane vesicles with adequate stability towards nuclease activity or can be vesicle free and accompanied with either AGO proteins only or transported by HDL particles (**Figure 13**). On the other hand, these circulating small RNA are found in blood plasma, serum, and other body fluids such as; saliva, peritoneal fluid urine, breast milk, seminal plasma, amniotic fluid, cerebrospinal fluid, and pleural fluid [73].

Extracellular mi-RNA are encapsulated in three kinds of membrane vesicles these are: apoptotic bodies, shedding vesicles, and exosomes. Shedding vesicles, and exosomes both are known as microvesicles, which are produced by all types of body cells, made up of lipid layers and usually enclosing cellular components including protein, mRNA and mi-RNA. Shedding vesicles are created by budding towards the outer side and fission of the plasma membrane. Exosomes are much minor membrane particles that normally exist within the lumen of multivesicular bodies (MVBs). After fusion of (MVBs) with the plasma membrane, exosomes will be released. Apoptotic bodies from its name are by products and remaining particles of apoptosis [73, 74, 75].

A number of latest research have revealed that some circulatory mi-RNA (exosomes) can play a crucial role during viral infection in intercellular, immune response, tumor progression, and neurological processes. There are two hypothesis regarding extracellular mi-RNA secretion into blood circulation. First, these circulatory mi-RNA are passively secreted as byproducts of routine microvesicle secretion and cell death. Second that they are released specifically to perform a certain function such as cell-cell communication [73]. Worth mentioning, the number of specific mechanisms for mi-RNA release from the cell are much more than passive release means. This in turn strengthens the belief that circulatory mi-RNAs are associated with regulatory and pathophysiologic mechanisms [76].

Circulatory mi-RNAs expression levels have been recently implicated to hepatitis C infection and HCC. For instance; expression levels of miR-134, miR-198, miR-320c and miR-483-5p have been found to be up-regulated in the serum HCV infected patients of genotypes 1 and 3 [70]. Serum levels of miR-20a and miR-92a in HCV infected fibrosis cases were considerably up-regulated compared with that of healthy subjects. Subsequently, they can be used as markers of HCV mediated liver disease progression [77].

Circulatory mi-RNA could be utilized as prognostic biomarkers in cases of HCC. Elevated serum levels of miR-122, miR-155 and decreased levels of miR-199a have been correlated to HCC pathogenesis. It has been postulated that miR-122 and miR-155 are oncogenic mi-RNAs that induce liver carcinogenesis in HCV infected patients [78].

Serum levels of miR-16 and miR-199a were significantly down-regulated in HCC than in chronically infected patients or control subjects. In addition to this, miR-16 has been proposed to be used in association of conventional biomarkers for HCC diagnosis [79]. In another study serum levels of miR-19a, miR-195, miR-192, and miR-146a have been revealed to have high accuracy in the differentiation between healthy controls and HCC affected individuals. Besides being proposed to be utilized as a biomarker incases of HCC, miR-19a has been claimed to be used for the study of liver prognosis from fibrosis to cirrhosis and finally HCC [80].

In 2007 a group of researchers in Germany suggested that serum miR-21 level can be used as a marker for necro-inflammatory activity, but wouldn’t differentiate between HCV and HCV-induced HCC infected patients [81]. MicroRNA-141 and microRNA-

200a serum levels have been found to be down-regulated in HCC patients compared to HCV infected ones, so these 2 mi-RNAs have been proposed to discriminate between chronic infection of HCV and HCC [82].

## 2.14 Role of mi-RNA 122 in HCV infection, liver disease and metabolism

### 2.14.1 Role of mi-RNA 122 in HCV infection

Besides being one of the firstly discovered tissue specific mi-RNAs, miR-122 is the most prevalent liver mi-RNA, accounting for nearly 70% of mi-RNA pool in hepatocytes. It is released from chromosome 18 in humans. Many researchers are interested in studying miR-122 owing to its potential role in HCV replication, transcription regulation and translation, HCC and cholesterol metabolism. miR-122 regulates virus C translation, increasing its' rate through the interaction with the HCV IRES [37]. On one hand, hepatic levels of miR-122 have been found to be higher in HCV infected patients of genotype-3 than those of genotype-1. On the other hand, serum levels of miR-122 have been found to up-regulated in HCV-1 while in HCV-3 there has been no correlation between serum and hepatic levels [83].

In addition to the former roles, miR-122 has been known to play a significant role in anti-HCV therapy. Accordingly a pharmaceutical company (Santaris Pharma) has developed an anti-viral therapy in the form of antisense oligonucleotide. Miravirsen is an antisense oligonucleotide of a complementary sequence to miR-122 that plays an important role in miR-122 inhibition which subsequently would alter viral replication [84]. When the former therapy was targeted to the liver through intravenous injection, it effectively inhibits miR-122. A pilot study has been performed on four chimpanzees and has given promising results by reducing the viral load in these animals effectively and with no toxicity [37].

### 2.14.2 Role of mi-RNA 122 in HCC

Among the recent advances, miR-122 has been found to be down-regulated in cases of HCC compared to normal liver. This down expression is correlated with poor prognosis of the cancer which suggests that miR-122 is a tumor suppressor [85]. On the other hand, several studies have revealed that the expression level of serum miR-122 is down-regulated in cases of HCC compared to healthy subjects [86].

### 2.14.3 Role of mi-RNA 122 in Liver diseases

Liver injury due to acetaminophen toxicity has induced a noticeable change in the expression levels of several mi-RNAs. Among these, miR-122 expression levels has been found to be implicated to the dose- and exposure duration of acetaminophen. These changes have been observed in the plasma and have been correlated to serum aminotransferase levels and the histopathology of liver degeneration. Worth mentioning, the changes in miR-122 levels have been detected significantly earlier than aminotransferase levels. These findings propose the possibility of utilizing miR-122 as sensitive biomarkers for drug-induced liver injury [87, 88].

### 2.14.4 Role of mi-RNA 122 in cholesterol metabolism and iron homeostasis

miR-122 has been found to play an important role in cholesterol metabolism and systemic iron homeostasis. On one hand, the mechanism by which miR-122 regulate cholesterol metabolism is not yet established. On the other hand, miR-122 control systemic iron homeostasis by acting upon the target mRNAs hemochromatosis (Hfe) and hemojuvelin (Hjv). These mRNAs are translated to activators of the hormone hepcidin, which standardize iron availability. Studies have shown that mice with reduced miR-122 expression experience iron deficiency [37].

## 2.15 Role of mi-RNA 155 in HCV infection and liver disease

### 2.15.1 Role of mi-RNA 155 in immune response

Recent advances in mi-RNA studies have focused on miR-155, an immune system mi-RNA. In human, miR-155 is found at a region within chromosome 21q21.3 known as B cell integration cluster (BIC) [89]. When correctly regulated, this mi-RNA is found to play an important role in protective immunity. While when dysregulated, it may contribute to malignant conditions [90]. Studies have shown that miR-155 directly induced interferon stimulating genes (ISG) expression in macrophages and stimulate a direct antiviral response in mRNA HCV replicon [91].

### 2.15.2 Role of mi-RNA 155 in HCV infection

Hepatic miR-155 expression was significantly up-regulated in serum of HCV-infected patients [92, 93]. Chronic HCV subjects that haven't started any therapeutic regimen were shown to have higher expression of miR-155 in their circulating monocytes when evaluated against people who cleared HCV infection after therapy.

This finding hypothesized a potential correlation between elevated miR-155 in monocytes and HCV viral existence and/or replication [90, 92]. In 2013, a study in China has postulated that miR-155 plays a crucial role in hepatic fibrosis through the regulation of the pathological network involved in fibrosis process. These findings suggest the role of miR-155 in the pathogenesis and progression of chronic HCV infection to HCV-induced HCC [94]. Moreover, miR-155 expression levels were increased in serum, liver tissues, and peripheral blood mono nuclear cells (PBMCs) of genotypes 1, 2 and 3 HCV-infected patients [92, 93]. Conversely, in another study it was reported that miR-155 expression did not change in the PBMCs of Egyptian patients genotype 4 with chronic infection of HCV when being evaluated against healthy subjects [95].

### 2.15.3 Role of mi-RNA 155 in HCC

It is consistently postulated that miR-155 acts as an oncogenic mi-RNA (oncomiR) in human tumors. This notion is based on the evidence that this mi-RNA expression has been reported to be increased in several human cancers. In hepatitis C infected patients, miR-155 levels were found to be significantly up-regulated, in addition to fostering liver cells proliferation and carcenogenesis by modulating Wnt signaling. The former pathway plays a vital role in the regulation of cell apoptosis and proliferation. [93]. A study in Shanghai had postulated that miR-155 can be used as a powerful biomarker for cancer relapse and survival of HCC patients following orthotopic liver transplantation (OLT). This hypothesis was reached after finding that the expression of miR-155 was higher in tumor tissues in patients with post-OLT HCC relapse compared with those in patients with non-recurrence [96].

## 2.16 Role of mi-RNA 21in HCV infection and liver disease.

### 2.16.1 Role of mi-RNA 21 in HCV infection

A study published in 2013 has studied the role of miR-21 during HCV infection. The results of this study revealed that miR-21 suppresses triggered type I IFN production in hepatocytes of HCV transfected Huh7 cell line. Besides, it proposed that miR-21 enhances HCV replication as well as facilitating the replication of other viruses by reducing antiviral activity of IFN-α. This study pointed out that miR-21 controls the expression of components of the Toll-like receptor signaling cascade too [97]. A study performed in Germany compared the expression levels of

miR-21 in serum of healthy controls, chronically infected HCV, cirrhosis and HCC patients. The results revealed that miR-21 expression is up-regulated in chronic hepatitis C patients (CHC). Moreover this study reported that the expression levels of miR-21 wasn't different between patients with cirrhosis and HCC [81]. Plasma levels of miR-21 wasn't different between HCV induced cirrhosis and HCC. On the other hand, compared to healthy volunteers and CHC, miR-21 is highly expresses in HCC [98].

### 2.16.2 Role of mi-RNA 21 in HCC

Recent studies have revealed that miR-21 expression levels is altered in multiple types of cancers among these is HCC. miR-21 had been found to be up-regulated in HCC compared to normal liver tissue. The increased expression of miR-21 has been associated with malignant cell proliferation, invasion and metastasis. In addition, it plays an important role in cellular transformation via suppression of apoptotic signaling which support the hypothesis that miR-21 acts as an onco-miR [78, 99, 100]. A study in China had demonstrated that serum miR-21 is elevated in patients with HCC or chronic hepatitis and hypothesized that miR-21 can act as a novel biomarkers for liver injury generally and not particularly for HCC [81, 101]. On the other hand, another study made on Egyptian patients of genotype 4 had revealed that miR-21 is overexpressed in sera of HCC patients compared to patients with chronic hepatitis [78].

# Chapter 3 SUBJECTS AND METHODS

## 3.1 Subjects

This study was performed after receiving IRB approval from the American University in Cairo CASE #2015-2016-185 and the approval of the National Hepatology and Tropical Medicine Research Institute (NHTMRI) in Cairo serial No: 10-2016.

Thirty seven Egyptian patients with chronic hepatitis C (CHC), 39 Egyptian patients with hepatitis C induced cirrhosis, 40 Egyptian patients with hepatitis C induced hepatocellular carcinoma (HCC) and 40 healthy controls were included in this study. CHC patients (male=26, female=11, mean age=45±9.64; range: 25-64). Cirrhotic patients (male=20, female=19, mean age=57± 5.65; range: 43-76). HCC patients (male=23, female=17, mean age=60 ± 7.69; range: 41-75).

All diseased patients were having HCV positive antigen and were genotype 4. Anti-HCV antibody assessment, viral load and genotyping were performed using real time pcr assay.

Patients were excluded from the study if they had hepatitis B virus (HBV) antigen or antibody, human immune deficiency virus (HIV) infection. Additionally, in cases of HCC patients, they were excluded if they had started a chemotherapy regimen or had undergone a surgical operation.

A total of 40 healthy control participated in this study (male=24, female=16, mean age=47 ± 10.51; range: 21-65). None of the samples were known to be having a history of hepatic disorder and all of them had normal liver function tests and negative serological findings for viral liver diseases.

### 3.1.1 Laboratory assays

Samples were gathered from National Hepatology and Tropical Medicine Research Institute Hospital, Cairo. For healthy control patients, samples were collected at the Biochemistry lab at the British University in Egypt from healthy volunteers. Written informed consent was obtained from all patient and healthy population. Whole blood was collected from patients and control and plasma was

separated for routine assessment. The later included whole blood picture, HCV qualitative assay and liver function tests; alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), direct bilirubin, albumin, prothrombin time, platelets' count and leukocytes' count using commercially available kits. A portion of the plasma was separated and stored at -80 ºC until molecular assays.

### 3.1.2 Histological investigations and assessment of fibrosis, cirrhosis and HCC

In viral hepatitis C infected patients, CT-abdominal ultrasonography and liver biopsy were done to determine the stage of fibrosis using Metavir scoring system in which F= fibrosis (F0= no fibrosis, F1= portal fibrosis without septa, F2= portal fibrosis with rare septa, F3= numerous septa without cirrhosis, and F4= cirrhosis) [102]. In addition to the former tests, magnetic resonance imaging (MRI), RBC, bleeding profile (Prothrombin time (PT) and International Normalized Ratio (INR)) creatinine, urea, viral load, hemoglobin, hemoglibinA1C, Packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cells' distribution width, alfa feto protein (AFP) assessment was achieved in cases of CHC, cirrhosis and HCC. Barcelona-Clínic Liver Cancer (BCLC) assessment was utilized to determine HCC staging in which (A= early stage, B=intermediate stage, C=moderate stage, D=terminal stage). Eight HCC patients were diagnosed to be in an early stage, 12 patients were intermediate stage, 12 patients were moderate stage and 8 patients were in terminal stage.

## 3.2 Plasma microRNA assay

### 3.2.1 RNA extraction

To separate the plasma, blood samples were centrifuged using bench-top centrifuge (EBA 20, Hettich, Germany) at 4000rpm for 10mins. To completely remove cellular components, the supernatant was separated and centrifuged at 13000rpm using a cooling centrifuge (Pro-Research, Centurion scientific, UK) at 4ºC for 10mins.

Total RNA including mi-RNAs' extraction was performed using Direct-zol™ RNA MiniPrep (cat. no. R2050, Zymo Research, USA) according to the protocol provided by the manufacturer which involved lysis using trizol reagent TRI Reagent® then

washing of the lysate in a spin column; the Zymo-Spin™ IIC Column. Briefly, 3 times' plasma volume (600 µl) of TRI Reagent® (cat. no: R2050-1-50) was added to the plasma volume (200 µl) to disrupt proteins and plasma components while maintaining RNA integrity. The mixture was mixed well by vortexing for 2mins and then incubated for 5mins at room temperature. To remove the particulates, the mixture was centrifuged at 13000 rpm for 1min using cooling centrifuge (Pro-Research, centurion scientific, USA). Following this step, synthetic 3.75µl (25 fmol) ***Caenorhabditis elegans*** miR-39 (cel-miR-39) was spiked in the supernatant of sample TRI homogenate. One and half volume ethanol was then added to and mixed well by vortexing for 2mins. miR-39 was selected as a reference owing to the absence of any similarity between its' sequence and human mi-RNAs and being not changed by any diseases which is the case in housekeeping genes [103, 104]. The mixture was then loaded into Zymo-Spin™ IIC Column and centrifuged at 13000rpm for 1min. The column was then transferred to a new collection tube and 400µl of Direct-zol™ pre-wash Buffer were added to the column and the column was centrifuged at 13000rpm for 1min. The former step was repeated twice then 700µl of RNA wash buffer were added to the column and centrifuged at 13000rpm for 1min. The column was then centrifuged in an emptied collection tube for additional 2mins. RNA and mi-RNAs were then eluted using DNase/RNase free water. Various elution volumes/methods have been tested for 10x dilution of synthetic miR-39 (100pg/ µl), for optimization purposes, using either 1X50µl, 2x25µl or 3x16.5µl DNase/RNase free water. The quality and quantity of extracted RNA was determined using Nanodrop (Q5000 UV-Vis Spectrophotometer, Quawell, USA) through measurement of A260/A280 ratio.

### 3.2.2 Poly Adenylation

Poly Adenylation was carried out on 100ng of total extracted RNA using E. coli Poly (A) Polymerase | NEB (New England Biolabs). The former step was firstly optimized trying different concentrations of total extracted RNA as an input (50, 100, 250 and 500 ng). The reaction mixture volume was 10µl and prepared by mixing a volume of extracted RNA equivalent to 100ng concentration with 1µl of *E. coli* Poly (A) Polymerase. Reaction Buffer, 1µl (5mM) ATP, 0.2µl E. coli Poly (A) Polymerase and Dnase/Rnase free water. The reaction was incubated at a dry bath incubator (Boeco, Germany) at 37ºC for 30mins.

### 3.2.3 Reverse transcription

Reverse transcription (RT) was carried out on 4µl of poly adenylated RNA using GoScript Reverse Transcription (Cat. no.A5001, Promega, USA). The reaction mixture volume was 20µl and prepared by mixing 4µl of poly adenylated RNA with 1 µl (20pmole) gene specific primer. The reaction was then placed into a dry bath incubator at 70 ºC for 5mins. Then immediately the reaction was chilled in ice-water for at least 5mins. Another tube of reverse transcription was prepared by adding 4µl GoScript™ 5X Reaction Buffer, 4µl MgCl (5mM), 1µl PCR Nucleotide Mix (0.5mM each dNTP), 1µl Recombinant RNasin®Ribonuclease Inhibitor, 1µl GoScript™ Reverse Transcriptase and 4µl Dnase/Rnase free water. The two reaction tubes were then added to each other and then were incubated at 25 ºC for 5mins for annealing. To allow strand extension, the reaction tube was then incubated at 42 ºC for 60mins. Finally for reaction inactivation, the reaction was incubated at 70 ºC for 15mins.

### 3.2.4 Quantitative real-time PCR

Real-time pcr was firstly carried out on 2µl of either stock, 2.5x, 5x or 10x dilution of cDNA obtained from reverse transcription step using Gotaq qpcr master mix (Cat. no.A6001, Promega, USA). The master mix includes a SYBR® Green I like fluorescent DNA-binding dye that presents excessive fluorescence enhancement when it binds to double-stranded DNA (dsDNA). The reaction mixture volume was 20µl and prepared by mixing 2µl of cDNA with 0.8µl (0.4µmol) gene specific forward primer and 0.8µl (0.4µmol) of gene specific reverse primer, 0.2µl carboxy-X-rhodamine (CXR) reference dye, 10µl master mix and 4.2µl Dnase free water. To ensure that taq polymerase enzyme is not having a reverse transcriptase activity, a trial was performed on a prepared poly adenylated miR-39 without adding reverse transcriptase enzyme in the RT-step. The amplification plot of this step gave undetectable Ct, this result ensured that taq-polymerase catalyze PCR without having reverse transcriptase activity. The mi-RNA-specific primers are designed by Dr.Abdullah Gibriel depending on miRNA sequences obtained from the miR-Base database (http://www.mirbase.org/) and he has the proprietary right for the sequence. The annealing temperatures of the 3 selected miRNAs in addition to miR-39 were optimized at different temperatures (50 ºC, 55 ºC, 60 ºC, 65 ºC, 70 ºC and 75 ºC). The reaction was then placed into real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Singapore). The instrument was operated with the following

conditions: 95 ºC for 2mins for activation followed by 95 ºC for 5secs denaturation then 60 ºC for 30secs for annealing step and finally 72 ºC for 20secs for extension. The device was adjusted to perform 50 cycles of the previous conditions followed by running a melt curve.

## 3.3 Data analysis

The results of the real-time PCR system is expressed in term of cycle threshold (Ct), which is the number of cycles needed for the fluorescent signal to cross the automated threshold. First, ΔCt was estimated by subtracting the Ct value of mi-RNA-39 from the Ct of each targeted mi-RNA for the same sample. Then ΔΔCt was calculated by subtracting the mean ΔCt of the reference group (Chronic HCV infected category) from ΔCt of each sample for each mi-RNA.

ΔΔCt of each mi-RNA= [for each sample (Ct target mi-RNA- Ct miR-39) – mean (Ct target mi-RNA- Ct miR-39) of the reference group] [105].

This is then followed by calculating the fold change, which is equal to 2-ΔΔCT (2 to the power of minus Delta Delta CT). Then transforming 2-ΔΔCt to log2 fold change. The value of log2 $\geq 0.5$ or $\leq -0.5$, were considered as notable differentially expressions.

## 3.4 Statistical analysis

Supplementary comparisons were carried out between HCC group and Non-HCC (Control, CH and cirrhosis). Quantitative values were expressed by mean ± SE. They were compared by one way anova (ANOVA), post hoc test and chi-square test when appropriate with P value $\leq 0.05$ is considered significant. Pearson correlation was used to study the correlation between studied mi-RNAs in each category. Receiver operator characteristic (ROC) curves were drawn to investigate the accuracy and area under the curve (AUC) was calculated. All statistical analysis were done using computer program Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) software 15 and MedCalc (MedCalc Software, Ostend, Belgium) version 15.0 for Microsoft Windows.

# Chapter 4 RESULTS

## 4.1 Demographic data and clinical features of study subjects

Demographic and clinical data records are summarized in (**Tables 2, 3**) and (**Figures 14-29**). There was no significant differences in gender distribution among the four groups (*P*=0.4622). Cirrhotic and HCC patients had significant increased levels in ALT (*P*=0.042 and *P*<0.0001 respectively) and AST (*P*=0.03 and *P*<0.0001 respectively) compared to healthy control. However, elevated levels of ALT and AST appeared to non-significant in CHC patients compared to healthy control (*P*=0.124 and P=0.513 respectively). ALP and total bilirubin levels were significantly elevated among the three diseased groups compared to healthy control subjects (*P*<0.0001, *P*=0.004 respectively). On the other hand, Albumin was significantly reduced in the three diseased groups compared to healthy control (*P*<0.0001). There was no significant difference in prothrombin time between the four categories (*P*=0.108). However, Total leukocyte and platelet counts appeared to be significantly deregulated among the four categories (*P*=0.014, *P*=0.004 respectively).

There were no significant differences in creatinine, HBA1C, MCV, MCH, MCHC, RDW, AFP and log10-viral load among the cirrhotic (*P*=0.956, *P*=0.999 respectively) and HCC groups (*P*=0.790, *P*=0.276 respectively) when compared to CHC. However, hemoglobin, RBC's levels and PCV were significantly dysregulated among the three diseased categories (*P*<0.0001, *P*=0.001 and *P*<0.0001 respectively). There had been a marginal significance in urea levels between the three diseased groups ( *P*=0.042)

## 4.2 Optimization steps

Several trials had been made on pure synthetic miRNA (mir-39) sample dissolved in DNase/RNase free water to optimize all our study steps and to ensure obtaining the most optimum results. Extraction step had been optimized using different volumes of DNase/RNase free water as shown in (**Table 4**). Elution with 50µl for one time yielded 16.1% with purity of 2.147. However, when the same column was eluted for a second time with 50µl, the yield was 5.56% with purity of 1.706, to have a total yield of these two steps= 21.66%. On the other hand, when a new column was eluted three times with 25µl, the first two times yielded 14% with

purity= 1.969. While the third time elution yielded 27.5% with purity=1.493, to have a total yield for the three steps= 41.5%. Finally when a new column was eluted four times with 16.5µl, the first three times yielded 132.68% with purity=1.642. Yet, the last elution yielded 3.5% with purity=1.655, to have a total yield for the four steps=136.18%. Our results revealed that eluting the column with 16.5µl of Dnase/Rnase free water for three times would enable the elution of most (132.68%) of the RNA attached to the column with A260/A280 value of (1.642).

Furthermore, our results (**Table 5**) indicated that plasma had a high content of mi-RNA (33.5 ng/µl±0.9), yet of low purity (0.9) because of the protein components. This was obvious in the difference in purity measured for optimized samples that were processed in Dnase/Rnase free water that yielded (purity=1.67±0.01) and the actual plasma samples (purity=0.9). These results are in agreement with the data supplied by Liping Wang et al. [106] and Inés Moret et al. [107] that pointed out that several mi-RNA plasma extraction practices gave rise to low 260/280 ratios around 1. Real time pcr data analysis of the extracted samples with high yield and relatively low purity had given cycle threshold at comparable and dynamic ranges, verifying that we could progress to polyadenylation step with this yield and purity.

When we reached the poly adenylation step, serial concentrations (50ng, 100ng, 250ng, 500ng) as shown in (**Table 6)** had been tested to investigate the optimum concentration of total extracted RNA that would be sensitive at the lowest cycle threshold value. Data indicated that there hadn't be a significant difference in Cts of (100ng, 250ng and 500ng) that gave (27.67, 27.74 and 27.92 respectively). These results indicated that the enzyme saturates after utilizing a concentration of 100ng. However, utilizing 50ng gave a large Ct of 30.1. Our results indicated that the utilization of 100ng of total extracted RNA in poly-adenylation step is considered to be the optimum concentration (**Figures 30**).

Regarding the RT-qPCR optimization, several dilutions were tested (stock, 2.5x dilution, 5x dilution and 10x dilution) as shown in (**Table 7)**. The cycle threshold obtained for stock, 2.5x, 5x and 10x was 23.56, 23.65, 22.94, and 23.7 respectively. Our data revealed that utilizing 2µl of 5x dilution of cDNA would be the optimum choice (**Figures 31**). Despite the fact that the four trials gave an acceptable dynamic and comparable Ct, but we didn't select a high concentration owing to the fact that the

extracted plasma samples contain PCR inhibitors [108]. Besides highly diluted samples would not have enabled us to detect mi-RNAs in samples if present in very low concentrations.

Moreover, the best annealing temperature (**Table 8)** was selected after performing RT qPCR for the 4 miRNAs (miR-39, miR-122, miR-21 and miR-155) at different temperatures (50ºC, 55ºC, 60ºC, 65ºC and 70ºC). miR-39 gave (22.67, 23.1, 25.66, 30.96 and undetected Ct) at (50ºC, 55ºC, 60ºC, 65ºC and 70ºC respectively) (**Figures 32**). miR-122 gave (33.1, 32.31, 34.26, 39.98 and undetected Ct) at (50ºC, 55ºC, 60ºC, 65ºC and 70ºC respectively) (**Figures 33**). miR-21 gave (27.19, 28.15,33.92, 41.86and undetected Ct) at (50ºC, 55ºC, 60ºC, 65ºC and 70ºC respectively) (**Figures 34**). miR-155 gave (38.93, 42.17, undetected, and undetected Ct) at (50ºC, 55ºC, 60ºC, 65ºC and 70ºC respectively) (**Figures 35**). Our results have demonstrated that 50 ºC is the optimum annealing temperature for all miRNAs as it gave the lowest Ct value at comparable and dynamic range with a single peak upon performing melt curve analysis.

## 4.3 Differential expression of plasma mi-RNAs in healthy controls, Cirrhotic subjects and HCC patients

Plasma mi-RNA profiles of miR-122, miR-155 and miR-21 were analyzed in healthy control, cirrhotic and HCC patients compared to chronic hepatitis C patients (**Table 9**). The mean levels of mi-R-122 were significantly dysregulated ($P$<0.0001) in healthy control, cirrhotic and HCC group compared to CHC with log2 fold change= (0.88±0.65 vs 0.00±0.57 vs 5.45±1.31, log2 fold change± standard error) (**Figures 36)**. Noting that log2 fold scale increase is equivalent to 32x increase in linear scale. Interestingly, miR-122 was significantly up-regulated in HCC patients compared to healthy controls and cirrhotic subjects ($P$=0.002 and $P$<0.0001, respectively). The log2 fold down regulation in cirrhotic group compared to healthy control was non-significant ($P$=0.772).

The mean levels of mi-R-21 were significantly dysregulated (P=0.005) in healthy control, cirrhotic and HCC group compared to CHC with log2 fold change= (3.52±1.07 vs 0.88±0.90 vs 6±1.08, log2 fold change± standard error, respectively) (**Figures 37)**. However, miR-21 was significantly up-regulated in HCC patients compared to cirrhotic patients with Log2 fold change of 6 (p=0.03) (which is

equivalent to 64 times linear increase) but not compared to healthy control (P=0.093). Although Control and HCC in mir-21 increased by 3.52 and 6 log2 scale they were in marginal insignificance (*p*=0.093) that might be due to limited number of samples used in this study.

The mean level of miR-155 was non-significantly (P=0.064) dysregulated in healthy control, cirrhotic and HCC group compared to CHC (4.12±1.13 vs -2.42±0.93 vs 2.72±0.96, log2 fold change± standard error, respectively) (Figures 38).

## 4.4 Evaluation of mi-RNAs as potential diagnostic markers

ROC curves (**Table 10**) and (**Figures 39, 40**) were conducted and the results revealed that miR-122 differentiated HCC patients from healthy control and cirrhotic patients with an AUC of 0.705 (*P*=0.002) and 0.767 (*P* <0.0001) respectively. The best specificity and sensitivity to differentiate HCC from control were 57.50% and 65.52% (at a cutoff >1.86, positive predictive value=52.75%, negative predictive value=57.15%, likelihood ratio positive (LR+) = 1.3 and likelihood ratio negative (LR−) = 0.6)). The best specificity and sensitivity to differentiate cirrhotic from HCC patients were and 61.54% and 71.43% (at a cutoff> 0.43, positive predictive value=57.15%, negative predictive value=75%, LR+ =1.83 and LR− = 0.464) to discriminate cirrhotic patients from HCC subjects.

Additionally, analyzing ROC curve (**Table 11**) and (**Figures 41**) indicated that miR-21 differentiated HCC patients from cirrhotic patients with AUC of 0.767 (*P*<0.0001). The best sensitivity and specificity to differentiate HCC patients from cirrhotic were 72.97% and 50% (at a cutoff >-0.43, positive predictive value=65.9%, negative predictive value=58.3% LR+ =1.4 and LR− = 0.54).

## 4.5 Correlations between studied mi-RNAs in healthy control, cirrhotic patients and HCC subjects

Correlation between plasma levels of the selected mi-RNAs were examined in healthy control, cirrhotic and HCC subjects using Pearson correlation (**Tables 12-14**). Correlation is considered significant if *P*≤0.05.On one hand, in healthy control subjects a correlation was found only between miR-122 and miR-155 (r=0.472, *P*=0.020). On the other hand, no correlation between studied mi-RNAs were found in the cirrhotic group. However, in HCC group, several significant positive correlations

were found between the tested mi-RNAs. miR-122 was correlated with miR-21 (r=0.789, *P*<0.0001), miR-122 was correlated with miR-155 (r=0.681, *P*=0.004) and miR-21 was correlated with miR- 155 (r=0.684, *P*=0.05).

## 4.6 Logistic regression analysis of the three miRNAs combination

Logistic regression analysis was performed to assess the diagnostic power of combing mi-RNAs to differentiate between healthy control and HCC subjects (**Table 15)**, cirrhotic and HCC patients (**Table 16)** and healthy control and cirrhotic patients (**Table 17)**. Results revealed that combining the three mi-RNAs to differentiate healthy control from HCC subjects would relatively increase the AUC to a value of 0.723 (*P*=0.049) compared to an AUC=0.705 (*P*=0.002) upon utilizing miR-122 as a standalone biomarker. On the other hand, combining two mi-RNAs (miR-122 and miR-21) to differentiate cirrhotic patients from HCC subjects would relatively increase the AUC to a value of 0.809 (*P*<0.0001). Interestingly, logistic regression data analysis revealed that combining the three mi-RNAs may have a promising diagnostic potential to differentiate healthy control from cirrhotic subjects with AUC of value 0.922 (*P*=0.0007).

## 4.7 Correlation between plasma mi-RNAs and clinical data in Cirrhotic and HCC patients

Correlations between alfa feto protein levels and mi-RNA levels were examined in cirrhotic and HCC groups. No significant correlation between the studied mi-RNAs and AFP levels in cirrhotic group. Interestingly, in HCC group, several significant correlations were found between AFP levels and each studied mi-RNA. Plasma miR-122 was significantly correlated with AFP levels (r=0.572, *P*=0.026) in the positive direction (**Figures 42-A)**. There was a significant positive correlation between plasma miR-21 and AFP levels (r=0.556, *P*=0.036) (**Figures 42-B)**. Plasma miR-155 was positively correlated with AFP (r=0.993, *P*=0.002) (**Figures 42-C).**

# Chapter 5 DISCUSSION

Hepatitis C virus (HCV) infection is a devastating health challenge that affects more than 185 million individuals globally [6]. Unresolved chronic HCV infection could develop to more worsening stages such as cirrhosis and/or HCC. The latter is the fifth most common cancer that leads to death between men [109]. The lack of reliable biomarkers for HCC early diagnosis and prognosis is a current challenge that quest the finding of new sensitive and specific diagnostic tools.

Egypt has the top-most incidence for chronic HCV infection worldwide [110]. This fact made our decision in selecting genotype4 to be investigated in our study. All enrolled patients were genotype4 with detectable HCV RNA viral load and anti-HCV antibody positive results. Our demographic data analysis indicated that ALP and total bilirubin levels are significantly elevated in all three diseased categories; chronic hepatitis, cirrhosis and HCC compared to healthy control. On the other hand, albumin levels were significantly reduced in the all three diseased categories compared to healthy control. Creatinine, urea and viral load data indicated that there had been no significant difference in their levels between the three diseased categories. There hadn't been significant difference in AFP levels among the three diseased categories which confirm the results of Di Bisceglie et al. that AFP levels are elevated in advanced CHC and that serum AFP values are frequently elevated, even in the nonexistence of HCC [110]. This as well confirm the idea that AFP is lacking specificity [2, 110].

Recent studies have shed light on miRNAs and their role in different hepatological disorders. Studies have revealed the important role of these short sequenced miRNA in different clinical conditions including protein regulation, liver cells' apoptosis and hepatic carcinogenesis through the regulation of oncogenes' transcription factors and tumor suppressor genes [111]. Besides, researches have revealed their role in obstructive jaundice [112] and hepatic stellate cells fibrosis [113].

The main goal of our study was to investigate the expression of plasma miR-122, miR-155 and miR-21 as potential and reliable non-invasive biomarkers that can differentiate between different stages of HCV infection in Egyptian population and used as a sensitive and specific biomarker for HCC. Our data revealed that miR-122 results and miR-21 are significantly des-regulated among the four selected categories;

healthy control, CHC, cirrhosis and HCC. However, miR-155 was des-regulated but with non-significance.

Analysis of miR-122 expression have demonstrated significant fold increase in plasma expression level in HCC group compared to healthy control, CHC and cirrhosis. This result coincides with previous studies showing up-regulation in miR-122 in HCC [101, 114, 115]. The up-regulation of miR-122 may be attributed to its' induction with severe hepatocytes' injury in cases of HCC [116]. However, in other studies miR-122 was down-regulated in HCC human tissue and cell lines [117,118,119,120]. This conflict in results may be attributed to the leakage of miR-122 from the injured liver cells to the blood stream. As a result elevating miR-122in serum and plasma and reducing miR-122 levels in human tissue [116]. Another conflicting study have demonstrated that miR-122 levels were non-significantly down-regulated in human serum compared to healthy control [120]. The discrepancy between the results of our study and this study might be due to multiple reasons; first the difference in samples used; plasma vs serum samples. Second this might be due to the use of a different normalizer which is RNU48 in that case not synthetic miR-39 as the one used in our study. There has been conflicting issues concerning the use of proper normalizers such as housekeeping genes, small nucleolar rna SNORD, RNU48, etc [104].The miRNA length is very short and should not be normalized to large RNA such as housekeeping gene. Even SNORD and RNU48 are not favored anymore for normalization as their expression have been reportedly to be affected with various disease conditions and not to be stably expressed [121]. Moreover this might be due to using different reference group which is healthy control in that case. Fourthly, this could be due to different etiology and origin of HCC cases as they might arise from various HCV genotypes that could have different molecular pathogenesis [122,123].

In addition, the fold change of miR-122 had been shown to be non-significant in cirrhotic stage compared to healthy control. Yet, this result was different from another study made on 75 HCV chronically affected patients; 25 were CHC, 25 were cirrhotic and 25 were HCC that had demonstrated significant down regulation in cirrhotic samples compared to healthy group [124]. Again this might be attributed to the factors listed above or due to lack of representative number of samples in that study. Another study had pointed out that miR-122 expression levels are downregulated in late fibrotic stages in mice liver biopsies [125]. On the other hand,

our results have demonstrated significant up regulation in HCC samples compared to cirrhotic patients. This comes in agreement with several studies that have shown significant elevated levels in miR-122 in HCC compared to cirrhotic group [116,124,126]. Besides an Egyptian study performed on serum samples came in confirmation of these results [115]. In contrast to our results, the study of Köberele et al. have demonstrated non-significant elevation in serum miR-122 expression levels in HCC group compared to cirrhotic patients [127]. The inconsistency in cirrhotic result might be attributed to large ethnic and geographic variability in the prevalence of HCC and cirrhosis between the different populations. In addition to the difference in samples investigated in our study and other studies.

Regarding the diagnostic performance of miR-122 in HCC. Our results revealed that miR-122 was having a good diagnostic power to differentiate HCC patients from healthy control subjects with (AUC=0.705), sensitivity=65.52% and specificity=57.50%. Besides having a good diagnostic power differentiating HCC patients from cirrhotic patients with (AUC=0.767), sensitivity=71.43% and specificity=61.54%. This comes in agreement with the results obtained from Jian Xu et al. confirming that ROC curve results obtained from miR-122 expression could discriminated between HCC and healthy control and those suffering from chronic hepatitis [101]. The former study has shown ROC analysis with (AUC= 0.79), sensitivity=71% and specificity=86%. An Egyptian study had partially agreed with our results, revealing that serum miR-122 have a good diagnostic accuracy in differentiating HCC patients from healthy control with (AUC=0.617) [115]. The previous data in addition to knowing that miR-122 is a liver specific mi-RNA would make miR-122 a very promising diagnostic marker for HCC. All miR-122 studies are listed in (**Table 18**).

Des-regulation of miR-21 had been found to play an important role in different malignancies. The former include up-regulation in tissue [128] and serum levels [129] in large B-cells lymphoma. Besides being up-regulated in tissue in case of glioblastoma tumor [119, 120]. Moreover, miR-21 experiences up-regulation in cases of breast cancer [130], colon, lung, pancreas, prostate, stomach [131] pancreatic cancer and adenocarcinomas [132].

The present study demonstrated up-regulation in miR-21 expression levels in HCC patients compared to cirrhotic patients and also in comparison to healthy control group. This upregulation was reported to be significant in comparison to cirrhotic

group with a p-value of (0.005). This upregulation was found to be of marginal insignificance when compared to healthy control group with a p-value of (0.093). This coincides with several studies that have shown elevated expression levels of miR-21 in HCC plasma [98, 101] and tissue [99, 119]. Additionally our results are in partial agreement with an Egyptian study carried on three groups; healthy control, CHC and HCC [133]. Their data revealed that miR-21 is significantly up-regulated in HCC plasma samples compared to CHC which involved cirrhotic samples which is the case in our study. On the other hand, their data demonstrated significant elevation in plasma miR-21 expression in HCC compared to healthy control which is contrary to our results that have shown up-regulated levels but with a marginal non-significance values. Furthermore, our data were contradicted with Verena Bihrer et al. who have demonstrated that miR-21 up-regulation levels in HCC tissues and plasma weren't significant compared to CHC samples of German patients [83]. Noting that the previously mentioned study has involved chronic hepatitis and HCC patients of different etiologies.

Our results have shown down-regulation in miR-21 expression levels in CHC and non-significant decrease in the expression levels of cirrhotic patients compared to healthy control. In contrast to our results, several studies have demonstrated up-regulated expression levels in miR-21 in serum, plasma [81, 133] and hepatocytes [97] of CHC compared to healthy control. Again this inconsistency in the results might be attributed to variability in the prevalence of HCC and cirrhosis between the different populations. In addition to the presence of different genotypes that might show different expression in every mi-RNA.

Regarding the diagnostic performance of miR-21 in HCC. Our results revealed that miR-21 was having a good diagnostic power to differentiate HCC patients from cirrhotic patients with (AUC=0.775), sensitivity=72.29% and specificity=50%. This comes in agreement with the results obtained from Yoshito Tomimaru et al. confirming that ROC curve results obtained from miR-21 expression could discriminated between HCC and those suffering from chronic hepatitis [98]. The former study has shown ROC analysis with (AUC= 0.773), sensitivity=61.61% and specificity=83.3%. The previous data suggest that miR-21 can make a powerful marker investigating the prognosis from cirrhosis to HCC.

Interestingly half of the total number of samples (n=78 out of n=156) gave undetectable cycle threshold Ct, even with multiple input of 2x dilution and

5microliter cDNA, with miR-155 after q RT-PCR operation regardless the category classification of the sample. Worth mentioning that several approaches were made to enhance the detection of this mi-RNA. First, higher concentration of extracted RNA had been used, instead of using 100ng of extracted RNA in poly-adenylation step, 250ng, 300ng and 500ng of the undetectable samples were investigated with undetectable results in q RT-PCR as well. Additionally another trial was made elevating the concentration of utilized cDNA in the q RT-PCR step, rather than using 2µl of 5x dilution of prepared cDNA, 5µl of 2x dilution of prepared cDNA was utilized with non-significant change in the primary results. Accordingly, these undetected samples were ignored and only samples that gave detectable values of Ct were utilized in calculations.

Des-regulation of miR-155 had been investigated in different cancers. These include breast, colon, lung [115] and large B-cell lymphoma (DLBCL) [129]. miR-155 is proposed to play an important for host antiviral innate immune response [90, 91]. Therefore, studying of the role of these microRNAs in HCV infection could help in understanding HCV molecular pathogenesis. Our study revealed non-significant up regulation in miR-155 expression in HCC and healthy control compared to CHC and cirrhotic categories. Additionally, results illustrated non-significant down-regulation in miR-155 expression in cirrhotic category compared to CHC, healthy control and HCC. These results coincides with Nada El-Ekiaby et al study [95] on PBMC. Among the results of this Egyptian study that no significant difference was found regarding miR-155 expression levels in PBMCs of Egyptian patients genotype 4 with chronic infection of HCV when being evaluated against healthy subjects. On the other hand, this was in contrary to another Egyptian study that was performed on serum samples of HCC and chronic hepatitis patients [78]. On one hand, a reason for this variation could be that the study conducted used serum rather than human plasma for their investigation. In addition, this might be due to the smaller no of samples employed for each category; utilizing around 20 samples for each category rather than employing around 40 samples. Finally, this study utilized RNU48 rather than synthetic miR-39 as reference for the samples. Moreover our results were opposite to others studies' findings[134] performed on serum and monocytes samples using 18S as a normalizer and [135] performed on liver tissue that have reported significant up-regulation in miR-155 expression in serum or plasma of CHC samples compared to

healthy control. The study that had been performed on liver tissue utilized 30 HCC samples and 8 control, in addition to using U6 RNA as a normalizer.

Logistic regression analysis revealed that combining miR-122, miR-21 and miR-155 might have a slight increase in the differentiation potential (AUC=0.723) between healthy control subjects and hepatocellular carcinoma patients. Additionally, combining miR-122 and miR-21 (AUC=0.809) might have a good increase in the differentiation potential between cirrhotic subjects and HCC patients. Finally, combing the three mi-RNAs (AUC=0.922) would have a promising diagnostic power to differentiate healthy control from cirrhotic patients.

Our data demonstrated positive correlation between the three studied miRNAs in HCC patients only. Besides, our results have shown on one hand significant positive correlations between alfa feto protein levels and the expression levels of the three miRNAs. On the other hand, no correlations were found between the expressed miRNAs and AFP levels in cirrhosis category. This suggests concurrent expression of immune response related miRNAs in hepatocellular carcinoma subjects. Whether this concurrent expression of miR-122, miR-155 and miR-21 in HCC particular to HCV associated HCC remain unclear and needs more investigation.

Finally, our study is limited by relatively moderate number of patients; however, it suggests that miR-122 can be utilized as a potential specific diagnostic biomarker for HCV associated HCC cases. Additionally, that miR-21 can investigate the prognosis from cirrhosis to HCC. Moreover, miR-155 would not be ideal as a prognostic marker in plasma of HCV related HCC patients of genotype-4. Additional studies are required to validate these data.

# Chapter 6 CONCLUSIONS AND FUTURE PERSPECTIVES

Current biomarkers for HCC diagnosis are lacking both sensitivity and specificity. This necessitates the development of reliable biomarkers that are sensitive, specific and non-invasive for early diagnosis and rapid intervention of HCC to increase the survival rate of patients.

In this study, we investigated mi-RNA expression profile from the plasma of Egyptian patients with chronic hepatitis C, cirrhosis and HCV associated HCC compared with healthy control. We aimed to assess the diagnostic potential of the selected miRNAs to differentiate between HCC and other HCV associated disorders. In addition to studying the expression pattern in the different studied stages.

Using real time PCR, we compared the levels of circulating mir-122, miR-21 and miR-155 in plasma from healthy control (n=40), cirrhosis (n=39) and HCC (n=40) to CHC (n=37). Synthetic miR-39 was spiked in the samples to be used as a normalizing control for the samples. Plasma miR-122 was significantly up-regulated in HCC than in three other categories ($P<0.001$). The plasma level of miR-122 in HCC patients was significantly higher than healthy control and cirrhotic patients ($P = 0.002$ and $P<0.001$, respectively). The plasma level of miR-21 was significantly up-regulated in HCC compared to cirrhotic patients ($P=0.03$). This upregulation was found to be of marginal insignificance when compared to healthy control group. There had been no significant des-regulation in the expression levels of miR-155 between the 4 categories.

ROC curves' analysis revealed that miR-122 differentiated HCC patients from healthy control with a specificity and sensitivity 57.50% and 65.52% respectively (at a cutoff >1.86). To discriminate cirrhotic patients from HCC subjects, a specificity and sensitivity of 61.54% and 71.43% (at a cutoff> 0.43) were determined. Additionally, analyzing ROC curve indicated that miR-21 differentiated HCC patients from cirrhotic patients with best sensitivity and specificity 72.97% and 50% (at a cutoff >-0.43).

In conclusion, plasma miR-122, miR-21 may be used as sensitive and specific biomarkers for screening HCV associated HCC. Further studies are necessary to confirm the diagnostic value of the miR-122 and miR-21 as accurate plasma markers for HCC-associated HCV.

# TABLES

**Table 1. Examples of direct and indirect biomarkers utilized to assess liver diseases' severity**

| Indirect biomarkers | Direct biomarkers |
|---|---|
| 1. Aminotransferase (AST and ALT) | 1. ECM proteins (hyaluronic acid) |
| 2. Bilirubin | 2. Matrix metalloproteases (MMPs) |
| 3. Gamma-glutamyl transpeptidase (GGT) | 3. MMP inhibitors [tissue inhibitors of matrix metalloproteases (TIMPs)] |
| 4. Prothrombin time | 4. Fragments of procollagen III |
| 5. Albumin | |
| 6. platelet (PLT) counts | |

**Table 2. Demographic and clinical data of Healthy control, CHC, Cirrhosis and HCC**

| Parameter | Healthy control | CHC | Cirrhosis | HCC | *P* value |
|---|---|---|---|---|---|
| Age (years)<br>Range (years) | 46.82±1.68<br>21-65 | 45.43±1.54<br>25-64 | 57.46±0.91<br>43-76 | 47.10±1.68<br>21-65 | <0.0001 |
| Sex | | | | | 0.4622 |
| Male, n (%) | 24 (60%) | 25 (69.4%) | 20 (51.3%) | 24 (60%) | |
| Female, n (%) | 16 (40%) | 11(30.6%) | 19 (48.7%) | 16 (40%) | |
| ALT (IU/l) | 9.86±1.17 | 48.65±8.67 | 46.61±6.8 | 74.48±12.07 | <0.0001 |
| AST (IU/I) | 13.60±2.08 | 40.74±6.42 | 64.31±8.2 | 118.56±19.41 | <0.0001 |
| ALP (IU/I) | 40.29±3.39 | 113.33±8.77 | 168.71±17.8 | 187.62±24.6 | <0.0001 |
| Total bilirubin (mg/dl) | 0.73±0.09 | 1.00±0.19 | 4.07±0.98 | 3.44±0.85 | 0.004 |
| Albumin (g/dl) | 4.37±0.11 | 3.92±0.13 | 2.65±0.13 | 3.27±0.13 | <0.0001 |
| Prothrombin time (sec) | 16.10±0.22 | 13.68±0.32 | 18.8±0.8 | 17.71±1.80 | 0.108 |
| Total Leukocyte count ($x10^3/mm^3$) | 4.92±0.22 | 4.82±0.45 | 7.15±0.65 | 7.35±0.42 | 0.014 |
| Platelet count ($x10^3/mm^3$) | 197.90±9.84 | 176.09±25.26 | 124.39±12.98 | 142.83±11.26 | 0.004 |

Data are expressed by mean± standard error, or number (percentage) for gender. Statistical significance ($P \leq 0.05$).

**Table 3. Clinical and virological data of CHC, Cirrhosis and HCC**

| Parameter | CHC | Cirrhosis | HCC | *P*-Value |
|---|---|---|---|---|
| Creatinine (mg/dl) | 0.79±0.05 | 1.18±0.1 | 1.13±0.10 | 0.105 |
| Urea (mg/dl) | 25±1.73 | 60.98±7.8 | 57.33±9.10 | 0.052 |
| INR | 1.13±0.05 | 1.61±0.08 | 1.55±0.14 | 0.104 |
| AFP (IU/ml) | 7.15±1.26 | 7.38±1.92 | 20.96±3.91 | 0.194 |
| Viral load log10 (IU/ml) | 5.65±0.20 | 5.68±0.13 | 4.50±0.06 | 0.232 |
| Hemoglobin (mg/dl) | 14.66±0.77 | 10.65±0.39 | 11.61±0.31 | <0.0001 |
| HbA1C (%) | 5.40±0.23 | 6.71±0.24 | 6.03±0.17 | 0.305 |
| RBC's ($10^6$/mm³) | 4.75±0.24 | 3.64±0.11 | 4.14±0.12 | 0.001 |
| PCV (%) | 41.57±2.35 | 30.77±0.94 | 35.04±0.99 | <0.0001 |
| MCV (µm³) | 84.74±3.19 | 85.36±1.64 | 81.77±2.14 | 0.465 |
| MCH (pg) | 27.39±1.43 | 27.71±0.65 | 27.52±0.55 | 0.969 |
| MCHC (g/dl) | 32.09±0.06 | 32.42±0.40 | 32.43±0.30 | 0.928 |
| RDW (%) | 19.85±3 | 17.85±0.55 | 17.54±0.69 | 0.729 |

Data are expressed by mean± standard error. Statistical significance ($P \leq 0.05$).

**Table 4. Water elution step trials' for mi-RNA extraction optimization**

| Trials on 10x dilution of synthetic miR-39 (100pg/ µl) | Yield in % | A260/A280 |
|---|---|---|
| 1-a)Elution with 50 µl of dnase/rnase free water | 16.1 | 2.147 |
| 1-b)Elution with 50 µl of dnase/rnase free water on the same column | 5.56 | 1.706 |
| **Total yield** | **21.66** | |
| 2-a) Elution with 2 times 25 µl of dnase/rnase free water | 14 | 1.969 |
| 2-b) Elution with 1 time 25 µl of dnase/rnase free water on the same column | 27.5 | 1.493 |
| **Total yield** | **41.5** | |
| 3-a) Elution with 3 times 16.5 µl of dnase/rnase free water | 132.68 | 1.642 |
| 3-a) Elution with 1 time 16.5 µl of dnase/rnase free water on the same column | 3.5 | 1.655 |
| **Total yield** | **136.18** | |

**Table 5. Representative data of extracted RNA from different categories**

| Sample no. | Sample ID | 260/280 | Conc. (ng/µl) |
|---|---|---|---|
| 33 | Control | 1.381 | 13.04 |
| 36 | Control | 1.149 | 27.2 |
| 2 | Control | 0.994 | 39.96 |
| 23 | Control | 0.939 | 19.32 |
| 10 | Fibrotic | 0.728 | 24.44 |
| 26 | Fibrotic | 0.73 | 47.84 |
| 27 | Fibrotic | 0.703 | 28.12 |
| 5 | Fibrotic | 0.842 | 49.28 |
| 17 | Cirrhotic | 0.86 | 37.92 |
| 23 | Cirrhotic | 0.867 | 16.08 |
| 21 | Cirrhotic | 0.855 | 67.2 |
| 10 | Cirrhotic | 0.862 | 13.28 |
| 13 | HCC | 0.837 | 26.64 |
| 10 | HCC | 1.001 | 44.28 |
| 20 | HCC | 0.82 | 34.24 |
| 21 | HCC | 0.839 | 47.6 |

Mean± standard error of extracted RNA purity 260/280 is 0.9±0.01.
Mean± standard error of extracted RNA concentration is 33.5±0.9.

**Table 6. Poly-adenylation step optimization**

| Concentration in ng | Cycle threshold |
|---|---|
| 50 | 30.1 |
| 100 | 27.67 |
| 250 | 27.74 |
| 500 | 27.92 |

Different concentrations of total extracted RNA were investigated with corresponding cycle threshold.

**Table 7. q RT-PCR step optimization.**

| Dilution | Cycle threshold |
|---|---|
| Stock | 23.56 |
| 2.5x | 23.65 |
| 5x | 22.94 |
| 10x | 23.7 |

Different concentrations of cDNA were investigated with corresponding cycle threshold.

**Table 8. mi-RNA annealing temperature optimization**

| mi-RNA | Ct at 50 ºC | Ct at 55ºC | Ct at 60 ºC | Ct at 65 ºC | Ct at 70 ºC |
|---|---|---|---|---|---|
| miR-39 | 22.67 | 23.1 | 25.66 | 30.96 | Undetected |
| miR-122 | 33.1 | 32.31 | 34.26 | 38.98 | Undetected |
| miR-21 | 27.19 | 28.15 | 33.92 | 41.86 | Un detected |
| miR-155 | 38.93 | 42.17 | Undetected | Undetected | Undetected |

Gradient temperatures were investigated for every mi-RNA to select the optimum annealing temperature.

**Table 9. Log2 fold change of plasma mi-RNA expression levels in healthy control, cirrhotic patients and HCC compared to CHC patients.**

| mi-RNA | Healthy control | Cirrhosis | HCC | P value |
|---|---|---|---|---|
| miR-122 | 0.88±0.65 | 0.00±0.57 | 5.45±1.31 | <0.0001 |
| miR-155 | 4.12±1.13 | -2.42±0.93 | 2.72±0.96 | 0.064 |
| miR-21 | 3.52±1.07 | 0.88±0.90 | 6±1.08 | 0.005 |

Data are expressed by mean± standard error and were analyzed by one way anova. Statistical significance ($P \leq 0.05$).

**Table 10. ROC curve analysis of plasma miR-122 as a diagnostic marker in patients with HCC**

| miR-122 | AUC | *P*-Value | Best cutoff value (Log2 fold change) | Sensitivity % | Specificity % | PPV % | NPV % |
|---|---|---|---|---|---|---|---|
| Control | 0.705 | 0.002 | >1.86 | 65.52 | 57.50 | 52.75 | 69.72 |
| Cirrhotic | 0.767 | <0.0001 | >0.43 | 71.43 | 61.54 | 57.15 | 75 |

PPV; positive predictive value. NPV; negative predictive value.

**Table 11. ROC curve analysis of plasma miR-21 as a diagnostic marker in patients with HCC**

| miR-21 | AUC | *P*-Value | Best cutoff value (Log2 fold change) | Sensitivity % | Specificity % | PPV % | NPV % |
|---|---|---|---|---|---|---|---|
| Cirrhotic | 0.775 | <0.0001 | >-0.46 | 72.97 | 50 | 65.9 | 58.3 |

PPV; positive predictive value. NPV; negative predictive value.

**Table 12. Significant correlation between studied plasma mi-RNA in healthy control**

| mi-RNA | miR-122 | miR-155 | miR-21 |
|---|---|---|---|
| miR-122 | - | r=0.472<br>P=0.020 | NS |
| miR-155 | r=0.472<br>P=0.20 | - | NS |
| miR-21 | NS | NS | - |

Association between mi-RNA levels were determined in healthy control (n=40) using Pearson correlation. r: Pearson rho coefficient, NS: non-significant (*P*>0.05).

**Table 13. Significant correlation between studied plasma mi-RNA in Cirrhotic patients**

| mi-RNA | miR-122 | miR-155 | miR-21 |
|---|---|---|---|
| miR-122 | - | NS | NS |
| miR-155 | NS | - | NS |
| miR-21 | NS | NS | - |

Association between mi-RNA levels were determined in Cirrhotic patients (n=39) using Pearson correlation. r: Pearson rho coefficient, NS: non-significant (*P*>0.05).

**Table 14. Significant correlation between studied plasma mi-RNA in HCC patients**

| mi-RNA | miR-122 | miR-155 | miR-21 |
|---|---|---|---|
| miR-122 | - | r=0.681<br>P=0.004 | r=0.789<br>p<0.001 |
| miR-155 | r=0.681<br>P=0.004 | - | r=0.684<br>p=0.005 |
| miR-21 | r=0.789<br>p<0.001 | r=0.684<br>P=0.005 | - |

Association between mi-RNA levels were determined in HCC patients (n=40) using Pearson correlation. r: Pearson rho coefficient, NS: non-significant (*P*>0.05).

**Table 15. Logistic regression analysis to differentiate healthy control subjects from HCC patients utilizing mi-RNAs combinations**

| Selected miRNA | Area under the curve | *P*-Value |
|---|---|---|
| miR-122 | 0.705 | 0.002 |
| miR-21 | 0.567 | 0.0064 |
| miR-122 and miR-21 | 0.703 | 0.0002 |
| miR-122 and miR-155 | 0.688 | 0.0261 |
| miR-21 and miR-155 | 0.575 | 0.8632 |
| miR-122, miR-21 and miR-155 | 0.723 | 0.049 |

**Table 16. Logistic regression analysis to differentiate cirrhotic subjects from HCC patients utilizing mi-RNAs combinations**

| Selected miRNA | Area under the curve | *P*-Value |
|---|---|---|
| miR-122 | 0.767 | <0.0001 |
| miR-21 | 0.775 | <0.0001 |
| miR-122 and miR-21 | 0.809 | <0.0001 |
| miR-122 and miR-155 | 0.760 | 0.0428 |
| miR-21 and miR-155 | 0.792 | 0.0272 |
| miR-122, miR-21 and miR-155 | 0.839 | 0.0575 |

**Table 17. Logistic regression analysis to differentiate healthy control subjects from cirrhotic patients utilizing mi-RNAs combinations**

| Selected miRNA | Area under the curve | *P*-Value |
|---|---|---|
| miR-122 | 0.599 | 0.3077 |
| miR-122 and miR-21 | 0.745 | 0.0147 |
| miR-122 and miR-155 | 0.765 | 0.0474 |
| miR-122, miR-21 and miR-155 | 0.922 | 0.0007 |

**Table 18. Collection of studies performed on miR-122**

| No of patients | Type of sample | Category | Genotype /Region | Assay-used | Results | Reference |
|---|---|---|---|---|---|---|
| 190 | Serum | HCC, chronic hepatitis B | China | Quantitative real time pcr | miR-122 was upregulated in HCC compared to healthy control. miR-122 was upregulated in CHC compared to HCC. | 101 |
| 384 | Serum | CHC, cirrhosis and HCC | Egypt* | Quantitative real time pcr | miR-122 was significantly upregulated in HCC compared to healthy control and cirrhotic patients. | 115 |
| - | Cell line and liver tissue | HCC | Germany | Quantitative real time pcr | miR-122 was up regulated in HCC samples compared to healthy control | 116 |
| 75 | serum | CHC, cirrhosis and HCC | Egypt* | Quantitative real time pcr | miR-122 was significantly upregulated in the three diseased groups compared to healthy control | 124 |
| - | Liver tissue | CHC | USA | Quantitative real time pcr | miR-122 was downregulated in CHC. | 125 |
| - | Liver tissue | CHC | USA | Microarray | miR-122 was down regulated in HCC. | 126 |
| 249 | Serum | Cirrhosis and HCC | | Quantitative real time pcr | miR-122 levels didn't differ significantly between cirrhosis and HCC | 127 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 116 | Plasma | CHC, Cirrhosis and HCC | Egypt* | Quantitative real time pcr | miR-122 was significantly up regulated in HCC cases compared to healthy control and cirrhotic. There had been non-significant up regulation in cirrhotic group compared to healthy control. | Our study |

*Studies that were performed in Egypt. Our study was listed at last in the table.

# FIGURES

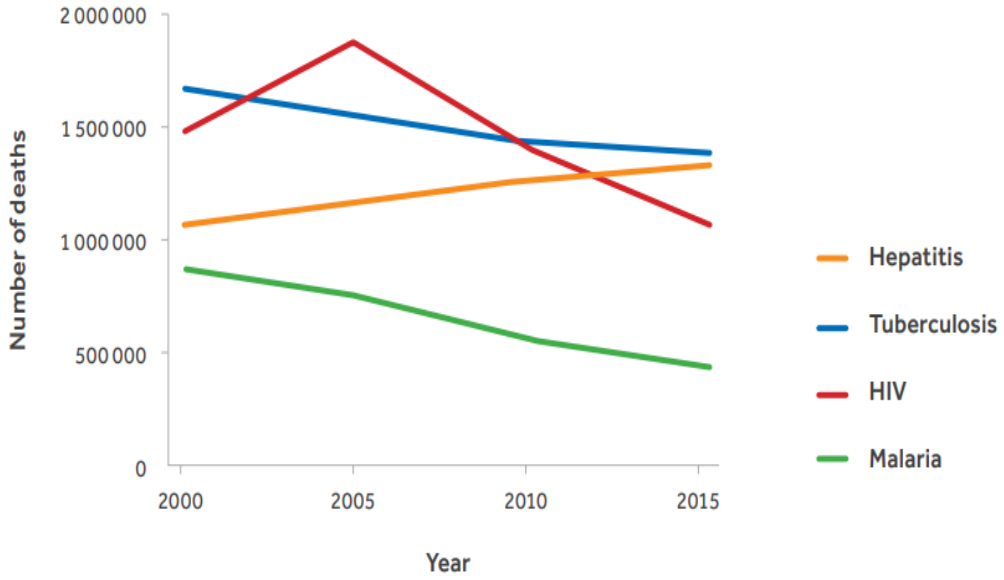



**Figure 1. Annual deaths from viral hepatitis, tuberculosis, HIV and Malaria. The incidence of viral hepatitis deaths is increasing over time. Retrieved from [8].**

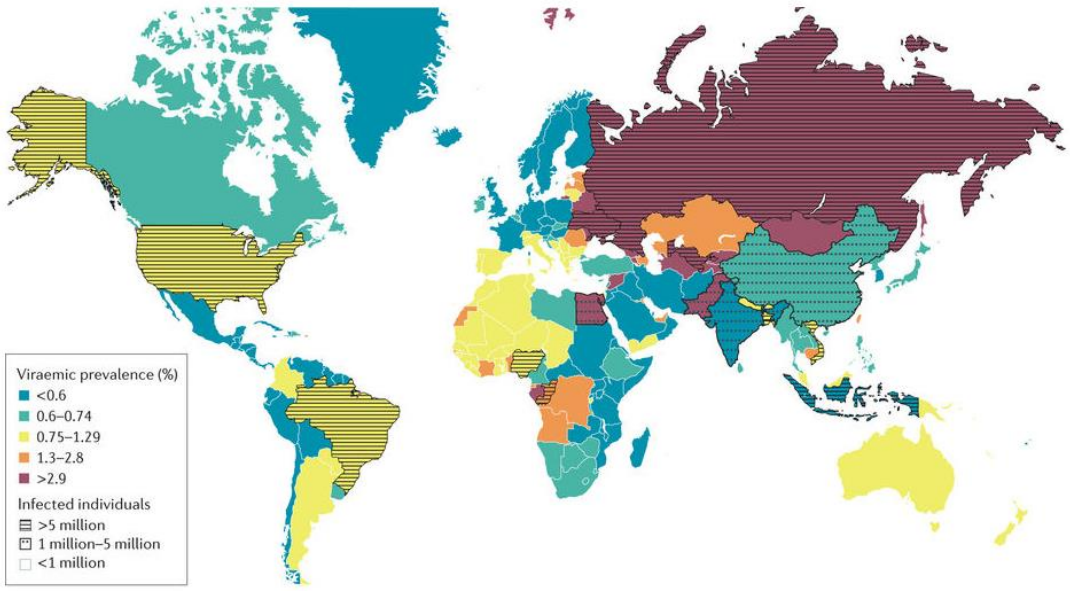



**Figure 2. An illustration of the viraemic HCV prevalence and the concluded total HCV cases for each country. Retrieved from [9].**


**Figure 3. Global prevalence of HCV infection by WHO. The Eastern Mediterranean region has the highest prevalence of HCV. Retrieved from [8].**

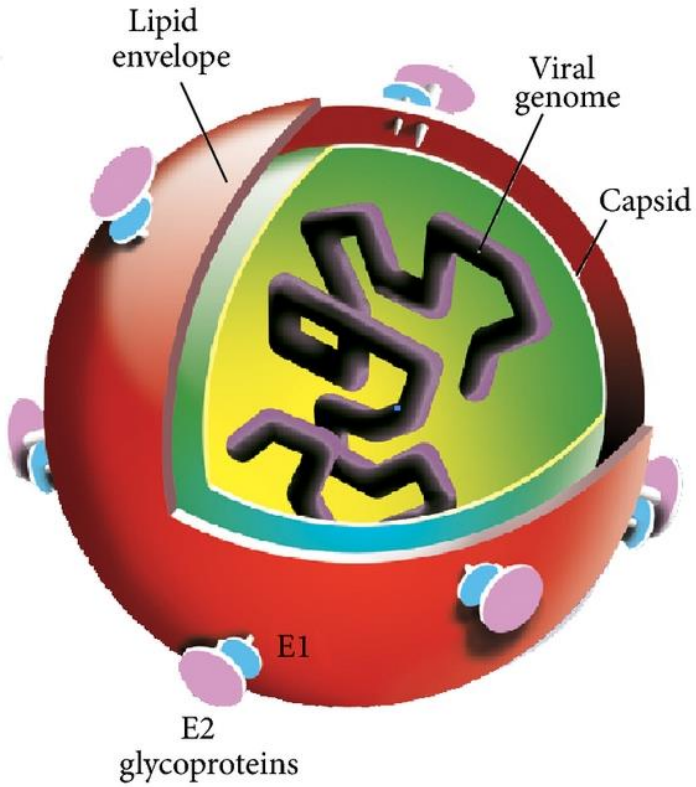



**Figure 4. HCV particle structure. HCV particle is made up of an envelope with glycoproteins, a nucleocapsid and the viral genome. Retrieved from [136].**

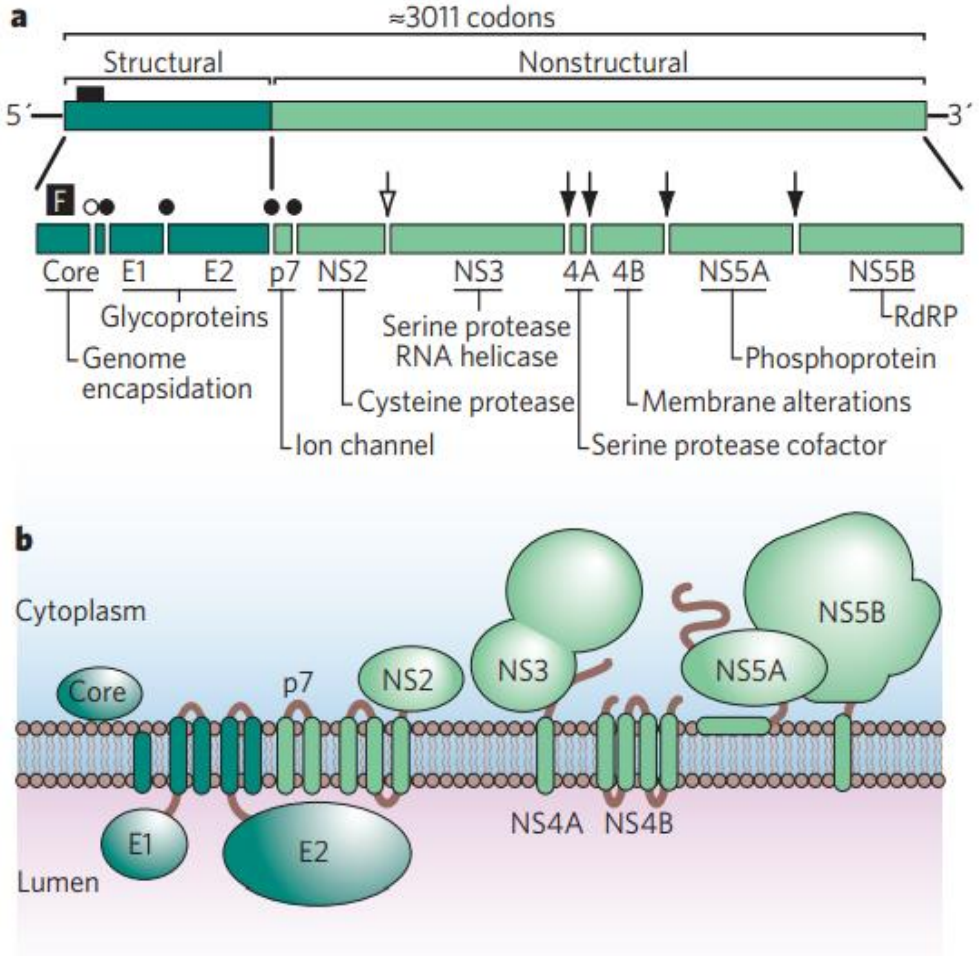



**Figure 5. HCV Virology. (a) Structure of the HCV genome and (b) summary of HCV polyprotein processing. Retrieved from [17].**

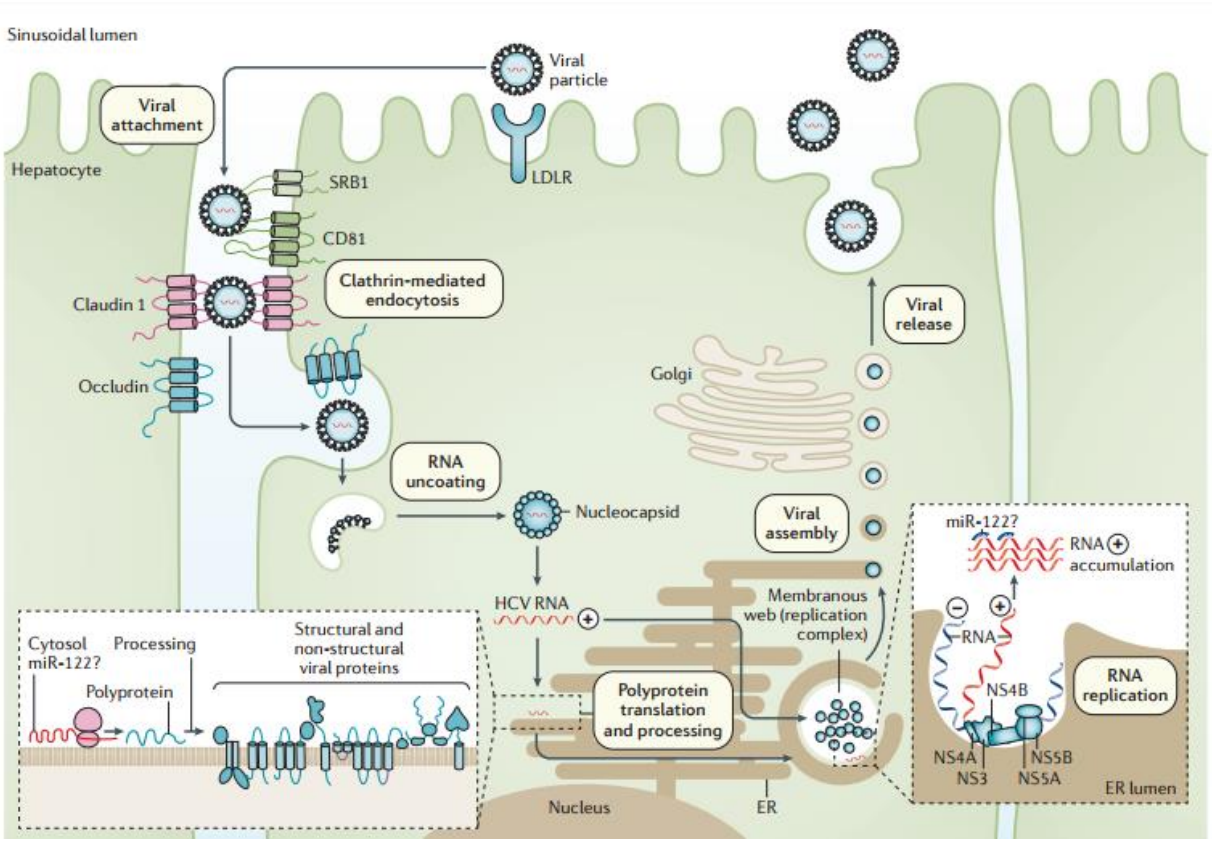



**Figure 6. Hepatitis C virus life cycle. Retrieved from [9]**

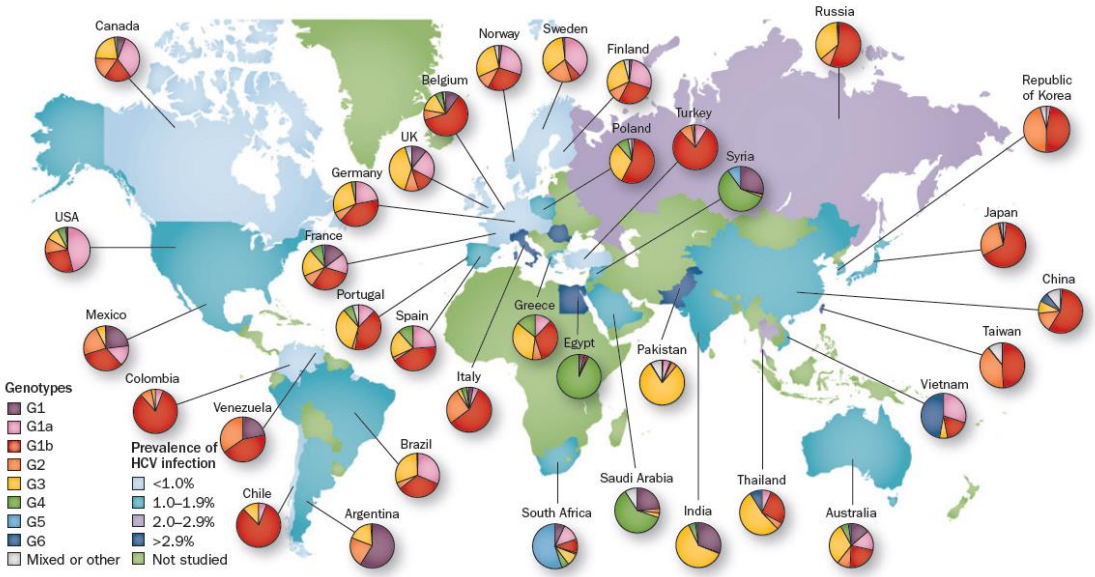



**Figure 7. HCV genotypes global prevalence. Retrieved from [26]**

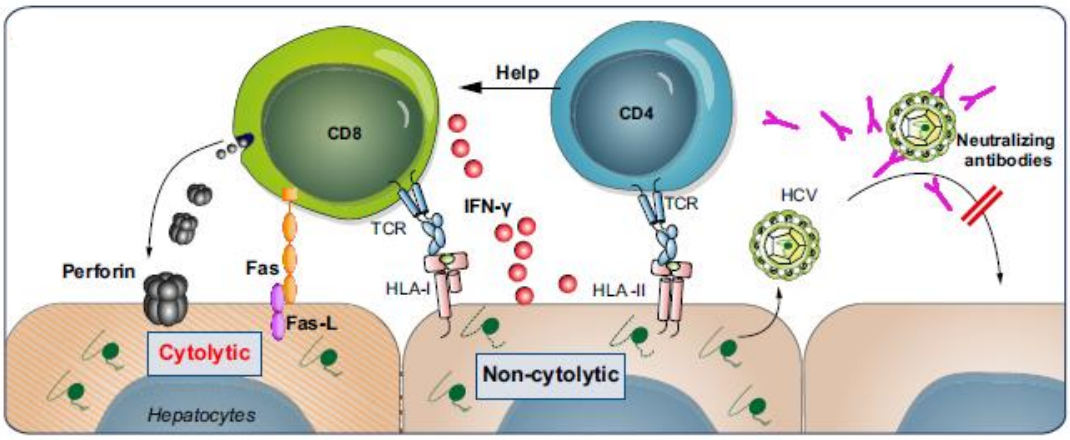



**Figure 8. HCV and immune response. Adaptive immune response, $CD8^+$ cells have antiviral activity through either cytolysis or non-cytolysis activity. Retrieved from [32].**

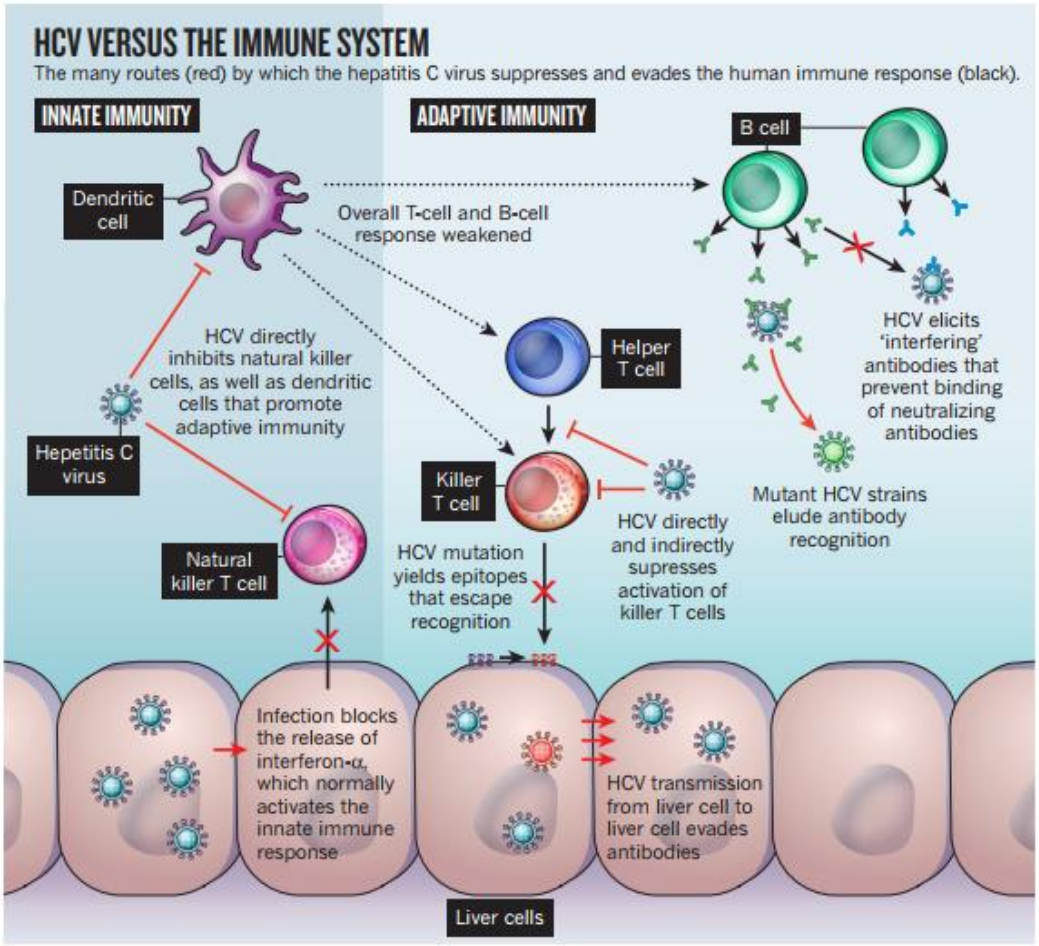



**Figure 9. HCV ability to evade host immune response. Retrieved from [137].**

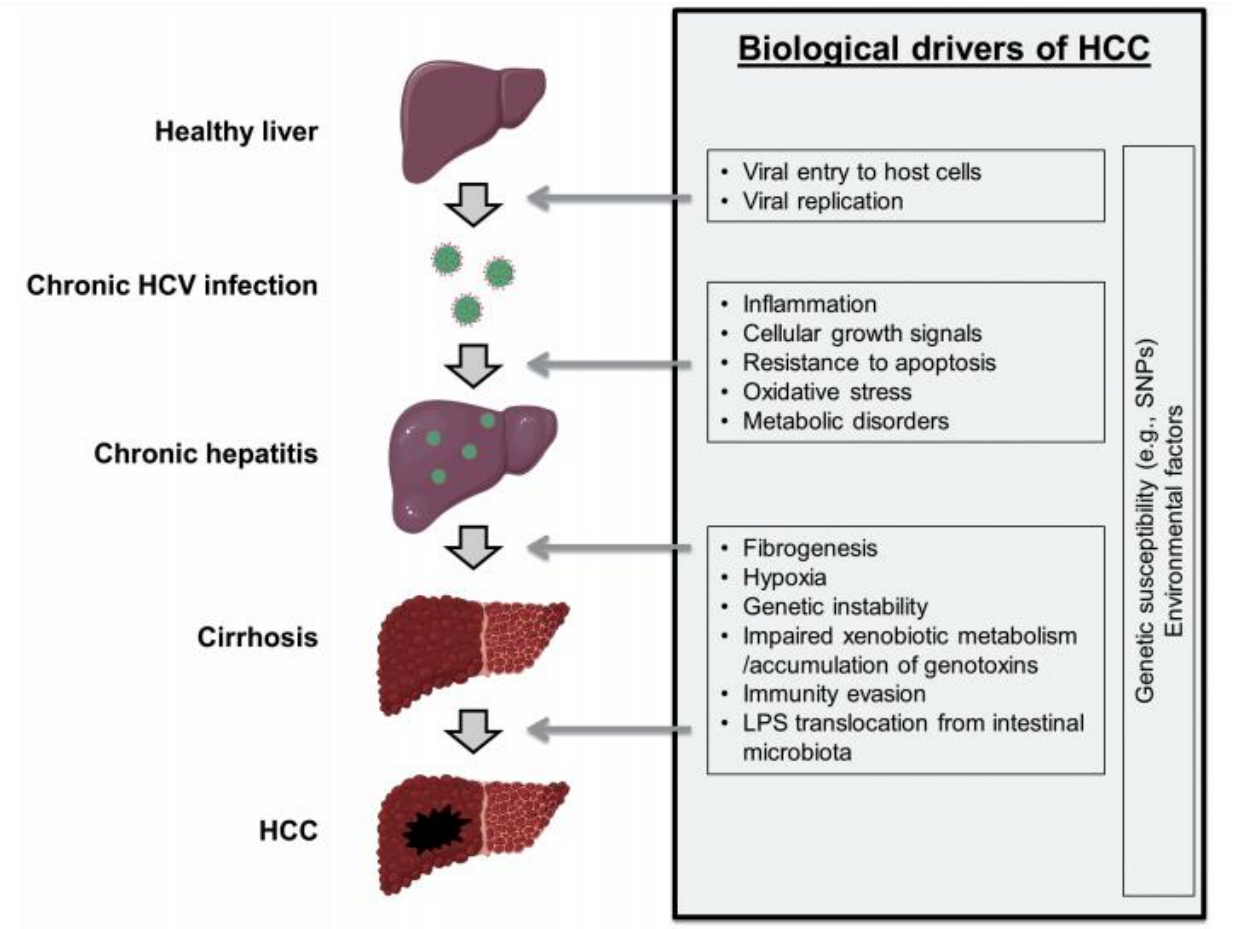



**Figure 10. HCV prognosis to HCC. Pathogenesis of HCC derived HCV involves multiple steps and different associated biological drivers. Retrieved from [40]**

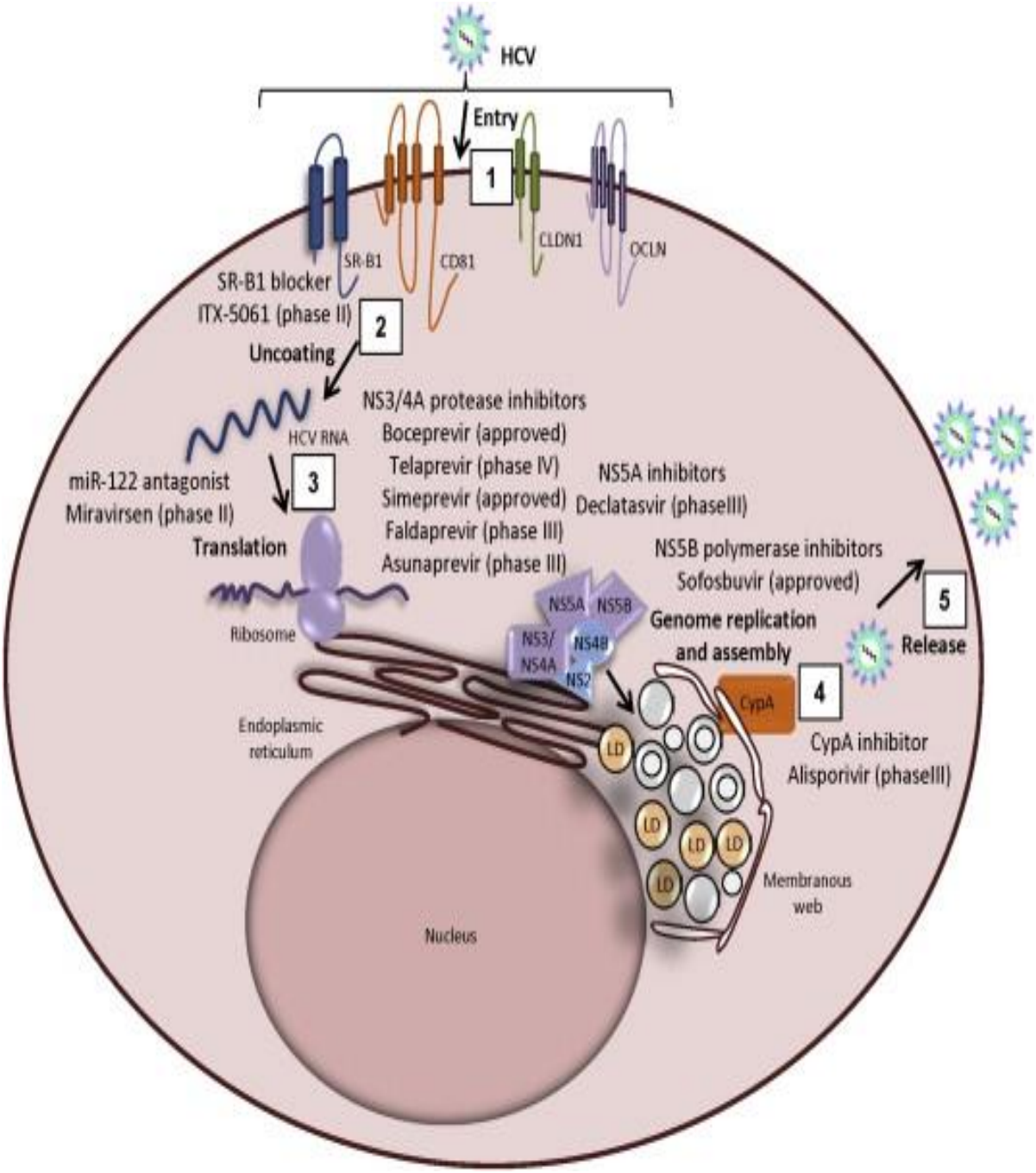



**Figure 11. Different available DAA. Retrieved from [53]**

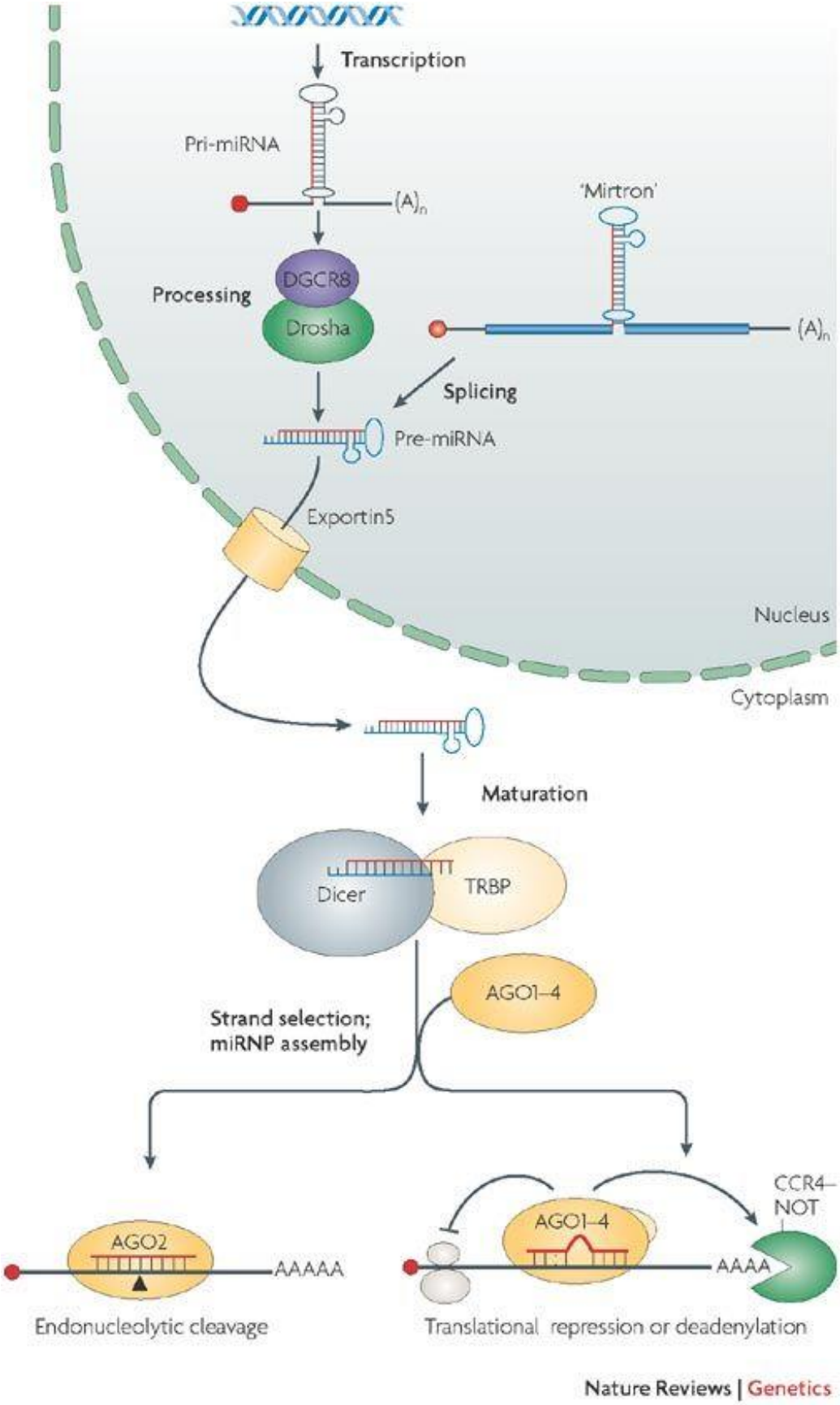



**Figure 12. mi-RNA biogenesis. Retrieved from [57]**

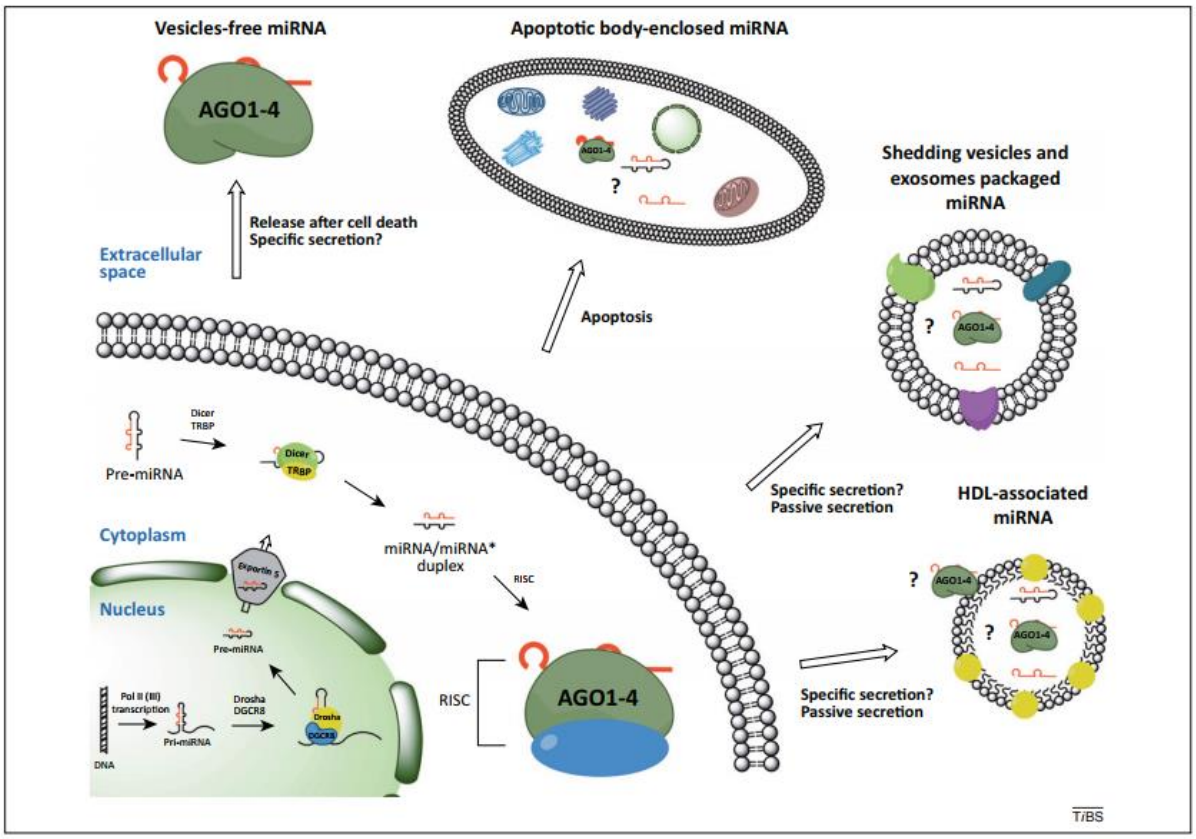
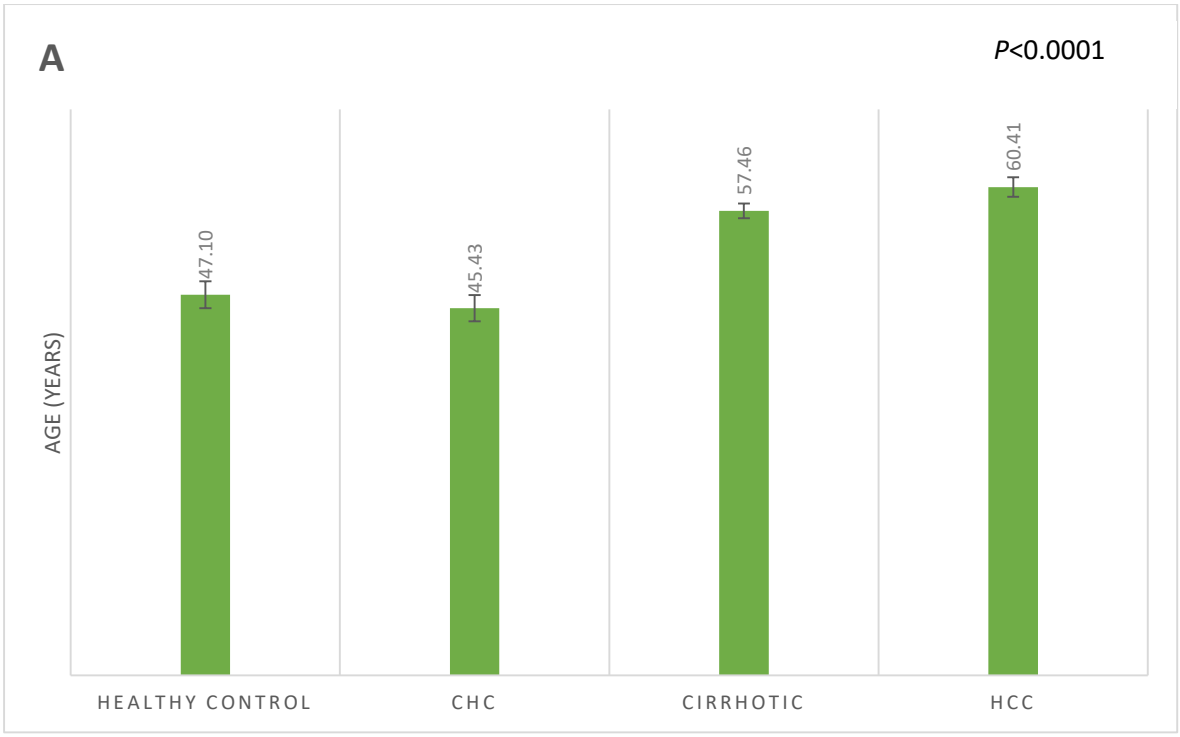



**Figure 13. Modes of mi-RNA packaging after their biogenesis. There are three packs of extracellular mi-RNAs, either packed in apoptotic bodies, shedding vesicles or exosomes. Retrieved from [73]**



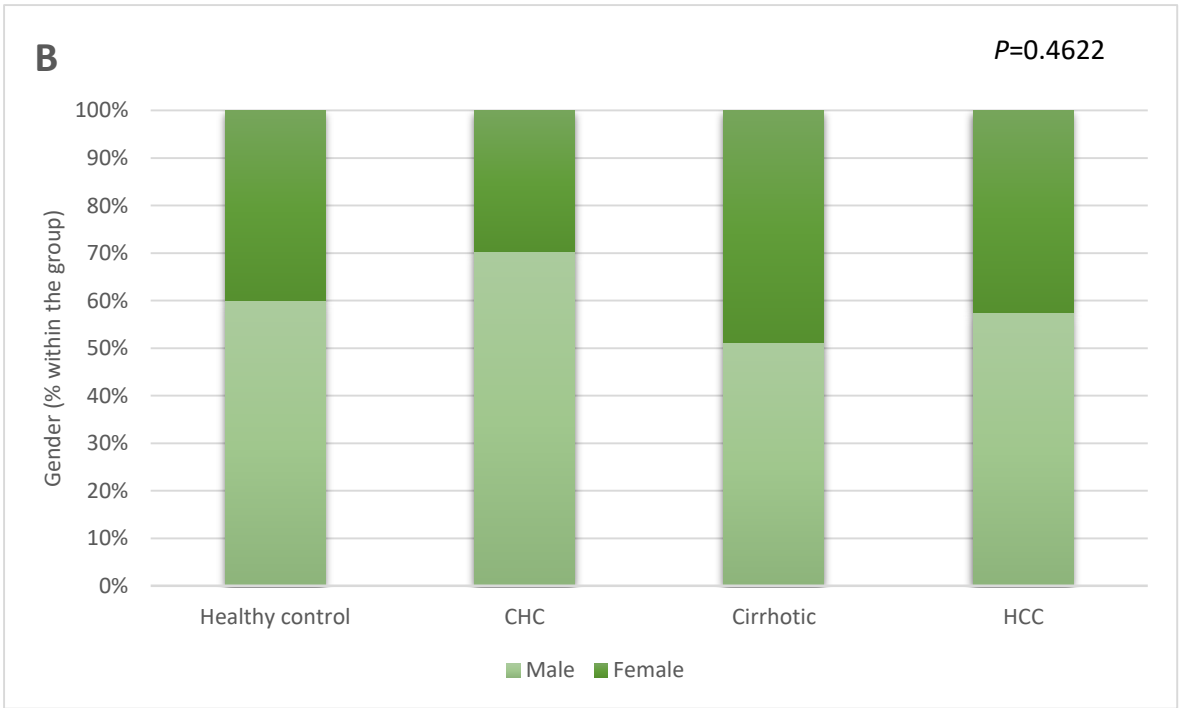




**Figure 14. Demographic data of healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). A) Age (mean ± standard error) and B) Gender (% within the group).**

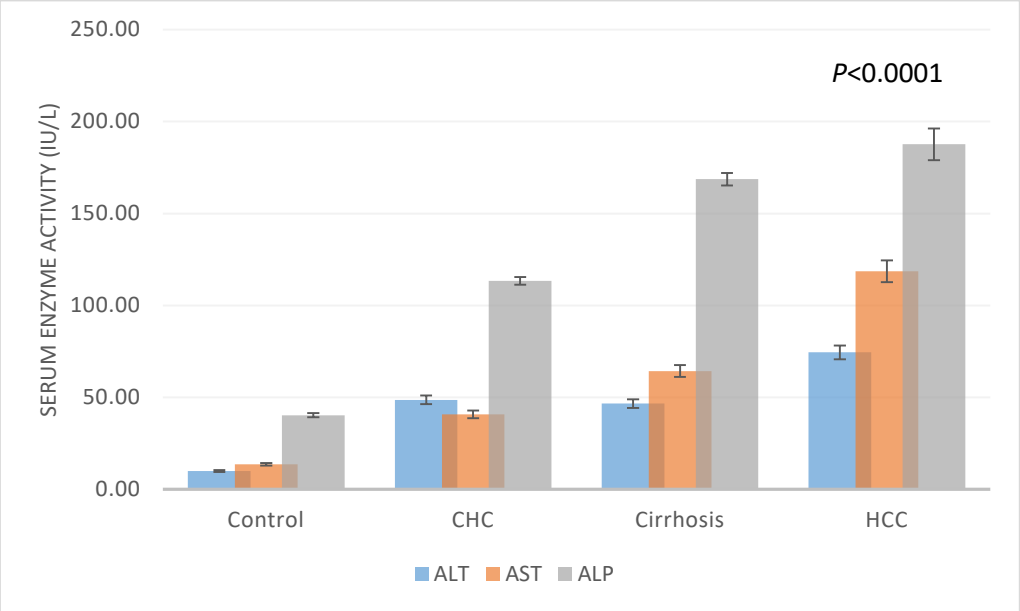



**Figure 15. Serum activities of liver enzymes (ALT, AST and ALP) in healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

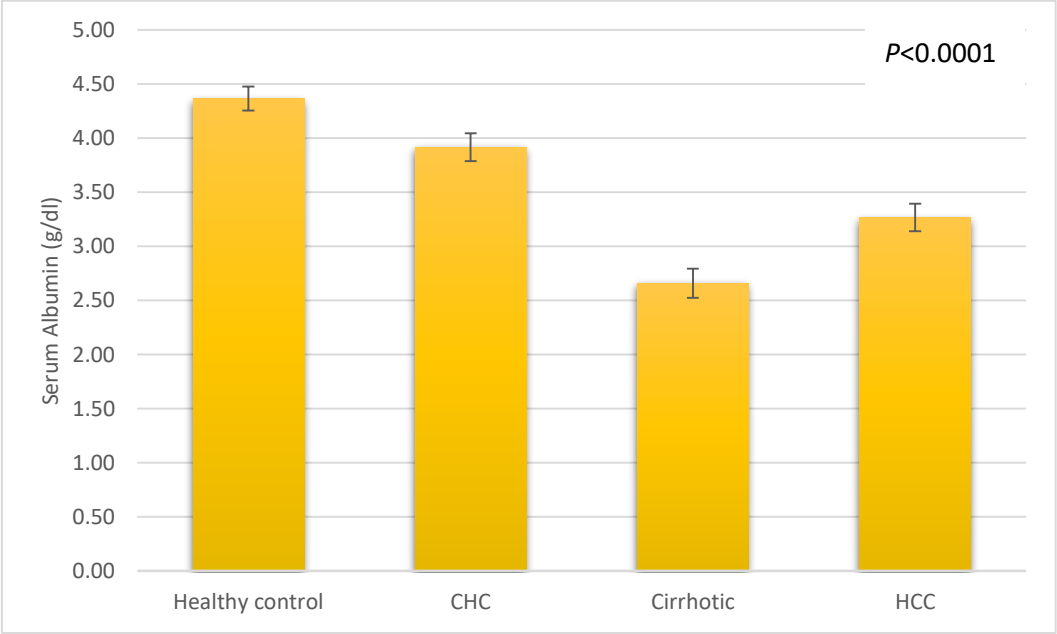



**Figure 16. Serum Albumin levels in healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

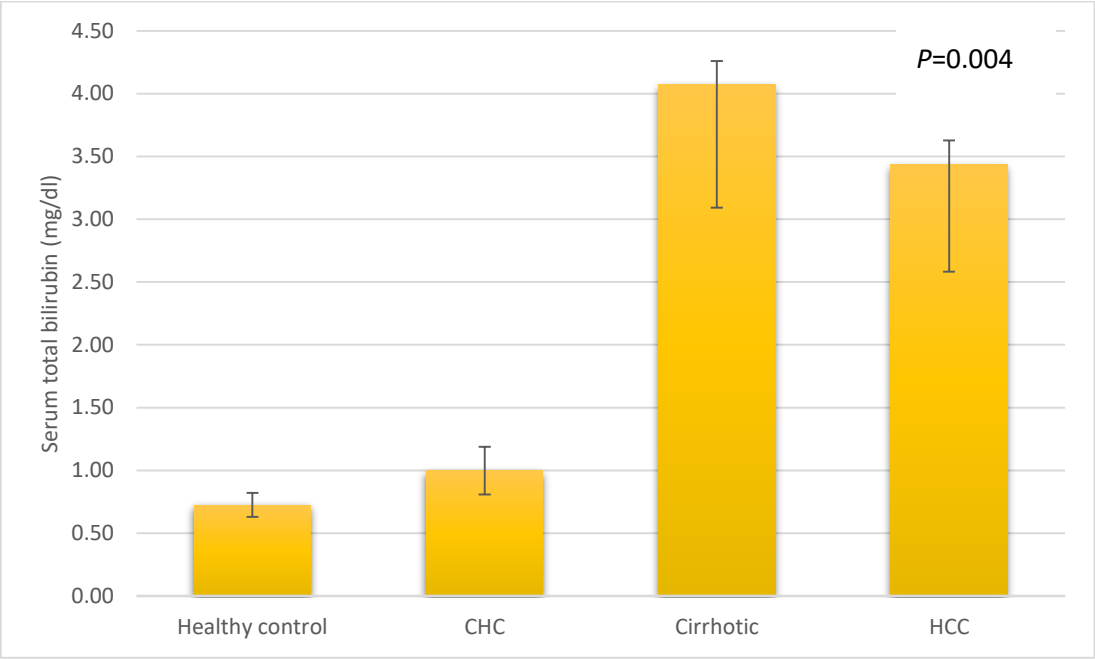



**Figure 17. Serum total bilirubin levels in healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

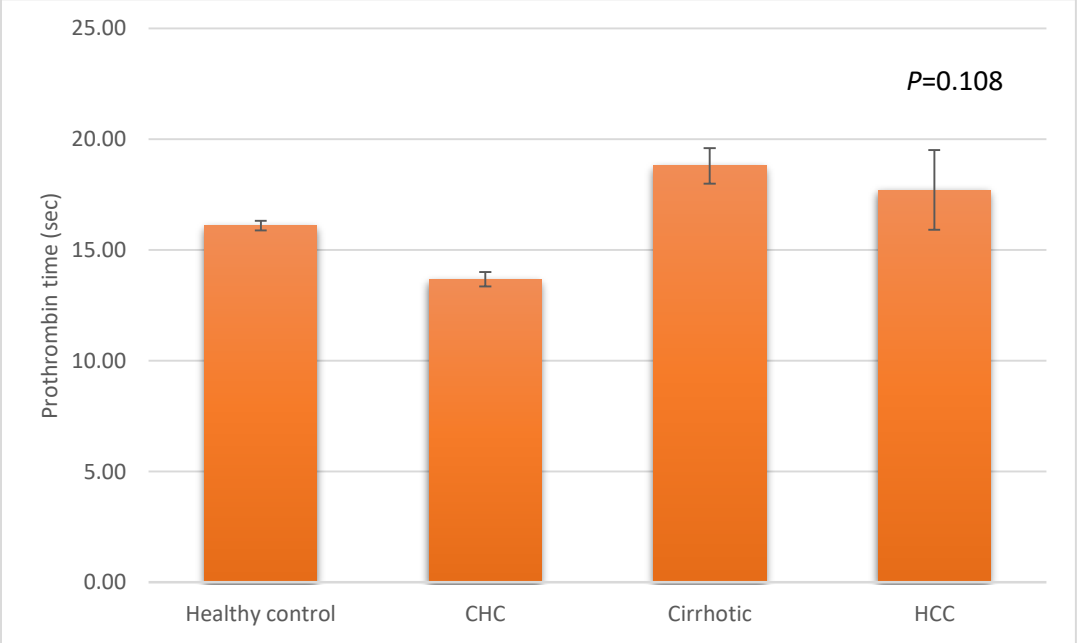



**Figure 18. Prothrombin time in healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

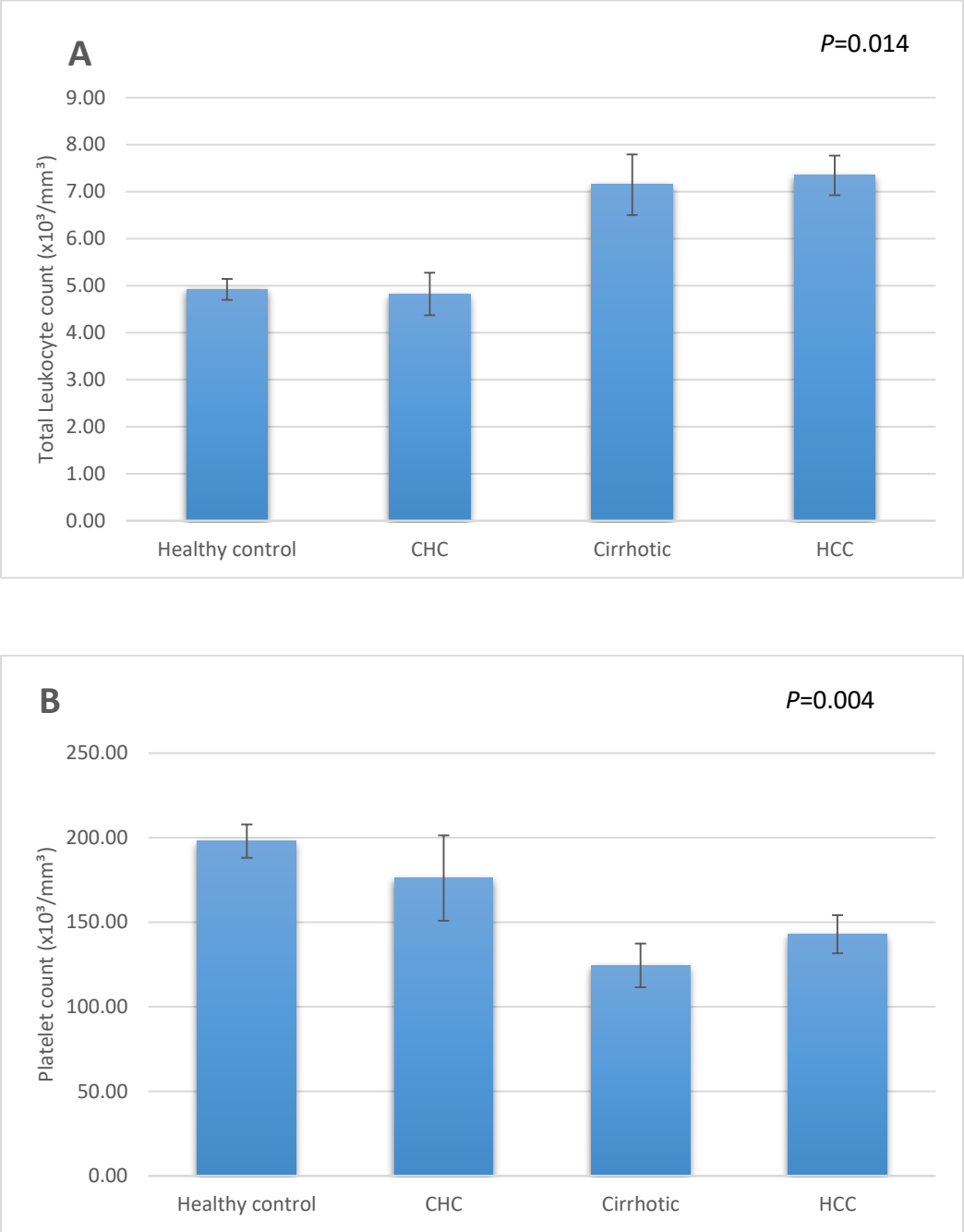



**Figure 19. Hematological data in healthy controls (n=40), CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

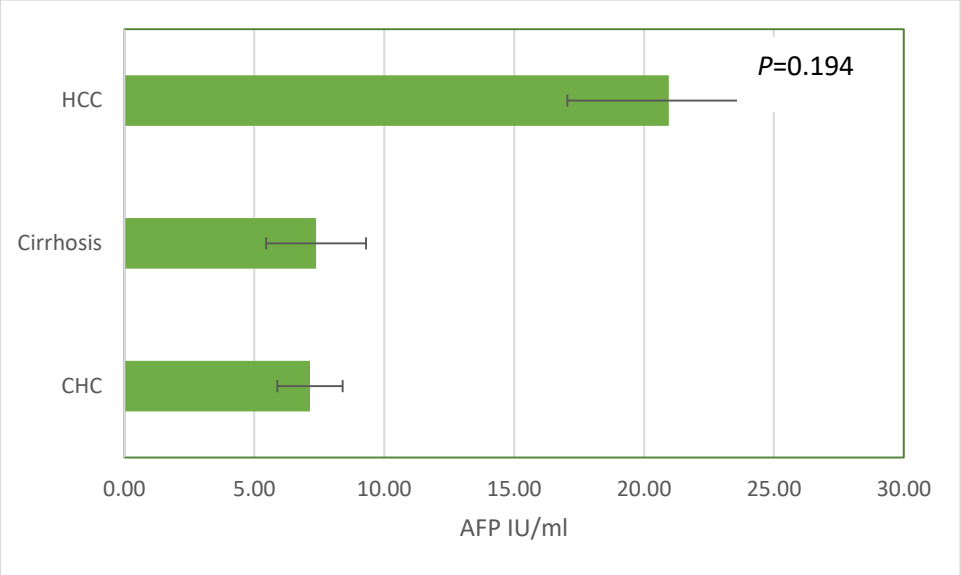



**Figure 20. Alfa feto protein levels in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

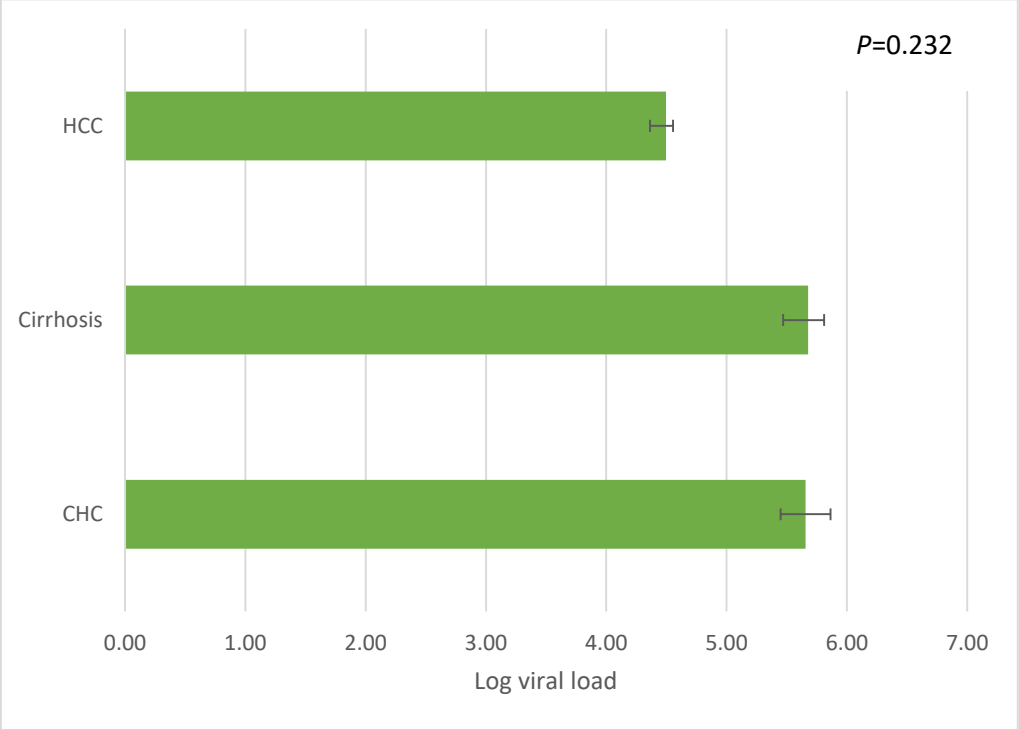



**Figure 21. Log10 viral load levels in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

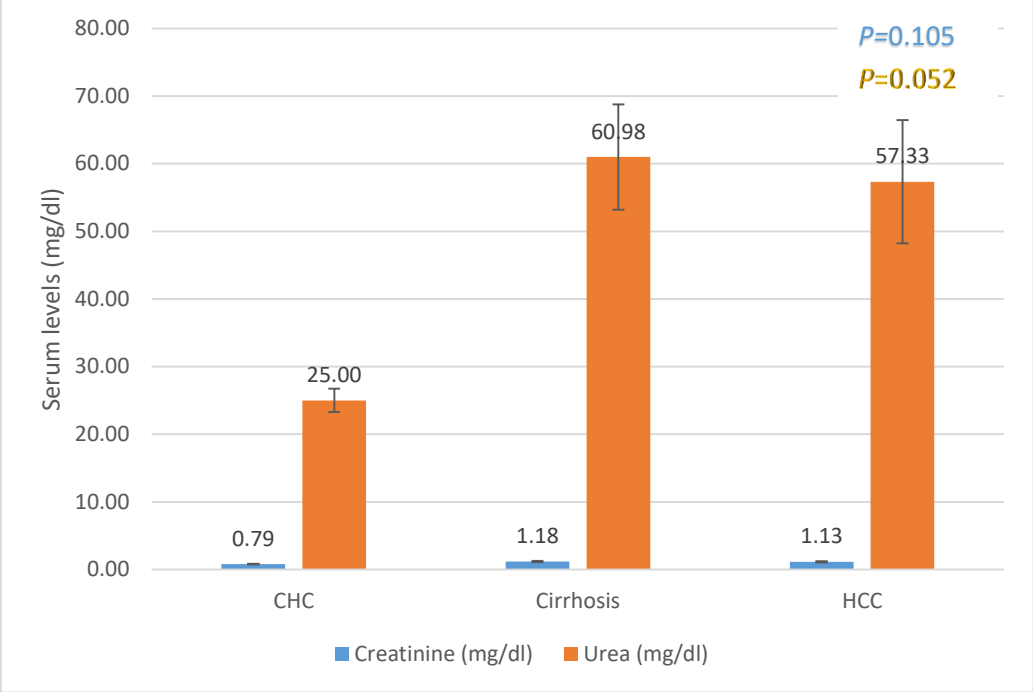



**Figure 22. Serum creatinine and urea levels CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

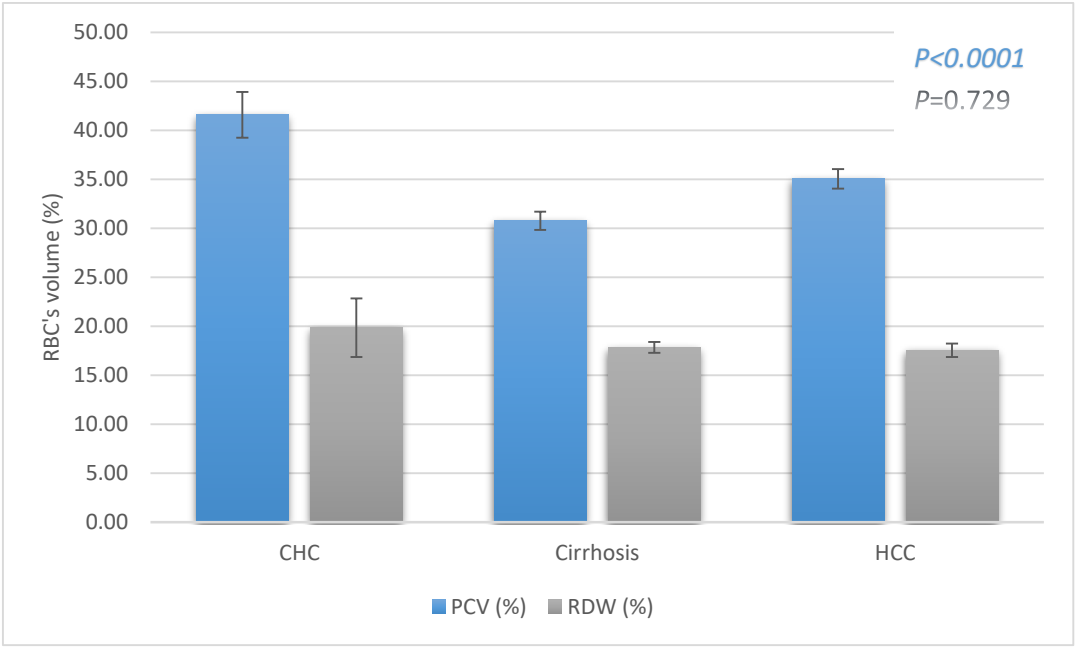



**Figure 23. Packed cell volume and red blood cell distribution width distribution in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

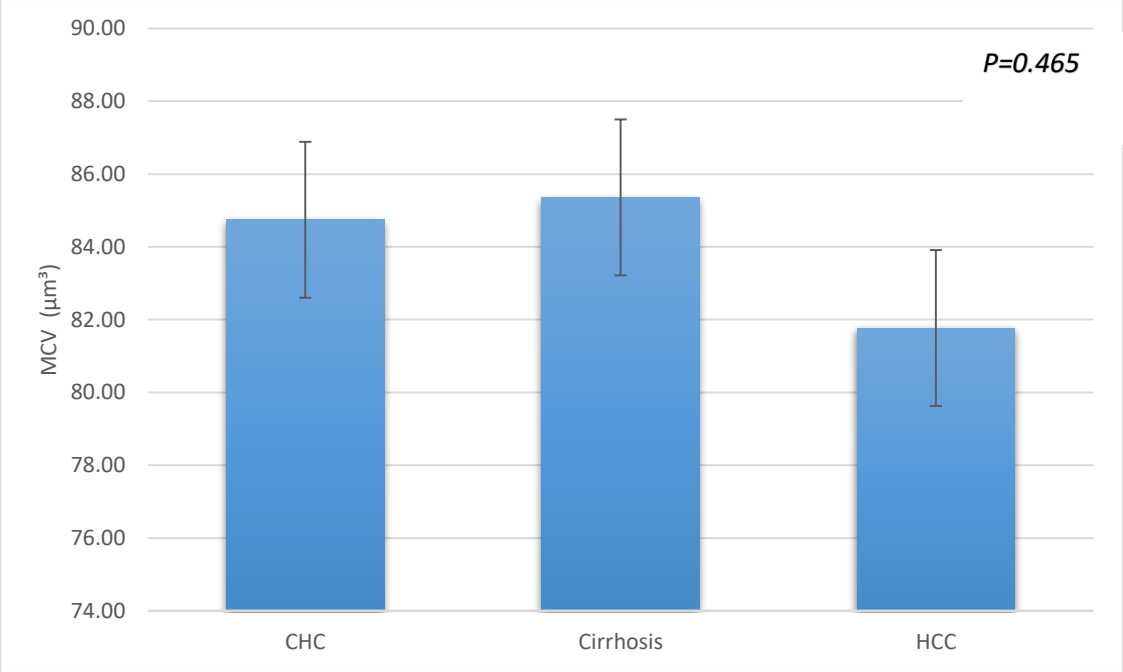



**Figure 24. Mean corpuscular volume in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

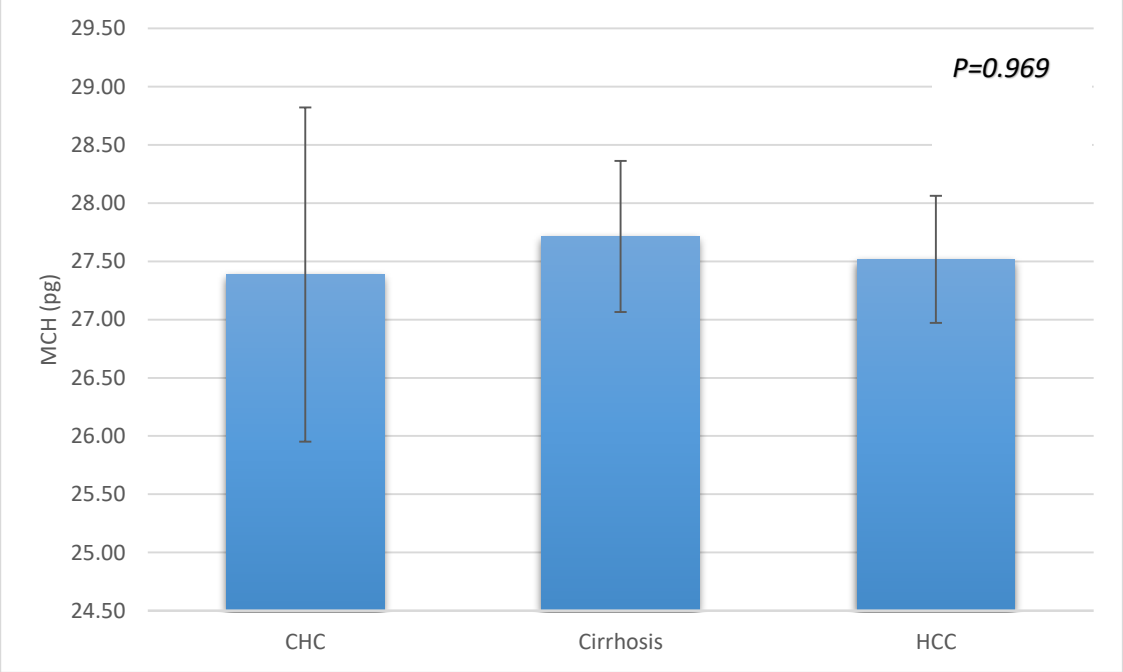



**Figure 25. Mean corpuscular hemoglobin in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

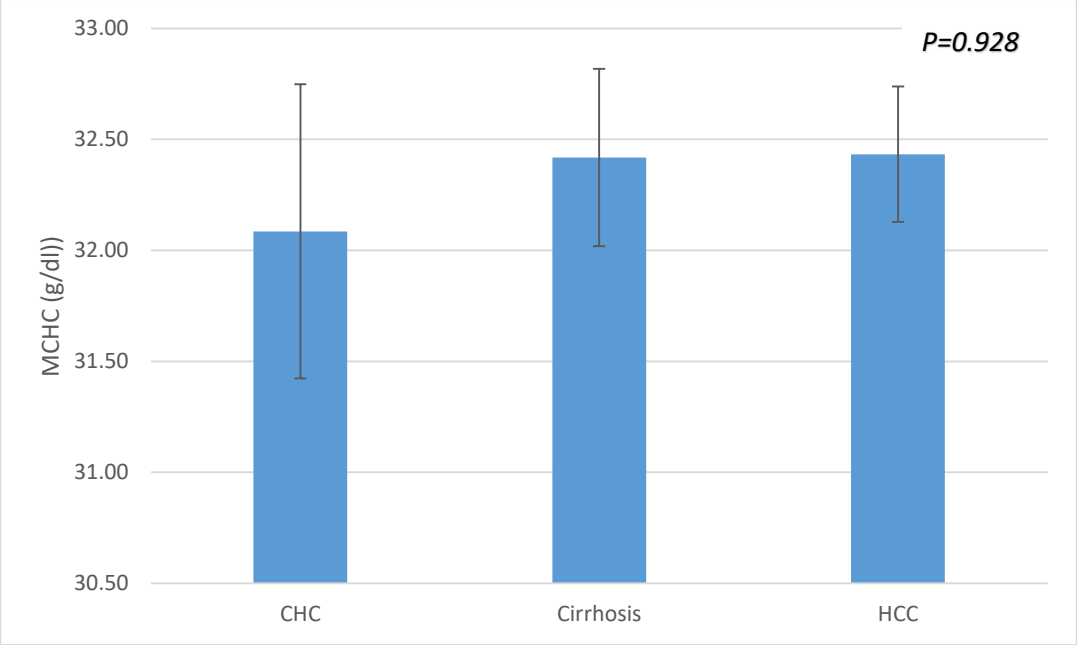



**Figure 26. Mean corpuscular hemoglobin concentration in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

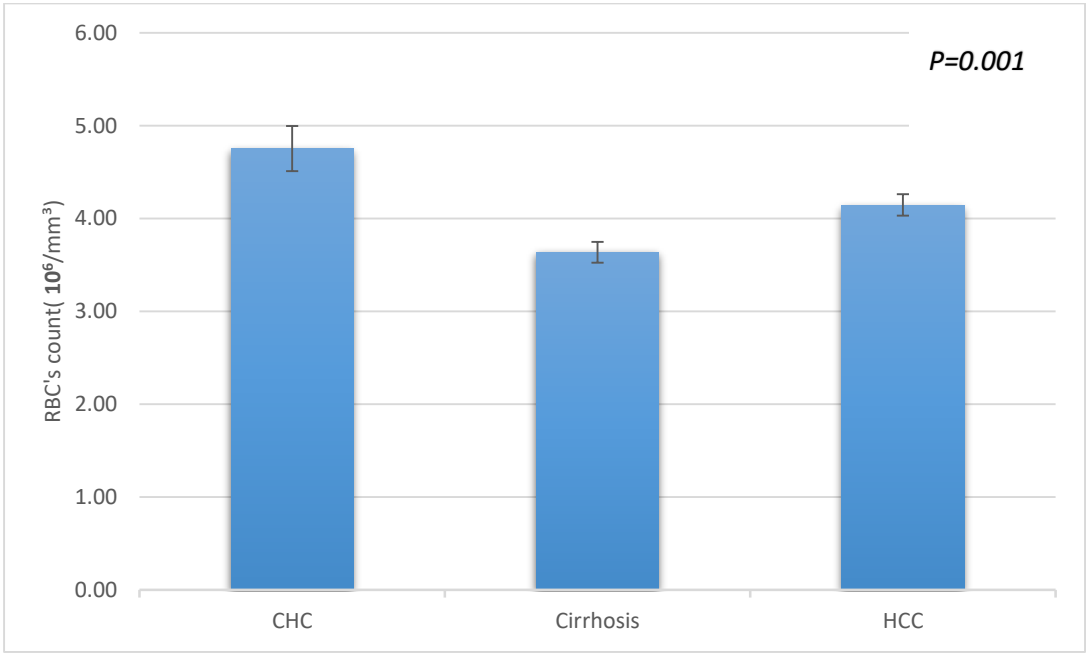



**Figure 27. RBC's count and hemoglobin level in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

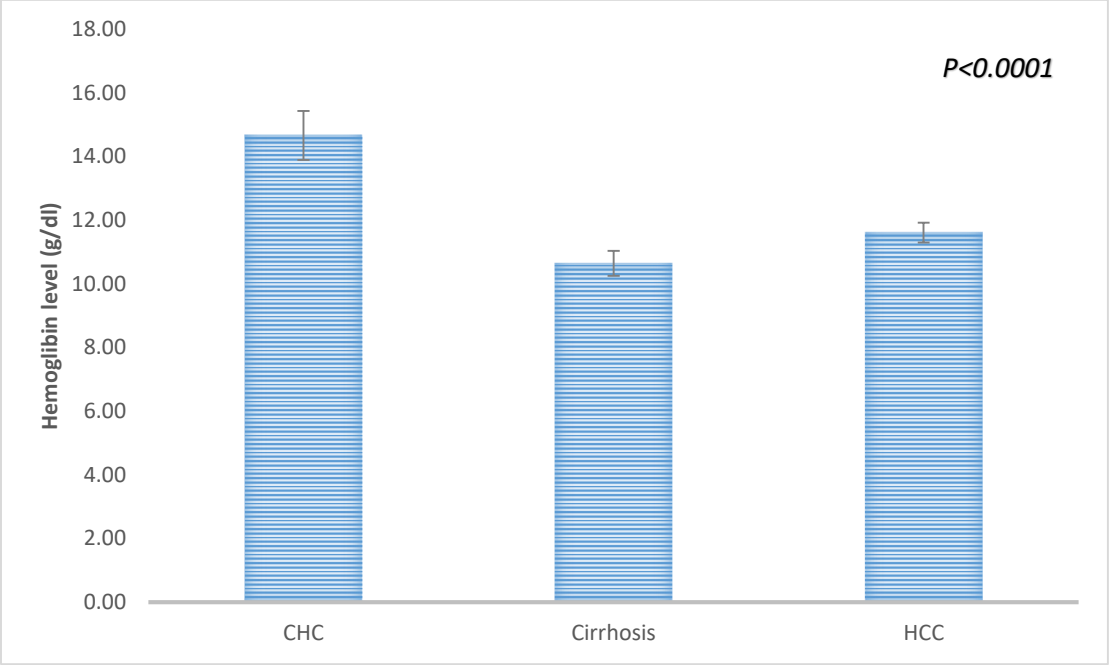



**Figure 28. Hemoglobin level in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

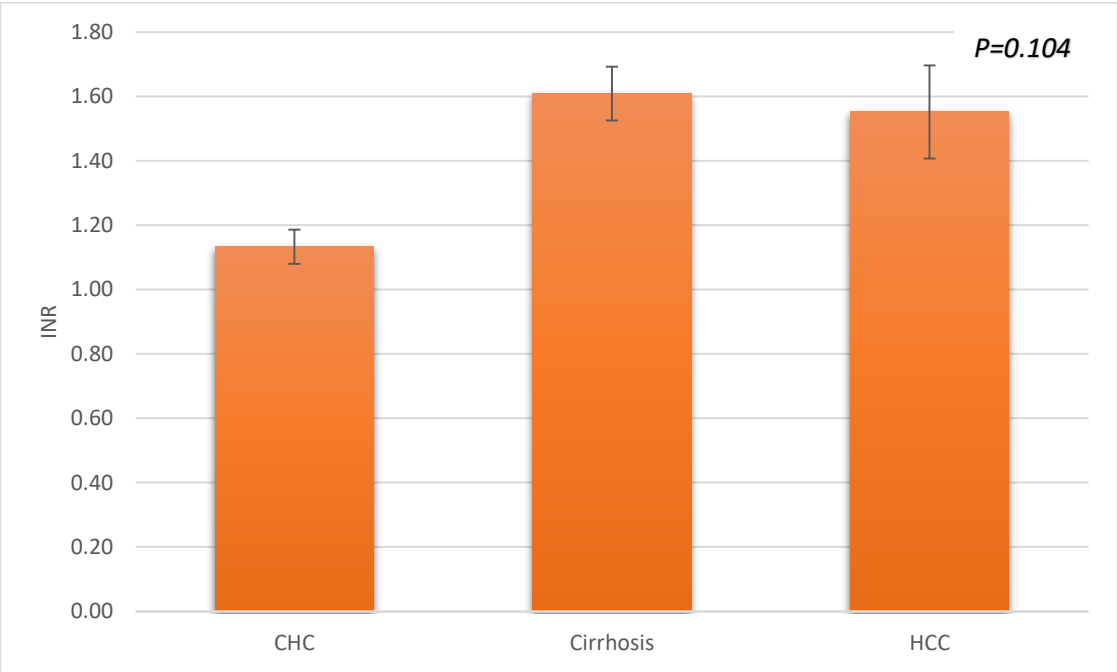



**Figure 29. Assessment of extrinsic coagulation pathway using international normalized ratio in CHC (n=37), Cirrhosis (n=39) and HCC (n=40). Data are expressed by means ± standard error.**

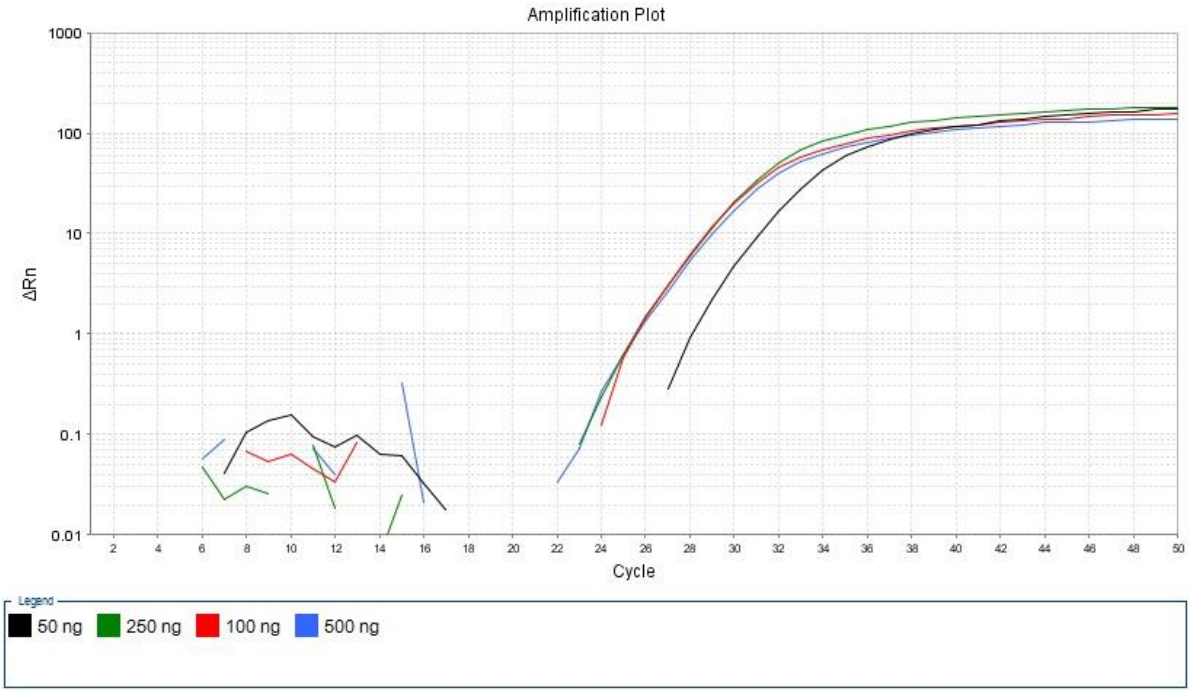
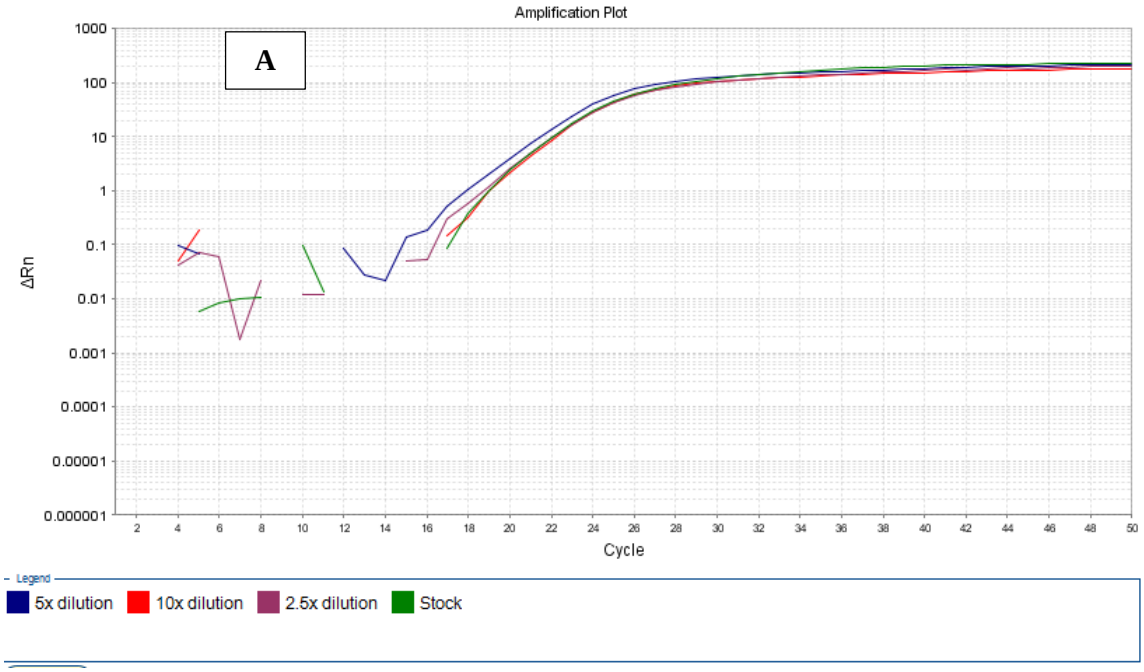



**Figure 30. Poly adenylation step optimization. Serial concentrations of total extracted RNA had been tried (50ng, 250ng, 100ng and 500ng).**



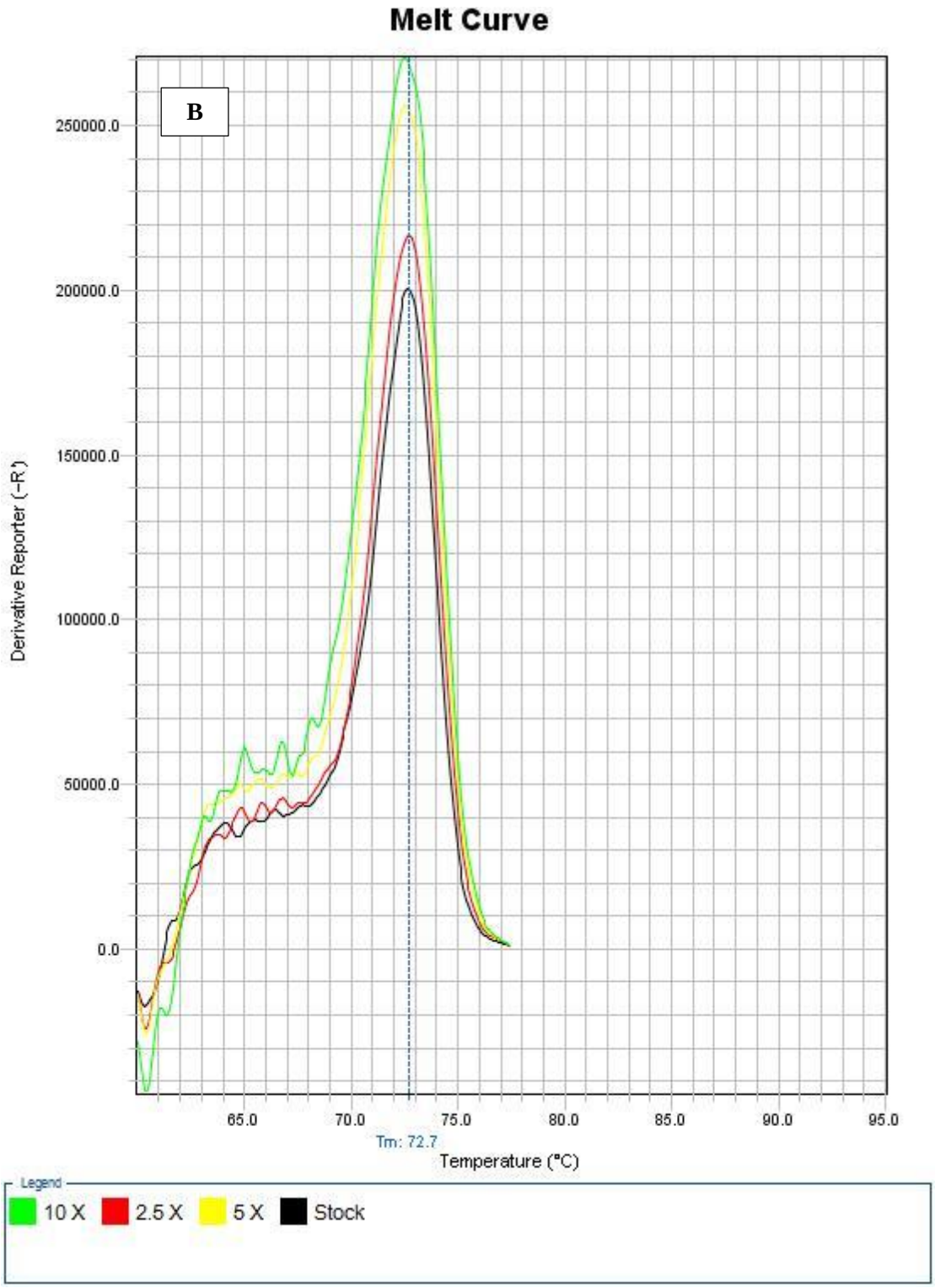
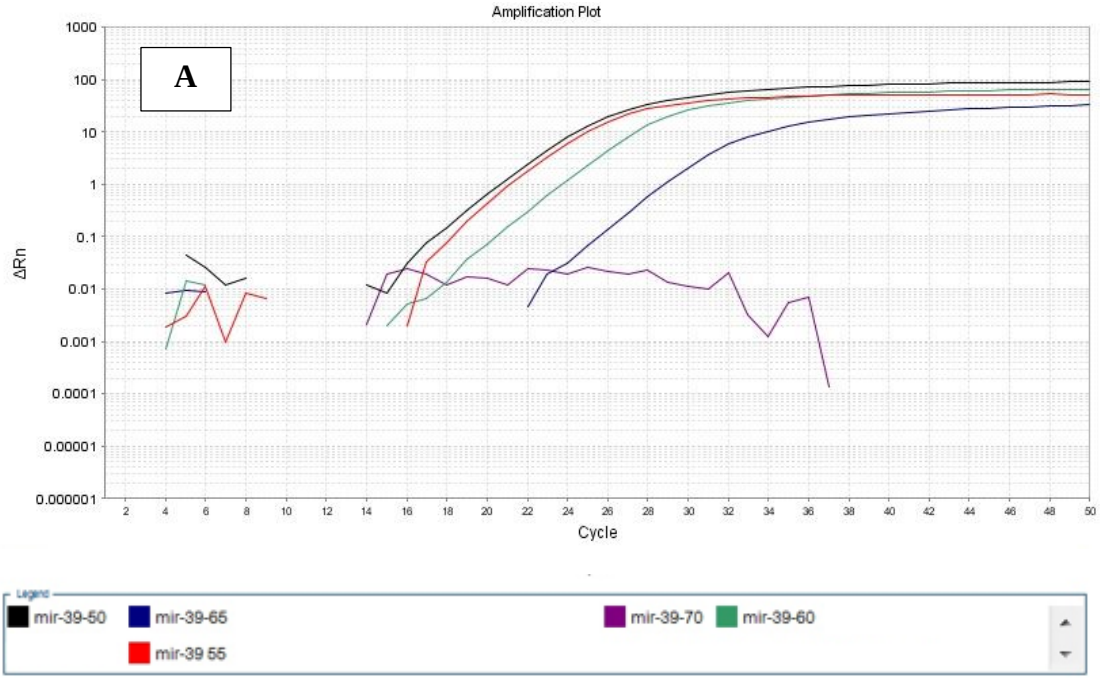



**Figure 31. q RT-PCR step optimization. (A) Amplification plot of different cDNA dilutions (stock, 2.5x, 5x and 10x). (B) The melt curve of these dilutions. The melt curve is showing single peaks for all dilutions.**


Amplification Plot
A
ΔRn
Cycle
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50


Legend
mir-39-50
mir-39-65
mir-39 55
mir-39-70
mir-39-60

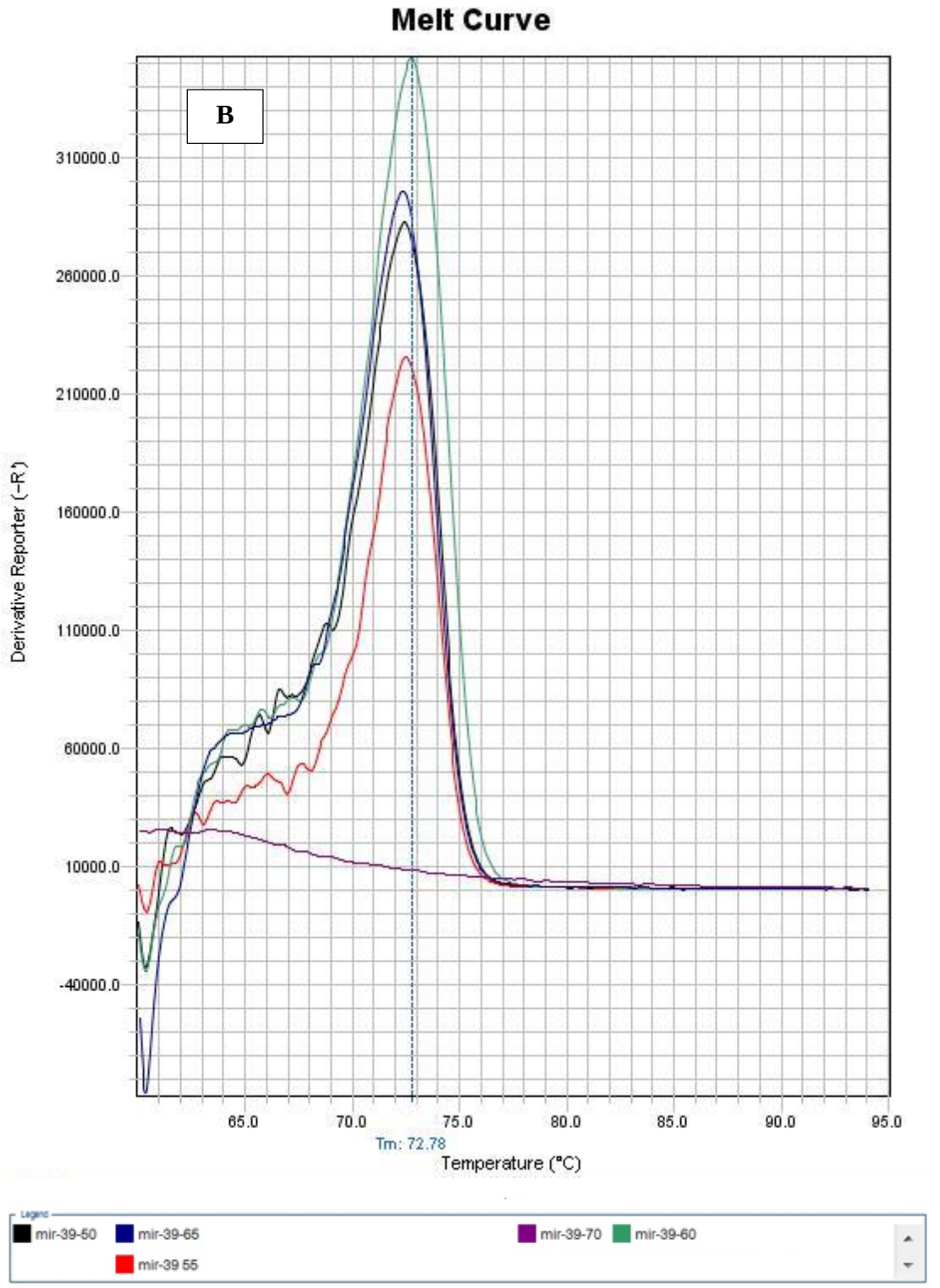
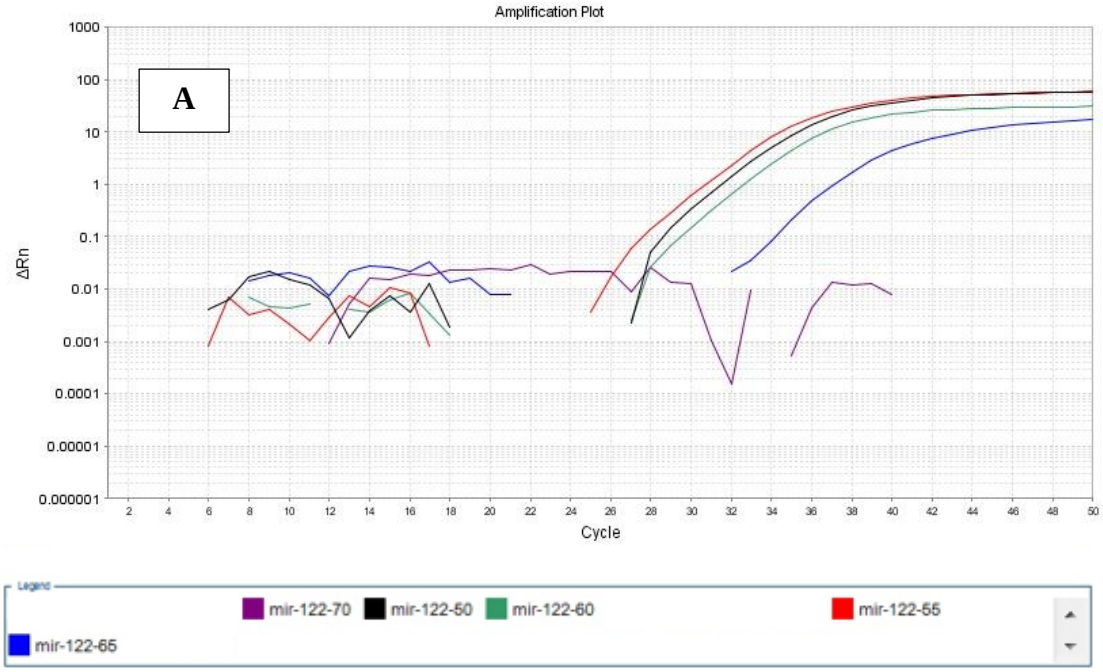



**Figure 32. miR-39 annealing temperature optimization. (A) Amplification plot of miR-39 at different annealing temperatures. (B) The melt curve of these temperatures. The melt curve is showing single peaks for all temperatures except for temp 70 ºC that have shown undetectable Ct.**

.


Amplification Plot
A
ΔRn
Cycle
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Legend
mir-122-70
mir-122-50
mir-122-60
mir-122-55
mir-122-65

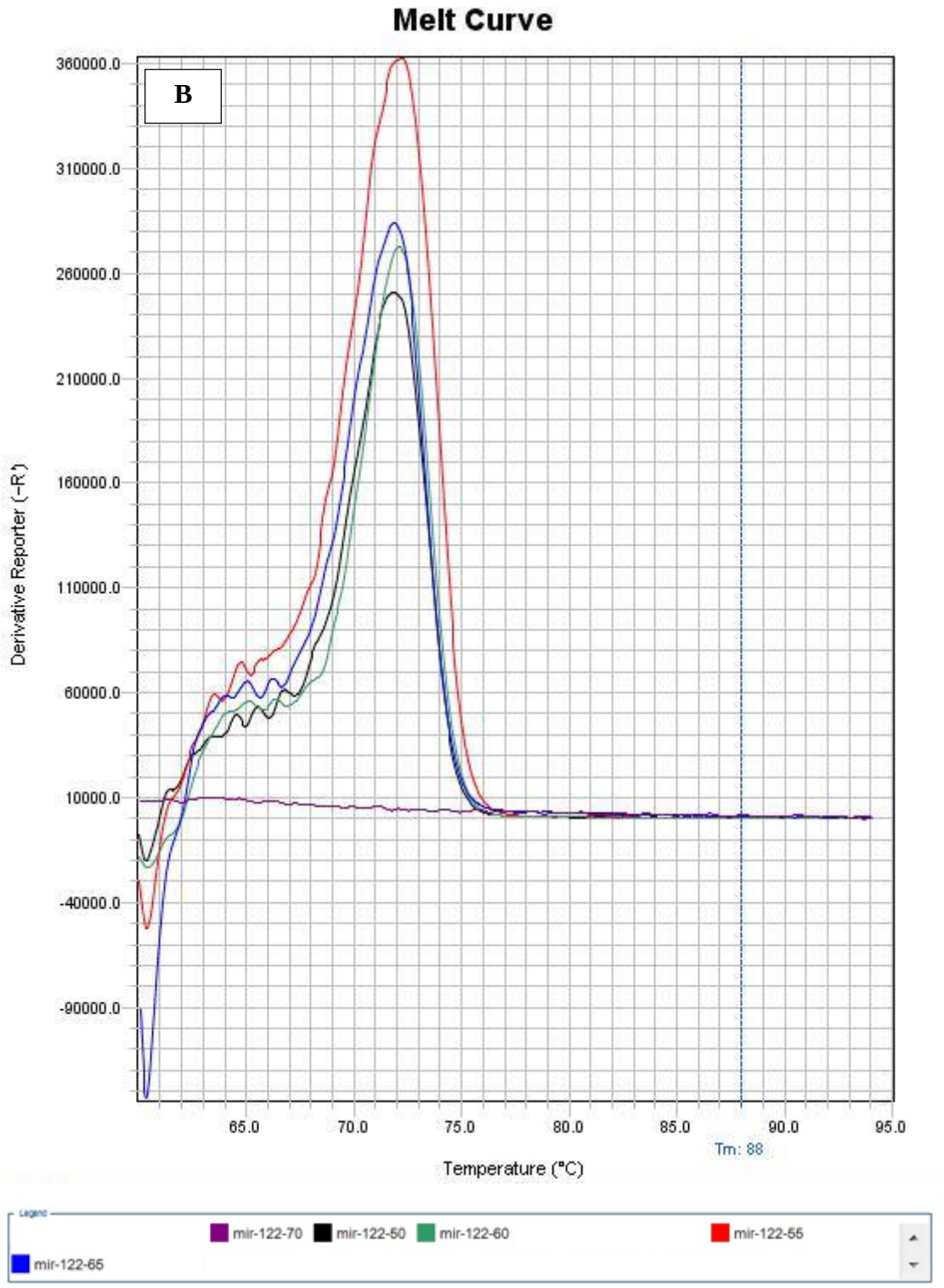
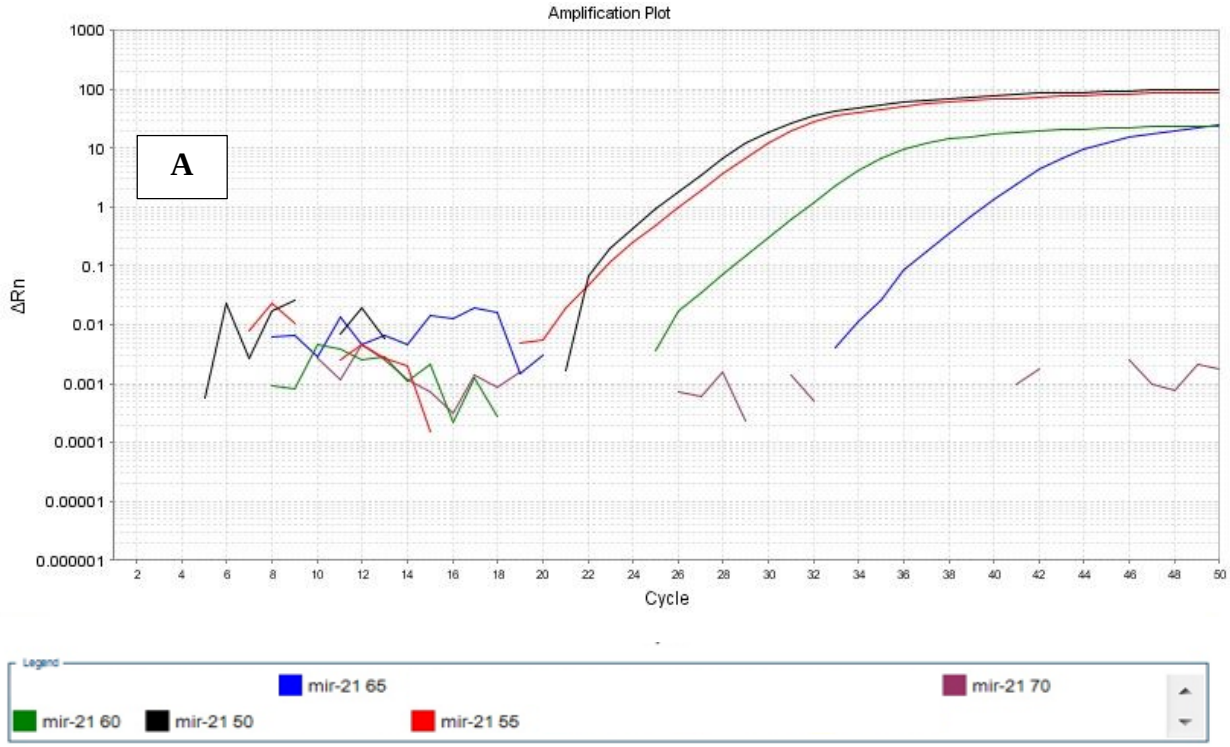



**Figure 33. miR-122 annealing temperature optimization. (A) Amplification plot of miR-122 at different annealing temperatures. (B) The melt curve of these temperatures. The melt curve is showing single peaks for all temperatures except for temp 70 ºC that have shown undetectable Ct.**


Amplification Plot
A
ΔRn
Cycle
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50


Legend
mir-21 65
mir-21 70
mir-21 60
mir-21 50
mir-21 55

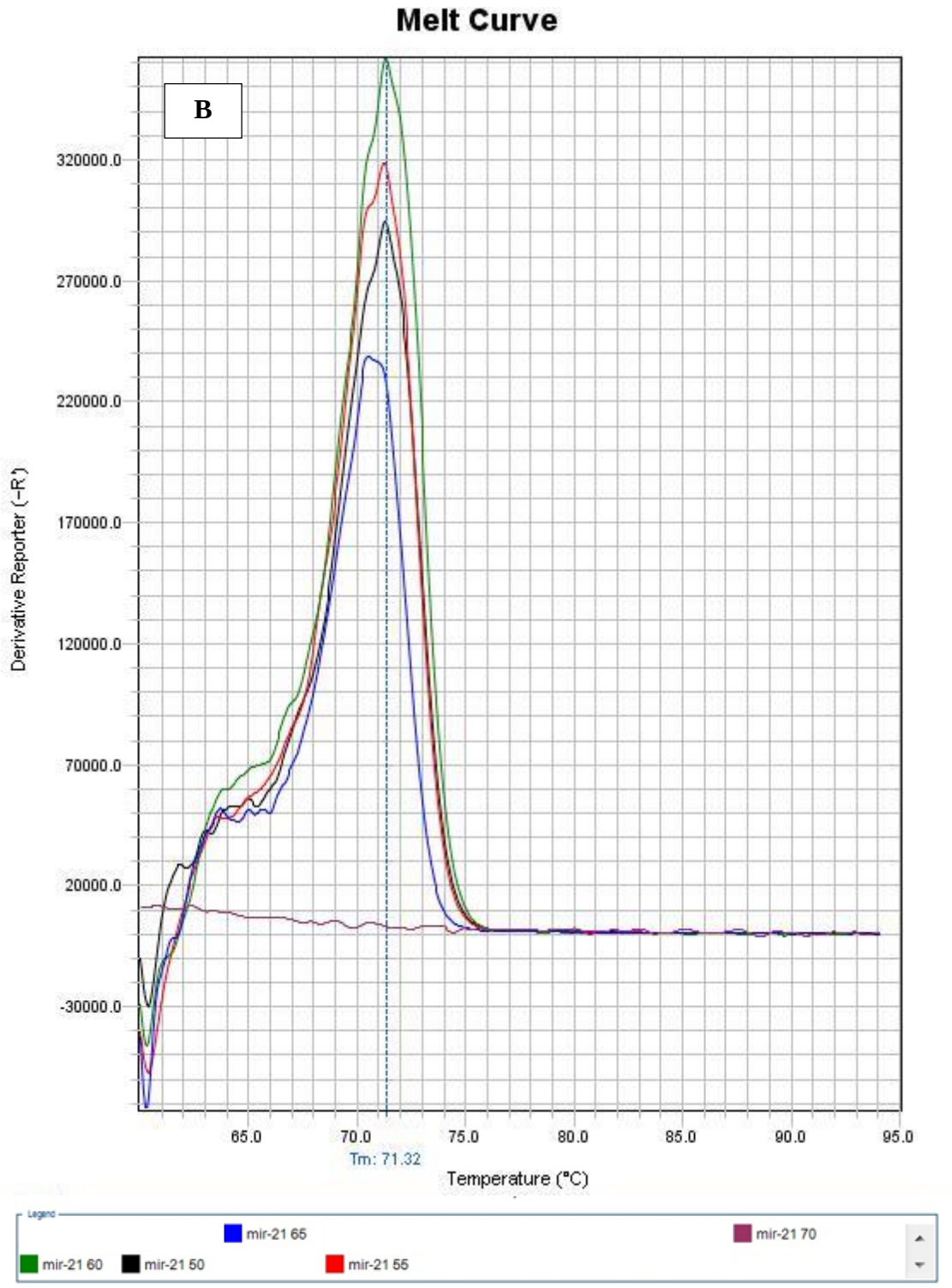
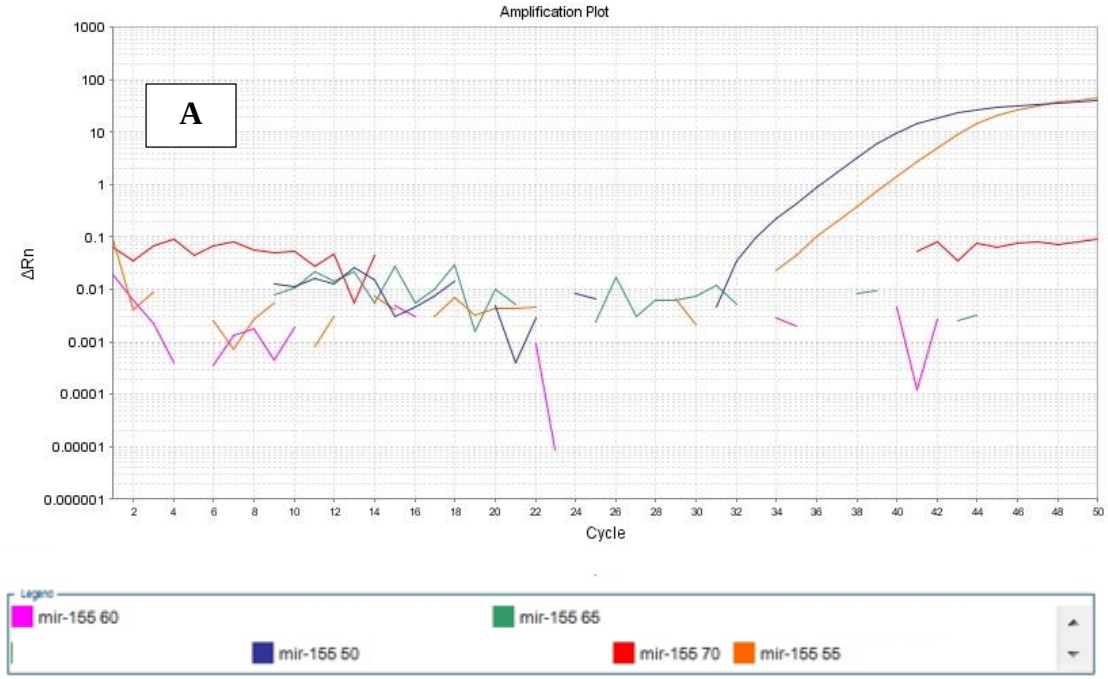



**Figure 34. miR-21 annealing temperature optimization. (A) Amplification plot of miR-21 at different annealing temperatures. (B) The melt curve of these temperatures. The melt curve is showing single peaks for all temperatures except for temp 70 ºC that have shown undetectable Ct.**


Amplification Plot
A
ΔRn
Cycle
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50


Legend
mir-155 60
mir-155 65
mir-155 50
mir-155 70
mir-155 55

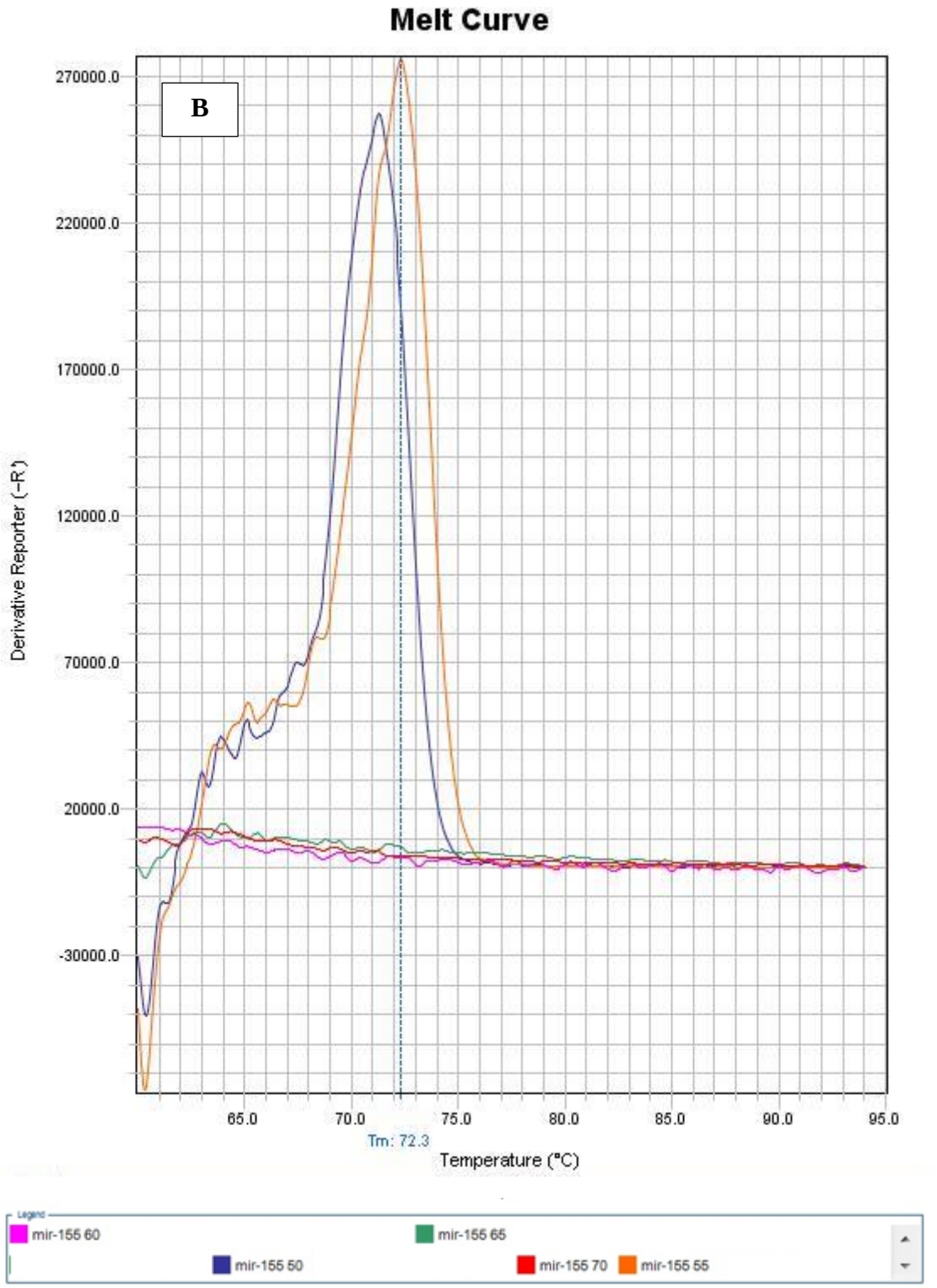



**Figure 35. miR-155 annealing temperature optimization. (A) Amplification plot of miR-155 at different annealing temperatures. (B) The melt curve of these temperatures. The melt curve is showing single peaks for all temperatures except for temp 60 ºC., 65 ºC and 70 ºC that have shown undetectable Ct.**

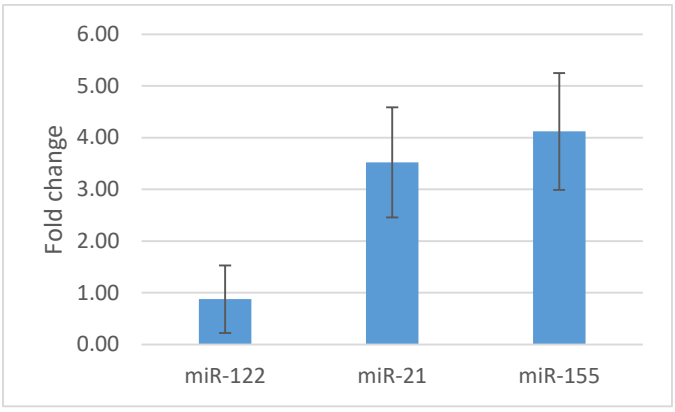



**Figure 36. Differential expression of plasma mi-RNAs in healthy control subjects (n=40) compared to CHC (n=37). Data are presented as mean± standard error.**

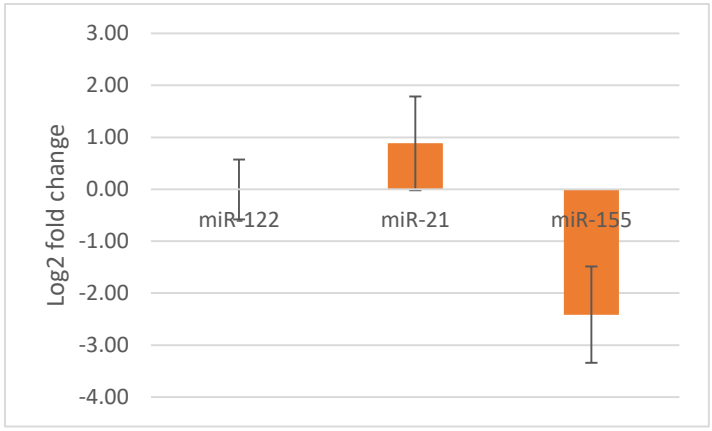



**Figure 37. Differential expression of plasma mi-RNAs in Cirrhotic subjects (n=39) compared to CHC (n=37). Data are presented as mean± standard error.**

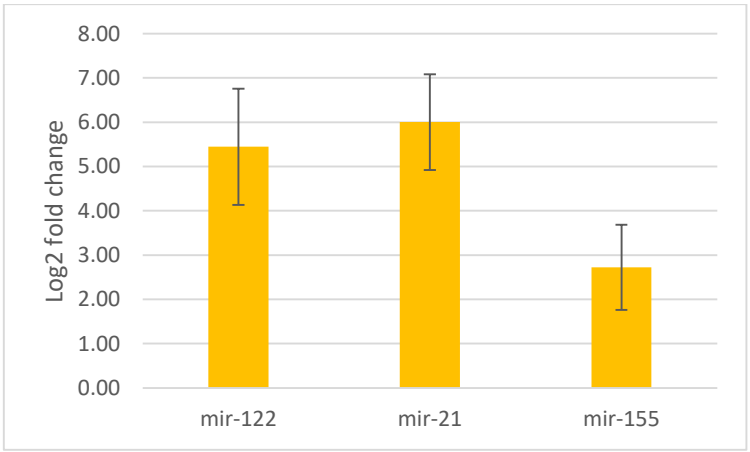



**Figure 38. Differential expression of plasma mi-RNAs in HCC subjects (n=40) compared to CHC (n=37). Data are presented as mean± standard error.**

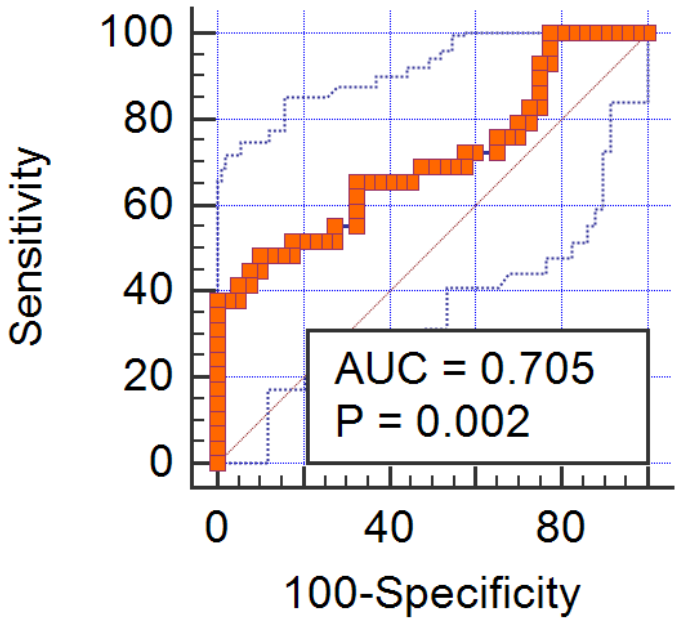



**Figure 39. Plasma miR-122 as diagnostic marker of HCC. ROC curve analysis of plasma mi-122 expression as diagnostic biomarker to differentiate HCC patients (n=40) from healthy control (n=40).**

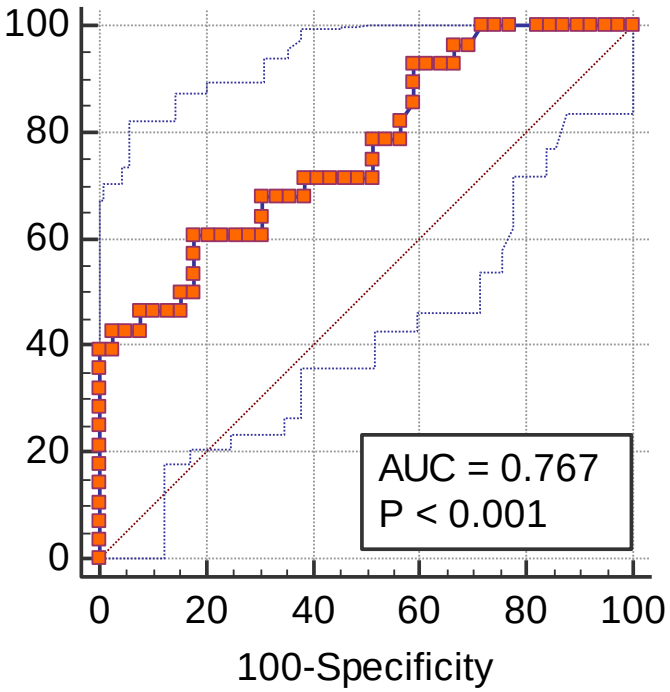



**Figure 40. Plasma miR-122 as diagnostic marker of HCC. ROC curve analysis of plasma mi-122 expression as diagnostic biomarker to differentiate HCC patients (n=40) from cirrhotic patients (n=39).**

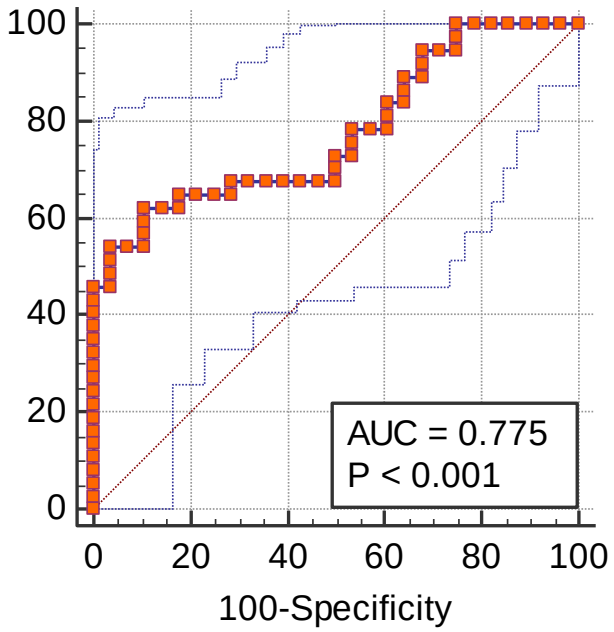



**Figure 41. Plasma miR-21 as diagnostic marker for cirrhosis. ROC curve analysis of plasma mi-21 expression as diagnostic biomarker to differentiate cirrhotic patients (n=39) from HCC patients (n=40).**

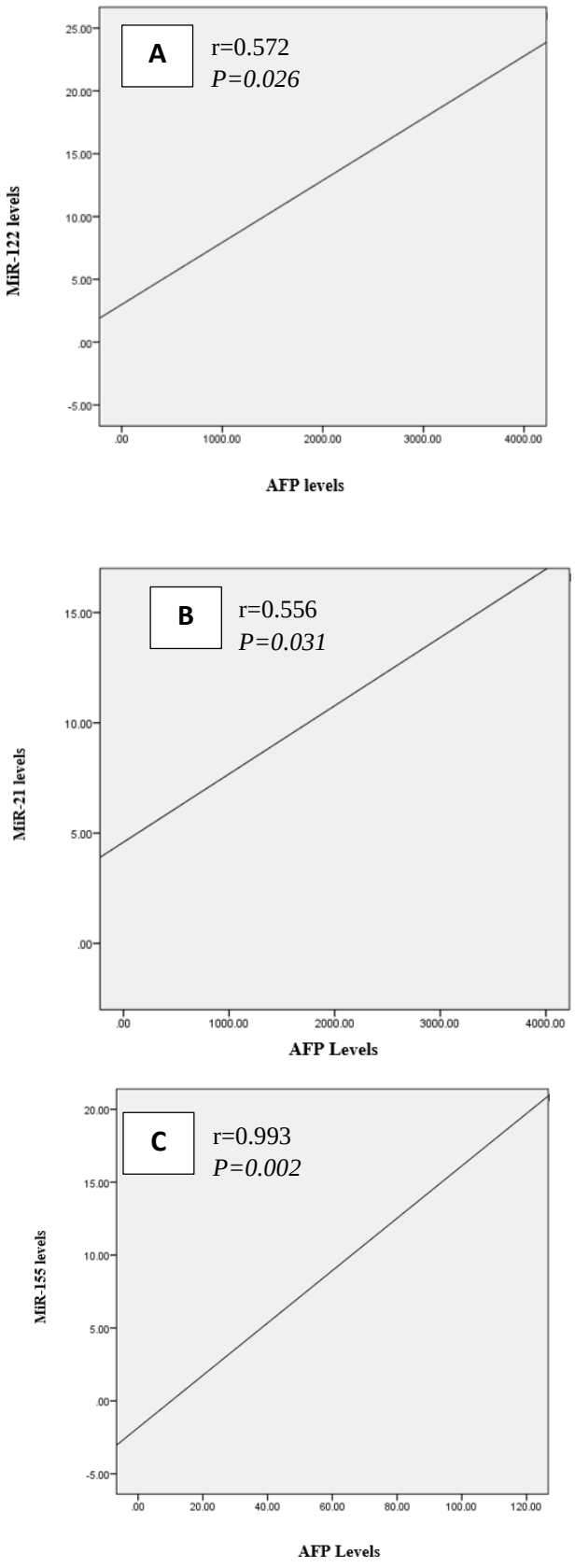



**Figure 42. Correlation between plasma miRNA levels and AFP in HCC patients. Association were determined using Pearson correlation, r: Pearson rho coefficient.**

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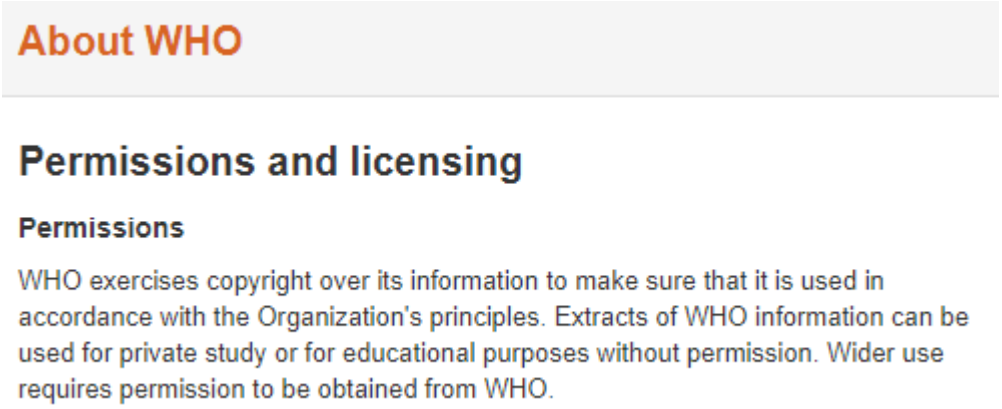

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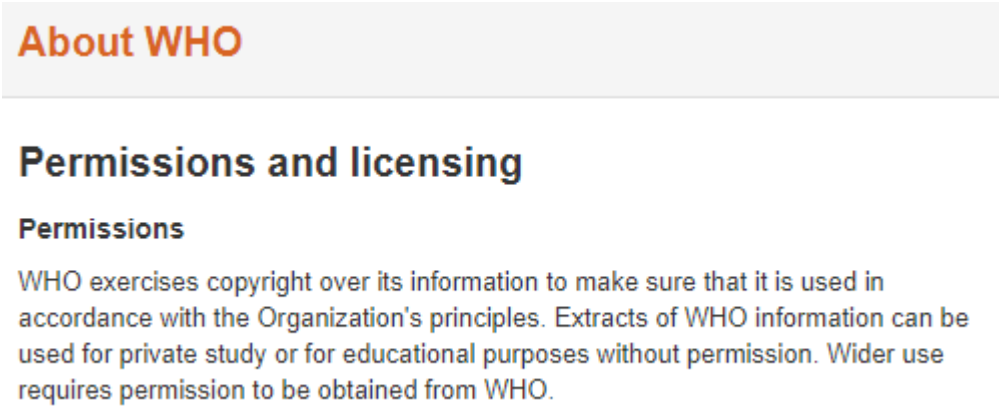

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