American University in Cairo

AUC Knowledge Fountain

Theses and Dissertations

2-1-2017

MicroRNAs as noninvasive biomarkers for the diagnosis and prognosis of liver fibrosis in HCV genotype 4 patients

Amanda Abdel-Al

Follow this and additional works at: https://fount.aucegypt.edu/etds

Recommended Citation

APA Citation

Abdel-Al, A. (2017).*MicroRNAs as noninvasive biomarkers for the diagnosis and prognosis of liver fibrosis in HCV genotype 4 patients* [Master's thesis, the American University in Cairo]. AUC Knowledge Fountain.
https://fount.aucegypt.edu/etds/309

MLA Citation

Abdel-Al, Amanda. *MicroRNAs as noninvasive biomarkers for the diagnosis and prognosis of liver fibrosis in HCV genotype 4 patients*. 2017. American University in Cairo, Master's thesis. *AUC Knowledge Fountain*.
https://fount.aucegypt.edu/etds/309

This Thesis is brought to you for free and open access by AUC Knowledge Fountain. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of AUC Knowledge Fountain. For more information, please contact mark.muehlhaeusler@aucegypt.edu.

THE AMERICAN UNIVERSITY IN CAIRO

School of Sciences and Engineering

MicroRNAs as Noninvasive Biomarkers for the Diagnosis and Prognosis of Liver Fibrosis in HCV Genotype 4 Patients

A Thesis Submitted to

The Biotechnology Graduate Program

In partial fulfillment of the requirements for

The degree of Master of Science in Biotechnology

By:

Amanda Abdel-Alim Abdel-Al

BSc in Biology, California State University Northridge, CA 2012

Under the supervision of:

Professor Suher Zada, Ph.D.

Professor of Immunology, Biology Department, The American University in Cairo

Professor Eman El-Ahwany, Ph.D.

Professor of Immunology, Immunology and Evaluation Therapy Department, Theodor Bilharz Research Institute

Fall/2017

Dedication

To my dear parents, your endless support, patience, guidance, and love is the reason I am able to achieve such milestones in my life. I humbly believe I have been blessed with the greatest parents anyone could ever have, and I thank God every single day for this blessing. Thank you for all the time you invested to raise me, guide me, advise me, discipline me, love me, pray for me, and be there for me throughout every single day of my life.

To my loving husband, thank you for always believing in me and putting my needs and happiness above yours. I couldn't imagine a more perfect person for me, and I look forward to spending the rest of my life being by your side.

To my precious siblings, whom I miss being around every day, thank you for constantly keeping me grounded and supporting me by any means. I hope I continue to be the big sister whom you are always proud of and look up to.

To my extended family, in Cairo and LA, thank you for your love, support, and encouragement.

To my dear friends whom I consider family, thank you for always being there for me whenever I needed someone to vent to and cheer me up.

This accomplishment is only possible because of all of your love, support, and prayers. Thank You! I love you all.

Acknowledgements

My sincere appreciation and gratitude goes first and foremost, to my thesis advisor Dr. Suher Zada. Thank you for allowing me to be a part of your team, and for your continuous supervision, help, and support. I would also like to thank my thesis co-adviser, Dr. Eman El-Ahwany, for all her help throughout this long process. Thank you for helping throughout every step of the way, from collecting blood samples to analyzing the data to completing my thesis dissertation, and for all your support and effort. I would like to thank Dr. Hoda Abu-Taleb for helping me with the statistical analysis and patiently answering and explaining all of my questions. Special thanks to Mr. Amged Ouf for helping me throughout all my laboratory work and always being available whenever I needed him. A sincere and deep appreciation goes to my colleague and now friend, Lobna Abul-Dahab, for guiding and advising me throughout the entire process. Thank you for all the encouragement and emotional support you provided for me, and for always making time to help me whenever I needed it. This project would not have been possible without the alliance of Theodor Bilharz Research Institute (TBRI), which provided me with the patient samples necessary for this study. Thank you to AUC for providing the research grant which aided in funding this project. Finally, I would like to thank everyone who contributed and helped with this project including staff and lab members at AUC and TBRI.

Abstract

The American University in Cairo

MicroRNAs as noninvasive biomarkers for the diagnosis and prognosis of liver fibrosis in HCV genotype 4 patients

By: Amanda Abdel-Al

Under the supervision of: Dr. Suher Zada and Dr. Eman El-Ahwany

---

Hepatitis C virus (HCV) is a major world health problem affecting millions of people worldwide. HCV causes fibrosis of the liver; untreated, it leads to complications such as hepatic cirrhosis, decompensation, and hepatocellular carcinoma (HCC). Current methodologies used to determine the progression of hepatic fibrosis rely heavily on liver biopsy, a dangerous and invasive procedure, with subjective analysis of the results of the biopsy. Liver biopsies are also difficult to perform in the developing world, where the strain of HCV infection is great. A new methodology, that is both convenient and inexpensive, is needed for monitoring the progression of liver fibrosis in HCV patients. Small noncoding RNAs known as microRNAs are up-regulated or down-regulated when damage occurs in the liver. miRNAs are stable and present in almost all body fluids, therefore the measurement of circulating miRNAs in serum of liver fibrosis as a noninvasive method to evaluate disease severity and progression is promising. Currently, miRNAs have been found to play essential roles in hepatic stellate cell (HSC) differentiation, proliferation, apoptosis and migration linking them to aberrant expression variations in the development of liver fibrosis. Several microRNAs have shown promise as noninvasive biomarkers of hepatic fibrosis, and some even in the treatment of HCV. To study regulation of genes at the miRNA level is a huge advantage as gene expression is regulated at an epigenetic level before even the formation of proteins.

Hepatitis C-genotype 4 infected patients were selected to detect and study the progression of liver fibrosis. The study consisted of three patient groups: 42 cases of chronic hepatitis C (CHC) with early stage fibrosis, 45 cases of CHC with late stage fibrosis, and 40 healthy patients with no CHC or fibrosis as controls. Blood samples were taken from each patient and RNA was extracted using the miRNeasy extraction kit. Expression patterns of 5 miRNAs (miR-16, miR-146a, miR-214-5p, miR-221, miR-222) were measured in each group using TaqMan real-time

reverse transcription-polymerase chain reaction. MiRNA analysis was performed to determine the most specific and sensitive miRNA to be used as a diagnostic biomarker.

Serum levels of miRNA-16, miRNA-146a, miRNA-221, and miRNA-222 were all significantly upregulated in early and late stage fibrosis compared to the control ($p<0.001$). MiRNA-222 had the highest sensitivity and specificity values in both early and late stage fibrosis with values of (69.23 %, 83.83%) and (100%, 96.77%) respectively. MiRNA-221 had the second highest sensitivity and specificity values with the late stage fibrosis group having values of 100% and 88.24% respectively. MiRNA-222 and miRNA-221 suggest promising potential as biomarkers for HCV-induced liver fibrosis as they had the highest sensitivity and specificity values. MiRNA-221 showed significant positive correlations with both miRNA-16 and miRNA-146a in the early and late stage fibrosis groups, with the early stage having a stronger correlation (at the 0.01 level). These correlations have great substantial values for future uses in formulating liver fibrosis diagnostic assays.

Table of Contents

1 **Introduction** 1

2 **Literature Review** 3

2.1 Liver Fibrosis 3

2.1.1 Background 3

2.1.2 The liver and fibrosis 4

2.1.3 Pathogenetic roadmap of liver fibrosis. 6

2.1.4 Prognosis of liver fibrosis. 11

2.2 HCV 11

2.2.1 Epidemiology & Etiology. 12

2.2.2 Statistics Worldwide 13

2.2.3 HCV in Egypt. 15

2.2.4 Relation of HCV to Liver Fibrosis. 17

2.3 MicroRNAs 17

2.3.1 Background 17

2.3.2 miRNA-16. 21

2.3.3 miRNA-146a. 22

2.3.4 miRNA 214-5p. 23

2.3.5 miRNA-221 24

2.3.6 miRNA-222. 26

2.4 Current methods involved in staging fibrosis 27

2.5 Importance 34

3 **Study Objectives** 36

4 **Materials and Methods** 37

4.1 Patients 37

4.2 Liver Histopathology 37

4.3 Collection of Sera 38

4.4 Laboratory Tests 38

4.4.1 Detection of Circulating Anti-Schistosome Antibodies 38

4.4.2 Detection of Hepatitis B Surface Antigen (HBs Ag) 38

4.4.3 Detection of Anti-HCV (genotype 4) Antibodies 39

4.5 Serum miRNA assay 39

4.5.1 miRNA Extraction 39

4.5.2 Reverse Transcription (RT) and Quantitative PCR (qPCR) 40

4.6 Statistical Analysis 42

5 Results 43
5.1 Demographic, biochemical, & liver histopathology investigations of patients. 45
5.2 MicroRNAs expression profiles by qPCR 46
5.3 Diagnostic performance of circulating miRNAs in predicting liver fibrosis 48
5.4 Correlations 52

6 Discussion 54

7 Conclusion 59

8 Future Prospective 60

9 References 62

10 Appendix 71
10.1 Copyright Approval 71
10.1.1 Copyright for Figures 1 & 3 71
10.1.2 Copyright for Figure 6 72
10.1.3 Copyright for Figure 7 73
10.1.4 Copyright permission for Figure 11 74
10.2 Institutional Review Board (IRB) 75
10.3 Consent Form 76

List of Tables

Table 1: Assay IDs and mature miRNA sequence for the 5 chosen miRNAs 41

Table 2: Demographic and biochemical characterization of early/late stage fibrosis patients and controls 44

Table 3: Expression levels of the 5 miRNAs in each patient group 46

Table 4: AUC, CI, and p-values for the miRNAs in early stage fibrosis 48

Table 5: The sensitivity and specificity values for miRNAs in early stage fibrosis 48

Table 6: AUC, CI, and p-values for the miRNAs in late stage fibrosis 50

Table 7: The sensitivity and specificity values for the miRNAs in late stage fibrosis 50

Table 8: Correlations between miRNAs in early stage fibrosis 52

Table 9: Correlations between miRNAs in late stage fibrosis 53

List of Figures

Figure 1: Functions of HSCs....5

Figure 2: Changes in hepatic architecture (A) associated with advanced hepatic fibrosis (B)..7

Figure 3: Activated HSCs via extracellular stimuli....8

Figure 4: Changes in the extracellular matrix (ECM) during development of fibrosis....9

Figure 5: ECM structural components in HCV-induced liver fibrosis progression from blood sample....10

Figure 6: Global distribution of HCV genotype....14

Figure 7: Number of HCV-infected individuals in highest prevalence countries....16

Figure 8: Prevalence of HCV infection around the world....16

Figure 9: Biogenesis of miRNAs and its cellular release....20

Figure 10: Fibrosis staging using METAVIR scoring method....30

Figure 11: A comparison of the Ishak and METAVIR scoring systems for liver fibrosis....31

Figure 12: Ziols transient elastography (TE) cutoffs correlated with traditional METAVIR fibrosis scores....32

Figure 13: Gene expression in fibrosis stages....33

Figure 14: Global annual mortality from hepatitis, tuberculosis (TB), HIV and malaria....35

Figure 15: Number of people infected, diagnosed and treated for HCV....35

Figure 16: HCV-Induced liver fibrosis stages....45

Figure 17: Box Plots....47

Figure 18: ROC Curves in early stage fibrosis....49

Figure 19: ROC Curves for late stage fibrosis....51

List of Abbreviations

ACR	Acute Cellular Rejection
ADAMTS	A Disintegrin-like And Metalloprotease with Thrombospondin
AFP	Alpha Fetoprotein
ALT	Alanine Aminotransferase
APO A2	Apolipoprotein A2
APO C3	Apolipoprotein C3
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
B-CLL	Beta-cell Chronic Lymphocytic Leukemia
BMF	Bcl-2-Modifying Factor
CDC	Centers for Disease Control and Prevention
CDS2	CDP- Diacylglycerol Synthase 2
CD1	Cyclin D1
CD44	Cluster of Differentiation 44
CFH	Complement Factor H
CHC	Chronic Hepatitis C
CI	Confidence Interval
CLD	Chronic Liver Disease
COLI-A1	Collagen la1
CT	Cycle Threshold
CYP2C8	Cytochrome P_{450}2C8
Dnm3	Dynamin3
ECM	Extracellular Matrix
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HGF	Hepatocyte Growth Factor
HSC	Hepatic Stellate Cells
IARC	International Agency for Research on Cancer
ICAM1	Intracellular Adhesion Molecule One
IFN	Interferon
Ig	Immunoglobulin

IRB	Institutional Review Board
ITGA	Integrated Trace Gas Analyzer
ITIH1	Inter-alpha-trypsin Inhibitor H1
Kbp	Kilo base pairs
LAMB1	Laminin Subunit Beta 1
LAMC1	Laminin Subunit Gamma 1
LC	Liver Cirrhosis
LSM	Liver Stiffness Measurement
MAP7	Microtubule-Associated Protein 7
MiRNA	Micro RNA
MMPs	Matrix Metalloproteinase
MOH	Egyptian Ministry of Health
mRNA	messenger Ribonucleic Acid
NF-κB	Nuclear Factor-Kappa B
NIH	National Institutes of Health
NPV	Negative Predictive Value
OPTN	Organ Procurement and Transplantation Network
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PPV	Positive Predictive Value
PRDM4	PR Domain containing 4
Pri-miRNA	Primary miRNA
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristics
RT	Reverse Transcription
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCFR	Stem Cell Growth Factor Receptor
SERPINF2	Serpin Family F Member 2
SOCS	Suppressor of Cytokine Signaling
SPSS	Statistical Package for the Social Science

SRTR	Scientific Registry of Transplant Recipients
TB	Tuberculosis
TBRI	Theodor Bilharz Research Institute
TE	Transient Elastography
TGF- β	Transforming Growth Factor- β
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF	Tumor Necrosis Factor
TTR	Transthyretin
UTR	Untranslated Region
WHO	World Health Organization

1 Introduction

Infection by the Hepatitis C virus (HCV) has reached global epidemic proportions, with 2016 research estimating 180 million chronically infected individuals, a global prevalence of 2.35%, and three to four million new infections every year (Lavanchy, 2011; Mohd-Hanafiah *et al.,* 2013; Sheiko & Rosen, 2016). Liver fibrosis is the inevitable pathophysiological conclusion of chronic liver injury, such as is the case in chronic HCV infections (Moreira, 2007). Although initially reversible, if left untreated, liver fibrosis will progress to liver cirrhosis, which often presents with the associated complications of hepatocellular carcinoma (HCC), end-stage liver disease, and death, however, this process typically takes many years (Sheiko & Rosen, 2016). HCC's high mortality rates mean a poor prognosis for patients diagnosed with HCC, however, diagnoses are typically only made after hepatic fibrosis has progressed an alarming amount, due to limitations in current diagnostic methods and the delayed onset of fibrotic symptoms (Ahmad *et al*., 2011). HCV infection is notable for the way in which it presents by being asymptomatic at first, followed by a significantly delayed onset of acute symptoms, and for this reason is rarely detected at the time of infection (Taneja *et al*., 2016).

Identifying infected patients, and monitoring and managing the progression of their liver fibrosis in a standardized and measurable way is therefore a chief concern in the treatment of patients with chronic HCV infections. The development and progression of hepatic fibrosis, necro-inflammation, and histological staging is typically assessed by liver biopsy, which is currently the most commonly employed protocol for fibrosis assessment. The results of these liver biopsies are then evaluated using semi-qualitative scoring systems such as Ishak or METAVIR scores (Shiha & Khaled, 2011). Unfortunately, liver biopsies have many potential drawbacks, not the least of which is their invasiveness and the subjective nature of their interpretation. Liver biopsies also require full hospital amenities, and highly trained specialists to perform them, limiting their feasibility in the developing world, where high HCV infection rates are prevalent (Sheiko & Rosen, 2016).

Other methods of classifying liver fibrosis fall under the umbrella of non-invasive protocols, which include serum, genetic, and imaging techniques. Serological (serum) marker and genetic techniques involve measuring one or more molecules in the patients' blood or serum correlating to hepatic fibrosis. Scanning or imaging techniques, on the other hand, detect changes in hepatic parenchyma and ideally differentiate between moderate and severe

fibrosis. However, this methodology is often nearly as expensive as liver biopsies, and few have been thoroughly validated (Ahmad *et al.*, 2011). There exists a clear need to identify specific biomarkers of liver fibrosis and develop non-invasive methodologies for interpreting them (Nallagangula *et al.*, 2017).

MicroRNAs (miRNAs) have shown great potential as novel biomarkers for the detection and assessment of hepatic fibrosis progression. Additionally, miRNAs can be analyzed via noninvasive blood tests, and these tests can be performed repeatedly on patients to gain sequential data as the disease progresses unlike (Khattab *et al.*, 2016). Furthermore, because miRNAs have been suggested to be involved in the multi-step process of chronic HCV infection they are proving to be great diagnostic markers for progression of liver fibrosis (Nallagangula *et al.*, 2017). Some miRNAs have even shown promise in the treatment of HCV in conjunction with other treatment methods such as Interferon (IFN). With a proper and efficient prevention, screening and treatment method, total viremic infections can decline rapidly. Therefore, it is of upmost importance to diagnose liver fibrosis as early as possible while making such diagnostic assays accessible to the majority of infected individuals. This includes being cost effective for the infected individuals of low-income countries.

2 Literature Review

2.1 Liver Fibrosis

2.1.1 Background

Liver (or hepatic) fibrosis is defined as the accumulation of excessive extracellular matrix proteins that occurs as a result of chronic liver injury (Bataller & Brenner, 2005). If not treated, hepatic fibrosis will progress to hepatic cirrhosis, which is associated with serious complications such as hepatocellular carcinoma (HCC), end-stage liver disease, imperative liver transplant, or death (Sheiko & Rosen, 2016). Hepatic cirrhosis secondary to chronic HCV infection is the single most prevalent reason given for liver transplantation in the world (Wiesner *et al.,* 2003).

In the United States, HCV is the leading cause of liver transplantation, with over 40% of liver transplants occurring as a result of HCV-associated hepatic deterioration (Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR): OPTN/SRTR, 2010). Liver transplants are dangerous, invasive, advanced surgical procedures with countless associated risks. Worsening conditions of patient and graft survival in HCV liver transplant patients are frequently reported (Wiesner *et al*., 2003). Furthermore, HCV infection frequently recurs after transplantation, necessitating retransplantation, and the pathology of the recurrence of HCV after liver transplantation is not yet fully understood, and cannot be easily predicted (Wiesner *et al.*, 2003).

In the United Kingdom, a typical example of a developed country with a high-quality, modern healthcare system, hepatic disease is currently identified in the general population only through the use of liver enzyme blood tests, which are non-specific markers of hepatic injury (Harman *et al*., 2015). The degree of fibrosis influences both patient prognosis and remediation strategies, therefore the measurement of fibrous progression is critical to proper treatment of HCV patients (Taneja *et al*., 2016). If hepatic fibrosis is identified early, it is potentially reversible (Ahmad *et al*., 2011). Unfortunately, the liver enzyme blood test strategy is known to be inadequate because of its tendency to result in late diagnosis. The absence of symptoms in the early ages of liver disease means that many patients, even with comprehensive healthcare available to them, do not give reason to their healthcare providers to perform an analysis or investigation of liver in the early stages of hepatic deterioration. Furthermore, the liver enzyme blood tests are insufficiently sensitive to detect hepatic fibrosis in its early stages (Harman *et al.*, 2015). Liver biopsies remain the gold standard for the evaluation of liver fibrosis (Taneja *et al.*, 2016). However, the associated costs of liver

biopsies are significant and pose serious barriers to their application in any country without universal healthcare (Klevens *et al.*, 2016). Indeed, liver cirrhosis is now the third most common cause of premature death in the UK and its prevalence has increased even as other major causes of death decrease (Harman *et al.*, 2015). Liver biopsies are not practical standard procedures even in modern healthcare systems, as they are invasive and require highly-trained specialist surgeons to perform them (Harman *et al.*, 2015).

In Egypt, the rate of infection by HCV approaches 15% and treatment methods (such as those available in more developed countries) are often unfeasible for the general population (Khattab *et al.*, 2016).

2.1.2 The liver and fibrosis

The liver is one of the largest organs in the body, second only to the skin, and is vital for metabolic function, playing a critical role in the metabolization of both fats and proteins. The liver processes, stores, alters, detoxes and returns to the bloodstream, or eliminates, nutrients, medication, or toxic substances introduced to the system. It also creates proteins important in blood clotting, breaks down injured, old, or apoptotic blood cells, and maintains blood sugar levels (Insititute for Quality and Efficiency in Health Care, 2016).

Fibrosis is a medical term used to refer to scarring, or the formation of fibrous extracellular matrix (ECM) on an organ (Schiffman & Cunha, 2016). An extracellular matrix (ECM) is also known as “nonfunctional connective tissue” (Highleyman, 2014). When cells and tissues are injured, fibrosis is the natural response of the body’s immune system as it attempts to repair the damage; in other words, it is the body’s wound-healing or protective response. Pro-fibrogenic pathways are observed across a variety of tissues in the body, including the liver, lungs, and heart (Sheiko & Rosen, 2016). The liver’s functional cells are known as hepatocytes. Injury or necrosis of hepatocytes stimulates the release of cytokines, growth factors, and other chemical markers that command the liver’s support cells, hepatic stellate cells (HSCs), to activate their fibrogenic properties and produce collagen, fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans (Figure 1) (Bataller & Brenner, 2005; Tsuchida & Friedman, 2017). These substances are deposited in the liver as extracellular matrix. At the same time, the breakdown of collagens by normal processes is impaired. Fibrosis is the consequence of an imbalance between the rates of fibrogenesis and fibrous degeneration (Highleyman, 2014). Thusly, hepatic fibrosis is the inevitable consequence of the liver’s persistent wound-healing response to repeated injury (Bataller & Brenner, 2005).

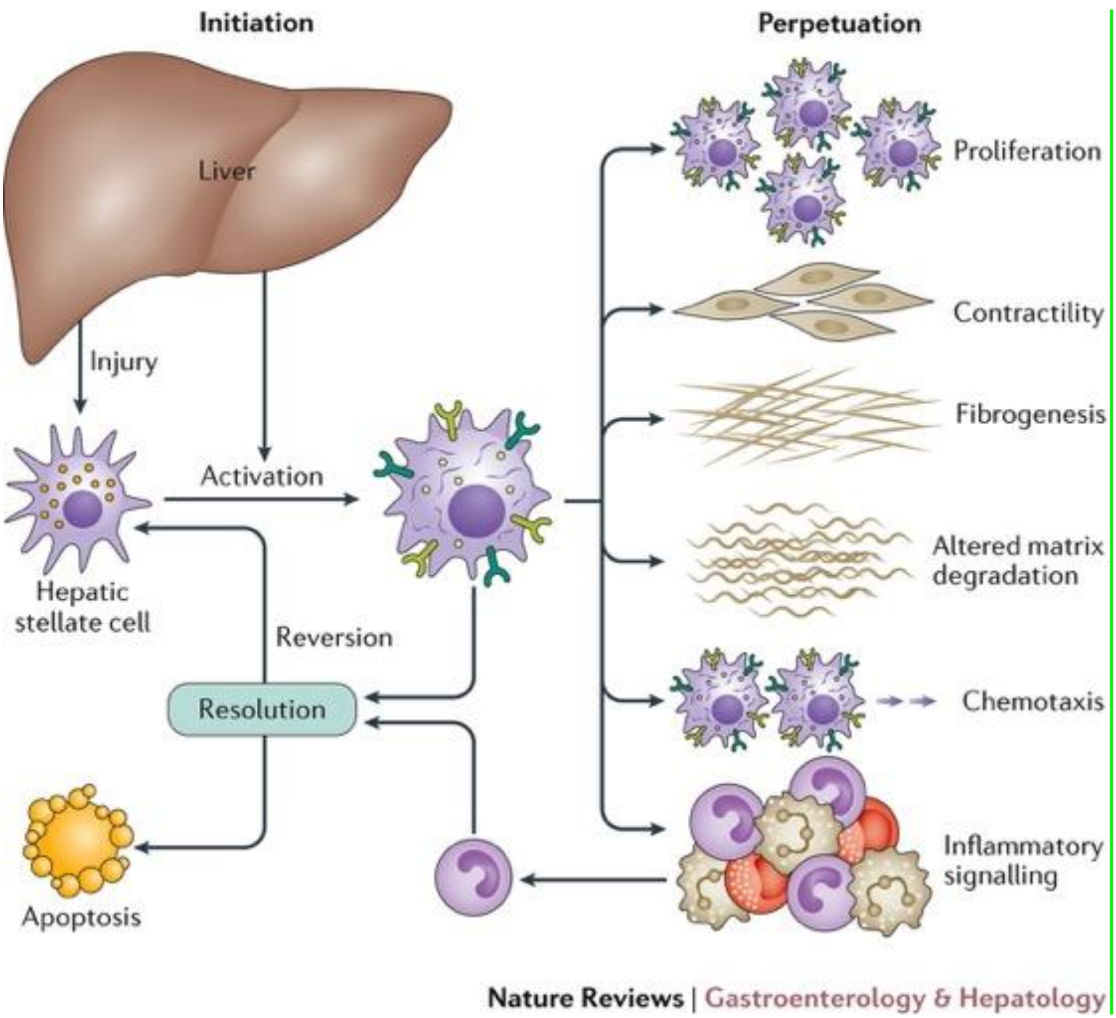


Figure 1: Functions of HSCs.

Liver injury initiates the activation of quiescent HSCs to activated HSCs. Activated HSCs are responsible for proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis and inflammatory signaling. HSCs can be resolved by apoptosis or by going back to the inactivated, quiescent state. Adapted with permission from Tsuchida & Friedman, 2017.

2.1.3 Pathogenetic roadmap of liver fibrosis.

Following acute liver injury, liver parenchymal cells first regenerate, and then replace, the apoptotic or necrotic cells. This process is accompanied by an inflammatory biological response and results in the limited deposition of ECM proteins, and is initially reversible (Bataller & Brenner, 2005; Sheiko & Rosen, 2016). If, however, the hepatic injury persists, such as in the case of viral hepatitis, then liver regeneration will fail, and hepatocytes are replaced with copious amounts of ECM, including fibrous collagen, deposited into the space of Disse (Sheiko & Rosen, 2016).

HSCs are the primary producers of ECM in the injured liver, and are critical to understanding hepatic fibrosis (Figure 2A & 2B). In a healthy liver, HSCs are the primary storage sites of Vitamin A and are located in the space of Disse (Bataller & Brenner, 2005). In cases of chronic liver injury, HSCs transdifferentiate or activate into myofibroblast-like cells, gaining their associated pro-inflammatory, fibrogenic, and contractile properties (Bataller & Brenner, 2005). Through this transdifferentiation, HSCs gain the ability to develop ECM. This transdifferentiation is incited by the presence of mediators created by cellular damage and recruited immune cells, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and interleukin-13, which activate mesenchymal precursors (Sheiko & Rosen, 2016). The majority mitogen for activate HSCs is PDGF, and is primarily produced by Kupffer cells (Bataller & Brenner, 2005).

Chronic HCV infections prompt the release of TGF-β-1, which is critical in the release of the primary markers for HSC activation and proliferation: type-1 collagen and α-smooth muscle actin (Presser *et al.*, 2013). Phagocytosis of apoptotic bodies from hepatocytes infected by HCV can also trigger the activation of HSCs (Figure 3) (Jiang *et al.,* 2008; Tsuchida & Friedman, 2017). Activated HSCs expressing the myogenic markers c-myb, α-smooth muscle actin, and myocyte enhancer factor–2 migrate to and accumulate at the sites of tissue injury and repair, secreting abundant ECM and inhibiting ECM degradation (Bataller & Brenner, 2005).

In a healthy liver, the hepatic sinusoids typically contain collagen IV and VI, in contrast, hepatic fibrosis is marked by the dominant presence of collagens I and III and elastin (Figures 4 & 5) (Karsdal *et al.*, 2015; Baiocchini *et al.*, 2016). The altered ECM can also act as a reservoir for growth factors and MMPs (Olaso *et al.*, 2001). The diseased liver becomes trapped in a vicious cycle, as inflammatory and fibrogenic cells continuously stimulate each other (Maher, 2011). As the deposition of scar tissue continues, normal hepatic parenchyma

are replaced by ECM, and the hepatic fibrosis will progress to cirrhosis of the liver, which is characterized by nodules of regenerative parenchyma surrounded by extracellular matrix (Sheiko & Rosen, 2016).

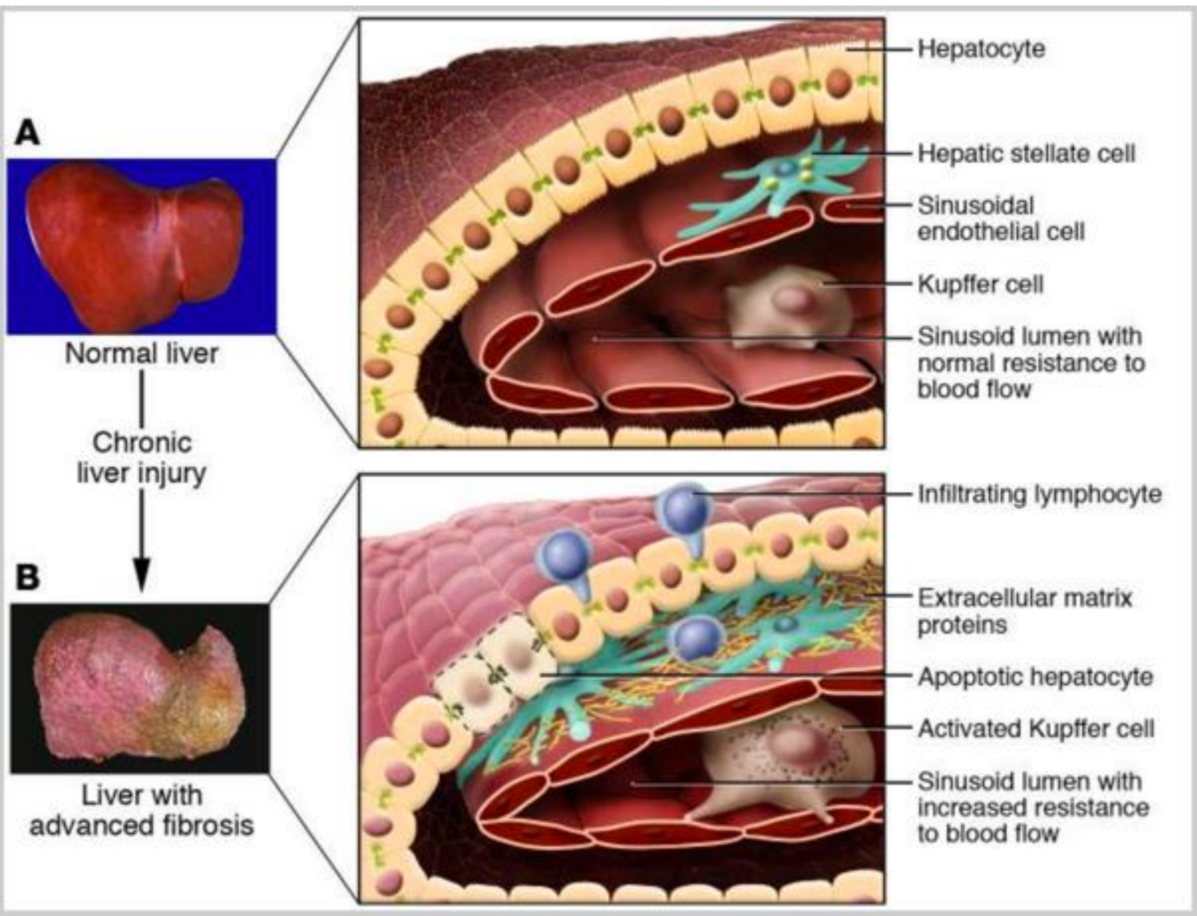


Figure 2: Changes in the hepatic architecture (A) associated with advanced hepatic fibrosis (B).

Inflammatory lymphocytes invade the hepatic parenchyma, causing activation of Kupffer cells. Some hepatocytes undergo apoptosis. HSCs multiply aggressively and secrete ECM proteins (including fibrous collagen) and are deposited into the space of Disse. Sinusoidal endothelial cells lose their fenestrations, and vigorously contracting HSCs obstruct blood flow in the hepatic sinusoid. Adapted from Bataller & Brenner, 2005, open access article, no permission.

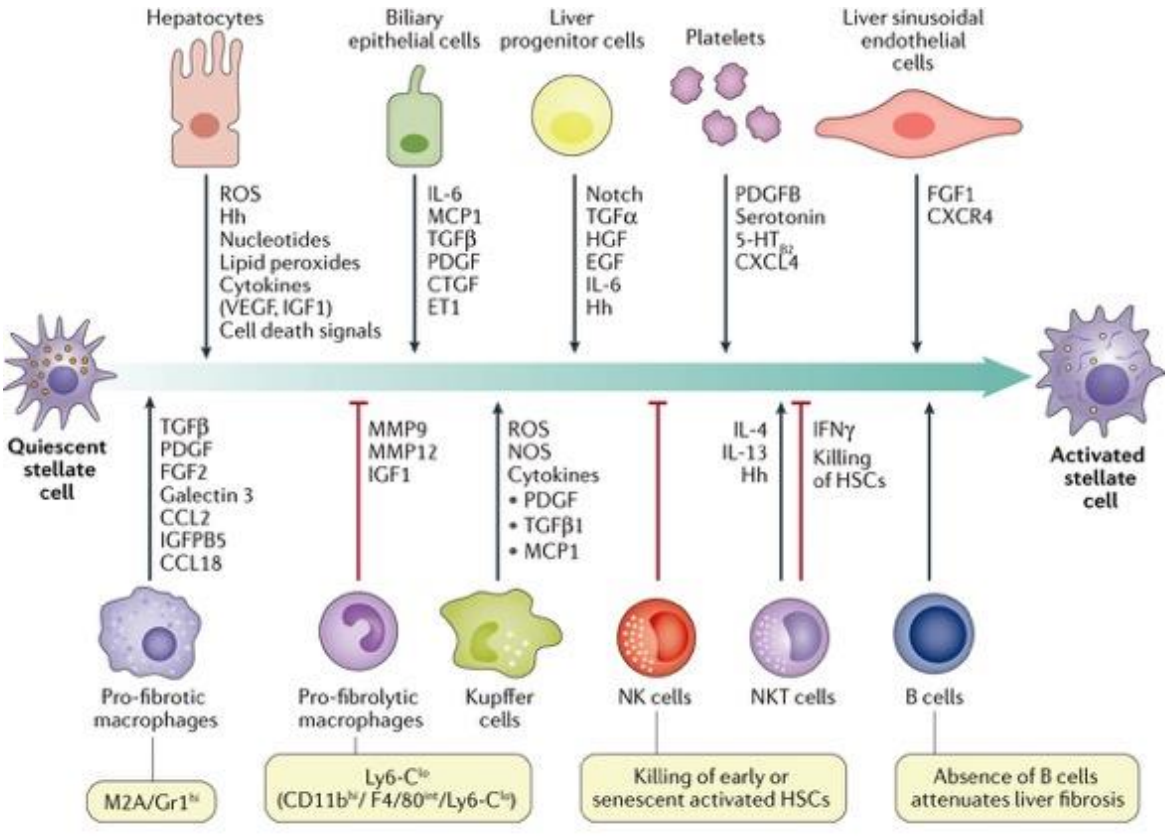


Figure 3: Activated HSCs via extracellular stimuli.

Hepatic cells including hepatocytes, macrophages, biliary epithelial cells, liver progenitor cells, liver sinusoidal endothelial cells, natural killer cells, natural killer T cells, platelets and B cells promote or inhibit the activation of HSCs through the production of hormones, cytokines and other signaling molecules. Adapted with permission from Tsuchida & Friedman, 2017.

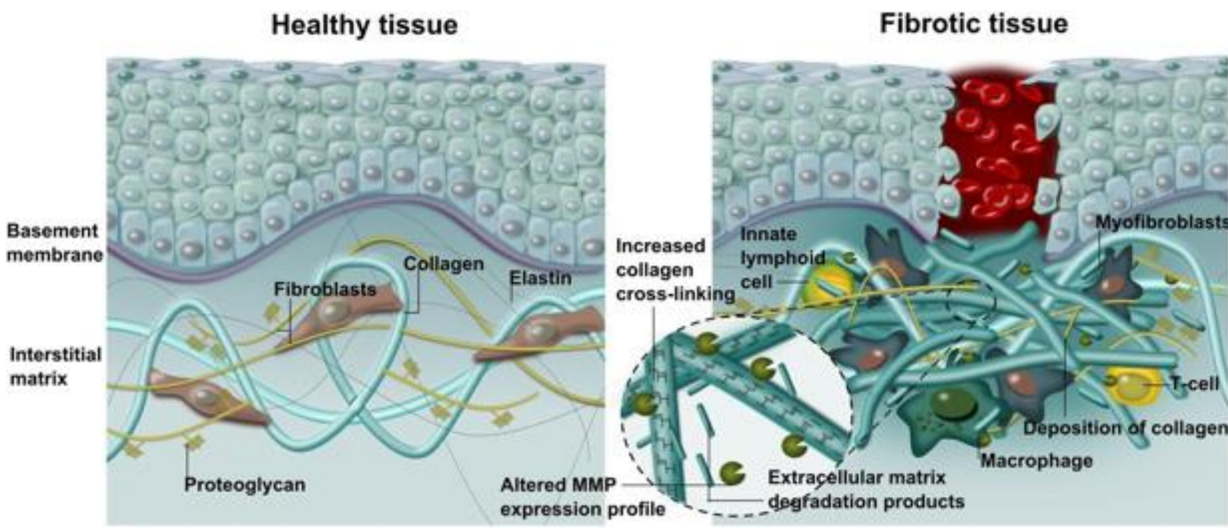


Figure 4: Changes in the extracellular matrix (ECM) during development of fibrosis.

The basement membrane mainly consists of type IV collagen, proteoglycans, laminins and entactin. Interstitial matric mainly consists of fibrillary collagen, glycoproteins, proteoglycans and elastin. Chronic wound healing instigates a profibrotic response. HSCs differentiate into myofibroblasts which deposit excess levels of interstitial collagens. Matrix metalloproteinases (MMPs) are initially enhanced to degrade ECM however as stages progress most MMPs are downregulated. Adapted from Karsdal *et al.*, 2015, open access article, no permission.

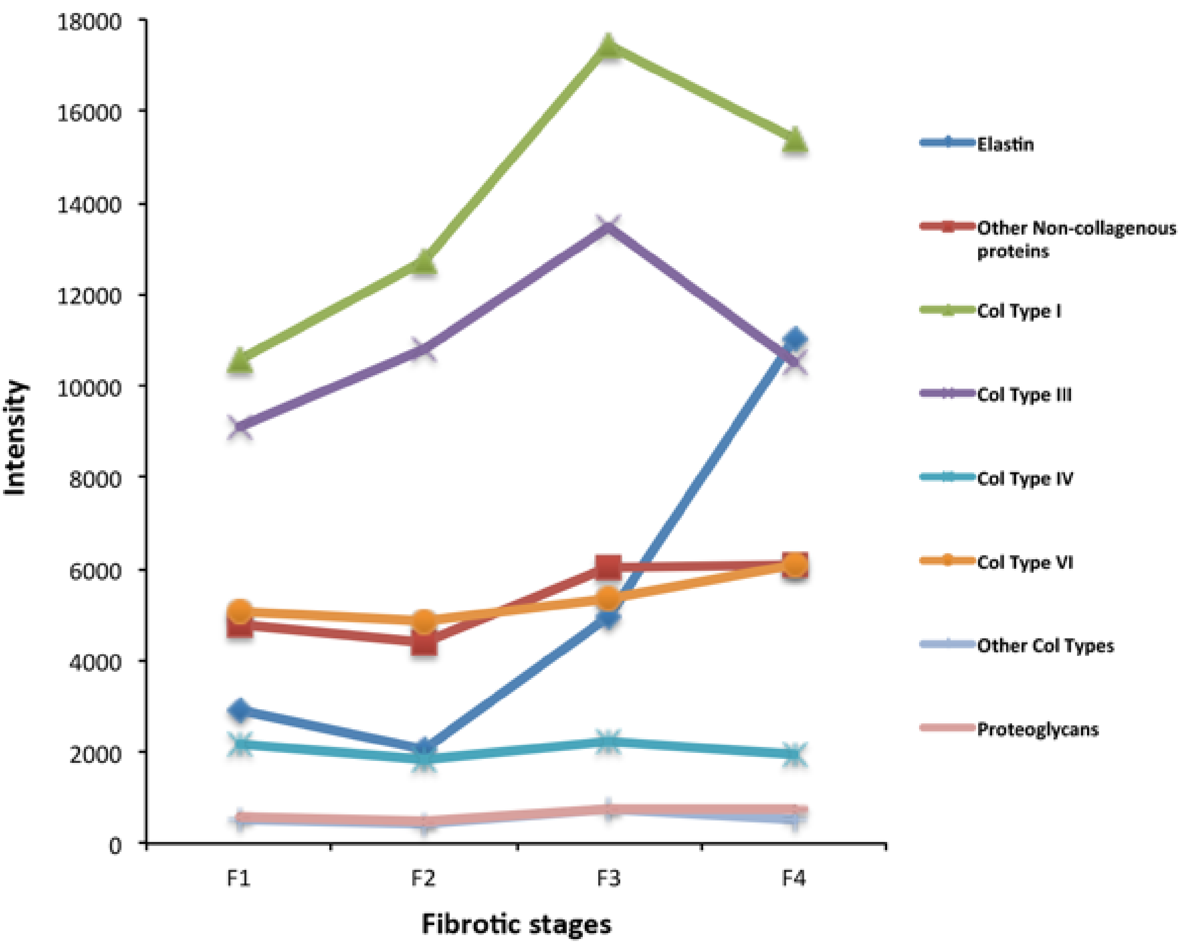


Figure 5: ECM structural components in HCV-induced liver fibrosis progression from blood sample.

Collagens type I and III are the most abundant in hepatic fibrosis followed by elastin. As fibrosis progresses towards cirrhosis collagen levels drop and elastin levels rise significantly. It concludes that in progression towards cirrhosis, liver stiffness is composed of collagen types I and III and elastin fibers. Adapted from Baiocchini *et al.*, 2016, open access article, no permission.

2.1.4 Prognosis of liver fibrosis.

HCV is typically not fatal on its own. Similarly, liver fibrosis does not have associated morbidity and mortality factors until it progresses to cirrhosis. The ultimate, inevitable conclusion of chronic liver fibrosis, liver cirrhosis often presents with major clinical complications such as variceal bleeding, ascites, hepatic encephalopathy, renal failure, and hepatocellular carcinoma (HCC) (Bataller & Brenner, 2005). Some patients with cirrhosis remain free from major complications for several years, in these cases they are deemed as having compensated cirrhosis (Bataller & Brenner, 2005). Decompensated cirrhosis, on the other hand, has high associated morbidity and mortality, and liver transplantation is frequently the only effective therapy (Davis *et al.*, 2001). Because of a typical lack of external symptoms, liver fibrosis is insidious, rarely detected at the onset, and typically takes many years to progress to cirrhosis (Davis *et al.*, 2001).

In one clinical review of over two-thousand patients chronically infected with HCV, the mean length of time for the development of cirrhosis from fibrosis was 30 years (Poynard *et al.*, 2000). According to Bataller & Brennan (2005), in the majority of patients, the progression to hepatic cirrhosis occurs after approximately 15-20 years. Current clinical procedures frequently fail to note the onset of fibrosis in a timely manner. There are a variety of external factors, both genetic and non-genetic, which can affect the advancement of fibrosis in HCV patients, including coinfection of hepatitis B virus and/or HIV, age at the time of severe infection, the hereditary hemochromatosis gene (HFE), angiotensinogen, TGF-β-1, tumor necrosis factor (TNF), and more. Some of the most significant factors on fibrosis progression have been found to be advanced age (≥40 years), alcohol intake (≥30gm), and diabetes mellitus (Taneja *et al.*, 2016).

2.2 HCV

Hepatitis C is one form of infectious hepatitis, meaning inflammation of the liver, and is caused by the hepatitis C virus (HCV) which is a part of the *Flaviviridae* family of viruses; the other types are Hepatitis A and Hepatitis B (Gompf *et al.*, 2016). HCV is a single-stranded RNA that consists of 9.6 kb. HCV RNA encodes a single polyprotein that is split into structural and non-structural proteins. The structural proteins consist of three components (core, E1, and E2) and the non-structural proteins consist of seven components seven (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Ferragamo, 2013). The condition of Hepatitis C is a systemic viral infection that primarily comprises and affects the liver, and can cause both acute and chronic liver diseases. HCV primarily infects hepatocytes; however, it has

also been found in peripheral blood, especially mononuclear cells (Xiong, 2016). This extrahepatic store of HCV is the primary cause of recurrent hepatitis C in orthopedic liver transplant recipients (Xiong, 2016).

2.2.1 Epidemiology & Etiology.

Before the hepatitis C virus was discovered in 1989, it was understood to be associated with blood transfusions, but known only as "non-A, non-B Hepatitis" (Centers for Disease Control and Prevention (CDC), 2014). After the discovery and isolation of the HCV virus, routine testing of the U.S. blood supply became compulsory (Xiong, 2016). As a result of these new protocols, as well as more advanced testing methodologies, HCV was virtually eliminated from blood banks in the United States by 1992, and in the decade after its discovery, the annual number of new HCV infections is reported to have dropped by more than 80% (CDC, 2014). The CDC and US Preventative Services Task Force have advocated for one-time screening of persons born between 1945-1965, or, the "baby boomer" generation, to try and identify previously infected individuals who might be unaware of their infection (Klevens *et al.*, 2016). This testing is particularly important because patient age >50 is one of the factors affecting the rate of progression of hepatic fibrosis most strongly (Klevens *et al.*, 2016).

Unfortunately, although stricter testing protocols, increased awareness, and greater public understanding of the threat of HCV has led to an overall decrease in the number of new infections, there are still 160 million chronically infected individuals worldwide (Lavanchy, 2011). Because of the long timeline of its progression, HCV is still a global health concern affecting every country in the world, necessitating sweeping, active interventions by governmental agencies and health organizations to prevent its spread, control its progression in the infected populations, and to find a safe, reliable, and affordable vaccine (Lavanchy, 2011). In the United States, the "baby boomer" generation accounts for 75% of all HCV infections, and 73% of HCV-associated mortality (CDC, 2014), with old age a particularly complicating factor in the progression and prognosis of hepatic fibrosis to cirrhosis (Sheiko & Rosen, 2016).

Prior to its discovery and isolation in the 1980's, the primary cause of HCV infection was the transfusion of infected blood (Barrera *et al.*, 1995). In the present day, injected recreational drug use accounts for approximately 60% of new infections (Xiong, 2016). The other most common methods of HCV infection in the 21st century are sexual transmission, vertical transmission, and occupational exposure (National Institutes of Health (NIH), 2002).

2.2.2 Statistics Worldwide

Recent estimates put global HCV infection rates at 2.35% of the world's population (Khattab *et al.*, 2016). Approximately three to four million new infections occur worldwide every year (Mohd-Hanafiah *et al.*, 2013). The numerous varieties of HCV have been divided into seven genotypes and several geno-subtypes, each with distinct geographic distributions, and varying responses to anti-HCV agents (Li *et al.*, 2016). A genotype is considered distinct when 30-35% of the nucleotides in the complete genome differ, and several subtypes also exist within each genotype (Thrift *et al.*, 2017). Because treatment regimens and the clinical courses of the disease can vary considerably between each genotype, it is critical that global treatment policies reflect the understanding of the various genotypes that are prevalent in different areas of the world (Thrift *et al.*, 2017).

A systematic review conducted in 2015, that took into account more than a thousand studies on HCV and 90% of the world's population, provides a clear overview of the various genotypes of HCV and their distribution globally (Figure 6) (Blach *et al.,* 2016). HCV Genotype 1 is the most common HCV genotype in the world, comprising 46.2% of all HCV cases, of which approximately one-third are located in East Asia (Messina *et al.*, 2015). HCV Genotype 1 is the predominant type of HCV in 85 of the 115 countries studied by Messina *et al.* (2015) and dominated in high-income and upper-middle income countries (60% of all infections) (Blach *et al.*, 2016). HCV subtypes 1a and 1b are most prevalent in the United States and Europe, while in Japan, 73% of HCV-infected persons carry subtype 1b (Thrift *et al.*, 2017). HCV Genotype 1 is associated with the more aggressive development of severe liver disease and typically displays an inferior response to antiviral therapy (Lavillette *et al.*, 2005).

HCV Genotype 3 is the next most prevalent genotype worldwide, comprising 30.1% of cases globally (Thrift *et al.*, 2017) and is common in lower-middle income countries (Blach *et al.*, 2016). HCV Genotype 3 is prevalent in south Asia and comparatively rare in Africa (Messina *et al.*, 2015). Genotype 3 typically indicates an increased risk of serious hepatic complications and has a higher rate of overall mortality than the average for all patients infected with HCV (Thrift *et al.*, 2017). HCV Genotype 3a is particularly prevalent amongst intravenous drug users in the United States and Europe (Zein, 2000).

HCV Genotypes 2, 4, and 6 together account for 22.8% of global HCV cases (Thrift *et al.*, 2017). Genotype 4 is the most common HCV genotype in the Middle East and low-

income countries, and is overwhelmingly the dominant genotype in central sub-Saharan Africa (Blach *et al.*, 2016; Thrift *et al.*, 2017). Although subtypes 2a and 2b are relatively equally dispersed across North America, Japan, and Europe, subtype 2c is particularly common in northern Italy (Zein, 2000). HCV Genotype 6 is present only as a minority genotype in East and Southeast Asia, and is the dominant genotype only in Laos (Thrift *et al.*, 2017).

HCV Genotype 5 makes up less than one percent of all global infections of HCV (Thrift *et al.*, 2017). It is also geographically limited, and is only prevalent as an endemic genotype in Southern Africa (Messina *et al.*, 2015). Only one confirmed case of HCV genotype 7 infection has been reported globally, found in a Central African patient who had emigrated to Canada (Murphy *et al.*, 2007).

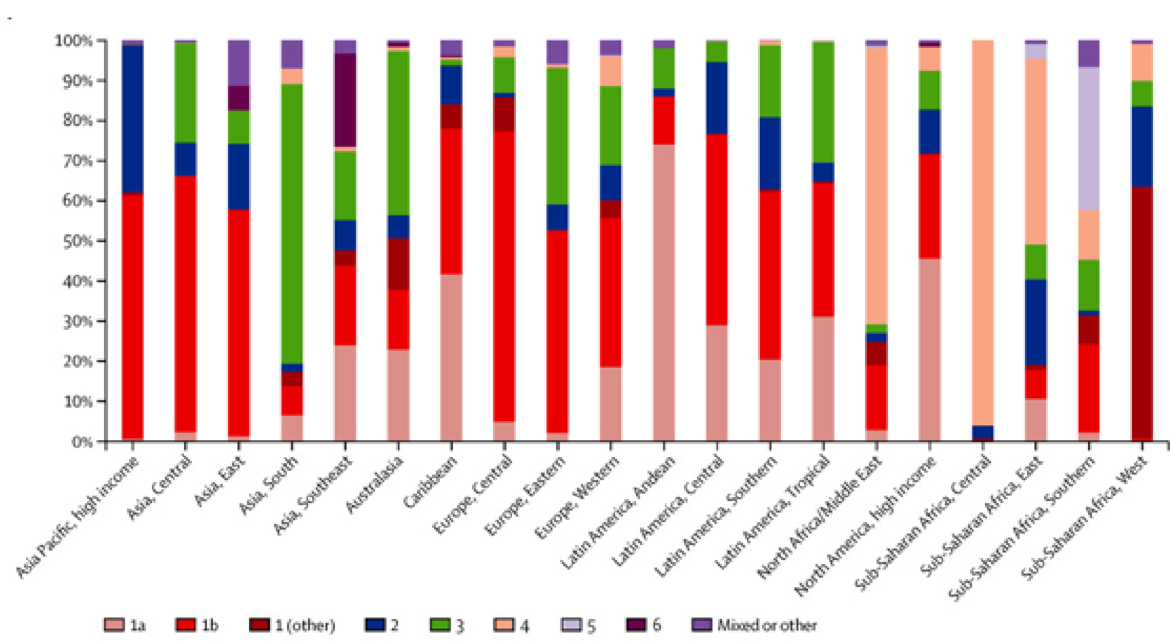


Figure 6: Global distribution of HCV genotype.
Genotypes 1 and 3 are the most prevalent worldwide (44% and 25% of all infections respectively). However, infection rate of genotypes 2, 4, 5 and 6 is particularity high in unique geographical regions with genotype 4 coming in third at 15% mainly composed of low-income countries. Egypt, grouped under North Africa/Middle East, is predominantly composed of genotype 4. Adapted with permission from Blach *et al.*, 2016.

2.2.3 HCV in Egypt.

Egypt has one of the highest rates of prevalence of HCV infection worldwide, estimated at 14.9% by the Egypt Demographic and Health Survey (Khattab *et al.*, 2016). Egypt has the fifth-highest rates of HCV infection in the world, after China, Pakistan, Nigeria, and India (Figures 7 & 8) (Daw *et al.*, 2016; Thrift *et al.*, 2017). New cases of HCV peaked in 1970 in Egypt, an unfortunate consequence of governmental efforts to eradicate schistosomiasis (Elgharably *et al.*, 2017; Thrift *et al.*, 2017).

Historically, and as far back as the ancient Egyptian empires, Egypt's foremost health problem was the schistosomiasis parasite (*Schistosoma mansoni* and *Schistosoma haematobium*), with *S. mansoni* previously being the primary cause of liver disease in the country (Barakat, 2013). In 1918, it was discovered that an injection of the antimony salt, tartar emetic, could cure schistosomiasis, and mass treatment of the parasite in this way was introduced via primary healthcare services (Elgharably *et al.*, 2017). From the 1950s to the 1980s, more than 2 million tartar emetic injections were given annually to an average of 250,000 patients, as a part of a sweeping national health effort organized by the Egyptian government with advice and support from the WHO (Elgharably *et al.*, 2017). Unfortunately, in the process of this mass treatment of schistosomiasis, the Egyptian population was simultaneously infected by HCV, a virus unknown until decades later, and other blood-borne diseases. Three main factors influenced the high infection rates of HCV during this time period as a part of the schistosomiasis treatment: patients were injected multiple times, increasing the likelihood of pathogen transmission; sterilization techniques were very poor to non-existent; and equipment was often reused, a protocol not considered important until the HIV epidemic of the 1980s (Elgharably *et al.*, 2017). Furthermore, the delayed onset of acute symptoms of HCV infection meant that infections went overwhelmingly unnoticed until years, or even decades, later (Elgharably *et al.*, 2017). In the present day, nosocomial infections, or infections originating in hospitals, are a primary source of infection in high-prevalence countries such as Egypt (Thrift *et al.*, 2017).

HCV-genotype 4 is the most common genotype in Egypt (Khattab *et al.*, 2016). Twenty percent of Egyptian blood donors are seropositive for HCV antibodies, and approximately 90% of Egyptian HCV patients are infected with HCV-genotype 4 (El-Guendy *et al.*, 2016). Egyptian geographical origin and insulin resistance are the major predicting factors of liver fibrosis, and associated response to therapy, in HCV-genotype 4 (Khattab *et al.*, 2016).

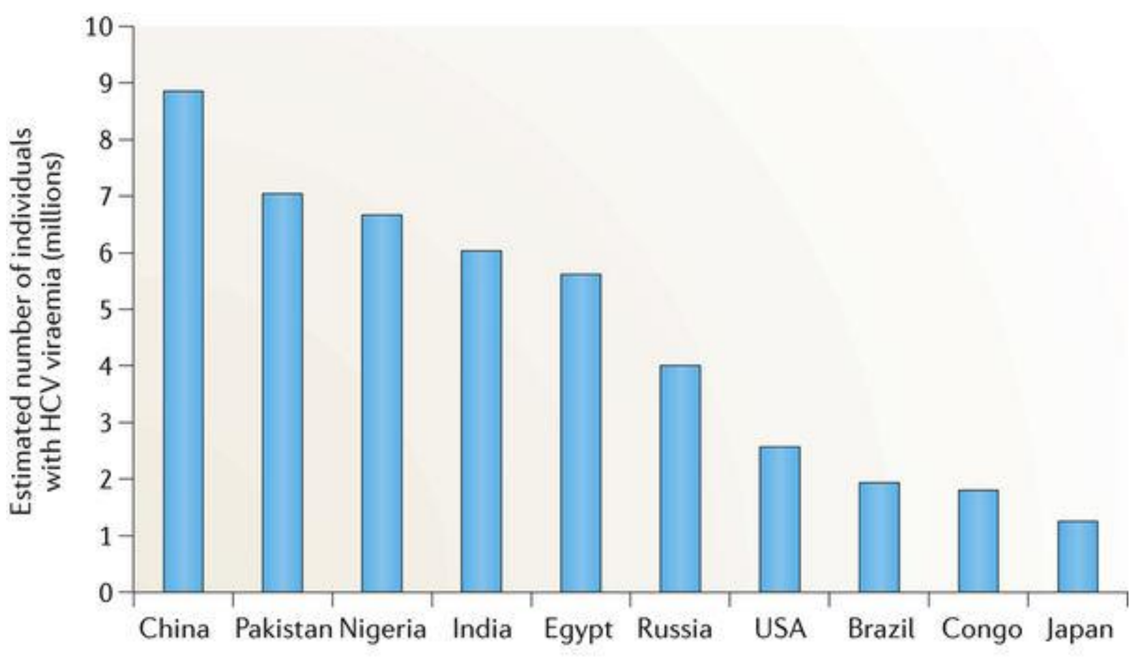


Figure 7: Number of HCV-infected individuals in highest prevalence countries.
Top 10 countries with the highest numbers of individuals with HCV viremia. Egypt is 5th highest following China, Pakistan, Nigeria and India. Adapted with permission from Thrift *et al*., 2017.

Figure 8: Prevalence of HCV infection around the world.
Egypt has the highest prevalence of HCV with more than 14% of people infected. Adapted from Daw *et al*., 2016, open access article, no permission.

2.2.4 Relation of HCV to Liver Fibrosis.

Chronic HCV infection is one of the most common causes of hepatic fibrosis, distortion of the hepatic architecture, and ultimate progression to cirrhosis of the liver (Ahmad *et al.*, 2011). HCV infection inflames the liver which, if unmanaged or untreated, will inevitably result in fibrosis, and then progress to cirrhosis, which has many comorbid complications such as hepatic decompensation or hepatocellular carcinoma (HCC) (Sheiko & Rosen, 2016; Thrift *et al.*, 2017). HCV was declared carcinogenic to humans in 1993 by the International Agency for Research on Cancer (IARC), and HCC is the third most common cause of cancer-related death globally (Thrift *et al.*, 2017). Chronic HCV infections are also associated with insulin resistance and type-2 diabetes mellitus, which are more commonly seen accompanying HCV infections compared to liver diseases of other etiology or healthy controls (Khattab *et al.*, 2016). Liver biopsies have long been the "gold standard" diagnostic tools to determine the progression of hepatic fibrosis of any etiology, but their semi-subjective scoring systems are not ideal, they are extremely costly, and the potential for pain and serious complications associated with such invasive surgical procedures are large (Klevens *et al.*, 2016). The most common scoring methods, Ishak & METAVIR scores, are evaluated by individual physician opinion, and are not quantitative (Ahmad *et al.*, 2011) (Figure 11). It is clear that a better methodology is needed to manage this global health concern.

2.3 MicroRNAs

2.3.1 Background

MicroRNAs (miRNAs) are small, non-coding ribonucleic acids (Li *et al.*, 2016). MiRNAs are found in most eukaryotes, including humans (MacFarlane & Murphy, 2010). Thousands of mature miRNAs have been identified across the spectrum of plants, animals, and even viruses, and more than 2,500 have also been identified in humans alone (Li *et al.*, 2016). Typically, miRNAs negatively modulate gene expression through partial or full complementary binding to target sequences in the 3' untranslated region (3'UTR) of mRNA (Guo *et al.*, 2009). The study of miRNAs has revealed a huge variety of functions that they perform in the body. These include critical biological processes such as cell proliferation, differentiation, tumorigenesis, and apoptosis, and the translation, transcription, and regulation of many genes, particularly members of the signal transduction networks (Guo *et al.*, 2009; Cermelli *et al.*, 2011). Furthermore, absent and/or aberrant expression of miRNA is often

associated with the development of serious pathophysiological disorders, including various types of cancers (Cermelli *et al.*, 2011).

Two forms of miRNA regulation have been identified: in the first, miRNAs pair with target sequences of mRNA by full or partial complementarity, and then trigger the degradation of the target mRNA; in the second, miRNAs pair imperfectly with target sequences and typically inhibit the translation of mRNA (Ambros, 2004). However, precise or nearly precise complementarity is not necessary, meaning that any given miRNA could theoretically bind to a wide variety of mRNAs. Although some miRNAs are uniquely created from individualized transcription units, many more miRNAs are created from units capable of creating various end products (Bartel, 2004). Particular transcripts may encode clusters of distinct miRNAs, or they can encode combinations of miRNA and proteins, the latter of which are organized so that the miRNA sequences are located inside introns (Carthew & Sontheimer, 2009). Many new animal miRNAs are thought to be created by nucleotide sequence changes, rather than through gene duplication (Lu *et al.*, 2004). If a new miRNA sequence appears within an existing transcription unit, it immediately expresses its corresponding product without the creation or replication of enhancers or promoters, which means that new miRNA genes can grow without the process of gene duplication, and may explain why there are so many miRNA genes with multiple products (Carthew & Sontheimer, 2009). Single-stranded miRNAs have been associated with biological effector assemblies known as RNA-induced silencing complexes (RISCs) (Carthew & Sontheimer, 2009).

The biogenesis of miRNAs has been studied in detail, and it is a complicated mechanism. Nuclear transcription of miRNAs leads to capped and polyadenylated pri-miRNAs. In plants, Dc11 processes the RNA in succession, whereas in animals, the pri-miRNA is processed by the enzyme Drosha with the aid of DGCR8, resulting in a pre-miRNA species which is expelled from the nucleus and processed by the enzyme Dicer into the mature miRNA-miRNA duplex (Carthew & Sontheimer, 2009). After this process has completed, the miRNA duplexes are assembled into miRISCs via miRISC loading (Carthew & Sontheimer, 2009) (Figure 9).

MiRNAs have the potential to disturb expansive regulatory networks, but their flexibility also means they have great potential in targeted therapies (Li *et al.*, 2016). Because miRNAs are small, specific, and relatively stable in plasma, they also show great promise as diagnostic and prognostic biomarkers (Motawi *et al.*, 2016).

HCV does not encode viral miRNA, as it is a single-stranded, positive-sense RNA virus, however, it does alter the expression of host miRNAs, both in cellular culture and in patients with progressive, chronic liver diseases, such as fibrosis, cirrhosis, and HCC (Li *et al*., 2016). Current research continues to bring evidence that miRNAs are one of the most important factors to consider in the interactions between virus and host, as host miRNAs regulate the life cycle of the hepatitis C virus directly or indirectly. Host miRNAs regulate the life cycle of HCV directly by binding to HCV RNAs or indirectly by targeting cellular miRNAs, which in turn up- or down-regulate HCV replication (Li *et al*., 2016). Li *et al*. (2016) proposed that this "competitive viral and host RNA hypothesis" indicates a hidden cross-regulation pattern between host miRNAs and HCV RNAs. In cases of HCV infection, high loads of HCV RNA co-opt host miRNAs from their healthy host targets, disturbing host gene expression and allowing HCV to survive and establish a persistent infection (Li *et al*., 2016). The expressions of certain miRNAs are closely matched to hepatic disease progression, and because the changes in levels of these proteins are measurably specific using techniques such as PCRs, more so than in traditional proteins, they can potentially serve as novel diagnostic biomarkers for chronic HCV patients with associated liver diseases (Li *et al*., 2016).

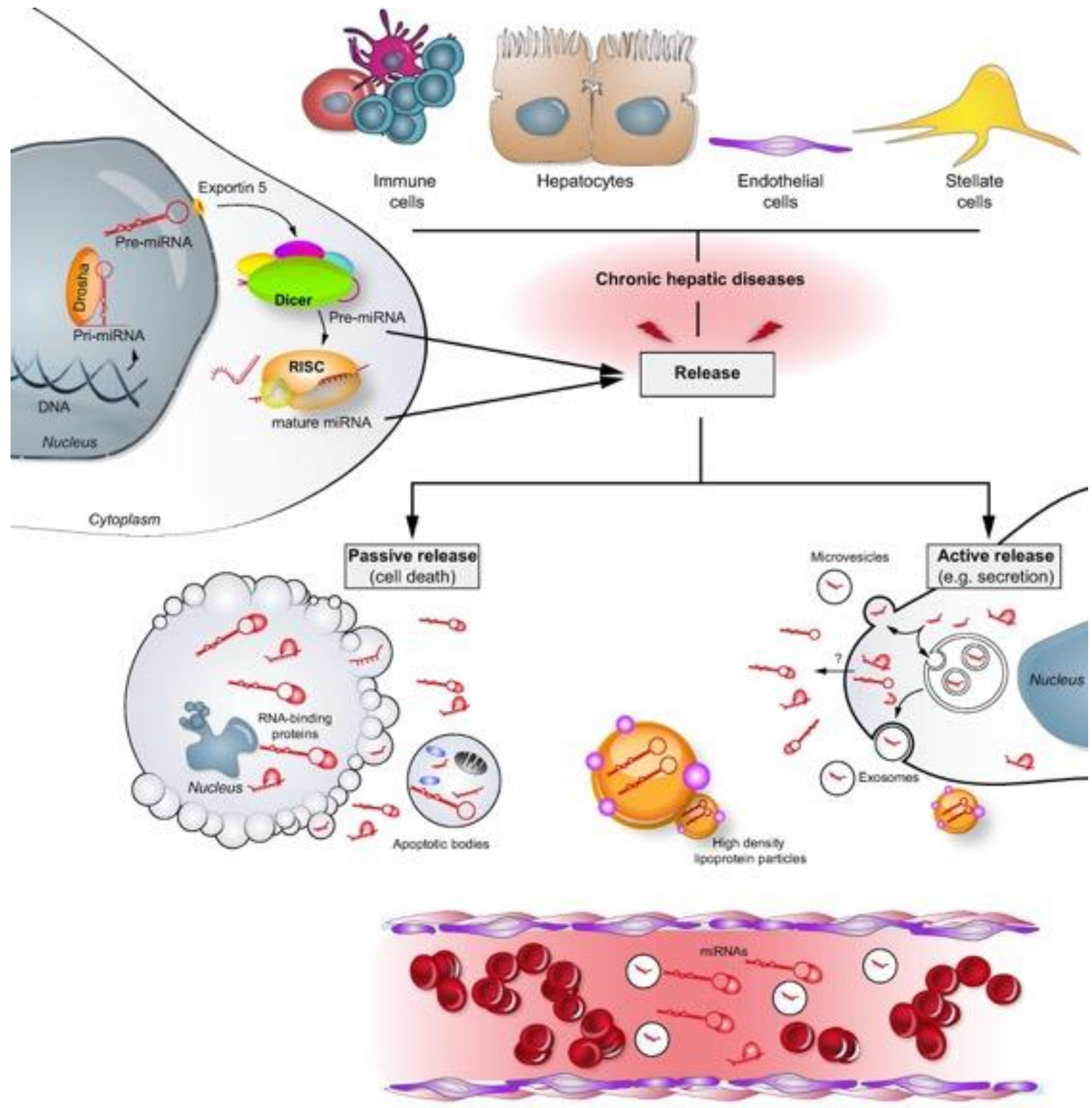


Figure 9: Biogenesis of miRNAs and its cellular release.

Pri-miRNAs are cleaved by Drosha enzyme and turned into pre-miRNAs which are transported into the cytoplasm via Exportin 5. In the cytoplasm, they are cleaved into ~20 nucleotide mature miRNAs and join RISC to prevent translation of mRNA into proteins. During chronic hepatic diseases, miRNA-protein complexes can be released passively or actively. Adapted from Roderburg & Luedde, 2014, open access article, no permission.

2.3.2 miRNA-16.

MiRNA-16 is an important eukaryotic miRNA that is involved in numerous biological processes, including tumorigenesis (Yan *et al.*, 2013). In humans, miRNA-16 is known to be clustered within a 0.5 kilo base pair (kbp) region in Chromosome 13 and to have specific gene targets (Yan *et al.*, 2013). MiRNA-16 is known to be involved in the proliferation, clonogenicity, and anchorage-independent growth of HBx-expression HepG2 cells by G1 phase arrest and the induction of apoptosis (Wu *et al.*, 2011). MiRNA-16 has been shown to directly target microtubule-associated protein 7 (MAP7), PR domain containing 4 (PRDM4), and CDP- diacylglycerol synthase 2 (CDS2) (Yan *et al.*, 2013). MiRNA-16 has also been shown to be critically involved in the regulation of the proliferation of cancer cells, which makes sense as its location on the chromosome is a region known to be downregulated or absent entirely in cases of beta-cell chronic lymphocytic leukemia (B-CLL), the most common form of leukemia in adults (Döhner *et al.*, 2000).

Several miRNAs, including miRNA-16, have been found to be frequently deregulated in cases of HCV, indicating the potential for the development of protocols to detect HCV and the progression of hepatic fibrosis using these miRNAs. In 2009, Guo *et al.* showed that miRNA-16 is downregulated during the activation of HSCs by microarray hybridization. They also showed that miR-16 participates in the apoptosis of HSCs by targeting Bcl- 2 and the downstream protease cascade. In 2011, Bihrer *et al.* and Cermelli *et al.* were among the many researchers who have shown that miRNA-16 is differentially regulated in patients with HCV. Cermelli *et al.* (2011) performed a study that showed that serum levels of miRNA-16 were 3.0-fold and 6.3-fold higher, respectively, in two study groups of patients with chronic HCV infection, compared to healthy controls. They also showed that miRNA-16 was more sensitive than alanine aminotransferase (ALT) in detecting the early stages of HCV. In 2015, Zhu *et al.* further investigated miRNA-16, and found that miRNA-16 levels were increased in patients with HCV infection, and that these values correlated inversely with Smad7 and hepatocyte growth factor (HCF) expression levels. They also showed that both HGF and Smad7 were targets of miRNA-16, and were downregulated as miRNA-16 expression increased, promoting liver fibrosis.

In 2016, El-Abd *et al.* expanded on this previous research to investigate the potential of miRNA-16 as a biomarker for the development of HCC. Their work showed that the combination of serum miRNA-16 and serum alpha fetoprotein (AFP) made for a marked

improvement in the early identification of HCC in Egyptian patients with chronic HCV infections, compared to current best practices which indicate the use of AFP alone.

MiRNA-16 also has potential in the treatment of hepatic fibrosis. Guo *et al.* (2009) expanded upon previous work which had shown that miRNA-16 is downregulated during the activation of HSCs to a potential therapeutic application. In their work, they showed that restoring intracellular miRNAs by the administration of miRNA-16 significantly decreased the expression levels of cyclin D1 (CD1), and also encouraged cell-cycle arrest and apoptosis in activated HSCs. They concluded that the transduction of miRNA-16 is a promising method for inhibiting HSC proliferation and increasing the apoptosis index, thereby potentially providing a treatment methodology for hepatic fibrosis.

2.3.3 miRNA-146a.

MiRNA-146 is a family of miRNA precursors found in mammals, including humans, and has been shown to be directly involved in the regulation of inflammation and other processes of the innate immune system (Sonkoly *et al.*, 2008). MiRNA-146 is located on Chromosome 5 in humans (Lagos-Quintana *et al.*, 2002). The altered expression of miRNA-146 in chronic inflammatory diseases has suggested its involvement in immune-mediated diseases (Sonkoly *et al.*, 2008). Inflammatory factors such as interleukin-1 and tumor necrosis factor (TNF) have been shown to upregulate miRNA-146, both of which are key players in the pathological processes associated with hepatic fibrosis (Sheedy & O'Neill, 2008). Another key player in the development of hepatic fibrosis is the primary stimulus factor responsible for the activation of HSCs, transforming growth factor-β1 (TGF-β1). Recent research has shown that the expression of miRNA-146a in HSC is downregulated in HSCs in a dose-dependent manner in response to TGF-β1 stimulation (He *et al.*, 2012).

He *et al.* (2012) showed that miRNA-146a is downregulated in fibrotic liver tissues, and that the overexpression of miRNA-146a suppressed TGF-β1-induced HSC growth increase and encouraged HSC apoptosis, that is, miRNA-146a expression is inversely correlated with HSC cell proliferation. They also showed that the SMAD4 gene is the likely target of miRNA-146a, and that miRNA-146a modulates SMAD4 in HSCs. Therefore, they concluded that miRNA-146a modulates HSC proliferation induced by TGF-β1 by targeting SMAD4 (He *et al.*, 2012).

It has been shown that miRNA-146a is acutely involved in not only the progression, but the replication and amplification of the HCV virus in infected patients, and its potential as a

novel noninvasive biomarker for the progression of HCV-associated hepatic fibrosis and HCC is great. Bandiera *et al*. (2004) worked with miRNAs and HCV on the theory that HCV may directly induce miRNAs that contribute to hepatic injury and carcinogenesis. They reached a conclusion that HCV-induced increases in miRNA-146a expression both promote further viral infection, and are relevant to the pathogenesis of hepatic disease. It was shown that miRNA-146a overexpression modulated pathways related to hepatic disease and HCC development. The level of miRNA-146a was consistently shown to have increased across multiple different models of HCV-infection, both in primary human hepatocytes and HCV-infected hepatocyte-like cells, and in HCV patient-derived hepatic tissue (Bandiera *et al*., 2004). Overexpressed miR-146a also increased overall HCV infection through positive impact on the late stages of the HCV replication cycle indicating that miRNA-146a-5p is a promising biomarker for virus-induced liver disease and HCC (Bandiera *et al*., 2004).

Joshi *et al.* (2013) further explored the potential for miRNA-146a to act as a novel noninvasive biomarker, particularly as it pertains to the severity of HCV recurrence and acute cellular rejection (ACR) after liver transplantation. They worked on determining an appropriate methodology for distinguishing recurrent HCV from ACR in liver transplant patients, which is a frequently problematic task that can lead to incorrect conclusions, ill-advised treatments, and adverse outcomes. They hypothesized that intragraft miRNA expression profiles could not only differentiate recurrent HCV from ACR, but could potentially also identify the severity of recurrent HCV. Joshi *et al.* (2013) studied patients with both slow HCV fibrosis progression (Ishak stage <F2) and fast HCV fibrosis progression (Ishak stage ≥F2), and found that microRNAs were a promising source of potential resolution for this problem. Increased miRNA-146a expression was seen intragraft in slow progressors versus fast progressors, and key fibrogenic mediators were down-regulated in response to the upregulation of miRNA-146a. They concluded that miRNA-146a plays an important role in the pathogenesis of HCV recurrence after liver transplant.

2.3.4 miRNA 214-5p.

The miRNA-214 family is a vertebrate-specific family of miRNA precursors, and is located on the opposite strand of an intron of the Dynamin3 (Dnm3) gene where it is related via expression cluster to miRNA-199 (Desvignes *et al*., 2014). MiRNA-214 is located on Chromosome 1 in humans, specifically location 1q24.3 (NCBI, 2017). MiRNA-214 has been shown to play an important role in a broad variety of both developmental processes and developmental diseases. Given its vertebrate-specificity, it is unsurprising that miRNA-214

has been shown to play a critical part in skeletogenesis, particularly the development of the craniofacial skeletal elements, as well as several types of cancer (Desvignes *et al*., 2014).

MiRNA-214-5p is one of the most promising novel noninvasive biomarkers for the detection of hepatic fibrosis progression in patients with HCV. In 2012, Iizuka *et al.* showed that miRNA-214-5p was upregulated in both human and mouse livers in a fibrosis progression-dependent manner. In the activation of mouse primary stellate cells, miRNA-214-5p expression markedly increased, and was shown to be significantly higher in stellate cells than in hepatocytes (Iizuka *et al*., 2012). The researchers showed that there was a direct relation between the overexpression of miRNA-214-5p in LX-2 cells and the expression of factors associated with the development of fibrosis, such as matrix metalloproteinase (MMP)-2, MMP-9, α-smooth muscle actin, and TGF-β1 (Iizuka *et al*., 2012).

In 2014, Chen *et al.* expanded upon this previous research to show that miRNA-214 in LX-2 cells is transferred via exosomes to recipient LX-2 cells or human HepG2 hepatocytes. Furthermore, this connective tissue growth factor (CCN2) is another factor critical in driving the fibrogenesis of HSCs, and it has been associated with a reciprocal downregulation of miRNA-214. The downregulation of CCN2 expression is consistent with a conserved binding of miRNA-214 to the CCN2 3'-UTR site, and the downregulation of miRNA-214 results in the suppression of CCN2 3'-UTR activity and expression of CCN2 downstream fibrogenetic targets alpha smooth muscle actin and collagen (Chen *et al*., 2014). It has also been shown that Twist-1 is increased in fibrotic livers, and induced during HSC activation (Iizuka *et al*., 2012). It can be concluded that miRNA-214-5p plays a crucial role in the activation of hepatic stellate cells and the progression of hepatic fibrosis, and that Twist-1 may be the regulator of miRNA-214-5p in hepatic stellate cells (Iizuka *et al*., 2012), and in 2015, *Okada et al.* confirmed that miRNA-214-5p directly affects the development of hepatic fibrosis by modulating both TGF-β signaling pathways and the epidermal growth factor receptors.

2.3.5 miRNA-221.

MiRNA-221 is an oncogenic miRNA, also known as a carcinogenic or tumorigenic miRNA, that is involved in the formation of cancer cells. It is located on chromosome band Xp11, specifically Xp11.3 (Tabasi & Erson, 2008). MiRNA-221 targets the mast/stem cell growth factor receptor (SCFR) also known as proto-oncogene c-Kit or CD117, which then prevents cell migration, proliferation, and angiogenesis in endothelial cells (Urbich *et al*., 2008). The upregulation of miRNA-221 promotes cell cycle progression, angiogenesis, and invasion; miRNA-221 has been shown to be an anti-apoptotic RNA, and its silencing can lead to

increased cell death (Gupta *et al.*, 2014). One of the targets of miRNA-221 is cyclin-dependent kinase inhibitor CDKN1B/p27, and the downregulation of this protein impacts HCC prognosis (Tannapfel *et al.*, 2000). Another target of miRNA-221 is CDKN1C/p57 (Fornari *et al.*, 2008). The Bcl-2-modifying factor (BMF) is a pro-apoptotic protein that modulates the susceptibility of HCC cells to apoptotic stimuli through a caspase 3-dependent pathway, and BMF has been identified as another direct functional target of miRNA-221 (Gupta *et al.*, 2014). MiRNA-221 was also shown to be upregulated in activated LX-2 HSC cells and expression was enhanced by pro-fibrotic proteins including TGFα and tumor necrosis factor (TNF)-α. It was also conversely shown to have a reduced expression by NF-κB inhibitor, which is one signaling pathway that regulates HSCs activation (Kitano & Bloomstom, 2016). Ogawa *et al.* (2012) also showed that miRNA-221 was upregulated in the human liver in a fibrosis progression-dependent manner.

Quite a bit of research has been done on miRNA-221 and HCV, and it is one of the most promising avenues for potential biomarkers, therapies, and more effective treatments for HCV. El-Garem *et al.* (2014) identified miRNA-221 as one of the most promising non-invasive biomarkers for the development of HCC in Egyptian patients with HCV-HCC. The fold changes in serum miRNA expression were considerable across the different patient groups. MiRNA-221 was elevated in the interferon-naïve chronic HCV patients and the post-HCV compensated cirrhosis patients, and decreased in the treatment-naïve HCC patients (El-Garem *et al.*, 2014). Receiver operating characteristic (ROC) curve analysis for miRNA-221 showed a sensitivity of 87% and a specificity of 40% for the differentiation of HCC from non-HCC patients at cutoff 1.82 (El-Garem *et al.*, 2014).

In addition to its potential as a biomarker, miRNA-221 has shown promise as a potential partner in HCV treatment therapies. Xu *et al.* (2014) showed that miRNA-221 is upregulated by HCV infection and helps to repress HCV replication. Furthermore, it was shown that miRNA-221 could work in parallel with Interferon (IFN), (one of the most common treatment therapies for HCV), amplifying IFN's anti-HCV effect (Xu *et al.*, 2014). They also showed that miRNA-221 targets two members of the suppressor of cytokine signaling (SOCS) family, SOCS1 and SOCS3, both of which are well-studied inhibitory factors on the IFN pathway.

2.3.6 miRNA-222.

Like miRNA-221, miRNA-222 is located on chromosome band Xp11 at site Xp11.3 (Tabasi & Erson, 2008). Also like mi-RNA-221, miRNA-222 inhibits endothelial cell migration, proliferation, and angiogenesis by targeting CD117 and endothelial nitric oxide synthase expression (Urbich *et al*., 2008). MiRNA-222 is also an oncogenic miRNA just like miRNA-221, and its upregulation has been observed in several different kinds of human tumors (Pang *et al*., 2010). The AKT (also known as Protein Kinase B) protein-coding, protein phosphatase 2A subunit B (PPP2R2A) has been identified as a direct functional target of miRNA-222 (Wong *et al*., 2010).

MiRNA-222 is upregulated in patients with chronic HCV, as well as patients with NAFLD, in a manner dependent upon the progression of hepatic fibrosis in the patient (Murakami & Kawada, 2016). Chang *et al.* (2014) showed that miRNA-222 has significantly higher expression levels in patients infected with chronic HCV than those of healthy controls. Upregulation of miRNA-222 has also been closely correlated with the miRNA expression of collagen α-1 and α-SMA (Murakami & Kawada, 2016). It has also been shown that miRNA-222 expression levels differ significantly between chronic hepatitis C (CHC) patients with HCV genotype 1 and non-genotype 1, and significant correlations between miRNA-222 levels and AST and ALT levels have also been identified (Chang *et al*., 2014).

MiRNA-222 is known to be involved in oncogenesis; it is an oncogenic miRNA (Gupta *et al*., 2014). It has shown promise as a diagnostic biomarker for monitoring patients infected with HCV in the progression to HCC. Motawi *et al.* (2016) performed a study on Egyptian patients of HCV, which showed that serum levels of miRNA-222 were significantly upregulated in liver fibrosis patients, but not in liver cancer patients. ROC analysis confirmed that miRNA-222 has great diagnostic potential as a biomarker to determine the progression of liver fibrosis, although the authors postulated its application to identifying HCC was limited (Motawi *et al*., 2016). However, other studies performed have found that miRNA-222 expresses at consistently high levels in HCC tumors when compared to adjacent cirrhotic liver and normal hepatic tissue (Pineau *et al*., 2010). MiRNA-222 upregulation in cases of HCC in turn downregulates antitumor immune response (Murakami & Kuwada, 2016). Furthermore, it has been shown that there is a progressive, analogous increase in miRNA-222 overexpression from cirrhotic livers to early-stage HCC (Wong *et al*., 2010). Wong *et al.* (2010) proposed that ability of miRNA-222 to activate AKT signaling in HCC is the reason for its notable pro-metastatic effect, a conclusion with which Gupta *et al.* (2014) agreed.

2.4 Current methods involved in staging fibrosis

The current most commonly applied protocol for the histological assessment of liver disease activity and fibrosis is liver biopsy. Liver biopsies involve the histological evaluation of a liver tissue sample removed from the body and examined under a microscope. Liver biopsies have been practiced in formal modern medical settings since 1883 (Strassburg & Manns, 2006). There are three types of liver biopsies: percutaneous, laparoscopic, and transvenous (Johns Hopkins, 2017). Percutaneous liver biopsies involve local anesthetizing of the patient, and the sample is taken via a needle inserted directly into the liver through the skin of the abdomen (Johns Hopkins, 2017). Percutaneous biopsies are most commonly employed in cases of HCV infection or suspected rejection in a transplant patient (Strassburg & Manns, 2006). Laparoscopic biopsies necessitate the general anesthetization of the patient, after which a laparoscopic camera is inserted into the abdominal cavity through an incision. A second tube guides the surgical needle to remove the tissue sample from the organ (Johns Hopkins, 2017). Laparoscopic biopsies are typically employed to investigate the impact of diseases which are characterized by a zonal affectation of the liver, such as sclerosing cholangitis or suspected metastatic disease (Strassburg & Manns, 2006). Transvenous, or transjugular liver biopsies involve an incision in the patient's neck, whereby the biopsy needle is inserted into the liver via the hepatic vein (Johns Hopkins, 2017). Transjugular biopsies are used when percutaneous biopsy is contraindicated due to patient clotting problems or ascites (Keshava *et al*., 2008).

Liver biopsies are limited by many factors such as the potential for sampling variability and serious complications from such an invasive technique (Everhart *et al*., 2010). Liver biopsies are also assessed through subjective scoring techniques such as the Ishak and METAVIR scores. Such numerical scoring techniques for evaluating liver biopsies were developed in the 1990s as the medical community studied and understood Hepatitis C and developed treatments (Everhart *et al*., 2010). These scoring systems have not changed in any dramatic way since their development, and rely on the knowledge and expertise of the doctor assessing the biopsy for accuracy. The METAVIR score describes four broad stages of fibrosis: portal, few septa, numerous septa, and cirrhosis (Everhart *et al*., 2010). The Ishak score, on the other hand, defines six stages of fibrosis, with finer distinctions in hepatic fibrosis analysis and architectural restructuring (Everhart *et al*., 2010) (Figure 10 & 11). Although these figures clearly show the distinctions between stages of fibrosis progression, these are idealized examples that do not reflect the frequently unclear realities of biopsy

results. In real-world applications, it has been shown that fragmented biopsies often result in histological understaging (Everhart *et al.*, 2010).

Other methods of classifying liver fibrosis fall under the umbrella of non-invasive protocols, which include serum, genetic, and imaging techniques. Serological (serum) marker techniques involve measuring one or more molecules in the patients' blood or serum correlating to hepatic fibrosis. Direct serum markers are associated with ECM generation and degeneration and the rates of hepatic fibrogenesis and fibrotic deterioration. Indirect serum markers are associated with the disturbance of hepatic function or architecture. Combinations of both direct and indirect serum markers are being established as potential alternatives to liver biopsies (Lurie *et al.,* 2015). The cytokines TGF- β1 and PDGF are directly involved in the deposition of ECM, have been shown to significantly correlate with the degree of liver fibrosis, and thereby show promise as direct serum markers (Sheiko & Rosen, 2016). The Fibrosis Index is an indirect serum marker methodology that combines platelet count with albumin contents, and has shown the ability to differentiate mild fibrosis, significant fibrosis, and cirrhosis (Ahmad *et al.*, 2011; Lurie *et al.,* 2015). The Apricot, or FIB-4 assay combines four direct and indirect markers, and is an example of a composite test. The Apricot assay combines AST, ALT, platelet count, and patient age, and has been validated to predict significant fibrosis in HCV patients (Ahmad *et al.*, 2011). These three examples are only a tiny fraction of the serum markers known to be at least somewhat useful for identifying the progression of hepatic fibrosis, however, of the many markers, tests, and assays identified thus far, few are universally applicable to patients of chronic HCV infection (Ahmad *et al.*, 2011).

Another avenue being explored is scanning or imaging techniques, which can detect changes in hepatic parenchyma and ideally differentiate between moderate and severe fibrosis. However, these methodologies are often nearly as expensive as liver biopsies, and few have been thoroughly validated (Lurie *et al.*, 2015). One of the most promising scanning techniques is transient elastography (TE) which measures liver stiffness (Taneja *et al.*, 2016). Liver stiffness measurements (LSM) have been shown to closely relate to fibrosis stage (Ahmad *et al.*, 2011) (Figure 12). When using LSM in combination with the serum marker FIB-4, fair reliability was shown in predicting hepatic fibrosis in patients with chronic HCV (Taneja *et al.*, 2016).

The final branch of potential noninvasive evaluation techniques for hepatic fibrosis lies with genetic markers. Because the metabolism of ECM is a very complex process that

involves specific balances between rates of ECM deposition and degradation, several genetic polymorphisms have been identified that are influenced by cytokines or other factors and affect the progression of hepatic fibrosis (Ngo *et al.*, 2006). The primary advantage of using genetic markers over liver biopsy is their long life and potential for retesting, whereas liver biopsy represents only one point in time (Ahmad *et al.*, 2011; Lurie *et al.,* 2015). The role of several genes, such as PDGF, TGF-β1, various collagens, interlukin, and their up- or down-regulation in the progression of hepatic fibrosis has been investigated and determinations have been made regarding their usefulness in predicting fibrotic progression (Figure 13) (Ahmad *et al.*, 2011).

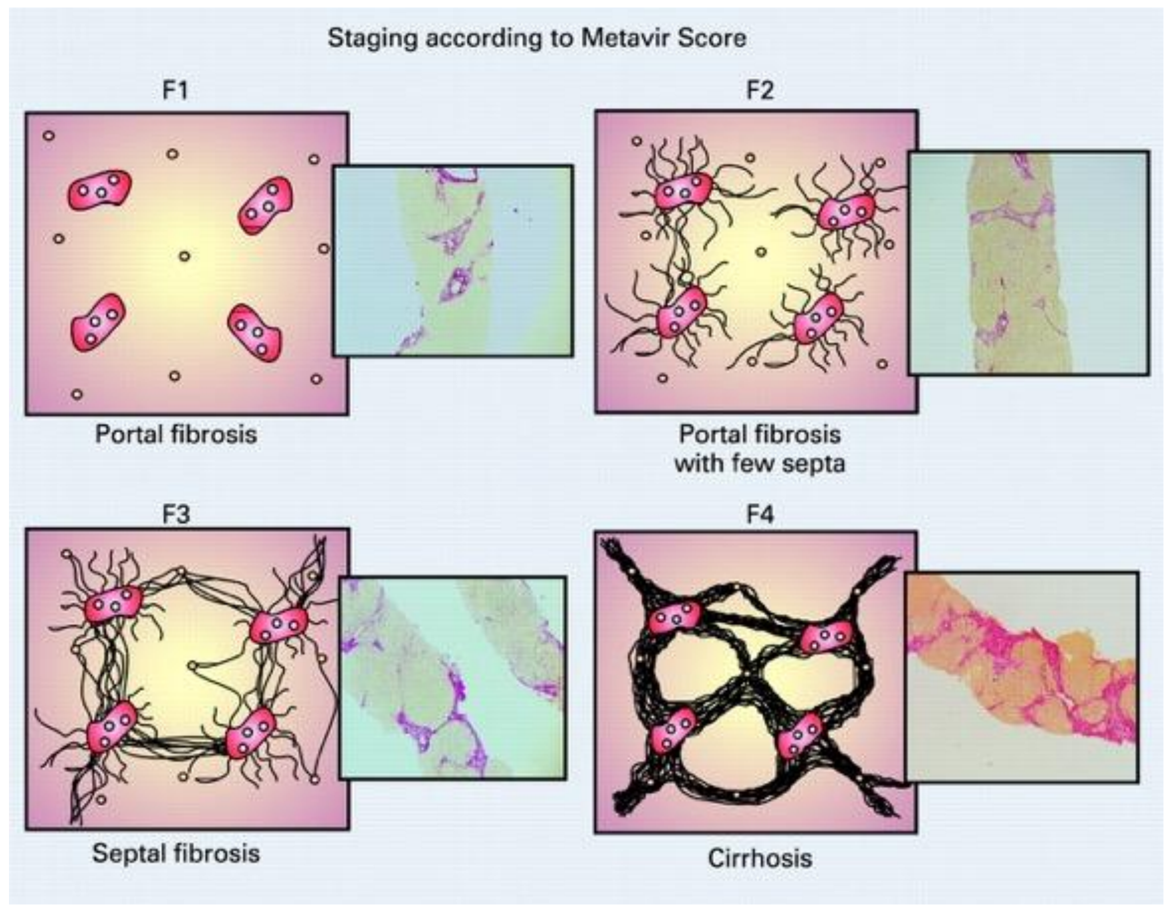


Figure 10: Fibrosis staging using METAVIR scoring method.

The Metavir grading method evaluates two structures, periportal necrosis and lobular necroinflammatory activity. Progression of fibrosis is composed of four stages (F1 minimal scaring/portal fibrosis, F2 scarring has occurred/portal fibrosis with few septa, F3 bridging fibrosis is occurring/septal fibrosis and F4 advanced scarring of the liver/cirrhosis). Adapted from Asselah *et al*., 2009, open access article, no permission.

Appearance	Ishak stage: Categorical description	ISHAK	METAVIR
	No fibrosis (Normal)	0	F0
	Fibrosis expansion of some portal areas ± short fibrous septa	1	F1
	Fibrosis expansion of most portal areas ± short fibrous septa	2	F2
	Fibrosis expansion of most portal areas with occasional portal to portal(P-P) bridging	3	
	Fibrosis expansion of portal areas with marked portal to portal(P-P) bridging as well as portal to central(P-C)	4	F3
	Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5	
	Cirrhosis, probable or definite	6	F4

Figure 11: A comparison of the Ishak and METAVIR scoring systems for liver fibrosis.

The left-most column shows type-defining examples of what would be typically expected to see in thin section during liver biopsy analysis, the next column contains verbal descriptions of the fibrotic progression, and these are then correlated to their corresponding Ishak and METAVIR scores. Adapted with permission from Standish *et al*., 2006.

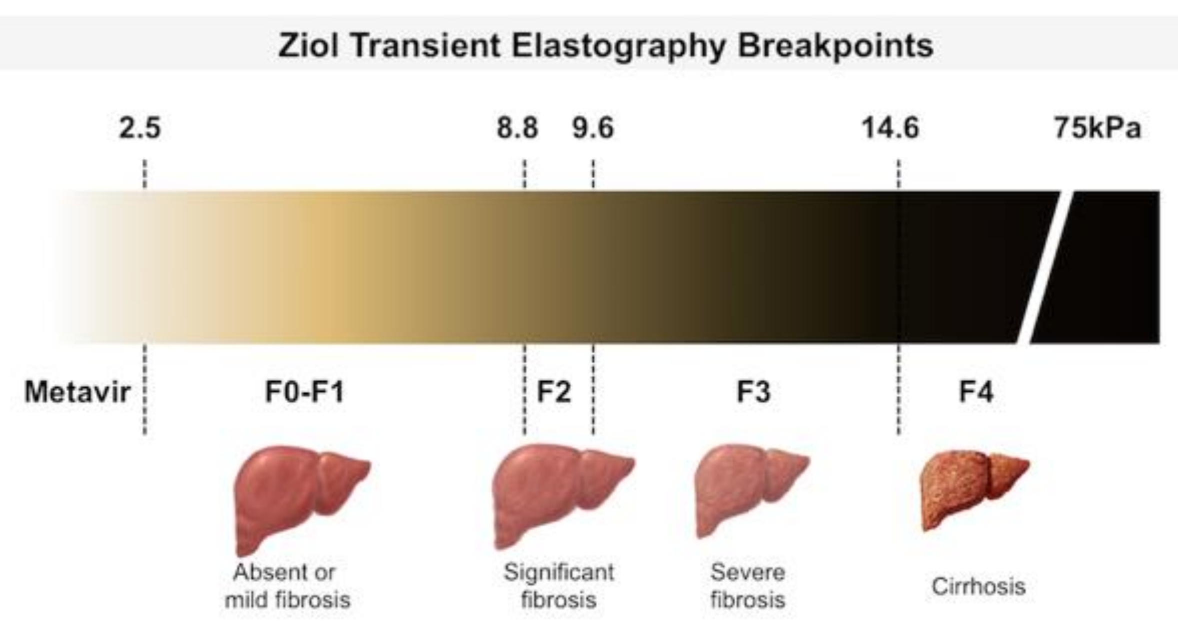


Figure 12: Ziols transient elastography (TE) cutoffs correlated with traditional METAVIR fibrosis scores.

TE, used to measure liver stiffness, is just one of many proposed non-invasive methodologies for measuring the progression of hepatic fibrosis. Adapted from Cox-North & Shuhart, 2015, open access article, no permission.

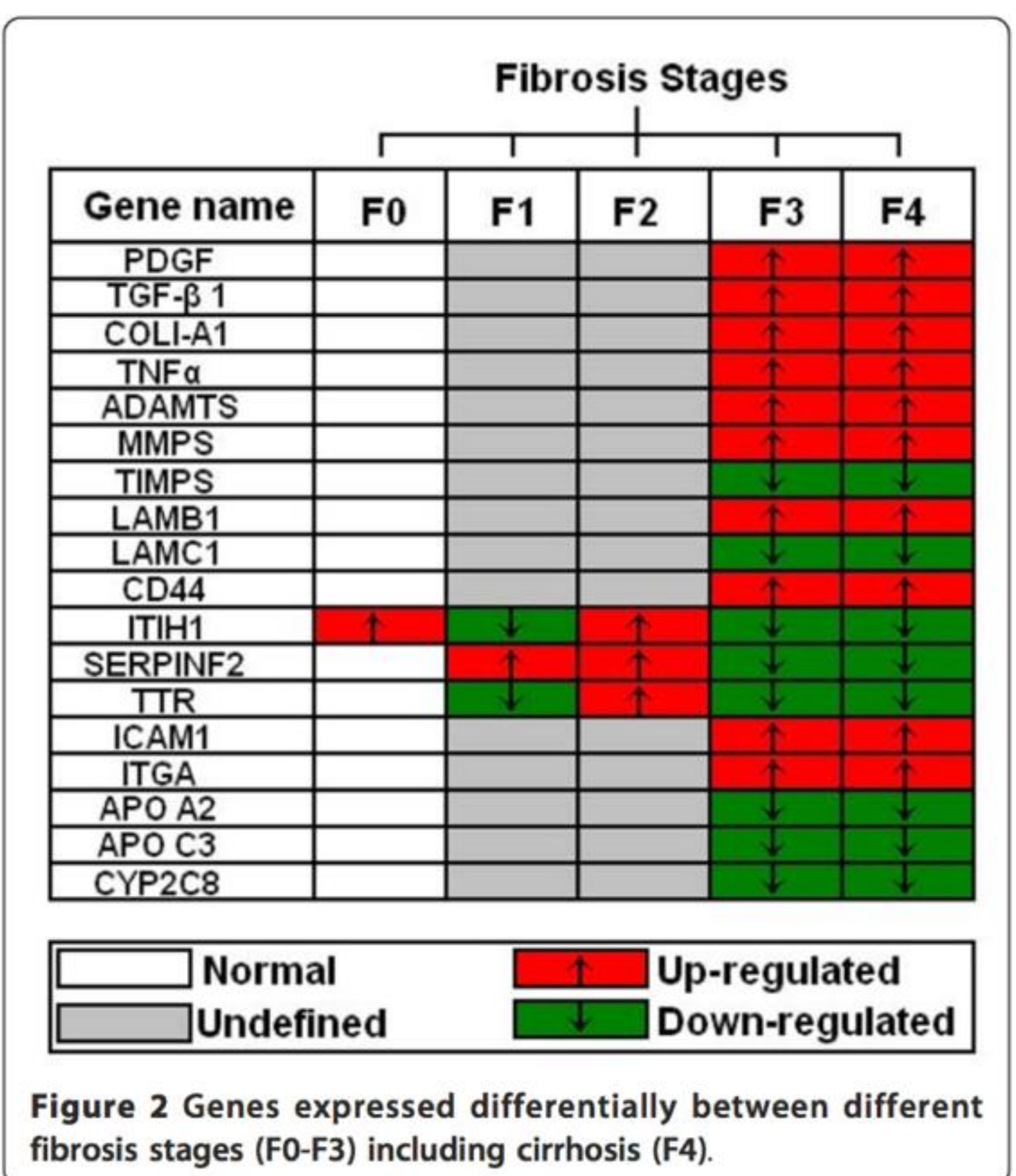


Figure 2 Genes expressed differentially between different fibrosis stages (F0-F3) including cirrhosis (F4).

Figure 13: Gene expression in fibrosis stages.

Different genes are shown to be differentially expressed between various stages of hepatic fibrosis, including cirrhosis. Adapted from Ahmad *et al.*, 2011, open access article, no permission.

(PDGF= Platelet-Derived Growth Factor; TFG-β1= Transforming Growth Factor- β; COLI-A1= Collagen la1; TNFα= Tumor Necrosis Factorα; ADAMTS= A Disintegrin-like And Metalloprotease with Thrombospondin; MMPs= Matrix Metalloproteinase; TIMPs= Tissue Inhibitors of Metalloproteinases; LAMB1= Laminin Subunit Beta 1; LAMC1= Laminin Subunit Gamma1; CD44= Cluster of Differentiation 44; ITIH1= Inter-alpha-trypsin Inhibitor H1; SERPINF2= Serpin Family F Member 2; TTR= Transthyretin; ICAM1= Intracellular Adhesion Molecule One; ITGA= Integrated Trace Gas Analyzer; APO A2= Apolipoprotein A2; APO C3= Apolipoprotein C3; CYP2C8= Cytochrome P_{450}2C8)

2.5 Importance

Chronic HCV infection affects huge swaths of the global population, with hundreds of millions of people currently infected, and millions more infected every year (Lavanchy, 2011). Furthermore, the burden of HCV on healthcare systems will only increase in the coming decades, as the disease typically takes years to develop and progress. Chronic HCV infection will inevitably result in hepatic fibrosis, which, if unmanaged for years, will progress to hepatic cirrhosis, which is a major indicator for the development of hepatocellular carcinoma (HCC) (Li *et al.*, 2016).

HCV is the leading cause of liver disease and liver cancer in the modern world (Bandiera *et al.*, 2004). HCC is the sixth most common cancer in the world (El-Abd *et al.*, 2015), and the third most deadly cancer in the world (Thrift *et al.*, 2017). According to WHO, tuberculosis (TB), HIV and malaria have decreased the mortality numbers between 2000-2015, however viral hepatitis has increased (Figure 14) (WHO, 2017). HCC's high mortality rates mean a poor prognosis for patients diagnosed with HCC, however, diagnoses are typically only made after hepatic fibrosis has progressed an alarming amount, due to limitations in current diagnostic methods and the delayed onset of acute symptoms in HCV infections (Ahmad *et al.*, 2011) (Figure 15).

There is also growing evidence that shows that a viral cure does not eliminate the risk of HCC development (Bandiera *et al.*, 2004). Even after liver transplantation, HCV reoccurrence is frequent, and its recurrence patterns are poorly understood. Histological findings have shown HCV to reoccur after liver transplant at wildly varying time points and through many different mechanisms of hepatocyte damage (Wiesner *et al.*, 2003). Therefore, it is of upmost importance to diagnose liver fibrosis as early as possible while making such diagnostic assays accessible to the majority of infected individuals. This means cost effective for the infected individuals of low-income countries.

MiRNAs are small, specific, relatively stable in plasma, and present in almost every bodily fluid, and these qualities make them excellent potential novel biomarkers (Motawi *et al.*, 2016). Assessment of miRNA expression can be done using noninvasive techniques such as blood tests, potentially eliminating, or at least alleviating, the need for dangerous, expensive tests such as liver biopsies. Furthermore, liver biopsies cannot be performed sequentially, while serum tests can be, allowing for progressive monitoring and analysis in chronic HCV patients (Khattab *et al.*, 2016). Because miRNAs have been suggested to be involved in the multi-step process of chronic HCV infection they are proving to be great

diagnostic markers for progression of liver fibrosis (Nallagangula *et al.*, 2017). With a proper and efficient prevention, screening and treatment method, total viremic infections can decline rapidly (Nallagangula *et al.*, 2017).

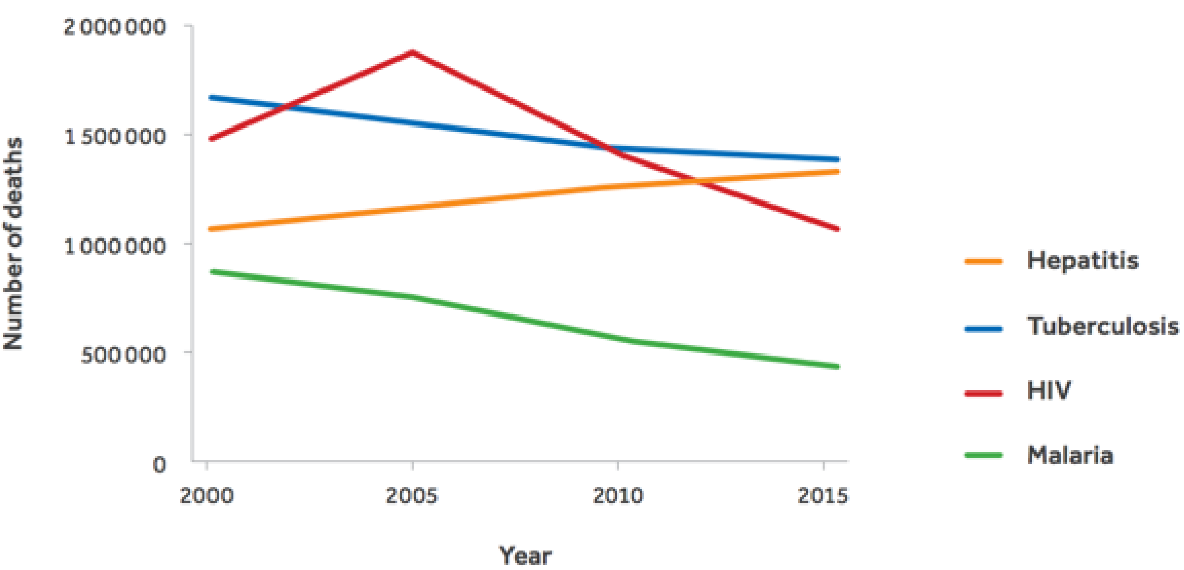


Figure 14: Global annual mortality from hepatitis, tuberculosis (TB), HIV and malaria.

Mortality numbers for hepatitis have increased while TB, HIV and malaria have decreased between the year of 200-2015. Adapted from Global Hepatitis Report 2017, WHO, 2017, open access article, no permission.

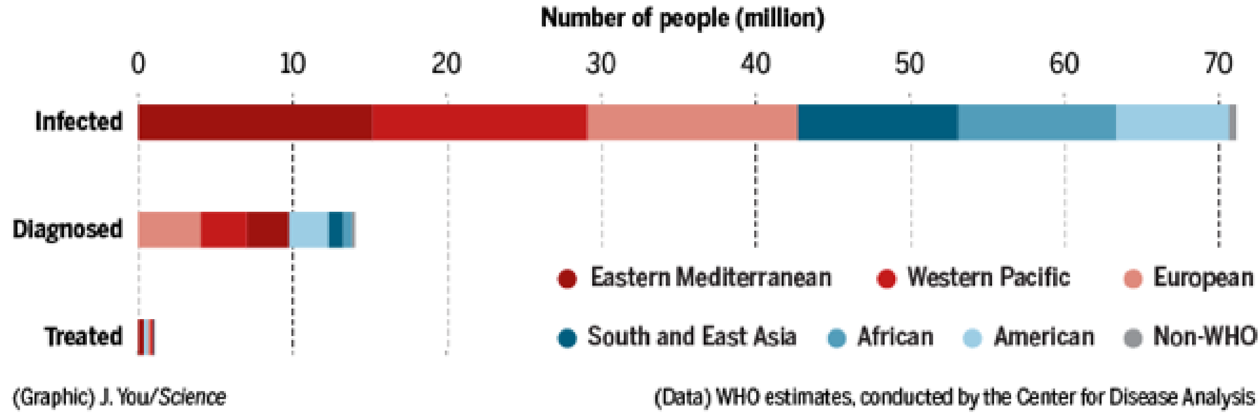


Figure 15: Number of people infected, diagnosed and treated for HCV.
The number of people infected by HCV from all different parts of the world significantly outweigh the numbers treated of even diagnosed for HCV. Adapted from Cohen, 2017, open access article, no permission.

3 Study Objectives

The purpose of this study is to find suitable miRNAs that can be used to develop a simple diagnostic assay to detect different stages of liver fibrosis. The results generated from this study may add information critical to understanding the role of the five main miRNAs (miR-16, miR-146a, miR-214-5p, miR-221, miR-222) during the development of liver fibrosis and their possible use as biomarkers to detect progression from liver fibrosis to cirrhosis. This study will specifically enhance the data available on liver fibrosis induced by HCV in the Egyptian population consisting primarily of the genotype 4 strain. The goal is to find highly specific and sensitive miRNA biomarkers to diagnose liver fibrosis as early as possible.

4 Materials and Methods

4.1 Patients

The patients selected for this research study are patients who were admitted to the hepato-gastreoenterology department of Theodore Bilharz Research Institute (TBRI) in Cairo, Egypt. Full medical history was obtained and thorough clinical investigations were performed to evaluate and select eligible candidates.

Patients were excluded from the study if they had any discoveries of other etiologies of chronic liver diseases alongside HCV. Other etiologies of chronic liver diseases comprised Schistosoma infection, hepatitis B virus, dual hepatitis B and C viral infection, biliary disorders and other malignancies. These were identified using serological, parasitological, histopathological, or ultrasonographic methods. Patients were also excluded if they were HCV infected but were receiving immunomodulatory interferon-α therapy.

The patients that were included in this study had evidences of chronic hepatitis C, circulating anti-HCV genotype 4 antibodies, and histopathological features of fibrosis in liver biopsy specimens. Patients were selected and divided into three groups: 42 HCV infected early stage fibrosis patients (F1-F2), 45 HCV infected late stage fibrosis patients (F3-F4) and 40 normal patients as controls. Fibrosis grading was based on the Metavir scoring system (Cox-North & Shuhart, 2015).

Patients written consent were obtained according to the IRB regulations and the guidelines of the TBRI Institute’s Human Research Ethics Committee.

4.2 Liver Histopathology

A liver biopsy sample was taken from each patient on 5 micrometer thick serial sections of paraffin-embedded, formalin-fixed blocks, stained with Masson trichrome and hematoxylin/eosin stains. Fibrotic stage was evaluated using the Metavir scoring system (Cox-North & Shuhart, 2015). Metavir scoring system was designed to assess liver damage specifically affected by hepatitis C virus. Fibrosis is staged based on the length in expansion of fibrotic areas between portal tracts. The stages correlate as follows: F0= no fibrosis, F1= portal fibrosis without septa, F2= portal fibrosis with rare septa (>1 septum), F3= numerous septa and F4= cirrhosis (Cox-North & Shuhart, 2015). Accordingly, patient samples were divided into early stage (F1-F2), and late stage (F3-F4) fibrosis groups.

4.3 Collection of Sera

Blood serum was extracted from each patient (using sterile disposable syringes) in complete aseptic conditions. From each patient, 5 mL of blood were withdrawn intravenously into uncontaminated test tubes and allowed to clot. Blood clot was removed by centrifuging the test tubes at low speeds for 10 minutes at room temperature. The resulting supernatant (serum) was then aliquoted into 1.7 ml Eppendorf tubes and were stored at -80˚C until used.

4.4 Laboratory Tests

Liver function tests were conducted using commercially available reagents and standard kits. These tests included aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, albumin, and prothrombin time (PT).

4.4.1 Detection of Circulating Anti-Schistosome Antibodies

ELISA technique was used to detect anti-schistosome antibodies. Microtiter plate (Immulon II, Dynatech Laboratories, USA) was coated with 100µL/well of 10µg/mL of *S. mansoni* soluble egg antigens (SEA) in 0.06 M carbonate buffer with pH 9.6. After the plate was incubated overnight at room temperature, it was then washed with 0.01 M phosphate buffer saline (PBS). 200 mL/well of 1% bovine serum albumin (Sigma Chemicals, St. Louis, USA) was used in carbonate buffer to block free sites. It was left to incubate for one hour at 37˚C, followed by thorough washing with PBS. The sera was then diluted 1:500 in 0.01M PS and 100µL were dispensed per well. The plate was left to incubate again for another 30 minutes at 37˚C. The plate was then washed with PBS again and 100µL of 1:1000 dilution of polyclonal goat anti-human horseradish peroxidase-labeled IgG antibody (Sigma Chemicals, St. Louis, USA) was delivered per well. For one last time, the plate was left to incubate at 37˚C for 30 minutes, then was washed with PBS. 100µL of ortho-phenylenediamine-H_2O_2 substrate solution was added per well and was left covered to incubate in the dark for 20 minutes. Finally, 50µL of 8N H_2SO_4 was added to each well to stop the reaction. Using the ELISA microplate reader, the absorbance of each well was read at 492 nm wavelength. A cut off value of 0.23 was used to differentiate positive from negative samples which was based on the mean value of healthy subjects +3 SD.

4.4.2 Detection of Hepatitis B Surface Antigen (HBs Ag)

Solid phase sandwich ELISA kit (Axiom Diagnostics, Burstadt, Germany) was used to detect hepatitis B surface antigen in patients’ sera. The wells of the microtiter plate were coated with antibodies specific to HBsAg and incubated with unknown serum samples including a mixture of anti-HBs Ag horseradish peroxidase-conjugated mouse monoclonal

antibodies. The wells were thoroughly washed to remove excess unbound material and a substrate solution contained 3, 3', 5, 5' tetramethylbenzidine and H_2O_2 was added. 8 N H_2SO_4 was added to each well to terminate the enzyme-substrate reaction. The wells that contained HBsAg in the serum changed from a purple to an orange colors. The ELISA microplate reader was used to determine the amount of color in the wells photometrically at 450/620 nm wavelength, which was directly proportional to the amount of bound conjugate and equal to the concentration of HBsAg.

4.4.3 Detection of Anti-HCV (genotype 4) Antibodies

For the detection of anti-HCV genotype 4 antibodies a Version V anti-HCV solid phase indirect ELISA kit (Axion Diagnostics, Burstadt, Germany) was used. Micro-wells were coated with extremely purified antigens which contained sequences from the putative core, NS3, NS4 and NS5 regions of HCV. The sera were then added to each well, left to incubate and then thoroughly washed. The captured anti-HCV antibodies were incubated with goat anti-human peroxidase-labeled IgG antibody and then washed again to remove unbound excess conjugate. Substrate solution containing H_2O_2 and 3, 3', 5, 5'-tetramethylbenzidine was added to visualize the bound enzyme. The wells containing anti-HCV positive samples developed a purple color. The reaction was terminated by the addition of 8 N H_2SO_4 and caused the samples to change from a purple color to an orange color. The ELISA microplate reader was used to determine the absorbance of each well which was read at 450 nm wavelength photometrically. The color of the wells was directly proportional to the amount of bound conjugate and therefore directly proportional to the concentration of anti-HCV antibody.

4.5 Serum miRNA assay

4.5.1 miRNA Extraction

Total RNA was extracted using the miRNeasy serum/plasma extraction kit (Qiagen, Valencia, CA, USA) which specifies the use of QIAzol lysis reagent in the manufacturer's protocol. For each reaction 200µL of serum was mixed with 1000µL of QIAzol. 3.5µL of miRNeasy serum spike-in control and 200µL of chloroform were added and vortexed respectively. The serum was then centrifuged for 15 minutes at 12,000 xg at 4˚C. The upper aqueous phase which contains the RNA was transferred to a new collection tube and 900µL of ethanol was added and mixed in. The sample was then pipetted into a miRNeasy elute spin column and centrifuged at 10,000 rpm for 15 seconds at 35˚C. 700µL of RWT buffer and 500µL of RPE buffer were added and centrifuged for 15 seconds at 10,000 rpm respectively.

500µL of 80% ethanol was added and centrifuged for 2 minutes at 10,000 rpm. The spin column was placed in a new 2 mL collection tube and centrifuged at full speed for 5 min. The spin column was then placed in a new 1.5 mL tube and 15µL of RNase-free water was added and centrifuged at full speed for 1 minute to elute the RNA. This was step was repeated again to obtain a final elution volume of 30µL. RNA concentration was determined using the NanoDrop2000 (Thermo scientific, USA). DNase treatment (Qiagen, Valencia, Ca) was done to remove any contaminating DNA. Concentration of RNA was obtained for all reactions using the NanoDrop 2000 (Thermo Scientific, USA). Approximately 300 ng/ µL of RNA was obtained from each reaction.

4.5.2 Reverse Transcription (RT) and Quantitative PCR (qPCR)

Reverse Transcription (RT) and quantitative PCR (qPCR) kits designed specifically for accurate miRNA analysis in serum samples were used to evaluate the selected miRNAs. RT reactions were performed using the TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Each RT reaction had a final volume of 15µL which was composed of: 7µL RT master mix, 3µL specific miRNA RT primer, and 5µL total extracted RNA with a concentration between 1 to 10 ng. The RT reactions were incubated for 30 minutes at 16˚C degrees Celsius, 30 minutes at 42˚C degrees Celsius, 5 minutes at 85˚C degrees Celsius and then maintained at 4˚C degrees Celsius.

1.33µL of the RT product was mixed with 10µL of TaqMan® 2X Universal PCR master mix, 1µL of the specific TaqMan® microRNA assay (Table 1) and 7.67µL of nuclease-free water for a final reaction volume of 20µL. The reactions were run on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95˚ C for 10 minutes, followed by 40 cycles at 95˚ C for 15 seconds, and 60˚ C for 1 minute. The comparative cycle threshold (C_T) $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2002) was used to calculate the relative expression of miRNA with miRNA-39 as the endogenous control to normalize the data. $2^{-\Delta\Delta CT}$ is the most common method used for relative quantitation. The C_T is the number is number of cycles required for the fluorescent signal to cross the threshold. ΔC_T was calculated by subtracting the C_T value of miR-39 from the C_T value of the targeted miRNA. $\Delta\Delta C_T$ was then calculated by subtracting ΔC_T of the control samples from the ΔC_T of the tested samples. The negative value of this value ($-\Delta\Delta C_T$) is used as the exponent of 2 in the equation and this represents that fold change of the targeted miRNA relative to the control.

Table 1: Assay IDs and mature miRNA sequence for the 5 chosen miRNAs

MicroRNA	Assay ID	Mature miRNA Sequence
miR-16	000391	UAGCAGCACGUAAAUAUUGGCG
miR-146a	000468	UGAGAACUGAAUUCCAUGGGUU
miR-214-5p	002293	UGCCUGUCUACACUUGCUGUGC
miR-221	000524	AGCUACAUUGUCUGCUGGGUUUC
miR-222	002276	AGCUACAUCUGGCUACUGGGU

Assay IDs are composed of a 6-digit number code uniquely chosen for each TaqMan® assay design which include the RT & qPCR primers and probe sequence. The mature miRNA sequence is the sequence of the RNA targeted by the assay.

4.6 Statistical Analysis

Statistical Package for the Social Science (SPSS) version 24 (IBM SPSS, Chicago, Il, USA) was utilized to analyze the data. The variables were expressed as the mean ± standard deviation (SD). The Mann-Whitney U-test was used to analyze and compare the demographic and biochemical data. The Mann-Whitney test was chosen because the data did not have a normal distribution meaning the data was considered to be nonparametric. The analysis of variance (ANOVA) test was used to determine whether there was any significance in the expression of miRNAs between each group of patients (controls, early stage fibrosis and late stage fibrosis). The expression was considered to be significant if the probability was less than 0.01 ($p < 0.01$).

Receiver operating characteristic (ROC) curve analysis was done to evaluate the diagnostic accuracy (sensitivity and specificity) of the 5 miRNAs. The area under the ROC curve (AUC) and the 95% confidence interval (CI) were calculated to determine the degree of significance of this data. A p value < 0.05 was considered significant, while $p < 0.001$ was considered highly significant.

Pearson correlation method was used to correlate miRNA expression patterns within each group of patients. A minimum p value < 0.05 was used to define a significant correlation between miRNAs.

5 Results

5.1 Demographic, biochemical, and liver histopathology investigations of patients

Clinically and biochemically healthy individuals served as the control group which consisted of 40 individuals. Demographic and biochemical profiles of the three groups are presented in Table 2. Demographic data included: age, gender and number of patients. Whereas the biochemical data included: albumin, bilirubin, ALT, AST and PT values. The control group were all within in the expected range/concentrations for the biochemical properties tested. The early and late stage fibrosis included were all elevated.

The early stage fibrosis group consisted of 42 patients with F1-F2 fibrotic stages, while the late stage fibrosis group consisted of 45 patients with F3-F4 fibrotic stages. Fibrosis is staged based on the length in expansion of fibrotic areas between portal tracts. The stages correlate as follows: F0= no fibrosis, F1= portal fibrosis without septa, F2= portal fibrosis with rare septa (>1 septum), F3= numerous septa and F4= cirrhosis (Cox-North & Shuhart, 2015) (Figure 16).

Table 2: Demographic and biochemical characterization of early/late stage fibrosis patients and controls

Parameters	Control (n=40)	CHC with early stage fibrosis (n=42)	CHC with late stage fibrosis (n=45)
Age	47.0 ± 3.5	46.3 ± 7.6	51.3± 4.6
Male / female ratio	3 / 1	4 / 3	6 / 3
Albumin (g/dL)	4.1 ±0.9	3.7 ±0.6	2.6 ± 0.2*
Bilirubin (mg/dL)	1<	> 1 – 3	> 3
ALT (IU/L)	36.9 ± 5.2	69.1 ± 17.3*	57.8 ± 15.3*
AST (IU/L)	29.7 ± 8.3	52.8 ±13.9*	49.7 ± 11.1*
PT (%)	95.6 ± 3.4	89.6 ± 4.8	41.5 ±11.1*

Data expressed as mean ± standard deviations (SD).
* $p < 0.01$ significant change compared to control group.
Albumin: Normal range = 3.5-5 g/dL,
Alanine aminotransferase (ALT) and aspartate aminotransferase (AST): Normal range is up to 40 IU/L.
Prothrombin time (PT): Normal range for concentration = 80-100%.

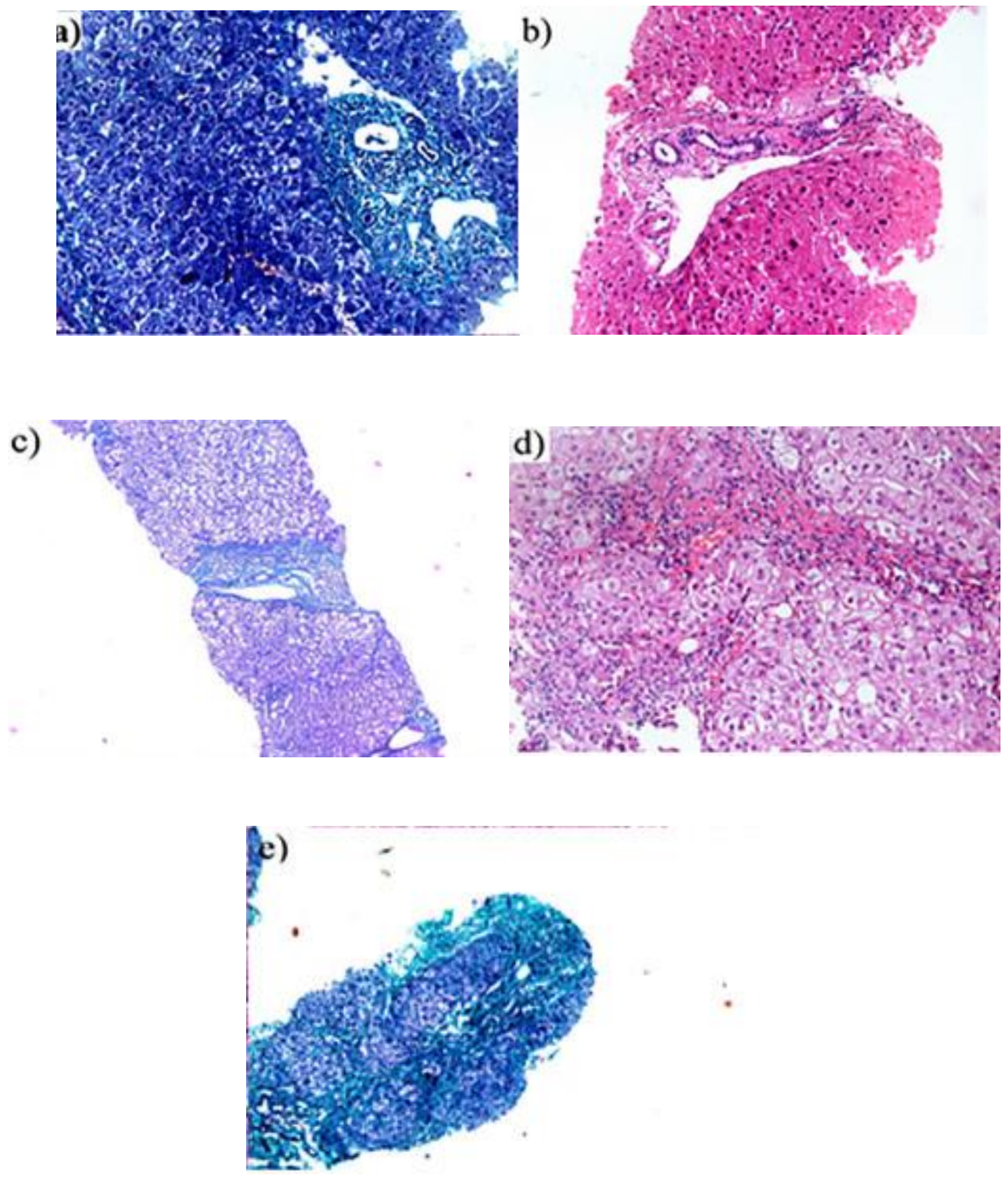


Figure 16: HCV-Induced liver fibrosis stages.

Different stages of fibrosis are illustrated in the figure above as follows: a) F0- no fibrosis; b) F1-portal fibrosis without septa; c) F2- portal fibrosis with rare septa; d) F3- numerous septa; e) F4-cirrhosis.

5.2 MicroRNAs expression profiles by qPCR

MiRNA-16, miR-146a, miR-221 and miR-222 all showed a very highly significant increase in the early and late fibrosis stages compared to the control/normal with a p value <0.001. MiRNA-221 also showed a very highly significant increase in the late stage fibrosis than the early stage ($p<0.001$). MiRNA-214-5p is the only miRNA that did not show any significance in any of the stages.

Table 3: Expression levels of the 5 miRNAs in each patient group

MiRNAs	Normal	Early Stage Fibrosis	Late Stage Fibrosis
miRNA-16	14.26 ± 0.69	24.25 ± 0.50**	23.29 ± 0.47**
miRNA-146a	21.75 ± 0.34	28.75 ± 0.42**	27.84 ± 0.52**
miRNA-214-5p	40.69 ± 1.24	37.85 ± 0.93	38.98 ± 2.02
miRNA-221	20.95 ± 0.61	74.38 ± 2.16**	96.01 ± 4.36$^{**, a}$
miRNA-222	19.99 ± 0.67	63.74 ± 1.67**	85.31 ± 0.53$^{**, a}$

$^{**}p<0.001$ very highly significant increase than normal;
$^{a}p<0.001$ very highly significant increase than early stage fibrosis.

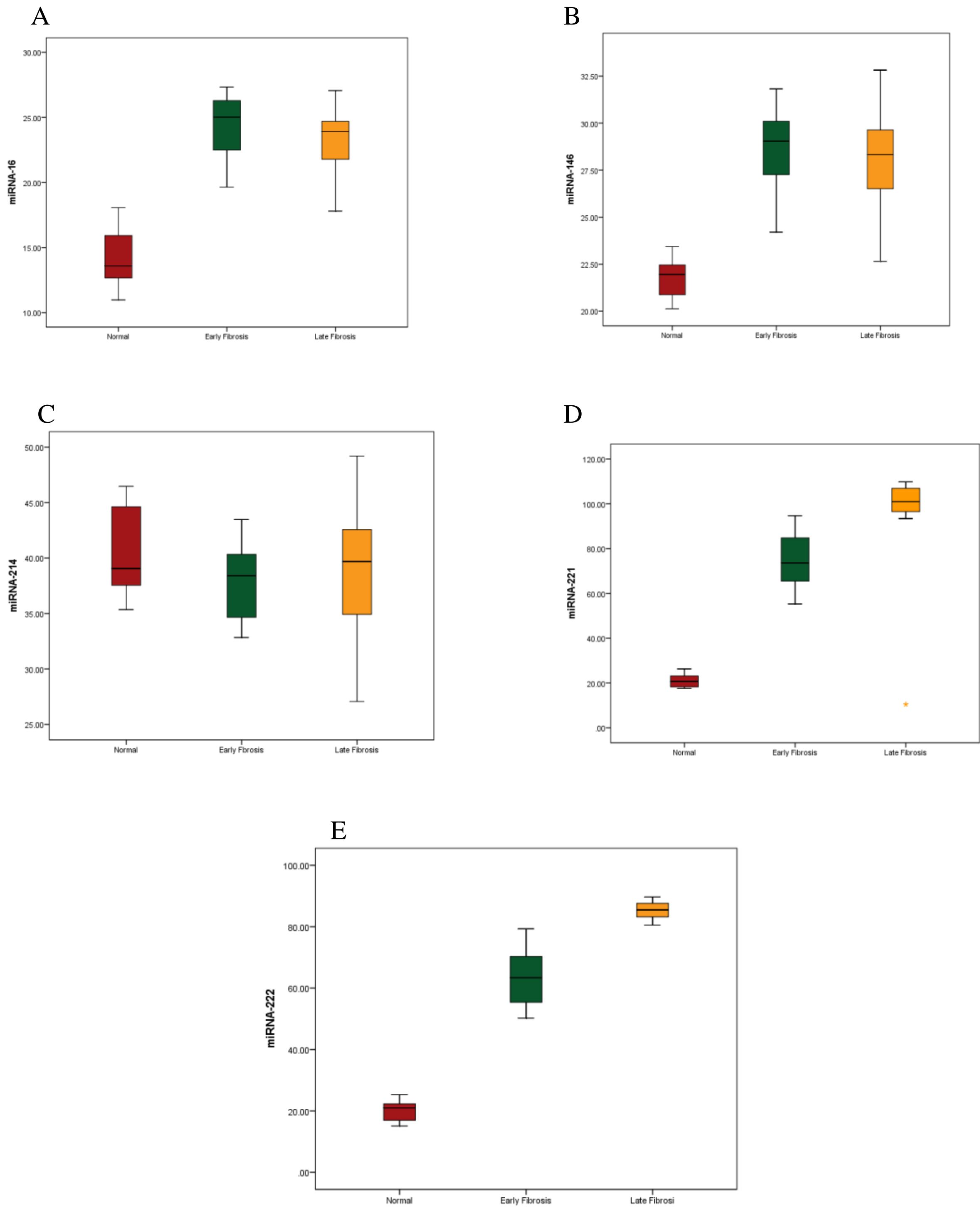


Figure 17: Box Plots.
Box plot diagrams show the expression of miR-16 (A), miR-146a (B), miR-214-5p (C), miR-221 (D), and miR-222 (E) in early and late stage fibrosis via HCV. The box includes the values for the 25th and 75th percentile of the data as well as the median. The vertical line extends from the minimum to the maximum value.

5.3 Diagnostic performance of circulating miRNAs in predicting liver fibrosis

MiRNA expression levels were assessed by calculating the area under the curve (AUC), confidence interval (CI), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The AUC, CI and p-values for early and late stage fibrosis are presented in Table 4 and Table 6, respectively. The AUC values are usually between 0.5 and 1.0 with 1.0 theoretically being the perfect scenario and 0.5 indicating no discriminative value. The sensitivity, specificity, PPV and NPV values for early and late stage fibrosis are presented in Table 5 and Table 7, respectively. The receiver operator characteristic (ROC) curve is used calculates the specificity and sensitivity. MiRNA-221 and miRNA-222 had the highest AUC values in both early and late stage fibrosis. They both showed extremely high sensitivity and specificity in late stage fibrosis with values of (100, 88.4) and (100, 96.77), respectively, indicating potential significance in diagnosis.

Early Stage Fibrosis:

Table 4: AUC, CI, and p-values for the miRNAs in early stage fibrosis

MiRNAs	AUC	CI 95%	*p-value*
miRNA-16	0.716 ± 0.05	0.604 - 0.829	<0.01
miRNA-146a	0.470 ± 0.08	0.317 - 0.623	>0.623
miRNA-214-5p	0.379 ± 0.09	0.197-0.561	>0.216
miRNA-221	0.738 ± 0.06	0.609-0.867	<0.001
miRNA-222	0.759 ± 0.06	0.631-0.887	<0.001

Table 5: The sensitivity and specificity values for miRNAs in early stage fibrosis

MiRNAs	Sensitivity (%)	Specificity (%)	PPV	NPV
miR-16	68.75	65.22	73.33	60.0
miR-146a	60.0	57.14	55.56	61.54
miR-214-5p	40.0	60.0	45.45	54.55
miR-221	65.22	78.13	68.18	75.76
miR-222	69.23	83.83	78.26	75.76

PPV: Positive Predictive value; NPV: Negative Predictive Value

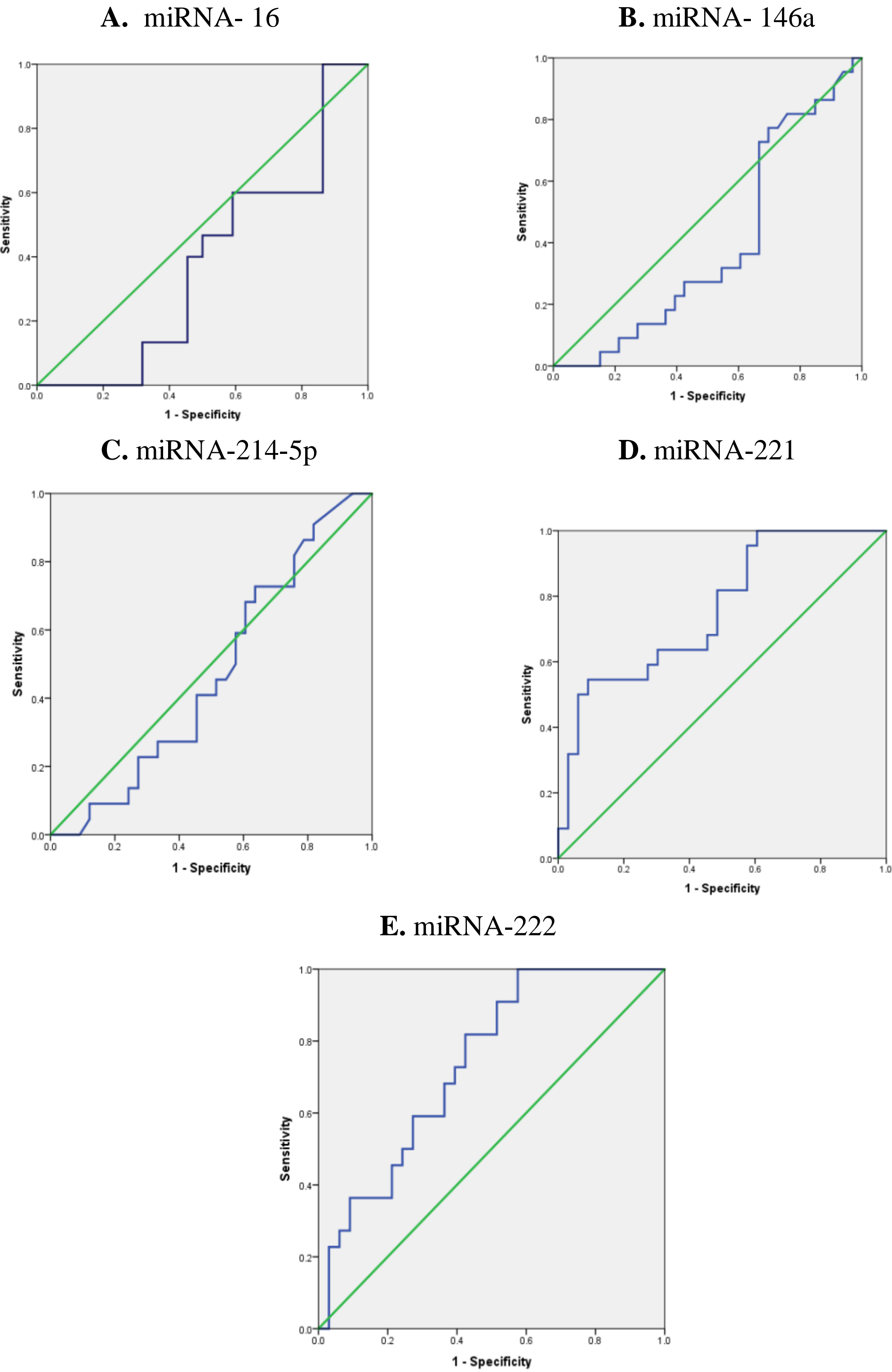


Figure 18: ROC Curves in early stage fibrosis.
ROC curve analysis was performed for each of the 5 tested miRNAs in early stage fibrosis. The ROC curve is used in comparing specificities and sensitives of each miRNA to determine its significance as a diagnostic and screening biomarker.

Late Stage Fibrosis:

Table 6: AUC, CI, and p-values for the miRNAs in late stage fibrosis

MiRNAs	AUC	CI 95%	*p*-value
miRNA-16	0.760 ± 0.06	0.641 - 0.879	<0.05
miRNA-146a	0.788 ± 0.06	0.676-0.900	<0.05
miRNA-214-5p	0.510 ± 0.121	0.274-0.747	>0.921
miRNA-221	0.929 ± 0.04	0.840 - 0.1.000	<0.001
miRNA-222	0.893 ± 0.04	0.809-0.976	<0.001

Table 7: The sensitivity and specificity values for the miRNAs in late stage fibrosis

MiRNAs	Sensitivity (%)	Specificity (%)	PPV	NPV
miR-16	71.43	75.0	83.33	60.00
miR-146a	73.68	90.0	93.33	64.29
miR-214-5p	63.16	47.06	72.73	36.36
miR-221	100.0	88.24	84.0	100.00
miR-222	100.0	96.77	96.00	100.00

PPV: Positive Predictive value; NPV: Negative Predictive Value

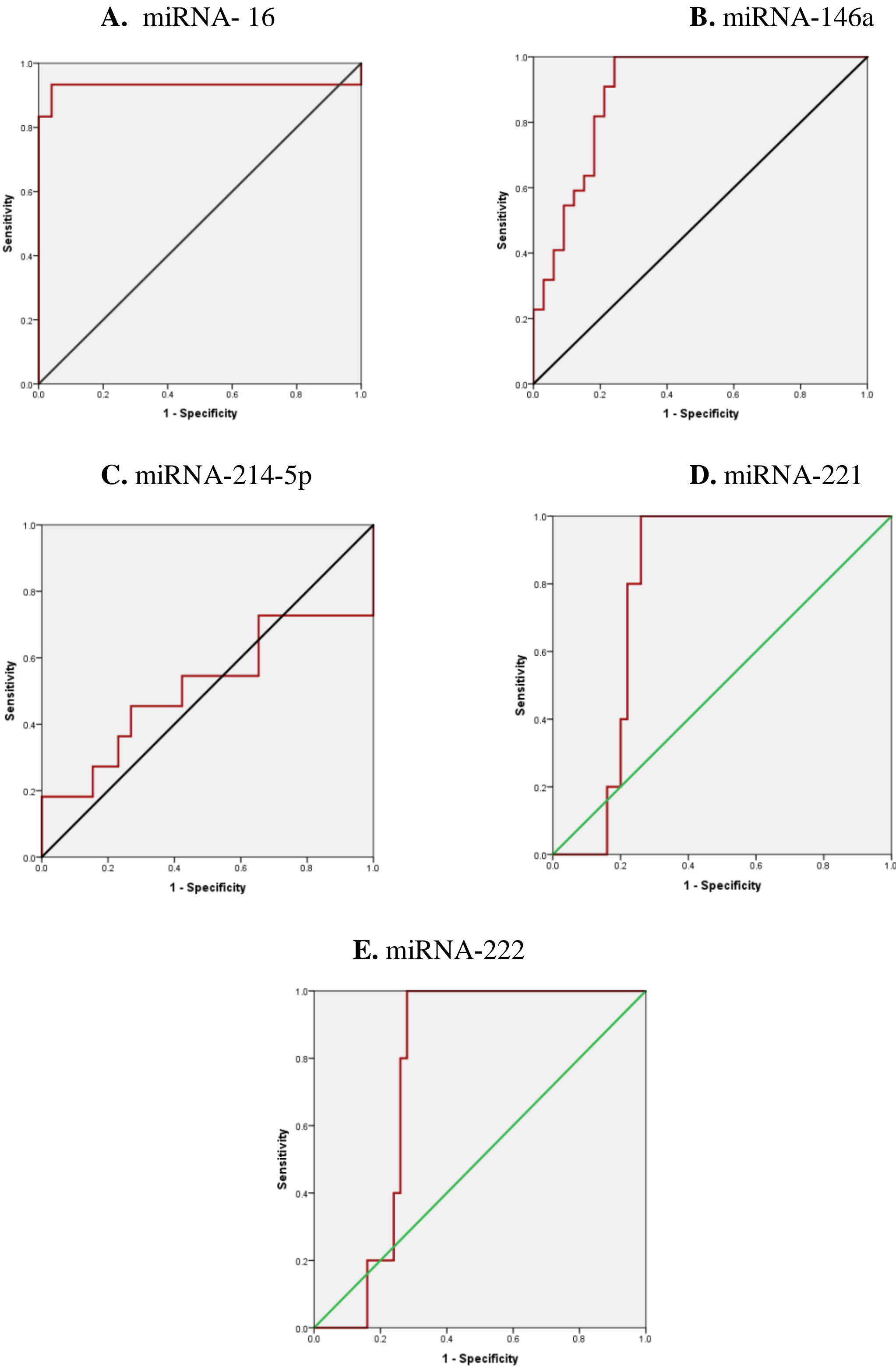


Figure 19: ROC Curves for late stage fibrosis.
ROC curve analysis was performed for each of the 5 tested miRNAs in late stage fibrosis. The ROC curve is used in comparing specificities and sensitives of each miRNA to determine its significance as a diagnostic and screening biomarker.

5.4 Correlations

Analysis was done to study the relationship between the miRNAs in both early and late stage fibrosis using the Pearson correlation. Pearson correlation coefficient (r) is expressed as a value between -1 to +1. A value of r= -1 indicates a negative correlation, r=0 indicates no correlation and r= +1 indicates a positive correlation. MiRNA-221 showed positive correlation with miRNA-146a and miRNA-16 in early and late stage fibrosis, with a higher significance in early stage. In late stage fibrosis, miRNA-146a showed the strongest correlation with miRNA-16.

Early Stage Fibrosis:

Table 8: Correlations between miRNAs in early stage fibrosis

		miR-16	miR-146a	miR-214-5p	miR-221	miR-222
miR-16	Pearson Correlation	1	.397	-.169	.570**	-.009
	Sig. (2-tailed)		.067	.452	.006	.969
miR-146a	Pearson Correlation	.397	1	.269	.634**	.183
	Sig. (2-tailed)	.067		.225	.002	.414
miR-214-5p	Pearson Correlation	-.169	.269	1	.127	.033
	Sig. (2-tailed)	.452	.225		.574	.885
miR-221	Pearson Correlation	.570**	.634**	.127	1	-.128
	Sig. (2-tailed)	.006	.002	.574		.499
miR-222	Pearson Correlation	-.009	.183	.033	-.128	1
	Sig. (2-tailed)	.969	.414	.885	.499	

** Correlation is significant at the 0.01 level (2-tailed).

Late Stage Fibrosis:

Table 9: Correlations between miRNAs in late stage fibrosis

		miR-16	miR-146a	miR-214-5p	miR-221	miR-222
miR-16	Pearson Correlation	1	.757**	-0.087	.446*	-0.019
	Sig. (2-tailed)		.000	.699	.037	.933
miR-146a	Pearson Correlation	.757**	1	.048	.493*	.073
	Sig. (2-tailed)	.000		.833	.020	.748
miR-214-5p	Pearson Correlation	-.087	.048	1	-.180	-.125
	Sig. (2-tailed)	.699	.833		.423	.512
miR-221	Pearson Correlation	.446*	.493*	-.180	1	.309
	Sig. (2-tailed)	.037	.020	.423		.162
miR-222	Pearson Correlation	-.019	.073	-.125	.309	1
	Sig. (2-tailed)	.933	.748	.512	.162	

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

6 Discussion

One hundred and seventy million individuals worldwide are affected by chronic hepatitis C which is a serious multistep health problem as it causes persistent liver inflammation eventually leading to liver fibrosis, cirrhosis, hepatocellular carcinoma and ultimately death (Appourchaux *et al*., 2016). Liver fibrosis is a wound-healing response liver cells undergo due to prolonged liver injury. Liver injuries cause the cells to die triggering regeneration of parenchymal cells to replace necrotic or apoptotic cells. If injury persists, regeneration may fail and hepatocytes are replaced by extracellular matrix (ECM) which is the start of fibrosis. In early stages of fibrosis via viral hepatitis, fibrotic cells are formed around portal tracts that grow and cause bridging of collagen bands as stages progress (Roy *et al*., 2015). Chronic liver inflammation involves changes in the gene expression in immune cells which led scientists to prove miRNAs are involved in liver inflammation and hepatic fibrosis (Bandiera *et al.,* 2016). MiRNAs can be detected and quantified in the blood (plasma and serum), saliva and urine and are extremely stable under conditions that would usually degrade most RNAs. For this reason, they have emerged as optimal biomarkers and a lot of research is being done to use miRNAs as biomarkers for the detection and staging of different diseases. In the liver, there have been miRNAs associated with HCV induced liver inflammation, cirrhosis and HCC (Bandiera *et al*., 2016). HCV is one of the main causes of liver injury leading to liver fibrosis (Zhu *et al*., 2015). However, there is not a lot of information present about miRNAs with different expressions in the intermediate stages of fibrosis (Appourchaux *et al*., 2016). Therefore, in this study, miRNAs were used to provide expression patterns that correlated with the different stages of fibrosis to distinguish between patients with advanced fibrosis/cirrhosis (F3-F4) and those with mild to moderate fibrosis (F1-F2). This information is necessary as it will then help in assessing the prognosis and treatment plan for each patient individually.

Results in this study showed that miRNA-16 was upregulated in early and late stage fibrosis and showed a highly significant increase (p<0.001) in both patient groups compared to the normal (control) group. This was also confirmed by Zhu *et al.* (2015) in which they also confirmed the upregulation of miRNA-16 in patients with chronic HCV infection. They specifically investigated the role of miRNA-16 and found that it downregulated both HGF and Smad7 expression. In previous studies, it has been shown that HGF mRNA expression is upregulated in the livers of different animals with various types of liver injuries (Zarnegar *et al*., 1991). HGF inhibits liver fibrosis by inhibiting the expression of

TGF-β. TGF-β is a pro-fibrogenic cytokine that transmits intracellular signaling via Smad proteins (Zhu *et al.*, 2015). There are seven Smad proteins and specifically Smad7 is an inhibitor that represses Smad2 and Smad3 phosphorylation and subsequent downstream signaling events (Dooley *et al.*, 2003; Zhu *et al.*, 2015). Inhibiting the TGF-β signal transduction by overexpressing Smad7 reduces the amount of fibrosis in vivo (Dooley *et al.*, 2003). Therefore, HCV-induced liver fibrosis may happen through repression of the HGF and TGF-β/Smad7 signaling pathways (Zhu *et al.*, 2015). Cermelli *et al.* (2011) performed a study that showed that serum levels of miRNA-16 were 3.0-fold and 6.3-fold higher, respectively, in two study groups of patients with chronic HCV infection, compared to healthy controls. They also showed that miRNA-16 was more sensitive than alanine aminotransferase (ALT) in detecting the early stages of HCV.

It has been shown that miRNA-146a is acutely involved in not only the progression, but the replication and amplification of the HCV virus in infected patients, and its potential as a novel noninvasive biomarker for the progression of HCV-associated hepatic fibrosis and HCC is great. The results of this study indicated that miRNA-146a was upregulated with a highly significant increase in early and late fibrosis stages than with the normal, control group. Joshi *et al.* (2013) studied patients with both slow HCV fibrosis progression (Ishak stage <F2) and fast HCV fibrosis progression (Ishak stage ≥F2), and found that microRNAs were a promising source of potential resolution for this problem. Increased miRNA-146a expression was seen intragraft in slow progressors versus fast progressors, and key fibrogenic mediators were down-regulated in response to the upregulation of miRNA-146a. It was shown that miRNA-146a plays an important role in the pathogenesis of HCV recurrence after liver transplant (Joshi *et al.*, 2013).

The upregulation of miRNA-146a observed in this study agreed with Bandiera *et al.* (2016) results, where they indicated that miRNA-146a was upregulated in liver tissue from HCV infected patients. They used microarray and computational analysis to show that miR-146a over expression regulates pathways that cause liver disease and HCC development. Specifically, they showed that miR-146a effects late steps of the viral replication cycle causing HCV infection to escalate. This research done by Bandiera *et al.* (2016) suggests that miRNA-146a-5p is a promising biomarker for virus-induced liver fibrosis and HCC.

MiRNAs control expression of more than one target mRNAs and thus regulate expression of hundreds of genes, making them complicated in defining their precise role in

biological processes and progression of human diseases (Felekkis *et al*., 2010). A defined mechanism of the differential regulation of miR-214-5p is still being studied. Several studies show that miRNA-214-5p is upregulated in in liver injuries (Chen *et al*., 2011; Maubach *et al*., 2011; Okada *et al*., 2015), while other studies show contradicting results with miRNA-214-5p being downregulated (Chen *et al*., 2014). MiRNA-214 expression has been shown to be upregulated in other carcinogenic injuries in the body including in the ovaries, stomach, pancreas, cervix, lung, and mouth (Yang *et al*., 2008; Ueda *et al*., 2009). Upregulation of miRNA-214-5p was also noticed to be linked to decreased liver function induced by alcohol and glutathione metabolism (Okada *et al*., 2015). In 2012, Iizuka *et al.* showed that miRNA-214-5p was upregulated in both human and mouse livers in a fibrosis progression-dependent manner. During the culture-dependent activation of mouse primary stellate cells, miRNA-214-5p expression markedly increased, and was shown to be significantly higher in stellate cells than in hepatocytes (Iizuka *et al*., 2012). In 2015, Okada *et al.* confirmed that miRNA-214 directly affects the development of hepatic fibrosis by modulating both TGF-β signaling pathways and the epidermal growth factor receptors. They reported results of upregulation of miRNA-214-5p in liver fibrosis and inhibition of miRNA-214-5p ameliorated hepatic fibrosis in mice (*in vivo*) and reduced the occurrence of hepatic tumors.

Unlike Iizuka *et al.* (2012) and Okada *et al.* (2015), Chen *et al.* (2014) showed that miRNA-214 in LX-2 cells is transferred via exosomes to recipient LX-2 cells or human HepG2 hepatocytes. Furthermore, this connective tissue growth factor (CCN2) is another factor critical in driving the fibrogenesis of HSCs, and it has been associated with a reciprocal downregulation of miRNA-214 (Chen *et al*., 2014).

The results from this study showed a minor downregulation in both early and late staged fibrosis compared to the control. However, the data was considered to have no significant value throughout any stage of fibrosis. This contradicts all published studies (regardless if it is up/down-regulated) which concluded miRNA-214-5p is one of the most promising novel noninvasive biomarkers for the detection of hepatic fibrosis progression in patients with HCV. The poor outcome of miRNA-214-5p in this study could have been due to the fact that it was performed on genotype 4 patients. MiRNA-214-5p has not been studied on this genotype before.

Quite a bit of research has been done on miRNA-221 and HCV, and it is one of the most promising avenues for potential biomarkers, therapies, and more effective treatments for HCV. In correlation to other studies, miRNA-221 was upregulated and showed highly

significant increase in early and late stage fibrosis compared to the control group as well as a highly significant increase between the late and early stage fibrosis groups ($p < 0.001$). The upregulation of miRNA-221 promotes cell cycle progression, angiogenesis, and invasion; miRNA-221 has been shown to be an anti-apoptotic RNA, and its silencing can lead to increased cell death (Gupta *et al*., 2014). El-Garem *et al.* (2014) showed miRNA-221 was upregulated in fibrosis patients than normal patients with a significant fold increase (<0.01) in CH and cirrhosis groups compared to the control. In this study, miRNA-221 had high sensitivity and specificity values of 100% and 88.24% respectively in late stage fibrosis. It is safe to deduce that the progression of liver fibrosis is associated with an increase in miRNA-221 expression level, thus providing great potential for it being used as a biomarker to detect HCV-induced liver fibrosis.

MiRNA-222 was also very highly significantly upregulated ($p < 0.001$) in early and late stage fibrosis groups compared to the control group. According to the ROC curve analysis, miRNA-222 had the highest sensitivity and specificity values in both early and late staged fibrosis groups with values of (69.23%; 83.83%) and (100%; 96.77%) respectively. Both, miRNA-222 and miRNA-221, could therefore be used to detect progression of liver fibrosis in later stage of fibrosis (F3-F4) due to their high sensitivity and specificity value, with miRNA-222 also being a reliable candidate to detect fibrosis even earlier. Ogawa *et al.* (2012), also showed that miR-221 and miR-222 were upregulated in the human liver that had been affected with fibrosis and continued to increase as fibrosis progression increased.

Lastly, Pearson correlation was used measure the association between miRNAs. Pearson correlation is denoted by the coefficient (r) with range values between +1 and -1 (indicating a positive or negative correlation respectively) (Glen, 2017). MiRNA-146a showed a significant positive correlation with miRNA-16 at the 0.01 level in the late stage fibrosis group. This means that if miRNA-146a is upregulated, miRNA-16 will also be upregulated and vice versa. Therefore, only one miRNA (either miR-146a or miR-16) needs to be tested, which is beneficial diagnostically. A positive correlation was also discovered between miRNA-221 and miRNA-16 and between miRNA-221 and miRNA-146a in both the early and late staged fibrosis groups. However, in the early stage group both correlations had significant values at the 0.01 level, while in the late stage group both correlations had significant values at the 0.05 level. The results of this correlation allow the assumption that if an increase in miRNA-221 was detected at any stage of fibrosis, miRNA-16 and miRNA-146a would also increase. Also, it can be deduced that although miRNA-16 and miRNA-146a

on their own didn't have high sensitivity or specificity values and miRNA-221 had high sensitivity and specificity values at the late stage fibrosis, this strong correlation (miRNA-221 and miRNA-16; miRNA-221 and miRNA-146a) detects early stages of fibrosis. Results, such as this strong correlation, will allow researchers to develop diagnostic assays to detect fibrosis as early as possible as no one miRNA could be used to detect progression of fibrosis alone.

MiRNAs have shown great promise as biomarkers for hepatic fibrosis, HCV, and even HCC, and are specific enough to provide greater diagnostic detail than currently available from biological tests. Because most miRNAs are also stable or relatively stable in serum, they can be analyzed through simple blood tests, and these noninvasive tests can be sequentially repeated on patients throughout their courses of treatment. MiRNAs are becoming more biologically significant (especially in liver diseases) and are being used for both diagnostics purposes as well as for therapeutic purposes. Studying and understanding the role of miRNA in cellular functions of the liver and its development of fibrosis is crucial in order to use and benefit from them in the best possible way. MiRNAs can be up-regulated or down-regulated in liver damage and this change in quantity along with its quality can be utilized in assessing how far the damage has gone. Developing a repeatable, accurate, noninvasive test for liver fibrosis using miRNA expressions would aid in early detection, precise analysis, and better treatment methods. Furthermore, such a test could be administered repeatedly throughout the treatment, giving clinicians a better understanding of the advancement of liver disease, and patients better prognoses.

The expectancy of using miRNA in therapy is very likely to occur and soon hepatologists will be using them in their therapeutic regimen. Some miRNAs, such as miRNA-221, have even shown promise in the treatment of HCV, potentially alleviating the strain on healthcare systems currently posed by the disease, particularly in developing countries where IFN is not readily accessible or pragmatically affordable. There is definitely still more work to be done on these promising miRNAs that shall surely result in the development of protocols that can accurately determine the progression of liver fibrosis in HCV patients, without the need for biopsy. Such methodologies will improve the prognoses of millions of patients, and perhaps even unravel the mechanisms by which we might find a cure for HCV.

7 Conclusion

HCV poses a great health problem to the world today, one which will only grow as the disease progresses in infected patients over the next years and decades. Chronic HCV infection causes liver fibrosis, which causes hepatic cirrhosis, HCC, and often, death. Liver biopsy, currently the standard methodology for the detection and assessment of hepatic fibrosis, is subjective, invasive, and with significant potential complications. Nor can liver biopsies be performed repeatedly to follow-up on HCV patients, limiting their diagnostic and prognostic potentials. Furthermore, liver biopsies are not easily performed in developing countries, or even developed ones, as they require modern hospital facilities and highly trained specialists to perform. There exists a clear need to develop alternative, noninvasive detection techniques to identify and assess the progression of liver fibrosis in patients with HCV.

This study aimed at testing five specific miRNAs to aid in the diagnosis and prognosis of HCV (genotype4)- induced liver fibrosis. The results indicated that miRNA-16, miRNA-146a, miRNA-221, and miRNA-222 can be used to detect the presence of liver fibrosis as serum levels were all significantly upregulated in both early and late stage fibrosis than the control ($p<0.001$), proving to have important prognostic values. MiRNA-222 and miRNA-221 were shown to have the highest sensitivity and specificity values in late stage fibrosis indicating promising potential biomarkers for HCV-induced liver fibrosis. MiRNA-222 had sensitivity and specificity values of 100% and 96.77% (respectively) in late stage fibrosis, while miRNA-221 had sensitivity and specificity values of 100% and 88.24% (respectively). Additionally, miRNA-221 showed significant positive correlations with both miRNA-16 and miRNA-146a in the early and late stage fibrosis, with the early stage having a stronger correlation (at the 0.01 level). These correlations have high significant values for future uses in formulating liver fibrosis diagnostic assays.

8 Future Prospective

HCV is a global health concern affecting hundreds of millions of people worldwide (Sheiko & Rosen, 2016). Because HCV typically takes years, if not decades, to progress, the burden it places on healthcare systems will only increase in coming years (Lavanchy, 2011). Current methodologies for identifying hepatic fibrosis are insufficient, and often result in late diagnoses because acute symptoms do not present at the onset of infection. Furthermore, the standard scoring systems for hepatic fibrosis depend on invasive liver biopsies for samples and subjective sample interpretation by the attending physicians. Serum analyses, imaging tests, and genetic markers have all been explored as alternative potential avenues for the identification and analyses of hepatic fibrosis progression.

In recent years, more attention has been paid to microRNAs as a source of potential diagnostic information for hepatic fibrosis, HCV, and HCC. Since some miRNAs are known to be deregulated in cases of liver fibrosis and HCC, they are therefore promising candidates for biomarkers of liver disease associated with chronic HCV infection (Motawi, 2016). MiRNA-16, -146a, 214-5p, 221and 222 were studied as potential biomarkers for the progression of liver disease. Some miRNAs, such as miRNA-221, have even shown promise in the treatment of HCV. Researchers around the world are working to develop a reliable serum miRNA assay for the detection and monitoring of hepatic fibrosis. Developing a repeatable, accurate, noninvasive test for liver fibrosis using miRNA expressions would aid in early detection, precise analysis, and better treatment methods. Furthermore, such a test could be administered repeatedly throughout the treatment, giving clinicians a better understanding of the advancement of liver disease, and patient’s better prognoses.

Unfortunately, there are several challenges to the development of a serum assay test for the liver deterioration of chronic HCV patients. The first difficulty for researchers was identifying which miRNAs are involved in these situations. There are thousands of miRNAs, and only some are involved in the progression of hepatic architecture distortion and/or the progression and proliferation of the Hepatitis C virus. Some of the dynamics of miRNA release in the early stages of acute HCV infection still remain unclear (El-Diwany, 2015). However, progress has been made, and thanks to the work that has already been done, a range of promising miRNA candidates have been identified for use in diagnostic assays, such as those studied here. The next challenge has been to discern which miRNAs are the most useful and relevant to diagnostic tests. Of the miRNAs directly involved in HCV and associated hepatic distortions, not all of these miRNAs are up- or downregulated distinctly enough in

cases of HCV and hepatic fibrosis to provide quality data on the progression of the conditions (Motawi *et al.*, 2016). Thirdly, the variance within the Hepatitis C virus itself presents serious challenges for researchers. There are seven different genotypes of HCV, and are all distinct enough to progress differently and respond differently to treatments. Differences in expressed levels of the same miRNA have been observed between different HCV genotypes, meaning that any diagnostic assays developed must be specialized for each genotype (Chang *et al.*, 2014). This can be seen in a great deal of previous research that has been completed on the subject, wherein researchers often choose a particular genotype of HCV to study when attempting to identify feasible miRNA assays (El-Abd *et al.*, 2015; El-Diwany *et al.*, 2015; Motawi *et al.*, 2016; Elemeery *et al.*, 2017). More research must be done to identify specific assays that can correctly identify the progression of hepatic fibrosis in each of the seven genotypes of HCV. The high number of miRNAs and seven HCV genotypes means that the potential combinations are extremely numerous, however, the work already done on miRNAs has begun to narrow down viable options.

With the development of a safe, effective, repeatable, non-invasive diagnostic test for the progression of hepatic fibrosis, the management of the HCV epidemic may be possible. One of the greatest challenges posed by chronic HCV infection is the long progression of the disease, and monitoring patient progress is difficult in developing areas where liver biopsies are not readily available. The development of a diagnostic assay, or unique diagnostic assays for each genotype, would help doctors and patients alike.

9 References

Ahmad, W., Ijaz, B., Gull, S., Asad, S., Khaliq, S., Jahan, S., Hassan, S. (2011). A brief review on molecular, genetic, and imaging techniques for HCV fibrosis evaluation. *Virology Journal, 8*(53), 1-16.

Ambros, V. (2004, September 16). The functions of animal microRNAs. *Nature, 431*(7006), 350-355.

Appourchaux, K., Dokmak, S., Resche-Rigon, M., Treton, X., Lapalus, M., Gattolliat, C.-H., Asselah, T. (2016, October 12). MicroRNA-based diagnostic tools for advanced fibrosis and cirrhosis in patients with chronic hepatitis B and C. *Scientific Reports, 6*, 34935 .

Asselah, T., Bièche, I., Sabbagh, A., Bedossa, P., Moreau, R., Valla, D., Marcellin, P. (2009, June 1). Gene expression and hepatitis C virus infection (article) Author. *Gut, 58*(6), 846-858.

Baiocchini, A., Montaldo, C., Conigliaro, A., Grimaldi, A., Correani, V., Mura, F., Mancone, C. (2016, March 21). Extracellular Matrix Molecular Remodeling in Human Liver Fibrosis Evolution. (U. o. Matias A. Avila, Ed.) *PLoS ONE*.

Bandiera, S., Pernot, S., El Saghire, H., Durand, S. C., Thumann, C., Crouchet, E., Zeisel, M. B. (2004). Hepatitis C Virus-Induced Upregulation of MicroRNA miR-146a-5p in Hepatocytes Promotes Viral Infection and Deregulates Metabolic Pathways Associated with Liver Disease Pathogenesis. *Journal of Virology, 431*(7006), 350-355.

Bandiera, S., Pernot, S., El Saghire, H., Durand, S. C., Thumann, C., Crouchet, E., Zeisel, M. B. (2016). Hepatitis C Virus-Induced Upregulation of MicroRNA miR-146a-5p in Hepatocytes Promotes Viral Infection and Deregulates Metabolic Pathways Associated with Liver Disease Pathogenesis. *Journal of Virology, 90*(14), 6387-6400.

Barakat, R. M. (2013). Epidemiology of Schistosomiasis in Egypt: Travel through Time: Review. *Journal of Advanced Research, 4*(5), 425-432.

Barrera, J. M., Bruguera, M., Ercilla, M. G., Gil, C., Celis, R., Gil, M. P., Ordinas, A. (1995). Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C. *Hepatology, 21*(3), 639-644.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell, 116*(2), 281-297.

Bataller, R., & Brenner, D. (2005). Liver fibrosis. *Journal of Clinical Investigation, 115*(2), 209-218.

Bihrer, V., Friedrich-Rust, M., Kronenberger, B., Forestier, N., Haupenthal, J., Shi, Y., Piiper, A. (2011). Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. *The American Journal of Gastroenterology, 106*(9), 1663-1669.

Blach, S., Zeuzem, S., Manns, M., Altraif, I., Duberg, A.-S., Muljono, D. H., Malu, A. O.-S.-S.-O.-S.-S.-S.-T.-C.-D.-H.-S.-f. (2016, December 15). Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *The Lancet Gastroenterology & Hepatology, 2*(3), 161-176.

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell., 136*(4), 642-655.

Centers for Disease Control and Prevention (CDC). (2014, August 22). *Hepatitis C: 25 Years of Discovery.* Retrieved September 22, 2017, from Know More Hepatitis: https://www.cdc.gov/knowmorehepatitis/timeline.htm

Cermelli, S., Ruggieri, A., Marrero, J. A., Ioannou, G. N., & Beretta, L. (2011). Circulating MicroRNAs in Patients with Chronic Hepatitis C and Non-Alcoholic Fatty Liver Disease. *PLoS ONE, 6*(8), e23937.

Chen, C., Wu, C.-Q., Zhang, Z.-Q., Yao, D.-K., & Zhu, L. (2011, July 15). Loss of expression of miR-335 is implicated in hepatic stellate cell migration and activation. *Experimental Cell Research, 317*(12), 1714-1725.

Chen, L., Charrier, A., Zhou, Y., Chen, R., Yu, B., Agarwal, K., Brigstock, D. R. (2014, January 27). Epigenetic regulation of connective tissue growth factor by MicroRNA-214 delivery in exosomes from mouse or human hepatic stellate cells. *Hepatology, 59*(3), 1118-1129.

Chen, W., Rock, J. B., Yearsley, M. M., Ferrell, L. D., & Frankel, W. L. (2014). Different collagen types show distinct rates of increase from early to late stages of hepatitis C-related liver fibrosis. *Human Pathology, 45*, 160-165.

Cohen, J. (2017, April 2017). *New report halves the number of people infected with hepatitis C worldwide*. Retrieved from Science: http://www.sciencemag.org/news/2017/04/new-report-halves-number-people-infected-hepatitis-c-worldwide

Cox-North, P. P., & Shuhart, M. C. (2015, October 21). *Evaluation and staging of liver fibrosis.* Retrieved September 26, 2017, from Hepatitis C Online: https://www.hepatitisc.uw.edu/go/evaluation-staging-monitoring/evaluation-staging/core-concept/all

Döhner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Krober, A., Bullinger, L., Lichter, P. (2000). Genomic aberrations and survival in chronic lymphocytic leukemia. *New England Journal of Medicine, 343*(26), 1910-1916.

Davis, G. L., Albright, J. E., Cook, S. F., & Rosenberg, D. M. (2001, April). Projecting future complications of chronic hepatitis C in the United States. *Liver Transplantation, 9*(4), 331-338.

Daw, M. A., El-Bouzedi, A. A., Ahmed, M. O., Dau, A. A., Agnan, M. M., & Drah, A. M. (2016, November 12). Geographic integration of hepatitis C virus: A global threat. *World Journal of Virology, 5*(4), 170-182.

Desvignes, T., Contreras, A., & Postlethwait, J. H. (2014). Evolution of the miR199-214 cluster and vertebrate skeletal development. *RNA Biology, 11*(4), 281-294.

Dooley, S., Hamzavi, J., Breitkopf, K., Wiercinska, E., Said, H. M., Lorenzen, J., Gressner, A. M. (2003, July). Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats . *Gastroenterology, 125*(1), 178-191.

El-Abd, N. E., Fawzy, N. A., El-Sheikh, S. M., & Soliman, M. E. (2015). Circulating miRNA-122, miRNA-199a, and miRNA-16 as Biomarkers for Early Detection of Hepatocellular Carcinoma in Egyptian Patients with Chronic Hepatitis C Virus Infection. *Molecular Diagnosis and Therapy, 19*(4), 213-220.

El-Diwany R, Wasilewski LN, Witwer KW, Bailey JR, Page K, Ray SC, Cox AL, Thomas DL, Balagopal A. 2015. Acute hepatitis C virus infection induces consistent changes in circulating microRNAs that are associated with nonlytic hepatocyte release. J Virol 89:9454–9464. doi:10.1128/JVI.00955-15

Elemeery, M. N., Badr, A. N., Mohamed, M. A., & Ghareeb, D. A. (2017). Validation of a serum microRNA panel as biomarkers for early diagnosis of hepatocellular carcinoma post-hepatitis C infection in Egyptian patients. *World Journal of Gastroenterology, 23*(21), 3864-3875.

El-Garem, H., Ammer, A., Shehab, H., Shaker, O., Anwer, M., El-Akel, W., & Omar, H. (2014, November 27). Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma. *World Journal of Hepatology, 6*(11), 818-824.

Elgharably, A., Gomaa, A., Crossey, M. M., Norsworthy, P. J., Waked, I., & Taylor-Robinson, S. D. (2017). Hepatitis C in Egypy - past, present, and future. *International Journal of General Medicine, 10*, 1-6.

El-Guendy, N. M., Helwa, R., El-Halawany, M. S., Ali, S. A., Aly, M. T., Alieldin, N. H., Abdel-Wahab, A.-H. A. (2016, April). The Liver MicroRNA Expression Profiles Associated With Chronic Hepatitis C Virus (HCV) Genotype-4 Infection: A Preliminary Study. *Hepatitis Monthly, 16*(4).

Everhart, J. E., Wright, E. C., Goodman, Z. D., Dienstag, J. L., Hoefs, J. C., Kleiner, D. E., Litman, H. (2010). Prognostic Value of Ishak Fibrosis Stage: Findings from the HALT-C Trial. *Hepatology, 51*(2).

Felekkis, K., Touvana, E., Stefanou, C., & Deltas, C. (2010). microRNAs: a newly described class of encoded molecules that play a role in health and disease. *Hippokratia, 14*(4), 236-240.

Ferragamo, V. (2013, February 7). *Hepatitis C Virus*. Retrieved from flipper e nuvola: http://flipper.diff.org/app/items/5762

Fornari, F., Gramantieri, L., Ferracin, M., Veronese, A., Sabbioni, S., Calin, G. A., Negrini, M. (2008). MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. *Oncogene, 27*, 5651-5661.

Glen, S. (2017, November 14). *Correlation Coefficient: Simple Definition, Formula, Easy Steps*. Retrieved from Statistics How To : http://www.statisticshowto.com/probability-and-statistics/correlation-coefficient-formula/

Gompf, S. G., Bansal, S., & Singal, A. (2016, April 12). *Hepatitis C Infection (Hep C): Causes, How It's Spread, and Treatment Guidelines for the Cure*. (J. W. Marks, Editor) Retrieved from MedicineNet.com: https://www.medicinenet.com/hepatitis_c/article.htm

Guo, C.-J., Pan, Q., Ding-Guo, L., Sun, H., & Liu, B.-W. (2009). miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: An essential role for apoptosis. *Journal of Hepatology, 50*, 766-778.

Guo, C.-J., Pan, Q., Jiang, B., Guang-Yu, C., & Ding-Guo, L. (2008). Effects of upregulated expression of microRNA-16 on biological properties of culture-activated hepatic stellate cells . *Apoptosis, 14*(1331).

Gupta, P., Cairns, M. J., & Saksena, N. K. (2014). Regulation of gene expression by microRNA in HCV infection and HCV–mediated hepatocellular carcinoma. *Virology Journal, 11*(64).

Harman, D. J., Ryder, S. D., James, M. W., Jelpke, M., Ottey, D. S., Wilkes, E. A., Guha, I. N. (2015). Direct targeting of risk factors significantly increases the detection of liver cirrhosis in primary care: a cross-sectional diagnostic study utilising transient elastography. *BMJ Open, 5*(e007615).

He, Y., Huang, C., Sun, X., Long, X., & Li, J. (2012). MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4. *Cellular Signalling, 24*(10), 1923-1930.

Hernandez-Gea, V., & Friedman, S. L. (2011). Pathogenesis of liver fibrosis. *Annual Review of Pathology, 6*, 425-456.

Highleyman, L. (2014, August). *HCV Advocate.* (A. Franciscus, Ed.) Retrieved September 12, 2017, from HSCP Fact Sheet: What is Fibrosis?: http://hcvadvocate.org/hepatitis/factsheets_pdf/Fibrosis.pdf

Iizuka, M., Ogawa, T., Enomoto, M., Motoyama, H., Yoshizato, K., Ikeda, K., & Kawada, N. (2012). Induction of microRNA-214-5p in human and rodent liver fibrosis. *Fibrogenesis & Tissue Repair, 5*(12).

Insititute for Quality and Efficiency in Health Care. (2016, August 22). *How does the liver work?* Retrieved September 12, 2017, from PubMed Health: https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0072577/

Jiang, J. X., Mikami, K., Shah, V. H., & Torok, N. J. (2008). Leptin induces phagocytosis of apoptotic bodies by hepatic stellate cells via a Rho guanosine triphosphatase-dependent mechanism . *Hepatology, 48*, 1497-1505.

Johns Hopkins. (2017). *Liver Biopsy.* Retrieved October 24, 2017, from Health Library: http://www.hopkinsmedicine.org/healthlibrary/test_procedures/gastroenterology/liver_biopsy_92,p07696

Joshi, D., Salehi, S., Brereton, H., Arno, M., Quaglia, A., Heaton, N., Aluvihare, V. (2013). Distinct microRNA profiles are associated with the severity of hepatitis C virus recurrence and acute cellular rejection after liver transplantation. *Liver Transplantation, 19*, 383-394.

Karsdal, M. A., Manon-Jensen, T., Genovese, F., Kristensen, J. H., Nielsen, M. J., Sand, J. B., Schuppan, D. (2015, May 15). Novel insights into the function and dynamics of extracellular matrix in liver fibrosis . *American Journal of Physiology - Gastrointestinal and Liver Physiology, 308*(10), G807-G830.

Keshava, S. N., Mammen, T., Surendrababu, N. R., & Moses, V. (2008). Transjugular liver biopsy: What to do and what not to do. *Indian Journal of Radiology and Imaging , 18*(3), 245-248.

Khattab, M., Sakr, M. A., Fattah, M. A., Mousa, Y., Soliman, E., Ashraf, B., Motawea, I. (2016, November 18). Novel non-invasive biological predictive index for liver fibrosis in hepatitis C virus genotype 4 patients. *World Journal of Hepatology, 8*(32), 1392-1401.

Kitano, M., & Bloomston, P. M. (2016). Hepatic Stellate Cells and microRNAs in

Pathogenesis of Liver Fibrosis. *Journal of Clinical Medicine*, *5*(3), 38. http://doi.org/10.3390/jcm5030038

Kim, V. N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. *National Review of Molecular Cell Biology, 6*(5), 376-385.

Klevens, R. M.; Canary, L.; Huang, X.; Denniston, M. M.; Yeo, A. E.; Pesano, R. L.; Ward, J. W.; Holmberg, S.;. (2016). The Burden of Hepatitis C Infection–Related Liver Fibrosis in the United States. *Clinical Infectious Diseases, 63*(8), 1049-1055.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., & Tusch, T. (2002). Identification of tissue-specific microRNAs from mouse. *Current Biology, 12*(9), 735-739.

Lavanchy, D. (2011). Evolving epidemiology of hepatitis C virus. *Clinical Microbiology and Infection, 17*, 107-115.

Lavillette, D., Tarr, A. W., Voisset, C., Donot, P., Bartosch, B., Bain, C., Cosset, F. (2005). Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology, 41*(2), 265-274.

Li, H., Jiang, J.-D., & Peng, Z.-G. (2016, January 28). MicroRNA-mediated interactions between host and hepatitis C virus. *World Journal of Gastroenterology, 22*(4), 1487-1496.

Livak, K. J., & Schmittgen, T. D. (2002, February 27). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods, 25*(4), 402-408.

Lu, J., Shen, Y., Wu, Q., Kumar, S., He, B., Shi, S., Wu, C. I. (2004). The birth and death of microRNA genes in drosophilia. *Nature Genetics, 116*(2), 281-297.

Lurie, Y., Webb, M., Cytter-Kuint, R., Shteingart, S., & Lederkremer, G. Z. (2015). Non-invasive diagnosis of liver fibrosis and cirrhosis. *World Journal of Gastroenterology*, *21*(41), 11567–11583. http://doi.org/10.3748/wjg.v21.i41.11567

MacFarlane, L., & Murphy, P. R. (2010). MicroRNA: Biogenesis, Function and Role in Cancer. *Current Genomics, 11*(7), 537-561.

Maher, J. J. (2011, August 21). Interactions between hepatic stellate cells and the immune system. *Seminars in Liver Disease, 3*, 417-426.

Mannan, A. A. (2015, April 3). *Liver and intrahepatic bile ducts- nontumor, Hepatitis.* (S. Yuan, Ed.) Retrieved September 26, 2017, from PathologyOutlines.com: http://www.pathologyoutlines.com/topic/liverchronichepgrading.html

Maubach, G., Lim, M. C., Chen, J., Yang, H., & Zhuo, L. (2011, June 14). miRNA studies in in vitro and in vivo activated hepatic stellate cells. *World Journal of Gastroenterology, 17*(22), 2748-2773.

Messina, J. P., Humphreys, I., Flaxman, A., Brown, A., Cooke, G. S., Pybus, O. G., & Barnes, E. (2015). Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology, 61*(1), 77-87.

Mohd Hanafiah, K., Groeger, J., Flaxman, A. D., & Wiersma, S. T. (2013). Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology, 57*(4), 1333-1342.

Moreira, R. K. (2007). Hepatic stellate cells and liver fibrosis. *Archives of Pathology & Laboratory Medicine, 131*(11), 1728-1734.

Motawi, T. M., Sadik, N. A., Shaker, O. G., & Ghaleb, M. H. (2016). Elevated serum microRNA-122/222 levels are potential diagnostic biomarkers in Egyptian patients with chronic hepatitis C but not hepatic cancer. *Tumor Biology, 37*(7), 9865-9874.

Murphy, D. G., Willems, B., Descenes, M., Hilzenrat, N., Mousseau, R., & Sabbah, S. (2007). Use of Sequence Analysis of the NS5B Region for Routine Genotyping of Hepatitis C Virus with Reference to C/E1 and 5′ Untranslated Region Sequences. *Journal of Clinical Microbiology, 45*(4), 1102-1112.

Nallagangula, K. S., Nagaraj, S. K., Venkataswamy, L., & Chandrappa, M. (2017, October 5). Liver fibrosis: a compilation on the biomarkers status and their significance during disease progression. *Future Science OA*, FSO250.

National Institutes of Health (NIH). (2002). NIH Consensus Statement on Management of Hepatitis C: 2002. *NIH Consensus and State-of-the-Science Statements, 19*(3), 1-46.

NCBI. (2017, August 29). MIR 215 microRNA 214 [Homo sapiens (human)]. *Gene Resources*.

Ngo, Y., Munteanu, M., Messous, D., Charlotte, F., Imbert-Bismut, F., Thabut, D (2006). A prospective analysis of the prognostic value of biomarkers (FibroTest) in patients with chronic hepatitis C. *Clinical Chemistry, 52*, 1887-1896.

Ogawa, T., Enomoto, M., Fujii, H., Sekiya, Y., Yoshizato, K., Ikeda, K., & Kawada, N. (2012). MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis . *Gut, 61*(11), 1600-1609.

Okada, H., Honda, M., Campbell, J. S., Takegoshi, K., Sakai, Y., Yamashita, T., Kaneko, S. (2015, September 1). Inhibition of microRNA-214 ameliorates hepatic fibrosis and tumor incidence in platelet-derived growth factor C transgenic mice. *Cancer Science, 106*(9), 1143-1152.

Olaso, E., Ikeda, K., Eng, F. J., Xu, L., Wang, L.-H., Lin, H. C., & Friedman, S. L. (2001, November 1). DDR2 receptor promotes MMP-2–mediated proliferation and invasion by hepatic stellate cells. *Journal of Clinical Investigation, 108*(9), 1369-1378.

Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR): OPTN/SRTR. (2010, December 19). *Annual data report.* Retrieved September 13, 2017, from Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation: https://srtr.transplant.hrsa.gov/annual_reports/2010/chapter_index.htm

Pang, Y., Young, C. Y., & Yuan, H. (2010). MicroRNAs and prostate cancer. *Acta Biochimica et Biophysica Sinica, 42*(6), 363-369.

Pineau, P., Volinia, S., McJunkin, K., Marchio, A., Battiston, C., Terris, B., Dejean, A. (2010). miR-221 overexpression contributes to liver tumorigenesis. *Proceedings of the National Academy of Sciences, 107*, 264-268.

Poynard, T., Ratziu, V., Benhamou, Y., Opolon, P., Cacoub, P., & Bedossa, P. (2000, April). Natural history of HCV infection. *Baillieres Best Practice & Research Clinical Gastroenterology, 14*(2), 211-228.

Presser, L. D., McRae, S., & Waris, G. (2013). Activation of TGF-β1 promoter by hepatitis C virus-induced AP-1 and Sp1: role of TGF-β1 in hepatic stellate cell activation and invasion. *PLoS One, 8*(2).

Roderburg, C., & Luedde, T. (2014, December). Circulating microRNAs as markers of liver inflammation, fibrosis and cancer. *Journal of Hepatology, 61*(6), 1434-1437.

Roy, S., Luedde, T., Benz, F., & Roderburg, C. (2015, January 5). The role of miRNAs in the regulation of inflammatory processes during hepatofibrogenesis. *Hepatobiliary Surgery and Nutrition, 4*(1), 24-33.

Sage, C., Nagel, R., Egan, D. A., Schrier, M., Mesman, E., Mangiola, A., Agami, R. (2007). Regulation of the p27Kip1 tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *The EMBO Journal, 26*(15), 3699-3708.

Santhekadur, P. K., Das, S. K., Gredler, R., Chen, D., Srivastava, J., Robertson, C., Sarkar, D. (2012). Multifunction Protein Staphylococcal Nuclease Domain Containing 1 (SND1) Promotes Tumor Angiogenesis in Human Hepatocellular Carcinoma through Novel Pathway That Involves Nuclear Factor κB and miR-221. *Journal of Biological Chemistry, 287*, 13952-13958.

Schiffman, G., & Cunha, J. (2016, September 14). *Pulmonary Fibrosis.* (M. C. Stöppler, Ed.) Retrieved September 12, 2017, from MedicineNet: http://www.medicinenet.com/pulmonary_fibrosis/article.htm#what_is_pulmonary_fibrosis

Sheedy, F. J., & O'Neill, L. A. (2008). Adding fuel to fire: microRNAs as a new class of mediators of inflammation. *Annals of the Rheumatic Diseases, 67*(3).

Sheiko, M. A., & Rosen, H. R. (2016). Hepatic Fibrosis in Hepatitis C. In T. Miyamura, S. M. Lemon, C. M. Walker, & T. Wakita, *Hepatitis C Virus II* (pp. 79-108). Tokyo, Japan: Springer Japan.

Shen, J., Wang, Q., Gurvich, I., Remotti, H., & Santella, R. M. (2016, November 18). Evaluating normalization approaches for the better identification of aberrant microRNAs associated with hepatocellular carcinoma . *Hepatoma research, 2*, 305-315.

Shiha, G., & Khaled, Z. (2011). Ishak versus METAVIR: Terminology, Convertability and Correlation with Laboratory Changes in Chronic Hepatitis C. In H. Takahashi (Ed.), *Liver Biopsy.* InTech.

Sonkoly, E., Stähle, M., & Pivatcsi, A. (2008). MicroRNAs and immunity: Novel players in the regulation of normal immune function and inflammation. *Seminars in Cancer Biology, 18*(2), 131-140.

Standish, R. A., Cholongitas, E., Dhillon, A., Burroughs, A. K., & Dhillon, A. P. (2006). An appraisal of the histopathological assessment of liver fibrosis. *Gut, 55*(4), 569-578.

Strassburg, C. P., & Manns, M. P. (2006). Approaches to liver biopsy techniques - revisited. *Seminars in Liver Disease, 26*(4), 318-327.

Tabasi, S. A., & Erson, A. E. (2008, September). *MIR221 (microRNA 221).* Retrieved October 22, 2017, from Atlas of Genetics and Cytogenetics in Oncology and Haematology: http://atlasgeneticsoncology.org/Genes/GC_MIR221.html

Tabasi, S. A., & Erson, A. E. (2008, September). *MIR222 (microRNA 222).* Retrieved October 22, 2017, from Atlas of Genetics and Cytogenetics in Oncology and Haematology: http://atlasgeneticsoncology.org/Genes/GC_MIR222.html

Taneja, S., Tohra, S., Duseja, A., Dhiman, R. K., & Chawla, Y. K. (2016). Noninvasive Assessment of Liver Fibrosis By Transient Elastography and FIB4/APRI for Prediction of Treatment Response in Chronic Hepatitis C—An Experience from a Tertiary Care Hospital . *Journal of Clinical and Experimental Hepatology, 6*(4), 282-290.

Tannapfel, A., Grund, D., Katalinic, A., Uhlmann, D., Kockerling, F., Haugwitz, U., . . . Wittekind, C. (2000). Decreased expression of p27 protein is associated with advanced tumor stage in hepatocellular carcinoma. *International Journal of Cancer, 89*, 350-355.

Thrift, A. P., El-Serag, H. B., & Kanwal, F. (2017). Global epidemiology and burden of HCV infection and HCV-related disease. *Nature Reviews Gastroenterology & Hepatology, 14*(2), 122-132.

Tsuchida, T., & Friedman, S. L. (2017, May 10). Mechanisms of hepatic stellate cell activation. *Nature Reviews Gastroenterology & Hepatology, 14*(7), 397-411. Retrieved from Nature: https://www.nature.com/nrgastro/journal/v14/n7/abs/nrgastro.2017.38.html

Ueda, T., Volinia, S., Okumura, H., Shimizu, M., Taccioli, C., Rossi, S., . . . Croce, C. M. (2009, December 21). Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *The Lancet Oncology, 11*(2), 136-146.

Urbich, C., Kuehbacher, A., & Dimmeler, S. (2008). Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovascular Research, 79*(4), 581-588.

WHO. (2017, April 21). *Global hepatitis report, 2017.* Retrieved from World Health Organization : http://apps.who.int/iris/bitstream/10665/255016/1/9789241565455-eng.pdf?ua=1

Wiesner, R. H., Sorrell, M., & Villamil, F. (2003). Report of the First International Liver Transplantation Society Expert Panel Consensus Conference on Liver Transplantation and Hepatitis C . *Liver Transplantation, 9*(11, Suppl 3), S1-S9.

Wong, Q. W., Ching, A. K., Chan, A. W., Choy, K. W., To, K. F., Lai, P. B., & Wong, N. (2010). MiR-222 overexpression confers cell migratory advantages in hepatocellular carcinoma through enhancing AKT signaling. *Clinical Cancer Research, 16*, 867-875.

Wu, G., Yu, F., Xiao, X., Xu, K., Tang, W., Wang, J., & Song, E. (2011). Hepatitis B virus X protein downregulates expression of the miR-16 family in malignant hepatocytes in vitro. *British Journal of Cancer, 105*(1), 146-153.

Xiong, W. (2016, October 22). *Chronic Hepatitis C Pathology.* (M. Younes, Ed.) Retrieved September 22, 2017, from MedScape: http://emedicine.medscape.com/article/1610728-overview?pa=iWO0LjxjTtc0n%2BX5oTJndp9IBYfmawOXbQacMybNbjp5ZsNc3QCpOvjLVlQTzmTUJyGvMX%2Fu%2BWdIXoARf%2FT0zw%3D%3D#a2

Xu, G., Yang, F., Ding, C.-L., Wang, J., Zhao, P., Wang, W., & Ren, H. (2014). MiR-221 accentuates IFN's anti-HCV effect by downregulating SOCS1 and SOCS3. *Virology, 462-463*, 343-350.

Yan, X., Liang, H., Deng, T., Zhu, K., Zhang, S., Wang, N., Chen, X. (2013). The identification of novel targets of miR-16 and characterization of their biological functions in cancer cells. *Molecular Cancer, 12*(92).

Yang, H., Kong, W., He, L., Zhao, J.-J., ODonnell, J. D., Wang, J., Cheng, J. Q. (2008, January 15). MicroRNA Expression Profiling in Human Ovarian Cancer: miR-214 Induces Cell Survival and Cisplatin Resistance by Targeting PTEN. *Cancer Research, 68*(2), 425-433.

Yang, Y., & Wang, J.-K. (2016). The functional analysis of MicroRNAs involved in NF-kB signaling. *European Review for Medical and Pharmacological Sciences, 20*, 1764-1774.

Zarnegar, R., DeFrances, M. C., Kost, D. P., Lindroos, P., & Michalopoulos, G. K. (1991, May 31). Expression of Hepatocyte Growth Factor mRNA in regenerating rat liver after partial hepatectomy. *Biochemical and Biophysical Research Communications, 177*(1), 559-565.

Zein, N. N. (2000). Clinical Significance of Hepatitis C Virus Genotypes. *Clinical Microbiology Reviews, 13*(2), 223-235.

Zhu, B., Wei, X. X., Wang, T. B., Zhou, Y. C., Liu, A. M., & Zhang, G. W. (2015). Increased miR-16 expression induced by hepatitis C virus infection promotes liver fibrosis through downregulation of hepatocyte growth factor and Smad7. *Archives of Virology, 160*(8), 2043-2050.

10 Appendix

10.1 Copyright Approval

10.1.1 Copyright for Figures 1 & 3

Title: Mechanisms of hepatic stellate cell activation

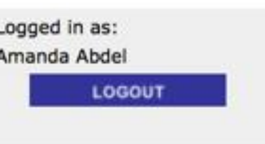
Author: Takuma Tsuchida, Scott L. Friedman

Publication: Nature Reviews Gastroenterology and Hepatology

Publisher: Nature Publishing Group

Date: May 10, 2017

Copyright © 2017, Rights Managed by Nature Publishing Group



Order Completed

Thank you for your order.

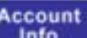
This Agreement between Ms. Amanda Abdel-Al ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4218700485555
License date	Oct 30, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Gastroenterology and Hepatology
Licensed Content Title	Mechanisms of hepatic stellate cell activation
Licensed Content Author	Takuma Tsuchida, Scott L. Friedman
Licensed Content Date	May 10, 2017
Licensed Content Volume	14
Licensed Content Issue	7
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
High-res required	no
Figures	Figures 1, 2, and 3.
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Using miRNAs as noninvasive biomarkers for the detection of the progression of liver fibrosis in HCV-genotype 4 patients
Expected completion date	Dec 2017
Estimated size (number of pages)	70

10.1.2 Copyright for Figure 6



Title: Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study

Author: Sarah Blach,Stefan Zeuzem,Michael Manns,Ibrahim Altraif,Ann-Sofi Duberg,David H Muljono,Imam Waked,Seyed M Alavian,Mei-Hsuan Lee,Francesco Negro,Faisal Abaalkhail,Ahmed Abdou,Maheeba Abdulla,Antoine Abou Rached,Inka Aho,Ulus Akarca,Imad Al Ghazzawi et al.

Publication: The Lancet Gastroenterology & Hepatology

Publisher: Elsevier

Date: March 2017

© 2017 Published by Elsevier Ltd.

Logged in as:
Amanda Abdel-Al
Account #:
3001211726
LOGOUT

Order Completed

Thank you for your order.

This Agreement between Ms. Amanda Abdel-Al ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4218930757456
License date	Oct 30, 2017
Licensed Content Publisher	Elsevier
Licensed Content Publication	The Lancet Gastroenterology & Hepatology
Licensed Content Title	Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study
Licensed Content Author	Sarah Blach,Stefan Zeuzem,Michael Manns,Ibrahim Altraif,Ann-Sofi Duberg,David H Muljono,Imam Waked,Seyed M Alavian,Mei-Hsuan Lee,Francesco Negro,Faisal Abaalkhail,Ahmed Abdou,Maheeba Abdulla,Antoine Abou Rached,Inka Aho,Ulus Akarca,Imad Al Ghazzawi et al.
Licensed Content Date	Mar 1, 2017
Licensed Content Volume	2
Licensed Content Issue	3
Licensed Content Pages	16
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 4
Title of your thesis/dissertation	Using miRNAs as noninvasive biomarkers for the detection of the progression of liver fibrosis in HCV-genotype 4 patients
Expected completion date	Dec 2017
Estimated size (number of pages)	70

10.1.3 Copyright for Figure 7

Title:	Global epidemiology and burden of HCV infection and HCV-related disease
Author:	Aaron P. Thrift, Hashem B. El-Serag, Fasiha Kanwal
Publication:	Nature Reviews Gastroenterology and Hepatology
Publisher:	Nature Publishing Group
Date:	Dec 7, 2016

Copyright © 2016, Rights Managed by Nature Publishing Group

Logged in as:
Amanda Abdel-Al
Account #:
3001211726
LOGOUT

Order Completed

Thank you for your order.

This Agreement between Ms. Amanda Abdel-Al ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4246440539722
License date	Dec 12, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Gastroenterology and Hepatology
Licensed Content Title	Global epidemiology and burden of HCV infection and HCV-related disease
Licensed Content Author	Aaron P. Thrift, Hashem B. El-Serag, Fasiha Kanwal
Licensed Content Date	Dec 7, 2016
Licensed Content Volume	14
Licensed Content Issue	2
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Using miRNAs as noninvasive biomarkers for the detection of the progression of liver fibrosis in HCV-genotype 4 patients
Expected completion date	Dec 2017
Estimated size (number of pages)	70

10.1.4 Copyright permission for Figure 11

Title: An appraisal of the histopathological assessment of liver fibrosis

Author: R A Standish,E Cholongitas,A Dhillon,A K Burroughs,A P Dhillon

Publication: Gut

Publisher: BMJ Publishing Group Ltd.

Date: Apr 1, 2006

Copyright © 2006, BMJ Publishing Group Ltd and the British Society of Gastroenterology

Logged in as:
Amanda Abdel-A
Account #:
3001211726
LOGOUT

Order Completed

Thank you for your order.

This Agreement between Ms. Amanda Abdel-Al ("You") and BMJ Publishing Group Ltd. ("BMJ Publishing Group Ltd.") consists of your license details and the terms and conditions provided by BMJ Publishing Group Ltd. and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4246441301206
License date	Dec 12, 2017
Licensed Content Publisher	BMJ Publishing Group Ltd.
Licensed Content Publication	Gut
Licensed Content Title	An appraisal of the histopathological assessment of liver fibrosis
Licensed Content Author	R A Standish,E Cholongitas,A Dhillon,A K Burroughs,A P Dhillon
Licensed Content Date	Apr 1, 2006
Licensed Content Volume	55
Licensed Content Issue	4
Type of Use	Dissertation/Thesis
Requestor Type	Individual
Format	Print and electronic
Portion Used	Figure/table/extract
Number of figure/table/extracts	1
Description of figure/table/extracts	Figure 1
Will you be translating?	No
Circulation/distribution	1
Title of your thesis / dissertation	Using miRNAs as noninvasive biomarkers for the detection of the progression of liver fibrosis in HCV-genotype 4 patients
Expected completion date	Dec 2017
Estimated size(pages)	70

10.2 Institutional Review Board (IRB)

CASE #2016-2017-138

THE AMERICAN UNIVERSITY IN CAIRO
INSTITUTIONAL REVIEW BOARD

To: Amanda Abd-Al

Cc: Hind Helaly

From: Atta Gebril, Chair of the IRB

Date: May 18 , 2017

Re: Approval of study

This is to inform you that I reviewed your revised research proposal entitled "**MicroRNAs as Noninvasive Biomarkers for the Detection of Different Stages of Liver Fibrosis in HCV Patients**" and determined that it required consultation with the IRB under the "expedited" heading. As you are aware, the members of the IRB suggested certain revisions to the original proposal, but your new version addresses these concerns successfully. The revised proposal used appropriate procedures to minimize risks to human subjects and that adequate provision was made for confidentiality and data anonymity of participants in any published record. I believe you will also make adequate provision for obtaining informed consent of the participants.

This approval letter was issued under the assumption that you have not started data collection for your research project. Any data collected before receiving this letter could not be used since this is a violation of the IRB policy.

Please note that IRB approval does not automatically ensure approval by CAPMAS, an Egyptian government agency responsible for approving some types of off-campus research. CAPMAS issues are handled at AUC by the office of the University Counsellor, Dr. Amr Salama. The IRB is not in a position to offer any opinion on CAPMAS issues, and takes no responsibility for obtaining CAPMAS approval.

This approval is valid for only one year. In case you have not finished data collection within a year, you need to apply for an extension.

Thank you and good luck.

Atta Gebril

Dr. Atta Gebril
IRB chair, The American University in Cairo
2046 HUSS Building
T: 02-26151919
Email: agebril@aucegypt.edu

Institutional Review Board
The American University in Cairo
AUC Avenue, P.O. Box 74
New Cairo 11835, Egypt.
tel 20.2.2615.1000
fax 20.2.27957565
Email: **aucirb@aucegypt.edu**

10.3 Consent Form

استمارة موافقة مسبقة للمشاركة في دراسة بحثية

عنوان المشروع: الميكرو أر ان أيه كمؤشرات حيوية للكشف عن مراحل مختلفة من تليف الكبد في مرضى التهاب الكبد الوبائي

الباحث الرئيسي: أماندا عبد العال
البريد الالكتروني: aabdelal89@aucegypt.edu
الهاتف: 01092450926

يطلب منك المشاركة في دراسة بحثية. والغرض من البحث هو العثور على ميكرو أر ان أيه جديدة من شأنها أن تعمل كمؤشرات حيوية غير للكشف عن تطور تليف الكبد على مستوى جيني، ويمكن نشر النتائج وعرضها.
المدة المتوقعة لمشارتك هي شهر واحد.

سوف تكون إجراءات البحث على النحو التالي:
-٢٠ حالة التهاب الكبد المزمن C مع التليف في مرحلة مبكرة.
-٢٠ حالة من التهاب الكبد المزمن C مع التليف المرحلة الرابعة.
- ٢٠ حالة أصحاء مع عدم وجود التهاب الكبد المزمن C أو التليف كضوابط.
- ٣ ميكرو أر ان أيه الرئيسية سوف تستخدم لإثبات نمط تعبير ملحوظ خلال تطور تليف الكبد. سيتم أخذ عينات الدم من جميع المرضى وسيتم استخراج الذي ان أيه باستخدام مجموعات مخصصة. سيتم قياس الميكرو أر ان أيه عبر مجموعات متخصصة باستخدام جهاز .Real time PCR سيتم تحليل الميكرو أر ان ايه لتقييم التعبير عن الميكرو أر ان ايه المختار. سوف يطلب منك إعطاء ٥ مل من الدم كمساهمتك في هذا المشروع. وسوف نحتاج أيضا إلى الوصول إلى السجلات الطبية الأخيرة لتحديد ما إذا كنت مرشحا جيدا لهذا البحث.

- بتوقيع هذه الموافقة فإنك توافق على السماح بالدخول في هذه الدراسة.
- هناك مخاطر أو مضايقات مرتبطة بهذا البحث هي عملية الحصول على دمك ومن المتوقع عدم الراحة الصغرى للعملية.
- سيكون هناك فوائد لك من هذا البحث. قد تساعد الدراسة للعثور على مرشح مناسب يتيح استخدامه لتطوير مقايسة تقدم تليف الكبد.
- المعلومات التي تقدمها لأغراض هذا البحث سرية.
- يجب توجيه الأسئلة حول البحث أو الحقوق أو البحوث المتعلقة بالإصابات إلى:
  - أماندا عبد العال 01092450926
  - د. إيمان الأهواني 01002050805.
- المشاركة في هذه الدراسة طوعية. ولا ينطوي رفض المشاركة على أي عقوبة أو خسارة من المزايا التي يحق لك الحصول عليها. يجوز لك التوقف عن المشاركة في أي وقت دون عقوبة أو فقدان المزايا التي يحق لك الحصول عليها.

الإسم:______________________________

التوقيع:______________________________

التاريخ:______________________________