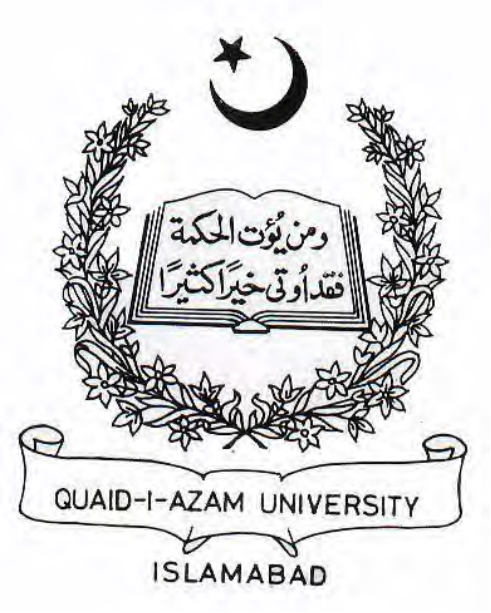
Use of ATR-FTIR to screen for Hepatocellular Carcinoma in Patients with Sustained Virologic Response



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2023

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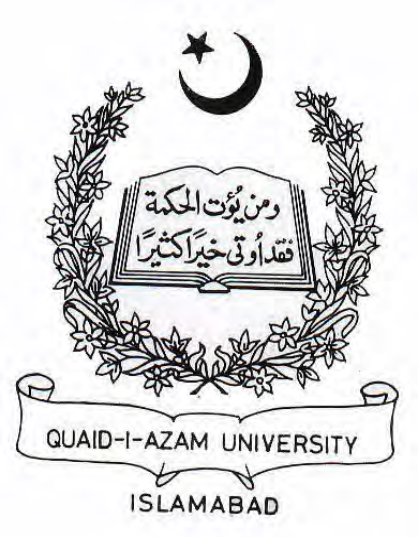

Thesis submitted to the Department of Biotechnology, Quaid-i-Azam University Islamabad, in partial fulfillment of the degree of MPhil in Biotechnology.

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CERTIFICATE

It is certified that the research work presented in this thesis titled "Use of ATR-FTIR to screen for Hepatocellular Carcinoma in Patients with Sustained Virologic Response" was conducted by **Mr. Muhammad Tahir Hayat**. This thesis is accepted in its present form by the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the requirements for the thesis necessary for the partial fulfillment of the degree of Master of Philosophy in Biotechnology.

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Dated: 21/07/2023

Declaration

I hereby declare that the work accomplished in this thesis is the result of my own research carried out in Molecular Virology Lab, Department of Biotechnology, Quaid-i-Azam University, Islamabad. This thesis has not been published previously nor does it contain any material from the published resources that can be considered as a violation of international copyright law.
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Muhammad Tahir Hayat

Department of Biotechnology,
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Islamabad.

Dedicated to my parents

for their

unconditional love and support

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Acknowledgements

All praises and thanks to Allah Almighty, Lord of All creations, the beneficent and the most merciful, who is the source of entire knowledge and wisdom endowed to humanity. So is His Prophet, Hazrat Muhammad (Peace Be Upon Him), who is forever a source of guidance and knowledge for humanity as a whole.

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Muhammad Tahir Hayat

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List of Abbreviations

AFP	Alpha-Fetoprotein
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
BCLC	Barcelona Clinic Liver Cancer
CLDN1	Claudin-I
CD	Cluster Of Differentiation
CT	Computed Tomography
DAAs	Direct-Acting Antiviral Agents
ELISA	Enzyme-Linked Immunosorbent Assay
HCV	Hepatitis C Virus
HCC	Hepatocellular Carcinoma
HIV	Human Immunodeficiency Virus
IR	Infrared
IBC	Institutional Biosafety Committee
IRES	Internal Ribosome Entry Site
LDA	Linear Discriminant Analysis
LDL	Low Density Lipoproteins
MRI	Magnetic Resonance Imaging
ORF	Open Reading Frame
PC	Principal Component
PCA	Principal Component Analysis
RCF	Relative Centrifugal Field
SNV	Standard Normal Variate
SVM	Support Vector Machine
SVR	Sustained Virologic Response
TGF-β	Transforming Growth Factor-B

TN	True Negative
TP	True Positive
UTRs	Untranslated Regions
VLDL	Very Low-Density Lipoproteins
WHO	World Health Organization

Abstract

Advanced approaches such as attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy have been developed and adopted due to the growing need for non-invasive diagnostics. This study aims to utilize ATR-FTIR spectroscopy combined with chemometric analysis to screen for hepatocellular carcinoma (HCC) in patients with sustained virologic response (SVR) following direct-acting antiviral (DAA) therapy for chronic hepatitis C virus (HCV) infection. The study population included 57 individuals, comprising 21 healthy subjects, 16 cirrhotic SVR patients, and 20 HCC SVR patients. Serum samples were collected, processed, and subjected to ATR-FTIR spectral acquisition. The resulting spectra were analyzed using pre-processing techniques, i.e., baseline correction, smoothing, and normalization. Principal component analysis (PCA) was employed to identify general trends in the spectral data, revealing distinct variations in the lipid and bio-fingerprint regions. Subsequently, a PCA-linear discriminant analysis (LDA) model was developed for enhanced classification, achieving an accuracy of 89.47% in distinguishing cirrhotic SVR, HCC SVR, and healthy individuals, whereas the confusion matrix created by the PCA-LDA model shows the sensitivity and specificity of 100% and 91.3% respectively. The findings demonstrate the ability of ATR-FTIR spectroscopy coupled with chemometric analysis as a non-invasive, cost-effective, and time-efficient technique for HCC screening in patients with SVR. This technique holds promise for disease management, treatment response assessment, and prognosis, offering a valuable tool in the field of liver disease diagnostics. Further studies with larger and more diverse populations are necessary to validate these results and enhance their clinical applicability.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

This section briefly overviews the Hepatitis C virus (HCV), its structure, genome, pathogenesis, prevalence, diagnosis, treatment, and its association with hepatocellular carcinoma (HCC). It also describes HCC, its screening, treatment, and detection techniques with their limitations. ATR-FTIR is a non-invasive and effective approach with its basic working principle, multivariate analysis, and performance evaluation.

1.1 Hepatitis C virus (HCV)

Blood-borne viral infection HCV, initially identified in 1989 (Choo et al., 1989), primarily affects the liver (Prati, 2006). It belongs to the *Flaviviridae* family and is categorized as a positive-sense single-stranded RNA virus. HCV is mainly spread via contact with infected blood, such as sharing needles among people who inject drugs, receiving contaminated blood transfusions or organ transplants, and needle-stick injuries in healthcare settings (Alter, 2007).

HCV infection presents a significant challenge for public health and contributes significantly to liver-related morbidity and mortality, placing a burden on healthcare systems globally. Around 58 million people worldwide have chronic HCV infection, with an estimated 1.5 million worldwide contracting the virus yearly. According to estimates, 0.8% of the world's population is infected with HCV. The Eastern Mediterranean Region has the highest HCV prevalence at 1.6%. Hepatitis C-related causes account for an estimated 290,000 deaths annually, and only 21% of HCV-infected individuals are diagnosed, with 62% receiving treatment (World Health Organization [WHO], 2021).

Pakistan bears the burden of having the second-highest worldwide prevalence of HCV infection, affecting around 5% of its population, which is equivalent to 8 million people infected (Mahmood et al., 2017; Qasim, 2017). The delayed diagnosis of this infectious disease contributes to the development of chronic liver disease, hepatocellular carcinoma, and cirrhosis and further strains the already economically disadvantaged nation (Moin et al., 2018).

Symptoms of acute HCV infection may include fatigue, fever, nausea, and abdominal pain, while chronic HCV infection may cause few or no symptoms until liver damage has occurred. There are several different genotypes of HCV, which can vary in their geographic distribution and response to treatment. Antiviral medications can effectively treat HCV, and new medications with improved rates of cure and fewer adverse effects have become available in recent years. Prevention measures include using sterile injection equipment and screening blood donations for HCV. There is currently no vaccine for HCV (Echeverría et al., 2021).

1.2 Structure and genome of HCV

The genus *Hepacivirus* of the *Flaviviridae* family contains the tiny, enveloped, positive-sense single-stranded RNA virus known as HCV. Viral envelopes have an icosahedral shape and a diameter of 56–65 nm, whereas the viral core is roughly 45 nm in size (Gastaminza et al., 2010; Kaito et al., 1994). The 6 nm-long viral spikes on the virion membrane are composed of E1 and E2 glycoprotein heterodimers. The size, buoyant density, and infectiousness of the extracellular population of HCV particles are all different. The majority of these particles are not contagious (Gastaminza et al., 2010; Kaito et al., 1994). Figure 1.1 illustrates the particle structure of HCV.

The HCV genome shown in Figure 1.2 is composed of approximately 9600 nucleotides (Drexler et al., 2013; Kapoor et al., 2011). The 5′ and 3′ non-translated regions (NTRs), which are crucial for viral replication, surround the viral genome's single open reading frame (ORF), which codes for 3010 amino acids (Tanaka et al., 1995) and translation (Tsukiyama-Kohara et al., 1992). An internal ribosome entry site (IRES) can be found in the 5′ NTR (Honda et al., 1999).

1.3 Pathogenesis and viral entry

HCV is a hepatotropic non-cytopathic virus because after attacking liver cells and initiating replication, it can escape the immune system as a mechanism to survive in infected hosts. Ultimately HCV leads to cell death by several mechanisms, including immune-mediated cytolysis, alongside other processes like hepatic steatosis, oxidative stress, and insulin resistance (Irshad et al., 2013).

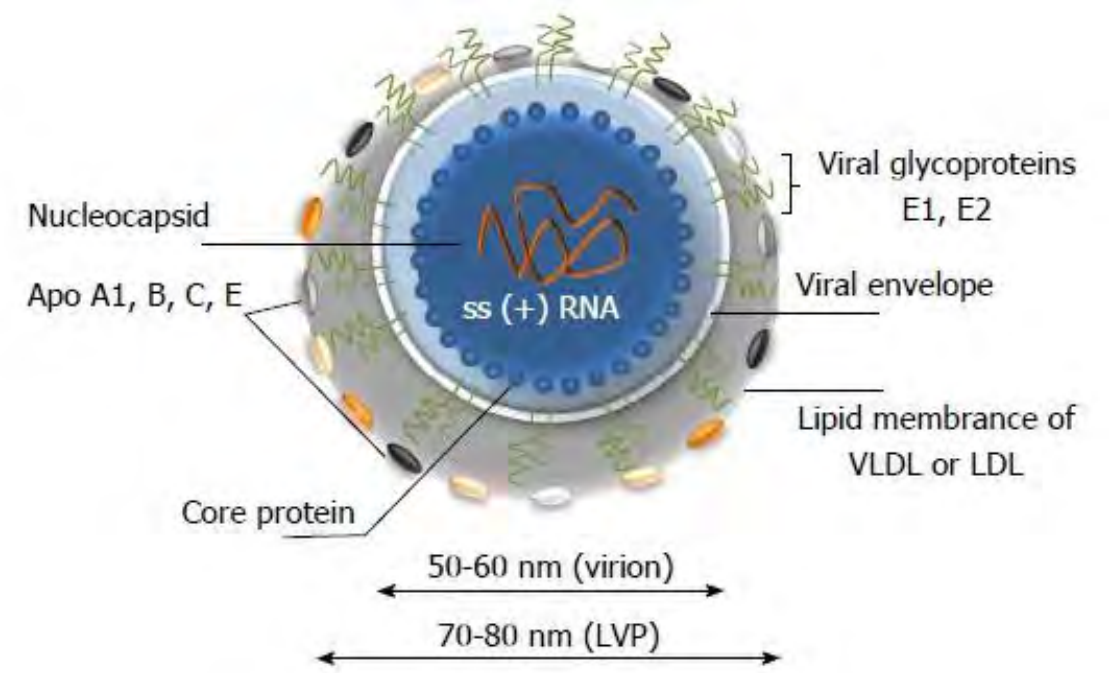

Figure 1.1 Particle structure of HCV

On the surface of the virion (shown in grey), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) combine to create the lipid membrane. Viral RNA is displayed in orange, while the viral core is presented in blue. The lipid bilayer contains heterodimers of the glycoproteins E1 and E2, which create 6 nm-long spikes (projections) on the virion's surface. The virion's shape is not icosahedral due to the connection with LDL and VLDL. The particles' size and form may change depending on the viral source (Morozov & Lagaye, 2018).

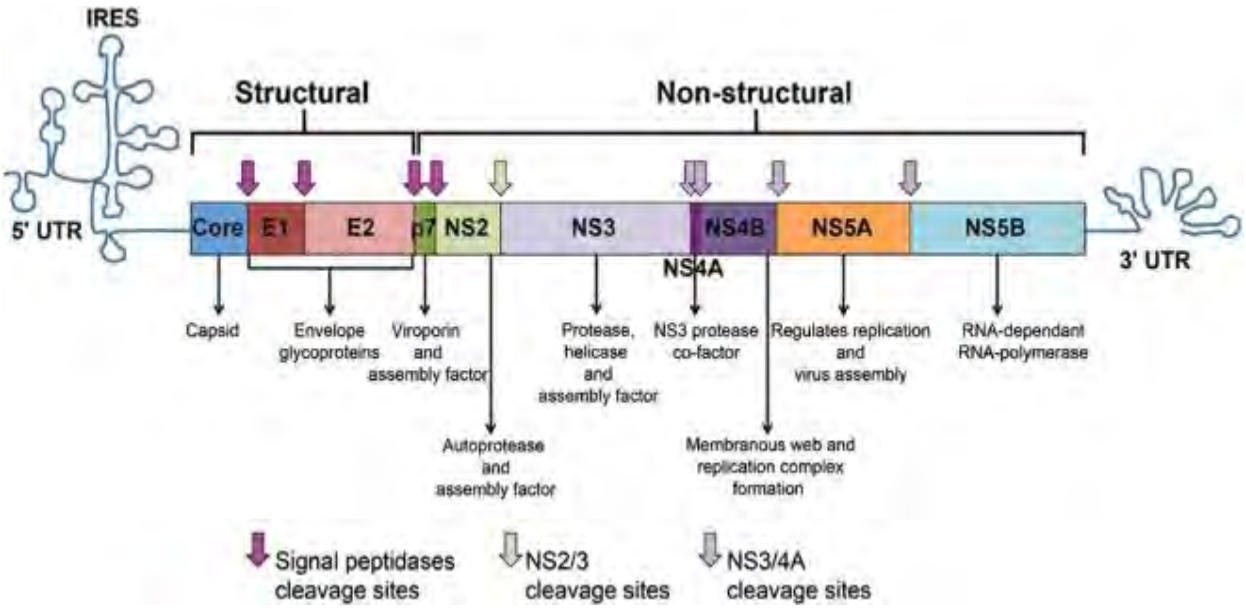

Figure 1.2 Representation of HCV

HCV is made up of a continuous series of nucleotides with ORF and 5′ and 3′ untranslated regions (UTRs) on each side. The IRES makes translating the ORF into a polyprotein easier. A big precursor molecule is created due to this process, and it is later converted into ten useful viral proteins. Cellular signal peptidases (shown by pink arrows) cleave the core protein from E1 and E1, E2, and p7 from the polyprotein. An auto cleavage of the NS2-NS3 protease is present (a green arrow denotes this). A membrane-bound cofactor called NS4A is needed for the NS3 protease, found in the N-terminal region of NS3. These proteases cleave the remaining viral proteins, including the NS3 and the NS4A, NS4B, NS5A, and NS5B (shown by a violet arrow) (Abdel-Hakeem & Shoukry, 2014).

These events are significantly affected by the proteins and peptides generated by various sub-genomic regions of the HCV genome and their genetic variants. As a result, they have an important function in the emergence and spread of HCV-related illnesses. A brief explanation of HCV pathogenesis concerning these factors is shown in Figure 1.3. HCV enters liver cells through a multistep process involving several host variables. Scavenger receptor class B type I, Occludin, Claudin-I (CLDN1), and CD81 are necessary factors for HCV entry. In addition, additional proteins like CLDN6 and CLDN9 can serve as replacement entry factors in human non-liver cells. To help the viral particle enter liver cells, CD81 functions as the primary viral receptor on host cells. Nearly all nucleated cells express CD81, which interacts with other cell-surface receptors, including CD19 and CD21, to communicate with other cells (Bruening et al., 2018).

The main extracellular loop of CD81 is where the viral envelop protein, E2, interacts. It may also bind to other molecules, including the LDL receptors. Because the RNA-dependent RNA polymerase of the virus lacks proofreading abilities and frequently undergoes mutations in the hyper-variable areas of the E2 protein, the HCV quasi-species has a high mutation rate. These mutations may be brought on by HCV-specific cytolytic T cells and virus-neutralizing antibodies. As a result, HCV interacts specifically with CD81 and binds to many sites (Zeisel et al., 2013).

1.4 Diagnosis and treatment of HCV

Virological testing is essential in the management of HCV infection, serving multiple purposes, such as diagnosing infection, predicting antiviral therapy response, and guiding treatment decisions (Elmasry et al., 2017). The initial diagnostic tests for HCV infection involve serologic assays that detect antibodies produced by the body in response to HCV infection (Elbasha et al., 2017; Majid & Gretch, 2002; Pawlotsky, 2002).

Enzyme immunoassay (EIA) is a one-step diagnostic method that detects the presence of specific antigens related to HCV infection in the serum. The third-generation EIA test is highly sensitive and specific, making it a valuable tool in the initial screening of viremic HCV infection in individuals, particularly those coinfected with human immunodeficiency virus (HIV) (Vermeersch et al., 2008).

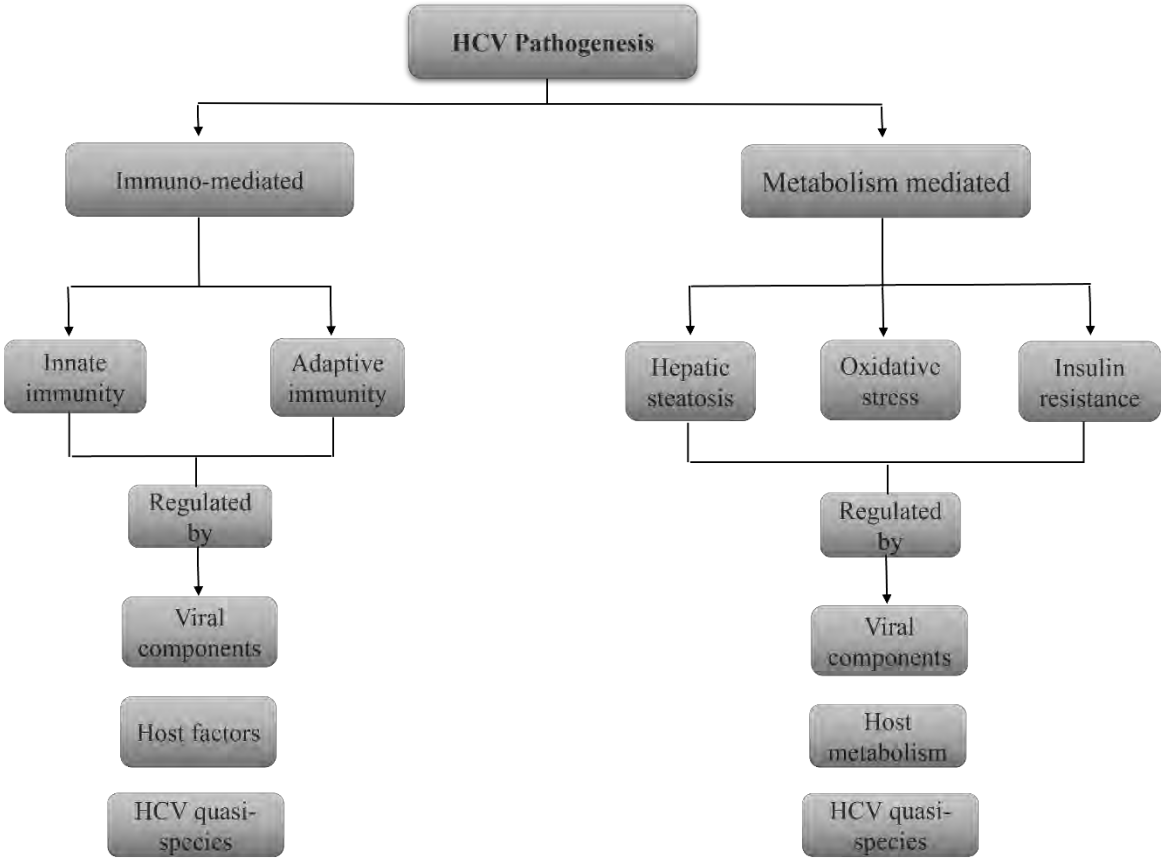

Figure 1.3 Regulation of HCV pathogenesis

HCV enters liver cells and replicates, resulting in the death of the cells through immune system-mediated and metabolism-mediated cytolysis. It also leads to hepatic steatosis, increased oxidative stress, and insulin resistance. The specific proteins and peptides produced by various regions of the HCV genome, along with their quasi-species, significantly contribute to the development of these mechanisms and ultimately drive the progression of HCV infection (Irshad et al., 2013).

Chemiluminescence Immunoassay (CIA) is an antibody-based diagnostic test that shares similarities with EIA in terms of its operational mechanism and results but is less commonly utilized. Its sensitivity and specificity are comparable to third-generation EIA when used as a diagnostic method for HCV (Dufour et al., 2003; Roger et al., 2021).

Nucleic acid test (NAT) is used to identify HCV RNA. NAT test becomes positive a week or two after infection (Berger et al., 2017). This test has become a gold standard as an additional test for people having a positive HCV EIA (Krajden, 2000). Results are given in international units (IU). HCV core antigen has been used as a diagnostic marker either alone or anti-HCV antibody (Czerwinski et al., 2017; Hullegie et al., 2017). The use of HCV core antigen is less costly than HCV RNA testing (van Tilborg et al., 2018).

The introduction of IFN-free regimens depending on direct-acting antiviral agents (DAAs) has marked a significant milestone in HCV therapy. These antiviral drugs, including telaprevir and boceprevir, have demonstrated remarkable efficacy, with response rates reaching up to 98%. Furthermore, the treatment duration has been significantly reduced to just 8-12 weeks of oral medication, providing patients with a more convenient and tolerable option (Li & Chung, 2019).

However, despite these advancements, there are several challenges associated with HCV treatment. The cost and practical considerations related to accessing treatment pose a significant barrier. Access to these therapies remains unequal across different countries, particularly in underdeveloped countries where HCV treatment may not be readily available. This lack of access perpetuates health disparities and prevents the effective control of HCV in high-risk populations (Bailey et al., 2019).

The appearance of resistance to DAAs has become a major issue through their formation and utilization. Resistance-related substitutions have been found both before and after receiving therapy with DAAs. This highlights the need for continuous monitoring of treatment response and the development of strategies to overcome or prevent the emergence of drug resistance (Wyles, 2017).

Persistent challenges in HCV treatment also extend beyond viral clearance. Although DAAs can effectively eliminate HCV infection, they do not entirely remove the

risk of getting liver cancer. Additionally, protective immunity following natural or treatment-induced viral clearance is often insufficient, leaving individuals susceptible to reinfection. This poses a substantial challenge in achieving HCV elimination, particularly among high-risk populations (Abdel-Hakeem & Shoukry, 2014).

1.5 Hepatocellular carcinoma

HCC is a primary liver cancer frequently found in cirrhosis and chronic liver disease individuals. HCC is a significant liver cancer and the fifth most frequent cancer globally. Due to a lack of helpful screening techniques, it is an essential cause of cancer-related mortality and is frequently discovered at an advanced stage. HCV is a significant risk factor for developing HCC, especially in patients with cirrhosis. The development of HCC resulting from HCV is a gradual process spanning 20 to 40 years (Vescovo et al., 2016). The host's immunological response and viral-induced factors can lead to the carcinogenic process of HCV (Ghouri et al., 2017).

Hepatocarcinogenesis represents a complex and prolonged process characterized by the sequential acquisition of genetic alterations leading to the development of HCC, as illustrated in Figure 1.4. The transformation of hepatocytes into malignant cells occurs due to augmented liver cell turnover, triggered by chronic liver damage and subsequent regeneration in the presence of inflammation and oxidative stress. HCV proteins have the ability to enhance mitogenic signaling pathways directly, inhibit cell death, and stimulate the production of reactive oxygen species (ROS) (Machida et al., 2006).

Additionally, HCV induces persistent inflammation, characterized by the infiltration of lymphocytes into the liver and the release of various cytokines, such as LTα and LTβ, which are closely linked with HCC development. Long-lasting inflammation worsens the formation of ROS, which is known to be the main cause of genetic abnormalities. Through the stimulation of the transforming growth factor-β (TGF-β) pathway, ROS also helps to promote fibrogenesis and the activation of hepatic stellate cells (Vescovo et al., 2016; Zhang et al., 2017).

The interaction between TGF-β and TLR4 facilitates the epithelial-mesenchymal transition, a key process in HCC progression. HCV disrupts the host lipid metabolism,

accumulating fat in the liver, a condition frequently observed in patients with HCC. Furthermore, HCV can induce angiogenic and metastatic pathways, contributing to the aggressive nature of HCC. Recent studies have highlighted the role of the genetic polymorphisms, particularly in the DEPDC5 and MICA genes, in increasing the susceptibility to HCC development (Hai et al., 2017).

HCC is a major health concern globally. Around the world, 782,000 fatalities and 841,000 new cases were reported in 2018, with Asia and Africa having the highest incidences (Bray et al., 2018). The incidence of HCC has been steadily increasing in the United States over the past few decades, with a predicted 42,230 new cases and 30,230 deaths in 2020 (Elshami et al., 2022).

HCV infection is a significant risk factor for HCC development, with approximately 15-20% of patients with chronic HCV infection developing cirrhosis, which is a precursor to HCC (El-Serag, 2012). The introduction of DAAs has altered the treatment of HCV, with Sustained virologic response (SVR) rates exceeding 90% (Nash et al., 2010). SVR has been associated with a reduced risk of HCC development, but the risk is not entirely eliminated (Singal et al., 2013).

Most infected patients cannot eliminate the infection and acquire HCV (Heim & Thimme, 2014). Inflammation-induced liver tumors brought on by the chronic HCV are usually accompanied by hepatic fat buildup (steatohepatitis) and progressive fibrosis, and over the course of 20 to 40 years, these lesions may proceed to cirrhosis (10–20% of patients) or HCC (1–5% of patients) (Figuring 1.4) (Arzumanyan et al., 2013; Westbrook & Dusheiko, 2014). According to estimates, HCV infection causes 25% of HCC cases and 27% of cirrhosis cases globally. SVR to antiviral therapy is linked with a decreased risk of HCC development in HCV cirrhotic patients. However, HCC surveillance in these patients is still necessary, and current screening methods have limitations.

1.6 Screening and detection of HCC in HCV

Early detection of HCC is crucial due to its high mortality rate. Patients with HCV-induced cirrhosis should undergo regular liver ultrasonography screening, possibly with adjunct serum alpha-fetoprotein (AFP) level measurement (Fitzmorris & Singal,

2015). Ultrasonography is the preferred screening modality since it can detect lesions as small as 0.5-1 cm with greater sensitivity and specificity than AFP (Ye & Chen, 2011). However, its accuracy in detecting nodules smaller than 0.5 cm is limited, and its sensitivity depends on the operator. Alternative screening techniques include computed tomography (CT) and magnetic resonance imaging (MRI) (Parikh & Taouli, 2013).

Figure 1.5 depicts the globally preferred Barcelona Clinic Liver Cancer (BCLC) staging system, which can be used to stage the HCC once confirmed (Tellapuri et al., 2018). Before the beginning of HCV therapy, liver ultrasonography is required within 12 weeks of its conclusion and every 24 weeks after that. Currently, ultrasound is only advised as a backup screening method when it is unavailable or of poor quality (Daniele et al., 2004). However, the sensitivity of HCC identification rose from 63% to 69% in some instances when AFP was used in conjunction with ultrasound imaging (A. Singal et al., 2009).

Cirrhosis is the most common scenario for HCV-associated HCC; bridging fibrosis can also infrequently be present (Crissien & Frenette, 2014). As a result, individuals with cirrhosis and F3 fibrosis are advised to follow the current guidelines for HCC surveillance (liver ultrasonography with or without AFP twice a year) (Jacobson et al., 2017). The facts about the risk of getting HCC after receiving therapy with DAA are still debatable. Cirrhosis has significant psychological and economic repercussions. There is evidence that, with the development of new treatment modalities, fibrosis may begin to regress after attaining SVR (Yoo et al., 2022). There is little evidence to link the risk of HCC and regression in cirrhosis as measured histologically (D'Ambrosio et al., 2018).

Early detection of HCC is crucial for successful treatment and improved patient outcomes. Several methods are available for detecting and diagnosing HCC, including imaging techniques and blood tests. Ultrasound is a commonly used imaging technique for detecting HCC. It is a non-invasive, painless, and low-cost method that uses high-frequency sound waves to create images of the liver. It can detect small lesions in the liver that other imaging techniques may miss.

The CT scan uses X-rays to produce detailed images of the liver. It is a more

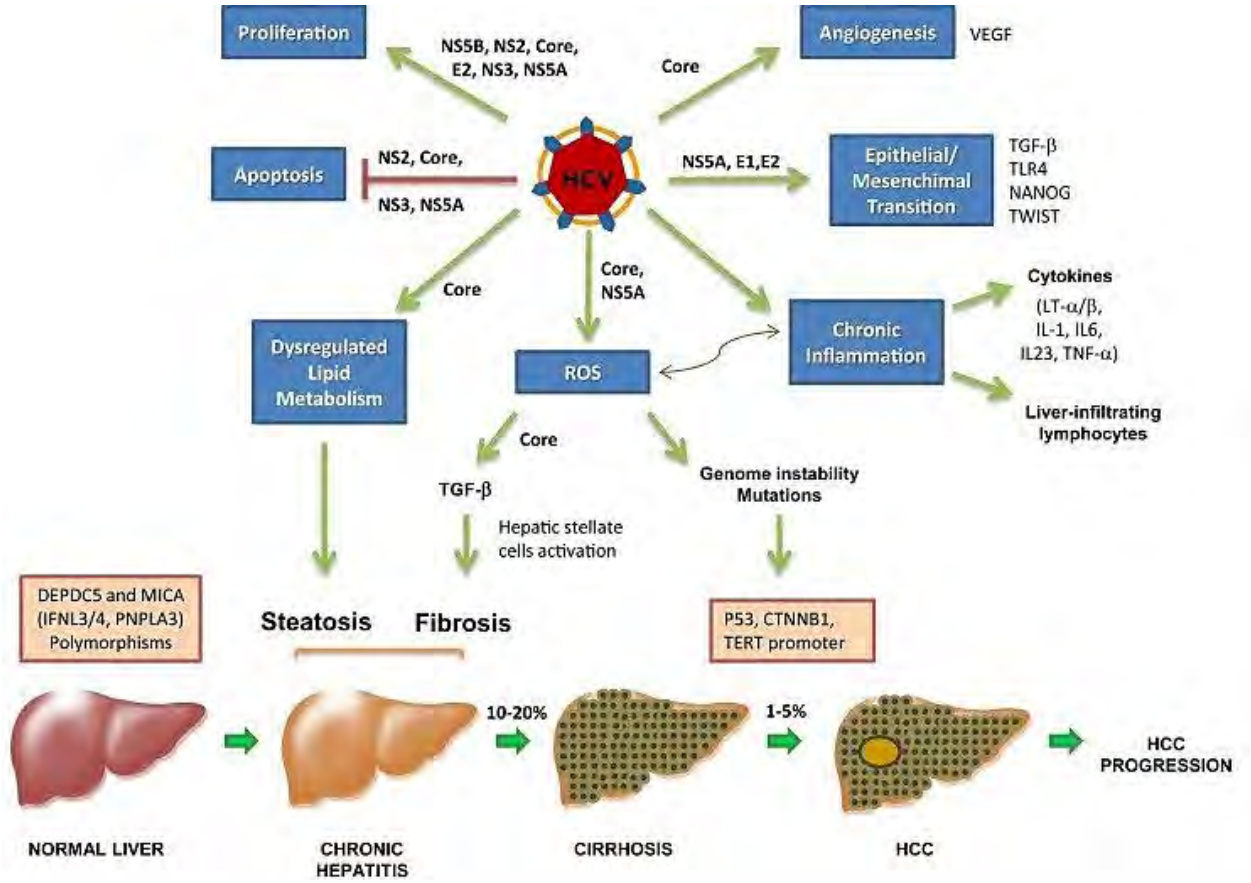

Figure 1.4 Mechanism of progression of HCV infection to HCC

Chronic HCV and associated liver cirrhosis are significant risk factors for developing HCC through the multistep process known as hepatocarcinogenesis. This process involves genetic alterations, chronic inflammation, oxidative stress, dysregulation of lipid metabolism, and activation of angiogenic and metastatic pathways. Collectively, these factors contribute to the malignant transformation of liver cells and the progression toward HCC (Vescovo et al., 2016).

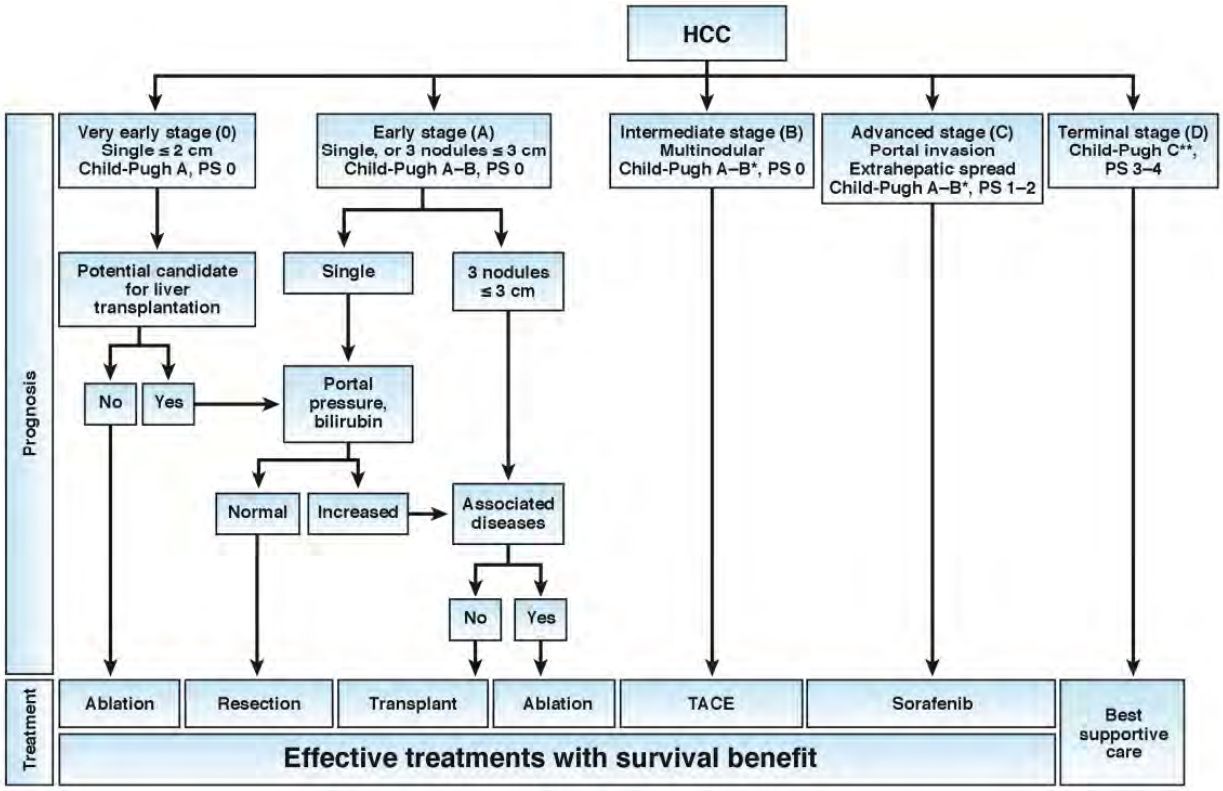

Figure 1.5 BCLC staging system

The Barcelona-Clinic Liver-Cancer (BCLC) Staging System considers both the overall state of health and fitness, or performance status (PS), and the number and size of liver tumours. HCC: hepatocellular carcinoma; PS: performance status; TACE: transarterial chemoembolization adapted from (Bruix et al., 2016).

sensitive imaging technique than ultrasound and can detect smaller lesions. A CT scan can also provide information about the blood vessels in the liver. A strong magnet and radio waves are used in MRI to provide detailed pictures of the liver. It is more sensitive than a CT scan and can detect small lesions in the liver. MRI can also provide information about the blood vessels in the liver (Bartolozzi et al., 2009).

Patients with HCC frequently have high levels of AFP, a protein the liver produces. AFP is a blood test that can be used as a screening tool for HCC. However, AFP levels can also be elevated in patients with other liver diseases, so it is not a reliable diagnostic tool on its own. Imaging biomarkers are specific imaging features that can be used to identify HCC. These include arterial phase hyperenhancement, washout, and capsule appearance. These features can be identified on CT or MRI and can help to differentiate HCC from other liver lesions (Mavilia et al., 2018).

A tiny piece of liver tissue is taken and examined under a microscope during a biopsy. The biopsy is the most reliable method of identifying HCC. However, it is an invasive procedure with some risk of bleeding and infection. HCC screening in patients with cirrhosis is crucial for early detection and improved outcomes. Current guidelines recommend ultrasound surveillance every six months in patients with cirrhosis, but this approach has limitations, including operator dependence, low sensitivity, and high false-positive rates (Marrero et al., 2018). Alternative methods, such as serum biomarkers and imaging modalities, have been investigated, but their sensitivity and specificity remain suboptimal (Choi et al., 2019).

1.7 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is an effective non-invasive tissue diagnosis tool. In recent years a great deal of attention has been gathered by FTIR biological studies, especially clinical investigations related to cancer and malignancy detection. Several research papers have been published on the diagnostic importance of FTIR (Talari et al., 2017).

1.7.1 Basic Working Principle

FTIR is a technique that is used to acquire absorption or emission of solid, liquid, or gas in the infrared region. The spectrometer simultaneously collects spectral resolution

data over a wide spectral range. This is comparatively advantageous over the dispersive spectrometer that measures intensity over a narrow range. Fourier transform is a mathematical procedure that is used to convert raw fat into spectral form (Bombardi, 2017; Vieira et al., 2018).

The basic aim of every absorption spectrometer is to check how well a sample absorbs light at any given wavelength. The most simplistic approach is to shine a beam of monochromatic light on a sample and measure the amount of light absorbed. FTIR is basically the same procedure with little alteration. Instead of shining one particular wavelength at a time, the spectrometer shines a broadband/polychromatic light (beam containing multiple wavelengths) source and measures the corresponding absorption by the samples (Movasaghi et al., 2008).

The source shines the light onto the Michelson interferometer (configuration of mirrors moved by motor), which blocks each light source periodically by the phenomenon of wave interference generating a second data point. This procedure is repeated several times to obtain the raw data, known as an interferogram. In the end, the computer takes all the data, which then works backward to infer absorption at each wavelength. The raw data is converted to a spectrum by a mathematical procedure known as Fourier transform (Morota et al., 2009; Movasaghi et al., 2008).

1.7.2 Biomedical Applications of FTIR Spectroscopy

There has been enormous progress in the vibrational spectroscopic analysis of biological samples. It has a major advantage that only one tissue section is sufficient to obtain the biochemical information. In comparison, the use of standard histologic techniques requires vigorous preparation of the individual tissue sections for staining each component.

FTIR spectroscopic imaging is indeed a powerful tool for studying biological samples. It is rapidly obtaining popularity in advanced clinical research. The technique is useful in widely understanding its application of it, from quality control of many compounds to characterization and biomedicine. Organic materials, for instance, lipids, nucleic acids, carbohydrates, and proteins, have unique and distinct structures that make it

possible to get significant spectral fingerprints that correspond to their respective functional groups. FTIR analysis has been done to a wide range of biological samples; these studies include gastrointestinal tissue, lymphocytes (childhood leukemia), oral tissues, cervix, breast, lung, skin, lymphoid tissue, fibroblasts, prostate, bacteria, DNA, tissue preservation, cancer detection, tissue processing, cytotoxicity and heating, gallstones, bone and glucose measurement (Addison, 2017; Movasaghi et al., 2008).

FTIR spectroscopic techniques produce an instant appeal in medicine and biology due to their quick and non-invasive nature. That enables simple characterization of cellular components on the basis of their chemical composition and intrinsic properties. FTIR provides a significant route for screening diagnostic markers for diseases such as cancer. It has been considered a reliable and useful tool for analyzing the chemical composition of biological materials such as human calculi. Further, stone recurrence in repeated stone formation can also be prevented by analyzing the samples using FTIR (Ntovas et al., 2018). The high sensitivity of FTIR to the detection of alterations in functional groups of tissue components (such as nucleic acids, lipids, and proteins) makes it a potentially reliable technique in the fields of biology and medicine (Kotoulas et al., 2018).

The Infrared spectra of these tissues can obtain information on the molecular structure of human and animal tissues. It has also been widely used for the determination of biochemical metabolites present in biological fluids. Diagnosis of different cancers, including lung, colon, cervical, skin, and breast malignancies, are already published in the literature (Guleken et al., 2021; Mostaco-Guidolin et al., 2009; Sitnikova et al., 2020; Wood et al., 1996; Yang et al., 2021).

Variations in the level of biomolecules, such as DNA, RNA, carbohydrate, and phosphates, can be detected by analysis of the spectra. Changes in RNA/DNA ratios measured at 1121/1020 cm^{-1} usually show a lower ratio for non-malignant tissues in comparison to their malignant counterparts. Variations in the infrared spectra correspond to changes in sugars and bases and redistribution of the Hydrogen bonds in the genetic

material. The spectra also detect any loss or changes in the covalent bonds caused by damage in the structure of nucleic acids at primary, secondary, and tertiary levels.

FTIR spectroscopy has also been used to identify specific drug-targeting molecular pathways that cause myeloid leukemia. Drug compounds are being tested in ex vivo cancerous cells through chemometric data analysis. Several computational methods have been incorporated into the system to enhance the sensitivity of the overall setup making it a suitable sensitive technique for automation to fulfill the required demand of the scientific community (Morota et al., 2009).

FTIR can provide the details on molecular structure and composition on the level of a single cell within a few seconds to perform quantitative and qualitative multiple component analysis. It helps in the objective classification and recognition of samples in an automated pattern with label-free and minimal sample treatment. The improvements related to this technique and its potential are progressively increasing in clinical diagnosis and cancer research. It can be assumed that future preclinical and clinical trials will be of sample evolution through FTIR in order to generate the necessary data to approve the use of FTIR spectroscopy in clinical applications, particularly cancer research, and diagnosis (Movasaghi et al., 2008).

1.8 ATR-FTIR spectroscopy

ATR-FTIR spectroscopy is a novel technique that has shown potential in the early detection of various cancers, including breast, colon, and lung cancer (Sala et al., 2020). This technique analyzes the molecular composition of tissue samples using infrared radiation and provides a fingerprint-like spectrum that can be used to distinguish between normal and abnormal tissues (Talari et al., 2017). A few studies have investigated the use of ATR-FTIR spectroscopy in HCC diagnosis, but its potential as a screening tool for HCC in HCV cirrhotic patients with SVR has not been explored.

1.8.1 Pre-processing

Spectra obtained are composed of analytic information and noise. Noise can be due to chemical interference, leading to band superposition and additive effects. Baseline deviations may also arise from physical interference. Moreover, random noise from the

environment is present in spectra. Pre-processing is applied before data analysis to improve and correct the signal-to-noise ratio (Santos et al., 2017).

In the first-order spectra, baseline deviation occurs. Every sample has certain unidentified wavenumbers; hence, absorbance must have zero value. Mie scattering typically causes spectra to be elevated above zero. These effects can be reduced by standard normal variate (SNV), derivative analysis techniques, and multiplicative scatter correction (MSC) (Bassan et al., 2010; Hibbert, 2016; Kelly et al., 2011; Salman et al., 2014). Sometimes only a small spectra region is used to build a chemometric model. Biological fingerprinting depends on the technique being used (Kelly et al., 2011; Trevisan et al., 2012).

1.9 Multivariate Analysis

These analyses are used to examine the data having more than one variable. A few of the widely used multivariate algorithms in bio-spectroscopy are discussed below.

1.9.1 Principal Component Analysis (PCA)

A popular technique for unsupervised data analysis in biological investigations is PCA. It is used to lower data dimensionality while retaining useful information. The original variables are linearly transformed, and orthogonal variables are generated. These orthogonal variables are known as principal components (PC). PC1 explains the biggest variance in the data, and PC2 explains the next biggest difference. This helps choose the smallest number of PCs that explain the largest variation. Therefore, we can say that PCA helps visualize datasets in compact size where class separation can be exposed (Fogarty et al., 2014; Geladi & Kowalski, 1986; Martin et al., 2010).

When biological samples are separated into classes, PCA is executed to identify clusters and main contributors. The cluster vector approach is used in combination with PCA to analyze these samples. A median is calculated for every PC, and loading vectors are weighted against the median scores. Therefore, a loading vector is generated in the form of an effective loading plot. The disadvantage associated with PCA is that there is a risk of artificial discrimination as the selection of optimal PC is a difficult process (Bro & Smilde, 2014; Martin et al., 2010).

1.9.2 Linear Discriminant Analysis (LDA)

This is a supervised technique that is used to discriminate class. This enhances the inter-class differences over intra-class variation so that a linear decision boundary is made. The performance of LDA is enhanced when combined with other classification algorithms like PCA or partial least square regression (PLSR). For using PCA-LDA, a cluster vector is obtained by ambiguous problems that provide more reliable class differences (Kelly et al., 2011; Lee-Montiel et al., 2011; Petisco et al., 2011; Wang et al., 2014).

1.9.3 Performance Evaluation

Sensitivity is the measure of a sample's positive confidence outcome for a class that has been assigned a label. Confidence that a sample's negative result in an unlabeled class has been achieved is known as specificity. The positive likelihood ratio describes the chance of calculating samples as positive when it is not positive. The ratio between the chance of predicting a sample to be negative when it is really positive and the probability of predicting a sample to be negative when it is actually negative is known as the negative likelihood ratio (Siqueira & Lima, 2016). Mathematical formulas are given below:

$$\text{Sensitivity (\%)} = (TP/ (TP + FN)) \times 100$$

$$\text{Specificity (\%)} = (TN/ (TN + FP)) \times 100$$

1.7 Aim and objectives

- To use ATR-FTIR to identify the major biochemical markers expressed differentially in blood sera of cirrhotic SVR, HCC SVR, and healthy individuals.
- To differentiate between healthy and diseased sera samples through chemometric analysis.
- To establish ATR-FTIR as an alternative diagnostic tool.

CHAPTER 2

MATERIALS AND METHODS

This section describes the materials, instruments, and techniques/software used in the research study. It provides information on the study population, sample collection, ATR-FTIR spectral acquisition, and chemometric data analysis. A syringe, tourniquet, vacutainer, Eppendorf, centrifuge, Freeze-dryer, ethanol, tissue papers, and ATR-FTIR were used in the study. Figure 2.1 shows the methodology used in this study. A detailed description of the method utilized in the study is presented below:

2.1 Study population

This study included 57 individuals, of which 21 were healthy, 16 were cirrhotic SVR, and 20 were HCC SVR diagnosed with chronic hepatitis C and underwent antiviral therapy with DAAs. All participants achieved SVR after treatment, defined as undetectable HCV RNA 12 weeks after the end of therapy. Individuals were recruited from the Pakistan Kidney and Liver Institute and Research Center (PKLI & RC). Informed consent was obtained from all individuals before enrollment in the study.

Enzyme-Linked Immunosorbent Assay (ELISA) determined that healthy people tested negative for HCV, HBV, and HIV. Patients were diagnosed by serum makers (i.e., AFP), molecular techniques, imaging techniques, and physical conditions.

2.2 Sample Collection

Whole blood samples were collected using vacutainers designed for serum separation from the study population. After collection, the blood was left undisturbed in the vacutainers for 30 minutes. Subsequently, the vacutainers were centrifuged at 1500 RCF to isolate the serum. The protocol was conducted on ice throughout the process, and the resulting serum was stored at -20˚C (Ali et al., 2019; Naseer et al., 2019). All samples were processed while adhering to the standard biosafety protocols. Prior to the ATR-FTIR analysis, the serum samples were freeze-dried to remove all the moisture content utilizing a method optimized by Ali et al. (2019) before spectral acquisition.

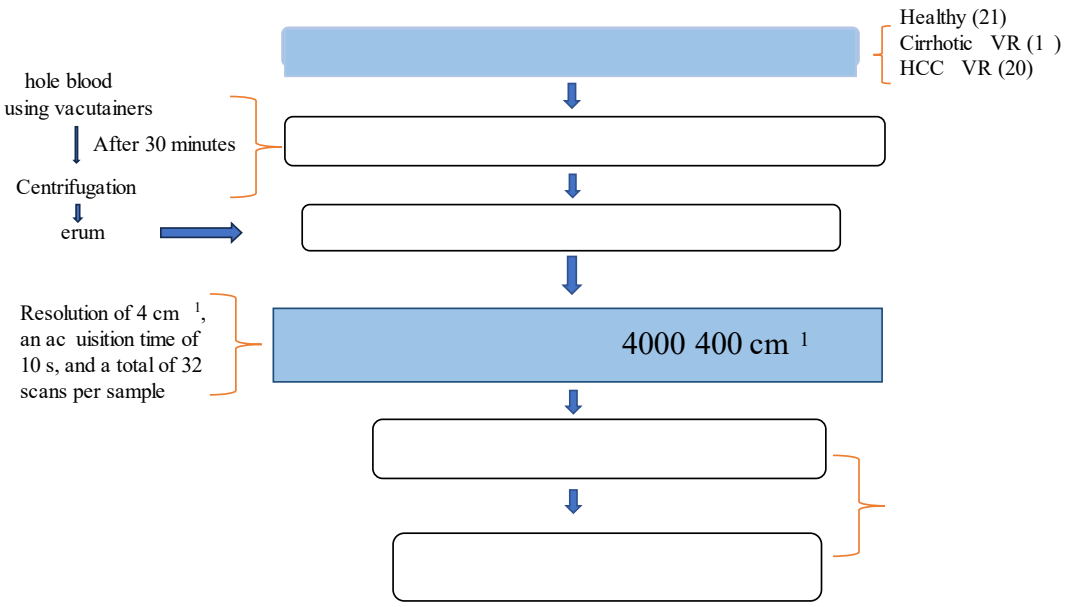

Figure 2.1 Schematic illustration of the methodology used

The freeze-dried sera from 57 individuals categorized into healthy, cirrhotic SVR, and HCC SVR were processed through ATR-FTIR for spectral acquisition and later for chemometric data analysis.

2.3 ATR-FTIR spectral acquisition

The freeze-dried samples were processed using the ATR-FTIR Bruker alpha (Bruker, USA) at Lahore University of Management Sciences (LUMS). The air background at a temperature of 20˚C was subtracted prior to analysis. A small quantity of freeze-dried sera (approximately 50 mg) was then applied to the ATR crystal. Spectra have been acquired within the infrared (IR) region spanning from 4000 to 400 cm^{-1}, employing a resolution of 4 cm^{-1}, an acquisition time of 10 s, and a total of 32 scans per sample (Ali et al., 2019; Naseer et al., 2019).

2.4 Chemometric data analysis

Utilizing Unscrambler X version 10.4 (CAMO Software AS, Oslo, Norway), chemometric data analysis was carried out (figure 2.2). Baseline correction, smoothing, and normalization were crucial pre-processing processes that were used to reduce the impact of scattering effects and background noise. The whole ATR-FTIR spectrum covering the 4000-510 cm^{-1} wavenumber range was captured. Both the lipid area (3500-2800 cm^{-1}) and the bio fingerprint region (1800-900 cm^{-1}) showed significant differences. As a result, these particular areas were chosen for the subsequent multivariate study.

PCA-LDA, a multivariate data analysis method, was used to improve class separation. LDA, a supervised classification technique, was employed to increase inter-class variability and utilize improved sample categorization after PCA, an unsupervised multivariate statistical tool, was initially used to reduce dimensionality. To improve diagnostic accuracy and obtain better categorization, the supervised method PCA-LDA was applied.

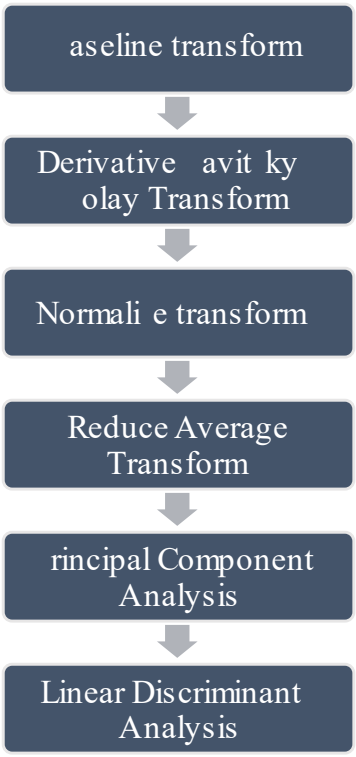

Figure 2.2 Pre-processing and multivariate data analysis

Pre-processing was performed using baseline correction, Savitzky-Golay filtering, and mean normalization. After pre-processing, multivariate data analysis, PCA and LDA, was conducted on the reduced data.

CHAPTER 3

RESULTS

This chapter describes the application of ATR-FTIR spectroscopy and subsequent analysis techniques for the characterization and classification of healthy individuals, cirrhotic SVR, and HCC SVR patients.

3.1 ATR-FTIR spectral analysis

The increased demand for non-invasive diagnostics has significantly contributed to the advancement and widespread adoption of ATR-FTIR spectroscopy (Ali et al., 2019, 2021, 2022; Huang et al., 2003). A total of 32 spectra were recorded for each sample of the three classes of sera samples from healthy, and individuals with HCC SVR and cirrhotic SVR, and in this way for 57 samples 1824 spectral records were recorded, baseline correction was performed and line plot was created to show the results of baseline correction (Figure 3.1).

Figure 3.2 illustrates the average spectrum of 57 samples, wherein the red color signifies the average spectrum of sera samples from individuals with HCC and SVR, the blue color denotes the average spectrum of cirrhotic SVR sera samples, and the green color represents the average spectrum of healthy sera samples.

The analysis of these spectra revealed discernible variations in metabolic intensity within two specific regions: the lipid region spanning 3500–2800 cm^{-1} and the bio fingerprint region ranging from 1800 to 900 cm^{-1}. These variations served as indicative markers of biochemical and metabolic intensity variations among the three sample types.

Baseline transformation was performed using the method of baseline offset. Savitzky-Golay smoothing, specifically a 15-smoothing point, 2nd order polynomial order, was applied. Subsequently, the second derivative of the smoothed spectra was calculated. The resulting spectra were then averaged (as shown in Figure 3.3). Finally, mean normalization was applied to the averaged spectra that normalized the features of the data.

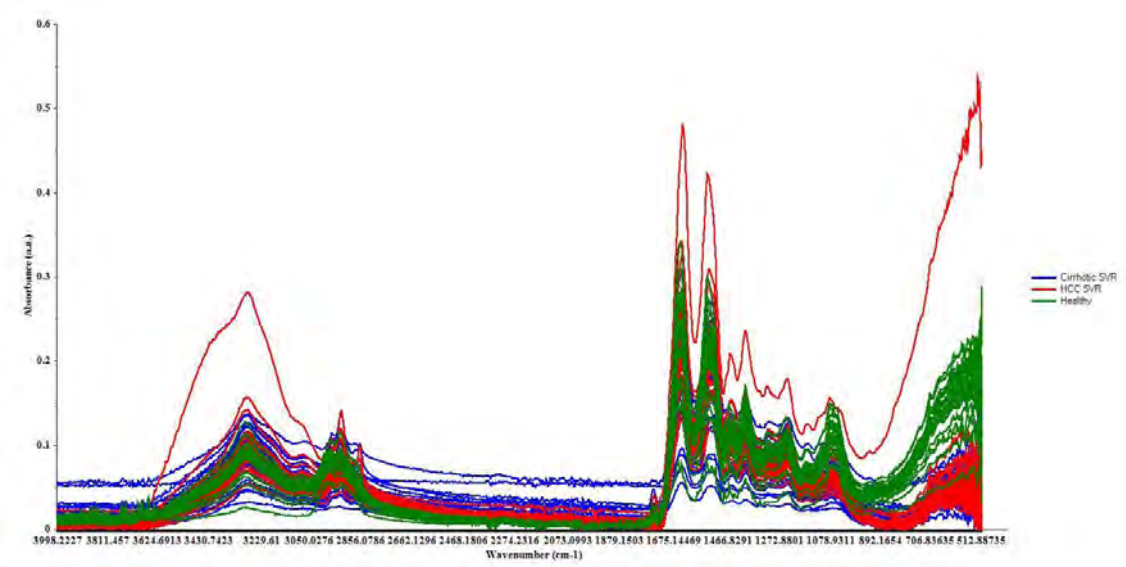

Figure 3.1 Baseline corrected spectra of freeze-dried sera samples

1824 spectra (32 per sample) were recorded for the three classes of sera samples from cirrhotic SVR (blue), HCC SVR (red), and healthy individuals (green), and the results of baseline correction are shown in the line plot.

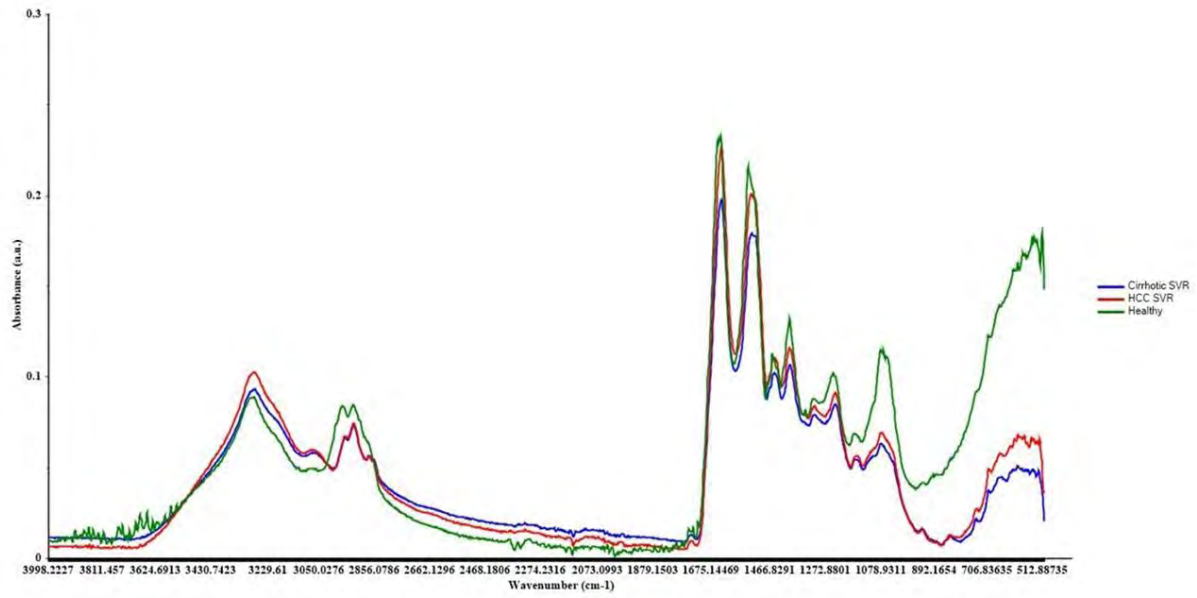

Figure 3.2 Average ATR-FTIR spectra of freeze-dried sera samples

The average (reduced) spectrum of 57 samples for each class; cirrhotic SVR (blue), HCC SVR (red), and healthy individuals (green) was performed and the results are shown in a line plot with clear variations in the spectral peaks of these samples based on the absorbance.

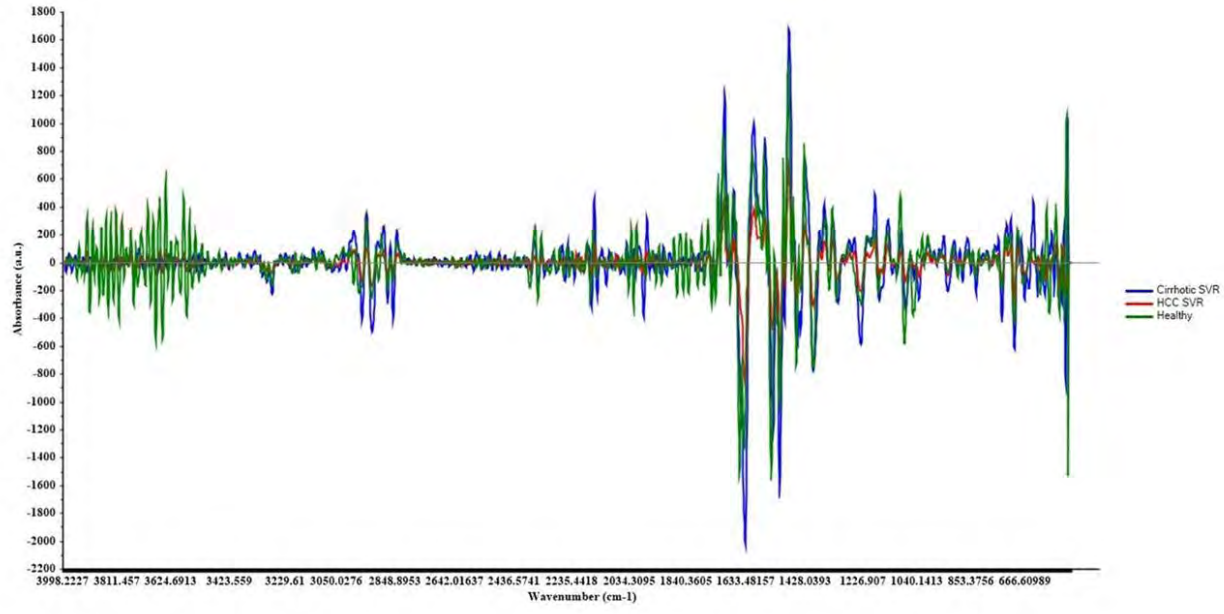

Figure 3.3 Average line plot of samples after Savitzky-Golay smoothing

The second derivative Savitzky-Golay smoothing (15 smoothing points, two polynomial order) was performed to visualize the average trend of cirrhotic SVR (blue), HCC SVR (red), and healthy individuals (green).

3.2 Principal component analysis (PCA)

PCA was utilized to analyze the vector-normalized second derivative spectra of cirrhotic SVR, HCC SVR, and healthy individuals. A spectrum represented each individual, and a total of 32 spectra were replicated. The purpose was to identify general trends in the spectral data. Figure 3.4 illustrates the results of the PCA-based classification, where blue dots denote cirrhotic SVR samples, HCC SVR samples by red dots, and healthy individuals by green dots. It was observed that the first two principal components (PCs) yielded the most significant classification outcomes. The PCA model accounted for 75% of the variation between these datasets when using only 2 PCs. In the PCA scatter plot, PC1 explained 61% of the variation, while PC2 explained 14% of the variation within the dataset.

3.3 Linear discriminant analysis (LDA)

The PCA-LDA model was developed to further classify the study population into cirrhotic SVR, HCC SVR, and healthy individuals. Figure 3.5 shows that cirrhotic SVR, HCC SVR, and healthy samples were classified using 6 PC components for the development of the PCA-LDA model, and 89.47% classification accuracy was obtained.

Table 3.1 shows the confusion matrix generated using 2 PCs. It can be seen that ten individuals were correctly classified as Cirrhotic SVR (true positives or TP). None of the Cirrhotic SVR individuals were incorrectly classified as HCC SVR or Healthy (false negatives or FN). Four individuals were incorrectly classified as Cirrhotic SVR (False positive or FP). Twenty individuals were correctly classified as HCC SVR (TP). None of the HCC SVR individuals were incorrectly classified as Healthy (FN).

Two individuals were incorrectly classified as Cirrhotic SVR (FP). None of the healthy individuals were incorrectly classified as HCC SVR (FN). Twenty-one individuals were correctly classified as Healthy (TP). The overall sensitivity and specificity were calculated:

$$\text{Sensitivity} = (TP / (TP + FN)) \times 100$$

$$\text{Specificity} = (TN / (TN + FP)) \times 100$$

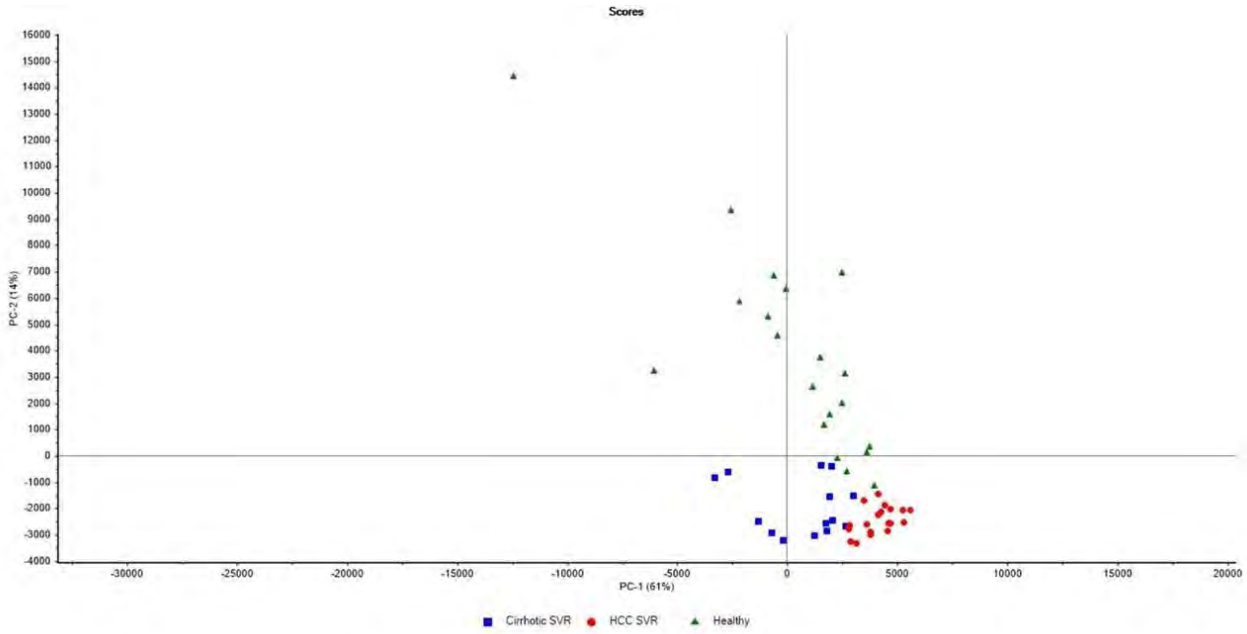

Figure 3.4 PCA analysis score plot of freeze-dried sera samples

PCA score plot of cirrhotic SVR (blue), HCC SVR (red), and healthy individuals (green) freeze-dried sera samples showing 75% variation. PC1 contributes 61% variation, while PC2 represents 14% variation in the dataset.

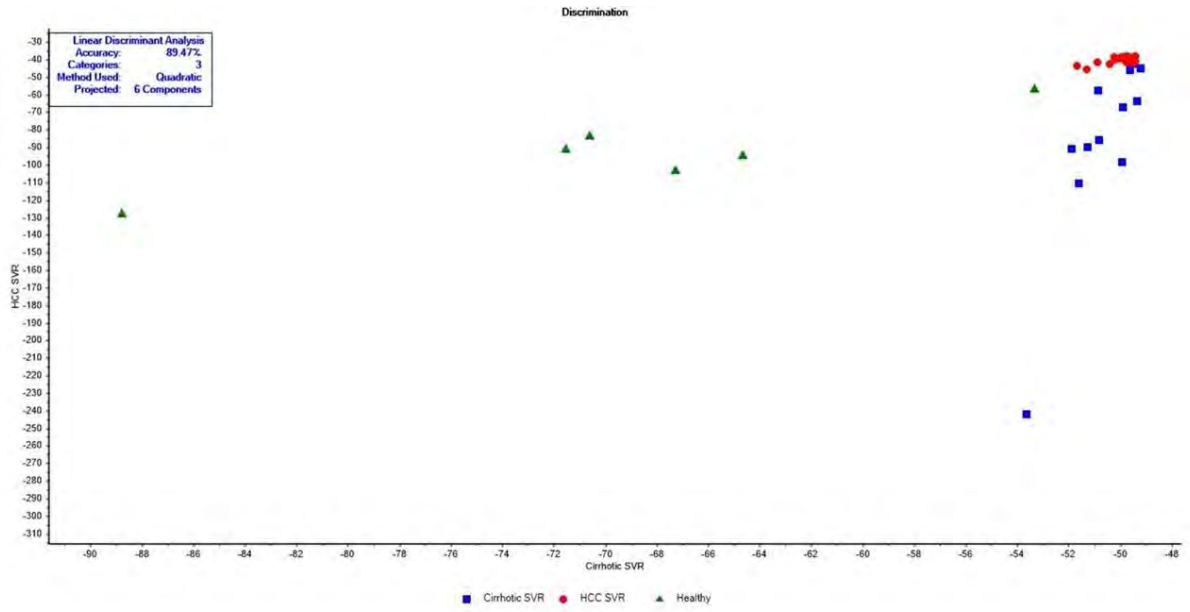

Figure 3.5 PCA-LDA-based classification of data

The PCA-LDA shows data classification based on cirrhotic SVR (blue), HCC SVR (red), and healthy individuals (green). This PCA-LDA model was generated by projecting 6 PC components. The LDA classifier reports 89.47% discrimination accuracy between the three sample types.

Applying these formulas to the confusion matrix:

$$\text{Sensitivity} = (10 / (10 + 0)) \times 100 = 100\%$$

$$\text{Specificity} = (21 / (21 + 2)) \times 100 = 91.3\%$$

Therefore, the overall sensitivity of the PCA-LDA model is 100%, and the overall specificity is approximately 91.3%.

Table 3.1 Confusion Matrix of PCA-LDA of healthy and diseased individuals

Actual Class	Predicted Class		
	Cirrhotic SVR	**HCC SVR**	**Healthy**
Cirrhotic SVR	10	0	0
HCC SVR	4	20	0
Healthy	2	0	21

Table 3.2 Statistical Values of Sensitivity and Specificity

Results obtained from © 2018 MedCalc Software bvba

Statistic	Formula	Value
Sensitivity (%)	(TP/ (TP + FN)) × 100	100%
Specificity (%)	(TN/ (TN + FP)) × 100	91.3%

CHAPTER 4

DISCUSSION

HCC is a serious complication that can arise from HCV infection, with a significant proportion of HCC cases, approximately 70-90%, being linked to HCV. In 2017, the World Health Organization (WHO) issued recommendations regarding the evaluation of SVR following DAA treatment for HCV infection. According to the WHO guidelines, SVR should be assessed 12 or 24 weeks after the administration of DAA treatment using a NAT capable of detecting HCV RNA (WHO guidelines, 2018). The purpose of the SVR assessment is twofold: to confirm the absence of HCV replication and to evaluate the long-term effectiveness of the treatment. Achieving SVR indicates a high likelihood of SVR for the patient, significantly reducing the risk of HCC development and other complications associated with HCV infection.

It is important to note that regular monitoring for HCC is still recommended for individuals with a history of HCV infection, even after achieving SVR. This is because the risk of developing HCC persists, although it is significantly reduced after successful HCV treatment. Common surveillance methods such as liver imaging techniques (ultrasound, magnetic resonance imaging, or computed tomography scan) and AFP tumor marker test are typically employed to detect early signs of HCC in high-risk individuals. HCC surveillance is recommended every six months (WHO guidelines, 2018).

However, these surveillance methods can be challenging and costly for patients, requiring multiple tests and visits and imposing psychological pressure. Therefore, there is a need for a non-invasive, cost-effective, and time-efficient technique to improve HCC screening. Over the past few decades, optical diagnostic methods such as Raman and ATR-FTIR spectroscopy have gained significant popularity and garnered attention from the scientific community. These techniques have been extensively studied to assess their potential in diagnosing pathogenic infections through the analysis of biofluids (Bilal et al., 2016; Khan et al., 2016; T. Mahmood et al., 2018; Nawaz et al., 2017; Roy et al.,

2019; Saleem et al., 2013; Silva et al., 2020). Recently, ATR-FTIR has been used to discriminate HCV-infected sera samples from healthy sera samples and to further discriminate sera of cirrhotic individuals from those of non-cirrhotic individuals (Ali et al., 2022, 2023).

This study aimed to utilize ATR-FTIR spectroscopy to screen for HCC in patients with SVR by demonstrating the spectral signature of HCV-related HCC. Additionally, the study aimed to employ chemometric analysis to distinguish healthy samples from cirrhotic SVR and HCC SVR samples. The developed models, particularly the PCA-LDA model, exhibited superior performance with a discrimination accuracy of 89.47%.

The ATR-FTIR spectra obtained in this study revealed significant variations in metabolic intensity within the lipid region (3500-2800 cm^{-1}) and the bio fingerprint region (1800-900 cm^{-1}). These variations served as indicative markers of metabolic intensity variation among the three sample types, suggesting changes in the structure and content of lipids, proteins, and nucleic acids.

PCA was employed to analyze the ATR-FTIR spectra and identify general trends in the spectral data. The PCA-based classification successfully discriminated between cirrhotic SVR, HCC SVR, and healthy individuals. The first two principal components (PC1 and PC2) accounted for 75% of the variation within the datasets, with PC1 contributing 61% and PC2 contributing 14% of the variation (Figure 3.4). These findings suggest that the ATR-FTIR spectra contain valuable information for distinguishing the different sample types.

The LDA model was developed based on the PCA results to improve the classification accuracy further. The PCA-LDA model effectively classified the study population into cirrhotic SVR, HCC SVR, and healthy individuals, achieving a classification accuracy of 89.47% using six principal components (Figure 3.5).

The findings of this study have significant implications for non-invasive diagnostics in liver diseases. As a non-invasive method for analyzing serum samples, ATR-FTIR spectroscopy shows promise in the early detection, monitoring, and characterization of liver diseases such as cirrhosis and HCC. The ability to differentiate

between cirrhotic SVR, HCC SVR, and healthy individuals based on metabolic variations holds potential clinical applications in disease management, treatment response assessment, and prognosis. Furthermore, the optimized algorithms utilized in ATR-FTIR spectroscopy and HCV-related HCC diagnosis demonstrate promising potential. This technique offers the advantages of being cost-effective and non-invasive, making it suitable for repeated clinical findings or for monitoring HCV patients at high risk of developing HCC.

It is crucial to recognize some of this study's shortcomings, though. First off, this study was conducted as a pilot phase with a relatively small sample size of 57 individuals. The generalizability of the findings would be strengthened by increasing the sample size and integrating more varied groups. The study also concentrated on a particular demographic from a single research center, which may have introduced bias. Future studies should consider including larger and more diverse cohorts from multiple centers to validate the findings and enhance the robustness of the results.

CHAPTER 5

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