Molecular characterization of blood-circulating protein biomarkers for non-invasive and rapid diagnosis of Hepatocellular Carcinoma (HCC)

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MS Industrial Biotechnology

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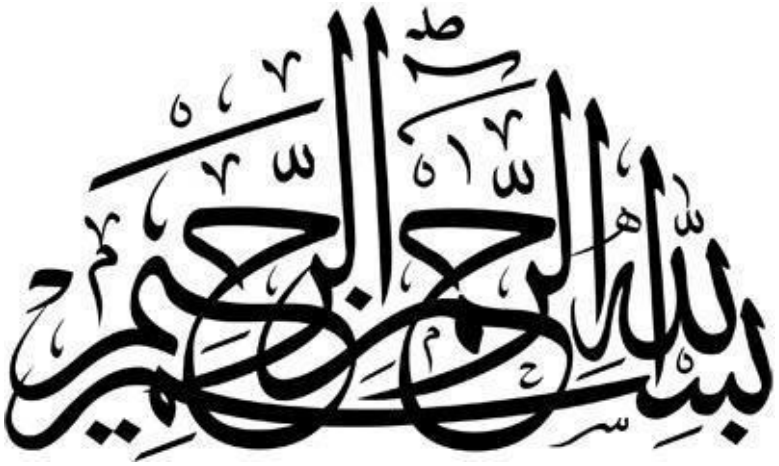

IN THE NAME OF ALLAH, THE MOST BENEFICENT

THE MOST MERCIFUL

Read! And thy Lord is Most Honourable and Most Benevolent,

Who taught (to write) by pen, He taught man that which he knew not

(Surah Al-Alaq 30: 3-5)

Al-Quran

FORM TH-4

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DECLARATION

I, **Arslan Ali**, declare that this research work titled **"Molecular characterization of blood-circulating protein biomarkers for non-invasive and rapid diagnosis of HCC"** is my own work. The research work here in was carried out while I was a post-graduate student at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of **Dr. Hussnain A. Janjua**. The work has not been presented for assessment elsewhere. The materials which have been used from other sources, have been properly acknowledged / referred.

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DEDICATION

I dedicate this thesis to my parents,

Mr. & Mrs. Shafqat Ali Ranjha,

who led me to believe that everything was possible
and supported me throughout the way

Acknowledgment

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“My success is only by ALLAH” Qur’an [11:88]

Arslan Ali

Table of contents

List of Figures

List of Figures

List of Tables

ABSTRACT

Major burden of cancer is attributed to Hepatocellular carcinoma. Third world countries are more prone to HCC because they possess poor healthcare systems. Annually around 7.6 persons per 100,000 suffer from HCC in Pakistan. Hepatitis C is a major contributor towards HCC in Pakistan contributing 60-70% of HCC incidences. Lack of proper healthcare systems in the poor countries lead to late diagnosis of HCC because the imaging techniques employed to diagnose HCC i-e MRI, Ultrasound and histopathology require expensive healthcare systems. Various studies have been focused on developing biomarkers for HCC to detect HCC at an early stage. Circulating proteins can easily be detected in the body fluids, therefore offer excellent potential for the development of biomarker for the disease. For easy, and early detection of HCC, the need of the hour is to develop blood-based serum biomarkers. Alpha Fetoprotein (AFP) possess less clinical efficacy because it is used in conjunction with imaging techniques. Multiple circulating proteins are used in various research to check their potential to act as efficient biomarker candidates for HCC detection previously in our lab. These findings are in continuation of our previous studies by (Awan et al., 2015) in which bioinformatics pipeline was established. Subsequently, the blood samples were collected from HCC patients and ELISA was performed on blood serum of C8A, SERPINC1, HSD11B1 and MBL2 along with Western blot of C8A (Abdullah, MS Thesis 2021). Mr. Abdullah Ahmed performed ELISA of the first four proteins namely C8A, SERPINC1, HSD11B1 and MBL2. Among them C8A showed excellent ability to act as a potential biomarker for HCC having sensitivity and specificity values far better than AFP while other proteins did not show significant potential to act as biomarker for HCC. He then qualitatively validated the positive results of C8A through Western Blot.

In the current study, ELISA of the remaining three proteins along with Western Blot analysis and Multiplex ELISA is performed. Proteins namely Cyp2a6, UPB1 and ADH6 were checked in the serum of 150 HCC patients and 50 control samples via quantitative ELISA. Among these, ADH6 poses decent biomarker potential with 70.67% sensitivity and 64% specificity values and Cyp2a6 showed 64% sensitivity and 54% specificity whereas UPB1 did not show significant biomarker potential. These results were then confirmed via quantitatively and qualitatively through Western blot of selected serum samples of all three proteins i-e Cyp2a6, UPB1 and ADH6. Qualitative validation was performed to determine the antibody's specificity to that particular protein while

quantitative validation was performed by setting the first band in the gel as a reference band and then finding the relative intensity of all the remaining bands in comparison to the reference band by using ImageLab software. Multiplex ELISA was performed to check whether the combination of two proteins yielded better results. The best performing biomarker ADH6 was combined with AFP (the current gold standard biomarker for diagnosing HCC). The combination yielded better sensitivity (88.68%) and specificity (80%). Conclusively, the combination of two biomarkers ADH6 and AFP showed excellent potential to serve as a circulating blood-based protein biomarker panels for non-invasive and rapid detection of HCC.

Chapter 1 Introduction

Approximately 2 million causalities occur every year around the globe owing to increased mortality of liver diseases (Paik *et al.,* 2020). About 1 million of these deaths are caused collectively by Liver cirrhosis is responsible for half of these deaths and other half include Hepatocellular carcinoma and other viral diseases (Paik *et al.,* 2020). Since liver diseases greatly interfere with body's normal function such as drug metabolism, fat metabolism and maintenance of homeostasis, any disturbance in these vital functions can prove to be fatal for human body.

HCC (Hepatocellular carcinoma) is one of the deadliest form of cancers in Pakistan, it is the fourth most common malignancy affecting men and seventh common malignancy affecting women (Adnan *et al.,* 2020). The death rate of HCC is increasing 2-3% annually because of the late diagnosis which usually leaves the patient with limited treatment options (Wang and Wei, 2020) The only potential treatment options available for HCC are liver transplant, surgical resection, and tumor ablation (Lurje *et al.,* 2019). HCC reoccurs in almost two-third of patients which are treated with surgical resection that ultimately would limit the survival of the patients (Kim *et al.*, 2020). HCC is also associated with hepatitis B and C which are responsible for inflammation and cirrhosis leading to HCC (Zamor and Russo, 2017). HCC is a lethal form of cancer around the globe and second major cause of deaths in Asia and Africa and sixth major cause in western countries (Rawla *et al.,* 2018). Adults are mostly exposed to HCC making it most common cause of cancer among them as hepatitis C & B viruses can be transmitted from one person to another through sharing contaminated needles, unprotected intercourse, or childbirth. also the viruses may pass through blood transfusion as well (Bhatti *et al.*, 2016). In Pakistan 7.6 persons per 100,000 persons per year suffer due to HCC and this percentage falls significantly in females only i-e 2.8 persons per 100,000 persons (Bhatti *et al.*, 2016). Social factors like less women on the streets, less alcohol consumption and smoking habits in women may be related to this less HCC incidence in Pakistan (Adnan *et al.,* 2020). It is expected that sixty to seventy percent (60–70%) HCC in Pakistan is because of hepatitis C by Bhatti et al (2016). Hepatitis C patients with high HCC progression rate leads to a high rate of mortality in Pakistan, for which early diagnosis is the only option to limit HCC prevalence in Pakistan.

Introduction

Early diagnosis leads to early treatment of disease and survival rate of cancer patients is increased significantly (Z.-M. Zhang *et al.*, 2020). Early diagnosis is possible only if the means for detection of cancer is convenient, easy, and cheap. For this purpose, blood-based biomarkers possess significant potential as they are easy to detect, patient undergoes non-invasive procedure for the detection of biomarkers and techniques are relatively cheap to detect biomarkers. Hepatocellular carcinoma is currently diagnosed via histopathology and expensive imaging techniques like MRI and Ultrasound (Violi *et al.*, 2021). Blood- based novel biomarkers are the need of time that have higher sensitivity and specificity to serve the purpose of early diagnostics in case of HCC. To effectively combat HCC, it is vital to develop non-invasive blood-based biomarkers for early, easy, non-invasive, and cheap diagnosis of HCC.

During Hepatocellular carcinoma, Alpha-fetoprotein (AFP) an oncofetal protein is produced in the liver. This protein is elevated during both neo-plastic as well as in non-neoplastic condition (K *et al.*, 2018). This situation can be highly suggestive for diagnosing hepatocellular carcinoma at the level of >200 ng/ml for AFP. Thus, confirms that the possibility for HCC is greater than ninety percent (>90%). Currently, serum AFP level is the standard biomarker for the detection of HCC in patients with specificity from 76% to 94% and a sensitivity of 39% to 65% (EDOO *et al.*, 2019) which is controversial. Currently, HCC is also diagnosed via level of serum alpha-fetoprotein (AFP) along with ultrasonography for every 6 to 12 months (K *et al.*, 2018). Because of its low specificity and sensitivity, AFP's diagnostic performance is severely limited and thus is not an ideal choice for the diagnosis of HCC but only for surveillance and should be used in conjunction with ultrasound, according to AASLD guidelines (Lim and Singal, 2019). AFP is inclined to false negative results rendering it ineffective for the Hepatocellular Carcinoma diagnosis although AFP based test is commonly used to identify potential liver cancer. Alcoholic hepatitis, chronic hepatitis or cirrhosis can also result in AFP elevation other than hepatocellular carcinoma (F *et al.*, 2018). Moreover, in some cases, AFP levels are not elevated at and normal AFP levels diagnosed at the time of diagnosis tend to stay the same throughout the course of the disease (Carr *et al.*, 2018). Therefore, AFP cannot be maintained as a standard diagnostic biomarker for HCC because of specific AFP negative HCC cases, where there is no marked difference in AFP levels even in the presence of HCC (Carr *et al.*, 2018). As a result, multidisciplinary research concentrating on the characterization of circulating biomarkers, which have good diagnostic ability (sensitivity and

specificity) for early HCC detection can have a major impact on improving patient survival rates (F *et al.*, 2018). This, project is focused on the characterization of biomarkers with high specificity and sensitivity compared to Alpha fetoprotein (AFP) and to increase the diagnostic accuracy of HCC.

Several biomarkers have been revealed with the advancement in cancer biology such as CK-19, GP-73 and PIVKA-2 (protein induced by vitamin K absence) (Zacharakis, Aleid and Aldossari, 2018). These biomarkers are currently under study to determine their efficiency in reducing the initiation of new tumors and inhibiting the reoccurrence of tumors in the liver transplanted patients. Many tumor-related proteins, such as iso-zymes are reported to be expressed by Hepatocellular carcinoma therefore it is mandatory to define tumor detailed biomarkers for the diagnostics of HCC in patients (Lou *et al.*, 2017). Numerous technological advancements have led the way for a greater interest in characterizing new and improved biomarkers by examining many potential biomarkers.

Despite efforts to enhance early hepatocellular carcinoma (HCC) diagnosis, still there is a lack of efficient blood-based biomarkers for the detection of cancer. Large expression data analysis utilizing published pipelines created by Awan et al., 2015 at the Nanobiotechnology lab ASAB-NUST that would incorporate multiple bioinformatics databases/tools and literature is required to uncover highly sensitive and specific protein biomarkers. Compared to any other field of basic medical research, tumor-specific blood-based biomarkers occupy key significance and may be viewed as a viable medium for improving cancer treatment. To efficiently treat HCC, active research should concentrate on documenting novel blood-based biomarkers. As numerous tumor-related genes, enzymes, proteins, and microRNAs (miRNAs) are released into bodily fluids like blood or urine by cancer cells, effective biomarkers with greater sensitivity and specificity for identifying HCC are required. (Zacharakis, Aleid and Aldossari, 2018).

A computational biology approach was adapted in our lab for the circulating protein biomarkers' identification through a computational pipeline. These candidate biomarkers were **C8A, SERPINC1, MBL2, HSD11B1, Cyp2a6, UPB1 and ADH6** (Awan *et al.*, 2015). These proteins were selected by using the following pipeline.

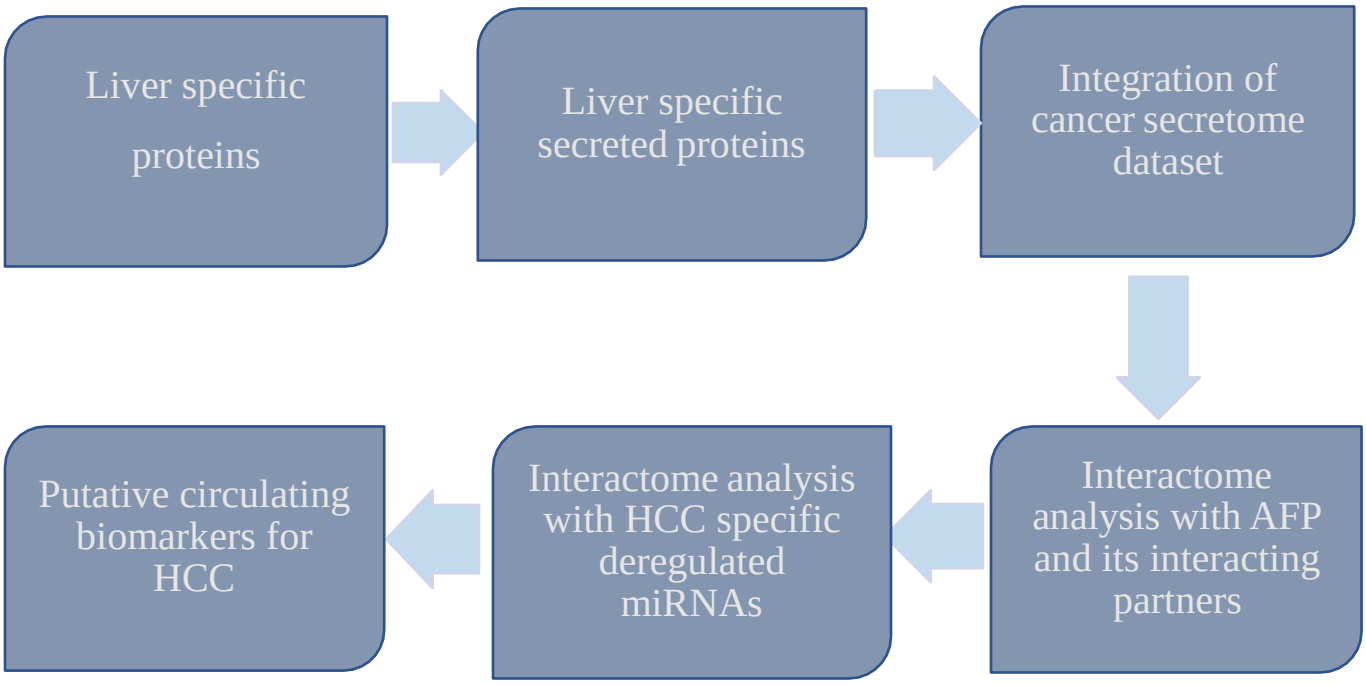

Figure 1.1: Liver specific proteins were checked in multiple databases and matched with secretome datasets; liver specific secreted proteins were shortlisted (Awan et al., 2015)

Afterwards, interaction of these proteins was confirmed with AFP and related proteins which is i.e., the current diagnostic biomarker for HCC. Further these shortlisted proteins were matched with HCC specific deregulated miRNAs and via statistical analysis, 7 protein biomarkers for HCC were shortlisted (Awan et al., 2015).

Mr. Abdulah Ahmed (Adullah Ahmed MS Thesis 2021) completed the first phase of the project. He performed ELISA on 4 proteins namely, C8A, SERPINC1, HSD11B1 and MBL2 after collection of blood samples from HCC patients. The ELISA kits were provided by Nanjing Biotech. Among these C8A showed excellent ability to function as efficient biomarker for HCC diagnosis with sensitivity and specificity values of 85.33% and 100% respectively. Among other three proteins SERPINC1 showed results that were similar but not better than the already established biomarker for HCC which is AFP. The remaining two proteins, MBL2 and HSD11B1 did not show any significant ability to act as biomarkers for HCC diagnosis, with sensitivity and specificity values less than 60%. Mr. Abdullah Ahmed further qualitatively validated the ELISA results of the best performing protein i-e C8A through Western blot analysis (Abdullah Ahmed, MS Thesis 2021).

In the current study, three protein biomarkers **(Cyp2a6, UPB1 and ADH6)** were validated in the serum of both HCC patients and controls to see their performance as candidate biomarkers, which was further validated through wet lab approach (ELISA, Western blot analysis and Multiplex ELISA) to check the presence of HCC. The aims and objectives of this research as follow.

I. Wet lab validation of three potential biomarkers Cyp2a6, UPB1 and ADH6 in the blood of 150 HCC patients against 50 healthy controls through ELISA test.
II. Using a student t-test the selection of the biomarker with a significant concentration difference and a p value less than 0.05.
III. Using the ROC curve method, further analysis of the diagnostic ability of the candidate biomarker, and only that biomarker candidate was selected having AUC value of >0.90.
IV. Western blot qualitative and qualitative validation of biomarker results (AUC >90%).
V. Multiplex ELISA to confirm whether a combination of two or more proteins can enhance sensitivity and specificity of a candidate biomarker.

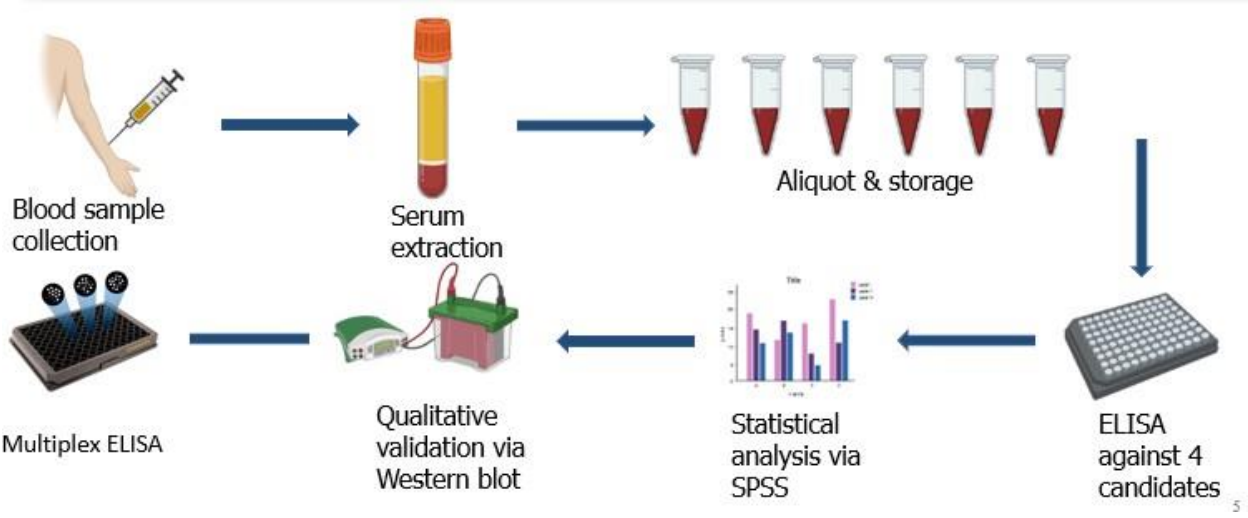

Figure 1.2: Flow sheet Diagram of the represents the strategies adopted for the characterization of 3 blood circulating biomarkers (Cyp2a6, UPB1 and ADH6) in 150 HCC patients while 50 are healthy controls.

To assess the concentrations of biomarker candidates' serum was extracted from the collected blood samples of patients and quantitative ELISA was performed on these serum samples Statistical analysis was performed via SPSS version 24 and GraphPad Prism to assess the ability of these biomarker candidates to diagnose HCC. The proteins were further qualitatively validated via Western blot analysis. The proteins were subjected to multiplex ELISA approach to confirm

whether combination of proteins provide better sensitivity and specificity values when compared to the individual proteins

Chapter 2 Literature Review

2.1. Liver Diseases

The largest gland of the human body is liver and is involved in a variety of key processes i.e., protein synthesis, detoxification of metabolites and drugs, metabolism of fats and is also involved in the bile production (Hansel *et al.*, 2014). The liver is composed of a variety of cells with 70-80% volume composed of parenchymal cells and 6.5% of non-parenchymal tissue (Hansel *et al.*, 2014).

Liver is thus an essential component of human body in maintaining homeostasis and maintaining overall body function. Thus, any abnormality in liver can lead to major disturbances in the overall normal body function. Hepatitis, hemochromatosis, cirrhosis, fatty liver disease and Hepatocellular carcinoma (HCC) that is a end stage liver disease, are the major diseases of liver (Marcellin and Kutala, 2018).

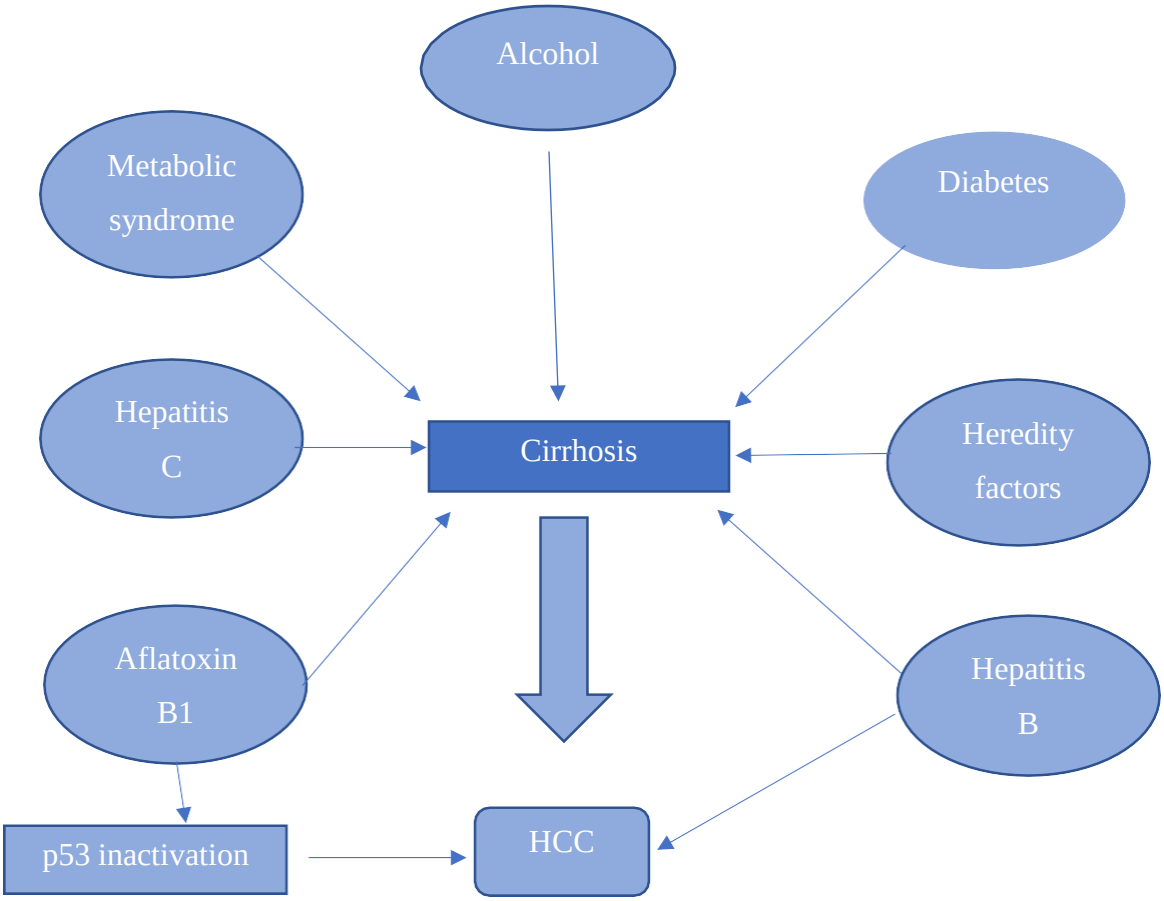

Figure 2.1 Various liver diseases that can lead to HCC (SK et al., 2019)

Among all these issues, Cirrhosis is one of the biggest contributors to disturbing global health in recent years (SK et al., 2019). It progresses over years and once the liver has sustained considerable damage, it is named as End-Stage Liver Disease (ESLD) (Peng et al., 2019)**.** Cirrhosis and Hepatocellular carcinoma (HCC) are both considered as different stages of ESLD. Hepatocellular carcinoma (HCC) can also arise from a multitude of other factors apart from cirrhosis. Over the globe, Hepatocellular carcinoma (HCC) is the most common type of liver cancer and fourth leading cause of death (Kim and Viatour, 2020)**.** Viral hepatitis (B and C) are also major contributors of HCC (Axley et al., 2018)**,** especially in Pakistan where the rate of viral hepatitis is fairly high (Kim et al., 2020)**.**

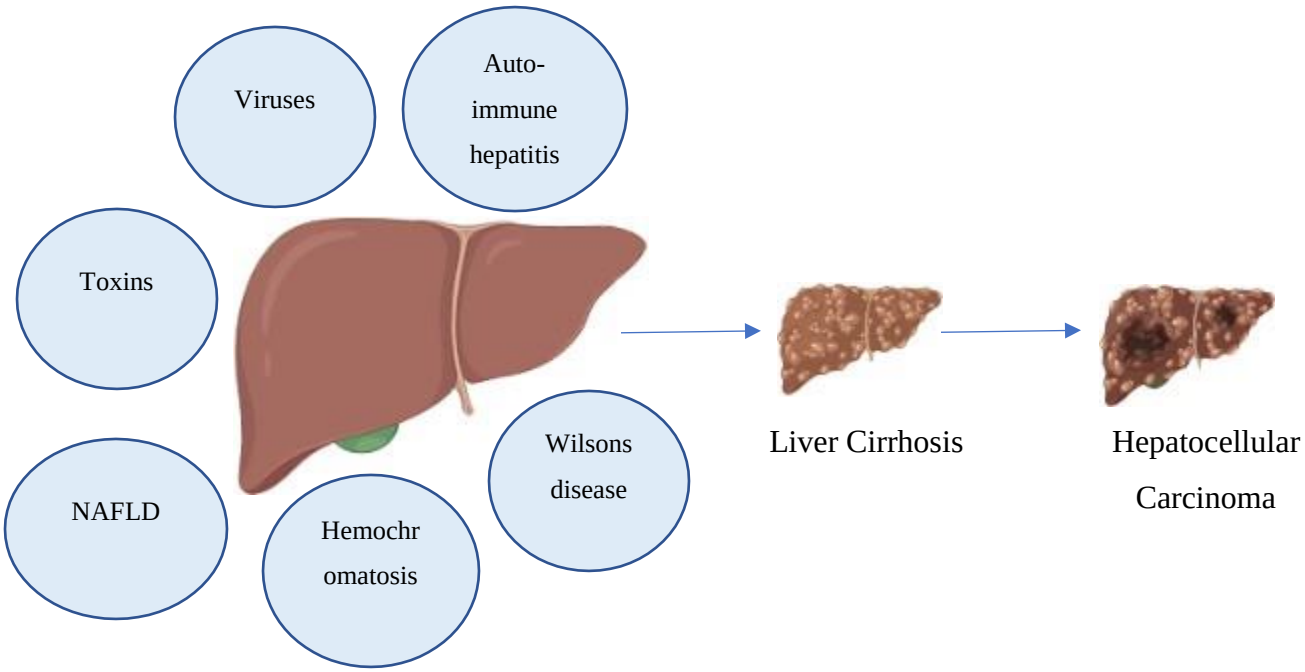

Figure 2.2: Risk factors that potentially lead to liver cirrhosis and ultimately HCC. Adapted from (Uhl et al., 2014)

2.2. Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer and is estimated to be 90% of all forms of liver cancer (Llovet et al., 2021)**.** The most common cause of HCC is viral hepatitis or liver cirrhosis (Axley et al., 2018)

A higher incidence of HCC is found in developing countries i.e. Asia and Africa in contrast to the developed countries (Rawla et al., 2018)**.** This can be attributed to late diagnosis, inadequate health monitoring and management facilities resulting in limited treatment options for HCC patients. Pakistan has a higher HCC incidence compared to rest of the world mainly due to prevalent viral hepatitis, late diagnosis, and poor health management. According to the data provided by PubMed, Pakistan published 38 publications in HCC research thus lagging far behind from China, US and India with 6976, 1825 and 268 publications respectively

However rate of HCC incidence is also increasing in developed countries across Europe and America (Singal, Lampertico and Nahon, 2020)**.** On a global scale, the research on Hepatocellular carcinoma lags behind other forms of cancer like breast cancer and lung cancer. Most of the published data consists of clinical observations, with randomized trials few and far in between.

The second major root of death globally, amid all types of cancer is Hepatocellular Carcinoma (Rawla et al., 2018). In Pakistan, adult males are most exposed thus making it the most common cause of cancer among them (Bhatti et al., 2016). Pakistan contributes a huge sum towards the global weight of hepatitis C which is also a major cause contributing towards HCC therefore the prevalent rate of HCC is high in Pakistan (Mahmood and Raja, 2017). Absence of screening programs along with the lack of patient's data in the form of national cancer registry are making the issues worse related to the treatment of HCC.

2.2.1. Viral HCC

2.2.1.1 Chronic Hepatitis C infection

Hepatitis C is an infection that primarily affects liver, causing inflammation and damage that can progress to cirrhosis. Virus that causes infection is a single-stranded RNA virus that spreads by coming into contact with infected blood (Mahmood and Raja, 2019). So far, six genotypes of the Hepatitis C Virus (HCV) have been identified, four of which are known to cause infections that progress to chronic diseases (Al-Salama and Deeks, 2017). Cirrhosis raise likelihood of developing malignant liver illnesses like HCC, especially if the patient drinks alcohol or has a dual infection with both the hepatitis C and B viruses (Li et al., 2020). The risk of HCC is significantly lowered in treated patients who have aviremia or a sustained viral response (SVR) (Li et al., 2020). In Pakistan, HCV is the major cause of cirrhosis as well as HCC (Mahmood and Raja, 2019).

2.2.1.2 Chronic Hepatitis B and D:

Hepatitis B Virus (HBV) is become the cause of Hepatitis B, a double-stranded DNA molecule that spreads through blood, sperm, and other bodily fluids, and can even be passed from a woman to her unborn child (Chan and Smith, 2018). Due to the severity of the disease and the virus's integrative capacity, people infected with HBV develop cirrhosis sooner than those infected with HCV.

However, even if no indications of cirrhosis are present, carriers of this virus have a major risk of developing HCC (Xue, Liao and Xing, 2020). This increased risk is more frequently related with HBV genotype C and several markers have been categorized as indicators for later progression to HCC (Sarma et al., 2018). Despite the fact that infants and adults are inoculated against HBV, the virus has eight genotypes, and affects around 1/3rd of the world's population (Jefferies et al.,

2018). HBV is hazardous on its own, but people who also have Hepatitis D virus (HDV) can develop severe viral hepatitis. HDV relies on HBV for reproduction, and the co-infections of these two can cause liver cancer and death (Puigvehí et al., 2019).

2.2.2. Non-viral causalities of HCC

2.2.2.1 Nonalcoholic Fatty Liver Disease (NAFLD) or NASH:

Excess fat stored in the liver can harm the tissues, resulting in a condition known as NAFLD. It is the major type of chronic liver disease in persons with metabolic disorders such as diabetes, coronary artery disease, protein deficiency, and obesity, as it usually develops to HCC and cirrhosis (Peng et al., 2021). Normally 0.5 to 2.6% of patients with NASH cirrhosis develop HCC but non-cirrhotic NAFLD rarely leads to HCC i.e. 0.1 to 1.3 patients per 1000 patients (Huang, El-Serag and Loomba, 2020)

2.2.2.2 Alcohol-Related Liver Disease:

Excessive and frequent alcohol intake is another cause of cirrhosis and HCC. The amount of alcohol required to produce chronic liver disease differs from person to person. Cirrhosis is strongly associated with alcohol consumption and the risk is almost two-thirds higher in people that consume seven or more drinks in a week (Simpson et al., 2019). Cirrhosis develops in 10-20% of heavy drinkers, resulting in scar tissue. Because of its widespread usage in European countries, alcohol is one of the most common causes of HCC and cirrhosis (Ganne-Carrié and Nahon, 2019).

2.2.2.3 Chronic biliary diseases:

Certain disorders damage the bile ducts, causing harmful bile acids to build up in the liver. As inflammation develops to scarring, as seen in liver cirrhosis, this affects the liver's normal function. The most frequent type of cirrhosis is primary biliary cirrhosis, which arises when the bile duct becomes inflamed and then vanishes (Li et al., 2017). Primary sclerosing cholangitis causes bile duct scarring and is strongly associated with Chronic Liver Disease (CLD) (Liao et al., 2019).

2.2.3 Inherited diseases:

Hemochromatosis is a condition that causes the development of cirrhosis in the liver, which can lead to HCC (Jayachandran et al., 2020). Other hereditary illnesses that can lead to cirrhosis or DCLD include cystic fibrosis, Wilson disease, and alpha-1 antitrypsin deficiency (Ponzetto, Holton and Lucia, 2018; Brandi et al., 2020; Narayanan and Mistry, 2020). Furthermore, long-

term exposure to hazardous chemicals, aflatoxins, and parasite infections can cause liver failure or cancer (Dash et al., 2020).

2.2.4 Autoimmune hepatitis:

Autoimmune hepatitis, a chronic condition characterized by autoantibodies circulating in the system attacking normal liver cells, causes inflammation and chronic liver disease which eventually progresses into cirrhosis and is an uncommon cause of cirrhosis and HCC (Tansel et al., 2017). Susceptibility to this condition is thought to be influenced by genetic factors.

2.2.5. HCC incidence in Pakistan

HCC, as described previously, has a higher incidence in low and middle income i.e., developing countries. In Pakistan, the HCC and cirrhosis incidence trend differs from rest of the world. In Pakistan, cirrhosis is one of the biggest reasons behind mortality and hospital visits (Majid *et al.*, 2019)**.** HBV and HCV-related cirrhosis are the most common among numerous etiologies. In chronic HCV or HBV infected patients, HCC is most common in the post-cirrhotic liver, with 96 percent prevalence (Bhatti *et al.*, 2016). In Pakistan, HCV is the most common etiological cause, accounting for roughly 58 percent of HCC patients, while HBV-related liver cancer is estimated to account for 25.3 percent of HCC patients (Bhatti et al., 2016).

2.2.6. Causalities of HCC

HCC as described previously is caused by several reasons. These causalities can be divided into 2 main categories i.e., viral, and non-viral.

2.3. Diagnosis

Early detection is critical for improved illness management, especially in the case of cancer, when treatment options become limited as the disease progresses. Healthcare providers must be able to distinguish late-stage cirrhosis from early-stage cirrhosis in order to intervene quickly before major consequences emerge. Various procedures are used for this goal, but the symptoms are usually too complex for straightforward separation. Early on in the course of HCC, patients may appear with similar symptoms or stay asymptomatic, making it difficult to adjust treatment as the illness progresses.

In various parts of the world the incidence of HCC is increasing drastically. For early diagnosis, surveillance with or without serological (serum) testing and the use of proper imaging techniques

are important methods to identify those at higher risks. Surgical interventions which include translation of liver, local ablation and resection offer chances of prolonged survival to liver cancer patients (Lang, 2021).

One of the reasons for HCC's low curability is its late diagnosis, which leads to severe consequences that, in the majority of instances, are permanent. More than 85% of HCC patients in Pakistan present to the clinic at a later stage (C-D) (Bhatti *et al.*, 2016). Because of the delayed diagnosis, the odds of survival are considerably lowered.

The American Association for the Study of Liver Diseases and Disorders (AASLD) makes recommendations for disease surveillance, diagnosis, staging, and treatment of liver cirrhosis and liver cancer (Heimbach *et al.*, 2018). Serum biomarkers, CT scans, MRIs, and abdominal ultrasonography are currently being used for this differentiation.

In all cases HCC is considered as end point of a sequence which start with chronic injury of liver then progresses to cirrhosis of liver and at the end after many years confirm the results of liver cancer (Lafaro, Demirjian and Pawlik, 2015). miRNA (microRNA) has a therapeutic role in infection of hepatitis c that acts as a risk factor for developing liver cirrhosis and HCC. The treatment of liver cirrhosis and HCC still have limited applications in clinical management as patients suffering with these diseases have no proper pharmacological treatment. However, miRNA exhibits the potential to regulate the genes network and can be used as model therapeutics in HCC patients and also serve for characterization as biomarkers (Awan *et al.*, 2015; Lou *et al.*, 2019; Pascut *et al.*, 2019).

2.3.1. Role of cancer biomarkers in detection of HCC

Liver disease is confused easily with other problems of health due to which these diseases are difficult to diagnose and have vague symptoms. However, specific markers have been used by physicians which help in diagnosing and lead to follow up the disease related to liver. In the liver, some metabolic pathway and enzymes are occurring which are sensitive for any abnormality due to which these are considered as biochemical biomarkers of liver dysfunction (Awan *et al.*, 2015).

In a study published by Awan et al., 2015, it was discussed that after the emergence of omics technology several putative biomarkers have been identified as well as published which increased dramatically the opportunities for developing therapeutics more effectively (Awan *et al.*, 2015).

Such opportunities can have well-known benefits for healthcare's economics and for patients. However, transferring of biomarker from the phase of discovery to practice at clinical stage is still under the process. For becoming clinically approved test, a biomarker should be validated and confirmed using hundreds of HCC patients' blood specimens to provide specific, sensitive, and reproducible results based on the detection of blood circulating biomarkers in HCC patients (Awan et al., 2015).

Improved and efficient detection and effectiveness in the management and treatment of cancer patients has been made possible due to the increasing knowledge of cancer biomarkers. Examination of many potential biomarkers due to the advancements in molecular biology during the last decade have strengthen the development of new and improved biomarkers. A potential biomarker of interest would include several biological entities such as proteins, sugar, small metabolites, cytogenetic and cytokinetic parameters as well as tumor fluids found in the body (Umekar, 2021). The importance of biomarkers can be determined by the understanding of its prevalence and can be used for the earlier disease diagnostics and for multiple alternative therapies being used currently to treat patients in an effective way (Umekar, 2021).

Hepatocellular carcinoma (HCC) produces increased level of Alpha-fetoprotein (AFP) which is an oncofetal protein (Luo *et al.*, 2020). An elevated level of AFP provides highly significant diagnostic situation for HCC at the level of >400ng/ml which means HCC have probability of greater than ninety percent (>90%) (J. Zhang et al., 2020a). A study reported that there was no reoccurrence of HCC in patients who had their hepatic tumor surgically removed but were still having significantly high levels of AFP after the surgery for a period of two years (Rungsakulkij et al., 2018).

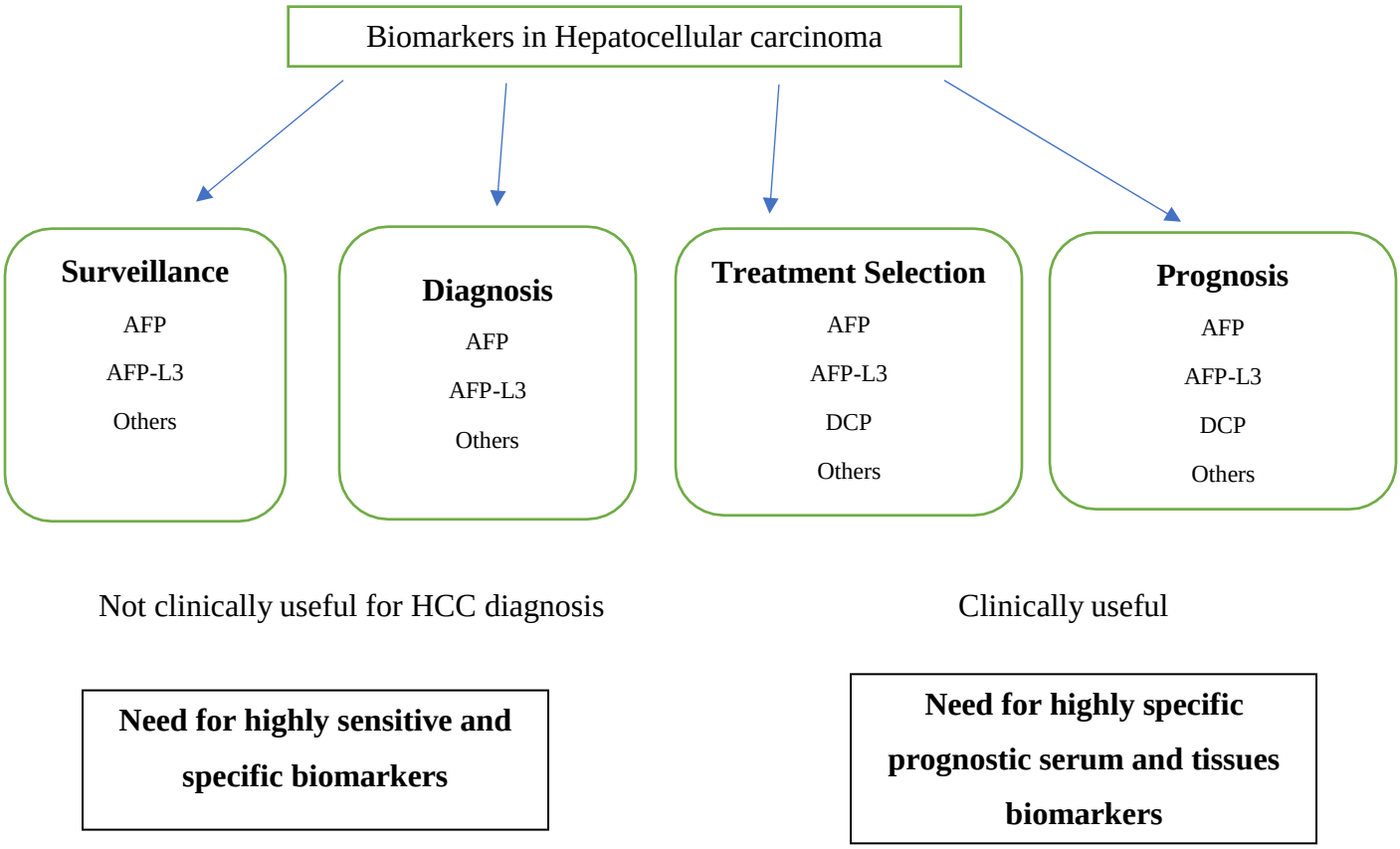

Figure 2.4: Classification of already established biomarkers for HCC. But the need for highly sensitive and specific biomarkers is highlighted because these biomarkers do not have clinically diagnostic utility. Source: (Piñero et. al, 2020).

The Tumorigenesis of hepatocellular carcinoma is mostly examined through magnetic resonance imaging, ultrasonography, and tomography scans (Heimbach et al., 2018) however these techniques are not useful for the detection of initial tumor growth. A detailed review on current situation of HCC biomarkers highlights enzyme and protein biomarkers for HCC diagnostics. These include α-L-fucosidase (AFU), glypican-3 (GPC3), γ-glutamyl transferase (GGT), α-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP), Golgi protein 73 (GP73), and (SCCA) squamous cell carcinoma antigen as candidate protein biomarkers for HCC (Zhao et al., 2017).

Wang et al., 2018 explained that HCC with high rate of mortality considered as type of malignant tumor. In regulation of gene as well as in cellular processes the non-coding RNAs define significant roles, while for the treatment and monitoring of HCC, the identification of novel

prognostic biomarkers has significant value however only a small number of biomarkers with specificity and sensitivity are used in clinical practices. Authors of this study aimed to study serum LRB1 level as a novel HCC biomarker. LRB1 expression level were linked to HCC in three hundred twenty-six (326) patients and in seventy-three (73) healthy controls by using LncRNA expression microarrays and analysis of quantitative polymerase reaction. The results showed that level of LRB1 serum was increased significantly in HCC patients compared to the control group. In addition, the combination of AFP, DCP (des-y-carboxy prothrombin) and LRB1 showed increased diagnostic accuracy compared to the use of LRB1 alone. It is considered that this serum biomarker has the potential to act not only as a regulator but also as a predictor for diagnosing HCC patients (Wang et al., 2018).

In an earlier study conducted by our group (Awan et al., 2015) a computational pipeline was developed for the discovery of candidate biomarkers for HCC early detection. The focus was on identifying and characterizing blood-based secretary proteins that can be applied for the early diagnostics of HCC. Proteome analysis related to cancer and liver proteins were extracted through proteome mining and compared. The matched proteins in both proteomes were then shortlisted and further compared with the secretome, so that only those proteins were selected which were secretory in HCC patients and secreted in blood. As a result of this pipeline, seven novel protein biomarker candidates were shortlisted namely **MBL2, ADH6, UPB1, SERPINC1, HSD11B1, CYP2A6 and C8A** (Awan et al., 2015). The current research project related to the *''Molecular characterization of blood-based protein biomarkers for non-invasive and rapid diagnosis of HCC''* is focused on the ELISA and western blot analysis characterization of the three biomarker candidates (Cyp2a6, UPB1 and ADH6) that have been identified in previous work published by (Awan *et al.*, 2015) for which we need to apply this analysis on the blood serum of HCC patients. Validations through wet lab experiments (ELISA, Western Blot analysis and Multiplex ELISA) will strengthen our hypothesis that all seven proteins or combination of few of these or any of these secreted proteins would work as a biomarker for the early detection of HCC.

Chapter 3 Methodology

3.1. Overview

Following is the flow chart representing the methodology used during this project.

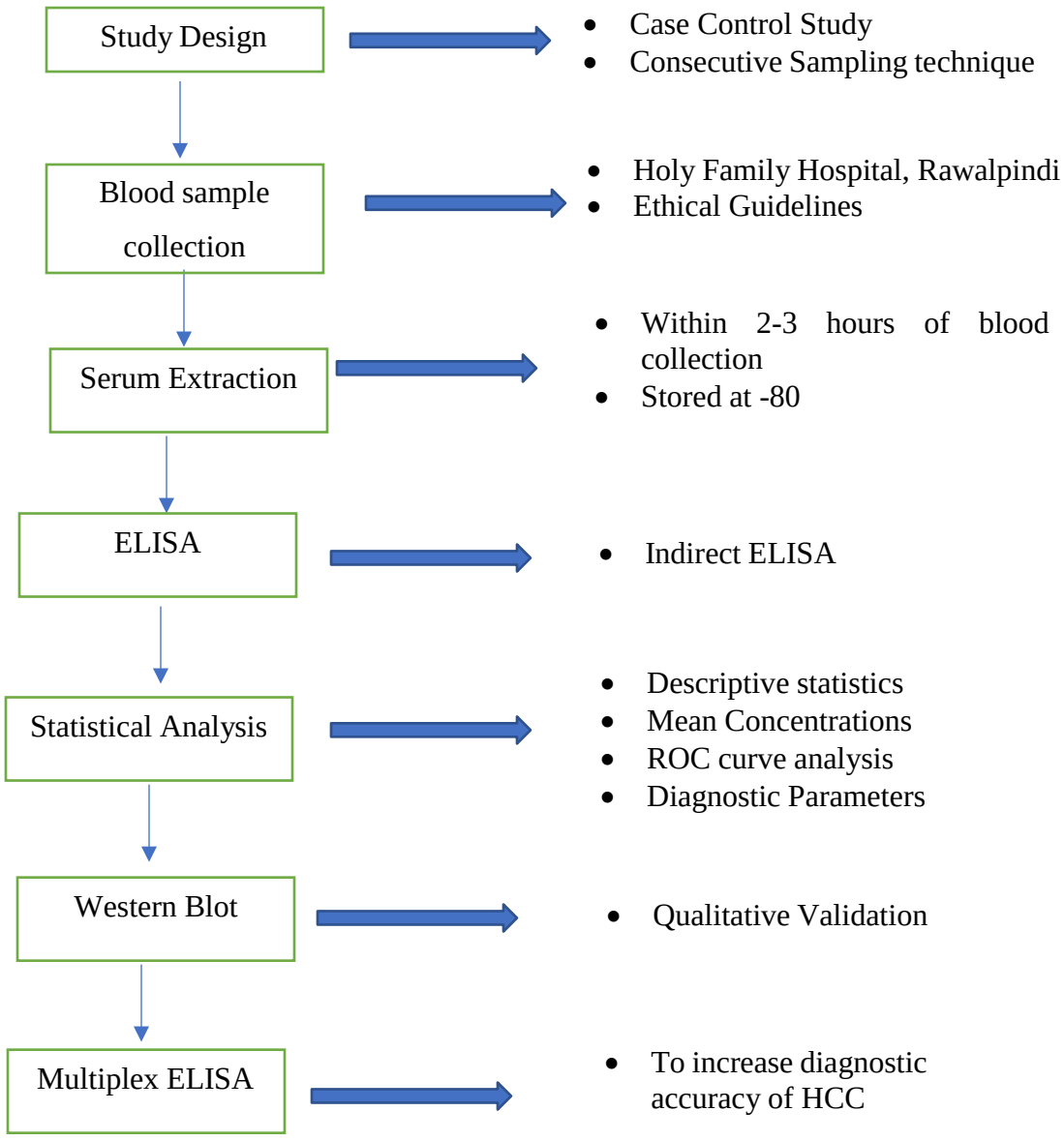

Figure 3.1: Flowchart showing the methodology followed during the project.

3.2. Study design

Consecutive sampling was utilized in this study, a "case control study," to circumvent randomization.

3.3. Collection of blood samples

A partnership was established between the Nanobiotechnology Lab, ASAB, and Rawalpindi Medical University for the collecting of blood samples from HCC patients (RMU). For this reason, the Vice Chancellor of RMU oversaw the presentation of our summary to a panel of doctors and researchers in order to secure an ethical permission from the Institutional Review Board of Holy Family Hospital, RMU, and Rawalpindi. All the ethical standards were established during the meeting, and the research partner at RMU made sure that they were strictly followed. . Mr. Abdullah Ahmed and I collected blood samples from 150 HCC patients at The Holy Family Hospital in Rawalpindi's Center for Liver Diseases (CLD), Medicine Wards 1 and 2, and Gastrointestinal Ward after gaining IRB approval. The patients' verbal agreement was obtained before blood samples were taken. The patients were given access to a questionnaire, which they voluntarily decided to complete after being informed of the purpose, nature, and content of the data being collected from them.

Considering all ethical norms, a 3 ml blood sample was taken from the HCC patients and healthy controls. To reduce the likelihood that the patient would sustain harm, a trained phlebotomist was employed. On a questionnaire, the patient's age, sex, history of HBV and HCV, as well as the time and location of blood collection, were gathered. Blood samples from 150 HCC patients and 50 samples from healthy controls were collected. Within two to three hours of the blood sample collection, all samples were delivered to the ASAB Nanobiotechnology Lab for additional processing.

The following criterion was used to choose patients and healthy controls:

3.3.1. Inclusion and exclusion criterion

At the location of blood collection, the research assistant and the on-call doctors screened the patient’s using inclusion and exclusion criteria.

3.3.1.1. Inclusion criterion

- HCC was confirmed using imaging methods including MRI and ultrasound.

- Imaging examinations, including X-rays, bone scans, abdominal and pelvic CT scans were used to verify the non-metastatic stage of HCC.
- HCC diagnosis was confirmed from the attending physician.
- Only those individuals were included in the study that possess no prior history of viral hepatitis along with no history of liver disease.

3.3.1.2. Exclusion criterion

- Exclusion criteria included comorbidities associated with the liver.
- Any individual with a prior history of liver disease was not included in the study.
- The presence of one or more tumors.
- Imaging studies, such as X-rays, bone scans, abdominal and pelvic CT scans that confirm the tumor's metastatic stage.
- Those individuals were excluded that had liver condition of any kind, had any form of cancer along with occurrence of viral hepatitis (B&C).

3.4. Serum Extraction

Blood samples were collected into serum vacutainers (yellow caps) and placed vertically on stands. To obtain the serum, the blood serum were centrifuged at 2000 rpm for 2 minutes. The gel that separates the blood clot in the serum vacutainer enables us to scoop up pure serum from the top. All the secreted proteins found in the blood are present in the serum. With 1.5 ml of the extracted serum being aliquoted into Eppendorf tubes, which were then placed in a freezer set at -80 °C. Blood samples were collected between September 2020 and July 2021.

3.5 Descriptive Statistics on sample population

3.5.1. Test sample population; HCC patients

It is important to categorize patient population in terms of gender, age group, and HCC causalities by applying various statistical analyses on the study population. At the first stage, test sample population, varying on the age grouping, is categorized in terms of frequencies and proportions. The greatest percentage of people belonged to the age range of 50-59 years according to the age distribution of our sample population with HCC (43.3%), followed by 60–69 years (29.3%). Following them were the lowest population (6.7%) and a mediocre percentage (20.7%) of people aged 40 to 49. Figure 3.2 shows these data in the graphic form.

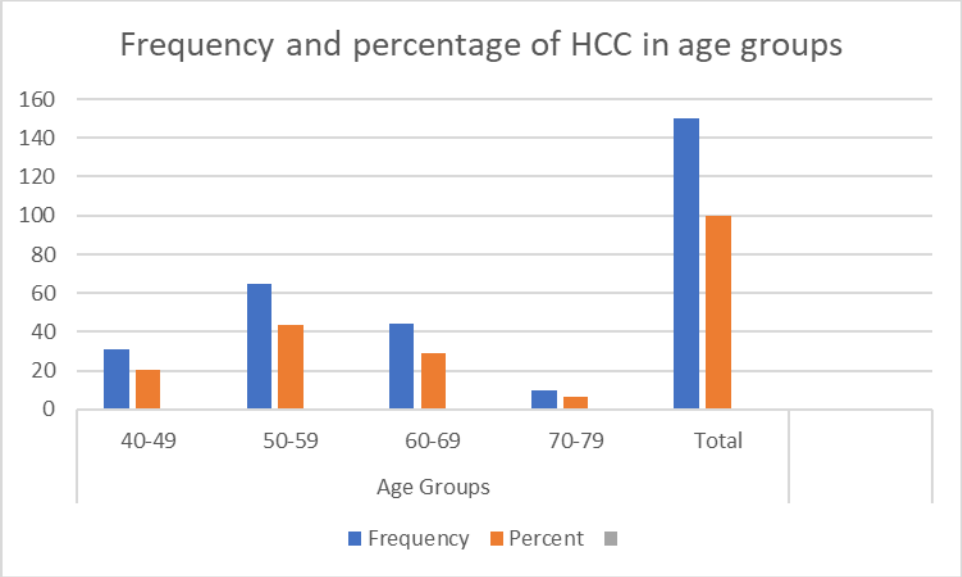

Figure 3.2: Frequency and percentage of HCC patients in age groups.

Table 3.1 displays the population's distribution by gender the majority of the HCC sample population was made up of men (92 cases), with the remaining cases being women (58 cases).

Gender distribution in HCC patients

Table 3.1 Frequency and percentage of HCC patients in terms of Gender

		Frequency	Percent
Gender	Male	92	61.3
	Female	58	38.7
	Total	150	100.0

Cross tabulation can be employed to deduce the gender-wise distribution in various age groups from the age group and gender divisions. Such information can shed light on important

understandings related to age-wise demographic distribution of gender in HCC patients, as illustrated in Figure 3.3.

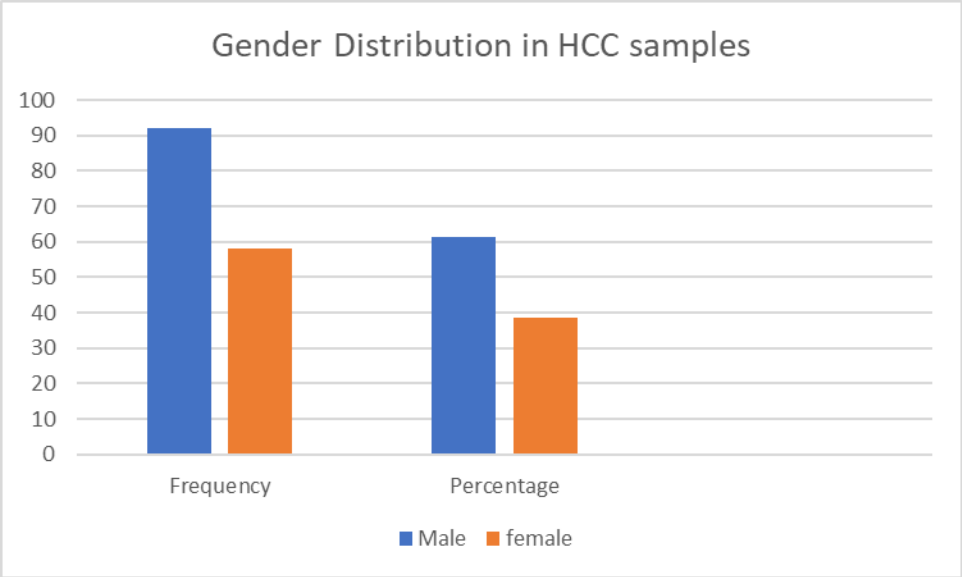

Figure 3.3: Distribution of gender in different age groups is represented in the form of clustered bar chart. Majority of the population was male in every age group when compared to females.

3.5.1.1. Co-morbidities (Causalities) of HC

The most common cause of HCC around the globe is through HCC viral hepatitis (HBV and HCV) and Pakistan is no different (D'souza et al., 2020). Therefore, HCC population is distributed in viral and non-viral categories. The major burden of HCC came from a viral source contributing to 133 viral HCC cases overall as demonstrated by Figure 3.4, whereas 17 of the cases came from a source other than viral.

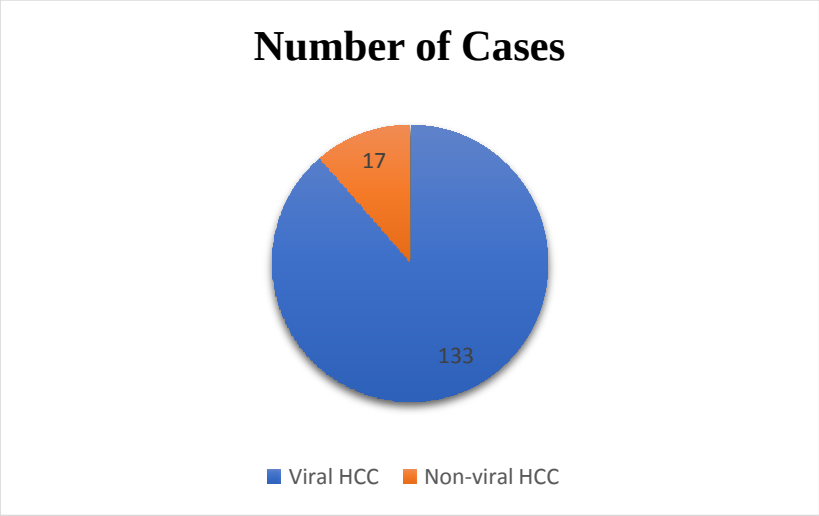

Figure 3.4: Viral and non-viral groups of HCC. The main cause of HCC in Pakistan is due to high incidence of HCV and HBV related HCC.

A different pool of test population was created depending on the presence of HBV or HCV. According to the research, there were 103 HCV cases and 23 HBV cases in all, whereas five instances reported both HCV and HBV as depicted by Figure 3.5.

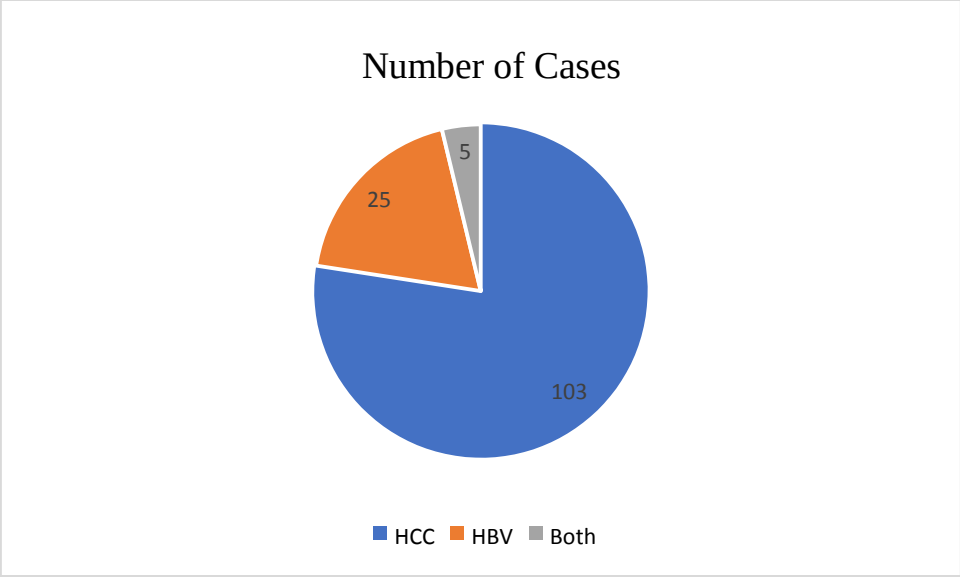

Figure 3.5 Division of HCC population in HCV, HBV and both HCV and HBV incidence, 103 samples belonged to HCV incidence alone, whereas 25 cases belonged to HBV. 5 cases had a history of both HCV and HBV.

3.6. Control Samples

Total number of 50 control samples were also used in the study, in addition to the 150 test samples mentioned above. The samples were taken after verbal consent from the healthy individuals. These individuals were chosen from a variety of age groups and localities and had no history of HCC, HCV, or HBV.

To compare the control samples with the HCC patient population, they are additionally separated by age categories. The age range of the control samples with the highest percentage of people in it was 30–39 years old (36%) and 40–49 years old (30%). The distribution of age groups in the population of HCC patients and this tendency are similar.

Figure 3.6 represents this information graphically as a pie chart displaying the percentage of controls in various age categories.

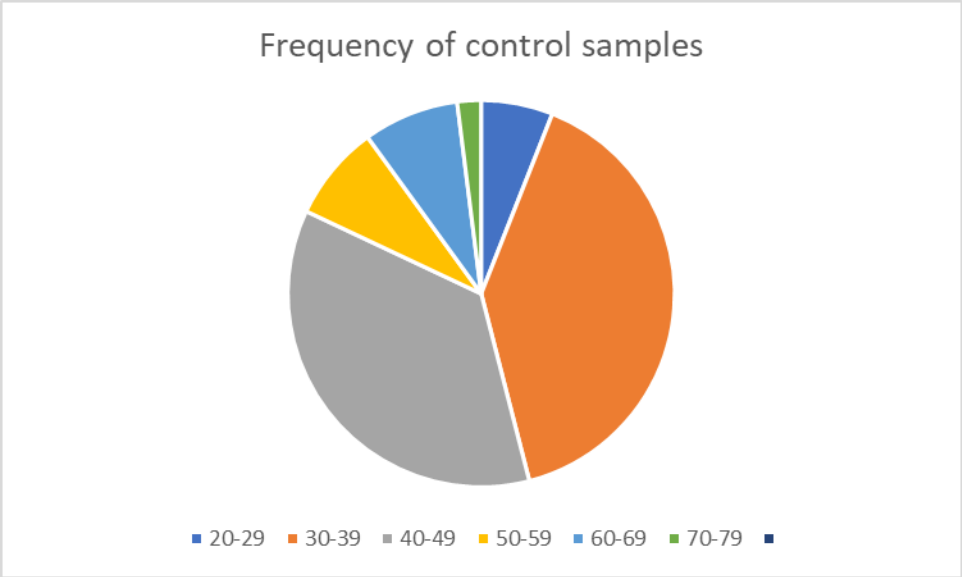

Figure 3.6 Breakdown of control samples in terms of frequency in different age groups. The highest percent is present in 30-39 age group followed by 40-49 years of age.

3.7. ELISA

Using an ELISA test, patients' collected serum was examined. Using protein-specific antibodies, ELISA is a quantitative study that quantifies the amount of protein in a sample. Depending on the type of ELISA being performed, the bound antibodies produced a colorimetric or chemiluminescent signal, which is compared to known standards to determine the amount of

protein present in the sample. Chemiluminescence was employed in our ELISA test to determine the concentration of the target protein in a sample.

We ordered ELISA kits from Nanjing Pars Biochem Ltd. against three potential protein biomarkers: Cyp2a6, UPB2 and ADH6. The absorption values from ELISA were read using a Bio-Rad Ltd. ELISA reader (Model PR4100).

3.7.1. ELISA procedure

- The 96-well ELISA plate has wells on it for the primary antibody to be coated in during the ELISA method. In our instance, the primary antibody was pre-coated on the ELISA kits from Nanjing Pars Biochem Ltd.
- Standard wells were initially placed on the ELISA plate. By serially dilutions with the standard diluent, ELISA standards with known concentration values from the ELISA kit were applied in duplicate to these wells. In order to do serial dilutions, 100 µl of the standard sample was added to the first two wells (duplicates), subsequently 50 µl of the standard diluent was added to both the wells. The 50 µl was then removed and thrown from these first and second wells. The third and fourth wells were then added with 50 ml from these wells. The third and fourth wells were then each given 50 µl of normal diluent. The fifth and sixth wells were then added 50 µl from the third and fourth wells. After that, the fifth and sixth wells each received 50 µl of standard diluent, and so on. This protocol should be elaborated in a simple way, it’s difficult to understand
- Two wells were left unfilled which can be served as "blanks". All ELISA phases were carried out on blanks, except for adding the sample and HRP conjugate reagent. The remaining wells will be filled with 40 µl of sample diluent.
- The wells of the ELISA plate, which already had 40 ml of sample diluent in the were filled with 10 µl each of the test and control samples. As a result, test sample concentration was diluted five times.
- To enable the coated antibody that bind with the sample' proteins, the plate was then incubated for 30 minutes at 37 degrees Celsius. The plates were incubated by having a sealing membrane placed over them. The wells were dried by swinging once the closure membrane was opened and the samples inside the wells were removed after 30 minutes.

- After that, washing buffer was applied to the wells five times and then the HRP-conjugated secondary antibody (20 µl) was added. Blank wells did not have HRP conjugated antibody added to them.
- The plate was again allowed to stand for 30 minutes for allowing the secondary antibody to bind with the protein bound primary antibodies.
- After 30 minutes all wells were washed with washing buffer (5 times) subsequently chromogen solutions A and B (HRP substrates) were added.
- The plate was incubated again at 37°C for 15 minutes. With the combination of HRP and chromogen solutions, the resulting reaction will be producing the color.
- Using an ELISA plate reader, absorbance was instantly measured at 450 nm after adding sulfuric acid to stop the reaction after 15 minutes (Bio Rad Model No: PR4100).

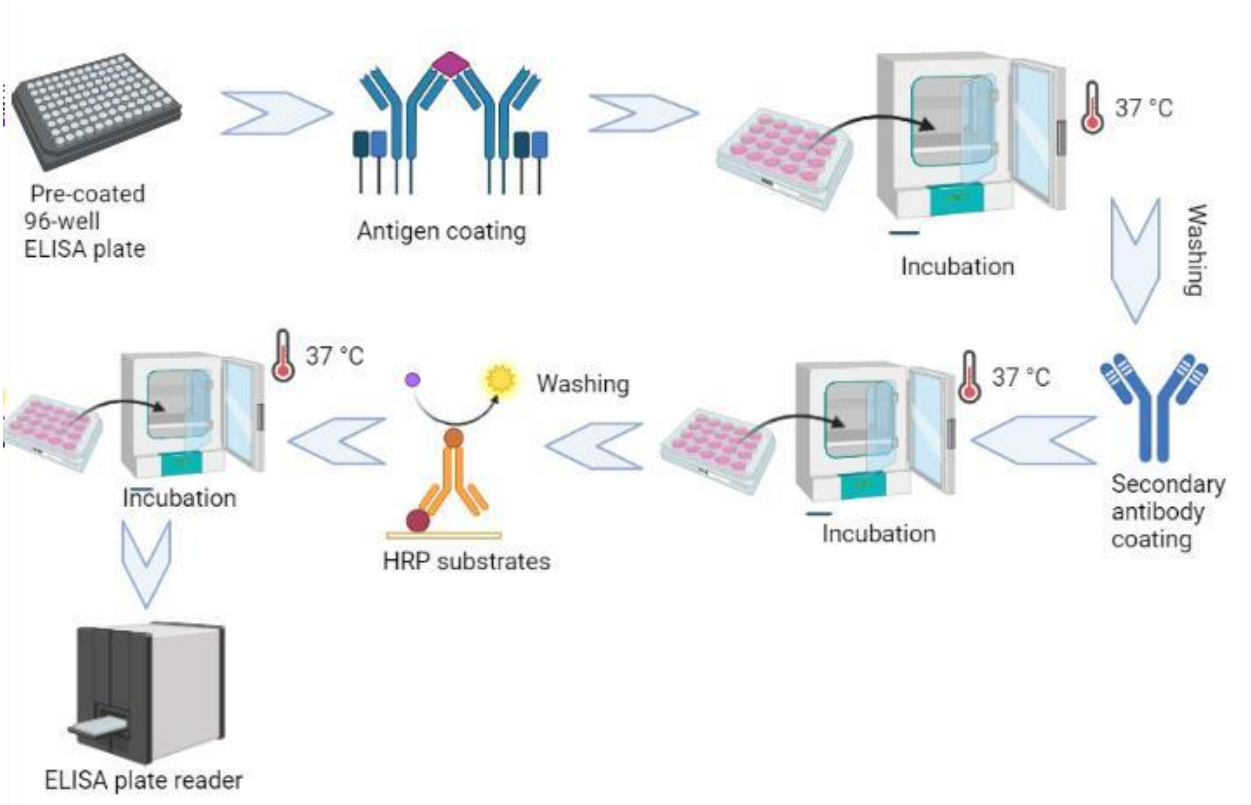

Figure 3.7 Flow chart of ELISA procedure.

3.7.2. ELISA results analysis

MyAssays Online (https://myassays.com/index.html) and their desktop application "MyAssays Desktop" were used to assess ELISA data. For OD values acquired for the standard samples, for which the concentration was known, the standard curve was created. The "Best Fit" tool in the MyAssays desktop application automatically generates standard curve by analyzing the standard values and drawing the best curve in accordance with the appropriate curve fitting model identified by our sample data. According to the "abbexa" corporate website (https://www.abbexa.com/elisa-standard-curve), a good standard curve has an R2 value over 0.95. Access: August 24, 2021. (abbexa, 2021) and after comparing the sample OD values to the standard curve, the sample OD values are calculated. The "dilution factor," or 5, was first multiplied by the OD values of our samples. The concentration of proteins is calculated from the OD values by extrapolating the values outside of the curve based on the trendline derived from the standardurve.

3.8 Statistical analysis

Statistical Package for Social Sciences (SPSS) version 26 (IBM Corp., 2016) was used to conduct the statistical analysis. Armonk, NY: IBM Corp., IBM SPSS Statistics for Windows, Version 24.0. P values of 0.05, 0.005, and 0.0005 with a confidence interval of 95% were regarded as significant for all the statistical tests. The quantitative results from the ELISA as well as demographic information of the patient and control populations were subjected to subsequent statistical tests.

3.8.1. Descriptive statistics

In SPSS, descriptive statistics were calculated to ascertain sample frequencies and percentages by age and gender. The SPSS "Chart Builder" tool was used to create bar charts and pie charts as visual representations. To further understand the study population, cross tabulations of age groups vs. gender were also carried out in both the test and control populations. The patient's information was also separated into "causalities of HCC" based on the proportion of viral and non-viral cases, and further separated into HCV and HBV for viral instances. Although the frequency and percentage were determined in SPSS, Graph Pad Prism version 8 was used to visualize this data using bar charts.

3.8.2. Mean concentration values

The 'Compute means' tool in SPSS version 26 was used to calculate the mean concentration values and standard deviations of each protein proposed as a possible biomarker. The graphs, however,

were created using Graph Pad Prism version 8 to compare mean values of the test and control samples.

3.8.3. ROC curve analysis

With the use of the "ROC analysis tool" in SPSS version 26, a Receiver Operator Characteristic (ROC) curve was created. A biomarker's capacity to discriminate between test and control samples is determined by the ROC curve (Hoo, Candlish and Teare, 2017). For each potential cut off value, a graph between "sensitivity" and "1-specificity" is drawn. The choice of the cut off value is highly arbitrary and determined by the study's goals. The cut off value with the highest sum of sensitivity and specificity is chosen to assess a biomarker's diagnostic potential. (Choi *et al.*, 2019). Using ROC curve analysis, area under the curve (AUC) was also calculated. A biomarker's diagnostic potential increases with the value of AUC (Hoo, Candlish and Teare, 2017c). AUC levels close to 0.5 imply no or very little promise for biomarkers, whereas AUC values close to 1 indicate excellent potential for biomarkers. All AUC values were calculated with 95% confidence level.

3.8.4. Determination of diagnostic ability

Number of True Positive (TP), True Negative (TN), False Positive (FP), and False Negative (FN) cases from the data set can be calculated by calculating the cut off value. If a greater mean concentration of the biomarker was found in patient samples, all values of the biomarker over the cut off were considered positive results and values below the cut off were reflected negative results. This is equivalent to a biomarker's diagnostic capacity to differentiate between test and control samples.

3.8.5. Determination of diagnostic parameters

After counting the number of True Positives (TP), True Negatives (TN), False Positives (FP), and False Negatives (FN), diagnostic metrics including Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value can be determined (FN). The values of the diagnostic parameters were calculated using these numbers in the Medcalc online diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic test.php). The following formulas can be used to manually calculate the values of sensitivity, specificity, and accuracy.

$$Sensitivity = TP/P$$

$$Specificity = TN/N$$

$$Accuracy = TP + TN / P + N$$

3.9. Western blot analysis

Given that the antibodies employed in ELISA are specific to one protein, Western blot was utilized to confirm the results qualitatively and quantitatively. For qualitative validation, the first band in the first gel was set as a reference band and the conv=centration of remaining bands was then calculated in comparison to the reference band by using ImageLab software. Western blotting was performed on putative biomarkers that showed positive results as a biomarker using selected samples (test = 12; control = 4). Its methodology and optimization can be divided into the phases given below:

3.9.1. Performance and optimization of SDS-PAGE gel electrophoresis

- Using gel casting stands, casting frames, etc. that Wix Technologies Ltd. procured, SDS page gels were casted based on the selected gel concentration. Wet transfer equipment for SDS PAGE and western blot was bought from Wix Technologies Ltd.
- By casting gels with various concentrations, such as 8%, 12%, and 16%, the SDS Page gel concentration was optimized. The general guideline is that a higher concentration of gel must be cast because the target protein has a smaller molecular weight. In our case, 12% gel was selected following electrophoresis on a variety of gel types.
- Samples were loaded mixed with loading dye and diluted in PBS and then the resolving and stacking gels were placed in an electrophoretic tank using "Tris-glycine" buffer or electrophoresis buffer (Phosphate buffer saline).
- Multiple dilutions, such as 2X, 4X, 10X, etc., were loaded in various wells of the same gel in order to optimize the sample concentrations. To find the ideal loading concentration, bands were afterwards detected using Ponceau S stain. Since serum is highly dense mixture of proteins, it was directly employed as a sample in our case. The greatest results, however, came from combining 1 µl of serum with 19 µ of PBS and 5 µl of loading dye. The loading dye was bought from Thermo Fisher Scientific Ltd.
- The materials were placed into the remaining wells after a molecular ladder that was acquired from Thermo Fisher Scientific Ltd. was loaded into the first well.
- Bands moved to the bottom of the gel during 40 minutes of 170V electrophoresis.

- Following electrophoresis, the gels were removed and placed in Transfer buffer, which was purchased from Thermo Fisher Scientific Ltd. A blotting sandwich was prepared and loaded in the blotting chamber, consisting of the following components in the order: sponge, filter paper, gel nitrocellulose membrane, filter paper, and sponge.
- The blotting tank was filled with transfer buffer because the wet transfer method was being used, and an ice pack was also added to regulate temperature. 20V of blotting voltage was used for 2.5 hours.
- The sandwich was opened after 2.5 hours, and nitrocellulose membranes were exposed to Ponceau S solution for 5 minutes in order to gauge the effectiveness of the blotting.
- If clear bands are seen, the membranes were rinsed in water to remove Ponceau S and then immersed overnight in blocking buffer, which is composed of 5% bovine serum albumin (BSA) in PBS.
- The membrane was taken out of the blocking solution the following day and subjected to primary antibody treatment. The main antibody was diluted using an antibody diluent (PBS-Tween with sodium azide or some other anti-microbial agent). The user manual for the antibody, which the manufacturer provided, determined dilution concentration. Primary antibody dilution concentrations typically vary from 1:1000 to 4:1000.
- The membrane was placed on a rotating platform and immersed in primary antibody solution for one hour to ensure constant shaking.
- After discarding the primary antibody solution, the membrane was vigorously shaken for 5 minutes while being immersed in wash buffer (TBST). The same antibody diluent used for the primary antibody was then utilized to treat the membrane with an HRP-conjugated secondary antibody.
- The concentration of the secondary antibody dilution is determined using the manufacturer's user manual. The secondary antibody's dilution concentration normally ranges from 1:1000 to:10000.
- Membrane was coated with secondary antibody and placed on a rotating platform for an hour in order to maintain steady shaking. After removing the secondary antibody solution membrane was vigorously shaken five times for five minutes each while being rinsed with wash buffer (TBST).

- Thermo Fisher Scientific Ltd.-purchased ECL reagents (A & B) were applied in equal amounts to the membranes for 5 minutes while it was dark.

  After placing the membranes under Chemi-Doc XRS+, which was obtained from Bio Rad Laboratories Ltd., the signal was discovered using Chemi-"Auto-exposure" Doc's settings.

3.10 Multiplex ELISA:

Multiplex ELISA was performed to confirm whether a combination of two or more proteins yielded better sensitivity and specificity values when compared with individual proteins.

3.11 Multiplex Procedure:

Following procedure was adopted in our lab for detection of two antibodies simultaneously i-e ADH6 and AFP:

- The 96-well ELISA plate has wells on it for the primary antibody to be coated in during the ELISA method. In our case, firstly, we coated antigen on the ELISA plate. 10 µl blood serum form 150 HCC patients and 50 controls were added in each well separately. As a result the test sample concentrations are diluted five times.
- Standard wells were initially placed on the ELISA plate. Through serial dilutions with the standard diluent, provided in the antibody buffer kit. These wells received repeated applications of ELISA standards with established concentration values from the ELISA kit that had previously been obtained from Nanjing Par Biochem Ltd. 100 µl of the standard sample was added to the first two wells (duplicates) in order to do serial dilutions, and then 50 µl of the standard diluent was added to both wells. The 50 µl was thereafter taken out and dumped into the first and second wells. Then, 50 µl each from the third and fourth wells were added. Then, 50 µl of typical diluent were delivered to the third and fourth wells, respectively. The fifth and sixth wells were added 50 µl from the third and fourth wells. After that, the fifth and sixth wells each received 50 µl of standard diluent, and so on.
- Two wells were left unfilled so that these might serve as "blanks". All ELISA phases were carried out on blanks, except for adding the sample and HRP conjugate reagent. The remaining wells were all then filled with the 40 µl of sample diluent.

- After adding sample diluent, primary antibody was coated on 96 well ELISA plate. The primary antibody was diluted by using a dilution ratio of 1:3000, according to the manufacturer's guidelines. To enable the coated antibody to bind with the samples' proteins, plate was then incubated for 30 minutes at 37 degrees Celsius. The plates were incubated by having a sealing membrane placed over them. The wells were dried by swinging once the closure membrane was opened and the samples inside the wells were removed after 30 minutes.
- Subsequently, washing buffer was applied to the wells five times, and subsequently HRP conjugated secondary antibody was added. The dilution ratio recommended for secondary antibody is 1:5000 according to the user manual provided by the manufacturer. Blank wells did not have the HRP conjugated antibody added to them.
- The plate was again allowed to stand for 30 minutes for allowing the secondary antibody to bind with the protein bound primary antibodies.
- After 30 minutes all wells were washed with washing buffer (5 times) and subsequently chromogen solutions A and B (HRP substrates) were added.
- The plate was once again left to stand at 37°C for 15 minutes. When HRP and chromogen solutions are combined, color is produced.
- Using an ELISA plate reader, absorbance was instantly measured at 450 nm after adding stop solution-sulfuric acid to stop the reaction after 15 minutes (Bio Rad Model No: PR4100).

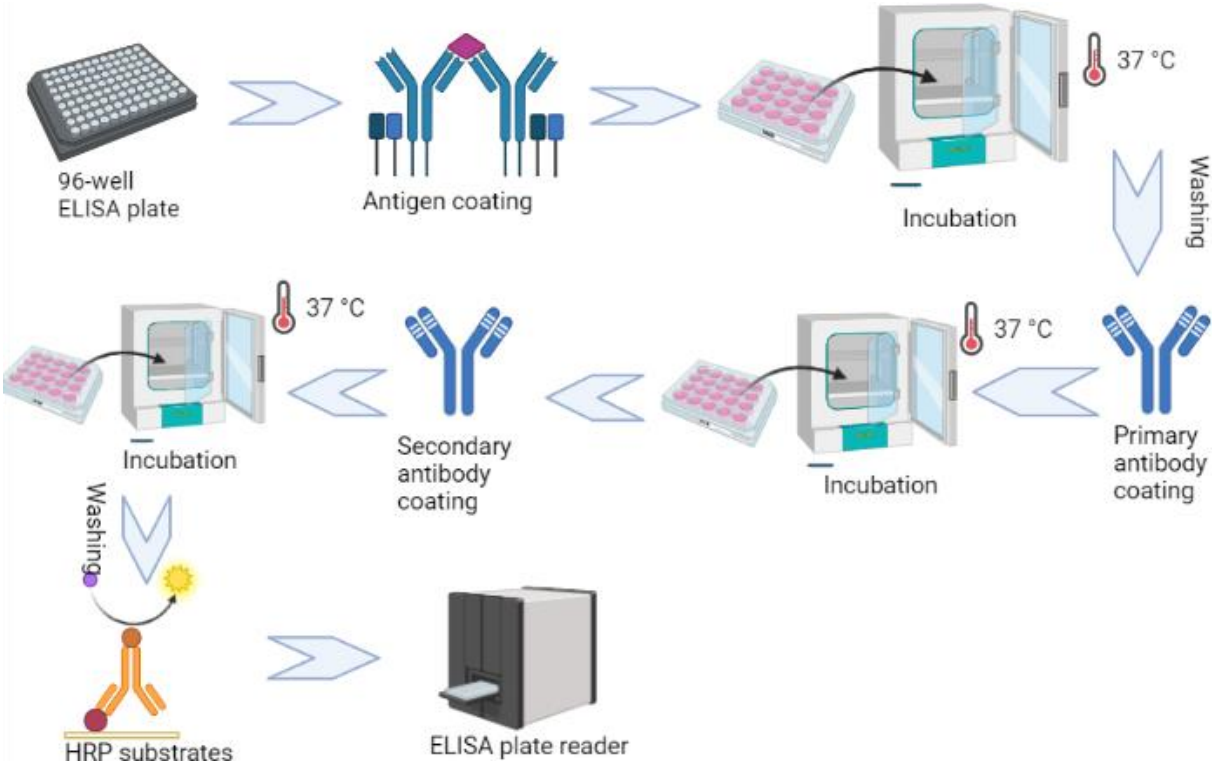

Figure 3.8 Flow chart of procedure adopted to perform Multiplex ELISA.

Chapter 4 Results

In the first phase of the project (Abdullah Ahmed MS Thesis 2021), ELISA was performed on four proteins C8A, SERPINC1, HSD11B1 and MBL2. Among these four proteins, C8A showed excellent potential to function as biomarker for HCC diagnosis with sensitivity and specificity values (85.33% and 100%), these values are better than the already established gold standard biomarker for HCC diagnosis i-e AFP. Among other three proteins, SERPINC1 showed moderate potential to act as efficient biomarker for HCC diagnosis but sensitivity and specificity values were similar to AFP while the remaining two proteins did not show any significant ability to act as efficient biomarkers for HCC diagnosis. The results were qualitatively validated for C8A via Western Blot analysis. The current study focusses on the ELISA of the remaining three proteins i-e Cyp2a6, UPB1 and ADH6, qualitatively and quantitatively validation by performing Western Blot and then Multiplex ELISA to enhance sensitivity and specificity values when different combinations of protein biomarkers are employed.

4.1 ELISA results analysis:

The aforementioned sample population was subjected to an ELISA analysis to determine the presence of the three proposed biomarkers CyP2A6, UPB1 and ADH6 Myassays.com was used to examine ELISA results and SPSS version 26 and GraphPad Prism were used to compute clinical value of these biomarkers. Following repeated statistical analyses, concentrations acquired via ELISA were utilized to determine the sensitivity and specificity as shown in figure 4.1 below.

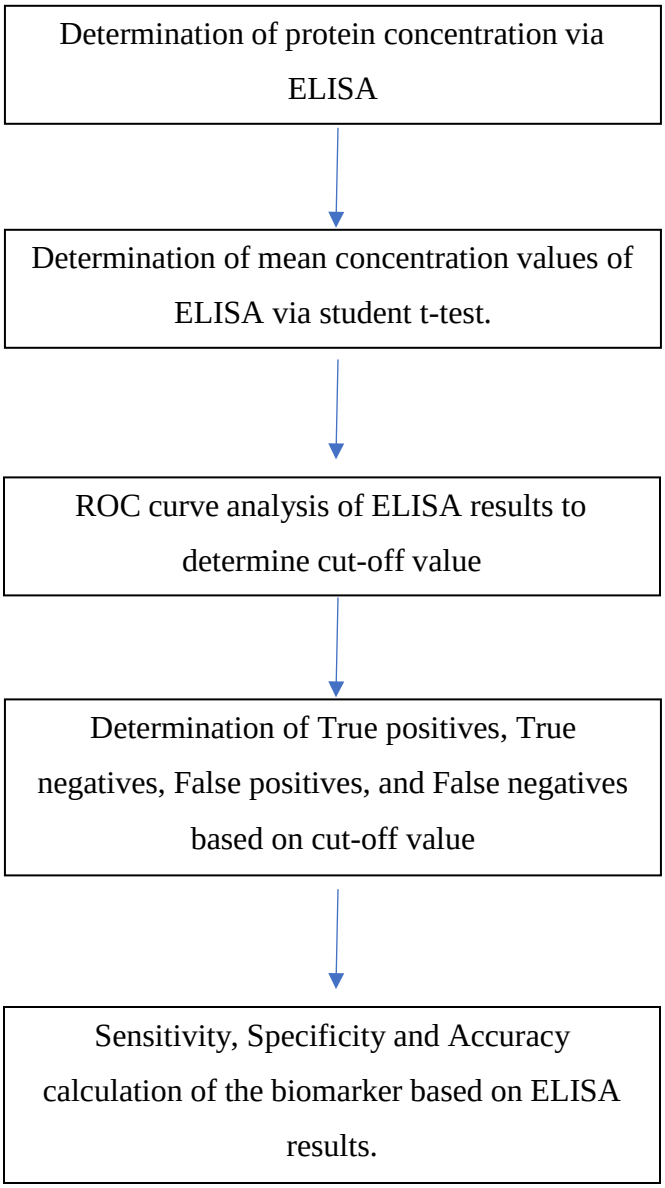

Figure 4.1. Flowsheet diagram of statistical analysis from ELISA results to determine sensitivity, specificity and accuracy of a biomarker.

Grouping of data plays avital role when dealing with large data. Usually, a bar chart is used to display such data. Individual observations of a variable are grouped together to create data, which may then be easily analysed or summarised by looking at the frequency distribution of the groups. Group statistics of all three proteins under test are summarized in the following figure 4.2. This includes the mean values of the protein concentrations obtained as well as the standard deviation of these biomarkers.

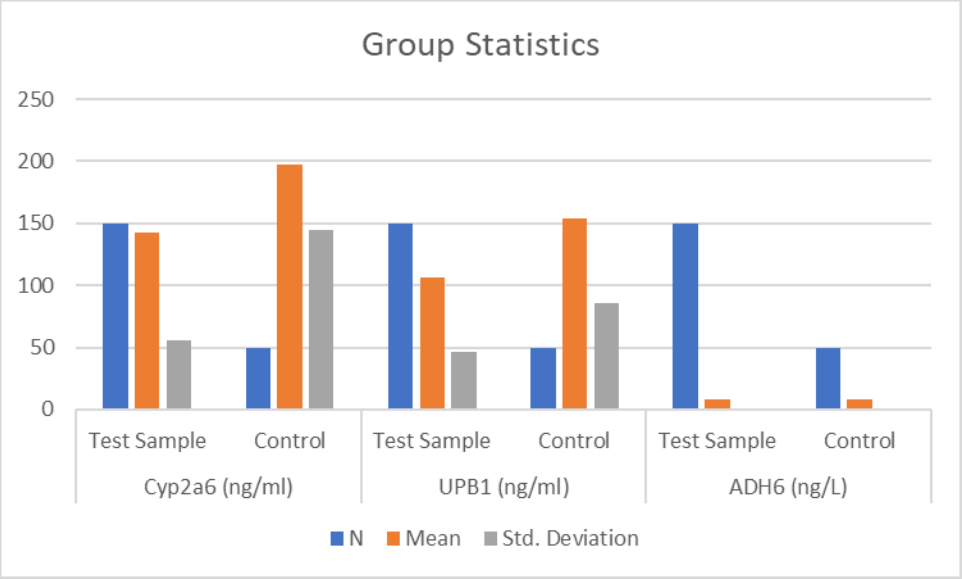

Figure 4.2: Group statistics of 3 proteins (all potential candidate biomarkers) computing the mean values and standard deviations in both test samples and controls. This figure highlights the mean concentrations of these proteins found in both HCC patients (n=150) and healthy individuals (n=50) along with the standard deviation.

These proteins are individually discussed as follows.

4.1.1. Cyp2a6

4.1.1.1. Mean concentration values

Cyp2a6, the first biomarker candidate was checked in test and control samples. The mean concentration values in control samples (healthy patients) were elevated as compared to test samples (HCC patients) as shown in the Figure 4.3.

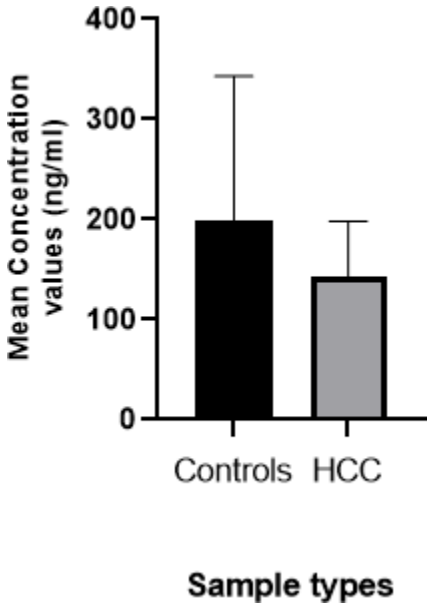

Figure 4.3: Mean values of controls and test samples for Cyp2a6**.** Mean concentration values (ng/ml) of Cyp2a6 in 150 HCC patient's blood samples and healthy controls. The mean value in control samples is slightly elevated (198.2 ng/ml) compared to test sample population (142.1 ng/ml).

4.1.1.2. ROC curve analysis of Cyp2a6

Based upon the difference in mean values, the diagnostic ability of Cyp2a6 was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure. The confidence interval (CI) was set at 95% and the ROC curve was obtained. The AUC was found to be 0.554. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 160.67 ng/ml. The ROC curve for Cyp2a6 is shown in Figure 4.4. ROC curve determines the distinguishing ability of a biomarker between test and control samples (Hoo, Candlish and Teare, 2017)**.** A graph is plotted between 'sensitivity' and '1-specificity' for every possible cut off value. The choice of cut off value is very subjective, as it depends upon the objectives of the study. For determining the diagnostic ability of a biomarker, cut off value with the highest sum of sensitivity and specificity is selected (Choi et al., 2019).

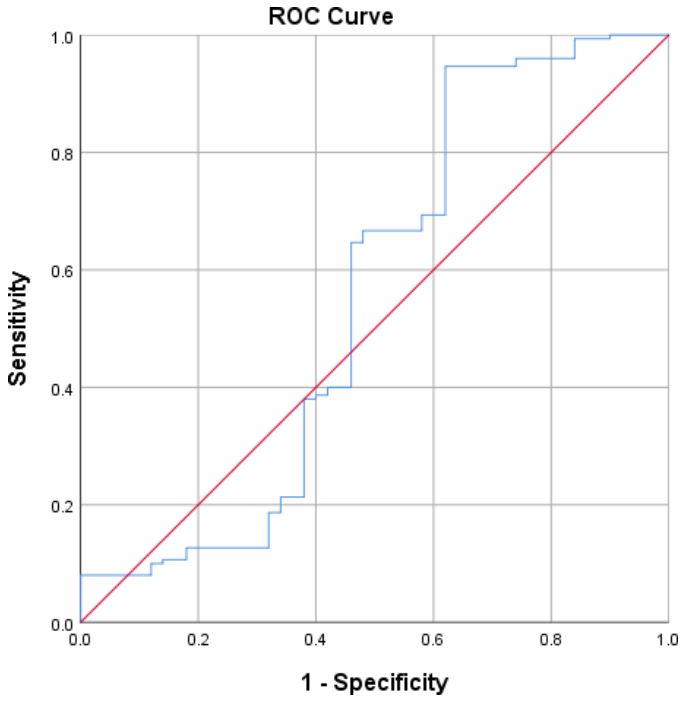

Figure 4.4: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.554 and the cut off value of 160.67ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.1.1.3. Determination of diagnostic ability of Cyp2a6

Based upon the cut-off value, sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers give an idea of the diagnostic potential of biomarker. Following is the Table 4.4 containing the numbers of True positives, false positives, True negatives, and false negatives.

Table 4.1: Diagnostic ability of Cyp2a6 to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False negatives (FN). Positive cases in control are False positives (FP) whereas negative cases in controls are True negatives (TN).

Cyp2a6's diagnostic ability to distinguish HCC and control samples

Count

		Biomarker Cyp2a6		
		Present	Absent	Total
Case Type	Test Sample	96	54	150
	Control	23	27	50
Total		119	81	200

4.1.1.4. Determination of diagnostic parameters

Upon determination of the number of True positives, True negatives, False positives and False negatives, different parameters relating to a biomarker's diagnostic ability were computed via MedCalc diagnostic test evaluation calculator. The sensitivity was determined as 64.00% whereas the specificity was determined at 54.00%. The accuracy of the test was determined at 61.57%. These parameters are given in the Table 4.2.

Table 4.2: Diagnostic parameters of Cyp2a6 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	Value	95% Confidence Interval
Sensitivity	64.00%	55.77% to 71.67%
Specificity	54.00%	39.32% to 68.19%
Positive Predictive Value (*)	80.67%	75.13% to 85.22%
Negative Predictive Value (*)	33.33%	26.38% to 41.10%
Accuracy (*)	61.50%	54.38% to 68.28%

4.2.2. UPB1

4.2.2.1. Mean concentration values

UPB1 also showed a lower mean concentration value in test samples (142.11 µg/ml) compared to the control samples (198.2 µg/ml) as shown in the Figure 4.5. The mean values are obtained by conducting a student t-test on the concentration values of HCC patients and healthy controls obtained via the quantitative ELISA test.

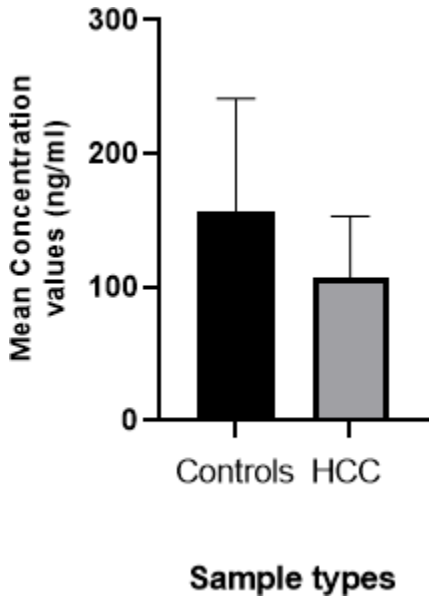

Figure 4.5: Mean values of controls vs test samples for UPB1.

Mean concentration values (µg/ml) of UPB1 in 150 HCC patient's blood samples and 50 healthy controls. The mean value in test samples is lower in test samples (142.11 µg/ml) compared to the control sample population (198.2 µg/ml). This low mean value of UPB1 in HCC patients hints at the disturbance of complement system in HCC patients. Further research in HCC molecular pathways will pave the way to determine pathways that interfere with the complement system.

4.2.2.2. ROC curve analysis of UPB1

Based upon the difference in mean values, diagnostic ability of UPB1 was calculated via performing ROC curve analysis on SPSS version 26 as shown in the Figure 4.10. The Confidence Interval (CI) was set at 95% and the ROC curve was obtained. The AUC was found to be 0.665. The cut off value was determined by selecting the value with the highest sum of sensitivity and specificity at 117.58 µg/ml. The ROC curve for UPB1 is given in Figure 4.6.

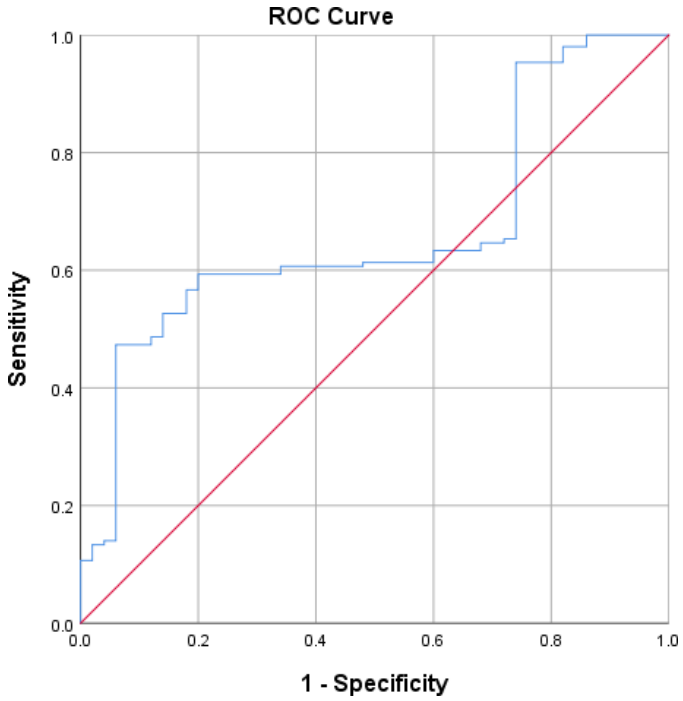

Figure 4.6: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.665 and the cut off value of 117.58 µg/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.2.2.3. Determination of diagnostic ability of UPB1

True positives, True negatives, False positives and False negatives in both HCC and Control population is determined by using cut-off-value. Diagnostic ability of the candidate numbers is evaluated by using these numbers. The following Table 4.3 demonstrates the numbers of True positives, True negatives, False positives and False negative in both Control and Test samples.

Table 4.3: Diagnostic ability of UPB1 to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False negatives (FN). Positive cases in control are False- positives (FP) whereas negative cases in controls are True negatives (TN). The term "Present" in the figure below indicate that the disease is present in both groups while the term "Absent" means that the disease is not found in both groups.

UPB1's diagnostic ability to detect HCC and control samples

Count

		Biomarker UPB1		
		Present	Absent	Total
Case Type	Test Sample	89	61	150
	Control	17	33	50
Total		106	94	200

4.2.2.4. Determination of diagnostic parameters of UPB1

MedCalc diagnostic test evaluation calculator was used to determine different parameters concerning the diagnostic ability of the candidate biomarker by using the number of True negatives, False positives, and False negatives The sensitivity was determined as 59.73% whereas the specificity was determined at 66%. The accuracy of the test was determined as 61.31%. These parameters are given in the Table 4.4. All of these values are computed from ELISA results.

Table 4.4 Diagnostic parameters of UPB1 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	Value	95% Confidence Interval
Sensitivity	59.73%	51.39% to 67.68%
Specificity	66.00%	51.23% to 78.79%
Positive Predictive Value (*)	83.96%	77.68% to 88.73%
Negative Predictive Value (*)	35.48%	29.38% to 42.10%
Accuracy (*)	61.31%	54.16% to 68.11%

4.2.3. ADH6

4.2.3.1. Mean concentration values

ADH6, the last of the three candidate biomarkers showed a higher mean value in test samples (8.632 ng/ml) compared to control samples (8.010 ng/ml) as shown in the Figure 4.7.

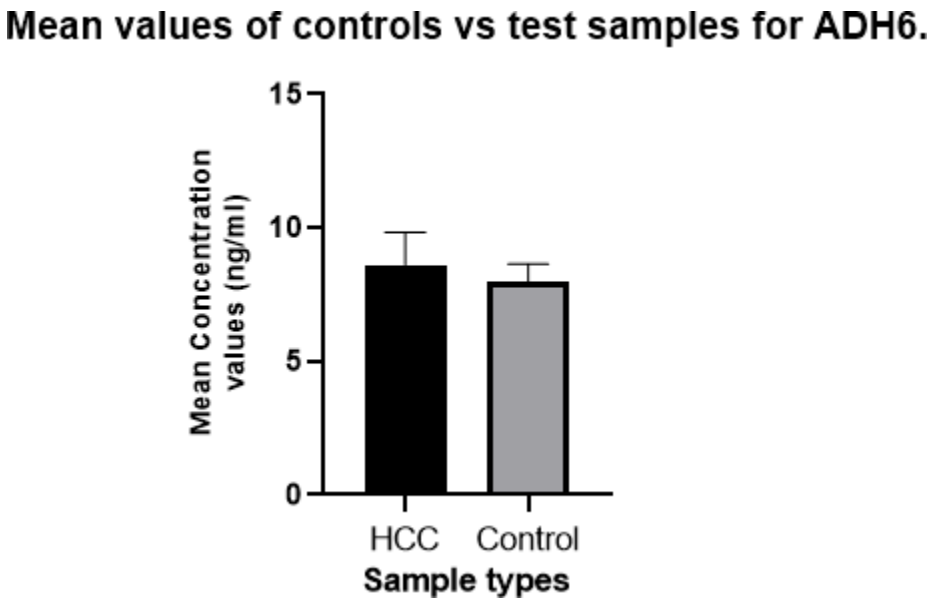

Figure 4.7: Mean concentration values (ng/ml) of ADH6 in 150 HCC patient's blood samples and healthy controls. The mean value in test samples is slightly elevated (8.632 ng/ml) compared to control sample population (8.010 ng/ml). The expression is increased in this case.

4.2.3.2. ROC curve analysis of ADH6

Based upon the difference in mean values, the diagnostic ability of ADH6 was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure 4.12. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.674. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 117.5845 ng/ml. The ROC curve for ADH6 is shown in Figure 4.8.

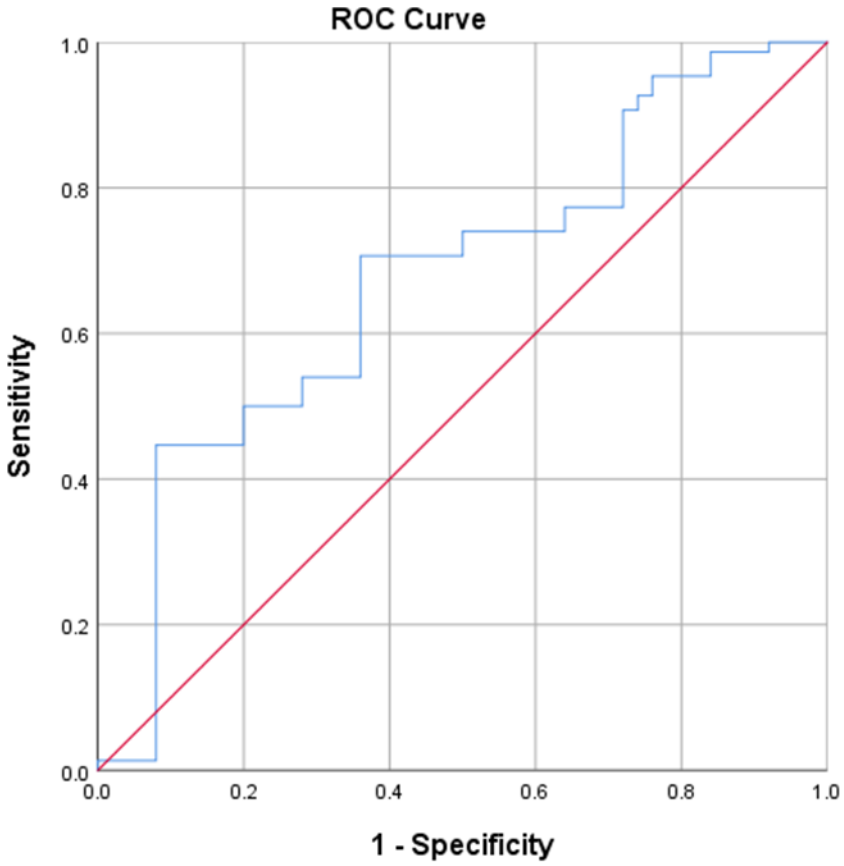

Figure 4.8: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.674 and the cut off value of 117.58 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.2.3.3. Determination of diagnostic ability of ADH6

Based upon the cut-off value, sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers provide potential diagnostic of the biomarker. Following is the Table 4.5 containing the numbers of True positives, False positives, True negatives, and False negatives based upon the cut-off value established previously.

Table 4.5: Diagnostic ability of ADH6 to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False negatives (FN). Positive cases in control are False positives (FP) whereas negative cases in controls are True negatives (TN).

ADH6's diagnostic ability to distinguish HCC and control samples

Count

		Biomarker ADH6		
		Positive	Negative	Total
Case Type	Test Sample	106	44	150
	Control	18	32	50
Total		124	76	200

4.2.3.4. Determination of diagnostic parameters of ADH6

Upon determination of the number of True positives, True negatives, False positives and False negatives, different parameters relating to a biomarker's diagnostic ability were computed via MedCalc diagnostic test evaluation calculator. The sensitivity was determined as 70.67% whereas the specificity was determined at 64.00%. The accuracy of the test was determined at 69.00%. These parameters are given in the Table 4.6.

Table 4.6 Diagnostic parameters of ADH6 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	Value	95%Confidence Interval
Sensitivity	70.67%	62.69% to 77.81%
Specificity	64.00%	49.19% to 77.08%
Positive Likelihood Ratio	88.32%	80.05% to 89.63%
Negative Likelihood Ratio	53.97%	34.47% to 50.14%
Accuracy	69.00%	62.09% to 75.33%

4.3. Combined diagnostic parameters of three proteins

The above-mentioned diagnostic parameters of all the three proteins namely Cyp2a6, UPB1 and ADH6 are given in Figure 4.9.

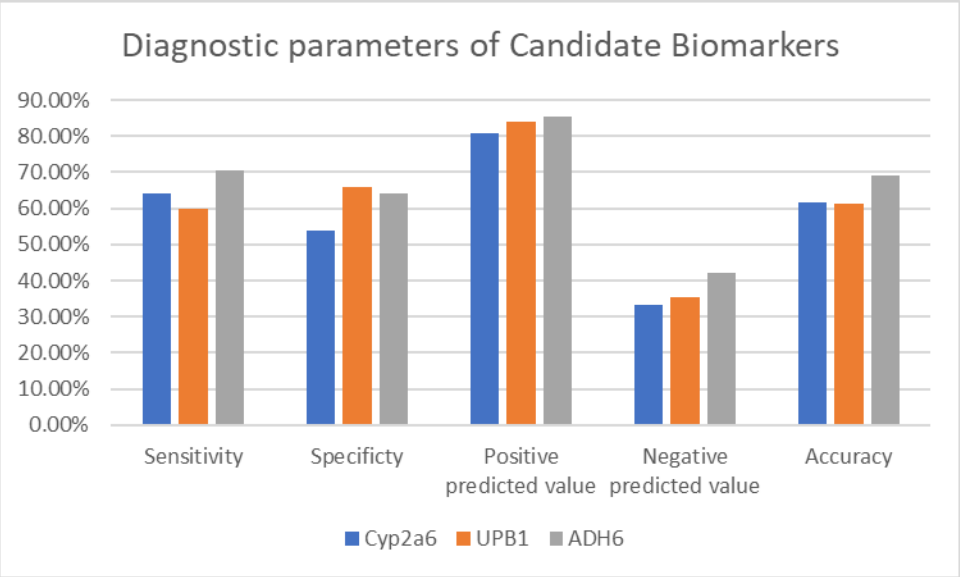

Figure 4.9: Combined diagnostic parameters of Cyp2a6, UPB1 and ADH6. Among them ADh6 shows the highest sensitivity and specificity values followed by UPB1 and Cyp2a6 which shows moderate potential as compared to ADH6.

4.4. Western Blot analysis

Western blot was performed to qualitatively and quantitatively to verify the ELISA results to check if the antibodies used are specific to one protein. The kit we aim to design will be ELISA based and the specificity of those antibodies is verified here by performing Western blotting.

4.4.1. Cyp2a6 Western Blot

Cyp2a6 showed moderate potential to serve as biomarker for HCC upon ELISA. To validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of Cyp2a6 in these samples. The existence of a continuous band exhibits the presence of Cyp2a6 and the antibody specific reactivity to Cyp2A6, thus qualitatively confirming the validity of our ELISA results for Cyp2a6The results of western blot analysis are shown in the Figure 4.10.

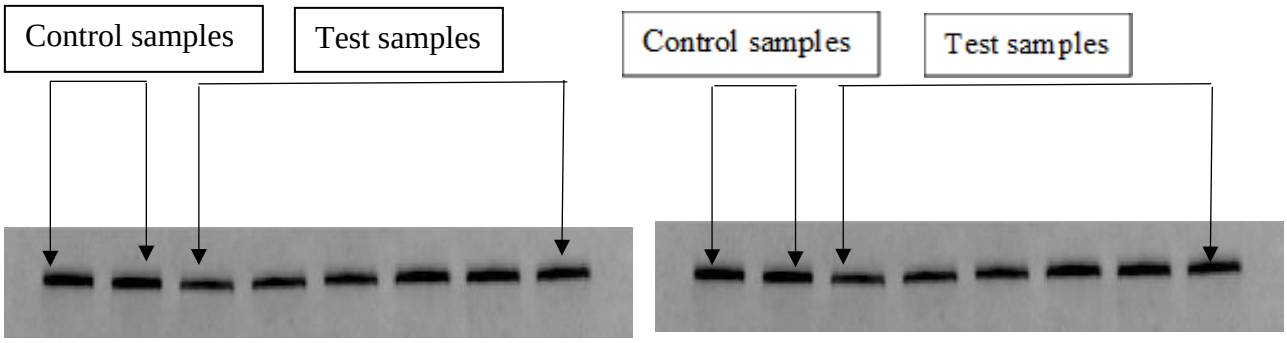

Figure 4.10: Western blots of 4 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of Cyp2a6 and the antibody specific reactivity to Cyp2A6, thus qualitatively confirming the validity of our ELISA results for Cyp2a6. The age range of test samples lies between 50-60 years while majority of the patients were male.

4.4.1.1. Determination of mean concentration for Cyp2a6

Cyp2a6 showed a higher relative mean concentration in control samples when compared with test samples. The concentration was obtained by setting a band in the second lane as reference and subsequently comparing these with the rest of the samples by using ImageLab software. Significant difference (t=5.293, p=0.0018) is found between mean intensity in control and test

samples where the p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The graph showing the concentration of control as well as test sample is shown in figure 4.11 below.

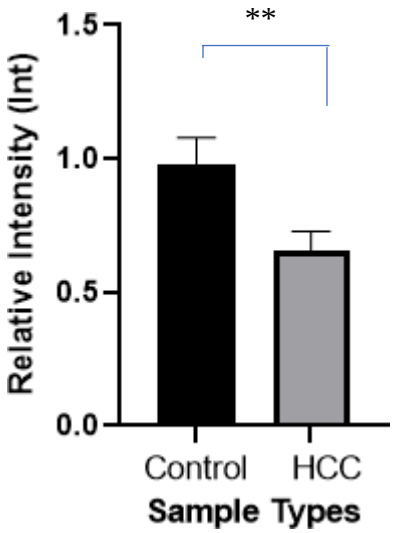

Figure 4.11 The graph showing relative intensities of all the bands in control as well as test samples. The relative intensity is observed to be greater in control samples (0.9787 ng/ml) compared to test samples (0.6787). Significant difference ($t=5.293$, $p=0.0018$) is found between mean intensity in control and test samples is observed. The p value is observed to be $p<0.05$ which indicates significant difference. The quantitate validation was performed via ImageLab software. These results are in accordance with the data obtained from ELISA. 4.4.2 Western blot of UPB1

UPB1 showed relatively lower potential compared to other two protein biomarkers. To validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of UPB1 in these samples. The results of western blot analysis are shown in the Figure 4.12.

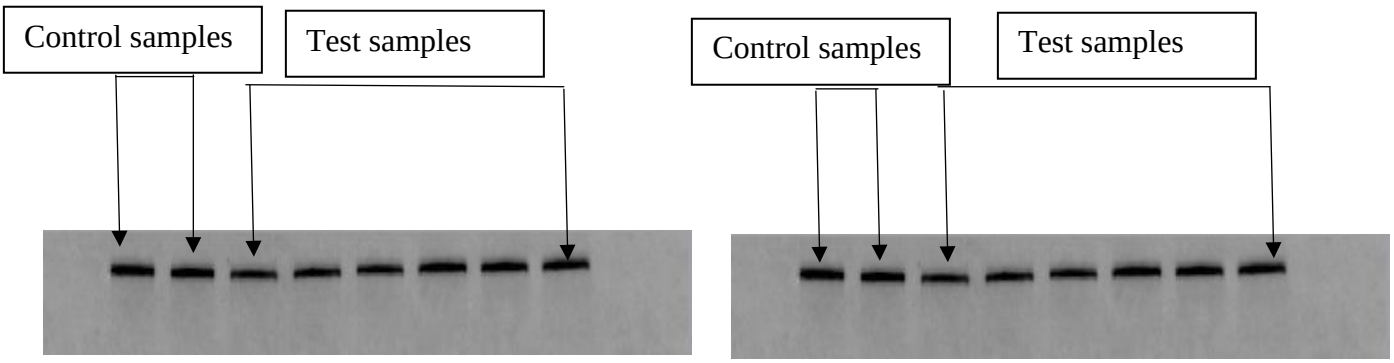

Figure 4.12 western blot of 04 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of Cyp2a6 and the antibody specific reactivity to UPB1, thus qualitatively confirming the validity of our ELISA results for UPB1. The age range of the test samples was between 50-60 years and majority were male.

4.4.2 Determination of mean concentration of UPB1

UPB1 showed a higher relative mean concentration in control samples on comparison with the test samples. The concentration was obtained by setting a band in the second lane as reference and then comparing them with the rest of the samples. The relative intensity is observed to be greater in control samples (1.22 ng/ml) compared to test samples (0.98). The p value is observed $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The graph showing the concentration of control as well as test sample is shown in figure 4.13 below.

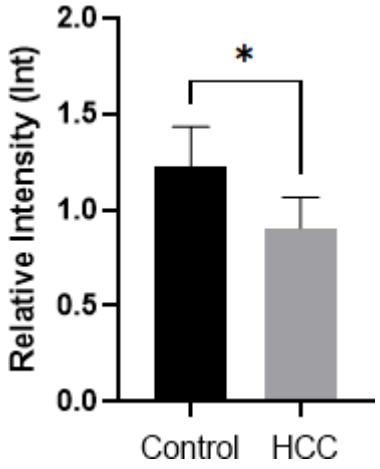

Figure 4.13 The graph representing the relative intensities of control samples compared to test samples. The relative intensity is observed to be greater in control samples (1.22 ng/ml) compared to test samples (0.98). Significant difference (t=2.450, p=0.0498) is found between mean intensity in control and test samples. The p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA.

4.4.3 Western blot of ADH6

Among all the three potential protein biomarker candidates, ADH6 showed relatively greater potential to act as an efficient biomarker. Therefore to validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of ADH6 in these samples. The existence of a continuous band exhibits the presence of ADh6 and the antibody specific reactivity to Adh6, thus qualitatively confirming the validity of our ELISA results for ADH6. The results of western blot analysis are shown in the Figure 4.14.

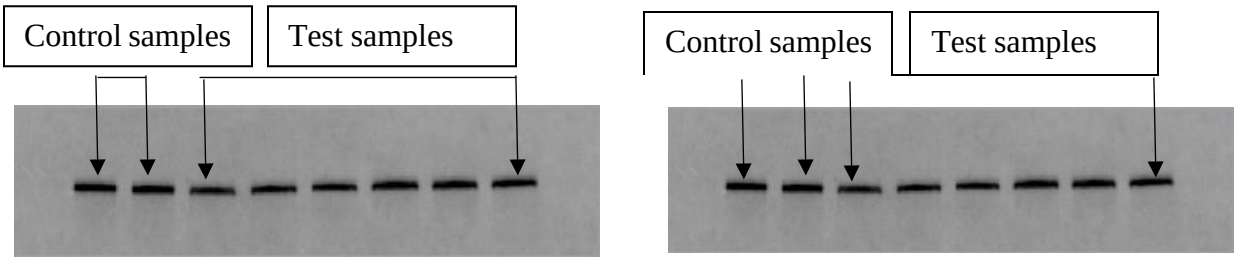

Figure 4.14 Western blot for ADH6 along with protein ladder. Western blot was conducted on a total of 04 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-

4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of ADh6 and the antibody specific reactivity to Adh6, thus qualitatively confirming the validity of our ELISA results for ADH6.

4.4.3.1 Determinartion of relative intensities for ADH6

ADH6 showed a higher relative mean concentration in control samples when compared with test samples. The concentration was obtained by setting a band in the second lane as reference and comparing them with the rest of samples. The graph showed the concentration of control as well as test samples (figure 4.15)

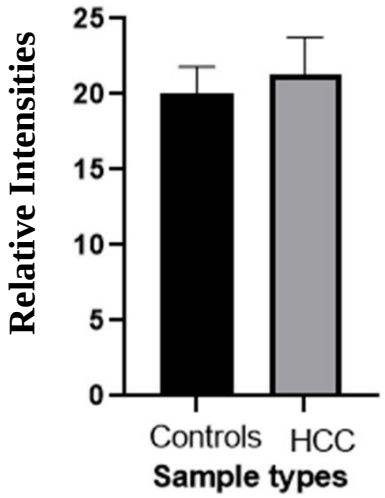

Figure 4.15 The graph representing the relative intensities of control samples compared to test samples. The relative intensity is observed to be greater in test samples (21.25 ng/ml) compared to the control samples (20.3). Significant difference (t=0.8076, p=0.045) was observed between mean intensity in control and test samples. The p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The software used to measure the concentration of the protein was ImageLab software.

4.4.4. SERPIINC1 Western Blot

SERPINC1 showed moderate potential to serve as biomarker for HCC upon ELISA almost as similar to AFP. To validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of SERPINC1 in these samples. The existence of a continuous band exhibits the presence of SERPINC1 and the antibody specific reactivity to SERPINC1, thus qualitatively confirming the

validity of our ELISA results for SERPINC1.The results of western blot analysis are shown in the Figure 4.10.

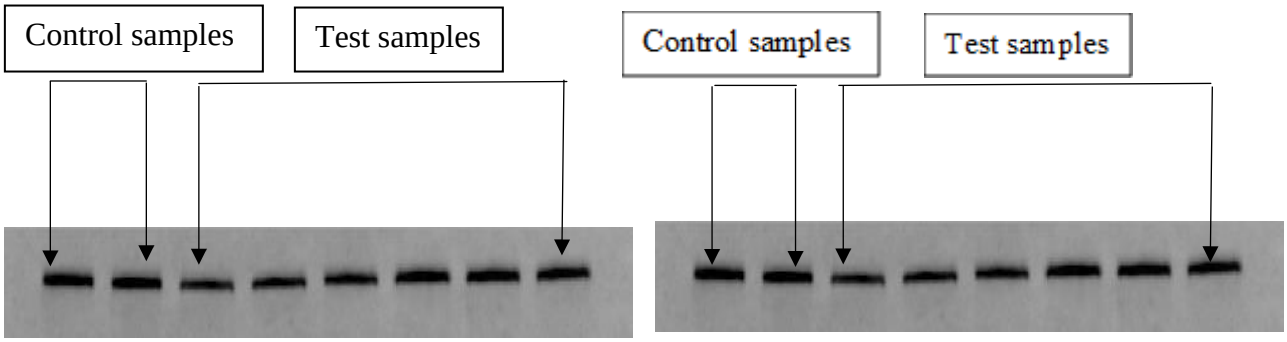

Figure 4.16: Western blots of 4 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of SERPINC1 and the antibody specific reactivity to SERPINC1, thus qualitatively confirming the validity of our ELISA results for SERPINC1. The age range of test samples lies between 50-60 years while majority of the patients were male.

4.4.1.1. Determination of mean concentration for SERPINC1

SERPINC1 showed a higher relative mean concentration in control samples when compared with test samples. The concentration was obtained by setting a band in the second lane as reference and subsequently comparing these with the rest of the samples by using ImageLab software. Significant difference ($t=5.393$, $p=0.0028$) is found between mean intensity in control and test samples where the p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The graph showing the concentration of control as well as test sample is shown in figure 4.17 below.

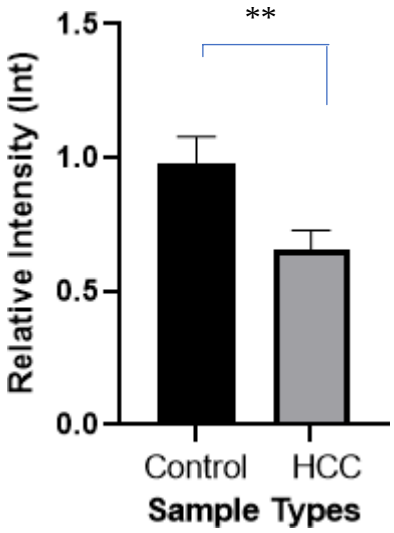

Figure 4.17 The graph showing relative intensities of all the bands in control as well as test samples. The relative intensity is observed to be greater in control samples (0.8787 ng/ml) compared to test samples (0.5787). Significant difference (t=5.093, p=0.0028) is found between mean intensity in control and test samples is observed. The p value is observed to be $p<0.05$ which indicates significant difference. The quantitate validation was performed via ImageLab software. These results are in accordance with the data obtained from ELISA.

4.4.2 Western blot of HSD11B1

HSD11B1 showed relatively lower potential compared to other protein biomarkers. To validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of HSD11B1 in these samples. The results of western blot analysis are shown in the Figure 4.18.

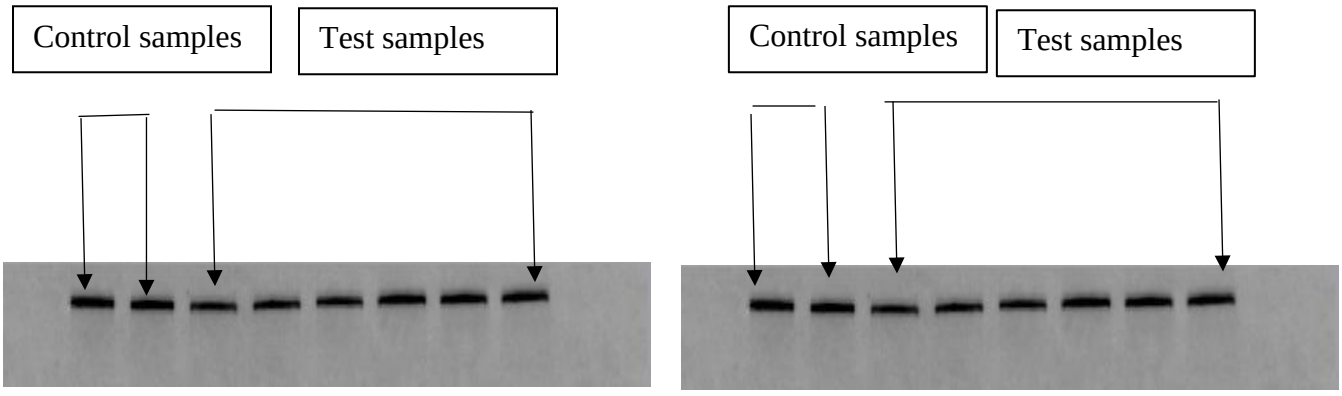

Figure 4.18 western blot of 04 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of HSD11B1 and the antibody specific reactivity to HSD11B1, thus qualitatively confirming the validity of our ELISA results for HSD11B1. The age range of the test samples was between 50-60 years and majority were male.

4.4.2 Determination of mean concentration of HSD11B1

HSD11B1 showed a higher relative mean concentration in control samples on comparison with the test samples. The concentration was obtained by setting a band in the second lane as reference and then comparing them with the rest of the samples. The relative intensity is observed to be greater in control samples (1.42 ng/ml) compared to test samples (0.88). The p value is observed $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The graph showing the concentration of control as well as test sample is shown in figure 4.19 below.

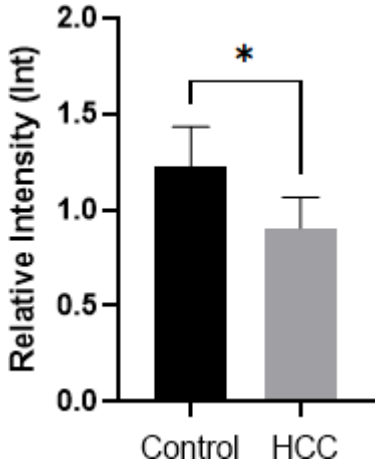

Figure 4.19 The graph representing the relative intensities of control samples compared to test samples. The relative intensity is observed to be greater in control samples (1.42 ng/ml) compared to test samples (0.88). Significant difference ($t=2.350$, $p=0.0398$) is found between mean intensity in control and test samples. The p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA.

4.4.3. MBL2 Western Blot

MBL2 did not show any potential to serve as biomarker for HCC upon. To validate the ELISA results, western blot analysis was conducted on a total of 16 samples (test samples=12; control= 4) in duplicates to qualitatively confirm the presence of MBL2 in these samples. The existence of a continuous band exhibits the presence of MBL2 and the antibody specific reactivity to MBL2, thus qualitatively confirming the validity of our ELISA results for MBL2. The results of western blot analysis are shown in the Figure 4.20.

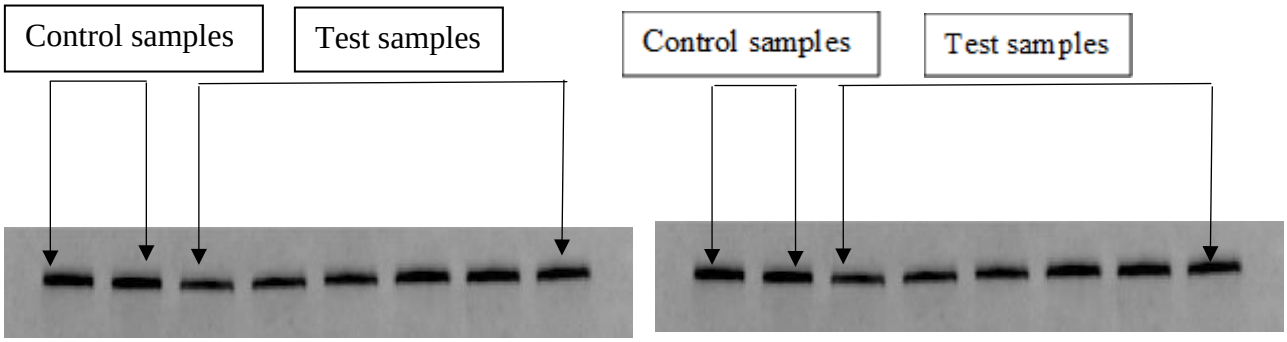

Figure 4.20: Western blots of 4 control samples and 12 test samples. All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of MBL2 and the antibody specific reactivity to MBL2, thus qualitatively confirming the validity of our ELISA results for MBL2. The age range of test samples lies between 50-60 years while majority of the patients were male.

4.4.3.1. Determination of mean concentration for MBL2

MBL2 showed a higher relative mean concentration in control samples when compared with test samples. The concentration was obtained by setting a band in the second lane as reference and subsequently comparing these with the rest of the samples by using ImageLab software. Significant difference ($t=5.793$, $p=0.0018$) is found between mean intensity in control and test samples where the p value is observed to be $p<0.05$ which indicates significant difference. These results are in accordance with the data obtained from ELISA. The graph showing the concentration of control as well as test sample is shown in figure 4.21 below.

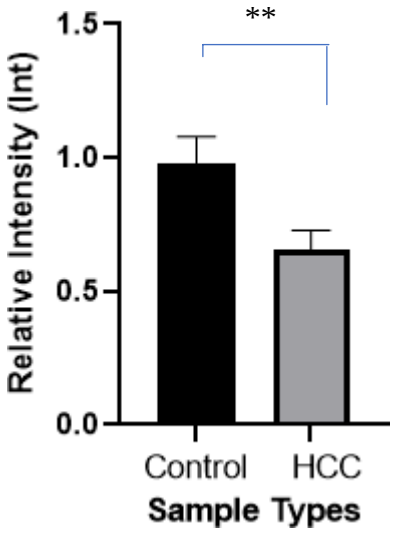

Figure 4.21 The graph showing relative intensities of all the bands in control as well as test samples. The relative intensity is observed to be greater in control samples (0.6787 ng/ml) compared to test samples (0.6787). Significant difference (t=5.793, p=0.001s8) is found between mean intensity in control and test samples is observed. The p value is observed to be $p<0.05$ which indicates significant difference. The quantitate validation was performed via ImageLab software. These results are in accordance with the data obtained from ELISA.

4.5 Multiplex ELISA

Multiplex ELISA was performed, the best performing biomarker ADH6 was combined with the gold standard biomarker for diagnosing HCC i-e AFP to check whether their combined effect can increase the diagnostic accuracy of HCC.

4.5.1 ROC curve analysis of ADH6 combined with AFP

Based upon the difference in mean values, the diagnostic ability of ADH6 in combination with AFP was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure 4.21. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.70. The cut off value was determined by selecting the value with the highest sum of sensitivity and specificity at 11.40 ng/ml. The ROC curve for ADH6 combined with AFP is shown in Figure 4.22.

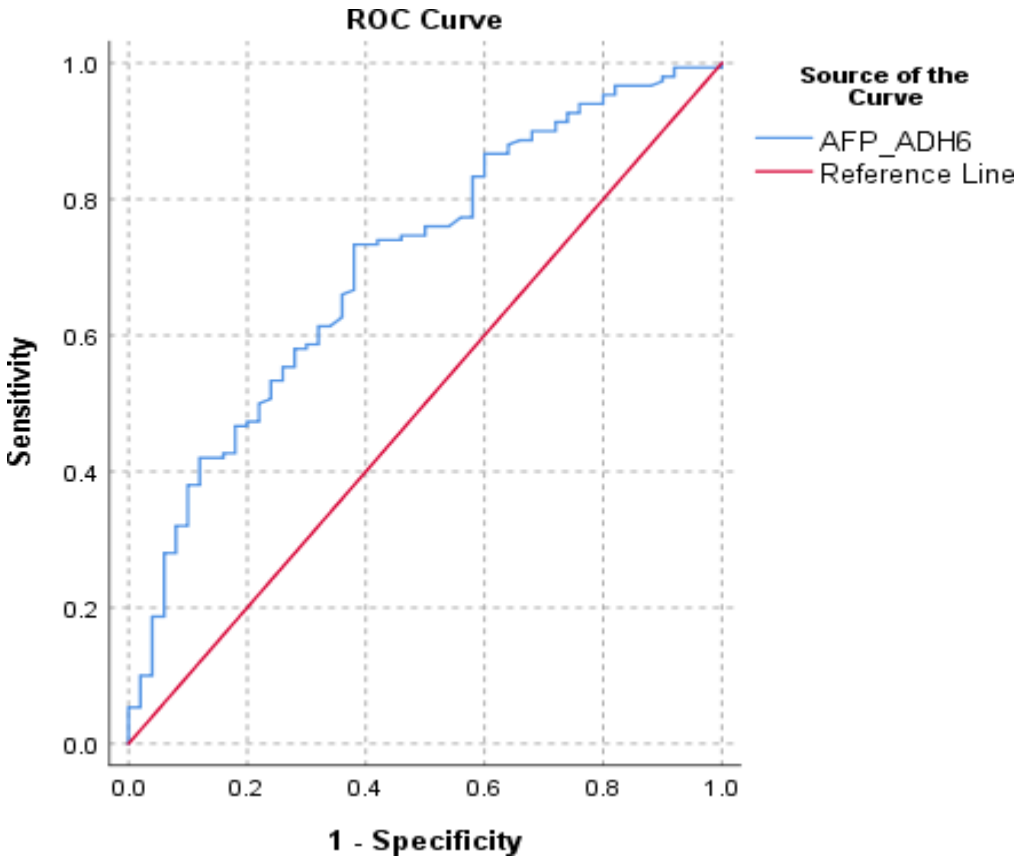

Figure 4.22: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.70 and the cut off value of 11.40 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.5.2 Determination of diagnostic ability of ADH6 combined with AFP

Based upon the cut-off value, sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate

positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers give an insight into the diagnostic ability of the combined biomarkers. Following is the Table 4.7 containing the numbers of True positives, False positives, True negatives, and False negatives.

Table 4.7: Diagnostic ability of ADH6 combined with AFP to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False negatives (FN). Positive cases in control are False- positives (FP) whereas negative cases in controls are True negatives (TN).

Diagnostic ability of combined biomarkers	
True Positives	141
False positives	08
True negatives	32
False negatives	18

4.5.3 Determination of diagnostic parameters of ADH6 combined with AFP

MedCalc diagnostic test evaluation calculator was used to determine different parameters concerning the diagnostic ability of the biomarker panel by using the number of True negatives, False positives, and False negatives The sensitivity was determined as 88.68% whereas the specificity was determined at 80%. The accuracy of the test was determined at 86.93%. These parameters are given in the Table 4.8. All of these values are computed from ELISA results.

Table 4.8 Diagnostic parameters of ADH6 in combination with AFP calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	Value	95%Confidence Interval
Sensitivity	88.68%	82.70% to 93.15%
Specificity	80.00%	64.35% to 90.95%
Positive Likelihood Ratio	94.63%	90.44% to 97.04%
Negative Likelihood Ratio	53.97%	52.84% to 73.83%
Accuracy	64.00%	81.44% to 91.28%

4.6 Multiplex ELISA of C8A and Cyp2a6

The combination of C8A (the best performing biomarker among the pool) was combined with Cyp2a6, (possessing little to no utility to function as efficient biomarker) to see whether this combination can increase sensitivity and specificity of Cyp2a6 compared to individual results.

4.6.1 ROC curve analysis of C8A and Cyp2a6:

Based upon the difference in mean values, the diagnostic utility of C8A in combination with Cyp2a6 was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure 4.21. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.724. The cut off value was determined by selecting the value with the highest sum of sensitivity and specificity at 130.98 ng/ml. The ROC curve for C8A combined with Cyp2a6 is shown in Figure 4.23

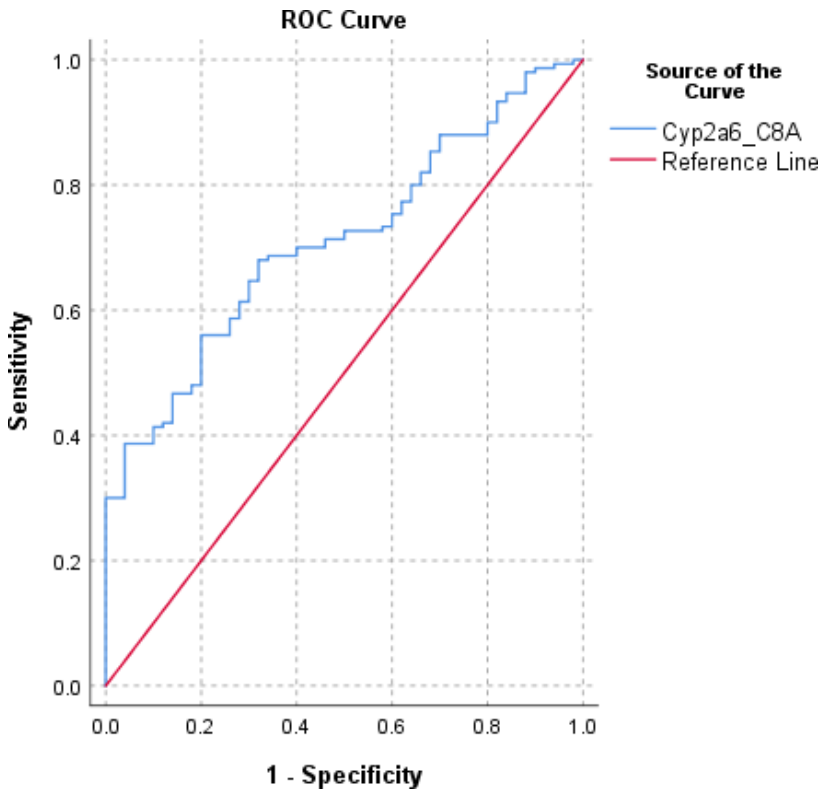

Figure 4.23: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.70 and the cut off value of 11.40 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.6.2 Determination of diagnostic ability of C8A combined with Cyp2a6

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers give an idea on the diagnostic potential of combined biomarkers. Following is the Table 4.9 containing the numbers of True positives, False positives, True negatives, and False negatives.

Table 4.9: Diagnostic ability of C8A combined with Cyp2a6 to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False negatives (FN). Positive cases in control are False- positives (FP) whereas negative cases in controls are True negatives (TN).

Diagnostic ability of combined biomarkers	
True Positives	108
False positives	16
True negatives	34
False negatives	42

4.6.3 Determination of diagnostic parameters of C8A combined with Cyp2a6

MedCalc diagnostic test evaluation calculator was used to determine different parameters concerning the diagnostic ability of the biomarker panel by using the number of True negatives, False positives, and False negatives The sensitivity was determined as 72.68% whereas the specificity was determined at 68%. The accuracy of the test was determined at 71%. These parameters are given in the Table 4.10. All of these values are computed from ELISA results.

Table 4.10 Diagnostic parameters of ADH6 in combination with AFP calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95%Confidence Interval**
Sensitivity	72.68%	64.09% to 79.02%
Specificity	68.00%	53.30% to 80.48%
Positive Likelihood Ratio	87.10%	81.66% to 91.10%
Negative Likelihood Ratio	44.74%	37.04% to 52.70%
Accuracy	71.00%	64.18% to 77.18%

4.7 Multiplex ELISA of C8A combined with UPB1

The third combination was performed by combining C8A with UPB1. Individually UPB1 showed moderate ability to function as efficient biomarker with sensitivity and specificity values of 59.73% and 66% respectively but was better than Cyp2a6. In order to validate further UPB1 was combined with C8A to see whether this combination has the ability to function as efficient biomarker panel.

4.7.1 ROC curve analysis of C8A combined with UPB1

Based upon the difference in mean values, the diagnostic utility of C8A in combination with UPB1 was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure 4.21. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.724. The cut off value was determined by selecting the value with the highest sum of sensitivity and specificity at 130.98 ng/ml. The ROC curve for C8A combined with Cyp2a6 is shown in Figure 4.24.

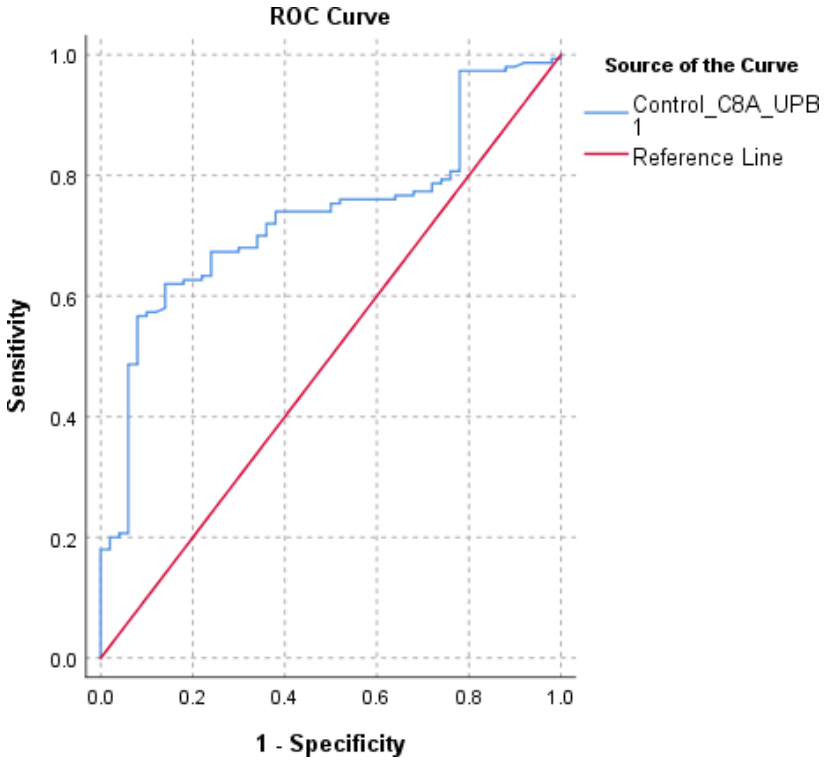

Figure 4.24: ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.724 and the cut off value of 130.98 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.7.2 Determination of diagnostic ability of C8A combined with Cyp2a6

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers give an idea into the diagnostic ability of the combined biomarkers. Following is the Table 4.11 containing the numbers of True positives, False positives, True negatives, and False negatives.

Table 4.11: Diagnostic ability of C8A combined with Cyp2a6 to act as a biomarker for HCC. Positive cases in Test sample indicate True positives (TP). Negative cases in test samples are False

negatives (FN). Positive cases in control are False- positives (FP) whereas negative cases in controls are True negatives (TN).

Diagnostic ability of combined biomarkers	
True Positives	116
False positives	14
True negatives	30
False negatives	40

4.7.3 Determination of diagnostic parameters of C8A combined with UPB1

MedCalc diagnostic test evaluation calculator was used to determine different parameters concerning the diagnostic ability of the biomarker panel by using the number of True negatives, False positives, and False negatives The sensitivity was determined as 74.36% whereas the specificity was determined at 68.18%. The accuracy of the test was determined at 73%. These parameters are given in the Table 4.12. All of these values are computed from ELISA results.

Table 4.12 Diagnostic parameters of C8A in combination with UPB1 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	Value	95%Confidence Interval
Sensitivity	74.36%	66.76% to 81.01%
Specificity	68.18%	52.42% to 81.39%
Positive Likelihood Ratio	89.23%	84.19% to 92.80%
Negative Likelihood Ratio	42.86%	34.92% to 51.18%
Accuracy	73.00%	66.28% to 79.02%

Chapter 5 Discussion

One of the biggest contributors to the global burden is Hepatocellular carcinoma, leaving only a few potential treatment options available (Lurje et al., 2019). The treatment options available to treat HCC are invasive and expensive therefore the need of the hour is to develop strategies and treatment options which can help in early diagnosis of HCC. Previously, HCC is being diagnosed with the help of techniques that are invasive, and overpriced, this includes technologies such as MRI, ultrasound, and histopathology (Ayuso et al., 2018). Blood based biomarker detection have solved this problem by diagnosing HCC using techniques that are not only non-invasive but are cost-effective. Alpha-fetoprotein (AFP), the current gold standard biomarker to diagnose HCC suffers a great deal owing to its low sensitivity and specificity as reported in various studies, and possesses poor clinical relevance (Ahmed Mohammed and Roberts, 2017a; Carr et al., 2018). Three protein biomarkers were selected in our study: Cyp2a6, UPB1 and ADH6, by following a previous published bioinformatics pipeline (Awan et al., 2015). Blood samples were collected from Holy Family Hospital, RMU, Pakistan to determine the ability of these biomarkers to act as efficient biomarkers to diagnose HCC. Among the 3 potential biomarkers, ADH6 has shown significant potential to serve as the biomarker for HCC, but not better than AFP whereas Cyp2a6 has also shown a fair biomarker potential for HCC but less than ADH6. The remaining candidate UPB1 did not show significant ability to distinguish HCC from healthy individuals.

Due to the ease with which liver illness can be mistaken with other health issues, many diseases are challenging to detect and have ambiguous symptoms. Medical professionals have, however, employed certain indicators that aid in the diagnosis and progression of liver disease. Some metabolic pathways and enzymes in the liver exist, and since they are sensitive to any alteration, they are regarded as biochemical biomarkers of liver dysfunction (Awan et al., 2015). In a paper published in 2015 by Awan et al., it was highlighted how the development of omics technology led to the identification and publication of various potential biomarkers, which significantly enhanced the prospects for creating medicines more successfully (Awan et al., 2015). These possibilities may have well-known advantages for patients and the financial side of healthcare. However, the process of moving a biomarker from the stage of discovery to use at the clinical

stage is still ongoing. To offer precise, accurate, and repeatable findings based on the detection of blood circulating biomarkers in HCC patients, a biomarker must be verified and confirmed using hundreds of blood specimens from HCC patients (Awan et al., 2015.

50 healthy individuals belonging to different age ranges and backgrounds were engaged in this study to create a diverse group. Only those individuals were engaged that possess no history of HCC, HBV, and HCV incidence. By carefully analyzing people's ages it was revealed that most people falling into the 30-39 and 40-49 age brackets, as shown in Figure 4.4. It was revealed upon gender distribution analysis that 64% of population were male and the female comprised of 36% of total population as illustrated in Figure 4.5.

After performing several demographic and medical history analysis of our sample populations (both HCC patients and controls), the candidate biomarkers were subjected to ELISA. ELISA is the standard for measuring the quantity of proteins in a sample. ELISA is a potent tool in order to quantify proteins in a sample (Hosseini et al., 2018) and in this current study, ELISA was performed on serum of both HCC and control samples and their results were analyzed via SPSS version 26.

In case of Cyp2a6, the first biomarker candidate, exhibited very little clinical utility for diagnosis of HCC, the mean value in control samples is slightly elevated (198.2 ng/ml) compared to test sample population (142.1 ng/ml). In case of Cyp2a6 as shown in Figure 4.7. However, the standard deviation in control samples is much greater compared to test samples indicating the increased spread of Cyp2a6 values and is in line with previous studies on HCC diagnostic potential of Cyp2a6 (Luo et al., 2020)**.** The area under the ROC curve (AUC) at $p<0.05$ was determined to be 0.554 which gives a good biomarker potential for HCC as shown in Figure 4.8. With the help of ROC curve, the cut off value at 160.67 ng/ml was determined for our HCC population. This value was obtained by analyzing the ROC curve results, providing sensitivity and specificity at every possible cut off value. The cut off value is selected at the point where there is a maximum sum of sensitivity and specificity (Choi et al., 2019)**.** On the basis of this cut off, the number of True positives (TP), True negatives (TN), False positives (FP) and False negatives (FN) were determined. These numbers were then analyzed via MedCalc diagnostic test evaluation calculator and the values of sensitivity (64.00%), specificity (54.00%) and accuracy (61.50%) were determined as shown in Table 4.5. These values are less when compared to AFP hinting at its

moderate potential to act as efficient biomarker for HCC. A similar pattern was expressed in a study conducted by (Ren, at all, 2018) which showed the downregulation of Cyp2a6 in their study. Any change in the values of Cyp2a6 resulted in metabolism and bio inactivation of clinical therapeutics and carcinogens and possess important clinical considerations (Ren et al., 2018). The values of Cyp2a6 can be attributed to an advance staging of cancer, metastasis, and an increase in value of AFP (Ren et al., 2018). Another study conducted by (Jiang et al., 2021) showed that Cyp2a6 is an important factor in diagnosing HCC and possess fair potential to serve as potential biomarker of HCC.

UPB1, our second biomarker candidate exhibited a low mean concentration value in test samples i.e., (142.11 µg/ml) compared to the control sample population (198.2 µg/ml) as shown in Figure 4.9, thus pointing towards dysregulation of complement component pathway in HCC, as has been suggested in many studies, due to chronic and ectopic inflammatory states underlying the origins of HCC (Malik et al., 2021; Zhang et al., 2021)**.** The standard deviation in the mean concentration values does not overlap thus indicating good results. The ROC curve analysis at $p<0.05$ was performed by keeping the 'lower values as positive results' since the mean concentration in test samples was less than control samples. The AUC was determined at 0.665 which exhibits fair potential to serve as a biomarker for HCC. With the help of ROC curve, the cut off was determined at 117.5854 µg/ml at maximum sum of sensitivity and specificity. The sensitivity was determined at 59.73% and specificity at 66% which are moderate values for a biomarker of HCC. The accuracy of the test was also determined at 61.31%. The expression of UPB1 suffers significantly in HCC patients compared to normal tissues in a study conducted by (Li et al.., 2018) which was in accordance with above results. UPB1 was amongst three proteins reported in TCGA (The Cancer Genome ATLAS Programme) to be used as prognostic signature (Li et al.,2018). However, the role of UPB1 in relation to cancer is not well defined as fewer studies were conducted to validate this role and there is a need to estimate the significance of UPB1 in tumors (Li et al.., 2018).

Another protein biomarker candidate ADH6 showed a higher concentration in test samples as compared to controls as shown in Figure 4.11 but there is no considerable difference among the two and there is a high standard deviation which shows increased spread of data for both test and control samples. The area under the curve (AUC) at $p<0.05$ was determined as 0.674 which shows a moderate biomarker potential, a bit higher than UPB1 and a cutoff value (8.3632 ng/ml)

determined as described previously gave sensitivity, specificity, and accuracy values of 70.67%, 64.00% and 69% respectively as shown in Table 4.9. A study conducted by (X Liu et al.., 2020). Showed that ADH6 can play a significant role in prognosis of HCC. A study conducted by (Yu et al., 2020) confirmed the role of ADH6 in pathogenesis of HCC and is important in controlling retinol metabolism. Another study conducted by (Liu et al., 2020) confirmed that the high expression of ADH6 associated with HCC revealed through numerous oncogenic signaling pathways.

A qualitative as well as quantitative confirmation was done via performing western blot analysis of all three biomarker candidates. Western blot was performed to qualitatively confirm the antibody's specific reactivity to the three biomarker candidates and clear bands were observed on 4 gels with 4 samples each (in duplicate). One of the gels had 4 control samples, and the rest three had 12 test samples in total. This result verifies our ELISA's results since only one band at a similar position was observed in all samples. Furthermore, quantitative validation was performed by using ImageLab software. One of the bands in second lane was set as a reference and the mean intensity of the rest of bands was computed compared to reference band. Western Blotting was performed in a study conducted by (D Guo et al., 2020) who explored the expression of Thrombospondin 4 (THBS4) in HCC samples. Their study concluded that the expression of THBS4 is important in regulating HCC development. (H Song et al., 2020) used Western Blotting and in-situ Hybridization in HCC patients to analyze the prognostic effects of long non-coding RNA CASC11. Their study validated that CASC11 have the potential to aid in progression of HCC through EIF4A3 mediated activation.

Multiplex ELISA was performed to see if the combinations of different proteins yielded better results in terms of diagnostic accuracy of biomarker. The best performing biomarker from the current pool, ADH6, was paired with AFP, the current gold standard biomarker, to see if the combinations can enhance the sensitivity and specificity of the reaction. The area under the curve (AUC) at $p<0.05$ was determined as 0.706 which shows an excellent biomarker potential and a cutoff value (11.40 ng/ml) determined as described previously gave sensitivity, specificity, and accuracy values of 88.88%, 80.00% and 86.93% respectively. The combined sensitivity and specificity are higher compared to individual results thus increasing the diagnostic ability of these protein biomarkers to detect HCC at an early stage. A similar study conducted by (Saitta et al.,

2017) combined AFP with PIVKA-2 (protein induced by vitamin K absence). Their study confirmed that the combination of two biomarkers possess increased diagnostic accuracy to detect HCC, validating our results. Another study conducted by (Hu et al., 2021) combined AFP with Golgi protein 73 (GP73) developed through Nano-biosensors, showed that the combination of these two proteins can enhance the diagnostic accuracy of HCC. Furthermore, a study conducted by (Bayard et al., 2018) showed that the expression of both ADH6 and AFP are increased during tumor advancement. This finding is in line with the results we obtained by combining ADH6 and AFP.

The second combination that was tested via multiplex ELISA was of C8A (the best performing biomarker) with Cyp2a6 (exhibiting relatively moderate results). This combination was tried based on the first combination that yielded excellent results when two biomarkers i-e ADH6 and AFP were combined. The combination of biomarker panels yielded good results with AUC of 0.70 and a cut-off value of 11.40 ng/ml. The biomarker showed good potential to act as biomarker for HCC diagnosis with sensitivity, specificity and accuracy values of 72.18%, 68% and 71% respectively. A similar study was conducted by Wu et al. (2020), they computed the sensitivity and specificity of AFP with other GPC3 and DCP. Their study showed that the combination of two or more proteins can significantly enhance the values of sensitivity and specificity, similar to our results.Less performing biomarkers in our study were compared with better performing ones to validate whether their combination has an effect on overall values of sensitivity and specificity.

The third combination that was tested via multiplex ELISA was of C8A with UPB1. C8A performed significantly well compared to all other potential protein biomarkers whereas UPB1 showed fair ability to act as biomarker for HCC, better than Cyp2a6. The combination yielded better results with AUC of 0.73 and a cut-off value of 135.64 ng/ml. The values of sensitivity, specificity and accuracy were 72.38%, 68.18% and 73% respectively. The role of UPB1 in HCC prognosis is well documented according to a study conducted by (Y Zheng et al.., (2018). The study discovered biomarker with a four-gene signature to assess the prognosis of HCC namely UPB1, SOCS2, and RTN3. However, the role of UPB1 in combination with C8A needs further validation.

Chapter 6 Conclusion and Prospects

This study has validated three serum protein biomarker candidates namely Cyp2a6, UPB1 and ADH6 out of seven proposed biomarkers previously by Awan et al., 2015. These biomarkers have the potential to provide an alternative to expensive, invasive detection of HCC via imaging techniques and also pave the way towards convenient, early detection of HCC. This study has looked into the demographic analysis of a 150 HCC patient sample size from and looked into the viral and non-viral causalities of HCC in Pakistan. The diagnostic abilities of the candidate biomarkers are compared with each other along with comparison between combinations. One biomarker (ADH6) exhibited good results and one (UPB1) with a moderate biomarker potential in comparison to Cyp2a6 is determined. Moreover, the detection techniques (ELISA) used was convenient, economical, and easy to access so that if a positive result is obtained, it is easy to establish it as a diagnostic test for HCC in the future. Multiplex protein assays were employed to detect strong biomarker candidates simultaneously in the blood samples of HCC patients and controls. The current study provides an insight into the performance of ADH6 when combined with AFP, additionally this study also tested two more combinations to confirm whether the combination of biomarker panels have the potential to act as efficient biomarker for HCC. These two combinations included UPB1 combined with C8A and Cyp2a6 combined with C8A.Individually in the current study, ADH6 showed good ability to act as efficient biomarkers of HCC but when less performing biomarkers are combined with C8A and AFP the values of sensitivity and specificity were significantly enhanced.

Future prospects include the designing a diagnostic kit featuring biomarker combinations that can increase the diagnostic accuracy of HCC. Based on this multiplex analysis, and follow up of proposed candidate biomarkers, an HCC detection kit can be established which can totally change the way of diagnosing HCC today. There is still a need to conduct more bioinformatics analysis to determine candidate biomarkers specific for different stages of HCC as well as biomarkers for different causalities like viral and non-viral HCC. Such prognostic biomarkers can pave the way in the future to monitor HCC development and treatment regimens with the aid of a biomarker toolkit which will be specific for specific stages and treatment regiments of HCC.

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