Characterization of Serum Protein Biomarkers for Diagnosis of Hepatocellular Carcinoma (HCC)

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Industrial Biotechnology

Supervisor

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Islamabad, Pakistan

2021

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

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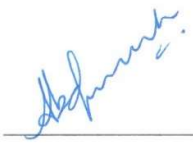
DECLARATION

I, **Abdullah Ahmad**, declare that this research work titled **"Characterization of serum protein biomarkers for diagnosis of Hepatocellular carcinoma (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. Zahoor Ahmad Siddiqi,

who made me believe that ‘Sky is the limit’

Acknowledgment

All praise and glory to Almighty ALLAH, who bestowed me my mere existence and then conferred me all the individual experiences that led me to this day. Indeed, nothing would have been possible without His will, and I have experienced His presence throughout my academic career. Peace and blessings be upon the Holy Prophet Muhammad, the sole source of religion and divine guidance.

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Abdullah Ahmad.

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ABSTRACT

Hepatocellular carcinoma contributes excessively to the global burden of cancer. HCC affects developing countries due to poor healthcare systems and recently its incidence is on the rise globally. In Pakistan, HCC occurs on average in 7.6 persons per 100,000 annually. Moreover, HCC is associated with Hepatitis C, another prevalent infection in Pakistan, which contributes to about 60-70% of HCC incidence here. Poorly developed healthcare systems in the Third World countries result in late detection of HCC since it is diagnosed via Imaging techniques like MRI, Ultrasound and histopathology which often require expensive, healthcare systems. Significant research is being done to develop novel HCC biomarkers for its early detection of the disease. These include development of biomarkers based on transcriptomic and proteomic profiles of HCC patients. Circulating proteins can therefore pose an opportunity for biomarker development of HCC as these can be easily detected in the body fluids among which blood serum is the ideal choice. There is a need to develop blood-based serum biomarkers for easy, non-invasive, and early detection of HCC. Alpha Fetoprotein (AFP) has been used to diagnose HCC in conjunction with imaging techniques, but it has very less clinical utility. In recent years, research has focused on other multiple circulating proteins as biomarker candidates for HCC detection. In this study, blood serum was used to validate 4 novel protein biomarker candidates previously predicted in our study via a bioinformatics pipeline (Awan *et al.*, 2015). Proteins namely C8A, SERPINC1, HSD11B1 and MBL2 were checked in the serum of 150 HCC patients and compared with the AFP levels via quantitative ELISA. Among these, C8A poses significant biomarker potential with 85.33% sensitivity and 100% specificity values and SERPINC1 showed 80.67% sensitivity and 68% specificity whereas MBL2 and HSD11B1 did not show significant biomarker potential. These results were then confirmed via Western blot of selected serum samples of C8A. Conclusively, C8A showed excellent potential to serve as a circulating blood-based protein biomarker for detection of HCC. SERPINC1 also showed moderate results but not better than AFP

Chapter 1 Introduction

Liver Diseases are the biggest causes of increased mortality worldwide and responsible for approximately 2 million deaths a year (Paik *et al.*, 2020). About 1 million of these deaths are caused collectively by Hepatocellular carcinoma and viral diseases whereas rest of 1 million are the result of liver cirrhosis (Paik *et al.*, 2020). The high mortality rate due to liver diseases is attributed to its major role in normal body function i.e., drug metabolism, fat metabolism, detoxification of harmful waste and maintenance of homeostasis. Liver diseases result in the disturbance of these vital functions which often prove to be fatal.

Cancer comprises of a series of disorders caused by the unregulated growth of the body cells (Yadav and Mohite, 2020). This unregulated growth is caused by genetic mutations, abnormal production of proteins involved in cell proliferation, angiogenesis etc and in some cases such as liver cancer (HCC) due to prolonged inflammation caused by activation of adaptive immune response (Dash *et al.*, 2020). Cancer can be of different types, when a tumor is localized in the body and shows limited growth and does not spread then it is termed as benign tumor however when it spreads to other body segments and affect the healthy neighbor organs or cells it is termed as malignant tumor (Patel, 2020).. More than 70% cancer related deaths occur in the developing countries and the reason for this increased number is the lack of timely diagnostics of cancer along with the limited resources for its prevention, treatments, and managements (Dain, 2018)

HCC (Hepatocellular carcinoma) is one of the deadliest form of cancers in Pakistan, it is the fourth most common malignancy affecting men and seventh most 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 60–70% HCC in Pakistan is because of hepatitis C (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.

. 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. In order 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). 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 diagnosis of Hepatocellular Carcinoma 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 all, 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 detection of HCC 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).

With the advancement in cancer biology research several biomarkers for HCC have been revealed such as CK-19 and GP-73 (Zacharakis, Aleid and Aldossari, 2018). These biomarkers are currently under study to prove their significance in early diagnosis thus optimizing therapy, reducing the advent of new tumors and preventing 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). Examination of many potential biomarkers due to several technological advancements have paved the way for a greater interest in characterizing new and improved biomarkers. Tumor protein markers when excessively released in blood results in liver cancer.

For early analysis of hepatocellular carcinoma (HCC), various steps have been taken, however, still there is a lack of precise blood-based biomarkers to carry out screening and diagnostics of the cancer. To find highly sensitive and specific protein biomarkers there is a need to analyze large expression data using already published pipelines developed by Awan et al., 2015 at Nanobiotechnology lab ASAB-NUST that would assimilate numerous bioinformatics databases/tools and literature). In order to improve cancer treatment, tumor specific blood-based

biomarkers hold key status and can be looked upon as effective medium compared to any other area of fundamental medical research. Active research, therefore, should focus on the documentation of new blood-based biomarkers resulting in the analysis and handling of HCC. Efficient biomarkers with higher sensitivity and specificity for diagnosing HCC are needed to diagnose HCC on time as many tumor-related genes, proteins, enzymes, and microRNAs (miRNAs) are released into body fluids such as blood or urine by cancer tissues. (Zacharakis, Aleid and Aldossari, 2018).

A computational pipeline was designed and used in our lab for the identification of seven blood circulating, serum protein biomarkers through computational biology approach. These candidate biomarkers were C8A, SERPINC1, HSD11B1, MBL2, CYP2A6, ADH6 and UPB1 (Awan *et al.*, 2015). The pipeline designed for shortlisting these protein candidate biomarkers is shown in Figure 1.1.

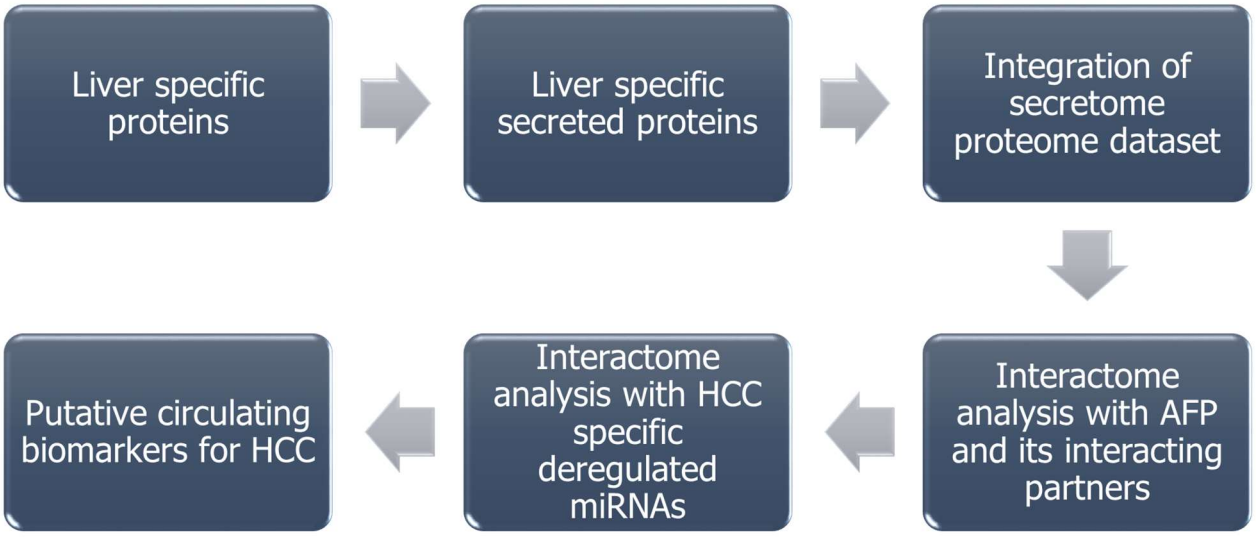

Figure 1.1: Computational pipeline for discovery of biomarker candidates of HCC. Liver specific proteins were checked in multiple databases and matched with secretome datasets; liver specific secreted proteins were shortlisted. Afterwards, interaction of these proteins was confirmed with AFP and related proteins 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)

Thus, in this project we are now aiming to confirm the presence of four protein biomarkers in the serum of HCC patients and their performance as candidate biomarkers was evaluated through wet

lab approach (ELISA and Western blot analysis) for detection of HCC. Currently, we have analyzed four protein biomarker candidates (SERPINC1, C8A, HSD11B1, MBL2) out of seven candidates and checked them in blood serum of 150 HCC patients and 50 healthy controls. Following are the steps involved in this research in terms of aims and objectives of this project

I. Wet lab validation of four potential biomarkers C8A, SERPINC1, HSD11B1, and MBL2 in the blood of HCC patients against 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 selection of a candidate biomarker with an AUC value of >0.90.
IV. Western blot qualitative validation of positive biomarker results (AUC >90%).

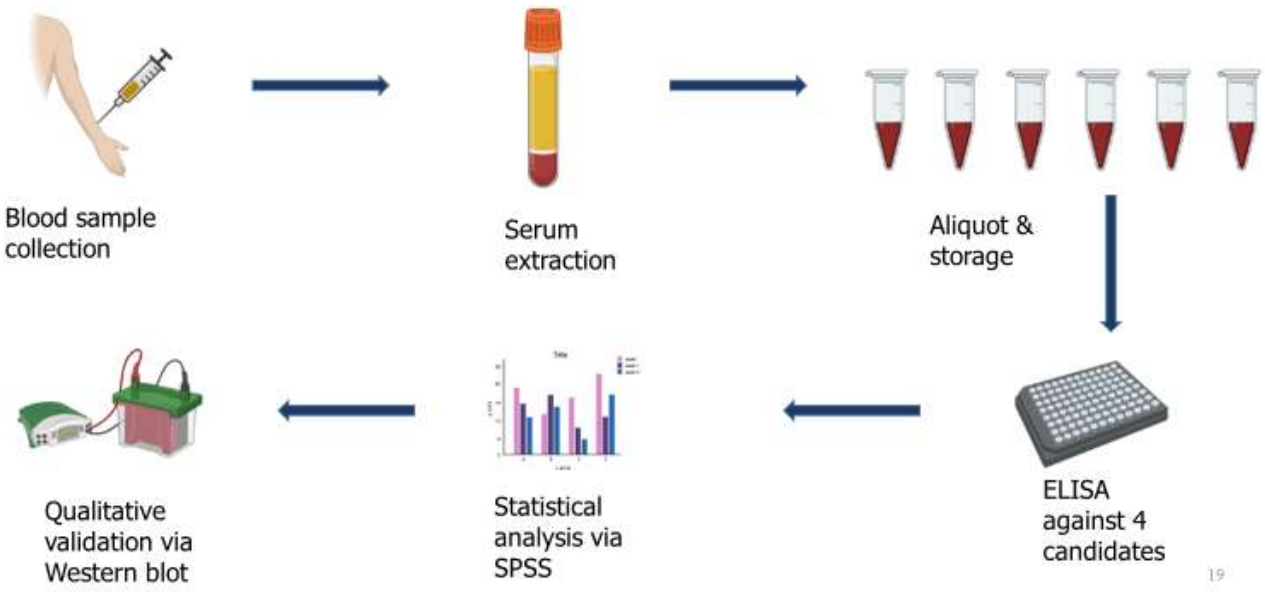

Figure 1.2: Flowsheet diagram depicting overview of methodology. Flow sheet diagram shows the strategies adopted for the characterization of 4 blood circulating biomarkers (SERPINC1, C8A, HSD11B1, MBL2) in 150 HCC patients and 50 healthy controls. Serum was extracted from the collected blood samples of patients and quantitative ELISA was performed on these serum samples to assess the concentrations of biomarker candidates in these 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 with positive results were further qualitatively validated via Western blot analysis.

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 production of bile (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 end stage liver disease including Hepatocellular carcinoma (HCC) are the major diseases of liver (Marcellin and Kutala, 2018)

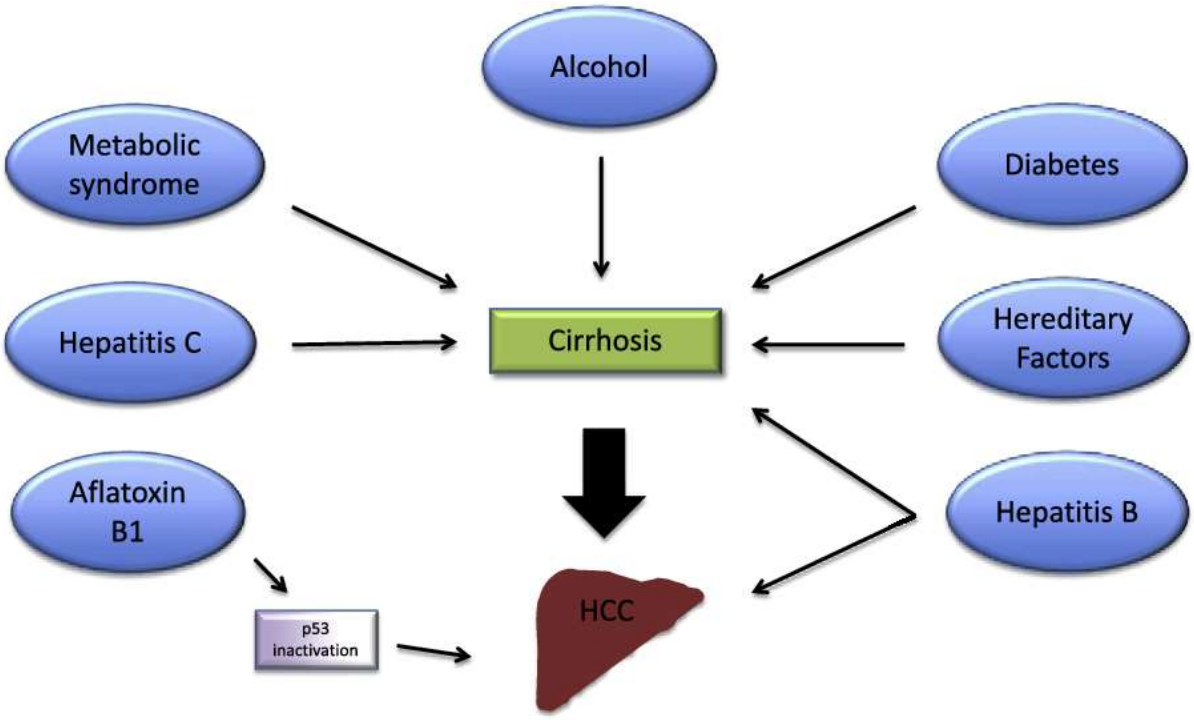

Figure 2.1 HCC as an end point of a sequence of multiple diseases of liver. Source: (Lafaro, Demirjian and Pawlik, 2015)

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. HCC is the most common type of liver cancer and fourth leading cause of deaths all over the globe (Kim and Viatour, 2020). Viral hepatitis (B &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).

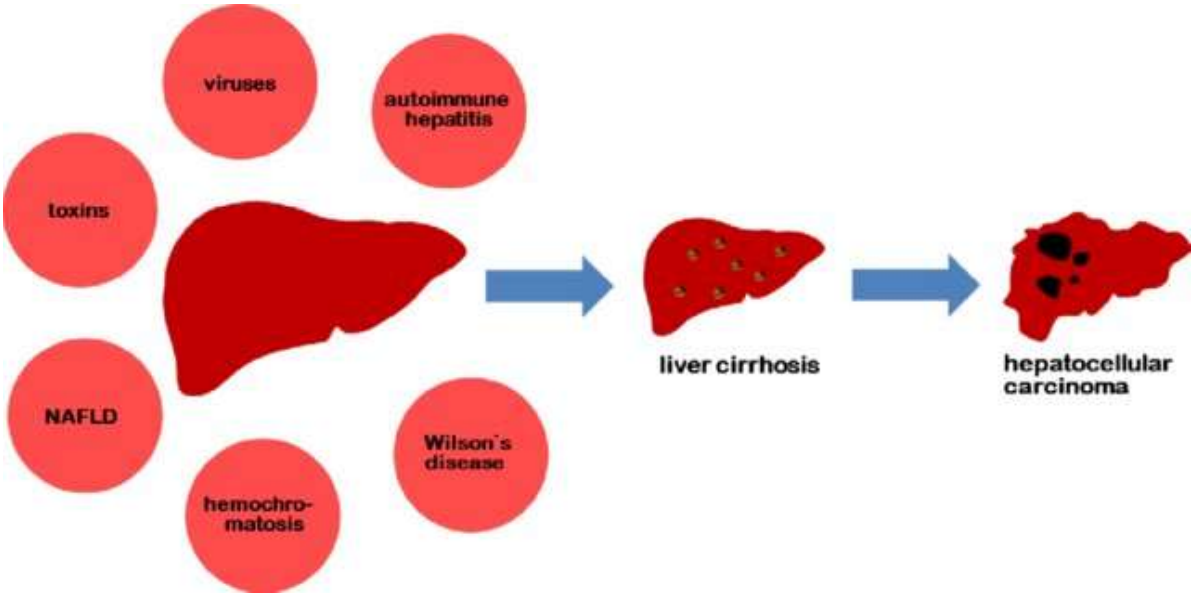

Figure 2.2: Risk factors that potentially lead to liver cirrhosis and ultimately HCC. Adapted from (Uhl et al., 201*4)*

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)

Sometimes during cirrhosis, the damaged liver tissue starts multiplying uncontrollably leading to

HCC development. The dysplastic nodules present in cirrhosis lead to cancerous nodules thus liver function starts declining and other complications arise.

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 Following figure illustrates the research contribution regarding HCC in terms of publications (Bhatti *et al.*, 2016)

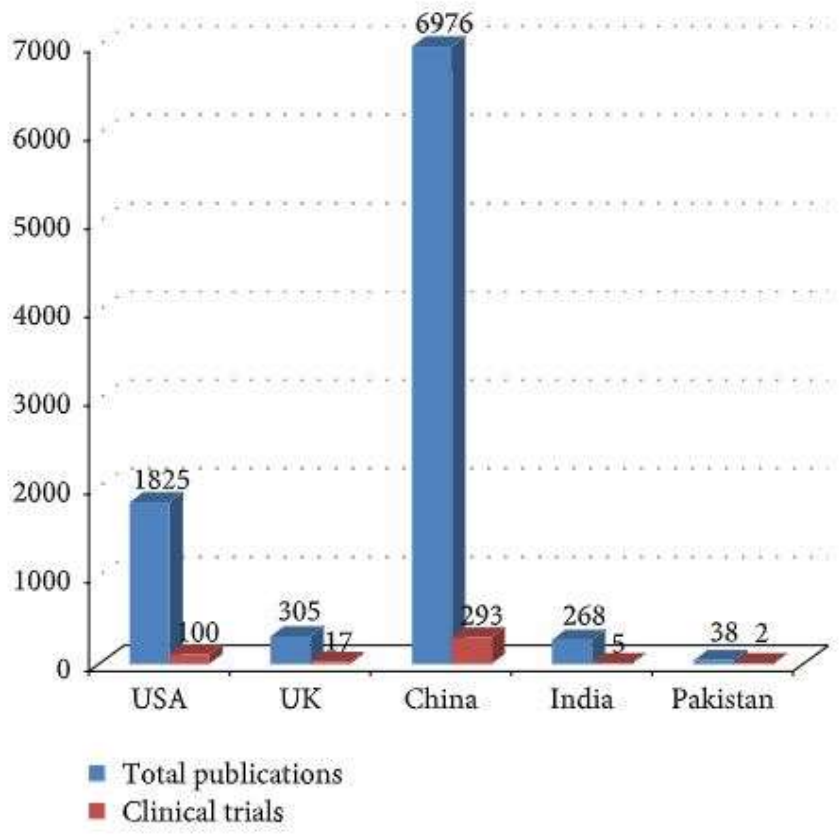

Figure 2.3: Published research and number of clinical trials on HCC from 1990-2015. Source: (Bhatti et al., 2016)

But recently, 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 burden 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. 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).

2.2.2. Causalities of HCC

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

2.2.2.1. Viral HCC

Chronic Hepatitis C infection

Hepatitis C is an infection that primarily affects the liver, causing inflammation and damage that can progress to cirrhosis. The virus that causes the 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 raises the 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. 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)

Chronic Hepatitis B And D:

Hepatitis B is caused by the Hepatitis B Virus (HBV), 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 about 1/3rd of the world population (Jefferies *et al.*, 2018). HBV is hazardous enough 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 two combined can cause liver cancer and death (Puigvehí *et al.*, 2019).

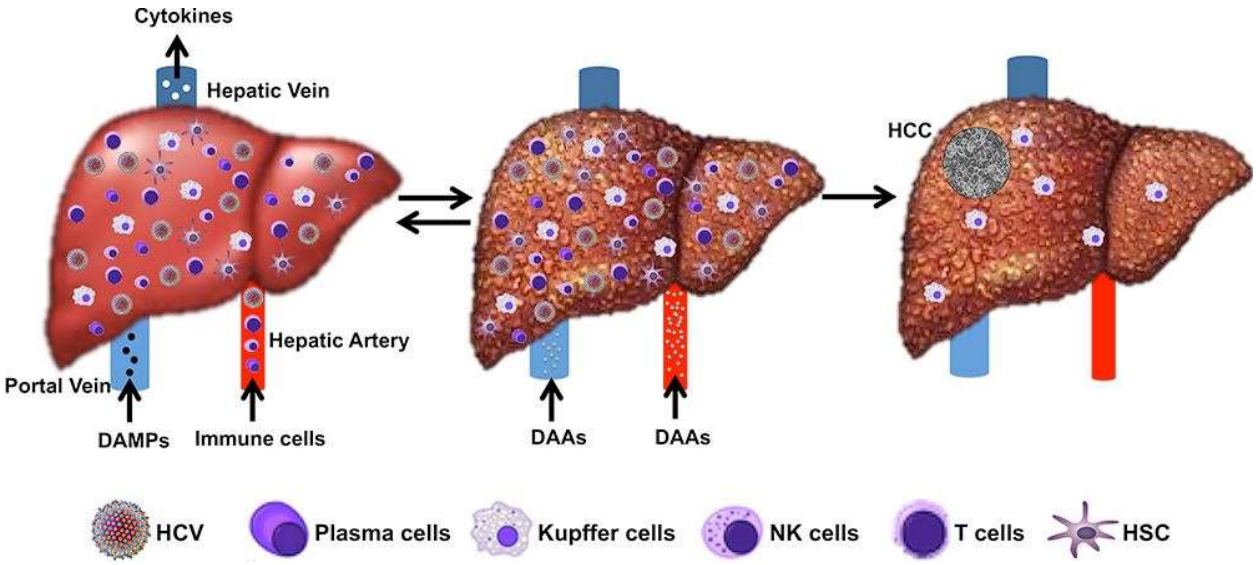

Figure 2.4: HCC resulting from viral hepatitis (HCV and HBV) and other pathogens leading to adaptive immune response. Viruses like HBV and HCC enter the liver via blood supplied through hepatic artery. Activation of adaptive immune response like Kupffer cells, sinusoidal endothelial cells, new adaptive immune cells (CD4 and CD8 T cells) and B cells lead to inflammation. Prolonged inflammation ultimately leads to cirrhosis, and HCC. Source: (Dash et al., 2020)

2.2.2.2. Non-viral causalities of HCC

*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 diabetes, coronary artery disease, protein deficiency, and obesity, and 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)

*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).

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.

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).

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)

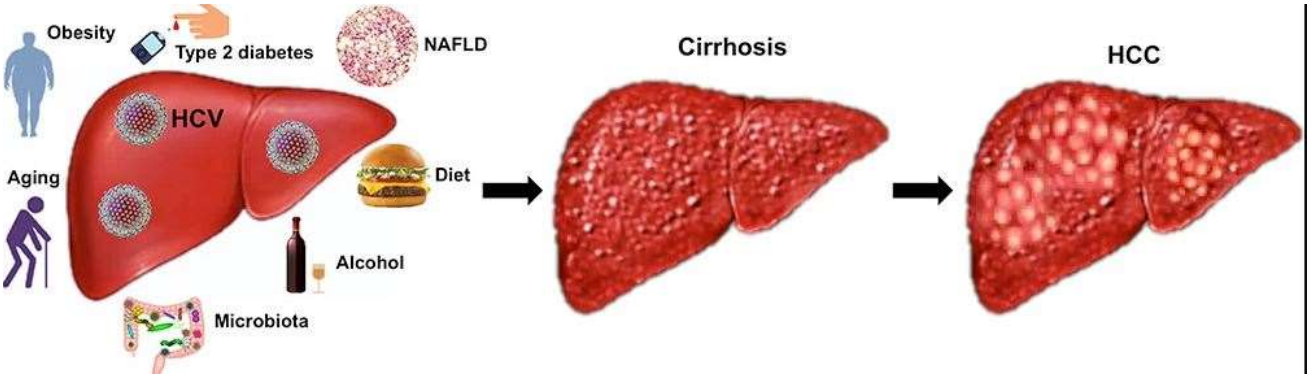

Figure 2.5: HCC development via non-viral routes. Hepatic stress induced via multiple host related factors like metabolic syndrome, type 2 diabetes, NAFLD linked to high calorie diet and obesity, autoimmune diseases and alcohol, leads to inflammation. This inflammation prolonged over a long time can lead to cirrhosis and HCC. Source: (Dash et al., 2020)

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).

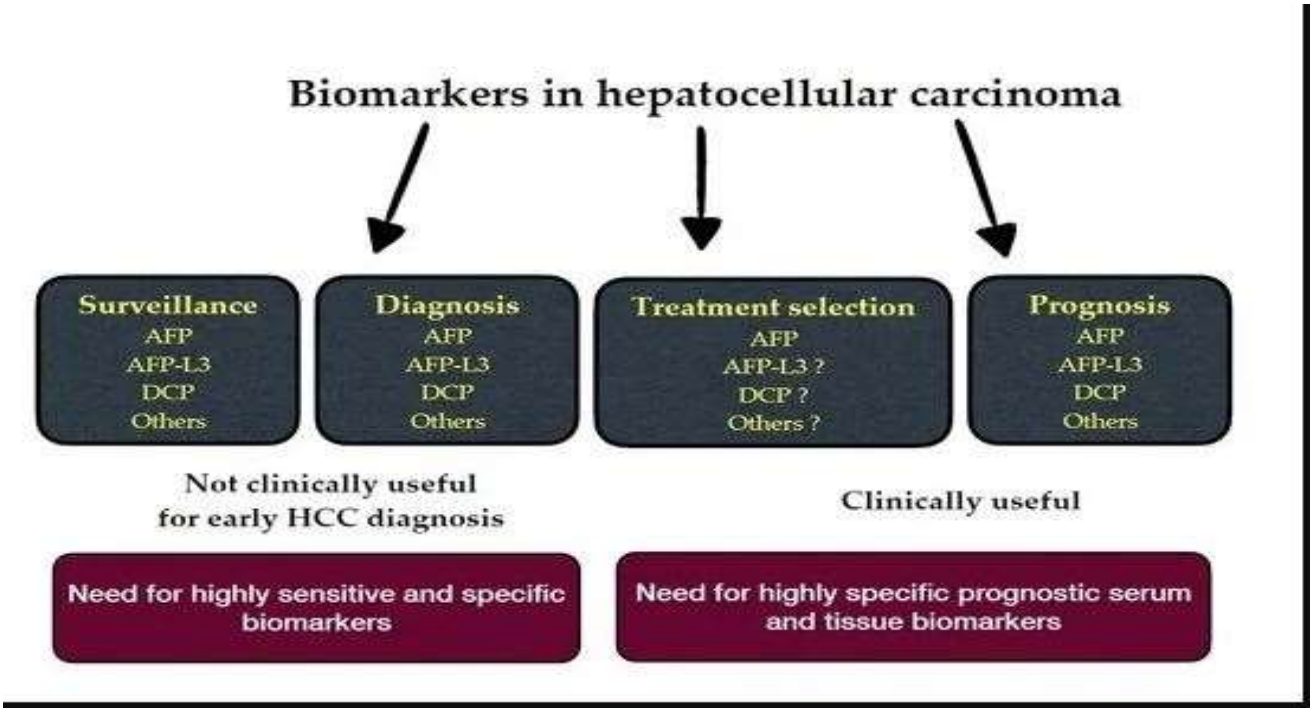

Figure 2.6: Classification of already established biomarkers for HCC. But the need for highly sensitive and specific biomarkers is highlighted because these biomarkers do not have clinically diagnostic utility. Source: (Piñero et. al, 2020).

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 *''Characterization of serum protein biomarkers for diagnosis of Hepatocellular carcinoma (HCC)''* is focused on the ELISA and western blot analysis characterization of the biomarkers that have been identified in previous work published by our work (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 and Western Blot analysis) 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.

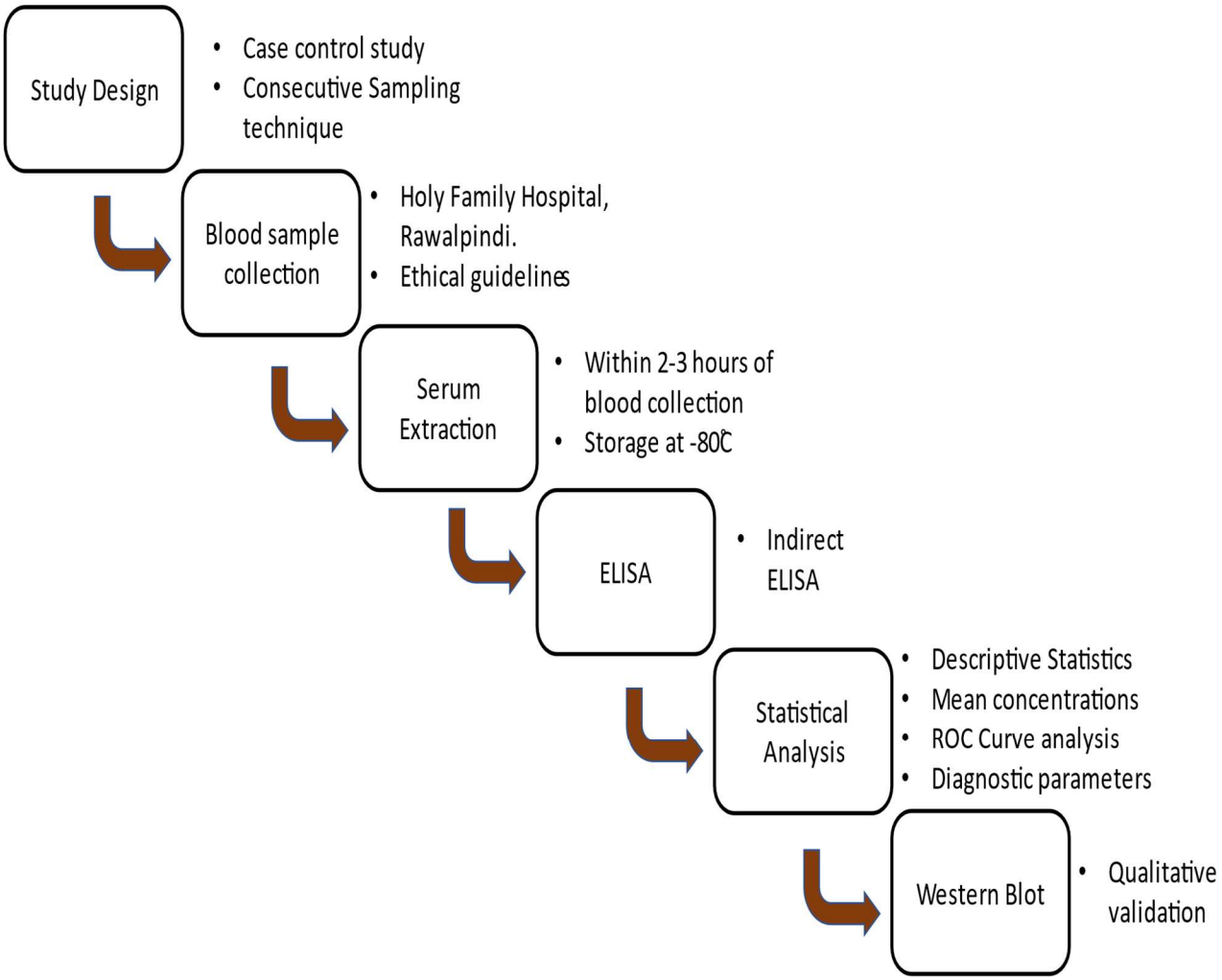

Figure 3.1: Flowchart showing the methodology followed during the project.

3.2. Study design

This study is a 'case control study' and consecutive sampling technique was used to avoid randomization.

3.3. Collection of blood samples

For the collection of HCC patients' blood samples, a collaboration was established between Nanobiotechnology Lab, ASAB and Rawalpindi Medical University (RMU). For this purpose, an ethical approval was obtained from the Institutional Review Board of Holy Family Hospital, RMU, Rawalpindi by defending our synopsis in front of a panel of doctors and researchers under the supervision of Vice Chancellor RMU. All the ethical guidelines were setup during the meeting and a stringent following of the ethical guidelines was ensured by the research collaborator at RMU. After obtaining the IRB approval, blood samples of 150 HCC patients were collected from the Centre of Liver Diseases (CLD), Medicine wards 1 and 2 and Gastrointestinal ward at The Holy Family Hospital, Rawalpindi. Before obtaining blood samples, verbal consent was taken from the patients. A questionnaire was shared with the patients whom they chose to fill out voluntarily after being briefed about the content, nature and use of data being obtained from them.

A 3 ml blood sample was drawn from the HCC patients and healthy controls considering all the ethical guidelines. Trained Phlebotomist was hired to minimize the risk of injury to the patient. Patient's age, sex, HBV and HCV history and date and site of blood collection were obtained on a questionnaire. A total of 150 HCC patients' blood samples and 50 healthy control samples were obtained. All the samples were brought to Nanobiotechnology Lab, ASAB within 2-3 hours of blood sample collection for further processing.

Patients and healthy controls were selected based on the following criterion:

3.3.1. Inclusion and exclusion criterion

The inclusion criterion and exclusion criterion for test and control samples is given in the following Tables 3.1 and 3.2. The patients were screened based on inclusion and exclusion criteria by the Research assistant and the on-duty doctors at the site of blood collection.

3.3.1.1. Inclusion criterion

Table 3.1. Inclusion criterion of HCC patients and healthy controls.

Hepatocellular carcinoma patients	Healthy controls
• Confirmed HCC detection via imaging techniques such as ultrasound, MRI etc. • Non-metastatic stage of HCC confirmed via imaging investigations including X-rays, bone scan, abdominal and pelvic CT scan. • Confirmed diagnosis of HCC by the on-duty doctor	• Controls from diverse age groups • No liver disease history • No viral hepatitis (B & C) history

3.3.31.2. Exclusion criterion

Table 3.2. Exclusion criteria for HCC patients and healthy controls.

Hepatocellular carcinoma patients	Healthy controls
• Liver related comorbidities • Other forms of cancer • Metastatic stage of tumor confirmed via imaging investigations including X-rays, bone scan, abdominal and pelvic CT scan.	• With any form of liver disorder • With any type of cancer • With any viral hepatitis (B&C) incidence

3.4. Serum extraction

The blood samples were drawn in a serum vacutainer (yellow cap) and placed vertically in a stand for the blood to clot. These were then centrifuged at 2000 rpm for 2 minutes to obtain the blood serum. The blood clot in the serum vacutainer is separated by a gel and allows to extract pure

serum from the top. The serum contains all the secreted proteins present in the blood. with 1.5 ml serum extracted and aliquoted in Eppendorf tubes and these tubes were stored in a -80˚C freezer. Blood samples were collected from September 2020- July 2021.

3.5. ELISA

The collected serum of patients was analyzed via Enzyme Linked Immunosorbent Assay (ELISA) test. ELISA is a quantitative analysis which determines the amount of protein present in a sample via protein specific antibodies. The bound antibodies give a colorimetric or a chemiluminescent signal based upon the type of ELISA used and that signal is compared with the known standards to calculate the amount of protein present in the sample. In our ELISA test, chemiluminescent approach was used to detect the amount of target protein in a sample.

ELISA kits against five candidate protein biomarkers namely C8A, SERPINC1, HSD11B1, MBL2 and AFP were ordered from Nanjing Pars Biochem Ltd. AFP was used as a reference for our four candidate protein biomarkers. Pipettes used were purchased from Dragon Lab Co. Ltd. The ELISA reader from Bio-Rad Ltd. (Model PR4100) was used to read the absorption values from ELISA.

3.5.1. ELISA procedure

- ELISA is conducted by coating of primary antibody in the wells of 96-well ELISA plate. In our case, the ELISA kits from Nanjing Pars Biochem Ltd. were pre-coated with the primary antibody.
- At first, standard wells set on the ELISA plate. ELISA standards with known concentration values provided in the ELISA kit were added in these wells in duplicates by performing serial dilutions with the standard diluent. Serial dilutions were performed by adding 100 µl standard sample in the first two wells (duplicates) and afterwards addition of 50 µl of standard diluent in both wells. The 50 µl was then taken out from these first and second wells and discarded. Then, 50 µl from these wells was taken and added in the third and fourth well. subsequently, 50 µl standard diluent was added in the third and fourth well. Again 50 µl from third and fourth well was then added to fifth and sixth well. Afterwards 50 µl of standard diluent was then added to fifth and sixth well and so on.
- Two wells were left empty to serve as blanks. In blanks, all steps of ELISA were performed except addition of sample and HRP conjugate reagent.

- The 40 µl of sample diluent was then added to all the remaining wells. Both test and control samples (10 µl) were added in the wells of the ELISA plate, already containing 40 µl of sample diluent. This results in a five-fold dilution of test samples.
- The plate was then incubated for 30 mins at 37˚C, for allowing the coated antibody to bind with the proteins in the samples. The plates were incubated by covering with a closure membrane.
- After 30 minutes, the closure membrane was opened, samples present inside the well were discarded and wells were dried by swinging.
- Afterwards, wells were washed with washing buffer (5 times) followed by the addition of the HRP conjugated secondary antibody (20 µl). The HRP conjugated antibody was not added to blank wells.
- 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 then chromogen solutions A and B (HRP substrates) were added.
- The plate was again allowed to stand for 15 minutes at 37˚C. The chromogen solutions react with HRP and emits color.
- The reaction was stopped after 15 minutes through the addition of stop solution (Sulfuric acid) and absorbance was immediately read at 450 nm on an ELISA plate reader (Bio Rad Model No: PR4100).

3.5.2. ELISA results analysis

ELISA results were analyzed via an online ELISA result analysis tool; MyAssays Online (https://myassays.com/index.html) and their desktop application 'MyAssays Desktop'. The application drew a standard curve for the OD values obtained for the standard samples, for which concentration was known. The standard curve is obtained automatically by use of 'Best Fit' tool in MyAssays desktop application which analyzes the standard values and draws the best curve according to the suitable curve fitting model determined by our sample values. The R^2 value above 0.95 represent a good standard curve as per ''abbexa'' company website (https://www.abbexa.com/elisa-standard-curve) *Accessed: 24th August 2021*. (abbexa, 2021) and the OD values of the samples are then computed via comparing with the standard curve. The OD

values of our samples were first multiplied with the 'dilution factor' i.e., 5. The values outside of the curve are extrapolated based on the trendline obtained from the standard curve and thus concentration of proteins is determined from the OD values.

3.6. Statistical analysis

Statistical analyses were performed via Statistical Package for Social Sciences (SPSS) version 26 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). For all statistical tests, $p<0.05$*, $p<0.005$** and $p<0.0005$*** and a confidence interval =95% was considered significant. Following statistical tests were performed on the quantitative data obtained via ELISA and demographic data of the patient and control population.

3.6.1. Descriptive statistics

Descriptive statistics to determine frequencies and percentages of samples according to age groups and gender were computed in SPSS. Visual representations in the form of bar charts and pie charts were performed via 'Chart Builder' tool in SPSS. Cross tabulations of age groups vs gender were also performed in both test and control samples to understand the study population.

The patient's data was also divided into 'causalities of HCC' based on number of viral and non-viral cases and further division of viral cases into HCV and HBV. The frequency and percent were calculated in SPSS, but visual representation of this data was performed via bar charts in Graph Pad Prism version 8.

3.6.2. Mean concentration values

Mean concentration values of all proposed candidate biomarker proteins were computed along with their standard deviation values from the 'Compute means' function in SPSS version 26. The graphs however, for comparison of mean values of test and control samples were drawn via Graph Pad Prism version 8.

3.6.3. ROC curve analysis

Receiver Operator Characteristic (ROC) curve was drawn in SPSS version 26 with the help of 'ROC analysis tool'. 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, and

it depends upon the objectives of the study. For determining the diagnostic ability of a biomarker, the cut off value with the highest sum of sensitivity and specificity is selected (Choi *et al.*, 2019).

Area under the curve (AUC) was also computed with ROC curve analysis. The higher the value of AUC, the greater diagnostic potential a biomarker possesses (Hoo, Candlish and Teare, 2017c). AUC values near 0.5 indicates zero to very low biomarker potential and AUC values near 1 indicate excellent biomarker potential. All the values of AUCs were computed at 95% confidence interval.

3.6.4. Determination of diagnostic ability

By determining the cut off value, we can determine the number of True Positive (TP), True Negative (TN), False positive (FP) and False negative (FN) cases from the data set. All the values of the biomarker above cut off were considered positive results and below cut off were considered negative results, if a higher mean concentration of biomarker was observed in patient samples and vice versa. This corresponds to diagnostic ability of a biomarker to distinguish between test and control samples.

3.6.5. Determination of diagnostic parameters

Diagnostic parameters like Sensitivity, specificity, accuracy, positive predictive value, negative predictive value can be computed after determining the number of True Positive (TP), True Negative (TN), False positive (FP) and False negative (FN). These numbers were put into Medcalc online diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php) and the values of diagnostic parameters were computed. The values of sensitivity, specificity and accuracy can manually be computed by the following formulas

$$Sensitivity = TP/P$$

$$Specificity = TN/N$$

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

3.7. Western blot analysis

Western blot was performed to qualitatively verify the ELISA results as the antibodies used are specific to one protein. Selected samples were used (test =12; control =4) on candidate biomarkers

showing positive results as a biomarker and western blotting was performed on them. Its optimization and protocol can be divided into several phases as given below:

3.7.1. Performance and optimization of SDS-PAGE gel electrophoresis

- SDS page gels were cast according to chosen gel concentration with the help of gel casting stand, casting frames etc. purchased by Wix Technologies Ltd. The whole SDS PAGE and western blot equipment (wet transfer) was purchased from Wix Technologies Ltd.
- SDS Page gel concentration was optimized by casting gels with different concentrations i.e., 8%, 12%, 16% etc. The general rule is that lower the molecular weight of the target protein, the higher concentration of gel is required to be cast. In our case, after running electrophoresis on multiple gel types, 12% gel was decided upon.
- After preparing resolving and stacking gels, the gels were places in a 'Tris-glycine' buffer or Electrophoresis buffer in an electrophoretic tank and samples were loaded mixed with loading dye and diluted in PBS (Phosphate buffer saline).
- The sample concentrations were also optimized by loading multiple dilutions i.e., 2X, 4X, 10X etc. in different wells of the same gel. The bands obtained were later detected via Ponceau S stain to determine the best loading concentration. In our case, serum was directly used as a sample and since serum is a highly dense mixture of proteins, the best results were obtained only after using 1µl serum in 19µl PBS and 5µl of loading dye. Loading dye was purchased from Thermo Fisher Scientific Ltd.
- A molecular ladder purchased from Thermo Fisher Scientific Ltd. was loaded in the first well and the samples were loaded in the remaining wells.
- Electrophoresis was run for 170V for 40 minutes and till then, bands moved to the bottom of the gel.
- After performing electrophoresis, the gels were removed and put in Transfer buffer purchased from Thermo Fisher Scientific Ltd. and blotting sandwich composed of these components in sequence: sponge, filter paper, gel nitrocellulose membrane, filter paper and sponge, was prepared and loaded in the blotting chamber.
- Since wet transfer method was employed, the blotting tank was filled with transfer buffer and an ice pack was also added to maintain temperature. Blotting voltage was set at 20V and ran for 2.5 hours.

- After 2.5 hours, the sandwich was opened, and nitrocellulose membranes were treated with Ponceau S solution for 5 minutes in order to detect the blotting efficiency.
- If clear bands were visible, the membranes were washed with water to remove the Ponceau S and it was placed in blocking buffer; 5% Bovine Serum Albumin (BSA) in PBS; overnight.
- The next day, membrane was removed from blocking buffer and treated with primary antibody. The dilution of the primary antibody was done in an antibody diluent (PBS-Tween with sodium azide or some other anti-microbial agent). The dilution concentration depended upon the antibody's user manual provided by the company. The usual dilution concentrations for primary antibodies range between1:1000-1:4000.
- The membrane was dipped in primary antibody solution for 1 hour and put on a rotating platform to ensure continuous shaking
- The primary antibody solution was discarded, and the membrane was dipped into wash buffer (TBST) and treated for 5 mins for 5 times with rigorous shaking.
- The membrane was then treated with HRP conjugated secondary antibody in the same antibody diluent used for primary antibody. The secondary antibody dilution concentration is selected according to user's manual provided by the manufacturer. The secondary antibody's dilution concentration usually ranges from 1:1000-1:10000.
- The membrane was dipped in secondary antibody and placed on a rotating platform for 1 hour to ensure continuous shaking
- The secondary antibody solution was washed off and the membrane was washed with wash buffer (TBST) for 5 times for 5 minutes each with rigorous shaking.
- The membranes were then treated with equal amounts of ECL reagents (A & B) purchased from Thermo Fisher Scientific Ltd. for 5 minutes in the dark.
- The membranes were immediately placed afterwards under Chemi-Doc XRS+ purchased from Bio rad laboratories Ltd. and the signal was detected under 'Auto -exposure' settings of Chemi-Doc.

Chapter 4 Results

4.1. Collection of blood samples of HCC patients and controls

In this study, a total of 200 samples (test samples= 150; control=50) were collected from HCC patients and healthy controls. The data of the participants was collected through verbal consent and following ethical guidelines (Alshehri *et al.*, 2020). After obtaining verbal consent, a structured questionnaire (Annex-I) was shared with the patients and their names and data were not shared with anyone other than the researchers involved in this research project. In the questionnaire, patients' history, age, date of collection, viral or non-viral HCC etc. were included. All the HCC samples were collected from Holy Family Hospital, Rawalpindi Medical University (RMU), Rawalpindi after obtaining an approval letter (Annex 2) of research from their Institutional Review Board (IRB).

4.2. Descriptive Statistics on sample population

4.2.1. Test sample population; HCC patients

In order to understand the patient population in terms of gender, age group and HCC causalities, there is a need to conduct various statistical analysis on the study population.

At first, we need to determine the frequencies and percentages of our test sample population (HCC patients) based on their age groups. The division of our sample population in age groups showed that the majority of HCC population was in the age group 50-59 years (43.3%) followed by 60-69 years (29.3%). These were followed by a mediocre percentage in 40-49 years (20.7%) and the least population in 70-79 years (6.7%) of age. This data is depicted in Table 4.1.

Table 4.1: Frequency and percentage of HCC patients in age groups.

Distribution of HCC patients in Age Groups

		Frequency	Percent
Age Groups	40-49	31	20.7
	50-59	65	43.3
	60-69	44	29.3
	70-79	10	6.7
	Total	150	100.0

After determining the division of testing sample population in age groups, the population was also distributed in terms of Gender as shown in Table 4.2. Majority of the HCC sample population was comprised of the male population (92 cases) while the rest were females (58 cases).

Table 4.2 Frequency and percentage of HCC patients in terms of Gender

Gender distribution in HCC patients

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

The above-mentioned divisions into age groups and gender can be cross tabulated in order to infer the gender wise distribution in different age groups which can provide valuable insight into the age wise demographic distribution of gender in HCC patients as shown in the Figure 4.1.

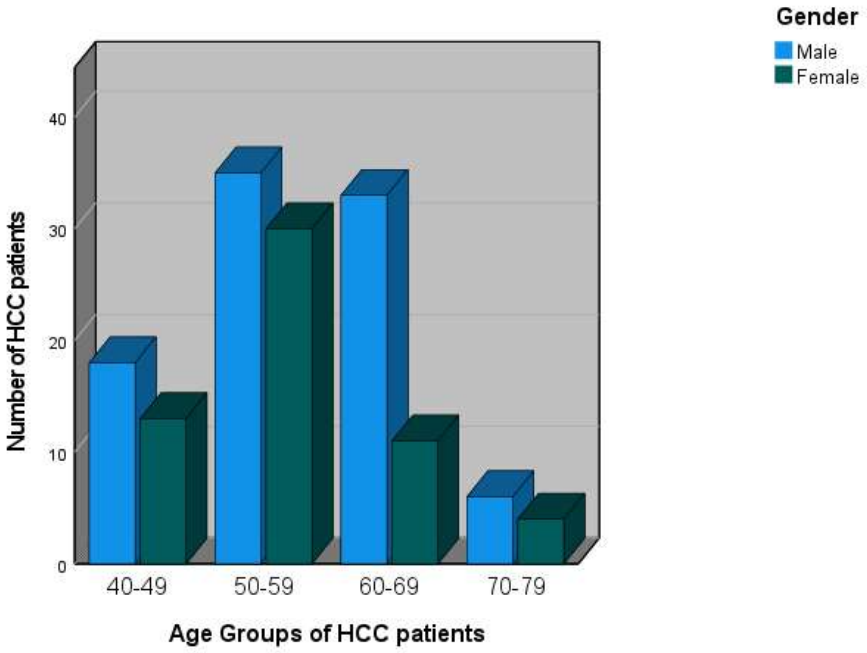

Figure 4.1: **Clustered bar chart of distribution of gender in different age groups**. Males are dominant in every age group as compared to females; however, the highest proportion of females is found in the 50-59 years of age.

4.2.1.1. Co-morbidities (Causalities) of HCC

In Pakistan and other parts of the world, viral hepatitis (HBV and HCV) is the biggest contributor to HCC (D'souza *et al.*, 2020). Therefore, we determined the causalities of our HCC population and divided into viral and non-viral groups. A total of 133 viral HCC cases were present in the total population and 17 cases were due to non-viral causes as shown in the Figure 4.2.

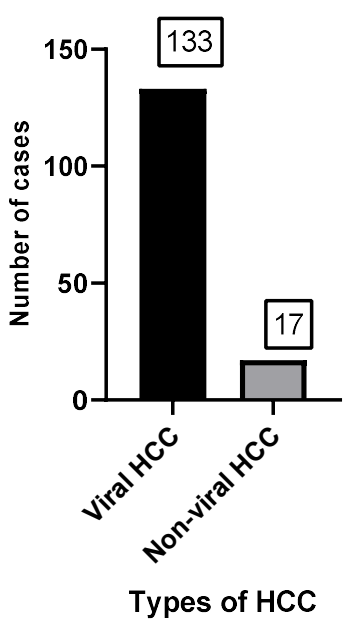

Figure 4.2: Division of HCC patients in viral and non-viral groups. The presence of 133 viral HCC cases is due to high incidence of HCV and HBV related HCC in Pakistan; the rest (only 17 cases) were of non-viral nature.

Among the viral cases, a test sample population was divided based on the presence of HBV and HCV. The total number of cases with HCV alone were found to be 103 and with HBV were 23. Both HBV and HCV were present in 5 cases as shown in the Figure 4.3

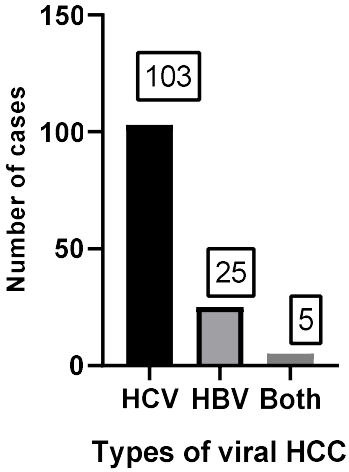

Figure 4.3 Types viral hepatitis cases among HCC patients. Breakdown of viral HCC population in HCV, HBV and both HCV and HBV incidence. 103 samples belonged to HCV incidence alone, whereas 25 cases belonged to HBV. 5 cases had a history of both HCV and HBV.

4.3. Control Samples

Apart from 150 test samples discussed above, 50 control samples were also included in the study and the samples were collected after obtaining verbal consent. Healthy individuals with no history of HCC, HCV and HBV from different age groups and locations were selected and blood was collected from them to serve as healthy control.

The control samples are also divided into different age groups so that these can be compared with the HCC patient population. The highest proportion of control samples belonged to 40-49 years of age (36%) followed by 50-59 years of age (30%). This trend is quite similar to age group distribution found in HCC patient population. The following Figure 4.4 indicates the age group

division of control samples.

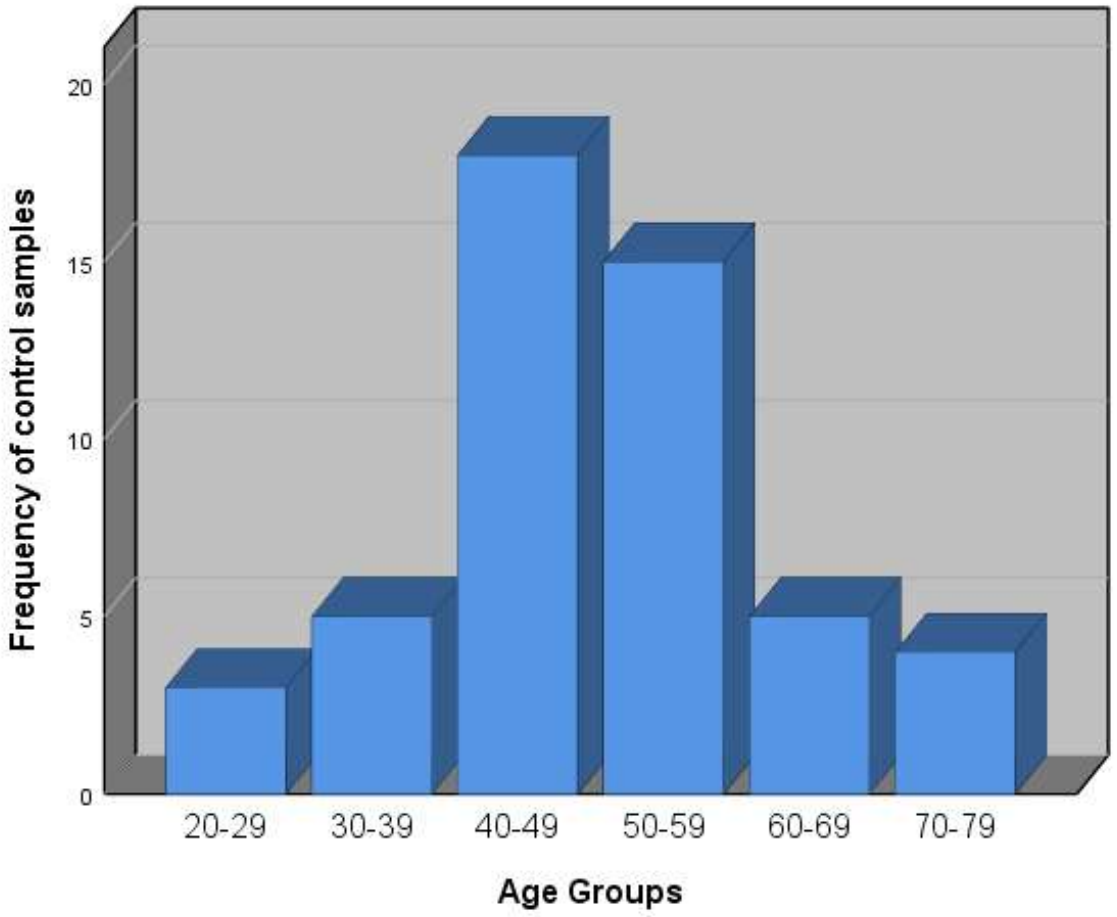

Figure 4.4 Frequency of control samples in different age groups. The highest percent is present in 40-49 age group followed by 50-59 years of age. Individuals within this age group were selected as control samples to match the age distribution of HCC patients i.e., test sample population.

Furthermore, the gender distribution of control samples was also performed in order to compare them to HCC sample population. The dominant gender here was also male population with 32 cases followed by females with 18 cases and this trend is once again very similar to HCC patient population with a majority of male population as compared to females, as shown in the Figure 4.5

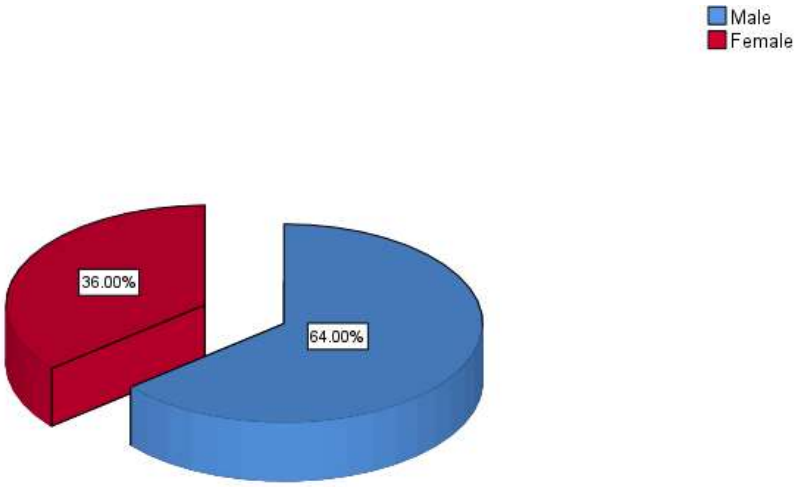

Figure 4.5: Percent gender distribution in control samples. The male population formed 64% of the control sample population followed by females i.e., 36%.

4.4. ELISA results analysis

The above-mentioned sample population (test samples=150; control=50) were analyzed by ELISA to check for the presence of 4 proposed biomarkers i.e., C8A, SERPINC1, MBL2 and HSD11B1 and 1 currently accepted biomarker i.e., Alpha Fetoprotein (AFP). ELISA results were analyzed via 'Myassays.com' and the clinical utility of these biomarkers was computed out via SPSS version 26 and Graph Pad Prism. The concentrations obtained via ELISA were then used to perform multiple statistical analysis and finally determination of sensitivity and specificity as shown in Figure 4.6.

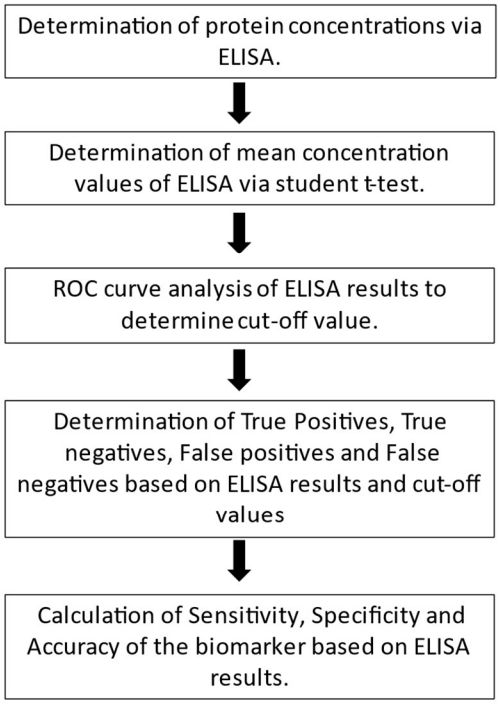

Figure 4.6. Overview of statistical analysis. Flow-sheet diagram of statistical analysis from ELISA results to determine the sensitivity, specificity, and accuracy of a biomarker.

Group statistics of all the five proteins under test (1 standard AFP and 4 candidate biomarkers) are summarized in the following Table 4.3. This includes the mean values of the protein concentrations obtained as well as the standard deviation of these biomarkers.

Table 4.3: Group statistics of 5 proteins (1 currently accepted AFP and 4 candidate biomarkers) computing the mean values and standard deviations in both test samples and controls. This table highlights the mean concentrations of these proteins found in both HCC patients (n=150) and healthy individuals (n=50) along with the standard deviation.

Group Statistics

	Case Type	N	Mean	Std. Deviation
AFP (ng/ml)	Test Sample	150	6.7589	.14005
	Control	50	6.6444	.32960
SERPINC1 (ng/ml)	Test Sample	150	13.2669	2.03313
	Control	50	12.8129	3.81568
MBL2 (ng/L)	Test Sample	150	78.01306867	52.857922698
	Control	50	67.76956600	27.535723580
C8A (µg/ml)	Test Sample	150	13.5116	4.58855
	Control	50	19.3097	1.00022
HSD11B1 (pg/ml)	Test Sample	150	215.6540	75.78212
	Control	50	207.3540	40.86932

These proteins are individually discussed as follows.

4.4.1. AFP

4.4.1.1. Mean concentration values

Alpha Fetoprotein (AFP) the currently prevalent biomarker for HCC was checked in test and control samples. The mean concentration values in test samples (HCC patients) were elevated as compared to control samples as shown in the Figure 4.7. The higher concentration of AFP in HCC samples with a significant p-value indicates its utility as an HCC biomarker.

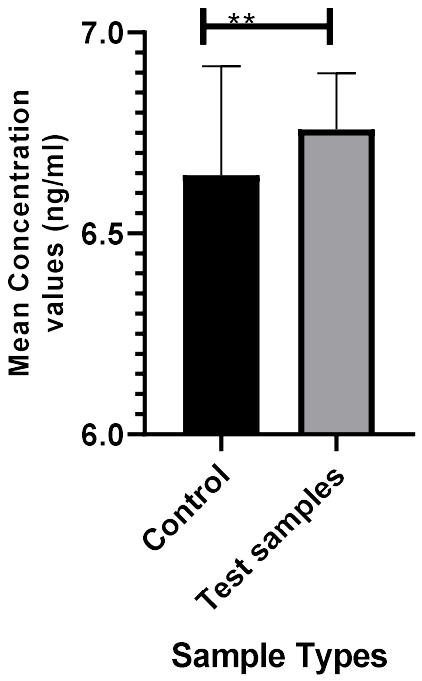

Figure 4.7: Mean values of controls and test samples for AFP. Mean concentration values (ng/ml) of AFP in 150 HCC patient's blood samples and healthy controls. The mean value in test samples is slightly elevated in test samples (6.7589 ng/ml) compared to control sample population (6.6444 ng/ml).

4.4.1.2. ROC curve analysis of AFP

Based upon the difference in mean values, the diagnostic ability of AFP was calculated via performing ROC curve analysis on SPSS v26 as shown in the Figure 4.8. The confidence interval (CI) was set at 95% and the ROC curve was obtained. The AUC was found to be 0.752. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 6.6504 ng/ml. The ROC curve for AFP is shown in Figure 4.8. 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, and it depends upon the objectives of the study. For determining the diagnostic ability of a biomarker, the cut off value with the highest sum of sensitivity and specificity is selected (Choi *et al.*, 2019)

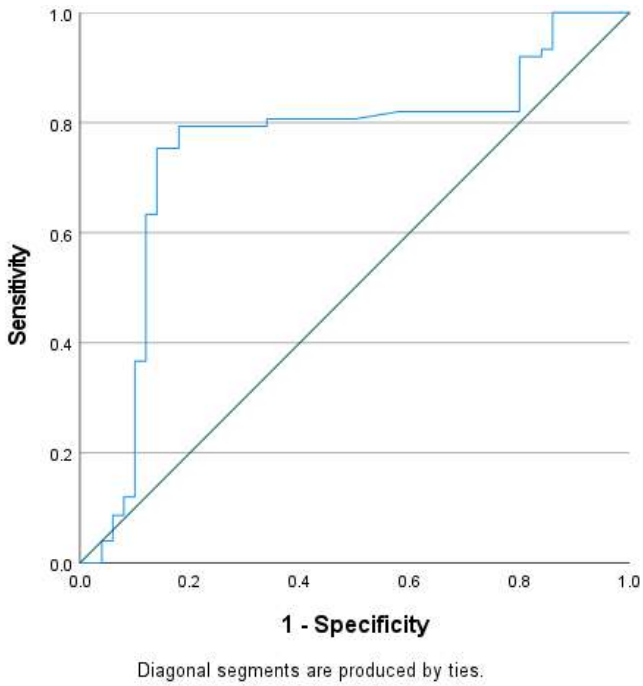

Figure 4.8: ROC curve for AFP. ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.752 and the cut off value of 6.6504 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.4.1.3. Determination of diagnostic ability of AFP

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 supports the diagnostic ability of the biomarker. Following is the Table 4.4 containing the numbers of True positives, False positives, True negatives and False negatives.

Table 4.4: Diagnostic ability of 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)

AFP's diagnostic ability to distinguish HCC and control samples

Count

		Biomarker AFP		
		Present	Absent	Total
Case Type	Test Sample	114	9	150
	Control	36	41	50
Total		114	86	200

4.4.1.4. 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 76.00% whereas the specificity was determined at 82.00%. The accuracy of the test was determined at 77.50%. These parameters are given in the Table 4.5.

Table 4.5: Diagnostic parameters of AFP calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	76.00%	68.35% to 82.59%
Specificity	82.00%	68.56% to 91.42%
Positive Predictive Value (*)	92.68%	87.44% to 95.84%
Negative Predictive Value (*)	53.25%	45.44% to 60.90%
Accuracy (*)	77.50%	71.08% to 83.09%

4.4.2. C8A

4.4.2.1. Mean concentration values

C8A showed a lower mean concentration value in test samples (13.5 µg/ml) compared to control samples (19.3 µg/ml) as shown in the Figure 4.9. 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. The lower mean values in HCC patients as compared to healthy individuals hints at the disturbance of the complement pathway in HCC. This lower concentration with a significant p-value hints at C8A's excellent potential to serve as an HCC biomarker.

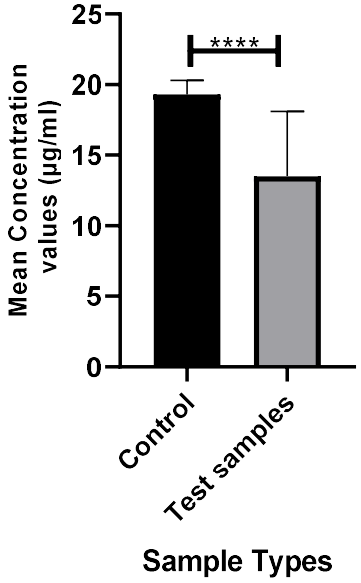

Figure 4.9: Mean values of controls vs test samples for C8A. Mean concentration values (µg/ml) of C8A in 150 HCC patient's blood samples and healthy controls. The mean value in test samples is lower in test samples (13.5 µg/ml) compared to control sample population (19.3 µg/ml). This low mean value of C8A in HCC patients hints at the disturbance of complement system in HCC patients. There is a need to conduct further research on the molecular pathways of HCC which interfere with the complement system.

4.4.2.2. ROC curve analysis of C8A

Based upon the difference in mean values, the diagnostic ability of C8A was calculated via performing ROC curve analysis on SPSS version 26 as shown in the Figure 4.10. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.926. The cut off value was determined by selecting the value with the highest sum of sensitivity and specificity at 17.5099 µg/ml. The ROC curve for C8A is given in Figure 4.10.

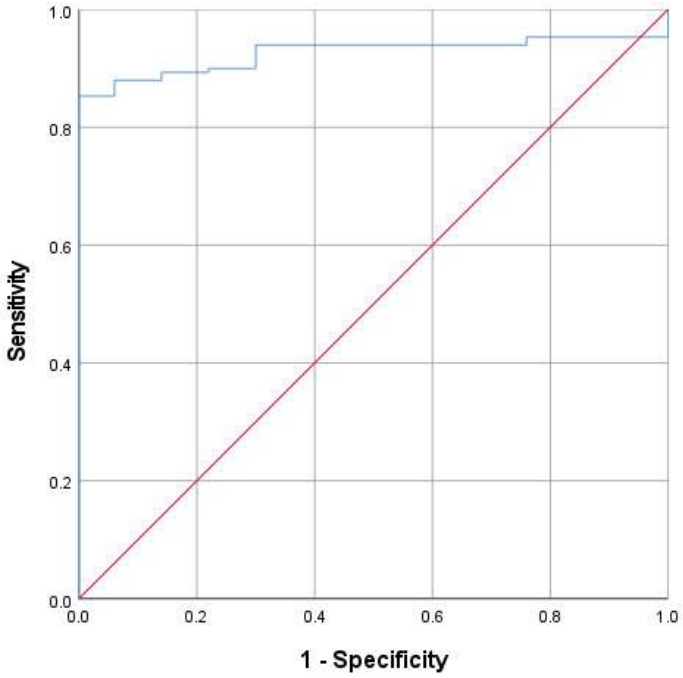

Figure 4.10: **ROC curve for C8A.** ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.926 and the cut off value of 17.5099 µg/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.4.2.3. Determination of diagnostic ability of C8A

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the number of True positives, True negatives, False positives and False negatives. These numbers give an idea on the diagnostic ability of the biomarker. Following is the Table 4.6 containing the numbers of True positives, True negatives, False positives and False negative.

Table 4.6: Diagnostic ability of C8A 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)

Count

		Biomarker C8A		
		Present	Absent	Total
Case Type	Test Sample	128	22	150
	Control	0	50	50
Total		128	72	200

4.4.2.4. Determination of diagnostic parameters of C8A

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 85.33% whereas the specificity was determined at 100.00%. The accuracy of the test was determined at 89.00%. These parameters are given in the Table 4.7. All of these values are calculated from ELISA results.

Table 4.7 Diagnostic parameters of C8A calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	85.33%	78.64% to 90.57%
Specificity	100.00%	92.89% to 100.00%
Positive Predictive Value (*)	100.00%	
Negative Predictive Value (*)	69.44%	60.71% to 76.98%
Accuracy (*)	89.00%	83.82% to 92.98%

4.4.3. SERPINC1

4.4.3.1. Mean concentration values

SERPINC1, one of the four candidate biomarkers showed a higher mean value in test samples (13.2 ng/ml) compared to control samples (12.8 ng/ml) as shown in the Figure 4.11. Even though there is a difference in mean values of HCC patients and controls, a non-significant p-value shows there is quite an overlap between the concentration of SERPINC1 in HCC and controls. This shows that SERPINC1 is an unreliable candidate biomarker for HCC.

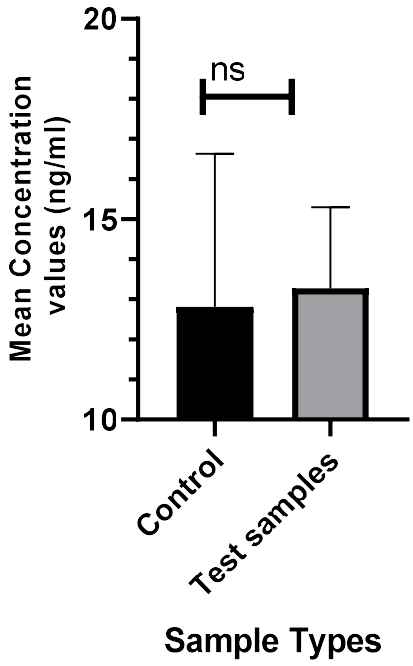

Figure 4.11: Mean values of SERPINC1 for HCC and healthy controls. The mean value in test samples is slightly elevated in test samples (13.2 ng/ml) compared to control sample population (12.8 ng/ml).

4.4.3.2. ROC curve analysis of SERPINC1

Based upon the difference in mean values, the diagnostic ability of SERPINC1 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 0.677. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 12.5818 ng/ml. The ROC curve for SERPINC1 is shown in Figure 4.13. The AUC value of SERPINC1 shows a moderate potential of SERPINC1 to distinguish between HCC patients and controls.

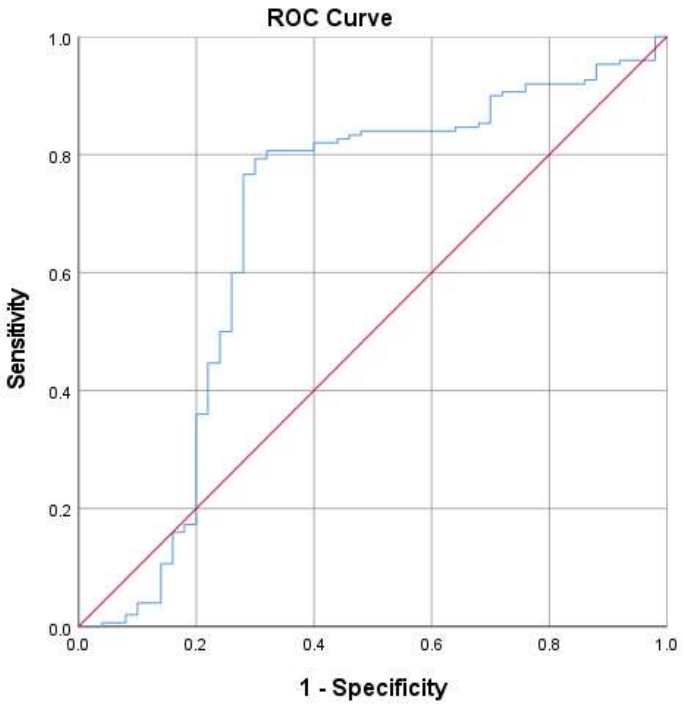

Figure 4.12: ROC curve for SERPINC1. ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.677 and the cut off value of 12.5818 ng/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.4.3.3. Determination of diagnostic ability of SERPINC1

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 biomarker. Following is the Table 4.8 containing the numbers of True positives, False positives, True negatives and False negatives.

Table 4.8: Diagnostic ability of SERPINC1 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)

SERPINC1's diagnostic ability to distinguish HCC and control samples

Count

		Biomarker SERPINC1		
		Positive	Negative	Total
Case Type	Test Sample	121	29	150
	Control	16	34	50
Total		137	63	200

4.4.3.4. Determination of diagnostic parameters of SERPINC1

Upon the 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 80.67% whereas the specificity was determined at 68.00%. The accuracy of the test was determined at 77.50%. These parameters are given in the Table 4.9.

Table 4.9 Diagnostic parameters of SERPINC1 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95%Confidence Interval**
Sensitivity	80.67%	73.43% to 86.65%
Specificity	68.00%	53.30% to 80.48%
Positive Likelihood Ratio	88.32%	83.36% to 91.94%
Negative Likelihood Ratio	53.97%	44.54% to 63.12%
Accuracy	77.50%	71.08% to 83.09%

4.4.4. HSD11B1

4.4.4.1. Mean concentration values

HSD11B1 showed a higher mean concentration value in test samples means HCC positive (215.65 pg/ml) compared to control samples (207.35 pg/ml) as shown in the Figure 4.13. The p-value is greater than 0.05 which shows that there is considerable overlap between the concentration values of HSD11B1 in HCC patients and controls and depicts its poor potential to serve as an HCC biomarker.

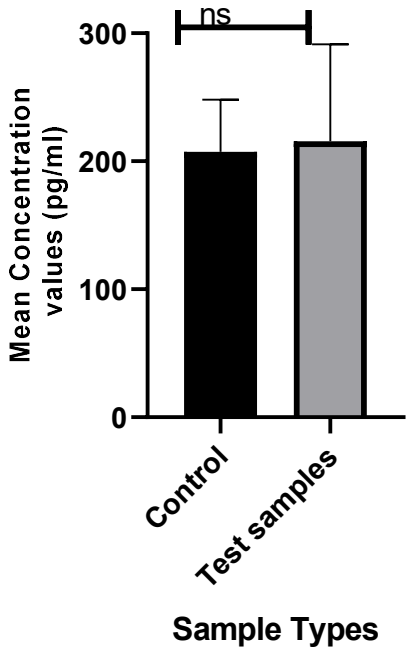

Figure 4.13: Mean values of controls and HCC samples for HSD11B1. Mean concentration values (pg/ml) of HSD11B1 in 150 HCC patient's blood samples and healthy controls. The mean value in test samples is higher in test samples (215.65 pg/ml) compared to control sample population (207.35 pg/ml).

4.4.4.2. ROC curve analysis of HSD11B1

Based upon the difference in mean values, the diagnostic ability of HSD11B1 was calculated via performing ROC curve analysis on SPSS version 26 as shown in the Figure 4.14. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.583. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 190.1 pg/ml. The ROC curve analysis of HSD11B1 is given in Figure 4.14. The AUC of 0.583 is very low and shows HSD11B1's poor potential to distinguish between HCC patients and healthy individuals.

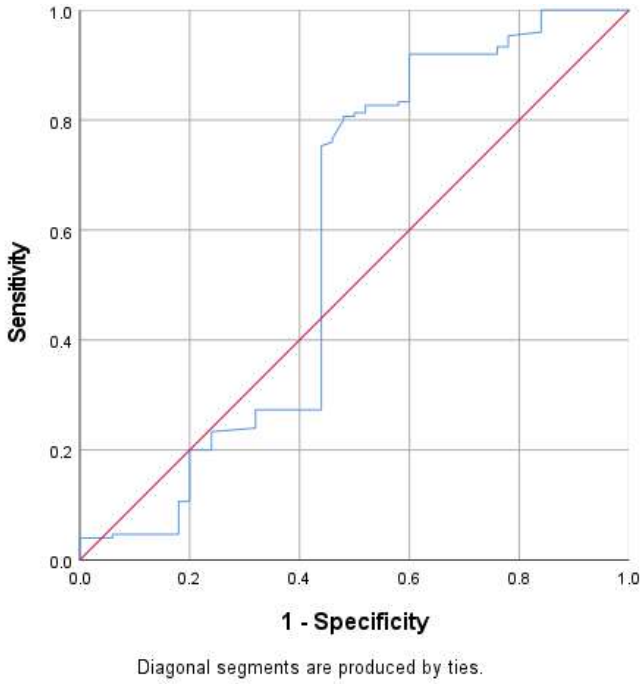

Figure 4.14: ROC curve for HSD11B1. ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The AUC is 0.583 and the cut off value of 190.1 pg/ml is selected as it gave the maximum possible sum of sensitivity and specificity.

4.4.4.3. Determination of diagnostic ability of HSD11B1

Based upon the ELISA results and cut off value, the sample population including both HCC patients and controls were checked to determine the number of True positives, False positives, True negatives, and False negatives. These numbers give an idea into the diagnostic ability of the biomarker. Following is the Table 4.10 containing the numbers of True positives, False positives, True negatives, and False negatives.

Table 4.10 Diagnostic ability of HSD11B1 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)

HSD11B1's diagnostic ability to detect HCC and control samples

Count

		Biomarker HSD11B1		
		Present	Absent	Total
Case Type	Test Sample	113	37	150
	Control	22	28	50
Total		135	65	200

4.4.4.4. Determination of diagnostic parameters of HSD11B1

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 75.33% whereas the specificity was determined at 56.00%. The accuracy of the test was determined at 70.50%. These parameters are given in the Table 4.11.

Table 4.11 Diagnostic parameters of HSD11B1 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	75.33%	67.64% to 82.00%
Specificity	56.00%	41.25% to 70.01%
Positive Predictive Value (*)	83.70%	78.76% to 87.68%
Negative Predictive Value (*)	43.08%	34.28% to 52.34%
Accuracy (*)	70.50%	63.66% to 76.72%

4.4.5. MBL2

4.4.5.1. Mean concentration values

MBL2 showed a higher mean value in test samples (78.01 ng/L) compared to control samples (67.7 ng/L) as shown in the Figure 4.15. There is a higher mean concentration of MBL2 in HCC patients as compared to healthy individuals, but the p value is non-significant (>0.05) which shows that the values are overlapping and MBL2 does not possess a good potential to serve as an HCC biomarker.

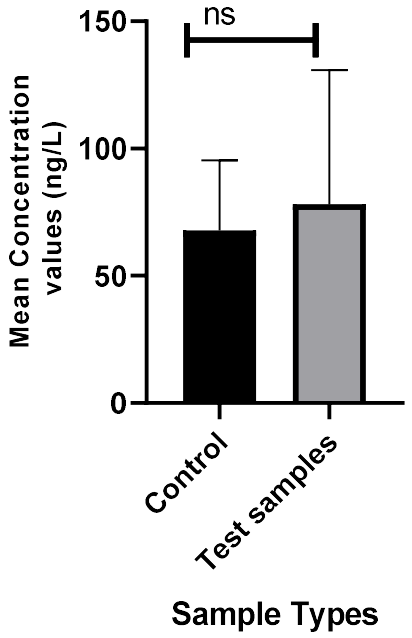

Figure 4.15: Mean values of controls vs Test samples for MBL2. Mean concentration values (ng/L) of MBL2 in 150 HCC patient's blood samples and healthy controls. The mean value in test samples is elevated in test samples (78.01 ng/L) compared to control sample population (67.7 ng/L).

4.4.5.2. ROC curve analysis of MBL2

Based upon the difference in mean values, the diagnostic ability of MBL2 was calculated via performing ROC curve analysis on SPSS version 26 as shown in the Figure 4.16. The CI was set at 95% and the ROC curve was obtained. The AUC was found to be 0.571. The cut off value was then determined by selecting the value with the highest sum of sensitivity and specificity at 56.627 ng/L. The ROC curve analysis of MBL2 is given in Figure 4.16. The low AUC value 0.571 shows MBL2 is not suitable candidate biomarker to distinguish between HCC patients and controls.

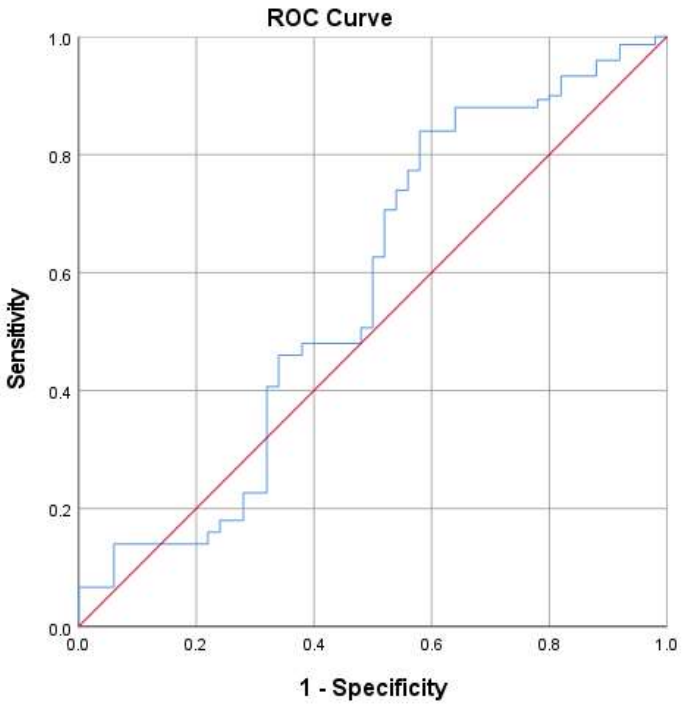

Figure 4.16 ROC curve for MBL2. ROC curve obtained via plotting Sensitivity against 1-Specificty at every possible cutoff value. The area under the curve (AUC) is 0.571 and the cut off value of 56.627 ng/L is selected as it gave the maximum possible sum of sensitivity and specificity.

4.4.5.3. Determination of diagnostic ability of MBL2

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the True positives, False positives, True negatives, and False negatives. These numbers give an idea into the diagnostic ability of the biomarker. Following is the Table 4.12 containing the numbers of True positives, False positives, True negatives and False positives.

Table 4.12: Diagnostic ability of MBL2 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)

MBL2's diagnostic ability to distinguish HCC and Control samples

Count

		BiomarkerMBL2		
		Present	Absent	Total
CaseType	Test Sample	94	56	150
	Control	25	25	50
Total		119	81	200

4.4.5.4. Determination of diagnostic parameters of MBL2

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 62.67% whereas the specificity was determined at 50.00%. The accuracy of the test was determined at 59.50%. These parameters are given in the Table 4.13

Table 4.13 Diagnostic parameters of MBL2 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% CI**
Sensitivity	62.67%	54.40% to 70.42%
Specificity	50.00%	35.53% to 64.47%
Positive Predictive Value (*)	78.99%	73.52% to 83.59%
Negative Predictive Value (*)	30.86%	24.00% to 38.69%
Accuracy (*)	59.50%	52.35% to 66.37%

4.5. Combined diagnostic parameters of six proteins

The above-mentioned diagnostic parameters of all the six proteins including 5 candidate biomarkers and 1 AFP as a currently accepted standard are given in Figure 4.17.

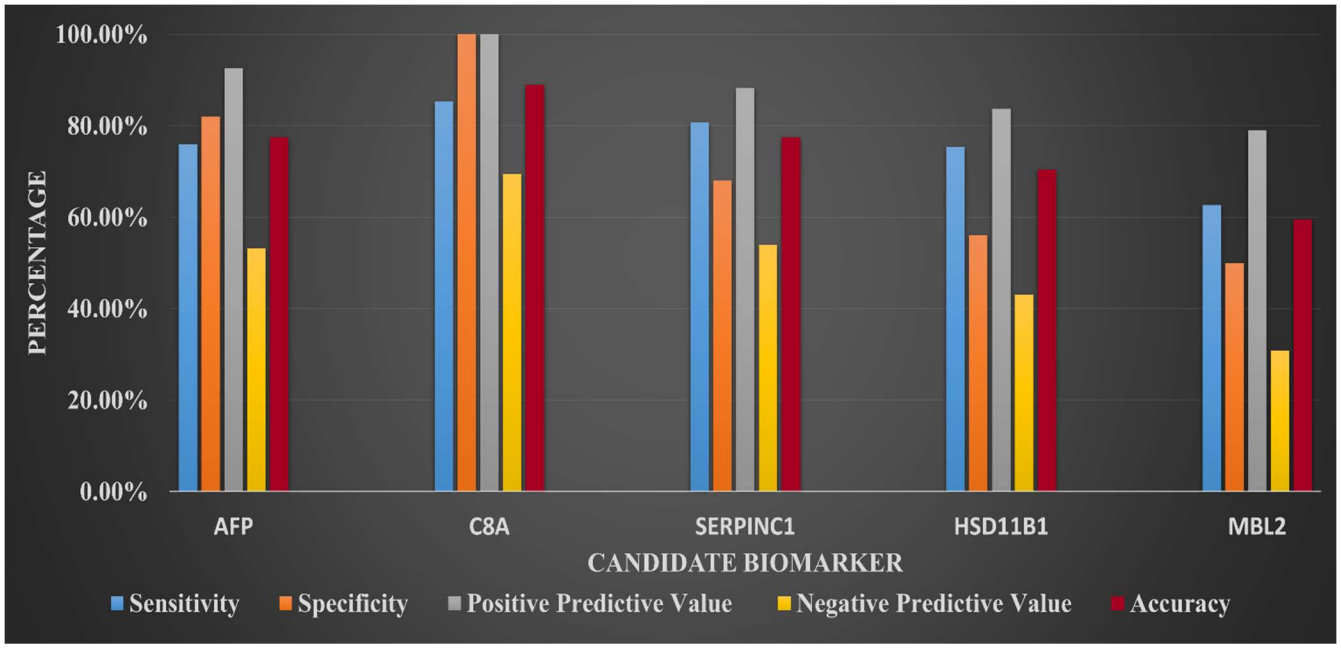

Figure 4.17: Combined diagnostic parameters of AFP, C8A, SERPINC1, HSD11B1 and MBL2. C8A shows the highest sensitivity and specificity values followed by SERPINC1 which shows moderate potential as compared to AFP.

4.6. Western blot analysis

Western blot was performed to qualitatively 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. We aimed to achieve a single continuous band of C8A among all the samples so that we can qualitatively confirm the presence of C8A in the samples and thus validating our ELISA results.

4.6.1. C8A Western blot

C8A shows a significant potential to serve as biomarker for HCC. So, in order 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 C8A in these samples. The results of western blot analysis are shown in the Figure 4.18

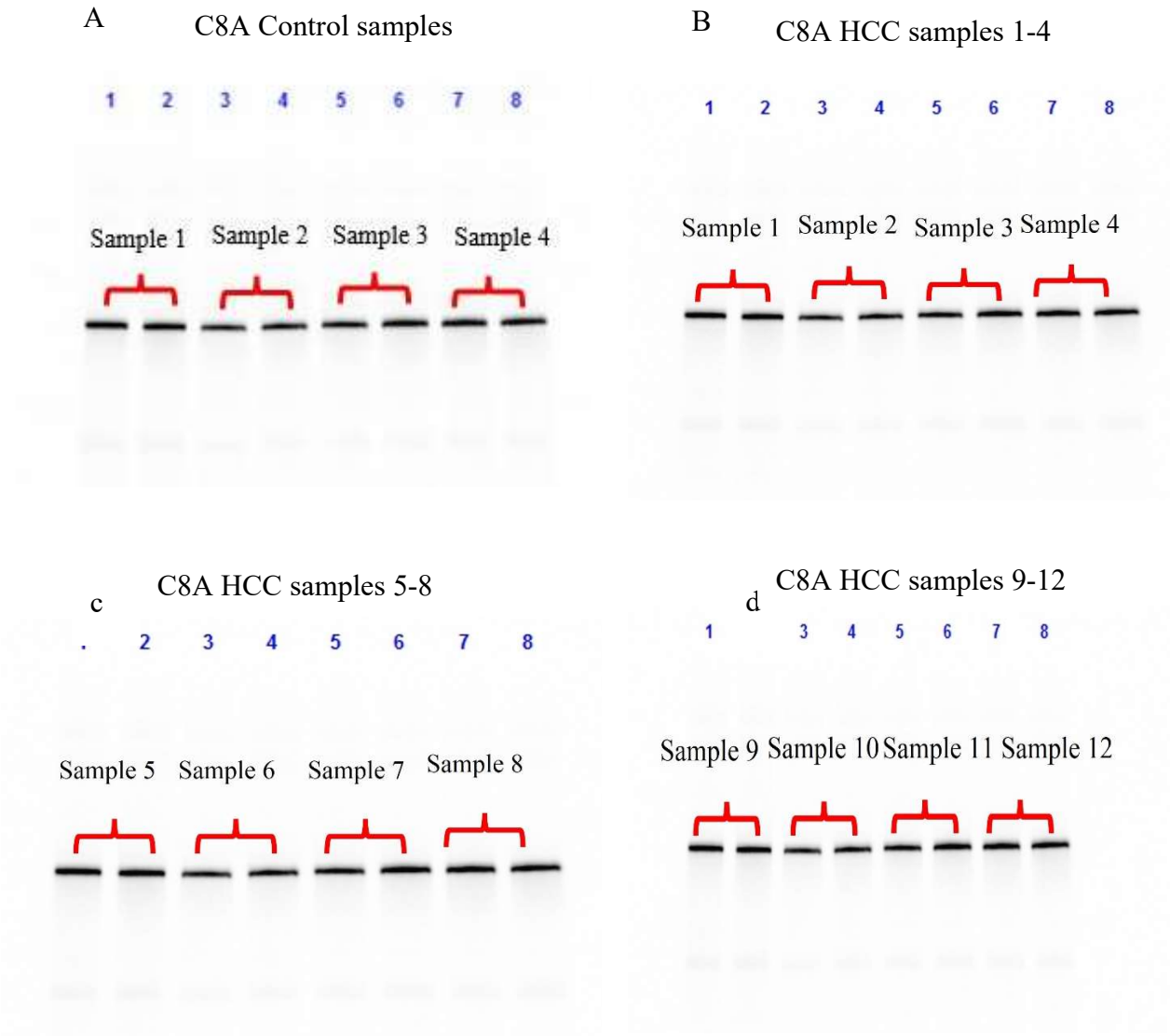

Figure 4.18: Western blots of 4 control samples (A) and 12 test samples (B, C and D). All the samples are in duplicates and lanes 1-2, 3-4, 5-6 and 7-8 show replicates of same samples. The existence of a continuous band exhibits the presence of C8A and the antibody specific reactivity to C8A, thus qualitatively confirming the validity of our ELISA results for C8

Chapter 5 Discussion

Hepatocellular carcinoma is one of the biggest contributors to the global burden of cancer. The only potential treatment options available for HCC are liver transplant, surgical resection, and tumor ablation (Lurje *et al.*, 2019). Due to expensive and invasive treatment options, early detection is the most convenient way to combat HCC. However, the detection methods for Hepatocellular carcinoma are also invasive and expensive techniques like MRI, Ultrasound and histopathology (Ayuso *et al.*, 2018). There is a need to diagnose HCC via non-invasive and inexpensive techniques like detection via blood-based biomarkers. The currently used biomarker against HCC, alpha fetoprotein has very less clinical utility due to its varied and low sensitivity and specificity reported in different studies and can only serve to complement ultrasound (Ahmed Mohammed and Roberts, 2017a; Carr *et al.*, 2018). In this study, we have validated 4 biomarker candidates shortlisted via a previously published bioinformatics pipeline (Awan *et al.*, 2015) namely C8A, SERPINC1, HSD11B1 and MBL2.

The potential of these biomarkers is analyzed in comparison to AFP in serum from HCC patients. The blood samples were collected from Holy Family Hospital, RMU, Pakistan. Among the 4 potential biomarkers, C8A has shown significant potential to serve as the biomarker for HCC, much better than AFP whereas SERPINC1 has also shown moderate biomarker potential for HCC almost equally as AFP. The remaining two candidates MBL2 and HSD11B1 did not show significant ability to distinguish HCC from healthy individuals.

A total of 200 people (150 HCC patients; 50 healthy individuals) were recruited in this study after obtaining their verbal consent and their names were not noted in order to keep their privacy. The data obtained from them in a questionnaire (Annex-1) was shared only with the Principal Investigator and the Research Assistants involved in the study keeping in view of the ethical guidelines (Igoumenidis and Zyga, 2011). For this purpose, the synopsis was defended in front of a panel of doctors and researchers at the Rawalpindi Medical University, Rawalpindi and their approval was obtained via Institutional Review Board (IRB), during which the ethical practices

were scrutinized, and clear guidelines were provided to follow complete ethical procedures while collecting a blood sample from the HCC patients.

In our HCC sample population, the majority of the patients fell into the age group 50-59 (43.3%) followed by 60-69 (29.3%) and 40-49 (20.7%) as shown in Table 4.1. This is in line with previous studies and observations which stress that HCC is most prevalent in the fourth, fifth and sixth decades of life (Mittal *et al.*, 2018). Similarly, division of HCC patients on the basis of gender reflected a majority of male population (61.3%) followed by female population (38.7%) as shown in Table 4.2. This observation is also in line with previous studies which report a higher incidence rate of HCC in males as compared to females (Wu *et al.*, 2018). Upon doing a gender vs age group cross tabulation, it was revealed that in the highest HCC incidence age group i.e., 50-59 years of age, there was not a significant difference in number of male and female patients i.e., 35 and 30 respectively as shown in Figure 4.1. However, a great difference in numbers of HCC was found in 60-69 years of age i.e., 33 male cases and only 11 female cases.

In Pakistan and other parts of the world, viral hepatitis (HBV and HCV) is the leading cause of HCC (D'souza *et al.*, 2020). Therefore, it is important to determine the viral and non-viral history of our HCC population so that the data on causalities of HCC can be determined. As shown in Figure 4.2, among 150 HCC patients, 133 (88.6%) had a viral history with either HBV or HCV. This is in line with previous observations that viral hepatitis is major cause of HCC (Goto *et al.*, 2020). Among the viral cases, the HCC population was divided into HCV and HBV history, and it was found that 103 patients (77.4%) among the 133 viral hepatitis cases were HCV, and 25 patients (18.7%) had a history with Hepatitis B (HBV) as shown in Figure 4.3. This is clearly due to high incidence of HCV in Pakistan (Kanaani *et al.*, 2018) and resultantly higher rate of HCV induced HCC. HBV incidence is also supportive of previous studies that have demonstrated similar percentages of HBV incidence in Pakistan as compared to HCV (Samo *et al.*, 2021) 5 cases (3.7%) had both HCV and HBV incidence history.

50 healthy individuals from different locations ang age groups were recruited in the study so as to obtain a diverse group and their serum samples were obtained after taking verbal consent and following all the ethical guidelines. Individuals were selected on the basis of absence of HCC, HBV and HCV incidence. An analysis into the age groups of these individuals showed a mixed distribution in age groups but closely matches with HCC with most individuals in 40-49 and 50-

59 years of age as shown in Figure 4.4**.** A gender distribution analysis of healthy individuals showed a male majority with 64% population and female population with 36% as shown in Figure 4.5 to keep the gender numbers in line with the test sample population.

After the demographic and medical history analysis of our sample populations (both HCC patients and controls), the candidate biomarkers were quantified via ELISA. ELISA is one of the most powerful tools to quantify proteins in a sample (Hosseini *et al.*, 2018) and in this case, ELISA was performed on serum of both HCC and control samples and their results were analyzed via SPSS version 26.

In case of Alpha Fetoprotein (AFP), the currently used but with very little clinical utility for diagnosis of HCC the mean concentration of AFP was elevated in test samples as compared to control samples as shown in Figure 4.7. An elevated AFP level is usually a characteristic of hepatocellular carcinoma (Özdemir and Baskiran, 2020)**.** However, the standard deviation in control samples is much greater compared to test samples indicating the increased spread of AFP values and is in line with previous studies on HCC diagnostic potential of AFP (Luo *et al.*, 2020)**.** The area under the ROC curve (AUC) at $p<0.05$ was determined to be 0.752 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 6.6504 ng/ml was determined for our HCC population. This value was obtained by analyzing the ROC curve results, which provides 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 (76.00%), specificity (82.00%) and accuracy (77.50%) were determined as shown in Table 4.5.

These values were in line with previous studies conducted on AFP's sensitivity and specificity which are usually in the range of 41% to 84% sensitivity and 80-94% specificity (Mehinovic *et al.*, 2018; Rojas *et al.*, 2018; J. Zhang *et al.*, 2020b; Wang and Zhang, 2020) which is a very broad range due to different expression levels and cut-off values in different populations**.** These diverse values obtained in different studies can be attributed to different populations under study which usually affect the number of AFP positive and negative HCCs (Mehinovic *et al.*, 2018; Luo *et al.*, 2020)**.** Moreover, biomarkers usually tend to differ in different areas and populations and these

varied results can thus be attributed only to specific localities (Ahmed Mohammed and Roberts, 2017b)

C8A, our first biomarker candidate exhibited a low mean concentration value in test samples i.e., 13.5 µg/ml compared to the control sample population 19.3 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 0.926 which exhibits excellent potential to serve as a biomarker for HCC. With the help of ROC curve, the cut off was determined at 17.5099 µg/ml at maximum sum of sensitivity and specificity as shown in Figure 4.10. The sensitivity was determined at 85.33% and specificity at 100.00% which are excellent values for a biomarker of HCC, and these values are far better than reported for AFP (Mehinovic *et al.*, 2018; Rojas *et al.*, 2018; J. Zhang *et al.*, 2020b; Wang and Zhang, 2020). The accuracy of the test was also determined at 89.00% which further enhances confidence in the diagnostic ability of this candidate biomarker as shown in Table 4.7. Due to such positive results, a qualitative confirmation was done via performing western blot analysis as shown in Figure 4.18. Western blot was done just to qualitatively confirm the antibody's specific reactivity to C8A, 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 validating the presence of C8A in our samples and the antibody's specificity to C8A.

Another protein biomarker candidate SERPINC1 showed a higher concentration in test samples as compared to controls as shown in Figure 4.11 but just like AFP results, 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 0.677 which shows a moderate biomarker potential, a bit lower than AFP and a cutoff value (12.5818 ng/ml) determined as described previously gave sensitivity, specificity, and accuracy values of 80.67%, 68.00% and 77.50% respectively as shown in Table 4.9**.** These values are

comparable to the values obtained for AFP in this study and the reported literature on AFP (Mehinovic *et al.*, 2018; Rojas *et al.*, 2018; J. Zhang *et al.*, 2020b; Wang and Zhang, 2020), and thus exhibits a quite similar moderate biomarker potential for HCC. These close values for SERPINC1 and AFP provide an opportunity to investigate a combined biomarker panel of AFP and SERPINC1 to diagnose HCC.

The remaining two biomarker candidates (HSD11B1 and MBL2) showed higher mean concentrations in test samples but there was a huge spread of data which indicated their poor biomarker potential. The results of the t-test did not show significant p value, and this showed the overlap of values for these biomarkers in HCC patients and in controls, thus ruling them out as potential biomarkers of HCC. This was further verified via their ROC curve analysis in which AUCs at $p<0.05$ gave 0.583 and 0.571 values which highlight their poor diagnostic potential. The sensitivity for HSD11B1 was in the moderate range (75.33%) but very poor specificity at 56.00 %. MBL2 showed even poorer numbers with 62.67% sensitivity and only 50.00 % specificity which highlight little to no biomarker potential for both HSD11B1 and MBL2.

Chapter 6 Conclusion and Prospects

This study has validated four serum protein biomarker candidates namely C8A, SERPINC1, HSD11B1 and MBL2 out of seven proposed biomarkers previously by Awan et al., 2015. These biomarker studies 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. It also provides an insight into the performance of AFP as a diagnostic marker for HCC in Pakistani population. The diagnostic abilities of the candidate biomarkers are compared with AFP and one biomarker (C8A) with a very good result and one (SERPINC1) with a moderate biomarker potential in comparison to AFP 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.

Future prospects include the testing of the remaining three proteins (ADH6, CYP2A6 and UPB1) out of seven candidates proposed by Awan et al., 2015. 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. Moreover, there is a need to conduct multiplex protein assays to detect strong biomarker candidates simultaneously in the blood samples of HCC patients. 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 we diagnose HCC today.

Chapter 7 References

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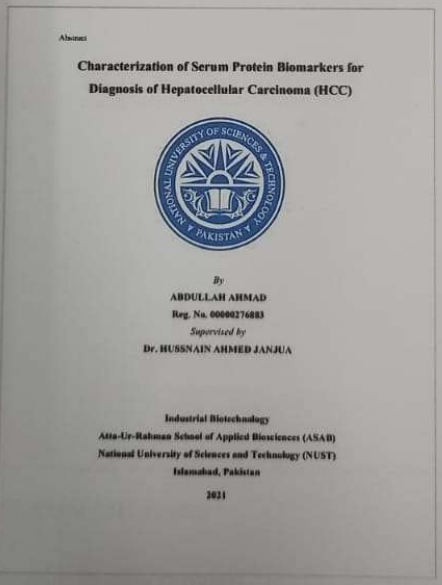

Abstract

Characterization of Serum Protein Biomarkers for Diagnosis of Hepatocellular Carcinoma (HCC)

By

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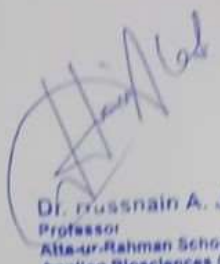

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