ASSOCIATION OF PIVKA II WITH HEPATOCELLULAR CARCINOMA

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06-117222-001

BAHRIA UNIVERSITY ISLAMABAD

PAKISTAN

ASSOCIATION OF PIVKA II WITH HEPATOCELLULAR CARCINOMA

By

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APPROVAL FOR EXAMINATION

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ABSTRACT

Primary liver tumours are significantly influenced by hepatocellular carcinoma, a diverse liver disease. Clinically, this carcinoma presents with obstructive jaundice, pyogenic liver abscess, and hepatic encephalopathy in addition to variceal or intraperitoneal hemorrhage. In hepatocellular cancer, metastasis outside liver frequently in the lung, abdominal lymph nodes, bone, and adrenal glands. The prevalence of HCC in Pakistan is estimated to be between 3.7% and 16.7%. Exposure to aflatoxin B, hepatitis B or C viruses' infections (HBV or HCV), and alcoholic cirrhosis are the major factors in the HCC development. In Pakistan the causes of HCC such as hepatitis B and hepatitis C virus are common therefore it is a potential burden. Alpha fetoprotein is used as biomarker for detection of HCC, but its specificity and sensitivity are less and also its level is increased in other liver disease. Current diagnostic tools for HCC suffer from a lack of practical and acceptable biomarkers with high specificity and sensitivity. Therefore, it is critical to investigate potential new biomarkers for HCC that can improve the precision of early diagnosis and improve the chances of successful outcomes. Through a comparative cross-sectional study conducted over the course of six months, 55 subjects were included in the study who were diagnosed with hepatocellular carcinoma. Subjects ranged in age from 18 to 65 years old and male or female has been included in the study. Blood samples of the patients were taken and then stored in the laboratory. AFP and PIVKA II levels in the serum were then analyzed by enzyme-linked immunoassay (ELISA). PIVKA II sensitivity and specificity with the alpha fetoprotein has been compared through ROC curve. The sensitivity of PIVKA II was 79.4% and specificity was 72.9% at the level of >40m AU/ml. the sensitivity of AFP was 75.7% and specificity was 71.5% at the level of 10ng/ml. a combination of PIVKA II and AFP give the sensitivity of 80.3% and specificity of 75.2. % sensitivity and specificity of PIVKA II was more in HCC cases as compared to alpha

fetoprotein. Hence it was concluded that the sensitivity and specificity of protein induced vitamin K since II (PIVKA II) is higher than the Alpha Fetoprotein (AFP).

Key words: hepatocellular carcinoma, PIVKA II, cirrhosis, hepatitis B, hepatitis C

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HCC	Hepatocellular Carcinoma
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic SteatoHepatitis
SNP	Single Nucleotide Polymorphism
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
AFB1	Aflatoxin B1
CYP450	Cytochrome P450
NKT	Natural Killer T Cells
BCLC	Barcelona Clinic Liver Cancer
CT	Computed Tomography
MRI	Magnetic Resonance Imaging

AFP	Alpha Fetoprotein
PIVKA II	Protein Induced Vitamin K Absence II
PT	Prothrombin
ER	Endoplasmic Reticulum
DCP	Des Gamma Carboxyprothrombin
US	Ultrasonography
TACE	Trans arterial Chemoembolization
ALT	Alanine Transaminase
GGT	Gamma Glutamyl Transferase
ROC	Receiver Operating Characteristics Curve
AUC	Area under the curve
AU/mL	Arbitrary unit per milliliter

LIST OF ANNEXURES

CHAPTER 1

INTRODUCTION

1.1 Background

Hepatocellular carcinoma is a multifaceted liver disease that has a substantial impact on primary liver tumors as shown in fig. 1.1(Luna-Cuadros *et al*., 2022). In addition to variceal or intraperitoneal hemorrhage, this cancer clinically manifests as obstructive jaundice, pyogenic liver abscess, and hepatic encephalopathy. Extrahepatic metastasis in lung, abdominal lymph nodes, bone, and adrenal glands is common in hepatocellular carcinoma (Asafo-Agyei *et al*., 2023).

Aflatoxin rich diet, frequent alcohol usage, infection by hepatitis virus C (HCV) and hepatitis B virus (HBV) are the main causes of HCC (Hadi *et al*., 2022). The risk of HCC increases with nonalcoholic steatohepatitis (NASH), or Nonalcoholic fatty liver disease (NAFLD) as shown in fig. 1.2 (Yang *et al*., 2021, Coffin *et al*., 2023).

In the past several years, it has been recognized that HCC is one of the most common prevalent malignancies diagnosed worldwide. Due to its increasing prevalence, HCC is currently a significant part of the global rates of deaths due to cancer (Piñero *et al*., 2020). It ranks third worldwide in terms of mortality and fifth in terms of morbidity among malignant tumors, posing a serious threat to life and health (Tian *et al* 2023). In 2020, 830000 fatalities worldwide were caused by primary liver cancer, or 8.3% of cancer-related

deaths, and 906,000 new cases globally in 2020, accounting for 4.7% of all cancer cases (Hadi *et al* 2022).

Hepatocellular carcinoma (HCC) incidence in Pakistan is also a concern and it thought to vary between 3.7% to 16.7% (Tahir, 2021). In Pakistan hepatocellular carcinoma is also increasing due to its causes such as viral hepatitis B and C which are common in Pakistan (Tahir, 2022, Afzal 2017).

Hepatocellular carcinoma can develop at any age, most common at the old age group. Its occurrence varies in different age groups and nations. It is generally acknowledged that, worldwide, men are more likely than women to get HCC (Zhang *et al.,* 2022) The development of HCC influenced differently by sex hormones; while estrogen have a preventive effect, androgen have a stimulating effect (Yip *et al*., 2019).

Hepatocellular carcinoma is a complex illness with a varied pathophysiology that often begins with liver injury and the inflammatory response that follows. Chronic liver inflammation triggers the fibrotic response, which ultimately results in cirrhosis-related changes to the architecture of the liver tissue the root causes of precancerous illnesses that eventually manifest as cancer include cirrhosis and persistent inflammation. These conditions aid in the slow addition of epigenetic and genetic alteration that eventually result in the development of HCC (Daniela *et al.,* 2024).

HCCs can range widely in size, from little tumors (diameter < 2cm) to enormous tumors with a diameter of more than 20 cm that can completely replace a liver lobe. Macroscopic patterns can be broadly classified into three categories: diffuse or cirrhotomimetic, enormous, and nodular. The Liver Cancer Study Group of Japan (LCSGJ) suggested splitting the nodular type into two subclasses: clearly nodular and vaguely nodular, with relation to the hepatectomy specimens (Andrea *et al*., 2022).

People are predisposed to chronic liver disease by numerous genetic abnormalities. The main reasons for this are protein deficiency (alpha-1 antitrypsin deficiency due to SERPINA1 mutations), iron overload (haemochromatosis due to C282Y/C282Y mutations of HFE), or metabolic issues (tyrosinemia due to FAH mutations).

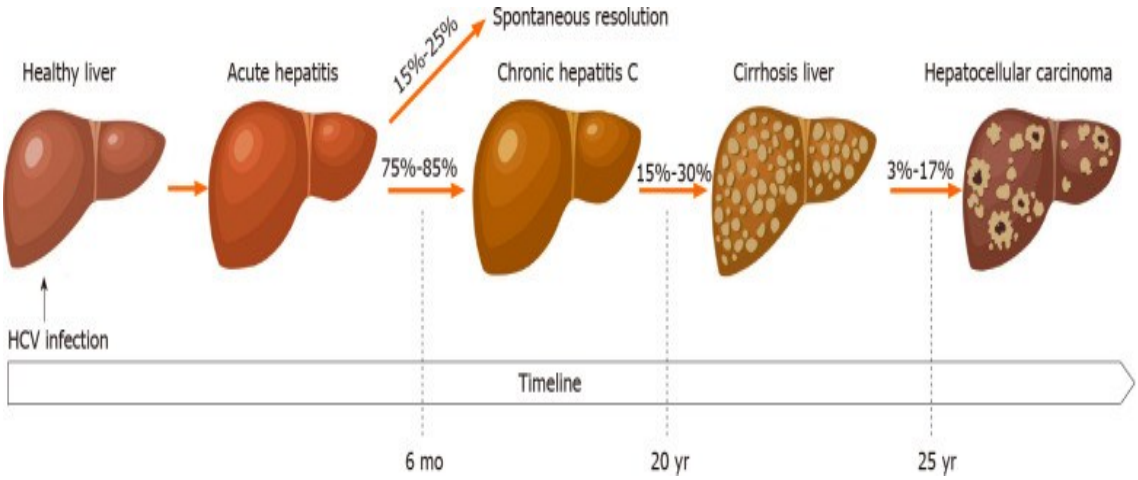


Figure 1.1 Hepatocellular Carcinoma (Maria Alejandra Luna-Cuadros et al., 2022)

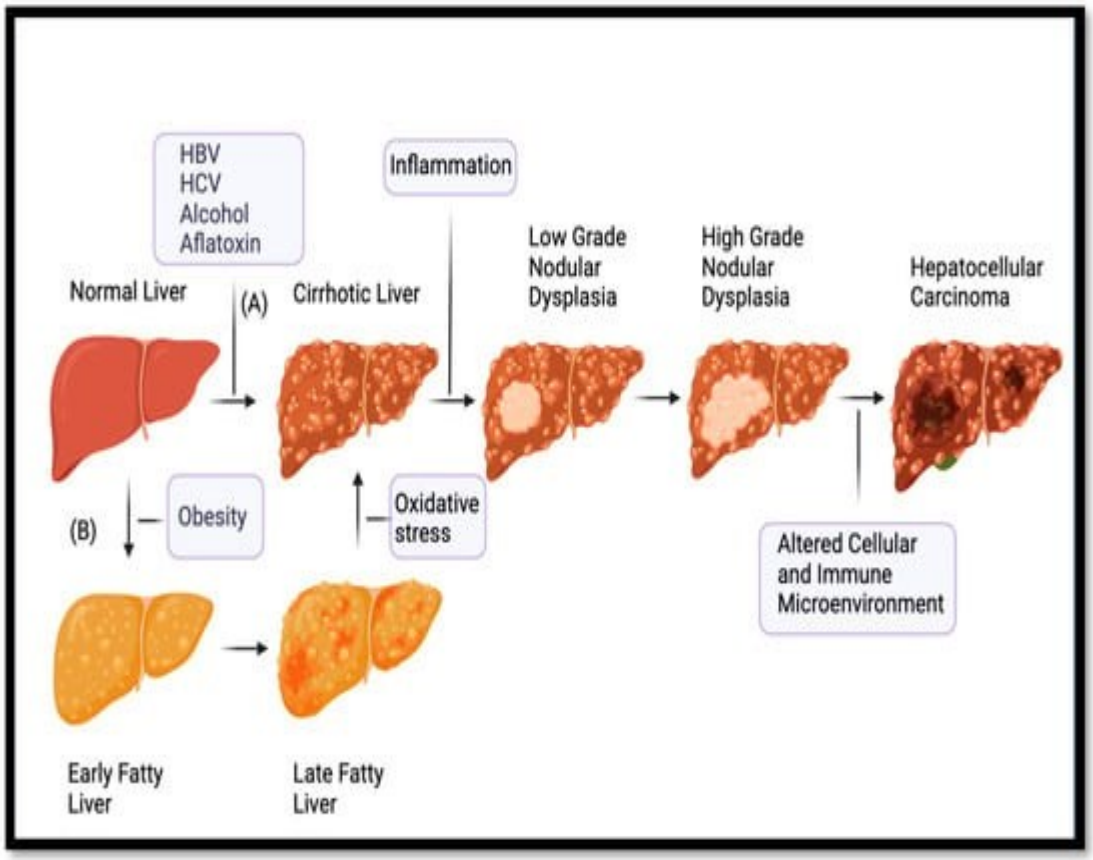


Figure 1.2 Hepatocellular carcinoma (Philip coffin *et al*., 2023)

All of these inherited mutations take part in HCC development, with the exception of glycogenosis type 1A (caused by G6PC inactivating mutations) and germline HNF1A mutations, which both predispose to benign liver tumors with potential for malignant change in a non-fibrotic liver (Miryam *et al*., 2020). Numerous single-nucleotide polymorphisms (SNPs) connected to multiple pathways such as growth factors, oxidative stress, DNA repair, cell cycle regulation, inflammation, and iron metabolism have been associated to the development of HCC (Miryam *et al*., 2020).

When examining malignant transforming foci in lesions under a microscope, different characteristics can be seen, such as faintly nodular, multicentric, expansile, cirrhotomimetic, nodular with peri nodular extension, and infiltrative types (Doreen *et al*., 2022).

The two types of small HCCs, less than 2 cm, are as follows: early HCCs are generally more cellular compared the cirrhotic tissue around it with irregular trabeculae, pseudo acini formation, increased nuclear to cytoplasmic ratio, vague nodules, and indistinct margins. More advanced morphologically, traditional HCC shows four basic architectural growth patterns: trabecular, solid, pseudo glandular/acinar and macro trabecular. HCC are obviously nodular with distinct edges, often capsulated and exhibiting an infiltrative or expansile growth pattern. A number of cytological characteristics often occur in combination (clear cell, steatosis, pleomorphism, multinucleation, foamy cells, oncocyte cells, spindle cells) (Mukul *et al*., 2021).

HCC deaths were caused by HBV, HCV, Alcohol, NAFLD, and related disorders (Sarin *et al* 2020). In Pakistan there are variations in the relative frequency of risk factors; the most common being hepatitis C. Pakistan is one of the few countries in the world where anti-HCV antibody prevalence is more than 3% (Bhatti *et al*., 2016).

Hepatitis C virus is a Positive-sense single-stranded RNA virus. Globally, liver illnesses are mostly caused by the hepatitis C virus (HCV virus) (David *et al*., 2021). Chronic hepatitis C infection, hepatocellular carcinoma and liver cirrhosis are among the possible outcomes by Hepatitis C virus. Infection with HCV is responsible for 25% of HCC cases worldwide. Numerous host factors including gender, age and length of infection, as

well as viral factors, including genotype/subtype and viral load affect the rate of progression from CHC to HCC. Being an RNA virus, HCV has a very variable genomic sequence (Pin *et al*., 2021).

Seven major genotypes and 67 subgroups have been identified in isolates. While genotypes 1 and 3 along with subtypes 1a, 1b, 2a, and 3a are believed to be the common worldwide mostly. The most common genotype in Asia is genotype 1, while in South Asia which includes Bangladesh, Pakistan, and India, the frequency of genotype 3 is approximately 66.7%. It is estimated that eight to 10 percent of Pakistanis have HCC (Ul-Abideen *et al*., 2019).

The most recent statistics indicate that HBV infection accounts for a large HCC case globally. It has also been demonstrated that a number of pathogenic variables work in tandem with HBV to increase the prevalence of HCC (Sarin *et al*., 2020).

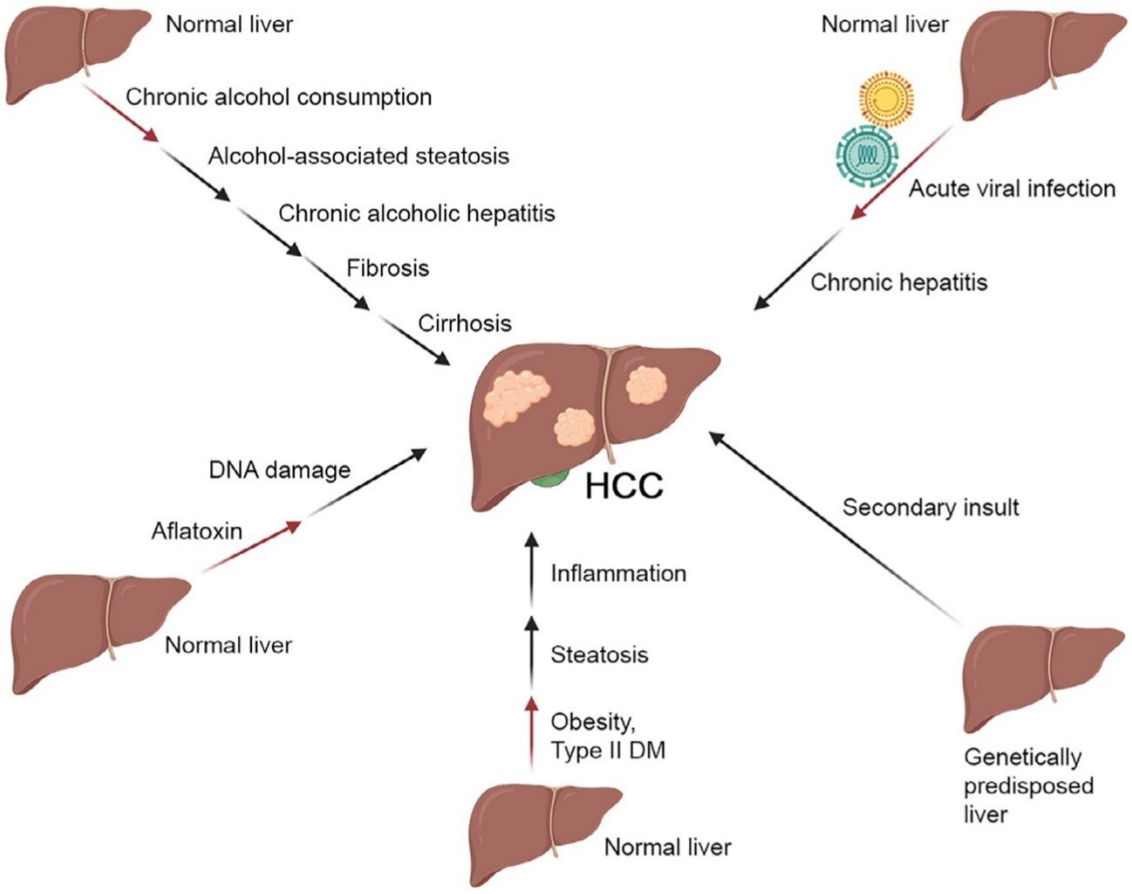


Figure 1.3 Etiology of Hepatocellular carcinoma (Paul B Fisher *et al* 2021)

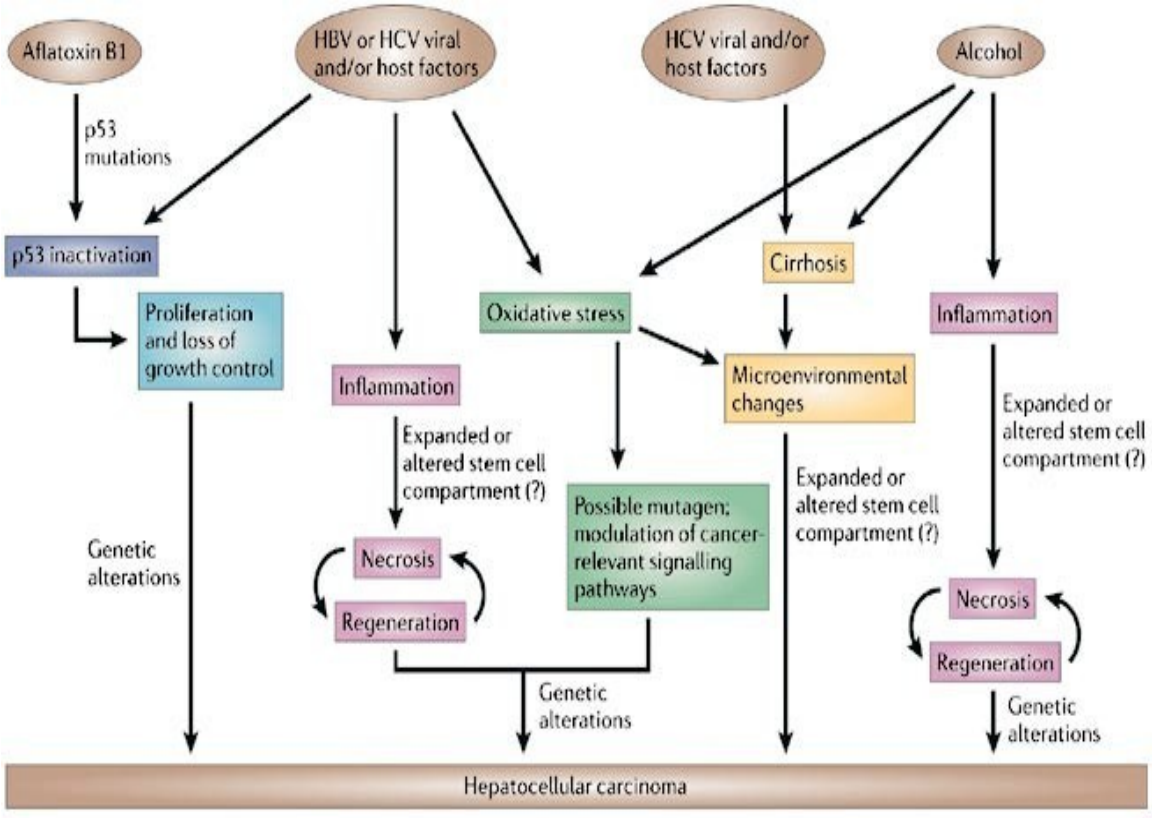


Figure 1.4 Pathophysiology of HCC (Ronald A. Depinho *et al*., 2006)

A DNA virus known as hepatotropic HBV possesses relaxed circular DNA (rcDNA), which is partially double-stranded. This increases the risk of HCC in people who have an infection in multiple ways. Currently the bulk of viral exposures occur during the early years of life or during vertical transmission from mother to child. Sexual intercourse or contact with tainted blood or body fluids can potentially spread viruses (Giacomo *et al*., 2022).

Based on the varying levels of the entire HBV genome sequence, there are ten genotypes of HBV. The expression of the four primary antigens that the HBV gene encodes—HBsAg, HBcAg, HBeAg, and HBxAg—has been associated to the emergence of HCC. HBV infection may contribute directly or indirectly to the development of hepatocellular carcinogenesis. HBV can cause chromosomal rearrangements, aberrant oncogene and tumor suppressor gene expression, and increased genome instability in the host cell by integrating into or causing mutations in host genes. It can also promote epigenetic remodeling of host DNA (Yuqin *et al*., 2020).

Additionally, it may cause liver cells to undergo a malignant change through the control of cell metabolism, the activation of several signaling pathways linked to cancer, and other processes. The liver microenvironment is altered by HBV infection, nevertheless, as a result of the virus's interactions with both innate and adaptive immune cells as well as persistent inflammation. This facilitates the virus's ability to evade immune monitoring and quickens the disease's progression from inflammation to tumor formation (Jiang *et al*., 2021).

Some Aflatoxin have also been linked to HCC e.g. Aspergillus flavus and Aspergillus parasiticus. These "fungal molds" grow on grains, including groundnuts, corn, and cereals. It is thought that aflatoxin-B1 (AFB1) is the most dangerous kind of aflatoxin. Prolonged exposure to AFB1 causes many genetic abnormalities and epigenetic modifications that lead to hepatocellular cancer (HCC) (Mungamuri *et al*., 2020). By reacting with guanine's N7 in RNA and DNA to form AFB1-N7-guanine. AFB1 is activated to its 8,9-epoxide isoform by CYP450 enzymes. Via DNA adduction and epoxidation, these processes start the carcinogenesis process. AFB1 genotoxic damage is

most likely to affect hepatocytes because of their inherent expression of microsomal cytochrome P450 enzymes. AFB1-adducts typically result in G → T transversions at the molecular level (Laura *et al*., 2022).

Approximately one-third of incidence instances of primary liver cancer worldwide are related to alcohol, with regional and national differences being notable. Alcohol is known to be carcinogenic, and the chance of liver cancer death increases with consumption of alcohol. It is a separate risk factor for the growth of HCC. Furthermore, alcohol interacts with other HCC risk factors such diabetes, viral hepatitis and obesity (Nathalie, 2019).

A variety of pathologic elements, including 1) the synthesis of acetaldehyde and its direct destruction to proteins and DNA, are unique to hepatic alcohol-mediated carcinogenesis. 2) The breakdown of antioxidant defenses and DNA repair mechanisms, which exacerbates the increased production of reactive oxygen species (ROS) induced by cytochrome P450 family and/or iron; 3) immune system modifications and the induction of chronic inflammation; 4) disruption of methyl group transfer and modifications to gene expression (Makiko, 2020).

In many countries, HCC is expected to be primarily caused by non-alcoholic fatty liver disease (NAFLD) and NASH. Numerous NAFLD risk factors, such as obesity, diabetes, Hispanic ethnicity, and genetic polymorphisms, are also independently linked to HCC. Those patients who have cirrhosis that is linked with NAFLD have a higher incidence of HCC (George *et al*., 2021).

Changes in lipid metabolism cause hepatic lipid accumulation, increased lipogenesis, and decreased triglyceride outflow, which culminates in steatosis, a characteristic of nonalcoholic fatty liver disease. Adipose tissue, gut microbiota, several immune systems strike, and persistent inflammation all play a part in the complicated progression to NASH. Both chronic systemic inflammation and insulin resistance are influenced by these factors (Pierre *et al*., 2020).

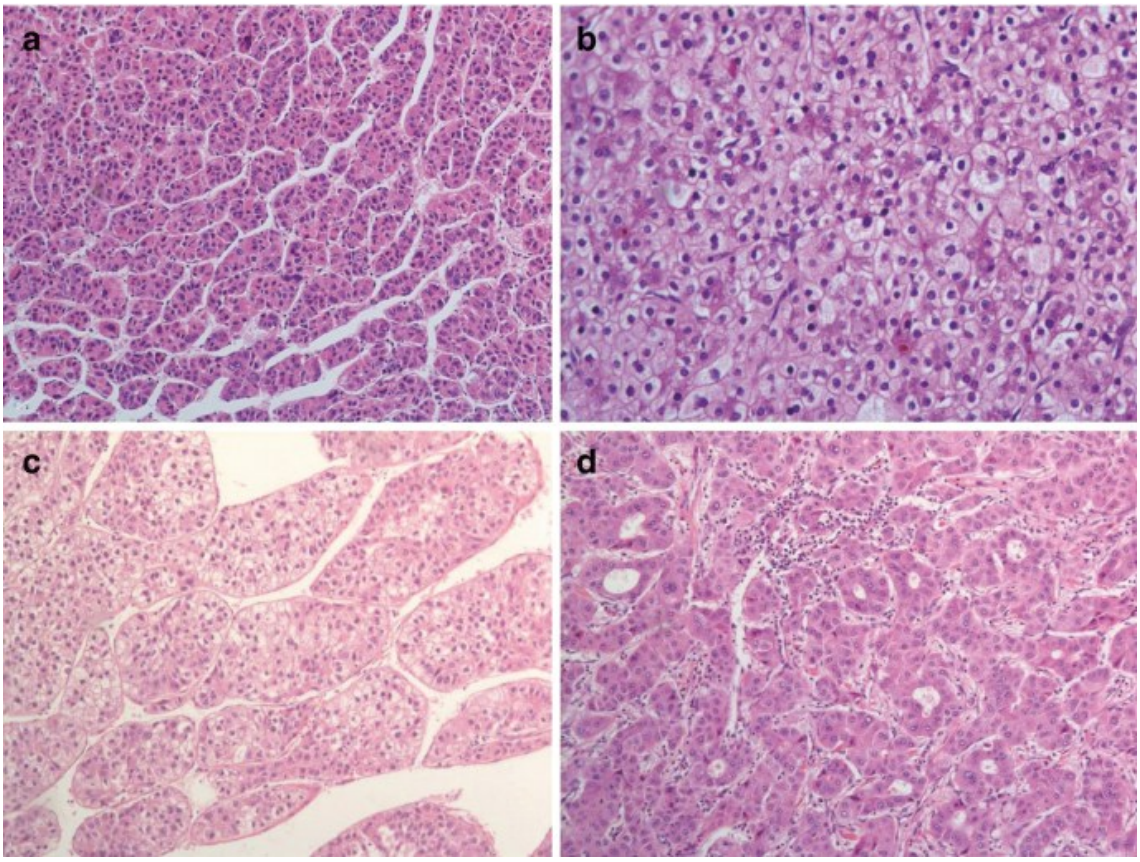


Figure 1.5 Histopathology of HCC (Andrea Baiocchini *et. al*., 2022)

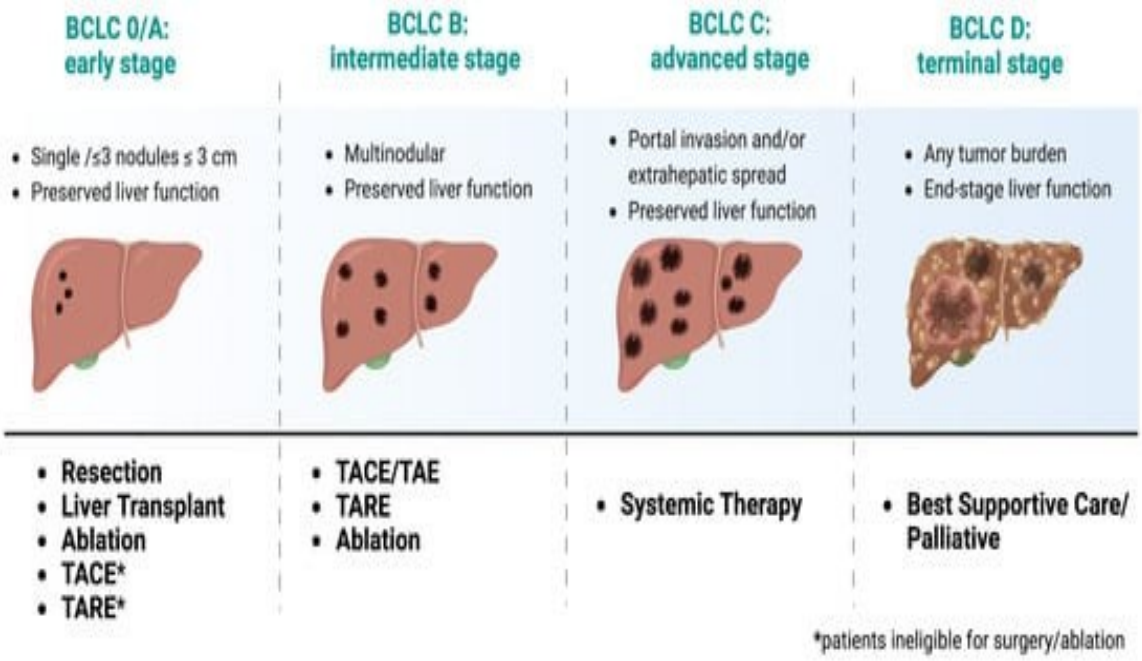


Figure 1.6 BCLC Staging (Cody R. Criss *et al* 2023)

A major player in the pathophysiology of NASH, oxidative stress causes fibrosis and inflammation in the liver. The metabolism of fats and carbohydrates is constantly dysregulated during carcinogenic processes. The interaction between NKT-cells and CD8+ T-cells accelerates the development of cancer and liver damage (Jorge *et al*., 2023).

Many staging systems have been established in order to determine the HCC patient's prognosis and guide therapy recommendations. The Barcelona Clinic Liver Cancer (BCLC) approach is distinctive in that it uses the stage of the HCC tumor to define the treatment algorithm recommendations and to assess the prognosis of the patient (Diamantis *et al*., 2019).

The goal of the 1999 proposal for the BCLC system was to enhance the therapeutic algorithm for the care of patients suffering from hepatocellular cancer (HCC). With implications for prognosis and treatment, the BCLC staging algorithm has been approved by the American and European Guidelines on the Treatment of HCC (Quirino *et al*., 2020).

Although several staging systems have been proven effective in assessing the prognosis of HCC, no single methodology has gained widespread acceptance. However, the Barcelona Clinic Liver Cancer (BCLC) approach is widely employed for the purpose of comparing treatment results. Similar to the European Association for the Study of Liver (EASL) and the American Association for the Study of Liver Disease (AASLD) systems, the BCLC system advises surgical resection for patients with solitary tumors, Stage 0 (very early), and Stage A (early). However, due to the low 5-year overall survival rates, it does not recommend surgical resection for patients with Stage B (intermediate) and C (advanced) tumors (Wee *et al*., 2022).

There are 5 stages of BCLC staging system as shown in fig. 1.6. Stage 0: Extremely preliminary (the tumor is smaller than 2 cm.) Phase A: Initial phase (This indicates the presence of one tumor, regardless of size, or up to three tumors, each smaller than 3 cm.) The Intermediate Stage, or Stage B (This indicates that the liver has several tumors.) Advanced stage, or stage C (This indicates that the cancer has progressed to other body

organs, lymph nodes, or blood arteries.) Stage D. (This indicates that your liver damage is significant). (Criss *et al*., 2023).

Imaging is essential for diagnosing, staging, monitoring, and assessing the efficacy of treatment for hepatocellular carcinoma (HCC). Clinical imaging techniques include CT scan, MRI, and abdominal ultrasound according to practice guidelines. These techniques are mainly used for imaging-guided biopsies, HCC screening and surveillance. Typical imaging features include hyperenhancement during the arterial phase in comparison to the surrounding liver parenchyma, and washout in the portal venous or delayed venous phases post-contrast on CT and MRI., the appearance of qualitative HCC on imaging is directly associated with its vascular characteristics (Azeez *et al*., 2020).

The use of quantitative imaging to evaluate tissue qualities has gained popularity recently. In order to obtain accurate and individualized care of HCC, a number of quantitative imaging techniques have been developed with the shared objective of improving tumor characterization and aggressiveness prediction. Novel methods, such radiomics, have been suggested as a stand-in for histologic analysis, mitigating the hazards associated with tissue collection and enabling repeated measurements of the whole tumor volume. Furthermore, noninvasive imaging approaches that enable an early evaluation of therapeutic success are essential in light of the advent of molecular targeted therapy (Christian *et al*., 2022).

Patients with space-occupying lesions that exhibit distinctive imaging features and are evaluable using the clinical criteria for the diagnosis of HCC typically do not require a diagnostic liver biopsy. Preoperative liver biopsy is not advised in order to lower the risk of tumor dissemination in patients with resectable HCC or those who are planned for liver transplantation. A clear pathologic diagnosis for space-occupying lesions lacking diagnostic imaging features can be obtained through liver biopsy. A liver biopsy can help with the molecular categorization of HCC, offer recommendations for therapy selection and prognosis, and provide important information about the nature of the lesion and the origin of the disease (Jian *et al*., 2020).

Surgical resection is the most effective curative treatment for individuals with HCC in early stages. However, liver transplantation (LT) can be a better course of treatment for the majority of people who develop HCC in conjunction with an underlying liver condition. Owing to the organ shortage, people who will benefit from Liver Transplantation must be carefully chosen. Even with strict selection criteria, 8–20% of patients had HCC recurrence following Liver Transplantation (Monique *et al*., 2023).

For individuals with treatable diseases, surgery—such as liver transplantation, resection, or ablation—remains the cornerstone of care which provides the cure of the disease. (Diamantis *et al*., 2019).

Biomarkers with a high degree of sensitivity and specificity that could identify HCC early on may be extremely important for both disease diagnosis and treatment. Tumor biomarkers have a critical role in early tumor detection, diagnosis, treatment assessment, recurrence, and prognosis prediction. Because of its high incidence and death rates, HCC, one of the most common malignant tumors, poses a serious risk to human health. The development of laboratory identified HCC biomarkers, including blood and histochemical biomarkers, is essential for pathological classification, patient outcomes, early detection, and therapy decisions (Li *et al*., 2019).

A well-known oncological marker, alpha-fetoprotein (AFP) is a serum protein that is indicative of the fetal development stage. It is typically produced by the liver and yolk sac of a developing fetus. In fetal life, albumins eventually replace the common predominance of AFP among blood proteins as its functions gradually diminish after birth. Diagnostic methods have evolved dynamically over time, moving from qualitative methods employed only in research projects to widely applied radioimmunoassay and immunoenzymatically assays Certain genetic mutations results in the total suppression of fetal ability to produce AFP. Pregnancy-related screening test results are impacted by this, and it also causes adults to have persistently elevated levels of AFP that are unrelated to oncogenesis (Joanna *et al*., 2021).

Prolongedly increased AFP levels have been established as a risk factor for the development of HCC and may help classify at-risk individuals. Early identification of HCC

may also enhance outcomes. It has proven difficult to screen the general population for HCC using AFP alone because elevated AFP levels can also occur in other benign liver conditions. As a screening method to find people in the general population who are more likely to develop HCC, AFP by itself is not advised. There are genetic pathways that could concomitantly dysregulate the expression of AFP and a number of other known and undiscovered angiogenesis-related genes as HCC progresses. Similarly, it has been demonstrated that AFP silencing inhibits VEGF synthesis in vitro in HCC cells. When considered collectively, these results are in line with the hypothesis that AFP expression is linked to more angiogenic tumors and, as previously said, may identify specific HCC subtypes (Galle *et al*., 2019).

Since approximately half of HCC tumors secrete AFP, it is the primary serological marker employed in the diagnosis of HCC. Generally speaking, a pathogenic threshold for human plasma concentrations of AFP is set at 20 ng/mL. It is generally accepted that a concentration of more than 400 ng/mL is a valid indicator of HCC (Jian *et al.,* 2019).

A common biomarker for HCC diagnosis is α-fetoprotein (AFP), although due to its high false-negative rate for tiny and early-stage tumor detection, its diagnostic accuracy is limited. Moreover, AFP perhaps increased in certain benign liver conditions, like cirrhosis without HCC and chronic hepatitis (Feng *et al*., 2021).

Alpha-fetoprotein (AFP) is the most often utilized biomarker for hepatocellular carcinoma (HCC) in the globe; yet, because of its subpar sensitivity and specificity in surveillance (varying low levels that might be caused by a viral hepatitis flare-up and a small percentage of early-stage tumors secreting (Sagar *et al*., 2021).

The majority of AFP-negative hepatic cancers (ANHCs) are tiny, early-stage HCCs that frequently lack normal imaging features, making their diagnosis more difficult. Additionally, liver nodular lesions may present with imaging features resembling HCC, leaving ANHC patients susceptible to misdiagnosis. However, because ANHC patients have a better prognosis than those with AFP-positive HCC (APHC), the identification of ANHC is significant in clinical practice (Choi *et al*., 2019).

AFP in serum has been widely employed as a tumor biomarker for the diagnosis of HCC and for tracking the progression of illness. However, it is still not the finest biomarker at this time for the purpose of diagnosis. To that aim, there is a significant deal of interest in discovering and putting forth novel biomarkers (Svobodova *et al*., 2018).

In 1984, Liebman and associates discovered elevated levels of PIVKA II in serum among individuals with HCC. These results imply that PIVKA II could be a new marker for HCC. Researchers think PIVKA II may take the place of AFP in the diagnosis of HCC (Feng *et al*., 2021).

Protein induced by vitamin K absence or antagonist-II (PIVKA II), commonly referred to as des gamma-carboxyprothrombin (DCP), is an aberrant prothrombin molecule that arises from an acquired defect in the prothrombin precursor's post-translational carboxylation in malignant cells. It has been established that the existence of HCC and a vitamin K deficit are related to the release of this marker. Previous research on the correlation between different clinicopathological variables and serum PIVKA II level in HCC has demonstrated that increased PIVKA II may be linked to poor tumor behavior and prognosis in HCC patients. Because PIVKA II can stimulate tumor cell proliferation and enhance tumor angiogenesis, serum PIVKA II level may more accurately represent the course of extrahepatic illness following liver transplantation than AFP level (Francisco *et al*., 2023).

Prothrombin (PT) is produced from the precursor through an enzymatic reaction catalyzed by c-glutamyl carboxylase, which is dependent on Vitamin K. This enzymatic pathway fully transforms all ten glutamic acid (Glu) residues to γ-carboxylated glutamic acid under physiological circumstances. The production of PIVKA II, which contains Glu residues, occurs when this process is disrupted (Takuya *et al*., 2024).

One aberrant protein produced in hepatocellular carcinoma (HCC) is PIVKA II. High PIVKA II levels in HCC have been linked to increased invasion, metastasis, and proliferation (Yang *et al*., 2021). For HCC cases that are AFP-positive or negative, DCP demonstrates similar diagnostic efficacy, making it a valuable addition to AFP evaluations

(Wang & Wei, 2020) it can detect 3 cm HCC tumors as well as AFP. PIVKA II works better against viral HCC than non-viral HCC (Sagar *et al*., 2021).

The Individuals with higher PIVKA II have a higher risk of metastasis and recurrence (Tian *et al*., 2023). PIVKA II has the potential to forecast the risk of microvascular invasion and aid in the selection of patients for liver transplantation. It is therapeutically useful for tracking the efficacious and intra-arterial locoregional therapies, outcomes, and recurrence. (Kim *et al*., 2023).

Patients with AFP-negative HCC can use abnormal prothrombin (PIVKA II, protein generated by vitamin K absence/antagonist-II) as a prognostic biomarker. PIVKA II was first proposed by Liebman *et al*. in 1984, and since then, its use as a particular biomarker for liver cancer has grown. PIVKA II is currently included in the guidelines of the Japan Society of Hepatology as a significant biological marker for the identification of liver cancer. The Chinese Guidelines for Prevention and Treatment of Chronic Hepatitis B advocate PIVKA II, which can be used in conjunction with AFP to facilitate early identification. as a significant indicator for HCC diagnosis (Tian *et al*., 2022).

It was shown that PIVKA II can be employed as a screening or surveillance biomarker in HCC high-risk groups. PIVKA II displayed good diagnostic performances in patients with liver disorders of various etiologies. Additionally, the study found that PIVKA II exhibited a lower degree of toxicity from patients with liver illness due to HBV, indicating its higher diagnostic efficacy in comparison to AFP. Combining AFP and PIVKA II, in our opinion, will enhance HCC patient diagnosis and recurrence detection rates, raise the percentage of early treatment, and eventually increase survival and patient longevity (Ji *et al*., 2021).

Patients with HCC and those without tumors could be distinguished with a fair degree of sensitivity and specificity using a cut-off value of > 40 mAU/mL, and the measurement of serum PIVKA II demonstrated acceptable performance for HCC identification. Lastly, PIVKA II seemed helpful in stratifying patients with cirrhosis under surveillance's risk of developing HCC (Caviglia *et al* l., 2023).

Hepatocellular carcinoma (HCC) is a primary cause of cancer-associated mortality on a global scale. With its high sensitivity and specificity, PIVKA-II is a useful biomarker for hepatocellular carcinoma diagnosis, especially when it comes to differentiating HCC from chronic liver disorders. In high-risk populations for HCC, PIVKA-II can be utilized as a screening or monitoring biomarker. This study shows PIVKA II as strong diagnostic performance and less affected by other liver diseases unlike AFP. (JI *et al*., 20201).

1.2 Research Gap/ Rationale of the Study

Multiple studies have shown that PIVKA II can be used as a potential biomarker in early detection and prognosis of hepatocellular carcinoma as compared to alpha fetoprotein. Research on PIVKA II level in hepatocellular carcinoma in Pakistan is needed due to the country's burden of the disease, high prevalence of hepatitis B and C which are leading causes of hepatocellular carcinoma, the need for early detection, improved prognosis, limited screening resources and potential for better treatment plan. By this study we may provide a more specific and sensitive diagnostic tool for hepatocellular carcinoma. This kind of study may help in improving of early detection and better management of hepatocellular carcinoma in Pakistan.

1.2.1 Theoretical Gap

There are several different genes that can contribute to the development of hepatocellular carcinoma. Hepatocellular carcinoma biomarkers require additional study and development for better understanding. There is a disconnect between our current cancer knowledge and our capacity to make good use of biomarkers in the diagnosis and monitoring of HCC. To fill this void, researchers must systematically scour the globe for untapped data sets that may provide novel biomarkers for the early detection and monitoring of hepatocellular carcinoma.

1.2.2 Contextual Gap

There is lack of knowledge and application of hepatocellular carcinoma related biomarkers in clinical settings. Some HCC-related biomarkers have been identified and studied, however their actual implementation in clinical practice has lagged behind. To close this knowledge gap, researchers must first validate biomarkers in a variety of patient populations, establish standardized methods for biomarker analysis, and set clinically meaningful cutoff values. More studies are needed to place biomarker results in the context of the intricate clinical landscape of HCC diagnosis, prognosis, and treatment in order to fill this informational void.

1.2.3 Methodological Gap

Hepatocellular carcinoma (HCC) biomarker research needs improved methods due to a methodological gap. At present HCC biomarkers are not standardized. This lack of standard protocols makes data comparison and integration difficult. Biomarker studies would be more credible if standardised. In biomarker research, selection and measurement bias must be minimized. To represent the target population, participant selection and recruiting must be careful. Validated and reliable measurement procedures reduce bias and improve biomarker readings. Standardised methods and bias reduction in HCC biomarker research are needed to address these difficulties. Researchers can improve HCC biomarker research by increasing methodological rigor.

1.3 Problem Statement

Hepatitis B and C are very common in Pakistan. These are the main causes of hepatocellular carcinoma which is a major health risk because of the difficulties associated with diagnosis, therapy, and prognosis. As a result, patients with hepatocellular carcinoma have a lower chance of receiving an accurate diagnosis and benefiting from effective

treatment. Therefore, it is crucial to develop a more sensitive and specific diagnostic tool for early detection, maximize treatment success, and ultimately improve survival prospects for people with hepatocellular carcinoma

1.4 Research Question/ Hypothesis of Study

A) Null Hypothesis

There is no significant difference in sensitivity and specificity of PIVKA II (protein induced by vitamin K absence) in patients with hepatocellular carcinoma than alpha fetoprotein.

B) Alternate Hypothesis

There is significant difference in sensitivity and specificity of PIVKA II (protein induced by vitamin K absence) in patients with hepatocellular carcinoma than alpha fetoprotein.

1.5 Objectives

The objectives of this study were:

- To investigate the levels of PIVKA II (Protein Induced by Vitamin K Absence or Antagonist-II) and alpha fetoprotein in hepatocellular carcinoma (HCC).
- To compare the diagnostic accuracy of PIVKA II (Protein Induced by Vitamin K Absence or Antagonist-II) and alpha-fetoprotein (AFP) in the hepatocellular carcinoma (HCC).

1.6 Significance of the Study

The high incidence of chronic liver diseases, such as viral hepatitis B and C infections, aflatoxin exposure, and non-alcoholic fatty liver disease (NAFLD), all contribute to the high risk of hepatocellular carcinoma (HCC) in Pakistan. The incidence of HCC in a given area is proportional to the frequency with which certain risk factors are present there. Liver-related illnesses and deaths in Pakistan are largely attributable to HCC. Inadequate screening programs, delayed diagnosis, and restricted access to healthcare facilities are all factors that complicate HCC management. PIVKA II (protein implicated in vitamin K deficiency or antagonist-II) studies are particularly relevant to the investigation of HCC. The diagnosis, prognosis, and treatment of HCC can all benefit from the information gleaned from studying PIVKA II. This biomarker is essential for the diagnosis of HCC at an early stage, allowing for prompt treatment. In addition, increased PIVKA II levels in the blood can be used as a predictive indicator of tumour characteristics such size and stage. Therefore, progress in both research and clinical strategies for treating HCC can be attributed to a deeper familiarity with PIVKA II's function in this disease.

CHAPTER 2

LITERATURE REVIEW

The most common primary liver cancer and a major contributor to cancer-related deaths globally is hepatocellular carcinoma (HCC). Cirrhosis, in which liver cells go through recurrent cycles of necrosis and regeneration, is the root cause of 90% of HCC cases. To establish links between frequently mutated pathways and patient prognosis, there has been a lot of research done on the intricate genomic landscape of HCC (Eunsun *et al*., 2020).

It can be linked to specific risk factors that lead to liver cirrhosis, a premalignant state that precedes the development of preneoplastic hepatocellular lesions and ultimately liver cancer (mostly chronic viral hepatitis and alcoholic/nonalcoholic steatohepatitis). Even in the majority of biopsy specimens, early HCC may be distinguished from its precursor lesions and other highly differentiated hepatic lesions by using stringent morphological criteria and a panel of immunohistological markers. Histological characteristics and molecular subgroups of human HCC were linked through integrative characterization (Fisher *et al*., 2020).

The primary risk factors for the development of HCC are consumption of food contaminated with aflatoxin, diabetes, obesity, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), cirrhosis caused by hepatitis B virus (HBV) and/or hepatitis C virus (HCV), steatohepatitis, and chronic liver disease (Shu *et al*., 2020).

Hepatocellular Carcinoma (HCC) is a very aggressive cancer with limited therapeutic alternatives and a death rate that parallels its occurrence. HCC is largely influenced by chronic liver inflammation and damage in its onset and progression. The incidence rates of HCC are influenced by various factors, including gender, age, ethnicity, and demographic regions (Fisher *et al*., 2020).

The elements of risk for HCC comprise carcinogens, such as smoke, food contaminants, and environmental toxins, as well as inherited diseases and the hepatitis C and hepatitis B virus causing chronic infection. Evidence from recent years indicates that the main causes of HCC worldwide are metabolic syndrome (obesity and diabetes), excessive alcohol use (alcoholic fatty liver disease), and high calorie intake (nonalcoholic fatty liver disease) which are associated with a westernized sedentary lifestyle. Although cirrhosis and chronic liver disease account for 80% of HCC incidence, 20% of cases are known to occur in patients without cirrhosis. Most people agree that there may be interactions between many etiological factors, which could include distinct processes in the pathophysiology of HCC. It is essential to comprehend the molecular mechanisms behind the onset and spread of HCC in order to create tailored treatments that effectively treat this fatal illness (Tateishi *et al*., 2020).

Chronic inflammation is well acknowledged to be a major factor in the development, propagation, and advancement of cancer. Hepatocellular carcinomas (HCCs) exhibiting elevated inflammatory response levels have been found to exhibit heightened immune cell infiltration and other pro-cancerous signals. Elevations in EMT, KRAS signature, hypoxia, and angiogenesis are linked to elevated inflammation and are thought to be negatively correlated with prognosis (Kanda *et al*., 2024).

Diverse etiological factors can cause diverse inflammatory reactions in the liver. Depending on the etiology and several other circumstances, the inflammatory process may resolve or remain persistent. Genetic alterations, extended inflammation, aberrant cellular signaling and tissue remodeling are thought to be important mechanisms that support immunosuppression. The anti-tumor immunity implicated in the genesis and development of HCC carcinogenesis is rendered inactive by the increasing immunosuppression. Both

inflammation that promotes cancer and immune surveillance are affected by major cellular processes such as stress to ER (endoplasmic reticulum), damage to DNA and necrosis indicating a reciprocal dependency. Numerous inflammatory processes in the liver microenvironment have been identified, along with the molecules that regulate them. This has led to a great deal of translational research and encouraging clinical studies including novel immunomodulatory drugs (Carla *et al*., 2023).

Globally, the prevalence of liver cancer is rising. According to World Health Organization forecasts, liver cancer would claim the lives of over a million individuals by 2030. One or more risk factors are the primary source of the diversity in the racial and geographic distribution of HCC that has been documented. Hepatocellular carcinoma (HCC) has a unique prognosis that depends on both the tumor's stage and the severity of the underlying liver disease. Because of its high mortality ratio, liver cancer ranks as the fourth most prevalent cause of cancer-related death globally. In addition to the 782,000 (8.2%) deaths associated with HCCs, 2018 is expected to have witnessed 841,000 (4.7%) new cases of HCCs. The varied overall incidence of HCC is probably caused by fluctuations in both the hepatitis virus prevalence and environmental factors (Samant *et al*., 2021).

About 80% of HCC cases are seen in Sub-Saharan Africa and Eastern Asia, both of which have significant rates of chronic hepatitis B virus carriers. While hepatitis C is the main risk factor for HCC, Japan, North and South America, the majority of Europe, Australia, and the Middle East are reported to be low-incidence regions (less than 3 cases recorded per 100,000 inhabitants annually) (Sarin *et al*., 2020).

In Pakistan, 10.7% of all cancers in men are hepatobiliary malignancies. In Pakistan, the prevalence of HCC ranges between 3.7 to 16.7% of all cancers, with chronic hepatitis B, C, and D-related cirrhosis being the main contributing factor (Tahir, 2022). According to two distinct studies, the prevalence of HCC in Pakistan as a result of HCV and HBV is 87% and 66%, while the frequency is 22% and 43%. Most patients present when there are less than 1% odds of resection remaining and extremely few therapy

alternatives for cure. In Pakistan, screening results in fewer than 10% of HCC cases being diagnosed (Hafeez *et al*., 2020).

Hepatocellular carcinoma (HCC) requires interdisciplinary management, and the influence of underlying liver disease and function must be considered, predicting the prognosis and selecting a treatment plan are particularly challenging. Even in patients with otherwise early malignancies, the frequent occurrence of advanced liver disease may significantly worsen the prognosis and restrict the application of potentially curative treatments and psychiatric comorbidities (Strazzabosco *et al*., 2022).

The poor prognosis of HCC is largely caused by a delayed diagnosis. Nonetheless, the unsatisfactory prognosis resulting from a delayed diagnosis of hepatocellular carcinoma (HCC) has led to a current focus on detecting individuals with early-stage HCC. Molecular biomarkers may offer pertinent and extra details regarding the biological activity of these malignancies. Further investigation into biomarkers sensitivity, combinations may yield useful and more precise data for future customized HCC diagnosis and/or prognosis. Numerous biomarkers that are significant for prognosis have been found, however they have all only been examined in retrospect. Furthermore, only a small percentage of the many molecular signatures that have been published have undergone external validation (Singal *et al*., 2023)

A difficult diagnostic issue arises from the significant prognostic difference between hepatocellular carcinoma (HCC) in its early and late stages. the creation and use of several biotechnologies, such as next-generation sequencing and a variety of "omics" data, including transcriptomics, proteomics, metabolomics, genomics, and metagenomics, have been utilized in the screening of HCC diagnostic biomarkers. At various stages of the clinical process, successful biomarkers, panels, and models have been identified and verified. Many of these are useful as HCC diagnostic tools, especially in the initial phase of illness. In order to facilitate early detection and treatment of HCC, screening is crucial. There are several methods of diagnosing HCC, including imaging, molecular marker, omics, and clinical methods. Molecular biomarkers, imaging diagnostics, and clinical symptoms are the most widely used indicators for HCC identification (Liu *et al*., 2019).

In order to treat and detect HCC early, screening is crucial. There are several methods of diagnosing HCC, including imaging, molecular marker, omics, and clinical methods. Molecular biomarkers, imaging diagnostics, and clinical symptoms are the most widely used indicators for HCC identification. To confirm the diagnosis of hepatocellular carcinoma, MRI and multiphasic CT scan should be performed if the surveillance test yields questionable results. Hepatocellular carcinoma may be confirmed by liver biopsy or repeat imaging if radiologic testing yields equivocal results (Tayob *et al*., 2020).

Insufficient use of HCC screening in actual practice, with only 24% of at-risk patients obtaining the test that society recommendations prescribe. Stage migration and a worse prognosis can arise from downstream care continuum failures, even in patients who have undergone screening. Conceptually straightforward, the HCC care continuum comprises multiple steps: screening, abnormal result recognition, appropriate diagnostic testing, accurate test interpretation, treatment referral, clinic visit adherence, and appropriate treatment recommendations. At any stage, a mix of patient-, provider-, and system-level factors can lead to failures (Rao *et al*., 2021).

The most successful curative procedure for those with early-stage hepatocellular carcinoma (HCC) is surgical resection. Liver transplantation (LT), however, can be a better course of treatment for the majority of people who develop HCC in conjunction with an underlying liver condition. Owing to the organ shortage, people who will benefit from Liver Transplantation must be carefully chosen. Even with strict selection criteria, 8–20% of patients had HCC recurrence following Liver Transplantation. (Devillers *et al*., 2023). For individuals with treatable diseases, surgery—such as liver transplantation, resection, or ablation—remains the cornerstone of care (Tsilimigras *et al*., 2019).

Disease management is a complicated process. A multidisciplinary team should evaluate patients, and when choosing a course of treatment, variables like the number of tumors present, the degree of liver impairment, coexisting conditions, local knowledge, and patient preferences should all be taken into account. The best course of action for early-stage hepatocellular carcinoma is curative therapy, which can involve transplantation, ablation, or resection. Treatment for patients with intermediate stage illness is frequently

locoregional. Patients with advanced disease are the only ones eligible for systemic therapy. The systemic treatment options for advanced hepatocellular carcinoma have been expanded by a number of positive phase III randomized controlled trials, with promising long-term outcomes. These trials, particularly those that used combination treatments, may have implications for the treatment of earlier stages of the disease in the future (Nishidaet *et al*., 2020).

For individuals with cirrhosis, the recommended course of treatment for early-stage hepatocellular carcinoma (HCC) is liver resection, and it is also one of the main curative alternatives for patients without cirrhosis. To balance the danger of postoperative liver failure with the possible benefit on long-term outcomes, cautious patient selection is necessary. The list of indications for liver resection has been enlarged in the past several decades due to advancements in surgical methods, perioperative care, and patient selection. The primary goals of this to outline the liver resection indications for the treatment of HCC, its place in the therapeutic algorithm relative to liver transplantation and percutaneous ablation, and the latest developments in liver surgery that may help patients with HCC (Saillard *et al*., 2020).

Resection's chance of curing HCC demonstrated that a cure is possible and gets more likely the longer a patient goes without a recurrence. The information provided here can be utilized to give patients accurate information and to guide decision-making (Cucchetti *et al*., 2019).

Independent risk variables for late recurrence of HCC included cirrhosis, multiple tumors, satellite nodules, sex, and microscopic and macroscopic vascular invasion in addition to the maximum tumor size of more than 5 cm. According to the patterns of late recurrence, after two years of surgery, testing for extrahepatic metastases using skeletal emission computed tomography and chest CT is not necessary, before diagnosing intrahepatic recurrence. When a late recurrence of HCC was diagnosed, regular surveillance increased the likelihood that patients would receive potentially curative treatment, which enhanced their chances of survival (Xu *et al*., 2019).

The majority of people diagnosed with hepatocellular carcinoma (HCC) do not have long to survive following diagnosis—on average, less than two years. In contrast, those with early diagnosis are amenable to surgical cures. Nevertheless, there is still a 70% chance of postoperative HCC recurrence even following curative resection. Over the previous five years, significant adjustments have been made to the care of HCC, especially for patients with advanced stages. In spite of the plethora of novel treatments available, there are insufficiently clear biomarkers to direct healthcare professionals in selecting sequence therapy that optimize effectiveness while reducing side effects. In order to increase patient survival overall, new biomarkers should be developed and put into use for a number of clinical managements of HCC areas that are particularly difficult. Here, we summarize the key developments in liquid biopsy biomarkers for minimum residual illness, early HCC identification, and treatment response predictions (Song *et al*., 2021).

Early discovery greatly increases survival, as it does for many malignancies. For individuals diagnosed at a severe stage, the median survival is less than 15 months, but it is greater than 60 months for those with early HCC. Although monitoring at-risk patients improves outcomes, existing surveillance techniques have low sensitivity and specificity, and less than 20% of patients at risk for HCC get surveillance. Blood-based biomarkers with sufficient sensitivity or specificity would be ideal for early HCC identification; unfortunately, alpha fetoprotein, the most widely used biomarker for HCC, has insufficient performance characteristics. Many prospective sera proteomic, glycomic, and genetic indicators have demonstrated promise in the early diagnosis of HCC during the biomarker validation phase. However, validation of these markers in carefully selected cohorts is necessary. Phase III biomarker study retrospective longitudinal validation of biomarkers will be made possible by ongoing prospective cohort studies (Parikh *et al*., 2021).

Promising studies indicate that a number of serum-based biomarkers and biomarker panels have good sensitivity and specificity for HCC in cirrhosis patients. The only serum biomarker to complete all five stages of biomarker validation for HCC surveillance is alpha fetoprotein (AFP); however, due to tumor heterogeneity, single biomarkers perform less well than ideal for early HCC identification. Although most data only assess biomarkers at one time point, the inclusion of longitudinal data may enhance biomarker performance by

detecting earlier elevations in biomarker levels signaling early-stage HCC and lowering the chance of false positive results (Marrero *et al*., 2023).

HCC is still associated with a bad prognosis in patients with an advanced stage of the disease despite recent advancements in therapeutic interventions. Prior research has documented the positive impact of routine HCC surveillance in high-risk groups on early detection of HCC. The debate over whether a surveillance program should include ultrasonography and tumor markers for HCC surveillance continues because the most often used tumor marker for HCC is AFP, has insufficient sensitivity or specificity on its own. The multifactorial character of HCC makes it challenging to employ a single biomarker to predict its occurrence. Thus, it's critical to combine several biomarkers to increase diagnostic precision. To enhance the diagnostic precision of AFP, other tumor markers have been suggested thus far, including des-gamma-carboxyprothrombin (DCP) and the Lens culinaris agglutinin-reactive portion of AFP (AFP-L3). Data on tumor markers are frequently available in daily clinical practice, in addition to data on liver function (total bilirubin [TB] and albumin), fibrosis (platelet count), and biomarkers of liver inflammation (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]), as well as the hepatitis virus status. These biomarkers are helpful in predicting the existence of HCC because they change the pretest likelihood for an HCC diagnosis using a tumor marker (Sato *et al*., 2019).

Hepatocellular carcinoma (HCC) early and differential diagnosis using noninvasive techniques is a significant clinical problem. Finding biomarkers can be accomplished by using new high-throughput techniques to analyze low-molecular-weight compounds (Kim *et al*., 2019).

There aren't many options for treating late-stage HCC, despite significant advancements in curative therapies for early-stage lesions. Unfortunately, the vast majority of HCC cases are discovered at a later stage, which worsens the disease's prognosis. To maximize the prognosis of individuals with HCC, efficient screening and precise early diagnosis are therefore essential. These days, radiologic exams and serum biomarkers are the main tools used in the diagnosis of HCC. Ultrasonography (US), computed

tomography, angiography, and magnetic resonance imaging (MRI) are frequently used in radiologic tests. The US's operator dependence and the CT, MRI, and CT's contrast/radiation dose have limited the utility of these diagnostics for routine screening and early diagnosis. Therefore, finding a trustworthy serum biomarker, or combination of indicators, is critical to achieving early diagnosis and improving clinical outcomes (Lee *et al*., 2019).

Many different metabolic activities that are vital to maintaining bodily homeostasis depend on the liver. Hence, it would make sense to speculate that harm to this organ would show up in serum as an imbalance in metabolites that results from chemicals leaking out of damaged cells or from cancer cells with changed metabolic activity. Finding precise serum biomarkers is made more difficult by the heterogeneity of cancer cells and the possibility of tumor development in livers with various underlying illnesses (Banales *et al*., 2019).

The high fatalities are due to metastasis, recurrence, and late diagnosis. In clinical settings, an early diagnosis of HCC is both desirable and critical. AFP has primarily been utilized to diagnose primary HCC up till recently. The specificity and sensitivity, however, are unsatisfactory. In order to do this, a lot of research revealed that circulating serum exosomal miRNAs were suggested as a possible source of biomarkers for numerous illnesses. While the precise therapeutic implications of these results remain unclear, those investigations showed that serum exosomal miRNAs may represent novel biomarkers for prognosis or diagnosis of cancerous diseases like HCC (Xue *et al*., 2019)

Early detection of HCC can increase survival and enable more effective therapy. Traditionally, serum α-fetoprotein (AFP) and hepatic ultrasound imaging are used in tandem to diagnose HCC. AFP, however, is not very specific as a serum biomarker. The development of biomarkers that could offer excellent diagnosis accuracy and support efficient therapy and surveillance has received a lot of attention. Researchers' attention has long been piqued by the genetic underpinnings of HCC (Boonkaew *et al*., 2023).

The expression of miRNA is aberrant in a number of human malignancies, including HCC. The investigation of these miRNAs as predictive and diagnostic indicators

for HCC is prompted by the hallmarks of aberrant miRNA expression in HCC tissues. Serum AFP is one less intrusive marker than tissue miRNA expression, making tissue miRNA expression less useful for diagnosis. Rather, miRNAs that are in circulation in bodily fluids like urine and plasma are thought to be potential biomarker candidates. Since miRNAs have been found in bodily fluids, particularly plasma, at comparatively constant concentrations, they may be able to identify cancer patients from healthy people. While circulating miRNAs as non-invasive biomarkers have just recently come to light, several studies have already shown their promise as biomarkers for a range of malignancies, including stomach, prostate, and lung cancers (Jin *et al*., 2019).

Concern over the rise of HCC-related fatalities is mounting. Out of all HCC patients, approximately 50–70% survive for five years, despite notable advancements in the early surgical treatment of these patients. World Health Organization estimate predicts that by 2030, liver cancer will claim the lives of over a million people. Thanks to thorough genomic research and developments in bioinformatics technology, additional molecular events have been identified to investigate potential novel biomarkers and treatment targets for hepatocellular carcinoma. Precision medicine's use of molecular therapeutics has not yet resulted from molecular pathogenesis research accomplishments. Thus, the search for additional potent prognostic and diagnostic markers is urgent (Hu *et al* l., 2020).

For HCC surveillance, alpha-fetoprotein (AFP) is the most commonly used biomarker. This 70 kD glycoprotein, which is produced by the fetal liver and yolk sac during the first trimester of pregnancy, rapidly diminishes following birth. With the exception of a slight alteration in their N terminal sequence, AFP and albumin have very similar structures. More than 50 years ago, it was first identified as a helpful biomarker for HCC in research involving populations in Siberia and Africa as well as murine models. Its usefulness has been questioned ever since it was first introduced as a screening tool for HCC (Jakub *et al*., 2022).

It is commonly acknowledged that serum alpha-fetoprotein (AFP) has a clinical role in patients with hepatocellular carcinoma (HCC). The molecular characteristics of

people with AFP-high tumors and the mechanisms behind AFP overexpression are unclear, nevertheless (Montal *et al*., 2019).

AFP cutoff levels in HCC vary, ranging from 20, 200, 400, 1,000, to 10,000 ng/mL. The wide range of thresholds demonstrates how challenging it is to choose the best cut-off. However, as soon as the AFP positive threshold is crossed, the prognosis for HCC deteriorates (Musca *et al*., 2020).

The only tumor biomarker that is regularly utilized to treat hepatocellular carcinoma (HCC) is alpha-fetoprotein (AFP). Despite the fact that this marker has been known for almost 50 years and that 32% to 59% of patients with HCC have normal AFP levels, it is not a reliable diagnostic or screening tool (Musca *et al*., 2020).

The under expression of AFP levels, which remain undiagnosed because they fall within the normal range happens in as many as 30% of HCC patients. Furthermore, upregulation of AFP levels has been observed in certain patients with non-malignant chronic liver sickness, including 15–58% with chronic hepatitis and 11–47% with liver cirrhosis. When used as a screening tool for HCC diagnosis, these fluctuations in AFP levels seen in both benign and malignant individuals can cause diagnostic challenges. In order to obtain early diagnosis and a better prognosis, this has created a prospective study area for the detection of biomarkers to supplement AFP (Edoo *et al*., 2019).

Hepatocellular carcinoma is frequently diagnosed with α-Fetoprotein. However, in many patients with HCC the α-Fetoprotein is negative, the diagnostic utility of α-fetoprotein has been questioned. Therefore, new noninvasive approaches must be developed for the diagnosis of hepatocellular carcinoma early, especially in cases when the level of α-fetoprotein is low or negative. Diagnosing HCC is still challenging, particularly in its early stages. Patients with HCC will have a much higher 5-year survival percentage if early diagnosis is successful. Therefore, it is crucial to find new biomarkers for the diagnosis of early-stage HCC in order to benefit patients, especially those who do not have AFP (Luo *et al*., 2019).

Prior research has indicated a connection between the degree of AFP elevation in HCC patients and their ethnicity. The hepatocellular carcinoma (HCC) biomarker serum alpha-fetoprotein (AFP) improves the sensitivity of the early diagnosis of HCC.A number of factors, such as the amount of tumor growth, can impact AFP levels in HCC patients. Racial/ethnic background and the cause of liver illness are examples of non-tumoral factors that have been linked to increased AFP levels. We predicted that the shift from viral to non-viral etiology would lower the AFP level at HCC diagnosis (Vipani *et al*., 2021)

In 1984, des-gamma-carboxyprothrombin (DCP) also known as prothrombin induced by vitamin K absence II (PIVKA II) was discovered. This is another blood biomarker of HCC. PIVKA II, an abnormal form of prothrombin, is released into the bloodstream when the vitamin K-dependent post-translational carboxylation of glutamic acid residues is inhibited for any reason—for instance, by the absence of vitamin K or the presence of vitamin K antagonists. It has undergone extensive research as a diagnostic biomarker for HCC (Sagar *et al*., 2021).

As a novel marker for HCC therapy, the Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA II) has been evaluated. It is characterized by aberrant prothrombin, sometimes referred to as Des-g-carboxy prothrombin, which is caused by antagonist-II of vitamin K or by its absence. Moreover, it might result from an acquired malfunction in the prothrombin precursor's post-translational carboxylation (Basile *et al*., 2020).

PIVKA II is a retroactive indication of vitamin K level that has been extensively researched in human medicine. It has been shown that high blood concentrations of PIVKA II are indicative of certain neoplastic illnesses, including HCC, and coagulative pathologies. Because PIVKA II was expressed even in small-sized or well-differentiated neoplastic cells, but not in adenomatous hyperplasia, it can be inferred that PIVKA II is a useful immunohistochemical marker for HCC. Additionally, immunohistochemical examination of paraffin-embedded tissues was used to investigate PIVKA II expression and the different grades of HCC (Maniscalco *et al*., 2019).

PIVKA II models effectively distinguish NBNC-HCC from precancerous phases such as hyperplasia and dysplastic nodules, which can advance to HCC in a matter of months to years (Phan *et al*., 2023).

Greater tumor size over 5 cm, higher stages of BCLC, and portal venous thrombosis are associated with higher blood PIVKA II levels. Elevations in serum PIVKA II levels were linked to the development and oncogenesis of HCC. Patients with BCLC stage B had different serum PIVKA II levels than those with stages C and D (Darmadi *et al*., 2021).

PIVKA-II level can distinguish between patients who have a higher risk of HCC recurrence and those who do not. Patients with low PIVKA-II may be excused from HCC surveillance following Liver Transplantation (Kim *et al*., 2022).

In addition, PIVKA II outperforms AFP in terms of discriminative power when it comes to predicting explant microvascular invasion. This means that in order to select Liver Transplantation candidates who are at a high risk of having their HCC recur, PIVKA II should be included in future models. When it comes to HCC diagnosis, PIVKA II outperforms AFP as a biomarker. Additionally, PIVKA II is a predictor of HCC recurrence following curative resection (Devillers *et al*., 2023).

The most specific diagnosis was made using PIVKA II. Additionally, there is evidence linking PIVKA II to the prognosis of hepatocellular carcinoma patients (Li *et al*., 2020)

PIVKA II levels rose gradually prior to the diagnosis of HCC, but they were constant in individuals who did not develop HCC, suggesting that rising PIVKA II levels over time could be an early warning indicator of developing HCC risk. A 40 mAU/mL cut-off PIVKA II value for the diagnosis of HCC in patients (SU *et al*., 2022).

Levels of PIVKA II expression with a few clinicopathological characteristics, including tumor size and the quantity of trans arterial chemoembolization (TACEs) performed prior to transplantation. Following LT, serum levels of AFP and PIVKA II dramatically dropped, while those with a recurrence of the tumor had higher levels. Serum PIVKA II levels might be a significant factor in determining how severe an illness will be.

In addition, PIVKA II level monitoring in recipients of HCC transplants represents the early tumor recurrence following transplantation and may serve as a unique biomarker of this pathology in addition to AFP and imaging testing (Villalba-López *et al*., 2023).

The pre-transplant PIVKA II concentration in peripheral blood in patients with HCC is significantly correlated with clinical factors like larger tumour size and the number of TACEs completed prior to transplantation; however, the levels of this biomarker are unrelated to the underlying liver disease. Because elevated levels of this tumor marker indicate a greater tumor volume and a poorer clinical stage, they may be useful in forecasting the severity of the disease. In several Asian nations, PIVKA II is already covered by clinical practice standards (Villalba-López *et al*., 2023).

For a number of years, researchers have been looking for a tumor biomarker in HCC that offers outstanding diagnostic accuracy. The most widely used test, AFP, is generally less sensitive and specific for diagnosing HCC, which is why the most recent guidelines do not include it. However, a meta-analysis that shown the inadequate sensitivity of US for early HCC identification has revealed the need for a biomarker to boost the sensitivity of abdominal ultrasound in surveillance of HCC, emphasizing the need for alternative surveillance measures. the normal values of PIVKA II in a sizable group of compensated Caucasian cirrhotic patients on NUC-suppressed therapy who did not develop HCC for over five years, offering early evidence that PIVKA II is more accurate than AFP in detecting HCC early on (Loglio *et al*., 2020).

Operational Definitions

Hepatocellular Carcinoma: hepatocellular carcinoma present with inflammation and cirrhosis of liver.

PIVKA II: Prothrombin induced by vitamin K absence II (PIVKA II), also referred to as des-gamma-carboxyprothrombin (DCP) is an abnormal form of prothrombin that is released into the bloodstream when the carboxylation of glutamic acid residues, a process dependent on vitamin K, is hindered.

Alpha Fetoprotein: α-Fetoprotein (AFP) is a fetal glycoprotein produced by most human hepatocellular carcinoma.

Biomarker: The easiest and least invasive method for illness screening and diagnosis is the detection of disease-related chemicals in blood knowns as biomarkers.

Jaundice: this is the discoloration of eyes and different body parts due to excess of bilirubin.

Hepatitis C: Hepatitis C can cause Hepatocellular carcinoma which is a rare but serious kind of cancer. The disease is transmitted through blood. Its virus is an RNA.

Hepatitis B: hepatitis B is the most common cause of hepatocellular carcinoma in west.it is a DNA type of virus and spread through blood.

Vitamin K: Liver coagulation factors II, VII, IX, and X require vitamin K. Vitamin K-dependent carboxylase is hindered in the absence of vitamin K, forming abnormal coagulation factors e.g., PIVKA II which cannot clot.

Bilirubin: Heme oxygenase-1 breaks down heme into biliverdin, which is subsequently transformed to unconjugated bilirubin and further to conjugated bilirubin in the live organism. Bilirubin is a consequence of heme metabolism.

Alkaline phosphatase: One of the four human isozymes expressed in bone, liver, and kidney tissues is alkaline phosphatase which is essential for ectopic calcification and bone mineralization.

Gamma Glutamyl Transferase (GGT): The liver, kidney, and pancreas contain the majority of the enzyme gamma glutamyl transferase (GGT), which is present in the cell membranes of several tissues.

Normally Alanine Transaminase (ALT): is present in the hepatocytes, whenever there is any damage to the liver cells the alanine transaminase (ALT) is released in the blood stream.

Classification of BMI: Body mass index (BMI) is a basic weight-for-height metric frequently used to categorize persons who are overweight or obese. It may be calculated by dividing the square of an individual's height in meters by their weight in kilograms.

The Body Mass Index (BMI) was classified according to the Asia-Pacific categorization into the following categories: underweight (< 18.5 kg/m2), normal weight (18.5-22.9 kg/m2), overweight (23.0-24.9 kg/m2), obesity class I (25.0-29.9 kg/m2), or obesity class II (≥ 30.0 kg/m2)

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study Design

Analytical cross sectional study

3.2 Subjects

Total 55 individuals Male and Female patients between 18 to 65 years of age who had diagnosed with the hepatocellular carcinoma were included in the study.

3.3 Place of sample collection/ Setting

The study was conducted in out-patient department (OPD) of Gastroenterology department of the Jinnah Postgraduate Medical Centre (JPMC), Karachi

3.4 Inclusion Criteria

- Patients diagnosed with hepatocellular carcinoma
- Male and female Patients between the ages of 18 to 65 years

3.5 Exclusion Criteria

- Patients with hepatorenal syndrome
- Those who have taken anticoagulants
- Those who have taken vitamin K
- Patients with known extrahepatic metastasis
- Patients having refractory ascites
- Patients with 3-4 grade encephalopathy.
- Patients with solid organ transplants.

3.6 Duration of Study

3.6.1 Individual study period: N/A

3.6.2 Total study period: 06 months

3.7 Sample Size Estimation

Total 55 subjects were included in this study. Sample size was calculated by OpenEpi, Version 3. 0 with a 95% confidence interval and 5% margin of error. The prevalence of hepatocellular carcinoma in Pakistan is estimated to be between 3.7% and 16.7% (Tahir, 2021).

Sample Size for Frequency in a Population

Population size(for finite population correction factor or fpc)(*N*):	1000000
Hypothesized % frequency of outcome factor in the population (*p*):	3.7%+/-5
Confidence limits as % of 100(absolute +/- %)(*d*):	5%
Design effect (for cluster surveys-*DEFF*):	1

Sample Size(*n*) for Various Confidence Levels

ConfidenceLevel(%)	Sample Size
95%	55
80%	24
90%	39
97%	68
99%	95
99.9%	155
99.99%	216

Equation

Sample size $n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]$

Results from OpenEpi, Version 3, open source calculator--SSPropor
Print from the browser with ctrl-P
or select text to copy and paste to other programs.

3.8 Sampling Technique

Non-probability consecutive sampling

3.9 Human Subject and Consent

This study received approval from the institutional research committee (IRB) of Bahria University Health Science Campus, Karachi (FRC/BUHS-07/2024), with the letter approval number (BUHS-IRB # R-O39/24).

Permission was acquired from the director and head of the medical institute Jinnah Postgraduate Medical Center Karachi (NO.F.2-81/2023-GENL/104/JPMC) to carry out the research at the gastrointestinal outpatient department (OPD) of the respective hospital.

3.10 Materials

3.10.1 Questionnaire

Attached as annex.

3.10.2 Consent Form

Attached as annex. Participants who agreed with the study procedures were enrolled upon signing informed consent forms in Urdu and English

3.10.3 Drugs

N/A

3.10.4 Equipment

3.10.4.1 ELISA plate reader

Analysis of baseline samples was performed according to manufacturer's protocol. The ELISA plate reader was employes using sandwich technique. This sandwich kit is used for the accurate quantitative detection of protein induce vitamin K absence (PIVKA II) and alpha fetoprotein (AFP) in a biological sample such as serum.

Figure 3.1 Multidisciplinary Research Laboratory (MDRL) BUHSC Karachi.

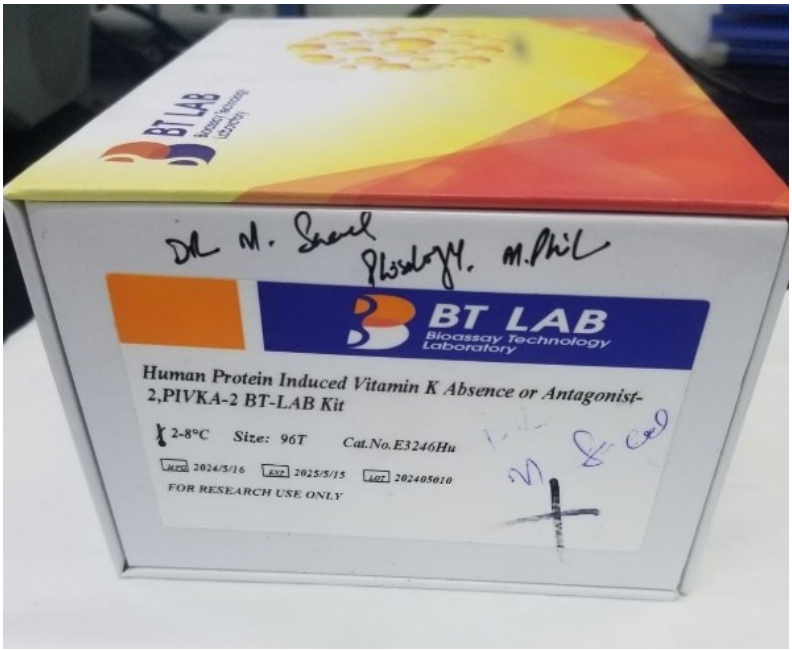


Figure 3.2 ELISA Kits

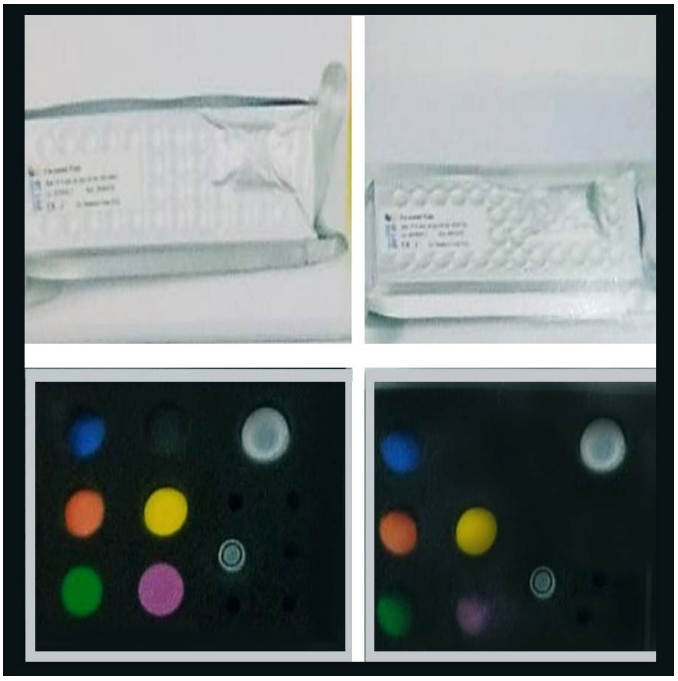


Figure 3.3 PIVKA II and AFP ELISA Kit

Figure 3.4 Eppendorf Tube

Figure 3.5 ELISA plate reader

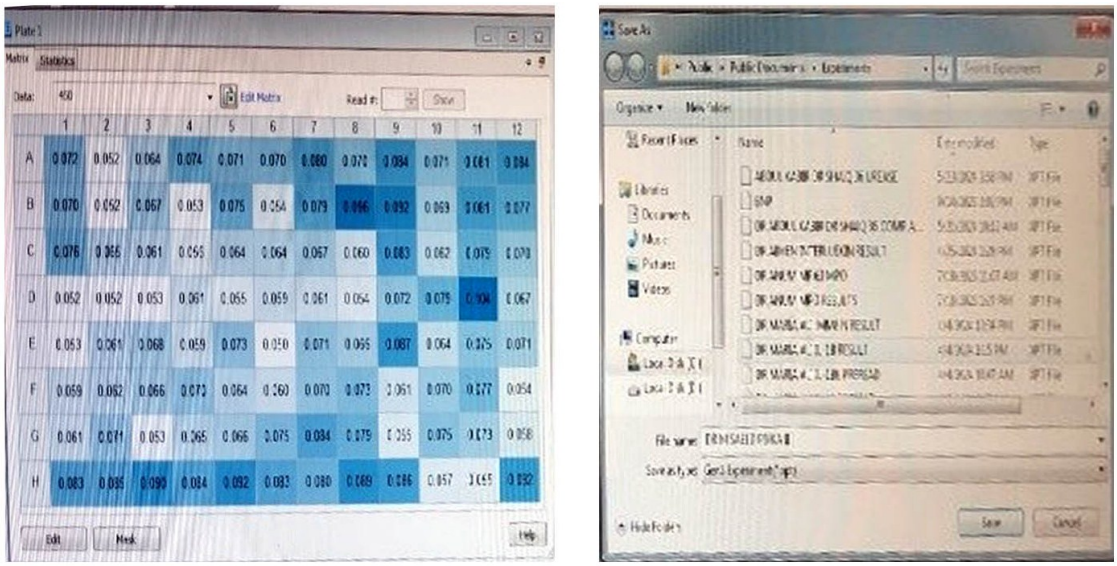


Figure 3.6 Pre reading taken

Figure 3.7 Adding standard Solution

Figure 3.8 Sample Taking

Figure 3.9 Adding Antibody

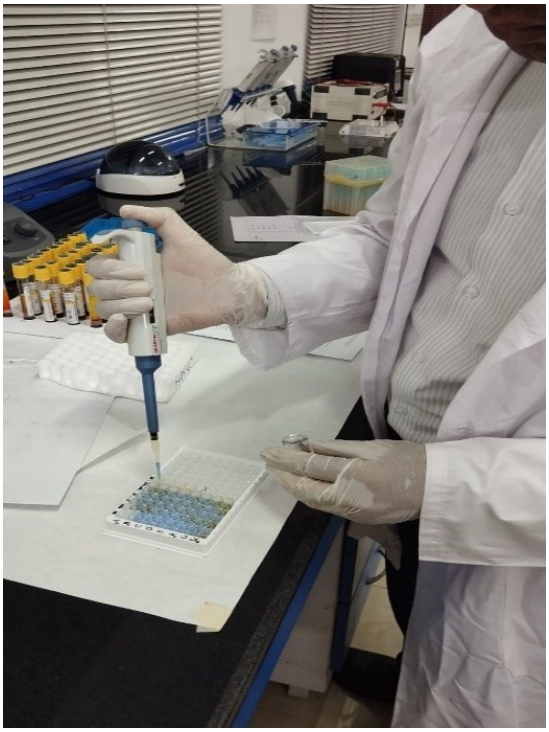


Figure 3.10 Adding HRP Enzyme

Figure 3.11 Plate Incubation

Figure 3.12 Adding Substrate A solution

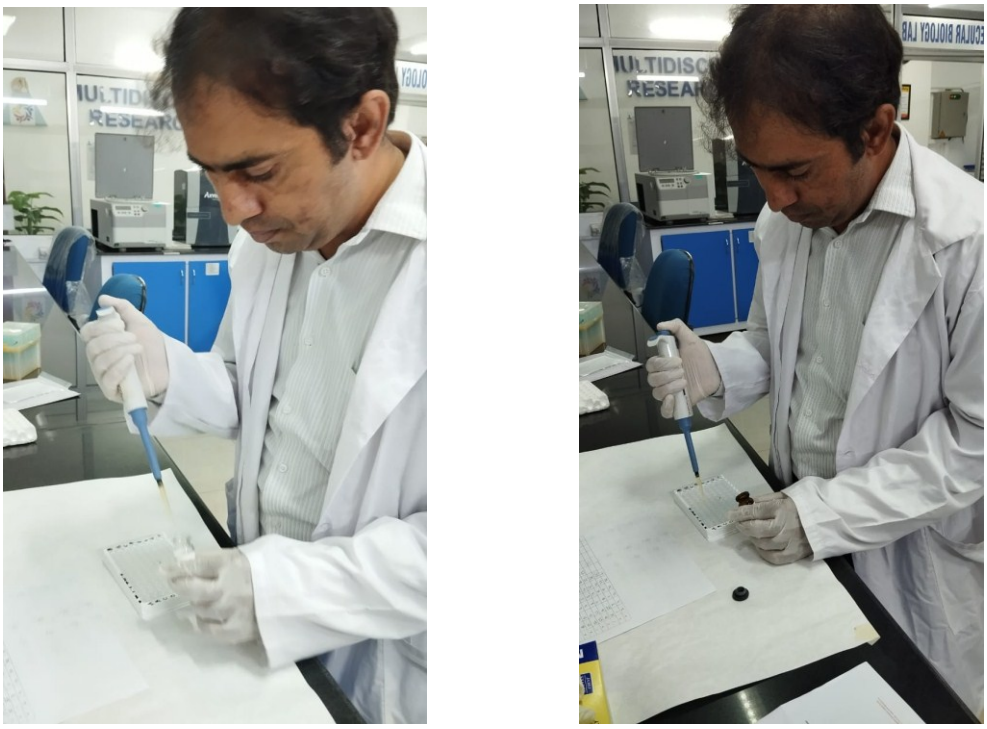


Figure 3.13 Adding Substrate B Solution

Figure 3.14 Adding Stop solution

Figure 3.15 Plate Running

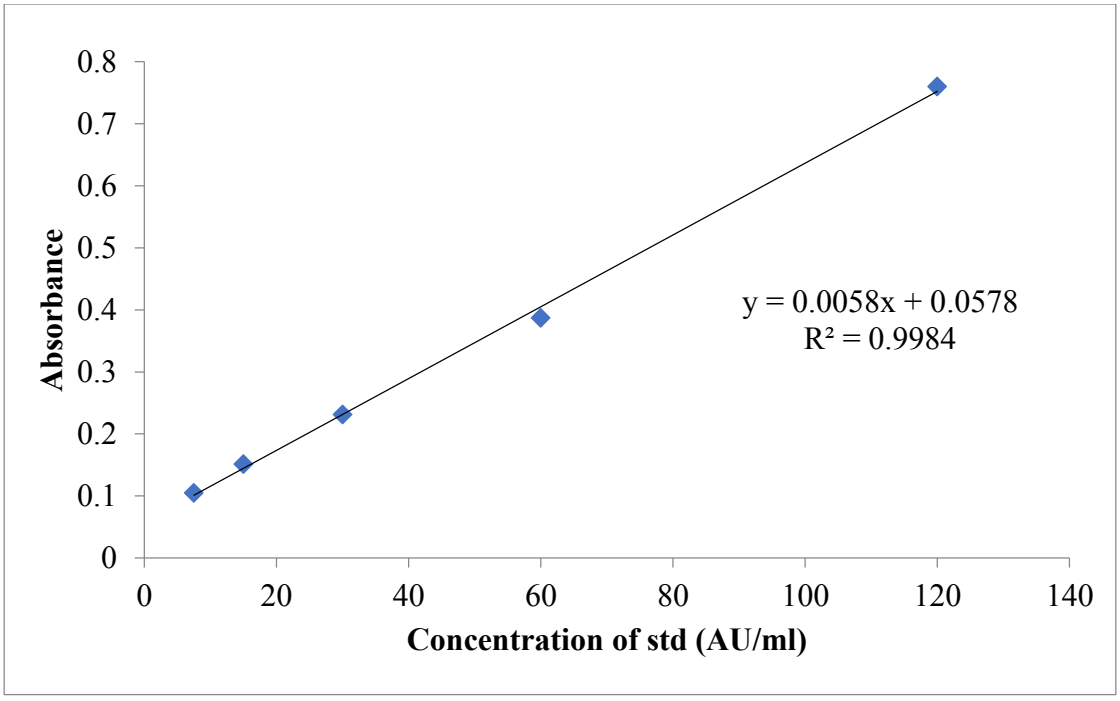


Figure 3.16 Standard Curve of Human Protein Induced Vitamin K Absence or Antagonist-2 plotted by Regression Analysis

3.10.4.2 Kit Used

Commercially available solid phase sandwich enzyme linked immunosorbent assay ELISA kits: PIVKA II kits and AFP kits (bioassay technology laboratory, China)

3.10.4.3 Others

- 1-5ul color coded Hirschmann's microcapillary pipettes
- 0.5 ml Eppendorf
- 5cc disposable syringes
- Centrifuge tubes

3.11 Parameters of Study

3.11.1 Biochemical markers: Protein induce vitamin k absence (PIVKA II) and alpha fetoprotein (AFP) were assessed in human serum using ELISA kits

3.11.2 Human serum sample collection for PIVKA II and AFP:

- Blood samples of patients were taken by sterile method using disposable syringe.
- Blood samples of patients were kept in test tubes.
- Centrifugation of blood samples was done by centrifuge machine to make and separate serum from blood cells.

- The serum collected was carefully dispensed into airtight and sterilized, 0.5ml Eppendorf bearing 398ul of sample diluent and a blast of air was passed through the micro capillary pipettes to enable complete removal of contamination.
- All the samples were kept in cryobox and stored at -80c until analyzed
- Thawing of the samples and analysis was done only once for the assay (using a solid phase sandwich enzyme linked immunosorbent assay)
- Concentration of PIVKA II and AFP in a sample was determined by a commercially available enzyme linked immunosorbent (ELISA) kit (Bioassay Technology Laboratory China) in accordance with manufacturer's instructions.
- Results were analyzed statistically using SPSS version.

3.11.3 Analysis of human serum levels of PIVKA II by enzyme linked immunosorbent assay

- Concentration of PIVKA II in a sample was determined by a commercially available enzyme linked immunosorbent (ELISA) kit (Bioassay Technology Laboratory China) in accordance with manufacturer's instructions.
- Prior to use, all reagents were brought at room temperature.
- A standard solution of 120 AU/ml was made by mixing of standard dilute (120 μl) with 120 μl of the standard (240AU/ml). Before creating the dilution, let the standard sit with mild agitation for 15 minutes.
- Serial dilution of stock solution (120 AU/ml) 1:2 with standard dilute, duplicate standard points were prepared, yielding 60 AU/ml, 30 AU/ml, 15 AU/ml, and 7.5 AU/ml.

120AU/ml	Standard no. 5	120 µl Original standard + 120 µl standard diluent
60AU/ml	Standard no. 4	120 µl standard no.5 + 120 µl standard diluent
30AU/ml	Standard no. 3	120 µl standard no.4 + 120 µl standard diluent
150AU/ml	Standard no. 2	120 µl standard no.3 + 120 µl standard diluent
7.50AU/ml	Standard no. 1	120 µl standard no.2 + 120 µl standard diluent

Standard Concentration	Standard no. 5	Standard no. 4	Standard no. 3	Standard no. 2	Standard no. 1
240AU/ml	120AU/ml	60AU/ml	30AU/ml	15AU/ml	7.5AU/ml

- Addition of standard well of 50µ were done.
- 40µl of the sample was added to each well, followed by 10µl of the anti PIVKA II antibody, 50µl of streptavidin-HRP, and a standard well (not the blank control well). After thoroughly combining, seal the plate, and let it sit at 37°C for 60 minutes.
- Took off the sealant and used wash buffer to wash the plate five times wells that are soaked with 30µl washed buffer for 30seconds to 1 minutes for each wash. Blotted the plate on to paper towels.
- First, 50µl of substrate solution A was applied to each well, and then 50µl of substrate solution B. The plate was incubated for ten minutes at 37°C in the dark with a fresh sealer covering it.
- When 50µl of stop solution was added to each well, the blue color instantly turned yellow.

- Within ten minutes of injecting the stop solution, each well's optical density (OD value) was ascertained using a microplate reader set to 450 nm.
- Plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis allowed for the construction of a standard curve, and a best fit curve was created by connecting the points on the graph.

3.11.4 Analysis of human seum levels of Alpha Fetoprotein by enzyme linked immunosorbent assay

- Concentration of PIVKA II in a sample was determined by a commercially available enzyme linked immunosorbent (ELISA) kit (Bioassay Technology Laboratory China) in accordance with manufacturer's instructions.
- Prior to use, all reagents were brought at room temperature.
- A standard solution of 120 AU/ml was made by mixing of standard dilute (120 µl) with 120 µl of the standard (240AU/ml). Before creating the dilution, let the standard sit with mild agitation for 15 minutes.
- By serially diluting the stock solution (120 AU/ml) 1:2 with standard dilute, duplicate standard points were prepared, yielding 60 AU/ml, 30 AU/ml, 15 AU/ml, and 7.5 AU/ml.

120AU/ml	Standard no. 5	120 µl Original standard + 120 µl standard diluent
60AU/ml	Standard no. 4	120 µl standard no.5 + 120 µl standard diluent
30AU/ml	Standard no. 3	120 µl standard no.4 + 120 µl standard diluent
150AU/ml	Standard no. 2	120 µl standard no.3 + 120 µl standard diluent
7.50AU/ml	Standard no. 1	120 µl standard no.2 + 120 µl standard diluent

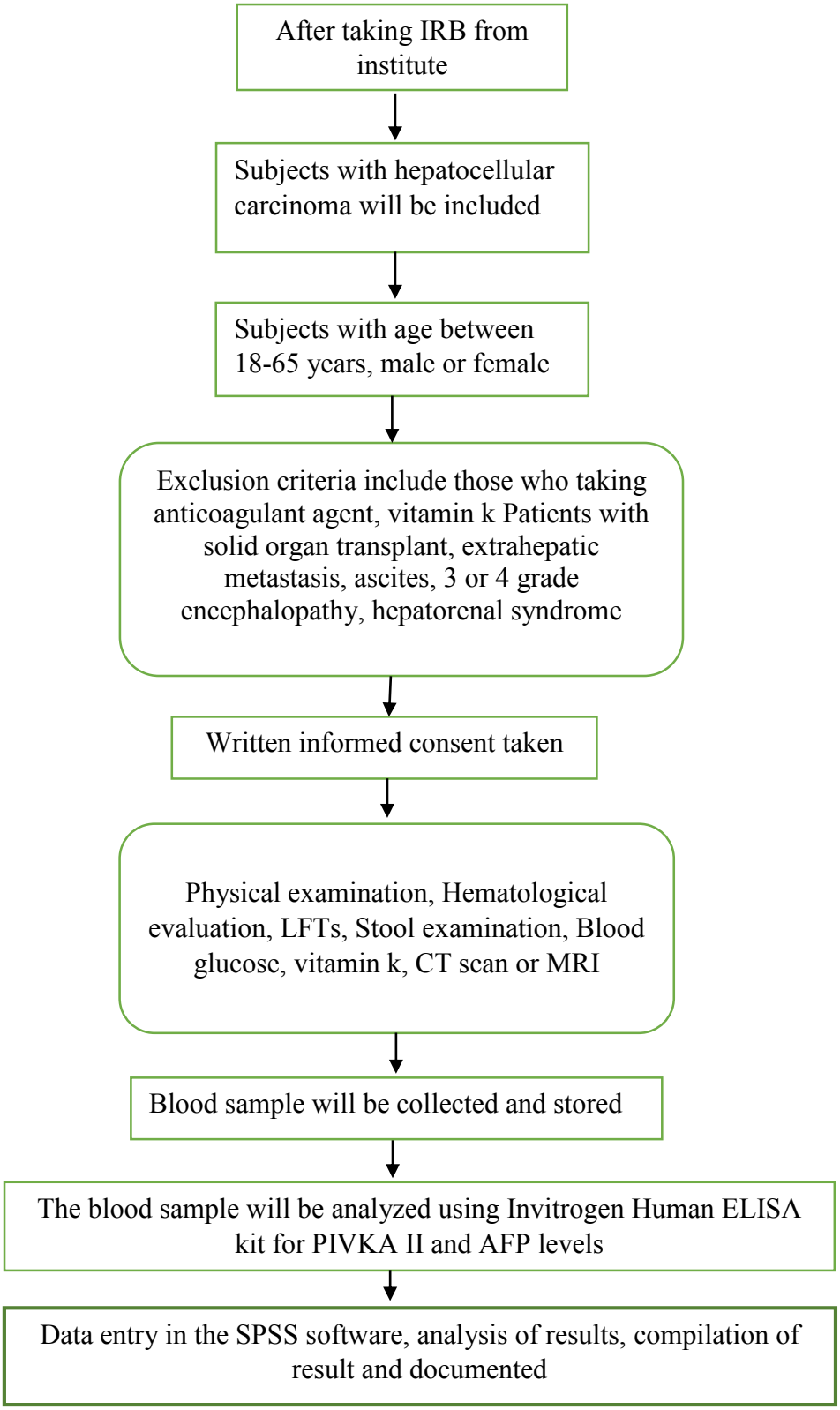
Standard Concentration	Standard no. 5	Standard no. 4	Standard no. 3	Standard no. 2	Standard no. 1
240AU/ml	120AU/ml	60AU/ml	30AU/ml	15AU/ml	7.5AU/ml

- Addition of standard well of 50µ were done.
- 40µl of the sample was added to each well, followed by 10µl of the anti PIVKA II antibody, 50µl of streptavidin-HRP, and a standard well (not the blank control well). After thoroughly combining, seal the plate, and let it sit at 37°C for 60 minutes.
- Took off the sealant and used wash buffer to wash the plate five times wells that are soaked with 30µl washed buffer for 30seconds to 1 minutes for each wash. Blotted the plate on to paper towels.
- First, 50µl of substrate solution A was applied to each well, and then 50µl of substrate solution B. The plate was incubated for ten minutes at 37°C in the dark with a fresh sealer covering it.
- When 50µl of stop solution was added to each well, the blue color instantly turned yellow.
- Within ten minutes of injecting the stop solution, each well's optical density (OD value) was ascertained using a microplate reader set to 450 nm.
- Plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis allowed for the construction of a standard curve, and a best fit curve was created by connecting the points on the graph.

3.12 Protocol of Study

This analytical cross sectional study was conducted after obtaining FRC and IRB of Bahria University of Health Sciences (BUHS). Written informed consent from all the study participants was taken. Patients diagnosed with hepatocellular carcinoma were taken in the study. The patients were recruited from OPD of Gastroenterology Department of Jinnah Post Graduate Medical Center Karachi (JPMC). Demographic details, comorbidities, disease history were recorded by questionnaire and subject evaluation form. Blood samples of patients were taken by sterile method using disposable syringe. Blood samples of patients were kept in test tube. Centrifugation of blood samples were done by centrifuge machine to separate serum from blood cells. The serum collected was carefully dispensed into airtight and sterilized, 0.5ml Effendorfs bearing 398ul of sample diluent and a blast of air was passed through the micro capillary pipettes to enable complete removal of contamination. All the samples were kept in cryobox and stored at -80c until analyzed. Thawing of the samples and analysis was done only once for the assay (using a solid phase sandwich enzyme linked immunosorbent assay) Concentration of PIVKA II and AFP in a sample was determined by a commercially available enzyme linked immunosorbent (ELISA) kit (Bioassay Technology Laboratory China) in accordance with manufacturer's instructions. Results were analyzed statistically.

3.13 Algorithm/FLOW CHART of Study



3.14 Statistical Analysis

3.14.1 Statistical Analysis

Data analysis was done using SPSS version 23. Continuous variables were analyzed as mean and standard deviation. Categorical data was analyzed as frequencies and percentages. Appropriate statistical tests such as sensitivity, specificity, receiver operating characteristic (ROC) curve, Spearman rank correlation test and Pearson correlation test was applied to see the relation between different parameters. The statistically significant value of p was set at < 0.05 for all comparisons. Pie diagrams and bar charts are also used to give graphical presentation of study outcomes.

CHAPTER 4

RESULTS

4.1 Descriptive Statistics of Parameters

Table 4.1.1 reports the spearman rank correlation analysis of marker PIVKA II with demographic, biochemical and other studied parameters, results showed it gives negative association with Age (r=-15.9%, p=0.24), negative Association with Sex (r=-25.2%, p=0.06), positive Association with Ethnicity (r=15.4%, p=0.26), negative Association with Duration of HCC (Months) (r=-16.3%, p=0.23), negative Association with Co morbidities (r=-34.7%, p=0.009*), positive Association with BMI (r=24.4%, p=0.07), negative Association with Type of HCC (r=-7.4%, p=0.59), positive Association with type of hepatitis (r=13.5%, p=0.32), positive Association with Pain (r=18.4%, p=0.17), positive Association with Fatigue (r=25.1%, p=0.06), positive Association with Nausea, Vomiting (r=7.2%, p=0.6), positive Association with Abdominal Swelling (r=1.6%, p=0.9), positive Association with Constipation (r=17.7%, p=0.19), positive Association with loss of appetite (r=9.8%, p=0.47), positive Association with Weight loss (r=24.6%, p=0.06), positive Association with AFP (r=20.4%, p=0.13), positive Association with TB (r=6.2%, p=0.65), positive Association with ALT (r=16.7%, p=0.22), positive Association with ALK-P (r=10.7%, p=0.43), and positive Association with GGT (r=7.8%, p=0.57). Association of PIVKA II with co morbidities was considered statistically significant with $p<0.05$.

Table 4.1.2 reports the spearman rank correlation analysis of marker AFP with demographic, biochemical and other studied parameters, results showed it gives negative Association with Age (r=-1.2%, p=0.92), negative Association with Sex (r=-26.8%, p=0.04*), positive Association with Ethnicity (r=24.3%, p=0.07), negative Association with Duration of HCC (Months) (r=-15.4%, p=0.26), negative Association with Co morbidities (r=-14%, p=0.3), negative Association with BMI (r=-9.2%, p=0.5), positive Association with Type of HCC (r=17.7%, p=0.19), positive Association with type of hepatitis (r=12.4%, p=0.36), positive Association with Pain (r=19.9%, p=0.14), positive Association with Fatigue (r=23.3%, p=0.08), negative Association with Nausea, Vomiting (r=-1.6%, p=0.9), negative Association with Abdominal Swelling (r=-15.4%, p=0.26), positive Association with Constipation (r=13.3%, p=0.33), positive Association with loss of appetite (r=31.8%, p=0.01*), positive Association with Weight loss (r=38%, p=<0.01*), positive Association with TB (r=23.8%, p=0.08), positive Association with ALT (r=15.5%, p=0.25), positive Association with ALK-P (r=24.5%, p=0.07), positive Association with GGT (r=4.4%, p=0.74), and positive association with GGT (r=7.8%, p=0.57). Association of AFP with sex, loss of appetite and weight loss was found statistically significant ($p<0.05$).

Table 4.1.3 shows that the levels of four biomarkers total bilirubin, alkaline phosphatase, alanine transaminase and gamma glutamyl transferase were higher than normal in Hepatocellular Carcinoma. It shows that when there is pathology of liver occur certain biochemical enzymes were released to show the severity of disease.

Table 4.1.4 represents the results of Pearson correlation analysis which shows the relationship between four biomarkers total bilirubin, alkaline phosphatase, alanine transaminase and gamma glutamyl transferase. Positive correlation was found between total bilirubin with alkaline phosphate which has statistically significant p value (<0.05). This indicates that higher levels of total bilirubin are associated with higher alkaline phosphatase. There is no significant correlation between total bilirubin with the other two enzymes alanine transaminase and gamma glutamyl transferase. There is a positive correlation between alkaline phosphate and gamma glutamyl transferase with a statistically significant p value (<0.005). There is no significant

correlation between alanine transaminase and alkaline phosphatase. Also, no significant relationship was found between alanine transaminase and gamma glutamyl transferase.

Table 4.1.5 presents the sensitivity and specificity of three diagnostic approaches for HCC, using two biomarkers: AFP and PIVKA II both individually and in combination. At cut off value of >10ng/ml the sensitivity of AFP is 75.7% which means the test correctly identifies 75.7% of true positive case (patients with the disease) and the specificity of AFP is 71.5% depicting that test correctly identifies 71.5% of true negative cases (patients without the disease). This means AFP alone has moderate accuracy. At cut off value of >40mAU/ml the sensitivity of PIVKA II is 79.4 % which means the test has slightly higher sensitivity compared to AFP meaning it is better at identifying patients with the disease and the specificity of PIVKA II is 72.9% which mean the test has slightly higher specificity compared to AFP indicating better performance in identifying patients without disease. Combination of AFP and PIVKA II at cut off value 69.3 the sensitivity is 80.3% and specificity is 75.2% means combining both biomarkers improve the sensitivity and specificity compared to using either marker alone, making this combination a more accurate diagnostic approach.

Table 4.1.6 shows the diagnostic performance of the biomarker AFP, PIVKA II and their combination (AFP and PIVKA II). According to their area under the curve (AUC) from a receiver operating characteristic (ROC) study shows the diagnostic accuracy of biomarkers. AFP has AUC of 0.758 indicating moderate accuracy while PIVKA II has AUC of 0.821 which shows a good accuracy and better than AFP alone. Their combination (AFP and PIVKA II). has an AUC value of 0.885 which shows better accuracy than both biomarkers alone. The PIVKA II has standard error of 0.093 which is less than AFP (0.102) and their combinations (AFP and PIVKA II) (0.109) suggests better precision of the AUC estimate than AFP and their combination (AFP and PIVKA II). The p value of AFP is 0.39, PIVKA II is 0.34 and their combination is o.026 which is statistically significant (<0.05).

Figure 4.1.1 shows the receiver operating curve (ROC) which was used to assess the diagnostic performance of biomarkers AFP, PIVKA II and their combination (AFP and PIVKA II). The higher the curve above the reference line indicates better diagnostic performance. The closer to the curve to the top left corner, the higher the accuracy. AFP has moderate diagnostic accuracy while PIVKA II shows better diagnostic accuracy than AFP combination of AFP and PIVKA II has better diagnostic accuracy than both biomarkers alone.

Table 4.1.1: Association of PIVKA II Marker with Demographic and Biochemical Parameters

Parameters	Correlation	p-value
Age	-0.159	0.24
Sex	-0.252	0.06
Ethnicity	0.154	0.26
Duration of HCC(Months)	-0.163	0.23
Co morbidities	-0.347	0.009*
BMI	0.244	0.07
Type of HCC	-0.074	0.59
type of hepatitis	0.135	0.32
Pain	0.184	0.17
Fatigue	0.251	0.06
Nausea, Vomiting	0.072	0.60
Abdominal Swelling	0.016	0.90
Constipation	0.177	0.19
loss of appetite	0.098	0.47
Weight loss	0.246	0.06
Jaundice	0.087	0.52
AFP	0.204	0.13
TB	0.062	0.65
ALT	0.167	0.22
ALK-P	0.107	0.43
GGT	0.078	0.57

*Spearman rank correlation considered statistically significant with $p<0.05$

Table 4.1.2: Association of AFP Marker with Demographic and Biochemical Parameters

Parameters	Correlation	p-value
Age	-0.012	0.92
Sex	-0.268	0.04*
Ethnicity	0.243	0.07
Duration of HCC(Months)	-0.154	0.26
Co morbidities	-0.140	0.30
BMI	-0.092	0.50
Type of HCC	0.177	0.19
type of hepatitis	0.124	0.36
Pain	0.199	0.14
Fatigue	0.233	0.08
Nausea, Vomiting	-0.016	0.90
Abdominal Swelling	-0.154	0.26
Constipation	0.133	0.33
loss of appetite	0.318	0.01*
Weight loss	0.380	<0.01*
Jaundice	0.200	0.14
TB	0.238	0.08
ALT	0.155	0.25
ALK-P	0.245	0.07
GGT	0.044	0.74

*Spearman rank correlation considered statistically significant with $p<0.05$

Table 4.1.3 Biochemical Characteristics of Patients

Characteristics	Values
Total bilirubin	3.024mg/dl (0.1-1.2mg/dl)
ALT	65.4909IU/L (19-33IU/L
Alkaline phosphatase	486.4364IU/L (44-147IU/L)
Gamma GT	126.8182U/L (5-40U/L)

Table 4.1.4 Correlations between biochemical characteristics

		TB	ALT	ALK_P	GGT
TB	**Pearson Correlation**	1	.053	.343*	.167
	Sig. (2-tailed)		.702	.010	.224
ALT	**Pearson Correlation**	.053	1	-.030	-.037
	Sig. (2-tailed)	.702		.830	.789
ALK_P	**Pearson Correlation**	.343*	-.030	1	.579**
	Sig. (2-tailed)	.010	.830		.000
GGT	**Pearson Correlation**	.167	-.037	.579**	1
	Sig. (2-tailed)	.224	.789	.000	

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

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

Table 4.1.5 Sensitivity and Specificity of each biomarker and their Combination

	SENSITIVITY	SPECIFICITY
AFP (cut of value >10ng/ml)	75.7%	71.5%
PIVKA II cut of value >40mAU/ml)	79.4%	72.9%
Combination (AFP + PIVKA II) (cut of value 69.31)	80.3%	75.2%

Table 4.1.6 AUC in evaluating PIVKA-II and AFP in the diagnosis of Hepatocellular carcinoma

Biomarkers	AUC	SE	P value	95 % CI
AFP	0.758	0.102	0.039	0.157-0.759
PIVKA II	0.821	0.093	0.034	0.115-0.831
AFP with PIVKA II	0.885	0.109	0.026	0.075-0.889

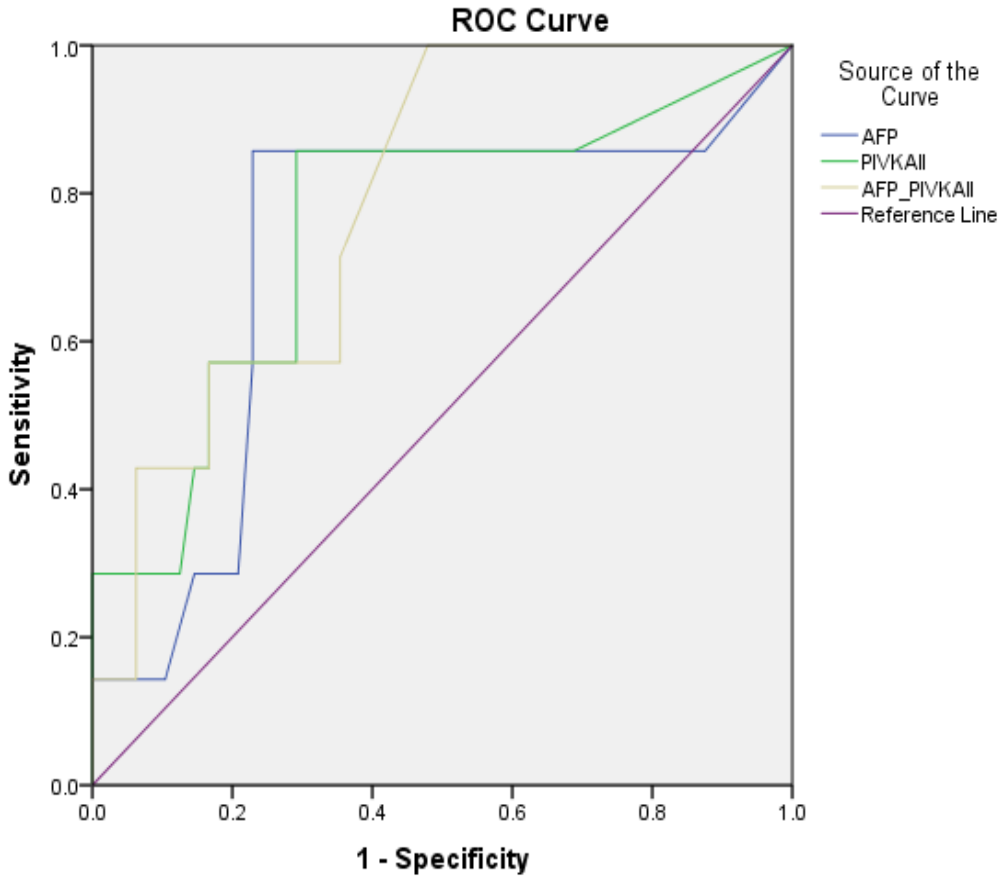


Figure 4.1 ROC of biomarkers AFP, PIVKA II and AFP with PIVKA II

4.2 Demographic Profile of the Patients

4.2.1 Gender Distribution

The study was conducted on a total of 55 subjects who had been diagnosed with hepatocellular carcinoma. In the study frequency of males was 41 (74.55%) and females was 14 (25.45%) as shown in figure 4.2.1

4.2.2 Age Distribution

The mean age of patients along with the corresponding standard deviation was 58.61±10.00 years. The age distribution of targeted subjects lies between the 18 years to 65 years. It is observed that 11% of the subjects were in age range of 35 to 45, 25% of the subjects were in age range of 45 to 55 years and 64% of the cases were in age range of 55 to 65 years having higher number of patients shown in figure 4.2.2

4.2.3 Classification of BMI

Figure 4.2.3 shows a graphical presentation, with BMI <18kg/m² being more prevalent among subjects with 30(56%), patients with BMI 18-22.9 kg/m² were 21(36%), patients with BMI 23-24.9 kg/m² were 2(4%). and patients with BMI 25-29.9 kg/m² were 2(4%).

4.2.4 Ethnicity

Of all, 15 (27.3%) were members of the Punjabi community. Following this with 10 (18.2%) were the Sindhi community, followed by Urdu speaking with 9 (16.4%), Pathan with 9 (16.4%), Baloch with 3 (5.5%), Hindko with 3 (5.5%), Bangali with 2(3.6%),

Kachhi with 2(3.6%). Memon with 1(1.8%) and Saraiki with 1(1.8%). The proportion of Punjabi and Sindhi patients were higher as compared to other ethnicities as shown in figure 4.2.4.

4.2.5 Comorbidities

As shown in figure 4.2.5 subjects exhibited comorbidities such as hypertension and diabetes, with 40 (72.7%) participants who did not report any other ailments. Hypertension is present in 7 (12.7%) patients. Diabetes mellitus is present in 5 (9.1%) patients. Hypertension and diabetes mellitus both are present in 3 (5.5%) patients as comorbidity.

4.2.6 Most Common Symptoms

As shown in figure 4.2.6 pain was the most common symptoms in 35(63.6%) patients which were followed by Ascites in 8(14.5%) patients, constipation in 6(10.9%) patients, hematemesis in 3 (5.5%), diarrhea in 1 (1.8%), jaundice in in 1(1.8%) and weight loss in 1(1.8%) patient.

4.2.7 Type of Hepatitis

As shown in figure 4.2.7 among all subjects Hepatitis C was among the highest type of hepatitis is present in 39 (71%) subjects. After that Hepatitis B is present in 7 (13%) patients and Hepatitis D is present in 1 (1.8%) patient. There was not any type of hepatitis present in 8(14%) patients

4.2.8 Alcohol drinking

54 (98%) patients are non-alcohol drinking and 1 (2%) are alcohol drinker.

4.2.9 Marital Status

54 (98%) patients were married and 1 (2%) was unmarried.

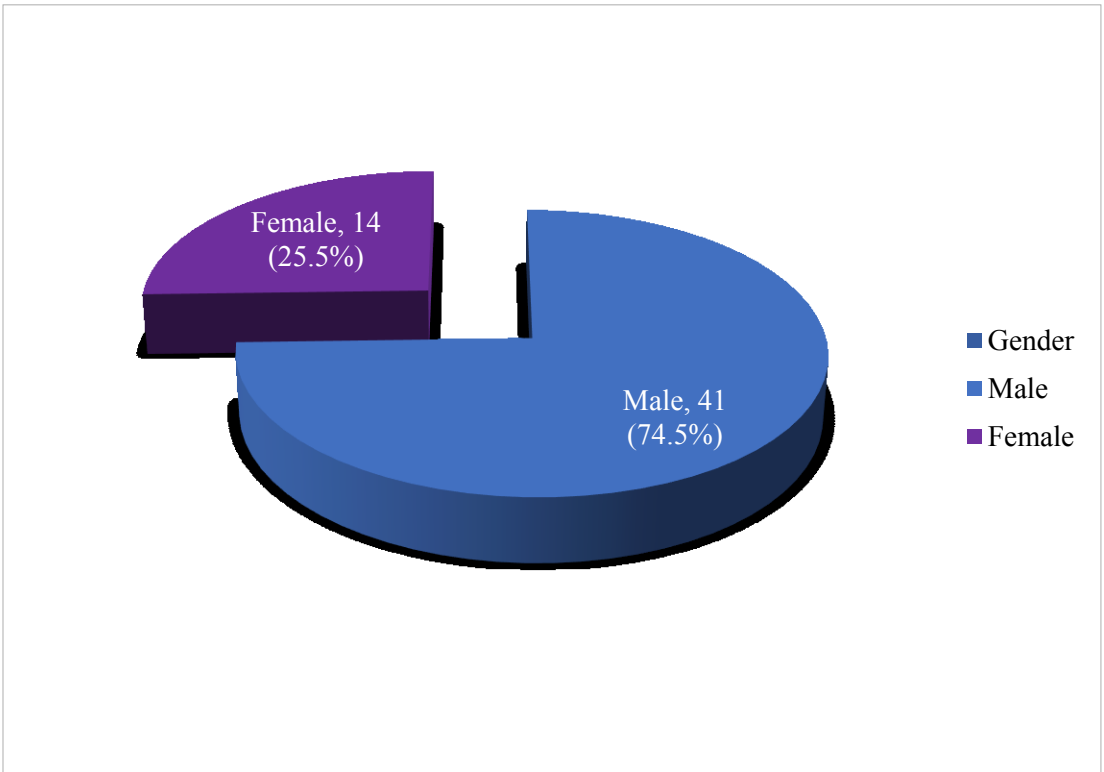


Figure 4.2.1 Gender Classification of subjects

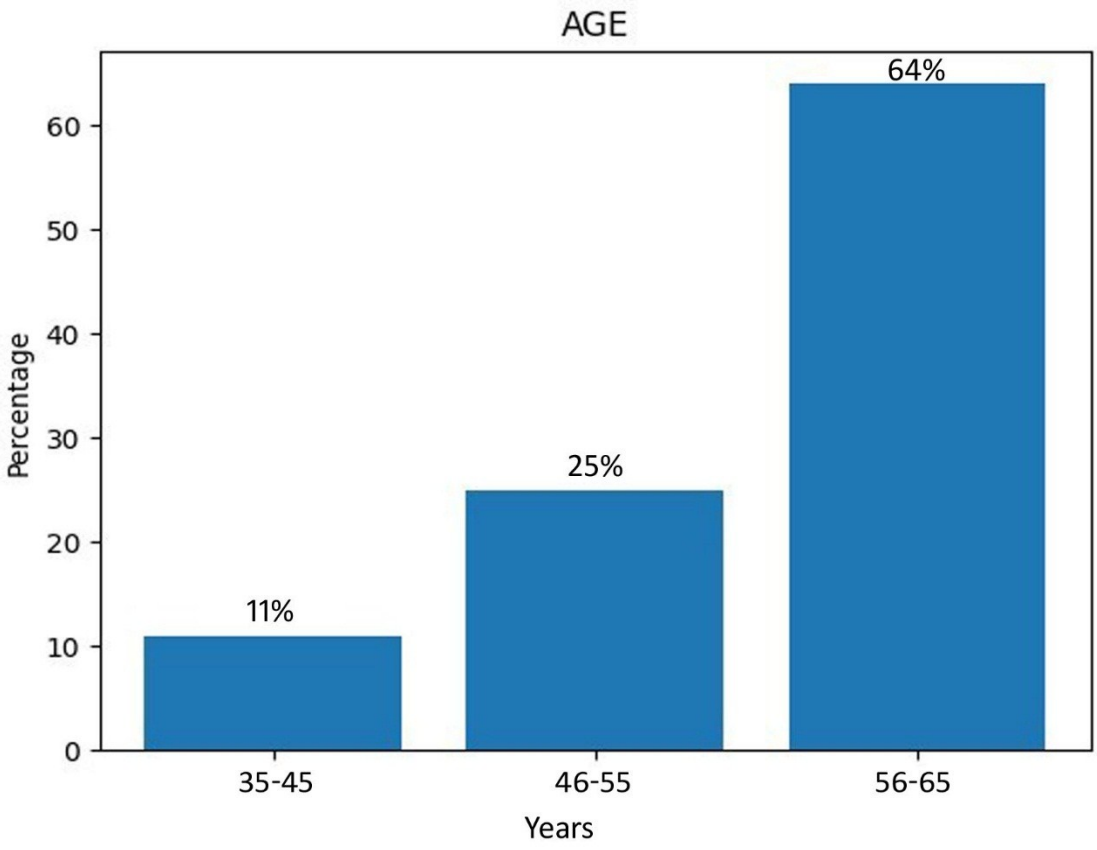


Figure 4.2.2 Age Classification of subjects

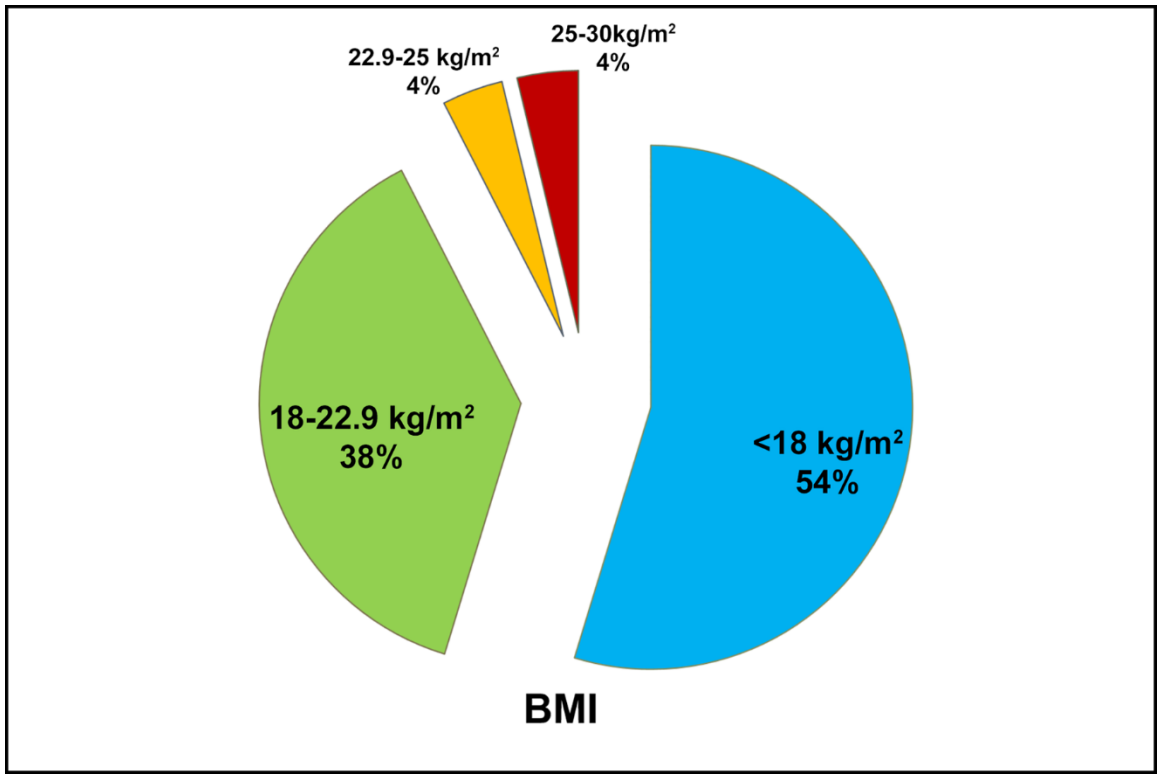


Figure 4.2.3 BMI Classification of subjects

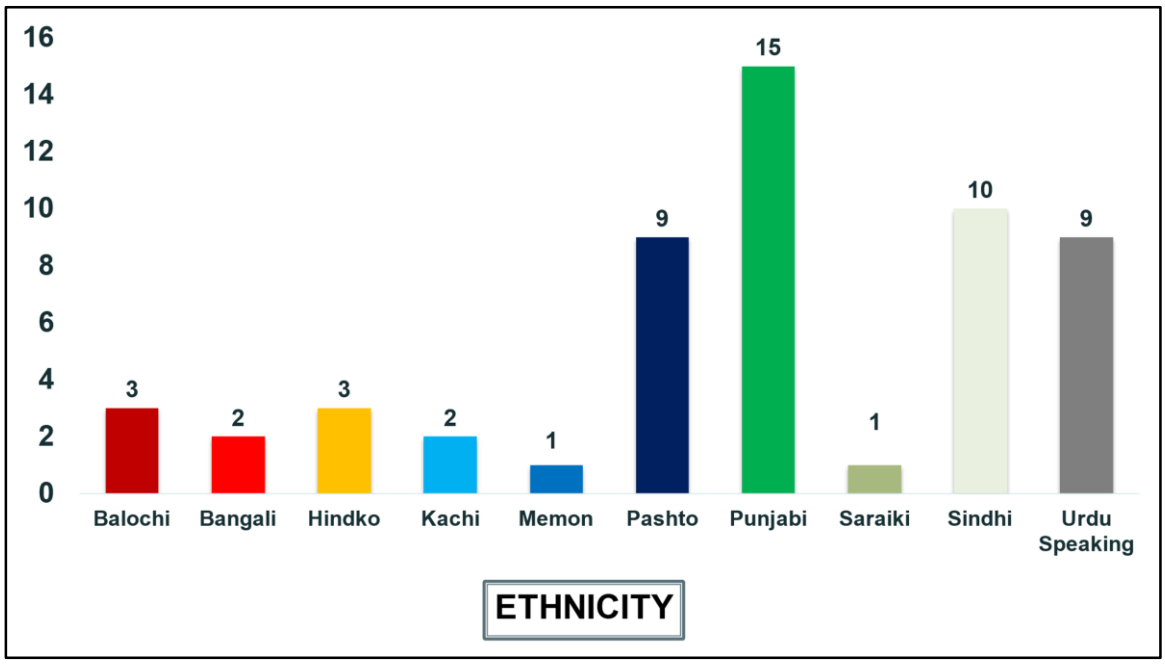


Figure 4.2.4 Common Symptoms of subjects

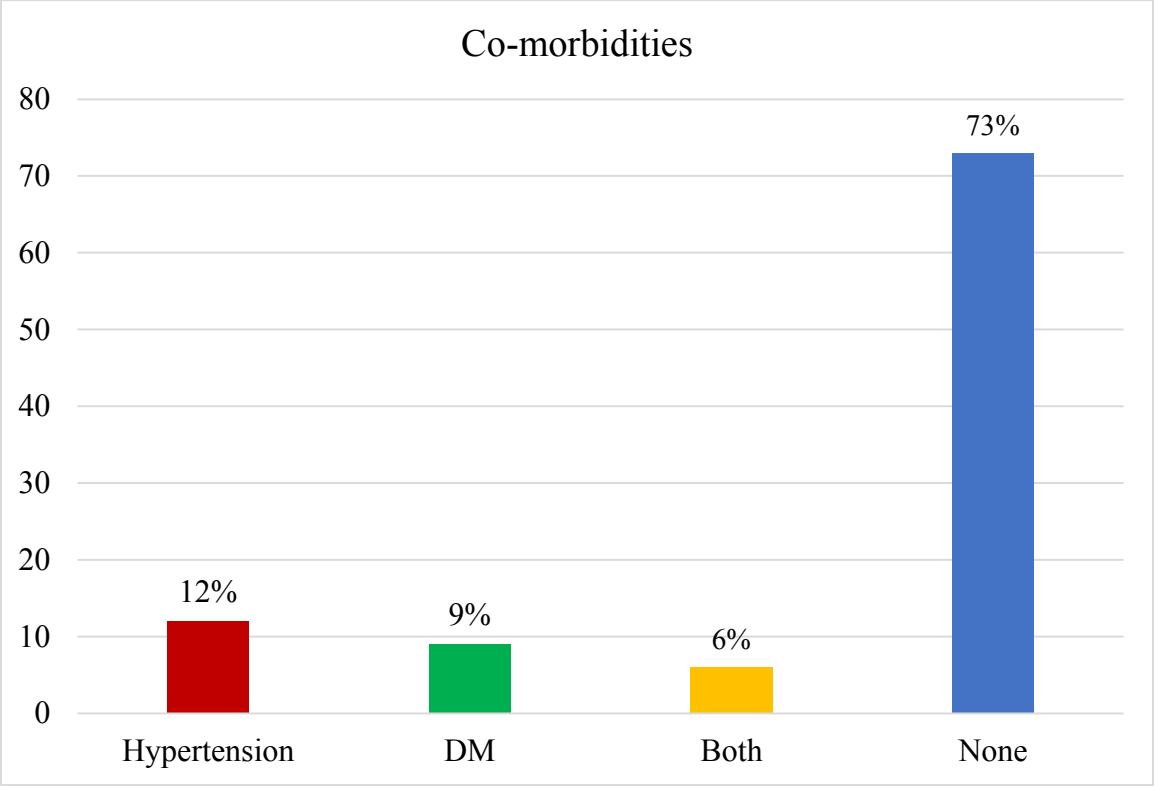


Figure 4.2.5 Comorbidities of subjects

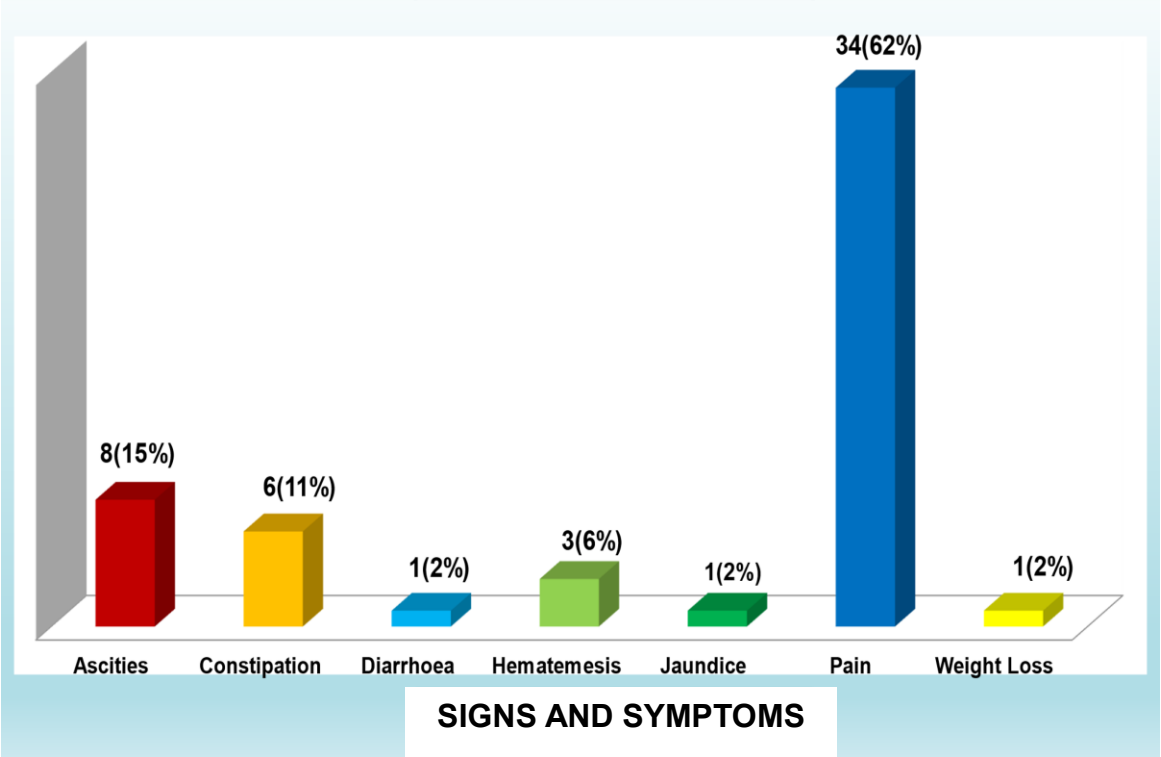


Figure 4.2.6 Common Symptoms of subjects

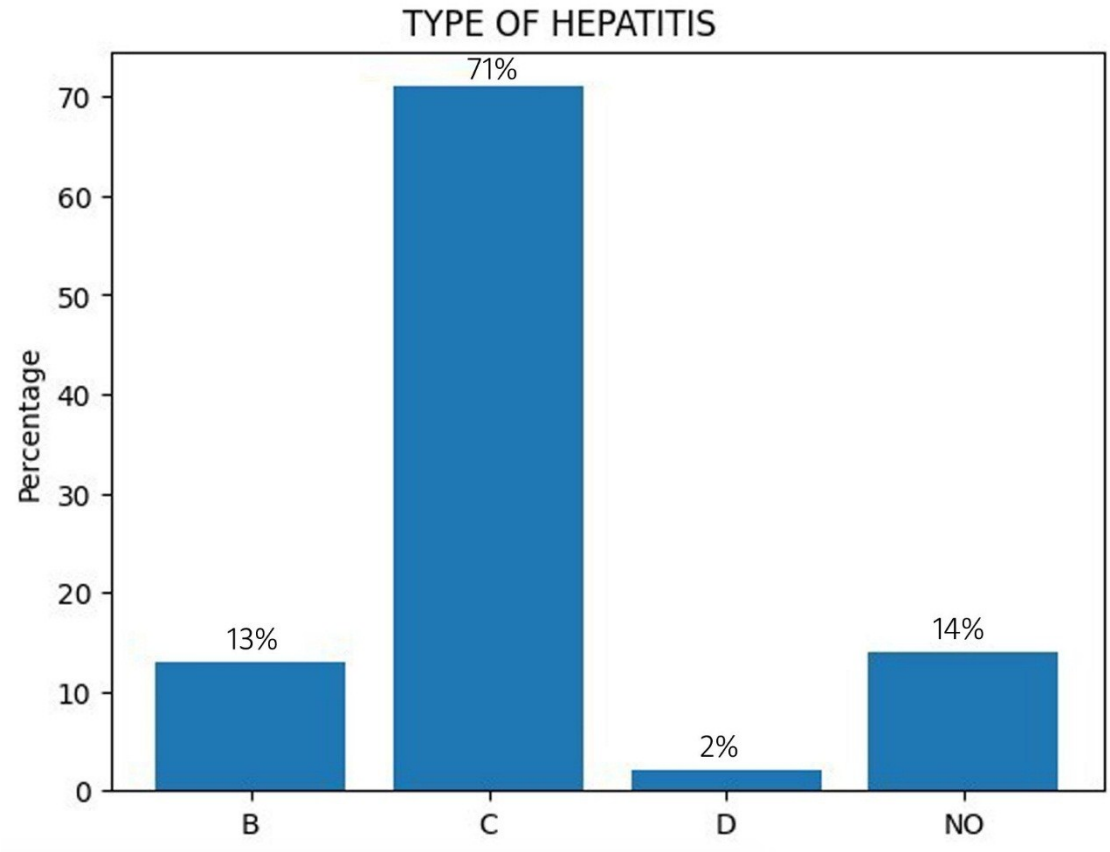


Figure 4.2.7 Common Symptoms of subjects

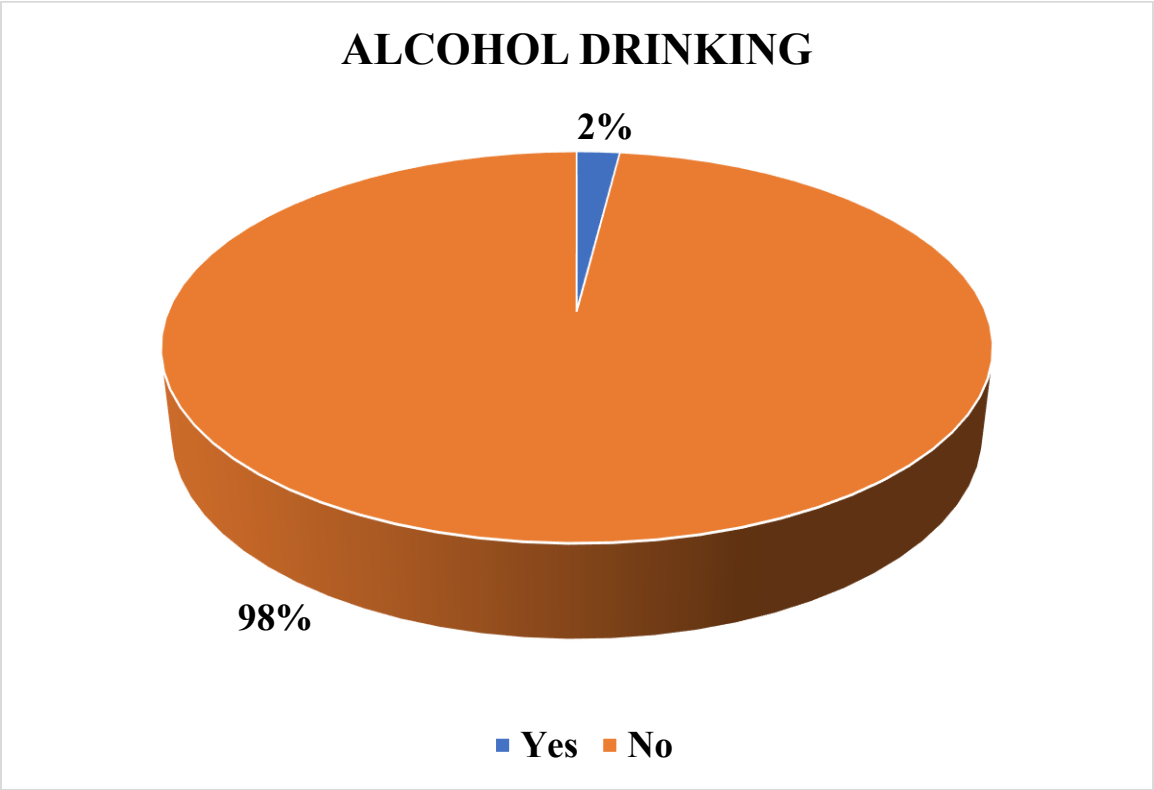


Figure 4.2.8 alcohol use

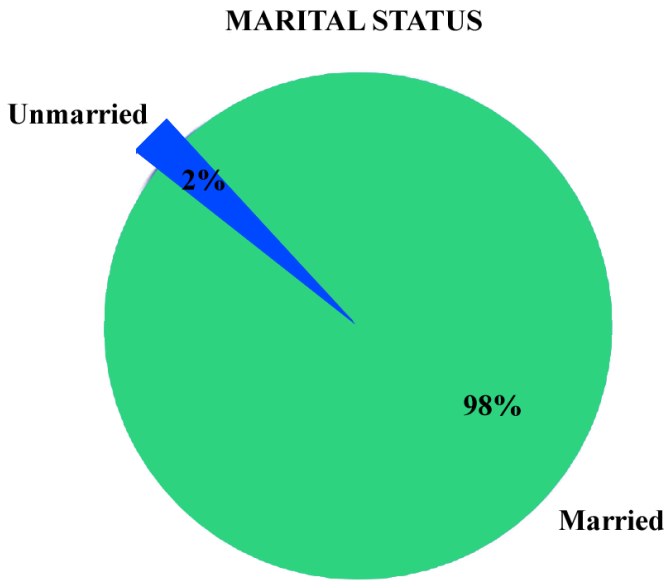


Figure 4.2.9 Marital Status

CHAPTER 5

DISCUSSION

5.1 Sequence

In our research, Hepatocellular Carcinoma was found to be more prevalent in males than females as out of 55 patients, 74.5% were male, compared to 25.5% female. This was consistent with the studies (Liao *et al*., 2022) in which patients suffering from Hepatocellular Carcinoma were predominantly male. In another study the Hepatocellular Carcinoma is also predominant in male (Rich *et al*., 2021). There are other multiple studies in which Hepatocellular Carcinoma is predominant in males as compared to female (Faith Ajayi1 *et al*., 2021; Yi Shen *et al*., 2020). Based on different data incidence of HCC in Pakistan is also predominant in males as compared to females (Bhatti *et al*., 2016). The exact reasons for higher male prevalence remain unclear. There are complicated molecular processes behind the differences in gender in HCC. The development of HCC influenced differently by sex hormones; while estrogen have a preventive effect, androgen have a stimulating effect. Higher blood testosterone levels are linked to higher HCC risk. Estrogen appears to be involved in the synthesis of IL6 and the regulation of gene expression which inhibits the growth of HCC (Yip *et al*., 2019).

Based on our research, we found that the average age of participants was 58.62 years, indicating that Hepatocellular carcinoma is prevalent in older demographics. In Pakistan, HCC is common in 5^{th} and 6^{th} decade (Zaigham, 2013) which is consistent with

our study. A study conducted reported a mean age of 58 years for Hepatocellular Carcinoma prevalence (Le *et al*., 2019). A study conducted in Egypt, reported a mean age for HCC to be 57 years (Kesuma *et al*., 2021). Compared to HCC in younger groups, HCC in elderly is linked to distinct clinical features. Metabolic illnesses and other medical comorbidities are more common among the elderly (Wong *et al*., 2022).

The mean body mass index (BMI) in our study is in normal range 20.05 kg/m^2 which is consistent with the study conducted in Pakistan in which mean BMI is also in normal range 24.55 kg/m^2 (Butt *et al*., 2013) an elevated BMI was linked to high risk of HCC. There are differences in the relationship between BMI and HCC risk based on age, sex and liver disease (Kim *et al*., 2023) There was a non-linear relationship between BMI and HCC: in the range of ≥25 kg/m^2, but not in the range of <25 kg/m2, BMI was positively linked with the risk of HCC (Jun *et al*., 2022)

In our research most common type of hepatitis was Hepatitis C which was present in 38 (69.09%) patients which is consistent with the study in which the cause of approximately 87% of HCC cases in Pakistan is a viral hepatitis C (68%). (Parkash, *et al*., 2016) also in another study in Pakistan in Pakistan, HCC is primarily caused by diseases associated with the hepatitis C virus (HCV) (Zaigham abbas 2013; Bhatti *et al.,* 2016). In a study conducted in America hepatitis C causes 31% of HCC (Shiels *et al*., 2019). Another study conducted in which in North America, Europe, Japan, areas of central Asia, including Mongolia, and northern Africa and the Middle East, especially Egypt, HCV is the primary virus-related cause of HCC (Yang *et al*., 2019) in another study which was conducted Hepatitis C is the most prevalent hepatitis in Americans with HCC (McGlynn *et al*., 2021). There is another study which is contradictory to our study that HBV is more common cause of HCC (Yeh *et al*., 2023).

In our research the mean value of total bilirubin was 3.023mg/dl and mean value of direct bilirubin was 1.83 which were on higher side. It was consistent with a study conducted in China in which total bilirubin was higher in HCC patients (Xu *et al*., 2023). In another study was conducted showed that total bilirubin was higher in HCC. A higher level of total bilirubin showed the damage of liver parenchyma and inflammation and even

liver failure (Carr *et al*., 2016) a study conducted showing an elevated level of the total bilirubin in HCC also enhances the performance of some biomarkers in diagnosing of HCC (Qian *et al*., 2023). In another study it showed that there is a rise in the level of total bilirubin in HCC patients. Which can also be a prognostic marker of the disease along with other biomarkers. (Antkowiak, *et al*., 2019). In another study it was shown that there are elevated levels of bilirubin in HCC which are due to the damage to the liver cells by different causes (Lei *et al*., 2023).

The mean value of alanine transaminase (ALT) was 65.50IU/L in our study which was higher than normal. This is consistent with a study conducted in China in which there is an elevated level of alanine transaminase (ALT) was shown. Normally alanine transaminase (ALT) is present in the hepatocytes, whenever there is any damage to the liver cells the alanine transaminase (ALT) is released in the blood stream. (Zhang, *et al*., 2019) In another study it was shown that there is elevated level of alanine transaminase (ALT) in hepatocellular carcinoma. (Wang, *et al*., 2023). there is a study conducted in China showed that there is high level of alanine transaminase (ALT) in hepatocellular carcinoma patients due to the damage of liver cells which releases the alanine transaminase (ALT) in blood (Zhao *et al*., 2021).

In our study the mean value of alkaline phosphatase was 486.44IU/L which is higher than the normal value. This is consistent with the study in which there was a high level of alkaline phosphatase in HCC patients (Huang *et al*., 2022). Alkaline phosphatase is distributed throughout the liver cells. It is also present in cancerous cells. Alkaline phosphatase is also associated with many vascular changes that occur during the initial stage of HCC (Su *et al*., 2023). Alkaline phosphatase elevation shows that there is a pathology in the liver. In HCC there is an increase in the alkaline phosphatase level which shows the poor prognosis of the disease (Yu *et al*., 2022).

In our research the value of Gamma Glutamyl Transferase (GGT) was 126.82U/L which was higher than the normal value. This result is consistent with the study in which there is elevated level of GGT in HCC in Turkey. (Carr, *et al*., 2021) In another study there is also a higher level of GGT seen in HCC. (Tian, *et al*., 2021) GGT is linked with the pre

neoplastic lesions of HCC. In the growth of HCC there is involvement of GGT (Carr *et al.,* 2021) in another study in Turkey there has higher level of GGT seen in HCC patients (Ince, *et al*., 2020).

There is no significant relationship between total bilirubin level and PIVKA II in our study. This was consistent with the study (Lei, *et al.,*2023) which shows PIVKA-II's diagnostic performance for HCC declines as bilirubin levels rise, indicating an inverse relationship in their predictive values.in another study there is There was no discernible link between PIVKA-II levels and bilirubin concentrations (Ivanko, *et al.,*2017). In another study Patients with normal total bilirubin levels are more likely to have HCC detected by PIVKA-II than those with increased levels (Qian, *et al.,* 2023). There is another study which is contradictory to our study shows that Increased levels of PIVKA-II may be linked to abnormal liver function, which can result in hyperbilirubinemia. This can then impact the absorption of vitamin K and trigger the creation of PIVKA II (Parkash, *et al.,* 2021).

In our study there is no significant association between PIVKA II and alanine transaminase, PIVKA II and alkaline phosphatase. This result is contradictory to a study which shows Serum alkaline phosphatase is positively correlated with elevated PIVKA-II levels in people with chronic liver disease, which affects how well the enzyme detects hepatocellular cancer (Qian, *et al.,* 2023). There is also no significant association between was seen between PIVKA II and gamma glutamyl transferase. However, another study which is contradictory to our study shows PIVKA-II and γ-GT levels are higher in patients with HCC, and they correlate with the size and stage of the tumor. (Wang, *et al.,* 2020).

There is a positive correlation between total bilirubin with alkaline phosphate with a significant (P value= 0.01) in our study which is consistent with another study which shows the severity of the disease and alterations in the liver parenchyma are reflected in the elevated bilirubin level linked to alkaline phosphate elevation in HCC which suggest liver dysfunction and possible biliary blockage (Huang *et al.,*2022).in our study there is a positive correlation between alkaline phosphatase and gamma glutamyl transferases (P value= <0.001) which is consistent with a study which shows that In patients with HCC, elevated ALP and GGT levels were risk predictors which shows poor survival outcomes

(Ouyang *et al.,*2022). There is another study which is contradictory to this that level of alkaline phosphatase and gamma glutamyl transferases does not show consistent correlation suggesting they may reflect different aspect of liver pathology (Ouyang *et al.,*2014). There is not any significant correlation between total bilirubin and gamma glutamyl transferases. There is not any significant correlation between gamma glutamyl transferases and alanine transaminase. It has been seen that liver diseases can be evaluated by certain biochemical enzymes such as alkaline phosphatase, alanine transaminase, total bilirubin and gamma glutamyl transferase. It has been observed that these enzymes were released by liver cells (Jain et al., 2022).

A prospective study evaluating the sensitivity and specificity of alpha fetoprotein (AFP) and protein-induced vitamin K absence (PIVKA II) in hepatocellular carcinoma was carried out in Thailand in high risk patients showed that the sensitivity of protein induced vitamin K absence (PIVKA II) at the level of 40mAU/ml is 80.80% and the sensitivity of alpha fetoprotein (AFP) at the level of 10ng/ml is 75.80%. The specificity of protein induced vitamin K absence (PIVKA II) at the level of 40mAU/ml is 75.60% and the specificity of alpha fetoprotein (AFP) at the level of 10ng/ml is 65.90%. Alpha fetoprotein (AFP) and protein-induced vitamin K deficiency (PIVKA II) together produced a sensitivity of 60.30% and specificity of 92.70%. Though not statistically different from PIVKA II alone (0.855 vs. 0.832 p value = 0.130), the area under the ROC curve for PIVKA II plus AFP was larger than that of AFP alone (0.855 vs. 0.795, respectively, p value = 0.027) (Suttichaimongkol *et al*., 2023)

In another study which was conducted in China for the comparison of Alpha fetoprotein (AFP), protein-induced vitamin K deficiency (PIVKA II), and their combination in hepatocellular carcinoma and other conditions. AFP's area under the ROC curve was 0.901, PIVKA II's was 0.938, and their combination was 0.972. The ideal AFP cut-off value was determined to be 6ng/ml. In the HCC group, the AFP's sensitivity and specificity were 75.9% and 96%, respectively. For PIVKA II, the ideal cutoff value was determined to be 49.74mAUng/mL. In the HCC group, PIVKA II's sensitivity and specificity were 86.2% and 98%, respectively. In the HCC group, the combination's sensitivity and specificity were 91% and 100%, respectively (Tian *et al*., 2022).

A study was conducted in surveillance and monitoring of PIVKA II, AFP and their combination in Hepatocellular Carcinoma in Asia Pacific region showed that sensitivity of PIVKA II at the level of 40mAU/ml is 69% and the sensitivity of AFP at the level of 10ng/ml is 65%. The specificity of PIVKA II at the level of 40mAU/ml is 89% and the specificity of AFP at the level of 10ng/ml is 88%. The combination of PIVKA II and AFP gave the sensitivity of 82% and specificity of 85%. The area under ROC curve was 0.880 for AFP, 0.75 for PIVKA II and 0.90 for their combination (Kim *et al*., 2023).

Another study conducted in postoperative patients of HCC to see the early recurrence showed that the positivity of PIVKA II was 78.4% than the AFP which have passivity of 66.3 (Wang *et al*., 2022).

In a clinical trial that assessed the comparison between PIVKA II and AFP in hepatocellular carcinoma. In this study the area under ROC curve for PIVKA II was 0.98 and for AFP was 0.87. Sensitivity of PIVKA II at the level of 37.5mAU/ml is 94% and the sensitivity of AFP at the level of 6.5ng/ml is 79%. The specificity of PIVKA II at the level of 37.5mAU/ml is 100% and the specificity of AFP at the level of 6.5ng/ml is 100% (Prakash *et al*., 2021).

In our study we included 55 patients diagnosed with hepatocellular carcinoma based on inclusion criteria**.** Sensitivity and specificity of PIVKA II and AFP (Alpha Fetoprotein) was determined. The sensitivity of PIVKA II was 79.4% and specificity was 72.9% at the level of >40m AU/ml. the sensitivity of AFP was 75.7% and specificity was 71.5% at the level of 10ng/ml. a combination of PIVKA II and AFP give the sensitivity of 80.3% and specificity of 75.2.% **.**The area under roc curve for biomarker PIVKA II for diagnosing hepatocellular carcinoma shown that (0.821, 95% confidence interval) with significant p value (0.034) was higher than that of AFP (0.758, 95% confidence interval) with significant p value (0.039). The area under roc curve for biomarker PIVKA II and AFP combine was (0.885, 95% confidence interval) with significant p value (0.026).

5.2 Implication

5.2.1 Theoretical Implication

These findings have several theoretical implications that have driven the hypothesis of the study. Firstly, from a methodological perspective, this study contributes to the literature on the enhancement of the diagnostic accuracy of the disease. It can reduce the unnecessary tests in patients who don't have the disease. This approach adds another biomarker for the physicians to consider for the disease. Theoretically, this research adds to the existing literature by the role of this biomarker in the disease to explore its mechanism and therapeutic targets more effectively. This leads to more precise and appropriate use of medical interventions. Additionally, this study specifically focuses on another biomarker with more sensitivity and specificity as a diagnostic tool, thereby filling a gap in the literature.

5.2.2 Practical Implication

Our study provides new guidelines for physicians regarding the use of another biomarker which has more sensitivity so that more patients are diagnosed before the disease progression and timely intervention to better the survival rates and outcomes. Higher specificity reduces the likelihood of false positive. This could reduce the need of other diagnostic tests and save the resources This can also become a primary marker for screening in high risk populations, especially where hepatitis B and C is more common. By better diagnostic accuracy it can also become more cost effective. This could help physician to better intervention in patients, enhance the quality care and outcomes. Accurate and less invasive testing builds confidence among patients for screening and monitoring programs. However, integrating this new biomarker may require careful consideration and evidence-based policy adjustments.

5.2.3 Policy Implication

N/A

5.3 Limitations and Strengths of the Study

(A) Limitations Strengths

- Due to time constraints, a random sampling procedure was not feasible, and therefore a non-probability consecutive sampling method was utilized, which resulted in selection bias.
- The study was performed in a single center.
- The sample size of the study is small.
- We did not follow up longer to dynamically observe the correlation between changes in serum PIVKA II and AFP

(B) Strengths

Following strengths can be expected in the study:

- This study is a good fit for investigating PIVKA II's potential involvement as a marker in HCC.
- This study is pioneering in nature and aims to uses a new marker PIVKA II which is not commonly used.

5.4 Future Research Direction/ Recommendations

- Further studies should be done in future that work on new biomarkers in detection of hepatocellular carcinoma.
- More multicenter, large-scale studies across diverse patient's populations should be done to validate the improved sensitivity and specificity of PIVKA II. Performance of the biomarker should be evaluated in at risk groups especially with hepatitis B and C.
- Future research should focus on use of PIVKA II in combination with other biomarkers to determine that the combining PIVKA II with other biomarkers can further improve the diagnostic accuracy and reduces false positive and negatives.
- Further studies should be done to monitor the role of PIVKA II in disease progression, treatment response and recurrence. Also, should investigate the utility of PIVKA II to distinguish HCC from other liver diseases.
- Further studies should be done to explore potential genetic polymorphism that may affect PIVKA II production and its effectiveness as a biomarker.

5.5 Conclusion

In conclusion PIVKA II has better sensitivity and specificity than AFP in the detection and diagnosis of Hepatocellular Carcinoma. This enhanced performance makes PIVKA II a more reliable biomarker for early detection, improving the chances of successful treatment outcomes. The greater sensitivity of PIVKA II allows for earlier identification of HCC cases, while its higher specificity reduces the likelihood of false positive, thereby minimizes unnecessary interventions.

REFERENCES

Ajayi, f., jan, j., singal, a. G., & rich, n. E. (2020). Racial and sex disparities in hepatocellular carcinoma in the usa. *Current hepatology reports*, *19*(4), 462–469. Https://doi.org/10.1007/s11901-020-00554-6

Antkowiak, M., Gabr, A., Das, A., Ali, R., Kulik, L., Ganger, D., Moore, C., Abecassis, M., Katariya, N., Mouli, S., Mahalingam, D., Lewandowski, R. J., Salem, R., & Riaz, A. (2019). Prognostic Role of Albumin, Bilirubin, and ALBI Scores: Analysis of 1000 Patients with Hepatocellular Carcinoma Undergoing Radioembolization. *Cancers*, *11*(6), 879. https://doi.org/10.3390/cancers11060879

Banales, J. M., Iñarrairaegui, M., Arbelaiz, A., Milkiewicz, P., Muntané, J., Muñoz-Bellvis, L., La Casta, A., Gonzalez, L. M., Arretxe, E., Alonso, C., Martínez-Arranz, I., Lapitz, A., Santos-Laso, A., Avila, M. A., Martínez-Chantar, M. L., Bujanda, L., Marin, J. J. G., Sangro, B., & Macias, R. I. R. (2019). Serum Metabolites as Diagnostic Biomarkers for Cholangiocarcinoma, Hepatocellular Carcinoma, and Primary Sclerosing Cholangitis. *Hepatology (Baltimore, Md.)*, *70* (2), 547–562. https://doi.org/10.1002/hep.30319

Basile, U., Miele, L., Napodano, C., Ciasca, G., Gulli, F., Pocino, K., De Matthaeis, N., Liguori, A., De Magistris, A., Marrone, G., Biolato, M., Marino, M., Di Giacinto, F., Gasbarrini, A., Grieco, A., & Rapaccini, G. L. (2020). The diagnostic performance of PIVKA II in metabolic and viral hepatocellular carcinoma: a pilot study. *European review for medical and pharmacological sciences*, *24*(24), 12675–12685. https://doi.org/10.26355/eurrev_202012_24165

Boonkaew, B., Satthawiwat, N., Pinjaroen, N., Chuaypen, N., & Tangkijvanich, P. (2023). Circulating Extracellular Vesicle-Derived microRNAs as Novel Diagnostic and Prognostic Biomarkers for Non-Viral-Related Hepatocellular Carcinoma. *International journal of molecular sciences*, *24*(22), 16043. https://doi. org/10.3390/ijms242216043

Butt, A. S., Abbas, Z., & Jafri, W. (2012). Hepatocellular carcinoma in pakistan: where do we stand?. *Hepatitis monthly*, *12*(10 HCC), e6023. https://doi.org/10.5812/hepatmon.6023

Butt, A. S., Hamid, S., Wadalawala, A. A., Ghufran, M., Javed, A. A., Farooq, O., Ahmed, B., Ul Haq, T., & Jafri, W. (2013). Hepatocellular carcinoma in Native South Asian Pakistani population; trends, clinico-pathological characteristics & differences in viral marker negative & viral-hepatocellular carcinoma. *BMC research notes*, *6*, 137. https://doi.org/10.1186/1756-0500-6-137

Carr, B. I., Akkiz, H., Bag, H. G., Karaoğullarından, U., Yalçın, K., Ekin, N., Özakyol, A., Altıntaş, E., Balaban, H. Y., Şimşek, H., Uyanıkoğlu, A., Balkan, A., Kuran, S., Üsküdar, O., Ülger, Y., Güney, B., & Delik, A. (2021). Serum levels of gamma-glutamyl transpeptidase in relation to HCC human biology and prognosis. *Journal of translational science*, *7*(3), 10.15761/jts.1000446. https://doi.org/10.15761/jts.1000446

Carr, B. I., Guerra, V., Giannini, E. G., Farinati, F., Ciccarese, F., Rapaccini, G. L., Di Marco, M., Benvegnù, L., Zoli, M., Borzio, F., Caturelli, E., Masotto, A., & Trevisani, F. (2016). A Liver Index and its Relationship to Indices of HCC Aggressiveness. *Journal of integrative oncology*, *5*(4), 178. https://doi.org/10.4172/2329-6771.1000178

Caviglia, G. P., Abate, M. L., Troshina, G., Carucci, P., Rolle, E., Risso, A., Burlone, M. E., Albè, A., Crevola, M., Musso, E. C., Rosso, C., Armandi, A., Olivero, A., Minisini, R., Saracco, G. M., Bugianesi, E., Pirisi, M., Ciancio, A., & Gaia, S. (2023). Identification of the Best Cut-Off Value of PIVKA-II for the Surveillance

of Patients at Risk of Hepatocellular Carcinoma Development. *Biology*, *12*(1), 94. https://doi.org/10.3390/biology12010094

Chakraborty, E., & Sarkar, D. (2022). Emerging Therapies for Hepatocellular Carcinoma (HCC). *Cancers*, *14*(11), 2798. https://doi.org/10.3390/cancers14112798

Chen, T. C., Hsiao, S. W., Chen, Y. Y., Yen, H. H., Su, W. W., Hsu, Y. C., Huang, S. P., & Su, P. Y. (2024). Increased prevalence but decreased survival of nonviral hepatocellular carcinoma compared to viral hepatocellular carcinoma in recent ten years. *Scientific reports*, *14*(1), 9068. https://doi.org/10.1038/s41598-024-59668-2

Chidambaranathan-Reghupaty, S., Fisher, P. B., & Sarkar, D. (2021). Hepatocellular carcinoma (HCC): Epidemiology, etiology and molecular classification. *Advances in cancer research*, *149*, 1–61. https://doi.org/10.1016/bs.acr.2020.10.001

Choi, J., Kim, G. A., Han, S., Lee, W., Chun, S., & Lim, Y. S. (2019). Longitudinal Assessment of Three Serum Biomarkers to Detect Very Early-Stage Hepatocellular Carcinoma. *Hepatology (Baltimore, Md.)*, *69*(5), 1983–1994. https://doi.org/10.1002/hep.30233

Darmadi, D., & Ruslie, R. H. (2021). Association between prothrombin induced by Vitamin K absence-II (PIVKA-II) and barcelona clinic liver cancer (BCLC) stage, tumor size, portal venous thrombosis in hepatocellular carcinoma patients. *Sains Malaysiana*, *50*(2), 475-480.

De Battista, D., Zamboni, F., Gerstein, H., Sato, S., Markowitz, T. E., Lack, J., Engle, R. E., & Farci, P. (2021). Molecular Signature and Immune Landscape of HCV-Associated Hepatocellular Carcinoma (HCC): Differences and Similarities with HBV-HCC. *Journal of hepatocellular carcinoma*, *8*, 1399–1413. https://doi.org/10.2147/JHC.S325959

Devillers, M. J. C., Pluimers, J. K. F., van Hooff, M. C., Doukas, M., Polak, W. G., de Man, R. A., Sonneveld, M. J., Boonstra, A., & den Hoed, C. M. (2023). The Role

of PIVKA-II as a Predictor of Early Hepatocellular Carcinoma Recurrence-Free Survival after Liver Transplantation in a Low Alpha-Fetoprotein Population. *Cancers*, *16*(1), 4. https://doi.org/10.3390/cancers16010004

Dhanasekaran, R., Bandoh, S., & Roberts, L. R. (2016). Molecular pathogenesis of hepatocellular carcinoma and impact of therapeutic advances. *F1000Research*, *5*.

Edoo, M. I. A., Chutturghoon, V. K., Wusu-Ansah, G. K., Zhu, H., Zhen, T. Y., Xie, H. Y., & Zheng, S. S. (2019). Serum Biomarkers AFP, CEA and CA19-9 Combined Detection for Early Diagnosis of Hepatocellular Carcinoma. *Iranian journal of public health*, *48*(2), 314–322.

El-Halawany, F., Mahmoud, S., Oun, M. R., Kadry, M., Tarek, H., Hanna, R., & Osama, A. (2020). Detection of Serum Biomarkers In Hepatocellular Carcinoma Patients. *Maghreb-Machrek*, *2*(2), 01-12.

Feng, H., Li, B., Li, Z., Wei, Q., & Ren, L. (2021). PIVKA-II serves as a potential biomarker that complements AFP for the diagnosis of hepatocellular carcinoma. *BMC cancer*, *21*(1), 401. https://doi.org/10.1186/s12885-021-08138-3

Gabbia, D., & De Martin, S. (2024). Insights into Hepatocellular Carcinoma: From Pathophysiology to Novel Therapies. *International journal of molecular sciences*, *25*(8), 4188. https://doi.org/10.3390/ijms25084188

Galle, P. R., Foerster, F., Kudo, M., Chan, S. L., Llovet, J. M., Qin, S., & Zhu, A. X. (2019). Biology and significance of alpha- fetoprotein in hepatocellular carcinoma. *Liver international*, *39*(12), 2214-2229.

Ganne-Carrié, N., & Nahon, P. (2019). Hepatocellular carcinoma in the setting of alcohol-related liver disease. *Journal of hepatology*, *70*(2), 284–293. https://doi.org/10.1016/j.jhep.2018.10.008

Giannini, E. G., Sammito, G., Farinati, F., Ciccarese, F., Pecorelli, A., Rapaccini, G. L., Di Marco, M., Caturelli, E., Zoli, M., Borzio, F., Cabibbo, G., Felder, M., Gasbarrini, A., Sacco, R., Foschi, F. G., Missale, G., Morisco, F., Svegliati Baroni, G., Virdone, R., Trevisani, F., Italian Liver Cancer (ITA.LI.CA) Group (2014). Determinants of alpha-fetoprotein levels in patients with hepatocellular carcinoma: implications for its clinical use. *Cancer*, *120*(14), 2150–2157. https://doi.org/10.1002/cncr. 28706

Gisder, D. M., Tannapfel, A., & Tischoff, I. J. H. R. (2022). Histopathology of hepatocellular carcinoma-when and what. *Hepatoma Res*, *8*(4), 10-20517.

Gramantieri, L., Gnudi, F., Vasuri, F., Mandrioli, D., Fornari, F., Tovoli, F., Suzzi, F., Vornoli, A., D'Errico, A., Piscaglia, F., & Giovannini, C. (2022). Aflatoxin B1 DNA-Adducts in Hepatocellular Carcinoma from a Low Exposure Area. *Nutrients*, *14*(8), 1652. https://doi.org/10.3390/nu14081652

Gutiérrez-Cuevas, J., Lucano-Landeros, S., López-Cifuentes, D., Santos, A., & Armendariz-Borunda, J. (2022). Epidemiologic, Genetic, Pathogenic, Metabolic, Epigenetic Aspects Involved in NASH-HCC: Current Therapeutic Strategies. *Cancers*, *15*(1), 23. https://doi.org/10.3390/cancers15010023

Hadi, H., Wan Shuaib, W. M. A., Raja Ali, R. A., & Othman, H. (2022). Utility of PIVKA-II and AFP in Differentiating Hepatocellular Carcinoma from Non-Malignant High-Risk Patients. *Medicina (Kaunas, Lithuania)*, *58*(8), 1015. https://doi.org/ 10.3390/medicina58081015

Hafeez Bhatti, A. B., Dar, F. S., Waheed, A., Shafique, K., Sultan, F., & Shah, N. H. (2016). Hepatocellular Carcinoma in Pakistan: National Trends and Global Perspective. *Gastroenterology research and practice*, *2016*, 5942306. https://doi. org/10.1155/ 2016/5942306

Hafeez, M., Nadeem, M., Ahmed, M., & Faheem-Ur-Rehman (2020). Hepatocellular Carcinoma (HCC), where do we stand? Current situation. *Pakistan journal of medical sciences*, *36*(3), 344–348. https://doi.org/10.12669/pjms.36.3.1594

Ho, S. Y., Liu, P. H., Hsu, C. Y., Hsia, C. Y., Huang, Y. H., Lei, H. J., Su, C. W., Lee, R. C., Hou, M. C., & Huo, T. I. (2020). Evolution of etiology, presentation, management and prognostic tool in hepatocellular carcinoma. *Scientific reports*, *10*(1), 3925. https://doi.org/10.1038/s41598-020-61028-9

Hu, X., Bao, M., Huang, J., Zhou, L., & Zheng, S. (2020). Identification and Validation of Novel Biomarkers for Diagnosis and Prognosis of Hepatocellular Carcinoma. *Frontiers in oncology*, *10*, 541479. https://doi.org/10.3389/fonc.2020.541479

Huang, C. W., Wu, T. H., Hsu, H. Y., Pan, K. T., Lee, C. W., Chong, S. W., Huang, S. F., Lin, S. E., Yu, M. C., & Chen, S. M. (2022). Reappraisal of the Role of Alkaline Phosphatase in Hepatocellular Carcinoma. *Journal of personalized medicine*, *12* (4), 518. https://doi.org/10.3390/jpm12040518

Ince, V., Carr, B. I., Bag, H. G., Ersan, V., Usta, S., Koc, C., Gonultas, F., Sarici, B. K., Karakas, S., Kutluturk, K., Baskiran, A., & Yilmaz, S. (2020). Liver transplant for large hepatocellular carcinoma in Malatya: The role of gamma glutamyl transferase and alpha-fetoprotein, a retrospective cohort study. *World journal of gastrointestinal surgery*, *12*(12), 520–533. https://doi.org/10.4240/wjgs.v12.i12.520

Ioannou G. N. (2021). Epidemiology and risk-stratification of NAFLD-associated HCC. *Journal of hepatology*, *75*(6), 1476–1484. https://doi.org/10.1016/j.jhep.2 021.08.012

Ivanko, O. G., Solianik, A. V., & Pashchenko, I. V. (2017). PIVKA-II Ontogenetic view on PIVKA-II in the development of prothrombin synthesis in infants *Pathologia*, (3).

Jain, P., Batta, A. K., & Singh, P. (2022). Comparative Study of Serum Levels of Gamma-glutamyl Transferase, Aspartate Aminotransferase (AST), Alanine Transaminase (ALT), AST: ALT, and Bilirubin in Patients with Chronic Hepatitis. *Indian Journal of Medical Biochemistry*, *26*(3), 74.

Ji, J., Liu, L., Jiang, F., Wen, X., Zhang, Y., Li, S., Lou, J., Wang, Y., Liu, N., Guo, Q., Jia, Y., & Gao, C. (2021). The clinical application of PIVKA-II in hepatocellular carcinoma and chronic liver diseases: A multi-center study in China. *Journal of clinical laboratory analysis*, *35*(11), e24013. https://doi.org/10.1002/jcla.24013

Jiang, Y., Han, Q., Zhao, H., & Zhang, J. (2021). The Mechanisms of HBV-Induced Hepatocellular Carcinoma. *Journal of hepatocellular carcinoma*, *8*, 435–450. https://doi.org/10.2147/JHC.S307962

Jin, Y., Wong, Y. S., Goh, B. K. P., Chan, C. Y., Cheow, P. C., Chow, P. K. H., Lim, T. K. H., Goh, G. B. B., Krishnamoorthy, T. L., Kumar, R., Ng, T. P., Chong, S. S., Tan, H. H., Chung, A. Y. F., Ooi, L. L. P. J., Chang, J. P. E., Tan, C. K., & Lee, C. G. L. (2019). Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma. *Scientific reports*, *9*(1), 10464. https://doi.org/10.1038/s41598-019-46872-8

Jun, B. G., Kim, M., Shin, H. S., Yi, J. J., & Yi, S. W. (2022). Impact of overweight and obesity on the risk of hepatocellular carcinoma: a prospective cohort study in 14.3 million Koreans. *British journal of cancer*, *127*(1), 109–115. https://doi.org/10. 103 8/s41416-022-01771-0

Kanda, T., Sasaki-Tanaka, R., & Terai, S. (2024). Inflammation of the liver, HCC development and HCC establishment. *Hepatology international*, 10.1007/s12072-024-10707-0. Advance online publication. https://doi.org/10.1007/s12072-024-10707-0

Khan, M. R., & Begum, S. (2021). Mandatory multidisciplinary management of hepatocellular carcinoma: Need of the hour in a country with high prevalence of hepatitis C virus infection. *JPMA. The Journal of the Pakistan Medical Association*, *71*(5), 1455–1457. https://doi.org/10.47391/JPMA.705

Kim, D. J., Cho, E. J., Yu, K. S., Jang, I. J., Yoon, J. H., Park, T., & Cho, J. Y. (2019). Comprehensive Metabolomic Search for Biomarkers to Differentiate Early Stage Hepatocellular Carcinoma from Cirrhosis. *Cancers*, *11*(10), 1497. https://doi.org/10.3390/cancers11101497

Kim, D. Y., Toan, B. N., Tan, C. K., Hasan, I., Setiawan, L., Yu, M. L., Izumi, N., Huyen, N. N., Chow, P. K., Mohamed, R., Chan, S. L., Tanwandee, T., Lee, T. Y., Hai, T. T. N., Yang, T., Lee, W. C., & Chan, H. L. Y. (2023). Utility of combining PIVKA-II and AFP in the surveillance and monitoring of hepatocellular carcinoma in the Asia-Pacific region. *Clinical and molecular hepatology*, *29*(2), 277–292. https://doi.org/10.3350/cmh.2022.0212

Kim, E., & Viatour, P. (2020). Hepatocellular carcinoma: old friends and new tricks. *Experimental & molecular medicine*, *52*(12), 1898–1907. https://doi.org/10.1038/s12276-020-00527-1

Kim, M. J., Kang, W. H., Hwang, S., Ahn, C. S., Moon, D. B., Ha, T. Y., Song, G. W., Jung, D. H., & Park, G. C. (2022). Expression Patterns of Tumor Markers in Liver Transplant Recipients Showing Complete Pathological Response of Hepatocellular Carcinoma. *Journal of clinical medicine*, *11*(19), 5897. https://doi.org/10.3390/jcm11195897

Kim, M., Jun, B. G., Shin, H. S., & Yi, S. W. (2023). The impact of high body mass index on hepatocellular carcinoma in viral hepatitis and cirrhosis Lai, Q., & Vitale, A. (2021). BCLC staging system and liver transplantation: From a stage to a therapeutic hierarchy. *Hepatobiliary & pancreatic diseases international : HBPD INT*, *20*(1), 4–5. https://doi.org/10.1016/j.hbpd.2020.12.014

Krishnan, M. S., Rajan Kd, A., Park, J., Arjunan, V., Garcia Marques, F. J., Bermudez, A., Girvan, O. A., Hoang, N. S., Yin, J., Nguyen, M. H., Kothary, N., Pitteri, S., Felsher, D. W., & Dhanasekaran, R. (2021). Genomic Analysis of Vascular Invasion in HCC Reveals Molecular Drivers and Predictive Biomarkers.

Hepatology (Baltimore, Md.), *73*(6), 2342–2360. https://doi.org/10.1002/hep.31614

Le, P. H., Kuo, C. J., Hsieh, Y. C., Chen, T. H., Lin, C. L., Yeh, C. T., & Liang, K. H. (2019). Ages of hepatocellular carcinoma occurrence and life expectancy are associated with a UGT2B28 genomic variation. *BMC cancer*, *19*(1), 1190. https://doi.org/10.1186/s12885-019-6409-3

Lee, C. W., Tsai, H. I., Lee, W. C., Huang, S. W., Lin, C. Y., Hsieh, Y. C., Kuo, T., Chen, C. W., & Yu, M. C. (2019). Normal Alpha-Fetoprotein Hepatocellular Carcinoma: Are They Really Normal?. *Journal of clinical medicine*, *8*(10), 1736. https://doi.org/10.3390/jcm8101736

Lei, Y., Lu, X., Duan, X., Tang, W. & Wang, Q. (2023). Bilirubin is a superior biomarker for hepatocellular carcinoma diagnosis and for differential diagnosis of benign liver disease. *Journal of Laboratory Medicine*, *47*(5), 233-241. https://doi.org/10.1515/labmed-2023-0023

Li, H., Liu, Z., & Han, C. (2020). Role of AFP, AFP-L3, PIVKA-II, and CA199 in diagnosis and prognosis evaluation of liver cancer. *Int J Clin Exp Med*, *13*(12), 9778-9785.

Liao, C. Y., Lee, C. Y., Wei, C. Y., Chao, Y., Huang, Y. H., Hou, M. C., Su, Y. H., Wu, J. C., & Su, C. W. (2022). Differential prognoses among male and female patients with hepatocellular carcinoma. *Journal of the Chinese Medical Association : JCMA*, *85*(5), 554–565. https://doi.org/10.1097/JCMA.0000000000000722

Liu, X. N., Cui, D. N., Li, Y. F., Liu, Y. H., Liu, G., & Liu, L. (2019). Multiple "Omics" data-based biomarker screening for hepatocellular carcinoma diagnosis. *World journal of gastroenterology*, *25*(30), 4199–4212. https://doi.org/10.3748/wjg.v25.i30.4199

Loglio, A., Iavarone, M., Facchetti, F., Di Paolo, D., Perbellini, R., Lunghi, G., Ceriotti, F., Galli, C., Sandri, M. T., Viganò, M., Sangiovanni, A., Colombo, M., & Lampertico, P. (2020). The combination of PIVKA-II and AFP improves the detection accuracy for HCC in HBV caucasian cirrhotics on long-term oral therapy. *Liver international: official journal of the International Association for the Study of the Liver*, *40*(8), 1987–1996. https://doi.org/10.1111/liv.14475

Luo, C. L., Rong, Y., Chen, H., Zhang, W. W., Wu, L., Wei, D., Wei, X. Q., Mei, L. J., & Wang, F. B. (2019). A Logistic Regression Model for Noninvasive Prediction of AFP-Negative Hepatocellular Carcinoma. *Technology in cancer research & treatment*, *18*, 1533033819846632. https://doi.org/10.1177/1533033819846632

Maniscalco, L., Varello, K., Morello, E., Montemurro, V., Olimpo, M., Giacobino, D., Abbamonte, G., Gola, C., Iussich, S., & Bozzetta, E. (2022). Investigating a Prognostic Factor for Canine Hepatocellular Carcinoma: Analysis of Different Histological Grading Systems and the Role of PIVKA-II. *Veterinary sciences*, *9*(12), 689. https://doi.org/10.3390/vetsci9120689

McGlynn, K. A., Petrick, J. L., & El- Serag, H. B. (2021). Epidemiology of hepatocellular carcinoma. *Hepatology*, *73*, 4-13.

Montal, R., Andreu-Oller, C., Bassaganyas, L., Esteban-Fabró, R., Moran, S., Montironi, C., Moeini, A., Pinyol, R., Peix, J., Cabellos, L., Villanueva, A., Sia, D., Mazzaferro, V., Esteller, M., & Llovet, J. M. (2019). Molecular portrait of high alpha-fetoprotein in hepatocellular carcinoma: implications for biomarker-driven clinical trials. *British journal of cancer*, *121*(4), 340–343. https://doi.org/10.1038/s41416-019-0513-7

Müller, M., Bird, T. G., & Nault, J. C. (2020). The landscape of gene mutations in cirrhosis and hepatocellular carcinoma. *Journal of hepatology*, *72*(5), 990–1002. https://doi.org/10.1016/j.jhep.2020.01.019

Mungamuri, S. K., & Mavuduru, V. A. (2020). Role of epigenetic alterations in aflatoxin-induced hepatocellular carcinoma. *Liver Cancer International*, *1*(2), 41-50.

Munson, P. V., Adamik, J., & Butterfield, L. H. (2022). Immunomodulatory impact of α-fetoprotein. *Trends in immunology*, *43*(6), 438–448. https://doi.org/10.1016/j.it.2022.04.001

Muscari, F., & Maulat, C. (2020). Preoperative alpha-fetoprotein (AFP) in hepatocellular carcinoma (HCC): is this 50-year biomarker still up-to-date?. *Translational gastroenterology and hepatology*, *5*, 46. https://doi.org/10.21037/tgh.2019.12.09

Nahon, P., Allaire, M., Nault, J. C., & Paradis, V. (2020). Characterizing the mechanism behind the progression of NAFLD to hepatocellular carcinoma. *Hepatic oncology*, *7*(4), HEP36. https://doi.org/10.2217/hep-2020-0017

Nishida N. (2020). Long-term prognosis and management of hepatocellular carcinoma after curative treatment. *Clinical and molecular hepatology*, *26*(4), 480–483. https://doi.org/10.3350/cmh.2020.0208

Osho, A., Rich, N. E., & Singal, A. G. (2020). Role of imaging in management of hepatocellular carcinoma: surveillance, diagnosis, and treatment response. *Hepatoma research*, *6*, 55. https://doi.org/10.20517/2394-5079.2020.42

Parikh, N. D., Mehta, A. S., Singal, A. G., Block, T., Marrero, J. A., & Lok, A. S. (2020). Biomarkers for the Early Detection of Hepatocellular Carcinoma. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, *29*(12), 2495–2503. https://doi.org/10.1158/1055-9965.EPI-20-0005

Parkash, A., Singla, P., & Ahirwar, A. K. (2021). Assessment of PIVKA II as tumour marker in hepatocellular carcinoma. *Int J Cancer Clin Res*, *8*, 151.

Parkash, O., & Hamid, S. (2016). Next big threat for Pakistan Hepatocellular Carcinoma (HCC). *JPMA. The Journal of the Pakistan Medical Association*, *66*(6), 735–739.

Piñero, F., Dirchwolf, M., & Pessôa, M. G. (2020). Biomarkers in Hepatocellular Carcinoma: Diagnosis, Prognosis and Treatment Response Assessment. *Cells*, *9*(6), 1370. https://doi.org/10.3390/cells9061370

Qian, X. J., Wen, Z. M., Huang, X. M., Feng, H. J., Lin, S. S., Liu, Y. N., Li, S. C., Zhang, Y., Peng, W. G., Yang, J. R., Zheng, Z. Y., Zhang, L., Zhang, D. W., Lu, F. M., Liu, L. J., & Pan, W. D. (2023). Better performance of PIVKA-II for detecting hepatocellular carcinoma in patients with chronic liver disease with normal total bilirubin. *World journal of gastroenterology*, *29*(8), 1359–1373. https://doi.org/10.3748/wjg.v29.i8.1359

Rao, A., Rich, N. E., Marrero, J. A., Yopp, A. C., & Singal, A. G. (2021). Diagnostic and Therapeutic Delays in Patients with Hepatocellular Carcinoma. *Journal of the National Comprehensive Cancer Network: JNCCN*, *19*(9), 1063–1071. https://doi.org/10.6004/jnccn.2020.7689

Reig, M., Forner, A., Rimola, J., Ferrer-Fàbrega, J., Burrel, M., Garcia-Criado, Á., Kelley, R. K., Galle, P. R., Mazzaferro, V., Salem, R., Sangro, B., Singal, A. G., Vogel, A., Fuster, J., Ayuso, C., & Bruix, J. (2022). BCLC strategy for prognosis prediction and treatment recommendation: The 2022 update. *Journal of hepatology*, *76*(3), 681–693. https://doi.org/10.1016/j.jhep.2021.11.018

Renne, S. L., Sarcognato, S., Sacchi, D., Guido, M., Roncalli, M., Terracciano, L., & Di Tommaso, L. (2021). Hepatocellular carcinoma: a clinical and pathological overview. *Pathologica*, *113*(3), 203–217. https://doi.org/10.32074/1591-951X-295

Rich, N. E., Murphy, C. C., Yopp, A. C., Tiro, J., Marrero, J. A., & Singal, A. G. (2020). Sex disparities in presentation and prognosis of 1110 patients with hepatocellular carcinoma. *Alimentary pharmacology & therapeutics*, *52*(4), 701–709. https://doi.org/10.1111/apt.15917

Rizzo, G. E. M., Cabibbo, G., & Craxì, A. (2022). Hepatitis B Virus-Associated Hepatocellular Carcinoma. *Viruses*, *14*(5), 986. https://doi.org/10.3390/v14050986

Sagar, V. M., Herring, K., Curbishley, S., Hodson, J., Fletcher, P., Karkhanis, S., Mehrzad, H., Punia, P., Shah, T., Shetty, S., & Ma, Y. T. (2021). The potential of PIVKA-II as a treatment response biomarker in hepatocellular carcinoma: a prospective United Kingdom cohort study. *Oncotarget*, *12*(24), 2338–2350. https://doi.org/10.18632/oncotarget.28136

Saillard, C., Schmauch, B., Laifa, O., Moarii, M., Toldo, S., Zaslavskiy, M., Pronier, E., Laurent, A., Amaddeo, G., Regnault, H., Sommacale, D., Ziol, M., Pawlotsky, J. M., Mulé, S., Luciani, A., Wainrib, G., Clozel, T., Courtiol, P., & Calderaro, J. (2020). Predicting Survival after Hepatocellular Carcinoma Resection Using Deep Learning on Histological Slides. *Hepatology (Baltimore, Md.)*, *72*(6), 2000–2013. https://doi.org/10.1002/hep.31207

Samant, H., Amiri, H. S., & Zibari, G. B. (2021). Addressing the worldwide hepatocellular carcinoma: epidemiology, prevention and management. *Journal of gastrointestinal oncology*, *12*(Suppl 2), S361–S373. https://doi.org/10.21037/jgo.2020.02.08

Sarin, S. K., Kumar, M., Eslam, M., George, J., Al Mahtab, M., Akbar, S. M. F., Jia, J., Tian, Q., Aggarwal, R., Muljono, D. H., Omata, M., Ooka, Y., Han, K. H., Lee, H. W., Jafri, W., Butt, A. S., Chong, C. H., Lim, S. G., Pwu, R. F., & Chen, D. S. (2020). Liver diseases in the Asia-Pacific region: a Lancet Gastroenterology & Hepatology Commission. *The lancet. Gastroenterology & hepatology*, *5*(2), 167–228. https://doi.org/10.1016/S2468-1253(19)30342-5

Sato, M., Morimoto, K., Kajihara, S., Tateishi, R., Shiina, S., Koike, K., & Yatomi, Y. (2019). Machine-learning Approach for the Development of a Novel Predictive Model for the Diagnosis of Hepatocellular Carcinoma. *Scientific reports*, *9*(1), 7704. https://doi.org/10.1038/s41598-019-44022-8

Shi, Y., Yang, H., Bai, X., Liu, X., Li, Q., & Du, W. (2024). Female and diabetes are risk factors for alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II negative in hepatocellular carcinoma. *Medicine*, *103*(42), e40100. https://doi.org/10.1097/MD.0000000000040100

Shen, Y., Risch, H., Lu, L., Ma, X., Irwin, M. L., Lim, J. K., Taddei, T., Pawlish, K., Stroup, A., Brown, R., Wang, Z., Jia, W., Wong, L., Mayne, S. T., & Yu, H. (2020). Risk factors for hepatocellular carcinoma (HCC) in the northeast of the United States: results of a case-control study. *Cancer causes & control : CCC*, *31*(4), 321–332. https://doi.org/10.1007/s10552-020-01277-1

Shiels, M. S., Engels, E. A., Yanik, E. L., McGlynn, K. A., Pfeiffer, R. M., & O'Brien, T. R. (2019). Incidence of hepatocellular carcinoma among older Americans attributable to hepatitis C and hepatitis B: 2001 through 2013. *Cancer*, *125*(15), 2621–2630. https://doi.org/10.1002/cncr.32129

Shiha, G., Mousa, N., Soliman, R., Nnh Mikhail, N., Adel Elbasiony, M., & Khattab, M. (2020). Incidence of HCC in chronic hepatitis C patients with advanced hepatic fibrosis who achieved SVR following DAAs: A prospective study. *Journal of viral hepatitis*, *27*(7), 671–679. https://doi.org/10.1111/jvh.13276

Singal, A. G., Lampertico, P., & Nahon, P. (2020). Epidemiology and surveillance for hepatocellular carcinoma: New trends. *Journal of hepatology*, *72*(2), 250-261.

Singal, A. G., Llovet, J. M., Yarchoan, M., Mehta, N., Heimbach, J. K., Dawson, L. A., Jou, J. H., Kulik, L. M., Agopian, V. G., Marrero, J. A., Mendiratta-Lala, M., Brown, D. B., Rilling, W. S., Goyal, L., Wei, A. C., & Taddei, T. H. (2023). AASLD Practice Guidance on prevention, diagnosis, and treatment of hepatocellular carcinoma. *Hepatology (Baltimore, Md.)*, *78*(6), 1922–1965. https://doi.org/10.1097/HEP.0000000000000466

Singal, A. G., Tayob, N., Mehta, A., Marrero, J. A., El-Serag, H., Jin, Q., Saenz de Viteri, C., Fobar, A., & Parikh, N. D. (2022). GALAD demonstrates high sensitivity for HCC surveillance in a cohort of patients with cirrhosis. *Hepatology (Baltimore, Md.)*, *75*(3), 541–549. https://doi.org/10.1002/hep.32185

Song, M., He, J., Pan, Q. Z., Yang, J., Zhao, J., Zhang, Y. J., Huang, Y., Tang, Y., Wang, Q., He, J., Gu, J., Li, Y., Chen, S., Zeng, J., Zhou, Z. Q., Yang, C., Han, Y., Chen,

H., Xiang, T., Weng, D. S., … Xia, J. C. (2021). Cancer-Associated Fibroblast-Mediated Cellular Crosstalk Supports Hepatocellular Carcinoma Progression. *Hepatology (Baltimore, Md.)*, *73*(5), 1717–1735. https://doi.org/10.1002/hep.31792

Strazzabosco, M., Cabibbo, G., & Colombo, M. (2022). Adjusting Barcelona Clinic Liver Cancer Staging System to the Evolving Landscape of Hepatocellular Carcinoma: A Look to the Future. *Gastroenterology*, *162*(7), 2106–2108. https://doi.org/10.1053/j.gastro.2022.01.035

Su, K., Huang, W., Li, X., Xu, K., Gu, T., Liu, Y., Song, J., Qian, K., Xu, Y., Zeng, H., Yang, Y., Guo, L., & Han, Y. (2023). Evaluation of Lactate Dehydrogenase and Alkaline Phosphatase as Predictive Biomarkers in the Prognosis of Hepatocellular Carcinoma and Development of a New Nomogram. *Journal of hepatocellular carcinoma*, *10*, 69–79. https://doi.org/10.2147/JHC.S398632

Su, T. H., Peng, C. Y., Chang, S. H., Tseng, T. C., Liu, C. J., Chen, C. L., Liu, C. H., Yang, H. C., Chen, P. J., & Kao, J. H. (2022). Serum PIVKA-II and alpha-fetoprotein at virological remission predicts hepatocellular carcinoma in chronic hepatitis B related cirrhosis. *Journal of the Formosan Medical Association = Taiwan yi zhi*, *121*(3), 703–711. https://doi.org/10.1016/j.jfma.2021.08.003

Suresh, D., Srinivas, A. N., & Kumar, D. P. (2020). Etiology of Hepatocellular Carcinoma: Special Focus on Fatty Liver Disease. *Frontiers in oncology*, *10*, 601710. https://doi.org/10.3389/fonc.2020.601710

Suttichaimongkol, T., Mitpracha, M., Tangvoraphonkchai, K., Sadee, P., Sawanyawisuth, K., & Sukeepaisarnjaroen, W. (2023). PIVKA-II or AFP has better diagnostic properties for hepatocellular carcinoma diagnosis in high-risk patients. *Journal of Circulating Biomarkers*, *12*, 12.

Svobodova, S., Karlikova, M., Topolcan, O., Pecen, L., Pestova, M., Kott, O., Treska, V., Slouka, D., & Kucera, R. (2018). PIVKA-II as a Potential New Biomarker for

Hepatocellular Carcinoma - A Pilot Study. *In vivo (Athens, Greece)*, *32*(6), 1551–1554. https://doi.org/10.21873/invivo.11413

Tang, Y., Zhang, Y., & Hu, X. (2020). Identification of Potential Hub Genes Related to Diagnosis and Prognosis of Hepatitis B Virus-Related Hepatocellular Carcinoma via Integrated Bioinformatics Analysis. *BioMed research international*, *2020*, 4251761. https://doi.org/10.1155/2020/4251761

Taniai M. (2020). Alcohol and hepatocarcinogenesis. *Clinical and molecular hepatology*, *26*(4), 736–741. https://doi.org/10.3350/cmh.2020.0203

Tateishi, R., & Koike, K. (2020). Changing etiology of hepatocellular carcinoma. *Journal of gastroenterology*, *55*(1), 125–126. https://doi.org/10.1007/s00535-019-01622-5

Tayob, N., Kanwal, F., Alsarraj, A., Hernaez, R., & El-Serag, H. B. (2023). The Performance of AFP, AFP-3, DCP as Biomarkers for Detection of Hepatocellular Carcinoma (HCC): A Phase 3 Biomarker Study in the United States. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*, *21*(2), 415–423.e4. https://doi.org/10.1016/j.cgh.2022.01.047

Tian, S., Chen, Y., Zhang, Y., & Xu, X. (2023). Clinical value of serum AFP and PIVKA-II for diagnosis, treatment and prognosis of hepatocellular carcinoma. *Journal of clinical laboratory analysis*, *37*(1), e24823. https://doi.org/10.1002/jcla.24823

Tran, V. T., Phan, T. T., Nguyen, T. B., Le, T. T., Tran, T. T., Nguyen, A. T., Nguyen, H. T., Nguyen, N. B., Ho, T. T., Pho, S. P., Nguyen, T. T., Nguyen, H. T., Mai, H. T., Pham, B. T., Nguyen, K. D., Le, B. T., Nguyen, T. T., & Nguyen, S. T. (2023). The diagnostic performance of AFP and PIVKA-II models for non-B non-C hepatocellular carcinoma. *BMC research notes*, *16*(1), 317. https://doi.org/10.1186/s13104-023-06600-y

Tsilimigras, D. I., Bagante, F., Sahara, K., Moris, D., Hyer, J. M., Wu, L., Ratti, F., Marques, H. P., Soubrane, O., Paredes, A. Z., Lam, V., Poultsides, G. A., Popescu, I., Alexandrescu, S., Martel, G., Workneh, A., Guglielmi, A., Hugh, T., Aldrighetti, L., Endo, I., Pawlik, T. M. (2019). Prognosis after Resection of Barcelona Clinic Liver Cancer (BCLC) Stage 0, A, and B Hepatocellular Carcinoma: A Comprehensive Assessment of the Current BCLC Classification. *Annals of surgical oncology*, *26*(11), 3693–3700. https://doi.org/10.1245/s10434-019-07580-9

Tümen, D., Heumann, P., Gülow, K., Demirci, C. N., Cosma, L. S., Müller, M., & Kandulski, A. (2022). Pathogenesis and Current Treatment Strategies of Hepatocellular Carcinoma. *Biomedicines*, *10*(12), 3202. https://doi.org/10.3390/biomedicines10123202

Ul Abideen, Z., Siddique, S., Nasrullah, I., Khan, J. S., Rehman, S., Tong, Y., Qamar, R., & Butt, A. M. (2019). A 2-year retrospective study of viral and host-associated risk factors in Pakistani hepatocellular carcinoma patients. *European journal of gastroenterology & hepatology*, *31*(9), 1103–1109. https://doi.org/10.1097/MEG.0000000000001384

Van der Pol, C. B., McInnes, M. D. F., Salameh, J. P., Chernyak, V., Tang, A., Bashir, M. R., LI-RADS IPD Group, & LI-RADS IPD Group Collaborators (2022). Impact of Reference Standard on CT, MRI, and Contrast-enhanced US LI-RADS Diagnosis of Hepatocellular Carcinoma: A Meta-Analysis. *Radiology*, *303*(3), 544–545. https://doi.org/10.1148/radiol.212340

Villalba-López, F., Sáenz-Mateos, L. F., Sánchez-Lorencio, M. I., De La Orden-García, V., Alconchel-Gago, F., Cascales-Campos, P. A., García-Bernardo, C., Noguera-Velasco, J. A., Baroja-Mazo, A., & Ramírez-Romero, P. (2023). Usefulness of PIVKA-II for monitoring after liver transplantation in patients with hepatocellular carcinoma. *Scientific reports*, *13*(1), 5621. https://doi.org/10.1038/s41598-023-32879-9

Vipani, A., Lauzon, M., Luu, M., Roberts, L. R., Singal, A. G., & Yang, J. D. (2022). Decreasing Trend of Serum α-Fetoprotein Level in Hepatocellular Carcinoma. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*, *20*(5), 1177–1179.e4. https://doi.org/10.1016/j.cgh.2021.08.011

Wang, F., Gao, S., Wu, M., Zhao, D., Sun, H., Yav, S., Chen, Y., Zhang, Z., Yang, M., Dong, Y., Wang, J., Wang, X., Yan, Z., & Liu, L. (2023). The prognostic role of the AST/ALT ratio in hepatocellular carcinoma patients receiving thermal ablation combined with simultaneous TACE. *BMC gastroenterology*, *23*(1), 80. https://doi.org/10.1186/s12876-023-02719-1

Wang, T., & Zhang, K. H. (2020). New Blood Biomarkers for the Diagnosis of AFP-Negative Hepatocellular Carcinoma. *Frontiers in oncology*, *10*, 1316. https://doi.org/10.3389/fonc.2020.01316

Wang, W., & Wei, C. (2020). Advances in the early diagnosis of hepatocellular carcinoma. *Genes & diseases*, *7*(3), 308–319. https://doi.org/10.1016/j.gendis.2020.01.014

Wee, I. J. Y., Moe, F. N. N., Sultana, R., Ang, R. W. T., Quek, P. P. S., Goh, B. K. P., Chan, C. Y., Cheow, P. C., Chung, A. Y. F., Jeyaraj, P. R., Koh, Y. X., Mack, P. O. P., Ooi, L. L. P. J., Tan, E. K., Teo, J. Y., Kam, J. H., Chua, J. S. S., Ng, A. W. Y., Goh, J. S. Q., & Chow, P. K. H. (2022). Extending Surgical Resection for Hepatocellular Carcinoma Beyond Barcelona Clinic for Liver Cancer (BCLC) Stage A: A Novel Application of the Modified BCLC Staging System. *Journal of hepatocellular carcinoma*, *9*, 839–851. https://doi.org/10.2147/JHC.S370212

Wong, L. L., Lee, L. Y., Karasaki, K., Ogihara, M., & Tran, C. (2022). Management of hepatocellular carcinoma in patients who are 70 years or older. *Surgery open science*, *10*, 53–58. https://doi.org/10.1016/j.sopen.2022.07.003

Wu, G., Wu, J., Pan, X., Liu, B., Yao, Z., Guo, Y., & Ding, Y. (2019). Racial disparities in alpha- fetoprotein testing and alpha- fetoprotein status associated with the diagnosis and outcome of hepatocellular carcinoma patients. *Cancer medicine*, *8*(15), 6614-6623.

Xia, F., Ndhlovu, E., Liu, Z., Chen, X., Zhang, B., & Zhu, P. (2022). Alpha-Fetoprotein+Alkaline Phosphatase (A-A) Score Can Predict the Prognosis of Patients with Ruptured Hepatocellular Carcinoma Underwent Hepatectomy. *Disease markers*, *2022*, 9934189. https://doi.org/10.1155/2022/ 9934189

Xu, F., Zhang, L., He, W., Song, D., Ji, X., & Shao, J. (2021). The Diagnostic Value of Serum PIVKA-II Alone or in Combination with AFP in Chinese Hepatocellular Carcinoma Patients. *Disease markers*, *2021*, 8868370. https://doi.org/10.1155/2021/8868370

Xu, J., An, S., Lu, Y., Li, L., Wu, Z. Q., & Xu, H. G. (2023). Preoperative alpha fetoprotein, total bilirubin, fibrinogen, albumin, and lymphocytes predict postoperative survival in hepatocellular carcinoma. *Cancer medicine*, *12*(12), 13319–13328. https://doi.org/10.1002/cam4.6030

Xu, X. S., Wan, Y., Song, S. D., Chen, W., Miao, R. C., Zhou, Y. Y., Zhang, L. Q., Qu, K., Liu, S. N., Zhang, Y. L., Dong, Y. F., & Liu, C. (2014). Model based on γ-glutamyltransferase and alkaline phosphatase for hepatocellular carcinoma prognosis. *World journal of gastroenterology*, *20*(31), 10944–10952. https://doi.org/10.3748/wjg.v20.i31.10944

Xue, X., Zhao, Y., Wang, X., Qin, L., & Hu, R. (2019). Development and validation of serum exosomal microRNAs as diagnostic and prognostic biomarkers for hepatocellular carcinoma. *Journal of cellular biochemistry*, *120*(1), 135–142. https://doi.org/10.1002/jcb.27165

Yang, J. D., Hainaut, P., Gores, G. J., Amadou, A., Plymoth, A., & Roberts, L. R. (2019). A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nature reviews. Gastroenterology & hepatology*, *16*(10), 589–604. https://doi.org/10.1038/s41575-019-0186-y

Yang, J. D., Hainaut, P., Gores, G. J., Amadou, A., Plymoth, A., & Roberts, L. R. (2019). A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nature reviews. Gastroenterology & hepatology*, *16*(10), 589–604. https://doi.org/10.1038/s41575-019-0186-y

Yang, Y., Li, G., Lu, Z., Liu, Y., Kong, J., & Liu, J. (2021). Progression of prothrombin induced by vitamin K absence-II in hepatocellular carcinoma. *Frontiers in Oncology*, *11*, 726213.

Yeh, S. H., Li, C. L., Lin, Y. Y., Ho, M. C., Wang, Y. C., Tseng, S. T., & Chen, P. J. (2023). Hepatitis B virus DNA integration drives carcinogenesis and provides a new biomarker for HBV-related HCC. *Cellular and Molecular Gastroenterology and Hepatology*, *15*(4), 921-929.

Yin, L., He, N., Chen, C., Zhang, N., Lin, Y., & Xia, Q. (2019). Identification of novel blood-based HCC-specific diagnostic biomarkers for human hepatocellular carcinoma. *Artificial cells, nanomedicine, and biotechnology*, *47*(1), 1908–1916. https://doi.org/10.1080/21691401.2019.1613421

Yip, T. C., Wong, G. L., Chan, H. L., Tse, Y. K., Liang, L. Y., Hui, V. W., Lee, H. W., Lui, G. C., Kong, A. P., & Wong, V. W. (2020). Elevated testosterone increases risk of hepatocellular carcinoma in men with chronic hepatitis B and diabetes mellitus. *Journal of gastroenterology and hepatology*, *35*(12), 2210–2219. https://doi.org/10.1111/jgh.15079

Yoo, J., Lee, M. W., Lee, D. H., Lee, J. H., & Han, J. K. (2020). Evaluation of a serum tumour marker-based recurrence prediction model after radiofrequency ablation for hepatocellular carcinoma. *Liver international : official journal of the International*

Association for the Study of the Liver, *40*(5), 1189–1200. https://doi.org/10.1111/liv.14406

Yu, M. C., Huang, C. W., Hsu, H. Y., Chong, S. W., & Huang, S. F. (2022). The Role of Alkaline Phosphatase in Hepatocellular Carcinoma: A Special View from Liver Regeneration. *HPB*, *24*, S183.

Zhang, C. H., Cheng, Y., Zhang, S., Fan, J., & Gao, Q. (2022). Changing epidemiology of hepatocellular carcinoma in Asia. *Liver International*, *42*(9), 2029-2041.

Zhang, L. X., Lv, Y., Xu, A. M., & Wang, H. Z. (2019). The prognostic significance of serum gamma-glutamyltransferase levels and AST/ALT in primary hepatic carcinoma. *BMC cancer*, *19*(1), 841. https://doi.org/10.1186/s12885-019-6011-8

Zhao, P., Malik, S., & Xing, S. (2021). Epigenetic Mechanisms Involved in HCV-Induced Hepatocellular Carcinoma (HCC). *Frontiers in oncology*, *11*, 677926. https://doi.org/10.3389/fonc.2021.677926

Zhao, Z., Zhu, Y., Ni, X., Lin, J., Li, H., Zheng, L., Zhang, C., Qi, X., Huo, H., Lou, X., Fan, Q., Bao, Y., & Luo, M. (2021). Serum GGT/ALT ratio predicts vascular invasion in HBV-related HCC. *Cancer cell international*, *21*(1), 517. https://doi.org/10.1186/s12935-021-02214-1

Zhou, J., Sun, H., Wang, Z., Cong, W., Wang, J., Zeng, M., Zhou, W., Bie, P., Liu, L., Wen, T., Han, G., Wang, M., Liu, R., Lu, L., Ren, Z., Chen, M., Zeng, Z., Liang, P., Liang, C., Chen, M., Fan, J. (2020). Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma (2019 Edition). *Liver cancer*, *9*(6), 682–720. https://doi.org/10.1159/000509424

Zhu, Q., Ma, Y., Liang, J., Wei, Z., Li, M., Zhang, Y., Liu, M., He, H., Qu, C., Cai, J., Wang, X., Zeng, Y., & Jiao, Y. (2021). AHR mediates the aflatoxin B1 toxicity associated with hepatocellular carcinoma. *Signal transduction and targeted therapy*, *6*(1), 299. https://doi.org/10.1038/s41392-021-00713-1

A

Bahria University
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BUHS-IRB # R-039/24
Date:03- 01-24
Name of PI: Dr. Muhammad Saeed
Affiliation & Department: MPhil Candidate: Physiology
Address-BUHS,C Sailor Street, DHA Phase 2, Karachi

Subject: **APPROVAL OF YOUR RESEARCH PROPOSAL:**

Title of the research
"ASSOCIATION OF PIVKA II WITH HEPATOCELLULAR CARCINOMA"

Dear Dr. Muhammad Saeed,

I am writing this letter at your request to confirm that we support the research project "ASSOCIATION OF PIVKA II WITH HEPATOCELLULAR CARCINOMA." I know the research compares the diagnostic accuracy of PIVKA-II (Protein Induced by Vitamin K Absence or Antagonist-II) and alpha-fetoprotein in hepatocellular carcinoma (HCC). The IRB will support the project under the proposed guidelines in the IRB application for six ***months***.

Suppose any unanticipated problems or adverse advents occur. In that case, it is up to Dr. Muhammad Saeed to report these events to the IRB as promptly as possible. The research will contribute to providing a more sensitive diagnostic tool for the early detection of hepatocellular carcinoma. We will be happy tosupport this endeavor.

Sincerely,

Prof Dr. Inayat H. Thaver
FCPS (Community Medicine); PhD (Public Healt
Chairperson, Institutional Review Board (IRB)
Bahria University Health Sciences Campus
Karachi

Prof. Dr. Inayat H. Thaver
Chair, Institutional Review Board.

B

FACULTY RESEARCH COMMITTEE
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LETTER OF APPROVAL

Date: 02-01-2024

To,
Dr. Muhammad Saeed
MPhil – Student
Department of Physiology
BUHSCK

Subject: **Faculty Research Committee**
FRC-BUHSCK Approval of Research Study

Title of Study: Association of PIVKA II with hepatocellular carcinoma.

Name of Student: **Dr. Muhammad Saeed**
Reference No: **FRC-BUHS 07/2024**

Dear Dr. Muhammad Saeed

Thank you for submitting research proposal to FRC-BUHSCK. The committee has approved your project.
Regards

PROF. DR. Shehla M. Baqai HI (M)
Maj. Gen (R)
Chairperson FRC-BUHSCK

Cc:
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C

WRITTEN INFORMED CONSENT FORM OF PATIENT

You are giving your consent to participate voluntarily and at your own will in this research clinical trial project that aims for detection of hepatocellular carcinoma.

You are willing to give your blood sample (3-5ml) during which you might experience some pain. In this procedure we follow the protocols to avoid spread of any kind of infection.

You have been explained in detail the nature and significance of participating in the project and you understand the provided explanation.

You have been told that findings of your disease and your data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.

You also agree to give all relevant information needed and to the best of your knowledge to the researcher. It is clarified to you that no incentive will be provided to you for participating in the study except the cost of lab investigations, whereas you do have the right to withdraw from the study at any time.

You are advised to contact **Dr. Muhammad Saeed** on mobile number: **0322-2212903** or visit Jinnah postgraduate medical center Hospital, Karachi in case of any query/ emergency related to disease.

I am voluntarily taking part in this study.

Name of Patient: _____

S/D/ W/o _____

Signature / Thumb impression of patient: _____

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

مریض کے لئے اجازت فارم

آپ رضاکارانہ طور پر اور اپنی مرضی سے اس تحقیق میں حصہ لے رہا ہیں جس کا مقصد جگر کے کینسر کی بیماری کا علاج کرنا ہے.

آپ اس تحقیق میں اپنے خون کا نمونا (۵-۳ ملی لیٹر) دیں گے جس سے آپ کو تھوڑے درد کا احساس ہو سکتا ہے۔ یہ عمل میں ہم مکمل جراثیم کش طریقہ کار کا استعمال کریں گے تاکہ کسی قسم کے جراثیم کے پھیلنے کا خطرہ نہ ہو۔

آپ کو اس تحقیقاتی عمل میں حصہ لینے کے نوعیت اور اہمیت کے بارے میں تفصیل سے وضاحت کی گئی ہے اور آپ فراہم کردہ وضاحت کو سمجھتے ہیں.

آپ کو بتایا گیا ہے کہ آپ کی بیماری اور آپ کے اعداد و شمار کو سختی سے خفیہ رکھا جائے گا اور صرف کمیونٹی کے فائدے، اشاعتوں اور آرٹیکل اشاعت کے لئے استعمال کیا جائے گا۔

آپ پوری طرح سے اور بہتر علم کے ساتھ محقق کو تمام متعلقہ معلومات دینے پر اتفاق کرتے ہیں. یہ آپ کو واضح کیا گیا ہے کہ لیبارٹری کی تحقیقات کے علاوہ مطالعہ میں حصہ لینے کے لئے آپ کو کوئی ادائیگی نہیں کی جائے گی، جبکہ آپ کو کسی بھی وقت تحقیقاتی عمل کو چھوڑنے کا حق حاصل ہے.

کسی بھی ہنگامی صورت حال کے معاملے میں اور آپ کی بیماری سے متعلق کسی بھی سوال کیلئے آپ کو مشورہ دیا جاتا ہے کہ **ڈاکٹر محمد سعید** موبائل نمبر: **0322- 2212903** پر رابطہ کریں یا **جناح پوسٹ گریجویٹ میڈیکل سینٹر اسپتال کراچی** سے رابطہ کریں.

میں رضاکارانہ طور پر اور اپنی مرضی سے اس تحقیق میں حصہ لے رہا ہوں

مریض کا نام: _____

والد/شوہر کا نام: _____

مریض کے دستخط/انگوٹھے تاثر: _____

محقق کا نام: _____

محقق کا دستخط: _____

تاریخ: _____

D

SUBJECT EVALUATION FORM

S.NO: _____

Name _____ Father/ Husband Name _____

Age _____Years Gender: Male / Female

Ethnicity _____ Marital status Married / Unmarried

Weight _____ kg Height _____ m BMI _____

Co-Morbid

Hypertension Yes / No Diabetes Yes / No

Duration of HCC _____ Type of HCC _____

Most Common Symptoms _____

Frequency of Loose Stool _____ Severity of Pain _____

Associated Symptoms _____

Any psychological issues _____

Any family member having same illness _____

Currently taking any medicine _____

Other co-existing medical conditions _____

MEDICATION HISTORY: Please list any medications you are currently taking.

MEDICATION	**DOSE**	**REASON**

QUESTIONNAIRE

Name _____ Father/ Husband Name _____

Age _____Years Gender: Male / Female

Weight _____ kg Height _____ m BMI _____

Co-Morbid

Hypertension Yes / No Diabetes Yes / No

1.PAIN

Not at all a little quite a bit very much

2.FATIGUE

Not at all a little quite a bit very much

3.NAUSEA AND VOMITING.

Not at all a little quite a bit very much

4.ABDOMINAL SWELLING

Not at all a little quite a bit very much

5.DIARROHEA

Not at all a little quite a bit very much

6.LOSS OF APPETITE

Not at all a little quite a bit very much

7.WEIGHT LOSS

Not at all a little quite a bit very much

8.JAUNDICE

Not at all a little quite a bit very much

9.HAIR LOSS

Not at all a little quite a bit very much

10.PHYSICAL FUNCTIONING

Not at all a little quite a bit very much

11.ROLE FUNCTIONING

Not at all a little quite a bit very much

12.EMOTIONAL FUNCTIONING

Not at all a little quite a bit very much

13.SOCIAL FUNCTIONING

Not at all a little quite a bit very much

14.COGNITIVE FUNCTIONING

Not at all a little quite a bit very much

15.concern about disease progression

Not at all a little quite a bit very much

16.CONCERN ABOUT THE FUTURE

Not at all a little quite a bit very much

17.DIFFICULTIES RELATED TO FINANCES DUE TO ILNESS

Not at all a little quite a bit very much

E

IRB

NO.F.2-81/2023-GENL/104/JPMC
JINNAH POSTGRADUATE MEDICAL CENTRE
KARACHI.75510.

Dated the 24-07-2023

Dr. Muhammad Saeed
Department of Physiology
Bahria University Health Sciences
Campus Karachi

Subject: ***Association of PIVKA II with Hepatocellular Carcinoma***

With reference to your application / letter dated 22nd June, 2023, on the subject noted above and to say that the Institutional Review Board has approved your subject proposal.

Dr. Nazish Butt, Associate Prof. & Head Department of Gastroenterology, JPMC, Karachi is the co-supervisor of this study.

Prof. Syed Masroor Ahmed
Prof. of Medicine / Dean and
Chairman, Institutional Review Board Committee
JPMC, Karachi.

Copy forwarded for information and necessary action to:

- ***Prof. Dr. Shazia Shakoor, Prof. Department of Physiology, Bahria University health Sciences, Karachi.***
- ***Dr. Nazish Butt, Associate Prof. & Head Department of Gastroenterology, JPMC, Karachi.***

F

PLAGIARISM REPORT

Dr M Saeed thesis.docx

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