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Validation of Protein Biomarker Candidates for Diagnosis of HBV induced HCC

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Abstract

Hepatocellular carcinoma is a major contributor to the global cancer burden. It affects millions of people in Pakistan on a yearly basis. Furthermore, HCC is linked to viral infections Hepatitis B and C, which account for roughly 87 percent of HCC cases in Pakistan. HCC is identified using imaging techniques such as MRI, Ultrasound, and histology, which have radiation hazards and frequently need expensive healthcare systems that are less available in most of the developing countries. Novel HCC biomarkers are being developed as part of a large research project aimed at detecting the disease early. These include the creation of biomarkers based on HCC patients' transcriptome and proteomic profiles. Circulating proteins, which are easily detected in body fluids, including blood serum, may thus provide an opportunity for the development of HCC biomarkers. Blood-based serum biomarkers must be developed for easy, non-invasive, and early detection of HCC. In conjunction with imaging techniques, alpha-fetoprotein (AFP) has been used to detect HCC, although it has little clinical usefulness. Also, the reported AFP negative results make its utility meager. Multiple circulating proteins have been studied as biomarker possibilities for HCC diagnosis in recent years.

In this study, Blood serum was used to validate three novel protein biomarker candidates to detect HBV induced HCC that had previously been predicted using a bioinformatics methodology. Proteins named C6, C8A and C8B were measured in the serum of 22 HCC patients infected with HBV in Pakistani population and compared to AFP levels using quantitative ELISA. C8A possesses considerable biomarker potential, with 95.45 percent specificity and 77.27% sensitivity with 0.933 Area Under the Curve (AUC), whereas C6 and C8B showed poor biomarker potential. Hence, C8A demonstrated great promise as a circulating blood-based protein biomarker for HBV induced HCC diagnosis.

Keywords: HCC; Serum proteins; AFP; biomarker; diagnosis; ELISA

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1.1. Introduction:

Hepatocellular carcinoma (HCC) is one of Pakistan's deadliest malignancies, accounting for the fourth most common malignancy in men and the seventh most common malignancy in women (1). Because of the late diagnosis, which usually leaves the patient with limited treatment options, the fatality rate of HCC is increasing by 2% to 3% per year (2). HCC therapy options are limited to liver transplantation, surgical resection, and tumor ablation (3). HCC recurs in about two-thirds of patients who are treated with surgical resection, putting the patients' lives at risk (4). HCC is also linked to hepatitis B and C, which cause inflammation and cirrhosis, resulting in HCC (5). HCC is a cancer that kills people all over the world. It is the second leading cause of death in Asia and Africa, and the sixth leading cause in Western countries (6).

HCC affects 7.6 people per 100,000 people in Pakistan each year, with a slightly lower rate of 2.8 people per 100,000 people in females (7). Less women on the streets, less alcohol usage, and women's smoking behaviors may all be contributing to Pakistan's lower HCC incidence (1). Hepatitis C is thought to be responsible for 60–70% of HCC in Pakistan (7). Also, despite the fact that HBV vaccine programs and availability of effective antiviral therapies in many countries that lead to cures to HBV replication suppression, more than 240 million people are infected with HBV and continue to be at risk for liver cirrhosis and HCC (8,9).

Early detection leads to early treatment, and the survival rate of cancer patients is greatly improved (10). Early cancer detection is only achievable if the methods are simple, quick, and inexpensive. Blood-based biomarkers have tremendous potential for this purpose since they are easy to detect, patients undergo non-invasive procedures for biomarker identification, and biomarker detection techniques are very inexpensive. Histopathology and costly imaging techniques such as MRI and Ultrasound are being used to identify hepatocellular cancer (11). Blood-based new biomarkers with greater sensitivity and specificity are urgently needed for

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early detection of HCC. It is critical to discover non-invasive blood-based biomarkers for early, easy, non-invasive, and low-cost diagnosis of HCC in order to successfully combat the disease.

Alpha-fetoprotein (AFP), an oncofetal protein, is generated in the liver during hepatocellular carcinoma. Both neoplastic and non-neoplastic conditions cause an increase in this protein (12). At an AFP level of >200 ng/ml, this scenario can be very indicative of hepatocellular carcinoma. As a result, the likelihood of HCC is larger than ninety percent (>90 percent). Currently, blood AFP levels are the gold standard biomarker for detecting HCC in patients, with specificity ranging from 76% to 94% and sensitivity ranging from 39% to 65%. (13). HCC is now diagnosed every 6 to 12 months using a combination of serum alpha-fetoprotein (AFP) levels and ultrasonography (12). According to AASLD standards, AFP's diagnostic performance is severely limited due to its low specificity and sensitivity. As a result, it is not an ideal candidate for the diagnosis of HCC but only for surveillance and should be used in conjunction with ultrasound (14). Although AFP-based tests are routinely used to identify probable liver cancer, they are prone to false negative results, making them unhelpful for the diagnosis of Hepatocellular Carcinoma. Other than hepatocellular carcinoma, AFP increase can be caused by alcoholic hepatitis, chronic hepatitis, or cirrhosis (15). Furthermore, in some situations, AFP levels are not increased at all, and normal AFP levels diagnosed at the time of diagnosis tend to remain stable over time (16). Because of unique AFP negative HCC instances, where there is no discernible difference in AFP levels despite the presence of HCC, AFP cannot be maintained as a conventional diagnostic biomarker for HCC (17). In comparison to Alpha fetoprotein, this study is focused on the characterization of biomarkers with better specificity and sensitivity (AFP).

Several biomarkers for HCC, such as CK-19 and GP-73, have been discovered due to advancements in cancer biology research (18). These biomarkers are now being investigated to determine their utility in early diagnosis, hence optimizing therapy, minimizing the formation of new tumors, and preventing tumor recurrence in liver transplant recipients. However, there is still a shortage of accurate blood-based biomarkers for cancer screening and diagnosis. There is a

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need to evaluate big expression data utilizing already published pipelines created by Li et el. (9) that would incorporate multiple bioinformatics databases/tools and literature to uncover sensitive and specific protein biomarkers. Tumor specific blood-based biomarkers hold key importance in improving cancer treatment and can be considered an effective medium when compared to any other area of fundamental medical research. As a result, active research should focus on the recording of new blood-based biomarkers that may be used to analyze and treat HCC. Because many tumor-related genes, enzymes, microRNAs (miRNAs) and proteins are released into body fluids such as blood or urine by cancer tissues, efficient biomarkers with improved sensitivity and specificity for identifying HCC are needed to diagnosis HCC on time. (18).

Materials and Method

2.1. Study design

This is a 'case control study,' and to circumvent randomization, a consecutive sampling strategy was adopted.

2.2. Calculation of Sample Size

The sample size was calculated using the following formula keeping the sensitivity 31.47% and specificity 99.13% for AFP for the diagnosis of HCC in a population under study (19)

$$n = Z^2 * P(1-P) / \Delta 2 \quad \text{..........................} (1)$$

n will be (a+c) if we use Sensitivity as P, and n will be (b+d) if we use Specificity as P in formula (1).

$$N = (a + c)/\text{Prevalence} \quad \text{..................................} (2)$$

$$N = (b + d)/(1\text{-Prevalence}) \quad \text{............................} (3)$$

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2.3. Collection of blood samples

A collaboration between the Microbiology department at GCUF and Chaudhary Hospital Gujranwala was formed to collect blood samples from HCC patients. By defending our summary in front of a panel of doctors and researchers under the supervision of Chairman Chaudhary Hospital, we were able to acquire ethical permission from the Chaudhary Hospital's Institutional Review Board. During the conference, all of the ethical rules were established, and the research collaborator at the hospital verified that they were strictly followed. Blood samples from 22 HCC patients infected with HBV were taken from Chaudhary Hospital's liver ward after receiving IRB approval. Patients' verbal agreement was obtained prior to collecting blood samples. The patients were given a questionnaire to complete out willingly after being instructed on the content, nature, and use of the data collected from them.

Following all ethical criteria, a 3 mL blood sample was taken from HCC patients and healthy controls. To reduce the danger of injuries to the patient, a trained phlebotomist was employed. A questionnaire was used to collect information about the patient's age, gender, HBV and HCV history, as well as the date and location of blood collection. Blood samples from 22 HCC patients and 22 healthy controls were collected. Within 2-3 hours of collecting the blood samples, all of the samples were returned to the lab for additional processing. The following criteria were used to choose patients and healthy controls:

2.4. Inclusion and exclusion criterion

Tables 3.1 and 3.2 show the inclusion and exclusion criteria for test and control samples, respectively. The Research assistant and on-duty doctors at the blood collection site examined the patients based on inclusion and exclusion criteria.

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2.4.1. Inclusion criterion

Table 1 Inclusion criterion of HCC patients and healthy controls.

Hepatocellular carcinoma patients	Healthy controls
• • HCC has been confirmed using imaging techniques such as ultrasonography and MRI. • • Imaging studies, such as X-rays, bone scans, and abdominal and pelvic CT scans, verified the non-metastatic stage of HCC. • • The on-duty doctor confirmed the diagnosis of HCC.	• Controls from diverse age groups • No liver disease history • No viral hepatitis (B & C) history

2.4.2. Exclusion criterion

Table 2 Exclusion criteria for HCC patients and healthy controls.

Hepatocellular carcinoma patients	Healthy controls
• Liver related comorbidities • Other types of cancer • Metastatic stage of tumor established through imaging investigations such as X-rays, bone scans, and abdominal and pelvic CT scans.	• With any form of liver disorder • With any type of cancer • With any viral hepatitis (B&C) incidence

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2.5. Serum extraction

The blood was collected into a serum vacutainer (yellow cap) and then placed vertically in a stand to clot. The blood serum was then obtained by centrifuging the samples at 2000 rpm for 2 minutes. A gel separates the blood clot in the serum vacutainer, allowing us to extract pure serum from the top. All of the secreted proteins in the blood are found in the serum. 1.5 mL serum was extracted and aliquoted into Eppendorf tubes, which were then frozen at -80°C. From September 2021 until December 2021, blood samples were taken.

2.6. ELISA

The serum of the individuals was tested using an Enzyme Linked Immunosorbent Assay (ELISA). ELISA is a quantitative study that uses protein specific antibodies to quantify the amount of protein present in a sample. Depending on the type of ELISA utilized, the bound antibodies produce a colorimetric or chemiluminescent signal, which is compared to known standards to determine the amount of protein present in the sample. The amount of target protein in a sample was detected using a chemiluminescent technique in our ELISA test. Nanjing Pars Biochem Ltd provided ELISA kits against four potential protein biomarkers: C6, C8A, C8B and AFP. For our three potential protein biomarkers, we used AFP as a reference. The absorption values from ELISA were read using an ELISA reader from Bio-Rad Ltd. (Model PR4100).

2.6.1. ELISA results analysis

MyAssays Online (https://myassays.com/index.html) and MyAssays Desktop (https://myassays.com/index.html) were used to examine the ELISA data. For the OD values acquired for the standard samples, for which the concentration was known, the application created a standard curve. The standard curve is automatically generated by using the 'Best Fit' function in the MyAssays desktop application, which analyses the standard values and creates the best curve based on the appropriate curve fitting model given by our sample values. According to the "abbexa" corporate website (https://www.abbexa.com/elisa-standard-curve), an R2 value greater than 0.95 indicates a strong standard curve. Accessed on 5th January, 2022. (20). The samples' OD values are then calculated by comparing them to the standard curve. Our samples' OD values were first multiplied by the 'dilution factor,' which was 5. The values outside

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of the curve are extrapolated using the standard curve's trendline, and the concentration of proteins is calculated using the OD values.

2.7. Statistical analysis

Statistical analyses were carried out using IBM Corporation's Statistical Package for Social Sciences (SPSS) version 26 (IBM Corp. Released 2016). IBM SPSS Statistics for Windows, Version 24.0.Armonk, NY: IBM Corp). For all statistical tests, p<0.05*, p<0.005** and p<0.0005*** were considered significant, with a confidence interval of 95 percent. The quantitative data obtained via ELISA, as well as demographic data from the patient and control populations, were subjected to the following statistical tests.

2.7.1. Descriptive statistics

In SPSS, descriptive statistics were calculated to identify the frequencies and percentages of samples by age groups and gender. The 'Chart Builder' feature in SPSS was used to create visual representations in the form of bar charts and pie charts. To further understand the study population, cross tabulations of age groups vs gender were undertaken in both the test and control populations. The data was also separated into 'causalities of HCC to check how much HCV was also presented among the patient population. The frequency and % were calculated in SPSS, while bar charts in Graph Pad Prism version 8 were used to visualize the data.

2.7.2. Mean concentration values

The 'Compute means' tool in SPSS version 26 was used to calculate the mean concentration values of all proposed potential biomarker proteins, as well as their standard deviation values. The graphs for comparing the mean values of the test and control samples, on the other hand, were created using Graph Pad Prism version 8.

2.7.3. ROC curve analysis

The Receiver Operator Characteristic (ROC) curve was created using the 'ROC analysis tool' in SPSS version 26. The ROC curve determines a biomarker's capacity to discriminate between test

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and control samples (21). For each conceivable cut off value, a graph is plotted between 'sensitivity' and '1-specificity.' The cut-off value is highly subjective, and it is determined by the study's objectives. The cut off value with the highest sum of sensitivity and specificity is chosen to determine a biomarker's diagnostic capacity (22).

With ROC curve analysis, the area under the curve (AUC) was also calculated. The better a biomarker's diagnostic potential, the higher its AUC value (Hoo, Candlish and Teare, 2017c). AUC values of less than 0.5 suggest no to very low biomarker potential, whereas AUC values of more than 1 indicate good biomarker potential. All AUC values were calculated using a 95% confidence interval.

2.7.4. Determination of diagnostic ability

We can ascertain the number of True Positive (TP), True Negative (TN), False positive (FP), and False negative (FN) cases from the data set by establishing the cut off value. If a greater mean concentration of biomarker was observed in patient samples, all values of the biomarker above cut off were deemed positive results, while all values of the biomarker below cut off were considered negative results. This relates to a biomarker's diagnostic ability to discriminate between test and control samples.

2.7.5. Determination of diagnostic parameters

After identifying the number of True Positive (TP), True Negative (TN), False positive (FP), and False negative (FN), diagnostic parameters such as sensitivity, specificity, accuracy, positive predictive value, and negative predictive value can be computed. The values of diagnostic parameters were obtained using the Medcalc online diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic test.php). The following formulas can be used to manually calculate the values of sensitivity, specificity, and accuracy.

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

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RESULTS AND DISCUSSION

3.1. Collection of blood samples of HCC patients and controls

In this study, a total of 44 blood samples were taken from HCC patients and healthy controls (test samples = 22; control samples = 22). This sample size was calculated in accordance with the sensitivity and specificity values of AFP for HCC diagnosis in a study (19). The participants' data was obtained with their verbal consent and in accordance with ethical rules (Alshehri et al., 2020). Patients were given a structured questionnaire once verbal consent was obtained, and their identities and data were not disclosed with anyone other than the researchers involved in this study. Patients' histories, ages, collection dates, and whether they had viral or non-viral HCC were all included in the questionnaire. After getting an approval letter from their Institutional Review Board (IRB), all HCC samples were collected from Chaudhary Hospital in Gujranwala.

3.2. Descriptive Statistics on sample population

3.2.1. Test sample population; HCC patients

It is necessary to conduct numerous statistical analyses on the study population in order to understand the patient population in terms of gender, age group, and HCC causalities.

To begin, we must first establish the frequencies and percentages of our test sample population (HCC patients) by age group. Our sample population was divided into age groups, and the majority of HCC patients were in the 50–59-year age group (45.5 percent), followed by the 40–49-year age group (27.2 percent). These were followed by a mediocre percentage in the 60–69-year age group (18.2%) and the smallest population in the 70–79-year age group (9.1%). Table 3 depicts this information.

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Table 3 Frequency and percentage of HCC patients in age groups.

Distribution of HCC patients in Age Groups

		Frequency	Percent
Age Groups	40-49	6	27.2
	50-59	10	45.5
	60-69	4	18.2
	70-79	2	9.1
	Total	22	100.0

Following the age split of the testing sample population, the population was also divided into gender groups, as indicated in Table 4. The male population (17 cases) made up the majority of the HCC sample population, while the rest were females (5 cases).

Table 4 Frequency and percentage of HCC patients in terms of Gender

Gender distribution in HCC patients

		Frequency	Percent
Gender	Male	17	77.27
	Female	5	22.27
	Total	22	100.0

As illustrated in Figure 8, the above-mentioned divisions into age groups and gender can be cross tabulated to infer the gender wise distribution in different age groups, providing useful insight into the age wise demographic distribution of gender in HCC patients.

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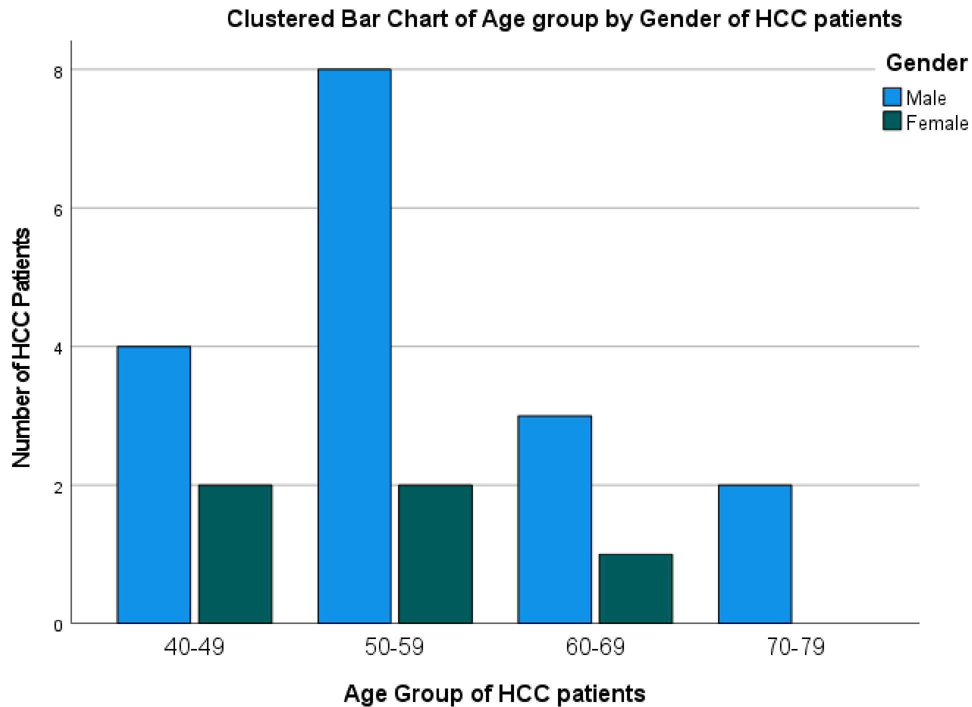

Figure 1 Clustered Bar chart of distribution of gender in different age groups.

3.2.2. Co-morbidities (Causalities) of HCC

In Pakistan and other parts of the world, viral hepatitis (HBV and HCV) is the biggest contributor to HCC (23). All 22 of our HCC patients were infected by HBV, but some of them had both HCV and HBV. Therefore, we determined the causalities of our HCC population and divided into HBV and HCV groups. A total of 18 HCC patients infected with HBV were present in the total population and 4 patients were infected by both HBV and HCV as shown in the Figure 9. This is in line with previous observations that viral hepatitis is major cause of HCC (24,25). HBV infection being one of the major causes of HCC development is also emphasized by Ayub et. el (26).

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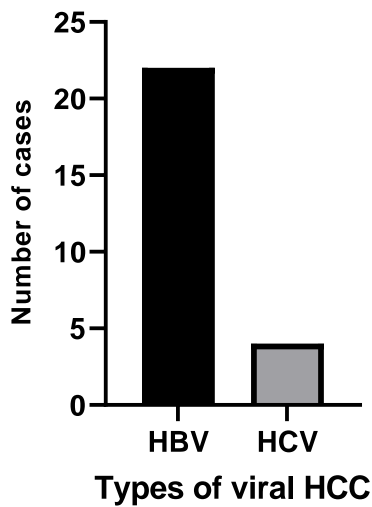

Figure 2 Breakdown of viral HCC population in HCV and HBV incidence.

3.3. Control Samples

Aside from the 22 test samples mentioned above, the study also included 22 control samples, which were collected after verbal agreement was obtained. Healthy individuals without a history of HCC, HCV, or HBV were chosen from various age groups and places and blood was taken from them to act as healthy controls.

The HCC patient population is compared to the control samples, which are separated into different age groups. The age group 50-59 years old had the highest proportion of control samples (45.45 percent), followed by 40-49 years old (22.7 percent). This pattern resembles the age distribution in the HCC patient population. The age group division of control samples is depicted in Figure 10.

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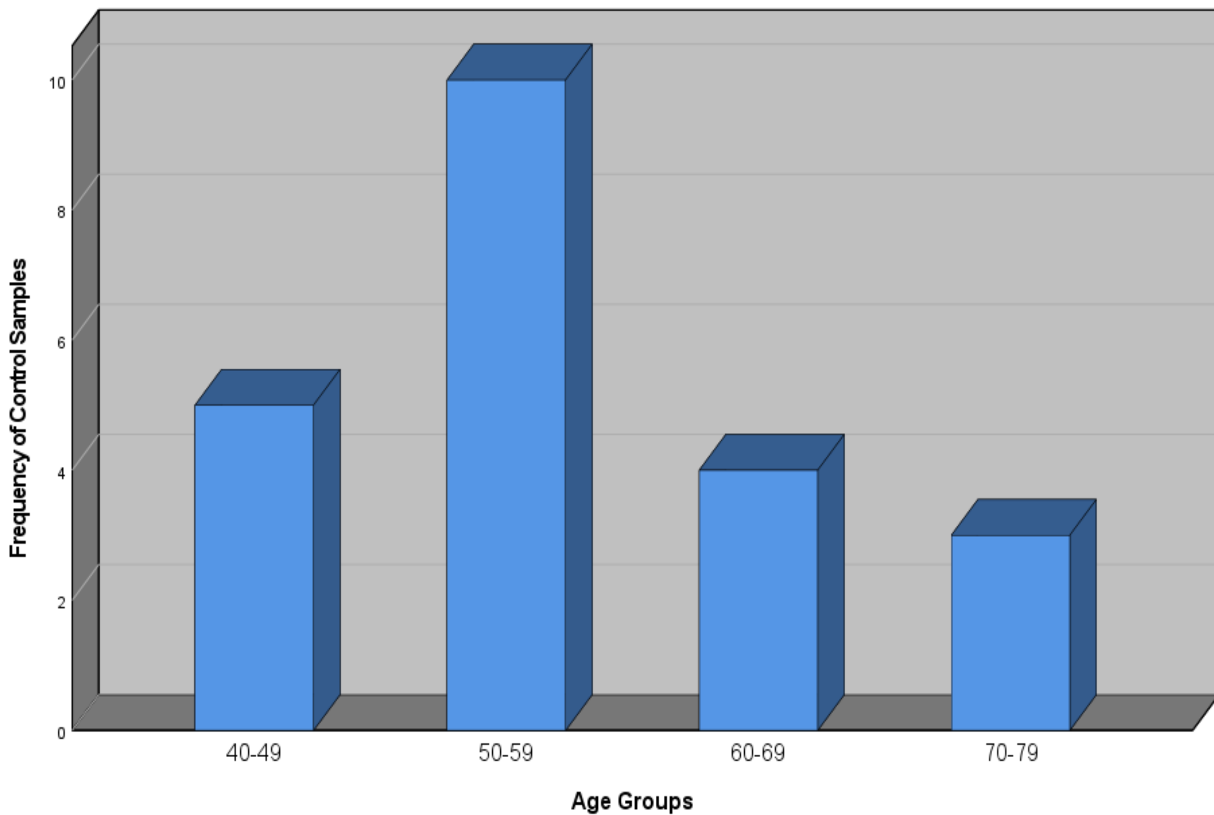

Figure 3 Division of frequency of control samples in different age groups.

In order to compare control samples to the HCC sample population, the gender distribution of control samples was also examined. Males were the dominating gender here, with 16 cases, followed by females with 6 cases, and this tendency is quite similar to the HCC patient population, which has a predominance of males as compared to females, as seen in Figure 11.

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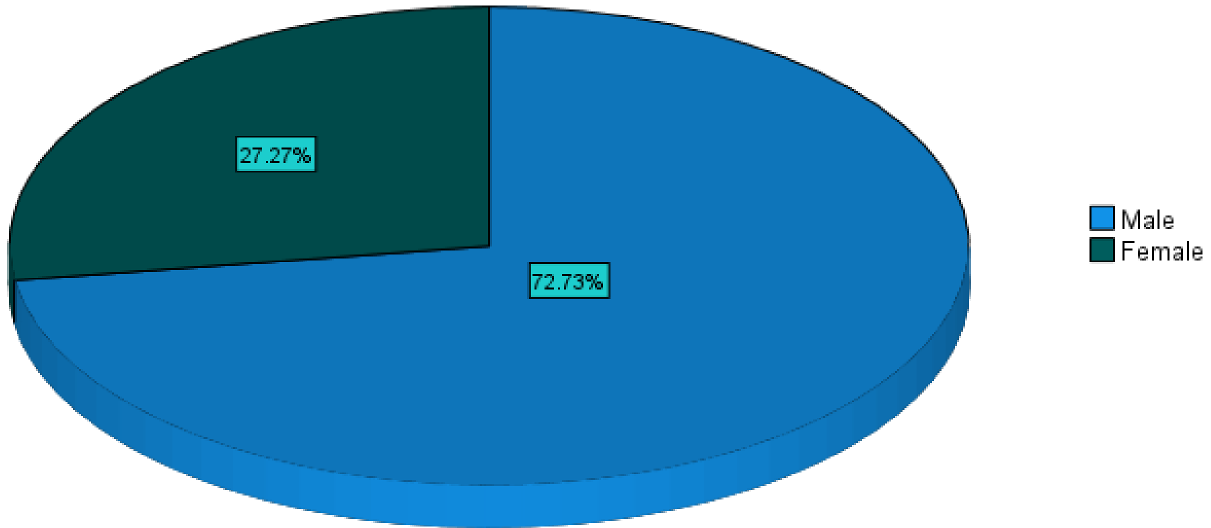

Figure 4 Percent gender distribution in control samples

3.4. ELISA results analysis:

The above-mentioned sample population (test samples=22; control samples=22) was tested using ELISA to look for three proposed biomarkers, C6, C8A, and C8B, as well as one currently accepted biomarker, Alpha Fetoprotein (AFP). ELISA is one of the most powerful tools to quantify proteins in a sample (27). The clinical utility of these biomarkers was calculated using SPSS version 26 and Graph Pad Prism. ELISA findings were evaluated using 'Myassays.com,' and the clinical utility of these biomarkers was calculated using SPSS version 26 and Graph Pad

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Prism. As illustrated in Figure 12, the quantities acquired via ELISA were subsequently utilized to undertake various statistical analyses and eventually determine sensitivity and specificity.

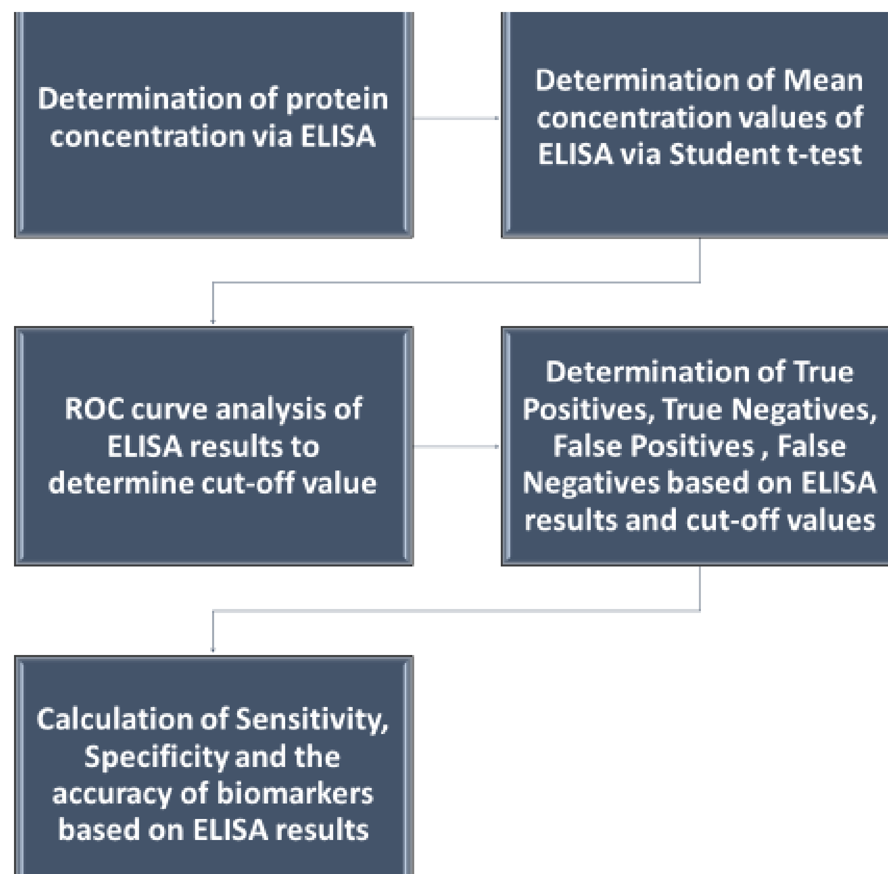

Figure 5 Flowsheet diagram of statistical analysis from ELISA results to determination of sensitivity, specificity, and accuracy of a biomarker.

3.4.1. AFP

3.4.1.1. Mean concentration values

Alpha Fetoprotein (AFP) the currently prevalent biomarker for HCC was checked in test and control samples. The mean concentration values in test samples (HCC patients) were almost

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same or slightly elevated as compared to control samples. Also, the P-value is greater than 0.005 that make AFP results insignificant as shown in the Figure 13.

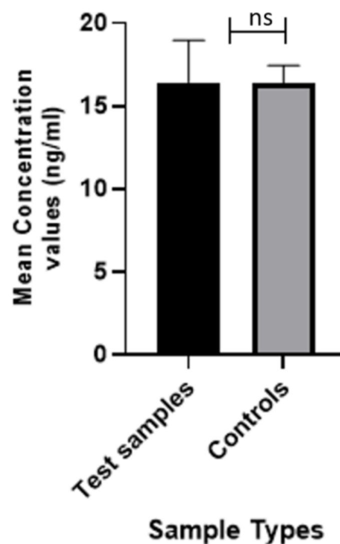

Figure 6 Mean values of test samples and controls for AFP**.** The mean value in test samples is slightly elevated in test samples (16.42 ng/ml) compared to control sample population (16.40 ng/ml).

3.4.1.2. ROC curve analysis of AFP

The diagnostic ability of AFP was estimated using ROC curve analysis on SPSS v26, as shown in the Figure, based on the difference in mean values. The 95 percent confidence interval (CI) was used to create the ROC curve. The AUC value was discovered to be 0.443 which makes it a poor potential biomarker. The cut off value of 16.25 ng/ml was chosen as the value with the highest sum of sensitivity and specificity. Figure 14 depicts the ROC curve for AFP. The ROC

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curve determines a biomarker's capacity to discriminate between test and control samples (21). For each conceivable cut off value, a graph is plotted between 'sensitivity' and '1-specificity.' The cut-off value is highly subjective, and it is determined by the study's objectives. The cut off value with the highest sum of sensitivity and specificity is chosen to determine a biomarker's diagnostic capacity (22).

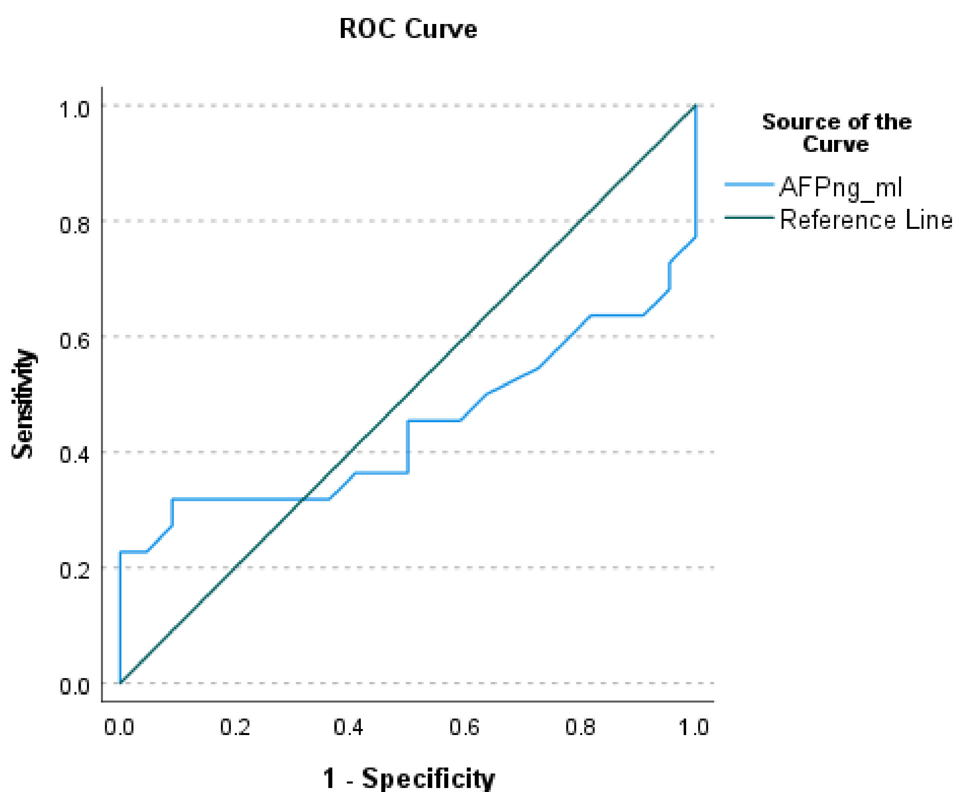

Figure 7 ROC curve obtained via plotting Sensitivity against 1-Specificity at every possible cut-off value.

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3.4.1.3. Determination of diagnostic ability of AFP

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the accurate positively diagnosed i.e. (True positive, TP), inaccurate positively diagnosed (False positives, FP), accurately negatively diagnosed (True negative, TN) and inaccurately negatively diagnosed (False negatives, FN). These numbers give an idea into the diagnostic ability of the biomarker. Following is the Table 5 containing the numbers of True positives, False positives, True negatives and False negatives.

Table 5 Diagnostic ability of AFP to act as a biomarker for HCC.

AFP's diagnostic ability to distinguish HCC and control samples

Count

		Biomarker AFP		
		Present	Absent	Total
Case Type	Test Sample	10	12	22
	Control	11	11	22
Total		21	23	44

3.4.1.4. Determination of diagnostic parameters

Upon determination of the number of True positives, True negatives, False positives and False negatives, different parameters relating to a biomarker's diagnostic ability were computed via MedCalc diagnostic test evaluation calculator. The specificity was determined as 50.00% whereas the sensitivity was determined at 45.45%. The accuracy of the test was determined at 47.73%. These values were in line with previous studies conducted on AFP's sensitivity which are usually in the range of 41% to 84% (10,28–30) which is a very broad range due to different

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expression levels and cut-off values in different populations**.** These diverse values obtained in different studies can be attributed to different populations under study which usually affect the number of AFP positive and negative HCCs (29,31)**.** The majority of our test samples came out to be AFP negative since they did not show elevated amount of AFP. These AFP negative results are in accordance with the previous researches (17,32)

The accuracy of the test was determined at 47.73%. These parameters are given in the Table 6.

Table 6 Diagnostic parameters of AFP calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	45.45%	24.39% to 67.79%
Specificity	50.00%	28.22% to 71.78%
Positive Likelihood Ratio	0.91	0.49 to 1.69
Negative Likelihood Ratio	1.09	0.62 to 1.92
Disease Prevalence (*)	50.00%	34.56% to 65.44%
Positive Predictive Value (*)	47.62%	32.85% to 62.82%
Negative Predictive Value (*)	47.73%	32.24% to 61.75%
Accuracy (*)	47.73%	32.46% to 63.31%

3.4.2. C6

3.4.2.1. Mean concentration values

As demonstrated in Figure 15, C6 have a 14.54 ng/ml mean concentration value in test samples and 11.95 ng/ml in control samples, but just like AFP results, there is no considerable difference among the two and there is a high standard deviation which shows increased spread of data for

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both test and control samples. A student t-test on the concentration values of HCC patients and healthy controls obtained from the quantitative ELISA test yielded the mean values.

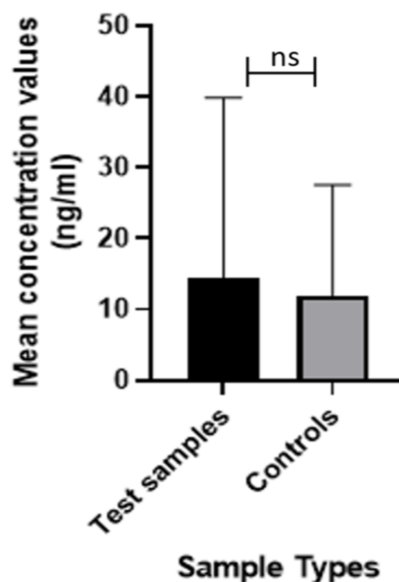

Figure 8 Mean values of controls vs test samples for C6.

3.4.2.2. ROC curve analysis of C6

The diagnostic ability of C6 was evaluated using ROC curve analysis using SPSS version 26 based on the difference in mean values, as shown in Figure 16. The ROC curve was created using a 95% confidence interval. The AUC at $p<0.05$ was determined as 0.499 which shows a poor biomarker potential. The cutoff value was then calculated by choosing 7.00 ng/ml as the value with the highest sum of sensitivity and specificity. Figure 16 shows the ROC curve for C6.

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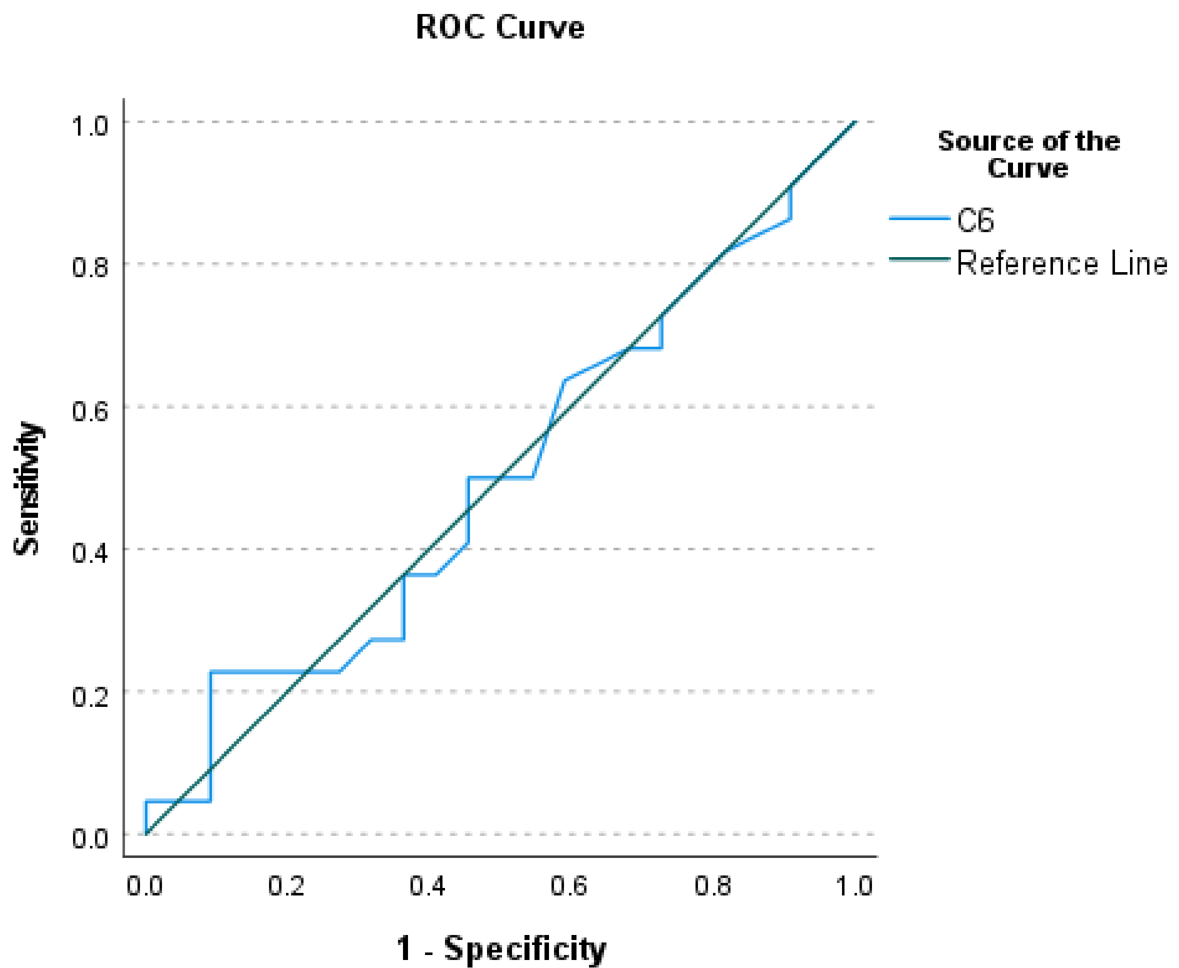

Figure 9 ROC curve obtained via plotting Sensitivity against 1-Specificity at every possible cutoff value.

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3.4.2.3. Determination of diagnostic ability of C6

The sample population, which included both HCC patients and controls, was evaluated to determine the number of True positives, True negatives, False positives, and False negatives based on the cut-off value. These figures provide insight into the biomarker's diagnostic capacity. The numbers of True positives, True negatives, False positives, and False negatives are listed in Table 7.

Table 7 Diagnostic ability of C6 to act as a biomarker for HCC

C6's diagnostic ability to detect HCC and control samples

Count

		Biomarker C6		
		Present	Absent	Total
Case Type	Test Sample	14	8	22
	Control	13	9	22
Total		27	17	44

3.4.2.4. Determination of diagnostic parameters of C6

After determining the number of True positives, True negatives, False positives, and False negatives, numerous metrics related to a biomarker's diagnostic capacity were evaluated using the MedCalc diagnostic test evaluation calculator. The sensitivity was 63.64 percent while specificity was found to be 40.91 percent. The accuracy of the test was determined to be 52.27 percent. Table 8 contains these figures. All of these data are based on ELISA results.

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Table 8 Diagnostic parameters of C6 calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	63.64%	40.66% to 82.80%
Specificity	40.91%	20.71% to 63.65%
Positive Likelihood Ratio	1.08	0.67 to 1.72
Negative Likelihood Ratio	0.89	0.42 to 1.88
Disease Prevalence (*)	50.00%	34.56% to 65.44%
Positive Predictive Value (*)	51.85%	40.14% to 63.27%
Negative Predictive Value (*)	52.94%	34.77% to 70.36
Accuracy (*)	52.27%	36.69% to 67.54%

3.4.3. C8A

3.4.3.1. Mean concentration values

C8A had a lower mean concentration value in test samples (33.41 µg/ml) than in control samples (42.84 µg/ml), thus pointing towards dysregulation of complement component pathway in HCC, as has been suggested in many studies, due to chronic and ectopic inflammatory states underlying the origins of HCC (33,34)**.** The standard deviation in the mean concentration values does not overlap thus indicating good results. A student t-test on the concentration values of HCC patients and healthy controls obtained from the quantitative ELISA test yielded the mean values as shown in Figure 17.

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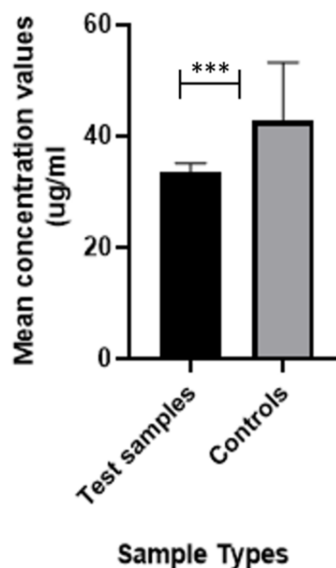

Figure 10 Mean values of controls vs test samples for C8A.

3.4.3.2. ROC curve analysis of C8A

The diagnostic ability of C8A was evaluated using ROC curve analysis using SPSS version 26 based on the difference in mean values, as shown in Figure 4.10. The ROC curve was created using a 95% confidence interval. The ROC curve analysis at $p<0.05$ was performed by keeping the ‘lower values as positive results’ since the mean concentration in test samples was less than control samples. It was discovered that the AUC was 0.933 which exhibits excellent potential to serve as a biomarker for HCC. The cutoff value was then calculated by choosing 36.70 g/ml as

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the value with the highest sum of sensitivity and specificity. Figure 18 shows the ROC curve for C8A.

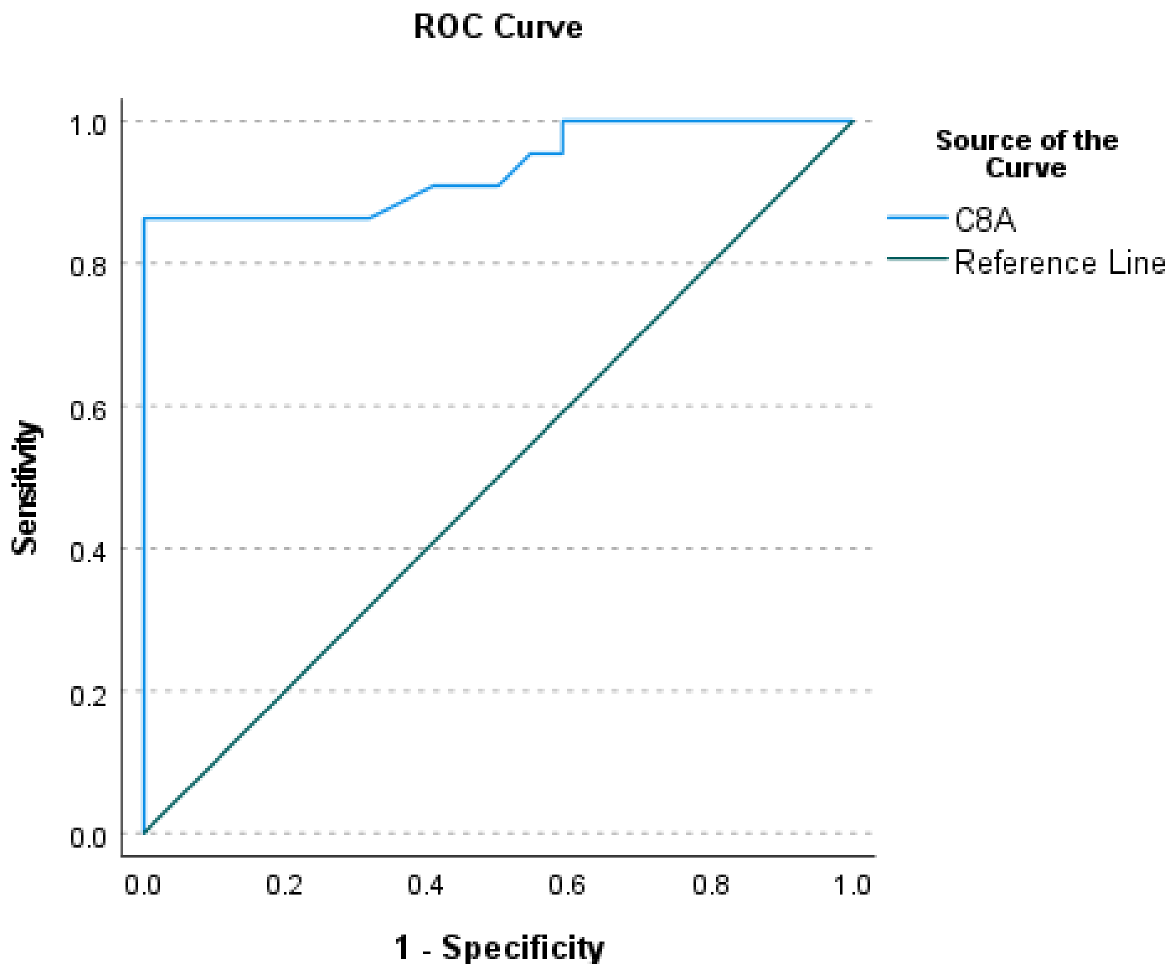

Figure 11 ROC curve obtained via plotting Sensitivity against 1-Specificity at every possible cutoff value.

3.4.3.3. Determination of diagnostic ability of C8A

Based upon the cut-off value, the sample population including both HCC patients and controls were checked to determine the number of True positives, True negatives, False positives and

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False negatives. These numbers give an idea into the diagnostic ability of the biomarker. Following is the Table 9 containing the numbers of True positives, True negatives, False positives and False negative.

Table 9 Diagnostic ability of C8A to act as a biomarker for HCC.

C8A's diagnostic ability to detect HCC and control samples

Count

		Biomarker C8A		
		Present	Absent	Total
Case Type	Test Sample	17	5	22
	Control	1	21	22
Total		18	26	44

3.4.3.4. Determination of diagnostic parameters of C8A

Using the MedCalc diagnostic test evaluation calculator, different metrics linked to a biomarker's diagnostic capacity were computed after the number of True positives, True negatives, False positives, and False negatives were determined. The specificity was found to be 95.45 percent, while the sensitivity was 77.27 percent which are excellent values for a biomarker of HCC, and these values are far better than reported for AFP (29,30). The test's accuracy was determined to be 86.36 percent which further enhances confidence in the diagnostic ability of this candidate biomarker. These values can be found in Table 10. These figures are all derived from ELISA results.

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Table 10 Diagnostic parameters of C8A calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	77.27%	54.63% to 92.18%
Specificity	95.45 %	77.16% to 99.88%
Positive Likelihood Ratio	17.00	2.47 to 116.92
Negative Likelihood Ratio	0.24	0.11 to 0.52
Disease Prevalence	50.00%	34.56% to 65.44%
Positive Predictive Value	94.44%	72.10% to 99.15%
Negative Predictive Value	80.77%	65.91% to 90.12
Accuracy	86.36%	72.65% to 94.83%

3.4.4. C8B

3.4.4.1. Mean concentration values

C8B had a mean concentration of 1.518 ng/ml in test samples and 1.586 ng/ml in control samples, as shown in Figure 19. The mean results were determined using a student t-test on the concentration values obtained from the quantitative ELISA test in HCC patients and healthy controls.

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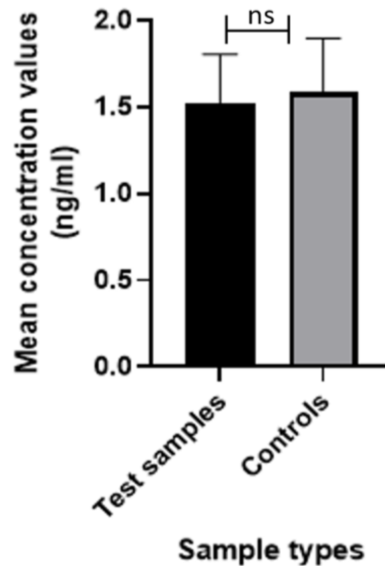

Figure 12 Mean values of controls vs test samples for C8B

3.4.4.2. ROC curve analysis of C8B

As shown in Figure 20, the diagnostic ability of C8B was assessed using ROC curve analysis in SPSS version 26 based on the difference in mean values. A 95 percent confidence interval was used to produce the ROC curve. The AUC at $p<0.05$ was discovered to be 0.448 which highlight their poor diagnostic potential. The cutoff value was then computed by taking the number with the maximum sum of sensitivity and specificity, which was 1.3500 ng/ml. The ROC curve for C8B is shown in Figure 20.

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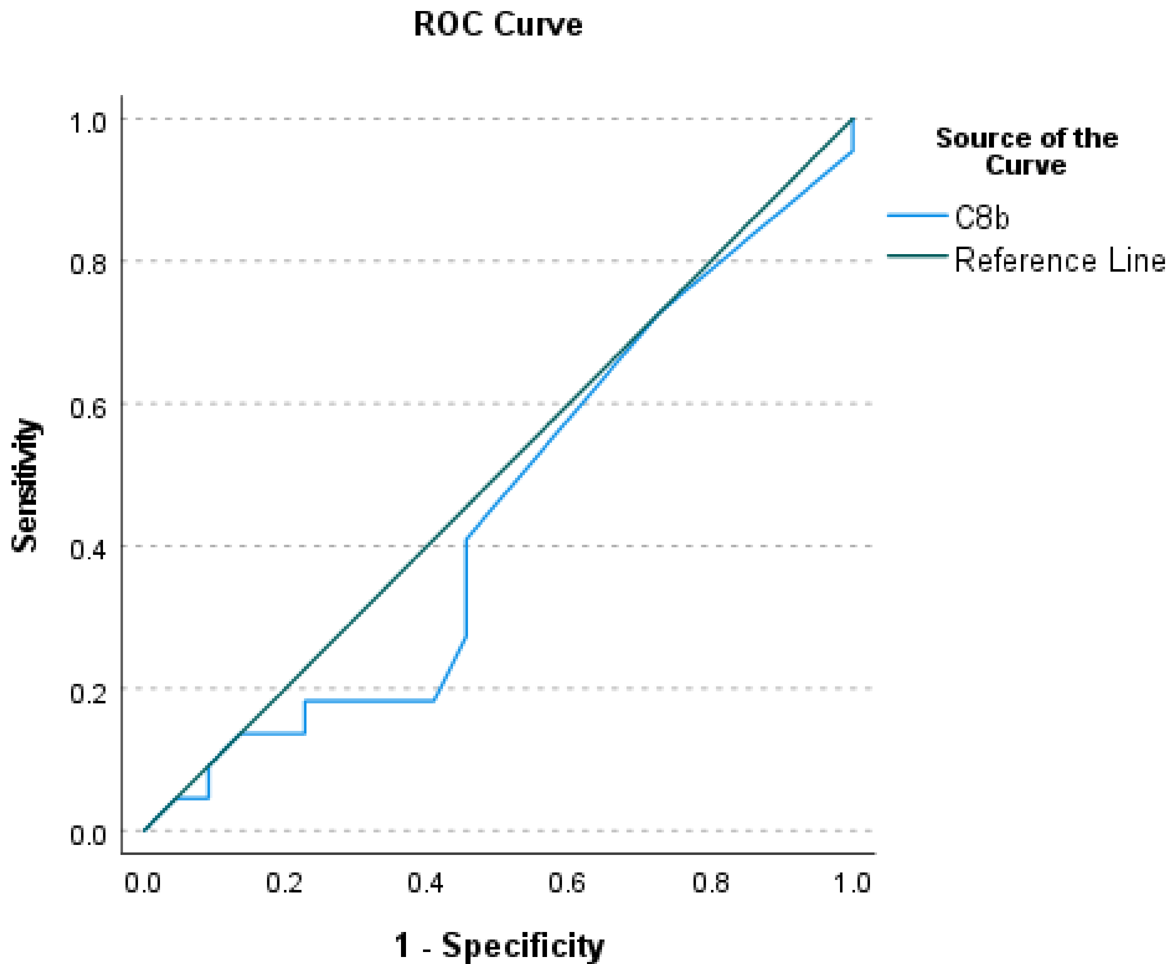

Figure 13 ROC curve obtained via plotting Sensitivity against 1-Specificity at every possible cutoff value.

3.4.4.3. Determination of diagnostic ability of C8B

The sample population, which included both HCC patients and controls, was evaluated to determine the number of True positives, True negatives, False positives, and False negatives based on the cut-off value. These figures provide insight into the biomarker's diagnostic capacity.

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The numbers of True positives, True negatives, False positives, and False negatives are listed in Table 11.

Table 11 Diagnostic ability of C8B to act as a biomarker for HCC

C8B's diagnostic ability to detect HCC and control samples

Count

		Biomarker C8B		
		Present	Absent	Total
Case Type	Test Sample	16	6	22
	Control	16	6	22
Total		32	12	44

3.4.4.4. 4.4.2.4. Determination of diagnostic parameters of C8B

After determining the number of True positives, True negatives, False positives, and False negatives, numerous metrics related to a biomarker's diagnostic capacity were evaluated using the MedCalc diagnostic test evaluation calculator. The sensitivity was found to be moderate i.e., 72.73 percent while the specificity was very poor i.e., 27.27. The accuracy of the test was determined to be 50.00 percent. These results show almost little to no biomarker potential. Table 12 contains these figures. All of these data are based on ELISA results.

Table 12 Diagnostic parameters of C8B calculated via the MedCalc diagnostic test evaluation calculator.

Statistic	**Value**	**95% Confidence Interval**
Sensitivity	72.73%	49.78% to 89.27%
Specificity	27.27%	10.73% to 50.22%

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Positive Likelihood Ratio	1.00	0.70 to 1.44
Negative Likelihood Ratio	1.00	0.38 to 2.62
Disease Prevalence (*)	50.00%	34.56% to 65.44%
Positive Predictive Value (*)	50.00%	41.05% to 58.95%
Negative Predictive Value (*)	50.00%	27.59% to 72.41
Accuracy (*)	50.00%	34.56% to 65.44%

4.1. Conclusion

Out of several potential biomarkers by Li et El, 2020, this study validated three serum protein biomarker candidates: C6, C8A, and C8B based on the complement system. These biomarker studies have the potential to provide a cost-effective, non-invasive alternative to imaging-based HCC identification, as well as open the path for easy, early detection of HCC. This study looked at the demographics of 22 blood samples of HBV induced HCC patients in Pakistan. It also sheds light on the efficacy of AFP as an HCC diagnostic marker in the Pakistani community. The possible biomarkers' diagnostic capacities are compared to AFP, and one biomarker (C8A) with a very good outcome in contrast to AFP is determined. Furthermore, the detection technique (ELISA) used was simple, inexpensive, and accessible, making it simple to establish it as a diagnostic test for HCC in the future if a positive result is acquired.

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