

Assessment of Serum Cytokine Levels in Patients with Hepatocellular Carcinoma and those with Chronic Liver Disease: Cross-Sectional Study

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Abstract The diagnosis of hepatocellular carcinoma (HCC) is currently limited by the use of Alpha-fetoprotein (AFP) levels and medical imaging. To improve the diagnostic process, this study aimed to compare cytokine levels in patients with HCC, liver cirrhosis, and chronic hepatitis. Sixty participants were consecutively enrolled in a cross-sectional study and divided into three groups: 20 with Chronic Hepatitis, 20 with Liver Cirrhosis, and 20 with HCC. The study was conducted in Yaoundé from December 2020 to April 2022. Blood samples were collected from each participant and analysed using a Luminex instrument to quantify cytokine markers. The data were analysed using the Statistical Package for Social Sciences (SPSS) Version 25.0. Significant variations in proinflammatory (IL-2 and IL-6) and angiogenic growth factor (VEGF) cytokines were observed among the three groups, with p-values of <0.0001, 0.050, and <0.0001 respectively. VEGF showed high sensitivity (80%) and moderate specificity (70%) at a threshold of 200 pg/ml, while in the one hand, AFP showed high sensitivity (80%) and low specificity (35%) at a threshold of 100 ng/ml. On another hand, IL-6 showed moderate sensitivity (60%) and high specificity (80%) at a threshold of 25 pg/ml. The findings of this study suggest that combining AFP levels, medical imaging, and cytokine markers such as IL-6 and VEGF can improve the early diagnosis of HCC.

Keywords: AFP, IL-6, VEGF, chronic hepatitis, cirrhosis, HCC

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1. Introduction

Hepatocellular carcinoma (HCC) is a critical public health concern, ranking as the sixth most common cancer and the fourth leading cause of cancer mortality globally [1,2]. The incidence of HCC is on the rise, with over 900,000 new cases and a corresponding number of fatalities each year [3,4]. East Asia and Africa are the hardest hit regions [5], with a higher incidence among men compared to women [6]. The development of HCC is linked to various risk factors, including liver cirrhosis, chronic infection with hepatitis B, and C viruses, aflatoxin exposure, excessive alcohol consumption, smoking, type II diabetes, and metabolic disorders [1,2,5,6,7,8]. We can see a range of molecular malfunctions in addition to these

key risk factors [3]. Despite the use of imaging exams such as ultrasonography in the diagnosis and monitoring of HCC [9,10,11], a number of molecular malfunctions associated with the disease remain unknown [10].

Treatment options for HCC include both curative and non-curative approaches [10,12], and the pathological characteristics of the tumor, such as size, grade, stage, and vascular invasion, have been the most well researched predictors of prognosis [13,14,15]. However, the search for additional serum prognostic markers ongoing [16].

Recent advancements in biomedical research have identified potential markers for the early detection of HCC, including Vascular Endothelial Growth Factor (VEGF), non-coding RNAs, DCP, vitamin K deficiency-induced protein or antagonist-II (PIVKA-II), lymphocytes, tumour infiltrating, programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2 [17-24].

The purpose of this study was to compare cytokine levels in individuals with HCC, liver cirrhosis, and chronic hepatitis.

2. Methods

2.1. Ethical Considerations

This study was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [25] and was approved by the ethics committee of the Faculty of Science, University of Yaoundé 1 (No: BTC-JIRB2022-044), the Administration of Yaoundé General Hospital (N/Ref: 213-21/HGY/DG/DPM/MA-TR), and the University Teaching Hospital of Yaoundé (N° 3235/AR/CHUY/DG/DGA/CAPRC). All participants provided written informed consent.

2.2. Design and Setting

This cross-sectional study was conducted at the hepatogastroenterology department of the Yaoundé General Hospital (YGH) and the internal medicine department of the University Teaching Hospital of Yaoundé (UTHY) between December 2020 and April 2022.

2.3. Study Population

The study population consisted of participants with Chronic Liver Disease and Hepatocellular Carcinoma (HCC). Participants were divided into three groups: Group 1 with chronic hepatitis (CH) who were negative for cirrhosis and HCC; Group 2 with cirrhosis who were negative for HCC; and Group 3 with HCC. Participants were 21 years or older and were chosen regardless of their sex, age, or nationality. Participants with Human Immunodeficiency Virus (HIV) or comorbidities were excluded from this analysis. These data were published elsewhere [26,27].

2.4. Measurement of Serum Cytokine Levels

Peripheral venous blood was collected from each participant via venepuncture, and the serum was obtained after centrifugation of the blood sample. The sera were frozen and stored at -20°C until analysis. Concentrations of cytokines were determined using a Luminex multiplex technique, with reagents purchased from Merck Millipore (Billerica, Massachusetts, USA). The experiments were performed and read on the Bio-Plex platform (Bio-Rad, USA), with the Bio-Plex Manager Software version 6.1 used for bead acquisition and analysis.

2.5. Data Analyses

The data were analysed using SPSS Version 25.0. Quantitative variables were presented as median ($\pm$ interquartile interval). The ANOVA test was used to compare quantitative variables between the three groups, and the Spearman correlation coefficient test was used to find the association between two variables. P-values ≤ 0.05

were considered significant. To determine the optimal cut-off point for serum VEGF in diagnosing HCC, a receiver operating characteristic (ROC) curve was constructed to plot sensitivity against 1-specificity at each point. A significance level of less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinical Characteristics of the Study Population

Among the 40 non-HCC patients, 20 had chronic hepatitis B, and 20 had chronic hepatitis C. Among the 20 HCC patients, 10 had chronic hepatitis C, and the remaining 10 had chronic hepatitis B (Figure 1).

Out of the 60 participants in our study (HCC group: $n=20$, Cirrhosis group: $n=20$, and Chronic Hepatitis group: $n=20$), the majority were male (82%) with a median age of 56 years (range: 26-71 years) and the most represented age group was 61-70 years (35%). There was no significant difference between the three groups in terms of age and gender. However, the mean value of AFP was significantly different between the three groups, with a value of 63.6 ± 21.8 for Chronic Hepatitis, 88.41 ± 19.2 ng/ml for Cirrhosis, and 226.5 ± 66.4 ng/ml for HCC (Table 1).

3.2. Cytokines Profile in the Study Groups

Sera from the study participants were analysed for the presence of Pro-inflammatory (IL-2, IL-6, IL-17A and TNF- α), Anti-inflammatory (IL-4 and IL-10), Antiviral (IFN α 2 and IFN- γ) chemokines (RANTES and MIP-1) and growth factors (VEGF and G-CSF). Table 2 shows the mean ($\pm$ SD) concentrations of these cytokines in the three groups. Participants with HCC showed higher levels of cytokines such as IL-2, IL-6, IFN- γ , G-CSF, and VEGF compared to participants with Cirrhosis and Chronic Hepatitis, with p-values all below 0.05. There were no significant differences in cytokine concentrations such as IL-15, IL-17A, IL-4, IL-10, IFN α 2, RANTES, and G-CSF.

3.3. Correlation between DNA HBV, IFN-g, IL-2, IL-6 and VEGF

No correlation was found between serum AFP and VEGF levels among participants. Also, no correlation was detected between serum VEGF and IL-2 levels among participants. However, a significant correlation was observed between serum VEGF and IFN- γ , serum VEGF and DNA HBV, and serum VEGF and IL-6 levels among participants (Table 3).

3.4. Assessment of Diagnostic Accuracy of DNA HBV, IFN-g, IL-2, IL-6, and VEGF Using ROC

Curve Analysis We evaluated the sensitivity and specificity of five biomarkers (IFN-g, DNA HBV, IL-6, VEGF) compared to that of AFP, which was considered as

the reference standard. The diagonal line represents a random classification model, and a good performance index is indicated by curves above the line, with a performance index of 0.5. As shown in Figure 2, DNA HBV, IL-6, AFP, and VEGF demonstrated a good performance index as they are all above the random classification model.

Serum AFP at a cut-off value of 80 ng/mL showed an accuracy of 80% in diagnosing HCC, with a sensitivity of 80% and specificity of 35%. At a cut-off value of 200 pg/mL, serum VEGF showed an accuracy of 70%

(sensitivity of 80% and specificity of 70%) for HCC diagnosis. Serum IL-6 at a cut-off value of 25 pg/mL demonstrated a sensitivity and specificity of 60% and 80%, respectively (Table 4).

The results revealed that VEGF and AFP had the highest sensitivity among the studied serum tumor markers. Furthermore, the ROC curve analysis indicated that both VEGF and IL-6 had high specificity. The area under the ROC curve (AUC) for serum VEGF was 70%, while the AUC values for serum AFP and IL-6 were 80% respectively (Table 4).

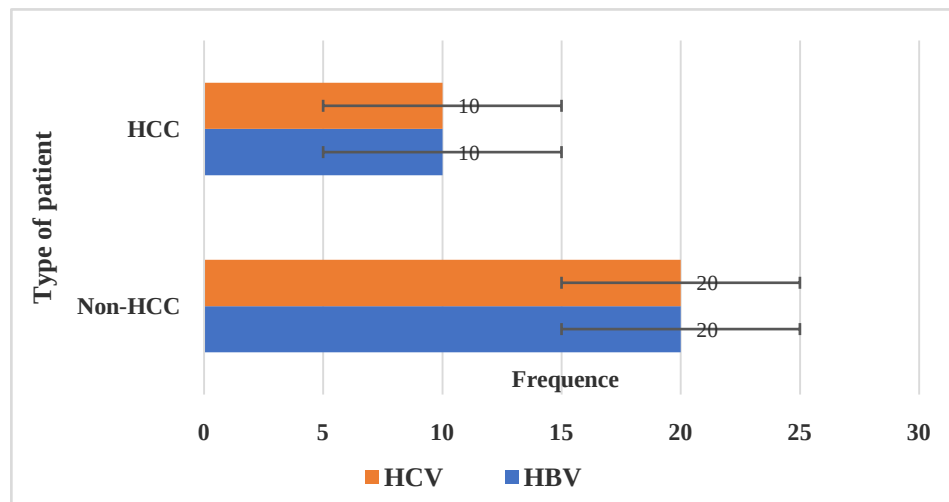


Figure 1. Distribution of study participants

Table 1. Demographic and clinical characteristics of the three study groups

		Chronic Hepatitis n=20	Cirrhosis n=20	HCC n=20	P-value
Gender	Female	3(27.3%)	3(27.3%)	5(45.5%)	0.6
	Male	17(34.7%)	17(34.7%)	15(30.6%)	
Age	Mean $\pm$ SD	48.1 $\pm$ 15.8	50.0 $\pm$ 15.4	53.8 $\pm$ 12.5	0.4
	Median	51.0	53.0	56.0	
	Range	26-67	26-70	28-71	
AFP ng/ml	Mean $\pm$ SD	63,6 $\pm$ 21,8	88,41 $\pm$ 19,2	226,5 $\pm$ 66,4	<0.0001
	Median	88.3	95.0	245.0	
	Range	64.0-107.0	84.0-135.0	90.0-360.0	

Table 2. Cytokine concentrations in study groups

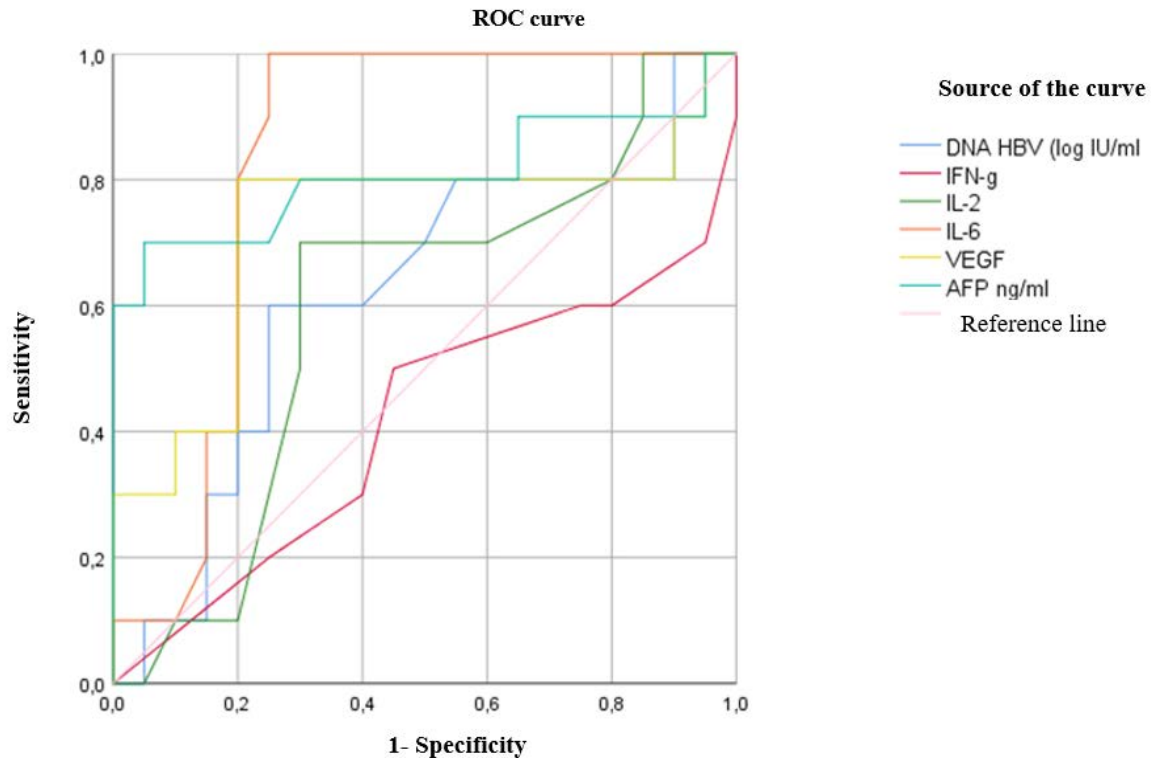
Cytokines	Chronic hepatitis n=20	Cirrhosis n=20	HCC n=20	P-value
Pro-inflammatory pg/ml				
IL-2	20,0 $\pm$ 6,9	16,6 $\pm$ 5,9	12,9 $\pm$ 4,1	<0,0001
IL-12	94,1 $\pm$ 84,9	93,02 $\pm$ 130,2	109,4 $\pm$ 100,4	0,8
IL-17A	24,6 $\pm$ 4,1	30,8 $\pm$ 10,7	42,8 $\pm$ 74,9	0,4
IL-6	11,2 $\pm$ 1,2	12,3 $\pm$ 2,7	25,0 $\pm$ 3,0	0,050
IL-15	27,5 $\pm$ 9,1	29,9 $\pm$ 8,9	38,4 $\pm$ 54,1	0,534
Anti-inflammatory pg/ml				
IL-4	22,2 $\pm$ 10,5	24,5 $\pm$ 16,3	28,1 $\pm$ 15,02	0,4
IL-10	102,7 $\pm$ 80,3	112,1 $\pm$ 73,5	118,1 $\pm$ 53,5	0,7
Antiviral pg/ml				
IFN α_2	15,8 $\pm$ 6,4	17,6 $\pm$ 8,01	24,6 $\pm$ 35,8	0,399
IFN- γ	13,3 $\pm$ 1,4	13,5 $\pm$ 0,9	12,9 $\pm$ 1,6	0,4
Chemokine pg/ml				
RANTES	88,3 $\pm$ 74,3	126,05 $\pm$ 111,3	120,9 $\pm$ 99,1	0,410
Growth Factor pg/ml				
VEGF	168,8 $\pm$ 24,2	187,3 $\pm$ 34,4	406,0 $\pm$ 72,7	<0,0001
G-CSF	12,8 $\pm$ 2,5	12,9 $\pm$ 2,9	13,1 $\pm$ 1,8	0,9

Table 3. Correlations between AFP, IL-2, IL-6 and VEGF

		IL-2 r (p-value)	VEGF r (p-value)
Chronic Hepatitis n=20	IFN-g (pg/ml)		0,5* (0,04)
Cirrhosis n=20	DNA HBV (log IU/ml)		-0,6* (0,04)
HCC n=20	DNA HBV (log IU/ml)	-0,7* (0,012)	
Hole participants n=60	IL-6 (pg/ml)		0,3* (0,02)

*. The correlation is significant at the 0.05 level (two-sided).

The correlation coefficient (r) varies between -1 and +1, 0 reflecting a zero relationship between the two variables, a negative value (negative correlation) meaning that when one of the variables increases, the other decreases; while a positive value (positive correlation) indicates that the two variables vary together in the same direction.

**Figure 2. ROC curves of IFN-g; DNA HBV; IL6; VEGF and AFP****Table 4. Diagnostic performance of AFP, DNA HBV INF-g, IL-2, IL-6, VEGF, in the prediction of hepatocellular carcinoma**

Test result variable(s)	Thresholds	Se%	Sp %	TP	FN	PPV%	NPV%	Area under the curve			
								Area	ES	95% CI	p
HBV DNA (log IU/ml)	//	//	//	//	//	//	//	0.6	0.1	[0.3-0.8]	0.3
IFN-g pg/ml	//	//	//	//	//	//	//	0.4	0.1	[0.1-0.6]	0.4
IL-2 pg/ml	//	//	//	//	//	//	//	0.5	0.1	[0.3-0.8]	0.4
AFP ng/ml	80	80	35	16	32	66.7	88.9	0.8	0.07	[0.6-1.0]	0.007
IL-6 pg/ml	25	60	80	16	32	66.7	88.9	0.8	0.1	[0.6-0.9]	0.004
VEGF pg/ml	200	80	70	15	28	55.6	84.8	0.72	0.1	[0.4-0.9]	0.048

The test result variables: DNA HBV (log IU/ml), IFN-g, AFP ng/ml, IL-2 include at least one link between the real positive state group and the real negative state group; ES: Standard error; CI: Confidence interval; Se: Sensitivity; Sp: Specificity. TP: true positive; FN: false negative; PPV: positive predictive value; NPV: negative predictive value.

4. Discussion

In this part, we examined the profile of the following 12 analytes (IL-2; IL-4; IL-6; IL-10; IL-12; IL-15; IL-17A; IFN α 2; IFN - γ ; VEGF; G-CSF; RANTES) in 60 participants with chronic liver disease and those with HCC. Overall, we observed High levels of proinflammatory cytokines (IL-2 and IL-6) whose levels varied significantly ($p < 0.0001$ and $p = 0.050$) between participants with Chronic Hepatitis, Liver Cirrhosis and HCC respectively. We also observed significant variation

($p < 0.0001$) for VEGF (factor of angiogenic growth) between the different groups studied.

The significant variation of IL-2 is explained by the fact that IL-2 is a powerful immunoregulator of the stimulation, proliferation and activation of T lymphocytes, natural killer cells and lymphocytes. B [28]. The effect of inflammation and inflammatory cells on the process of tumor development and progression is increasingly recognized. It is now clear that cases of cancer result from chronic infections and inflammation. IL-2 therefore plays a central role in the interplay between innate resistance

and adaptive immunity by generating T helper 1 (Th1) and CTL responses. In this study, we found that serum IL-2 levels were lower in patients with HCC compared with patients with Cirrhosis and Chronic Hepatitis. Our results are comparable to those of Peng et al; who in his study obtained the following amounts of IL-2 for HCC patients (238.2 ± 136.7 ng/L) compared to controls (288.6 ± 177.1 ng/L) [29]. Our results suggest that decreasing levels of IL-2 may play a role in the etiology of HCC by reducing Th1 cytotoxic responses [30].

In this study, the levels of IFN- γ and IL-17A were investigated in patients with Chronic Hepatitis, Liver Cirrhosis, and HCC. Although no significant difference was found in IFN- γ and IL-17A. IL-17A levels between the three groups, the levels were higher in patients with HCC compared to those with Chronic Hepatitis and Liver Cirrhosis. These findings are in line with previous studies that showed an increase in IL-17A levels with disease severity or stage. Compared to our study, Ribeiro et al, found higher amounts of IL-17A in patients with chronic liver disease compared to acute liver disease [31]. These results could mean that IL-17A levels increase with disease severity or stage. This is further supported by studies previously performed by Du et al in 2013 which showed that IL-17A expression in patient liver tissue was positively correlated with the degree of inflammation and the stage of fibrosis [32] as well as Studies by Du et al and Elkhawaga et al have found that the highest levels of IL-17A were observed in patients with cirrhosis of the liver compared to those with chronic hepatitis. This suggests that IL-17A may play a role in the pathogenesis and progression of HCC [33]. IFN- γ is known to be associated with chronic liver disease and HCC through its interaction with other pro-inflammatory cytokines. These findings suggest that the balance of cytokines plays a crucial role in the development and progression of liver diseases and HCC. Further research is necessary to fully understand the role of IFN- γ and IL-17A in the pathogenesis and progression of HCC [31].

Our results indicated that patients with HCC (406.0 ± 72.7 pg/ml) had significantly elevated serum VEGF levels compared to those without liver cirrhosis (187.3 ± 34.4 pg/ml) and chronic hepatitis (168.8 ± 24.2 pg/ml). Our findings were consistent with those of Alzamzamy et al, who reported higher VEGF levels in the HCC group compared to the cirrhosis group (1409 pg/ml vs 233.3 pg/ml) and the control group (1409 pg/ml vs 204 pg/ml) [34]. Youssef et al also observed higher VEGF levels in HCC patients ($2180.8 \pm 1,621.4$ pg/ml) compared to patients with cirrhosis ($1605.3 \pm 4,082.4$ pg/ml) and healthy individuals (561.0 ± 251.7 pg/ml) [35]. These findings suggest that VEGF may play a role in liver angiogenesis and carcinogenesis through hypoxia-induced upregulation of VEGF gene expression. VEGF has been proposed as a potential tumor marker for HCC, as HCC is a highly vascular tumor that requires angiogenesis for growth and survival, and elevated VEGF levels have been linked to poor tumor clinical features and prognosis. In line with previous studies, our results showed that higher serum VEGF levels were associated with poor HCC prognosis. Thus, monitoring serum VEGF levels could aid in early HCC diagnosis and provide clinicians with valuable information about disease prognosis [34]. These

findings suggest that VEGF may play a role in liver angiogenesis and carcinogenesis through hypoxia-induced upregulation of VEGF gene expression. VEGF has been proposed as a potential tumor marker for HCC, as HCC is a highly vascular tumor that requires angiogenesis for growth and survival, and elevated VEGF levels have been linked to poor tumor clinical features and prognosis [36]. In line with previous studies, our results showed that higher serum VEGF levels were associated with poor HCC prognosis [37]. Thus, monitoring serum VEGF levels could aid in early HCC diagnosis and provide clinicians with valuable information about disease prognosis [39].

Besides its role in the diagnosis of HCC, higher serum AFP levels were also observed in participants with HCC. AFP is currently the most widely used serum biomarker for screening and early diagnosis of HCC, as well as for evaluating the efficacy and prognosis of HCC treatment. However, not all HCC tumours contribute to an increase in AFP [36]. In this study, we found that higher serum VEGF levels were significantly associated with HCC compared to patients without liver cirrhosis and chronic hepatitis. This finding is in line with previous studies that have suggested that VEGF could serve as a potential biomarker for HCC diagnosis. Our results also showed that there was no correlation between VEGF and AFP, which is in contrast to previous studies that reported a correlation between the two markers. However, this discrepancy could be due to the role of VEGF in the pathogenesis and spread of HCC, as not all HCC tumours result in an increase in AFP levels [37,38].

At a threshold of 200 pg/ml, VEGF had a sensitivity of 80% and a specificity of 70%. On the other hand, AFP had a sensitivity of 80% and a specificity of 35% for HCC diagnosis. These results are similar to previous studies, such as Alzamzamy et al who found a threshold of 250 pg/ml VEGF had a sensitivity of 80% and a specificity of 81.7% [40]. Our results were also comparable to Mukozu et al who reported a sensitivity of 86.4% and a specificity of 96.2% for VEGF with a threshold value of 108 pg/ml [41]. However, our results contrast with those of Sadik et al who found a high specificity (100%) and a weak sensitivity (23.8%) for AFP in HCC diagnosis [42]. In comparison, Yvamoto et al found that VEGF levels had a sensitivity and specificity of 65% and 85%, respectively, while AFP had a sensitivity and specificity of 28% and 99% for HCC diagnosis [43]. These findings suggest that VEGF could be a more accurate biomarker for the diagnosis of HCC compared to AFP.

In our study, we found that the sensitivity and specificity of VEGF in the diagnosis of HCC were 80% and 70% respectively, at a threshold of 200 pg/ml. On the other hand, the sensitivity and specificity of AFP in the diagnosis of HCC were 80% and 35% respectively, at a threshold of 100 ng/ml. These results are in line with previous studies by Alzamzamy et al and Mukozu et al who also found high sensitivity and specificity of VEGF for HCC diagnosis [41,43]. However, our results are not in line with the findings of Atta et al, Sadik et al and Yvamoto et al, where higher sensitivity and specificity were found for AFP, in the diagnosis of HCC [42,43]. This discrepancy highlights the need for further research in this area to establish the most reliable biomarker for HCC diagnosis.

As limitations, it's worth mentioning the absence of staging for participants who had cirrhosis and HCC in the study. Additionally, we did not analyze the genetic variations of the biomarkers in our investigation to examine the genetic relationships. Another limitation is that we did not examine the relationship between VEGF levels and the effectiveness of antiviral therapies for viral hepatitis B and C, as well as the impact of HCC antitumor therapies on VEGF levels, as it is possible that VEGF levels could predict the response to these treatments when used in combination with other treatments in the patient groups studied.

5. Conclusion

In conclusion, our study showed that the immunological biomarkers IL-6 and VEGF, both pro-inflammatory and growth factors, demonstrated good diagnostic performance. These biomarkers have proven to be effective in assisting with the diagnosis of HCC in patients with chronic liver disease. To improve early diagnosis of HCC, we recommend to use these biomarkers in conjunction with AFP and medical imaging.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability Statement

The data supporting the results of this study are available on request from the corresponding author. The data is not publicly available because it contains information that could compromise the confidentiality of research participants.

List of Abbreviations

AFP: Alpha foetoprotein
 AUC: Area Under the ROC curve
 CI: Confidence interval
 FN: False Negative
 G-CSF: Granulocyte Colony Stimulating Factor
 HCC: Hepatocellular Carcinoma
 HIV: Human Immunodeficiency Virus
 IFN: Interferon
 IL: Interleukin
 MIP-1: Macrophage Inflammatory Protein-1
 NPV: Negative Predictive Value
 PPV: Positive Predictive Value
 PD-1: Programmed Death 1
 PIVKA-II: Protein Induced by Vitamin K Absence or Antagonist
 ROC: Receiver Operating Characteristic
 RANTES: Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
 Se: Sensitivity
 r: Spearman's correlation coefficient
 Sp: Specificity
 ES: Standard Error
 TP: True Positive
 VEGF: Vascular Endothelial Growth Factor

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