RESEARCH ARTICLE

Determination of correlation between cytokines IL 6, IL8, IL10, IL12, IL17 and severity of hepatocellular carcinoma in patients having hepatitis C

Ishtiaq Ahmad[1], Hamidullah[2], Ashraf[3], Murad Ali[4], Manzoor Hussain[5], Rehman Ud Din[6]

Abstract

Objective: To determine the correlation between the levels of interleukins 6, 8, 10, 12 and 17 and the severity of hepatocellular carcinoma in patients positive for hepatitis C.

Method: The retrospective, cross-sectional, analytical study was conducted at the Department of Medicine, Mardan Medical Complex, Mardan, Khyber Pakhtunkhwa, Pakistan, and comprised data of hepatocellular carcinoma patients who were positive for hepatitis C on polymerase chain reaction test from March 2021 to December 2022. A control group of healthy patients was also included. The severity of hepatocellular carcinoma was assessed using the Barcelona Clinic Liver Cancer method, and the patients were classified into five stages: very early stage 0, early-stage A, intermediate-stage B, advanced-stage C, and end-stage D. Interleukin 6, 8, 10, 12 and 17 levels and disease severity were correlated using Pearson correlation coefficient. Data was analysed using SPSS 26.

Results: Of the 115 subjects, 67(58.3%) were patients and 48(41.7%) were controls. There were 72(62.6%) males and 43(37.4%) females. The median age of patients was 56 years (interquartile range: 38-64 years), and among the cases it was 36 years (interquartile range: 32-44 years). Among the patients, 8(11.94%) were at stage 0, 11(16.41%) stage A, 24(35.82%) stage B, 13(19.40%) stage C, and 11(16.41%) stage D. Intergroup differences indicated a significant difference, with interleukin levels being higher in patients with more advanced stages ($p<0.05$).

Conclusion: The use of interleukin levels as biomarkers could facilitate earlier detection of advanced-stage disease, leading to more timely interventions and potentially improving patient outcomes.

Key Words: Interleukins, Hepatitis C, Hepatocellular carcinoma, Cytokines.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer globally, accounting for 75-85% of all occurrences.[1] It is a primary liver cancer that arises from hepatocytes. HCC is the fourth leading cause of cancer-related fatalities worldwide, accounting for roughly 700,000 deaths each year.[2] Pakistan significantly adds to the global burden of hepatitis C, a recognised risk factor for HCC, and has one of the highest prevalence rates in the world, exceeding 3%.[3] Infection with the hepatitis C virus (HCV) is a substantial risk factor for the development of HCC, with an estimated 3% of the world's population affected.[4] Several variables influence the progression of HCC, including viral, host, and environmental factors. Among these, the immunological response of the host has been linked to the progression of HCC and its response to treatment.[5] The Barcelona Clinic Liver Cancer (BCLC) staging method is a commonly used approach for stratifying HCC patients based on tumour features, liver function and overall performance.[6] The system can help determine prognosis and guide treatment recommendations.

Interleukins (ILs) are cytokines, which are proteins that are required for cell signalling and immunological responses. They are produced by a wide range of cell types, including immune cells, like T cells, macrophages, and dendritic cells, as well as non-immune cells, including endothelial cells and fibroblasts.[7] ILs play a role in several aspects of immunological control, including immune cell activation and differentiation, inflammation and tissue repair.[8] ILs, because of their function in immunological responses, have been linked to the development and progression of several malignancies, including HCC.

Several ILs have been implicated in HCC growth and the host's immunological response to the tumour. IL6 is a pleiotropic cytokine with both pro- and anti-inflammatory actions, and it has been linked to HCC development via the Janus Kinase 2/ Signal Transducer and Activator of Transcription 3 (JAK2/STAT3) signalling

[1,]Department of Accident and Emergency, Mardan Medical Complex, Bacha Khan Medical College, [2,4,5]Department of Medicine, Mardan Medical Complex, Bacha Khan Medical College, [3]Pro-Gene Diagnostics and Research Laboratory, [6]Department of Gastroenterology, Mardan Medical Complex, Bacha Khan Medical College, Mardan, Pakistan.

Correspondence: Hamidullah. **Email:** dr.hamidullah2013@gmail.com

ORCID ID: 0009-0008-1796-1816

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pathway.[9] IL8 is a pro-inflammatory cytokine and a powerful neutrophil chemoattractant, and its overexpression has been linked to cancer development.[10] IL10 is an anti-inflammatory cytokine that suppresses the immune response to tumours, and high levels of IL10 have been linked to a poor prognosis in cancer patients.[11] IL12 is a heterodimeric cytokine that stimulates T-helper 1 (Th1) cell development, and increases the cytotoxic activity of natural killer (NK) cells. By boosting the host's immune response against the tumour, IL12 has been demonstrated to have anti-tumour effects in a variety of malignancies.[12] IL17 is a pro-inflammatory cytokine that is primarily generated by Th17 cells, and has been linked to angiogenesis, tissue inflammation, and tumour growth.[13]

Despite the established role of cytokines in the progression of HCC, significant gaps in human understanding remain. Specifically, it is unclear how cytokine profiles differ between HCC arising from various aetiologies, such as chronic hepatitis C versus alcohol-related HCC. Previous studies have largely focussed on cytokines as biomarkers in a general context,[14] without distinguishing between different underlying causes of HCC. This gap highlights the need for targetted research to delineate the specific cytokine patterns associated with different aetiologies of HCC.

Understanding these differences is crucial for developing targetted diagnostic and therapeutic strategies. The current study was planned to examine the correlation of IL-6, IL-8, IL-10, IL-12 and IL-17 levels with the BCLC stage of HCC in HCV-positive individuals.

Patients and Methods

The retrospective, cross-sectional, analytical study was conducted at the Department of Medicine, Mardan Medical Complex, Mardan, Khyber Pakhtunkhwa (KP), Pakistan, and comprised data from March 2021 to December 2022 of HCC patients who were HCV-positive on polymerase chain reaction (PCR) test. A control group of healthy patients was also included.

The sample size calculation for the study was determined using the OpenEpi software, employing a two-sided test with a significance level of 0.05 and a power of 80%. Based on the expected effect size and prevalence rates, the minimum sample size required was calculated to ensure reliable and valid results. This calculation considered the proportion of patients with advanced hepatocellular carcinoma (HCC) stages and healthy controls, aiming to achieve adequate statistical power to detect significant differences in interleukin levels and their correlation with HCC severity. The final sample size was set to 115 participants, including 67 HCC patients and 48 healthy controls, to meet these criteria.[15]

Approval was obtained from the institutional ethics review board, while informed consent had been taken from all patients or their legal representatives. The sample was raised using convenience sampling technique.

Patients were included if they had a confirmed HCC diagnosis based on histopathological and radiological criteria, had serological determination of tumour marker alpha fetoprotein (AFP), were positive for HCV infection on PCR, and had complete clinical and laboratory data. Data of patients having a history of other liver illnesses, autoimmune disorders, concomitant malignancies was excluded, and so were cases with insufficient clinical and laboratory data.

Data was retrieved from the medical records section. Patients' ascites was classified using the International Club of Ascites (ICA) system as mild (detectable only by ultrasound), moderate (symmetrical abdominal distension) and severe (marked abdominal distension).[16] Patients' health state was defined using the Eastern Cooperative Oncology Group (ECOG) performance status testing, and the patients were divided into five stages; PS 0: fully functional as before the illness, PS 1: unable to perform strenuous physical labour, but able to perform any other task, PS 2: spending more than half of the day awake, can take care of oneself but cannot work, PS 3: spending more than half of the waking hours in bed or a chair, and require help for personal care, and PS 4: always in bed or a chair and require 24-hour care.[6]

The Child-Pugh classification system was used to see how well the liver was working based on bilirubin levels in the blood, albumin levels in the blood, prothrombin time (PT), ascites, and encephalopathy. Each factor was assigned a number score, and the patients were divided into 3 groups depending on that value; class A: the liver operating normally, class B: mild to moderate damage, and class C: severe liver damage.[6]

The BCLC staging method was used to identify HCC severity, and the patients were classified into very early stage 0, early stage A, intermediate stage B, advanced stage C, and end stage D.[6]

Different blood biomarkers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase transferase (ALP), total bilirubin (T.Bil), direct bilirubin (D.Bil), indirect bilirubin (I.Bil) and total protein, were measured using Cobas C11 biochemistry analyser made in Switzerland, while PT, activated partial thromboplastin time (APTT) and international normalised ratio (INR) were measured using

Helena C-4 series coagulometer analyser made in United kingdom. Serum levels of IL-6, IL-8, IL-10, IL-12, IL-17 and AFP were measured using enzyme-linked immunosorbent assay (ELISA) kits as per the manufacturer's instructions by ELK biotechnology made in the united states of America.

Data was analysed using SPSS 26. Continuous variables were expressed as median and interquartile range (IQR), while categorical variables were reported as frequencies and percentages. Kruskal Wallis test was used to analyse intergroup differences in HCC severity stages and ILs with 95% confidence interval (CI). The correlation between IL levels and HCC severity was assessed using Spearman correlation coefficient. Relationship between HCC severity and ILs was studied using Linear regression in scatter plots. The discriminatory ability of IL levels for predicting advanced HCC was evaluated using receiver operating characteristic (ROC) curve analysis and calculating the area under the curve (AUC). The AUC ranged from 0.5 to 1.0, where an AUC of 0.5 indicated no discriminative ability, and values >0.5 indicated a greater fit of the model and higher predictive accuracy. P<0.05 was considered statistically significant.

Results

Of the 115 subjects, 67(58.3%) were patients and 48(41.7%) were controls. The control group consisted of individuals without HCC and HCV, who were matched with the patient group for age, sex, and other relevant demographic factors to serve as a baseline comparison. There were 72(62.6%) males and 43(37.4%) females. The median age of the patients was 56 years (IQR: 38-64 years), and among the cases it was 36 years (IQR: 32-44 years). Among the patients, 8(11.94%) were at BCLC stage 0, 11(16.41%) stage A, 24(35.82%) stage B, 13(19.40%) stage C, and 11(16.41%) stage D. Clinical and laboratory data was noted in detail (Table 1).

There were 46(68.56%) patients with mixed-sized tumours, 46(68.56%) had >3 nodules, 17(25.37%) had vascular invasion, and 7(10.44%) had extrahepatic dissemination. The HCC group's liver function values and AFP level revealed considerable departure from normal values (Table 2).

Intergroup differences indicated a significant difference, with I: levels being higher in patients with more advanced stages ((Table 3).

There was a positive and robust relationship between each IL level and HCC severity (Table 4).

Varying degrees of positive relationship between the different ILs and BCLC stages were noted, with the

Table-1: Characteristic of study participants

Characteristics	HCC group	Control group
Patients	67(100)	48(100)
Gender		
Male	44(65.67)	28(58.33)
Female	23(34.32)	20(41.66)
Age	56(38-64)	36(32-44)
Symptoms		
Ascites		
Mild	18(26.86)	0.00
Moderate	24(35.82)	0.00
Severe	25(37.31)	0.00
Encephalopathy		
Absent	12(17.91)	0.00
Mild	31(46.26)	0.00
Severe	24(35.82)	0.00
Weight loss	58(86.56)	0.00
Jaundice	67(100)	0.00
Nausea	52(77.61)	0.00
Fever	62(92.53)	0.00
Performance status of hepatocellular carcinoma hepatitis c positive patients		
P0	35(52.23)	0.00
P1	03(4.47)	0.00
P2	05(7.46)	0.00
P3	13(19.40)	0.00
P4	11(16.41)	0.00
Child-Pugh system		
Class A	15(22.38)	0.00
Class B	35(52.53)	0.00
Class C	17(25.37)	0.00
BCLC staging system for severity of hepatocellular carcinoma hepatitis c positive patients		
Stage 0	8(11.94)	0.00
Stage A	11(16.41)	0.00
Stage B	24(35.82)	0.00
Stage C	13(19.40)	0.00
Stage D	11(16.41)	0.00

Data is presented as frequency and percentage or as the median with interquartile range (IQR). BCLC: Barcelona Clinic Liver Cancer.

Table-2: Diagnostic confirmation of HCC and HCV.

Characteristics	HCC group	Control group
Patients	67(100)	48(100)
Anti-HCV Antibodies		
Positive	67(100)	0.00
Negative	0.00	0.00
Viral load by PCR log10 IU/mL	6.35(4.86-8.32)	0.00
Diagnostic confirmation of HCC		
Radiological and histopathological	67(100)	0.00
Tumour Size		
≤ 2cm	08(11.94)	0.00
≤ 3 cm	13(19.40)	0.00
Mixed Size	46(68.56)	0.00

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Nodules		
Single	13(19.40)	0.00
Up To 3	08(11.94)	0.00
More Than 3	46(68.56)	0.00
Vascular Invasion	17(25.37)	0.00
Extrahepatic Spread	07(10.44)	0.00
Alpha-fetoprotein (AFP) Normal: <20ng/ml	1329(349-3042)	3.91(1.22-4.51)
Liver Function Tests		
Alanine Aminotransferase (ALT) Normal: <43 u/l	199(171-286)	25.50(18.25-34.75)
Aspartate Aminotransferase (AST) Normal: <40 u/l	155(122-231)	31.00(26.25-35.00)
Alkaline Phosphatase Transferase (ALP) Normal: 44-147 iu/l	464(398-872)	119(94-143)
Albumin (Alb) Normal:3.4-5.4 g/dl	2.10(1.52-3.45)	4.35(4.12-4.61)
Total bilirubin (T.Bil) Normal: 0.1-1.2 mg/dL	5.61(4.74-6.44)	0.90(0.89—0.91)
Direct Bilirubin (D.Bil) Normal: 0..0-0.3 mg/dL	3.48(2.60-4.42)	0.60(0.59-0.61)
In- Direct Bilirubin (I.Bil) Normal: 0.2-0.8 mg/dL	2.00(1.91-2.31)	0.30(0.29-0.31)
Prothrombin time (PT) Normal: 11-15 seconds	138(108-157)	13(12-14)
Activated partial thromboplastin time (APTT) Normal: 21-35 seconds	107(81-132)	29(26-31)
international normalized ratio (INR) Normal: <1.0	9.2(7.2-10.4)	0.86(0.80-0.93)

Data is presented as frequency and percentage or as the median with interquartile range (IQR).

HCC: Hepatocellular carcinoma, HCV: Hepatitis C virus, PCR: Polymerase chain reaction.

strongest relationship being with IL-6, followed by IL-17, IL-8, IL-12 and IL-10 (Figure 1).

AUC values indicated that IL-6, IL-8, IL-10, IL-12 and IL-17 had a good predictive accuracy in identifying advanced-stage HCC, with IL-6 exhibiting excellent predictive accuracy, IL-10 and IL-12 displaying good predictive accuracy, and as IL8 and IL17 showing acceptable predicative accuracy (Figure 2).

Discussion

The current study investigated the correlation between various IL levels with HCC severity in HCV-positive patients, using the BCLC staging system. The findings revealed potential correlations between the examined IL levels and HCC severity, contributing to the understanding of the role of cytokines in HCC progression.

Findings related to ECOG scale and liver function testing demonstrated a wide range of illness severity in the current sample. This variation in disease presentation was consistent with literature.[17]

The presence of anti-HCV antibodies in all HCC patients was noteworthy, as it confirmed the well-established link between HCV infection and HCC development. The median viral load (6.35 log10 IU/mL) identified in the current study is within the range reported in other studies, suggesting HCV's significance as a risk factor for HCC.[18] In addition, when compared to normal ranges, HCC patients had elevated levels of AFP, ALT, AST, ALP, T.Bil, D.Bil, I.Bil, PT, APTT and INR. These findings were consistent with an earlier study that showed the potential of these biomarkers for the diagnosis and monitoring of liver illnesses, such as HCC. Although the sensitivity and specificity of all liver function tests and AFP are variable and not always ideal, elevated AFP has been frequently employed as a diagnostic biomarker for HCC.[19] As a result, histological and radiographic results were used to confirm HCC diagnosis in the current study, which is considered the gold standard for HCC diagnosis.[20]

Table-3: Level of interleukins based on the Barcelona Clinic Liver Cancer (BCLC) staging system.

HCC severity	All	Stage0	Stage A	Stage B	Stage C	Stage D	p-value
Interleukins							
IL-6	85.95(52.62-121.21)	10.39(5.39-17.67)	48.49(29.00-66.57)	72.92(63.25-108.54)	123.00(108.43-168.18)	118.95(98.00-226.02	0.00
IL-8	340.77(151.27-496.09)	40.94(29.57-43.89)	330.24(181.25-366.02)	399.08(187.46-494.24)	346.67(266.25-542.16)	694.94(259.05-996.13)	0.00
IL-10	51.11(16.00-222.00)	5.29(3.98-27.25)	13.33(5.13-18.18)	49.18(22.50-197.00)	172.50(70.61-376.50)	163.00(64.63-267.00)	0.00
IL-12	81.09(28.30-168.13)	22.20(18.31-27.07)	38.06(12.35-55.02)	123.26(63.66-165.49)	132.14(66.35-318.46)	245.03(44.94-256.45)	0.00
IL-17	58.42(26.51-92.21)	15.86(6.72-18.27)	30.81(24.61-47.78)	60.37(30.53-68.81)	89.56(39.71-123.51)	134.06(76.26-247.29)	0.00

Data is presented as frequency and percentage or as the median with interquartile range (IQR). IL: Interleukin. p<0.05 was statistically significant.

Table-4: Spearman correlation coefficients between interleukin levels and severity of hepatitis C virus (HCV) infection

Interleukins	Correlation Coefficient	p-value	95%cl	
			Lower bound	Upper bound
IL-6	0.775	0.00	77.37	106.29
IL-8	0.733	0.00	300.25	426.21
IL-10	0.691	0.00	86.38	155.18
IL-12	0.696	0.00	97.66	173.46
IL-17	0.741	0.00	55.08	88.20

IL: Interleukin. p<0.05 was statistically significant.

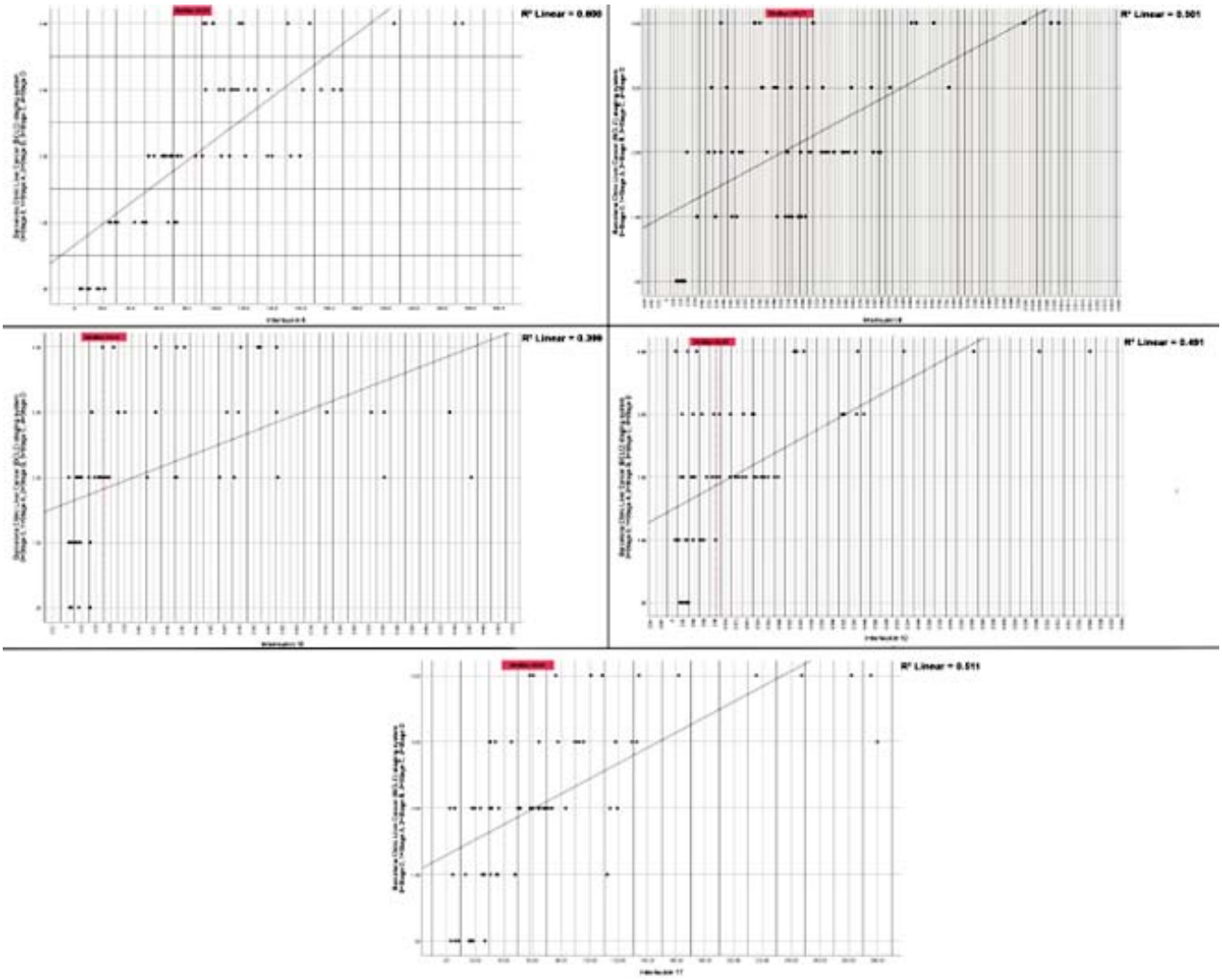

Figure-1: Scatter plot analysis of interleukins.

The current study found a substantial correlation coefficient of 0.775 between IL-6 levels and the severity of HCC, as well as a link between high IL-6 levels and advanced-stage HCC along with inferior clinical outcomes. Shakiba E. et al. in a meta-analysis discovered that elevated IL-6 levels in HCC patients compared with hepatitis and cirrhosis patients, and healthy controls showed a significant association.[21] As a result, IL-6 may be a useful predictive biomarker for HCC severity in HCV-positive individuals. The substantial relationship between IL-6 and HCC severity emphasises the necessity of early detection and intervention in high-risk patient populations. The adoption of IL-6 as a predictive marker for HCC patients could contribute to the development of targetted medicines and individualised treatment regimens.

As for the involvement of IL-8 in the progression of HCC, Wang Z et al. emphasised the importance of angiogenesis in cancer progression and therapy, emphasising the role of IL-8 as a pro-angiogenic cytokine in cancers.[22] The significance of IL-8 in stimulating angiogenesis and tumour growth is well known. IL-8 increases angiogenesis by activating the Phosphoinositide 3-Kinase / Protein Kinase B (PI3K/AKT) signalling pathway. IL-8 is involved in HCC cell migration and invasion, contributing to disease development.[23] The current findings were in line with such findings.

The findings are consistent with previous studies on the involvement of IL-10 in the evolution of HCC. Gao L. et al. discovered that IL-10 promoter polymorphisms were substantially associated with the likelihood of developing HCC, particularly in individuals infected with hepatitis B

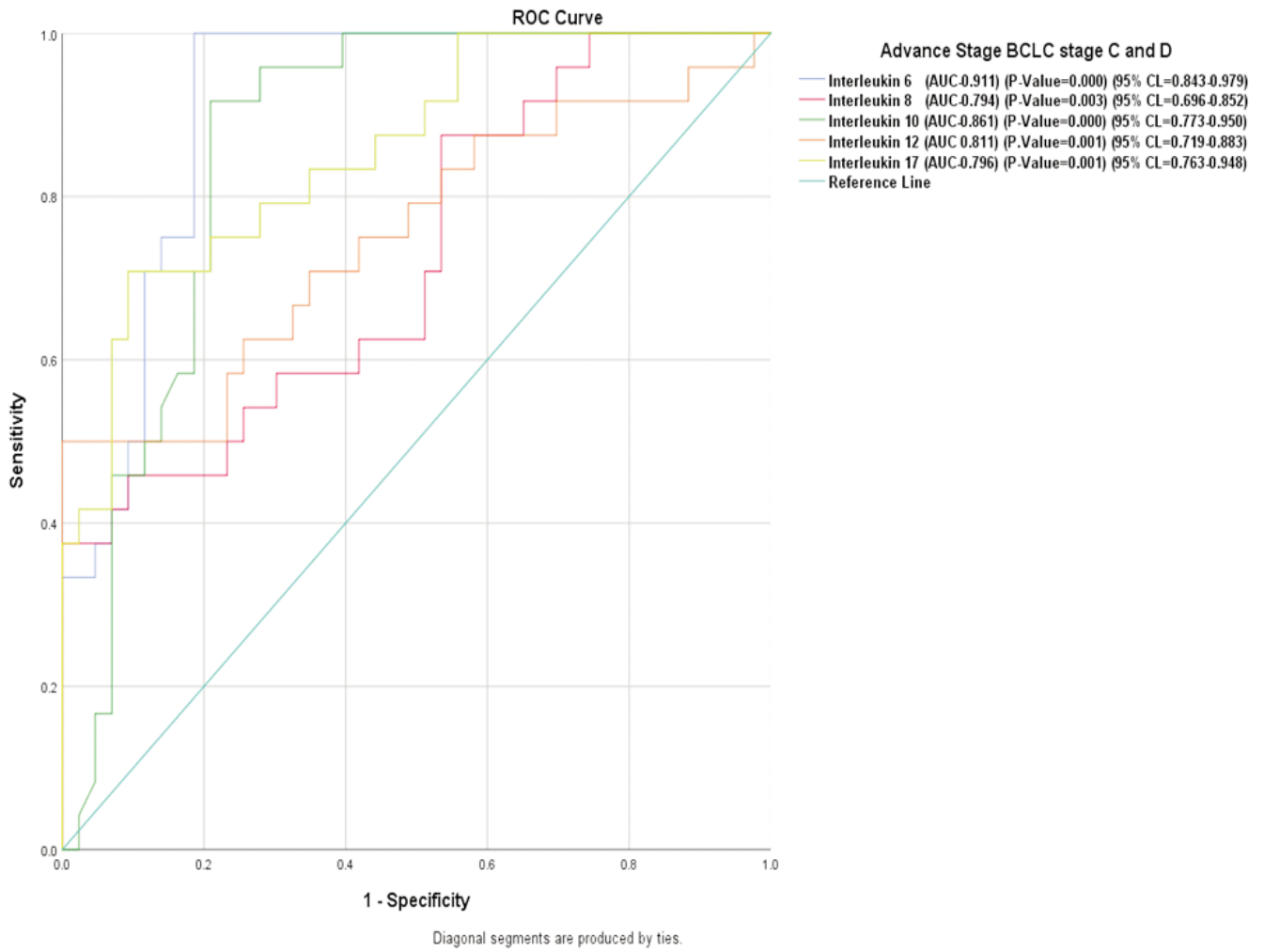

Figure-2: Receiver operator characteristic (ROC) curve analysis of Barcelona Clinic Liver Cancer (BCLC) advanced stages C and D.

virus (HBV).[24] Furthermore, the immunosuppressive characteristics of IL-10 have been extensively established. For example, study investigated the significance of IL-10 in immune response regulation, specifically its role in suppressing pro-inflammatory cytokines and lowering tissue damage. The link between IL-10 and HCC severity in hepatitis C patients, as established in the current study and corroborated by prior research[25], emphasises the importance of IL-10 as a possible biomarker for HCC progression. This could be especially helpful in the case of HCV-associated HCC, allowing for more precise staging and more tailored therapy methods.

In the current study, the increased IL-12 levels was associated with a poor outcome in HCC patients, implying that IL-12 may contribute to HCC aggressiveness. Similarly, Yaojie Fu et al. discovered that the overexpression of AFP by HCC cells indirectly impaired the production of IL-12 by dendritic cells (DCs), which in turn reduced the release of cytotoxic effector molecules, the expression of NK group 2 member D (NKG2D), an activating receptor on NK cells, and the ability of NK cells to activate.[26] The r2 value of 0.49 obtained from the linear regression analysis of the scatter plot supported the significant association between IL-12 levels and HCC severity seen in the current study. IL-12 levels could explain about 49% of the diversity in HCC severity, underlining IL-12's potential as a good biomarker for HCC progression. Furthermore, the AUC of 0.81 for stages C and D indicated that IL-12 had a good diagnostic accuracy in distinguishing between advanced and less advanced HCC stages, which may benefit physicians in prognostic assessment and treatment decision-making for HCC patients.

The AUC of 0.79 for stages C and D indicated that IL-17 had a good diagnostic accuracy in distinguishing between advanced HCC stages C and D. The current

findings were consistent with a prior study that found a link between elevated IL-17 levels and HCC development.[27]

The current study has limitations. First, the study employed a cross-sectional design, which only provided a snapshot of the data at a specific point in time, and limited the ability to establish causal relationships or track changes over time. Longitudinal studies would be beneficial in understanding the dynamics of IL levels and their association with HCC progression in HCV-positive patients. Second, the limited sample size and the single-centre nature of the study may have reduced the generalisability of the findings. Future studies should not only involve multi-centre collaborations, but also enrol all patients presenting to the study site. Third, the current study did not include patients with comorbidities. Future research should investigate the impact of comorbidities on IL levels in HCV-positive HCC patients. Fourth, the current study did not analyse the genotype of the HCV patients, as this information was not available for all patients at the time of the study. Fifth, the current study did not explore the potential for IL-targetting therapies in HCC patients with HCV infection. Sixth, the current study used linear regression for analysis, which is typically based on means, but the data was non-parametric and had to be reported in median terms. Seventh the study utilized a convenience sampling technique to select participants, which allowed us to include individuals who were readily available and accessible. While this approach facilitated the inclusion of participants within a practical timeframe, it also introduced potential biases. Convenience sampling may not fully represent the broader population, limiting the generalizability of the findings. The sample may not capture the diversity of characteristics present in a more randomized population, and thus, results should be interpreted with caution when applying them to different settings or populations.

Conclusion

A positive correlation was noted between specific cytokines and HCC stages in patients with chronic hepatitis C. Elevated levels of these interleukins were associated with more advanced stages of HCC, indicating their potential as biomarkers for disease progression.

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Conflict of Interest: None.

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IA: Data collection, analysis, interpretation, design, revision, drafting and final approval.
H: Data collection, analysis, interpretation, design, revision and drafting.
A: Data collection, analysis, interpretation, design and drafting.
MA, MH: Data collection, revision and drafting.
RUD: Drafting and final approval.