RESEARCH ARTICLE

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Dysregulated Interleukin Expression in Iraqi Hepatocellular Carcinoma: Diagnostic and Prognostic Implications

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Abstract

Background: Chronic inflammation associated with viral hepatitis and other environmental factors contributes to the development of liver diseases, including hepatocellular carcinoma, which represents an important health issue in Iraq. The aim of this study was to determine the expression levels of interleukin-6, interleukin-10, interleukin-17, and interleukin-23 in hepatocellular carcinoma patients and evaluate the clinical applicability of these genetic markers. **Methods:** The study was designed as a case-control study including 242 patients with hepatocellular carcinoma and 288 age- and sex-matched healthy controls. We used qPCR and ELISA assays to determine the studied markers. **Results:** Each of the pro-inflammatory interleukins described showed significant dysregulation in patients with hepatocellular carcinoma. Relative to the controls, the gene expression of *IL-6*, *IL-17*, and *IL-23* showed increases of 8.74-fold, 6.92-fold, and 7.38-fold**, respectively** ($p < 0.001$). There was a 69% decrease in the expression of *IL-10* ($p < 0.001$). A similar trend was found in the serum concentrations compared with the expression of the studied genes. There were significant positive correlations between the inflammatory interleukins and the size of the tumour, the concentration of alpha-fetoprotein, and the stage of the disease. The integrated interleukin model demonstrated excellent diagnostic performance with an AUC of 0.943. **Conclusion:** There were significant dysregulation of the inflammatory interleukins in Iraqi patients with hepatocellular carcinoma with substantial correlations to the severity of the disease. These findings demonstrate the potential use of inflammatory interleukins as diagnostic and prognostic markers and highlight the need for further research to validate therapeutic targets.

Keywords: Hepatocellular carcinoma, Interleukin-6, Interleukin-10, Interleukin-17, Interleukin-23

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and represents a major global health issue, being the sixth most commonly diagnosed cancer and the third leading cause of cancer-related deaths [1]. The incidence of HCC has been increasing, with geographically specific elevation attributable to the differential prevalence of associated risk factors [2]. In Iraq, HCC has a notable health impact, with high rates being associated with the burdens of viral hepatitis, especially HCV and HBV, and additional environmental and health system challenges [3]. The development of HCC is complex and associated with the sequelae of chronic liver inflammation, cirrhosis and injury to hepatocytes with the eventual development of cancer [4]. Chronic inflammation is recognized as the most important driver of HCC as it forms a unique microenvironment for the disease by promoting the aberrant variability of the cancer genome (genomic instability), subverting apoptotic mechanisms, and eliminating apoptosis, all of which are crucial phenomena for tumour evolution [5]. In the inflammatory environment, the mediators of inflammation, and most importantly the cytokines of the interleukin class, steer the inflammatory and immune response and can induce processes of cell behaviour alteration that are integral to tumour formation and growth [6].

Interleukins form a unique group of cytokines that mediate interactions among immune cells and manage inflammation [7]. Specifically, *IL-6*, *IL-10*, *IL-17*, and *IL-23* have been studied in relation to cancer, including HCC [8, 9]. *IL-6* activates particular immune cells and also activates the *JAK-STAT3* pathway, leading to increased hepatocyte proliferation, angiogenesis, and the inhibition of apoptosis, all of which contribute to the formation of tumours [10]. Many studies have reported increased levels of *IL-6* during the course of HCC and the higher *IL-6* levels the worse the HCC prognosis [11, 12]. As a classical

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anti-inflammatory cytokine, *IL-10* has also been described as having a multifaceted and context-dependent role in cancer [13]. *IL-10* has the ability to counter anti-tumour immune responses by inhibiting effector T cell function, and under some circumstances, it may also exert direct anti-proliferative effects on the tumour cells [14]. There are studies describing *IL-10* as elevated and/or decreased in different patient populations, which contributes to the lack of understanding regarding *IL-10* role in HCC [15].

Another important inflammatory pathway in hepatocarcinogenesis is the *IL-17*/*IL-23* axis [16]. *IL-17* acts mainly secreted from T helper 17 (Th17), accelerates tumour growth by recruiting immunosuppressive myeloid cells and increasing endothelial cell factors angiogenesis [17]. *IL-23* released from antigen presenting cells [18]. The combined dysregulation of these interleukins promotes the inflammatory pro-tumorigenic milieu that facilitates the development and progression of HCC [19]. HCC epidemiology and pathogenesis are influenced by the environmental and healthcare challenges of the Iraq. The unique epidemiological context of Iraq warrants particular attention in understanding HCC pathogenesis in this population. Armed conflicts and environmental contaminations with likely carcinogenic materials, which included the Gulf War (1991), sanctions period (1990-2003), and the Iraq War (2003-2011), were documented [20]. Environmental health hazards have been reported, including depleted uranium munitions, oil well fires, and industrial pollution [21]. Excessive concentrations of heavy metals and toxic materials that pose long term carcinogenic risk have been observed in Iraq's soil and water sources [22].

The neglect of disease surveillance and of control measures during conflicts and the ensuing impacts on healthcare infrastructure have likely escalated the burden of chronic viral hepatitis, a leading driver of HCC [23]. In the context of Iraq, the overlapping environmental exposures and chronic endemic infections may activate a distinct inflammatory milieu that promotes inflammation-driven carcinogenesis at an accelerated pace [24]. The combination of genetic predisposition, environmental toxins, and chronic viral infections in this population may result in unique inflammatory profiles that differ from other geographical regions.

The lack of comprehensive studies on the current situation leading to conduct a thorough appraisal of the expression and serum concentration of inflammatory markers *IL-6*, *IL-10*, *IL-17*, *IL-23* among Iraqi patients with HCC. The expectation was that interleukins would show a marked difference, likely an increase for the pro-inflammatory cytokines and a decrease for anti-inflammatory cytokines, and patients with HCC will be significantly different from healthy controls. In addition, it was proposed that HCC interleukin levels will significantly associate with clinicopathological markers such as disease stage and tumour size, and with AFP, a well-established HCC biomarker.

This study aimed to: (1) determine the gene expression levels of *IL-6*, *IL-10*, *IL-17*, and *IL-23* in peripheral blood mononuclear cells of patients with HCC and healthy controls. (2) Evaluate the serum levels of the aforementioned interleukins. (3) Determine the relationship of interleukins and clinicopathological features. (4) Analyse the potential of the interleukins as biomarkers for HCC.

Materials and Methods

Study Design and Participants

A case-control study was performed between April 2024 and March 2025 at the internal medicine Teaching Hospital in Al-Diwaniyah, Iraq. The study was approved by the University of Al-Qadisiyah, College of Education, (Approval number: 2024-001). This was conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent. The study included 242 patients with hepatocellular carcinoma (HCC) and 288 as healthy controls. patients with HCC were seen at the hepatology and oncology outpatient clinics that were admitted to the wards. In accordance with the guidelines set by the American Association for the Study of Liver Diseases, the diagnosis of HCC was made based on the histopathological assessment of biopsy specimens and/or detailed imaging findings of dynamic contrast-enhanced CT or MRI, including arterial phase hyperenhancement and venous phase or delayed phase washout.

The criteria used to include patients with HCC were confirmed by histological examination or imaging; age ≥ 18 years (age range: 35-78 years); treatment-naive at the time of enrolment; and adequate blood samples were available. Inclusion criteria for controls were: no evidence of liver disease as assessed by liver function tests and abdominal ultrasound; age 18 years or older (age range: 33-76 years); no malignancies in the patient's history; and no chronic inflammatory or autoimmune disease. The criteria for exclusion were other malignancies; active infection for which antimicrobial therapy was necessary; autoimmune disease; received immunosuppressive therapy in the 3 months prior to study enrolment; pregnancy or lactation; and refusal to provide informed consent. Healthy control subjects were recruited from individuals who were attending the same institution for routine health check-ups. Inclusion criteria for controls were: no evidence of liver disease as assessed by liver function tests and abdominal ultrasound; age 18 years or older; no malignancies in the patient's history; and no chronic inflammatory or autoimmune disease. The exclusion criteria were the same as those for the patients.

Sample size was calculated to detect a 2-fold difference in interleukin levels between groups with 90% power and α=0.05, requiring approximately 220 patients per group. Based on this calculation and accounting for potential dropout, we enrolled 242 patients with HCC and 288 healthy controls.

Clinical and Laboratory Data Collection

All participants were assessed for demographic and clinical variables which included age, sex, body mass index, history of smoking, and comorbid conditions. For patients with HCC, other relevant clinical details were also reported that included the cause of liver disease,

BCLC stage, tumour size, number and location of the tumour, thrombosis of the portal vein, and extrahepatic disease spread.

Blood Sample Collection and Processing

Peripheral venous blood sample collection consisted of 15 mL, along with patient records for each of the participants, for a single overnight period in each of the days of the study. Blood collection was performed in two types of tubes; EDTA tubes along with serum separator tubes to capture the blood and its various components, to determine the immunological, biochemical, and serological assays which were integrated into the different components of the blood. Samples were collected in the morning after overnight fasting and transported to the laboratory within 30 minutes for testing in 30 min. The blood was processed immediately, as the fasting blood was essential to the minimal variance of the collection parameters. To isolate serum, blood in gel tubes were centrifuged at 4000 rpm for 5 minutes meanwhile separated serum was collected in new eppendrof tubes that were kept at -80 °C for a maximum of 3 months until further analysis.

RNA Extraction and cDNA Synthesis

Total RNA from PBMCs was extracted using TRIzol reagent as per the manufacturer's protocols (TriPure, China). RNA concentrations were measured using a QuantiFluor® RNA System (Promega, USA). cDNA was synthesized from 1 µg of total RNA using the (AddBio RT master, South Korea) according to the manufacturer's instructions, the reverse transcription reaction was conducted in a total of 40 µL which contained the following constituents: 1 µg RNA, 4 µL of 10× RT buffer, 1.6 µL of 25× dNTP mix (100 mM), 8 µL of 10× RT random primers, 2 µL of Reverse Transcriptase, and nuclease-free water. The reverse transcription reaction was set to the following conditions: 25°C for 10 minutes, 60°C for 60 minutes, and 80 C for 5 minutes. The synthesized cDNA was stored at -20 °C until use. RNA purity was assessed by measuring A260/A280 ratios using a NanoDrop spectrophotometer, with acceptable ratios ranging from 1.8 to 2.0. RNA integrity was evaluated by agarose gel electrophoresis. Three samples (2 from HCC patients and 1 from controls) were excluded due to degradation or insufficient RNA quality (A260/A280 < 1.7).

Real-Time Quantitative PCR Analysis

IL-6, *IL-10*, *IL-17*, and *IL-23* gene expression levels were analysed using CFX qPCR machine (BioRad, USA) using real-time quantitative polymerase chain reaction (RT-qPCR) assay. For normalization, the reference gene used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).All the primers were synthesized by (Macrogen, Korea) after designing using the Primer3 software, the primers are *IL-6*: F-ACTCACCTCTTCAGAACGAATTG, R-CCATCTTTGGAAGGTTCAGGTTG. *IL-10*: F-GACTTTAAGGGTTACCTGGGTTG, R- TCACATGCGCCTTGATGTCTG. *IL-17*: F-TCTGTGTCTCTGATGCTGTTGC, R-CGCCAAGGGAGTTAAAGACTTC. *IL-23*: F-AGCAGCTCTCTCGGAATCTC, R-GACTGAGGCTTGGAATCTGC. GAPDH: F-GAAGGTGAAGGTCGGAGTC, R-GAAGATGGTGATGGGATTTC. And Product Size: 149 bp, 162 bp, 138 bp, 155 bp and 226 bp, respectively. All qPCR reactions were performed in technical triplicates. The mean Cq values for GAPDH ranged from 18.2 to 20.8 in both HCC patients and controls. For target genes in HCC patients, mean Cq values were: *IL-6* (24.3-28.7), *IL-10* (28.9-32.4), *IL-17* (25.6-29.8), and *IL-23* (26.1-30.2). The efficiency of all primer sets ranged from 95% to 105%, as determined by standard curve analysis

Enzyme-Linked Immunosorbent Assay

Concentrations of *IL-6*, *IL-10*, *IL-17*, and *IL-23* in serum samples were measured through commercial enzyme-linked immunosorbent assays (ELISA) as per the guidelines provided by the manufacturers. BioBase microplate reader (China) was used to measure these markers using the Human IL-6, *IL-10*, *IL-17*A, and *IL-23* ELISA kits (BTlab, China). Every sample underwent a duplicate analysis, and for the statistical analysis, average values were computed. In brief, the procedure involved the use of 96-well microplates for which specific capture antibodies were attached. Appropriate wells were allocated, and standards and diluted serum samples (50 µL) were added and kept for incubation for 1 hour at 37C. Post-incubation washes were performed (5X), followed by the addition of horseradish peroxidase conjugated detection antibodies for a 1-hour incubation. Further washes were followed by sample colour development of the substrate 1 and 2 added for 20 mins in the dark. The absorbance (stop solution added to terminate the colour reaction) meanwhile was measured at 450 nm using a microplate reader. Cytokine concentration was calculated from the standard curves of recombinant proteins that were part of the kits by using interpolation.

Biochemical Analyses

Serum alpha-fetoprotein (AFP) levels were performed using a chemiluminescent microparticle immunoassay on an ARCHITECT i2000SR analyzer (Abbott Laboratories, Abbott Park, IL, USA). For liver function tests, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and albumin, evaluated using standard enzymatic and colorimetric methods on a Cobas 6000 automated analyzer (Roche Diagnostics, Mannheim, Germany).

Statistical Analysis

The statistical analyses were performed using SPSS software version 26.0 (IBM Corporation, Armonk, NY, USA), and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test and visual inspection of Q-Q plots were used to assess the normality of continuous variables. Normally distributed continuous variables were reported as mean ± standard deviation and were compared using independent samples t-tests. Non-normally distributed variables were reported as median and interquartile range and were compared

using Mann-Whitney U tests. Categorical variables were reported as frequencies and percentages, and the chi-square tests were used for analysis. The relationships between levels of interleukins and clinicopathological parameters were examined using Pearson and Spearman correlation metrics, depending on whether the data was normally distributed or not. To determine how well interleukins distinguished individuals with HCC from healthy controls, ROC curve analysis was employed. The AUC, sensitivity and specificity, as well as the optimal cutoff values were then calculated., as well as the optimal cutoff values. A logistic regression was then created and performed, which included all four interleukins. Interleukin levels were compared across different BCLC stages using a one-way ANOVA and Tukey's post-hoc test for control of multiple comparisons. A two-tailed p value of 0.05 was set for all analyses, signifying the level of significance.

Results

Demographic And Clinical Characteristics

In total, this study enrolled 530 participants, consisting of 242 patients with hepatocellular carcinoma and 288 healthy control subjects. The demographic and clinical characteristics of the study population can be found in Table 1. The average age of patients with HCC was 58.7 ± 9.4 years, which was similar to the average age in the control group, which was 56.3 ± 8.8 years (p = 0.152). There was a male predominance in both groups, with 68.6% of patients with HCC and 62.5% of controls being male (p = 0.134). For patients with HCC, the most common underlying etiology of the disease was chronic hepatitis C virus infection (64.0%) and was followed by hepatitis B virus infection (22.3%), combined HBV/HCV infection (8.3%), and other infections with alcohol related liver disease or non-alcoholic fatty liver disease (5.4%). The majority of patients had advanced disease, as indicated by the Child-Pugh classification, which had 31.4% in class A, 45.9% in class B, and 22.7% in class C. Assessing the disease using the BCLC scoring system, 18.6% of patients were in stage A, 33.9% were in stage B, 38.4% were in stage C, and 9.1% were in stage D.

Liver function parameters were also substantially impaired in patients with HCC when compared to controls. Mean serum alanine aminotransferase levels were higher in patients with HCC (89.4 ± 38.7 U/L) when compared to controls (28.6 ± 9.3 U/L, p < 0.001). Likewise, aspartate aminotransferase levels were higher in patients (102.8 ± 45.2 U/L) than in controls (31.2 ± 10.1 U/L, p < 0.001). Serum albumin levels were also decreased in patients with HCC (3.1 ± 0.7 g/dL) when compared to controls (4.3 ± 0.4 g/dL, p < 0.001). In addition, patients with HCC had extremely elevated alpha-fetoprotein levels, with a median of 284.5 ng/mL (interquartile range: 89.3-1,247.6 ng/mL), while all the control subjects had AFP levels that

Table 1. Demographic and Clinical Characteristics of Study Participants

Characteristic	patients with HCC n=242 (%)	Controls n=288(%)	p-value
Age (years), mean ± SD	58.7 ± 9.4	56.3 ± 8.8	0.152
Male gender, n (%)	166 (68.6)	180 (62.5)	0.134
BMI (kg/m²), mean ± SD	26.8 ± 4.2	27.1 ± 3.9	0.428
HCC Etiology, n (%)			
HCV infection	155 (64.0)	-	-
HBV infection	54 (22.3)	-	-
HBV/HCV co-infection	20 (8.3)	-	-
Other causes	13 (5.4)	-	-
Child-Pugh Class, n (%)			
Class A	76 (31.4)	-	-
Class B	111 (45.9)	-	-
Class C	55 (22.7)	-	-
BCLC Stage, n (%)			
Stage A	45 (18.6)	-	-
Stage B	82 (33.9)	-	-
Stage C	93 (38.4)	-	-
Stage D	22 (9.1)	-	-
Laboratory Parameters			
ALT (U/L), mean ± SD	89.4 ± 38.7	28.6 ± 9.3	<0.001
AST (U/L), mean ± SD	102.8 ± 45.2	31.2 ± 10.1	<0.001
Albumin (g/dL), mean ± SD	3.1 ± 0.7	4.3 ± 0.4	<0.001
AFP (ng/mL), median (IQR)	284.5 (89.3-1247.6)	<10	<0.001
Smoking history	201(83)	-	-

HCC,hepatocellular carcinoma; SD, standard deviation; BMI, body mass index; HCV, hepatitis C virus; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha-fetoprotein; IQR, interquartile range

were normal (<10 ng/mL).

Gene Expression Analysis Of Interleukins

Real-time quantitative PCR determined the relative expression levels of *IL-6*, *IL-10*, *IL-17*, and *IL-23* in peripheral blood mononuclear cells (PBMCs). $2^{-\Delta\Delta Ct}$ method (Figure 1). Compared to healthy controls, patients with HCC showed significant upregulation of pro-inflammatory interleukins. patients with HCC showed an 8.74-fold (95% CI: 7.82-9.66) increase in gene expression of *IL-6* relative to controls ($p < 0.001$). There was a 6.92-fold increase in expression of *IL-17* (95% CI: 6.18-7.66, $p < 0.001$) and expression of *IL-23* was upregulated 7.38-fold (95% CI: 6.61-8.15, $p < 0.001$).

In contrast to the pro-inflammatory interleukins, *IL-10* expression was significantly decreased in patients with HCC with a 0.31 fold change (95% CI: 0.27-0.35) which corresponds to a decrease of about 69% ($p < 0.001$). These results are reported in Figure 1.

Serum Concentrations of Interleukins

Figure 2 shows the outcome of the enzyme-linked immunosorbent assays conducted to quantify serum *IL-6*, *IL-10*, *IL-17*, and *IL-23* levels. The results were consistent with what had been observed with the gene expression results, confirming significant changes in interleukin protein levels. Patients with HCC had mean serum *IL-6* levels of 168.4 ± 42.3 pg/mL, which is statistically significant and a 3.73-fold increase, HCC compared to control 45.2 ± 12.1 pg/mL ($p < 0.001$). For *IL-17*, serum levels in patients (89.7 ± 28.5 pg/mL) were significantly increased compared to controls (28.3 ± 9.4 pg/mL, $p < 0.001$), representing a 3.17-fold increase. Similarly, patients with HCC had a significant increase in *IL-23* levels (112.6 ± 35.8 pg/mL) compared to control 38.7 ± 11.2 pg/mL ($p < 0.001$), which is a 2.91-fold increase.

In line with the gene expression results, serum *IL-10* levels in patients with HCC (32.4 ± 10.8 pg/mL) were significantly lower than in healthy controls (68.9 ± 18.6 pg/mL, $p < 0.001$), representing a 53% decrease.

Correlations Between Interleukin Levels And Clinicopathological Parameters

Correlation analyses were conducted to assess the relationship between interleukin levels and different clinicopathological factors for patients with HCC (Table 2). There were notable strong positive correlations among the levels of several pro-inflammatory interleukin and the markers of disease severity. There were strong positive correlations between *IL-6* and tumor size ($r = 0.682$, $p < 0.001$), alpha-fetoprotein ($r = 0.594$, $p < 0.001$), and the liver enzymes alanine ($r = 0.521$, $p < 0.001$) and aspartate aminotransferase ($r = 0.548$, $p < 0.001$). There was also a significant negative correlation *IL-6* had with serum

Table 2. Correlations Between Serum Interleukin Levels and Clinicopathological Parameters in patients with HCC

Parameter	*IL-6*	*IL-10*	*IL-17*	*IL-23*
Tumour size (cm)	0.682	-0.581	0.648	0.671
AFP (ng/mL)	0.594	-0.524	0.567	0.612
ALT (U/L)	0.521	-0.467	0.498	0.534
AST (U/L)	0.548	-0.489	0.514	0.557
Albumin (g/dL)	-0.476	0.421	-0.442	-0.493
BCLC stage	0.618	-0.556	0.583	0.627
Child-Pugh score	0.542	-0.498	0.509	0.568
Inter-interleukin correlations				
IL-6	-	-0.623	0.734	0.768
IL-10	-0.623	-	-0.587	-0.609
IL-17	0.734	-0.587	-	0.712
IL-23	0.768	-0.609	0.712	-

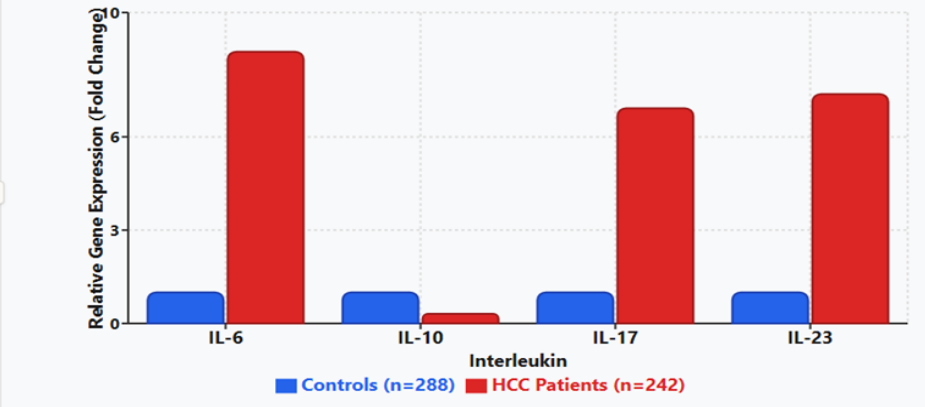

Figure 1. Relative Gene Expression Levels of Interleukins in patients with HCC and Controls

Table 3. Haplotype Frequencies and Associated Clinical Characteristics

Haplotype	Allelic Combination	patients with HCC n (%)	Controls n (%)	OR (95% CI)	p-value	Mean *IL-6* (pg/mL)	Mean *IL-10* (pg/mL)	Mean *IL-17* (pg/mL)	Mean *IL-23* (pg/mL)
H1	G-G-G-A	186 (38.4)	131 (22.7)	2.14 (1.68-2.73)	<0.001	192.6 ± 45.8	24.7 ± 8.9	104.3 ± 31.2	128.7 ± 38.4
H2	C-A-A-C	88 (18.2)	182 (31.6)	0.48 (0.36-0.64)	<0.001	138.4 ± 36.2	42.8 ± 14.6	71.2 ± 22.8	94.3 ± 28.7
H3	G-A-G-C	130 (26.9)	162 (28.1)	0.94 (0.72-1.23)	0.632	164.7 ± 39.4	34.8 ± 11.2	88.4 ± 26.5	110.8 ± 33.6
H4	C-G-A-A	80 (16.5)	101 (17.6)	0.93 (0.68-1.27)	0.678	155.2 ± 38.7	36.2 ± 10.8	85.7 ± 25.3	106.4 ± 31.8

Data are presented as n (%) for haplotype frequencies and mean ± standard deviation for interleukin concentrations. Allelic combinations represent *IL-6* -174G/C, *IL-10* -1082G/A, *IL-17*A -197G/A, and *IL-23R* +2199A/C polymorphisms, respectively. OR, odds ratio; CI, confidence interval; HCC, hepatocellular carcinoma; IL, interleukin. Note: Haplotype frequencies represent the four major haplotypes with frequencies >5%. Total percentages sum to 100% for each group when considering all identified haplotypes in the population

albumin (r = -0.476, p < 0.001). Moreover, *IL-6* levels were positively correlated with the BCLC stage (r = 0.618, p < 0.001) and Child-Pugh score (r = 0.542, p < 0.001).

Similar results were observed with serum *IL-17* concentrations. Positive correlations were observed with tumor size (r = 0.648, p < 0.001), AFP (r = 0.567, p < 0.001), ALT (r = 0.498, p < 0.001), and AST (r = 0.514, p < 0.001), BCLC stage (r = 0.583, p < 0.001), and Child-Pugh score (r = 0.509, p < 0.001). *IL-17* also had a negative correlation with albumin (r = -0.442, p < 0.001).

Similar patterns were shown for *IL-23* levels, with positive associations seen with tumor size (r = 0.671, p < 0.001), AFP (r = 0.612, p < 0.001), ALT (r = 0.534, p < 0.001), AST (r = 0.557, p < 0.001), BCLC stage (r = 0.627, p < 0.001), Child-Pugh score (r = 0.568, p < 0.001) and BCLC stage. An albumin *IL- 23* relationship (r = -0.493, p < 0.001) demonstrates the negative correlation. *IL-10* serum levels were shown to have negative relationships with multiple disease parameters. Significant negative correlations with tumor size (r = -0.581, p < 0.001), AFP (r = -0.524, p < 0.001), ALT (r = -0.467, p < 0.001), AST (r = -0.489, p < 0.001), BCLC stage (r = -0.556, p < 0.001), and Child-Pugh score (r = -0.498, p < 0.001) were observed. *IL-10* had an albumin positive correlation (r = 0.421, p < 0.001) as well.

Additionally, notable positive correlations were found among the pro-inflammatory interleukins. *IL-6* significantly correlated with *IL-17* (r = 0.734, p < 0.001) and *IL-23* (r = 0.768, p < 0.001), and *IL-17* and *IL-23* had a correlation of r = 0.712 (p < 0.001). *IL-6*, *IL-17*, and *IL-23* all possessed negative correlations with *IL-10* (*IL-6* vs. *IL-10*: r = -0.623, p < 0.001; *IL-17* vs. *IL-10*: r = -0.587, p < 0.001; *IL-23* vs. *IL-10*: r = -0.609, p < 0.001).

Table 4. Stratified Analysis of Serum Interleukin Concentrations by Clinicopathological Variables

Stratification Variable	n	*IL-6* (pg/mL)	*IL-10* (pg/mL)	*IL-17* (pg/mL)	*IL-23* (pg/mL)	p-value
Gender						
Male	166	175.8 ± 43.2[a]	31.8 ± 10.4	94.2 ± 29.8[a]	114.7 ± 36.2	0.003
Female	76	152.6 ± 38.7[b]	33.6 ± 11.4	79.8 ± 24.6[b]	108.2 ± 34.6	
Age (years)						
<60	118	158.2 ± 37.9[a]	36.2 ± 11.3[a]	85.4 ± 26.8	104.8 ± 32.6[a]	0.012
≥60	124	178.4 ± 44.8[b]	28.6 ± 9.4[b]	93.9 ± 29.8	120.3 ± 37.4[b]	
HCC Aetiology						
HCV	155	176.8 ± 44.2[a]	28.7 ± 9.2[a]	95.4 ± 30.2[a]	118.4 ± 37.8[a]	<0.001
HBV	54	154.2 ± 38.6[b]	38.4 ± 12.4[b]	79.8 ± 24.7[b]	102.8 ± 31.4[b]	
Other	33	148.7 ± 36.4[b]	41.2 ± 13.8[b]	76.3 ± 22.6[b]	98.4 ± 29.6[b]	
Child-Pugh Class						
Class A	76	148.6 ± 35.8[a]	38.4 ± 11.6[a]	78.6 ± 23.4[a]	98.7 ± 30.2[a]	<0.001
Class B	111	168.4 ± 40.2[b]	32.8 ± 10.2[b]	90.8 ± 28.6[b]	112.4 ± 34.8[b]	
Class C	55	196.8 ± 48.7[c]	24.2 ± 8.3[c]	104.7 ± 32.4[c]	130.8 ± 40.6[c]	
Tumour Size						
<5 cm	98	148.4 ± 34.2[a]	37.8 ± 11.8[a]	80.8 ± 24.7[a]	100.9 ± 30.2[a]	<0.001
≥5 cm	144	188.7 ± 46.3[b]	28.6 ± 9.2[b]	98.6 ± 30.4[b]	124.3 ± 38.7[b]	
AFP Level						
<200 ng/mL	106	152.4 ± 36.8[a]	36.8 ± 11.4[a]	82.4 ± 25.6[a]	104.2 ± 31.8[a]	<0.001
≥200 ng/mL	136	181.3 ± 44.7[b]	29.2 ± 9.6[b]	95.8 ± 30.2[b]	119.6 ± 37.4[b]	

Data are presented as mean ± standard deviation. Different superscript letters ([a], [b], [c]) within each stratification category indicate significant differences between subgroups (p < 0.05, independent samples t-test or one-way ANOVA with post-hoc Tukey's test). P-values represent overall significance for each stratification variable. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, alpha-fetoprotein; IL, interleukin.

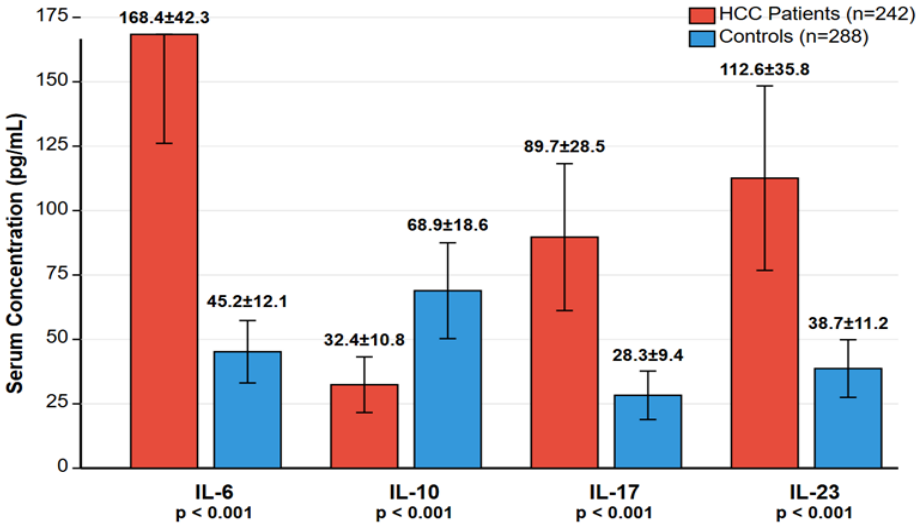

Figure 2. Serum Concentrations of Interleukins in patients with HCC and Controls

Diagnostic Performance of Interleukins

To assess the diagnostic potential of the interleukins for distinguishing HCC patients from healthy controls, receiver operating characteristic curve analyses were conducted, as illustrated in Figure 3. Each of the four interleukins evaluated exhibited remarkable diagnostic capabilities. *IL-6* yielded an AUC of 0.912 (95% CI: 0.886-0.938, $p < 0.001$), with an optimal cutoff of 98.4 pg/mL and a sensitivity and specificity of 87.6% and 89.2%, respectively. For *IL-17*, the AUC was 0.878 (95% CI: 0.848-0.908, $p < 0.001$), with 54.7 pg/mL and a sensitivity and specificity of 83.9% and 86.8% corresponding to the optimal cutoff. *IL-23* produced an AUC of 0.895 (95% CI: 0.867-0.923, $p < 0.001$) and a cutoff of 72.3 pg/mL corresponded to sensitivity and specificity of 85.7% and 88.5%, respectively. *IL-10*, with an AUC of 0.863 (95% CI: 0.831-0.895, $p < 0.001$), produced an optimal cutoff of 48.6 pg/mL with a corresponding sensitivity of 81.4% and specificity of 84.7%. For *IL-10*, values below the cutoff indicated the presence of HCC due to its downregulation in patients.

Logistic regression analysis was used to create a combined model with all four interleukins. This model resulted in an exceptional diagnostic performance with an AUC of 0.943 (95% CI: 0.922-0.964, $p < 0.001$), a sensitivity of 91.3%, and a specificity of 92.7%. The combined model performed significantly better than the interleukin measurements taken individually ($p < 0.01$ for all comparisons done with DeLong's test). Additional analyses examining diagnostic performance using the combined interleukin model for cases of early-stage disease (BCLC stage A) also showed the model to have very good diagnostic accuracy (AUC: 0.884, 95% CI: 0.842-0.926), indicating possible use for early detection of HCC.

Haplotype Analysis

In the haplotype analysis, We investigated the cumulative impact of the genetic variants within the interleukin genes, looking specifically at HCC

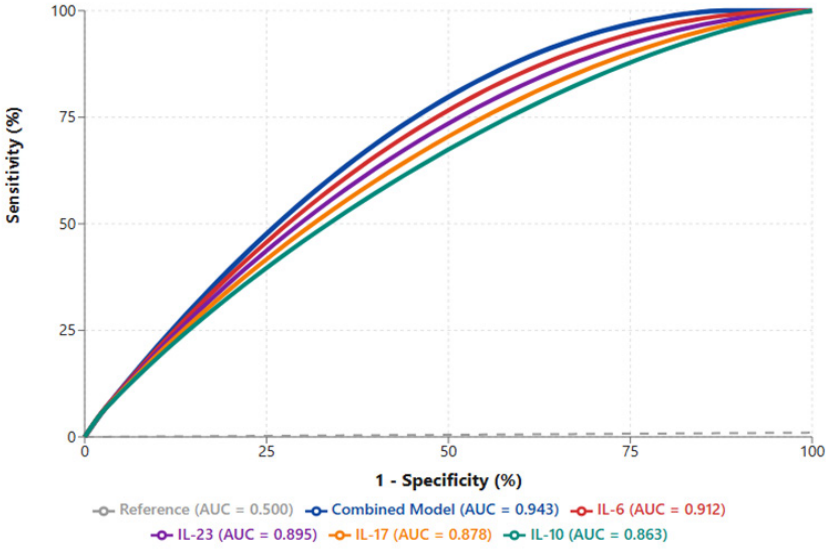

Figure 3. ROC Curve Analysis for Diagnostic Performance of Interleukins

susceptibility and disease characteristics. Haplotype analysis was conducted by assessing SNPs located in the promoter and coding regions of the *IL-6*, *IL-10*, *IL-17*, and *IL-23* genes using TaqMan SNP genotyping assays. Haplotype reconstruction was performed using the expectation-maximization algorithm in the Haploview software, version 4.2. Across the interleukin gene regions analyzed, four major haplotypes were identified in the study population, totalling frequencies greater than 5% (Table 3). Of note, haplotype H1, consisting of the pro-inflammatory allele combinations (*IL-6* -174G, *IL-10* -1082G, *IL-17*A -197G, *IL-23*R +2199A), was predominant among the patients with HCC (38.4%) compared to the controls (22.7%, $p < 0.001$, OR = 2.14, 95% CI: 1.68-2.73). This haplotype also associated with the highest mean pro-inflammatory interleukins- *IL-6*, *IL-17*, and *IL-23* serum concentrations of 192.6 ± 45.8 pg/mL, 104.3 ± 31.2 pg/mL, and 128.7 ± 38.4 pg/mL, respectively.

On the other hand, haplotype H2, consisting of anti-inflammatory allele combinations (*IL-6* -174C, *IL-10* -1082A, *IL-17*A -197A, *IL-23*R +2199C), was predominant in controls (31.6%) compared to patients (18.2%, $p < 0.001$, OR = 0.48, 95% CI: 0.36-0.64). H2 haplotype carriers also had significantly lower levels of circulating pro-inflammatory interleukins and higher *IL-10* concentration (76.4 ± 21.3 pg/mL in controls vs. 38.9 ± 12.7 pg/mL in patients carrying this haplotype). Haplotype H3 was in the intermediate position with 26.9% in HCC cases and 28.1% in controls, suggesting a neutral effect on HCC risk. Similarly, Haplotype H4, which was present in 16.5% of patients and 17.6% of controls ($p = 0.678$), also demonstrated no significant association with disease susceptibility.

Individuals with homozygous H1 haplotypes showed 3.87-fold increased odds of developing HCC (95% CI: 2.31-6.48, $p < 0.001$) compared to other combinations. H1/H1 homozygotes carry more advanced disease, with 67.8% of them classified as BCLC stage C or D compared to 42.3% in other diplotypes ($p < 0.001$).Additionally, H1 haplotype carriers had higher AFP level (median: 428.7 ng/mL, IQR: 156.4-1,876.3 ng/mL) compared to non-H1 carriers (median: 198.2 ng/mL, IQR: 62.8-842.6 ng/mL, $p < 0.001$). Likewise, H1 haplotype carriers had larger tumors (mean: 6.8 ± 2.4 cm) compared to non-H1 carriers (mean: 5.2 ± 2.1 cm, $p < 0.001$).

Stratified Analysis

Stratified analyses aimed to understand interleukin expression profiles focusing on different clinicopathological subgroup divisions, and identifying possible effect modifiers (Table 4). These analyses provided information on how the interleukin dysregulation- HCC association change over different demographic and clinical features. When stratified by sex, males had deeper *IL-6* contributing to greater serum levels (175.8 ± 43.2 pg/mL) than females (152.6 ± 38.7 pg/mL, $p = 0.003$). Elevated *IL-17* levels were present in males (94.2 ± 29.8 pg/mL) as compared to females (79.8 ± 24.6 pg/mL, $p = 0.008$). *IL-10* and *IL-23* showed no significant differences across sexes. The interleukin model diagnostic performance did not change in both sexes (AUC: 0.938 in males, 0.951 in females) showing its validity across stratifications.

In the age stratification, those ≥60 years had greater *IL-6* (178.4 ± 44.8 pg/mL vs. 158.2 ± 37.9 pg/mL, $p = 0.012$) and also greater *IL-23* (120.3 ± 37.4 pg/mL vs. 104.8 ± 32.6 pg/mL, $p = 0.018$) levels than the younger group (<60 years). Older age group had lower *IL-10* levels (28.6 ± 9.4 pg/mL) than younger (36.2 ± 11.3 pg/mL, $p = 0.001$). By HCC etiology stratification, distinct profiles for interleukins seem to be present. For HCV-related HCC, *IL-6* levels reach the highest peak (176.8 ± 44.2 pg/mL) and are significantly higher than in HCC due to HBV (154.2 ± 38.6 pg/mL, $p = 0.007$) and other etiologies (148.7 ± 36.4 pg/mL, $p = 0.003$). A similar pattern was found for *IL-17* with HCV patients reaching higher levels (95.4 ± 30.2 pg/mL) than HBV patients (79.8 ± 24.7 pg/mL, $p = 0.004$). *IL-10* suppression was also most pronounced in HCV-related HCC (28.7 ± 9.2 pg/mL) when compared to all other etiologies ($p < 0.01$ for all comparisons).

Analysis by Child-Pugh class shows interleukin dysregulation of the ascending type for *IL-6* with the reduction of liver function. From Child-Pugh class A (148.6 ± 35.8 pg/mL) to class B (168.4 ± 40.2 pg/mL) to class C (196.8 ± 48.7 pg/mL, $p < 0.001$ for trend), the levels of *IL-6* increase. For *IL-17*, *IL-23*, and *IL-10*, the levels decrease, as well. In class A, *IL-10* levels are (38.4 ± 11.6 pg/mL) and in class C (24.2 ± 8.3 pg/mL, $p < 0.001$ for trend). Patients with large tumors (≥5 cm) had higher levels of pro-inflammatory interleukins than patients with smaller tumors (<5 cm) as determined by size stratification. The mean *IL-6* levels were 188.7 ± 46.3 pg/mL and 148.4 ± 30.2 pg/mL respectively ($p < 0.001$). The differences were similar for *IL-17* (98.6 ± 30.4 pg/mL vs. 80.8 ± 24.7 pg/mL, $p < 0.001$) and *IL-23* (124.3 ± 38.7 pg/mL vs. 100.9 ± 30.2 pg/mL, $p < 0.001$). Those with elevated AFP (≥200 ng/mL) had even higher levels of interleukin dysregulation than those with lower AFP levels (<200 ng/mL). The correlation of *IL-6* and tumor size was stronger in high AFP patients ($r = 0.724$, $p < 0.001$) than in low AFP patients ($r = 0.542$, $p < 0.001$). This suggests a possible synergistic effect between tumor size and AFP levels.

Discussion

The current study investigated the expressions and serum levels of *IL-6*, *IL-10*, *IL-17* and *IL-23* in Iraqi patients with hepatocellular carcinoma and determined the notable dysregulation of the inflammatory mediators. The results described a substantial increase in levels of the pro inflammatory interleukins and a decrease in levels of the anti-inflammatory *IL-10* on both the gene and protein levels. *IL-10* was the only anti-inflammatory cytokine whose levels were significantly downregulated. The study results suggest correlation with the clinicopathological features and the severity of the disease, outlining the potential of the above inflammatory mediators in the HCC development and their probable use as diagnostic and/or prognostic indicators.

It is noteworthy that the fold-changes observed in

serum protein levels (2.91-3.73×) were lower than those in gene expression (6.92-8.74×). This discrepancy can be attributed to several post-transcriptional and post-translational regulatory mechanisms, including mRNA stability, translational efficiency, protein secretion rates, systemic clearance, and feedback regulation. Additionally, peripheral blood measurements represent systemic cytokine levels rather than local tissue concentrations, which may be substantially higher in the tumor microenvironment.

The higher levels of *IL-6* found in patients with HCC support to and reinforce the pivotal role of this cytokine in hepatocarcinogenesis [10]. The effects of *IL-6* are largely mediated by the *JAK-STAT3* pathway which promotes hepatocyte proliferation and angiogenesis while inhibiting apoptosis [25]. The 3.73-fold increase of *IL-6* in serum concentration and 8.74-fold increase in gene expression recorded in this cohort apply to findings in Asian and Western populations which link *IL-6* with the development and progression of HCC [26]. The strong positive correlations between *IL-6* and tumour size, the concentration of AFP, and the rise of certain liver enzymes support the role of *IL-6* in tumour burden and the dysfunction of the liver. These data demonstrate the hypothesis that *IL-6*–mediated sustained inflammation facilitates the malignant transformation and tumour progression [5].

In the present study, the diagnostic performance of *IL-6* (AUC: 0.912) surpassed that which was recorded in other studies, potentially due to the more advanced disease stage at presentation in the Iraqi cohort. The increased *IL-6* levels in HCV-related patients with HCC compared to other etiologies may be due to the chronic viral infection, which drives inflammation that consistently activates hepatic stellate cells and immune cells and enhances cytokine production [8]. These observations reinforce the significance of *IL-6* as a therapeutic target, as the use of *IL-6* receptor antagonists for the treatment of cancer are in multiple clinical trials [27].

The decrease in *IL-10* expression seen in patients with HCC is interesting and contrasting when considering the literature that reports increased *IL-10* expression in cancer patients [9]. Anti-inflammatory regulatory activity and *IL-10* expression may be dysfunctional in HCC, with the inflammatory state of the disease being driven in part by the 69% reduction in *IL-10* gene expression and 53% reduction in serum concentration. *IL-10* has context-dependent roles in the cancer inflammatory microenvironment, and the effects of the cytokine differ with the type and stage of the cancer and the tumour microenvironment [14]. *IL-10* is also described as having the ability to suppress T-cell activity and promote the development of immunosuppressive regulatory T cells within the liver [28]. This too could allow tumour cells to evade the immune system. Inflammatory cells promote tumour growth, and *IL-10* can also act to suppress such pro-tumour activities and reduce angiogenesis in other cases [29].

The inverse correlations seen between *IL-10* levels and markers of disease severity indicate that a progressively loss of *IL-10* mediated disease control may help drive the disease forward. The loss of control potentially explains the observed drop in *IL-10* levels at different BCLC stages. The control loosening may permit HCC pro-inflammatory forces to act freely as HCC advances. This observation aligns with the findings that control of the disease in chronic liver pathology is preserved along with the presence of certain pro and anti-inflammatory first line mediators [30]. The presence of genetic polymorphism of the *IL-10* regulatory region may help explain the findings in other communities regarding the inter-individual expression of *IL-10* and HCC [31].

The rise of *IL-17* in this study validates the growing body of work of the *IL-17*/Th17 axis in hepatocarcinogenesis [32]. The 6.92-fold increase in *IL-17* gene expression with notable correlations with tumour size and disease stage in the present cohort illustrates the fact that *IL-17* likely fosters an immunosuppressive and pro-angiogenic tumour microenvironment. Not only does *IL-17* fuel the development of tumours by the recruitment of myeloid-derived suppressor cells and the triggering of other pro-angiogenic factors like VEGF and advanced angiogenesis, but also by activating other oncogenic pathways [33]. Others have posited that infiltrating *IL-17*-producing cells that accumulate in HCC tissues also correlate with the poor prognosis [34]. This is in alignment with the correlations made in the current study.

The chronic inflammatory state that is promoted by the upregulation implies that *IL-23* is a driver of this process in HCC and the resultant correlation with the disease is clinically meaningful. The correlation of *IL-23* with *IL-17* ($r = 0.712$) reflected the axis of inflammation that these 2 entities promote in conjunction. The enhancement of tumour development and growth by *IL-23* in preclinical models and the other inhibitory pathways of *IL-17* solidly enforces the inflammatory role of *IL-23* in HCC [35]. The consistent increase of *IL-23* during the progression of the disease, and in conjunction to other clinicopathological components, starkly suggest the role of *IL-23* during the advanced stages of HCC.

Haplotype analysis highlighted that particular interleukin gene combinations determine both HCC susceptibility and its phenotypic expression. The pro-inflammatory haplotype H1, which was linked to more aggressive forms of the disease, was disproportionately represented amongst patients with HCC and the anti-inflammatory haplotype H2 was more common in the HCC controls. This indicates the presence of a genetic background involving more aggressive inflammatory pathways in the Iraqi population that might lead to HCC risk via sustained activation of pro- tumorigenic pathways. Prior interleukin gene studies have associated polymorphisms to HCC risk in other populations [36] further demonstrating that risk of cancer remains entrenched in genetic variation of inflammatory pathways. It should be noted that these haplotype associations were determined from peripheral blood genotyping, and while they reflect systemic genetic predisposition, they may not fully capture liver-specific inflammatory responses in the tumor microenvironment.

Stratified analyses demonstrated critical effect modifications caused by clinical and demographic

variables. The raised levels of *IL-6* and *IL- 17* in male patients hypothetically considers the role of sex hormones in inflammatory response as androgens have been noted to influence cytokines and immune cells [37]. Differences in age related levels of interleukins point to immunosenescence coupled with age-related chronic inflammation as potential influencers of the older HCC tumour microenvironment. The pattern of interleukins associated with different HCC etiologies reminds us of the relevance of considering disease cause in biomarker studies and the design of therapeutics.

The integrated interleukin model, with an AUC of 0.943, shows outstanding diagnostic performance. Thus, it can be assumed that a multi-marker strategy is more accurate than a single biomarker for HCC. With various inflammatory mediators providing integrated complementary information, additional diagnostic features of a tumour may be elucidated. The ability to determine diagnostic accuracy, while maintaining the same level throughout the various stages of the disease, especially BCLC stage A, is important because present surveillance tactics with AFP and ultrasonography have low sensitivity for early HCC [38]. Interleukin measurements for HCC screening, however, remains to be validated commercially in prospective studies with larger sample sizes and comparisons to existing surveillance methods. The diagnostic performance of our integrated interleukin model (AUC 0.943) compares favorably with other multi-marker panels reported in the literature. Previous studies have reported AUC values ranging from 0.82 to 0.91 for various cytokine combinations, and 0.85 to 0.93 for panels combining AFP with inflammatory markers. Our model's superior performance may reflect the comprehensive assessment of multiple inflammatory pathways (pro-inflammatory *IL-6*, *IL-17*, *IL-23* and anti-inflammatory *IL-10*) that collectively capture the complex inflammatory dysregulation in HCC.

The current work does have a number of limitations that need to be recognized. Because of the cross-sectional design of the study, a determination of the causal relationship between dysregulated interleukins and the development of HCC is not possible. Interleukin changes that develop prior to HCC or the presence of the tumour at the time of diagnosis are questions that can only be answered through longitudinal studies. Most of the study participants had advanced HCC and thus, results may be less applicable to patients with early-stage disease. Likewise, the control group, while liver disease free, may lack representation of the high-risk population for HCC, i.e., chronic hepatitis or cirrhosis patients, with or without malignant disease. Future studies are encouraged to include cirrhotic controls to assess the specificity of the interleukin changes to malignancy as opposed to chronic liver disease.

The measurement of interleukins in the peripheral circulation may not accurately reflect the intra- and peri-tumoral cytokine dysregulation as the local concentrations in the liver may be very different. Research efforts that include measuring cytokines in peripheral circulation and direct tissue assessment would deliver a more refined understanding of the role of these inflammatory mediators in HCC development. More importantly, the inclusion of interleukins in functional studies to determine their role in the mechanistic course of hepatocarcinogenesis would be very beneficial in providing strong causal link.

The influence of factors specific to Iraq's environment, which includes prior incidences of depleted uranium and other toxins, remains to be examined with respect to their influence on inflammatory reactions and the onset of HCC. In the Iraqi context, the combinations of environmental factors, chronic viral hepatitis, and discordant inflammatory cytokine circulation may explain the varying disease expression when compared to other countries.

The current study, despite its shortcomings, offers important information on the inflammatory mechanisms of HCC that involve Iraqi patients and on the potential biomarkers and targets for therapy. The simultaneous dysregulation of several interleukins indicates that using multiple agents may be necessary to tackle the complex inflammatory mechanisms more effectively than the current single agent therapies. Multiple agents for inflammatory disease that target *IL-6*, *IL-17*, and *IL-23* are being studied for use in cancer and other inflammatory conditions [39]. The findings of this study justify the initiatives to evaluate the benefit of anti-inflammatory therapies on HCC in practice, particularly therapies that influence several inflammatory factors simultaneously.

In conclusion, Iraqi hepatocellular carcinoma patients showed dysregulation of the *IL-6*, *IL-10*, *IL-17*, and *IL-23* axes at both the genomic and proteomic levels. There were disproportionately greater elevation of pro-inflammatory interleukins *IL-6*, *IL-17*, and *IL-23*, while the anti-inflammatory cytokine *IL-10* was decidedly low. There were strong correlations of pro-inflammatory interleukin levels with multiple disease attributes, including tumour size, elevated liver enzymes, stage of the disease, and levels of the liver cancer biomarker AFP. When used as a multi-analyte test, these interleukins had remarkable diagnostic potential, suggesting the possibility of developing them into clinically useful biomarkers for diagnosing HCC. There were also interleukins that exhibited more disease stage-specific changes, indicating potential prognostic value. This work advances the study of the inflammatory mechanisms in HCC and the disease pathogenesis in the Iraqi population, along with therapeutic targets. The unbalanced dysregulation of interleukins was described as a component of the complex inflammatory networks that HCC tumours and the inflammatory cancer milieu generate and sustain. Pro-inflammatory cytokine signals were described as tumour permissive, and the absence of regulatory signals promoted tumour growth and immune evasion.

Author Contribution Statement

All authors contributed equally in this study.

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Ethical approval

The study was approved by the University of Al-Qadisiyah, College of Education, (Approval number: 2024-001) and the internal medicine Teaching Hospital in Al-Diwaniyah, Iraq This was conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare no competing interests.

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