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Defensins (HAD-1/HBD-1) as novel immune biomarkers in chronic hepatitis C: Association with inflammatory and antiviral cytokines and ROC-based diagnostic accuracy

Ahmed T. Enad[1], Al-Zahraa J. Jassim[1], Thuraya K. Alwandawi[2], and Ruqaya K. Abbas[1]

[1]Ibn Sina University of Medical & Pharmaceutical Sciences, Iraq, Baghdad.
[2]Al-Iraqia University, Baghdad, Iraq.

Corresponding author: Ahmed T. Enad, 1Ibn Sina University of Medical & Pharmaceutical Sciences, Iraq, Baghdad.
Email: ahmed.talal.g@ibnsina.edu.iq

Abstract

Chronic hepatitis C virus (HCV) infection is associated with prolonged immune activation and systemic inflammation, which may be reflected in circulating immune mediators. Defensins and cytokines have emerged as potential biomarkers for monitoring immune dysregulation in chronic viral hepatitis. This study aimed to monitoring serum levels of human α-defensin-1 (HAD-1) and human β-defensin-1 (HBD-1) with selected cytokines (IL-6, IL-28, TNF-α, and IFN-γ) in Iraqi patients with chronic HCV and to evaluate their diagnostic performance by the receiver operating characteristic (ROC) analysis. This cross-sectional case–control study was conducted in Iraq during February–November 2025. It included 100 laboratory-confirmed chronic HCV patients and 50 normal controls. Serum HAD-1, HBD-1, IL-6, IL-28, TNF-α, and IFN-γ were measured by ELISA. Hematological indices and liver function markers were also analyzed. HCV patients showed significantly increased HAD-1 and HBD-1 levels compared with controls (p< 0.01). Cytokine profiling revealed markedly elevated IL-6, IL-28, TNF-α, and IFN-γ levels in patients, indicating strong activation of inflammatory and antiviral immune pathways (p<0.01). ROC curve analysis demonstrated excellent diagnostic performance of defensins and cytokines to distinguish chronic HCV patients from controls, with area under the curve values ranging from 0.849 to 0.950 units. In conclusion, the circulation of defensins and inflammatory cytokines is markedly up-regulated in chronic HCV and has high diagnostic utility indicating their potential as immune biomarkers for chronic HCV-related inflammation and immune dysregulation.

Keywords: Chronic hepatitis C; HAD-1; HBD-1; Inflammatory cytokines; IL-28; ROC curve analysis.

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Introduction

Hepatitis C virus (HCV) infection is currently a global health consequence due to its capacity for chronic infection and progression to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Although direct-acting antivirals are highly effective, chronic HCV continues to place a considerable clinical burden on systems and organizations, and reliable biomarkers

addressing immune activity and inflammatory status associated with such disorders remain challenging to obtain.[1,2] Chronic HCV pathogenesis is profoundly induced by chronic immune activation and host-virus interactions that underline inflammation and ultimately hepatic injury. Chronic inflammation affects both innate and adaptive immune pathways and induces systemic immune changes. As such, assaying circulating immune mediators could be relevant for the immunopathogenesis and clinical surveillance of chronic HCV.[3,4] Cytokines are essential regulators of antiviral immunity and inflammation. Previous investigations showed a heterogeneous pattern of cytokines in viral hepatitis and hepatocellular carcinoma and associated inflammatory cytokines with liver inflammatory activity and response to therapy. These results are consistent with circulating cytokines being useful immune biomarkers as important clinic educational markers in chronic HCV infection.[5,6] Within the inflammatory mediating cell lines, interleukin-6 (IL-6) is a predominant pro-inflammatory cytokine with recognized roles in chronic liver inflammation and carcinogenesis, and clinical evidence is available on its relationship with HCV clinical course. Furthermore, tumor necrosis factor alpha (TNF-α) is also central in systemic inflammation and involved in systemic immune and metabolic pathways in chronic HCV infection. Thus, it is believed that the IL-6 and TNF-α measurement might represent inflammation and immune dysregulation induced inflammation burden in chronic HCV patients .[7,8]

Interferons are natural proteins, important for resistance of antiviral defense. IFN-γ, a pro-inflammatory cytokine, plays a role in viral control via macrophage activation and increased antigen presentation; however, chronic infections may lead to chronic immune activation and immune impairment. IL-28 (IFN-λ family), also known to exert antiviral action and to determine innate and adaptive immune responses and could be one of the biomarkers on the antiviral immune status in chronic hepatitis.[9,10,11]

Defensins are other important innate immune peptides that display generalized antimicrobial activity and immune-modulatory functions in addition to cytokines. Human α-defensin-1 (HAD-1) is primarily produced by neutrophils, involved in inflammation and the activation of innate immunity. Human β-defensins like HBD-1 play roles in epithelial defense and immune signaling. A growing number of sources of evidence indicated that these substances are involved in chronic inflammatory processes; in addition to this, their serum levels may be altered for infections such as HCV.[12] Viral infections can cause persistent post-transcriptional regulatory impairments by host- and viral-specific microRNAs that could contribute to the immune dysregulation and disease risk.[13,14] The objective of this study was monitoring serum HAD-1 and HBD-1 and certain inflammatory cytokines (IL-6, IL-28, TNF-α, and IFN-γ) and to examine the assessment of their performance by the receiver operating characteristic (ROC) curve analysis in Iraqi HCV infected patients.

Materials and Methods

Study design: This was a cross-sectional case–control study, performed during February to November 2025 in Tikrit, Iraq to determine serum levels of HAD-1 and HBD-1 in patients infected with chronic HCV and to analyze the associations of certain inflammatory cytokines with these defensins and their diagnostic accuracy.

The study included two groups. The patient group included 100 HCV patients from Tikrit General Hospital. Their HCV infection was confirmed by anti-HCV antibody positivity and detection of HCV ribonucleic acid (RNA) by the real-time polymerase chain reaction (RT-PCR). Chronic HCV infection was defined as detectable HCV RNA persistence for ≥ 6 months based on clinical records. All participants in the HCV group were treatment-naive and had not been treated with direct-acting antivirals before their recruitment. The control group comprised 50 apparently healthy individuals, randomly selected from those attending the Main Blood Bank in Tikrit, who had no serological evidence of HCV infection. All samples and data were coded and analyzed anonymously the authors

did not have access to information that could identify individual participants during or after data collections.

Collection of Blood Sample and Serum Preparation

Blood samples were collected aseptically from controls and patients with HCV based on the mandate of Iraqi Ministry of Health. The venipuncture area was thoroughly disinfected by 70% ethanol, and a blood sample (5 ml) was drawn using sterile disposable plastic syringes. Each blood sample was divided into two parts. The first portion (2 ml) was placed into an ethylenediaminetetraacetic acid (EDTA) tube for hematological studies including complete blood count (CBC) and for molecular detection and quantitation of HCV by the RT-PCR. The second half of the sample (3 ml) was allowed to clot at room temperature for 20–30 min and then centrifuged for 5–10 min at 5000 g. Serum was separated into aliquots of 250 µl and kept at -20°C for enzyme-linked immunosorbent assay (ELISA) and biochemical assay purposes.

Serological and Molecular Confirmation of HCV Infection

All HCV cases were confirmed by anti-HCV antibody positivity using ELISA, and molecular detection as well as viral load quantification performed using the RT-PCR technique.

Inclusion and Exclusion Criteria

Patients with laboratory-confirmed chronic HCV infection (anti-HCV positive and HCV RNA detectable by RT-PCR) were enrolled, while apparently healthy HCV-negative individuals served as controls. Participants were excluded if they had HBV or HIV co-infection, autoimmune or inflammatory diseases, acute infection at sampling, malignancy, immunosuppressive drug use, or metabolic conditions that may affect inflammatory biomarkers (uncontrolled diabetes or obesity).

Quantification of Serum Defensins and Cytokines

Serum concentrations of HBD-1, HAD-1, interleukin-6 (IL-6), interleukin-28 (IL-28), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) were measured using quantitative ELISA kits (supplied by My BioSource, San Diego, CA, USA), according to the manufacturer's instructions. The concentrations were expressed as pg/ml.

Hematological Investigations

Each blood sample was divided into two parts. The first portion was collected into an EDTA tube for complete blood count (CBC) analysis. A separate portion was collected into a separate tube containing sodium citrate for measurement of erythrocyte sedimentation rate (ESR) using the Westergren method.

Liver Function Tests

liver function parameters were assessed using a colorimetric method based on commercial kits (supplied by Spinreact, Spain), according to the manufacturer's instructions. These included total serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS) software (version 9.1). Comparisons between HCV patients and controls were carried out using the T-test and Mann-Whitney test according to data distribution. For comparisons among more than two groups, we applied the one-way Analysis of Variance (ANOVA) followed by the Fisher's Least Significant Difference (LSD) post hoc test. Statistical significance was considered at $p< 0.05$.

Results

Demographic characteristics

Table 1 revealed the demographic patterns, which showed that all the study groups were similar in terms of age. The mean age of the HCV patients was 38.4 ± 4.2 y, did not differ significantly than the control group (40.9 ± 3.8y) (p=0.45). Regarding the distribution of residence, 61% of HCV patients came from rural areas, and 39% were from urban areas as shown in Table 1.

Table 1. Demographic characteristics of the study participants.

Variable	HCV patients (n=100)	Controls (n=50)	*p*-value
Age (years), mean ± SD	38.4 ± 4.2	40.9 ± 3.8	NS
Residence (patients only), n (%)			
Rural	61 (61.0)	--	--
Urban	39 (39.0)	--	--

$p > 0.05$ is not significant (NS).

Hematological Parameters and Inflammatory Indices

Table 2 shows that in HCV patients there were also striking hematological and biochemical signs, which confirmed systemic inflammation and hepatic injury. In HCV patients, ESR were significantly high (24.95±4.98 mm/1 hr.) compared with controls (5.30 ±3.08 mm/1 hr.; $p<0.001$).

The total white blood cell (WBC) count was significantly increased in the HCV group (8.67±5.88 $\times10^3$/µl), while controls had a count of 4.58 ± 3.83 $\times10^3$/µl ($p<0.009$), which indicated leukocytosis consistent with an inflammatory response. In contrast, the neutrophils/granulocytes were much lower in HCV patients (4.74 ± 0.91 $\times10^3$/µl) than controls (5.66 ± 1.87 $\times10^3$/µl; $p<0.003$), indicating possible immune cell redistribution or disease-initiated hematological modulation. The HCV patients exhibited significantly elevated lymphocyte levels (33.50±10.09%) compared with controls (17.61±4.91%; $p=0.007$). As for erythrocyte indices, hemoglobin levels decreased significantly in HCV patients (12.00±3.38 g/dl) when compared with controls (12.80 ± 2.30 g/dl; $p<0.001$), which were suggestive of mild anemia in the study patients. Platelet count was significantly increased in HCV patients (270.40±107.76 $\times10^3$/µl) as compared to controls (180.92 ± 67.90 $\times10^3$/µl; $p<0.009$). Liver function tests showed pronounced hepatic injury in HCV patients with significantly higher ALT (80.76 ± 26.90 vs 10.36 ± 5.88 U/L; $p<0.001$), AST (73.76 ± 19.86 vs 9.60 ± 4.78 U/L; $p< 0.001$), and total bilirubin than controls.

Table 2. Hematological parameters and liver function tests in HCV patients and controls.

Parameter	HCV patients (n=100)	Controls (n=50)	*p*-value
ESR (mm/1 hr)	24.95 ± 4.98	5.30 ± 3.08	<0.001
WBC count ($\times10^3$/µl)	8.67 ± 5.88	4.58 ± 3.83	<0.009
Neutrophils/Granulocytes ($\times10^3$/µl)	4.74 ± 0.91	5.66 ± 1.87	<0.003
Lymphocytes %	33.50 ± 10.09	17.61 ± 4.91	0.007
Hemoglobin (g/dl)	12.00 ± 3.38	12.80 ± 2.30	<0.001
Platelets ($\times10^3$/µl)	270.40 ± 107.76	180.92 ± 67.90	<0.009
ALT (U/L)	80.76 ± 26.90	10.36 ± 5.88	<0.001
AST (U/L)	73.76 ± 19.86	9.60 ± 4.78	<0.001
ALP (U/L)	123.19 ± 16.80	70.66 ± 10.45	0.009
Total bilirubin	2.18 ± 1.29	0.56 ± 0.37	<0.001

$p \leq 0.05$ is significant.

Serum Defensins in HCV Patients and Controls

As presented in Table 3, serum defensins were markedly elevated in chronic HCV patients compared with controls. Serum HAD-1 levels were significantly higher in the HCV group (24.65 ± 12.20 pg/ml) than in controls (1.65±1.54 pg/ml; $p<0.004$). Likewise, concentrations of serum HBD-1 were significantly increased among HCV patients (18.56±9.76 pg/ml) compared with controls (1.99 ± 1.09 pg./ml; $p<0.001$).

Table 3. Serum levels of defensins (pg/ml) in HCV patients and controls.

Biomarker (pg/ml)	HCV patients (n=100)	Controls (n=50)	*p*-value
HAD-1	24.65 ± 12.20	1.65 ± 1.54	<0.004
HBD-1	18.56 ± 9.76	1.99 ± 1.09	<0.001

$p \leq 0.05$ is significant.

Serum Cytokine Profile

In Table 4, cytokine analysis showed a pronounced systemic inflammatory response of chronic HCV patients compared with controls. HCV patients showed significantly increased serum IL-6 levels (109.65 ± 10.40 pg/ml) in comparison to controls (3.72±1.48 pg/ml), ($p<0.003$). In the HCV group, serum IL-28 levels were also elevated (568.48±54.61 pg/ml) relative to controls (29.48±8.76 pg/ml; $p<0.001$). Also, the TNF-α level was significantly increased in the HCV patients (419.55±19.18 pg/ml) compared to controls (33.23±8.90 pg/ml; $p<0.001$). Besides, IFN-γ levels were significantly higher among HCV patients (679.21±25.89 pg/ml) versus controls (11.54±8.17 pg/ml), ($p<0.007$).

Table 4. Serum levels of cytokines (pg/ml) in HCV patients and controls.

Cytokine (pg/ml)	HCV patients (n=100)	Controls (n=50)	*p*-value
IL-6	109.65 ± 10.40	3.72 ± 1.48	<0.003
IL-28	568.48 ± 54.61	29.48 ± 8.76	<0.001
TNF-α	419.55 ± 19.18	33.23 ± 8.90	<0.001
IFN-γ	679.21 ± 25.89	11.54 ± 8.17	<0.007

$p \leq 0.05$ is significant.

Correlation between Defensins and Cytokines among HCV Patients

As indicated in Table 5, correlation analysis of the HCV group (n=100), showed weak associations between defensins with inflammatory cytokines. HAD-1 showed very weak negative linkages with IL-6 (r=−0.110) and IL-28 (r=−0.200), and its correlations with TNF-α (r=0.048) and IFN-γ (r=−0.089) were negligible, evidencing very weak linkages. Again, HBD-1 were weakly negatively correlated to IL-6 (r=−0.193) and IL-28 (r=−0.161). Notably, the strongest association was a moderate inverse relationship between HBD-1 and TNF-α (r=−0.349), which was accompanied by a weaker negative correlation between HBD-1 and IFN-γ (r=−0.231).

Table 5. Correlation (r) between defensins and cytokines in the 100 HCV patients.

Correlation (r)	IL-6	IL-28	TNF-α	IFN-γ
HAD-1	-0.110	-0.200	0.048	-0.089
HBD-1	-0.193	-0.161	-0.349	-0.231

Diagnostic Performance of the Studied Biomarkers

The ROC curve analysis showed high diagnostic accuracy for the investigated defensins and inflammatory cytokines in differentiating chronic HCV patients from normal controls. As demonstrated in Figure 1, defensins performed best according to the diagnostic test, and Table 6 showed that HAD-1 had outstanding discrimination (AUC = 0.950; 95% CI: 0.910–0.990; p<0.001) followed by HBD-1 (AUC = 0.856; 95% CI: 0.780–0.932; p<0.005). Among cytokine biomarkers, IL-6 showed a high level of diagnostic accuracy (AUC=0.901; 95% CI: 0.845–0.957; p<0.001), while IFN-γ showed great performance (AUC=0.867; 95% CI: 0.805–0.929; p<0.009). Moreover, IL-28 revealed a strong discriminative power (AUC=0.880; 95% CI: 0.820–0.940; p<0.05), and TNF-α also showed similarly good diagnostic power (AUC=0.849; 95% CI: 0.807–0.940; p< 0.03).

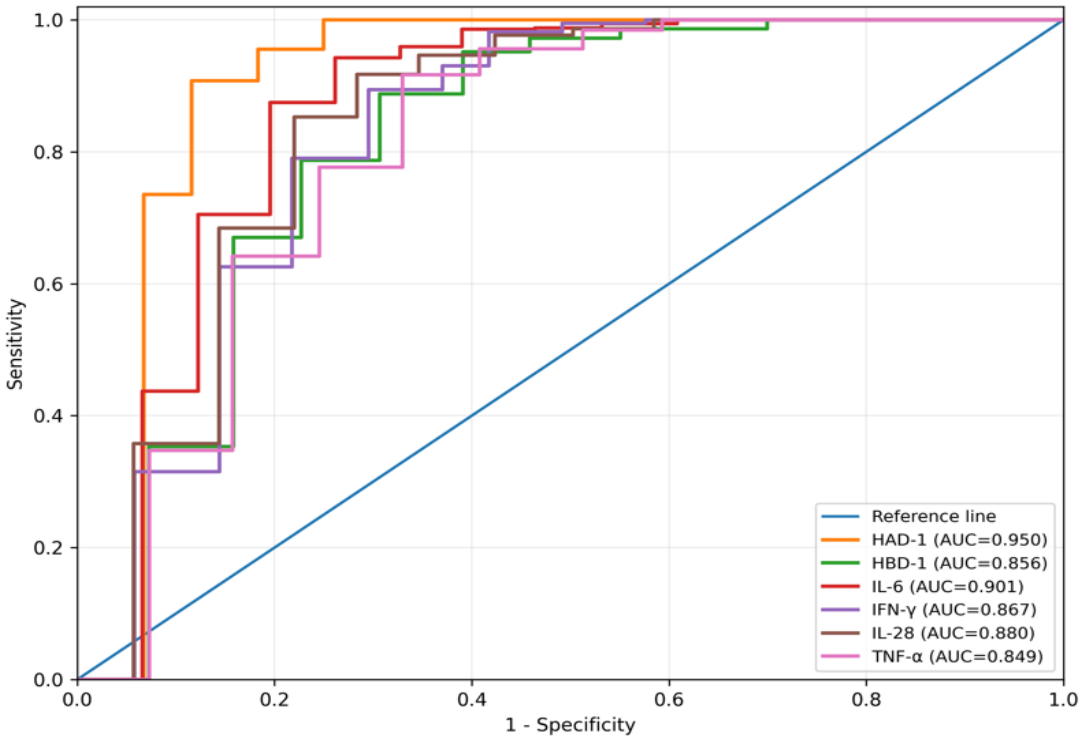

Figure 1. Receiver operating characteristic (ROC) curves of defensins and inflammatory cytokines for diagnosis of chronic HCV. ROC analysis shows the diagnostic performance of serum HAD-1, HBD-1, IL-6, IL-28, TNF-α, and IFN-γ in discriminating HCV patients (n=100) from normal controls (n=50). The diagonal line indicates the reference line (AUC=0.5).

Table 6. Receiver operating characteristic (ROC) curve performance (AUC and 95% CI) of defensins and cytokines for discrimination of chronic HCV.

Biomarker	AUC	95% CI	*p*-value
HAD-1	0.950	0.910 – 0.990	<0.001
HBD-1	0.856	0.780 – 0.932	<0.005
IL-6	0.901	0.845 – 0.957	<0.001
IFN-γ	0.867	0.805 – 0.929	<0.009
IL-28	0.880	0.820 – 0.940	<0.05
TNF-α	0.849	0.807 – 0.940	<0.03

$p \leq 0.05$ is significant.

Discussion

In the current research, immune and antiviral mediators played a pivotal role during chronic HCV infection where persistent stimulation of the virus leads to ongoing activation of host defense responses. In this context, IL-28 (IFN-λ family) was shown to be a key antiviral cytokine, recently identified as a master regulator of the viral replication response as well as modulating mechanisms of immune signaling, and hence its importance as a biological parameter during chronic viral diseases.[15] Consistent with this approach, elevated serum IFN-λ was reported in HCV patients, suggesting that IFN-λ responses are still functional during the phase of chronic infection, and may reflect persistent immune modulation of viral activity, rather than total elimination of the virus. Thus, the increased IL-28 cytokine in our study agrees with the immunologic profile in chronic HCV and provides for diagnostic and prognostic significance.[16] Mechanistically, prophylaxis via IFN-λ is mediated by a cytokine receptor complex other than type I interferons that turns on downstream antiviral gene programs. Hence, the continued activity of these IFN-λ-related pathways in HCV-infected subjects is thought to underpin the high immunomodulatory profile recorded in serum.[17] This interpretation of immunological findings should also be placed in the context of the historical and physiological characterization of hepatitis viruses and their disease behavior as viral persistence and chronic inflammation are established features of the HCV pathophysiology. Thus, prolonged cytokine production has become a classic consequence of untreated hepatotropic viral infections, which are constantly stimulating immune surveillance mechanisms.[18] From a clinical immunopathological point of view, persistent HCV infection is well described to provoke systemic immune activation beyond hepatic inflammation, and significant alterations of circulating inflammatory mediators are observed. This is why cytokines IL-6, TNF-α, and IFN-γ may stay elevated in chronic patients compared with normal controls due to their continued immune stimulation.[19] Moreover, current epidemiological data shows that chronic HCV is still a global health problem and has long-lasting inflammatory effects. This enduring disease burden is also consistent with the chronic inflammatory signature described in the present study, in which the multiple immune biomarkers in the patients were significantly elevated.[20] Moreover, geographic and genotype distinctions of HCV were found to affect immune and clinical outcome in infected individuals. This could partially account for differences in absolute levels of cytokines and innate immune markers between studies, despite an overall similar trend, supporting that genotype diversity may have implications on the host immune dynamics.[21]

The markedly elevated TNF-α levels observed in this study are consistent with

previous evidence of association between TNF-α with chronic HCV disease and its systemic complications, such as glucose metabolism disturbances. TNF-α has the potential to act as an inflammatory amplifier and mediator between viral-driven inflammation and more general metabolic and immune responses.[8] Concurrently, intracellular antiviral response pathways are a hallmark of HCV infection, in which host cells mediate the loss and reinstatement of viral replication through interference of interferon-stimulated pathways and immune gene activation.[23] Hence, the excessive cytokine profile shown in this study corresponds biochemically to the enduring HCV-induced antiviral defense state.[22] The increased IL-6 in chronic HCV patients observed in this study was attributed to its key role in immune modulation of inflammatory cascades and hepatic immune function. IL-6 was consistently linked to chronic inflammatory diseases and the normal course of hepatitis C, indicating that it is a consequence of disease activity and immune-mediated tissue damage.[23] Regarding defensins, significant rise in HAD-1 and HBD-1 indicates for the modern immunological role of these proteins beyond the antimicrobial defense role. Defensins can stimulate innate immunity and adaptive immunity, and activate important immune effector cells[12], and their up regulation in chronic viral inflammation is therefore highly likely and meaningful. Furthermore, the defensins can involve chemotaxis to induce cells such as naïve T cells and immature dendritic cells to be recruited and further induce immune cell accumulation and extended immunological activation. This accounts for the elevated defensins in chronic HCV, where sustained immune cells migratory and activate is predicted.[24] Immunologically chronic HCV infection is characterized by the complex dysregulation of both innate versus adaptive immunity, including sustained secreted cytokines and immune modulators. Thus, the simultaneous rise of cytokines and defensins in this study represents widespread immunopathogenesis processes and not biomarker shifts.[3] The elevated IFN-γ in the study patient samples could be regarded as sustained T helper 1/natural killer (Th1/NK)-cell elevation also, consistent with chronic immune induction in viral hepatitis condition. Such persistent increase of IFN-γ may foster chronic inflammation while sustaining antiviral immune pressure.[25]

The ROC curve analysis is an established method for validating diagnostic biomarkers for viral infections, and similar diagnostic advantage of molecular diagnostic methods compared with serology was observed in cytomegalovirus-associated pregnancy loss.[26] Variability should be noted between population and among clinical cohorts, and risk factors and background variables may affect immuno-compromising markers. Differences in demographics, exposure history and local clinical context can differ significantly in biomarker magnitude, even though the inflammatory trajectory is similar in most studies.[27] Finally, the increase of TNF-α could in part be attributed to virally induced direct infection with innate immune receptors, namely toll-like receptor 7 (TLR7) and TLR8, leading to induced release of TNF-α, which in turn could aid in interferon signaling through autocrine signaling loops. This mechanism offers strong biological support for the intimate role of pro- and anti-inflammatory cytokines and antiviral interferon signaling in chronic HCV.[28]

In conclusion, we demonstrate the association between chronic HCV infection and significant immune dysregulation through the increased expression of circulating defensins (HAD-1 and HBD-1) and inflammatory/antiviral cytokines (IL-6, IL-28, TNF-α, and IFN-γ). Concurrent elevation of these biomarkers is indicative of ongoing systemic inflammation and persistent antiviral immune activation in the chronic HCV population. Notably, the ROC curve analysis demonstrated excellent diagnostic accuracy for defensins and cytokines, highlighting their strong potential as reliable noninvasive biomarkers for discriminating against chronic HCV patients from normal individuals. Altogether, the results suggested that a composite defensin-cytokine immune signature could be used as a diagnostic panel of choice for early detection and immunological monitoring of chronic HCV infection.

Author Contributions

ATE: Conceptualization, literature review, manuscript writing, editing, and final revision. AJJ: Data collection, literature review, and manuscript preparation. TKA: Supervision, scientific review, and manuscript editing. RKAs: Data analysis, manuscript review, and formatting. All authors reviewed and approved the final version of the manuscript.

Declaration of Conflicting Interests

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Ethical approval

The study protocol was reviewed and approved by the Ethics Committee of Ibn Sina University of Medical and Pharmaceutical Sciences (approval number ISU6.1.25).

Informed consent

Each study participant provided a verbal informed consent.

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