Elevated Co-infection in Hepatitis C Virus and Toxoplasmosis Patients With IL-32, IL-33 and TNF-α

Amjad T. Hameed [1*], Nazar S. Mohammed [1], Amani M. Jasim [1]

Abstract

Background: Hepatitis C virus (HCV) infection is a major global health concern, often leading to chronic liver diseases such as hepatitis, cirrhosis, and hepatocellular carcinoma. Toxoplasmosis, caused by Toxoplasma gondii, primarily affects the central nervous system but can also cause severe symptoms when co-infected with HCV, particularly in the liver. The relationship between these two infections and their impact on cytokine levels, especially interleukins (IL-32, IL-33) and tumor necrosis factor alpha (TNF-α), remains poorly understood. Methods: A case-control study was conducted involving 100 HCV-infected patients, 20 of whom were co-infected with both HCV and Toxoplasma. Blood samples were collected, and serological assays were performed to measure levels of HCV and Toxoplasma antibodies (IgM and IgG). Additionally, cytokine levels (IL-32, IL-33, TNF-α) were quantified using enzyme-linked immunosorbent assays (ELISA). DNA sequencing was performed to analyze gene variations associated with cytokine production. Results: Co-infected patients exhibited significantly higher levels of HCV and Toxoplasma antibodies compared to HCV-infected individuals without Toxoplasma co-infection ($p < 0.001$). Furthermore, levels of IL-32, IL-33, and TNF-α were elevated in co-infected patients, indicating a potential role of these cytokines in disease severity. The mean levels of IL-32, IL-33, and TNF-α were 10.110±0.596, 24.914±2.308, and 12.356±1.369, respectively, in co-infected patients. Conclusion: Co-infection with HCV and toxoplasmosis is associated with elevated levels of pro-inflammatory cytokines IL-32, IL-33, and TNF-α, suggesting a dysregulated immune response. These findings highlight the importance of understanding cytokine dynamics in co-infected patients for targeted therapeutic interventions and improved clinical management. DNA sequencing revealed variations in genes encoding cytokines, suggesting a genetic predisposition to elevated cytokine levels and disease progression.

Keywords: Hepatitis C Virus (HCV), Toxoplasmosis, Cytokine Levels, Interleukins (IL-32, IL-33), Tumor Necrosis Factor Alpha (TNF-α)

Significance | The co-occurrence of HCV and toxoplasmosis presents severe health risks, including liver damage and exacerbated symptoms. Understanding cytokine dynamics is crucial for targeted therapies.

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Introduction

Hepatitis C virus (HCV) infection has been conclusively linked to acute and chronic hepatitis, as well as liver cancer, establishing its significant impact on global health (Fahey et al., 2014). Chronic liver disease has various etiologies, with the most prevalent being alcohol, nonalcoholic fatty liver disease, and chronic infections by hepatitis C and B viruses. HCV, in particular, is a major global health concern, leading to chronic infections that can progress to severe liver conditions such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Al-Azzawi et al., 2022; Rasheed et al., 2022).

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HCV's pathogenicity primarily results from cytokine dysregulation rather than direct cytopathic effects (Dhefer et al., 2021). The diagnosis of HCV involves identifying the presence of the virus through serological assays and molecular assays, which detect HCV antibodies and genetic material, respectively (Alkhuder, 2023). Accurate diagnosis is crucial due to the similarity of clinical signs of HCV infection to those of other diseases, necessitating a high degree of clinical suspicion (Greigert et al., 2019).

An intriguing aspect of HCV infection is its co-occurrence with toxoplasmosis, although this is rare. Toxoplasmosis, caused by the parasite Toxoplasma gondii, when combined with HCV, can lead to severe complications. Symptoms of co-infection include elevated body temperature, fatigue, muscle pain, joint rigidity, abdominal discomfort, nausea, vomiting, and jaundice. The co-infection exacerbates the risk of developing cirrhosis and liver failure (Akoolo et al., 2022; Ifijen et al., 2023).

Diagnosing such co-infections typically requires a combination of blood tests, imaging techniques, and medical sample analysis. Treatment often involves a combination of antiretroviral and antiparasitic medications, and in severe cases, surgical intervention may be necessary to remove damaged tissues (Naranje et al., 2022). The regulation of cytokines, essential for a healthy immune response, can become dysregulated in these infections, leading to either excessive pro-inflammatory responses known as cytokine storms, or inadequate immune responses seen in immunodeficiency diseases. Both scenarios result in significant tissue damage and impaired functionality (Bulut et al., 2022; Yang et al., 2021).

Understanding the molecular basis of these infections and their interactions is critical for developing effective diagnostic and therapeutic strategies. This involves determining the sequence of nucleotides within DNA molecules, a process known as sequence determination, which is fundamental for advancing our knowledge of HCV and its co-infections.

Materials and Methods

2.1 Study Design

This research employed a case-control study design conducted at three medical facilities: Medical City Gastroenterology & Hepatology Centre, Ibn Al Balady Children and Maternity Hospital, and Balad General Hospital. The study period extended from May 2023 to the end of July 2023.

2.2 Participants

A total of 100 blood samples were collected from patients infected with HCV. Among these, twenty individuals were found to have co-infections with both HCV and Toxoplasmosis. The cohort consisted of 52 males and 48 females, with ages ranging from 5 to 65 years. To facilitate a structured comparison of outcomes, an unrelated control group of fifty healthy individuals was included. This control group consisted of twenty-five males and twenty-five females aged between 5 and 50 years.

2.3 Ethical Approvals

The study received approval from the Discussion Committee, Scientific Committee, and Postgraduate Studies Division of the College of Health and Medical Technologies at Middle Technical University. Additional approvals were secured from the Medical City Gastroenterology & Hepatology Centre, Ibn Al Balady Children & Maternity Hospital, and Balad General Hospital to ensure adherence to local ethical standards. Verbal consent was obtained from all participants after explaining the study's objectives, procedures, potential risks, and benefits. Confidentiality, anonymity, and the right to withdraw at any time were guaranteed. The study adhered to ethical standards, including the Declaration of Helsinki, and was conducted with precise laboratory tests.

2.4 Data Collection

To ensure precision and reliability, meticulous planning was involved in data collection. Serologic tests were primarily used to identify toxoplasmosis. Co-infection with HCV and toxoplasmosis, although rare, can result in severe consequences. Toxoplasmosis is caused by the parasite Toxoplasma gondii, while HCV primarily affects the liver. Concurrent infections often lead to exacerbated symptoms compared to separate infections (Akoolo et al., 2022). Indicators of co-infection include increased body temperature, fatigue, muscle discomfort, joint stiffness, abdominal pain, nausea, vomiting, and jaundice. Chronic co-infection can increase the risk of cirrhosis and liver failure (Ifijen et al., 2013). Diagnosis involves multiple blood tests, imaging studies, and medical sample analyses. Standard treatment includes antiretroviral and antiparasitic medications administered concurrently.

2.5 Diagnostic Assays and Analysis

2.5.1 HCV-IgM Investigation

The Enzyme-Linked Immunosorbent Assay (ELISA) method was used for HCV-IgM investigation. Blood samples were placed on microtiter plates pre-coated with HCV antigens (HCV-Ag). The reaction was then visualized through a color change, measured using a spectrophotometer at a wavelength of 450 nm with an accuracy standard deviation of 2 nm. This wavelength was selected for its high accuracy in detecting the specific color changes necessary for the assay.

2.5.2 Toxo IgM Investigation

For the Toxoplasma gondii IgM (Toxo IgM) investigation, the ELISA test was employed. A diluted serum sample from each participant was used in the assay. The level of IgM-specific antibodies was directly proportional to the intensity of the color developed during the enzyme-substrate reaction. The color intensity was measured using a spectrophotometer, which quantified the amount of IgM present in the samples.

2.5.3 Toxo IgG Investigation

Similarly, the Toxoplasma gondii IgG (Toxo IgG) levels were determined using the ELISA technique. Diluted serum samples were added to microplates coated with antigens. The intensity of the color developed in this assay correlated directly with the concentration of IgG-specific antibodies in the serum. The spectrophotometric readings provided a quantitative measure of the IgG antibodies.

2.5.4 Cytokine Measurements

Serum levels of cytokines IL-32, IL-33, and TNF-α were quantitatively assessed using sandwich ELISA tests. Microplates pre-coated with specific capture antibodies were used. During the assay, the serum samples were added, and the target cytokines bound to the antibodies. An enzyme-linked secondary antibody facilitated the detection of the bound cytokines through a substrate reaction, resulting in a color change. The intensity of the color change was measured with a spectrophotometer. Cytokine concentrations were calculated based on standard curves prepared according to the manufacturer's instructions.

2.5.5 Sequencing

The sequencing process involved several steps: DNA extraction, PCR amplification, sequencing, and assembly. DNA was extracted from the blood samples, amplified using polymerase chain reaction (PCR), and sequenced. The data obtained from sequencing were analyzed using the Geneious software program to identify sequence variations within a specific gene.

2.5.6 Primer Preparation

Lyophilized primers were obtained from Macrogen Company. The primers listed in Table 1 were reconstituted by dissolving them in nuclease-free water to achieve a stock concentration of 100 pmol/µl. For the working solution, 10 µl of the primer stock was diluted with 90 µl of nuclease-free water, resulting in a concentration of 10 µmol/µl. The primer solutions were stored at -20°C.

2.5.7 DNA Extraction

Genomic DNA was extracted from blood samples using the ReliaPrep™ Blood gDNA Miniprep System from Promega. The procedure followed the manufacturer's protocol, ensuring high-purity DNA suitable for downstream applications. The DNA samples showing clear bands upon gel electrophoresis were stored at 4°C for further use (Sehree MM et al., 2023).

2.5.8 DNA Quantitation

To determine the concentration of the extracted DNA, the Quantus™ Fluorometer was used. In this procedure, 200 µl of diluted Quantifluor Dye was mixed with 1 µl of DNA sample. The mixture was incubated at room temperature for five minutes before measurement. The fluorometer provided a quantitative value for the DNA concentration, ensuring the samples' suitability for further applications.

2.5.9 Primer Optimization

To determine the optimal annealing temperatures for the primers, DNA templates were amplified using identical forward and reverse primer pairs at annealing temperatures of 55°C, 58°C, 60°C, 63°C, and 65°C. PCR amplification was carried out in a 20 µl reaction volume, consisting of 10 µl of GoTaq Green Master Mix (2X), 1 µl of each primer (10 pmol), 6 µl of nuclease-free water, and 2 µl of template DNA. The PCR cycling was executed using the PCR Express (Thermal Cycler, BioRad, USA) with the following parameters: an initial denaturation at 94°C for four minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at the specified temperatures for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for seven minutes, followed by a termination step at 4°C for ten minutes.

2.5.10 Agarose Gel Electrophoresis

Following PCR amplification, agarose gel electrophoresis was employed to confirm the presence of amplified DNA. The process involved horizontal agarose gel casting, where agarose solution was poured into a gel tray with sealed edges, and left to solidify at room temperature for 30 minutes. After solidification, the comb was gently removed, and the gel was placed in an electrophoresis tank filled with 1X TAE buffer until the buffer covered the gel surface by 3-5 mm. PCR products (5 µl) were directly loaded into the wells of the gel. Electrophoresis was conducted at 100 volts/50 mA for 60 minutes, allowing the DNA to migrate from the cathode to the anode. The ethidium bromide-stained bands were visualized using a gel imaging system (Chen S et al., 2022).

2.5.11 Standard Sequencing

The PCR products were sent to Macrogen Corporation in Korea for Sanger sequencing using ABI3730XL automated DNA sequencers. The sequencing results were received via email and analyzed using Geneious software.

Standard sequencing was conducted using the ABI3730XL automated DNA sequencers by Macrogen Corporation in Korea. The results of the Sanger sequencing were received via email and subsequently analyzed using Geneious software.

The analysis of genes using Sanger sequencing focused on the SNPs rs2283468, rs12922880, and rs12934561 of IL-32, as shown in Supp. Table 1 and Figures 1, 2, and 3. The IL-32 gene, which is part of the cytokine family, contains sites for tyrosine sulfation, potential N-myristoylation, multiple putative phosphorylation, and RGD cell attachment sequences. Expression of this protein is increased by the activation of T cells with mitogen or NK cells with IL-2, and it induces TNFα production from macrophages. Multiple isoforms of IL-32 are produced via alternate transcriptional splice variants, with the gene located at 16p13.3 and consisting of six exons.

2.5.12 Statistical Analysis

Data analysis was conducted using SPSS software version 20.0. Quantitative data were summarized using mean and standard error,

while qualitative data were presented as numbers and percentages. The Student's t-test was utilized to determine the statistical significance of the results, with a p-value of 0.01 considered significant (Metwally M et al., 2021).

3. Results

The study revealed distinct differences in HCV IgM levels across the control group, HCV patients, and individuals with co-infections. In the control group, the mean HCV IgM level was 0.081±0.022. Among HCV-infected patients, this mean increased significantly to 13.498±0.965, and even more markedly in patients co-infected with other pathogens, where the mean was 19.077±2.085. Statistical analysis confirmed a highly significant difference between these groups ($p<0.001$).

The analysis of Toxo IgM levels showed that the control group had a mean of 0.111±0.024. In contrast, HCV patients exhibited a slightly lower mean of 0.108±0.018, while co-infected individuals had a substantially higher mean of 2.289±0.839. This difference was statistically significant ($p<0.001$), highlighting a considerable disparity in Toxo IgM levels between the groups.

Similarly, Toxo IgG levels varied significantly among the groups. The control group had a mean of 0.096±0.022, HCV patients had a slightly lower mean of 0.086±0.0165, and co-infected individuals had a mean of 1.350±0.233. The statistical analysis underscored a very significant difference between these groups ($p<0.001$).

The investigation into cytokine levels revealed that the control group had an average IL-32 level of 3.919±0.226. In HCV patients, the mean IL-32 level rose significantly to 10.508±0.265, and in those with co-infections, it was 10.110±0.596. The differences between these groups were statistically significant ($p<0.001$).

For IL-33, the control group showed an average level of 4.775±0.249. HCV patients had a much higher mean level of 24.037±0.884, while individuals with co-infections exhibited a mean level of 24.914±2.308. This substantial variation was also statistically significant ($p<0.001$).

TNF-α levels were similarly variable, with the control group having a mean of 4.655±0.271. HCV patients showed a significantly higher mean of 15.146±0.998, and co-infected individuals had a mean of 12.356±1.369. The differences between these groups were highly significant ($p<0.001$).

The analysis of the IL-33 gene, specifically SNP rs7044343, is detailed in Supp. Table 2 and Supp. Figure 3. IL-33, like IL-32, belongs to the cytokine family and shares similar structural features and functional properties. It is located at 16p13.3 and has six exons, with various isoforms resulting from alternate splicing (Supp. Figure 1, Supp Figure 2).

Lastly, the analysis of the TNF-A gene focusing on SNP rs2430561 is presented in Supp Table 3 and Supp Figure 4. TNF-A, another cytokine family member, also shares structural and functional characteristics with IL-32 and IL-33, including its role in TNFα production and its response to immune cell activation.

4. Discussion

The results of this study revealed a significant variation in HCV IgM levels among different patient groups, with the highest levels observed in those with co-infections, followed by HCV patients, and the lowest levels in the control group. This finding aligned with previous research indicating that co-infections can exacerbate the severity of individual infections, leading to higher antibody levels (Whittaker et al., 2018; Backmund et al., 2005). The seroprevalence of triple co-infection with HIV, HCV, and Toxoplasma in a Chinese study was notably high at 7.7% among HIV-positive plasma donors (Zhang et al., 2008). This supported the notion that co-infections were not only prevalent but also significantly impacted immune response and disease progression.

The study further highlighted the significant differences in Toxo IgM and IgG levels among the control, HCV, and co-infected groups. These findings suggested a complex interplay between Toxoplasma gondii and HCV infections, potentially exacerbated by overlapping risk factors such as injectable drug use (Backmund et al., 2005). The significant correlation between anti-HCV antibodies and Toxoplasma IgG observed in previous studies (El-Nahas et al., 2014) underscored the need for integrated screening protocols for individuals at risk. This was particularly relevant given the overlapping transmission routes and risk factors for both infections.

A deeper understanding of the relationship between Toxoplasma gondii and HCV infections was critical. El-Nahas et al. (2014) suggested that chronic hepatitis C patients exhibited higher levels of anti-T. gondii IgG/IgM, indicating a potential immunological link. The presence of Toxoplasma antigenic proteins in current infections could influence the immune response to HCV, leading to variations in viral load and disease severity. This was supported by findings that negative results for Toxoplasma antigen (Tag) were associated with lower levels of HCV Nonstructural protein (HCV-NS4) (Medina et al., 2021).

The correlation between T. gondii-specific antibodies and HCV viral load was further emphasized by Mohamed et al. (2021), who found a strong statistical relationship between blood TAg and serum HCV-NS4 levels. This indicated that the immune response to one pathogen could significantly influence the course and severity of the other infection. Consequently, it was recommended that individuals with positive toxoplasmosis antibodies should also be tested for HCV to ensure comprehensive disease management and control (Bazmjoo et al., 2023).

Table 1. Name of primers and sequences used in the Molecular study

Primer Name	Sequence 5'-3'	Annealing Temp. (°C)	Product Size (bp)
rs7044343-F	TGTAAAACGACGGCCAGTTGTCTCACCAGAGGGATTT	60	996
rs7044343-R	CAGGAAACAGCTATGACCATCAACACCGTCACCTTAC		
rs12934561-F	TGTAAAACGACGGCCAGTCCTCCAAATCTCGGGTTTAAG		1025
rs12934561-R	CAGGAAACAGCTATGACGCAAAGGTGGTGTCAGTATC		
rs2430561-F	TGTAAAACGACGGCCAGTCGTTGCTCACTGGGATTT	55	1029
rs2430561-R	CAGGAAACAGCTATGACCATGTCTTCCTTGATGGTCTC		

Table 2. Distribution of HCV IgM among controls, hepatitis C patients and other co-infections

Parameter	Groups	N	Mean±S.E	P value
HCV IgM	Control	50	0.081±0.022	p<0.0001 (HS)
	Hepatitis Patients	100	13.498±0.965	
	Co-infection patients	20	19.077±2.085	

Table 3. Distribution of Toxo IgM and IgG among the controls, HCV patients and other co-infections

Parameter	Groups	N	Mean±S.E	P value
Toxo IgM	Control	50	0.111±0.024	<0.0001**
	Hepatitis Patients	100	0.108±0.018	
	Co-infection patients	20	2.289±0.839	
Toxo IgG	Control	50	0.096±0.022	<0.0001**
	Hepatitis Patients	100	0.086±0.016	
	Co-infection patients	20	1.350±0.233	

Table 4. Distribution of IL-32, IL-33 and TNF-α among the controls, HCV patients and other co-infections

Parameter	Groups	N	Mean±S.E	P value
IL-32	Control	50	3.919±0.226	p<0.0001 (HS)
	Hepatitis Patients	100	10.508±0.265	
	Co-infection patients	20	10.110±0.596	
IL-33	Control	50	4.775±0.249	p<0.0001 (HS)
	Hepatitis Patients	100	24.037±0.884	
	Co-infection patients	20	24.914±2.308	
TNF-α	Control	50	4.655±0.271	p<0.0001 (HS)
	Hepatitis Patients	100	15.146±0.998	
	Co-infection patients	20	12.356±1.369	

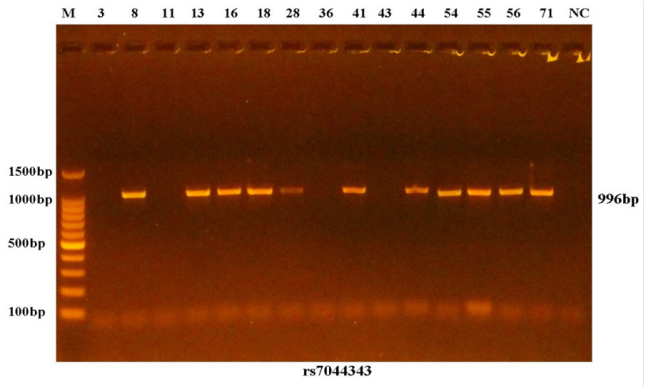

Figure 1. Fractionation of the results of the amplification of the rs7044343 specific region of human samples species was performed on 1.5% agarose gel electrophoresis and stained with Eth.Br. M: 100bp ladder marker. The 996-bp PCR products are similar to the lanes 3-71.

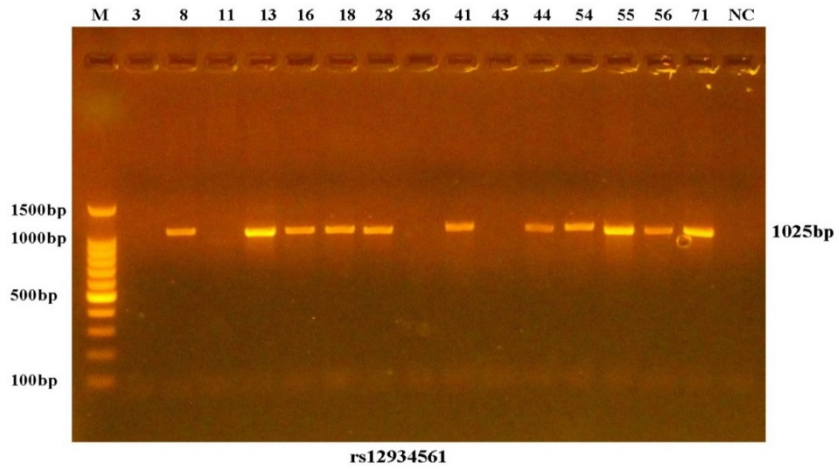

Figure 2 shows the results of the amplification of the rs12934561 specific area of human samples. The samples were fractionated on 1.5% agarose gel electrophoresis and stained with Eth.Br. M: 100bp ladder marker. The 1025-bp PCR products are similar to the lanes 3-71.

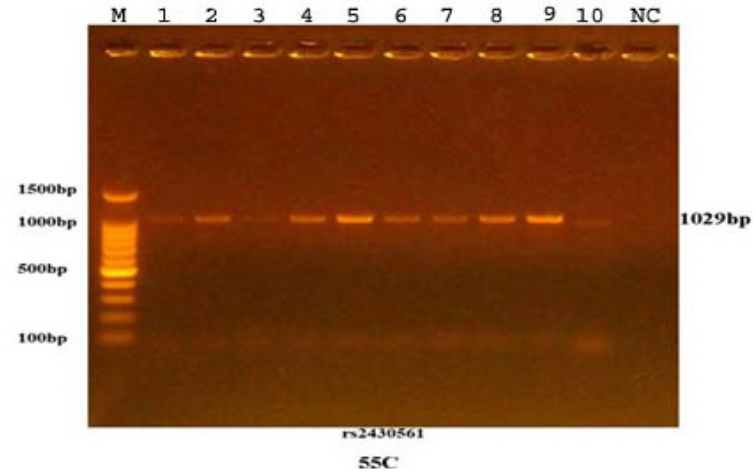

Figure 3 shows the results of the amplification of the rs1800797 specific gene region of human samples. The samples were fractionated on 1.5% agarose gel electrophoresis and stained with Eth.Br. M: 100bp ladder marker. It is similar to 969bp PCR products that Lanes B41-B71 areData Analysis

The study also delved into the cytokine profiles of different patient groups, revealing significant differences in IL-32, IL-33, and TNF-α levels. These cytokines played crucial roles in mediating inflammatory responses and were indicative of disease severity and progression. Higher levels of IL-32 in HCV patients and those with co-infections suggested a heightened inflammatory response, which could contribute to liver damage and fibrosis (Bae et al., 2023). This was supported by findings that IL-32 co-localized with pro-inflammatory cytokines in inflamed tissues (Si Yr et al., 2022).

IL-33 levels were also elevated in HCV and co-infected patients compared to controls, indicating its role in chronic inflammation and immune regulation. The cytokine's involvement in the recruitment and activation of various immune cells underscored its importance in managing chronic infections (Yang et al., 2023). Elevated IL-33 levels in co-infected patients may have reflected a compounded inflammatory response, necessitating targeted therapeutic strategies.

TNF-α, a well-known pro-inflammatory cytokine, was significantly higher in HCV and co-infected patients. TNF-α is produced by various cell types, including monocytes and macrophages, and plays a pivotal role in mediating inflammatory and immune responses (Liang et al., 2023; Yang et al., 2020). The association of TNF-α with advanced stages of liver disease, including cirrhosis and liver cancer, highlighted its relevance in the pathogenesis of chronic HCV infection (Zheng et al., 2023).

The genetic polymorphisms associated with TNF-α and IFN-γ also contributed to the variability in immune responses and disease outcomes among patients. Studies had shown that specific gene variations could influence the severity of liver disease and the risk of developing complications such as cirrhosis and hepatocellular carcinoma (Zhong et al., 2023). These findings underscored the importance of genetic screening and personalized medicine in managing chronic HCV infection and associated co-infections.

Overall, this study provided valuable insights into the immunological and genetic factors influencing HCV and Toxoplasma gondii co-infections. The significant differences in antibody levels and cytokine profiles among different patient groups highlighted the need for comprehensive screening and tailored therapeutic approaches. The findings supported the integration of HCV and Toxoplasma testing in high-risk populations to improve disease management and patient outcomes. Further research was needed to elucidate the underlying mechanisms of these interactions and to develop effective strategies for preventing and treating co-infections.

Furthermore, our investigation revealed critical insights into the presence and elevation of interleukins IL-32 and IL-33, as well as tumor necrosis factor (TNF-α), in the bloodstreams of patients. The findings demonstrated that individuals diagnosed with both HCV and Toxoplasma had significantly elevated levels of IL-32, IL-33, and TNF-α. This elevation in cytokine levels suggests a heightened inflammatory response in co-infected patients. In contrast, the control group, which comprised healthy individuals, exhibited significantly lower levels of these interleukins and TNF-α. This comparison underscores the impact of co-infections on the immune system, highlighting the importance of monitoring cytokine levels in managing and understanding the progression of these diseases.

5. Conclusions

Our study underscored the significant impact of co-infections with HCV and Toxoplasma gondii on cytokine profiles and immune responses. The investigation revealed that patients with both HCV and Toxoplasma exhibited markedly elevated levels of interleukins IL-32 and IL-33, as well as tumor necrosis factor (TNF-α), compared to those infected with HCV alone and healthy controls. These findings suggest that co-infection exacerbates inflammatory responses, potentially leading to increased liver damage and disease severity. The control group, composed of healthy individuals, showed significantly lower levels of these cytokines, highlighting the stark contrast in immune activation. The elevated cytokine levels in co-infected patients emphasize the need for comprehensive diagnostic and therapeutic strategies to manage such infections effectively. Monitoring cytokine levels can provide valuable insights into the progression and management of HCV and Toxoplasma co-infections, aiding in the development of targeted treatments to mitigate their severe complications.

Ethics

The authors sincerely thanked the dedicated staff of Medical City/Gastroenterology & Hepatological Centre, Ibn Al-Balady Children & Maternity Hospital, and Balad General Hospital for their invaluable support and cooperation throughout the duration of the study. Additionally, heartfelt appreciation was extended to the participants of the study for generously providing their samples, enabling the advancement of understanding of these complex medical conditions.

Author contributions

A.T. H. performed the experiments, wrote the manuscripts. N. S. M., A. M. J., designed the study, reviewed, edited and wrote the article.

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The authors expressed their sincere gratitude to the dedicated staff of Medical City/Gastroenterology & Hepatological Centre, Ibn Al-Balady Children & Maternity Hospital, and Balad General Hospital for their invaluable support and cooperation throughout the duration of this study. Additionally, we extend our heartfelt

appreciation to the participants of this study for generously providing their samples, enabling us to advance our understanding of these complex medical conditions. The project was approved by the local ethical committee in Middle Technical University, Code Number [number 1036/18 on 4/17/2023].

Competing financial interests

The authors have no conflict of interest.

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