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Serum levels of interleukin-10 in both responders and non-responders to DAA therapy for the hepatitis C virus

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ABSTRACT

Hepatitis C virus (HCV) infection represents a significant global health challenge, with the advent of direct-acting antivirals (DAAs) substantially transforming its therapeutic landscape. Nevertheless, it is noteworthy that not all individuals achieve a sustained virologic response (SVR) post-treatment with DAAs. This study seeks to elucidate the function of interleukin-10 (IL-10) in the immune response to HCV infection and its association with therapeutic success. According to the recommendations from the Egyptian National Committee for Control of Viral Hepatitis (NCCVH), 4,300 patients were selected for treatment with the novel DAA therapy aimed at managing HCV infection. Comprehensive assessments were conducted on all participants, including evaluations of liver function profile, creatinine levels, complete blood count, prothrombin time, serum Alpha-Fetoprotein (AFP), and RT-PCR for HCV diagnosis. Additionally, serum human IL-10 levels were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Non-responder patients had significantly elevated levels of alanine (ALT), aspartate aminotransferase (AST), ALP, and total bilirubin. They exhibited considerably lower albumin levels and platelet count. Furthermore, non-responding patients had significantly high values of two common fibrosis markers fibrosis-4 score (FIB-4) and AST to platelet ratio index (APRI). There was no association ($P > 0.05$) between treatment failure and IL-10 (18.99±7.24 vs. 18.82±4.19 pg/mL for non-responder and responder patients, respectively). The ROC analysis indicated that IL-10 demonstrated an AUC of 0.546 in distinguishing non-responder patients, showing low sensitivity and specificity values. Regardless of DAA HCV treatment, the serum level of IL-10 doesn't differ significantly. DAA therapy alone may not result in substantial variations in IL-10 levels between responders and non-responders.

Keywords: Hepatitis C Virus, interleukin 10, Direct-acting antivirals and therapy response.

1. INTRODUCTION

Around 71 million people worldwide contracted HCV in 2015. HCV is a significant contributor to mortality from infectious illnesses and continues to be the primary reason for liver transplantation in several regions globally [1, 2]. Consequently, promptly identifying and efficiently handling the illness can alter its inherent progression [3]. Effective antiviral therapy can avoid both the immediate and long-lasting consequences of HCV infection in a significant number of individuals [4]. There have been major improvements in HCV therapy, including the introduction of novel treatments. As a result, the likelihood of achieving a cure has greatly improved. Boceprevir and telaprevir, as pioneering direct-acting antivirals (DAAs), initially played a significant role in treating individuals infected with Hepatitis C Virus (HCV) genotype 1. These agents achieved sustained virologic response (SVR) rates exceeding 70%, demonstrating notable efficacy in managing the condition. The response was mostly influenced by viral kinetics and hepatic fibrosis. The most recent generations of pan-genotypic antiviral medicines have resulted to considerably greater rates of sustained virological response (SVR) [3]. Nevertheless, a minority of individuals encounter recurrence after receiving therapy [5]. There is limited knowledge of factors that might predict the inability to achieve a sustained virologic response (SVR) using direct-acting antiviral agents (DAAs). Several clinical factors, including age, race, multiple HIV infections, insulin resistance, and the interleukin (IL)-28b genotype, have been linked with a poor response to pegylated IFN therapy. Nevertheless, none of these parameters exhibited any correlation with the recurrence of a virus following DAA-based treatment [2, 5, 6]. IL-10 has demonstrated a protective function in two models of acquired hepatitis caused by Concavalin A, galactosamine, and lipopolysaccharide. When someone gets an anti-IL-10 antibody before starting ConA therapy, their hepatitis gets worse, and their blood levels of IL-12, TNFα, and IFNγ rise. Mice lacking IL-10 have exhibited comparable outcomes [7]. Interleukin-10 is pivotal in modulating the immune response to (HCV) infection [8,9]. Increased levels of the substance can support the long-term existence of viruses and have a detrimental effect on the effectiveness of conventional interferon-based therapy for treating hepatitis C [10]. Gaining insight into the function of IL-10 in HCV infection and treatment has significant implications for the development of more effective therapeutic approaches [11]. IL-10 levels have become a new biomarker for assessing the extent of inflammation in hepatocellular carcinoma, taking into account the potential role of IL-10 in the progression of hepatitis C virus-related HCC [12]. Sharafeldin et al. [12] suggested that combining alpha-fetoprotein (AFP) with interleukin-10 (IL-10) could make it much easier to diagnose and predict hepatocellular carcinoma (HCC). Future research should prioritize assessing these biomarkers' clinical validity, predictive utility, and compatibility with other therapeutic methods, such as immunotherapy [12]. The purpose of this study is to assess and compare the levels of IL-10 in the serum of individuals who have successfully eliminated the Hepatitis C virus (HCV) management following therapy with direct-acting antivirals (DAAs) (referred to as DAA HCV responders), and those who have not been able to eliminate the virus (referred to as DAA HCV non-responders). The study seeks to ascertain any notable disparities in the serum IL-10 levels between these two groups.

2. MATERIALS AND METHODS

2.1. Ethical approval

The Ethics Committee of the Faculty of Medicine at Port Said University, designated by serial number 92 (BIO_003), granted ethical approval in compliance with the ethical standards outlined in the Helsinki Declaration. Informed consent was obtained from all participants involved in the study.

2.2. Study Population

This prospective study included two subgroups: 1) DAA-responder HCV patients; and 2) DAA-non-responder HCV patients. For this prospective study, we collected blood samples (3-5 mL) from patients in various groups who underwent clinical examinations at the Viral Diseases outpatient clinic at Sherbin Central Hospital, Sherbin, Dakahlia Governorate, Egypt. From 2017 to 2023, the Egyptian national

campaign for HCV screening and treatment, also known as the 100 million seha period, took place from 2018 to 2019. Throughout the entire treatment duration, we documented demographic, clinical, and laboratory data for every patient using a specialized electronic case report form. The main DAA treatment consisted of a 12-week course of sofosbuvir (SOF)/daclatasvir (DCV), without ribavirin, tailored to each patient's needs. After the treatment procedure, we measured the viral load using qPCR, with the detection threshold is established at 15 IU/mL. A sustained virological response (SVR) is characterized by the absence of detectable viral RNA in blood serum for at least three months after stopping DAA therapy. The criteria for inclusion encompassed the confirmed diagnosis of HCV infection by HCV RNA assessment, as well as ages between 18 and 65 years. The patients are willing and able to provide informed consent forms. Additionally, the patients are either treatment-naive or have undergone prior treatments involving interferon therapy. Exclusion criteria were included. Some people who were not eligible were pregnant, had both HBV and HIV, had failed DAA treatment, had a history of HCC or unclassified nodular lesions, were pediatric patients with HCC, had non-hepatic cancers, were actively bleeding in their intestines, had neoadjuvant chemotherapy, microwave/radiofrequency ablation, or Sorafenib before surgery, or had a liver transplant or were waiting for one.

2.3 Biochemical assessment

To examine the liver profile, we performed lab tests on fresh serum utilizing commercial kits and an automated biochemistry analyzer (Response 920, DiaSys Diagnostic Systems, GmbH, Germany) per the manufacturer's recommendations. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin, alkaline phosphatase (ALP), and a creatinine test were performed to assess kidney function. To determine prothrombin time in citrate-anticoagulated blood samples, we utilized commercial kits (Spectrum, Egypt) and a semi-automatic coagulation analyzer (Thrombosta, Behnk Elektronik, Germany). We also performed a full blood count on the Sysmex automated analyzer (Sysmex, Japan) using EDTA-K3-treated blood.

2.4 Measurement of Alpha-Fetoprotein (AFP)

The study involved determining AFP using a commercial ELISA (PerkinElmer Health Science, Inc. US10 Divsison, Hayward CA, USA, Cat# 10101) following the manufacturer's instructions. The assay method employs an anti-AFP antibody for solid-phase immobilization within microtiter wells, alongside a mouse monoclonal anti-AFP antibody integrated into the enzyme conjugate solution containing horseradish peroxidase. The test specimen, which is serum, was introduced to the microtiter wells coated with the AFP antibody and subsequently incubated with the zero buffer. A specimen with human AFP will combine with a good antibody. After washing the well to eliminate any remaining test specimens, we added AFP antibodies labeled with horseradish peroxidase (conjugate). In this procedure, IgG immunologically binds to AFP present in the well, positioning the AFP molecules between the solid phase and the enzyme-linked antibodies. Following an incubation period at room temperature, the wells are rinsed with water to eliminate any unbound labeled antibodies. A TMB solution is then introduced and incubated for 20 minutes, producing a blue coloration. The development of color is halted by the addition of 2N HCl, which alters the color to yellow. This change in color is subsequently measured spectrophotometrically at a wavelength of 450 nm. The intensity of the color in the test sample is directly proportional to the concentration of AFP.

2.5 Measurement of HCV RNA

RT-PCR has three main steps for diagnosing HCV: removing the viral RNA, changing the HCV-RNA into complementary DNA (cDNA), and amplifying the product and finding it. We performed HCV-RNA extraction using the Artus® HCV RG RT-PCR kit (Qiagen GmbH, Germany), following the manufacturer's instructions. The viral load was quantified utilizing the Rotor-Gene Q MDx Light Cycler

Real-Time PCR System from Qiagen, Germany. This procedure employed the Rotor-Gene 3000 software, version 6.23.

2.6 Measurement of Serum level of Interleukin 10 (IL-10)

The study involved the determination of (IL-10) using a commercial ELISA kit (Bioneovan Co., Ltd, Nova, No. 18, Keyuan Road, DaXing Industry Zone, Beijing, China). The Sandwich-ELISA method is used in the ELISA kit. This kit coats the Microelisa strip plate with antibodies specific to the Human IL-10 Standards. Specimens are introduced into the designated wells of the Microelisa strip plate and subsequently combined with the specified antibody. Following this, each well receives an addition of horseradish peroxidase (HRP)-conjugated antibody, which is specific for human interleukin-10 (IL-10), before undergoing incubation. Any unbound elements are thereafter removed through a washing process. The TMB substrate solution is then applied to each well. Wells that contain IL-10 alongside the HRP-conjugated Human IL-10 antibodies will exhibit a blue coloration, transitioning to yellow upon the introduction of the stop solution. The optical density (OD) is determined using spectrophotometry at a wavelength of 450 nm. This OD measurement correlates with the concentration of human interleukin-10 (IL-10). By comparing the OD values of the samples to a standard calibration curve, one can ascertain the concentration of human IL-10 present in the samples. The assay is designed to measure concentrations ranging from 1.2 pg/mL to 100 pg/mL.

2.7 Statistical methods

Statistical analyses were conducted utilizing SPSS version 20 and GraphPad Prism version 9.0. Continuous numerical data were represented by the mean, while categorical variables were expressed with the standard deviation (SD). The data was compared using the student's t-test or chi-squared test ($\chi 2$). We assessed each cytokine's diagnostic ability for SVR prediction in terms of sensitivity, specificity, positive predictive (PPV) and negative predictive values (NPV), as well as AUC. Statistical significance was established at $p < 0.05$.

Fibrosis (FIB-4) indices were calculated after Naga et al. [13] according to the following equation:

$$\text{FIB} - 4 = \text{Age ([yr] x AST [U/L]) / ((PLT [}10^9\text{/L]) x (ALT [U/L])(1/2))}$$

Aspartate aminotransferase (AST) to platelet ratio index (APRI), was determined according to Amernia et al. [14].

3. RESULTS AND DISCUSSION

3.1 Characteristics of patients

We conducted the current study on a total of 175 CHC patients. We enrolled them from a real-world population (n=4300) who received treatment at Sherbin Central Hospital, Dakahlia Government, Ministry of Health, Egypt, as part of the national initiative to eliminate HCV. The study included all patients who did not obtain SVR (n = 75; non-responder; response rate was 98.26%), and randomly selected 100 patients from those who obtained SVR (responder). Comparing the median age of DAA-responders (49.5±6.9) and DAA-non-responders (51.56±7.0) in HCV patients with each other revealed non-significant differences ($P < 0.069$). Also, male/female genders showed no significant differences ($p < 0.252$), with the respondent being male/female (40/60) and the non-respondent being male/female (30/45) as shown in Table (1).

Table 1. **Gender and age variables in the DAA responder and non-responder groups**

Variables	Responder	Non-Responder	*P* value
Gender (male/female)	40/60	30/45	0.252
Age (years)	49.5±6.9	51.56±7.0	0.069

3.2 The biochemical parameters of both responders and non-responders to DAA therapy for HCV.

Patients who failed to exhibit a reaction demonstrated significantly elevated levels of ALT, AST, ALP, and total bilirubin (P<0.05), while displaying markedly reduced levels of albumin and platelet count (P<0.05). Additionally, these individuals presented with substantially higher levels of the widely recognized fibrosis markers, FIB-4 and APRI (Figures 1,2,3).

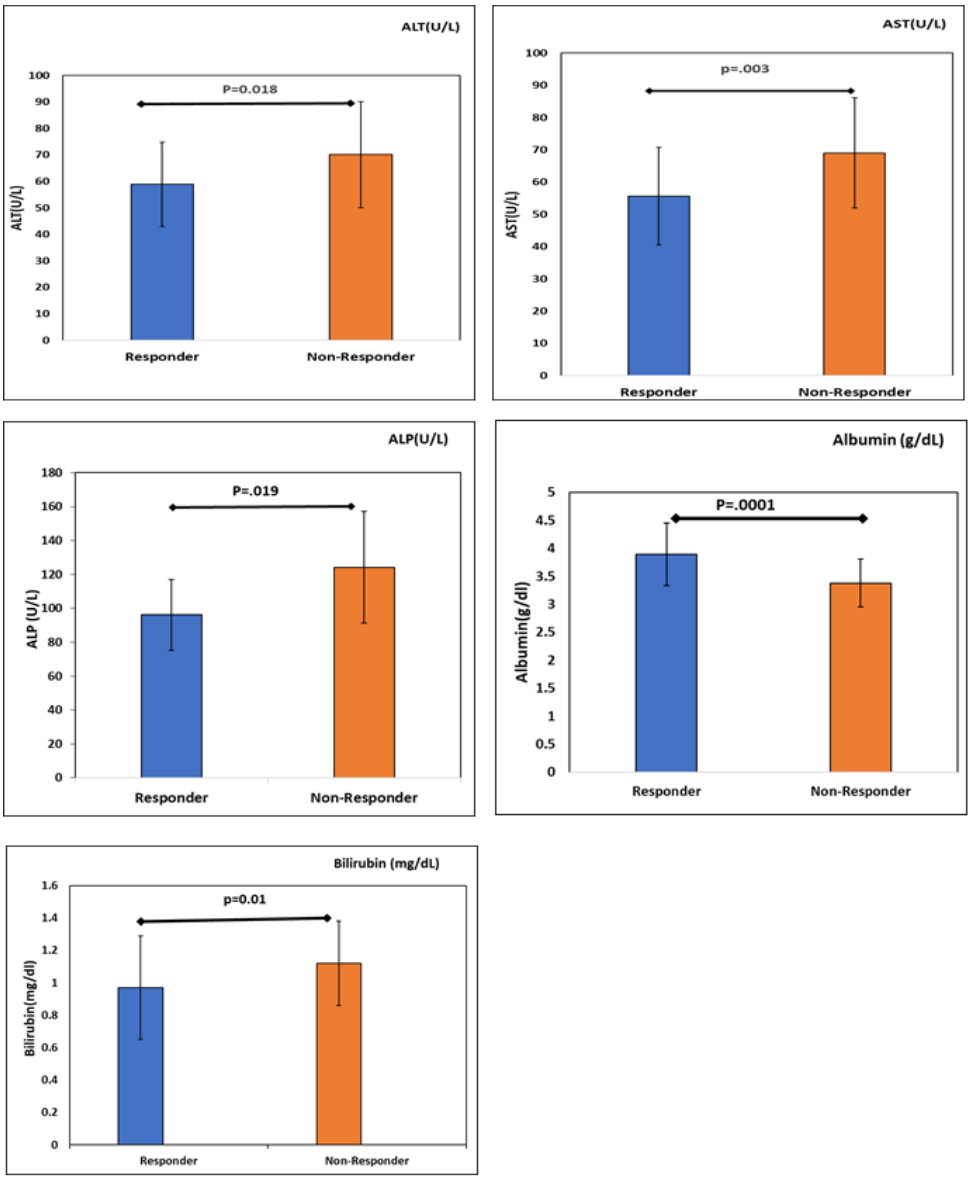

Fig. 1. The biochemical parameters of Liver function tests (ALT, AST, ALP, Albumin, and Bilirubin) in both responders and non-responders to DAA therapy for HCV.

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase

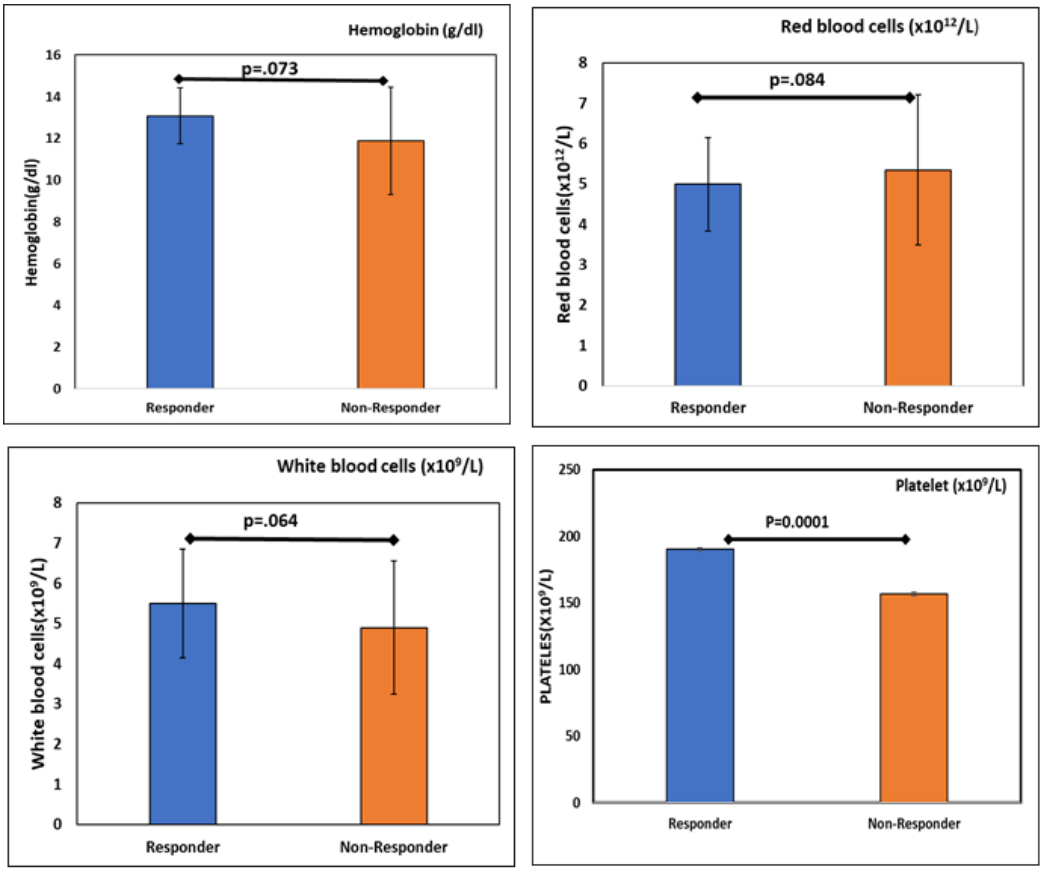

Fig. 2. Complete blood count (CBC) test (HB, RBCs, WBCs, and Platelets) in the DAA HCV therapy responder and non-responders

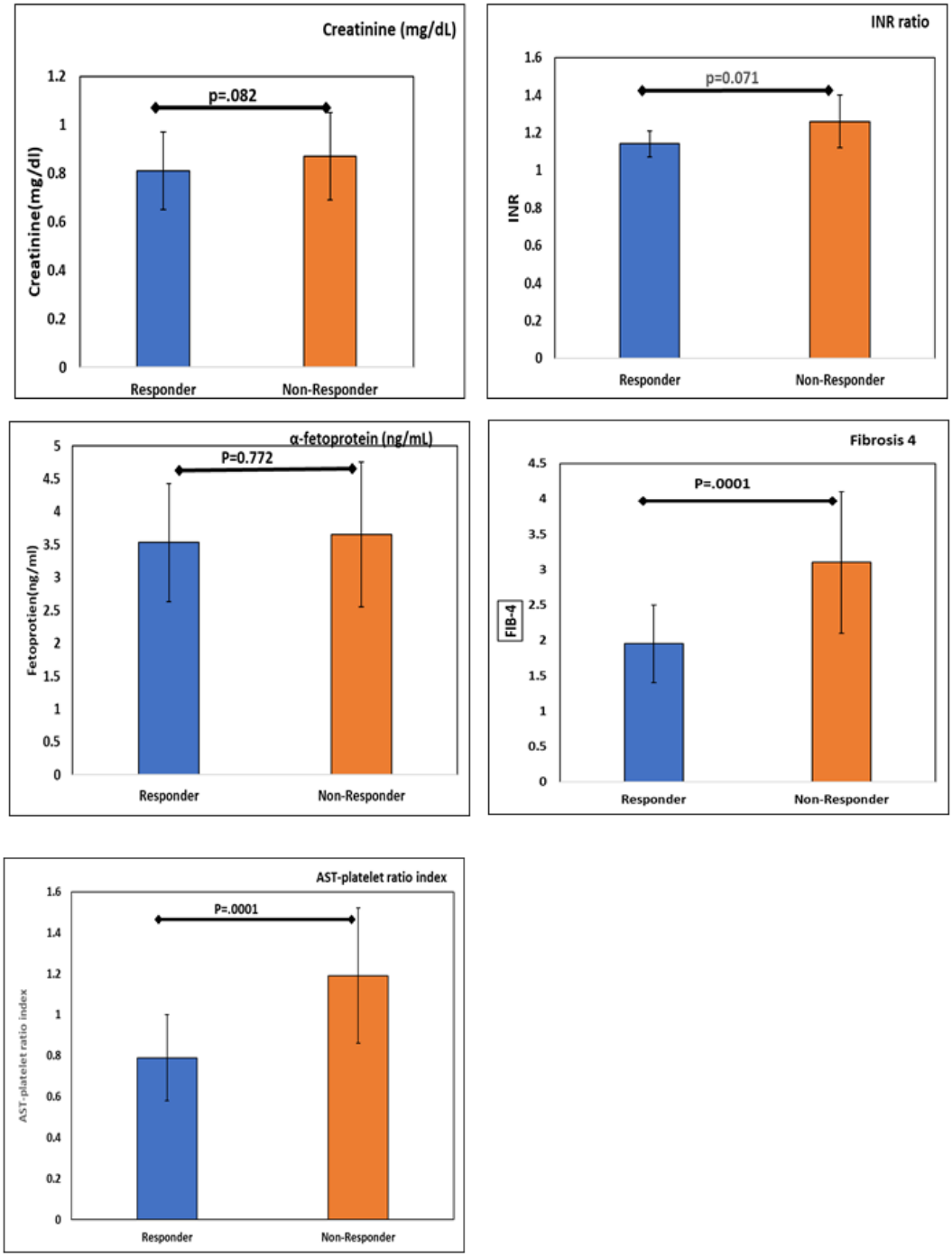

Fig. 3. The biochemical parameters (Creatinine, INR, α fetoprotein, FIB-4, (fibrosis-4 score); Aspartate aminotransferase (AST) to platelet ratio index (APRI), in both responders and non-responders to DAA therapy for HCV.

3.3 Differential expression of serum IL-10 in both responders and non-responders to DAA therapy for HCV.

There was no association (P >0.05) between treatment failure and IL-10 (18.99±7.24 *vs.* 18.82±4.19 pg/mL for non-responder and responder patients, respectively). We assessed its ability to differentiate non-responders from responders using the ROC curve analysis. The ROC revealed that IL-10 achieved an Area Under the Curve (AUC) of 0.546 for differentiating between patients who do not respond to treatment (Figure 4, Table 2), with poor sensitivity and specificity values.

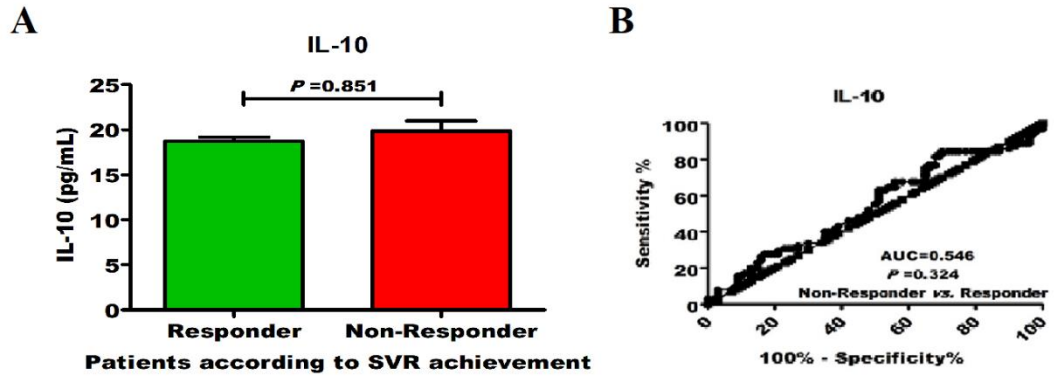

Fig. 4. Comparison of the IL-10 levels in the blood of responders and non-responders (4A). Figure 4B shows the area under the receiver-operating characteristic curve of IL-10 for telling the difference between non-responders and responders.

Table 2. Response to DAA HCV Therapy, Sensitivity, Specificity, Accuracy, PPV, and NPV of Serum IL10

Categories	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV(%)	NPV(%)	Accuracy(%)
IL-10≥ 18 pg/mL	0.546 (0.45-0.64)	50.7	50	43.2	57.5	50.3

Cutoff values were established through Receiver Operating Characteristic (ROC) analysis. In the study, patients who did not respond to the treatment were compared to those who did. The analysis considered the Positive Predictive Value (PPV) or Negative Predictive Value (NPV). Additionally, the Area Under the Curve (AUC) was calculated to assess the effectiveness of the ROC curve.

HCV is a hepatotropic RNA virus, classified under the family Flaviviridae Hepacivirus genus. A 50 nm structure encapsulates the virus, containing a positive RNA strand. Each of the six main genotypes of HCV has a minimum of 30% variation in nucleotide sequence compared to the others. The presence of genetic diversity within the population plays an important role in the development of resistance to treatment and the immune system's ability to avoid detection. Hepatitis C virus genotype 4 is the most common in Egypt, accounting for 92.5% of cases, whereas type 1 is present in 3.6% of cases [15]. Since 2014, Egypt has effectively treated over 2 million patients, yet when their program began in 2018, HCV infection remained a major challenge. At that time, 4.6% of the adult population who had not previously received treatment tested positive for the virus. Consequently, Egypt ranked among the top ten countries worldwide with the highest incidence of HCV, leading to significant implications for public health and the economy [16]. Standard treatment for hepatitis C virus (HCV) involves the use of peginterferon, ribavirin, and Harvoni, each associated with the regression of liver fibrosis. Recently, the FDA has

authorized a few of new HCV treatments, such as Epclusa® (sofosbuvir) and OLYSIO® (simeprevir). Direct-acting antiviral drugs (DAAs) have a high rate of sustained virologic response (SVR), which means that the hepatitis C virus (HCV) can't be found in blood plasma 24 weeks after the end of antiviral treatment. Nevertheless, around 30% of patients exhibit no amelioration in fibrosis during sustained virologic response (SVR) [17]. Cytokines are proteins that are either released or attached to the cell membrane. They play a crucial role in controlling the growth, specialization, and activation of immune cells. They are a component of the immune system that aids in the host's defense against invading pathogens. Cellular stressors such as infection, inflammation, and carcinogen-induced damage trigger the release of cytokines. Baraka et al., categorize Cytokines, particularly those produced by CD4+ T helper (Th) cells, are broadly categorized into two types: Th1 and Th2. The Th1 cytokines are primarily composed of interleukins (ILs). Examples of Th1 cytokines include IL-1, IL-2, IL-12p35, and IL-12p40, along with IL-15. Additionally, non-interleukin cytokines such as tumor necrosis factor (TNF) and interferon (IFN) are also integral components of the Th1 response, collectively contributing to inflammatory processes.Th2 cytokines, on the other hand, cause anti-inflammatory responses [18]. As a significant hepatocellular carcinoma (HCC) marker, alpha-fetoprotein (AFP) dependability was low. IL-10, likely involved in the development of HCV-HCC, can serve as a new biomarker to gauge the level of inflammation in HCC [12]. Sharafeldin et al. [12] recommend the combination of AFP and IL-10 to significantly enhance the diagnostic and prognostic efficacy of HCC. Future research should emphasize these biomarkers' clinical validity, predictive utility, and compatibility with other treatment methods, like immunotherapy.

This study encompassed 175 patients diagnosed with chronic hepatitis C (CHC). Participants were selected from a larger Egyptian population cohort (n = 4300) undergoing treatment at Sherbin Central Hospital, Dakahlia Governorate, under the auspices of Egypt's Ministry of Health for the national HCV elimination initiative. Among these, patients failing to achieve a sustained virological response (SVR) numbered 75, reflecting a response rate of 98.26%. Additionally, 100 patients were randomly chosen from those who successfully attained SVR, ensuring they were age- and gender-matched with the non-responders ($P > 0.05$). The non-responder group exhibited significantly elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin ($P<0.05$), alongside significantly reduced albumin and platelet counts ($P<0.05$). Furthermore, non-responders demonstrated notably higher levels of two prevalent fibrosis markers ($P<0.05$).Several variables contribute to the regression of fibrosis, including the age of the individual, genetic and epigenetic factors, the speed at which fibrosis progresses (whether it is gradual or quick), and illness-related factors such as the cause and stage of chronic liver disease [19-21]. The AST and ALT levels showed a substantial decrease, which aligns with the results of previous research [22-25]. The observed decrease in ALT levels may primarily attribute the drop in FIB-4 values to a reduction in necroinflammation. Hsu et al. hypothesized that the quick reduction in AST and ALT levels, along with a slight rise in platelet count, might be responsible for the decrease in FIB-4 values [25].

The cytokine IL-10 has anti-inflammatory effects. It has a significant impact on HCV infection and its therapy [26]. IL-10 assists in suppressing the host's inflammatory immunological response to HCV, which, if left unchecked, can lead to liver damage [27]. Nevertheless, elevated levels of IL-10 can also inhibit the immune response against viruses, which could potentially lead to the virus persisting [28]. Chronic HCV infection often leads to increased IL-10 levels, which are linked to higher viral loads and the persistence of the virus [8]. IL-10 can impede the generation of antiviral cytokines like IFN-γ, hence restricting the host's capability to eliminate the virus [27]. Higher levels of IL-10 have been linked to interferon-based HCV treatments like pegylated interferon and ribavirin not working as well [29]. Individuals presenting with elevated interleukin-10 (IL-10) levels at the initiation of treatment exhibit a reduced likelihood of achieving a sustained virological response (SVR) during standard hepatitis C virus (HCV) therapies [30]. New developments in treating the hepatitis C virus, especially direct-acting antivirals, have made IL-10 less important in determining the success of treatment [31]. Nevertheless, IL-

10 might still impact the immune response and the advancement of liver disease when undergoing DAA-based treatment [32].

The present study revealed that there was no association (P >0.05) between treatment failure and IL-10 (18.99±7.24 vs. 18.82±4.19 pg/mL for non-responder and responder patients, respectively). We assessed its ability to differentiate non-responders from responders using ROC curve analysis. The ROC analysis indicated that interleukin-10 (IL-10) exhibited an AUC value of 0.546, reflecting its limited sensitivity and specificity in differentiating non-responder patients. However, a reduction in IL-10 levels at week 72 correlates with a sustained virologic response in patients possessing either the CC genotype or other genotypes. Both these elements are significant in assessing the treatment's efficacy [33]. Research on the association between IL-10 and direct-acting antiviral (DAA) responses in the treatment of the hepatitis C virus (HCV) has been interesting. The anti-inflammatory cytokine IL-10 has previously been linked to pegylated interferon-alpha (PEG-IFN) and ribavirin-based HCV treatment outcomes [34]. Therefore, how IL-10 influences or predicts the response to DAA-based HCV treatment is unclear. In the current study, the present study revealed no association (P >0.05) between treatment failure and IL-10. The lack of a clear correlation between IL-10 and the DAA response could be due to a few different factors. The mechanisms of action of DAAs and PEG-IFN-based treatments are essentially dissimilar. PEG-IFN therapy relies on the host's immune system, which includes cytokines like IL-10, to control the virus. DAAs, on the other hand, target specific viral proteins to stop HCV replication directly [35, 36]. This variation in the method of action could lower IL-10's DAA response prediction value. The rates of sustained virological response (SVR) achieved through direct-acting antiviral (DAA)-based regimens are significantly greater than those of previous treatments like as PEG-IFN and ribavirin [37]. The enhanced effectiveness of DAAs may mitigate the potential impact of host variables, such as IL-10, on treatment outcomes. According to Sølund et al. [38], host factors such as IL-10 may have less of an impact on the response to DAA therapy than viral parameters such as baseline viral burden, resistance-associated mutations, and HCV genotype. Patients with varied genetic backgrounds, clinical circumstances, and other variables may react differently to PEG-IFN and DAA therapy. This might alter the relationship between treatment response and IL-10 [39]. Considering that IL-10 levels can change during HCV infection and therapy, the timing of the assessment could be quite important. The current trials may not have provided a clear definition for the ideal time point to evaluate IL-10 regarding DAA response. Additionally, this investigation may require a larger number of patients.

4. CONCLUSION

IL-10 production does not change significantly regardless of the DAA HCV therapy. The DAA treatment alone may not cause significant differences in IL-10 levels between those who respond and those who do not. The sample size and study strategy may influence the lack of a substantial disparity in IL-10 levels. If the study has a low number of participants or lacks statistical power, it may not be able to identify modest variations in IL-10 levels between the two groups. So, we recommend using a larger sample size for further study.

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