European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 02, 2020

Study the role of cytokines (IL-6 and TGF$_\beta$-1) and apoptosis-related proteins (PD-1 and STAT3) in the outcome of HCV infection in Wasit Province/Iraq

Nada M. Sadoon[1], Kadhum J. Gattia[2] and Ahmed D. Jabbar[3]

[1]Ph.D. student. Department of Biology, College of Science, University of Wasit, Iraq
[2]Professor. Department of Biology, College of Science, University of Wasit, Iraq
[3]Assist. Professor. Department of Biology, College of Science, University of Wasit, Iraq

Abstract Hepatitis C virus is one of the public health disorders in the world. It's blood-borne, causes acute disease and may extend to chronic infection, if HCV is untreated, it can progress and lead to fibrosis, cirrhosis and cancer of the liver and then death. Since the virus targets the immune system, here in this finding two cytokines (IL-6 and TGF$_\beta$-1) and two apoptosis-related proteins (PD-1 and STAT3) were tested serologically by Enzyme Linked-Immunosorbant Assy (ELISA) technique to detect the changes in their levels during the infection. The results revealed that IL-6 and TGF$_\beta$-1were abnormally raised in patients with HCV compared with healthy peoples ($P<0.001$). Apoptosis-related proteins involved in this study were PD-1 and STAT3, both were also elevated in HCV patients rather than control ($P<0.001$). The age group (46-55) of patients was the most group displayed higher levels in IL-6, TGF$_\beta$-1, PD-1 and STAT3. In conclusion, IL-6 was good indicator on the liver inflammation during HCV, TGF$_\beta$-1stimulates the transformation of the infected hepatocytes to be fibrotic, cirrhotic or cancer cells. PD-1 considered as a checkpoint for the impairment of T cell during infection, continuous stimulation of STAT3 by IL-6 and other factors contribute to HCV progression.

Keywords: HCV, IL-6, PD-1, STAT3, TGF$_\beta$-1, Tcell.

Introduction

Hepatitis C is an overall health problem, cause of liver disorders and widely distributed. Globally, it affects more than 170 million individual which about 3% (Moosavy *et al.*, 2017). Equal to annual tuberculosis and over an HIV deaths, hepatitis virus caused death of 1.34 million person in 2015, annually about 400,000 deaths caused by HCV alone (Bartenschlager *et al*., 2018). The highest prevalence of HCV was between 6-28% in Egypt. However, HCV is considered low-endemic in Iraq, with a prevalence of 0.5% in a blood donors (WHO, 2008). Causative agent of this disease is positive stranded RNA genome, which is typed as *Flavivirus* genus. Primarily, this virus infects liver cells, and then establishes a persistent infection in the liver, leading to the progression of chronic hepatitis, cirrhosis and malignant of the liver cells, even though a few numbers of peoples are able to clear the virus spontaneously (Zaltron *et al*., 2012). The transmission of HCV is occurred firstly via the skin or from unsafely use of injected needles that contaminated with HCV, or by sexual and vertical contact that causing little spreading of the virus, this virus access directly to the hepatocytes, that are considered as major target (Belouzard *et al*., 2017). The largest part of the patients who initially infected with virus are without symptoms, hence, the clinical investigations couldn't be utilized as markers on this

disease. Serological methods routinely used to indicate on the presence of HCV, that includes tests performed in direct or indirect methods. Indirect method used to detect on antibodies that stimulated against the virus, involving (IgM) for newly diagnoses and (IgG) for new and past, and the direct method involve the isolation of virus, then detecting on nucleic acids and antigens. In last few years, diagnosis of antibodies, viral antigens added to the RNA detection all performed as routine examination (Li and Lo, 2015). No vaccine has been discovered until now. The standard current treatment for HCV is ribavirin combined with pegylated IFN-α, which known as (Direct Acting Antivirals DAAs) or (Sofosbuvir. SOF) that firstly introduced in wide range. Clinical experiments demonstrated the large efficacy of the regimens based on SOF that used by peoples infected with 1-6 HCV genotypes (Gentile *et al*., 2015 and Tsertsvadze *et al*., 2020). Programmed cell death 1 (PD-1) is a transmembrane protein of immunoglobulin superfamily, which expressed on the surface of T cells. PD-L1 is the main ligands of PD-1 protein called programmed cell death ligand-1 (Zhu and Lang, 2017). In the blood, T cell expressed a huge levels of PD-1 receptors that are more susceptible to the apoptosis (Radziewicz *et al*., 2008). PD-1 (CD279) is a co-inhibitory agent expressed by pathogen-stimulated T cells and serves as a significant negative controller of T cell immunity to regulate immune homeostasis and peripheral tissue tolerance (Okazaki *et al*., 2013)**.** At the level of chronic HCV, T cell subjected to excessive antigen loads, here the PD-1/ PD-L1 ligand stimulated to overexpression leading to viral-specific CD8+ T cell inhibition. However, blocking the PD-1 / PD-L1 combination can allow exhausted T cells to be restored (Salem and El-Badawy, 2015). The signal transducer and activator of transcription 3 (STAT3), is the substrate protein of Janas kinases (JAK) STAT-pathway, serve as a transcription factor, which combined with the cytokine receptor directly to regulate the gene expression (Page *et al*., 2011). JAK is a major cascade of STAT3 signaling pathway, four JAK proteins are found: JAK1, JAK2, JAK3 and Tyk2. These cascades are associated with the signal transduction to cytokines and several growth factors involving: interleukin 22 (IL22), oxidative stress, type I and II interferons (IFNs), and even signals from epigenetic regulation. In addition to interferon signaling in liver cells, STAT3 protein was initially found to be a transcription factor related to the genes regulated by IL-6. (He, G. and Karin, 2011; Delgoffe *et al*., 2013). IL-6 activation, for instance, contributes to the phosphorylation of STAT3, JAK1 and JAK2 accompanied by STAT3 homodimer nuclear translocation. The interactions between cytokines and the unique surface receptors cause JAK and then STAT to activate. This activation relies on the phosphorylation at the particular residues of tyrosine (Atsushi-Hosui, 2003). Interleukin-6 (IL-6) of human has a molecular weight of 26 kD and is a glycosylated protein. The precursor peptide of IL-6 is consist of 212 amino acids. The gene promoter region of IL-6 includes important transcriptional organizing elements that are controlled by activating protein-1 (AP1) among other proteins and nuclear factor kappa B (NF-κB) (Tian Lan *et al*., 2015). During HCV infection, the activation of TNFα contributes to the activation of NF-κB, which is the transcription factor for IL-6 gene expression activation. TNF an induces IL-6 expression in the liver, especially in Kupffer cells and also in endothelial and hepatic stellate cells , resulting in activation of Jak-STAT in liver cells (Friedman, 2000; Mangnall and Majeed, 2003). Another cytokine member is TGFβ-1, a highly pleiotropic protein. It seems that inhibitory cytokines including TGFβ-1 have an inhibitory role in acute and chronic viral infection, and little is understood about the effect of inhibitory receptors and cytokines on CD4+ T-cell virus-specific

impairment throughout chronic HCV infection (Raziorrouh *et al*., 2011). Hematopoiesis ,Embryogenesis, angiogenesis, immune responses, fibrosis, carcinogenesis, wound healing and transplantation are the mechanisms in which TGF-β-1 plays an important role (Neuzillet *et al*., 2015).This study aimed to detect the changing in the levels of cytokines (IL-6 and TGF_{β}-1) and proteins (PD-1 and STAT3) in peoples infected with HCV infection and to investigate their role in disease development.

Material and method

This work was carried out in the University of Wasit, College of Science, Department of Biology and in the unit of Molecular & immunology at Al-Karama Teaching Hospital in Wasit Province/Iraq. Blood and serum samples were taken from 60 HCV patients (36 males and 24 females) with an age ranging between 16 and 75 years, as well as 29 samples used as control cases. Some clinical information or data were taken, involving the medical full history; complete of clinical tests and some other data such as age and gender of patients. However, other unavailable options were excluded, such as the stages of HCV infection and following up the line of treatment.

Molecular test

Detection of HCV viral load in patients with HCV conducted by using qPCR technique after RNA isolation by using Quantiphor Viral RNA Extraction Kit. Cat. No: MB276v2f. Anatolia. Turkey. Bosphore® Viral RNA Extraction Spin Kit is highly compatible with Bosphore® Real-Time PCR kits. Starting sample volume is 400 µl and RNA recovery (elution) volume is 60 µl. qPCR viral load test performed by using Bosphore® HCV Quantification Kit Cat. No: MB226v3f. Anatolia. Turkey. The protocol of the HCV quantification kit was composed of two steps of activation the HotStar Tag DNA polymerase by initial denaturation amplification cycle and a final hold. And two steps of amplification cycle and final hold. Table (1.1).

Table(1.1): HCV quantification PCR program.

Steps	Temperature	Time	No. of Cycles
Reverse Transcription	50℃	30:00 min.	1cycle
Initial Denaturation	95℃	14:30 min.	1 cycle
Denaturation	97℃	00:30 min.	40 cycles
Annealing(data collection)	55℃	01:20 min.	
Synthesis	72℃	00:15 min	
Hold	22℃	05:00 min.	1 cycle

Serological test

This test used in this study represented by Enzyme-Linked Immunosorbent Assay (ELISA) technique for detecting and quantifying some human substances such as cytokines and proteins (IL-6, TGF-β1, PD-1 and STAT3). SinoGeneClon ELISA kit.(IL-6) Cat. No: SG-10267, (TGF-B1) Cat. No: SG-10060, (PD-1) Cat. No: SG-13297 and (STAT3) Cat. No: SG-11700.Biotech Corporation. The Principle of this method is using for quantitative levels of IL-6,TGF-β1, PD-1 and STAT3 in human serum sample. Depend on the purification antibody belongs to each sample, antibody used to coat micro-titer plate by making solid phase antibody. Then protein add to wells, combining antibody with labeled enzyme called (HRP) to form Antibody-Antigen-Enzyme-Antibody complex (Sandwich ELISA).After washing complete, the TMB substrate solution add and becomes blue in color because the HRP enzyme-catalyzed, then the stop solution add after that to limit the reaction, here the color changing occurs and measured at a wavelength of 450 nm. The concentration of each protein determined by comparing (O.D) of the samples with the standard curves.

Statistical analysis

The statistical analysis of the results was conducted by the GeneStat Discovery program, Version 2012, Edition 4. t-Test, Mean, SD and SE were expressed in analysis of the data. Analysis of Variance (ANOVA) was used to detect Least Significant Difference (L.S.D) among different parameters, F-correlation of viral load with other parameters and the probability of ($p< 0.05$) and ($p<0.001$) was adopted to be a significant difference value.

Results

Patients samples involved 36 males (60%) and 24 females (40%), their mean age was 34.9 with a range of 16 to 75 years, compared with 17 males (58%) and 12 females (41%) of control group, their mean age was 42.8 with a range of 18 to 66 years, as shown in (Table 1.2).

Table (1.2): Characteristics of patients with HCV and control group

Characteristics	**No. of Samples**	**Gender (n) (%)**		**Ages Min-Max / Mean±SD.**	
HCV $^{+}$ (Patients)	60	Males	(36) 60%	(16-75)	34.9±14.1
		Females	(24) 40%		
Control	29	Males	(17) 59%	(18-66)	42.8±13.8
		Females	(12) 41%		

Ages of patients infected with HCV depend on indirect ELISA assay were taken randomly during collection of samples; these ages were classified to 6 groups as in table (1.3) as follows:(16-25) [n=8 (13.3%)], (26-35) [n=14 (23.3%)], (36-45) [n=14 (23.3%)], (46-55) [n=10 (16.7%)], (56-65) [n=12 (20%)] and (66-75) [n=2 (3.4%)]. Two groups (26-35) and (36-45) years were constituted the largest proportion and highly percentage [n=14(23.3%)], while lowest percentage was in group with (66-75) years old.

Table (1.3): Seroprevalence of HCV according to the age groups

Age groups	Number	Percentage
16-25	8	13.3%
26-35	14	23.3%
36-45	14	23.3%
46-55	10	16.7%
56-65	12	20%
66-75	2	3.4%
Total	60	100

The result of viral load test:

A molecular assay based on HCV-RNA detection by quantitative real time PCR to find the concentration of viral nucleic acid in the blood of patients and to confirmed the serological HCV test results, as well as this test used to assessment the chronicity of this disease and if it developed to late stages. The results revealed that (39) sample of patients were RNA positive (65%) while (21) were RNA negative (35%) from total numbers of 60 samples, as shown in table (1.4) below.

Table (1.4): Viral load assessment in patients with HCV

Viral load results	No.	Percentage
RNA+	39	65%
RNA-	21	35%
Total tested Samples	60	100%

Minimum value of viral load in patients with HCV RNA+ was (54 IU /ml) and maximum was (36,840,000 IU /ml). The HCV RNA+ patients were classified into three groups depending on the viral concentration in their blood as following: low viremia if HCV RNA (< 400,000 IU/ml), intermediate if HCV RNA (400,000-800,000 IU/ml) and High represented by HCV RNA(> 800,000 IU/ml). This category was adopted according to Velosa *et al*., (2011).

Results of serological test

Enzyme Linked-Immunosorbant Assay (ELISA) test result of IL-6 in patients with HCV was significantly high (P value < 0.001) with mean level at (268.9) as compared to control at (174.7). Same test was also performed to detect the activation or inhibition of the TGFβ-1 cytokine in patients with this disease. The data revealed highly significant difference among HCV patients and control cases by increasing level of TGFβ-1 in patients with mean at (33.03) and low in healthy individuals with mean at (0.32), P value was (< 0.001). Table (1.5) shows the levels of IL-6 and TGF-β1in patients compared with control group.

Table (1.5): Prevalence of IL-6 & TGFβ-1in HCV patients and control group

Parameter	Sample	No.	Mean	S.D	S.E	t Test	P value
IL_6	Patients	60	268.9	125.4	16.18	3.55	< 0.001
	control	29	174.7	98.3	18.26		
TGFβ-1	Patients	60	33.03	28.55	3.686	8.87	< 0.001
	Control	29	0.32	0.28	0.051		

The test results of PD-1 protein demonstrated high level in HCV patients group, the mean was (1178.2), (P value at < 0.001) and low in control group, the mean was (296.1). In the analysis of STAT3, a significant increase (P value < 0.001) of this protein expression level was identified among individuals with HCV(28.06), compared with control (6.05). As shown in table (1.6).

Table (1.6): Prevalence of PD-1& STAT3 in HCV patients and control group

Parameter	Sample	No.	Mean	S.D	S.E	T Test	P value
PD_1	Patients	60	1178.2	950.4	122.7	7.01	< 0.001
	control	29	296.1	149.2	27.71		
STAT3	Patients	60	28.06	19.02	2.455	8.9	< 0.001
	control	29	6.05	1.57	0.291		

Distribution of cytokines (IL-6 and TGFβ-1) and (PD-1and STAT3) proteins according to the gender of HCV patients.

The overall distribution of cytokine and other proteins according to the gender was different in mean values. Although men tended to possess higher levels compared to females, but there was no statistical significant found in this study as shown in table (1.7) below.

Table (1.7): Prevalence of IL-6, TGFβ-1, PD-1and STAT3 according to gender of patients

Gender	IL-6	TGFB-1	PD-1	STAT3
Males	280.8122	34.55958	1085.589	28.45647
Females	238.982	30.72871	1317.106	27.47519
t -Test	-0.9	-0.51	0.92	-0.19
P Value	0.372	0.615	0.36	0.847

Distribution of IL-6, TGFB1, PD-1and STAT3 according to age groups of HCV patients

According the statistical analysis of the STAT3, TGFβ-1, PD-1 and IL-6 with age of HCV infected patients, the age group of HCV patients at (46-55) years displayed high levels of concentration to all parameters more than other age groups. The mean value of (46-55) age group was (103.5) with STAT3, (60.6) with TGFβ-1, (2101.8) with PD-1 and (352.41) with IL-6. This analysis was done by statistical Fisher Exact Test. Table (1.8).

Table (1.8): Prevalence of IL-6, TGFβ-1, PD-1and STAT3 according to age groups of HCV patients

Age groups	IL-6	TGFB1	PD1	STAT3
16-25	209.37	39.59	934.2	25.64
26-35	243.01	24.25	676.2	18.26
36-45	231.5	25.16	1022.9	22.645
46-55	*352.41	*60.6	*2101.8	*103.5
56-65	326.11	30.07	1328.5	37.298
66-75	189.4	3.16	1235.7	13.055
L.S.D 0.05	127.1	28.01	931.1	18.43

Correlation among cytokines (IL-6 and TGFβ-1) and proteins (PD-1and STAT3) in patients with HCV

The statistical analysis of the correlation among IL-6,TGFβ-1, PD-1and STAT3 parameters test showed a positive correlation among all of them at different values. IL-6 correlation with other factors recorded as: (0.5516) with PD-1, (0.5986) with STAT3 and (0.5345) with TGFβ-1. The correlation values among PD-1with STAT3 and TGFβ-1 were (0.7183) and (0.4849). Finally, the correlation result between STAT3 and TGFβ-1 was (0.6157). Table (1.9).

Table (1.9): Correlation among IL-6, TGFβ-1, PD-1 and STAT3 in patients with HCV

	IL_6	PD_1	STAT3
IL_6			
PD_1	0.5516		
STAT3	0.5986	0.7183	
TGFB1	0.5345	0.4849	0.6157

The value of standard correlation between (1and-1)

Correlation of cytokines and proteins with viral load of HCV patients

The results of the correlation among cytokines and proteins with viral load had been listed in table (1.10) which demonstrated the positive correlation among HCV viral load and all parameters. IL_6 showed high significant in correlation with viral load at (0.501) and P value was ($p<0.05$). PD1 also demonstrated significant difference ($p<0.05$) at (0.321). STAT3 was correlated at (0.374) with p value ($p<0.05$). The last one was TGFβ-1 which correlated with viral load positively at high significant difference (0.4017), p value ($p<0.05$).

Table (1.10): Correlation of IL-6, TGF-$_\beta$1, PD-1 and STAT3 with patients' viral load

Parameters	Correlation with viral load
IL_6	0.501
PD1	0.321
STAT3	0.374

TGFB1	0.4017

Discussion

According to the gender of HCV infected individuals, this virus affects males more than females, and if they become chronically infected, women have slower rates of liver disease development than men , women are also more likely to spontaneously clear the virus (Baden *et al*., 2014). The current study was agreed with Fedeli *et al*., (2019) from Italy, who found the high distribution of HCV infection was in male 45% than in females 39%. In Iraq, Sharif *et al*., (2017) from Mosul, reported in their study the rate of HCV infection was higher in male than in female among hemodialysis patient (14%) and (6%) respectively. This also corresponds to the studies of Al-Zuheiry, (2016) from Diyala, and Mukharmash *et al*., (2017) from Wasit province. While current result disagreed with some studies also in Iraq; Jamil & Ahmad, (2015) from Baghdad, and Muslim, (2014) from Wasit province showed a higher percentage of HCV among females than in males. The vast majority of patients with HCV in this study were aged between 26 to 45 years with percentage of (23.3%), this outcome was agreed with the study of Petruzziello *et al*., (2019) when reported that the prevalence of HCV in the age range 31- 40 years was significantly higher in 2012 and above than in previous period. The study conducted by Saleh, (2012) reported that more than half of HCV patients were found to be in the third and fourth decades, i.e. 30-49 years, with 52.4% and 55.6% respectively for pre-treatment and post-treatment patients., this may also comparable to current data. Alao *et al*., (2009) showed the 41-50 age group had the highest seroprevalence of 10.9 percent , followed by 10-20, 21-30 and 31-40 age groups with a prevalence of 8.8 percent, 7.7 percent, 4.7 percent , respectively, while the above 50 age groups shows no indication of HCV antibodies. In study of Buseri *et al*., (2009); the highest rates of seroprevalence were found among the 18-47 years of age group. However, a lot of studies reported that HCV can infect people at any age but appears most common in adult especially the elderly above 50 years, but this statement not compatible with current study such as in study of Niu *et al*., (2016) which demonstrated that the highest distribution of HCV illness was at the age group 50-59 years. The similar findings were reported by Janahi *et al*., (2015) and Umutesi *et al*., (2019). In the early immune response to viruses and the chronic development of disease, cytokines play an significant role. Pro-inflammatory cytokines such as TNF-, IL-1, and IL-3 are developed by liver Kupffer cells. In addition , the liver generates acute-phase proteins by inducing serum cytokine production (IL-6) (Metanat *et al*., 2016). Interleukin 6, developed immediately and transiently in response to infections and tissue injuries, by stimulating acute phase responses, hematopoiesis, and immune reactions, contributes to host defense. While transcriptional and posttranscriptional mechanisms strictly control its expression, dysregulated continuous IL-6 synthesis plays a pathological effect on persistent inflammation and autoimmunity (Tanaka, 2014). Via different mechanisms, over-expression of IL-6 during the viral immune response could induce viral persistence by impairing the polarization and functionality of Th1 cells and the lytic ability of CD8 T cells, leading to chronic phase (Velazquez-Salinas et al. 2019). Moreover, degree of IL-6 can be used as an independent indicator of CHC patients' response to anti-HCV therapy. When determining the suitability of the patient to receive combined PEG-IFN / RBV therapy,

the IL-6 level, fibrosis stage, and HCV viral load should be taken into account. These explanations confirms by current result of IL-6, when it appeared high level in patients more than in healthy peoples, and this fact also accords with other studies (Afzal *et al*., 2011 and Abd El Salam *et al*., 2017).

TGFβ-1 preserves the homeostasis of the naive T-cell pool under steady-state conditions. In response to immune challenges, TGFβ-1 suppress the differentiation of cytotoxic T lymphocytes (CTL), Th1, and Th2 cells whereas, enhancing peripheral Treg, Th17, Th9 (Sanjabi *et al*., 2017). In the present study, high mean TGFβ-1 concentration observed in HCV patients compared with control group with significant P-value (< 0.001). This suggested that TGFβ-1 is induced by the inflammation or damage of hepatocytes during HCV infection as a result of inhibition of immune response represented by T-cell escape. Thus inhibitory action may exacerbates the disease and develop chronic infection of the liver to HCC. This opinion may confirmed by the study of Pereira *et al*., (2008) which was explained that Tregs have been found to inhibit T cell immune responses via cytokine secretion, especially TGFβ-1 infection, and HCV is characterized by impairment of HCV specific effector T cell responses, indicating that TGFβ-1 may contribute to the long-term persistence of HCV as an effector cytokine. Another finding made by Mehmedovic *et al*., (2013) found in the malignant and toxic hepatitis community, the highest mean concentration of TGFβ-1 was noted. This cytokine was therefore considered an important parameter of inflammatory activity and fibrosis assessment in chronic liver damage assessment. As well as, Presser *et al.*, (2011) demonstrated that TGFβ-1 positively regulates HCV RNA replication. Furthermore, studies of Benzoubir *et al*., (2013); Schon and Weiskirchen, (2014) concluded that In the liver of transgenic mice and hepatoma cells, the HCV core protein elevates the portion of active TGFβ-1 and is also capable of activating HSCs in the same culture as TGFβ-mediated. The analysis of PD-1 in this study showed increasing level of this protein in patients comparing with normal volunteers, P value (< 0.001). As mentioned previously, primary infection with the hepatitis C virus can be cleared or become chronic spontaneously and may lastly progress to HCC, as known the inhibitory signal pathway (PD-1 / PD-L1) was confirmed to be involved in creating persistent viral infections. A possible therapeutic system is expected to be a blockade of PD-1/PD-L1 commitment to reinvigorate T cell development. Since some patients under study have indicators to disease progression and their PD-1 level was high, this indicates were identical to previous narration, such as studies of Golden-Mason *et al*., (2008) and Dolganiuc *et al*., (2006) when they have been shown that PD-1 is normally expressed in normal individuals only at low levels in most forms of immune cells, but is up-regulated in the presence of HCV infection. These findings are also consistent with the hypothesis that HCV can cause global suppression of immunity. The studies of Kasprowicz *et al*., (2008) and McMahan *et al*., (2011) also observed that in patients with more viral enrichment in their hepatic compartment, the amount of PD-1 was significantly increased and did not vary significantly between those in acute or chronic infection., this concept is completely agreed with current result in this study. The result of this research also had been shown the obviously increase the activation of STAT3 protein in patients compared with control group, this indicated that some of patients have immune activation mediated inflammation caused by HCV, this activation represented by response to such ligands such as growth factor, interferons, interleukins (IL-5) and (IL-6). These responses lead to the

stimulation of some indicators such as STAT3 depending on disease stage, development and immune susceptibility of the patient. This observation could agree with previous studies were reported that active form of STAT3 could enhance HCV replication by the response of IFN type I, in turn the replication of HCV could promote more activation of STAT3. Furthermore, STAT3 activated extracellular HCV core on human macrophages and STAT3 activation induced by HCV core depended on cytokine IL-6 manufacturing (Tacke *et al*., 2011; McCartney *et al*., 2013 and Xiong *et al*., 2015). Contrary to the current and several previous results about the activation of STAT3 by HCV core protein, study of He and Karin, (2011) and study of Svinka *et al*., (2014) even in the presence of cytokines, STAT3 activation has been shown to be intermittent in non-transformed cells, and STAT3 activating mutations are rarely present in HCC. As well as, the result of Larrea *et al*., (2006) observed in HCV contaminated livers, the expression and activation of STAT3 is decreased. The influence of HCV patients' gender on their IL-6, TGFβ-1, PD-1and STAT3 did not show any significant differences. With regarding the cytokines; gender did not represent the risk factor to the IL-6 as found in study of Bautmans *et al*., (2011) and also had no effect on the level of TGFβ-1 as found in study of Mousa *et al*., (2019). As well as, the distribution of PD-1 and STAT3 proteins did not show any significant difference as reported in studies of Botticelli *et al*., (2017) and Liang *et al*., (2018) respectively. Statistical analysis revealed that the levels of TGFβ-1 and IL-6 or PD-1 and STAT3 were high in the age group of (46-55), this group considered being slightly larger than medium age of all patients, it can be said that these cytokines and proteins were high in older patients, some liver diseases such as hepatitis are more commonly found than in younger patients, and different physiological changes due to aging can affect the pathogenesis of liver disease. As known, in the case of liver damage , especially fibrosis, which may occur in older people, liver enzymes increase and the age group of (46-55) years may include patients with liver damage progression, but also this result is not inevitable and it subject to the changes. Few studies have focused on the influence of age on the levels of these parameters, for example; study of Hussein *et al*., (2013) found that TGFβ-1 was high in (≤ 40) age group of HCV patients. Respect to STAT3 and IL-6, Chazaud and Mouchiroud, (2014) showed that the STAT3 level was changed according to the age of people included in their study. As well as, the recent finding of Piber *et al*., (2019) for the first time observed that the older ages with inflammations had increasing levels in STAT signaling. However, the most recent study of Nilsson *et al*., (2020) demonstrated that higher systemic levels of IL-6 and STAT3 were associated with increasing of age as in their association to the size of body in patients with breast cancer. Finally, the study of Daste *et al*., (2017) stated that increasing levels of PD-1 may considered as a predictive factor about finding of tumors and these levels were not influenced by any age group. At the level of cytokines, Ahn *et al*., (2018) discovered PD-1 expression is immediately triggered after signaling via the T cell receptor (TCR) and controlled by cytokines; this may support what the result about the correlation of PD-1with IL-6 (0.5516) and PD-1 with TGFβ-1 (0.4849) that appeared in this study. While the correlation of PD-1 and STAT3 that documented in this research was (0.7183); this study is the first one demonstrated the positive relationship between PD-1 and STAT3 in HCV patients in Iraq, in order to study these two measures more broadly together and to know the impact of each of them in HCV patients, this result gives a new perspective. Other researchers studied the correlation between these two parameters such as Song *et al*., (2018) when they found that strongly binding

activated STAT3 to the PD-L1 gene promoter, STAT3 induced PD-L1 expression and activation of STAT3 affords high PDL1 expression, which can facilitate tumor immune evasion. A potential treatment strategy for NK/Tcell lymphoma (NKTL) and probably peripheral T-cell lymphoma (PTCL) may be a combination of PD-1 / PD-L1 antibodies and STAT3 inhibitors. This approach can be used to apply it to HCV in future. Zerdes *et al*., (2019) observed that STAT3 was positively associated with PD-L1 expression by STAT3 inhibition and gene silencing, leading to decreased in vitro PD-L1 expression levels, as well as decreased tumor growth and metastatic dissemination in the mouse model of mammary carcinoma. The current results of correlation also revealed positive correlation among STAT3 protein and cytokines (IL-6 and TGFβ-1) in significant values. Effective activation of STAT3 is achieved under the influence of specific cytokines and selected growth factors showing janus-kinase signaling, as is well known and stated earlier. Phosphorylation at Tyr705 is known to be a STAT3 activation hallmark for protein dimerization, nuclear translocation, and gene expression control (Qi and Yang, 2014). Therefore any over-expression levels of these cytokines lead to more induction of STAT3 protein. According to the viral load results; high significant increasing of IL-6 was established in this study in related with patient's viral load; this observation of increasing in IL-6 levels indicated that the persistence of virus overcomes on the immune system of the body. Scientific experimental evidence also indicated the possible negative impact of this interleukin increasing level on the cellular immune response through viral infection and then preference the establishment of virus with persistence situation in infected body (Velazquez-Salinas *et al*., 2019). Similar findings which reported the same results such as of Cussigh *et al*., (2011) and Deeb *et al*., (2019) when they found the correlation between increasing expression of IL-6 with viral load of patients. PD-1 protein analysis also showed significant correlation with viral load of HCV in patients. Any defect or abnormal increase in this protein levels may lead to inhibition of T-cells more than required and thus loss of ability to attack the virus. This simple interpretation is consistent with Neil Shah *et al*., (2019) study which explained that PD-1 up-regulation is associated with functional exhaustion of T-cell CD8+ during persistent infection of HBV, HIV and even HCV. According to the positive correlation of viral load with STAT3, this protein activated through virus infection especially in CHCV phase, but it could prevent the disease develop to HCC as in study of Song *et al*., (2019) which observed that STA3 in the other hand with activation of IL-6 may enhancing the progression of HCV, this discrepancy in the results indicate that the precise role of this protein in HCV infection exactly still unknown and more investigations are required. Ultimately, TGFβ-1 also demonstrated high positive correlation with viral load in this study; this is in line with a lot of information about TGFβ-1 that mentioned in other studies for example: Liu *et al*., (2011) reported that the core protein of HCV plays a crucial role in changing the signaling pathways of several factors such as TGFβ -1 particularly during CHCV and HCC and this changing lead to more secretion of this cytokine which cause inhibition in the synthesis of IFN-γ. In addition to that most recent study of de-Brito *et al*., (2020) suggested that the other factors with TGFβ -1 can influence on viral load of HCV and this cytokine alone is not sufficiently influence the increasing level of viral load.

Conclusions

Hepatitis C is the common type of viral hepatitis, which affects both males and females in Wasit Province/Iraq. In this study, males infected more than females with HCV, among them the age between (26-45 years) infected with virus more than others. Two cytokines (IL-6 and TGF_{β}-1), proteins (PD-1 and STAT3) levels were significantly high in patients with HCV compared with control group, from this results we concluded that IL-6 considered as a strong predictor for the liver inflammation while TGF_{β}-1induces the conversation of hepatocytes to fibrotic, cirrhotic and cancer cells. As well as, PD-1is considered as a checkpoint for T cell dysfunction during the infection with HCV. The continuous activation of STAT3 can enhance the disease to progressed stages because of permanent stimulation to this protein by different cell pathways such as more secretion of IL-6.

References

1. Afzal, M. S., Tahir, S., Salman, A., Baig, T. A., Shafi, T., Zaidi, N. U. S. S., & Qadri, I. (2011). Analysis of interleukin-10 gene polymorphisms and hepatitis C susceptibility in Pakistan. *The Journal of Infection in Developing Countries*, *5*(06), 473-479.
2. Ahn, E., Araki, K., Hashimoto, M., Li, W., Riley, J. L., Cheung, J., et al. (2018). Role of PD-1 during effector CD8 T cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 115, 4749–4754.
3. Alao, O., Okwori, E., Araoye, M.(2009). The Sero-Prevalence of Hepatitis C Virus (HCV) Infection Among Prospective Blood Donors In a Nigerian Tertiary Health Institution. *The Internet Journal of Epidemiology*, 7 (2).
4. Al-Zuheiry, M. S. (2016). The Prevalence and the Quantification of Hepatitis C Virus among Thalassemia Patients using ELISA and PCR in Diyala Province. *Diyala Journal of Medicine*, *10*(2), 63-69.
5. Baden, R., Rockstroh, J. K., & Buti, M. (2014). Natural history and management of hepatitis C: does sex play a role?. *The Journal of infectious diseases*, *209*(suppl_3), S81-S85.
6. Bartenschlager, R., Baumert, T. F., Bukh, J., Houghton, M., Lemon, S. M., Lindenbach, B. D., ... & Thimme, R. (2018). Critical challenges and emerging opportunities in hepatitis C virus research in an era of potent antiviral therapy: considerations for scientists and funding agencies. *Virus research*, *248*, 53-62.
7. Bautmans, I., Onyema, O., Van Puyvelde, K., Pleck, S., & Mets, T. (2011). Grip work estimation during sustained maximal contraction: validity and relationship with dependency and inflammation in elderly persons. *The journal of nutrition, health & aging*, *15*(8), 731-736.
8. Belouzard, S., Danneels, A., Fénéant, L., Séron, K., Rouillé, Y., & Dubuisson, J. (2017). Entry and release of hepatitis C virus in polarized human hepatocytes. *Journal of Virology*, *91*(18).
9. Benzoubir, N., Lejamtel, C., Battaglia, S., et al. (2013). HCV coremediated activation of latent TGF-β via thrombospondin drives the crosstalk between hepatocytes and stromal environment. *J Hepatol* ,59,1160-8.

10. Botticelli, A., Onesti, C. E., Zizzari, I., Cerbelli, B., Sciattella, P., Occhipinti, M., ... & Vici, P. (2017). The sexist behaviour of immune checkpoint inhibitors in cancer therapy?. *Oncotarget*, *8*(59), 99336.

11. Buseri, F. I., Muhibi, M. A., & Jeremiah, Z. A. (2009). Sero-epidemiology of transfusion-transmissible infectious diseases among blood donors in Osogbo, south-west Nigeria. *Blood Transfusion*, *7*(4), 293.
12. Chazaud, B., & Mouchiroud, G. (2014). Inflamm-aging: STAT3 signaling pushes muscle stem cells off balance. *Cell stem cell*, *15*(4), 401-402.
13. Cussigh, A., Falleti, E., Fabris, C., Bitetto, D., Cmet, S., Fontanini, E., ... & Pirisi, M. (2011). Interleukin 6 promoter polymorphisms influence the outcome of chronic hepatitis C. *Immunogenetics*, *63*(1), 33-41.

14. Daste, A., Domblides, C., Gross-goupil, M., Chakiba, C., Quivy, A., Cochin, V., ... & Ravaud, A. (2017). Immune checkpoint inhibitors and elderly people: a review. *European Journal of Cancer*, *82*, 155-166.

15. de- Brito,W.B., Queiroz,M.A. , Amoras,E.G., Lima,S.S., Conde,S.R., Santos,E.J., Cayres-Vallinoto,I.M., Ishak,R. , Vallinoto,A.C. (2020). The TGFB1 -509C/T polymorphism and elevated TGF-β1 levels are associated with chronic hepatitis C and cirrhosis. *Immunobiology*, 225, 152002.
16. Deeb, A. S. A., Nasr, M. Y., Badra, G., & El, I. H. (2019). The Relationship between Interleukin-6 Polymorphism and Susceptibility to Hepatitis C-virus Infected Patients
17. Delgoffe, G. M., & Vignali, D. A. (2013). STAT heterodimers in immunity: A mixed message or a unique signal?. *Jak-Stat*, *2*(1), e23060.
18. Dolganiuc, A., Chang, S., Kodys, K., Mandrekar, P., Bakis, G., Cormier, M., & Szabo, G. (2006). Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-α and plasmacytoid dendritic cell loss in chronic HCV infection. *The Journal of Immunology*, *177*(10), 6758-6768.

19. Fedeli, U., Avossa, F., Ferroni, E., De Paoli, A., Donato, F., & Corti, M. C. (2019). Prevalence of chronic liver disease among young/middle-aged adults in Northern Italy: role of hepatitis B and hepatitis C virus infection by age, sex, ethnicity. *Heliyon*, *5*(7), e02114.

20. Friedman, S. L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *Journal of Biological Chemistry*, *275*(4), 2247-2250.

21. Gentile, I., Maraolo, A. E., Buonomo, A. R., Zappulo, E., & Borgia, G. (2015). The discovery of sofosbuvir: a revolution for therapy of chronic hepatitis C. *Expert opinion on drug discovery*, *10*(12), 1363-1377.
22. Golden-Mason, L., Madrigal-Estebas, L., McGrath, E., Conroy, M. J., Ryan, E. J., Hegarty, J. E., ... & Doherty, D. G. (2008). Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. *Gut*, *57*(8), 1121-1128.

23. He, G., & Karin, M. (2011). NF-κB and STAT3–key players in liver inflammation and cancer. *Cell research*, *21*(1), 159-168..

24. Hosui, A., Ohkawa, K., Ishida, H., Sato, A., Nakanishi, F., Ueda, K., ... & Hayashi, N. (2003). Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-γ stimuli. *Journal of Biological Chemistry*, *278*(31), 28562-28571.

25. Hussein,A.A., Khashman,B.M., Hussain,S.M. (2013). Role of Tgf-β1and Gremlin-1in the Pathogenesis of Chronic HCV Infection and Hepatocellular Carcinoma. *INDIAN JOURNAL OF APPLIED RESEARCH*, 3(9),2249-555.

26. Jamil, N. F., & Ahmad, M. J. (2015). Seroprevalence of Hepatitis C and Associated Risk Factors in Hemodialysis Units in Baghdad. *IRAQI JOURNALOF COMMUNITY MEDICINE*, *28*(4), 162-167.

27. Janahi, E. M., Al-Mannai, M., Singh, H., & Jahromi, M. M. (2015). Distribution of hepatitis C virus genotypes in Bahrain. *Hepatitis monthly*, *15*(12).

28. Kasprowicz, V., Zur Wiesch, J. S., Kuntzen, T., Nolan, B. E., Longworth, S., Berical, A., ... & Kwok, W. W. (2008). High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *Journal of virology*, *82*(6), 3154-3160.

29. Larrea, E., Aldabe, R., Molano, E., Fernandez-Rodriguez, C. M., Ametzazurra, A., Civeira, M. P., & Prieto, J. (2006). Altered expression and activation of signal transducers and activators of transcription (STATs) in hepatitis C virus infection: in vivo and in vitro studies. *Gut*, *55*(8), 1188-1196.

30. Li, H. C., & Lo, S. Y. (2015). Hepatitis C virus: Virology, diagnosis and treatment. *World journal of hepatology*, *7*(10), 1377.

31. Liang, C., Xu, Y., Ge, H., Li, G., & Wu, J. (2018). Clinicopathological significance and prognostic role of p-STAT3 in patients with hepatocellular carcinoma. *OncoTargets and therapy*, *11*, 1203.

32. Liu, J., Ding, X., Tang, J., Cao, Y., Hu, P., Zhou, F., ... & Zhang, B. (2011). Enhancement of canonical Wnt/β-catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. *PLoS One*, *6*(11), e27496.

33. Mangnall, D., Bird, N. C., & Majeed, A. W. (2003). The molecular physiology of liver regeneration following partial hepatectomy. *Liver international*, *23*(2), 124-138.

34. McCartney, E. M., Helbig, K. J., Narayana, S. K., Eyre, N. S., Aloia, A. L., & Beard, M. R. (2013). Signal transducer and activator of transcription 3 is a proviral host factor for hepatitis C virus. *Hepatology*, *58*(5), 1558-1568.

35. McMahan, R. H., Golden-Mason, L., Nishimura, M. I., McMahon, B. J., Kemper, M., Allen, T. M., ... & Rosen, H. R. (2011). Tim-3 expression on PD-1+ HCV-specific

human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *The Journal of clinical investigation*, *121*(2), 821-821.

36. Mehmedović, A., Mesihović, R., Prnjavorac, B., Vanis, N., Vukobrat-Bijedić, Z., Borovac, N., ... & Mujarić, E. (2013). Non-invasive liver fibrosis markers: use of serum levels of cytokines IL 1α and TGF β1 in management of chronic liver diseases. *Medicinski Glasnik*, *10*(1).

37. Metanat, M., Tabatabaei, S. M., Alenabi, A., Mojarad, M. H. A., Rad, N. S., & Pahlavani, E. (2016). Diagnostic Value of the Quantitative Titer of High-Sensitivity C-Reactive Protein in the Detection of Clinical Stages of Chronic Hepatitis B Infection. *International Journal of Infection*, *4*(2).

38. Moosavy, S. H., Davoodian, P., Nazarnezhad, M. A., Nejatizaheh, A., Eftekhar, E., & Mahboobi, H. (2017). Epidemiology, transmission, diagnosis, and outcome of Hepatitis C virus infection. *Electronic physician*, *9*(10), 5646.

39. Mousa, A. O., Saliem, S. S., Abdullah, B. H., & Raad, H. (2019). Age Gender and Site Effect on Immunohistochemical Expression of TGF-β1 and IFN-γ in Hereditary Gingival Fibromatosis.*Journal of Global Pharma Technology*, 11(02),542-547.
40. Mukharmash, J. H., Mutlag, A. M., & Abdul-Rudha, S. A.(2017) .Epidemiological survey for the identification and diagnosis of viral hepatitis B and C, using Enzyme Linked Immunosorbent Assay (ELISA) technique at Al-Kut city/Iraq, and the surrounding area.
41. Muslim, T. M. (2014). Epidimiologic Study of Hepatitis B and C Virus Aong Thalassemia Patients in Wassit Governarate/Iraq. *Al-Taqani*, *27*(2), E1-E6.

42. Neil Shah, N. J., Al-Shbool, G., Blackburn, M., Cook, M., Belouali, A., Liu, S. V., ... & Kim, C. (2019). Safety and efficacy of immune checkpoint inhibitors (ICIs) in cancer patients with HIV, hepatitis B, or hepatitis C viral infection. *Journal for immunotherapy of cancer*, *7*(1), 1-8.

43. Neuzillet, C., Tijeras-Raballand, A., Cohen, R., Cros, J., Faivre, S., Raymond, E., & de Gramont, A. (2015). Targeting the TGFβ pathway for cancer therapy. *Pharmacology & therapeutics*, *147*, 22-31.

44. Nilsson, L., Sandén, E., Khazaei, S., Tryggvadottir, H., Nodin, B., Jirström, K., ... & Jernström, H. (2020). Patient Characteristics Influence Activated Signal Transducer and Activator of Transcription 3 (STAT3) Levels in Primary Breast CancerImpact on Prognosis. *Frontiers in Oncology*, *10*.
45. Niu, Z., Zhang, P., & Tong, Y. (2016). Age and gender distribution of Hepatitis C virus prevalence and genotypes of individuals of physical examination in WuHan, Central China. *Springerplus*, *5*(1), 1557.

46. Okazaki, T., Chikuma, S., Iwai, Y., Fagarasan, S., & Honjo, T. (2013). A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nature immunology*, *14*(12), 1212-1218.

47. Page, B. D., Ball, D. P., & Gunning, P. T. (2011). Signal transducer and activator of transcription 3 inhibitors: a patent review. *Expert opinion on therapeutic patents*, *21*(1), 65-83.

48. Pereira, F. A., da Silva, N. N. P., Rodart, I. F., Carmo, T. M. A., Lemaire, D. C., & Reis, M. G. (2008). Association of TGF- β1 codon 25 (G915C) polymorphism with hepatitis C virus infection. *Journal of medical virology*, *80*(1), 58-64.

49. Petruzziello, A., Sabatino, R., Loquercio, G., Guzzo, A., Di Capua, L., Labonia, F., ... & Botti, G. (2019). Nine-year distribution pattern of hepatitis C virus (HCV) genotypes in Southern Italy. *PloS one*, *14*(2), e0212033.

50. Piber, D., Olmstead, R., Cho, J. H. J., Witarama, T., Perez, C., Dietz, N., ... & Irwin, M. R. (2019). Inflammaging: age and systemic, cellular, and nuclear inflammatory biology in older adults. *The Journals of Gerontology: Series A*, *74*(11), 1716-1724.

51. Presser, L. D., Haskett, A., & Waris, G. (2011). Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-β1: role of TGF-β1 in HCV replication. *Virology*, *412*(2), 284-296.

52. Qi, Q. R., & Yang, Z. M. (2014). Regulation and function of signal transducer and activator of transcription 3. *World journal of biological chemistry*, *5*(2), 231.

53. Radziewicz, H., Ibegbu, C. C., Hon, H., Osborn, M. K., Obideen, K., Wehbi, M., Grakoui, A. (2008). Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. *Journal of virology*, *82*(20), 9808-9822.

54. Raziorrouh, B., Ulsenheimer, A., Schraut, W., Heeg, M., Kurktschiev, P., Zachoval, R., ... & Wächtler, M. (2011). Inhibitory molecules that regulate expansion and restoration of HCV-specific CD4+ T cells in patients with chronic infection. *Gastroenterology*, *141*(4), 1422-1431.

55. Saleh, M. A. (2012). Study the expression level of beta 2 microglobulin gene on hepatitis C patients before and after treatment with Interferon. *Baghdad Science Journal*, *9*(3), 504-510.

56. Salem, M. L., & El-Badawy, A. (2015). Programmed death-1/programmed death-L1 signaling pathway and its blockade in hepatitis C virus immunotherapy. *World journal of hepatology*, *7*(23), 2449.

57. Sanjabi, S., Oh, S. A., & Li, M. O. (2017). Regulation of the immune response by TGF-β: from conception to autoimmunity and infection. *Cold Spring Harbor perspectives in biology*, *9*(6), a022236.

58. Schon, H. T., & Weiskirchen, R. (2014). Immunomodulatory effects of transforming growth factor-β in the liver. *Hepatobiliary surgery and nutrition*, *3*(6), 386.

59. Sharif, H. A., Yousef, A. M & Khalid Mohammed Al-Adeeb, M. (2017). Seroprevalence of (HBV and HCV) and studying the effect of some risk factors among Hemodialysis patient in Mosul city. *Kirkuk University Journal-Scientific Studies*, *12*(3), 607-625.

60. Song, T. L., Nairismägi, M. L., Laurensia, Y., Lim, J. Q., Tan, J., Li, Z. M., ... & Nagarajan, S. (2018). Oncogenic activation of the STAT3 pathway drives PD-L1 expression in natural killer/T-cell lymphoma. *Blood, The Journal of the American Society of Hematology*, *132*(11), 1146-1158.

61. Song, Y., Yang, X., Shen, Y., Wang, Y., Xia, X., & Zhang, A. M. (2019). STAT3 signaling pathway plays importantly genetic and functional roles in HCV infection. *Molecular genetics & genomic medicine*, *7*(8), e821.

62. Svinka, J., Mikulits, W., & Eferl, R. (2014). STAT3 in hepatocellular carcinoma: new perspectives. *Hepatic oncology*, *1*(1), 107-120.

63. Tacke, R. S., Tosello-Trampont, A., Nguyen, V., Mullins, D. W., & Hahn, Y. S. (2011). Extracellular hepatitis C virus core protein activates STAT3 in human monocytes/macrophages/dendritic cells via an IL-6 autocrine pathway. *Journal of Biological Chemistry*, *286*(12), 10847-10855.

64. Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor perspectives in biology*, *6*(10), a016295.

65. Tian Lan, T., Chang, L., Wu, L., & Yuan, Y. F. (2015). IL-6 plays a crucial role in HBV infection. *Journal of clinical and translational hepatology*, *3*(4), 271.

66. Tsertsvadze, T., Gamkrelidze, A., Nasrullah, M., Sharvadze, L., Morgan, J., Shadaker, S., ... & Ezugbaia, M. (2020). Treatment outcomes of patients with chronic hepatitis C receiving sofosbuvir-based combination therapy within national hepatitis C elimination program in the country of Georgia. *BMC Infectious Diseases*, *20*(1), 30.

67. Umutesi, G., Shumbusho, F., Kateera, F., Serumondo, J., Kabahizi, J., Musabeyezu, E., ... & Nsanzimana, S. (2019). Rwanda launches a 5-year national hepatitis C elimination plan: A landmark in sub-Saharan Africa. *Journal of Hepatology*, *70*(6), 1043-1045.

68. Velazquez-Salinas, L., Verdugo-Rodriguez, A., Rodriguez, L. L., & Borca, M. V. (2019). The role of interleukin 6 during viral infections. *Frontiers in microbiology*, *10*, 1057.

69. Velosa, J., Serejo, F., Bana, T., Redondo, I., Simão, A., Vale, A., ... & Sarmento, J. (2011). Chronic hepatitis C treated with peginterferon alfa plus ribavirin in clinical practice. *Hepatogastroenterology*, *58*, 1260-1266.

70. World Health Organization. (2008). Monitoring and evaluation for viral hepatitis B and C: recommended indicators and framework.

71. Xiong, Y., Jia, M., Yuan, J., Zhang, C., Zhu, Y., Kuang, X., ... & Wang, X. (2015). STAT3-regulated long non-coding RNAs lnc-7SK and lnc-IGF2-AS promote hepatitis C virus replication. *Molecular medicine reports*, *12*(5), 6738-6744.

72. Zaltron, S., Spinetti, A., Biasi, L., Baiguera, C., & Castelli, F. (2012). Chronic HCV infection: epidemiological and clinical relevance. *BMC infectious diseases*, *12*(2), 1-7.

73. Zerdes, I., Wallerius, M., Sifakis, E. G., Wallmann, T., Betts, S., Bartish, M., ... & Rassidakis, G. Z. (2019). STAT3 activity promotes programmed-death ligand 1 expression and suppresses immune responses in breast cancer. *Cancers*, *11*(10), 1479.

74. Zhu, X., & Lang, J. (2017). Soluble PD-1 and PD-L1: predictive and prognostic significance in cancer. *Oncotarget*, *8* (57), 97671.