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Assessment of CD4+CD25+ Regulatory T Lymphocytes Cells in Chronic Hepatitis C, Chronic Hepatitis C– Associated Cirrhosis and Hepatocellular Carcinoma Patients

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ABSTRACT

CD4+CD25+ regulatory T lymphocytes are a subset of circulating CD4+ T cells with suppressive properties. CD4+CD25+ regulatory T cells suppress HCV-specific T cell responses and it has been suggested that they may play a role in viral persistence. Our aim was to assess the frequency of regulatory T cells in the different clinical presentations of hepatitis C virus infections in a chosen Egyptian population. Peripheral blood CD4+CD25+ regulatory T cells from patients with different progression of HCV, was conducted and analyzed using flow cytometry. For each stage of HCV progression, twenty patients (n=20) were subjected for analysis in comparison with normal healthy control subjects (n=20). Results showed a significantly higher frequency of CD4+CD25+ regulatory T cells in chronic HCV (3.0 ± 0.9%), HCV related liver cirrhosis (٣.٣±0.8%), HCV related-HCC (3.9 ±1.6%), when compared to normal healthy controls (2.3 ±0.8%). No statistical significant differences were found when comparing HCV related cirrhotic patients with chronic HCV infected patients (P=0.95). Meanwhile, statistical significant differences were detected when comparing HCV related HCC patients with chronic HCV patients (P=0.047). In conclusion, our results indicated the presence of significant higher than normal frequency of peripheral blood CD4+CD25+ regulatory T cell among Egyptian patients that may help to early detect the viral disease and open new field to find a way to disease recovery or protection. Conversely, within different clinical presentation of hepatitis C virus infection no significant differences were detected. Severity of liver affection was significantly correlated with viral load.

Key words: Chronic Hepatitis, Flow cytometry, Hepatitis C infection, Hepatitis associated cirrhosis, Hepatocellular Carcinoma, Liver disease, Regulatory T cells.

INTRODUCTION

$CD4^+CD25^+$ regulatory T lymphocytes (T_{reg}) are a subset of circulating $CD4^+$ T cells with suppressive properties (Jonuleit *et al.*, 2001; Stephens *et al.*, 2001). There are two general categories of $CD4^+CD25^+$ T cells. One T_{reg} cell subset develops during the process of T cell maturation in the thymus, resulting in the generation of a naturally occurring population of T_{reg} cells poised to prevent autoimmune responses by suppressing auto-reactive T cells (Sakaguchi, 2000; Shevach, 2000). The second subset of T_{reg} cells develops as a consequence of ex *vivo* peripheral activation of naive $CD4^+CD25^-$ T cells (Bluestone and Abbas 2003). These induced $CD4^+CD25^+$ which have been shown to suppress T cell responses to tumors, acute and chronic bacterial and viral infections (Woo *et al.*, 2002; Belkaid *et al.*, 2002). T_{reg} cells suppress the proliferation, cytokine-production (IFN-γ, IL-2) and cytolytic activity of naïve and antigen specific CD4+ and CD8+ cells. In addition, T_{reg} cells are able to suppress the functions of antigen presenting cells and B cells (von Boehmer, 2005).

T_{reg} cells may mediate their suppressive activity either through the secretion of anti-inflammatory cytokines like IL-10 or TGF-ß, direct killing of the target cells or distinct cell-cell contact dependent mechanisms (von Boehmer, 2005). However, the mechanisms and the antigen-specificity of T_{reg} cell mediated immune-suppression are still largely unknown. Indeed, the virus specific induction of T_{reg} cells may have two very different consequences: first, it may help the virus to establish viral persistence and second, it may be an important process that occurs to prevent excessive immuneopathological damage (Mills, 2004; Belkaid and Rouse 2005).

Hepatitis C virus (HCV) is a disease with a significant global impact. According to the World Health Organization (WHO) there are 200 million people infected with HCV (WHO, 2012). In Egypt, HCV is considered a serious problem due to its propensity to chronicity, which often resulting in liver cirrhosis and hepatocellular carcinoma, and the high prevalence of antibodies to HCV among apparently healthy Egyptian population (Perz and Alter, 2006; Miller and Laith, 2010). It also has extremely high HCV seroprevalence among Egyptian children (Nahla *et al.*, 2011). Acute infection usually is not spontaneously cleared in part due to immune escape by emerging quasispecies and virus-induced immune dysfunction.

In particular, it has been reported that T_{reg} cells suppress HCV-specific T cell responses and suggested that they may play a role in viral persistence (Cabrera *et al.*, 2004). Many investigators reported a higher frequency of T_{reg} cells in the blood of chronically HCV infected persons versus recovered or healthy persons (Sugimoto *et al.*, 2003; Boettler *et al.*, 2005). It has also been reported an increment of T_{reg} cells in peripheral blood mononuclear cells (PBMCs) of Hepatocellular

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carcinoma patients which was positively correlation with tumour burden (Mengde *et al.,* 2007).

Therefore, the aim of this study was to assess the frequency of T_{reg} cells in different clinical presentation of chronic hepatitis C virus infection, in a chosen Egyptian population, which may be linked to choronicity and severity or progression of HCV disease. Furthermore, may these findings may facilitate the understanding of the mechanisms underlying the pathogenesis of liver damage and of hepatic immune cells during inflammation.

MATERIALS AND METHODS

Study Subjects

Sixty patients, receiving care at Suez Canal University Hospital, were categorized into 3 groups, depending on progression of HCV disease. First group was patients with chronic hepatitis C virus (CHCV), second group included patients with HCV related liver cirrhosis and third group included patients with HCV related-HCC. However, reference group was selected from healthy blood donor without a history of HCV infection**.** Each group was represented by twenty individuals.

CHCV was diagnosed if the following criteria were present after a 6-month follow-up: a) significant and persistent symptoms, b) fluctuating or persistently elevated ALT and AST (> 1.5 fold of normal levels), normal serum albumin and prothrombin time, c) ultrasonography revealed an enlarged bright texture ± portal tract thickening ± normal spleen. Cases were clinically diagnosed as cirrhosis if they had coarse texture, attenuated hepatic veins; irregular surface, shrunken liver, enlarged spleen ± dilated portal and splenic veins ± hypoalbuminemia ± prolonged prothrombin time and ascites. HCC was diagnosed according to the diagnostic guidelines of the AASLD (Bruix *et al.,* 2005).

Patients and control subjects were subjected to a detailed history taking and clinical examination. None of the patients had fever, evidence of other infectious diseases, hepatitis B virus co-infection (excluded serologically), or any other autoimmune inflammatory disorders, at the time of the blood samples were obtained. No patients had received previous anti-viral, anti-cancer or immuno-modulatory treatment. Informed consent was obtained from each patient included in the study. CHCV patients were 4 females and 16 males, their ages ranged between 35 and 50 (45.5±9.3) years. Cirrhotic patients were 5 females and 15 males, their ages ranged between 40 and 60 (51±7.7) years. HCC patients were 3 females and 17 males, their ages ranged between 45 and 60 (52±8.4) years. Control subjects were 6 females and 14 males and their ages ranged between 35 and 48 (43±8.5) years.

Venous blood samples (7ml in EDTA and plain tubes) were withdrawn from each subject. Serum samples were obtained after clotting of the blood. For PCR testing, serum samples were freezed-stored until the time of assay. All samples were subjected to the following laboratory investigations:

1-Complete blood count using automated cell counter Cell-DYN170 (Abbot, USA). 2-Liver function tests including, alanine aminotransferas (ALT), aspartate aminotransferase (AST) total bilirubin,direct bilirubin,and serum albumin (Cobas integra autoanalyzer,Roche). Alpha-fetoprotein was assessed by enzyme linked fluorescent assay (ELFA) using VIDAS autoanalyzer, according to the manufacture's instructions (Biomerieux, France). 3-Viral hepatitis markers: HCV antibodies were assessed by microparticle enzyme immunoassay (MEIA) and HBsAg by chemiluminescent immunoassay (AXSYM (Abbott laboratories, USA) according to the manufacture's instructions. 4-Quantitative real-time PCR: Quantitative PCR was performed for chronic HCV patients by TaqMan amplification system with an internal positive control for HCV RNA quantitation according to manufacture instructions (Applied Biosystem). 5- Flow cytometry analysis: T_{reg} cells were assessed by flow cytometry using FACS Calibur (BD Bio-sciences, San Jos´e, CA, USA). Monoclonal antibodies against CD4 and CD25 were purchased from IQ Product (Groningen, Nertherlands), 100 µl of whole blood was incubated with 20 µl FITC-conjugated anti-CD25 monoclonal antibodies (mAb) and PE-conjugated anti-CD4 (mAb) for 20 min at room temperature, in the dark. This was followed by red cell lysis and wash. Cells were then re-suspended in 300 µl of PBS. Data were analyzed using Paint-A-Gate software, the typical forwards and side scatters were used for lymphocytes gating. $CD4CD25^{High}$ T cells were assessed as percentage of gated $CD4^{+}$ lymphocytes. High CD25 expression ($CD25^{high}$) was defined at a threshold where most CD4-negative cells lose CD25 expression according to Baecher-Allan *et al.,* (2000).

Statistical analysis

Data were analyzed using computer program SPSS, ver.11. Values were given as mean ± SD. Statistical analysis was performed using t-test. Correlation analysis was carried out using Spearman's test and P-values less than 0.05 were considered to be significant.

RESULTS

Studied HCV-population characteristics

Clinical characteristics and laboratory parameters of the three studied groups enrolled in this study are listed in (Table 1)**.** Data analysis demonstrated a statistical significant difference in the liver function of the three studied groups when compared with controls. The hematological features of the three studied groups, including white blood cells, haemoglobin and platelets showed a statistical significant difference when compared with controls.

Frequency of $CD4CD25^{High}$ T cells in the studied groups

In control subjects, the percentage of $CD4CD25^{High}$ T cells ranged between 1-3.5% (2.3 ±0.8), while for

CHCV patients, HCV-related cirrhosis patients and HCC patients the percentages were 2-5% (3 ± 0.9), 2.3-4.7% (3 ±0.8) and 2- 8 % (3.9 ±1.6), respectively as shown in (Table 2). A significantly higher proportion of CD4CD25 ^{High} T cells were found in those with chronic infection when compared with normal controls (P ≤.034). Also, comparing HCV related cirrhosis or HCV related HCC patients with controls showed a statistical significant differences (P≤0.023) and (P≤0.001) respectively. No statistical significant differences were detected when comparing HCV related cirrhotic patients with chronic HCV infected patients (P≤0.95). Meanwhile, high frequency of CD4CD25^{High} T cells of HCV related HCC patients (Table 3) showed a statistical significant differences when compared with chronic HCV patients (P≤0.047) or HCV-related cirrhosis patients (P≤0.036). The flow cytometric analysis of CD4CD25 ^{High} T cells for a control subject and a patient of each group are shown in Figure 1**.** A significant positive correlation between the frequency of CD4CD25^{High} T-cells and HCV viral load of chronic hepatitis C group was also detected (r= 0.47, p<0.05) (Figure 2)**.**

Table (1): Clinical characteristics and laboratory parameters of study population.

Variables	HCVgroups#						Control
	CHCV		Cirrhosis		HCC		
	N	%	N	%	N	%	
Risk factors:							
Blood transfusion	3	15	3	15	3	15	0
Anti-Bilharazial	4	20	12	60	11	55	0
Previous surgery	3	15	3	15	4	20	0
Tattoos	1	5	3	15	3	15	0
Tooth extraction	14	70	5	25	4	20	0
Liver function :	**mean ±SD**		**mean ±SD**		**mean ±SD**		**mean±SD**
ALT(U/L)	45.3 ±11§		55.2 ±23§		49 ±130†		16±7
AST(U/L)	58.7±14§		64.1±21§		123±263†		18 ±7
T-Bilirubin(mg/dl)	0 .6±0.8		3.2 ±1.9§		2.9 ±1.3§		0.6 ±0.2
D-Bilirubin(mg/dl)	2.03±0.2§		1.7±1.3†		1.1±1.0§		0.08±0.03
Albumin(g/l)	4 .0±0.7		2.4±1.3		3.3±0.4§		4.0±0.3
Haematological parameters:	**mean ±SD**		**mean ±SD**		**mean ±SD**		**mean ±SD**
Hemoglobin(g/dl)	11.9±3.2		10.3±2.3§		9.7±1.9§		14.9±0.8
WBCs (cells/ul)	6625±1657		9910±8679		5605±2384		5910±1848
Platelets countx10^{3}	130±78†		105±41§		80±74§		265±64
Viral load:(IU/mlx103)	1313±146		-		-		-

#Number of individuals/HCV groups= 20.
* Significant *p < 0.05* compared with Controls.
† Significant *p < 0.01* compared with Controls.
§ Significant *p < 0.001* compared with Controls.

Table (2): Frequency of CD4CD25 ^{High} T cells in the studied groups compared with controls

Variable	Control group	CHCV group		Cirrhotic group		HCC group	
	Range (mean± SD)	Range (mean± SD)	P- value	Range (mean± SD)	P- value	Range (mean±SD)	P- value
% Treg cells	1-3.5 % (2.3 ± 0.8)	2-5 % (3.0 ± 0.9)	0.034	2.3-4.7% (3.0 ± 0.8)	0.023	2.0-8.0 (3.9 ±1.6)	0.001

Table (3): Illustrate the 2-tailed significance values of t-test of the frequency of CD4CD25 ^{High} T cells in the studied groups.

Variables	CHCV group	Cirrhotic group	HCC group
CHCV group	-	0.95	0.047
Cirrhotic group	0.95	-	0.036
HCC group	0.047	0.036	-

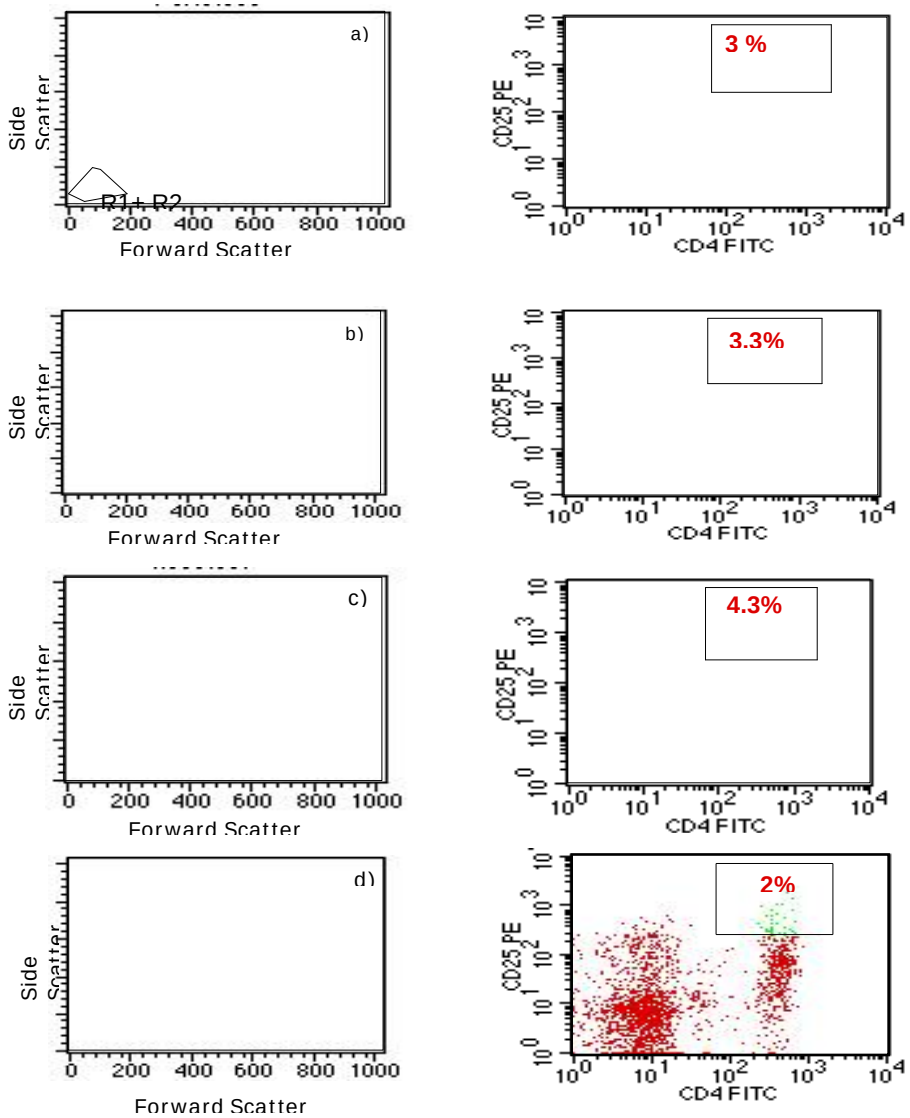

Figure (1): Flow cytometry analysis of CD4CD25 ^{High} T cells.
a) Representative dot plots of CD4CD25 ^{High} T cells in chronic hepatitis subject C
b) Representative dot plots of CD4CD25 ^{High} T cells in HCV related cirrhotic subject.
c) Representative dot plots of CD4CD25 ^{High} T cells in HCV related HCC subject
d) Representative dot plots of CD4CD25 ^{High} T cells in normal healthy subject.

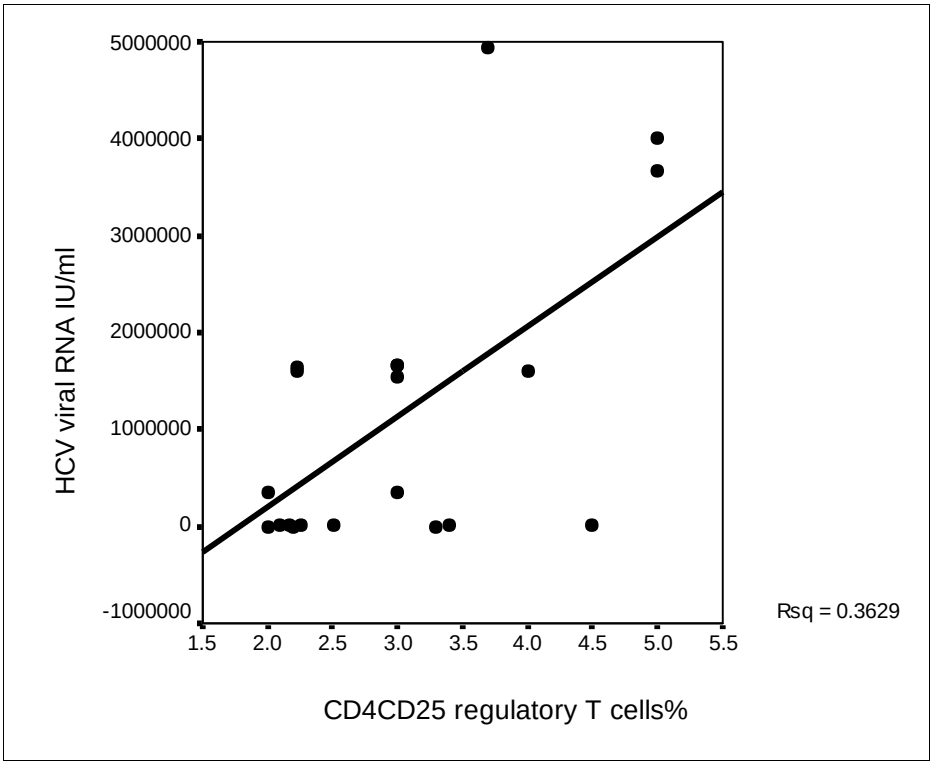

Figure (2): Regression line drawn on a scatter diagram shows a positive correlation between the frequency of CD4CD25 regulatory T cells and HCV viral load.

DISCUSSION

About 3% of the world populations are HCV carriers, a proportion of whom will eventually develop cirrhosis, cancer, or liver failure. Persistent HCV infection is associated with a weak, narrow cell-mediated immune response that is characterized by a low frequency, typically <0.3%, of HCV-specific IFN-γ-producing T cells (Cooper *et al.*,1999 , Thimme *et al.*,2001 , Chisari ,2005) and an even lower frequency of IL-2-producing cells (Semmo *et al.*,2005).

In this study, the frequency of $CD4CD25^{High}$ T-cells in peripheral blood of patients with different clinical presentation of chronic hepatitis C virus infection was found to be increased. These results agreed with that reported by Sugimoto *et al.*,(2003) and Cabrera *et al* .(2004). They reported the presence of a higher proportion of circulating $CD4^{+}CD25^{+}$ cells in the blood of HCV carriers than in convalescent patients and uninfected control individuals. Also, Rushbrook *et al.*,(2005) and Ward *et al.*,(2007) found a much higher percentages of $CD4^{+}CD25^{+}$ cells in the infected liver than in the blood. Dolganiuc *et al.,* (2008) indicated a role for myeloid dendritic cells in expansion of T_{reg} to promote chronic infection of patients with HCV. The $CD4^{+}CD25^{+}$ T-cell fraction isolated from PBMC of infected patients suppressed virus-specific $CD8^{+}$ T-cell proliferation and IFN-γ production, and depletion of these cells resulted in increased IFN-γ production by the remaining cells in response to HCV proteins (Boettler *et al.*,2005 and Rushbrook *et al.*,2005). These studies suggested an important role for T_{reg} in establishing and/or maintaining HCV persistence.

In contrast to the results described above, T_{reg} deficiency was reported in patients with persistent HCV infection. These patients often develop mixed cryoglobulinemia (MC), an autoimmune B-cell proliferative disorder (Boyer *et al.,* 2004). In a comparison of HCV/MC patients to healthy donors, circulating T_{reg} frequency was found to be significantly reduced in HCV/MC patients, and these T_{reg} showed reduced immunosuppressive activity. In addition, Ormandy *et al.,* (2005) reported absences of any significant differences in the number of regulatory T cells between healthy controls and patients with chronic HCV/HBV infection.

Comparing HCV related cirrhotic patients with chronic HCV infected patients, we did not find any statistically significant differences in the two groups, these results agreed with the finding of Mengde *et al.*,(2007) who reported that the presence or absence of cirrhosis did not change the level of $CD4^{+}CD25^{+}$ T cells in patients with chronic HCV infection. However, Delhem *et al.,* (2008) evaluated the existence of T_{reg} cells in liver biopsies of chronically infected patients including patients without liver lesions, with cirrhosis and with Hepatocellular carcinoma of HCV-1b infected patients. They observed installation of T_{reg} cells in the liver of chronically infected patients, with increased frequency in cirrhosis and HCC; that suggested a significant role for these cells in the aggravation of liver pathology. The contradiction between these results may

be attributed to a compartmentalization of T_{reg} cells to liver, the site of infection.

A significant increase of circulating regulatory T cells was seen in HCV related HCC patients compared with chronic HCV patients. These results agreed with the data reported by Mengde *et al.,* (2007). They showed that T_{reg} are increased in peripheral blood mononuclear cells from HCC patients and positively correlated with tumor burden. This also agreed with the data reported by Ormandy *et al.,* (2005) & Yang *et al.,* (2006) that the frequencies of T_{reg} are increased in both peripheral blood and the tumor microenvironment in HCC, and T_{reg} from HCC can suppress proliferation and perforin expression of autologous T cells in vitro.

T_{reg} have also been suggested to impede immune surveillance against cancer. (Linehan and Goedegebuure 2005).Their inactivation improves tumor-specific immunity and leads to more efficient tumor rejection (Yu *et al.,* 2005). In addition, Sasaki *et al.,* (2008) indicated that the high number of hepatocellular carcinoma -infiltrating T_{reg} cells is an independent predictive factor of tumor recurrence after hepatic resection for HCC patients .The increase of the peripheral blood T_{reg} cells may not be related to the aggravation of liver pathology in HCV infection, this may be explained by Mengde *et al.,* (2007) who reported that HCC culture supernatants promoted expansion of T_{reg} and enhanced their suppressive function. This supports the hypothesis that the proliferation of T_{reg} is tumor driven through the secretion of soluble factors and explains absence of their increase in HCV related cirrhosis.

The significant positive correlation between the frequency of T_{reg} and HCV viral load of chronic hepatitis C patients agrees with the results reported by Cabrera *et al.,* (2004). In contrast to previous study, Zarife *et al.,* (2009) observed a parallel increase of CD4CD25 ^{High} T-cells in the low viral load blood donors; they suggest that CD4CD25 ^{High} T-cells might play an important role in controlling viremia.

In conclusion, our results indicated that the higher frequency of peripheral blood $CD4CD25^{High}$ regulatory T cell in Egyptian patients with different clinical presentation of chronic hepatitis C virus infection , that was not related to the aggravation of liver pathology and its positive correlation with HCV viral load further.

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