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GENETIC DIVERSITY AND PRO INFLAMMATORY MEDIATORS IL-1β AND IL-6 IN SERA OF *HEPATITIS C VIRUS* INFECTED PATIENTS IN BABYLON PROVINCE, IRAQ

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Abstract

Hepatitis C virus (HCV) is a member of Flavi viridae family, *is* characterized with positive sense RNA and as an envelope containing virus that specifically propagates in the liver, with worldwide classification as the genus *Hepacivirus*. Recently, there are 71 million people infected with different subtypes *HCV*. In this study, we aimed to figure out the possible relation of *hepatitis C virus (HCV)* genotypes, evaluation of serum cytokines including Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) in Chronic liver disease (CLD) patients, evaluation of clinical significance of these cytokines in different genotypes of *HCV* infection, as well as their possible use as markers of *HCV*- CLD progression. In this prospective descriptive study, genotyping of *HCV* based on Reverse Hybridization Strip Assay was achieved for 30 chronic liver disease (CLD) patients and 26 subjects negative for *HCV* markers served as controls by reverse transcriptase polymerase chain reaction (RT-PCR). All cases were examined for *HCV* viral load test and *HCV* RT-PCR during the period from 1st May to 1st December 2019. Interleukins (1β and 6) values were also measured by Enzyme-Linked Immuno sorbent Assay (ELISA). Results of *HCV* genotyping revealed that genotypes were found to be positive in 28:30 (93.3%), while two cases (6.7%) were undetected. All healthy persons (26:26) were *HCV* negative. Chronic liver disease caused by *HCV* genotype-4 infection was the most dominant comprising 14:30 (46.6%) of the total *HCV* positive cases, followed by *HCV* genotype 1a that comprise 9:30 (30%), genotype-6 that resemble 3:30 (10 %), genotype-7a was detected in 2:30 (6.7 %), while 2 cases (6.7%) were undetected. Results of IL-1β and IL-6 levels according to the viral load were showing preferentially elevation in patients with viral load of ≥ 20,000 IU\mL, comprising 28.58±6.23 (pg/mL) and 45.15± 27.84 (pg/mL) respectively. Nearly fifty seven percent of the total *HCV* infected cases included in this study were from females' population. Chronic liver disease caused by genotype-4 *HCV* is the most dominant type of *Hepatitis* caused by different subtypes. Other cross sectional or prospective studies may be required to confirm the attitude of IL-6 and IL-1β as prognostic inflammatory potential mediators for HCV infection in Babylon province/iraq.

Key words: HCV Genotype, CLD, IL-6, IL-1β

Introduction

Hepatitis C virus (HCV) is a member of Flaviviridae family, *is* characterized with positive sense RNA and as an envelope containing virus that specifically propagates in the liver, with worldwide classification as the genus *Hepacivirus*. Recently, there are 71 million people infected with different subtypes *HCV*, (World Health Organization, 2020). These viruses are regarded as main risky causative agents of hepatic illnesses involving liver cirrhosis and hepatocellular carcinoma. There is about 3% of worldwide prevalence of *HCV* infection and this prevalence is remarkable in countries with high endemic infection rates (may reach 20%), Hence, *HCV* infection is considered as a public health challenge that a combined with serious problems concerning a prominent diversity of different subtypes in combination with *HCV* non-trivial pattern of replication cycle, (Morozov and Lagaye, 2018).

The RNA genome numerical content of *HCV* is nearly 9600 nucleotides (nts) long that coding for single long sequence of polyprotein that is further processed in co- and post-translationally events to reach the mature gene configuration, (Niepmann and Gerresheim, 2020). Chronic *HCV* infection is an infection that affects large number of population world- wide that primarily leads to valuable morbidity and mortality rates through its tendency

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to cause variable illnesses such as cirrhosis, liver fibrosis and liver cancer, (Morozov and Lagaye, 2018).

Hepatitis C virus is primarily transmitted via parenteral exposures to risky materials including body fluids or infectious blood and the most commonly transmission route through injection drug use. The problem is also dormant in the lacking of vaccine against hepatitis C and absence of effective pre- or post-exposure prophylaxis. Hence, More than 50% of *HCV* infected persons will develop a complicated chronic infection, (Schillie, 2020).

The most inflammatory mediators including cytokines are soluble extracellular proteins or peptides with small molecular weight, that are produced by every nucleated cell in response to any injurious stimuli as a pattern of body metabolism, inflammation, infection control and neuronal or tissue damage; hence acting as mediators that carrying the signal between tissues and the immune system; and give participation in many physiological processes by their pro-inflammatory or anti-inflammatory characteristics. Cytokines have variable cellular sources and targets, as well as many natural enhancers and inhibitory factors. The induction of cytokines secretion at high levels is occur in pathophysiological conditions, during the early phase of CLD, as well as infections like viral, bacterial, parasitic, also in the presence of ethanol, or toxins, (Mannaa and Abdel-Wahhab, 2016).

The immune-regulatory cytokines may affect the persistency of *HCV* chronic infection and the severity of liver damage. Interleukin-1 plays an important role in host immune process and the viral clearance, (Tawfik *et al.*, 2018); also has 2 isoforms, IL-1α and IL-1β, both have similar biological functions. Interleukin-1 can exert numerous biological effects on diverse cell types and previous data suggests that viral persistence might be facilitated by insufficient IL-1β production by sinusoidal cells in chronic hepatitis C, (Dumoulin *et al.*, 1999). Interleukin-6 is a multifunctional and pro-inflammatory cytokine which is located at 7p21 chromosome, which plays an important role in defense against viral infection, the immune response and hematopoiesis, (Adnan *et al.*, 2020), also plays a key role in the early acute phase response and in the transition or shifting from acute hepatic response to infections and systemic inflammation, (Tanaka, Narazaki and Kishimoto, 2014). In many aspects that allows to making a great step forward towards antivirals design, that result in direct-acting antiviral (DAA) medications. However, even with the new therapeutic drugs, the *HCV*-associated problems of both fundamental and applied origin are not solved. Here, this study was planned for monitoring *HCV* genetic diversity as well as proinflammatory mediators IL-1β and IL-6 in Chronic liver disease (CLD) patients in Babylon province/Iraq.

Materials and Methods

Patients and controls

This study was conducted on 30 CLD patients (*i.e.*, chronic active *hepatitis* with or without cirrhosis); their ages ranging from 27 to 59 years. All patients were presented before treatment to the specialized liver clinic of Babylon gastrointestinal tract and liver diseases center/ Babylon. All cases were examined for *HCV* viral load by *HCV* RT-PCR during the period from 1st May to 1st December 2019. The study also included 26 control subjects as negative controls (negative for *HCV* by RT-PCR). Viral RNA was firstly extracted from sera sample using Magno-Virus kit (Sacace Biotechnologies, Italy) according to the standard protocol, (Abdulhassan *et al.*, 2018). Then amplification of HCV specific RNA genes via using of Cepheid GeneXpert System, (USA) and application of GEN-C 2.0 kit based on Reverse Hybridization Strip Assay (Nuclear Laser Medicine (NLM), (Italy), (Nuclear Laser Medicine, 2014). The nucleic acid purity and concentration of viral RNA estimation was achieved as mentioned previously by (Al-kelaby *et al.*, 2020), using Scan-drop spectrophotometer (Biodrop co, UK). The RNA was considered with acceptable purity if the absorbance(A) at 260nm/A at 230nm was equal to A260/A280 and >1.8< 2.1, (Imbeaud *et al.*, 2005; Usman *et al.*, 2014). Proinflammatory mediators including Interleukins (1β and 6) values were also evaluated by using Enzyme-Linked Immuno-Sorbent Assay (ELISA), using Human IL-1β and IL-6 ELISA kits (Elabscience co., USA).

Inclusion and exclusion criteria

The criteria that were mentioned by Zekri *et al.*, (16) were adapted for inclusion in this study as follows: (1) CLD group: Those with persistent elevation of the Alanine aminotransferase (ALT) values of more than three folds the normal value for at least 6 months due to *HCV* infection (2) exclusion of CLD cases that caused by hepatotoxic drugs or alcoholism. (3) A history and physical examination details of patients were achieved with special emphasis on bilharzias history, infective hepatitis and jaundice, prior parenteral therapy, or other signs of hepatic failure. Other clinical examinations, which include the manifestations of hepatomegaly, ascites, tenderness in the right hypochondrium, lower limb edema splenomegaly, as well as abdominal ultrasonography were also done side by side with routine laboratory investigations including kidney function tests and complete

blood picture (data were not shown), (Zekri *et al.*, 2005).

Statistical analysis

Results statistical analysis was performed using SPSS-18.0. Data were expressed as Mean ± SD. The interassay coefficient of variation or (Between-Component Variance) values was also applied by analysis of variance (ANOVA). In all tests, P- value ≤ 0.05 was regarded statistically significant.

Ethical considerations

This study received clearance from the respective institutional review board at the Babylon gastrointestinal tract and liver diseases center/Babylon. All participants provided written informed consent in line with good clinical practice.

Results

Results of *HCV* genotyping revealed that genotypes were found to be positive in 28:30 (93.3%), while two cases (6.7%) were undetected. All healthy persons (26:26) were *HCV* negative. Chronic liver disease caused by *HCV* genotype-4 infection is the most dominant comprising 14:30 (46.6%) of the total *HCV* positive cases, followed by *HCV* genotype 1a that comprise 9:30 (30%), genotype-6 that resemble 3:30 (10 %), genotype-7a was detected in 2:30 (6.7%), while 2 cases (6.7%) were undetected, Table 1. In this study, individuals with *HCV* infection of viral load of < 20.000 IU/mL were comprising 21:30 (70%), while those revealing viral load of ≥ 20.000 IU/mL were comprising 9:30 (30%), table 2.

Results of IL-1β were showing that infection of viral load less than 20,000 IU\ml was prevalent in patients with IL-1β levels mean ± SD of 25.25±3.16, while the infection of viral load ≥ 20,000 IU\mL was prevalent among patients with IL-1β levels mean ± SD of 28.58±6.23, table 3. While results of IL-6 were also showed that infection of viral load less than 20,000 IU\ml was prevalent in patients with IL- 6 levels mean ± SD of 37.85± 22.45, while the infection of viral load ≥ 20,000 IU\mL was prevalent among patients with IL- 6 levels mean ± SD of 45.15± 27.84, table 4.

Table 1: Prevalence of *HCV* genotypes presented by number and percentage.

Genotypes		Frequency	Percent
HCV genotypes	1a	9:30	30%
	4	14:30	46.6%
	6	3:30	10%
	7a	2:30	6.7%
	Undetected	2:30	6.7%
	Total	30	100.0

Table 2: Prevalence of *HCV* genotypes according to the viral load presented by number and percentage.

Viral Load (IU/mL)		Frequency	Percent
HCV infection	< 20.000	21:30	70%
	≥ 20.000	9:30	30%
	Total	30	100.0

Table 3: Proinflammatory mediator IL- 1β levels according to the Viral Load in *HCV* infected patients presented by Mean ± SD.

Proinflammatory mediator	Viral Load (IU/mL)	No.	Mean (pg/mL) ± SD	Between-Component Variance
IL- 1β	< 20.000	21	25.25±3.16	0.104
	≥ 20.000	9	28.58±6.23	
	Control	26	27.21±4.08	
	Total	56	26.69±4.28	

Table 4: Proinflammatory mediator IL- 6 levels according to the Viral Load in *HCV* infected patients presented by Mean ± SD.

Proinflammatory mediator	Viral Load (IU/mL)	No.	Mean (pg/mL) ± SD	Between-Component Variance
IL-6	< 20.000	21	37.84± 22.45	0.134
	≥ 20.000	9	45.15± 27.84	
	Control	26	30.52± 12.67	
	Total	56	35.62± 19.95	

The results showed that 56.7% of *HCV* infected cases were females, while 43.3% of the total infected cases were from males' population, table 5. Proinflammatory mediators IL-1β and IL-6 levels in HCV infected patients and control presented by Mean ± SD were also shown in table 6. These results showed non-significant variation

Table 5: Prevalence of *HCV* genotypes according to the patient's gender presented by number and percentage.

Gender		Frequency	Percent
HCV infection	Male	13:30	43.3%
	Female	17:30	56.7%
	Total	30	100.0

Table 6: Pro inflammatory mediators IL-1β and IL-6 levels in HCV infected patients and control group presented by Mean ± SD.

Pro inflammatory mediators	Groups	N	Mean (pg/mL) ± SD	P-Value
IL- 1B	*HCV* infected	30	26.25± 4.47	0.407
	Control	26	27.21± 4.083	
IL-6	*HCV* infected	30	40.04± 23.94	0.075
	Control	26	30.52±12.67	

at p value equal to 0.05. Genotypes of HCV and proinflammatory mediator IL-1β levels presented by Mean ± SD and Between- Component Variance (0.804 and 0.165) were shown in table 8 and 9 respectively. Results were also reflected non-significant correlation coefficient between IL-1β and IL-6 (Pearson correlation coefficient equal to 0.245 and p value > 0.05 (P=0.068). Non-significant variation was also noticed regarding the proinflammatory levels IL-1β, IL-6 according to different HCV genotypes, or gender, but there was slightly elevation of IL-6 in HCV patients who were infected with genotype 6 (51.47± 47.07 pg/mL), as shown in table 7, 8 and 9.

Table 7: Pro inflammatory mediators IL-1β and IL-6 levels in *HCV* infected patients according to the gender presented by Mean ± SD.

Pro inflammatory mediators	No.	Male (No. 13) Mean (pg/ml) ± SD	Female (No. 14) Mean (pg/ml)	Total Mean (pg/ml) ± SD	P-Value
IL- 1B	30	26.71±5.46	25.89±3.68	26.69±4.29	0.625
IL-6		39.22±22.19	40.67±25.85	35.62±19.95	0.203

Table 8: Genotypes of *HCV* and pro inflammatory mediator IL-1β levels presented by Mean ± SD and Between-Component Variance.

Pro inflammatory mediators	*HCV* geno type	N	Mean (pg/mL) ± SD	Between-Component Variance
IL-1β	1a	9	26.56± 4.79	0 .804
	4	14	26.45± 5.18	
	6	3	23.92± .58	
	7a	2	26.51± 5.32	
	Undetected	2	26.22± 3.12	
	Control	26	27.21± 4.08	
	Total	56	26.69± 4.29	

Table 9: Genotypes of *HCV* and pro inflammatory mediator IL-6 levels presented by Mean ± SD and Between-Component Variance.

Pro inflammatory mediators	*HCV* geno type	N	Mean (pg/mL) ± SD	Between-Component Variance
IL-6	1a	9	43.42±25.55	0.165
	4	14	39.49± 20.84	
	6	3	51.47± 47.07	
	7a	2	25.80± 2.22	
	Undetected	2	26.3± 2.08	
	Control	26	30.52± 12.67	
	Total	56	35.62± 19.95	

Discussion

The genome of *HCV* is containing approximately 9600 nts long, that contains double highly conserved sequences of untranslated regions (UTR) 5‘-UTR and 3‘-UTR. These sequences are flanking a single open reading frame (ORF). The ORF might contain nucleotides from 9030 to 9099 depending on the genotype and it is coding for a single chain of polyprotein precursor comprising of 3010 - 3033 amino acids, respectively, (Morozov and Lagaye, 2018; Niepmann and Gerresheim, 2020).

This study was depending on the use of Gen-C 2.0 genotyping kit based on the use of line- probe assay for *HCV* genotyping *in vitro*. This assay discriminates between *HCV* genotypes on the basis of variations in the 5‘ untranslated region (5‘-UTR) and Core regions. The procedure for amplicons was certified with NLM (code number AA910\48 or AA896\48). According to the fundamentals of the *in silico* method for binding energy index and sequence analysis, this assay is also able to differentiate among genotypes *HCV* 1, 2, 3, 4, 6 and also subtypes 1 (1a and 1b), 2 (2a\2c and 2b), 3 (3a, 3b, 3c and 3k), 4 (4a, 4b, 4c\4d, 4e, 4f and 4h), 5a, 6 (6a\6b and 6g), f, q, 6 m and 7a, (Nuclear Laser Medicine, 2014).

The results that obtained from this study may be depended as a guide for the determination of type and antiviral therapy duration period for CLD patients. This procedure is based on the principles of reverse-hybridization of the biotinylated amplicons that generated by RT-PCR of the 5‘-UTR and Core regions of *HCV* RNA to the probes that are bound specifically to the strip of nitrocellulose by a poly-T tail, (Hraber *et al.*, 2006; Ansari, Lingaiah and Irshad, 2012).

Results of *HCV* genotyping positivity was seen in 93.3 %, while two cases (6.7%) were undetected. Chronic liver disease caused by *HCV* genotype-4 infection was revealing dominance comprising 14:30 (46.6%) of the total *HCV* positive cases, followed by *HCV* genotype 1a that comprise 9:30 (30%), genotype-6 that resemble 3:30 (10 %), genotype-7a was detected in 2:30 (6.7 %), while 2 cases (6.7%) were undetected. In similar study done by Najim and Hassan in 2018 in Basra, they were also mentioned that 50.7% of the examined specimens were having *HCV* positivity as detected by PCR and the most dominant genotype was 4 as detected in 43 patients with a percentage of 56.5%, followed by genotype-1 in 31 specimens that comprise 40.7% of the total investigated patients, genotype 1a was comprising 21.1%, (Najim OA, 2018). These results were again came in agreement with that mentioned by Sallam *et al* in similar study in Jordan, (Sallam *et al.*, 2020), that the dominant *HCV* genotype is

genotype 4 (54.0%), followed by genotype 1(33.1%). The recent study was also fitted with that mentioned by Abdulhassan *et al* in 2018 for *HCV* genotyping in Baghdad governorate, in which they were mentioning that the most dominant HCV genotypes were genotype 4 and genotype 1; genotype 4 was the predominant (27\50 and a percentage of 54%), then genotype 1 (23\50 and a percentage of 46%). Subtype 1a was of a highest percentage, since comprise 28%, followed by 4e (24%), 1b (18%), 4a (14%) and 4b (12%), (Abdulhassan *et al.*, 2018). Sievert *et al*, were also concluded genotype 4 dominance in Middle Eastern countries such as Egypt, Saudi Arabia and Syria. Other study from Egypt in 2019 also revealed HCV genotype 4 dominance accounted 94.1% of the total HCV genotypes, (Sievert *et al.*, 2011). Genotype 4 was reported to be dominant in Iraq, Palestine, Saudi Arabia, Qatar, Egypt, Jordan and Syria, (Mahmud and Raddad, 2016).

The current study results were came in agreement with that mentioned by many authors that important variations have been observed in the epidemiology of the *HCV* infection, including the prevalence of distinct risk factors around the world, (El-Ghitany, 2019). Different pattern of genotype dominance was recorded by another study that was achieved by Petruzziello *et al*., (2016), who were mentioned that genotype 1 of HCV was the most prevalent worldwide with a percentage of 49.1%, followed by genotype 3 that comprised 17.9%, genotype 4 (16.8%) and genotype 2 (11%). While genotype 4 was dominant in North Africa and the Middle East (71%), (Petruzziello *et al.*, 2016), however, genotyping results revealed HCV genotypes diversity in CLD patients in Babylon Province, table 1.

Among the healthy Iraqi population, the prevalence of *HCV* infection was reported to be 0.4%, (Akram and Al-naaimi, 2013). This frequency is also comparable to those reported in Kuwait, 0.44% and Qatar, 0.51%. However, the same study reported lower frequencies in the United Arab Emirates (0.24%) and in Saudi Arabia for *HCV* among nationals (1.65%), (Mohamoud, Riome and Abu-raddad, 2016).

About 56.7% of *HCV* infected cases were females, while 43.3% of the total infected cases were from males' population. These results were similar to that mentioned by Olmedo *et al* in similar study that 57.7% of *HCV* infected cases were females, (Olmedo *et al.*, 2017), also come in agreement with that mentioned by Najim and Hassan in 2018 in Basra, about non-significant variance in distribution of *HCV* according to the gender. These results were also consisted with another study achieved by Radhi *et al*., who were mentioned that from 37 *HCV* infected patients, there were 22 (59.46%) females, while 15 (40.54%) were from male population, (Radhi *et al.*, 2019).

According to the assay information; the range of 15 to 100,000,000 IU\L that equal to 1.18 log to 8.0 log IU\L) was regarded as detectable for the measurement of HCV RNA in serum. While an "Undetected" result indicates absence of *HCV* examined genotypes in the patient's serum specimen. Two cases were undetected; these cases may be related to *HCV* genotype 8 as recently mentioned by other similar studies (Borgia *et al.*, 2018; Hedskog *et al.*, 2019).

Results of viral load revealed that *HCV* infected patients viral load of < 20.000 IU\L comprised 21:30 (70%), while the infection of viral load e™ 20.000 IU\L was recorded in 9:30 (30%) of the total investigated *HCV* infected patients. Results of IL-1β and IL-6 levels according to the viral load were showing preferentially elevation in patients with viral load of e™ 20,000 IU\mL, comprising 28.58±6.23 (pg/mL) and 45.15± 27.84 (pg/mL) respectively. The optimal results were strictly preferred to use samples of viral load ranging between 5×10^3 and 8×10^6, hence, preferentially sometimes to make dilution plasma of high viral load before the *HCV* RNA extraction. History of previous surgery, blood transfusion, invasive dental procedures, circumcision at home and wet cupping therapy (hijama) and delivery at home may be regarded as the most important self-risk factors for *HCV* acquisition, (Sallam *et al.*, 2020).

Hepatitis C virus RNA is known to be detectable within short period of infection; hence, the nucleic acid-based detection assays are regarded with efficiency in an early diagnosis of acute *HCV* infection and should be considered as mandatory. The *HCV* RNA quantification is also important in the *HCV* genotyping, therapy duration determination, treatment strategy selection and evaluation of the treatment success, (Sarrazin *et al.*, 2010).

Values of IL-6 and IL-1β levels (means) were variable in CLD caused by HCV patients than in healthy persons, but without statistically significant differences ($p<0.05$). These results were also consistent with that mentioned by Mourtzikou *et al*., (Mourtzikou *et al.*, 2014). However, the immune-regulatory cytokines affects the persistency of *HCV* chronic infection and the severity of liver damage, since Interleukin-1 may affecting host immune process and viral clearance; can exert different biological effects on diverse cell types and the viral persistence might be facilitated by depletion of IL-1β expression by sinusoidal cells in chronic hepatitis C. While interleukin-6 is a multifunctional and pro-inflammatory

cytokine, which plays an important role in the early acute phase response against viral infection and in the transition or shifting from acute hepatic response to infections and systemic inflammation, (Tanaka, Narazaki and Kishimoto, 2014; Tawfik *et al.*, 2018; Adnan *et al.*, 2020). The necessity of viral clearance gave enhancement toward the passive immunization and cytokine therapy strategies development. In viral infections with prolonged persistency, such as HCV infection, the use of cytokine therapy is preferred as a powerful immunotherapy, (Nguyen *et al.*, 2015; Shoukry, 2018). This may be regarded as preferred therapeutic approach for the immune response formation of and viral elimination.

Recently, Centers for Disease Control and Prevention (CDC) was mentioned two new recommendations; Firstly: according to adults of 18 years or more; *Hepatitis C* screening should be applied at least once in a lifetime, unless the prevalence *of HCV* infection is £ 0.1% of the total population investigated and secondly: concerning pregnant women; *HCV* screening should be applied during the period of each pregnancy, again unless the prevalence of *HCV* infection is £ 0.1% (Schillie *et al.*, 2020). *Hepatitis CV* periodically screening should be done to all persons with risk factors while the latter persist. Hepatitis C testing should be received by any person who requests it, regardless of being in disclosure of risk, (Schillie *et al.*, 2020). Genotyping of *Hepatitis CV* and a better knowledge about viral diversity within target populations at regional and national level, might also critically favoring the information about the rational design and evaluation of future *HCV* vaccines, (Petruzziello *et al.*, 2016).

Conclusions and recommendations

We concluded that *HCV* genotype-4 infection is the most dominant in CLD cases, followed by *HCV* genotype 1a and genotype-6. We also recommended that more précised knowledge via advanced steps toward *HCV* genotyping and monitoring will be surely helpful as a guide to improve access to the new therapeutic approaches and best inform national healthcare models.

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Conflict of Interest

Both authors were accept announcement of no conflict of interest and approving of the final article.

Abbreviations

HCV	Hepatitis C virus
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
CLD	Chronic liver disease
RT-PCR	reverse transcriptase polymerase chain reaction
NLM	Nuclear Laser Medicine
ALT	Alanine aminotransferase
SD	Standard deviation
IU	International unit
5' UTR	5‘ untranslated region
CDC	Centers for Disease Control and Prevention
Nts	Nucleotides

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