

# Comparison of biomarkers between genotypes 1a and 3a in hepatitis C virus patients with control group

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## History

- Received: Oct 24, 2018
- Accepted: Dec 11, 2018
- Published: Apr 30, 2019

## DOI :

<https://doi.org/10.15419/bmrat.v6i4.537>



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## ABSTRACT

**Introduction:** Three percent of people worldwide are infected with Hepatitis C virus (HCV). A few studies have been performed to evaluate the biochemical markers of the disease. In the current study, biochemical markers were evaluated in HCV patients and the control group. **Methods:** Two sex- and age-matched healthy individuals (n = 100) and HCV positive patients (n = 100) were included (mean age of 20-75, 26.0% females and 74.0% males). Biochemical markers, including liver enzymes (ALT, AST and ALP), lipid profiles (cholesterol, LDL, and HDL) and triglyceride (TG) were investigated in both groups. HCV genotyping was also performed by Polymerase Chain Reaction (PCR) and OHNO methods. **Results:** The biochemical markers between HCV patients and controls were compared (cholesterol, ALP, AST, ALT, LDL: p = 0.0001, HDL: p = 0.002, TG: p = 0.003), and statistically significant difference was found between two groups. The biochemical markers between HCV patients and the control group in terms of age was compared and no differences was observed (p = 0.741), however, there was a significant difference in sex between HCV patients and control group (26.0% females, 74.0% males in control group, and x% females and y% males in HCV patients) (p = 0.032). The results of HCV genotyping showed that 39 patients were genotype 1a, and 43 patients were genotype 3a, and 1 patient was genotype 2a. Evaluation of biochemical markers in patients with genotype 1a and 3a showed that there were significant differences in cholesterol (p = 0.001), LDL (p = 0.001) and HDL (p = 0.003) levels, but there were no significant differences in liver enzymes and TG levels in both genotypes. **Conclusion:** In the present study, we found significant difference in biochemical markers between HCV patients and controls. In HCV patients, the biochemical markers were dependent on HCV genotypes, and their levels in genotype 1a were higher than genotype 3a. In conclusion, biochemical markers are one of the most important factors for the identification of treatment.

**Key words:** Biochemical markers, Genotype 1a, Genotype 3a, Hepatitis C Virus, Lipid profiles, Liver enzymes, Photometric method

## INTRODUCTION

Hepatitis C virus (HCV) is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) in the world<sup>1</sup>. This virus is a single positive-stranded RNA genome of 9.6 Kb and belongs to the Flaviviridae family. Hepacivirus genus contains one long open reading frame (ORF). This single ORF encodes a polyprotein of about 3000 amino acids that produces viral proteins after cleavage by the host and viral encoded proteases<sup>2</sup>. In addition, core-encoding region of this virus expresses a protein entitled alternative reading frameshift protein (ARFP) or frame-shift protein (F protein)<sup>3,4</sup>. It has been shown that in chronic HCV patients, 82% were positive for anti-F antibodies, showing a significant difference from the healthy individuals<sup>5</sup>. This protein increases the frequency of CD4+CD25+ Treg cells, which may be associated with persistent and chronic infection

through subversion of the host immune response<sup>6</sup> F proteins could also enhance liver cell proliferation<sup>7</sup>, which is subsequently associated with the disease progression towards cirrhosis<sup>8</sup>. The protease function of HCV NS3 protein is also a crucial factor for the induction of liver fibrosis via interaction with cellular miRNAs and down-regulation of miRNA gene expressions<sup>9,10</sup>.

HCV is transmitted through several ways such as drug injection, blood transfusion and sexual exposure<sup>11</sup>. It is estimated that more than 170 million people worldwide are infected with hepatitis C virus<sup>12</sup>. In Iran, approximately 200,000 people are infected with this virus (less than 1%). Also, the prevalence of HCV infection is low among Iranian blood donors<sup>13,14</sup>. There are six genotypes and more than 70 subtypes of Hepatitis C virus in the world<sup>15,16</sup>. Epidemiological studies have shown the relationship between HCV geno-

**Cite this article :** Yazdansetad S, Nikoo H R, Azimi S M, Mohebbi A, Niazi M, Ajourloo M. **Comparison of biomarkers between genotypes 1a and 3a in hepatitis C virus patients with control group.** *Biomed. Res. Ther.*; 6(4):3121-3130.

types and geographic regions. Predominate genotype in Europe and North America is 1b, in Asia and Mediterranean region is 2 and in Europe is 3, whereas genotype 4 is often found in the Middle East, genotype 5 is only in South Africa, and genotype 6 is dominant in Southeast Asia<sup>17,18</sup>. Studies have shown that predominate genotypes in Iran are 1a and 3a<sup>19</sup>.

Hepatitis C virus attachment to hepatocytes is mediated by LDLr, which describes the role of lipids in HCV pathogenesis<sup>12,20</sup>. Many studies have shown that there is a relationship between serum cholesterol and LDL levels with the development of liver damage and HCV associated-cirrhosis<sup>16,20,21</sup>. Also, other findings have shown that the association between hepatitis C virus and LDL can protect the virus against antibodies<sup>22,23</sup>. This approach leads to the design of new drugs for HCV patients. Ledipasvir and Sofosbuvir are used for chronic HCV treatment<sup>21,24-26</sup>.

Fewer studies have been performed to evaluate and compare biochemical markers in HCV patients and healthy individuals (or control group). In the current study, biochemical markers in HCV patients and control group were evaluated and compared. We measured 3 groups of biomarkers: lipid profiles (total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL)), liver enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphate (ALP)), and triglyceride (TG). Our research objectives included the evaluation of biomarkers in HCV patients and control group, finding the relationship between biomarkers in HCV patients and control group, evaluation of biomarkers in HCV patients with genotypes 1a and 3a, and finding relationship between biomarkers in HCV patients with genotypes 1a and 3a.

## MATERIALS AND METHODS

In this study, information such as age, sex, marital status, HCV genotype, biochemical markers (ALP, AST, ALT, HDL, LDL, TG, and cholesterol), and transmission ways were collected. The information was recorded in a questionnaire.

### Study area and subjects

200 volunteers of 100 healthy individuals (control group) and 100 HCV patients were included. The serum samples were collected in a period of 18 months from 2011 to 2013 from HCV patients referred to a special clinic of Digestive Disease Research Center of Ghaem hospital, Mashhad, Iran. Before sampling, all patients were informed and signed the consent form that was approved by Ethics Committee of Mashhad University of Medical Sciences. The

serum samples were kept at  $-70^{\circ}\text{C}$  freezer until examination.

### Isolation of HCV RNA, cDNA synthesis and genotyping

HCV RNA was isolated from each patient's serum using the total RNA extraction kit following instructions by the manufacturer (Pars Tous biotechnology, Mashhad, Iran). HCV RNA was then detected by nested reverse transcription PCR using 5' UTR primers, as previously described by Krekulova *et al*<sup>27</sup>. Briefly, 10 $\mu\text{l}$  extracted RNA were reverse-transcribed into cDNA, using Maloney murine leukemia virus reverse transcriptase (M-MLV RT, 100 units) (Thermo Fisher Scientific) and 1M of downstream primer (outer antisense). The products generated from each sample were subjected to two separate PCR amplifications, one with primer set A and the other with primer mix B, in a final 20 $\mu\text{l}$  reaction volume containing 4.0 $\mu\text{L}$  of 5x First Strand Buffer, 1.0 $\mu\text{L}$ , dNTPs (10mM), 3.0 $\mu\text{L}$  dH<sub>2</sub>O and Taq polymerase (Fermentas Life Sciences, USA). The resulting specific HCV bands were visualized by ethidium bromide following electrophoretic separation on 2% agarose gel. Primer set A included primers for genotypes 1a, 1b, 1c, 3a, 3c and 4, while set B included primers for genotypes 2a, 3c, 3b, 5a, and 6a. To determine the sensitivities of PCR assays, prepared plasmids were quantified by UV spectroscopy, and the viral copy number was calculated. The 10-fold serial dilutions of all HCV cDNA-containing plasmids were made. For sensitivity, PCR was performed on a 10-fold dilution of plasmids ranging from 10<sup>1</sup> to 10<sup>8</sup> copies/reaction. The specificity was tested by determining the ability of primers to discriminate plasmids with different genotypes of HCV types. There was no specific signal for the independent PCR assays (data not shown). The ability of all types of PCR primers to amplify HCV cDNA-containing plasmids was analysed by using a dilution series that started from 10<sup>1</sup> plasmid copies to 10<sup>8</sup> plasmid copies. This assay showed significant sensitivity of about 100 viral copies for all HCV genotypes.

**Table 1: The normal and non-normal percent of biomedical markers between HCV patients and control group**

Variables	Groups	Control group		HCV patients		Total	
		subjects	percent	subjects	percent	subjects	percent
Cholesterol	Normal	62	62.00%	77	77.00%	139	69.50%
	Non-normal	38	38.00%	23	23.00%	61	30.50%
	Total	100	100.00%	100	100.00%	200	100.00%
Alanine	Normal	99	99.00%	28	28.00%	29	14.50%
	Non-normal	1	1.00%	72	72.00%	171	85.50%
	Total	100	100.00%	100	100.00%	200	100.00%
Triglyceride (TG)	Normal	83	83.00%	83	83.00%	166	83.00%
	Non-normal	17	17.00%	17	17.00%	34	17.00%
	Total	100	100.00%	100	100.00%	200	100.00%
High-density lipoprotein (HDL)	Normal	86	86.00%	57	57.00%	143	71.50%
	Non-normal	14	14.00%	43	43.00%	57	28.50%
	Total	100	100.00%	100	100.00%	200	100.00%
Low-density lipoprotein (LDL)	Normal	76	76.00%	82	82.00%	158	79.00%
	Non-normal	24	24.00%	18	18.00%	42	21.00%
	Total	100	100.00%	100	100.00%	200	100.00%
Aspartate	Normal	98	98.00%	37	37.00%	135	67.50%
	Non-normal	2	2.00%	63	63.00%	65	32.50%
	Total	100	100.00%	100	100.00%	200	100.00%
Alkaline	Normal	99	99.00%	0	0.00%	1	0.50%
	Non-normal	1	1.00%	100	100.00%	199	99.50%
	Total	100	100.00%	100	100.00%	200	100.00%

### Comparison of biochemical marker levels between patient and healthy groups

Clinical chemistry kits (Human, Pars Azemoun Kit) and an automatic biochemistry analyzer (Micro Lab 300, Merck Germany) were used to analyze biochemical markers such as aspartate aminotransferase (AST), alanine transaminase (ALT), -glutamyl transferase (-GT), alkaline phosphatase (ALP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), cholesterol and triglycerides (TG) in the serum samples. These biomarkers were evaluated and compared between HCV patients and control group, and between HCV patients with genotypes 1a and 3a.

### Statistical analysis

All data were recorded in the previously designed form and analyzed by statistic software SPSS 20. For analysis of biochemical markers between HCV patients and control group, we used Kolmogorov-Smirnov, Asti dent, Man Whitney, Pearson Chi-Square, Fisher's Exact Test and Cochran. At first the Kolmogorov-Smirnov was used, if data were normal, the Student t-test was then used, but if data were not normal, the Man-Whitney test was used. Statistical significance was considered as  $p < 0.05$ .

## RESULTS

### Patients

The mean age was  $47.3 \pm 10.2$  for patients and  $47.8 \pm 9.7$  for control group. Information based on age between HCV patients and control group was compared and the difference in age distribution among patient groups was not significant. **Figure 1** shows the percentage of HCV patients and control group in four age subgroups. In case of sex (26.0% females, 74.0% males), there was a significant difference between control group (75.0% married, 25.0% single), and HCV patients (81.0% married, 19.0% single) ( $p = 0.032$ ).

In investigation for other diseases, 4.0% of patients had hemophilia and 1.0% had thalassemia, but there was no hemophilia or thalassemia in control group. Percentage of individuals with diabetes was 4.0% in control group and 5.0% in HCV patients. Percentage of people had other diseases were 6.0% in HCV patients and 3.0% in control group. Transmission ways among HCV patients were different. The most prevalent transmission way of HCV was observed among injective drug users (62%). Other forms of transmission included blood transfusion in patients with hemophilia, thalassemia, and surgery (11.0%), sexual transmission (3.0%), and 20% of the causes were unknown.

### HCV typing

In 83% (83/100 in patient group) of clinical specimens positive for HCV, genotype 3a was the most prevalent among the patients (52% or 43/83 patients), followed by genotype 1a and 2a (47% and 1.2%, respectively). No genotypes were obtained for 17% of the patients.

### Evaluation of biomarkers in control group and HCV patients

Analysis of associations between HCV genotypes and bio-chemical markers showed that the levels of cholesterol, HDL and LDL significantly differed ( $p < 0.005$ ) in patient group (cholesterol = 77.0%, LDL = 82.0%, HDL = 57.0%) compared to control group (cholesterol = 62.0%, LDL = 76.0%, HDL = 86.0%). However, no significant differences between two groups in other biomarkers were detected. The normal percentage of TG were identical in two groups (TG = 83.0%) (**Table 1**).

The mean levels of lipid profile in HCV patients were: cholesterol = 149.6, LDL = 101.6, HDL = 41.1, and in control group were: cholesterol = 193.3, LDL = 108.6, HDL = 45.0. Mean TG levels in HCV patients was 125.0 and in control group was 144.1. The mean levels of liver enzymes in HCV patients were: ALT = 205.4, AST = 209.3, ALP = 401.5, and in control group were: ALT = 19.0, AST = 21.8, ALP = 166.3 (**Table 2**). The levels of biochemical markers between HCV patients and control group were compared and there was a significant difference between two groups (cholesterol, ALP, AST, ALT, LDL:  $p = 0.0001$ , HDL:  $p = 0.002$ , TG:  $p = 0.003$ ).

### Evaluation of biomarkers in HCV patients with genotypes 1a and 3a

The evaluation between genotypes 1a and 3a in HCV patients showed that the normal percentage of lipid profiles in genotype 1a was: cholesterol = 74.4%, LDL = 74.4%, HDL = 64.1%, and in genotype 3a was: cholesterol = 88.4%, LDL = 93.0%, HDL = 39.5%. The normal percentage of liver enzymes in genotype 1a was: ALT = 28.2%, AST = 30.8%, ALP = 0.0%, and in genotype 3a was: ALT = 25.6%, AST = 46.5%, ALP = 0.0%. Also, the normal percentage of TG in genotype 1a was 79.5% and in genotype 3a was 93.0% (**Table 3**). The mean levels of biomedical markers in HCV patients with genotype 1a was: cholesterol = 161.9, LDL = 122.3, ALT = 222.1, TG = 127.8, HDL = 47.7, AST = 259.1, ALP = 441.0, and in HCV patients with genotype 3a was: cholesterol = 127.9, LDL = 78.9, ALT = 183.5, TG = 102.4, HDL = 34.2, AST = 141.6, ALP = 385.4 (**Table 4**). There were significant differences

**Table 2: Minimum, maximum, mean, SD and p-value amounts of biochemical markers between HCV patients and control group**

Variable	Groups	Minimum	Máximum	Mean	SD	P-Value
Cholesterol	HCV patient	54	456	149.6	70.2	Mann-Whitney U=-6.01 P-Value=0.0001**
	control	118	310	193.3	41.2	
	total	54	456	171.6	61.4	
ALT	HCV patient	10	3502	205.4	545.2	Mann-Whitney U=-8.73 P-Value=0.0001**
	control	4	129	19	14.9	
	total	4	3502	111.8	394.9	
TG	HCV patient	30	541	125	97	Mann-Whitney U=-2.97 P-Value=0.003**
	control	45	503	144.1	84.7	
	total	30	541	134.6	91.3	
HDL	HCV patient	14	125	41.1	22.8	Mann-Whitney U=-3.17 P-Value=0.002**
	control	22	98	45	14.1	
	total	14	125	43	19	
LDL	HCV patient	22	945	101.6	110.2	Mann-Whitney U=-3.88 P-Value=0.0001**
	control	12	231	108.6	40.2	
	total	12	945	105.1	82.6	
AST	HCV patient	11	3158	209.3	454	Mann-Whitney U=-9.31 P-Value=0.0001**
	control	7	106	21.8	11.7	
	total	7	3158	115.1	333.1	
ALP	HCV patient	55	2713	401.5	475	Mann-Whitney U=-6.25 P-Value=0.0001**
	control	34	354	166.3	76.7	
	total	34	2713	283.3	358.5	

in the levels of cholesterol (p-value = 0.001), LDL (p-value = 0.001) and HDL (p-value = 0.003) in genotypes 1a and 3a, but there were no significant differences in liver enzymes and TG in both of genotypes.

### DISCUSSION

In this study, the samples were selected among patients referred to a special clinic of Digestive Disease Research Center of Ghaem hospital in Mashhad, Iran. One hundred HCV positive patients were collected and 82% of them had genotypes 1a and 3a, and 100 healthy individuals were collected as control group. Some factors influence on severity of HCV disease

such as: sex, age, other diseases and increased levels of biomarkers. For example the risk of HCV increases with age<sup>28,29</sup>.

For the evaluation of demography results, we collected male patients three times more than female patients, due to a higher risk of HCV in males in comparison with females. Jamalidoust *et al.*<sup>30</sup> and Alavian *et al.*<sup>20</sup> showed similar results.

Sixty two percent of our cases were drug addicted. Jamalidoust and her colleagues had similar samples to the current study and 62.2% of their cases were drug addicted<sup>30</sup>. Education range was higher in the control group, which can influence on HCV infection,

**Table 3: The normal and non-normal percentage of biomedical markers between genotypes 1a and 3a in HCV patients**

Variable	Groups	Genotype 1a		Genotype 3a		Total	
		HCV patients	percent	HCV patients	percent	HCV patients	percent
Cholesterol	Normal	29	74.40%	38	88.40%	67	81.70%
	Non-normal	10	25.60%	5	11.60%	15	18.30%
	Total	39	100.00%	43	100.00%	82	100.00%
ALT	Normal	11	28.20%	11	25.60%	22	26.80%
	Non-normal	28	71.80%	32	74.40%	60	73.20%
	Total	39	100.00%	43	100.00%	82	100.00%
TG	Normal	31	79.50%	40	93.00%	71	86.60%
	Non-normal	8	20.50%	3	7.00%	11	13.40%
	Total	39	100.00%	43	100.00%	82	100.00%
HDL	Normal	25	64.10%	17	39.50%	42	51.20%
	Non-normal	14	35.90%	26	60.50%	40	48.80%
	Total	39	100.00%	43	100.00%	82	100.00%
LDL	Normal	29	74.40%	40	93.00%	69	84.10%
	Non-normal	10	25.60%	3	7.00%	13	15.90%
	Total	39	100.00%	43	100.00%	82	100.00%
AST	Normal	12	30.80%	20	46.50%	32	39.00%
	Non-normal	27	69.20%	23	53.50%	50	61.00%
	Total	39	100.00%	43	100.00%	82	100.00%
ALP	Normal	0	0.00%	0	0.00%	0	0.00%
	Non-normal	39	100.00%	43	100.00%	82	100.00%
	Total	39	100.00%	43	100.00%	82	100.00%

therefore, there is a relationship between education and lower rate of HCV infection. Detection and rapid assessment of chronic liver disease that lead to liver fibrosis is important for monitoring and evaluating the risk of liver cancer. The first step after detection of positive HCV is to measure the levels of liver enzymes. If these markers are higher than normal levels, the individual is infected with chronic liver disease.

Hepatitis C virus enters host cells via LDL and cholesterol receptors, thus, the levels of these biomarkers

increase in HCV patients. Agnello<sup>31</sup> and Jine<sup>32</sup> researched about this topic. Results of our study were opposite to their findings. In the current study, the levels of cholesterol and LDL in HCV patient were lower than that of the control group due of drug consumption and the types of diet, which were given to patients.

The results of our study showed the percentage of normal cholesterol, LDL and HDL levels in patients, which were lower than the levels in control group, and

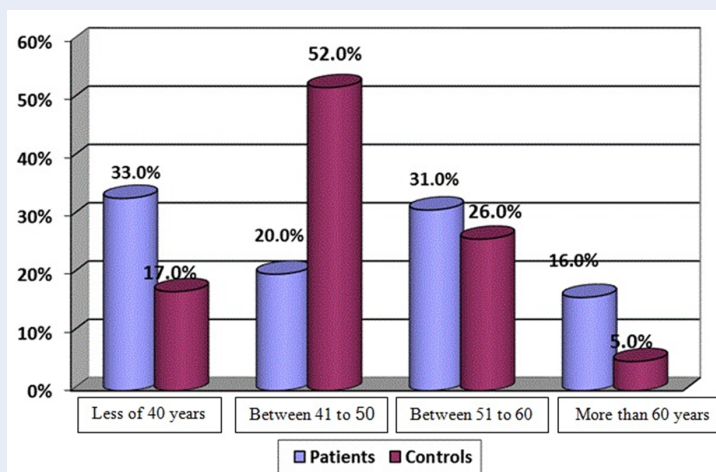
**Table 4: Minimum, maximum, mean, SD and p-value amounts of biochemical markers between genotypes 1a and 3a in HCV patients**

Variable	Groups	Minimum	Maximum	Mean	SD	P-Value
Cholesterol	1a	54	362	161.9	66.6	Mann-Whitney U=-3.19
	3a	65	456	127.9	72.7	P-Value=0.001**
	total	54	456	144.1	71.5	
ALT	1a	11	3012	222.1	537	Mann-Whitney U=-0.757
	3a	10	3502	183.5	551.2	P-Value=0.449
	total	10	3502	201.9	541.5	
TG	1a	30	448	127.8	108.2	Mann-Whitney U=-0.306
	3a	30	298	102.4	59.8	P-Value=0.759
	total	30	448	114.5	86.7	
HDL	1a	17	120	47.7	26.5	Mann-Whitney U=-2.926
	3a	14	125	34.2	20.1	P-Value=0.003**
	total	14	125	40.6	24.2	
LDL	1a	31	945	122.3	143.3	Mann-Whitney U=-3.668
	3a	22	625	78.9	91	P-Value=0.0001**
	total	22	945	99.5	120	
AST	1a	11	3158	259.1	555	Mann-Whitney U=-1.57
	3a	12	2099	141.6	337	P-Value=0.115
	total	11	3158	197.4	454.9	
ALP	1a	98	2713	441	582.5	Mann-Whitney U=-0.664
	3a	93	1771	385.4	384.7	P-Value=0.507
	total	93	2713	411.8	486.5	

percentage of normal TG was equal in two groups. Orangi et al. evaluated the percentage of normal LDL and HDL in patients and control groups and showed the same results<sup>33</sup>. However, in a study by Alavian et al., the percentage of normal cholesterol, LDL, HDL and TG levels was lower than those from our patients. One reason for these differences in the results may be due to thalassemia patients in their study. Also, in the current study, the levels of normal ALT, AST and ALP in control group were higher than HCV patients. Alavian et al. showed similar findings to our results in two groups, but Pontisso et al. findings were opposite. Their results showed that percentage of normal

ALT was more than 50%<sup>34</sup> compared to 28% of normal ALT in our study. This difference may be related to the reason that some of our patients were at the beginning of the treatment, and the medicine had not exerted a full impact. In a study, Peymani et al. used chloroquine drug in non-responder patients for antiviral treatment. They showed that HCV RNA loads and ALT levels were decreased and normalized during treatment<sup>35</sup>. In the other study, Peymani and his colleagues showed that the use of chloroquine drug could reduce AST, ALT, cholesterol, and triglyceride levels and help to normalize some biochemical markers<sup>36</sup>. Riaz et al. evaluated the association of HCV





**Figure 1: Age distributions of HCV patients and control group.** The age group analysis of HCV-positive cases revealed that the highest infection rate was among patients less than 40 years of age with 33.0% prevalence. Patient between 51-60 years of age were the most affected (31.0%), while those within the age bracket of more than 60 years were the least affected (16.0%).

genotypes with biomarkers in HCV infected patients. They found that infection with genotype 1a was associated with significantly higher ALT, AST and ALP levels compared to genotype 3a<sup>37</sup>. Moallemi *et al.* determined serum levels of IP-10 (as a new biomarker) in HCV patient and control groups. They found that the levels of IP-10 in patient group was higher than control group, and the difference was statistically significant between two groups. They also checked the levels of this biomarker between HCV genotypes and revealed that the levels of IP-10 were higher in genotype 1a than 3a<sup>38</sup>. According to the results, we found that the mean levels of biochemical markers in genotype 1a were higher than genotype 3a, also, the percentage of normal biochemical markers were different in genotype 1a and 3a. In evaluation of biochemical markers in the patient group, we found their relationship with genotypes. The new studies have shown a relationship between genotypes and HCV severity. In a research, Jhaveri *et al.* showed similar results to our study<sup>39</sup>. There were differences in the levels of ALT, AST and ALP between two groups. One reason for our different was due to different demography features. In the current study, sampling was randomly performed within 18 months. Some patients had finished their treatments, but some were being treated and the others had not started treatment. We used 7 biomarkers (cholesterol, HDL, LDL, TG, ALT, AST, and ALP), and our patients had special diets and have used drugs. Thus, triglyceride, cholesterol, LDL and HDL levels in these patients were lower than the controls.

The present study assessed the correlation between HCV genotypes with lipid profiles in a small sample size. Thus, it is be important to continue the analysis of lipid profile and characterization of HCV genotypes in a larger population, and to follow their changes over time. Genotyping by PCR is highly specific and sensitive for the determination of HCV genotype, but it is a time-consuming and costly technique. On the other hand, in association with blood born viruses, we need a highly trained technician.

## CONCLUSION

The results of this study showed that there is a significant relationship between biomarkers in both control and patient groups. In HCV patients, the biochemical markers were dependent on HCV genotypes, and their levels in genotype 1a were higher than genotype 3a. There was a significant correlation between cholesterol, HDL, and LDL in patients with genotype 1a and 3a. Biochemical markers may be one of the most important factors that will help in the formulation of treatment strategies. We examined all the demographic information of our patients and control group, however, we did not check the demographic information of other family members. Evaluations of all family members are useful to prevent disease transmission and to help families to improve the patient care.

## COMPETING INTERESTS

Authors declare no conflict of interest



## ACKNOWLEDGEMENTS

This work has supported by the School of Medicine, Golestan University of Medical Sciences, Gorgan, Iran. The authors are greatly thankful to Digestive Disease Research Center of Ghaem hospital in Mashhad, Iran. This study was conducted as an MSc project in Damghan Branch, Islamic Azad University, Damghan, Iran.

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