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**Research Article**

# Relative and combined Effects of Ethanol and Hepatitis C Virus Infection on Serum Interleukin-17 Levels

## Abstract

**Background:** Interleukin-17 (IL-17) is involved in neutrophil recruitment and may contribute to the progression from fatty liver to steatohepatitis. Its role in alcoholics and in HCV-infected patients is not clear, lower- or higher-than normal values having been reported.

**Aim:** To study the behavior of IL-17 among alcoholics with or without Hepatitis C virus infection (HCV), given the frequent association of both entities and the apparently opposite results reported in these two groups of patients.

**Results:** Among 96 alcoholics, 10 with co-existing HCV infection, 15 non-alcoholic HCV-infected patients and 21 controls, IL-17 showed significant differences between patients (median=2.76; IQ=0.85-5.50) and controls (median=0.50; IQ= 0.30-1.54; Z=3.06, p=0.002). Also, IL-17 was significantly different when the sample was classified in 4 groups (alcoholics without HCV infection, alcoholics with HCV infection, non-alcoholics, HCV infected patients, and controls (KW= 11.86; p=0.008).

**Main findings**: By two way variance analysis we disclosed that both hepatitis C (F=7.36; p=0.008) and alcohol consumption (F=6.16; p=0.014) exerted significant effects on IL-17, a negative interaction existing between both variables (F=4.97; p=0.028). Inverse correlations were observed between left leg fat and IL-17 (ρ= -0.28; p= 0.009). Patients with IL-17 over the median showed less trunk fat than those with IL-17 below the median (Z=2.072; p=0.038), and also less total body fat (Z=1.99; p=0.046). No relations were observed between IL-17 and viral load, ethanol consumption or liver function impairment.

**Conclusion**: Ethanol and HCV infection lead to increased IL-17 levels, similar to what is observed when both factors coexist. An inverse relationship exists between body fat and IL-17.

**Brief Summary**: Interleukin-17 is a cytokine produced by lymphocytes involved in neutrophil recruitment that may have a role in the progression from fatty liver to steatohepatitis. Its role in alcoholics and in HCV-infected patients is controversial. We here report higher IL-17 levels in alcoholic and HCV-infected patients. Additionally, we also describe an inverse relationship between IL-17 levels and fat mass which supports an inhibitory role of this cytokine on adipogenesis. To our knowledge, no other study has analysed the relative and combined effects of HCV and ethanol on IL-17 levels, as we have done in this study.

## Abbreviations

BMI: Body Mass Index; HCV: Hepatitis C Virus; HIV: Human Immunodeficiency Virus; IFNG: Interferon-γ; IL: Interleukin; IQ: Interquartile; KW: Kruskall-Wallis; SNS: Subjective Nutritional Score; TGF: Transforming Growth Factor; Th: T helper

## Background

Interleukin (IL)-17A (IL-17) is the main product of a subset of CD4+ T-lymphocytes (T helper (Th)-17 lymphocytes) [1], but it can also be secreted by other cells, including γδ-T cells, natural killer and natural killer T cells, macrophages, Paneth cells and other cell types involved in innate immunity [2]. Differentiation of CD4+ cells into Th-17 lymphocytes is promoted by several cytokines, such as IL-6, transforming growth factor (TGF)-β, and IL-21, (in mice) and IL-1, IL-6 and IL-23 in humans [3], whereas IL-4 and interferon-γ (IFNG) exert inhibitory effects [4]. Although Th-17 lymphocytes regulate the immune response mainly via secretion of IL-17A and IL-17F, orchestrating the defence especially against fungal species [5] and several bacteria, such as *Listeria*

*monocytogenes* [6], *Mycobacterium tuberculosis* [7], *Klebsiella* or *Staphylococcus aureus* [8], they are also involved in the pathogenesis of immune-mediated diseases, such as systemic lupus erythematosus, among others [9].

Interleukin 17 is involved in neutrophil recruitment. Receptors for IL-17 are found in all liver cells [10]. In this sense, IL-17 activates Kupffer cells, stellate cells, epithelial cells and hepatocytes, in which stimulation by IL-17 induces the production of acute phase reactants [11] and the expression of several cytokines, including IL-8, which is a potent chemoattractant for neutrophils. Th-17 lymphocytes infiltrate the liver in alcoholic liver disease, and IL-17 levels are markedly increased in plasma in these patients. In liver preparations, there is a relationship between the intensity of fibrosis and Th-17 infiltration, as shown in a study by Lemmers, et al., [12]. In that study, the authors also reported that plasma IL-17 levels were increased in alcoholics, but not in patients with hepatitis C virus (HCV) - related liver cirrhosis [12]. Controversy exists, indeed, regarding the role of IL-17 in HCV-infected patients. Some authors report low IL-17 levels among these patients [13], but others an opposite result: increased IL-17 in HCV- infected patients in association with progression to cirrhosis and with viral RNA titer [14]. In a similar sense, it has been postulated that Th-17 cells may be also involved in the progression from fatty liver to steatohepatitis, at least in non-alcoholic patients [15].

Therefore, the behaviour of IL-17 in alcoholic or HCV-infected patients is unclear, and it is not known what happens when both factors, HCV infection and alcoholism, co-exist in the same patient, a clinically common situation.

Based on these facts, in this study we aim to analyse the behaviour of plasma IL-17 in alcoholic patients with or without HCV infection, and non-alcoholic HCV- infected patients, comparing them with a control group of non-alcoholic, non-HCV infected patients.

## Patients and Methods

### Patients and controls

We included 111 patients (98 men); 96 of them were alcoholics, who drank a median amount of 150 g ethanol per day (interquartile range (IQ) =96-212) during 32 years; IQ=21-40 years. Twenty five patients were affected by chronic HCV infection, 15 of them non-drinkers and 10 alcoholics. We also included 21 controls (19 men, p=0.54 using the exact Fisher´s test when sex was compared in patients and controls). Therefore, there were 4 groups: non-HCV infected alcoholics (86 patients); HCV-infected alcoholics (10 patients); HCV-infected non alcoholics (15 patients) and 21 controls. The mean age of the patients was 54.14 ± 12.48 years (median=55; IQ=47-61 years), while controls had a mean age of 51.86 ± 7.11 years (median=51; IQ=48-58 years; t=1.18; NS). HCV infection was assessed by the presence of anti-HCV antibodies and/or HCV RNA measured using reverse transcriptase polymerase chain reaction. HCV genotypes included 10 genotype 1a, 4 genotype 1b, 3 genotype 1, 1 genotype 1a and 1b, 2 genotype 3,

and 3 genotype 3a. All of these patients were recruited before a treatment for virus C hepatitis was administered. Serological tests for detection of human immunodeficiency virus (HIV) infection were performed in all patients; those patients with a positive test were excluded from this study.

Eighty-nine patients underwent an abdominal ultrasound examination. We recorded the presence (38) or not (51) of liver steatosis and the presence (38) or not (51) of data of liver cirrhosis (heterogeneous liver, portal dilatation, splenomegaly). An inverse association was found between the presence of steatosis and cirrhosis ($\chi^2$ =6.15; p=0.013).

### Nutritional evaluation

Nutritional evaluation was assessed by

1. – Body mass index (BMI) was calculated as weight (in kg)/height$^2$ (in m).

2. – Subjective nutritional evaluation, according to the following protocol: the muscle masses of the upper and lower limbs and of the temporal muscle were examined , defining absence of atrophy and two degrees of atrophy (moderate or severe), and assigning 0, 1 and 2 points to each category, respectively. We also measured fat loss by physical examination. We recorded fat loss on the cheek and abdomen, Bichat´s fat and subcutaneous abdominal fat atrophy, and classified them in a similar way, defining a score (SNS), based on the sum of the assigned points, for which the poorest value was 10 and 0 the best one. We further classified our patients in well-nourished (Global score= 0-2 points), moderately undernourished (3-4 points) and severely undernourished (5-10 points), since this classification is related to prognosis [16].

3. – Whole body composition by densitometry

After informed consent, patients underwent assessment of lean mass and fat mass at different body parts, such as arms, legs, trunk, and total body, with a HOLOGIC QDR-2000 (Waltham, MA, USA). This procedure was performed in 90 patients.

### Cytokines and biochemical parameters

Blood samples were taken at 8.00 a.m. in fasting conditions and were immediately frozen at -20°C. Serum IL-17 levels were determined by Luminex® Performance Assay (R&D Systems, Minneapolis, MN, USA). Sensitivity stated by the manufacturer was less than 15 pg/ml, whereas the intra-assay precision was 6.2%.The detection limit assessed in our laboratory was 0.42 pg/ml, based on measurement of the intensity of the color of the solution contained in the well that includes a monoclonal antibody specific for human IL-17 and the serum sample to be analyzed.

In addition to IL-17 determination, routine laboratory evaluation was also performed. Main results of this laboratory evaluation are shown in Table 1.

Table 1: Some data of the individuals included in the study. Data are given either as means ± standard deviations or medians (interquartile ranges).

| | HCV- alcoholics (n=86; 80 men) | HCV + alcoholics (n=10; 9 men) | HCV+ , non-alcoholics (n=15; 9 men) | Controls (n=21; 19 men) |
|---|---|---|---|---|
| Serum IL-17 (pg/ml) | 2.5 (0.81-5) | 2.68 (2-5.88) | 4.2 (1.1-7.30) | 0.5 (0.3-1.55) |
| Age | 57.08 ± 11.54 | 49.40 ± 13.66 | 41.87 ± 9.50 | 51.86 ± 7.10 |
| ASAT (U/L) | 47.5 (22-94.25) | 90.5 (42-189) | 42 (32-65) | Normal 7-40 |
| ALAT (U/L) | 29.5 (19-56.25) | 75 (46.5-104.75) | 47 (36-68) | Normal 7-40 |
| GGT (U/L) | 186 (51-361) | 198.5 (78.75-388.5) | 56 (24-106) | Normal 7-40 |
| Alkaline phosphatase (U/L) | 84 (58-120) | 106.5 (82.5-165) | 78 (58-87) | Normal= 40-129 |
| Prothrombin (%) | 79.85 (58.30-99.0) | 100 (60.5-100) | 100 (100-100) | Normal 80-100 |
| Serum albumin (g/dL) | 3.60 ± 0.72 | 3.61 ± 0.78 | 4.47 ± 0.32 | Normal 3.8-4.5 |
| Serum bilirubin (mg/dL) | 1 (1-2.85) | 1.1 (0.68-1.75) | 0.6(0.4-0.7) | Normal <1.4 |
| Total neutrophils (per mm3) | 4170 (3075-6298) | 4138 (2993-5982) | 3519 (2917-5324) | Normal=1700-7000 |
| Total lymphocytes (per mm3) | 1165 (828-1423) | 1662 (923-2217) | 2217 (1819-2992) | Normal=1000-4800 |
| Mean corpuscular volumen (fl) | 101.30 ± 9.03 | 99.29 ± 5.59 | 90.7v1 ± 2.68 | Normal= 80-100 |
| Daily ethanol (g) | 150 (100-225) | 110 (80-200) | --------------- | ------------- |
| Years of addiction | 32 ± 14 | 33 ± 19 | ------------ | ------------ |
| Body mass index | 25.72 (23.85-28.54) | 23.02 (21.19-28.01) | 22.86 (21.0-24.31) | 25 (23-28) |
| Viral load (Log) | ----------------- | 5.13 ± 2.31 | 6.28 ± 0.64 | --------------- |
| Total fat mass (g) | 19638 (14393-29368) | 18509 (10172-22545) | 18804 (9572- 24857) | ------------- |
| Trunk fat mass (g) | 11577 (9069-18100) | 10734 (5539-13693) | 9380 (4937-13480) | ------------- |
| Total lean mass (g) | 48125 (45087-53911) | 43594 (35133- 45838) | 22877 (18543-42030) | -------------- |

## Statistics

The Kolmogorov-Smirnov test was used to test for normal distribution, a condition not fulfilled by most of the variables. Therefore, non-parametric tests, such as Mann-Whitney´s U test and Kruskall-Wallis were used to analyse differences of these parameters between groups. Student´s t test, variance analysis and Pearson´s correlation analysis were used with the few variables with a normal distribution, whereas Spearman´s rho (instead of Pearson´s correlation) was utilised in the case of non-parametric variables. Non parametric variables are given as median and interquartile range, whereas parametric variables are shown as means ± standard deviations.

In order to assess whether alcohol or HCV infection exerted an independent effect on IL-17 levels, and whether or not there was an interaction between both parameters, we used a two-way variance analysis. These analysis were performed with the SPSS program (Chicago, Ill., USA).

The study protocol was approved by the local ethical committee of our Hospital (2014-11) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

## Results

IL-17 showed highly significant differences between patients (median= 2.76; IQ=0.85-5.50 pg/ml) and controls (median=0.50; IQ= 0.30-1.54 pg/ml; Z=3.06, p=0.002). Also, IL-17 was significantly different when the sample was classified in 4 groups (alcoholics without HCV infection, alcoholics with HCV infection, non-alcoholics, HCV infected patients, and controls (KW= 11.86; p=0.008, Table 1, Figure 1). By two way variance analysis we disclosed that both hepatitis C (F=7.36; p=0.008) and alcohol consumption (F=6.16; p=0.014) exerted significant effects on IL-17, an interaction existing between both variables (F=4.97; p=0.028). Similar results were observed when only men were included (KW=10.44, p=0.015 when IL-17 was compared among the 4 groups; significant effects of both HCV infection (F=6.51; p=0.012 and ethanol (F=5.14; p=0.025; and significant interaction (F=4.21; p=0.043) between ethanol and HCV infection). There were no differences among men (median=2.98; IQ=1.20-5.50 pg/ml) and women (median=2.50; IQ=0.17-5 pg/ml; Z=0.96; non-significant (NS)), and IL-17 values were not associated with age ($\rho$=-0.07; NS). When only women were subjected to statistical analysis, no differences were observed among the 4 groups, and we failed to find any significant effects of HCV and/or ethanol on IL-17 levels (probably because the short number of women included).

### Ethanol consumption and liver function

No association was observed between IL-17 and the amount of ethanol consumed ($\rho$=0.06), the duration of the drinking habit ($\rho$=0.011) or the levels of GGT ($\rho$=0.02) and MCV values ($\rho$=0.04), either by Spearman´s correlation or by Mann Whitney´s U test comparing patients with IL-17 over the median or below the median.

Among patients with HCV infection the negative correlation between IL-17 and viral load (23 patients) was not statistically significant ($\rho$=-0.32; p=0.14), and there were no differences in viral load when patients with IL-17 over the median were compared with those with IL-17 values below the median (Z=1.13; NS).

We also failed to find any association between IL-17 and prothrombin activity ($\rho$=-0.04), serum albumin ($\rho$=-

0.02) or bilirubin (ρ=0.02), or the presence of ascites (24 cases; median=3.48; IQ=1.43-5.95 vs 2.50; IQ=0.5-4.83 pg/ml; Z=1.25; NS) or encephalopathy (14 cases; median=4; IQ=1.98-9.24 vs 2.5; IQ=0.81-4.83 pg/ml; Z=1.39; NS). There were also no differences in IL-17 between patients with liver steatosis (median=3.5; IQ=1.4-6.5 pg/ml) and those without liver steatosis (median=2.63; IQ=0.5-5.33 pg/ml; Z=1.11; NS), or among patients with ultrasonographic features of liver cirrhosis (median=2.76; IQ=0.81-5.65 pg/ml)or without these features (median=2.27; IQ=0.85-4.83 pg/ml; Z=0.4; NS).

We also failed to find any significant relationship between IL-17 and the variables mentioned before when only men were considered.

### Relationships with lean and fat mass

Fifty patients had normal nutritional status according to subjective nutritional score, 30 were moderately undernourished and 27, severely undernourished. No relationship existed between IL-17 levels and subjective nutritional evaluation (KW=0.06; NS). Also, no relationship was observed between IL-17 and BMI (ρ=0.09; NS).

No associations were observed with body lean mass. In general, inverse correlations were observed with fat distribution, but these correlations were statistically significant only between left leg fat and IL-17 (ρ= -0.28; p= 0.009). Patients with IL-17 over the median showed less trunk fat than those with IL-17 below the median (Z=2.072; p=0.038), and also less total body fat (Z=1.99; p=0.046; Figures 2a,b). These differences were also observed when only men were analysed (Z=2.14, p=0.032 for total fat, and Z=2.16, p=0.031 for trunk fat).

## Discussion

In this study we have shown that both alcoholism and HCV infection lead to increased IL-17 levels, but when both conditions coexist, the degree of increase is less remarkable. As mentioned previously, Lemmers, et al., reported for the
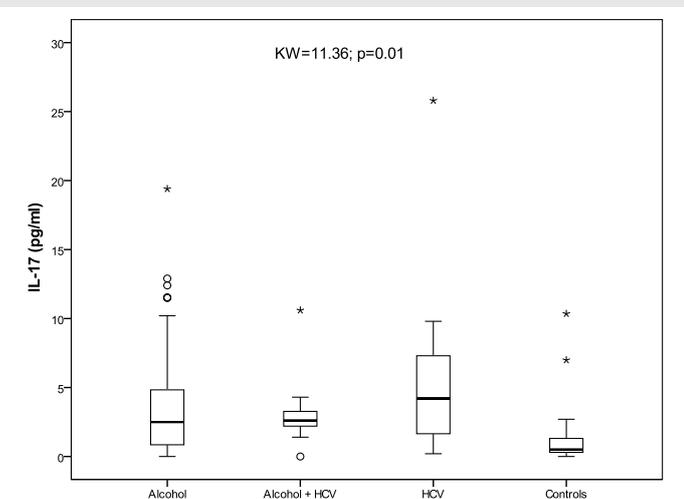


**Figure 1:** IL-17 among the four groups of individuals included in the study (Controls, pure alcoholics, pure HCV-infected patients and alcoholic + HCV infection).
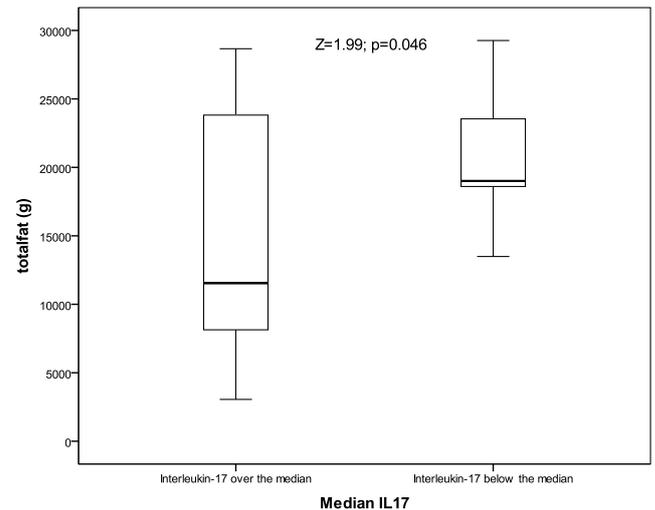


**Figure 2a:** Differences in total fat comparing patients with serum IL-17 values over the median or below the median.
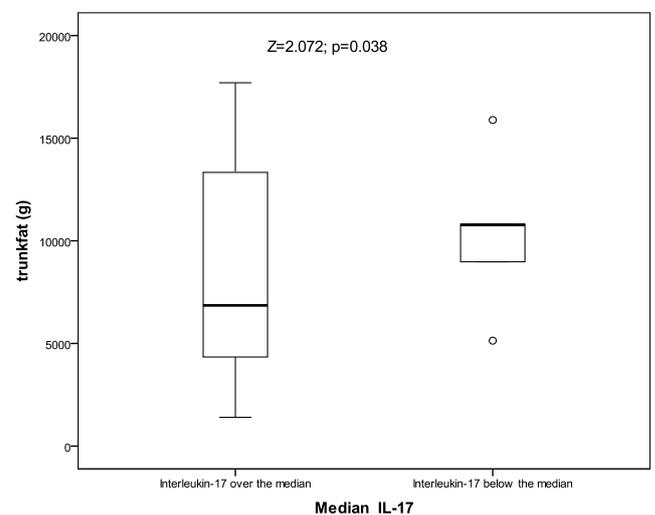


**Figure 2b:** Differences in trunk fat comparing patients with serum IL-17values over the median or below the median.

first time high IL-17 levels among alcoholics, accompanied by an increase in peripheral Il-17 producing mononuclear cells (PBMC), and by infiltration of the liver by IL-17 secreting cells. Liver infiltration by IL-17 secreting cells correlated with liver function impairment assessed by MELD score, but a paradoxical inverse correlation was observed between plasma levels of IL-17 and liver function impairment [12]. In the same study the authors also failed to find any differences in IL-17 values between patients with HCV infection and controls. On the contrary other authors have found higher plasma IL-17 values among patients with HCV infection. In this sense, our data for controls and HCV patients are similar to those reported by Hammad, et al., (1.2 ± 0.4 pg/mL for controls and 6.42 ± 1.7 pg/mL for non-complicated HCV-infected patients) [14]. In Hammad´s study the development of liver cirrhosis or hepatocellular carcinoma led to an enormous rise in IL-17 levels. Other researchers have found that IL-17 plays a key role in the progression of HCV infection, and also a marked decrease in the proportion of IL-17 producing peripheral PBMC after

interferon therapy [17]. Reda, et al., have found significantly increased IL-17 values in HCV-infected patients that were significantly related to liver function impairment [18]. Other authors report normal values in HCV-infected patients compared with controls (8.35 ± 3.42 pg/ml vs 13.15 ± 6.80 pg/ml, $p$ = 0.088 in El Bassuoni study), [19] and Sousa, et al., also reported even lower IL-17 levels in HCV-infected patients than in controls, in the range of those reported in our study [13]. Hao, et al., also failed to find differences between controls and 114 HCV –infected patients [20]. Therefore, a great deal of controversy exists regarding the role of Th17 cells in HCV infection. A prevailing hypothesis states that IL-17 would increase in these patients as a compensatory mechanism, rather than as a mechanism directly involved in liver damage [13]. To our knowledge no other study has analysed the relative and combined effects of HCV and ethanol on IL-17 levels, as we have done in this study. Although we did find that HCV infection was accompanied by an increase in IL-17 levels, no association was found with viral load, in accordance with other authors [13,20]. Similarly, although IL-17 was also increased in alcoholics, we also failed to find any association with daily ethanol intake or duration of alcoholism. Moreover, differences between the group of patients in whom alcoholism and HCV infection coexisted and pure alcoholics and non-alcoholic HCV infected patients were small, probably lacking clinical relevance.

The role of IL-17 in liver disease is still controversial [21]. Most studies have been performed among patients with nonalcoholic fatty liver disease (NAFLD). This entity nowadays constitutes the most common form of liver injury [22]. Both in alcoholic and non-alcoholic patients, fatty liver can be considered as a multifactorial process, in which an initial feature, biochemically characterized by an increased deposition of triglycerides within the hepatocyte triggers a cascade of events ultimately leading to increased inflammation, liver fibrosis, cirrhosis, and hepatocellular carcinoma [23].

The obesity pandemic is strongly related to the increasing prevalence of fatty liver [22]. Adipose tissue is an active endocrine organ, able to secrete proinflammatory cytokines, among other adipokines. Interleukin-6, produced by adipocytes, is a key inducer of the transformation of T-helper naive cells into Th-17, IL-17 secreting cells [24]. This fact probably contributes to the increased expression of IL-17 observed in obese individuals, [25] and it has been postulated that the neutrophils´chemoattractant properties of IL-17 may play a role in the progression of liver steatosis to steatohepatitis [26,27]. Indeed, several authors have reported increased plasma IL-17 levels in obese patients [28], but there are exceptions to this finding. Zapata- Guerrero, et al., studied morbidly obese women and found lower IL-17 levels than in controls [29]. Jung, et al., in 2016 also report lower levels of IL-17 in overweight adolescents than in lean controls [30]. These authors also report lower IL-17 levels in mice in whom obesity was induced by a high fat diet consumption than in controls. These last results are in accordance with what we observed in this study: there was an inverse relationship between total fat and trunk fat, assessed by whole body densitometry, and IL-17

levels. Moreover, there are data that support an inhibitory role of IL-17 on adipogenesis [31].

Therefore we conclude that IL-17 is increased in alcoholic patients and HCV –infected patients, and also when both factors coincide in the same patient. However, this increase is not related to ethanol consumption, viral load, or liver function impairment, but, inversely, with total body fat and trunk fat, a result in accordance with some observations that support an inhibitory role of IL-17 on adipogenesis.

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