Evidence for Increased Inflammatory Cytokine Profile in Hepatitis E Virus-Infected Obese Patients: Implications for Chronic Liver Disease

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Abstract

We aimed to characterize the contribution of hepatitis E virus (HEV) in perpetuating the cytokine-mediated inflammatory setting related to liver damage in the context of obesity. Herein, serum samples from patients with liver disease were retrospectively analyzed and categorized as normal-weight patients (NW), overweight patients (OW), obese patients (ObP), and high alcohol consumer patients (HAC), and biochemical, anthropometrical, and transient elastography measurements were obtained. The positivity for immunoglobulin M (IgM) and immunoglobulin G (IgG) anti-HEV antibodies in samples was determined by enzyme-linked immunosorbent assay. Available samples from ObP were tested by reverse transcription-nested polymerase chain reaction for the presence of HEV-RNA. Cytokine profile in the serum of ObP was identified using a multiplexed immune assay. Globally, the highest frequency of IgG anti-HEV was found in ObP (57.5%), followed by HAC (20%), OW (15%), and NW (7.5%). A strong association between HEV serology and obesity was found (odds ratio=4.21, confidence interval=1.91.9.27) with a cutoff of 29.3 kg/m² (area under curve [AUC]=0–66; $p=0.003$) and, a 23.7% of available samples of ObP provided amplification of HEV genome. Cytokine analysis revealed significantly higher levels of proinflammatory cytokines (interleukin [IL]-12, interferon [IFN]- γ , and IL-1 β) in IgG anti-HEV-positive ObP than in IgG anti-HEV-negative ObP. Moreover, a high proportion of patients with positive serology showed advanced liver damage. In conclusion, the high percentage of anti-HEV antibodies and viral RNA detection in the setting of an excess of fat, along with an associated proinflammatory cytokine profile found in IgG anti-HEV-positive ObP with more severe liver disease, support an interplay between HEV and obesity.

Keywords: hepatitis E virus, obesity, inflammation, chronic liver disease

Introduction

OBESITY IS DEFINED as an excessive or abnormal accumulation of fat. This condition is considered a 21st century epidemic, with a prevalence of 1.9 billion cases worldwide (42).

Obesity is intimately related to chronic, low-grade inflammation generated by pathologic adipose tissue (30). Accumulating evidence suggests that obesity-associated inflammation is a risk factor for other chronic diseases, in-

cluding nonalcoholic fatty liver disease (NAFLD), which in turn predisposes individuals to nonalcoholic steatohepatitis (NASH) and liver fibrosis (32).

In normal physiological conditions, adipose tissue is characterized by the presence of resident immune cells that operate in a T helper (Th) 2 state with a predominant concentration of interleukin (IL)-4, IL-33, IL-10, and IL-13 (1,23). In contrast, excessive accumulation of free fatty acids or pathologic antigen-triggered inflammation is characterized by the release of a large amount of proinflammatory cytokines, such as tumor

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necrosis factor alfa (TNF- α) and IL-1 β (22). Moreover, CD4⁺ T cells are also activated in adipose tissue during inflammatory processes (30).

Antigen-presenting cells have been demonstrated to be able to activate the Th1 phenotype in CD4⁺ T cells (10,43), promoting increased levels of interferon (IFN)- γ and IL-12 (31,39). These cytokines and IL-17F, produced mainly by Th17 cells, are related to the development of chronic inflammatory disease (8,13). Furthermore, the inability of adipose tissue to store energy excess induces fat deposition in the liver, releasing proinflammatory cytokines that activate Kupffer cells from the liver into a proinflammatory stage, inducing genes related to *de novo* lipogenesis, contributing to hepatic steatosis, which is a risk factor for NAFLD and NASH, perpetuating the inflammatory state (22).

Similarly, the liver plays a key role in immune homeostasis. The disruption of cellular homeostasis by inflammation as a consequence of obesity or infectious diseases is a risk factor for liver damage (3). During viral hepatic infection (hepatitis B virus [HBV] or hepatitis C virus [HCV] infections), excess of fat acts synergistically with liver damage progression through chronic inflammation (9). Moreover, during HCV genotype 3 (Gt3) infection, the development of NAFLD is enhanced fivefold in conditions of obesity (26), increasing the concern of liver damage induction in this population, particularly in the setting of secondary viral hepatitis infections.

Hepatitis E virus (HEV) has recently emerged as the leading causative agent of liver disease, with 20 million HEV infections worldwide each year. This virus belongs to the genus *Orthohepevirus* in the *Hepeviridae* family and is endemic in many low-income countries, where it is mainly transmitted through the fecal/oral route by contaminated water (14). In addition, HEV is also recognized as an emerging infection in developed regions, where it has been associated with zoonotic transmission by the consumption of raw pig meat (41).

HEV usually causes self-limiting hepatitis, but in immunocompromised patients, the infection may progress to chronic disease, potentially evolving into liver cirrhosis. In this regard, several reports indicate that in the context of alcoholic or viral hepatitis comorbidities, the infection with HEV promotes the promptly advance of liver fibrosis and rapid decompensated cirrhosis (5,21). Nevertheless, it remains unclear whether HEV may influence the risk of progression to chronic liver disease in obese patients (ObP), and we lack conclusive evidence that would allow us to elucidate the immune mechanisms responsible for this process. Herein, we aimed to study the interplay between HEV infection and cytokine secretion in the context of obesity and liver disease.

Methods

Study population

In this study, 136 serum samples from unrelated patients (>16 years of age) with liver disease, determined by clinical history and biochemical markers as previously reported (38) were retrospectively analyzed. Liver disease was considered when clinical findings, including hepatomegaly and jaundice and at least one abnormality in conjugated bilirubin (CB >0.3 mg/dL), gamma-glutamyl transferase (GGT >30 IU/L), aspartate aminotransferase (AST >38 IU/L), and

alanine aminotransferase (ALT >35 IU/L) values was found. Moreover, taking into account that alcohol consumption is a major etiology for liver damage, high alcohol consumption (>200 g/week) was retrospectively analyzed in accordance with the fifth edition Diagnostic and Statistical Manual of Mental Disorders for Alcohol Use Disorder (29). In addition, total cholesterol (TC) and triglycerides (TG) values were retrospectively analyzed. Participants were recruited at the Centro de Referencia de Hepatitis Virales del Occidente de Mexico in the Servicio de Biología Molecular, Hospital Civil de Guadalajara Fray Antonio Alcalde (HCGFAA), Guadalajara, Jalisco, Mexico, between 2011 and 2016. The study was conducted at the Unidad de Inmunovirología in the Servicio de Biología Molecular, HCGFAA. Serum levels of CB, GGT, AST, ALT, TC, and TG were previously measured using routine clinical laboratory procedures by using an Ortho Chemistry System Clinical Vitros Chemistry System (GMI, Ramsey, MN).

All serum samples analyzed were seronegative for hepatitis A virus (HAV), HBV, HCV, and human immunodeficiency virus (HIV), and negative for HBV and HCV viral genomes. None of the participants included in this study had been vaccinated against HAV or HBV, and none was diagnosed with decompensated cirrhosis as defined previously (37). Patients with liver disease who were undergoing treatment with immunosuppressive or anti-inflammatory drugs; hepatotoxic drugs; those with acute or chronic HAV, HBV, HCV, or HIV infections; and those diagnosed with autoimmune hepatitis were excluded from the study.

This study was approved by the Ethics Committee of the HCGFAA and the Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara (IRB: CI-00215).

Body mass index analysis

The height of patients was previously determined using a stadiometer (Rochester Clinical Research, New York, NY). The body composition was evaluated by electrical bioimpedance with an InBody 3.0 instrument (Analyzer Body Composition; Biospace, Seoul, Korea), which evaluated body fat, total weight, and body mass index (BMI; kg/m²) as previously reported (34). Using the BMI results, we classified the subjects according to the criteria of the WHO for obesity and overweight as follows: normal-weight patients (NW; 18.5–24.9 kg/m²), overweight patients (OW; 25–29.9 kg/m²), and ObP (>30 kg/m²) (42).

Serological diagnosis of HEV

The serum samples were tested for the presence of immunoglobulin M (IgM) and immunoglobulin G (IgG) anti-HEV antibodies using the commercial Enzyme-Linked Immunosorbent Assay Kit following the manufacturer's instructions (MIKROGEN, Neuried, Germany). The final reading was obtained with a WHYM201 microplate reader (Poweam Medical Co., Ltd., Nanjing, China).

Multiplex immunoassay for serum cytokines

The cytokine profile was determined in serum from 46 ObP (23 samples positive and 23 samples negative for the presence of IgG anti-HEV antibodies) and 5 NW (3 samples positive and 2 samples negative for the presence of IgG

anti-HEV antibodies). An 8-plex Kit (IL-1 β , IFN- γ , IL-12p70, IL17F, IL-23, IL-13, IL-4, and IL-10) from Merck Millipore (Darmstadt, Germany) for cytokine detection was used. A fraction of serum samples stored at -80°C until the day of the assay was thawed and processed according to the manufacturer's instructions. Briefly, following plate pre-wetting, interleukin IL-1 β , IFN- γ , IL-12p70, IL17F, IL-23, IL-13, IL-4, and IL-10 cytokine beads were combined and then added to the plate. After the plate was washed twice, 25 μ L of serum from each patient (diluted 1:2 with the assay buffer) was added to the plate. The plate was incubated overnight at room temperature and then the plate was washed twice. Subsequently, detection antibody was added per well and incubated for 30 min at room temperature. Finally, the plate was washed three times, sheath fluid was added to each well, and precombined beads for each cytokine from the plate were read in a MAGPIX machine driven by xMAP[®] Luminex Technology. The exponent software EMD (Merck Millipore) was used for analysis. The detected values were expressed as median fluorescence intensity.

Evaluation of liver stiffness

The liver stiffness in patients was previously assessed by transient elastography (FibroScan[®]; Echosens, Paris, France). Liver fibrosis stages were classified according to the specific cutoff values by each etiology according to the manufacturer's instruction. The cutoff values for patients with liver disease were F0; <5.8 kPa, F1; 5.9–7.0 kPa, F2; 7.1–8.8 kPa, F3; 8.9–11.8 kPa, and F4; >11.9 kPa, and for those patients with high alcohol consumption F0; <5.8 kPa, F1; 5.9–7.8 kPa, F2; 7.9–11.5 kPa, F3; 11.6–19.5 kPa, and F4; >19.6 kPa (34,37).

Nested polymerase chain reaction for HEV RNA detection

RNA was extracted from 300 μ L of patient's serum samples using a QIAamp Viral RNA Mini Kit (QIAGEN) following the manufacturer's specifications.

HEV-RNA detection was carried out with a two-step reverse transcription-nested polymerase chain reaction (RT-nPCR) targeting the ORF-1 region (5' end methyltransferase gene) (40) using 5 μ L of purified RNA. The reverse transcription reaction was carried out in a 12 μ L reaction mix of 1.75 μ M of reverse primer (R: 5'-CCATCRARRCAGTA AGTGCGGTC-3'), 8.5 U of M-MLV RT (Promega), and 870 μ M of dNTP mix (Promega). For the first round of amplification of nPCR, a total volume of 20 μ L was used. The mix contained 4 μ L of the complementary DNA, 250 μ M of dNTP mix, 0.25 μ M of each primer (F: 5'-CTGGCATYACTACTGTCYATTGAGC-3'), and 2.5 U Taq Polymerase. Thirty-five cycles of 94°C for 1 min, 48°C for 45 sec, and 72°C for 1 min, with a final extension of 72°C for 10 min, were used for amplification. For the second round, 2 μ L of the first round was used under the same conditions as the first round and 0.25 μ M of each primer (F: 5'-CTGCCYTKGCGAATGCTGTGG-3' and R: 5'-GGCA GWRTACCARCGCTGAACATC-3'). The first-round external primers amplified 418 bp, and the second-round internal primer amplified 287 bp.

Statistical analyses

The description of the categorical (qualitative) variables is represented by frequencies and percentages. A Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. The continuous (quantitative) variables are summarized using the mean $\pm$ standard deviation. Differences between groups were assessed with Student's *t*-test and analysis of variance for variables normally distributed and with nonparametric tests, Mann-Whitney U and Kruskal-Wallis, for variables not normally distributed. Statistical associations were analyzed by Chi-square and Fisher's test to compare proportions. The results of the previous analysis were used for univariate analyses as appropriate. Receiver operating characteristic (ROC) curve analysis was used to establish the cutoff point that best distinguished the serology result. Spearman analysis was used for statistical correlations. A *p*-value <0.05 was considered statistically significant. All analyses were performed using the program IBM SPSS statistics, version 24 for Windows (IBM Corp, Inc., Chicago, IL).

Results

Demographic and clinical data of the study population

As shown in Table 1, samples were mostly obtained from male patients. The mean age of patients was 40.88 $\pm$ 12.55 years. Based on the criteria mentioned above relative to BMI, globally, the cohort was classified as overweight. In addition, a high percentage of subjects were high alcohol consumer patients (HAC). Even though the mean GGT, ALT, AST, and CB presented values in the normal range, the subjects individually had altered values, indicating liver

TABLE 1. DEMOGRAPHIC AND BIOCHEMICAL CHARACTERISTICS OF PATIENTS

Variable	Reference values (29,34,42)	Patients, mean $\pm$ SD (n = 136)
Sex % (M/F)	—	63.2/36.8
Age (years)	—	40.88 $\pm$ 12.55
CB (mg/dL)	<0.3	0.14 $\pm$ 0.24
GGT (IU/L)	<30	33.54 $\pm$ 29.88
ALT (IU/L)	7–40	32.51 $\pm$ 20.66
AST (IU/L)	10–38	30.45 $\pm$ 15.76
TC (mg/dL)	<200	199.67 $\pm$ 36.43
TG (mg/dL)	<150	198.29 $\pm$ 116.50
BMI (kg/m ²)	<25	28.78 $\pm$ 4.91
		n (%)
NW (kg/m ²)	18.5–24.9	15 (11)
OW (kg/m ²)	25–29.9	34 (25)
ObP (kg/m ²)	>30	46 (33)
Alcohol consumption (g/week)	<200	629.92 $\pm$ 421.97
HAC	—	41 (30)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CB, conjugated bilirubin; F, female; GGT, gamma-glutamyl transferase; HAC, high alcohol consumer patients; M, male; NW, normal-weight patients; ObP, obese patients; OW, overweight patients; SD, standard deviation; TC, total cholesterol; TG, triglycerides.

insult in this cohort. In addition, TC and TG were close to reaching the abnormally increased range supporting metabolic disorders in patients.

Highly frequent IgG anti-HEV antibodies in obese individuals

We previously reported a high HEV seroprevalence (26.5%) in serum samples from cirrhotic patients from west Mexico with no other etiological agent present, suggesting the potential role of HEV in the development of liver damage (28). This piece of information led us to compare the IgM and IgG anti-HEV frequency in serum samples from patients with liver disease. The cohort presented an IgM anti-HEV prevalence of 1.47% ($n=2$), and 40 patients (29.4%) presented IgG anti-HEV antibodies (Fig. 1A). These results suggest that either (1) the underlying liver damage represents a risk factor for HEV infection or (2) liver disease might be associated with HEV infection in these patients.

Data from a previous study by our group revealed an IgG anti-HEV seroprevalence of 17% in individuals with obesity and diabetes mellitus type 2 (28). However, little is known about the role of HEV in the obese state. Given the high incidence of obesity in Mexico (12) and considering that this condition is a recognized risk factor for NAFLD and thus a predisposing factor for NASH, we hypothesized that HEV might play a role in the development of liver disease in conditions of excess of fat. To test this hypothesis, we evaluated IgM and IgG anti-HEV frequencies in patients categorized according to their BMI. In addition, because high alcohol consumption is a major etiology for liver damage (16), the HEV seroprevalence according to alcohol consumption pattern was also analyzed. Of the patient groups examined, ObP showed the highest proportion of IgG reactivity to HEV

in the overall analysis, followed by HAC, OW, and NW with 57.5% ($n=23$), 20% ($n=8$), 15% ($n=6$), and 7.5% ($n=3$), respectively. Furthermore, when the groups were analyzed independently, ObP exhibited a significantly higher IgG anti-HEV prevalence compared with the patients in the other groups (ObP vs. NW, $p=0.005$; ObP vs. OW $p=0.001$; ObP vs. HAC, $p=0.005$). No significant differences were found among NW, OW, and HAC. ObP and HAC presented only one patient with IgM anti-HEV antibodies (Fig. 1B).

Because of the high seroprevalence of IgG found in ObP, we hypothesized a potential association between obesity and HEV infection. To test this hypothesis, patients were categorized into NW/OW and ObP to estimate the influence of this condition on HEV seropositivity, grouped as IgG anti-HEV negative or positive. The univariate analysis showed that obesity was associated (odds ratio [OR]=4.21, confidence interval [CI]=1.91–9.27; $p<0.001$) with increased HEV seropositivity. The ROC curve analysis for BMI identified 29.3 kg/m² (area under curve [AUC]=0.66; $p=0.003$) as the cutoff value that best distinguished IgG anti-HEV positive from negative.

A direct relationship between liver damage and excess fat has been well established. To analyze whether our population follows the same behavior, a comparison of the demographic and biochemical variables among the stages of liver damage was carried out but did not reveal significant differences. Moreover, of 134 patients with available transient elastography data, 68 (50.7%) had no liver fibrosis and 19 (14.2%), 20 (14.9%), 12 (9%), and 15 (11.2%) were in stage F1, F2, F3, and F4, respectively. However, after subcategorization according to BMI and alcohol consumption, the highest proportion of patients with liver damage in the F4 stage was found among HAC (20%), followed by ObP, OW, and NW with 8.9%, 8.8%, and 0%, respectively, but no significant differences were found (data not shown).

FIG. 1. Seroprevalence of anti-HEV antibodies in patients with liver disease. (A) A total of 136 serum samples from patients were analyzed for anti-HEV IgM and IgG antibody determination. (B) ObP showed a high frequency of anti-HEV IgG antibodies; the total number of patients included in this study was grouped by BMI (NW, $n=15$; OW, $n=34$; ObP, $n=46$) and high alcohol consumption ($n=41$) and analyzed for proportion differences. Differences between groups were determined by chi-square and Fisher's test with an $\alpha<0.05$. * $p<0.05$; ** $p<0.01$. BMI, body mass index; HAC, high alcohol consumer patients; HEV, hepatitis E virus; IgG, immunoglobulin G; IgM, immunoglobulin M; NW, normal-weight patients; ObP, obese patients; OW, overweight patients.

TABLE 2. DEMOGRAPHIC AND BIOCHEMICAL CHARACTERISTICS OF PATIENTS ACCORDING TO THE BODY MASS INDEX AND ALCOHOL CONSUMPTION

	NW, mean $\pm$ SD (n = 15)	OW, mean $\pm$ SD (n = 34)	ObP, mean $\pm$ SD (n = 46)	HAC, mean $\pm$ SD (n = 41)	p
Sex: M/F (%)	60/40	70.6/29.4	69.6/30.4	51.2/48.8	—
Age (years) ^{a-c}	30.93 $\pm$ 11.29	41.62 $\pm$ 11.69	42.28 $\pm$ 11.85	42.32 $\pm$ 13.18	0.01
CB (mg/dL)	0.13 $\pm$ 0.09	0.08 $\pm$ 0.07	0.10 $\pm$ 0.06	0.20 $\pm$ 0.37	N/S
GGT (mg/dL) ^{a-c}	15.18 $\pm$ 5.74	24.52 $\pm$ 14.85	37.79 $\pm$ 31.94	42.17 $\pm$ 36.94	<0.001
ALT (UI/dL) ^{a-d}	19.50 $\pm$ 8.45	28.64 $\pm$ 15.00	40.77 $\pm$ 24.42	31.11 $\pm$ 20.19	<0.001
AST (UI/dL)	27.21 $\pm$ 13.76	29.33 $\pm$ 13.22	33.23 $\pm$ 16.69	29.51 $\pm$ 17.30	N/S
TC (mg/dL)	190.40 $\pm$ 33.12	207.65 $\pm$ 37.50	200.11 $\pm$ 29.34	195.54 $\pm$ 43.84	N/S
TG (mg/dL)	171.73 $\pm$ 88.73	196.94 $\pm$ 146.70	197.89 $\pm$ 106.28	210.78 $\pm$ 109.73	N/S
BMI ^{c,f}	22.87 $\pm$ 1.68	27.68 $\pm$ 1.38	33.53 $\pm$ 3.25	26.33 $\pm$ 4.56	<0.001

Differences between groups were analyzed by Kruskal–Wallis test and Mann–Whitney U-test for *post hoc* differences with an $\alpha = <0.05$. Statistically significant values are shown in bold.

^aNW/OW; ^bNW/ObP; ^cNW/HAC; ^dObP/OW; ^eOW/HAC; ^fObP/HAC.

ALT, alanine aminotransferase.

Likewise, when the biochemical variables were compared according to the subcategorization by BMI and alcohol consumption, we observed that hepatic biomarkers as GGT and ALT were significantly increased in Ob and HAC groups (Table 2); nonetheless, the levels of ALT remain in the limit of the normal ranks and GGT levels were slightly increased.

HEV serology correlates with a proinflammatory cytokine profile in ObP

Obesity is a condition that predisposes individuals to a proinflammatory state, promoting an increased risk for liver damage in the presence of a pathogen trigger. To evaluate whether HEV influences the immune inflammatory state during obesity, a cytokine profile evaluation in ObP was performed. Compared with ObP negative for IgG anti-HEV, ObP seropositive for IgG anti-HEV exhibited a different cytokine pattern.

Significantly higher levels of proinflammatory cytokines IL-12, IFN- γ , and IL-1 β were found in IgG anti-HEV-positive ObP than in IgG anti-HEV-negative ObP. In contrast, those ObP with negative detection of IgG anti-HEV showed significantly higher levels of cytokines IL-4, IL-10, and IL-13. IL-17F exhibited a trend toward an increase in HEV-positive patients, whereas IL-23 had no significant differences. A trend toward increased levels of IL-12, IFN- γ , and IL-1 β was found in NW positive for IgG anti-HEV relative to individuals negative for antibodies (Fig. 2 and Supplementary Table S1).

Because the cytokine profile can differentiate the HEV contact, quantitative correlations between the cytokines and the optical density (OD) for IgG anti-HEV antibodies were analyzed. Positive linear correlations were found between OD values and IL-12, IFN- γ , and IL-1 β levels, whereas negative correlations were exhibited with IL-13 and IL-4. However, the R^2 value of the data was low, indicating low linearity. Moreover, when the correlation data of the cytokines from the NW group was evaluated, we observed a positive linear correlation among IL-1 β and IFN- γ (Table 3).

Liver damage in IgG anti-HEV-seropositive patients

To analyze the influence of the IgG seropositivity for HEV in liver damage, a comparison of the liver biomarkers was carried out according to the serology results. The patients positive for IgG anti-HEV were significantly older than those nonreactive (46.08 $\pm$ 11.39 vs. 38.71 $\pm$ 12.43, $p = 0.002$). A univariate analysis showed an OR = 1.05 (CI = 1.01–1.09; $p = 0.008$) for HEV infection and age. Moreover, ROC curve analysis identified 40.5 years (AUC = 0.67; $p = 0.002$) as the cutoff value that best distinguished IgG anti-HEV positive from negative. It is important to point out that, considering this cutoff value, the BMI did not show significant differences in this cohort ($p > 0.2$). Additionally, patients who were IgG anti-HEV reactive had increased levels of GGT and AST (40.41 $\pm$ 34.53 vs. 30.83 $\pm$ 27.60, $p = 0.033$; 32.63 $\pm$ 13.00 vs. 29.52 $\pm$ 16.78, $p = 0.049$), suggesting that patients with IgG anti-HEV antibodies might have a worse liver condition than those without these antibodies. Moreover, the mean comparison of the BMI demonstrated that patients positive for HEV were mostly obese (30.5 $\pm$ 4.57 vs. 28.07 $\pm$ 4.89, $p = 0.003$) (Table 4). The demographic and biochemical comparisons between the seroprevalence results according to the BMI and alcohol consumption subcategorization did not show significant differences.

To test if the increase in liver biomarkers was reflected in the fibrosis stage, an analysis of the proportion of IgG anti-HEV-positive samples according to the stage of liver damage is shown in Figure 3A and B. In this study, 52.5% of patients with IgG anti-HEV antibodies had liver damage; similarly, 50% of those with negative results for IgG anti-HEV antibodies had liver damage. Interestingly, HEV contact showed a trend toward an increased proportion of patients in stage F4 liver damage, at a proportion of 15% compared with 9.5% in those with no HEV contact, whereas patients negative for IgG anti-HEV antibodies exhibit a trend toward an increased proportion of patients in stage F3 relative to HEV-positive patients. Moreover, liver damage analysis according to BMI and alcohol consumption showed a higher proportion of those with stage F4 liver damage

FIG. 2. HEV serology was correlated with a proinflammatory cytokine profile in serum. (A) Heatmap showing the analysis of cytokine profile determination from HEV-negative ($n=23$) and HEV-positive ($n=23$) serum samples from ObP by MAGPIX technology. Moreover, an $n=2$ of HEV-negative and $n=3$ of HEV-positive NW group were also included in the analysis. Comparative analysis was carried out with Mann-Whitney U with an $\alpha=<0.05$. * $p<0.05$; *** $p<0.001$. (B) Venn-Euler diagram summarizing the differential cytokine profile in ObP according to the serological result.

among IgG anti-HEV-positive patients than among patients overall and those nonreactive for HEV; however, these differences were not statistically significant (Fig. 3C). In addition, statistical associations among risk factors for liver damage and liver fibrosis were not significant (data not shown). Furthermore, screening of the 5'-end ORF-1 region of HEV-RNA in 38 IgG anti-HEV-positive ObP with available serum samples showed that 23.7% ($n=9$) were positive for HEV-RNA, which indicates an active HEV infection (data not shown).

Discussion

To our understanding, this is the first report that provides evidence of a relationship between HEV infection and obesity. A meta-analysis of the country-level socio-epidemiological factors associated with IgG anti-HEV seroprevalence in north and south America (15) revealed a negative correlation between BMI and the presence of HEV serology; however, these results did not reach statistical significance. Furthermore, the analysis was only conducted

TABLE 3. QUANTITATIVE CORRELATIONS AMONG CYTOKINES MEDIAN FLUORESCENCE INTENSITY AND OPTICAL DENSITY

Cytokines	ObP ($n=46$)			NW ($n=5$)		
	Correlation coefficient	R ²	p	Correlation coefficient	R ²	p
IL-10	-0.180	0.07	NS	-0.671	0.58	NS
IL-4	-0.361	0.09	0.016	-0.3	0.02	NS
IL-13	-0.332	0.05	0.028	0.9	0.75	0.037
IL-23	-0.362	0.05	0.015	-0.8	0.72	NS
IL-17F	-0.063	0.01	NS	0.1	<0.001	NS
IL-12	0.537	0.29	<0.001	0.7	0.5	NS
IFN- γ	0.412	0.13	0.005	0.9	0.77	0.037
IL-1 β	0.315	0.07	0.037	0.9	0.79	0.037

Spearman analysis was carried out with an $\alpha=<0.05$. Statistically significant values are shown in bold. IFN, interferon; IL, interleukin; NS, nonsignificant.

TABLE 4. DEMOGRAPHIC AND BIOCHEMICAL CHARACTERISTICS OF PATIENTS ACCORDING TO IMMUNOGLOBULIN G SEROLOGY

Variable	HEV-, mean $\pm$ SD (n=96)	HEV+, mean $\pm$ SD (n=40)	p
Sex (M/F)	59.4/40.6	72.5/27.5	NS
Age (years)	38.71 $\pm$ 12.43	46.08 $\pm$ 11.39	0.002
CB (mg/dL)	0.15 $\pm$ 0.28	0.13 $\pm$ 0.07	NS
GGT (IU/L)	30.83 $\pm$ 27.60	40.41 $\pm$ 34.53	0.033
ALT (IU/L)	30.64 $\pm$ 20.53	36.7 $\pm$ 20.58	0.0830
AST (IU/L)	29.52 $\pm$ 16.78	32.63 $\pm$ 13.00	0.049
TC (mg/dL)	197.95 $\pm$ 34.24	203.77 $\pm$ 37.35	NS
TG (mg/dL)	199.06 $\pm$ 118.75	196.44 $\pm$ 112.45	NS
BMI	28.07 $\pm$ 4.89	30.5 $\pm$ 4.57	0.003
Alcohol consumption (g/week)	684 $\pm$ 472.13	665.29 $\pm$ 387.60	NS

Differences between groups were analyzed by *t*-test and Mann–Whitney U-test with an $\alpha = <0.05$. Statistically significant values are shown in bold.

HEV, hepatitis E virus.

among overweight and obese individuals, and no comparisons were performed between normal-weight and obese populations. In addition, a recent report revealed a high prevalence of NAFLD in patients with anti-HEV IgG antibodies in China (35). Although other studies have evaluated the relation of these variables, with no significant differences, these studies have not been conducted in the context of obesity (11,17) or were carried out with a resulting low (2.6%) HEV seroprevalence (20) compared with the present study (29.4%).

Herein, a strong association between obesity and the HEV seroprevalence was found, identifying a BMI cutoff of 29.3 kg/m² that differentiates the anti-HEV reactivity. Interestingly, a high percentage of ObP was reactive to IgG anti-HEV (57.5%). This may be explained by socioeconomic

characteristics of the analyzed population living in west Mexico, a region where a high IgG anti-HEV seroprevalence has been reported in swine samples suggesting a potential zoonotic transmission of the infection (24). However, future studies analyzing HEV strains from animal and human sources from this geographical region may support this possibility. In addition, as previously reported (4), in the present study the highest frequency of anti-HEV antibodies was found in older patients supporting that age and lifestyle might predispose for the infection.

A synergistic effect on liver inflammation by obesity and viral infection has been described, as in chronic HCV-infected patients, and this inflammation is related to increased hepatic fibrosis (18). A higher intrahepatic

FIG. 3. An increased proportion of IgG anti-HEV-seropositive patients presented advanced liver damage. Available data of transient elastography from 134 patients were included for analysis. Statistical differences were analyzed by Chi-square with an $\alpha < 0.05$. (A, B) Proportion analysis of the liver damage stage according to IgG anti-HEV serology. (C) Comparison of the proportion of patients in liver damage stages according to BMI/alcohol consumption and IgG anti-HEV serology.

concentration of proinflammatory cytokines (as IFN- γ) has been previously reported in patients with a BMI $>30\text{ kg/m}^2$ during chronic infection with HCV (27). In fact, during the liver fibrosis process, systemic inflammation is present in patients and is characterized by the increased production of activation antigens in T cells accompanied by the augmentation of IFN- γ and IL-17 (2,3,25). In the present study, a proinflammatory cytokine profile (elevation of IL-12, IFN- γ , and IL-1 β) was found in ObP reactive to IgG anti-HEV; also, a trend of a positive correlation between the OD for IgG anti-HEV and these cytokines was observed but with low linearity. The cytokine profile from patients nonreactive to IgG was more likely to be present in the lean population or obese people with nonpathologic adipose tissue, characterized by the presence of IL-4, IL-13, and IL-10. It is important to note that no high alcohol consumers were included in this analysis; therefore, alcohol did not influence the inflammatory process found. Moreover, a trend toward increased levels of IL-12 IFN- γ , and IL-1 β in NW reactive to IgG anti-HEV relative to NW patients nonreactive to IgG supporting that in the context of obesity HEV promotes an exacerbated proinflammatory state.

Recent studies have revealed that patients exposed to HEV (IgG anti-HEV positive) present an upregulation of proinflammatory cytokines IFN- γ and IL-12 (7,36); in fact, this inflammation is present even once the infection was resolved (33). Thus, inflammation due to HEV exposure and the well-documented systemic inflammation during chronic liver disease and NAFLD in obesity (1,2) might create a vicious circle that contributes to the progression of fibrosis in HEV-positive patients, resulting in the greater percentage of patients with advanced liver damage observed in this study. Herein, patients who were IgG anti-HEV reactive had increased levels of liver enzymes suggesting a more altered liver dysfunction than those without these antibodies. Moreover, although patients nonreactive to IgG showed a trend toward an increased proportion of patients in stage F3 liver damage, those individuals positive for IgG anti-HEV antibodies exhibit a trend toward an increased proportion of patients in stage F4 suggesting a more accelerated progression toward liver disease in the context of HEV infection. However, future prospective studies are required to evaluate this possibility.

Genomic variability influences the HEV outcomes. The HEV causing chronic liver disease is predominantly HEV Gt3 (19). Although this viral genotype is mostly related to chronic liver damage in immunosuppressed patients, HEV-Gt3 chronic liver infection in an apparently immunocompetent patient has been recently reported in an obese individual with liver cirrhosis (6). In this study, viral RNA was detected in 23.7% of obese individuals but genomic variability was not determined due to the limited volume of samples available. However, taking into account that we previously reported the circulation of HEV-Gt3 in patients with chronic liver disease from the same geographical region (38), our study underscores that the genetic characterization of circulating HEV strains in risk groups, including the obese population, is necessary for handling the infection on specific geographic regions.

In conclusion, the high percentage of ObP with antibodies against HEV and viral RNA detection, along with an asso-

ciated proinflammatory cytokine profile found in this study, support an interplay between HEV and obesity. Although the nature of our study does not make it possible to establish which one is a risk for the development of the other (obesity as a risk factor for HEV infection or HEV infection causing obesity); the information obtained in this study, will help to elucidate the inflammatory machinery involved in the development of chronic liver disease during HEV in the setting of the obesity pandemic.

Authors' Contributions

O.V.-S.: main author; processing of samples; analysis and interpretation of data; drafting and critical revision of the article. A.P.: coordinator of the Viral Hepatitis Clinic; medical liver disease assessment; acquisition of samples; clinical database files; critical revision of the article. J.L.T.-O.: counseling in the cytokine profile determination; interpretation of the immunity data; critical revision of the article. E.C.-V.: serology analysis for IgM and IgG anti-HEV determination. R.T.-V.: acquisition of samples; serum bank organization for patients with high alcohol consumption; determination of alcohol consumption; analysis of liver damage data; critical revision of the article. M.S.-V.: acquisition of samples; serum bank organization for ObP; anthropometric analysis; critical revision of the article. S.R.: serum bank organization for patients with obesity and high alcohol consumption; acquisition of samples; clinical database files; critical revision of the article. N.A.F.: study concept and design; critical revision of the article for important intellectual content.

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Author Disclosure Statement

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Supplementary Material

Supplementary Table S1

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