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Occult Hepatitis C Virus Infection in Patients with Diabetic Nephropathy: Epidemiological and Clinical Implications

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***Abstract*— Background:A high prevalence of hepatitis C virus (HCV) infection in patients with diabetic kidney disease (DKD) has been reported. However, the epidemiology and relationship between DKD and occult HCV infection (OCI)are unknown.Objetives: To determine the prevalence of OCI in a population without conventional markers of HCV infection diagnosed with DKD, and to study its possible clinical implications.Study design: This prospective study included 125 anti-HCV and serum HCV-RNA-negative patients with DKD for the presence of OCI. HCV-RNA was tested by real-time reverse transcription PCR in peripheral blood mononuclear cells and in plasma after ultracentrifugation. Results: OCI was positive in 10 patients (8%). The patients with OCI had significantly higher ferritin levels (p=.002) and monoclonal gammopathy (30% [3/10] *vs.* 0.87% [1/115] than the patients without OCI [p=.003]). We found similar plasma ALT and GGT levels and HbA1C in both groups. At the end of the follow-up, the progression rate of renal disease tended to be faster in the group with OCI relative to the negative one, but without a significant difference. We did not find an association between OCI and cardiovascular morbidity. Conclusions: There was an 8% prevalence of OCI in patients with chronic renal failure secondary to DKD, higher than in the general population. This occult infection does not appear to play a role in the control of diabetes, cardiovascular risk or steatosis. However, the progression rate of renal disease tended to be faster, and the incidence of associated monoclonal gammopathy was significant.**

Index Terms— Diabetes Kidney Disease, Type 2 diabetes mellitus, Occult hepatitis C virus.

I. INTRODUCTION

Type 2 diabetes mellitus (T2DM) and hepatitis C virus (HCV) are two major public health problems worldwide. Previous reports have shown a high prevalence of HCV infection in patients with diabetic kidney disease (DKD) [1,2]. The association between these two diseases is a matter of debate [3].On one hand, this association is supported by two meta-analyses [4,5],longitudinal studies, and by described pathophysiological mechanisms by which HCV infection causes insulin resistance [6–10]. Both entities share metabolic complications such as resistance to insulin, fatty liver, subclinical inflammation and perhaps cardiovascular disease, resulting in a significant increase in morbidity and premature mortality [3]. On the other hand, a recent cohort study of 15,128 American patients (NHANES) has challenged this dogma because it found that the prevalence of diabetes or prediabetes was not associated with HCV infection status[11].

Occult HCV infection (OCI) is characterized by the presence of HCV-RNA in the liver or in peripheral blood mononuclear cells in the absence of serological markers [12]. Complementary studies have demonstrated that OCI can also be diagnosed by concentrating 2ml of serum by ultracentrifugation, followed by HCV-RNA detection by real-time PCR [13]. OCI, as a state of nonapparent but real HCV infection, could explain these differences between HCV infection and T2DM. Furthermore, our group has demonstrated an association between OCI and a large spectrum of immune-mediated glomerular diseases, including a role in the progression of the disease [14].

II. OBJETIVES

We have aimed to better understand the epidemiological and clinical implications of OCI in a population of T2DM and DKD. We also reviewed the progression of DKD according to OCI status, and its association with steatosis, diabetes control and cardiovascular complications.

A. Study design

This prospective study included 125 adult patients visiting the nephrology departmentdue to DKDbetween 2012 and 2013. Diabetes was defined using the standard American Diabetes Association criteria, and was confirmed by laboratory testing. DKD was characterized as long-term diabetes with albuminuria (ratio of albumin to creatinine >30 mg/g), impaired renal function (estimated glomerular filtration rate [eGFR] <60 ml/minute per 1.73 m2), or both. Other nephropathies were ruled out by an expert

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nephrologist. All the included patients tested negative for anti-HCV, hepatitis B surface antigenand anti-HIV using a routine commercial test (INVITROS Anti-HCV Assay, Ortho Clinical Diagnostics, Raritan, NJ; Enzygnost hepatitis B surface antigen 5.0and Enzygnost Anti-HIV ½ Plus Siemens Healthcare Diagnostics, Marburg, Germany).

The study was approved by the Research Ethics Committee of University Hospital La Paz and was conducted according to the Declaration of Helsinki. Each patient provided written informed consent and was then tested for occult HCV infection. An average follow-up of 17.5 ± 9 months was used to explore outcomes of kidney function and incidence of cardiovascular complications. Demographic, epidemiological, clinical and laboratory parameters of the 125 patients with DKD at OCI testing are shown in Table 1.

Hematuria was defined as a red blood cell count greater than 5 per high-power field in urinary sediment. Waist circumference ≤102 cm for men and ≤88 cm for women were considered normal. Previous blood transfusions, surgical procedures, household contacts and partners diagnosed with HCV infection, as well as the presence of a tattoo or piercing were recorded as potential risk factors for HCV infection

Table I: Demographic and clinical characteristics of the patients at inclusion.

Age (years; mean ± SD)	73.7±10.04
Male (n; %)	115; 92
Type of diabetes: (n; %)	
I	10; 8
II	115; 92
Risk Factors for HCV infection: (n; %)	
Previous blood transfusion	35; 28
Tattoo/drug addiction	2; 1.6
Household HCV contact	2; 1.6
HBV infection (n; %)	9; 15
ALT (IU/L; mean ± SD)	24.3 ± 23.4
AST (IU/L; mean ± SD)	43.2 ± 22
GGT (IU/L; mean ± SD)	47.8 ± 66.7
Creatinine (mg/dl)	1.91 ± 0.83
Creatinine clearance (ml/min per 1,73 m^2)	44.2 ± 26.8
Proteinuria (g/24h)	1.24± 1.86

HBV: hepatitis B virus; ALT: alanine transaminase; AST: aspartate transaminase; GGT: gamma-glutamyl transferase

B. HCV-RNA detection

Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated blood samples collected from all patients at the time of inclusion in the study. The plasma samples were stored at -80°C and the PBMCs at -20°C in RNAlater solution (Ambion, Austin, TX) until detection of HCV-RNA.

Two milliliters of plasma were ultracentrifuged over a 10% sucrose cushion for 17 h at 100,000 x g and 4°C to concentrate HCV particles [13]. The pellet was dissolved in TE buffer (Tris-HCl 10 mM, EDTA 10 mM; pH 7.5). Total RNA was isolated with the Trizol LS Reagent (Invitrogen, Carlsbad, CA), then precipitated, and the RNA pellet was dissolved in diethyl-pyrocarbonate-treated water. Total RNA from the PBMCs was isolated with SV Total RNA Isolation System (Promega, Madison, WI). After precipitation, the pellets were dissolved in diethyl-pyrocarbonate-treated water, and the RNA concentration was determined by spectrophotometry.

Detection of HCV-RNA was performed using 5 µl of total RNA isolated from 2ml of ultracentrifuged plasma or using 0.5 µg of total RNA from PBMCs by real-time reverse transcription polymerase chain reaction (RT-PCR) with the Tth enzyme for cDNA synthesis as described [13]. Real-time PCR was performed with FRET probes in a LightCycler (Roche Diagnostics, Mannheim, Germany) with 2 µl of cDNA in a final volume of 20 µl, using the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics), as reported [13]. For HCV-RNA quantification, a standard curve constructed with 10-fold dilutions of a synthetic HCV-RNA was used. The sensitivity of this assay was of three HCV-RNA copies per reaction [13].

Negative controls (repeated HCV-RNA-negative plasma and PBMC samples from four healthy volunteers) and reagent blanks, in which total RNA was replaced with PCR-grade water, were included in each PCR run. All these negative controls were coprepared with the samples and accompanied the samples through the entire RT-PCR process.

C. Statistical analysis

The categorical variables were compared using the chi-squared test or Fisher's exact test, as appropriate. The continuous variables were compared using Student's t-test or the Mann-Whitney U-test.

III. RESULTS

Of the 125 studied patients, 7tested positive for the presence of HCV-RNA in PBMCs, with a median concentration of 1980 HCV-RNA copies per µg total RNA (range 110–4820). In addition, HCV-RNA was detected in plasma after ultracentrifugation of 2 ml of plasma in another three patients at concentrations of 120, 135 and 140 HCV-RNA copies per ml plasma. No patient was simultaneously positive for HCV-RNA in PBMCs and in plasma after ultracentrifugation. Thus, we found a prevalence of OCI in our series of 10/125 (8%); these patients had detectable viral RNA in PBMCs or in plasma after ultracentrifugation despite the absence of anti-HCV antibodies.

Table 2 displays the demographic characteristics, clinical features and cardiovascular risk factors of the two groups of patients with DKD according to the presence or not of OCI. Most of the variables were similar.

Table II: Demographic, clinical and analytical data according to occult negative/positive HCV infection.

	Occult **negative** HCV infection (n = 115)	Occult **positive** HCV infection (n=10)	Signif
Age (years; mean ± SD)	73.7 ± 10	74.9 ± 8.6	
Male (n; %)	78; 70.9	60; 61. 3	
Risk factors for acquisition of HCV (%)	1.4	1.1	
Smoking (n; %)	7; 11.8	2; 9.5	
Systolic blood pressure (mmHg)	134.6 ± 12.1	135.5 ± 22.6	
Diastolic blood pressure (mmHg)	64.0 ± 17.9	66.8 ± 10.9	
BMI (kg/m^2)	31.7 ± 6.5	30.8 ± 6	
Waist circumference (cm)	114.5 ± 13.3	107.1 ± 14	
Cholesterol(mg/dl)	140.4 ± 27.1	147.6 ± 33.1	
HbA1C (%)	7.1 ± 1.31	6.6 ± 0.70	
Myocardial infarction (n; %)	23; 20.9	2; 20	
Cerebrovascular ischemia (n; %)	12; 10.9	1; 10	

HCV: hepatitis C virus; BMI: body mass index; HbA1c: hemoglobin glycosylate.

At the beginning of the follow-up, plasma levels of liver enzymes, renal function and proteinuria were the samefor both groups; however, a significantly higher plasma ferritin level (mean ± SD: 325.8 ± 194.4 ng/ml) in patients with OCI was demonstrated (patients with no OCI showed 139.1 ± 136.8 ng/ml; p=.002). The number of patients who reported risk factors for acquisition of HCV infection (blood transfusions, tattoos or piercings, or household contacts with a chronic HCV carrier) was similar in both groups.At the end of the follow-up (mean ± SD:17.5 ± 9 months), there was no difference in plasma levels of liver enzymes, diabetes control, cardiovascular events (myocardial infarction or cerebrovascular ischemia), renal function and proteinuria, as shown in Table 3.

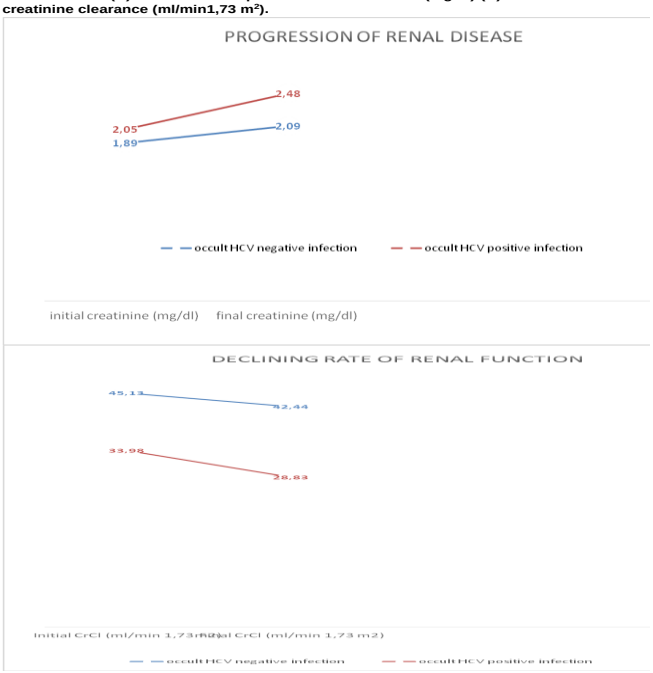
le III: Kidney and liver parameters at the end of the follow-up.

	Occult **negative** HCV infection (n=115)	Occult **positive** HCV infection (n=10)	Significance
Serum creatinine (mg/dl; mean ± SD)	2.1 ± 1.1	2.5 ± 1.3	0.282
CrCl (ml/min per 1.73 m^2; mean ± SD)	42.4 ± 23.4	28.8 ± 15	0.076
Proteinuria (gr/24 h; mean ± SD)	1.3 ± 1.8	0.5 ± 0.5	0.179
Hematuria (n; %)	13; 11.8	1; 1	0.67
ALT (IU/L; mean ± SD)	22.1 ± 11.4	16.6 ± 4.5	0.136
GGT (IU/L; mean ± SD)	50.9 ± 39.9	29.7 ± 24.2	0.271
Ferritin (ng/ml; mean ± SD)	150.2 ± 130.8	163.2 ± 81.4	0.02*

ALT: alanine aminotransferase
GGT: gamma-glutamyl transpeptidase

Although there were no significant differences in the liver and kidney parameters, the renal function decline rate tended to be more rapid in the group with OCI, as demonstrated by plasma creatinine and clearance values (Figure 1).

Figure 1. Evolution of renal function in patients with occult negative/positive HCV infection. (A) Initial and final plasma creatinine levels (mg/dl) (B) Initial and final creatinine clearance (ml/min1,73 m^2).



We did not identify an association between occult HCV infection and cardiovascular morbidity, but interestingly, we found that the patients with occult HCV infection had an incidence of 30% (3/10) monoclonal gammopathy vs. 0.87% (1/115) of the patients without OCI (p=.003), over the follow-up.

IV. DISCUSSION

OCI is a situation characterized by the absence of serological markers and the presence of HCV-RNA in the liver or in PBMCs. The presence of OCI has been documented in various populations, including patients with immune-mediated glomerulonephritis, cryptogenic hepatitis, NAFLD (non-alcoholic fatty liver disease) and lymphoproliferative disorders, as well as those undergoing hemodialysis, HIV-infected patients, drug users andthe healthy population [12].

Previous studies from our group had reported a high prevalence of OCI in groups of chronic kidney disease patients (hemodialysis 45%, immune-mediated glomerulonephritis 39%) [14, 15].The prevalence of anti-HCV in Spain is 1.7 % (0.4%–2.6%) [16]. HCV infectiondetected by conventional serological markers is known to be highly prevalent in patients with DKD, reaching 19.5% [17,18]. On these bases, this prospective study aimed to determine the prevalence of OCI and the possible clinical implications in a population of patients with DKD.

The prevalence of OCI found in patients with DKD in our study was 8%, as demonstrated by the detection of HCV-RNA in PBMCs or in plasmaafter ultracentrifugation—higher than that found in the group with hereditary glomerulonephritis (3.8 %) [14]. In our study, risk factors for HCV infection were greater than those previously found in hereditary nephropathies (4%), and less than those described in immune-mediated glomerulonephritis.

Chronic HCV infection is recognizably associated with liver steatosis [19]. Both HCV and metabolic syndrome are frequent disorders, and there is the possibility of overlapping.

We did not find any difference in the liver parameters according to the presence of OCI.The only parameter we found with significant differences was serum ferritin,which could reflect liver inflammation.

The overweight patients were encouraged to reach ideal body weight because weight loss reduces liver steatosis and albuminuria and improves kidney function in patients with diabetes [20].For most patients, glycated hemoglobin (A1C) goals were ≤7.0% to avoid diabetic comorbidities. Although BMI and waist circumference tended to be higher in the OCI group, we did not find significant differences in the HbA1C insulin requirement for either group.

Whether HCV is an independent risk factor for cardiovascular disorders remains controversial [19]. The presence of HCV infection increased the risk of coronary artery disease in several studies [21–27].In addition, two community-based studies found that HCV infection was an independent predictor of cerebrovascular death, and also noted an association between serum HCV RNA levels and cerebrovascular mortality, hinting at a dose effect mechanism [28]. Other studies, however, have failed to identify an association between HCV infection and cardiovascular morbidity [29–30].We did not identify an association between OCI and cardiovascular morbidity, but we found that the patients with OCI had an incidence of 30% (3/10) monoclonal gammopathy vs. 0.87% (1/115) of the patients without OCI (p=.003). The incidence of HCV infection and other non-hepatic malignancies was examined in a Swedish cohort of patients infected with HCV. The study showed that the risk of B-cell non-Hodgkin's lymphoma and multiple myeloma were significantly increased [31]. OCI has also been described in patients with lymphoproliferative disorders, with a prevalence between 1.9% and 20% [32–33]. Another study reported that the HCV-NS3 antigen is frequently expressed in the lymph nodes of anti-HCV- and serum HCV-RNA-negative patients with non-Hodgkin's lymphoma, suggesting the possibility of OCI in these patients[34].Although these findings remain to be confirmed, they suggest that HCV infection could be important in the pathogenesis of posttransplant lymphoproliferative disorder and other hematological malignancies.The lymphotropism of this virus could explain the relationship between infection by HCV and some lymphoproliferative disorders, particularly mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma [35]. In our cohort, it is difficult to establish a connection between, OCI, DKD and monoclonal gammopathy, particularly given the small number of patients with OCI; but the remarkable incidence (30%) suggests new areas for research.The primary clinical manifestations of DKD are albuminuria, occasionally microscopic hematuria, and in manypatientsprogressive kidney disease [36–38]. We did not find a higher prevalence of hematuria in the OCI group.At the end of the follow-up period, the progression of kidney disease tended to be more rapid in the group with occult HCV infection; however, the difference was not statistically significant. Proteinuria was similar in both groups,as was the use of RAAS therapy. Our group has also described the tendency for patients with immune-mediated glomerulonephritis and OCI to progress to end stage renal disease more rapidly than patients OCI negative ones. [14].One of the limitations of our study is the size of the series. However, the trends that are complementary with previous findings justify a preliminary recognition of these associations, specifically those related to the more rapid progression of kidney disease in patients with OCI.

V. CONCLUSIONS

In conclusion, for patients with chronic renal failure secondary to DKD, there is a lower prevalence (8%) of OCI compared with the prevalence in patients with immune-mediated glomerular nephropathies (39%), but OCI is higher than in the general population and in hereditary nephropathies [14]. These data indicate that the presence of OCI plays a limited role in the control of diabetes, cardiovascular risk or liver steatosis in these patients. In the group with OCI, however, the progression of renal disease tended to be more rapid than in the non-OCI group and showed a remarkable and unexpected prevalence of monoclonal gammopathy.

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