

VIRAL HEPATITIS

## Chronic hepatitis C virus infection: Prevalence of extrahepatic manifestations and association with cryoglobulinemia in Bulgarian patients

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production (37.5%), thrombocytopenia (31.6%), and autoantibodies: anti-nuclear (18.4%), anti-smooth muscle (16.9%), anti-neutrophil cytoplasm (13.2%) and anti-cardiolipin (8.8%). All extrahepatic manifestations showed an association with cryoglobulin-positivity, with the exception of thyroid dysfunction, sicca syndrome, and lichen planus. Risks factors for the presence of extrahepatic manifestations (univariate analysis) were: age  $\geq$  60 years, female gender, virus transmission by blood transfusions, longstanding infection ( $\geq$  20 years), and extensive liver fibrosis. The most significant risks factors (multivariate analysis) were longstanding infection and extensive liver fibrosis.

**CONCLUSION:** We observed a high prevalence of extrahepatic manifestations in patients with chronic HCV infection. Most of these manifestations were associated with impaired lymphoproliferation and cryoglobulin production. Longstanding infection and extensive liver fibrosis were significant risk factors for the presence of extrahepatic manifestations in HCV patients.

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**Key words:** Hepatitis C; Liver cirrhosis; Extrahepatic manifestations

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### Abstract

**AIM:** To assess the prevalence of extrahepatic manifestations in Bulgarian patients with chronic hepatitis C virus (HCV) infection and identify the clinical and biological manifestations associated with cryoglobulinemia.

**METHODS:** The medical records of 136 chronically infected HCV patients were reviewed to assess the prevalence of extrahepatic manifestations. Association between cryoglobulin-positivity and other manifestations were identified using  $\chi^2$  and Fisher's exact test. Risk factors for the presence of extrahepatic manifestations were assessed by logistic regression analysis.

**RESULTS:** Seventy six percent (104/136) of the patients had at least one extrahepatic manifestation. Clinical manifestations included fatigue (59.6%), kidney impairment (25.0%), type 2 diabetes (22.8%), paresthesia (19.9%), arthralgia (18.4%), palpable purpura (17.6%), lymphadenopathy (16.2%), pulmonary fibrosis (15.4%), thyroid dysfunction (14.7%), Raynaud's phenomenon (11.8%), B-cell lymphoma (8.8%), sicca syndrome (6.6%), and lichen planus (5.9%). The biological manifestations included cryoglobulin

### INTRODUCTION

Hepatitis C virus (HCV) is associated with a wide spectrum of clinical and biological extrahepatic manifestations<sup>[1-3]</sup>. In chronically infected patients, the virus can trigger an impairment in lymphoproliferation with cryoglobulin production<sup>[3]</sup>. Mixed cryoglobulinemia with its complications (skin, neurological, renal, and rheumatologic) is the most significant extrahepatic manifestation of HCV

infection<sup>[4,5]</sup>. Mixed cryoglobulinemia can evolve into B-cell lymphoma in up to 10% of the patient<sup>[6,7]</sup>.

Various non-organ specific auto-antibodies, present in low titers, are noted during the course of chronic HCV infection<sup>[8,9]</sup>, but they do not influence the clinical profile of the disease<sup>[10]</sup>. Chronic HCV infection has been linked to two skin disorders, porphyria cutanea tarda and lichen planus, particularly with the involvement of the oral cavity<sup>[11,12]</sup>. Other possible HCV-associated diseases are type 2 diabetes mellitus<sup>[13]</sup>, thrombocytopenia<sup>[14]</sup> and pulmonary fibrosis<sup>[15]</sup>. The sicca syndrome, although different from the typical Sjogren's syndrome, also appears to be associated with hepatitis C virus infection<sup>[16,17]</sup>.

It is unclear whether HCV plays a pathogenic role in the development of thyroid dysfunction<sup>[18]</sup>. It is possible, that this extrahepatic manifestation of HCV is related to treatment with interferon rather than the virus<sup>[19]</sup>.

The most common risk factors associated with extrahepatic manifestations of HCV infection are older age, female sex, and extensive liver fibrosis<sup>[8]</sup>. Several reports from different parts of the world suggest that hepatitis C virus affects not only the liver, but other tissues, organs and systems as well. Lymphoproliferative disorders, triggered by the virus, are the most significant extrahepatic manifestations in South-Eastern Europe, where Bulgaria is located.

The aim of the present study was to determine the prevalence of various extrahepatic manifestations of chronic HCV infection in our country, to analyze which extrahepatic manifestations are associated with impaired lymphoproliferation and cryoglobulin production, and to identify which patients are at greater risk of developing extrahepatic manifestations.

In our clinical practice, several patients present with extrahepatic manifestations even in the absence of a clearly defined clinical picture of hepatic illness. It is important to recognize these manifestations in order to make an early diagnosis and to initiate therapy in a timely manner.

## MATERIALS AND METHODS

### Patients

We included 136 Bulgarian patients who were referred to the Department of Internal Diseases and Gastroenterology of the University Hospital Alexandrovska during the period from 1996 to 2004. The main reason for patient referral was elevated liver enzymes. The diagnosis of HCV infection was made by the presence of anti-HCV antibodies (third generation ELISA) and a positive test for HCV-RNA (Cobas Amplicor HCV Monitor Test, v. 2.0, Roche Diagnostics). HCV genotypes were identified by direct sequence analysis (Immunogenetics, Belgium) in 60 patients<sup>[20]</sup>. HBsAg and HIV positive patients were not included in the study. Liver biopsy was performed in all patients. The data was collected before the patients were started on any specific treatment.

### Methods

Patient records were reviewed to assess the presence of the following clinical manifestations: fatigue, arthralgia,

Raynaud's phenomenon, palpable purpura, paresthesia, renal impairment (proteinuria, creatinine above the upper normal limit, and hypertension), sicca syndrome (mouth and eyes), thyroid dysfunction (TSH level below or above the normal range of 0.31-5.00 mU/L), lichen planus (skin and oral lesions), type 2 diabetes mellitus (hyperglycemia, treated by hypoglycemic drugs), pulmonary fibrosis (X-ray examination), lymphadenopathy, and lymphoma. The enlargement of peripheral lymph nodes was detected by palpation. The enlargement of mediastinal lymph nodes was detected by X-ray examination, and abdominal lymphadenopathy was detected by abdominal ultrasonography. Chest X-ray and abdominal ultrasonography were obligatory exams carried out routinely in all patients admitted to the hospital. Lymph node enlargement was confirmed by computerized tomography. The diagnosis of lymphoma was based on morphologic evaluation of lymph node tissue in the patients with co-existing peripheral lymphadenopathy, and by bone marrow biopsy. Histological analysis of the enlarged mediastinal and/or abdominal lymph nodes was not performed because patients refused to provide informed consent for invasive diagnostic surgical procedures.

Biological data obtained from for each patient included the presence or absence of cryoglobulins, thrombocytopenia, and auto-antibodies: anti-nuclear (ANA), anti-smooth muscle (ASMA), anti-neutrophil cytoplasm (ANCA), and anti-cardiolipin (ACL). The platelet count was obtained from the patients' files. Thrombocytopenia was defined as platelet count  $\leq 110.10^9/L$ . Cryoglobulins were detected by the Winfield method<sup>[21]</sup>. Twenty milliliters of venous blood was obtained from each patient in a pre-warmed (37°C) syringe, allowed to clot at 37°C and the serum was separated by centrifugation. The supernatant was incubated at 4°C for 8 d and examined daily for cryoprecipitate. Indirect immunofluorescence was used for the detection of ANA, ASMA, and ANCA (IFA/ Binding Site). A positive test for ANA and ASMA was defined as a titer  $\geq 1/40$ , and for ANCA  $\geq 1/20$ . Anticardiolipin antibodies (ACL-IgG and ACL-IgM) were detected by ELISA (Orgentec).

The following demographic and epidemiologic data was collected: age (less or  $\geq 60$  years), sex, the suspected duration of the infection (less or  $\geq 20$  years), an alcohol intake (less or  $\geq 50$  g/d), and the mode of infection. Questions regarding the most relevant identifiable HCV risks factors were asked, including transfusion of blood and blood products, intravenous drug use, surgical procedures, dental manipulation associated with bleeding, and needle stick injury in health workers. In patients with a past history of transfusions of blood and blood products, it was assumed that HCV infection was caused by contaminated blood and blood products.

The histological abnormalities in liver biopsy specimens, obtained blindly (Hepafix 1.4 mm, B. Braun, Germany), were scored according to the METAVIR system<sup>[22]</sup>. Each liver biopsy sample was assessed for the stage of fibrosis and grade of histological activity. Liver fibrosis was staged on a scale of 0 - 4, where 0 = no fibrosis, 1 - portal fibrosis without septa, 2 = few septa,

**Table 1** Prevalence of clinical and biological extrahepatic manifestations in 136 patients with chronic HCV infection

Clinical manifestations	n (%)
Fatigue	81 (59.6)
Renal involvement	34 (25.0)
Type 2 diabetes mellitus	31 (22.8)
Paresthesia	27 (19.9)
Arthralgia	25 (18.4)
Purpura	24 (17.6)
Pulmonary fibrosis	21 (15.4)
Thyroid dysfunction	20 (14.7)
Raynaud's phenomenon	16 (11.8)
B-cell lymphoma	12 (8.8)
Sicca-syndrome (xerostomia)	9 (6.6)
Lichen planus	8 (5.9)
Biological manifestations	
Cryoglobulinemia	51 (37.5)
Thrombocytopenia	4 (31.6)
ANA	25 (18.4)
ASMA	23 (16.9)
ANCA	18 (13.2)
Anticardiolipin antibodies	12 (8.8)
Overall	
At least 1 extrahepatic manifestation	104 (76.5)

ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; ANCA: Anti-neutrophil cytoplasm antibodies.

3 = numerous septa without cirrhosis, and 4 = cirrhosis. Necroinflammatory activity was graded on a scale of A0-A3, where A0 = no histological activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity.

### Statistical analysis

Quantitative data was expressed as mean  $\pm$  SD. Univariate analysis used the  $\chi^2$ -square or Fisher's exact test for comparison of qualitative values. An assessment of the characteristics of HCV infection (demographic, epidemiologic, histologic), associated with the presence of extrahepatic manifestations, was performed using univariate and multivariate logistic regression analysis. Statistical significance was assessed at  $P < 0.05$ . Adjusted odds ratio (OR) and 95% confidence intervals (CI) were derived from the coefficient of the final multivariate logistic model. The analysis was performed with SPSS v.12.0 statistical software.

## RESULTS

A total of 136 patients with chronic HCV infection were included in the study, comprising of sixty-two (45.6%) males and seventy-four (54.4%) females. The mean age of the patients was  $50.16 \pm 16.08$  years (range 20-80 years). Forty-six patients (33.8%) were  $\geq 60$  years of age. Thirty-four patients (25.0%) had a history of alcohol intake ( $\geq 50$  g/d). The suspected duration of infection was  $\geq 20$  years in 70 patients (51.5%) and  $< 20$  years in 66 patients (48.5%). Genotyping was performed in sixty patients, fifty-two (87%) had genotype 1, 4 (6.5%) genotype 3, and 4 (6.5%) had mixed genotypes. Metavir scores for inflammatory activity were: A0 in 5 patients (3.7%), A1 48 (35.3%), A2 46 (33.8%), and A3 37 patients (27.2%). Metavir scores for fibrosis were:

F0 in 12 patients (8.8%), F1 16 (11.8%), F2 25 (18.4%), F3 27 (19.9%), and F4 in 56 patients (41.1%). Twenty patients (14.7%) were intravenous drug users. Thirty-seven patients (27.2%) were possibly infected during surgical procedures. History of dental manipulation with bleeding was detected in 18 patients (13.2%). Needle stick injury was found in 11 health workers (8.1%). Fifty of the 136 patients (36.8%) had previously been transfused with blood or blood products. Thirty-eight of these 50 patients were women, who received blood transfusions during childbirth, and in 33 of these patients the duration of infection was  $\geq 20$  years. Nineteen of these 38 women were  $\geq 60$  years of age at the time of presentation. Twenty of these patients had Metavir stage F4, and 10 (10/38) were Metavir F3.

The overall prevalence of individual extrahepatic manifestations is shown in Table 1. At least one extrahepatic manifestation was identified in 104 patients (76.5%). The clinical manifestations, in descending order of prevalence were: fatigue (59.6%), kidney impairment (25.0%), type 2 diabetes mellitus (22.8%), paresthesia (19.9%), arthralgia (18.4%), palpable purpura predominantly of the lower extremities (17.6%), lymphadenopathy (16.2%), pulmonary fibrosis (15.4%), thyroid dysfunction (14.7%), Raynaud's phenomenon (11.8%), B-cell non-Hodgkin's lymphoma (8.8%), sicca-syndrome (6.6%), and lichen planus (5.9%). All patients with sicca-syndrome had xerostomia, but none had xerophthalmia. Three patients had skin lesions of lichen planus and five had oral lesions. The biological manifestations, in descending order of prevalence were: cryoglobulin (37.5%), thrombocytopenia (31.6%), ANA (18.4%), ASMA (16.9%), ANCA (13.2%), anti-cardiolipin antibodies (8.8%). The ANA, ASMA and ANCA were present in low titers ( $\leq 1/160$ ). None of the patients in the study had clearly defined clinical features of autoimmune liver disease.

The relationship between the presence of cryoglobulins and other extrahepatic manifestations is shown in Table 2. Cryoglobulin-positivity was related to the following clinical manifestations: fatigue, purpura, Raynaud's phenomenon, kidney impairment, type 2 diabetes mellitus, arthralgia, paresthesia, pulmonary fibrosis, lymphadenopathy, and B-cell lymphoma. Cryoglobulin-positivity was also associated with the following biological manifestations: thrombocytopenia, and positive ANA, ASMA, ANCA, and anti-cardiolipin autoantibodies. Three female patients ( $\geq 60$  years at the time of the study) with advanced liver fibrosis and history of blood transfusions during childbirth, first became cryoglobulin positive and subsequently developed lymphadenopathy over two, three, and five years respectively. Histological analysis of the removed lymph nodes showed low-grade non Hodgkin B-cell lymphoma. The present study did not show any association between the presence of cryoglobulins and thyroid dysfunction, sicca syndrome, and lichen planus (Table 2).

The demographic, epidemiological and liver histological features associated with the extrahepatic manifestations were analyzed by univariate and multivariate logistic regression analysis. The results are shown in Table 3. Using univariate logistic regression analysis there was a positive correlation

**Table 2** Relationship between cryoglobulin-positivity and the presence (%) of extrahepatic manifestations in patients with chronic HCV infection

Extrahepatic manifestations	Cryoglobulin-positive patients ( <i>n</i> = 51)	Cryoglobulin-negative patients ( <i>n</i> = 85)	<i>P</i>	Odds ratio (95% confidence interval)
Fatigue	92.2	40.0	0.000	17.6 (5.8-53.4)
Purpura	41.2	3.5	0.000	19.1 (5.3-68.8)
Raynaud' phenomenon	25.5	3.5	0.000	9.3 (2.5-34.7)
Arthralgia	33.3	9.4	0.001	4.8 (1.8-12.2)
Paresthesia	41.2	7.1	0.000	9.2 (3.3-25.0)
Renal involvement	45.1	12.4	0.000	5.5 (2.3-12.7)
Type 2 diabetes	35.3	15.3	0.011	3.0 (1.3-6.8)
Lymphadenomegaly	35.3	4.7	0.000	11.0 (3.4-35.1)
Lymphoma	17.6	3.5	0.015	5.8 (1.5-22.7)
Pulmonary fibrosis	25.5	9.4	0.015	3.2 (1.2-8.6)
Thyroid dysfunction	15.7	14.1		
Xerostomia	11.8	3.5		
Lichen planus	7.8	4.7		
Thrombocytopenia	58.8	15.3	0.000	7.9 (3.5-17.8)
ANA	29.4	11.8	0.013	3.1 (1.2-76.0)
ASMA	33.3	7.1	0.000	6.5 (2.3-18.1)
ANCA	25.5	5.9	0.002	5.4 (1.9-16.4)
ACL-antibodies	17.6	3.5	0.010	5.8 (1.5-22.7)

ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; ANCA: Anti-neutrophil cytoplasm antibodies; ACL: Anti-cardiolipin.

**Table 3** Univariate and multivariate logistic regression analysis of factors associated with the presence of extrahepatic manifestations in patients with chronic HCV infection

Factors	Patients with the factor (%) in the presence of extrahepatic manifestations ( <i>n</i> = 104)	Patients with the factor (%) in the absence of extrahepatic manifestations ( <i>n</i> = 32)	Uni-variate analysis	Multi-variate analysis	Odds ratio (95% confidence interval)
Female sex	59.6	37.5	0.042		
Age $\geq$ 60 yr	43.3	3.1	0.000		
Duration of infection $\geq$ 20 yr	66.3	3.1	0.000	0.001	36.3 (4.6-284.9)
Transfusion of blood and blood products	43.3	15.6	0.006		
Metavir A3	30.8	15.6			
Metavir F4	52.9	3.1	0.000	0.011	15.4 (1.8-125.9)
Alcohol $\geq$ 50 g/d	24.0	28.1			

between the presence of extrahepatic manifestations and female sex, older age ( $\geq$  60 years), duration of the infection ( $\geq$  20 years), transfusion of blood and blood products, and extensive liver fibrosis (Metavir F4). Univariate analysis did not show any correlation between extrahepatic manifestations and the mode of transmission: intravenous drug use (8.7% *vs* 34.4% for the patients without extrahepatic manifestations), surgical procedures (27.9% *vs* 25.5%), dental manipulation with bleeding (12.5% *vs* 15.6%), and needle stick injury in health workers (7.7% *vs* 9.4%). Univariate logistic regression analysis did not show an association between extrahepatic manifestations and high grade of inflammatory activity in the liver (Metavir A3) and alcohol intake of  $\geq$  50 g/d (Table 3). Multivariate logistic regression analysis showed that the most significant association was between extrahepatic manifestations and long duration of infection and advanced liver fibrosis (Table 3).

## DISCUSSION

The present study on 136 patients with chronic HCV infection, showed a high prevalence of extrahepatic clinical

and biological manifestations. At least one manifestation was present in 76.5% of the patients. These results are similar to those reported in a large prospective French study, in which 74% of 1614 patients with chronic HCV infection had at least one extrahepatic clinical symptom<sup>[23]</sup>. By univariate analysis (Table 3) five risks factors were found to be related with clinical and biological extrahepatic manifestations in our patients: female sex, age  $\geq$  60 years, transfusion of blood and blood products, duration of the infection  $\geq$  20 years, and extensive liver fibrosis. By multivariate analysis (Table 3) the most significant risks factors were the longstanding infection and extensive liver fibrosis.

Fatigue was the most frequent non-specific clinical symptom in chronic HCV infection<sup>[2]</sup>. About 60% patients in this study considered fatigue as the initial or worst symptom of their disease (Table 1). In a prospective study of 1614 individuals with chronic HCV infection, fatigue was present in 53% of patients<sup>[24]</sup>. It is not known what causes fatigue in chronic HCV infection. The elevated fatigue score<sup>[25]</sup> is probably related to an increase in serum leptin levels which may interact with serotonin neurotransmission<sup>[26]</sup>. Fatigue can be considered a part of

the clinical picture of HCV-associated cryoglobulinemia, since this symptom was seen more frequently in cryoglobulin-positive than in cryoglobulin-negative patients (92.2% *vs* 40.0%, OR 17.6, 95% CI 5.8-53.4).

Hepatitis C virus escapes immune elimination in chronically infected patients<sup>[5]</sup>. In such patients, CD81-mediated activation of B cells triggers mono-oligoclonal B-lymphocyte expansion and the appearance of various HCV-related autoimmune disorders, including the syndrome of mixed cryoglobulinemia<sup>[6,27]</sup>. A recent meta-analysis showed that 44% patients with chronic HCV infection had circulating immune complexes with cryoprecipitating properties<sup>[28]</sup>. In the present study, cryoglobulins were isolated in 51 of the 136 (37.5%) patients, but we were not able to define the type of cryoglobulin in all the cases. However, there was considerable evidence to suggest that HCV was associated with type II mixed cryoglobulinemia, with clinical features of vasculitis which mainly affects the small sized blood vessels of the skin, joints, nerves, and kidneys<sup>[5,29]</sup>. Skin is the most frequently involved target organ<sup>[30]</sup>. In previous studies, palpable purpura was observed in 10% to 21% patients with clinically manifested cryoglobulinemic syndrome<sup>[11]</sup>, and in 7% of all HCV infected patients<sup>[8]</sup>. We observed palpable purpura, predominantly over the lower extremities, in 24 of the 136 patients (17.6%), with much higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (41.2% *vs* 3.5%, OR 19.1, 95% CI 5.3-68.8). Symptoms related to Raynaud's phenomenon were observed in 16 of the 136 patients (11.8%), with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (25.5% *vs* 3.5%, OR 9.3, 95% CI 2.5-34.7). Pruritus, Raynaud's phenomenon, and palpable purpura of the lower extremities are the main skin manifestations of chronic HCV infection, which is consistent with the findings of previous studies<sup>[11,31,32]</sup>. However, considering the high frequency of cryoglobulins in HCV patients, severe symptomatic mixed cryoglobulinemia with the clinical presentation of diffuse vasculitis was rare, seen in only 1% of cryoglobulin-positive patients<sup>[23]</sup>. Polyarteritis nodosa-type of clinical presentation was not observed in the present study.

Previous studies have shown that arthralgia is present in 44.7% patients with HCV-associated mixed cryoglobulinemia<sup>[33]</sup> and in 19% of all HCV infected patients<sup>[8]</sup>. The clinical picture may mimic rheumatoid arthritis, especially since rheumatoid factor was present in 71% of cases<sup>[54]</sup>, but there was no joint destruction, and antibodies to cyclic citrullinated peptides, which are highly specific for rheumatoid arthritis, were absent<sup>[35]</sup>. In the present study, arthralgia was present in 18.4% patients, with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (33.3% *vs* 9.9%, OR 4.8, 95% CI 1.8-12.2). None of the patients in the present study met the diagnostic criteria for rheumatoid arthritis.

Peripheral nervous system involvement is a characteristic feature of the more severe forms of clinically apparent HCV-cryoglobulinemia<sup>[3]</sup>. Peripheral neuropathy, manifested by paresthesia, was noted in 17% of all HCV patients<sup>[23]</sup> and in 37% to 80% of the patients with HCV-associated mixed cryoglobulinemia<sup>[36,37]</sup>. We

observed paresthesia in 19.9% of all HCV infected patients, with a higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (41.2% *vs* 7.1%, OR 9.2, 95% CI 3.3-25.0). Sensory nerves are mainly affected in patients with HCV-associated mixed cryoglobulinemia<sup>[38]</sup>. Neuropathological data shows axonal degeneration, differential fascicular loss of axons, signs of demyelination, and small-vessel vasculitis with mononuclear cell infiltrates in the perivascular areas<sup>[38,39]</sup>.

Chronic HCV infection can trigger the immune complex syndrome of cryoglobulin deposition and type-1 membranoproliferative glomerulonephritis<sup>[40]</sup>. Diffuse membranoproliferative glomerulonephritis is found in 83% of cryoglobulinemic renal disease<sup>[41]</sup>. Misiani *et al*<sup>[42]</sup> found a high prevalence of HCV antibodies (66%) and HCV RNA (81%) in the serum of patients with cryoglobulinemic glomerulonephritis. Only 2% of the controls (patients with noncryoglobulinemic glomerulopathies) had HCV antibodies<sup>[42]</sup>. In Japan, the virus was found in 60% patients with membranoproliferative glomerulonephritis<sup>[43]</sup>. Clinically obvious renal disease was present in 20% to 30% of cryoglobulin-positive patients with HCV infection<sup>[7,23,44]</sup>. In 55% of these patients, the findings include mild proteinuria, mild microscopic hematuria and mild renal insufficiency<sup>[45,46]</sup>. Arterial hypertension is present in 80% patients<sup>[45,47]</sup>. In the present study, 34 of the 136 HCV patients (25%) had mild proteinuria, hypertension and serum creatinine levels above the upper limit of normal. These symptoms are related to HCV-cryoglobulinemia, since their prevalence was higher in cryoglobulin-positive than in cryoglobulin-negative patients (45.1% *vs* 12.4%, OR 5.5, 95% CI 2.3-12.7). Renal abnormalities during the course of HCV infection are usually diagnosed in most patients between the fifth and sixth decades of life, and occur slightly more frequently in women than men<sup>[45,48]</sup>. Risk factors for the development of severe renal failure at follow-up of these patients include age, serum creatinine level, and proteinuria at the onset of renal disease<sup>[41]</sup>. Cryoglobulin-related nephropathy has been reported to progress to chronic renal failure requiring dialysis in 10% patients<sup>[44]</sup>, but the overall survival at 10 years was 80%<sup>[41]</sup>.

Case-control studies show an increase in the prevalence of type 2 diabetes mellitus (14.5% to 24%) in patients with chronic HCV infection<sup>[13,49,50]</sup>. These findings have been confirmed in a representative sample of the general population in the USA<sup>[51]</sup>. Reports from diverse geographic regions have shown a 2- to 10- fold increase in the prevalence of diabetes in patients with HCV infection compared to liver disease controls<sup>[51-53]</sup>. The highest prevalence (up to 50%) was noted in patients with HCV-associated liver cirrhosis<sup>[54-56]</sup>. Our findings are consistent with these studies. We found type 2 diabetes in 31 of the 136 HCV patients (22.8%), which is higher than the prevalence (8.0%) in the general population of our country. Antonelli *et al*<sup>[57]</sup> found a higher prevalence (14.4%) of type 2 diabetes in HCV patients with mixed cryoglobulinemia compared to HCV-negative age-matched controls (6.9%) and the general population in northern Italy (2.5%-3.3%). We also found a higher prevalence of type 2 diabetes in cryoglobulin-positive than in cryoglobulin-negative patients (35.3% *vs* 15.3%, OR 3.0,

95% CI 1.3-6.8). Given the biology of HCV, which is both hepatotropic and lymphotropic, an immune-mediated mechanism may explain the raised prevalence of type 2 diabetes in HCV-patients with mixed cryoglobulinemia<sup>[57]</sup>. Recent studies suggest that insulin resistance mediated by proinflammatory cytokines, rather than a deficit in insulin secretion, is the primary pathogenic mechanism involved in the development of type 2 diabetes in HCV infection<sup>[58]</sup>.

Soresi *et al*<sup>[59]</sup> detected abdominal lymphadenopathy in 22% patients with HCV infection and persistently normal transaminases, and in 38% of those with high alanine aminotransferase values. Multiple logistic regression analysis showed a significant relationship between abdominal lymphadenopathy and histological abnormalities of the liver, presence of HCV RNA in the serum and gamma-globulin levels<sup>[59]</sup>. Lymphadenopathy in chronic HCV infection indicates a possible interaction between viral antigens and the immune system<sup>[60]</sup>. This interaction may be complicated by autoimmunity, cryoglobulinemia and B cell malignancy<sup>[60]</sup>. In the present study, lymphadenopathy was seen in 22 of the 136 HCV patients (16.2%), with a higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (35.3% *vs* 4.7%, OR 11.0, 95% CI 3.4-35.1). Epidemiological studies have confirmed a link between HCV infection and B-cell non Hodgkin's lymphoma<sup>[61-63]</sup>. In a recent meta-analysis, the prevalence of HCV infection in patients with B-cell non Hodgkin's lymphoma was approximately 15%, which is much higher than the prevalence of HCV in the general population<sup>[64]</sup>. Clonal B cell proliferation was observed in patients with a longer duration of HCV infection, type II cryoglobulin, and vasculitis<sup>[5,65,66]</sup>. Over 90% patients who developed non-Hodgkin lymphoma had type II cryoglobulins<sup>[64]</sup>. About 10% patients with HCV mixed cryoglobulinemia evolved into lymphoma<sup>[6]</sup>. In a series of 231 Italian patients with mixed cryoglobulinemia, 20 developed B-cell lymphoma after a mean of 8.8 years<sup>[7]</sup>. The overall risk of non-Hodgkin lymphoma in patients with cryoglobulinemic syndrome was 35 times higher than that in the general population<sup>[64]</sup>. Our findings are in agreement with the results reported from Italy<sup>[67]</sup>. In the present study, B-cell non Hodgkin's lymphoma was diagnosed in 12 of the 136 HCV patients (8.8%) with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (17.6% *vs* 3.5%, OR 5.8, 95% CI 1.5-22.7). The exact prevalence of HCV associated lymphoma in our country may be much higher, since patients with abdominal and/or mediastinal lymphadenopathy (without enlargement of peripheral lymph nodes) did not provide consent for invasive diagnostic surgical procedures. The appearance of cryoglobulins, peripheral lymphadenopathy and low-grade B-cell lymphoma in 3 women during follow-up suggested that benign lymphoproliferation, triggered by the virus, can evolve into malignant B cell lymphoma. It is possible that genetic and environmental factors<sup>[68]</sup> play a role in the higher prevalence of HCV-associated lymphoma in South-East Europe, including our country, compared to Northern Europe<sup>[69]</sup>. Based on the available evidence it appears that HCV is an important risk factor for B-cell malignancy in areas with a high prevalence of HCV infection<sup>[5]</sup>, which in

our country is 1.3%.

Ueda *et al*<sup>[70]</sup> noted a higher prevalence of anti-HCV antibodies in Japanese patients with idiopathic pulmonary fibrosis compared to the general population. The prevalence of idiopathic pulmonary fibrosis in anti-HCV positive patients was 9.4%<sup>[71]</sup>. HCV patients with mixed cryoglobulinemia have been found to have asymptomatic interstitial lung fibrosis diagnosed by chest X-ray and high-resolution computed tomography<sup>[15]</sup>. Pulmonary fibrosis in HCV-related cryoglobulinemia is perhaps triggered by local deposition of circulating HCV-containing immune complexes<sup>[72]</sup>. In the present study 21 of the 136 HCV patients (15.4%) showed pulmonary fibrosis on routine X-ray examination; the frequency of pulmonary fibrosis was higher in cryoglobulin-positive than in the cryoglobulin-negative patients (25.5% *vs* 9.4%, OR 3.2, 95% CI 1.2-8.6). These findings need further confirmation with the use of more sophisticated radiological techniques.

Serum autoantibodies are commonly seen in patients with chronic HCV infection. In a study by Lenzi *et al*<sup>[9]</sup> the overall prevalence of non-organ specific autoantibodies was significantly higher in anti-HCV positive patients compared to healthy subjects, and HBsAg positive controls (25% *vs* 6% and 7% respectively). Several studies have evaluated the prevalence of ANA and ASMA, with figures ranging from 4.4% to 41%<sup>[8,10,34]</sup> and 7% to 66% respectively<sup>[23,34,73]</sup>. In the present study, positive ANA and ASMA tests were seen in 18.4% and 16.9% patients respectively. The anti-ANA and ASMA antibodies were found in low titers ( $\leq 1/160$ ), and were detected predominantly in the cryoglobulin-positive patients (Table 2). However, none of the patients had a clearly defined clinical picture of autoimmune liver disease. Recent data suggests that the HCV core particles concentrated in the cryoprecipitate play a role in the interaction between the cryoglobulins, endothelial cells and neutrophil granulocytes<sup>[74]</sup>. The main antigens in HCV patients with a positive ANCA test are proteinase 3 and dihydroliipoamine dehydrogenase<sup>[75]</sup>. A positive ANCA has been found in 10% patients with HCV-associated mixed cryoglobulinemia<sup>[76]</sup>. In the present study, ANCA was present in 18 of the 136 patients (13.2%), with higher prevalence in cryoglobulin-positive than cryoglobulin-negative patients (25.5% *vs* 5.9%, OR 5.4, 95% CI 1.9-16.4). HCV patients with ANCA had a higher prevalence of skin involvement, anemia, abnormal liver function tests and elevated alpha feto-protein levels<sup>[75]</sup>. Anticardiolipin antibodies have been observed in 3.3% to 22% of HCV patients<sup>[77-79]</sup>. These antibodies were found more frequently in patients with HCV-associated cryoglobulinemia<sup>[80]</sup>. Our findings are consistent with previously reported data. In the present study, 12 of the 136 patients (8.8%) had anticardiolipin antibodies, with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (17.6% *vs* 3.5%, OR 5.8, 95% CI 1.5-22.7). According to a recent study, these antibodies do not have any clinical significance in HCV patients, since they are anti- $\beta$ 2-glycoprotein I independent<sup>[78]</sup>. The auto-antibodies seen in patients with HCV infection appear to resemble those seen in chronic viral infections - the autoantibody titers are low, frequently found during the clinical course

of cryoglobulinemia, but do not produce a typical autoimmune disease<sup>[29,80]</sup>.

The prevalence of thrombocytopenia is higher in patients with chronic HCV infection than in the general population<sup>[14]</sup>. In a series of 368 patients with chronic HCV infection, Nagamine *et al*<sup>[81]</sup> detected 151 cases (41%) of thrombocytopenia. Our findings also suggest a high prevalence of thrombocytopenia in patients with chronic HCV infection (31.6%). This can be explained by the large number of patients with cirrhosis in the present study. Platelet sequestration and destruction in the spleen<sup>[82,85]</sup>, along with low thrombopoietin production<sup>[83,84]</sup> play an important role in the pathogenesis of thrombocytopenia. However, a direct viral infection of the megakaryocytes<sup>[85]</sup> and autoimmune mechanisms<sup>[86,87]</sup> cannot be ruled out. The higher prevalence of thrombocytopenia in cryoglobulin-positive than in cryoglobulin-negative patients (58.8% *vs* 15.3%, OR 7.9, 95% CI 3.5-17.8) in the present study may reflect the role of immune dysregulation in causing both abnormalities.

In previous studies, thyroid specific antibodies were observed in 22.1% patients with chronic HCV infection, but the presence of thyroid dysfunction was not different compared to age and gender matched controls<sup>[18]</sup>. Thyroid dysfunction was seen in 14.7% of our 136 untreated HCV patients, and there was no association with the presence of cryoglobulins. However, up to 12.6% HCV patients developed thyroid dysfunction during or after IFN therapy<sup>[88-90]</sup>, with a significant association with female gender<sup>[19]</sup>.

Lymphocytic infiltration of the salivary glands has been described in 57% patients with chronic HCV infection compared to 5% in deceased controls<sup>[16]</sup>. A direct role of the virus is suggested by studies on mice expressing HCV envelope proteins E1 and E2 in the liver and salivary glands<sup>[91]</sup>. Sialadenitis developed in 84% HCV infected mice compared to 2% in control littermates and 0% in HCV core transgenic animals<sup>[91]</sup>. Up to 10% patients with chronic HCV infection may develop symptoms of xerostomia and xerophthalmia<sup>[8]</sup>, whereas less than 5% of patients with confirmed Sjogren syndrome are carriers of HCV<sup>[17]</sup>. In the present study, xerostomia was seen in 6.6% patients, but none had xerophthalmia. Our results show a much higher prevalence of xerostomia in cryoglobulin-positive patients, however there was no difference compared to those without cryoglobulins (Table 2). In a French study, the classic Sjogren syndrome (xerostomia, xerophthalmia, histological stages III and IV in the Chishom scale, and anti-SSA or anti-SSB antibodies) was observed only in 1% of patients with chronic HCV infection<sup>[32]</sup>.

Clinical studies, predominantly from the Mediterranean area and Japan, suggest that lichen planus, mainly the oral form, is associated with HCV infection<sup>[92-94]</sup>. The overall prevalence of anti-HCV antibodies in Spanish patients with lichen planus was 29.2%<sup>[12]</sup>. Nagao *et al*<sup>[92]</sup> found serum anti-HCV antibodies in 28 (62%) and serum HCV RNA in 27 (60%) of 45 Japanese patients with oral lichen planus. However, these results have not been confirmed by reports from other regions<sup>[32,95]</sup>. The geographical differences could be related to immunogenetic factors since the allele HLA-DR6 is expressed in a significant

proportion of Italian patients with oral lichen planus and HCV infection<sup>[94]</sup>. The frequency of lichen planus in our HCV patients (5.9%) is similar to that reported by Pawlowsky *et al*<sup>[32]</sup> in France (5%). We found oral lesions of lichen planus in 5 patients and skin lesions in 3 patients. In the present study, we did not find any association between cryoglobulinemia and the presence of lichen planus (Table 2). Pilli *et al*<sup>[96]</sup> detected HCV-specific T cells with phenotypic and functional characteristics of terminally differentiated effector cells at the site of the oral lesions. These findings and the detection of HCV RNA strands in the lichen tissue<sup>[97]</sup> suggests a possible role for HCV-specific T-cell responses in the pathogenesis of lichen planus associated with HCV infection<sup>[98]</sup>.

Our study has demonstrated a high prevalence of extrahepatic manifestations in Bulgarian patients with chronic HCV infection. Most of these manifestations are the consequence of impaired lymphoproliferation and show an association with cryoglobulin production. Clinical features such as fatigue, palpable purpura, Raynaud's phenomenon, arthralgia, paresthesia, kidney impairment, type 2 diabetes, pulmonary fibrosis, lymphadenopathy, B cell lymphoma, thrombocytopenia and non-organ specific autoantibodies can be regarded as a part of the clinical spectrum of HCV-associated cryoglobulinemia. On the other hand, thyroid dysfunction, sicca-syndrome and lichen planus are also seen in patients with chronic HCV infection, but were not associated with the presence of cryoglobulins in the present study. Extrahepatic manifestations were observed more frequently in older women with longstanding infection, advanced liver fibrosis and HCV infection acquired through transfusion of blood and blood products during childbirth. These findings suggest that the dose of the virus and the route of transmission may be important factors associated with extrahepatic manifestations. The long duration of HCV infection and extensive liver fibrosis were the most frequent findings associated with extrahepatic manifestations, but the pathogenesis of such an association is unclear<sup>[1,23]</sup>. Progression of liver fibrosis and the development of systemic-portal venous anastomosis allows the passage of antigens directly into the systemic circulation, thus bypassing the liver filter and resulting in chronic interaction with the immune system<sup>[23]</sup>. Some of the immunological defects are the consequence of HCV interaction with dendritic cells and a shift from Th1 to Th2 cytokine profile<sup>[99,100]</sup>.

Patients with chronic HCV infection have a high prevalence of extrahepatic manifestations. These manifestations are found more frequently in the patients with a long duration of infection and liver cirrhosis, even in the absence of a clearly defined clinical picture of hepatic illness. Most of these manifestations are associated with impaired lymphoproliferation and cryoglobulin production, triggered by hepatitis C virus. The benign lymphoproliferation, associated with cryoglobulinemia, can evolve into malignant B cell lymphoma. Physicians should be aware of the extrahepatic signs and symptoms of HCV infection. HCV should be tested in all patients with these manifestations. This may lead to early diagnosis and successful treatment of chronic HCV infection.

## COMMENTS

### Background

Hepatitis C virus (HCV) affects not only the liver, but other tissues, organs, and systems as well. In our practice, several patients with chronic HCV infection present with extrahepatic manifestations, even in the absence of a clearly defined clinical picture of hepatic illness. Lymphoproliferative disorders, triggered by the virus, are frequently seen in South-Eastern Europe, where Bulgaria is located.

### Research frontiers

The aim of the present study was to assess the prevalence of different extrahepatic manifestations in Bulgarian patients with chronic HCV infection, to identify extrahepatic manifestations associated with impaired lymphoproliferation and cryoglobulin production, and to determine which patients are at greater risk of developing extrahepatic manifestations of chronic HCV infection.

### Innovations and breakthroughs

We observed a high prevalence of extrahepatic manifestations (76.5%) in patients with chronic HCV infection. Patients with longstanding infection and cirrhosis had a higher risk of developing such manifestations. In the present study, most of the extrahepatic manifestations were associated with impaired lymphoproliferation and cryoglobulin production, with the exception of sicca syndrome, thyroid dysfunction and lichen planus. The presence of B-cell non Hodgkin's lymphoma was observed in 8.8% patients, with a higher prevalence in cryoglobulin-positive compared with cryoglobulin-negative patients (17.6% vs 3.5%, OR 5.8, 95% CI 1.5-22.7).

### Applications

Physicians should be aware of the extrahepatic signs and symptoms of HCV infection. HCV should be tested in patients who have any of these manifestations. This may lead to early diagnosis and successful treatment of chronic HCV infection. The treatment of lymphoproliferative disorders, including B-cell non Hodgkin's lymphoma poses a challenge, and requires new therapeutic strategies.

### Terminology

Hepatitis C virus (HCV) escapes immune elimination. HCV interacts with hepatocytes and B lymphocytes through a common (CD81) receptor. CD81-mediated activation of B cells in chronically infected patients can trigger clonal B-lymphocyte proliferation with cryoglobulin production, and evolution of disease into B-cell non Hodgkin's lymphoma. HCV can interact, with or without immune mediated mechanisms, with different tissues, organs, and systems.

### Peer review

The authors assessed the prevalence of extrahepatic manifestations in Bulgarian patients with chronic HCV infection and identified the clinical and biological manifestations associated with cryoglobulinemia.

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