Background: Hepatitis C is a serious problem in Greece, where a high prevalence of antibodies against hepatitis C (anti-HCV) has recently been reported. This article reports the findings of a study carried out in Crete, which investigated the prevalence of serum autoantibodies in patients with chronic hepatitis C.

Patients and Methods: One hundred and forty two patients (59 men and 83 women), who were found anti-HCV seropositive in two hospitals and two Primary Health Care Centres in Crete, were eligible. Sixty healthy blood donors (46 men, 14 women), which were negative to anti-HCV, were used as the control group. They were randomly selected from those attending Rethymnon Hospital. Autoantibodies were identified using the indirect immunofluorescence (IFL) technique on human epithelial cells from larynx cancer (HEp-2 cells), rat liver-kidney-stomach substrate (CT3) and Chritidia Luciliae (CL).

Results: Serum autoantibodies were detected in 104 HCV patients, yielding an overall prevalence of 73.2%. The most frequent autoantibodies were antinuclear antibodies (ANA), positive in 72 patients (50.7%). Anti-smooth muscle antibodies (ASMA) were detected in 33 patients (23.2%). Only one patient was positive for LKM1 autoantibodies. No autoantibodies were found in 38 patients (26.7%). Autoantibodies were also found in 5 out of the 60 examined healthy blood donors (8.3%).

Conclusions: Autoantibodies, mainly ANA and ASMA are very common in HCV seropositive patients from Crete. By contrast LKM1 autoantibodies are exceptionally rare in these patients.
LKM<sub>1</sub>, an autoantibody reacting with liver and kidney microsomes, was also reported to be associated with HCV chronic hepatitis [14, 15]. Unfortunately, similar studies are not available in Greece.

The present study was therefore designed to establish the prevalence of serum autoantibodies in HCV seropositive patients in Crete. This article reports the findings in patients with chronic hepatitis C attending a number of primary and secondary health care units on the island.

**Patients and methods**

**Patients**

Patients who were found to be anti-HCV seropositive in two Hospitals and two Primary Health Care Centres were eligible. Criteria for selection were when the presence of HCV RNA and an ALT elevation was above the formal limits. The study group comprised of 142 patients (59 men and 83 women) from: (a) The University Hospital of Heraklion (Department of Gastroenterology, 75 subjects), (b) The General Hospital of Rethymnon (17 subjects), (c) The Perama Health Centre (25 subjects) and (d) The Spili Health Center (25 subjects). The University Hospital was chosen because it is the Regional Reference Centre for Hepatitis C. The General Hospital of Rethymnon was chosen because it serves a county in which a very high prevalence of hepatitis C has been reported [1, 3]. The Perama and Spili Health Centres are both primary care units affiliated to Rethymnon Hospital and they have participated in large epidemiological studies, previously reported [1, 2, 3].

Patients included 83 women (58.5%) who had a median age of 61 years (range: 27-96 years). A liver biopsy was performed in 54 HCV seropositive (38%), Liver biopsy was performed only on those patients who consented. Histological sections were classified according to the Ishak grading and staging system [16]. Forty-five of the 54 were classified as chronic Active Hepatitis (different types), 6 classified as Cirrhosis, 2 as Primary Biliary Cirrhosis, and one as Acute Viral Cholestatic Hepatitis. A HCV RNA test was done by the Amplicore test (Roche Diagnostics) Genotyping was done by the Inno Lipa test (Innogenetics, Belgium).

Sixty healthy blood donors negative for anti-HCV formed the control group. They had been randomly selected from those attending the Rethymnon Hospital. This group consisted of 46 (76.7%) males and 14 (23.3%) females and had a median age of 35 years (range 20-55 years).

**Serological tests**

Serum from patients and controls was stored at -70°C after being tested for hepatitis C markers. Anti-HCV was tested by the IMX Enzyme Immune Assay (Abbott Laboratories). HCV RNA was identified by the Amplicor test (Roche). The samples were examined at the Department of Immunology of the General Hospital of Rethymnon and autoantibodies were identified using the indirect immunofluorescence (IFL) technique on human epithelial cells from larynx cancer (HEp-2 cells, Kallestad, USA), rat liver-kidney-stomach (triple substrate - Kallestad-USA/Byosystems-Spain) and the Crithidia Luciliae flagellate (Kallestad-USA/Biosystems-Spain). The following autoantibodies were determined: (a) by IFL on HEp-2 cells, antinuclear antibodies (ANA), antibodies against Golgi Apparatus, Spindle Apparatus, Intermediate Filaments (IMF) and Microfilaments (MF); (b) by IFL on Crithidia L., antibodies to double-stranded DNA; (c) by IFL on triple substrate, antibodies to mitochondria (AMA), ribosomes, Liver-Kidney Microsomes (LKM<sub>1</sub>) and Liver Cytosol, antibodies against Smooth Muscle (ASMA), Parietal Cell Antibodies and Reticulin. The initial dilutions were: for ANA, Golgi Apparatus and Spindle Apparatus 1:80; for ASMA, anti-Parietal Cell and anti-Reticulin antibodies 1:40; for AMA, anti-ribosomes, LKM<sub>1</sub>, anti-Liver Cytosol and anti-ds DNA 1:10. Positive samples were diluted up to the highest positive titre.

**Statistical analysis**

The prevalence and the 95% confidence intervals were estimated for serum antibodies. Confidence intervals for the proportions were estimated using the normal approximation to the binomial distribution. The differences in prevalence were tested for statistical significance by the \( \chi^2 \) test. Multivariable analysis by stepwise logistic regression was also done.

**Results**

**Patients’ descriptives**

Demographics of patients and controls are found in Table 1. All anti-HCV positive subjects were also positive for serum HCV RNA. Genotype 1 was identified in 65% of patients.

| Gender | Healthy blood donors |
|--------|----------------------|
| Male   | 59 (41.5%)           |
| Female | 83 (58.5%)           |

| Age group | Healthy blood donors |
|-----------|----------------------|
| 27-44 years | 15 (10.6%)          |
| 45-64 years | 63 (44.4%)          |
| > 64 years   | 64 (45.1%)           |
Prevalence of autoantibodies in HCV seropositive patients

As shown in Table 2, 104 HCV positive patients were identified with serum autoantibodies, yielding an overall prevalence of 73.2%. The most frequent autoantibodies were ANA, positive in 72 patients (50.7%). ASMA were detected in 33 patients (23.2%, 95% CI. 9-38%). No autoantibodies were found in 38 (95% CI. 36-62%) cases (26.8%, 95% CI. 13-41%). Speckled immunofluorescence of both coarse and fine type was the most frequent pattern of ANA, accounting for 79% (57/72) ANA positive cases. Homogeneous immunofluorescence was rare, occurring in only 11% (8/72) ANA positive cases. Details of ANA fluorescence are given in Table 3. Almost all (94% - 31/33) ASMA positive sera had the SMA-V pattern, the remaining two cases exhibiting the SMA-T pattern. Only one patient (0.7%) was LKM₁ and ANA positive.

Of the minor reactivities, antibodies to gastric parietal cells and anti-IMF were the most common, although at low frequencies (14.1% and 9.1% respectively). Antibodies to double-stranded DNA occurred in 4 patients (2.8%), who were also all ANA positive and anti-MF in 2 cases (1.4%), both positive for ASMA with the SMA-T pattern. One of these two was positive for homogeneous ANA as well.

Prevalence of autoantibodies in healthy blood donors

Autoantibodies were found in 5 of the 60 (8.3%) healthy blood donors who were examined. No autoantibodies were detected in 55 cases (91.7%) (Table 2). Of the 5 autoantibody-positive controls, 4 had ANA of the speckled type and one ASMA with the SMA-V pattern. ANA, ASMA, antibodies to parietal cells and anti-IMF turned out to be significantly more frequent in anti-HCV patients than in healthy controls.

Multivariate analysis

None of the variables considered, including age, genotype, gender and histological staging, was significantly associated with any autoantibody.

Discussion

This study confirms that the overall prevalence of serum autoantibodies is high in HCV seropositive subjects. In
Two distinct features, however, must be outlined: Greek carriers of HCV infection are characterized by a low prevalence of LKM₁ and a high prevalence of ANA. In this study, LKM₁ was detected in less than 1% of cases (only 1 patient was positive out of the 104 enrolled). Studies from Italy have reported a 6% prevalence [4] raising as high as 22% when children are considered [6]. The LKM₁ prevalence in our Greek series is even lower than that reported in other countries where LKM₁ is only rarely found such as Sweden [18] and the United States [7,19]. Moreover, in contrast these countries, autoimmune hepatitis is quite rare on Crete. From a group of more than 850 patients with chronic hepatitis, under observation by the Department of Gastroenterology, only 3 cases of autoimmune etiology could be identified, all with type 1 and none with type 2 autoimmune hepatitis (unpublished observations). Therefore, in Crete, LKM₁ antibodies seem to be an exceptional finding in the setting of both autoimmune and HCV-related chronic hepatitis.

The prevalence of ANA in our Greek series is definitely higher than that reported by studies from other countries. The highest ANA rate so far published comes from a Northern Italian study by Lenzi et al. [5] where ANA occurred in 16% of cases and represented a more frequent finding than ASMA. Such a figure however, is still lower than that reported here (50.7%). Such a difference could, in part, have two explanations: 1. instead of the traditional tissue sections, we have used the more sensitive HEp-2 cells as IFL substrate for ANA screening. This methodological approach could account for a higher ANA prevalence, in spite of the different starting serum dilution (1:80 rather than 1:40). 2. Greek patients are older than those from other countries and certainly older than the blood donor controls. They are thus more likely to show autoimmune features including ANA. Further studies are needed to shed light on this point.

Several concerns should also be discussed and those mainly focused on our sampling procedures. Patients and controls were not matched for age and sex. In most series, patient selection was based on liver biopsy and patients with chronic hepatitis were then selected. Only 54 of the 142 patients had liver biopsy. To explore if any selection bias had been introduced, we performed a comparison of ANA and ASMA prevalence between those which had liver biopsy and those which had not, with no statistical difference being found. The prevalence of anti-LKM₁ only slightly changed by 2.4% when we considered as a sample those patients who had liver biopsy.

In this study not only ANA and ASMA, which had already been investigated, but other previously untested reactivities such as antibodies to parietal cells and IMF were significantly more frequent in HCV patients than healthy controls. The reason for such a high prevalence of different autoantibodies during HCV infection is still a matter of speculation. It has been suggested that the virus may disrupt the immunological tolerance to various self-antigens such as the cyp2D6 molecule which is the target of LKM₁ antibodies [8, 20, 21]. On the other hand, molecular mimicry may be involved [8, 20, 21]. A striking homology between E₁ and NS regions of the HCV polyprotein and cyp2D6 has been reported [22, 23]. Whether similar homologies exist between HCV-related proteins and the molecular targets of such heterogeneous antibodies as ANA, ASMA, anti-parietal cells and anti-IMF antibodies, remains to be clarified. The hypothesis that viral genotypes may be critical for the development of autoimmunity is inconsistent with the fact that the occurrence of LKM₁ antibodies is independent of any particular HCV genotype [24].

The type of autoimmunity detected in our HCV patients from Greece closely resembles that already reported from other countries in the same setting: low-medium titre ANA and SMA mainly with the speckled and respectively SMA-V pattern. This autoimmune status is distinct from that marking type 1 autoimmune hepatitis and characterized by high titre homogeneous ANA and/or high titre actin-related ASMA mainly with the SMA-T pattern. In particular, the concomitant positivity for both the above reactivities has been claimed to be absolutely specific for autoimmune hepatitis [17]. However, among our HCV patients, one case was detected which exhibited such an autoantibody status. A serological overlap between autoimmune and HCV-related chronic hepatitis may occur, although to a minimal extent. Correct discrimination between the two liver disorders is of paramount importance because of their quite distinct therapeutic approach. However, in Crete, such a diagnostic problem is not as compelling as in other geographical areas due to the already mentioned rarity of autoimmune hepatitis on the island.

**Conclusions**

In conclusion, serum autoantibodies, namely ANA and ASMA, are frequently found in HCV seropositive subjects from Crete. By contrast, LKM₁ antibodies are virtually absent. General Practitioners and Hospital Physicians in Crete should be aware that these antibodies are more likely to be associated with HCV infection than true autoimmune hepatitis.

**Competing interests**

None declared
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