

## Idiopathic dilated cardiomyopathy: lack of association with hepatitis C virus infection

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### Abstract

**Objective**—To determine whether there is an association between hepatitis C virus (HCV) infection and dilated cardiomyopathy in a well defined area of north western Greece; such an association has been reported elsewhere.

**Design**—Evaluation of consecutive patients with chronic HCV infection for the presence of clinical or subclinical manifestations of dilated cardiomyopathy by history, physical examination, and non-invasive laboratory procedures (ECG, chest x ray, and echocardiography) before the initiation of interferon  $\alpha$  treatment; investigation for HCV infection markers in patients with dilated cardiomyopathy by enzyme and immunoblot assays (antibodies to HCV) and the reverse transcriptase polymerase chain reaction (HCV RNA).

**Setting**—A tertiary referral centre for patients with chronic hepatitis and dilated cardiomyopathy.

**Patients**—102 patients with well defined chronic HCV infection and 55 patients with well established dilated cardiomyopathy were evaluated.

**Main outcome measures**—The need for HCV testing in patients with dilated cardiomyopathy, or follow up for heart disease in patients with chronic HCV infection.

**Results**—None of the patients with chronic HCV infection had clinical or subclinical evidence of dilated cardiomyopathy from history and laboratory findings. None of the patients with dilated cardiomyopathy was positive for antibodies to HCV or viraemic on HCV RNA testing.

**Conclusions**—The study neither confirms the findings of other investigators, nor indicates a pathogenic link between HCV and dilated cardiomyopathy. For this reason, at least in Greece, testing for HCV in patients with dilated cardiomyopathy or follow up for heart disease in HCV patients appears unnecessary. Genetic or other factors could be the reason for this discrepancy if previously reported associations between HCV and dilated cardiomyopathy or hypertrophic cardiomyopathy were not coincidental.

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Idiopathic dilated cardiomyopathy is a primary myocardial disease of unknown cause characterised by left ventricular dilatation and impaired myocardial contractility.<sup>1</sup> At least four basic mechanisms have been proposed in the pathogenesis of the disease. These are familial and genetic factors, immune abnormalities, metabolic, energetic and contractile abnormalities, and the effects of viral myocarditis and other cytotoxic agents.<sup>1-5</sup> Viral myocarditis is thought to be caused by a range of viruses including enteroviruses (particularly coxsackie B virus), adenoviruses, influenza, human immunodeficiency virus (HIV), and cytomegalovirus, suggesting that dilated cardiomyopathy may be a long term sequel of this viral heart disease in some patients.<sup>1 2 5-9</sup> The incidence of biopsy proven myocarditis, however, varies among reported series of patients with dilated cardiomyopathy from a low of 1-2% to a high of 67%, being highest in cases with the shortest time from the onset of symptoms to biopsy.<sup>1 8</sup>

The development of molecular biological techniques and their ability to detect viral nucleic acid in small endomyocardial tissue samples have strengthened the pathogenic link between viral myocarditis and dilated cardiomyopathy in experimental animals as well as in humans.<sup>7 9-13</sup> However, other studies have failed to show the presence of viruses in the myocardium.<sup>14</sup> Furthermore, when using molecular hybridisation assays it was shown that enteroviral nucleic acids were present in the myocardium of control subjects as well as patients with dilated cardiomyopathy,<sup>1 15-17</sup> while the more highly specific and sensitive polymerase chain reaction (PCR) technique has generally failed to detect enteroviral genomic sequences.<sup>14 16 18 19</sup> The wide discrepancy in results reported is probably the result of the different detection procedures adopted by the various investigators, indicating the need for a reliable, sensitive, and specific assay.

Matsumori *et al* have recently found that hepatitis C virus (HCV) infection was often present in patients with dilated cardiomyopathy,<sup>20</sup> suggesting that HCV may be an important cause of this disease. In addition, they proposed that antiviral treatment against HCV might be considered in patients with dilated cardiomyopathy. However, since there are discrepancies in the reports of the associations between chronic HCV infection and several diseases or syndromes,<sup>21-26</sup> this proposed involvement of HCV in the pathogenesis of dilated cardiomyopathy—although attractive—needs to be confirmed. Our study was conducted in an attempt to replicate these

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intriguing findings. We investigated 102 consecutive patients with chronic HCV infection for the presence of clinical or subclinical findings of dilated cardiomyopathy and 55 consecutive patients with well defined dilated cardiomyopathy for the presence of HCV infection markers.

## Methods

### PATIENTS

#### HCV infection

One hundred and two consecutive patients (52 male and 50 female, median age 48 years, range 32 to 65 years) with well defined chronic HCV infection were investigated for the presence of clinical and laboratory manifestations of dilated cardiomyopathy before the initiation of interferon  $\alpha$  treatment. All these patients were followed up at the gastroenterology outpatients clinic of the University Hospital of Ioannina and consented to participate in the study. The diagnosis of chronic HCV infection was based on clinical, laboratory, and histological evaluation. All these patients met the following criteria:

- (1) Serological evidence of chronic infection with HCV for at least six months before their enrolment into the study, as indicated by the presence of antibodies to HCV (anti-HCV) detected using a third generation enzyme immunoassay (Murex Diagnostics, Temple Hill, Dartford, UK) and a third generation recombinant immunoblot assay (RIBA 3.0, Ortho Diagnostics System, Raritan, New Jersey, USA) for confirmation.
- (2) Active virus replication determined by measurement of HCV RNA using an assay based on a combination of reverse transcriptase PCR (RT-PCR) and a DNA enzyme immunoassay (DEIA) (Sorin Biomedica, Saluggia, Italy) as described previously.<sup>27, 28</sup>
- (3) Raised serum concentrations of alanine aminotransferase (at least twofold the upper limit) for at least six months before the initiation of the study.
- (4) Histologically proven chronic viral hepatitis.

All patients with HCV infection were evaluated for a history of chest pain, arrhythmias, and symptoms of cardiac failure according to the New York Heart Association (NYHA) classification. Routine ECG, chest *x* ray, and transthoracic echocardiography (M mode, cross sectional, and colour Doppler) were also performed on all HCV patients. The echocardiographic assessment was done blindly by two independent observers (KA and GK). The interobserver agreement was 97%. A diagnosis of clinical or subclinical dilated cardiomyopathy was accepted according to the World Health Organisation (WHO) criteria,<sup>29</sup> and the recommended criteria for classifying dilated cardiomyopathy and subclinical cardiac impairment of the National Heart, Lung and Blood Institute Workshop.<sup>8</sup> Thus patients were considered to have clinical or subclinical evidence of dilated cardiomyopathy if they met the following non-invasive criteria: ejection

fraction by echocardiography of < 45% or M mode fractional shortening of < 30%, or both, in the presence of a cardiothoracic ratio of > 0.50 to 0.55 or an end diastolic left ventricular diameter of > 5.7 cm/m<sup>2</sup>, with or without the presence of symptoms or signs on physical examination.<sup>8, 29</sup>

#### Dilated cardiomyopathy

Fifty five patients with well established dilated cardiomyopathy (42 male, 13 female, median age 53 years, range 35 to 75 years) who are followed at the cardiology division of the University Hospital of Ioannina consented to participate in the investigation for HCV infection markers. All these patients were investigated for anti-HCV antibodies by a third generation enzyme immunoassay (Murex Diagnostics) and the RIBA 3.0 confirmatory test (Ortho Diagnostics), while negative samples were further examined for the presence of HCV RNA by DEIA (Sorin Biomedica) to avoid false negative results arising from using only the enzyme immunoassay for the detection of anti-HCV. The diagnosis of dilated cardiomyopathy was based on a history of symptoms of congestive heart failure (particularly left sided heart failure), physical examination, non-invasive laboratory examination (including chest *x* ray, ECG, and echocardiography), normal coronary vessels after cardiac catheterisation and angiocardiology (Judkin's method was used in all patients with dilated cardiomyopathy at the time of diagnosis), and in the absence of a known cause of cardiomyopathy (inflammatory, metabolic, infiltrative, fibroplastic, haematological, genetic, and toxic or drug hypersensitivity reactions). The standard criteria outlined above<sup>8, 29</sup> were used to define dilated cardiomyopathy.

#### HEPATITIS C VIRUS ANTIBODY DETECTION

Blood was collected early in the morning under aseptic conditions from all patients, and the separated sera were stored after coding at -80°C until tested. Anti-HCV antibodies were detected by a third generation solid phase enzyme immunoassay (Murex Diagnostics), which uses microplate wells coated with a combination of HCV antigens from the putative core (structural), protease/helicase (NS3, non-structural; NS4, non-structural), and replicase (NS5, non-structural) regions of the HCV genome. All serum samples were run in duplicate. A serum sample was considered reactive when the value of the optical density was higher than the cut off point. The reactive samples were confirmed by the RIBA 3.0 method (Ortho Diagnostics), which uses two recombinant antigens (c33c produced by *Escherichia coli* and NS5 produced by yeast) and two synthetic peptides (C22 and C100, derived from the nucleocapsid and NS4 regions of the HCV genome, respectively), immobilised as separate bands on nitrocellulose strips. In this assay, the C100 band is a mixture of peptides from both the 5-1-1 region and the immunodominant regions of C100 that do not contain 5-1-1. A response of 1+ to 4+ on a band of the nitrocellulose strip

indicates reactivity of the sample to a given antigen or peptide. When reactivity against two or more bands was observed, the serum sample was considered to be reactive (positive), while when a single band was observed the sample was considered indeterminate. A negative result was recorded when no bands appeared after immunoblot.

#### HEPATITIS C VIRUS RNA DETECTION

Six to eight millilitres of venous blood from the morning collection were rapidly separated in a laminar flow instrument using pyrogen-free tubes previously sterilised by ultraviolet light and subsequently by heating at 250°C for 30 minutes. Sterile pipettes and tips were also used, and appropriate precautions were taken during the whole assay in order to protect samples from subsequent environmental contamination, as described previously for other sensitive determinations.<sup>30-31</sup>

#### Primers and probes

All primers and probes were from the 5' untranslated region of the HCV-RNA genome and were provided by Sorin Biomedica. The sequence of oligonucleotides in primer 1CH used in the cDNA synthesis was 5'GGT-GCACGGTCTACGAGACCTC3' (nt from -1 to -21). The sequence of oligonucleotides in primer 2CH used in the first round of PCR was 5'AACTACTGTCTTCACGCAGAA3' (nt from -289 to -269). The two primers 1TS and 4CH used in the second round of PCR were 5'GCGACCCAACACTACTCGGCT3' (nt from -70 to -90), and 5'ATGGCGTTAG-TATGAGTG3' (nt from -257 to -240).<sup>32</sup>

The 5' biotinylated probe 3CH used in DEIA was 5'CGGTGAGTACACCGCAATT-GCCAGGACGACCGGGTCCTTTCT3' (nt from -185 to -143). A complementary sequence to 3CH was used as positive control for DEIA.<sup>27-28, 32</sup>

#### Preparation of RNA and cDNA synthesis

Total RNA was extracted from 100 µl of sera from the patients and from positive and negative controls by thermic shock (heating at 95°C for 30 seconds and quick freezing on ice for five minutes) and subsequently by brief centrifugation as previously described.<sup>33</sup> The extracted RNA in the supernatant was transcribed to cDNA using the 1CH primer by avian myeloblastosis virus reverse transcriptase (25 U per sample), according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin, USA).

#### Nested PCR

The cDNA produced was amplified in a two round PCR. In the first round, amplification was done for 34 cycles, using the 2CH primer and including denaturation at 94°C for two minutes, annealing at 50°C for one minute, and extension at 72°C for two minutes. Three microlitres of the first round product were then amplified for 24 cycles by using the primers 1TS and 4CH. Each cycle included denaturation at 94°C for one minute, annealing at 50°C

for one minute, and extension at 72°C for one minute.

#### DNA enzyme immunoassay

The method is described by Mantero *et al.*<sup>27</sup> Avidin coated plates from GEN-ETI-K DEIA (Sorin Biomedica) were incubated overnight at 4°C with 15 ng per well of biotinylated oligonucleotide probes in 100 µl of TE (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0). The wells were washed five times with 300 µl of washing buffer (6.7 mM phosphate buffer (PBS, pH 6.4), 0.13 M NaCl, 0.004% Cialit (Sigma), and 0.01% Tween-20 (Sigma)). Crude PCR mixtures (amplified products) were denatured by incubation at 95°C for 15 minutes and then quickly cooled on ice. Twenty microlitres of the PCR products from the patients and the positive and negative controls were diluted in hybridisation solution (1× SSG, 2× Denhard's solution, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) in total volume of 100 µl and were then added in duplicate to the coated wells and incubated at 50°C for one hour. After washing, the wells were incubated with 100 µl of a standard dilution of murine monoclonal anti-double stranded DNA antibodies in phosphate buffered saline/10% fetal calf serum at room temperature for one hour. After a further washing process, 100 µl of the substrate solution (tetramethylbenzidine hydrochloride/hydrogen peroxide) were added. The mixture was kept dark at room temperature for additional 30 minutes and the reaction was then stopped by 20 µl of 1N sulphuric acid. The absorbance (A) of the samples was determined in a microtitre plate reader at 400 nm and 630 nm. The A<sub>630nm</sub> was subtracted from the A<sub>400nm</sub> automatically. The DEIA cut off value was then calculated as the mean value of four negative controls plus 0.2 A. This provides a cut off value between 0.25 and 0.30 A. The lower detection limit by DEIA is between 10 and 10<sup>2</sup> HCV RNA copies present in the initial sample used for reverse transcription.<sup>34</sup>

#### STATISTICAL ANALYSIS

All statistical calculations were performed using Statview software. Results are expressed as mean (SD). Data were analysed by paired and unpaired *t* tests where applicable.

#### Results

We excluded pregnant or postpartum women with HCV infection from study; in an attempt to avoid possible bias in the tested hypothesis, we also excluded patients with chronic HCV infection suffering from the following: HIV, arterial hypertension, significant coronary artery disease including previous myocardial infarction, angina or > 50% obstruction in a major coronary vessel with accompanying myocardial dysfunction, present or past history of alcohol or drug abuse, neoplastic disease, haemoglobinopathies, hypocalcaemia, hypophosphataemia, renal failure, or a past history of viral, rickettsial, spirochaetal, or parasitic diseases.

The mean (SD) duration of chronic hepatitis C was 9.2 (3.1) years. None of the patients with

Table 1 Observed and predicted echocardiographic characteristics, after adjustment for body weight and age, according to Henry *et al.*,<sup>35</sup> in patients with chronic hepatitis C and dilated cardiomyopathy

	Patients with hepatitis C (n = 102)			Percentage of the calculated normal size†	Patients with dilated cardiomyopathy (n = 55)			Percentage of the calculated normal size†
	Observed values	Predicted values	Significance*		Observed values	Predicted values	Significance*	
LVIDd (mm)	49.3 (5.7)	48.2 (2.8)	NS	101.8 (6.6)	70.8 (9.6)	47.5 (1.7)	p < 0.0005	148.8 (17.8)
LVIDs (mm)	30.4 (4.0)	30.5 (1.8)	NS	99.5 (10.3)	56.9 (10.1)	30.2 (1.1)	p < 0.0005	189.9 (32.2)
IVSd (mm)	9.8 (1.4)	10.1 (0.8)	NS	97.1 (11.8)	8.3 (1.6)	10.6 (0.5)	p < 0.001	76.8 (15.1)
LVPWd (mm)	9.3 (1.2)	9.5 (0.8)	NS	97.9 (9.0)	7.9 (1.4)	9.6 (0.4)	p < 0.001	74.6 (13.4)
AO (mm)	28.9 (3.1)	29.4 (1.3)	NS	98.3 (9.3)	ND	ND		ND
LAIAs (mm)	32.1 (3.8)	31.2 (2.9)	NS	102.9 (7.8)	49.0 (1.5)	31.5 (1.3)	p < 0.00005	124.2 (3.8)
LVEF (%)‡	76.1 (5.2)	—	—	—	39.6 (8.2)	—	—	—
FS (%)‡	38.2 (4.6)	—	—	—	19.8 (4.0)	—	—	—

Data are given as mean (SD).

\*Difference by paired *t* test between the observed and predicted values.

†Normal ranges: LVIDd 88–112%, LVIDs 82–118%, IVSd 82–118%, LVPWd 84–116%, AO 82–118%, and LAIDs 82–118%.<sup>35</sup>

‡LVEF and FS are independent of both body weight and age.<sup>35</sup>

AO, aortic root internal diameter; FS, fractional shortening; IVSd, interventricular septal wall thickness, end diastole; LAIDs, left atrial internal dimension, end systole; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal dimension, end diastole; LVIDs, left ventricular internal dimension, end systole; LVPWd, posterior left ventricular wall thickness, end diastole; ND, not determined.

chronic HCV infection had a history of symptoms of congestive heart failure according to NYHA criteria.<sup>29</sup> Routine ECG and chest *x* ray were also normal in all. Echocardiography failed to reveal any sign of subclinical dilated cardiomyopathy according to the standard criteria used in the study.<sup>8</sup>

The variables studied by echocardiography and colour Doppler among HCV patients and patients with dilated cardiomyopathy are shown in table 1 after adjustment for age and body weight according to Henry *et al.*<sup>35</sup> In contrast to the patients with dilated cardiomyopathy, in the HCV patients the values for the echocardiographic variables were not significantly different from predicted control values after adjustment for body weight and age (table 1). After adjustment for body weight and age, there was no significant difference in the predicted values between HCV and dilated cardiomyopathy patients (table 1). In contrast, there were significant differences between HCV and dilated cardiomyopathy patients in mean percentage of predicted value, assessed by the method of Henry *et al.*,<sup>35</sup> for left ventricular internal dimension at end diastole ( $p < 0.0005$ ) and end systole ( $p < 0.0005$ ), interventricular septal wall thickness at end diastole ( $p < 0.001$ ), posterior left ventricular wall thickness at end diastole ( $p < 0.0005$ ), and left atrial internal dimension at end diastole ( $p < 0.0005$ ). In addition, the mean values of left ventricular ejection fraction and fractional shortening—both independent of body weight and age<sup>35</sup>—were significantly decreased in the cardiomyopathy patients compared with the HCV patients ( $p < 0.0005$  for both; table 1).

All patients with dilated cardiomyopathy had an enlarged left ventricle on echocardiography (mean left ventricular end diastolic dimension 70.8 mm, table 1) when expressed as a percentage of the calculated normal size<sup>35</sup> (mean 148.8%, normal range < 112%). On functional classification according to the NYHA criteria,<sup>29</sup> 17 patients were in class I, 11 in class II, 14 in III, and 13 in class IV. The mean duration of cardiomyopathy following the initial presentation in the cardiology division of the University Hospital of Ioannina

was 13.3 (3.8) months. One of the 55 patients with well established dilated cardiomyopathy (1.8%) was positive for anti-HCV by the enzyme immunoassay. However, the confirmatory test by RIBA 3.0 was negative, indicating a false positive result with the enzyme immunoassay. In addition, none of the cardiomyopathy patients was found to be viraemic for HCV RNA with the sensitive RT-PCR used in our study.

## Discussion

This study in the northwestern part of Greece failed to show any association between HCV and dilated cardiomyopathy, either in patients with chronic HCV infection, or among a cohort of patients with well established dilated cardiomyopathy. Although the development of dilated cardiomyopathy following myocarditis cannot definitely be excluded by studies that analyse only blood samples, the absence of HCV viral markers in dilated cardiomyopathy patients and the absence of any evidence of dilated cardiomyopathy in a large cohort of patients with chronic HCV infection tend to rule out HCV as an important causal agent in the pathogenesis of dilated cardiomyopathy, at least in our country. Additional analysis of myocardial tissue specimens for HCV RNA detection in our patients might be considered unethical. In contrast, Matsumori *et al* have shown a 16.7% incidence of HCV in Japanese patients with dilated cardiomyopathy.<sup>20</sup> These investigators also isolated strands of HCV in myocardial tissues, supporting the hypothesis of HCV replication by the myocardium.<sup>20</sup> To the best of our knowledge, however, there is no other study confirming these Japanese findings.

The discrepancy between our findings and those of the Japanese series could be explained on the basis of the high background prevalence of HCV infection in the Japanese population,<sup>36</sup> or by as yet undefined factors. Invasive diagnostic and therapeutic procedures, as well as folk remedies such as acupuncture and cutting the skin using non-sterilised knives, have been implicated as a route for transmission of HCV.<sup>37–39</sup> These procedures, and particularly folk remedies, might be the reason for the high prevalence of HCV in Japanese patients with

dilated cardiomyopathy, and the two conditions could be merely coincidental. The same Japanese group has reported a similar prevalence HCV infection (17.1%) in another cohort of patients suffering from hypertrophic cardiomyopathy,<sup>40</sup> further supporting this explanation. There is also disagreement between European and Japanese centres over the aetiology of non-A, non-B fulminant hepatic failure.<sup>41-42</sup> Indeed, unlike Yanagi *et al*,<sup>42</sup> other investigators from Europe<sup>41-43</sup> and the United States<sup>44</sup> were unable to find evidence for a significant contribution of HCV infection in any of their cases with non-A, non-B fulminant hepatitis.

HCV infection has been found to be associated with many diseases and syndromes.<sup>21-23</sup> However, associations in clinical medicine do not necessarily imply causation and additional extended investigation is generally required.<sup>45</sup> For example, in contrast to what has been observed by others,<sup>21</sup> we have already shown the absence of any relation between HCV and idiopathic thrombocytopenic purpura.<sup>24</sup>

In conclusion, we were unable either to confirm the findings of others<sup>20</sup> or show a trend for a pathogenic link between HCV and dilated cardiomyopathy. For this reason, at least in our country, testing for HCV in patients with dilated cardiomyopathy or follow up for heart disease in HCV patients cannot be recommended. Genetic or other factors reflecting known differences in our population<sup>24-46-50</sup> could explain the discrepancy, providing the previously reported associations between HCV and dilated cardiomyopathy<sup>20</sup> or hypertrophic cardiomyopathy<sup>40</sup> were not simply coincidental. More studies, perhaps in experimental animals or in cell culture, are required to evaluate the possible relation between HCV and dilated cardiomyopathy.

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