

Hepatitis C Virus Genotype 3 Is Cytopathic to Hepatocytes: Reversal of Hepatic Steatosis After Sustained Therapeutic Response

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On the basis of cross-sectional studies, it has been proposed that hepatic steatosis is a cytopathic effect of hepatitis C virus (HCV) genotype 3 but not genotype 1 infections. We tested this hypothesis by examining whether antiviral treatment altered hepatic steatosis in chronic hepatitis C. In 28 patients with genotype 1 and 34 with genotype 3 HCV, we determined the severity of steatosis in pre- and posttreatment liver biopsies using computer-assisted morphometric image analysis as well as conventional semiquantitative scoring. Before treatment, hepatic steatosis was present in 16 (57%) patients infected with HCV genotype 1 and 21 (62%) of those with genotype 3. Sustained viral response (SVR) was achieved in 9 (32%) patients with genotype 1 and 22 (65%) with genotype 3. In neither group were there significant changes in body weight or alcohol consumption between pre- and posttreatment biopsies. In patients with HCV genotype 1, there was no change in hepatic steatosis after treatment, irrespective of the treatment response. Among those infected with genotype 3, SVR significantly reduced steatosis ($P < .001$), but there was no change in steatosis among those without a SVR. By logistic regression analysis, SVR was the only variable predictive of improvement in hepatic steatosis (OR = 36, 95% CI = 2.7-481, $P = .007$). In conclusion, these data provide strong support for a direct causal association between HCV genotype 3 infection and hepatic steatosis. (HEPATOLOGY 2002;36:1266-1272.)

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Hepatic steatosis is a common feature on liver biopsy specimens from patients with chronic hepatitis C, and its presence is associated with fibrotic progression.^{1,2} The pathogenesis is complex; host factors including alcohol consumption, exposure to other hepatotoxins, obesity, insulin resistance, type 2 diabetes, and hypertriglyceridemia have been identified as determinants.³ We and others have recently found that viral factors, particularly hepatitis C virus (HCV) genotype, may

be a critical determinant of steatosis in chronic hepatitis C.^{2,4,5} Thus, patients with HCV genotype 3 infection and chronic hepatitis C are more likely to have steatosis and more likely to have extensive hepatic steatosis than those infected with HCV genotype 1. Furthermore, hepatic steatosis in genotype 3 chronic hepatitis C correlates directly with serum and intrahepatic titers of HCV RNA and inversely with apolipoprotein B levels.^{2,5,6} In contrast, steatosis in HCV genotype 1 infection appears independent of virus levels but correlates with measures of obesity, including body mass index and visceral fat distribution.^{2,4} We hypothesized that, if the genotype-specific associations between HCV and hepatic steatosis are real, then a sustained response to antiviral therapy should be accompanied by improvement in steatosis in those infected with genotype 3 but not in those with genotype 1 HCV infection.

The effect of viral eradication on hepatic steatosis in relation to HCV genotype has not been evaluated. Published data on steatosis after antiviral treatment of hepatitis C are limited by a failure to examine the role of genotype and host factors^{6,7} or by the small numbers studied (2 subjects with serial biopsies).⁵ In the present study,

Abbreviations: HCV, hepatitis C virus; SVR, sustained viral response; NSVR, nonsustained viral response.

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we employed a longitudinal design and carefully monitored body composition changes and alcohol intake during and after treatment. Furthermore, we used quantitative computerized morphometric image analysis to quantify steatosis accurately, as well as conventional histologic grading. We report here the changes in hepatic steatosis in relation to treatment outcomes in patients with chronic hepatitis due to genotypes 1 and 3 HCV infection.

Patients and Methods

Patients and Data Collection. We considered for inclusion all patients cared for at Westmead Hospital Liver Clinics with chronic hepatitis C who had received a course of antiviral therapy between 1988 and 1998 and in whom both pre- and posttreatment liver biopsy specimens had been obtained. To minimize confounding factors, only patients with chronic hepatitis C because of genotypes 1 and 3 were included; as reported elsewhere, these cases were approximately 85% of all those treated at the host institution.⁸ Patients with concurrent liver disorders or comorbidity, including hepatitis B virus infection, autoimmune hepatitis, hemochromatosis, α -1 antitrypsin deficiency, Wilson's disease, uncontrolled diabetes, other severe medical disorders, and those having less than 6 months follow-up after completion of therapy were excluded. Patients taking corticosteroids, other drugs associated with hepatic steatosis, or lipid-lowering drugs were excluded. Antiviral treatment was generally by interferon alfa monotherapy for 6 to 12 months, using doses and products as previously reported⁹⁻¹¹; 2 patients received interferon/ribavirin combination therapy. HCV RNA was determined in appropriately collected serum by reverse transcriptase-polymerase chain reaction using a commercial kit (Amplicor HCV, Roche Diagnostics, Branchburg, NJ). HCV genotyping was performed with a second-generation reverse hybridization, line probe assay (Inno-LiPA HCV II; Innogenetics, Zwijndrecht, Belgium).

The following data were entered on a clinical database: age, gender, height, source and duration of HCV infection, alcohol intake during the 12 months preceding biopsy, presence or absence of diabetes, and body weight (kg) at the time of each liver biopsy. We also recorded the dose and duration of antiviral therapy, the viral and biochemical responses to treatment, and the time from end of therapy to first posttreatment biopsy. Portal and lobular necroinflammatory activity scores as well as fibrosis stage were determined in biopsy specimens according to the Scheuer system.¹² Changes in body weight, alcohol in-

take, histologic activity, fibrosis score, and steatosis between the pre- and posttreatment liver biopsies were assessed.

Sustained viral response (SVR) was defined as undetectable serum HCV RNA 6 months after completion of treatment. Nonsustained viral response (NSVR) was defined as detectable serum HCV RNA at 6 months after completion of treatment (this included both nonresponders as well as response relapsers). The study protocol was approved by the Human Ethics Committee of the Western Sydney Area Health Service.

Assessment of Hepatic Steatosis. Liver sections stained with H&E were coded and assessed blind for steatosis by a single hepatologist (D.K.) without knowledge of genotype, treatment response, or the timing of biopsy in relation to antiviral treatment. Macrovesicular steatosis was graded semiquantitatively (modified from Brunt et al.¹³) as follows: 0 (<1% of hepatocytes affected), 1 (1%-33% of hepatocytes affected), 2 (33%-66% of hepatocytes affected), or 3 (>66% of hepatocytes affected). Steatosis was also quantified continuously by computer-assisted morphometric image analysis, using a Leica DML microscope (Leica Microsystems AG, Germany) linked to an imaging system (SPOT Software V2.2, Spot Diagnostic Instruments Inc., Sterling Heights, MI). On each slide, all the tissue was imaged using a 10 \times magnification objective. The only areas excluded were those with sectioning artefacts or containing large blood vessels. The pixel area of biopsy specimen and that of steatosis in each field imaged was measured using Image Proplus Software Version 4.0 (Media Cybernetics, Silver Spring, MD). For each image, windows on the image analyzer grey scale were adjusted so that all clear spaces (including areas of the image field not occupied by biopsy specimen core, fat globules, sinusoids, and empty blood vessels) were selected in contrast to the surrounding parenchyma and triads (Fig. 1A and B). The contribution of sinusoids was reduced by excluding structures with a high-aspect ratio (structures that were more linear than circular in shape). Larger vessels, bile ducts, and areas of the image field not occupied by biopsy specimen core were measured and subtracted manually. Among the 139 biopsy specimens analyzed from the 62 patients [15 (8 with genotype 1 [4 SVR, 4 NSVR] and 7 with genotype 3 [4 SVR, 3 NSVR]) of whom had 2 follow-up biopsies], the grade of steatosis determined semiquantitatively by grading correlated closely ($r_s = 0.877$, $P < .001$) with the percentage area occupied by steatosis as assessed by morphometry (Fig. 2).

Statistical Analyses. Data are nonparametric and have therefore been expressed as median (range). All analyses were carried out using SPSS software (SPSS, Inc,

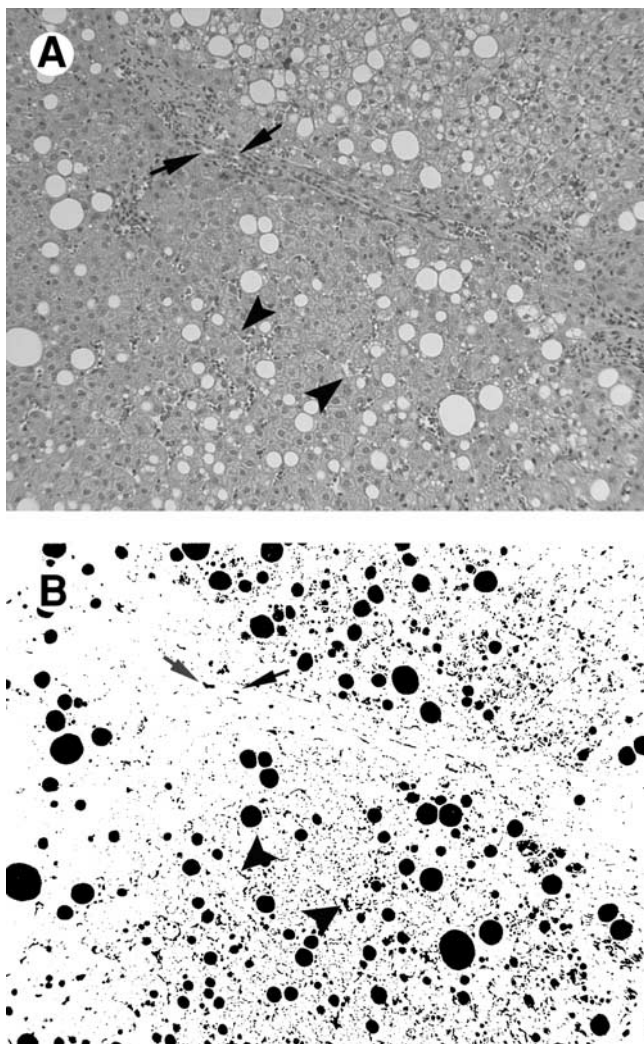


Fig. 1. Hematoxylin-eosin section (original magnification $\times 10$) of liver biopsy (A) and the same section after conversion on the image analyzer to grey scale (B). The area selected for morphologic quantitation of steatosis is outlined in **black**. The area contributed by sinusoids (**arrowheads**) was excluded from analysis by using a high-aspect ratio (ratio between the major axis and the minor axis; hence structures that were more linear than circular in shape were excluded), whereas the areas comprising bile ducts (**arrows**) and blood vessels were manually subtracted. In this example, steatosis occupied 8.9% of the area shown.

Chicago, IL). A significance level of 5% was used throughout. Mann-Whitney and χ^2 tests were used to compare continuous and categorical variables, respectively. The Wilcoxon signed ranks test was used to compare paired variables before and after treatment. The histologic grades of steatosis and morphometric percentage of steatosis were correlated using the Spearman rank correlation coefficient. Multiple logistic regression analysis was performed to identify independent predictors for steatosis reversal.

Results

Characteristics of Patients Studied and Outcomes of Antiviral Treatment. Sixty-two patients fulfilled the criteria for this study, 28 with HCV genotype 1 and 34 with genotype 3. As shown in Table 1, patients with genotype 1 and genotype 3 were comparable on all baseline parameters except that patients with genotype 1 were older. Steatosis (grade 1 or worse) was present on the pretreatment liver biopsy specimen of 16 (57%) patients with genotype 1 and 21 (62%) with genotype 3. As indicated by the median and distribution of steatosis grades, the severity of steatosis between the 2 groups appeared comparable (Table 1). By the more discriminatory and continuous determination of steatosis by morphometric assessment, steatosis appeared to be more extensive in biopsy specimens from patients with genotype 3 than genotype 1 (1.5% vs. 0.3%, respectively), but this apparent difference was not significant ($P = .1$).

Following antiviral therapy, SVR was obtained in 9 (32%) patients with genotype 1 and 22 (65%) with genotype 3. The duration of HCV infection was significantly longer in patients with genotype 1 without SVR as compared with those with SVR. Other baseline characteristics including age, gender, and alcohol intake during the preceding 12 months, presence or absence of diabetes, pretreatment weight, and pretreatment steatosis grade were comparable between patients with SVR and NSVR in both genotype 1- and genotype 3-infected patients (Table 2). Within each genotype group, the pretreatment assessment of steatosis was comparable in those who later obtained SVR and those who did not (Table 2).

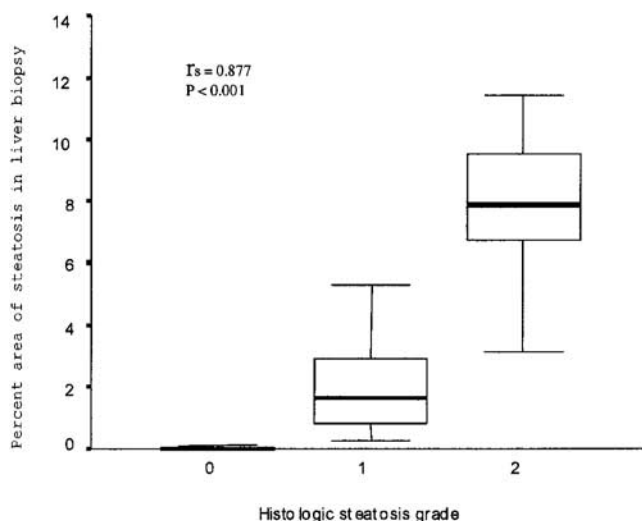


Fig. 2. Correlation between the histologic grade of steatosis and morphometric percentage area occupied by steatosis.

Table 1. Characteristics of Patients With Genotype 1 and Genotype 3 HCV Infection at Entry Into the Study

Characteristics	Genotype 1 (n = 28)	Genotype 3 (n = 34)	P
Age (yr)*	40 (26-69)	35 (23-71)	.04
Males (%)	18 (64)	24 (71)	.8
Alcohol intake (g/d)*	0 (0-80)	10 (0-100)	.1
Diabetes (%)	1 (3.6)	4 (11.8)	.4
Country of birth, Australia (%)	18 (64)	26 (76)	.3
Risk factors (BT/IDU/other/NK)	7/13/3/5	7/18/2/7	.9
Duration of infection (yr)*	12 (1-35)	10 (1-27)	.2
Pretreatment weight (kg)*	76 (48-111)	71 (47-123)	.3
Portal activity (score)*	2 (2-3)	2 (1-4)	.4
Lobular activity (score)*	2 (1-3)	2 (1-3)	.4
Fibrosis (score)*	2 (0-4)	2 (0-4)	.8
Patients with steatosis grade ≥ 1 (%)	16 (57)	21 (62)	.8
Distribution of steatosis grade (0/1/2/3)	12/15/1/0	13/18/3/0	.8
Pretreatment steatosis grade*	1.0 (0-2)	1.0 (0-2)	.6
Pretreatment % area of steatosis*	0.3 (0.0-9.1)	1.5 (0.0-9.4)	.1

Abbreviations: BT, blood transfusion; IDU, injecting drug use; NK, not known.

*Results expressed as median (range).

Change in Hepatic Steatosis After Antiviral Treatment. The median time from the end of treatment to the first posttreatment biopsy considered in this study was comparable between cases grouped by HCV genotype [genotype 1, 1.0 (0-51) (median [range]) month; genotype 3, 1.0 (0-63), $P = 0.9$] and for patients with and without SVR within each genotype (data not shown). There was no significant change in body weight or alcohol consumption between the pre- and posttreatment liver biopsies. As expected, portal and lobular necroinflammatory activity scores decreased among sustained responders irrespective of HCV genotype but not in those without an SVR (Table 3).

Among patients with genotype 1, there was no difference in either the median grade or morphometric extent

of steatosis after treatment whether or not SVR was achieved (Table 3 and Fig. 3). In contrast, there were significant reductions in the steatosis grade (median improvement of 1 grade) as well as the relative area of steatosis determined morphometrically [median improvement by 1.86% (range, -8.96% to $+1.99\%$)] in patients with HCV genotype 3 with SVR (Table 3 and Fig. 3). There was no change in the grade or percentage area of steatosis in those patients with genotype 3 when SVR was not achieved (Table 3 and Fig. 3).

Among 14 patients with HCV genotype 3 and steatosis (grade ≥ 1) in whom SVR was obtained, steatosis completely disappeared in 10 (71%), whereas it reduced by 1 grade in another 2 (14%). Two of the former patients had an additional follow-up biopsy at 15 and 72 months,

Table 2. Baseline Characteristics According to Treatment Response in Patients With Genotype 1 and Genotype 3 HCV Infection

	Genotype 1		Genotype 3	
	SVR (n = 9)	NSVR (n = 19)	SVR (n = 22)	NSVR (n = 12)
Age (yr)*	34 (26-67)	44 (30-69)	33 (23-71)	37 (23-56)
Males (%)	5 (56)	13 (68)	14 (64)	10 (83)
Alcohol intake (g/d)*	10 (0-20)	0 (0-80)	10 (0-100)	7.5 (0-100)
Diabetes (n)	0	1	1	3
Acquisition (BT/IDU/others/NK)	3/5/1/0	4/8/2/5	7/10/0/5	0/8/2/2†
Duration of infection (yr)*	8 (1-35)	17 (5-27)‡	10 (1-27)	10.5 (1-24)
Pretreatment weight (kg)*	77 (57-111)	75 (48-107)	69 (47-108)	73 (66-123)
Patients with steatosis grade ≥ 1 (%)	6 (67)	10 (53)	14 (71)	7 (58)
Pretreatment steatosis grade*	1 (0-1)	1 (0-2)	1 (0-2)	1 (0-2)
Pretreatment % of steatosis*	0.3 (0-0.8)	0.7 (0-9.1)	2.0 (0-9.4)	1.0 (0-9.4)

Abbreviations: SVR, sustained viral response; NSVR, nonsustained viral response; BT, blood transfusion; IDU, injecting drug use; NK, not known.

*Results expressed as median (range) unless otherwise indicated.

† $P = .03$, compared with genotype 3 SVR.

‡ $P = .03$, compared with genotype 1 SVR.

Table 3. Median Change in Steatosis and Related Indices at Posttreatment Biopsy Compared With Pretreatment Biopsy in Patients With Genotype 1 and Genotype 3 HCV Infection

	Genotype 1		Genotype 3	
	SVR (n = 9)	NSVR (n = 19)	SVR (n = 22)	NSVR (n = 12)
Weight (kg)	-2.6	-0.7	-0.4	-0.4
Alcohol intake (g/d)	0	0	0	0‡
Portal activity (score)	-1.0†	0	-0.5†	0
Lobular activity (score)	-1.0†	0	-1.0†	0‡
Fibrosis (score)	0	0	0	0
Steatosis (histologic grade)	0	0	-1.0*	0
Steatosis (% area)	-0.04	0	-1.86*	0

NOTE. Minus sign indicates reduction (improvement) compared with the pretreatment values. *P* values refer to comparisons between median pre- and posttreatment values for the indicated parameter using the Wilcoxon signed ranks test.

Abbreviations: SVR, sustained viral response; NSVR, nonsustained viral response.

**P* = .001.

†*P* ≤ .01.

‡*P* ≤ .05.

respectively, in which there was no longer any evidence of steatosis. In the remaining 2 (14%) patients who did not show any reduction of histologic steatosis grade after treatment, morphometric assessment indicated a reduction in steatosis from 2.6% to 0.7% in 1 and an increase from 1.1% to 3.1% in the other. The latter had gained 3 kg in weight between the pre- and posttreatment biopsies. Among the 7 patients with HCV genotype 3 and steatosis who did not experience SVR, steatosis disappeared in only 1 (14%). The treatment outcome in this individual was an end of treatment response followed by relapse, and it is noteworthy that the posttreatment biopsy specimen was obtained only 10 days after completion of therapy. In a

subsequent liver biopsy performed 22 months later, steatosis was again evident.

Predictors for Reversal of Hepatic Steatosis. To determine independent predictors for reversal of steatosis, a multiple logistic regression model was constructed to include the following input variables: age, gender, pretreatment weight, change in weight and alcohol consumption between biopsies, time from end of treatment to first posttreatment biopsy, and response to treatment (SVR vs. no SVR). In patients with genotype 3 chronic hepatitis C, SVR was the only independent predictor of steatosis reversal (OR = 36, 95% CI = 2.7-481.2, *P* = .007). However, among patients with genotype 1 chronic hepatitis C, no input variable predicted steatosis reversal.

Discussion

HCV Genotype 3 but Not Genotype 1 Causes Steatosis by a Cytopathic Effect. The present study demonstrates almost invariable and complete reversal of hepatic steatosis after the eradication of HCV genotype 3 infection by successful antiviral therapy. However, steatosis was unaltered in genotype 3-infected patients in whom a SVR was not achieved, and in those with genotype 1 infection, irrespective of the treatment outcome. In the only similar study, 2 patients with HCV genotype 3 infection and SVR had complete resolution of steatosis 1 year after the completion of interferon therapy.⁵ Two other studies have reported overall reductions in steatosis in patients achieving an SVR to treatment, but genotype-specific data were not provided.^{6,7} The present findings, therefore, extend earlier studies by examining a larger cohort with more complete viral characterization and by using morphometric analysis to improve the accuracy

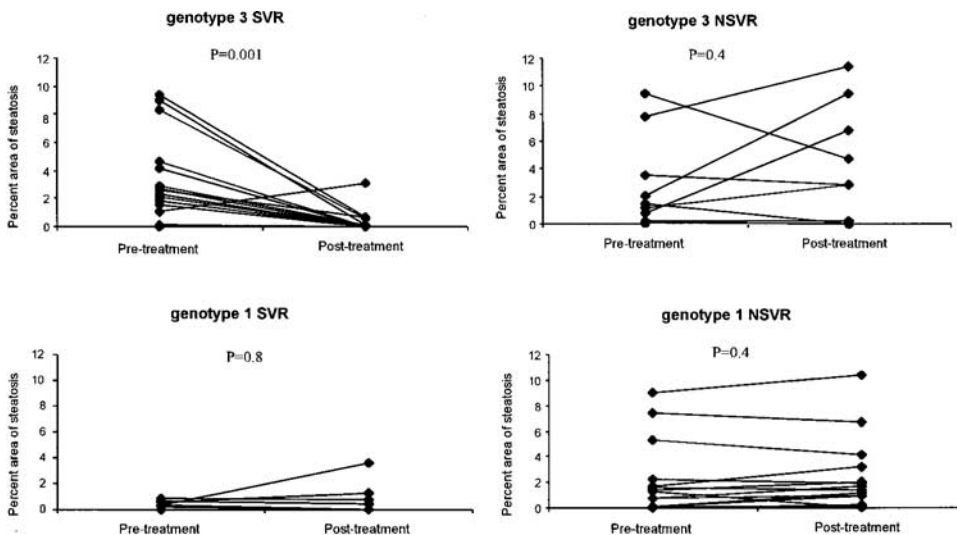


Fig. 3. Change in percentage area of steatosis after treatment in liver biopsy specimens of patients with chronic hepatitis because of genotype 1 and genotype 3 HCV infection. **Dots** represent individual patients and **lines** connect pretreatment biopsy of each patient with their posttreatment biopsy. SVR, sustained viral response; NSVR, nonsustained viral response.

compared with semiquantitative, categorical assessment of hepatic steatosis. The results provide strong support for a direct role for HCV genotype 3 but not genotype 1 in the pathogenesis of hepatic steatosis.

Possible Confounding Variables. Apart from viral factors, host characteristics and alcohol consumption are important determinants of hepatic steatosis.³ However, neither weight loss nor systematic reductions in alcohol consumption (which was required to be minimal before antiviral therapy was started) accounted for the change in steatosis among patients with HCV genotype 3 after an SVR. Conversely, there was no increase in alcohol consumption among those with genotype 1 to account for the failure of hepatic steatosis to resolve after SVR. Indeed, both alcohol consumption and the percentage of patients with diabetes appeared to be marginally higher (not significant) in the genotype 3 group. Another factor that could influence the steatosis grade is the time from the end of treatment to the posttreatment liver biopsy. Again, this did not differ among sustained responders with either HCV genotype. Finally, none of the potential confounding variables considered here were independent predictors of steatosis reversal by multiple logistic regression.

We also considered the possibility that steatosis reversal could be related to improvements in hepatic inflammation. However, as shown in Table 3, the improvements in hepatic inflammation among patients achieving an SVR with either genotype 1 or genotype 3 HCV infection were similar, yet reversal of steatosis was observed only in those with genotype 3 chronic hepatitis C.

Durability of Resolution of Steatosis. Steatosis reversal appears to persist in those with genotype 3 HCV infection in whom SVR had been achieved. Thus, steatosis did not reappear in 2 patients who had a third liver biopsy 15 and 72 months after treatment. In another patient with genotype 3 whose initial treatment response was followed by relapse, fat had completely disappeared from the biopsy specimen taken immediately after completion of therapy (month 0) but reappeared in a follow-up biopsy 22 months later without any accompanying weight change. A similar case has been reported in the transplantation literature,¹⁴ further highlighting the direct prosteatotic effect of HCV genotype 3 infection.

Morphometric Quantitation Improves Assessment of Hepatic Steatosis. We used morphometric quantitation of liver biopsy specimens to assess hepatic steatosis in addition to routine histologic grading. The particular advantage of the morphometric approach was that the results are continuous rather than categorical. This is important because of the wide range of steatosis severity (33% of hepatocytes) within each conventional "grade" as

determined semiquantitatively. It also partly overcomes the nonuniform distribution of steatosis within a biopsy specimen core, which can make it difficult to assess accurately steatosis grade and thus confound attempts to estimate changes in response to therapy. However, some general limitations of biopsy specimen-based methods to quantitate hepatic steatosis are not necessarily overcome by the morphometric approach. For example, fat in the liver may sometimes be difficult to differentiate from other clear areas in hepatocytes because of glycogen deposition or hydropic degeneration. Furthermore, although macrovesicular steatosis is easily recognized, microvesicular steatosis may be more subtle. Despite these potential problems, morphometric quantitation of steatosis has been shown to correlate well with hepatic triglyceride content in human liver when the latter was assessed non-invasively by ¹³C NMR spectroscopy.¹⁵

Why Is HCV Genotype 3 Pathogenic? The pathogenesis of hepatic steatosis caused by genotype 3 chronic hepatitis C is not resolved by this study. A correlation between steatosis grade and intrahepatic HCV RNA titers as well as intrahepatic core protein expression in genotype 3 chronic hepatitis C suggests a direct viral effect.^{5,16} Further support is provided by the finding of hepatic steatosis and mitochondrial dysfunction in transgenic mice expressing 1 or more of the structural HCV proteins alone or in combination with nonstructural viral proteins.^{17,18}

Another possibility relates to interactions with hepatic triglyceride turnover. Thus, HCV entry into hepatocytes may be mediated by the LDL receptor.¹⁹ Likewise, interactions between HCV core protein and apoA2, as well as NS5A with apoA1 and apoA2, have been demonstrated.²⁰ In addition, HCV induces hypobetalipoproteinemia (apoB), an effect more commonly seen with genotype 3 infection than genotype 1.⁶ HCV core protein likewise inhibits microsomal triglyceride transfer protein activity and modifies hepatic VLDL secretion in transgenic mice who develop steatosis.²¹⁻²³ However, the precise nature of interactions between the virus proteins, genotype-specific determinants, and pathways of lipid flux through the hepatocyte require further study.

In conclusion, the results of this longitudinal study provide the strongest evidence to date that implicates HCV genotype 3 but not 1 as a modulator of hepatic steatosis. This appears to be a direct action of the virus on hepatic lipid homeostasis rather than a consequence of changes in host variables (*e.g.*, body mass), exogenous factors (*e.g.*, alcohol consumption), or a reduction in hepatic inflammation.

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