Early Menopause Is Associated With Lack of Response to Antiviral Therapy in Women With Chronic Hepatitis C

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BACKGROUND & AIMS: Chronic hepatitis C (CHC) and liver fibrosis progress more rapidly in men and menopausal women than in women of reproductive age. We investigated the associations among menopause, sustained virologic response (SVR), and liver damage in patients with CHC. **METHODS:** We performed a prospective study of 1000 consecutive, treatment-naïve patients 18 years of age and older with compensated liver disease from CHC. Liver biopsy samples were analyzed (for fibrosis, inflammation, and steatosis) before patients received standard antiviral therapy. From women (n = 442), we collected data on the presence, type, and timing of menopause; associated hormone and metabolic features; serum levels of interleukin-6; and hepatic tumor necrosis factor (TNF)-α. **RESULTS:** Postmenopausal women achieved SVRs less frequently than women of reproductive age (46.0% vs 67.5%; $P < .0001$) but as frequently as men (51.1%; $P = .283$). By multivariate regression analysis, independent significant predictors for women to not achieve an SVR were early menopause (odds ratio [OR], 8.055; 95% confidence interval [CI], 1.834–25.350), levels of γ-glutamyl transpeptidase (OR, 2.165; 95% CI, 1.364–3.436), infection with hepatitis C virus genotype 1 or 4 (OR, 3.861; 95% CI, 2.433–6.134), and cholesterol levels (OR, 0.985; 95% CI, 0.971–0.998). Early menopause was the only independent factor that predicted lack of an SVR among women with genotype 1 hepatitis C virus infection (OR, 3.933; 95% CI, 1.274–12.142). Baseline levels of liver inflammation, fibrosis, steatosis, serum interleukin-6 ($P = .04$), and hepatic TNF-α ($P = .007$) were significantly higher among postmenopausal women than women of reproductive age. **CONCLUSIONS: Among women with CHC, early menopause was associated with a low likelihood of SVR, probably because of inflammatory factors that change at menopause.**

Keywords: HCV Therapy; Prognostic Factors; Antiviral Therapy; Menopause.

Appraisal of the clinical course of chronic hepatitis C (CHC) has revealed several striking differences between men and women. The progression of fibrosis is more than twice as fast in men,[1,2] even when potential confounding factors such as age, duration of infection, or metabolic features are accounted for by multivariate analysis.[1,3] There are conflicting data about sustained virologic response (SVR) rates in women; some studies report that response rates are not significantly different from those of men,[4] whereas others have identified female sex as an independent factor associated with SVR.[5] This inconsistency might arise from the fact that the female cohorts have always been evaluated as a whole, without taking into account differences in responses due to hormonal state.

The reduced rate of fibrosis among women disappears after menopause; in fact, postmenopausal women have accelerated progression of fibrosis[6,7] compared with men that is slowed by long-term estrogen exposure with hormone replacement therapy (HRT).[7] Postmenopausal women are also at higher risk for developing hepatocellular carcinoma; there is a more balanced ratio of men/women in later life that results from the higher incidence of hepatocellular carcinoma in older women.[8,9]

Antiviral therapy greatly improves the natural course of CHC when it results in an SVR,[10] even in patients with established cirrhosis.[11] Negative predictive factors for SVR include older age,[12–14] which might coincide with

Abbreviations used in this paper: CHC, chronic hepatitis C; CI, confidence interval; GGT, γ-glutamyl transpeptidase; HOMA, homeostasis model assessment; HRT, hormone replacement therapy; IL, interleukin; OR, odds ratio; SVR, sustained virologic response; TNF, tumor necrosis factor.

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menopause. However, no studies have evaluated the impact of menopause itself on response to antiviral therapy.

We therefore evaluated the impact of menopause on SVR and on histologic features in a prospective study. We collected data on the type and timing of menopause, parity, and the use and duration of HRT in a cohort of 1000 patients with CHC undergoing standard antiviral therapy with pegylated interferon and ribavirin.

Patients and Methods

Patients

From January 2002 to December 2008, 1000 consecutive patients with CHC were recruited to receive standard antiviral treatment at the Gastrointestinal and Liver Units of the University Hospitals of Modena and Bari. Eligible patients were 18 years of age or older with compensated liver disease due to chronic hepatitis C virus (HCV) infection (any fibrosis stage, including compensated cirrhosis), with a detectable plasma HCV RNA level, and who had not been previously treated for hepatitis C. All patients had undergone liver biopsy within 1 year before enrollment. All biopsy specimens were reviewed and scored according to Ishak et al[15] by a single pathologist (L.L.) who was unaware of patient identity or history. The percentage of hepatocytes containing macrovesicular fat was determined for each 10× field, and steatosis was classified as absent-mild (<5%), moderate (≥5 to <20%), or severe (≥20%).

Patients were excluded if they were coinfected with human immunodeficiency virus or hepatitis B or had any other cause of liver disease, severe depression or psychiatric disorder, or active substance or alcohol consumption <20 g/day in the last year or more, evaluated by a specific questionnaire.

The study was approved by the institutional review boards at the 2 centers and was conducted in accordance with provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Clinical and Laboratory Assessment

The following data were collected at the time of liver biopsy: age, sex, weight, height, and body mass index. In women only, the following data were collected: occurrence, type (spontaneous, surgical), and age at menopause; length of estrogen deprivation; use and duration of HRT; and number of full-term pregnancies and abortions. Menopause was defined as no menstrual periods for 12 consecutive months and was defined as "early" when present for <5 years.

At the time of biopsy, serum levels of alanine aminotransferase, γ-glutamyl transpeptidase (GGT), alkaline phosphatase, ferritin, glucose, and insulin as well as platelet count were obtained. Insulin resistance was determined with the homeostasis model assessment (HOMA) method.[16] HCV RNA was quantified by Abbott RealTime HCV assay (Abbott Molecular Inc, Des Plaines, IL) and genotyped by INNO-LiPA (Innogenetics, Gent, Belgium).

Antiviral Treatment Schedule and Outcomes

Standard antiviral treatment consisted of either (1) pegylated interferon alfa-2a (Pegasys; Roche, Basel, Switzerland) 180 μg/wk or (2) pegylated interferon alfa-2b (PegIntron; Schering–Plough, Kenilworth, NJ) 1.5 $\mu g \cdot kg^{-1} \cdot wk^{-1}$ for 48 weeks for genotype 1 and 4 and for 24 weeks for genotype 2 and 3. Ribavirin was always used at a dosage of 1000 or 1200 mg/day according to body weight (1000 mg/day if ≤75 kg, 1200 mg/day if >75 kg). SVR was defined as undetectable HCV RNA on polymerase chain reaction (detection limit, 12 IU/mL) 6 months after stopping antiviral therapy. Patients with an insufficient virologic response at 12 weeks (detectable HCV RNA and a decrease <2 $\log_{10}$ IU from baseline) or at 24 weeks (detectable HCV RNA) discontinued therapy.

Serum Cytokine Concentrations

Serum interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels were measured at enrollment in 638 patients with CHC (442 women and 196 men with characteristics comparable to the entire male cohort) and in 80 age-matched (±3 years) controls (40 women and 40 men without HCV infection) using quantitative sandwich immunoassays (R&D Systems, Minneapolis, MN) with sensitivities of 0.7 pg/mL and 0.12 pg/mL, respectively.[17]

Immunohistochemistry

Immunohistochemistry was performed on 177 baseline liver biopsy specimens from female patients (93 of reproductive age, 84 menopausal). Paired liver biopsy specimens were collected from 39 women (for 20 patients, both biopsy specimens were obtained at reproductive ages; for 19 patients, the biopsy specimens were obtained before and after menopause). Only 2 of the latter group (both nonresponders) received antiviral treatment in the time between the 2 biopsies. Liver tissue from 5 subjects undergoing elective cholecystectomy (no liver disease; negligible alcohol consumption; normal alanine aminotransferase values; and no evidence of HCV, hepatitis B virus, or human immunodeficiency virus infection) served as control. Immunohistochemistry with standard streptavidin-biotin and immunoperoxidase staining procedures was performed on 4-μm serial sections with a monoclonal anti-human TNF-α antibody (R&D Systems) or a rabbit polyclonal anti-human SOCS3 antibody (Abcam, Cambridge, England) diluted 1:100 and incubated overnight at 4°C after antigen retrieval in 10 mmol/L citrate buffer, pH 6.0, at 90°C for 45 minutes.[18,19] Antibody binding was detected using the HRP Polymer Detection Kit (Biocare Medical, Concord, CA) for TNF-α, while the Dako REAL Detection System (Dako, Glostrup, Denmark) was used for SOCS3. Staining was scored as negative/weak (−/+) or moderately/

Table 1. Baseline Demographic, Laboratory, Metabolic, and Histologic Features of 442 Female Patients With CHC According to the Presence or Absence of Menopause

Variables	Women of reproductive age (n = 168)	Menopausal women (n = 274)	*P* value
Mean age at enrollment (*y*)	40.3 ± 8.4	59.0 ± 5.4	<.001
Source of infection, n (%)			
Community acquired	118 (70.2)	191 (69.7)	.90
Posttransfusional	28 (16.7)	52 (19.0)	.68
Drug addiction	11 (6.5)	0 (0)	<.001
Parenteral exposure	11 (6.5)	31 (11.3)	.16
Estimated duration of HCV infection (*y*), n (%)	11.5 (3.3)	14.7 (4.2)	.0001
Mean age at menopausal onset (*y*)			
Overall	NA	47.7 ± 4.7	—
Surgical (n = 54)		42.5 ± 6.9[a]	
Spontaneous (n = 220)		49.0 ± 2.7[a]	
Length of estrogen deprivation (*y*)			
Overall	NA	11.4 ± 6.3	—
Surgical (n = 54)		15.6 ± 8.1[a]	
Spontaneous (n = 220)		10.3 ± 5.3[a]	
History of pregnancies, n (%)	103 (61.3)	242 (88.3)	<.001
HRT, n (%)	NA	54 (19.7)	—
Mean period on HRT (*y*)	NA	5.5 ± 3.2	—
Mean body mass index (kg/m^2)	23.9 ± 3.5	25.1 ± 3.9	.001
Platelet count ($\times 10^3/mm^3$)	229 ± 63.9	187 ± 63.1	<.001
Alanine aminotransferase (*IU/L*)	65.3 ± 58.2	78.3 ± 70.0	.03
GGT (*IU/L*)	30 ± 21.5	42 ± 43.8	<.001
Cholesterol (*mg/dL*)	167 ± 38	185 ± 37	.017
Triglycerides (*mg/dL*)	93 ± 45.3	80 ± 32.8	.050
Ferritin (*ng/mL*)	76 ± 92.0	172 ± 158.5	.001
Blood glucose (*mg/dL*)	83.8 ± 8.9	98.0 ± 23.8	.002
Insulin ($\mu U/mL$)	9.9 ± 7.9	10.2 ± 7.2	.931
HOMA score	2.0 ± 1.5	2.3 ± 2.0	.646
HCV RNA ($IU/mL \times 10^3$)	1.426 ± 4.230	1.455 ± 3.402	.88
HCV genotype, n (%)			
1–4	97 (57.7)	169 (61.7)	.425
2–3	71 (42.39)	105 (38.3)	
TNF-α (*pg/mL*)	28.6 ± 2.4	27.7 ± 2.5	.106
IL-6 (*pg/mL*)	2.6 ± 1.5	11.8 ± 7.5	.040
Histology at biopsy, n (%)			
Steatosis			
<5%	117 (70.4)	158 (58.5)	.034
≥5% to <20%	40 (24.0)	86 (31.8)	
≥20%	9 (5.4)	26 (9.6)	
Grade of inflammation			
0–5	138 (86.7)	194 (76.0)	.021
6–11	20 (12.5)	54 (21.1)	
12–18	1 (0.6)	7 (2.7)	
Stage of fibrosis			
0–3	155 (97.4)	217 (84.7)	<.0001
4–6	4 (2.5)	39 (15.3)	
Cirrhosis	3 (1.7)	28 (10.8)	.0018

NOTE. Values are expressed as mean ± SD unless otherwise noted.
NA, not applicable.
[a]$P < .0001$.

strongly positive (+/++). Colocalization of TNF-α and SOCS3 was calculated as the percentage of hepatocytes showing positive cytoplasmic staining for both proteins.

Statistics

Continuous variables were summarized as mean ± SE and categorical variables as frequency and percentage. Student *t* test and analysis of variance were used when appropriate. Multiple logistic regression models were used to assess the relationship between (1) SVR, (2) severe fibrosis (Ishak staging ≥3), (3) severe necroinflammation (Ishak grading ≥8), and (4) presence of steatosis. In the statistical models, the dependent variables were coded as 1 (present) versus 0 (absent). We included all patients

Table 2. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Severe Fibrosis (F4–F6) in 442 Female Patients With CHC

	Univariate analysis		Multivariate analysis	
Variables	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Mean age (*y*)	1.066 (1.016–1.119)	.009	1.084 (0.882–1.334)	.442
Menopause	6.964 (2.438–19.891)	<.0001	1.261 (0.009–18.899)	.926
Length of estrogen deprivation by menopausal length (*y*)				
<5	2.576 (0.556–11.927)	.226	0.848 (0.135–5.317)	.860
5–10	7.110 (2.099–24.082)	.002	4.930 (1.176–20.668)	.029
≥10	9.038 (3.079–26.530)	<.0001	6.377 (1.912–21.265)	.003
Estimated duration of HCV infection (*y*)	1.070 (0.988–1.160)	.097	1.135 (0.931–1.383)	.211
Mean body mass index (kg/m^2)	1.084 (1.006–1.168)	.034	0.941 (0.732–1.211)	.637
Platelet count ($\times 10^3/mm^3$)	0.975 (0.970–0.979)	<.0001	0.982 (0.955–1.010)	.982
Alanine aminotransferase (*IU/L*)	1.005 (1.003–1.007)	<.0001	0.996 (0.982–1.010)	.588
GGT (*IU/L*)	1.017 (1.009–1.026)	<.0001	1.020 (0.997–1.043)	.082
Cholesterol (*mg/dL*)	0.992 (0.984–1.000)	.063	0.999 (0.950–1.049)	.954
Triglycerides (*mg/dL*)	0.999 (0.993–1.006)	.817		
Ferritin (*ng/mL*)	1.001 (1.000–1.002)	.010	0.992 (0.982–1.003)	.143
HOMA score	1.040 (0.91–1.850)	.890		
HCV RNA ($IU/mL \times 10^3$)	1.000 (1.000–1.000)	.740		
HCV genotype				
1–4 vs 2–3	0.810 (0.547–1.200)	.293		
Histology at biopsy				
Steatosis	1.295 (0.876–1.915)	.195		
Grade of inflammation	1.429 (1.326–1.539)	<.0001	2.471 (1.116–5.471)	.026

who received at least one dose of pegylated interferon (intention-to-treat analysis).

In the female group, we also considered age at menopause, type of menopause (spontaneous, surgical), length of estrogen deprivation (3 different periods were evaluated: <5 years, 5–10 years, and more than 10 years), use and duration of HRT, and number of full-term pregnancies and abortions. Variables associated with the dependent variable in univariate analyses (probability threshold, $P < .10$) were included in the multivariate regression models.

To avoid colinearity effects, menopause and length of estrogen deprivation were not included in the same multivariate model.

Regression analyses were performed using PROC LOGISTIC, PROC REG, and subroutines in SAS (SAS Institute, Inc, Cary, NC).[20]

Results

Baseline features of all 1000 patients according to sex are shown in Supplementary Table 1. Ninety-nine patients (9.9%) had a diagnosis of cirrhosis; cirrhosis was more frequent in men (12.3%) than women (6.7%) ($P = .003$), despite the latter being significantly older (52 vs 48 years; $P < .001$).

Characteristics of Female Patients

At enrollment, 274 of 442 women (62.0%) were menopausal. Table 1 reports the baseline characteristics of the female group stratified by reproductive status. In 220 women (80.2%) menopause was spontaneous, whereas in 54 (19.8%) it resulted from surgery. Mean age at the time of menopause was 49.0 ± 2.7 years for spontaneous and 42.5 ± 6.9 years for surgical menopause ($P < .0001$). Length of estrogen deprivation was significantly longer in women with surgical menopause versus those with spontaneous menopause (Table 1). Fifty-four of the women (19.7%) had a history of past estrogen therapy or HRT starting soon after the onset of menopause and continuing for a median of 5 years (range, 1–20 years). In 51 of these women (94.5%), HRT was stopped a mean of 8.2 ± 3.2 years (median, 7 years) before standard antiviral therapy was started.

At baseline, menopausal women had significantly more frequent metabolic alterations (blood glucose, cholesterol) and significantly more histologic liver damage than their counterparts who were of reproductive age (Table 1). An increase in the severity of fibrosis was evident between nonmenopausal and early menopausal women (staging score, 1.4 [1.0] vs 2.0 [1.0]; $P = .002$) and between early menopausal and late menopausal women (staging score, 2.0 [1.0] vs 2.4 [1.2]; $P = .009$); cirrhosis was present in 1.7% of nonmenopausal women, 6.0% of early menopausal women, and 11.0% of late menopausal women (nonmenopausal vs late menopausal, $P = .0014$). Multivariate analysis showed that, in addition to necroinflammatory activity (odds ratio [OR], 1.464; 95% confidence interval [CI], 1.256–1.707; $P < .001$), low platelet levels (OR, 0.976; 95% CI, 0.966–0.986; $P < .001$), and

Table 3. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Severe Necroinflammatory Activity in 442 Female Patients With CHC

	Univariate analysis		Multivariate analysis	
Variables	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Mean age (*y*)	1.045 (1.016–1.076)	.002	0.981 (0.894–1.076)	.682
Menopause	3.873 (1.967–7.627)	<.0001	2.594 (0.381–17.645)	.330
Length of estrogen deprivation by menopausal length (*y*)				
<5	4.115 (1.707–9.923)	.002	3.490 (0.881–13.921)	.075
5–10	2.993 (1.246–7.189)	.014	11.823 (2.779–50.302)	.001
≥10	3.778 (1.822–7.833)	<.0001	9.292 (2.531–34.111)	.001
Estimated duration of HCV infection (*y*)	1.044 (0.981–1.111)	.174		
Mean body mass index (kg/m^2)	1.032 (0.965–1.103)	.362		
Platelet count ($\times 10^3/mm^3$)	0.002 (0.987–0.997)	.001	0.991 (0.979–0.998)	.019
Alanine aminotransferase (*IU/L*)	1.007 (1.003–1.010)	<.0001	1.002 (0.995–1.009)	.642
GGT (*IU/L*)	1.018 (1.008–1.028)	<.0001	1.023 (0.998–1.049)	.075
Cholesterol (*mg/dL*)	0.990 (0.980–1.000)	.055	.991 (0.739–1.009)	.991
Triglycerides (*mg/dL*)	1.003 (0.995–1.012)	.459		
Ferritin (*ng/mL*)	1.004 (1.001–1.008)	.006	1.003 (0.999–1.008)	.642
Blood glucose (*mg/dL*)	1.030 (1.005–1.056)	.018	1.014 (0.967–1.063)	.573
HOMA score	1.021 (0.990–1.850)	.760		
HCV RNA ($IU/mL \times 10^3$)	1.000 (1.000–1.000)	.515		
HCV genotype				
1–4 vs 2–3	1.054 (0.603–1.841)	.854		
Histology at biopsy				
Steatosis	3.920 (1.777–8.646)	.001	0.842 (0.242–2.930)	.787
Stage of fibrosis	2.470 (1.901–3.209)	<.0001	3.524 (1.794–6.923)	<.0001

elevated GGT levels (OR, 1.010; 95% CI, 1.003–1.018; $P = .008$), a longer duration of estrogen deprivation (5–10 years: OR, 4.078 [95% CI, 1.013–16.409]; $P = .048$; more than 10 years: OR, 4.867 [95% CI, 1.476–16.042]; $P = .009$) was independently linked with severe fibrosis (Table 2). Other factors influencing exposure to estrogen, including past pregnancies (OR, 0.764; 95% CI, 0.464–1.259; $P = .29$), more than 2 lifetime pregnancies (OR, 0.914; 95% CI, 0.607–1.377; $P = .66$), and HRT use (OR, 0.454; 95% CI, 0.059–3.508; $P = .44$) were unrelated to fibrosis severity. Menopausal women also had significantly higher liver necroinflammatory activity and a higher rate of steatosis compared with women of reproductive age (Table 1). Multivariate analysis for severe necroinflammatory activity showed that stage of fibrosis (OR, 3.610; 95% CI, 1.785–7.301; $P < .001$), GGT levels (OR, 1.032; 95% CI, 1.003–1.062; $P = .030$), and longer duration of estrogen deprivation (5–10 years: OR, 11.823; 95% CI, 2.779–50.302; $P = .001$; >10 years: OR, 9.292; 95% CI, 2.531–34.111; $P = .001$) were independently linked with higher inflammatory activity (Table 3). Similarly, age (OR, 1.133; 95% CI, 1.059–1.213; $P < .0001$), baseline cholesterol level (OR, 0.981; 95% CI, 1.026–1.333; $P < .0001$), body mass index (OR, 1.170; 95% CI, 1.003–1.062; $P = .019$), and duration of estrogen deprivation (<5 years: OR, 3.726; 95% CI, 1.219–11.385; $P = .021$; 5–10 years: OR, 2.648; 95% CI, 1.117–6.276; $P = .027$; more than 10 years: OR, 1.474; 95% CI, 0.732–2.969; $P = .278$) were independently associated with steatosis by multivariate analyses.

Paired liver biopsy specimens taken shortly before and after menopause were available for 19 women (median interval 3 years before and 2 years after); in 20 other women, paired biopsy specimens were obtained while the women were still of reproductive age, at a median interval of 4 years. Analysis of the pairs of menopausal women and those of reproductive age revealed that inflammation had worsened in 8 women (42.1%), increasing by 2 points in 4 women, by 3 points in 3, and by 4 points in 1 of them. It was unchanged in 5 women (26.3%) and improved in 6 (31.6%), decreasing by 2 points in 2 women and 4 points in 4 of them. Fibrosis had progressed in 7 (36.8%), with fibrosis scores increasing by 1 point in 4 women, 2 points in 2, and 3 points in 1 of them. Two women improved by 1 point each and 10 women (52.6%) had no change in fibrosis stage. In the pairs of women who were both of reproductive age, inflammation had worsened in 6 women (30.0%), increasing by 1 point in 3 women and by 2 points in 3 of them. It was unchanged in 5 women (25.0%) and improved in 9 (45.0%), decreasing by 1 point in 4 women and 2 points in 5 of them. Fibrosis had progressed by 1 point in 7 (35.0%), improved by 1 point in 6 (30.0%), and was unchanged in 7 (35.0%).

Results of Antiviral Treatment

Results are reported as intention-to-treat analysis. A total of 838 patients completed the antiviral treatment program; 49 (4.9%; 34 men and 15 women) withdrew because of side effects. SVR was achieved in 511 individuals (51.1%).

Table 4. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for SVR Failure in 442 Female Patients With CHC

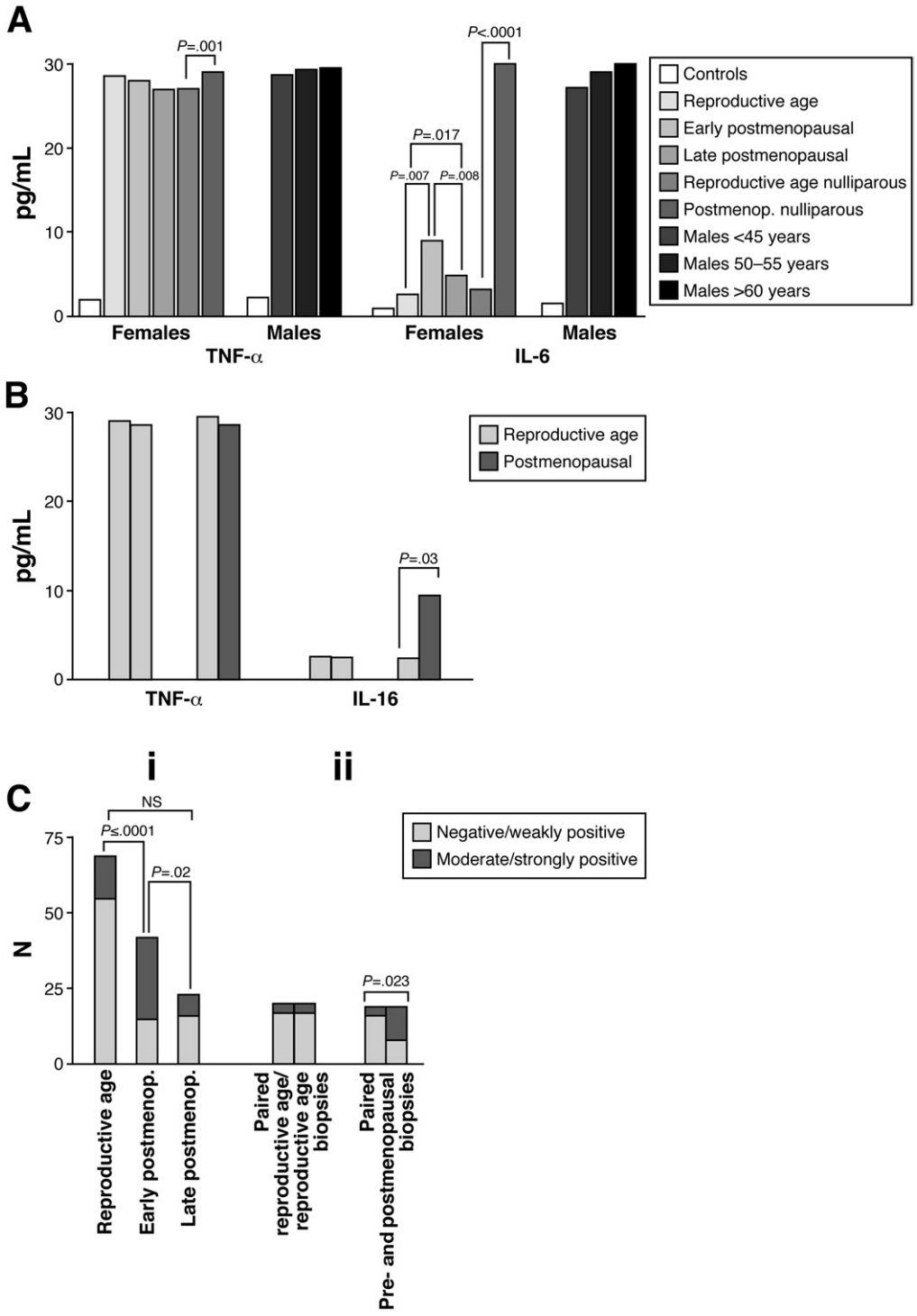
	Univariate analysis		Multivariate analysis	
Variables	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Entire female cohort (n = 442)				
Mean age (*y*)	1.033 (1.015–1.052)	<.0001	0.983 (0.950–1.068)	.686
Menopause	2.436 (1.620–3.662)	<.0001	1.884 (1.177–3.016)	.008
Length of estrogen deprivation (*y*)	1.042 (1.015–1.070)	.002	1.115 (1.048–1.185)	.001
Length of estrogen deprivation by menopausal length (*y*)				
<5	2.497 (1.010–8.172)	.047	8.055 (1.834–25.390)	.006
5–10	1.295 (0.497–3.375)	.597	1.683 (0.335–8.458)	.527
≥10	2.374 (1.137–4.354)	.021	4.277 (0.747–24.503)	.103
Estimated duration of HCV infection (*y*)	1.089 (1.040–1.140)	<.0001	1.047 (0.973–1.126)	.221
Mean body mass index (kg/m^2)	1.009 (0.960–1.060)	.719		
Platelet count ($\times 10^3/mm^3$)	0.992 (0.989–0.995)	<.0001	0.997 (0.993–1.001)	.119
Alanine aminotransferase (*IU/L*)	0.999 (0.996–1.001)	.346		
GGT (*IU/L*)	1.017 (1.008–1.026)	<.0001	2.165 (1.364–3.436)	.001
Cholesterol (*mg/dL*)	0.992 (0.983–1.001)	.074	0.985 (0.971–0.998)	.026
Triglycerides (*mg/dL*)	0.999 (0.990–1.007)	.731		
Ferritin (*ng/mL*)	1.000 (0.998–1.003)	.925		
HOMA score	1.007 (0.988–1.045)	.381		
HCV RNA ($IU/mL \times 10^3$)	1.000 (1.000–1.000)	.049	1.000 (1.000–1.000)	.279
HCV genotype				
1–4 vs 2–3	3.690 (2.427–5.617)	.000	3.875 (2.444–6.134)	<.0001
Histology at biopsy				
Steatosis	1.402 (0.940–2.091)	.097	3.053 (0.925–10.076)	.067
Grade of inflammation	1.131 (1.045–1.225)	.002	1.110 (1.021–1.224)	
Stage of fibrosis	1.494 (1.246–1.793)	<.0001	1.079 (0.839–1.388)	.063
Cirrhosis	0.823 (0.206–3.292)	.783		.553
Women with genotype 1 HCV infections (n = 252)				
Mean age (*y*)	1.032 (1.010–1.055)	.005	0.972 (0.911–1.037)	.386
Menopause	3.625 (1.562–5.699)	.003	2.908 (1.544–5.478)	.001
Length of estrogen deprivation (*y*)	1.040 (1.008–1.086)	.048	1.088 (1.006–1.177)	.035
Length of estrogen deprivation by menopausal length (*y*)				
<5	4.833 (81.83–21.561)	.038	3.933 (1.274–12.142)	.017
5–10	2.071 (0.565–7.593)	.272	2.300 (0.982–5.386)	.055
≥10	2.201 (0.865–5.602)	.098	1.437 (0.743–2.781)	.282
Estimated duration of HCV infection (*y*)	1.017 (0.961–1.077)	.559		
Mean body mass index (kg/m^2)	0.970 (.878–1.072)	.552		
Platelet count ($\times 10^3/mm^3$)	0.992 (0.985–0.999)	.030	0.998 (0.989–1.006)	.564
Alanine aminotransferase (*IU/L*)	1.000 (0.995–1.004)	.851		
GGT (*IU/L*)	1.021 (1.007–1.035)	.004	1.012 (0.999–1.025)	.062
Cholesterol (*mg/dL*)	0.990 (0.977–1.004)	.162		
Triglycerides (*mg/dL*)	0.995 (0.982–1.008)	.454		
Ferritin (*ng/mL*)	1.000 (0.996–1.003)	.839		
HOMA score	1.010 (0.966–1.039)	.399		
HCV RNA ($IU/mL \times 10^3$)	1.000 (1.000–1.000)	.168		
Histology at biopsy				
Steatosis	1.776 (0.757–4.166)	.187		
Grade of inflammation	1.224 (1.037–1.444)	.017	1.039 (0.299–3.605)	.952
Stage of fibrosis	1.220 (0.886–1.679)	.223		
Cirrhosis	1.680 (0.293–9.632)	.560		

Significant independent predictors of SVR failure by multivariate analysis were genotype 1/4, necroinflammatory activity, and GGT levels (Supplementary Table 2).

Influence of menopause on response to antiviral therapy. Overall, SVR was achieved by 231 of 442 women (52.2%). SVR occurred in 121 of 263 menopausal women (46.0%) versus 110 of 163 women of reproductive age (67.5%; OR, 2.436; 95% CI, 1.620–3.662; $P < .0001$). The rate of SVR was similar among women of reproductive age who were stratified for parity (nulliparous vs parous, 42 of 65 [64.6%] vs 69 of 103 [66.9%]; OR, 1.200; 95% CI, 0.521–2.724; $P = .668$). Nulliparous menopausal women had worse SVR compared with postmenopausal women

with any number of pregnancies (nulliparous vs parous, 4 of 32 [12.5%] vs 130 of 242 [53.7%]; OR, 0.132; 95% CI, 0.029–0.607; $P = .009$).

The probability of SVR failure between men and unstratified women was similar (OR, 0.827; 95% CI, 0.615–1.113; $P = .210$). Instead, women of reproductive age had a significantly lower risk of not achieving an SVR (women of reproductive age vs men: OR, 0.452; 95% CI, 0.295–0.693; $P < .0001$; menopausal women vs men: OR, 1.212; 95% CI, 0.853–1.722; $P = .283$). Among men, the risk of not achieving an SVR was similar after stratification for age groups and comparable to that of women of reproductive age (younger than 45 years) or menopausal (older than 55 years) women (men aged younger than 45 years vs men older than 55 years: OR, 1.204; 95% CI, 0.964–1.502; $P = .101$). The percentage of SVR in men younger



than 45 years was 59.2% and in those older than 55 years was 50.0% ($P = .114$).

By multivariate analysis, significant independent baseline predictors of SVR failure in women were the presence of menopause (OR, 1.884; 95% CI, 1.177–3.016; $P = .008$), cholesterol level (OR, 0.985; 95% CI, 0.971–0.998; $P = .026$), high GGT levels (OR, 2.165; 95% CI, 1.364–3.436; $P = .001$), and genotype 1/4 (OR, 3.875; 95% CI, 2.444–6.134; $P < .0001$) (Table 4, *top*). Substituting "duration of estrogen deprivation" for "menopause" in the multivariate model, the OR of SVR failure for women who were postmenopausal for <5 years was 8.055 (95% CI, 1.834–25.390; $P = .006$); longer periods of estrogen deprivation were not significantly associated with SVR failure. In addition, when replacing length of estrogen deprivation as a categorical variable with the linear variable, the latest remained significantly associated with lower SVR rate (OR, 1.115; 95% CI, 1.048–1.185).

Restricting analysis to genotype 1–infected women, logistic regression analysis identified only menopause as an independent predictive factor for SVR failure (OR, 2.908; 95% CI, 1.544–5.478, $P = .001$) (Table 4, *bottom*). Substituting "duration of estrogen deprivation" for "menopause" in the multivariate model revealed that the OR of SVR failure decreased in parallel with increasing time from the menopausal event: less than 5 years, 3.933 (95% CI, 1.274–12.142; $P = .017$); 5–10 years, 2.300 (95% CI, 0.982–5.386; $P = .055$); more than 10 years, 1.437 (95% CI, 0.743–2.781; $P = .282$). Similar to the entire population, in G1, when replacing length of estrogen deprivation as a categorical variable with the linear variable, the latest remained significantly associated with lower SVR rate (OR, 1.088; 95% CI, 1.006–1.177).

Cytokine Levels

Regardless of reproductive status, HCV-positive women had significantly higher serum levels of both TNF-α and IL-6 compared with controls ($P < .0001$ for all combinations) (Figure 1*A*). IL-6 levels in women of reproductive age were significantly lower than those in early postmenopausal women (2.6 ± 1.5 vs 8.9 ± 10.3 pg/mL; $P = .007$) and were lower, although to a lesser degree, than those in late postmenopausal women (2.6 ± 1.5 vs 4.8 ± 8.2 pg/mL; $P = .017$). When only nulliparous women were considered, the difference in IL-6 levels between women of reproductive age and postmenopausal women was greater (3.2 ± 2.2 vs 42.3 ± 18.2 pg/mL; $P < .0001$) and a small but significant difference was found also in TNF-α levels (27.0 ± 3.1 vs 29.2 ± 2.5; $P = .001$) (Figure 1*A*). Among menopausal women, but not women of reproductive age, baseline levels of IL-6 correlated with higher baseline necroinflammatory scores (menopausal: OR, 3.571; 95% CI, 1.494–8.536, $P = .004$; reproductive age: OR, 0.727; 95% CI, 0.305–1.736; $P = .473$).

Analysis of the paired serum samples taken shortly before and after menopause revealed significantly different levels for IL-6 (before vs after: 2.0 ± 1.2 vs 9.2 ± 2.2; $P = .03$) but not for TNF-α (29.6 ± 2.4 vs 28.7 ± 3.5; $P = .395$) (Figure 1*B*). The postmenopausal increase in IL-6 levels correlated with fibrosis progression (OR, 3.333; 95% CI, 1.293–8.591; $P = .011$) and with worsened necroinflammatory score (OR, 3.667; 95% CI, 1.397–9.624; $P = .004$).

Levels of TNF-α and IL-6 in men were stratified to have age groups comparable to those of female patients stratified by hormonal status: younger than 45 years (reproductive age), 50–55 years (early menopause), and older than 60 years (late menopause). Levels found for both cytokines did not differ significantly among the 3 age groups (TNF-α: 28.7 ± 16.2, 29.2 ± 21.2, and 29.5 ± 7.5; IL-6: 27.2 ± 35.0, 29.0 ± 42, and 34 ± 51, respectively; not significant for all combinations). Levels of TNF-α did not differ significantly between men and women (not significant for all combinations), whereas levels of IL-6

←

Figure 1. (*A*) Serum levels of TNF-α and IL-6 in 682 patients with CHC (442 women, 196 men) and in 80 controls (40 age-matched women and 40 age-matched men without HCV infection). The level of cytokines was assessed as described in Patients and Methods. Differences between controls and each group of patients with CHC were significant at the <.0001 level. For levels of TNF-α, there were no significant differences between women of reproductive ages and postmenopausal women; the difference was significant when only nulliparous women were considered ($P = .001$). In men, levels of TNF-α were similar in all 3 age groups (difference not significant among all 3 combinations). For IL-6, there was a significant difference between women of reproductive age and women at early postmenopause ($P = .007$), those at early and late postmenopause ($P = .004$), and women of reproductive age and women at late postmenopause ($P = .017$). Levels of IL-6 were also significantly higher among postmenopausal women with and without a history of pregnancies in comparison with women of reproductive age ($P < .0001$). In men, IL-6 levels were high in all 3 age groups considered without significant differences between each group. They were also significantly higher than in each female group evaluated ($P < .0001$ for all combinations) but nulliparous postmenopausal women ($P = .025$, $P = .098$, and $P = .393$ for men younger than 45 years, men 50–55 years old, and men older than 60 years, respectively). (*B*) Serum levels of TNF-α and IL-6 in paired sera from 20 women, in whom sera were both obtained during reproductive age, and 19 women before menopause and after menopause. Sera were obtained at a median interval of 3 years before menopause and 2 years after menopause. (*C*) *Panel i* shows hepatic TNF-α expression. Women with CHC were stratified by reproductive status into reproductive age, early postmenopause, or late postmenopause groups. A significant and marked increase in hepatic levels of TNF-α was observed in women at early postmenopause compared with women of reproductive age and with women at later stages of menopause. The difference between women of reproductive age and women at late stages of menopause was not statistically significant. *Panel ii* shows TNF-α expression in paired liver biopsy specimens. In 20 women, both biopsy samples were collected while they were of reproductive age, whereas for the other 19 women, the first biopsy sample was collected at a median of 3 years before menopause and the other was collected 2 years after menopause. Biopsy samples were obtained at the same time points as the sera collected for analysis of cytokine levels (*B*).

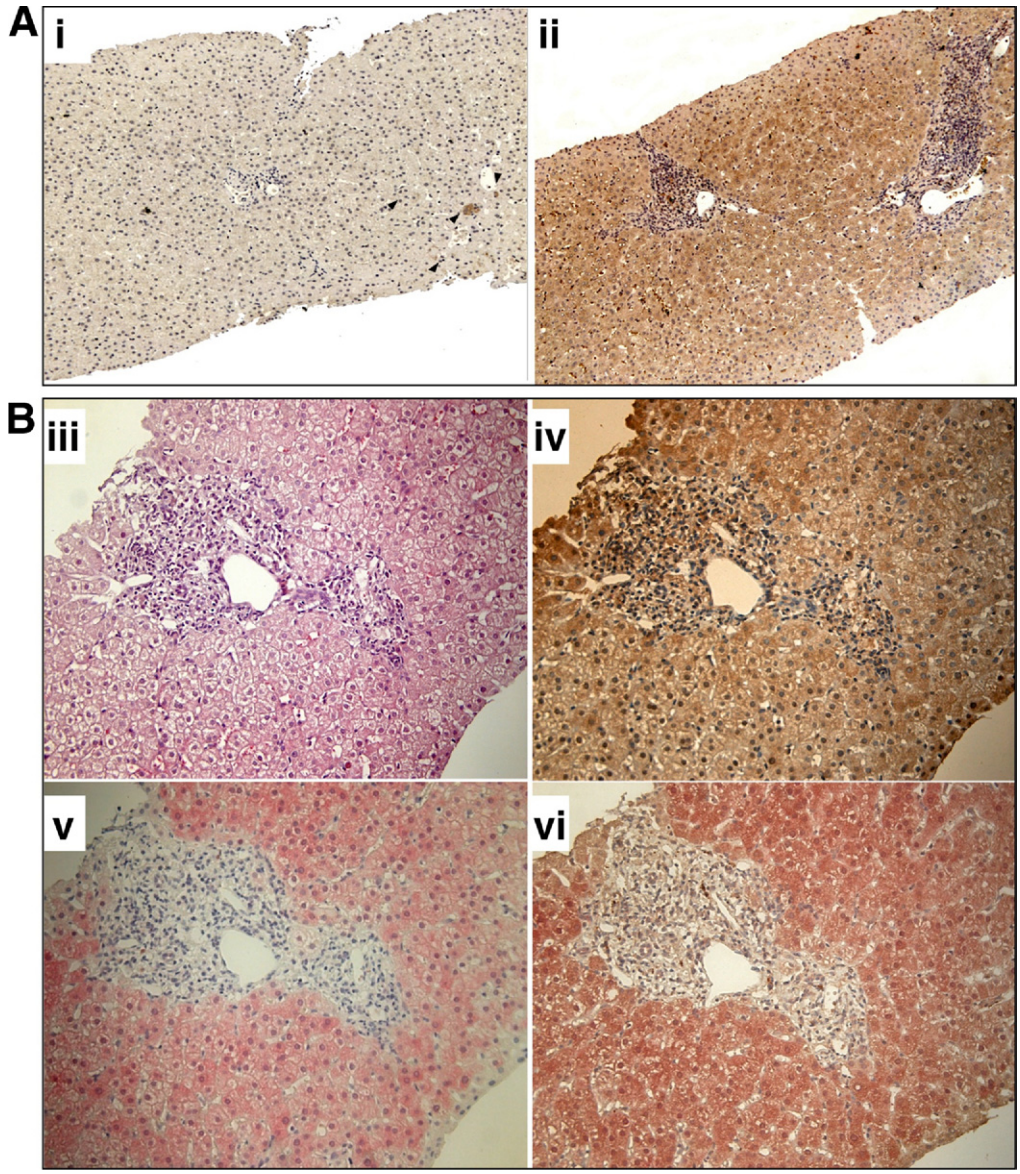


Figure 2. Immunohistochemical evaluation of TNF-α in liver biopsy specimens from women with CHC. (*A*) Immunohistochemical evaluation of TNF-α. Representative staining for TNF-α in 2 biopsy specimens from the same patient with CHC, obtained (*i*) 3 years before and (*ii*) 2 years after menopause. In the first biopsy specimen, there are few positive hepatocytes (*arrowheads*), whereas in the second there was strong TNF-α expression in the cytoplasm of hepatocytes, spread over the entire lobule. (*B*) Representative staining of TNF-α and SOCS3 expression in the liver of a postmenopausal, genotype 1 nonresponder (biopsy specimen was obtained 2 years after menopause). (*iii*) H&E staining, (*iv*) TNF-α, and (*v*) SOCS3. There was strong and diffuse immunohistochemical positivity for both TNF-α and SOCS3; double staining for both proteins showed colocalization of TNF-α and SOCS3 in about 60% of hepatocytes (*vi*).

were significantly higher among men ($P < .0001$ for all combinations but vs nulliparous menopausal female patients: $P = .025$, $P = .098$, and $P = .393$ for men younger than 45 years, men 50–55 years old, and men older than 60 years, respectively).

Immunohistochemical Evaluation of TNF-α and SOCS3

Control liver tissue was negative or had very slight TNF-α staining. TNF-α positivity increased significantly after menopause, with the highest values present in biopsy specimens taken shortly after the occurrence of menopause (liver biopsy specimens scored +/++ for TNF-α: nonmenopausal vs menopausal: 21 of 93 [22%] vs 36 of 84 [43%]; $P = .007$) (Figure 1*C*, *panel i*).

No significant differences were observed in levels of TNF-α between 20 paired liver biopsy specimens collected from women of reproductive age (Figure 1*C*, *panel ii*), whereas in the 19 paired liver specimens taken before and after menopause, staining intensity and the number of positive hepatocytes increased after menopause (Figure 1*C*, *panel ii*; Figure 2*A*). Differences in TNF-α scores correlated with circulating levels of

IL-6 (2.5 ± 1.0 vs 20.6 ± 41.8 pg/mL in specimens staining negative vs positive for TNF-α; $P = .045$).

Staining for SOCS3 was absent from normal liver samples; it was also absent, or very weak, in samples from women of reproductive age and strong to very strong in samples from postmenopausal women (reproductive age vs menopausal: 17 of 93 [18.2%] vs 39 of 84 [46.6%]; $P < .0001$). TNF-α and SOCS3 were colocalized in 60% ± 10% of hepatocytes (Figure 2*B*).

Discussion

In this prospective study of 1000 consecutive patients with CHC, we found that menopause is independently associated with the severity of liver damage and with a remarkably lower likelihood of achieving SVR.

Various lines of evidence support a link between menopause and the severity of fibrosis in patients with CHC. Recent studies suggest that postmenopausal women have accelerated progression of fibrosis,[6,7] which is prevented by long-term estrogen exposure from HRT.[7] In this large cohort of female patients with HCV, we confirmed that liver inflammation and higher levels of GGT (a known surrogate marker of metabolic alterations and of TNF-α up-regulation)[21] are independently associated with severe fibrosis. We also identified the length of estrogen deprivation as a strong independent risk factor for fibrosis; the longer the menopausal period, the higher the risk of severe fibrosis. It was 5-fold higher in women who had been menopausal for more than 10 years in comparison with early menopausal women.

We found menopause to be significantly correlated with necroinflammation, steatosis, and metabolic alterations (high cholesterol and glucose levels). Accordingly, a recent clinical study showed a higher prevalence of steatosis in postmenopausal patients with CHC who were older than 55 years.[13] Experimental studies are in line with our clinical data on the association between menopause and liver damage, showing that menopause is associated with a proinflammatory state that can drive fibrosis progression and lead to hepatocellular carcinoma.[8] In addition, some experimental data also indicate a potential role of estrogen insufficiency in steatogenesis.[22,23]

Menopause coincides with older age, which has been identified as a negative predictive factor for an SVR.[13,14] However, the influence of menopause itself on response to antiviral therapy has not been investigated. We examined age and menopause in multivariate analysis and found that age was not independently correlated with SVR, whereas menopause was. Our findings indicate that menopause is associated with a remarkable and unrecognized resistance to antiviral therapy, especially in carriers of genotype 1 HCV. Moreover, our data indicate that early stages of menopause (estrogen deprivation for <5 years) correlate with failure of antiviral therapy. This is particularly evident in women with genotype 1 HCV, who have less resistance to antiviral therapy with the passage of years after the onset of menopause. A possible explanation for this finding resides in the marked up-regulation of hepatic TNF-α and circulating IL-6 occuring at the time of menopause (potentially but unlikely due to occasional NSAID assumption or hidden alcohol use). Levels of both cytokines decrease after menopause, and hepatic expression of TNF-α levels become nonsignificantly different from those of women of reproductive age (Figure 1*C*, *panel i*). A similar phenomenon has been described regarding bone loss and levels of IL-6, which affect bone loss less with time after menopause, becoming insignificant after 10 years.[24] It is interesting that in men, who had levels of cytokines that were constant yet greater than those of women at all age groups tested, the OR of not achieving an SVR was not significantly different among those younger than 45 years or older than 55 years (ie, in 2 age groups similar to those of women of reproductive age or menopausal women).

Although our study was not designed to clarify the pathogenesis of this association, the results do suggest several hypotheses. We focused our attention on levels of TNF-α and IL-6; these cytokines undergo large changes during menopause[25] and in HCV infection, during which their levels are greatly up-regulated,[26–29] and they are able to interfere with antiviral response. Our data show that the occurrence of menopause is associated with a large additional increase in circulating IL-6 levels, a striking increase in hepatic TNF-α levels, and an increase in the expression of SOCS3 in the liver. Although there are no reports linking serum IL-6 levels with resistance to IFN, TNF-α has been implicated as an independent factor associated with response to IFN,[21,29,30] and hepatic SOCS3 is reportedly the strongest factor influencing the outcome of interferon-based antiviral therapy,[31–34] especially in patients with genotype 1.[33] SOCS3 is induced by HCV core protein[35] and also by IL-6 and TNF-α.[31,32] These data support the hypothesis that menopause determines a switch to a systemic and hepatic proinflammatory state[25] in which increased IL-6 and TNF-α production contributes to the observed resistance to IFN-based therapies. Women entering menopause would rapidly go from an estrogen-protected environment, where HCV-mediated inflammation is limited, to an estrogen-deprived one in which inflammation becomes less controlled and resistance to antiviral therapy increases remarkably, thus increasing the risk of developing severe fibrosis.

We have also confirmed that genotype 1 and higher GGT levels are independent negative predictors of SVR, both for the entire cohort and when women are considered as a group. Although hidden alcohol or drug abuse cannot be excluded in principle to explain changes in GGT levels, the bias, if present, was equally shared by all groups, with the methodology of ascertainment the same

for all. More relevant, in this context, is the demonstrated relationship between GGT levels and hepatic TNF-α messenger RNA, which in turn is strongly related to nonresponse in patients with HCV.[21]

The study has some limitations. In particular, we cannot rule out that lack of data on vitamin D serum levels, a recently recognized factor influencing achievement of an SVR,[36] could affect the interpretation of our results, as well as the absence of data about other adipokines/cytokines.

In conclusion, our data from a large cohort of European women with CHC show that menopause is associated with profound changes in TNF-α and IL-6 levels. These changes are greater than those resulting from HCV infection in women of reproductive age and result in a more pronounced inflammatory state, more rapid progression to fibrosis, and a hitherto unrecognized resistance to antiviral therapy. This suggests that CHC in women should be treated early, disregarding the fact that liver disease is milder in women of reproductive age, as this condition will last only as long as the estrogen-exposed period. Alternative strategies should be tested for HCV-positive women presenting in early postmenopause. We are currently examining the combination of HRT with pegylated interferon/ribavirin therapy in a controlled randomized trial (EudraCT 2008-001260-36).

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.12.027.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Table 1. Baseline Demographic, Laboratory, Metabolic, and Histologic Features of 1000 Patients With CHC According to Sex

Variables	Men (n = 558)	Women (n = 442)	*P* value
Mean age at enrollment (*y*)	47.9 ± 11.6	51.9 ± 11.3	<.001
Mean body mass index (kg/m^2)	26.3 ± 3.6	24.7 ± 3.8	<.001
Platelet count ($\times 10^3/mm^3$)	179.0 ± 56.5	203.4 ± 66.5	<.001
Alanine aminotransferase (*IU/L*)	98.6 ± 87.4	73.4 ± 67.4	<.001
GGT (*IU/L*)	57.1 ± 52.4	37.1 ± 37.3	<.001
Cholesterol (*mg/dL*)	161.9 ± 38.0	175 ± 36.5	<.001
Triglycerides (*mg/dL*)	103.4 ± 52.8	85.9 ± 39.3	<.001
Ferritin (*ng/mL*)	332.6 ± 328.0	139.5 ± 139.5	<.001
Blood glucose (*mg/dL*)	103.5 ± 19.9	99.9 ± 36.2	.713
Insulin ($\mu U/mL$)	6.1 ± 3.5	10.0 ± 6.4	.093
HOMA score	1.5 ± 0.7	2.6 ± 2.2	.124
Source of infection, n (%)			
Community acquired	370 (67.4)	309 (69.5)	.54
Posttransfusional	64 (11.7)	80 (18.1)	.002
Drug addiction	85 (15.4)	11 (2.5)	<.001
Parenteral exposure	30 (5.5)	42 (9.5)	.01
HCV RNA ($IU/mL \times 10^3$)	1418 ± 128	1436 ± 183	.92
Length of HCV infection (*y*)	14.1 ± 1.6	13.5 ± 2.2	.073
HCV genotype, n (%)			
1	313 (56.4)	252 (57.0)	.80
2	132 (23.8)	149 (33.7)	<.001
3	84 (15.1)	27 (6.1)	<.001
4	26 (4.7)	14 (3.2)	.29
Histology at biopsy, n (%)			
Steatosis			
<5%	328 (63.1)	261 (63.5)	.95
≥5% to <20%	150 (28.8)	116 (28.2)	.99
≥20%	42 (8.0)	34 (8.2)	.99
Grade of inflammation			
0–5	391 (71.89)	332 (80.2)	.018
6–11	128 (24.5)	74 (17.9)	
12–18	4 (0.8)	8 (1.9)	
Stage of fibrosis			
0–3	443 (84.4)	372 (80.6)	.020
4–6	82 (15.6)	43 (10.4)	
Cirrhosis	69 (12.3)	30 (6.7)	.003

NOTE. Data are given as mean ± SD unless otherwise noted.

Supplementary Table 2. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for SVR in 1000 Patients With CHC

	Univariate analysis		Multivariate analysis	
Variables	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (*y*)	1.020 (1.009–1.031)	<.001	1.010 (0.991–1.031)	.362
Sex (male/female)	0.882 (0.684–1.137)	.332		
Mean body mass index (kg/m^2)	1.003 (0.971–1.037)	.854		
Platelet count ($\times 10^3/mm^3$)	2.039 (1.483–2.803)	<.001	0.999 (0.995–1.000)	.761
Alanine aminotransferase (*IU/L*)	1.317 (1.023–1.695)	.030	0.996 (0.992–1.000)	.031
GGT (*IU/L*)	2.256 (1.745–2.916)	<.001	1.013 (1.006–1.021)	.001
Cholesterol (*mg/dL*)	0.997 (0.992–1.003)	.293		
Triglycerides (*mg/dL*)	0.999 (0.994–1.003)	.599		
Ferritin (*ng/mL*)	1.000 (1.000–1.001)	.379		
HOMA score	1.008 (0.998–1.018)	.118		
HCV RNA ($IU/mL \times 10^3$)	1.000 (1.000–1.000)	.048	1.000 (1.000–1.000)	.868
HCV genotype				
1–4 vs 2–3	4.784 (3.597–6.369)	<.0001	3.448 (2.538–6.896)	<.0001
Histology at biopsy				
Steatosis	1.104 (0.849–1.434)	.461		
Grade of inflammation	1.147 (1.086–1.211)	.000	1.076 (0.968–1.196)	.173
Stage of fibrosis	1.409 (1.260–1.575)	.000	1.071 (0.858–1.336)	.546
Cirrhosis	2.885 (0.979–8.501)	.055	1.261 (0.447–3.552)	.661