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Early Menopause is Associated with Lack of Response to Antiviral Therapy in Women with Chronic Hepatitis C

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Calogero Cammà: statistical analysis, analysis and interpretation of data, critical revision of the manuscript

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Abstract

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 virological response (SVR), and liver damage in patients with CHC.

Methods: We performed a prospective study of 1000 consecutive, treatment-naive patients ≥18 years old 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 (IL-6); and hepatic tumor necrosis factor-α (TNF-α).

Results: Post-menopausal 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*=.178). 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, 1.3643–.436), infection with hepatitis C virus (HCV) genotypes 1 or 4 (OR=3.861; 95% CI, 2.433–6.134), and cholesterol levels (OR=0.967; 95% CI 0.943–0.991). Early menopause was the only independent factor that predicted lack of an SVR among women with genotype 1 HCV infection (OR=3.933; 95% CI, 1.274–12.142). Baseline levels of liver inflammation, fibrosis, steatosis, serum IL-6 (*P*= .04), and hepatic TNF-α (*P*= .007) were significantly higher among post-menopausal 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.

KEY WORDS: HCV therapy; Prognostic factors; anti-viral therapy; menopause

Introduction

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 like age, duration of infection, or metabolic features are accounted for by multivariate analysis.[1,3] There are conflicting data about 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 whole, without taking into account differences in responses due to hormonal state.

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

Antiviral therapy greatly improves the natural course of CHC when it results in a sustained virological response (SVR),[10] even in patients with established cirrhosis.[11] Negative predictive factors for SVR include older age,[12-14] which might coincide with 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 histological features in a prospective study. We collected data on the type and timing of menopause, parity, and the use and duration of hormone replacement therapy (HRT) in a cohort of 1000 patients with CHC undergoing standard antiviral therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV).

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 HCV infection (any fibrosis stage, including compensated cirrhosis), a detectable plasma HCV RNA level and had not been previously treated for hepatitis C. All patients had undergone liver biopsy within 1 year prior to enrollment. All biopsies were reviewed and scored according to Ishak et al.[15] by a single pathologist (L.L.) unaware of patient identity or history. The percentage of hepatocytes containing macrovesicular fat was determined for each 10x 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, any other cause of liver disease, severe depression or psychiatric disorder, or active substance or alcohol consumption of <20g/day in the last year or more, evaluated by a specific questionnaire.

The study was approved by the institutional review boards at the two 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, body mass index (BMI), and in women only: occurrence, type (spontaneous, surgical) and age at menopause, length of estrogen deprivation, use and duration of HRT, number of full term pregnancies and of abortions. Menopause was defined as no menstrual periods for 12 consecutive months and was

defined as "early" when present for less than 5 years.

At the time of biopsy, serum levels of ALT, γ-glutamyl transpeptidase (GGT), alkaline phosphatase, platelet count, ferritin, glucose, and insulin were obtained. Insulin resistance (IR) 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 α-2a (Pegasys, Roche, Basel, Switzerland) 180 μg /week, or (2) pegylated interferon α-2b (Peg-Intron, Schering-Plough) 1.5 μg/kg/week 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). Sustained virological response (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 virological 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 IL-6 and TNF-á 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 biopsies from female patients (93 of reproductive age, 84 menopausal). Paired liver biopsies were collected from 39 women (for 20,

both biopsies were obtained at reproductive ages, and for 19, they were before and after menopause). Only 2 of the latter group (both non-responders) 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 ALT values and no evidence of HCV, HBV or HIV 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, Minneapolis, MN) or a rabbit polyclonal anti-human SOCS3 antibody (Abcam, Cambridge, UK), diluted 1:100 and incubated overnight at 4 C, after antigen retrieval in 10 mM citrate buffer pH 6.0 at 90°C for 45 min.[18,19] Antibody binding was detected using the HRP-polymer detection Kit (Biocare Medical, Concord, CA) for TNF-α, while the Dako REAL™ Detection Systems (Dako, Glostrup, Denmark) was used for SOCS3. Staining was scored as: negative/weak (-/+-) or moderately/strongly positive (+/++). Co-localization 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 ± standard error and categorical variables as frequency and percentage. The 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), 4) presence of steatosis and presence and the demographic, metabolic, and histological characteristics of patients. In the statistical models, the dependent variables were coded as 1=present versus 0=absent. We included all patients 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: less than 5 years, 5 to 10 years and more than 10 years), use and duration of hormone replacement therapy (HRT), 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 1.000 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 out 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 while 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 vs. those with spontaneous menopause (Table 1). Fifty-four of the women (19.7%) had a history of past estrogen or HRT starting soon after the onset of menopause and continuing for a median period of 5 years (range 1-20 years). In 51 of these women (94.5%), HRT was stopped a mean 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 histological liver damage than their counterparts who were of reproductive age (Table 1). An increase in the severity of fibrosis was evident between non-menopausal 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) to 2.4 (1.2), P = .009]; cirrhosis was present in 1.7% of non-menopausal women, 6.0% of early- and 11.0% of late-menopausal women (non menopausal vs late menopausal: P = .0014). Multivariate analysis showed that, in addition to necroinflammatory activity (OR 1.464, 95% CI 1.256–1.707, P < .001), low platelet levels (OR 0.976, 95% CI 0.966–0.986, P < .001) and elevated GGT (OR 1.010, 95% CI 1.003–1.018, P = .008), also a longer duration of estrogen deprivation (5 to 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 .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 necro-inflammatory activity, and a higher rate of steatosis compared with women of reproductive age (Table 1). Multivariate analysis for severe necro-inflammatory 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 to 10 years: OR 11.823, 95%CI 2.779-50.302, *P*= .001; more than 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 (OR 0.981, 95%CI 1.026-1.333, P P<.0001), BMI (OR 1.170, , 95%CI 1.003-1.062, P=.019) and duration of estrogen deprivation (< than 5 years OR 3,726, 95%CI 1.219-11.385, P=.021; 5 to 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 biopsies taken shortly before and after menopause were available for 19 women (median interval 3 years before and 2 years after); in other 20 women, paired biopsies were obtained while they were still of reproductive ages, 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. 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 unchanged in 7 (35.0%).

Results of antiviral treatment

Results are reported as Intention-to-treat analysis. Eight hundred thirty-eight patients completed the antiviral treatment program; forty-nine (4.9%) (34 men/15 women) withdrew because of side effects. SVR was achieved in 511 individuals (51.1%).

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%) vs. 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 ere 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 to 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, $P = .009$).

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 SVR (women of reproductive age vs. men: OR 0.452, 0.295-0.693, $P < .0001$; menopausal women vs. men: OR: 1.212, 95% CI 0.853-1.722, $P = .283$). Among men, risk of not achieving an SVR was similar after stratification for age groups and comparable to that of women of reproductive age (<45 years) or menopausal (>55 years) women (men aged <45 years vs. men>55 years: OR 1.204, 95% CI 0.964-1.502, $P = .101$). Percentage of SVR in men younger than 45 years was 59.2% and in those older than 55, 50.0% (p=.114).

By multivariate analysis significant independent baseline predictors of SVR failure in women were the presence of menopause (OR 1.802; 95% CI 1.154-2.813, $P = .01$), cholesterol

level (OR 0.997; 95% CI 0.943-0.991, *P* = .008), high GGT levels (OR 2.165; 95% CI 1.364-3.436, *P* = .001) and genotype 1/4 (OR 3.861, 95% CI 2.433-6.134) *P* = .006)(Table 4, top). Substituting "duration of estrogen deprivation" for "menopause" in the multivariate model, the OR of SVR failure for women who were post-menopausal for less than 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 categorical variable with the linear variable the latest remained significantly associated with lower SVR rate (OR 1.115; C.I. 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 to 10 years: 2.300 (95% CI 0.982-5.386, *P* = .055); more than 10 years 1.437 (0.743-2.781), *P* =.282. Similar to the entire population, in G1, when replacing length of estrogen deprivation as categorical variable with the linear variable the latest remained significantly associated with lower SVR rate (OR 1.088; C.I. 1.006-1.177).

Cytokines levels

Regardless of reproductive status, HCV-positive women had significantly higher serum levels of both TNF-α and IL-6 compared to controls (*P* < .0001 for all combinations) (Figure 1A). IL-6 levels in women of reproductive age were significantly lower than those in early post-menopausal 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 post-menopausal 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 post-menopausal 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 1A). Among menopausal women, but not women of reproductive age, baseline levels of IL-6

correlated with higher baseline necro-inflammatory scores (menopausal: OR 3.571, 1.494-8.536, P=.004; reproductive age OR .727, .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* = 0.395)(Figure 1B). The post-menopausal increase in IL-6 levels correlated with fibrosis progression (OR 3.333, 95%CI 1.293-8.591, *P* = .011) and with worsened necro-inflammatory 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 females stratified by hormonal status: <45 years (reproductive age); 50–55 years (early menopause); > 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, 29.5±7.5 and IL-6: 27.2±35.0, 29.0±42, 34±51 respectively, NS for all combinations). Levels of TNF-α did not differ significantly between men and women (NS for all combinations), whereas levels of IL-6 were significantly higher among men (p<.0001 for all combinations but vs. nulliparous menopausal females: p=.025, p=.098, p=.393 for <45 years; 50–55 and > 60 year-old men 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, the highest values being present in biopsies taken shortly after the occurrence of menopause [liver biopsies scored +/++ for TNF-α: non menopausal vs menopausal: 21 of 93 (22%) vs 36 of 84 (43%), *P* = .007)] (Figure 1C, panel a).

No significant differences were observed in levels of TNF-α between 20 paired liver biopsies collected from women of reproductive ages (Figure 1C, panel b), whereas in the 19 paired liver specimens taken before and after menopause, staining intensity and the number of positive hepatocytes increased after menopause (Figure 1C, panel b; Figure 2, panel 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 post-menopausal women [reproductive age vs menopausal: 17 of 93 (18.2%) vs. 39 of 84 (46.6%), $P < .0001$]. TNF-α and SOCS3 were co-localized in 60% ± 10% of hepatocytes (Figure 2, Panel 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 CHC patients. Recent studies suggest that post-menopausal 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 HCV patients, 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 necro-inflammation, steatosis and metabolic alterations (high cholesterol and glucose). Accordingly, a recent clinical study showed a higher prevalence of steatosis in post-menopausal CHC patients who were more than 55 years old.[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 pro-inflammatory state that can drive fibrosis progression and lead to HCC.[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 a 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 less than 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 is that hepatic levels of TNF-α and circulating IL-6 are upregulated at the time of menopause. Another, unlikely, explanation is that occasional consumption of NSAIDs or unreported use of alcohol, causes levels of both cytokines to decrease after menopause, and hepatic expression of TNF-α levels become non-significantly different from those of women of reproductive age. (Figure 1C, panel a). 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 (i.e. in 2 age groups similar to those of women of reproductive age or menopausal females).

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 in 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, a striking increase in hepatic TNF-α, and an increase in the expression of SOCS3 in the liver. While 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 pro-inflammatory state[25] in which increased IL-6 and TNF-α production contribute 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 GGT alteration, the bias, if present, was equally shared by all groups, the methodology of ascertainment being the same for all. More relevant, in this context, is the demonstrated relationship between GGTs levels and hepatic TNF-α mRNA, which in turn strongly related with non response in HCV patients[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 SVR achievement [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 post-menopause. We are currently examining the combination of HRT with PEG IFN/Ribavirin therapy in a controlled randomized trial (EudraCT 2008-001260-36).

1. Legends to Figures

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 Methods. Differences between controls and each group of CHC patients were significant at the <0.0001 level.

For levels of TNF-α, there were no significant differences between women of reproductive ages and post-menopausal 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 non significant between all 3 combinations).

For IL-6, there was a significant difference between women of reproductive age and women at early post-menopause (P = .007), those at early and late post-menopause (P = .004), and women of reproductive age and women at late post-menopause (P = .017). Levels of IL-6 levels were also significantly higher among post-menopausal women, with and without 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 post-menopausal women (p=.025, p=.098, p=.393 for <45 years, 50-55 and > 60 years-old men respectively).

Figure 1 - B

Serum levels of TNF-α and IL-6 in paired sera from 19 women before menopause and after menopause. Sera were obtained at a median interval of 3 years before menopause and 2 years after

menopause.

Figure 1 - C

Panel (a): Hepatic TNF-α expression. Women with CHC were stratified by reproductive status into those of reproductive age, early post-menopause, or late post-menopause groups. A significant and marked increase in hepatic levels of TNF-α was observed in women at early post-menopause, compared to women of reproductive age and to 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 (b) TNF-α expression in paired liver biopsies. 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 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 (Figure 1B).

Figure 2

Immunohistochemical evaluation of TNF-α in liver biopsies from women with CHC. Panel A: Immunohistochemical evaluation of TNF-α. Representative staining for TNF-α in 2 biopsies from the same patient with CHC, obtained 3 years before (a) and 2 years after (b) menopause. In the first biopsy there are few positive hepatocytes (arrowheads) while in the second there is strong TNF-α expression in the cytoplasm of hepatocytes, spread over the entire lobule.

Panel B: Representative staining of TNF-α and SOCS3 expression in the liver of a post-menopausal, genotype 1 non responder (biopsy was obtained 2 year after menopause). H&E staining (c), TNF-α (d) and SOCS3 (e) There is strong and diffuse immunohistochemical positivity for both TNF-α and SOCS3; double staining for both proteins show co-localization of TNF-α and SOCS3 in about 60% of hepatocytes (f).

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Table 1 – Baseline Demographic, Laboratory, Metabolic and Histological Features of 442 Female Patients with Chronic Hepatitis C According to the Presence or Absence of Menopause.

Variables	Women of Reproductive Age (n=168)	Menopausal Women (n=274)	*P*
Mean Age at enrolment -y	40.3±8.4	59.0±5.4	< .001
Source of infection			
Community-acquired	118 (70.2)	191 (69.7)	.90
Post-transfusional	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	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*	
Spontaneous (n=220)		49.0±2.7*	
Length of Estrogen deprivation -y			
Overall	NA	11.4±6.3	
Surgical (n=54)		15.6±8.1*	-
Spontaneous (n=220)		10.3±5.3*	
History of Pregnancies	103 (61.3)	242 (88.3)	< .001
HRT	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
Platelets count X 10^3/mm^3	229±63.9	187±63.1	< .001
Alanine Aminotransferase – IU/L	65.3±58.2	78.3±70.0	.03
γ-glutamyl transpeptidase – 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 – µU/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 X 10^3	1.426± 4.230	1.455± 3.402	.88
HCV Genotype			
1-4	97 (57.7)	169 (61.7)	
2-3	71 42.39	105 (38.3)	.425
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			
Steatosis:			
<5%	117 (70.4)	158 (58.5)	
≥5% to <20%	40 (24.0)	86 (31.8)	.034
≥20%	9 (5.4)	26 (9.6)	
Grade of Inflammation	138 (86.7)	194 (76.0)	

0-5	20 (12.5)	54 (21.1)	.021
6-11	1 (0.6)	7 (2.7)	
12-18			
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

Abbreviation: y, years; HRT, hormonal replacement therapy; IU, international units; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid; NA: not applicable.

Data are given as mean ± standard deviation or as number of case (%).

* $P < .0001$

Table 2. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Severe Fibrosis (F4-F6) in 442 Female Patients with Chronic Hepatitis C.

Variables	Univariate Analysis		Multivariate Analysis	
	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 (.009-18.899)	.926
Length of Estrogen deprivation by menopausal length -y				
<5 years	2.576 (0.556-11.927)	.226	0.848 (0.135-5.317)	.860
5-10 years	7.110 (2.099-24.082)	.002	4.930 (1.176-20.668)	.029
≥10 years	9.038 (3.079-26.530)	<.0001	6.377 (1.912-21.265)	.003
Estimated duration of HCV infection	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
Platelets count X 10^3/mmc	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 X 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

Abbreviation: y, years; IU, international units; GGT: γ-glutamyltranspeptidase; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid.

Table 3. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Severe Necroinflammatory activity in 442 Female Patients with Chronic Hepatitis C.

Variables	Univariate Analysis		Multivariate Analysis	
	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 years	4.115 (1.707-9.923)	.002	3.490 (0.881-13.921)	.075
5-10 years	2.993 (1.246-7.189)	.014	11.823 (2.779-50.302)	.001
≥10 years	3.778 (1.8227.833)	<.0001	9.292 (2.531-34.111)	.001
Estimated duration of HCV infection	1.044 (0.981-1.111)	.174		
Mean Body Mass Index – Kg/m^2	1.032 (0.965-1.103)	.362		
Platelets count X 10^3/mmc	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 X 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 (1901-3.209)	<.0001	3.524 (1.794-6.923)	<.0001

Abbreviation: y, years; IU, international units; GGT: γ-glutamyltranspeptidase; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid.

Table 4. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Sustained Virological Response Failure in 442 Female Patients with Chronic Hepatitis C.

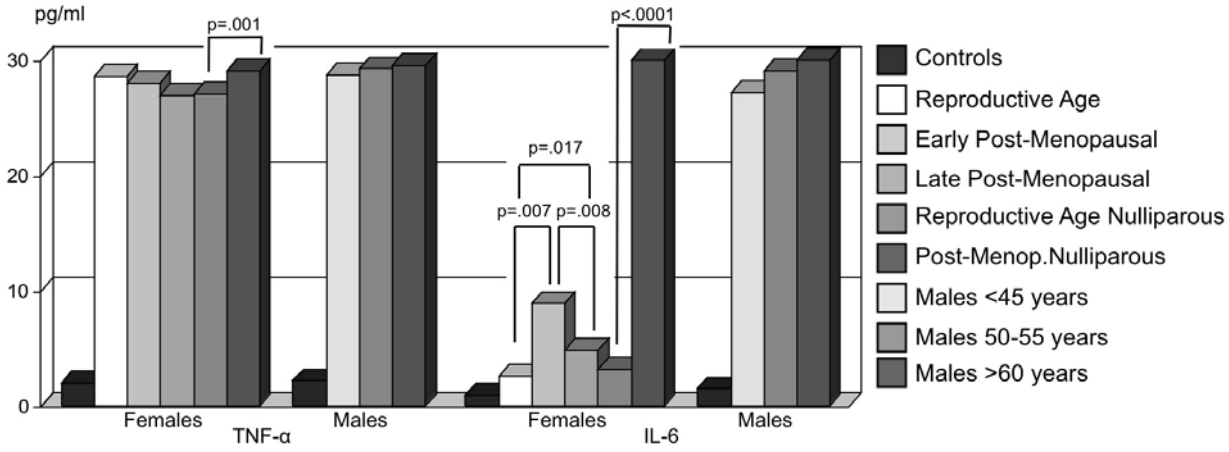
Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Entire Female Cohort (n=442)				
Mean Age - y	1.033 (1.015-1.052)	<.0001	0.983 (0.950-1.068)	.686
Menopause	2.436 (1.620-3.662)	<.0001	1.884 (1.177-3.016)	.008
Length of Estrogen deprivation -y	1.042 (1.015-1.070)	.002	1.115 (1.048-1.185)	.001
Length of Estrogen deprivation by menopausal length -y				
<5 years	2.497 (1-010-8.172)	.047	8.055 (1.834-25.390)	.006
5-10 years	1.295 (0.497-3.375)	.597	1.683 (0.335-8.458)	.527
≥10 years	2.374 (1.137-4.354)	.021	4.277 (0.747-24.503)	.103
Estimated duration of HCV infection	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		
Platelets count X 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 X 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) .063
Stage of Fibrosis	1.494 (1.246-1.793) <.0001	1.079 (0.839-1.388) .553
Cirrhosis	0.823 (0.206-3.292) .783	
Women with Genotype 1 HCV Infections (n=252)		
Mean Age -y	1.032 (1.010-1.055) .005	0.972 (.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 (1.008.1.0086) .048	1.088 (1.006-1.177) .035
Length of Estrogen deprivation by menopausal length -y		
<5 years		
5-10 years	4.833 81.83-21.561) .038	3.933 (1.274-12.142) .017
≥10 years	2.071 (0.565-7.593) .272	2.300 (0.982-5,386) .055

	2.201 (0.865-5.602)	.098	1.437 (0.743-2,781)	.282
Estimated duration of HCV infection	1.017 (0.961-1.077)	.559		
Mean Body Mass Index – Kg/m^2	0.970 (.878-1.072)	.552		
Platelets count X 10^3/mmc	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 X 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		

Abbreviation: y, years; IU, international units; GGT: γ-glutamyl transpeptidase; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid.

A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p<.0001
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30
20
10
0
Females
Males
Females
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TNF-α
IL-6
Controls
Reproductive Age
Early Post-Menopausal
Late Post-Menopausal
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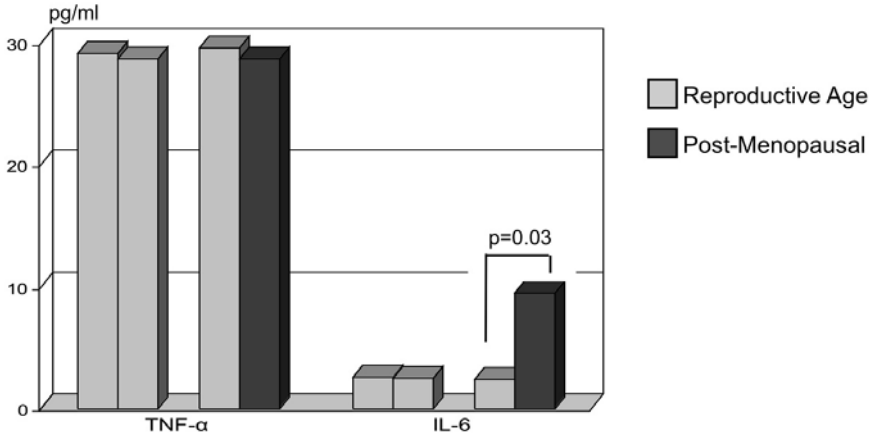
Post-Menop.Nulliparous
Males <45 years
Males 50-55 years
Males >60 years

B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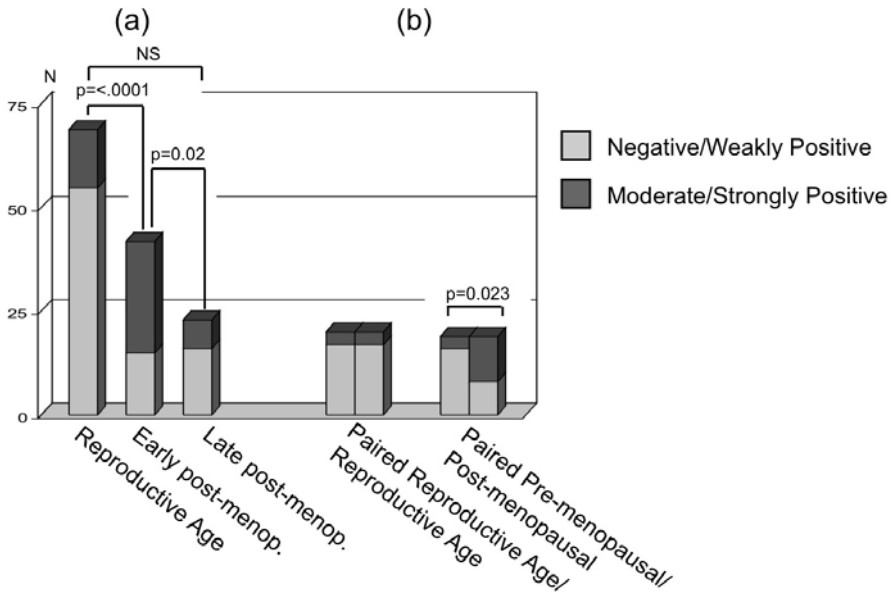
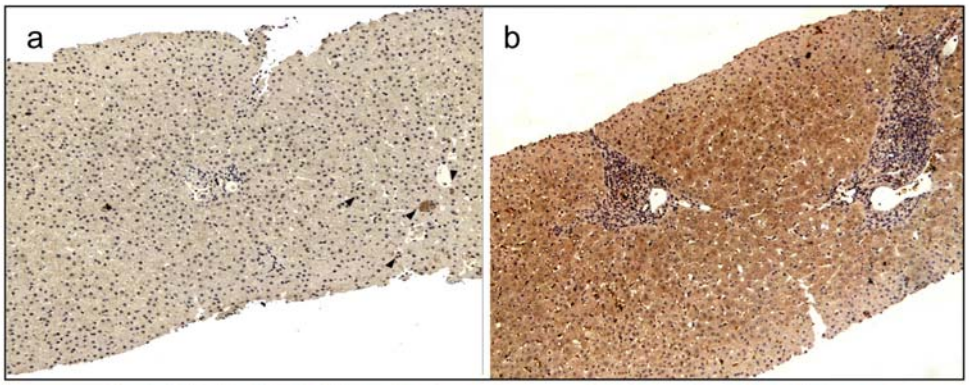
20
10
0
p=0.03
TNF-α
IL-6
Reproductive Age
Post-Menopausal

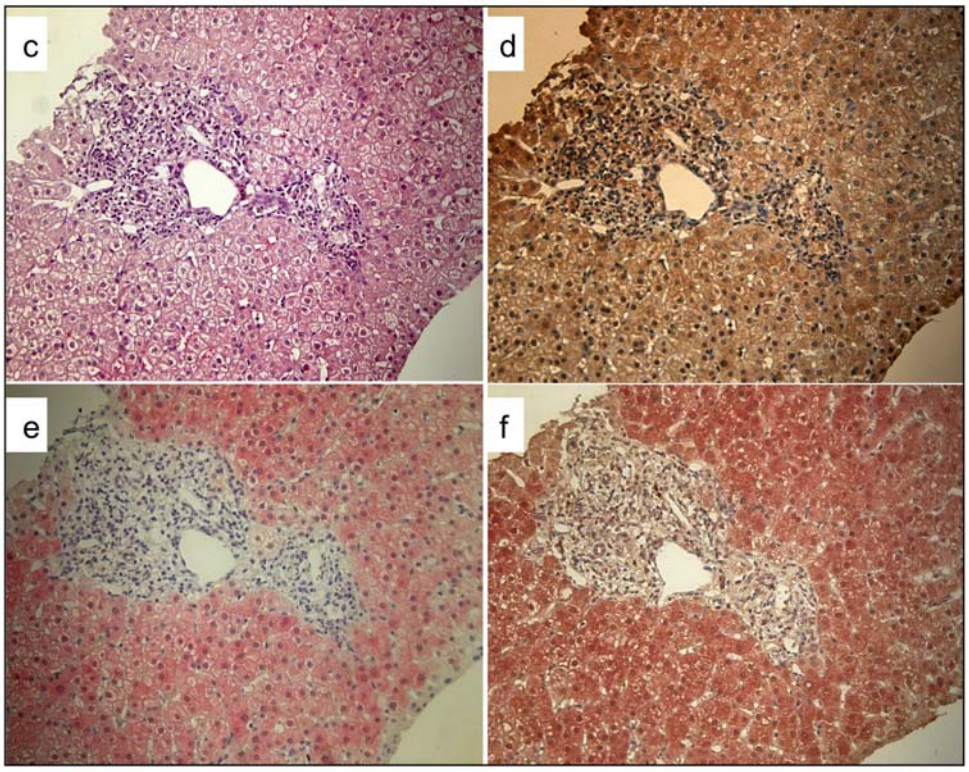
C


(a)
(b)
N
NS
p=<.0001
p=0.02
p=0.023
75
50
25
0
Reproductive Age
Early post-menop.
Late post-menop.
Paired Reproductive Age
Paired Reproductive Age/ Post-menopausal
Paired Pre-menopausal/
Negative/Weakly Positive
Moderate/Strongly Positive

Panel A



Panel B



Supplementary Table 1. Baseline Demographic, Laboratory, Metabolic and Histological Features of 1000 Patients with Chronic Hepatitis C According to Gender.

Variables	Men (n=558)	Women (n=442)	p
Mean Age at enrolment - years	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
Platelets count X 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 – µU/mL	6.1±3.5	10.0±6.4	.093
HOMA-score	1.5±0.7	2.6±2.2	.124
Source of infection			
Community-acquired	370 (67.4)	309 (69.5)	.54
Post-transfusional	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 X 10^3	1,418 ±	1,436 ±	.92
Length of HCV infection (years)	14,1 ±1.6	13,5 ±2.2	.073
HCV Genotype			
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			
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)	
6-11	128 (24.5)	74 (17.9)	.018

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

Abbreviation: y, years; IU, international units; GGT, γ-glutamyltranspeptidase; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid. Data are given as mean ± standard deviation or as number of cases (%).

Supplementary Table 2. Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Sustained Virological Response in 1000 Patients with Chronic Hepatitis C.

Variables	Univariate Analysis OR (95% CI)	*P* value	Multivariate Analysis OR (95% CI)	*P* value
Age - y	1.020 (1.009-1.031)	<.001	1.010 (0.991-1.031)	.362
Gender Male/Female	0.882 (0.684-1.137)	.332		
Mean Body Mass Index – Kg/m^2	1.003 (0.971-1.037)	.854		
Platelets count X 10^3/mm^3	2.039 (1.483-2.803)	<.001	0.999 (.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 X 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

Abbreviation: y, years; IU, international units; GGT: γ glutamyltranspeptidase; HOMA, homeostasis model assessment; HCV-RNA, hepatitis C virus ribonucleic acid.