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Hepatocyte steatosis in patients infected with genotype 1 hepatitis C virus

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Abstract

Background: Recent findings suggest a higher prevalence of hepatic steatosis in patients with chronic hepatitis C, estimated at 50%. Both host and viral factors contribute to the development of steatosis in chronic hepatitis C. Steatosis is an initial stage, which promotes hepatic fibrosis through oxidative stress. *Aim*: To assess the pathogenic mechanism of genotype 1 hepatitis C virus in steatosis and to evaluate the correlation between the degree of steatosis and the level of oxidative stress. *Patients and Methods*: The study was carried out on 50 patients (29 males, 21 females) with genotype 1 HCV and liver biopsy proven chronic hepatitis C. Patients with other etiology of chronic liver disease were excluded. We statistically correlated the degree of steatosis with clinical (age, sex, waist circumferences) and biological parameters (alaninaminotransferase, gammaglutamyltranspeptidase – GGT, insulin, ferritin, serum viral load, oxidative stress). Insulin resistance (IR) was determined by the homeostasis model assessment (HOMA) method. The oxidative stress was estimated by serum malondialdehyde (MDA) and glutathione (GSH). *Results*: 27 patients presented steatosis (57%): 14 out of 29 men (48%) and 14 out of 21 women (66%); in two thirds of them, steatosis was moderate. Univariate analysis identified five parameters that significantly influenced steatosis: age >45 years, sex – female, IR (HOMA>2.5), BMI, central adiposity (as reflected by waist circumferences and high GGT-values). Multivariate analysis identified four significant parameters: sex – female, insulin resistance (HOMA>2.5), BMI>30 kg/m^2 and GGT>2N. No relationship was found between steatosis and viral replication. The study demonstrated a significant correlation between steatosis and IR on the one hand and between steatosis and liver fibrosis on the other hand (p<0.05). Liver fibrosis was significant correlated with the increase levels of free radicals (MDA>250 nmol/dL). *Conclusions*: The pathogenic mechanism of genotype 1 HCV in steatosis is independent from viral replication and it may be linked to virus induced metabolic abnormalities such as IR. More women (66%) than men (48%) developed steatosis. Increased levels of free radicals, correlated with moderate and severe steatosis suggest the intervention of oxidative stress in determining the hepatic lesions associated with steatosis.

***Keywords*:** hepatic steatosis, chronic hepatitis C, insulin resistance, viral steatosis, oxidative stress.

Introduction

Hepatitis C virus infection (CHC) represents a major cause of mortality and morbidity all over the world, at present 170 000 000 of people bearing the chronic infection given by this virus. The prevalence of the hepatitis C virus (HCV) in Romania is 4.9%.

Hepatic steatosis is a histological feature that is frequently encountered in C virus chronic infections. There are two forms of steatosis that may be found in patients infected with hepatitis C virus: metabolic steatosis and virus-induced steatosis [1–3].

Metabolic steatosis was described in patients infected with C virus who also possessed other metabolic disorders such as obesity, dyslipidemia and diabetes mellitus [4, 5]. This form is the consequence of the mitochondrial dysfunction involved in the process of beta-oxidation of the free fatty acids. Metabolic steatosis is not induced by the C virus itself, but the combination between this form of steatosis and the presence of the virus is associated with an accelerated progression towards fibrosis.

C virus-induced steatosis is determined by the direct effect of the virus, more frequently genotype 3a, and the severity of the steatosis is proportional with the level of viral replication [6, 7]. It has been noticed that in heaptitis caused by the genotype 3a C virus, steatosis improves and has a sustained virologic answer, a consequence of the combined antiviral treatment. After the virus is eradicated, the steatosis not only disappears but, furthermore, the recurrence of the disease is associated with the histological recurrence of steatosis [8].

It is difficult to estimate if the C virus induced steatosis and metabolic steatosis equally contribute to the progression of the disease in these patients. It is assumed that there is a significant correlation between the severity of steatosis and the extent of hepatic fibrosis. There are no studies at present that analyze the contribution of each form to the progression of the disease.

The purpose of the study is to establish the particular features of the C virus chronic infection, especially of the pathogenic mechanisms involved in the progression of hepatic lesions, the presence of steatosis, as well as its contribution to hepatic fibrosis.

Clinical studies based on large number of enrolled patients have indicated that hepatic steatosis is negatively influencing the answer of HCV to the antiviral treatment. It is assumed that the mechanism involved in the progression of fibrosis, process that is associated with steatosis, may imply the lipid peroxidation processes, with subsequent activation of the stellate cells that are mainly responsible for the intrahepatic collagen synthesis.

The first hypothesis that we seek to test is that steatosis arising in HCV chronic hepatitis is a multifactorial process that is significantly linked with all the factors that determine changes in lipid metabolism, independently of the viral infection. These elements include obesity, diabetes mellitus, alcohol consumption, dyslipidemia, cryoglobulins and metabolic syndrome with insulin resistance. Besides metabolic steatosis, the accumulation of triglycerides inside hepatic cells in HCV is the consequence of the direct cytopathogen effect of the virus on mitochondrial metabolism, effect that is correlated with specific features of the C virus: genotype, subtype, viral loading. No matter the cause behind it, steatosis is probably an initial stage in the development of the disease that acts as a trigger mechanism for hepatic fibrosis.

The first objective is to determine the risk factors associated with steatosis occurring in C virus chronic infection by assessing the correlation between the degree of steatosis and clinic parameters (age, sex, body weight, and alcohol consumption), biological parameters (insulin resistance, serum glucose level, lipid profile, cryoglobulins) and viral parameters (genotype, viral load).

The second hypothesis is that in all patients having a HCV chronic infection steatosis is an initial stage, which promotes hepatic fibrosis through oxidative stress. In order to test this hypothesis we wish to discover a correlation between the degree of steatosis and the level of oxidative stress.

Patients and Methods

Patients with HCV chronic infections were selected out of 308 patients who addressed to the University Hospital in Bucharest for hepatic cytolysis; 75 of them showed positive viral markers for hepatitis C virus and had the necessary investigations in order to classify their hepatic disorder. Inclusion criteria were:

1. Patients with chronic C virus infection without previous antiviral treatment;
2. Hepatic cytolysis (ALT>1.5×N);
3. Liver punction biopsy compatible with the histological diagnosis of chronic hepatitis regardless of the degree of fibrosis;
4. Hematological tests: ♀/♂ Hg>12/13 g/dL, leukocytes >3000/mm^3, PMN>1200, thrombocytes >100 000/mm^3.

HCV infection was defined by the presence in serum of anti HCV antibodies using the 3rd generation ELISA (enzyme-linked immunoabsorbent assay). C virus infection was confirmed by performing the COBAS TaqMan HCV-test – the test of *in vitro* amplification of the C virus nucleic acid, which uses the High Pure System Viral nucleic acid kit for manual preparation and the COBAS TaqMan 48 analyzer for automatic amplification and detection. The RNA titter was expressed in international units (IU/mL). The detection limit is >10 IU/mL with a positive rate of 95%.

Patients with other forms of chronic hepatic disorders (viral, metabolic and of autoimmune etiology) were excluded from the study.

All the patients included in the study were informed about the study protocol and all of them gave their consent in a written form in order to take part in the study. The research was carried out in conformity with the international provisions regarding medical ethics.

Study protocol

A research form was filled in for every patient, containing information on common risk factors, medical history, and lifestyle and associated conditions: smoking habit, hypertension, diabetes, overweight, hypercholesterolemia.

All the patients were classified according to the calculated body mass index (BMI [kg/m^2]) as follows: normal, BMI 18.5–24.9; weight excess, BMI 25.0–29.9; moderate obesity, BMI 30.0–34.9; severe obesity, BMI 35.0–39.9. In obese patients, the distribution of the body fat was established using waist circumference measurements. The waist was considered to be the lowest circumference between the inferior edge of the thoracic cage and the iliac crest when the measurements are performed in orthostatic position. According to the classification of Lean MEJ *et al.*, patients have been marked as having visceral obesity if the waist circumference was in women >88 cm, and in men >102 cm [9].

The biological parameters were prelevated from the blood of the patients after 24 hours of fasting and consisted of ALT, AST, glucose, gamma-glutamyltranspeptidase (GGT), HDL- and LDL-cholesterol, triglycerides, INR, bilirubin. All were measured on an automatic analyzer (Dimension). Serum ferritin levels were measured by chemiluminescence (Immulite).

Insulin determinations were performed using the method of indirect chemiluminescence (MEIA). Insulin resistance was calculated according to the HOMA score (homeostasis model assessment): the product between the serum glucose and insulin levels in basal conditions were divided at 22.5. For insulin resistance the cut-off value was considered to be 2.5.

The oxidative stress was estimated by determining the lipid peroxides for which the marker is malondialdehyde (MDA) using the method of high performance liquid chromatography (HPLC) which measures MDA in its free state. Also, another marker of oxidative stress was glutathione, which is a tripeptide considered to be the main non-enzymatic antioxidant. Thus, the Ellman's method was used. It is used to detect non-protein thiolic groups by measuring the mercapto-benzene acid with

the help of the spectrophotometer color reaction (with 2,4-dinitrobenzene).

In order to determine the genotype of the C virus the Linear Array Hepatitis C Genotyping test was used with AMPLICOR R HCV-test and COBAS AMPLICOR HCV Test version 2. The test uses the RNA reverse transcriptase to produce complementary RNA (cADN), the Polymerase Chain Reaction (PCR) to amplify the cDNA and hybridization methods to determine the HCV genotype. Serum RNA was detected with EDTA as an anticoagulant. The serum detection limit is >500 IU/mL with a positive response rate >95%; the clinic specificity of the test is >99.99%.

Histological assessment

The analysis of a liver sample obtained by liver biopsy punction (using a Menghini needle) was performed. The section was fixed in tamponated 10% formalin and immersed in paraffin in the laboratories of Pathologic Anatomy of the University Hospital in Bucharest. The dimension of the hepatic fragment was at least of 15 mm in order to examine at least 10 portal spaces. The diagnosis was established using Hematoxylin–Eosin stained cross-sections and was made complete with the help of special staining methods such as Van Gieson and Prussia blue. The histological examination was aimed to detect:

- The necroinflammatory activity (histological activity index was established according to the Knodell's classification;
- Fibrosis was stadialized according to the METAVIR system F0–F4;
- Steatosis was assessed according to the percent of hepatic cells that included macrovesicular drops of fat on each field, as follows:
  – 0, absent;
  – 1, light, 1–20% of the hepatocytes;
  – 2, moderate, 21–40% of the hepatocytes;
  – 3, severe, >40% of the affected hepatocytes.
- The iron deposits were determined using the Pearls staining method and were graded according to Brissot following a scale of 0–4.

Statistical analysis

For data interpretation and result comparison, statistical analysis was used. This established the contribution of clinical, biological and virologic parameters to the determination of steatosis and it correlated the degree of steatosis with the severity of hepatic fibrosis and the oxidative stress level. For this purpose, a database and a questionnaire were used. They were created with the help of Epi Data v. 3.0 ©Jens Lauritsen. The statistical package used was "SPSSv13 for Mac OSX" (©SPSS Inc., 1989–2006). The chosen statistic significance threshold was $p<0.05$.The statistic tests were used for:

- Testing the continuous, normally distributed variable means and estimating the confidence intervals 95%: two-samples, two-tailed *t*-test (pooled variances or separate variances).
- Mann–Whitney U-test for ordinal, non-normal distributed variables.
- One Way ANOVA for multiple average testing.
- For nominal and ordinal variables: Pearson χ^2-test; Fisher for expected values <5.
- Pearson χ^2-test for proportion testing and estimation of confidence levels 95%
- *R*-Pearson coefficient for correlations between continuous variables and Spearman's *rho* for non-parametric correlations.
- Cox-regression analysis used to estimate the Hazard Ratio for the risk factors as well as for the protection factors in the occurrence of the endpoints.

Results

Patients with HCV chronic infections were selected out of 308 patients who addressed to the University Hospital in Bucharest for hepatic cytolysis; 75 of them showed positive viral markers for hepatitis C virus and had the necessary investigations in order to classify their hepatic disorder. Fifty patients that fulfilled the inclusion criteria in the study were selected taking into consideration the criteria created in order to establish the particular features of the C virus and the pathogenic mechanisms involved in the occurrence of hepatic lesions. Thus, the incidence of HCV chronic infection represented 4.1% of the total hepatic disorders.

Epidemiologic features: among the patients under study, the ratio between genders was: 29 men and 21 women, and the average age 45 years (21–66 years). The highest incidence of the C virus chronic infection was encountered at the age interval ranging from 41–55 years.

Steatosis was present in 27 patients (57%), the distribution was the following: grade 1 – light 11/50 (22%), grade 2 – moderate 10/50 (20%), grade 3 – severe – 6/50 (12%).

Note that in the studied group, steatosis was a frequent histological feature, which was present in more than 50% of the patients, fact confirmed by the samples obtained by hepatic punction biopsies.

A number of 16/50 (32%) patients had an accompanying metabolic syndrome: seven patients (14%), obesity – BMI>30 kg/m^2, two (4%) – alcohol consumption, 4 (8%) – type 2 diabetes mellitus, 3 (6%) dyslipidemia.

Among the patients disclosing a metabolic syndrome, hepatic steatosis was present in 12 of the cases (24%): five (10%) – obesity, four (8%) diabetes mellitus, one (2%) alcohol consumption, two (4%), dyslipidemia (triglycerides >180mg/dL). The proportion of steatosis of metabolic origin was of 12/27 (44%).

Excluding the patients with associated metabolic factors, a number of 15 patients (30%) presented hepatic steatosis. The proportion of viral steatosis was of 66%. All the patients of the study had genotype 1. Note the fact that although all the patients had genotype 1, viral-induced steatosis was leading (66%) regardless of the metabolic status of the host.

The clinical, epidemiologic and biological features of the patients depending on the degree of steatosis are listed in Table 1.

Table 1 – *Patient' characteristics according of steatosis' degree*

Variables	Absence	Light steatosis	Moderate steatosis	Severe steatosis
No. of cases	N=23	N=11	N=10	N=6
Average age [years]	45.3±10.6	43.2±11.2	52.3±9.9	54.1±11.9
Age [years]				
<45	12 (52%)	4 (36%)	2 (20%)	0
>45	11 (48%)	7 (44%)	8 (80%)	6 (100%)
Sex				
Men	15 (52%)	8 (27%)	4 (14%)	2 (7%)
Women	7 (33%)	2 (10%)	6 (28%)	6 (28%)
BMI [kg/m^2]				
<25	15 (66%)	7 (44%)	5 (50%)	0
25–29	8 (34%)	3 (27%)	3 (30%)	2 (23%)
>30	2 (9%)	0	1 (10%)	4 (67%)
Waist [cm]				
<88	8	1	0	0
>88	0	2	6	4
<102	15	1	0	0
>102	0	7	4	2
ALT	3×N	3.1×N	2.9×N	3.2×N
GGT	1.2×N	1.5×N	1.9×N	2.2×N
Glucose [mmol/L]	4.63±0.82	4.52±0.91	4.62±0.85	4.86±0.76
Ferritin [ng/mL]	250±165	307±182	326±176	332±297
HOMA	2.3±2.0	2.4±2.1	2.5±2.2	3.4±3.1
Cholesterol [mg/dL]	195±32.7	192±31.4	195±36.9	184±34.2
Triglycerides [mg/dL]	103±43.4	105±46.1	108±44.2	117±52.3
Log_{10} HCV RNA [IU/mL]	12.5±2.1	12.7±2.4	12.8±1.8	12.5±2.3

As observed from the table, there were no significant differences of the biological values between the different forms of steatosis. The only exceptions were the ferritin, GGT values and the HOMA net superior scores in patients with steatosis, proportional with its degree. Also, the total cholesterol was not modified and neither the LDL or HDL fractions were different among the groups of steatosis. Liver steatosis was in 81% (22/27) of cases light or moderate.

Two patients with high triglyceride levels in the serum have shown moderate steatosis at hepatic biopsies. There were no significant differences of the other hepatic samples and 12 (24%) of the patients revealed the presence of cryoglobulins in their serum.

Insulin resistance was present in 16 patients (32%) and out of the 27 cases of steatosis 13 (48%) disclosed hyperinsulinemia through peripheral resistance. The cut-off value of the HOMA test was considered 2.5. The HOMA score was net superior in patients with steatosis and it was directly proportional with its severity. The HOMA score estimates insulin resistance. An important pathogenic mechanism involved in the occurrence of steatosis in HCV chronic infections would be hyperinsulinemia that is secondary to peripheral insulin resistance.

Steatosis was more frequent in women (66%) than in men (48%). Also in women, moderate forms were prevalent (28%) and severe (28%) while in men the predominant form was the light one (27%). Also, steatosis was not associated with viral replication, viral RNA having comparable values regardless of the presence or absence of hepatic steatosis and its severity.

Histological features

The distribution of histological scores is shown in Table 2.

Table 2 – *Patient distribution according to fibrosis stage, activity grade and steatosis*

Features	Score	Total no.	%
Inflammation	8	11	22
	9	18	36
	10	10	20
	11	8	16
	12	3	6
Fibrosis	0	0	0
	1	4	8
	2	35	70
	3	10	20
	4	1	2
Steatosis	0	23	46
	1	11	22
	2	10	20
	3	6	12

It is noted that the majority of patients had moderate necroinflammatory activity (score 9) and the prevalent degree of fibrosis was 2 (70%). Iron deposits were absent in 23 (63%) of the patients, grade 1 in 10 (20%) patients, grade 2 in eight (16%) of the patients, grade 3 in five (10%) of the patients and grade 4 in four (8%) of the patients.

Factors associated with steatosis

In order to establish the predictive factors for steatosis in patients with hepatitis C virus, a univariate and multivariate analysis were performed. Univariate analysis compared the basal values between the patients with steatosis and the ones without steatosis. After the univariate analysis the predictive factors of the steatosis were consider to be the following: age >45 years, female sex, raised body mass index (BMI>30 kg/m^2), visceral obesity (waist >88 cm for women and >102 cm for men), HOMA score and raised values of GGT (Table 3).

Table 3 – *Predictive factors for steatosis in C virus chronic infection (univariate analysis)*

Variables	No. of patients	Steatosis (moderate and severe)	*p*
Age [years]			
<45	18	2 (11%)	<0.05
>45	32	14 (43%)	
Sex			
Men	29	6 (21%)	<0.05
Women	21	12 (67%)	
BMI [kg/m^2]			
<30	43	10 (23%)	<0.05
>30	7	5 (71%)	
Visceral obesity			
<88/102	9/16	0 (0) / 0 (0)	<0.05
>88/102	12/13	10 (83%) / 6 (46%)	

Variables	No. of patients	Steatosis (moderate and severe)	p
HOMA			
<2.5	23	3 (13%)	<0.05
>2.5	27	13 (49%)	
ALT			
<3×N	33	10 (33%)	ns
>3×N	17	6 (35%)	
Log_{10} HCV RNA [IU/mL]			ns
<12.7	24	7 (29%)	
>12.7	26	9 (34%)	
Cholesterol [mg/dL]			
>260	15	6 (40%)	ns
<260	35	10 (35%)	
GGT			
<1.8×N	34	0 (0)	<0.05
>1.8×N	16	16 (100%)	

Multivariate analysis confirmed as predictive factors the following: *female sex*: OR 5.2 95% CI (1.9–14.1), *HOMA score* >2.5 OR 4.1 95% CI (1.2–11.4), *BMI* >30 kg/m^2 OR 5.1 95% CI (1.3–12) and increased basal values of *GGT* >2 N (visceral obesity: OR 1.36; 95% CI 1.08–2.6) (Table 4).

Table 4 – ***Predictive factors for steatosis in C virus chronic infection (multivariate analysis)***

Variables	OR	95% CI	p
Female sex	5.2	1.9–14.1	<0.05
HOMA score >2.5	4.1	1.2–11.4	
BMI>30 kg/m^2	5.1	1.3–16	
Visceral obesity: Values > GGT >1.8×N	1.36	1.08–4.8	

Factors associated with fibrosis

Factors associated with fibrosis in HCV chronic infection were established using the uni- and multivariate analysis. According to the univariate analysis, the factors associated with fibrosis were: age >45 years, HOMA >2.5, moderate or severe steatosis, Log_{10} HCV RNA >12.7 UI/mL, GSH<40 μmol/dL, MDA>250 nmol/dL (Table 5).

Table 5 – ***Predictive factors for fibrosis in C virus chronic infection (univariate analysis)***

Variables	No. of patients	Fibrosis (moderate/severe)	p
Age [years]			
<45	18	1 (5%)	<0.05
>45	32	10 (30%)	
Sex			
Men	29	7 (24%)	*ns*
Women	21	4 (19%)	
HOMA			
<2.5	23	2 (9%)	<0.05
>2.5	27	9 (33%)	
ALT			
<3×N	33	9 (27%)	*ns*
>3×N	17	2 (12%)	
Log_{10} HCV RNA [UI/mL]			
<12.7	24	1 (4%)	<0.05
>12.7	26	10 (38%)	
Steatosis			
0.1	34	3 (9%)	<0.05
2.3	16	8 (50%)	
MDA [nmol/dL]			
<250	28	2 (7%)	<0.05
>250	22	9 (40%)	
GSH [μmol/dL]			
>40	31	3 (9%)	<0.05
<40	19	8 (42%)	

Multivariate analysis has shown as predictive factors for fibrosis: severe steatosis, GSH<40 μmol/dL and MDA>250 nmol/dL (Table 6).

Table 6 – ***Predictive factors for fibrosis in C virus chronic infection (multivariate analysis)***

Variables	OR	95% CI	P
Severe steatosis	15	3.3–44	<0.05
MDA>250 nmol/dL	5.2	1.9–22	
GSH<40 μmol/dL	6.9	6.2–21	

Discussion

In this study, factors associated with steatosis in patients bearing chronic hepatitis induced by the genotype 1 C virus were analyzed. The mechanism by which hepatitis C virus induces chronic, progressive hepatic lesions is not known. The absence of connection between the intrahepatic level of viral RNA and the necroinflamatory activity suggests an immunologic mediated pathogenic mechanism [10].

Hepatic steatosis, a common feature that was encountered in our study with a prevalence of 57%, supports the cytopathic effect of the C virus. All the randomized patients had genotype 1, a well-known condition for our country. The factors associated with hepatic steatosis described up to present include: chronic alcohol consumption, obesity, dyslipidemia, diabetes mellitus, drugs, and genotype 3 of the C virus. Metabolic factors were encountered in 16 patients (32%) and 12/27 (44%) of them had hepatic steatosis.

In our study, the measured Body Mass Index (BMI) was independently associated with steatosis. Similar results were reported by Hourigan LF *et al.* [11] while Adinolfi LE *et al.* [4] discovered a link between steatosis and central (visceral) obesity which was estimated by making waist measurements. In our study, both obesity as well as its visceral distribution were predictive factors for steatosis in the univariate analysis as well as in the multivariate one.

In order to estimate visceral obesity we have used as indicators the waist circumference: >88 cm in women and >102 cm in men, as well as the high values of gamma glutamiltranspeptidase (GGT). Stranges S *et al.* have proven that in both women and men there is a more tight association between abdominal circumference, which is a simple anthropometric indicator of visceral obesity, and the GGT values, compared to the association between visceral obesity and the global body mass index [12]. In our study, it was shown that patients with increased basal values of GGT had a higher probability for developing moderate or severe steatosis. The association between GGT and steatosis is probably linked to the association between the regional distribution of the fat and hepatic steatosis, regardless of the body mass global index. In a study that included 69 healthy, randomly selected patients [13] significant correlations were reported between the GGT values and the waist/hips ratio, regardless of the BMI. We have considered as well that GGT was a superior marker for the visceral and liver fat especially since it was present in patients with normal values of BMI, but with high values of the waist circumference. Our randomized

patients had no recent history of alcohol or drug consumption, which might have been responsible for the etiology of the increased values of GGT.

Diabetes mellitus plays a certain role in the etiology of hepatic steatosis [14, 15]; in our study, steatosis was present in all four diabetic patients, but the small number explains why the two conditions were not significantly associated after the analysis of Cox regression. Obesity represents a causal factor of the type 2 diabetes; the link between obesity and diabetes is so tight that it can conceal the link between diabetes and steatosis, independently of obesity. Diabetes determined by obesity induces hepatic steatosis and finally fibrosis and hepatic fibrosis may generate changes in lipid metabolism [16, 17].

Ceasing the significant alcohol consumption six months prior to the study was one of the inclusion criteria applied to the patients that were hospitalized in order to receive antiviral treatment, in order to achieve a better therapeutic compliance. Thus, it makes sense why there was no association between alcohol and steatosis, alcoholic steatosis being reversible after temperance.

After excluding all recognized cofactors, steatosis was present in 15/27 patients (66%). Previous studies have proved the fact that patients bearing genotype 3 and who obtain a sustained virologic answer after being subjected to the antiviral treatment show a significant decrease of steatosis, fact that was confirmed in repeated biopsy examinations. The conclusion drawn from these observations is that steatosis in genotype 3 is a consequence of the viral infection [8, 18, 19]. All these considering, we still do not know whether steatosis associated with genotype 1 is the consequence of the viral infection or of other non-viral factors such as metabolic ones.

"Viral" steatosis associated with genotype 3 is determined by the cytopathic effects of the virus and its proteins [1]. The genotype segregation of the types of steatosis cannot be absolute and there is a possibility that viral steatosis may be present in other genotypes as well, genotypes such as genotype 1. Supporting this concept it was shown in transgenic mice experiments that the core viral protein can interact directly with the intracellular lipids and produce steatosis, even when these proteins originate from genotype 1 [20]. The unique effect of the hepatitis C virus on the lipid metabolism is also sustained by the genetic analysis of the hepatocytes after the acute viral infection with the C virus in chimpanzee [4]. These studies have shown the modulating effect exerted over the genes involved in lipid metabolism, immediately following the infection, which is an argument in favor of the direct action of the virus and does not sustain the theory of the C virus acting through the agency of the hepatic lesions induced by it. Several mechanisms by which the C virus and its proteins induce steatosis have been proposed. Interference with a microsomial protein which transfers triglycerides is a theory that counts among these. This is a heterodimeric protein localized in the smooth sarcoplasmic reticulum of the hepatocytes and enterocytes, which plays a key role in apo β-lipoproteins assembly and secretion [21, 22]. This mechanism is sustained by the fact that drugs inducing hepatic steatosis determine a decrease in this transfer protein's activity followed by a decrease in the liver secretion of lipoproteins. In favor of this mechanism, patients in the study presented low serum levels of cholesterol proportional to the degree of steatosis [22].

The link we have discovered between steatosis and insulin resistance through univariate analysis as well as multivariate analysis is suggestive for the hypothesis that in C virus induced chronic hepatitis; IR represents a risk factor for steatosis. Recent studies revealed a clear link between HCV chronic hepatitis and insulin resistance, mediated by metabolic factors as well as viral factors [23–25]. The prevalence of the IR in chronic hepatitis shows geographical variations between 20–70%. In our study, IR was present in 16 patients (32%) [25]. Among the patients with steatosis 13 (48%), disclosed insulin resistance. A tight association between IR and C virus infection was observed on one hand and on the other hand, an association between insulin resistance and steatosis was noticed. It is worth mentioning that IR was encountered in the presence as well as in the absence of metabolic factors. This last argument suggests a direct viral implication as well, not only through the agency of metabolic factors. Recent data reveal the fact that hepatic insulin resistance is responsible for the accumulation of free fatty acids in the liver cells, such as triglycerides [26–28]. Also, hepatic resistance to insulin is exacerbated by the systemic resistance to insulin and by inadequate usage of sugars and free fatty acids in the skeletal muscle and the adipose muscle. The consequence of the hepatic insulin resistance – inadequate signal transduction of insulin in the liver cell-leads to cellular and molecular changes that intensify the triglyceride depositing in the liver cell [29–31]. The association between insulin resistance/steatosis is important because of the cumulated risk for fibrosis and because of the reduction in the sustained virologic answer to antiviral treatment [32].

In women, hepatic steatosis was more frequent (66%) and more sever (28%) comparing to that occurring in men for whom the registered prevalence was (48%) and who suffered mild forms of the disease (27%). These differences regarding frequency and severity of steatosis between genders can be explained by the hormonal differences. Estrogens influence lipid homeostasis and adiposity distribution. In experiments with mice with estrogenic insufficiency, two conditions aroused: hepatic steatosis and obesity progressive with age. It is noted that out of the 12 patients with moderate and severe steatosis, eight had already reached menopause and did not receive any substitution treatments.

The lack of association between steatosis and viral replication encountered in our study suggests as a possible mechanism for steatosis the direct changes in lipid metabolism.

In this study, moderate and severe steatosis was independent risk factors for advanced fibrosis. Although recent studies have suggested that insulin resistance may contribute to the progression of fibrosis in HCV chronic hepatitis, in the study we have performed we have not found any direct association between the HOMA score and the severity of the hepatic fibrosis [32–34].

The studies performed in the last years tried to explain the mechanism by which steatosis associated with hepatitis C virus contributes to the emergence of hepatic lesions. The hypothesis by which progression towards fibrosis may occur through non-inflammatory mechanisms was postulated [35]. Such a mechanism might be represented by the C virus induced lipid peroxidation, followed by free radical arousal, secondary activation of the stellate cells and collagen production [36–38].

In order to support this hypothesis we have considered in our study that the increase of malondialdehyde (MDA), which is a marker of the oxidative stress, along with the decrease in glutathione (GSH), which is an antioxidant protector, to be suggestive for the process of lipid peroxidation. This process is situated at the origin of liver lesions induced by steatosis and is accompanied by the stimulation of fibrogenesis.

Conclusions

In conclusion, hepatic steatosis in chronic infection with C virus is a complex process to which both metabolic factors of the host with a confirmed role in steatosis induction as well as viral factors contribute. The predictive factors for steatosis in chronic infection with the C virus were: female gender, visceral obesity (raised values of GGT), global body mass index and insulin resistance. The significant relation between insulin resistance and chronic infection with the C virus genotype 1 sustains the implication of the virus in lipid metabolism of the hepatocytes having consequently secretion disorders of the lipid fractions and their accumulation within the liver cells. Increased levels of free radicals, correlated with moderate and severe steatosis suggest the intervention of oxidative stress in determining the hepatic lesions associated with steatosis. Moderate and severe steatosis have clinical importance, being associated with severe hepatic fibrosis and, implicitly, with an inefficient answer to antiviral therapy. Since hepatic steatosis represents a predictive indicator for the antiviral answer, getting to know the main causal factors and their mechanisms of action may create the premises for a pathogenic therapy along with the estrogenic one.

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References

[1] RUBBIA-BRANDT L, QUADRI R, ABID K, GIOSTRA E, MALÉ PJ, MENTHA G, SPAHR L, ZARSKI JP, BORISCH B, HADENGUE A, NEGRO F, *Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3*, J Hepatol, 2000, 33(1):106–115.

[2] ASSELAH T, RUBBIA-BRANDT L, MARCELLIN P, NEGRO F, *Steatosis in chronic hepatitis C: why does it really matter?*, Gut, 2006, 55(1):123–130.

[3] MORIYA K, YOTSUYANAGI H, SHINTANI Y, FUJIE H, ISHIBASHI K, MATSUURA Y, MIYAMURA T, KOIKE K, *Hepatitis C virus core protein induces hepatic steatosis in transgenic mice*, J Gen Virol, 1997, 78(Pt 7):1527–1531.

[4] ADINOLFI LE, GAMBARDELLA M, ANDREANA A, TRIPODI MF, UTILI R, RUGGIERO G, *Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity*, Hepatology, 2001, 33(6):1358–1364.

[5] MONTO A, ALONZO J, WATSON JJ, GRUNFELD C, WRIGHT TL, *Steatosis in chronic hepatitis C: relative contributions of obesity, diabetes mellitus, and alcohol*, Hepatology, 2002, 36(3):729–736.

[6] PERLEMUTER G, SABILE A, LETTERON P, VONA G, TOPILCO A, CHRÉTIEN Y, KOIKE K, PESSAYRE D, CHAPMAN J, BARBA G, BRÉCHOT C, *Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis*, FASEB J, 2002, 16(2):185–194.

[7] ABID K, PAZIENZA V, DE GOTTARDI A, RUBBIA-BRANDT L, CONNE B, PUGNALE P, ROSSI C, MANGIA A, NEGRO F, *An in vitro model of hepatitis C virus genotype 3a-associated triglycerides accumulation*, J Hepatol, 2005, 42(5):744–751.

[8] KUMAR D, FARRELL GC, FUNG C, GEORGE J, *Hepatitis C virus genotype 3 is cytopathic to hepatocytes: reversal of hepatic steatosis after sustained therapeutic response*, Hepatology, 2002, 36(5):1266–1272.

[9] LEAN MEJ, HAN TS, MORRISON CE, *Waist circumference as a measure for indicating need for weight management*, BMJ, 1995, 311(6998):158–161.

[10] WALSH MJ, JONSSON JR, RICHARDSON MM, LIPKA GM, PURDIE DM, CLOUSTON AD, POWELL EE, *Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signaling (SOCS-3) in patients with chronic hepatitis C, viral genotype 1*, Gut, 2006, 55(4):529–535.

[11] HOURIGAN LF, MACDONALD GA, PURDIE D, WHITEHALL VH, SHORTHOUSE C, CLOUSTON A, POWELL EE, *Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis*, Hepatology, 1999, 29(4):1215–1219.

[12] STRANGES S, DORN JM, MUTI P, FREUDENHEIM JL, FARINARO E, RUSSELL M, NOCHAJSKI TH, TREVISAN M, *Body fat distribution, relative weight, and liver enzyme levels: a population-based study*, Hepatology, 2004, 39(3):754–763.

[13] VAN BARNEVELD T, SEIDELL JC, TRAAG N, HAUTVAST JG, *Fat distribution and gamma-glutamyl transferase in relation to serum lipids and blood pressure in 38-year old Dutch males*, Eur J Clin Nutr, 1989, 43(12):809–818.

[14] PERRY IJ, WANNAMETHEE SG, SHAPER AG, *Prospective study of serum gamma-glutamyltransferase and risk of NIDDM*, Diabetes Care, 1998, 21(5):732–737.

[15] OHLSON LO, LARSSON B, SVÄRDSUDD K, WELIN L, ERIKSSON H, WILHELMSEN L, BJÖRNTORP P, TIBBLIN G, *The influence of body fat distribution on the incidence of diabetes mellitus. 13.5 years of follow-up of the participants in the study of men born in 1913*, Diabetes, 1985, 34(10):1055–1058.

[16] SANYAL AJ, CONTOS MJ, STERLING RK, LUKETIC VA, SHIFFMAN ML, STRAVITZ RT, MILLS AS, *Nonalcoholic fatty liver disease in patients with hepatitis C is associated with features of the metabolic syndrome*, Am J Gastroenterol, 2003, 98(9):2064–2071.

[17] RATZIU V, MUNTEANU M, CHARLOTTE F, BONYHAY L, POYNARD T; LIDO STUDY GROUP, *Fibrogenic impact of high serum glucose in chronic hepatitis C*, J Hepatol, 2003, 39(6):1049–1055.

[18] POYNARD T, RATZIU V, MCHUTCHISON J, MANNS M, GOODMAN Z, ZEUZEM S, YOUNOSSI Z, ALBRECHT J, *Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C*, Hepatology, 2003, 38(1):75–85.

[19] PATTON HM, PATEL K, BEHLING C, BYLUND D, BLATT LM, VALLÉE M, HEATON S, CONRAD A, POCKROS PJ, MCHUTCHISON JG, *The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients*, J Hepatol, 2004, 40(3):484–490.

[20] ASSELAH T, RUBBIA-BRANDT L, MARCELLIN P, NEGRO F, *Steatosis in chronic hepatitis C: why does it really matter?* Gut, 2006, 55(1):123–130.

[21] CAMMÀ C, BRUNO S, DI MARCO V, DI BONA D, RUMI M, VINCI M, REBUCCI C, CIVIDINI A, PIZZOLANTI G, MINOLA E, MONDELLI MU, COLOMBO M, PINZELLO G, CRAXÌ A, *Insulin resistance is associated with steatosis in nondiabetic patients with genotype 1 chronic hepatitis C*, Hepatology, 2006, 43(1):64–71.

[22] KAWAGUCHI T, YOSHIDA T, HARADA M, HISAMOTO T, NAGAO Y, IDE T, TANIGUCHI E, KUMEMURA H, HANADA S, MAEYAMA M, BABA S, KOGA H, KUMASHIRO R, UENO T, OGATA H, YOSHIMURA A, SATA M, *Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3*, Am J Pathol, 2004, 165(5):1499–1508.

[23] HUI JM, SUD A, FARRELL GC, BANDARA P, BYTH K, KENCH JG, MCCAUGHAN GW, GEORGE J, *Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]*, Gastroenterology, 2003, 125(6):1695–1704.

[24] ROMERO-GÓMEZ M, DEL MAR VILORIA M, ANDRADE RJ, SALMERÓN J, DIAGO M, FERNÁNDEZ-RODRÍGUEZ CM, CORPAS R, CRUZ M, GRANDE L, VÁZQUEZ L, MUÑOZ-DE-RUEDA P, LÓPEZ-SERRANO P, GILA A, GUTIÉRREZ ML, PÉREZ C, RUIZ-EXTREMERA A, SUÁREZ E, CASTILLO J, *Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients*, Gastroenterology, 2005, 128(3):636–641.

[25] FARTOUX L, POUJOL-ROBERT A, GUÉCHOT J, WENDUM D, POUPON R, SERFATY L, *Insulin resistance is a cause of steatosis and fibrosis progression in chronic hepatitis C*, Gut, 2005, 54(7):1003–1008.

[26] LEANDRO G, MANGIA A, HUI J, FABRIS P, RUBBIA-BRANDT L, COLLOREDO G, ADINOLFI LE, ASSELAH T, JONSSON JR, SMEDILE A, TERRAULT N, PAZIENZA V, GIORDANI MT, GIOSTRA E, SONZOGNI A, RUGGIERO G, MARCELLIN P, POWELL EE, GEORGE J, NEGRO F; HCV META-ANALYSIS (ON) INDIVIDUAL PATIENTS' DATA STUDY GROUP, *Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data*, Gastroenterology, 2006, 130(6):1636–1642.

[27] MUZZI A, LEANDRO G, RUBBIA-BRANDT L, JAMES R, KEISER O, MALINVERNI R, DUFOUR JF, HELBLING B, HADENGUE A, GONVERS JJ, MÜLLHAUPT B, CERNY A, MONDELLI MU, NEGRO F; SWISS HEPATITIS C COHORT STUDY, *Insulin resistance is associated with liver fibrosis in non-diabetic chronic hepatitis C patients*, J Hepatol, 2005, 42(1):41–46.

[28] HICKMAN IJ, POWELL EE, PRINS JB, CLOUSTON AD, ASH S, PURDIE DM, JONSSON JR, *In overweight patients with chronic hepatitis C, circulating insulin is associated with hepatic fibrosis: implications for therapy*, J Hepatol, 2003, 39(6):1042–1048.

[29] PETIT JM, MINELLO A, JOOSTE V, BOUR JB, GALLAND F, DUVILLARD L, VERGES B, OLSSON NO, GAMBERT P, HILLON P, *Decreased plasma adiponectin concentrations are closely related to steatosis in hepatitis C virus-infected patients*, J Clin Endocrinol Metab, 2005, 90(4):2240–2243.

[30] HICKMAN IJ, CLOUSTON AD, MACDONALD GA, PURDIE DM, PRINS JB, ASH S, JONSSON JR, POWELL EE, *Effect of weight reduction on liver histology and biochemistry in patients with chronic hepatitis C*, Gut, 2002, 51(1):89–94.

[31] ROMERO-GÓMEZ M, CASTELLANO-MEGIAS VM, GRANDE L, IRLES JA, CRUZ M, NOGALES MC, ALCÓN JC, ROBLES A, *Serum leptin levels correlate with hepatic steatosis in chronic hepatitis C*, Am J Gastroenterology, 2003, 98(5):1135–1141.

[32] WESTIN J, NORDLINDER H, LAGGING M, NORKRANS G, WEJSTÅL R, *Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients*, J Hepatol, 2002, 37(6):837–842.

[33] GIANNINI E, CEPPA P, TESTA R, *Steatosis in chronic hepatitis C: can weight reduction improve therapeutic efficacy?* J Hepatol, 2001, 35(3):432–433.

[34] PETIT JM, BENICHOU M, DUVILLARD L, JOOSTE V, BOUR JB, MINELLO A, VERGES B, BRUN JM, GAMBERT P, HILLON P, *Hepatitis C virus-associated hypobetalipoproteinemia is correlated with plasma viral load, steatosis, and liver fibrosis*, Am J Gastroenterol, 2003, 98(5):1150–1154.

[35] LONARDO A, ADINOLFI LE, LORIA P, CARULLI N, RUGGIERO G, DAY CP, *Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease*, Gastroenterology, 2004, 126(2):586–597.

[36] FIERBINŢEANU-BRATICEVICI C, MOHORA M, CREŢOIU D, CREŢOIU S, PETRIŞOR A, USVAT R, ION DA, *Role of oxidative stress in the pathogenesis of chronic hepatitis C (CHC)*, Rom J Morphol Embryol, 2009, 50(3):407–412.

[37] FARINATI F, CARDIN R, DEGAN P, DE MARIA N, FLOYD RA, VAN THIEL DH, NACCARATO R, *Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection*, Free Radic Biol Med, 1999, 27(11–12):1284–1291.

[38] DE MARIA N, COLANTONI A, FAGIUOLI S, LIU GJ, ROGERS BK, FARINATI F, VAN THIEL DH, FLOYD RA, *Association between reactive oxygen species and disease activity in chronic hepatitis C*, Free Radic Biol Med, 1996, 21(3):291–295.

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