Comparative Study of Hepatitis C Virus Genotypes 1 and 3 in Salvador, Bahia

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Hepatitis C virus displays a high degree of genetic mutation, with considerable heterogeneity, motivating clinical and biomolecular investigations. It is necessary to understand the effects of genotypes on the course of the disease, as well as their peculiarities at the regional level. Objective: The study objective was to compare epidemiological, biochemical and histological aspects of hepatitis C virus genotypes 1 and 3 in Salvador, Bahia. Study Design: Data were collected retrospectively from outpatient medical records. Materials and Methods: 127 patients with positive anti-HCV results were selected, based on detectable RNA-HCV (RT-PCR) of genotypes 1a, 1b and 3a. Results: Thirty-nine (30.7%) individuals were infected by subtype 1a, 45 (35.4%) by subtype 1b and 43 (33.9%) by subtype 3a. Most (73.2%) patients were male, with an average age of 47.8 years. The subtype 1b-infected patients had the highest average age (512 ±11.17; P=0.09). The use of illicit injected drugs was more frequent among subtype 3a infected individuals when compared with genotype 1 (6/43; 14% and 3/84; 3.6%, respectively; P=0,06). No significant differences were found for other epidemiological characteristics. Average values for GT, AST, ALT and ferritin did not differ between the groups (64, 78, 109, 276, respectively). Thyroid dysfunction occurred in 7/30 (23.3%) of those infected by genotype 3 (P=0.05). Cryoglobulinemia was also more frequent in this group (5/13, 38%, P=0.02). Most patients presented limited necro-inflammatory activity, stages 2 and 3 by the METAVIR Classification. In some cases, dissociation was noticed between inflammatory activity and fibrosis. No significant differences were found in the histopathological findings of the various genotypes. Younger patients had a significantly smaller degree of necrosis in stomatocytosis (P=0.032) and fibrosis (P=0.012). Intense parenchymatous activity and lymphoid follicles were more frequent among alcohol consumers (P=0.06 and P=0.04, respectively). Conclusions: In Bahia, genotype 3 dissemination seems to be associated with illicit drug use. The disease evolution depends on a function of complex interactions between virus and host. Age and alcohol consumption stand out as important variables in the development of cirrhosis.

Key Words: Hepatitis C, genotype.

Hepatitis C virus (HCV) is endemic worldwide and is considered a public health problem. It has a heterogeneous natural history, with several clinical and epidemiological aspects awaiting better definition. In most acutely infected individuals, HCV evolves toward a chronic stage. Among those that remain chronic carriers of HCV, there is a risk of disease progression, with progressive fibrosis and a possible evolution to cirrhosis after an average period of 20 to 30 years. [1]

Liver disease progression does not occur in a linear fashion. It is probably influenced by viral and host factors, such as viral load, period of infection, gender, age, alcohol consumption and concurrent infection by Hepatitis B virus (HBV) or human immunodeficiency virus (HIV). [2]

The genetic variability of Hepatitis C virus has biological implications that are still little understood.

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Most studies show that viral genotype influences treatment response. [3,4] It is necessary to better understand the effects of genotype on disease transmission, pathogenesis and natural history.

Epidemiological and clinical differences have been described for Hepatitis C virus infection in several areas of the world. Consequently, regional factors should be considered when evaluating virulence and epidemiological peculiarities of the HCV genotypes. We compared epidemiological, biochemical and histological aspects of hepatitis C virus genotypes 1 and 3 in Salvador,Bahia, northeastern Brazil.

Materials and Methods

We made a comparative study, based on a series of cases, in which the data was collected retrospectively from patient medical records. These patients were followed in liver referral units from January 1995 to June 2000, at the University Hospital of the Federal University of Bahia.

One hundred and twenty-seven hepatitis C patients were selected, based on anti-HCV positive results and RNA-HCV detection of genotypes 1a, 1b or 3. Second or third generation ELISA was used for anti-HCV testing (ABBOTT, Chicago IL or SANOFI-PASTEUR, Paris, France). An HCV-RNA exam was made of serum samples, using the polymerase chain reaction technique with reverse transcription (RT-PCR). This qualitative test produced results classified as "detectable" or "undetectable". Genotype determination was also based on nested-PCR of the Hepatitis C virus core region, using genotype-specific primers, as previously described. [5]

Patients with undetectable HCV-RNA – (without defined genotypes), and patients with mixed genotyping, were excluded. Due to the very low frequency of genotype 2 in northeast Brazil, these patients were not included in the study. Cases of concurrent infection by hepatitis virus B or HIV were also excluded.

All patients were undergoing antiviral treatment evaluation. The HCV-RNA (RT-PCR) genotyping and hepatic biopsies were routinely made before the onset of the antiviral treatment. These biopsies were all reviewed by the same pathologist, without prior knowledge of patient genotyping. The METAVIR System was used as the classification parameter of histopathological lesions. [6]

Hepatic steatosis was also evaluated, the intensity of which was defined as follows:

0 - absent,
1+ - involving less than 10% of the liver cells;
2+ - present in from 10 to 25%;
3+ - from 25 to 50%;
4+ - detected in more than 50% of the hepatic cells.

Sinusoidal fibrosis was also quantitatively evaluated and classified in the following manner:

0 - absent;
1 - mild;
2 - mild to moderate;
3 - moderate to intense;
4 - intense.

The same scale was used to measure liver cell iron overload. The presence of lymphoid follicles, ductule lesions and intra-sinusoidal lymphocytosis was registered. These variables were defined as present or absent.

The available information on the following variables was registered on an evaluation patient record sheet:

Epidemiological. Age, gender, time elapsed since diagnosis, alcohol use, blood transfusions, surgeries, tattoos, endovenous drug use, endovenous vitamin complex use with non-disposable needles, inhaled drug use, use of manicures or barbers, hemodialysis and health care work.

Clinical. Asthenia, arthralgia, myalgia, and other presentation forms.

Laboratory tests. Aminotransferase (AST and ALT), ferritin, gammaglutamyltransferase (GT), cryoglobulins, platelets, thyroid function testing (free T4, TSH), qualitative HCV-RNA exams, genotyping.

Histological. Portal inflammation, piecemeal necrosis, parenchymatous activity, stage, hepatic steatosis,

sinusoidal fibrosis, lymphoid follicles, ductule aggression, tissue iron overload, intra-sinusoidal inflammatory infiltration.

Genotype was classified as the main independent variable and the remaining variables as dependent. Age was considered a possibly confusing variable in the analysis of the hepatic disease stage.

This study was submitted to and approved by the Research Ethics Committee of the University Hospital of Bahia. Financial support was obtained from CNPq/ Inserm Cooperation Program.

RNA extraction. For RNA extraction, the guanidine thiocyanate (GuSCN) - phenol solution method was used, as previously described [7].

For reverse transcription, the following reagent mixture was prepared: DEPC-treated water; 5 µl of buffered solution 5 X; 2.5 µl of dNTPs, 2.5 µl of DTT; 0.7 µl of MMLV and 0.7µl of RNAsin; 50 uM random primer; 25 µl of this mixture was added to the tubes containing RNA. The reverse transcription was carried out at 37ºC for 1.5 hours.

After quickly centrifuging the cDNA, a polymerase chain reaction (PCR) was initiated. The following reagent mixture was used: 5 µl of 10 X; 1 µl of dNTPs; 5 µl of the SF1primer; 5 µl of the SR1primer; 1.5 µl of magnesium chloride; 0.2 µl of Taq DNA polymerase and Mill-Q treated water. 45 µl of the reagent mixture was placed in each tube and 5 µl cDNA added. The tubes were then placed in a thermocycler. After the polymerase chain reaction, the amplified product, in agar gel to 2% in TAE 1X, was visualized. The gel was photographed in an *Eagle Eye* apparatus, using the dynamic method of image integration. Detection of a 295-base-pair fragment was expected.

Statistical analysis. The continuous variables were expressed as an average value with a standard deviation; the categorical variables were calculated as ratios.

The Chi-square test, or Fisher's exact test (when necessary), was used to test differences between the ratios. The Mann-Whitney test was applied to evaluate differences between continuous variables. The Spearman coefficient was used to test for correlation between age and histopathological variables. The analyses were carried out with the aid of SPSS software (Statistical Package for the Social Sciences), version 10.0 for Windows. P values less than 5% ($P < 0.05$) were considered significant.

Results

Of the 127 patients included in the study, 39 (30.7%) individuals were infected by subtype 1a, 45 (35.4%) by subtype 1b and 43 (33.9%) by genotype 3.

Epidemiological and clinical variables and laboratory and histological exams of subtypes 1a and 1b were compared. Significant differences were not found, except for age groups. The individuals infected by subtype 1a had an average age of 43.9 ± 10.5 years and those with subtype 1b had an average age of 51.2 ± 11.2 years ($P=0.003$). Based on these results, and the analysis of the epidemiological and clinical data, laboratory and histological exams were conducted, considering genotype 1(subtypes 1a and 1b) as a unique group, compared with the genotype 3 group.

In the gender comparison, 93 (73.2%) were male and 34 (26.8%) were female. There was no significant gender ratio difference between the genotype 1 and 3 groups.

The patients had an average age of 47.8 years, with a minimum of 18 and a maximum of 75 years. No significant age difference was found between the genotype 1 and 3 groups.

Sixteen individuals (19.5%) infected with genotype 1 did not have any known parenteral risks, while 5 (11.9%) of those contaminated with genotype 3 did not have any identifiable percutaneous exposure. This difference was not significant ($P=0.29$).

Among the individuals with a possible infection source, 75 (59.1%) had a history of surgery, 48 (39.3%) had past blood transfusions, 16 (12.7%) patients had used intravenous vitamin complexes with reusable needles, 9 (7.1%) mentioned illicit injected drug use, while 7 (5.5%) had inhaled cocaine. Tattooing was considered a possible risk factor in 7 (5.5%) cases, manicuring in 6 (4.8%) and razor blade sharing in 3

Figure 1. Principal forms of Hepatitis C virus transmission in Salvador.

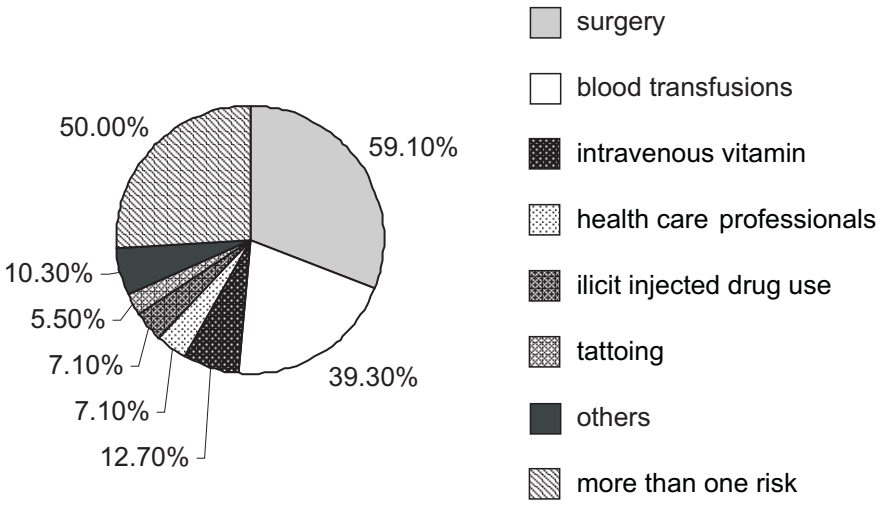

Table 1. Epidemiological characteristics by genotype

Epidemiological characteristics	Total (n=127)	Genotype 1 (n = 84)	Genotype 3 (n = 43)	p
Gender (male) (%)	93 (73.2%)	61 (72.6%)	32 (74.4%)	0.83
Age	47.8 ± 11.0	47.8 ± 11.4	47.5 ± 10.38	0.90
Absence of risks (%)	21 (16.9%)	16 (19.5%)	5 (11.9%)	0.29
Previous surgeries (%)	75 (59.1%)	49 (58.3%)	26 (60.5%)	0.82
Blood transfusions (%)	48 (39.3%)	33 (40.7%)	15 (36.6%)	0.66
Injected drug use (%)	9 (7.1%)	3 (3.6%)	6 (14%)	0.06
Vitamin complex (%)	16 (12.7%)	10 (11.9%)	6 (14.3%)	0.71
Inhaled cocaine use (%)	7 (5.6%)	3 (3.6%)	4 (9.5%)	0.17
Tattooing (%)	7 (5.5%)	5 (6.0%)	2 (4.7%)	1.00
Hemodialysis (%)	4 (3.1%)	3 (3.6%)	1(2.3%)	1.00
Health care professionals (%)	9 (7.1%)	6 (7.1%)	3 (7.0%)	1.00
Manicure (%)	6 (4.8%)	3 (3.6%)	3 (7.3%)	0.39
Barber shop services (%)	3 (2.4%)	2 (2.4%)	1 (2.4%)	1.00
Other risks (%)	63 (50%)	40 (47.6%)	23 (54.8)	0.45

Table 2. Laboratory characteristics by virus genotype

Laboratory characteristics	Total	Genotype 1	Genotype 3	p
AST (mean)	78	84	98	0.09
ALT (mean)	109	108	115	0.23
GT (mean)	64	85	66	0.46
Ferritin (mean)	276	304	276	0.75
Thrombocytopenia (%)	20 (16.5%)	16 (19.3%)	4 (10.5%)	0.23
Cryoglobulinemia (%)	9 (15.8%)	4 (9.1%)	5 (38.5%)	0.02
Thyroid dysfunction (%)	12 (12.5%)	5 (7.6%)	7 (23.3%)	0.05

Table 3. Histopathological characteristics by viral genotype

Histopathological characteristics	Total (%)	Genotype 1 (%)	Genotype 3 (%)	p
Portal inflammation				0.14
Limited	9 (16.0)	8 (20.5)	1 (5.9)	
Limited-moderate	40 (71.4)	28 (71.8)	12 (70.6)	
Moderate-intense	7 (12.5)	3 (7.7)	4 (23.5)	
Piece meal necrosis				0.27
Absent	10 (17.9)	9 (22.5)	1 (6.3)	
Limited	17 (30.4)	13 (32.5)	4 (2.5)	
Limited-moderate	21 (37.5)	14 (35.0)	7 (43.8)	
Moderate-intense	8 (14.3)	4 (10.0)	4 (25.0)	
Parenchyma activity				0.39
Limited	39 (73.6)	25 (67.6)	14 (87.5)	
Limited-moderate	9 (17.0)	7 (18.9)	2 (12.5)	
Staging				0.64
Stage I	12 (22.6)	8 (21.6)	4 (25.0)	
Stage II	20 (37.7)	16 (43.2)	4 (25.0)	
Stage III	13 (24.5)	8 (21.6)	5 (31.3)	
Stage IV	8 (15.1)	5 (13.5)	3 (18.8)	
Steatosis				0.37
Absent	15 (29.4)	13 (37.1)	2 (12.5)	
<10%	16 (31.4)	9 (25.7)	7 (43.8)	
10 to 25%	11 (21.6)	8 (22.9)	3 (18.8)	
>25%	2 (3.9)	1 (2.9)	1 (6.3)	
Sinusoidal fibrosis				0.20
Limited	32 (60.4)	22 (65.8)	7 (46.7)	
Limited-moderate	19 (35.8)	11 (28.9)	8 (53.3)	
Lymphoid follicles	19 (36.5)	12 (33.3)	7 (43.8)	0.47
Ductule lesions	37 (74)	23 (67.6)	14 (87.5)	0.18
Iron excess				0.13
Limited (%)	12 (30.8)	6 (21.4)	6 (54.5)	
Limited-moderate	4 (10.3)	3 (10.7)	1 (9.1)	
Sinusoidal lymphocytosis	12 (23.5)	11 (31.4)	1 (6.3)	0.08

(2.4%). Only 4 (3.1%) patients had undergone hemodialysis and 9 (7.1%) were health care professionals. More than one risk factor was reported by 63 (50%) individuals (Figure 1).

The Chi-square test showed no relevant differences in epidemiological variables, except for illicit injected drug use, which was more frequent in those infected by genotype 3 than in those infected by genotype 1 (14%, versus 3.6%, P=0.06).

Most of the patients (96/125; 76.8%) had an incidental hepatitis C diagnosis, detected through laboratory exams, while 19/125 (15.2%) sought medical assistance for asthenia and 9/125 (7.2%) for arthralgia. No association was found between genotype and clinical signs.

Alcoholic intake equal or superior to 40 grams/day was mentioned by 34/127 (26.8%) patients, with no difference between genotypes 1 and 3.

During the pre-treatment evaluation visit, the median values for ferritin, glutamyl transferase (GT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were 276, 64, 78, and 109, ui/l, respectively. No significant differences were found in the comparison between genotypes 1 and 3 (Table 2).

Thyroid function tests (free T4 and TSH) were made in 96 patients, before or during the use of anti-viral therapy. Abnormal results were found for 12 (12.5%) patients. Thyroid dysfunction (abnormal free T4 and/or TSH) was diagnosed in 7/30 (23.3%) individuals infected by subtype 3a and in 5/66 (7.6%) genotype 1 patients, including subtypes 1a or 1b (Table 2). The Fisher Exact Test showed this difference to be significant (P=0.05).

Genotype 3 patients also presented cryoglobulinemia at a higher frequency, when compared with genotype 1 patients. Cryoglobulinemia was investigated in 57 individuals. Of these, 9 (15.8%) had cryoglobulinemia, 4/44 (9.1%) were of genotype 1 and 5/13 (38%) were of genotype 3 (P=0.02) (Table 2). Only one patient with genotype 3 and cryoglobulinemia presented myalgia and arthralgia. The other patients were asymptomatic.

In the pre-treatment phase, thrombocytopenia (counts less than 100,000 cells/mm3) was observed in 20/121 (16.5%) cases, made up of 16/83 (19.3%) patients with genotype 1 and 4/38 (10.5%) with subtype 3a (Table 2) (P=0.23).

The liver biopsies were reviewed in 56/99 cases (56.5%). Hematoxylin-Eosin (HE), Reticulin, Picrosirius and Perls stains were used.

Most patients (71.4%) presented mild to moderate portal inflammation (Table 3). Piecemeal necrosis was mild or mild to moderate in most patients (17/56, 30.4%; 21/56; 37.5%, respectively). Limited lobular activity was more frequent (39/53; 73.6%).

With regard to staging, portal fibrosis was observed with rare septa (stage 2) in 20/53 cases (37.7%) and numerous septa without cirrhosis (stage 3) in 13/53 (24.5%) cases. In some cases, a dissociation was observed between inflammatory activity and fibrosis, as there was considerable fibrosis in biopsies with mild inflammation.

Steatosis was absent in 15/51 (29.4%) cases, present in less than 10% of the hepatocytes in 16/51(31.4%) biopsies and in 10 to 25% of the hepatocytes in 11/51 (21.6%) cases (Table 3). Macro and micro steatitis was identified.

Perisinusoidal fibrosis was limited in most of the liver biopsies (32/53; 60.4%), sometimes coinciding with areas of hepatic steatosis.

Ductule lesions were found in 37/50 (74%) cases, frequently associated with lymphoid follicles in 19/52 biopsies (36.5%).

Hepatocellular iron overload was detected in 39 biopsies. In most cases (12/39; 30.8%), when present, it was mild (Table 3). Iron overload restricted to Kupffer cells was found in 3/39 cases.

Intra-sinusoidal lymphocytosis was detected in some cases (12/51; 23.5%), even in areas of mild parenchymatous activity, being more frequent in genotype 1 patient's liver biopsies, but the difference was not significant (P=0.08).

Some biopsies (6/56; 10.7%) presented features compatible with steato-hepatitis, such as areas of neutrophilic infiltration, pericentrolobular distribution of steatosis, hepatocellular necrosis and/or balloonization – predominantly in Zone III, and perivascular collagen deposition.

The relation between alcohol intake and the histopathological findings was evaluated. Patients who consumed large amounts of alcohol (above 40g/dia) had more parenchymatous activity (33.3% of the cases with mild-to-moderate activity and 13.3% of those with moderate-intense activity). This contrasted with nondrinkers, with ratios of 10.5% and 2.6%, respectively (P=0.06). Lymphoid follicles were also more frequent among alcohol consumers, in comparison with non-consumers (66% and 24.3%, respectively; P=0.04). No association was found between alcohol consumption and other histopathological findings.

No significant differences in histopathological findings were observed between genotypes. Cirrhosis was not more frequent among individuals infected by genotype 1, compared to genotype 3, infection not being associated with specific histological lesions.

The Spearman Correlation Coefficient was applied to evaluate the association between age and histological variables. A significant positive correlation was observed between age and piecemeal necrosis, as well as between age and staging (r = 0.288 and 0.344; P=0.03 and 0.01; respectively). Stage 1 patients were on average 12 years younger than other stage patients and those without piecemeal necrosis were on average 11 years younger.

Discussion

Epidemiological and clinical differences have been observed in Hepatitis C virus infection in several areas of the world. An example is genotype 4, which presents a benign process of evolution in Central Africa, where the number of cases with abnormal aminotransferases does notn't exceed 10%. However, in Europe, cirrhosis and hepatocellular carcinoma are frequent. Host genetic factors and environmental factors possibly interact and determine the natural history of hepatitis C as a function of genotype. On the other hand, many other aspects could play important roles in the natural history of hepatitis C, regardless of genotype.

Our retrospective and comparative study had as its objective the evaluation of hepatitis C genotypes 1 and 3 in Salvador, northeastern Brazil, with emphasis on epidemiological, clinical, biochemical and histological aspects.

Our group previously presented data on this study area, characterized as a median endemic state in terms of HCV infection [8]. Later, we demonstrated that while HCV genotypes 1a and 1b are the most prevalent in the area, genotype 3 is highly prevalent, apparently unrelated to injected drug use. This situation is different from findings described for other countries, especially in Europe. In addition, Salvador has a peculiar population profile where racial miscegenation between Amerindians, Iberian Europeans and Africans is a natural consequence of the intermingling of these groups. The introduction of HCV into Brazil could be related to all these ethnic groups.

In agreement with other authors, the subtype 1b carriers were older than patients infected by other subtypes. However, our genotype 3 carriers were older than those described by other authors [9,10].

In our study, it was not possible to determine viral infection duration because many patients could not identify the contamination source or presented more than one parenteral risk factor; most had an incidental hepatitis C diagnosis.

Based on our results, it appears that genotype 3 dissemination in this region is only marginally influenced by the spread of intravenous drug use. This finding contrasts with European studies, where a clear association is described between genotype 3 infection and illicit drug use [11]. It is possible that the small sample size impeded an objective comparison, however injected drug use was far less important as an HCV risk factor in this area than has been reported for other countries [12].

Associations between other forms of transmission and specific genotypes were not found, however we note the importance of vitamin complex injections made with glass syringes and non-disposable needles, which was very popular in the seventies [13]. This could replace intravenous drug use in transmitting genotype 3 in this area. Another peculiar factor is tattooing, already described by our group as an HCV risk factor in this area [14].

As for clinical aspects, cryoglobulinemia was detected at a higher frequency in genotype 3 patients. The association between hepatitis C and cryoglobulinemia may be justified by the fact that this virus infects mononuclear cells. The presence of cryoglobulin in serum is not always associated with important clinical manifestations and this was the case in our study. Some authors have found higher frequencies of Hepatitis C virus genotype 2 infection among individuals with cryoglobulinemia, however others have not confirmed this finding [11]. The association between genotype and cryoglobulinemia remains controversial.

Higher frequencies of thyroid dysfunction were found among genotype 3 infected individuals, however most did not present clinical manifestations. We believe that these aspects need to be studied in greater detail, considering the relatively small number of individuals covered in this analysis.

High thyroid antibody prevalence has been described for hepatitis C patients [15]. The pathogenic mechanism for thyroid disease is not well known, though it is possible that Hepatitis C virus induces autoimmunity in genetically susceptible individuals. An association between Hepatitis C virus infection and thyroid abnormalities, especially auto-immune thyroiditis, is suggested by the high prevalence of anti-HCV among patients with Hashimoto thyroiditis [16]. Antiviral treatment with interferon is another risk factor, probably related to autoimmunity exacerbation [17].

No association of hepatic disease severity was found with viral genotype in our study, contrasting with some European studies, where genotype 1 was associated with more severe forms of hepatic disease [18, 19].

The histopathological analysis of the liver biopsies revealed steato-hepatitis coexistence in some cases. An attempt was made to evaluate the relation of these findings with alcohol consumption, however no significant association was encountered. Based on current knowledge, hepatitis C is not considered a cause of non alcoholic steato-hepatitis (NASH) per se [20], though it would be useful to investigate NASH risk factors, such as excess body weight, dyslipidemia and type 2 Diabetes Mellitus. Unfortunately, a systematic registration of this data was not made in the patient files, consequently we can not comment about this interesting finding.

Some authors have reported that steatosis accelerates hepatic lesion progression, associated with necro-inflammatory activity and with the degree of fibrosis. [21] In our series, Perisinusoidal fibrosis was documented in areas of hepatic steatosis, which suggests that fat infiltration in the liver is an important co-factor, implicated in disease evolution.

Hepatitis C virus infection consequences depend on complex interactions between the virus and the host. Our results suggest that differences in genotype are not a key variable in cirrhosis evolution. Age and alcohol consumption are, however, important factors in hepatitis C disease progression. Certainly, other aspects, such as infection duration and concurrent viral infections, also play a role in the course of hepatitis C infections.

In conclusion, genotype 3 dissemination in northeastern Brazil seems to be marginally associated with illicit drug use; however the existence of other sources of parenteral exposure may explain its high prevalence. No association was found between other forms of transmission and specific genotypes. Patients infected by subtypes 1a and 1b did not present significant differences, except by age. Extra-hepatic manifestations seem to be more frequent among genotype 3 carriers. Disease evolution occurs as a function of complex interactions between virus and host. The variables age and alcohol consumption are central to cirrhosis onset.

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