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ORIGINAL

Thyroid disturbance related to chronic hepatitis C infection: role of CXCL10

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Abstract. Association between autoimmune thyroid diseases (AITD) and hepatitis C is controversial, but may occur or worsen during alpha-interferon treatment. The mechanism responsible for autoimmune diseases in infected patients has not been fully elucidated. This study aims to evaluate the frequency of AITD in chronic hepatitis C and the association of chemokine (CXC motif) ligand 10 (CXCL10) and AITD. One hundred and three patients with chronic hepatitis C and 96 controls were prospectively selected to clinical, hormonal, thyroid autoimmunity and ultrasound exams, besides thyroxine-binding globulin (TBG) and CXCL10 measurements and hepatic biopsies. The frequency of AITD among infected subjects was similar to controls. TT3 and TT4 distributions were right shifted, as was TBG, which correlated to both of them. Thyroid heterogeneity and hypoechogenicity were associated with AITD. Increased vascularization was more prevalent in chronic hepatitis C.CXCL10 was higher in infected patients (p=0.007) but was not related to thyroid dysfunction. Increase in CXCL10 levels were consistent with hepatic necroinflammatory activity (p=0.011). In summary, no association was found between chronic hepatitis C and AITD. Infected subjects had higher TT3 and TT4 which were correlated to TBG. Increased CXCL10 was not associated to thyroid dysfunction in HCV-infected population.

Key words: Autoimmune thyroiditis, CXCL10, Hepatitis C, Thyroxine-binding globulin

THE ASSOCIATION between chronic hepatitis C (HCV) infection and thyroid autoimmune disorders (AITD) is controversial, but may occur or worsen during alpha-interferon treatment [1]. A large study observed slightly increased risk for thyroiditis in HCV-infected patients (hazard ratio = 1.06, 95% CI = 1.01-1.11) [2] and a recent review suggested a weak association in comparisons to hepatitis B (HBV) infected and control populations (odds ratio = 1.6, 95% CI=1.4-1.9). The authors suggested that differences in geographical distribution, genetic variability and environmental cofactors could be responsible for divergence among studies [3].

The mechanism responsible for autoimmune diseases in HCV-infected patients, especially thyroid disorders, has not yet been fully elucidated. The identification of HCV in thyroid tissue could suggest direct action of the virus [4]. However, HCV is an RNA virus unable to insert its genes into host genome and, therefore, indirect mechanisms may be responsible for autoimmune thyroid diseases. Antonelli *et al.* [3] proposed that high levels of endogenous interferon alpha (IFNα) may be involved in the development of thyroid autoimmune diseases in genetically predisposed patients. They also suggested possible involvement of interferon gamma (IFNγ) and IFNγ-inducible chemokines, such as chemokine (CXC motif) ligand 10 (CXCL10), in the pathogenesis of autoimmune thyroid diseases related to HCV infection. Increased expression of IFNγ and CXCL10 in hepatocytes and lymphocytes of HCV-

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positive patients has also been previously described [5, 6]. Increased expression of intrathyroidal IFNγ and CXCL10 secretion in thyrocytes could be conducive to the development of autoimmune thyroidal disorders [7, 8]. Increased CXCL10 levels is not associated with hyper- or hypothyroidism *per se*, but is specifically sustained by the autoimmune inflammatory event occurring both in Graves disease and autoimmune thyroiditis [9]. CXCL10 possibly acts by recruiting T-helper 1 lymphocytes which secrete IFNγ and TNF that in turn are responsible for CXCL10 secretion, potentially leading to perpetuation of the autoimmune process [10].

This aims of this study were to evaluate the frequency of thyroid diseases in chronic HCV-infected patients and to verify the association of CXCL10 and thyroid and liver disorders.

Materials and Methods

Patients

A total of 103 with the diagnosis of chronic hepatitis C and followed up at the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* between January 2007 and July 2009 patients, were prospectively selected. The diagnosis of chronic HCV infection was based on positive anti-HCV serology and confirmed by the presence of viral nucleic acid evaluated by polymerase chain reaction (PCR) using the commercial Amplicor HCV test (Roche Diagnostics Systems). None of the patients had been previously treated with interferon. Patients with HBV infection, HIV infection, those who were pregnant, or using amiodarone or lithium were excluded. Nine percent of the subjects had histological diagnosis of cirrhosis, all of them with compensated liver disease. None had hepatocarcinoma.

The control group was formed by selecting 96 blood donor volunteers from the *Hospital do Servidor Público Estadual* with negative HIV, HCV and HBV serologies. Subjects in use of levothyroxine, lithium, amiodarone or antithyroidal drugs were excluded.

Methods

Patients were submitted to clinical and hormonal evaluation. Total T3 (TT3), total T4 (TT4), free T4 (FT4), and TSH were measured using commercial fluoroimmunoassay kits (AutoDELFIA®, Upsala, Turku, Finland). Serum anti-thyroglobulin (anti-Tg) and anti-thyroperoxidase (anti-TPO) antibodies were determined using commercial indirect fluoroimmunoassay kits (AutoDELFIA®, Upsala, Turku, Finland). Anti-TSH receptor antibody (TRab) was evaluated by immunoradiometric assay (RSR, Cardiff, England). Serum thyroxine-binding globulin (TBG) was measured by commercial chemiluminescence immunoassay (DPC Biermann, Bad Nauheim, Germany).

Subclinical hypothyroidism and hyperthyroidism were diagnosed when TSH levels were increased (> 4.5 mUI/L) or decreased (<0.4 mUI/L), respectively, and FT4 levels were within normal range (0.7 – 1.5 ng/dL). Overt hypothyroidism and hyperthyroidism were diagnosed based on increased or decreased TSH levels, respectively, associated to respective decreased or increased FT4 levels. AITD was diagnosed when any anti-thyroid antibody was present.

Serum alanine (ALT) and aspartate (AST) aminotransferases were measured by conventional methods. Aminotransferase quotients, qALT and qAST, were established by the ratio of aminotransferase serum levels to their respective reference values. HCV genotyping was performed by a reverse hybridization assay, the Line Probe Assay (INNO-LiPA HCV / VersantTM HCV Genotype Assay - Bayer Corporation, Tarrytown, NY, USA).

Serum CXCL10 levels were assayed by a quantitative sandwich immunoassay using Invitrogen Human IP-10 ELISA kit for research (Invitrogen Corporation, Camarillo, USA). We measured CXCL10 levels only in controls without thyroid disturbance for comparisons.

Color-flow Doppler thyroid ultrasonography (CDUS) was performed by the same investigator (7.5–12 MHz, Philips HDI 5000 device, Philips Medical Systems, Bothell,Wash., USA). Images were obtained on B-mode, color and pulsed Doppler. We evaluated thyroid parenchyma and echogenicity, presence of nodules, glandular volume and vascularization. Glandular vascularization was classified according to Bogazzi *et al*. [11]: pattern 0: absent intraparenchymal vascularity or minimal spots; pattern I: presence of parenchymal blood flow with patchy uneven distribution; pattern II: mild increase of color flow Doppler signal with patchy distribution; pattern III: marked increase of color flow Doppler signal with diffuse homogeneous distribution, including the so-called "thyroid inferno."

Liver histological analysis was carried out by *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* Pathology Department using criteria validated by the Brazilian Hepatology and Pathology Society [12, 13]. Liver aggression stag-

ing was determined based on a necroinflammatory activity score ranging from A0 to A4 and a liver architectural change score also ranging from F0 to F4.

Statistical analysis

Data were processed using SPSS 13.0 software. Two-tailed *p*-values were used and *p*-values <0.05 were considered statistically significant.

Categorical variables are presented as absolute and relative (percentages) frequencies. Differences were evaluated by Pearson's χ^2-test and Fisher's exact test when appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Continuous variables are presented as mean ± standard deviation. The Kolmogorov-Smirnov or Shapiro-Wilk statistics were used to test for any deviation from normality. Differences among studied subgroups were determined using Student's *t*-test and one-way ANOVA if presenting normal distribution, and Mann-Whitney U, Wilcoxon, or Kruskal-Wallis tests for non-normal distributions. Correlations between two variables were ascertained by Spearman's correlation test.

Simple linear regression analysis was used to test the association between TBG and hormonal levels, and TBG and CXCL10 levels after logarithmic transformation. Multiple linear regression analysis was performed to determine the influence of absence or presence of thyroid disorders (AITD, non-autoimmune subclinical hypothyroidism or non-autoimmune subclinical hyperthyroidism), results of CDUS (thyroid parenchyma and echogenicity, glandular volume and vascularization pattern), logarithmic transformed qALT, qAST, TT3, TT4, FT4, TSH and TBG levels and liver biopsy inflammatory activity score and architectural change score on logarithmic transformed CXCL10 levels (dependent variable).

Ethics

The study was approved by the institutional research ethics committee and all subjects gave their informed written consent to participate.

Results

Thyroid Disorders in HCV group

Eleven HCV-infected subjects presented autoimmune thyroid disease on initial evaluation. Prevalence of autoimmune thyroid diseases (AITD) among HCV and control groups was similar (10.7% *vs*. 13.5%, *p*=0.545) (Table 1). After matching for age and sex, both groups still had a similar prevalence of AITD (6.2 % *vs*. 15%, *p*=0.139).

Four HCV-infected patients had non-autoimmune subclinical hypothyroidism (Table 2) and only 1.9% presented non-autoimmune subclinical hyperthyroidism.

Hormonal evaluation of the HCV group with no Thyroid Disorders

Eighty-six HCV-infected patients had normal thyroid function with absence of anti-thyroid antibodies (83.5%) (Table 2).

Among these patients with no thyroid disorders in the HCV group, TT3 and TT4 levels had distributions values superior to normal limits of hormonal assays (normal range TT3 40 to 180 ng/dL and TT4 4.5 to 12 µg/dL). Mean TT3 was 166 ± 37 ng/dL (73 to 274) and 95% confidence limits were 115 to 249 ng/dL, whereas

Table 1 Clinical features and prevalence of thyroid disorders in chronic HCV-infected patients and blood donors (controls).

	HCV-infected (n = 103)	Controls (n = 96)	*p*-value
Age (years)	46.4 ±12.8	35.6 ± 10.3	< 0.001
Sex (male/female)	40 / 63	44 / 52	0.318
Diagnosis (%)			
No thyroid disturbance	86 (83.5)	78 (81.3)	-
Autoimmune thyroid disorder	11 (10.7)	13 (13.5)	0.545
Anti-Tg and/or anti-TPO +	10 (9.7)	11 (11.5)	0.677
Subclinical hypothyroidism	0 (0)	1 (1.0)	0.479
Overt hypothyroidism	1 (1.0)	0 (0)	1.000
Graves disease	0 (0)	1 (1.0)	0.479
Subclinical hypothyroidism Ab-	4 (3.9)	4 (4.2)	1.000
Subclinical hyperthyroidism Ab-	2 (1.9)	1 (1.0)	1.000

+, positive; Ab-, antithyroidal antibody negative

Table 2 Clinical and color-flow Doppler thyroid ultrasonography (CDUS) characteristics of HCV-infected patients with no thyroid disorders, with autoimmune thyroid disorders (AITD), and non-autoimmune subclinical hypothyroidism (SH).

	No thyroid disorder (n=86)	AITD (n=11)	SH (n=4)
Age (years)	46.5 ± 12.6	42.2 ± 15.0	50.4 ± 10.3
Sex (male / female)	32 / 54	5 / 6	2 / 2
Race (caucasian/non-caucasian)	70 / 16	9 / 2	4 / 0
HCV genotype			
1	49	7	3
2	4	0	0
3	24	4	1
unknown	9	0	0
CDUS †:			
Heterogeneity (%)	54 (66)	9 (100)*	3 (100)
Hypoechogenicity (%)	10 (12)	4 (44)**	1 (33)
Glandular volume (grams)	10.8 ± 5.0	12.8 ± 6.3	11.3 ± 6.4
Nodules (%)	26 (32)	6 (67)	1 (33)
Vascularization (%)			
I	17 (27)	1 (12.5)	0 (0)
II	16 (25)	2 (25)	1 (50)
III	30 (48)	5 (62.5)	1 (50)

†, Frequency based on the number of exams with the parameter evaluated; *, p=0.052 for comparison between no thyroid disorder and autoimmune thyroid disorder; **, p=0.029 for comparison between no thyroid disorder and autoimmune thyroid disorder

mean TT4 was 11.5 ± 2.5 µg/dL (5.5 to 20.4) and 95% confidence limits were 7.8 to 15.5 µg/dL. Mean TBG level was 31 ± 9 mg/L and the 95% confidence limits were also superior to hormonal assay, at 18 to 47 mg/L (normal range 14 to 31 mg/L). TT3 and TT4 correlated to TBG levels, r=0.624 (p<0.001) and r=0.766 (p<0.001), respectively.

Mean FT4 level was 0.9 ± 0.1 ng/dL (0.6 to 1.4) and significantly below that of normal controls (1.0 ± 0.1 ng/dL, p<0.001). Nevertheless, 95% confidence limits of FT4 in HCV-infected subjects (0.7 to 1.2 ng/dL) remained within reference ranges of the assays (normal range 0.7 to 1.5 ng/dL).

TSH level was 1.7 ± 0.7 mUI/L (0.5 to 4.0), showing no statistically significant difference compared to the control group (1.8 ± 0.9 mUI/L, p=0.389). As it would be expected, FT4 levels correlated to TSH in HCV-infected subjects (r= - 0.245, p=0.023).

CDUS evaluation in HCV group

Eighty-two HCV-infected patients with no thyroid disturbance presented thyroid CDUS. Heterogeneous and hypoechoic parenchyma were observed in 66% and 12% of the exams, respectively. Increased glandular vascularization (pattern III) was revealed in 48% of exams (Table 2).

Compared to patients with no thyroid disorders, all patients with AITD had heterogeneous parenchyma (p=0.052) and 44% had hypoechogenicity (*OR* 5.8, *95%CI* 1.3-25.1, p=0.029).

CXCL10

Serum CXCL10 levels were significantly superior in the HCV group compared to normal controls, 407 ± 304 pg/mL and 222 ± 181 pg/mL (p=0.007), respectively.

In the HCV group, CXCL10 levels correlated significantly to qALT (r=0.283, p=0.042) and qAST (r=0.416, p=0.002). In addition, increases in CXCL10 levels were consistent with necroinflammatory activity score (295 ± 187 pg/mL, score A1, 328 ± 120 pg/mL, score A2, and 520 ± 276 pg/mL, score A3, p=0.011) (Fig. 1). CXCL10 levels were also significantly higher in HCV-genotype 1 infected patients (429 ± 228 pg/mL) compared to those with genotype 3 (273 ± 96 pg/mL) (p=0.026). CXCL10 did not differ significantly in liver architectural change score (fibrosis).

CXCL10 levels correlated to TT3 (r=0.417, p=0.002), TT4 (r=0.496, p<0.001) and TBG levels (r=0.562, p<0.001) (Fig. 2), but not to FT4 (r= -0.143, p=0.308) or TSH (r=0.019, p=0.894) levels. CXCL10 levels did not differ in patients with AITD compared to patients with no thyroid disorders (437 ± 366 pg/mL and 404 ± 305

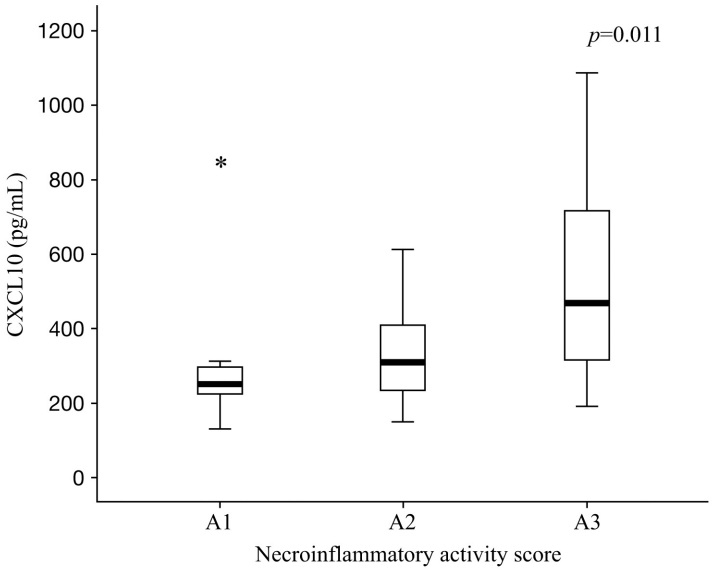

Fig. 1 Serum CXCL10 levels in HCV-infected patients with different necroinflammatory activity score on liver biopsy. Difference was assessed by the Kruskal-Wallis test. The asterisk represents an outlier.

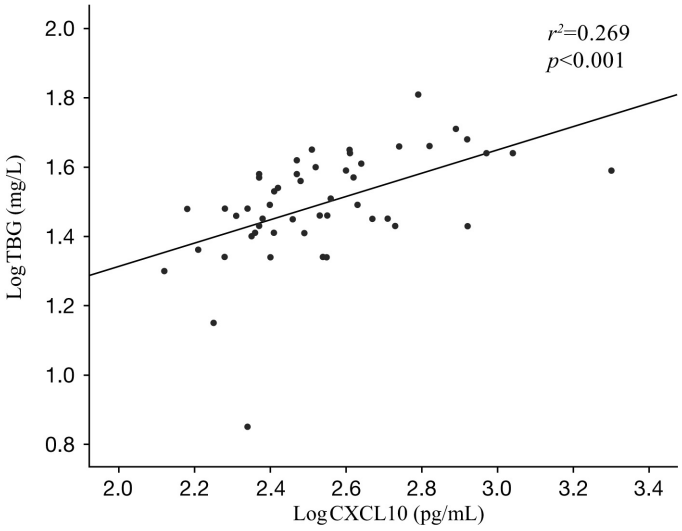

Fig. 2 Linear regression of natural logarithm of CXCL10 with natural logarithm of TBG.

pg/mL, respectively, p=0.904). CXCL10 levels were not related to any CDUS findings, including vascularization patterns. Even when comparing patients with similar hepatic necroinflammatory activity score and similar HCV genotypes infection, we found no association of CXCL10 to AITD or increased thyroid vascularization.

Multivariate linear regression analysis identified two major factors which were significantly associated to logarithmic CXCL10 levels (r^2=0.385): natural logarithmic TBG levels (*standard* β=+0.472, p<0.001) and natural logarithmic qAST (*standard* β=+0.328, p=0.007).

Discussion

Despite several studies suggesting an association between HCV infection and autoimmune thyroid disease, controversy remains over this issue [3]. Our study identified no difference in the frequency of thyroid disorders between HCV-infected patients and control pop-

ulation (10.7 *vs*. 13.5%), even after matching for sex and age. Several aspects may explain this finding. First, the Brazilian population had a different genetic background with characteristic race miscegenation, contrasting with the homogenous European [14-16] or Asian [17, 18] populations investigated in other studies. Second, Brazil is considered a iodine sufficient country [19]. Previously, our Institution had already observed a high prevalence of chronic autoimmune thyroiditis (16.9%) in the same metropolitan area of our study [20] reassuring a high frequency of AITD in control group (13.5%) in this study. Consequently, HCV infection as a predisposing risk could have been masked.

We emphasize that anti-thyroid antibodies, anti-Tg and anti-TPO, were measured by fluoroimmunoassay in the present study, yielding a higher sensitivity than that previously reported [21]. This may also have contributed to the higher frequency of AITD observed in the control group. It is noteworthy that we also selected HCV-infected patients with chronic disease, confirmed by viral RNA detection in sera, which is more representative of HCV's role in immune modulation than other studies with similar negative association [22-24].

Hormonal evaluation of the HCV group with no thyroid disturbances revealed increased TT3 and TT4 which were positively correlated to TBG levels, together with normal FT4 and TSH levels. Previous studies have suggested TT3 and TT4 increases in chronic liver disease, commensurate with severity of liver dysfunction due to high TBG, and that TT3 and TT4 serum levels were reduced in liver cirrhosis [25, 26].

Thyroid ultrasound evaluation confirmed that heterogeneous and hypoechoic parenchyma is more frequent in AITD. Increased vascularization was seen in AITD as previously described [27]; however, it was not statistically more frequent than in HCV-infected patients with no thyroid disturbance, because 48% of them also presented pattern III in their exams. Since our data represents an initial evaluation, prospective follow-up of this particular subgroup of HCV-infected patients with no thyroid disturbance might be necessary to exclude posterior development of AITD.

Associations of CXCL10 levels and AST and AST levels or necroinflammatory activity and grade of fibrosis in liver biopsies are variable in literature [28-31]. Nishioji *et al*. demonstrated that CXCL10 levels correlated with serum levels of AST and ALT in chronic hepatitis C [30], but other studies could not associate CXCL10 levels to higher grades of necroinflammatory activities in liver biopsies [28, 31]. Our data demonstrated that serum CXCL10 levels were higher in the HCV group and correlated to qAST and qALT, increasing concomitantly with liver biopsy inflammatory score. These findings highlight the role of CXCL10 in the liver inflammatory process in chronic active HCV infection. It is noteworthy that CXCL10 levels were higher in the HCV genotype 1 compared to genotype 3, denoting a more aggressive inflammatory course, where the former genotype may contribute to a higher proportion of treatment resistance [32].

In the present study, higher levels of CXCL10 were detected in HCV-infected subjects than in controls, regardless of thyroid dysfunction. Contrary to the hypothetical role of CXCL10 in the pathogenesis of AITD in HCV-infected patients [3], no association between CXCL10 levels and autoimmune thyroid disorders was identified in our HCV-infected population. Previously, Domberg *et al*. [33] also could not demonstrate significant association of CXCL10 and AITD, using a different technique for the measurement of CXCL10. It is possible that increased CXCL10 levels associated to hepatitis C masked the association of the chemokine with AITD in our HCV-infected subjects. Our subgroup analysis of different hepatic necroinflammatory activities scores was not able to prove it but other studies with larger subgroups analysis would elucidate it.

Apart from that, Corona *et al.* [27] have demonstrated higher CXCL10 levels in patients with autoimmune disease as well as in those with increased thyroid vascularization, and proposed that CXCL10 could play an important role in intrathyroid angiogenesis modulation. On the contrary, no association between CXCL10 levels and increased thyroid vascularization at CDUS was found in the present study, rejecting this hypothesis, particularly in the HCV-infected population. We speculate that other angiogenetic factors may play a part in the increased thyroid vascularization observed.

Correlation between CXCL10 and TBG levels was identified in this study in HCV-infected patients, independent of aminotransferase increase, as observed in multivariate analysis. Unfortunately, it was not possible to confirm these findings in normal controls that did not have their TBG or aminotransferases levels evaluated. We hypothesize that TBG levels could reflect HCV liver disease, independently of the hepatocellular destruction represented by AST levels. A previous study of acute hepatitis suggested that higher TBG

reflects synthesis from regenerating hepatocytes and not only leakage from damaged hepatocytes [34]. As CXCL10 is considered a marker of the liver inflammatory process, it has been proposed that pre-treatment CXCL10 levels could predict outcome of antiviral therapy in HCV-infected patients [35]. However, we propose TBG measurement, as an easier and more accessible alternative methodology to CXCL10 assessment.

In summary, our results allow us to confirm no association between chronic HCV infection and thyroid diseases. Chronic HCV-infected subjects present high TT3 and TT4 which is correlated to a rise in serum TBG levels. CXCL10 levels are increased in HCV-infected patients but not associated to thyroid dysfunction.

Based on these findings, we suggest that hormonal evaluation of chronic HCV-infected patients be carefully analyzed, given the important role played by TBG.

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