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CYTOKINES. THE MISSING SHACKLE IN CHRONIC HEPATITIS C DEVELOPMENT

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Abstract: *The immune mechanisms involved in chronic hepatitis C (CHC) development are still unclear, but pro-inflammatory cytokines definitely have an important role in the development of hepatic lesions and reparations. The aim of our retrospective study was to analyze TNF-α, Il-6, soluble receptors sTNFR-2 and sIL-6R in a cohort of 80 patients diagnosed with CHC and compared to a group of 14 healthy subjects. There were registered higher levels of cytokines in patients with CHC compared to controls. Cytokine receptors have a greater diagnostic power. Il-6 and its soluble receptor have a high predictive value for viral induced cirrhosis. The associations between TNF-α and IL-6 suggest a strong interrelationship in disease development.*

Key words: *chronic hepatitis C, pro-inflammatory cytokines, TNF-α, Il-6, sTNFR-2, sIL-6R*

1. Introduction

Cytokines are regulatory molecules with an important role in antiviral immune response [20]. Chronic hepatitis C (CHC) is known to have a Th1 type response, with synthesis of pro-inflammatory cytokines: IFN-γ, TNF-α and IL-2 in the liver as well as in mononuclear cells in the periphery [13], [20].

The TNF-α/TNF-α receptor (TNFR) system plays an important role in inflammation, apoptosis and in liver renewal [7]. TNFR found on the cell membrane surface are cleaved by proteolic enzymes, hence the increase of their soluble fragments in serum reflects their increase in expression at tisular level in vivo [17]. Il-6 is a pro-inflammatory cytokine, crucial for liver regeneration, acting upon the immune system, increasing immunoglobulin synthesis by B lymphocytes and activating T lymphocytes [2], [16]

An accepted hypothesis in literature is the fact that the release of pro-inflammatory cytokines in patients with CHC could represent a non-invasive marker of liver damage [8]. Moreover, there is a lack of information regarding the precise role of sTNFR-R2 in relationship to TNF- α and sIL-6R in relationship to Il-6 in CHC. Aspects regarding the host could increase the pro-inflammatory state and lead to an increase of pro-inflammatory cytokines, contributing to the extension of hepatic lesions.

Therefore, our aim was to evaluate seric levels of cytokines and their receptors in correspondance with clinical and biological features of patients with CHC.

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2. Material and Methods

80 patients diagnosed with CHC were included in our study. Infection with hepatitis C virus was defined as the persistence of anti HCV antibodies for a minimum of 6 months combined with HCV-ARN. Patients with other causes of chronic hepatitis (infection with HBV, autoimmune hepatitis, Wilson's disease, hemochromatosis, α1-antitripsin deficiency, coinfection with HIV, alcohol consumption of more than 20 g/day in women and more than 30 g/day in men) were excluded from the current study. Patient evaluation was compared with a control group of 14 healthy subjects with normal liver parameters and negative serological markers for HBV and HCV.

Blood samples were drawn a jeun in all subjects. Biochemical parameters were determined on Konelab 30 I Thermo Electron Corp Finland. Serum samples were stored at -80 0C and TNF-α, sTNFR-2, IL-6 and sIL-6R were measured using the ELISA method with specific antibodies (Quantikine, Human hsTNF-α and sTNFR-2 Immunoassay Quantikine, Human hsIL-6 and sIL-6R Immunoassay, R&D Systems, Minneapolis, USA). The presence of absence of HCV infection was confirmed using ELISA immunoenzimatic techniques (Dia Soin, Raritan, USA) for HCV-ARN. All 80 subjects had positive results using Cobas Amplicor HCV Monitor Test (version 3.0 Branchburg, USA) with a detection level of 600 IU.

Liver histology

Transcutaneous liver biopsies were performed under ultrasound guidance in order to confirm the diagnosis of CHC and for grading and staging according to the METAVIR scoring system [25]. Fibrosis was evaluated on scale from F0 to F4, F0 being absent fibrosis, F1 portal fibrosis with no septums, F2 portal fibrosis and the presence of some septums, F3 portal fibrosis and multiple septums but no cirrhosis and F4 representing cirrhosis. Irrelevant fibrosis was considered for stages F0 and F1, while significant fibrosis was allocated for stages F2, F3 and F4. Patients were stratified into CHC with no cirrhosis (stages F0-F3) and viral liver cirrhosis (F4) according to the METAVIR scoring system. Histological activity that evaluates the necro-inflammatory lesions was further classified as follows: A0- no activity, A1-minimal activity, A2-moderate activity, A3- severe activity. The degree of steatosis was expressed as a percent of hepatocytes affected, using the following scale: 0- no steatosis, 1- minimal steatosis (1-10%), 2-moderate steatosis (11-30%), 3-moderate/severe steatosis (31-70%) and finally 4- severe steatosis (71-100%) [1]. Scores of 0-2 were considered irrelevant steatosis and scores 3 and 4 were considered significant. Hematoxylin-eosin and tricrom Masson stainings were used.

Statistical analysis

Descriptive statistics was used to summarize patient data and was represented as median ± SD (standard deviation) for continuous variables and as number and percentage for categorical data. Differences between variables medians were determined using the ANOVA test.

Correlations between parameters were described using the Pearson correlation coefficient and confirmed after linear regression. To test for differences between groups, we used the student t test for continuous variables and X2 for categorical data. Parameters considered significant in univariate analysis were introduced into multivariate analysis, using logistic regression analysis for categorical data and multiple regressions for continuous data.

Sensibility (Se), Specificity (Sp), ROC curve ((Receiver Operating Characteristic Curve) and the area under the curve (AUC)

were determined using the MedCalc 8.0 statistical software (Belgium).

3. Results

Patient data regarding demographical parameters is depicted in Table 1 and histological features are presented in Table 2. Biochemical and virusological parameters of patients with CHC are depicted in Table 3. Demographical and biochemical data of subjects in the control group are presented in Table 4.

Demographical data of the CHC group Table 1

Demographical data		*CHC group (n=80) Median±SD, n (%)*
Age (years)		47.71 ± 10.51
Sex	Female (%)	46 (57.5%)
	Male (%)	34 (42.5%)

Histological data of patients diagnosed with CHC Table 2

Hystological activity (METAVIR)		***CHC group (n=80) (n, %)***
Activity	*A1*	15 (18.8%)
	A2	35 (43.7%)
	A3	30 (37.5%)
Fibrosis	*F0*	4 (5.0%)
	F1	17 (21.2%)
	F2	27 (33.7%)
	F3	18 (22.5%)
	F4	14 (17.5%)
Steatosis	*<30%*	62 (77.5%)
	≥30%	18 (22.5%)

Biochemical and virusological data of patients with CHC Table 3

Biochemical and virusological data	***CHC group (n=80) (Median±DS)***
ALAT (U/L)	111.60 ± 96.49
ASAT (U/L)	72.25 ± 60.22
Total Bilirubin (mg/dl)	0.79 ± 0.40
GGT (U/L)	77.98 ± 67.38
Viral load (UI/ml)	1391155.00 ± 1018942.33

Demographical and biochemical data of subjects in the control group Table 4

Parameters		**Control group *(n=14) Median±DS, n (%)***
Age (years)		36.71 ± 12.23
Sex	Female (%)	8 (57.14%)
	Male (%)	6 (42.86%)
ASAT (U/L)		18.64 ± 5.83
ALAT (U/L)		23.42 ± 6.41
Total Bilirubin (mg/dl)		0.59 ± 0.21
GGT (U/L)		23.71 ± 5.94

I. TNF-α and its receptor sTNFR-2:

Patients with CHC presented with significantly higher levels of TNF-α (2.44±1.59 pg/ml) compared to the control group (1.21±0.43 pg/ml), p=0.02 (fig. 1).

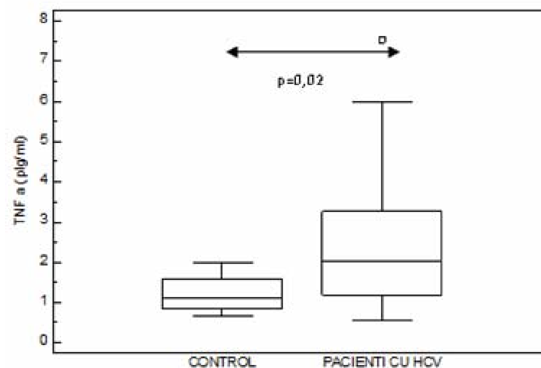

Fig.1. *Serum levels of TNF- α in patients with CHC compared to controls.*

Also, serum levels of sTNFR-2 were higher in patients with CHC when compared to the healthy subjects: 12.23±5.38 ng/ml vs 3.59±1.12 ng/ml, p<0.001 (fig. 2).

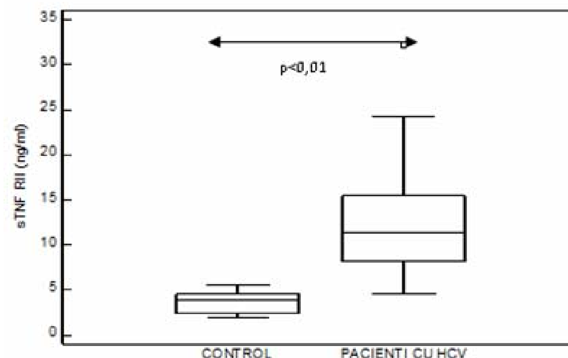

Fig. 2. *Serum levels of sTNFR-2 in patients with CHC compared to the control group.*

The accuracy of the diagnostic tests for the immune parameters performed was evaluated and we obtained a cut-off value of 1.7 pg/mL for TNF- α, with a Se of 60% and a Sp of 85.7%, and of 7.87 ng/mL for sTNFR-2, with a Se of 96.2% and a Sp of 92.9% (fig. 3).

AUROC - Area Under Receiver Operating Characteristics Curve area were calculated for TNF- α and sTNFR-2 to distinguish the patients with CHC and the control group and sTNFR-2 had a better diagnostic power than TNF- α with an AUC of 0.990 vs 0.763 (fig.3). Comparing the AUROCs for the two parameters in the group of patients diagnosed with CHC, we had a significantly higher diagnostic value for sTNFR-2 (ng/ml), compared to TNF- α (pg/ml), P<0.0001 (Table 5).

We further evaluated the relationship between TNF- α levels and histological parameters, but no significant correlation was noted between TNF-α, inflammatory activity, fibrosis score (METAVIR) or significant steatosis (>30%).

On the other hand we found a significant

correlation between sTNFR-2 levels and some hystological parameters, such as the activity score, higher values were detectable in patients with more severe activity scores (A3) (17.19 ±4.74 ng/ml), compared to those with minimal signs of activity (A1) (7.11±2.18 ng/ml) or moderate activity (A2) (10.15±2.89 ng/ml), p<0.05 (fig. 4).

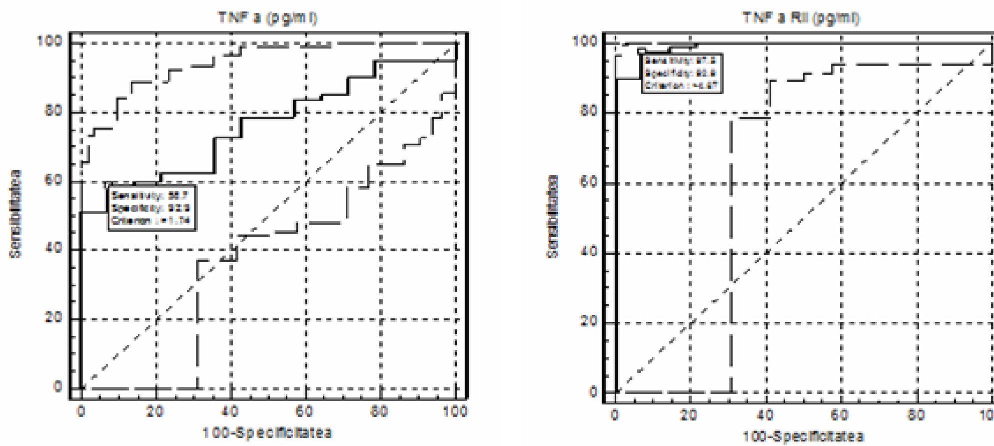

Fig. 3. *ROC curves for TNF-α (left) and sTNFR-2 (right) in patients with CHC.*

Diagnostic power of immune parameters Table 5

Pro-inflamatory cytokines	*AUC*	*SE*	*95% CI*	*p*
TNF-α (pg/ml)	0.76	0.059	0.66 - 0.84	
sTNF-R2 (ng/ml)	0.99	0.0084	0.94 – 0.99	**<0.0001**

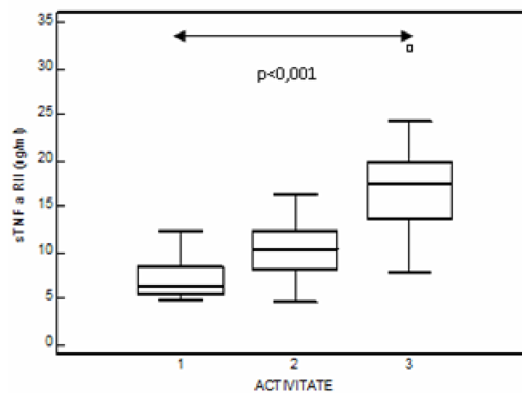

Fig. 4. *sTNFR-2 serum levels according to activity score (METAVIR)*

Moreover, we evaluated the serum levels of sTNFR-2 according to the presence or absence of liver cirrhosis and patients with cirrhosis had significantly higher levels of sTNFR-2 (F4, n=14, 17.32±4.00 ng/ml), compared to patients with CHC (F0-F3, n=66, 11.14±5.02 ng/ml), p<0.001 (fig. 5)

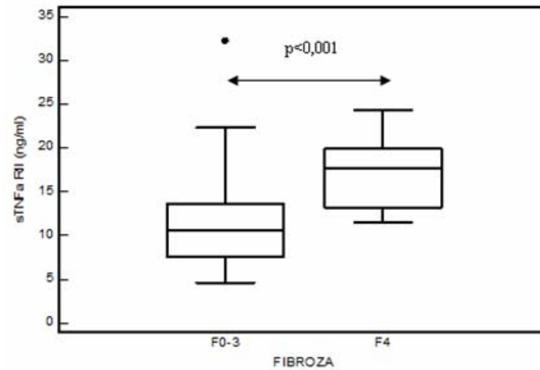

Fig. 5. *sTNFR-2 serum levels in patients with CHC with (n=66)/without (n=14) cirrhosis*

Univariate analysis showed no significant correlations between TNF-α and any studied biochemical markers, or viral load. On the other hand, sTNFR-2 serum levels positively correlated with ALAT values (r=0.28; p=0.01), ASAT (r=0.24; p=0.03) (fig. 6).

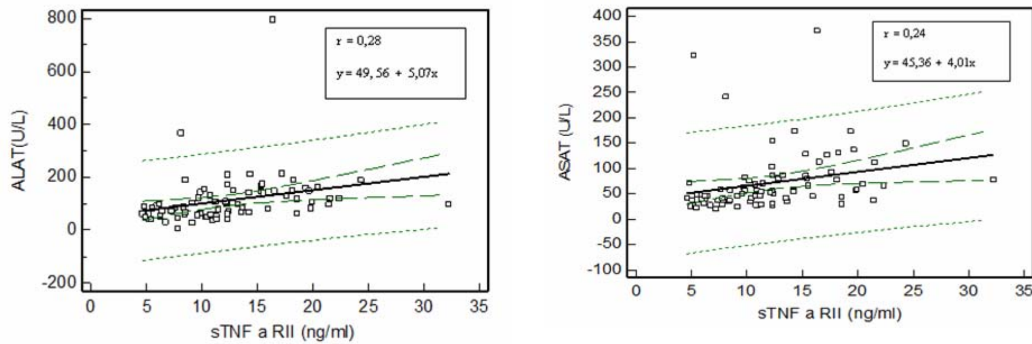

Fig. 6. *Relationship depiction between sTNFR-2 and ALAT (upper panel) and ASAT (lower panel) in patients with CHC*

Multiple regression analaysis for sTNFR-2 Table 6

Parameters	corelation coeficient	Standard error	t	P	r
ALAT (U/L)	0.015	0.0060	2.60	0.01	-0.28
R^2=0.10		p=0.01			

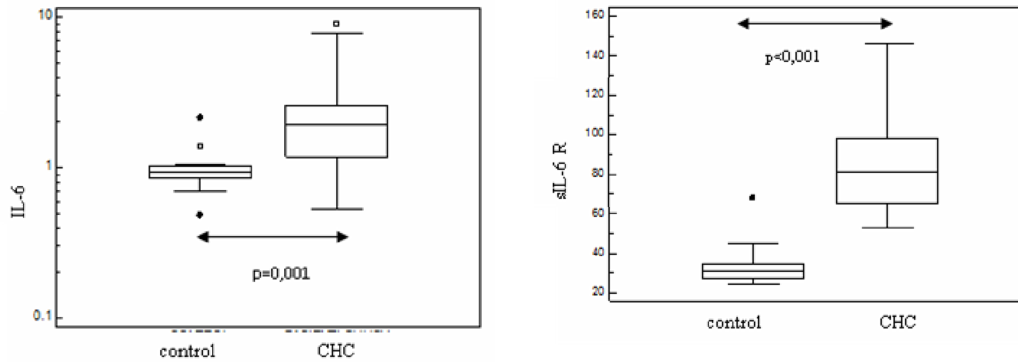

Fig.7. *Serum levels of IL-6 (left) and sIL-6R (right) in patients with CHC compared to controls*

IL-6 and sIL-6R serum levels according to activity score (METAVIR) Table 7

Activity (METAVIR)	N(%)	IL-6(pg/ml) (medie±SD)	p	sIL-6R(ng/ml) (medie±SD,)	p
			p<0.001*		p<0.001*
A1	15 (18.75%)	1.79±0.88	NS	79.76±21.43	p<0.05**
A2	35 (43.75%)	1.99±1.34		75.21±18.65	
A3	30 (37.5%)	2.89±2.00	p<0.001*	94.26±20.22	p<0.05**

However, in multiple regression analysis, only ALAT was independently associated to sTNFR-2 ($R2=0.10$, $p=0.01$)

II. Il-6 and its soluble receptor sIl-6R

Il-6 serum levels were significantly higher in patients with CHC (2.30±1.62 pg/ml) compared to controls (0.99±0.39 pg/ml), $p=0.001$, just like sIL-6R: 12.23±5.38 ng/ml vs 3.59 ± 1.12 ng/ml, $p<0.001$.

Moreover, Il-6 and sIl-6R were both significantly correlated to the inflammatory activity, patients with A3 scores having higher Il6 and sIl-6R levels compared to those with minimal activity.

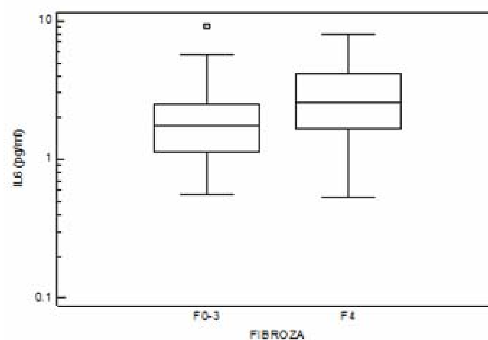

Fig. 8. *Serum levels of Il-6 in patients with CHC and cirrhosis(F4).*

We also determined serum levels of Il-6 and sIl-6R according to the presence or absence of cirrhosis and IL-6 levels were higher in patients with cirrhosis (F4, n=14,

3.21±2.14 pg/ml), compared to those with CHC only (F0-F3, n=66, 2.10±1.43 pg/ml), p<0.001 (fig. 8). A similar spectrum was noted for sIl-6R.

The diagnostic accuracy of tests performed for Il-6 and sIl-6R was performed and we obtained a cut-off value of 2.57 pg/mL for Il-6 with a Se of 57.1% and a Sp of 77.3% and a cut-off value of 76.3 ng/mL for sIl-6R with a Se of 92.9% and a Sp of 48.5%. sIl-6R, in a similar manner like sTNF-αR, had a higher diagnostic power with an AUC of 0.977 compared to Il-6 of 0.681 (fig. 9). No significant relationship was noted between Il-6 or sIl-6R and biochemical parameters or viral load. However, we found a positive correlation between Il-6 and sTNFR-2 and between sIl-6R and sTNFR-2 (r=0.33; p=0.001 r=0.39; p=0.001 respectively) (fig 10).

Multiple regression analysis showed that TNFR-2 levels, independent of TNF-α correlate to Il-6 and sIl-6R levels.

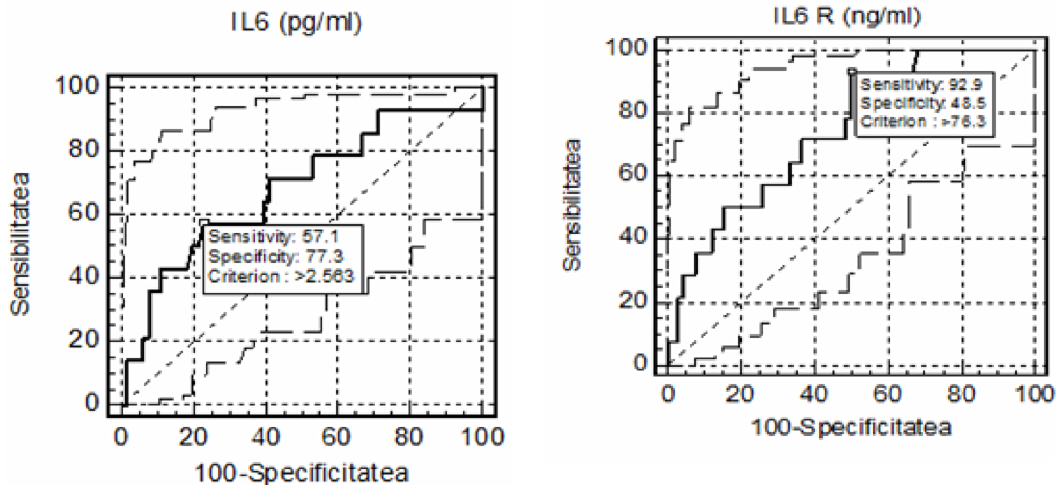

Fig. 9. *Se and Sp for Il-6 (left) and sIl-6R (right) in patients with CHC.*

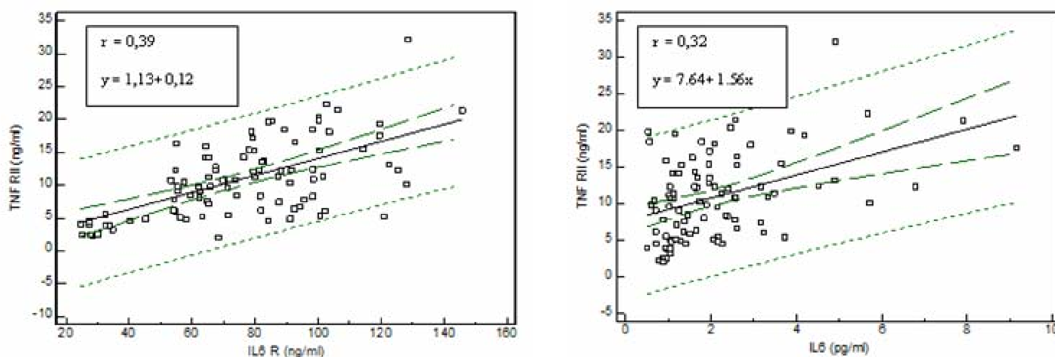

Fig.10. *Scatter diagram and graphic representation of the relationship between Il-6, sIl-6R and TNFR-2.*

Multiple regression analaysis for IL-6 (A) and sIL-6R (B) Table 8

A

Parameter	Correlation coeficient	Standard error	t	P	r
TNFR-2	0.097	0.032	3.039	0.003	0.32
R^2=0.10		p=0.003			

B

Parameter	Correlation coeficient	Standard error	t	P	r
TNFR-2	1.56	0.41	3.77	0.0003	0.39
R^2=0.15		p=0.003			

4. Discussion

In spite of a proper immune response, in 85% of cases hepatitis C virus turns into a chronic infection, due to the properties of the virus that allow an escape phenomena from the hosts defense mechanisms [6]. The immune pathway involved in the development of CHC is still debated, mostly the molecular mechanisms that get activated during the chronic inflammation process and are responsible for the cellular mediated lesions [19]. In our research we noted that TNF- α and its soluble receptor have increased levels in patients with CHC. Nonetheless, sTNFR-2 has a higher diagnostic power, suggesting the fact that it not only reflects a response to increasing expression levels and secretion of TNF-α, but could be induced by cytokines or direct viral action, independent of TNF-α.

TNF-α is involved in the pathogenesis of multiple hepatic diseases and it can be released by infected Kupffer cells, lymphocytes and hepatocytes, as a response to intracellular viral replication. In our study, TNF-α and sTNFR-2 levels were markedly increased in patients with CHC compared to the healthy subjects, in a similar way as in other studies [18, 23]. Although we have no obtained any significant correlation between TNF-α and the activity score (METAVIR) or hepatic steatosis other studies have noted a relationship between TNF-α levels and the histological activity index (HAI), an association supported by immun-histochemical stains on biopsy specimens, where the staining for TNF-α positively correlated with the inflammation degree [14, 15]. However, this connection was only present for moderate HAI, not for severe HAI. In another study TNF-α was correlated to A1 and A3 activity score, but not with A0 and A2 [24]. These conflicting results could be explained by the relatively small number of patients enrolled, the different scoring systems used to quantify inflammation and different ELISA kits for TNF-α determination. Hence, we still don't know whether the increased levels of TNF-α are the cause or the result of hepatic inflammation.

Other studies have described a positive relationship between TNF-α mARN levels and HAI, as well as viral genotype 1 and increased levels of mARN TNF-α were associated with a lack of response to IFN-α therapy [11]. We obtained similar results to other studies regarding TNF- α and fibrosis stage, with no association between the two [24]. sTNFR-2 levels however increased together with the activity grade, in accordance to other papers published [10], suggesting the fact that sTNFR-2 reflects the progression of hepatic disease in patients with CHC. Moreover, the circulating levels of soluble receptors could reflect the activation of the TNF- α signaling pathway, with receptors having a

longer persistence in the periphery [22]. Receptors are cleaved from the cell surface by proteolytic action, following PMN, monocyte and fibroblast activation as a response to different stimulants (LPS, IL-10, IL-2, GM-CSF) [9].

Soluble receptors are present in patients with CHC in concentrations 30-80 times higher than TNF- α, but their concentrations should be 500-1000 times higher in order to block 50% of TNF-α activity [4].

Il-6 is a multifunctional cytokine with an important role in hepatic regeneration and together with TNF- α and other growth factors plays an important step in the hepatic immune response [23]. Previous studies have showed increased levels of Il-6 in patients with acute hepatic lesions [21], alcoholic liver disease [3], but also in patients with CHC. The biological effects of Il-6 depend on its soluble receptor, being responsible of the trans signaling process. Our work has identified the presence of increased levels of Il-6 and Il-6R in patients with CHC. This fact supports the theory that Il-6/sIl-6R complex system is set off in chronic infections with HCV. The interesting fact about the Il-6/sIl-6R is their behavior in cirrhosis. In a previous study Il-6 levels were increasing with disease progression towards cirrhosis, probably representing a response of hepatic regeneration of residual cells [12]. On the contrary, sIl-6R levels decreased towards the advanced stages of liver disease, suggesting that the release of sIl-6R decreases with the reduction of viable hepatocytes [5]. In our study, sTNFR-2 and Il-6/sIl-6R were tightly correlated, suggesting an interrelationship between these immunological parameters.

The pro-inflammatory cytokines and cytokine receptors have a greater diagnostic power. Therefore, dynamic measurements of Il-6/sIl-6R and TNF-α/sTNFR-2 in patients with chronic viral hepatitis C, could be useful tools for disease progression monitoring.

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