TNF-α and CXCL-10 Correlation with Insulin Resistance in Patients with Chronic Hepatitis C Virus Infection

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There are increasing reports of association between HCV infection and type-2 diabetes mellitus. Although the mechanism by which this association remains uncertain, development of insulin resistance may explain this association. We investigated the association of TNF-α and CXCL-10 with insulin resistance in HCV infected patients. Forty-four non-diabetic chronic hepatitis C patients and twenty healthy individuals were included. Fasting blood was used for glucose and insulin measurements. Diagnosis of insulin resistance (IR) was based on a mathematical means by the homeostasis model assessment score-insulin resistance index (HOMA-IR). Serum insulin, TNF-α and CXCL-10 levels were measured by enzyme linked immunosorbent assay (ELISA). Quantitative measurement of hepatitis C virus was performed by a standardized real time PCR assay. The HCV patients demonstrated a significant increase in serum TNF-α, CXCL-10, and HOMA-IR values as compared to normal controls. HOMA-IR level positively correlated with hepatitis C viral load, TNF-α and CXCL-10. It is concluded that, TNF-α, CXCL -10 correlate with IR and may play a role in the development of type-2 diabetes mellitus in chronic hepatitis C infected patients.

Hepatitis C virus infection causes acute and chronic hepatitis and in addition to well established hepatic injury (Castera *et al.,* 2003), there are many examples of extra-hepatic disorders related to chronic hepatitis C such as, thyroditis, arthritis, essential mixed cryoglobulinemia and other immunological diseases (Hadziyannis, 1997). It has been shown that, hepatitis C virus (HCV) infection is associated with type 2 diabetes mellitus (Mehta *et al.,* 2003; Huang *et al.,* 2007). In addition, a high prevalence with both diabetes and impaired fasting glucose has been reported in HCV-infected patients in comparison with other chronic liver diseases (Lecube *et al.,* 2004). However, the specific mechanisms involved in the pathogenesis of diabetes associated with HCV infection remain to be elucidated; it seems that insulin resistance (IR) may play an essential role (Shintani *et al.,* 2004). There is increasing evidence suggesting the concept that chronic low-grade activation of the immune system may play a role in the pathogenesis of insulin resistance and type 2 diabetes mellitus (Pradhan *et al.,* 2001). The presence of insulin resistance in the setting of HCV infection is of particular importance because hyper-insulinemia appears to play a role in the HCV-liver disease (Hickman *et al.,* 2003; Hui *et al.,* 2003), as insulin resistance seems not only to accelerate the morbidity course of chronic hepatitis C, but also to influence the response to antiviral therapy (Romero-Gomez *et al.,* 2005). HCV and type 2 diabetes mellitus are associated more than just by chance, suggesting that HCV infection is an independent predictor of type 2 diabetes mellitus (White *et al.,* 2008), as HCV may alter glucose homeostasis by its direct action or via indirect mechanisms such as through cytokine stimulation (Knobler *et al.,* 2003). Chronic hepatitis C virus infection may

exacerbate insulin resistance, by increasing oxidative stress and intra-hepatic secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α,) (Knobler & Schattner, 2005).

Chemokines, a short neologism for "Chemotactic cytokines" that induce recruitment of leukocytes to the site of inflammation, as virus infections usually induce a massive chemokine and cytokine burst and therefore recruit a large plethora of leukocytes with the goal to restrict viral spread. There are currently four branches of the chemokine family: CXC, CC, CXC3 and C which can be divided on the basis of their molecular structure (Invernizzi *et al.,* 2008). CXC chemokines have been classified as "inflammatory" or "homeostatic" on the basis of their main functions. The inflammatory/ inducible chemokines, which are regulated by pro-inflammatory stimuli, such as lipopolysaccharide and primary cytokines (i.e., IL-1 and TNF-α), orchestrate innate and adaptive immune response and control the recruitment of effectors leukocytes in infection and inflammation sites (Rot & Von Andrian, 2004). The role of CXC chemokines in several types of inflammatory and autoimmune disorders has been largely investigated (Charo & Ransohoff, 2006).

CXCL-10 is one of three CXC chemokines sharing the properties to be strongly up-regulated by IFN-α. CXCL-10 was initially called "IFN-γ inducible protein 10" (IP-10). CXCL-10 binds to a receptor named CXCR3, which was first identified on activated T cells mainly on type 1 T helper cells (Th1), B cells, and natural killer cells (Bonecchi *et al.,* 1998). CXCR3 expression is associated with Th1-mediated immune responses, and may play a role in the pathogenesis of endocrine autoimmune diseases. Among the CXCR3 binding chemokines, (CXCL-9, CXCL-10, and CXCL-11), CXCL-10 is the most helpful and reliable serum marker of the therapeutic outcome in HCV patients (Christen *et al.,* 2003).

Despite the multiplicity of chemokines and cytokines released during inflammation and the likely redundancy of the system, many studies demonstrated a unique and non-redundant role CXCl-10 as it has been proposed that CXCL-10 is involved in recruitment and potentiation of Th1 mediated immune responses as CXCL-10 up-regulates the production of Th1 cytokines, (Romagnani *et al.,* 2005) as well as CXCL-10 is involved in the pathogenesis of allograft rejection, multiple sclerosis, diabetes mellitus types I and Grave's disease (Rotondi *et al.,* 2007).

The development of IR in chronic hepatitis C infected patients is due to virus-specific alteration in host metabolism as chronic low-grade activation of the immune system may play a role in the pathogenesis of IR and DM. In chronic hepatitis C infection, markers of inflammation, like, tumor necrosis factor-α (TNF-α) may play an essential role in the pathogenesis of IR as TNF-α represents an integral component of the inflammatory response to HCV infection (Elsammak *et al.,* 2005).

So, HCV, insulin resistance and type 2 diabetes mellitus are associated to an extent that can not be merely explained by chance, which suggests that HCV interferes with glucose metabolism, directly (through one or more of its proteins) and/or indirectly (by modulating the production of specific cytokines, like TNF-α and also other chemokines like CXCL-10.

In this study, we measured serum level of CXCL-10 and TNF-α in chronic hepatitis C positive patients and explored its link to IR and development of diabetes mellitus in hepatitis C infected patients.

Subjects and Methods

Subjects

The study included 44 non-diabetic patients with chronic hepatitis c virus infection. Chronic HCV infection is defined by persisting hepatitis c infection for more than six months which was evidenced by persistent increased alanine transferase (ALT). All patients were sero-positive for HCV antibody and HCV RNA quantitative by PCR. HCV patients were excluded if they had: a) A fasting blood glucose over 125 mg/dl or were on anti- diabetic treatment, b) Concurrent hepatitis B virus or human immunodeficiency virus infections or autoimmune-hepatitis, c) Antiviral or corticosteroid therapy, and d) Malignancies, liver fibrosis, cirrhosis, infections, renal insufficiency, significant respiratory or cardiac dysfunction, obesity and positive family history of diabetes mellitus

Ultrasound was done for every HCV patient to exclude cirrhosis, splenomagaly and ascites and upper endoscopy examination to exclude oesophageal varices. All patients were attending the outpatient unit of the Internal Medicine Department, Tanta University Hospital.

Twenty matched healthy non-diabetic volunteers, served as controls. All of them had normal medical history, physical examination, blood chemistry including [(-ve) HCV antibody, and normal liver function tests]. Also, none of them was obese.

The body mass index (BMI) was calculated by dividing body weight (Kilos) by the square of height (meters).

Informed written consent was obtained from all participants. The study was approved by Tanta University Hospital human ethics committee.

Methods

Laboratory data, include anti-HCV, routine chemistries as, (fasting glucose liver function tests, total cholesterol, and triglycerides) and specific tests as fasting insulin, tumor necrosis factor α (TNF-α), interferon gamma inducible protein 10 (IP-10) or CXCL-10 concentrations, and HCV - RNA levels.

Venous blood samples were collected after an overnight fast (about 12 hr.) Aliquots of these samples were used for HCV antibody and other routine biochemical analysis immediately, but aliquots for serum insulin, TNF-α, CXCL-10/IP-10, and HCV-RNA were stored at -80°C until tested. Anti- HCV was measured using automated chemiluminescence's system (ADVIA Centaur) provided by Bayer HealthCare. Fasting glucose, total cholesterol, triglycerides, and liver function tests were assayed by automated laboratory methods at the clinical chemistry laboratories using (Autohumalyzer 900 S, Human).

-Fasting serum insulin: was determined with a solid phase two- site enzyme immunoassay supplied with the kit [DRG International, Inc (USA)] according to manufacturer instructions.

-Tumor necrosis factor-a (TNF- α): was measured by the commercially available enzyme linked immunosorbent assay (ELISA) kit (Anogen, Canada), as described by the company. The results were read spectrophotometrically at 450 nm using LABTOP ELISA reader, (Aggarwal, 1985).

-Interferon gamma inducible protein 10(IP-10)/Chemokine (CXC motif) ligand 10 (CXCL-10): was measured by sandwich enzyme immunoassay technique as described by the manufacturer's protocol supplied by [R&D systems, Inc, (USA)], (Angiolillo, *et al.,* 1995).

-Quantitative measurement of hepatitis C virus: was performed by a standardized quantitative StepOne real time PCR assay by (Applied biosystems). HCV RNA of serum samples were extracted using QIAamp spin columns kit supplied by (Qiagen, Inc, USA). The extracted RNA of samples was amplified by specific primers using real time PCR master mix and enzyme (TaqMan reagents). The presence of specific viral RNA sequences in the reaction was detected by an increase in the fluorescence observed from the relevant dual-labelled probe, and was reported as a cycle threshold value (Ct) by the real time thermocycler (Applied biosystems). A standard curve is generated by plotting the known concentration of quantification standards (QS1; QS2; QS3 (Qiagen, Inc, USA); included in the test) against their Ct value. The Ct value of a patient sample was compared against the standard curve in order to obtain the viral load in the specimen, (Pawlotsky *et al,* 2000).

- Insulin resistance (IR)

IR was calculated using the homeostasis model assessment (HOMA) score, expressed in the following equation: IR = Fasting insulin (mU/L) x Fasting glucose (mmol/L) / 22.5, HOMA -IR = > 3.0, according to (Matthews *et al.,* 1985). With such method, high HOMA scores denote low insulin sensitivity (insulin resistance). In Egyptian study according to (Laila *et al,* 2008), they suggested Cut off point to define insulin resistance corresponds to

HOMA-IR ≥ 3.8 while the mean value in healthy non diabetic subjects was 2.8±5.2.

Statistical Analysis

Data were analyzed using the statistical package for social sciences (SPSS). Results were expressed as mean ± SD, and differences between the means of different variables were tested using the student t-test. Pearson's correlation coefficient was used to study the correlation between different variables. Significance was accepted at the level of $P<0.05$.

Results

The characteristics of the 44 HCV (+) patients and 20 HCV (-) healthy controls are shown in Table 1. HCV (+) patients include 33 male and 11 female with a mean age of 44.2 ± 8.5 years while the HCV (-) healthy controls include 14 male and 6 female with a mean age of 43.8 ± 9.0. Both groups were matched as regard to age, sex, body mass index and fasting blood glucose levels.

Table 1. Clinical and biochemical features of HCV (+) patients in comparison with HCV (-) controls.

	HCV (+) N=44	HCV (-) N=20	*P* =
Age (years)	44.2 ± 8.5	43.8 ± 9.0	NS
Sex (M/F)	33/11(75%)	14/6(70%)	NS
Body mass index (kg/m^2)	25.3 ± 3.5	24.8 ± 3.4	NS
HCV RNA (copies/ml)	606.0 ± 492.0	---	---
Fasting glucose(mg/dl)	94.2±8.5	91.0±8.2	NS

P> 0.05 is not significant. NS= not significant.

The mean value of serum TNF-α (pg/ml) was higher in HCV infected patients than controls (87.6±31.2 vs. 22.7±12.1, $P<0.001$). Similarly, there was a significant increase in CXCL-10 (pg/ml) in HCV infected patients as compared to healthy controls (152.0 ± 35.5 vs. 81.6 ± 20.2, $P < 0.001$), Table 2.

Table 2. TNF-α and CXCL-10 levels in HCV (+) patients in comparison with HCV (-) controls

	HCV (+) N=44	HCV (-) N=20	**P* =
TNF-α (pg/ml)	87.6 ± 31.2	22.7 ± 12.1	< 0.001
CXCL-10 (pg/ml)	152.0 ± 35.5	81.6 ± 20.2	< 0.001

**P*< 0.05 is significant

Table 3 shows that patients with HCV infection have higher fasting insulin (mU/L) level (19.5±5.9 vs 11.2±2.1, $P<0.05$) as compared to healthy controls, and that HOMA-IR values are higher in HCV positive patients than HCV negative group (4.5±1.77 vs 2.0±0.7, $P<0.01$).

Hepatitis C viral load and TNF-α correlated positively with HOMA-IR (Fig.1 & Fig.2 respectively). A positive correlation was also found between CXCL-10 and HOMA-IR in HCV (+) patients (Fig.3).

Table 3. Fasting insulin and HOMA-IR in HCV (+) patients in comparison with HCV (-) controls.

	HCV (+) N=44	HCV (-) N=20	*P* =
Fasting insulin (mU/L)	19.5 ± 5.9	11.2 ± 2.1	< 0.05*
HOMA-IR*	4.5 ± 1.77	2.0 ± 0.7	< 0.01*

P< 0.05 is significant. *Homeostasis model assessment- insulin resistance (HOMA-IR)

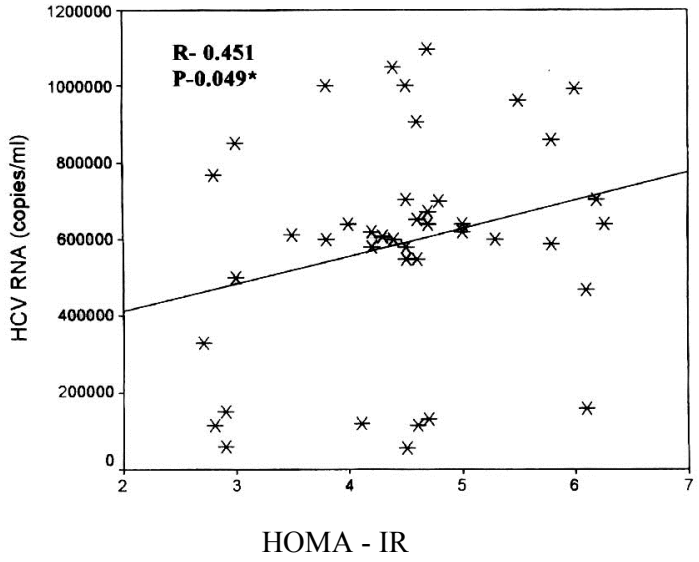

Figure 1. Correlation of HCV-RNA Levels with HOMA-IR in HCV (+) Patients

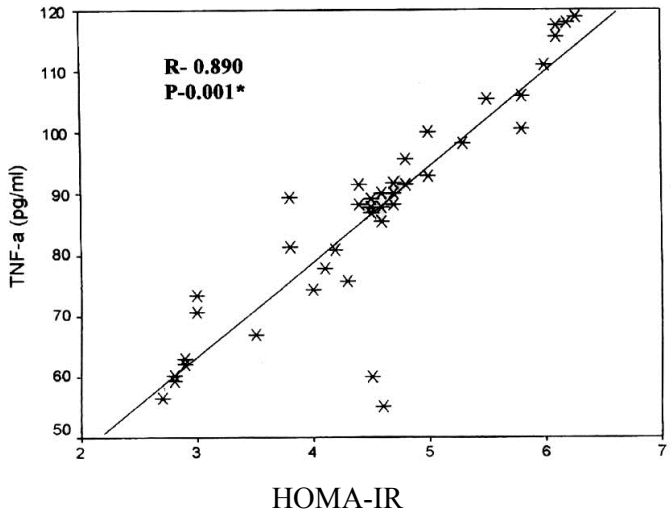

Figure 2. Correlation of TNF-α Levels with HOMA-IR in HCV (+) Patients.

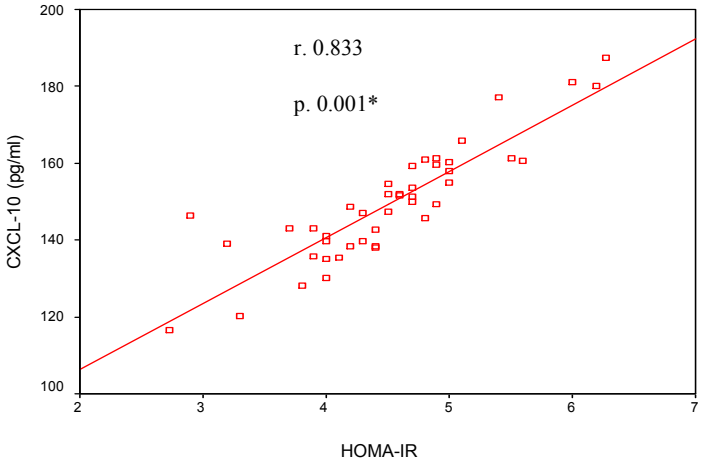

Figure 3. Correlation of CXCL-10 (pg/ml) with HOMA-IR in HCV (+) Patients

Discussion

Metabolic abnormalities are common in patients with hepatitis C virus (HCV) infection, there is considerable evidence that patients with chronic HCV infection are at a greater risk of developing insulin resistance (IR) and ultimately, diabetes mellitus (DM) compared with non-infected individual individuals or patients with hepatitis B virus (HBV) infection (Knobler *et al.,* 2000).

In the present study, we found that the patients with hepatitis C virus infection had significantly increased fasting insulin levels when compared with healthy controls. Also, insulin resistance indicated by HOMA-IR was significantly increased in HCV +ve patients than healthy controls matched for age, sex, BMI. These results are in agreement with the study of Lecube *et al.,* (2006), where insulin abnormalities including impaired fasting insulin levels, were significantly increased in HCV positive patients, compared to HCV negative patients.

Our results demonstrated a positive correlation between viral load and insulin resistance as we found that HCV +ve patients who had high viral load had significantly increased fasting serum insulin levels, and increased HOMA-IR scores when compared with patients of low viral load. These results are supported by the previous results of Yoneda *et al.,* (2007) and Hsu *et al.,* (2008). They reported a significant positive correlation between HCV mRNA levels and both fasting insulin levels and HOMA-IR score in patients with HCV infection with no history of diabetes mellitus or obesity.

Unlike our results, the studies were done by Imazeki *et al.,* (2008) and Tanaka *et al.,* (2008) where they did not find correlation between HCV replication level and HOMA-IR score, the lack of correlation between HCV RNA levels and HOMA-IR score may also be caused by the fact that score of IR depends on contribution from the muscle and fatty tissues and these two extra-hepatic organs are not infected by HCV.

In our study, IR was independently and significantly associated with high serum HCV RNA levels of non-diabetic HCV infected patients and this confirmed with the previous study of Moucari *et al.,* (2008), and Harrison, (2006) who reported that non diabetic HCV infected patients have significantly higher IR compared with age, sex and BMI matched

healthy subjects. These data suggested that IR represent not only a metabolic disorder related to obesity and adipose tissue inflammation with subsequent imbalance in the secretion of specific cytokines, but also a direct viral effect of HCV infection. Several mechanisms have been proposed to explain HCV induced IR, (Romero-Gomez, 2006) and all suggested direct impairment of the insulin – signaling pathway (Negro & Alaei, 2009).

TNF-α level was estimated in our study as it is an important cytokine in the inflammatory process of HCV infection and may have a role in IR in HCV infected patients. In our results, TNF-α was significantly increased in HCV infected patients as compared to control subjects. The study of Kawaguchic *et al.,* (2004) and Noto *et al.,* (2006), are in agreement with our results. They suggested that, HCV infection by increasing tumor necrosis factor-alpha (TNF-α) levels, changing the insulin signaling pathways, could result in insulin resistance. So, TNF-α may have a significant role in the development of insulin resistance in patients with HCV infection. Hepatitis C virus induces insulin resistance (IR) independently of body mass index, as HCV core protein has been found able to induce IR, and type 2 diabetes mellitus (Wang *et al.,* 2003).

The main mechanism seems to be the over production of TNF-α. This cytokine phosphorylates serine residues of insulin-receptor substrate 1 and 2 (IRS-1, 2) and enhances the production of suppressor of cytokines (SOC-3). The SOC-3 substance inhibits the phosphorylation of AKt and phosphatidyl inositol 3 kinase (PI3-K). All these impairments in the intracellular signaling of insulin could block the trans-activation of GLU-T, avoiding the uptake of glucose by cells (Aytug *et al.,* 2003). Other possible mechanisms of IR include direct cytotoxic effects of HCV on pancreatic islet cells (Masini *et al.,* 2005), by immune – mediated mechanisms (Antonelli et al., 2004), and elevated levels of CXCL-10 (Antonelli *et al.,* 2009).

Our results demonstrated that increased levels of TNF-α detected in HCV infected patients and their relationship with HOMA-IR strongly suggested that TNF-α is a mechanism by which HCV infected patients are more prone to develop IR and ultimately type 2 diabetes mellitus. Our results showed a positive correlation between increase of TNF-α levels and insulin resistance which was detected by HOMA-IR in HCV infected patients. Our results are supported by the previous results of Lecube *et al.,* (2006) and Hung *et al.,* (2009), they found that TNF-α levels were significantly up-regulated in the sera of those patients with HCV infection and diabetes mellitus. But unlike our results as in the part of +ve correlation between HOMA-IR and TNF-α levels, the previous study by Hung *et al.,* (2009) where they are in agreement with our results as these are significant high levels of TNF-α in the sera of HCV patient but they reported that serum TNF-α levels did not correlate with the extent of IR, suggesting that HCV-associated IR is not mediated by these only cytokines, but other cytokines and mechanisms may be involved.

So, TNF-α levels may be a biomarker for the development of DM and an unfavorable clinical course in patients with chronic HCV infection. So, IR in chronic HCV patients is mediated by pro-inflammatory cytokines expression with TNF-α being of particular importance.

In addition, Romero-Gomez *et al.,* (2005) demonstrated that the clearance of HCV in non-diabetic patients after antiviral therapy induced a decrease of insulin resistance but not in non-responder and also at the same study, in the relapsers, the HOMA- index increased. In addition, the study of D'Souza *et al.,* (2005) demonstrated that the HOMA-

index was significantly higher in non-responders than in patients with sustained response and they confirmed that the non-diabetic HCV patients treated with peginterferon and ribavirin, their insulin resistance is the most important host factor in the prediction of response to antiviral therapy, so the treatment of insulin resistance by decreasing hyperinsulineamia may improve sustained response in chronic hepatitis C treated with peginterferon and ribavirin.

Previous studies of Masini *et al.,* (2005) and Christen *et al.,* (2004) showed that infection of Beta-cells by HCV might induce an inflammatory or autoimmune reaction and the studies have linked Th1 immune response with HCV infection and endocrine disorders. Furthermore, the previous study of Patzwahl *et al.,* (2001) is in agreement with our study.

Their study has shown an increased expression of interferon-gamma (IFN-γ) and IFN-γ inducible protein in hepatocytes and lymphocytes of HCV-infected patients. In fact, HCV infection may up-regulates CXCL-10 secretion by beta-cells; this chemokine is responsible for Th1 lymphocyte recruitment. Then, these Th1 lymphocytes secret interferon-gamma (IFN-γ) and TNF-α with a synergistic effect induce CXCL-10 secretion by beta-cells, thus perpetuating the immune cascade leading to the appearance of beta-cell dysfunction in genetically predisposed subjects (Apolinario *et al.,* 2005).

Our results showed significant elevation of CXCL-10 in non-diabetic hepatitis C +ve patients compared to the control healthy subjects. These results are in agreement with the results of Itoh *et al.,* (2001) and Matskevich *et al.,* (2003). They showed an increased expression of interferon-gamma (IFN-γ) and IFN-γ inducible chemokines (CXCL-10) in hepatocytes and in lymphocytes of HCV-infected patients with an increase of circulating levels of IFN-γ and CXCL-10.

The role of CXCL-10 has emerged in the study done by Butera *et al.,* (2005) and Diago *et al.,* (2006), where they suggested that high plasma concentration of CXCL-10 may be a predictor of unresponsiveness to antiviral therapy with peginterfern-α and ribavirin in chronic HCV infected patients. So, CXCL-10 through its double action, the indirect one by stimulating the release of TNF-α which has an essential role of insulin resistance through impairment in insulin signaling pathway and by its direct action on beta-cells of pancreas which may lead finally to the appearance of beta cell dysfunction in genetically predisposed subjects. The fact that autoimmunity is responsible for most of endocrine disease indicates that the results obtained by assaying CXCL-10 in autoimmune non endocrine disorders as in hepatitis C virus infection may give idea about the possible transfer to endocrinopathies in the future. So, measurements of serum CXCL-10 represent a reliable marker of aggressive Th1-mediated autoimmune disease and consequent appearance of endocrine diseases.

So, early screening of patients with chronic HCV infection for detection of insulin resistance or diabetes mellitus is recommended as HCV infection is an independent predictor of type 2 DM. Moreover, hyperinsulinaemia and insulin resistance are independent risk factors for the response to antiviral therapy. So, early diagnosis and control of DM in HCV infected patients could result in better care and outcome of these patients. Also, better control of HCV infection could prevent the development of DM and IR in chronic hepatitis C patients. Furthermore early measuring of CXCL-10 and TNF-α levels in non-diabetic HCV patients is recommended to predict the occurrence of IR and DM and

moreover, better control of IR and hyperinsulinemia improve the response to antiviral therapy peginterferon and ribavirin.

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