

The plasma level of cathelicidin-LL37 in patients with chronic hepatitis C virus

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Abstract—The role undertaken by antimicrobial peptides in neutralizing immune inflammation in patients with infection remains unclear. Human cathelicidin LL37 is an antimicrobial peptide upregulated by 1,25-hydroxyvitamin D, which has not been previously studied in patients with HCV infection. The aim of our study was to evaluate the serum levels of LL37 as an anti-inflammatory factor in 46 subjects (25 HCV patients and 21 healthy controls) and to investigate the potential relationships between the LL37, 25-hydroxyvitamin D and necroinflammation in these patients. The serum concentrations of LL37 were significantly greater in patients with HCV infection by comparison with values obtained from controls (12.2405 ng/ml compared with 9.9930 ng/ml, $p=0.023$).

The highest LL37 levels were found in patients with low necroinflammatory activity (18.1426 ng/ml) and medium fibrosis (18.5578 ng/ml), compared to those recorded in controls (9.9930 ng/ml). The difference between the values found in HCV infected patients and controls was statistically significant ($p=0.023$).

The obtained data suggests a possible involvement of LL37 in chronic immune inflammation in patients infected with HCV.

Keywords—Cathelicidin-LL37, Chronic inflammation, Hepatitis C virus

I. INTRODUCTION

THE HCV infection remains a global health problem through its frequency, chronic potential and lack of an efficient prophylaxis [1]. HCV is an RNA virus with hepatic tropism [2]. Extra-hepatic replication also occurs in immune cells such as dendritic cells or lymphocytes, as well as in other additional sites, promoting immune evasion and a chronic infection. The HCV infection along with other inflammatory factors maintains the chronic immune activation, which ultimately leads towards hepatic fibrosis

and malignancy. Factors independently associated with fibrosis progression are numerous (age, male gender, various associated infections, low CD4 count and others) [4]. An important role could also be attributed to the breakdown of the gastrointestinal barrier. Thus, the lipopolysaccharide (LPS) released in the portal blood could increase, activating molecules of the innate immune system [5], [6]. Recently the low vitamin D level was correlated with hepatic fibrosis and poor liver function [7]. The anti-inflammatory effect of 1,25(OH)D, the active form of vitamin D is exerted via vitamin D receptors expressed by all cells of the immune system. Vitamin D receptors further up-regulate the synthesis of LL37, an immune modulating antimicrobial peptide with a high antiinflammatory potential. LL37 was particularly studied for its ability to neutralize LPS in sepsis. Other possible therapeutic uses have also been suggested, including chronic inflammatory diseases. [8]- [10].

The aim of our study was to evaluate the serum levels of LL37 as anti-inflammatory factor in patients with HCV infection and to investigate the potential relationships between the LL37, 25-hydroxyvitamin D [25(OH)D] (the best indicator of vitamin D status) and necroinflammatory activity at these patients.

II METHODS AND MATERIALS

Patients. The study recorded data obtained on 46 Caucasian subjects aged 43.6years (SD=16.14) out of which 17 males and 29 women, HIV negative. 25 subjects were diagnosed with HCV infection genotype 1 and 21 were healthy controls. The patients presented no underlying renal disease, cardiovascular disease, diabetes, autoimmunity, malignancy, or other coinfections, not taking vitamin D or calcium supplements.

Samples. Samples were collected between January and September 2009. Serum collection for 25(OH)D and LL37 level were obtained in EDTA tubes and centrifuged for 20 minutes at 1100 – 1300 rpm. The plasma was stored at -80°C prior to analysis.

Tests. Baseline laboratory tests, performed using standard hospital laboratory methods, included: the hemogram, creatinine, blood glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), hepatitis viral markers (Anti-HCV, HBsAg, Anti-HBs, HBeAg, Anti-HBe, Anti-HBc IgG/IgM), Anti-HIV, CD4+T cell and CD8+Tcell number (flow cytometric detection). The Fibrotest and Actitest score (BioPredictive) were used to assess the fibrosis and necroinflammatory liver activity. The viral load (RNA HCV) was detected with Real-time PCR assay (Roche Cobas TaqMan, limit of detection 45ui/mL).

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TABLE I

BASELINA DEMOGRAPHIC, LABORATORY AND ACTITEST/FIBROTEST RESULTS OF PATIENTS WITH HEPATITIS C VIRUS INFECTIONS

Variable	HCV patients	controls	p value
	mean±SD/median value (number cases)		
Age	50.58±14.87	35.61±13.90	0.0012
Sex (male/female)	8/17	9/12	0.4473
Alanine aminotransferase (ALT)	70	25	0.0000
Aspartate aminotransferase (AST)	59	23	0.0000
White blood cell count	6.328 x 103 ± 2.1248 x 103	7.5143 x 103 ± 1.7746 x 103	0.0483
Lymphocytes	2.2560 x 103 ± 0.8073 x 103	2.2857 x 103 ± 0.7023 x 103	0.8957
Platelet count	198.64 ± 90.6476	239.75 ± 73.1501	0.1075
CD4 number cells	836.136 ± 310.5243	899.615 ± 260.1405	0.5402
CD8 number cells	429.363 ± 183.9603	590.416 ± 198.8295	0.0239
CD4/CD8	2.1005 ± 0.6911	1.7658 ± 0.8659	0.2263
Serum 25(OH)D, nmol/l	29.5714 ± 7.5514	29.2701 ± 9.4078	0.9046
Serum LL37 level, ng/ml	12.2405	9.9930	0.0023
Stage of fibrosis	F1(1) F2(8) F3(11) F4(5)		
Grade of inflammation	A0(3) A1(10) A2(1) A3(11)		

Serum levels of 25(OH)D (expressed as nmol/L) were assessed using Elisa (IDS 25-Hydroxy Vitamin D EIA kit, Immunodiagnostic Systems Ltd, UK -detection range 6–360 nmol/l).

Serum levels of LL-37 (expressed as ng/ml, dilution factor 1/10) were determined with Elisa kit (HK 321 Humman LL37 Elisa Kit, Hycult biotechnology, Uden, The Netherlands- detection range 0.1-100 ng/mL).

All protocols followed the manufacturer’s Instructions. Each Elisa test was run in duplicate, with mean absorbance computed from the average for 2 wells normalized to a zero calibrator well. Levels of vitamin D in test samples were derived by fitting a 2-parameter logistic curve to 6 standard levels .The intra-assay CV for 25 (OH) D and LL37 was <8% and <10% respectively. The inter-assay CV for 25 (OH) D and LL37 was <10% in both cases .

Diagnosis. Diagnosis of HCV infections followed CDC criteria (Guidelines For Viral Hepatitis Surveillance And Case Management, 2009 : <http://www.cdc.gov/hepatitis/SurveillanceGuidelines.htm>).

The scores for necro-inflammatory activity range were considered from A0 to A3 (A0= no activity, A1 = minimal activity, A2 = moderate activity, A3 = severe activity). Fibrosis was scored from F0-F4 [11]-[13].

Vitamin D status was defined according to 25(OH)D serum levels. Vitamin D deficiency or insufficiency was defined as a 25(OH)D concentration <80nmol/L and <50 nmol/L respectively .

LL37 status was defined by comparing the obtained LL37 serum concentrations with values found in healthy controls.

The HCV status was classified according to the Fibrotest (F1-F4) and Actitest (A1-A3) results and was correlated with the immune status (CD4+Tcell, CD8+Tcell count and CD4/CD8 index). Active HCV infection was defined by the necroinflammatory activity A≥2 (A2+A3) and Inactive HCV infection by a score A<2 (A0-A1). Cirrhosis was defined by Fibrotest score (F≥3), abdominal ultrasonography and liver function tests.

Statistical analysis: Results were given as means or median. When Bartlett’s test indicated that the group comparisons had equal variances Student T or one-way ANOVA and Tukey’s multiple comparison post hoc tests were performed. When the group data showed unequal variances, nonparametric Mann-Whitney or Wilcoxon/Kruskal-Wallis and Dunn’s, multiple comparison post hoc tests were used. Correlations were evaluated for statistical significance with Pearson's test. P < 0.05 was considered significant. Statistical tests were performed using SPSS software (version 15).

The study was performed in accordance with the principles of the Declaration of Helsinki. Approval was obtained from the hospital’s Institutional Review Board and Ethics Committee and written informed consent was obtained from all patients and controls.

III. RESULTS

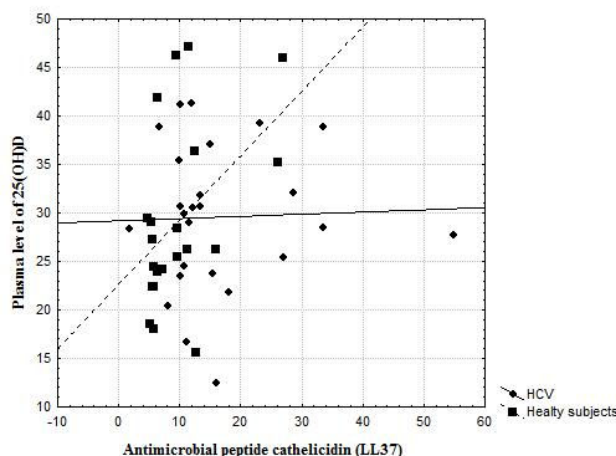
The study included 25 patients infected with HCV genotype 1 (RNA HCV between 22808 and 2770 x 10⁶ ui/ml) and 21 healthy controls. Patients were classified as having an active HCV infection form (12 patients) or an inactive HCV infection respectively (13 patients). 9 patients

were diagnosed with chronic hepatitis and 16 with cirrhosis. Table 1 presents demographic and biologically significant data of the selected HCV infected patients.

Serum LL37 and 25(OH)D in HCV infections and healthy controls.

Serum LL37 levels were significantly higher in HCV patients versus healthy controls (12.2405 ng/ml versus 9.9930 hg/ml, p=0023). Serum levels of 25(OH)D were not different between the two groups of subjects (HCV patients 29.5714 nmol/l versus controls 29.2701nmol/l) (Fig. 1)

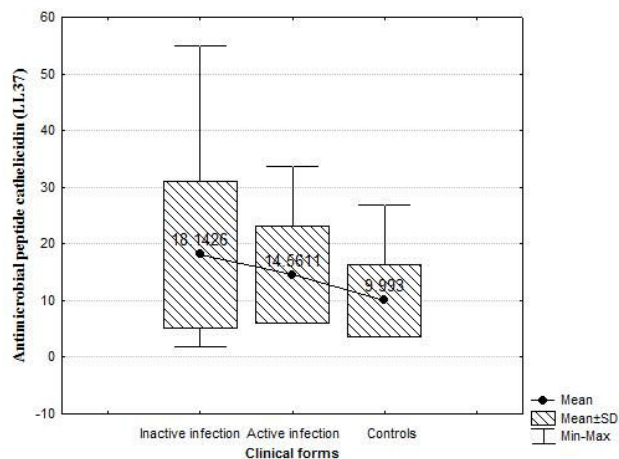
Fig. 1. Serum LL37 and 25(OH)D in HCV infections and healthy controls



Comparative status of LL37 in HCV active infection (A2-A3) versus inactive infection (A0-A1).

Inactive HCV patients had increased levels of LL37 compared with patients with active infection (18.1326 ng/ml versus 14.5611 ng/ml) or controls (9.9930 ng/ml) (Fig 2)

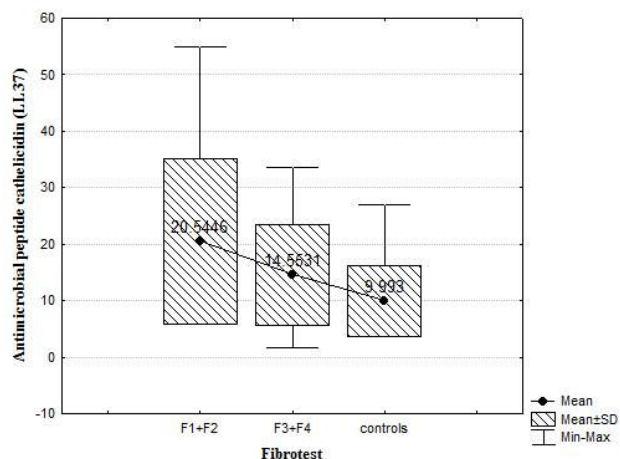
Fig 2. Comparative status of LL37 in HCV active infection versus inactive infection and controls



Comparative status of LL37 in HCV chronic infection versus cirrhosis.

LL37 serum level was higher in chronic infection versus cirrhosis (20.5446 ng/ml versus 14.5531 ng/ml) and controls (9.9930ng/ml). (Fig 3)

Fig 3. Comparative status of LL37 in HCV chronic infection versus cirrhosis and controls



IV. DISSCUTION

Removal of HCV requires a rapid, intense and coordinated immune response, present in only 20% of the untreated patient. There are various associated factors influencing the immune response and thus involved in chronic inflammation, worsening the fibrosis and increasing the risk of hepatic cancer [14]-[17]. HCV chronic inflammation is associated with lymphocytic inflammatory infiltrate, hepatic necrosis and fibrosis. The primary mechanism underlying chronic inflammation is the activation of a large number of immune cells and pro-inflammatory Th1 cytokines by HCV- derived products or endogenous molecules. LPS is one such endogenous

mediator and also a physiological constituent of portal-venous blood with pro-inflammatory effects in chronic hepatitis [18], [19].

This study evaluated the role of LL37, an essential antimicrobial peptide of the innate immune system, with a considerable immune modulating potential [20]. LL37 is known for its ability to neutralize LPS and to inhibit proinflammatory TH1 cytokines after stimulation with LPS [21]-[24]. LL37 also displays a broad spectrum of activity against mycobacteria, various bacteria, viruses and fungi [25]. However most effects have only been proven in vitro so far. LL37 exhibits other experimentally proved effects but none of these have been previously studied in connection with HCV activity. Thus, LL37 interacts with several receptors, out of which the purinergic P2X7 receptor [26] and the heparansulfate [27], [28], both being receptors of HCV. LL37 increase Natural Killer cells proliferation by activating the Toll like receptor 9, another receptor capable of recognizing HCV RNA [29]. LL37 increases proinflammatory cytokines at dendritic cells level, promoting CD4+Th1 cell response with major implications in HCV pathogenesis [30]. Although LL37 serum concentration has not been previously studied in HCV patients, LL37 release was observed in the biliary duct [31]. Since, vitamin D receptors are located in hepatic cells [32], [33], in the biliary epithelium [34] and also in certain immune cells involved in hepatic necroinflammation (dendritic cells, natural killer cells, CD8+T cells and CD4+T cells) there is an increased potential of releasing LL37 in patients suffering from chronic hepatitis.

The LL37 level recorded in the present study was significantly increased in HCV patients by comparison with controls (12.2405 ng/ml compared to 9.9930, $p=0.0023$). The patients with low or moderate fibrosis scores (F1-F2) as well as the patients with low necroinflammatory activity had increased levels of LL37 (18.5578.ng/ml and 18.1426.ng/ml respectively). The lowest LL37 value was recorded in advanced stages of cirrhosis (9.3189.ng/ml). Decreased concentrations of LL37 in severe infections were also observed in other studies and were likewise correlated with an unfavourable prognosis [35], [36].

Mentioning as LL37 serum level was increased in patients with HCV infection despite a marked deficiency of 25(OH)D in all selected subjects. No statistically significant correlations were found between LL37 and 25(OH)D and neither between LL37 and the other studied parameters (CD4+T cell, CD8+T cell, white blood cell count, sex, age).

Data recorded suggests the synthesis of LL37, a molecule with an important anti-inflammatory potential in HCV infected patients. The high LL37 levels in patients with moderate forms of hepatitis and low scores of cirrhosis could advance a possible association between increased values of LL37 and a favourable outcome. Unfortunately, the low number of cases was insufficient for obtaining statistically significant values in several groups. Moreover, the dynamic tracking of LL37 values has not been studied but could be useful to establish possible correlations of LL37 and cirrhosis.

The actual role of LL37 in HCV infected patients remains to be further investigated in what concerns not only the immunomodulating potential but also the mechanisms that

could interfere with the attachment of HCV to cellular receptors.

V. CONCLUSION

The immune mechanisms involved in the HCV infection are complex and partly still unknown. Numerous factors beside HCV could influence hepatic inflammation and evolution towards cirrhosis. One of these is persistent immune stimulation. Under these circumstances, the role of anti-inflammatory mediators like LL37 appears to have been underestimated. Understanding the role of these molecules appears extremely promising from a therapeutic point of view.

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