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Effect of Antiviral Therapy on Serum Activity of Angiotensin Converting Enzyme in Patients with Chronic Hepatitis C

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ABSTRACT

Introduction: Renin-angiotensin system (RAS) is frequently activated in patients with chronic liver disease. Angiotensin - II (AT-II), produced by angiotensin converting enzyme (ACE), has many physiological effects, including an important role in liver fibrogenesis. Combined antiviral therapy with PEG-IFN and ribavirin besides its antiviral effect also leads to a reduction in liver parenchyma fibrosis. **Aim of the study:** Determining the value of ACE in serum of patients with chronic hepatitis C before and after combined antiviral therapy, as well as the value of ACE activities in sera of the control group. **Materials and methods:** We studied 50 patients treated at Gastroenterohepatology Department, in the time-period of four years. Value of ACE in serum was determined by Olympus AU 400 device, with application of kit "Infinity TN ACE Liquid Stable Reagent". HCV RNA levels in sera were measured by real time PCR. HCV RNA test was performed with modular analysis of AMPLICOR and COBAS AMPLICOR HCV MONITOR test v2.0, which has proved infection and was used for quantification of the viruses and monitoring of the patients' response to therapy. Liver histology was evaluated in accordance with the level of necroinflammation activity and stage of fibrosis. **Results:** Serum activities of ACE in chronic hepatitis C patients is statistically higher than the values in the control group ($p=0.02$). Antiviral therapy in chronic hepatitis C patients statistically decreases serum activities of ACE ($p= 0.02$) and indirectly affects fibrogenesis of the liver parenchyma. Correlation between ACE and ALT activity after the therapy was proved (0.3934). **Conclusion:** Our findings suggest that the activity of ACE in serum is a good indirect parameter of the liver damage, and could be used as an indirect prognostic factor of the level of liver parenchyma damage. Serum activity of ACE can be used as a parameter for non-invasive assessment of intensity of liver damage.

Key words: Angiotensin-converting enzyme, interferon, antiviral response, chronic hepatitis C.

1. INTRODUCTION

Pathogenesis of liver fibrosis depends on the etiological factor which caused the fibrosis. In chronic hepatitis C, fibrosis caused by HCV has been insufficiently studied due to deficiencies of animal models with persistent HCV infection (1).

Renin-angiotensin system (RAS) is frequently activated in patients with chronic liver disease. Angiotensin - II (AT-II), produced by angiotensin converting enzyme (ACE), has many

physiological effects, including an important role in liver fibrogenesis. Local RAS in liver, i.e. ANG II, participates in the process of genesis of liver fibrosis by starting the proliferation and contraction of stellate cells in hepatic sinusoids (2, 3). Inhibition of RAS by clinically applied angiotensin-converting enzyme inhibitor (ACE-1) and AT II type 1 receptor blockers significantly slows down liver fibrosis in several experimental models (4, 5). In clinical practice, these agents for inhibition of

RAS also intensify antifibrotic effects on several types of chronic liver diseases, including CHC. Therapeutic aim of chronic hepatitis C (CHC) is eradication of hepatitis C virus, prevention of development of cirrhosis and hepatocellular carcinoma (6). In the last decade, ribavirin and pegylated interferon treatment significantly improved antiviral therapy for HCV.

It was already known that continuous inflammation along with HCV infection slowly leads to development of liver fibrosis and possibly leads to development of hepatocellular carcinoma (HCC). One of clinical aims in the treatment of refractory HCV infection is prevention of progression of liver fibrosis (7). IFN inhibits the activity of collagen promoter in activated stellate hepatic cells which play a critical role in the development of liver fibrosis (8). Elimination of the cause of fibrosis is the most effective approach in the treatment of liver fibrosis. This strategy has proved effective in many chronic liver diseases (9, 10, 11). There is no standard treatment for liver fibrosis. Majority of therapeutic approaches have not been proved in humans due to a need for serial liver biopsies in order to assess the changes of fibrosis stage.

Inhibition of renin-angiotensin system is probably the most promising therapeutic approach to liver fibrosis. Inhibitors of RAS are widely used as antifibrotic factors in treatment of chronic kidney and heart diseases, and have proved safe for long-term therapeutic approach (12). There are limited data on use of this therapeutic approach to chronic liver disease. Patients with liver transplant who are receiving inhibitors of renin-angiotensin system as antihypertensive therapy have shown lesser progression of fibrosis than patients who are receiving other type of therapy (13). Blockade of AT-II by inhibitors of angiotensin-converting enzyme or by AT-III blockers reduces the development of liver fibrosis, and leads to regression of fibrosis even in cases of developed fibrosis (14, 15). Although the results are still unsatisfactory, IFN combined with ribavirin is used in therapy for patients suffering from chronic hepatitis C for the purpose of virus eradication. In addition to its antiviral effect, there is abundant evidence that IFN is also effective in regression of liver fibrosis (16, 17).

Long-term administration of IFN improves the prognosis of chronic hepatitis in its entirety, together with regression of liver fibrosis (18). It has been proved that both ACE inhibitors and IFN lead to suppression of activated hepatic stellate cells which play a critical role in fibrogenesis of liver parenchyma (15, 19).

2. MATERIALS AND METHODS

Control group of respondents consisted of 30 persons (13 men and 17 women) ranging from 20 to 65 years of age, who did not have manifestations of liver disease based on subjective and objective indicators of general health. Group of respondents with chronic liver disease of viral C etiology consisted of 50 patients of both genders; 38 (75%) were male and 13 (25%) were female. Age of patients hospitalized at the Gastroenterohepatology Clinic of Clinical Center of University of Sarajevo ranged from 20 to 65 years. Patients were selected according to

genotype of hepatitis C viral infection and treated according to two current therapeutic protocols: Genotypes 1 and 4: Pegylated interferon alpha 2a 40 KD, 48 weeks with ribavirin (1000-1200 mg/daily depending on body weight). Genotypes 2 and 3: Pegylated interferon alpha 2a 40 KD, 24 weeks with ribavirin (800 mg/daily).

Biochemical analyses and other tests

The following laboratory tests using standard methods were performed for all respondents: functional liver tests (albumins with total proteinogram, prothrombin time, alanine aminotransferase, aspartate aminotransferase, bilirubin), serological analyses; detection of nucleic acid of hepatitis C virus by polymerase chain reaction (PCR) qualitatively and quantitatively along with genotyping of C virus which determines the length of therapy.

Routine hematological and biochemical tests were performed for all patients: Triiodothyronine (T3), thyroxine (T4), thyrotrophic hormone (TSH), markers of types of viral hepatitis, markers of autoimmune diseases, AFP concentration in serum. Serum activity of ACE was determined in all respondents.

As for assessment of stage of chronic liver disease, fibrosis and architectural disorder were analyzed by way of pathohistological examination of liver tissue sample obtained from liver biopsy under ultrasound control.

Percutaneous liver biopsy

Analysis of liver tissue in the cylinder of at least 20 mm in length was made possible by percutaneous liver biopsy. Routine preparation of sample for histopathological interpretation began with instant fixation in 10% neutral "buffered" formalin. Following standard and special methods of coloring (PAS, D-PAS, Ganori, Van Gieson, Trichrom Masson), grade of necroinflammatory activity and stage of fibrosis in the liver were determined using classification according to Ishak and associates (20).

Measurement of ACE concentration

Blood sampling for assessment of serum activity of ACE

Blood samples were taken by puncture of cubital vein in order to determine serum activity of ACE in all patients. After coagulation and centrifuging (5 minutes at 2000 g), the serum was removed and stored in a freezer at - 25°C until assessment of ACE activity.

Procedure for assessment of serum activity of ACE

Serum activity of ACE was determined by spectrophotometric method with use of synthetic substrate Hip-Gly-Gly (Filipović et al., 1983) (21). Results were expressed in units corresponding to one nmol of hippuric acid released in one minute by enzymatic hydrolysis of Hip-Gly-Gly substrate per milliliter of serum, and tissue activities in units corresponding to one nmol of hippuric acid released in one minute by enzymatic hydrolysis of Hip-Gly-Gly substrate per milligram of tissue and per milligram of protein. Serum activity of ACE is determined by Olympus AU 400 device with use of kit «Infinity™ ACE Liquid Stable Reagent» by Thermo Scientific Company to determine angiotensin-converting enzyme. This procedure for assessment of serum activity of ACE is based on a reaction described by Holmquist and associates (22): FAPGG FAP + glycylglycine, in which FAPGG is an abbreviation for N- β -(2-furyl)-acryloyl-L-phe-

nyl-alanyl-glycylglycine. Hydrolysis of FAPGG by angiotensin-converting enzyme leads to a decrease in ACE absorbancy at 340 nm wavelength. The device calculates ACE activity automatically as per formula:

$$ACE = \frac{\Delta Abs/min \text{ sample}}{\Delta Abs/min \text{ calibrator}} \times \text{calibrated value.}$$

Results are expressed in units per liter (U/L).

Serological analyses and monitoring of viremia

HCV infection markers imply detection of antibodies to HCV and viral RNA. Detection of HCV antibodies was done by Enzyme immunoassay EIA, and definitive confirmation of findings was done by Recombinant immunoblot assay RIBA. HCV RNA test was done by molecular analysis of AMPLICOR and COBAS AMPLICOR HCV MONITOR test v2.0.; this analysis was used to confirm infection and to monitor the outcome of therapy. AMPLICOR HCV MONITOR test v2.0. is industrial standardized test which was used for quantification of quantity of virus (viral load) and monitoring of patients' response to therapy. Qualitative AMPLICOR and COBAS AMPLICOR HCV test with the lowest detection level of 50 IU/ml was used for assessment of sustained virological response (SVR) (23). Virus genotyping was done by direct sequencing.

Statistical data processing

Results were statistically processed to determine existence of differences and also level of significance of differences, variance inside groups was analyzed. For vari-

atits C is statistically higher than the values in the control group (p=0,02).

Antiviral therapy in chronic hepatitis C statistically decreases serum activities of ACE (p= 0,02). Paired t-test for means of two samples was used and the extreme case in data was replaced by mean value of other data for ACE values both before and after therapy. Since the value of t-statistics is greater than t-critical value, hypothesis of parity of means of these two samples with significance threshold 0.05 was rejected. In contrast, test statistics and measures of effect for values of ACE before and after therapy with data in which extreme case was replaced by mean value of other data for ACE values both before and after therapy, have determined that ACE values vary significantly, and in such a way that values after therapy are reduced relative to values before start of therapy. We also measured the value of PCR prior and after the therapy is presented in Figure 2., End of Treatment Respond - ETR, was regardless the genotype achieved in 77% of the patients.

Mean values (X+SEM) of ACE concentration in control group of respondents, group of patients before and after therapy are presented (Figure 1 and 2). Control- respondents without liver disease manifestations (N=30). CHC before Th- patients with chronic hepatitis C before therapy (N=50). CHC after Th- patients with chronic hepatitis C after therapy (N=50). Results presented in Figure 3 show that ACE values in the group of respondents with CHC are statistically significantly higher than those in the

	AST	ALT1	ALT2	Albumins	INR	bilirubin	PCR1	PCR2	ACE1	ACE2
AST	1									
ALT1	0.7146	1								
ALT2	0.5142	0.5061	1							
Albumins	-0.2572	-0.0503	-0.0694	1						
INR	0.1915	-0.0783	-0.0938	-0.1079	1					
bilirubin	0.0612	0.0316	0.0200	0.1361	0.0391	1				
PCR1	-0.1535	-0.0779	-0.1046	-0.0634	-0.0701	-0.0484	1			
PCR2	0.0932	0.0120	0.1567	0.0165	0.0035	0.3083	-0.0455	1		
ACE1	0.0028	0.0251	0.0132	0.0740	-0.2281	0.0870	0.1399	-0.1133	1	
ACE2	0.4168	0.1492	0.3934	-0.1420	0.1658	0.2117	0.0129	0.2395	0.2197	1

Table 1. Table of correlations between data in group of treated patients

ables determined not to belong to the same population, Student t-test was employed to determine statistically significant difference between groups. Values of p<0.05 were taken as significant. Differentiation test was used to determine differences in parameters within a group.

3. RESULTS

We investigated total number of 50 patients, where the 70% were male, and 30% of patients were female. Among the different analyses, we tested presence of correlation between the different values (as is presented in table 1). It was proved correlation between ACE and ALT activity after the therapy (0.3934) as well the AST prior the treatment and ALT prior the treatment (0.71). In our investigation we measured serum activity of ACE in control group, in group of patients prior the therapy and the same group of patients after the therapy. As it is presented in figure 1 and 3. serum activities of the ACE in chronic hep-

control group of healthy respondents, and that ACE value after therapy is statistically significantly lower in respondents after therapy.

4. DISCUSSION

Fibrosis of liver tissue is the result of chronic liver damage, proliferation of connective tissue and accumulation of extracellular matrix (ECM) protein. These changes characterize most chronic liver diseases. Progressive accumulation of fibrillar extracellular matrix (ECM) in the liver is oftentimes a consequence of damage to liver tissue by viral agents (HCV) and activation of process of healing damaged parts of the tissue (24).

In patients with chronic liver disease which is progressing to liver cirrhosis, significant accumulation of fibrillar ECM is evident after clinical course which in some patients lasts for years and even decades. In most patients with chronic hepatitis C, there is a long peri-

od of latency (10-15 years) between HCV infection and detection of minimal stage of fibrosis in the presence of evident degree of necroinflammatory activity.

Progressive hepatic fibrosis and cirrhosis develop in 20-30% of patients with chronic hepatitis C (25).

These results are partially confirmed by results of our research, which determined that the largest number of patients have fibrosis stage 1 (60%) and fibrosis stage 2 (29%), only 6% of patients had fibrosis stage 3 while a total of 6% of patients had fibrosis stages 5 and 6 which indicate presence of incipient and already developed cirrhosis in liver tissue. Comparing to previously already determined ratio of patients who are developing significant fibrosis and cirrhosis in liver tissue, we can conclude

that in our group of respondents, most patients had a lesser stage of fibrosis of liver parenchyma at the time of diagnosis of the disease. Considering that natural course of chronic hepatitis C infection shows significant individual variability, identification of predictors of fibrosis progression is of exceptional significance (26). Several risk factors affecting fibrosis progression have been definitely confirmed thus far, and they include older age at the time of infection, male sex, consumption of alcohol and co-infection with other pathogens such as hepatitis B virus and HIV (26, 27). Research done so far has definitely shown elevated values of serum activity of ACE in liver cirrhosis and chronic hepatitis.

In literature, there are no data on mutual connection of ACE and combined antiviral therapy of pegylated interferon and ribavirin, nor data on their mutual effect in patients with chronic liver disease of viral C etiology.

Some isolated data suggested that angiotensin blockade inhibits fibrosis progression in recurrent HCV post-liver transplantation (28). Results of our research showed elevated values of serum activity of ACE in group of respondents with chronic hepatitis C relative to control group of respondents who did not have manifestations of liver disease. Mean value of ACE activity in group of respondents without liver disease was 30.8IU/L, while in the group of respondents with chronic hepatitis C, mean value was 38.96 IU/L. That difference was statistically significant. In concern to increase in serum activity of ACE in respondents with chronic hepatitis C before antiviral therapy our results are in line with research published so far regarding elevated serum activity of ACE in group of patients with chronic liver disease.

Angiogenesis plays an important role in many biological phenomena, including development of fibrosis. Studies have proved that neovascularization increases significantly during development of liver fibrosis.

It has already been determined that ACE and IFN suppress angiogenetic activity. Kardum and associates (2005) have determined that an increase in serum activity of ACE in respondents is proportional to severity of liver disease. Lowest activities have been recorded in serum of patients with liver steatosis and scarce fibrosis, while the activity of ACE was highest in serum of patients with the most severe form of fibrosis.

According to the results of this research, serum activity of ACE shows a noticeable increase during transition of liver fibrosis from scarce to moderate.

These results suggest that the changes in values of serum concentration of ACE might be early indicators of progression of chronic liver disease.

In our research, we have examined the effect of combined antiviral therapy on serum activity of ACE in a group of patients suffering from chronic hepatitis C before and after administered therapy, independent of the outcome of therapeutic response at the end of treatment (ETR) and we have determined that antiviral therapy for chronic hepatitis C significantly lowers serum activity of angiotensin-converting enzyme ($p < 0.05$). Combination treatment with low-dose interferon (IFN) and ACE-I exerts a stronger inhibitory effect than either single agent

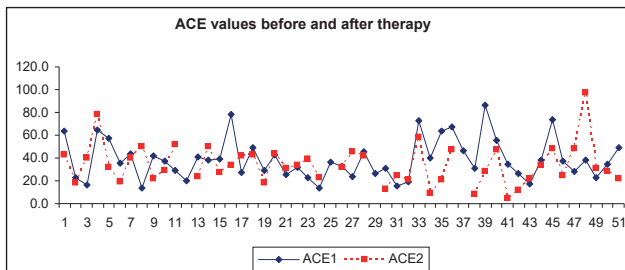


Figure 1. Line diagram of ACE values in patients measured before (ACE1) and after therapy (ACE2).

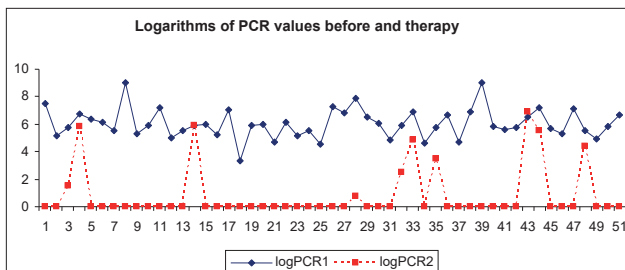


Figure 2. Line diagram of logarithms at base 10 for PCR values measured before (logPCR1) and after therapy (logPCR2). Negative findings have been replaced by zeroes. Correlations in a set of treated patients

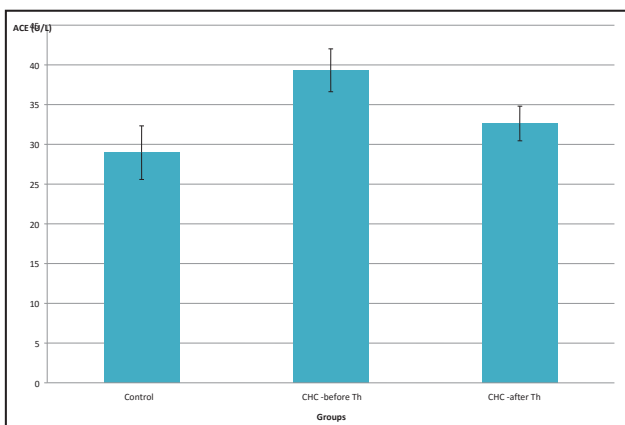


Figure 3. Concentration of ACE in serum of patients with chronic hepatitis C, before therapy, after therapy and in control group. Mean values ($\bar{X} \pm \text{SEM}$) of ACE concentration in control group of respondents, group of patients before and after therapy are presented. Control- respondents without liver disease manifestations (N=30). CHC before Th- patients with chronic hepatitis C before therapy (N=50). CHC after Th- patients with chronic hepatitis C after therapy (N=50) measured before (logPCR1) and after therapy (logPCR2). Negative findings have been replaced by zeroes. Correlations in a set of treated patients

on its own (29). Based on the results of our research where a decrease in serum activity of ACE under effect of antiviral therapy was determined, it was confirmed that besides antiviral effect, IFN may improve the condition of liver fibrosis, by inhibiting activated HSC which play a critical role in the development of liver fibrosis, and also by suppressing the activity of ACE as another major factor in fibrogenesis of liver parenchyma. These results allow for a conclusion that determining the serum activity of ACE in non-invasive assessment of a degree of damage to liver parenchyma in patients with various stages of chronic hepatitis C and after finished antiviral therapy is justified.

Determining ACE in a group of patients who fail to respond to therapy would have a special significance, with possible consideration given to prolongation of antiviral therapy or administration of other therapeutic methods.

5. CONCLUSION

Our findings suggested that activity of the ACE in serum is good indirect parameter of the liver damage, and could be used as an indirect prognostic factor of the level of liver parenchyma damage. Serum activity of ACE can be used as a parameter of non-invasive assessment of intensity of liver damage.

- **Author's contribution:** Azra Husic-Selimovic: substantial contribution to conception and design, final approval of the version to be published. Amela Sofic: substantial contribution to acquisition of data, substantial contribution to analysis and interpretation of data. Jasminko Huskic: substantial contribution to conception and design, substantial contribution to acquisition of data, substantial contribution to analysis and interpretation of data, drafting the article. Deniz Bulja: substantial contribution to acquisition of data and interpretation of data.
- **Conflict of interest:** There is no conflict of interest of any of listed authors.

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