Assay Value of Acid Mammalian Chitinase Activity in Chronic Hepatitis C Patients: Correlation with Non-invasive Markers of Hepatic Fibrosis

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Abstract— Chronic hepatitis C virus run the risk of developing fibrosis, cirrhosis and hepatocellular carcinoma in later life. Aim: The aim is to assay acid mammalian chitinase (AMCase) activity in chronic hepatitis C (CHC) patients using a simple colorimetric technique and Correlate such activity with those of non-invasive markers of hepatic fibrosis. Methods: Blood samples were withdrawn to characterize viral infection, to estimate AMC activity, to count blood cells and to evaluate liver function tests. Beside, clinical investigation and transient elastography were done. Results: The mean acid mammalian chitinase activity was significantly elevated (P<0.03) when compared with that of the control group. Such activity was significantly and positively correlated with leukocytes counts. The activity was negatively correlated with AST/ALT score (r= - 0.25 and P<0.033). The activity gave an AUC of 0.69 with higher specificity (Sp= 86.7) and PPV value (94.3) when tested to differentiate patients from control individuals. Thus, it was significantly differentiate patients from healthy control. Also, AUC of the enzyme activity was higher than those of AST/ALT and Age /AST. Further, its PPV was higher than that of the former indicating its tendency to differentiate CHC patients from healthy individuals. The lowering in its NPV may be due to the heterogeneity of liver pathology of CHC patients. In conclusion, the assay of AMCase activity can add more to the assessment of liver disorders in CHC patients; especially if a larger number of patients and detailed pathology of the liver is included in a latter study.

KEYWORDS—Acid mammalian chitinase activity, non-invasive scores, chronic hepatitis C, transient elastography.

1 INTRODUCTION

Hepatitis C virus is a hepatotropic virus. It is made up of core proteins. This protein is further encapsulated in lipid bilayer which contains E1/E2 glycoproteins [1].

Increased inflammatory response is a crucial physiological event that occurs during chronic HCV infection. This process is viral proteins-dependent. This process is defined by the persistence of inflammatory cells and destruction of liver cells [2].

In general, chronic liver damage and regeneration results in scarring of liver with the formation of liver fibrosis. The latter is characterized by the activation of hepatic stellate cells (HSCs), extracellular matrix (ECM) secretion and deposition. The disease is also enhanced due to promotion of activated HSCs survival in a NF-κB dependent manner by the KCs and recruited macrophages [2]. Subsequently, Kupffer cells (KCs) release ROS which stimulate NADPH oxidase in HSCs and hepatocytes followed by induction of oxidative stress leading to DNA damage, enhanced expression of proinflammatory genes, fibrogenesis with eventual loss of normal functionality of liver (cirrhosis), malignancy, variceal hemorrhage and hepatic encephalopathy[3].

Oxidative stress plays a significant role in HCV-induced liver damage. HCV infection has also been reported to activate the liver-residing macrophages- Kupffer cells (KC) and result in ROS production. The activated KCs enhance the production of TNF-α and ROS which are good player in HCV-induced liver damage [3]. During the long developing period from HCV infection to HCC, immune cells are recruited to the liver in an attempt to control viral replication. However; and in the majority of cases, chronic infection is established. Generally, the liver resident macrophages; KCs, play an important role in immune surveillance and immunoregulation [4]. However, viral proteins could activate hepatocytes, macrophage, dendritic cells and natural killer cells as well as stromal cells; including, stellate cells, and myofibroblasts to escape host immune defense in the inflammatory micro-environment, which might contribute to the development of disease [5],[6].

Chitin is a highly insoluble β,1-4-linked polymer composed primarily of N-acetylglucosamine (GlcNAc) and some glucosamine (GlcN) residues and is one of the most abundant organic substances in nature. There is no endogenous chitin in mammals but in lower
life forms act as a host-defence against chitin-containing organisms. Despite the absence of endogenous chitin, a number of chitinases and chitinase-like proteins (CLPs) have been identified in mammals. However, their roles have only recently begun to be elucidated [7].

Glycosyl hydrolases belong to family 18 chitinases and are found in a wide range of organisms, including bacteria, viruses and protozoan parasites, and more recently were identified in mammals [8]. The optimal pH and temperature for activity of these enzymes toward colloidal chitin were found to be 6 and 30 °C, respectively. The enzyme was found to hydrolyze chitin and oligomers of N-acetylglucosamine, generating N, N'-diacetylchitobiose and N-acetylglucosamine as products. [8][9].

Immunohistochemical examination showed that the AMCase is expressed in both epithelial cells and macrophages in lungs of sensitized mice. Expression of AMCase was preferentially stimulated by IL-13, a Th2 cytokine [10].

Liver biopsy is still the gold standard to assess liver fibrosis; its invasive nature prevents it from wide use. Therefore, it is an unmet clinical need to develop a non-invasive and quantitative measurement for the changes in liver fibrosis [11]. Therefore, the activity of AMCase will be tested to be a marker of inflammation and to be one of the non-invasive measures of viral infection.

2 MATERIALS AND METHODS
2.1 Patients and Blood sampling
2.1.1 Patients
This cross sectional observational study was performed on patients selected from the outpatient hepatology clinics, in Egyptian Liver Research Institute and Hospital (ELRIAH), Sherpin, Aldakhilia, Egypt. The study has been conducted on 77 participants. All patients were subjected to full medical history, complete clinical examination, and full basal laboratory and radiological investigations.

2.1.2 Blood sampling
Six ml venous blood sample were withdrawn from each individual; of whom:

**Serum sample collection**
Four milliliters of venous blood were obtained of which 4 ml were left to clot, centrifuged and the serum fraction was separated and either freshly used or stored at −80 °C until used. This sample was used to estimate HCV antibodies and polymerase chain reaction for detecting HCV-RNA and to confirm the infection, hepatitis B surface antigen (HBsAg); to exclude hepatitis B viral infection, AMCase activity and routine liver function tests.

**Plasma sample collection**
2 ml of whole blood were poured onto EDTA for the haematological assays.

2.2 Biochemical and immunological assays
2.2.1 Routine laboratory tests
Hematological markers were done using D-cell 60 automated hematology analyzer (Sysmex X 1800 incorporation, Japan), Liver function tests; including serum Aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin and bilirubin. They were done using automated Biochemistry analyzer (CobasIntegra 400, Roch, Switzerland).

2.2.2 Assay of acid mammalian chitinase
2.2.2.1 Principle
Acid mammalian chitinase hydrolyze colloidal chitin prepared from Shrimp shells into N-acetylglucosamine at pH 4.8. The latter react with 3, 5-dinitrosalicylic acid (DNS) to form orange color which absorbs at 550 nm.

Colloidal chitin + Enzyme/H2O → N-acetylglucosamine

2.2.2.2 Assay procedure of acid mammalian chitinase
50 μl of serum containing enzyme were added to 1.0 ml of acetate buffer (pH 4.8; 0.2 M) in a glass tube containing 10 mg/ml colloidal chitin. The solution was incubated at 370°C for 30 min, centrifuged and the supernatant 1.0 ml of DNS solution was added and the tube was boiled for 6.0 min. After cooling, the optical density was measured at 550 nm against that of the blank tube to which no enzyme was added. The concentration was then evaluated from the standard curve.

2.2.3 Serological markers
Serological markers for detecting HCV infection [hepatitis C antibodies (HCV Abs)] were estimated by enzyme linked immunosorbtent assay [ELISA, Merieux anti-HCV, version 4.0, Diasorin S.P.A. via Crescent no 13040 Saluggia (VC) - Italy].

2.2.4 Molecular Markers
Manual extraction of RNA from 100 µl of plasma, reverse transcribed, followed by its amplification using polymerase chain reaction (PCR). Then, HCV RNA was quantized by quantitative RT-PCR using fully automated analyzer (Cobas amplified, Taqman48 analyzer, Roche Switzerland).

2.3 Statistical analyses:

All statistical analyses were performed by Medcalc software (version 14.8.1.; Medcalc Software Bvba, Ostend, Belgium). Continuous variables were expressed as mean± standard deviation (SD). Comparisons of markers as well as routine laboratory tests and stages of fibrosis were analyzed using a two-sided P value. Person’s correlation coefficient was used in establishing correlation among parameters. Analyses were done for parametric quantitative variables using one way ANOVA test and for non-Parametric quantitative variables using Kruskal Wallis test. ROC curve was done to determine the cutoff point, AUC, sensitivity, specificity, PPV and NPV of presences of fibrosis. A value of P < 0.05 was considered statistically significant.

3 RESULTS

3.1 Hematological parameters in the whole blood of all HCV patients versus those of the healthy control group

3.1.1 Hemoglobin Concentration (gm%)

The mean hemoglobin concentration of the healthy control was 13.3 ± 1.1 gm% and that of all patients was 14.06 ± 1.64 gm%. The mean hemoglobin concentration of the patients showed no significant difference when compared with that of the control individuals (Table 1).

3.1.2 Red Blood Cells (RBCs) counts

The mean count of RBCs of the healthy control was 5.1 ± 0.63 x10^{12}/L and that of all patients was 4.83 ± 0.49 x10^{12}/L. The mean RBCs counts of the patients showed no significant difference when compared with that of the control individuals (Table 1).

3.1.3 White Blood Cells (WBCs) counts

The mean count of WBCs of the healthy control was 6.8 ± 1.97 x10^{9}/L and that of all patients was 6.83 ± 2.15 x10^{9}/L. The mean WBCs counts of the patients showed no significant difference when compared with that of the control individuals (Table 1).

1.4 Platelets counts

The mean platelets count of the healthy control was 238.9 ± 52.0 x10^{9}/L and that of all patients was 194.1 ± 83.98 x10^{9}/L. The latter count was significantly decreased (P< 0.05) when compared with that of the control (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>All Patients group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Hemoglobin (gm %)</td>
<td>10</td>
<td>13.3 ± 1.1</td>
</tr>
<tr>
<td>Red Blood Cells (x10^{12}/L)</td>
<td>10</td>
<td>5.1 ± 0.63</td>
</tr>
<tr>
<td>White Blood Cells (x10^{9}/L)</td>
<td>10</td>
<td>6.8 ± 1.97</td>
</tr>
<tr>
<td>Platelets (x10^{9}/L)</td>
<td>15</td>
<td>238.9 ± 52.0</td>
</tr>
</tbody>
</table>

N= number, P= probability, values were expressed as mean± standard deviation (mean ± SD) and P = the significance degree when the mean values of the individual groups were compared with those of the control.

3.2 Liver functions tests and Acid mammalian Chitinase (ACM) in the blood of all HCV patients versus those of the healthy control group

3.2.1 Serum Activities of transaminases

3.2.1.1 Activity of alanine amino-transferase (ALT)

The mean enzyme activity of the healthy control was 21.6 ± 7.3 IU/L and that of HCV patients was 37.96 ± 17.67 IU/L. The latter activity was significantly increased (P<0.006) when compared with the corresponding mean activity of the control group (Table 2).

3.2.1.2 Activity of aspartate amino transferase (AST)

The mean enzyme activity of the healthy control was 21.6 ± 7.3 IU/L and that of all patients was 37.96 ± 17.67 IU/L. The latter activity was significantly higher (P<0.0006) when compared with the corresponding mean activity of the control group (Table 2).

3.2.2 Serum total bilirubin levels

The mean level of serum total bilirubin of the healthy control was 0.52 ± 0.18 mg/dl and that of all patients was 0.85 ± 0.53 mg/dl. This change was
3.2.3 Serum albumin

The mean serum albumin concentration of the healthy control was 4.4 ± 0.7 g/dl and that of all patients was 4.24 ± 0.43 g/dl. The mean serum albumin level of the patients showed no significant difference (P= NS) when compared with that of the control individuals (Table 2).

### TABLE 2: The mean as well as the standard deviations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, total bilirubin levels and serum albumin as well as acid mammalian chitinase (AMCase) in sera of all patients and those of the healthy control group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>All Patients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGPT (IU/L)</td>
<td>15  21.2 ± 11.49</td>
<td>62  39.53 ± 21.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>15  21.6 ± 7.3</td>
<td>62  37.96 ± 17.67</td>
<td>&lt;0.0006</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>15  0.52 ± 0.18</td>
<td>62  0.85 ± 0.53</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>15  4.4 ± 0.7</td>
<td>62  4.24 ± 0.43</td>
<td>NS</td>
</tr>
<tr>
<td>AMCase (U)*</td>
<td>15  5.2 ± 1.2</td>
<td>63  6.6 ± 2.35</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

*One AMCase unit is defined as the enzyme activity necessary for production of one mg N-acetylglucosamine/ml serum/hour, N= number, values were expressed as mean±standard deviation (mean ± SD) and P= the significance when the mean values of the individual groups were compared with those of the control.

3.2.4. Acid Mammalian Chitinase (AMCase)

The mean serum AMCase activity of the healthy control was 5.2 ± 1.2 (U)* but that of all patients was 6.6 ± 2.35 (U)*. The mean serum acid mammalian chitinase activity of the patients was significantly increased (P<0.03) when compared with that of the control individuals (Table 2).

3.3 Correlation of Acid Mammalian Chitinase with the parameters of liver function tests and the hematological parameters in the blood of all individuals

Table 3 showed the correlations between the individual activities of AMCase with the parameters of liver function tests and the hematological parameters. The enzyme activity showed positive and significant correlation only with leukocytes counts (r=0.262 and P< 0.0276). The correlations with the other listed parameters were not significant.
3.4 Receiver-operating characteristic curve (ROC) of Acid Mammalian Chitinase (AMCase) activity

The enzyme activity gave an AUC of 0.69 with higher specificity (Sp= 86.7) and PPV of 94.3. Thus, it significantly differentiates patients from healthy control. Also, the activity gave an AUC of 0.69 that was higher than those of AST/ALT and Age /AST. Further, its PPV was higher than that of the former. The lowering in its NPV may be due to the heterogeneity of liver pathology of CHC patients (Table 4 & Figure 1).
3.5 Receiver-operating characteristic curves (ROC) of Acid Mammalian Chitinase and some fibrosis scores based on indirect serum markers

AMCase activity significantly differentiates patients from healthy control. Also, the activity gave an AUC that was higher than those of AST/ALT and Age /AST. Further, its PPV was higher than that of the former. The lowering in its NPV may be due to the heterogeneity of liver pathology of CHC patients (Table 5 & figure 2).

**TABLE 5: COMPARISON OF THE DIAGNOSTIC VALUES OF ACID MAMMALIAN CHITINASE (AMCase) AND SOME FIBROSIS SCORES BASED ON INDIRECT SERUM MARKERS.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cut off</th>
<th>AUC</th>
<th>SN</th>
<th>SP</th>
<th>PPV</th>
<th>NPV</th>
<th>P –Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMCase (U)*</td>
<td>&gt;6.04</td>
<td>0.69</td>
<td>52.4</td>
<td>86.7</td>
<td>94.3</td>
<td>30.2</td>
<td>P&lt; 0.03</td>
</tr>
<tr>
<td>AST/ALT</td>
<td>≤1.24</td>
<td>0.608</td>
<td>80.65</td>
<td>53.33</td>
<td>87.7</td>
<td>40</td>
<td>P=0.2258</td>
</tr>
<tr>
<td>Age/AST</td>
<td>≤1.42</td>
<td>0.633</td>
<td>58.8</td>
<td>90</td>
<td>96.8</td>
<td>30</td>
<td>P&lt; 0.0461</td>
</tr>
<tr>
<td>Age/PLT</td>
<td>&gt;0.17</td>
<td>0.89</td>
<td>80.39</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>APRI</td>
<td>&gt;0.29</td>
<td>0.84</td>
<td>77.42</td>
<td>80</td>
<td>94.1</td>
<td>46.2</td>
<td>P&lt; 0.0001</td>
</tr>
<tr>
<td>FIB 4</td>
<td>&gt;17.18</td>
<td>0.98</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>66.7</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

*One AMCase unit is defined as the enzyme activity necessary for production of one mg N-acetylglucosamine/ml serum/hour. AUC: Area under the ROC curve, Sp: specificity, Sn: sensitivity, PPV: positive predictive value, NPV: negative predictive value, P value: P > 0.05 non-significant, P < 0.05: significant, P < 0.001: more significant and P < 0.0001: extremely significant.

**FIGURE 2: RECEIVER-OPERATING CHARACTERISTIC CURVES (ROC) OF ACID MAMMALIAN CHITINASE (AMCase) AND SOME FIBROSIS SCORES BASED ON INDIRECT SERUM MARKERS.**
3.6 Correlation of Acid Mammalian Chitinase (AMCase) with some fibrosis scores based on indirect serum markers of all individuals

Table 6 showed the correlations between the individual activities of acid mammalian chitinase with

<table>
<thead>
<tr>
<th>Fibrosis Scores</th>
<th>AMCase</th>
<th>AST/ALT</th>
<th>Age/AST</th>
<th>Age/PLT</th>
<th>APRI</th>
<th>FIB 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation Coefficient (r)</td>
<td>0.25</td>
<td>0.18</td>
<td>-0.15</td>
<td>-0.12</td>
<td>-0.11</td>
<td></td>
</tr>
<tr>
<td>Significance Level (P)</td>
<td>0.033</td>
<td>0.158</td>
<td>0.244</td>
<td>0.297</td>
<td>0.397</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>76</td>
<td>60</td>
<td>60</td>
<td>76</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

**4 DISCUSSION**

In liver tissue more macrophages were infiltrated with the severity of liver fibrosis Kumagai et al. (2016) and [12]. Therefore, it is plausible that the increase in the number and/or activity of macrophages in the fibrous liver can enhance an increase in the levels of chitinase-like proteins and AMCase activity in sera of CHC patients as was reported in sera of patients with non-alcoholic fatty liver disease (AFLD) having severe fibrosis. These others added that, macrophage differentiation plays key roles in liver fibrogenesis, which may be the case in the present study.

In healthy human organism mRNA for chitinase was found in lymph nodes, lung, and bone marrow. In addition, the enzyme protein is restrictedly expressed to phagocytes [13]. In contrast, van Eijk et al. [14] added neutrophils to be another source of such enzyme in human. Also, mRNA for murine chitotriosidase is expressed in the gastrointestinal tract, fore-stomach and in small intestine [13]. Therefore, Kumagai et al. [15] reported that, chitotriosidase (or related enzymes; including AMCase) is indicator for macrophage-driven inflammatory processes in various organs during the phagocytic processes; including those of liver in the present study. They added that, in vitro and in the animal model the enzyme play a role as an innate immunity component in host response against chitin-containing pathogens [15] [14]. Thus, one cannot neglect the involvement of this enzyme in the immune defense against HCV infection in the present study. In this regard, Yao et al. [16] showed that, the exogenous HCV core protein mediates the interaction between the proliferation of human hepatocytes and macrophages. This interaction may be involved in persistence of HCV infection. However, when considering the diversity of HCV proteins and the complexity of the in vivo environment, the effects of other HCV proteins on macrophages, the interaction between macrophages and other cells; including, immune cells, primary hepatocytes, and stellate cells, and other intracellular downstream molecules, are all involved in the processes of persistence of HCV infection.

AMCase inhibits chitin-induced innate inflammation; augments chitin-free, allergen-induced Th2 inflammation; and mediates effector functions of IL-13. In accord with these findings, AMCase of serum are increased in any inflammatory disease, in remodeling disorders and often correlate with disease severity [9]. Thus one can suggest that, the continuous degeneration and/or regeneration accompanying viral infection may be one of the causes of AMCase elevation in the present study, possibly via activated macrophage accompanying such infection. Also, the correlation between AMCase activities with the count of the white blood in the blood of CHC patients, of the present study, may add more to the involvement of the immune cells in the production of AMCase enzyme activity (Table 3).

AMCase is expressed in gastrointestinal tract and to the less extent in the lung (Boot et al. 2001). In spite, significant increase of AMCase mRNA and protein was detected in lungs of ovalbumin-sensitised mice (aeroallergen asthma model) [10], indicating that the enzyme is of the inducible types. The elevations of enzyme activity after viral infection; possibly via viral proteins, confirm this nature of the enzyme [16].

The major cell types producing mammalian chitinases and chitinase-like proteins are macrophages, neutrophils, epithelial cells, chondrocytes and synovial cells, as well as tumor cells. In the same line of studies van Eijk et al. [14] showed that, the production of chitotriosidase in cultured macrophages is strongly stimulated by GM-CSF which is actually one of the upregulated signals during HCV infection [14]. This may be actually the case in the present study.

A significant correlation was only found between plasma chitotriosidase activity and those of ferritin and with mean number of transfusions per year. Further, the patient with the highest chitotriosidase had the highest
ferritin [17]. It was found that hepatocytes are able to secrete ferritin which can act as a pro-inflammatory cytokine in activated HSCs, in an iron independent manner [18]. The pro-inflammatory processes may be involved in the activation of HSCs, thus inducing ECM remodeling and mediate liver disorders. Thus, serum ferritin has been shown to be a predictor of mortality in patients with end-stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin-based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. Therefore, a ferritin -based simple stage liver disease, both before and after liver transplantation [18] [19]. 

The natural route of bacterial infection is through the intestine after consumption of contaminated food. After entry into the blood, most of the bacteria are trafficked to the liver and the spleen by macrophages. In the liver, the bacteria are taken up by resident macrophages (Kupffer cells) [21]. Thus, Kupffer cells become activated and secrete proinflammatory cytokines such as interleukin 6 (IL-6), IL-1β, IL-12 and tumor necrosis factor (TNF) that help in the recruitment and activation of macrophages and neutrophils [22]. However the remaining bacteria spread to the liver hepatocytes and replicate exponentially for the next 72 hours [23]. All of these can participate in elevation of AMCase activity with simultaneous hepatic damage. In this regards phagocytic cells; including macrophages and dendritic cells engulf the bacteria by the process in which the bacteria are entrapped in phagocytic vesicles or phagosomes [24]. Thus, bacterial flora in hepatic disorders can mediate phagocytic processes with further derangement in hepatic functions. To confirm, Marrie et al. [25] showed that concurrent HCV infection in patients with IPD is common, and HCV infection in this population portends an increased risk for death and more serious complication of IPD than for non-HCV infected patients.

5 CONCLUSION

The assay of AMCase activity can add more to the assessment of liver disorders in CHC patients; especially if

6 REFERENCES


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