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CLINICAL SIGNIFICANCE OF SERUM N-TERMINAL PRO C-TYPE NATRIURETIC PEPTIDE IN HEPATITIS C-RELATED CHRONIC LIVER DISEASES

By

NADIA A. ABDELKADER[1*], MONA ZAKI[2], WESSAM E. SAAD[2], GEHAN HAMDY[3] and DINA SABRY[4]

Departments of Tropical Medicine[1`] and Clinical and Chemical Pathology[2]; Faculty of Medicine, Ain Shams University; Cairo 11566[1] and Departments of Internal Medicine[3] and Biochemistry[4] , Faculty of Medicine, Cairo University, Cairo, Egypt (*Correspondence` Email: dr.nadiaabdelaaty@gmail.com Tel: 002-01091244734)

Abstract

To evaluate the clinical utility of serum levels of N- terminal pro C- type natriuretic peptide (NT-pro CNP) in patients with hepatitis C related chronic liver disease (CLD), in prospective to disease complications and progression. This study included 66 hepatitis C- related CLD patients with and without ascites and 15 healthy individuals (control group). Serum NT-pro CNP was measured by ELISA. A stepwise progressive increase in NT-pro CNP levels was recorded through controls, patients without ascites and patients with ascites ($p< 0.05$). In addition, patients with hematemesis or encephalopathy had more than its double values than those without ($p<0.01$). Moreover, a significant difference was observed in the marker levels among esophageal varcies stages 1, 2, 3 ($H=13.679$, $p=0.001$), with highest levels in grade 3. NT-pro CNP correlated positively with alpha fetoprotein ($r_s =0.455$, $p=0.008$) with no significant correlation neither with MELD nor Child scores ($p>0.05$). ROC curve analysis revealed the overall performance of the marker in discriminating CLD patients collectively from controls, the optimum cut-off level was 85 ng/L (AUC= 0. 803, sensitivity 84.8%& specificity 53.3%). An increased level of NT-pro CNP is a promising non-invasive marker of hepatitis C related CLD complications and disease progression.

Keywords: Natriuretic Peptide, NT-pro CNP, Chronic Liver Disease, Hepatitis C.

Introduction

Chronic liver diseases (CLD) represent a major public health problem, accounting for significant morbidity and mortality worldwide. Egypt has the highest country-wide prevalence of hepatitis C virus (HCV) in the world, estimated nationally at 14.7% (Mahmoud *et al,* 2013). On the other hand, accumulating evidence suggests that nonalcoholic liver disease (NAFLD)-related cirrhosis is rapidly becoming another important cause of CLD and HCC (Rafiq and Younossi, 2009; Neuschwander-Tetri *et al,* 2010). Prognosis and management greatly depend on the amount and progression of liver fibrosis with the risk of developing cirrhosis with severe impairments in the health-related quality of life (Afendy *et al,* 2009; Park *et al,* 2014).

Liver inflammation is commonly associated with hepatocyte necrosis and apoptosis. These forms of liver cell injury initiate a sequence of events that is independent of the etiological basis for the inflammation and can result in hepatic fibrosis. Apoptotic bodies derived from the damaged hepatocytes can activate the quiescent hepatic stellate cells and Kupffer cells, and these activated cell populations can in turn promote inflammatory and fibrogenic responses (Lee and Friedman, 2011; Czaja, 2014).

Natriuretic peptides, consisting of the three family member's atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP); C-type natriuretic peptide is widely expressed in the vasculature, especially on

the endothelium (Barr *et al,* 1996). CNP has anti-inflammatory, anti-proliferative, and anti-migratory properties (Kuehnl *et al,* 2013). N-Terminal pro C-type natriuretic peptide (NT-proCNP) circulates at higher concentrations than CNP, allowing a direct assay and use of smaller amounts of plasma (Del Ry *et al,* 2011). Therefore, circulating NT-proCNP was intensively studied as a possible biomarker for inflammatory conditions (Bahrami *et al,* 2010; Tomasiuk *et al,* 2014).

The plasma NT-proCNP concentration can potentially serve as an accurate predictor of sepsis in multiple-traumatized patients without brain injury (Bahrami *et al,* 2010). Because sepsis and cirrhosis are both characterized by the hyperdynamic circulation associated with a low systemic vascular resistance and the release of many pro-inflammatory mediators, which was speculated that NT-proCNP might be involved in the progression of chronic liver disease and portal hypertension (Vincent and Gustot, 2010).

The aim of this work was to study serum level of N- terminal pro C- type natriuretic peptide in hepatitis C related chronic liver disease patients with and without ascites to evaluate the clinical utility as a non-invasive marker of chronic liver disease complications and disease progression.

Subjects, Materials and Methods

This prospective case-control study was conducted on 66 Egyptian patients with chronic liver diseases (CLD) of chronic hepatitis C virus and 15 apparently healthy subjects serving as a control group, all of whom willingly participated in the study after an informed consent.

Patients and controls were recruited from Department of Tropical Medicine, Ain Shams University Hospitals and department of Internal Medicine, Cairo University Hospitals.

Patients' Groups (n=66): In total, 66 patients with CLD were recruited from the in- and outpatients' clinic. All patients were chronically infected with HCV. Patients were divided into 2 groups: GI: Non ascitic Group (n=33): This group included 33 patients without evidence of ascites or liver cirrhosis as shown by abdominal ultrasonography. GII: Ascitic group (n=33): included 33 patients with established ascites & cirrhosis. They were subdivided according to presence or absence of hematemesis, encephalopathy and esophageal varices stages.

Diagnosis of liver cirrhosis was based on composite presence of characteristic features such as typical complications (e.g. ascites, variceal bleeding, hepatic encephalopathy), imaging (ultrasound, CT scan, MRI-scan), and/or histological verification combined with the existence of an underlying chronic liver disease predisposing to cirrhosis (Zimmermann *et al,* 2010).

Staging of patients with established liver cirrhosis was performed following Child-Pugh's criteria (Child A, B and C) as well as the standard model for end- stage liver disease MELD score. The severity of each patient's liver disease was scored using the MELD score (Cocito *et al,* 2010).

Control Group (n=15): As controls cross matched healthy volunteers with negative serology for HBV and HCV as well as normal aminotransferase activity levels were enrolled.

Exclusion criteria: Patients with ongoing bacterial infections including spontaneous bacterial peritonitis were excluded. Also, patients with HIV, malignant tumors and those on systemic steroid medication were excluded.

All were subjected to the following**:** 1. Full history taking and thorough clinical examination**.** 2. Laboratory investigations including CBC, liver enzymes, albumin, and total bilirubin and renal profile, viral markers, INR and alpha fetoprotein (AFP). Assay of serum NT- pro CNP was done by ELISA technique. 3. Imaging diagnosis by abdominal ultrasonography. 4. Upper gastrointestinal endoscopy.

Ten milliliters of venous blood were collected under complete aseptic precautions from each subject. The blood was divided among an EDTA tube for CBC, a citrated tube for international normalized ratio (INR) estimation and finally a plain test tube for serum separation. After clotting, samples were centrifuged at 1000 xg for 15 minutes, and sera were separated. The separated serum was then divided into two aliquots; the first one was used for the subsequent assay of liver function tests (serum transaminases, ALP, albumin, total and direct bilirubin), kidney function tests {creatinine, sodium. (Na) and potassium (K)} and serological markers {Hepatitis B Surface Antigen (HbsAg) and anti-HCV antibodies (anti-HCV Ab)}, in addition to alpha fetoprotein (AFP). The second aliquot was stored at -20°C for later assay of sera NT- pro CNP. Hemolysed samples were discarded and other sample without hemolysis were used. One freeze and one thaw was done.

Analytical Methods: N-terminal C-type natriuretic peptide (NT-pro-CNP) was measured using a commercially available ELISA kit of Glory science (Glory science Co, Ltd, USA). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for NT-pro-CNP was pre-coated onto a micro plate. Standards and samples are pipetted into the wells and any NT-pro-CNP present bounded by immobilized antibody. After washing any unbound substances, an enzyme-linked monoclonal antibody specific for NT-pro-CNP was added to the wells. Following a wash step to remove any unbound antibody-enzyme reagent, a substrate solution was added and color developed in proportion to the amount of NT-pro-CNP bound in the initial step. The color development was stopped and its intensity was measured. The chroma of color and the concentration of the NT-pro-CNP of sample were positively correlated (Clerico *et al,* 2012).

To deduce the concentration of NT-pro-CNP in serum samples and control material, a standard curve was constructed by plotting the mean absorbance for each standard on y-axis against concentration on the x-axis. The best fit curve was drawn through the points on the graph. Zero standard absorbance was subtracted from all absorbance.

Statistical analysis: This was carried out using statistical soft- ware program IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013). Parametric data were expressed as mean and standard deviation (X±SD) while non-parametric data were expressed as median and interquartile range (IQR). Comparative statistics was done by Wilcoxon's rank sum and Kruskall Wallis test in case of non-parametric data whereas Student' s t test was used for parametric data. Correlation analysis was performed by Spearman's rank correlation. P = <0.05 were considered significant, whereas P= <0.01 were considered highly significant. Receiver operating characteristic curve (ROC) analysis was applied to assess the overall diagnostic performance of serum NT-pro CNP in discriminating the studied groups.

Results

Patients in GI were 26 males and 7 females whose ages ranged from 27 to 82 years (mean 52.1±11.5 years). While patients in GII) were 23 males and 10 females whose ages ranged from 24 to 76 years (mean 51.6±12.2 years). Controls were 10 males and 5 females whose ages ranged from 27 to 60 years (mean 48.8 ± 11 years).

Descriptive analysis of different (Tab. 1) parameters, showed that serum NT- pro CNP gave a significant stepwise increase starting as early as GI, non-ascitic patients {105 (83-138) ng/l} as compared to group II, *ascitic patients* {119 (104-211) ng/L, p=0.017}. But, in spite of its elevation in GI in respect to control G, it did not reach a significant level as p=0.356 (Tab. 2).

Table 1: Descriptive analysis of all studied parameters in all groups

Parameter	GII (n=33) M ±SD/ median (IQR)*	GI (n=33) M±SD/ median (IQR)*	Control G (n=15)M±SD/ median (IQR)*
ALT(U/L)	33(21.5-55.5)*	38(18.5-60)*	28(25-30)*
AST(U/L)	32(23.5-63)*	23(19-36.5)*	21(18-25)*
INR ratio	1.38 ±0.34	1.17 ±0.26	0.9 ±0.06
Albumin(g/dL)	2.2 ±0.48	2.5 ±0.63	4.08±0.4
Total Bilirubin(mg/dL)	1.6(1.05-2.7)*	0.9(0.6-1.3)*	0.9(0.6-1)*
ALP (U/L)	89(65-132)*	79(50.5-99)*	45(30-58)*
Hb(g/dL)	9.3±1.3	9.5±1.4	13.5±1.6
PLT(X 10^9/L)	90.5(72.2-101.75)*	110(100-145)*	241(180-300)*
Creatinine(mg/dL)	1.2(0.85-1.5)*	0.8(0.7-1.15)*	0.8(0.6-0.9)*
AFP(ng/mL)	2.5(2.1-2.95)*	2.1(1.75-3.1)*	2.5(1.5-3.2)*
NT -pro CNP(ng/L)	119(104-211)*	105(83-138)*	85(79-121)*
MELD score	14(9-19.5)	8(6-13.5)	-
Child score	9 (8-10)	7(6-7)	-

- Mean ±SD for parametric data, *Median & interquartile range (IQR) for skewed data

A significant elevation was in serum NT- pro CNP in ascitic patients compared to controls {119(104-211) ng/l vs. 85(79-121) ng/L, z=2.516, p=0.012; respect- ively}. A significant statistical difference was between GI versus GII as regards most of liver profile tests, serum creatinine, MELD score and Child score; being higher in GII.

Table 2: Comparative analysis of different studied parameters among 3 groups

Parameter	Control vs. GI		Control vs. GII		GI vs. GII	
	T z	p	T z	p	T z	p
ALT(U/L)*	-1.071	0.284	-1.169	0.243	-0.244	0.807
AST(U/L)*	-1.773	0.076	-3.507	0	-2.22	0.026
INR ratio	-4.836	0	-7.202	0	2.754	0.008
Albumin(g/dL)	9.621	0	13.083	0	-2.319	0.024
T.Bilirubin (mg/dL)*	-1.03	0.303	-3.567	0	-3.033	0.002
ALP(U/L)*	-2.829	0.005	-4.172	0	-1.996	0.046
Hb(g/dL)	8.017	0	8.577	0	-0.519	0.606
PLT(X 109/L)*	-5.174	0	-5.478	0	-4.589	0
Creatinine(mg/dL)*	-1.502	0.133	-3.354	0.001	-2.356	0.018
AFP(ng/mL)*	-0.089	0.929	-0.49	0.624	-1.304	0.192
NT- pro CNP(ng/L)*	-0.924	0.356	-2.516	0.012	-2.381	0.017
MELD score	-	-	-	-	-3.374	0.001
Child score	-	-	-	-	-6.625	0

P < 0.05=significant difference, P < 0.01=highly significant difference, P > 0.05- no significant difference
T: Student t test for parametric data, z: Wilcoxcon rank sum test for skewed data*

A highly significant progressive increase in marker level was recorded by stage of no hematemesis was more than its double levels in hematemesis stage in GII, ascitic patients {108 (97.25-120) ng/l vs. 270 (237.5-270) ng/l, p<0.01; respectively}. Ascetic patients with encepha-lopathy showed more than double values of NT-pro CNP compared to those without {253 (218-270) vs.110 (101-124), p<0.01; respectively}. At a cut-off level 175 ng/l, a clear discrimination occurred between patients with hematemesis or encephalopathy and patients without, with a sensitivity, specificity and accuracy of 100%

Table 3: Statistical comparison between NT–pro CNP values in GII

GII, Ascitic Patients	NT- pro CNP (ng/L)	Z	p-value
Hematemesis: -with, n= 9 -without, n=24	270 (237.5-270)* 108 (97.25-120)*	4.374	0.000
Encephalopathy: -with, n=6 -without, n=27	253 (218-270)* 110 (101-124)*	3.087	0.002

Patients with hematemesis and encephalopathy versus patients without
Z: Wilcoxcon rank sum test for skewed data, P < 0.01: highly significant difference

A statistically significant difference was in marker levels among esophageal varcies stages 1, 2, 3 (H=13.679, p=0.001) with a significant stepwise elevated levels from stage 1{103 (94.5-114.5) ng/l} compared to stage 2 {121 (110-270) ng/l, z=2.64, p=0.008} and stage 3{225(118-263) ng/l, z=3.443, p=0.001}.

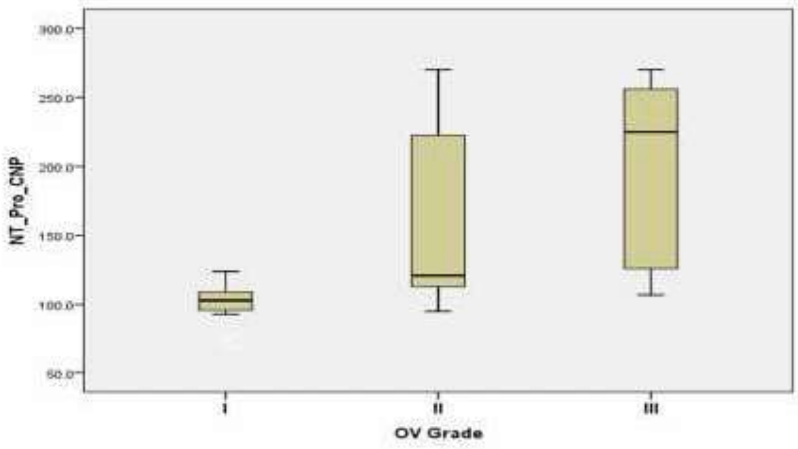
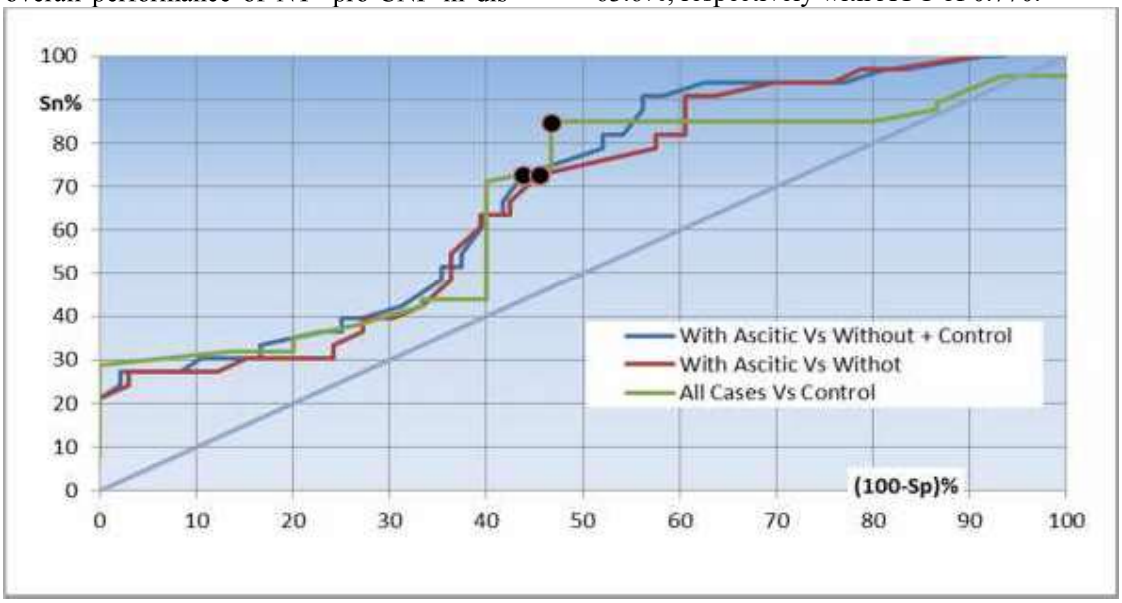


Fig. 1: Serum NT-proCNP Levels in Esophageal Varices Stages

A significant positive correlation was found between the marker values and AFP as well as Hb in GII (r_s = 0.455, p=0.008; r_s = 0.400, p= 0.021; respectively) whereas it was not significantly correlated with the other studied laboratory parameters. NT- pro-CNP show a significant positive correlation with serum creatinine in GI (r_s = 0.390, p=0.029).

ROC curve was constructed to examine the overall performance of NT- pro-CNP in discriminating studied groups (Fig. 2). At a cut-off level 85ng/l, the sensitivity, specificity and efficacy of NT- pro-CNP in discriminating patients from controls were 84.8%, 53.3% and 79%, respectively with AUC of 0.803. NT- pro-CNP at a cut-off value 105 ng/l could discriminate patients with ascites (GII) from those without (GI) achieving sensitivity, specificity and efficacy of 72.7%, 54.5% and 63.6%; respectively with AUC of 0.770.



AUC Patients with ascites vs. without & control 0.782**;** patients with ascites vs. without 0.770**,** all patients vs. control 0.803

Fig. 2: ROC curve analysis showing diagnostic performance of NT-pro CNP in discriminating studied groups

Discussion

Chronic liver diseases and its end-stages are worldwide leading causes of morbidi-ty and mortality with risky socioeconomic costs (Dooley and Ten Dijke, 2012). HCV is considered the most common etiology of CLD in Egypt, where prevalence of antibodies to HCV was approximately 10-fold greater than in the United States and Europe (Waked *et al,* 2014).

In clinical routine, non-invasive, longitudinally measurable biomarkers for local and systemic inflammation would be highly desirable, as they may allow early identification of patients at risk for the cirrhosis or even for fatal outcome. However, currently available laboratory parameters have limitations, because they either reflect hepatic biosynthetic capacity (as albumin, pseudocholinesterase, INR), hepatic cell injury (as ALT), cholestatic damage (as bilirubin, GT) or inflamm- atory activation restricted to distinct leucocyte subsets (as interleukin or IL-8) for neutrophils, CXCL10 for T-lymphocytes, monocyte chemo-attractant protein; MCP-1 for monocytes (Castera and Pinzani, 2010).

In the present study, the evaluated serum NT- pro-CNP in patients with hepatitis C related chronic liver disease with a statistically significant increase of its serum levels in patients with ascites when compared to control subjects was comparable with studies revealing that serum NT-pro CNP concentrations were significantly elevated in established hepatic cirrhosis with ascites compared to healthy controls (Koch *et al,* 2012). Moreover, the study results revealed a statistically significant increase in the marker levels in patients with ascites when compared to non- ascitic patients indicating its possible correlation with disease severity.

Generally, NT- pro-CNP is widely expressed throughout the vasculature and is found in particularly high concentrations in endothelium with anti-inflammatory, antiatherogenic and antiprolifrative properties (Bahrami *et al,* 2012). Many inflammatory cytokines including interleukin-1, TNF-α and endotoxin are known to trigger the release of C-type natriurectic peptide from endothelial cells (Koch *et al,* 2012). TNF-α has a variety of biological effects. The level of TNF-α was reflecting the capability of resisting viral infection, in addition to its association with immunopathological to damage hepatocytes (Liu *et al,* 2011).

The present study not only showed the presence of a statistically significant increase in NT- pro CNP serum levels in patient with ascites, but also a highly significant progressive increase in the marker levels in patients having hemate-mesis. The latter increase was exceeding its double values in respect to patients with no hematemesis with 100% accuracy in discrimination at a cut- off level 175 ng/l. Interestingly, the maker levels show a significant stepwise increase from stage 1 to stage 3 esophageal varices revealing the pathological implication of NT- pro-CNP in portal hypertension and its end results of varices and hematemesis which are integral part in liver cirrhosis.

Likewise, when comparing NT- pro-CNP serum levels in patient with or without encephalopathy, a statistically significant increase in the marker levels was noticed among patients with encephalopathy. Obviously, more than double values of NT-pro CNP were achieved with 100% accuracy in discrimination at a cut-off level 175ng/l. These findings were comparable to a previous study reported that NT-pro-CNP was associated with complications of liver diseases and portal hypertension, namely ascites, esophageal varices and hepatic encephalopathy (Koch *et al,* 2012). Specifically, CNP was suggested as an important mediator of vasodilatation as well as a regulator of fluid and sodium balance in cirrhosis (Gulberg *et al,* 2000).

In order to evaluate the significance of NT-pro CNP in progression of chronic liver diseases, NT- pro-CNP correlation with

liver function tests was studied. A significant positive correlation was found between the marker levels and AFP in patients with ascites; but without signi-ficant correlation detected with liver enzymes, total bilirubin and biomarkers reflecting liver synthesis function such as albumin or INR. Also, it was neither significantly correlated neither with MELD scores nor Child-Pugh- score in those patients, but showed a trend towards higher levels at advanced disease. On the other hand, studies showed that circulated NT-pro-CNP was inversely correlated to parameters reflecting hepatic biosynthetic capacity in critically ill patients, namely albumin and pseudocholinesterase acti-vity but did not differ significantly bet-ween stages of liver cirrhosis as assessed by Child-Pugh-score (Koch *et al,* 2011).

In the present study, NT-pro-CNP serum levels correlated positively with serum creatinine in CLD patients. This striking observation was noticed before (Koch *et al,* 2012). Previous authors reported that circulating NT-pro-CNP correlated inversely with glomerular filtration rate in CLD patients. In fact, CNP exerts natriuretic functions directly on renal tubular cells (Rubattu *et al,* 2008). This association could possibly hint at a negative influence of NT-pro-CNP on kidney function, potentially caused by reduced renal perfusion due to splanchnic vasodilatation and/or direct negative effects on tubules. Alternatively, as the clearance me-chanism of circulating NT-pro-CNP was not fully unraveled at present, it might also reflect altered renal elimination (Koh *et al,* 2012). Another study suggested the enhanced renal CNP production in cirrhosis. Thus, it may act as a paracrine mediator of renal function in patients with cirrhosis (Gulberg *et al,* 2000).

In the present study, the capacity of NT-pro-CNP to discriminate between CLD patients with ascites from patients without was examined with best ROC cut-off value of 105ng/l with AUC of 0.770 that yielded sensitivity, specificity and accuracy of 72.7%, 54.5% & 63.6%; respectively. No doubt, elevated NT-pro-CNP levels were identified as a predictor of mortality or necessity for transplantation. They added that NT-pro-CNP levels >2 pmol/l indicated adverse prognosis (sensitivity 66.7%, specificity 72.8%, RR 5.4 i.e., 95%-CI 2.6-11.2 (Koch *et al,* 2012). Therefore, serum NT-pro-CNP is elevated in advanced liver diseases and has a prognostic value in cirrhotic patients.

Fortunately, NT-pro-CNP concentration did not change significantly during clotted time and did not differ between serum, plasma, and whole blood samp- les. Also, NT-pro-CNP is stable for at least 2 hours, even when sample processing is delayed or blood samples are stored at room temperature. NT-pro-CNP assay showed more consistent and reliable data and should therefore be preferred for usage in clinical applications. However, as recommended for ANP and BNP, immunoassays for CNP should also be standardized or harmonized (Kuehnl *et al,* 2013).

Conclusion

The outcome data showed an increased level of NT-pro CNP as a promising non-invasive marker of hepatitis C related CLD complications and disease progression.

Conflict of Interest: The authors declare that they have no competing interests.

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