THE LEVEL OF CYTOKERATIN 18 IN PATIENTS WITH HIV AND VIRAL HEPATITIS C CO-INFECTION IN LATVIA

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Cytokeratin 18 (CK18) is a specific marker of hepatocellular apoptosis, which is a useful non-invasive indicator of liver fibrosis in the HIV/HCV group. However, data on the CK18 level in serum are limited for this group. This study demonstrated CK18 levels in serum in HIV/HCV co-infected and HIV mono-infected patients; investigated the association of CK18 levels with other non-invasive markers of liver fibrosis; and presents CK18 dynamics in a four-month-long period. The sample included 273 patients with HIV infection (128 of them were with HIV/HCV co-infection) aged from 23 to 65 (35% females). Levels of hyaluronic acid, CK18, ALT, and AST were determined in serum, and the FIB4 index was calculated. All markers had higher levels in the HIV/HCV group than in the HIV mono-infection group. The HIV/HCV group demonstrated coherent correlations among the markers and their associations with the level of CK18 than the HIV mono-infection group. During the four-month-long period, the CK18 level in serum showed no significant changes.

Key words: liver fibrosis, non-invasive test, hepatocyte apoptosis, CK18, HIV/HCV.

INTRODUCTION

Hepatitis C virus (HCV) is the second most common type of viral hepatitis that co-occurs with HIV infection. Of the 37 million people living with HIV worldwide, about 2.3 million are positive for antibodies to HCV (Platt *et al.*, 2016). HCV co-infection can affect from 6% (Platt *et al.*, 2016) to 30% of HIV patients (Rockstroh *et al.*, 2013), and up to 82% of people injecting drugs (Platt *et al.*, 2016). HCV co-infection with HIV promotes faster progression of liver fibrosis than HCV mono-infection (Mohsen *et al.*, 2003; de Lédighen *et al.*, 2008) or HIV infection without HCV (Kim *et al.*, 2016). As a result, mortality rates in HIV/HCV co-infected patients are higher than in patients with HIV infection without HCV (Rockstroh *et al.*, 2013; Scherzer *et al.*, 2017). Thus, it is important not only to detect liver fibrosis promptly but also to develop an optimal approach to reduce the progression of liver disease among HIV patients (Kim *et al.*, 2016).

The pathogenesis of liver fibrosis is in detail described by a variety of authors (Canbay *et al.*, 2004; Guicciardi and Gores, 2005; Friedman, 2010). Liver fibrosis occurs due to inflammation and hepatocyte apoptosis, which activate hepatic stellate cells (HSC) to produce collagen and other extracellular matrix molecules, as well as ferments, reducing the extracellular matrix degradation.

Hepatocyte apoptosis is an important pro-fibrogenic factor and, at the same time, the first response to different impacts, independently of their aetiology (Canbay *et al.*, 2004; Guicciardi and Gores, 2005). Phagocytosis of apoptotic bodies by Kupffer cells and HSC increases their expression of pro-fibrogenic cytokine as transforming growth factor (TGF- β), other pro-inflammatory cytokines, and death ligands, which promotes HSC activation and enhances hepatocyte apoptosis. Non-phagocytosed apoptotic bodies release their content into the tissues and further enhance hepatocyte apoptosis and HSC activation. It should be noted that the re-

relationship between apoptosis and fibrosis is bidirectional (Canbay *et al.*, 2004). Evidence of this was demonstrated in a recent experimental study (Ganesan *et al.*, 2018) and a clinical study (Viksna *et al.*, 2019).

Liver fibrosis in HIV/HCV co-infection associates with the cytopathic effect of HCV on hepatocytes and with the ability of HIV virus to induce direct and indirect liver injury (Chew and Bhattacharya, 2016). The most commonly described mechanisms of liver damage by HIV are the following. HIV envelop protein gp120 interacts with CCR5 and CXCR4 receptors — the main HIV co-receptors (Wilen *et al.*, 2012) — on hepatocyte and HSC and directly cause hepatocyte apoptosis (Blackard and Sherman, 2008) and HSC activation (Hong *et al.*, 2012). Moreover, HCV proteins activate HSC and increase the expression of both CXCR4 and CCR5 receptors on HSC (Hong *et al.*, 2009). An important effect on liver damage during HIV infection is related to microbial translocation, which extremely increases after the substantial loss of CD4⁺ T cells residing in the gut (Marchetti *et al.*, 2013). An increase of circulating lipopolysaccharides triggers Kupffer cells and induces their activation and production of TGF- β and other pro-fibrogenic cytokines. During HIV infection, fibrogenesis in the liver is enhanced by dysregulation of the immune system, leading to an imbalance between the production of anti-fibrogenic and pro-fibrogenic cytokines, as well as by oxidative stress and mitochondrial damage (Kaspar and Sterling, 2017). Prolonged antiretroviral treatment (ART) can also enhance mitochondrial toxicity and hepatocyte apoptosis. Different drugs of the classes nucleoside / nucleotide reverse transcriptase inhibitors (NRTI) (Maagaard *et al.*, 2009), protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) (Apostolova *et al.*, 2011; Selvaraj *et al.*, 2014) can affect liver injury.

Liver biopsy is traditionally used for the detection and assessment of liver fibrosis. At the same time, several studies in patients with HIV/HCV indicate that serum hyaluronic acid (HA), tissue inhibitors of metalloproteinase (Laurrouse *et al.*, 2007), AST-to-Platelets Ratio Index (APRI) (Schmid *et al.*, 2015; Klieman *et al.*, 2016), FIB4 index, and other non-invasive parameters and indexes (Schmid *et al.*, 2015) have a significant correlation with the stage of liver fibrosis. Additionally, APRI, FIB4 index, and HA are highly predictive of liver disease outcome in HIV/HCV co-infected individuals (Nunes *et al.*, 2010). Based on these studies, the use of these non-invasive tests for screening liver fibrosis is recommended in these patients, if transient fibroelastography is not available or impossible to perform (Schmid *et al.*, 2015). However, there is no consensus on the specificity and sensitivity of parameters or indices and their “cut-off” levels for HIV/HCV patients (Sterling *et al.*, 2006; Laurrouse *et al.*, 2007; Rohrbach *et al.*, 2013; Schmid *et al.*, 2015; Klieman *et al.*, 2016).

Cytokeratin 18 (CK18) is a specific indicator of hepatocyte apoptosis as it is mostly expressed by hepatocytes (Chu and Weiss, 2002). CK18 is elevated in patients with various liver diseases: acute and chronic viral hepatitis (Viksna *et*

al., 2009; Parfieniuk-Kowerda *et al.*, 2014), patients with acute alcoholic hepatitis (Viksna *et al.*, 2009) and non-alcoholic steatohepatitis (Feldstein *et al.*, 2009; Aida *et al.*, 2014; Kobayashi *et al.*, 2017). An increased CK18 level in blood was observed in patients with HIV mono-infection because of development of nonalcoholic steatohepatitis caused by longtime ART, dyslipidemia, and insulin resistance (Benmassaoud *et al.*, 2018). Benmassaoud *et al.* (2018) noted that data about CK18 levels are not available for HIV mono-infected patients from Western countries.

There are limited studies about the CK18 level in serum in HIV patients co-infected with HCV. Using the keywords “Cytokeratin 18” AND “HIV” AND “HCV”, only one publication was found in the PUBMED database regarding the level of CK18 in blood (Rohrbach *et al.*, 2013). Rohrbach *et al.* (2013) demonstrated a positive association between CK18 and AST level and between CK18 level and the stage of liver fibrosis in METAVIR classification. At the same time, there was no association between CK18 and other markers of liver fibrosis in this group. Higher levels of CK18 in HIV/HCV patients than in HIV mono-infected patients have been shown previously in two studies in Latvian cohorts (Viksna *et al.*, 2019; Madelāne *et al.*, 2019). Our study merges data from both Latvian studies and provides CK18 levels in a relatively large group of HIV/HCV co-infected and HIV mono-infected patients, and the association of CK18 levels with other non-invasive markers of liver fibrosis, and describes CK18 dynamics in a four-month-long period.

MATERIALS AND METHODS

This study combined two retrospective studies conducted in the frame of the National Research Programme “Biomedicine”, Sub-project 5.7.1, “Host organism’s determinants, predisposition and susceptibility to infections caused by RNA viruses (HIV, HCV, Tick-borne encephalitis), assessment of modulations of these determinants during the course of disease”. Both studies were approved by the Ethics Committee of Rīga Stradiņš University (Nr. 42/30.04.2015), Ethics Committee of Rīga East University Hospital (Nr. 18-A/15, 06.08.2015), and by the Rīga East University Hospital Support Foundation of Medical and Biomedical Studies Ethics Committee on 5 May 2016, with corresponding ethical approval code No. 6-A/16. The inclusion criteria were: age ≥ 18 , confirmed HIV mono-infection, confirmed HIV and HCV co-infection, and available serum for additional testing. The exclusion criteria were: opportunistic infection, viral hepatitis B, alcoholic liver diseases, and liver cirrhosis or neoplasia.

The study sample included 273 patients aged from 23 to 65 (mean age was 39 ± 9 years, 35% females, 100% Caucasian). The median CD4⁺ cell count was 335 cells/ μ l, and the interquartile range (IQR) was 192–483 cells/ μ l. The HIV/HCV group included 128 patients, of whom 73 received ART. The HIV mono-infection group included 145 patients (85 of them with ART). Two nucleoside reverse

transcriptase inhibitors (NRTI) combined with one protease inhibitors (PI) or with one non-nucleoside reverse transcriptase inhibitor (NNRTI) were used for treatment.

The HIV diagnosis was based on positive HIV1/2 antibodies in serum, confirmed by Western Blott, and on detection of HIV RNA by real-time polymerase chain reaction (COBAS AmpliPrep/COBAS TaqMan HIV-1 Test 2.0, Roche, USA, the lower limit of detection was 20 copies/ml). The HCV co-infection was confirmed by a positive HCV antibody test (ELISA, Bio-Rad, France or Ortho-Clinical Diagnostics Inc., USA) and by detection of HCV RNA (COBAS AmpliPrep/COBAS TaqMan HCV test, Roche, USA) or HCV Core Ag (ELISA Architect system HCV Ag, Abbot, USA) in plasma.

In patient's serum the following parameters were detected: hyaluronic acid (HA) by Hyaluronic Acid test Kit (Corgenix, USA), cytokeratin 18 neoepitope by M30 Apoptosense ELISA kit Peviva (VLV bio, Sweden) according to manufacturer's recommendations, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and platelet count by using validated methods. The FIB4 index was calculated as $FIB4 = (age \times AST) / (platelets (10^9/L) \times ALT^{1/2})$ (Sterling *et al.*, 2006). The CD4⁺ T lymphocyte count was determined by flow cytometry.

Statistical analysis was performed with IBM SPSS for Windows 22.0 and the R-package 'nparLD' 2.1 (Noguchi *et al.*, 2012) as an implementation of the nonparametric approach to the analysis of longitudinal data in factorial settings.

RESULTS

Shapiro-Wilk test values varied from 0.57 to 0.94, which indicated the non-normal distribution of markers under investigation. Therefore, the comparison of HIV and HIV/HCV was based on the Mann-Whitney U test. The results revealed significantly higher levels of all markers of liver fibrosis in the HIV/HCV co-infection group (Table 1).

In 21.5% patients with HIV mono-infection and 39.6% patients with HIV/HCV co-infection, the level of FIB4 was greater than 1.45 — the level of absence of advanced fibrosis, following Sterling *et al.* (2006). It also confirmed an association of liver fibrosis with HCV, $\chi^2(1) = 8.70$, $p = 0.003$.

The relationships among markers of liver fibrosis were assessed in HIV mono-infection group and HIV/HCV co-infection group separately, taking into account differences between the two groups. Table 2 presents correlation coefficients in the HIV mono-infection group.

The level of CK18 was correlated positively with the level of ALT. FIB4 index was correlated positively with the level of HA and AST, while AST and ALT demonstrated a strong positive association.

In the group with HIV/HCV co-infection, markers of liver fibrosis demonstrated stronger positive associations, and all of them were statistically significant (Table 3).

Table 1. Mann–Whitney U test on markers of liver fibrosis and CD4⁺ T lymphocytes in HIV mono-infection and HIV/HCV co-infection groups

Markers	HIV Group (n = 145) Median (IQR)	HIV/HCV Group (n = 128) Median (IQR)	U
CK18, U/l	156 (130–188)	199 (149–269)	6198.5***
FIB4 Index	0.94 (0.69–1.34)	1.05 (0.78–2.10)	5071.0*
HA, ng/ml	18.9 (14.4–27.6)	27.0 (17.0–45.9)	5794.0***
ALT, U/l	23 (16–36)	52 (31–91)	3352.5***
AST, U/l	22 (18–30)	44 (26–78)	3252.0***
CD4 ⁺ , cells/ μ l	357 (246–497)	308 (139–467)	7842.5*

HCV, viral hepatitis C; IQR, interquartile range; CK18, cytokeratin 18; HA, hyaluronic acid; ALA, alanine aminotransferase; AST, aspartate aminotransferase.

* $p < 0.05$; *** $p < 0.001$

Table 2. Spearman's rank correlations among markers of liver fibrosis in the HIV mono-infection group (n = 145)

Markers	CK18	FIB4	HA	ALT	AST
CK18	-				
FIB4	0.04	-			
HA	0.05	0.25**	-		
ALT	0.25**	0.16	0.10	-	
AST	0.17	0.47***	0.07	0.79***	-

CK18, cytokeratin 18; HA, hyaluronic acid; ALA, alanine aminotransferase; AST, aspartate aminotransferase.

** $p < 0.01$; *** $p < 0.001$

Table 3. Spearman's rank correlations among markers of liver fibrosis in the HIV/HCV co-infection group (n = 128)

Markers	CK18	FIB4	HA	ALT	AST
CK18	-				
FIB4	0.20*	-			
HA	0.19*	0.46***	-		
ALT	0.32**	0.41***	0.19*	-	
AST	0.28**	0.64***	0.24*	0.84***	-

CK18, cytokeratin 18; HA, hyaluronic acid; ALA, alanine aminotransferase; AST, aspartate aminotransferase.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The final step of analysis involved the assessment of the dynamics of the level of CK18 within four months. The comparison was performed in a subgroup of 36 patients, which were available for comparison (Table 4). The non-parametric, robust rank-based method for the analysis of longitudinal data (baseline vs four months) in two sub-groups (HIV mono-infection vs HIV/HCV co-infection) was applied following procedures suggested by Noguchi *et al.* (2012).

The results demonstrated no significant time effect and no interaction between HCV co-infection and time. The significant main effect was observed only for HCV co-

Table 4. CK18 dynamics in four-months-long follow-up (n = 36)

Group	CK18 baseline median (IQR)	CK18 after 4 months median (IQR)
HIV (n = 22)	141.3 (99.7–183.3)	116.5 (104.4–176.9)
HIV/HCV (n = 14)	202.8 (139.1–266.2)	199.2 (120.9–317.7)
Factor	ANOVA-type statistic	p
HCV	7.39	0.007
Time	0.76	0.382
HCV × Time	0.06	0.797

HCV, viral hepatitis C; IQR, range; CK18, cytokeratin 18

infection. It confirmed the difference between HIV and HIV/HCV groups, revealed at the first step of the analysis.

DISCUSSION

In general, all of the investigated liver damage parameters were higher in the HIV/HCV group than in patients with HIV mono-infection. Differences between HIV and HIV/HCV co-infection groups and the proportion of patients with FIB4 higher than 1.45 confirmed association of liver fibrosis with HCV co-infection (Kim *et al.*, 2016).

The median and the first quartile values of CK18 in both groups were higher than 122 U/l as the “cut-off” value suggested for this marker of hepatic apoptosis (Rohrbach *et al.*, 2013). This indicates that both groups faced significant hepatic apoptosis. Simultaneously, the higher CK18 level in patients with HIV/HCV co-infection than in patients with HIV mono-infection confirmed pronounced hepatocyte apoptosis during HIV/HCV co-infection (Macías *et al.*, 2005).

The HIV/HCV group demonstrated a more coherent pattern of correlations among HA, AST, ALT, and FIB4 index and their associations with the level of CK18. In the study of Rohrbach *et al.* (2013), CK18 was correlated with AST only. The difference in correlation patterns between the studies can be addressed to the greater amplitude of markers. For example, IQR for CK18 in HIV/HCV group in our study was from 149 U/l to 269 U/l, while in Rohrbach *et al.* (2013) it was from 83 U/l to 123 U/l (in a pre-ART group).

Previously, correlations between CK18 and AST or ALT were described in HIV mono-infected patients because of the presence of steatohepatitis developed due to insulin resistance, longtime ART, and dyslipidemia during the course of HIV infection (Benmassaoud *et al.*, 2018). The positive relationship between CK18 and ALT was described in patients with chronic HCV without HIV infection (Parfieniuk-Kowerda *et al.*, 2014). Both transaminases are ferments in hepatocytes, and the relationship between CK18 and transaminases confirms the damage of hepatocytes during HIV/HCV co-infection.

An increased HA level in serum is the marker of liver fibrosis (Neunman *et al.*, 2016). Correlation between CK18 and

HA confirms the theoretical relationship between hepatocyte apoptosis and liver fibrosis, described by Canbay *et al.* (2004), Guicciardi and Gores (2005) and Friedman (2010). A clinical study (Viksna *et al.*, 2019) also indicated that HIV/HCV co-infection impacts CK18 indirectly through the level of HA.

In our study, the investigation of CK18 level in serum during the four-month-long period revealed no significant changes. This is in line with Rohrbach *et al.* (2013), who found no significant changes in CK18 level within a year in HIV/HCV patients under successful ART and pre-ART. In our study, the higher level of CK18 in HIV/HCV patients than in HIV mono-infected patients remained during this period.

The retrospective nature of our study forms its significant limitation. A relatively small number of patients in a four-month-long longitudinal study limited the assessment of CK18 dynamics. A longer follow-up interval is requested for a more precise evaluation of the dynamics. The multi-factor model of liver fibrosis and a complex assessment of its markers remain the question for further investigation.

In general, our study provides information for a comparison of observed levels of CK18 and other non-invasive markers of liver fibrosis in a European country. The revealed levels of markers of liver fibrosis and apoptosis provide a basis for discussion on the improvement of the assessment of risk for patients with HIV mono-infection and HIV/HCV co-infection. Significant correlations of CK18 with markers of liver fibrosis and hepatocyte damage confirmed the significance of CK18 level in serum for the assessment of liver damage and fibrosis in patients with HIV/HCV co-infection.

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CITOKERATĪNA 18 LĪMENIS PACIENTIEM AR HIV INFEKCIJU UN VĪRUSA HEPATĪTA C KOINFEKCIJU LATVIJĀ

Citokeratīns 18 (CK18) ir hepatocelulāras apoptozes rādītājs, kas ir noderīgs aknu fibrozes novērtēšanā ar neinvazīviem marķieriem HIV/HCV grupā. Tomēr dati par CK18 līmeni serumā šajā pacientu grupā ir ierobežoti. Šis pētījums parāda CK18 līmeni serumā pacientiem ar HIV/HCV koinfekciju un HIV monoinfekciju, atklāj CK18 saistību ar citiem neinvazīviem aknu fibrozes marķieriem un novērtē CK18 dinamiku četru mēnešu ilgā periodā. Paraugkopā bija iekļauti 273 pacienti ar HIV infekciju (128 no tiem bija ar HIV/HCV koinfekciju) vecumā no 23 līdz 65 gadiem (35% bija sievietes). Serumā tika noteikta hialuronskābe, CK18, ALT un AST, un tika aprēķināts FIB4 indekss. Visi marķieri HIV/HCV grupā vairāk atšķīrās no normas nekā pacientiem ar HIV monoinfekciju. HIV/HCV grupā tika atklāts vairāk korelāciju starp izmeklētiem aknu fibrozes marķieriem un CK18 līmeni nekā grupā ar HIV monoinfekciju. Četru mēnešu ilgā periodā CK18 līmenis serumā neuzrādīja būtiskas izmaiņas.