

Markers of liver fibrosis and apoptosis in patients with HIV mono-infection and HIV/HCV co-infection

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Abstract

Introduction: Human immunodeficiency virus (HIV)/hepatitis C virus (HCV) co-infection has a significant impact on liver-related morbidity and mortality. Among many other pathogenic mechanisms leading to accelerated hepatic disease are liver fibrosis and hepatocyte apoptosis. Hence the objective of this investigator-initiated cross-sectional study was to analyse the plasma levels of hyaluronic acid, cytokeratin-18, and cytochrome c in order to assess their impact on progression of liver disease in patients with HIV/HCV co-infection comparing to those with HIV mono-infection.

Material and methods: There were 80 patients with HIV infection included in the study. HCV co-infection was in 46 (57.5%) patients and HIV mono-infection in 34 patients. Blood levels of hyaluronic acid were tested using a Hyaluronic Acid test Kit, cytokeratin-18 neopeptide levels using M30-Apoptose ELISA test, and cytochrome c using human Cytochrome c ELISA test.

Results: The levels of cytokeratin-18 and of hyaluronic acid were significantly higher in the group of patients with HIV/HCV co-infection. The differences in the level of cytochrome c were not significant. The analysis revealed that the main effect of HCV co-infection on the level of cytokeratin-18 is indirect. It is mediated by the level of hyaluronic acid.

Conclusions: The progression of liver disease in patients with HIV/HCV co-infection is more pronounced compared to those with HIV mono-infection. It is shown by the higher plasma levels of hyaluronic acid, which is a relevant liver fibrosis marker and hepatocyte apoptosis marker cytokeratin-18 in patients with HIV/HCV co-infection. The hyaluronic acid level is important in assessing the impact of HCV co-infection on liver apoptosis.

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Key words: apoptosis, hepatitis C virus, liver fibrosis, co-infection, human immunodeficiency virus (HIV).

Introduction

Due to similar routes of transmission, hepatitis C virus (HCV) and human immunodeficiency virus (HIV) co-infection is common in medical practice. According to

a systematic review and meta-analysis [1] there are approximately 2 278 400 HIV/HCV co-infections globally of which the majority, approximately 1 362 700, are through drug injection. These data indicate a co-infection rate of 6.2% in HIV-infected individuals. Comparing geographic regions,

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the majority of HIV/HCV co-infected people are in Eastern Europe – approximately 607 700 [1] – and sub-Saharan Africa [2]. Among countries with highest incidence of HIV infection are Estonia and Latvia. According to available data from January 1st 2018, there are 7343 known HIV-positive persons in Latvia [3]. In the time period from January 1, 2000 till December 31, 2017 there were 6135 HIV-positive patients registered in Latvia. From them 1025 (16.71%) were co-infected with HCV [4].

HIV/HCV co-infection has a significant impact on liver-related morbidity and mortality due to a number of pathogenic factors. The most relevant pathogenic mechanisms leading to accelerated hepatic fibrosis among HIV/HCV co-infected individuals are the following: direct viral effects, immune/cytokine dysregulation, altered levels of matrix metalloproteinases and fibrosis biomarkers, increased oxidative stress and hepatocyte apoptosis, HIV-associated gut depletion of CD4+ cells, and microbial translocation [5]. Other pathogenesis mechanisms include products of microbial translocation such as lipopolysaccharide (LPS) and toll-like receptor 4 (TLR4). Increased LPS blood levels are observed in patients with chronic HCV infection and in those with HIV infection. Consequently, in case of HIV/HCV co-infection, progression of liver disease may be faster due to increased levels of LPS [6]. In HIV/HCV co-infected patients higher METAVIR activity scores are observed compared to patients with HCV mono-infection [7]. In patients with low levels of peripheral CD4+ T lymphocytes (200 cells/ μ l) advanced liver fibrosis and liver cirrhosis develops more frequently [8], indicating the protective role of these important immune cells.

The risk of developing advanced or end-stage liver disease in co-infected patients is definitely increased [9, 10]. End-stage liver disease is found among the most frequent causes of death in HIV-infected, hospitalised patients [11]. Management of patients with co-infection poses a greater challenge due to the higher number of medicines used in cases of concomitant treatment of both infections [12]. Achieving sustained virologic response (SVR) is very important when treating chronic hepatitis C patients because in the liver stiffness stage (LSS), measured by elastometry, improvement is achieved only in those with SVR [13]. When comparing change in liver stiffness in patients with viral eradication, no significant differences were observed based on presence or absence of HIV coinfection [14].

HCV is also one of the leading reasons of liver transplantation worldwide. Because liver transplantation is done in more patients each year, it is important to properly manage HIV and HCV infections prior to the procedure, because it is known that outcomes are poorer in co-infected patients [15].

HIV infection leads to immune activation and increased inflammation, which may result in more significant multi-organ damage, including liver. There is evidence already from early studies that the HIV virus itself is capable of causing histological liver abnormalities [16]. Because there is no clear evidence that HIV infects hepatocytes, many studies

suggest that HIV may indirectly result in hepatocellular injury [17]. There are extremely complex immune interactions in viral co-infections including differential regulatory pathways that influence virus-specific T cells [18].

One of the mechanisms by which viral infections cause pathogenic changes is through initiating the process of programmed cell death or apoptosis. Apoptosis can occur via two fundamental pathways: extrinsic and intrinsic. In the case of extrinsic pathway cell death signals are transmitted by the death receptor (DR), while the intrinsic pathway is initiated by intracellular stimuli. The intrinsic apoptosis pathway is characterised by the release of cytochrome c or other caspase-activating factors from the mitochondria intermembrane space into the cytoplasm [19]. As a result, apoptosome complex is formed in the cytoplasm. This complex activates caspase-9, which further activates the effector caspase cascade [20]. While in acute HCV infection, intensive hepatocyte apoptosis serves as a key mechanism of virus elimination, in chronic hepatitis C hepatocyte apoptosis is accompanied by cell proliferation and ensures persistent infection [21]. In the case of HCV infection apoptosis is not strongly correlated with the viral load but is rather immune-oriented, as shown by correlation with histology grading and infiltration of CD8-positive immune cells [22]. There is evidence that HCV core protein regulates hepatocyte apoptosis. For example, the core protein could inhibit Fas-mediated apoptosis by inhibiting the release of cytochrome c and activating caspase-9, caspase-3, and caspase-7 [23]. Besides core protein, HCV structural proteins E1 and E2 also influence hepatocyte apoptosis; for example, E2 inhibits DR-induced apoptosis in hepatoma cells by inhibiting the release of mitochondrial cytochrome c [24].

There are increased levels of peripheral T-cell apoptosis observed in patients with chronic hepatitis C, and the extrinsic apoptosis pathway is upregulated in both HCV mono-infection and HIV/HCV co-infection, although this up-regulation is independent of liver disease severity [25]. In the case of HIV infection, among other mechanisms T-helper cells are depleted due to apoptosis, resulting from a series of biochemical pathways [26] such as deactivation of anti-apoptotic Bcl-2 by HIV enzymes. The Bcl-2-interacting protein Bid is cleaved by caspase-8, yielding a truncated-Bid that causes the loss of mitochondrial membrane potential and caspase-9 cleavage, further resulting in apoptosis [27].

Hepatocyte apoptosis plays an important protective role by removing external microorganisms, including viruses. At the same time apoptosis contributes to the development of acute and chronic liver diseases, such as chronic hepatitis C. Hepatocyte apoptosis is increased in the case of HIV/HCV co-infection compared to HCV infection alone, and this increase is mediated by up-regulation of death receptors 4 and 5 (DR4 and DR5), also known as tumour necrosis factor-related apoptosis-inducing ligands (TRAIL) – R1 and R2 [17].

In one study the authors concluded that HCV is a molecular-level determinant in HIV disease [28]. The authors

found that in HIV/HCV co-infected patients T-cells were found to be destroyed at a slower rate than in patients infected HIV alone. Interestingly, in the study CD4+ and CD8+ T-cell apoptosis was lower while CD19+ B cell apoptosis was higher in HIV/HCV co-infected patients compared with HIV mono-infection.

There are many patients worldwide with nonalcoholic fatty liver disease (NAFLD). Among other risk factors for hepatocyte apoptosis in NAFLD patients is mitochondrial dysfunction, characterised by increased plasma caspase-3 levels, which generates cytokeratin-18 fragments and soluble Fas [29].

HCV primarily attacks hepatocytes causing necrosis – especially during acute infection marked by extremely high values of alanine aminotransferase (ALT) activity. In addition, hepatitis C infection triggers inflammation through stimulation of production of pro-inflammatory cytokines, which is associated with insulin resistance and hepatic steatosis [30] and has an association with atherosclerosis [31]. Furthermore, chronic HCV infection causes liver cirrhosis and hepatocellular carcinoma (HCC) in a relatively large proportion of infected individuals. Chronic immune activation in HIV/HCV co-infected patients may also contribute to hepatic fibrogenesis [32]. Apoptosis triggers mechanisms of cell repair in the liver – regeneration that is accompanied by inflammation. These processes lead to liver fibrogenesis. Fibrogenic activity is stimulated further by hepatic stellate cells (HSC), which engulf apoptotic bodies [33]. Hepatic stellate cells synthesise hyaluronic acid (HA), a glycosaminoglycan component of the extracellular matrix (ECM) – the basis of liver fibrosis. Hyaluronic acid is regarded as a very good (class I) biomarker of advanced liver fibrosis and cirrhosis [34]. HA has very strong negative predictive value (98-100%), determined by its ability to rule out advanced liver fibrosis and cirrhosis [35]. More intense apoptotic processes correlate with more severe hepatic disease and more advanced [36] stage of fibrosis in patients with steatosis [37].

The aim of this investigator-initiated cross-sectional study was to analyse the plasma levels of hyaluronic acid (liver fibrosis marker), cytokeratin-18, and cytochrome c (hepatocyte apoptosis markers) to assess their impact on progression of liver disease in patients with HIV/HCV co-infection compared to those with HIV mono-infection.

Material and methods

Patients

There were 80 patients with HIV infection included in the study. HCV co-infection was in 46 (57.5%) patients while HIV mono-infection was in 34 (42.5%) patients. Additionally, patients were grouped according to stage of HIV infection and presence of anti-retroviral therapy (ART). The ages of the patients ranged from 24 to 62 years, and 26 (32.5%) were females.

Data

Data were collected from electronic patient records. The following data were registered: date of birth, gender, diagnosis (stage of HIV infection, co-infection with chronic hepatitis C), and blood serum laboratory test results (CD4+, insulin, insulin resistance [HOMA-IR], glucose, hyaluronic acid [HA], cytokeratin-18 [CK-18], cytochrome c [CYC]).

Laboratory methods

Hyaluronic acid levels (ng/ml), with normal values in the range 0-75 ng/ml, were measured using a Hyaluronic Acid test Kit (Corgenix, USA). Cytokeratin-18 neoepitope levels (U/l), with normal values in the range 47.1-103.9 U/l, were quantified using the M30-Apoptose ELISA test (PEVIVA, Sweden). Cytochrome c (ng/ml), with normal value 0 ng/ml, was detected using human Cytochrome c ELISA test (Bender MedSystems, Austria). HIV virus levels in plasma were measured using PCR HIV RNA test (Cobas AmpliPrep/Cobas TaqMan HIV-1 test, Roche, USA). The CD4+ lymphocyte counts were determined by flow cytometry. ALT and AST levels were detected using kinetic fermentation method. Plasma insulin was measured by Microparticle Enzyme Immunoassay (AxSym Insulin, Abbott, USA). Insulin resistance was calculated using homeostasis model assessment (HOMA-IR): fasting insulin (μ U/ml) \times fasting glucose (mmol/l)/22.5. Plasma glucose was measured by hexokinase method.

The study was initiated and monitored by the infectious diseases and dermatology department of Rīga Stradiņš University. Ethical approval was provided by Rīga Stradiņš University Ethics Committee on April 30, 2015 with corresponding ethical approval code No. 42/30.04.2015 and by the Medical and Biomedical Studies Ethics Committee “afonds.ētika” on May 5, 2016 with corresponding ethical approval code No. 6-A/16. The work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki of 1975, as revised in 2000). All included patients provided written, informed consent.

Statistical analysis

For statistical analyses IBM SPSS Statistics for Windows, Version 22.0 [38] and ‘lavaan’ (0.5-22) for R [39] were used. Demographic characteristics were summarised using standard descriptive methods. Data distribution was assessed using Shapiro-Wilk tests. Correlations among the markers were calculated using Spearman’s rank correlation test. Comparisons between the groups were made using the Mann-Whitney *U* test. A two-tailed *p*-value less than 0.05 was considered statistically significant. A path analysis was performed in order to assess a complex contribution of HCV to the level of the markers. Satorra-Bentler correction [40] was applied for accounting of the deviance from normal distribution of data included in the path analysis.

Results

Patients

Altogether, 80 patients were included in the study. To study the differences in hepatocyte apoptosis markers and hyaluronic acid in their relation to HIV/HCV co-infection, the HIV patients were divided into two groups (Table 1).

There were no statistically significant differences regarding patients' gender distribution, $\chi^2(1) = 2.17$, $p = 0.14$. In addition, there were no significant differences between the two groups associated with patients' age, $t(78) = 1.54$, $p = 0.13$, and HIV stage, $\chi^2(2) = 1.37$, $p = 0.51$.

The markers of liver fibrosis and apoptosis

Correlations among the markers

The relationships between plasma levels of cytokeratin-18, cytochrome c, hyaluronic acid, CD4+ lymphocytes, glucose, insulin, and insulin resistance (HOMA-IR) were assessed with Spearman's rank correlation coefficient (Table 2). It was selected on the basis of observed non-normality (a long-tail type) of data distribution.

The analysis demonstrated a significant positive correlation between the level of cytokeratin-18 and hyaluronic acid. Hyaluronic acid correlated positively with insulin resistance and the level of insulin. Cytochrome c demonstrated positive correlations with insulin resistance, the level of insulin, and glucose. As expected, correlations among insulin resistance, the level of insulin, and glucose were positive. Correlations between the level of cytochrome c

and the level of cytokeratin 18 or hyaluronic acid were not significant. Cytokeratin-18 demonstrated no significant correlation with insulin resistance, insulin, and glucose. Hyaluronic acid did not correlate with the level of glucose. There were also no significant correlations between the level of CD4+ lymphocytes and the markers under investigation.

Differences between HIV and HIV/HCV groups

Mann-Whitney *U*-test demonstrated that the levels of cytokeratin-18 and of hyaluronic acid were significantly higher in the group of patients with HIV/HCV co-infection (Table 3). The differences in the level of cytochrome c, insulin, glucose, CD4+ lymphocytes, and insulin resistance were not significant.

The revealed differences in groups with and without HCV co-infection and correlations between the markers of liver fibrosis, apoptosis, and insulin resistance raised a question about a complex effect of HCV co-infection on the processes under investigation. The following step was performed in order to assess this effect.

A complex contribution of HIV/HCV co-infection

In order to assess a complex contribution of HIV/HCV co-infection to the level of hyaluronic acid, cytokeratin-18, and insulin resistance, a path model was tested (Fig. 1). The path analysis was performed using 'lavaan' for R [39]. A deviance of measures from a normal distribution was accounted through Satorra-Bentler correction [40]. Cytochrome c was excluded from the model as having no significant independent contribution to the level of insulin or insulin resistance. The level of insulin was not included in the model because of its extremely high positive correlation with insulin resistance and the relatively small number of participants.

The results of the analysis demonstrated a direct effect of HCV co-infection on the level of hyaluronic acid. In the group with HIV/HCV co-infection, the estimated increase in the level of hyaluronic acid was 30.72 ng/ml (95% CI from 11.19 ng/ml to 50.25 ng/ml). The direct

Table 1. Distribution of HIV patients with or without HCV co-infection

| Gender | Without co-infection | With co-infection |
|--------|----------------------|-------------------|
| Male | 26 (76%) | 28 (61%) |
| Female | 8 (24%) | 18 (39%) |
| Total | 34 (100%) | 46 (100%) |

Values shown are number of patients (% of patients).

Table 2. Spearman rank correlations among the markers in HIV patients ($N = 80$)

| Marker | CK-18 | HA | CYC | IR | Ins | Glu | CD4+ |
|--------|-------|--------|---------|---------|---------|-------|------|
| CK-18 | 1.00 | | | | | | |
| HA | 0.28* | 1.00 | | | | | |
| CYC | 0.06 | 0.05 | 1.00 | | | | |
| IR | 0.09 | 0.33** | 0.25* | 1.00 | | | |
| Ins | 0.11 | 0.37** | 0.24* | 0.99*** | 1.00 | | |
| Glu | -0.05 | 0.00 | 0.39*** | 0.50*** | 0.39*** | 1.00 | |
| CD4+ | 0.03 | -0.09 | -0.13 | -0.05 | -0.07 | -0.02 | 1.00 |

CK-18 – cytokeratin-18, HA – hyaluronic acid, CYC – cytochrome c, IR – insulin resistance, INS – insulin, Glu – glucose, CD4+ – CD4+ lymphocytes
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Table 3. A comparison of the markers in HIV patients with and without HCV co-infection

| Marker | Without HCV co-infection (n = 34) | With HCV co-infection (n = 46) | p [#] |
|---|-----------------------------------|--------------------------------|----------------|
| Hyaluronic acid (ng/ml) | 156.00 ± 25.67 | 235.23 ± 45.78 | < 0.01 |
| Cytokeratin-18 (U/l) | 21.29 ± 3.86 | 52.01 ± 18.91 | < 0.01 |
| Cytochrome c (ng/ml) | 0.33 ± 0.29 | 0.54 ± 0.21 | 0.15 |
| Insulin resistance (Ins µIU/ml × Glu mmol/l /22.5) | 2.76 ± 0.42 | 2.81 ± 0.43 | 0.93 |
| Insulin (µIU/ml) | 10.91 ± 1.47 | 11.66 ± 1.51 | 0.76 |
| Glucose (mmol/l) | 5.44 ± 0.15 | 5.23 ± 0.10 | 0.36 |
| CD4+ lymphocytes (cells/mm ³) | 384.65 ± 39.16 | 342.46 ± 34.23 | 0.37 |

Values are number – mean with the 95% confidence interval

[#]p-values for Mann-Whitney U-test

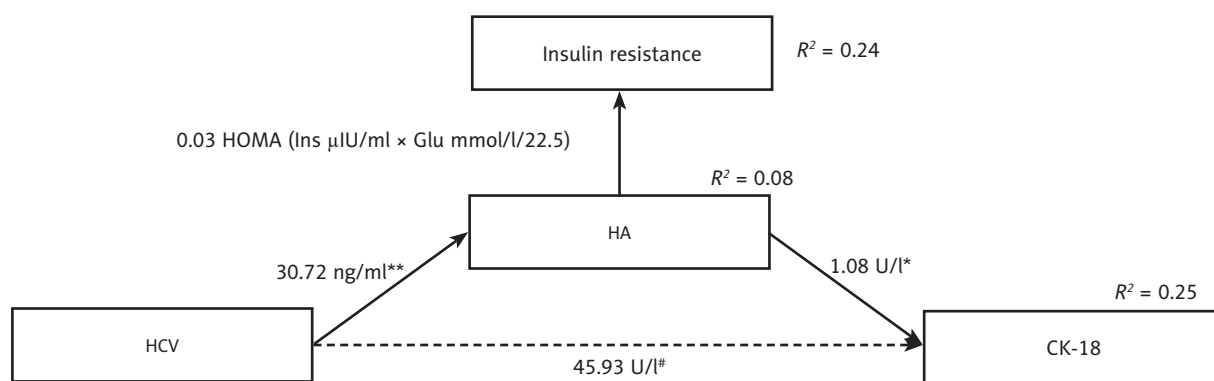


Figure 1. The path analysis on the contribution of HIV/HCV co-infection to the level of hyaluronic acid (HA), cytoke­ratin-18 (CK-18), and insulin resistance ($p < 0.01^{**}$, $p < 0.05^*$, $p < 0.10^{\#}$). Dashed line shows marginal significance. Path coefficients are non-standardized estimates

effect of HIV/HCV co-infection on the level of cytoke­ratin-18 was only marginally significant ($p = 0.08$). The estimated change in the level of cytoke­ratin-18 was 45.93 U/l (95% CI from -5.63 U/l to 97.49 U/l). The level of hyaluronic acid impacted the level of cytoke­ratin-18 significantly. An increase in the level of hyaluronic acid by 1.00 ng/ml was associated with an increase in the level of cytoke­ratin-18 by 1.08 U/l (95% CI from 0.10 U/l to 2.07 U/l). Hyaluronic acid impacted also insulin resistance. The estimated increase in insulin resistance was 0.03 HOMA (Ins µIU/ml x Glu mmol/l/22.5) and (95% CI from 0.01 to 0.04).

The analysis revealed that the effects of HIV/HCV co-infection on the level of cytoke­ratin-18 and insulin resistance are indirect. They are mediated by the level of hyaluronic acid. HIV/HCV co-infection and hyaluronic acid explained 25% of variance in the level of cytoke­ratin-18 and 24% of variance in insulin resistance, while HIV/HCV co-infection explained 8% of variance in the level of hyaluronic acid.

Discussion

The complex contribution of HIV/HCV co-infection analysis in this study indicates that the pathophysiology of accelerated liver fibrosis in the case of HIV infection and HIV/HCV co-infection is a complex and multifactorial process involving viral factors, the immune system, and inflammatory mediators. Advances in the understanding of fibrosis development mechanisms are very important for the planning of a liver fibrosis regulation strategy. It is well known that in the past 15-20 years progress has been made in understanding of the biology of HIV infection. However, the biochemical mechanisms of HIV-induced T-cell death, including death of uninfected CD4+ and CD8+ T cells, are not well defined.

The study confirmed a positive relationship between the markers of liver fibrosis and apoptosis – HA and CK-18. An impact of HIV/HCV co-infection on these two markers was also confirmed. As expected, the levels of HA and CK-18 were significantly higher in the group

with HCV co-infection. In addition, the study confirmed the relationship between liver fibrosis and insulin resistance.

In all patients included in the study a significant positive correlation between the level of cytokeratin-18 and hyaluronic acid confirmed that both processes – liver fibrosis and hepatocyte and other cell apoptosis – play a significant role in the progression of liver disease. The levels of cytokeratin-18 and of hyaluronic acid were significantly higher in the group of patients with HIV/HCV co-infection; this finding confirms that HCV presence impacts development and extent of fibrosis and apoptosis. Without a doubt the presence of HCV is the basis of the complex effect on the processes under investigation. In addition to pathogenetic effects of HIV/HCV, many other external and internal factors have a more or less prominent role in the disease progression. For example, major histocompatibility complex genes differ significantly between patients, as shown in many studies. Some patients may possess “protective gene alleles” while some possess genes that carry risk of faster disease progression.

While a direct effect of HCV on the level of hyaluronic acid is evident, the effect on the level of cytokeratin-18 is much less significant. That could be explained by different pathogenesis mechanisms in the case of HCV infection and nonalcoholic fatty liver disease with significant mitochondrial dysfunction. HIV/HCV co-infection impacts cytokeratin-18 indirectly through the level of hyaluronic acid.

The experience of clinicians at Riga East University Hospital Latvian Centre of Infectious Diseases over many years has shown that about one third of patients with chronic HCV infection have normal serum levels of ALT. The results disclosed in the present paper suggest that the marker of apoptosis CK-18, neoepitope, should be included in the HIV infection and HIV/HCV coinfection management algorithm. Circulation HA measurements have been proposed for operative monitoring of fibrotic lesion dynamics.

The extent of liver damage cannot be assessed only by ALT level measurements. Normal plasma ALT may be misleading because there could be intensive apoptosis processes ongoing.

An important limitation of the study was associated with the sample size. It allowed testing of a simple mediation model through the path analysis. At the same time, the size of subsamples limited an assessment of the model for HIV and HIV/HCV patient groups separately. Only in rare cases is it possible to determine the time and circumstances of HIV and/or HCV infection. Consequently, patients included in the study were already in different stages of disease progression and organ damage.

Future research in the field described should be aimed at a detailed study of cell injury at a molecular level. A more complex statistical model could be developed for representing the complexity of the processes of fibrosis and apoptosis and the relationships among their markers.

Conclusions

The progression of liver disease in patients with HIV/HCV co-infection is more pronounced comparing to those with HIV mono-infection. Besides many other parameters it is indicated by the higher plasma levels of hyaluronic acid, which is a relevant liver fibrosis marker, and hepatocyte apoptosis marker cytokeratin-18 in patients with HIV/HCV co-infection.

There is evidence for different mechanisms of impact of HIV/HCV co-infection on hyaluronic acid and CK-18. The presence of HCV impacts the level of HA directly, while the level of CK-18 is influenced through a change in the level of HA, so the stage of liver fibrosis is possibly an important factor in assessing the impact of HCV co-infection on liver apoptosis.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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