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LOXL-2 and TNC-C are markers of liver fibrogenesis in HCV/HIV-, HIV- and HCV-infected patients

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Background: Lysil oxidase like enzyme-2 (LOXL-2) and TNC-C play important roles in organ fibrosis. We assessed circulating LOXL-2 and TNC-C levels and their relationship to fibrosis severity in HIV- and/or HCV-infected individuals. **Methods:** Healthy controls (n = 22), HIV mono- (n = 15), HCV mono- (n = 52) and HCV/HIV-co-infected (n = 92) subjects were included. **Results:** LOXL-2 and TNC-C levels were significantly higher in HCV mono- and HCV/HIV-co-infected individuals with F0 compared to healthy controls. In addition, in HCV/HIV-co-infected individuals, LOXL-2 levels were higher in intermediate fibrosis compared to no/mild fibrosis. **Conclusion:** In HCV/HIV-co-infected study participants, both LOXL-2 and TNC-C were significantly higher in intermediate fibrosis compared to no/mild fibrosis, but did not further increase with advanced fibrosis. Furthermore, both markers were elevated among HCV/HIV-positive individuals with mild/no fibrosis.

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Chronic liver injury produces an imbalance between fibrogenesis and fibrolysis resulting in liver fibrosis (LF). Fibrosis begins as a homeostatic wound-healing response to injury but becomes deleterious after an accumulation of excess extracellular matrix over time [1]. Extracellular matrix deposition is a dynamic process, which may result in cirrhosis if the liver injury becomes persistent. However, it is still debatable if this dynamic process is reversible even if the underlying ongoing fibrosis reaches to the advanced fibrosis stage [2,3]. This process may be no more dynamic once the apoptotic cell death or apoptotic/necrotic cell death ratio in liver reaches plateau [5,6].

Lysil oxidase like enzyme-2 (LOXL-2), an extracellular matrix enzyme, plays an important role in inducing the fibrosis become more stable by binding collagen fibers to one another [4]. Thus, it could be further hypothesized that the more LOXL-2 is being produced in liver, the more irreversible liver fibrosis develops. Our recent study determined that macrophage activation marker, as a soluble marker of pro-inflammatory macrophages, also continued to increase until fibrosis stage 4 only (per Ishak) [7].

LOXL-2 is not expressed in healthy liver tissue, whereas *LOXL-2* gene expression is increased in fibrotic liver in the setting of etiologies ranging from Wilson's disease to chronic viral hepatitis [8]. Targeting the *LOXL-2* gene in an animal liver fibrosis model with CCL4 induction has demonstrated some promise [9,10]. On the other side, recent clinical trials targeting LOXL-2 with a specific LOXL-2 monoclonal antibody failed to show a promising result in terms of regression of liver fibrosis [11–13]. These unfortunate outcomes may be because of incompetent targeting of LOXL family. The clinical trials of interest focused on LOXL-2 only, instead of covering the other family members of LOXL [14]. Moreover, it is also still unknown that targeting LOXL-2 should be a part of treatment for mild/moderate liver or advanced fibrosis ones [15].

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TNC-C, another extracellular glycoprotein, is strongly expressed in fetal and tumor tissues as well as in fibrotic liver tissues [16]. Knocking out *TNC-C* in mice demonstrated the importance of TNC-C in liver fibrogenesis via promoting inflammation [17]. TNC-C values in serum are good indicators reflecting underlying cirrhosis among Hepatitis C virus (HCV)-induced subjects [18]. Its role in liver fibrogenesis is mostly related to polarization of macrophages to express a profibrogenic phenotype and stimulation of activated HSCs to secrete fibrogenic products including LOXL-2 [19].

HCV is one of the leading causes of cirrhosis and hepatocellular carcinoma, and HIV co-infection accelerates the progression of HCV-related LF [20,21]. HIV mono-infection may itself promote a fibrogenic environment in the liver. We and others have shown that HIV monoinfection can alter the liver milieu to a profibrogenic one, in addition to accelerating liver fibrosis in the setting of HCV infection [22–24].

As the role of LOXL-2 and TNC-C in HIV and/or HCV-induced liver fibrosis and the severity of fibrosis is not fully understood, we sought to assess the relationship between HIV, HCV and HIV/HCV infection and the severity of liver fibrosis and histologic activity index (HAI) on circulating LOXL-2 and TNC-C levels.

Materials & methods

In this cross-sectional analysis, we included 181 study participants, 22 of which were healthy controls. All healthy controls were HCV, hepatitis B surface antigen and HIV negative, and provided written informed consent (MGH protocol number 2013B000099). The healthy controls were enrolled from Massachusetts General Hospital (MA, USA); only serum samples were available for the study. Clinical data from the healthy controls were obtained from the electronic medical record.

All of the HCV monoinfected subjects (n = 52) were obtained from the Liver Disease Tissue Repository at Massachusetts General Hospital (MGH protocol number 1999P004983). Each of the subjects was negative for hepatitis B surface antigen, HIV, Wilson disease, hemochromatosis and autoimmune hepatitis. Each subjects underwent a liver biopsy and a serum sample was obtained before the study entry.

A subset of HCV/HIV-co-infected study participants (n = 72) came from the AIDS Clinical Trials Group (ACTG) A5071 study cohort [25]. Each of the participants had paired serum samples and undergone liver biopsy at baseline and at 6 months post peginterferon and ribavirin (PEG/RBV) therapy. Histologic evaluation of the liver biopsies was performed by a central hepatopathologist based on the Ishak scoring system. Liver fibrosis stages were stratified as no/mild (Ishak F0–1), intermediate (F2–3) and advanced (F4–6) fibrosis. Subjects positive for hepatitis B surface antigen and/or with and active infection which is associated with HIV, and/or who had significant cytopenia, kidney dysfunction, poorly controlled cardio-pulmonary disease, uncontrolled psychiatric problem were excluded from the study.

Twenty of the 92 HCV/HIV co-infected and 15 of the HIV monoinfected samples were obtained from an Observational Study of the Consequences of the HIV Protease Inhibitors Era at San Francisco General Hospital (SCOPE) [26]. These individuals were all naïve to antiviral therapy for both HCV and HIV. For this cohort study, each of the individuals were given ART for HIV, and follow up data and sera were obtained from participants at 24 weeks of ART. The subjects who were positive for hepatitis B surface antigen and/or with an active infection which is associated with HIV within the last 4 months (oral candidiasis acceptable), or with active treatment for a cancer or receiving interferon based or immunosuppressive medication were not included into the study.

All study participants in each of the study cohorts provided written informed consent, and each of the protocols was approved by the respective institutional review board. This retrospective study was also approved by the Massachusetts General Hospital ERB (Ethical Review Board).

LOXL-2 and TNC-C levels in serum were assessed using marketed sandwich ELISA kits (LifeSpan Biosciences and Immunobiological Laboratories, respectively). APRI and FIB-4 were calculated to reflect liver fibrosis noninvasively [27].

Statistics

Data among the groups were not distributed normally; therefore, they were presented as median with minimum and maximum values. SPSS version 17.0 was used for statistical analyses (SPSS Inc., IL, USA). Differences between the two groups were compared by using Mann–Whitney *U* or χ^2 test, whereas between the groups more than two were compared by Kruskall–Wallis test and χ^2 test; and for paired samples Wilcoxon test were used.

Table 1. Baseline characteristics of the subjects and healthy controls.

Parameters	Control (N: 22)	HIV alone† (N: 15)	HCV alone† (N: 52)	HCV/HIV with no treatment‡ (N: 20)	HCV/HIV under ART (N: 72)	p-value
Age, median, years old (min–max)	26.5 (22–62)	43 (23–53)	47 (19–77)	43 (28–53)	44 (27–65)	**0.003**
Sex, male, N (%)	8 (36.4)	12 (80)	39 (62.9)	39 (62.9)	55 (76.4)	**0.003**
ALT, median, IU/ml (min–max)	15.5 (7–32)	24 (11–105)	80 (19–573)	44 (12–287)	67 (23,5–346.5)	**<0.001**
HCV load, median, ($\times 10^{-6}$ IU/ml (min–max)	N/A	N/A	0.5 (0–1.5)	0.05 (0.01–0.4)	1.8 (0.06–0.7)	**<0.001**
HIV load, median, $\times 10^{-6}$ IU/ml (min–max)	N/A	99,683 (8723–402,295)	N/A	1,548,000 (195,618–7,692,310)	42.5 (6–29,646)	**<0.001**
CD4, median/mm^3 (min–max)	N/A	211 (10–661)	N/A	299.5 (16–606)	498.2 (131–1240)	**<0.001**
APRI, median (min–max)	0.24 (0.15–0.49)	0.43 (0.18–2.00)	0.88 (0.23–5.71)	0.69 (0.27–3.10)	0.75 (0.28–3.10)	**<0.001**
FIB-4, median (min–max)	0.76 (0.44–1.24)	0.97 (0.38–3.20)	1.52 (0.45–7.70)	1.41 (0.68–4.75)	1.35 (0.71–4.16)	**<0.001**
Mild/no fibrosis, N (%)	N/A	N/A	17 (32.6)	N/A	24 (33.3)	0.328
Intermediate, N (%)	N/A	N/A	19 (36.5)	N/A	25 (34.7)	
Advanced fibrosis, N (%)	N/A	N/A	16 (30.7)	N/A	23 (31.9)	
LOXL-2, median, pq/ml (min–max)	0.65 (0.14–1.31)	1.23 (0.80–1.62)	1.00 (0.44–2.16)	1.12 (0.52–1.49)	1.53 (0.73–3.42)	**<0.001**
TNC-C, median, pq/ml (min–max)	29.1 (9.53–63.8)	40.6 (14.4–91.9)	50.8 (18.4–250)	46.8 (18.0–109.5)	97.4 (26.9–197.0)	**<0.001**

p-values for variable values were performed by using Kruskal–Wallis non-parametric test; p-values for gender were by $\chi 2$ test.
†Treatment naïve HIV and HCV monoinfected subjects.
‡No specific viral treatment for HCV or HI.
ART: Anti-retroviral therapy; APRI: AST to platelet ratio index; FIB-4: Fibrosis-4; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; LOXL-2: Lysil oxidase like enzyme-2.

Table 2. The comparison of non-invasive serum markers between healthy individuals and the subjects with no fibrosis in liver.

Parameters	Healthy Controls (N: 22)	HCV and HCV/HIV-infected subjects with F0 (N: 8)	p-value
Age, median, years old (min–max)	26.5 (22–62)	39 (28–51)	0.113
ALT, median, IU/ml (min–max)	15.5 (7.0–32.0)	43 (22.0–152.0)	**0.001**
AST, median IU/ml (min–max)	20.5 (13.0–33.0)	34.0 (20.0–63.0)	**0.025**
APRI, median (min–max)	0.24 (0.15–0.49)	0.40 (0.23–0.64)	0.055
FIB-4, median (min–max)	0.77 (0.44–1.24)	0.83 (0.59–1.46)	0.364
LOXL-2, median, pq/mq (min–max)	0.71 (0.14–1.31)	1.12 (0.53–1.75)	**0.024**
TNC-C, median, pq/mq (min–max)	28.4 (9.53–63.8)	91.0 (50.8–250.0)	**<0.001**

p-values <0.05 accepted as statistical significance.
APRI: AST to Platelet ratio index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FIB-4: Fibrosis 4 score; PLT: Platelet count; Lysyl: Oxidase-like 2; TNC-C: Tenascin C

Binary logistic regression analysis was performed to predict histopathological advanced fibrosis among HCV mono- and HCV/HIV-co-infected subjects. The covariates were APRI, FIB-4 scores and LOXL-2 serum levels. p-values <0.05 were considered statistically significant.

Results

Baseline characteristics

We included 159 individuals (15 HIV mono-, 52 HCV mono- and 92 HCV/HIV co-infected) and 22 healthy controls in the study (Table 1). The highest levels of LOXL-2 were observed among HCV/HIV-co-infected ones. In contrast, TNC-C levels exhibited a statistically non-significant increasing trend from healthy controls toward HCV/HIV-co-infected individuals (p = 0.079).

The liver injury parameters including ALT, AST, APRI and FIB-4 scores were similar between HIV-monoinfected study participants and healthy controls (Table 1). However, there was a marked increase in serum levels of LOXL-2 and TNC-C not only in HIV-monoinfected individuals (p-values <0.001 and 0.039) (not shown in Table 1), but also in the HCV- and/or HIV-infected subjects with F0 fibrosis stage compared to healthy controls (p-values 0.024 and <0.001, respectively) (Table 2).

In addition, LOXL-2 and TNC-C were both found to be significantly higher in intermediate fibrotic individuals compared with no/mild fibrosis group among HCV/HIV-co-infected individuals (p-values 0.04 and 0.002,

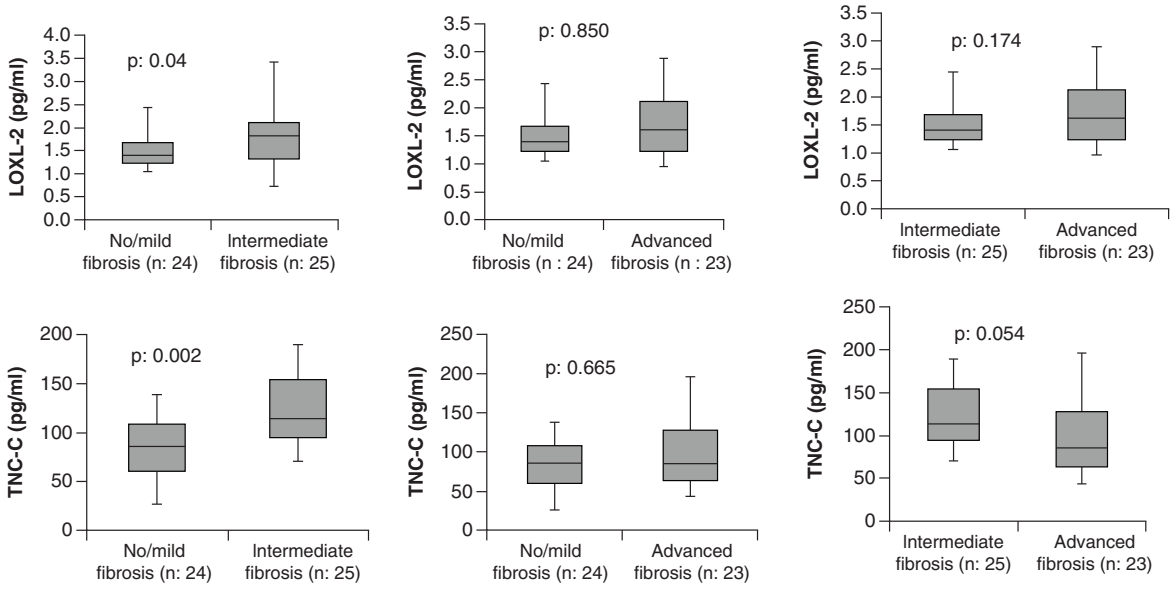

Figure 1. The changings in LOXL-2 and TNC-C levels in serum based on the severity of liver fibrosis among 72 HCV/HIV-infected subjects. Both LOXL-2 and TNC-C significantly increased in intermediate fibrosis group compared to no/mild fibrosis individuals. However, there was no other increase by worsening the underlying liver fibrosis. The values did not differ under same conditions in 52 HCV-monoinfected individuals (not-shown data). p-values given top of each separated figure represent the comparison of the regarding two groups base on Mann–Whitney *U* test. Top of the boxes indicates 75th percentile and bottom 25th percentile, and the line in the box represents median value. The lines originating from the box and reach top or bottom show the outliners.

respectively) (Figure 1). Moreover, even though the distribution of severity of fibrosis between HCV mono- (n = 52) and HCV/HIV-co-infected (n = 72) groups were similar (p = 0.128), overall LOXL-2 and TNC-C values were found to be higher in HCV/HIV-co-infected participants than that in HCV-monoinfected ones ($p < 0.001$ and <0.001, respectively) (Table 1).

As seen in Figure 2, effective ART had no effect on LOXL-2 or TNC-C levels; whereas LOXL-2 trended downward under PEG/RBV treatment, in contrast TNC-C trended upward. *Post-hoc* analysis revealed that LOXL-2 did not change in the subgroup of the subjects with no/mild fibrosis after PEG/RBV treatment (p: 0.310), even though the 47.8% of this group showed SVR with the treatment. On contrary, LOXL-2 levels decreased significantly among intermediate (with 34.8% SVR positivity) and advanced fibrotic individuals (with 17.4% SVR positivity) (0.009 and <0.001, respectively). In terms of TNC-C change, the only prominent increase with PEG/RBV treatment was apparent in the no/mild fibrosis group (p: 0.020). TNC-C levels remained stable in intermediate and advanced fibrotic groups (p-value: 0.655 and 0.290, respectively). FIB-4 values were similar in each group of SVR positive and negative before and after PEG/RBV treatment (p-value: 0.547 and 0.409, respectively); whereas APRI score showed a significant decline among the SVR-positive group (p-value: 0.023) and no change in SVR negative one (p-value: 0.596).

Discussion

LOXL-2 levels differ with severity of underlying liver fibrosis in chronically HBV monoinfected individuals [9]; however, studies of LOXL-2 in chronic HCV infection did not reveal major changes based on underlying liver fibrosis stage [28,29]. Similarly, TNC-C was determined not only to be increased in cirrhotic patients compared to healthy controls, but was also found to have a far higher serum level of LOXL-2 among cirrhotic individuals with an active HCV infection than serum levels of virological cured individuals [18].

LOXL-2 plays an important role in stabilizing fibrosis by binding the collagen fibers to one another; thus targeting LOXL-2 is promising way to slow down or even reverse the continued fibrosis [9]. However, it is still debatable, if LIXL-2 does play an important role in liver fibrogenesis, whether targeting LOXL-2 specifically could be an effective way to stop and/or reverse the underlying liver fibrosis. Our data could provide a new direction to

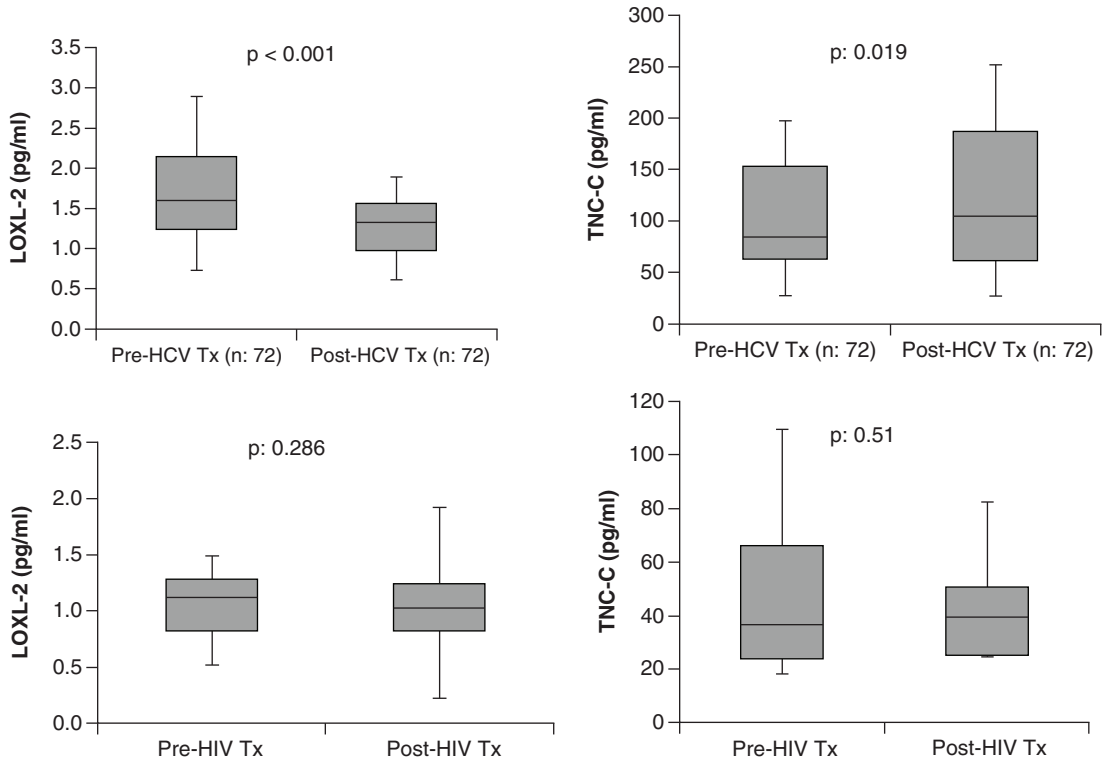

Figure 2. The impact of effective HCV treatment on co-infected individuals already on HIV medication and the impact of HIV therapy on HCV/HIV-co-infected subjects untreated for HCV. 62 HCV/HIV-co-infected individuals were already on HIV medication (ACTG cohort), 20 HCV/HIV-co-infected subjects remained untreated for HCV (SCOPE cohort). HCV treatment (PEG/RBV) decreased LOXL-2 levels at 6 months after stopping therapy. This decline was determined in both SVR and non-SVR groups accompanied with HCV viral load, APRI and FIB-4 values (not-shown data). On the other side, PEG/RBV medication increased TNC-C levels at the same time. HIV therapy had no impact on LOXL-2 and TNC-C at the 6 months of therapy in HCV/HIV-co-infected subjects. Similar finding was seen in 20 HIV monoinfected subjects as well (not-shown data). p-values given at the top of each separated figure represent the comparison of the two groups based on the Mann–Whitney *U* test. The top of the boxes indicates the 75th percentile and the bottom, the 25th percentile. The line in the box represents the median value. The vertical lines originating from the tops and bottoms of the boxes indicate the outliners.
Pre-HIV tx: Before ART initiation; Post-HIV tx: 6 months after ART initiation; Pre-HCV tx: Before PEG/RBV initiation; Post-HCV tx: 12 weeks after the completion of PEG/RBV treatment.

this controversial topic with its unique findings demonstrating that LOXL-2 may play an important role in the early settings of liver fibrosis.

In the current study, we found that in HCV mono- and HCV/HIV-co-infected individuals, LOXL-2 and TNC-C levels were significantly higher in study participants with no-to-mild fibrosis than in healthy individuals. In addition, even though the values continued to increase from no/mild fibrosis to intermediate fibrosis groups, no additional elevation was observed when it comes to transition to advanced fibrosis. These data collectively suggest that LOXL-2 and TNC-C could be markers of fibrogenesis rather than accumulated fibrosis. In the literature, there are unfortunate results in terms of targeting LOXL-2 in the setting of liver fibrosis [11–13]. Because of this unexpected outcome, the hope for clinical use of this promising modality is off the table for now. However, our current data demonstrated that both LOXL-2 and TNC-C are taking place in early and intermediate fibrosis stages in liver; and their role in advanced fibrosis may be more negligible. We have recently come to a similar conclusion with CD163, a pro-inflammatory macrophage activation marker, in the setting of liver fibrosis [7]. Therefore, it could be hypothesized that the rise in LOXL-2 and TNC-C levels are far more elevated in the early settings of liver fibrosis, even earlier than any pathological findings of liver fibrosis may appear; and then it continues to increase until advanced fibrosis becomes dominant.

Among HCV-monoinfected subjects, LOXL-2 showed a significant decline in people who were treated with direct acting antiviral agents [30]. In our study, we found that LOXL-2 levels decreased significantly overall in the

individuals treated with PEG/RBV for HCV in HCV/HIV co-infection, regardless of SVR status. However, the *post-hoc* analysis revealed that LOXL-2 levels among no/mild fibrosis group did not improve as expected, even in the SVR group. The main decline in LOXL-2 values was determined in intermediate and advanced groups. Unfortunately, we did not have the liver biopsy result after the PEG/RBV treatment in SVR positive and negative groups; thus, we cannot explain these findings based on histological data. On the other hand, a prominent decrease in HCV viral load was reported in both groups with the lack of a change of FIB-4 values. Therefore, only APRI score differed in the setting of SVR presence.

Interestingly, TNC-C levels in HCV/HIV-co-infected patients under PEG-RBV therapy increased at 6 months post therapy, irrespective of SVR. Considering the role of TNC-C in liver fibrogenesis, interferon may induce TNC-C on some level to lead macrophages. TNC-C favors the macrophage shifting into M1 phenotype through toll-like receptor 4 and by further suppressing M2 macrophage via IRF4 [19]. Interferon's immunomodulatory feature may be the reason of TNC-C increase, because the expected change in TNC-C values has declined even further with antiviral treatment, as shown recently [18].

The limitations of the current study are the following: first, all the subjects were obtained from different clinical trial repositories. Apparently, the inclusion and exclusion criteria of each clinical trial showed some variation, which may result in an unrecognized bias. As the LDTR repository, the details of the presence of non-alcoholic fatty liver disease or alcoholic hepatitis were not revealed in ACTG trial, even though each of the individuals underwent liver biopsy prior to study entry [25]. On the other hand, the subjects obtained from the SCOPE clinical trial did not undergo a liver biopsy and the information regarding non-alcoholic fatty liver disease and/or alcoholic liver disease were not given in the original manuscript [26]. The missing data could have led to various discussions based on our current results.

Conclusion

Our data suggest that both HCV and HIV contribute to LOXL-2 elevation, while HCV and, to a lesser extent, HIV are important drivers of TNC-C elevation. In HCV/HIV-co-infected subjects, both LOXL-2 and TNC-C may be markers of fibrogenesis rather than accumulated fibrosis.

Summary points

- Inflammation in the liver, which is initially protective, can eventually become harmful in the contribution to significant liver scarring.
- As the prior literature suggests, LOXL-2 may play a crucial role in ongoing liver fibrosis by binding collagen fibers to one another resulting in more stable liver fibrosis.
- We determined that LOXL-2 could be a marker of active fibrogenesis process rather than an accumulated fibrosis.
- Hepatitis C virus (HCV) and human immune deficiency virus (HIV) cooperatively induce liver fibrosis.
- Our data suggests that both HCV and HIV contribute to LOXL-2 elevation; however, targeting only HIV infection has no effect on LOXL-2 levels in serum in contrast to PEG/RBV treatment for chronic HCV infection.
- In the current study, we found that LOXL-2 and TNC levels in the serum could be helpful to define the stage of liver fibrosis among HCV with/without HIV-infected subjects.
- Monitoring LOXL-2 could be useful to follow the response of liver fibrosis to HCV treatment without the need for liver biopsy.
- In HCV/HIV-co-infected subjects, both LOXL-2 and TNC-C may be markers of active underlying fibrogenesis rather than an accumulated fibrosis.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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