

Correlations between plasma clusterin levels and liver fibrosis in people living with HIV

Nopparat Deesophon¹, Janya Khattiya², Warisara Sretapunya³, Chareeporn Akekawatchai^{4,5*}

¹Graduate Program in Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani Province, Thailand.

²Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Pathumthani Province, Thailand.

³Nakorn Nayok Hospital, Nakorn Nayok Province, Thailand.

⁴Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani Province, Thailand.

⁵Thammasat University Research Unit in Diagnostic Molecular Biology of Chronic Diseases related to Cancer (DMB-CDC), Pathumthani Province, Thailand.

ARTICLE INFO

Article history:

Received 2 March 2025

Accepted as revised 12 June 2025

Available online 20 June 2025

Keywords:

People living with HIV, PLWH, clusterin, liver fibrosis.

ABSTRACT

Background: Chronic liver disease has become a major health concern in people living with human immunodeficiency virus (PLWH). Clusterin has been involved in various pathologic conditions and its contributory role in liver complications in PLWH is still unclear.

Objective: This study aimed to detect plasma clusterin levels and assess their correlation with liver fibrosis in Thai PLWH.

Materials and methods: The study was conducted on 112 subjects, HIV-infected groups with (N=43) and without liver fibrosis (N=41), and uninfected controls (N=28). The subjects were evaluated for liver fibrosis using fibrosis-4 (FIB-4) score and aspartate aminotransferase to platelet ratio index (APRI), together with extracellular matrix fibrosis markers, laminin (LN), procollagen type III N-terminal peptide (PIIINP), hyaluronic acid (HA) and type IV collagen (IVC) and measured for plasma clusterin levels using an enzyme-linked immunosorbent assay.

Results: This study reported that medians of clusterin levels in uninfected controls, and the HIV patient groups without and with liver fibrosis were 1,779.38 (373.07-5,578.69), 1,602.73 (445.28-3,738.33) and 1,487.17 (0-3,111.33) µg/mL, respectively. Kruskal-Wallis test demonstrated a downward trend of clusterin levels in the HIV-infected groups without liver fibrosis and a significantly decreased level in the group with liver fibrosis ($p<0.05$), compared to those in uninfected controls. Pearson's correlation analysis indicated negative correlations of clusterin levels with fibrosis markers, FIB-4 score ($r=-0.224$, $p=0.018$), APRI ($r=-0.211$, $p=0.026$), LN ($r=-0.284$, $P=0.005$), HA ($r=-0.234$, $p=0.013$) and IVC ($r=-0.299$, $p=0.002$).

Conclusion: The study reported the lower levels of plasma clusterin in PLWH who develop liver fibrosis and their correlations with liver fibrosis assessed by the non-invasive markers.

Introduction

Human immunodeficiency virus (HIV) infection remains a major global health issue, with 39.9 million people living with HIV (PLWH) and 30.7 million people accessing anti-retroviral therapy at the end of 2023.¹ Effective ART has led to a decrease in acquired immunodeficiency syndrome (AIDS)-related mortality and morbidity. Life expectancy of PLWH has been closer to that of the general population. However, prolonged survival leads to increased evidence of non-AIDS-defining illnesses associated with age and premature ageing has been reported in PLWH.² Persistent viral replication in

* Corresponding contributor.

Author's Address: Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani Province, Thailand.

E-mail address: ejareepo@tu.ac.th

doi: 10.12982/JAMS.2025.083

E-ISSN: 2539-6056

PLWH causes chronic immune activation and inflammation leading to an elevation of proinflammatory cytokines and premature thymic atrophy.³ These increase the risk of developing non-AIDS-related comorbidities associated with immunosenescence in PLWH including chronic liver disease (CLD).^{3,4} Multiple risk factors mainly hepatitis B and C coinfection, anti-retroviral regimens, and HIV replication in the liver have been suggested to influence chronic liver damage and inflammation, potentially leading to CLD and hepatocellular carcinoma.^{4,5} Presently, molecular biomarkers associated with the progression of CLD in PLWH have been increasingly identified and require more investigation.²

Liver fibrosis is a common event caused by various etiologies, and its progression leads to advanced chronic liver disease, cirrhosis and eventually end-stage liver disease or hepatocellular carcinoma (HCC).^{6,7} Therefore, determination of the degree of liver fibrosis is crucial for evaluating liver disease severity and progression.^{6,7} Because the gold standard liver biopsy is an invasive procedure associated with the risk of complications, various non-invasive markers have been developed and suggested to facilitate clinical management of chronic liver diseases, including combinatorial serum marker, fibrosis-4 (FIB-4) score and aspartate aminotransferase (AST)-to-platelet ratio index (APRI), and direct liver fibrosis markers, hyaluronic acid (HA), type IV collagen (IVC), N-terminal polypeptide of type III procollagen (PIIINP) and laminin (LN).^{6,7} Particularly, the direct biomarkers, HA, IVC, PIIINP and LN, which reflect the deposition and removal of extracellular matrix (ECM) during the disease progression, have been evaluated in terms of their correlations with hepatic function indices in liver cirrhosis and hepatoma caused by different etiologies.^{7,8} The four direct biomarkers, together with tumor marker alpha fetoprotein (AFP) have also been utilized in predicting significant liver inflammation and fibrosis in chronic hepatitis B infection.^{9,10} As accumulating studies support the importance of chronic liver disease in PLWH, monitoring liver fibrosis in PLWH has also been suggested.^{4,5}

Clusterin (CLU) is synthesized as a highly glycosylated glycoprotein of 80 kDa, consisting of two polypeptide chains connected by disulfide bonds commonly found in the body fluid including serum and plasma. The secretory form of CLU, a heterodimeric complex of two 40-45 kDa subunits located in the extracellular space, exerts a chaperone-like activity by clearing cellular debris and misfolded stressed proteins.¹¹⁻¹³ Functionally, CLU is demonstrated to be cytoprotective and cytotoxic, resulting in conflicting consequences. Whereas cytoplasmic CLU expression was correlated with poor prognosis of

hepatocellular carcinoma (HCC),¹⁴ many studies supported the influence of CLU expression and function in the progression of infectious and non-infectious diseases.¹⁵⁻¹⁹ The expression was associated with good prognosis in pancreatic adenocarcinoma and no correlation with breast carcinoma.^{16,19} Moreover, the elevation of serum clusterin levels was reported in early rheumatoid arthritis,¹⁷ while markedly lower serum clusterin levels were observed in hepatitis B-mediated acute-on-chronic liver failure.¹⁸ Importantly, clusterin is considered one of the potential molecular markers of ageing with HIV² and a few studies suggest its association with disease progression and complications in PLWH.^{20,21} However, there is no direct evidence indicating the contribution of clusterin to chronic liver disease in PLWH. Therefore, this study aimed to examine the expression level of plasma clusterin and its correlation with liver fibrosis, assessed noninvasively by fibrotic markers, in PLWH. The study provides more understanding of disease progression mechanisms and potential biomarkers for liver complications in PLWH.

Materials and methods

Study population

A total of 84 patients infected with HIV attending the Antiretroviral Therapy Clinic in Nakorn Nayok Hospital, Thailand, from September 2017 to October 2020 (aged 20-80 years, 56.0% male and 44.0% female) were recruited into the present study. The inclusion criteria were as follows: 1) the patients aged 18 years or older, 2) the patients with documented HIV infection, and 3) the patients with available blood samples and clinical data. The exclusion criteria were 1) the patients consuming alcohol, herbal medicine and steroid drugs, and 2) the patients having opportunistic infections. The patients were divided into 41 patients without liver fibrosis (36.6%) and 43 patients with significant liver fibrosis (38.4%). Twenty-eight uninfected controls, aged 25-72 years, 57.1% male and 42.9% female, were recruited from the Healthcare Clinic at Police General Hospital, Bangkok, Thailand, in November 2022. Inclusion criteria for controls were individuals aged >18 years with available blood samples and clinical data, and seronegative for HIV, hepatitis B and C virus infection. Individuals with significant liver fibrosis, assessed by fibrosis-4 (FIB-4) score and aspartate aminotransferase (AST)-to-platelet ratio index (APRI) were excluded.²²⁻²⁴ The study protocol was reviewed and approved by the Human Ethics Committees No.3, Thammasat University, Pathumthani Province, Thailand (Approve No. 121/2565) and the Certificated Biological Safety Committee of Thammasat University, Pathumthani, Thailand (Approve No. 079/2565). Figure 1 showed the recruitment process of participants in this study.

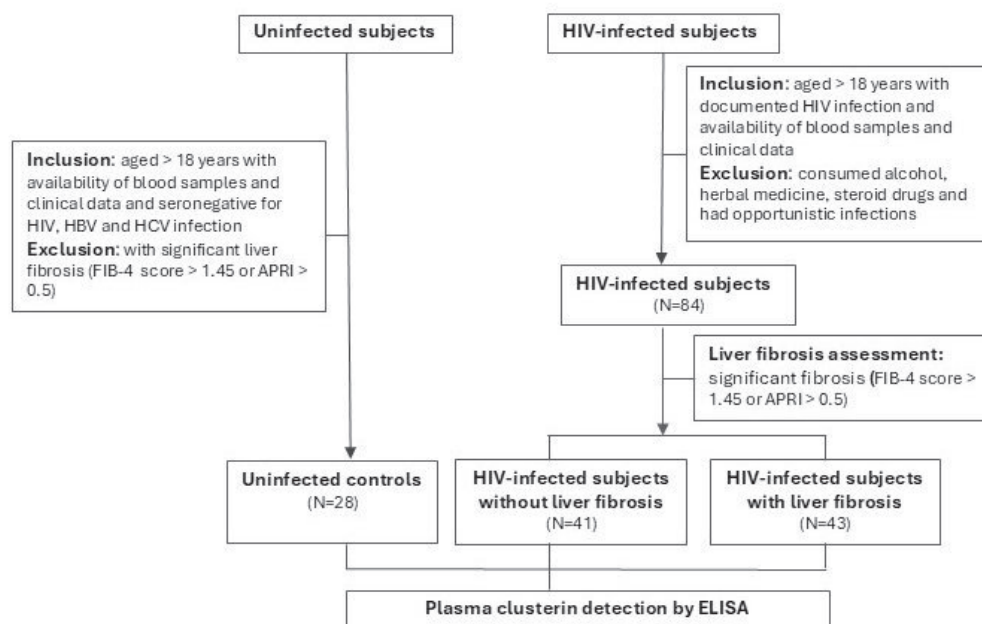


Figure 1. The recruitment process for participants in this study was based on a non-invasive assessment of liver fibrosis. FIB-4: fibrosis-4 score, APRI: AST to platelet ratio index, ELISA: enzyme-linked immunosorbent assay.

Clinical data and laboratory investigation

Clinical and laboratory data were obtained as described in the previous studies.²⁵⁻²⁷ Ethylene-diamine-tetra-acetic acid (EDTA) blood samples remaining after routine testing were subjected to plasma separation within 8 hours after blood collection and stored at -80°C until use. In this study, liver fibrosis in the subjects was determined by FIB-4 score, classified into class 1 (≤ 1.45), class 2 (1.46-3.25) and class 3 (> 3.25) and by APRI, classified into class 1 (≤ 0.5), class 2 (0.51-1.5) and class 3 (> 1.5). A significant liver fibrosis was defined as FIB-4 score > 1.45 or APRI > 0.5 .²²⁻²⁴ FIB-4 score was calculated using the formula: FIB-4 score = age [years] x aspartate aminotransferase (AST) level [U/L]/platelet count [$10^9/\text{L}$] x alanine aminotransferase (ALT)^{1/2} [U/L].²² APRI was calculated by the formula: APRI = (AST level [U/L]/upper limit of normal AST) x 100/platelet count [$10^9/\text{L}$].²⁴ In addition, levels of extracellular matrix (ECM) fibrotic markers, laminin (LN), procollagen type III N-terminal peptide (PIIINP), hyaluronic acid (HA) and type IV collagen (IVC), in addition to tumor marker α -fetoprotein (AFP) in plasma samples were measured using electrochemiluminescence immunoassay (Mindray CL-900i and LN, PIIINP, HA, IVC and AFP test kits, all Mindray Medical International Co., Ltd.)

Detection of clusterin levels by sandwich enzyme-linked immunosorbent assay

Clusterin levels in plasma samples were measured using a commercial ELISA kit (Human clusterin DuoSet ELISA kit, R&D Systems) according to the manufacturer's instructions. Clusterin concentrations were reported in $\mu\text{g}/\text{mL}$. The intra- and inter-assay coefficients of variability were less than 10% (4.85-9.22) and 15% (11.86), respectively.

Data analysis

Descriptive statistics, median and percentage were used to describe the characteristics of the study population. Chi-square and Fisher's exact tests analyzed differences of categorical variables between the study groups, whereas those with continuous variables were tested using one-way ANOVA, Mann-Whitney U, Kruskal-Wallis tests and Dunn's pos hoc tests. The $p < 0.05$ was considered to indicate a statistically significant difference. The PASW Statistic 18 software (SPSS Inc.) and GraphPad Prism 9.1.1 (Dotmatics) were used for statistical analysis.

Results

Characteristics of the study population.

Table 1 demonstrated the clinical characteristics of the three study groups, HIV-infected subjects with (N=43) and without liver fibrosis (N=41) and uninfected controls (N=28). The data indicated no difference in the sex distribution but significant age differences between the study groups. Patients without liver fibrosis had lower median ages than those with liver fibrosis and uninfected controls ($p=0.004$). Median levels of non-invasive fibrotic markers, FIB-4 score, APRI, LN, PIIINP, HA and IVC, and tumor marker AFP significantly differed among the three groups (all $p < 0.05$). Notably, analysis of the HIV-infected groups indicated that there is no difference between the distribution of patients with HBV and HCV coinfection in the HIV-infected groups with and without liver fibrosis. However, the data indicated significant differences in CD4⁺ cell count, ARV regimen and duration of ART between the two HIV-infected groups (Table 1).

Table 1. Demographic data and clinical characteristics of uninfected controls and PLWH with or without liver fibrosis recruited in this study.

Characteristics (N=112)	Uninfected control group (N=28)	HIV patient group without liver fibrosis (N=41)	HIV patient group with liver fibrosis (N=43)	p value
Subjects	28 (25.0)	41 (36.6)	43 (38.4)	
Age (years)^b	49.9±15.6)	41.2±9.2)	49.8±11.9)	0.004*
Gender^a				
Male	16 (57.1)	19 (46.3)	28 (65.1)	
Female	12 (42.9)	22 (53.7)	15 (34.9)	0.221
Liver fibrosis markers^c				
FIB-4 score	0.86 (0.28-1.40)	0.77 (0.35-1.45)	2.84 (0.80-52.62)	<0.001*
APRI	0.18 (0.12-0.32)	0.22 (0.12-0.46)	0.88 (0.22-36.44)	<0.001*
Liver fibrosis and tumor markers (ng/mL)^c				
Laminin (N=99)	32.20 (10.06-61.51)	35.24 (20.11-53.05)	41.73 (24.66-96.32)	0.004*
Procollagen III N-terminal peptides (N=112)	10.07 (4.85-85.13)	7.49 (4.12-23.95)	11.66 (4.33-118.30)	0.001*
Hyaluronic acid (N=112)	51.10 (14.01-455.90)	23.44 (8.89-491.42)	76.18 (7.09-2549.84)	<0.001*
Type IV collagen (N=108)	13.87 (2.30-75.24)	38.75 (23.38-123.83)	48.82 (19.26-458.64)	<0.001*
Alpha-fetoprotein (N=112)	2.54 (1.06-5.92)	1.85 (0.95-5.78)	2.38 (0.91-74.54)	0.041*
Clusterin levels (µg/mL)	1,779.38 (373.07-5,578.69)	1,602.73 (445.28-3,738.33)	1,487.17 (0-3,111.33)	0.038*
HBV and HCV coinfection^b (N, %)				
HIV monoinfection	ND	29 (82.9)	24 (70.6)	
HIV/HBV coinfection	ND	3 (8.6)	6 (17.6)	
HIV/HCV coinfection	ND	3 (8.6)	4 (11.8)	0.422
CD4⁺ cell count (cells/µL)^d (N=81)	ND	487 (4-873)	281 (2-1,282)	0.303
CD4⁺ cell count (cells/µL)^b (N=81)				
<350	ND	12 (30.0)	22 (53.7)	
≥350	ND	28 (70.0)	19 (46.3)	0.031*
Duration of ARV treatment^b (N=83)	ND	82.00 (40.50-123.50)	27.00 (0.00-97.00)	0.087
Duration of ARV treatment^a (N=83)				
Naive to ARV treatment	ND	2 (4.9)	9 (21.4)	
≤6 month	ND	3 (7.3)	5 (11.9)	
>6 months	ND	36 (87.8)	28 (66.7)	0.045*
ART^a (N=83)				
Naive to ART	ND	2 (4.9)	9 (21.4)	
ART	ND	39 (95.1)	33 (78.6)	0.026*
ARV regimens^a (N=72)				
Lamivudine/zidovudine/nevirapine	ND	10 (25.6)	3 (9.1)	
Tenofovir, efavirenz, lamivudine or emtricitabine	ND	20 (51.3)	14 (42.4)	
Others	ND	9 (23.1)	16 (48.5)	0.042*

Note: ^anumber (%), ^bmean±SD, ^cmedian (range). Some variables had missing data, and N is given in parentheses, *p<0.05, ART: antiretroviral therapy, ARV: antiretroviral, AST: aspartate aminotransferase, ALT: alanine aminotransferase, FIB-4: fibrosis-4 score, APRI: AST to platelet ratio index.

Plasma levels of FIB-4, APRI, ECM fibrotic markers, LN, PIIINP, HA and IVC, and tumor marker AFP were analyzed (Figure 1). Figure 1A-C indicated that levels of FIB-4 score, APRI and LN in the HIV-infected group without liver fibrosis were like those in the control group (Figure 1A-C). However, the levels in the infected group with liver fibrosis were significantly higher than those in the others. Figure 1D indicated that the levels of IVC were also significantly higher in the two HIV-infected groups, compared to the control group. However, the IVC levels

in the infected groups with and without liver fibrosis were similar. In Figure 1E, levels of HA were significantly lower in HIV-infected patients without liver fibrosis than in the control groups, whereas the levels in the infected patients with liver fibrosis were significantly higher than those without liver fibrosis. PIIINP levels in the three study groups were like those observed with HA levels (Figure 2F). Additionally, there is a significant difference only between AFP levels in HIV-infected patients without liver fibrosis and uninfected controls (Figure 2G).

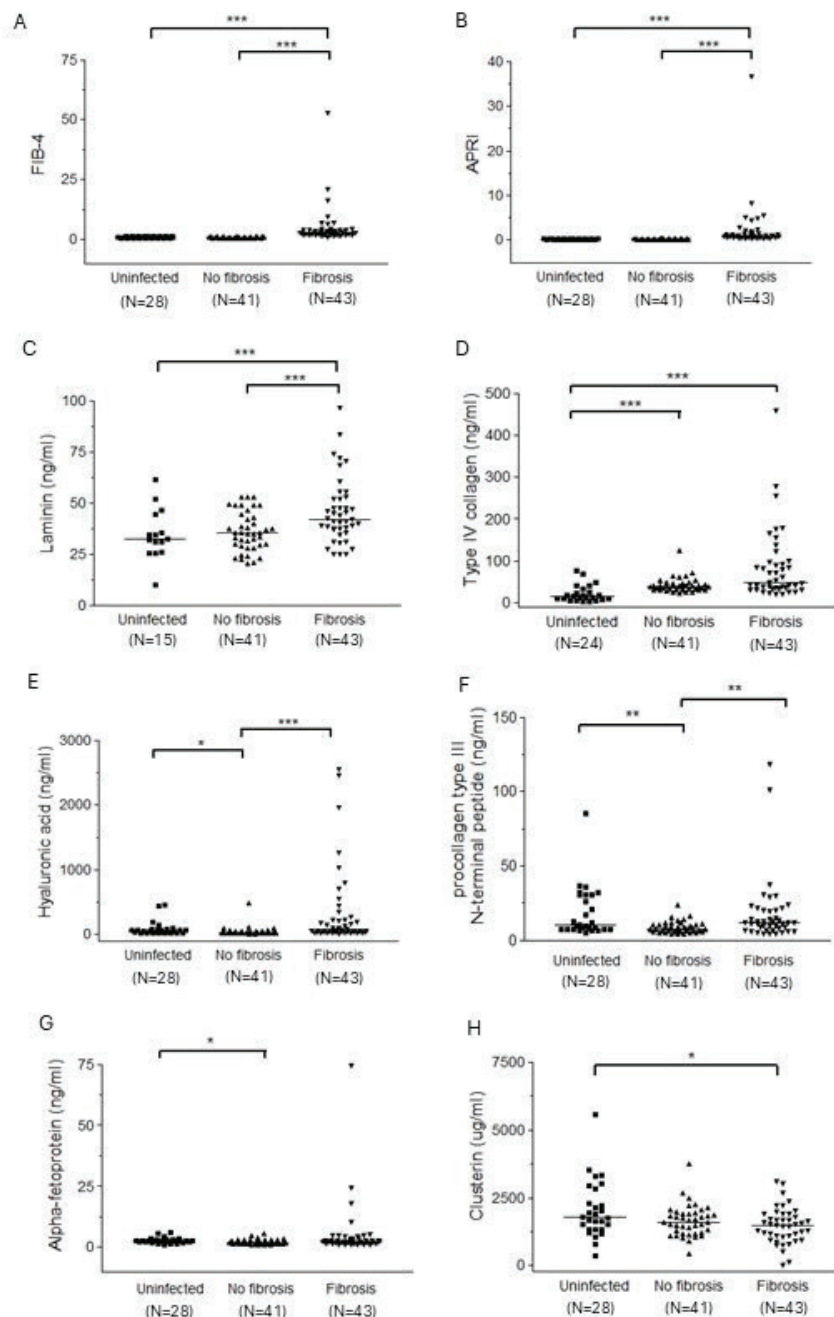


Figure 2. Plasma levels of FIB-4 (A), APRI (B), and fibrosis markers, laminin (C), type IV collagen (D) hyaluronic acid (E), procollagen type III N-terminal peptide (F), alpha-fetoprotein (G) and clusterin (H) in HIV-infected patients with and without liver fibrosis compared to those in uninfected control groups. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$, and ns, $p > 0.05$, FIB-4: fibrosis-4 score, APRI: AST to platelet ratio index

Plasma clusterin levels in the uninfected controls, HIV-infected patient groups with and without liver fibrosis

Clusterin levels were detected in plasma samples from three study groups. Median levels in the uninfected control, HIV-infected groups with and without liver fibrosis were 1,779.38 $\mu\text{g/mL}$ (373.07-5,578.69), 1,487.17 $\mu\text{g/mL}$ (0-3,111.33) and 1,602.73 $\mu\text{g/mL}$ (445.28-3,738.33), respectively. Statistical analysis indicated significant differences in clusterin levels between the three groups (Table 1). Figure 1H also showed a decreasing trend of clusterin levels in the HIV-infected groups, compared to uninfected controls. Importantly, a significant decrease in

clusterin levels was observed in the infected group having liver fibrosis compared to the uninfected control group. In addition, Pearson's correlation analysis in the study groups, uninfected and HIV-infected patients, indicated reverse correlations of clusterin levels with AST, FIB-4 score, APRI, LN, HA and IVC ($p=0.021$, $p=0.018$, $p=0.026$, $p=0.005$, $p=0.013$ and $p=0.002$, respectively) (Figure 3) and no correlation with ALT, PIIINP and AFP (Table 2). Interestingly, the analysis in the HIV-infected groups demonstrated no correlation between the clusterin levels and CD4^+ cell counts (Table 2).

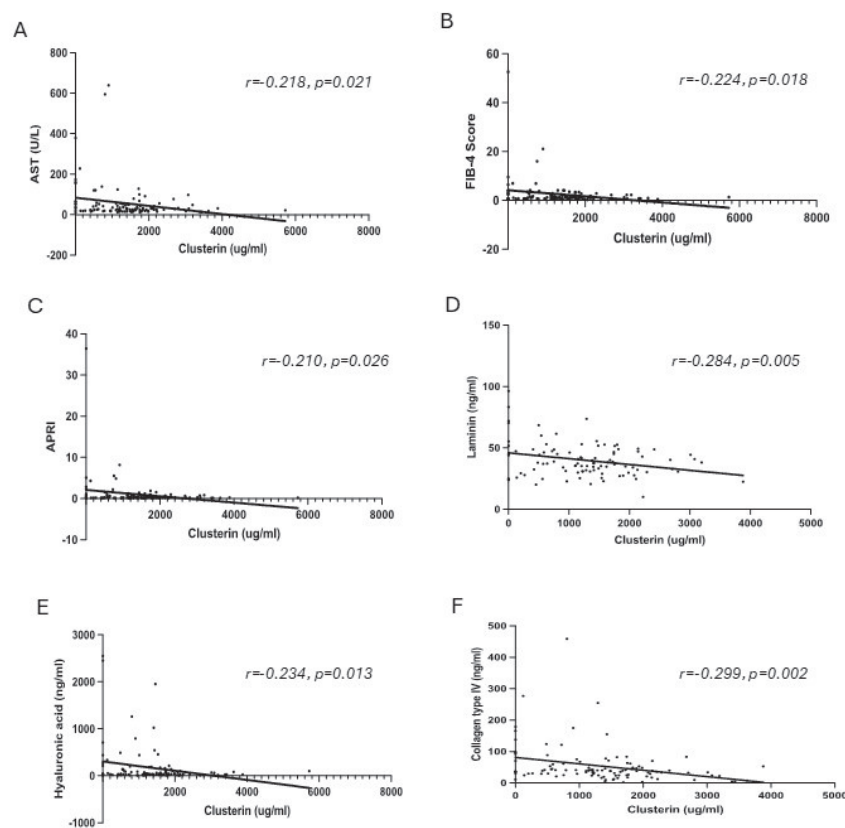


Figure 3. Pearson correlation analysis of plasma clusterin levels with abnormal liver parameters in the uninfected and HIV-infected patients. The correlations of clusterin levels with AST (A), FIB-4 (B), APRI (C), laminin (D), hyaluronic acid (E) and type IV collagen (F) were demonstrated ($p<0.05$). AST: aspartate aminotransferase, FIB-4: fibrosis-4 score, APRI: AST to platelet ratio index

Table 2. Pearson correlation of plasma clusterin levels with abnormal liver parameters and immune status in the uninfected and HIV-infected patients.

Abnormal liver parameters	Correlation coefficient (<i>r</i>)	<i>p</i> value
Aspartate aminotransferase	- 0.218	0.021*
Alanine aminotransferase	- 0.118	0.241
Fibrosis-4 score	- 0.224	0.018*
Aspartate aminotransferase to platelet ratio index	- 0.211	0.026*
Laminin	- 0.284	0.005*
Procollagen III (N-terminal peptides)	- 0.132	0.165
Hyaluronic acid	- 0.234	0.013*
Collagen type IV	- 0.299	0.002*
Alpha-fetoprotein	- 0.127	0.082
CD4 ⁺ cell count (N=81)	0.146	0.192

Note: *data shown as $p < 0.05$.

Discussion

Accumulating studies indicate an increased burden of chronic liver disease in PLWH,^{4,5} and monitoring for liver fibrosis in the PLWH has been suggested. Our previous studies have demonstrated the high prevalence of liver abnormalities and their risk factors in Thai PLWH.²⁵⁻²⁷ In this study, the two HIV-infected and uninfected control groups were recruited and classified according to significant liver fibrosis evaluated using the non-invasive FIB-4 score (>1.45) and APRI (>0.5). The classification was supported by the additional analysis of median FIB-4, APRI and the established ECM fibrotic markers, LN, HA, IVC, and PIIINP, in the three study groups. Our data indicated the elevation of LN and IVC levels in the HIV-infected group with liver fibrosis compared to those without fibrosis and the controls, supporting the assessment of significant liver fibrosis using FIB-4 and APRI. Although there is no evidence showing the evaluation of the ECM biomarkers in HIV-mediated liver fibrosis, these biomarkers have been demonstrated previously to predict significant liver inflammation and fibrosis in chronic hepatitis B.^{9,10} In this study, plasma clusterin levels and their correlation with significant liver fibrosis, as assessed by non-invasive fibrosis markers, have been reported in PLWH.

Hepatocyte apoptosis during liver injury is an initial event in all liver disease etiologies including chronic manageable HIV infection with ART.^{28,29} HIV itself and the adverse effects of ART appear to be a major cause of hepatic injury, liver inflammation and fibrosis.²⁹ Clusterin is known to exert extracellular chaperone function by clearing cell debris and abnormal proteins.¹¹⁻¹³ It also protects cells from apoptosis induced by many stressors.^{30,31} As hepatocytes are the major source of circulating clusterin,³² hepatocyte damage may directly cause the reduction of plasma clusterin levels observed in the HIV-infected groups. In this study, the clusterin level was significantly reduced in the HIV-infected group with liver fibrosis, and negatively correlated with the well-established liver fibrosis markers, FIB-4 and APRI, and ECM markers, LN, HA, IVC, and PIIINP. This finding was consistent with the previous study indicating the lower clusterin levels as a

predictor of the severity and prognosis of hepatitis B virus-related acute on chronic liver failure.¹⁸ However, a few studies indicate the upregulation of clusterin in the brains of HIV-mediated acquired immunodeficiency syndrome and the increased serum concentrations of clusterin in HIV positive pregnancies.^{20,21} This inconsistency may be due to specific mechanisms of complications in HIV disease. Notably, our analysis suggested no significant correlation of clusterin levels with the immune status of these HIV-infected patients assessed by CD4⁺ cell counts, possibly indicating no involvement of circulating clusterin in the severity of underlying HIV disease in this study group. The findings generated from this study suggest the potential contribution of clusterin in the progression of liver inflammation and fibrosis in PLWHIV.

Limitations

This study had limitations due to the characteristics of the study groups. Firstly, the study groups have differences in age, CD4⁺ cell count, ART, ARV regimens and ART duration. Our previous study in the HIV-infected groups suggested that age and CD4⁺ cell count, but not ART, were predictive factors for liver fibrosis.²⁷ Therefore, these risk factors may partially influence the impact of clusterin on CLD progression in the PLWH. Secondly, there was no data on the status and treatment of HBV and HCV infection, especially HBV and HCV viral loads in these study groups. Although there was no significant difference between the distribution of HBV and/or HCV coinfection in PLWH with and without liver fibrosis in this study, the effect of viral hepatitis on liver fibrosis in this study group could not be ruled out. Thirdly, the stability of plasma analytes during storage is a concern in bioanalytical methods. In this study, to avoid the effect of freeze/thaw cycles and temperatures, the uninfected- and HIV-infected plasma samples were aliquoted and stored at -80 °C before examination at the same time. Even though the stability in the samples with long-term storage, which may affect the clusterin levels reported in this study, has not been clear, the previous study reported that an ELISA could detect variants of clusterin in the serum samples stored at

-80°C up to 24 months.³³ Lastly, the study was conducted in a relatively small number of subjects, which may limit statistical significance for variables tested, and further studies in a larger HIV-infected group are warranted.

Conclusion

The present study demonstrated plasma clusterin levels in PLWH who developed liver fibrosis and their correlations with non-invasive markers, FIB-4 and APRI, and ECM markers, LN, HA, IVC, and PIIINP, suggesting the contributory role of clusterin in the progression of liver fibrosis. This finding may provide more understanding in the development of liver inflammation and fibrosis in PLWH.

Conflict of interest

The authors declare no conflict of interest.

Funding

The study was supported by the Thailand Science Research and Innovation Fundamental Fund fiscal year 2023 (Project no.2493097) and Thammasat University Research Unit in Diagnostic Molecular Biology of Chronic Diseases related to Cancer (DMB-CDC).

Ethical approval

The study protocol has been approved by the human Ethics Committees No. 3, Thammasat University, Thailand (Project no. 121/2565).

Credit authorship contribution statement

Chareeporn Akeawatchai contributed to funding acquisition resources, supervision, study design, manuscript preparation, review and editing. Nopparat Deesophon performed data collection and analysis, and manuscript preparation. Janya khattiya contributed to data collection and analysis. Warisara Sretapunya participated in collecting blood samples and clinical data. All authors read and approved the final version of the manuscript.

Acknowledgements

Special thanks to all patients and staff in Nakorn Nayok Hospital for their contribution to this study.

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