Comorbidity and ageing in HIV infection
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Liver fibrosis in HIV-infected individuals on long-term antiretroviral therapy: associated with immune activation, immunodeficiency and prior use of didanosine

Katherine W. Kooij, Ferdinand W.N.M. Wit, Rosan A. van Zoest, Judith Schouten, Neeltje Kootstra, Michèle van Vugt, Maria Prins, Peter Reiss, Marc van der Valk, on behalf of the AGE\textsubscript{i}IV Cohort Study Group

ABSTRACT

Background
It is unclear whether HIV infection is associated with liver fibrosis in the absence of chronic hepatitis B or C virus (HBV/HCV) coinfection. We compared prevalence of liver fibrosis, noninvasively assessed by the Fibrosis-4 (FIB-4) index, between HIV-infected patients and uninfected controls and explored determinants of a higher FIB-4 score, indicative of more liver fibrosis.

Methods
FIB-4 was assessed in HIV-uninfected and HIV-1-infected, predominantly virologically suppressed participants of the AGEuiV Cohort Study without HBV and/or HCV coinfection, and aged at least 45. Using multivariable regression, we investigated associations between FIB-4 and HIV-status, HIV-disease characteristics, antiretroviral drugs and markers of microbial translocation and immune activation.

Results
Prevalence of advanced liver fibrosis (FIB-4 ≥3.25) was low: 1.4% in HIV-infected and 1.0% in HIV-uninfected participants. After adjustment for age, sex, ethnicity, detectable anti-hepatitis B core/anti-HCV antibodies and excessive alcohol intake, HIV remained significantly associated with higher FIB-4 (+4.2%, \( P = 0.05 \)). Prior exposure to didanosine, longer duration of a CD4\(^+\) cell count below 500 cells/\( \mu l \) and a lower CD4\(^+\) cell count at enrolment were each associated with a higher FIB-4. Markers of immune activation (soluble CD163, activated CD8\(^+\) T-lymphocytes and regulatory T-lymphocytes) were associated with a higher FIB-4 in HIV-infected but not HIV-uninfected study participants.

Conclusion
HIV infection was independently associated with higher FIB-4 scores, indicating more advanced liver fibrosis, though the difference in FIB-4 scores between HIV-infected and HIV-uninfected was small. Higher levels of immune activation were associated with liver fibrosis in HIV-infected, even in the absence of HBV or HCV infection, but not in HIV-uninfected individuals.
INTRODUCTION

Liver disease causes a significant proportion of mortality in the HIV-infected population in the modern combination antiretroviral therapy (cART) era [1]. A large proportion of liver disease is related to chronic coinfection with hepatitis B or C virus (HBV and HCV), but other factors such as excessive alcohol intake and hepatotoxicity of (old) antiretroviral regimens, associated with mitochondrial toxicity and metabolic complications, may also contribute to the pathogenesis of liver disease [2–4]. Particularly in HIV-HCV coinfected patients, HIV-associated gut microbial translocation and resulting immune activation are thought to accelerate liver fibrosis, possibly mediated by Kupffer cells [5–7]. To what extent these processes are involved in the development of liver fibrosis in HIV infection without hepatitis coinfection is less clear [8,9].

In recent years, several non-invasive markers of liver fibrosis have been developed, facilitating disease staging without the need for liver biopsy. The Fibrosis-4 (FIB-4) index predicted significant and advanced liver fibrosis (Metavir score ≥F2 and ≥F3, respectively) in an HIV-HCV coinfected population [10]. Subsequently, the FIB-4 index has been validated in several cohorts, including patients with HIV-HCV coinfection, HCV monoinfection and nonviral hepatitis related liver disease [11–14]. Furthermore, liver fibrosis defined by FIB-4 (FIB-4 ≥3.25) has prognostic value, comparable to that of liver biopsy, for liver-related and all-cause mortality in HIV-HCV coinfected and in HCV monoinfected patients [15–17]. Several cross-sectional studies reported a higher FIB-4 in HIV-infected patients without chronic HCV coinfection, for a large part not receiving cART, compared to HIV-uninfected controls [18,19]. A higher HIV-1 RNA viral load and a lower CD4+ T-lymphocyte count (CD4+ cell count) have both been associated with a higher FIB-4 in HIV-infected individuals without chronic HBV or HCV infection [18–21].

We cross-sectionally investigated the severity of liver fibrosis, as reflected by a higher FIB-4 index, in HIV-uninfected and HIV-1-infected, virologically suppressed participants of the AGEnIv Cohort Study without HBV and/or HCV coinfection, all aged 45 years and older. We investigated whether HIV infection was independently associated with higher FIB-4 and explored HIV-related and ART-related possible determinants of FIB-4. Additionally, we explored the potential relationships of FIB-4 with markers of microbial translocation and immune activation.
METHODS

AGEsIV Cohort Study population
Five hundred ninety-eight HIV-1-infected individuals, aged 45 years and older, were recruited from the HIV outpatient clinic of the Academic Medical Center (AMC) of the University of Amsterdam, The Netherlands. Five hundred fifty HIV-uninfected individuals, aged 45 years and older, were recruited from the sexual health clinic and the Amsterdam Cohort Studies on HIV/AIDS at the Public Health Service of Amsterdam, as a control group from the same geographical region and with similar sociodemographic and behavioural (risk) factors. Participants undergo a biennial standardized screening for age-associated comorbidities and organ dysfunction, including physical examination, laboratory testing and an extensive questionnaire. Details concerning study procedures have previously been published [22]. Detailed information concerning HIV infection and ART history was obtained from the Dutch HIV Monitoring Foundation registry [23].

Written informed consent was obtained from all participants; the study was approved by the ethical review board of the AMC in Amsterdam and registered at ClinicalTrials.gov (identifier NCT01466582).

For this analysis participants with complete data to calculate a FIB-4 score at enrolment were selected. HIV-infected and HIV-uninfected individuals with detectable hepatitis B surface antigen (HBsAg) and/or HCV RNA, evidence of active HBV and/or HCV infection, were excluded from this analysis. HIV-infected individuals with a documented history of detectable HBsAg and/or detectable HCV RNA for at least 6 months, suggestive of a history of chronic HBV or HCV infection, were also excluded. Participants with detectable anti-hepatitis B core antibodies (anti-HBc) and no documented history of detectable HBsAg, as well as those with detectable anti-HCV antibodies and no recorded history of detectable HCV-RNA at least 6 months were assumed to have resolved HBV and/or HCV infection in the acute stage and were retained in the analysis. This was also applicable for HIV-uninfected participants, for whom no historic HBV / HCV serology was available.

Blood samples for peripheral blood mononuclear cell (PBMC) isolation were collected at enrolment from the first 100 HIV-uninfected and 100 HIV-infected participants with a plasma HIV-1 RNA viral load less than 40 copies/mL in the year prior to enrolment.

Clinical variables and definitions
Aspartate aminotransferase (AST) in U/L, alanine aminotransferase (ALT) in U/L and platelet count in $10^9$/L were determined centrally at the AMC; FIB-4 was calculated using the following formula:
Advanced fibrosis (Metavir ≥F3) was defined as a FIB-4 of at least 3.25, as previously described [10]. We defined a FIB-4 of at least 1.45 to less than 3.25 as suggestive of significant fibrosis (Metavir ≥F2), which it predicts fairly accurate in the context of HBV and HCV monoinfection [24,25].

Excessive alcohol intake was defined as self-reported daily or almost daily intake of at least three (females) or five (males) alcoholic consumptions in the past 6 months.

Soluble (s)CD163 and sCD14 levels were determined in stored plasma samples (-80°C) ELISA (Human CD14 DuoSet and Human CD163 DuoSet, R&D systems, Minneapolis, Minnesota, USA) according to the manufacturer’s instruction. The concentrations of sCD163 and sCD14 in plasma were quantified using the standard curve.

PBMCs were isolated from blood using Ficoll-Isopaque density gradient centrifugation. PBMC obtained from fresh blood samples were stained with monoclonal antibodies for 30 minutes at 4°C in the dark, to determine expression levels of different cell surface molecules. The following directly conjugated monoclonal antibodies were used for cell surface marker staining: CD4 PE-Cy7, HLA-DR Fitol, CD38 PE, CD25 APC, CD3 V500, CD8 Pacific Blue (BD Biosciences, Franklin Lakes, New Jersey, USA), CD127 APCFluor780 (eBioscience, San Diego, California, USA). Fluorescence was measured with the fluorescence-activated cell sorting (FACS) Canto II (BD Biosciences). The proportion of cells expressing each marker were determined using FlowJo (TreeStar, Ashland, Oregon, USA).

**Statistical analysis**

Statistical analysis was performed using Stata software (version 12; StataCorp LP, College Station, Texas, USA). Participants’ characteristics were summarized as median and interquartile range (IQR) or frequency and percentage; study groups were compared using Chi-squared, Student’s t test and rank sum test, as appropriate. Correlations were calculated using Spearman’s correlation coefficient (p). P values less than 0.05 were considered statistically significant, all tests were two-sided.

We performed a multivariable linear regression analysis with logarithmically transformed FIB-4 as a continuous outcome. Obtained coefficients were exponentiated to obtain the difference in FIB-4 on a multiplicative scale, which can be interpreted as the percentage difference in FIB-4. All multivariable models were adjusted for the following possible confounders: age, sex, ethnicity, detectable anti-HBc or anti-HCV antibodies, and excessive alcohol intake.
First, we investigated whether HIV was independently associated with FIB-4, adjusting for the abovementioned possible confounders. Subsequently, diabetes mellitus, BMI, waist-circumference and hip-circumference and the waist-to-hip ratio were explored as potentially lying on the causal pathway by analysing whether their addition to the multivariable model attenuated the coefficient of HIV-status by at least 10%.

Associations between HIV-related and ART-related potential determinants of FIB-4 were explored in a multivariable model including only HIV-infected participants.

Furthermore, the possible role of microbial translocation in the pathogenesis of liver fibrosis was investigated by exploring the associations between sCD14 and sCD163, markers of microbial translocation and immune activation, and FIB-4 in the multivariable regression model.

Finally, associations between activated CD8⁺ and CD4⁺ T-lymphocytes (CD38⁺HLA-DR⁺) and regulatory T-lymphocytes (CD25⁺CD127⁺), levels of which have been shown to increase as a result of microbial translocation [26,27], and FIB-4 were explored in the subset of HIV-uninfected and virologically suppressed HIV-infected participants for whom PBMC were available.

Statistical interactions between HIV status and covariates of interest were explored.

As a sensitivity analysis, multivariable regression was repeated excluding all individuals with detectable anti-HBc and/or anti-HCV antibodies.

We used multiple imputation to handle missing values of covariates, generating 10 imputed datasets.

RESULTS

Characteristics of study participants

FIB-4 scores and data on HCV and HBV (co)infection were available for 598 HIV-infected and 507 HIV-uninfected individuals. 80 HIV-infected and 8 HIV-uninfected individuals were excluded from the analysis because of current or historic chronic HCV (34 and 5, respectively) or HBV infection (46 and 3, respectively).

Overall, 86.4% of participants included in the analysis were male, of whom 83.4% were men who have sex with men. The median age was 52.6 years (IQR 48.2–58.9). HIV-infected
participants were more often of African descent than HIV-uninfected participants (14.5% vs. 6.4%, \( P < 0.001 \)). Excessive alcohol consumption was uncommon in both HIV-infected (4.7%) and HIV-uninfected (6.5%) participants. HIV-infected participants more often had detectable anti-HCV (3.3% vs. 0.6%, \( P = 0.002 \)) and anti-HBc antibodies (53.5% vs. 25.1%, \( P < 0.001 \)) (Table 1).

In HIV-infected participants, the median time since HIV-1 diagnosis was 11.6 years. 32.2% had a history of AIDS. 18.7% had been pre-treated with nucleoside reverse transcriptase inhibitor (NRTI) mono and/or dual therapy prior to start of cART. 94.8% were on cART at enrolment, 11.8% had an HIV-1 viral load above 200 copies/mL in the year prior to enrolment (Table 2).

Levels of sCD14 and sCD163 were higher in the HIV-infected participants. FACS data were available for 80 HIV-infected, all of whom on cART and all but one with HIV-1 RNA viral load below 200 copies/mL in the year prior to enrolment, and 85 HIV-uninfected participants without HBV/HCV coinfection. The median age of this subgroup was slightly higher (56.0 years, IQR 49.1–61.9) than that of the whole group; the HIV-infected subgroup had similar nadir and current CD4 counts compared to the whole HIV-infected group. Levels of activated CD8+ and CD4+ T-lymphocytes and regulatory T-lymphocytes were significantly higher in HIV-infected compared with HIV-uninfected participants (Table 1).

**Higher Fibrosis-4 in HIV-infected**

The prevalence of a FIB-4 score at least 3.25, suggestive of advanced liver fibrosis, was low in both HIV-infected [1.4%, 95% confidence interval (CI) 0.4 to 2.3%] and HIV-uninfected participants (1.0%, 95% CI 0.1 to 1.9%); the prevalence of a FIB-4 score more than 1.45 and less than 3.25, an indication for the presence of significant fibrosis was 38.2% (95% CI 34.0 to 42.4%) in HIV-infected and 29.5% (95% CI 25.5 to 33.5%) in HIV-uninfected (\( P = 0.01 \)) (Table 1).

Unadjusted, HIV infection was associated with a higher FIB-4 (+6.6%, 95% CI +1.9 to +11%, \( P = 0.005 \)). After adjustment for possible confounders (age, sex, ethnicity, detectable anti-HBc/anti-HCV antibodies, and excessive alcohol intake), being HIV-positive remained (borderline) significantly associated with a higher FIB-4 (+4.2%, 95% CI -0.05 to +8.7%, \( P = 0.05 \)). Additional adjustment for BMI, waist-to-hip ratio and diabetes mellitus did not attenuate the association between HIV and FIB-4 further (+4.9%, 95% CI +0.3 to +9.8%, \( P = 0.04 \)). We observed no significant interactions between HIV-status and the investigated covariates.
Table 1  Characteristics of individuals included in analysis

<table>
<thead>
<tr>
<th></th>
<th>HIV-infected participants</th>
<th>HIV-uninfected participants</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (% or median (IQR))</td>
<td>N (% or median (IQR))</td>
<td></td>
</tr>
<tr>
<td><strong>Demographic characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sex</td>
<td>457 (88.2%)</td>
<td>422 (84.6%)</td>
<td>0.09a</td>
</tr>
<tr>
<td>MSM^d</td>
<td>351 (73.6%)</td>
<td>330 (69.0%)</td>
<td>0.12a</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.8 (48.3 – 59.5)</td>
<td>52.4 (48.1 – 58.1)</td>
<td>0.30c</td>
</tr>
<tr>
<td>African descent^e</td>
<td>75 (14.5%)</td>
<td>32 (6.4%)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>Excessive alcohol intake^f</td>
<td>22 (4.7%)</td>
<td>31 (6.5%)</td>
<td>0.22a</td>
</tr>
<tr>
<td><strong>Hepatitis B and C coinfection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>detectable anti-HCV, undetectable HCV RNA</td>
<td>17 (3.3%)</td>
<td>3 (0.6%)</td>
<td>0.002a</td>
</tr>
<tr>
<td>detectable anti-HBc, undetectable HBsAg</td>
<td>277 (53.5%)</td>
<td>125 (25.5%)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td><strong>Metabolic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m^2</td>
<td>24.4 (22.4 – 26.8)</td>
<td>24.5 (22.9 – 27.1)</td>
<td>0.05c</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.97 (0.92 – 1.01)</td>
<td>0.91 (0.87 – 0.96)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Diabetes mellitus^g</td>
<td>30 (6.6%)</td>
<td>19 (4.0%)</td>
<td>0.09a</td>
</tr>
<tr>
<td><strong>Markers of immune activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soluble (s)CD14, ng/mL</td>
<td>1577 (1290 – 2012)</td>
<td>1338 (1071 – 1711)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>sCD163, ng/mL</td>
<td>282 (204 – 404)</td>
<td>242 (177 – 331)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>Activated CD8^+ T lymphocytes, %^h</td>
<td>5.9 (3.2 – 8.9)</td>
<td>4.3 (2.7 – 5.8)</td>
<td>0.01c</td>
</tr>
<tr>
<td>Activated CD4^+ T lymphocytes, %^h</td>
<td>1.5 (1.0 – 2.2)</td>
<td>1.0 (0.7 – 1.5)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>Regulatory T lymphocytes, %^h</td>
<td>7.9 (6.3 – 10.4)</td>
<td>6.1 (4.1 – 7.8)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td><strong>Markers of liver function/damage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>47 (45 – 49)</td>
<td>47 (45 – 49)</td>
<td>0.22c</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>29 (22 – 40)</td>
<td>26 (21 – 34)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>ALT ≥40 U/L</td>
<td>130 (25.1%)</td>
<td>76 (15.2%)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>28 (24 – 33)</td>
<td>26 (22 – 30)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>AST ≥40 U/L</td>
<td>50 (9.7%)</td>
<td>28 (5.6%)</td>
<td>0.02a</td>
</tr>
<tr>
<td>Platelets, 10^9 cells/μl</td>
<td>210 (184 – 247)</td>
<td>217 (185 – 255)</td>
<td>0.08c</td>
</tr>
<tr>
<td>FIB-4, median (IQR)</td>
<td>1.31 (1.05 – 1.67)</td>
<td>1.22 (0.99 – 1.55)</td>
<td>0.004c</td>
</tr>
<tr>
<td>&lt;1.45</td>
<td>313 (60.4%)</td>
<td>347 (69.5%)</td>
<td>0.01 a</td>
</tr>
<tr>
<td>1.45 – 3.25</td>
<td>198 (38.2%)</td>
<td>147 (29.5%)</td>
<td></td>
</tr>
<tr>
<td>≥3.25</td>
<td>7 (1.4%)</td>
<td>5 (1.0%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALT, alanine aminotransferase; anti-HBc, anti-hepatitis B core antibodies; AST, aspartate aminotransferase; FIB-4, Fibrosis-4; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; IQR, interquartile range; MSM, men who have sex with men.

a Chi-squared test.
b Student’s t-test.
c Rank sum test.
d Questionnaire derived data, available for 470 HIV-infected and 473 HIV-uninfected individuals.
e Birth country of an individual and at least 1 parent is Suriname(Creole)/Netherlands Antilles/sub-Saharan Africa or birth country of an individual/both parents is Suriname(Creole)/Netherlands Antilles/sub-Saharan Africa and AGE-reader measurement is not available because of low reflection as a result of a dark skin.
f Daily or almost daily intake of at least 5 (males) or at least 3 (females) alcoholic consumptions.
g HbA1c (IFCC) at least 48 mmol/mol and/or blood glucose (non-fasting) at least 11.1 mmol/L and/or blood glucose (fasting) at least 7.0 mmol/L, and/or in those taking antidiabetic medication.
h Data available for 85 HIV-infected and 80 HIV-uninfected individuals.
**Table 2** HIV-related and antiretroviral therapy-related characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-infected participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years since HIV-1 diagnosis</td>
<td>11.6 (6.1 – 16.9)</td>
</tr>
<tr>
<td>CD4⁺ cell count in year prior to enrolment, cells/μL</td>
<td>570 (433 – 745)</td>
</tr>
<tr>
<td>Nadir CD4⁺ cell count, cells/μL</td>
<td>180 (70 – 260)</td>
</tr>
<tr>
<td>Cumulative duration of CD4⁺ cell count &lt;500 cells/μL, years</td>
<td>4.0 (1.8 – 7.8)</td>
</tr>
<tr>
<td>History of AIDS</td>
<td>166 (32.2%)</td>
</tr>
<tr>
<td>HIV-1 load &gt;200 copies/mL in year before enrolment</td>
<td>61 (11.8%)</td>
</tr>
<tr>
<td>Cumulative exposure to ART, years</td>
<td>9.2 (3.7 – 14.0)</td>
</tr>
<tr>
<td>Exposed to NRTI mono/dual therapy prior to start of cART</td>
<td>97 (18.7%)</td>
</tr>
<tr>
<td>Using cART at enrolment</td>
<td>490 (94.8%)</td>
</tr>
<tr>
<td>Ever exposed to didanosine</td>
<td>143 (27.7%)</td>
</tr>
<tr>
<td>Cumulative duration of exposure to didanosine (of those ever exposed to</td>
<td>2.9 (0.8 – 7.3)</td>
</tr>
<tr>
<td>didanosine), years</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: (c)ART, (combination) antiretroviral therapy; IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitors.

**Immunodeficiency independently associated with higher Fibrosis-4**

Including only HIV-infected participants in the multivariable model, adjusting for age, sex, race, detectable anti-HBc/anti-HCV antibodies and excessive alcohol intake, associations between FIB-4 and HIV disease-related and ART-related characteristics were explored. A lower mean CD4⁺ cell count in the year prior to enrolment was independently associated with a higher FIB-4 (Fig. 1, Table 3). The duration of immunodeficiency, defined using CD4⁺ cell count cut-offs (below 200, 350 or 500 cells/μL) was also associated with a higher FIB-4. The duration of immunodeficiency, defined as a CD4⁺ cell count below 500 cells/μL was most strongly and independently associated with a higher FIB-4. Other markers related to HIV disease progression (years since HIV-1 diagnosis, nadir CD4⁺ cell count, prior AIDS, HIV-1 viral load above 200 copies/mL in the year prior to enrolment) were not independently associated with FIB-4. The duration of exposure to didanosine was independently associated with a higher FIB-4, but not the duration of exposure to any other antiretroviral, nor having been pre-treated with mono and/or dual NRTI before start of cART (Table 3).
Figure 1  Relation between log Fibrosis-4 and CD4+ cell count in the year prior to enrolment, HIV-infected participants only.

Table 3  HIV-related and antiretroviral therapy-related determinants of Fibrosis-4, HIV-infected participants only.

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Percentage difference in FIB-4 (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of exposure to a CD4+ cell count below 500 cells/μl (per year)</td>
<td>+0.7% (+0.02 to +1.5%)</td>
<td>0.05</td>
</tr>
<tr>
<td>CD4+ cell count in year prior to enrolment (per 100 cells/μl lower)</td>
<td>+1.8% (+0.6% to 3.1%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Duration of exposure to didanosine (per year)</td>
<td>+1.3% (+0.2% to +2.4%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; FIB-4, Fibrosis-4. Adjusted for age, sex, ethnicity, excessive alcohol use, detectable anti-HBc/anti-HCV antibodies.

Markers of microbial translocation

Adjusted for age, sex, race, detectable anti-HBc/anti-HCV antibodies, and excessive alcohol intake, a higher level of sCD163 was borderline significantly associated with a higher FIB-4 (+1.1% per 100 ng/mL, 95% CI -0.2% to +2.4%, P = 0.10), and this slightly attenuated the association between HIV-status and FIB-4 (adjusted coefficient: +3.8%, 95% CI -0.5% to +8.2%, P = 0.08). We found an interaction between the level of sCD163 and HIV-infected status (Pinteraction = 0.05); sCD163 was significantly associated with FIB-4 in HIV-infected (+2.2% per 100 ng/mL, 95% CI +0.5% to +3.9%, P = 0.01) but not in HIV-uninfected participants (-0.3% per 100 ng/mL, 95% CI -2.3% to +1.6%, P = 0.72). Soluble CD14 was not associated with the FIB-4 in either group. Introducing sCD163 into the model including only HIV-infected individuals (Table 3), did not attenuate the coefficients of the CD4+ cell count prior to enrolment, the duration of exposure to a CD4+ cell count less than 500 cells/μl or the duration of exposure to didanosine.
Activated and regulatory T lymphocytes

Within the 80 HIV-infected and 85 HIV-uninfected participants with available FACS data, activated T lymphocytes were significantly correlated with sCD163 (activated CD8+ T lymphocytes: Spearman’s ρ=0.21, P = 0.01, activated CD4+ T lymphocytes: ρ=0.27, P < 0.001) and with sCD14 (activated CD8+ T lymphocytes: ρ=0.14, P = 0.07, activated CD4+ T lymphocytes: ρ=0.21, P = 0.01), and regulatory T lymphocytes with sCD14 (P = 0.29, P < 0.001) but not sCD163 (p=0.07, P = 0.40). Each of these markers was individually included in the multivariable model, adjusting for sex, ethnicity, detectable anti-HBc/anti-HCV antibodies, and excessive alcohol intake. Activated CD4+ T lymphocytes were not significantly associated with FIB-4; activated CD8+ T lymphocytes (P_{interaction} = 0.02) and regulatory T lymphocytes (P_{interaction} = 0.06) were each associated with FIB-4 in the HIV-infected group only. Models were repeated, including HIV-infected individuals only; coefficients are shown in Table 4, first column. Within this subgroup of HIV-infected individuals, a lower CD4+ cell count in the year prior to enrolment was associated with a higher FIB-4, although the duration of exposure to a CD4+ cell count less than 500 cells/μl and the duration of exposure to didanosine were not (Table 4, first column). The covariates that were individually associated with FIB-4, were included in the combined model shown in Table 4, second column; activated CD8+ T lymphocytes were borderline significantly (P = 0.06) and regulatory T lymphocytes significantly (P = 0.006) associated with a higher FIB-4, whereas the association between the CD4+ cell count prior to enrolment was attenuated and no longer statistically significant.

Table 4 Markers of immune activation and Fibrosis-4 in a subset of HIV-infected participants (n = 80).

<table>
<thead>
<tr>
<th>Individually included in multivariable model</th>
<th>Combined multivariable model</th>
<th>P value</th>
<th>Percentage difference in FIB-4 (95% CI)</th>
<th>P value</th>
<th>Percentage difference in FIB-4 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD14 (per 100 ng/mL)</td>
<td>+0.7% (-0.2% to +1.6%)</td>
<td>0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sCD163 (per 100 ng/mL)</td>
<td>+2.2% (-2.0% to +6.6%)</td>
<td>0.31</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Activated CD8+ T lymphocytes (per percentage point)</td>
<td>+2.4% (+0.5% to +4.4%)</td>
<td>0.02</td>
<td>+1.9% (-0.02% to +3.9%)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Activated CD4+ T lymphocytes (per percentage point)</td>
<td>+5.4% (-2.1% to +13.5%)</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Regulatory T cells (per percentage point)</td>
<td>+4.3% (+1.7% to +7.0%)</td>
<td>0.002</td>
<td>+3.9% (+1.2% to +6.6%)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>CD4+ cell count in year prior to enrolment (per 100 cells/μl lower)</td>
<td>+4.4% (+1.3% to +7.6%)</td>
<td>0.007</td>
<td>+0.8% (-2.6% to +4.4%)</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Duration of exposure to a CD4+ cell count below 500 cells/μl (per year)</td>
<td>+1.5% (-0.1% to +3.1%)</td>
<td>0.08</td>
<td>+1.5% (+0.2% to +3.1%)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Duration of exposure to didanosine (per year)</td>
<td>+0.1% (-2.1% to +2.4%)</td>
<td>0.93</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; FIB-4, Fibrosis-4. * Adjusted for age, sex, ethnicity, excessive alcohol use, detectable anti-HBc/anti-HCV antibodies.
Sensitivity analyses
Multivariable models were repeated, including only participants without detectable anti-HBc and anti-HCV antibodies (233 HIV-infected and 374 HIV-uninfected). HIV-status remained associated with FIB-4 after adjusting for age, sex, ethnicity and excessive alcohol intake (+6.4%, 95% CI +1.1% to +12%, $P = 0.02$). Within the HIV-infected participants the association between sCD163 and FIB-4 was similar in magnitude though no longer statistically significant (+1.9%, 95% CI -0.9% to +4.9%, $P = 0.19$).

DISCUSSION

Summary of main results
In this cross-sectional study we observed higher FIB-4 scores, indicative of more advanced liver fibrosis, in HIV-infected individuals without active or historic chronic HBV or HCV coinfection compared with HIV-uninfected controls. Being HIV-infected was independently associated with a higher FIB-4, though the difference was small and FIB-4 values were mostly in the significant fibrosis range (FIB-4 1.45–3.25) rather than in the advanced fibrosis range (FIB-4 ≥3.25). Within the HIV-infected cohort, past exposure to didanosine, as well as current or historic low CD4$^+$ cell count were independently associated with a higher FIB-4. Our findings may suggest a role for microbial translocation and immune activation in the pathophysiology of liver fibrosis in HIV-infected individuals without chronic viral hepatitis coinfection. This is reflected by the observation that the level of monocyte activation marker sCD163 was independently associated with a higher FIB-4, as were higher percentages of regulatory T lymphocytes and activated CD8$^+$ T lymphocytes in a subgroup of virologically suppressed individuals.

Interpretation of results
The HIV-infected population which we studied was to a large extent on cART, enabling us to provide insight in liver fibrosis, specifically in well treated HIV-infected patients. The prevalence of advanced fibrosis, indicated by a FIB-4 score of at least 3.25, in HIV-infected individuals was 1.4%. This is comparable with previous studies assessing FIB-4 in HIV-infected populations, reporting prevalence rates of advanced fibrosis ranging from 0 to 4.3% [18–21]. However, a direct comparison of FIB-4 between studies is complicated because age is part of the FIB-4 calculation, resulting in lower values in younger populations. Compared with previous studies, we report a smaller difference in the prevalence of advanced fibrosis (1.4 vs. 1.0%) between HIV-infected and HIV-uninfected individuals. Blackard et al. reported 1.3 vs. 0.5% advanced fibrosis in HIV-infected vs. HIV-uninfected women, while Lim et al. found 4.3 vs. 1.8% advanced fibrosis in HIV-infected vs. HIV-uninfected individuals [18,19]. The majority of HIV-infected participants included
in these studies, in contrast to our study participants, however, had a detectable viral load, and relatively low CD4+ cell counts. Both these factors have been associated with higher FIB-4 values and may contribute to liver fibrosis and thus may explain the larger difference between HIV-infected and HIV-uninfected individuals in these earlier studies [18,20,21].

To improve our understanding of the possible pathogenic mechanisms underlying liver fibrosis in the context of HIV, we explored associations between several markers of immune activation and liver fibrosis. HIV infection, even when adequately suppressed, is associated with increased levels of microbial translocation, reflected by higher levels of monocyte activation markers sCD14 and sCD163, activated CD4+ and CD8+ T cells as well as regulatory T cells [26–28]. Several studies have reported associations between microbial translocation-related immune activation, possibly involving Kupffer cells, and accelerated liver fibrosis in HIV-HCV coinfection, and suggested that microbial translocation may be an important mechanism through which HIV accelerates liver fibrosis [5–7,9]. Thus far, few data are available on the contribution of microbial translocation to liver fibrosis in HIV monoinfection. A relatively small study found sCD163 to be associated with non-invasive markers of liver fibrosis in HIV-HCV coinfected patients, but did not find a statistically significant association in HIV-infected patients without chronic viral hepatitis coinfection [9]. In our larger cohort, we do show that sCD163 is associated with higher levels of liver fibrosis in HIV-infected individuals without chronic HBV or HCV coinfection. Furthermore, higher percentages of regulatory T cells and activated CD8+ T cells were associated with higher FIB-4 in HIV-infected individuals. These findings suggest that, like in HIV-HCV coinfection, microbial translocation-related immune activation may be involved in the development of liver fibrosis in HIV in the absence of chronic viral hepatitis coinfection. This may particularly be a relevant mechanism in severely immunodeficient individuals. We show that the duration and severity of immunodeficiency is associated with higher FIB-4 levels in HIV-infected individuals without chronic viral hepatitis coinfection, thereby confirming previous studies [18–21]. Our observation that the percentage of regulatory T cells in the model attenuates the association between CD4+ cell count and FIB-4, supports our hypothesis that immune activation may be one of the mechanisms underlying immunodeficiency related liver fibrosis.

Markers of immune activation were not associated with higher FIB-4 in HIV-uninfected controls. This might be a consequence of different mechanisms being involved in liver fibrosis in patients without HIV, HCV and HBV infection. We did not find an association between sCD14 and FIB-4. This is consistent with some studies including HIV-HCV coinfected patients [7,9,29], though other studies examining the association between sCD14 and liver fibrosis, mostly performed within HCV or HBV (co)infected patients,
have produced conflicting results [8,30,31]. Soluble CD163 may be a better marker of macrophage activation related to liver fibrosis than sCD14 [9].

Prior use of didanosine was associated with more liver fibrosis, suggesting a lasting effect of didanosine exposure. These findings confirm earlier studies reporting an association between didanosine exposure and liver fibrosis [4,32,33]. A potential mechanism for didanosine-associated liver fibrosis is mitochondrial toxicity, leading to hepatic steatosis [34,35]. Additionally, didanosine exposure has been associated with persistently increased levels of transaminases, or cryptogenic liver disease, possibly also an expression of liver fibrosis [36,37] and with nodular regenerative hyperplasia [38].

Limitations
Having a FIB-4 score above 3.25 is strongly correlated with liver fibrosis on liver biopsy and has been associated with adverse outcomes in several studies [10,15,16,39]. The proportion of individuals with a FIB-4 score of at least 3.25 in this study was low, as a consequence statistical power was insufficient to conduct a multivariable logistic regression analysis with FIB-4 at least 3.25 as the outcome. FIB-4 as a continuous variable has not been validated, nor has the FIB-4 score been validated in HIV-monoinfected patients. Furthermore, the clinical relevance of the small differences in FIB-4, as observed in the current study, is unknown. In this observational, cross-sectional study we merely are able to report associations and can only speculate on causative mechanisms. The observed associations between markers of immune activation and FIB-4, in particular, should be cautiously interpreted. Advanced liver fibrosis, particularly liver cirrhosis with portal hypertension, may be the cause rather than the consequence of increased microbial translocation and subsequent immune activation [40]. However, given the overall very low prevalence of cirrhosis, reflected by the small proportion of participants with a FIB-4 of at least 3.25, this seems an unlikely explanation for our findings. Conclusions regarding the impact of specific antiretroviral drugs should also be made with caution, considering the non-random allocation of these drugs in the population under study. Liver fibrosis resulting from chronic HBV or HCV infection may in part be irreversible. Therefore, in addition to excluding participants with active HBV or HCV infection at enrolment, we excluded those with a documented history of chronic HCV or HBV from the analysis.

Conclusions
In this cross-sectional comparative analysis of suppressed HIV-infected individuals and HIV-uninfected controls of similar age and background, advanced liver fibrosis was uncommon. HIV infection was independently associated with higher FIB-4 scores, indicating more advanced liver fibrosis, though observed levels of fibrosis were largely
within the moderate fibrosis range and differences in FIB-4 scores between HIV-infected and HIV-uninfected were small. These results may be considered reassuring. Exposure to harmful antiretroviral drugs, particularly didanosine, appears to have a lasting negative effect on liver fibrosis in those with HIV. This may be particularly clinically relevant for resource-limited settings, in which didanosine may still be prescribed. Previous studies in HIV-HCV coinfectected individuals have shown that HIV may aggravate HCV-associated liver fibrosis through microbial translocation and immune activation, possibly involving Kupffer cells. Our findings are suggestive of a similar process in HIV-infected patients without HBV or HCV coinfection; higher levels of immune activation were associated with higher FIB-4 scores in these individuals. Follow-up will show whether individuals with higher levels of immune activation may be more prone to develop clinically relevant liver fibrosis, even in the absence of HCV or HBV coinfection.

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PRESENDED IN PART

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AUTHORS’ CONTRIBUTIONS

PR conceived the study and together with FW, JS, MP and MV contributed to study design. KK, JS and RZ contributed to study coordination and data collection. KK and FW conducted the statistical analysis. KK, FW, NK, RZ, MP, PR, and MV contributed to data interpretation. KK drafted the manuscript. All authors critically reviewed and revised the manuscript, and approved the final version submitted for publication.

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