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NON-INVASIVE ASSESSMENT OF HEPATIC STEATOSIS AND FIBROSIS IN INDIVIDUALS LIVING WITH HIV

PhD thesis

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The List of Abbreviations

AIC: Aikake Information Criterion

ALD: alcoholic liver disease

anti-HBc: antibody against hepatitis B core antigen

APC: alcohol per capita consumption

ART: antiretroviral therapy

ARV: antiretroviral

BIC: Schwarz Bayesian Information Criterion

BMA: Bayesian Model Averaging

BMI: body mass index

CAP: controlled attenuation parameter

ART: antiretroviral therapy

cART: combined antiretroviral therapy

CI: confidence interval

CVD: cardiovascular

DM: diabetes mellitus

FLA: Facial lipoatrophy

GALT: Gut-associated lymphoid tissue

HBsAg: hepatitis B surface antigen

HBV: hepatitis B virus

HCV: hepatitis C virus

HDL: low density lipoprotein

HIV: human immunodeficiency virus

HS: hepatic steatosis

IQR: interquartile range

IVDU: intravenous drug user

LB: liver biopsy

LDL: high density lipoprotein

LF: liver fibrosis

LOWESS: locally weighted scatterplot smoothing

LPV/r: ritonavir boosted lopinavir

LSM: liver stiffness measurement
LS: liver stiffness

MSM: men having sex with men

NAFL: non-alcoholic fatty liver

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

PI: protease inhibitor

PLWH: people living with HIV

R: regression coefficient

SD: standard deviation

TcR: T-cell receptor

TE: transient elastography

VTCE: vibration controlled transient elastography

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1 Introduction

Following the widespread use of combined antiretroviral treatment (ART), the landscape of the mortality and morbidity of individuals living with HIV has undergone remarkable changes (1-3). The proportion of AIDS-defining events and bacterial infections have declined, while non-AIDS defining events have become more frequent (1, 2, 4). Although the life expectancy of an HIV-infected person has improved dramatically and is nearly equivalent to the uninfected population (5, 6), a significant decrease in health-related quality of life has been observed, even in virologically stable individuals (7). The leading causes of mortality are cardiovascular events, non-AIDS-defining malignancies, liver and pulmonary diseases (1). The association with a heightened risk for cardiovascular disease has been described by several investigators (2, 4, 8, 9), and the rates of osteoporosis (10), physical function impairments, sarcopenia, frailty (11, 12) and neurocognitive decline (13) have also been reported to be higher compared with the general population.

Individuals living with HIV have an increased risk of these age-related morbidities compared with HIV-negative matched controls (9, 14). Whether this increased risk is caused by differences in lifestyle, such as smoking (15), alcohol or drug consumption (16), viral replication or immunologic changes or long-term ART toxicities, remains unclear. However, these factors are not mutually exclusive (17, 18).

The adverse effects and long-term toxicities of antiretroviral drugs involve all major organ systems and may contribute to the etiology of the aforementioned observations. For example, regarding the increased risk of cardiovascular events, a nucleoside reverse transcriptase analogue, abacavir, represents a risk factor for acute myocardial infarction according to observational data (19); however, this association is unclear because of the potential biases in this study (20). In a recent study, abacavir was associated with a greater than two-fold increased risk of CVD, which was not explained by renal dysfunction or other CVD risk factors (20). Another example is the observed loss of bone mineral density in patients treated with tenofovir-based antiretroviral therapy (ART) (21). Regarding these age-related and/or metabolic changes, the effects of the mitochondrial toxicity of older ART drugs are most concerning (22). Older protease inhibitors, such as lopinavir, and nucleoside analogues, such as zidovudine, didanosine or zalcitabine, are well-known

for these effects, which mainly manifest as lipid metabolic changes and lipodystrophy (22-26).

Another non-mutually exclusive explanation for the increased risk for these conditions is a chronic HIV-induced inflammatory state (27-29). which can be characterized by numerous immune activation markers, such as elevated levels of plasma interleukin 6 (IL-6), soluble tumor necrosis factor receptor I and II , soluble CD14, the ratio of kynurenine-to-tryptophan, the levels of CD38+ CD+T cells, the ratio of CD4/CD8 and the levels of D-dimer (30-35).

However, this persistent, low-grade inflammation that is triggered by multiple factors (e.g., viral co-infections with hepatitis B, C virus, CMV, microbial translocation and lifestyle factors, such as intravenous drug use), likely has the pathogenesis of HIV-induced immune dysregulation (18, 27, 28). In addition to the immunocompromising effect of CD4 depletion, ongoing HIV replication and viral proteins represent a strong immune activating signal that results in numerous changes in nearly all of the cellular and non-cellular components of the immune system (27).

The function of HIV-infected CD4 cells is greatly altered early in the course of HIV infection, prior to any detectable numerical decline (36).In contrast, Tregs are relatively preserved, which likely limits immune activation while inhibiting antiviral responses (37). While Treg cell activation may promote tissue fibrosis, it may also contribute to the observed higher rates of lymph node and liver fibrosis in HIV patients (38).

Gut-associated lymphoid tissue (GALT) is the predominant site of HIV replication, and intestinal Th17 and lamina propria CD4 cells are especially affected and rapidly decrease in individuals living with HIV, even in the early stages of infection (39, 40). GALT is the largest lymphoid organ affected by HIV infection, and its dysregulation has a deleterious effect not only on lymphocytes but also on the number and functionality of epithelial tight junctions, resulting in destruction of the gastrointestinal mucosal barrier (41, 42). Bacterial metabolites and endotoxins are therefore chronically introduced into the bloodstream, resulting in and maintaining a strong proinflammatory environment (27, 42, 43).

Together with CD4 Th-cell depletion, the mixed effects of immune depletion and activation result in immune dysfunction, which is thought to be responsible for a chronic inflammatory state with various organ involvement that is often considered to be age-related or metabolic (28, 29).

The idea that chronic, ongoing inflammation is known to play an important role in several long-term pathologic conditions, including carcinogenesis, and has a long history; it was originally described by Virchow in 1863 (44). To date, the importance of the inflammatory state has not only been identified in other types of malignancies, such as gastric MALT lymphoma (45) or diffuse large B-cell lymphoma arising from cryoglobulinemic polyclonal B-cell activation in HCV-infected patients (46-48) but also in the pathogenesis of atherosclerosis and other age-related and metabolic conditions (49). Therefore, the initiation of early treatment seems to be appropriate for preventing viral replication and the consequent inflammatory state, which results in an increased incidence of non-AIDS defining malignancies and other age-related conditions (50). However, ART-related toxicity and unfavorable metabolic changes remain concerning (51).

The debate about the importance of ART-related toxicities versus ongoing viral replication, chronic inflammation and associated non-AIDS-defining morbidities seems to have ended. The recently published and early terminated INSIGHT START study has provided the most important evidence supporting the idea that control of viral replication, regardless of CD4 counts, is beneficial for patients, outweighing the concerns of ART toxicities. In that trial, participants (HIV-infected individuals with more than 500 CD4 cells per cubic millimeter) were randomized into two different groups. The immediate group received ART immediately, and the deferred group received ART only if the CD4 counts fell below 350 CD4 cells per cubic millimeter. The composite endpoint of non-AIDS-related SAEs was significantly lower in the immediate ART group, with a hazard ratio of 0.61 (95% CI, 0.38 to 0.97; p=0.04) (52). Justified mainly by these results, current major guidelines recommend the initiation of ART regardless of CD4 counts (53, 54), providing benefits not only at the individual level but also at an epidemiological level by reducing transmission (55).

However, concerns regarding complications from long-term ART toxicities, especially in the case of older ART, have not been completely resolved. For example, a substudy of

INSIGHT START reported that hip and spine bone mineral density loss was significantly higher in the immediate group than in the deferred group (56).

In low and middle income countries, in which the newest antiretroviral combinations are not always available or affordable, older drugs with less favorable toxicity profiles also contribute to the armamentarium against HIV. In Hungary, a significant proportion of patients receive zidovudine, lamivudine and tenofovir-containing combinations, or they have a history of treatment with older NRTIs or protease inhibitors (PIs) (Sulyok, unpublished 2015). Therefore, although no published data are available, there is likely a high proportion of patients with metabolic complications. In general, little is known about comorbidities of the Hungarian HIV-infected population. To date, only a few studies were published, focusing mainly on infectious complications (57-59).

In our study, we intended to shed light on hepatic conditions among non-AIDS-defining morbidities in the Hungarian HIV-infected population. Liver disease has become one of the most important causes of morbidity and mortality in individuals living with HIV (60), and liver-related deaths occur ten times more frequently in these individuals than in the general population (61). While hepatitis B or C co-infections remain the most significant cause of liver damage, liver-related mortality also affects those infected only with HIV (4, 62). Long-term antiretroviral and non-antiretroviral medications, HIV-induced long-term inflammation, metabolic complications and direct cytopathic effects may also contribute to liver damage and hepatotoxicity (63). While an increasing number of papers has been published on HIV/hepatitis virus co-infected patients (64-81), only a few studies have analyzed the data obtained from HIV-mono-infected individuals (3, 82-92). In addition to fibrosis, there has been increasing concern about the role of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (93). The pathogenesis of hepatic steatosis is still incompletely understood, and multiple factors have been hypothesized to be necessary for its development and progression (94).

NAFLD is the most common cause of liver disease worldwide, representing an enormous disease burden with an estimated prevalence of 25.24% (22.10-28.65). NAFLD is fueled by the global obesity epidemic (95).

Based on histology, NAFLD is divided into 2 categories (96, 97). Non-alcoholic fatty liver (NAFL) includes patients with hepatic steatosis with absent or mild inflammatory

changes (96). The rate of progression to liver cirrhosis is approximately 4%, but in a less well-defined subgroup of patients presenting with hepatic inflammation (but not fulfilling the criteria of NASH), this figure could be higher (98, 99). Non-alcoholic steatohepatitis (NASH) is characterized by hepatocellular injury and ongoing fibrosis, in addition to liver steatosis (96), and it is estimated to occur in 6% of the general US population (100). In contrast to NAFL, NASH is a more progressive condition, resulting in cirrhosis in an estimated 20% of cases (99). Moreover, NAFLD patients have an overall increased risk not only of liver-related mortality but also of malignancies and cardiovascular events (97).

The most important risk factors that are also involved in the pathogenesis of NAFLD are obesity and other metabolic factors, such as insulin resistance (101). However, numerous genetic, environmental and hormonal factors play an important role in the development of this condition (97, 101). Increased visceral adipose tissue generates a chronic inflammatory state and altered lipid and glucose metabolism (101). The ongoing lipotoxicity and proinflammatory state results in hepatic fat accumulation, hepatocellular damage and fibrosis (97). Why some patients only develop liver steatosis while others develop a marked inflammatory response that can result in cirrhosis or carcinogenesis remains unclear. Alterations in the intestinal microbiota appear to contribute to this difference and may be linked to the severity of NAFLD (102).

Currently, the gold standard for the diagnosis and assessment of HS is liver biopsy (LB) (103). Limiting factors of LB are cost and rare, but severe, complications, with the additional concerns of sampling error and difficulties related to reproducibility (104, 105). Furthermore, LB allows only semiquantitative grading. Given the high proportion of individuals with NAFLD in the general population, an invasive method, such as liver stiffness (LS), with possible life-threatening complications is impractical for diagnosis. Therefore, increasing attention has been paid to numerous non-invasive methods. Traditional radiologic approaches such as computed tomography (CT) and magnetic resonance imaging (MRI) for HS assessment have also been investigated, but their high cost, limited availability and lack of standardization prevent their widespread use (106). Another non-invasive and recently developed tool is the controlled attenuation parameter (CAP), which has demonstrated accurate and reliable measurement of HS and has been successfully validated in different patient groups (107, 108). CAP is based on ultrasound

attenuation by hepatic fat at the central frequency of the FibroScan M probe and is performed simultaneously with liver stiffness measurements (107).

The evaluation and grading of liver fibrosis is facing similar problems to those observed with hepatic steatosis. Large-scale screenings using LB in patient populations in which the benefit of the assessment of fibrosis is not well-established, such as in HIV-mono-infected individuals, is also impractical and places the subjects at risk of potentially life-threatening complications. However, additional data concerning liver fibrosis in this patient population are urgently needed.

Cross-sectional studies in HIV-mono-infected patients have reported high rates (8.3-41.9%) of significant liver fibrosis, suggesting that HIV itself may contribute independently to liver damage (63). To date, only limited data are available on the prevalence and risk factors for liver fibrosis among HIV-mono-infected patients. Viral replication, low CD4 cell counts and long-term exposure to antiretroviral regimes have been identified as risk factors for the development of significant liver fibrosis (62, 63, 109). Like hepatic steatosis, ongoing liver fibrosis is not always accompanied by elevated liver enzymes and a diagnosis of liver fibrosis. The prevention of progression to liver cirrhosis is an important challenge. As a result, adequate monitoring strategies for liver disease are needed to optimize the care of HIV-infected individuals (62, 63).
Noninvasive fibrosis determinations, such as liver stiffness measurements (LSM) with transient elastography, the aspartate aminotransferase (AST)-to-platelet ratio index (APRI) and the FIB-4 score, have facilitated cross-sectional and prospective studies to evaluate the prevalence and incidence of liver fibrosis in HIV-infected individuals. These tests are appropriate for predicting the absence of fibrosis or mild fibrosis (liver fibrosis <2 METAVIR score) and the presence of advanced fibrosis (liver fibrosis >3 METAVIR score) (110). Liver stiffness, which is a value that describes the grade of liver fibrosis, is also simultaneously determined by the CAP measurement using transient elastography. Therefore, LSM seems to be a practical method for evaluating liver diseases, especially in individuals living with HIV. To date, only a few studies using LSM have examined the prevalence and potential risk factors for hepatic fibrosis among HIV-mono-infected patients. The use of different cutoff values has resulted in a wide range of prevalence estimates (84, 85, 88, 90, 109).

While the main pathogenic factors resulting in the development of NAFLD are similar to the ones involved in the development of non-AIDS-defining morbidities in HIV-infected patients, such as chronic inflammation and microbiome changes (43, 101), an increased prevalence in the HIV-infected population is expected. ART may also contribute to hepatotoxicity. Older ART drugs (protease inhibitors and dideoxynucleoside analogs) cause hypertriglyceridemia and lipodystrophy and are considered to be especially harmful (76),(111). Furthermore, people living with HIV are vulnerable to environment-related hepatotoxic factors, such as alcohol consumption, illicit drug use, smoking and mental illnesses that require possible psychiatric medication (112-115). It should be emphasized that patients with normal liver enzymes can still have significant steatosis and fibrosis. Thus, early detection of HS in HIV-infected patients is the cornerstone of prevention of the silent progression of NAFLD to NASH and cirrhosis (116).

Surprisingly, data from this population are relatively scarce and are limited almost exclusively to hepatitis-co-infected subpopulations. To the date of the publication our first results (117), the only study that assessed HS with CAP in a large population of HIV patients is the one reported by Macías et al., who identified significant HS in 40% of the participants, and 60% of the study population was co-infected with HCV (74). Therefore, data from an unselected group of individuals living with HIV, including HIV-mono-infected patients, are urgently needed to develop better caring and treatment strategies for PLWH with NAFLD. To achieve this goal, the problem of selection and switching of ART in this population should be addressed. However, to our knowledge, the desperately needed large-scale prospective studies and clinical trials that focus on patients with NAFLD and HIV infection have not been performed.

As a first step, epidemiologic, cross-sectional studies are needed to better characterize the prevalence and disease burden. Therefore, we designed and performed a cross-sectional study in an unselected group of individuals living with HIV to assess the prevalence of NAFLD and to identify associated factors. The aim of our study was to assess the prevalence and severity of HS using a continuous CAP value in PLWH and to determine the association with different demographic, immunologic and metabolic factors. Given that the data for liver fibrosis in the HIV-mono-infected population are also limited, we planned to perform a predefined subgroup analysis in this patient population. In contrast

to the few published similar studies (84, 85, 88, 109, 118), we planned to use a continuous scale of LS and CAP values as regression endpoints to avoid the information loss and uncertainty that arises from cutoff values adopted from other patient populations (117).

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2 Study objectives

2.1 Primary objectives

- To identify a proportion of significant hepatic steatosis in individuals living with HIV
- To determine the associations between antiretroviral agents and hepatic steatosis in individuals living with HIV
- To determine the associations between HIV-related immunologic parameters and hepatic steatosis in individuals living with HIV
- To determine the associations between metabolic parameters and hepatic steatosis in individuals living with HIV

2.2 Secondary objectives

- To characterize the metabolic profile of the Hungarian HIV-infected population
- To identify a proportion of significant hepatic fibrosis HIV-mono-infected individuals

2.3 Exploratory objectives

- To determine the associations between antiretroviral agents and hepatic fibrosis in HIV-mono-infected individuals without significant alcohol consumption
- To determine the associations between HIV-related parameters and hepatic fibrosis in HIV-mono-infected individuals without significant alcohol consumption
- To determine the associations between metabolic parameters and hepatic fibrosis in HIV-mono-infected individuals without significant alcohol consumption

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3 Methods

3.1 Study population

The investigation was performed in accordance with the Helsinki Declaration and was approved by the Institutional Ethics Committee (approval number 34/EB/2013). From March 1, 2014 to October 30, 2014, all HIV-infected patients who attended the outpatient clinic at the HIV Center, St. Laszlo Hospital, Budapest, Hungary were invited to participate in the study. Patients were recruited by dedicated members of the study team during outpatient visits. Informational brochures were placed in the waiting hall of the HIV center, with the agreement of the proprietor.

After providing written informed consent, individuals older than 18 years of age were enrolled in the study (*n=139,* intended to treat population). Pregnant women and patients with unreliable transient elastography measurements (<10 valid measurements) were excluded. Taking these criteria into account, the final study population consisted of 136 individuals (per protocol population, **Figure 1.**). Following enrollment, all data were pseudonymized. The volunteers received an identification number, and no names, addresses, initials or other information that might identify the participant were stored in the database. Separate confidential files containing identifiable information were stored in secured locations that only the investigators and dedicated members of the study team could access.

Participation was entirely voluntary, and participants could verbally withdraw their consent from the study at any time without providing further explanation. Volunteers received an official copy of their own transient elastography results. Other forms of compensation were not provided.

A prespecified subgroup analysis of HIV-mono-infected individuals without documented or suspected significant alcohol consumption (defined by >50 g alcohol daily) was also performed. This subgroup consisted of 101 participants.

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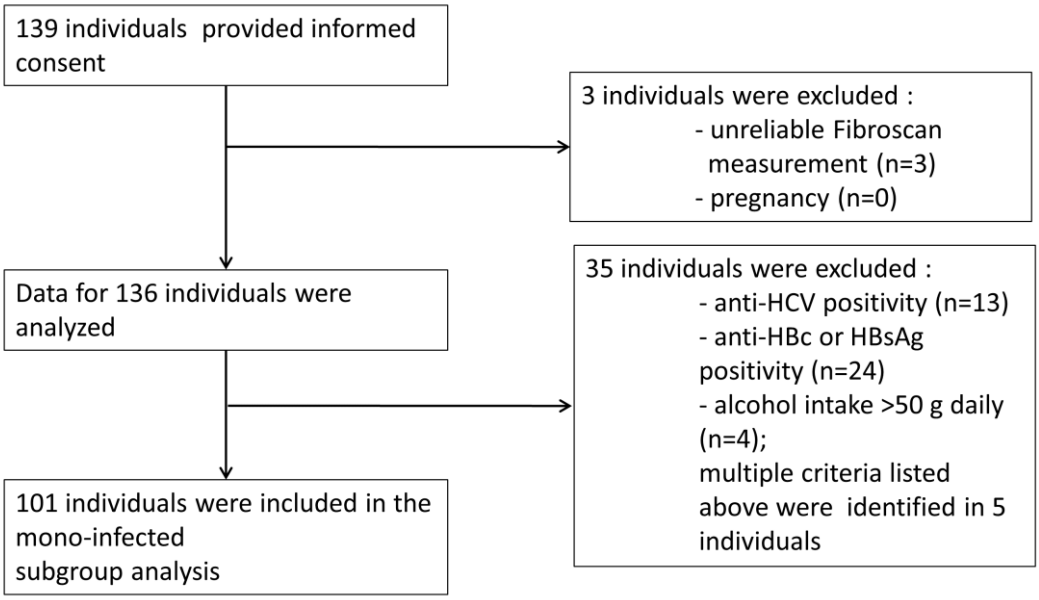

Figure 1. Recruitment Flow of the Study Participants.

3.2 Interview and clinical parameters

Interviews with the participants were conducted at the Hepatology Center of Buda, Budapest, Hungary, on the same day as the transient elastography. Information regarding the medical history was collected, including comorbidities, concomitant medications, mode of HIV transmission, known length of HIV positivity, history of antiretroviral therapy, alcohol and illicit drug intake and smoking habits. Anthropometric parameters, such as height, weight, gender, BMI and assessment of facial lipodystrophy, were gathered by an investigator experienced in HIV medicine. Facial lipodystrophy was assessed according to the FLA severity score using the 5-point Carruthers scale (119). The presence of facial lipodystrophy was defined according to a FLA severity score of 2 (deeper and longer central cheek atrophy, with the facial muscles beginning to show through) or higher (in grade 3, the atrophic area is even deeper and wider, with the

muscles clearly showing; in grade 4, atrophy covers a wide area and extends up toward the orbit, with the facial skin lying directly on the muscles over a wide area) (119).

Information obtained during the interviews regarding comorbidities, concomitant medications and history of antiretrovirals was also checked retrospectively in the patients' archived documentation. In the case of discrepancies between the written and oral information, a consultation with the patient's HIV specialist was initiated.

Biochemical and immunological parameters, serum cholesterol, serum triglyceride, blood count, CD4 and CD8 count, CD4/CD8, anti-HCV, HBsAg and anti-HBc serology were collected at the visit when informed consent was obtained (<4 weeks before the Fibroscan measurement).

3.3 Transient elastography

Vibration controlled transient elastography (VTCE) is a non-invasive method to quantify liver fibrosis. This technique uses both ultrasound (5 MHz) and low-frequency (50 Hz) elastic shear waves, with a propagation velocity that is related to tissue elasticity (120). Liver elasticity, or stiffness, is expressed in kPa and is equal to the Young-modulus of the examined tissue, or $E=3\rho v2$, where v is the shear velocity and ρ is the tissue density. The stiffer the tissue, the faster the shear wave propagates (121). Details of the technical background have been previously described (120). VTCE to assess hepatic fibrosis has been validated in different patient populations (122-132). VTCE is approved by the FDA (133) and is recommended by different international and national guidelines (121, 134, 135), e.g., Hungarian consensus guidelines for viral hepatitis (136, 137).

Controlled attenuation parameter measurements based on vibration controlled transient elastography were also simultaneously performed. CAP is the name of the algorithm that assesses the ultrasonic attenuation coefficient based on the ultrasonic properties of the radiofrequency back-propagated signals, or more precisely, it is an estimate of the total ultrasonic attenuation (go-and-return path) at 3.5 MHz (108). Technical details and validation with histological findings have also been extensively described (107, 108, 138).

To date, the CAP measurement has been validated for the diagnosis of hepatic steatosis in different patient groups (107, 134, 138-142). In NAFLD, this method is considered to be especially helpful (143-147). The inter-observer concordance of CAP has been demonstrated in HIV-infected individuals (148).

A cutoff of 238 dB/m was selected to define the presence of hepatic steatosis (S1), and cutoff values for more advanced steatosis of 260 dB/m (S2) and 292 dB/m (S3) have been applied (108). Because these cutoffs were adopted from a non-HIV-infected population, they cannot be reliably transferred to an HIV-infected population (117). Therefore, despite the defined cutoff values, for the univariate and multivariate analyses, a continuous scale of CAP values was used.

A similar problem was encountered for the liver stiffness cutoffs. Only a few studies using LS measurements have examined the prevalence and potential risk factors for hepatic fibrosis among HIV-mono-infected patients. Using different cutoff values resulted in a wide range of prevalence estimates (84, 88). Pre-defined cutoffs adopted from the HIV/HCV-co-infected population (significant liver fibrosis defined by liver stiffness >7.2 kPa and 14.6 kPa to identify the presence of cirrhosis) may lead to an underestimation of the number of HIV-mono-infected patients with clinically significant fibrosis because these cutoffs were determined for a population in which ongoing fibrosis is triggered by HCV co-infection (84, 149). A recently published observational study concluded that in HIV-mono-infected adults with biopsy-proven liver disease, LSM by VCTE is the best noninvasive method to predict fibrosis (85). However, LSM (with a cutoff value of 7.1 kPa) was performed in only 59 participants with elevated baseline aminotransferase levels.

However, using cutoffs from the general population in HIV-mono-infected individuals (84) may overestimate the prevalence of significant liver fibrosis.

To overcome this limitation, we used a continuous scale of LS values to interpret this variable in the uni- and multivariate analyses. To characterize the patient population, cutoffs both from the HIV/HCV population and the ones adopted from the normal population (84) were used.

Transient elastography examination and CAP measurements were performed by an experienced and qualified investigator at the Hepatology Center of Buda, Budapest, Hungary, using FibroScan 502 equipment (Fibroscan, EchoSens™, Paris, France). Measurements were performed using an M probe on the right lobe of the liver. Patients were placed in a supine position, with their right arms elevated behind their heads. The tip of the probe was placed in an intercostal region with contact gel in the 9th - 11th intercostal space on the right side. The examining investigator utilized a time-motion image that located the liver tissue (regions with large vessels were avoided), and measurements were collected. The software determined whether a measurement was valid. Examinations with 10 successful shots and an interquartile range (IQR) for liver stiffness less than 30% of the median value and a success rate (the ratio of valid shots to the total number of shots) above 60% were considered reliable and successful (118, 121, 150).

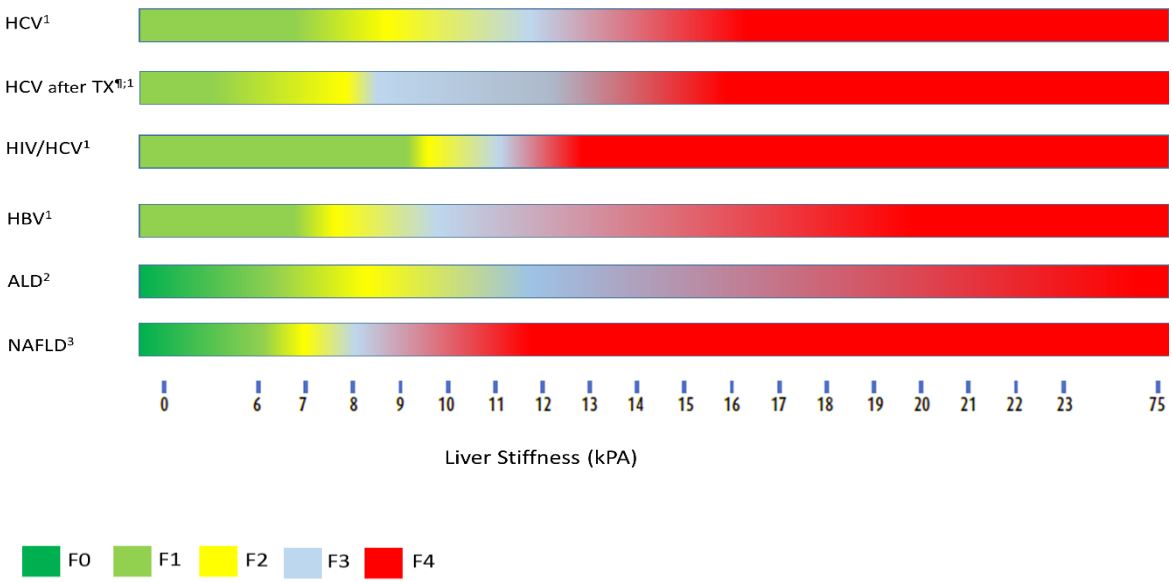

Figure 2. Staging of liver fibrosis according to liver stiffness values in liver disease. The different stages are coded in colors. Transient zones, such as F1/2 or F2/3, are shown in gradient colors. The large variation between the liver fibrosis grading in different patient populations is evident.

¶: Recurrence after liver transplantation 1: (132); 2: (130); 3: (131)

3.4 Sample size considerations

The sample size was calculated *a priori* using the function nBinomial in the gsDesign package of R software package version 3.1.2 (151). Regarding the first primary objective, to show with a power of 80% and a two-tailed alpha of 5% that significant HS is present in 25% of ART naive participants and 65% of ART experienced individuals (if an estimated 10% of the volunteers are ART naïve), the minimum participant number was calculated to be 132. If metabolic conditions (diabetes, hypertension, obesity) are estimated to be present in every third individual, and in this patient group the proportion of significant HS is also estimated to be 65%, then with 25% of the subjects lacking metabolic conditions, the required participant number is 64, with a power of 80% and a two-tailed alpha of 5%. The same estimation was applied to the third primary objective regarding immune dysregulation. Overall, the first and most stringent calculation was used, and the minimum number of 132 participants was utilized.

3.5 Statistical analysis

The primary outcome variable was the CAP value. The associations of the following factors were analyzed: sex, age, time from diagnosis of HIV infection, CD4 and CD8 cell counts, BMI, self-reported daily alcohol intake, smoking habits, fasting lipid profile (total cholesterol, triglycerides), blood pressure and ART exposure. The univariate association of CAP with categorical variables was assessed using a two-independent-sample Mann-Whitney U test. Stratified descriptive statistics are presented as the mean (median) ± SD (IQR) [min-max]. The univariate correlation of CAP with continuous variables was assessed using the Pearson (linear) and Kendall-τ rank-correlation coefficient. Visualization was performed with scattergrams, which indicated the best fitting linear curve and LOWESS-smoother for non-parametric regression. Cramér's V was used to describe multiple associations between categorical data and was visualized as a Circos plot (152). Bonferroni correction was performed to counteract problems related to multiple comparisons.

For the multivariate analysis, prespecified covariates were added to a linear regression model, with the CAP value as the response variable, as described by Sulyok et al. (117). Alterative models with predictor selection based on collinearity diagnostics and with a combined covariable of "Metabolic unfavorable antiretroviral therapy ever taken," which was defined based on a history of taking ritonavir-boosted lopinavir and/or zidovudine with a binary outcome, was introduced instead of different antiretroviral medications. In this analysis, the covariates serum triglyceride, cholesterol and HBV positivity (defined by HBsAg or anti-HBc positivity) were added to the model. A prespecified, but unpublished, subgroup analysis in HIV-mono-infected individuals without significant alcohol intake was also performed as described above using liver stiffness as the response variable. No interaction was added to the models. Categorical variables were added with Male/No as the reference category. The necessity of non-linearity was investigated by extending the model using restricted cubic splines for the continuous variables and the Wald-*F* test to assess joint significance. Aikake, Hurvich and Tsai's corrected Aikake and Bayes Information Criterion was used to determine the necessity of model penalization. The obtained model was checked and passed routine residual diagnostics. To internally validate the model, a calibration curve and optimism-corrected R^2 were calculated using the bootstrap method with 1000 replications (153). p-values less than 0.05 were considered significant. Multicollinearity diagnostics were also performed in the non-penalized models, and a variance inflation factor >10 was considered to indicate a high degree of multicollinearity. Calculations were performed using R software package version 3.1.2 (151) and library rms, with a custom script that is available upon request.

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4 Results

4.1 Study population characteristics

. Significant steatosis (>238 dB/m) was observed in 65 (47.8%) patients. Twenty-five (18.38%) patients had stage 1, 16 (11.75%) stage 2 and 24 (17.65%) stage 3. The median liver stiffness was 5.2 kPa (IQR 2). Fifty-two (36.76%) patients had a BMI greater than 25 kg/m^2, and obesity (defined by a BMI greater than 30 kg/m^2) was identified in 6 (4.55%) individuals. Hypertriglyceridemia (serum triglyceride levels >1.7 mmol/L) was detected in 57 (41.92%) patients, and hypercholesterolemia (serum cholesterol levels >5.2 mmol/L) or low serum HDL-C levels (<1 mmol/L in men, < 1.3 in women) were observed in 67 (49.26%) individuals. The mode of HIV transmission was reported to be sexual intercourse in 134 (98.5%) patients and transfusional or coagulation factor product in 2 (1.5%) individuals with hemophilia. Intravenous drug use was not reported by any patients. The study population characteristics are summarized in **Table 1.**

Anti-HCV antibodies were detected in 13 (9.56%) individuals. Eight patients had S0 stage HS, 2 had S1 and 3 had S3. The median CAP value was 237 dB/m and 216 dB/m in individuals without anti-HCV antibodies. HBsAg was observed in 11 (8.1%) study participants. The median CAP did not differ significantly between patients with (237 dB/m) and without (238 dB/m) HBsAg positivity. Five (2.94%) individuals reported more than 50 g of daily alcohol intake. One hundred and twenty-five patients were taking antiretroviral medication regularly, and 11 were treatment naive at the time of enrollment. In the subgroup of 11 naive patients, 4 (36.4%) had CAP values greater than 238 dB/m, and none had stage 2 or stage 3 steatosis. Compared with the ART-exposed patients, among which 66/125 (52.8%) had significant steatosis, 26/66 (39.4%) had stage 2 steatosis and 28/66 (42.4%) had stage 3 steatosis (p=0.465). The associations between antiretrovirals are shown in **Figure 3**.

Table 1. Study population characteristics. BMI: body mass index; CAP: controlled attenuation parameter; ART: antiretroviral therapy

Parameter	**Mean (Median) ± SD (IQR) [Min-Max]**
CD4 %	26.97 (27) ± 8.93 (12) [1 - 48]
CD8 %	45.59 (44.5) ± 12.3 (17) [20 - 78]
CD4/8 ratio	0.66 (0.63) ± 0.35 (0.39) [0.01 – 1.76]
Age (years)	44.51 (42.36) ± 11.06 (13.48) [24.35 - 78.13]
BMI (kg/m^2)	24.69 (24.16) ± 3.18 (3.62) [18.04 – 37.83]
Serum triglyceride (mmol/L)	2.543 (1.6) ± 2.32 (2.3) [0 – 13.1]
Serum cholesterol (mmol/L)	5.24 (5.2) ± 1.5 (1.8) [0 – 10.9]
Known HIV positivity (years)	9.13 (7) ± 6.71 (9) [0.75 - 28]
Liver Stiffness (kPa)	5.92 (5.2) ± 3.96 (2) [3 – 36.3]
CAP (dB/m)	245 (237) ± 52.61 (67) [160 - 385]
	N (%)
ART ever taken	125 (91.9)
Darunavir	27 (19.85)
Atanazavir	10 (7.35)
Lopinavir	27 (19.85)
Raltegravir	17 (12.5)
Lamivudine	120 (88.24)
Tenofovir	54 (39.7)

Abacavir	20 (14.7)
Zidovudine	47 (34.55)
Etravirine	9 (6.62)
Nevirapine	29 (21.32)
Efavirenz	33 (24.26)
Isolated anti-HBc positivity	13 (9.56)
anti-HCV positivity	13 (9.56)
HBsAg positivity	11 (8.08)
Smoking	16 (11.75)
Alcohol intake (>50g daily)	4 (2.94)
Sex (male)	133 (97.8)
Diabetes	13 (9.56)
Hypertension	29 (21.32)
Lipodystrophy	13 (9.56)

In the subgroup of HIV-mono-infected patients without significant alcohol consumption (n=101), LS ranged from 3.0 kPa to 34.3 kPa, with a median value of 5.1 kPa (IQR 1.7). According to the HIV/HCV co-infection LS cutoffs, significant liver fibrosis, defined as LS <7.2 kPa, was detectable in 10/101 individuals. The presence of cirrhosis (LS >14.6 kPa) was observed in 2 participants. Applying the cutoff value of 5.3 kPa from a healthy population as described in the study by Han et al., significant fibrosis was detected in 56/101 patients. CAP values in this subgroup ranged from 165 dB/m to 385 dB/m, with a median of 239 dB/m (IQR 74). Fifty-three (52.47%) participants had significant liver steatosis. Stage 1 steatosis was detected in 20 (19.8%) patients, stage 2 in 9 (8.91) and stage 3 in 24 (23.76%). The median BMI was 24.74 (IQR 3.32). A BMI greater than 25 kg/m^2 was found in 45 patients, and obesity was present in 5 patients. Age ranged from 24.35 to 71.33 years (median 42.36, IQR 13.4), and 99/101 (98.01%) participants were male. The median CD4% was 29 (IQR 11, min-max 1-46), median CD8 was 44 (IQR 17, min-max 20-78) and CD4/8 ratio was 0.6383 (IQR 0.4502, min-max 0.01282-1.76). The median known disease duration was 7 years (IQR 9, min-max 0.75-25). Eleven patients

were diabetic, 21 had hypertension, and facial lipodystrophy was identified in 12 individuals. The number of ART-experienced participants was 92 (91.09%). Twenty (19.8%) patients were taking darunavir, 7 (6.93%) atanazavir, 26 (25.74%) lopinavir, 8 (7.92%) raltegravir, 9 (8.91%) etravirine, 22 (21.78%) nevirapine, 27 (26.73%) efavirenz, 38 (37.62%) tenofovir, 13 (12.87%) abacavir, 39 (38.61%) zidovudine and 89 (88.11%) lamivudine. There were no significant differences in any of the parameters between this subgroup and the total study population.

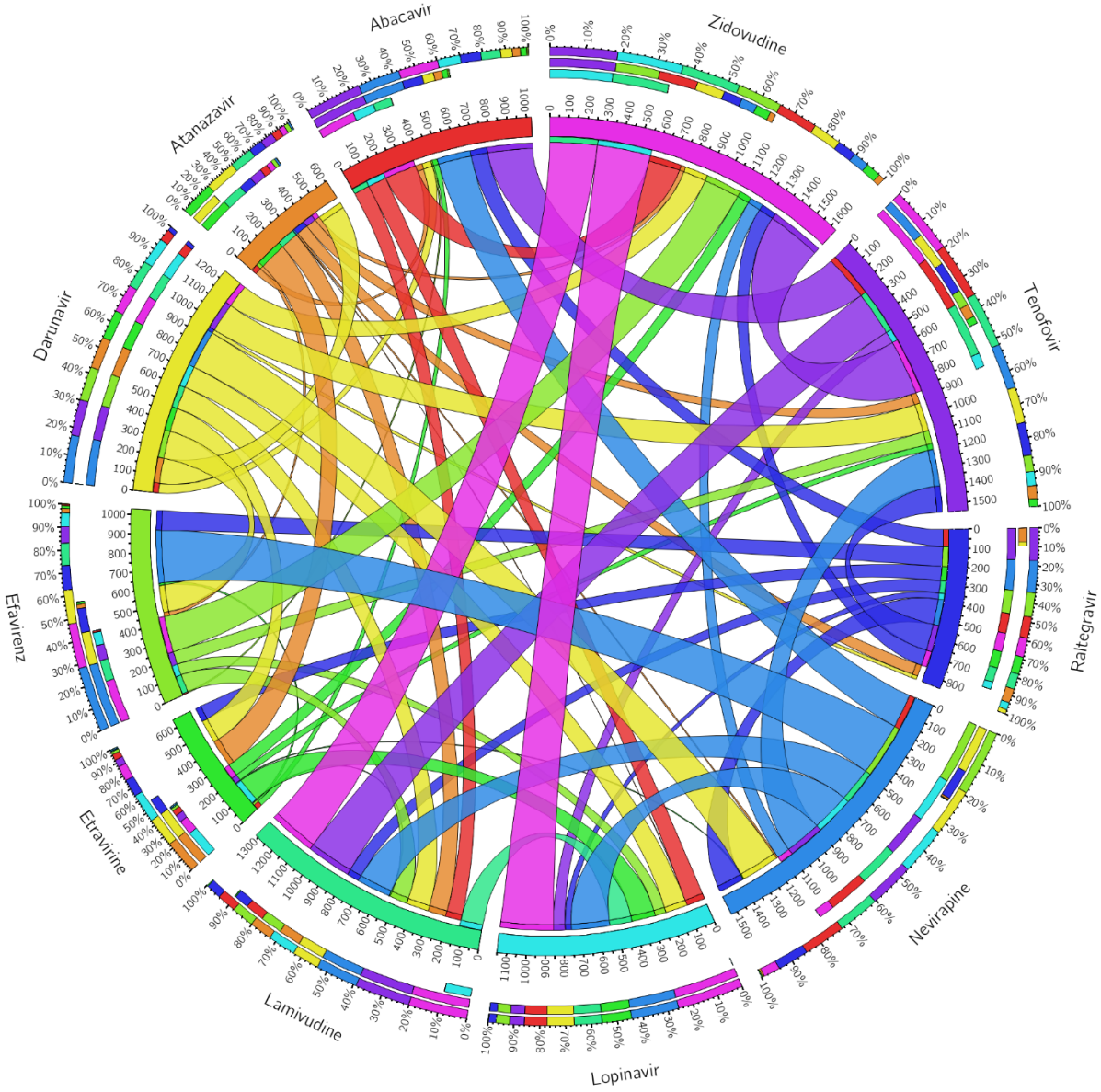

Figure 3. Associations between antiretroviral medications (ever taken) in the whole study population. The 11 most frequent antiretrovirals are coded in different colors.

Associations are expressed as Cramér's V value x 1000 and are displayed as 'two-way chords' between the antiretroviral panels. The thickness of the cords, denoting the strength of the association, is presented numerically for the inner ring and displayed as color-coded percentages for the outer rings. The only association that was found to be statistically significant after Bonferroni-correction was between zidovudine and tenofovir (Cramér's V value: 0.305, $p<0.001$.)

4.2 Univariate analysis of the association between the CAP value and different variables

The examined continuous variables showed a strong association with the CAP value following the Pearson and Kendall-τ-rank correlation. According to the Bonferroni-correction, the associations of age (adjusted $p<0.001$), serum triglyceride (adjusted $p<0.001$), BMI (adjusted $p<0.001$) and disease duration (adjusted $p<0.001$) using the Pearson and Kendall-τ-rank correlation and liver stiffness with the Kendall-τ-rank correlation were considered significant (adjusted $p<0.001$). The association was only negative for the CD8 percentage. Among the categorical variables, the presence of hypertension was considered to be significant (adjusted $p<0.001$). Associations of the CAP value and different continuous and categorical variables assessed by univariate analysis are shown in **Table 2**. **Figure 4** presents the assessed correlation of continuous variables with the CAP value in graphical form.

Table 2. Univariate analysis: associations between the CAP value and continuous (panel A) and categorical (panel B) variables.[a] Results are presented as the mean (median) ± SD (IQR) [min-max]; The p-value pertains to the null hypothesis of no correlation; *p*-values are unadjusted; BMI: body mass index; CAP: controlled attenuation parameter; ART: antiretroviral therapy; Lipodystrophy: facial lipodystrophy

Continuous variable	**Linear**		**Kendall**	
	r	*p*	τ	*p*
CD4%	0.18	0.0349	0.1304	0.027
CD8%	-0.21	0.016	-0.1366	0.0201

Continuous variable		**Linear**	**Kendall**	
	r	*p*	τ	*p*
CD4/8 ratio	0.2	0.0199	0.1435	0.0136
Age	0.39	<0.001	0.2456	<0.001
BMI	0.5	<0.001	0.3544	<0.001
Triglyceride	0.35	<0.001	0.2202	<0.001
Cholesterol	0.25	0.0036	0.1693	0.0042
Disease duration	0.36	<0.001	0.2363	<0.001
Liver stiffness	0.18	0.041	0.2076	<0.001

Panel B. Results are presented as the mean (median) ± SD (IQR) [minimum–maximum]. The p-value pertains to the null hypothesis of stochastic equivalence for the two populations (presence/absence). p-values are unadjusted.

[a] Antiretroviral therapy

Categorical	**Presence of variable[a]**	**Absence of variable[a]**	**p-value**
Darunavir	247 (237) ± 54.4 (64) [79 - 304]	236.9 (237) ± 44.7 (71.5) [171 - 332]	0.502
Atanazavir	243 (237) ± 51.3 (64.5) [160 - 385]	269.9 (263.5) ± 64.9 (89.25) [108 - 287]	0.185
Raltegravir	245.5 (238) ± 51.5 (63) [79 - 304]	241.9 (216) ± 61.8 (51) [92 - 297]	0.457
Etravirine	243.8 (237) ± 52.1 (66) [79 - 304]	262.6 (249) ± 60.2 (98) [116 - 287]	0.358
Nevirapine	243.8 (237) ± 54.3 (67.5) [160 - 385]	249.6 (239) ± 46.6 (49) [97 - 297]	0.394
Efavirenz	239.5 (235) ± 47.4 (58.5) [168 - 385]	262.2 (242) ± 64.2 (120) [79 - 287]	0.094

Categorical	**Presence of variable[a]**	**Absence of variable[a]**	**p-value**
Tenofovir	247 (237.5) ± 52.9 (69) [79 - 297]	241.9 (237) ± 52.5 (68.5) [165 - 385]	0.553
Zidovudine	239.4 (237) ± 49.2 (65) [79 - 304]	255.7 (237) ± 57.7 (79) [92 - 297]	0.208
Lamivudine	222.9 (231.5) ± 45.1 (60.5) [87 - 254]	248 (238) ± 53 (71.5) [160 - 385]	0.087
Lopinavir	240.1 (234) ± 50.4 (67) [79 - 304]	265.1 (245) ± 57.4 (89.5) [185 - 378]	0.042
ART[a] ever	247 (237) ± 53.7 (74) [79 - 304]	222.6 (236) ± 31.2 (32.5) [168 - 254]	0.264
HCV	244.4 (237) ± 51.7 (65.25) [160 - 385]	226.8 (216) ± 35.1 (47) [90 - 201]	0.320
HBV	246.16 (237) ± 53.78 (70) [79 - 304]	232 (238) ± 36.1 (64) [92 - 207]	0.632
Smoking	242.8 (237) ± 46.2 (57) [79 - 304]	235.6 (233.5) ± 50.1 (46.5) [92 - 287]	0.425
Alcohol	244.8 (237) ± 53.2 (66.25) [160 - 385]	251.5 (248) ± 29.2 (32) [140 - 208]	0.515
Sex	245.1 (237) ± 53.5 (66.5) [160 - 385]	244 (239) ± 22.6 (22) [132 - 191]	0.652
Diabetes	240.4 (236) ± 49.6 (61.5) [160 - 378]	288.6 (270) ± 62.3 (71) [121 - 304]	0.006
Hypertension	233.2 (229) ± 46.7 (49) [79 - 304]	279.5 (282) ± 49.8 (70) [107 - 297]	<0.001
Lipodystrophy	240.8 (237) ± 49.5 (62.25) [160 - 378]	281.5 (282.5) ± 66 (116.5) [118 - 304]	0.031

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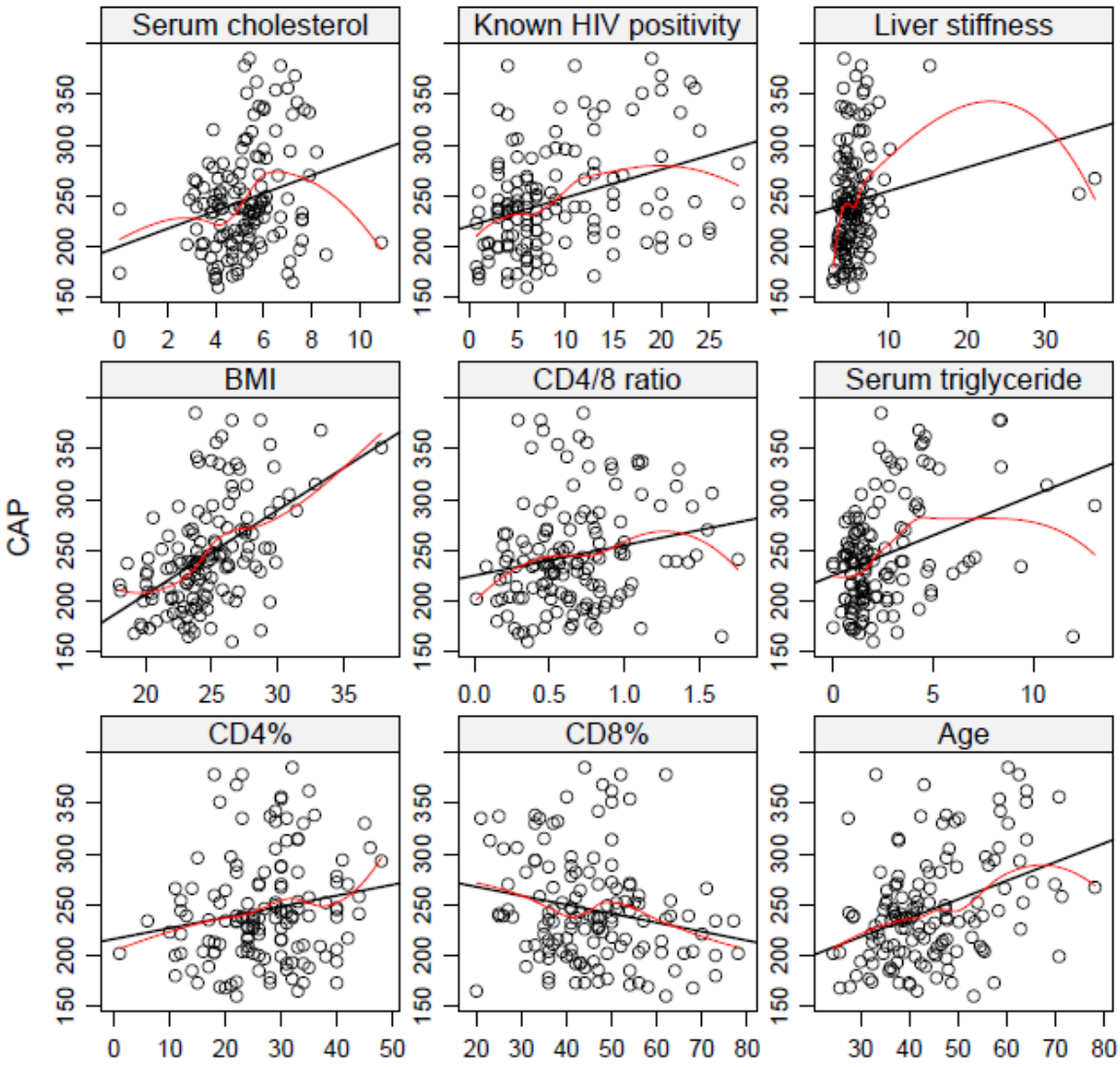

Figure 4. Correlations between continuous variables and the CAP value.

The black line shows the best fitting linear curve, and the halftone-line shows the LOWESS-smoother for the non-parametric regression. BMI: body mass index (kg/m2); CAP: controlled attenuation parameter (dB/m). Age and length of known HIV positivity are expressed in years, serum cholesterol and triglyceride in mmol/L and liver stiffness in kPa.

4.3 Multivariate regression models that predict the CAP value

To identify significant covariates of the CAP value, a non-linear multivariate model was created using restricted cubic splines for the continuous variables. All examined parameters were entered into the model with the exception of smoking (33.09% of the values were missing) and gender (97.8% of the participants were male). The Wald-F test showed that joint non-linearity was not significant (p=0.1787); therefore, a linear model could be established. The regression showed a strong association with BMI . The associations with other covariates (diabetes, hypertension) were also significant. Darunavir therapy as reported in the medical history was negatively associated with the CAP value. Covariates with their estimated regression coefficients and 95% confidence intervals are shown in **Table 3** and are graphically presented in a Forest plot in **Figure 5**. No significant collinearity was detected with the exception of the CD4/8 ratio (virtual influence factor 10.85). Although penalization was not deemed to be necessary (**Figure 6**), a large number of variables compared with the study population was concerning. Therefore, model calibration and validation were performed, which revealed poor fitting of the model (shown in **Figure 7**). To address this problem, we penalized the model. As a result, BMI and hypertension remained significant. The estimated regression coefficients and confidence intervals are shown in **Table 4** and are graphically presented in **Figure 8**. The penalized model calibration and validation are shown in **Figure 9**.

An alternative model after selection of the covariates revealed similar results. Based on variance inflation factors (VIFs; CD4% VIF=5.37; CD8%-VIF=4.8; CD4/8 ratio VIF=10.85), CD4% and CD8% were removed. A combined variable of "Metabolic favorable ART" with a binary outcome was introduced instead of the 11 covariates of different antiretrovirals. BMI (p<0.0001; regression coefficient: 6.208; 95% CI 3.736-8.681), diabetes (p=0.0509; regression coefficient 22.599; 95% CI 2.344-42.854) and hypertension (p=0.0291; regression coefficient: 28.0479; 95% CI -0.1092-56.205) remained significant positive predictors, but triglycerides also exhibited a significant, independent association (p=0.0262, regression coefficient 3.964, 95% CI 0.477-7.451). Significant negative predictors were not identified (**Figure 10**). Model fitting improved remarkably (R^2 = 0.403; optimism-corrected R^2 = 0.234), and non-linearity was not significant (p=0.907).

Table 3. Regression coefficients of covariates that predict the CAP value. S.E.: standard error.

Covariate	Regression coefficient	S.E.	t-value	95% CI	P-value
Liver Stiffness	-0.707	1.251	-0.570	-3.188 to 1.774	0.573
Age	-0.131	0.533	-0.250	-1.188 to 0.927	0.807
BMI	7.070	1.415	5.000	4.262 to 9.877	<0.0001
CD4%	0.558	0.958	0.580	-1.341 to 2.458	0.561
CD8%	-0.701	0.656	-1.070	-2.003 to 0.601	0.288
CD4/8 ratio	-24.729	34.868	-0.710	-93.897 to 44.439	0.480
Triglyceride	3.644	1.926	1.890	-0.177 to 7.465	0.061
Cholesterol	-0.201	2.971	-0.070	-6.095 to 5.694	0.946
Lipodystrophy	-7.629	16.236	-0.470	-39.836 to 24.579	0.640
Diabetes	32.868	16.059	2.050	1.011 to 64.724	0.043
Hypertension	26.328	10.976	2.400	4.554 to 48.103	0.018
Disease duration	1.145	0.901	1.270	-0.642 to 2.933	0.207
Nevirapine	-6.693	11.941	-0.560	-30.380 to 16.994	0.576
Efavirenz	-3.971	11.104	-0.360	-25.998 to 18.055	0.721
Etravirine	-4.149	17.407	-0.240	-38.680 to 30.382	0.812
Tenofovir	6.499	10.015	0.650	-13.368 to 26.366	0.518
Abacavir	12.228	11.811	1.040	-11.202 to 35.657	0.303
Zidovudine	-10.810	10.325	-1.050	-31.292 to 9.672	0.298
Lamivudine	12.565	14.936	0.840	-17.063 to 42.193	0.402
Raltegravir	-6.818	13.491	-0.510	-33.580 to 19.943	0.614
Atanazavir	-17.253	17.793	-0.970	-52.550 to 18.045	0.335

Covariate	Regression coefficient	S.E.	t-value	95% CI	p-value
Darunavir	-29.914	12.585	-2.380	-54.879 to -4.949	0.019
Lopinavir	16.644	12.059	1.380	-7.278 to 40.566	0.171
HCV positivity	-8.705	15.130	-0.580	-38.718 to 21.308	0.566
HBV positivity	-2.244	11.131	-0.200	-24.325 to 19.837	0.841
Alcohol	-11.426	24.403	-0.470	-59.835 to 36.984	0.641

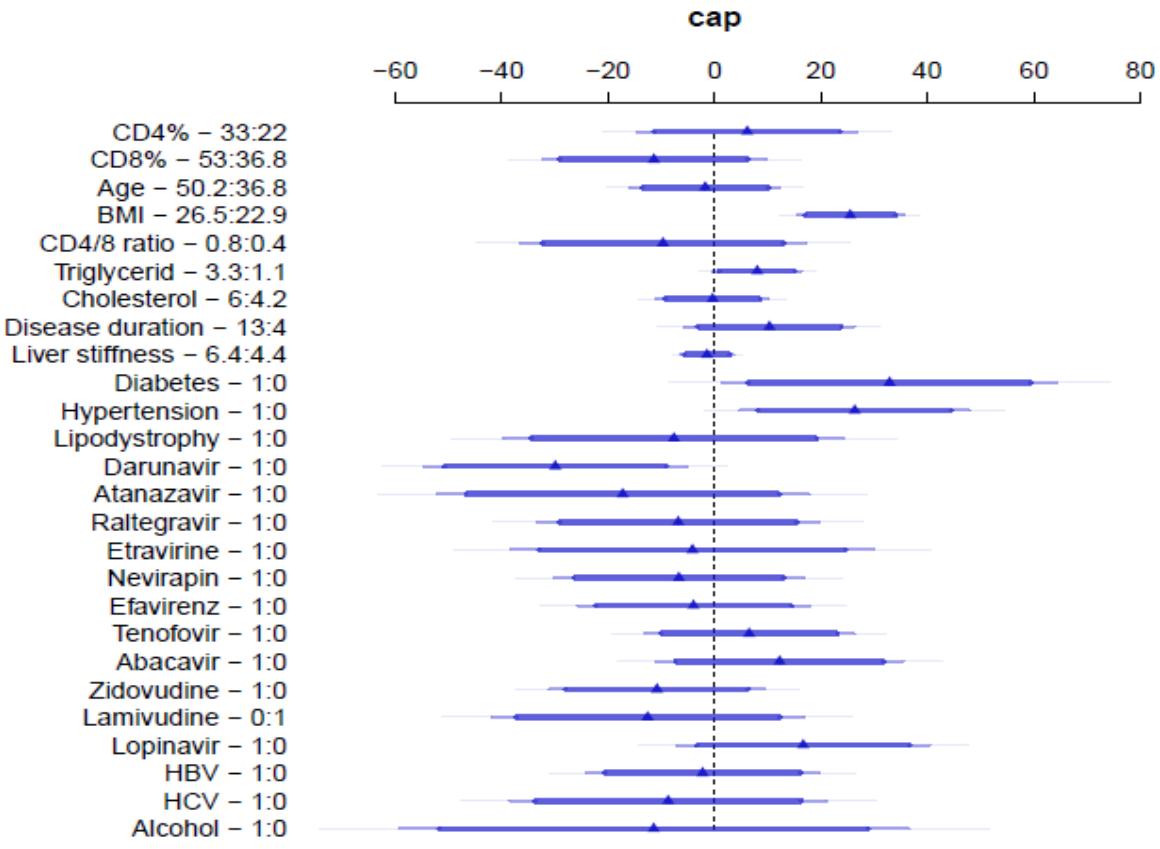

Figure. 5. Multivariate analysis: covariates with regression coefficients and confidence intervals of the model predicting the CAP value.

The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as being the change to the modal category, and for continuous

variables, it is a change of 1 IQR. In each case, this is explicitly indicated by two values that are separated by a colon after the variable. BMI is expressed in kg/m^2; age and length of known HIV positivity are expressed in years; and liver stiffness is expressed in kPa. ART, antiretroviral therapy; CAP, controlled attenuation parameter (dB/m); lipodystrophy, facial lipodystrophy. The thick dark blue lines represents 90% CIs, the thick light blue lines are 95% CIs and the narrow light blue lines are 99% CIs.

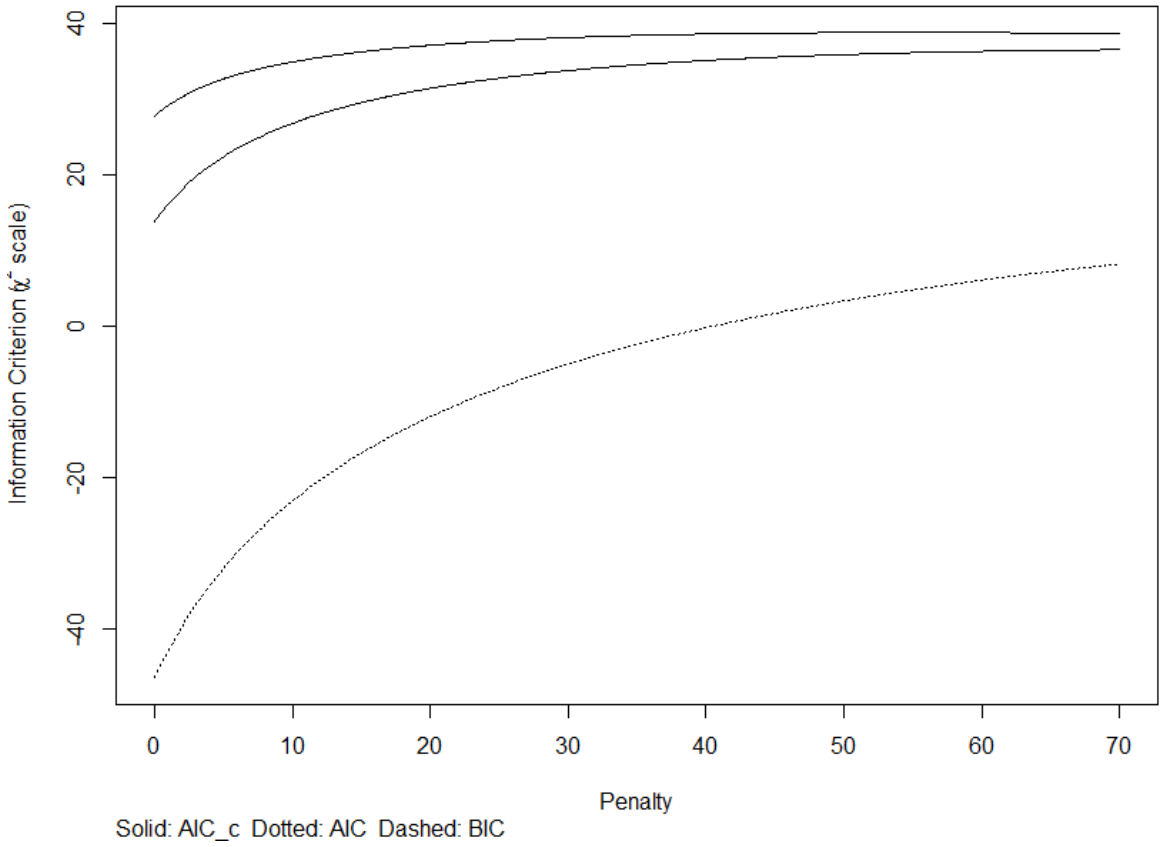

Figure 6. Information criterions for the multivariate model predicting the CAP value. Information criterions for the model predicting the CAP values are shown on the y-axis, and the degree of penalty is shown on the x-axis. The Aikake Information Criterion (AIC-upper curve), Hurvich and Tsai's corrected AIC (AIC_c-middle curve), and Schwarz Bayesian Information Criterion (BIC-lower curve) provided the lowest value without penalty.

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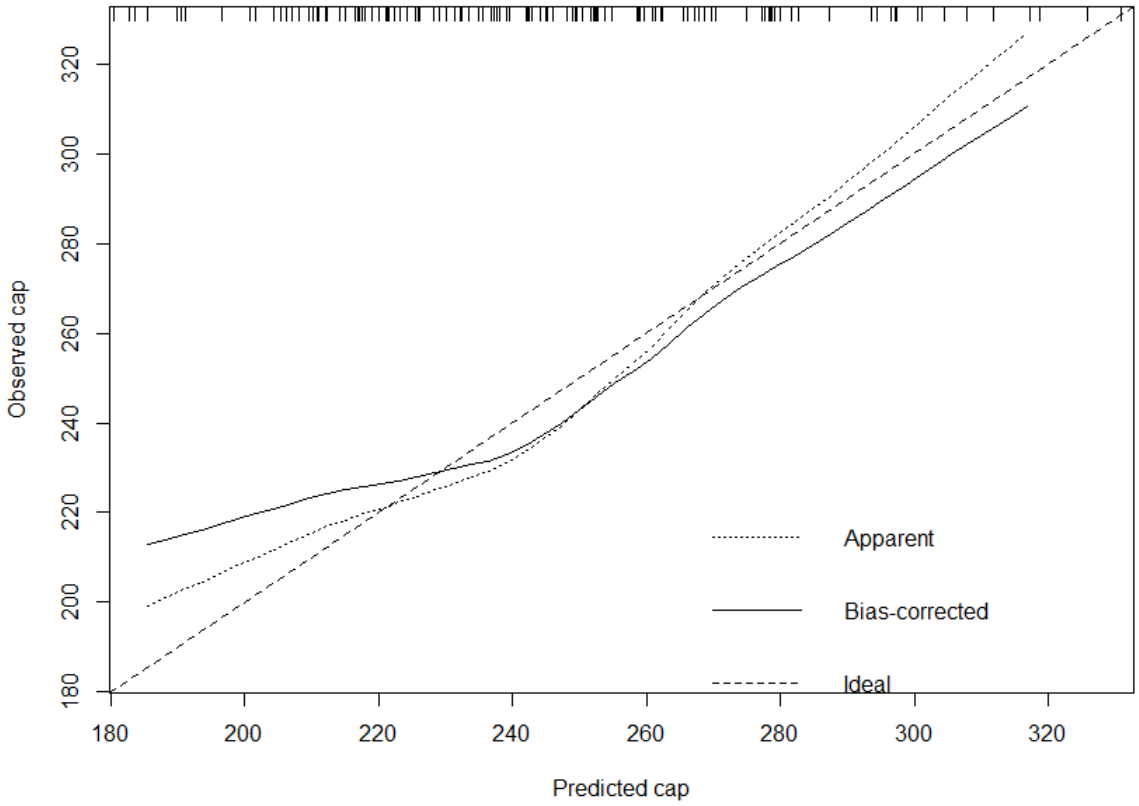

Figure 7. Bootstrap overfitting-corrected nonparametric calibration curve of the model predicting the CAP value. The horizontal axis is the prediction of CAP. The vertical axis is the observed CAP. The dashed line is the identity line. The dotted line is the apparent model performance. The solid line is the bias-corrected (overfitting-corrected) model performance. The optimism-corrected bootstrap validation with 1000 repeats displayed overfitting of the model (R^2=0.4635, optimism corrected R^2=0.0813).

Table 4. Regression coefficients of the covariates predicting the CAP value in the penalized model (R^2=0.41, adjusted R^2=0.322).

Covariate	Regression coefficient	S.E:	t	95% CI	p-value
CD4%	0.064	0.409	0.160	-0.764 to 0.873	0.877

CD8%	-0.231	0.294	-0.790	-0.813 to 0.35	0.433
Age	0.229	0.319	0.720	-0.401 to 0.856	0.474
BMI	3.940	0.996	3.960	1.969 to 5.910	<0.001
CD4/8 ratio	2.184	11.239	0.190	-20.053 to 24.422	0.846
Triglyceride	2.506	1.354	1.850	-0.171 to 5.185	0.067
Cholesterol	0.753	2.117	0.360	-3.435 to 4.942	0.723
Disease duration	0.611	0.537	2.160	-0.450 to 1.673	0.257
Liver Stiffness	0.359	0.802	0.450	-1.2265 to 1.945	0.655
Diabetes	19.097	11.136	1.710	-2.938 to 41.132	0.089
Hypertension	16.557	7.662	0.450	1.396 to 31.718	0.033
Lipodystrophy	3.397	11.027	0.310	-18.424 to 25.217	0.759
Darunavir	-9.986	8.277	-1.210	-26.364 to 6.393	0.230
Atanazavir	-2.841	12.724	-0.220	-28.021 to 22.338	0.824
Raltegravir	-4.150	9.463	-0.440	-22.876 to 14.576	0.662
Etravirine	-0.975	12.643	-0.080	-25.993 to 24.044	0.939
Nevirapine	1.397	7.698	0.180	-13.837 to 16.63	0.856
Efavirenz	0.459	7.420	0.060	-14.224 to 15.141	0.951
Tenofovir	2.086	6.585	0.320	-10.94 to 15.117	0.752
Abacavir	2.822	8.486	0.330	-13.971 to 19.615	0.740
Zidovudine	-1.635	6.867	-0.240	-15.222 to 11.953	0.812
Lamivudine	5.698	9.851	0.580	-13.769 to 25.191	0.564
Lopinavir	8.807	8.132	1.080	-7.285 to 24.898	0.281
HBV positivity	-2.810	8.061	-0.350	-18.761 to 13.144	0.728
HCV positivity	-7.747	10.423	-0.740	-28.373 to 12.879	0.459
Alcohol	-4.863	17.711	-0.270	-39.909 to 30.183	0.784

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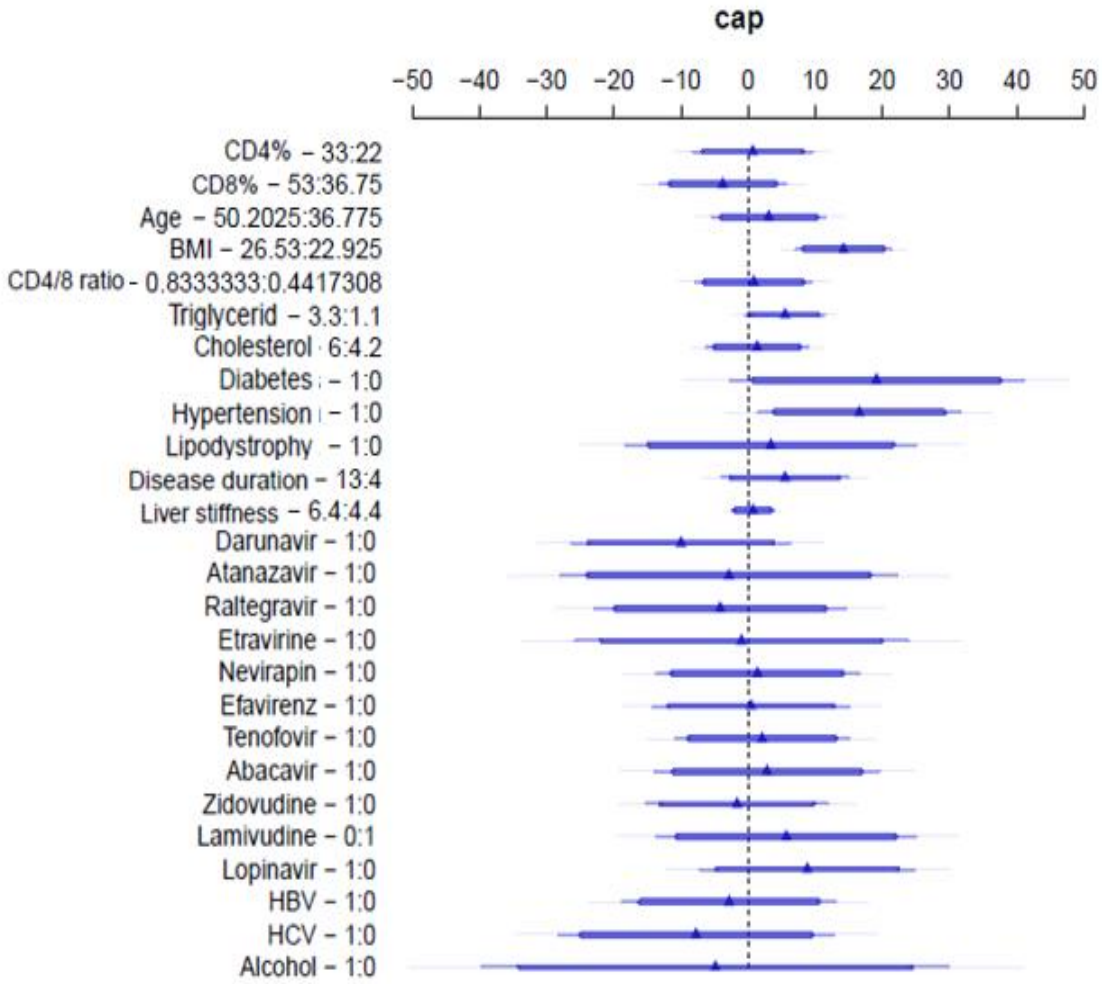

Fig. 8. Multivariate analysis: covariates with regression coefficients and confidence intervals for the penalized model. The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as the change in the modal category, and for continuous variables, it is a change of 1 IQR. In each case, this is explicitly indicated by two values that are separated by a colon after the variable. BMI is expressed in kg/m^2; age and length of known HIV positivity are expressed in years; and liver stiffness is expressed in kPa. ART, antiretroviral therapy; CAP, controlled attenuation parameter (dB/m); lipodystrophy, facial lipodystrophy. The thick dark blue lines represent 90% CIs, the thick light blue lines 95% CIs and the narrow light blue lines 99% CIs.

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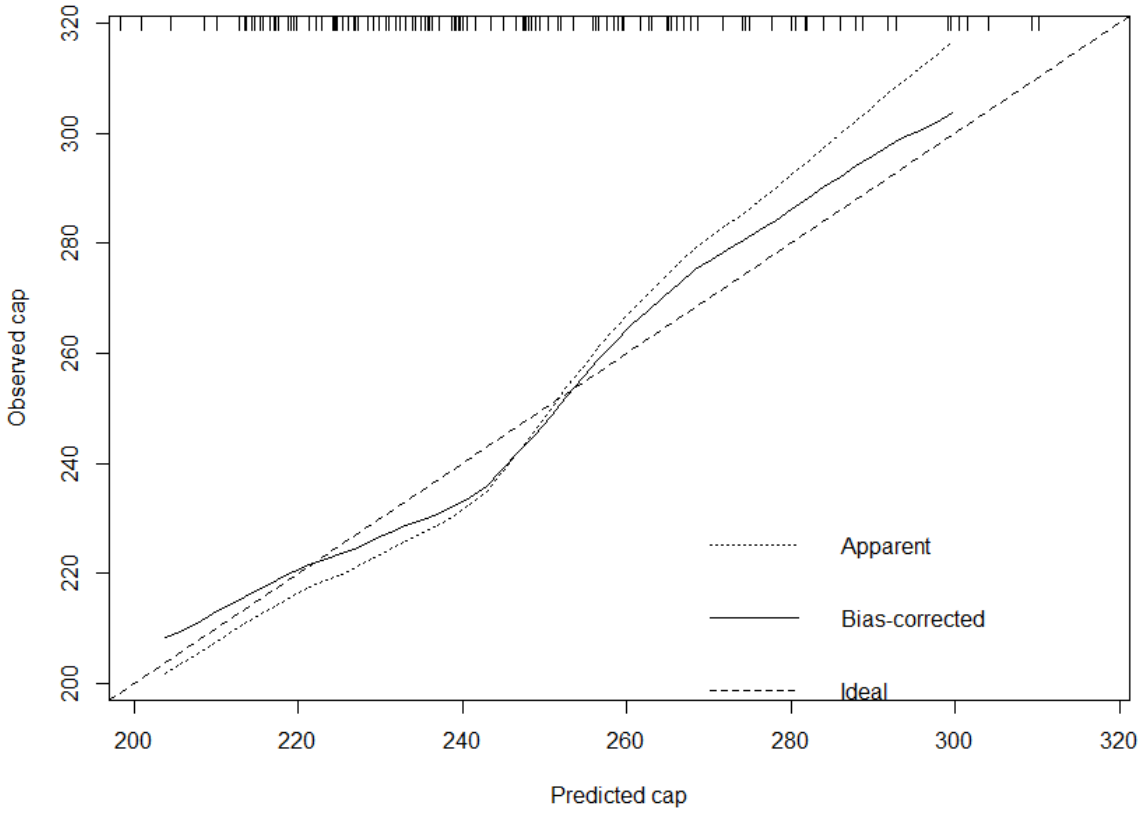

Figure 9. **Bootstrap overfitting-corrected nonparametric calibration curve of the penalized model predicting the CAP value**. The horizontal axis is the prediction of CAP. The vertical axis is the observed CAP. The dashed line is the identity line. The dotted line is the apparent model performance. The solid line is the bias-corrected (overfitting-corrected) model performance. Optimism-corrected bootstrap validation with 1000 repeats showed improvement in overfitting of the model (R^2=0.4104, optimism corrected R^2=0.2698)

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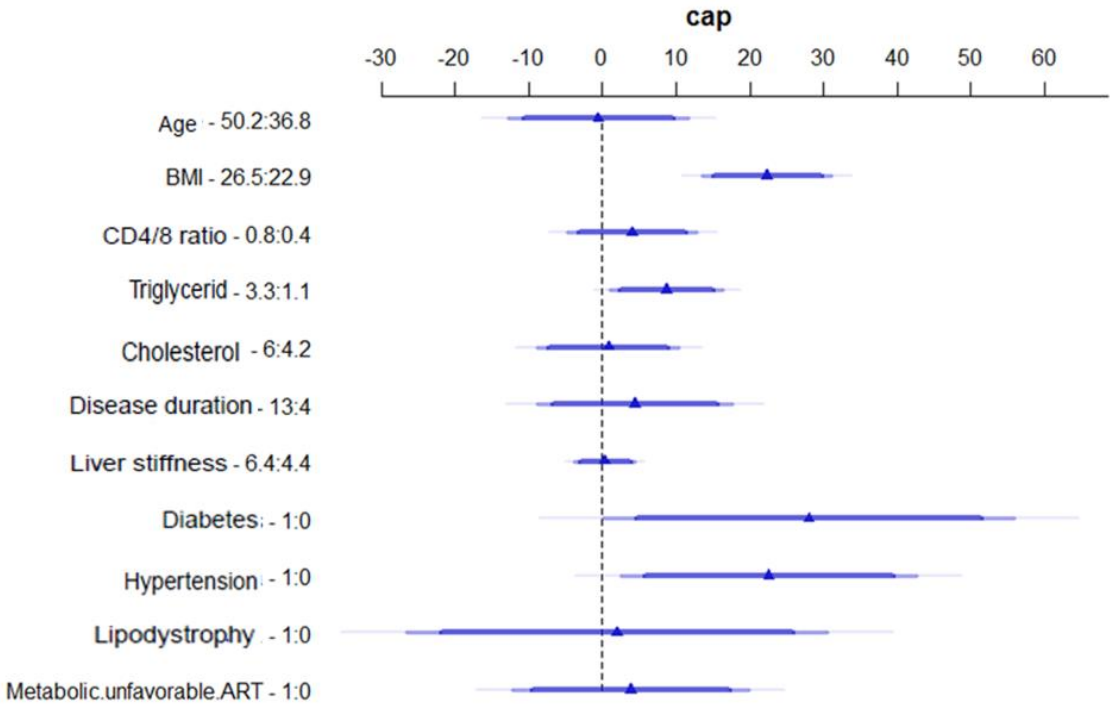

Figure 10. Multivariate analysis after covariate selection: covariates with regression coefficients and confidence intervals. The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as the change in the modal category, and for continuous variables, it is a change of 1 IQR. In each case, this is explicitly indicated by two values that are separated by a colon after the variable. BMI is expressed in kg/m^2; age and length of known HIV positivity are expressed in years; and liver stiffness is expressed in kPa. ART, antiretroviral therapy; CAP, controlled attenuation parameter (dB/m); lipodystrophy, facial lipodystrophy. The thick dark blue lines represents 90% CIs, the thick light blue lines 95% CIs and the narrow light blue lines 99% CIs.

4.4 Univariate analysis of the association between liver stiffness and different variables

A significant Kendall-τ-rank correlation was identified between the LS and CAP value (adjusted $p<0.0001$) and body mass index (adjusted $p<0.0001$). A non-significant but remarkable association was detected with age (adjusted $p=0.203$). With regard to categorical variables, no significant associations were identified, and the most

pronounced correlation was the presence of arterial hypertension (adjusted p>1). Associations between liver stiffness and different continuous and categorical variables are presented in **Table 5** and **Figure 11.**

Table 5. Univariate analysis: associations between the liver stiffness value and continuous (panel A) and categorical (panel B) variables.

Panel A. Results are presented as the mean (median) ± SD (IQR) [minimum–maximum]. The p-value pertains to the null hypothesis of no correlation; p-values are unadjusted.

[a] Controlled attenuation parameter

Continuous variable	**Linear**		**Kendall**	
	r	*p*	τ	*p*
CD4%	-0.087	0.387	0.008	0.902
CD8%	0.075	0.453	-0.018	0.789
CD4/8 ratio	-0.106	0.291	-0.003	0.960
Triglyceride	0.026	0.790	0.079	0.251
Cholesterol	0.028	0.782	0.059	0.392
Age	0.285	0.004	0.185	0.007
BMI	0.255	0.010	0.261	<0.0001
Disease duration	0.147	0.146	0.126	0.074
CAP[a]	0.226	0.023	0.295	<0.0001

Panel B. Results are presented as the mean (median) ± SD (IQR) [minimum–maximum]. The p-value pertains to the null hypothesis of stochastic equivalence for the two populations (presence/absence). p-values are unadjusted.

[a] Antiretroviral therapy

Categorical	**Presence of variable[a]**	**Absence of variable[a]**	**p-value**
Diabetes	6.96 (6.3) ± 3.31 (2.65) [3.9-15.3]	5.5 (5) ± 3.32 (1.6) [3-34.3]	0.063
Sex	4.66 (4.9) ± 0.58 (0.55) [4-5.1]	5.69 (5.15) ± 3.38 (1.85) [3-34,3]	0.446
Hypertension	6.11 (5.4) ± 2.44 (1.5) [4-15.3]	5.51 (4,9) ± 3.57 (1.77) [3-34.3]	0.045
Lipodystrophy	5.28 (5.05) ± 0.98 (1.55) [4-6.9]	5.71 (5.1) ± 3.53 (1.7) [3-34.3]	0.821
Darunavir	5.64 (5.3) ± 1.71 (1.97) [3.5-10.2]	5.66 (5) ± 3.63 (1.7) [3-34.3]	0.410
Atanazavir	5.32 (5.2) ± 1.26 (1.6) [3.6-7.3]	5.68 (5.05) ± 3.44 (1.7) [3-34.3]	0.840
Lopinavir	6.73 (5) ± 6.08 (1.8) [3.6-34.3]	5.29 (5.1) ± 1.41 (1.7) [3-10.2]	0.652
Raltegravir	6.15 (5) ± 3.74 (0.65) [3.9-15.3]	5.62 (5.2) ± 3.31 (1.9) [3-34.3]	0.914
Lamivudine	5.74 (5.1) ± 3.49 (1.8) [3.1-34.3]	5.06 (4.4) ± 1.77 (1.85) [3-9.3]	0.221
Tenofovir	6.33 (5.3) ± 5.13 (2.12) [3.1-34.3]	5.26 (5) ± 1.34 (1.65) [3-10.2]	0.548
Abacavir	5.59 (5.8) ± 1.83 (2) [3.6-10.2]	5.67 (5) ± 3.51 (1.75) [3-34.3]	0.819
Zidovudine	5.51 (4.9) ± 2.01 (1.65) [3.7-15.3]	5.76 (5.15) ± 3.96 (1.85) [3-34.3]	0.941

Etravirine	4.93 (4.8) ± 1.04 (1.9) [3.6-6.3]	5.73 (5.1) ± 3.47 (1.75) [3-34.3]	0.424
Nevirapine	5.28 (5.3) ± 1.14 (1.8) [3.6-7.4]	5.76 (5) ± 3.72 (1.7) [3-34.3]	0.853
Efavirenz	5.42 (5.3) ± 1.44 (2.35) [3.1-8.8]	5.75 (5.05) ± 3.80 (1.67) [3-34.3]	0.590
ART[a] ever taken	5.27 (5.1) ± 1.92 (1.62) [3-9.3]	5.69 (5.1) ± 3.43 (1.9) [3.1-34.3]	0.605

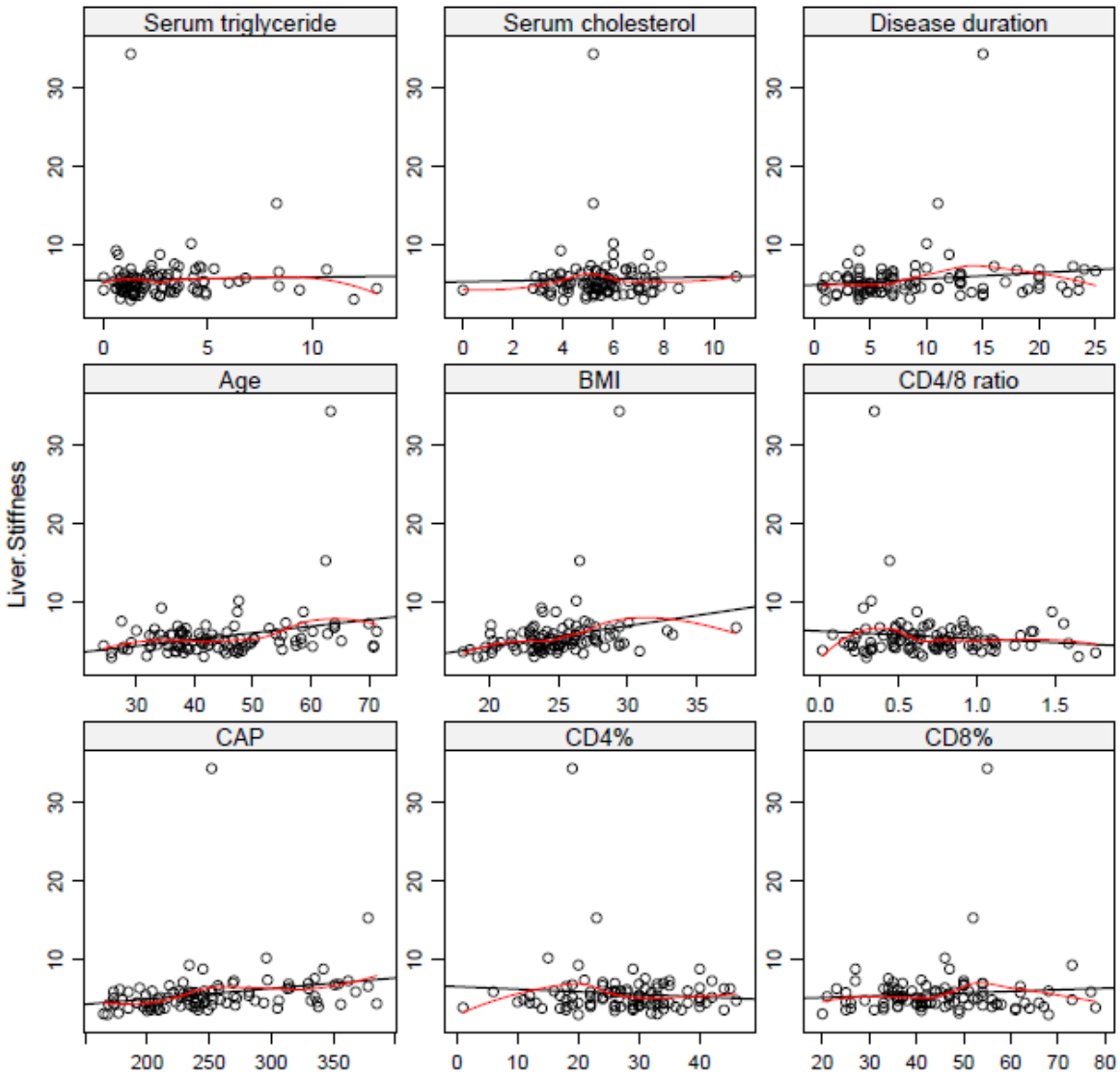

Figure 11. Correlations between continuous variables and liver stiffness. The black line shows the best-fitting linear curve, and the red line shows the LOWESS-smoother

for nonparametric regression. Liver stiffness is expressed in kPa, BMI in kg/m^2; age, length of known HIV positivity in years, and CAP in dB/m. Linearity is clearly justified by matching the LOWESS-smoother and the best-fitting linear curve.

4.5 Multivariate regression models predicting liver stiffness

Non-linearity was deemed unnecessary (p=0.5658) in the multivariate model. The linear regression identified BMI, age and the history of taking lopinavir as independent positive covariates. A history of taking zidovudine over the course of ART and the presence of lipodystrophy were independent negative covariates. Nevertheless, the model exhibited a weak fit. The regression coefficients and confidence intervals are summarized in **Table 6** and are presented graphically in **Figure 12.** To improve goodness-of-fit and to address the problem of multidimensionality (**Figure 13, Figure 14)**, we penalized the model. Although general model parameters improved, the model still exhibited poor fitting, and no significant covariates were identified (**Table 7** and **Figure 15 and Figure 16)**.

In the alternative model, CD4% and CD8% were removed (CD4% VIF=4.99; CD8%-VIF=4.86; CD4/8 ratio VIF=10.10 in the unpenalized model) and "Metabolic favorable ART" was introduced instead of different types of antiretrovirals. Age was identified as a significant positive factor (p=0.036; regression coefficient: 0.09; 95% CI 0.006-0.1736), and the CD4/8 ratio (p=0.0313; regression coefficient-2.1942; 95% CI -4.1886 to -0.2017) and the presence of lipodystrophy (p=0.0429; regression coefficient -2.7524; 95% CI -5.414 to 0.0898) were identified as significant negative covariates. However, the model again performed poorly (R2=0.2134, optimism-corrected R^2=-0.099). Therefore, penalization was conducted, which improved the goodness-of-fit (optimism-corrected R^2=0.0497), but significant covariables were no longer identifiable.

Table 6. Multivariate model predicting liver stiffness

[a] Controlled attenuation parameter

Covariate	Regression coefficient	S.E.	t	95% CI	p-value
CAP[a]	-0.005	0.009	-0.500	-0.0225 to 0.0134	0.615

Covariate	Regression coefficient	S.E.	t	95% CI	p-value
CD4	0.043	0.082	0.530	-0.1198 to 0.2055	0.601
CD8	0.010	0.057	0.170	-0.1036 to 0.1235	0.862
Age	0.096	0.047	2.030	0.0018 to 0.1899	0.046
BMI	0.336	0.145	2.320	0.0466 to 0.6262	0.024
CD4/8 ratio	-2.610	2.893	-0.900	-8.3810 to 3.1604	0.370
Triglyceride	-0.058	0.161	-0.360	-0.3787 to 0.2634	0.722
Cholesterol	-0.028	0.245	-0.120	-0.5170 to 0.4603	0.908
Diabetes	1.729	1.326	1.300	-0.9161 to 4.374	0.197
Hypertension	0.180	0.981	0.180	-1.7775 to 2.1365	0.855
Lipodystrophy	-3.694	1.384	-2.670	-6.4539 to -0.9334	0.010
Disease duration	0.046	0.889	0.460	-1.3629 to 2.1857	0.645
Darunavir	-2.097	1.134	-1.850	-4.3585 to 0.1651	0.069
Atanazavir	-1.813	1.606	-1.130	-5.0176 to 1.3912	0.263
Raltegravir	-1.035	1.372	-0.750	-3.7713 to 1.7008	0.453
Etravirine	-2.088	1.354	-1.540	-4.7894 to 0.6133	0.128
Nevirapine	-0.536	1.081	-0.500	-2.6922 to 1.6206	0.622
Efavirenz	-0.552	0.957	-0.580	-2.4619 to 1.3575	0.566
Tenofovir	0.972	0.873	1.110	-0.7687 to 2.7123	0.269
Abacavir	1.401	1.151	1.220	-0.8958 to 3.6975	0.228
Zidovudine	-2.016	0.901	-2.240	-3.8131 to -0.2178	0.029
Lamivudine	0.907	1.349	0.670	-3.5973 to 1.783	0.503
Lopinavir	2.459	0.993	2.480	0.4785 to 4.4387	0.016

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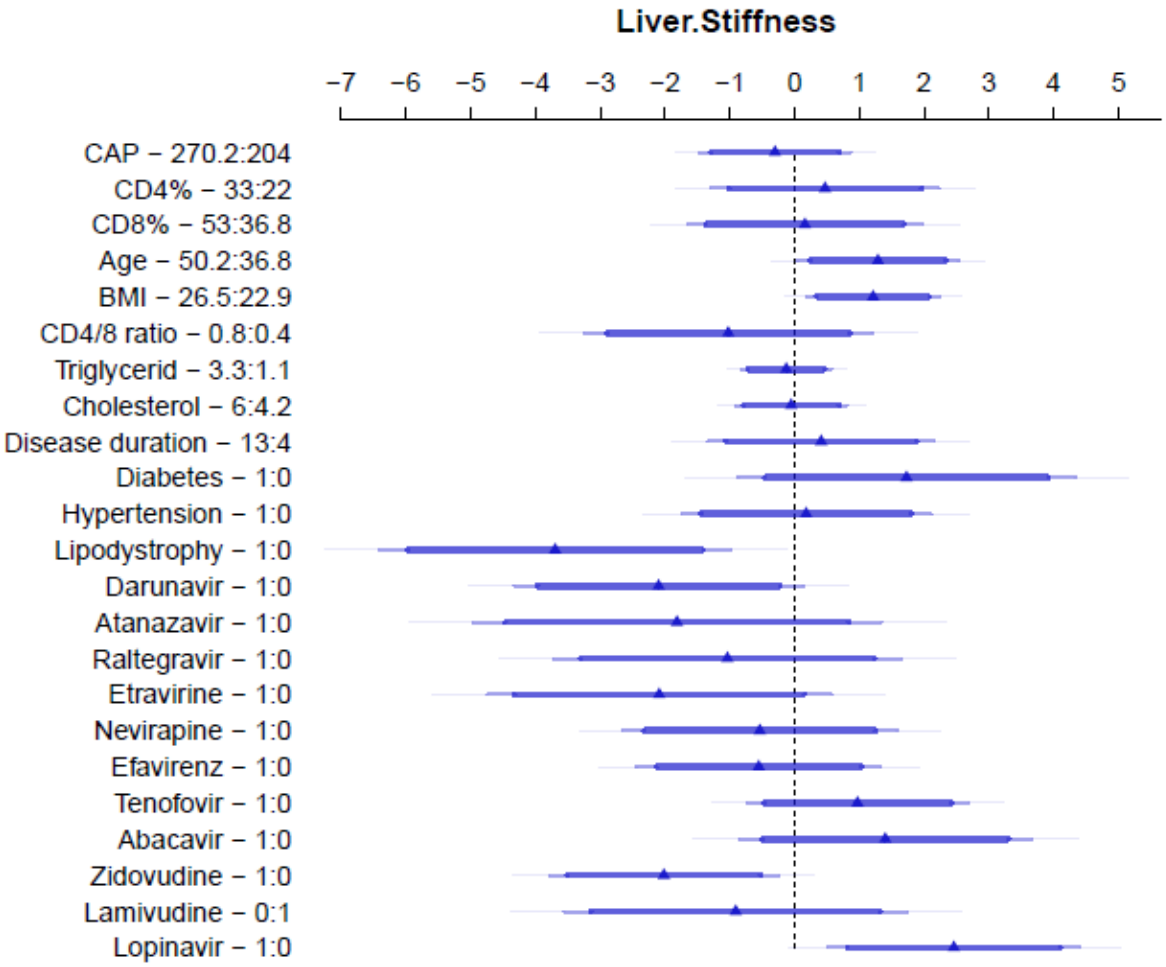

Figure 12. Multivariate analysis: covariates with regression coefficients and confidence intervals of the model predicting liver stiffness. The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as the change in the modal category, and for continuous variables, it is a change of 1 IQR. In each case, this is explicitly indicated by two values that are separated by a colon after the variable. BMI is expressed in kg/m^2; age and the length of known HIV positivity are expressed in years; and liver stiffness is expressed in kPa. ART, antiretroviral therapy; CAP, controlled attenuation parameter (dB/m); lipodystrophy, facial lipodystrophy. The thick dark blue lines represents 90% CIs, the thick light blue lines 95% CIs and the narrow light blue lines 99% CIs.

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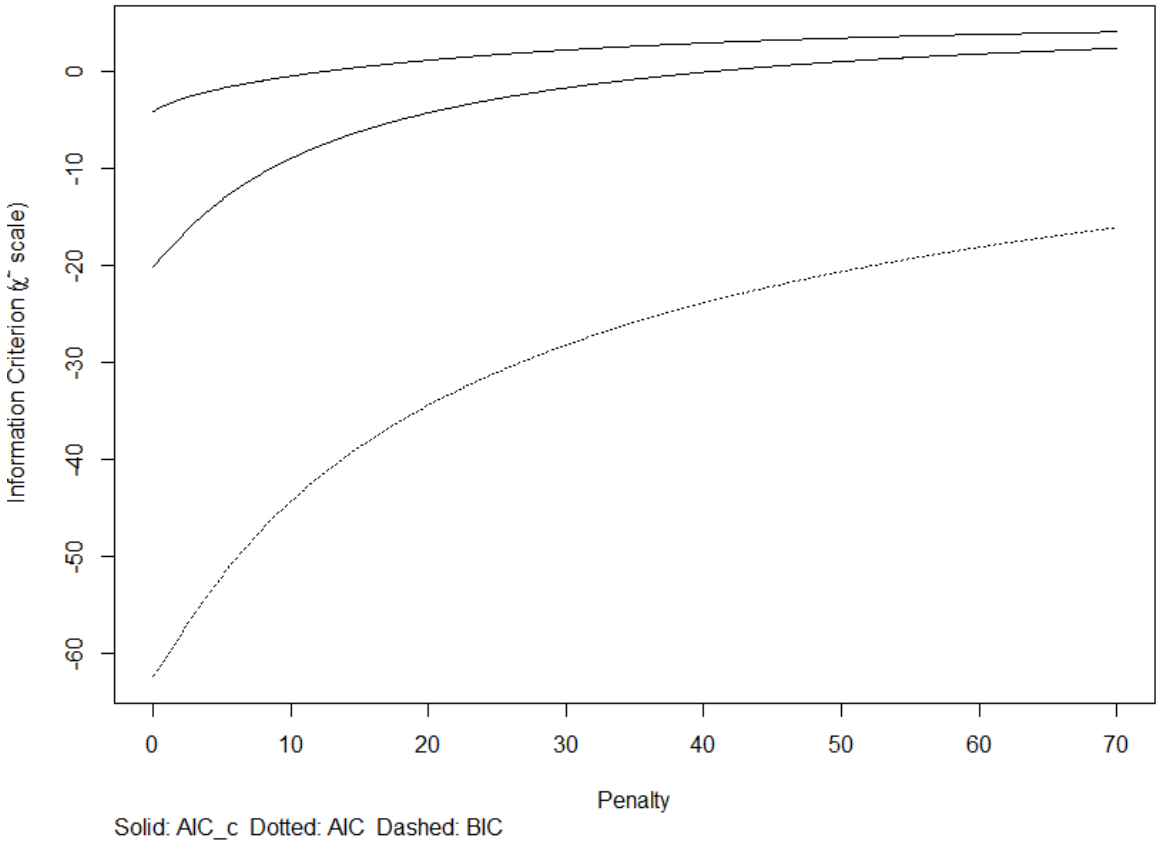

Figure 13. Information criterions for the multivariate model predicting liver stiffness. Information Criterions for the model predicting liver stiffness are shown on the y-axis, and the degree of penalty is shown on the x-axis. The Aikake Information Criterion (AIC-upper curve), Hurvich and Tsai's corrected AIC (AIC_c-middle curve) and Schwarz Bayesian Information Criterion (BIC-lower curve) show the lowest value without penalty.

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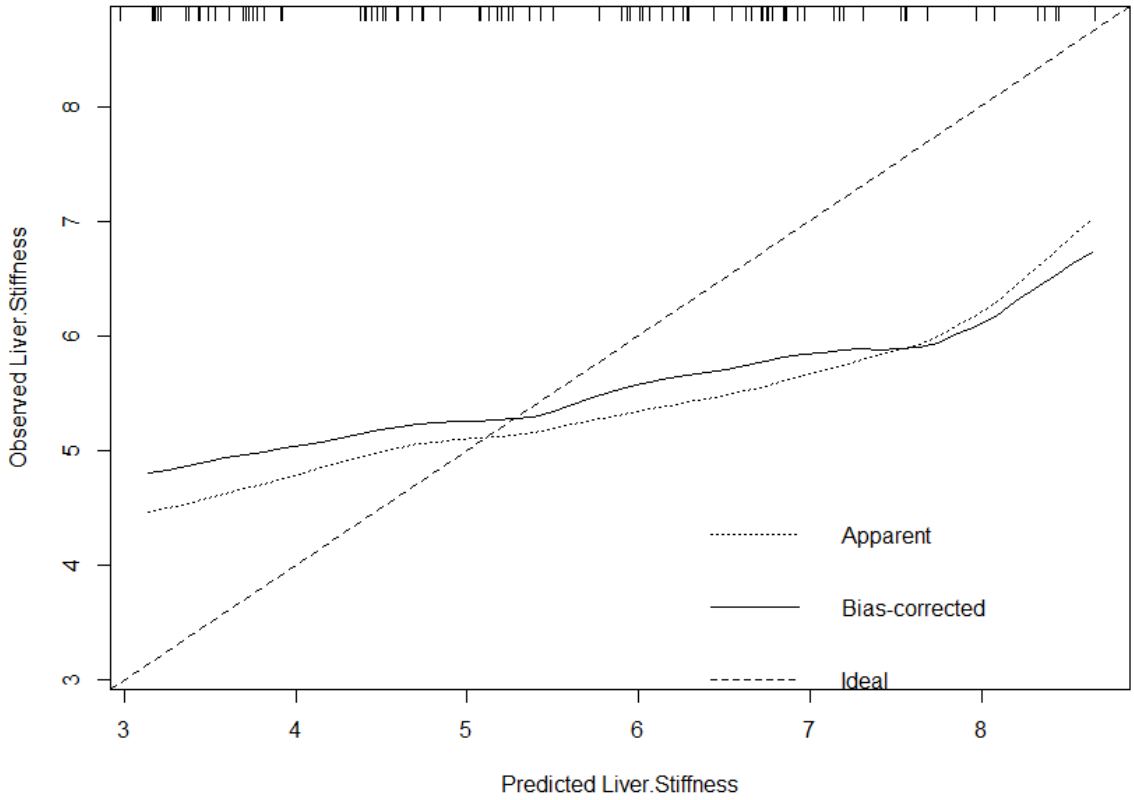

Figure 14. Bootstrap overfitting-corrected nonparametric calibration curve of the model predicting liver stiffness. The horizontal axis represents the prediction of liver stiffness. The vertical axis is the observed liver stiffness. The dashed line is the identity line. The dotted line is the apparent model performance. The solid line is the bias-corrected (overfitting-corrected) model performance. Optimism-corrected bootstrap validation with 1000 repeats showed overfitting of the model (R^2=0.362, optimism corrected R^2=-0.1856).

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Table 7. Multivariate penalized model predicting liver stiffness.

[a] Controlled attenuation parameter

	Regression coefficient	S.E.	t	95% CI	p-value
CAP	0.005	0.005	0.920	-0.0054 to 0.0146	0.362
CD4	-0.019	0.032	-0.600	-0.0836 to 0.0448	0.550
CD8	0.011	0.023	0.490	-0.0343 to 0.0567	0.625
Age	0.042	0.025	1.670	-0.0078 to 0.0909	0.098
BMI	0.122	0.084	1.460	-0.0438 to 0.2877	0.148
CD4/8 ratio	-0.644	0.851	-0.760	-2.3344 to 1.0461	0.451
Triglyceride	-0.018	0.102	-0.170	-0.2208 to 0.1853	0.863
Cholesterol	-0.011	0.169	-0.060	-0.3468 to 0.3257	0.950
Diabetes	0.162	0.489	0.330	-0.0779 to 0.1069	0.742
Hypertension	0.004	0.460	0.010	-0.8097 to 1.1334	0.994
Lipodystrophy	-0.366	0.493	-0.740	-0.9097 to 0.9171	0.460
Disease duration	0.015	0.047	0.310	-1.344 to 0.6128	0.756
Darunavir	-0.162	0.467	-0.350	-1.0893 to 0.7659	0.730
Atanazavir	-0.095	0.507	-0.190	-1.1019 to 0.9110	0.851
Raltegravir	-0.005	0.497	-0.010	-0.9916 to 0.9817	0.992
Etravirine	-0.177	0.493	-0.360	-1.1565 to 0.8017	0.720
Nevirapine	-0.159	0.454	-0.350	-1.0614 to 0.7432	0.727
Efavirenz	-0.182	0.446	-0.410	-1.0667 to 0.7031	0.684
Tenofovir	0.392	0.432	0.910	-0.4653 to 1.2495	0.366
Abacavir	0.091	0.475	0.190	-0.8516 to 1.0333	0.849
Zidovudine	-0.282	0.436	-0.650	-1.1468 to 0.5831	0.519
Lamivudine	0.063	0.483	0.130	-1.0223 to 0.8969	0.897
Lopinavir	0.420	0.451	0.930	-0.4760 to 1.3161	0.355

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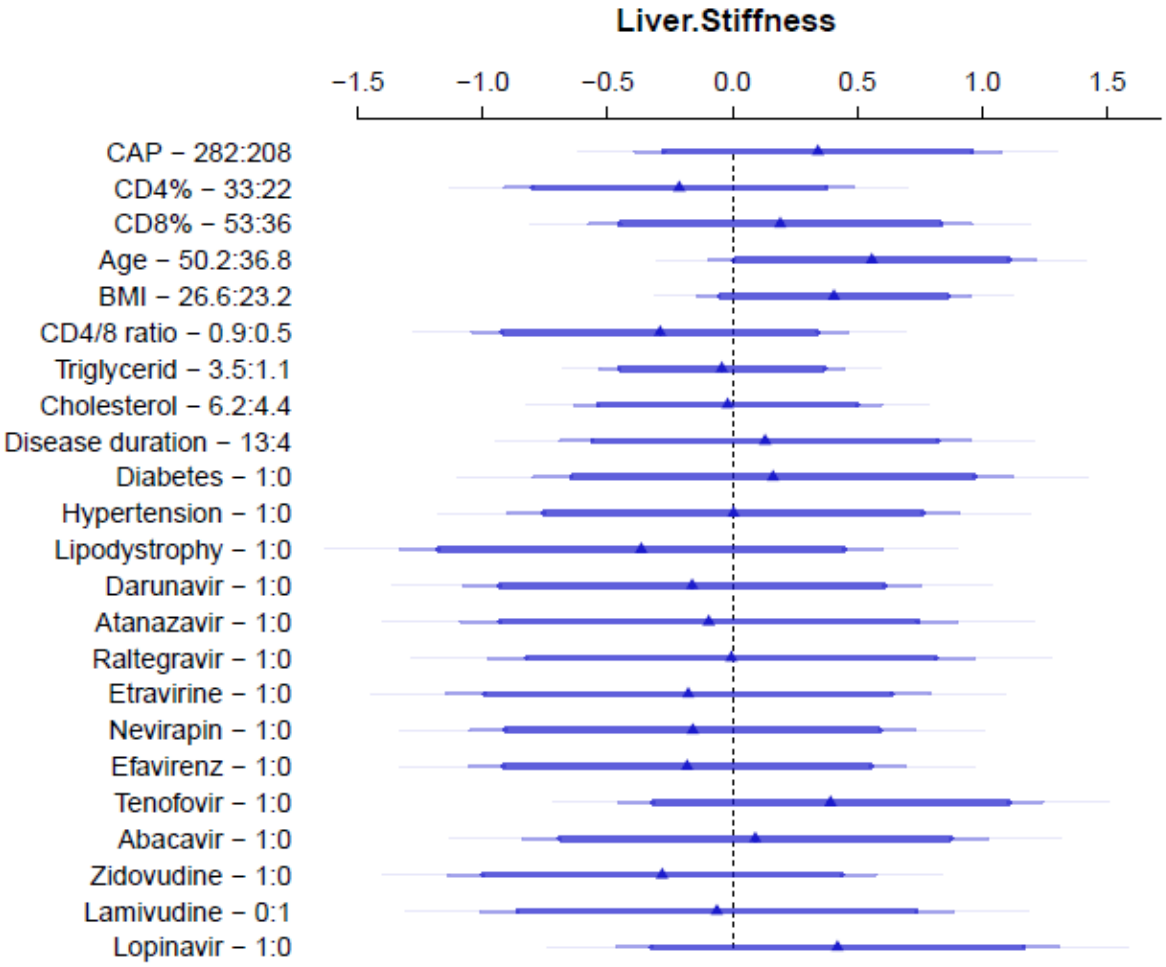

Figure 15. Multivariate analysis: covariates with regression coefficients and confidence intervals for the penalized model using liver stiffness as the response variable. The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as the change to the modal category, and for continuous variables, it is a change of 1 IQR. In each case, this is explicitly indicated by two values that are separated by a colon after the variable. BMI is expressed in kg/m^2; age and the length of known HIV positivity are expressed in years; and liver stiffness is expressed in kPa. ART, antiretroviral therapy; CAP, controlled attenuation parameter (dB/m); lipodystrophy, facial lipodystrophy. The thick dark blue lines represent 90% CIs, the thick light blue lines 95% CIs and the narrow light blue lines 99%.

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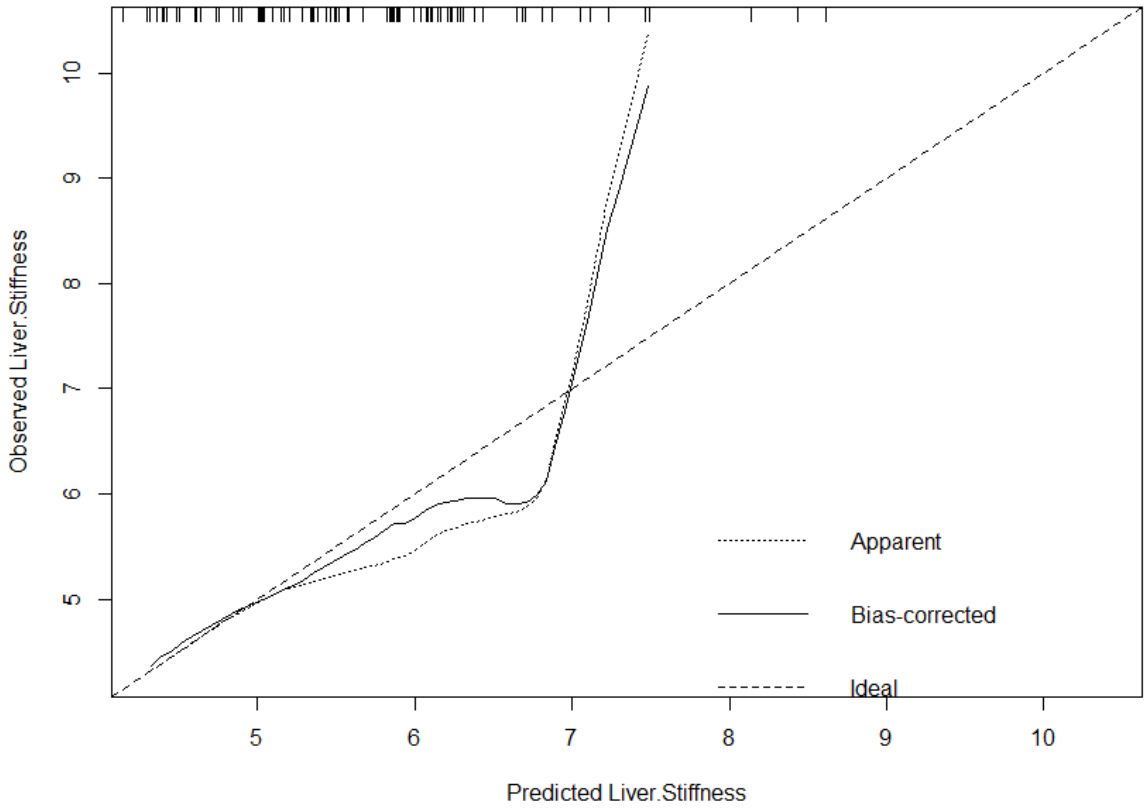

Figure 16. Bootstrap overfitting-corrected nonparametric calibration curve of the penalized model predicting liver stiffness. The horizontal axis shows the prediction of liver stiffness. The vertical axis is the observed liver stiffness. The dashed line is the identity line. The dotted line is the apparent model performance. The solid line is the bias-corrected (overfitting-corrected) model performance. Optimism-corrected bootstrap validation with 1000 repeats showing overfitting of the model (R^2=0.1949, optimism corrected R^2=0.0525).

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5 Discussion

To the best of our knowledge, CAP had only been used in one other study performed in a large, unselected group of HIV-infected individuals to evaluate HS at the time of the publication our results (118). Using the same cutoff value (238 dB/m) and a methodology similar to our analysis, they detected HS in 40% of the participants (118). Our cross-sectional design resulted in similar findings, and we identified significant HS in 47.8% of the individuals living with HIV. However, Macías *et al*. used a dichotomized endpoint (the presence of significant HS). In contrast, we used a continuous scale of CAP values in the multivariate regressions for an HIV-infected population. One of the main advantages of CAP in comparison to other methods is that the quantitative measurement of HS can be integrated without losing information (117). Most recently, a longitudinal cohort was performed in a population of 326 PLWH (154). After 12 months, the baseline 37% prevalence of significant HS (>238 dB/m) increased to 39% (154).

Most studies assessing HS in HIV-infected patients enrolled a selected population with HCV co-infection. These studies, based on LB, reported a wide range of HS prevalence (11-72%) (72, 74, 76, 155-157). A meta-analysis demonstrated HS in 40% of HIV/HCV patients assessed with LB (73). Of note, LB is more likely to be performed in patients who require anti-HCV treatment or have more progressed liver disease; therefore, the results could have been influenced by selection bias (118). Two recent studies using ultrasonography described a 54% and 52% prevalence in a subset of co-infected patients (71, 72).

Few studies have assessed HS in HIV-mono-infected patients. The prevalence of HS was 31% and 52% in five studies using ultrasonography (71, 94, 111, 158) and 37% in another study using CT (94). Ingiliz *et al*. reported a 60% prevalence that was evaluated with LB in participants with persistent liver enzyme elevation (159). However, there are considerable concerns regarding the heterogeneity of the study population and the methods applied in these surveys. In another recently published cross-sectional study in HIV-mono-infected individuals, NAFLD was identified in 48% of the participants using (160) CAP measurements.

Similar to hepatic steatosis, only a limited number of published studies have assessed liver stiffness in HIV-infected patients without HBV or HCV infection. In these publications the prevalence of liver fibrosis ranged from 11% to 42% using different cutoff values (84, 85, 88, 90, 92, 160). The highest proportion was reported by Han et al., who identified abnormal LS values in 39/93 (42%) patients on ART for at least 12 months without hepatitis virus co-infection (84). Using the same cutoff value, the proportion of individuals with abnormal LS was even higher in our subgroup of HIV-mono-infected patients without significant alcohol consumption (56/101; 55.44%). Of note, this cutoff was adopted from the general Korean population; therefore, ethnical differences may have influenced these results. In contrast, Merchante *et al.* (88) identified 29/258 (11.2%) patients in their study population with significant liver fibrosis (cutoff >7.2 kPa). In the prospective study of Rivero-Juarez *et al.*, the incidence of significant LF among HIV-infected individuals with liver damage of uncertain origin was reported to be 10.6% with the same cutoff (89). Applying their cutoff value (>7.2 kPa), we obtained similar results: abnormal LS values were detected in 10/101 (9.9%) individuals in the subgroup. In a large study published by Mohr et al., a 10% prevalence of significant liver fibrosis was obtained with VTCE among 343 HIV-mono-infected patients (cutoff of 7.1 kPa) and 432 individuals living with HIV (109). Applying the same cutoff, Vuille-Lessard et al. reported 15% prevalence (160). Our results for the prevalence of significant liver fibrosis were very similar to those of published values.

The observed outlier value in one participant in the HIV-mono-infected group refers to an advanced liver disease of unknown origin. Similarly, other observational studies in the HIV mono-infected population also identified individuals with high grade fibrosis and even with cryptogenic cirrhosis (88, 90). Recently, cirrhosis was identified in 5.2% percent of the HIV mono-infected patients (defined as LS>10.3 kPa) compared to the 0.6% of the uninfected control group (90).

Nevertheless, these diverse results underline the importance of identifying better cutoff values for HIV-mono-infected patients. The most reliable method to achieve this goal would be to perform a liver biopsy and compare the results with those of transient elastography. Nevertheless, to our knowledge, no such study has yet been conducted.

Morse et al. verified the cutoff of 7.1 kPa in HIV-mono-infected patients with elevated transaminases undergoing liver biopsy (85), but as previously mentioned, adopting this cutoff for an unselected HIV-mono-infected population may underestimate the true prevalence. The discrepancies in cutoff values may lead to an unreliable estimation of the rate and grade of liver fibrosis. Therefore, instead of dichotomizing our study population to patients with abnormal and normal LS values, we used a continuous scale of LS for further correlation and regression analyses to avoid uncertainty arising from using a pre-defined "abnormal" value as the cutoff.

Regarding the general patient characteristics of the study population, demographic and anthropometric parameters, namely age and BMI, were similar to the findings of observational VTCE studies of large unselected patient groups (84, 109, 118), with the notable exception of the high proportion of male participants in our participant population. Correspondingly, because the HIV transmission route was almost exclusively sexual, the prevalence of HCV co-infection remained low, similar to the reported values among MSM PLWH of 1-12% (161). In contrast, among HIV-infected IVDUs, 72-95% were found to be co-infected with HCV (161). Therefore, our findings are likely explained by the absence of intravenous drug users in our study population. Despite the 24% seropositivity of hepatitis C infection among Hungarian IVDUs (162), HIV remained relatively rare (163). Because illicit intravenous drug use was not reported, it was not included in the analysis. The absence of IVDUs may be related to a general mistrust of Hungarian HIV patients toward the healthcare system and may underscore the use of these addictive substances in this population.

Regarding HBV, the HBsAg seroprevalence (8.08%) was both comparable to the reported values in HIV-infected IVDU (7-10%) and the MSM (9-17%) populations (161). Nevertheless, the identified proportion of patients with HBsAg was slightly higher than the ones identified in the largest observational VTCE studies of unselected HIV-infected patient groups (5-6.7%) (109, 118). If isolated anti-HBc positivity was also taken into account, then roughly one-fifth of the study population had contracted HBV. Nevertheless, the clinical significance of isolated anti-HBc positivity is not well-defined in PLWH (164). However, it does not have an impact on HIV progression of liver-related mortality (165), and individuals with isolated anti-HBc positivity have a significantly

shorter survival than those with positive anti-HBs at baseline (166), suggesting a role of HIV-associated immunologic changes in the presence of this marker. The proportion of HBV DNA positivity among HIV-infected patients with isolated anti-HBc positivity was reported to be 8.3% (164). Based on these findings and the possibility of ongoing abnormal immunologic and inflammatory changes in the liver, we decided to incorporate this variable in the analysis. However, to avoid model overfitting, we combined it with the patient pool of HBsAg, which could be interpreted as an oversimplification of the immunologic effects of different serologic states of HBV infection in this patient population.

Although alcohol consumption in Hungary is one of the highest in the world, with an APC (alcohol per capita consumption) of 13.3 L of pure alcohol annually (167), only a small proportion (2.94%) of the patients reported significant regular alcohol intake. A high index of suspicion should be maintained about the generalizability, especially in terms of data about addictive substance use, of the mode of HIV transmission and hepatitis co-infections, given the possible absence of non-compliance in our study population.

The prevalence of diabetes (9.56%) was somewhat higher than the prevalence in the general adult Hungarian population (7.5%) (168), but it was comparable to the prevalence (7-13%) reported from PI, stavudine and zidovudine-experienced HIV patients. However, whether HIV infection itself increases the risk of diabetes remains unclear (169-173). Interestingly, our identified DM prevalence was higher than the reported values from similar studies (4.4-5%) (109, 118). Although assessing comparability is difficult given the different definitions of DM, our results may reflect an unfavorable metabolic profile of the Hungarian HIV-infected population compared with the Spanish and German HIV-infected populations reported in the aforementioned studies (109, 118), which could be partially explained by the high proportion of zidovudine and PI-exposed individuals and the potential high cumulative exposure to these ARVs.

The prevalence of hypertension portrays a similar profile. In our study, it was similar (21.32%) to the general Hungarian population (22.6%) (174), but we should note that our results likely constitute an underestimation because hypertension was defined by the regular intake of an antihypertensive. Therefore, patients with undiagnosed hypertension

and hypertension not requiring medication were not identified. Of note, studies have shown a wide range of results regarding the prevalence of hypertension in HIV–infected individuals; thus, whether it is more prevalent than in the general population remains unclear (175-181).

In our study, using univariate analysis, ART medications were not significantly associated with steatosis. In the multivariate analysis, darunavir exposure was a significant independent variable with lower CAP values. Darunavir has previously been shown to have a more favorable metabolic profile (especially with regard to serum lipid level changes) than older PIs such as LPV/r (182). Our findings may provide additional support for these observations. It must be emphasized, however, that this association disappeared after penalizing the model, and no other associations with ARV remained significant in the alternative model. Thus, the generalizability of this result remains questionable. In addition, the duration of ARV therapy was not assessed, which could influence the results. Associations between NRTI exposure, particularly dideoxynucleoside analogs, such as didanosine, stavudine or zalcitabine, and hepatic steatosis have been described in a few studies (74, 76, 111, 155, 159). In a study investigating HCV-co-infected patients, the cumulative dideoxynucleoside analogue exposure revealed a significant association with HS progression (74). According to another study, HS was associated with stavudine use in the multivariate analysis in HCV-co-infected patients (155). In our study population, the number of dideoxynucleoside-treated patients was negligible (n=2) (117); thus, we did not include them in our analysis. In contrast, Woreta *et al*. found an association between ART and reduced progression of HS in HIV/HCV-co-infected patients (156); however, most other investigators did not observe such an association (71, 73, 75, 118, 183).

Regarding immune dysregulation, we used the CD4/8 ratio as a surrogate marker. A lower ratio and a higher CD8 percentage are characteristics of ongoing inflammatory processes (184). Most other studies did not find an association between CD4 or CD8 cell counts, or the CD4/8 ratio, and HS (71, 73, 74, 94, 111, 118, 155, 183). Our results were similar because after adjustment, none of these values were significant. Another possible marker of immune activation is the length of known HIV positivity (referred to as ‘disease duration’), which may refer to a cumulative amount of viral replication and may refer to

triggered immunologic alterations. The association of CAP with this value remained significant after adjustment, but this variable may also reflect some other parameters (e.g., ART, lifestyle factors) that raise the possibility of the presence of confounders. With multivariate models and in the alternative model with improved fitting, the association with this value remained non-significant after penalization. Therefore, a reasonable link between CAP and ARVs, or markers of HIV-induced immune activation, cannot be confirmed. Overall, the relationship of immunologic markers, such as lymphocyte percentages and the CD4/8 ratio, length of known HIV positivity and antiretroviral exposure, with HS remains controversial. To better characterize these associations, clinical trials using the CAP value or other methods of liver steatosis detection as the endpoint are needed.

In contrast, metabolic factors showed a strong association with the CAP value. In the univariate analysis, BMI, age, hypertension and serum triglycerides were significantly associated with the CAP measurements. In the multivariate models, BMI remained significant with a narrow 95% CI of the regression coefficients not involving zero. These findings are in accord with the only other similarly published study from Spain performed by Macías *et al*., in which BMI was the only significant independent covariate, with an adjusted odds ratio of 1.34 (95% CI 1.22–1.47; $p<0.001$) (118). Later, BMI was also indentified as the only independent predictor (B (standard error): 9.03 (1.9); $p< 0.001$) of CAP value progression (154). Moreover, in the cross-sectional study performed by Vuille-Lessard et al.(160), BMI also showed the greatest effect size on significant CAP value (adjusted odds ratio 4.86, 95% CI 2.55-9.26

The results for hypertension were also convincing. This covariate was independently associated with CAP in all multivariate models. Nonetheless, in the alternative model, the 95% CI of the regression coefficient was too wide, incorporating zero. In this model, diabetes and serum triglycerides were detected as independent significant predictors. Similarly, a study performed by Li Vecchi et al. showed that triglyceride levels and the Framingham risk score were independently associated with HS when assessed by ultrasonography (71). Altogether, the association with these factors was not as impressive as with BMI, but they were still independently associated with CAP and should thus be

considered as a main driving force of HS in individuals living with HIV. However, as previously mentioned, prospective studies are needed to address causal relationships.

According to our findings, neither HCV nor HBV co-infection was associated with HS. Previous studies have reported similar results (71, 72, 118), with the notable exception of the survey performed by McGovern *et al*., in which HCV genotype 3 co-infection was identified as a significant covariate (76).

In the subgroup analysis of HIV-mono-infected patients without clinically significant alcohol consumption, significant positive correlations were observed for CAP and BMI in the univariate analysis. However, in the multivariate models, no significant association could be identified if the models were penalized to avoid overfitting. Therefore, an independent association with liver stiffness remains to be determined. Previous studies investigating HIV-mono-infected patients identified an association of metabolic factors, such as central obesity in HIV/HCV co-infection (64), elevated homeostasis model assessment-estimated insulin resistance levels (89), diabetes (160), and the presence of metabolic syndrome (92) with LS. A study using the non-invasive APRI score in 432 HIV-mono-infected patients enrolled in the Center for AIDS Research Database also identified diabetes (adjusted OR, 3.15; 95% CI, 1.12–10.10) and detectable HIV viremia (adjusted OR, 2.56; 95% CI, 1.02–8.87) as independent covariates for significant fibrosis after controlling for active alcohol use and site (87). These results shed light on the possible importance of metabolic conditions in the development of LF, which can be triggered by ongoing HIV replication. However, the relationship between unfavorable metabolic conditions, such as insulin resistance, is increasingly observed in persons infected with HIV, and viral replication is unclear and must be established (63).

Data suggesting direct HIV-induced effects on the pathogenesis of fibrosis generation have mainly been described in patients with HIV/HCV co-infection (63), but the mechanism has not been precisely determined. In HCV-infected patients, the CD4/CD8 ratio as a contributing factor to liver fibrosis has also been considered (185). CD4 cells can stimulate anti-fibrotic NK cell activity; thus, the loss and impaired activity of CD4 cells may contribute to the progression of liver fibrosis (63). Age has also been previously

identified as a predictor of fibrosis (88) (adjusted OR 1.05, 95% confidence interval [CI] 1.002-1.1; p=0.004). The CAP value has demonstrated a significant linear correlation in a univariate analysis, and it was also detected in the whole study population (117). However, in the other study by Macías *et al.* measuring CAP values in an HIV-infected population, no association was observed between LS and abnormal CAP values (>238 dB/m) (118). This finding was confirmed by others later (154, 160). The presence of steatosis is not unexpected in HIV-mono-infected patients with liver fibrosis. The prevalence of hepatic steatosis confirmed by LB was 66.6% in HIV-mono-infected patients with abnormal liver stiffness and liver damage of unknown origin (>7.2 kPa) (89).

With regard to ART, no ARV was found to be significantly associated with LS. Han *et al.* found that the cumulative exposure to boosted PIs was a significant independent negative predictor [OR 0.941, 95% CI, 0.889–0.997] (84). The authors concluded that ritonavir boosting may provide a protective effect. Considering the metabolic changes associated with boosted protease inhibitors, this result is surprising (63). Contrary, Vuille-Lessard et al. (160) identified current PI use as a risk factor (adjusted OR 3.96, 95% CI 1.64-9.54).

Associations between didanosine and stavudine and liver fibrosis have been previously described (88, 186, 187). An unexpected finding, the significant association between LS and previous abacavir exposure (adjusted OR 3.01, 95% CI 1.18-7.67; p=0.02), has also been obtained (88). The proinflammatory effect of abacavir has been previously described, but the exact mechanisms underlying the induction of liver damage are unclear (19). These results raise questions regarding this ARV that may have causal relationships with LF, but our findings could not support this observation. Clearly, further prospective, controlled trials will be needed to clarify the role of these side effects in the pathogenesis of LF in HIV-infected individuals.

A published analysis performed on the same subgroup of HIV-mono-infected individuals used an alternative approach (188). Bayesian Model Averaging (BMA) (189) provided a high support for age (Posterior Effect Probability-PEP: 84.5%), moderate for BMI (PEP: 49.3%), CD4/8 ratio (PEP: 44.2%) and lipodystrophy (PEP: 44.0%) (188). These findings

overall suggest that age and BMI have a positive association with LS, while CD4/8 ratio and lipodystrophy are rather negatively associated.

These results shed light on the possible importance of ageing, overweight and HIV-induced immune dysregulation in the development of liver fibrosis in the HIV-infected population. Nonetheless, these reported findings. clearly underscore the advantages of using alternative modelling strategies and variable selection methods in small datasets. As we concluded (188): "It is worth contrasting these result with those obtained using traditional linear regression (without variable selection). At 5%, age (p=0.0415), BMI (p=0.0204), presence of lipodystrophy (p=0.0131), history of taking zidovudine (p=0.0442) and lopinavir (p=0.0173) were significant. However while this model has an apparent R^2 of 36%, its realistic - overfitting-optimism corrected - R^2 is practically zero (obtained through bootstrap validation). Thus, regularization was applied - with the penalty parameter selected by Hurvich and Tsai's corrected AIC - which resulted in a realistic model, however, it had no significant variable at all (153). This experiment clearly illustrates the problems of modelling with so limited sample size, and the possible advantages of BMA. In particular for small datasets the effect of model uncertainty can be substantial - this is disrespected in the framework of traditional regression modelling. Variable selection is often employed, however, when it is non-blinded to the outcome, it leads to models that are biased in virtually all of their parameters. For small sample sizes, the sound alternatives - such as regularization - might lead to results that are clinically not meaningful. BMA is a relevant alternative, which avoids these issues by explicitly considering many models."

Our study has considerable limitations, the relatively small sample size of which is the most important**.** However, the proposed sample size was calculated based on a higher expected proportion of HS than observed. Moreover, the calculation did not aid in establishing the required participant numbers for the multivariate analysis, which was particularly problematic in the shrunken subgroup for liver stiffness assessment. Given the numerous covariates, two different approaches were utilized to address overfitting, namely the penalization and post-hoc predictor selection**,** but the models still performed relatively poor, especially in the subgroup analysis. Another important limitation is the possibility of selection bias and significant confounders. In addition, the presence of

diabetes and hypertension was determined by regular intake of antidiabetic and antihypertensive medication. As a result, it was difficult to identify non-compliant subjects and individuals with undiagnosed disease or those who were in the early stages of disease. Therefore, the prevalence of these conditions was likely underestimated. Basic liver function tests, such as transaminases, gamma-glutamyl transferase and alkaline phosphatase, were not assessed. Although it would be desirable to describe a patient subpopulation with biochemical evidence of ongoing liver damage, e.g., NASH patients, the high number of variables compared with the patient number in the multivariate analysis was already concerning. Thus, we focused on factors that could play an etiologic role in hepatic steatosis/fibrosis rather than biochemical markers of the ongoing liver damage. Moreover, other causes of liver disease were not explored in detail, but no patients with previously diagnosed liver diseases, other than viral hepatitis, NAFLD and ALD, were included. A further limitation is that genotype testing or viral load measurement was not performed in the HCV seropositive patients. Molecular methods to detect HBV DNA would be helpful, especially in HIV-infected individuals with isolated anti-HBc positivity. To determine associations with ARVs more accurately, a cumulative exposure to the drug would be more appropriate, but with the potential unreliable character of retrospective data collection, we decided to use the binary variables instead.

The main strength of our study is the use of a non-invasive quantitative assessment of HS in an unselected group of individuals living with HIV. In addition to the novelty of this method, especially in this population, to our knowledge, this was the first study in the literature that did not use CAP cutoff values resulting in information loss. Furthermore, this observational study provided insight into epidemiologic data about the non-AIDS defining metabolic conditions and characteristics of the Hungarian HIV-infected population.

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6 Conclusions

In conclusion, the prevalence of significant HS in HIV-infected patients was high, affecting every second patient in the study population. Significant independent covariates were metabolic factors (BMI, diabetes and hypertension, serum triglycerides). The association was unequivocally the most impressive with BMI. With the exception of an independent negative relation in the unpenalized model with darunavir exposure, ART and ARVs were not significantly associated with CAP values. Thus, our findings reflect the overwhelming importance of metabolic factors in HS. Lifestyle modification, dietary counseling and physical activity are paramount in fighting NAFLD (190) and should be included in the care of HIV patients (53). To better identify the target group of individuals with ongoing liver steatosis and fibrosis, non-invasive CAP and LS measurements with transient elastography could be considered as regular screening methods because TE is already recommended for the annual evaluation of HIV/hepatitis co-infected patients according to the EACS guideline (53). However, the role of other metabolic factors, such as HIV-induced chronic immune activation, in NAFLD should not be underestimated (191), and ART of all HIV-infected individuals, regardless of CD4 cell count, is the cornerstone of prevention of non-AIDS-defining morbidities (52, 53).

Regarding the secondary objectives, according to the general patient population characteristics, diabetes was more prevalent, and other demographic, metabolic and immunologic parameters were comparable to those reported in similar studies. Notable exceptions were the low proportion of HCV-co-infected individuals, patients with significant daily alcohol intake and the absence of IVDUs.

Furthermore, significant hepatic fibrosis (LS >7.1 kPa) was detectable in one out of every ten patients in the subgroup of HIV-mono-infected individuals without significant daily alcohol intake.

We were unable to show any meaningful statistical relationship between LS and any of the analyzed parameters as specified in the exploratory objectives. Therefore, further investigations in larger patient populations will be performed in future analyses.

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Using CAP and LS values as continuous variables offers a valuable tool in clinical studies for detecting even subtile changes in hepatic steatosis and fibrosis. In the everyday, clinical setting of HIV care the regular use of CAP and LS values without cutoffs might also help to coordinate preventive measures. Therefore, as future directions, longitudinal data collection would be desirable. Further prospective studies and the organization of patient registries are warranted to better understand the epidemiology, burden and possible clinical consequences of NAFLD, as this common cause of liver disease in HIV-infected and uninfected individuals is fueled by the ongoing, silent epidemic of obesity.

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7.A Summary

Following the widespread use of combined antiretroviral treatment (ART), the landscape of mortality and morbidity has shifted from AIDS-defining conditions to age-related, metabolic conditions. Liver-related deaths occur ten times more often in the HIV-infected population than in the general population, even in those who are devoid of hepatitis virus co-infection. Long-term antiretroviral medications, HIV-induced immune dysregulation, and metabolic complications may also contribute to liver damage. Fueled by the global epidemic of obesity, non-alcoholic fatty liver disease (NAFLD), which is the most frequent cause of liver disease worldwide, is expected to have a prominent impact on the HIV-infected population; however, available data concerning the prevalence in the unselected HIV-infected population are limited. The aims of this study were to determine the prevalence of hepatic steatosis, assess associated factors, and characterize the demographic, immunologic and metabolic profile of the Hungarian HIV-infected population. A prespecified subgroup analysis to determine the prevalence of significant fibrosis and the associated factors in HIV-mono-infected patients without significant daily alcohol intake were secondary and exploratory objectives. One hundred and thirty-six HIV-infected individuals were enrolled in this cross-sectional study. Patients underwent liver stiffness and CAP measurements to assess the degree of liver fibrosis and steatosis. We used a continuous scale of CAP values to identify significant covariates of hepatic fat accumulation. The prevalence of significant HS (defined as CAP >238 dB/m) in HIV-infected patients was high, affecting every second patient in the study population. A significant independent association with hepatic steatosis was found for hypertension ($p=0.0328$; R: 16.557; 95% CI 1.396 to 31.71) and more impressively for BMI ($p<0.0001$; R: 3.94; 95% CI 1.969 to 5.910). Darunavir use was a significant negative covariate with the CAP value in the unpenalized model ($p=0.0193$ R: -29.913; 95% CI -54.879 to -4.948). Furthermore, significant hepatic fibrosis (defined as LS >7.1 kPa) was detectable in 9.9% (10/101) of the subgroup of HIV-mono-infected individuals without significant daily alcohol intake, but no significant covariates could be identified. Our findings reflect the importance of metabolic factors in hepatic steatosis. Nonetheless, further prospective studies are warranted to clarify causal relations. The possible protective effect of darunavir should be determined in the future in randomized controlled trials.

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7.B Összefoglalás

A kombinált antiretrovirális kezelés (ART) elterjedése alapvetően változtatta meg a HIV fertőzöttek megbetegedéseit. A korábbi AIDS definiáló állapotok háttérbeszorulásával az időskorra jellemző anyagcserebetegségek kerültek előtérbe. A májjal összefüggő halálozás a normál populáció tízszerese HIV fertőzöttekben, és krónikus vírushepatitissel nem rendelkező HIV fertőzötteket is érinti. Ennek okai összetettek; a ART hepatotoxicitásától és kedvezőtlen metabolikus hatásaitól kezdve a HIV által indukált immundiszreguláción át, az életmódból fakadó faktorokig több tényező is okozhatja, azonban pontos szerepük a mai napig sem tisztázott. A világ leggyakoribb májbetegesége, a nem-alkoholos eredetű zsírmáj feltehetően különösen súlyosan érinti a HIV fertőzötteket, azonban erre vonatkozóan kevés klinikai adat áll rendelkezésre az irodalomban. Jelen vizsgálat célja a klinikailag jelentős májzsírosodás prevalenciájának, illetve az ezzel összefüggő faktorok megállapítása volt. További célkitűzésként szerepelt a magyar HIV fertőzöttek anyagcserebetegségeinek, demográfiai és immunológiai jellemzőinek felmérése, illetve a krónikus vírushepatitissel és jelentős alkoholfogyasztással nem rendelkező betegek alcsoportjában a májfibrózis előfordulásának és az azzal összefüggő faktorok meghatározása szerepelt. A vizsgálatban 136 beteg vett részt, akiknél “liver stiffness” (LS) és “controlled attenuation parameter” (CAP) meghatározás történt tranziens elasztográfiával a májfibrózis ás szteatózis számszerűsítésére. A többváltozós analízisekben a CAP és LS értékeket folyamatos változóként értelmeztem. Klinikailag jelentős májzsírosodás (CAP> 238 dB/m) minden második beteget érintett. A magas vérnyomás betegség jelenlétét ($p=0.0328$; R: 16.557; 95% CI 1.396 - 31.71) mint független szignifikáns kovariánst azonosítottam, azonban még erősebb összefüggés mutatkozott a testtömegindexszel ($p<0.0001$; R: 3.94; 95% CI 1.969 - 5.910). A darunavir használata független negatív asszociációt mutatott a CAP értékkel a nem penalizált modellben ($p=0.0193$; R: -29.913; 95% CI -54.879 to -4.948). Jelentős májfibrózis (LS >7.1 kPa) a vizsgált alcsoport 9.9%-ban volt kimutatható, szignifikáns, független faktort azonban nem sikerült azonosítani. A vizsgálat eredményei az elhízás és egyéb metabolikus betegségek HIV fertőzöttek májszírosodásban betöltött szerepére hívja fel a figyelemet. A darunavir esetleges protektív szerepét a későbbiekben randomizált, kontrollált klinikai vizsgálatokban kell meghatározni.

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8 Conflicts of interest

MS has been an investigator in clinical trials supported by Novartis, Bristol-Myers Squibb, Janssen-Cilag, Roche, Boehringer-Ingelheim, Merck Sharp & Dohme and AbbVie Pharmaceuticals.

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10 Bibliography of the candidate's publications

10.1 Related publications

Sulyok M, Makara M, Rupnik Z, Ferenci T, Újhelyi E, Kormos L, Gerlei Z, Szlávik J, Horváth G, Vályi-Nagy I. (2015) Hepatic steatosis in individuals living with HIV measured by controlled attenuation parameter: a cross-sectional study. Eur J Gastroenterol Hepatol, 27: 679-685.

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10.2 Unrelated publications

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