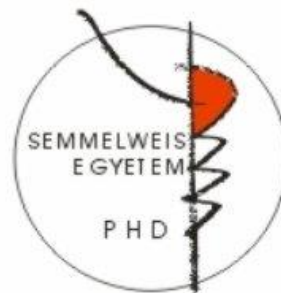


Non-invasive assessment of hepatic steatosis and fibrosis in individuals living with HIV

PhD theses

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1 Introduction

Following the widespread use of combined antiretroviral treatment (cART), the landscape of the mortality and morbidity of individuals living with HIV has undergone remarkable changes. The proportion of AIDS-defining events and bacterial infections have declined, while non-AIDS defining events have become more frequent. Although the life expectancy of an HIV-infected person has improved dramatically and is nearly equivalent to the uninfected population, a significant decrease in health-related quality of life has been observed, even in virologically stable individuals. Individuals living with HIV have an increased risk of these age-related morbidities compared with HIV-negative matched controls. Whether this increased risk is caused by differences in lifestyle, such as smoking, alcohol or drug consumption, viral replication or immunologic changes or long-term cART toxicities, remains unclear.

A possible, but non-mutually exclusive explanation for the increased risk for these conditions is a chronic HIV-induced inflammatory state. 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. 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. To date, the importance of the inflammatory state has not only been identified in other types of malignancies, such as gastric MALT lymphoma or diffuse large B-cell lymphoma arising from polyclonal B-cell activation in HCV-infected patients but also in the pathogenesis of atherosclerosis and other age-related and metabolic conditions. Therefore, the initiation of early treatment seems to be appropriate for preventing viral replication and the consequent inflammatory state. However, ART-related toxicity and unfavorable metabolic changes remain concerning.

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 risks of cART toxicities. However, concerns regarding complications from long-term ART toxicities, especially in the case of older ART, have not been completely resolved. 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 and lamivudine containing combinations, or they have a history of treatment with older NRTIs or protease inhibitors (PIs). 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

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, and liver-related deaths occur ten times more frequently in these individuals than in the general population. 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. 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. In addition to fibrosis, there has been increasing concern about the role of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).

Currently, the gold standard for the diagnosis and assessment of HS is liver biopsy (LB). Limiting factors of LB are cost and rare, but severe, complications, with the additional concerns of sampling error and difficulties related to reproducibility. 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. A 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.

The evaluation and grading of liver fibrosis is facing similar problems to those observed with HS. 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. 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. 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.

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. 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.

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, an increased prevalence in the HIV-infected population is expected. ART may also contribute to hepatotoxicity. 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.

Surprisingly, data from the HIV-mono-infected population are relatively scarce and are limited almost exclusively to hepatitis-co-infected subpopulations. 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. 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, 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.

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

3 Methods

3.1 Study population

The investigation was approved by the Institutional Ethics Committee. 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. After providing written informed consent, individuals older than 18 years of age were enrolled in the study. Pregnant women and patients with unreliable transient elastography measurements were excluded. Taking these criteria into account, the final study population consisted of 136 individuals (**Figure 1**). A subgroup analysis of HIV-mono-infected individuals without documented or suspected significant alcohol consumption (defined by >50 g alcohol daily) was also performed.

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 and demographic parameters, and assessment of facial lipodystrophy, were gathered by an investigator experienced in HIV medicine. Information obtained during the interviews regarding comorbidities, concomitant medications, biochemical and immunological parameters and history of antiretrovirals was also checked retrospectively in the patients' archived documentation.

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. VTCE is approved by the FDA and is recommended by different international and national guidelines e.g., Hungarian consensus guidelines for viral hepatitis.

CAP measurements based on VTCE 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 at 3.5 MHz. Technical details and validation with histological findings have also been extensively described. 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. Because these cutoffs were adopted from a non-HIV-infected population, they cannot be reliably transferred to an HIV-infected population. 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. To overcome this limitation, we used a continuous scale of LS values to interpret this variable in the uni- and multivariate analyses. VTCE were performed by an

experienced and qualified investigator at the Hepatology Center of Buda, Budapest, Hungary, using FibroScan 502 equipment (Fibroscan, EchoSens™, Paris, France).

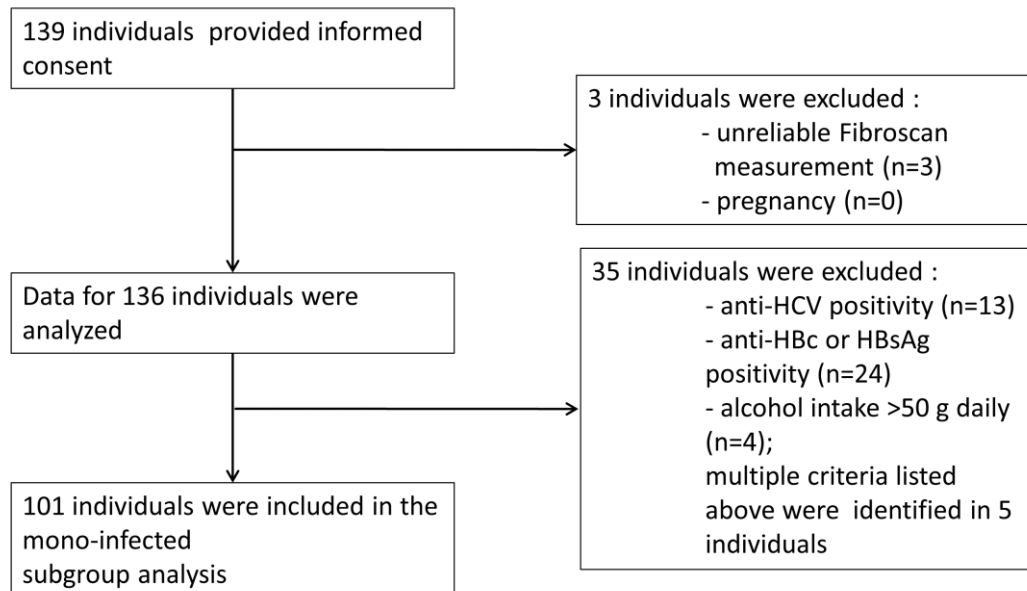


Figure 1. Recruitment Flow of the Study Participants.

3.4 Statistical analysis

The primary outcome variable was the CAP value. The univariate association of CAP with categorical variables was assessed using a two-independent-sample Mann-Whitney U test. 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. 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.* 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 and the Wald- F test to assess joint significance. Hurvich and Tsai's corrected Akaike 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. 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. Calculations were performed using R software version 3.1.2 with a custom script that is available upon request.

4 Results

4.1 Study population characteristics

Significant steatosis 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/m², and obesity (defined by a BMI greater than 30 kg/m²) 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 (47.8%) 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 (p=0.32). HBsAg was observed in 11 (8.1%) study participants. The median CAP did not differ significantly (p=0.632) between patients with (237 dB/m) and without (238 dB/m) HBsAg positivity. Four (2.94%) individuals reported more than 50 g of daily alcohol intake (median CAP 237 dB/m and 238 dB/m; p=0.515).

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. The median BMI was 24.74 (IQR 3.32). A BMI greater than 25 kg/m² 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, 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%).

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

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

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 ($p < 0.001$), serum triglyceride ($p < 0.001$), BMI ($p < 0.001$) and disease duration ($p < 0.001$) using the Pearson and Kendall- τ -rank correlation and liver stiffness with the Kendall- τ -rank correlation were considered significant ($p < 0.001$). The association was only negative for the CD8 percentage. Among the categorical variables, the presence of hypertension was considered to be significant ($p < 0.001$).

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 ($p<0.0001$; regression coefficient 7.0696; 95% CI 4.268 to 9.877). The associations with other covariates (disease duration, $p=0.0433$; regression coefficient 1.145; 95% CI -0.641 to 2.933; hypertension, $p=0.0183$; regression coefficient 26.328; 95% CI 4.554 to 48.103) were also significant. Darunavir therapy as reported in the medical history was negatively associated with the CAP value ($p=0.0193$ regression coefficient -29.913; 95% CI -54.879 to -4.948). A significant negative association was also detected for liver stiffness ($p=0.0183$), but a 95% CI interval of the regression coefficient involved 0 (95% CI -3.189 to 1.775). 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, 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 (adjusted R^2 with optimism-corrected bootstrap 0.0813, shown). To address this problem, we penalized the model (adjusted R^2 with optimism-corrected bootstrap 0.2698). As a result, BMI ($p<0.0001$; regression coefficient 3.94; 95% CI 1.969 to 5.910) and hypertension ($p=0.0328$; regression coefficient 16.557; 95% CI 1.396 to 31.71) remained significant. The estimated regression coefficients and confidence intervals are graphically presented in **Figure 2**.

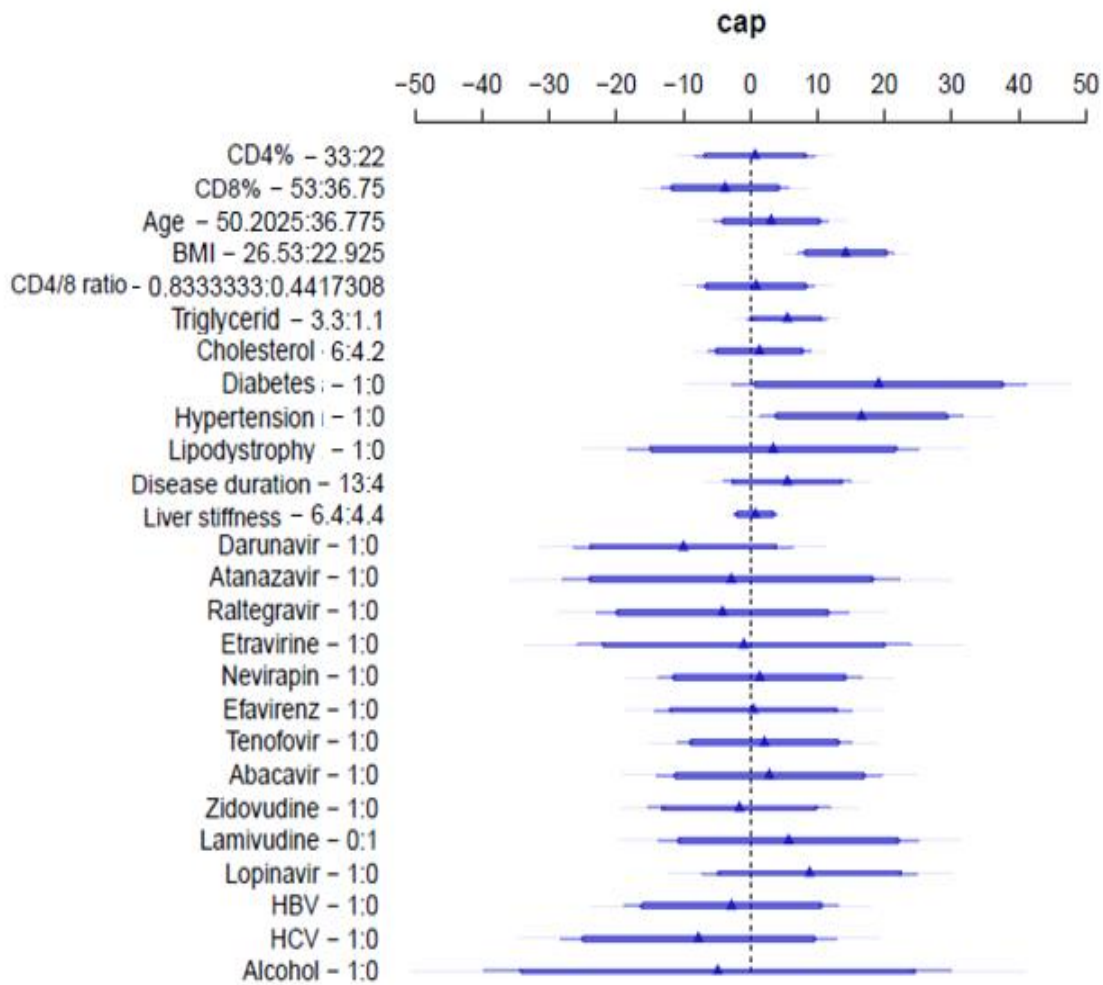


Fig. 2. Multivariate analysis: covariates with regression coefficients and confidence intervals for the penalized model. The figure shows the regression coefficients of the covariates. For categorical variables, the change is understood as the change in the modal category, and for continuous variables, 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/m²; 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.

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 ($p < 0.0001$) and body mass index ($p < 0.0001$). A non-significant but remarkable association was detected with age ($p = 0.007$, 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 ($p = 0.045$, adjusted $p > 1$).

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 ($p = 0.0236$; regression coefficient 0.336; 95% CI 0.0465 to 0.6262), age ($p = 0.0458$; regression coefficient 0.0959; 95% CI 0.0018 to 0.1899) and the history of taking lopinavir ($p = 0.0157$; regression coefficient 2.458; 95% CI 0.478 to 4.438) as independent positive covariates. A history of taking zidovudine over the course of ART ($p = 0.0285$; regression coefficient -2.015; 95% CI -3.813 to -0.217) and the presence of lipodystrophy ($p = 0.04$; regression coefficient -3.693; 95% CI -6.453 to -0.933) were independent negative covariates. Nevertheless, the model exhibited a weak fit ($R^2 = 0.362$, optimism-corrected $R^2 = -0.1856$). To improve goodness-of-fit and to address the problem of multidimensionality, we penalized the model. Although general model parameters improved (optimism-corrected $R^2 = 0.0525$), the model still exhibited poor fitting, and no significant covariates were identified.

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. Using the same cutoff value (238 dB/m) and a methodology similar to our analysis, they detected HS in 40% of the participants. Our cross-sectional design resulted in similar findings, and we identified significant HS in 49% 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. Most recently, a longitudinal cohort was performed in a population of 326 HIV-infected individuals. After 12 months, the baseline 37% prevalence of significant HS (>238 dB/m) increased to 39%. 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%).

Few studies have assessed HS in HIV-mono-infected patients. The prevalence of HS was 31% and 52% in five studies using ultrasonography and 37% in another study using CT. Ingiliz *et al.* reported a 60% prevalence that was evaluated with LB in participants with persistent liver enzyme elevation. 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 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. 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. 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%). In contrast, Merchante *et al.* 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. Applying their cutoff value (>7.2 kPa), we obtained similar results: abnormal LS values were detected using this cutoff 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. 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. 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. 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, 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, 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%. In contrast, among HIV-infected IVDUs, 72-95% were found to be co-infected with HCV. Therefore, our findings are likely explained by the absence of intravenous drug users in our study 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. 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%). 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. However, it does not have an impact on HIV progression of liver-related mortality, and individuals with isolated anti-HBc positivity have a significantly shorter survival than those with positive anti-HBs at baseline, suggesting a role of HIV-associated immunologic changes in the presence of this marker.

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, only a small proportion (2.94%) of the patients reported 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%), 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. Interestingly, our identified DM prevalence was higher than the reported values from similar studies (4.4-5%). 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 previous studies, 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%), but we should note that our results likely constitute an underestimation because hypertension was defined by the regular intake of an antihypertensive.

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. Our findings may provide additional support for these observations. It must be emphasized, however, that this association disappeared after penalizing the model. Thus, the generalizability of this result remains questionable. Regarding immune dysregulation, we used the CD4/8 ratio as a surrogate marker. Most other studies did not find an association between CD4 or CD8 cell counts, or the CD4/8 ratio, and HS. 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, which may refer to a cumulative amount of viral replication and may refer to triggered immunologic alterations. With multivariate models 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.

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; $p < 0.001$). Later, BMI was also identified as the only independent predictor (B (standard error): 9.03 (1.9); $p < 0.001$) of CAP value progression. Moreover, in the cross-sectional study of Vuille-Lessard *et al.*, 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 the multivariate models. Altogether, the association with this factor was not as impressive as with BMI, but it was still independently associated with CAP and should thus be considered as a main driving force of HS in individuals living with HIV. According to our findings, neither HCV nor HBV co-infection was associated with HS. Previous studies have reported similar results.

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. 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 and detectable HIV viremia as independent covariates for significant fibrosis after controlling for active alcohol use and site. These results shed light on the possible importance of metabolic conditions in the development of LF, which can be triggered by ongoing HIV replication. 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. The authors concluded that ritonavir boosting may provide a protective effect. Considering the metabolic changes associated with boosted protease inhibitors, this result is surprising. Associations between didanosine and stavudine and liver fibrosis have been previously described. 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 (Sulyok et al. 2017). Bayesian Model Averaging (BMA) 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%). 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 published findings by Sulyok et al. clearly underscore the advantages of using alternative modelling strategies and variable selection methods in small datasets.

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. 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. 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.

6 Conclusions

In conclusion, the prevalence of significant HS in HIV-infected patients was high, affecting every second patient in the study population. 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. 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, cART 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 and should be included in the care of HIV patients. 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. However, the role of other metabolic factors, such as HIV-induced chronic immune activation, in NAFLD should not be underestimated, and cART of all HIV-infected individuals, regardless of CD4 cell count, is the cornerstone of prevention of non-AIDS-defining morbidities. 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, therefore, further investigations in larger patient populations will be performed in future analyses. 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.

7 Bibliography of the candidate's publications

7.1 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.

Sulyok M, Ferenci T, Makara M, Horváth G, Szlávik J, Rupnik Z, Kormos L, Gerlei Z, Sulyok Z, Vályi-Nagy I. (2017) Hepatic fibrosis and factors associated with liver stiffness in HIV mono-infected individuals. *PeerJ*, 5: 2867.

7.2 Unrelated publications

Sulyok M, Rózsa L, Bodó I, Hardi R, Tappe D. (2014) Ocular Pentastomiasis in the Democratic Republic of the Congo. *PLoS Negl Trop Dis*, 8: 3041.

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Sulyok M; Sinkó J, Mihály I, Szalai B, Csire M, Dolgos J, Reményi P, Bobek I, Masszi T. (2014) Respiratory syncytial virus infections in hematological patients. *Hematológia és Transzfúziológia*, 47: 17-22.