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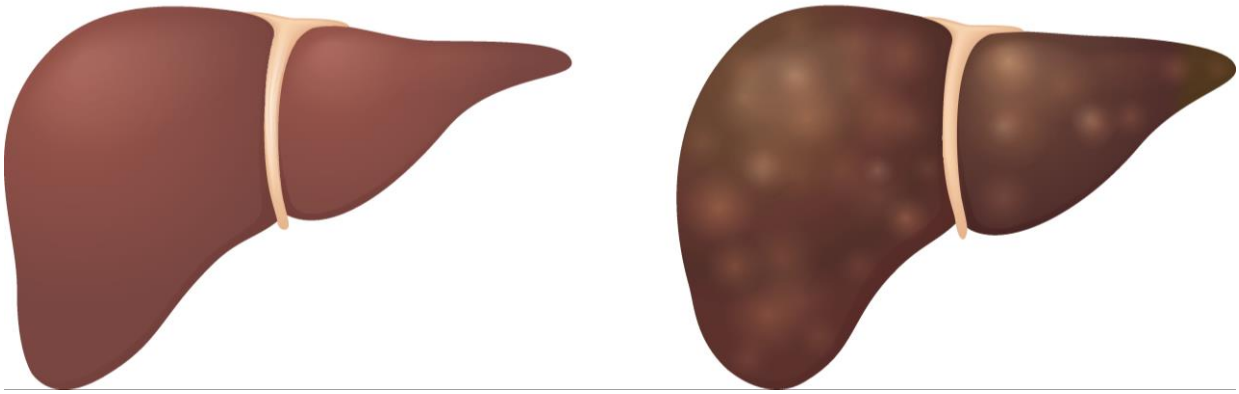
PhD Thesis



Non-Alcoholic Fatty Liver Disease and Fibrosis in HIV Infection

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This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen (1 November 2019).

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Preface

This PhD thesis is based on research carried out from the Department of Infectious Diseases, Amager Hvidovre Hospital, Rigshospitalet and University of Copenhagen from July 2015 to November 2019. It has been submitted to the Faculty of Health and Medical Sciences, University of Copenhagen, in order to obtain the PhD degree.

It has been a great privilege to work with my four supervisors, Thomas Benfield, Susanne Dam Nielsen, Jens Lundgren and Flemming Bendtsen. I sincerely appreciate your commitment, support, and scientific contributions throughout this thesis. A special thanks to my principal supervisor Thomas Benfield for your patience, your always open door, for the confidence you have shown me and for your guidance in both scientific and personal matters. Thanks to Susanne Dam Nielsen for introducing me to a research environment and for giving me the opportunity to work with a large-scale cohort study. I am very grateful for all that you have taught me.

Thanks to heads of department Bjarne Ørskov Lindhardt and Åse Bengård Andersen for making it possible to conduct these studies and for your support of young researchers. Thanks to all medical staff in the outpatient clinics at Hvidovre Hospital and Rigshospitalet for your support and participation in recruitment of study participants. Thanks to our team of medical students for your invaluable help with data collection. Thanks to all study participants for your time, engagement and your willingness to promote research. I am deeply grateful. A special thanks to Judith Haissman and Andreas Ronit for a fantastic collaboration with setting up the Copenhagen Co-Morbidity in HIV Infection (COCOMO) study and for your friendship and for your support. Thanks to all my lovely office colleagues for the past three years. You know who you are.

Finally, a very special thanks to my family, my friends and especially to Anders and our kids for all your love and support. You celebrated the happy moments with me, and you showed me the important things in life when everything seemed tough.

Ditte Marie Kirkegaard-Klitbo
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Abbreviations

ALT	Alanine aminotransferase
APRI	Aspartate-to-platelet ratio index
ART	Antiretroviral treatment
AUC	Area under curve
BMI	Body mass index
CGPS	Copenhagen General Population Study
COCOMO	Copenhagen Co-Morbidity in HIV Infection Study
CT	Computed tomography
FIB4	Fibrosis 4 index
HBsAg	Hepatis B surface antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HU	Hounsfield units
IL	Interleukin
IQR	Interquartile range
LPS	Lipopolysaccharide
LSM	Liver stiffness measurement
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFS	NAFLD fibrosis score
NPV	Negative predictive value
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
OR	Odds ratio
PPV	Positive predictive value
PLWH	People living with HIV
ROC	Receiver operatic characteristics
WHO	World Health Organization

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Studies

The thesis is based on four studies:

I. **Prevalence and risk factors of moderate to severe hepatic steatosis in HIV infection: Results from the Copenhagen Co-Morbidity Liver Study**

Ditte Marie Kirkegaard-Klitbo, Andreas Fuchs, Stefan Stender, Per Ejlstrup Sigvardsen, Jørgen Tobias Kühl, Klaus Fuglsang Kofoed, Lars Køber, Børge G. Nordestgaard, Flemming Bendtsen, Amanda Mocroft, Jens Lundgren, Susanne Dam Nielsen and Thomas Benfield (Manuscript submitted for publication in Journal of Infectious Diseases).

II. **Prevalence and risk factors of liver fibrosis in people living with HIV without viral hepatitis: Results from the Copenhagen Co-Morbidity Liver Study**

Ditte Marie Kirkegaard-Klitbo, Flemming Bendtsen, Jens Lundgren, Susanne Dam Nielsen, Thomas Benfield (Manuscript in circulation).

III. **Increased prevalence of liver fibrosis in people living with HIV without viral hepatitis compared to population controls**

Ditte Marie Kirkegaard-Klitbo, Flemming Bendtsen, Jens Lundgren, Susanne Dam Nielsen, Thomas Benfield (Manuscript submitted for publication in American Journal of Gastroenterology).

IV. **Poor concordance between liver stiffness and non-invasive liver fibrosis scores in people living with HIV without viral hepatitis: Results from the COCOMO Liver Study**

Ditte Marie Kirkegaard-Klitbo, Flemming Bendtsen, Jens Lundgren, Susanne Dam Nielsen, Thomas Benfield (Manuscript accepted as a Research Correspondence for publication in Clinical Gastroenterology and Hepatology).

Graphical abstract

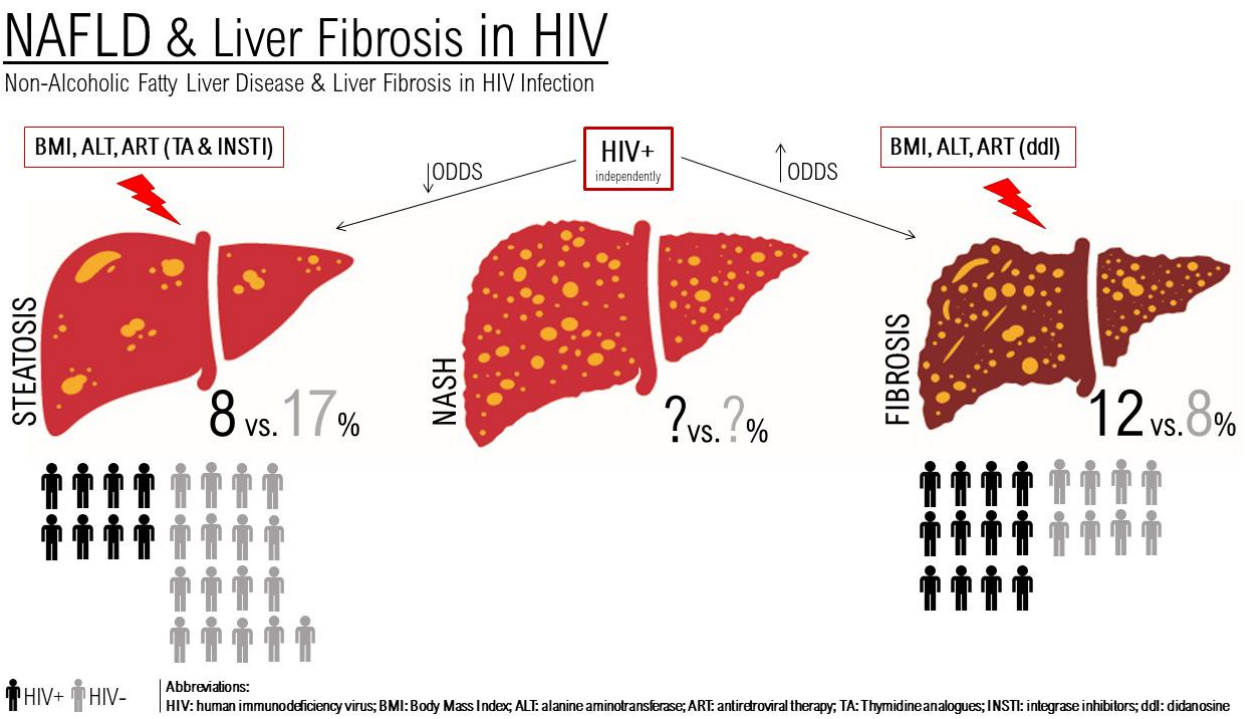


Figure adapted from (1)

Summary

The human immunodeficiency virus (HIV) epidemic has changed remarkably through the past three decades. In 1988, the World Health Organization (WHO) celebrated the first World AIDS day to raise awareness of the AIDS pandemic caused by the spread of HIV infection. The first global guidelines on how to manage HIV infection was published in 1990 and within one year, countries with a national AIDS programme increased from 7 to 130 countries worldwide. In 1996, the first highly active antiretroviral therapies were introduced and the United Nations programme on AIDS (UNAIDS) were founded. In 2000, the Millennium Development Goals 2015 were written by World leaders to fight poverty and included a goal to combat HIV/AIDS. The global initiative resulted in a 40% reduction of new HIV infections from 2003 to 2013, and a 45% reduction in HIV related deaths. Despite these initiatives, huge disparities still exit leaving key populations behind especially in Eastern Europe, central Asia, the Middle East and Northern Africa.

In the Western World, HIV is now considered a chronic disease and people living with HIV are ageing. Consequently, the number of non-AIDS diseases have increased, and focus have shifted to prevention and treatment of non-AIDS comorbidities. While the research has focused on cardiovascular disease, renal disease and bone disease, little is known about non-infectious liver diseases. Liver disease is a major cause of non-AIDS disease and mortality in people living with HIV (PLWH) and is especially caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) co-infection. However, the spectrum of liver disease most likely will change due to today's effective treatment of viral hepatitis and WHO's 2030 elimination plan for HCV. Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the Western World, with fat accumulation in the liver and a risk of liver inflammation, fibrosis, cirrhosis, liver cancer and eventually, liver transplantation. A large research effort has been put into this field to improve diagnosis, prevention and treatment of NAFLD and liver fibrosis in the general population. PLWH may be at increased risk of NAFLD and fibrosis due to i) chronic activation of the immune system by HIV itself, ii) potential liver toxic effects of antiretroviral treatment, and iii) adverse lifestyle behaviours among others. Most studies have focused on high-risk groups among PLWH such as individuals with obesity or elevated liver enzymes, and only very few studies have included an HIV-uninfected comparator group to test the effect of HIV itself.

In this PhD thesis we studied NAFLD and liver fibrosis in PLWH without viral hepatitis followed at two hospitals in Copenhagen. We included an HIV-uninfected comparator group to test the independent effect of HIV. We hypothesized that the prevalence of fatty liver disease and liver fibrosis

was higher in PLWH compared to uninfected controls, and that HIV itself was an independent risk factor for fatty liver disease and liver fibrosis. In the first paper, we present results on fatty liver. In this study, we showed that the prevalence of fatty liver was lower in PLWH compared to uninfected controls, and that HIV itself was independently associated with lower odds of fatty liver. Further we show, that higher body mass index (BMI), higher alanine aminotransferase (ALT) and cumulative duration of treatment with an integrase inhibitor and thymidine analogue were independently associated with higher odds of fatty liver. In the second paper, we present results on liver fibrosis in Fatty liver disease, higher age and non-Caucasian ancestry were associated with higher odds of liver fibrosis, while alcohol and smoking were not. In the third paper, we present results on liver fibrosis in PLWH compared to uninfected controls aged 50 to 70 years. In this study, we showed that the prevalence of liver fibrosis was higher in PLWH compared to HIV-uninfected controls, and that HIV itself was independently associated with higher odds of liver fibrosis. Further we showed, that higher BMI, higher ALT and previous exposure to didanosine were independently associated with higher odds of liver fibrosis. In the fourth paper, we present results on the agreement between different diagnostic methods for liver fibrosis used in routine clinical practice. We showed, that the agreement between the investigated methods was poor suggesting that these methods may not be used as independent diagnostic tools.

Conclusively, our results showed that PLWH may not be at an increased risk of NAFLD compared to the general population. However, integrase inhibitors seem to be an independent risk factor for fatty liver disease and should be investigated further in future studies. In contrast to fatty liver, PLWH seem to be at higher risk of liver fibrosis which may not only be driven by hepatotoxic effects of antiretroviral therapy such as didanosine but also by fatty liver disease.

Dansk Resume

Human immundefekt virus (HIV) epidemien har igennem de seneste tre årtier ændret sig markant. I 1988 blev den første internationale AIDS-dag afholdt af verdenssundhedsorganisationen (WHO) med det primære formål at skabe en global bevågenhed omkring den AIDS-pandemi der herskede som følge af smitte med HIV. De første retningslinjer for den sundhedsfaglige håndtering af HIV blev udgivet af WHO i 1990 og i løbet af det følgende år, steg antallet af lande med et nationalt AIDS program fra syv til 130 lande på verdensplan. I 1996 blev den første antiretrovirale behandling mod HIV tilgængelig og de Forenede Nationers program for AIDS (UNAIDS) blev grundlagt. I år 2000, formulerede verdens statsledere FN's Millenniumerklæring (FN's 2015 mål) med det overordnede formål at forbedre menneskers levevilkår og bekæmpe fattigdom. Det ene af de otte mål var dedikeret bekæmpelsen af HIV og AIDS. Denne internationale indsats medførte at antallet af nye HIV-smittede faldt med 40% og antallet af dødsfald relateret til HIV faldt med 45% fra 2003 til 2013. Trods disse globale indsatsområder er der fortsat en stor ulighed i verdens HIV-epidemi og særligt i Østeuropa, Centralasien, Mellemøsten og det nordlige Afrika er antallet af nye HIV-smittede stigende.

I den vestlige verden kan HIV nu betragtes som en kronisk sygdom. Mennesker der lever med HIV (HIV-positive), bliver ældre og 80% af de HIV-positive forventes at have minimum én følgesygdom i 2030. Fokus på forebyggelse og behandling af følgesygdomme er derfor steget betragteligt. Forskningen har i høj grad fokuseret på hjerte-kar sygdom, nyresygdom og knoglesygdom, hvorimod der kun er sparsom viden om ikke-infektiøse leversygdomme. Leversygdom er en hyppig årsag til ikke-AIDS relateret sygdom og død blandt HIV-positive og er særligt forårsaget af smitte med hepatitis B virus (HBV) og hepatitis C virus (HCV). Det forventes dog, at årsagerne til leversygdom vil ændre sig over de næste årtier grundet effektive behandlingsmuligheder for HBV og HCV samt WHO's eliminationsplan for HCV i 2030.

Non-alkoholisk fedtleversygdom (NAFLD) er den hyppigste årsag til kronisk leversygdom i den vestlige verden. Sygdommen forårsages af en fedtophobning i leveren med risiko for at der tilkommer inflammation, arvæv (fibrose), skrumpelever og i yderste konsekvens lever cancer med risiko for levertransplantation. En stor forskningsindsats er indledt med fokus på forebyggelse, diagnostik og behandling af NAFLD i baggrundsbefolkningen. Tidligere forskning har rejst mistanke om, at HIV-positive er i øget risiko for NAFLD og lever fibrose grundet en pågående aktivering af immunforsvaret forårsaget af HIV, potentielle leverskadelige virkninger af den anvendte antiretrovirale behandling, uhensigtsmæssig livsstil m.m. Tidligere undersøgelser blandt HIV-positive primært har fokuseret på høj-risiko grupper som f.eks. overvægtige eller patienter med forhøjede levertal, og kun ganske få studier har haft en HIV-negativ kontrolgruppe at sammenligne med, hvorved effekten af HIV i sig selv kan undersøges.

I denne PhD afhandling undersøgte vi NAFLD og lever fibrose blandt HIV-positive uden viral hepatitis og alkohol overforbrug. Vi inkluderede en HIV-negativ kontrolgruppe med henblik på at undersøge effekten af HIV i sig selv. Vores hypotese var, at forekomsten af NAFLD og lever fibrose var højere blandt HIV-positive sammenlignet med HIV-negative, og at HIV i sig selv var en risikofaktor for NAFLD og lever fibrose. I den første artikel præsenterer vi resultater for NAFLD. I denne undersøgelse fandt vi, at HIV-positive havde en lavere forekomst af NAFLD sammenlignet med den HIV-negative kontrolgruppe, og at HIV i sig selv var associeret med lavere odds for fedtlever. Derudover fandt vi at højere BMI, højere levertal og den kumulerede behandlingsvarighed af integrase hæmmere eller thymidin analoger var associeret med højere odds for fedtlever. I den anden artikel præsenterer vi resultater for leverfibrose. Vi fandt at fedtlever, højere alder og højere BMI var forbundet med højere forekomst af lever fibrose. Der var ingen sammenhæng mellem lever fibrose og henholdsvis alkohol og rygning. I den tredje artikel præsenterer vi resultater for lever fibrose blandt HIV-positive sammenlignet med HIV-negative i alderen 50 til 70 år. I denne undersøgelse fandt vi, at HIV-positive havde en højere forekomst af lever fibrose sammenlignet med HIV-negative, og at HIV i sig selv var associeret med højere odds for leverfibrose. Endvidere fandt vi, at et højere BMI, højere levertal og tidligere behandling med didanosine var forbundet med højere odds for lever fibrose. I den fjerde artikel præsenterer vi resultater for anvendelsen af forskellige diagnostiske metoder til vurdering af lever fibrose i klinisk praksis. I denne undersøgelse fandt vi, at de diagnostiske metoder identificerede forskellige mennesker i risiko for at have lever fibrose og stiller spørgsmålstegn ved om undersøgelserne kan bruges selvstændigt i vurderingen af lever fibrose hos HIV-positive.

Samlet set viste vores resultater at HIV-positive ikke var i øget risiko for fedt lever sygdom sammenlignet med HIV-negative. Behandling med integrase hæmmere, højere BMI og højere levertal var selvstændige risikofaktorer for fedt lever sygdom, og særligt sammenhængen mellem integrase hæmmere og fedt lever bør undersøges nærmere i fremtidige studier. Modsat fundene for fedtlever, fandt vi at HIV-positive var i øget risiko for lever fibrose. Den øget risiko skyldtes både leverskadelige effekter af didanosine, NAFLD, højere alder og højere BMI.

1. Non-alcoholic fatty liver disease and Liver Fibrosis in the General Population

1.1 Global Epidemiology of NAFLD and Liver Fibrosis

NAFLD is a leading cause of liver disease worldwide and caused by fat accumulation within the liver. The prevalence of NAFLD is estimated to be 25% in the global adult population with highest prevalence in the Middle East, South America and Asia (Figure 1) (2).

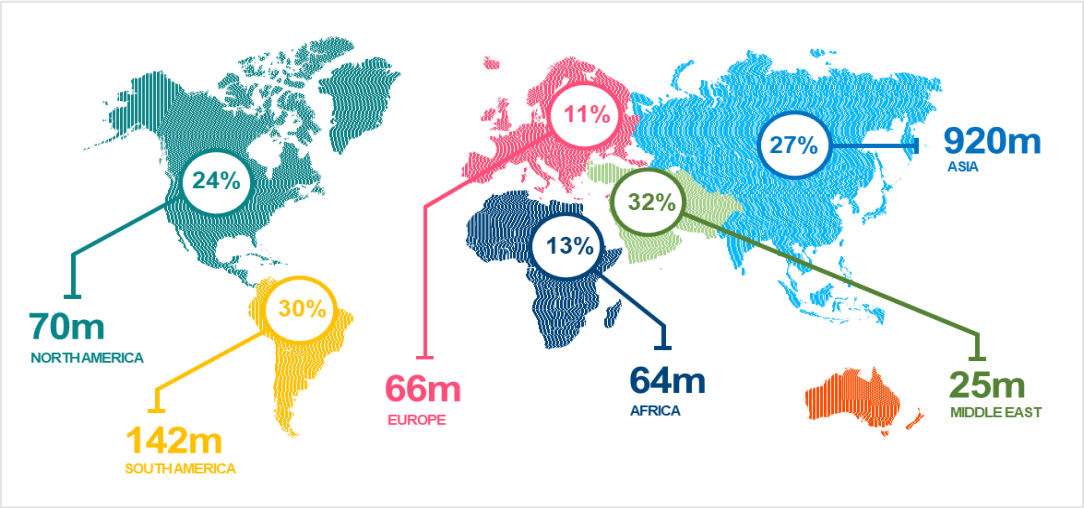


Figure 1 | The global prevalence of non-alcoholic fatty liver disease in 2016. Data adapted from (2).

Risk factors associated with NAFLD in the general population are male sex, higher age, ethnicity, obesity, type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome (3). Few studies have investigated the prevalence of liver fibrosis in the general population with unknown liver disease. Three studies have been conducted on European populations with reported prevalences of 5.6 to 7% (4–6). One of these studies included liver biopsies on a subset of the population; 88% of these had NAFLD (5). Of concern, NAFLD was the second leading etiology of liver transplantation in the United States in 2015 (7), and according to a Markov model the prevalence of NAFLD will continue to rise through 2030 (8). In Europe, the leading cause of liver transplantation in 2018 was decompensated liver cirrhosis related to viral hepatitis, alcohol abuse and primary liver tumors (9). However, according to the UK National Health Service Blood and Transplant Agency, the proportion of individuals undergoing liver transplantation due to NAFLD-associated liver cirrhosis has increased from 4% in 1995 to 12% in 2013 (10).

Conclusively, NAFLD and liver fibrosis is a major health concern worldwide and large international research consortiums have been established to address the urgent need for reliable and affordable diagnostic and treatment programs.

1.2 Natural history of NAFLD and Liver Fibrosis

NAFLD is considered to be a spectrum of liver diseases consisting of non-alcoholic fatty liver (NAFL) with at least 5% fat infiltration of the hepatocytes (11), and non-alcoholic steatohepatitis (NASH) with additional hepatocellular ballooning, lobular inflammation and potential liver fibrosis (12). The diagnosis requires exclusion of excessive alcohol intake and secondary causes of fat accumulation within the hepatocytes (e.g. steatogenic treatment, hepatitis virus infection). Traditionally, NAFL has been considered a benign and non-progressive condition, while NASH has been considered a more severe and progressive condition with a risk of developing liver fibrosis and cirrhosis. However, a recent study demonstrated that NAFL may also progress to liver fibrosis and emphasizes the potential variability of the natural history of NAFLD (13). Importantly, among those who progress to fibrosis 80% were diabetic (14). Accordingly, the natural history of liver fibrosis seems to vary. In a study based on paired biopsies with a mean time between biopsies of 22.8 ± 1.3 years, 53% had fibrosis progression, 34% were stable, and 13% regressed (15). The progression rate seems to be relatively slow with one fibrosis stage per 7.7 years (16), although individuals with NASH may progress twice as fast.

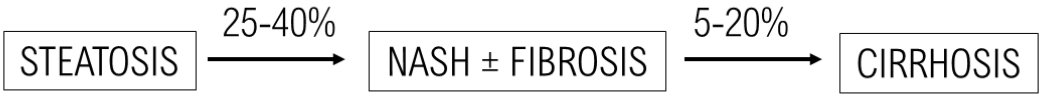


Figure 2 | Natural history of NAFLD. Figure adapted from (17)

Importantly, liver fibrosis is the strongest predictor of poor long-term liver-related outcomes and mortality in NAFLD (18,19), and early detection of liver fibrosis is extremely important.

1.3 Pathogenesis of NAFLD and Liver Fibrosis

Development of NAFLD is a multifactorial process driven by a complex interplay between hepatocytes, Kupffer cells and hepatic stellate cells (Figure 3) (20). Environmental factors such as a sedentary lifestyle and an inappropriate diet with high amounts of glucose, fructose, fat and cholesterol may contribute to increased lipogenesis within the hepatocytes, and to an increased uptake of fatty acids from the blood by the liver. Metabolic factors such as insulin resistance and obesity may also contribute by an impaired inhibition of lipolysis in the adipose tissue leading to an increased influx of free fatty acids into the liver. Finally, certain genotypes such as PNAPL3 and TM6SP2 may lead to accumulation of fat droplets within the hepatocytes leading to NAFL. The excessive lipid accumulation within the hepatocyte leads to stress of the endoplasmic reticulum and the mitochondria, which may induce hepatic cell death and production of hepatocyte-derived factors (e.g. pro-inflammatory cytokines). These factors as wells as dietary free fatty acids or lipid metabolites may induce activation of both Kupffer cells and hepatic stellate cells (HSC) leading to inflammation and fibrogenesis, respectively.

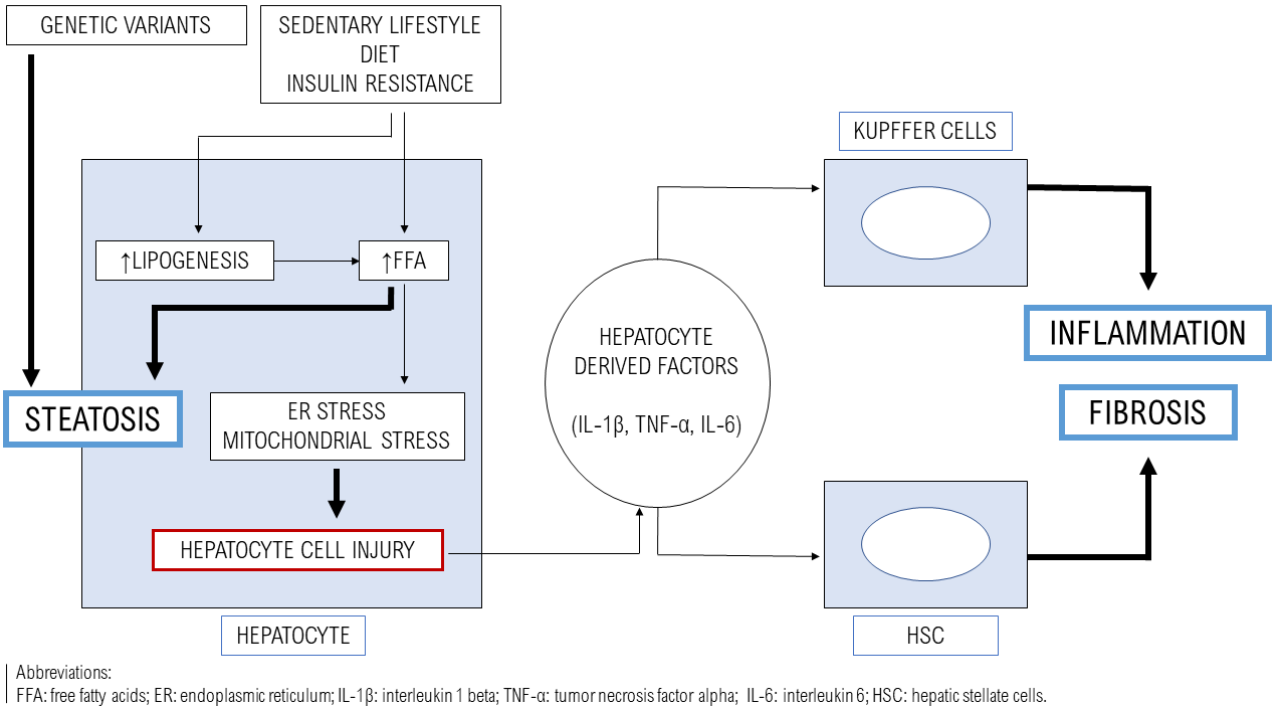
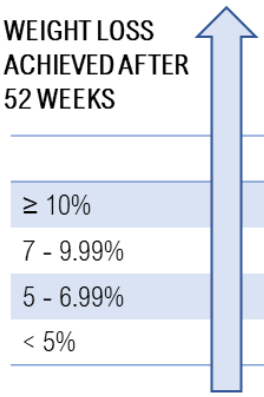


Figure 3 | The "multiple-parallel hit" model in the pathogenesis of NAFLD. Data adapted from (20).

1.4 Treatment of NAFLD and Liver Fibrosis

The cornerstone in the treatment of NAFLD is lifestyle intervention. Weight loss has the greatest effect on NAFLD with a potential of a >80% reduction in hepatic steatosis, while exercise has a more modest effect with a potential 20-30% reduction of hepatic steatosis (21). A weight loss of 7-10% will induce a significant improvement of both NAFL, NASH and liver fibrosis (Figure 4). Accordingly, The European Association for Study of the Liver (EASL) has published clinical practice guidelines recommending that a 7-10% weight loss should be the key target for lifestyle intervention in obese and overweight individuals with NAFLD (22). However, maintaining weight loss may be extremely challenging and only 10% maintained a weight loss of ≥10% after 52 weeks. Interestingly, it seems that diet interventions leading to weight loss may have long-lasting beneficial effects on hepatic steatosis even if there is weight regain (23).



	N	STEATOSIS (%)	NASH (%)	FIBROSIS (%)
≥ 10%	10%	100	90	81
7 - 9.99%	9%	76	64	50
5 - 6.99%	12%	65	26	38
< 5%	70%	35	10	45

Figure 4 | Results of lifestyle intervention in treatment of NAFLD. Data adapted from (24) (N: Number of individuals who maintained the given weight loss after 52 weeks)

No medical drugs have yet been approved for treatment of NASH, but several drugs have been tested (25). In the spring of 2019, results from the phase 3b study REGENERATE were presented at the EASL meeting in Vienna, Austria (26). Treatment with obeticholic acid (10 or 25 mg) was tested in an international, randomized, placebo-controlled study and showed a significant improvement of both NASH and fibrosis after 18 months in a dose-response manner. Thus, results have shown promising results and medical treatment for NASH may become available within the next few years.

2. NAFLD and Liver Fibrosis in HIV Infection

2.1 HIV infection in the Western World

After the introduction of antiretroviral therapy, the HIV epidemic in the Western World has improved dramatically, and HIV is now considered a chronic disease. Consequently, PLWH are ageing and three out of four are expected to be ≥50 years of age by 2030, and one out of three will have at least three co-morbidities (27,28). Thus, the research effort has shifted towards prevention and treatment of non-AIDS comorbidities. Chronic liver disease is the second leading non-AIDS cause of death in PLWH and has primarily been caused by HBV and HCV co-infection (29,30). However, the spectrum of liver disease most likely will change with current effective treatment for HBV and HCV, and WHO's 2030 HCV elimination plan (31).

2.2 Global Epidemiology of NAFLD in HIV Infection

In PLWH, the prevalence of NAFLD have been reported with a broad range from 10-70% (32–45) (Figure 5). In 2017, a meta-analysis on NAFLD in PLWH reported a pooled prevalence of 35% (34). When compared to the 25% NAFLD prevalence in the general population (2), PLWH seemed to be at increased risk of NAFLD and for the first time, guidelines for management of NAFLD in PLWH were included in the European AIDS Clinical Society (EACS) guidelines in October 2017 (46). However, studies conducted with an HIV-negative comparator group have shown either lower or comparable prevalences of fatty liver in PLWH, but higher prevalences of liver fibrosis (35,38,45,47). A study by Vodkin et al based on liver biopsies showed that HIV-associated NAFLD had more severe inflammation and liver cell injury compared to primary NAFLD, while no difference was observed in steatosis or fibrosis. In conclusion, PLWH may be at increased risk of NAFLD and especially more advanced disease may be more prevalent in PLWH.

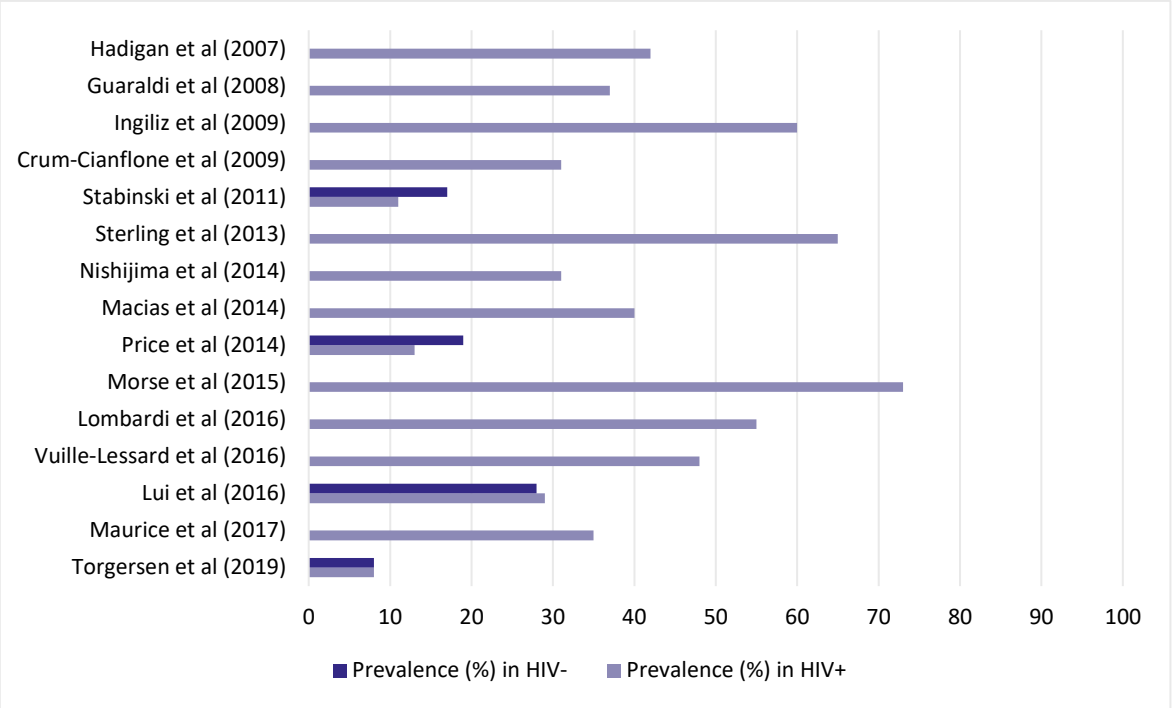


Figure 5 | Prevalence of NAFLD in people living with HIV (HIV+) and uninfected controls (HIV-) (32–45,47)

2.3 Pathogenesis of NAFLD and liver fibrosis in HIV infection

The pathogenesis of NAFLD and liver fibrosis in PLWH may be different from the general population. Besides traditional risk factors (e.g. obesity, diabetes, diet and sedentary lifestyle), HIV-related factors such as hepatotoxic effects of antiretroviral drugs (ART), chronic immune activation and microbial translocation may play an important role in the pathogenesis and progression of NAFLD and liver fibrosis.

Antiretroviral drugs may cause metabolic alterations and mitochondrial dysfunction, and thus contribute to the development and progression of NAFLD. Studies on ART exposure and NAFLD are limited and results so far have been conflicting. Early-generation nucleoside reverse transcriptase inhibitors (NRTIs), especially stavudine and didanosine, have been associated with development of NAFLD and liver fibrosis (33,38,48–50). The underlying mechanisms may be explained by i) hepatic mitochondrial dysfunction leading to fat accumulation within the hepatocytes (51), and ii) mitochondrial dysfunction in the peripheral fat tissue leading to insulin resistance, dyslipidemia and lipodystrophy (52). Protease inhibitors may promote an altered lipid profile, especially when combined with ritonavir or cobicistat (53), and have been associated with liver fibrosis by Vuille-Lessard et al (32). Integrase inhibitors may promote weight gain (54) but have not been associated with NAFLD or liver

fibrosis. Studies on non-nucleoside reverse transcriptase inhibitors (NNRTIs) are limited but does not seem to promote an altered lipid profile. However, nevirapine has been linked to chronically elevated liver enzymes, end-stage liver disease, and hepatocellular carcinoma in addition to didanosine, stavudine, tenofovir disoproxil fumarate, and emtricitabine (55,56).

Chronic immune activation is a hallmark of HIV infection, and e.g. interleukin-6 (IL-6) and D-dimer may be strongly related to non-AIDS comorbidities and all-cause mortality in PLWH (57). Interestingly, Price et al showed that different inflammatory markers were associated with NAFLD in PLWH and uninfected controls (58). In general, PLWH had higher levels of sCD14, sCD163, CRP, ICAM-1, and TNFα2. Despite this, the endothelial cell activation marker ICAM-1 was the only marker associated with higher risk of NAFLD in PLWH, while adiponectin was protective of NAFLD. This emphasizes, that different pathophysiologic pathways may be involved in the development of NAFLD in PLWH and uninfected controls.

Finally, HIV infection may cause depletion of CD4+ T-cells in the gut mucosa, leading to disruptions of the epithelial barrier, translocation of bacterial products (e.g. lipopolysaccharide (LPS)) into the systemic circulation, and systemic immune activation and inflammation. Circulating LPS may recruit inflammatory cytokines such as IL-1β and TNFα that binds to Kupffer cells and induce fibrogenesis. Microbial metabolites and food substrates from e.g. poor diet and alcohol may also affect the gut-liver axis and contribute to the development of NAFLD (59).

3. Aims

3.1 Objectives

1. To determine the prevalence and factors associated with hepatic steatosis in PLWH and population controls (Study I).
2. To determine the prevalence and factors associated with liver fibrosis in PLWH (Study II).
3. To determine the prevalence and factors associated with liver fibrosis in elder PLWH compared to population controls (Study III).
4. To compare the concordance between non-invasive liver fibrosis scores and vibration controlled transient elastography in PLWH (Study IV).

3.2 Hypotheses

1. The prevalence of hepatic steatosis is higher in PLWH compared to population controls.
2. Liver fibrosis in PLWH is associated with higher age and exposure to antiretroviral therapy.
3. The prevalence of liver fibrosis is higher in elder PLWH compared to population controls.
4. HIV status is independently associated with higher odds of hepatic steatosis and liver fibrosis.
5. The concordance rate of non-invasive liver fibrosis scores and vibration controlled transient elastography is high in PLWH.

4. Methods

4.1 Study populations

The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Study Cohort

The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Study is a non-interventional, longitudinal, observational cohort study designed to characterize non-AIDS co-morbidity in PLWH in the area of Copenhagen, Denmark. We invited all PLWH under care in the outpatient clinics at the Department of Infectious Diseases, Rigshospitalet and the Department of Infectious Diseases, Amager Hvidovre Hospital. Eligible were people with HIV-1 infection aged 18 years or above, with the ability to read and understand the written study information, and the ability to provide informed consent. There were no exclusion criteria. The study invitation was communicated through the HIV care-givers in the outpatient clinics, through posters in the mentioned outpatient clinics, and through the patient organization's magazine HIV Denmark. From March 2015 through November 2016, a total of 1,099 PLWH were consecutively enrolled in the COCOMO Study. This comprises approximately 20% of the 5,600 individuals living with HIV in Denmark, and >40% in the area of Copenhagen. The study protocol of the COCOMO study have been published (60).

The Copenhagen General Population Study (CGPS) Cohort

The Copenhagen General Population Study (CGPS) is an ongoing, population-based prospective study initiated in 2003 (61,62). Citizens aged 20 years and above living in the greater area of Copenhagen are randomly invited for study participation through the Danish Civil Registry System (63). In total, 25% of the inhabitants aged 20 to 40 years are invited for the study, and 100% of the inhabitants aged >40 years are invited. More than 100,000 individuals have been included, and a randomly selected subpopulation of approximately 10,000 inhabitants aged >40 years were invited to an unenhanced CT scan of the abdomen. The CGPS population is not routinely HIV-tested and were thus assumed to be HIV-negative as the prevalence of HIV in Denmark is 0.1% (64).

The Rotterdam Study Cohort

The Rotterdam Study is an ongoing, population-based study among elderly inhabitants from the Ommoord district of Rotterdam, The Netherlands (65). The study was initiated in 1990 and more than 15,000 inhabitants have been included through various cycles (Figure 6). The first cycle (RS-I) was initiated in 1990 and comprises approximately 8000 inhabitants aged > 55 years; the second

cycle (RS-II) was initiated in 2000 and comprises approximately 3000 inhabitants aged > 55 years; the third cycle (RS-III) was initiated in 2006 and comprises approximately 4000 inhabitants aged 45 to 54 years; and the fourth cycle was initiated in 2016 and is expected to comprise approximately 4000 inhabitants aged 44 to 55 years by the end of 2019. For this study, we included inhabitants from RS-II and RS-III. The Rotterdam study population is not routinely HIV tested and were assumed to be HIV negative as the prevalence of HIV in The Netherlands is 0.2% (66).

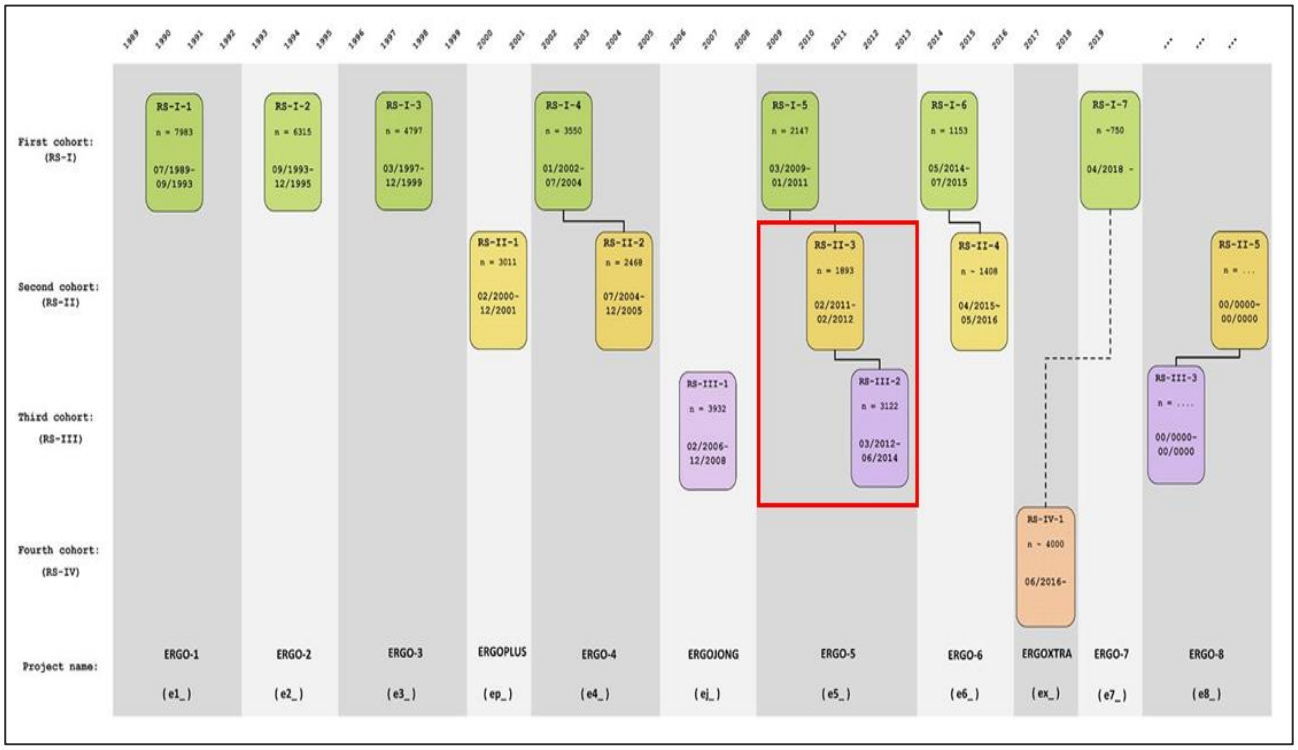


Figure 6 | Diagram of examination cycles in The Rotterdam Study (65). The red box illustrates cycles included for this study. Figure kindly provided by the Rotterdam Study.

4.2 Data Collection

Details on the data collection for the COCOMO study, the CGPS and the Rotterdam Study have been published previously (60,61,65). Data were uniformly collected in the COCOMO study and the CGPS. An overview of the data used from the three cohorts for this PhD thesis are provided in **Table 1**.

Table 1. Overview of data collected in the COCOMO study, the CGPS and the Rotterdam Study

	COCOMO	CGPS	Rotterdam
Questionnaires	✓	✓	✓
Anthropometrics	✓	✓	✓
Blood pressure	✓	✓	✓
Venous blood samples	✓	✓	✓
Fasting blood samples	-	-	✓
Hepatitis serology	✓	-	✓
Abdominal CT	✓	✓	-
Abdominal ultrasound	-	-	✓
Transient Elastography	✓	-	✓

Questionnaires and blood samples

Study participants from the COCOMO study and the CGPS completed identical questionnaires with more than 100 questions on e.g. lifestyle behaviours, dietary habits, alcohol consumption and medication use. In both cohorts, questionnaires were completed by the study participants and reviewed by a health care professional at the first study visit. Non-fasting, venous blood samples were drawn, and routine biochemical analyses performed using the same laboratory equipment for both cohorts. Study participants from the Rotterdam Study completed an extensive interview on e.g. lifestyle behaviours, alcohol consumption and medical history. Fasting venous blood samples were drawn, and routine biochemical analyses performed.

HIV specific information and hepatitis serology

For COCOMO study participants, all medical records were reviewed to extract HIV specific data (e.g. CD4 nadir, history of ART) and hepatitis serology data. We defined HBV as a positive hepatitis B surface antigen (HBsAg) and HCV as a positive anti-HCV antibody. For CGPS participants, hepatitis

serology was not available. However, the burden of HBV and HCV is very low with 234 incident cases of chronic HCV and 262 incident cases of chronic HBV in the total Danish general population in 2016 (67,68). The numbers are stable. For Rotterdam study participants, all individuals were tested for HBsAg positivity and anti-HCV antibody positivity (65).

Anthropometric measurements

For all three cohorts, anthropometric measurements were performed by trained personnel and included height (cm), weight (kg), waist circumference (cm), hip circumference (cm), and electronic blood pressure measurement (mmHg) in sitting position.

Abdominal computed tomography scan and ultrasonography scan

In the COCOMO study and the CGPS study, hepatic steatosis was assessed by unenhanced computed tomography (CT) scan of the upper abdomen. The CT scans were performed using identical scan protocols for both cohorts. CT scans were performed on an Aquillion One scanner (Toshiba Medical Systems, Tokyo, Japan) (69,70). Of those invited for the CT scan, 84% and 70% accepted the invitation and were scanned in the COCOMO study and the CGPS, respectively. Image analysis was uniformly conducted in both cohorts using Vitrea 3.1 imaging software (Vital Images, Minnetonka, USA). Image analysis of the 921 CT liver scans available from the COCOMO study cohort was performed by DK. A pilot study showed high degree of interrater correlation (Spearman rho = 0.95) between data processors in the two cohorts. The liver CT attenuation was estimated by placing a region of interest (ROI) with an area of 1500 mm^2 in the Coinaud liver segments 5 and 6. The mean liver CT-attenuation is inversely correlated with the fat content of the liver (71,72); thus, the liver CT attenuation decreases when the liver fat content increases. The physiologic liver CT attenuation is 55 to 65 Hounsfield Units (HU) (73). We used a pre-defined liver CT attenuation threshold of <48 HU to assess moderate-to-severe hepatic steatosis with a specificity of 100%, a sensitivity of 53.8%, a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 93.9% (71).
In the Rotterdam cohort, hepatic steatosis was assessed by abdominal ultrasonography of the liver (US). The US was performed on a Hitachi HI VISION 900 (Hitachi Medical Corporation, Tokyo, Japan). All images were reviewed by an experienced hepatologist and hepatic steatosis was defined as the presence of hyperechogenic liver tissue (74).

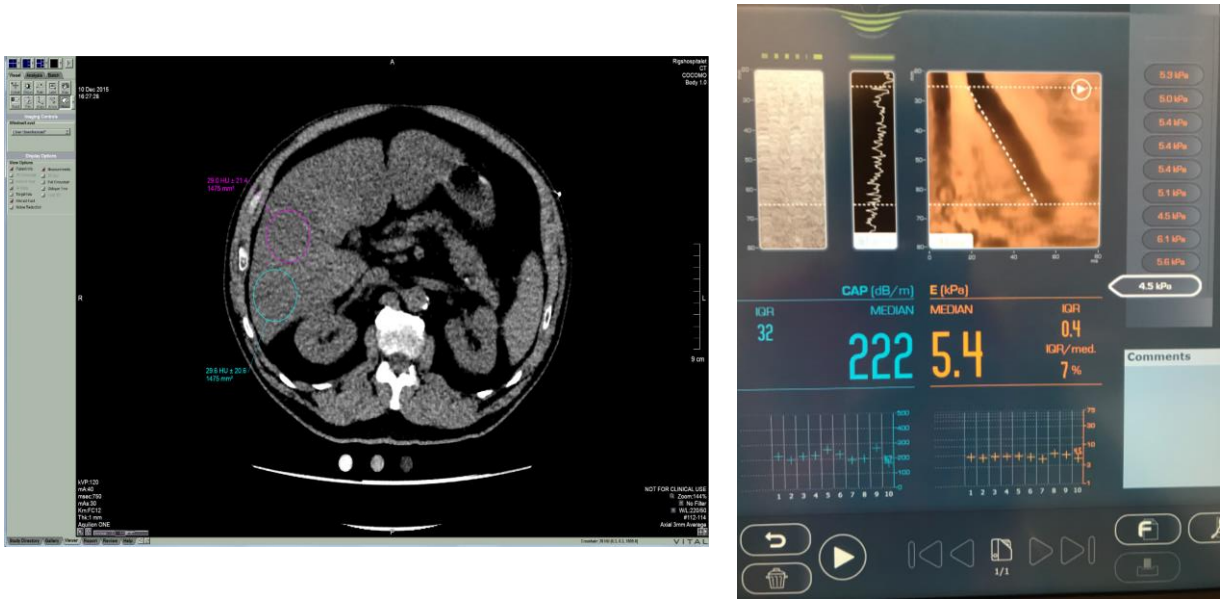


Figure 7 | CT scan with liver attenuation measurements (left) and vibration controlled transient elastography (right).

Vibration Controlled Transient Elastography (VCTE)

Liver fibrosis was assessed by VCTE (Fibroscan®, Echosens, Paris, France) in the COCOMO study and the Rotterdam study. VCTE was performed with the patient in supine position with the right arm placed under the head. The probe was placed in an intercostal space on the skin in the right mid-axillary line corresponding to the location of the liver. A 50 Hz shear wave was transmitted from the ultrasound transducer to the underlying tissue including the liver tissue. The velocity of the shear wave through the tissue was measured (in meters per second) and converted into a liver stiffness measurement (LSM) expressed in kilopascals (kPa). Using the medium (M) probe, VCTE measures the stiffness of the tissue located 25 to 65 mm below the surface of the skin with a cylinder-shaped volume of approximately one cm width and four cm length. The examination were considered valid when at least 10 valid measurements have been conducted with a success rate of ≥ 60% and an interquartile range (IQR) of ≤ 30% of the median LSM (75).

The LSM correlates with the degree of liver fibrosis and increases with higher degrees of liver fibrosis (76). In individuals without liver disease or elevated liver enzymes, the mean LSM has been reported to be 5.5 ± 1.6 kPa (6). In individuals with liver disease, the liver stiffness varies depending on aetiology and cut-offs to distinguish significant liver fibrosis from no to mild fibrosis may vary accordingly. Lowest cut-off points have been proposed in individuals with HBV and HCV and highest cut-off points

in individuals with NAFLD and chronic cholestatic diseases (77). In our study we used a predefined cut-off point of 7.6 kPa with a sensitivity of 76.3%, a specificity of 79.6%, a PPV of 71.7% and a NPV of 83.2% to distinguish F2-F4 fibrosis from F0-F1 fibrosis in NAFLD patients (76).

VCTE is a rapid and painless procedure for liver fibrosis assessment and can be performed bedside in the outpatient clinic and the scan result is provided immediately for clinical decision making. The LSM may be difficult to perform in individuals with narrow intercostal spaces and ascites, and reliable results may not be obtained in e.g. overweight or obese patients using the M probe. Thus, a XL probe has been launched that reaches tissue located 35-75 mm below the surface suitable for individuals with large waist circumference. However, this should only be used in individuals with a BMI >32 kg/m^2 (78). Liver inflammation, cholestasis and liver tumours may induce falsely elevated LSM and the result should therefore be interpreted with caution in those patients.

4.3 Statistical Analysis

Descriptive characteristics of PLWH and population controls were compared using Wilcoxon Rank test for continuous variables and χ^2-test for categorical variables.

In PLWH, univariate logistic regression models were used to estimate the association between single exposure variables and the probability (odds) of the binary outcomes moderate-to-severe hepatic steatosis (Study I) or liver fibrosis (Study II and III). Accordingly, multivariate logistic regression analysis was conducted to estimate the association between several exposure variables and the probability of the outcome, when the effect of potential confounders had been considered. Results from logistic regression analysis were presented as odds ratios (OR) with 95% confidence intervals (95% CI). Potential confounders were selected *a priori* based on previous literature and with support from Akaike's Information Criterion when e.g. two continuous variables were highly correlated. To test whether a positive HIV status was independently associated with moderate-to-severe hepatic steatosis (Study I) or liver fibrosis (Study III), we conducted the multivariate regression analysis on the total population of PLWH and population controls. Further, linear regression analysis was conducted to estimate factors associated with higher LSM (Study II). Liver fibrosis scores were calculated (Study IV) using traditional formulas (Table 2). Explorative C-statistics analysis was performed for the liver fibrosis scores with VCTE (LSM≥8.8 kPa) as reference (Study IV). Area under the curve (AUC) with 95% CI were calculated and considered excellent if AUC 1.00-0.90, good if AUC 0.90-0.80, fair if AUC 0.80-0.70, poor if AUC 0.70-0.60 and failure if AUC 0.60-0.50. Sensitivity (%),

specificity (%), NPV (%), and PPV (%) were calculated with cut-off points from the general population applied. Optimal cut-off points for liver fibrosis scores were calculated using Youden's Index.

Table 2. Calculation of liver fibrosis scores	
FIB4 (79)	(Age in years x AST in IU/L) / (Platelets in 10^9/L x $\sqrt{}$(ALT in IU/L))
APRI (80)	(AST in IU/L) / (AST Upper Limit of Normal in IU/L) / (Platelets in 10^9/L)
NFS (18)	-1.675 + (0.037 x age in years) + (0.094 x BMI in kg/m^2) + (1.13 x diabetes (yes = 1, no = 0)) + (0.99 x AST/ALT ratio) – (0.013 x Platelets in 10^9/L) – (0.66 x albumin in g/dl)

Abbreviations: APRI: aspartate-to-platelet ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; FIB4: fibrosis 4 index; NFS: NAFLD fibrosis score.

4.4 Ethical Considerations

The COCOMO study and the CGPS was approved by the Capital Region of Denmark (H-15017350 and H-KH-01-144/01, respectively) and the Danish Data Protection Agency. The Rotterdam study was approved by the Netherlands Ministry of Health, Welfare and Sports and by the Medical Ethics Committee of the Erasmus University Medical Center, Rotterdam, The Netherlands. All participants received written and oral information about the study before providing informed consent.

Study participants were exposed to low-dose radiation, which may increase the lifetime risk of cancer induced by radiation. The study participants were informed about this in written and oral study information before providing informed consent. Blood sampling, VCTE and the physical examination programme were not associated with any risk for the patient. Abdominal CT scans were analysed after completion of study inclusion, and COCOMO study participants were informed that results from this examination would not be provided. Results from blood samples were reviewed and abnormal results were communicated to the treating physician. Result from VCTE were communicated to the study participant at the time of examination, and the treating physician was informed if LSM ≥12.0 kPa in order to decide whether additional work-up was required.

5. Study Designs

Study I

Study I was a cross-sectional study aimed to estimate the prevalence of hepatic steatosis in PLWH compared to population controls, to estimate factors associated with hepatic steatosis, and to assess whether a positive HIV status was independently associated with hepatic steatosis. We included all PLWH >40 years with an available CT liver scan from the COCOMO study, and all population controls >40 years with an available CT liver scan from the CGPS and matched on sex and 5-years age strata in a ratio of 1:2. Individuals with HIV/HBV and HIV/HCV co-infection were excluded from the analysis. Individuals who reported an excessive alcohol consumption were excluded from the main analyses and only included for sensitivity analyses to test the effect of alcohol on hepatic steatosis. The final study population comprised 453 PLWH and 756 population controls.

Study II

Study II was a cross-sectional study aimed to estimate the prevalence of liver fibrosis in PLWH and to estimate factors associated with liver fibrosis. We included PLWH from the COCOMO study of all ages with a valid VCTE. Individuals with self-reported alcohol consumption above national recommendation, HBV infection and/or HCV infection were excluded from this study. The final study population comprised 473 PLWH.

Study III

Study III was a cross-sectional study aimed to estimate the prevalence of liver fibrosis in elder PLWH compared to population controls, to estimate factors associated with liver fibrosis, and to assess whether a positive HIV status was independently associated with liver fibrosis. We included PLWH from the COCOMO study and population controls from the Rotterdam Study with a valid VCTE and aged 50 to 70 years. Individuals with HBV or HCV infection were excluded from the analysis. The final study population comprised 342 PLWH and 2190 population controls.

Study IV

Study IV was a cross-sectional study aimed to estimate the concordance rate between subjects identified with advanced liver fibrosis assessed by VCTE and the simple non-invasive liver fibrosis scores FIB4, APRI and NFS, respectively. The diagnostic performance of the non-invasive fibrosis scores was estimated using VCTE as reference as supplementary analysis. All PLWH from the COCOMO study with a valid VCTE and without HBV or HCV were included for the analysis. The final study population comprised 743 PLWH.

6. Results

6.1 Study I

Key findings

Moderate-to-severe hepatic steatosis was less prevalent in PLWH compared to population controls aged >40 years (Figure 8). A positive HIV status was associated with a lower prevalence of moderate to severe hepatic steatosis (aOR: 0.44 (95% CI: 0.24;0.8)) in adjusted analysis (Figure 8). In PLWH, higher BMI and plasma ALT were independently associated with higher odds of moderate to severe hepatic steatosis (aOR: 1.58 (95% CI:1.35;1.85) and aOR: 1.76 (95%CI: 1.31;2.37), respectively). Cumulative duration of exposure to an integrase inhibitor and a thymidine analogue were associated with higher odds of moderate to severe hepatic steatosis (aOR: 1.28 (95%CI: 1.00;1.65) and aOR: 1.19 (95%CI: 1.03;1.37), respectively).

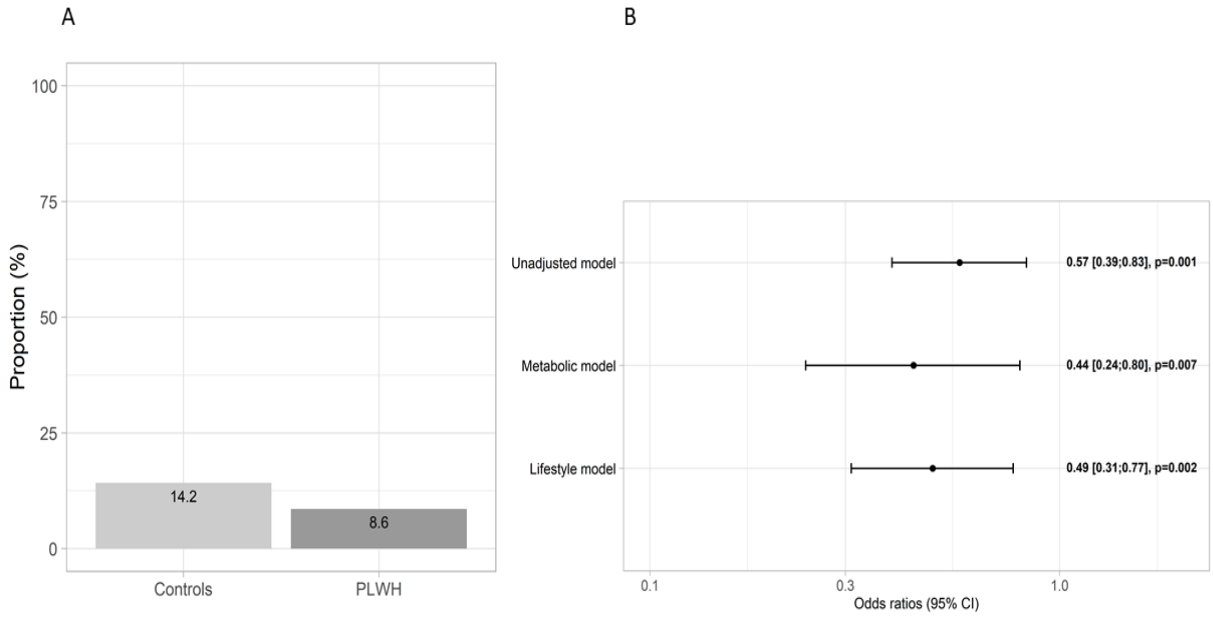


Figure 8 | (A) Prevalence of moderate-to-severe hepatic steatosis in PLWH and population controls and (B) the association between HIV and moderate-to-severe hepatic steatosis.

Supplementary results

In Study I, we found that cumulative exposure to an integrase inhibitor was associated with higher odds of moderate to severe hepatic steatosis. However, we did not have statistical power to test

whether the association differed by specific drug. Thus, we performed an explorative analysis and included all PLWH aged 20 to 84 years without excessive alcohol intake and viral hepatitis (N=516). Logistic regression analysis was performed to estimate the association between moderate to severe hepatic steatosis and dolutegravir, elvitegravir, and raltegravir, respectively. Moderate to severe hepatic steatosis was associated with any exposure to raltegravir (aOR 3.67 (95% CI: 1.29;10.46)), cumulative exposure to raltegravir (aOR 1.19 per year (95% CI: 1.01;1.41)) and cumulative exposure to elvitegravir (aOR 2.84 per year (95% CI: 1.58;5.10)). The association between moderate-to-severe hepatic steatosis and cumulative exposure to elvitegravir with emtricitabine/tenofovir disoproxil fumarate) and with emtricitabine/tenofovir alafenamide were comparable (aOR 3.06 (95% CI: 1.63;5.75) vs. aOR 3.62 (95% CI: 0.73;17.81), respectively). No association was found with dolutegravir, abacavir, didanosine, emtricitabine, lamivudine, tenofovir disoproxil fumarate, tenofovir alafenamide, zidovudine, efavirenz, etravirine, nevirapine, rilpivirine, atazanavir, darunavir, or lopinavir.

Considerations on methodology

An important hallmark for the COCOMO study was the uniform data collection in the two cohorts, including data collected for assessment of hepatic steatosis. A comprehensive CT scan protocol was designed for the CGPS to cover hepatic, cardiovascular and pulmonary outcomes in one scan session, and this scan protocol was applied on the COCOMO cohort. The same CT scanner and imaging software was used for both cohorts to avoid bias due to e.g. different scan calibrations.

CT liver scan is a reliable method for assessment of hepatic steatosis. We defined hepatic steatosis by a liver attenuation <48 HU based on a study by *Pickhardt et al* (71). In this study, 315 individuals from the general population underwent an unenhanced abdominal CT scan and an ultrasound guided liver biopsy on the same day. A liver attenuation <48 HU was 100% specific for moderate-to-severe hepatic steatosis and the PPV reached 100%. However, with a sensitivity of 53%, some individuals may have been missed in both cohorts. Further, liver CT scan only detects ≥30% fat content in the liver and no conclusions can be made on milder degrees of hepatic steatosis. Magnetic resonance imaging (MRI) is a very sensitive method for diagnosis and quantification of hepatic steatosis and may detect mild degrees of hepatic steatosis (>5%). Furthermore, it reflects the fat content of the whole liver in contrast to e.g. liver biopsy which represents only a very small amount of the total liver volume. In PLWH, liver MRI has been poorly validated. One study by *Lemoine et al* (81) showed an excellent performance of MRI to detect moderate-to-severe hepatic steatosis in PLWH (AUROC 0.98 (95% CI: 0.96;1.00)), however, the study group did not report performance characteristics for detection of mild hepatic steatosis. A meta-analysis by *Gu et al* (82) from the general population showed an excellent performance of MRI with AUROC 0.91 (95% CI: 9,88;0.93) for classification of none-to-mild vs. moderate-to-severe hepatic steatosis, and AUROC 0.90 (95% CI:

0.87;0.92)) for classification of none-to-moderate vs. severe hepatic steatosis. In both cases a sensitivity of approximately 75% and a specificity of 85% was reported. Despite the ability to accurately detect even mild degrees of hepatic steatosis, MRI may not be suitable for larger cohort studies due to high costs and a need of high expertise for image analysis. Similarly, liver biopsy may not be useful in cohort studies due to high costs, potential risk of serious complications for the study participants, and requirement of high expertise for histological evaluation. However, our results could have been strengthened if a diagnosis of CT-defined moderate-to-severe hepatic steatosis had been confirmed by either MRI or liver biopsy in a subgroup of individuals. If MRI or liver biopsy had been applied to all COCOMO study participants, and we would have expected a higher prevalence of hepatic steatosis as also milder degrees of steatosis would have been detected.

In the COCOMO study, all PLWH were invited for the CT liver scan and 84% accepted the invitation. When compared to PLWH with a CT liver scan, PLWH without a CT scan were more likely females (22 vs 14%) and with a longer duration of HIV infection (19 vs 16 years). No difference was observed in ancestry, educational level, physical activity level, BMI or diabetes. Selection bias cannot be precluded; some individuals did not wish to participate due to the risk of radiation, while others did not wish to participate due to e.g. a busy working life, limited resources to meet for scheduled appointments or serious illness. Importantly, we were not aware of any systematic selection bias that could have turned the results into one specific direction.

Consideration on results

This is the largest study of hepatic steatosis in European PLWH (n=453) with a HIV negative comparator group (n=765). While previous studies have suggested a high risk of hepatic steatosis in PLWH, our study adds to the controversial literature. The protective effect of HIV itself does not seem to have a biological explanation but may rather be explained by different metabolic phenotypes in the two cohorts. Due to regular HIV care, PLWH will most likely have more frequent physician encounters compared to healthy individuals from the general population. This may lead to early detection and treatment of e.g. high blood pressure, diabetes and dyslipidaemia. Consequently, the metabolic risk profiles may be favourable in PLWH even if obesity is present and one may need to distinguish between metabolic “healthy” obesity and metabolic “unhealthy” obesity (83). Few studies did include a HIV negative comparator group, but common to these studies were, that steatosis did not occur more frequently in PLWH compared to controls. Price et al found a lower prevalence of CT-defined moderate to severe hepatic steatosis in PLWH (n=465) compared to uninfected controls (n=254) from the US MACS cohort (13% vs 19%, p=0.02) and also found HIV to be independently associated with lower prevalence of moderate to severe hepatic steatosis (aOR: 0.44 (95% CI: 0.26;0.74), p=0.002). A study by Lui et al from an Asian cohort based on MRS-defined hepatic

steatosis found the prevalence of hepatic steatosis to be comparable in PLWH (n=80) and uninfected controls (n=160) (29% vs 28% (P=0.39), respectively).

We found higher BMI and higher ALT to be associated with higher odds of moderate to severe hepatic steatosis, similar to previous studies (32). We found no evidence of an association between duration of HIV infection; route of HIV transmission; blood CD4 T cell count; or plasma level of HIV RNA and moderate to severe hepatic steatosis. However, cumulative exposure to thymidine analogues and integrase inhibitors were associated with higher odds of moderate to severe hepatic steatosis in PLWH. While the association with – especially early-generation – NRTIs have been reported previously (33,84), the association between integrase inhibitor and moderate to severe hepatic steatosis is novel. Through recent years, there has been an increased focus on integrase inhibitors, as a number of studies have demonstrated that treatment with integrase inhibitor-based regimens may induce a weight gain (54,85). A prospective study by Zelber-Sagi et al found, that weight gain was associated with incident hepatic steatosis during 7 years of follow-up independently of BMI. Taken together, the association between integrase inhibitor and hepatic steatosis is of great concern as it suggests that integrase inhibitors may not only induce weight gain, but also liver manifestations caused by metabolic alterations. Thus, it is of great importance to explore whether hepatic steatosis is an adverse effect of integrase inhibitor treatment and if so; if it develops in a dose-response manner and if it is a reversible metabolic alteration. This could be explored in a randomized placebo-controlled study.

6.2 Study II

Key findings

In this study of 473 PLWH without viral hepatitis and excessive alcohol intake, we found a prevalence of liver fibrosis of 9.3% (95% CI: 7.0;12.2%). Liver fibrosis was associated with higher age (aOR: 1.42 (95% CI:1.04;1.94)), non-Caucasian ancestry (aOR: 2.17 (95% CI:1.03;4.56)), total cholesterol (aOR: 0.50 (95% CI: 0.34;0.75)), and any and cumulative exposure to atazanavir (aOR: 0.24 (0.07;0.84) and aOR: 0.79 (95% CI: 0.63;0.99), respectively). Moderate to severe hepatic steatosis was associated with higher odds of liver fibrosis (OR: 6.13 (95% CI: 2.44;15.44)) and aOR: 7.68 (95% CI: 2.70;21.81)). The predicted probability of liver fibrosis in PLWH increased with age and the association seemed to be stronger in PLWH with moderate to severe hepatic steatosis compared to PLWH without moderate to severe hepatic steatosis (Figure 9).

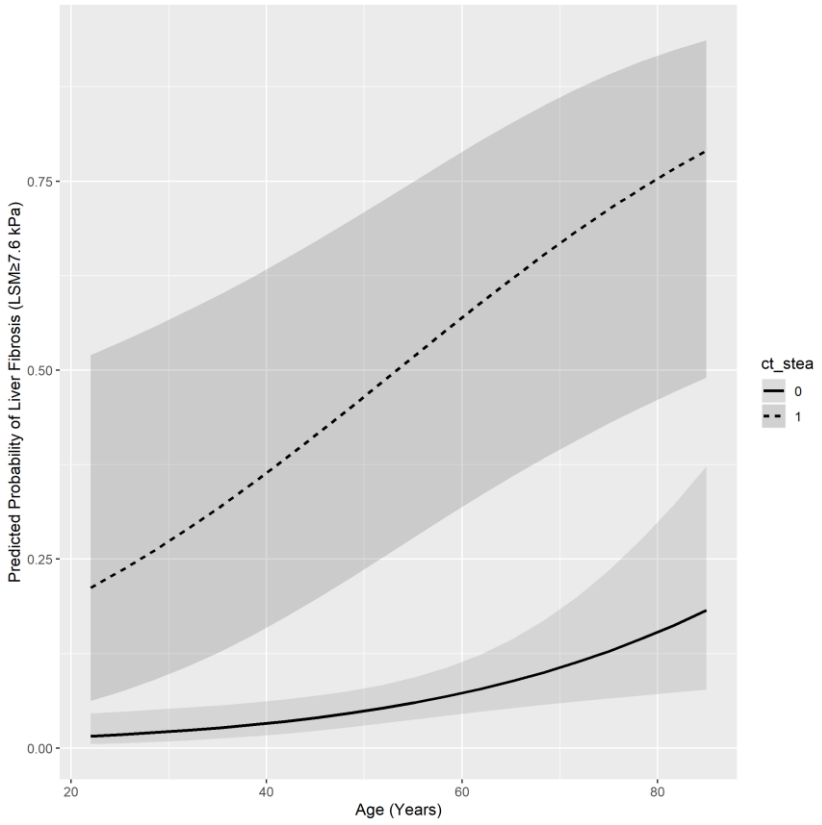


Figure 9 | Predicted probability of liver fibrosis with higher age grouped by presence of hepatic steatosis

Consideration on Methodology

Liver fibrosis was assessed by VCTE. We found a failure rate of 21% after applying traditional validity criteria (75), which is higher than reported from other studies (86,87). This may have various explanations. First, the physicians who performed the VCTE were not experienced operators although certified, and this may have led to a low number of valid measurements in e.g. patients with obesity or narrow intercostal spaces. Second, we used the M-probe for all study participants as the XL probe was not available. Among those with failure of VCTE, a higher proportion of individuals were obese (21% vs 7%) suggesting that the skin-to-capsule distance was too long to gain a valid measurement. However, a study by Berger et al showed that the XL probe should only be used in patients with BMI ≥32 kg/m^2, which in the COCOMO cohort applies to a total of 54 (5%) individuals (30 PLWH with an invalid LSM and 27 PLWH with a valid LSM).

The VCTE was performed non-fasting due to the overall COCOMO study design and feasibility. However, the liver stiffness may increase after a meal due to hyperemia in the liver. One study by Arena and al demonstrated an increase in liver stiffness of 0.8-4.7 kPa approximately 15-45 minutes after a meal, with the highest increment observed in individuals with histological defined F4 fibrosis stage (88). Similar, a study by Ratchatasettakul et al showed a peak increase in liver stiffness 15 minutes after a meal with a mean increment of 2.4 kPa. The LSM returned to baseline after 150 minutes (89). Thus, non-fasting VCTEs may imply an overestimation of liver fibrosis if the procedure is performed within three hours after a meal.

Several cut-offs points for liver fibrosis have been proposed but no official cut-off point has been decided for use in the general population or PLWH. We defined liver fibrosis as LSM ≥7.6 kPa based on a study of 230 individuals from the general population with biopsy-proven NAFLD (76). Morse et al proposed a cut-off of ≥7.1 kPa with a sensitivity of 93%, a specificity of 73%, a NPV of 97% and a PPV of 52% in PLWH monoinfection with elevated liver enzymes (90). VCTE performed well with an area under the receiver operating curve (AUROC) of 93% (95% CI: 86-99%) for identification of ≥F2 liver fibrosis using the Ishak scoring system (90,91). To meet the challenge of an arbitrary cut-off, we used log-transformed LSM as an outcome in addition to the binary outcome of liver fibrosis (LSM ≥7.6 kPa) and performed sensitivity analysis with liver fibrosis defined as LSM ≥8.8 kPa.

Lastly, VCTE may be used to detect significant fibrosis and identify patients where additional histological assessment may be needed. Other methods may have a higher diagnostic accuracy and e.g. magnetic resonance elastography (MRE) have been proved superior to VCTE (AUROC 0.92 (0.88-0.96) vs 0.97 (0.82-0.91)). However, due to costs and feasibility VCTE was considered the best method for this large-scale study.

Considerations on Results

The prevalence of liver fibrosis in this cohort of PLWH without viral hepatitis was lower than reported from previous studies. A meta-analysis published in 2017 by Maurice et al reported a pooled prevalence of liver fibrosis of 22% (95% CI: 13;34%). The higher numbers reported in other studies may be explained by different selection criteria, different methods used (e.g. non-invasive liver fibrosis scores, VCTE, biopsy) and different cut-off points for liver fibrosis. We found higher age, plasma total cholesterol, and non-Caucasian ancestry to be independently associated with liver fibrosis. Any exposure to ddI showed a trend towards higher prevalence of liver fibrosis, while any and cumulative exposure to atazanavir were protective of liver fibrosis. The pathogenesis behind the potential protective effect of atazanavir on liver fibrosis is unclear. However, Kovari et al reported a protective effect of atazanavir after two years treatment of the risk of developing chronic liver enzymes elevations as outcome, and the potential mechanisms for this should be explored in future studies. Previous results on factors associated with VCTE-defined liver fibrosis in PLWH have been controversial. Vuille-Lessard et al found higher ALT, diabetes and current use of protease inhibitors to be independent predictors of liver fibrosis. Lemoine et at showed that obesity, HOMA-IR, adipokines and sCD163 were independently associated with liver fibrosis. Anadol et al found an association between ddI exposure and liver fibrosis. Despite the variability in reported risk factors for liver fibrosis, our results seem to support previous findings. Our study emphasizes the importance of age as an independent risk factors of liver fibrosis. Whether this is explained by an increasing immunosenescent immuneprofile, a higher cumulative duration of ART or metabolic alterations among others are unclear and needs to be explored in future studies. Interestingly, we found a strong and independent association between moderate to severe hepatic steatosis and liver fibrosis in PLWH. This suggests that NAFLD should be considered as a cause of liver fibrosis in PLWH.

6.3 Study III

Key findings

The prevalence of liver fibrosis in individuals aged 50 to 70 years without HBV or HCV was higher in PLWH compared to population controls, and more severe fibrosis stages were observed in PLWH (Figure 10A). A positive HIV status was independently associated with higher odds of liver fibrosis (aOR: 1.84 (95% CI: 1.17;2.88)). Male sex and higher age, BMI and plasma ALT and previous exposure to didanosine were independently associated with higher odds of liver fibrosis in PLWH. The association between age and liver fibrosis was modified by HIV, with higher predicted probability in PLWH compared to uninfected controls (Figure 10B).

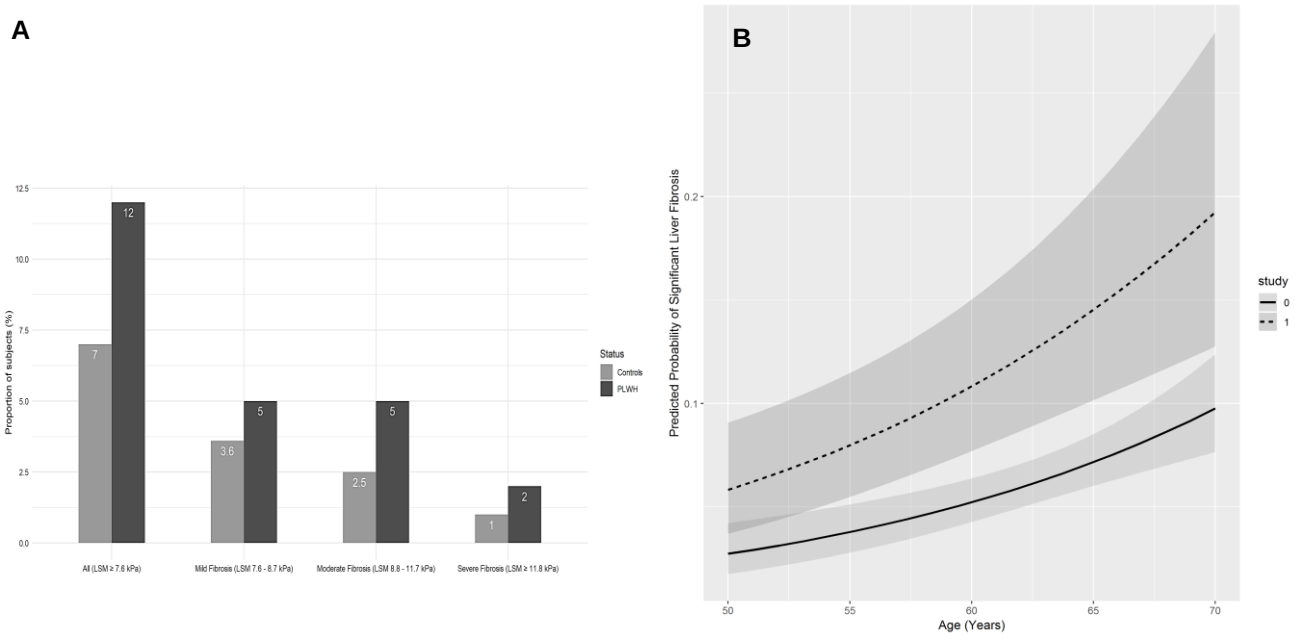


Figure 10 | (A) Proportion of PLWH and uninfected controls with liver fibrosis.
(B) Predicted probability of liver fibrosis with higher age in PLWH (dotted line) and uninfected controls

Considerations on methodology

A research collaboration with the Rotterdam Study was established to obtain an HIV negative comparator group with VCTE-defined liver fibrosis. However, use of a comparator group without uniformly collected data may introduce bias. Participants from the Rotterdam Study were older and with a larger proportion of individuals aged >70 years. To reduce potential age-related bias, we chose to focus on individuals aged 50 to 70 years. Use of different Fibroscans and laboratories between cohorts may introduce bias due to e.g. different calibrations. Different methods for assessment of hepatic steatosis limits the ability to compare these results. Fourth, different formulations in

questionnaires may provide different results on health and lifestyle questions. However, the Fibroscans used in the two cohorts are from the same manufacturer with uniform requirements for calibration and the medical laboratories used are required to comply with requirements of the current ISO certification. Thus, bias introduced to different calibrations should at least theoretically be less pronounced than e.g. potential bias caused by different Fibroscan operators.

Considerations on Results

This is the largest European study of liver fibrosis in elder PLWH with an HIV-uninfected comparator group. Twelve percent of PLWH aged 50 to 70 years had liver fibrosis, which is comparable to a meta-analysis by Maurice et al from 2017 with a pooled prevalence of biopsy-proven liver fibrosis of 22% (range: 4 - 36%) (34). Interestingly, HIV was independently associated with 84% higher odds of liver fibrosis compared to uninfected controls in our study. Only few previous studies included an HIV uninfected comparator group to allow this analysis. Similar to our study, HIV was identified as an independent risk factor for liver fibrosis in an Asian cohort (aOR: 4.00 (95% CI: 1.29-12.41), P=0.02) (35) and in an African cohort (adjusted prevalence risk ratio (aPRR): 1.5 (95% CI: 1.1-2.1), P=0.01) (47). Interestingly, PLWH seemed to have more advanced liver fibrosis and suggests, that HIV-associated factors may induce an accelerated fibrogenesis. Increased levels of proinflammatory cytokines (e.g. IL-1β, TNF-α and IL-6) may be higher in PLWH compared to uninfected controls (92), which may activate hepatic stellate cells and induce fibrogenesis in the liver. Further, antiretroviral drugs may induce liver fibrosis through mitochondrial toxicity (93), and we found that previous exposure to didanosine was associated with liver fibrosis. Finally, PLWH may parallel the immune profile of ageing and NASH with an increased proinflammatory- and ageing state (94,95). Taken together, this may explain the higher prevalence of liver fibrosis observed in PLWH as well as the stronger association between age and liver fibrosis in PLWH. The independent association between a positive HIV status and liver fibrosis suggests that HIV itself or factors associated with HIV infection may contribute to the complex pathogenesis of liver fibrosis. Unfortunately, no liver biopsies were available to confirm these results and to demonstrate whether liver inflammation is more pronounced in PLWH compared to uninfected controls.

6.4 Study IV

Key findings

Among 743 PLWH without HBV and HCV, 37 (5%) had liver fibrosis by VCTE. The concordance rate between VCTE and the simple non-invasive liver fibrosis scores FIB4, APRI and NFS were poor (Figure 10). Among those with VCTE defined liver fibrosis, 24% would not have been identified with potential fibrosis by FIB4>2.67, 76% by APRI>1.5 and 40% by NFS>0.676. A large proportion of PLWH had indeterminant liver fibrosis scores between the low and high cut-off points.

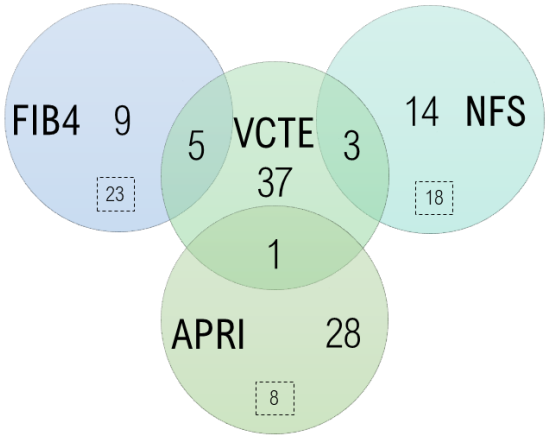


Figure 10 | Venn diagram showing the concordance between VCTE and the liver fibrosis scores FIB4, NFS and APRI. Number presented in dotted box represents proportion of individuals with indeterminant liver fibrosis scores between low and high cut-off.

Supplementary results

In Study IV we found a poor concordance between VCTE and the non-invasive liver fibrosis scores FIB4, APRI, and NFS, respectively. However, whether one of the fibrosis scores performed better compared to VCTE was unclear. We performed C-statistics to estimate the diagnostic performance of FIB4, APRI and NFS, respectively with VCTE as reference (Table 3). Optimized cut-offs for each fibrosis score were estimated. The concordance between VCTE and the liver fibrosis scores improved if those were applied; 26 (70%) were identified with fibrosis by FIB4>1.51, 14 (38%) by APRI>0.44, and 25 (71%) by NFS>-1.56.

Table 3. Diagnostic performance of low, high and optimized cut-off points for liver fibrosis scores in PLWH

	AUC (95% CI)	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
FIB4	0.72 (0.64;0.81)	1.30	76	58	9	98
		2.67	14	96	14	95
		1.51	70	70	11	98
APRI	0.65 (0.56;0.75)	0.5	24	91	13	96
		1.5	3	100	50	95
		*0.44	38	87	13	96
NFS	0.70 (0.61;0.79)	-1.46	60	64	8	97
		0.676	9	98	18	95
		*-1.56	71	61	9	97

Abbreviations: AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; FIB4: fibrosis-4 index; APRI: aspartate-to-platelet ratio index; NFS: NAFLD fibrosis score.

Considerations on Methodology

Non-invasive liver fibrosis scores were developed to provide a simple tool to identify patients with potential liver fibrosis that may require additional work-up such as histological evaluation. When cut-offs from the general population was applied, individuals with proposed liver fibrosis differed markedly. Importantly, no liver biopsies were available to determine which of the diagnostics methods performed best compared to histology, whether histological features such as inflammation and ballooning may affect the result of any of the non-invasive methods and whether one of the methods were strongly correlated to liver fibrosis. Thus, liver biopsies would have improved this study from this study to confirm the results and to answer the research question on whether non-invasive liver fibrosis scores can be used concomitantly with VCTE in routine clinical practice.

Considerations on Results

In this cross-sectional study of PLWH we showed a poor concordance rate between VCTE and the simple non-invasive liver fibrosis scores FIB4, APRI and NFS if cut-off points from the general population were applied. Our findings support previous literature. Cheng et al showed that 15% with VCTE-defined liver fibrosis would not have been identified with fibrosis by FIB4 in a population with HCV (96). Sagir et al showed a discrepancy of 36% between VCTE and FIB4, and 26% between VCTE and APRI in PLWH (97). This suggests, that simple non-invasive liver fibrosis scores should

be used with caution as single diagnostic tools in routine clinical practice. However, the fibrosis scores may be used to support additional work-up in PLWH suspected for liver fibrosis, and the high NPVs suggest, that they may be suitable to rule out potential liver fibrosis.

7. Strengths and Limitations

Studies conducted in this PhD thesis have several strengths. First, the size of the COCOMO cohort and the detailed information on a broad spectrum of confounders allowed us to make various analysis on factors associated with NAFLD and liver fibrosis and to estimate the independent effect of a positive HIV status. The identical procedures for data collection in the COCOMO study and the CGPS reduced the risk of bias. However, residual confounding cannot be excluded and information on e.g genotypes and microbial translocation were not available at time of this PhD. Questionnaires are associated with recall bias which may affect e.g. the self-reported alcohol use and thus confound the results in any direction. The cross-sectional design does not allow any conclusions on causality.

8. Conclusion and Perspectives

Based on results from this PhD thesis, we conclude that PLWH without viral hepatitis and excessive alcohol intake have a lower risk of moderate-to-severe hepatic steatosis compared to the background population but have a higher risk of liver fibrosis. Overall, NAFLD and liver fibrosis should be considered in patients with high BMI, high ALT and high age. Further, patients exposed to thymidine analogues, didanosine or integrase inhibitor may require special attention and additional work-up.

Several questions remain unanswered. Why are PLWH at lower risk of NAFLD compared to the background population? Is this explained by a more metabolic healthy phenotype or is it explained by residual confounding such as microbial translocation, genotype etc.? What drives the increased risk of liver fibrosis in PLWH? NAFLD was strongly associated with liver fibrosis, but is it NAFL, NASH or a combination of both that triggers the fibrogenesis? How do we screen, diagnose and monitor patients with NAFLD and liver fibrosis in routine clinical practice? Which available diagnostic methods are reliable and what cut-offs should be applied in PLWH? How do we treat PLWH diagnosed with NAFLD and liver fibrosis? Is the pathogenesis of NAFLD and liver fibrosis the same in PLWH and people not living with HIV?

The following studies may help address these research questions:

- Prospective studies to assess risk factors of incident NAFLD and liver fibrosis as well as risk factors for disease progression, regression and stability in PLWH diagnosed with NAFLD or fibrosis.
- Prospective studies based on liver biopsies to characterize the natural history of NAFLD and liver fibrosis in PLWH compared to uninfected controls
- Prospective studies that include various known and novel diagnostic methods for NAFLD and fibrosis to be compared with liver biopsies, to develop and validate an algorithm for screening, diagnosis and monitoring of NAFLD and liver fibrosis in PLWH.
- Observational and interventional studies to address the large gap in knowledge on how to treat PLWH with NAFLD and liver fibrosis

The COCOMO study may answer some of these questions, but new studies are needed to fully understand the spectrum of NAFLD and liver fibrosis in PLWH.

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10. Manuscripts

Manuscript I

Prevalence and Risk Factors of Hepatic Steatosis in HIV Infection: The COCOMO Liver Study

Prevalence and Risk Factors of Moderate to Severe Hepatic Steatosis in HIV Infection: The Copenhagen Co-Morbidity Liver Study

Running title: Hepatic steatosis in HIV infection

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ABSTRACT (Word count: 192/200)

Background

People living with HIV (PWH) may be at risk of non-alcoholic fatty liver disease (NAFLD). We compared the prevalence of moderate-to-severe hepatic steatosis (M-HS) in PWH with HIV-uninfected controls and determined risk factors for M-HS in PWH.

Methods

The Copenhagen Co-Morbidity in HIV infection Study included 453 participants and the Copenhagen General Population Study 765 participants. None had prior or current viral hepatitis or excessive alcohol intake. M-HS was assessed by unenhanced CT liver scan defined by liver attenuation ≤48 Hounsfield units. Adjusted odds ratios (aOR) were computed by adjusted logistic regression.

Results

The prevalence of M-HS was lower in PWH compared to uninfected controls (8.6% vs. 14.2%, p<0.01). In multivariable analyses, HIV (aOR:0.44, p<0.01); female sex (aOR:0.08, p=0.03); physical activity level (aOR 0.09 very active vs inactive, p<0.01); alcohol (aOR:0.89 per unit/week, p=0.02); BMI (aOR:1.58 per 1 kg/m^2, p<0.01); ALT (aOR:1.76 per 10 U/L, p<0.01); and exposure to integrase inhibitors (aOR: 1.28 per year, p=0.02) were associated with M-HS.

Conclusions

Moderate-to-severe hepatic steatosis is less common in PWH compared to demographically comparable uninfected controls. Besides BMI and ALT, integrase inhibitor exposure was associated with higher prevalence of steatosis in PWH.

Key words: NAFLD, NAFL, fatty liver disease, comorbidity, human immunodeficiency virus

Introduction

In the Western World, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in adults with an estimated overall prevalence of 25 % [1]. A high prevalence of NAFLD has been reported for people living with HIV (PWH), but with a wide range from 13-73% due to substantial differences in study populations and diagnostic methods used [2–6]. A recent meta-analysis found a prevalence of NAFLD in PWH without viral hepatitis of 35% based on imaging procedures [7].

NAFLD covers a wide spectrum of liver disease from hepatic steatosis with accumulation of fat within the hepatocytes to non-alcoholic steatohepatitis (NASH) with additional inflammation and injury of the hepatocytes to liver cirrhosis, liver failure and hepatocellular carcinoma. Hepatic steatosis has been considered a benign condition, but a recent study of HIV uninfected individuals with serial liver biopsies showed that 44% of individuals with baseline hepatic steatosis progressed to NASH and 22% progressed to advanced fibrosis [8]. As liver fibrosis is the only histological feature of long-term prognosis in NAFLD [9], this is of major concern and patients at risk of progression to NASH and liver fibrosis should be identified to prevent disease progression. Risk factors for NAFLD in HIV infection have differed in previous studies. A meta-analysis found that an increase in body mass index (BMI), waist circumference, type 2 diabetes mellitus, hypertension and high levels of total-cholesterol; high-density lipoprotein (HDL); low-density lipoprotein (LDL); triglycerides; fasting glucose; alanine transaminase (ALT); aspartate transaminase (AST) and CD4+ T-cell count were all associated with higher odds of NAFLD [7]. Further, antiretroviral treatment (ART) may contribute to the development of NAFLD due to adverse metabolic effects with mitochondrial dysfunction [10–12].

The aim of this study was to determine if the prevalence of hepatic steatosis was different between PWH and matched HIV uninfected individuals. We hypothesized that PWH had a higher prevalence of hepatic steatosis compared to HIV uninfected individuals. Factors associated with hepatic steatosis were assessed in PWH and the influence of HIV infection evaluated.

Methods

Study populations

The COCOMO Study has been described in detail elsewhere [13,14]. In short, the COCOMO study is an observational, longitudinal cohort study designed to estimate prevalence and incidence of non-AIDS comorbidity in PWH living in Copenhagen, Denmark. Adult PWH were recruited consecutively from the outpatient clinics of the Departments of Infectious Diseases at Rigshospitalet and Amager Hvidovre Hospital in Copenhagen, Denmark from March 2015 through November 2016. The comparator group was retrieved from the Copenhagen General Population Study (CGPS), a prospective cohort study of >100,000 randomly selected adult individuals from the area of Copenhagen initiated in 2003 [15–17]. The comparator group was

assumed to be HIV-uninfected as the prevalence of HIV infection was estimated to be 0.1% in the Danish, adult population in 2016 [18].

Data Collection

The data collection has been described in detail elsewhere [13]. In short, comprehensive questionnaires were completed comprising >100 items with information on health, dietary habits, and lifestyle. HIV-specific information and status of hepatitis B and C co-infection was retrieved from medical records. Data was >95% complete unless otherwise stated. All data was collected uniformly in the COCOMO study cohort and the CGPS study cohort with identical questionnaires, laboratory equipment and physical examination techniques.

CT scan of upper abdomen

CT scan of the upper abdomen was performed on a Aquillion One scanner (Toshiba Medical Systems, Otawara-shi, Tochigi-ken, Japan) using identical scan protocols for the two cohorts[13,19]. Liver attenuation was measured for all CT scans using Vitrea 3.1 imaging software (Vital Images Inc., Minnetonka, MN, USA). A region of interest (ROI) with an area of 1500 mm^2 (+/- 100 mm^2) was placed in Coinaud liver segments 5 and 6. The average liver attenuation was calculated from the two ROIs and results presented in Hounsfield Units (HU). All analyses were performed by trained physicians blinded to clinical and biochemical details of the study participants. A pilot study of 20 participants demonstrated a high interrater correlation (R^2=0.98 and spearman rho=0.99) with no bias.

All participants in the COCOMO study were invited to a CT scan; 921 participants (84%) attended. Participants from the CGPS aged 40 years or above were randomly invited to a CT scan; 70% accepted the invitation [20] .

Definitions of Outcome

The physiologic attenuation of the liver parenchyma ranges from 55 to 65 HU by unenhanced CT of the liver [21]. Liver attenuation is inversely correlated with liver fat content, yielding lower Hounsfield units with increasing amounts of hepatic steatosis. In this study we defined moderate-to-severe hepatic steatosis as a CT liver attenuation <48 HU with a specificity of 100%, sensitivity of 53.8%, positive predictive value of 100% and negative predictive value of 93.9% [22]. Sensitivity analyses were conducted to test a threshold of CT liver attenuation ≤40 HU, which has been used to exclude mild hepatic steatosis in previous literature [23,24].

Ethics

The study was approved by the regional ethics committee of the Capital Region of Denmark (H-15017350; H-KF-01-144/01)) and conducted in accordance with the declaration of Helsinki. All

participants provided informed consent. The study has been registered at clinicaltrials.gov (NTC02382822).

Statistical analyses

PWH and uninfected controls with a CT scan of the abdomen aged 40 years or older were matched on sex and 5-years age strata in a ratio of 1:2 except for men aged 40-55 was matched 1:1 due to availability (**Supplementary Figure S1**). Baseline clinical and demographic data of the two cohorts were compared by Fisher's exact test and Chi square test (categorical variables), and Kruskal Wallis and Mann-Whitney's U-test (continuous). Univariable and multivariable logistic regression models were conducted in PWH with moderate-to-severe hepatic steatosis as outcome. Two multivariable regression models were constructed with a priori selection of independent variables. Both models were adjusted for age (per decade), sex (female vs male), and Caucasian ethnicity (no vs yes). The metabolic model was further adjusted for: body mass index (BMI, per 1 kg/m^2), plasma total cholesterol (per 1 mM), plasma triglycerides (per 1 mM), diabetes (yes vs no), plasma glucose (per 1 mM) and plasma alanine aminotransferase (ALT, per 10 IU/L). The lifestyle model was further adjusted by: smoking status (never smoker, current smoker, previous smoker), alcohol consumption (per 1 unit per week) and level of physical activity (inactive, moderate inactive, moderate active, very active). The association between a positive HIV status and moderate-to-severe hepatic steatosis was estimated in the total population. The association between HIV specific variables including ART drug classes were estimated in univariable analyses and multivariable analyses after adjustment for sex, age, ethnicity, BMI and duration of HIV infection. Results are presented as crude and adjusted odds ratios (OR) with 95% confidence intervals (CI). A p-value <0.05 was considered statistically significant. The interaction between BMI (>25 kg/m^2) and HIV status was tested to determine whether HIV modifies the effect of BMI on moderate-to-severe hepatic steatosis. We defined hepatitis B virus infection (HBV) as presence of hepatitis B surface antigen (HBsAg); hepatitis C virus infection (HCV) as presence of anti-HCV antibodies (anti-HCV); excessive alcohol intake as an average consumption of >14 alcoholic units per week for men and >7 alcoholic units per week for women; abdominal obesity as a waist-to-hip ratio of ≥0.90 for men and ≥0.85 for women according to the International Diabetes Federation [25]; and metabolic syndrome as a minimum of three of the following 5 items: (1) Waist circumference waist circumference of ≥94 cm for men and ≥80 cm for women; (2) Systolic blood pressure ≥130 mmHg and/or antihypertensive treatment; (3) plasma HDL ≤1.036 mmol/l for men, and plasma HDL ≤1.295 mmol/l for women; (4) plasma triglycerides ≥ 1.693 mmol/l; (5) self-reported diabetes mellitus and/or antidiabetic treatment and/or non-fasting plasma glucose ≥11.1 mmol/l [25]. All analyses were conducted in R version 3.4.1.

Results

A total of 1,099 participants were included in the COCOMO study. Participants were excluded due to age below 40 years (n=191), CT scan unavailability (n=143), HBV (n=23), HCV (n=82), excessive alcohol consumption (n=174) or missing information on these parameters (n=52). The final study population comprised 453 PWH. A total of 1,192 participants from the CGPS were selected for the comparator group; participants were excluded due to excessive alcohol consumption (n=415). The final control population comprised 765 individuals (**Figure 1**).

Clinical and demographic characteristics

Clinical and demographic characteristics of PWH and uninfected controls are depicted in **Table 1**, and HIV specific characteristics of PWH in **Table 2**. In short, PWH were more likely males (86 vs 82%), of non-Scandinavian descent (25 vs 4%), with lower BMI (25 vs 26 kg/m2), less alcohol use (48 vs 72 grams/week) and higher physical activity level and educational level. The majority of PWH acquired HIV through sex between men (71%), received ART (99%), and were well-treated with HIV RNA < 50 copies/mL (97%) and a median CD4 T-cell count of 690 cells/µL (IQR: 520;884). Clinical and demographic characteristics stratified by presence of moderate-to-severe hepatic steatosis can be found in **Table 1** for PWH and in **Supplementary Table S1** for uninfected controls.

Prevalence of moderate-to-severe hepatic steatosis in PWH and uninfected controls

Thirty-nine (8.6% (95% CI: 6.4-11.6%)) of PWH had CT-defined moderate-to-severe hepatic steatosis compared to 109 (14.2% (95% CI: 11.9-196.9%)) of HIV uninfected controls (p<0.001) (**Figure 2A**). The distribution of liver attenuation in PWH and uninfected controls are depicted in the **Supplementary Figure S2**. The median CT liver attenuation was comparable in PWH and controls (61.3 HU (IQR: 56.5;65.6) vs 61.6 HU (IQR: 53.9;66.1), p=0.56).

HIV infection and moderate-to-severe hepatic steatosis

Compared to controls, PWH had lower odds of moderate-to-severe hepatic steatosis in unadjusted and adjusted analyses (**Figure 2B**). The association between BMI and moderate-to-severe hepatic steatosis was not modified by HIV status (p=0.91 for interaction). In PWH, neither current CD4 T cell count, nadir CD4 T cell count < 200 cells/µL, plasma HIV RNA ≥50 copies/mL, nor duration of HIV infection were associated with moderate-to-severe hepatic steatosis (**Table 3**). No association was found between moderate-to-severe hepatic steatosis and exposure to nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase

inhibitors (NNRTI), integrase inhibitors, protease inhibitors, didanosine, or thymidine analogues (stavudine and zidovudine) (**Table 3**). However, the cumulative duration of exposure to an integrase inhibitor was associated with higher odds of moderate-to-severe hepatic steatosis in univariate analyses (OR: 1.19 (95% CI: 1.02;1.39), per year, p=0.02) and the association persisted after adjustment for age, sex, BMI, and duration of HIV infection (aOR: 1.28 (95% CI: 1.00;1.65), per year, p=0.05). Cumulative duration of exposure to a thymidine analogue was not associated with higher odds of moderate-to-severe steatosis in univariate analyses, but after adjustment for age, sex, BMI and duration of HIV infection, a positive association was found (aOR: 1.19 (95% CI: 1.03;1.37) per year, p=0.02).

Factors associated with moderate-to-severe hepatic steatosis in PWH

Factors associated with moderate-to-severe hepatic steatosis in PWH can be found in **Table 4** and **Figure 3**. In PWH, abdominal obesity, diabetes, metabolic syndrome and higher BMI, waist circumference, plasma ALT, plasma AST, and plasma triglycerides were associated with higher odds of moderate-to-severe hepatic steatosis in unadjusted models (**Table 4**). Higher physical activity level, higher educational level, and higher plasma HDL concentration were associated with lower odds of moderate-to-severe hepatic steatosis. After adjusting for potential metabolic confounders, higher BMI and higher ALT were associated with higher odds of moderate-to-severe hepatic steatosis, while female sex was associated with lower odds. After adjusting for potential lifestyle confounders female sex and higher physical activity level were associated with lower odds of moderate-to-severe hepatic steatosis (**Figure 3**).

In PWH, a higher weekly alcohol consumption within the national recommendations for excessive alcohol intake was associated with lower odds of moderate-to-severe hepatic steatosis in univariate and multivariate analyses after adjusting for age, sex, ethnicity, BMI and physical activity level (**Table 4** and **Figure 3**). Type of alcohol, sugar-sweetened beverages, coffee, fast food and type of meat product were not associated with moderate-to-severe hepatic steatosis in adjusted analysis (**Supplementary Table S2**).

Sensitivity analyses

In sensitivity analyses with a CT liver attenuation threshold of ≤40HU, the lower prevalence of moderate-to-severe hepatic steatosis in PWH compared to HIV uninfected controls persisted (3.5% (95% CI: 2.2;5.7%) vs 6.4% (95% CI: 4.9;8.4), p=0.04). Accordingly, a positive HIV status was associated with lower odds of hepatic steatosis in univariable analyses (OR: 0.54 (95% CI: 0.30;0.95), p=0.03) and after adjusting for age and sex (aOR: 0.53 (95% CI: 0.30;0.95), p=0.03).

Discussion

In this study of 453 predominantly well-treated PWH without chronic hepatitis and excessive alcohol use and 765 HIV-uninfected controls, PWH had a lower prevalence of CT-defined moderate-to-severe hepatic steatosis than HIV uninfected controls. HIV infection was independently associated with lower odds of hepatic steatosis.

In our cohort of PWH without viral hepatitis or excessive alcohol intake, 8.6% had CT-evidence of moderate-to-severe hepatic steatosis, which was considerably lower than compared to a meta-analysis of NAFLD in PWH [7]. Several reasons may account for the discrepancy. Firstly, time may play a role as 1. generation antiretroviral drugs had more liver toxicity than currently used agents. Secondly, some studies included individuals with signs of liver disease (e.g. persistently elevated liver enzymes) [4,6,25–27] or individuals with metabolic disorders [3,28]. Thirdly, the prevalence of hepatic steatosis varies globally [29,30] due to increased adoption to a Western diet and sedentary lifestyle as well as genetic variation [20,31]. Finally, the presence of steatosis may differ due to different diagnostic methodology used. Our study supports the findings of *Price et al* who found a lower prevalence of hepatic steatosis in unselected PWH compared to HIV-uninfected controls assessed by CT liver scans (13 vs 19%) [10]. Overall, our study and Price et al. question the proposed higher risk of moderate-to-severe hepatic steatosis for PWH compared to a demographically similar group of HIV uninfected individuals. Future studies should explore this in more detail because our findings do not exclude the possibility of an increased risk of mild hepatic steatosis or of more progressive NAFLD in PWH. Our study design does not permit any distinction as to whether the difference in the proportion of hepatic steatoses between the two groups is related to HIV itself or factors associated with HIV infection. Finally, residual confounding of e.g. life style cannot be precluded.

Few studies have been able to investigate the association between HIV infection and hepatic steatosis due to lack of a HIV-uninfected comparator group. A key finding of this study was, that a positive HIV status independently was associated with lower odds of moderate-to-severe hepatic steatosis. The result was robust even after adjustment for age, sex, ethnicity, and potential metabolic and lifestyle confounders and when using a lower threshold for moderate-to-severe hepatic steatosis of ≤40HU. Interestingly, *Price et al* reported that HIV was independently associated with lower odds of hepatic steatosis (OR 0.44, $p<0.002$) [10], which is consistent with our findings. This may emphasize the complexity underlying the pathogenesis of hepatic steatosis in PWH [32] and warrants future studies.

Adipose tissue abnormalities leading to lipodystrophy and atrophy are associated with specific antiretroviral drugs, in particular with thymidine analogues *[34]*. Thymidine analogues and didanosine have hepatotoxic properties. Price et al. found an association between didanosine use and hepatic steatosis but this was not reproduced in our study *[10]*. A possible explanation may be

that only 1 of 6 PWH in our study had been exposed to didadosine and that the exposure time was less in the COCOMO cohort compared to MACS cohort (2 vs 4 years) *[10]*. We did, however, see an association between use of thymidine analogues and hepatic steatosis. Unfortunately, we were unable to show if the association was due to stavudine or zidovudine. Interestingly, use of thymidine analogues was discontinued approximately a decade prior to inclusion in COCOMO. Similarly, low visceral and subcutaneous adipose tissue density was associated with prior exposure to thymidine analogue and/or didanosine exposure in the cohort *[35]*. Collectively, this suggests that the hepatotoxic effects of thymidine analogues may be long-lasting in terms of moderate-to-severe hepatic steatosis. Individuals exposed to thymidine analogues may require additional work-up for hepatic steatosis. Further, we found an association between cumulative exposure to integrase inhibitor treatment and hepatic steatosis. Of note, use of integrase inhibitors has been associated with excess weight gain *[36]*. It is likely that there may be a direct link between weight gain and hepatic steatosis. Alternatively, integrase inhibitors may induce hepatic steatosis regardless of overall weight gain. Future studies are warranted to study if specific integrase inhibitors may infer an increased risk of hepatic steatosis and fibrosis.

Male gender, higher BMI and higher ALT were associated with higher odds of moderate-to-severe hepatic steatosis in PWH. These results are consistent with previous findings, and especially the association between BMI, insulin resistance and hepatic steatosis are well established [2,3,10,36]. Surprisingly, we did not find a significant association between diabetes and moderate-to-severe hepatic steatosis in PWH after adjusting for metabolic risk factors. In our study, PWH were more frequently on antidiabetic- and lipid-lowering treatment compared to controls, which may indicate more frequent physician encounters due to regular HIV care. One may speculate, that PWH initiate therapy for diabetes and dyslipidaemia at an earlier stage, which may cause a lower rate of hepatic fat accumulation. Further, there could be a synergistic effect of diabetes and increasing BMI on the development of hepatic steatosis, as the comparator group had higher BMI and more overweight individuals. A synergistic effect of excessive alcohol intake and increased BMI on liver disease has been reported previously [37], and future studies should explore these possible synergistic effects in NAFLD. Finally, no association was found between moderate-to-severe hepatic steatosis and alcoholic beverages, non-alcoholic beverages, fast-food or meat items in adjusted analyses. However, current international guidelines on treatment of NAFLD focus on changes in diet and lifestyle [38], and future studies should explore the role of different diets in randomized controlled trials.

To our knowledge, this is the largest study of moderate-to-severe hepatic steatosis in PWH with a comparable HIV-uninfected control group using identical methodologies. Our study is limited by a homogeneous population of PWH, which limits the generalizability to other settings. With a sensitivity of 54%, it cannot be precluded that PWH and uninfected controls may have been

missed in the diagnosis of moderate-to-severe hepatic steatosis. Further, conclusions on mild hepatic steatosis cannot be made using CT liver scans. Sampling errors cannot be avoided despite the attempt to minimize this, no information on inflammatory markers, gut microbiota or insulin resistance (e.g. HOMA-IR) were available, and unmeasured residual confounding cannot be excluded. No testing for HIV, HBV or HCV were available for the comparator group. Finally, causality cannot be inferred in a cross-sectional study.

In conclusion, the prevalence of moderate-to-severe hepatic steatosis in this cohort of well-treated PWH was lower compared to a demographically comparable cohort of HIV uninfected individuals, and HIV infection was independently associated with lower odds of moderate-to-severe hepatic steatosis. Male sex, higher BMI and higher ALT were associated with higher odds of hepatic steatosis. Exposure to integrase inhibitor treatment was associated with moderate-to-severe hepatic steatosis and should be explored in more detail.

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Figure 1 Flowchart of study participants

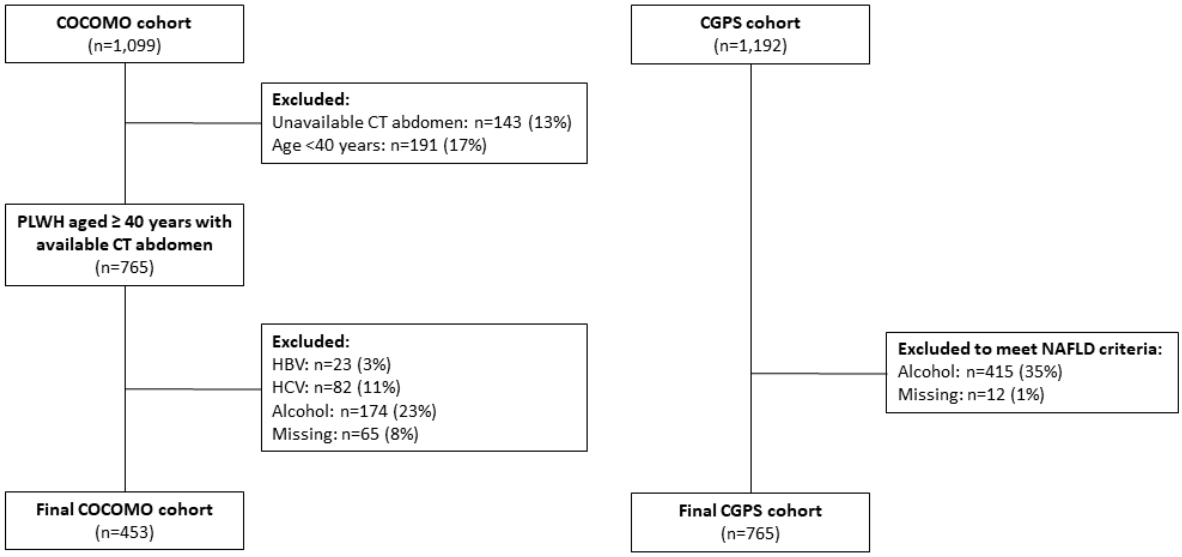
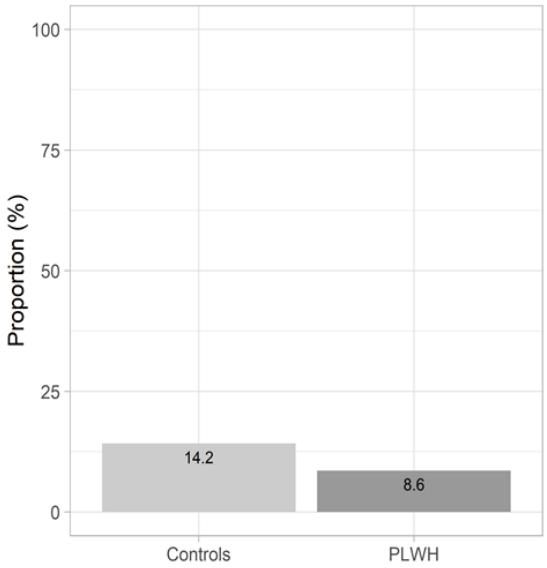


Figure 2 (A) Prevalence of moderate to severe hepatic steatosis in people living with HIV and uninfected controls. (B) Association between HIV infection and moderate to severe hepatic steatosis. Odds ratio (OR) and 95% confidence interval (CI) obtained from univariate and multivariable logistic regression analyses with results shown on a log10 scale. Metabolic model adjusted for age, sex, ethnicity, BMI, plasma total cholesterol, plasma triglycerides, diabetes, plasma glucose, and ALT. The lifestyle model adjusted for age, sex, ethnicity, smoking status, weekly alcohol consumption, and physical activity level.

A


100
75
50
25
0
Proportion (%)
14.2
8.6
Controls
PLWH

B

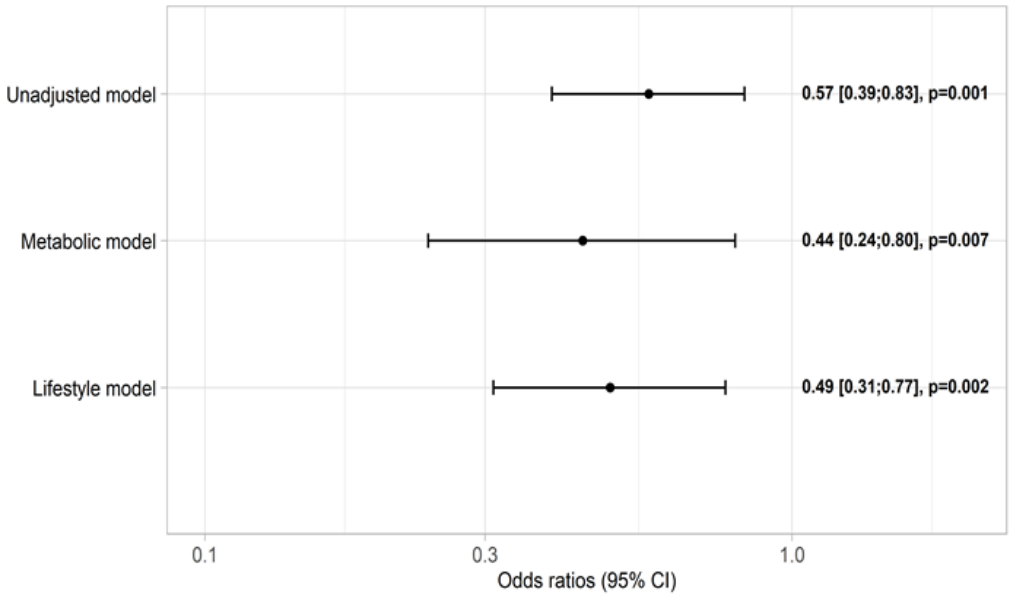
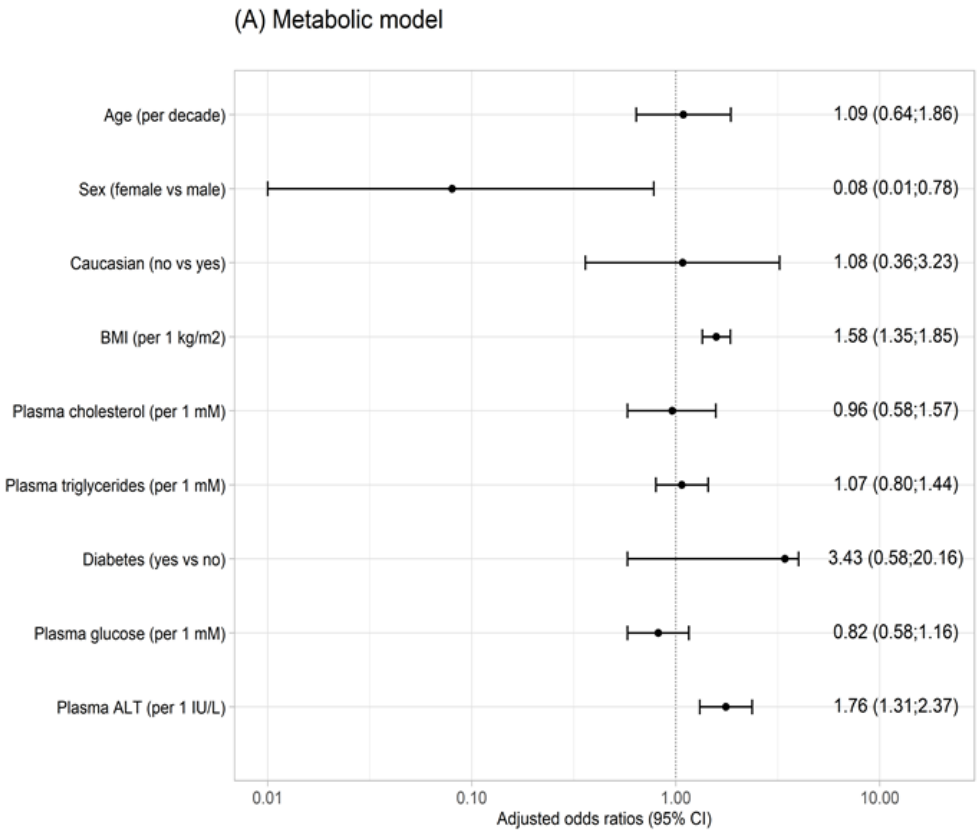

Unadjusted model
Metabolic model
Lifestyle model
0.57 [0.39;0.83], p=0.001
0.44 [0.24;0.80], p=0.007
0.49 [0.31;0.77], p=0.002
0.1
0.3
1.0
Odds ratios (95% CI)

Figure 3 Factors associated with moderate-to-severe hepatic steatosis in people living with HIV. Odds ratio (OR) and 95% confidence interval (CI) obtained from univariate logistic regression analyses with results shown on a log10 scale. (A) Metabolic model adjusted for age, sex, ethnicity, BMI, plasma total cholesterol, plasma triglycerides, diabetes, plasma glucose, and ALT. (B) The lifestyle model adjusted for age, sex, ethnicity, smoking status, weekly alcohol consumption, and physical activity level.



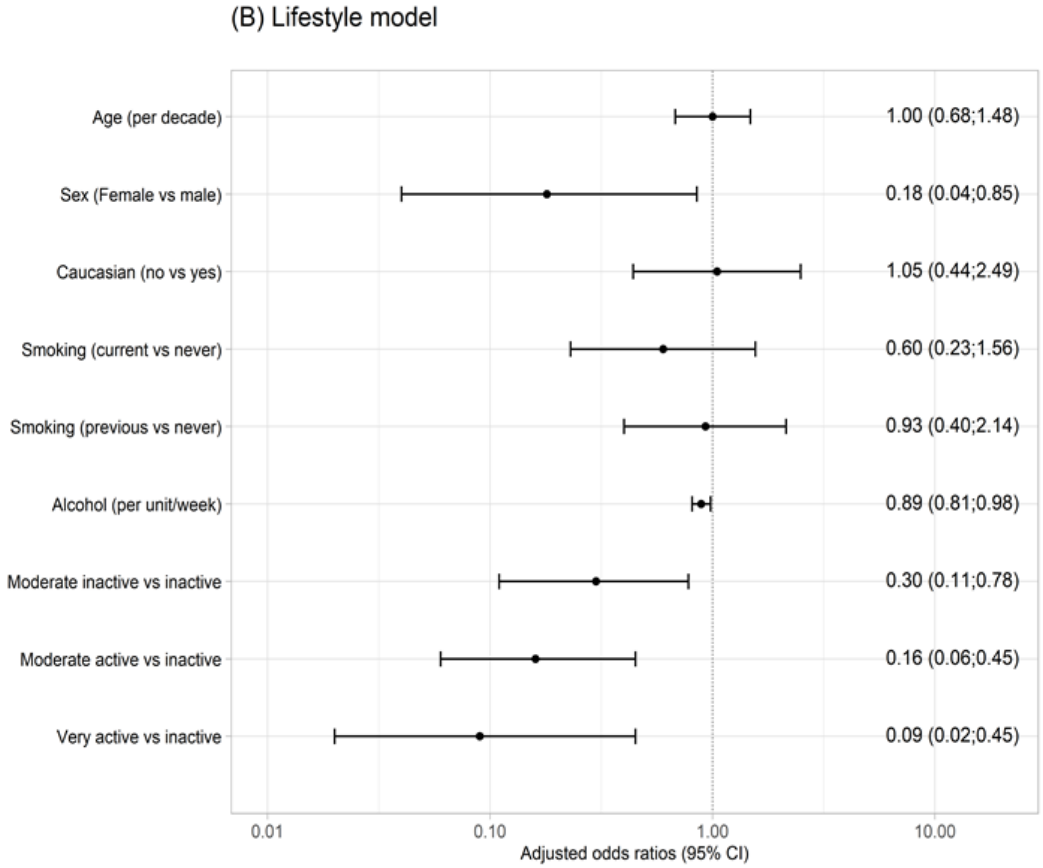

(B) Lifestyle model
Age (per decade)
Sex (Female vs male)
Caucasian (no vs yes)
Smoking (current vs never)
Smoking (previous vs never)
Alcohol (per unit/week)
Moderate inactive vs inactive
Moderate active vs inactive
Very active vs inactive
1.00 (0.68;1.48)
0.18 (0.04;0.85)
1.05 (0.44;2.49)
0.60 (0.23;1.56)
0.93 (0.40;2.14)
0.89 (0.81;0.98)
0.30 (0.11;0.78)
0.16 (0.06;0.45)
0.09 (0.02;0.45)
0.01
0.10
1.00
10.00
Adjusted odds ratios (95% CI)

Table 1 Clinical and demographic characteristics of people living with HIV (PLWH) and population controls.					
	PLWH			Controls	p-value
	Steatosis (n=39)	No steatosis (n=414)	Total (n=453)	Total (n=765)	
Age (years), median (IQR)	53.1 (46.2;63.3)	52.1 (46.8;60.8)	52.4 (46.8, 61.0)	53.4 (47.7, 61.4)	0.16
Sex (male), n (%)	37 (94.9)	351 (84.8)	388 (85.7)	625 (81.7)	0.09
Ancestry, n (%)					<0.01
Scandinavian	27 (71.1)	311 (76.0)	338 (75.6)	728 (96.0)	
Other European	7 (18.4)	45 (11.0)	52 (11.6)	28 (3.7)	
Middle East and Indian Subcontinent	1 (2.6)	3 (0.7)	4 (0.9)	0 (0.0)	
Other	3 (7.9)	50 (12.2)	53 (11.9)	2 (0.3)	
Educational level, n (%)					<0.01
None	11 (29.7)	39 (9.8)	50 (11.5)	91 (11.9)	
Short	8 (21.6)	96 (24.2)	104 (24.0)	209 (27.4)	
Middle Length	8 (21.6)	169 (42.7)	177 (40.9)	425 (55.7)	
University	10 (27.0)	92 (23.2)	102 (23.6)	38 (5.0)	
Smoking, n (%)					<0.01
Current smoker	8 (20.5)	108 (26.1)	116 (25.6)	72 (9.4)	
Previous smoker	14 (35.9)	159 (38.4)	173 (38.2)	331 (43.3)	
Never smoker	17 (43.6)	147 (35.5)	164 (36.2)	359 (46.9)	
Alcohol (g/week), median (IQR)	24 (0;60)	54 (12;108)	48.0 (0, 108)	72.0 (36, 108)	<0.01
Physical activity, n (%)					
Inactive	10 (27.0)	29 (7.2)	39 (8.9)	49 (6.4)	
Moderate inactive	15 (40.5)	132 (32.8)	147 (33.5)	250 (32.8)	
Moderate active	10 (27.0)	184 (45.8)	194 (44.2)	385 (50.5)	
Very active	2 (5.4)	57 (14.2)	59 (13.4)	78 (10.2)	0.07
Abdominal obesity, n (%)	35 (89.7)	279 (69.8)	314 (71.5)	469 (61.5)	<0.01
Waist circumference (cm), median (IQR)	114 (105;122)	93.5 (86;102)	94.0 (87.0, 104.0)	93.0 (86.0, 101.0)	0.04
Body mass index (kg/m^2), median (IQR)	31.6 (28.4;33.5)	24.3 (22.1;26.8)	24.7 (22.4, 27.5)	26.0 (23.7, 28.4)	<0.01

WHO BMI category, n (%)					
Underweight, < 18.4 kg/m^2	0 (0.0)	10 (2.4)	10 (2.2)	2 (0.3)	
Normal weight, 18.5-24.9 kg/m^2	3 (7.9)	226 (54.7)	229 (50.8)	287 (37.5)	
Overweight, 25-29.9 kg/m^2	10 (26.3)	148 (35.8)	158 (35.0)	349 (45.6)	
Obese ≥ 30 kg/m^2	25 (65.8)	29 (7.0)	54 (12.0)	127 (16.6)	<0.01
Diabetes, n (%)	9 (23.1)	24 (5.8)	33 (7.3)	31 (4.1)	0.02
Metabolic syndrome, n (%)	29 (80.6)	154 (39.9)	183 (43.4)	261 (35.1)	0.01
Lipid lowering treatment, n (%)	6 (16.7)	74 (18.3)	80 (18.2)	88 (11.5)	
Antidiabetic treatment, n (%)	4 (10.3)	21 (5.1)	25 (5.5)	26 (3.4)	0.10
Biochemistry, median (IQR)					
Plasma ALT (IU/L)	39.5 (27;55.8)	25 (20;3)	26 (20, 34)	22 (17, 29)	<0.01
Plasma total cholesterol (mM)	5.2 (4.5;6.1)	4.9 (4.2;5.7)	4.9 (4.2, 5.7)	5.4 (4.8, 6.1)	<0.01
Plasma triglycerides (mM)	3.1 (2.3;4.3)	1.7 (1.3;2.6)	1.8 (1.3, 2.8)	1.5 (1.0, 2.2)	<0.01
Plasma LDL (mM)	3.0 (2.3;3.7)	2.8 (2.2;3.4)	2.8 (2.2, 3.5)	3.2 (2.7, 3.9)	<0.01

Abbreviations: IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; LDL: low-density lipoprotein. Missing variables for COCOMO (CGPS): Ancestry: 6 (7); educational level: 20 (2); physical activity: 14 (3); abdominal obesity: 14 (2); waist circumference: 14 (2); BMI 2 (0); Metabolic syndrome 31 (21); lipid lowering treatment 13(1); ALT 30 (9); Cholesterol 21 (9).

Table 2 Characteristics of people living with HIV (n=453)	
Route of HIV transmission	
MSM	316 (70.7)
HSX	101 (22.6)
IDU	2 (0.4)
Other	28 (6.3)
Blood CD4 T-cell count (cells/μL), median (IQR)	690 (520, 884)
< 200	4 (0.9)
200-349	25 (5.6)
350-500	71 (15.8)
> 500	349 (77.7)
Blood CD4 nadir T-cell count (cells/ μL), median (IQR)	220 (110;320)
Plasma HIV RNA ≥50 copies/mL, n (%)	14 (3.1)
Duration of HIV infection (years), median (IQR)	16.0 (8.3, 23.1)
cART, n (%)	445 (98.9)
ART exposure, n (%)	
NRTI	441 (97.4)
NNRTI	353 (77.9)
Integrase inhibitors	141 (31.1)
Protease inhibitors	258 (57.0)
Didanosine	76 (16.8)
Thymidine analogue	261 (57.6)
Duration of ART exposure (years), median (IQR)	
NRTI	15.1 (7.2, 22.4)
NNRTI	7.4 (3.6, 11.5)
Integrase inhibitors	1.9 (0.9, 5.2)
Protease inhibitors	10.6 (4.8, 19.2)
Didanosine	2.5 (0.8, 5.8)
Thymidine analogue	6.2 (3.5, 9.1)

Abbreviations: HIV: human immunodeficiency virus; IQR: interquartile range; ART: antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor

Table 3 HIV specific factors associated with moderate-to-severe hepatics steatosis in PLWH				
	Crude OR (95% CI)	**p-value**	**aOR (95% CI)**	**p-value**
Blood CD4 T-cell count (per 50 cells/µl)	1.03 (0.98;1.09)	0.27	1.05 (0.98;1.12)	0.18
Blood CD4 nadir T-cell count < 200 cells/µl	1.33 (0.69;2.57)	0.39	1.52 (0.61;3.76)	0.37
Plasma HIV RNA ≥50 copies/mL	3.02 (0.81;11.33)	0.1	1.68 (0.36;7.96)	0.51
Duration of HIV infection (per 5 years)	1.03 (0.85;1.24)	0.76	1.04 (0.99;1.09)	0.16
ART exposure (yes vs no)				
NRTI	1.04 (0.13;8.25)	0.97	0.61 (0.06;6.36)	0.68
NNRTI	0.91 (0.38;1.72)	0.58	0.52 (0.19;1.38)	0.19
Integrase inhibitors	1.43 (0.73;2.81)	0.30	1.76 (0.73;4.22)	0.21
Protease inhibitors	1.57 (0.78;3.14)	0.20	1.49 (0.56;3.93)	0.42
Didanosine	1.31 (0.58;2.98)	0.51	1.90 (0.65;5.58)	0.24
Thymidine analogue	1.35 (0.68;2.67)	0.39	1.12 (0.35;3.55)	0.85
Stavudine	1.53 (0.72;3.28)	0.27	2.22 (0.79;6.27)	0.13
Zidovudine	1.29 (0.66;2.54)	0.46	1.14 (0.39;3.34)	0.81
Duration of ART exposure (per year)				
NRTI	1.02 (0.98;1.05)	0.35	1.03 (0.97;1.09)	0.35
NNRTI	1.01 (0.94;1.09)	0.73	1.00 (0.91;1.09)	0.99
Integrase inhibitors	1.19 (1.02;1.39)	0.02	1.28 (1.00;1.65)	0.05
Protease inhibitors	1.00 (0.96;1.05)	0.97	1.00 (0.94;1.07)	0.98
Didanosine	0.96 (0.78;1.18)	0.67	0.94 (0.75;1.19)	0.62
Thymidine analogue	1.07 (0.98;1.17)	0.14	1.19 (1.03;1.37)	0.02
Stavudine	1.42 (1.08;1.88)	0.01	1.13 (0.77;1.65)	0.54
Zidovudine	1.01 (0.92;1.12)	0.79	1.09 (0.94;1.25)	0.26

Abbreviations: OR: odds ratio; aOR: adjusted odds ratio; ART: antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor

Table 4 Factors associated with moderate-to-severe hepatic steatosis in people living with HIV

Variable	Crude OR (95% CI)	p-value
Sex (female vs male)	0.30 (0.07;1.28)	0.10
Age (per decade)	1.12 (0.80;1.57)	0.51
Age groups		
< 50 years	Ref	
50-60 years	1.12 (0.51;2.46)	0.78
61-70 years	1.06 (0.43;2.64)	0.89
> 70 years	1.80 (0.55;5.90)	0.33
Ancestry		
Scandinavian	Ref	
Other European	1.79 (0.74;4.36)	0.20
Middle East and Indian Subcontinent	3.84 (0.39;38.18)	0.25
Educational level		
None	Ref.	
Short	0.30 (0.11;0.79)	0.02
Middle length	0.17 (0.06;0.44)	<0.01
University	0.39 (0.15;0.98)	0.05
Smoking		
Never smoker	Ref	
Current smoker	0.64 (0.27;1.54)	0.32
Previous smoker	0.76 (0.36;1.60)	0.47
Alcohol (per 1 unit/week)	0.89 (0.81;0.97)	0.01
Physical activity		
Inactive	Ref	
Moderate inactive	0.33 (0.13;0.81)	0.02
Moderate active	0.16 (0.06;0.41)	<0.01
Very active	0.10 (0.02;0.50)	<0.01
Abdominal obesity (yes vs no)	3.79 (1.32;10.91)	0.01
Waist circumference (per 1 cm)	1.16 (1.11;1.20)	<0.01
BMI (per 5 kg/m^2)	6.84 (4.11;11.40)	<0.01
BMI ≥25 kg/m^2 (yes vs no)	15.56 (4.71;51.39)	<0.01
Diabetes (yes vs no)	4.86 (2.08;11.39)	<0.01
Metabolic syndrome (yes vs no)	6.24 (2.67;14.60)	<0.01
Biochemistry		
Plasma ALT (per 10 IU/L)	1.73 (1.42;2.10)	<0.01
Plasma AST (per 10 IU/L)	1.44 (1.16;1.78)	<0.01
Plasma total cholesterol (per 1 mM)	1.31 (0.97;1.76)	0.07
Plasma triglycerides (per 1 mM)	1.40 (1.18;1.65)	<0.01
Plasma HDL (per 1 mM)	0.10 (0.03;0.31)	<0.01
Plasma LDL (per 1mM)	1.17 (0.83;1.64)	0.38

Abbreviations: IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; LDL: low-density lipoprotein; OR: odds ratio.

SUPPLEMENTARY MATERIAL

Prevalence and Risk Factors of Hepatic Steatosis in HIV Infection: The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Liver Study

Figure S1 Number of controls for each 5-year age strata in men (A) and women (B).

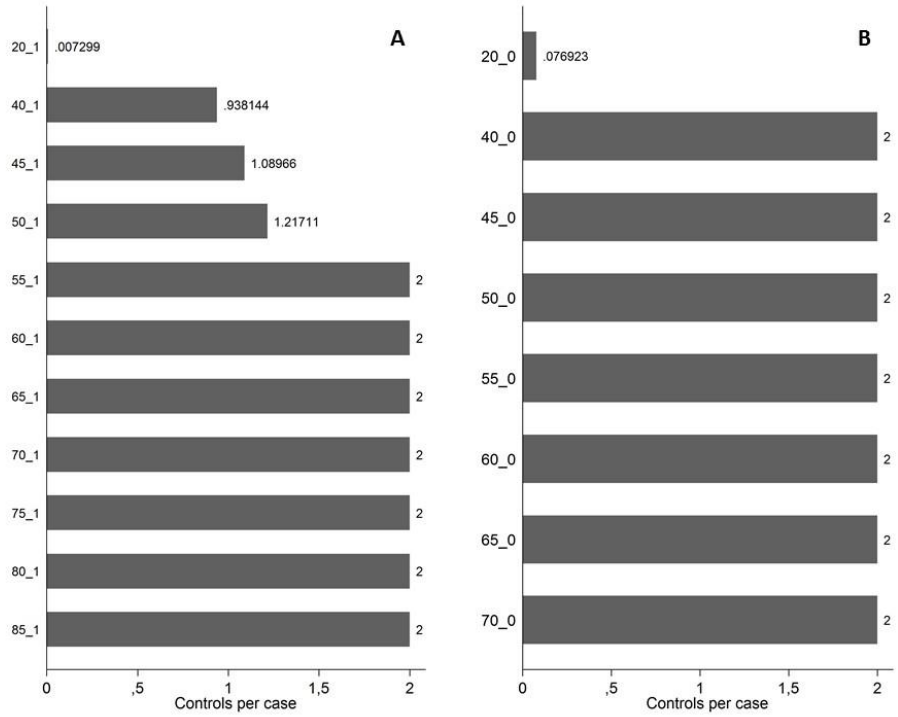


Figure S2 Histogram of liver attenuation in people living with HIV and uninfected controls.

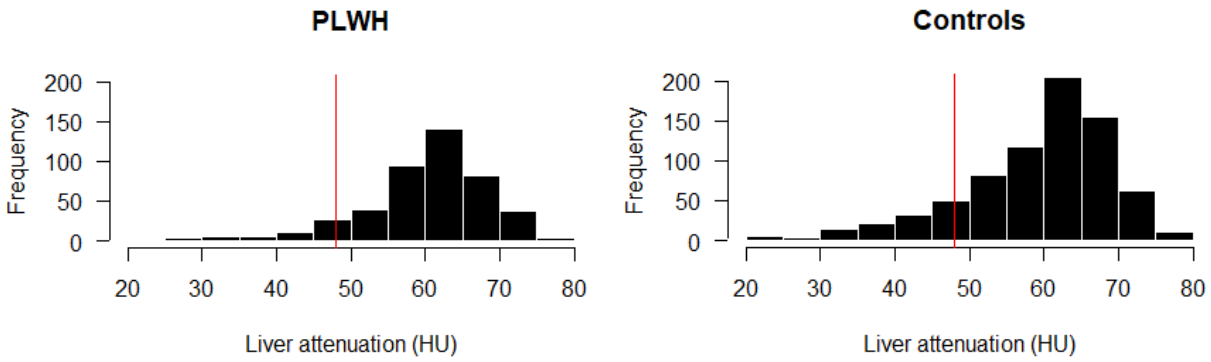


Table S1 Clinical and demographic characteristics of HIV uninfected controls stratified by presence of moderate to severe hepatic steatosis.

	Steatosis (n=109)	No steatosis (n=656)	p-value
Age (Years), median (IQR)	58.0 [50.9, 65.0]	52.7 [47.5, 60.1]	<0.01
Sex (Male), n (%)	101 (92.7)	524 (79.9)	<0.01
Ancestry, n (%)			0.74
Scandinavian	103 (97.2)	625 (95.9)	
Other European	3 (2.8)	25 (3.8)	
Educational level, n (%)			<0.01
None	28 (25.7)	63 (9.6)	
Short	33 (30.3)	176 (26.9)	
Middle Length	46 (42.2)	379 (58.0)	
University	2 (1.8)	36 (5.5)	
Smoking, n (%)			0.38
Current smoker	9 (8.3)	63 (9.6)	
Previous smoker	55 (50.5)	276 (42.1)	
Never smoker	45 (41.3)	314 (47.9)	
Alcohol (g/week), median (IQR)	72 [36, 120]	72 [36, 108]	0.73
Physical activity, n (%)			<0.01
Inactive	13 (11.9)	36 (5.5)	
Moderate inactive	47 (43.1)	203 (31.1)	
Moderate active	44 (40.4)	341 (52.2)	
Very active	5 (4.6)	73 (11.2)	
Abdominal obesity, n (%)	102 (93.6)	367 (56.1)	<0.01
Waist circumference (cm), median (IQR)	109 [102, 114]	91 [85, 99]	<0.01
Body mass index (kg/m^2), median (IQR)	30.4 [27.9, 34.2]	25.5 [23.4, 27.6]	<0.01
WHO BMI category, n (%)			<0.01
Underweight, < 18.4 kg/m^2	0 (0.0)	2 (0.3)	
Normal weight, 18.5-24.9 kg/m^2	1 (0.9)	286 (43.6)	
Overweight, 25-29.9 kg/m^2	46 (42.2)	303 (46.2)	
Obese ≥ 30 kg/m^2	62 (56.9)	65 (9.9)	
Diabetes, n (%)	18 (16.5)	13 (2.0)	<0.01
Metabolic syndrome, n (%)	78 (71.6)	183 (28.8)	<0.01
Lipid lowering treatment, n (%)	26 (23.9)	62 (9.5)	<0.01
Antidiabetic treatment, n (%)	16 (14.7)	10 (1.5)	<0.01
Biochemistry, median (IQR)			
Plasma ALT (IU/L)	30.0 [24.0, 40.0]	21.0 [17.0, 27.0]	<0.01
Plasma total cholesterol (mM)	5.4 [4.7, 6.2]	5.4 [4.8, 6.1]	0.78
Plasma triglycerides (mM)	2.2 [1.5, 3.1]	1.4 [0.9, 2.1]	<0.01
Plasma LDL (mM)	3.2 [2.5, 3.8]	3.3 [2.7, 3.9]	0.35

Abbreviations: COCOMO: Copenhagen Co-Morbidity in HIV infection study; CGPS: Copenhagen General Population Study; IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; LDL: low-density lipoprotein

Table S2 Dietary factors associated with moderate-to-severe hepatic steatosis in PLWH

Variable	Crude OR (95% CI)	p-value	aOR (95% CI)	p-value
Beer				
Never	Ref		Ref	
Monthly	0.73 (0.34;1.56)	0.42	0.54 (0.20;1.48)	0.23
Weekly	0.49 (0.16;1.48)	0.21	0.12 (0.02;0.68)	0.02
Daily	NA		NA	
Red wine				
Never	Ref		Ref	
Monthly	0.24 (0.09;0.65)	<0.01	0.60 (0.19;1.95)	0.40
Weekly	0.27 (0.09;0.80)	0.02	0.51 (0.15;1.78)	0.29
Daily	0.34 (0.04;2.66)	0.31	0.31 (0.02;4.24)	0.38
White wine				
Never	Ref		Ref.	
Monthly	0.59 (0.25;1.41)	0.24	0.63 (0.20;1.98)	0.43
Weekly	0.55 (0.16;1.89)	0.34	0.56 (0.11;2.76)	0.47
Daily	0.90 (0.11;7.31)	0.92	0.71 (0.03;14.91)	0.83
Liquor				
Never	Ref		Ref	
Monthly	1.01 (0.46;2.24)	0.97	0.90 (0.29;2.79)	0.85
Weekly	0.69 (0.16;3.06)	0.63	0.15 (0.00;4.23)	0.26
Daily	NA		NA	
Coke (per 0.5L/week)	1.07 (0.96;1.19)	0.22	0.98 (0.82;1.18)	0.82
Coke Light (per 0.5L/week)	0.99 (0.87;1.13)	0.94	0.88 (0.70;1.10)	0.26
Soda (per 0.5L/week)	0.77 (0.45;1.33)	0.35	0.44 (0.16;1.21)	0.11
Soda Light (per 0.5L/week)	0.93 (0.64;1.37)	0.73	0.94 (0.60;1.48)	0.79
Coffee (per cup/week)	0.99 (0.97;1.01)	0.37	1.00 (0.97;1.02)	0.89
Poultry (per times per week)	0.94 (0.71;1.25)	0.69	0.96 (0.65;1.42)	0.85
Fish (per times per week)	0.89 (0.61;1.29)	0.53	1.06 (0.65;1.71)	0.82
Pork (per times per week)	1.35 (1.03;1.76)	0.03	1.07 (0.72;1.60)	0.74
Fast-food (per times per week)	1.55 (1.12;2.15)	<0.01	1.39 (0.91;2.12)	0.12

Abbreviations: OR: odds ratio; aOR: adjusted odds ratio; CI: confidence interval; PLWH: people living with HIV. Adjustments: age, sex, ethnicity, body mass index, and physical activity level.

Manuscript II

Prevalence and risk factors of liver fibrosis in people living with HIV without viral hepatitis.

Prevalence and factors associated with liver fibrosis in people living with HIV without viral hepatitis: Copenhagen Comorbidity in HIV infection (COCOMO) study

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ABSTRACT

Objectives:

To estimate the prevalence of liver fibrosis and factors associated with liver stiffness in the COCOMO cohort.

Design:

Multicentre, observational cohort study

Methods:

PLWH without viral hepatitis or alcohol abuse (n=473) were recruited from the Copenhagen Co-morbidity in HIV infection (COCOMO) study. Liver fibrosis was assessed by vibration controlled transient elastography (VCTE) and defined as a valid liver stiffness measurement ≥7.6 kPa. Adjusted odds ratios (aOR) and 95% confidence intervals (95% CI) was obtained from logistic regression analysis.

Results:

Liver fibrosis was present in 44 (9%) of PLWH without viral hepatitis and alcohol abuse. Age (aOR: 1.42 per decade (95% CI: 1.04;1.94), non-Caucasian ancestry (aOR: 2.18 (95% CI: 1.01;4.67)), plasma total cholesterol (aOR: 0.50 (95% CI: 0.34;0.75)), and exposure to atazanavir (aOR:0.24 (95% CI: 0.07;0.84)) was associated with liver fibrosis. Blood CD4 nadir T-cell count was associated with log-transformed LSM (β -0.011 (95% CI: -0.020;-0.002)). Moderate-to-severe hepatic steatosis was strongly associated with liver fibrosis (aOR: 7.68 (95% CI: 2.70;21.81)) and the association increased with higher age. No association was found between liver fibrosis and duration of HIV infection, blood CD4 T-cell count, plasma HIV RNA, previous AIDS defining disease, any or cumulative exposure to non-nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs, protease or integrase inhibitor.

Conclusion:

Liver fibrosis was highly prevalent in PLWH without viral hepatitis or alcohol abuse. Hepatic steatosis was strongly associated with liver fibrosis, and liver fibrosis assessment should be considered in PLWH with hepatic steatosis.

The human immunodeficiency virus (HIV) epidemic has changed dramatically in the Western World through the past three decades. Effective antiretroviral therapy (ART) has improved the survival of HIV infection, and HIV is now considered a chronic disease in the Western World. Consequently, people living with HIV (PLWH) are ageing and non-AIDS co-morbidities are increasing[1]. Chronic liver disease is the second leading cause of non-AIDS co-morbidity in PLWH[2] and has mainly been driven by hepatitis B virus (HBV) and hepatitis C virus (HCV) co-infection. However, with effective treatment of HBV and HCV infection in combination with the UN's 2030 HCV elimination plan, the spectrum of liver disease most likely will change [3,4]. In the general population without known liver disease, liver fibrosis has been reported with a prevalence of 5-7%, and is mainly caused by excessive alcohol intake and non-alcoholic fatty liver disease (NAFLD)[5,6]. People living with HIV may be at increased risk of liver fibrosis[7–10] due to e.g. exposure to potential hepatotoxic antiretroviral drugs[11–13], chronic immune activation and microbial translocation[14,15]. Few studies have investigated liver fibrosis in PLWH without viral hepatitis and without excessive alcohol use. We aimed i) to estimate factors associated with higher liver stiffness, ii) to estimate the prevalence of liver fibrosis, iii) to estimate factors associated with liver fibrosis, and iv) to estimate the independent association between hepatic steatosis and liver fibrosis.

Methods

Study population

The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Study is a non-interventional, longitudinal cohort study of adult people living with HIV-1 infection in Copenhagen, Denmark [16]. All PLWH in HIV care at the Department of Infectious Diseases, Rigshospitalet and Amager Hvidovre Hospital, Copenhagen, Denmark were invited to the study. Study participants were consecutively enrolled from March 2015 through November 2016. In Denmark, all PLWH have access to ART free of charge. At time of study initiation, PLWH initiated ART at blood CD4 T cell counts < 350 cells/µL but changed during the inclusion period to include any PLWH regardless of CD4 T cell counts.

Data collection

Vibration controlled transient elastography (VCTE) (Fibroscan, Echosens, Paris, France) was performed in all PLWH with the study participant in the supine position using the M probe placed on the surface of the skin in the right mid-axillary line. The median liver stiffness was measured in kilopascals (kPa) and a valid VCTE defined as at least 10 valid measurements, a success rate

of at least 60% and an interquartile range (IQR) of less than 30% of the median liver stiffness measurement (LSM). Blood was drawn and routine biochemical analyses were performed including liver enzymes and lipid profiles. Blood sampling and VCTE was performed on the same day. Hepatic steatosis was assessed by unenhanced abdominal CT scan[16]. A liver attenuation <48 Hounsfield Units (HU) defined moderate-to-severe hepatic steatosis [17,18]. Questionnaires on health and lifestyle including weekly alcohol consumption were filled out and reviewed by a healthcare professional. Information on HIV-specific factors (e.g. plasma HIV RNA, CD4 T-cell count, current and previous exposure to ART) and hepatitis serology was retrieved from medical records. Hepatitis B virus infection (HBV) was defined as presence of HBsAg. Hepatitis C virus infection (HCV) was defined as presence of anti-HCV antibodies. Excessive alcohol intake was defined as a weekly alcohol consumption of >14 units for men and >7 units for women.

Statistics

Clinical and demographic characteristics were compared by Wilcoxon rank test for continuous variables, and by students t-test or χ^2-test for categorical variables. Linear regression was performed with LSM as dependent variable to estimate factors associated with higher liver stiffness. The LSM was log-transformed to reduced skewness of data. Logistic regression was performed with LSM≥7.6 kPa as dependent variable, indicative of clinically relevant liver fibrosis. Two multivariate logistic regression models were built; a metabolic model and a lifestyle model. The metabolic model was adjusted for age (per decade), sex (male vs female), Caucasian (yes vs no), body mass index (BMI, per 1 kg/m2), plasma total cholesterol (per 1 mM), plasma triglycerides (per 1 mM), diabetes (yes vs no), plasma glucose (per 1 mM), plasma alanine aminotransferase (ALT, per 10 IU/L). The lifestyle model was adjusted for age (per decade), sex (male vs female), Caucasian (yes vs no), smoking status (current vs never, and previous vs never), weekly alcohol consumption (per unit/week), physical activity (inactive vs moderate inactive, moderate active, very active, respectively). Logistic regression analysis was used to estimate the independent association between liver fibrosis and HIV related factors, any- and cumulative exposure to ART and moderate-to-severe hepatic steatosis, respectively. The multivariate model was adjusted for factors found to be associated with liver fibrosis in the metabolic and lifestyle model. Results from regression analysis are presented as β-coefficients and odds ratios (OR) as appropriate with 95% confidence intervals (95% CI). P-value <0.05 is considered statistically significant. Analysis were performed using R (version 3.5.2, Vienna, Austria).

Ethics

The study was approved by the regional ethics committee of the Capital Region of Denmark (H-8-2014-004). All participants provided informed consent after written and oral study information. The COCOMO study was registered at clinicaltrials.gov (NTC02382822).

Results

Of 863 COCOMO participants with a valid VCTE, 473 PLWH without HBV (n=32), HCV (n=94), or excessive alcohol intake (n=264) were included in this study. Clinical and demographic characteristics are presented in Table 1. In short, 86% were males, the median age was 50 years, 72% acquired HIV through homosexual transmission and 99% were on ART. The median LSM were 4.7 kPa (IQR: 3.9; 5.8) and liver fibrosis (LSM ≥7.6 kPa) was found in 44 (9.3% (95% CI: 7.0;12.2%)) of PLWH. Characteristics of individuals with and without liver fibrosis are presented in Table 1.

Factors associated with liver stiffness

The distribution of LSM in PLWH is depicted in Supplementary Figure S1. A weak to moderate negative relationship was observed between LSM and liver attenuation measurements (r=-0.38, p<0.001). Results from univariate regression analysis are presented in Supplementary Table S1. Log-transformed liver stiffness was positively associated with age (β: 0.030 (95% CI: 0.004;0.056) per decade), age 61-70 years compared to age <40 years (β: 0.127 (95% CI: 0.029;0.225), p<0.01), no higher education compared to university education (β: 0.189 (95% CI: 0.073;0.305), p<0.01), waist circumference (β: 0.005 (95% CI: 0.002;0.008), p<0.01 per 1 cm), BMI (β: 0.011 (95% CI: 0.002;0.0179), p=0.02 per 1 kg/m^2), presence of obesity (β: 0.317 (95% CI: 0.192;0.443), p<0.01), diabetes (β: 0.260 (95% CI: 0.130;0.389), p<0.01), metabolic syndrome (β: 0.115 (95% CI: 0.051;0.179), p<0.01) and moderate-to-severe hepatic steatosis (β: 0.376 (95% CI: 0.241;0.511), p<0.01), higher plasma levels of ALT (β: 0.040 (95% CI: 0.017;0.063), p<0.01 per 10 IU/L), total-cholesterol (β: -0.050 (95% CI: -0.078;-0.022),p<0.01 per 1 mM), and triglycerides (β: 0.038 (95% CI: 0.014;0.061), p<0.01 per 1 mM). No association was found with sex, ancestry, smoking status, level of physical activity, abdominal obesity, or overweight compared to normal weight. After adjustment for potential metabolic confounders, log-transformed liver stiffness remained associated with plasma levels of total-cholesterol, triglycerides, and ALT. After adjustment for potential lifestyle confounders, log-transformed liver stiffness remained associated with higher age (Table 2).

Factors associated with liver fibrosis

Results from univariate and multivariate logistic regression analysis are shown in Supplementary Table S1 and Table 2, respectively. Liver fibrosis was associated with higher age (OR: 1.56 per decade (95% CI: 1.25;1.94)), age 61-70 years compared to age <40 years (OR: 6.25 (95% CI: 2.05;19.10)), waist circumference (OR: 1.05 per 1 cm (95% CI: 1.02;1.08)), BMI (OR: 1.13 per 1 kg/m^2 (95% CI: 1.04;1.23)), plasma ALT (OR: 1.22 per 10 IU/L (95% CI: 1.00;1.48)), total-cholesterol (OR: 0.63 per 1 mM (95% CI: 0.46;0.86)), presence of obesity (OR: 6.16 (95% CI: 2.37;16.03)), diabetes (OR: 6.20 (95% CI: 2.58;14.93)) and metabolic syndrome (OR: 2.63 (95% CI: 1.38;4.98)). No association was found with sex, ancestry, smoking status, physical activity level, educational level, abdominal obesity, overweight compared to normal weight, or plasma levels of triglycerides. After adjustment for potential metabolic confounders liver fibrosis remained associated with higher age, higher total-cholesterol and non-Caucasian ancestry (Table 2). After adjustment for potential lifestyle confounders, liver fibrosis remained associated with higher age and non-Caucasian ancestry (Table 2). In sensitivity analysis, LSM ≥8.8 kPa was associated with higher BMI (aOR: 1.20 per 1 kg/m^2 (95% CI: 1.03;1.39)); higher plasma levels of total cholesterol (aOR: 0.40 per 1 mM (95% CI: 0.22;0.74)) and triglycerides (aOR: 1.55 per 1 mM (95% CI: 1.13;2.13)) after adjustment for metabolic confounders (Supplementary Table X). After adjustment for lifestyle confounders, LSM ≥8.8 kPa was associated with higher age (aOR: 1.87 per decade (95% CI: 1.18;2.96)).

HIV associated factors, liver stiffness and liver fibrosis

Results on the association between HIV related factors and log-transformed LSM are shown in Table 3. In univariate analysis, log-transformed LSM was associated with duration of HIV infection (β: 0.004 per year (95% CI: 0.001;0.008), p=0.02), and blood CD4 nadir T-cell count (β: -0.011 per 50 cells/µl (-0.019;0.002), p=0.01). After adjustment for age, ancestry and plasma total cholesterol, blood CD4 nadir T-cell count was independently associated with log-transformed LSM (β: -0.011 per 50 cells/µl (95% CI: -0.020;0.002), p=0.02).

Results on the association between HIV related factors and liver fibrosis are presented in Table 3. In univariate logistic regression analysis, duration of HIV infection (OR:1.05 per year (95% CI: 1.01;1.10)) and blood CD4 nadir T-cell count (OR: 0.90 per 50 cells/µl (95% CI: 0.81;0.99)) were associated with liver fibrosis. After adjustment for age, ancestry and plasma total cholesterol, the associations did not reach statistical significance. Route of HIV transmission, blood CD4 T-cell count <200 cells/µl, plasma HIV RNA and previous AIDS defined disease were not associated with liver fibrosis in univariate and multivariate logistic regression analysis.

Results on the association between ART and liver fibrosis are shown in Supplementary Table S2. In univariate logistic regression analysis, any exposure to stavudine (OR: 2.53 (95% CI: 1.25;5.10)), any exposure to didanosine (OR: 2.74 (95% CI: 1.35;5.54)), cumulative exposure to nucleoside reverse transcriptase inhibitors (NRTI) (OR: 1.05 (95% CI: 1.02;1.08)) and cumulative exposure to integrase inhibitors (OR: 1.25 (95% CI: 1.02;1.53)) were associated with higher odds of liver fibrosis. After adjustment for age, ancestry, total-cholesterol, moderate-to-severe hepatic steatosis and duration of HIV infection, any exposure to atazanavir (OR: 0.26 (95% CI: 0.07;0.92)) and cumulative exposure to atazanavir (OR: 0.80 per year (95%CI: 0.64;1.00) was independently associated with lower odds of liver fibrosis. Results from analysis of any and cumulative exposure to a NRTI, non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor or integrase inhibitor as well as specific drugs in each drug class are shown in Supplementary Table S2.

NAFLD and liver fibrosis

Among 473 PLWH, 23 (4.9% (95%CI: 3.2;7.2%)) had moderate-to-severe hepatic steatosis among which 8 (35%) individuals had liver fibrosis. In univariate analysis, moderate-to-severe hepatic steatosis was associated with higher odds of liver fibrosis (OR: 6.13 (95% CI: 2.44;15.44), p<0.01). After adjusting for age, ancestry, and plasma total cholesterol, moderate-to-severe hepatic steatosis was independently associated with liver fibrosis (aOR: 7.68 (95% CI: 2.70;21.81), p<0.01). Sensitivity analysis was conducted with liver fibrosis defined as LSM ≥8.8 kPa. The association between liver fibrosis and moderate-to-severe hepatic steatosis persisted in both univariate (OR: 7.08 (95% CI: 2.35;21.32) and multivariate analysis (OR: 8.10 (95% CI: 2.34;28.03)). We calculated the predicted probability of having LSM ≥7.6 kPa in PLWH with and without NAFLD, after adjusting for age, ancestry and plasma total-cholesterol (Figure 1). The predicted probability of liver fibrosis increased with age and was higher in PLWH with NAFLD. Characteristics of PLWH with fibrosis and with and without moderate-to-severe hepatic can be found in Supplementary Table S3. In short, PLWH with fibrosis and moderate-to-severe hepatic steatosis had a higher waist circumference (114 vs 97 cm, p<0.01), higher BMI (31 vs 25 kg/m2, p<0.01), higher proportion of obese individuals (63 vs 8%, p<0.01), higher proportion of metabolic syndrome (100 vs 49%, p=0.02), higher plasma levels of ALT (44 vs 26 IU/L, p<0.01), total-cholesterol (5.3 vs 4.1 mM, p=0.05), and triglycerides (3.2 vs 2.0 mM, p<0.01).

Discussion

In this cross-sectional study of 473 PLWH without viral hepatitis and excessive alcohol intake, 44 (9%) individuals had VCTE-defined liver fibrosis. Higher age, non-Caucasian ancestry and presence of moderate-to-severe hepatic steatosis were associated with higher prevalence of liver

fibrosis, while higher plasma total cholesterol, and any and cumulative exposure to atazanavir were independently associated with lower prevalence of liver fibrosis. Blood CD4 nadir T-cell count was negatively associated with log-transformed LSM.

The prevalence of VCTE-defined liver fibrosis has been reported to be between 11% and 19% in HIV monoinfected individuals without excessive alcohol use[8,19–22]. *Pembroke et al* found a prevalence of significant liver fibrosis (LSM >7.1 kPa) of 19% in the Canadian LIVEHIV cohort of 561 HIV monoinfected individuals. Compared to the COCOMO cohort, the LIVEHIV cohort comprises PLWH with a higher proportion of individuals of non-Caucasian ancestry (25 vs 39%) and hepatic steatosis (5 vs 36%), and a lower proportion of MSM (73 vs 35%), which may explain the difference in prevalence of liver fibrosis[20]. Similar, *Lombardi et al* found a prevalence of liver fibrosis (LSM >7.4 kPa) of 18% in a Greek cohort of 125 HIV monoinfected individuals. In this study, individuals with excessive alcohol use were included, 55% had hepatic steatosis and only 68% were on ART compared to 5% and 99%, respectively in the COCOMO cohort[19]. Our findings are in line with previous studies despite different cohort characteristics[23,24]. However, the prevalence of liver fibrosis in PLWH is higher than reported from the general population (9% vs 5-7%)[5,6]. Importantly, studies from the general population did not exclude individuals with excessive alcohol intake and the prevalence of liver fibrosis may thus be even lower if individuals with alcohol abuse had been excluded. The potential higher risk of liver fibrosis in PLWH may have several explanations. First, PLWH is exposed to potential hepatotoxic antiretroviral drugs which may cause long lasting metabolic disturbances or mitochondrial toxicity and endoplasmic reticulum stress, which may lead to hepatic cell injury and apoptosis, production of cytokines (e.g. tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-1β), activation of hepatic stellate cells and Kupffer cells and ultimately increased fibrogenesis and inflammation in the liver parenchyma[25]. Results from previous studies on ART and liver fibrosis have been conflicting and both protease and integrase inhibitors and NRTIs such as didanosine, stavudine and zidovudine have been related to hepatotoxic effects[8,13,20,26,27]. We found a trend between any exposure to didanosine and higher prevalence of liver fibrosis (p-value 0.06), and the association became stronger with more advanced liver fibrosis (LSM ≥8.8 kPa). Interestingly, any and cumulative exposure to atazanavir was protective of liver fibrosis, which supports previous literature by *Kovari et al,* reporting a lower risk of chronic liver enzyme elevation after >2 years treatment of atazanavir[12]. However, we found no strong evidence of an association between ART and liver fibrosis. Second, immunodeficiency may play a role in the development of liver fibrosis. We found that higher blood CD4 nadir T-cell count was protective of liver fibrosis and indicates a direct link between HIV, immunodeficiency and liver fibrosis. Third, hepatic steatosis may progress to non-alcoholic steatohepatitis (NASH) and liver fibrosis through a multifactorial and complex

pathophysiological pathway that has not yet been fully understood[28,29]. In this study, moderate-to-severe hepatic steatosis was independently associated with liver fibrosis. The association was stronger than other associations observed in this study, and it increased with higher age. We found a weak negative relationship between LSM and liver attenuation, but whether the LSM may be affected by other features than fibrosis remains unclear. Results from previous studies have been conflicting [30,31], and future studies should explore this in more detail in PLWH. However, *Pembroke et al* showed that PLWH had a higher rate of steatosis progression compared to HIV/HCV coinfected individuals, Further, they showed that PLWH monoinfection had a higher probability of liver fibrosis if hepatic steatosis was present. This emphasizes the importance of diagnosing hepatic steatosis in PLWH, as it may progress to liver fibrosis, which is considered to be the most important histological feature related with poor long-term outcomes[32]. Fourth, HIV itself may induce fibrogenesis. The HIV envelope protein gp120 may induce chemotaxis in the hepatic stellate cells as well as increased expression of proinflammatory cytokines[33], and binding of HIV to the HIV coreceptor CXCR4 on the hepatocytes may cause apoptosis and hepatic fibrogenesis[34]. Further epidemiological studies have demonstrated an independent association between HIV itself and liver fibrosis [21,35,36].

This study is limited by the absence of an HIV-negative comparator group to estimate the independent association between HIV itself and liver fibrosis. The study did not include histology or MRI to confirm the diagnose of liver fibrosis. Causality cannot be inferred by the cross-sectional study design and unmeasured confounding cannot be precluded.

Conclusion

The prevalence of liver fibrosis in PLWH without viral hepatitis or alcohol abuse was 9%. Presence of moderate-to-severe hepatic steatosis was strongly associated with liver fibrosis and the association became stronger with higher age. Liver fibrosis assessment should be performed in PLWH with hepatic steatosis.

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Author contributions: Study design: DK, SD, JL, KK and TB. Data collection: DK for the COCOMO cohort. Data analysis: DK with supervision by TB, SD and statistical support from AM. Manuscript drafting: DK. All authors have contributed with revisions of the manuscript and all authors have read and approved the final manuscript.

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Figure 1 Predicted probability of liver fibrosis according to age in people living with HIV with NAFLD (dotted line) and without NAFLD (solid line)

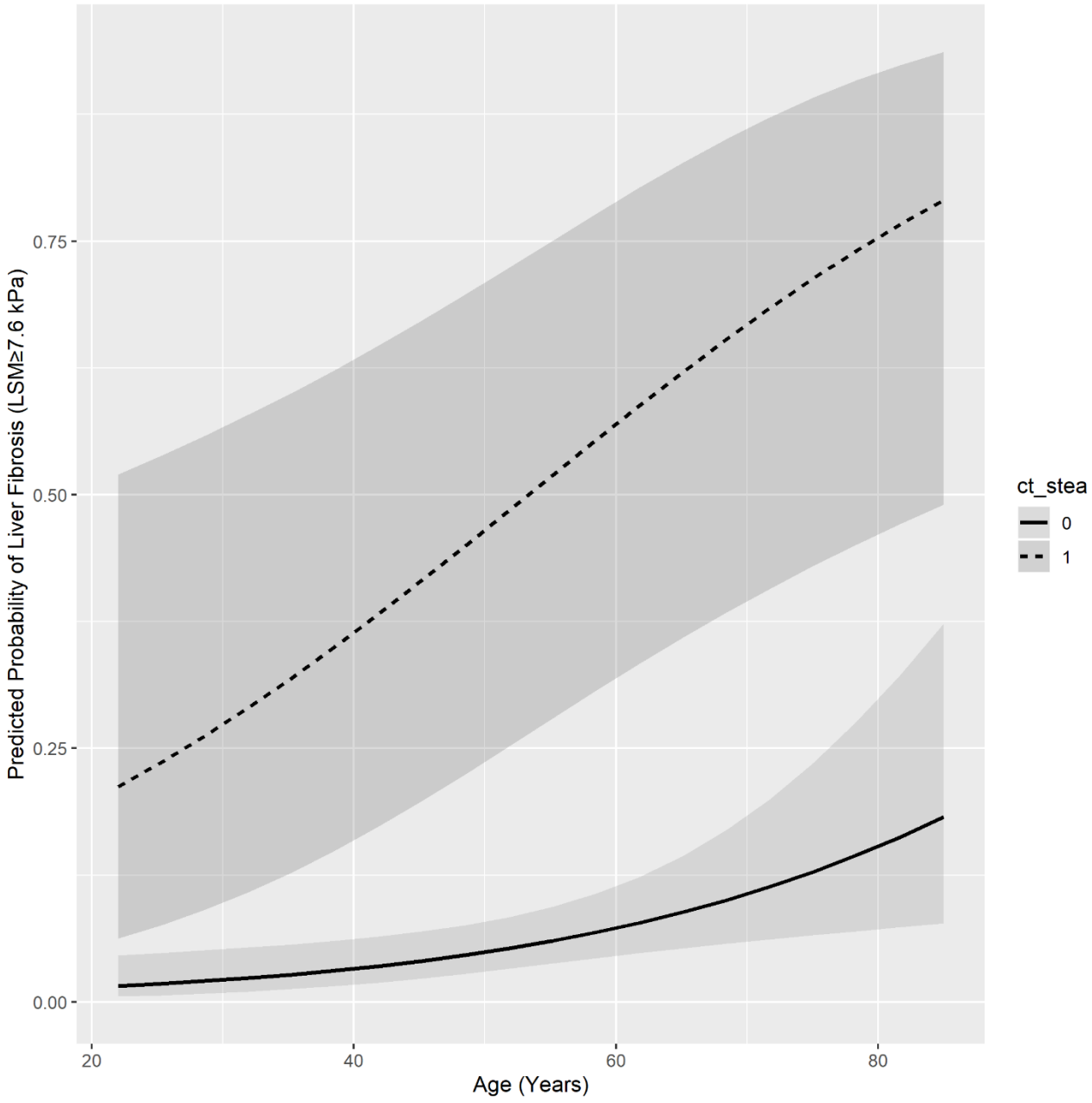

Table 1 Clinical and demographic characteristics of people living with HIV

	LSM <7.6 kPa (n=429)	LSM ≥7.6 kPa (n=44)	Total (n=473)	p-value
Age (years), median (IQR)	49.0 (42.0, 57.0)	61.5 (46.8, 65.0)	50.0 (42.0, 59.0)	<0.01
Age groups (years), n(%)				<0.01
< 40	81 (18.9)	4 (9.1)	85 (18.0)	
41-50	152 (35.4)	12 (27.3)	164 (34.7)	
51-60	112 (26.1)	5 (11.4)	117 (24.7)	
61-70	68 (15.9)	21 (47.7)	89 (18.8)	
>70	16 (3.7)	2 (4.5)	18 (3.8)	
Male sex, n(%)	368 (85.8)	38 (86.4)	406 (85.8)	1.00
Caucasian, n(%)	326 (76.2)	27 (64.3)	353 (75.1)	0.13
Smoking status, n(%)				0.27
Current Smoker	116 (27.0)	10 (22.7)	126 (26.6)	
Previous smoker	152 (35.4)	21 (47.7)	173 (36.6)	
Never smoker	161 (37.5)	13 (29.5)	174 (36.8)	
Alcohol** (per week), n(%)				NA
None	107 (24.9)	11 (25.0)	118 (24.9)	
Within recommendations	322 (75.1)	33 (75.0)	355 (75.1)	
Educational level*, n(%)				0.55
None	35 (8.7)	6 (14.0)	41 (9.2)	
Short	86 (21.3)	10 (23.3)	96 (21.5)	
Middle Length	166 (41.2)	18 (41.9)	184 (41.3)	
University	116 (28.8)	9 (20.9)	125 (28.0)	
Abdominal obesity, n(%)	239 (57.7)	30 (68.2)	269 (58.7)	0.24
Waist circumference (cm), median (IQR)	90.0 (85.0, 98.0)	99.5 (85.8, 106.5)	91.0 (85.0, 99.0)	<0.01
BMI (kg/m^2), median (IQR)	23.9 (22.0, 26.1)	25.9 (22.6, 28.5)	24.0 (22.0, 26.3)	0.03
WHO BMI category, n(%)				0.01
Underweight, <18.4 kg/m^2	10 (2.3)	2 (4.5)	12 (2.5)	
Normoweight, 18.5-24.9 kg/m^2	262 (61.2)	17 (38.6)	279 (59.1)	

Overweight, 25-29.9 kg/m^2	136 (31.8)	17 (38.6)	153 (32.4)	
Obese ≥30 kg/m^2	20 (4.7)	8 (18.2)	28 (5.9)	
Diabetes, n(%)	17 (4.0)	9 (20.5)	26 (5.5)	<0.01
Metabolic syndrome, n(%)	137 (34.6)	25 (58.1)	162 (36.9)	<0.01
Hepatic steatosis, n(%)	15 (3.5)	8 (18.2)	23 (4.9)	<0.01
Biochemistry, median (IQR)				
Plasma ALT (IU/L)	24.0 (19.0, 33.0)	27.0 (22.2, 38.0)	25.0 (19.0, 34.0)	0.07
Plasma total cholesterol (mM)	4.8 (4.2, 5.6)	4.3 (3.7, 5.2)	4.8 (4.1, 5.5)	<0.01
Plasma triglycerides (mM)	1.7 (1.2, 2.5)	2.1 (1.2, 3.2)	1.7 (1.2, 2.6)	0.11
HIV specific characteristics				
ART, n(%)	422 (99.1)	44 (100.0)	466 (99.1)	1.00
Duration of HIV infection (years), median (IQR)	13.3 (6.5;21.2)	18.8 (11.5;26.6)	13.8 (6.8;21.6)	0.02
MSM, n(%)	311 (73.5)	26 (61.9)	337 (72.5)	0.15
Plasma HIV RNA ≥50 copies/ml, n(%)	19 (4.5)	1 (2.3)	20 (4.3)	0.76
Blood CD4 T-cell count (cells/µl), n(%)				0.04
< 200	3 (0.7)	1 (2.3)	4 (0.9)	
200-349	14 (3.3)	5 (11.4)	19 (4.1)	
350-500	61 (14.4)	4 (9.1)	65 (13.9)	
> 500	346 (81.6)	34 (77.3)	380 (81.2)	

*Highest achieved educational level after primary school. **National alcohol recommendations: <14 units/week for men and <7 units/week for women. Metabolic syndrome defined as a minimum of three of the following 5 items: (1) Waist circumference waist circumference of ≥94 cm for men and ≥80 cm for women; (2) Systolic blood pressure ≥130 mmHg and/or antihypertensive treatment; (3) plasma HDL ≤1.036 mmol/l for men, and plasma HDL ≤1.295 mmol/l for women; (4) plasma triglycerides ≥ 1.693 mmol/l; (5) self-reported diabetes mellitus and/or antidiabetic treatment and/or non-fasting plasma glucose ≥11.1 mmol/l (Alberti, Zimmet, and Shaw 2006). Abbreviations: HIV: human immunodeficiency virus; LSM: liver stiffness measurement; kPa: kilopascal; IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; MSM: men sex with men; ART: antiretroviral therapy. Missing variables: Caucasian: 3; alcohol: 88; educational level: 27; waist circumference: 15; BMI:1; diabetes:2; Metabolic syndrome:86; ALT: 36; CHOL: 22; TRIG: 22; cART:3: CD4: 5; HIV RNA: 6

Table 2 Factors associated with (log transformed) liver stiffness and liver fibrosis (LSM ≥7.6 kPa) by multivariate regression analysis (n=473).

	Linear regression		Logistic regression	
Metabolic model	**β-coeff. (95% CI)**	**p-value**	**aOR (95% CI)**	**p-value**
Age (per decade)	0.025 (-0.002;0.053)	0.07	1.42 (1.04;1.94)	0.03
Male sex	0.011 (-0.079;0.100)	0.82	0.79 (0.27;2.33)	0.66
Caucasian (no vs yes)	0.028 (-0.043;0.100)	0.44	2.18 (1.01;4.67)	0.05
BMI (per 1 kg/m2)	0.007 (-0.003;0.016)	0.17	1.10 (0.99;1.22)	0.06
Total cholesterol (per 1 mM)	-0.070 (-0.100;-0.039)	<0.01	0.50 (0.34;0.75)	<0.01
Triglycerides (per 1 mM)	0.044 (0.018;0.070)	<0.01	1.25 (0.97;1.61)	0.08
ALT (per 10 IU/L)	0.028 (0.005;0.051)	0.02	1.15 (0.92;1.42)	0.22
Diabetes	0.090 (-0.077;0.258)	0.29	1.64 (0.42;6.44)	0.48
Glucose (per 1 mM)	-0.002 (-0.028;0.051)	0.87	0.97 (0.76;1.23)	0.79
Lifestyle model	**β-coeff. (95% CI)**	**p-value**	**aOR (95% CI)**	**p-value**
Age (per decade)	0.030 (0.002;0.058)	0.04	1.58 (1.15;2.16)	<0.01
Male sex	0.086 (-0.005;0.177)	0.07	1.03 (0.38;2.78)	0.96
Caucasian (no vs yes)	0.034 (-0.039;0.108)	0.36	2.17 (1.03;4.56)	0.04
Physical activity				
Inactive	Ref		1.0	
Moderate inactive	-0.087 (-0.208;0.035)	0.16	0.52 (0.17;1.56)	0.24
Moderate active	-0.091 (-0.211;-0.028)	0.13	0.55 (0.19;1.60)	0.27
Very active	-0.092 (-0.229;0.044)	0.19	0.38 (0.09;1.57)	0.18
Alcohol (per unit/week)	-0.004 (-0.011;0.004)	0.33	1.02 (0.94;1.10)	0.61
Smoking				
Never smoker	Ref		1.0	
Current smoker	-0.026 (-0.104;0.051)	0.50	0.90 (0.35;2.31)	0.83
Previous smoker	0.008 (-0.064;0.080)	0.83	1.24 (0.57;2.68)	0.59

Abbreviations: LSM: liver stiffness measurement; CI: confidence interval; BMI: body mass index; ALT: alanine aminotransferase.

Adjustments: Metabolic model: age (per decade), sex (male vs female), Caucasian (yes vs no), BMI (per 1 kg/m2), plasma total cholesterol (per 1 mM), plasma triglycerides (per 1 mM), diabetes (yes vs no), plasma glucose (per 1 mM), plasma ALT (per 10 IU/L). Lifestyle model: age (per decade), sex (male vs female), Caucasian (yes vs no), smoking status (current vs never, and previous vs never), weekly alcohol consumption (per unit/week), physical activity (inactive vs moderate inactive, moderate active, very active, respectively).

Table 3. HIV related factors associated with liver stiffness (log transformed) and liver fibrosis (LSM ≥7.6 kPa) in univariate and multivariate linear and logistic regression analysis.

	Linear regression				Logistic regression			
	β-coeff. (95% CI)	**p-value**	**Adj β-coeff. (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**aOR (95% CI)**	**p-value**
Duration of HIV Infection (per year)	0.004 (0.001;0.008)	0.02	0.003 (-0.01;0.008)	0.14	1.05 (1.01;1.10)	0.02	1.04 (0.99;1.10)	0.09
MSM HIV acquisition	-0.000 (-0.068;0.067)	0.99	-0.040 (-0.109;0.029)	0.25	0.59 (0.30;1.13)	0.11	1.74 (0.85;3.57)	0.13
Blood CD4 nadir T-cell count (per 50 cells/µl)	-0.011 (-0.019;-0.002)	0.01	-0.011 (-0.020;-0.002)	0.02	0.90 (0.81;0.99)	0.04	0.91 (0.82;1.02)	0.12
Blood CD4 nadir T-cell count <200 cells/µl	0.050 (-0.012;0.112)	0.11	0.032 (-0.033;0.097)	0.33	1.45 (0.77;2.72)	0.25	1.10 (.054;2.23)	0.79
Blood CD4 T-cell count (per 50 cells/µl)	-0.005 (-0.010;0.001)	0.10	-0.003 (-0.009;0.002)	0.24	0.97 (0.91;1.03)	0.29	0.99 (0.93;1.05)	0.81
Plasma HIV RNA ≥50 copies/ml	0.012 (-0.137;0.161)	0.87	-0.009 (-0.161;0.142)	0.91	0.49 (0.06;3.78)	0.48	0.63 (0.08;5.12)	0.66
AIDS defining event	-0.034 (-0.114;0.047)	0.41	-0.040 (-0.123;0.044)	0.36	0.75 (0.31;1.85)	0.54	0.77 (0.30;1.96)	0.58

Abbreviations: HIV: human immunodeficiency virus; LSM: liver stiffness measurement; kPa: kilopascal; IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; MSM: men sex with men; ART: antiretroviral therapy

SUPPLEMENTARY MATERIAL

“Prevalence and factors associated with liver fibrosis in people living with HIV without viral hepatitis: Copenhagen Comorbidity in HIV infection (COCOMO) study”

Figure S1. Distribution of liver stiffness measurements in people living with HIV

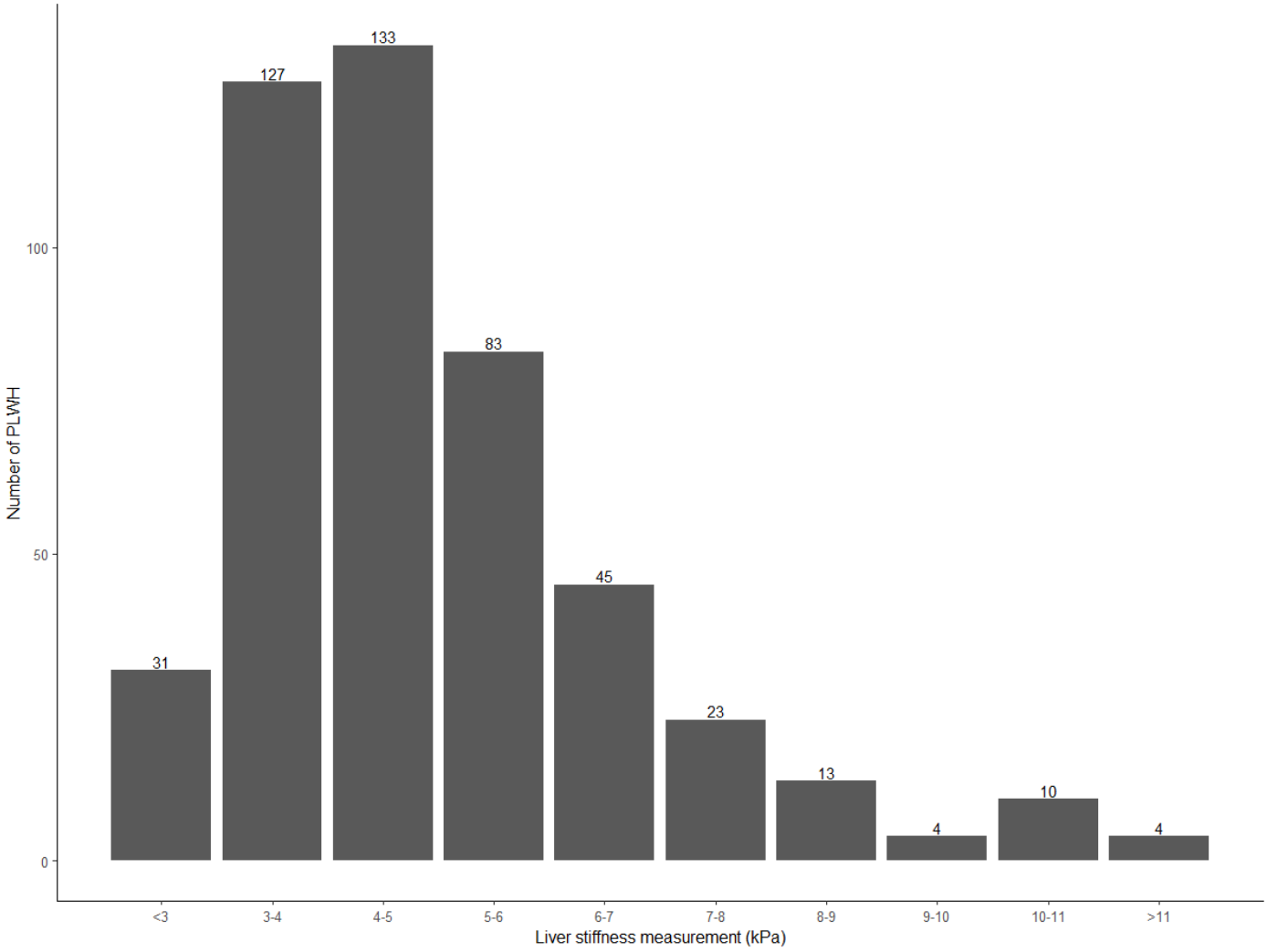

Table S1. Factors associated with liver stiffness (log transformed) and liver fibrosis (LSM ≥7.6 kPa) in univariate linear and logistic regression analysis.

	Linear Regression		Logistic Regression	
	β-coeff. (95% CI)	**p-value**	**OR (95% CI)**	**p-value**
Age (per decade)	0.030 (0.004;0.056)	0.03	1.56 (1.25;1.94)	<0.01
Age groups (years)				
< 40	Ref.		1.0	
41-50	0.016 (-0.071;0.102)	0.72	1.60 (0.50;5.12)	0.43
51-60	0.029 (0.063;0.121)	0.54	0.90 (0.24;3.47)	0.88
61-70	0.127 (0.029;0.225)	0.01	6.25 (2.05;19.10)	<0.01
>70	0.021 (-0.146;0.189)	0.80	2.53 (0.43;15.01)	0.31
Male sex	0.068 (-0.017;0.154)	0.12	1.05 (0.43;2.59)	0.92
Caucasian (no vs yes)	0.020 (-0.049;0.089)	0.58	1.78 (0.91;3.47)	0.09
Smoking status				
Never smoker	Ref.		1.0	
Previous smoker	0.024 (-0.045;0.094)	0.49	1.71 (0.83;3.54)	0.15
Current Smoker	-0.013 (-0.089;0.063)	0.73	1.07 (0.45;2.52)	0.88
Alcohol (per 10 units/week)	-0.028 (-0.097;0.041)	0.43	1.10 (0.54;2.25)	0.79
Physical activity				
Inactive	Ref		1.0	
Moderate inactive	-0.090 (-0.211;0.032)	0.15	0.49 (0.17;1.40)	0.18
Moderate active	-0.098 (-0.216;0.020)	0.10	0.47 (0.17;1.28)	0.14
Very active	-0.100 (-0.234;0.034)	0.14	0.30 (0.08;1.13)	0.08
Educational level*				
University	Ref		1.0	
Middle Length	0.034 (-0.041;0.108)	0.38	1.40 (0.61;3.22)	0.43
Short	0.086 (-0.002;0.173)	0.06	1.50 (0.58;3.85)	0.40
None	0.189 (0.073;0.305)	<0.01	2.21 (0.74;6.64)	0.43
Abdominal obesity	0.045 (-0.017;0.107)	0.15	1.57 (0.81;3.05)	0.18
Waist circumference (per cm)	0.005 (0.002;0.008)	<0.01	1.05 (1.02;1.08)	<0.01
BMI (per 1 kg/m^2)	0.011 (0.002;0.0179)	0.02	1.13 (1.04;1.23)	<0.01
WHO BMI category				
Normoweight 18.5-24.9 kg/m^2	Ref		1.0	
Overweight 25-29.9 kg/m^2	0.013 (-0.051;0.077)	0.68	1.93 (0.95;3.89)	0.07
Obese ≥30 kg/m^2	0.317 (0.192;0.443)	<0.01	6.16 (2.37;16.03)	<0.01
Diabetes	0.260 (0.130;0.389)	<0.01	6.20 (2.58;14.93)	<0.01
Metabolic syndrome	0.115 (0.051;0.179)	<0.01	2.63 (1.38;4.98)	<0.01
Hepatic steatosis	0.376 (0.241;0.511)	<0.01	6.13 (2.44;15.44)	<0.01
Plasma ALT (per 10 IU/L)	0.040 (0.017;0.063)	<0.01	1.22 (1.00;1.48)	0.05
Plasma total cholesterol (per MM)	-0.050 (-0.078;-0.022)	<0.01	0.63 (0.46;0.86)	<0.01
Plasma triglycerides (per mM)	0.038 (0.014;0.061)	<0.01	1.19 (0.98;1.45)	0.08

*Highest achieved educational level after primary school. Metabolic syndrome defined as a minimum of three of the following 5 items: (1) Waist circumference waist circumference of ≥94 cm for men and ≥80 cm for women; (2) Systolic blood pressure ≥130 mmHg and/or antihypertensive treatment; (3) plasma HDL ≤1.036 mmol/l for men, and plasma HDL ≤1.295 mmol/l for women; (4) plasma triglycerides ≥ 1.693 mmol/l; (5) self-reported diabetes mellitus and/or antidiabetic treatment and/or non-fasting plasma glucose ≥11.1 mmol/l [1]. Abbreviations: HIV: human immunodeficiency virus; LSM: liver stiffness measurement; kPa: kilopascal; IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase.

Table S2. Association between antiretroviral drugs and liver fibrosis (LSM ≥7.6 kPa)

			Any exposure (yes vs no)				Cumulative exposure (per year)			
	N_{EXP}	N_{FIB}	Crude OR (95% CI)	p-value	aOR (95% CI)	p-value	Crude OR (95% CI)	p-value	aOR (95% CI)	p-value
NRTI	461 (97.5)	43	1.13 (0.14;8.98)	0.91	0.49 (0.05;4.53)	0.53	1.05 (1.02;1.08)	<0.01	1.00 (0.99;1.01)	0.77
TDF	367 (77.6)	30	0.58 (0.30;1.15)	0.12	0.65 (0.27;1.59)	0.35	1.01 (0.93;1.10)	0.74	1.01 (0.91;1.12)	0.90
TAF	32 (6.8)	1	0.30 (0.04;2.24)	0.24	NA		0.73 (0.11;5.04)	0.75	NA	
FTC	229 (48.4)	20	0.88 (0.47;1.64)	0.68	0.86 (0.38;1.91)	0.70	1.07 (0.95;1.19)	0.26	1.00 (0.86;1.17)	1.00
3TC	379 (80.1)	4	2.65 (0.93;7.61)	0.07	1.52 (0.40;5.83)	0.54	1.00 (0.99;1.01)	0.96	1.00 (0.99;1.01)	0.73
ABC	240 (50.7)	24	1.18 (0.63;2.21)	0.60	0.95 (0.40;2.28)	0.91	1.04 (0.98;1.10)	0.18	1.00 (0.98;1.01)	0.75
AZT	240 (50.7)	28	1.79 (0.94;3.41)	0.08	1.69 (0.56;5.03)	0.35	1.05 (0.98;1.13)	0.16	1.01 (0.90;1.13)	0.85
D4T	74 (15.6)	13	2.53 (1.25;5.10)	<0.01	1.41 (0.50;4.00)	0.51	1.17 (1.04;1.32)	<0.01	1.12 (0.94;1.33)	0.20
ddI	70 (14.8)	13	2.74 (1.35;5.54)	<0.01	2.70 (0.95;7.67)	0.06	1.06 (0.90;1.25)	0.50	1.16 (0.90;1.49)	0.26
NNRTI	353 (74.6)	33	1.02 (0.50;2.09)	0.95	1.00 (0.40;2.50)	1.00	1.07 (1.00;1.15)	0.06	1.07 (0.96;1.19)	0.23
EFV	301 (63.6)	26	0.81 (0.43;1.52)	0.51	1.06 (0.47;2.41)	0.89	0.99 (0.92;1.06)	0.69	0.98 (0.90;1.07)	0.70
RPV	20 (4.2)	1	0.50 (0.07;3.84)	0.51	NA		0.83 (0.40;1.73)	0.62	NA	
NPV	96 (20.3)	12	1.54 (0.76;3.12)	0.23	1.18 (0.47;2.98)	0.73	1.09 (1.02;1.16)	0.01	1.06 (0.97;1.16)	0.17
ETV	13 (2.7)	2	1.81 (0.39;8.44)	0.45	0.81 (0.09;7.26)	0.85	1.05 (0.83;1.31)	0.69	1.00 (0.76;1.32)	0.99
PI	251 (53.1)	27	1.45 (0.77;2.74)	0.25	0.71 (0.28;1.79)	0.47	1.01 (0.97;1.05)	0.66	1.00 (0.94;1.06)	0.99
LPV	55 (11.6)	5	0.97 (0.37;2.58)	0.95	0.30 (0.07;1.42)	0.13	1.04 (0.90;1.20)	0.60	0.84 (0.65;1.11)	0.22
ATV	122 (25.8)	8	0.61 (0.28;1.36)	0.23	0.24 (0.07;0.84)	0.03	0.93 (0.82;1.06)	0.29	0.79 (0.63;0.99)	0.04
DRV	111 (23.5)	10	0.96 (0.46;2.00)	0.90	0.94 (0.38;2.35)	0.90	1.07 (0.96;1.19)	0.21	1.06 (0.93;1.20)	0.37
INSTI	151 (31.9)	10	0.60 (0.29;1.25)	0.17	0.57 (0.22;1.52)	0.26	1.25 (1.02;1.53)	0.03	1.26 (0.95;1.67)	0.11
DOL	68 (14.5)	3	0.41 (0.12;1.36)	0.15	0.26 (0.03;1.98)	0.19	0.77 (0.35;1.70)	0.52	0.64 (0.17;2.45)	0.52
EVG	58 (12.3)	1	0.15 (0.02;1.12)	0.06	NA		0.31 (0.07;1.41)	0.13	NA	
RAL	52 (11.0)	7	1.61 (0.68;3.83)	0.28	1.38 (0.47;4.09)	0.56	1.11 (0.96;1.28)	0.15	1.11 (0.94;1.32)	0.21

Abbreviations: N_{exp}: Number exposed to each treatment; N_{fib}: Number exposed with liver fibrosis; OR: odds ratio; CI: confidence interval; NRTI: nucleoside reverse transcriptase inhibitor; TDF: tenofovir disoproxil fumarate; TAF: tenofovir alfenamide; FTC: emtricitabine; 3TC: lamivudine; ABC: abacavir; AZT: zidovudine; D4T: stavudine; ddI: didanosine; NNRTI: non-nucleoside reverse transcriptase inhibitor; EFV: efavirenz; RPV: rilpivirine; NPV: nevirapine; ETV: entecavir; PI: protease inhibitor; LPV: lopinavir; ATV: atazanavir; DRV: darunavir; INSTI: integrase inhibitor; DOL: dolutegravir; EVG: elvitegravir; RAL: raltegravir.

Table S3. Characteristics of PLWH with fibrosis with or without moderate-to-severe hepatic steatosis (M-HS) (n=44)

	Fibrosis without M-HS (n=36)	Fibrosis with M-HS (n=8)	p-value
Age (years), median (IQR)	57.0 (45.5, 64.0)	63.5 (58.0, 68.0)	0.12
<40 years	4 (11.1)	0 (0.0)	0.33
41-50 years	11 (30.6)	1 (12.5)	
51-60 years	3 (8.3)	2 (25.0)	
61-70 years	17 (47.2)	4 (50.0)	
>70 years	1 (2.8)	1 (12.5)	
Sex (male), n(%)	30 (83.3)	8 (100.0)	0.50
Caucasian, n(%)	22 (64.7)	5 (62.5)	1.00
Smoking, n(%)			0.15
Current Smoker	10 (27.8)	0 (0.0)	
Ex smoker	15 (41.7)	6 (75.0)	
Never smoker	11 (30.6)	2 (25.0)	
Alcohol use (units/week), median (IQR)	4.0 (2.0;9.2)	4.0 (0.0;6.2)	0.61
Abdominal obesity, n(%)	22 (61.1)	8 (100.0)	0.09
Waist circumference, median (IQR)	97.0 (84.0, 103.2)	113.5 (107.2, 119.0)	<0.01
BMI (kg/m^2), median (IQR)	25.1 (22.3, 26.9)	31.4 (29.3, 33.3)	<0.01
WHO BMI category (kg/m^2), n(%)			<0.01
Normoweight, 18.5-24.9	16 (44.4)	1 (12.5)	
Overweight, 25-29.9	15 (41.7)	2 (25.0)	
Obese, ≥30	3 (8.3)	5 (62.5)	
Diabetes, n(%)	7 (19.4)	2 (25.0)	
Metabolic syndrome, n(%)	17 (48.6)	8 (100.0)	0.02
Plasma ALT (IU/L), median (IQR)	26.0 (19.2, 32.8)	43.5 (36.8, 52.0)	<0.01
Plasma AST, median (IQR)	31.0 (25.5, 35.5)	38.0 (31.0, 49.8)	0.08
Plasma total-cholesterol, median (IQR)	4.1 (3.6, 4.9)	5.3 (4.5, 5.7)	0.05
Plasma triglycerides (mM), median (IQR)	2.0 (1.1, 2.8)	3.2 (2.9, 3.4)	<0.01
Duration of HIV infection (years), median (IQR)	18.3 (9.0;26.4)	21.5 (17.1;25.8)	0.55
MSM HIV acquisition, n(%)	19 (55.9)	7 (87.5)	0.21
Blood CD4 T cell count (cells/µl), n(%)			0.53
< 200	1 (2.8)	0 (0.0)	
200-349	3 (8.3)	2 (25.0)	
350-500	3 (8.3)	1 (12.5)	
> 500	29 (80.6)	5 (62.5)	
Plasma HIV-RNA ≥50 copies/mL	1 (2.8)	0 (0.0)	1.00

Abbreviations: IQR: interquartile range; BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; MSM: men sex with men; HIV: human immunodeficiency virus. Metabolic syndrome defined

as a minimum of three of the following 5 items: (1) Waist circumference waist circumference of ≥94 cm for men and ≥80 cm for women; (2) Systolic blood pressure ≥130 mmHg and/or antihypertensive treatment; (3) plasma HDL ≤1.036 mmol/l for men, and plasma HDL ≤1.295 mmol/l for women; (4) plasma triglycerides ≥ 1.693 mmol/l; (5) self-reported diabetes mellitus and/or antidiabetic treatment and/or non-fasting plasma glucose ≥11.1 mmol/l [1]

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Manuscript III

Increased prevalence of liver fibrosis in people living with HIV without viral hepatitis compared to uninfected population controls

Increased prevalence of liver fibrosis in people living with HIV without viral hepatitis compared to population controls

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Study highlights:

WHAT IS KNOWN

- People living with HIV are at high risk of liver fibrosis
- The effect of HIV itself remains unclear

WHAT IS NEW HERE

- HIV is independently associated with higher odds of liver fibrosis
- Higher age, BMI, ALT and previous exposure to didanosine was independently associated with liver fibrosis
- PLWH with high age, BMI, ALT or previously exposed to didanose may require additional work-up

Abstract

Background Liver fibrosis is associated with poor liver related outcomes and mortality. People living with HIV (PLWH) may be at increased risk of liver fibrosis. Our objective was to estimate the prevalence of and factors associated with liver fibrosis in a cohort of PLWH without viral hepatitis compared to population controls.

Methods

Cross-sectional cohort study. We analyzed data from 342 PLWH and compared them with 2,190 population controls aged 50 to 70 years. Liver fibrosis was assessed by transient elastography and defined as a liver stiffness measurement ≥7.6 kilopascals. Odds ratios (aOR) and 95% confidence intervals were computed by logistic regression after adjustment for age, sex, body mass index (BMI), and plasma levels of alanine aminotransferase (ALT), triglyceride and cholesterol.

Results

The prevalence of liver fibrosis was higher in PLWH than in uninfected controls (12% vs 7%), p<0.01). HIV infection was independently associated with liver fibrosis (aOR:1.84 (95% CI:1.17;2.88), p<0.01). Presence of liver fibrosis was associated with higher age (per decade, aOR:3.34 (95% CI:1.81;6.18), p<0.01); ALT (per 10 IU/L, aOR:1.25 (95% CI:1.05;1.49), p<0.01); BMI (per 1 kg/m^2, aOR:1.17 (95% CI: 1.05;1.29), p<0.01) and in PLWH with previous exposure to didanosine (aOR:2.26 (95% CI:1,01;5.06), p=0.05).

Conclusions: The prevalence of liver fibrosis was higher in PLWH compared to population controls. Higher age, BMI, ALT, previous exposure to didanosine and a positive HIV status was independently associated with higher odds of significant liver fibrosis.

After the introduction of combination antiretroviral therapy (ART), the HIV epidemic in high- and middle-income countries has changed markedly with a substantial reduction in mortality among people living with HIV (PLWH) (1). Today, HIV infection is a chronic disease and the population of PLWH is ageing. Consequently, the number of age-related comorbidities have increased, and by 2030 more than 80% of PLWH is predicted to have at least one age-related comorbidity (2). Liver disease is the second leading cause of death among PLWH with high mortality rates among PLWH co-infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) (3). However, the spectrum of liver disease among PLWH likely will change due the ability to suppress HBV replication with ART, direct-acting antiviral therapy for HCV infection, and the World Health Organization's 2030 HCV elimination plan (4,5). In PLWH, the prevalence of liver fibrosis has been reported to be 15% in unselected PLWH (6). This is higher than in the general population where the prevalence has been reported to be 2 to 9% (7,8), and PLWH seem to be at higher risk of liver fibrosis. Possible explanations may be adverse lifestyle behavior, microbial translocation (9,10), immune activation or immunosenescence (11,12), ART induced liver toxicities (13–16), and non-alcoholic fatty liver disease (NAFLD) (17). However, studies that assess the prevalence and risk of liver fibrosis among unselected PLWH without viral hepatitis and with an uninfected comparator group are few (18,19). In this study, we aimed to estimate the prevalence of and factors associated with liver fibrosis in PLWH without viral hepatitis compared to HIV-uninfected controls, and to estimate if a positive HIV status was independently associated with liver fibrosis. We hypothesized that PLWH had a higher prevalence of liver fibrosis compared to the general population, and that a positive HIV status was independently associated with liver fibrosis.

Methods

Study populations

The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Study is an observational, prospective cohort study of PLWH aged 20 years and above in the area of Copenhagen, Denmark (20). From March 2015 through November 2016, 1,099 PLWH were consecutively recruited from the outpatient clinics of the Department of Infectious Diseases, at Rigshospitalet and Amager-Hvidovre Hospital, both in Copenhagen, Denmark. For this study, PLWH aged 50 to 70 years were included for further analysis. The Rotterdam Study is a prospective, population-based study of adult people living in the area of Ommoord, Rotterdam, The Netherlands (21). The study was initiated in 1989 and comprises two cohorts with inhabitants aged 55 years and above (RS-I, RS-II), and one cohort with inhabitants aged 45 years and above (RS-III). For this study, participants aged 50 to 70 years enrolled from March 2011 to November 2014 from RS-II and RS-III were used as a comparator group. The

comparator group was assumed to be HIV uninfected, as the prevalence of PLWH in the Netherlands in 2017 was 0.1% (22). Individuals with HBV infection and/or HCV infection were excluded from the analyses.

Data collection

Data collection for the COCOMO Study and the Rotterdam Study has been described in detail elsewhere (20,21). For the COCOMO study, data were collected through blood sampling and comprehensive questionnaires on health and lifestyle. Information on HIV specific parameters (e.g. CD4 T-cell count, HIV RNA, exposure and duration of antiretroviral therapy) and hepatitis serology (HBsAg and anti-HCV) were retrieved from medical records. Hepatic steatosis was assessed by unenhanced computed tomography (CT) scan of the upper abdomen in the COCOMO study as described previously (20). In short, CT scans were analyzed by a trained physician and the average liver attenuation in the Couinaud liver segments five and six was estimated. Moderate-to-severe hepatic steatosis was defined as an average liver attenuation ≤48 Hounsfield units (HU) according to Pickhardt et al (23).

For the Rotterdam study, data were collected through blood sampling including hepatitis serology (HBsAg and anti-HCV antibody), and comprehensive questionnaires. Hepatic steatosis was assessed by abdominal ultrasonography (24). In short, abdominal ultrasonography images were re-evaluated by an experienced hepatologist, and hepatic steatosis defined as presence of hyperechogenic liver parenchyma according to Hamaguchi et al (25).

Transient elastography

Transient elastography was performed by trained personnel using Fibroscan, Echosens^{TM}, Paris, France in both cohorts. With the non-fasting participant in supine position, the transducer was placed on the skin in an intercostal space in the right midaxillary line at the level of the right liver lobe. The liver stiffness was measured by the standard M-probe and results expressed in kilopascal (kPa). The transient elastography was considered valid if at least 10 valid measurements were obtained; the interquartile range (IQR) was less than 30% of the median liver stiffness measurement (LSM); and the success rate was at least 60% (26). The transient elastography was considered failed if no valid measurements were obtained after at least 10 attempts.

Definitions

The physiologic stiffness of the liver parenchyma is 5.5 ±1.6 kPa by transient elastography (7). Liver stiffness is positively correlated with liver fibrosis, yielding higher LSM with higher amounts of liver fibrosis. In this study we defined significant liver fibrosis as LSM ≥7.6 kPa with an area under the receiver operating characteristics (AUROC) of 87% (95% CI: 82 – 91%) for discriminating F2-F4 fibrosis from F0-F1 fibrosis and with a specificity of 80%, a sensitivity of 75%, a positive predictive value of 72% and a negative predictive value of 83% (27).

We defined metabolic syndrome as a minimum of three of the following 5 items: (1) waist circumference of ≥94 cm for men and ≥80 cm for women; (2) systolic blood pressure ≥130 mmHg and/or antihypertensive treatment; (3) plasma high density lipoprotein (HDL) ≤1.036 mmol/l for men, and plasma HDL ≤1.295 mmol/l for women; (4) plasma triglycerides ≥ 1.693 mmol/l; (5) self-reported diabetes mellitus and/or antidiabetic treatment and/or non-fasting plasma glucose ≥11.1 mmol/l (28). We defined HBV infection as the presence of hepatitis B surface antigen (HBsAg); HCV infection as presence of hepatitis C antibodies (anti-HCV); elevated alanine aminotransferase (ALT) as plasma ALT ≥70 IU/L for males and ALT ≥45 IU/L for females.

Ethics

The COCOMO Study has been approved by the regional ethics committee of the Capital Region of Denmark (protocol no. H-8-2014-004). The Rotterdam study has been approved by the Netherlands Ministry of Health, Welfare and Sports and by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, Netherlands. The studies were conducted in accordance with the Helsinki declaration. All study participants provided informed consent.

Statistical analyses

Clinical and demographic characteristics were summarized as median with IQR for continuous variables and as number with percentage for categorical variables. Comparison between the study cohorts were performed by Fisher's exact test and chi-square test for categorical variables and by Mann-Whitney's U-test or Kruskal Wallis for continuous variables. Uni- and multivariate logistic regression analyses were performed to assess factors associated with significant liver fibrosis (dependent variable). Covariates included in the adjusted model were age (continuous), sex (binary), plasma ALT (continuous), body mass index (BMI) (continuous), plasma triglycerides (continuous) and plasma cholesterol (continuous). Sensitivity analyses was performed excluding individuals with elevated ALT, as this may be induced by liver inflammation and lead to falsely higher liver stiffness measurements. Results are presented as crude and adjusted odds ratios (aOR) with 95% confidence

intervals (95% CI). P-values <0.05 are considered statistically significant. Missing values of ALT, aspartate aminotransferase (AST), albumin and platelets were imputed by predictive mean matching for the COCOMO Study cohort. All statistical analyses were conducted in R 3.4.1.

Results

Clinical and demographic characteristics

A total of 342 PLWH from the COCOMO Study, and 2,190 controls from the Rotterdam Study were included for this study (**Figure 1**). Clinical and demographic characteristics are shown in **Table 1** and HIV specific characteristics in **Table 2**.

Liver fibrosis in PLWH and population controls

Forty-one (12%) of PLWH without viral hepatitis had significant liver fibrosis assessed by transient elastography compared to 154 (7%) population controls (p<0.01). The proportion of PLWH with mild, moderate and severe fibrosis was higher compared to population controls (p<0.01) (**Figure 2**). The proportion of PLWH with CT-defined moderate-to-severe hepatic steatosis was 24 (8%), while the proportion of population controls with ultrasound-defined steatosis was 776 (35%). In sensitivity analyses individuals with elevated ALT were excluded, and the prevalence of liver fibrosis remained higher in PLWH with normal ALT compared to population controls (11% vs 7%, p<0.01). Compared to PLWH without fibrosis, PLWH with significant liver fibrosis were older (62 vs 56 years, p<0.01), with higher waist circumference (102 vs 93 cm, p<0.01), BMI (26 vs 24 kg/m^2, p=0.02), and AST (31 vs 28 IU/L, p=0.02). They were more frequently diabetic (23% vs 5%, p<0.01), overweight and obese (56% vs 39%, p=0.02), had more frequently metabolic syndrome (62% vs 40%, p=0.02) and hepatic steatosis (27% vs 6%, p<0.01).

HIV infection and liver fibrosis

HIV infection was associated with higher odds of significant liver fibrosis (aOR: 1.84 (95% CI: 1.17; 2.88), p<0.001). The association between HIV infection and liver fibrosis increased with age (**Figure 3**); individuals aged 57-63 years had higher odds of liver fibrosis (aOR: 4.35 (95% CI: 1.27;14.88), p=0.02) when compared to individuals aged 50-52 years, and the odds were even higher in individuals aged 63-79 years (aOR: 8.67 (95% CI: 2.56;29.35), p<0.01).

Factors associated with liver fibrosis in PLWH

In univariate regression analysis, higher age, BMI, waist circumference, ALT, triglycerides, total cholesterol, and presence of diabetes, and steatosis were all associated with higher odds of liver fibrosis in PLWH (**Table 2**). CD4 T-cell count >350 cells/µL were associated with lower odds of fibrosis, while previous exposure (but not cumulative exposure time) to didanosine (ddI) was associated with higher odds of fibrosis (OR: 2.57 (95% CI: 1.29;5.12), p<0.01) in univariate analyses. Neither duration of HIV infection, route of HIV transmission, plasma HIV-RNA, previous or cumulative exposure to nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors or thymidine analogues were associated with liver fibrosis and thus were not tested in multivariate analysis.

In multivariate analyses, higher age, ALT and BMI were independently associated with liver fibrosis in PLWH (**Figure 4**). The effect of previous exposure to ddI remained statistically significant in multivariate analyses (aOR: 2.26 (95% CI:1.01;5.06), p=0.05) in the total population of PLWH. To test whether this was an independent effect of ddI exposure or an effect of longer duration of HIV infection, we tested the effect of ddI in a subgroup of PLWH with comparable duration of HIV infection (≥20 years). In this population, 62 PLWH were previously exposed to ddI and 95 PLWH were not exposed to ddI (median duration of HIV infection: 25 vs 27 years, p=0.13). Although statistically non-significant, the effect of ddI exposure remained associated with higher odds of liver fibrosis in univariate analysis and after adjustment for sex and age (OR: 2.26 (95% CI: 0.92;5.53), p=0.08 and aOR: 2.26 (95% CI: 0.90;5.63), p=0.08).

Discussion

In this cross-sectional study of 342 unselected PLWH aged 50 to 70 years without viral hepatitis, we show that significant liver fibrosis was more prevalent in PLWH compared to population controls. Interestingly, a positive HIV status in individuals without viral hepatitis was independently associated with higher odds of significant liver fibrosis. Further, age, higher BMI, and plasma ALT were associated with liver fibrosis, as well as previous exposure to ddI.

Our results are comparable with previous studies, where the prevalence of liver fibrosis has been reported to range from 8% to 18% in adult PLWH without viral hepatitis when assessed by transient elastography (6,18,29–35). Two studies included an HIV negative comparator group. Lui et al found a prevalence of significant liver fibrosis in 14% of PLWH mono-infected compared to 3% of HIV-uninfected controls in a cohort from Hong Kong (18); and Stabinski et al reported a prevalence of fibrosis of 18% in PLWH compared to 11% in uninfected controls in a cohort from Uganda (19).

However, these studies were conducted in Asian and African settings and results may not be comparable to a European setting. Further, Stabinski et al included individuals with HBV, which may contribute to the fibrogenesis, and no information on HCV was provided. Interestingly, an independent association between HIV infection and significant liver fibrosis was identified in these studies as well as in ours suggesting that HIV itself may play a role in the pathogenesis of liver fibrosis. Several studies have demonstrated a direct effect of HIV on the hepatic cells. Hepatic fibrogenesis may be induced by HIV entering the hepatic stellate cells (36); oxidative stress and hepatic apoptosis may be triggered by the HIV gp120 signaling pathway (37); and immune-mediated liver injury may be triggered by HIV through alterations of the functions of the stellate cells and Kupffer cells (38). However, future studies are needed to directly link HIV-induced alterations in the hepatic cells to liver fibrosis development.

HIV-associated factors may also contribute to the development of liver fibrosis. Although duration of HIV infection, low CD4 T-cell counts, high plasma HIV RNA or route of HIV transmission was not associated with significant liver fibrosis, previous exposure to ddI was independently associated with higher odds of fibrosis. The association persisted in PLWH with comparable duration of HIV infection. This finding supports the existing literature from both HIV mono-infected and HIV/HCV co-infected individuals, where ddI has been associated with liver fibrosis, variceal bleeding, non-cirrhotic portal hypertension , cirrhosis and end-stage liver disease (15,16). Several mechanisms for this potential hepatotoxic effect of ddI have been proposed and includes mitochondrial toxicity, hepatic steatosis and hepatocellular injury (13). These findings emphasize, that PLWH without viral hepatitis who was previously exposed to ddI should be monitored for their liver function including development of liver fibrosis.

Age was associated with significant liver fibrosis, and the association between HIV infection and liver fibrosis increased with age, although this may partly be explained by the fact that older PLWH tended to have been treated with more hepatotoxic agents no longer used. In the general population, the pathogenesis of non-alcoholic steatohepatitis (NASH) and liver fibrosis is described as a “multiple-parallel hit” model (39). In short, environmental factors (e.g. diet, sedentary lifestyle), metabolic factors (e.g. obesity, insulin resistance) and genetic factors (e.g. PNAPL3) contribute to lipid accumulation of the hepatocyte (40); production of pro-inflammatory cytokines (e.g. tumor necrosis factor α (TNFα), interleukin 6 (IL-6)); activation of Kupffer cells; and secretion of inflammatory cytokines (e.g. TNFα, IL-1β). Kupffer cells and recruited innate immune cells may induce inflammation in the liver, and especially IL-1β secretion plays a crucial role in the progression of NAFLD/NASH. Eventually, hepatic stellate cells (HSCs) are activated by e.g. proinflammatory

cytokines and IL-1β resulting in fibrogenesis in the liver parenchyma. In PLWH, increased levels of proinflammatory cytokines (including TNFα, IL-6 and IL-1β) have been demonstrated as well as reduced production of naïve CD4 T cells, increased numbers of late differentiated CD4+ and CD8+ T-cells and shortened telomere length (41,42). Thus, the immune profile of PLWH is similar to the ageing- and immunosenescent immune profiles as in NASH and may contribute to both increased inflammation and increased fibrogenesis in the liver. Interestingly, ALT and AST levels were higher in PLWH compared to uninfected controls and have been linked to liver fibrosis by several study groups including ours (6,43). Prospective studies are needed to explore these age-specific differences in more detail and to characterize specific phenotypes of PLWH at risk of liver fibrosis.

Excessive alcohol consumption and drug-use are known causes of liver disease. In this study, fewer PLWH reported an excessive alcohol consumption compared to population controls, and no association was found between alcohol consumption and liver fibrosis in either of the two populations. Individuals with HBV and HCV co-infection were excluded from the analysis, excluding many individuals with potential drug-use. Thus, these adverse lifestyle behaviors do not seem to contribute to the high prevalence of fibrosis observed in PLWH.

Higher BMI was independently associated with liver fibrosis. Given the close correlation between high BMI and hepatic steatosis it is possible that liver fibrosis is at least partly induced by the NAFLD pathway. However, the number of overweight and obese individuals was highest in population controls. The prevalence of hepatic steatosis was also higher in population controls. On the other hand, the effect of hepatic steatosis on liver fibrosis in PLWH seemed stronger than in population controls, and suggests that a positive HIV status may induce a pathway of synergistic effects leading to accelerated fibrogenesis (44). This hypothesis should be assessed in future studies.

To our knowledge, this is the largest study to date to evaluate liver fibrosis by transient elastography in an unselected population of PLWH mono-infection with a large comparator group of population controls. The main limitation of this study is the lack of liver biopsies, and that PLWH and population controls were included from different countries, which may introduce bias due to methodological differences and country-specific differences. However, Denmark and The Netherlands are believed to be very similar in terms of e.g. ethnicity and life style. Given the control population being in the age range of 50-70 years, we limited the PLWH cohort to those at similar age, and hence is unable to comment on fibrosis stage in younger PLWH which tends to have shorter duration of HIV infection than those studied here.

In conclusion, HIV infection was independently associated with higher odds of significant liver fibrosis, and the prevalence of liver fibrosis was higher in PLWH compared to population controls. Higher age,

BMI, ALT and previous exposure to ddI was independently associated with liver fibrosis and suggests that liver fibrosis may be induced by a combination of hepatotoxic drugs, ageing and steatosis. Future studies on the pathogenesis of liver fibrosis in PLWH without viral hepatitis are warranted.

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Author contributions: Study design: DK, FB, SD, JL and TB. Data collection: DK (COCOMO), RK (Rotterdam Study) Data analysis: DK with supervision by TB, SD, FB. Manuscript drafting: DK. All authors have contributed with revisions of the manuscript and all authors have read and approved the final manuscript.

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Figure 1 Flowchart of study participants. Selection of study participants from The COCOMO study cohort and the Rotterdam Study cohort. Hepatitis B co-infection (HBV) was defined as the presence of hepatitis B surface antigens (HBsAg). Hepatitis C virus (HCV) infection was defined as the presence of anti-HCV antibodies (anti-HCV).

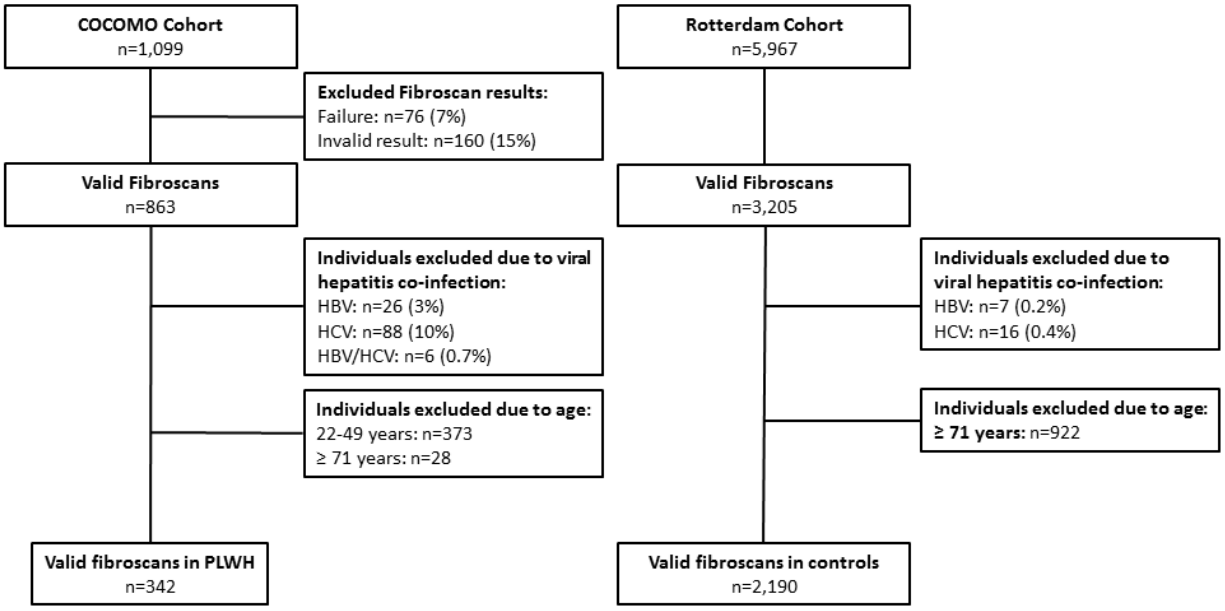

Figure 2 Proportion of subjects with fibrosis in people living with HIV (PLWH) (dark grey) compared to population controls (light grey).

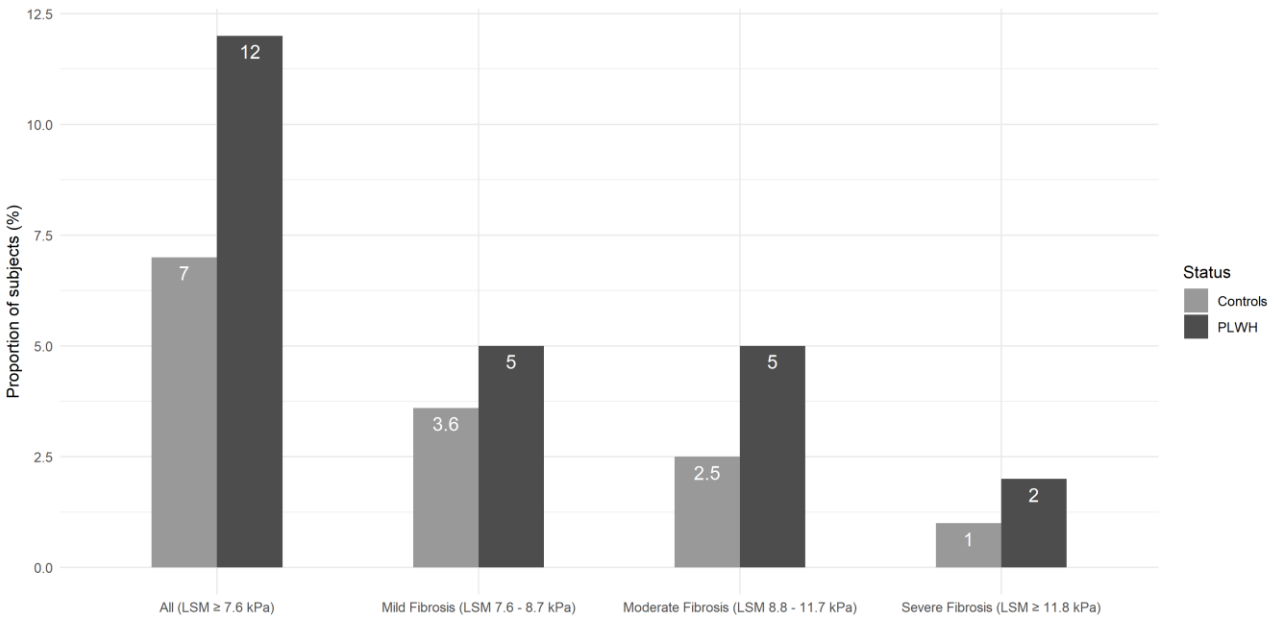

Figure 3 Predicted probability of significant liver fibrosis according to age in people living with HIV without viral hepatitis (dotted line) compared to population controls (solid line). The predicted probabilities are adjusted by age, and plasma alanine aminotransferase, triglycerides, and cholesterol.

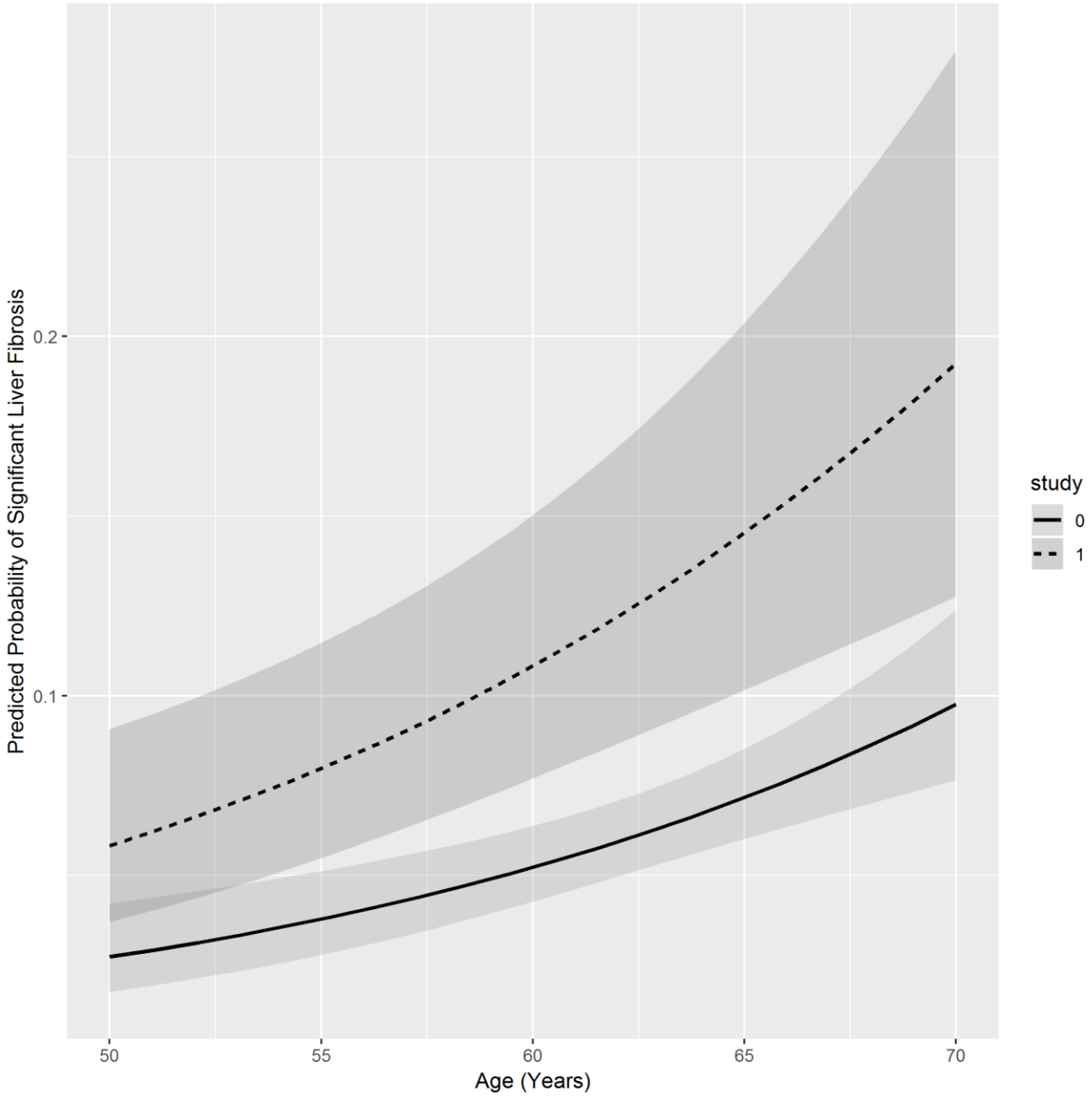

Figure 4 Factors associated with significant liver fibrosis in people living with HIV (PLWH) (black), population controls (grey). Adjusted odds ratios (aOR) with 95% confidence intervals (CI) assessed by multivariate logistic regression model adjusted for sex, age, alanine aminotransferase (ALT), body mass index (BMI), triglycerides, and cholesterol.

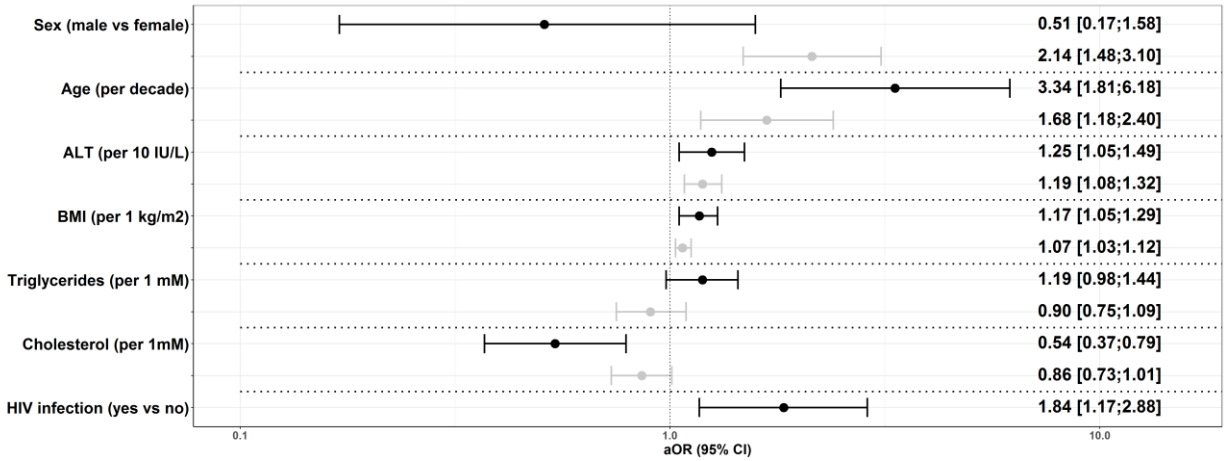

Table 1 Clinical and demographic characteristics of people living with HIV (PLWH) and population controls.

	PLWH (n=342)	Controls (n=2190)	p-value
Age (years), median (IQR)	57.0 (52.0, 63.0)	63.0 (58.1, 66.4)	<0.01
Sex (male), n (%)	296 (86.5)	1059 (48.4)	<0.01
Caucasian, n (%)	264 (78.3)	1905 (96.7)	<0.01
Smoking, n (%)			0.20
Current Smoker	86 (25.1)	558 (27.1)	
Previous smoker	151 (44.2)	805 (39.1)	
Never smoker	105 (30.7)	698 (33.9)	
Alcohol, n (%)			0.03
None	63 (20.3)	304 (17.7)	
Within recommendations	159 (51.3)	787 (45.9)	
Above recommendations	88 (28.4)	623 (36.3)	
Abdominal obesity, n (%)	252 (75.9)	1327 (60.6)	<0.01
Waist circumference (cm), median (IQR)	94.0 (87.0, 101.0)	93.0 (84.0, 101.0)	0.02
BMI (kg/m^2), median (IQR)	24.3 (22.1, 26.5)	26.6 (24.3, 29.3)	<0.01
BMI WHO categories, n (%)			<0.01
Underweight, < 18.4 kg/m^2	7 (2.1)	11 (0.5)	
Normal weight, 18.5-24.9 kg/m^2	192 (56.5)	659 (30.1)	
Overweight, 25-29.9 kg/m^2	119 (35.0)	1062 (48.5)	
Obese, ≥ 30 kg/m^2	22 (6.5)	458 (20.9)	
Diabetes, n (%)	23 (6.9)	226 (10.3)	0.06
Metabolic syndrome, n (%)	132 (43.0)	689 (31.5)	<0.01
Plasma ALT (IU/L), median (IQR)	25.5 (20.0, 34.8)	20.0 (15.0, 26.0)	<0.01
Plasma AST (IU/L), median (IQR)	29.0 (25.0, 35.0)	24.0 (21.0, 28.0)	<0.01
Plasma total cholesterol (mM), median (IQR)	5.0 (4.3, 5.7)	5.5 (4.8, 6.3)	<0.01
Plasma triglycerides (mM), median (IQR)	1.8 (1.3, 2.8)	1.3 (0.9, 1.7)	<0.01
Route of HIV transmission, n (%)		NA	
MSM	240 (70.8)		
HSX	77 (22.7)		
IDU	3 (0.9)		
Other	19 (5.6)		
Blood CD4 T-cell count (cells/µL), n (%)	0.40	NA	
< 200	3 (0.9)		
200-349	17 (5.0)		
350-500	57 (16.8)		
> 500	263 (77.4)		
Blood CD4 Nadir (cells/µL), median (IQR)	200.0 (84.2, 290.0)	NA	
Plasma HIV RNA ≥50 copies/mL, n (%)	10 (2.9)	NA	
Duration of HIV infection (years), median (IQR)	19.3 (11.5, 25.8)	NA	
ART (yes), n (%)	334 (97.7)	NA	

Abbreviations: PLWH: People living with HIV; HIV: human immunodeficiency virus; IQR: interquartile range; BMI: Body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; MSM: men sex with med; HSX: heterosexual; IDU: intravenous drug-use; ART: antiretroviral therapy; LSM: liver stiffness measurement; kPa: kilopascal.
Missing variables (n) for COCOMO (RS): Caucasian 5 (220), smoking 0 (129), alcohol 32 (476), education 15 (10), abdominal obesity 10 (1); waist circumference 10 (1); BMI 2 (0); diabetes 8 (0); metabolic syndrome 35 (4); total cholesterol 19 (0); triglycerides 19 (0).

Table 2 Factors associated with liver fibrosis in people living with HIV (PLWH) and population controls

	PLWH		Controls	
	Crude OR (95% CI)	**p-value**	**Crude OR (95% CI)**	**p-value**
Age (years), per decade	2.39 (1.41;4.06)	<0.01	1.67 (1.18;2.37)	<0.01
Age groups (quartiles)				
50-52 years	Ref		Ref	
52-57 years	0.68 (0.21;2.23)	0.53	1.44 (0.84;2.47)	0.18
57-63 years	2.14 (0.81;5.64)	0.13	1.27 (0.73;2.18)	0.4
63-70 years	3.43 (1.33;8.87)	0.01	2.08 (1.25;3.45)	<0.01
Sex (male vs female)	1.14 (0.42;3.06)	0.80	2.51 (1.77;3.57)	<0.01
Caucasian (no vs yes)	1.06 (0.48;2.33)	0.89	0.60 (0.18;1.92)	0.39
Smoking				
Never smoker	Ref		Ref	
Current smoker	1.25 (0.49;3.16)	0.64	1.24 (0.81;1.91)	0.32
Previous smoker	1.53 (0.69;3.41)	0.29	0.90 (0.59;1.36)	0.62
Alcohol				
None	Ref		Ref	
Within recommendations	0.77 (0.31;1.81)	0.54	0.74 (0.44;1.23)	0.25
Above recommendations	0.77 (0.29;2.02)	0.59	0.89 (0.53;1.49)	0.65
BMI (per 1 kg/m2)	1.13 (1.03;1.23)	<0.01	1.08 (1.04;1.12)	<0.01
BMI category (yes vs no)				
Normal weight, 18.5-24.9 kg/m^2	Ref.		Ref.	
Overweight, 25-29.9 kg/m^2	0.93 (0.11;8.26)	0.95	1.13 (0.75;1.71)	0.56
Obese, ≥ 30 kg/m^2	2.80 (0.28;27.91)	0.38	2.01 (1.29;3.14)	<0.01
Diabetes (yes vs no)	5.81 (2.32;14.51)	<0.01	3.92 (2.67;5.76)	<0.01
Waist circumference (per 1 cm)	1.06 (103;1.10)	<0.01	1.04 (1.02;1.05)	<0.01
Abdominal obesity (yes vs no)	1.36 (0.60;3.07)	0.46	2.16 (1.47;3.15)	<0.01
Plasma ALT (per 10 IU/L)	1.26 (1.07;1.47)	<0.01	1.21 (1.11;1.33)	<0.01
Plasma triglyceride (per 1 mM)	1.21 (1.03;1.42)	0.02	1.05 (0.90;1.23)	0.54
Plasma total cholesterol (per 1 mM)	0.63 (0.45;0.88)	<0.01	0.74 (0.63;0.86)	<0.01
Hepatic steatosis†	6.00 (2.37;15.16)	<0.01	2.69 (1.93;3.75)	<0.01
Duration of HIV infection (per 5 years)	1.19 (0.98;1.46)	0.08	NA	
MSM HIV transmission (no vs yes)	1.30 (0.65;2.60)	0.46	NA	
Plasma HIV RNA ≥ 50 (yes vs no)	0.82 (0.10; 6.67)	0.86	NA	
Blood CD4 T-cell count (cells/µL)			NA	
< 200	Ref			
200-349	0.15 (0.01;2.18)	0.17		
350-500	0.04 (0.00;0.51)	0.01		

> 500	0.07 (0.01;0.76)	0.03	
Previous ART exposure (yes vs no)			NA
NRTI	1.38 (0.47;4.07)	0.56	
NNRTI	0.79 (0.41;1.51)	0.47	
Protease inhibitor	1.22 (0.64;2.35)	0.54	
Integrase inhibitor	0.37 (0.08;1.58)	0.18	
Thymidine analogue	1.74 (0.80;3.79)	0.16	
Didanosine	2.57 (1.29;5.12)	<0.01	
Cumulative ART exposure (per 5 years)			NA
NRTI	1.00 (0.98;1.02)	0.98	
NNRTI	0.94 (0.66;1.34)	0.74	
Protease inhibitor	1.08 (0.86;1.35)	0.5	
Integrase inhibitor	2.88 (0.95;8.70)	0.06	
Thymidine analogue	1.20 (0.78;1.84)	0.4	
Didanosine	1.08 (0.44;2.70)	0.86	

Abbreviations: PLWH: people living with HIV; OR: odds ratio; CI: confidence interval; BMI: body mass index; ALT: alanine aminotransferase; MSM: men who have sex with men; ART: antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; NA: not applicable. † Moderate to severe hepatic steatosis assessed by CT scan (COCOMO) or ultrasound (Rotterdam).

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	5
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-7

Bias		9	Describe any efforts to address potential sources of bias	9
Study size		10	Explain how the study size was arrived at	9
Quantitative variables		11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7-9
Statistical methods		12	(*a*) Describe all statistical methods, including those used to control for confounding	8-9
			(*b*) Describe any methods used to examine subgroups and interactions	
			(*c*) Explain how missing data were addressed	
			(*d*) If applicable, explain how loss to follow-up was addressed	
			(*e*) Describe any sensitivity analyses	
Results				
Participants		13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9
			(b) Give reasons for non-participation at each stage	
			(c) Consider use of a flow diagram	
Descriptive data		14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9-10
			(b) Indicate number of participants with missing data for each variable of interest	
			(c) Summarise follow-up time (eg, average and total amount)	
Outcome data		15*	Report numbers of outcome events or summary measures over time	10-11
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95%		10-11

		confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	11
Discussion			
Key results	18	Summarise key results with reference to study objectives	11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-15
Generalisability	21	Discuss the generalisability (external validity) of the study results	11-15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for exposed and unexposed groups

Manuscript IV

Poor concordance between liver stiffness and non-invasive liver fibrosis scores in people living with HIV without viral hepatitis: Results from the COCOMO Liver Study

Poor concordance between liver stiffness and non-invasive fibrosis scores in HIV infection without viral hepatitis.

Short title: Fibrosis assessment in HIV infection.

Ditte Marie Kirkegaard-Klitbo[1], Flemming Bendtsen[2], Jens Lundgren[3], Susanne Dam Nielsen[4], Thomas Benfield[1]; on behalf of the COCOMO study group.

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Grant support: This study was supported by Simonsen Foundation, Novo Nordic Foundation, Augustinus Foundation and Rigshospitalet Research Council.

Abbreviations: APRI: Aspartate-to-platelet ratio; COCOMO: Copenhagen Co-Morbidity in HIV infection; FIB4: Fibrosis-4 Index; IQR: interquartile range; PLWH: People living with HIV; kPa: Kilopascal; LSM: Liver stiffness measurement; NAFLD: Non-alcoholic fatty liver disease; NFS: NAFLD fibrosis score; TE: Transient elastography.

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Introduction

People living with HIV (PLWH) are at higher risk of liver fibrosis compared to the general population. As liver fibrosis is independently associated with poor long-term liver related outcomes and mortality[1], early detection of is extremely important. Liver biopsy is considered gold standard for the diagnosis of liver fibrosis. However, this invasive procedure is only suitable for a selected group of patients due to risk of serious complications. We aimed to estimate the concordance between vibration controlled transient elastography (VCTE) and the simple non-invasive liver fibrosis scores FIB-4 index (FIB4), aspartate-to-platelet ratio index (APRI) and NAFLD fibrosis score (NFS), respectively in PLWH without viral hepatitis.

Methods

The Copenhagen Co-Morbidity (COCOMO) in HIV Infection Study is a longitudinal, observational cohort study[2]. In total, 1,099 PLWH were consecutively enrolled from March 2015 through November 2016. Physical examinations were performed, blood samples drawn, and questionnaires filled in. HIV specific factors and hepatitis serology were retrieved from medical records. Liver fibrosis was assessed by VCTE (Fibroscan (Echosens, Paris)) with a M-probe for all participants. A valid VCTE was defined as an interquartile range (IQR) less than 30% of the median liver stiffness measurement (LSM), ≥10 valid measurements, and a success rate ≥60%. Non-invasive liver fibrosis scores were

calculated using formulas previously described [3–5]. We defined significant liver fibrosis as LSM≥8.8 kPa; hepatitis B virus infection as positive HbSAg; hepatitis C infection as positive anti-HCV antibodies. Low and high cut-offs points were defined as <0.5 and >1.5 for APRI; <1.30 and >2.67 for FIB4; and <-1.455 and >0.676 for NFS. We presented continuous variables as medians with IQR, and concordance as frequency with percentage. χ^2-test were performed between categorical variables. P-value <0.05 was considered statistically significant. Analysis was performed in R (version 3.5.2, Vienna, Austria).

Results

A total of 743 PLWH with a valid VCTE and without viral hepatitis were included in the study. The majority were males (85%) with a median age of 49 years (IQR: 42;57). The majority acquired HIV through homosexual transmission (72%), 98% were on antiretroviral therapy, 95% had plasma HIV RNA <50 copies/mL, and the median CD4 T-cell count was 690 cells/µL (IQR: 530;890). In total, 37 (5%) of all PLWH had LSM ≥8.8 kPa by VCTE. Of these, five (14%) were classified with fibrosis by FIB4 >2.67, one (3%) by APRI >1.5, and three (9%) by NFS >0.676 (**Table 1**). Of 706 PLWH without VCTE-defined fibrosis, 408 (58%) were also classified without fibrosis by FIB4 <1.30; 644 (91%) by APRI <0.5; and 412 (65%) by NFS <-1.455. Nine (24.3%) subjects would not have been identified with potential fibrosis if FIB4 had been used alone, 28 (76%) subjects if APRI score had been used alone, and 14 (40%) if NFS had been used alone. The concordance between VCTE and fibrosis scores improved (p<0.01 for all scores) using a higher threshold of LSM ≥10kPa. All authors had access to study data and reviewed and approved the final manuscript.

Discussion

In this cross-sectional study of 743 PLWH without viral hepatitis, we found a marked discrepancy between subjects identified with liver fibrosis by VCTE and by non-invasive liver fibrosis scores. Of 37 subjects with VCTE defined fibrosis, 24%, 76% and 40% would not have been identified with fibrosis by FIB4, APRI or NFS, respectively, if the scores had been used as single diagnostic tools. Cheng et al found a similar discrepancy between VCTE and FIB4 in a population infected with HCV; 15% of subjects with VCTE-defined fibrosis (LSM ≥7.1 kPa) were not classified with liver fibrosis by FIB4[6]. Similarly, Sagir et al found a discrepancy of 26% between VCTE and APRI, and a discrepancy of 36% between VCTE and FIB4 in a population with HIV infection[7]. Our results support these findings and suggest that VCTE and simple fibrosis scores should be used with caution individually. A combination of VCTE and a fibrosis score may be useful as a screening tool among PLWH[8]. In

conclusion, VCTE and simple non-invasive liver fibrosis scores were discordant in PLWH without viral hepatitis and may not be used as single diagnostic tools in routine clinical practice.

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Table 1 Disconcordance between transient elastography and non-invasive liver fibrosis scores in people living with HIV (n=743)

		Cut-off	LSM <8.8 kPa (n=706)	LSM ≥8.8 kPa (n=37)	LSM ≥10.0 kPa (n=20)	p-value*
FIB4	Low	<1.30	408 (57.8)	9 (24.3)	6 (30.0)	<0.01
	Indeterminant	1.30 – 2.67	268 (38.0)	23 (62.2)	10 (50.0)	
	High	>2.67	30 (4.2)	5 (13.5)	4 (20.0)	
APRI	Low	<0.5	644 (91.2)	28 (75.7)	5 (26.3)	<0.01
	Indeterminant	0.5 - 1.5	61 (8.6)	8 (21.6)	12 (63.2)	
	High	>1.5	1 (0.1)	1 (2.7)	2 (10.5)	
NFS	Low	<-1.455	412 (64.5)	14 (40.0)	15 (75.0)	<0.01
	Indeterminant	-1.455 - 0.675	213 (33.3)	18 (51.4)	4 (20.0)	
	High	>0.676	14 (2.2)	3 (8.6)	1 (5.0)	

Abbreviations: HIV: human immunodeficiency virus; kPa: kilopascals; LSM: liver stiffness measurement; APRI: Aspartate aminotransferase to Platelet Ratio Index; FIB4: FIB4-Index; NFS: NAFLD fibrosis score. *P-value when LSM 8.8-10 kPa compared to LSM ≥10 kPa