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Lifestyle and Biological Risk Factors for Liver Fibrosis in the Miami Adult Studies on HIV (MASH) Cohort: An HIV Infected and HIV/HCV Co-infected Population

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LIFESTYLE AND BIOLOGICAL RISK FACTORS FOR LIVER FIBROSIS IN THE MIAMI ADULT STUDIES ON HIV (MASH) COHORT: AN HIV INFECTED AND HIV/HCV CO-INFECTED POPULATION

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by

Tiffanie S. Stewart

2016

To: Dean Tomàs R. Guillarte
R.Stempel College of Public Health and Social Work

This dissertation, written by Tiffanie S. Stewart, and entitled Lifestyle and Biological Risk Factors for Liver Fibrosis in the Miami Adult Studies on HIV (MASH) Cohort: An HIV Infected and HIV/HCV Co-Infected Population, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Fatma Huffman

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Adriana Campa, Major Professor

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The dissertation of Tiffanie. S. Stewart is approved.

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Dean Tomàs R. Guillarte
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Dean Andrés G. Gil
Vice President for Research and Economic Development
And Dean of the University Graduate School

Florida International University, 2016

DEDICATION

This dissertation is dedicated to the millions of HIV positive survivors worldwide, and those who have shared their lives with me - I am forever grateful. I also dedicate this work to my loving parents, sister, family and friends because it would not exist without them. Your love and faith in me provided the strength to complete a lifelong dream, and I am honored to share the dedicated work with you.

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A doctoral degree is more than a test of intelligence. Of course, the ability to learn is the keystone to a path in academia, however, at its heart, a doctoral degree is a quest to develop resiliency, adaptability, and a true passion for a field. In my case, the field is science. Many student embark on this journey wide-eyed and unaware of the challenges they will face, which is exactly what happened to me, and exactly the reason I could not have succeeded without the help of the people mentioned in the acknowledgements (and many more, too many to mention in this document).

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ABSTRACT OF THE DISSERTATION

LIFESTYLE AND BIOLOGICAL RISK FACTORS FOR LIVER FIBROSIS IN THE MIAMI ADULT STUDIES ON HIV (MASH) COHORT:

AN HIV INFECTED AND HIV/HCV CO-INFECTED POPULATION

by

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Florida International University, 2016

Miami, Florida

Professor Adriana Campa, Major Professor

Liver disease is now a leading cause of non-AIDS related morbidity and mortality in people living with HIV (PLWH). The present study investigated the interplay between adverse lifestyle factors that are prevalent in PLWH, biological mediators of liver pathogenesis, and a non-invasive measure of liver fibrosis (FIB-4 index) in HIV mono- and HIV/HCV co-infected individuals.

The results of this investigation in the Miami Adult Studies of HIV (MASH) cohort show that the odds of liver fibrosis progression significantly increased over two years for HIV mono-infected participants who drank alcohol hazardously (OR 3.038, P=0.048), and had BMI ≥ 28kg/m^2 (OR 2.934, P=0.027). Cocaine use reduced the odds of advancing one stage of liver fibrosis (OR 0.228, P=0.038), but an interaction between high BMI and cocaine use slightly raised the odds by 4.8% of liver fibrosis progression (P=0.072). HIV/HCV co-infected participants showed interactions between cocaine use

and high BMI with increased FIB-4 stage (OR 4.985, *P*= 0.034), however no lifestyle factors could independently predict FIB-4 stage in this group.

Biological mediators previously associated with liver pathogenesis were associated with higher FIB-4 index over 2 years in a subset of (n=65) HIV mono-infected participants. Plasma measures of oxidative stress (% oxidized glutathione: OR 4.342, *P*= 0.046), hepatocyte-specific apoptosis (Cytokeratin-18 (CK-18): OR 1.008, *P*=0.021), and microbial endotoxin (lipopolysaccharide (LPS): OR 1.098, *P*= 0.097) were associated with having higher odds of progressing at least one stage of FIB-4 over 2 years.
The same biological mediators were also associated with liver fibrosis within HIV infected people who also had a harmful lifestyle characteristic. FIB-4 index was significantly associated with % oxidized glutathione in obese subjects (β=0.563, *P*=0.018), TGF-β1 in cocaine users (β=0.858, *P*=0.027), and CK-18 in HIV infected individuals without any adverse lifestyle factors (β=0.435, *P*=0.015).

Taken together, the findings of these studies describe interrelationships between HIV disease status, lifestyle, and biological mediators of liver fibrosis. The results show interactions between lifestyle conditions and the mediators of liver fibrosis may account for higher rates of liver disease in HIV infection. Research is warranted to develop personalized therapeutics for PLWH to curb the burden of liver disease.

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CHAPTER I: INTRODUCTION

Statement of Problem

The use of combined antiretroviral therapy (ART) to treat HIV infection has prolonged lifespan by reducing opportunistic infections, [1] but brings new challenges for survival. [2] Liver disease is now a major cause of morbidity and mortality in HIV infection in the United States and Europe. [3-7] In addition to adverse lifestyle factors (obesity, alcohol dependence, and cocaine use) [5,8-11] that contribute to liver fibrosis, co-infection with the hepatitis C virus (HCV) has become an important prognostic factor of liver disease in people living with HIV (PLWH). [12] The risk of liver-related mortality is predominantly attributed to HIV/HCV co-infection; however, HIV mono-infected persons are at higher risk for liver disease than non-infected persons. [13,14] Several studies suggest that HIV may directly interact with liver cells, even infecting hepatocytes, suggesting that liver disease is a rising issue for both HIV mono- and HIV/HCV co-infected persons. [15] Other studies suggest that by reducing HCV-specific CD4 T-cell counts in HIV/HCV co-infected people, HIV infection supports a strong response of HCV-specific interferon-gamma (IFN-α) producing CD8 T-cells, which favor the destruction of HCV-infected hepatocytes and may play a role in accelerating liver fibrosis. [16-20]

Liver fibrosis is a cellular mechanism initiated by presence of injury to hepatocytes. It can be classified into sequential phases based on the activation of hepatic stellate cells (HSCs): initiation phase and perpetuation phase. [21] The initiation phase refers to early events that render the quiescent HSCs responsive to a range of growth factors. [21]

Important fibrogenic stimuli in this stage include: reactive oxygen species, leading to oxidative stress; apoptotic fragments, generated from hepatocyte cell death, and the bacterial endotoxin lipopolysaccharide (LPS), resulting from bacterial products crossing from the gut into the blood stream. [21] The perpetuation phase involves proliferation and migration of HSCs to the extra cellular matrix (ECM), where they synthesize and secrete elevated levels of collagen and fibronectin, promoting fibrosis. [22,23]

The upregulation of the cytokine transforming growth factor beta-1 (TGF-β1) is characteristic of the perpetuation phase because it is released by Kupffer cells, platelets, and HSCs themselves in the presence of fibrogenic stimuli. [24] TGF-β1 is a key regulator of liver fibrosis because it is required by HSCs to lay down fibrotic tissue. [25] Anti-TGF-β1 strategies have been proposed to reduce liver fibrosis by blocking its fibrogenic properties, [26] including direct neutralization of TGF-β1 and indirectly reducing TGF-β1 by targeting biological risk factors such as oxidative stress, [27] microbial translocation, [28] and apoptosis. [29] For example, neutralizing activated TGF-β1 could potentially reduce fibrosis by preventing the activation of HSCs. [26] These biological risk factors for liver fibrosis may be different within the lifestyle risk factors of obesity, [9] alcohol dependence, [9,30] and cocaine use, [10,11] thus there is a need to identify mediators within these lifestyle conditions.

Fibrosis can be resolved in both the initiation phase and the perpetuation phase when fibrogenic stimuli are cleared and degrading enzymes remove fibrotic tissue from the ECM. [31] However, HIV mono- and HIV/HCV co-infection expose hepatocytes to continuously higher levels of fibrogenic stimuli, which propagates the development of

fibrosis. Higher levels of oxidative stress, [32] microbial translocation, [33] TGF-β1, [34] and hepatocyte apoptosis [35] are observed in HIV infection, and have been proposed as factors that accelerate liver disease in HIV/HCV co-infection. [8,35,36] Lifestyle factors such as high body mass index, [9,37,38] hazardous alcohol use, [9,30] and cocaine use [10,11] further increase risk of liver fibrosis. It is important to understand, first, how lifestyle factors affect liver fibrosis in HIV mono- and HIV/HCV co-infection, and then investigate which biological factors pose a greater risk for liver fibrosis within adverse lifestyle conditions.

The relationship between oxidative stress, [39,40] microbial translocation, [41-43] TGF-β1 [22,44,45] and hepatocyte-specific apoptosis [46-48] has been established in cell and animal models, however, relationships between these factors have not been thoroughly investigated in HIV+ patients with lifestyle conditions that put them at risk for liver fibrosis. More recent pharmaceutical developments in direct acting antivirals (DAAs) may potentially provide high cure rates (between 67% to 84% in initial trials) [49] for HIV/HCV co-infected participants, making lifestyle factors and biological mediators of liver fibrosis even more relevant factors to study in people living with HIV. However, access to newly developed DAAs remains limited in PLWH who often experience barriers to health care. [50,51] As such, the study's aim was to characterize adverse lifestyle factors (alcohol use, body mass index, and cocaine use) associated with liver fibrosis in HIV mono- and HIV/HCV co-infection, respectively. Secondly, we sought to identify biological factors that may contribute to liver fibrosis within the adverse lifestyle factors for HIV mono-infection cross-sectionally, and overtime.

Summary

Cellular and animal studies elucidate possible mediators contributing to liver fibrosis, [22,39-43,45-48] however, these may not necessarily explain the pathophysiology of liver fibrosis in humans with adverse lifestyle conditions. The increased morbidity associated with progressive liver fibrosis in HIV mono-infected and HIV/HCV co-infected persons, [52,53] combined with lack of access to the new effective HCV therapies for the study population, [50,51,54] highlight the importance of identifying significant etiological drivers of liver fibrosis (both lifestyle and biological factors) to identify potential therapeutic targets that may prevent or delay liver fibrosis in a population at high risk for rapid advancement to liver disease. The Miami Adult Studies on HIV (MASH) Cohort is unique because it provides a large sample (n=464) followed for two to four years with 25% of the cohort co-infected with HIV/HCV. The aim of this study was to examine the biological mediators (oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1) related to lifestyle factors (obesity, alcohol dependence, cocaine use) that contribute to liver fibrosis in a cohort of HIV-infected persons in Miami, FL.

Specific Aims and Hypotheses

Specific Aim 1: Examine the effects of lifestyle factors (high BMI, alcohol use, and cocaine use) on liver fibrosis in HIV mono- and HIV/HCV co-infection subjects cross-sectionally, and investigate whether high baseline measures of BMI, alcohol intake, and cocaine use affect liver fibrosis progression over time.

Rationale for Specific Aim 1: Hepatitis C infection may not fully account for liver fibrosis development in HIV infection. This study examined lifestyle factors that

contribute to liver fibrosis and identified useful lifestyle changes that can be made by this population to prevent or delay liver fibrosis development.

Hypothesis 1: Adverse Lifestyle factors (High BMI, alcohol use and cocaine use) are associated with higher liver fibrosis measured with FIB-4, in HIV-mono and HIV/HCV co-infection.

Specific Aim 2: Examine the effect of biological factors at baseline and on changes in liver fibrosis over time.

Rationale for Specific Aim 2: Biomarkers at baseline may predict onset of liver fibrosis over time. Although a cross-sectional biomarker may not correlate to fibrosis, it may predict liver fibrosis over time. This study aimed to identify important biomarkers that may lead to early therapeutic targets before the progression of liver fibrosis.

Hypothesis 2: Increased oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1 independently increase the risk of liver fibrosis over time (2 years) in HIV mono-infected individuals.

Specific Aim 3: Explore the potential biological factors associated with individual lifestyle factors that contribute to liver fibrosis at baseline.

Rationale for Specific Aim 3: Lifestyle factors that contribute to liver fibrosis may have different underlying biological mechanisms to promote liver fibrosis. This aim identifies whether there exists different types of biomarkers linked to liver fibrosis in people with high BMI, who drink alcohol hazardously, use cocaine.

Hypothesis 3: Oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1 are increased in participants living with HIV who have high BMI, drink or abuse alcohol, or use cocaine compared to controls.

Table 1: Methods and statistical analyses for each hypothesis.

Hypothesis	Methods	Statistical Analysis
1. High BMI, alcohol use and cocaine use are associated with higher liver fibrosis measured with FIB-4, in HIV-mono and HIV/HCV co-infection.	We set cut-off points to identify participants with lifestyle factors linked to liver fibrosis: High Body Mass Index: ≥ 28 kg/m^2 Alcohol: AUDIT score ≥ 8 indicated hazardous/binge drinking Cocaine use: Self-report and/or positive urine. Dependent variable: FIB-4 index: calculated by patient lab values using [(AgeXAST)/(PlateletsX√(ALT))] FIB-4 is categorized as follows: None/mild fibrosis = FIB-4 <1.45 Moderate fibrosis = 1.45≤ FIB4 ≤3.25 Advanced fibrosis/cirrhosis = FIB-4 >3.25	T-test and one-way ANOVA with post-hoc analysis were used to analyze differences between groups with two and three levels, respectively. Multiple linear regression analyzed each lifestyle factor independently for significance on FIB-4 index as the dependent variable controlling for age, gender, ART use, CD4 cell count, and HIV viral load. Logistic regression analyzed the odds of progressing at least on stage of fibrosis over 24 months based on having a risky lifestyle factor (i.e., high BMI, alcohol use, or cocaine use). 3-way ANOVA was used to investigate associations between lifestyle factors and FIB-4 index. Analysis will include interactions between lifestyle factors. Logistic Regression were used to measure the odds of progressing at least one FIB-4 category over 2 years, for those with high levels vs. low levels of independent variables at baseline.
2. Increased oxidative stress, microbial translocation, hepatocyte apoptosis, and TGFβ1 independently increase the risk of liver fibrosis over time (2 years) in HIV mono-infection.	Baseline cut-off points for independent variables to grouped participants as having low/high biomarkers. We assigned participants to groups who decreased, stayed the same, or increased in FIB-4 category over time (2 years). Variables: Biological mediators Microbial translocation, oxidative stress, TGFβ1, apoptosis. Liver fibrosis FIB-4 is categorized as follows: None/mild fibrosis = FIB-4 <1.45 Moderate fibrosis = 1.45≤ FIB4 ≤3.25 Advanced fibrosis/cirrhosis = FIB-4 >3.25	Proportion test determined the proportion of participants that progressed in each group. Logistic Regression measured the odds of progressing at least one FIB-4 category over 2 years, for those with high levels vs. low levels of independent variables at baseline. GEE models analyzed repeated measured to describe relationships with biological factors, FIB-4 (continuous) with time (three time points) as an interaction.
3. Oxidative stress, microbial translocation, hepatocyte apoptosis, and	We categorized participants into groups of lifestyle factors (see methods in hypothesis 1 for categorization): High BMI group, Alcohol group, Cocaine group, Control group to assess	Student's T-test directly compared the mean of each biological factor within a lifestyle group to the control group.

TGFβ1 are increased HIV-infected participants who have high BMI, drink or abuse alcohol, or use cocaine compared to controls.	biological factors between groups. Variables: Biological mediators Oxidative stress measured by malondialdehyde (MDA) Colorimetric TBARS assay (Northwest Life Science) and % Oxidized Glutathione Colorimetric assay (Arbor Assays). Microbial Translocation measured by LPS Colorimetric Lonza Limulus assay (Lonza). Hepatocyte-specific apoptosis measured by CK-18 protein ELISA assay, M30 Apoptosense kit (Peviva®). TGFβ1 measured by Quantikine ELISA (R&D Systems). Liver fibrosis FIB-4 index calculated by patient lab values using [(AgeXAST)/(PlateletsX√(ALT))].	One-Way ANOVA with post-hoc analysis established differences in biological factors between lifestyle groups and the control group. Three-way ANOVA was employed to assess interactions between lifestyle groups for each biological factor. Multivariate linear regression run with control variables determined which biological factors were associated with liver fibrosis within lifestyle groups. Logistic regression was used to associate odds of having an adverse lifestyle with high biological mediators of fibrosis.

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CHAPTER II: LITERATURE REVIEW

The Centers for Disease Control and Prevention estimates that approximately 1.2 million people are infected with HIV in the United States.[1] Those most seriously affected by HIV in the recent years are African American men who have sex with men (MSM).[1] According to the Florida Department of Health, the State of Florida ranked third in 2013 with the highest number of people living with HIV, and first in the US in incidence of HIV.[2] Miami-Dade specifically bears the highest burden in the State in number of new cases (n=1,411), and is listed among the top ten in the nation for metropolitan divisions with new cases of HIV in 2013.[2]

HIV remains a heavy burden on the health care system. An earlier study found that direct economic costs of HIV in the United States in the era of antiretroviral therapy (ART) is approximately $36.4 billion annually.[3] Co-morbidities account for productivity losses and direct medical costs, and liver fibrosis is a leading co-morbidity in HIV-infection.[4-7] The prevalence of liver fibrosis in HIV infection is apparent by the substantial increase in liver-related morbidity, accounting for 14-18% of all deaths in HIV+ patients.[8,9] While hepatitis C plays a major role in contributing to liver fibrosis, it does not account for all of the liver fibrosis cases. A study conducted in 2010 of 432 HIV mono-infected patients found that 8.3% developed severe liver fibrosis and suggested that future studies explore the mechanisms of fibrosis in HIV mono-infection.[10] Miami-Dade is an excellent location to study the relationship between liver fibrosis and HIV since it has high rates of HIV/HCV co-infection (over 2,000 cases reported in 2014),[11] and county services offer access to care for both HIV mono- and co-infected

individuals. More recently, direct acting antivirals against hepatitis C show high cure rates in HIV/HCV co-infection are being developed.[12,13] Although they are not widely available to the HIV community,[14] their development justifies more research into the pathogenesis of liver disease in HIV mono-infection as well as HIV/HCV co-infection.

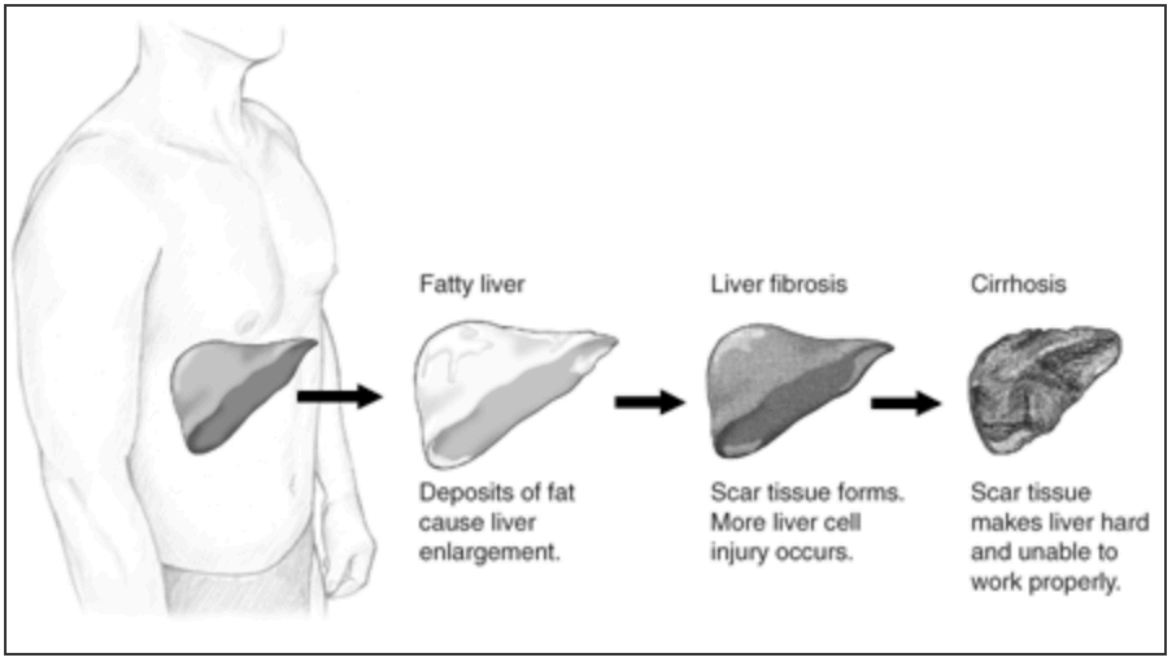
Lifestyle and Risk Factors in Liver Fibrosis

Pathogenesis of liver disease. Liver fibrosis is an important stage of liver disease because it serves as the main determinant of prognosis and of most therapeutic decisions.[15] Liver fibrosis is part of a series of stages of liver disease which include fatty liver (steatosis), inflammation combined with fatty liver (steatohepatitis), liver fibrosis (varying degrees), cirrhosis, and possibly hepatocarcinoma (Figure 1).

There are several liver conditions associated to fibrotic changes: Non Alcoholic Fatty Liver Disease (NAFLD) is associated with early stages of liver pathogenesis (steatosis) that cannot be attributed to heavy alcohol use, and Non Alcoholic Steatohepatitis (NASH) is associated with inflammation of the liver.[16] Alcoholic Liver Disease (ALD) encompasses more than one stage of liver pathogenesis, and its etiology is attributed to excessive alcohol consumption.[16,17]

HIV destroys the immune system, and is associated with chronic immune activation[18,19] and systemic inflammation.[20] The virus itself is able to infect hepatocytes, potentially causing damage to liver cells.[21,22] HIV itself may be a potential risk factor for fatty liver,[23] and HIV-infected persons are prone to accumulating known epidemiological risk factors for NAFLD (and subsequent fibrosis), such as lipodystrophy, increased triglycerides, glucose abnormalities, microbial translocation, and immune dysregulation.[24,25]

Figure 1. Stages of liver damage



Source credit: *National Institute of Diabetes and Digestive and Kidney Diseases*[26]

High Body Mass Index. Obesity is a major cause of liver fibrosis in the general population, commonly leading to non-alcoholic fatty liver disease NAFLD.[27] According to the Centers for Disease Control and Prevention, obesity is characterized by BMI greater or equal to 30 kg/m^2, and is associated with the development of liver disease.[28] However, a lower BMI may also be associated with liver fibrosis. The development of the BARD score uses a BMI cutoff of 28 kg/m^2 to assess liver fibrosis development in the general population.[29] Considering other fibrogenic contributing factors in HIV such as increased inflammation, poor immune function, ART and oxidative processes, the BMI cutoff may even be lower. In fact, a cohort study of 1176 patients found that a BMI range of 25-29 significantly contributed to liver fibrosis in HIV infection.[30] In HIV mono-infected patients on ART, high BMI, waist-to-hip ratio, and glucose intolerance

were strongly associated with liver pathology than HIV-specific parameters, suggesting that obesity may be a significant driver of fibrosis in this population.[31] In the era of ART, different from the pre-ART times, HIV-infection is associated with higher rates of obesity,[32] a condition that could further put patients at risk for liver disease. Examining the range in which BMI contributes to liver fibrosis in HIV may serve as an indicator for establishing a cutoff for a population at risk of liver fibrosis.

The mechanisms by which obesity causes liver fibrosis are mainly due to altered fatty acid metabolism and the development of hepatic steatosis.[33,34] The liver is involved in manufacturing, storing, and exporting lipids.[35] Chronically increased levels of circulating free fatty acids (FFAs) in obese patients accounts for a build up of lipids in liver, leading to hepatic steatosis in NAFLD.[36] Lipid accumulation in the liver is followed by proinflammatory mediators to induce inflammation, hepatocellular injury, leading to fibrosis.[37]

Oxidative stress is associated with increased hepatic fatty acid storage and obesity. A study in 31 NAFLD patients with increased BMI showed decreased antioxidant capacity and increased oxidative stress.[38] A similar study showed higher levels of lipid peroxidation and lower levels of reduced glutathione in patients with NAFLD compared to controls, regardless of obesity, type II diabetes, and hyperlipidemia.[39] Mouse models show a link between obesity, oxidative stress and liver fibrosis. In obese mice fed a high fructose diet, increased oxidative stress markers were associated with lipogenesis and hepatic fibrosis, whereas antioxidant supplementation attenuated fibrotic markers.[40] In Ldlr knockout mice (a model of NAFLD) fed a Western diet, mRNA expression of oxidative stress genes Nrf2, Hmox1, and NADPH oxidase

subunits and hepatic fibrosis were increased compared to controls.[41] TGF-β1 was also induced in these mice compared to controls.[41]

TGF-β1 is a pro-fibrogenic cytokine involved in inflammatory signaling associated with fibrosis.[42-44] It is suggested that in both HIV and obesity, TGF-β1 levels are elevated,[45-47] making it an important cytokine to investigate in the context of liver fibrosis. Microarray gene expression in 38 obese patients with and without fibrosis showed that those with fibrosis displayed significantly higher expression of fibrotic cytokines, including TGF-β1 expression, compared to controls.[48] While studies in humans are limited, circulating TGF-β1 in plasma has not consistently shown to reflect the hepatic microenvironment; one study in obese patients shows that plasma TGF-β1 does not correlate to degree of fibrosis using the Brunt's system of fibrosis classification.[49] On the other hand, in an HCV infected cohort, BMI was significantly correlated to TGF-β1 levels.[50] However, in HIV, TGF-β1 has mixed uses as a biomarker of fibrosis. In a small study (n=66), higher levels of TGF-β1 were associated with disease progression in HIV-infected patients,[51] and impaired immune function could lead to liver dysfunction.[52] Sanvisens et al.[53] showed that serum TGF-β1 was not predictive of fibrosis measured by liver biopsy in HIV/HCV co-infected patients, and Rallon et al.[54] found that elevated TGF-β1 levels were, in fact, associated with lower liver fibrosis.[54] Both studies were conducted cross-sectionally in HIV/HCV co-infected cohorts with relatively small samples sizes (n=69, n=88 respectively). To date, there are no human studies that investigate TGF-β1 in HIV mono-infection to predict liver fibrosis and the role that obesity plays in its development.

Recently, new insights into the gut microbiome links obesity to increased microbial dysfunction leading to microbial translocation. Microbial translocation is the crossing over of endotoxin and bacterial antigens into the blood stream that elicits an immune response.[54] The liver serves as a first pass of venous blood from the intestine, and is highly exposed to microbial translocation products,[55] which can induce liver fibrosis by binding Toll-like receptors (TLR-4) on hepatic stellate cells and Kupffer cells in the liver, inducing a pro-fibrogenic response. A popular marker of microbial translocation is lipopolysaccharide (LPS) and its surrogate markers LPS binding protein (LBP) and soluble CD14 (sCD14), which are used as markers of microbial translocation in plasma. A study in morbidly obese patients (n=40) found that obesity alone can induce high levels of LBP, and it was correlated with markers of liver inflammation.[56] A similar study found elevated levels of LPS and gut permeability in 16 obese patients compared to12 healthy controls, and these levels were significantly associated with liver disease.[57] These studies offer evidence that microbial translocation markers are elevated in those with high BMI. HIV and obesity both contribute to downregulation of tight junctions in gut mucosa, which leads to an increase in gut permeability.[58] HIV also specifically depletes CD4+ T cells in the gastrointestinal tract, rendering the gut associated lymphoid tissue more permeable to microbial products.[59] However, few studies have investigated the dual role of HIV and obesity on liver fibrosis in relation to microbial translocation in humans.

It is hypothesized that as a result of increased oxidative stress,[60] TGF-β1,[61] and microbial translocation,[62] apoptotic cascades are eventually triggered, and apoptotic fragments further increase hepatic fibrosis.[63] The association between hepatic apoptosis

and liver fibrosis has been linked to obesity.[64] In a group of 62 patients categorized as having BMI in obese range, a serum marker of hepatic fibrosis and hepatic apoptosis, cytokeratin-18 fragment level (CK-18), a protein secreted by liver cells undergoing cell death and a validated surrogate marker of hepatocyte apoptosis, was correlated with increased degree of liver fibrosis.[65] A cross-sectional study conducted in 229 obese youth looked at liver biopsies separated by ethnicity. Results showed that CK-18 levels are significantly associated with steatosis in obese Caucasian and Hispanic youth, but not in African American youth, suggesting that apoptosis is associated with liver damage and may be affected by race/ethnicity.[66] In HIV, the gp120 viral protein independently induced apoptosis in hepatocytes.[67] HIV infection increased hepatocyte apoptosis in an HCV model.[68]

Alcohol Use. It is well established that excessive alcohol consumption causes liver disease, commonly known as alcoholic liver disease (ALD).[17] Alcohol is the second most common cause of liver disease after hepatits C, accounting for 20-25% of the cases in the US.[69] ALD is characterized by a spectrum of pathological conditions, including steatosis, steatohepatisis, which can progress to hepatic fibrosis that may lead to cirrhosis, and hepatocellular carcinoma.[70] Liver fibrosis is often the main determinant of prognosis and of most therapeutic decisions.[15] A commonly used tool to classify alcohol use is the Alcohol Use Disorders Identification Test (AUDIT), developed by the World Health Organization to screen for excessive alcohol consumption and to help health-care professionals identify individuals who would benefit from abstinence or reduction.[71,72] AUDIT scores range from 0 to 40, and can classify alcohol consumption from abstinence/no drinking (score of 0), mild/moderate drinking (score < 8), hazardous/binge

drinking (score ≥ 8), to alcohol dependence (score ≥ 13 in women and ≥ 15 in men).[72-74] Higher AUDIT scores have been associated with liver pathogenesis. For example, an early study to validate the AUDIT tool found that a cutoff ≥ 8 could predict liver disease and gastrointestinal bleed in an ambulatory setting (n=350) with a sensitivity of 63% and a specificity of 74%.[75] AUDIT is often used to study the effects of alcohol in HIV infection because of the extra burden placed on the liver by the disease, increasing fibrotic events and rapid progression of ALD pathogenesis, including fibrosis.[76,77] A large-scale study of the Veterans Aging Cohort (701 HIV/HCV co-infected, 1410 HIV mono-infected, 296 HCV infected, and 1158 uninfected adults) associated higher odds of having liver fibrosis with higher AUDIT category in HIV mono-infection, HIV/HCV co-infection, and HCV infection compared to non-infected controls.[77] Even low levels of alcohol consumption were associated with advanced hepatic fibrosis in these groups suggesting that small quantities of alcohol may lead to liver fibrosis in HIV and HIV/HCV infection compared to uninfected controls.[77]

Mechanisms of liver pathogenesis are described in cell and animal models. Decreased glutathione antioxidant capacity is also a consequence of excessive alcohol consumption, leading to greater reactive oxygen species (ROS) in murine models.[78-81] Excessive alcohol consumption that creates ROS triggers elevated lipid peroxidation products,[82] such as malondialdehyde (MDA), and these products lead to activation of hepatic stellate cells (HSCs) and the TGF-beta system that initiates fibrosis.[83-85] Alcohol use influences cross-talk among intracellular events in the liver involving TGF-β1 signaling in the perpetuation phase of fibrosis.[86] More specifically, when cultured HSCs are treated with acetaldehyde, a by-product of alcohol metabolism, it stimulates

activation of TGF-β1 and induces its expression, upregulating collagen expression that leads to fibrosis.[87] HSCs are the key regulators of liver fibrosis because they produce the intracellular matrix required for fibrotic tissue.[44] Cell and animal models of alcoholic liver disease (ALD) also show endotoxin (LPS) plays a significant role in liver fibrosis. Treating Caco2 cells (a model for colorectal epithelial cells) with acetaldehyde opens tight junctions, suggesting a mechanism for increased gut permeability for microbial endotoxins.[88] Rat HSCs treated with combined LPS, ethanol and its metabolite acetaldehyde showed high levels of oxidative stress and collagen deposition, compared to single ethanol, acetaldehyde, or LPS exposure.[89] Another study conducted *in vivo* suggested that LPS potentiates the fibrotic effects of alcohol through increasing oxidative stress levels, activating HSCs, and increasing collagen production.[90] Apoptosis is also investigated as a mechanism of fibrosis in mouse models of ALD.[91] Alcohol infusion in the mouse for 4 weeks lead to a marked increase in the number of apoptotic nuclei in the liver compared to controls.[92]

Human studies investigating the relationship between oxidative stress, TGF-β1, LPS and apoptosis in liver fibrosis show similar trends. Grasselli et al.[93] showed that alcohol dependence is associated with higher BMI, increased levels of oxidative stress measured by the TBARS assay, and more severe steatosis, which could lead to liver fibrosis, in 60 alcoholic subjects compared to 58 controls.[93] A study matching HIV- and HIV+ alcohol users (n=10) compared oxidative stress levels measured by oxidized/reduced glutathione.[94] HIV+ alcohol users had higher oxidative stress levels than HIV- alcohol users, suggesting that the combined effect of HIV and alcohol may lead to failure of antioxidant systems to maintain crucial redox homeostasis.[94] These

investigators also reported that oxidative stress was associated with the CYP2E1 system, which is involved in alcohol metabolism that generates alcohol-induced oxidative stress in the liver.[95] Higher levels of oxidative stress in people living with HIV who drink excessive alcohol suggest that oxidative stress may be one of the mechanism to advance fibrosis in this population.

Circulating TGF-β1 is not always a reliable correlate to liver fibrosis in humans; however, a study showed that 41 patients with alcohol dependence had significantly higher levels of circulating TGF-β1 than healthy controls.[96] Notably, within the alcohol dependent group, those with or without liver pathology showed no difference in TGF-β1 levels, suggesting that TGF-β1 may be higher in alcohol users with or without the presence of liver damage.[96] Alcohol disrupts gut permeability and LPS is associated with alcohol intake in human studies. A small study of healthy volunteers (n=25) showed that acute binge drinking significantly increased circulating LPS within hours of intake, and triggered an inflammatory cascade via the TLR-4 system.[97] A larger study in patients with liver cirrhosis found that microbial endotoxin levels were significantly higher in patients with alcoholic cirrhosis compared to patients with non-alcoholic cirrhosis.[98] The results indicate that alcohol abuse contributes to endotoxemia and may exacerbate alcohol-induced liver disease.[98] HIV adds a level of complexity to this phenomenon, as it also destroys the protective barrier in the gut by specifically depleting Th17 CD4+ T-cells and tight junctions.[58,99]A cross-sectional study examined whether unhealthy drinking influenced microbial translocation in people living with HIV before initiating ART. The study revealed significantly higher sCD14, a surrogate marker of immune activation by endotoxin, in HIV+ patients with unhealthy alcohol use compared to those

who did not drink alcohol, indicating that the combined effect of HIV and heavy alcohol use increases microbial translocation in patients.[100]

In HIV/HCV co-infected women (n=44), sCD14 was higher in those who progressed from no fibrosis to cirrhosis or liver-related death, compared to non-progressors.[101] This finding remained significant after controlling for alcohol use.[101] The relationship between circulating LPS, alcohol intake, liver fibrosis in HIV mono-infection and HIV/HCV co-infection warrants further investigation in human populations. Another variable that warrants further investigation in HIV+ alcohol user is liver-related apoptosis. As previously discussed, mouse models show that alcohol increases apoptotic bodies in the liver.[92] In humans, circulating CK-18, a marker for hepatic apoptosis, is elevated in heavy alcohol drinkers with fibrosis compared to those without fibrosis.[102] A study compared hepatic apoptosis in 134 HIV/HCV co-infected patients and 130 HCV mono-infected patients using liver biopsies.[103] The results indicated that HIV/HCV co-infection had the highest odds of increasing hepatic apoptosis compared to HCV alone, and low CD4 cell counts contributed to apoptosis.[103] Heavy alcohol consumption was slightly associated with apoptosis in the entire cohort ($p=0.06$); however, within the HIV/HCV group, significance was lost, which the authors attributed to unreliable interview methods.[103]

Alcohol use warrants investigation in the MASH cohort because it is prevalent among people living with HIV.[104,105] Alcohol is associated with a multitude of co-morbidities, and is a risk factor for lack of viral control and non-adherence to ART,[106] accelerated liver disease in HCV infection,[57] and HIV liver-related mortality.[107]

Cocaine Use. According to the National Institute on Drug Abuse, in 2013 there were 1.5 million cocaine and/or crack-cocaine users in the US.[108] In the same year, Miami-Dade had the highest number of cocaine-related deaths (n=238) among the State's metropolitan areas.[109] Collecting drug use data can be accomplished through self-report or toxicology screening. Self-reporting is effective during in-person interviews in a private and confidential setting, and that data are more robust when it is confirmed by toxicology.[110] Cocaine is known to have deleterious effects in HIV infection. In cell models, cocaine can facilitate HIV-1 progression by impairing macrophage and CD4 T-cell function, modulating the levels of cytokines and increasing the level of viral replication.[111] Mice that expressed human leukocytes displayed higher HIV replication when exposed to cocaine compared to HIV-infected mice not exposed to cocaine, suggesting that there is an interaction between HIV, cocaine, and immunity *in vivo*.[112] Early studies with cocaine showed that it significantly depresses the immune system of mice, specifically thymocytes and leukocytes in a dose-dependent manner.[113] While few studies investigating the effects of cocaine have been conducted in HIV populations, its use is associated with poor outcomes. Parikh et al.[114] used multivariate modeling to show that cocaine use alters cytokine profiles, leading to an imbalance in Th1/Th2 T-cells in African Americans and may decrease CD4 cell counts and increase viral load over time.[114] Analysis of a large HIV+ cohort for all-cause mortality indicates that cocaine users are at a higher risk for ART non-adherence (2.60 times higher odds, $p<0.001$), and that hard drug use (cocaine, heroine, amphetamine) had significant risk (2.58, $p=0.03$) of an AIDS outcome or death.[115] In this multi-center HIV+ cohort, cocaine was the most prevalent drug used in those who used hard drugs.

In the MASH cohort, Baum et al.[116] reported that crack-cocaine accelerates HIV disease progression in HIV+ drug users. The cohort of 222 drug users showed that only crack-cocaine use was associated with time-to-event (CD4 decline to 200 cells/mL) with a relative risk of 2.145, p=0.029.[116] The current MASH cohort preliminary data describes that cocaine use increases mitochondrial DNA oxidative damage in HIV, which is positively correlated to liver fibrosis.[117] Oxidative stress may trigger hepatic fibrosis by activating hepatic stellate cells and increasing TGF-beta systems.[83,84] In a small cross sectional study, HIV+ cocaine users have dysfunctional microbial profile compared to non-users, which may induce microbial translocation. The authors attribute microbial dysfunction to food insecurity among their HIV+ drug cocaine users.[118] Exposure to the microbial endotoxin (LPS) augments cocaine's hepatotoxicity. In mice pre-exposed to cocaine, those exposed to LPS showed higher levels of hepatic nitric oxide and ROS compare to controls, leading to liver damage.[119] Only when nitric oxide was neutralized were the effects reversed, suggesting that cocaine sensitizes the liver to the hepatotoxic effects of LPS.[119] Cocaine is processed in the liver to norcocaine by the cytochrome CYP2E1 system that generates oxidative stress and lipid peroxidation in hepatocytes.[120,121] The oxidative stress generated from CYP2E1 has been linked to apoptosis in hepatocytes in cell models, which could lead to fibrosis.[122,123] Preliminary data of the MASH cohort presented at the International AIDS conference showed that HIV+ frequent cocaine users (at least 3-4 times/week) were 5.5 times more likely to have higher hepatocyte apoptosis (CK-18), compared to those who used it less frequently.[124]

Antiretroviral Treatment (ART). A confounding factor for fibrogenesis in people living with HIV is the chronic use of ART and its related hepatotoxicity. A number of studies have highlighted the hepatotoxic effect of ART in this population;[125-129] however, ART use in advanced HIV disease has been associated with lower levels of hepatic fibrosis.[130] Another part of this problem is that a large proportion of patients in the United States do not adhere to ART,[131]which needs to be considered in future studies.

Biological Risk Factors in Liver Fibrosis

Oxidative Stress. A family of established mediators in liver fibrosis includes reactive oxygen species (ROS) leading to increased oxidative stress. Oxidative stress occurs when ROS generated from exogenous sources (i.e., alcohol) and endogenous sources (i.e., liver enzyme P-450 system and mitochondrial oxidative phosphorylation) are not balanced by endogenous and exogenous antioxidants.[132] High levels of oxidative stress contribute to the development of liver fibrosis by disrupting lipids, proteins, and DNA, inducing necrosis and apoptosis, amplifying the inflammatory response, and stimulating HSCs to initiate fibrosis.[133,134] Oxidative stress may also contribute to the perpetuation phase of liver fibrosis through the enhancement of TGF-β1 as observed in an HCV cell culture model,[135] a relationship that has not been investigated in humans.

Markers of oxidative stress are present in fibrotic areas of the liver, linking ROS directly to liver damage.[136] Specifically, in biopsies from 39 patients with liver disease showed malondialdehyde (MDA), a marker of lipid peroxidation associated with ROS, is significantly associated with areas of hepatic steatosis ($p<0.04$) and fibrosis ($p<0.05$).[137] Notably, circulating markers of oxidative stress suggested to be indicative of the hepatic microenvironment because they are highly correlated with liver fibrosis. For example,

titers of antibody against circulating albumin with MDA adducts in 167 NAFLD patients showed that having high levels of MDA independently predicted advanced liver fibrosis/cirrhosis compared to those within the control range.[138] These findings, along with those of other investigators,[139,140] suggest that circulating markers of oxidative stress correlate with liver fibrosis.

Prior studies report that HIV infection is associated with higher levels of oxidative stress, and HIV/HCV co-infection increases oxidative stress levels even more.[141] Common measures of oxidative stress include lipid peroxidation (MDA levels),[142] redox status (i.e., oxidized:reduced glutathione ratio),[142] and protein modifications.[143] Circulating levels of MDA and glutathione are relatively inexpensive commercial kits that have been validated and are readily available for measurement in human plasma. In experimental models, circulating MDA correlates to liver MDA levels in the presence of liver fibrosis. Intra-abdominal sepsis rat model showed that circulating MDA levels correspond to tissue MDA levels in liver.[144] Another study showed that liver MDA in hamsters who were fed a high cholesterol fibrogenic diet correlated significantly with levels of circulating MDA (raw data in Chen YY, 2015).[145] Circulating glutathione is another biomarker that correlates well with liver glutathione levels. In patients with chronic hepatitis C, the levels of hepatic glutathione, plasma glutathione, and PBMC glutathione, were highly correlated and significantly depleted in HIV/HCV co-infected patients.[146] In summary, MDA and glutathione are good measures of oxidative stress in the MASH cohort because they reliably and significantly correlate with liver fibrosis, and represent the microenvironment of the liver.

Microbial Translocation. HIV damages the gastrointestinal mucosal barrier[99] and facilitates microbial translocation[147] into the bloodstream from the gut. Microbial translocation increases sensitivity of HSCs to fibrotic mediators, such as oxidative stress and the cytokine TGF-β1.[148-150] In cell models, the bacterial endotoxin lipopolysaccharide (LPS) induces oxidative stress that leads to liver injury.[151] In mice, the presence of LPS enhances production of TGF-β1 from Kupffer cells and primes HSCs to secrete TGF-β1, leading to increased fibrogenesis.[149,152] People living with HIV have high levels of LPS due to the depletion of gut-associated lymphoid tissue.[153] Few human studies have investigated the impact of LPS on liver fibrosis in HIV infection, although a compelling study conducted by Balagopal et al.[148] showed that HIV/HCV co-infected subjects with elevated plasma LPS had a 2.7-fold higher risk of liver disease, and in the overall cohort (n=28 HIV/HCV co-infected, n=88 HCV mono- and n=60 uninfected controls) elevated LPS was associated with 18.7-fold higher risk of cirrhosis when adjusting for confounding factors.[148] Although microbial translocation can be measured by multiple methods, such as 16S rDNA, sCD14, and lipoprotein binding protein (LBP), LPS is considered the cardinal and most direct measure of endotoxin.[154] Recently, measurement of LPS in plasma has been debated because of an inhibitory effect that has yet to be identified. However, the inhibitory effect can be overcome by processing the plasma with heat-inactivation[155] and by diluting plasma to mitigate inhibitory effects.[154] LPS is a direct stimulus for liver fibrosis, by binding to the TLR-4 receptor on hepatic macrophages (known as Kupffer cells) that activate HSCs to produce a matrix rich in collagen leading to fibrosis.[62] It is the most direct measure of endotoxin and an acute stimulus of liver fibrosis.

TGF-β1. The fibrogenic cytokine TGF-β1 is one of the key regulators of liver fibrosis.[156,157] During times of tissue injury, elevated levels of TGF-β1 are produced in the liver by hepatic stellate cells (HSCs), Kupffer cells and platelets. The presence of TGF-β1 activates HSCs to proliferate and migrate to the ECM where they synthesize and secrete elevated levels of matrix proteins, promoting fibrosis.[44,158] Thus, circulating TGF-β1 has been suggested as a biomarker and target in the treatment of liver fibrosis because it is elevated in HIV and HCV infection,[159] and because it is a potent inducer of fibrogenesis.[42,86] The TGF-β1 producing polymorphism is associated with increased fibrosis, supporting the hypothesis that increasing TGF-β1 will increase fibrosis in the liver microenvironment.[160]

Serum levels of TGF-β1 do not always reflect liver the hepatic microenvironment,[49] because a number of studies show an inconsistent relationship between circulating TGF-β1 and degree of liver fibrosis. Nonetheless, it is often cited as a biomarker of liver fibrosis.[161,162] TGF-β1 levels are often high in early stages of liver fibrosis, in patients who are at risk of developing liver disease, such as HIV,[51] and HIV/HCV[54] so it may be an important predictor of hepatic fibrosis.

Apoptosis. Elevated levels of cytokines that induce liver fibrosis are associated with hepatocyte apoptosis, or programmed cell death. Apoptotic fragments released from hepatocytes are fibrogenic in cultured HSCs,[63] and hepatocyte apoptosis in experimental animals has also been shown to be fibrogenic, and induces HSCs to secrete TGF-β1.[163] In cell models, TGF-β1 induces hepatocyte cell death,[61] generating pro-fibrogenic apoptotic fragments that may further increase TGF-β1, leading to a cycle of increasing fibrosis. The association between hepatic apoptosis and liver fibrosis has been linked to obesity[64] and

heavy alcohol drinking,[102] in people without HIV, and exposure to cocaine in mice.[164] A validated biomarker of hepatocyte-specific apoptosis is the protein cytokeratin-18 (CK-18).[64,165] During the apoptotic process, intracellular caspases cleave a number of substrates, including CK-18, a major intracellular filament in hepatocytes, resulting in the characteristic and morphologic changes of apoptosis.[166] The specificity of the filament coming from hepatocytes makes it a reliable biomarker of hepatocyte-specific apoptosis. Several studies have associated CK-18 with fibrosis. A multicenter validation study in patients with NAFLD showed that the odds of having liver fibrosis based on biopsy was significantly associated with plasma CK-18 levels.[64] Circulating CK-18 was also an independent predictor of NASH after adjusting for variables for variables associated with NASH.[64] Plasma CK-18 can also predict liver fibrosis in heavy alcohol drinkers, and is especially sensitive at predicting severe fibrosis in this population.[102] In HIV/HCV populations, CK-18 predicts liver fibrosis, especially when CD4 cells count is low, most likely due to poor adherence to ART or pre-ART administration.[130,167]

Literature Review Summary

HIV is a disease that destroys the immune system and is associated with disease states that contribute to liver dysfunction such as chronic immune activation,[36,37] systemic inflammation,[38] and direct hepatocyte infection by HIV.[39,40]

The literature identifies obesity,[44,31] alcohol drinking,[81,83,90] and cocaine use[130-134] as significant factors contributing to liver fibrosis. The interplay between lifestyle and biological factors on liver fibrosis have not thoroughly been studied in human patients with HIV mono- and HIV/HCV co-infection. While hepatitis C infection significantly increases the risk of advancing the degree of liver fibrosis, hepatitis C virus itself cannot

account for all of the liver damage,[168,169-170] nor can it account for liver disease progression in HIV mono-infection.[33]

Theoretical Model: Liver Fibrosis Development in HIV Involving Lifestyle Factors

Based on the literature review, we developed a conceptual model that explains the development of liver fibrosis through lifestyle factors, and the associated biological factors. The model was adapted from graphical interpretations in the scientific literature. The basis of this figure was developed from Meyer et al.[171] because it contained many of the core variables of interest. However, we modified and added microbial endotoxin, Kupffer cells, and the specific biomarkers we are measuring: oxidized GSH, MDA, TGF-β1, LPS, and CK-18. We also added the lifestyle factors of interest to the model.

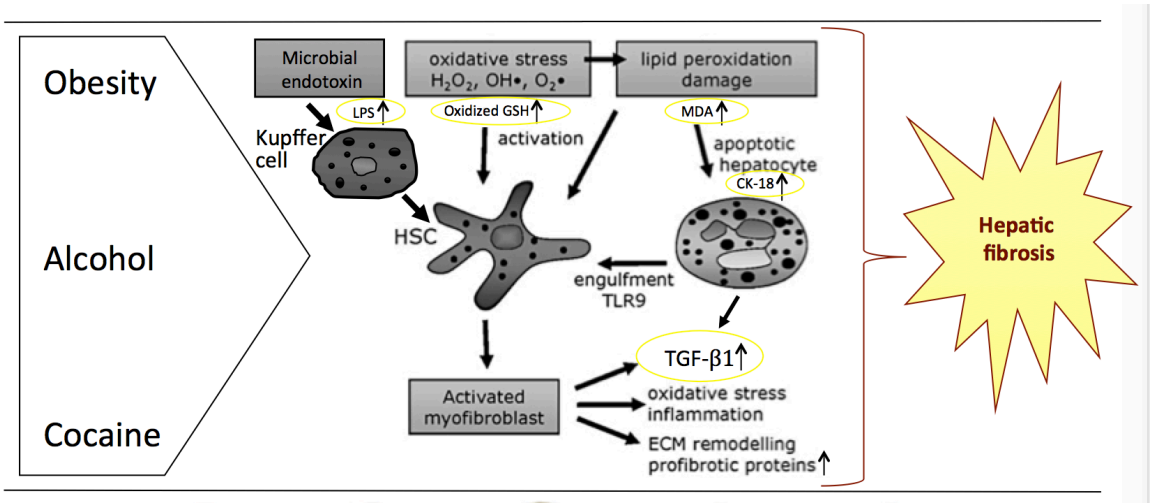


Figure 2: Theoretical model of liver fibrosis development in HIV infection

Image adapted from: *TGF-beta signaling in alcohol induced hepatic injury*[171]

There is evidence that these lifestyle conditions advance liver fibrosis through biological factors that include oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1 to perpetuate fibrosis.[33,172,173] This study identified the effect of

lifestyle factors on the development of liver fibrosis in HIV mono- and HIV/HCV co-infection in the MASH cohort. It also characterized important biomarkers of liver fibrosis linked to lifestyle factors and associated biomarkers with fibrosis in an HIV infected population.

Table 1: Literature Review: Lifestyle Factors Associated with Increased Liver Fibrosis.

Author/Study design	Study Purpose	Population/Methods	Findings
HIGH BODY MASS INDEX			
Harrison SA et al., 2008[29] *Retrospective study*	Develop and validate a simple NAFLD clinical scoring system for identifying patients without advanced disease.	827 patients with biopsy evidenced to have NAFLD (no infection or heavy alcohol use) were categorized based on NASH diagnosis, and then fibrosis stage. Demographic and metabolic factors were analyzed as determinants for liver fibrosis.	When comparing people with no fibrosis or mild fibrosis, to advanced fibrosis, being older (>50 years), having a higher aminotransferase ration (≥0.8), having diabetes and BMI ≥28 kg/m^2 were individually associated with an odds ratio of ≥2.4 for advanced fibrosis.
Kirk GD et al., 2013[30] *Prospective, observational*	Investigate whether persons with HIV develop hepatitis-related liver disease at younger ages than similar persons without HIV.	1176 current and former injection drug users with antibodies to HCV enrolled longitudinally. Fibrosis was measured with transient elastography and compared to demographic, hematological, and metabolic factors.	Liver fibrosis was associated with older age and was greater in persons with HIV than those without HIV. Having a BMI greater than 25 kg/m^2 is a significant determinant of liver fibrosis.

Morse CG et al., 2015[31] *Cross-sectional, observational*	Examine the prevalence and clinical correlates of antiretroviral-associated liver disease, including steatosis, steatohepatitis, and fibrosis.	Sixty-two HIV-infected participants with no previous hepatitis infection who displayed elevated liver enzymes for ≥6 months while receiving ART underwent metabolic laboratory testing.	NAFLD was found in 73% of liver biopsies in HIV mono-infected participants on ART with elevated liver enzymes. Higher BMI (29.1 kg/m^2), waist-to-hip ratio (1.01), and risk factors for diabetes (glucose fasting, OGTT, hemoglobin A1C).
Videla LA et al., 2004[38] *Case control,* *Cross-sectional study*	Investigate the molecular mechanism underlying liver oxidative stress in NAFLD in patients.	Thirty-one patients ranging from 18-55 years old who underwent gastric bypass surgery enrolled as case subjects and twelve patients with healthy BMI who had anti-reflux surgery enrolled as controls. Liver biopsies were obtained at the time of surgery. Both liver and plasma samples generated oxidative stress samples.	Patients with NAFLD and high BMI exhibited substantial pro-oxidant condition in the liver compared to normal weight controls. This finding is also significant in the plasma.
Cayon A et al., 2008[48] *Cross-sectional study*	Determine the specific intrahepatic gene expression profiles associated with histological severe NASH.	Obese patients with NASH (n=38) with liver biopsies and categorized as NASH with fibrosis, without fibrosis, and with obese patients without NASH (control group).	Patients with NASH, but without fibrosis, showed increased expression of pro-apoptotic and inflammatory genes. However, patients with NASH and fibrosis also showed an elevated expression of the TGF-beta1 gene.

Farhadi A et al., 2008[57] *Case control,* *Cross-sectional study*	Study gut leakiness in morbidly obese NASH patients versus morbidly obese patients without NASH and determine its association with liver disease.	Gastrointestinal permeability was measured using lactulose/mannitol (L/M) ratio in urine samples (n=16 NASH patients, n=12 "healthy" patients), and was repeated after challenge with aspirin to measure whether gut leakiness is affected by a slight challenge to GI tract. Circulating LPS was also measured in a subgroup of patients. Liver biopsy diagnosed histological damage using the Brunt system.	Serum endotoxin levels (LPS) were significantly higher in patients with NASH. L/M ratio was significantly higher in patients with NASH after the aspirin challenge. Results suggest endotoxin serves as a second hit after altered lipid metabolism that leads to liver damage.
Civera M et al., 2010[65] *Cross-sectional study*	Investigate the relationship between insulin resistance and caspase-generated CK-18 fragments in patients with severe obesity.	Sixty-two patients selected for bariatric surgery were categorized based on liver histology (degree of fibrosis) and 70th percentile of insulin resistance (based on HOMA).	Patients with greater HOMA had significantly higher CK-18 fragments. There was a significant correlation between CK-18 fragments and degree of liver fibrosis.
Santoro N et al., 2013[66] *Cross-sectional study*	Explore association between hepatosteatosis and circulating CK-18 fragments, and assess the impact of ethnicity, insulin resistance and SNPs associated with steatosis.	Two-hundred and twenty-nine obese youths with various ethnicities underwent MRI, oral glucose tolerance test, and CK-18 level measurements.	CK-18 levels are significantly associated with hepatic steatosis in obese Caucasian and Hispanic youth, but not in African American youth, suggesting that apoptosis associated with liver damage may be affected by ethnicity.
ALCOHOL USE			
Conigrave	Examine the performance	A cohort of 330 subjects was followed for 2-	Cutoff point of 7 and lower

KM et al., 1995[75] *Prospective, observational*	of the AUDIT questionnaire as a predictor of harm, over the full range of its scores.	3 years to study the prevalence of alcohol consumption. The baseline interview consisted of medical questionnaires and an AUDIT score. The follow-up interviews examined alcohol-related harm with endpoints including social problems, alcohol-related hospitalizations, liver disease, gastrointestinal bleeding, and established cutoff points to maximize discrimination.	predicted most alcohol-related disorders with maximum discrimination. For more serious and specific alcohol-related problems, cutoffs of 15 and 22 provided higher specificity, although lower sensitivity.
Lim JK et al. 2014[77] *Cross-sectional study*	Evaluate associations between alcohol use AUDIT categories and advanced hepatic fibrosis by HIV and chronic HCV status.	The Veterans Aging Cohort Study enrolled participants who reported alcohol consumption (n=3565; 701 HIV/HCV co-infected, 1410 HIV mono-infected, 296 HCV mono-infected, 1158 uninfected). FIB-4 index >3.25 defined advanced fibrosis. AUDIT cutoffs determined nonhazardous drinking or hazardous drinking.	Within HIV/HCV co-infected group, advanced hepatic fibrosis increased as alcohol used category increased. Both HIV mono- and HCV mono-infection showed associations between advanced liver fibrosis and higher AUDIT categories compared to healthy individuals.
Grasselli EE et al. 2014[93] *Case control,* *Cross-sectional study*	Investigate the correlation between oxidative stress and antioxidant proteins in serum related to liver steatosis grade.	A total of 118 cases (60 alcoholic subjects and 58 controls) were assessed for BMI, lever steatosis (using ultrasound), and blood chemistry. Oxidative stress was determined by TBARS assay and MDA analysis. Antioxidant status measured of superoxide dismutase activity. Alcohol consumption was assessed by trained interview.	Alcoholic subjects had a higher BMI and moderate/severe hepatic steatosis. Alcoholic subjects had higher TBARS and SOD activity, indicating increased production of oxidative stress in alcoholic individuals.
Ande A et al. 2015[94]	Examine the effects of mild to moderate alcohol use on	HIV+ alcohol users (n=4) who reported mild/moderate alcohol use, and HIV-	Compared to HIV- alcohol users, HIV+ alcohol users had

Case control, *Cross-sectional study*	oxidative stress and the CYP2E1 pathway in HIV+ ART-naïve patients.	mild/moderate alcohol users (n=6) had oxidative stress (DNA 8-oxo-dG, % oxidized glutathione),serum alcohol levels, mRNA of antioxidant enzymes, and CYP2E1 mRNA levels measured.	increased oxidative DNA damage, and increased % oxidized glutathione. HIV+ individuals had lower levels of plasma alcohol levels, and higher levels of CYP2E1, suggesting that there is significantly enhanced antioxidant requirements for HIV+ alcohol users to maintain homeostasis.
Kim YK et al. 2009[96] *Case control,* *Cross-sectional study*	Verify the relationship between alcohol dependence and TGF-beta1.	Alcohol use was assessed in 41 healthy and 41 alcohol dependent males using DSM-IV criteria. Demographics and anthropometrics were collected, and circulating TGF-beta1 levels were analyzed using ELISA. Liver fibrosis was measured by ultrasonography after consultation with a physician.	Patients with alcohol dependency showed significantly higher circulating TGF-beta1 than healthy controls, however, within the alcohol dependent group, those with or without liver fibrosis did not show a difference in TGF-beta1 levels, suggesting that TGF-beta1 may be higher in alcohol users with or without the presence of liver damage.
Bode C et al. 1987[98] *Case control,* *Cross-sectional*	Evaluate the presence of endotoxemia in peripheral blood in alcoholic cirrhotic patients compared to non-alcoholic cirrhotic patients.	Patients with liver cirrhosis (88 with alcoholic cirrhosis "AC", and 42 with non-alcoholic cirrhosis "NAC" had endotoxemia evaluated by LPS. History of alcohol use was obtained and patients were categorized into subgroups based on acute and chronic	Higher levels of LPS were found more frequently in patients with AC compared to NAC. Acute alcohol consumption in patients without chronic liver disease lead to an

study		liver disease.	increase of LPS, however when measured 5-8 days after the consumption, no LPS could be measured. This suggests that heavy alcohol use leads to transient endotoxemia, which may aggravate liver disease.
Lavallard VJ et al. 2011[102] *Cross-sectional study*	Quantify and correlate the circulating levels of CK-18 to liver disease severity in heavy alcoholics.	CK-18 was evaluated in the serum of 143 heavy alcoholics. All patients were HIV and hepatitis B and C negative. Liver disease was evaluated by biopsy.	Circulating CK-18 is elevated in heavy alcohol drinkers who have liver fibrosis, compared to heavy alcohol drinker without fibrosis. AUROC score of CK-18 predict liver fibrosis is 0.84, suggesting that it is a useful biomarker.
COCAINE USE			
Cohn SE et al. 2011[115] *Prospective, longitudinal cohort study*	Describe the impact of self-reported alcohol and drug use in HIV+ patients on ART enrolled in a longitudinal study.	Subjects were assessed at baseline, week 4, week 8, then at 8-32 week intervals until the end of the study at week 512. Data was collected for medical history, drug and alcohol use, cardiovascular events, DC4 cell counts, HIV viral loads, and ART medications. Drug use was assessed by frequency and heavy use, and by drug type.	HIV+ cocaine users are at a higher risk of ART non-adherence and hard drug use. They also had significant risk of an AIDS outcome or death. Cocaine was the most prevalent drug of choice of the hard drugs used.
Baum, MK et al. 2009[116] *Prospective,*	Evaluate the relationship between substances of abuse and HIV disease progression in a cohort of	A cohort of 222 HIV-infected drug users were recruited for a 30-month study. Medical history and blood draws were taken bi-annually. Drug use (type, frequency, mode	The use of crack-cocaine significantly predicted a decline in CD4 and an increase in viral load, independent of other risk

longitudinal cohort study	HIV+ active drug users.	of administration) was assessed by trained staff using a questionnaire. CD4 cell count and viral load were the main outcomes to determine disease progression.	factors (i.e., other drugs, age, gender, years since diagnosis, ART use). This suggests that cocaine may have physiological action unrelated to ART adherence that accelerates disease progression in HIV infected individuals.
Labib R et al. 2009[119] *In vivo, animal* *Experimental*	Examine the role of Kupffer cells and the modulating effects of nitric oxide and reactive oxygen species on the LPS potentiation of cocaine-mediated hepatotoxicity.	Four groups of mice, consisting of 4 mice in each group (total n=16) were exposed to LPS, cocaine, cocaine+LPS, or no exposure (control). Cocaine was administered for 5 consecutive days, LPS was given intraperitoneally. Glutathione, antioxidant status, nitric oxide, and liver damage were determined 18 hours after final LPS administration.	Mice exposed to LPS showed higher levels of hepatic nitric oxide and reactive oxygen species compared to controls. Cocaine exacerbated these effects in the presence of LPS.

Table 2: Literature Review: Biological Factors Associated with Increased Liver Fibrosis.

Author/Study design	Study Purpose	Model or Population	Findings
MacDonald GA et al. 2001[137] *Cross-sectional study*	Assess the distribution of lipid peroxidation in association with these factors and the relationship of this to the fibrogenic cascade.	Thirty-nine patients with hepatic steatosis, and no history of heavy alcohol drinking or any form of hepatitis were enrolled in the study. Liver biopsies were taken to confirm fibrosis staging and MDA-adduct staining.	Levels of MDA, a marker of lipid peroxidation, are significantly associated with areas of hepatic steatosis and liver fibrosis *in situ*.
Barbaro G et al. 1996[146] *Cross-sectional, convenience sample*	Quantitate the amount of hepatic and plasma glutathione (GSH) in patients with chronic hepatitis who were HIV+ or HIV- and examine correlations between activity of liver disease	One hundred and five patients with hepatitis C (HCV) were recruited and categorized as being HIV+ or HIV- based on serological markers. GSH concentrations were measured by HPLC. Liver biopsies were obtained to measure degree of fibrosis. Circulating GSH levels were also measured in a healthy control group.	Antioxidant GSH concentrations were significantly reduced in patients with chronic hepatitis C compared to healthy controls. Circulating GSH significantly correlated to hepatic GSH levels in HIV/HCV co-infected patients. Lower levels of GSH were significantly associated with higher liver fibrosis in HIV/HCV.
Balagopal A et al, 2008[148] *Retrospective, case-control study*	Study the effect of microbial translocation on liver disease in HIV and hepatitis C infection using a repository of samples.	One-hundred consecutive samples with paired liver biopsies were chosen. Cases were defined as having end stage liver disease or cirrhosis, and controls had no or minimal fibrosis. Microbial translocation was defined by measuring LPS in plasma using the Lonza LAL assay and sCD14 using ELISA.	HIV infection itself increases microbial translocation compared to uninfected controls, however HCV mono-infection does not. HIV/HCV co-infected subjects with elevated plasma LPS had a 2.7-fold higher risk

			of liver disease, and in the overall cohort elevated LPS is associated with 18.7-fold higher risk of cirrhosis when adjusting for confounding factors.
Sanvisens A et al. 2009[53] *Cross-sectional, convenience sample*	Analyze the prognostic value of hyaluronic acid and TGF-beta1 for liver fibrosis using liver biopsies in HIV/HCV co-infection.	Sixty-nine consecutive HIV/HCV co-infected patients had liver fibrosis assessed using Sheuer's fibrosis score. Serum hyaluronic acid and TGF-beta1 were assessed as diagnostic markers using AUROC curves.	TGF-beta1 was not predictive of fibrosis in HIV/HCV co-infected patients treated with HAART. The levels of TGF-beta1 dropped in the later stages of fibrosis.
Rallon N et al. 2011[54] *Case-control,* *Cross-sectional study*	Examine the levels of T-regulatory cells and circulating TGF-beta1 in HCV mono-infected and HIV/HCV co-infected according to the stage of liver fibrosis.	42 HIV/HCV co-infected patents, 20 HCV mono-infected patients, and 26 HIV- and HCV-seronegative control subjects had T-reg cells measured using flow cytometry and circulating TGF-beta1 levels measured using ELISA. Liver fibrosis was measured using Fibroscan transient elastography.	There was an inverse correlation between liver stiffness and TGF-beta1, however, it was not statistically significant. High levels of TGF-beta1 was significantly associated with not having advanced liver fibrosis.
Connoy A et al. 2011[130] *Cross-sectional study*	Test the expression of serum markers of inflammation and apoptosis and explore their relationship with hepatic fibrosis in HIV infected patients.	Samples from 50 subjects (10 patients in each group) were collected: HIV ART naïve, HIV on ART, HCV mono infected HIV/HCV ART naïve, and HIV/HCV on ART. M30 apoptosense measured circulating CK-18 for heapatic apoptosis. Liver fibrosis was characterized by expression of the fibrotic enzymes MMP-2 and TIMP-1.	Hepatocyte apoptosis was higher and significantly associated with markers of liver fibrosis in patients with HIV who were not taking antiretroviral medication, and had lower CD4 cell counts.

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CHAPTER III: METHODS

Study Participants

Participants recruited from the Borinquen Health Care Center in Miami, FL, who participated in the observational Miami Adult Studies on HIV (MASH) cohort funded by R01 AA018011 and R01 DA 023405 (P.I. Marianna K. Baum) were included in this study. After assessing for eligibility criteria for the parent study and obtaining informed consent, baseline and follow-up data for demographics, drug use, alcohol use, medication use and ART adherence, along with 24-hour dietary recall and anthropometrics were collected by trained interviewers, and were used in this study. In a cohort of 464 people living with HIV (PLWH), 25.4% (118/464) were HIV/HCV co-infected, and none of the participants were HBV infected. The remainder of the cohort (346 participants) consisted of HIV mono-infected participants. HIV, HBV and HCV status were collected using proof of positive test in medical records. Subjects were referred to Borinquen Health Center for HIV or hepatitis testing if they did not have proof of viral status. The FIU Institutional Review Board approved this proposed study, and study participants agreed during baseline consent to have their data and lab specimens used for future research. The investigator only used de-identified data and specimens linked by a code number.

Variables Obtained by the MASH Cohort:

Demographics, anthropometrics (including BMI), and ART use were obtained from the parent MASH study.

Alcohol use information was gathered during patient interviews using the Alcohol Use Disorders Identification Test (AUDIT) tool. AUDIT was developed by the World Health Organization to screen for alcohol consumption.[1,2] Moderate alcohol use or abstinence is

AUDIT score < 8, while scores ≥ 8 indicate hazardous drinking and ≥ 13 in women and 15 in men indicates possible dependence.[2,3]This tool was used in the parent study to assess alcohol use.

Body Mass Index was obtained by measuring height using a stadiometer and weight on a calibrated scale when subjects were dressed in light clothing and after removing shoes. Height was recorded at the baseline visit only, and weight was collected at each visit throughout the study to measure BMI in kg/m^2.

Cocaine use was determined by a self-report questionnaire that included type and frequency of drug of abuse and was confirmed by urine toxicology.

FIB-4 index is a non-invasive measure of liver fibrosis. Although liver biopsy is the current gold standard for liver fibrosis staging using METAVIR scores, it is highly invasive and it is not feasible to obtain for research purposes.[4] Specifically in HIV patients, it is contraindicated because of the increased risk of internal hemorrhage due to thrombocytopenia.[4,5] FIB-4 index was calculated using results collected by the parent study from common laboratory reports generated by a commercial laboratory using the formula: [(AgeXAST)/(PlateletsX√(ALT))]. It has been used in previous studies in HIV and validated in HCV[6,7] and HIV/HCV[6] with specificity of 94.7% and 90%, and sensitivity of 74.3% and 70%, respectively. METAVIR categories of liver fibrosis using FIB-4 values are as follows: F0-F1 (FIB-4 <1.45) none/mild fibrosis, F2 (1.45≤ FIB4 ≤3.25) moderate fibrosis, and F3-F4 (FIB-4 >3.25) advanced fibrosis/cirrhosis.[6,7]

Control variables included factors known to impact liver fibrosis: age, gender, CD4 cell count (baseline), HIV viral load (baseline), ART use, lifestyle factors (obesity, alcohol use, cocaine use) and HCV disease status, when appropriate.

Laboratory Sample Analysis Performed for this Study:

Laboratory tests for oxidative stress, microbial translocation, TGF-β1, and hepatocyte-specific apoptosis were generated specifically for this study. Whole blood samples were collected at baseline and annually for the participants in the MASH cohort, and transported to the FIU laboratory for processing and storage within 4 hours of collection. All laboratory tests were performed in plasma samples. The assays used in this analysis required that plasma samples were collected and processed within 4 hours of venipuncture for accurate results. After separating plasma from red blood cells and the buffy coat by centrifugation in vacutainers with an anticoagulant, approximately 8 X 1 mL aliquots were stored in cryovials at -80C for future research. This study did not use samples that had undergone repeated freeze-thaw cycles.

i. Malondialdehyde (MDA)

MDA is widely accepted as a marker of lipid peroxidation, indicating oxidative stress, and correlates well with liver fibrosis in other populations.[8-10] MDA was measured using a Thiobarbituric Acid Reactive Substance (TBARS) assay (Northwest Life Science Specialties, Portland, OR, USA). The test principle is based on the reaction between MDA and thiobarbituric acid (TBA), forming a stable MDA-TBA adduct that absorbs light strongly at 532nm, and can be read spectrophometrically. Samples that were "cloudy" upon thawing were centrifuged at 3,000 X g for 5 minutes prior to the addition of reagents. All reagents were reconstituted according to manufacturer's protocol. Butylated hydroxytoluene (BHT) with excess precipitate in solution was warmed in a water bath at 60 C for 1 minute. Ten microliters of BHT preservative was added to 250 uL of plasma sample or a known standard (provided by Northwest Life Science) in

microcentrifuge tubes. Acid reagent (250 uL) and TBA (250 uL) were added to the sample or standards and vortexed vigorously for 5 seconds, then incubated in a water bath at 60C for 60 minutes. After incubation, tubes were centrifuged at 10,000 X g for 3 minutes. Each sample was transferred into a disposable cuvette and Absorbance was read individually at 532nm in a spectrophotometer (Beckman Coulter DU530). A calibration curve generated a linear equation from which concentration of samples were estimated.

ii. Glutathione (GSH)

The reduced form of GSH, is the most abundant endogenous intracellular antioxidant, and it directly participates in quenching harmful peroxide radicals as well as recycling other antioxidants.[11] GSH represents antioxidant status by measuring the ratio of oxidized glutathione to total glutathione (oxidized GSSG + reduced GSH). Measuring levels of reduced GSH alone does not indicate oxidative stress levels, however, a higher ratio of oxidized glutathione to total glutathione is indicative of higher oxidative stress. Healthy values of oxidized glutathione are under 10% of the total glutathione pool.[12] Percent oxidized glutathione is measured using DetectX colorimetric assay that measures total and oxidized glutathione (Arbor Assays, Ann Arbor, MI, USA). The principle behind DetectX colorimetric assay is to bind the free thiol groups in oxidized GSSG to yield a highly colored product.

Prior to long term storage of plasma for glutathione analysis, the samples were treated with equal parts 5% sulfosalicylic (SSA) solution and centrifuged at 14,000 RPM for 5 min at 4C to prevent further oxidation of the sample by precipitating proteins that may cause damage. Then samples were aliquoted and stored at -80C until use. To prepare samples for the assay, each sample was diluted in assay buffer to 1:5 as per

manufacturer's instruction and 50uL was distributed in two 96 well plates in duplicate (i.e. 2 X 50 uL diluted sample in plate 1, and 2 X 50 uL sample in plate 2). Plate 1 represented % oxidized GSH, and plate 2 represented total GSH measurement. A standard curve was created by making six serial dilutions of the provided standard in each plate. Before adding the colorimetric reagent, 1.2 uL of 2-vinylpyridine was added to each well in plate 1 only to block reduced GSH from binding to the colorimetric substrate. Colorimetric reagent was added (25 uL) and subsequently, 25 uL of the reaction mixture was added using a multichannel pipette. The sides of the plates were tapped gently to mix the samples and incubated at room temperature for 20 minutes then immediately read using a microplate reader at 405nm using Gen5 Software (BioTek, EL800). Absolute values of reduced and total GSH were estimated using standard curves. In order to generate the final values for % oxidized GSH, the values of oxidized GSH were divided by 2 because each thiol group in the GSSG disulfide bond, can bind to the colorimetric agent, generating twice the absorbance of the original GSH molecule. The equation to calculate % oxidized GSH was $\frac{(\text{Oxidized GSSG} \div 2)}{\text{Total GSH}}$. Both MDA and % oxidized glutathione kits have been used in previous MASH cohort studies and results were presented in publications.[13,14]

iii. Lipopolysaccharide (LPS)

The bacterial endotoxin LPS is a major component of Gram-negative bacterial cell walls and can be quantitatively assessed to determine the degree of microbial translocation.[15] Plasma LPS levels are directly associated with the degree of intestinal permeability following invasive surgery.[16] The limulus amebocyte lysate (LAL) assay has been widely used for the detection of endotoxin for approximately 30 years,[17] and in

HIV-related research.[15,18-20] Recently, methods have been developed to measure LPS using the LAL Lonza kit in plasma of HIV+ patients.[18] Circulating LPS was measured using the Endpoint Chromogenic LAL Assay (Lonza, Walkersville, MD, USA) that utilizes Factor C, a protease zymogen that is cleaved and activated in the presence of endotoxin. The assay utilizes the cascade to ultimately activate a synthetic chromogenic peptide that exhibits a yellow color and can be read at 405 nm. A greater absorbance directly corresponds to a higher level of LPS endotoxin present in samples. Plasma samples require extra processing for this assay. Samples were thawed and diluted 1:5 in LAL reagent water, then heated to 70C in a heating block for 15 minutes to inactivate proteins. Samples were aliquoted into a microplate on a pre-warmed heating block to 37C. A series of endotoxin standard dilutions was used as a reference curve, and LAL water served as a blank. All samples and standards were plated in duplicate on 96 well plates. At time zero, 50 uL of LAL reagent water was added using a microchannel pipette. After 10 minutes of incubation at 37C, the pre-warmed (37C) chromogenic substrate was added in the same order as the LAL reagent water and incubated at 37C for 16 min. The stop solution (25% acetic acid) was added to stop the reaction in the same order as the previous reagents, and the samples were read on a microplate reader at 405nm (BioTek EL800) using Gen5 software. A "best fit" linear standard curve was generated to estimate the values of LPS in the samples.

iv. Transforming Growth Factor-Beta 1 (TGF-β1)

Measurement of the fibrogenic cytokine TGF-β1 uses ELISA methods from a commercially available kit. The Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) measures the activated form of TGF-β1 circulating in plasma samples, and

has been used on previous studies in human plasma.[21,22] Although circulating TGF-β1 shows mixed results when correlating with FIB-4 index,[23] its levels are often high in the early stages of fibrosis.[24,25] The sandwich ELISA assay quantifies TGF-β1 using pre-coated wells with monoclonal antibodies that specifically bind to TGF-β1 in samples and standards. After washing away excess sample, the bound TGF-β1 is immobilized by the antibody and treated with and enzyme-linked polyclonal antibody to bind with TGF-β1 in the well, the excess is washed away. Finally, a substrate solution that reacts to the presence of the enzyme of the polyclonal antibody is introduced to generate a color proportional to the quantity of TGF-β1in the well. For plasma samples, latent TGF-β1 was activated by adding 20 uL of 1N HCl to 40 uL of plasma and vortexed for 3 counts. After 10 minutes of incubation at room temperature, 1.2 NaOH/0.5 M HEPES was added to neutralize the mixture. The sample was diluted with the calibrator diluent to a final dilution factor of 40 based on the manufacturer's protocol. A standard curve was generated with several serial dilutions, and the calibrator diluent served as a blank. Fifty microliters of assay diluent was added to each well using a multichannel pipette, then 50 uL of sample, standard, or blank was added to each individual well. The well plate was covered and placed inside a foil pouch during a 2 hour incubation period at room temperature. The wells were washed manually with a squirt bottle using 400 uL of wash buffer with complete removal of liquid and bubbles at each step. TGF-β1 conjugate (100 uL) was added to each well and incubated in the foil pouch with gentle rocking at room temperature for 2 hours. The wash step was repeated, then 100 uL of substrate solution was added to each well and the plate was incubated at room temperature for 30 minutes and protected from light. Stop solution (100 uL) was added and plate was tapped gently

to ensure mixing. The absorbance of each sample was measured (in duplicate) at 450 nm on a microplate reader (BioTek EL800) using Gen5 software. TGF-β1 values were estimated from the linear equation generated by the standard curve (blank 450nm).

v. Cleaved Cytokerain-18 protein (CK-18)

Programmed cell death, or apoptosis, in hepatocytes has been suggested as a mechanism for liver fibrosis because apoptotic bodies initiate inflammation and activate hepatic stellate cells.[26]Hepatocyte-specific apoptosis was quantified using Apoptosense ELISA assay (Peviva, Nacka, Sweden) that uses the M30 antibody specific for the cleaved cytokeratin-18 (CK-18) protein. Hepatocyte cells undergoing cell death produce cleaved CK-18 and an increase circulating levels are associated with hepatic injury and fibrosis.[27-32]The protocol for the sandwich ELISA assay requires 25 uL of plasma in duplicate. Standards provided in kit (did not require dilutions) were pipetted in 96 well plate along with a "high" control and "low" control. Samples were dispensed directly into wells, and 75 uL of the conjugate solution was added to the plate using a multichannel pipette. The plate was sealed and incubated for four hours at room temperature on a shaker. After, the wells were rinsed with a squirt bottle (400 uL with overflow) using the wash solution 4-5 times. TMB substrate (200 uL) was added to each well and the plate was incubated at room temperature for 20 minutes with no exposure to light. Fifty microliters of the stop solution was added and the plate was placed on the shaker for 5 minutes before reading the absorbance on the microplate reader at 450 nm using Gen5 software. The standard curve provided a linear estimate of CK-18 values.

Acceptable results for all sample analyses had coefficient of variation (CVs) within 10%, that is, the value of each duplicate must be within 10% of the mean for that sample, indicating a small variance between replicates; otherwise the samples were reanalyzed.

Statistical Methods

A convenience sample from the parent study was included in this analysis. We selected participants that had complete liver enzyme and platelet information to calculate FIB-4 index, and with BMI, AUDIT, and drug use information completed. The laboratory data for hypothesis 2 was conducted in a subset of the mono-infected population to factor out any deleterious effects from hepatitis C virus on liver fibrosis. Data were generated at three time points throughout the parent study (baseline, 12 months, and 24 months) with n=60 participants included in the analysis. The participants included for hypothesis 3 testing were selected based on completeness of data and comprehensive laboratory data at baseline. A total of n=65 participants met this criteria and were included in the analysis.

Sample Size: Sample size was calculated using G*Power Program for all hypotheses. The first hypothesis analyzing lifestyle factors had the largest sample size (464 participants), mainly because it had the most complete data. The sample for hypothesis 2 included HIV mono-infected participants only to increase power and exclude the powerful confounder of hepatitis C infection. We reduced the sample size to 65 participants and included 3 time points for a more robust analysis of the biological samples (65 participants X 3 time points = 195 samples analyzed). A post-hoc power analysis of 65 HIV-mono infected participants generated 80% power to detect an alpha of 0.05 with three time points (baseline, 12 months, 24 months) with a correlation between

variables at r=0.2. Therefore a sample size of 65 participants for hypothesis 2 provided 80% power to detect predictive value of biomarkers. When calculating the power for logistic regression with a samples size of n=65, we had 80% power to see a statistically significant difference when the odds of fibrosis progressing one stage of fibroses increases by a factor of 2.3 times.

Statistical Analysis: The variables that required transformations to meet normality of distribution for the study included $\log_{10}$ transformation for the absolute HIV viral load, squaring CD4 cell count, and log10 TGF-β1. All other variables met the requirements of normal distributions. Descriptive characteristics for each hypothesis were generated using chi-square tests to compare categorical variables, and continuous variables were compared using student's t-test for two groups or one-way ANOVA for multiple groups. Multivariate linear regression was used to assess independent predictor variables on FIB-4 for specific aim 1, and for biological factors in specific aim 2 and 3. Logistic regression examined the odds of progressing at least one category of FIB-4 over two years when being at risk for a lifestyle factor at baseline (high BMI, drinking alcohol, using cocaine) for specific aim 1, or for having a high level of a fibrogenic biomarker (oxidative stress, LPS, TGF-β1, hepatocyte apoptosis) for specific aim 2. When sample size permitted, analyses were conducted separately in HIV mono- and HIV/HCV co-infected participants to avoid confounding effects of the HCV virus on liver fibrosis. $P < 0.05$ set the significance level for all analyses. Statistical analysis were performed using SPSS software versions 21 and 22.

Statistics for Specific Aim 1: Examine the effect of lifestyle factors (high BMI, alcohol use, and cocaine use) on liver fibrosis in HIV mono- and HIV/HCV co-

infection cross-sectionally, and investigate whether high baseline measures affect liver fibrosis progression over time. Lifestyle groups were separated based on cutoff points generated by AUROC analyses of body mass index, alcohol AUDIT score, and cocaine use frequency. A BMI cutoff of BMI≥28 kg/m^2, an AUDIT cut off of ≥8 and positive cocaine use (self-reported cocaine use in the past 6 weeks or a positive urine test) were the cutoff values that best fit the data and were used as cutoff points in the analysis. Three-way ANOVA investigated associations between lifestyle factors and FIB-4 index and included interactions between lifestyle factors. Significant interactions were further investigated with supplementary statistics, by stratifying the data and including plots to evaluate interaction on FIB-4 index. T-test and one-way ANOVA with post-hoc analysis was used to determine differences between groups with two and three levels, respectively. Multiple linear regression was run to analyze each lifestyle factor independently for significance on FIB-4 index as the dependent variable controlling for age, gender, ART use, CD4 cell count, and HIV viral load. Logistic regression was used to analyze the odds of progressing at least on stage of fibrosis over 24 months based on having a risky lifestyle factor (i.e., high BMI, alcohol use, or cocaine use).

Statistics for Specific Aim 2: Examine the effect of biological factors at baseline on changes in liver fibrosis over time. Sixty-five HIV-mono infected participants were selected for longitudinal analyses to test whether high levels of biomarkers at baseline predict liver fibrosis progression over two years. Biomarkers were separated into tertiles, and biomarkers that fell into higher tertile value was considered "high". FIB-4 values for participants were evaluated from baseline to 24 months and categorized as (1) nonprogressor – did not advance in FIB-4 category, and (2) progressor – progressed at

least one FIB-4 category in two years. A test of proportions was initially conducted to determine the proportion of participants that progressed in each group biomarker group (high vs. low). The odds of progressing at least one category of fibrosis (FIB-4 index) based on having a high fibrogenic biomarker (oxidative stress, LPS, TGF-β1, or CK-18), was examined using logistic regression, and controlled for variables associated with liver fibrosis. Mixed model analysis used repeated measures to describe the associations between biological factors and liver fibrosis (FIB-4 index) with time as an interaction.

Statistics for Specific Aim 3: Exploring the biological factors within lifestyle factors that contribute to liver fibrosis cross-sectionally. Participants were selected based on having one identifying lifestyle factor (i.e. only high BMI, or only hazardous drinking, or only using cocaine) in order to isolate any association between a specific lifestyle factor and biomarkers associated with liver fibrosis. Student's T-test compared the mean of each biological factor within a lifestyle group to a control group (no adverse lifestyle factors). One-way ANOVA was used to determine whether the means of biological factors were significantly different between each (High BMI vs. Hazardous drinking vs. Cocaine use vs. Control Group). Post-hoc analysis revealed specific differences between groups. Three-way ANOVA assessed interactions between lifestyle groups for each biological factor. Multiple linear regressions determined associations between individual biomarkers and FIB-4 within lifestyle groups, controlling for covariates known to affect liver fibrosis.

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CHAPTER IV: HAZARDOUS ALCOHOL USE, HIGH BODY MASS INDEX, AND COCAINE USE ARE ASSOCIATED WITH LIVER FIBROSIS IN HIV MONO- AND HIV/HCV CO-INFECTED ADULTS

Abstract

Objective: Liver fibrosis is a leading cause of morbidity and mortality in people living with HIV (PLWH). Co-infection with HIV and Hepatitis C (HCV) is a major contributor to liver disease progression, because of the ability of HCV and HIV to specifically infect hepatic stellate cells. Adverse lifestyle factors that are prevalent in PLWH include hazardous alcohol use, high body mass index (BMI), and illicit drug abuse, such as crack/cocaine use also may be significant contributors to liver fibrosis in HIV disease. This research aims to investigate harmful lifestyle factors that contribute to liver fibrosis using FIB-4 index as a non-invasive indicator in an HIV mono- and HIV/HCV co-infected people.

Methods: Baseline data was collected in 310 HIV mono-infected and 134 HIV/HCV co-infected participants in the Miami Adults Studies on HIV (MASH) cohort in Miami, FL. Longitudinal data was collected in a subset of participants to evaluate liver fibrosis progression after 24 months of study enrollment. Hazardous drinking was assessed by AUDIT score ≥ 8, high BMI was considered ≥ 28 kg/m^2, and positive cocaine use was collected using participant self-report, and confirmed with urine toxicology test. Participants were grouped into categories of liver fibrosis at baseline according to their FIB-4 score generated by the equation that uses common laboratory values: [(AgeXAST)/(PlateletsX√(ALT))]. FIB-4 index < 1.45 ruled out advanced stage of hepatic fibrosis (METAVIR stage F0-F1), FIB- 4 score that fell between 1.45 and less

than 3.25 was considered and indeterminate score (METAVIR stage = F2), and severe hepatic fibrosis or cirrhosis was defined by FIB-4 index ≥ 3.25 (METAVIR ≥ F3-F4). Multivariate linear regressions were used to find associations between lifestyle factors and FIB-4 index cross-sectionally. Univariate and multivariate analyses were used to assess whether hazardous alcohol use, high BMI, and/or positive cocaine use were significantly associated with increased liver fibrosis. A longitudinal analysis explored odds ratios of increasing one category of hepatic fibrosis after completing 2 years of study enrollment and having an adverse lifestyle factor. Interactions between lifestyle factors were explored in the analyses.

Results: Within the HIV mono-infected group, higher AUDIT score was independently associated with increasing FIB-4 index at baseline in regression models (P=0.023) and multivariate analysis of variance (P=0.023), after adjusting for age, gender, HIV viral load, CD4 cell count, and ART use. The odds of progressing at least one stage of hepatic fibrosis in HIV mono-infected participants was independently associated with high BMI (≥ 28 kg/m^2), after adjusting for covariates (P=0.015). Hazardous drinking was also associated with a 3 times increased odds of liver fibrosis progression over 2 years when controlling for covariates and high BMI (P=0.048). Cocaine use significantly decreased the odds of progressing in hepatic fibrosis stage when controlling for BMI (P=0.034), however, significance was lost in participants with low BMI, whereas CD4 cell count predicts FIB-4 in HIV mono-infected participants with lower BMI. There is an interaction between BMI, cocaine use, and liver fibrosis progression in both HIV mono- and HIV/HCV co-infection (P=0.072 and P=0.034, respectively). Hepatic fibrosis was

associated to clinical outcomes in HIV/HCV co-infected participants. Age (P= 0.004) was the major predictor of higher FIB-4.

Conclusions: Adverse lifestyle factors in HIV mono-infection associated with hepatic fibrosis may account for the high prevalence of liver disease not directly related to HIV/HCV co-infection. Hazardous drinking was associated with higher FIB-4 index in HIV mono-infection. BMI increased the odds of progressing FIB-4 category over 2 years. There was an interaction between cocaine use and BMI in both HIV mono- and HIV/HCV co-infected participants, and CD4 cell count may play a protective role in HIV mono-infected participants at risk for liver fibrosis with lower BMI.

Keywords: HIV infection, HIV/HCV co-infection, liver fibrosis, alcohol, AUDIT, cocaine, body mass index, FIB-4 index

Introduction

Liver fibrosis is a leading cause of morbidity and mortality in people living with HIV (PLWH).[1-3] Co-infection with HIV and Hepatitis C (HCV) is a major contributor to liver disease progression, because of the ability of HCV and HIV to specifically infect hepatic stellate cells.[4,5] This is evidenced by poor HIV disease status association with more advanced liver disease.[6,7] People living with HIV in the United States may also be exposed to adverse lifestyle factors that further contribute to the development of liver disease,[8-11] and in the United States harmful lifestyle factors that are prevalent in PLWH include hazardous alcohol use,[12,13] high body mass index (BMI),[8,12,14] and illicit drug abuse, such as crack/cocaine use.[15-17] Alcohol use is studied in HIV infection because of the extra burden it places on the liver, and leads to rapid progression of alcoholic liver disease in PLWH.[18] Hazardous drinking is associated with binge drinking and not necessarily alcohol dependence.[19,20] Binge drinking alcohol can lead to mechanisms of liver damage in healthy individuals,[21] so it is believed that hazardous alcohol use is associated with liver fibrosis in HIV mono- and HIV/HCV co-infection.[11] According to recent NHANES data, 2 in 5 HIV- infected women and 1 in 5 HIV-infected men are obese (BMI$\geq$ 30kg/m^2) in the United States. Obesity and being overweight is associated with advanced liver fibrosis in the general population, leading to conditions such as non-alcoholic fatty liver disease (NAFLD)[22,23] Illicit drug use is associated with HIV-infection, and there is a growing prevalence of non-injection drug users who use crack/cocaine in PLWH.[24] Cocaine is also associated with hepatotoxicity[25] through oxidative mechanisms[26] that cause liver fibrosis.

These lifestyle factors are associated with liver disease, and their association with increased liver fibrosis is also observed in the general population,[22,23,27,28] and may significantly affect liver fibrosis in HIV infection.

Stage of liver fibrosis serves as an important prognostic factor for liver disease because it defines the extent of liver disease advancement for therapeutic decisions.[29] FIB-4 is a non-invasive index of liver fibrosis. Liver biopsy is the current gold standard for liver fibrosis, it is highly invasive, carries a high risk for research purposes.[30] Specifically in PLWH, it is contraindicated because there is an increased risk of internal hemorrhage due to thrombocytopenia.[30,31] Therefore, non-invasive methods of determining liver fibrosis, such as FIB-4 index[32,33] have been used. Sterling et al.[34] have described the FIB-4 index, which consists of alanine aminotransferase (ALT) level, AST level, PLT counts, and age, for assessing fibrosis in a large cohort of patients with HIV/HCV co-infection. The FIB-4 index has also been validated as an inexpensive and accurate marker of fibrosis in the context of HCV mono-infection with a sensitivity of 74.3% and specificity of 80.1% (FIB-4 cut off< 1.45 to rule out advanced liver fibrosis).[35] This noninvasive index constitutes an inexpensive yet accurate prediction of hepatic fibrosis and can be correlated with liver biopsy METAVIR stage of F0-F1 based on a FIB-4 value <1.45, representing no/mild fibrosis, and METAVIR stage ≥F2 (FIB-4≥ 1.45) representing advanced fibrosis and METAVIR stage F3-F4 (FIB-4 ≥ 3.25) representing advanced fibrosis/cirrhosis.[6,36,37]

Most studies have attributed the advancing liver fibrosis in PLWH to high rates of HIV/HCV co-infection, however, with an effective use of antiretroviral therapy, PLWH are aging,[38] and this is accompanied by an increase in co-morbidities (e.g. cardiovascular

disease and liver fibrosis) both due to aging, risky lifestyle factors as well as the HIV and HCV virus infections.[39,40] Due to the strong confounding impact that HCV infection has on liver disease,[41-43] it is important to investigate factors associated with liver fibrosis in both HIV mono- and HIV/HCV co-infection as separate populations. This research aims to investigate harmful lifestyle factors that contribute to liver fibrosis using FIB-4 as a non-invasive indicator in an HIV mono- and HIV/HCV co-infected people.

Methods

The aim was to determine the association of harmful lifestyle factors with liver fibrosis in HIV mono-infected and HIV/HCV co-infected subjects participating in the Miami Adult Studies on HIV (MASH) cohort.

Study design and setting

A total of 454 HIV-infected adults, a subsample of MASH, participated in the study and were followed for two years. Data analysis was conducted separately for HIV mono-infected and HIV/HCV co-infected people as indicated in the results section. All participants gave written consent and completed each clinic visit with trained interviewers who also collected anthropometrics at each time point. Blood was collected annually via venipuncture by the study nurse and sent to a central laboratory (Quest Diagnostics, Miami, FL) for analysis and blind reporting. Participants visited the clinic every three months for follow-up interviews that included data collection for information on lifestyle, risk factors for disease, and blood samples for laboratory assessment. The study was approved by the Institutional Review Board at Florida International University.

Measurements

Dependent variable. Laboratory data to obtain FIB-4 index was sent to a central laboratory (Quest Diagnostics, Miami, FL) for blinded analysis. FIB-4 index was calculated using the validated equation [(AgeXAST)/(PlateletsX√(ALT))].[34,35] For baseline groups, participants were categorized as having no/mild fibrosis (FIB-4 index <1.45, METAVIR stage F0-F1), indeterminate fibrosis (1.45 ≥ FIB-4 < 3.25) or severe (FIB-4 ≥ 3.25 METAVIR stage ≥ F3-F4).[36] The absence of advanced fibrosis was determined with the cut off value of FIB-4 < 1.45 (METAVIR stage F0-F1) for cross-sectional and longitudinal analyses.[7,35,44] A participant qualified as progressing in at least one METAVIR stage or category of liver fibrosis if their baseline FIB-4 score increased from < 1.45 to greater than 1.45, or from between 1.45 – 3.25 to ≥ after 2 years of study enrollment.

Lifestyle factors. Interviewers collected information about alcohol consumption. The Alcohol Use Disorders Identification Test (AUDIT), a questionnaire developed by the World Health Organization to identify patients who drink hazardously or who are alcohol dependent and may benefit from abstinence was used.[45,46] AUDIT generates a score from 0 to 40 and can classify patients into abstinence from alcohol (AUDIT = 0), mild drinking habits (AUDIT < 8), hazardous drinking (AUDIT ≥ 8), and alcohol dependence (AUDIT ≥ 13 in women and ≥ 15 in men).[19,45,47] Body mass index (BMI) was obtained by measuring height using a stadiometer and weight on a calibrated scale when subjects were dressed in light clothing and after removing shoes. Height was recorded at the baseline visit only, and weight was collected throughout the study. Established cut off points of BMI for being overweight (BMI ≥ 25kg/m^2) and obese (BMI

$\geq 30 kg/m^2$) were used since they are associated with liver fibrosis,[14,48]however, previous research suggests that using a BMI cut off $\geq 28 kg/m^2$ predicted fibrosis.[23,49] The interviewer utilized a drug-frequency questionnaire to obtain self-reported crack/cocaine use, and the information was confirmed with urine toxicology.

Covariates. Age, gender, and ART use, along with HIV disease parameters (HIV viral load, and CD4 cell count) were obtained from interviews and primary care physicians with the consent of the participants at each visit.

Statistical Analysis

Descriptive statistics were used to describe the overall study sample in HIV mono- and HIV/HCV co-infected groups. Baseline characteristics were compared across groups using Chi-square/Fisher's exact test for categorical variables and analysis of variance for continuous variables. Receiver Operating Curve (ROC) analysis guided decisions to choose cut off points for primary analyses of harmful lifestyle factors with FIB-4 index. Using statistical analyses we evaluated (1) the associations between drinking (AUDIT score), anthropometrics (body mass index), and crack/cocaine use (self-report and/or urine sample) with liver fibrosis (FIB-4 index) and (2) whether participants experienced a progression in liver fibrosis over two years. Analyses were controlled for covariates that are known to affect liver fibrosis (age, gender, HIV viral load, CD4 cell count, and ART use). Spearman's correlations were used to evaluate potential collinearity between covariates. No pair of covariates included in the same regression model were highly correlated ($r>0.50$). Multivariate linear regressions were used to evaluate associations between AUDIT scores, BMI, and cocaine use independently with FIB-4 index. Univariate and Multivariate ANOVA models were

used to test the effect of adverse lifestyle groups on FIB-4 index. Three-way ANOVA Levene's test failed, but univariate analysis modeling is robust to heterogeneity, which allowed three-way ANOVA to proceed because the ratio of variance between the largest group and the smallest group was less than 3. Associations between the odds of progressing at least one category of FIB-4 index and having an adverse lifestyle were evaluated using logistic regression models. Lifestyle groups were evaluated in statistical models as independent factors, and subsequent analyses identified the impact of interactions between lifestyle groups on liver fibrosis. HIV viral load was log transformed, CD4 cell count was transformed by square root to meet assumptions of normality. The assumptions of linearity, independence of errors, homoscedasticity, and normality of residuals were met and independent variables showed no multicollinearity. A P-value < 0.05 was considered statistically significant.

Results

Characteristics of study population (Table 1)

In the MASH cohort, HIV mono-infected individuals were younger, had lower HIV viral load but no difference in CD4 cell count or ART use, and had slightly higher BMI. As expected, HIV/HCV co-infected participants had significantly higher FIB-4 index, which places them in a higher clinical category of liver fibrosis compared to those who are HIV mono-infected (advanced fibrosis or METAVIR stage $\geq$ F2 vs. mild fibrosis or METAVIR stage F0-F1, respectively).

Factors associated with categories of fibrosis (Table 2a and 2b)

ANOVA analysis revealed that mean FIB-4 value fell within clinical range for mild, indeterminate and severe liver fibrosis in HIV mono-infection (mild 0.96±0.26, vs.

indeterminate 1.83±0.38, vs. severe 5.32±3.44) and HIV/HCV co-infection (mild 1.09 ± 0.25 vs. indeterminate 2.07 ± 0.51, vs. severe 4.66 ± 1.84). Only 5.6% of HIV mono-infected participants qualified as having severe liver fibrosis at baseline visit compared to 18.9% in the co-infected group. A higher percent of the HIV mono-infected participants with higher stages of liver fibrosis at baseline were men (77% vs 58%, $P<0.001$, and significantly older (44.5± 8.3 vs. 49.4± 4.9 years $P=0.001$). The mean BMI was in the "overweight" category for all categories of liver fibrosis. In HIV/HCV co-infection, only age differed significantly and HIV viral load trended towards significance between FIB-4 categories such that participants in later stages of liver fibrosis tended to be older (49.1±5.6 vs. 51.3±5.2 years, $P=0.003$) and had a higher proportion of uncontrolled HIV viral load (55.4% vs. 80.0%, $P=0.056$) (Table 2b).

Cut off points in within lifestyle groups

Each lifestyle parameter had at least three useful cut off values that could be potentially used for analysis. Figure 1a and b show ROC analysis curves that determined the single best cut off point generated for each lifestyle variable to predict liver fibrosis progression over 2 years. Body mass index (BMI) was the only variable in HIV mono-infection that had an acceptable AUROC value (0.650 ± 0.059, $P=0.023$). The cut off of 28 kg/m^2 had the highest sensitivity and specificity combination (68.2% and 59.1%, respectively). AUDIT score and cocaine frequency had poor AUROC curves, however, the cut offs that showed the highest sensitivity and specificity were an AUDIT score of 8 and positive cocaine use (Table 3). HIV/HCV co-infection showed no significant AUROC curves, and similar sensitivity and specificity to HIV mono-infection values, so

the cut off values from HIV mono-infection were applied to HIV/HCV co-infection for the analysis.

Due to the diverse etiologies of liver disease in HIV mono- and HIV/HCV co-infection, adverse lifestyle variables alone may not be associated with FIB-4 liver disease progression. Even so, this analysis is necessary for the generation of cut off points to investigate the relationship and interactions between lifestyle factors and other confounding factors that affect liver fibrosis progression.

Factors associated with liver fibrosis in HIV mono-infection

After removing participants with missing data in the various categories of variables used, analysis was conducted in HIV mono- and HIV/HCV co-infection, respectively. Data from 300 HIV mono-infected participants were analyzed at baseline. Simple correlations showed FIB-4 index was significantly correlated to alcohol consumption (r= 0.118, P=0.021), BMI (r=-0.127, P=0.014), age (r=0.377, p<0.001), and CD4 cell count (r= -0.152, P=0.004). Multivariate linear regression used lifestyle factors (AUDIT score, BMI, cocaine use) as predictors of FIB-4 index and included covariates known to influence liver fibrosis into the model (age, gender, HIV viral load, CD4 cell count, ART use). The model significantly predicted FIB-4 index $F(8, 300)$= 5.202, P<0.001, adjusted R^2 = 0.099; however AUDIT score was the only lifestyle factor that was an independent predictor of liver fibrosis (β =0.003, P=0.023) when adjusting for confounding factors. Regression coefficients are shown in Table 4.

Three-way ANOVA investigated the main effects and interactions of harmful lifestyle factors on FIB-4 index as a continuous variable.

The results for main effects of lifestyle factors (shown in Table 5) describe an association between hazardous drinking and higher FIB-4 index when adjusting for age, gender, viral load, CD4 cell count, and ART use $F(6,300)=5.216$, $P=0.023$. Correspondingly, an alternative model that was adjusted for main effects and interactions between lifestyle groups was significant $F(7,300)=2.721$, $P=0.009$, with a significant association of increasing liver fibrosis with drinking hazardously, AUDIT score ≥8 $F(1, 300)= 3.925$, $P=0.048$, and with being overweight BMI ≥ 28 kg/m^2 $F(1, 300)= 4.429$, $P=0.036$. No significant association was found between liver fibrosis and cocaine use $F(1, 300)=1.955$, $P=0.163$, however, there was interaction between BMI and cocaine use $F(1, 300)=4.509$, $P=0.035$ that is shown graphically (Figure 2).

In the ANOVA model, HIV mono-infected participants who were overweight (BMI ≥ 28kg/m^2) and who engaged in hazardous alcohol drinking (AUDIT score ≥ 8) had significantly higher liver fibrosis; however there was an inverse association relationship between BMI and FIB-4 index in regression models. To explore alternative factors leading to liver fibrosis in HIV mono-infection, we selected for participants with healthy BMI (<28 kg/m2) and low AUDIT score (<8). In participant with healthy lifestyle factors, having a low CD4 cell count was significantly associated with higher FIB-4 value, ($\beta= -0.227$, $P=0.037$) adjusting for age, gender, and ART use. Interestingly, the significant relationship between CD4 cell count and FIB-4 index is not present in participants with high BMI ($\beta= 0.115$, $P=0.173$), which may be related to protective effect that high BMI has on CD4 cell count, however, the fibrogenic effects of BMI overshadows any protective effects of CD4 cell count on FIB-4 (Figure 3).

A total of n=176 HIV mono-infected participants had complete data for baseline and 24-month visits. Figure 4 describes the proportion of participants who progressed at least one category of fibrosis over 2 years. Within BMI category, a significantly higher proportion of participants progressed in liver fibrosis over 2 years compared to those with low BMI ($p<0.001$).

Logistic regression analysis describes an association between liver fibrosis progression of at least one fibrosis category over 24 months and high BMI (≥ 28 kg/m^2) at baseline (OR 2.934 [95% CI 1.132 -7.605]; *P*= 0.027, that became more significant after adjusting for age, gender, HIV viral load, CD4 cell count, (OR 3.797 [95% CI 1.301 - 11.077]; *P*=0.015). Hazardous drinking and crack/cocaine use became significantly associated with progressive liver fibrosis when adjusting for BMI in the model (alcohol $\geq$ 8 (OR 3.038 [95% CI 1.010 - 9.135]; *P*=0.048) and crack/cocaine use (OR .228 [95% CI 0.057 - 0.918]; *P*=0.038).

On the other hand, crack/cocaine use was associated with a 4.39 lower odds of progressing in liver fibrosis in the adjusted model that includes BMI. To further investigate paradoxical relationship between crack/cocaine use and liver disease, the participants were stratified by "high" BMI (≥ 28 kg/m^2) and "low" BMI (< 28 kg/m^2) and the adjusted odd ratio was re-evaluated. When stratifying by BMI, the "protective" effect of crack/cocaine was reversed although the finding was not significant (n= 95, OR 1.105 [95% CI 0.187 – 6.508]; *P*=0.912). In participants with "high" BMI, cocaine cannot predict the odds of progressing in FIB-4 category with an insignificant and extremely low odds ratio (n=80, OR <0.000 [95% CI 0.000 - <0.001]; *P*= 0.998. Liver fibrosis progression is slightly associated with the interaction between BMI and crack/cocaine use

(OR 0.959 [95% CI 0.913 – 1.006]; *P*=0.085; aOR 1.048 [95% CI 0.907 – 1.006]; *P*= 0.072), where the interaction between BMI and cocaine is associated with a 4.8% increase in the odds of progressing in liver fibrosis. It is important to note that here is a 2.22 greater odds of having a lower BMI when using crack/cocaine (OR 0.450 [95% CI 0.229 – 0.889]; *P*=0.021) in HIV mono-infection.

Factors associated with liver fibrosis in HIV/HCV co-infection

A total of n=125 HIV/HCV co-infected participants had complete data at baseline. Pearson correlation indicated FIB-4 index was significantly correlated to age (*r*=0.258, p<0.002), HIV viral load (*r*= 0.144, *P*=0.051), and CD4 cell count (*r*= -0.152, *P*=0.004). Multivariate linear regression significantly predicted FIB-4 index $F(7, 125)= 2.883$, *P*=0.008, adjusted $R^2 = 0.09$, however, unlike the results from the HIV mono-infected group, no lifestyle factors were associated with FIB-4 index in HIV/HCV co-infection. Age (β=0.296, *P*=0.001) was the only significant predictor of FIB-4 index in HIV/HCV co-infection. Regression coefficients are listed in Table 7.

Multivariate analysis (three-way ANOVA) showed no interaction between lifestyle factors and FIB-4 index in HIV/HCV co-infection, however age was independently associated with FIB-4, $F(1, 125)= 8.456$, *P*=0.004. The results for main effects of harmful lifestyle factors are shown in Table 8. The alternative model adjusting for main effects and interactions between lifestyle groups was not significant F(7,125) 0.841, *P*=0.555.

Although there were no significant effect or statistically significant interactions of FIB-4 with crack/cocaine use, when the data is plotted, an interaction between BMI and cocaine use was observed (Figure 5). This graphical representation differs from HIV

mono-infection because high BMI and cocaine use increase the estimated marginal association with FIB-4 index in HIV/HCV co-infection, whereas this association is attenuated in HIV mono-infection.

Age was the only significant factor that predicted liver fibrosis in HIV/HCV co-infection, therefore we performed the analysis stratifying subjects by age and running regression analysis separately in HIV/HCV co-infected individuals with an age cut off of 51 years. In participants who were 51 years old or less, age remained a significant factor that predicted FIB-4 value (β =0.022, P=0.016), after adjusting for gender, HIV viral load, CD4 cell count, AUDIT score, BMI, and cocaine use. In HIV/HCV co-infected participants older than 51 years old, age lost significance, but BMI significantly predicted FIB-4 value (β=0.012, P=0.029) controlling for the same variables and with a comparable sample size. Notably, BMI was not significantly different between the age groups in HIV/HCV co-infection, however CD4 cell count was significantly lower in younger age group (453 vs. 617, P=0.004) and AUDIT score was significantly higher (10 vs. 6, P=0.002).

A total of n=61 HIV/HCV co-infected participants had complete longitudinal data for baseline and 24-month visits. Figure 6 shows that although there was a greater proportion of cocaine users that progressed in at least one FIB-4 category, the result was not statistically significant.

Table 9 shows the logistic regression analyses, adjusting for age, gender, HIV viral load, CD4 cell count and ART use in HIV/HCV co-infection. None of the models, including the full model containing all predictors was statistically significant, χ^2(8,

61)=5.071 *P*=0.750, indicating that the model could not distinguish between those who progressed and those who did not progress one category of FIB-4.

Using the plotted interaction generated from the three-way ANOVA analysis, further investigation with logistic regression analysis showed that liver fibrosis progression was significantly associated with the interaction of BMI and crack/cocaine in HIV/HCV co-infection. Being overweight BMI ($\geq 28 kg/m^2$) and using crack/cocaine had a statistically significant association with odds of progressing in FIB-4 category (OR 3.800 [95% CI .982 – 14.698]; *P*=0.053; adjusted OR 4.985 [95% CI 1.130 – 21.993]; *P*= 0.034). Unlike HIV mono-infection, the interaction of BMI and crack/cocaine increased the odds of progression of fibrosis by a factor of nearly five in the HIV/HCV co-infected group.

Discussion

The findings from this study indicate that adverse lifestyle factors influence the progression of liver fibrosis in both the HIV mono-infected and the HIV/HCV co-infected subjects in this study. Moreover, these findings support the premise that there is a great amount of negative reinforcing interaction among these adverse influences.

A relatively small percentage of HIV mono-infected individuals in the MASH cohort were categorized as having severe liver fibrosis at baseline (5.6%), however, when combined with the number of participants with FIB-4 ≥ 1.45 liver fibrosis that percentage increased to 25.5% of participants with advanced liver fibrosis or METAVIR stage ≥ F2. In studies with comparable sample size, the prevalence of advanced liver fibrosis was 8.3%[40] and 10.0%[50] in HIV mono-infection, indicating that the MASH cohort has a greater proportion of HIV mono-infected participants with advanced liver fibrosis

compared to the findings from other studies. On the other hand, the literature for HIV/HCV co-infected populations shows a prevalence of advanced liver fibrosis ranging from 37.4% to 60.0%.[37,51,52] HIV/HCV co-infected participants in the MASH cohort had proportions of advanced liver fibrosis (58.8%) similar to a higher prevalence of advanced fibrosis in HIV/HCV co-infected populations in the literature.

Choosing appropriate cut off values was important for conducting analysis of variance and logistic regression. AUROC analysis indicated that an AUDIT score ≥ 8, BMI ≥ 28kg/m^2 and positive cocaine use were the best cut off points to predict advancing liver fibrosis using the FIB-4 index in this cohort, and these values correspond with cut off points associated with liver disease and poor HIV disease in the literature.[17,21,23] An AUDIT score ≥ 8 distinguishes between hazardous drinking, typically associated with binge drinking that has been linked to liver damage.[21] High BMI (≥ 28kg/m^2) may be a stronger predictor of liver fibrosis in HIV-infection than other lifestyle factors. Initiation of ART improves HIV prognosis and is associated with increasing BMI. A majority of the participants in this study were ART adherent (82.6% in HIV mono-infected group and 82.7% in HIV/HCV co-infected group) which is associated with higher BMI, better CD4 cell counts.[53-55]

Furthermore, BMI in HIV infection may have a paradoxical effect on liver fibrosis because it has been shown to be protective of the immune response[56] and it is associated with better HIV disease outcomes,[54] which in turn protects against liver disease.[40] Our results highlight the relationship between BMI, CD4 cell count and liver fibrosis in HIV mono-infection because CD4 cell count is a significant predictors of liver fibrosis only in those with lower BMI ≤ 28 kg/m^2, and The relationship between CD4 cell

count and FIB-4 index is lost in those who have higher BMI, indicating that the protective effect that higher CD4 cell count has on the liver is negated in those who are overweight/obese (β= 0.115, *P*=0.173).

Our results also show that BMI has a significant interaction with cocaine. In fact, there are 2.22 greater odds of having a lower BMI when using crack/cocaine in HIV mono-infection, which is likely attributed to the anorectic effect of cocaine in HIV infection, and in non-infected populations, leading weight loss that may prevent odds of developing liver fibrosis from being overweight (i.e. preventing non-alcoholic fatty liver disease).[57-59]

We have found that alcohol use is associated with liver fibrosis in HIV mono-infection, but not in HIV/HCV co-infection, perhaps because the participants who tend to have higher AUDIT scores in this population are younger, and the HIV/HCV co-infected participants with advanced liver fibrosis tended to be older than 50 years.

The attenuation of the interaction of BMI and cocaine effects on FIB-4 index was not seen in HIV/HCV co-infection. While an interaction existed between the two variables, they significantly increased the odds of liver fibrosis progression by a factor of nearly five. In this case, the combined effect of high BMI and cocaine use may damage the liver because it is even more susceptible to higher levels inflammation and oxidative stress resulting from cocaine use and being overweight, combined with the deleterious effect of cocaine in CD4 cell count.[17,60-62]

Overall, BMI and cocaine showed a deleterious interaction on FIB-4 index in both HIV mono- and HIV/HCV co-infected participants. Although the anorectic effects of cocaine use initially masked harmful effect of BMI, the average of crack/cocaine users

still fell into the "overweight" BMI category (Figure 7), and this study shows that combined effects of cocaine use and being overweight are damaging to the liver.

Age is one of the most important factors associated with liver fibrosis in HIV/HCV co-infected participants in the MASH cohort. When stratifying for age, increased BMI was significantly associated with FIB-4 index in participants older than 51 years; however, it is important to note that BMI was not significantly different between the age groups in HIV/HCV co-infection, but CD4 cell count was significantly lower in younger age group (453 vs. 617, P=0.004) and AUDIT score was significantly higher (10 vs. 6, P=0.002), which may account for the effect on FIB-4 values. Although CD4 cell count and AUDIT were independently significant in the model they contribute to the overall direction of the relationship between BMI and FIB-4 in younger HIV/HCV-infected persons.

Conclusion

Adverse lifestyle factors prevalent in in HIV mono- and HIV/HCV co-infection may act together to magnify their individual effect on liver fibrosis, and contribute independently or through their interaction to advance liver fibrosis over time. Hazardous alcohol use, high BMI, and the interaction between crack/cocaine use and BMI are adverse lifestyle conditions that are associated with liver fibrosis, and are of concern in this population. These findings will be useful to help guide future research into lifestyle factors that affect the HIV population, and guide healthcare choices to prevent and ameliorate liver disease as a comorbidity in people living with HIV.

Tables and Figures

Table 1: Descriptive characteristics of HIV mono- and HIV/HCV co-infected groups at baseline.

	HIV n=310 Mean±S.D. or %(n)	**HIV/HCV n=134 Mean±S.D. or %(n)**	***P*-value**
Descriptive Characteristics	n=310	n=134	
Age	**45± 7.8**	**50± 5.2**	**<0.001**
Gender (%male)	64%(198)	62%(83)	0.585
Ethnicity			0.327
White Non-Hispanic	6.0(19)	9.6(12)	
White Hispanic	17.7(55)	18.4(25)	
Black Non-Hispanic	68.1(211)	65.4(88)	
Black Hispanic	4.7(14)	5.1(7)	
Other	3.7(11)	1.4(2)	
HIV Parameters			
Log_{10}(HIV viral load)	2.68± 1.30	2.76± 1.32	0.587
CD4 cell count	516.4± 424.6	528.5± 335.4	0.617
HCV viral load	N/A	12712808 (n=61)	N/A
ART use (% yes)	**82.6%(256)**	**82.7%(111)**	**<0.001**
Lifestyle Factors			
AUDIT	9.25± 10.0	8.34± 10.3	0.386
BMI	27.9± 5.22	26.9± 5.24	0.057
Cocaine use (% yes)	29.7(92)	29.2(39)	0.925
Liver Fibrosis			
FIB-4 index	**1.32± 1.15**	**2.14± 1.54**	**<0.001**
FIB-4 ≥ 1.45 (% yes)	**25.5(79)**	**58.8(79)**	**<0.001**

P-value <0.05 is significant.

Table 2a: Descriptive characteristics within FIB-4 categories in HIV mono-infection.

	Degree of fibrosis			
	Mild FIB-4< 1.45 Mean± S.D. or %(n)	**Indeterminate FIB-4≥ 1.45-3.24 Mean± S.D. or %(n)**	**Severe FIB-4≥ 3.25 Mean± S.D. or %(n)**	***P*- value**
	n=230	n=67	n=13	
Descriptive Characteristics				
Age	**44.5 ± 8.3[a]**	**48.0 ± 6.0[a,b]**	**49.4 ±4.9[b]**	**0.001**
Gender (% Male)	**58(138)[a]**	**84(57)[b]**	**77(10)[a,b]**	**<0.001**
Ethnicity, n(%within FIB-4 category)	4.2(10)	11.8(8)	7.7(1)	0.509
White Non-Hispanic	20.8(49)	10.3(7)	0.0(0)	
White Hispanic	68.2(161)	66.2(45)	76.9(10)	
Black Non-Hispanic	4.2(10)	7.4(5)	0.0(0)	
Black Hispanic	2.5(6)	4.4(3)	15.4(2)	
Other				
Disease Parameters				
Log_{10}(HIV viral load)	2.62± 1.27	2.83± 1.36	2.97± 1.49	0.371
Uncontrolled HIV viral load (>75 copies/ml)	49.8(114)	51.5%	69.2%	0.392
Uncontrolled HIV viral load (>200 copies/ml)	41.8(96)	47.1%	53.8%	0.548
CD4 cell count (cells/mm^3)	**549.04 ± 371.38[a,b]**	**359.76 ± 235.60[b]**	**776.50 ± 1267.70[a]**	**0.001**
CD4 cut off of 200 cells/ml (% < 200)	**13.9(32)[a,b]**	**30.9(21)[b]**	**15.4(2)[a]**	**0.005**
FIB-4 index	0.96 ± 0.26[a]	1.83 ± 0.38[b]	5.32 ± 3.44[c]	<0.001
Lifestyle Characteristics				
Drinks alcohol (%Y)	78.1(179)	83.8	69.2	0.402
AUDIT score	8.7 ± 9.8	10.2 ± 10.4	13.5 ± 12.4	0.164
AUDIT cut off of 8 or higher (%Y)	39.7(91)	45.6	69.2	0.089
BMI (kg/m^2)	**28.32 ± 5.22[a,b]**	**26.49 ± 5.00[a]**	**27.09 ± 5.61[a]**	**0.034**
BMI cut off ≥25 kg/m^2	**71.7(170)**	**54.4(37)**	**61.5(8)**	**0.024**
BMI cut off ≥ 28 kg/m^2	**53.2(126)**	**36.8(25)[b]**	**38.5(5)**	**0.043**
BMI cut off ≥ 30 kg/m^2	**35.0(83)**	**20.6(14)[b]**	**15.4(2)**	**0.035**
Cocaine use (Y/N)	27.7(64)	39.4(26)[b]	15.4(2)	0.096
Cocaine frequency, n(% in each category)				
1. No cocaine use	72.6(172)	60.3(41)	84.6(11)	0.749
2. Uses crack/cocaine	3.8(9)	8.8(6)	0.0(0)	
a) Once per week	13.3(31)	14.7(10)	0.0(0)	
b) > once per week	3.0(7)	8.8(6)	0.0(0)	
c) Once per day	3.0(7)	2.9(2)	7.7(1)	
d) > once per day	4.6(11)	4.4(3)	7.7(1)	

P-value <0.05 is significant.

Table 2b: Descriptive characteristics within FIB-4 categories in HIV/HCV co-infection

	Degree of fibrosis			
	Mild FIB-4< 1.45 Mean± S.D. or %(n)	**Indeterminate FIB-4≥ 1.45-3.24 Mean± S.D. or %(n)**	**Advanced FIB-4≥ 3.25 Mean± S.D. or %(n)**	***P*- value**
	n=56	n=54	n=24	
Descriptive Characteristics				
Age	**49.1 ± 5.6[a]**	**52.5 ± 4.5[b]**	**51.3 ± 5.2[b]**	**0.003**
Gender (% Male)	31(55.4%)	35(63.6%)	18(72.0%)	0.339
Ethnicity, n(%within FIB-4 category)	16.1(9)	5.5(3)	4.0(1)	0.344
White Non-Hispanic	17.9(10)	14.5(8)	28.0(7)	
White Hispanic	57.1(32)	76.4(42)	60.0(15)	
Black Non-Hispanic	5.4(3)	3.6(2)	8.0(2)	
Black Hispanic	3.6(1)	0.0(0)	0.0(0)	
Other				
Disease Parameters				
Log_{10}(HIV viral load)	2.72 ± 1.32	2.58 ± 1.29	3.26 ± 1.34	0.107
Uncontrolled HIV viral load (>75 copies/ml)	**55.4(31)[a]**	**52.7(29)[a]**	**80.0(20)[b]**	**0.056**
Uncontrolled HIV viral load (>200 copies/ml)	44.6(25)	43.6(24)	68.0(17)	0.097
CD4 cell count (cells/mm^3)	538.79 ± 319.67	525.62 ± 350.19	516.36 ± 350.15	0.966
CD4 cut off of 200 cells/ml (% < 200)	16.1(9)	20.0(11)	16.0(4)	0.839
FIB-4 index	**1.09 ± 0.25[a]**	**2.07 ± 0.51[b]**	**4.66 ± 1.84[c]**	**<0.001**
Lifestyle Characteristics				
Drinks alcohol (%Y)	66.1(37)	65.5(36)	64(16)	0.984
AUDIT score	8.3 ± 10.4	7.8 ± 9.8	9.6 ± 11.7	0.777
AUDIT cut off of 8 or higher (%Y)	37.5(21)	40.0(22)	40.0(10)	0.958
BMI (kg/m^2)	26.55 ± 5.04	27.3 ± 5.48	26.56 ± 5.32	0.329
BMI cut off ≥25 kg/m^2	50.0(28)	65.5(36)	52.0(13)	0.227
BMI cut off ≥ 28 kg/m^2	42.9(24)	47.3(26)	32.0(8)	0.440
BMI cut off ≥ 30 kg/m^2	25.0(14)	29.1(16)	20.0(5)	0.660
Cocaine Use n(% in each category)				
1. No cocaine use	72.2(40)	65.5(35)	73.9(18)	0.636
2. Uses crack/cocaine	27.8(15)	34.5(18)	26.1(6)	
a) Once per week	12.5(7)	20.0(11)	12.5(3)	
b) > once per week	1.8(1)	1.8(1)	4.0(1)	
c) Once per day	5.4(3)	0.0(0)	4.0(1)	
d) > once per day	1.8(1)	5.5(3)	4.0(1)	

P-value <0.05 is significant.

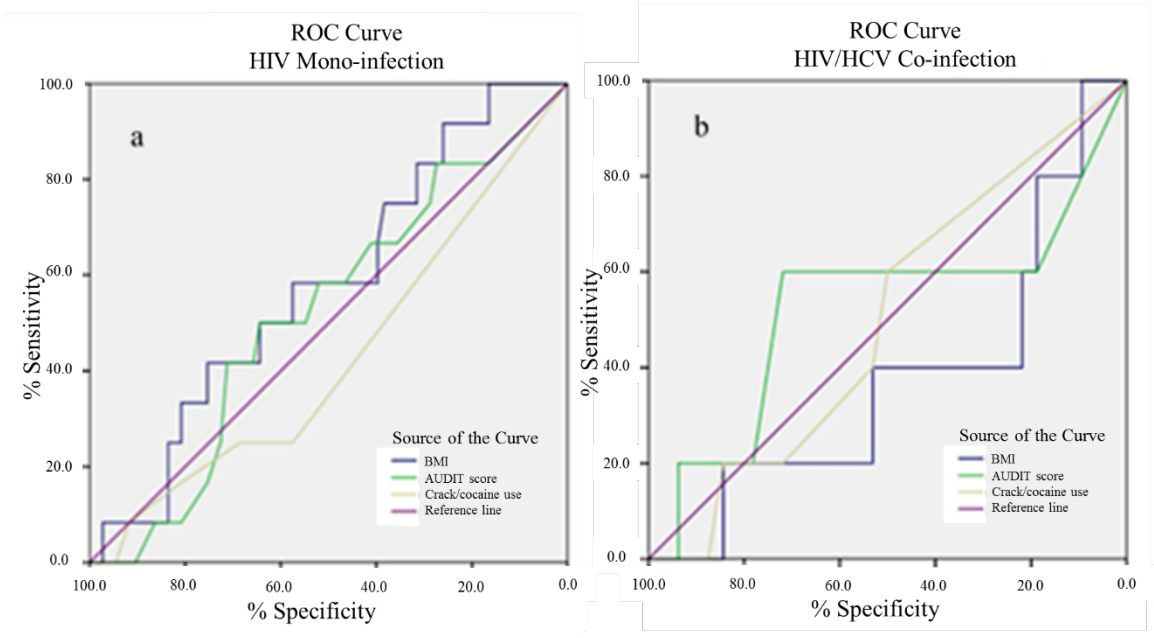

Figure 1: ROC curves for adverse lifestyle factors in HIV mono- and HIV/HCV co-infection. The curves indicate the sensitivity and specificity of each parameter (BMI, AUDIT, and crack/cocaine use) to predict progression in FIB-4 over 24 months. The values with the best combination of sensitivity and specificity were chosen as cut off points for individual lifestyle factors.

Table 3: AUROC analysis for adverse lifestyle factors in HIV mono- and HIV/HCV co-infection to predict progression of FIB-4 category over 24 months.

	Lifestyle factor	Area under ROC (S.E)	*P*-value (95% CI)	Cut off tested	Sensitivity	Specificity
HIV mono-infection	BMI	.650 (.059)	.023 (.536, .765)	Overweight[a] (25 kg/ m^2) **Overweight[b] (28 kg/ m^2)*** Obese (30 kg/ m^2)	86.4%, **68.2%** 59.1%	31.8% **59.1%** 68.2%
	AUDIT	.565 (.069)	.325 (.430, .700)	Mild drinking (score of 1) **Hazardous drinking (score of 8)*** Alcohol dependence (score of 13F/15M)	72.7% **45.5%** 31.8%	32.0% **66.0%** 79.1%
	Cocaine Frequency	.428 (.089)	.423 (.252,.603)	**Cocaine use Yes/No** Uses crack/cocaine a) Once per week b) > once per week c) Once per day	**25.0%** 16.7% 8.3% 0.0%	**68.5%** 80.8% 91.8% 94.5%
HIV/HCV co-infection	**Lifestyle factor**	**Area under ROC (S.E)**	***P*-value (95% CI)**	**Cut off tested**	**Sensitivity**	**Specificity**
	BMI	.425 (.103)	.436 (.223, .627)	Overweight[a] (25 kg/ m^2) Overweight[b] (28 kg/ m^2) Obese (30 kg/ m^2)	86.4% 68.2% 59.1%	31.8% 57.8% 68.2%
	AUDIT	.486 (.103)	.888 (.285, .688)	Mild drinking (score of 1) Hazardous drinking (score of 8) Alcohol dependence (score of 13F/15M)	54.5% 36.4% 26.4%	38.0% 66.0% 80.0%
	Cocaine Frequency	.500 (.128)	.999 (.249, .751)	Cocaine use Yes/No Uses crack/cocaine a) Once per week b) > once per week c) Once per day	60.0% 20.0% 20.0% 0.0%	50.0% 71.9% 84.5% 87.5%

[a]Overweight category based on CDC cut off points[42]
[b] Overweight category based on previous publications[23,48,49]
*indicates the cutoff value that best predicted progression in FIB-4 category over 24 months.

Table 4: Association with FIB-4 index and continuous variables BMI, AUDIT score, and cocaine use in HIV mono-infection.

	Unadjusted model			Adjusted model*		
Variable	**β**	**t**	***P*-value**	**β**	**t**	***P*-value**
BMI (kg/m^2)	-0.005	-2.211	0.028	-0.003	-1.294	0.197
AUDIT score	0.002	2.047	0.042	0.003	2.291	0.023
Cocaine (Y/N)	-0.005	-0.183	0.855	0.006	0.154	0.800

*Multivariate linear regression model was adjusted for age, gender, viral load, CD4, and ART use.
P-value <0.05 is significant.

Table 5: ANOVA analysis showed independent effects of being overweight, drinking hazardously, and using cocaine on FIB-4 index in HIV mono-infection.

Variable	**F unadjusted**	***P*-value**	**F adjusted***	***P*-value**
AUDIT score ≥8	4.202	.041	5.216	.023
BMI≥28kg/m^2	4.239	.040	3.543	.061
Cocaine use (Y/N)	0.493	.483	2.812	.095

*Model was adjusted for age, gender, viral load, CD4 cell count, and ART use.
P-value <0.05 is significant.

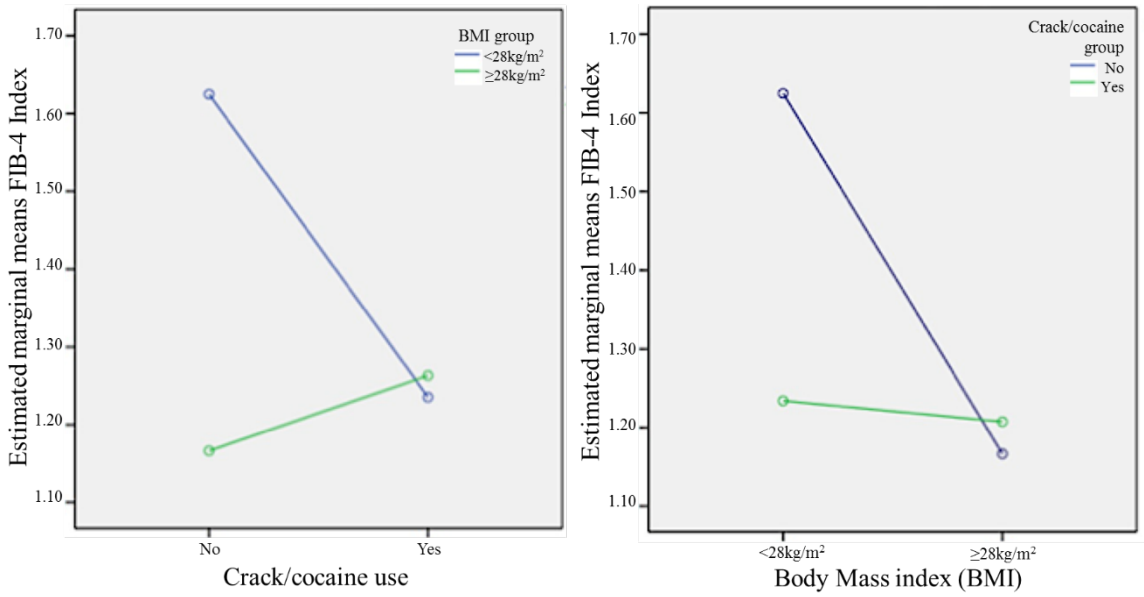

Figure 2: ANOVA analysis shows and interaction between BMI ≥ 28 kg/m^{2}and crack/cocaine use on FIB-4 index in HIV mono-infection.

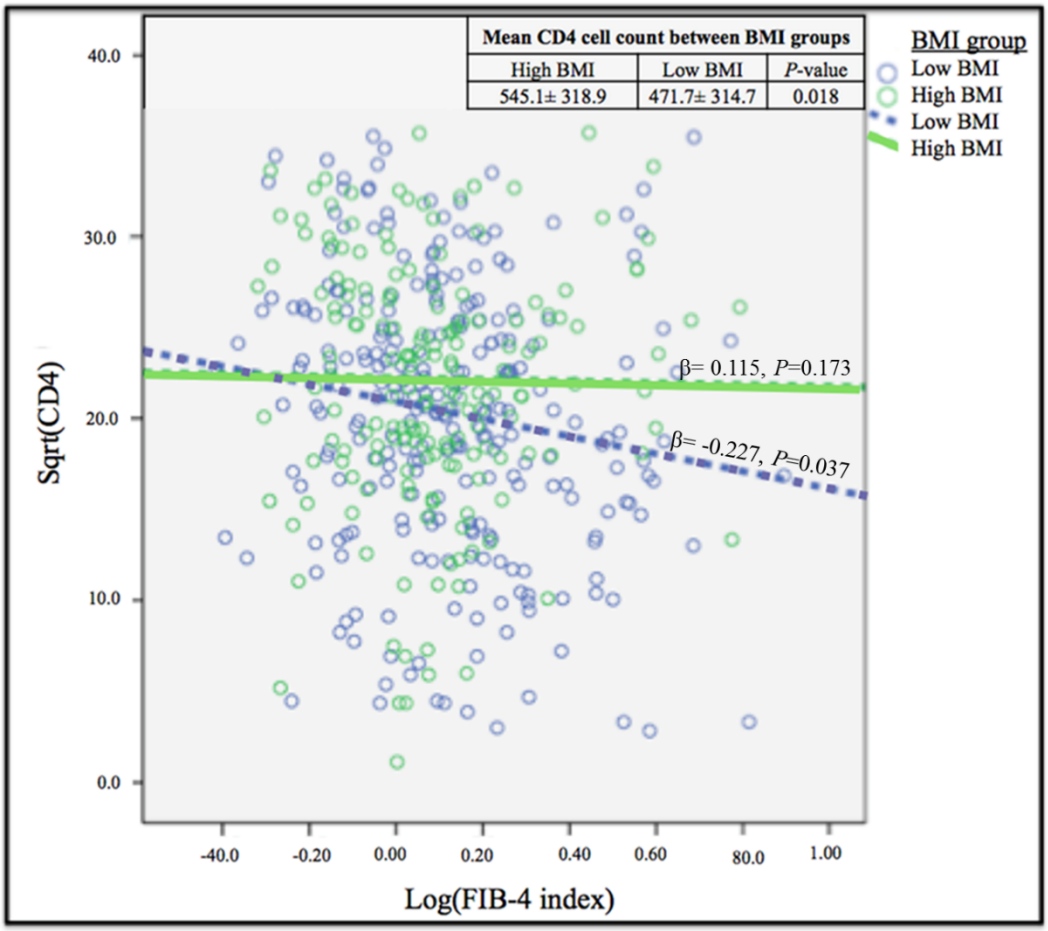

Figure 3: Associations between CD4 cell count and FIB-4 index within BMI categories. An association can be observed between lower CD4 cell count and increasing liver fibrosis (FIB-4 index) in participants in the low BMI. However, there is no association between CD4 cell count and FIB-4 in participants with high BMI due to the protective effect that BMI has on CD4 cell count. Participants with low BMI had significantly lower mean CD4 values than those with high BMI.

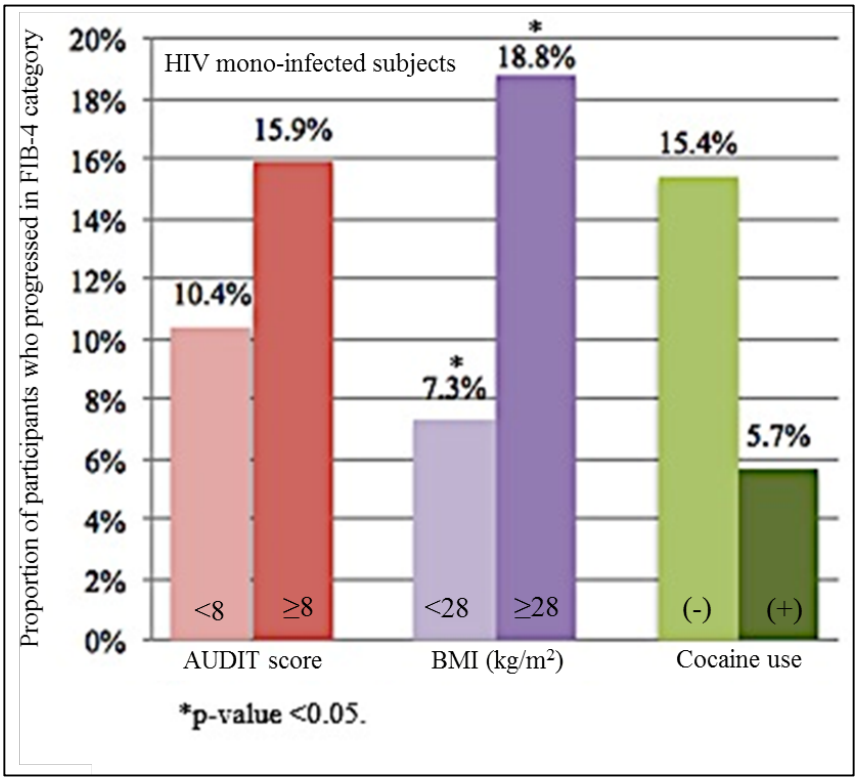

Figure 4: Proportion of HIV mono-infected participants who progressed one category of liver fibrosis over 2 years in each lifestyle group. Significantly more participants who were overweight progressed a stage of liver fibrosis than those who had a lower BMI.

Table 6: HIV mono-infection. Association between adverse lifestyle factors and the odds of progressing at least one category of fibrosis over 2 years.

Variables	**Unadjusted OR (95% CI) for progressing one FIB4 category**	***P*-Value**	**Adjusted OR (95% CI) for progressing one FIB4 category**	***P*-Value**
Model 1				
Alcohol ≥ 8	1.638 (.668 - 4.018)	.281	2.085 (.758 - 5.735)	.155
Model 2				
BMI ≥ 28 kg/m^2	**2.934 (1.132 - 7.605)**	**.027**	**3.797 (1.301 - 11.077)**	**.015**
Model 3				
Crack/Cocaine Use (Y/N)	.328 (.093 - 1.162)	.084	.288 (.076 - 1.095)	.068
Model 4[†]				
Alcohol ≥ 8	2.450 (.935 - 6.423)	.068	**3.038 (1.010 - 9.135)**	**.048**
BMI ≥ 28 kg/m^2	**2.907 (1.087 - 7.777)**	**.034**	**4.108 (1.360 - 12.403)**	**.012**
Crack/Cocaine Use (Y/N)	.308 (.082 - 1.161)	.082	**.228 (.057 - .918)**	**.038**

*Models were adjusted for age, gender, HIV viral load, CD4 cell count, and ART use.
[†]Model controlled for age, gender, HIV viral load, CD4 cell count, ART use, and BMI.

Table 7: Association with FIB-4 index and continuous variables BMI, AUDIT score, and cocaine use in HIV/HCV co-infection.

	Unadjusted model			Adjusted model*		
Variable	β	t	*P*-value	β	t	*P*-value
BMI (kg/m^2)	0.003	0.725	0.470	0.004	0.981	0.329
AUDIT score	0.001	0.484	0.629	0.001	0.607	0.545
Cocaine (Y/N)	-0.028	-0.569	0.571	-0.048	-0.993	0.323

*Model was adjusted for age, gender, viral load, CD4 cell count, and ART use.

Table 8: ANOVA analysis showed independent effects of being overweight, drinking hazardously, and using cocaine on FIB-4 index in HIV/HCV co-infection.

Variable	**F unadjusted**	***P*-value**	**F adjusted***	***P*-value**
AUDIT score ≥ 8	.931	.336	1.046	.303
BMI ≥ 28 kg/m^2	1.591	.209	.390	.533
Cocaine use (Y/N)	0.101	.751	.442	.507

*Model was adjusted for age, gender, viral load, CD4 cell count, and ART use.

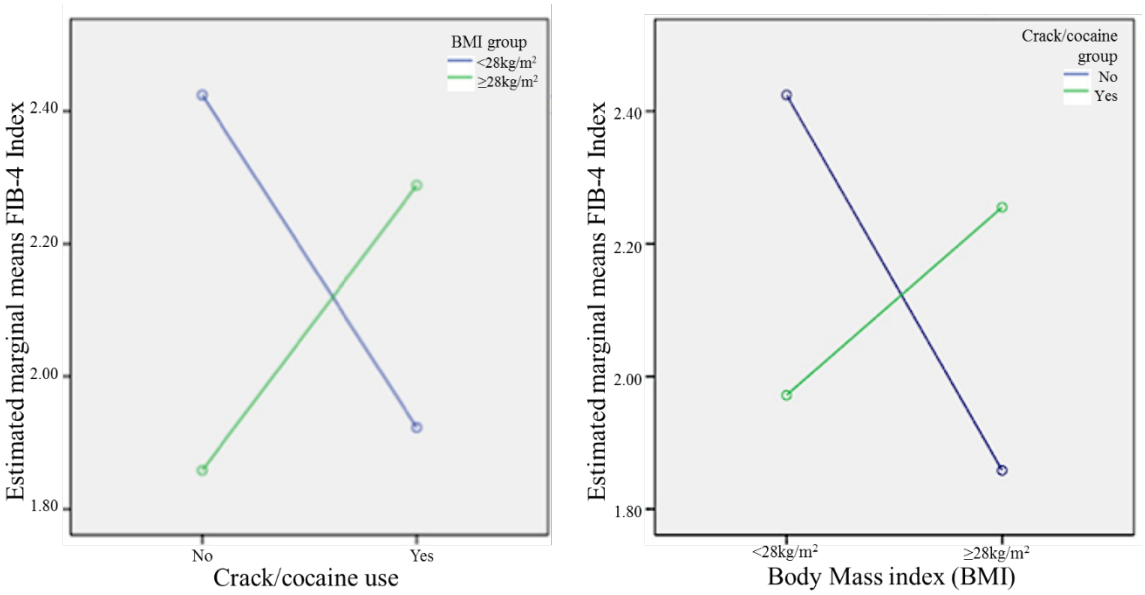

Figure 5: ANOVA analysis shows an interaction between BMI ≥ 28 kg/m^{2}and crack/cocaine use on FIB-4 index in HIV mono-infection.

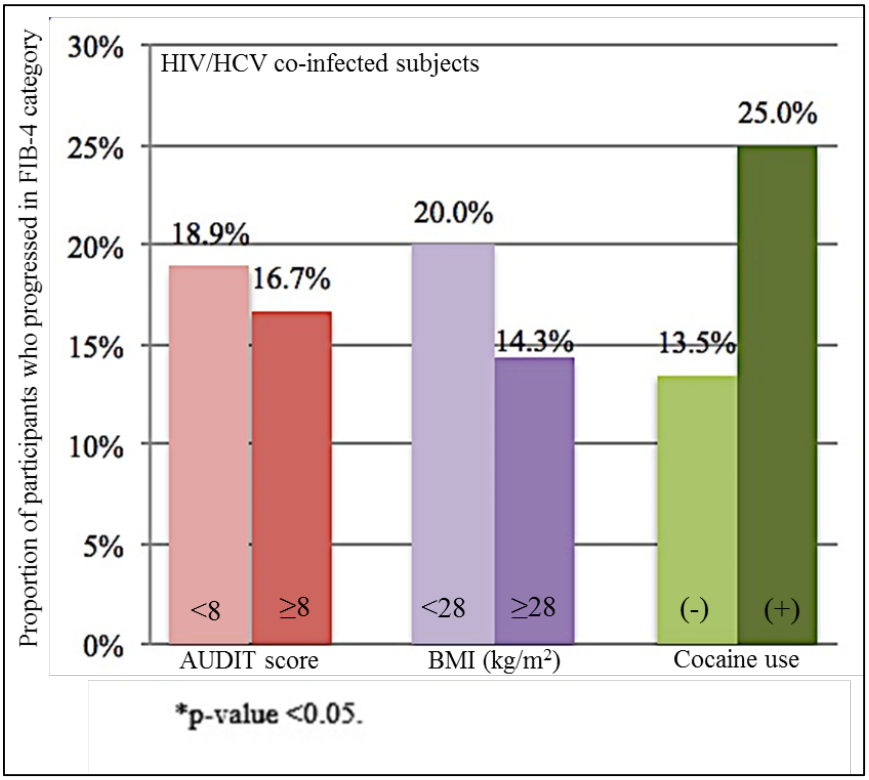

Figure 6: Proportion of HIV/HCV co-infected participants who progressed one category of liver fibrosis over 2 years in each lifestyle group. None of the lifestyle groups showed a statistically significant higher proportion of participants progressing in fibrosis stage over 2 years, perhaps due to the smaller sample size of HIV/HCV co-infected participants analyzed longitudinally (n=61).

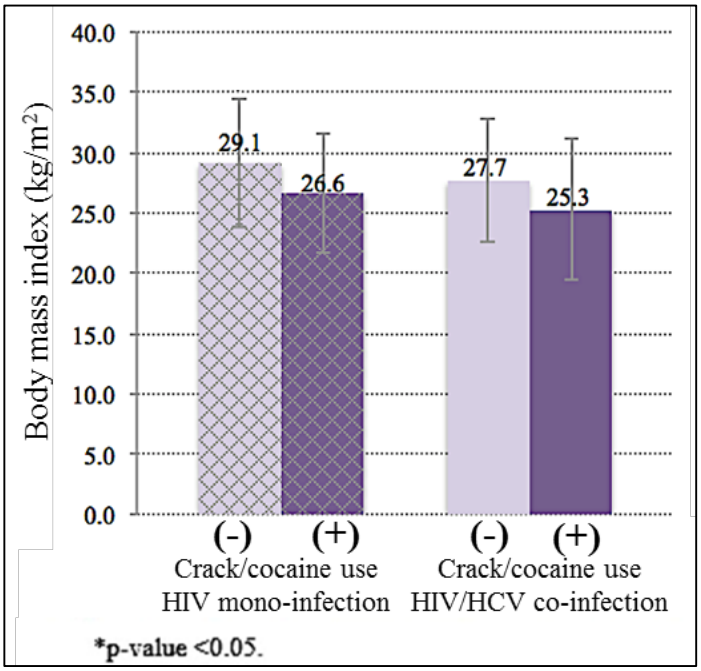

Figure 7: Mean BMI between cocaine groups in HIV mono- and HIV/HCV co-infection. Cocaine users show no difference in mean BMI values. Mean BMI values for both cocaine users and non-users fall into the "overweight" category.

Table 9: HIV/HCV co-infection. Association between adverse lifestyle factors and the odds of progressing at least one category of fibrosis over 2 years.

Variables	Unadjusted OR (95% CI) for progressing one FIB4 category	*P*-Value	Adjusted OR (95% CI) for progressing one FIB4 category	*P*-Value
Model 1				
Alcohol ≥ 8	0.857 (0.222 – 3.315)	0.823	0.766 (0.177 – 3.325	0.722
Model 2				
BMI ≥ 28 kg/m^2	0.671 (0.174 – 2.584)	0.562	0.581 (0.128 – 2.644)	0.482
Model 3				
Crack/Cocaine Use (Y/N)	2.133 (0.570 – 7.985)	0.261	2.687 (0.627 – 11.513)	0.183
Model 4†				
Alcohol ≥ 8	0.540 (0.112 – 2.597)	0.442	0.412 (0.068 – 2.492)	0.334
BMI ≥ 28 kg/m^2	0.818 (0.813 – 3.662)	0.793	0.775 (0.158 – 3.809)	0.754
Crack/Cocaine Use (Y/N)	2.735 (0.588 – 12.711)	0.199	3.764 (0.675 – 20.972)	0.130

*all adjusted models controlled for age, gender, HIV viral load, CD4 cell count, and ART use.
†Model controlled for age, gender, HIV viral load, CD4 cell count, ART use, and BMI.

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CHAPTER V: BIOLOGICAL MEDIATORS PREDICT LIVER FIBROSIS PROGRESSION OVER TIME IN AN HIV MONO-INFECTED COHORT

Abstract

Background: As the HIV infected population is aging with the advent of effect antiretroviral therapies (ART), significant morbidity and mortality can be attributed to non-AIDS defining illnesses in people living with HIV (PLWH). While co-infection with HIV and Hepatitis C (HCV) is a major contributor to liver disease progression, higher prevalence of liver fibrosis is observed in HIV mono-infected populations. New and effective direct acting antivirals that target HCV infection may also reduce the risk of liver decompensation in HIV/HCV co-infection, encouraging research into independent mediators of liver disease related to HIV mono-infection. Biological mediators that are routinely high in HIV disease and associated with liver fibrosis in cell and animal models include oxidative stress, hepatocyte apoptosis, transforming growth factor-beta1 (TGF-β1), and microbial endotoxin. The aim of this study is to associate biological mediators of liver fibrosis in HIV mono-infected participants to predict development of liver fibrosis over 2 years.

Methods: A subset of the Miami Adults Studies on HIV (MASH) cohort in Miami, FL (n=65) was chosen for longitudinal analysis. Participants were grouped into categories of liver fibrosis at baseline according to their FIB-4 score. Laboratory values for malondialdehyde (MDA), percent oxidized glutathione (GSH), hepatocyte apoptosis marker Cytokeratin 18 (CK-18), TGF-β1, and the microbial endotoxin lipopolysaccharide (LPS) were measured in plasma using commercially available kits. Multivariate linear regressions were used to find associations between biological mediator and FIB-4 index

cross-sectionally. Logistic regression and General Estimating Equations were used to describe associations between liver fibrosis progression and biological mediators of fibrosis over 2 years. Mixed model analysis used repeated measures to describe the associations between biological factors and liver fibrosis (FIB-4 index) with time as an interaction. All models were adjusted for covariates that are known to influence liver fibrosis.

Results: In this subset of 65 participants of the MASH cohort, 13.8% of HIV mono-infected participants progressed at least one FIB-4 stage from baseline to 2 years. High % oxidized glutathione (≥ 24% at baseline) was associated with 4.342 times higher odds of progressing in fibrosis category over time, after adjusting for covariates (adjusted OR 4.342 [95% CI 1.026 – 18.144]; *P*= 0.046). LPS showed a trend of increasing the odds of progressing in liver fibrosis (OR 1.098 [95% CI 0.794 – 5.741]; *P*= 0.097], indicating a small (9%) increase in the odds of liver fibrosis progression. CK-18 protein was significantly associated with an increase in the odds of progressing FIB-4 category over 2 years (OR 1.008, [95% CI 1.001 – 1.015]; *P*=0.021), and a trend in progressing at least one category of liver fibrosis over two years (OR 4.311 [95% CI 0.881 – 21.089]; *P* = 0.071). Percent oxidized glutathione was significantly associated with small probability of progression of liver fibrosis in the adjusted model (OR 1.063 [95% CI 1.101 – 3.432]: *P*=0.003). There were no significant associations in this subset between FIB-4 and MDA, and TGF-β1.

Conclusions: Biological mediators associated with increased liver fibrosis may serve as potential targets for future therapy, so it is important to identify biomarkers associated with the progression of liver fibrosis in HIV mono-infection. Establishing cut off values

for some biological mediators of liver fibrosis, such as LPS, may be useful to predict liver fibrosis. Larger studies are warranted to investigate mediators of fibrosis in PLWH.

Keywords: HIV infection, liver fibrosis, FIB-4 index, liver disease, oxidative stress, hepatocyte apoptosis, transforming growth factor-beta1, microbial endotoxin, malondialdehyde, glutathione, MDA, GSH, TGF-β1, CK-18, LPS

Introduction

Early initiation of antiretroviral therapy (ART) has significantly increased life expectancy in people living with HIV (PLWH) in the United States and Canada.[1] As this population is aging, morbidity and mortality from non-AIDS defining illnesses has increased.[2,3] Liver disease is a major cause of mortality in PLWH, contributing to between 14-18% of the deaths ascribed to non-AIDS related causes, mainly attributed to HIV/hepatitis C (HCV) co-infection.[2,4,5]

Infection with HCV increases the odds of liver fibrosis progression and mortality, however investigators have also reported increased liver-related mortality in HIV mono-infected populations when compared to non-infected populations,[6-8] indicating that HIV mono-infection may be an independent risk factor for liver disease. Furthermore, recent developments in HCV direct acting antivirals (DAAs) offer a new line of defense against liver decompensation in the HIV/HCV co-infected population,[9,10] making it more compelling to investigate factors associated with liver fibrosis in HIV mono-infected populations.[11] Liver fibrosis is used as a determinant of the seriousness of liver disease and its prognosis.[12] A useful tool for determining liver fibrosis in PLWH is the FIB-4 index that takes into account age, liver enzymes, and platelet count.[13,14] Although with lower sensitivity and specificity than liver biopsy, FIB-4 can distinguish between none or mild fibrosis (equivalent to METAVIR stage F0 –F1), and presence of advance fibrosis/cirrhosis (equivalent to METAVIR stage ≥ F2), and its main advantage is that it is not invasive and uses biomarkers that are collected routinely to follow HIV-infected patients.[15,16]

Pathogenesis of liver fibrosis in HIV is associated with biological mediators that have been investigated in cellular and animal models. Liver fibrosis is a cellular mechanism initiated by presence of injury to hepatocytes. It can be classified into sequential phases based on the activation of hepatic stellate cells (HSCs): initiation phase and perpetuation phase.[17] The initiation phase refers to early events that render the quiescent hepatic stellate cells (HSCs) responsive to a range of growth factors.[17] Important fibrogenic stimuli in this stage include: reactive oxygen species,[18-20] leading to oxidative stress; apoptotic fragments, generated from hepatocyte cell death,[21,22] and the bacterial endotoxin lipopolysaccharide (LPS),[23] resulting from bacterial products crossing from the gut into the blood stream.[17] The perpetuation phase involves proliferation and migration of HSCs to the extra cellular matrix (ECM), where they synthesize and secrete elevated levels of collagen and fibronectin, promoting fibrosis.[24,25] The upregulation of the cytokine transforming growth factor beta-1 (TGF-β1) is characteristic of the perpetuation phase because it is released by Kupffer cells, platelets, and HSCs themselves in the presence of fibrogenic stimuli.[26] TGF-β1 is a key regulator of liver fibrosis because it is required by HSCs to lay down fibrotic tissue.[27] Anti-TGF-β1 strategies have been proposed to reduce liver fibrosis by blocking its fibrogenic properties,[28] including direct neutralization of TGF-β1 and indirectly reducing TGF-β1 by targeting biological risk factors such as oxidative stress,[29] microbial translocation,[30] and apoptosis.[31]

While clinical markers have been associated with liver fibrosis in HIV, such as liver enzymes,[32] HIV viral load,[33] CD4 cell count,[34] and age,[35] few research studies have investigated biological mediators that are increased in HIV mono-infected persons and their impact on liver fibrosis over time. Biological mediators of advancing liver fibrosis

and liver pathogenesis have been determined in animal and in vitro models,[17] however, human studies have not been conducted, although increased levels of individual markers of fibrogenesis, such as increased oxidative stress,[36,37] hepatocyte apoptosis,[38] transforming growth factor-beta 1 (TGF-β1),[39]and bacterial endotoxin[40] have been demonstrated in HIV-infected populations. This study aims to investigate associations between biological mediators that contribute to liver fibrosis in HIV mono-infection longitudinally.

Methods

Study Design and Participants

The Miami Adult Studies on HIV (MASH) cohort is an ongoing research project conducted in Miami, FL that follows HIV mono- and HIV/HCV co-infected participants longitudinally. Participants signed informed consent upon screening and agreed to have their data and biological samples stored for future analyses. A convenience sample that included 65 HIV-mono infected participants was selected for longitudinal analyses at baseline, 12 month, and 24 months. Out of 464 participants enrolled at the time of the present study, 118 were excluded due to HCV co-infection, because the known contribution of HCV to the progression of liver fibrosis. Participants were selected for analysis if they had complete data sets, including all covariates and FIB-4 data at baseline, 12 months, and 24 months. Subjects were excluded if baseline FIB-4 index was ≥3.25. Laboratory data for biological mediators were generated at Florida International University using a repository of samples labeled with an identification number that was not associated with personal or identifying patient information. The study was approved by the Institutional Review Board at Florida International University.

Biomarker Analysis

1. Measures of oxidative stress:

1.a. Malondialdehyde (MDA) is a widely accepted marker of lipid peroxidation, which correlates well with liver fibrosis in other populations.[37,41,42] MDA is measured using a TBARS assay (Northwest Life Science Specialties, Portland, OR, USA). MDA reacts with thiobarbituric acid (TBA) to form a complex that is highly absorbed at 532nm, which can be read spectrophometrically.[43]

1.b. Glutathione (GSH) is an important part of the endogenous antioxidant system because it is the most abundant intracellular antioxidant, and directly participates in quenching harmful peroxide radicals as well as recycling other antioxidants.[18] GSH represents antioxidant status by measuring the ratio of oxidized glutathione (GSSH) to total glutathione (oxidized GSSH + reduced GSH). Measuring levels of reduced GSH alone does not indicate oxidative stress levels because GSH measures vary across time in an individual; however, a higher ratio of oxidized glutathione to total glutathione is indicative of higher oxidative stress. Healthy values of oxidized glutathione are under 10% of the total glutathione pool.[44] Percent oxidized glutathione is measured using a colorimetric assay that measures total and oxidized glutathione (Arbor Assays, Ann Arbor, MI, USA). Both MDA and % oxidized glutathione kits have been used in our previous studies with the MASH cohort.[36,43]

2. Measures of Fibrogenesis:

2.a. The Apoptosense ELISA assay (Peviva, Nacka, Sweden) quantifies hepatocyte-specific apoptosis. The assay uses the M30 antibody specific for the cytokeratin-18 (CK-

18) protein in a sandwich ELISA kit with monoclonal antibodies specific for the caspase-cleaved CK-18 protein.[21,45-49]

2.b.The fibrogenic cytokine TGF-β1 is determined using sandwich ELISA methods from a commercially available kit. The Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) measures the activated form of TGF-β1 in plasma samples, and has been used in previous studies.[50,51]

3. Measures of Immune Activation

The bacterial endotoxin lipopolysaccharide (LPS) is a major component of Gram-negative bacterial cells walls and can be quantitatively assessed to determine the degree of microbial translocation.[52] Plasma LPS levels are directly associated with the degree of intestinal permeability following invasive surgery.[53]

3.a. The limulus amebocyte lysate (LAL) assay has been widely used for the detection of endotoxin for approximately 30 years,[54] and in HIV-related research.[52,55-57] Recently, methods have been developed to measure LPS using the LAL Lonza kit in plasma of HIV+ patients.[55] Circulating LPS is measured using a chromogenic assay (Lonza, Walkersville, MD, USA) using the EL800 microplate reader and GEN5 software.

Laboratory values were measured using residual plasma samples stored at -80°C until analysis was conducted in duplicate. Acceptable results had coefficient of variation (CVs) within 10% indicating a small variance between replicates; otherwise they were reanalyzed.

Two groups (low/high) were generated using highest tertile value as a cut off pointfor each biological marker. For example, a value that fell within the highest tertile was placed in the “high” group for that biomarker.

Index of Liver fibrosis

FIB-4 index is a non-invasive measure of liver fibrosis. Although liver biopsy is the current gold standard for liver fibrosis staging using METAVIR scores, it is highly invasive and unless needed for clinical diagnosis is associated with a high risk for research purposes[16] Specifically in HIV-infected patients, it is contraindicated because of the increased risk of internal hemorrhage due to thrombocytopenia.[15,16] FIB-4 index was calculated using data collected by the MASH cohort using the formula: [(AgeXAST)/(PlateletsX√(ALT))]. It has been used in previous studies in HIV infection and validated in patients with HCV infection [13,14] and HIV/HCV co-nfection[13] with specificity of 94.7% and 90%, and sensitivity of 74.3% and 70%, respectively. For baseline groups, participants were categorized as having no/mild fibrosis (FIB-4 index <1.45, METAVIR stage F0-F1), indeterminate fibrosis (1.45 ≥ FIB-4 < 3.25) or severe (FIB-4 ≥ 3.25 METAVIR stage ≥ F3-F4).[58] The absence of advanced fibrosis was determined with the cut off value of FIB-4 < 1.45 (METAVIR stage F0-F1) for cross-sectional and longitudinal analyses.[7,14,59] A participant qualified as progressing in at least one METAVIR stage or category of liver fibrosis if their baseline FIB-4 score increased from < 1.45 to greater than 1.45, or from between 1.45 – 3.25 to ≥ after 2 years of study enrollment (Figure 1).

Statistical Analysis

Baseline characteristics for progressors and non-progressors were assessed using Chi-square or Mann-Whitney U for non-parametric measures due to the unequal variances between progressors and non-progressors. Student's t-test evaluated differences between descriptive characteristics for each biomarker at different time points. A test of

proportions was conducted to determine the percent of participants who progressed in each biomarker group (high vs. low) over 2 years. Logistic regressions were used to determine the association between progressing at least one FIB-4 category over 2 years with high/low levels of biological mediators at baseline. Mixed model analysis used repeated measures to describe the associations between biological factors and liver fibrosis (FIB-4 index) with time as an interaction. FIB-4 was the dependent variable and the biomarkers of oxidative stress, fibrogenesis and immune activation were the independent variables. Significant interactions were evaluated in sub-analyses and predicted FIB-4 values were derived from General Estimating Equations and Mixed Models when appropriate. All models were adjusted for covariates that are known to influence liver fibrosis.

Covariates

Covariates included in the analysis are those associated with liver fibrosis in HIV infection. They include: age,[60] gender,[61,62] ethnicity,[35] CD4 cell count (baseline),[34] HIV viral load (baseline),[33] and ART use.[63,64]

Results

Descriptive characteristics

Biological mediators were separated into tertiles and categorized as "high" if values fell in the highest tertile. Table 1 shows the values for the tertile cutoff points for each biomarker associated with liver fibrosis. Table 2 describes the baseline characteristics for HIV mono-infected participants. In this HIV mono-infected subset, 13.8% (n=9) participants progressed at least one fibrosis category over 2 years of enrollment in the study. The participants who progressed over 24 months were older at

baseline, which trended towards significance (46.6±6.9 vs. 51.3+6.6 years, *P*=0.063). There were no significant differences in biological factors associated with liver fibrosis between FIB-4 category progressors and non-progressors at baseline. Table 3 shows descriptive characteristics for participants within each biomarker group at baseline and 24 months. Participants with low MDA had significantly higher viral load at the end of the study, and those with the high hepatocyte apoptosis (CK-18) were older. In participants with the highest levels of oxidized glutathione, more people were obese, and a higher percent identified as black non-Hispanic.

Biological markers associated with liver fibrosis progression over 2 years

The proportion of participants who progressed in FIB-4 stage over time is depicted in Figure 2. A greater proportion of participants with high MDA (21.7%), and TGF-β1 (18.2%) progressed compared to those with low MDA (9.3%) and TGF-β1 (11.6%); however, the differences in proportions are not statistically significant (*P*=0.138 and *P*=0.469, respectively).

The major aim of the study was to determine associations between high levels of biomarkers at baseline and the odds of progressing in FIB-4 category over 2 years. Logistic regressions were used to describe the association between baseline biomarkers (high/low) and the odds of progressing at least one category of FIB-4 (mild fibrosis: FIB-4 >1.45, moderate fibrosis: FIB-4 = 1.45-3.24, severe fibrosis: FIB-4 ≥ 3.25) over 24 months. Results in Table 4 show that high % oxidized glutathione (≥ 24% at baseline) was associated with 4.342 times higher odds of progressing in fibrosis category over time, after adjusting for covariates (OR 4.342 [95% CI 1.026 – 18.144]; *P*= 0.046). LPS showed a trend of increasing the odds of progressing in liver fibrosis (OR 1.098 [95% CI

0.794 – 5.741]; *P*= 0.097], indicating a small (9%) increase in the odds of liver fibrosis progression.

To evaluate the impact that biological mediators have on liver fibrosis progression at each time point, a model was developed using logistic regression to predict the odds of progressing in FIB-4 category. Figure 3a-e shows the predicted probabilities of progressing at least one category of fibrosis based on having high and low biomarkers at baseline, 1 year, and 2 years. Although none of the odds of progressing were statistically significant, the figure illustrates that the estimated probabilities of progressing in liver fibrosis are generally higher for participants with higher levels of biomarkers at each time point.

Biomarkers may impact liver fibrosis progression linearly, i.e. the range of biomarker values may matter more than having a high or low value. Logistic regression models assessed the effect of biomarkers as continuous variables on the odds of liver fibrosis progression over time. Results in Table 5 show that CK-18 protein is significantly associated with an increase in the odds of progressing FIB-4 category over 2 years (OR 1.008, [95% CI 1.001 – 1.015]; *P*=0.021, however, this significance was lost for CK-18 after adjusting for covariates.

Percent oxidized glutathione was significantly associated with small probability of progression of liver fibrosis in the adjusted model, suggesting that there is an interaction between glutathione and at least one of the covariates in the model that contributes to progression over time (OR 1.063 [95% CI 1.101 – 3.432]: *P*=0.003). Besides oxidized GSH, age was the only other variable in the model that contributed to the model to increasing the odds of progressing by one FIB-4 category (OR 1.120 [95%

CI -.989 – 1.292]; *P*=0.072); however, there was no significant interaction between oxidized GSH and age on progression of liver fibrosis (OR 1.031 [95% CI 0.836 – 1.271]; *P*=0.778).

Generalized Estimating Equation models adjusting for baseline values of biomarkers were used to determine associations between odds of progressing at least one fibrosis stage over 2 years. Table 6 shows that percent oxidized GSH is associated with progressing at least one stage of liver fibrosis on a linear scale (OR 1.072 [95% CI 1.017 – 1.131]; *P* = 0.010) and also when using a high cut off value (OR 4.897 [95% CI 1.080 – 22.206]; *P* = 0.039). High levels of hepatocyte-specific apoptosis marker CK-18 were associated with a trend in increasing odds of progressing in FIB-4 over 2 years (OR 4.311 [95% CI 0.881 – 21.089]; *P* = 0.071). CK-18 was not associated with liver fibrosis progression on a linear scale, indicating that higher levels of CK-18 have a stronger influence on liver fibrosis progression than lower levels.

Biological markers associated with FIB-4 index over 2 years

A mixed model approach to repeated measures was used to determine changes in the biological variables and FIB-4 index over time. The mean values for each variable are displayed in Table 6 at each time point. In this HIV mono-infected cohort, mean FIB-4 values increased from baseline to 24 months. Although the result was not statistically significant (*P*=0.403), the mean FIB-4 increased from mild fibrosis to moderate fibrosis, which represents a clinical increase in liver disease. The biomarkers that significantly increased over time were hepatocyte apoptosis (CK-18), pro-fibrogenic cytokine TGF-β1, and bacterial endotoxin (LPS). ART use and ethnicity (being Black non-Hispanic)

significantly interacted with time, which decreased the significance of the biomarkers, suggesting that these covariates may interact with biomarkers in repeated measures.

Mixed models with time as a covariate analyzed whether biomarkers were good predictors for FIB-4 index over 2 years. Figure 4a-e shows the predicted values of FIB-4 index between high and low biomarkers, while Table 7 displays the association between biomarkers as continuous predictors on FIB-4 index. The most significant increase in FIB-4 index was observed with hepatocyte apoptosis CK-18 as a predictor (β= 2.011, S.E. 0.985, *P*=0.043) (Figure 5), indicating that another covariate significantly contributes to liver fibrosis progression and supersedes any the effect of hepatocyte apoptosis. In the adjusted model (β = 0.218. S.E. = 0.094, *P*= 0.023), HIV viral load (log_{10}(copies/mL) was the only significant variable that contributed to advanced FIB-4 index. A sub-analysis was used to investigate the relationship between CK-18 and HIV viral load. The result showed an interaction, although not significant, between HIV viral load and CK-18 hepatocyte apoptosis on FIB-4 index over time (β = 0.040, S.E. = 0.126, *P*=0.075), which may highlight the relationship between HIV's ability to infect hepatocytes and cause apoptosis that leads to liver fibrosis.

Discussion

The aim of this study was to investigate biological mediators associated with liver fibrosis in HIV mono-infected participants. Liver fibrosis is a growing concern in PLWH,[5] and cannot fully be attributed to co-infection with the hepatitis virus.[6-8] The ability of the HIV virus to infect hepatocytes,[65,66] damage the intestinal barrier by depleting gut associated lymphoid tissue,[67,68] and cause immune dysregulation[69,70] contribute to liver fibrosis, and may explain higher rates of liver disease in this

population.[2,4] Biological mediators associated with increased liver fibrosis may serve as potential targets for future therapy, so it is important to identify biomarkers associated with the progression of liver fibrosis in HIV mono-infection.

In this subset of 65 participants of the MASH cohort, 13.8% of HIV mono-infected participants progressed in FIB-4 stage from baseline to 2 years (i.e. from no fibrosis and mild fibrosis, to indeterminate or severe, or from indeterminate to severe fibrosis or cirrhosis). Our results are in line with previous research studies that use more advanced categories of liver disease as outcomes. A study conducted by Sebastiani et al.[35] reported that 4% of HIV mono-infected subjects developed severe liver fibrosis over a median of 4.9 years. Another study that included 132 HIV mono-infected subjects reported 6.2% incidence of liver cirrhosis over 2.6 years.[71]

In the present study, high baseline values of oxidized glutathione and circulating microbial endotoxin (LPS) were associated with increased odds of progression in liver fibrosis stages after 2 years. Higher levels of oxidative stress, including excess glutathione is associated with liver fibrosis in HIV-infection regardless of HCV disease status.[43] Our findings show higher % oxidized glutathione associated liver fibrosis progression over time in HIV mono-infected participants. Notably, LPS was not associated with FIB-4 index when analyzed as a continuous variable, but showed a trend to predict liver fibrosis progressed with higher cut off values of endotoxin. This finding is supported by Balagopal et al.[68] who observed that the highest quartile values of LPS cutoff values increased the odds of developing liver cirrhosis 19.0 times compared to lower levels of LPS. However, a subset of this analysis showed that high LPS was not associated with cirrhosis 12 months prior to progression,[68] whereas our data show a trend

of high levels of LPS and progression of liver fibrosis over 2 years. Establishing cut off values may be important for certain biological mediators when predicting liver fibrosis progression, because once a "tipping point" is reached, it may ultimately lead to liver fibrosis progression. This phenomenon has been described by Cani et al.[72,73] in metabolic endotoxemia, whereby a threshold of LPS is reached that is associated with inflammatory triggers, insulin insensitivity, and liver disease. In the context of HIV, LPS levels could reach a dangerously high threshold in some patients that may trigger chronic immune activation.[52] Consistently high levels of LPS may decrease the ability of Kupffer cells to clear endotoxin, making the liver more susceptible to fibrosis development over time.[74,75]

On the other hand, certain biological mediators may be more useful predictors of liver fibrosis without the use of cut off points and liver fibrosis progression with cut off points. Our results show that the marker for hepatocyte apoptosis (CK-18) was associated with progressing in liver fibrosis category using a high cut off value and linearly associated with FIB-4 index over 2 years as a continuous measure. Interestingly, there was a slight interaction between CK-18 values and HIV viral load on FIB-4 index, which may indicate that with high HIV viral load, infection of hepatocytes is most likely more rapid, leading to apoptosis, and subsequently fibrosis.[38]

Average values of CK-18, TGF-β1, and LPS significantly increased in HIV mono-infected participants over 24 months, however, the interaction between time, ART use and black ethnicity decreased the statistical significance of the biomarkers. Mean values for FIB-4 index increased from baseline to 24 months in the HIV mono-infected cohort, and although the result was not statistically significant, there may be clinical

implications because the average value of FIB-4 advanced from mild/no fibrosis to moderate fibrosis.

A major limitation of this study is the small number of participants that progressed in the stage of fibrosis determined with FIB-4. This is not surprising however, as 24 months is not sufficient amount of time for a significant progression of liver fibrosis to occur.[71] Our data revealed trends that associated biological mediators with liver fibrosis progression in HIV mono-infected persons with a small sample of liver fibrosis progressors. A larger sample of HIV mono-infected participants followed for a longer period of time may provide a larger number of people who progress in liver fibrosis stage, and improve statistical significance. However, investigating these biomarkers in humans offers a great insight into potential therapeutic targets for HIV mono-infected persons at risk for developing liver fibrosis.

Conclusion

Our results indicate that the progression of liver fibrosis occurs in HIV mono-infection over time at least in a relatively small number of participants, in a fairly rapid manner. In addition, higher levels of oxidative stress, hepatocyte apoptosis, and microbial endotoxin are associated with liver fibrosis progression and FIB-4 index over time in HIV mono-infected participants. These findings elucidate potential biological processes that contribute to liver fibrosis progression in HIV mono-infection.

Tables and Figures

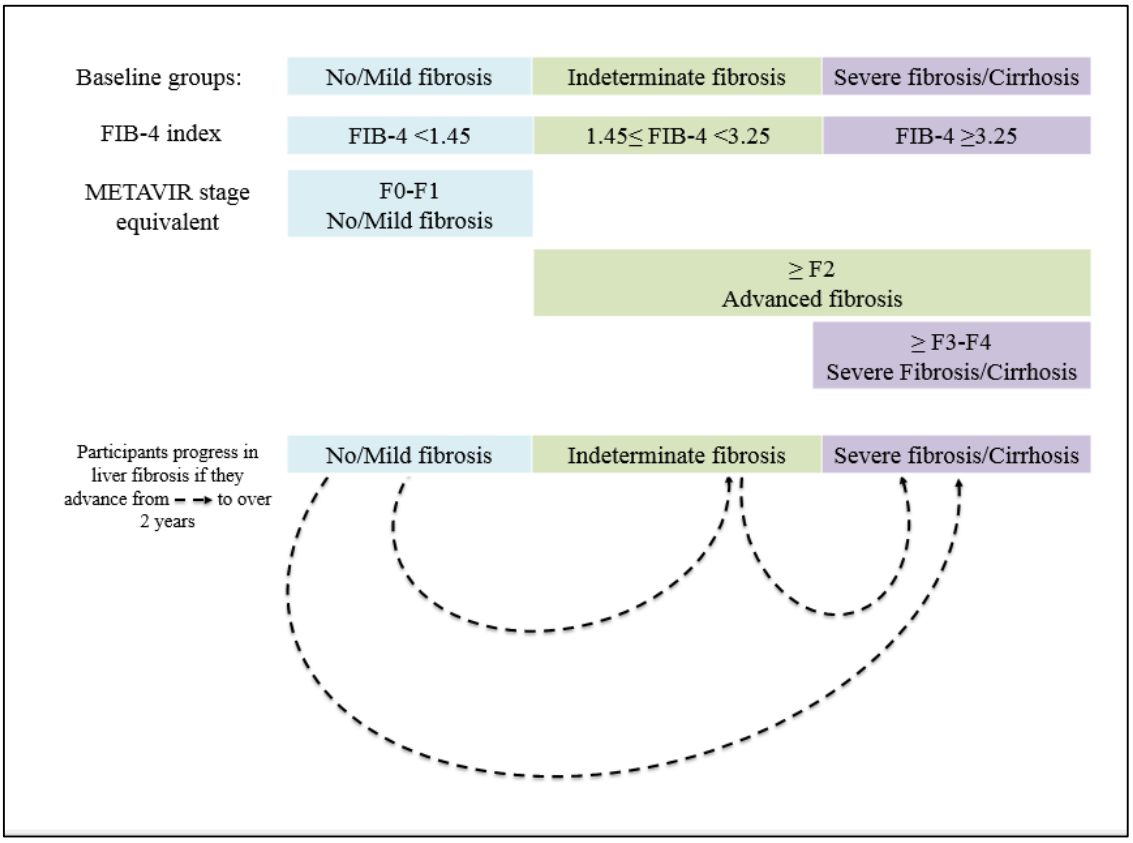

Figure 1: Schematic describing FIB-4 index and METAVIR stage equivalent used for the study. Participants who progressed at least one category of liver fibrosis over 2 years were considered "progressors". The cut off value used for statistical analyses was FIB-4 index ≥ 1.45 to establish presence or absence of advanced fibrosis.

Table 1: Tertile cutoff values for biomarkers associated with liver fibrosis at baseline (n=65)

	MDA (uM)	GSH (% oxidized)	CK-18 (pM)	TGF-β1 (pg/mL)	LPS (EU/mL)
Low tertile	0.438	19.6	90.0	1556.9	0.186
High tertile	0.617	27.9	100.4	5304.0	0.223

Table 2: Baseline characteristics of participants who did and did not progress in FIB-4 category.

	Did not progress in FIB-4 category Mean±(S.D) or %(n)	Progressed at least one FIB-4 category Mean±(S.D) or %(n)	*P*-value
	n=56	n=9	
Age (years)	46.6± 6.9	51.3± 6.6	0.063
Gender (% male)	69.1(38)	66.7(6)	0.884
Ethnicity (% non-Hispanic)	83.6(46)	66.7(6)	0.227
Log(HIV viral load)	2.41± 1.21	2.22± 0.90	0.644
CD4 cell count (cells/mm^3)	553.7± 398.3	396.5± 169.2	0.250
ART use (% yes)	89.1(49)	88.9(8)	0.986
BMI (kg/m^2)	27.8± 5.1	29.6± 6.3	0.348
Alcohol intake (AUDIT score)	6.0± 7.4	8.4± 8.7	0.392
Crack/cocaine use (% yes)	26.8(15)	11.1(1)	0.311
FIB-4 index	1.30± 0.55	1.35± 0.29	0.814
Biological Markers			
MDA (uM)	0.522± 0.196	0.641±0.269	0.115
GSH (% oxidized)	24.1± 9.2	28.6± 7.8	0.173
CK-18 (pM)	106.5± 47.5	129.5± 88.9	0.469
TGF-β1 (pg/mL)	4496.3± 4764.7	4863.3± 3495.3	0.826
LPS (EU/mL)	0.212±0.063	0.190± 0.059	0.334

P-value <0.05 is significant.

Table 3: Characteristics of biological mediators of liver fibrosis from baseline to 24 months.

	MDA Mean±(S.D.) or %(n)		% oxidized GSH Mean±(S.D.) or %(n)		CK-18 Mean±(S.D.) or %(n)		TGF-β1 Mean±(S.D.) or %(n)		LPS Mean±(S.D.) or %(n)	
Variable	Low n=43	High n=22	Low n=46	High n=19	Low n=43	High n=22	Low n=43	High n=22	Low n=43	High n=22
Age (years, baseline)	46.4±7.2	49.1±6.3	45.9±7.4	48.1±6.1	**45.6±7.0***	**50.4±5.8***	47.5±6.3	46.7±8.2	46.8±7.7	48.1±5.5
Gender (% male)	67.4(29)	71.4(15)	75.6(34)	52.6(10)	66.7(28)	72.7(16)	71.4(30)	63.6(14)	69.0(29)	68.2(15)
Ethnicity (% Black Non-Hispanic)	83.7(36)	72.7(16)	**71.7(33)***	**100(19)***	81.4(35)	72.7(16)	76.7(33)	86.4(19)	79.1(34)	81.8(18)
Log_{10}(HIV viral load)										
Baseline	2.48±1.19	2.21±1.12	2.34±1.06	2.50±1.42	2.43±1.16	2.29±1.20	2.29±1.08	2.57±1.32	**2.60±1.32***	**1.96±1.60***
24 months	**3.06±1.47****	**1.58±0.31****	2.62±1.43	2.42±1.37	2.62±1.39	2.39±1.45	2.60±1.32	2.35±1.74	2.32±1.26	2.97±1.58
CD4 cell count (cells/mm^3)										
Baseline	532±423	530±277	548±408	492±296	524±418	545±290	523±408	549±317	**460±270***	**672±508***
24 months	788±1085	424±348	573±599	816±1315	520±588	952±1347	518±553	1247±1709	688±1012	614±741
ART use (%Y)										
Baseline	90.7(39)	85.7(19)	91.1(41)	84.2(16)	90.5(38)	86.4(19)	100(43)	100(22)	83.7(36)	100(22)
24 months	100(43)	100(22)	100(46)	100(19)	100(43)	100(22)	100(43)	100(22)	100(43)	100(22)
BMI (kg/m^2)										
Baseline	27.6±5.2	29.1±5.4	27.8±5.2	28.7±5.7	27.8±5.0	28.7±5.9	28.0±5.5	28.3±4.9	28.4±5.6	27.5±4.7
24 months	27.8±5.5	30.1±6.4	**26.7±4.5***	**32.1±6.7***	28.6±5.7	28.1±6.4	28.7±6.5	27.8±4.3	28.7±6.1	27.6±5.4
FIB-4										
Baseline	1.39±0.58	1.15±0.42	1.28±0.46	1.37±0.65	1.29±0.54	1.34±0.47	1.28±0.41	1.36±0.68	1.28±0.49	1.37±1.58
24 months	1.34±0.66	2.27±5.08	1.82±3.55	1.30±0.89	1.77±3.65	1.44±0.73	1.75±3.63	1.48±0.95	1.83±3.67	1.31±0.50

P-values <0.05 are considered significant; *p<0.05, **p<0.001

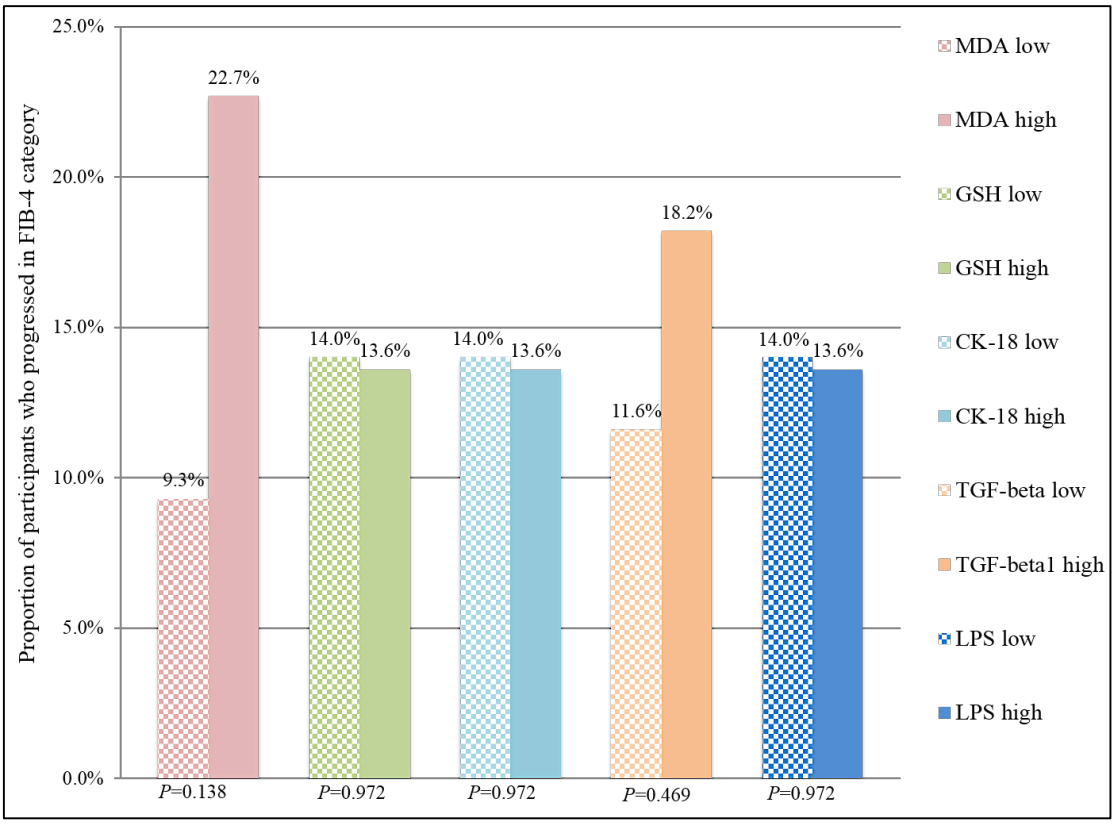

Figure 2: Proportion of liver fibrosis "progressors" within markers of oxidative stress, hepatocyte apoptosis and immune activation. A higher percent of participants with high MDA and high TGB-β1 at baseline progressed at least one category of FIB-4 over 24 months, however these results were not statistically significant.

Table 4: Odds of progressing in FIB-4 category over 24 months with high baseline biomarkers in (n=65) HIV mono-infected participants (n=9) progressed over 24 months.

	Unadjusted model			Adjusted model*		
Variable	β	OR	*p*-value	β	aOR	*p*-value
High MDA	0.332	1.394	0.646	0.569	1.766	0.488
High % oxidized GSH	1.035	2.815	0.097	**2.121**	**4.342**	**0.046**
High CK-18	-0.332	0.717	0.646	-0.727	0.483	0.403
High TGF-β1	0.497	1.644	0.496	0.973	2.647	0.250
High LPS	0.182	1.200	0.801	**0.914**	**1.098**	**0.097**

*Controlled for age, gender, $\log_{10}$(HIV viral load) and CD4 cell count, and ethnicity.

Note: High baseline levels of oxidized GSH and microbial endotoxin (LPS) were associated with increased odds of progressing at least one category of fibrosis over time.

Table 5: Logistic regression associations between biomarkers (as continuous variables) and odds of progressing FIB-4 category over 2 years.

	Unadjusted			Adjusted*		
Parameter (all time points)	Estimate	OR	*P*-value	Estimate	OR	*P*-value
MDA (uM)	1.648	5.197	0.147	1.079	2.942	0.465
GSH (% oxidized)	0.022	1.022	0.293	**0.066**	**1.063**	**0.003**
CK-18 (pM)	**0.008**	**1.008**	**0.021**	0.002	1.002	0.600
TGF- β1 (pg/mL)	0.001	1.001	0.431	0.001	1.001	0.738
LPS (EU/mL)	1.038	2.824	0.374	-2.485	0.083	0.230

*Controlled for age, gender, Log_{10}(HIV viral load) and CD4 cell count, and ethnicity.

Table 6: GEE associations between biomarkers and odds of progressing FIB-4 category over 2 years.

	Unadjusted			**Adjusted***		
Parameter (continuous)	**Estimate**	**OR**	***P*-value**	**Estimate**	**OR**	***P*-value**
MDA (uM)	-0.117	0.889	0.945	0.285	1.329	0.903
GSH (% oxidized)	**0.047**	**1.048**	**0.064**	**0.070**	**1.072**	**0.010**
CK-18 (pM)	0.004	1.004	0.533	0.008	1.008	0.299
TGF- β1 (pg/mL)	0.000	1.000	0.953	0.000	1.000	0.731
LPS (EU/mL)	-0.398	0.672	0.887	0.023	1.023	0.994
Parameter (high cut off)	**Estimate**	**OR**	***P*-value**	**Estimate**	**OR**	***P*-value**
High MDA $\geq$ 0.617 EU/mL	0.498	1.646	0.390	1.057	2.877	0.201
High % oxidized GSH $\geq$ 27.9%	0.879	2.409	0.128	**1.589**	**4.897**	**0.039**
High CK-18 $\geq$ 100.4 pM	0.884	2.421	0.123	**1.464**	**4.311**	**0.071**
High TGF-β1 $\geq$ 5304.0 pg/mL	-0.857	0.424	0.400	-1.571	0.208	0.221
High LPS $\geq$ 0.223 EU/mL	0.670	1.955	0.292	1.026	2.790	0.185

*Controlled for age, gender, Log_{10}(HIV viral load) and CD4 cell count, and ethnicity, and baseline parameter values.

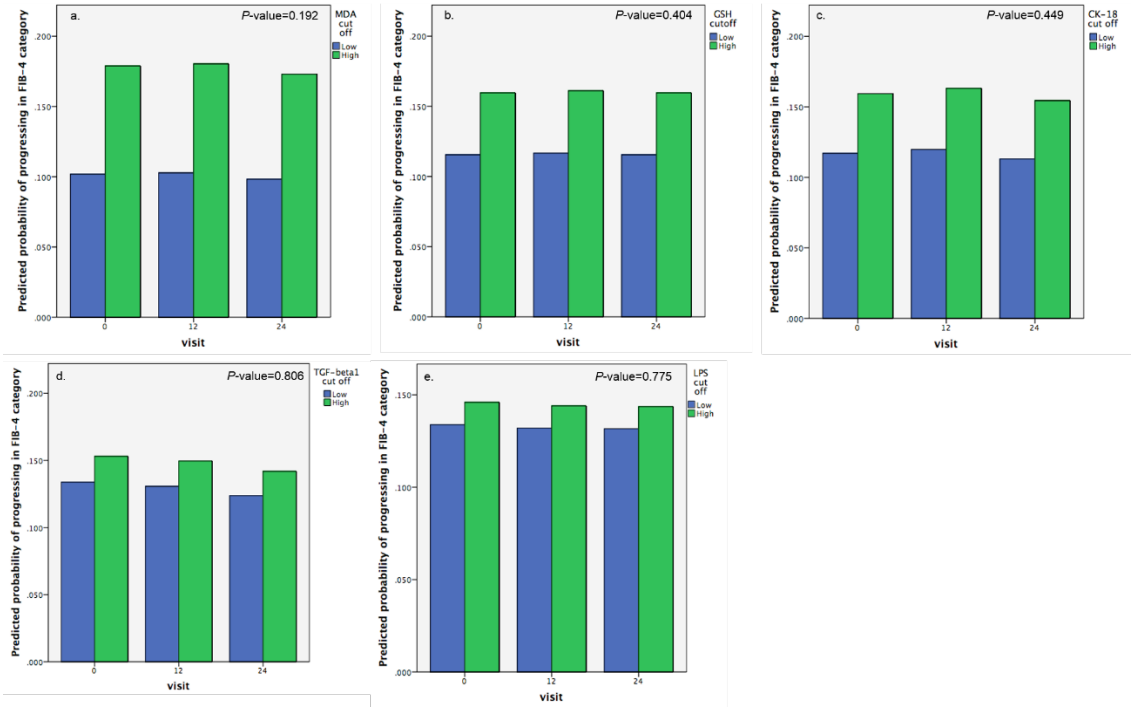

Figure 3a-e: Predicted probability of fibrosis progression at each time point associated with low/high values of biomarkers at baseline. The figure shows that at each visit the predicted probability of progressing in FIB-4 category over time based on having high/low biomarkers (MDA, GSH, CK-18, TGF-β1, LPS) at each time point. Participants categorized with high biomarkers have a higher predicted probability of progressing in FIB-4 category over time, compared to lower biomarker groups, however, these results did not reach statistical significance.

Table 7: Comparison of biomarkers at different time points (n=65).

Variables	Unadjusted	Adjusted*
FIB4		
Baseline	1.245 ± 0.530	1.245 ± 0.530
12 months	1.431 ± 1.514	1.431 ± 1.514
24 months	1.528 ± 2.851	1.528 ± 2.851
	P-value time = 0.403	*P*-value time = 0.743
MDA		
Baseline	0.556 ± 0.223	0.556 ± 0.223
12 months	0.574 ± 0.230	0.574 ± 0.230
24 months	0.581 ± 0.218	0.581 ± 0.218
	P-value time = 0.700	*P*-value time = 0.687
GSH		
Baseline	24.949 ± 8.879	24.949 ± 8.879
12 months	22.832 ± 10.046	22.832 ± 10.046
24 months	23.810 ± 10.262	23.810 ± 10.262
	P-value time = 0.412	*P*-value time = 0.283
CK-18		
Baseline	108.426 ± 52.632[a]	108.426 ± 52.632
12 months	95.628 ± 37.032[b]	95.628 ± 37.032
24 months	117.625 ± 78.615[c]	117.625 ± 78.615
	***P*-value time = 0.006**	*P*-value time = 0.438
TGF-β1		
Baseline	5122.520 ± 6379.338[a]	5122.520 ± 6379.338
12 months	10175.168 ± 8348.009[b]	10175.168 ± 8348.009
24 months	15840.438 ± 11117.780[c]	15840.438 ± 11117.780
	***P*-value time = 0.001**	*P*-value time = 0.834
LPS		
Baseline	0.211 ± 0.063[a]	0.211 ± 0.063
12 months	0.226 ± 0.096[b]	0.226 ± 0.096
24 months	0.319 ± 0.195[c]	0.319 ± 0.195
	***P*-value time = 0.001**	*P*-value time = 0.380

CK-18 *P*-value[b,c]=0.007
TGF-β1: *P*-value[a,b]=0.001,
P-value[a,c]=0.001, *P*-value[b,c]=0.003
LPS: *P*-value[a,c]=0.001, *P*-value[b,c]=0.002

* Adjusted for age, gender, ethnicity, HIV viral load $\log_{10}$ copies/mL, CD4 cell count, and ART use.

Table 8: Mixed model associations between biomarkers (as continuous variables) and FIB-4 index over 2 years.

	Unadjusted			Adjusted*		
Parameter (all time points)	Estimate	S.E.	p-value	Estimate	S.E.	p-value
MDA (uM)	0.513	0.651	0.431	0.747	0.522	0.156
GSH (% oxidized)	-0.102	0.014	0.472	-0.001	0.011	0.899
CK-18 (pM)	**2.011**	**0.985**	**0.043**	0.235	0.816	0.773
TGF-β1 (pg/mL)	0.044	0.148	0.765	-0.019	0.313	0.637
LPS (EU/mL)	0.298	0.777	0.701	0.434	0.739	0.558

*Controlled for age, gender, HIV viral load $\log_{10}$ copies/mL and CD4 cell count, and ethnicity.

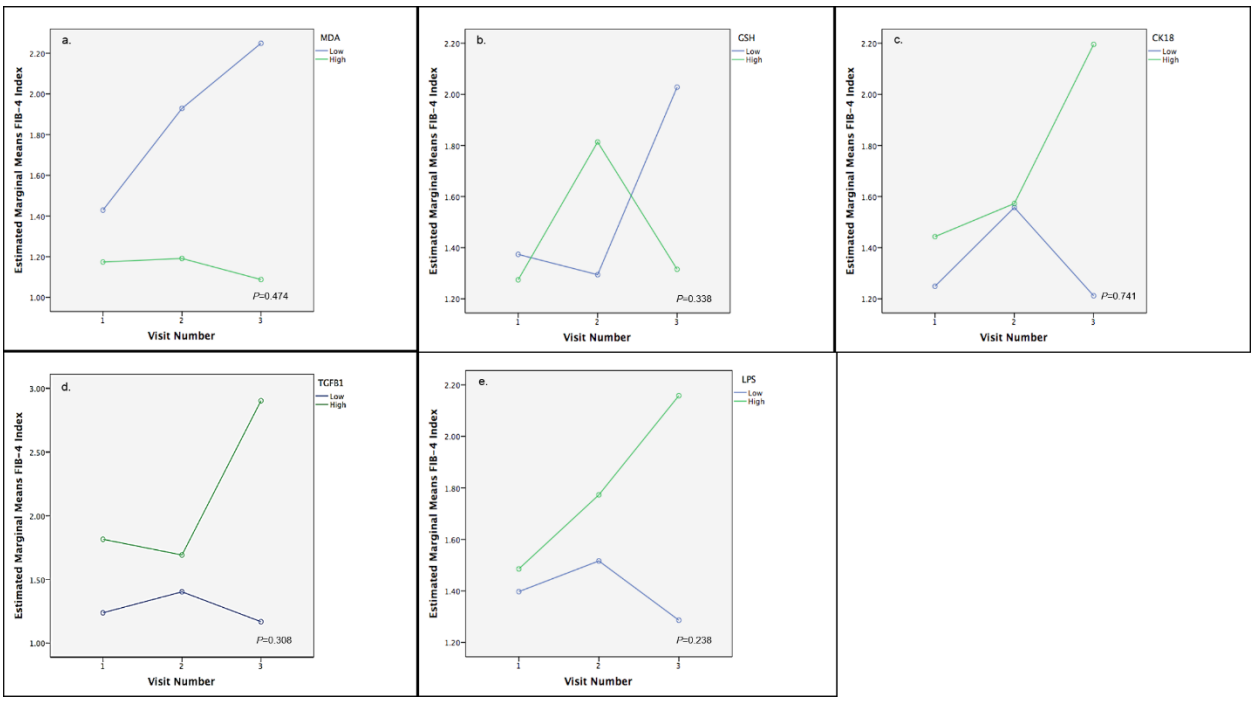

Figure 4: Mixed models predict FIB-4 values based on high/low biomarkers at each time point. Part a) shows low levels of MDA increasing FIB-4 index, b) shows interactions between high/low GSH groups, c, d, and e) show high levels of CK-18, TGF- β1, and LPS show trends of increasing estimated marginal means of FIB-4 index over time. Although the p-values did not reach statistical significance, there are clear trends for high levels of CK-18, TGF- β1, and LPS to predict an increase in FIB-4 index over 2 years.

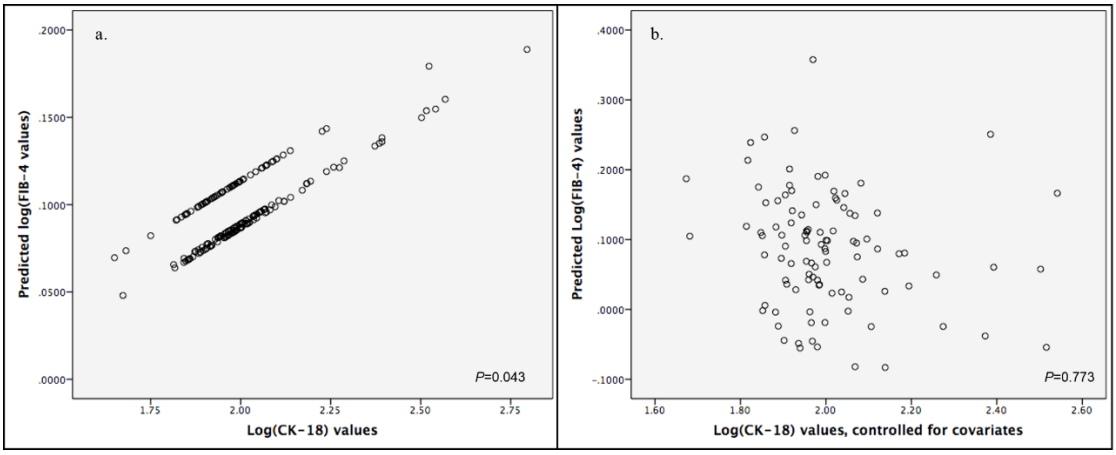

Figure 5a and b: Association between CK-18 values and Predicted FIB-4 with time as a covariate. The figure shows a) the significant relationship between the hepatocyte apoptosis marker CK-18 and predicted FIB-4 index, however, b) the significance is lost when controlling for covariates in the model.

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CHAPTER VI: MEDIATORS OF LIVER FIBROSIS ASSOCIATED WITH HAZARDOUS ALCOHOL USE, OBESITY, AND COCAINE USE IN HIV-INFECTION

Abstract

Objective: Liver fibrosis is a leading cause of morbidity and mortality in people living with HIV (PLWH). Co-infection with HIV and Hepatitis C (HCV) is a major contributor to liver disease progression, because of the ability of HCV and HIV to specifically infect hepatic stellate cells. Adverse lifestyle factors that are prevalent in PLWH include hazardous alcohol use, high body mass index (BMI), and illicit drug abuse, such as crack/cocaine use also may be significant contributors to liver fibrosis in HIV disease. Hazardous lifestyle factors are associated with increased levels of biological mediators associated with pathogenesis of liver fibrosis. This research aimed to investigate levels of biological mediators of liver fibrosis that contribute to liver fibrosis within harmful lifestyle factors using FIB-4 index as a non-invasive indicator in an HIV mono- and HIV/HCV co-infected people.

Methods: After excluding participants with multiple adverse lifestyle factors, baseline data was collected in n=67 HIV infected participants in the Miami Adults Studies on HIV (MASH) cohort in Miami, FL. Hazardous drinking was assessed by AUDIT score ≥ 8, obesity was considered ≥ 30 kg/m^2, and positive cocaine use was collected using participant self-report, and confirmed with urine toxicology test. Biological mediators of liver fibrosis including malondialdehyde (MDA), percent oxidized glutathione (GSH), hepatocyte apoptosis marker Cytokeratin 18 (CK-18), TGF-β1, and the microbial endotoxin lipopolysaccharide (LPS) were measured in plasma using commercially

available kits. Participants were grouped into categories of liver fibrosis at baseline according to their FIB-4 score generated by the equation that uses common laboratory values: [(AgeXAST)/(PlateletsX√(ALT))]. FIB-4 index < 1.45 ruled out advanced stage of hepatic fibrosis (METAVIR stage equivalent F0-F1), or advanced liver fibrosis FIB-4 index ≥ 1.45 (METAVIR stage equivalent ≥F2). Student t-test and one-way ANOVA were used to assess relationships between biological mediators of fibrosis between lifestyle groups. Multivariate analyses and logistic regression were used to evaluate associations between biological mediators and FIB-4 index within lifestyle groups.

Results: In the current study, higher levels of TGF-β1 was associated with higher liver fibrosis in HIV+ cocaine users (β=0.858, *P*=0.027). High % oxidized GSH was associated with liver fibrosis in obese subjects (β=0.563, *P*=0.018). Hepatocyte apoptosis (CK-18) independently predicted a higher FIB-4 index in the group without harmful lifestyle factors (β=0.435, *P*=0.015). MDA and TGF-β1 on liver fibrosis (F=3.171, *P*=0.071), indicating that mechanisms of hepatic pathogenesis may be elucidated in larger sample sizes of PLWH. There were no significant associations between MDA and LPS and liver fibrosis within lifestyle groups, and liver fibrosis did not differ significantly between lifestyle groups.

Conclusions: This study offers new insight into biological mediators of liver fibrosis and their interaction with harmful lifestyle factors associated with HIV-infection. Strategies to develop therapeutic targets, treatment options, and the utilization of diagnostic markers may become more personalized when examining the manner in which potential biomarkers are affected by individual lifestyle factors.

Keywords: HIV infection, HIV/HCV co-infection, liver fibrosis, alcohol, AUDIT, cocaine, body mass index, FIB-4 index, oxidative stress, hepatocyte apoptosis, transforming growth factor-beta1, microbial endotoxin, malondialdehyde, glutathione, MDA, GSH, TGF-β1, CK-18, LPS

Introduction

People living with HIV (PLWH) have longer lifespans with the successful use of antiretroviral therapies.[1] However, PLWH experience higher prevalence of non-infectious co-morbidities,[2] which negatively impacts the quality of life of the HIV-infected patient, increases morbidity and mortality, and unduly burdens health care systems.[3-6] A major co-morbidity identified in HIV infection is liver disease,[7-10] and a main diagnostic component of liver disease is liver fibrosis.[11] Hepatitis C co-infection (HIV/HCV) is attributed to the rapid progression of liver fibrosis in HIV-infected patients, and, there is increasing evidence that HIV mono-infection itself contributes to the development of liver fibrosis.[12-16] Thus, it is important to investigate the biological mediators of liver fibrosis in both HIV mono- and HIV/HCV co-infected persons at risk for developing liver fibrosis. Proposed biological mediators implicated in liver fibrosis and HIV infection include increased markers of oxidative stress,[17,18] pro-fibrogenic cytokine TGF-β1,[19-21] `microbial endotoxin lipopolysaccharide (LPS),[22,23] and hepatocyte apoptosis marker CK-18.[24,25]

Unhealthy lifestyle factors disproportionately affect PLWH in the United States, including increased hazardous drinking,[26,27] obesity,[26,28,29] and cocaine use.[30-32] Increased liver fibrosis is associated with having a high body mass index (BMI), drinking hazardously and using cocaine in the general population, as well as in the HIV-infected patients.[33-36] Furthermore, hazardous lifestyle factors are associated with increased levels of biological mediators associated with pathogenesis of liver fibrosis. For example, alcohol use and obesity independently increase circulating oxidative stress levels, which

stimulate the hepatic stellate cells to produce TGF-β1, which perpetuates the cellular framework of fibrosis.[37,38]

The combination of HIV infection and harmful lifestyle factors may advance biological markers of liver fibrosis. The aim of this research was to investigate specific biological markers associated with liver fibrosis in people with distinct harmful lifestyle factors in the Miami Adult Studies in HIV (MASH) cohort.

Methods

Study participants and design

Data from a subsample of the MASH cohort consisting of 454 HIV infected adults, with 29% of participants co-infected with HCV at the time of this cross-sectional secondary analysis was used for these analyses. Participants attended visits at FIU-Borinquen Research Clinic, Miami, FL every 3 months for the parent study. All participants gave written consent to participate and have agreed to their information used for future research at their baseline visit. Trained interviewers conducted patient interviews and collected anthropometric information. Blood samples were collected at baseline and annually via venipuncture by the study nurse and sent to a central laboratory (Quest Diagnostics, Miami, FL) for common laboratory data, and to FIU laboratory for processing and for specialized assays. The data used for this study was the cross-sectional baseline information the MASH cohort. A subset of the cohort participants was used for this study, who were categorized into different groups that included (i) Group 1: hazardous drinking (not obese and no cocaine use); (ii) Group 2: obese only (mild alcohol use, and no cocaine use); (iii) Group 3: cocaine use (mild alcohol use and not obese); and (iv) Group 4: control group (low alcohol use, not obese, and no cocaine use). Baseline

descriptive statistics and biological markers were characterized within and throughout the groups and associated with FIB-4, an index of liver fibrosis. The study was approved by the Institutional Review Board at Florida International University.

Measurements

The primary outcome of this study is stage of liver fibrosis. Laboratory data to obtain FIB-4 index was sent to a central laboratory (Quest Diagnostics, Miami, FL) for blinded analysis. FIB-4 index was calculated using the validated equation that include age, liver enzymes (AST, ALT) and platelets [(AgeXAST)/(PlateletsX√(ALT))].[39,40] Stage of liver fibrosis serves as an important prognostic factor for liver disease because it defines the extent of liver disease advancement for therapeutic decisions.[11] Although liver biopsy is the current gold standard for liver fibrosis, it is highly invasive, carries a high risk for research purposes.[41] Specifically in HIV infected populations, it is contraindicated because there is an increased risk of internal hemorrhage due to thrombocytopenia.[41,42] Sterling et al.[39] have described the FIB-4 index, which consists of alanine aminotransferase (ALT) level, AST level, PLT counts, and age, for assessing fibrosis in a large cohort of patients with HIV/HCV co-infection. The FIB-4 index has also been validated as an inexpensive and accurate marker of fibrosis in the context of HCV mono-infection with a sensitivity of 74.3% and specificity of 80.1% (FIB-4 cut off< 1.45) to rule out advanced liver fibrosis.[40] FIB-4 index also correlates to METAVIR stage of fibrosis that corresponds to to liver biopsy measurements such that FIB-4 <1.45 is associated with a METAVIR stage of F0-F1 (no/mild liver fibrosis) and FIB-4 ≥ 1.45 is associated with METAVIR stage ≥ F2 (advanced liver fibrosis).[43] FIB-4 index was examined as a continuous variable or participants were categorized having the presence

or absence of advanced hepatic fibrosis, as determined by the cut off value of FIB-4 index ≥1.45 (METAVIR stage ≥ F2) or FIB-4 < 1.45 (METAVIR stage F0-F1, respectively).

Lifestyle groups were determined using data collected during the baseline interview of the study. Information about alcohol consumption was obtained using the Alcohol Use Disorders Identification Test (AUDIT), a questionnaire developed by the World Health Organization to identify patients who drink hazardously with an AUDIT score ≥8.[44-47] Body mass index (BMI) was obtained by measuring height using a stadiometer and weight on a calibrated scale when subjects were dressed in light clothing and after removing shoes. Participants were categorized as being obese with BMI values ≥ 30 kg/m^2.[48] Interviewers utilized a drug-frequency questionnaire to obtain self-reported crack/cocaine use, and the information was confirmed with urine toxicology. Positive self-report or positive urine toxicology indicated that participants may be categorized in the cocaine use group.

Covariates in analyses included variables that are known to be associated with liver fibrosis in HIV, including age, gender, and ART use. HIV disease parameters (HIV viral load, and CD4 cell count) were obtained from primary care physicians with the consent of the participants at each visit.

Biological Mediators

Biological mediators served as independent predictors of liver fibrosis (FIB-4 index) within lifestyle groups. A secondary aim of the study was to investigate the impact of lifestyle factors on biological markers as dependent variables.

Malondialdehyde (MDA) is a widely accepted marker of lipid peroxidation, which correlates well with liver fibrosis in other populations.[49-51] MDA is measured using a TBARS assay (Northwest Life Science Specialties, Portland, OR, USA) whereby it reacts with thiobarbiuric acid (TBA) to form a complex that is highly absorbed at 532nm, which can be read spectrophometrically.

Glutathione (GSH) is an important part of the endogenous antioxidant system because it is the most abundant intracellular antioxidant, and directly participates in quenching harmful peroxide radicals as well as recycling other antioxidants.[18] GSH represents antioxidant status by measuring the ratio of oxidized glutathione (GSSH) to total glutathione (oxidized GSSH + reduced GSH). Measuring levels of reduced GSH alone does not indicate oxidative stress levels because GSH measures vary across individual, however, a higher ratio of oxidized glutathione to total glutathione is indicative of higher oxidative stress. Healthy values of oxidized glutathione are close to 10% of the total glutathione pool in tissues and 16% in plasma.[52-54] Percent oxidized glutathione is measured using a colorimetric assay that measures total and oxidized glutathione (Arbor Assays, Ann Arbor, MI, USA).

Bacterial endotoxin lipopolysaccharide (LPS) is a major component of Gram-negative bacterial cells walls and can be quantitatively assessed to determine the degree of microbial translocation.[55] The limulus amebocyte lysate (LAL) assay has been widely used for the detection of endotoxin for approximately 30 years,[56] and in HIV-related research.[55,57-59] Recently, methods have been developed to measure LPS using the LAL Lonza kit in plasma of HIV+ patients.[57] Circulating LPS was measured in plasma using a chromogenic endpoint assay (Lonza, Walkersville, MD, USA), Samples were heated to

75 C for 15 min to reduce protein interference and diluted (1:10) then spiked with endotoxin when values were undetectable, and read using the EL800 microplate reader and GEN5 software.

Fibrogenic cytokine TGF-β1 uses ELISA methods from a commercially available kit. The Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) measures the activated form of TGF-β1 circulating in plasma samples, and has been used in previous studies in human plasma with a sensitivity of 15.4 pg/mL.[60,61]

Hepatocyte-specific apoptosis (CK-18) is quantified with the M30 Apoptosense ELISA assay (Peviva, Nacka, Sweden) that uses the M30 antibody specific for the cytokeratin-18 (CK-18) protein. Programmed cell death, or apoptosis, in hepatocytes has been suggested as a mechanism for liver fibrosis because apoptotic bodies initiate inflammation and activate hepatic stellate cells.[62] Hepatocytes undergoing cell death produce CK-18 and circulating levels are associated with hepatic injury and fibrosis.[24,25,63-66]

Laboratory values for biological mediators were measured using residual blood samples stored at -80°C until analysis in duplicate. Acceptable results had coefficient of variation (CVs) within 10% indicating a small variance between replicates; otherwise they were reanalyzed.

Statistical Analysis

Descriptive statistics were conducted by t-test or one-way ANOVA for continuous variables, and Chi-square for categorical variables. Non-parametric equivalent tests were employed when equality of variances were not met. Multivariate linear regression was used to find associations within lifestyle groups for individual biological

markers (MDA, % oxidized GSH, CK-18, TGF-β1, LPS) and liver fibrosis (FIB-4 index) as the dependent variable. Logistic regression was used to examine binary outcomes of biological mediators categorized into high/low categories and categories of lifestyle groups.

Statistical models were adjusted for covariates that are known to affect liver fibrosis in HIV infection including age, gender, CD4 cell count, HIV viral load, ART use, and co-infected status. Covariates that were significantly associated with dependent variables (i.e., FIB-4 index or biological mediators) were kept in the model for analyses.

Results

After removing subjects with missing variables, participants were categorized into lifestyle groups that identified one single main characteristic as inclusion criterion. All participants were HIV-positive and qualified for 1 out of 4 lifestyle groups. Group 1 eligibility criterion was drinking alcohol hazardously (AUDIT ≥8) but were not obese and did not use cocaine. Group 2 main characteristic was having high BMI (≥30kg/m^2), had an AUDIT≤8, and did not use cocaine. Group 3 inclusion criterion was using cocaine (self-report and urine toxicology), had BMI≤30kg/$m^2$and AUDIT≤8. Group 4, the control group, did not have any of the unhealthy lifestyle characteristics of the other three groups.

The purpose of identifying participants with a single lifestyle factor was to investigate the effect of one adverse lifestyle factor rather than a combination of two or more, in order to isolate the main effect of that lifestyle factor on biomarkers and liver fibrosis. Participants who did not fall into a lifestyle category or who had a combination

of them were excluded from the analysis. A total of n=67 participants were included in the study, shown in Table 1.

There were no statistical differences in baseline characteristics between the lifestyle groups in this subset of the MASH cohort. After separating the groups into categories, the sample size for the cocaine group was small due to the difficulty in finding participants who had low AUDIT scores but reported using cocaine. Based on the descriptive characteristics, there were significantly more women in the obese category, and more men who drank hazardously (P=0.003). Mean MDA, % oxidized GSH, CK-18, TGF-β1, and LPS did not vary between lifestyle groups (Table 2).

Biological markers associated with FIB-4 index within adverse lifestyle groups

According to multivariate linear regressions, age, gender and being co-infected with HCV were significantly associated with liver fibrosis (Table 3). Regression models were controlled for these covariates, along with CD4 cell count to consider disease status when exploring associations with biological mediators and FIB-4 index within groups. Biological mediators that increased with FIB-4 index included TGF-β1, hepatocyte apoptosis marker CK-18, and % oxidized GSH.

TGF-β1 was significantly associated with FIB-4 in people who used cocaine (β=0.858, P=0.027) when the model was controlled for covariates. TGF-β1 also showed a trend in predicting FIB-4 index in the group that did not use unhealthy behaviors and was not obese (β=0.326, P=0.064). Also in this group, CK-18 was independently associated with FIB-4, and remained significant after controlling for covariates (β=0.435, P=0.015), suggesting that apoptosis can predict FIB-4 in HIV+ subjects without unhealthy lifestyle characteristics. In people who are obese, high oxidative stress (GSSH) is independently

associated with increased FIB-4 index when controlling for covariates (β=0.563, *P*=0.018).

Table 7 describes univariate and multivariate analyses examining the influence of adverse lifestyle factors on biological mediators of fibrosis. Among the adverse lifestyle groups, only the obese group showed trends of higher biomarkers of liver fibrosis. Percent oxidized GSH and TGF-β1 both trended towards significance in obese participants ($F(6, 67)=2.940$, $P=0.092$ and $F(6,67)=3.24$, $P=0.077$, respectively) after adjusting for significant covariates. Obese participants had higher estimated means of % oxidized GSH (28.2% vs. 23.5%) compared to the combined average of other groups. Obesity does not have a direct influence on TGF-β1, instead, there is a significant interaction between BMI ≥ 30 kg/m2 and being co-infected with HIV/HCV that significantly increases TGF-β1 $F(7, 67)=7.478$, $P<0.001$, which may reveal a relationship between these medical conditions and liver disease in HIV. Participants in the control group (who did not exhibit a harmful lifestyle factor) had a lower association with percent oxidized GSH $F(6, 67)= 3.29$, $P=0.075$; (estimated means: 23.0% vs. 26.9%); however, the unadjusted FIB-4 index was significantly higher in participants without a harmful lifestyle factor $F(1, 67)=5.04$, $P=0.028$. The estimated mean FIB-4 index was higher in the control group compared to the combined means of the adverse lifestyle groups (1.16±0.15 vs. 1.19 ± 0.14), however the effect was lost after adjusting for covariates which indicates that another factor is significantly associated with FIB-4 index in people without an adverse lifestyle factor. In fact, FIB-4 index is significantly influenced by HCV coinfection in this model $F(6, 67)=10.954$, $P=0.002$, and there is a slightly higher proportion of HIV/HCV co-infected participants in the control group

(32.3%) compare to the combined average of the other groups (13.9%), *P*=0.072. The higher proportion of HIV/HCV co-infected participants in the control group may account for significant associations with higher FIB-4 index in the unadjusted model.

The association between high levels of circulating biological mediators on FIB-4 index in people experiencing a harmful lifestyle factor was investigated using univariate and multivariate analyses. In the obese group, the estimated mean FIB-4 index was lower for those with low GSH vs high GSH (0.83± 0.20 vs. 1.33± 0.12), which was in agreement with the linear regression data.

TGF-β1 showed a trend in predicting FIB-4 index in those who drank alcohol hazardously (*P*=0.061) and are those who were obese (*P*=0.088). The estimated mean FIB-4 values were lower in participants who drank alcohol hazardously and who had low TGF-β1 compared to hazardous drinkers with high TGF-β1 (1.59±0.16 vs. 1.37± 0.12). Conversely, the estimated mean for FIB-4 was higher for participants with high TGF-β1 levels in the obese group (0.43± 0.46 vs 1.20± 0.31). These results indicate that TGF-β1 can influence FIB-4 index differently under different harmful lifestyle conditions.

Associations between harmful lifestyle factor and odds of high biological mediators of liver fibrosis

There were no significant associations between having high levels of biological mediators and the odds of having advanced liver fibrosis within any lifestyle group, likely due to the small sample sizes in the groups, however, factors associated with liver fibrosis were associated with increased odds of having a harmful lifestyle (Tables 8 a-d).

Not having a harmful lifestyle condition was associated with 65% lower odds of being in the control group, and this result remained slightly significant after adjusting for

age, gender, co-infection status, and CD4 cell count (OR 0.350 [95% CI 0.122 – 1.007]; *P*=0.051), indicating that HIV+ subjects with harmful lifestyle factors had more oxidative stress. Hazardous alcohol use decreased the odds of advanced liver fibrosis classification by 87.1% and there was a trend in association between high lipid peroxidation (MDA) and using cocaine in the adjusted model, however, it did not reach significance (OR 7.919 [95% CI 0.742 – 84.519]; *P*=0.087).

There was also slight interaction between high MDA and high TGF-β1 on FIB-4 index (*F*=3.171, *P*=0.071) in the control group with no adverse lifestyle factors. Figure 1 shows that FIB-4 index increased in participants with high MDA and high TGF-β1, with the opposite trend in people with low levels of TGF-β1. No other significant interactions between biological mediators were associated with FIB-4 index within lifestyle groups.

Discussion

Liver disease has now surpassed AIDS-defining illnesses as a leading cause of morbidity and mortality in PLWH.[67,68] Liver disease progression can be monitored using measures of liver fibrosis.[11] Harmful lifestyle factors tend to be more prevalent in HIV-infected populations, including alcohol abuse,[26,27] obesity,[26,28,29] and cocaine use,[30-32] and these conditions are also associated with increased progression of liver disease.[34,35,48,69-71] Our findings show that biological mediators of liver fibrosis that are high in HIV are also associated with liver fibrosis among PLWH with distinct harmful lifestyle characteristics.

Although baseline characteristics do not vary significantly between lifestyle groups, the ratio of oxidized GSSH:total GSSH (% oxidized GSH) is one of the few biomarkers with established values in healthy subjects in the literature.[52-54] Our data

show that baseline measures of % oxidized GSH in plasma were significantly higher than reported values in healthy individuals A one-sample t-test utilized a reported value of % oxidized GSH in plasma healthy individuals taken from the literature (16.6%) compared to this HIV+ sample. Glutathione values were significantly higher in all groups (hazardous drinking P=0.002, obese group P<0.001, cocaine use P=0.004, control group P=<0.001) compared to the healthy reference in the literature.[53,54] Glutathione is characteristically low in HIV populations, and research suggest replenishment of the glutathione pool as a therapeutic target to improve HIV status.[72]

In the current study, higher levels of TGF-β1 was associated with higher liver fibrosis in HIV+ cocaine users. In *in vitro* models, cocaine induces TGF-β1 production, and in mice exposed to cocaine, TGF-β1 receptors are upregulated.[73,74] Cocaine has also been shown to increase HIV replication and inflammation in peripheral blood mononucleocytes in a humanized mouse model,[75] which may contribute to liver fibrogenesis.

Hepatocyte apoptosis determined with circulating CK-18 independently predicted a higher FIB-4 index in the group without harmful lifestyle factors, indicating that hepatic apoptosis occurs in the context of HIV with the absence of harmful lifestyle conditions, and may lead to liver fibrosis. This finding is not unexpected, because HIV-infection itself may cause hepatocyte apoptosis due to its ability to infect hepatocytes through the CCR5 receptor,[14] and this pathogenesis is enhanced in HIV/HCV.[76] Also in the control group, an interaction between oxidative stress marker MDA and TGF-β1 showed a trend to contribute to higher FIB-4 index, that is, high MDA and high TGF-β1 are associated with higher FIB-4 values. The control group also had an association with increased odds

of advanced stages of liver fibrosis, most likely due to the higher proportion of HIV/HCV co-infected participants in the group. Taken together, these findings may represent the interplay between oxidative stress as an importance initiator of fibrosis through the activation of TGF-β1 to promote fibrosis in persons with HIV infection.[77-79]

This cross-sectional analysis of the MASH cohort may provide an indirect narrative of the population. The finding that hazardous drinking is associated with a decreased probability of having advanced liver fibrosis is counterintuitive at first glance, however, it may be related to a higher proportion of HIV/HCV co-infected participants and the higher FIB-4 value in the control group. Since the participants in the control group, on average, have advanced stages of liver fibrosis (1.64 ± 1.11) and greater than 30% are co-infected with hepatitis C, it is likely that they were strongly advised by a medical professional to stop drinking hazardously, and that they are aware of the damage hazardous drinking has on the liver. In fact, a study that compared reported alcohol consumption between HIV mono- and HIV/HCV co-infected patients showed that a co-infected patients reported restricted drinking habits to avoid medical problems.[80] Rather than conclude that probability of having advanced fibrosis is lower in hazardous alcohol drinkers with HIV, we may reason that PLWH in the MASH cohort are less likely to drink alcohol hazardously if they are co-infected with hepatitis C and have advanced stages of fibrosis.

Our findings consistently demonstrate that people who were obese had a significant and independent association between % oxidized GSH and liver fibrosis. Obesity is significantly associated with oxidative stress in HIV negative cohorts;[38,80] however few studies have investigated the combination of obesity and HIV with the

impact of oxidative stress on liver fibrosis. Interestingly, a high level of oxidized glutathione [GSSH/GSH+GSSH] is associated with a significantly lower odds of having a healthy lifestyle, which supports the concept that unhealthy lifestyle factors significantly contribute to higher oxidative stress in HIV-infected persons.

Although this study design had the advantage of isolating the effects of specific lifestyle conditions on biological mediators of liver fibrosis, it also limited the sample size of the study, which was fixed in this secondary analysis of specimens collected by the parent grant. Few participants met the criteria to be in the group that only used cocaine, even though high proportion of MASH cohort participants use cocaine (29%),[70] the majority used it in combination with alcohol AUDIT scores ≥ 8, which disqualified them for the group. Ultimately, the sample size limited the statistical analyses, however, even with a small sample, our data shows differences in biological mediators between lifestyle groups.

Conclusion

This study offers new insight into biological mediators of liver fibrosis and their interaction with harmful lifestyle factors associated with HIV-infection. Strategies to develop therapeutic targets, treatment options, and the utilization of diagnostic markers may become more personalized when examining the manner in which potential biomarkers are affected by individual lifestyle factors. More research may be warranted to further investigate the impact of lifestyle factors on the interpretation of predictive biomarkers of liver fibrosis, which is one of the most important co-morbidities associated with non-AIDS related death in PLWH.

Tables and Figures

Table 1: Descriptive characteristics of lifestyle groups.

	Hazardous Drinking Group (mean±SD) or %(n)	**Obese Group (mean±SD) or %(n)**	**Cocaine Use Group (mean±SD) or %(n)**	**Control Group (mean±SD) or %(n)**	
Covariate	n=13	n=15	n=7	n=31	*P*-value
Age (years)	45.1± 8.6	48.1± 6.1	45.7± 6.6	46.7± 8.0	0.758
Gender (% male)	**92.3(12)[b]**	**26.7(4)[a]**	**75.0(6)[a,b]**	**67.7(21)[a,b]**	**0.003**
Ethnicity (% Black Non-Hispanic)	92.3(12)	86.7(13)	62.5(5)	71.0(22)	0.244
Co-infection status (% HIV/HCV)	15.4(2)	13.3(2)	12.5(1)	32.3(10)	0.353
Log_{10}(HIV viral load)	2.34± 1.08	2.22± 0.76	3.01± 1.64	2.25± 0.98	0.361
CD4 cell count (cells/mm^3)	425.6± 246.9	726.2± 596.0	352.0± 261.6	594.4± 292.4	0.083
ART use (%yes)	83.3(10)	93.3(14)	87.5(7)	80.6(25)	0.722
FIB-4 index	1.17± 0.39	1.19± 0.44	1.46± 0.74	1.64± 1.11	0.241

Table 2: Mean biological mediators between lifestyle groups.

	Hazardous Drinking Group (mean±SD) or %(n)	**Obese Group (mean±SD) or %(n)**	**Cocaine Use Group (mean±SD) or %(n)**	**Control Group (mean±SD) or %(n)**	
Biomarker	n=13	n=15	n=7	n=31	*P*-value
MDA (uM)	0.613± 0.213	0.545±0.228	0.632± 0.140	0.546± 0.232	0.636
GSH (% oxidized)	24.9± 8.7	23.6± 6.9	27.0± 9.4	21.9± 6.8	0.217
CK-18 (pM)	141.9± 126.9	135.2± 74.4	99.1± 35.8	114.6± 89.7	0.651
TGF-β1 (pg/mL)	2662.4± 2939.0	1988.1±1599.9	4344.7± 3878.1	2507.9± 3147.3	0.329
LPS (EU/mL)	0.231± 0.060	0.239± 0.069	0.266±0.042	0.259± 0.074	0.444

Table 3: Significant covariates associated with FIB-4 index in the regression model.

Covariate	**β**	**t**	***P*-value**
Age	**0.256**	**2.086**	**0.042**
Gender (1 is Male)	**0.265**	**2.201**	**0.032**
Ethnicity (Black Non-Hispanic)	-0.112	-0.989	0.327
HCV Co-infected	**0.420**	**3.667**	**0.001**
Log_{10}(HIV viral load)	0.052	0.387	0.701
CD4 cell count (cells/mm^3)	-0.081	-0.714	0.478
ART use	0.020	0.155	0.877

Table 4: Multivariate regression within lifestyle groups – associations between biological mediators and FIB-4 index as the dependent variable.

4a. Hazardous alcohol use group (AUDIT score ≥8).

	Unadjusted model			Adjusted model*		
Parameter	**β**	**t**	**P-value**	**β**	**t**	**P-value**
MDA (μM)	-0.054	-0.465	0.643	0.278	0.835	0.428
[GSSH/GSSH+GSH]	-0.286	-0.990	0.344	-0.240	-0.789	0.453
CK-18 (pM)	-0.148	-0.148	0.629	-0.218	-0.712	0.497
TGF-β1 (pg/mL)	0.118	0.394	0.701	1.137	0.744	0.478
LPS (EU/mL)	-0.059	-0.197	0.848	-0.694	-1.393	0.201

4b. Obese group (BMI ≥30 kg/m^2).

	Unadjusted model			Adjusted model*		
Parameter	**β**	**t**	**P-value**	**β**	**t**	**P-value**
MDA (μM)	-0.193	-1.657	0.102	-0.403	-1.694	0.121
[GSSH/GSSH+GSH]	**0.568**	**2.489**	**0.027**	**0.563**	**2.821**	**0.018**
CK-18 (pM)	-.312	-1.186	0.257	-0.259	-1.080	0.306
TGF-β1 (pg/mL)	0.377	1.468	.166	0.240	0.847	0.417
LPS (EU/mL)	-0.028	-0.100	0.922	-0.127	-0.449	0.663

4c. Cocaine use group (Self-report and positive urine toxicology).

	Unadjusted model			Adjusted model*		
Parameter	**β**	**t**	**P-value**	**β**	**t**	**P-value**
MDA (μM)	-0.045	-0.290	0.773	-0.058	-0.306	0.762
[GSSH/GSSH+GSH]	-0.066	-0.445	0.659	-0.059	-0.137	0.900
CK-18 (pM)	-0.308	-0.794	0.457	-0.008	-0.017	0.988
TGF-β1 (pg/mL)	0.536	1.679	0.137	**0.858**	**4.049**	**0.027**
LPS (EU/mL)	0.191	0.477	0.650	-0.277	-0.503	0.650

4d. Control group (low BMI, low AUDIT score, no cocaine use).

	Unadjusted model			Adjusted model*		
Parameter	**β**	**t**	**P-value**	**β**	**t**	**P-value**
MDA (μM)	-0.168	-1.960	0.311	-0.071	-0.352	0.727
[GSSH/GSSH+GSH]	-0.081	-0.942	0.348	-0.159	-0.925	0.364
CK-18 (pM)	**0.593**	**3.967**	**<0.001**	**0.435**	**2.593**	**0.015**
TGF-β1 (pg/mL)	**0.326**	**1.922**	**0.064**	0.278	1.603	0.121
LPS (EU/mL)	0.176	0.960	0.345	0.113	0.635	0.531

*Model controlled for age, gender, co-infection status, and CD4 cell count.

Table 5: Binary covariates that influence biological mediators and FIB-4 index (n=67).

	MDA (μM)		**GSH (% oxidized)**		**CK-18 pM**		**TGF-β1 (pg/mL)**		**LPS (EU/mL)**		**FIB-4 index**	
Covariate	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**
Age ≥50 years	0.14	0.708	0.25	0.618	0.69	0.407	2.16	0.147	2.41	0.126	1.93	0.170
Gender (male)	3.31	0.074	1.20	0.277	**3.83**	**0.055**	**7.83**	**0.007**	1.26	0.265	**4.38**	**.041**
Ethnicity (Black Non-Hispanic)	0.20	0.653	**3.84**	**0.055**	0.01	0.928	0.16	0.683	0.01	0.936	0.89	0.348
Co-infection status (HIV/HCV)	**8.15**	**0.006**	0.00	0.922	1.37	0.245	**15.49**	**<0.001**	1.38	0.245	**14.10**	**<0.001**
HIV viral load (≥75 RNA copies/mL)	1.18	0.281	0.23	0.630	0.09	0.763	0.02	0.883	0.41	0.525	0.58	0.448
CD4 cell count (<200 cells/mm^3)	0.09	0.754	0.07	0.791	1.46	0.232	**5.70**	**0.020**	1.00	0.321	0.01	0.948
ART use (yes)	**4.00**	**0.050**	0.40	0.527	**4.17**	**0.046**	0.96	0.330	0.01	0.940	0.27	0.606

Table 6: Cut off values for biological mediators of liver fibrosis.

	MDA (uM)	**% oxidized GSH**	**CK-18 (pM)**	**TGF-β1(pg/mL)**	**LPS (EU/mL)**
50% percentile (median value)	0.525	24.0	93.27	1539.2	0.228

Any value that fell below the median qualified as a "low" biological marker. Values that were greater than or equal to the median were considered a "high" biological marker.

Table 7: Influence of adverse lifestyles on biomarkers of liver fibrosis.

Outcome variable	Hazardous drinking group				Obese group				Cocaine use group				Control Group			
	Unadjusted		Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*	
	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value
MDA (μM)	0.37	0.545	0.13	0.716	0.39	0.535	0.02	0.885	1.28	0.263	2.39	0.128	0.42	0.519	0.63	0.429
[GSSH/GSSH+GSH]	0.01	0.988	0.06	0.813	**3.64**	**0.061**	**2.94**	**0.092**	0.08	0.786	0.17	0.678	**3.25**	**0.076**	**3.29**	**0.075**
CK-18 (pM)	0.77	0.383	0.66	0.420	0.24	0.629	1.56	0.216	0.45	0.503	0.87	0.356	0.46	0.501	1.04	0.312
TGF-β1 (pg/mL)	0.07	0.782	2.45	0.123	0.87	0.354	**3.24**	**0.077**	0.98	0.327	2.24	0.140	0.02	0.965	0.56	0.457
LPS (EU/mL)	0.03	0.866	0.02	0.878	1.24	0.269	1.50	0.225	0.82	0.37	0.17	0.685	2.79	0.100	1.22	0.275
FIB-4 index	1.23	0.271	1.95	0.168	1.25	0.268	0.01	0.973	0.33	0.567	0.41	0.525	**5.04**	**0.028**	2.16	0.147

*Model controlled for gender, ethnicity, co-infection status, CD4 cell count, and ART use.
Outcome variables included biological mediators of liver fibrosis.

Table 8: High levels of biomarkers that influence FIB-4 index within lifestyle groups.

Parameter	Hazardous drinking group				Obese group				Cocaine use group				Control Group			
	Unadjusted		Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*	
	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value
High MDA ≥ 0.525 EU/mL	0.70	0.418	0.50	0.502	3.12	0.101	1.69	0.225	1.46	0.272	3.44	0.204	2.77	0.106	0.73	0.420
High % oxidized GSH ≥24.0%	3.42	0.910	3.25	0.121	**4.38**	**0.056**	**3.43**	**0.097**	0.12	0.740	5.64	0.254	0.47	0.495	0.01	0.960
High CK-18 ≥93.27 pM	0.03	0.871	0.05	0.819	0.02	0.964	0.16	0.700	0.81	0.400	0.33	0.667	1.65	0.208	0.13	0.724
High TGF-β1 ≥1539.2 pg/mL	0.03	0.871	**5.27**	**0.061**	1.74	0.210	**3.67**	**0.088**	1.27	0.302	3.44	0.204	0.52	0.473	0.95	0.339
High LPS ≥0.228 EU/mL	0.33	0.574	0.52	0.495	0.62	0.444	0.57	0.470	0.19	0.896	0.66	0.564	2.06	0.161	1.56	0.224

*Model controlled for gender, ethnicity, co-infection status, CD4 cell count, and ART use.
FIB-4 is dependent variable, each biological mediator was assessed separately within the respective lifestyle group.

Table 8: Associations between adverse lifestyle groups and odds of having higher biological factors of liver fibrosis.

8a. Hazardous alcohol use group (AUDIT score ≥8)

	Unadjusted model			Adjusted model*		
Variable	**OR**	**95% CI**	***P*-value**	**aOR**	**95% CI**	***P*-value**
High MDA ≥ 0.525 EU/mL	1.600	0.462 – 5.543	0.458	1.175	0.289 – 4.780	0.822
High % oxidized GSH ≥24.0%	0.926	0.274 – 3.131	0.901	1.114	0.304 – 4.085	0.871
High CK-18 ≥93.27 pM	0.536	0.155 – 1.857	0.325	0.412	0.106 – 1.604	0.201
High TGF-β1 ≥1539.2 pg/mL	1.260	0.373 – 4.262	0.710	1.705	0.439 – 6.620	0.441
High LPS ≥0.228 EU/mL	0.794	0.235 – 2.684	0.710	0.788	0.213 – 2.915	0.788
High FIB-4 ≥ 1.45	0.316	0.063 – 1.578	0.160	**0.129**	**0.017 – 0.953**	**0.045**

8b. High BMI group (BMI ≥30 kg/m^2)

	Unadjusted model			Adjusted model*		
Variable	**OR**	**95% CI**	***P*-value**	**aOR**	**95% CI**	***P*-value**
High MDA ≥ 0.525 EU/mL	0.524	0.162 – 1.695	0.280	0.569	0.143 – 2.261	0.423
High % oxidized GSH ≥24.0%	**4.125**	**1.151 – 14.786**	**0.030**	**3.743**	**0.899 – 15.587**	**0.070**
High CK-18 ≥93.27 pM	1.143	0.360 – 3.631	0.821	1.908	0.480 – 7.576	0.359
High TGF-β1 ≥1539.2 pg/mL	0.615	0.191 – 1.988	0.417	0.390	0.088 – 1.721	0.214
High LPS ≥0.228 EU/mL	0.393	0.117 – 1.318	0.130	0.347	0.862 – 27.998	0.135
High FIB-4 ≥ 1.45	0.706	0.195 – 2.522	0.595	2.084	0.400 – 10.856	0.383

8c. Cocaine use group (Self-report and positive urine toxicology)

	Unadjusted model			Adjusted model*		
Variable	**OR**	**95% CI**	***P*-value**	**aOR**	**95% CI**	***P*-value**
High MDA ≥ 0.525 EU/mL	**6.429**	**0.728 – 56.798**	**0.094**	**7.919**	**0.742 – 84.519**	**0.087**
High % oxidized GSH ≥24.0%	1.531	0.314 – 7.457	0.598	1.684	0.330 - 8.593	0.531
High CK-18 ≥93.27 pM	1.333	0.274 – 6.492	0.722	1.439	0.272 – 7.617	0.669
High TGF-β1 ≥1539.2 pg/mL	1.429	0.293 – 6.957	0.659	1.364	0.251 – 7.393	0.719

High LPS ≥0.228 EU/mL	1.333	0.274 – 6.492	0.722	1.882	0.353 – 10.040	0.459
High FIB-4 ≥ 1.45	0.821	0.146 – 4.627	0.823	0.785	0.119 – 5.187	0.802

8d. Control group (low BMI, low AUDIT score, no cocaine use)

	Unadjusted model			**Adjusted model***		
Variable	**OR**	**95% CI**	***P*-value**	**aOR**	**95% CI**	***P*-value**
High MDA ≥ 0.525 EU/mL	0.656	0.246 – 1.751	0.400	0.676	0.231 – 1.980	0.475
High % oxidized GSH ≥24.0%	**0.333**	**0.121 – 0.921**	**0.034**	**0.350**	**0.122 – 1.007**	**0.051**
High CK-18 ≥93.27 pM	1.014	0.356 – 2.883	0.980	1.210	0.456 – 3.213	0.702
High TGF-β1 ≥1539.2 pg/mL	1.059	0.399 – 2.808	0.909	1.100	0.380 – 3.182	0.860
High LPS ≥0.228 EU/mL	2.000	0.743 – 5.387	0.170	1.640	0.578 – 4.654	0.352
High FIB-4 ≥ 1.45	**2.581**	**0.886 – 7.521**	**0.082**	2.738	0.752 – 9.971	0.127

*Model controlled for age, gender, co-infection status, and CD4 cell count.

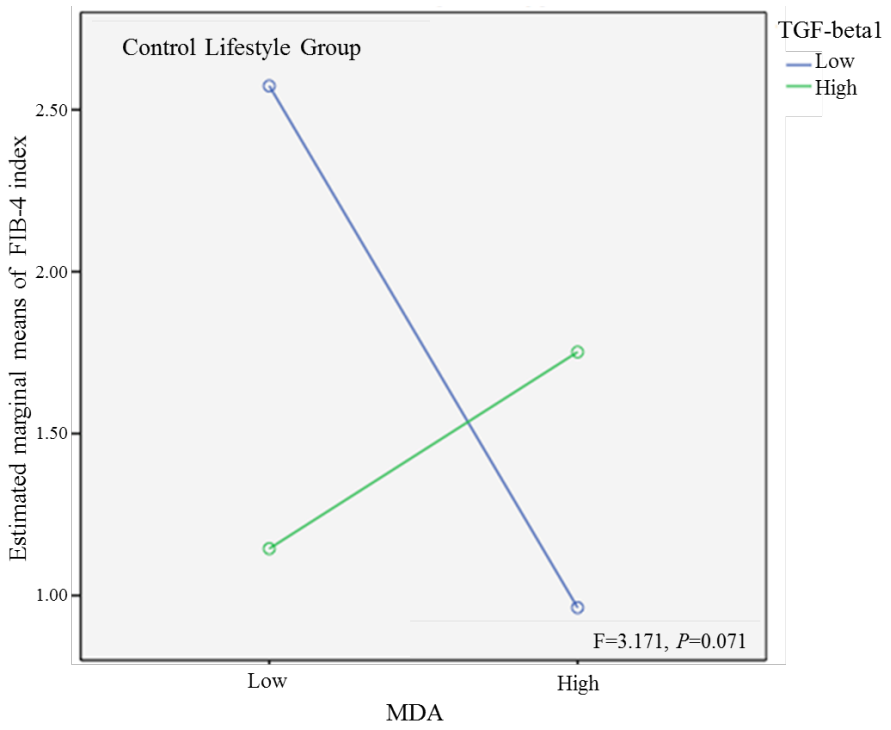

Figure 1: Interaction between MDA and TGF-β1 on estimating FIB-4 index. In HIV-infected persons with no adverse lifestyle conditions, the combination of high oxidative damage (MDA) and high TGF-β1 and may increase liver fibrosis.

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CHAPTER VII: SUMMARY AND CONCLUSIONS

With the introduction of effective antiretroviral therapy (ART),[1] and an aging HIV+ population,[2] liver disease is now a leading cause of non-AIDS related morbidity and mortality in PLWH,[3-6] creating new health burdens in this population and for the society.

The present study investigated the interplay between adverse lifestyle factors that are prevalent in people living with HIV (PLWH), biological mediators of liver damage, and a non-invasive measure of liver fibrosis (FIB-4 index) in HIV mono- and HIV/HCV co-infected study participants.

Adverse lifestyle factors that disproportionately affect morbidity and mortality among PLWH include hazardous drinking,[7,8] high body mass index (BMI),[7,9,10] and illicit drug use including crack/cocaine.[11-13] These lifestyle factors have been previously shown in both HIV-infected and HIV- non-infected populations to be to cause liver disease as reflected by increased liver fibrosis.[14-17]

The results of this investigation in the Miami Adult Studies of HIV (MASH) cohort show that, in people with HIV mono-infection, the odds of progression of liver fibrosis significantly increase over 2 years and that the process was associated with harmful lifestyle factors. Having an Alcohol Use Disorders Identification Test (AUDIT) score ≥8 was independently associated with higher liver fibrosis (FIB-4) index in HIV mono-infection and 3.04 higher odds of increasing at least the FIB-4 equivalent of one METAVIR stage of liver fibrosis over 2 years after adjusting for BMI, age, gender, CD4 cell count, HIV viral load, and ART use (OR 3.038 [95% CI 1.010 – 9.135]; *P*=0.048).

The results of the present study in the MASH cohort show that having a high body mass index (≥ 28 kg/m^2) was also associated with higher FIB-4 index in HIV mono-infection (OR 2.934 [95% CI 1.132 – 7.605]; *P*=0.027). The relationship between BMI and liver fibrosis in HIV infection has not been thoroughly explored. Studies have shown that high BMI may be protective in HIV, a phenomenon known as the "obesity paradox" whereby high BMI is associated with higher CD4 cell counts.[18,19] Additionally, higher CD4 cell count is associated with better outcomes of liver fibrosis because of improved immune function.[20,21] The results of the present study demonstrate the combined effect of BMI and CD4 cell count on liver fibrosis in HIV mono-infection in the MASH cohort. Decreased CD4 cell count was associated with higher liver fibrosis in participants with lower BMI (< 28 kg/m^2). On the other hand, CD4 cell count showed no association with liver fibrosis in participants with higher BMI, likely due to the strong fibrogenic influence of BMI on the liver. It is possible that any beneficial effect of CD4 cell count to lower liver fibrosis are masked by the fibrogenic effect of high BMI when participants are close to obesity.

The potent impact of BMI on liver fibrosis was revealed through the paradoxical associations between cocaine use and FIB-4 index. There is a significant association between the use of crack/cocaine and liver fibrosis (OR 0.228 [95% CI 0.057 – 0.918]; *P*=0.038). However, PLWH are less likely to be obese if they use crack/cocaine due to the anorectic effect of this illicit drug. Since higher BMI is strongly associated with liver fibrosis, lower BMI in those who use crack/cocaine leads to a seemingly paradoxically lower liver fibrosis, compared to PLWH who do not use crack/cocaine. When the analyses were stratified by BMI, there is was a strong interaction between BMI and

crack/cocaine use that shows that cocaine increases FIB-4 index, a marker of liver fibrosis, when adjusting for this interaction, such that the combined impact of BMI and cocaine use is associated with advancing liver damage. To our knowledge, this is the first time a relationship between crack/cocaine use, BMI, and liver fibrosis has been reported in HIV mono-infected subjects.

Although there were no independent associations with liver fibrosis and hazardous alcohol use, high BMI, or crack/cocaine use in the HIV/HCV co-infected group, BMI could predict higher FIB-4 in participants who were older than 50 years. BMI also had a significant interaction with crack/cocaine use in HIV/HCV co-infected subjects, and this interaction significantly increased the odds of progressing FIB-4 stage by a factor of nearly five (OR 4.985 [95% CI 1.130 – 21.993];P=0.034). The impact of crack/cocaine and BMI on liver in HIV/HCV co-infection may be enhanced compared to HIV mono-infection because of the underlying pathophysiology of HIV/HCV co-infection, so that the liver is more susceptible to facilitators of fibrogenesis.

The biological mediators of liver fibrosis were also investigated and we found them to be elevated in HIV infection, including oxidative stress, such as malondialdehyde (MDA) and % oxidized glutathione (GSH),[22,23] the hepatic apoptosis marker Cytokeratin 18 (CK-18),[24] transforming growth factor-beta1 (TGF-β1),[25] and the microbial endotoxin lipopolysaccharide (LPS).[26] Our results show that high glutathione and LPS are associated with progression of liver fibrosis over time in HIV mono-infection. This is a unique finding because these biological mediators have not been found to predict liver fibrosis progression over time in HIV mono-infected subjects in previous studies. Interestingly, the effect that oxidative stress and microbial translocation had on liver

fibrosis was only revealed in combination with the clinical and demographic factors that contribute to liver fibrosis. This suggests an interaction between biological mediators and covariates of disease stage, treatment and host characteristics, such as age, gender, CD4 cell count, and ART use that enhance liver fibrosis. Further research needs to be conducted to investigate these interactions and target conditions that increase the effect of the fibrogenic biomarkers on liver fibrosis. The mean values of TGF-β1 (*P*-value time = 0.001), CK-18 (*P*-value time = 0.006), and LPS (*P*-value time = 0.001) increased significantly from baseline to 24 months. This indicates that biological mediators of fibrosis may increase over time in HIV mono-infection. Furthermore, mean FIB-4 index increased from mild fibrosis (1.24 ± 0.53) or the equivalent METAVIR stage F0-F1 to more advanced stage of fibrosis (1.52 ±2.85) or the METAVIR equivalent ≥ F2 over 24 months in 65 mono-infected participants, and although the result was not statistically significant, clinically, it indicates a trend in liver fibrosis progression in HIV mono-infection.

A cross-sectional analysis revealed the individual adverse lifestyle characteristics with specific biological mediators that increased liver fibrosis. Subjects who were obese had a significant positive association between % oxidized glutathione and higher FIB-4 index (β=0.563, *P*=0.018).

Obesity is associated with higher oxidative stress even in HIV-negative populations,[27,28] and this is the first report of the significant effect of increased oxidative stress on liver fibrosis specifically in obese HIV mono-infected subjects. The marker for hepatocyte apoptosis was independently associated with liver fibrosis in people who did not have any of the investigated adverse lifestyle conditions (β=0.435, *P*=0.015),

indicating that hepatic apoptosis may be an important driver of liver fibrosis in HIV mono-infection.

Taken together, the findings of these studies describe interrelationships between HIV disease status, lifestyle, and biological mediators of fibrosis that are elevated in HIV. In addition, the results show that biological mediators of fibrogenesis were elevated among PLWH with different lifestyle conditions, and that there may be important interactions between lifestyle conditions and the biomarkers of mediators of liver fibrosis that may account for higher rates of liver disease in HIV mono-infection. More research is warranted to develop personalized therapeutics for this population to curb the growing burden of liver disease as a co-morbidity.

Table 1: Summary of hypotheses and major findings

Hypothesis #	Hypothesis	Major Findings
1	Adverse Lifestyle factors (high BMI, drinking alcohol, and cocaine use) are associated with higher liver fibrosis measured with FIB-4, in HIV-mono and HIV/HCV co-infection.	AUDIT score was an independent predictor of liver fibrosis (FIB-4 index) in HIV mono-infection. Being overweight (BMI $\geq$ 28kg/m^2) and drinking alcohol hazardously were independently associated with increased FIB-4 in HIV mono-infection, but not co-infection. Having a high BMI independently increased the odds of progressing in FIB-4 category by a 3.797 times (*P*=0.015) in the adjusted model. Drinking hazardously also increased the odds of progressing FIB-4 index significantly by 4.108 times over 2 years in HIV mono-infection (*P*=0.048). Crack/Cocaine use was associated with a 4.390 significantly lower odds of progressing in FIB-4 index, (*P*=0.038) due to an interaction with BMI in HIV mono-infection. When controlling for BMI, the effect of cocaine loses significance. None of the lifestyle factors were independently associated with FIB-4 index in HIV/HCV co-infection, however, the interaction with cocaine and BMI significantly increased FIB-4 index by a factor of nearly 5 (*P*=0.034). We can reject the null hypothesis that states there is no association with adverse lifestyle factors and liver fibrosis in HIV mono-infection; however, we cannot reject the null hypothesis in HIV/HCV co-infection.
2	Increased oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1 independently increase the risk of liver fibrosis over time (2 years) in HIV mono-infection.	None of the biological mediators could independently predict liver fibrosis progression over 2 years. High baseline measures of % oxidized glutathione (aOR 4.342, P=0.046) and circulating microbial endotoxin LPS (aOR 1.098, *P*=0.097) are associated with increased odds of progressing at least one category of liver fibrosis over 2 years, however, GSH was only significant in models adjusting for covariates and LPS only showed a trend towards statistical significance. Increasing levels of hepatocyte apoptosis marker CK-18 are significantly associated with progressing in stage of liver fibrosis and FIB-4 index over 2 years (β= 2.011, *P*=0.043), and high levels are associated with odds of progressing in FIB-4 category (OR 1.008, *P*=0.021) but these findings lose significance in multivariate models adjusting for age, gender, HIV viral load, CD4 cell count, and ethnicity. We cannot reject the null hypothesis that high levels of biological mediators of fibrosis care associated with increased liver fibrosis over 2 years. Secondary analyses show associations between increasing biomarkers over time, and FIB-4 index in HIV mono-infected population.
3	Oxidative stress, microbial translocation, hepatocyte apoptosis, and TGF-β1 are increased HIV-infected participants who have high BMI, drink or abuse alcohol, or use cocaine compared to controls.	Circulating levels of oxidative stress were significantly and independently associated with an increase in liver fibrosis index (FIB-4) in obese HIV+ subjects. Higher hepatocyte apoptosis marker CK-18 independently predicts an increase in liver fibrosis in HIV+ subjects with no adverse lifestyle conditions. TGF- β1 is associated with a trend in the increase of liver fibrosis in cocaine users (β=0.858, *P*=0.027) and a trend in interaction between MDA and TGF-β1 (F=3.171, *P*=0.071) on increasing FIB-4 index in the control group. We cannot reject the null hypothesis, because there were significant biomarkers associated with FIB-4 index in the control group that did not have adverse lifestyle factors, suggesting that HIV infection and/or HIVHCV co-infection contributes to liver fibrosis; however, our results suggest that biological mediators that potentiate liver fibrosis may differ slightly between subjects with different lifestyle conditions.

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CHAPTER VIII: STRENGTHS AND LIMITATIONS

The results for this study may guide future research to develop personalized diagnostic and therapeutic targets of liver fibrosis in people living with HIV. This is the first study to examine the relationships between adverse lifestyle factors that disproportionately affect the HIV population and biological mediators that are higher in PLWH with liver fibrosis, a non-AIDS co-morbidity.

The strengths of this study include capturing longitudinal data of an HIV+ cohort who use cocaine and drink hazardously, which are lifestyle factors associated with low study retention.[1] HIV mono-infection is associated with higher rates of liver disease and mortality compared to the non-infected population,[2] and this study highlights significant lifestyle factors and biological mediators that may generate new therapeutic approaches to treatment. Further investigations are needed to confirm these findings in a larger cohort with adequate sample size.

Design and Sample Size

The main limitation of this research are derived from its design as a secondary analysis of data. The laboratory data for biological mediators, however, were generated for the purpose of this work. Ideally, an experimental design for this research would include more participants who progressed in the FIB-4 categories, and who use cocaine combined with a low AUDIT score to be able to separate the effects of cocaine from that of the alcohol, and have adequate sample size for testing each hypothesis.

Methods

Plasma samples were used for laboratory analyses of biological mediators. The limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD, USA), has been

widely used for the detection of endotoxin for approximately 30 years,[3] and in HIV-related research, however, plasma is not the recommended sample type due to possible presence of inhibitory proteins.[4-7] Recently, methods have been developed to measure LPS using the LAL Lonza kit in plasma of HIV+ patients.[4] A large coefficient of variance (CV > 20%) was initially observed, for our samples, suggesting that the assay was not generating accurate results for the samples. After developing methods to treat samples with high CVs, the results improved, but it would be beneficial to corroborate LPS results with a surrogate marker of microbial translocation such as sCD14 that is associated with the LPS-binding protein.[8]

Although liver biopsy is the current gold standard for staging liver fibrosis by using METAVIR scores, it is highly invasive and it is risky and ethical to perform the procedure for research purposes only.[9] Specifically in HIV patients, repeated biopsies are contraindicated because of the increased risk of internal hemorrhage due to thrombocytopenia.[9,10] The FIB-4 index is a non-invasive measure of liver fibrosis that is calculated using data collected by common laboratory values using the formula: [(AgeXAST)/(PlateletsX√(ALT))]. Cut off points for liver fibrosis categories are: (FIB-4 <1.45) none/mild fibrosis, (1.45≤ FIB4 ≤3.25) moderate to severe fibrosis, and (FIB-4 >3.25) cirrhosis that correspond to METAVIR scores of liver fibrosis F0-F1, F2-F3 and F4, respectively.[11,12] The study may have benefitted by using a second non-invasive index of fibrosis, such as APRI, to reveal different associations for risk factors of liver fibrosis in HIV-infected subjects. For example, Mendeni et al.[13] found that progression of liver fibrosis occurred in 8.3% of their HIV-infected sample when using FIB-4 categories of

liver fibrosis. In the same sample, the proportion of subjects who were categorized as having progressed in stage of liver fibrosis increased to 31% using APRI score.

Hepatitis C infection (HCV) is a significant contributor of liver fibrosis in HIV-infection.[14-16] The data in the parent study had limited information about HCV treatment history, genotype, and viral load. Future studies should take into account HCV variables when developing a research design. Despite these limitations, our results offer new insights into associations and interactions between biological mediators of liver fibrosis and adverse lifestyle factors and their relationship with HIV-infection.

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CHAPTER IX: FUTURE RESEARCH

A greater part of the literature in HIV and liver fibrosis examines hepatitis C (HCV) as a primary source of fibrosis,[1-3] however, our study strongly suggests that adverse lifestyle factors play a role in increasing prevalence of liver fibrosis in HIV mono-infection and may have interactions to increase fibrosis in HIV/HCV co-infection.

Biological mediators of liver damage that are elevated in people living with HIV (PLWH) are associated with increased liver fibrosis development over time, and this relationship may be further examined for time-dependent associations with liver disease. Our research suggests that oxidative stress, hepatocyte-specific apoptosis, profibrogenic cytokine TGF-β1, and microbial translocation contribute to liver fibrosis differently under different lifestyle conditions, which provides a link between elevated biological mediators in HIV, adverse lifestyle conditions prevalent in HIV+ populations, and the development of liver fibrosis. Future research could aim to identify specific markers of liver fibrosis concerning PLWH who are experiencing adverse lifestyle conditions, and to elucidate and explain specific mechanisms by which these interactions drive liver fibrosis.

Initial studies using new direct acting antivirals (DAAs), such as Sofosbuvir and Ribavirin, show high cure rates in HIV/HCV co-infected populations,[4,5] and although DAAs are not widely available to PLWH due to multiple barriers,[6,7] future research could study the effects of DAAs on liver hepatotoxicity, especially in combination with HIV antiretroviral therapies. A high cure rate of HCV in HIV-infected populations may warrant more research into liver fibrosis in HIV mono-infected populations in future studies.

Although the size of this study was relatively small, our significant findings may encourage research into complex biological relationships arising from health and behavioral conditions in HIV. Finally, this research highlights the potential for personalized treatment of risk factors associated with FIB-4, a non-invasive liver fibrosis index in HIV mono- and HIV/HCV co-infection. Studies examining best approaches to translate these findings into recommendations for PLWH are warranted.

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