Monocyte-Macrophage activation is associated with NAFLD and liver fibrosis in HIV mono-infection independently of the gut microbiome and bacterial translocation.

Running Title: Non-alcoholic fatty liver disease in HIV.

James B MAURICE[1], Lucy GARVEY[2], Emmanuel A. TSOCHATZIS[3], Matthew WILTSHIRE[1], Graham COOKE[4], Naomi GUPPY[5], Julie MCDONALD[1], Julian MARCHESI[1], Mark NELSON[6], Peter KELLEHER[7], Robert GOLDIN[8], Mark THURSZ[1] and Maud LEMOINE[1].

Affiliations

1 Hepatology Unit, Department of Surgery and Cancer, Imperial College London, London UK

2 Department of Infectious Disease, Imperial College Healthcare NHS Trust, London UK

3 UCL Institute for Liver and Digestive Health, Royal Free Hospital and University College London, UK

4 Division of Infectious Disease, Department of Medicine, Imperial College London, London UK

5 University College London Advanced Diagnostics, London, UK

6 Department of Infectious Disease, Chelsea & Westminster Hospital NHS Trust, London UK

7 Centre for Immunology and Vaccinology, Department of Medicine, Imperial College London UK

8 Histopathology Unit, Department of Medicine, Imperial College London, UK

Corresponding Author

Corresponding author

Dr James Maurice: james.maurice@imperial.ac.uk. The Liver Unit, 10th Floor QEQM, St Mary's Hospital, South Wharf Road, London W21NY

Author Statement:

Guarantor of the article: Dr Maud Lemoine

JBM: Designed the study, collected data, analysed data, wrote the manuscript.

LG, GC, PK, MN, MT: Designed the study and critically reviewed the manuscript.

ET, MW, NG, JAKM, JM, RG: collected and analysed data, critically reviewed the manuscript.

ML: Designed the study, analysed data, wrote the manuscript.

Word Count: 3716

Structured Summary (Word Count: 250)

Background: Non-alcoholic fatty liver disease (NAFLD) is common among people living with HIV. There is limited data available on the pathophysiology of NAFLD and the development of fibrosis in this population.

Objectives: to investigate the association of bacterial translocation, adipose tissue dysfunction, monocyte activation and gut dysbiosis in patients with HIV mono-infection and NAFLD.

Methods: Cases with biopsy-proven NAFLD and HIV mono-infection were age and sex-matched to HIV+ and HIV- controls. Markers of bacterial translocation (lipopolysaccharide-binding protein (LBP), bacterial DNA and lipopolysaccharide (LPS)), adipose tissue dysfunction (leptin, adiponectin) and monocyte activation (sCD14 and sCD163) were measured by ELISA.

Hepatic patterns of macrophage activation were explored with immunohistochemistry. 16s rRNA sequencing was performed with stool.

Results: Thirty-three cases were included (≥F2 fibrosis n=16), matched to HIV+ (n=29) and HIV- (n=17) controls. Cases with NAFLD were more obese (BMI 31.0±4.4 kg/m^2 vs 24.1 ±2.8 kg/m^2 p<0.001) and had significantly increased levels of sCD14, sCD163 and higher leptin to adiponectin ratio versus HIV+ controls. Cases with ≥F2 verses <F2 fibrosis had increased sCD14 (1.4 ±0.4 vs 1.1 ±0.3 µg/ml, p=0.023) and sCD163 (1.0 ±0.3 vs 0.8 ±0.3 µg/ml, p=0.060) which correlated with waist circumference (sCD14 p=0.022, sCD163 p=0.011). Immunohistochemistry showed increased hepatic portal macrophage clusters in patients with fibrosis. No markers of bacterial translocation or changes to the microbiome were associated with NAFLD or fibrosis.

Conclusion: NAFLD fibrosis stage in HIV mono-infected patients is associated with monocyte activation in the context of obesity, which may be independent of bacterial translocation and gut microbiome.

Key Words: NAFLD; NASH; fibrosis; HIV; translocation; monocyte.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease worldwide with an estimated prevalence of 25%.[1] NAFLD encompasses a spectrum of disease pathology, ranging from non-alcoholic fatty liver (NAFL) i.e. steatosis without hepatic injury, to non-alcoholic steatohepatitis (NASH), a more severe entity defined by liver steatosis with lobular inflammation and hepatocytes ballooning, and fibrosis.[2] Liver fibrosis is the most important stage of disease progression in NAFLD, as it is the key predictor of increased

liver-related mortality.[3] Although only a minority of patients with NAFLD will develop cirrhosis, such is the scale of the problem that NASH is projected to become the leading indication for liver transplant in the next 5-10 years in developed countries.[4] [5]

NAFLD, NASH and fibrosis in HIV mono-infected subjects have only been investigated with a limited number of liver biopsy-based analyses.[6][7][8][9] A recent systematic review by our group found a prevalence of NAFLD of 35% in populations mainly investigated with imaging for abnormal liver function tests, and about 20% of patients who had a liver biopsy had significant fibrosis (>=F2).[10]

Obesity and the metabolic syndrome are strongly associated with NAFLD and progression to NASH and fibrosis in populations both without[11] [12] and with HIV. [10][13][14][15] In the non- HIV population this may in part be mediated by a complex interaction of adipose tissue dysfunction, bacterial translocation and changes to the structure of the gut microbiome[16][17][18]. However, this has been poorly investigated in patients with HIV. The loss of gut-associated lymphoid tissue (GALT) following HIV infection, bacterial translocation and systemic immune activation has been an important paradigm in our understanding of HIV disease progression,[19] and even in patients established on effective antiretroviral therapy (ART), restoration of the GALT is slower than the peripheral CD4 cell count. Therefore an incomplete resolution of the gut mucosal barrier may contribute to persistent immune activation in these patients,[20] in turn leading to chronic hepatic inflammation and the development of NASH. Furthermore, research on the gut microbiome has demonstrated changes associated with HIV infection that may further modulate the host immune response.[21] Therefore there may be a synergy between HIV and NAFLD driving liver inflammation and fibrosis.

Our study aimed to explore the role of bacterial translocation, adipose tissue dysfunction, immune activation and gut dysbiosis in the development of NAFLD, NASH and fibrosis in HIV mono-infected patients treated with ART.

Methods

Study Population

Patients were prospectively recruited in clinics specialising in HIV and liver disease at three main HIV centres in London, UK (Imperial College Healthcare NHS Trust, Chelsea & Westminster NHS Trust, Royal Free NHS Trust). Controls were prospectively recruited from the same institutions.

Cases were defined as patients with controlled HIV-1 mono-infection i.e. undetectable viral load (<50 cp/ml) and CD4 cell count> 200/mm^3) on ART and liver biopsy proven NAFLD.

Liver biopsy was performed in cases of persistent ALT≥80 IU/l and/or transient elastography (Fibroscan®) ≥7.1kPa according to the treating physician's discretion. Fibroscans were performed after an overnight fast according to standard protocol as previously described, reporting data on both liver stiffness and controlled attenuation parameter (CAP).[22] Exclusion criteria were: patients with alcohol excess within the last 6 months defined as >21units/week for men and >14units/ week for women; CD4 cell count <200/mm3 and/or active AIDS- defining illness; other known causes of chronic liver disease (positive HBs antigen or HCV antibody, autoimmune disease, biliary disease, haemochromatosis or Wilson's disease); current use of steatogenic medication such as methotrexate or long- term steroids.

HIV positive age and sex- matched controls were defined as non-obese subjects with normal liver function tests on at least two occasions over the last 12 months prior the start of the

study, alcohol intake less than 21 units per week and no history of liver disease. HIV negative controls were age, sex and body mass index (BMI)- matched to HIV positive controls.

Metabolic syndrome was defined by established international guidelines.[23]

Histopathology and Immunohistochemistry

Liver biopsies were formalin- fixed and paraffin- embedded. Sections were stained with trichrome and Haematoxylin & Eosin (H&E) and reported by liver histopathologists blinded to the study data. NASH was defined as the presence of steatosis with ballooning and lobular inflammation, and cases were graded according to the NASH Clinical Research Network (CRN) scoring system.[2] Clinically significant liver fibrosis was defined as at least F2 by the Brunt criteria (pericellular and periportal fibrosis).[24]

Liver biopsy slides were stained with antibodies for CD14 and CD163 and reviewed at x100 magnification. Clusters were defined as ≥3 positively stained macrophages in a single group. Clusters were identified as portal or lobular, manually counted and divided by the aggregate length of the biopsy cores.[25]

Laboratory Assays

Overnight fasted blood samples were drawn in clinic. Serological markers of bacterial translocation (lipopolysaccharide- binding protein (LBP) (RND Systems, Abingdon, UK) and lipopolysaccharide (LPS) (Cusabio, Wuhan, China)) and monocyte activation (soluble CD14 (sCD14) and soluble CD163 (sCD163), RND Systems, Abingdon, UK)), inflammatory cytokines (Interleukin-6 (IL-6) (Life Technologies, Paisley, UK)), tumour necrosis factor alpha receptor 2 (TNFαR2) (RND Systems, Abingdon, UK)) and adipokines (adiponectin and leptin (Life Technologies, Carlsbad, USA)) were measured by ELISA following the manufacturer's

instructions. Leptin to adiponectin ratio was used as a marker of adipose tissue dysfunction and insulin resistance.[26][27]

DNA was extracted from whole blood using QIAamp DNA Blood Midi kit (Qiagen Ltd, Manchester, UK) and bacterial DNA quantified by qPCR as previously reported[28] (supplementary methods).

Stool microbial DNA Extraction and 16s rRNA Sequencing

Stool samples were collected at the same time as blood samples or within the following 2 weeks. Faecal DNA extraction was performed as previously described.[29]

Sequencing was performed on an Illumina Miseq instrument (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) and paired-end 300bp chemistry. The 16s rRNA sequencing data generated on MiSeq was processed on Mothur v.1.39.5 using the MiSeq SOP Pipeline.[30] Further detail is described in the supplementary methods.

Statistical Analysis

Quantitative variables were presented as mean ±SD or median (95% CI) in cases of parametric and non-parametric distribution respectively. Two- group comparisons of continuous data were performed using independent sample t-tests for parametric data and Mann-Whitney U for non-parametric data, and chi-square for proportions. Multiple- group comparisons were conducted using ANOVA or Kruskall-Wallis tests. Associations between laboratory data and liver disease classification were explored using logistic regression and bivariate analyses conducted using biologically relevant variables. Associations between laboratory variables and obesity were explored using linear regression. P values <0.05 were considered significant.

Statistical analyses were conducted using GraphPad Prism and IBM SPSS Statistics Software version 23. Microbiome analysis was conducted on Statistical Analysis of Metagenomic Profiles (STAMP) and the R statistical package (Supplementary methods).

Results

Characteristics of cases and controls

Thirty-three cases, 29 HIV-positive and 17 HIV-negative controls were included in the study. The characteristics are described in table 1 and supplementary table 1.

The mean age of cases was 46 ±12.2 years, BMI 31.0±4.4 kg/m^2 and waist circumference 104.1±11.4 cm. Twenty-two (66.7%) patients had metabolic syndrome and 5 (15.2%) patients were on treatment for type 2 diabetes. HIV+ and HIV- healthy controls were 48.3 ±11.0 years and 48.0 (36.5-53.5) years respectively, and slim (BMI 24.1 ±2.8 kg/m^2 and 25.2 ±3.5 kg/m^2; waist circumference 85.7 ±8.0 cm and 86.0 ±7.1 cm).

Nine (27%) patients had non-alcoholic fatty liver (NAFL), and 24 (73%) patients had NASH;17 (51.5%) had none or mild liver fibrosis (F0-1), 3 (9.1%) had significant fibrosis (F2) and 13 (39.4%) advanced fibrosis (F3), including 4 patients with historical liver biopsies but with no significant weight change since biopsy. No patients had cirrhosis (Supplementary Table 2). The median time between biopsy and peripheral blood sample collection was 1 month (IQR 0-5).

NAFLD and liver fibrosis are not associated with markers of bacterial translocation

There was no difference either in levels of LBP (5.9 ±2.0 vs 5.3 ±1.7 μg/ml, p=0.330), bacterial DNA (0.01 ±0.01 vs 0.01 ±0.00 pg/ml, p=0.566) or LPS (30.2 (0.0-63.1) pg/ml vs 11.3 (0.0-49.7) pg/ml, p=0.269) between NAFLD cases compared to HIV+ controls. There was no difference

in any of these markers between HIV+ and HIV- controls (Supplementary Figure 1). These markers of translocation also did not distinguish NASH from NAFL (Supplementary Table 3) or significant fibrosis (Supplementary Figure 1).

NAFLD and liver fibrosis are associated with monocyte activation and adipose tissue dysfunction

NAFLD cases had significantly higher levels of sCD14 (1.3 ±0.4 vs 1.1 ±0.4 μg/ml, p=0.031), sCD163 (0.9 ±0.3 vs 0.7 ±0.2 μg/ml, p=0.002) and leptin (11.8 (3.8-20.2) vs 3.5 (2.1-5.5) ng/ml, p<0.0001), lower levels of adiponectin (1.1 (0.5-2.4) vs 2.5 (1.1-4.6) μg/ml, p=0.005), and higher leptin to adiponectin ratio (9.5 (2.5-27.6) vs 1.6 (0.6-4.6), p<0.0001) compared to HIV+ controls. IL-6 (7.2 ±2.0 vs 7.1 ±1.8 pg/ml, p=0.821) and TNFαR2 (1.1 ±0.5 vs 1.1 ±0.6 ng/ml, p=0.687) levels did not differ between HIV+ NAFLD cases and HIV+ controls. There was no difference in any of the markers between HIV+ and HIV- controls (Figure 1 and Supplementary Table 3).

Cases with F2-F3 fibrosis had significantly higher levels of sCD14 (1.4 ±0.4 vs 1.1 ±0.3 μg/ml, p=0.023, ANOVA p=0.008) compared to cases with F0-F1 fibrosis (Figure 1), whereas there was no difference in sCD14 levels between cases with F0-F1 fibrosis compared to HIV+ controls (1.1 ±0.3 v 1.1 ±0.4 μg/ml, p=0.521). There was a trend to increased sCD163 (1.0 ±0.3 vs 0.8 ±0.3 μg/ml, p=0.060) and leptin to adiponectin ratio (12.2 (7.5-37.3) vs 5.3 (1.8-21.7), p=0.063) from cases with F0-F1 fibrosis as compared to cases with F2-F3 fibrosis, but a significant increase in these markers by fibrosis stage compared to controls (ANOVA p=0.001 and p<0.0001 respectively, Figure 1). There was a significant increase in levels of IL-6 in cases with F2-F3 compared to cases with F0-F1 fibrosis (8.0 ±2.4 vs 6.4 ±1.0 pg/ml, p=0.022), but

there was no statistical difference in TNFαR2 levels between both groups (1.2 ±0.6 vs 1.0 ±0.4 ng/ml, p=0.341).

Systemic markers of monocyte activation and adipose tissue dysfunction correlate with central obesity

We next explored the impact of obesity and metabolic disorders on NAFLD and liver fibrosis. Cases had higher BMI (31.0 ±4.5 vs. 24.1 ±2.8 kg/m2, p<0.001), waist circumference (104.1 ±11.4 vs. 85.7 ±8.0 cm, p<0.001), more type 2 diabetes (15% vs. 0% p=0.037), hypertension (61% vs. 28%, p=0.012) and metabolic syndrome (67% vs. 10%, p<0.001) compared to HIV+ controls. We correlated markers associated with NAFLD and fibrosis with waist circumference, a surrogate marker for visceral adiposity. Soluble CD14 (r=0.297, p=0.022), sCD163 (r=0.413, p=0.001) and leptin to adiponectin ratio (r=0.487, p<0.0001) all positively correlated with waist circumference (Supplementary Figure 2). Similar results were observed with BMI, although sCD14 did not reach significance (sCD14 r=0.190, p=0.093; sCD163 r=0.371, p=0.001; leptin to adiponectin ratio r=0.534 p=<0.0001).

Bivariate logistic regression models were used to assess for an association of these markers with liver fibrosis independent of obesity in all HIV+ subjects (Table 2). Interestingly, sCD14 and sCD163 remained significantly associated with fibrosis when adjusted for BMI (OR 1.003 (1.001-1.005) p=0.016 and OR 1.003 (1.001-1.006) p=0.016) and waist circumference (OR 1.002 (1.000-1.005) p=0.049 and OR 1.003 (1.000-1.006) p=0.034), although the effect was blunted, whereas the association with leptin to adiponectin ratio was lost. Age and duration of ART did not affect the associations of these markers with fibrosis. This suggests that obesity contributes to but is not the sole factor in the increased monocyte activation associated with fibrosis.

Liver fibrosis is associated with macrophage clustering in the portal tracts

To investigate the relationship between peripheral monocyte activation and intra-hepatic macrophage activity in HIV-NASH with fibrosis, we performed immunohistochemistry on the liver tissue (n=28; NASH n=21; ≥F2 fibrosis n=14). Clusters of macrophages in the lobules were observed in patients with and without fibrosis. However, there were significantly more CD163- stained portal clusters in ≥F2 versus <F2 fibrosis (0.13 (0.00-0.22) vs 0.0 (0.00-0.04) clusters/mm, p=0.014), which was not observed with CD14 (0.01 (0.00-0.09)vs 0.00 (0.00-0.02) clusters/mm, p=0.122) (Figure 2), although the overall staining with CD14 was weaker than with CD163. There was a significant correlation between both sCD163 with CD163-stained portal clusters (r=0.504, p=0.010), and sCD14 with CD14-stained portal clusters (r=0.431, p=0.029). Neither portal clusters of sCD14 or sCD163 stained macrophages distinguished NASH from NAFL (CD14 0.00 (0.00-0.06) vs 0.00 (0.00-0.04) clusters/mm, p=0.492; CD163 0.04 (0.00-0.16) vs 0.00 (0.00-0.06) clusters/mm, p=0.101).

Gut microbiota

NAFLD is not associated with a distinct gut microbial profile

Fifty-seven stool samples (cases n=27/33, HIV+ controls n=20/29, HIV-controls n=10/17) were analysed using 16s rRNA sequencing. The characteristics of this subpopulation are shown in Supplementary Table 4. Analysis of the 16s rRNA gene sequencing showed no difference in the relative abundance of bacteria at all levels of the taxanomic classification between HIV+ patients with NAFLD and HIV+ controls. Community structures did not differ between groups on the non- metric multidimensional scaling (NMDS) plot (PERMANOVA p=0.809, Supplementary Figure 3A). Similarly, there was no distinct microbiota associated with NASH

or significant fibrosis (PERMANOVA p=0.858 and p=0.093, Supplementary Figure 3B and Figure 3A).

HIV infection is associated with a *Prevotella*- enriched enterotype

Given the lack of associations observed within all the HIV positive patients when stratified by NAFLD, NASH or fibrosis, these patients were grouped and compared to HIV negative controls. Interestingly, there were marked differences observed in the microbiome of subjects when stratified by HIV serostatus. NMDS plot demonstrated distinct clustering of microbial communities according to HIV serostatus (PERMANOVA p=0.001, Figure 3B), which remained when only HIV+ controls were compared to HIV- controls, confirming this was not a function of increased BMI or metabolic co-morbidities (data not shown). Significant changes between the groups emerged at the class level, with significantly higher abundance of *Negativicutes* (Mean difference (MD) 7.2% 95%CI 4.9-9.5, corrected p=0.002, Figure 3C). The most striking feature was an enrichment of *Prevotellaceae* (MD 28.0% (19.7-35.6), corrected p=0.011) and *Prevotella* (MD 25.7% (17.6-33.1), corrected p=0.013) at the family and genus level respectively. This was associated with an expected depletion in *Bacteroidaceae* (MD -22.9% (-15.1- -30.1), corrected p=0.022) and *Bacteroides* (MD -22.9% (-15.4- -30.3), corrected p=0.026) compared to HIV- subjects, who are known to compete in the same environmental niche (Figure 3C and supplementary Figure 4).

Discussion

We first explored bacterial translocation according to the biopsy-confirmed severity of liver disease, which has not previously been documented in this population, and found that neither

LBP,16s rDNA or LPS were associated with NAFLD and liver fibrosis stage. This was in contrast to a strong association with increased levels of sCD14, which in other studies has been used as a surrogate marker of bacterial translocation as CD14 is a co-receptor for LPS and is cleaved from the cell surface of circulating monocytes following activation by LPS.[31] However, sCD14 is not specific to LPS and may be released following monocyte stimulation by multiple ligands and as such also represents a non-specific marker of monocyte activation.[31] Given the lack of association with three other markers of bacterial translocation (LBP, bacterial DNA and LPS), monocyte activation more likely explains the increased circulating levels of sCD14 in our patients, which is consistent with the increase in sCD163 levels in cases with NAFLD and fibrosis.

There is an extensive literature supporting a role for bacterial translocation in NAFLD, although this is predominantly in animal models.[32] Clinical studies have also demonstrated associations between NAFLD and markers of increased gut permeability, but the results are more inconsistent.[33][34][35] This may be a function of methodological limitations, with LPS in particular lacking robust and reproducible assays.[36] However, it may also be that the absolute levels of systemic bacterial products are much less than in patients with more advanced liver disease (e.g. decompensated cirrhosis)[37][28] and beyond the limit of detection, especially when sampled peripherally rather than in portal blood. Moreover, the similar results between the HIV+ and HIV- control groups suggests there may in fact be restoration of the gut barrier in patients treated with effective ART.[20]

Biomarkers of monocyte activation in NAFLD have been investigated in both HIV and general populations. A study from the Multicentre AIDS Cohort Study (HIV+ n=329, NAFLD n=44) found an association between sCD14 and sCD163 with NAFLD which was lost following

adjustment for study site, age, race and *PNPLA3* genotype. However, cases were defined by liver steatosis on CT scan rather than biopsy, without stratification by NASH or fibrosis stage, so a detailed analysis of these markers in progressive disease could not be performed.[38] Another study in HIV mono-infected patients with or without metabolic syndrome (n=405) used Fibroscan to stratify by liver fibrosis, and found higher levels of circulating sCD14 and sCD163 in patients with metabolic syndrome, with sCD163 levels significantly associated with fibrosis stage independent of metabolic syndrome. Since clinical features of obesity were also associated with fibrosis stage, the authors concluded adipose tissue dysfunction was important but not the sole factor in monocyte activation and hepatic fibrogenesis. [13] In the non-HIV population, a study combining an Australian (n=157) and Italian (n=174) cohort of biopsy-confirmed NAFLD demonstrated a significant association between serum sCD163 levels and fibrosis stage, obesity and insulin resistance, which remained independently associated with liver fibrosis after adjustment for metabolic parameters.[25] Overall, these studies have consistently shown that biomarkers of monocyte activation, especially sCD163, are strongly linked to but not entirely explained by the metabolic complications of obesity, and appear to be key players in the development of NAFLD and fibrosis, regardless of HIV infection. This is consistent with our data: sCD14, sCD163 and leptin to adiponectin ratio (a marker of adipose tissue dysfunction and insulin resistance)[26] increased with fibrosis stage and significantly correlated with waist circumference, but the association between sCD14 and sCD163 with fibrosis remained after adjustment for waist circumference.

To investigate the link between peripheral markers of monocyte activation and intra-hepatic macrophages we performed immunohistochemistry in the liver tissue. CD163 - stained portal tract clusters of activated macrophages increased in patients with significant liver fibrosis (≥F2). This pattern was not so clearly seen with CD14, although the staining was weaker

throughout the biopsy suggesting it may be a less sensitive marker. Previous studies in non-HIV patients have found clustering of CD163-stained macrophages in NASH compared to NAFL patients, although they did not distinguish portal from lobular clusters, or look specifically at fibrosis.[25][39]

Here, the immunohistochemistry data, which significantly correlated with peripheral markers, further supports the notion that monocyte-macrophage activation is associated with progressive fibrosis stage, and the marked differences in peripheral markers between cases and controls is not solely a reflection of obesity rates in the groups. However, the demographic data clearly also highlights how obesity is an important contributor. This is consistent with experimental studies mechanistically linking central obesity to NASH in a disease model where inflamed, insulin resistant adipose tissue enriched with activated macrophages secretes leptin and other pro-inflammatory cytokines into the systemic circulation, in turn stimulating hepatic immune cell infiltration and fibrogenesis.[16] However, additional triggers independent of obesity such as hepatocyte injury from lipotoxicity and oxidative stress may also contribute to local monocyte activation.[40] Therefore, targeting monocyte recruitment is an emerging therapeutic option in NASH clinical trials; a phase 3 trial is underway evaluating Cenicriviroc, a CCR2/CCR5 antagonist targeting chemokine signalling important for monocyte infiltration and activation (NCT 03028740),[41] and similarly an early proof-of-concept trial is investigating the potential benefit of Maraviroc, a CCR5 receptor antagonist and licensed antiretroviral, in HIV-associated NASH (ISRCRN15410818).[42]

The role of the gut microbiome in NAFLD pathogenesis is an area of significant research interest, and its role in mediating complex metabolic and inflammatory pathways influencing

the development of NASH has been elegantly demonstrated in many pre-clinical models, opening new avenues for possible therapeutic targets.[17] However, human studies have often produced inconsistent results.[32] Our study has not observed an association between markers of bacterial translocation or the microbiota with NAFLD, NASH or fibrosis, contrasting with previous studies in the non-HIV population of patients with NAFLD.[43] This may reflect our small sample size, but the fact that associations of specific bacterial populations with NAFLD are rarely repeated in subsequent studies[44] demonstrates the difficulty in exploring a highly complex system in a disease that is slow to evolve.

One striking finding was the significant difference in gut microbial communities between cases with HIV and age and sex- matched healthy controls. This was driven principally by an enrichment in the genus *Prevotella* (family *Prevotellaceae*), mirrored by a converse depletion of its competitor *Bacteroides*. Interestingly, *Prevotella* enrichment has been a relatively consistent finding in previous studies investigating the impact of the microbiome in people living with HIV, although this may be a function of lifestyle factors, particularly sexual practices, rather than HIV infection per se.[45] The reasons for this are incompletely understood but may be linked to local environmental perturbations associated with microtrauma and tissue healing.[46] Further mechanistic work is required to investigate a possible role for *Prevotella* in mucosal healing, and whether this affects an individual's susceptibility to acquiring HIV infection.

Our study has some limitations. First, the small sample size. The gold standard for diagnosing NASH and fibrosis remains liver biopsy, an invasive procedure and currently only indicated in patients who meet specific criteria following assessment with non-invasive markers. This limits the sample size, restricts analyses to an enriched group with few cases of mild liver

disease, and some smaller associations with specific biomarkers may have been missed by lack of statistical power. This may explain why none of the biomarkers could distinguish NASH from NAFL, and negative results in the microbiota analysis. However, there is currently no validated diagnostic marker of NASH, and non-invasive markers have not been well validated in the HIV population, therefore a small study with well-characterised liver histology might be superior to larger studies based on non-invasive markers when investigating mechanisms of NAFLD. Second, some of the results may have been a function of the control group selection, whose BMI was much lower than the cases. However, our bivariate analysis demonstrated an association of monocyte markers independent of BMI and waist circumference. Finally, we were unable to collect Fibroscan values in HIV+ and HIV- controls. However, all had exclusion of acute or chronic liver disease and normal liver function tests and biochemistry.

In conclusion, monocyte activation associated with central obesity seems to be a key player in the development of NAFLD and significant liver fibrosis in HIV mono-infected patients independent of dysbiosis and gut translocation.

Acknowledgements: We would like to thank St Stephens AIDS Trust for funding this work, and Mike Liu and Fouzia Sadiq for their technical support in the laboratory.

References

1 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**:73–84.

2 Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**:1313–1321.

3 Hagström H, Nasr P, Ekstedt M, Hammar U, Stål P, Hultcrantz R, *et al.* Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in

biopsy-proven NAFLD. *J Hepatol* 2017; **67**:1265–1273.

4 Flemming JA, Kim WR, Brosgart CL, Terrault NA. Reduction in liver transplant wait-listing in the era of direct-acting antiviral therapy. *Hepatology* 2017; **65**:804–812.

5 Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and Outcomes of Liver Transplantation for Nonalcoholic Steatohepatitis in the United States. *Gastroenterology* 2011; **141**:1249–1253.

6 Morse CG, McLaughlin M, Matthews L, Proschan M, Thomas F, Gharib AM, *et al.* Nonalcoholic Steatohepatitis and Hepatic Fibrosis in HIV-1-Monoinfected Adults With Elevated Aminotransferase Levels on Antiretroviral Therapy. *Clin Infect Dis* 2015; **60**:1569–1578.

7 Vodkin I, Valasek M a, Bettencourt R, Cachay E, Loomba R. Clinical, biochemical and histological differences between HIV-associated NAFLD and primary NAFLD: a case-control study. *Aliment Pharmacol Ther* 2014; :1–11.

8 Ingiliz P, Valantin MA, Duvivier C, Medja F, Dominguez S, Charlotte F, *et al.* Liver damage underlying unexplained transaminase elevation in human immunodeficiency virus-1 mono-infected patients on antiretroviral therapy. *Hepatology* 2009; **49**:436–442.

9 Benmassaoud A, Ghali P, Cox J, Wong P, Szabo J, Deschenes M, *et al.* Screening for nonalcoholic steatohepatitis by using cytokeratin 18 and transient elastography in HIV mono-infection. *PLoS One* 2018; **13**:1–13.

10 Maurice JB, Patel A, Scott AJ, Patel K, Thursz M, Lemoine M. Prevalence and risk factors of nonalcoholic fatty liver disease in HIV-monoinfection. *AIDS* 2017; **31**:1621–1632.

11 Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis Progression in Nonalcoholic Fatty Liver vs Nonalcoholic Steatohepatitis: A Systematic Review and Meta-analysis of Paired-Biopsy Studies. *Clin Gastroenterol Hepatol* 2015; **13**:643–654.

12 Parikh ND, Marrero WJ, Wang J, Steuer J, Tapper EB, Konerman M, *et al.* Projected increase in obesity and non-alcoholic steatohepatitis-related liver transplantation waitlist additions in the United States. *Hepatology* Published Online First: 17 August 2017. doi:10.1002/hep.29473

13 Lemoine M, Lacombe K, Bastard JP, Sébire M, Fonquernie L, Valin N, *et al.* Metabolic syndrome and obesity are the cornerstones of liver fibrosis in HIV-monoinfected patients. *Aids* 2017; **44**:1.

14 C. D, A. P. Central obesity and non-alcoholic fatty liver disease in people living with HIV: A pragmatic approach. *HIV Med* 2018; **19**:S74.

15 G. G, N. S, C. S, G. O, R. D, G. L, *et al.* Nonalcoholic fatty liver disease in HIV-infected patients referred to a metabolic clinic: Prevalence, characteristics, and predictors. Clin. Infect. Dis. 2008; **47**:250–257.

16 Bijnen M, Josefs T, Cuijpers I, Maalsen CJ, van de Gaar J, Vroomen M, *et al.* Adipose tissue macrophages induce hepatic neutrophil recruitment and macrophage

accumulation in mice. *Gut* 2017; :gutjnl-2016-313654.

17 Marra F, Svegliati-Baroni G. Lipotoxicity and the gut-liver axis in NASH pathogenesis. *J Hepatol* 2018; **68**:280–295.

18 Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 2016; **65**:1038–1048.

19 Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, *et al.* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 2007; **12**:1365–1371.

20 Mudd JC, Brenchley JM. Gut Mucosal Barrier Dysfunction, Microbial Dysbiosis, and Their Role in HIV-1 Disease Progression. *J Infect Dis* 2016; **214**:S58–S66.

21 Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, *et al.* Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci Transl Med* 2013; **5**:193ra91.

22 Cassinotto C, Boursier J, de L??dinghen V, Lebigot J, Lapuyade B, Cales P, *et al.* Liver stiffness in nonalcoholic fatty liver disease: A comparison of supersonic shear imaging, FibroScan, and ARFI with liver biopsy. *Hepatology* 2016; **63**:1817–1827.

23 Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, *et al.* Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International . *Circulation* 2009; **120**:1640–1645.

24 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**:2467–2474.

25 Kazankov K, Barrera F, Møller HJ, Rosso C, Bugianesi E, David E, *et al.* The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease. *Liver Int* 2016; **36**:1549–1557.

26 Finucane FM, Luan J, Wareham NJ, Sharp SJ, O'Rahilly S, Balkau B, *et al.* Correlation of the leptin: Adiponectin ratio with measures of insulin resistance in non-diabetic individuals. *Diabetologia* 2009; **52**:2345–2349.

27 Tsochatzis E, Papatheodoridis G V, Archimandritis AJ. The Evolving Role of Leptin and Adiponectin in Chronic Liver Diseases. *Am J Gastroenterol* 2006; **101**:2629–2640.

28 Vergis N, Atkinson SR, Knapp S, Maurice J, Allison M, Austin A, *et al.* In Patients With Severe Alcoholic Hepatitis, Prednisolone Increases Susceptibility to Infection and Infection-Related Mortality, and Is Associated With High Circulating Levels of Bacterial DNA. *Gastroenterology* 2017; **152**:1068–1077.e4.

29 Mullish BH, Pechlivanis A, Barker GF, Thursz MR, Marchesi JR, McDonald JAK. Functional microbiomics: Evaluation of gut microbiota-bile acid metabolism interactions in health and disease. *Methods* Published Online First: 26 April 2018. doi:10.1016/j.ymeth.2018.04.028

30 Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl Environ Microbiol* 2013; **79**:5112–5120.

31 Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. doi:10.1097/QAD.0000000000000735

32 Brandl K, Schnabl B. Intestinal microbiota and nonalcoholic steatohepatitis. *Curr Opin Gastroenterol* 2017; **33**:128–133.

33 Pang J, Xu W, Zhang X, Wong GLH, Chan AWH, Chan HY, *et al.* Significant positive association of endotoxemia with histological severity in 237 patients with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2017; **46**:175–182.

34 Du Plessis J, Korf H, Van Pelt J, Windmolders P, Vander Elst I, Verrijken A, *et al.* Pro-inflammatory cytokines but not endotoxin-related parameters associate with disease severity in patients with NAFLD. *PLoS One* 2016; **11**:1–15.

35 Farhadi A, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, *et al.* Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. *Liver Int* 2008; **28**:1026–33.

36 Gnauck A, Lentle RG, Kruger MC. Chasing a ghost? – Issues with the determination of circulating levels of endotoxin in human blood. *Crit Rev Clin Lab Sci* 2016; **53**:197–215.

37 Wiest R, Lawson M, Geuking M. Pathological bacterial translocation in liver cirrhosis. *J Hepatol* 2014; **60**:197–209.

38 Price JC, Wang R, Seaberg EC, Budoff MJ, Kingsley LA, Palella FJ, *et al.* The Association of Inflammatory Markers With Nonalcoholic Fatty Liver Disease Differs by Human Immunodeficiency Virus Serostatus. *Open Forum Infect Dis* 2017; **4**:1–9.

39 Kazankov K, Tordjman J, Møller HJ, Vilstrup H, Poitou C, Bedossa P, *et al.* Macrophage activation marker soluble CD163 and non-alcoholic fatty liver disease in morbidly obese patients undergoing bariatric surgery. *J Gastroenterol Hepatol* 2015; **30**:1293–300.

40 Peterson KR, Cottam MA, Kennedy AJ, Hasty AH. Macrophage-Targeted Therapeutics for Metabolic Disease. *Trends Pharmacol Sci* 2018; **39**:536–546.

41 AURORA: Phase 3 Study for the Efficacy and Safety of CVC for the Treatment of Liver Fibrosis in Adults With NASH - Full Text View - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03028740 (accessed 25 Sep2018).

42 Clinical Trials register - Search for 2017-003172-32. https://www.clinicaltrialsregister.eu/ctr-search/search?query=2017-003172-32 (accessed 30 May2018).

43 Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, *et al.* The severity of NAFLD is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. *Hepatology* 2015; :1–32.

44 Brenner DA, Paik Y-H, Schnabl B. Role of Gut Microbiota in Liver Disease. *J Clin Gastroenterol* 2015; **49**:S25–S27.

45 Noguera-Julian M, Rocafort M, Guillén Y, Rivera J, Casadellà M, Nowak P, *et al.* Gut Microbiota Linked to Sexual Preference and HIV Infection. *EBioMedicine* 2016; **5**:135–146.

46 Kelley CF, Kraft CS, De Man TJB, Duphare C, Lee HW, Yang J, *et al.* The rectal mucosa and condomless receptive anal intercourse in HIV-negative MSM: Implications for HIV transmission and prevention. *Mucosal Immunol* 2017; **10**:996–1007.

521 **Table 1**

Parameters	Cases n=33	HIV+ Controls n=29	HIV- Controls n=17	P value*
Age (years)	46.4 (12.4)	48.3 (11.0)	48.0 (36.5-53.5)	0.523
Male Gender (%)	32 (97.0)	29 (100)	15 (88.2)	0.525
Ethnicity				
Caucasian (%)	27 (81.8)	22 (75.9)	10 (58.8)	0.756
Black (%)	2 (6.1)	3 (10.3)	0 (0.0)	0.658
Asian (%)	3 (9.1)	2 (6.9)	5 (29.4)	1.000
Other (%)	1 (3.0)	2 (6.9)	2 (11.8)	0.595
Transmission Risk Factor		29 (100)	0 (0.0)	0.241
MSM (%)	30 (90.9)	0 (0)	17 (0.0)	0.494
Heterosexual (%)	2 (6.1)	0 (0)	N/A	1.00
Vertical Transmission (%)	1 (3.0)			
BMI (kg/m2)	31.0 (4.5)	24.1 (2.8)	25.2 (3.5)	**<0.001***
Waist circumference (cm)	104.1 (11.4)	85.7 (8.0)	86.0 (7.1)	**<0.001***
Type 2 Diabetes (%) [1]	5 (15.2)	0 (0)	0 (0)	**0.037***
Hypertension (%) [2]	22 (66.7)	9 (31.0)	0 (0)	**0.010***
High serum Triglycerides (%) [3]	22 (66.7)	8 (27.6)	0 (0)	**0.003***
Low serum HDL (%) [4]	22 (66.7)	9 (31.0)	0 (0)	**0.010***
Metabolic Syndrome [5]	22 (66.7)	3 (10.3)	0 (0)	**<0.001***
Time since HIV Diagnosis (years)	9.0 (5.0-15.0)	12.0 (5.5-18.5)	N/A	0.385
CD4 cell count Nadir	262.1 (168.4)	292.5 (225.7)	N/A	0.536
Duration ART (years)	7.6 (6.5)	10.2 (8.0)	N/A	0.221
Cumulative duration of ART Class (years)				
NRTI	8.2 (6.5)	20.3 (8.0)	N/A	0.290

NNRTI	4.6 (4.6)	6.4 (6.9)	N/A	0.233
PI	0.0 (0.0-3.8)	0.0 (0.0-2.3)	N/A	0.584
II	0.0 (0.0-1.2)	0.0 (0.0-0.0)	N/A	**0.027***
ALT (IU/L)	104.7 (62.1)	28.3 (8.3)	-	**<0.001***
AST (IU/L)	56.9 (42.0-59.5)	27.8 (24.0-31.0)	-	**0.002***
ALP (IU/ml)	91.1 (25.1)	76.4 (16.3)	-	**0.011***
Cholesterol (mmol/L)	5.0 (0.9)	4.8 (1.4)	-	0.617
LDL (mmol/L)	3.1 (1.1)	3.0 (1.1)	-	0.795
HDL (mmol/L)	1.3 (1.2)	1.3 (0.4)	-	0.873
Triglycerides (mmol/L)	2.2 (1.1)	1.5 (0.8)	-	**0.011***
Glucose (mmol/L)	5.4 (1.2)	5.0 (0.8)	-	0.253
CD4 (cells/mm^3)	815.5 (309.2)	765.7 (235.1)	-	0.506
CD8 (cells/mm^3)	1048.8 (417.3)	830.8 (317.0)	-	**0.046***
Liver Stiffness (kPa)	8.7 (3.7)	-	-	-
CAP (dB/min)	308.8 (36.2)	-	-	-

Table 1: Demographic data of cases compared to age- and sex- matched HIV+ and HIV- controls. Categorical variables are expressed as raw numbers and percentages, continuous variables are reported as mean (SD) or median (IQR). [1] Active treatment with anti-diabetic medications; [2] Systolic BP ≥130mmHg, diastolic BP ≥85mmHg or active treatment anti-hypertensive medication; [3] Serum triglycerides >1.7mmol/L or active treatment with a fibrate; [4] Serum HDL < 1.0 or active treatment with a statin. [5]As per international guidelines.[23] *Cases vs HIV+ controls, P value<0.05. MSM: men who have sex with men; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; ART: antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse

532 transcriptase inhibitors; PI: protease inhibitors; II: integrase inhibitors; ALT: alanine
533 aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; CAP:
534 controlled attenuation parameter.

535

536

537

538

539

540

541

542

543

544

545

546

547

548

Biomarker	Unadjusted OR	P Value	Model	Adjust NAFLD
sCD14	1.003 (1.001-1.005)	***0.006***	+Age	1.003 (1.005)
			+Waist Circumference	1.002 (1.005)
			+BMI	1.003 (1.005)
			+ Duration of ART	1.003 (1.005)
sCD163	1.004 (1.001-1.006)	***0.003***	+Age	1.003 (1.006
			+Waist Circumference	1.003 (1.006)
			+BMI	1.003 (1.006)
			+ Duration of ART	1.003 (1.006)
Leptin:Adiponectin	1.059 (1.016-1.104)	**0.007**	+Age	1.057 (1.101)
			+Waist Circumference	1.034 (1.081)
			+BMI	1.033 (1.081)
			+ Duration of ART	1.061 (1.108)

Table 2: Odds ratios for sCD14, sCD163 (per 1ng/ml increase) and leptin to adiponectin ratio as biomarkers for significant fibrosis in all subjects with HIV (n=62), adjusted for either age, waist circumference, BMI or duration of antiretroviral therapy (ART). Italics indicate p<0.05.

Figure Legends

Figure 1: Markers of monocyte activation and adipose tissue function in cases and controls. A-E: NAFLD; F-J: sub-categorised by fibrosis stage. sCD14: soluble CD14; sCD163: soluble CD163.

Figure 2: Liver immunohistochemistry**.** A-B: Sample liver sections (Magnification x100) without (A)

and with (B, arrow) portal clusters of CD163-stained macrophages; C-D: Portal clusters/mm liver tissue with CD163 (C) and CD14 (D) staining; E-F: Correlation between liver portal macrophage clusters and peripheral markers of monocyte activation.

Figure 3: Gut microbial communities in liver fibrosis and HIV infection. Non-metric dimensional scaling (NMDS) plot comparing microbial community structures between A). HIV positive cases with NAFLD and ≥F2 Fibrosis vs NAFLD and <F2 Fibrosis vs HIV+ controls. PERMANOVA p=0.093; B). HIV+ (all) vs HIV- subjects. PERMANOVA p=0.001. C). Extended error bar plots comparing the mean difference of significantly altered proportions at Class, Order, Family and Genus taxonomic classification between HIV+ subjects vs HIV- subjects (White's non-parametric t-test with Benjamini-Hochberg FDR correction).