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

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

**Article Type:** Original paper (Basic Science)

**Keywords:** NAFLD; NASH; Fibrosis; HIV; translocation; monocyte; macrophage; microbiome

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**Abstract:**

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/m2 vs 24.1 ±2.8 kg/m2 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.
4th December 2018

To the editor

Re: Manuscript reference number: AIDS-D-18-00956
Title: Monocyte-Macrophage activation is associated with NAFLD and liver fibrosis in HIV mono-infection independently of the gut microbiome and bacterial translocation
Article type: Original paper (Basic Science)

Thank you for reviewing our paper and considering it for publication. The reviewers’ comments have been extremely constructive, and we believe the subsequent changes have added to the quality of the manuscript. Please see our specific responses to each comment below.

We look forward to hearing from you.

Yours sincerely,

Dr James Maurice

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Reviewers’ comments:

Reviewer #1: In this manuscript Maurice et al. investigate the association of bacterial translocation (lipopolysaccharide-binding protein (LBP), 16s rDNA), adipose tissue dysfunction (leptin, adiponectin), monocyte/macrophage activation (sCD14, sCD163 and immunohistochemistry) and gut dysbiosis (16s rRNA sequencing in stool) in patients with HIV monoinfection and NAFLD biopsy-proven compared with HIV monoinfected patients without liver disease and healthy donors.

Major comments

The manuscript is well written and results are interesting to the field. There are two major concerns and or doubts. i) It is not clear for this reviewer whether the real impact of bacterial translocation in NAFLD is limited because of the measurement used to assess this phenomenon or because bacterial translocation has no effect per se. For this reason, I suggest to soften even the negative result of bacterial translocation in relation to NAFLD. ii) In NAFLD, inflammation caused by obesity and monocyte/macrophage activation are two processes difficult to separate, this is even more difficult when HIV-control group is different in terms of obesity associated parameters. Although these two processes are concomitant authors may try to isolate these two effects with the appropriate statistical approach.

Association of NAFLD with markers of bacterial translocation. Figure 1 does not make reference to these markers, please correct for Supplementary Figure 1. The values of 16s rDNA are very low, are these values reliable? Did the authors compared with positive controls for this measurement; e.g. patients naïve for ART in chronic phase of infection? If this measurement is not consistent may be eliminated of the manuscript. If this is the case, the only measurement for bacterial translocation is the indirect parameter LBP, authors may have tried to assayed LPS which is a direct measurement of bacterial translocation. I understand the limitation to perform this measurement. In this sense, the independence of bacterial translocation with NAFLD based in only one parameter is provocative. Authors may focus the main message of the work in monocyte/macrophage activation.
Authors’ responses:

1. We have included additional LPS data using a commercial assay used in another paper on liver disease (Roberto Carnevale et al J Hep 2017;67;950-956). This was omitted on the original submission to simplify the paper and given it did not change the conclusions. We also have some concerns about LPS assays in general, but with mention of this in the discussion we agree it is helpful to include. Please see amended supplementary figure 1 and the text on p.8 line 171-172.

2. Therefore, with three negative assays, we think this is sufficient to maintain a relatively clear message about the lack of evidence for association of bacterial translocation with disease stage, albeit within the limitations of a small observational study.

3. We have corrected the reference to supplementary figure 1 on p.9 line 171.

4. We agree that the levels of bacterial DNA are very low. The assay has previously been used by our group to report on a large cohort of patients with alcoholic hepatitis, a population with more severe disease. Within this study higher levels of bDNA had worse outcomes, demonstrating the ability of the assay to identify clinically relevant patient populations (Nikhil Vergis et al Gastroenterology 2017;152:1068-1077). We believe this study acts as a good ‘positive control’ for the assay and supports our finding that in systemically well patients with NAFLD/NASH but not decompensated liver disease that the role of bacterial translocation is likely to be of diminished significance.

It should be interesting to analyze whether soluble markers of monocyte activation are associated with NAFLD after adjusting for obesity parameters. If this is the case this table may be part of the main text. Meanwhile Supplementary table 4 may be part of the main text.

Authors’ response:

1. We have made the suggested change and moved the table into the main text (Table 2) and moved Figure 3 (correlation of markers with waist circumference) to supplementary

2. We have added an additional sentence and reference in the discussion (p.16 line 335-337) to illustrate potential mechanisms leading to macrophage activation independent of obesity.

Importantly, sCD163 and sCD14 are surrogate markers of monocyte activation in the periphery; authors may not consider these markers of macrophage activation. Macrophage activation is better assessed in tissues. Please rephrase this expression throughout the manuscript including the title. Please, show the correlation between sCD163 and CD163 tissue staining and the same for sCD14 and CD14 staining.

Authors’ response:

1. We have amended the nomenclature throughout as suggested.

2. The manuscript already includes the data correlating peripheral monocyte and tissue macrophage markers (p.11 line 235-237). We have now included this data also in Figure 2.

The differences in soluble markers are clear. Authors stated that "There was no difference in any of the markers between HIV+ and HIV- controls". This is not true for leptin levels according to supplementary Table 3. What is the meaning of the asterisk in this table?

Authors’ Response:

1. There was an error in supplementary table 3- the leptin data is heavily negatively skewed, so this should have been presented as median (IQR) data with Mann-Whitney tests, which showed no difference between these groups. The table has been amended accordingly.
Why tumor necrosis factor alpha receptor 2 (TNFαR2) is measured instead of TNFα?

Authors’ Response:
1. We measured TNFαR2 as it can be a more sensitive marker of inflammation than TNFα (Spinas GA et al J Clin Invest 1992;90:533-6) and has been associated with fatty liver in previous studies and (Price et al Open Forum Infectious Diseases 2017). This latter paper is referenced in the discussion.

Minor comments

In the description of Table 1, some values from line 155 to 157 are different to those of the table. PI and II are expressed as median (IQR) while NRTI and NNRTI are in percentages, please correct this inconsistency.

In line 161, please indicate that the sample refers to peripheral blood samples.

In the first paragraph of the results authors may highlight the differences between cases and HIV-control group.

In general the font sizes of the axis legends in the figures are very small what makes the figure difficult to follow.

The meaning of the sentence in lines 182-184 is not clear.

Authors’ Response:
1. We previously had NRTI and NNRTI durations as mean (SD) and PI/II as median (IQR), because the latter were skewed. These have all now been changed to median (IQR) for clarity.
2. ‘Samples’ changed to ‘Peripheral blood samples’ on p.9 line 175.
3. We have now included in the results (p.8 line 168-170) the difference in BMI and waist circumference which we feel is the most important factor.
4. The figure format has been changed to PNG files in which the axes are now much clearer. We ask the editors to inform us if there are remaining issues with clarity.
5. We have amended this sentence to make it clearer.

Reviewer #2: The authors present a comparison of various markers of bacterial translocation, macrophage activation, and adipose tissue dysfunction in individuals with well-controlled HIV and biopsy-proven NAFLD as compared to a group of individuals with HIV and no known NAFLD and a group of individuals without HIV. The results are interesting and demonstrate that NAFLD in the individuals with HIV may be associated with monocyte activation independent of bacterial translocation/changes to the gut microbiome.

In my opinion the study has strengths and weaknesses, many of which the authors acknowledge. I appreciate that NAFLD was proven by biopsy and the methods for assessing bacterial translocation, macrophage activation, and adipose tissue dysfunction seem sophisticated and the statistical analysis appropriate. I also think this is an area in which further study/literature is
needed and our understanding is actively evolving, so studies and literature on the topic are appreciated, and I believe the authors provide a very useful discussion of both their findings and review of previous findings and the literature.

I have several concerns and questions for the authors. My primary concern has to do with matching of the cases and controls, which the authors acknowledge as a limitation. My principal concern is conclusions that are made despite the NAFLD/HIV+ cases being quite different from both groups of controls in terms of BMI, waist circumference, metabolic syndrome, etc. I see that the authors tried to control for this with bivariate logistic regression adjusting for BMI and waist circumference, and associated with markers such as sCD14 and sCD163 technically did remain significant, but the OR's are quite modest (in the range of 1.000 to 1.006) - this is on page 10. My concern is that many of the findings about the significance of macrophage activation may be secondary to what is largely a comparison between a group with metabolic syndrome/obesity versus a group without this. Despite the efforts to analyze results controlling for obesity, I still think this is a significant limitation and wording of results needs to be careful to reflect this. For example, in the abstract, the following sentence is included: "Cases with NAFLD had significantly increased levels of sCD14 (1265.0 v 1062.0ng/ml, p=0.038), sCD163 (894.2 v 668.7ng/ml, p=0.0016) and higher leptin to adiponectin ratio (9.4 v 1.6, p<0.0001) versus HIV+ controls," yet it is not clear that cases had significantly elevated BMI/waist circumference as compared to controls and that may be largely responsible for the findings. The conclusion of the abstract does focus on the association with macrophage activation in the HIV/NAFLD cases and not on the comparisons and I do agree that is probably the most important conclusion.

My suggestions and questions:
1. I think wording in the abstract and in the discussion/conclusions needs to make clear that the findings are in the setting of important differences between cases and controls, as discussed above (I know this is highlighted as a limitation, but I think readers need to be clear when reading the abstract and in other parts of the manuscript when reading about the results).

Authors’ response:
1. This is a very important point. As you say, we recognise the limitations of our control groups and have included this in the discussion. However, considering your comments we have also amended the abstract to include the differences in BMI between cases and controls, and focussed on the fibrosis data which makes our point more clearly. We have also added text to the discussion (p.15 324-331 and p.16 line 335-337). We think the differences between fibrosis stage in both peripheral and tissue markers shows the relevance of these findings which are can’t simply reflect control selection.

2. I would appreciate more clarity on the methods including it says that cases were recruited prospectively but were liver biopsies done prospectively or had cases already had biopsies that were reviewed retrospectively? Also were controls recruited prospectively as well or how were controls recruited and how was control data collected?

Authors’ response:
1. Please see amended methods (p.5 line 98-101) and results (p9 line 173-174).

3. Please clarify what you mean by "HIV/liver clinics" as the sites for recruitment.

Authors’ response:
1. Please see amended methods (p.5 line 98-99) to clarify this.

4. In table 1, the rows for risk factor for transmission do not line up and it would be useful/more clear to make them line up.

Authors’ response:
1. This has been corrected.

5. Also in table 1, I found the presentation of ART regimen confusing. I interpret this to mean that the majority of patients were on NNRTI+2 NRTI regimens? Were there any differences in the markers of interest by ART regimen or by specific NNRTI (which may be relevant given NNRTI’s especially nevirapine can cause liver dysfunction and even efavirenz has caused cases of AST/ALT dysfunction). If there is more detail you can add to the text or table about specific regimens used or even the years over which patients were recruited that could be helpful to the reader to get a sense of the most frequent ARV regimens used.

Authors’ response:
1. The table refers to cumulative exposure to each class, rather than current regimen. This has been clarified on the table.
2. Given the small sample size, we have not been able to do a detailed multivariate analysis of these factors, which we agree would be very interesting. However, we have now included ‘Duration of ARV’ in the bivariate analysis (table 2), and it does not seem to influence the biomarker results.
3. All patients were recruited in the timeframe stated and therefore were on modern regimens at the time of sample collection.

6. At the end of the discussion it is mentioned that "macrophages are an emerging therapeutic option" but does this mean to say that macrophage activation blockers are a potential therapeutic option?

Authors’ response:
1. We have amended the text to clarify this (see p.16 line 337).
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 its advanced forms (non-alcoholic steatohepatitis (NASH) and fibrosis) in this population.

**Objectives:** to investigate the association of bacterial translocation, adipose tissue dysfunction, macrophage-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), 16s rDNA, lipopolysaccharide (LPS)), adipose tissue dysfunction (leptin, adiponectin) and macrophage-monocyte activation (sCD14 and sCD163) were measured by ELISA. Hepatic patterns of macrophage activation were explored with immunohistochemistry. 16s rRNA sequencing was performed using stool.

**Results:** Thirty-three cases were included (NASH n=24; ≥F2 fibrosis n=16; BMI 31.0±4.5 kg/m², metabolic syndrome n=22), matched to HIV+ (n=29) and HIV- (n=17) controls. Cases with NAFLD were more obese (BMI 31.0±4.4 kg/m² vs 24.1±2.8 kg/m² p<0.001) and had significantly increased levels of sCD14 (1265.0 vs 1062.0 ng/ml, p=0.038), sCD163 (894.2 vs 668.7 ng/ml, p=0.0016) and higher leptin to adiponectin ratio (9.4 vs 1.6, p<0.0001) versus HIV+ controls. These markers increased with fibrosis stage and positively correlated with waist circumference. 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 distribution of macrophage activation in patients with fibrosis. LBP and 16s rDNA levels were not markers of bacterial translocation or changes to the microbiome were associated with NAFLD or fibrosis. Immunohistochemistry showed a portal distribution of macrophage activation in patients with fibrosis. There were no associated changes to the intestinal microbiome with NAFLD, but HIV infection was associated with enrichment of Prevotella.

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

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Structured Summary (Word Count: 250250)

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

**Key Words:** NAFLD; NASH; fibrosis; HIV; translocation; macrophage 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.\cite{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.\cite{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 dedicated HIV-liver 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/mm³) 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).\cite{22}
Exclusion criteria were: patients with alcohol excess within the last 6 months defined as
>21 units/week for men and >14 units/ 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) \textit{(RND Systems, Abingdon, UK)} and lipopolysaccharide (LPS) \textit{(Cusabio, Wuhan, China)}) and macrophage-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 multivariate 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/m² 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/m² and 25.2 ±3.5 kg/m²; 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; 451 (45.1%) had none or mild liver fibrosis (F0-1), 3 (9.1%) had significant fibrosis (F2) and
134 (38.4%) 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) 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).

NAFLD and liver fibrosis are associated with macrophage-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 macrophagemonocyte 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 (Supplementary Table 4Table 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 macrophage-monocyte activation associated with fibrosis.

Liver fibrosis is associated with macrophage clustering in the portal tracts

To investigate the relationship between peripheral macrophage-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 3), 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 45. Analysis of the 16s rRNA gene sequencing showed no difference in the relative abundance of bacteria at all levels of the taxonomic classification between HIV+ patients with NAFLD and HIV+ controls. Community structures also did not differ between groups on the non-metric multidimensional scaling (NMDS) plot (PERMANOVA p=0.809, Supplementary Figure 3A1). Similarly, there was no distinct microbiota associated with NASH or significant fibrosis (PERMANOVA p=0.858 and p=0.093, Supplementary Figure 2-3B and Figure 34Aa).

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 43Bb), 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, Supplementary Figure 2 and Figure 34C). The most striking feature was an enrichment of *Prevotellaceae* (MD 28.0% 95%CI (-19.7-35.6), corrected p=0.011) and *Prevotella* (MD 25.7% 95%CI (-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% 95%CI (−15.1−30.1), corrected p=0.022) and *Bacteroides* (MD -22.9% 95%CI (−15.4−30.3), corrected p=0.026) compared to HIV- subjects (Fig 4c), 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, or 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 two–three other markers of bacterial translocation (LBP, and 16s r 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 macrophagemonocyte 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 macrophagemonocyte 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 macrophagemonocyte 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] significantly correlated with both 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 and intrahepatic markers of macrophage 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) and correlated with peripheral levels. 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

Overall our data shows macrophage activation is important in the development of NAFLD and
fibrosis in patients with HIV mono-infection, to which obesity and insulin resistance are likely
to be important contributors. This supports 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, macrophage-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 macrophage-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 species 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 is limited with a small number of patients with advanced liver disease. 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, invariably 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 by definition much lower than the cases. However, our bivariate analysis demonstrated an association of macrophage-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, macrophage-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.

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**References**


20. Mudd JC, Brenchley JM. Gut Mucosal Barrier Dysfunction, Microbial Dysbiosis, and


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<th>HIV- Controls n=17</th>
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<td>ALP (IU/ml)</td>
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<td>Cholesterol (mmol/L)</td>
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<td>Triglycerides (mmol/L)</td>
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<td>1.5 (0.8)</td>
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<td>CD4 (cells/mm³)</td>
<td>815.5 (309.2)</td>
<td>765.7 (235.1)</td>
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<td>CD8 (cells/mm³)</td>
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<td>830.8 (317.0)</td>
<td>-</td>
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<td>Liver Stiffness (kPa)</td>
<td>8.7 (3.7)</td>
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<td>CAP (dB/min)</td>
<td>308.8 (36.2)</td>
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</table>

**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
transcriptase inhibitors; PI: protease inhibitors; II: integrase inhibitors; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; CAP: controlled attenuation parameter.
<table>
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<th>Biomarker</th>
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<th>P Value</th>
<th>Model</th>
<th>Adjusted OR for NAFLD (95% CI)</th>
<th>P Value</th>
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<td>sCD14</td>
<td>1.003 (1.001-1.005)</td>
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<td>1.003 (1.001-1.005)</td>
<td><strong>0.011</strong></td>
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<td>+Waist Circumference</td>
<td>1.002 (1.000-1.005)</td>
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<tr>
<td></td>
<td></td>
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<td>+BMI</td>
<td>1.003 (1.001-1.005)</td>
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<td>+ Duration of ART</td>
<td>1.003 (1.001-1.005)</td>
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<td>sCD163</td>
<td>1.004 (1.001-1.006)</td>
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<td>Leptin:Adiponectin</td>
<td>1.059 (1.016-1.104)</td>
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<td>+Waist Circumference</td>
<td>1.034 (0.989-1.081)</td>
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**Table 2**: Odds ratios for sCD14, sCD163 (per 1 ng/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).

<table>
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<tr>
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<td>+ Duration of ART</td>
<td>1.061 (1.016-1.108)</td>
<td>0.008</td>
</tr>
</tbody>
</table>
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; IL-6: interleukin-6; TNFαR2: TNFα receptor-2.

**Figure 2:** Correlation between sCD14, sCD163, Leptin to adiponectin ratio and waist circumference.

**Figure 3:** Liver immunohistochemistry. A-B: Sample liver sections (Magnification x100) without (A) and with (B, arrow) portal clusters of CD163-stained macrophages; C-D: Portal CD163-stained clusters/mm liver tissue with CD163 (C) and CD14 (D) staining; D: Portal CD14 clusters/mm liver tissue. E-F: Correlation between liver portal macrophage clusters and peripheral markers of monocyte activation.

**Figure 4:** 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=0.858093; B). HIV+ (all) cases and controls vs HIV- controls, 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).
Figure 3

A

≥F2 Fibrosis
<F2 Fibrosis
HIV+ Controls
P=0.093

B

HIV+
HIV-
P=0.001

C

Class
- Hepatitis
- HIV+es
- 0.022

Order
- 0.0029

Family
- Bacteroidaceae
- Prevotellaceae
- Succinivibrionaceae
- Verrucomicrobiaceae
- 0.045
- 0.015

Genus
- Megaplasma
- Prevotella
- Succinivibrio
- Faecalibacterium
- Mitsukella
- Bacteroides
- 0.011
- 0.013
- 0.015
- 0.019
- 0.038
- 0.017
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Supplemental Data File (.doc, .tif, pdf, etc.)
Revised Supplementary Tables 1-4.docx