Leukocyte extracellular vesicle concentration is inversely associated with liver fibrosis severity in NAFLD.

Summary Sentence: In a small preliminary study, leukocyte extracellular vesicles show an inverse association with liver fibrosis in NAFLD, warranting their further investigation as biomarkers.

Joshua A. Welsh [*], Eleonora Scorletti [*], Geraldine F. Clough [*], Nicola A. Englyst [*, ||], Christopher D. Byrne [*, †, ||]

[*] *Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, UK*

† *National Institute for Health Research Southampton Biomedical Research Centre, University of Southampton, University Hospital Southampton NHS Foundation Trust, Southampton, UK*

|| joint last author

Key words: Extracelllular vesicles, NAFLD, liver fibrosis

Total characters (excluding methods and references) – 20,290

Tables - 5

Figures – 1

Supplementary Tables - 3

Supplementary Figure - 4

References - 29

Abstract – 256 words

Abbreviations

AST; Aspartate aminotransferase

ALT; Alanine transaminase

AUC; area under the curve

BMI; body mass index

dfHBS; double-filtered HEPES buffered saline

EEV; endothelial extracellular vesicles

EVs; extracellular vesicles

HA; hyaluronic acid

LEV; leukocyte extracellular vesicles

LFS; liver fibrosis score

NAFLD; non-alcoholic fatty liver disease

NFS; NAFLD fibrosis score

NICE; National Institute for Care Excellence

PEV, platelet extracellular vesicles

PIIINP; type III procollagen peptide)

ROC; receiver operator characteristic

TIMP1; tissue inhibitor of metalloproteinase-1

WELCOME; **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD (non alcoholic fatty liver disease) with **OM**acor therapy

Abstract

The Enhanced Liver Fibrosis (LFS) score and the non-alcoholic fatty liver disease (NAFLD) Fibrosis score (NFS) are algorithmic-derived scores for diagnosing severe (F3/F4) liver fibrosis. In a pilot, sub-study of the WELCOME trial, we tested whether measurements of plasma platelet-, endothelial- and leukocyte-derived extracellular vesicles (EVs) counts are: a) associated with, and predict, F3/F4 fibrosis; and b) able to improve risk prediction of F3/F4 fibrosis in NAFLD, building upon LFS or NFS algorithms. 26 individuals with NAFLD had liver fibrosis severity determined by Kleiner scoring after liver biopsy. Plasma samples stained with CD41a, CD42b, CD31, CD105, CD14, CD16, and CD284 antibodies were analysed using flow cytometry to measure platelet-, endothelial- and leukocyte-derived EVs counts. The independence of associations between EVs and F3/F4 fibrosis were tested using logistic regression. Receiver operator characteristic (ROC) curves were used to evaluate F3/F4 fibrosis prediction models. LFS was more strongly associated with F3/F4 fibrosis than NFS (χ^2=15.403, p<0.0001 and χ^2=6.300, p=0.012 respectively). The association between LFS and F3/F4 fibrosis was further improved by addition of $CD14^+$ EVs ($\chi^2 = 20.847$, p=0.016 versus $\chi^2 = 12.803$, p=0.015, respectively) or $CD16^+$ EVs ($\chi^2 = 22.205$, p=0.009 versus $\chi^2 = 17.559$, p=0.001, respectively), and the area under the ROC for LFS (AUC=0.915, SE=0.055, p=0.001) was increased by the addition of $CD14^+$ or $CD16^+$ EVs (AUC=0.948, SE=0.042, p<0.001 and AUC=0.967, SE=0.055, p<0.001, respectively) as predictor variables. In this small preliminary study, $CD14^+$ and $CD16^+$ EV counts show potential to predict liver fibrosis severity with either marker improving the ability of the LFS to identify F3/F4 fibrosis in this small preliminary cohort study.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic progressive liver condition for which there is no licensed treatment. In NAFLD, liver fibrosis severity is the most important predictor of liver morbidity and mortality.[1] Currently the gold standard for diagnosis of liver fibrosis in NAFLD is liver biopsy. However, liver biopsy is an invasive and risky procedure that precludes its use for regular monitoring of liver disease progression over time.[2] Consequently, non-invasive precise, reproducible tests are needed that not only can be used to monitor disease progression, but can also be used to monitor responses to treatment.

Algorithm-derived scores have been developed utilising measurement of biomarkers of liver disease and anthropometric measurements, in order to develop simple, non-invasive tests to assess liver fibrosis severity in NAFLD. The Enhanced Liver Fibrosis Score (LFS) utilises concentrations of the circulating serum biomarkers PIIINP (type III procollagen peptide), hyaluronic acid, and TIMP1 (tissue inhibitor of metalloproteinase-1); and measurements of these biomarkers are fitted into an algorithm to produce a score to assess liver fibrosis.[3] This fibrosis score performs well (area under the receiver operator curve (AUROC) 0.9) for diagnosing severe fibrosis and recently this test was identified by the National Institute for Care Excellence (NICE) as the best of the non-invasive tests for identifying patients with severe (F3 and F4) fibrosis. The LFS score was recommended by NICE in the NICE NAFLD Guideline published in 2016 (https://www.nice.org.uk/guidance/ng49).[3, 4] The LFS was associated with a sensitivity of 80% and specificity of 90% at the optimum threshold, with a positive predictive value of 71%, and a negative predictive value of 94%. There is still therefore room to improve upon the specificity and

specificity of LFS and also other scores such as the NFS, (which utilises age, hyperglycaemia, BMI, platelet count, albumin, and AST/ALT ratio, as predictive variables).[5] In an attempt to improve the clinical utility of the non-invasive tests further, the LFS has been combined with the NFS and these data suggest that ~80% of liver biopsies could be potentially avoided in establishing a diagnosis of severe fibrosis.[4]

Extracellular vesicles (EVs) are small membrane-bound vesicles (30-1000nm) that are shed from cells. These include exosomes (30-100nm) which are derived from multivesicular intracellular bodies, and microvesicles (30-1000nm) which are shed directly from the plasma membrane. EVs are released upon cell activation and senescence, and their presence in blood has been shown to correlate with a wide range of diseases.[6] The relationship between EVs, NAFLD and fibrosis severity has had little investigation with only one published study to date. That study did not quantify EVs by absolute count but by staining percentage and this in turn meant that the utility of EVs in risk prediction models could not be evaluated.[7] Due to the inflammatory nature of NAFLD in steatohepatitis that precedes development of severe fibrosis, it is likely that the increased cell activation leads to modulation of EV shedding from cells derived from a leukocyte origin. EVs surface protein expression can be used to a degree to determine their cellular origin and an investigation of the amount and type of EV surface protein expression may give insight into the assessment of liver disease progression in NAFLD.[8]

In a proof of concept pilot sub-study within the WELCOME trial[9], (**WELCOME;** (**W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD (non-

alcoholic fatty liver disease) with **OM**acor th**E**rapy), we have tested whether platelet-, endothelial- and leukocyte-derived EV counts are: a) associated with severe (F3 and F4) fibrosis and b) if EV measurements improve risk prediction for F3 and F4 fibrosis, provided by measurement of either the LFS or the NFS.

Materials & Methods

Study participants

The WELCOME study was approved by the Southampton and South West Hampshire local research ethics committee (08/H0502/165). All participants gave informed written consent. The primary outcomes of the WELCOME trial have been reported previously.[10] EVs were measured in 46 participants of 105 on the WELCOME trial. Only 26 of 46 individuals had undergone liver biopsy, thereby limited the sample number to 26.

Sample Collection & Preparation

Venous blood samples were drawn into citrated tubes using a 21G needle. Blood was centrifuged twice at 2000x*g* for 10 minutes. Plasma was collected and aliquoted into 1mL tubes and stored at -80°C for 4.95±0.36 years (mean±SD) to minimise EV count changes before analyses.[11] Prior to analysis the frozen samples were stored on dry ice before incubation at 37°C for 10 minutes. 30 µL of plasma were added to 12x75mm sterile polystyrene test tubes containing 50 µl of 0.1 µm double-filtered HEPES buffered saline (dfHBS). Platelet EV (PEV) and endothelial EV (EEV) phenotyping tubes contained 0.73 µg mL^{-1} milk fat globulin (MFG) E8-FITC (Cambridge Bioscience, Cambridge, UK), 1.75 µg mL^{-1} anti-CD42b-PerCP (Biolegend, London, NW5 1LB), 0.44 µg mL^{-1} anti-CD41a PerCP-Cy5.5, 0.22 µg mL^{-1} anti-CD105 BV421, 0.11 µg mL^{-1} anti-CD31 PE (Becton Dickinson Bioscience, Oxford, UK). Leukocyte EV (LEV) phenotyping tubes contained 0.73 µg mL^{-1} anti-MFGE8-FITC (Cambridge Bioscience, Cambridge, UK), 0.45 µg mL^{-1} anti-CD14-PerCP-Cy5.5, 0.23 µg mL^{-1} anti-CD16 BV421 , 0.23 µg mL^{-1} anti-CD284-PE (Becton Dickinson Bioscience, Oxford, UK) Isotype-matched controls (Becton Dickinson

Bioscience, Oxford, UK) were run for each tube at the concentration of either the platelet and endothelial phenotyping tube, or leukocyte phenotyping tube. Further information on the antibodies and isotypes used can be found in **Supplementary Table 1**. Stained samples were incubated on ice in the dark for 20 minutes.

Flow Cytometry

A BD Special Order LSRFortessa X-20 using FACSDiva 8.0 software (BD Biosciences, Oxford, UK) was used for EV sample acquisition and analysis. 100, 300, 500, 900 nm fluorescent polystyrene beads (Megamix Plus FSC, Biocytex, France) were analysed on FSC-H, SSC-H. SSC and FSC voltages were increased until the 900 nm population appeared in the upper right corner of a plot without detector saturation. A SSC threshold was set using the median SSC-H parameter of 100 nm polystyrene fluorescent beads. 100, 200, 400, 700, 1000 nm polystyrene and 500, 700, 1000 nm silica NIST beads (ThermoFisher Scientific, Loughborough, UK) were then recorded at set thresholds and voltages for Mie scatter reference, **Supplementary Figure 1**. CompBeads (BD Bioscience, Oxford, UK) independently stained with each fluorophore at the same concentration as samples, stained for 20 minutes before acquisition, were analysed for each fluorophore. All samples were analysed initially for 30 seconds at the lowest flow rate attainable, allowing the core stream to stabilise. Samples were diluted until a stable event rate with confidence in single particle analysis was achieved (dilution median 1057-fold, minimum 44-fold, maximum 6147-fold) and transferred to TruCount counting bead tubes (Becton Dickinson Bioscience, Oxford, UK) before events were recorded for 5 minutes. This individualised dilution method minimises risk of more than one EV passing the laser at one time, improving accuracy of counting.

Flow Cytometry Data Analysis

Single particles were gated on SSC-H vs. SSC-A dot plots before sub-gating. EVs were gated on SSC from 100nm to 203nm polystyrene beads SSC-H median values based on refractive index normalisation using Mie theory[12], **Supplementary Figure 1.** PEV phenotypes were interpreted to be either $CD105^-$ $CD31^+$ $CD41/CD42^+$ or $CD105^-$ $CD31^-$ $CD41/CD42^+$. EEV phenotypes were interpreted to be either $CD105^+$ $CD31^+$ $CD41/CD42^-$, $CD105^+$ $CD31^-$ $CD41/CD42^-$, or $CD105^-$ $CD31^+$ $CD41/CD42^-$. $CD105^+$ EVs are defined as $CD105^+$ events, with the same practice applied to $CD284^+$, $CD16^+$, and $CD14^+$ EVs. EV populations used in our analysis include: PEVs, EEVs, $CD105^+$ EVs, $CD31^+$ EVs, $CD284^+$ EVs, $CD14^+$ EVs, $CD16^+$ EVs. LEVs refer collectively to $CD284^+$ EVs, $CD14^+$ EVs, $CD16^+$ EVs. All EV phenotype units are EV count $x10^{10}$ mL^{-1}. Gating strategy shown in **Supplementary Figure 1** with representative staining for each panel shown in **Supplementary Figure 2**.

Liver Fibrosis Score, Liver Biopsy & Biochemical Measurements

Two histologically validated algorithmically-derived biomarker scores for liver fibrosis were calculated for each patient.[1, 4] The LFS published algorithm utilises measurement of circulating serum biomarkers PIIINP (type III procollagen peptide) hyaluronic acid (HA), and TIMP1 (TIMP metallopeptidase inhibitor 1). The NAFLD fibrosis (NFS) score utilises age, hyperglycaemia, BMI, platelet count, albumin, and AST/ALT ratio. Both scores were calculated using the published algorithms shown in **Appendix 1**. Liver fibrosis severity was assessed and scored by an independent histopathologist who was blinded to the EV data. Scoring was from 0 to 4 points, F0-

F4, according to the original Kleiner classification. [13, 14] Fibrosis was identified using a reticulum stain of liver biopsy tissue. Briefly, fibrosis scoring was undertaken according to the following histological characteristics. None=0 points. Periportal (zone 1) or perisinusoidal (zone 3) fibrosis= 1 point. Perisinusoidal and periportal fibrosis =2 points. Bridging fibrosis (linking both periportal and perisinusoidal zones= 3 points and cirrhosis=4 points (bridging fibrosis plus nodules of fibrosis). [13, 14] An overview of the Kleiner scoring system, histology and histopathologiest report can be found in **Supplementary Figure 3**. Biopsies were taken 1.62±1.75 years (mean±SD) before blood was sampled for analyses. Participants were then stratified into two groups: severe fibrosis (scores 3 or 4 points i.e. F3/F4, n=9) and non-severe or no fibrosis (scores 0 points, 1 point or 2 points, i.e. F0-F2, n=17).[15, 16]

Biochemical measurements, including PIIINP, TIMP1, HA, along with lipoproteins and tryglycerides were undertaken using autoanalysers, according to standard operating procedures, used for clinical samples within the National Health Service. Further details can be found in previously described methods.[9]

Statistical Analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS) Version 23.0 (IBM, New York, USA). Samples were tested for normality using Shapiro-Wilk tests with a $p< 0.05$ marking significance. Group differences were tested by Mann Whitney U and Kruskal Wallis tests. Univariate associations were investigated using Spearman correlation. Normalisation of EVs and LFS was done using Box-Cox transformations[17]. Binary logistic regression was used to test for independent associations for relevant exposures with liver fibrosis severity, stratified

according to the presence or absence of F3/F4 fibrosis, as the binary outcome variable. Tested exposure variables in the models included EVs, LFS, NFS and the following potential explanatory/confounding factors were: age, sex, diabetes status, smoking status, sample storage time, and treatment with statins, anti-hypertensives, insulin, beta blockers, calcium anatagonists, ACE inhibitors, alpha 2 blockers, metformin, glitazone, sulphonylureas, thiazide diuretics, fibrates, gliptins, ezetimibe, anti-depressants, levothyroxine, and orlistat. The effects of these factors were investigated using backwards elimination regression models.

Individual EV phenotypes were added to a binary logistic regression models to determine if they were able to improve the associations of the LFS and NFS with F3/F4 fibrosis. Omnibus test model coefficient χ^2 significance values, $p<0.05$, were used to determine if the added EV population had significantly improved the NFS and LFS models. Models showing significant improvement had their variables merged into a single score using binary logistic regression model β coefficients. These calculated scores were then used in receiver operator characteristic (ROC) curve analysis to evaluate the sensitivity and specificity for predicting/excluding F3/F4 fibrosis.

Results

Characteristics of the trial participants

Table 1 shows the baseline characteristics of participants with severe (F3/F4 fibrosis) and non-severe fibrosis (F0-F2 fibrosis), determined by liver biopsy using the Kleiner score. The mean ± SD age was 48.9 ± 12.8 years for the 17 individuals (10 men) with non-severe fibrosis and 51.8 ± 13.8 years for the 9 individuals (3 men) with severe fibrosis. In the severe versus non-severe liver fibrosis groups (**Table 1**), there were differences between concentrations of aspartate aminotransferase (p=0.004), hyaluronic acid (p=0.001), PIIINP (p=0.001), and γ-glutamyl transpeptidase (p=0.002).

EVs, liver fibrosis scores and histological assessment of liver fibrosis severity

All EV populations showed no significant (p>0.05) differences by group when stratified by: smoking status, diabetes status, sex, carotid plaque presence, hypertension, statin usage, antihypertensive usage, oral antidiabetics usage and insulin usage. **Supplementary Table 2** summarises univariate correlations between EV populations and participant characteristics.

Stratifying by liver fibrosis severity, all EV absolute counts were lower in the F3/F4 fibrosis group, except PEVs (p=0.500), and $CD31^+$ EVs (p=0.138) **(Table 2).** Univariate associations between EV populations and liver function markers were then investigated **(Table 3**). Hyaluronic acid was significantly and inversely correlated with EEVs (r=-0.438, p=0.025), $CD105^+$ EVs (r=-0.481, p=0.013), $CD31^+$ EVs (r=-0.400, p=0.043), $CD16^+$ EVs (r=-0.633, p=0.001), $CD14^+$ EVs (r=-0.481, p=0.013). PIIINP showed an inverse association with $CD16^+$ EVs (r=-0.404, p=0.041), whilst aspartate aminotransferase and γ-glutamyl transpeptidase showed no significant associations with any of the EV populations tested. NFS showed a borderline significant inverse association with $CD284^+$ EVs (r=-0.388, p=0.05) and a significant association with $CD14^+$ EVs (r=-0.521, p=0.006). Finally, LFS was inversely associated with $CD105^+$ EVs (r=-0.433, p=0.027), $CD16^+$ (r=-0.595, p=0.001), $CD14^+$ EVs (r=-0.410, p=0.038). In summary, leukocyte EV counts are inversely associated with severe liver fibrosis and positively correlated with liver function markers.

Associations between EVs, NFS, LFS, and F3/F4 liver fibrosis

289 Binary logistic regression was undertaken to test the independence of associations
290 between: a) EV populations and histological fibrosis severity; b) NFS and histological
291 fibrosis severity; and c) LFS with histological fibrosis severity. Variables showing a
292 significant association included: NFS (χ^2=6.300, *p*=0.012), LFS (χ^2=15.403,
293 *p*<0.0001), EEVs (χ^2=7.768, *p*=0.005), CD284$^+$ EVs (χ^2=4.353, *p*=0.037), CD14$^+$
294 EVs (χ^2=9.613, *p*=0.002), and CD16$^+$ EVs (χ^2=14.132, *p*<0.001). In order to
295 investigate the independence of these associations further, potential confounding
296 factors/covariates were included in the models, including: age, sex, diabetes status,
297 smoking status, sample storage time, and various treatment groups. None of these
298 variables were independently associated with fibrosis severity and did not affect the
299 independence of the association between EVs and F3/F4 fibrosis severity. CD14$^+$
300 and CD16$^+$ EV counts therefore showing a larger association with liver fibrosis than
301 the NFS score and are independent of important potential confounding factors.
302
303 ***Calibration and discrimination of EVs to predict F3/F4 fibrosis.***
304 The χ^2 value of the LFS model ($\chi^2 = 15.403$) was increased by the addition of
305 various EV populations to the model. These additive models with EV populations
306 included: EEVs ($\chi^2 = 19.357$, *p*=0.041), CD16$^+$ EVs ($\chi^2 = 22.205$, *p*=0.009), and
307 CD14$^+$ EVs ($\chi^2 = 20.847$, *p*=0.016), (**Table 4**). EV populations also significantly
308 increased the χ^2values of the NFS model [NFS model alone= χ^2=6.300)], and these
309 additive models included: EEVs ($\chi^2 = 13.242$, *p*=0.012), CD16$^+$ EVs ($\chi^2 =$
310 17.559, *p*=0.001), and CD14$^+$ EVs ($\chi^2 = 12.803$, *p*=0.015). All NFS model χ^2 values
311 were lower than the comparable LFS additive models, **Supplementary Table 3**, and
312 therefore the NFS models showed weaker association with F3/F4 fibrosis than the
313 LFS models. Furthermore, the addition of NFS to LFS did not further improve the χ^2

($\chi^2 = 15.410$, p=0.936) and therefore we did not further examine the effect of adding NFS to LFS in ROC curve analysis. From the analyses of the LFS additive models χ^2 values, we selected the two models (LFS+CD14$^+$, and LFS+CD16$^+$) that had the strongest associations with F3/F4 fibrosis for further analysis. We used ROC curve analysis to test whether the addition of these EVs counts to LFS improved risk prediction for F3/F4 fibrosis, compared with LFS alone.

An evaluation of discrimination (how well each model discriminated between a participant having or not having F3/F4 fibrosis) was investigated by calculating the c statistic or AUROC. A comparison between the LFS (reference algorithm), LFS+CD14$^+$ and LFS+CD16$^+$ models was conducted in order to test their ability to predict F3/F4 fibrosis. (See **Appendix 1** numbers 6 and 7 for the equations used to calculate a combined LFS+EV score).

Figure 1 shows the ROCs and AUROCs for each model. For LFS alone the AUROC=0.915, SE=0.055, p=0.001). The AUROC for LFS was increased by the addition of CD14$^+$ or CD16$^+$ as predictor variables (AUC=0.948, SE=0.042, p<0.001) and (AUC=0.967, SE=0.055, p<0.001, respectively). A threshold of -0.8687 for LFS+CD16$^+$, had the best combination of high sensitivity (88.9%) and high specificity (94.1%). At this threshold, the positive-likelihood ratio was 15.07 and the negative likelihood ratio was 0.24. For comparison, a threshold of -0.3435 for LFS+CD14$^+$, the sensitivity was 88.9% and the specificity was 88.2%. There was a positive-likelihood ratio of 7.53 and a negative likelihood ratio of 0.13. (See **Table 5** for a full list of thresholds associated with the different LFS models). At a threshold value of -0.8687 for the LFS+CD16$^+$ model, with an LFS value of 10.97 which was the median LFS

score in F3/F4 fibrosis (see **Table 1**), using the equation shown in **Appendix 1**, the calculated $CD16^+$ EV count was 7.71 x 10^{10} mL^{-1}. The addition of $CD14^+$ and $CD16^+$ EV counts to the LFS algorithm therefore show a better ability to predicted F3/F4 fibrosis than LFS alone in this pilot study.

In summary, we have undertaken a proof of concept pilot study in subjects with biopsy-proven NAFLD to test whether measurement of EVs could be used to predict F3/F4 liver fibrosis, and then to investigate whether measurement of any EVs phenotype improves risk prediction for F3/F4 fibrosis, when added to either the LFS or the NFS. Our novel data show that measurement of $CD16^+$ EV counts specifically, improves calibration and discrimination of the LFS in risk prediction models for identifying F3/F4 fibrosis in NAFLD. Our results show circulating EEVs and LEVs are decreased in count in individuals with F3/F4 fibrosis, while PEV counts are unchanged in these subjects. We have shown several EV markers are more strongly associated with F3/F4 fibrosis than the NFS, including: EEVs, $CD14^+$ EVs and $CD16^+$ EVs. LEVs; specifically those expressing $CD16^+$ (and also $CD14^+$) were also able to increase the ability of LFS to identify and exclude F3/F4 fibrosis.

We have shown that $CD16^+$ EVs are inversely associated with several markers of liver function/fibrosis, e.g. aspartate aminotransferase (r=-0.388, p=0.05), CK-18 (r=-0.409, p=0.038), hyaluronic acid (r=-6.33, 0.001), and PIIINP (r=-0.404, p=0.041). Furthermore, we have demonstrated that the addition of $CD16^+$ EVs to the LFS model increases the strength of the association with fibrosis severity ($\chi^2 = 22.205$, p=0.009); more so, than adding NFS score to the LFS model ($\chi^2 = 15.410$, p=0.936).

Discussion

Currently only one other translational study has investigated the association between EVs and NAFLD progression[7]. In that study and ours, CD14 shows potential as a biomarker for liver fibrosis. However in the study by Kornek et al only 2 of 56 participants were classified as having F3/F4 fibrosis, making it difficult to examine any relationship between CD14 and F3/F4 fibrosis which is important because F3/F4 fibrosis predicts liver-related outcomes.[1,7] EVs have been shown to play a role in thrombosis, and are sensitive to isolation conditions.[18-21] Our study isolated platelet poor plasma in citrate tubes as suggested best practice according to ISTH guidelines.[22] EV counts and phenotypes may have differed between both studies, due to Kornek et al allowing the blood to clot before the plasma was isolated.[22] A major strength of our study was the use of reference beads and laser scatter physics models to allow determination of our flow cytometer scatter resolution limit for EVs, **Supplementary Figure 1**. This provides reassurance that the flow cytometer had the correct settings and sensitivity to detect single EVs. Kornek et al did not use reference markers that would allow a comparison of their results, or provide reassurance the flow cytometer used had the sensitivity to detect single EVs.[7] We therefore consider that our pilot study improves understanding of the relationship between EV count and liver fibrosis severity in the progression of NAFLD.

An inverse association was observed between membrane proteins $CD284^+$, $CD14^+$ and $CD16^+$ EVs and liver fibrosis severity (see **Table 3**) in our study. Both CD14 and CD16 also exist in soluble (sCD14, sCD16) forms and studies investigating sCD14 in individuals with NAFLD have shown an increase in sCD14 concentration with F3/F4 fibrosis.[23-25] A positive correlation has also been demonstrated between sCD14

release and hepatic CD14 mRNA expression.[24] It is plausible that the $CD14^+$ EVs observed in our study may be derived from hepatic macrophages. When hepatic CD14 mRNA expression increases, cells may undergo increased LPS-induced cleavage of membrane CD14 to sCD14.[26] This in turn would reduce the surface expression of the membrane CD14 whilst increasing sCD14 and would explain the CD14 results we have observed and the sCD14 results previously published. The ratio of sCD14 to $CD14^+$ EVs may therefore be an interesting avenue for future research for monitoring NAFLD liver fibrosis progression.

CD16 (FcγRIIIa) is a low affinity Fc receptor of IgG, expressed by monocyte/macrophages. Its modulation in NAFLD and in particular in Kupffer cells is poorly understood. CD284 (TLR4) is also a Kupffer cell marker whose expression has previously been implicated in hepatic fibrosis progression shown to contribute to hepatic inflammation. [27, 28] Research into circulating monocytes in individuals with NAFLD have shown a modest percentage increase in the non-classical monocyte phenotype ($CD14^+$, $CD16^+$).[29] However the absolute count of monocytes and how the monocyte sub-population percentage varies with NAFLD progression has not yet been demonstrated. Understanding the source of $CD284^+$ and $CD16^+$ EVs, and why their count is strongly associated with fibrosis severity, requires further research into hepatic and circulating monocyte phenotype modulation across the spectrum of liver disease severity in NAFLD.

There are various strengths and limitations of our study that need to be mentioned. One limitation of our proof of concept study is that our sample size is small and we do not have a control group without NAFLD. We only had access to this number of participants' data in the WELCOME trial for which we had measurement of EVs and histological data of liver disease severity, and within the context of the trial study design there was no control group without NAFLD. Moreover, it is important to now verify our findings in another cohort. This preliminary study however provides rational for further investigation of EVs in larger cohorts.

In conclusion, the results of our pilot proof of concept study show that addition of measurement of either $CD14^+$ or $CD16^+$ EVs adds value to the measurement of the LFS or the NFS, and may have clinical utility for predicting F3/F4 fibrosis in NAFLD. We suggest our novel data emphasise that larger studies are now needed to replicate and test our findings further.

Authorship

Authors contributed equally to the manuscript.

Acknowledgments

We thank Professor Clive Osmond, Professor of Medical Statistics, MRC Environmental Epidemiology Unit, University of Southampton, for his advice and expertise.

Conflict of Interest

NAE holds a collaboration agreement with ThermoFisher Scientific.

Finacial Support

This work was supported by the National Institute for Health Research through the NIHR Southampton Biomedical Research Unit grant and by a Diabetes UK grant (BDA 09/0003937). JAW was supported by the University of Southampton Vice-Chancellors Scholarship. CDB and ES are supported in part by the National Institute for Health Research Southampton Biomedical Research Centre.

Appendix 1.

1. LFS Algorithm

$$\text{Discriminating Score} = -7.412 + (\ln(\text{HA}) * 0.681) + (\ln(\text{PIIINP}) * 0.775) + (\ln(\text{TIMP1}) * 0.494)$$

2. NAFLD Fibrosis Score Algorithm

$$\text{Discriminating Score} = -1.675 + 0.037 * \text{age (years)} + 0.094 * \text{BMI}(\text{kg/m}^2) + 1.13 * \text{IFG/diabetes}(\text{yes} = 1, \text{no} = 0) + 0.99 * \text{AST/ALT ratio} - 0.013 * \text{platelet } \left(\frac{10^9}{\text{L}}\right) - 0.66 * \text{albumin (g/dl)}.$$

3. CD14 Normalisation

Normalisation was based on Box-Cox transformation, with a lambda value equal to -0.6. The normalisation output had 1.666 subtracted before multiplying by 1000 to allow statistical comparison in SPSS.

$$\text{nCD14} = ((((\text{CD14}(\text{count}/(\mu\text{L}))^{-0.6}) - 1)/-0.6) - 1.666) * 1000)$$

4. CD16 Normalisation

Normalisation was based on Box-Cox transformation, with a lambda value equal to 0.3.

$$\text{nCD16} = (((\text{CD16}(\text{count}/(\mu\text{L})^{0.3} - 1)/0.3)$$

5. LFS Normalisation

$$\text{nLFS} = ((((\text{LFS})^{-2} - 1)/-2)$$

6. LFS+CD14^{+} Algorithm

$$\text{Score} = -999.357 + ((\text{nLFS} + 10) * 2.262) + (\text{nCD14} * -188.714)$$

7. LFS+CD16^{+} Algorithm

$$\text{Score} = -1163.134 + ((\text{nLFS} + 10) * 2.372) + (\text{nCD16} * -0.018)$$

Reference List

1. Angulo, P., Kleiner, D. E., Dam-Larsen, S., Adams, L. A., Bjornsson, E. S., Charatcharoenwitthaya, P., Mills, P. R., Keach, J. C., Lafferty, H. D., Stahler, A., Haflidadottir, S., Bendtsen, F. (2015) Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 149, 389-97 e10.

2. Byrne, C. D. and Targher, G. (2016) Time to Replace Assessment of Liver Histology With MR-Based Imaging Tests to Assess Efficacy of Interventions for Nonalcoholic Fatty Liver Disease. Gastroenterology 150, 7-10.

3. Rosenberg, W. M., Voelker, M., Thiel, R., Becka, M., Burt, A., Schuppan, D., Hubscher, S., Roskams, T., Pinzani, M., Arthur, M. J., European Liver Fibrosis, G. (2004) Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology 127, 1704-13.

4. Guha, I. N., Parkes, J., Roderick, P., Chattopadhyay, D., Cross, R., Harris, S., Kaye, P., Burt, A. D., Ryder, S. D., Aithal, G. P., Day, C. P., Rosenberg, W. M. (2008) Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology 47, 455-60.

5. Angulo, P., Hui, J. M., Marchesini, G., Bugianesi, E., George, J., Farrell, G. C., Enders, F., Saksena, S., Burt, A. D., Bida, J. P., Lindor, K., Sanderson, S. O., Lenzi, M., Adams, L. A., Kench, J., Therneau, T. M., Day, C. P. (2007) The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 45, 846-54.

6. Barteneva, N. S., Fasler-Kan, E., Bernimoulin, M., Stern, J. N., Ponomarev, E. D., Duckett, L., Vorobjev, I. A. (2013) Circulating microparticles: square the circle. BMC Cell Biol 14, 23.

7. Kornek, M., Lynch, M., Mehta, S. H., Lai, M., Exley, M., Afdhal, N. H., Schuppan, D. (2012) Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. Gastroenterology 143, 448-58.

8. Yanez-Mo, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borras, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colas, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N. H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Kramer-Albers, E. M., Laitinen, S., Lasser, C., Lener, T., Ligeti, E., Line, A., Lipps, G., Llorente, A., Lotvall, J., Mancek-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E. N., Nyman, T. A., O'Driscoll, L., Olivan, M., Oliveira, C., Pallinger, E., Del Portillo, H. A., Reventos, J., Rigau, M., Rohde, E., Sammar, M., Sanchez-Madrid, F., Santarem, N., Schallmoser, K., Ostenfeld, M. S., Stoorvogel, W., Stukelj, R., Van der Grein, S. G., Vasconcelos, M. H., Wauben, M. H., De Wever, O. (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4, 27066.

9. Scorletti, E., Bhatia, L., McCormick, K. G., Clough, G. F., Nash, K., Calder, P. C., Byrne, C. D., Investigators, W. T. (2014) Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long

chainomega-3 fatty acid treatment in non-alcoholic fatty liver disease [corrected]. Contemp Clin Trials 37, 301-11.

10. Scorletti, E., Bhatia, L., McCormick, K. G., Clough, G. F., Nash, K., Hodson, L., Moyses, H. E., Calder, P. C., Byrne, C. D., Study, W. (2014) Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology 60, 1211-21.

11. Jayachandran, M., Miller, V. M., Heit, J. A., Owen, W. G. (2012) Methodology for isolation, identification and characterization of microvesicles in peripheral blood. J Immunol Methods 375, 207-14.

12. van der Pol, E., van Gemert, M. J., Sturk, A., Nieuwland, R., van Leeuwen, T. G. (2012) Single vs. swarm detection of microparticles and exosomes by flow cytometry. J Thromb Haemost 10, 919-30.

13. Scheuer, P. J. (1991) Classification of chronic viral hepatitis: a need for reassessment. J Hepatol 13, 372-4.

14. Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., Sanyal, A. J., Nonalcoholic Steatohepatitis Clinical Research, N. (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41, 1313-21.

15. Nojiri, S., Kusakabe, A., Fujiwara, K., Shinkai, N., Matsuura, K., Iio, E., Miyaki, T., Joh, T. (2013) Noninvasive evaluation of hepatic fibrosis in hepatitis C virus-infected

patients using ethoxybenzyl-magnetic resonance imaging. J Gastroenterol Hepatol 28, 1032-9.

16. Batheja, M., Vargas, H., Silva, A. M., Walker, F., Chang, Y. H., De Petris, G., Silva, A. C. (2015) Magnetic resonance elastography (MRE) in assessing hepatic fibrosis: performance in a cohort of patients with histological data. Abdom Imaging 40, 760-5.

17. Box, G. E. P. and Cox, D. R. (1982) An Analysis of Transformations Revisited, Rebutted. J Am Stat Assoc 77, 209.

18. Lannan, K. L., Phipps, R. P., White, R. J. (2014) Thrombosis, platelets, microparticles and PAH: more than a clot. Drug Discov Today 19, 1230-5.

19. Mooberry, M. J. and Key, N. S. (2016) Microparticle analysis in disorders of hemostasis and thrombosis. Cytometry A 89, 111-22.

20. Chandler, W. L. (2013) Microparticle counts in platelet-rich and platelet-free plasma, effect of centrifugation and sample-processing protocols. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis 24, 125-32.

21. Szatanek, R., Baran, J., Siedlar, M., Baj-Krzyworzeka, M. (2015) Isolation of extracellular vesicles: Determining the correct approach (Review). Int J Mol Med 36, 11-7.

22. Lacroix, R., Judicone, C., Mooberry, M., Boucekine, M., Key, N. S., Dignat-George, F., The, I. S. S. C. W. (2013) Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost.

577 23. Harte, A. L., da Silva, N. F., Creely, S. J., McGee, K. C., Billyard, T., Youssef-Elabd, E.
578 M., Tripathi, G., Ashour, E., Abdalla, M. S., Sharada, H. M., Amin, A. I., Burt, A. D.,
579 Kumar, S., Day, C. P., McTernan, P. G. (2010) Elevated endotoxin levels in non-
580 alcoholic fatty liver disease. J Inflamm (Lond) 7, 15.

581 24. Ogawa, Y., Imajo, K., Yoneda, M., Kessoku, T., Tomeno, W., Shinohara, Y., Kato, S.,
582 Mawatari, H., Nozaki, Y., Fujita, K., Kirikoshi, H., Maeda, S., Saito, S., Wada, K.,
583 Nakajima, A. (2013) Soluble CD14 levels reflect liver inflammation in patients with
584 nonalcoholic steatohepatitis. PLoS One 8, e65211.

585 25. Mueller, J. L., Feeney, E. R., Zheng, H., Misdraji, J., Kruger, A. J., Alatrakchi, N., King, L.
586 Y., Gelrud, L., Corey, K. E., Chung, R. T. (2015) Circulating Soluble CD163 is Associated
587 with Steatohepatitis and Advanced Fibrosis in Nonalcoholic Fatty Liver Disease. Clin
588 Transl Gastroenterol 6, e114.

589 26. Lin, S. M., Frevert, C. W., Kajikawa, O., Wurfel, M. M., Ballman, K., Mongovin, S.,
590 Wong, V. A., Selk, A., Martin, T. R. (2004) Differential regulation of membrane CD14
591 expression and endotoxin-tolerance in alveolar macrophages. Am J Respir Cell Mol
592 Biol 31, 162-70.

593 27. Von Hahn, T., Halangk, J., Witt, H., Neumann, K., Muller, T., Puhl, G., Neuhaus, P.,
594 Nickel, R., Beuers, U., Wiedenmann, B., Berg, T. (2008) Relevance of endotoxin
595 receptor CD14 and TLR4 gene variants in chronic liver disease. Scand J Gastroenterol
596 43, 584-92.

597 28. Guo, J. and Friedman, S. L. (2010) Toll-like receptor 4 signaling in liver injury and
598 hepatic fibrogenesis. Fibrogenesis Tissue Repair 3, 21.

29. Wang, Y., Oeztuerk, S., Kratzer, W., Boehm, B. O., Group, E. M.-S. (2016) A Nonclassical Monocyte Phenotype in Peripheral Blood is Associated with Nonalcoholic Fatty Liver Disease: A Report from an EMIL Subcohort. Horm Metab Res 48, 54-61.

Table 1. Baseline characteristics according to liver fibrosis stage.
Data are means ± standard deviation (SD), or median (25th, 75th percentiles), *p≤0.05, **p≤0.01. **Abbreviations**: LDL; low density lipoprotein, HDL; high density lipoprotein, CK-18 M65, cytokeratin-18 M65 subfraction; MRS, magnetic resonance spectroscopy; Carotid IMT, intima- media thickness; PIIINP, type III procollagen peptide; TIMP-1, tissue inhibitor of metalloproteinase-1.

Table 2. Comparison of EV absolute counts per mL stratified by fibrosis severity. Data are median (25th, 75th percentiles), *p≤0.05, **p≤0.01.
Abbreviations: EEV; endothelial extracellular vesicles, PEV; platelet extracellular vesicles.

Table 3. Univariate associations between EV populations and measures of liver function and disease severity in NAFLD. *p≤0.05, **p≤0.01
Abbreviations: EEV; endothelial extracellular vesicles, PEV; platelet extracellular vesicles, CK-18 M65, cytokeratin-18 M65 subfraction; PIIINP, type III procollagen peptide; TIMP-1, tissue inhibitor of metalloproteinase-1.

Table 4. Associations between LFS and histological diagnosis of liver fibrosis severity with addition of EV measurement to LFS. Exp(B) (95% CI) = Odds ratios. *p≤0.05, **p≤0.01. **Abbreviations:** EEV; endothelial extracellular vesicles, PEV; platelet extracellular vesicles.

Table 5. Diagnostic performance of LFS models at different thresholds to predict F3/F4 fibrosis.
Abbreviations: PLR; positive-likelihood ratio, NLR; negative-likelihood ratio.

Figure 1. Receiver operating characteristic (ROC) curves showing the change in area under the curve (AUC) for combining CD14 or CD16 with liver fibrosis score for predicting a diagnosis of F3/F4 liver fibrosis. Liver Fibrosis Score (LFS) was compared to LFS + $CD14^+$ EVs and LFS + $CD16^+$ EVs. Summarised are area under curve (Area), standard error, significance, and 95% confidence intervals; *p≤0.05, **p≤0.01.