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

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Abstract

The enhanced liver fibrosis (LFS) score and the nonalcoholic fatty liver disease (NAFLD) fibrosis score (NFS) are algorithmic-derived scores for diagnosing severe (F3/F4) liver fibrosis. In a pilot, substudy of the Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy (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. Twenty-six 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 analyzed 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 ($\chi^2 = 15.403$, $P < 0.0001$, and $\chi^2 = 6.300$, $P = 0.012$, respectively). The association between LFS and F3/F4 fibrosis was further improved by addition of CD14$^+$ EVs ($\chi^2 = 20.847$, $P = 0.016$ vs. $\chi^2 = 12.803$, $P = 0.015$, respectively) or CD16$^+$ EVs ($\chi^2 = 22.205$, $P = 0.009$ vs. $\chi^2 = 17.559$, $P = 0.001$, 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, and $P < 0.001$ 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.

KEYWORDS
extracelllular vesicles, liver fibrosis, NAFLD

1 | INTRODUCTION

Nonalcoholic 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, noninvasive 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 utilizing measurement of biomarkers of liver disease and anthropometric measurements, in order to develop simple, noninvasive tests to assess liver fibrosis severity in NAFLD. The enhanced liver fibrosis score (LFS) utilizes 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 noninvasive 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 utilizes age, hyperglycemia, BMI, platelet count, albumin, and aspartate aminotransferase (AST)/alanine transaminase (ALT) ratio, as predictive variables).[5] In an attempt to improve the clinical utility of the noninvasive 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 (30–1000 nm) that are shed from cells. These include exosomes (30–100 nm), which are derived from multivesicular intracellular bodies, and microvesicles (30–1000 nm), 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 1 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 substudy within the WELCOME trial[9], (WELCOME; (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD (nonalcoholic fatty liver disease) with OMacor thErapy), 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.

2 | MATERIALS AND METHODS

2.1 | 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.

2.2 | Sample collection and preparation

Venous blood samples were drawn into citrated tubes using a 21G needle. Blood was centrifuged twice at $2000 \times g$ for 10 min. Plasma was collected and aliquoted into 1 ml tubes and stored at –80°C for 4.95 ± 0.36 years (mean $\pm$ SD) to minimize EV count changes before analyses.[11] Prior to analysis, the frozen samples were stored on dry ice before incubation at 37°C for 10 min. A total of 30 μl plasma was added to 12×75 mm sterile polystyrene test tubes containing 50 μl of 0.1 μm double-filtered HEPES buffered saline. Platelet EV (PEV) and endothelial EV (EEV) phenotyping tubes contained 0.73 μg/ml milk fat globulin (MFG) E8-FITC (Cambridge Bioscience, Cambridge, UK), 1.75 μg/ml anti-CD42b-PerCP (Biolegend, London, NW5 1LB), 0.44 μg/ml anti-CD41a PerCP-Cy5.5, 0.22 μg/ml anti-CD105 BV421, 0.11 μg/ml anti-CD31 PE (Becton Dickinson Bioscience, Oxford, UK). Leukocyte EV (LEV) phenotyping tubes contained 0.73 μg/ml anti-MFGE8-FITC (Cambridge Bioscience, Cambridge, UK), 0.45 μg/ml anti-CD14-PerCP-Cy5.5, 0.23 μg/ml anti-CD16 BV421, 0.23 μg/ml 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 min.

2.3 | 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. Fluorescent polystyrene beads (100, 300, 500, and 900 nm; Megamix Plus FSC, Biocytex, France) were analyzed on FSC-H and 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. Polystyrene (100, 200, 400, 700, and 1000 nm) and silica NIST beads (ThermoFisher Scientific, Loughborough, UK) were then recorded at set thresholds and voltages for Mie scatter reference, (Supplementary Fig. 1). CompBeads (BD Bioscience, Oxford, UK) independently stained with each fluorophore at the same concentration as samples, stained for 20 min before acquisition, were analyzed for each fluorophore. All samples were analyzed initially for 30 s at the lowest flow rate attainable, allowing the core stream to stabilize. 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 min. This individualized

dilution method minimizes risk of more than one EV passing the laser at one time, improving accuracy of counting.

2.4 | Flow cytometry data analysis

Single particles were gated on SSC-H versus SSC-A dot plots before sub-gating. EVs were gated on SSC from 100 nm to 203 nm polystyrene beads SSC-H median values based on refractive index normalization using Mie theory,[12] (Supplementary Fig. 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, and CD16⁺ EVs. LEVs refer collectively to CD284⁺ EVs, CD14⁺ EVs, and CD16⁺ EVs. All EV phenotype units are EV count $\times 10^{10}$ per ml. Gating strategy shown in Supplementary Fig. 1 with representative staining for each panel shown in Supplementary Fig. 2.

2.5 | Liver fibrosis score, liver biopsy, and biochemical measurements

Two histologically validated algorithmically derived biomarker scores for liver fibrosis were calculated for each patient.[1,4] The LFS published algorithm utilizes measurement of circulating serum biomarkers PIIINP (type III procollagen peptide) hyaluronic acid (HA), and TIMP1 (TIMP metallopeptidase inhibitor 1). The NAFLD fibrosis (NFS) score utilizes age, hyperglycemia, 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 Fig. 3. Biopsies were taken 1.62 ± 1.75 years (mean ± SD) before blood was sampled for analyses. Participants were then stratified into the following two groups: severe fibrosis (scores 3 or 4 points, i.e., F3/F4, $n = 9$) and nonsevere or no fibrosis (scores 0, 1, 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 autoanalyzers, according to standard operating procedures, used for clinical samples within the National Health Service. Further details can be found in previously described methods.[9]

2.6 | Statistical analysis

Data were analyzed 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. Normalization 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, and NFS, and the following potential explanatory/confounding factors were age, sex, diabetes status, smoking status, sample storage time, and treatment with statins, antihypertensives, insulin, beta blockers, calcium anatagonists, ACE inhibitors, alpha 2 blockers, metformin, glitazone, sulphonylureas, thiazide diuretics, fibrates, gliptins, ezetimibe, antidepressants, 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.

3 | RESULTS

3.1 | Characteristics of the trial participants

Table 1 shows the baseline characteristics of participants with severe (F3/F4 fibrosis) and nonsevere 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 nonsevere fibrosis and 51.8 ± 13.8 years for the 9 individuals (3 men) with severe fibrosis. In the severe versus nonsevere 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 ($P = 0.002$).

3.2 | 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 summarizes univariate correlations between EV populations and participant characteristics.

TABLE 1 Baseline characteristics according to liver fibrosis stage

	Nonsevere Fibrosis (F0–F2) (n = 17)	Severe Fibrosis (F3–F4) (n = 9)	P value
Scheuer fibrosis stage 0 (%)	5 (29.4%)	–	–
Scheuer fibrosis stage 1 (%)	5 (29.4%)	–	–
Scheuer fibrosis stage 2 (%)	7 (41.2%)	–	–
Scheuer fibrosis stage 3 (%)	–	5 (55.6%)	–
Scheuer fibrosis stage 4 (%)	–	4 (44.4%)	–
Age (years)	48.9 ± 12.8	51.8 ± 13.8	0.792
Male (%)	10 (58.8)	3 (33.3)	–
Type 2 diabetes (%)	7 (41.2)	5 (55.6)	–
Total cholesterol (mmol/l)	5.25 ± 1.17	4.58 ± 0.93	0.426
LDL cholesterol (mmol/L)	3.27 ± 0.98	2.86 ± 0.79	0.466
HDL cholesterol (mmol/l)	1.11 ± 0.34	1.06 ± 0.27	–
Serum triglyceride (mmol/l)	1.86 ± 0.90	1.66 ± 0.57	0.751
Fasting glucose (mmol/l)	5.50 (5.15, 7.55)	6.50 (5.35, 9.85)	0.339
Haemoglobin A1c (% total Hb)	6.1 (5.6, 8.0)	6.8 (5.85, 9.8)	0.396
Fasting insulin (μU/l)	14.38 ± 10.98	22.71 ± 15.52	0.075
Alanine transaminase (iu/l)	58.0 (46.5, 84.5)	94 (58, 144.5)	0.133
Aspartate aminotransferase (iu/l)	48.0 (33.0, 55.5)	87 (59.5, 153.5)	0.004**
Platelet Count ($\times 10^9$ l^{-1})	230.7 ± 75.6	173 ± 60.8	0.058
Albumin (g/L)	40.7 ± 3.4	38.8 ± 3.8	0.312
Weight (kg)	95.42 ± 16.61	99.16 ± 20.45	0.672
Body mass index (kg/m^2)	32.91 ± 4.55	35.95 ± 7.66	0.560
Systolic blood pressure (mmHg)	140.04 ± 15.59	144.80 ± 22.23	0.672
Diastolic blood pressure (mmHg)	87.85 ± 11.78	84.25 ± 12.18	0.458
CK-18 M65 (U/L)	569.41 ± 378.00	815.78 ± 373.28	0.148
MRS liver fat (%)	21.0 (11.0, 48.5)	18 (11, 23.3)	0.396
Carotid IMT (mm)	0.67 ± 0.11	0.66 ± 0.09	0.916
Presence of any carotid plaque (%)	47.1	88.9	–
Hyaluronic acid (μg/l)	20.0 (13.5, 44.0)	91 (38, 223)	0.001**
PIIINP (μg/l)	6.00 (4.65, 7.30)	12.8 (8.45, 14.95)	0.001**
TIMP-1 (ng/ml)	288.34 (249.98, 414.86)	340.95 (318.09, 484.60)	0.058
γ-Glutamyl Transpeptidase (U/l)	49.0 (39.0, 122.0)	213.0 (98.0, 382.5)	0.002**
Liver Fibrosis Score (LFS)	8.89 (8.51, 9.41)	10.97 (9.49, 11.25)	0.001**
NAFLD Fibrosis Score	−1.59 (−2.39, 0.33)	0.10 (−0.75, 2.00)	0.025*
Sample Storage Time	4.96 (4.53, 5.27)	4.92 (4.66, 5.28)	0.916

Data are means ± SD, or median (25th, 75th percentiles), *$P \leq 0.05$, **$P \leq 0.01$. Abbreviations: CK-18 M65, cytokeratin-18 M65 subfraction; MRS, magnetic resonance spectroscopy; Carotid IMT, intima–media thickness; PIIINP, type III procollagen peptide; and TIMP-1, tissue inhibitor of metalloproteinase-1.

TABLE 2 Comparison of EV absolute counts per ml stratified by fibrosis severity

	Nonsevere Fibrosis (F0–F2) (n = 17)	Severe Fibrosis (F3-F4) (n = 9)	P value
EEV ($\times 10^{10}$ ml^{-1})	8.62 (5.87, 14.64)	3.47 (2.68, 6.80)	0.010**
PEV ($\times 10^{10}$ ml^{-1})	9.84 (4.10, 13.13)	7.77 (5.54, 10.26)	0.500
CD105$^+$ ($\times 10^{10}$ ml^{-1})	4.03 (2.60, 7.36)	0.77 (0.32, 2.39)	0.006**
CD31$^+$ ($\times 10^{10}$ ml^{-1})	8.26 (6.57, 12.26)	7.18 (3.54, 9.71)	0.138
CD284$^+$ ($\times 10^{10}$ ml^{-1})	7.78 (4.66, 11.36)	4.61 (1.14, 5.69)	0.025*
CD16$^+$ ($\times 10^{10}$ ml^{-1})	6.66 (3.89, 12.80)	1.26 (0.52, 3.83)	0.001**
CD14$^+$ ($\times 10^{10}$ ml^{-1})	12.16 (8.51, 20.93)	8.99 (4.89, 8.88)	0.005**

Data are median (25th, 75th percentiles), *$P \leq 0.05$, **$P \leq 0.01$
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

		EEV	PEV	CD105+	CD31+	CD284+	CD16+	CD14+
Alanine transaminase (iu/L)	r	−0.075	0.144	−0.111	0.039	0.02	−0.281	−0.03
	P-value	0.717	0.483	0.59	0.849	0.922	0.164	0.883
Aspartate aminotransferase (iu/L)	r	−0.126	0.082	−0.177	−0.009	−0.149	−0.388*	−0.16
	P-value	0.539	0.69	0.387	0.964	0.469	0.05	0.436
CK-18 M65 (U/L)	r	−0.148	0.123	−0.287	0.079	−0.104	−0.409*	−0.129
	P-value	0.47	0.548	0.155	0.701	0.615	0.038	0.53
Hyaluronic Acid (μg/L)	r	−0.438*	−0.147	−0.481*	−0.400*	−0.358	−0.633**	−0.481*
	P-value	0.025	0.472	0.013	0.043	0.072	0.001	0.013
PIIINP (μg/L)	r	−0.053	0	−0.238	0.011	−0.301	−0.404*	−0.277
	P-value	0.797	0.999	0.241	0.956	0.135	0.041	0.17
TIMP-1 (ng/mL)	r	−0.154	0.196	−0.264	−0.016	0.037	−0.136	−0.055
	P-value	0.452	0.338	0.192	0.938	0.859	0.506	0.789
MRS liver fat (%)	r	0.389*	0.430*	0.315	0.27	0.374	0.075	0.193
	P-value	0.05	0.028	0.117	0.183	0.06	0.716	0.345
γ-Glutamyl Transpeptidase (U/L)	r	−0.189	−0.059	−0.26	−0.145	−0.262	−0.356	−0.34
	P-value	0.355	0.774	0.199	0.481	0.196	0.074	0.089
Liver Fibrosis Score (LFS)	r	−0.322	0.035	−0.433*	−0.247	−0.283	−0.595**	−0.410*
	P-value	0.108	0.867	0.027	0.223	0.161	0.001	0.038
NAFLD Fibrosis Score (NFS)	r	−0.142	−0.19	−0.088	−0.17	−0.388*	−0.346	−0.521**
	P-value	0.489	0.351	0.67	0.407	0.05	0.084	0.006

*$P \leq 0.05$, **$P \leq 0.01$

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.

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 ($r = −0.404$, $P = 0.041$), while 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+ ($r = −0.595$, $P = 0.001$), and 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.

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

Binary logistic regression was undertaken to test the independence of associations between (a) EV populations and histological fibrosis severity; (b) NFS and histological fibrosis severity; and (c) LFS with histological fibrosis severity. Variables showing a significant association included NFS ($\chi^2 = 6.300$, $P = 0.012$), LFS ($\chi^2 = 15.403$, $P < 0.0001$), EEVs ($\chi^2 = 7.768$, $P = 0.005$), CD284+ EVs ($\chi^2 = 4.353$, $P = 0.037$), CD14+ EVs ($\chi^2 = 9.613$, $P = 0.002$), and CD16+ EVs ($\chi^2 = 14.132$, $P < 0.001$). In order to investigate the independence of these associations further, potential confounding factors/covariates were included in the models, including age, sex, diabetes status, smoking status, sample storage time, and various treatment groups. None of these variables were independently associated with fibrosis severity and did not affect the independence of the association between EVs and F3/F4 fibrosis severity. CD14+ and CD16+ EV counts therefore showing a larger association with liver fibrosis than the NFS score and are independent of important potential confounding factors.

3.4 | Calibration and discrimination of EVs to predict F3/F4 fibrosis

The χ^2 value of the LFS model ($\chi^2 = 15.403$) was increased by the addition of various EV populations to the model. These additive models with EV populations included EEVs ($\chi^2 = 19.357$, $P = 0.041$), CD16+ EVs ($\chi^2 = 22.205$, $P = 0.009$), and CD14+ EVs ($\chi^2 = 20.847$, $P = 0.016$), (Table 4). EV populations also significantly increased the χ^2 values of the NFS model [NFS model alone = $\chi^2 = 6.300$], and these additive models included EEVs ($\chi^2 = 13.242$, $P = 0.012$), CD16+ EVs ($\chi^2 = 17.559$, $P = 0.001$), and CD14+ EVs ($\chi^2 = 12.803$, $P = 0.015$). All NFS model χ^2 values were lower than the comparable LFS additive models (Supplementary Table 3) and therefore, the NFS models

TABLE 4 Associations between LFS and histological diagnosis of liver fibrosis severity with addition of EV measurement to LFS

	χ^2	*P*-value	Exp(B)	95% CI
LFS	15.403	–	7.08	(1.74, 28.76)
LFS+EEVs	19.357	0.047*	8.51	(1.27, 56.77)
LFS+CD284+ EVs	17.07	0.197	6.68	(1.55, 28.72)
LFS+CD16+ EVs	22.205	0.009**	10.72	(0.893, 128.75)
LFS+CD14+ EVs	20.847	0.02*	9.61	(1.29, 71.357)

Exp(B) (95% CI) = odds ratios. *$P \leq 0.05$, **$P \leq 0.01$.
Abbreviations: EEV, endothelial extracellular vesicles; PEV, platelet extracellular vesicles.

showed weaker association with F3/F4 fibrosis than the 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 among 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, and $P = 0.001$. 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 7.71×10^{10} per ml. 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
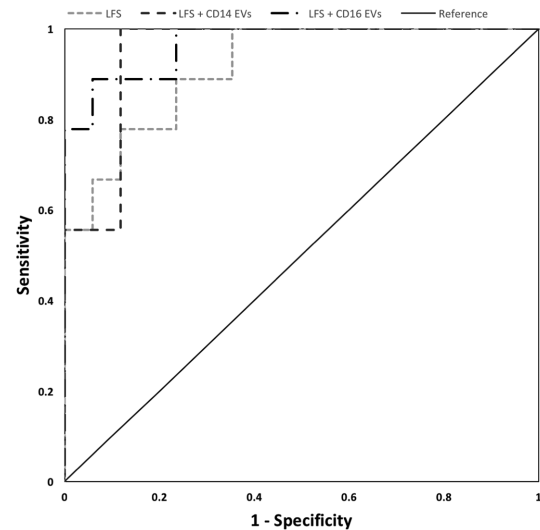
Test Result Variable(s)	Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval Lower Bound	Upper Bound
Liver Fibrosis Score (LFS)	0.915	0.055	0.001**	0.808	1
LFS + CD14+ EVs	0.948	0.042	<0.001**	0.866	1
LFS + CD16+ EVs	0.967	0.031	<0.001**	0.906	1

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. Summarized are area under curve (Area), standard error, significance, and 95% confidence intervals; *$P \leq 0.05$, **$P \leq 0.01$

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, for example, 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 ($\chi^2 = 15.410$, $P = 0.936$).

4 | 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

TABLE 5 Diagnostic performance of LFS models at different thresholds to predict F3/F4 fibrosis

Test result variable(s)	Discriminant score threshold	Specificity (%)	Sensitivity (%)	PLR	NLR
LFS	490.7306	100	0	1.00	–
	493.8796	100	64.7	2.83	0.00
	494.3406	88.9	76.5	3.78	0.15
	494.3945	77.8	76.5	3.31	0.29
	495.2127	66.7	94.1	11.31	0.35
	495.7398	55.6	100	–	0.44
	497.1416	0	100	–	1.00
LFS + CD14+ EVs	−10.2117	100	0	1.00	–
	−1.2882	100	88.2	8.47	0.00
	−0.3435	88.9	88.2	7.53	0.13
	0.2827	66.7	88.2	5.65	0.38
	0.6518	55.6	88.2	4.71	0.50
	1.5465	55.6	100	–	0.44
	6.3379	0	100	–	1.00
LFS + CD16+ EVs	−14.7571	100	0	1.00	–
	−2.5217	100	76.5	4.26	0.00
	−1.9110	88.9	76.5	3.78	0.15
	−1.2725	88.9	88.2	7.53	0.13
	−0.8687	88.9	94.1	15.07	0.12
	0.4538	77.8	100	–	0.22
	9.8894	0	100	–	1.00

Abbreviations: PLR, positive-likelihood ratio; NLR, negative-likelihood ratio.

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 Fig. 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 while 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 nonclassical 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. N.A.E. and C.D.B. are joint last author.

DISCLOSURE

NAE holds a collaboration agreement with ThermoFisher Scientific.

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SUPPORTING INFORMATION

Additional information may be found online in the supporting information tab for this article.

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

$$\text{Discriminating Score} = -1.675 + 0.037 * \text{age (years)} +$$

$$0.094 * \text{BMI(kg/m}^2) + 1.13 * \text{IFG/diabetes(yes} = 1, \text{no} = 0) +$$

$$0.99 * \text{AST/ALT ratio} - 0.013 * \text{platelet} \left(\frac{10^9}{L}\right) - 0.66 * \text{albumin (g/dl)}.$$

3. CD14 Normalization

Normalization 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.

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

4. CD16 Normalization

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

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

5. LFS Normalization

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

6. LFS + CD14$^+$ Algorithm

$$\text{Score} = -999.357 + ((\text{nLFS} + 10) * 2.262) + (n\text{CD14} * - 188.714)$$

7. LFS + CD16$^+$ Algorithm

$$\text{Score} = -1163.134 + ((\text{nLFS} + 10) * 2.372) + (n\text{CD16} * - 0.018)$$