## ORIGINAL ARTICLE

# Serum vascular cell adhesion molecule-1 predicts significant liver fibrosis in non-alcoholic fatty liver disease

S Lefere[1], F Van de Velde[2], L Devisscher[1], M Bekaert[2], S Raevens[1], X Verhelst[1], Y Van Nieuwenhove[3], M Praet[4], A Hoorens[4], C Van Steenkiste[1,5], H Van Vlierberghe[1], B Lapauw[2] and A Geerts[1]

**BACKGROUND:** Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide and is strongly associated with obesity, dyslipidemia and insulin resistance. NAFLD often presents as simple steatosis (NAFL) but can progress to non-alcoholic steatohepatitis (NASH) and fibrosis. Current non-invasive biomarkers are not tailored to identify significant ($\geqslant$F2) fibrosis, although recent guidelines recommend a stringent follow-up of this patient population. We and others have reported on the role of pathological angiogenesis in the pathogenesis of NAFLD, highlighting pro-angiogenic factors as potential diagnostic markers.
**OBJECTIVE:** To investigate the applicability of angiogenic and endothelial dysfunction markers as non-invasive diagnostic tools for NASH or NASH-associated fibrosis in obese patients.
**METHODS:** In a prospective cross-sectional study, male patients undergoing bariatric surgery ($n = 61$) and control patients ($n = 35$) were recruited. Serum protein levels and visceral adipose tissue gene expression of endothelial dysfunction and angiogenic markers were analyzed by multiplex bead-based assay and quantitative RT-PCR, respectively. For validation, we recruited a second cohort of patients undergoing bariatric surgery ($n = 40$) and a cohort of NAFLD patients from our outpatient clinic ($n = 30$).
**RESULTS:** We identified serum vascular cell adhesion molecule-1 (VCAM-1) as an independent predictor for $\geqslant$F2 fibrosis (median 14.0 vs 8.7 ng ml$^{-1}$ in patients with and without significant fibrosis; $P < 0.0001$) with an area under the receiver-operating characteristics (AUROC) curve of 0.80. The cutoff point of 13.2 ng ml$^{-1}$ showed a sensitivity of 80% and specificity of 83%. In line with these results, VCAM-1 visceral adipose tissue gene expression was also elevated in patients with fibrosis ($P = 0.030$). In the bariatric surgery and clinical validation cohorts, VCAM-1 displayed similar AUROCs of 0.89 and 0.85, respectively.
**CONCLUSIONS:** VCAM-1 levels are able to accurately predict significant ($\geqslant$F2) fibrosis in NAFLD patients.

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## INTRODUCTION

As a result of the obesity pandemic, non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease worldwide. NAFLD is the hepatic manifestation of obesity and a precursor of and independent risk factor for type 2 diabetes.[1–3] NAFLD comprises a spectrum of disease that ranges from hepatocellular steatosis without necro-inflammation (NAFL) to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and even hepatocellular carcinoma. In addition, NAFLD is an independent risk factor for cardiovascular disease, with recent studies unequivocally showing an increased cardiovascular mortality in NAFLD patients.[4,5] The global prevalence of NAFLD and NASH is around 25% and 3%, respectively, although this rises to an estimated 90% and 25%, respectively, in severely obese patients.[3,6]

Liver biopsy is still considered as the gold standard for the diagnosis of NASH and the assessment of disease activity and fibrosis, although it has important disadvantages such as its high cost, invasive nature and the risk of sampling error.[7] This has inspired the search for non-invasive disease markers, including both serum biomarkers and imaging methods. Nevertheless, there

are currently no non-invasive markers that can adequately distinguish NAFL from NASH.[8] Similarly, while many markers have shown an acceptable accuracy for the exclusion of advanced fibrosis/cirrhosis (F3-F4),[9,10] the identification of advanced disease is less accurate, and the distinction between significant ($\geqslant$F2) or any ($\geqslant$F1) fibrosis vs no fibrosis remains difficult.[7,9] The latter represents an unmet need, as recent guidelines recommend a closer follow-up of patients with significant fibrosis,[7] and the long-term prognosis of patients with fibrosis, even F1, is worse compared to NAFLD patients without fibrosis.[5]

The progression of NAFL to NASH, fibrosis and cirrhosis has previously been linked to endothelial dysfunction and pathological angiogenesis. Indeed, NAFLD strongly associates with various indices of endothelial dysfunction, such as a reduced brachial artery flow-mediated vasodilatation and a lower peripheral tonometry ratio.[11,12] Increases in serum levels of soluble markers of endothelial dysfunction and atherosclerosis, such as asymmetric dimethyl arginine and plasminogen activator inhibitor 1, have been reported in patients with NAFLD.[13,14] Similarly, neo-angiogenesis is increased in NASH patients and correlates with the severity of fibrosis.[15] Research in animal models has suggested a role for vascular endothelial growth factor (VEGF)-coordinated

[1]Department of Gastroenterology and Hepatology, Ghent University Hospital, Ghent, Belgium; [2]Department of Endocrinology, Ghent University Hospital, Ghent, Belgium; [3]Department of Gastrointestinal Surgery, Ghent University Hospital, Ghent, Belgium; [4]Department of Pathology, Ghent University Hospital, Ghent, Belgium and [5]Department of Gastroenterology and Hepatology, Maria Middelares Hospital, Ghent, Belgium. Correspondence: Professor Dr Anja Geerts, Department of Gastroenterology and Hepatology, Ghent University, De Pintelaan 185, 1K12IE, B-9000 Ghent, Belgium.
E-mail: anja.geerts@ugent.be

angiogenesis in fibrosis progression.[16] Our group has subsequently demonstrated that treatment with anti-VEGF antibodies reduced experimental steatohepatitis.[17] Endothelial dysfunction and pathological angiogenesis in turn predispose the liver to further injury as they increased intrahepatic vascular resistance, distorted the sinusoidal microvascular architecture, modulated leukocyte infiltration and caused local tissue hypoxia.[17–19] Indeed, both processes seem to be early events that precede the development of inflammation and fibrosis[18,20] and further substantiate the links between NAFLD and cardiovascular disease.[21] As such, angiogenesis and endothelial dysfunction are important mediators of NAFLD progression, although their role as diagnostic markers has not been thoroughly investigated.

Therefore, we investigated whether markers of endothelial dysfunction and angiogenesis could distinguish between various NAFLD disease stages in a well-characterized cohort of severely obese men undergoing bariatric surgery. Our results point to vascular cell adhesion molecule 1 (VCAM-1) as a promising marker for ⩾F2 fibrosis. We have validated these findings in an independent cohort of patients undergoing bariatric surgery, and in a cohort of outpatients with NAFLD recruited in our outpatient hepatology clinic.

## PATIENTS AND METHODS

### Patient cohort

A cohort of 61 consecutive male patients scheduled for bariatric surgery was recruited at the Ghent University Hospital between 2011 and 2016 within the context of a previous study.[22] All obese patients had biopsy-confirmed NAFLD. Appropriate exclusion of liver disease of other etiologies, including alcohol-induced or drug-induced liver disease, viral or auto-immune hepatitis, metabolic and cholestatic liver diseases, was performed using specific clinical, biochemical, histological and/or radiographic criteria. All patients had a negative history of alcohol abuse as indicated by an average daily consumption of ⩽20 g. None of the subjects were on treatment with corticosteroids or insulin, while other oral glucose-lowering medications were discontinued before surgery. For comparison, 35 non-obese male controls were recruited. These were healthy volunteers ($n = 17$) or patients who underwent elective abdominal surgery for adhaesiolysis, hernia diaphragmatica, sigmoidectomy or Nissen fundoplication ($n = 18$). All control subjects had an overall good health, with normal results on liver function tests (patients with aspartate aminotransferase (AST), alanine aminotransferase (ALT) $> 1.5$ times the upper normal value for 3 months or longer were excluded) and a negative history of alcohol abuse.

Blood samples were collected from patients and controls after overnight fasting, before surgery. All samples were centrifuged, fractionated and stored at $-80\,°C$ until further analysis. Liver and visceral adipose tissue (VAT) biopsies were obtained only in those patients undergoing bariatric surgery. Laboratory evaluation included standard liver biochemistry (ALT, AST, γ-glutamyl transpeptidase (GGT)), complete blood count, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and total serum cholesterol, glucose and insulin. Cytokeratin (CK)-18 M30 fragments were measured using the M30 apoptosense ELISA kit (TECOmedical, Nijkerk, The Netherlands). Anthropometric measurements were performed during a preoperative examination. Body weight was measured to an accuracy of 0.1 kg in light indoor clothing without shoes, and height was measured using a wall-mounted stadiometer. Body mass index (BMI) was calculated as body weight/height$^2$ (kg m$^{-2}$). Waist circumference was measured at the umbilicus. Insulin resistance was estimated by calculating the homeostasis model assessment-insulin resistance (HOMA-IR) using the formula: Insulin (µU ml$^{-1}$) × glucose (mg dl$^{-1}$)/405.[23] The presence of diabetes mellitus (according to the American Diabetes Association criteria[24]) and hypertension (blood pressure of ⩾140 mm Hg

systolic, ⩾90 mm Hg diastolic or treatment of previously diagnosed hypertension) was also recorded.

The FIB-4 ((age (years) × AST(U l$^{-1}$))/(thrombocytes (10$^9$ per l) × ALT$^{1/2}$ (U l$^{-1}$))) and BAAT (1 point for each item present: BMI $> 28$ kg m$^{-2}$, age $> 50$ years, triglycerides $> 150$ mg dl$^{-1}$ and ALT $> 2\times$ upper limit of normal) fibrosis scores were calculated as previously described.[25,26]

In order to validate our results, we recruited two additional cohorts. The first validation cohort comprised 40 patients undergoing bariatric surgery at the Ghent University Hospital between 2009 and 2017. Liver biopsy was obtained during surgery. The second validation cohort consisted of 30 NAFLD patients followed-up in our hepatology clinic. The majority of patients in the latter cohort ($n = 19$) underwent liver biopsy and blood sampling during the last 3 months; 11 patients were already included in a database and had serum and liver biopsy available (collected within a 1 year time span). The exclusion criteria and sample handling for both cohorts were identical to the initial cohort.

The study protocol was approved by the Ethical Committee of the Ghent University Hospital and conducted according to the principles of the Declaration of Helsinki. Participants gave their written informed consent, which was validated by the Ethical Review Board.

### Liver histology

Formalin-fixed liver biopsies were routinely processed and stained with hematoxylin–eosin and Masson's trichrome. An experienced pathologist (MP/AH) read the biopsies, blinded to the patient characteristics. Only biopsies with at least six complete portal tracts were deemed appropriate for adequate histological evaluation. Histological features were scored according to the NASH clinical research network (CRN) scoring system.[27] A diagnosis of NAFLD was made if ⩾5% of hepatocytes contained macrovesicular fat droplets, whereas the diagnosis of NASH was based on the joint presence of steatosis, hepatocyte ballooning and lobular inflammation.[28,29] Fibrosis was evaluated using the NASH CRN fibrosis staging system.[27]

### Measurement of serum endothelial dysfunction markers

The serum levels of several endothelial dysfunction markers were measured using multiplex bead-based assays (Bio-Plex MAGPIX Multiplex Reader, Bio-Rad, Temse, Belgium). VCAM-1 and intercellular adhesion molecule 1 (ICAM-1) were measured with the human cytokines 2-plex assay (#YF000000AY, Bio-Rad); the respective sensitivity, intra- and inter-assay coefficients of variation were 0.6 pg ml$^{-1}$, 6.7% and 5.5% for VCAM-1 and 2.4 pg ml$^{-1}$, 4.3% and 3.8% for ICAM-1. VEGF-A, VEGF-D, placental growth factor (PlGF) and endoglin were measured with analytes from the human cancer biomarker assays panel 2 (Bio-Rad). The sensitivity, intra- and inter-assay coefficients of variation were respectively 0.4 pg ml$^{-1}$, 2.4% and 8.6% for VEGF-A; 11.5 pg ml$^{-1}$, 3.5% and 8.2% for VEGF-D, 0.2 pg ml$^{-1}$, 2.5% and 6.8% for PlGF and 1.0 pg ml$^{-1}$, 2.9% and 9.6% for endoglin. Vascular endothelial (VE)-cadherin serum levels were measured using a commercially available enzyme linked immunosorbent assay (ELISA) kit (DY938-05; R&D Systems, Abingdon, UK).

### Visceral adipose tissue real-time quantitative PCR

Gene expression of the endothelial dysfunction markers was determined in the VAT samples, obtained at the end of the surgical intervention of 52 NAFLD patients, using real-time quantitative PCR (RT-qPCR). First, RNA was isolated out of up to 100 µg of VAT with the RNAeasy lipid tissue mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's protocol. The concentration and quality of the resulting RNA was evaluated using spectrophotometry and the A260/A280 ratio. cDNA synthesis was performed starting from 1 µg RNA, using the SensiFAST cDNA synthesis kit (Bioline, London, UK), according to the manufacturer's instructions. cDNA was added to a 384-well plate with the primers (Biolegio, Nijmegen, The Netherlands)

(Supplementary Table 1) and Sensimix SYBR No-ROX Mastermix (Bioline). Samples were run and analyzed using the Lightcycler 480 II (Roche, Belgium) according to manufacturer's protocols. PCR reactions using water instead of template showed no amplification. Measurements were performed in duplicate and Cp values were calculated with the second derivative maximum method. Average Cp values were normalized to the Cp of the housekeeping genes hydroxymethylbilane synthase (HMBS) and succinate dehydrogenase complex, subunit A (SDHA), which had the most stable expression patterns among four tested housekeeping genes, according to analysis by GeNorm (Biogazelle, Ghent, Belgium). Fold differences for patients with NASH are expressed relative to NAFL patients.

Statistical analysis

Statistical analysis was performed using SPSS 24.0 (SPSS Software, IBM Corp., Armonk, NY, USA) graphs were made using Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data distribution of continuous variables was evaluated with the Kolmogorov–Smirnov test and the appropriate parametric or non-parametric tests were applied. A two-sided $P$-value $\leqslant 0.05$ was considered statistically significant. Continuous variables are presented as median (interquartile range) or mean ± s.e.m., depending on the normality of distribution.

After univariate analysis, significant correlations were determined by calculating the Spearman's rank correlation coefficient. Multivariate binary logistic regression analysis was performed to identify independent factors associated with the presence of significant fibrosis. Bootstrapping was performed to determine the

robustness of the regression analysis. When applicable, the diagnostic performance was determined by constructing a receiver-operating characteristic (ROC) curve and by calculating the area under the ROC (AUROC) curve. Sensitivity and specificity were calculated for the optimal cutoffs determined by ROC analysis. The Ghent University Hospital Statistics Unit contributed to the statistical analysis.

## RESULTS

Patient characteristics

The main clinical and biochemical characteristics of the initial study cohort are summarized in Table 1; the histological characteristics of these patients are shown in Supplementary Table 2.

The bariatric surgery patients were older than the control subjects ($P = 0.002$) and, as expected, had a higher BMI ($P < 0.001$), waist circumference ($P < 0.001$), fasting glucose ($P = 0.009$) and insulin ($P < 0.001$), ALT ($P < 0.001$), AST ($P < 0.001$) and GGT ($P < 0.001$), and had more type 2 diabetes ($P < 0.001$) and hypertension ($P < 0.001$).

Patients with NASH were older than those with NAFL ($P = 0.041$) and had a higher fasting glucose level ($P = 0.049$), and more often had type 2 diabetes ($P = 0.016$), a well-known risk factor for NAFLD progression.[30] Patients with NAFL and NASH did not differ significantly in BMI, serum triglycerides, cholesterol, LDL cholesterol, HDL cholesterol, fasting insulin, HOMA-IR, ALT, AST, GGT,

**Table 1.** Clinical and biochemical characteristics and serum endothelial dysfunction marker levels of healthy controls and obese patients with NAFL and NASH

| Characteristic | Control population (n = 35) | NAFL (n = 24) | NASH (n = 37) | P-value NAFL vs NASH |
|---|---|---|---|---|
| Age, years | 34 (31–44) | 43 (30–49) | 46 (40–52) | 0.041*$ |
| BMI, kg m$^{-2}$ | 23.7 (22.4–25.1) | 41.5 (39.7–45.8) | 40.6 (36.0–43.1) | 0.100# |
| Waist circumference, cm | 84.5 (78.6–92.9) | 133 (130–140) | 131 (124–141) | 0.390# |
| Hypertension, % | 14.3 | 63.2 | 80.6 | 0.140# |
| Type 2 diabetes, % | 0 | 20.8 | 51.4 | 0.016*# |
| Fasting glucose, mg dl$^{-1}$ | 93.5 (86.1–105) | 96.5 (88.5–108.8) | 117 (93–150.5) | 0.049*$ |
| Fasting insulin, mU l$^{-1}$ | 5.4 (3.5–8.6) | 12.3 (8.5–15.5) | 11.6 (7.8–26.1) | 0.294# |
| HOMA-IR | 1.3 (0.8–2.1) | 2.8 (2.0–4.0) | 4.0 (1.9–8.6) | 0.227# |
| Triglycerides, mg dl$^{-1}$ | 154 (115.5–200.7) | 190.5 (145.8–224.2) | 188 (147–257) | 0.647§ |
| Total Cholesterol, mg dl$^{-1}$ | 175.5 (143.3–202.3) | 186 (164.3–206.6) | 169 (141–220.5) | 0.312 |
| LDL, mg dl$^{-1}$ | 96 (74.8–117.3) | 103.5 (78.7–126.3) | 87.8 (70.8–120.5) | 0.238 |
| HDL, mg dl$^{-1}$ | 41 (35.8–47) | 37.5 (28.3–44.0) | 35 (27.5–41) | 0.391§ |
| Thrombocytes, × 10$^3$ per μl | 220 (173–255) | 242 (200–270) | 213 (195–242) | 0.113 |
| AST, U l$^{-1}$ | 20 (15–25) | 25 (23–30) | 27 (24–42) | 0.061# |
| ALT, U l$^{-1}$ | 18 (12–26) | 39 (30–50) | 37 (32–58) | 0.647# |
| GGT, U l$^{-1}$ | 21 (15–28) | 29 (21–37) | 41 (25–61) | 0.065# |
| CRP, mg l$^{-1}$ | 0.6 (0.4–1.1) | 4.1 (2.0–6.3) | 3.5 (1.3–5.8) | 0.438# |
| CK-18 M30, U l$^{-1}$ | 178.8 (117–224.4) | 264.3 (182.4–361.6) | 277 (181.5–500.8) | 0.524# |

| Serum endothelial dysfunction marker | Control population (n = 35) | NAFL (n = 24) | NASH (n = 37) | P-value overall |
|---|---|---|---|---|
| VEGF-A (pg ml$^{-1}$) | 101.4 (75.9–126.2) | 90.5 (60.8–146.6) | 93.8 (52.1–148.3) | 0.762 |
| VEGF-D (pg ml$^{-1}$) | 20.5 (17–27) | 19.5 (15.8–24) | 18 (16–23.8) | 0.439 |
| PlGF (pg ml$^{-1}$) | 11.2 (8.8–13.8) | 8.1 (6.5–10.4) | 7.5 (6.0–10.5) | 0.003** |
| Endoglin (pg ml$^{-1}$) | 705 (389–1184) | 530 (348–710) | 580 (437–928) | 0.335 |
| VCAM-1 (ng ml$^{-1}$) | 10.4 (8.1–12.7) | 9.0 (7.9–11.6) | 13.3 (7.8–15.4) | 0.117 |
| ICAM-1 (pg ml$^{-1}$) | 10.7 (7.7–14.5) | 10.3 (8.1–15.6) | 14.2 (7.3–16.4) | 0.288 |
| VE-Cadherin (pg ml$^{-1}$) | 0.25 (0.25–0.78) | 0.94 (0.64–1.7) | 0.99 (0.59–1.8) | < 0.001*** |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CK-18 M30, cytokeratin-18 M30 apoptotic fragments; CRP, C-reactive protein; GGT, γ-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, Homeostasis Model Assessment Insulin Resistance; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; PlGF, placental growth factor; VCAM-1, vascular cell adhesion molecule 1; VE-Cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor. Results are expressed as median (interquartile range). The Mann–Whitney $U$ and $\chi^2$ tests were used to compare patient characteristics between NAFL and NASH patients, or controls and NAFLD/NASH patients, whereas the Kruskal–Wallis test was used to compare serum levels of endothelial dysfunction and angiogenic markers between controls, NAFL and NASH patients. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ for $P$-values shown in the table. §$P < 0.05$; $$P < 0.01$ and #$P < 0.001$ for patient characteristics between controls and patients with NAFLD/NASH.

**a**



**b**
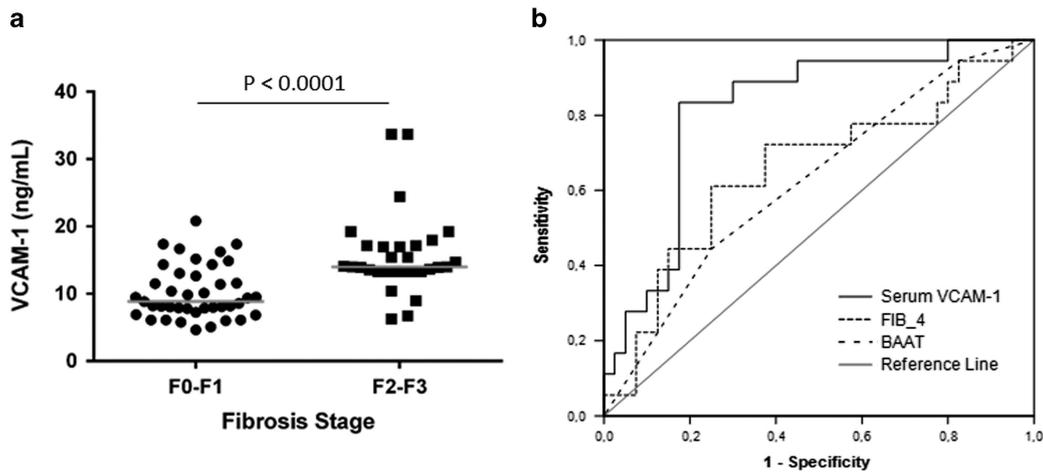


Figure 1. (**a**) Serum vascular cell adhesion molecule-1 (VCAM-1) levels according to the presence or absence of significant liver fibrosis ($\geqslant$ F2 fibrosis). *P*-values are calculated using the Mann–Whitney *U*-test. (**b**) Receiver-operating characteristic (ROC) curves for the diagnosis of significant fibrosis in morbidly obese patients. Curves are shown for VCAM-1, FIB-4 and BAAT.

| Table 2. | Univariate analysis according to the presence of significant ($\geqslant$ F2) fibrosis | | |
|---|---|---|---|
| | $\leqslant$ F1 fibrosis (n = 41) | $\geqslant$ F2 fibrosis (n = 20) | P-value |
| Age, years | 45 (37–50) | 47 (39–59) | 0.162 |
| BMI, kg m$^{-2}$ | 40.7 (37.0–43.4) | 42.3 (40.2–44.2) | 0.160 |
| Type 2 diabetes, % | 26.8 | 65 | 0.005** |
| HOMA-IR | 2.6 (1.9–4.7) | 5.5 (1.8–9.0) | 0.099 |
| ALT, U l$^{-1}$ | 41 (33–55) | 36 (26–45) | 0.148 |
| AST, U l$^{-1}$ | 27 (24–36) | 26 (21–41) | 0.741 |
| AST/ALT ratio | 0.70 (0.57–0.76) | 0.84 (0.59–1.02) | 0.073 |
| Thrombocytes, $\times 10^3$ per µl | 230 (196–270) | 215 (196–240) | 0.440 |
| Total cholesterol, mg dl$^{-1}$ | 188.0 (161.4–222) | 160.5 (119.8–197.5) | 0.029* |
| LDL, mg dl$^{-1}$ | 100.5 (78.1–100.5) | 86.5 (69.7–100.9) | 0.049* |
| HDL, mg dl$^{-1}$ | 37.0 (28.5–44.5) | 31.5 (24.3–37.8) | 0.056 |
| VCAM-1, ng ml$^{-1}$ | 8.4 (7.0–11.6) | 14.0 (13.3–17.1) | < 0.001*** |
| VEGF-A, pg ml$^{-1}$ | 87.3 (57.1–137.3) | 105.3 (58.7–165.2) | 0.442 |
| Endoglin, pg ml$^{-1}$ | 535 (402–696) | 713 (466–1035) | 0.075 |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment insulin resistance; LDL, low-density lipoprotein; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor. Results are expressed as median (interquartile range). *P < 0.05; **P < 0.01; ***P < 0.001.

thrombocytes, C-reactive protein, CK-18 M30 fragments or the presence of hypertension (Table 1).

Univariate analysis of serum markers

In univariate analysis (Table 1), vascular endothelial (VE)-cadherin levels were higher in NAFL and NASH patients compared to the control subjects (*P* < 0.001). Unexpectedly, PlGF levels were lower in NAFL and NASH patients compared to the non-obese controls (*P* = 0.003). Nevertheless, there were no significant differences in serum markers of angiogenesis and endothelial dysfunction between the NAFL and NASH groups.

Analysis according to the NASH CRN grading of steatosis, inflammation and ballooning similarly showed no significant differences in any of the serum markers (Supplementary Table 3). In contrast, serum VCAM-1 levels were higher in patients with $\geqslant$ F2 fibrosis compared to the patients with $\leqslant$ F1 fibrosis (median 14.0 and 8.7 ng ml$^{-1}$, *P* < 0.0001) (Figure 1a). Apart from VCAM-1, only the presence of type 2 diabetes, and serum LDL and total cholesterol were significantly associated with $\geqslant$ F2 fibrosis in the obese patients (Table 2). The latter two were lower in the group with significant fibrosis, likely due to a higher statin use in these patients (27% vs 55% of patients, *P* = 0.047).

To assess the predictive value of serum VCAM-1 levels, a ROC curve for prediction of $\geqslant$ F2 fibrosis was generated for VCAM-1 and for the FIB-4 and BAAT, two widely used non-invasive fibrosis scores. Only VCAM-1 had a good predictive value, with AUROCs for VCAM-1, FIB-4 and BAAT of 0.80 (95% CI: 0.673–0.919, *P* < 0.001), 0.65 (95% CI: 0.487–0.806, *P* = 0.075) and 0.63 (95% CI: 0.473–0.781, *P* = 0.124), respectively (Figure 1b). The best cutoff for VCAM-1 was 13.2 ng ml$^{-1}$, with a sensitivity of 80% and specificity of 83%. Given the prevalence of significant fibrosis of 0.33 in our population, this corresponded to a positive and negative predictive value of 70% and 89%, respectively.

Correlations and multivariate analysis for $\geqslant$ F2 fibrosis

Variables that were significantly associated with $\geqslant$ F2 fibrosis in univariate analysis were further subjected to multivariate analysis. As expected, LDL and total cholesterol correlated strongly with each other (*r* = 0.886, *P* < 0.001). Therefore, we built two separate models. Analysis for significant fibrosis in a model combining VCAM-1, type 2 diabetes and total cholesterol revealed that only serum VCAM-1 and type 2 diabetes were independently associated with significant fibrosis. Similarly, in a model combining VCAM-1, type 2 diabetes and LDL cholesterol, only serum VCAM-1

**Table 3.** Multivariate analysis according to the presence of significant (⩾ F2) fibrosis

| | ⩾F2 fibrosis (n = 20) vs ⩽F1 fibrosis (n = 41) | | |
|---|---|---|---|
| | OR | 95% CI | P-value |
| *Model 1* | | | |
| Cholesterol, mg dl$^{-1}$ | 0.987 | 0.970–1.005 | 0.159 |
| Type 2 diabetes, % | 7.190 | 1.564–33.049 | 0.011* |
| VCAM-1, ng ml$^{-1}$ | 1.372 | 1.128–1.668 | 0.002** |
| *Model 2* | | | |
| LDL, mg dl$^{-1}$ | 0.985 | 0.962–1.009 | 0.231 |
| Type 2 diabetes, % | 5.251 | 1.065–25.904 | 0.042* |
| VCAM-1, ng ml$^{-1}$ | 1.336 | 1.101–1.620 | 0.003** |
| *Model 3* | | | |
| Age, years | 1.038 | 0.967–1.113 | 0.305 |
| BMI, kg m$^{-2}$ | 1.020 | 0.906–1.149 | 0.738 |
| Fasting insulin, mU l$^{-1}$ | 1.032 | 0.991–1.076 | 0.130 |
| Thrombocytes, ×10$^3$ per μl | 1.000 | 0.983–1.017 | 0.994 |
| AST/ALT ratio | 1.920 | 0.113–32.743 | 0.652 |
| VCAM-1, ng ml$^{-1}$ | 1.296 | 1.073–1.566 | 0.007** |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; LDL, low-density lipoprotein; OR, odds ratio; VCAM-1, vascular cell adhesion molecule-1. *$P < 0.05$; **$P < 0.01$.
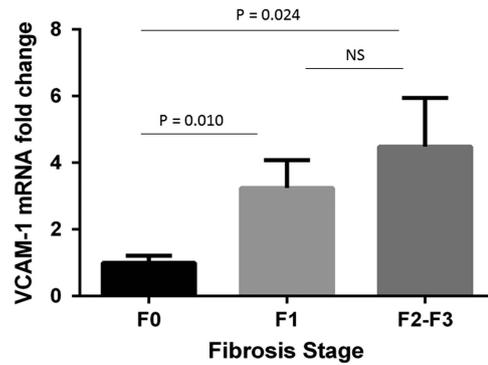


**Figure 2.** Visceral adipose tissue vascular cell adhesion molecule 1 (VCAM-1) expression according to fibrosis stage. Kruskal–Wallis P-value was 0.030. Represented P-values were calculated using the Mann–Whitney U-test.

and type 2 diabetes were independently associated with significant fibrosis (Table 3). These results remained similar after performing bootstrapping.

Serum VCAM-1 levels correlated moderately with fasting serum insulin levels ($r = 0.319$, $P = 0.015$) and thrombocytes ($r = -0.266$, $P = 0.042$), but not with other clinical and biochemical variables such as age, BMI, HOMA-IR, AST, ALT or cholesterol (Supplementary Table 4). To confirm that VCAM-1 is a predictor of significant fibrosis independent of the patient's characteristics and simple markers of fibrosis, we performed a multivariate analysis with age, BMI, fasting insulin, thrombocytes, and AST/ALT ratio as co-factors. In this model, VCAM-1 was the only variable independently associated with significant fibrosis (OR = 1.296 (95% CI: 1.073–1.566, $P = 0.007$)) (Table 3). These results did not change significantly after bootstrapping.

## Visceral adipose tissue gene expression

Recent studies have demonstrated the role of adipose tissue inflammatory and adipokine gene expression in the pathogenesis of NAFLD.[31] Furthermore, adipose tissue angiogenesis may influence insulin resistance and metabolic dysfunction.[32] Therefore, we analyzed the mRNA expression of angiogenic and endothelial dysfunction markers in the VAT of patients undergoing bariatric surgery. When comparing NASH ($n = 32$) to NAFL ($n = 20$) patients, endoglin (1.93 ± 0.31 fold vs NAFL, $P = 0.008$) and VE-cadherin (2.66 ± 0.51-fold versus NAFL, $P = 0.001$) expression was significantly higher in NASH patients (Supplementary Table 5). The other investigated genes showed a tendency toward increased expression in patients with NASH, yet these differences were not statistically significant, probably due to the high variance in expression levels. Endoglin and VE-cadherin also differed significantly when patients were stratified according to the grading of inflammation ($P = 0.037$ and 0.002, respectively) and ballooning ($P = 0.038$ and 0.020, respectively). Interestingly, only VCAM-1 gene expression was higher in patients with liver fibrosis than in those without, and this difference was already apparent in patients with F1 fibrosis (3.25 ± 0.83-fold F1 vs F0 fibrosis and 4.48 ± 1.46-fold F2 vs F0, Kruskal–Wallis $P = 0.030$) (Figure 2). The

serum levels and adipose tissue gene expression of these markers did not correlate significantly (Supplementary Table 6), indicating that adipose tissue endothelial dysfunction does not strongly influence the serum protein level.

## External validation of VCAM-1

As the initial cohort consisted of a selected patient population, namely obese males undergoing bariatric surgery, we set out to validate VCAM-1 in independent patients cohorts. We recruited a second cohort of 40 obese patients, a majority of which were female, undergoing bariatric surgery. The patient characteristics are shown in Supplementary Table 7. Although only three patients had histological evidence of F2 fibrosis, serum VCAM-1 levels were higher compared to patients without significant fibrosis (13.9 in ⩽F1 vs 21.6 ng ml$^{-1}$ in F2, $P = 0.007$) (Figure 3a). The AUROC for F2 fibrosis was 0.89 (95% CI: 0.71–1, $P = 0.02$). A low cutoff (15.6 ng ml$^{-1}$) had a sensitivity of 100% and specificity of 68.4%, whereas a higher cutoff of 18.4 ng ml$^{-1}$ had a sensitivity and specificity of 66.7% and 84.2%, respectively.

Next, we recruited 30 NAFLD patients from our outpatient hepatology clinic to test the potential of VCAM-1 as a marker in a more general NAFLD population. The main clinical characteristics are shown in Supplementary Table 8. VCAM-1 serum levels were higher in patients with significant fibrosis compared to those without (14.1 in ⩽F1 vs 21.3 ng ml$^{-1}$ in ⩾F2, $P = 0.001$) (Figure 3b), translating into an AUROC for the prediction of ⩾F2 fibrosis of 0.85 (95% CI: 0.70–0.99, $P = 0.002$). The low cutoff of 15.6 ng ml$^{-1}$ had a sensitivity of 89.5% and specificity of 63.3%, whereas the higher cutoff of 18.4 ng ml$^{-1}$ had a sensitivity and specificity of 73.3% and 90.9%, respectively.

## DISCUSSION

At present, the non-invasive diagnosis of moderate stages of fibrosis in NAFLD represents an important unmet clinical need as these patients have a worse prognosis[5] and could benefit from the novel pharmacological agents under development.[33] In this study, we have identified serum VCAM-1 as a promising marker of significant (⩾F2) fibrosis in a well-characterized cohort of obese men undergoing bariatric surgery. VCAM-1 had an AUROC of 0.80 for significant fibrosis, which was considerably higher than the AUROC of the FIB-4 and BAAT scores. Moreover, we have validated VCAM-1 in an independent cohort of patients undergoing bariatric surgery, and in a cohort of NAFLD patients from our outpatient clinic. VCAM-1 displayed an AUROC of respectively 0.89 and 0.85 in these cohorts, indicating extrapolability to other patient populations.

Recent guidelines recommend a close follow-up in patients with NASH and/or significant (⩾F2) liver fibrosis.[7] Due to the
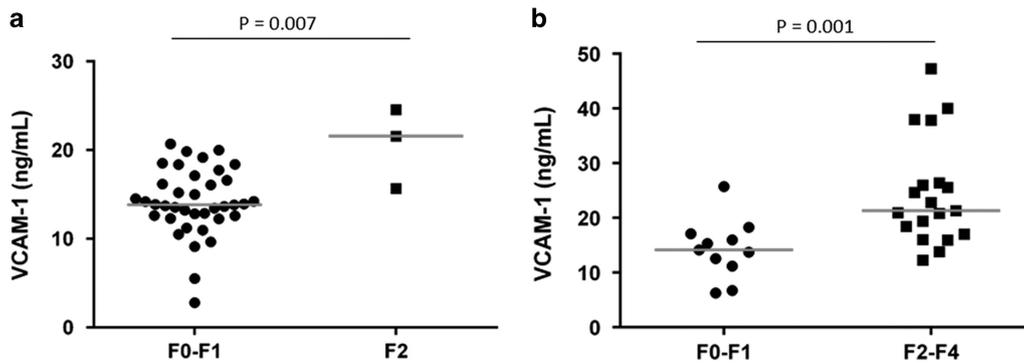
**Figure 3.** Serum vascular cell adhesion molecule-1 (VCAM-1) levels according to the presence or absence of significant liver fibrosis in a gastric bypass validation cohort (**a**) and a clinical validation cohort (**b**). *P*-values were calculated using an unpaired *t*-test (**a**) or Mann–Whitney *U*-test (**b**).

disadvantages of liver biopsy and the large population at risk for NASH and fibrosis, non-invasive diagnostic tools are increasingly being investigated and applied in clinical practice. Nevertheless, simple fibrosis risk scores are not accurate enough to diagnose or exclude moderate stages of fibrosis, as they are tailored to discriminate between patients with F0–F1 and F3–F4 fibrosis. Liver stiffness measurement with transient elastometry is another attractive approach, although morbid obesity is associated with a high technical failure rate. A meta-analysis concluded that transient elastometry is excellent in diagnosing cirrhosis (F4) and advanced (F3) fibrosis, yet only has moderate accuracy in significant (F2) fibrosis.[34] Therefore, accurate serum markers for ⩾ F2 fibrosis could aid in the identification of patients in need of closer follow-up.

Endothelial dysfunction and pathological angiogenesis have been implicated in the pathogenesis of NASH. They may also provide a link between adipose tissue dysfunction and NAFLD, as animal studies showed that improved adipose tissue angiogenesis through VEGF upregulation resulted in positive metabolic effects, including improved hepatic fat accumulation.[32,35] These mechanisms may also underlie the increased cardiovascular morbidity and mortality in patients with NAFLD.[21] We therefore evaluated the discriminating value of endothelial dysfunction and angiogenesis markers for NAFL, NASH and associated liver fibrosis. We chose to focus on VCAM-1, ICAM-1 and VE-cadherin because these endothelial adhesion molecules are commonly implicated in vascular and inflammatory diseases, as they mediate leukocyte adhesion and infiltration.[36,37] We further determined serum levels of key molecules in physiological and/or pathological angiogenesis, namely VEGF-A, VEGF-D, PlGF and endoglin.[38,39] Serum levels of these proteins correlate with tumor growth and aggressiveness in various organs, yet their expression levels in NAFLD are unclear. Notably, serum VEGF-A had already been determined in NAFLD patients, with conflicting results.[38,40,41] In our cohort, serum VEFG-A did not differ between NAFL, NASH and control patients. No marker could predict NASH. Of note, CK-18 M30 levels also did not differ between patients with NAFL and NASH, contradicting earlier reports,[42] yet corroborating more recent studies that casted doubt on the potential use of CK-18 fragments as a biomarker for NASH.[34,43]

On the other hand, we found that serum VCAM-1 predicted the presence of significant fibrosis, independent from clinical and biochemical patient characteristics. A recent paper by Yoshimura et al.,[44] in which a data mining approach to biological events was adopted in a Japanese cohort, identified VCAM-1 as a promising biomarker to diagnose F3–F4 fibrosis. Importantly, we have additionally shown that VCAM-1 can also reliably identify patients with F2 fibrosis, in contrast to other biomarkers, a finding we could replicate in two validation cohorts. Interestingly, our group previously found that VCAM-1 was an excellent predictor of

hepatopulmonary syndrome in a cohort of patients with liver cirrhosis, with an AUROC of 0.93.[45] VCAM-1 levels have also been shown to correlate with the severity of liver cirrhosis.[46] This suggests that VCAM-1 may be a useful biomarker in patients with chronic liver disease, both for the diagnosis of liver fibrosis as well as its associated complications. Moreover, evidence has mounted for a role for endothelial adhesion molecules in fibrotic diseases in general, as serum VCAM-1 levels were elevated in patients with idiopathic pulmonary fibrosis and were predictive of overall and post-transplant survival.[47]

Altered adipose tissue blood supply in obese subjects has also been implemented in the pathogenesis of NAFLD as it can lead to hypoxia, inflammation and insulin resistance.[19,32] We aimed to characterize VAT expression of angiogenic mediators and found that endoglin and VE-cadherin were significantly upregulated in patients that had progressed to NASH. Moreover, VCAM-1 expression in VAT correlated with fibrosis stage as well, and was already upregulated in patients with minimal fibrosis.

One weakness of our study is the cross-sectional design, which means we could not collect data on the relationship between serum VCAM-1 levels and the temporal evolution of fibrosis. Another limitation is the discrepancy in serum levels of VCAM-1 between the initial and validation cohorts, as the concentrations were higher in the latter. Nevertheless, VCAM-1 was an adequate predictor in each cohort and could therefore be a good candidate to facilitate the identification of patients with significant fibrosis that benefit from a more stringent follow-up in clinical practice. Further large-scale validation studies will need to standardize the analytic procedures, identify the optimal cutoff values, and elucidate if VCAM-1 can indeed be applied as a routine screening tool in clinical practice.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

SL, LD, BL and AG conceived and designed the study; SL, FVdV, MB, YVN, MP and AH were involved in data acquisition; SL, LD, SR, CVS, BL and AG were

involved in the data analysis and interpretation; SL drafted the paper; LD, XV, HVV, BL and AG critically revised the manuscript for intellectual content.

## REFERENCES

1 Yki-Jarvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol* 2014; **2**: 901–910.

2 Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Dig Liver Dis* 2015; **47**: 181–190.

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

4 Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015; **61**: 1547–1554.

5 Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology* 2015; **149**: 389–397.e10.

6 Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274–285.

7 European Association for the Study of the Liver, European Association for the Study of Diabetes, European Association for the Study of Obesity. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388–1402.

8 Machado MV, Cortez-Pinto H. Non-invasive diagnosis of non-alcoholic fatty liver disease. A critical appraisal. *J Hepatol* 2013; **58**: 1007–1019.

9 Guha IN, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S et al. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008; **47**: 455–460.

10 McPherson S, Anstee QM, Henderson E, Day CP, Burt AD. Are simple noninvasive scoring systems for fibrosis reliable in patients with NAFLD and normal ALT levels? *Eur J Gastroenterol Hepatol* 2013; **25**: 652–658.

11 Villanova N, Moscatiello S, Ramilli S, Bugianesi E, Magalotti D, Vanni E et al. Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 473–480.

12 Long MT, Wang N, Larson MG, Mitchell GF, Palmisano J, Vasan RS et al. Nonalcoholic fatty liver disease and vascular function: cross-sectional analysis in the Framingham heart study. *Arterioscler Thromb Vasc Biol* 2015; **35**: 1284–1291.

13 Kasumov T, Edmison JM, Dasarathy S, Bennett C, Lopez R, Kalhan SC. Plasma levels of asymmetric dimethylarginine in patients with biopsy-proven nonalcoholic fatty liver disease. *Metabolism* 2011; **60**: 776–781.

14 Verrijken A, Francque S, Mertens I, Prawitt J, Caron S, Hubens G et al. Prothrombotic factors in histologically proven nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Hepatology* 2014; **59**: 121–129.

15 Kitade M, Yoshiji H, Noguchi R, Ikenaka Y, Kaji K, Shirai Y et al. Crosstalk between angiogenesis, cytokeratin-18, and insulin resistance in the progression of nonalcoholic steatohepatitis. *World J Gastroenterol* 2009; **15**: 5193–5199.

16 Kitade M, Yoshiji H, Kojima H, Ikenaka Y, Noguchi R, Kaji K et al. Leptin-mediated neovascularization is a prerequisite for progression of nonalcoholic steatohepatitis in rats. *Hepatology* 2006; **44**: 983–991.

17 Coulon S, Legry V, Heindryckx F, Van Steenkiste C, Casteleyn C, Olievier K et al. Role of vascular endothelial growth factor in the pathophysiology of nonalcoholic steatohepatitis in two rodent models. *Hepatology* 2013; **57**: 1793–1805.

18 Francque S, Laleman W, Verbeke L, Van Steenkiste C, Casteleyn C, Kwanten W et al. Increased intrahepatic resistance in severe steatosis: endothelial dysfunction, vasoconstrictor overproduction and altered microvascular architecture. *Lab Invest* 2012; **92**: 1428–1439.

19 Lefere S, Van Steenkiste C, Verhelst X, Van Vlierberghe H, Devisscher L, Geerts A. Hypoxia-regulated mechanisms in the pathogenesis of obesity and non-alcoholic fatty liver disease. *Cell Mol Life Sci* 2016; **73**: 3419–3431.

20 Pasarin M, Abraldes JG, Rodriguez-Vilarrupla A, La Mura V, Garcia-Pagan JC, Bosch J. Insulin resistance and liver microcirculation in a rat model of early NAFLD. *J Hepatol* 2011; **55**: 1095–1102.

21 Francque SM, van der Graaff D, Kwanten WJ. Non-alcoholic fatty liver disease and cardiovascular risk: pathophysiological mechanisms and implications. *J Hepatol* 2016; **65**: 425–443.

22 Bekaert M, Ouwens DM, Horbelt T, Van de Velde F, Fahlbusch P, Herzfeld de Wiza D et al. Reduced expression of chemerin in visceral adipose tissue associates with hepatic steatosis in patients with obesity. *Obesity* 2016; **24**: 2544–2552.

23 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–419.

24 American Diabetes Association. Standards of Medical Care in Diabetes-2016. *Diabetes Care* 2016; **39** (Suppl 1): S1–112.

25 Ratziu V, Giral P, Charlotte F, Bruckert E, Thibault V, Theodorou I et al. Liver fibrosis in overweight patients. *Gastroenterology* 2000; **118**: 1117–1123.

26 Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317–1325.

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

28 Kleiner DE, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis* 2012; **32**: 3–13.

29 Bedossa P, Consortium FP. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology* 2014; **60**: 565–575.

30 Loomba R, Abraham M, Unalp A, Wilson L, Lavine J, Doo E et al. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. *Hepatology* 2012; **56**: 943–951.

31 du Plessis J, van Pelt J, Korf H, Mathieu C, van der Schueren B, Lannoo M et al. Association of adipose tissue inflammation with histologic severity of nonalcoholic fatty liver disease. *Gastroenterology* 2015; **149**: 635–648.e14.

32 Sung HK, Doh KO, Son JE, Park JG, Bae Y, Choi S et al. Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis. *Cell Metab* 2013; **17**: 61–72.

33 Ratziu V. Novel pharmacotherapy options for NASH. *Dig Dis Sci* 2016; **61**: 1398–1405.

34 Kwok R, Tse YK, Wong GL, Ha Y, Lee AU, Ngu MC et al. Systematic review with meta-analysis: non-invasive assessment of non-alcoholic fatty liver disease--the role of transient elastography and plasma cytokeratin-18 fragments. *Aliment Pharmacol Ther* 2014; **39**: 254–269.

35 Sun K, Wernstedt Asterholm I, Kusminski CM, Bueno AC, Wang ZV, Pollard JW et al. Dichotomous effects of VEGF-A on adipose tissue dysfunction. *Proc Natl Acad Sci USA* 2012; **109**: 5874–5879.

36 Flemming S, Burkard N, Renschler M, Vielmuth F, Meir M, Schick MA et al. Soluble VE-cadherin is involved in endothelial barrier breakdown in systemic inflammation and sepsis. *Cardiovasc Res* 2015; **107**: 32–44.

37 Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2292–2301.

38 Coulon S, Francque S, Colle I, Verrijken A, Blomme B, Heindryckx F et al. Evaluation of inflammatory and angiogenic factors in patients with non-alcoholic fatty liver disease. *Cytokine* 2012; **59**: 442–449.

39 Dewerchin M, Carmeliet P. PlGF: a multitasking cytokine with disease-restricted activity. *Cold Spring Harb Perspect Med* 2012; **2**: a011056.

40 Tarantino G, Conca P, Pasanisi F, Ariello M, Mastrolia M, Arena A et al. Could inflammatory markers help diagnose nonalcoholic steatohepatitis? *Eur J Gastroenterol Hepatol* 2009; **21**: 504–511.

41 Yilmaz Y, Yonal O, Kurt R, Alahdab YO, Ozdogan O, Celikel CA et al. Circulating levels of vascular endothelial growth factor A and its soluble receptor in patients with biopsy-proven nonalcoholic fatty liver disease. *Arch Med Res* 2011; **42**: 38–43.

42 Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. *World J Gastroenterol* 2007; **13**: 837–844.

43 Cusi K, Chang Z, Harrison S, Lomonaco R, Bril F, Orsak B et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. *J Hepatol* 2014; **60**: 167–174.

44 Yoshimura K, Okanoue T, Ebise H, Iwasaki T, Mizuno M, Shima T et al. Identification of novel noninvasive markers for diagnosing nonalcoholic steatohepatitis and related fibrosis by data mining. *Hepatology* 2016; **63**: 462–473.

45 Raevens S, Coulon S, Van Steenkiste C, Colman R, Verhelst X, Van Vlierberghe H et al. Role of angiogenic factors/cell adhesion markers in serum of cirrhotic patients with hepatopulmonary syndrome. *Liver Int* 2015; **35**: 1499–1507.

46 Lo Iacono O, Rincon D, Hernando A, Ripoll C, Catalina MV, Salcedo M et al. Serum levels of soluble vascular cell adhesion molecule are related to hyperdynamic circulation in patients with liver cirrhosis. *Liver Int* 2008; **28**: 1129–1135.

47 Richards TJ, Kaminski N, Baribaud F, Flavin S, Brodmerkel C, Horowitz D et al. Peripheral blood proteins predict mortality in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2012; **185**: 67–76.