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ORIGINAL RESEARCH—BASIC

# Obese Patients With Nonalcoholic Fatty Liver Disease Have an Increase in Soluble Plasma CD163 and a Concurrent Decrease in Hepatic Expression of CD163

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**BACKGROUND AND AIMS:** Macrophages play an important role in the development of nonalcoholic fatty liver disease (NAFLD) and its progression to nonalcoholic steatohepatitis (NASH). In this study, we investigated the hepatic expression of the macrophage scavenger receptor CD163 and the plasma level of its shed soluble form (sCD163) in patients with obesity and NASH, non-NASH NAFLD (NAFL), or healthy livers (no NAFLD). **METHODS:** Paired liver biopsies and plasma samples were collected from 61 patients with obesity (body mass index $\geq$35). Hepatic expression of CD163 was analyzed by immunohistochemistry and data-independent acquisition mass spectrometry, whilst plasma levels of sCD163 were determined by enzyme-linked immunosorbent assay and data-independent acquisition mass spectrometry. NAFLD stage and activity were assessed using the Kleiner fibrosis and NASH Clinical Research Network (NAS-CRN) scoring system. **RESULTS:** sCD163 turned out as a promising predictor of NASH with an area under the receiver-operating characteristic curve of 0.78 [0.65;0.92] ($P = .0008$). sCD163 increased with more severe NAFLD both in univariate (odds ratio [OR] = 3.31[1.80;6.11], $P < .001$) and multivariable ordinal logistic regression adjusting for NAFLD risk factors (OR = 2.02 [1.03;3.97], $P = .042$). On the other hand, hepatic expression of CD163 was negatively associated with more severe NAFLD in univariate ordinal logistic regression determined by immunohistochemistry (OR = 0.91[0.84;0.98], $P = .015$) and proteomics (OR = 0.13[0.02;0.80], $P = .028$). Taking NAFLD risk factors into account, hepatic expression of CD163 was only associated with the fibrosis stage (OR = 0.01 [0.0003;0.21], $P = .004$). Accordingly, hepatic CD163 surface expression and sCD163 were negatively correlated (rho = −0.478, $P = .0001$). **CONCLUSION:** An increased plasma sCD163 and a concurrent decreased hepatic expression of CD163 are strongly associated with NAFLD in obese patients.

*Keywords:* CD163; NASH; NAFLD; Immunohistochemistry; Proteomics; Translational

## Introduction

Nonalcoholic fatty liver disease (NAFLD) comprises a continuum of disease phases from mild hepatic steatosis to severe nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis with increased risk of hepatocellular carcinoma.[1,2] In the hepatic inflammatory process seen in NASH, especially macrophages have received increasing attention as drivers of the pathophysiology.[3–6] In the NASH liver parenchyma, the macrophages form characteristic aggregates termed lipogranulomas or hepatic crown-like structures, which presence is associated with the progression of simple steatosis to NASH.[7,8] Distinct macrophage phenotypes have been identified in NASH livers in both humans and mice,[5] however, their selective impact on the pathogenesis remains to be understood in-depth. In line with their pathological role, macrophages are potential targets for the treatment of NASH.[4,9]

***Abbreviations used in this paper:*** **ADAM17, ADAM metallopeptidase domain 17; AST, aspartate transaminase; AUROC, area under the receiver-operating characteristic curve; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; IHC, immunohistochemical; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; sCD163, soluble CD163; sTREM2, soluble triggering receptor expressed on myeloid cells 2.**

**Most current article**



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The scavenger receptor CD163 is exclusively expressed on monocytes and macrophages, especially the largely anti-inflammatory (M2-like) macrophages including Kupffer cells.[10] Targeting anti-inflammatory drugs to CD163-positive macrophages using antibodies have shown promising results in a rat NASH model.[11] In addition, the receptor has obvious diagnostic properties because, during inflammation-induced macrophage activation, ADAM metallopeptidase domain 17 (ADAM17) sheds CD163 from the cell surface as soluble CD163 (sCD163).[12,13] This shedding of CD163 is regarded as a part of the reprogramming of the anti-inflammatory macrophages to a more pro-inflammatory or at least less anti-inflammatory phenotype.[13] Circulating sCD163 has been linked to a range of inflammatory conditions including obesity,[14] insulin resistance,[15,16] and type 2 diabetes.[17–19] Elevated levels of sCD163 have also been associated with NAFLD/NASH in multiple studies[20–24] and have been proposed as a potential noninvasive biomarker.[4,23–25] The clinical use of sCD163 has been underlined in other liver diseases recently (including viral hepatitis,[26,27] alcoholic hepatitis,[28,29] cholangitis,[30,31] liver failure,[32,33] and hepatocellular carcinoma[34,35]), and intervention studies have shown that elevated sCD163 levels decrease after intervention in viral hepatitis[36–38] and NAFLD.[39,40]

Although never evidenced, the liver is a likely origin of sCD163 in NASH patients because it contains the major pool of tissue-resident macrophages,[41] however, both human and animal studies have surprisingly indicated decreased hepatic expression of *CD163* in NASH at the transcriptional level.[5,42–47] To further elucidate this relation in humans, we have analyzed the expression of CD163 in liver biopsies from patients with obesity with varying stages of NAFLD and compared it to matched plasma levels of sCD163.

## Methods

### Study population

In this unique cohort, cross-sectional baseline data collected approximately 6 months before bariatric surgery was used. Liver biopsies and fasting blood samples were collected from 61 patients with obesity of an ongoing biopsy-controlled, single-center, prospective interventional study (PROMETHEUS) between June 2018 and February 2020 at the gastroenterology and hepatology unit at The Hospital of Southwest Jutland, Esbjerg, Denmark. PROMETHEUS is approved by the committee on health research ethics for Southern Denmark (S-20170210) and is registered at ClinicalTrials.gov (NCT03535142). Patients were included if body mass index [BMI] $\geq$35 and age $\geq$18 upon screening for eligibility. Furthermore, exclusion criteria were active viral hepatitis, excessive alcohol consumption, cancer, cholestasis, and thrombocytopenia.

### Clinical and laboratory assessment

Liver biopsies and blood sampling were collected on the same day in a fasting state. EDTA plasma was stored at −80 °C before further use. Percutaneous liver biopsies were obtained during a sterile procedure using a 17-G Menghini needle (Hepafix, Braun, Germany). The tissue was formalin-fixed, paraffin-embedded, or snap-frozen, and kept at −80 °C until further use. Routine biochemical analyses were performed on Cobas6000 (Roche). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR=([fasting insulin in pmol/L]/6.945)*[blood glucose in mM]/22.5).[48] Liver fibrosis was quantified by histology as well as elastography.

The plasma concentration of sCD163 was determined in triplicates using an in-house enzyme-linked immunosorbent assay kindly provided by Holger Jon Møller (Aarhus University, Denmark).[49] Standards (6.25–200$\mu$g/L) and controls were included in each run with an inter-assay coefficient of variance of 8.5% at 1.40 mg/L and 8.6% at 3.77 mg/L. The normal range of sCD163 has previously been determined to be 0.7–3.9 mg/L in 240 healthy individuals.[13]

Plasma levels of soluble triggering receptor expressed on myeloid cells 2 (sTREM2) were analyzed using a commercial enzyme-linked immunosorbent assay kit (Abcam, #ab224881) and performed according to the manufacturer's instructions.

### Histological staging and activity scoring of NAFLD

Liver sections (3 $\mu$m) were stained by hematoxylin and eosin and Sirius Red by standard procedures at the Department of Pathology at Odense University Hospital, Denmark. An expert liver pathologist assessed and scored characteristic features of NAFLD according to the NASH Clinical Research Network (NAS-CRN) scoring system[50] blinded to clinical patient data. Unweighted summation of steatosis (0–3), lobular inflammation (0–3), and hepatic ballooning (0–2) gave rise to the NAFLD activity score (NAS). NASH was diagnosed according to the FLIP algorithm[51,52] with 'no NAFLD' in case of <5% steatosis (steatosis grade 0), 'non-NASH NAFLD (NAFL)' in case of >5% steatosis (steatosis grade >0) with or without either lobular inflammation or ballooning and 'NASH' in case of joint presence of steatosis, inflammation, and ballooning regardless of fibrosis stage. The stage of fibrosis (0–4) was evaluated according to the Kleiner fibrosis score.[50]

### Hepatic expression of CD163 by immuno histochemistry

Formalin-fixed, paraffin-embedded liver biopsies (3$\mu$m) were analyzed by immunohistochemical (IHC) staining of CD163 and CD68. For CD163 IHC, antigen demasking was performed in cell conditioning solution 1 (pH 8.5, Ventana Medical Systems) for 32 minutes at 100 °C and a monoclonal mouse anti-human CD163 antibody (clone: MRQ-26; Roche Tissue Diagnostics, Hvidovre, DK (ready to use)) was used for 32 minutes at 36 °C on the BenchMark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ) with the OptiView Detection Kit (Ventana Medical Systems, Tucson, AZ, United States). For CD68 IHC, antigen demasking was performed in TEG buffer (10mM Tris, 0.5mM EGTA, pH 9) for 15 minutes in the microwave oven and a monoclonal mouse anti-human CD68 antibody (clone: PG-M1; Roche Tissue Diagnostics, Hvidovre, DK) was incubated for 60 minutes at room temperature on the Dako Autostainer Link 48 instrument (Agilent, Santa Clara, United States) with the Dako EnVision FLEX (Agilent, Santa Clara, United States) detection system.

Stained slides were scanned using a 40x objective on a NanoZoomer 2.0HT whole-slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). IHC expression was quantified by an expert

**Table.** Patient Demographics and Histological and Biochemical Measurements

| | Total | No NAFLD | NAFL | NASH | |
|---|---|---|---|---|---|
| | 61 (100%) | 12 (19.7%) | 33 (54.1%) | 16 (26.2%) | $P^a$ |
| Demographics | | | | | |
| Female sex | 41 (67.2%) | 9 (75.0%) | 21 (63.6%) | 11 (68.8%) | .874 |
| Age, years | 47 (18–68) | 43.5 (23–51) | 47 (19–68) | 50 (18–62) | .422 |
| BMI, kg/m$^2$ | 42.5 (33.3–79.4) | 44.4 (36.5–49.6) | 42.5 (33.3–79.4) | 41.5 (36.1–56.4) | .670 |
| Waist-hip ratio | 0.93 ± 0.11 | 0.86 ± 0.11 | 0.94 ± 0.11 | 0.96 ± 0.11 | **.029** |
| Biochemical measurements | | | | | |
| ALT, U/L | 31 (11–173) | 19.5 (12–38) | 30 (11–128) | 58.5 (18–173) | **.0002** |
| AST, U/L | 25 (11–154) | 18.5 (12–27) | 25 (11–75) | 39 (14–154) | **.012** |
| GGT, U/L | 32.0 (10–198) | 15 (10–54) | 30 (16–192) | 80 (26–198) | **.0001** |
| TE, kPa[b] | 7.7 (2.0–61.1) | 4.4 (2.0–13.8) | 7.7 (3.7–45.6) | 14.4 (6.1–61.1) | **.0001** |
| HDL, mmol/L | 1.0 (0.6–1.7) | 1.6 (0.6–1.7) | 1.0 (0.7–1.7) | 1.0 (0.6–1.7) | .538 |
| LDL, mmol/L | 2.9 ± 1.0 | 2.5 ± 1.0 | 3.0 ± 1.1 | 3.1 ± 0.9 | .229 |
| Triglyceride, mmol/L | 1.4 (0.6–4.6) | 0.8 (0.7–1.7) | 1.6 (0.6–3.9) | 2.0 (1.07–4.6) | **.0001** |
| Cholesterol, mmol/L | 4.5 ± 1.0 | 3.9 ± 1.0 | 4.5 ± 1.1 | 4.7 ± 0.9 | .121 |
| Glucose intolerance[c] | 42 (68.9%) | 5 (41.7%) | 25 (75.8%) | 12 (75.0%) | .085 |
| Insulin, pmol/L | 154.5 (22–518) | 95.5 (50–155) | 165 (22–430) | 251.5 (116–518) | **.0001** |
| B-glucose, mmol/L | 6.3 (5.2–12.9) | 5.5 (5.4–6.6) | 6.3 (5.2–11) | 6.8 (5.5–12.9) | **.0141** |
| HOMA-IR | 6.7 (1.1–42.8) | 3.3 (2.0–6.5) | 7.8 (1.1–18.9) | 12.0 (5.3–42.8) | **.0001** |
| Hypertension[d] | 41 (67.2%) | 6 (50.0%) | 22 (66.7%) | 13 (81.3%) | **.016** |
| sCD163, mg/L | 2.8 (0.8–7.9) | 2.2 (0.8–3.3) | 2.8 (1.8–4.7) | 3.8 (2.2–7.9) | **.0005** |
| sTREM2, ng/ml | 43.1 (12.0–226.1) | 18.4 (12.0–27.7) | 39.6 (16.0–133.6) | 85.1 (43.5–226) | **.0001** |
| NAS-CRN score, *n* (*0/1/2/3/4/5/6/7/8*) | 10/5/17/8/8/10/1/1/1 | 10/2/0/0/0/0/0/0/0 | 0/3/17/7/4/2/0/0/0 | 0/0/0/1/4/8/1/1/1 | **.0001** |
| Kleiner fibrose stage, *n* (*0/1/2/3/4*) | 14/32/12/3/0 | 8/4/0/0/0 | 6/22/5/0/0 | 0/6/7/3/0 | **.0001** |
| CD163, positive % area of ROI | 17.8 ± 6.4 | 19.9 ± 5.7 | 18.9 ± 6.5 | 14.2 ± 5.6 | **.024** |
| CD68, positive % area of ROI | 8.1 ± 2.8 | 8.6 ± 3.3 | 8.1 ± 2.6 | 7.8 ± 3.1 | .758 |
| Omics | | | | | |
| Relative expression of CD163 in plasma (proteomics)[e] | 1.43 ± 0.54 | 1.09 ± 0.47 | 1.59 ± 0.54 | 1.47 ± 0.50 | .166 |
| Relative expression of CD163 in liver (proteomics)[f] | 0.97 ± 0.28 | 1.02 ± 0.15 | 1.03 ± 0.32 | 0.79 ± 0.20 | **.014** |
| Hepatic *CD163* expression (transcriptomics)[g] | 9.23 ± 0.63 | 9.93 ± 0.55 | 9.20 ± 0.57 | 8.93 ± 0.51 | **.009** |

Categorical data are represented as frequency (%), and continuous data are represented as mean ± SD if normally distributed otherwise as median (range).
BMI, body mass index; sCD163, soluble CD163; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma glutamyltransferase; TE, transistent elastography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance, NAS-CRN, NASH Clinical Research Network Score; ROI, region of interest.
[a]Differences between No NAFLD, NAFL, and NASH were determined by Fisher's exact test, Kruskal-Wallis rank test, or one-way ANOVA as appropriate, with a level of significance at $P < .05$ indicated by bold.
[b]Transistent elastography by Fibroscan.
[c]Fasting glucose level is > 5.6 mmol/L and/or diagnosed diabetes requiring pharmaceutical treatment.
[d]Hypertension is present if systolic blood pressure is > 130mmHg or diastolic blood pressure is > 85 mmHg or receiving antihypertensive treatment.
[e]Fold change of sCD163 compared to the median in No NAFLD patients (No NAFLD n = 9, NAFL n = 17, and NASH n = 7).
[f]Fold change of CD163 compared to the median in No NAFLD patients (No NAFLD n = 12, NAFL n = 31, and NASH n = 15).
[g]Normalized expression of hepatic CD163 mRNA (No NAFLD n = 5, NAFL n = 15, and NASH n =10).

liver pathologist using the open-source digital pathology software QuPath version 0.3.0[53] by detecting the positively stained area relative to the total liver biopsy area (region of interest).

## *Quantitation of CD163 by mass-spectrometry*

CD163 and sCD163 were quantified in snap-frozen liver biopsies and plasma by data-independent acquisition (DIA) mass spectrometry using an liquid chromatography with tandem mass spectrometry (LC-MS/MS) system. Plasma samples were diluted 1:10 in 0.5M HEPES buffer (pH 8.0) and the protein concentration was measured on the Implen NanoPhotometer N60. Snap-frozen liver biopsies were bisected and homogenized in denaturing buffer (8M guanidine hydrochloride in 25mM ammonium bicarbonate or 5% SDS) by 3–10 seconds of sonication (20% intensity) and 5 minutes incubation at 95 °C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and lysates were pooled 1:1 by volume and protein content before a 4-fold dilution. Proteins from liver lysates were purified using protein aggregation capture on microparticles[54] and then resuspended in 100$\mu$L of 50mM ammonium bicarbonate.

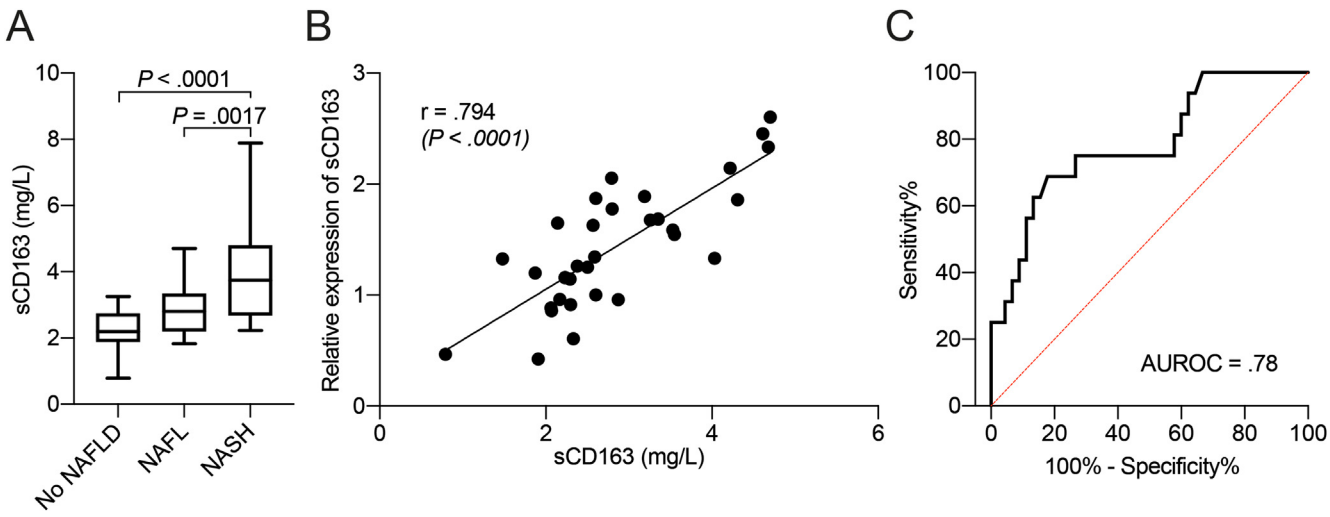


**Figure 1.** Soluble CD163 (sCD163) increases with NAFLD severity and predicts NASH. (A) Plasma levels of sCD163 increase with NAFLD severity (No NAFLD = 12, NAFL = 33, NASH n = 16). (B) Correlation between sCD163 determined by enzyme-linked immunosorbent assay and proteomics. (C) Receiver-operator characteristic (ROC) curve with sCD163 as a predictor for NASH (No NASH n = 45, NASH n = 16).

Protein digest and preparation for LC-MS/MS were done as previously described.[55]

Approximately 800 ng of peptide mixture was processed on an LC-MS/MS system, as previously described.[56] Briefly, we used an EASY-nLC 1000 nanoflow liquid chromatograph coupled with either a Q Exactive HF-X mass spectrometer or an Orbitrap Exploris 480 mass spectrometer (all Thermo Fisher Scientific). The mass spectrometers were operated using DIA by SWATH-MS.[57] First performing a 350–1400 m/z survey scan at a resolution of 120,000, maximum ion injection time of 45 ms, and normalized automatic gain control of 300%. Followed by sequential fragmentation of the precursor ions in the 360.5–1000.5 m/z range using a 13 m/z isolation window, a 1 m/z overlap between sliding windows, 30,000 resolution, maximum ion injection time of 54 ms, and collision energy of 28%. DIA raw files were analyzed in Spectronaut 14 (Biognosys) using our in-house generated spectral library and in Spectronaut 16 using DirectDIA for the liver and plasma samples, respectively. We used default settings with imputation disabled and single-hit filtering applied.

### *Hepatic expression of CD163 by RNA sequencing*

Publicly available whole-liver transcriptomes were retrieved from the NCBI Gene Expression Omnibus repository (GSE207310).[58] The patients in this libary origniate from the same cohort and include a subset of patients from the present study (No NAFLD n = 5, NAFL n = 15, and NASH n = 10) . Reads were aligned to the human genome (GRCh38, Ensemble release 101) using STAR (v.2.7.8a).[59] Exon read counts were quantified using FeatureCounts (v.2.0).[60] Counts were normalized by variance stabilizing transformation using DESeq2 (v.1.36.0).[61]

### *Statistical analysis*

Categoric variables are represented as frequency (%) and continuous variables are represented as mean ± standard deviation if normally distributed otherwise as median (range). Normality was assessed graphically using quantile-quantile plots. Comparison of 2 categories was performed with Student's t-test or Mann-Whitney U test for parametric or nonparametric data, respectively, while one-way analysis of variance or Kruskal-Wallis H test was used when more than 2 categories were compared. Fisher's exact test was used when categorical variables were compared. Pearson's correlation was used for normally distributed variables otherwise Spearman's rank correlation was used.

Univariate ordinal logistic regression was used to determine the association between primary outcomes (diagnosis of NAFLD determined by the FLIP algorithm or NAS) or secondary outcomes (steatosis, inflammation, ballooning, or fibrosis stage) and either hepatic expression of CD163 determined by IHC or proteomics or circulating level of sCD163 (Model 1). Ballooning was grouped as 'no ballooning' or 'ballooning' due to low frequency in some categories. In multivariable ordinal logistic regressions, the associations were adjusted by sex (Model 2) or by sex, age, BMI, HOMA-IR, triglycerides, and hypertension (Model 3). The proportional odds assumption was tested. Receiver operating characteristic (ROC) analysis was performed using pROC (v.1.18.0)[62] to determine sCD163 ability alone or in combination with aspartate transaminase (AST) to identify 'NASH' from 'No NASH' which includes all 'No NAFLD' and 'NAFL'. *P*-values of <.05 were considered significant. STATA (version 16.0, StataCorp LP, College Station, TX, USA) and RStudio (2022.02.3, build 492) were used for data analysis, and GraphPad Prism (version 8.2.1) was used for illustrations.

## Results

### *Patient characteristics*

In total, 61 matched plasma samples and liver biopsies from patients with obesity (BMI ≥35) were included. Table 1 describes the patient characteristics. Histological

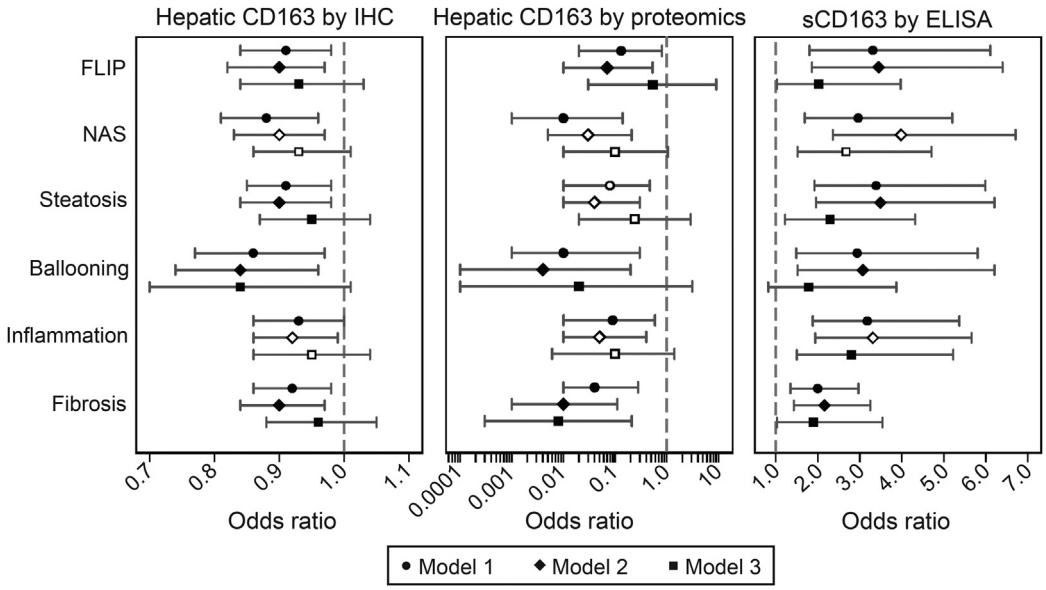


**Figure 2.** Association between hepatic CD163 and soluble (sCD163) with primary and secondary outcomes in univariate and 2 multivariable ordinal logistic regressions. Outcome variables were diagnostic scores according to the fatty liver inhibition of progression (FLIP) algorithm or NASH activity score (NAS) as well as histological features of NASH individually assessed according to NAS-CRN.[50] Model 1: Univariate logistic regression with hepatic expressed CD163 and sCD163 as the explanatory variables. Model 2: Multivariable logistic regression with hepatic expressed CD163 or sCD163 as explanatory variable and sex as covariate. Model 3: Multivariable logistic regression with hepatic expressed CD163 or sCD163 as explanatory variable and NAFLD risk factors (sex, age, body mass index (BMI), insulin resistance (HOMA-IR), triglycerides and hypertension) as covariates. Odds ratios are represented for the association of hepatic expressed CD163 or sCD163 to the respective outcome variables. Empty symbols indicate violated proportional odds assumption and therefore not significant.

assessment of liver biopsies diagnosed non-NASH NAFLD in 33 (54%) and NASH in 16 (26%) of patients according to the NAS-CRN scoring system. Only NASH patients had ballooned hepatocytes, while steatosis and lobular inflammation were observed in 49 (80%) and 48 (79%) of the liver biopsies, respectively. The NASH patients had mild to severe fibrosis (F1-F3) and elevated liver stiffness measures using Fibroscan. None of the included patients had cirrhosis (F4). The liver enzymes alanine transaminase, AST, and gamma-glutamyl transferase increased with increasing NAFLD severity as did triglycerides, and insulin resistance as determined by HOMA-IR.

## Soluble CD163 identifies patients with NASH

The plasma level of sCD163 was significantly increased from 2.2 mg/L in patients without NAFLD to 3.8 mg/L in NASH patients (Figure 1A). Soluble CD163 levels correlate significantly with the relative expression of sCD163 as determined by proteomics (Figure 1B). Circulating levels of sCD163 identified NASH patients from patients without NASH with an area under the receiver-operating characteristic curve (AUROC) of 0.78 [0.65;0.92] (Figure 1C). Including AST in the ROC analysis only improved the AUROC slightly to 0.82 [0.69;0.94].

In univariate logistic regression, the odds ratio (OR) of a more severe NAFLD diagnosis was 3.31 [1.80;6.11] ($P < .001$) as sCD163 increased (Figure 2). Additionally, increased sCD163 levels were associated with increased NAFLD activity score (NAS) (OR = 2.96 [1.69;5.20], $P < .001$), exacerbated histological scores of steatosis (OR = 3.39 [1. 92;5.99], $P < .001$), inflammation (OR = 3.18 [1.88;5.37], $P < .001$) and ballooning (OR = 2.94 [1.49;5.81], $P = .002$) as well as fibrosis stage (OR = 2.00 [1.35;2.97], $P = .001$) (*Model 1* in Figure 2). There was no sex difference in the levels of sCD163 ($P = .356$). However, sCD163 levels have previously been associated with obesity and insulin resistance and, consequently, NAFLD risk factors (sex, age, BMI, HOMA-IR, triglycerides, and hypertension) were included in a multivariable ordinal logistic regression (*Model 3* in Figure 2). sCD163 remained associated with NAFLD severity with an OR of 2.02 [1.03;3.97] ($P = .042$) using NAFLD diagnosis as the outcome variable. Steatosis, lobular inflammation, and fibrosis also remained associated with sCD163 levels after adjustment (OR = 2.29 [1.22;4.32] ($P = .010$)), OR = 2.80 [1.50;5.22] ($P = .001$)) and OR = 1.90 [1.03;3.54] ($P = .041$), respectively).

## Hepatic CD163 expression decreases with NAFLD severity

Bulk proteomic analysis of liver needle biopsies revealed a reduced hepatic expression of CD163 in NASH as compared to NAFL (Figure 3A). This was confirmed by IHC staining of CD163-positive macrophages in the liver specimens (Figure 3B). Furthermore, hepatic *CD163* was also

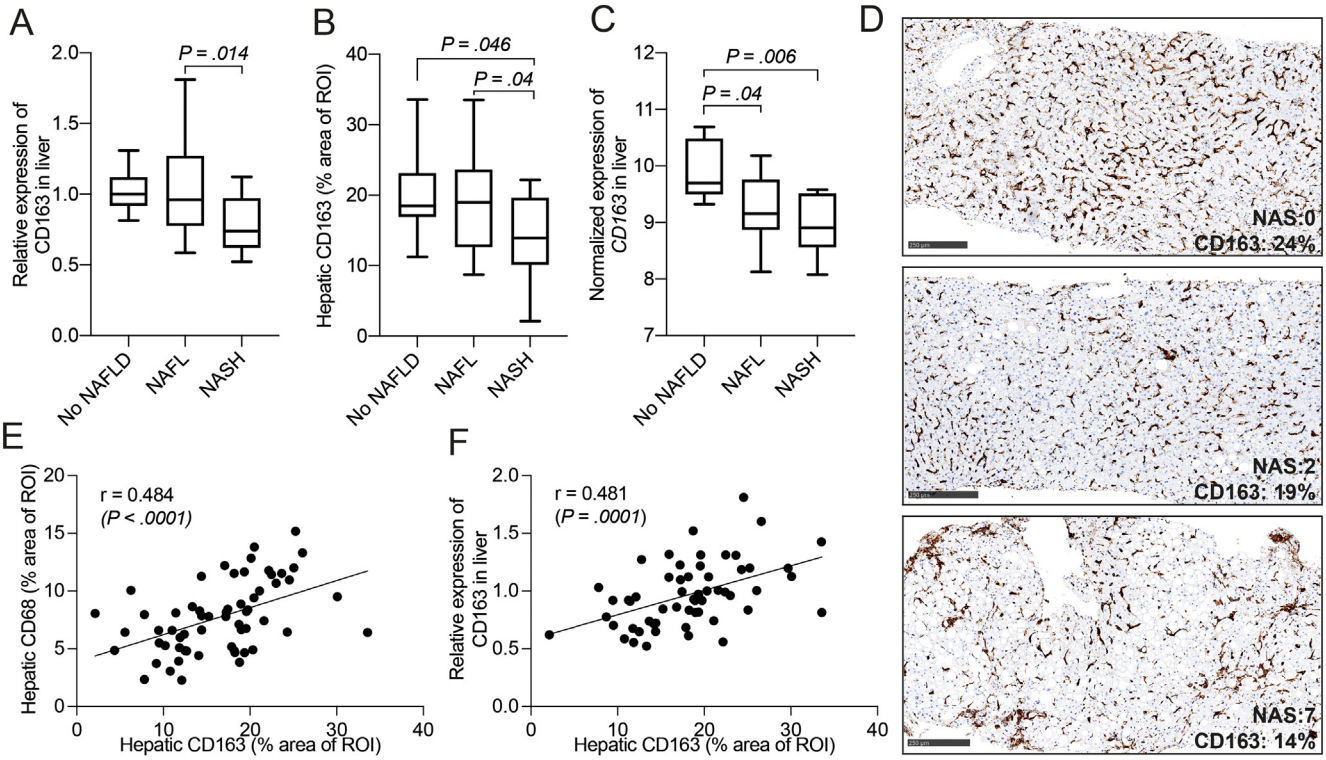


**Figure 3.** Hepatic expression of CD163 decreases with increased NAFLD severity. (A) Fold change of CD163 measured in liver biopsies using proteomics (No NAFLD n = 12, NAFL n = 31, and NASH n = 15). (B) Relative positively stained area of CD163 in liver biopsies using IHC quantified by QuPath (No NAFLD n = 12, NAFL n = 33, and NASH n = 16). (C) Normalized expression of *CD163* at mRNA level in liver biopsies using bulk RNA sequencing from a publicly available library[58] (GSE207310, No NAFLD n = 5, NAFL n = 15, and NASH n = 10). (D) Representative IHC staining of CD163 in liver specimens obtained by whole slide scanner NanoZoomer 2.0HT. NASH activity score (NAS) was determined[50] by an expert pathologist and the positive area of CD163 staining in the total region of interest was quantified using QuPath. Scale bars represent 250$\mu$m. (E) Correlation between hepatic expression of CD68 and CD163 determined by IHC. (F) Correlation between CD163 as per IHC compared to proteomics.

downregulated at the transcriptional level (Figure 3C). CD163-positive macrophages were evenly distributed in the sinusoids in healthy livers. However, in diseased livers from patients with NASH, the number of CD163-positive macrophages were strongly reduced in the sinusoids in areas with steatosis, and they were present in microgranulomas and hepatic crown-like structures (Figure 3D). Furthermore, CD163-positive macrophages accumulated in areas with interface hepatitis. The relative positively steained area of CD163 was quantified and it correlated positively with the macrophage pan marker CD68 (r = 0.48, $P < .0001$) and CD163 determined by proteomics (r = 0.48, $P = .0001$) in the liver specimens (Figure 3E and F).

The negative correlation between CD163 and NAFLD severity was supported by a OR of 0.91 [0.84;0.98] ($P = .015$) in univariate ordinal regression (Figure 2). Similarly, a lower hepatic expression of CD163 measured by IHC was significantly associated with higher NAS (OR = 0.88 [0.81;0.96], $P = .005$), steatosis (OR = 0.91 [0.85;0.98], $P = .012$), hepatocellular ballooning (OR = 0.86 [0.77;0.97], $P = .012$), and fibrosis (OR = 0.92 [0.86;0.98], $P = .013$). Hepatic CD163 expression determined by bulk proteomic analysis was also associated with NAFLD diagnosis (OR = 0.13 [0.02;0.80], $P = .028$) and its histological features (except steatosis) in a univariate logistic regression analysis (*Model 1* in Figure 2). Lobular inflammation was only significantly associated with CD163 expression determined by proteomics (OR = 0.09 [0.01;0.59], $P = .012$) and not by IHC (OR = 0.93 [0.86;1.00], $P = .060$).

The only demographic variable (among sex, age, BMI, and waist-hip ratio) that influenced the hepatic expression of CD163 was sex, where a lower expression was observed in females ($P = .040$ for IHC and $P = .003$ for proteomics, see Figure A1). Adjusting for sex in the ordinal logistic regression lowered the OR for hepatic CD163 determined by IHC and proteomics to 0.89 [0.82;0.97] ($P = .009$) and 0.07 [0.01;0.53] ($P = .010$), respectively (*Model 2* in Figure 2). In the advanced 'Model 3' adjusting for NAFLD risk factors, fibrosis was the only histologic parameter significantly negatively associated with hepatic CD163 expression as determined by proteomics (OR = 0.01 [0.0003;0.21] ($P = .004$)) (*Model 3* in Figure 2). In more advanced regression models, both sCD163 and hepatic CD163 expression failed to associate with NAFLD.

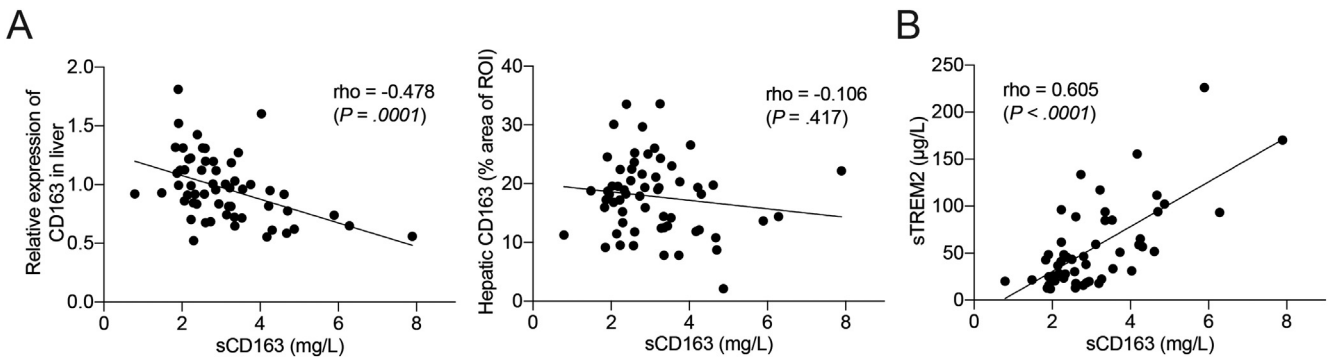


**Figure 4.** (A) Correlation between hepatic expressed CD163 and sCD163 levels in plasma in patients with obesity. (B) Correlation between ADAM17-shed soluble TREM2 (sTREM2) and sCD163 levels in plasma (No NAFLD n = 11, NAFL n = 26, and NASH n = 15). Correlations were assessed by Spearman's rank correlation.

### *Shedding of hepatic CD163 to the circulation by ADAM17*

Soluble CD163 correlates negatively with hepatic CD163 expression as determined by proteomics (rho = −0.478, $P = .0001$), but does not significantly associate with hepatic expressed CD163 as determined by IHC (rho = −0.106, $P = .417$) (Figure 4A). Protein sequences of ADAM17, which cleaves off the CD163 ectodomain, were only found in 11 out of the 58 samples analyzed by proteomics. Instead, as a surrogate marker, we measured sTREM2, which is also highly expressed in NASH at the transcriptional level[5,44] and shed by ADAM17 to release its soluble ectodomain to the circulation.[63] We found that sTREM2 was increased from 18.4 ng/ml in patients with no NAFLD to 85.1 ng/ml in patients with NASH and that it strongly and positively correlates with sCD163 levels in circulation (rho = 0.605, $P < .0001$) (Figure 4B).

## Discussion

In this cross-sectional study with access to plasma and liver biopsies in a well-defined cohort covering the full spectrum of NAFLD and liver healthy patients with obesity, we have thoroughly investigated the plasma level of sCD163 and the hepatic expression of CD163.

In accordance with previous studies,[4,23,24] we found sCD163 as a potential noninvasive biomarker of NASH with an AUROC of 0.78 [0.65;0.92]. The sCD163 levels were validated by mass spectroscopy with a high level of correlation. Soluble CD163 remained associated with NAFLD diagnosis, steatosis grade, and lobular inflammation grade as well as fibrosis stage when adjusting for NAFLD risk factors (sex, age, BMI, HOMA-IR, triglycerides, and hypertension). This cohort was limited to patients with obesity, however, sCD163 has also been considered as a potential biomarker in pediatric NAFLD[39,64] and nondiabetic nonobese (BMI<35) patients,[21] which suggests sCD163 as a potential biomarker of NASH. Presumably, it cannot be used as a single diagnostic biomarker as it is also elevated in several other inflammatory diseases,[13,19] including various hepatic inflammatory conditions.[27,29] Instead, it might be a valuable component in a panel of biomarkers for the clinical assessment to identify patients with NASH.

The increased level of sCD163 in circulation could indicate an increased expression of CD163 and/or enhanced shedding of CD163 by ADAM17. However, the hepatic expression of CD163 is decreased in more severe disease stages both at the transcriptional and protein level. Furthermore, hepatic expression of CD163 is negatively associated with the pathohistological features of NASH (steatosis, hepatocellular ballooning, and fibrosis) in simple ordinal logistic regressions and it remains negatively associated with fibrosis stage after adjusting for NAFLD risk factors. These findings comparing obese patients with and without NAFLD correspond well with very recent data on more severe NASH in patients with cirrhosis (F4).[65] Using multiplex immunofluorescence, they show a reduced number of $CD163^{+}$ macrophages in the parenchyma and a tendency of reduced mean intensity for CD163 staining indicating lower expression on macrophages. The inverse relation between hepatic CD163 and disease severity might be explained by the depletion of CD163-positive tissue-resident Kupffer cells and their replacement by short-lived monocyte-derived macrophages during progression, as recently described in NAFLD animal models.[43,44,65,66]

Sex was the only demographic variable that influenced hepatic CD163 expression which was significantly lower in women. However, hepatic expression of CD163 remained significantly negatively associated with disease stage after adjusting for sex. Future studies in larger cohorts with more men are needed to explore the sex difference observed in hepatic CD163 expression. In this study, fewer men (33%) than women were included in the cohort, although the prevalence of NAFLD is higher in men.[67] The explanation is probably that more women undergo bariatric surgery.[68]

The liver has previously been proposed as the main source of sCD163, as its levels were increased in the systemic vs portal blood,[22] and because it positively correlates with aggregates of CD163-expressing hepatic macrophages in NAFLD.[21–23] Aggregates of CD163-positive macrophages were also present in the stained liver biopsies in this study,

but the relative CD163 expression using IHC was lower in patients with NASH compared to NAFL and no NAFLD. In fact, the relative expression of hepatic expressed CD163 correlates negatively with the sCD163 concentration. Accordingly, transcription analysis showed accordingly that CD163 expression is downregulated during NASH and NAFL as compared to no NAFLD. Other sources of sCD163 might also contribute such as adipose tissue macrophages.[69,70]

If the liver, as suggested, is the main source of sCD163 during NAFLD although CD163 expression is downregulated, it might be owed to an increased ADAM17-mediated cleavage of CD163. Rosso et al[21] have shown such an association between hepatic mRNA expression of *ADAM17* and sCD163 in the circulation of patients with NAFLD, however, no such association was found in the online available transcriptomic data set used in this study. It is not possible to assess the ADAM17 activity in vivo, instead increased ADAM17 activity was indicated by increased plasma levels of sTREM2 that also derives from ADAM17 cleavage.[63] This finding is consistent with previous studies showing elevated sTREM2 levels in patients with NASH.[71,72] Tumor necrosis factor alpha, the most well-known product of ADAM17-mediated cleavage, has also been reported to be increased in NASH patients.[73] Finally, in these speculations on ADAM17 cleavage vs hepatic CD163 expression concerning the increased sCD163 in plasma, it should be considered that most NAFLD patients have an enlarged liver that, in terms of the absolute expression of CD163 in the liver, to some extent might 'compensate' for a lower CD163 expression measured per area.

## Conclusion

In conclusion, sCD163 in plasma is increased in NASH and the concentration is negatively correlated to the CD163 expression in the liver. An increased hepatic inflammatory activity leading to increased ADAM17 activity and shedding of hepatic CD163 seems to be a likely explanation for this observation, although extrahepatic contributions cannot be excluded. sCD163 differentiate between NASH and no NASH with an AUROC of 0.78 in patients with obesity. Further studies are needed to validate sCD163 use in a biomarker panel to identify NASH patients.

## Supplementary materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2023.03.006.

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**Conflicts of Interest:**
The authors disclose no conflicts.

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**Ethical Statement:**
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

**Data Transparency Statement:**
Study material will not be available due to legislation. However, transcriptomic data are publicly available at NCBI GEO (GSE207310) and MS-based proteomics will be publicly available at the ProteomeXchange Consortium, but is not ready yet.

**Reporting Guidelines:**
Helsinki Declaration, SAGER.