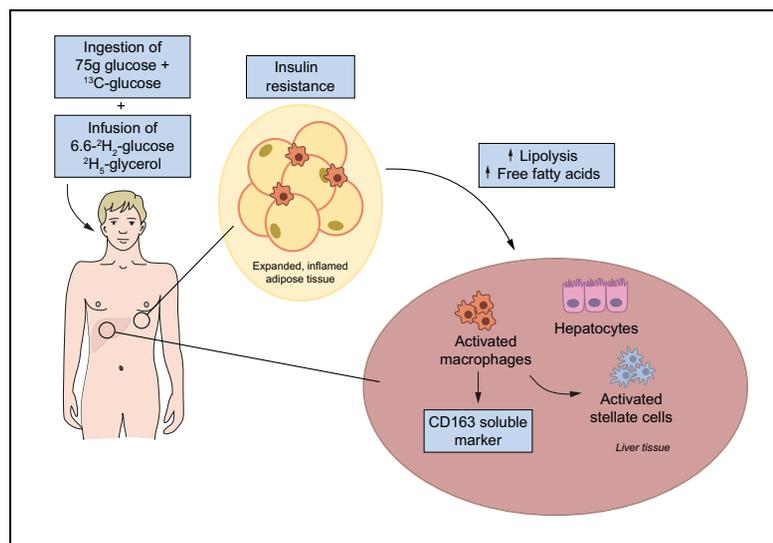


# Crosstalk between adipose tissue insulin resistance and liver macrophages in non-alcoholic fatty liver disease

## Graphical abstract



## Highlights

- Insulin resistance (IR) plays a pivotal role in the onset and progression of NAFLD.
- Adipose tissue IR seems to be the main metabolic determinant of the presence and degree of liver fibrosis in NAFLD.
- sCD163 is a specific macrophage activation marker that increases according to the degree of hepatic fibrosis in NAFLD.
- This study suggests a link between activation of resident macrophages in the liver and alterations in adipose tissue.

## Authors

Chiara Rosso, Konstantin Kazankov, Ramy Younes, ..., Jacob George, Henning Grønbaek, Elisabetta Bugianesi

## Correspondence

henngroe@rm.dk (H. Grønbaek)  
elisabetta.bugianesi@unito.it  
(E. Bugianesi)

## Lay summary

The pathogenesis of non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) is likely due to the interaction between a deranged metabolic milieu and local mediators of hepatic inflammation and fibrosis in the insulin resistant state. This study provides *in vivo* support for a possible link between deranged metabolism in the adipose tissue and activation of hepatic macrophages in patients with NAFLD, most likely in response to free fatty acid overflow and independent of obesity and diabetes.



## Crosstalk between adipose tissue insulin resistance and liver macrophages in non-alcoholic fatty liver disease

Chiara Rosso<sup>1</sup>, Konstantin Kazankov<sup>2</sup>, Ramy Younes<sup>1</sup>, Saeed Esmaili<sup>3</sup>, Milena Marietti<sup>1</sup>, Marco Sacco<sup>1</sup>, Fabrizia Carli<sup>4</sup>, Melania Gaggini<sup>4</sup>, Federico Salomone<sup>6</sup>, Holger Jon Møller<sup>5</sup>, Maria Lorena Abate<sup>1</sup>, Hendrik Vilstrup<sup>2</sup>, Amalia Gastaldelli<sup>4</sup>, Jacob George<sup>3</sup>, Henning Grønbaek<sup>2,\*</sup>, Elisabetta Bugianesi<sup>1,\*</sup>

<sup>1</sup>Division of Gastroenterology and Hepatology, Department of Medical Sciences, University of Turin, Turin, Italy; <sup>2</sup>Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark; <sup>3</sup>Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and the University of Sydney, Sydney, NSW, Australia; <sup>4</sup>Cardiometabolic Risk Unit, Institute of Clinical Physiology, CNR, Pisa, Italy; <sup>5</sup>Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark; <sup>6</sup>Division of Gastroenterology, Ospedale di Acireale, Azienda Sanitaria Provinciale di Catania, Catania, Italy

**Background & Aims:** The pathogenesis of non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) is likely due to the interaction between a deranged metabolic milieu and local mediators of hepatic inflammation and fibrosis. We undertook this study to elucidate the interplay between macrophage activation, insulin resistance (IR) in target organs/tissues and hepatic damage.

**Methods:** In 40 non-diabetic patients with biopsy-proven NAFLD we assessed: i) endogenous glucose production (EGP), glucose clearance and indexes of IR in the adipose tissue (Adipo-IR and Lipo-IR) and in the liver (Hep-IR) by tracer infusion ([6,6-2H<sub>2</sub>]glucose and [2H<sub>5</sub>]glycerol); ii) macrophage activity (by soluble sCD163) and iii) hepatic expression of CD163 (hCD163).

**Results:** We found that sCD163 levels paralleled both the plasma free fatty acid (FFA) levels and lipolysis from adipose tissue. Consistently, sCD163 significantly correlated with adipose tissue IR (Adipo-IR:  $r = 0.32$ ,  $p = 0.042$ ; Lipo-IR:  $r = 0.39$ ,  $p = 0.012$ ). At multiple regression analysis, sCD163 levels were associated with FFA levels ( $r_p = 0.35$ ,  $p = 0.026$ ). *In vitro* exposure of human monocyte-derived macrophages to palmitate enhanced sCD163 secretion. Conversely, sCD163 did not correlate with EGP or with Hep-IR. In the liver, hCD163 positively correlated with sCD163 ( $r = 0.58$ ,  $p = 0.007$ ) and the degree of steatosis ( $r = 0.34$ ,  $p = 0.048$ ), but not with EGP or Hep-IR ( $r = -0.27$  and  $r = 0.11$ , respectively,  $p > 0.10$ , both).

**Conclusions:** Our findings suggest a link between deranged metabolism in the adipose tissue and activation of hepatic macrophages in patients with NAFLD, possibly in response to FFA overflow and independent of obesity and diabetes.

Conversely, our findings do not support a link between activated hepatic macrophages and glucose metabolism (EGP or Hep-IR). The relationship between adipose tissue IR and hepatic macrophages should be considered to define therapeutic targets for NAFLD.

**Lay summary:** The pathogenesis of non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) is likely due to the interaction between a deranged metabolic milieu and local mediators of hepatic inflammation and fibrosis in the insulin resistant state. This study provides *in vivo* support for a possible link between deranged metabolism in the adipose tissue and activation of hepatic macrophages in patients with NAFLD, most likely in response to free fatty acid overflow and independent of obesity and diabetes.

© 2019 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

### Introduction

The recent epidemic of chronic liver disease is related to the burden of non-alcoholic fatty liver disease (NAFLD), paralleling the worldwide increase of obesity.<sup>1</sup> NAFLD is a complex condition related to metabolic derangements in insulin resistance (IR), but in a subset of patients the liver becomes the target of multiple hits leading to non-alcoholic steatohepatitis (NASH), the histological phenotype that may progressively lead to the development of liver fibrosis, cirrhosis and possibly hepatocellular carcinoma.<sup>1,2</sup> Understanding the biological and environmental factors that drive the progression to NASH and beyond in some individuals is fundamental to the development of robust methods for diagnosis, risk stratification and therapy.<sup>2</sup>

The prevailing notion of NASH pathogenesis is that a deranged metabolic milieu specifically interacts with local mediators of hepatic inflammation and fibrosis, but the nature of these interactions has not been fully elucidated.<sup>3,4</sup> It is generally believed that adipose tissue IR plays a pivotal role in the onset and progression of NAFLD.<sup>5,6</sup> Briefly, weight gain leads to expansion of adipose tissue and recruitment of macrophages through the secretion of various chemo- and cytokines.<sup>7</sup> Inflamed and dysfunctional adipose tissue actively releases free

Keywords: Steatohepatitis; Macrophage; Insulin resistance; Adipose tissue; Fibrosis. Received 12 September 2018; received in revised form 29 May 2019; accepted 27 June 2019; available online 10 July 2019

\* Corresponding authors. Address: Division of Gastroenterology and Hepatology, Department of Medical Sciences, University of Turin, Turin, Italy. Tel.: +39 011 6333541, fax: +39 011 6333976, or Department of Hepatology & Gastroenterology, Aarhus University Hospital, DK-8000 Aarhus C, Denmark. Tel.: +45 2167 9281, fax: +45 78462820.

E-mail addresses: [henngroe@rm.dk](mailto:henngroe@rm.dk) (H. Grønbaek), [elisabetta.bugianesi@unito.it](mailto:elisabetta.bugianesi@unito.it) (E. Bugianesi).

† Prof. Elisabetta Bugianesi and Prof. Henning Grønbaek share co-last authorship.



fatty acids (FFAs) into the bloodstream, promotes lipotoxicity in the liver, muscle and pancreas,<sup>3</sup> and contributes to systemic inflammation. In the normal liver, resident macrophages or Kupffer cells (KCs) play important regulatory roles through crosstalk with the different cell types and particularly with hepatocytes.<sup>8</sup> The pro-inflammatory polarization of hepatic macrophages is considered a hallmark of progressive disease, in the livers of patients with NASH, and an attractive therapeutic target as recently reviewed.<sup>8</sup> Hepatic lipid accumulation facilitates pro-inflammatory KC polarization, possibly as a consequence of FFA excess, or signals from surrounding steatotic hepatocytes, such as histidine-rich glycoprotein, extracellular vesicles or damage-associated molecular patterns.<sup>8</sup> More recently, data derived from animal models and *in vitro* studies suggest that both pro-inflammatory KCs and recruited hepatic macrophages (Ly6C<sup>hi</sup>) contribute to decreased hepatic insulin sensitivity by inhibiting insulin signaling and activating hepatic glucose production.<sup>9</sup> However, most data available have been derived from mouse models, which are not fully representative of human NASH, since they reflect certain aspects of the pathogenesis and rarely incorporate the full spectrum of etiology-specific mechanisms.<sup>8</sup>

Soluble CD163 (sCD163) is the ecto-domain of the hemoglobin-haptoglobin scavenger receptor which is exclusively expressed on macrophages and monocytes. It is shed to the circulation during macrophage activation by metalloprotease activity (e.g. tumor necrosis factor- $\alpha$  converting enzyme (TACE/ADAM17)).<sup>10,11</sup> CD163-positive macrophages are highly expressed in human adipose tissue from obese individuals, while sCD163 levels are associated with hepatic inflammation and fibrosis in patients with NAFLD<sup>12,13</sup> and decrease after successful life-style intervention and bariatric surgery.<sup>14–16</sup>

Against this background, we carried out this study to further elucidate the complex interplay between IR in target organs/tissues, macrophage activation and hepatic damage in a well-characterized cohort of non-diabetic patients with biopsy-proven NAFLD.

## Material and methods

### Study participants

The study was performed in 40 patients with NAFLD, selected from consecutive patients who had a liver biopsy for the evaluation of suspected NAFLD between June 2010 and June 2013 in the Liver Unit of the University of Torino, according to the absence of type 2 diabetes mellitus (T2DM) and morbid obesity (body mass index [BMI] >35); the absence of the 2 latter conditions in our patients unveils the independent contribution of NAFLD to metabolic and inflammatory alterations. Other etiologies of liver disease, including viral, autoimmune, cholestatic, genetic, metabolic, alcoholic and drug-induced were excluded. Past and current ethanol intake <20 g/day had been confirmed through direct questioning of patients and a close relative. A complete medical history and physical examination was undertaken and anthropometric data collected at the time of liver biopsy. BMI was calculated on the basis of weight (in kilograms) and height (in meters), waist circumference (to the nearest half-centimetre) was measured at the midpoint between the lower border of the rib cage and the iliac crest. Central obesity was defined according to the International Diabetes Federation criteria (>94 cm men, >80 cm women).

### Histological features

All liver biopsies were centrally read at the University of Torino soon after the procedure had been performed. Liver specimens were stained with haematoxylin and eosin, Masson's trichrome, and special stains for iron and copper and examined by a local expert liver pathologist blinded to patient clinical information. The average length of liver tissue was 25 mm (range 14–45 mm), with a minimum of 11 portal tracts. Histological features of NAFLD, *i.e.* steatosis, inflammation, hepatocyte ballooning, and fibrosis were assessed and scored as described by Kleiner *et al.*<sup>17</sup> Diagnosis of NASH was established according to the joint presence of steatosis, hepatocyte ballooning, and lobular inflammation with or without fibrosis.

### Analytical determinations

Serum samples for laboratory investigations were collected at the time of liver biopsy and stored at  $-80^{\circ}\text{C}$ . Biochemical exams included the complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, alkaline phosphatase and gamma-glutamyl-transferase. Serum glucose was measured by the glucose oxidase method (Sentinel, Milan, Italy). FFA concentrations were determined by enzymatic colorimetric assays (WAKO diagnostic, Richmond, VA). Triglycerides (TG), total cholesterol (Chol) and high-density lipoprotein cholesterol (HDL-Chol) levels were assessed by enzymatic colorimetric assays (Sentinel), the latter after precipitation of low-density lipoprotein (LDL) and very low-density lipoprotein. LDLs were measured with a standardized homogeneous enzymatic colorimetric method in order to avoid the effects of triglycerides (Sentinel, Milan, Italy).

### Genotyping

Genomic DNA was isolated from peripheral blood using the EZ1 DNA Blood 350  $\mu\text{l}$  kit (Qiagen) according to the manufacturer's instructions. Genotyping for *PNPLA3* was performed with the TaqMan Single Nucleotide Polymorphism allelic discrimination assay (Applied Biosystems, CA).

### Study ethics

The study, designed in accordance with the Helsinki Declaration, was approved by the ethics committee of the University Hospital "Città della Salute e della Scienza" of Torino. All patients gave signed consent for collection of personal data in the database, use of blood samples and liver tissue for research and for participation in the tracer study.

### "In vivo" tracer protocol

All tracer studies were performed within 1 month of liver biopsy, in the absence of significant weight loss (>5%) or medications for NASH. *In vivo* hepatic (Hep)-IR and AT-IR were assessed by the stable isotope technique in the fasting state and during a 3-hour oral glucose tolerance test (OGTT), after an overnight fast. A primed-continuous infusion of 6,6-D<sub>2</sub>-glucose (bolus 22  $\mu\text{mol}/\text{kg}$ , infusion rate 0.22  $\mu\text{mol}/\text{kg}/\text{min}$ ) and of U-D<sub>5</sub>-glycerol (bolus 1.5  $\mu\text{mol}/\text{kg}$ , infusion rate 0.1  $\mu\text{mol}/\text{kg}/\text{min}$ ) was administered for 2 h in fasting conditions (equilibration period) to assess endogenous glucose production (EGP) and lipolysis through the rate of appearance (Ra) of Glycerol, as previously described.<sup>18</sup> After reaching isotopic steady state for 6,6-D<sub>2</sub>-glucose and U-D<sub>5</sub>-glycerol (after 2 h infusion), the patients drank 75 g of glucose with 1.5 g of U-13C-glucose (double tracer-OGTT) while continuing stable isotope infusion

at a constant rate. Plasma samples were collected every 30 min for 3 h.

Tracer enrichment of 6,6-D<sub>2</sub>-glucose and U-D<sub>5</sub>-glycerol was determined by gas chromatography-mass spectrometry system (5975; Agilent, Palo Alto, CA), using electron impact ionization and selectively ion monitoring, and integrating peaks of mass to charge ratios of 200, 201, 202, and 205 for glucose and 145, 148 for glycerol. Tracer enrichments were calculated as tracer/trace ratios (TTR) from peak area-ratios (R) at each time point (t) corrected for baseline (bk, pre-infusion) as previously described;<sup>18,19</sup> briefly:

$$\text{TTR}_{6,6\text{-D}_2\text{-glucose}}(t) = \frac{R_{202/200}(t) - R_{202/200}(\text{bk})}{R_{201/200}(t) - R_{201/200}(\text{bk})}$$

$$\text{TTR}_{\text{U-D}_5\text{-glycerol}}(t) = \frac{R_{148/145}(t) - R_{148/145}(\text{bk})}{R_{145/145}(t) - R_{145/145}(\text{bk})}$$

Glucose fluxes and glycerol Ra were calculated as infusion rate/TTR during fasting and using non-steady state mathematical models during the OGTT, as previously reported.<sup>18,19</sup> Glucose clearance represents the amount of glucose taken up and metabolized by the muscle in the unit of time, which is a measure of insulin sensitivity in the muscle. Because the insulin concentration is a strong inhibitory stimulus for lipolysis,<sup>20,21</sup> adipose tissue IR can be calculated as FFA × fasting insulin (Adipo-IR) or as glycerol Ra × fasting insulin (Lipo-IR). Specifically, Adipo-IR takes into consideration the FFAs that are released into the circulation (*i.e.* not re-esterified intracellularly after TG hydrolysis) while Lipo-IR is calculated using glycerol Ra as index lipolysis since it measures the complete hydrolysis of TG. Insulin is also a strong inhibitory stimulus for endogenous glucose production<sup>20</sup> thus hepatic-IR is calculated as EGP × fasting insulin.<sup>18,19</sup>

### Fat quantification in specific sites

Liver fat was assessed by liver biopsy as percentage of steatosis in hepatocytes and by standard magnetic resonance imaging (MRI). The MRI estimates of liver fat content highly correlated with hepatic fat at biopsy ( $r = 0.80$ ,  $p < 0.0001$ ). Since we do not have MRI estimates of inflammation and fibrosis, all analysis was carried out based on histological features to avoid a potential source of bias. Visceral fat (VF) and subcutaneous fat (SCF) were measured by MRI with a specific volumetric reconstruction software in all individuals.<sup>5</sup>

### Circulating sCD163 measurement

Plasma concentrations of sCD163 were determined in duplicate by an in-house sandwich enzyme-linked immunosorbent assay (ELISA) using a BEP-2000 ELISA-analyzer (Dade Behring), essentially as previously described.<sup>22</sup> The sCD163 measurements were part of a combined biobank study investigating the association between sCD163 and liver morphology in patients with NAFLD.<sup>13</sup> We previously established a reference range (0.69–3.86 mg/L) for sCD163 in a large cohort ( $n = 240$ ) of healthy individuals using the same assay.<sup>23</sup>

### Hepatic expression of CD163

A total of 20 liver biopsies were available for RNA extraction. Biopsies had been collected in RNAlater (Ambion Inc., Austin, TX) and stored at  $-80^\circ\text{C}$  until processing. Total RNA extraction was performed using TRIzol reagent (Invitrogen, CA). For each sample, 1  $\mu\text{g}$  of total RNA was treated with deoxyribonuclease I (Invitrogen, CA) prior to reverse transcription, in turn performed with random primers and the SuperScript<sup>®</sup> II Reverse Transcriptase (Invitrogen, CA). As housekeeping genes,

succinate dehydrogenase subunit A (SDHA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used. Primers for the target genes *CD163* and *ADAM-17* were derived from the literature.<sup>24</sup> The control sample used as calibrator was a cDNA derived from an individual without features of NASH. Quantitative PCR (qPCR) was performed using the SsoAdvanced<sup>™</sup> Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the instrument protocol:  $98^\circ\text{C}$  for 30 s for polymerase activation,  $95^\circ\text{C}$  for 15 s for cDNA denaturation followed by 39 cycles of  $60^\circ\text{C}$  for 30 s for annealing and extension. All samples and controls were analyzed in duplicate. The relative expression of *CD163* and *ADAM-17* was calculated as  $2^{-\Delta\text{Ct}}$  and expressed as fold increase over the calibrator sample. Negative controls (no template cDNA) were run in every experimental plate to exclude potential contamination. Specificity of the amplified PCR products was determined by melting curve analysis. Gene expression analysis was performed by CFX Manager<sup>™</sup> Software (Bio-Rad Laboratories, CA).

### Cell culture

Human CD14 monocyte-derived macrophages were matured in M-CSF (50 ng/ml) media supplemented with 10% AB serum for 7 days. On day 7, cells were washed twice with serum free RPMI and replaced with RPMI containing 1% FBS and treated with either 500  $\mu\text{M}$  or 750  $\mu\text{M}$  palmitate (made fresh on the day) for 24 h. Supernatant was collected and centrifuged for 2,000 rpm for 5 min to remove cells or cell debris. Supernatant was then assayed using a human CD163 ELISA (R&D systems Cat no: DC1630).

### Immunohistochemistry

Immunohistochemical analyses were performed in all biopsies using a mouse monoclonal anti-human CD163 antibody (Cell Marque, Rocklin, CA, USA; MRQ-26). Cell clustering in liver tissues was quantified as previously reported.<sup>13</sup>

### Statistical analysis

Data are reported as mean  $\pm$  SD for continuous normally distributed variables, as median and interquartile range for continuous non-normally distributed variables and number and frequency (%) for categorical variables. Comparison between more than 2 groups was performed with Kruskal-Wallis test for continuous not normally distributed variables and by ANOVA for continuous normally distributed variables. Similarly, comparisons between 2 groups were performed by Mann-Whitney *U* test for not normally distributed variables and by *t* test for normally distributed variables. For categorical data, the Fisher's exact test or Chi-square test were used as appropriate. Spearman or Pearson correlation were used as appropriate to evaluate the correlation between sCD163 levels with anthropometric, metabolic and histological features. Multivariable regression analysis was performed to assess the association between sCD163 levels and glucose and lipid fluxes from tracer studies ( $r_p$ ). Values of  $p < 0.05$  were considered statistically significant. All calculations were performed with MedCalc Software bvba version 12 (Mariakerke, Belgium).

## Results

### Characteristics of the study patients

The anthropometric, biochemical, and histological characteristics of the study participants ( $n = 40$ ) are reported in [Table 1](#).

**Table 1. Anthropometric, biochemical, genetic and histological characteristics of the study participants.**

Variables	Study participants (n = 40)
Age, years	41.9 ± 11.2
M/F, n	31/9
BMI, kg/m <sup>2</sup>	26.9 ± 4.4
Normal weight, n (%)	12 (30)
Overweight, n (%)	14 (35)
Obese, n (%)	14 (35)
Waist circumference, cm	93.5 ± 10.9
AST, IU/ml (range)	33 (18–77)
ALT, IU/ml (range)	62 (26–154)
GGT, IU/ml (range)	54 (18–317)
Platelets, ×10 <sup>9</sup> /L (range)	216 (111–319)
INR	0.98 ± 0.05
Total bilirubin, mg/dl	0.76 ± 0.29
Albumin, g/L	4.55 ± 0.39
Creatinine, mg/dl	0.86 ± 0.14
sCD163, mg/L (range)	1.62 (1.07–4.42)
PNPLA3, n (%)	
CC	8 (20)
CG	23 (57.5)
GG	9 (22.5)
<b>Histological features</b>	
Steatosis, % (range)	25 (5–85)
NAS, n (%)	
1–2	6 (15)
3–4	21 (52.5)
5–6	13 (32.5)
Ballooning, n (%)	
0	5 (12.5)
1	16 (40)
2	19 (47.5)
Lobular Inflammation, n (%)	
0	10 (25)
1	30 (75)
Fibrosis Score, n (%)	
F0/F1	18 (45)
F2	11 (27.5)
F3/F4	11 (27.5)
NASH, n (%)	27 (67)

Data are reported as mean ± SD, median (range) and frequencies (%). ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein; HOMA, homeostasis model of assessment; IR, insulin resistance; M, male; NAS, non-alcoholic fatty liver disease activity score; NASH, non-alcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain-containing protein 3; sCD163, soluble CD163.

Overall, only one-third of patients (35%) were obese according to BMI, but central obesity was found in more than half (60%) and it was the most common feature of the metabolic syndrome (MetS), followed by hypertension (38%), low HDL-Chol (35%), hyperglycemia (23%) and hypertriglyceridemia (10%). Nevertheless, only 32% of patients met the criteria for the diagnosis of MetS. As per selection criteria, no patient had T2DM but 35% of them had a family history of T2DM and 48% had IR defined as homeostatic model assessment (HOMA)-IR >2.7. The PNPLA3 GG genotype was found in 22.5% of patients, but the clinical and histological features did not differ according to genetic variants (Table S1). Among histological features, hepatic steatosis was mild (<33%) in 52% of the study participants, moderate (33–66%) in 25% and severe (≥66%) in 23%, respectively. A diagnosis of NASH was made in 67% of cases according to the joint presence of steatosis, lobular inflammation and ballooning. Less than half of the patients had no/negligible fibrosis, while moderate and severe fibrosis was evenly distributed in the remaining patients (Table 1).

### Metabolic variables and histological features of liver damage

Table 2 summarizes the metabolic variables, including glucose and lipid fluxes from tracer studies, in all individuals according to the degree of fibrosis. The plasma levels of glucose, triglycerides, total and HDL cholesterol were unchanged across the spectrum of liver damage, while insulin levels, FFA levels and HOMA-IR significantly increased in relation to the severity of fibrosis (Table 2). Adipose tissue IR, calculated based on both FFA levels (Adipo-IR) and the rate of lipolysis (Lipo-IR), rose stepwise by fibrosis staging (Table 2 and Fig. 1A and B), confirming its putative role in liver damage as previously described.<sup>21,25</sup> Adipose tissue IR (Adipo-IR and Lipo-IR) also showed a strong correlation with hepatic fat (Fig. 1C and D). Notably, the correlation with steatosis was stronger for Lipo-IR, which considers body weight (since lipolysis is expressed as μmol/min normalized by body weight) (Table 2), suggesting that this correlation was not mediated by BMI. In order to further explore the latter possibility, we confirmed the association of Lipo-IR and steatosis by linear regression in the subgroup of non-obese patients with NAFLD (n = 25), whereas no association was found between BMI and liver fat (p = 0.146). Adipose tissue IR was correlated with SCF (Adipo-IR vs. SCF r = 0.48, p = 0.008 and Lipo-IR vs. SCF r = 0.43, p = 0.018), but not with VF (p = 0.22 and 0.23, respectively) assessed by MRI (Table S2); this finding was not unexpected since VF can at most account for ~20% of the total pre-hepatic FFA load.<sup>5</sup> Overall, these results suggest that increased lipolysis from the adipose tissue (mainly subcutaneous) is linked to both fibrosis and fat accumulation in the liver of patients with NAFLD, independently of BMI and T2DM. Conversely, glucose fluxes from the liver (EGP) and into the muscle (Glucose clearance) were unrelated to the severity of hepatic fibrosis (Table 2). Hepatic-IR (Hep-IR) paralleled the increasing degree of liver fat (Fig. 1E), but not the severity of fibrosis (Fig. 1F). In individuals with NAFLD and a NAFLD activity score (NAS) ≥4, both Hep-IR and adipose tissue IR (Adipo-IR and Lipo-IR) were higher (Fig. S1A–C), mainly because of their strong relationship with liver fat. When the glucose and lipid substrates/fluxes were sub-divided according to the presence or absence of NASH, we did not find a significant difference in all the parameters assessed (Table S3).

### Macrophage activation and fat depots

Overall, sCD163 levels increased proportionally to BMI, waist circumference and hepatic fat (r = 0.40, p = 0.01; r = 0.38, p = 0.015 and r = 0.53, p = 0.006, respectively) (Fig. 2A–C). However, BMI was not associated with sCD163 levels, while the relationship with steatosis stayed significant both at univariate and multivariate analysis (Table S4). Accordingly, in the subgroup of non-obese NAFLD (n = 25), sCD163 levels strongly correlated with liver fat (p <0.0001) but not with BMI (p = 0.383), suggesting an independent role of steatosis and BMI in NAFLD. Since CD163-positive macrophages are also highly expressed in human adipose tissue from obese individuals, we evaluated the relationship between sCD163 and different measures of adiposity, including VF and subcutaneous SCF fat depots by standard MRI. However, we did not observe any correlation between sCD163 levels and either VF or SCF (r = 0.28, p = 0.12 and r = 0.19, p = 0.30, respectively). After linear multivariate regression analysis, hepatic steatosis at biopsy was significantly associated with sCD163 levels, independently of BMI, WC, VF and SCF (Table S4).

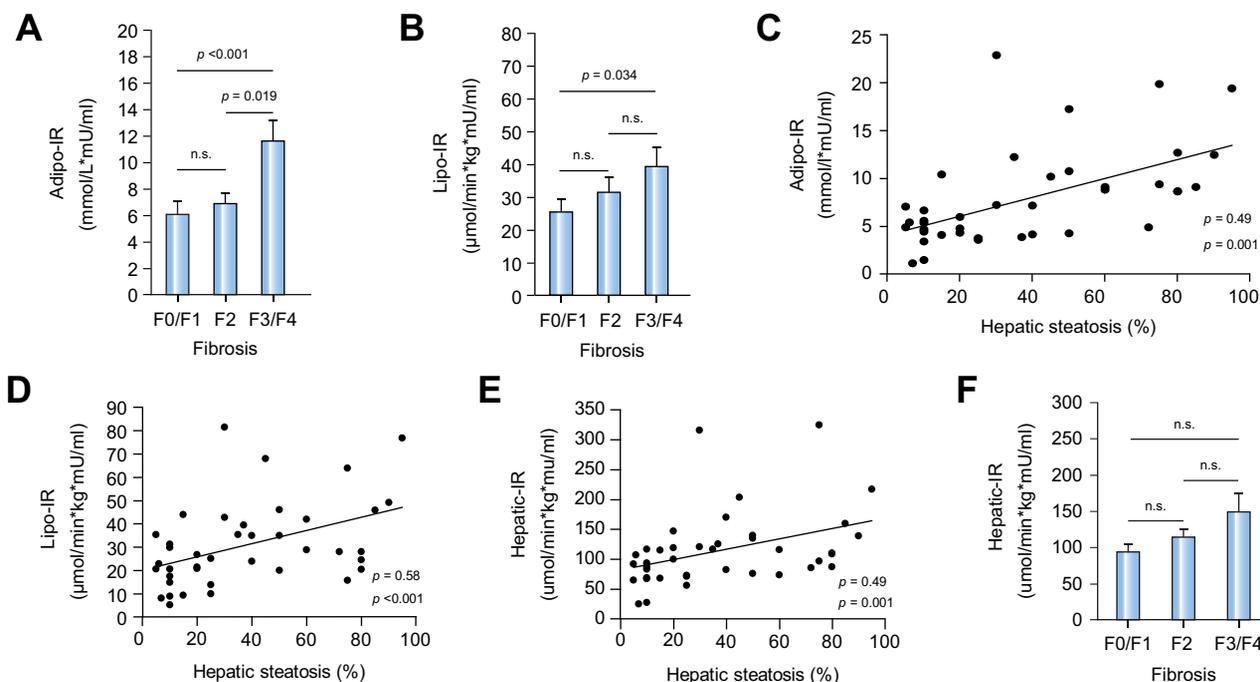
**Table 2. Metabolic features and direct measures of insulin resistance by tracer studies according to the degree of fibrosis.**

Variables	Study cohort (n = 40)	F0/F1 (n = 18)	F2 (n = 11)	F3/F4 (n = 11)	p <sup>#</sup> value
<b>Glucose metabolism</b>					
Fasting glucose, mg/dl	92 (89–99)	92 (87–99)	91 (89–98)	96 (90–120)	0.396
Fasting insulin, IU/ml	10.7 (9.2–14.2)	9.8 (8.2–10.5)	11.6 (10.1–14.4)	12.7 (10.9–14.6)	0.018
HOMA-IR	3.06 ± 1.86	2.4 ± 1.3	3.2 ± 1.6	4.1 ± 2.4	0.012
EGP (μmol/min/kg)	9.15 ± 1.28	9.3 ± 1.1	8.4 ± 1.5	9.3 ± 1.2	0.296
Glucose clearance (μmol/kg/min)	1.74 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	1.7 ± 0.4	0.392
Hep-IR (μmol/min/kg * IU/ml)	104 (75–130)	85 (68–119)	94 (84–144)	117 (109–135)	0.061
<b>Lipid metabolism</b>					
Total cholesterol (mg/dl)	191 (168–212)	193 (162–212)	188 (170–203)	199 (170–233)	0.770
HDL cholesterol (mg/dl)	43 (37–53)	47 (41–53)	40 (29–51)	41 (35–58)	0.322
Triglycerides (mg/dl)	86 (70–124)	78 (71–120)	89 (60–133)	96 (78–107)	0.872
FFAs (mmol/L)	0.616 ± 0.213	0.573 ± 0.188	0.527 ± 0.164	0.774 ± 0.224	0.035
Glycerol Ra (μmol * min <sup>-1</sup> )	2.43 ± 0.73	2.42 ± 0.69	2.29 ± 0.64	2.59 ± 0.91	0.747
Adipo-IR (mmol/L * IU/ml)	7.8 ± 5.0	6.1 ± 4.7	6.9 ± 2.9	11.6 ± 5.4	<0.001
Lipo-IR (μmol * min <sup>-1</sup> *IU/ml)	27 (20–41)	22 (14–35)	28 (20–42)	35 (25–47)	0.034

Data are reported as mean ± SD and median (interquartile range). Adipo-IR, adipose tissue insulin resistance by FFAs plasma levels (FFAs\* insulin); Lipo-IR, adipose tissue insulin resistance by tracers (glycerol Ra\* insulin); EGP, endogenous glucose production; FFAs, free fatty acids; Hep-IR, hepatic insulin resistance by tracers (EGP\* insulin); IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; Ra, rate of appearance.

A p value less than 0.05 was considered significant.

# Differences among the 3 groups were assessed by the Kruskal-Wallis test.



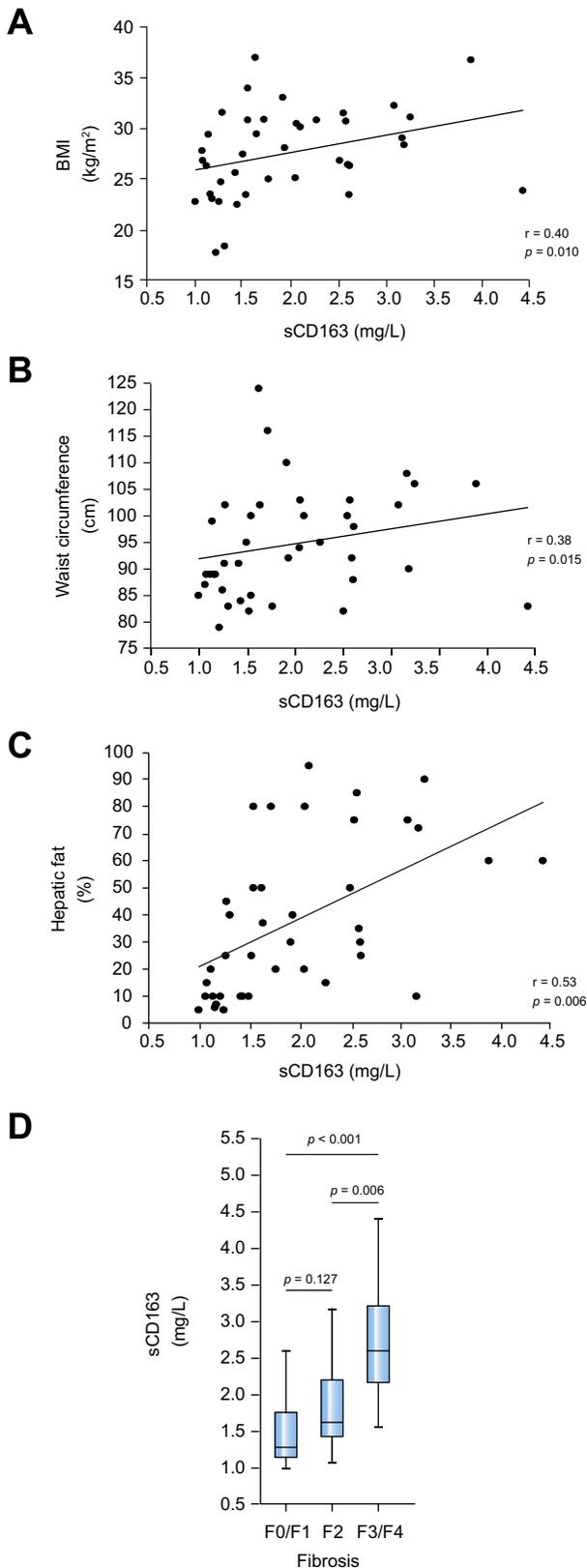
**Fig. 1. Adipose tissue- and hepatic-insulin resistance according to the degree of fibrosis and to the content of hepatic fat by liver histology.** Adipose tissue insulin resistance by (A) free fatty acids (Adipo-IR) and by (B) tracers (Lipo-IR), according to fibrosis staging. Correlation between adipose tissue insulin resistance (Adipo-IR and Lipo-IR) (C and D, respectively) and hepatic steatosis. (E) Correlation between hepatic insulin resistance and hepatic steatosis. (F) Hepatic insulin resistance according to fibrosis staging.

In order to further confirm that circulating sCD163 levels were predominantly related to macrophage activation in the liver, hepatic CD163 expression was investigated by immunohistochemistry (IHC CD163) and by qPCR (*hCD163*) in a subgroup of 20 individuals with NAFLD, where liver tissue had been collected and stored (clinical data summarized in Table S5). In the liver, we observed significant IHC CD163 staining, predominantly in patients with inflammation and fibrosis (Fig. 3A), while *hCD163* had a significant, direct correlation with the circulating levels of sCD163 ( $r = 0.58, p = 0.007$ ) (Fig. 3B). This result was strengthened by the association between hepatic expression of *ADAM-17* (*hADAM-17*), a specific metallopro-

tease involved in the cleavage and shedding of CD163, with both *hCD163* ( $r = 0.64, p = 0.003$ ) and *sCD163* ( $r = 0.47, p = 0.047$ ) (Fig. 3C and D, respectively). Although we cannot exclude the contribution of adipose tissue macrophages to circulating sCD163, taken together these results may suggest that in our patients with NAFLD, circulating sCD163 mainly represents hepatic macrophage activation.

**Macrophage activation and histological features of liver damage**

Among other histological features, serum levels of sCD163 increased according to fibrosis stage (ANOVA  $p < 0.001$ )



**Fig. 2. sCD163.** sCD163 and its association with (A) BMI, (B) waist circumference, (C) hepatic fat content and (D) liver fibrosis. BMI, body mass index; sCD163, soluble CD163.

(Fig. 2D) and were able to distinguish moderate (F = 2) from severe fibrosis (F3/F4). No association was observed between sCD163 levels and lobular inflammation or ballooning ( $p > 0.1$  for both), but in patients with a NAS score  $\geq 4$ , sCD163 levels were 1.3-fold higher than in NAS  $< 4$  ( $2.18 \pm 0.74$  vs.  $1.68 \pm 0.88$ ,  $p = 0.05$ ), as a consequence of the strong relationship with liver fat. Although there was a positive trend in the correlation between CD163 mRNA (hCD163) and the total CD163+ hepatic macrophage scored by IHC (IHC CD163), statistical significance was not reached, probably because of a lack of statistical power for categorical analysis (N = 20) (Fig. S3).

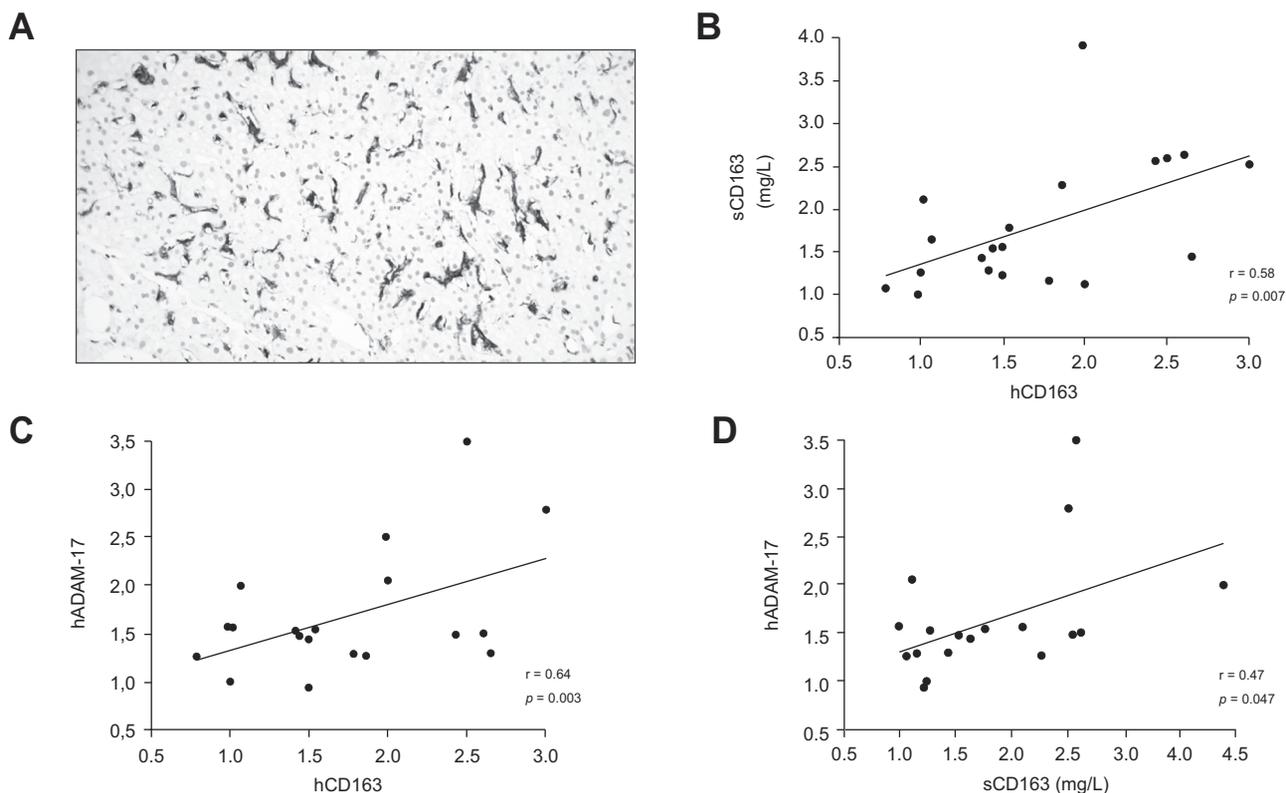
**Relationship between macrophage activation and insulin resistance at different sites**

Next, we sought to understand the relationship between macrophage activation and impaired insulin sensitivity in the target tissues (liver, adipose tissue and muscle). Circulating sCD163 levels were not directly related to insulin levels (Table 3), but they were significantly higher in individuals with NAFLD and HOMA-IR  $\geq 2.7$  ( $2.23 \pm 0.57$  vs.  $1.71 \pm 0.97$ ,  $p = 0.002$ ) and in those with MetS ( $2.29 \pm 0.69$  vs.  $1.8 \pm 0.75$ ,  $p = 0.024$ ) compared to their counterparts. Notably, the activation of macrophages, expressed by sCD163 levels, paralleled both the plasma FFA levels and the flux of glycerol, mainly representing lipolysis from adipose tissue (Table 3). Regression analysis confirmed the significant association between sCD163 levels and FFA concentration ( $r_p = 0.35$ ,  $p = 0.026$ ). In keeping with this finding, sCD163 was significantly associated with adipose tissue IR (Adipo-IR:  $r_p = 0.32$ ,  $p = 0.042$ ; Lipo-IR:  $r_p = 0.39$ ,  $p = 0.012$ ). Thus, resident macrophage activation may be triggered by the overflow of FFAs to the liver and may be one of the missing links between adipose tissue IR and liver fibrosis.

On the contrary, sCD163 levels were not associated with alterations in glucose metabolism, both as muscle insulin sensitivity (expressed as glucose clearance) or with glucose production by the liver (EGP) (Table 3). In our non-diabetic individuals with NAFLD, at multivariate regression analysis, the major determinant of hepatic-IR was fat infiltration at liver biopsy, supporting the concept that steatosis *per se* can alter the regulation of glucose production by the liver. In keeping with the relationship between sCD163 and hepatic fat in NAFLD, hCD163 also significantly correlated with the degree of steatosis ( $r = 0.34$ ,  $p = 0.048$ ) (Fig. 3C). However, again no correlation was found between hCD163 and EGP ( $r = -0.27$ ,  $p = 0.269$ ) or Hep-IR ( $r = 0.11$ ,  $p = 0.650$ ).

**Potential mechanisms for hepatic macrophage activation by adipose tissue insulin resistance: *in vitro* experiments**

As described earlier, sCD163 levels were associated with circulating FFAs, lipid flux and adipose tissue IR, suggesting that alterations in lipid metabolism *per se*, such as FFA overflow may favor macrophage activation in the liver. To investigate this possibility, we treated human monocyte-derived macrophages (hMDMs) with palmitate and measured sCD163 levels in culture media. As shown in Fig. 4, exposure of hMDMs to palmitate increases sCD163 secretion, which is consistent with data in our study participants. In fact, palmitic acid levels were significantly higher in patients with NAFLD and severe fibrosis compared to those with absent/mild fibrosis ( $184 \mu\text{mol} \pm 57$  vs.  $121 \mu\text{mol} \pm 42$ ,  $p < 0.001$ ) and they were directly related to both

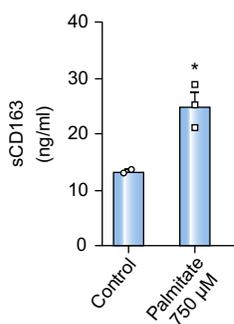


**Fig. 3. CD163 and ADAM-17 in liver biopsies.** (A) CD163 staining by immunohistochemistry, (B) correlation between hepatic mRNA expression of *CD163* (*hCD163*) with circulating sCD163, correlation between hepatic mRNA expression of (C) *ADAM-17* (*hADAM-17*) and *hCD163*, and between (D) *hADAM-17* and sCD163. sCD163, soluble CD163.

**Table 3. Correlations between sCD163 levels and insulin resistance components.**

sCD163	r	p value
Insulin, IU/ml	0.29	0.072
FFAs, mmol/L	0.35	<b>0.026</b>
Glucose clearance, $\mu\text{mol}/\text{min}/\text{kg}$	-0.01	0.950
EGP, $\mu\text{mol}/\text{min}/\text{kg}$	0.06	0.712
Hep-IR, $\mu\text{mol}/\text{min}/\text{kg} * \text{IU}/\text{ml}$	0.22	0.060
Glycerol Ra, $\mu\text{mol} * \text{min}^{-1}$	0.35	<b>0.028</b>
Adipo-IR, $\text{mmol}/\text{L} * \text{IU}/\text{ml}$	0.32	<b>0.042</b>
Lipo-IR, $\mu\text{mol} * \text{min}^{-1} * \text{IU}/\text{ml}$	0.39	<b>0.012</b>

Adipo-IR, adipose tissue insulin resistance by FFAs plasma levels (FFAs \* insulin); Lipo-IR, adipose tissue insulin resistance by tracer (glycerol Ra \* insulin); EGP, endogenous glucose production; FFAs, free fatty acids; Hep-IR, hepatic insulin resistance by tracers (EGP \* insulin); IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; Ra, rate of appearance; sCD163, soluble CD163.



**Fig. 4. Palmitic acid promotes the release of sCD163 in human monocyte-derived macrophages.** sCD163, soluble CD163.

sCD163 concentration and hepatic fat ( $r = 0.30, p = 0.05$ ;  $r = 0.38, p = 0.017$ , Fig. S2A and B, respectively).

**Discussion**

The present study indicates that macrophage activation in the liver of patients with NAFLD, possibly in addition to macrophage activation in the adipose tissue, stems from impaired lipid metabolism in the setting of IR. While the role of adipose tissue inflammation in the onset of IR is not under discussion, it is likely that the overflow of FFAs to the liver is one of the main metabolic sources of activation of resident hepatic macrophages in patients with NAFLD and may be one of the mechanisms linking adipose tissue IR and liver fibrosis in these patients. This is the first study to confirm in humans and *in vivo* a major mechanism of progression to NASH, that has previously been postulated in animal models;<sup>8</sup> further, it provides several potentially useful insights for targeted therapies in NASH.

First, adipose tissue IR appears to be the main metabolic determinant of the presence and degree of liver fibrosis in NAFLD, independent of the presence of T2DM and obesity, as our patients with NAFLD had been carefully selected not to be diabetics and the majority of them were non-obese.

Then, the main metabolic parameters of lipid metabolism, *i.e.* circulating levels of FFAs, rate of lipolysis and degree of IR in the adipose tissue, were all associated with macrophage activation as expressed by sCD163 levels. However, it is well known that obesity is characterized by accumulation of pro-inflammatory M1 macrophages in adipose tissue.<sup>26</sup> Hypertrophy and

hyperplasia of dysfunctional adipose tissue evoke cellular stress and necrotic adipocyte cell death, activation of innate immune cells, including (tissue) macrophages, and production of chemokines such as the chemokine (C-C motif) ligand 2 (CCL2) that recruit monocytes from the bloodstream, boosting the release of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6). The end result is an obesity-driven chronic state of low-grade adipose tissue inflammation.<sup>27–29</sup>

Different findings in obese individuals have been presented on sCD163 levels and adipose tissue. In one study, sCD163 was associated with visceral adipose tissue (VAT) but not with subcutaneous adipose tissue (SAT) or intrahepatic lipid, while CD163 mRNA was significantly upregulated in VAT compared with SAT at baseline and significantly downregulated in SAT after bariatric surgery.<sup>30</sup> Another study showed that sCD163 was associated directly with CD163 mRNA expression in SAT and inversely with peripheral insulin sensitivity.<sup>31</sup> Hence, the second question was whether sCD163 stemmed from fat depots or from the liver. We are not able to separate the different sources of circulating sCD163, but our findings suggest that in our patients, sCD163 levels predominantly mark macrophage activation in the liver. In fact, sCD163 levels were not related to the amount of VAT or SAT assessed by MRI, but they were tightly associated with the degree of hepatic fat and with CD163 mRNA expression in the liver, independently of BMI. These results were also confirmed in the subgroup of non-obese patients with NAFLD. The correlation between hepatic CD163 and ADAM-17, the metalloprotease involved in the cleavage of CD163, strengthen the hypothesis of a link with macrophage activation. Further, in our patients, sCD163 levels increased according to the severity of fibrosis at liver biopsy, supporting the intriguing role of macrophage activation in the progression of hepatic damage.<sup>8</sup> In recent studies we observed a sCD163 gradient across the liver in patients with NASH and cirrhosis.<sup>14,32</sup> In both children and adults, sCD163 is reduced independently of changes in body weight and after life-style intervention and bariatric surgery.<sup>14–16</sup> Although a contribution of circulating macrophages stemming from adipose tissue cannot be excluded, our findings support the notion that in NAFLD, adipose tissue IR can favor the activation of hepatic macrophages, thus promoting fibrosis. This interaction is likely to be an early event in the progression to NASH. In fact, macrophage expansion was the first difference seen in liver biopsies of patients with steatosis compared to control patients and it was the first step of liver inflammatory activation, preceding the recruitment of other inflammatory cells.<sup>33</sup> The pathways involved are not completely understood. According to a recent report, hepatic lipid accumulation may induce an inflammatory macrophage phenotype through the release of extracellular vesicles from hepatocytes.<sup>34</sup> In our patients, the amount of hepatic fat at liver biopsy was proportional to adipose tissue IR and it was related to both soluble and hepatic CD163. In keeping with the *in vivo* correlation between lipid fluxes and both sCD163 and hCD163, the exposure of human monocyte-derived macrophages to palmitate enhanced sCD163 shedding, which suggests a direct pathway of macrophage activation by saturated FFAs, of which palmitate is usually the most abundant. Similarly, *in vitro* stimulation of murine KCs with palmitic acid increases the expression of pro-inflammatory cytokines and toll-like receptor 4.<sup>35</sup>

The results of our study challenge the hypothesis that hepatic macrophage activation can induce hepatic and systemic IR. This hypothesis was based on observations from animal models and *in vitro* experiments. *In vitro*, co-culture of hepatocytes with M1-polarized KCs increased triglyceride accumulation in hepatocytes and fatty acid esterification and decreased fatty acid oxidation and insulin responsiveness; these effects were blocked by TNF- $\alpha$ -neutralizing antibodies.<sup>9</sup> A short-term high-fat diet model in mice demonstrated early Hep-IR and steatosis concurrent with macrophage activation. Furthermore, selective KC depletion obtained by intravenous clodronate significantly improved hepatic insulin sensitivity *in vivo*.<sup>10</sup> However, human patients are more heterogeneous than inbred mouse strains, with respect to genetic or environmental factors that might influence macrophage activation. In our patients, both plasma sCD163 and liver CD163 expression were not related either to Hep-IR or to EGP, suggesting a secondary role of macrophages in the onset of local IR. Conversely, our data confirm that Hep-IR in humans is mainly driven by liver steatosis, as repeatedly reported.<sup>36</sup> The discrepancy with previously published data<sup>26</sup> is likely explained by the different study population. At early stages of metabolic alterations and in the absence of morbid obesity and diabetes, there is no association between derangements of glucose metabolism and macrophage activation in patients with NAFLD. However, the relationship between hepatic insulin sensitivity and hCD163 can be different in morbidly obese and diabetic individuals, when EGP increases and actively contributes to hyperglycemia. Thus, in obese and diabetic patients a possible contribution of macrophage activation to the inhibition of insulin signaling in hepatocytes cannot be completely excluded.

A limitation of this study is that conclusions mostly rely on cross-sectional associations and some are rather weak as gauged from the correlation analysis plots. Experiments have been undertaken with state of the art techniques to allow a careful and reliable measurement of metabolic parameters in humans and *in vivo*.<sup>20,21,37</sup> Overall our results are consistent, in the sense that none of them contradict what we expected from our line of interpretation, or refute this line as the most plausible explanation; this explanation is not fully proven, although it is the best we could achieve *in vivo* and also rules out the overlapping contribution of diabetes and morbid obesity. However, we could not find any clear association with necroinflammation. One possible explanation is that liver biopsy is a snapshot in time of liver damage. While fibrosis is built up over a long period, the rapid changes over time in necro-inflammatory activity may explain the lack of association with several biomarkers and parameters. Another challenge with the interpretation of the results is the current histological scoring of ballooning (by definition only 2 max) and lobular inflammation (which is very mild in our patient cohort). According to the immunohistochemistry, there was an increased presence of activated macrophages, hence inflammation, but the latter can be poorly reflected by the very rough scoring of ballooning and lobular inflammation. It may be unusual that the inflammation was not more pronounced even in patients fulfilling NASH criteria, but *de facto*, mild lobular inflammation is common in NASH, a slowly evolving disease. In 3 large independent cohorts of patients with NAFLD and NASH previously studied by our group, the lobular inflammation score did not exceed 2 (2–4 foci per 200x field).<sup>13,14</sup>

Notably, the findings of our study represent a paradigm shift regarding the role of macrophages in human hepatic IR. These results have implications for our understanding of pathogenesis and therapy, by highlighting the strong link between metabolic derangement and local inflammatory responses. Hepatic macrophages are an attractive target for novel therapeutic approaches to treat fibrotic NASH, as underscored by the antifibrotic effects recently reported by drugs in phase II clinical trials that target macrophages by acting on monocyte recruitment (dual CCR2/CCR5 inhibitor cenicriviroc),<sup>38</sup> their inflammatory activation (inhibitor of the serine/threonine kinase ASK1, selonsertib), or on hepatic stellate cells.<sup>39</sup> However, none of these studies showed a significant effect on metabolic parameters independent of BMI, thus leaving unaffected the major source of macrophage activation. Another attractive option could be the combination with agonists of the fatty acid sensor peroxisome proliferator activator-receptor gamma (PPAR $\gamma$ ), for their favorable effects on adipose tissue IR, or delta (PPAR $\delta$ ), involved in alternative activation of macrophages and favoring glucose metabolism and fatty acid oxidation.<sup>40</sup> Clinical trials with a double PPAR $\alpha$  and PPAR $\delta$  agonist (elafibranor)<sup>41</sup> and pan- PPAR $\alpha$ , PPAR $\delta$  and PPAR $\gamma$  agonist (lanifibranor)<sup>42</sup> are currently ongoing. Interestingly, we have previously reported that direct targeting macrophages with an anti-CD163-IgG-dexamethasone conjugate significantly improved fructose diet-induced NASH changes *e.g.* inflammation, hepatocyte ballooning, fibrosis, and glycogen deposition.<sup>43</sup>

In summary, this *in vivo* study in non-diabetic patients with NAFLD may suggest a link between activation of resident macrophages in the liver and alterations in adipose tissue IR, while it does not support a link with hepatic glucose metabolism. Liver macrophages represent an attractive therapeutic target, but it is necessary to consider the potential role of adipose tissue IR in their activation in order to obtain a prolonged and sustainable effect on liver damage in NASH.

### Financial support

This work was funded by FP7/2007-2013 under grant agreement n.HEALTH-F2-2009-241762, project FLIP and by Horizon 2020 under grant agreement n. 634413, project EPoS for EB and AG. HG received funding from The NOVO Nordisk Foundation, The Danish Strategic Research Council (10-092797) and “Savværksejer Jeppe Juhl og hustru Ovita Juhls mindelegat”.

### Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying [ICMJE disclosure](#) forms for further details.

### Authors' contributions

CR provided data collection, statistical analysis, interpreted data and drafted the manuscript, KK, RY, SE, MM and MS provided the acquisition of data, interpreted data and critically reviewed the manuscript, CB, MG and FS provided the acquisition of data, HJM, MLA, HV, AG and JG interpreted data and critically reviewed the manuscript, EB and HG led the development of the study concept and design, interpreted data, drafted and finalized the manuscript.

### Acknowledgement

Professor Stephen Hamilton-Dutoit, Aarhus University Hospital, is acknowledged for CD163 IHC staining of liver biopsies.

### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2019.06.031>.

### References

- [1] Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018;15:11–20.
- [2] Marengo A, Jouness RI, Bugianesi E. Progression and natural history of nonalcoholic fatty liver disease in adults. *Clin Liver Dis* 2016;20:313–324.
- [3] Bugianesi E, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010;16:1941–1951.
- [4] Caligiuri A, Gentilini A, Marra F. Molecular pathogenesis of NASH. *Int J Mol Sci* 2016;17, pii: E1575.
- [5] Positano V, Gastaldelli A, Sironi M, Santarelli MF, Lombardi M, Landini L. An accurate and robust method for unsupervised assessment of abdominal fat by MRI. *J Magn Reson Imaging* 2004;20:684–689.
- [6] Lomonaco R, Ortiz-Lopez C, Orsak B, Webb A, Hardies J, Darland C, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. *Hepatology* 2012;55:1389–1397.
- [7] Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev* 2006;6:772–783.
- [8] Kazankov K, Jørgensen SMD, Thomsen KL, Møller HJ, Vilstrup H, George J, et al. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol* 2019;16:145–159.
- [9] Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol* 2016;13:316–327.
- [10] Lanthier N, Molendi-Coste O, Horsmans Y, van Rooijen N, Cani PD, Leclercq IA. Kupffer cell activation is a causal factor for hepatic insulin resistance. *Am J Physiol Gastrointest Liver Physiol* 2010;298:G107–G116.
- [11] Weaver LK, Hintz-Goldstein KA, Pioli PA, Wardwell K, Qureshi N, Vogel SN, et al. Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. *J Leukoc Biol* 2006;80:26–35.
- [12] Møller HJ. Soluble CD163. *Scand J Clin Lab Invest* 2012;72:1–13.
- [13] Kazankov K, Barrera F, Møller HJ, Rosso C, Bugianesi E, David E, et al. The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease. *Liver Int* 2016;36:1549–1557.
- [14] Kazankov K, Tordjman J, Møller HJ, Vilstrup H, Poitou C, Bedossa P, et al. Macrophage activation marker soluble CD163 and non-alcoholic fatty liver disease in morbidly obese patients undergoing bariatric surgery. *J Gastroenterol Hepatol* 2015;30:1293–1300.
- [15] Kazankov K, Møller HJ, Lange A, Birkebaek NH, Holland-Fischer P, Solvig J, et al. The macrophage activation marker sCD163 is associated with changes in NAFLD and metabolic profile during lifestyle intervention in obese children. *Pediatr Obes* 2015;10:226–233.
- [16] Rødgaard-Hansen S, St George A, Kazankov K, Bauman A, George J, Grønbaek H, et al. Effects of lifestyle intervention on soluble CD163, a macrophage activation marker, in patients with non-alcoholic fatty liver disease. *Scand J Clin Lab Invest* 2017;77:498–504.
- [17] 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.
- [18] Gastaldelli A, Coggan AR, Wolfe RR. Assessment of methods for improving tracer estimation of non-steady-state rate of appearance. *J Appl Physiol* 1999;87:1813–1822.
- [19] Gastaldelli A, Gaggini M, Daniele G, Ciociaro D, Cersosimo E, Tripathy D, et al. Exenatide improves both hepatic and adipose tissue insulin resistance: a dynamic positron emission tomography study. *Hepatology* 2016;64:2028–2037.

- [20] Groop LC, Bonadonna RC, DelPrato S, Ratheiser K, Zyck K, Ferrannini E, et al. Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *J Clin Invest* 1989;84:205–213.
- [21] Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* 2007;133:496–506.
- [22] Moller HJ, Hald K, Moestrup SK. Characterization of an enzyme-linked immunosorbent assay for soluble CD163. *Scand J Clin Lab Invest* 2002;62:293–299.
- [23] Moller HJ, Frikke-Schmidt R, Moestrup SK, Nordestgaard BG, Tybjaerg-Hansen A. Serum soluble CD163 predict risk of type 2 diabetes in the general population. *Clin Chem* 2011;57:291–297.
- [24] Fjeldborg K, Møller HJ, Richelsen B, Pedersen SB. Regulation of CD163 mRNA and soluble CD163 protein in human adipose tissue in vitro. *J Mol Endocrinol* 2014;53:227–235.
- [25] Bugianesi E, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* 2005;48:634–642.
- [26] Kristensen MD, Lund MT, Hansen M, Poulsen SS, Ploug T, Dela F, et al. Macrophage area content and phenotype in hepatic and adipose tissue in patients with obesity undergoing Roux-en-Y gastric bypass. *Obesity* 2017;25:1921–1931.
- [27] Catzigeorgiou A, Chavakis T. Immune cells and metabolism. *Handb Exp Pharmacol* 2016;233:221–249.
- [28] Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adypocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005;46:2347–2355.
- [29] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958–969.
- [30] Fjeldborg K, Pedersen SB, Møller HJ, Rask P, Danielsen AV, Stødkilde-Jørgensen H, et al. Intrahepatic fat content correlates with soluble CD163 in relation to weight loss induced by Roux-en-Y gastric bypass. *Obesity* 2015;23:154–161.
- [31] Kračmerová J, Rossmeislová L, Kováčková Z, Klimčáková E, Polák J, Tencerová M, et al. Soluble CD163 is associated with CD163 mRNA expression in adipose tissue and with insulin sensitivity in steady-state condition but not in response to calorie restriction. *J Clin Endocrinol Metab* 2014;99:E528–E535.
- [32] Holland-Fischer P, Grønbaek H, Sandahl TD, Moestrup SK, Riggio O, Ridola L, et al. Kupffer cells are activated in cirrhotic portal hypertension and not normalised by TIPS. *Gut* 2011;60:1389–1393.
- [33] Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, et al. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology* 2014;59:1393–1405.
- [34] Hirsova P, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, et al. Lipid-induced signaling causes release of inflammatory extracellular vesicles from hepatocytes. *Gastroenterology* 2016;150:956–967.
- [35] Tang T, Sui Y, Lian M, Li Z, Hua J. Pro-inflammatory activated Kupffer cells by lipids induce hepatic NKT cells deficiency through activation-induced cell death. *PLoS ONE* 2013;8 e81949.
- [36] Yki-Jarvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol* 2014;2:901–910.
- [37] Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006;355:2297–2307.
- [38] Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology* 2018;67:1754–1767.
- [39] Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, et al. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: a randomized, phase 2 trial. *Hepatology* 2017. <https://doi.org/10.1002/hep.29514> (Epub ahead of print).
- [40] Samuel VT, Shulman GI. Nonalcoholic fatty liver disease as a nexus of metabolic and hepatic diseases. *Cell Metab* 2018;27:22–41.
- [41] Ratziu V, Harrison SA, Francque S, Bedossa P, Leher P, Serfaty L, et al. Elafibranor, an agonist of the peroxisome proliferator-activated receptor- $\alpha$  and - $\delta$ , induces resolution of nonalcoholic steatohepatitis without fibrosis worsening. *Gastroenterology* 2016;50:1147–1159.
- [42] Avouac J, Konstantinova I, Guignabert C, Pezet S, Sadoine J, Guilbert T, et al. Pan-PPAR agonist IVA337 is effective in experimental lung fibrosis and pulmonary hypertension. *Ann Rheum Dis* 2017;76:1931–1940.
- [43] Svendsen P, Graversen JH, Etzerodt A, Hager H, Røge R, Grønbaek H, et al. Antibody-directed glucocorticoid targeting to CD163 in M2-type macrophages attenuates fructose-induced liver inflammatory changes. *Mol Ther Methods Clin Dev* 2016;4:50–61.