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

Crosstalk between adipose tissue insulin resistance and liver macrophage activation in patients with Non-Alcoholic Fatty Liver Disease

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Abbreviations: ADAM-17, tumor necrosis factor-α-converting enzyme; Adipo-IR, adipose tissue insulin resistance by free fatty acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; EGP, endogenous glucose production; FFA, free fatty acids; GGT, gamma-glutamyl transpeptidase; hCD163, hepatic CD163; Hep-IR, hepatic insulin resistance; hMDMs, human monocyte derived macrophages; HOMA, homeostasis model of assessment; IR, insulin resistance; Lipo-IR, adipose tissue insulin resistance by tracers; MRI, magnetic resonance imaging; OGTT, oral glucose tolerance test; PNPLA3, patatin-like phospholipase domain-containing 3; sCD163, soluble CD163; SCF, subcutaneous fat; VF, visceral fat.

Abstract

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 1) 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 tracers infusion ([6,6-2H2]glucose and [2H5]glycerol); 2) macrophage activity (by soluble sCD163) and 3) hepatic expression of CD163 (hCD163).

Results: We found that sCD163 levels paralleled both the plasma free fatty acids (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 to FFA (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 may 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 in defining 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 insulin resistant state. This study provides *in vivo* support of a possible link between deranged metabolism in the adipose tissue and activation of hepatic macrophages in patients with NAFLD, most likely in response to FFA overflow and independent of obesity and diabetes.

Introduction

The recent epidemic in chronic liver disease is related to the burden of Non-Alcoholic Fatty Liver Disease (NAFLD), paralleling the worldwide increase of obesity.[1] 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 develop liver fibrosis, cirrhosis and possibly hepatocellular carcinoma.[1,2] Understanding the biological and environmental factors that in some individuals drive the progression to NASH and beyond is fundamental to develop robust methods for diagnosis, risk stratification and therapy.[2]

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.[3,4] It is generally believed that adipose tissue IR plays a pivotal role in the onset and progression of NAFLD.[5,6] Briefly, weight gain leads to expansion of adipose tissue and recruitment of macrophages through the secretion of various chemo- and cytokines.[7] An inflamed and dysfunctional adipose tissue actively releases free fatty acids (FFA) to the bloodstream, promotes lipotoxicity in the liver, muscle and pancreas[3], and contributes to systemic inflammation. In the normal liver, resident macrophages or Kupffer cells (KCs) play important regulatory roles through the crosstalk with the different cell types and particularly with hepatocytes.[8] The pro-inflammatory polarization of hepatic macrophages is considered a hallmark feature of progressive disease in liver from NASH patients and an attractive therapeutic target as recently reviewed.[8] Hepatic lipid accumulation facilitates pro-inflammatory KC polarization possibly as a consequence of FFA excess, signals from surrounding steatotic hepatocytes like histidine-rich glycoprotein or extracellular vesicles and damage associated molecular patterns (DAMPs) induced KCs activation.[8] More recently, data derived from animal models and *in vitro* studies suggest that both pro-inflammatory KCs and recruited hepatic macrophages ($Ly6C^{hi}$)

contribute to decreased hepatic insulin sensitivity by inhibiting insulin signaling and activating hepatic glucose production.[9] However, most data available have been derived from mouse models, which are, by far, not fully representative of human NASH since they reflect certain aspects of the pathogenesis and rarely incorporate the full spectrum of etiology-specific mechanisms.[8]

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. tumour necrosis factor-alpha converting enzyme (TACE/ADAM17)).[10-11] CD163-positive macrophages are highly expressed in human AT from obese individuals and in NAFLD patients sCD163 levels are associated with hepatic inflammation and fibrosis[12-13] and decrease after successful life-style intervention and bariatric surgery.[14-16]

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

Material and Methods

Study subjects.

The study was performed in forty 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 (T2DM) and morbid obesity (body mass index [BMI] > 35); the absence of the two latter conditions in our patients unveils the independent contribution of NAFLD to metabolic and inflammatory alterations. Other aetiologies 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 IDF 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.[17] 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°C. Biochemical exams included the complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, alkaline phosphatase and gamma-glutamyl-transpeptidase. Serum glucose was measured by the glucose oxidase method (Sentinel, Milan, Italy*).* Free fatty acid (FFA) concentration was 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 (VLDL). Low-density lipoproteins 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 µl 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 one 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-D2-glucose (bolus 22 µmol/kg, infusion rate 0.22 µmol/kg min) and of U-D5-glycerol (bolus 1.5 µmol/kg, infusion rate 0.1 µmol/kg min) was administered for 2 hours in fasting conditions (equilibration period) to assess endogenous glucose production (EGP) and lipolysis through the rate of appearance (Ra) of Glycerol, as previously described.[18] After reaching isotopic steady state for 6,6-D2-glucose and U-D5-glycerol (after 2h infusion), the patients drank 75g of glucose with 1.5g of U-13C-glucose

(double tracer-OGTT) while continuing stable isotope infusion at constant rate and plasma samples were collected every 30 minutes for 3 hours.

Tracer enrichment of 6,6-D_2-glucose and U-D_5-glycerol were determined by gas chromatography-mass spectrometry system (5975; Agilent, Palo Alto, CA), using electron impact ionization and selectively ion monitoring (SIM), 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;[18-19] briefly:

$TTR_{6,6\text{-}D2\text{-}glucose}(t) = R_{202/200}(t) - R_{202/200}(bk) - R_{202/200}(t) \times (R_{201/200}(t) - R_{201/200}(bk))$

$TTR_{U\text{-}D5\text{-}glycerol}(t) = R_{148/145}(t) - R_{148/145}(bk)$

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. [18-19] 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,[20-21] adipose tissue IR can be calculated as FFA x fasting insulin (Adipo-IR) or as Glycerol-Ra x fasting insulin (Lipo-IR). Specifically, Adipo-IR takes into consideration the FFA 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[20] thus hepatic-IR is calculated as EGP x fasting insulin.[18-19]

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 had been carried on 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 subjects.[5]

Circulating sCD163 measurement.

Plasma concentration of sCD163 was determined in duplicate by an in-house sandwich enzyme-linked immunosorbent assay (ELISA) using a BEP-2000 ELISA-analyser (Dade Behring) essentially as previously described.[22] The sCD163 measurements were part of a combined biobank study investigating the association between sCD163 and liver morphology in NAFLD patients. [13] 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.[23]

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°C until processing. Total RNA extraction was performed using TRIzol reagent (Invitrogen, CA). For each sample, 1 μg of total RNA was treated with deoxyribonuclease I (Invitrogen, CA) prior to reverse transcription, in turn performed with random primers and the SuperScript® 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 andADAM-17 was derived from the literature.[24] The control sample used as calibrator was a cDNA derived from a subject without features of NASH. Quantitative polymerase chain reaction (qPCR) was performed using the SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the instrument protocol: 98°C for 30 seconds for polymerase activation, 95°C for 15 seconds for cDNA denaturation followed by 39 cycles of 60°C for 30 seconds for annealing and

extension. All samples and controls were analyzed in duplicate. The relative expression of CD163 and ADAM-17 was calculated as $2^{-\Delta Ct}$ 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™ Software (Bio-Rad Laboratories, CA).

Cell culture

Human CD14 monocyte derived macrophages were matured in M-CSF (50ng/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 uM or 750 uM Palmitate (made fresh on the day) for 24 hours. Supernatant was collected and centrifuged for 2000 rpm for 5 minutes 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.[13]

Statistical analysis. Data are reported as mean ± standard deviation (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 two groups was performed with Kruskal-Wallis test for continuous not normally distributed variable and by ANOVA for continuous normally distributed variables. Similarly, comparison between two

groups was performed by Mann-Whitney test for not normally distributed variables and by t-test for normally distributed variables. For categorical data, the Fisher exact test or χ^2 test was used as appropriate. Spearman or Pearson correlation was 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 subjects (n = 40) are reported in Table 1. 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 cholesterol (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 and 48% had insulin resistance (IR) defined as 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 (Supplementary Table 1). Among histological features, hepatic steatosis was mild (<33%) in 52% of the study subjects, 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 subjects 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, FFAs levels and HOMA-IR significantly increased in relation to the severity of fibrosis (Table 2). Adipose tissue-IR, calculated taking into account both FFA levels (Adipo-IR) and the rate of lipolysis (Lipo-IR), rose stepwise by fibrosis staging (Table 2 and Figures 1A and B), confirming its putative role in liver damage as previously described.[21,25] Adipose tissue-IR (Adipo-IR and Lipo-IR) also showed a strong correlation with hepatic fat (Figures 1C and D). Notably, the correlation with steatosis was stronger for Lipo-IR, that takes into account body weight (since lipolysis is express as umol/min normalized by body weight) (Table2), 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 NAFLD patients (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 (Supplementary Table 2); this finding was not unexpected since VF can at most account for ~20% of the total pre-hepatic FFA load.[5] Overall, these results suggest that an increased lipolysis from the adipose tissue (mainly subcutaneous) is linked to both fibrosis and fat accumulation in the liver of NAFLD patients independent of BMI and of Type 2 Diabetes. 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 (Figure 1E), but not the severity of fibrosis (Figure 1F). In NAFLD subjects with a NAS score ≥ 4 both Hepatic-IR and Adipose tissue-IR (Adipo-IR and Lipo-IR) were higher (Supplementary Figure 1A-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 (Supplementary Table 3).

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) (Figure 2A-C). However, BMI was not associated with sCD163 levels, while the relationship with steatosis stayed significant both at univariate and multivariate analysis (Supplementary Table 4). 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 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 to sCD163 levels independent of BMI, WC, VF and SCF (Supplementary Table 4).

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) and by qPCR (hCD163) in a subgroup of 20 NAFLD subjects where liver tissue had been collected and stored (clinical data summarized in Supplementary Table 5). In the liver, we observed significant hCD163 staining most predominant in patients with inflammation and fibrosis (Fig 3A) and hCD163 had a significant, direct correlation with the circulating levels of sCD163 (r=0.58, p=0.007) (Figure 3B). This result was strengthened by the association between hepatic ADAM-17, a specific metalloprotease 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)(Figure 3B and C respectively).

Although we cannot exclude the contribution of adipose tissue macrophages to circulating sCD163, taken together these results may suggest that in our NAFLD subjects circulating sCD163 mainly mark 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 $p < 0.001$) (Figure 2D) and was 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 ≥ 4, sCD163 levels were 1.3 fold higher than in NAS<4 (2.18 ± 0.74 *vs* 1.68 ± 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 and the total CD163+ hepatic macrophage (scored by IHC), statistical significance was not reached probably due to lack of statistical power for categorical analysis (N=20)(Supplementary Figure 3).

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 NAFLD subjects with HOMA-IR ≥ 2.7 (2.23 ± 0.57 vs 1.71 ± 0.97, $p=0.002$) and in those with MetS (2.29 ± 0.69 vs 1.8 ± 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 had a significant association 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 FFA to the liver and may be one of the missing link 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 NAFLD subjects, 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, also hCD163 significantly correlated with the degree of steatosis (r=0.34, p=0.048) (Figure 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 FFA, lipid flux and AT-IR, suggesting that alterations in lipid metabolism *per se* such as FFA overflow may favor macrophage activation in the liver. To investigate for this possibility, we treated human monocyte derived macrophages (hMDMs) with palmitate and measured sCD163 levels in culture media. As shown in Figure 4, exposure of hMDMs to palmitate increases sCD163 secretion, which is consistent with data in our study subjects. In fact, palmitic acid levels were significantly higher in NAFLD patients with severe fibrosis compared to those with absent/mild fibrosis (184 umol ± 57 vs 121 umol ± 42, p<0.001) and they were directly related to both sCD163 concentration and hepatic fat (r=0.30, p=0.05; r=0.38, p=0.017, Supplementary Figure 2 A and B respectively).

Discussion.

The present study may suggest a different perspective in considering also macrophage activation in the liver of patients with NAFLD, along with and possibly in addition to macrophage activation in the adipose tissue, stemming from an impaired lipid metabolism in the setting of insulin resistance. While the role of adipose tissue inflammation in the onset of IR is not under discussion, it is likely that the overflow of FFA to the liver is one of the main metabolic sources of activation of resident hepatic macrophages in patients with NAFLD and can provide 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 been previously postulated in animal models[8]; further, it provides several insights potentially useful for targeted therapies of 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 NAFLD patients 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 FFA, 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.[26] Hypertrophy and hyperplasia of a dysfunctional adipose tissue evoke cellular stress and necrotic adipocyte cell death, activation of innate immune cells, among which (tissue) macrophages, and production of chemokines such as the chemokine (C-C motif) ligand 2 (CCL2) to recruit monocytes from the bloodstream boosting the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). The final result is an obesity-driven chronic state of low-grade adipose tissue inflammation.[27-29]

Different findings in obese subjects 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 and CD163 mRNA was significantly up-regulated in

VAT compared with SAT at baseline and significantly down-regulated in SAT after bariatric surgery.[30] Another study showed that sCD163 was associated directly with CD163 mRNA expression in SAT and inversely with peripheral insulin sensitivity.[31] Hence, the second question was whether sCD163 was stemming 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 to CD163 mRNA expression in the liver, independent of BMI and also confirmed in the subgroup of non-obese 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.[8] In recent studies we observed a sCD163 gradient across the liver in NASH and cirrhotic patients,[14, 32] and in children and adults sCD163 is reduced independent of changes in body weight and after life-style intervention and bariatric surgery.[14-16] 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.[33] 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.[34] 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 FFA of which palmitate is usually the most abundant. Similarly, *in vitro* stimulation of murine KCs with palmitic acid increases the expressions of pro-inflammatory cytokines and toll-like receptor 4.[35]

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-a-neutralizing antibodies.[9] A short-term high-fat diet model in mice demonstrated early Hep-IR and steatosis concurrent with macrophage activation. Furthermore, selective KCs depletion obtained by intravenous clodronate significantly improved hepatic insulin sensitivity *in vivo*.[10] 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.[36] The discrepancy with previously published data[26] 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 NAFLD patients. However, the relationship between hepatic insulin sensitivity and hCD163 can be different in morbid obese and diabetic subjects, when EGP increases and actively contribute to hyperglicemia. 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 by state of the art techniques to allow a careful and reliable measurement of metabolic parameters in humans and *in vivo*.[20-21, 37] Overall our results are consistent, in the sense that none of them goes clearly opposite of what we expected from our line of interpretation or refute this line as the most plausible explanation; however, it is not fully proven although it is the best that we can achieve *in vivo* and also rules out the overlapping contribution of diabetes and morbid obesity. On the other hand, 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 three 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 200· field).[13-14]

Notably, the findings of our study represent a paradigm shift in thinking on the role of macrophages in human hepatic insulin resistance and has implications for understanding 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)[38], their inflammatory activation (inhibitor of the serine/threonine kinase ASK1, selonsertib), or on hepatic stellate cells.[39] 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 (PPARγ), for their favorable effects on adipose tissue-IR, or delta (PPARδ), involved in alternative activation of macrophages and favoring glucose metabolism and fatty acid oxidation.[40] Clinical trials with a double PPARα and PPARδ agonist (elafibranor)[41] and pan- PPARα, PPARδ and PPARγ (lanifibranor)[42] are currently ongoing. Interestingly, we have previously reported that direct targeting of macrophages by an anti-CD163-IgG-dexamethasone conjugate significantly improved fructose diet induced NASH changes e.g. inflammation, hepatocyte ballooning, fibrosis, and glycogen deposition.[43]

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 insulin resistance, 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.

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Figures legend

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

Figure 2. Soluble CD163. Soluble CD163 and its association with body mass index (A), waist circumference (B), hepatic fat content (C) and liver fibrosis (D).

Figure 3. Hepatic expression of CD163. Correlation between hepatic expression of CD163 with circulating sCD163 (A), hepatic expression of ADAM-17 (B) and hepatic steatosis (C).

Figure 4. Palmitic acid promotes the release of sCD163 in human monocyte derived macrophages.

Supplementary Figure 1. Adipose tissue and hepatic insulin resistance according to the non-alcoholic fatty liver disease activity (NAS) score.

Supplementary Figure 2. Palmitic acid levels according to sCD163 concentration (A) and hepatic fat (B).