Article

Adiponectin involved in portal flow hepatic extraction of ¹³C-metacethin in obesity and non-alcoholic fatty liver.

Agostino Di Ciaula[1,*,°], Federico Carbone[2,3,°], Harshitha Shanmugham[1], Emilio Molina Molina[1], Leonilde Bonfrate[1], Stefano Ministrini[4], Fabrizio Montecucco[2,3,°], Piero Portincasa[1]

Affiliations

[1] Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, Bari 70124, Italy. agostinodiciaula@tiscali.it (A.D.C.); harshithashanmugam993@gmail.com (H.S.); emmolin.ugr@gmail.com (E..M.); leonilde.bonfrate@uniba.it (L.B.); piero.portincasa@uniba.it (P.P.)

[2] First Clinic of internal Medicine, Department of Internal Medicine, University of Genoa, 6 viale Benedetto XV, 16132 Genoa, Italy. federico.carbone@edu.unige.it (F.C.); fabrizio.montecucco@unige.it (F.M.)

[3] IRCCS Ospedale Policlinico San Martino, Genoa – Italian Cardiovascular Network, 10 Largo Benzi, 16132 Genoa, Italy. federico.carbone@edu.unige.it (F.C.); fabrizio.montecucco@unige.it (F.M.)

[4] Internal Medicine, Department of Medicine, Università degli Studi di Perugia, 1 piazzale Gambuli, 06129, Perugia, Italy stefano.mns@gmail.com (S.M.)

*Correspondence: agostinodiciaula@tiscali.it; piero.portincasa@uniba.it
° share equal contribution

Abbreviations

NAFLD	non-alcoholic fatty liver disease
NASH	Non-alcoholic steato-hepatitis
uNAFLD	Ultrasonographic Nonalcoholic Fatty Liver
(13C)-MBT	(13C)-methacetin breath test
ARFI	acoustic radiation force impulse
HOMA	Homeostatic Model Assessment for Insulin Resistance
hsCRP	high-sensitivity C-reactive protein
DOB_{15}	delta over baseline after 15 minutes
$cPDR_{30}$	cumulative percent dose recovery after 30 minutes

Abstract:

Obesity and non-alcoholic fatty liver disease (NAFLD) are high prevalence, inter-related conditions at increased risk for advanced liver diseases and related mortality. Adiponectin and leptin have divergent roles in the pathogenesis of fat accumulation and NAFLD. However, the relationships between body and liver fat accumulation, early modification of liver function and unbalanced adipokine levels are still scarcely explored. We studied by (13C)-methacetin breath test ((13C)-MBT) 67 adults stratified according to body mass index, and to presence/absence of ultrasonographic nonalcoholic fatty liver disease (uNAFLD). uNAFLD was detected in 20%, 73% and 96% of normal weight, overweight and obese subjects, respectively. The delta over baseline after 15 min (DOB_{15}), a marker of hepatic extraction efficiency from portal blood flow, was lower in obese than in normal weight subjects, and in subjects with-, as compared to those without uNAFLD. The cumulative percent dose recovery after 30 min ($cPDR_{30}$), a marker of liver microsomal function, was lower in uNAFLD patients. DOB_{15} was positively correlated with adiponectin levels in obese and in uNAFLD patients. uNAFLD patients also showed a positive correlation between $cPDR_{30}$ values and adiponectin. Our data indicate the existence of early alterations of liver function in obese and in patients with uNAFLD. These dysfunctions are linked to altered leptin/adiponectin balance and can be identified noninvasively by (13C)-MBT.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease with a worldwide prevalence of about 30% [1]. NAFLD frequently occurs in overweight and obese individuals [2], and is often associated with dyslipidaemia, and insulin resistance [3, 4]. Subjects with NAFLD have no signs of acute or chronic liver disease other than an excessive (>5%) accumulation of lipids – mainly triglycerides - in the liver and no other causes for secondary hepatic fat accumulation, including heavy alcohol consumption [5, 6]. NAFLD encompasses a full spectrum of conditions ranging from nonalcoholic fatty liver (NAFL, i.e., simple steatosis) to the more progressive type with liver inflammation, fibrosis and necrosis, i.e., non-alcoholic steatohepatitis (NASH), to "cryptogenic" cirrhosis, and hepatocellular carcinoma [7, 8]. Therefore, NAFLD increases the risk of liver-related morbidity and mortality [9]. Furthermore, NAFLD is often associated to non-liver-related complications, including cardiovascular disease and malignancy [10]. Adipocytokines are strongly involved in the pathogenesis and evolution of NAFLD, and adipocytokines play a major role in the mechanisms linking systemic insulin resistance to fat accumulation in the hepatocyte [11]. Adiponectin is a 244-amino-acid-long polypeptide hormone produced mainly in adipocytes and involved in a number of metabolic processes, which include regulation of glucose homeostasis and fatty acid oxidation. Serum adiponectin levels are inversely related to insulin resistance [12], and adiponectin has anti-inflammatory, anti-oxidative and protective effects on the liver [13-15]. Low serum levels of adiponectin, however, might play a role in the progression to NASH [16]. Leptin is another hormone predominantly secreted by adipose cells and enterocytes in the small intestine, and helps to regulate energy balance by inhibiting hunger. This step diminishes fat storage in adipocytes. Serum levels of leptin are associated with whole-body fat mass (in particular subcutaneous adipose tissue[17]), are higher in overweight and obese subjects than in normal weight subjects [18]. Increased serum levels of leptin have pro-inflammatory and atherogenic properties [19], and elevated concentrations of this adipokine can promote the progression of NAFLD [11, 20]. In this scenario, an unbalanced adipokine homeostasis could be linked with altered liver function in patients with NAFLD, a multifactorial and multi-organic disease. In particular, NAFLD patients could develop functional hepatic alterations without significant fibrosis due to inter-relations between low adiponectin levels [21], (i.e., low protective effects on the liver), and increased leptin levels [22] (i.e., high pro-inflammatory effects).

Currently available imaging techniques (i.e. ultrasonography, computed tomography, nuclear magnetic resonance) are able to detect liver steatosis or advanced liver diseases, but miss the ongoing necro-inflammatory changes typical of NASH [23]. Such imaging techniques also cannot diagnose the early alterations of liver function, and liver biopsy is still considered the gold standard for diagnosing and staging NAFLD/NASH. Validated and non-invasive diagnostic tools able to identify the "dynamic" functional ability of the liver and early modifications of liver function depending on overweight/obesity, NAFLD, and unbalanced adipokine levels are therefore strongly needed. In this respect, breath tests employing specific substrates labelled with the naturally occurring (13C) stable (non-radioactive) isotope are a promising tool to measure the hepatic clearance of oral-ingested, metabolically active substances and, thus, liver extraction efficiency from the portal flow [24, 25]. The (13C)-methacetin breath test ((13C)-MBT) has been previously used to evaluate liver extraction efficiency and microsomal function in chronic liver diseases [26] as chronic HCV infection [27], liver cirrhosis [28], and NAFLD [25, 29, 30]. In particular, (13C)-MBT has been validated as a useful technique to distinguish patients with NASH from those with normal or fatty liver, and to predict the level of liver fibrosis [31-33].

Recent observations by (13C)-MBT indicate that fat accumulation, NAFL and insulin-resistance can be linked with decreased hepatic extraction efficiency [25]. Although mechanisms leading to a disturbed portal extraction in NAFLD patients need to be fully elucidated, prior animal [34-36] and human studies [37] point to a role for increased liver stiffness secondary to fat accumulation and, likely, to mild fibrosis. Both changes contribute to increased intrahepatic vascular resistance to blood flow [25]. In addition, NAFLD patients may exhibit an altered intracellular demethylation of (13C)-methacetin due to decreased CYP 1A2 expression [38] which parallels NAFLD progression [39]. Furthermore, animal models suggest that CYP 1A2 has a protective role versus NAFLD onset and progression by limiting the oxidative stress and inflammation generated by toxic substances [40].

Thus, the aim of the present study was to explore the existence of possible relationships between serum adipokine levels, insulin resistance, chronic systemic inflammation, NAFLD, and hepatic extraction efficiency evaluated by (13C)-MBT in obese, overweight and normal weight subjects.

2. Materials and Methods

2.1. *Subjects*

Subjects were consecutively enrolled on an outpatient basis at the Division of Internal Medicine, Regional Hospital "Policlinico", Bari, Italy. The reason for referral (either from general practitioners or other specialists) was depended on underlying metabolic disorders and/or the suspicion regarding the liver steatosis. Controls were age-matched healthy academic or hospital employees. All subjects were classified according to body mass index (BMI) (**see supplementary material**) and the presence of ultrasonographic liver steatosis. Chronic liver disease other than NAFLD (viral, alcoholic, drug-induced liver damage, autoimmune diseases) were carefully excluded with clinical history, physical examination, and blood samples for the determination of hepatitis B/C viral markers and autoantibodies. None of enrolled subjects had diabetes. The protocol included anthropometric evaluation (see Supplementary materials), assessment of liver function by (13C)-MBT, abdominal ultrasound exact profile of fatty liver, and blood analyses. All subjects underwent the first screening visit at the outpatient clinic and the complete evaluation required about 4-hrs.

2.2. *Study approval*

The protocol was approved by the local Ethics Committee (study number 5408, protocol number 0013869; AOUCPG23/COMET/P). Before the study, all subjects gave full written informed consent to allow all authors to access and use the data for research purposes.

2.3. *Measurement of liver function by (13C)-MBT*

The protocol of (13C)-MBT is described elsewhere in details [25, 29, 41-44]. In brief, the test relies on the capacity of hepatic cytochrome P450 1A2 to demethylate the ingested dose of (13C)-labelled methacetin into acetaminophen and (13C)-formaldehyde. Then, $(13C)CO_2$ is produced and becomes detectable in expired breath [45]. Subjects perform the test in the morning after fasting for at least 8 hours. Half an hour before and during the whole test, the participant refrains from smoking, or from vigorous physical exercise [30, 46]. We recorded information about current medical prescription and drugs able to affect liver function, to rule out any potential interfering factor. Samples of expired air were collected at baseline in duplicate with a straw into glass exetainers. After, each participant ingested a solution containing 75 mg of (13C)-methacetin (AB-(13C) Metacetina®, AB Analitica srl, Padua, Italy) diluted in 25 mL of still water. Breath samples were collected again at 15 and 30 min (**Figure 1**).
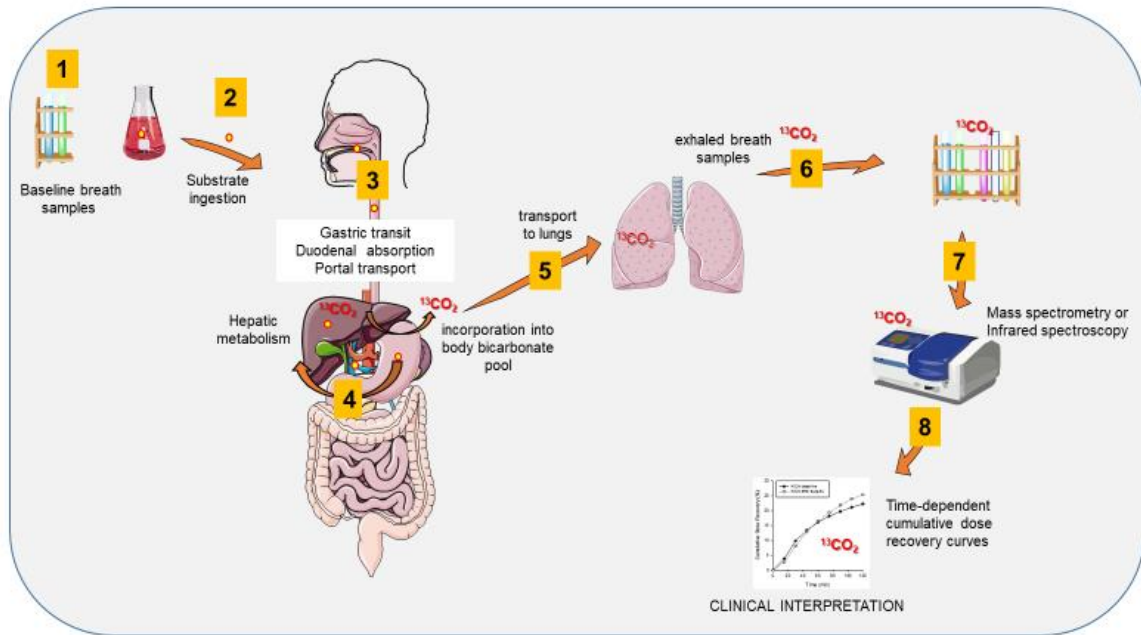
Figure 1. General methodology of breath-test analysis employing (13C)-substrates for assessment of liver function. The protocol consists of eight different steps [47-49]. Cartoons partly provided from https://smart.servier.com/

For the interpretation of results, as stated by the manufacturer, a value of delta over baseline (DOB) of <14.5‰ after 15 minutes (DOB_{15}) indicates limited hepatic function and extraction ability from portal blood flow [41, 43]. A value of cumulative percent dose recovery (cPDR) of <8.1% after 30 minutes ($cPDR_{30}$) reflects methacetin cumulative oxidation percentage over time [50], and is expression of reduced liver microsomal function [29, 41, 43, 44] **(Figure 2)**.
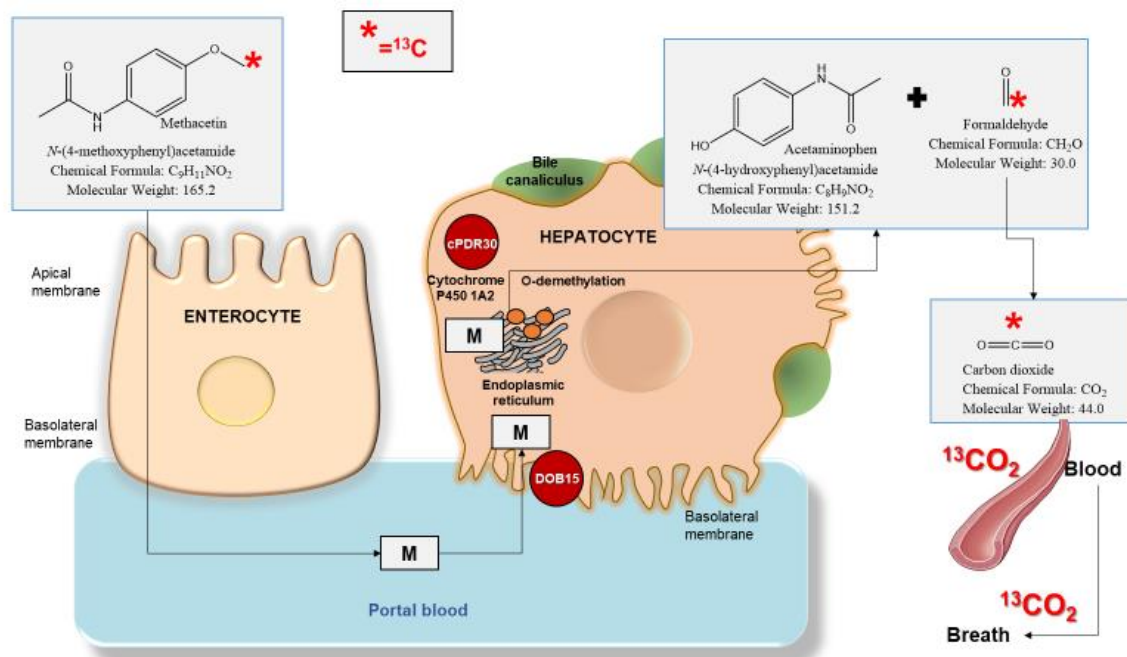

Figure 2. Event leading to metabolization of methacetin (M) for the study of liver function. Following upper intestinal absorption and portal transport, M enters the hepatocyte and is metabolised into the endoplasmic reticulum and microsomes enriched with cytochrome P450 1A2. O-demethylation generates acetaminophen and formaldehyde-derived CO2 which is promptly transferred to blood and breath. The 13C-labelled M (*) provides 13C throughout the process. Delta-over-baseline at 15 minutes (DOB_{15}) indicates the hepatic extraction

efficiency from portal blood flow. Cumulative percent dose recovery at 30 minutes (cPDR$_{30}$) indicates the efficiency of hepatic microsomal function.

2.4. *Ultrasonographic assessment of liver steatosis*

Liver steatosis was assessed by "real-time" ultrasonography using the Noblus-E equipment (Hitachi Medical, Tokyo, Japan), and a 3.5 MHz convex probe. All examinations were performed by expert operators, and according to precisely defined criteria, leading to optimal inter-observer agreement [51]. Briefly, kidney cortex echogenicity was taken as the control parenchyma, against the echogenicity of the liver parenchyma (i.e., isoechoic normal liver or hyperechoic "bright" steatotic liver). Ultrasonography reliably detects a hyperechoic texture upon diffuse fatty infiltration. This finding is a sensitive marker of liver steatosis, although its accuracy is poor for mild steatosis (<30%), and for the detection of underlying inflammation [52]. Therefore, we defined this condition as Ultrasonographic Nonalcoholic Fatty Liver Disease (uNAFLD).

2.5. *Ultrasonographic assessment of liver fibrosis*

The degree of liver fibrosis was assessed non-invasively by acoustic radiation force impulse (ARFI) technology, using the Logiq E9 (GE, Healthcare) equipment with a 3.5 MHz convex probe. All examinations were performed by skilled operators, and according to precisely defined criteria to minimize inter-observer variability. The protocol requires that the operator performs 10 measurements in each subject, focusing on the liver parenchyma, and cut-off values are calculated from the mean of measurements (<1.19=F0, no fibrosis; 1.19-1.32=F1, portal fibrosis without septa; 1.32-1.71=F2, few septa; 1.71-2.0=F3, numerous septa without cirrhosis; >2.0=F4, cirrhosis) [53].

2.6. *Blood analyses*

All blood analyses were measured in the fasting subject. Peripheral venous blood (2.5 mL) was drawn into serum separating test tubes. Within 30 min, samples were centrifuged at 3000rpm for 10 min at room temperature, generating the one-step centrifugation serum sample (about 1 mL). The Homeostatic Model Assessment for Insulin Resistance (HOMA index) was used to measure the severity of insulin resistance. The HOMA index was calculated by the following formula: [plasma glucose (mg/dL) ∗ plasma insulin (μU/mL)] / 405 [54]. Serum levels of adiponectin, leptin and high-sensitivity C-reactive protein (hsCRP) were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D Systems, Minneapolis, MN). The lower limit of detection was 62.5 pg/ml for adiponectin, 31.25 pg/mL for leptin and 15.625 for hs-CRP. The intra- and inter-assay coefficients of variation were invariably <8% for all.

2.7. *Statistics*

Data are presented as medians (range) or percentages. Kruskal-Wallis Multiple-comparison Z-value test assessed inter-group differences. Differences between two groups were tested by the non-parametric Mann-Withney U test. Differences between proportions were tested by the χ^2 test. The Spearman correlation coefficient estimated the correlations. Graphic representation of data is provided by SigmaPlot software (https://systatsoftware.com/products/sigmaplot/). Statistical analyses were performed with NCSS10 Statistical Software (NCSS, LLC. Kaysville, UT).

To calculate odds ratios (OR) and confidence intervals (CI) for serum adipokine levels associated with measurement of liver function by (13C)-MBT, separate logistic regression models were fitted. DOB$_{15}$ as markers of extraction efficiency from portal blood flow, and cPDR$_{30}$ as marker of liver microsomal function were the dependent variables, and serum adipokine levels were considered as the independent variables. The models were adjusted according to possible confounders. Models were fitted using R software version 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

A total of 67 Caucasian adults entered the study (mean age 48±3 years, 25 males, mean BMI 22.8±0.4 Kg/m2).

3.1. *Stratification of subjects according to Body Mass Index (BMI)*

General and anthropometric measurements of enrolled subjects stratified according to BMI appear in **supplementary Table 1**. Results show that age, and male to female ratios were similar across normal weight and obese subjects, while females were less represented in the overweight group (23% vs 77% in female and males, respectively). Both BMI and waist circumferences increased significantly with body weight in both sexes. **Table 1** shows that NAFLD was detected by ultrasound in 20%, 73% and 96% of normal weight, overweight and obese subjects, respectively (p=0.01 normal weight vs. other subgroups). In normal weight subjects, waist circumference was similar in subjects with and without NAFLD (92.3±3.2 and 85.7±2.1 cm, respectively, P=NS). Fibrosis, when present, was mild (score range F0-F2). In obese subjects, the fibrosis score was significantly higher than in overweight and normal weigh subjects (p=0.01).

The extent of insulin resistance, as measured by the HOMA index, increased significantly (p=0.0005) in obese and in overweight subjects, as compared to normal weight subjects. Serum levels of leptin were significantly higher in obese subjects (p=0.02), with a trend to increased leptin/adiponectin ratio in obese subjects, as compared to both normal weight and overweight. hs-CRP was also significantly higher in obese than both normal weight and overweight subjects (p=0.002).

The analysis of (13C)-MBT revealed that DOB_{15} (the marker of liver extraction for metacethin) tended to decrease in overweight subjects and decreased significantly (p=0.0039) in obese subjects, as compared to normal weight subjects. An abnormal DOB_{15} value was recorded in 33%, 54% and 62% of normal weight, overweight and obese subjects, respectively.

Values of $cPDR_{30}$ (the marker for liver microsomal function) were remarkably similar across the three subgroups. An abnormal $cPDR_{30}$ value was recorded in 7%, 8% and 15% of normal weight, overweight and obese subjects, respectively.

Table 1. Ultrasonographic, biohumoral and 13C-metacethin breath test [(13C)-MBT] measurements, according to Body Mass Index

	Normal weight (n= 15)	Overweight (n=26)	Obese (n=26)	p-value
Presence of uNAFLD, n. (%)	3 (20)	19 (73%)*	25 (96%)**	0.01
Fibrosis score (ARFI)	0 (0-1)	1 (0-2)	2 (0-2)**	0.01
HOMA index	1.0 (0.33-4.6)	2.3 (0-10.9) *	3.5 (0-9.9) *	0.0005
Leptin (ng/mL)	6.3 (1.7-73.1)	8.0 (1.3-60.3)	21.5 (3.0-67.0) **	0.02
Adiponectin (ng/mL)	5.5 (1.7-17.3)	5.7 (1.4-13.8)	6.8 (2.9-12.7)	NS
Leptin/Adiponectin ratio	1.3 (0.3-13.2)	1.5 (0.4-5.9)	2.8 (0.4-14.0)	NS
hsCRP (µg/mL)	1.07 (0.2-20.4)	2.4 (0.2-60.8)	5.5 (0.5-47.2) **	0.002
DOB_{15} (‰)	18.3 (2.5 – 37.0)	13.2 (5.5 – 28.1)	10.4 (83.8-28.1) *	0.0039
$cPDR_{30}$ (%)	12.8 (2.3-21.9)	11.1 86.1-18.3)	10.6 (5.8-20)	NS

Data are presented as median (range) or ratio, as appropriate. The p-values refer to comparisons drawn with Kruskal-Wallis Multiple-comparison Z-value test or with chi-square test, as appropriate *p<0.01 vs normal weight; **p<0.01 vs normal weight and overweight. Abbreviations: uNAFLD, ultrasonographic non-alcoholic fatty liver; HOMA, Homeostatic Model Assessment for Insulin Resistance hsCRP, high-sensitivity C-reactive protein; DOB, delta over baseline; cPDR, cumulative percent dose recovery.

Considering the population as a whole, we found that HOMA index and DOB15 (but not $cPDR_{30}$) were inversely related (P=0.04, ϱ -0.33). DOB15 and $cPDR_{30}$ di not correlate with serum levels leptin and adiponectin, nor with hsCRP (data not shown). However, by separate sub-analysis, in obese subjects DOB15 (but not $cPDR_{30}$) showed a significant positive correlation with adiponectin serum levels (**Figure 3**). This correlation was not confirmed in overweight, nor in normal weight subjects (data not shown).
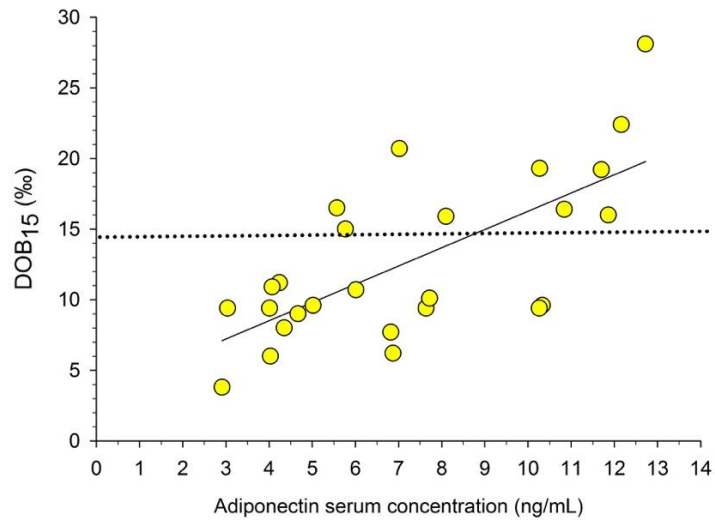
Figure 3. Correlation analysis between hepatic extraction efficiency of 13C-metacethin from portal blood flow (DOB$_{15}$ as ‰ delta over baseline after 15 minutes) and adiponectin serum concentrations in 26 obese subjects (P=0.0001; ϱ = 0.63). The horizontal dotted line indicates the normal value for DOB$_{15}$ (<14.5‰), as stated by the manufacturer.

In obese subjects, DOB$_{15}$ values progressively increased according to tertiles of serum adiponectin concentrations (P=0.0003 ANOVA, **Figure 4**).
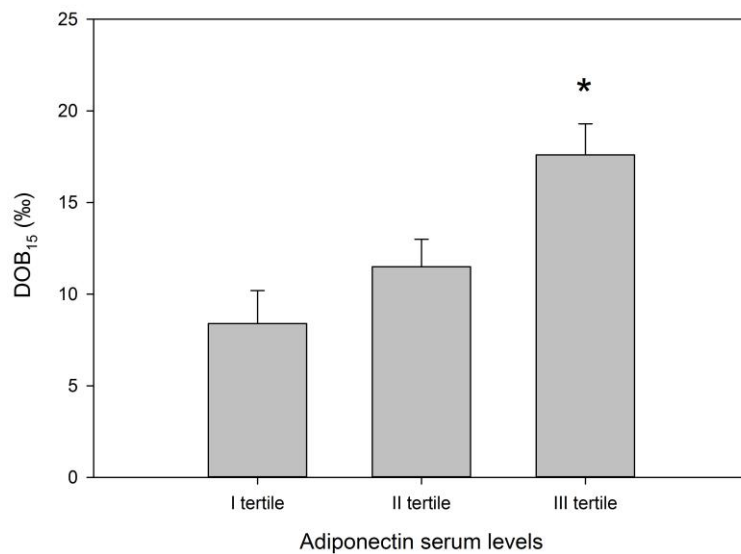

Figure 4. Results from the breath-test analysis after orally-administered (13C)-methacetin in obese subjects, stratified according to tertiles of serum adiponectin concentrations. Vertical bars represent average DOB$_{15}$ values (‰delta over baseline after 15 minutes), a marker of hepatic extraction efficiency from portal blood flow. Vertical lines indicate standard errors. *P=0.003 vs I and II tertiles (ANOVA followed by Fisher's LSD Multiple-Comparison Test).

In obese, we also used logistic regression models to calculate the ORs relating the spectrum of adiponectin and leptin serum levels according to DOB$_{15}$ and cPDR$_{30}$. The OR for DOB$_{15}$ increase changed with the tertiles of adiponectin, and was significantly higher in the case of obese subjects in the III tertile (OR 1.67 [95%CI 1.08-2.59]), than in those in the I tertile. Results persisted after adjusting for serum levels of hsCRP and for HOMA index, which were considered as covariates (OR 1.77 [95%CI 1.06-2.97]) in the III tertile, **Figure 5**).

No significant variations were showed in the ORs relating the spectrum of adiponectin serum levels according to $cPDR_{30}$ values, nor the spectrum of leptin levels, according to both DOB_{15} and $cPDR_{30}$ values.
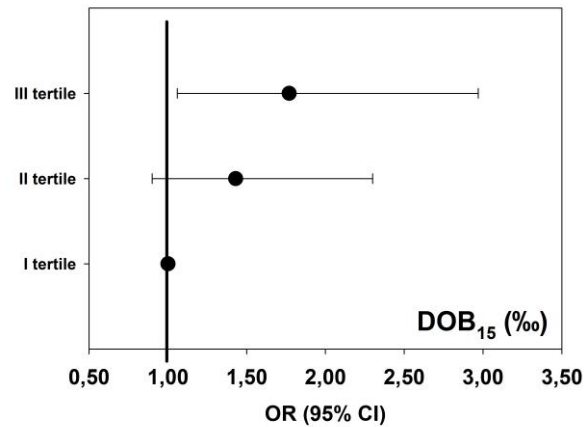

Figure 5. Odds Ratios and 95% Confidence Interval (CI) relating the spectrum of DOB_{15} values (‰delta over baseline after 15 minutes), a marker of hepatic extraction efficiency from portal blood flow, with tertiles of serum adiponectin concentrations in obese subjects. Values were calculated by logistic regression models, with DOB_{15} as dependent variable and tertiles of serum adiponectin concentrations as independent variables. The model was adjusted according to hsCRP and HOMA index, considered as confounders.

3.2. *Stratification of subjects according to presence or absence of uNAFLD*

General and anthropometric measurements of enrolled subjects stratified according to the presence or absence of uNAFLD are reported in **supplementary Table 2** and show that both BMI and waist circumference were significantly increased in subjects with NAFL, as compared to subjects without uNAFLD

In addition, **Table 2** shows that patients with uNAFLD had significantly increased liver fibrosis score, HOMA index, leptin and adiponectin serum levels, and hsCRP, as compared to patients without uNAFLD. By contrast, DOB_{15} and $cPDR_{30}$ values were significantly lower in uNAFLD subjects, as compared to those without uNAFLD. An abnormal DOB_{15} value was recorded in 35% and 60% of subjects without or with uNAFLD, respectively. Abnormal $cPDR_{30}$ values were found in 5% and 13% of subjects without or with uNAFLD.

Table 2. Liver fibrosis score, biohumoral and 13C-metacethin breath test [(13C)-MBT] measurements according to the presence of uNAFLD

	uNAFLD absent (n=20)	uNAFLD present (n=47)	p-value
Liver fibrosis score by ARFI	0 (0-1)	1 (0-4)	0.02
HOMA index	1.0 (0.3-3.1)	3.4 (0-10.9)	0.00001
Leptin (ng/mL)	4.4 (1.7-42.7)	12.4 (1.3-73.0)	0.004
Adiponectin (ng/mL)	5.0 (1.4-17.3)	6.2 (1.7-13.8)	0.03
Leptin/Adiponectin ratio	1.5 (0.3-4.5)	2.2 (0.4-14.0)	NS
hsCRP (μg/mL)	2.9 (0.2-20.4)	3.4 (0.2-60.8)	0.001
DOB_{15} (‰)	18.5 (2.5-37)	11.2 (3.8-28.1)	0.007
$CPDR_{30}$ (%)	12.5 (2.3-21.9)	10.9 (5.8-20)	0.04
Liver fibrosis score by ARFI	0 (0-1)	1 (0-4)	0.02

Data are presented as median (range). The p-values refer to comparisons drawn with Mann-Withney U test. uNAFLD, ultrasonographic non-alcoholic fatty liver disease; HOMA, Homeostatic Model Assessment for Insulin Resistance; hsCRP, high-sensitivity C-reactive protein; DOB, delta over baseline; cPDR,: cumulative percent dose recovery.

In subjects with uNAFLD, DOB_{15} and $cPDR_{30}$ values increased with adiponectin serum concentrations (P=0.02, ϱ 0.24, **Figure 6A** and P= 0.02, ϱ 0.28, **Figure 6B**, respectively), but not with leptin and hsCRP serum levels, nor with the HOMA index (data not shown). In subjects without uNAFLD, DOB_{15} and $cPDR_{30}$ did not correlate with adiponectin, leptin, hsCRP serum levels, nor with HOMA index.
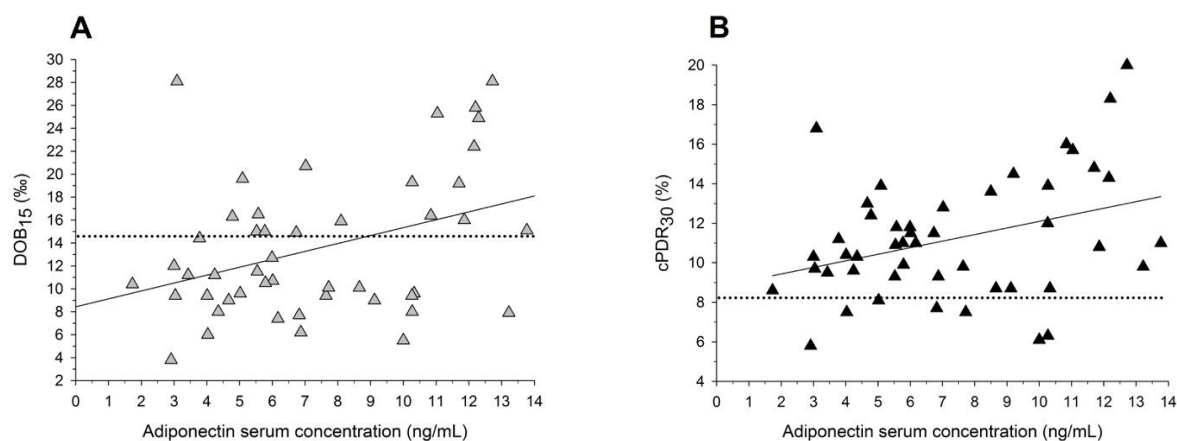

Figure 6. A) Correlation analysis between hepatic extraction efficiency of 13C-metacethin from portal blood flow (DOB_{15} as ‰ delta over baseline after 15 minutes) and adiponectin serum concentrations in 47 subjects with NAFL, as diagnosed by abdominal ultrasound (P=0.02, ϱ 0.24). The horizontal dotted line indicates the normal value for DOB_{15} (<14.5‰), as stated by the manufacturer. B) Correlation analysis between liver microsomal function by of 13C-metacethin ($cPDR_{30}$ as %), and adiponectin serum concentrations in 47 subjects with NAFL, as diagnosed by abdominal ultrasound (P= 0.02, ϱ 0.28). The horizontal dotted line indicates the normal value for $cPDR_{30}$ (<8.1%), as stated by the manufacturer.

4. Discussion

In the present study we employed (13C) breath test to study the liver extraction efficiency from the portal flow and microsomal function of the labelled substrate metacethin, as marker of liver function. We explored the potential role of two adipokines, namely leptin and adiponectin, characterized by divergent roles in the pathogenesis of NAFLD. Results show that the ongoing unbalance of both adipokines in obese and patients with NAFLD, participates in the determination of subtle alterations of the hepatic function.

We confirm that NAFLD prevalence increases in obese, compared to normal weight subjects [55], i.e. 96% vs 20% respectively. For keeping the study totally noninvasive and easily accepted by the subjects enrolled, we used ultrasonography to diagnose fat accumulation in the liver, namely uNAFLD. Technical limitations linked with this technique might have caused an underestimation of the NAFLD prevalence in lean subjects, due to the poor accuracy of liver ultrasound in diagnosing the presence of a mild steatosis (i.e. <30%) [52]. In the present series, however, normal weight subjects have been also characterized in terms of insulin resistance and adipokine levels, and results indicate the absence of significant metabolic alterations (i.e. the presence of a "metabolic obesity"[56]) in these subjects. Furthermore, normal weight subjects showed no evidence of liver fibrosis according to results by ARFI, and abdominal circumference was similar in normal weight subjects with or without uNAFLD. Although this last result is limited by the low number of normal weight subjects with uNALFD, data indicate that they should be classified as having no excess visceral fat [57]. A large meta-analysis showed that, in the general population, 5.1% of lean subjects have NAFLD [58]. This condition seems mainly linked with visceral (not general) fat, unhealthy dietary habits and genetic predisposition [59]. However, a full comprehension of liver steatosis in apparently lean, healthy subjects requires further studies [59-61].

Visceral fat acts as an endocrine organ, secreting adipokines with pro-inflammatory and atherogenic properties (i.e. tumor necrosis factor-α, resistin, interleukin-6, leptin)[19] or with anti-inflammatory, anti-oxidative and protective effects, including adiponectin [13].

In the present series, obese had significantly increased insulin resistance, higher serum concentrations of leptin and hs-CRP, than normal weight and overweight subjects. Adiponectin levels were similar in normal weight, overweight and obese subjects. This result can be explained with previous evidence describing variable adiponectin levels in obese subjects, who can be characterized by a metabolically healthy phenotype (with elevated adiponectin levels) [62] or by a phenotype with metabolic dysregulation and elevated risk of metabolic syndrome [63]. On the other hand, results obtained by (13C)-MBT point to the existence, in our series of obese subjects, of lower liver extraction efficiency from portal blood in the presence of low adiponectin levels. In fact, the highest liver extraction efficiency was noticed in subjects with adiponectin levels in the highest tertile of serum concentration.

In the whole population of enrolled subjects, the presence of a negative correlation between DOB15 and HOMA index indicates that this relationship is also influenced by the extent of insulin resistance. However, in the subgroup of obese subjects, results from logistic regression models suggest that the odds of having a high DOB15 are the highest in those in the III tertile of adiponectin serum levels, independently from the extent of chronic inflammation (hsCRP levels), and insulin resistance. Results from this analysis are limited by the small number of subjects and, thus, should be confirmed in larger studies. However, taken together, these results from obese patients suggest that the link between reduced extraction from the portal flow and extent of insulin resistance is not far evident in the presence of elevated levels of adiponectin. This confirms the hepatoprotective effect exerted by this specific adipokine. Adiponectin, in fact, is able to decrease insulin resistance [64], to exert anti-inflammatory effects [65-67] and to prevent, in the liver, accumulation of fat and free radicals potentially harmful to hepatocytes [68].

We noticed increased insulin resistance and higher levels of leptin and hsCRP in subjects with uNAFLD, confirming previous evidence indicating leptin [22] and hsCRP [69] as predictors for NAFLD. Subjects with uNAFLD also presented, on average, higher adiponectin levels than subjects without ultrasonographic evidence of fatty liver. Adiponectin has well-known protective effects on the liver [15], and low adiponectin levels can be considered a predictor of progression from NAFL to steatohepatitis [16]. Reduced adiponectin levels, in fact, exist in NAFLD patients [21], and likely linked with a progression of disease, with the severity of hepatic inflammatory changes [15, 21] and with the risk of NAFLD-associated hepatocellular carcinoma [70]. Furthermore, adiponectin levels in patients with NAFLD seem to be independent from body mass index, insulin resistance and levels of other adipokines [15].

In the present study, we described significantly lower values of both DOB15 and cPDR30 in patients with uNAFLD, as compared to those without ultrasonographic evidence of fatty liver. In this subgroup of patients, both DOB15 and cPDR30 values positively correlated with adiponectin serum levels, but not with hsCRP and leptin serum concentrations, nor with the HOMA index. In subjects with steatosis, these results point to the existence of an altered liver extraction efficiency from portal blood, and of a deranged liver microsomal function, and to a potential protective role exerted by adiponectin on the liver. In accord with this possibility, uNAFLD subjects showed, on average, significantly higher adiponectin concentrations than subjects without uNAFLD. These findings deserve further observations, also considering possible confounders. However, the apparent discrepancy observed in our series of uNAFLD patients, between increased average adiponectin serum levels and scarce hepato-protective effects should consider previous findings in NAFLD showing an altered interaction between adiponectin and its liver receptors (i.e., possible adiponectin-resistance) [71], the effects of adiponectin on portal smooth muscle cells [72, 73] and on hepatic blood flow [74], and interplays between adiponectin and the endoplasmic reticulum-mitochondria axis [75].

Binding of adiponectin to AdipoR2 receptors (predominantly expressed in the liver [76]) activates peroxisome proliferator-activated receptor-α signaling pathways, stimulates fatty acid oxidation, reduces intrahepatic triglycerides content [77] and represses the pro-inflammatory genes expression [78]. A decreased hepatic expression of AdipoR2 occurs in NAFLD patients suggesting hepatic adiponectin resistance [71]. Furthermore, adiponectin receptors are also found in rat vascular smooth muscle cells. A mechanical stretch of the right portal vein decreases adiponectin gene and protein expression time-dependently in these cells, reducing the adiponectin/leptin ratio [72]. In morbidly obese humans, the adiponectin protein expression in the liver has

been mainly detected in the endothelial cells of portal vessels and liver sinusoids. Finally, liver biopsies from NASH patients display lower staining of adiponectin and AdipoR2, as compared with NAFL subjects [73]. Taken together, these evidences point to a major functional role of adiponectin on portal hemodynamics and, possibly, in affecting the hepatic extraction efficiency from portal blood flow. A reduced venous pulsatility index has been shown by Doppler ultrasound in subjects with histologically-confirmed NAFLD [79]. In human donor livers, microcirculation assessed by laser Doppler flowmetry is diminished in macroscopically steatotic livers, as compared with normal livers [80]. In patients with advanced liver cirrhosis, increased adiponectin levels parallel decreased adiponectin hepatic extraction efficiency, altered hepatic hemodynamics and increased hepatic vascular resistance, independently from BMI and body fat mass. Of note, in the same patients, adiponectin levels are positively associated with indocyanine green half-life, a measure of effective hepatic blood flow [74].

(13C)-MBT, tracing the metabolic pathway of (13C)-methacetin in the liver, allows the identification of markers representative of the functional "reserve" of hepatic extraction efficiency from portal blood (DOB_{15}) and of microsomal functionality ($cPDR_{30}$) [47, 81].

Previous results from our group showed by (13C)-MBT that fat accumulation, NAFL and insulin-resistance were associated with decreased hepatic extraction efficiency, and that liver microsomal function was impaired in moderate-severe liver steatosis [25].

As suggested by studies in animals [34, 36] and humans [35, 37], results pointing to a decreased hepatic extraction efficiency might derive from a fat-induced increase of the intrinsic liver "stiffness", resulting in increased intrahepatic resistance to blood flow, in the absence of apparent portal hypertension and splenomegaly. The fat-mediated effects on hepatic microcirculation can be linked to sinusoidal endothelial dysfunction [34, 35, 82], to increased thromboxane and liver endothelin-1 (ET-1) expression [35], to parenchymal hypoxia [36, 82], and to architectural derangement of sinusoidal anatomy [35]. From this point of view, favorable effects of adiponectin have been previously reported in the case of endothelial dysfunction [83, 84], increased ET-1 levels[85], and liver parenchymal hypoxia [86]. Furthermore, the expression of adiponectin in the liver is maximal in endothelial cells of portal vessels and liver sinusoids, with potential implications in the pathogenesis of NASH [73].

Besides the effects on liver microcirculation, results from our study are also in line with recent findings from animal models, which indicate the endoplasmic reticulum-mitochondria axis as essential in the NAFLD progression [75]. These findings describe favorable effects exerted by the adiponectin-based agonist JT003 on this axis, through a dual agonism on AdipoR1- and AdipoR2 receptors[75].

A point of strength of the present study is the comprehensive approach to subjects with different BMI, with or without uNAFLD. Combining anthropometric and bio-humoral measurements with non-invasive, instrumental techniques (i.e., ultrasonographic assessment of fatty liver, measurement of liver fibrosis, measurement of liver function by (13C)-MBT) allowed us to depict, for the first time, the existence of complex relationships linking body and liver fat accumulation, adipokine levels, efficiency of liver extraction from the portal flow and liver microsomal function. Despite the low number of enrolled subjects, results from the present study offer a novel, integrated study model of NAFLD, with the possibility to identify early liver dysfunctions by (13C)-MBT. Larger studies need to extend these observations, for example in normal weight NAFLD patients.

A limitation of the present study is that, in case of methacetin ingestion, the final (13C)-MBT results can be affected by the rate of gastric emptying and by the absorption of the substrate from the gastrointestinal tract [26, 87]. Although the presence of such abnormalities cannot be totally excluded, none of the enrolled subjects had clinical conditions (including diabetes) potentially linked with deranged gastrointestinal motility or absorption, nor evidence of any gastrointestinal symptom.

Finally, in the present series, females were less represented than males in the overweight group. This gender difference, however, should not influence the final results since, to our knowledge, no difference has been reported, so far, in terms of (13C)-MBT outcome between genders, and gender is not considered among factors influencing the test result [26].

Conclusions

Our data indicate the existence of early alterations of liver function in obese subjects and in patients with nonalcoholic liver steatosis. These dysfunctions are linked with an altered leptin/adiponectin balance, and can be identified noninvasively by (13C)-MBT, with relevant implications in future studies exploring the onset and development of NAFLD, and prevention and therapeutic strategies of fat-associated liver diseases.

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