Peripheral insulin resistance predicts liver damage in nondiabetic subjects with nonalcoholic fatty liver disease

HEPATOLOGY

Peripheral Insulin Resistance Predicts Liver Damage in Non-Diabetic Subjects with Non Alcoholic Fatty Liver Disease

Journal:	*Hepatology*
Manuscript ID:	HEP-15-0620
Wiley - Manuscript type:	Original
Date Submitted by the Author:	29-Mar-2015

Complete List of Authors:	Rosso, Chiara; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Mezzabotta, Lavinia; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Gaggini, Melania; Institute of Clinical Physiology-CNR, Cardiometabolic Risk Laboratory, PIsa, Italy; Dipartimento di Patologia Chirurgica, Molecolare Medica e di Area Critica, Università di Pisa, Italy, Salamone, Federico; University of Catania, Department of Internal Medicine, Catania, Italy Gambino, Roberto; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Marengo, Andrea; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Saba, Francesca; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Vanni, Ester; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Jouness, Rami; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Saponaro, Chiara; Institute of Clinical Physiology-CNR, Cardiometabolic Risk Laboratory, PIsa, Italy; Dipartimento di Biotecnologie, Chimica e Farmacia, University of Siena, Italy, Buzzigoli, Emma; Institute of Clinical Physiology-CNR, Cardiometabolic Risk Laboratory, Pisa, Italy Caviglia, Gian Paolo; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy Abate, Maria; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy, Smedile, Antonina; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy, Rizzetto, Mario; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy, Cassader, Maurizio; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy, Gastaldelli, Amalia; Institute of Clinical Physiology-CNR, Cardiometabolic Risk Laboratory, PIsa, Italy Bugianesi, Elisaetta; Division of Gastro-Hepatology, Department of Internal Medicine, San Giovanni Battista Hospital, University of Torino, Italy,

Keywords:	OGIS, Liver fibrosis, Hepatic complications, NAFLD, OGTT

Peripheral Insulin Resistance Predicts Liver Damage in Non-Diabetic Subjects with Non Alcoholic Fatty Liver Disease

Short title: OGIS and liver fibrosis in NAFLD

[1]Chiara Rosso, [1]Lavinia Mezzabotta, [3, 4,]Melania Gaggini, [2]Federico Salamone, [1]Roberto Gambino, [1]Andrea Marengo, [1]Francesca Saba, [1] Ester Vanni, [1]Rami I.K. Jouness, [3,5,]Chiara Saponaro, [3]Emma Buzzigoli, [1]Gian Paolo Caviglia, [1]Maria Lorena Abate, [1]Antonina Smedile, [1]Mario Rizzetto, [1]Maurizio Cassader, [3]Amalia Gastaldelli and [1]Elisabetta Bugianesi.

[1]Division of Gastroenterology and Hepatology and Lab. of Diabetology, Dept. of Medical Sciences, University of Turin, Turin, Italy; , [2] Division of Gastroenterology, Azienda Sanitaria Provinciale di Catania, Catania, Italy.[3] Cardiometabolic Risk Unit, Institute of Clinical Physiology, CNR, Pisa, Italy, [4] University of Pisa, Italy, [5] University of Siena, Italy.

Correspondence to:

Amalia Gastaldelli, PhD,
Head of Cardiometabolic Risk Unit
Institute of Clinical Physiology, CNR
via Moruzzi 1 56100 Pisa Italy
tel +39 050 3152679/80
fax +39 050 3152166
email amalia@ifc.cnr.it

Keywords: OGIS, liver fibrosis, hepatic complications, NAFLD

Grant support: The project was funded by FP7/2007-2013 under grant agreement n° HEALTH-F2-2009-241762 for the project FLIP (E. Bugianesi and A. Gastaldelli). E. Bugianesi received funds from PRIN 2009ARYX4T. A. Gastaldelli received funds from the European Foundation for the Study of Diabetes (EFSD-Pfizer grant). C. Saponaro is recipient of a fellowship from "Dottorato Pegaso Regione Toscana in Biochemistry and Molecular Biology".

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the receiver operating curve; BIGTT, β-cell function, insulin sensitivity index derived from oral glucose tolerance test; BMI, body mass index; DM, type 2 diabetes mellitus; EGP, endogenous glucose production; $eMCR^{dem}$, metabolic clearance rate estimation with (dem) or without (nodem) demographic parameters; FIRI, fasting insulin resistance index; HDL-Chol, high density lipoprotein cholesterol; FPI, fasting plasma insulin; G, glucose; HepIR-DF, De Fronzo hepatic insulin resistance index; HIRi, hepatic insulin resistance index derived from tracer analysis; HOMA, homeostasis model assessment; FPI, fasting plasma insulin; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index; PLT, platelets; QUICKI, quantitative insulin sensitivity check index; SD, standard deviation; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test.

Disclosures: nothing to disclose

Author Contributions: C. Rosso, E. Bugianesi, study concept and design; L. Mezzabotta, F. Salomone, R. Gambino, F. Saba, M. Gaggini, RI. Jouness, E. Buzzigoli, C. Saponaro, acquisition of data; C. Rosso, E. Vanni, GP. Caviglia, ML. Abate, A. Gastaldelli, E. Bugianesi, analysis and interpretation of data; C. Rosso, E. Vanni, E. Bugianesi, drafting of the manuscript; , E. Vanni, F. Salomone, A. Smedile, M. Rizzetto, M. Cassader, A. Gastaldelli and E. Bugianesi, critical revision of the manuscript for important intellectual content; G.P. Caviglia, M.L. Abate, administrative, technical, or material support; A. Gastaldelli, E. Bugianesi, study supervision.

Abstract

BACKGROUND & AIMS. Surrogate indexes of insulin resistance/sensitivity (IR/IS) are widely used in Non Alcoholic Fatty Liver Disease (NAFLD) although they have never been validated in this population. We aimed to validate the available indexes in NAFLD subjects and to test their ability to predict liver damage and hepatic/ metabolic complications.

METHODS. Surrogate indexes were validated by tracer technique (D2-glucose and U-13C-glucose) in the basal state and during an Oral Glucose Tolerance Test (OGTT). The best performing indexes were used in an independent cohort of 145 non-diabetic NAFLD subjects to identify liver damage (fibrosis and NASH) and the longitudinal risk of hepatic/metabolic complications.

RESULTS. In the validation NAFLD cohort, HOMA-IR, IGR and ISI Stumvoll had the best association with hepatic IR, while peripheral IS was most significantly related to OGIS, ISI Stumvoll and $eMCR^{nodem}$. In the independent cohort, only OGTT derived indexes were associated with liver damage and OGIS was the best predictor of moderate/severe fibrosis (OR=0.70, 95% CI=0.58-0.85, *P*=0.0002) and NASH (OR=0.75, 95% CI=0.63-0.90, *P*=0.002). Both OGIS and NAFLD Fibrosis Score (NFS) identified advanced (F3/F4) fibrosis, but only OGIS was able to discriminate F2 from F3/F4 (*P*<0.003). After an average follow up of 112 ± 42 months, OGIS ≤ 10 mg/Kg min predicted all the liver-related complications, while NFS < -1.455 only 20% of them. OGIS was also inversely related with the risk of metabolic events (OR 0.66, 95% CI 0.48-0.90, P=0.0096).

CONCLUSIONS. OGIS is associated with peripheral IS in NAFLD and is inversely associated with an increased risk of advanced liver damage and metabolic complications in non-diabetic subjects with NAFLD.

Non-alcoholic Fatty Liver Disease (NAFLD) embraces a spectrum of liver damage ranging from isolated hepatic triglyceride accumulation (steatosis) through hepatic triglyceride accumulation plus inflammation (non-alcoholic steatohepatitis, NASH) [1] ultimately progressing to fibrosis/cirrhosis and potentially hepatocellular carcinoma. [2-4] NAFLD has become the most common cause of chronic liver disease in the Western countries [5] owing to increasing rates of obesity and diabetes in both adult and childhood [6-9] and one of the top concerns for hepato-gastroenterologists and endocrinologists due to its potential hepatic and metabolic complications. [10] An increasing number of features of the metabolic syndrome (MS), particularly type 2 diabetes (DM) and obesity, are risk factors for the presence of NASH [11] and are important phenotype reflections of insulin resistance (IR), one of the main pathogenic mechanisms for the onset and progression of NAFLD.[12-13] In clinical practice, this translates into the wide use of HOMA-IR as a screening tool to detect IR in NAFLD [14] and in the development of several algorithms for the non-invasive prediction of severe liver damage based on multiple components of the MS [15-16].

HOMA-IR is a rather crude index reflecting the final effects of hepatic and peripheral IR on fasting glucose homeostasis [17], but from the physiological point of view discerning the sites of IR is not trivial. Insulin resistance in the skeletal muscle (or peripheral IR) is defined as a lower than expected effect of insulin on glucose disposal by the muscle, leading to hyperglycemia and compensatory hyperinsulinemia and favoring de novo lipogenesis (DNL) in the liver. Peripheral IR is tightly linked to IR in the adipose tissue (ARi), i.e. impaired suppression of lipolysis and increased fatty acid flux from the adipocytes to other organs, including the liver.[18] In the liver IR leads to impaired suppression of glucose production and high glucose as well as insulin levels, thus setting up a vicious cycle.While the association between ARi and liver damage has been recently established, the relative role of hepatic and peripheral IR is currently unclear. [18,19-21]

Techniques to directly measure insulin resistance/sensitivity (IR/IS) are time-consuming, expensive and often unavailable in daily routine; therefore, more simple tests have been developed based on insulin and/or glucose levels in the fasted state [22-27] or during an oral glucose tolerance test

(OGTT) combined with other metabolic and anthropometric parameters. [28-35] Although the use of surrogate indexes of IR is widely performed in NAFLD patients, none of them has been validated in this population against a golden standard for the assessment of peripheral and hepatic IR so far.

Some of these indexes (HOMA-IR, OGIS) have also been associated with increased liver damage in NAFLD,[13,36] but a comparison with the non-invasive algorithms for the detection of NASH or fibrosis has never been made. The NAFLD fibrosis score (NFS) is currently the best validated score for the prediction of advanced fibrosis, both in cross sectional and longitudinal cohorts,[15,37] but its performance is linked to the presence of obesity and diabetes and may be unsatisfactory in the absence of one or both these variables.

Thus, the aim of this study is two-fold: (1) to evaluate the diagnostic accuracy of the surrogate indexes of IR/IS by comparison with state-of-the-art technique in NAFLD patients and (2) to test the best IR/IS indexes in an independent cohort of non-diabetic NAFLD subjects for the prediction of histological liver damage and disease progression in comparison with NFS.

Patients and Methods

i. Validation of the Diagnostic Accuracy of the Surrogate Indexes of Insulin Resistance/Insulin Sensitivity

Subjects and Study Design.

In twenty-two non-diabetic subjects with biopsy-proven NAFLD, *in vivo* hepatic and peripheral IR were assessed by stable isotopes technique in the fasting state and during a 4-hours oral glucose load (OGTT). Eleven healthy subjects matched by age, sex and BMI served as controls. All of them had normal liver enzymes, normal liver at US-scan and low probability of hepatic fat according to non-invasive indexes (Fatty liver Index < 20 and NAFLD liver fat Score > 1.257). All experiments were performed after an overnight fast. A primed-continuous infusion of D2-glucose (bolus 22 μmol/kg, infusion rate 0.22 μmol/kg min), to assess Endogenous Glucose Production (EGP), was delivered for approximately 4 hours, i.e., 2 hours in the fasting state and 2 hours after administration of a 75 g glucose load enriched with U-13C-glucose to determine glucose clearance.[35, 38]

Analytical Methods and Calculations.

Serum glucose was measured by the glucose oxidase method (Sentinel, Milan, Italy). Triglycerides and total-cholesterol (Chol) were performed by enzymatic colorimetric assays (Sentinel, Milan, Italy). High Density Lipoprotein-Chol (HDL-Chol) was determined by enzymatic colorimetric assay after precipitation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL). LDL-Chol was measured with a standardized homogeneous enzymatic colorimetric method in order to avoid triglycerides effects on LDL determination (Sentinel, Milan, Italy).

Tracers enrichments of 6,6-2H-glucose and U-13C-glucose were determined by a gaschromatography-mass spectrometry system (Agilent 5975, Palo Alto, CA), selectively monitoring ions at m/z 200, 201, 202 and 205 and expressed as tracer/tracee ratios as previously described.[12, 38]

Metabolic indexes derived from tracers analysis included the following: in the fasting state, hepaticIR (HIRi) was calculated as the product [fasting EGP x Fasting Plasma Insulin]; during the OGTT, peripheral insulin sensitivity was derived from glucose clearance as [EGP/Glucose concentration]. [12, 35, 38]

Surrogate indexes of IR/IS were calculated according to published formulas (Supplementary table 1).

ii. Association of Surrogate Indexes of Insulin Resistance/Sensitivity with Liver Damage

Subjects and Study Design

An independent cohort of 145 consecutive NAFLD patients was considered to examine the relation of the best performing indexes with the histological liver damage. Patients were selected from a prospectively built database including biopsy-proven NAFLD patients, recruited since 2000 at the GI Division of the University Hospital of Torino, based on the following criteria: absence of DM, absence of overt cirrhosis, BMI<35 and liver biopsy performed before 2007 in order to have a follow up long enough to detect liver related and metabolic complications. One hundred and sixty-six patients met the above mentioned criteria. Starting in 2001, a standard 2-h 75g OGTT was part of the investigation protocol in patients without known diabetes; the tests had been performed at the time of liver biopsies (± 1 month) and blood samples were collected at 0, 30, 60, 90, 120 and 180 min for the measurement of plasma glucose and insulin levels. Nine patients were further excluded because OGTT was not performed according to the above mentioned protocol, therefore the independent cohort initially included 157 patients. As OGTT newly diagnosed DM in 12 of them, we excluded these patients from the analysis, that was finally carried out in 145 NAFLD subjects.

All patients had been previously diagnosed NAFLD based on the following criteria: (1) elevated aminotransferases for at least 6 months; (2) liver biopsy showing changes consistent with NAFLD; and (3) exclusion of other etiologies, including viral, autoimmune, cholestatic, genetic, metabolic,

alcoholic, or drug-induced liver diseases. All patients had current and past consumption of ethanol less than 20 g per day on direct questioning of both the patients and a close relative.

A complete medical history and physical examination had been undertaken. Clinical and anthropometric data were collected at the time of liver biopsy. BMI was calculated on the basis of weight (in kilograms) and height (in meters) and subjects were classified as normal weight (BMI, 18.5-24.9 kg/m^2), overweight (BMI 25-29.9) and obese (≥30). Waist circumference (to the nearest half centimeter) was measured at the midpoint between the lower border of the ribcage and the iliac crest. Laboratory investigations included routine liver biochemistry (alanine-aminotransferase [ALT] and aspartate-aminotransferase [AST] levels, total bilirubin, albumin, alkaline phosphatase, and gamma glutamyltranspeptidase), complete blood count, fasting glucose, fasting insulin, total cholesterol, HDL-Chol and total triglycerides.

The presence of the MS was detected according to the presence of any three out of five components (waist circumference>94 cm for men and >80 cm for women; fasting blood glucose>100 mg/dl; triglycerides >150 mg/dl or under treatment; blood pressure >130/85 mm Hg or under treatment; and HDL cholesterol <40 mg/dl in men or <50 mg/dl in women).[39]

The NAFLD patients participated in the tracers study after signing an informed consent. All the other investigations were carried out during regular follow-up of NAFLD patients, according to specific protocols, and all patients had given their consent for including their personal data in the database. The study was approved by the ethics committee of the University Hospital San Giovanni Battista of Torino and was in accordance with the Helsinki Declaration.

Liver Histology

Liver biopsies were stained with hematoxylin and eosin, Masson's trichrome and special stains for iron and copper and were read by a single liver pathologist. All biopsies were of appropriate size and included enough portal tracts for a confident pathological grading and staging of the histological features. Histological features of NAFLD, i.e. steatosis, inflammation, hepatocyte

ballooning, and fibrosis, were scored as according to Kleiner et al.[40] NASH was defined by the local pathologist according to the joint presence of steatosis, hepatocyte ballooning and lobular inflammation with or without fibrosis.

Statistical Analysis

Data are reported as mean and standard deviation (SD) for continuous normal variables or number and percentage (%) for categorical variables. Comparisons between groups were performed using the two-tailed Student's *t*-test for normal continuous variables and the Mann-Whitney non-parametric test for non-normal continuous variables. For categorical data, the Fisher exact test or the χ-square test were used as appropriate.

Logistic regression analysis was performed to identify the better IR/IS surrogate indexes able to predict $F \geq 2$ and histological diagnosis of NASH. All the analyses were adjusted for age, sex and BMI. To evaluate the performance of both OGIS and NFS in discriminating $F \geq 2$, Receiver Operating Curves (ROC) analysis was computed. Cox regression was used to assess the effects of OGIS and NFS on the metabolic events development probability.

Dot plots were used to represent the distribution of liver-related and metabolic events at follow up according to OGIS and NFS. Values of $P<0.05$ were considered statistically significant. All calculations were performed with MedCalc® Software bvba version 12 (Mariakerke, Belgium).

Results

Baseline Characteristics of the Validation Cohort and of the Independent Cohort of NAFLD Subjects

Clinical, biochemical and histological data of the validation cohort (n=22) and of the independent cohort (n=145) are reported in Table 1. The two cohorts were similar with regard to age, gender, BMI, liver function test, metabolic profile and liver histology (steatosis, fibrosis and NAS score), although a diagnosis of NASH was more frequent in the validation cohort. In the independent cohort of non-diabetic NAFLD the prevalence of obesity was low (19%) but central obesity was found in half the population and was the most common metabolic abnormality followed by hypertriglyceridemia (26%) and elevated fasting glucose (25%). Only 16% of the subjects met the criteria for diagnosis of the metabolic syndrome; nonetheless, 44% had at least two features and 76% at least one. HOMA-IR was >2.7 in the vast majority of them (72%).

Validation of Surrogate Indexes of Insulin Resistance/Insulin Sensitivity vs Direct Tracer Measurements.

Table 2 summarizes the data from the tracer studies and all the surrogate indexes calculated in the validation cohort of NAFLD patients and in the control group. Although well matched for age, sex and BMI with controls, NAFLD subjects were characterized by increased fasting EGP and hepatic IR (HIRi) and by decreased peripheral IR (glucose clearance) assessed by tracers.

HIRi was significantly related with all the surrogate indexes except for TG/HDL-Chol, SiOGTT and HepIR DF (Supplementary Table 2), but HOMA-IR, IGR and ISI Stumvoll had the best correlation (Fig.1 A-B-C). Glucose clearance was related to the majority of the surrogate indexes as well (Supplementary Table 2), but OGIS, ISI Stumvoll and $eMCR^{nodem}$ were the most significant (Fig.1 D-E-F). Of note, HOMA-IR and IGR are indexes of insulin resistance, while OGIS, ISI Stumvoll and $eMCR^{nodem}$ assess insulin sensitivity and show a positive correlation with glucose clearance and a negative correlation with HIRi.

Surrogate Indexes Associated with Moderate/Severe Fibrosis

Next we examined the association between all the surrogate indexes and fibrosis F≥2 in the independent cohort of NAFLD subjects (Table 3). At univariate analysis, only the indexes based on OGTT were significantly associated with moderate/severe fibrosis, but at logistic regression, adjusted for age, sex and BMI, OGIS was the sole independent predictor , having an inverse relationship with fibrosis (OR = 0.70, 95% CI = 0.58-0.85, *P* = 0.0002).

We further investigated the reason why OGTT derived indexes were correlated with fibrosis by splitting the independent cohort according to the degree of fibrosis at liver biopsy and to the presence of overweight/obesity (defined as BMI>27) (Figure 2). During OGTT the glucose curve was higher in subjects with moderate/severe fibrosis (F≥2) independent of BMI (Fig. 2 A), while the insulin curve was similarly elevated in all NAFLD patients except for the lean ones without fibrosis who displayed the lower levels of insulin (Fig. 2 B). This pattern was not influenced by steatosis, as the glucose curve was similar in all patients independent of the degree of hepatic fat and of BMI (Panel C), suggesting a direct association between fibrosis and glucose levels during OGTT.

Last of all, we tested the reliability of NFS as predictors of fibrosis in this population without DM and compared it with that of OGIS. Overall, the degree of fibrosis was negatively associated with OGIS and positively associated with NFS (ANOVA < 0.0001 and 0.007, respectively), but only OGIS was able to discriminate moderate (F2) from severe (F3/F4) fibrosis (Figure 3 A-B). The sensitivity and specificity of the two indexes by ROC analysis are shown in Supplementary Table 3. The AUROCs were similar (Supplementary Figure 1) but, compared with NFS, OGIS had a better PPV and a worse NPV. At logistic regression analysis both indexes significantly predicted moderate/severe fibrosis, but the association was more meaningful with OGIS (Supplementary Table 4). Patients with lower values of OGIS had a 31% increased risk of significant fibrosis.

Surrogate Indexes Associated with Diagnosis of NASH

The same analysis were performed in order to examine the relationship between all the surrogate indexes and the diagnosis of NASH. Once again, only indexes derived from OGTT (OGIS, SiOGTT, BIGTT, eMCR^{dem} and eMCR^{nodem}) had a significant association with NASH and OGIS was the best predictor at logistic regression analysis (OR = 0.75, 95% CI = 0.63-0.90, *P* = 0.0021) (Table 4).

Long-Term Outcomes Based on OGIS and NAFLD Fibrosis Score risk categories

Follow up data were available in most of the study subjects. Five of the 13 patients lost had an histological diagnosis of simple fatty liver (SFL) and had been referred back to their GP, the remaining had F1/F2 fibrosis (n=6) and F3 (n=2). Not unexpectedly, after an average follow up of 112 ± 42 months (range 42-240), the number of liver-related and metabolically-related events was very low in this cohort. Liver-related events developed in 5 subjects, all with F3/F4 fibrosis at index biopsy: only one patient died from sudden acute decompensation, one developed HCC, one encephalopathy and ascites and two thrombocytopenia that prompted for a second liver biopsy, documenting in both cases a transition from F3 to F4. Other complications developed in 20 subjects, including metabolically-related in 12 of them (diabetes in 6, and cardiovascular events in 6) and non-liver cancers in 3 of them. Besides the mentioned liver-related death, the only other one was due to hemorrhagic stroke.

We used the average value of OGIS in the control group of the tracers studies as a cut-off . OGIS ≤ 10 correctly identified all the subjects who developed liver-related events at follow up (Figure 4 A, left). Further, OGIS properly identified the development of diabetes in 5/6 and of cardiovascular complications in 4/6 cases (Figure 4 A, right).

On the contrary, only 1 liver-related event was correctly identified by NFS according to published cut-offs (Figure 4, Panel B left). Most subjects (85%) with severe fibrosis (F3/F4) fell into the

"indeterminate" or "not-significant" fibrosis category, including 17% of severe fibrosis incorrectly classified as "not-significant".

As expected for the low number of cases, none of the two indexes were predictors of liver events at Cox regression analysis. However, lower values of OGIS significantly predicted metabolic events (OR 0.66, 95% CI 0.48-0.90, P=0.0096).

Discussion

The results of this study have significant implications, both from the pathophysiological and clinical point of view. Firstly, it provides a rationale for the use of surrogate indexes of insulin resistance/sensitivity in NAFLD patients that extends beyond the bare identification of IR, secondly supports the role of IR *per se* as a mechanism of onset and progression of NAFLD and highlights the unexplored relevance of peripheral IR, and lastly provides an index able to identify NAFLD patients at risk for significant liver damage even in the absence of diabetes and overt obesity.

Although widely used in epidemiological studies of NAFLD, surrogate indexes of IR have been validated by state-of-the-art techniques in healthy controls, obese and diabetic subjects but never in a NAFLD population.[41] This is the first study to compare IR/IS in the liver and in the muscle in a group of patients with NAFLD, in the absence of cofactors such as diabetes and overt obesity (BMI>35) that greatly influence insulin sensitivity. The exclusion of diabetic subjects also comes from the consideration that these indexes are affected by exhaustion of insulin secretion by the pancreas, besides a questionable clinical utility. In this validation cohort, several indexes derived from either fasting values [22-27] or OGTT sampling times [28-35] resulted valid, but HOMA-IR, IGR and ISI Stumvoll had the best association with hepatic IR, while peripheral IS was most significantly related to OGIS, ISI Stumvoll and $eMCR^{nodem}$. HOMA-IR is the most used in population studies for its simplicity and wide applicability and is based on fasting glucose and insulin levels, where glucose concentration is mainly driven by hepatic glucose production. [13,22] However, the relationship between HOMA-IR, IGR and HIRi can be affected by collinearity and in

this sense the association of HIRi with OGTT-derived indexes, such as ISI Stumvoll, is certainly more meaningful. On the contrary OGIS, ISI Stumvoll and eMCR^{nodem} mostly reflect glucose uptake by the muscle, i.e. the degree of peripheral insulin sensitivity; as such, they directly correlate with glucose clearance measured by tracers technique.

When tested in the independent cohort of NAFLD patients, the strength by which most surrogate indexes correlate with moderate/severe fibrosis at liver biopsy is similar to their correlation with IR/IS, confirming that the degree of IR *per se* is an important mechanism of onset and progression of NAFLD independent of its phenotypic expression (diabetes first). All the OGTT-derived indexes were better predictors of fibrosis and NASH, and OGIS confirms its effectiveness in identifying patients with advanced disease [12,36] even in a cohort considered at low risk. This finding raises the issue of the actual contribution of peripheral IR to the onset and progression of liver damage, beyond that already established of ARi. In particular, the association with fibrosis appears to be mediated by post-load glucose metabolism independent of BMI and of the degree of steatosis. Adipose tissue and skeletal muscle IR are tightly linked. In obese subjects with NAFLD there is an abrupt early-on decline in insulin action on skeletal muscle by increasing degrees of Ari [42-43], suggesting that skeletal muscle is rapidly affected by dysfunctional adipose tissue. Peripheral IR can independently contribute to NAFLD and NASH by changing the pattern of ingested carbohydrate from skeletal-muscle glycogen synthesis to hepatic DNL [44] via activation of sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP). The contribution of DNL, which is less than 5% in healthy subjects, increases to 26% in NAFLD leading to a 3-fold higher rate of production of VLDL-triglycerides and increased lipid flux to the liver [45]. On the other side, newly synthesized fatty acids from DNL, mainly palmitic acid, are fully saturated and are more likely to promote lipotoxic injury. Evidence of this in mice was provided by recent studies demonstrating that generation of fatty acids in the liver through fructose-driven DNL was associated with endoplasmic reticulum stress and cellular injury [45-46].

The last implication is clinical: all the non-invasive indexes of fibrosis in NAFLD usually include BMI and diabetes along with other components of the MS, supported by a burden of cross-sectional and longitudinal data linking a worse clinical outcome to diabetes and frank obesity. Although not designed to explore the natural history of NAFLD, this study confirms that the risk of advanced liver damage and liver-related events is lower in the absence of these important co-factors, but certainly not negligible. At the time of liver biopsy, half of the patients in the independent cohort had significant (≥ F2) fibrosis and 25% had severe fibrosis; in the latter group, 15% developed liver-related events in a range between 42 and 240 months. Remarkably, OGIS was able to discriminate F2 from F3/4 better than NFS, which is an important goal. In fact, the main limitation of currently available tests is that they perform best at distinguishing severe *vs*. non-severe fibrosis but not advanced (F2-F4) or any (>F1) fibrosis *vs*. no fibrosis. However, while the negative prognostic value of F1 is questionable, patients with F2 should be identified for a closer follow up and possibly a pharmacological therapy. Longitudinally, all liver-related events were predicted by OGIS ≤ 10, but the majority were missed by NFS. Although having a good sensitivity the NPV of OGIS was far from excellent, but the low number of liver related events strongly influenced the analysis and confirmation in larger cohorts is necessary. Consistent with being a quantitative measure of insulin sensitivity, OGIS ≤ 10 also resulted an independent predictor of metabolic complications, correctly identified in 75% of cases. The increased risk of prevalent and incident diabetes linked with NAFLD is well documented across literature and requires screening procedures, based on fasting or random blood glucose or haemoglobin A1c. In case of prediabetes (fasting glucose, 100-126 mg/dl; HbA1c, 5.7-6.4%), a standardized 75-g OGTT is advisable [47] and calculation of OGIS from OGTT is clinically relevant as it can add the risk of fibrosis and NASH in these subjects.

Limitations of the present study include the risk of collinearity in the validation of surrogate indexes, which is more significant when comparing HIRi vs indexes based on fasting insulin levels such as HOMA-IR. Another limitation is the limited number of events in the longitudinal follow up,

which was somehow expected. Therefore, additional studies in wider cohorts are needed to validate our findings. An important issue is the lack of genetic data, particularly for *PNPLA3* variants that have been extensively linked to more severe liver damage and increased risk of HCC. [48-49] However, *PNPLA3* action is independent of IR both at the peripheral and hepatic levels, hence its impact on the usefulness of insulin resistance/sensitivity indexes should be negligible.

In conclusion, this proof-of-principle study provides the rationale for the use of OGIS as non invasive index of liver damage as well as predictor of metabolic complications in a low-risk population of NAFLD subjects, and its effectiveness should be validated both cross-sectionally and longitudinally in larger cohorts.

References

1. Kleiner DE, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. Semin Liver Dis 2012;32:3-13.
2. Caldwell SH, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH, Driscoll CJ. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. Hepatology 1999;29:664-666.
3. Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. J Hepatol 2012;56:1384-1391.
4. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002;123:134-140.
5. Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, Srishord M. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. Clin Gastroenterol Hepatol 2011;9:524-530.
6. **Ogden CL**, Yanovski SZ, Carroll MD, Flegal KM. The epidemiology of obesity. Gastroenterology 2007;132:2087-2102.
7. Ogden CL, Carroll MD, Flegal KM. High body mass index for age among US children and adolescents, 2003–2006. JAMA 2008;299:2401-2405.
8. Fazeli Farsani S, van der Aa MP, van der Vorst MM, Knibbe CA, de Boer A. Global trends in the incidence and prevalence of type 2 diabetes in children and adolescents: a systematic review and evaluation of methodological approaches. Diabetologia 2013;56:1471-1488.
9. Geiss LS, Wang J, Cheng YJ, Thompson TJ, Barker L, Li Y, Albright AL, et al. Prevalence and Incidence Trends for Diagnosed Diabetes Among Adults Aged 20 to 79 Years, United States, 1980-2012. JAMA 2014;312:1218-1226.
10. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and the natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274-285.

11. Hamaguchi M, Kojima T, Takeda N, Nakagawa T, Taniguchi H, Fujii K, Omatsu T, et al. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Intern Med 2005;143:722-728
12. Bugianesi E, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, Ponti V, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. Diabetologia 2005;48:634-642.
13. Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, Bertelli C, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. Hepatology 2008;48:792-798.
14. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care 2004:27(6):1487-95.
15. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007;45:846-854.
16. Harrison SA, Oliver D, Arnold HL, Gogia S, Neuschwander-Tetri BA. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. Gut 2008;57:1441–1447.
17. Matsuda M. Measuring and estimating insulin resistance in clinical and research settings. Nutrition, Metabolism & Cardiovascular Diseases 2010;20:79-86
18. Gaggini M, Morelli M, Buzzigoli E, De Fronzo RA, Bugianesi E, Gastaldelli A. Non-Alcoholic Fatty Liver Disease (NAFLD) and Its Connection with Insulin Resistance, Dyslipidemia, Atherosclerosis and Coronary Heart Disease. Nutrients 2013;5:1544-1560.
19. Gastaldelli A, Harrison SA, Belfort-Aguilar R, Hardies LJ, Balas B, Schenker S, Cusi K. Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. Hepatology 2009;50:1087-1093
20. Lomonaco R, Ortiz-Lopez C, Orsak B, Webb A, Hardies J, Darland C, Finch J, 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

21. Musso G, Cassader M, De Michieli F, Rosina F, Orlandi F, Gambino R. Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. Hepatology 2012;56:933-942
22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419.
23. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ. Quantitative insulin sensitivity check Index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 2000;85:2402-2410.
24. Duncan MH, Singh BM, Wise PH, Carter G, Alaghband-Zadeh J. A simple measure of insulin resistance. Lancet 1995;346:120-121.
25. Hanson RL, Pratley RE, Bogardus C, Narayan KM, Roumain JM, Imperatore G, Fagot-Campagna A, et al. Evaluation of simple indices of insulin sensitivity and insulin secretion for use in epidemiologic studies. Am J Epidemiol 2000;151:190-198.
26. Anderson RL, Hamman RF, Savage PJ, Saad MF, Laws A, Kades WW, Sands RE, et al. Explotation of insulin sensitivity measures derived from frequently sampled intravenous glucose tolerance (FSIGT) tests: the insulin Resistance Atherosclerosis Study. Am J Epidemiol 1995;142:724-732.
27. Kim-Dorner SJ, Deuster PA, Zeno SA, Remaley AT, Poth M. Should triglycerides and the triglycerides to high-density lipoprotein cholesterol ratio be used as surrogates for insulin resistance? Metabolism 2010;59:299-304.
28. Mari A, Pacini G, Murphy E, Ludvik B, Nolan JJ. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. Diabetes Care 2001;24:539-548.
29. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes 1999;22:1462-1470.
30. Bastard JP, Vandernotte JM, Faraj M, Karelis AD, Messier L, Malita FM, Garrel D, et al. Relationship between the hyperinsulinemic-euglycaemic clamp and a new simple index assessing insulin sensitivity in overweight and obese postmenopausal women. Diabetes Metab 2007;33:261-268.

31. Stumvoll M, Mitrakou A, Pimenta W, Jenssen T, Yki-Järvinen H, Van Haeften T, Renn W, Gerich J. Use of the oral glucose tolerance test to assess insulin resistance and insulin sensitivity. Diabetes Care 2000;23:295-301.

32. Abdul-Ghani MA, Matsuda M, Balas B, DeFronzo RA. Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. Diabetes Care 2007;30:89-94.

33. Vangipurapu J, Stančáková A, Kuulasmaa T, Paananen J, Kuusisto J; EGIR-RISC Study Group, Ferrannini E, et al. A novel surrogate index for hepatic insulin resistance. Diabetologia 2011;54:540-543.

34. Hansen T, Drivsholm T, Urhammer SA, Palacios RT, Vølund A, Borch-Johnsen K, Pedersen O. The BIGTT test: a novel test for simultaneous measurement of pancreatic beta-cell function, insulin sensitivity, and glucose tolerance. Diabetes Care. 2007;30(2):257-262.

35. Glass LC, Cusi K, Berria R, Petz R, Cersosimo E, Defronzo RA, Gastaldelli A. Pioglitazone improvement of fasting and postprandial hyperglycaemia in Mexican-American patients with Type 2 diabetes: a double tracer OGTT study. Clin Endocrinol 2010;73:339-345.

36. Svegliati-Baroni G, Bugianesi E, Bouserhal T, Marini F, Ridolfi F, Tarsetti F, Ancarani F, et al. Post-load insulin resistance is an independent predictor of hepatic fibrosis in virus C chronic hepatitis and in non-alcoholic fatty liver disease. Gut 2007;56:1296-1301.

37. Angulo P, Bugianesi E, Bjornsson ES, Charatcharoenwitthaya P, Mills PR, Barrera F, Haflidadottir S, et al. Simple noninvasive systems predict long-term outcomes of patients with Nonalcoholic Fatty Liver Disease. Gastroenterology 2013;145:782-789.

38. Gastaldelli A, Casolaro A, Pettiti M, et al. Effect of pioglitazone on the metabolic and hormonal response to a mixed meal in type II diabetes. Clin Pharmacol Ther 2007;81:205-212.

39. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120:1640–1645.

40. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

41. Qureshi K, Clements RH, Saeed F, Abrams GA. Comparative evaluation of whole body and hepatic insulin resistance using indices from oral glucose tolerance test in morbidly obese subjects with nonalcoholic fatty liver disease. J Obes 2010; 2010:741521.

42. Bugianesi E, Pagotto U, Manini R, Vanni E, Gastaldelli A, de Iasio R, Gentilcore E, et al. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. J Clin Endocrinol Metab 2005;40:3498-3504.

43. Gastaldelli A, Harrison SA, Belfort-Aguilar R, Hardies LJ, Balas B, Schenker S, Cusi K. Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. Hepatology 2009;50:1087-1093.

44. Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, Cline GW, et al. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A 2007; 104:12587-12594

45. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. Gastroenterology. 2014 Mar;146:726-35

46. Pickens MK, Ogata H, Soon RK, Grenert JP, Maher JJ. Dietary fructose exacerbates hepatocellular injury when incorporated into a methionine-choline-deficient diet. Liver Int 2010; 30:1229–1239

47. Ren LP, Chan SM, Zeng XY, Laybutt DR, Iseli TJ, Sun RQ, Kraegen EW, et al. Differing endoplasmic reticulum stress response to excess lipogenesis versus lipid oversupply in relation to hepatic steatosis and insulin resistance. PLoS One 2012; 7:e30816

48. Ortiz-Lopez C, Lomonaco R, Orsak B, Finch J, Chang Z, Kochunov VG, Hardies J, Cusi K. Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). Diabetes Care 2012;35:873-878

49. Liu YL, Patman GL, Leathart JB, Piguet AC, Burt AD, Dufour JF, Day CP, et al. Carriage of the *PNPLA3* rs738409 C>G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma. J Hepatol 2014;61:75-81.

50. Trépo E, Nahon P, Bontempi G, Valenti L, Falleti E, Nischalke HD, Hamza S, et al. Association between the *PNPLA3* (rs738409 C>G) variant and hepatocellular carcinoma: Evidence from a meta-analysis of individual participant data. Hepatology 2014;59:2170-2177.

Figure Legends

Fig. 1. Correlation of Hepatic Insulin Resistance Index (HIRi) (A-C) and Glucose Clearance (D-F) as a Function of the best Surrogate Indexes in the Validation Group of NAFLD patients (black circles) and in Controls (open circles). The diagonal indicates the linear regression line.

Abbreviations: AUC, area under the receiver operating curve; $eMCR^{nodem}$, metabolic clearance rate estimation without metabolic parameters; HIRi, hepatic insulin resistance index; HOMA, homeostasis model assessment; IR, insulin resistance; ISI, insulin sensitivity index; OGIS, oral glucose insulin sensitivity index.

Fig. 2. Glucose and Insulin curves during OGTT according to the stage of fibrosis (A-B) or the degree of steatosis (C-D) in obese and non-obese subjects with NAFLD (Independent Cohort, n=145)

Abbreviations: F<2, absent/mild fibrosis; F≥2, moderate/severe fibrosis; Ob, obese; S1/S2, mild/moderate steatosis (< 66%); S2, severe steatosis (≥ 66%).

Fig.3. Oral Glucose Insulin Sensitivity Index (A) and NAFLD Fibrosis Score (B) according to the degree of fibrosis (Independent Cohort, n=145). The boxes identify the median and the 25^{th} and 75^{th} percentiles, while the whiskers stretch to the 5^{th} and 95^{th} percentiles.

Abbreviations: F0/F1, absent/mild fibrosis; F2, moderate fibrosis; F3/F4, severe fibrosis; NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index.

Fig. 4. Long-Term Outcomes Based on OGIS and NAFLD Fibrosis Score Risk Categories.

The grey area is «high risk» according to the corresponding score (OGIS or NFS). Subjects have been divided according to the absence (no events) or development (events) of liver-related and metabolic complications. The horizontal lines represent the cut-offs.

Abbreviations: NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index.

Table 1. Characteristics of the Patient Population in the Tracer Study Group (Validation Cohort) and in the Database Cohort (Independent Cohort).

Variable	**Validation cohort (n = 22)**	**Independent cohort (n = 145)**	***P* value**
Age /years	42 ± 13	43 ± 10	ns
Gender (M), n (%)	20 (69)	117 (81)	ns
BMI (kg/m^2)	28 ± 4	27 ± 5	ns
Normal weight/Overweight/Obese, %	18.2/54.5/27.3	22.8/57.9/19.3	ns
Waist (cm)	96 ± 11	96 ± 11	ns
AST (UI/l)	39 (26)	33 (20)	ns
ALT (UI/l)	78 (53)	72 (54)	ns
Albumin (g/dl)	4.5 (0.4)	4.5 (0.5)	ns
Platelets (x 10^9/l)	224 (71)	215 (75)	ns
Fasting insulin (mU/ml)	12 (4)	15 (17)	ns
Fasting glucose (mg/dl)	91 (13)	93 (16)	ns
Fasting triglycerides (mg/dl)	106 (73)	112 (81)	ns
Total cholesterol (mg/dl)	203 (39)	205 (64)	ns
HDL-cholesterol (mg/dl)	46 (18)	50 (16)	ns
Systolic pressure (mmHg)	123 ± 8.1	127 ± 16.4	ns
Diastolic pressure (mmHg)	78 ± 6.3	81 ± 8.5	ns
HOMA-IR	3.3 ± 1.9	3.8 ± 3.1	ns
Histological features			
Steatosis %	43 ± 26	36 ± 26	ns
Steatosis, n (%)			ns
1	10 (45.5)	80 (55.2)	
2	7 (31.8)	38 (26.2)	

3	5 (22.7)	27 (18.6)	
Fibrosis, n (%)			ns
0	7 (31.8)	51 (35.2)	
1	1 (4.5)	22 (15.2)	
2	6 (27.4)	35 (24.1)	
3	7 (31.8)	29 (20.0)	
4	1 (4.5)	8 (5.5)	
NAS score			ns
0-2	2 (9.1)	24 (16.6)	
3-4	12 (54.5)	92 (63.4)	
5-6	8 (36.4)	29 (20.0)	
NASH, n (%)	20 (90.9)	88 (60.7)	0.0036

Note. Data are reported as mean ± SD for continuous normal variables, median (interquartile range) for continuous non-normal variables and number (%) for categorical variables. BMI categories were defined as follow: normal weight (BMI 18.5-24.9 kg/m^2), overweight (BMI 25-29.9 kg/m^2), obese (BMI≥ 30 kg/m^2). ALT, alanine aminotransferases; AST, aspartate aminotransferases; BMI, body mass index; HDL, high density lipoprotein; IR, insulin resistance; HOMA, homeostasis model of assessment; M, male; NAS, non-alcoholic fatty liver disease activity score; NASH, non-alcoholic steatohepatitis.

Table 2. Parameters of Hepatic IR and Peripheral Insulin Sensitivity from Tracers Studies and Surrogate Indexes in the Validation Group of NAFLD Patients.

Variables	NAFLD (n = 22)	Controls (n = 11)	*P* value
Age, years	42.3 (2.9)	39.1 (2.5)	ns
Gender M/F, n	16/6	8/3	ns
BMI, kg/m^2	27.4 (0.7)	26.2 (1.0)	ns
Tracer study			
EGP (μmol/min kg)	10.1 ± 1.51	8.81 ± 1.35	0.0145
HIRi (μmol/min kg*UI/mL)	140 ± 76	90 ± 25	0.0084
Glucose clearance (ml/min kg)	1.94 ± 0.64	2.41 ± 0.51	0.0391
Based on fasting measurements			
HOMA-IR	3.36 ± 1.94	2.37 ± 0.70	0.0491
QUICKI	0.327 ± 0.025	0.337 ± 0.014	ns
FIRI	54 ± 31	39 ± 11	ns
IGR	0.143 ± 0.069	0.111 ± 0.029	0.0267
ISI Bennett	0.086 ± 0.011	0.097 ± 0.011	0.0117
TG/HDL-C	2.75 ± 1.76	1.29 ± 0.35	0.0006
Based on OGTT measurements			
OGIS (mg/Kg min)	8.40 ± 2.18	10.03 ± 1.19	0.0282
ISI Matsuda	2.63 ± 1.09	4.03 ± 0.99	0.0013
SiOGTT	0.193 ± 0.012	0.203 ± 0.007	0.0177
ISI Stumvoll	0.055 ± 0.043	0.084 ± 0.016	0.0295
HepIR-DF	63 ± 21	41 ± 19	0.0082
LIRI	2.26 ± 0.15	2.12 ± 0.11	0.0117
BIGTT	3.98 ± 3.21	6.54 ± 2.43	0.0267
eMCR^{dem}	5.14 ± 4.13	7.91 ± 1.62	0.0392

$eMCR^{nodem}$	4.71 ± 3.90	7.37 ± 7.31	0.0295

Note. Data are reported as mean ± SD.

BMI, body mass index; FIRI, fasting insulin resistance index; HepIR, hepatic insulin resistance index; BIGTT, β-cell function, insulin sensitivity, glucose tolerance test derived index; $eMCR^{dem}$, metabolic clearance rate estimation including demographic parameters; $eMCR^{nodem}$, metabolic clearance rate estimation without demographic parameters; HIRi, hepatic insulin resistance index; HOMA, homeostasis model of assessment; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; M, male; NAFLD, non-alcoholic fatty liver disease; OGIS, oral glucose insulin sensitivity index; OGTT, oral glucose tolerance test; QUICKI, quantitative insulin sensitivity check index; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test; TG/HDL-C, triglycerides to HDL-cholesterol ratio.

Table 3. Univariate and Multivariate Analysis of Surrogate Indexes Associated with Moderate/Severe Fibrosis (F≥2).

	Univariate analysis			Multivariate analysis		
	Fibrosis < 2 (n = 73)	**Fibrosis ≥ 2 (n = 72)**	***P* value**	**OR**	**95% CI**	***P* value**
Age	41.2 ± 9.2	46.0 ± 12.4	0.0109	-	-	ns
Sex, M/F	62/11	55/17	0.2746	-	-	ns
BMI	26.3 ± 3.5	28.3 ± 3.4	0.0021	-	-	ns
HOMA-IR	4.01 ± 3.5	4.85 ± 3.9	ns	-	-	-
QUICKI	0.33 ± 0.04	0.32 ± 0.04	ns	-	-	-
FIRI	65 ± 56	78 ± 64	ns	-	-	-
IGR	0.19 ± 0.14	1.19 ± 0.12	ns	-	-	-
ISI Bennett	0.099 ± 0.046	0.093 ± 0.06	ns	-	-	-
TG/HDL-C	2.6 ± 1.8	3.3 ± 2.4	ns	-	-	-
OGIS (mg/Kg min)	9.24 ± 1.70	7.79 ± 1.88	<0.0001	0.70	0.58-0.85	**0.0002**
ISI Matsuda	3.44 ± 2.40	2.64 ± 2.32	0.0091	-	-	ns
SiOGTT	0.196 ± 0.011	0.190 ± 0.01	0.0002	-	-	ns
ISI Stumvoll	0.067 ± 0.049	0.047 ± 0.049	0.0044	-	-	ns
HepIR DF	74 ± 54	87 ± 73	ns	-	-	-
LIRI	2.25 ± 0.15	2.30 ± 0.12	0.0180	-	-	ns
BIGTT	4.54 ± 3.30	2.90 ± 2.44	0.0020	-	-	ns
$eMCR^{dem}$	6.89 ± 4.03	4.62 ± 3.34	<0.0001	-	-	ns
$eMCR^{nodem}$	5.71 ± 4.25	3.90 ± 4.01	0.0005	-	-	ns

Note. Data are reported as mean ± SD.

BMI, body mass index; CI, confidence interval; BIGTT, β-cell function, insulin sensitivity, glucose tolerance test derived index; $eMCR^{dem}$, metabolic clearance rate estimation including demographic parameters; $eMCR^{nodem}$, metabolic clearance rate estimation without demographic parameters; HDL-C, high density

lipoprotein cholesterol; HOMA, homeostasis model of assessment; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; OGIS, oral glucose insulin sensitivity index; OR, odds ratio; QUICKI, quantitative insulin sensitivity check index; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test; TG, triglycerides.

Table 4. Univariate and Multivariate Analysis of Surrogate Indexes Associated with the Diagnosis of NASH.

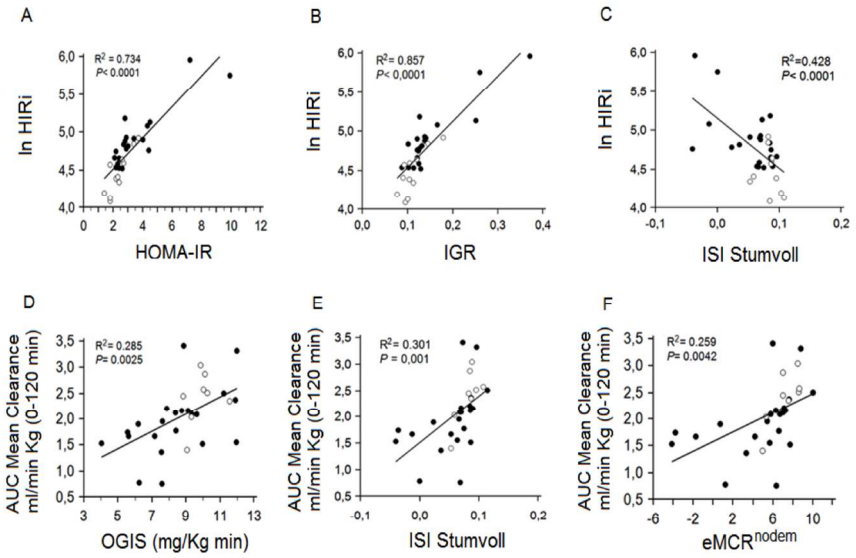
	Univariate analysis			Multivariate analysis		
	SFL (n = 57)	**NASH (n = 88)**	***P* value**	**OR**	**95% CI**	***P* value**
Age	40.9 ± 9.8	45.4 ± 12.1	0.0193	-	-	ns
Sex, M/F	62/11	55/17	ns	-	-	ns
BMI	26.6 ± 3.7	27.8 ± 3.4	0.0471	-	-	ns
HOMA-IR	4.34 ± 3.78	4.49 ± 3.70	ns	-	-	-
QUICKI	0.33 ± 0.04	0.32 ± 0.04	ns	-	-	-
FIRI	70 ± 61	73 ± 60	ns	-	-	-
IGR	0.20 ± 0.15	0.18 ± 0.12	ns	-	-	-
ISI Bennett	0.097 ± 0.047	0.095 ± 0.06	ns	-	-	-
TG/HDL-C	2.7 ± 1.9	3.1 ± 2.3	ns	-	-	-
OGIS (mg/Kg min)	9.20 ± 2.04	8.08 ± 1.94	0.0012	0.75	0.63-0.90	**0.0021**
ISI Matsuda	3.29 ± 2.22	2.88 ± 2.48	ns	-	-	-
SiOGTT	0.196 ± 0.011	0.191 ± 0.01	0.0176	-	-	ns
ISI Stumvoll	0.065 ± 0.053	0.052 ± 0.048	ns	-	-	-
HepIR DF	78 ± 58	83 ± 68	ns	-	-	-
LIRI	2.26 ± 0.15	2.28 ± 0.13	ns	-	-	-
BIGTT	4.40 ± 3.30	3.29 ± 2.73	0.0491	-	-	ns
$eMCR^{dem}$	6.81 ± 4.33	5.10 ± 3.40	0.0001	-	-	ns
$eMCR^{nodem}$	5.50 ± 4.56	4.37 ± 3.94	0.0143	-	-	ns

Note. Data are reported as mean ± SD.

BMI, body mass index; CI, confidence interval; BIGTT, □-cell function, insulin sensitivity, glucose tolerance test derived index; $eMCR^{dem}$, metabolic clearance rate estimation including demographic parameters; $eMCR^{nodem}$, metabolic clearance rate estimation without demographic parameters; HDL-C, high

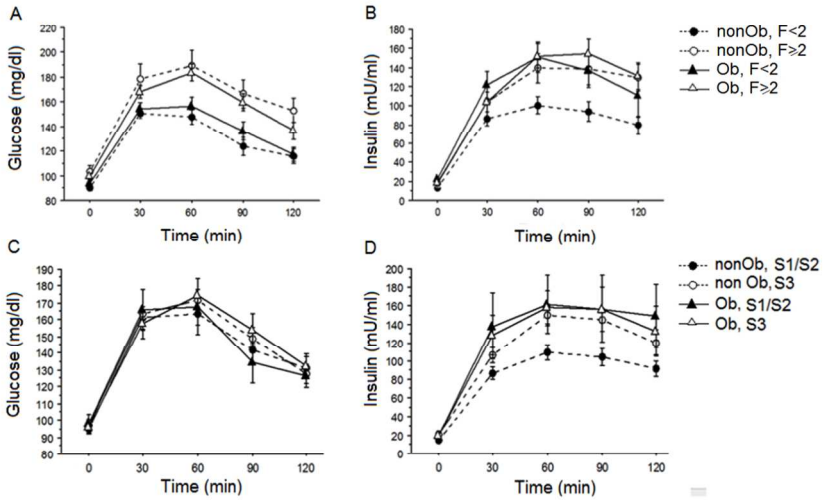
density lipoprotein cholesterol; HOMA, homeostasis model of assessment; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; NASH, non-alcoholic steatohepatitis; OGIS, oral glucose insulin sensitivity index; OR, odds ratio; QUICKI, quantitative insulin sensitivity check index; SFL, simple fatty liver; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test; TG, triglycerides.

Fig. 1. Correlation of Hepatic Insulin Resistance Index (HIRi) (A-C) and Glucose Clearance (D-F) as a Function of the Best Surrogate Indices.



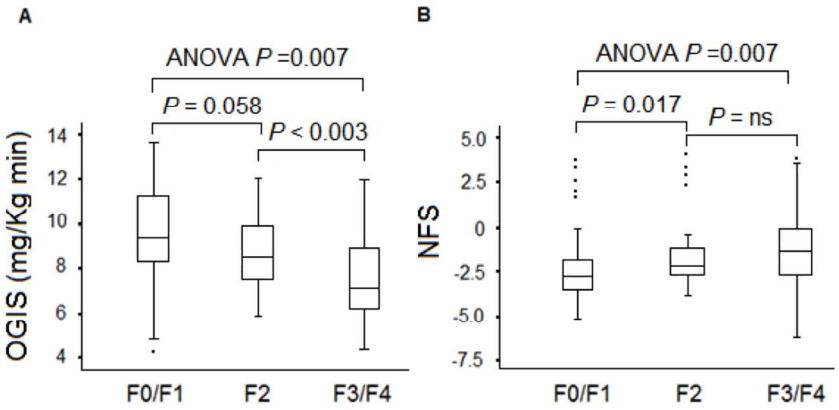
508x381mm (72 x 72 DPI)

Fig.2. Glucose and Insulin curves during OGTT according to the stage of fibrosis (A-B) or the degree of steatosis (C-D) in obese and non-obese subjects with NAFLD (Independent Cohort, n=145)



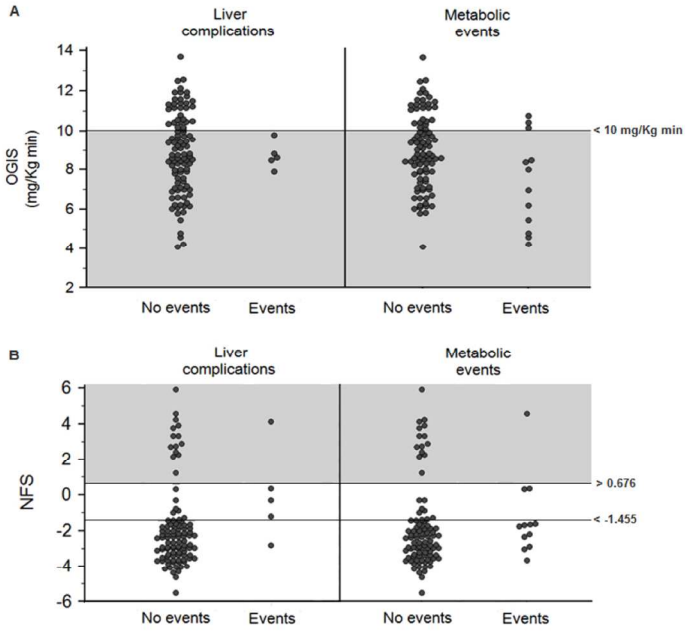
508x381mm (72 x 72 DPI)

Fig.3. Oral Glucose Insulin Sensitivity Index (A) and NAFLD Fibrosis Score (B) according to the degree of fibrosis.



508x381mm (72 x 72 DPI)

Fig.4. Long-Term Outcomes Based on OGIS and NAFLD Fibrosis Score Risk Categories.



508x381mm (72 x 72 DPI)

Supplementary Tables

Supplementary table 1. Formulas of the Surrogate Indexes of Insulin Resistance/Insulin Sensitivity.

	Formula
Based on fasting measurements	
HOMA-IR[22]	(I_0 mU/ml X G_0 mmol/l / 22.5
QUICKI[23]	1/(LOG I_0 mU/ml + LOG G_0 mg/dl)
FIRI[24]	(I_0 mU/ml X G_0 mg/dl) / 25
IGR[25]	I_0 mU/ml / G_0 mg/dl
ISI Bennett 26	1/(ln G_0 mg/dl X ln I_0 mU/l)
TG/HDL-Chol[38]	Tg/HDL-Chol
Based on OGTT measurements	
OGIS[27]	1/2 $\lfloor B+\sqrt{B^2+4}\ p5p6(G120-Gclamp)ClOGTT\rfloor^{\$}$
ISI Matsuda [28]	$10^4/\sqrt{}$ [(G_0 mg/dl X I_0 mU/ml) X (G_{mean} X I_{mean})]
SiOGTT[30]	1/[LOG(G_0 + G_{30} + G_{90} + G_{120}) mg/dl + LOG (I_0 + I_{30} + I_{90} + I_{120}) mU/ml]
ISI Stumvoll [31]	0.157 - 0.00004576 X I_{120}(pmol/l) - 0.000299 X I_0(pmol/l) - 0.00519 X G_{120}(mmol/l)
HepIR DF [32]	(G_0 mg/dl + G_{30} mg/dl) / 100 / 2 X (I_0 mU/ml + I_{30} mU/ml) / 2
LIRI[33]	- 0.091+LOG (I mean X 6) X 0.4 + LOG (FM / weight X 100) X 0.346 – LOG HDL-C mg/dl X 0.408 + LOG BMI X 0.435
BIGTT[34]	EXP (4.9-(0.00402 X I_0 pmol/l) – (0.000556 X I_{30} pmol/l) – (0.00127 X I_{90} pmol/l) – (0.152 X G_0 mmol/l) – (0.00871 X G_{30} mmol/l) – (0.0373 X G_{120} mmol/l) – (0.145 X Gender) – (0.0376 X BMI)
eMCR^{dem} [31]	18.8 – 0.271 X BMI – 0.0052 X I_{120} pmol/l – 0.27 X G_{90} mmol/l
eMCR^{nodem} [31]	13 – 0.0042 X I_{120} pmol/l – 0.384 X G_{90} mmol/l – 0.0209 X I_0 pmol/l

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the receiver operating curve; BIGTT, β-cell function, insulin sensitivity index derived from oral glucose tolerance test; BMI, body mass index; $eMCR^{dem}$, metabolic clearance rate estimation including demographic parameters; $eMCR^{nodem}$, metabolic clearance rate estimation without demographic parameters; FIRI, fasting insulin resistance index; HDL-C, high density lipoprotein cholesterol; G, glucose; HepIR DF, De Fronzo hepatic insulin resistance index; HOMA, homeostasis model of assessment; I, insulin; IFG, impaired fasting glucose; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index; QUICKI, quantitative insulin sensitivity check index; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test; TG, triglycerides.

$^{\$}$p5 and p6 are fixed rate constants; G_{120} is the plasma concentration of glucose measured at 120 min during OGTT; GCLAMP is the clamp glucose concentration (normally 90 mg x dl^{-1}); ClOGTT (the glucose clearance during the test) and B are obtained by means of the following equations: $B = [p_5 (G_{120}\text{-Gclamp})+1]$ ClOGTT.

Supplementary Table 2. Pearson Correlations of the Surrogate Indexes of Insulin Resistance/Sensitivity as a Function of Glucose Clearance and Hepatic Insulin Resistance Index.

	HIRi	Mean Clearance ml/min Kg (0-120 min)
HOMA IR	0.901***	-0.451*
QUICKI	-0.783***	0.414*
FIRI	0.901***	-0.451*
IGR	0.926***	-0.148
ISI Bennett	-0.617**	0.304
TG/HDL-C	0.428	-0.409*
OGIS	-0.556**	0.531**
ISI Matsuda	-0.545**	0.399*
SiOGTT	-0.519	0.408*
ISI Stumvoll	-0.655***	0.548**
HepIR DF	0.374	0.082
LIRI	0.575**	-0.279
BIGTT	-0.572**	0.402*
$eMCR^{dem}$	-0.564**	0.463**
$eMCR^{nodem}$	-0.623**	0.507**

Note. BIGTT, β-cell function, insulin sensitivity, glucose tolerance test derived index; EGP, endogenous glucose production; $eMCR^{dem}$, metabolic clearance rate estimation including demographic parameters; $eMCR^{nodem}$, metabolic clearance rate estimation without demographic parameters; FPI, fasting plasma insulin; HDL-C, high density lipoprotein-cholesterol; HepIR, hepatic insulin resistance index; HOMA,

homeostasis model of assessment; IGR, insulin to glucose ratio; IR, insulin resistance; ISI, insulin sensitivity index; LIRI, liver insulin resistance index; NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index; QUICKI, quantitative insulin sensitivity check index; SiOGTT, insulin sensitivity index derived from oral glucose tolerance test; TG, triglycerides.

*P value < 0.05

** P value < 0.001

*** P value < 0.0001

Supplementary table 3. Area Under the ROC Curve of OGIS and NAFLD Fibrosis Score for the Prediction of Advanced Fibrosis (F ≥ 2).

Index	Area under the ROC curve ± SE (95% CI)	PPV	NPV	*P* value
OGIS	0.71 ± 0.04 (0.62 - 0.78)	79	62	<0.0001
NFS	0.68 ± 0.04 (0.60 – 0.76)	63	74	<0.0001

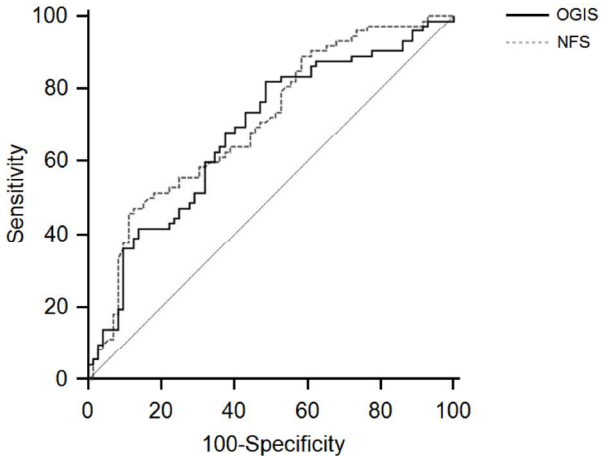
CI, confidence interval; NFS, non-alcoholic fatty liver disease fibrosis score; NPV, negative predictive value; OGIS, oral glucose insulin sensitivity index; PPV, positive predictive value; ROC, receiver operating curve; SE, standard error.

Supplementary table 4. Comparison Between OGIS and NFS as Predictors of Advanced Fibrosis (F ≥ 2) by Logistic Regression Analysis.

	OR	**95% CI**	***P* value**
Age	-	-	ns
Sex	-	-	ns
BMI	-	-	ns
OGIS (mg/Kg min)	0.69	0.57-0.84	<0.001
NFS	1.23	1.03-1.45	0.018

Note. BMI, body mass index; CI, confidence interval; NFS, non-alcoholic fatty liver disease fibrosis score; OGIS, oral glucose insulin sensitivity index; OR, odds ratio.

Supplementary Fig. 1. Receiver Operating Curve of the OGIS Index and NAFLD Fibrosis Score in the Prediction of Advanced Fibrosis (F ≥ 2).



supplementary figure
508x381mm (72 x 72 DPI)