Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes

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Research Article

Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes

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Non-alcoholic fatty liver (NAFL) is an independent risk factor for the development of type 2 diabetes (T2DM). We examined metabolic perturbations in patients with NAFL, patients with T2DM, and control (CON) subjects with normal intrahepatic lipid (IHL) content.
A two-step (10 mU/m^2 /min; 40 mU/m^2/min) hyperinsulinemic–euglycemic clamp was performed in 11 NAFL, 13 T2DM, and 11 CON subjects, all matched for BMI, and aerobic fitness. IHL content was measured using proton magnetic resonance spectroscopy. Because of high IHL content variability in T2DM patients, this group was separated into a high IHL content group (IHL $\geqslant$ 5.0%, T2DM+NAFL) and a normal IHL content group (IHL $<$ 5.0%, T2DM-non-NAFL) for further analysis.
IHL content was increased in NAFL and T2DM+NAFL subjects ($P<0.050$ versus CON and T2DM-non-NAFL subjects). Adipose tissue insulin sensitivity index (Adipo-IR$_i$) was higher in NAFL ($P<0.050$ versus CON and T2DM-non-NAFL subjects) and in T2DM+NAFL subjects ($P=0.055$ versus CON subjects, $P<0.050$ versus T2DM-non-NAFL subjects). Suppression of plasma-free fatty acids ($P=0.046$) was lower in NAFL compared with CON subjects, with intermediate values for T2DM-non-NAFL, and T2DM+NAFL subjects. Suppression of endogenous glucose production (EGP) and insulin-stimulated glucose disposal (ΔR_d) was comparable between NAFL, T2DM-non-NAFL, and T2DM+NAFL subjects (all $P>0.05$), and was lower in comparison with CON subjects (all $P<0.01$). Metabolic flexibility was lower in T2DM-non-NAFL subjects ($P=0.047$) and NAFL subjects ($P=0.059$) compared with CON subjects. Adipo-IR$_i$ ($r=0.652$, $P<0.001$), hepatic insulin resistance index (HIR$_i$) ($r=0.576$, $P=0.001$), and ΔR_d ($r=-0.653$, $P<0.001$) correlated with IHL content.
Individuals with NAFL suffer from metabolic perturbations to a similar degree as T2DM patients. NAFL is an important feature leading to severe insulin resistance and should be viewed as a serious health threat for the development of T2DM. ClinicalTrials.gov: NCT01317576

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Introduction

The number of individuals with non-alcoholic fatty liver (NAFL) is reaching epidemic proportions and, at present, is considered to be the most common liver disorder in western society [1,2]. NAFL affects up to one third of the adult population [3], and this number dramatically increases to 50–70% in obese individuals [4]. NAFL is closely related to insulin resistance, thus elevated intrahepatic lipid (IHL) content is

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considered to be an independent risk factor for the development of type 2 diabetes (T2DM) [2,5]. Importantly, the prevalence of prediabetes and diabetes in individuals with NAFL is as high as 85%, compared with only 30% in individuals with normal IHL content [6].

Elevated levels of alanine amino transferase (ALT) in plasma—a biomarker for liver disease—are known to be associated with the metabolic syndrome [7,8]. Cross-sectional studies using proton magnetic resonance spectroscopy (^{1}H-MRS)—the non-invasive gold standard to measure IHL content in humans—found that elevated IHL content directly correlates with progressive impairments of adipose tissue, hepatic, and skeletal muscle insulin sensitivity in lean, overweight and obese non-diabetic subjects, and in patients with T2DM [9-14]. Furthermore, patients with NAFL are characterized by lower adipose tissue, hepatic, and skeletal muscle insulin sensitivity compared with healthy individuals with normal IHL content [15-18]. However, the severity of insulin resistance and metabolic disturbances in individuals with elevated IHL content but without T2DM when compared with patients with T2DM is currently unknown.

Therefore, the purpose of the present study was to perform a very detailed metabolic characterization of patients with NAFL, and compare them with BMI-matched, non-insulin-dependent patients with T2DM, and individuals with normal IHL content (control subjects: CON). Based on the current scientific consensus, which considers NAFL a prediabetic state, and based on previous results obtained in other individuals with prediabetes [19,20], we hypothesized that metabolic disturbances, such as adipose tissue, hepatic, and skeletal muscle insulin resistance and impaired metabolic flexibility, would have intermediate values in NAFL subjects compared with insulin-resistant T2DM patients and insulin-sensitive CON subjects.

Experimental

Participants

In the study, 11 sedentary, middle-aged (40–70 years), BMI-matched (27–35 kg/m2), overweight/obese NAFL subjects; 13 T2DM patients; and 11 CON subjects were included. All subjects were male, with stable dietary habits. General exclusion criteria were unstable body weight, cardiac disease, impaired renal function, anemia (hemoglobin <7.5 mmol/l), use of β-blockers, use of anti-thrombotic medication, elevated blood pressure (>160/100 mmHg), claustrophobia, contra-indications for MRI, recent participation in a weight loss or vigorous exercise program, history of substantial alcohol use (>3 units per day), history of drug abuse, and use of insulin therapy. NAFL subjects were selected who had a high IHL content (all⩾5.0%) as measured with ^{1}H-MRS, and a fasting plasma glucose (FPG) concentration <7.0 mmol/l. CON subjects were selected who had a low IHL content (all⩽4.0%) as measured with ^{1}H-MRS, in the absence of clinical signs of liver disease or liver dysfunction (defined as alanine aminotransferase (ALAT) >2.5 times the normal value) and had to be normoglycemic as per WHO criteria. A 120-min 75 g oral glucose tolerance test (OGTT) was performed in NAFL and CON subjects. T2DM patients were treated for their hyperglycemia with metformin (n=5), sulfonyl urea (n=1), metformin + sulfonylurea (n=4), metformin + dipeptidyl peptidase-4 (DPP-4) inhibitors (n=1) or metformin + sulfonylurea + DPP-4 inhibitors (n=1) therapy for at least 6 months with a constant dose for at least 2 months or were on a dietary treatment (n=1) for at least 6 months prior to the onset of the study, with FPG concentration ⩾7.0 mmol/l, and <10.0 mmol/l at onset of the study. Participants were recruited via advertisements in local newspapers and gave written informed consent before participation. All data were collected at the Maastricht University Medical Center, Maastricht, The Netherlands. The local ethics committee approved the study, which was performed following the declaration of Helsinki principles.

Body composition and maximal aerobic capacity (VO_{2max})

Body composition was determined by dual X-ray absorptiometry (Hologic Discovery A, Waltham, MA, U.S.A.). Waist circumference was assessed at the end of exhalation, with the subjects standing erect, and relaxed with arms at their sides and their feet positioned close together. The waist circumference was measured to the nearest 0.1 cm in a horizontal plane around the abdomen at the level of the iliac crest. VO_{2max} and W_{max} were assessed during a graded cycling test with concurrent ECG until exhaustion, as previously described [21].

^{1}H-MRS

^{1}H-MRS was used to quantify IHL content, as described previously [22], but on a 3T whole-body scanner (Achieva 3Tx; Philips Healthcare, Best, The Netherlands) using a five-element coil, and a repetition time = 4000 ms, echo time = 32.5 ms and number of averages = 64. To minimize motion artifacts, subjects were asked to breathe in to the rhythm of the measurement and to be at end of expiration during acquisition of spectra. To determine the intensity of the lipid peak, the water signal was suppressed using frequency-selective pre-pulses. The unsuppressed water resonance

was used as an internal reference (number of averages = 32). Spectra were fitted with a home-written script [23] in MATLAB R2014b (Mathworks, Natick, MA, U.S.A.). The CH_2 resonance and the unsuppressed water resonance were corrected for T2 relaxation according to [24] and IHL content was calculated in weight/weight percentage, as described previously [25].

Hyperinsulinemic–euglycemic clamp

All participants underwent a two-step (10 mU/m^2/min; 40 mU/m^2/min) hyperinsulinemic–euglycemic clamp for 6 h [26], as described previously [27]. In short, after an overnight fast, participants received a primed continuous infusion of [6,6-$^2H_2$]-glucose (0.04 mg/kg/min). After 180 min, 10 mU/m^2/min insulin infusion was started for 4 h. Thereafter, 40 mU/m^2/min insulin infusion was started for 2 h. During insulin infusion, plasma glucose concentrations were maintained by a concomitant infusion of glucose that was enriched with [6,6-$^2H_2$]-glucose (hot-GINF) to minimize changes in plasma tracer enrichment [28]. Glucose steady state was reached during the last 30 min of both insulin infusion rates. Blood sampling and indirect calorimetry using ventilated hood system (Omnical, IDEE, Maastricht, The Netherlands) was performed in every final 30 min of steady state. Isotopic enrichment of plasma glucose was determined by electric impact ionization gas chromatography mass spectroscopy as previously described [29].

Calculations

Whole-body insulin sensitivity was assessed as the mean glucose infusion rate during 40 mU/m^2/min insulin infusion (*M*-value, μmol/min/kgFFM), and as *M*-value per mean plasma insulin concentration (I, mU/l) during 40 mU/m^2/min insulin infusion (M/I ratio). Steele's single-pool, non-steady-state equations were used to calculate glucose rate of appearance (R_a), and glucose rate of disappearance (R_d) [30]. Volume of distribution was assumed to be 0.160 l/kg for glucose. Insulin-stimulated glucose disposal (ΔR_d) was computed as the difference between R_d under insulin-stimulated conditions (40 mU/m^2/min) and R_d under basal conditions. Suppression of endogenous glucose production (EGP) was calculated as R_a—exogenous glucose infusion rate. The difference in respiratory quotient (RQ) under insulin-stimulated conditions (40 mU/m^2/min) and RQ under basal conditions was defined as metabolic flexibility (ΔRQ). Glucose and fat oxidation rates were calculated with the assumption that protein oxidation was negligible [31]. Non-oxidative glucose disposal (NOGD) was calculated as R_d (40 mU/m^2/min)—glucose oxidation (40 mU/m^2/min). The hepatic insulin resistance index (HIR_i) and adipose tissue insulin resistance index (Adipo-IR_i) were calculated as previously described [12]. HIR_i was calculated as the product of basal EGP and basal plasma insulin concentration before the start of the hyperinsulinemic–euglycemic clamp. Adipo-IR_i was calculated as the product of basal plasma free fatty acids (FFA) and basal plasma insulin concentration before the start of the hyperinsulinemic–euglycemic clamp.

Laboratory analysis

Arterialized blood samples were collected and immediately centrifuged at high speed. Plasma was frozen in liquid nitrogen and stored at −80°C until assayed. Plasma FFAs and plasma glucose were measured with enzymatic assays automated on a Cobas Fara/Mira (FFA: Wako Nefa C test kit; Wako Chemicals, Neuss, Germany) (plasma glucose: hexokinase method; La Roche, Basel, Switzerland). Fasting plasma triglycerides (TG) were measured colorimetrically (Roche, Vienna, Austria). Total cholesterol (Roche Diagnostics, Mannheim, Germany) and high-density lipoprotein (HDL) (Roche Diagnostics, Mannheim, Germany), after precipitation of apolipoprotein B-containing lipoproteins with phosphotungstic acid, and magnesium ions, were analyzed in serum enzymatically (GPO-Trinder; Sigma–Aldrich Corp., St. Louis, MO, U.S.A.). Low-density lipoprotein (LDL) was calculated according to the Friedewald equation [32], with no subjects having plasma TG levels above 4.5 mmol/l. Plasma insulin concentrations on the day of the hyperinsulinemic–euglycemic clamp were quantified using an immunometric assay (Advia Centaur, Siemens Diagnostics). Blood parameters during the screening visit were analyzed routinely.

Statistics

All values are reported as the mean ± SEM. Statistical significance was set at $P<0.05$. Normality was assessed by the Shapiro–Wilk test. Homogeneity of variance was assessed by Levene's test. Group comparisons were performed using one-way ANOVA with Tukey or Games-Howell *post-hoc* correction, or using Kruskal–Wallis H test with Dunn's (1964) procedure with Bonferroni correction for multiple comparison. For Pearson correlations, logarithmic transformation of IHL content, Glucose 120, HIR_i, and Adipo-IR_i was performed as the data were skewed. All statistic calculations were performed using IBM SPSS 21 (SPSS, Chicago, IL, U.S.A.).

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Table 1 Subject characteristics

	CON	NAFL	T2DM-non-NAFL	T2DM+NAFL	*P* value
Age (years)	57.6 ± 2.5	54.5 ± 2.0	63.0 ± 1.3	59.8 ± 3.2	0.095
IHL (%)	1.9 ± 0.4^{A}	9.7 ± 2.0^{B}	2.3 ± 0.4^{A}	16.6 ± 6.0^{B}	<0.001
AST (U/l)	22.1 ± 1.2	25.5 ± 2.4	24.0 ± 1.9	29.5 ± 3.4	0.176
ALT (U/l)	30.0 ± 3.6	34.5 ± 2.2	32.1 ± 3.5	45.5 ± 7.4	0.078
γ-GT (U/l)	34.3 ± 4.7	35.4 ± 3.7	39.0 ± 5.8	42.8 ± 9.1	0.726
Body weight (kg)	93.8 ± 3.6	102.2 ± 2.9	90.4 ± 2.7	97.5 ± 3.1	0.079
Height (cm)	178.0 ± 5.9	181.9 ± 7.2	177.7 ± 1.8	177.2 ± 3.0	0.330
BMI (kg/m^2)	29.5 ± 0.8	30.9 ± 0.8	28.8 ± 0.8	31.0 ± 0.6	0.187
Fat mass (kg)	27.5 ± 1.9	30.7 ± 1.2	25.4 ± 1.6	29.0 ± 1.0	0.134
Fat free mass (kg)	64.2 ± 1.9	69.0 ± 2.0	63.8 ± 1.3	66.3 ± 2.3	0.200
Fat percentage (%)	28.8 ± 1.0	30.0 ± 0.7	27.5 ± 1.2	29.7 ± 0.7	0.326
Waist circumference (cm)	106.2 ± 1.9	109.1 ± 2.0	105.2 ± 1.8	110.3 ± 1.2	0.332
Fasting glucose (mmol/l)	5.1 ± 0.2^{A}	5.5 ± 0.1^{A}	7.5 ± 0.4^{B}	7.4 ± 0.6^{B}	<0.001
Glucose 120 (mmol/l)	4.8 ± 0.3^{A}	6.7 ± 0.7^{B}	ND	ND	0.021
Fasting insulin (mU/l)	9.2 ± 0.7	15.2 ± 1.9	11.4 ± 3.1	19.1 ± 4.1	0.020
Triglycerides (mmol/l)	1.3 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.9 ± 0.3	0.136
Total cholesterol	4.7 ± 0.3	4.9 ± 0.3	4.0 ± 0.3	3.9 ± 0.4	0.144
LDL	3.2 ± 0.3	3.4 ± 0.3	2.9 ± 0.3	2.4 ± 0.3	0.184
HDL	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.283
VO_{2max} (ml/min)	2574.3 ± 128.1	2658.6 ± 151.5	2407.0 ± 126.1	2361.1 ± 115.9	0.443
VO_{2max} (ml/min/kg)	27.6 ± 1.4	26.1 ± 1.4	26.7 ± 1.5	24.2 ± 1.0	0.472
W_{max} (W/kg)	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	0.401

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; HDL: high-density lipoprotein; IHL: intrahepatic lipid; LDL: low-density lipoprotein; VAT: visceral adipose tissue; VO_{2max}: maximal oxidative capacity; W_{max}: maximal output; γ-GT: gamma-glutamyl transferase; ND, not determined. Different letters indicate significant differences between groups ($P<0.05$).

Results

Subject characteristics

Subject characteristics are summarized in Table 1. Because T2DM subjects had a wide spread in IHL content, we decided to analyze T2DM subjects with high IHL content (IHL ⩾ 5.0%, T2DM+NAFL, $n=6$), and with normal IHL content (IHL < 5.0%, T2DM-non-NAFL subjects, $n=7$) separately.

Age ($P=0.095$), BMI ($P=0.187$), fat mass ($P=0.134$), fat-free mass ($P=0.200$), fat percentage ($P=0.326$), and waist circumference ($P=0.332$) were not different among groups. By design, screening FPG was significantly different among groups ($P<0.001$). NAFL and CON subjects had similar screening FPG levels ($P=0.792$) in the normoglycemic range. Compared with NAFL and CON subjects, T2DM-non-NAFL, and T2DM+NAFL subjects had increased screening FPG levels (all $P<0.05$). Screening FPG levels in T2DM-non-NAFL and T2DM+NAFL subjects were comparable ($P=0.992$). Screening fasting insulin levels were different among groups ($P=0.020$), with a trend for higher fasting insulin levels in NAFL ($P=0.088$), and T2DM+NAFL subjects ($P=0.060$) compared with CON subjects. Plasma glucose levels upon OGTT (glucose 120) were higher in NAFL than in CON subjects ($P=0.021$). For T2DM-non-NAFL and T2DM+NAFL, no OGTT was performed. Fasting plasma TG levels ($P=0.136$), total cholesterol ($P=0.144$), LDL ($P=0.184$), and HDL ($P=0.283$) were not different among groups. Aerobic fitness was comparable among groups, with no differences in absolute VO_{2max} ($P=0.443$), VO_{2max} corrected for body weight ($P=0.472$), and W_{max} ($P=0.401$).

IHL content and liver enzymes

By design, IHL content was different among groups ($P<0.001$) (Figure 1A, Table 1). IHL content was comparable between NAFL and T2DM+NAFL subjects ($P=0.828$) and between CON and T2DM-non-NAFL subjects ($P=0.887$) (Figure 1A, Table 1). Glucose levels upon OGTT (glucose 120), which was only performed in CON and NAFL subjects, correlated with IHL content ($r=0.638$, $P=0.002$).

Plasma levels of aspartate aminotransferase (AST) ($P=0.176$), alanine aminotransferase (ALT) ($P=0.078$), and gamma-glutamyl transferase (γ-GT) ($P=0.726$)—markers of hepatocellular injury—were not different among groups (Figure 1B). ALT tended to be somewhat higher in T2DM+NAFL subjects due to a statistical but not clinical outlier

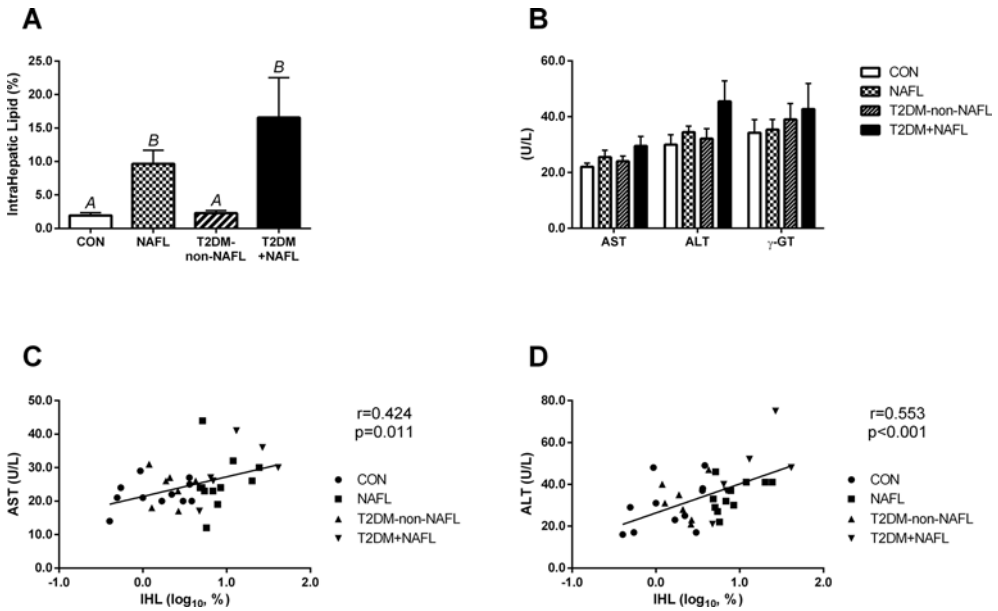

Figure 1. Intrahepatic lipid content and liver enzymes

(**A**) Intrahepatic lipid (IHL) content; (**B**) serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT) levels; (**C**) relation between serum AST levels and IHL content (n=35); (**D**) relation between serum ALT levels and IHL content (n=35). Results are mean ± SEM. Different letters indicate significant differences between groups (P<0.05).

that remained included in the analysis. Despite no significant group differences, AST (r=0.424, P=0.011, Figure 1C), and ALT (r=0.553, P<0.001, Figure 1D) both showed a positive correlation with IHL content in the whole study population.

Circulating metabolites during hyperinsulinemic–euglycemic clamp

Plasma insulin concentrations during both the 10 mU/m^2/min and 40 mU/m^2/min steady state period of the clamp were comparable among groups (Table 2). Plasma glucose values during 10 mU/m^2/min insulin infusion were different among groups (P=0.019, Table 2). T2DM+NAFL subjects had higher plasma glucose values during 10 mU/m^2/min insulin infusion than CON subjects (P=0.026), with NAFL and T2DM-non-NAFL subjects having intermediate values (Table 2). Plasma glucose values during 40 mU/m^2/min insulin infusion (P=0.143) and fasting plasma FFA levels were not significantly different among groups (P=0.574, Table 2).

Insulin sensitivity

Whole-body insulin sensitivity

Whole-body insulin sensitivity was assessed as M-value and M/I ratio during 40 mU/m^2/min insulin infusion. M-value was different among groups (P<0.001, Table 2). M-value was comparable between NAFL and T2DM-non-NAFL subjects (P=0.827); between NAFL and T2DM+NAFL subjects (P=0.553); and between T2DM-non-NAFL and T2DM+NAFL subjects (P=0.466) (Table 2). NAFL subjects (P<0.001), T2DM-non-NAFL subjects (P<0.001) and T2DM+NAFL subjects (P<0.001) were all characterized by a significantly lower M-value compared with CON subjects (Table 2). The M/I ratio was also different among groups (P=0.004, Table 2). M/I ratio was comparable between NAFL and T2DM-non-NAFL subjects (P=0.314); NAFL and T2DM+NAFL subjects (P=0.372); T2DM-non-NAFL and T2DM-NAFL subjects (P=0.100) and T2DM-non-NAFL and CON subjects (P=0.105) (Table 2). NAFL (P=0.003) and T2DM+NAFL subjects (P=0.001) were characterized by a significantly lower M/I ratio compared with CON subjects (Table 2).

Adipose tissue insulin sensitivity

Adipose tissue insulin resistance during basal conditions was determined by using the validated Adipo-IR$_i$ [12,33]. Adipo-IR$_i$ was different among groups (P=0.006, Figure 2A). Adipo-IR$_i$ in NAFL was comparable with Adipo-IR$_i$ in T2DM+NAFL subjects (P=0.947, Figure 2A). Adipo-IR$_i$ in T2DM-non-NAFL was comparable with Adipo-IR$_i$ in

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Table 2 Substrate kinetics during hyperinsulinemic–euglycemic clamp

	CON	NAFL	T2DM-non-NAFL	T2DM+NAFL	*P* value
Plasma insulin (mU/l)					
Basal	8.6 ± 1.0^{A}	17.0 ± 2.2^{B}	6.4 ± 0.8^{A}	18.9 ± 4.7^{B}	0.002
10 mU	29.1 ± 2.5	34.1 ± 2.2	30.3 ± 8.4	37.0 ± 3.5	0.335
40 mU	127.5 ± 10.2	132.7 ± 10.5	105.8 ± 11.1	147.8 ± 8.7	0.154
Plasma glucose (mmol/l)					
Basal	5.4 ± 0.2^{A}	5.6 ± 0.2^{A}	9.0 ± 0.8^{B}	8.3 ± 0.9^{B}	<0.001
10 mU	5.1 ± 0.2^{A}	5.2 ± 0.1^{AB}	5.6 ± 0.2^{AB}	5.9 ± 0.4^{B}	0.019
40 mU	5.2 ± 0.1	5.0 ± 0.2	4.8 ± 0.2	5.3 ± 0.1	0.143
M-value (μmol/min/kgFFM)	47.2 ± 2.6^{A}	27.0 ± 3.6^{B}	28.0 ± 2.5^{B}	24.2 ± 2.9^{B}	<0.001
M/I ratio (μmol/min/kgFFM)/(mU/l)	0.40 ± 0.04^{A}	0.23 ± 0.04^{B}	0.29 ± 0.06^{AB}	0.17 ± 0.03^{B}	0.004
EGP (μmol/min/kgFFM)					
Basal	11.6 ± 0.4	11.1 ± 0.4	13.4 ± 1.1	12.9 ± 0.6	0.123
10 mU	4.3 ± 0.5^{A}	7.9 ± 1.2^{B}	9.2 ± 0.9^{B}	9.2 ± 0.8^{B}	<0.001
δ %	−62.9 ± 4.0^{A}	−30.6 ± 8.2^{B}	−30.6 ± 6.2^{B}	−27.9 ± 7.6^{B}	<0.001
40 mU	−2.0 ± 0.5^{A}	0.17 ± 0.6^{AB}	1.8 ± 1.5^{B}	0.5 ± 0.8^{AB}	0.018
δ %	−-117.5 ± 4.4^{A}	−98.7 ± 5.9^{AB}	−86.2 ± 13.3^{B}	−96.5 ± 6.5^{B}	0.030
R_d (μmol/min/kgFFM)					
Basal	11.6 ± 0.3^{AC}	10.4 ± 0.4^{A}	14.5 ± 1.0^{B}	12.9 ± 0.7^{BC}	0.016
10 mU	16.5 ± 1.0^{A}	13.0 ± 1.5^{AB}	10.0 ± 0.5^{B}	11.3 ± 0.3^{B}	<0.001
δ	4.9 ± 1.1^{A}	2.6 ± 4.4^{AC}	−4.4 ± 0.8^{B}	−1.6 ± 0.8^{BC}	<0.001
40 mU	45.6 ± 2.3^{A}	25.7 ± 2.7^{B}	28.5 ± 1.9^{B}	24.3 ± 2.5^{B}	<0.001
δ	34.0 ± 2.3^{A}	15.3 ± 2.8^{B}	14.0 ± 2.1^{B}	11.4 ± 2.8^{B}	<0.001
Glucose oxidation (μmol/min/kgFFM)					
Basal	9.0 ± 2.4	8.1 ± 2.0	8.4 ± 1.0	4.9 ± 1.5	0.466
10 mU	13.8 ± 1.1^{A}	8.7 ± 1.8^{B}	11.5 ± 0.9^{AB}	6.9 ± 0.6^{B}	0.001
δ	4.8 ± 2.1	0.6 ± 1.4	3.1 ± 0.4	2.0 ± 1.9	0.381
40 mU	22.2 ± 2.3^{A}	16.7 ± 2.1^{AB}	16.4 ± 0.9^{AB}	13.5 ± 0.9^{B}	0.020
δ	13.2 ± 1.1^{A}	8.6 ± 1.4^{AB}	8.0 ± 1.2^{B}	8.5 ± 2.1^{AB}	0.037
NOGD (μmol/min/kgFFM)					
Basal	2.8 ± 2.2	2.0 ± 1.9	6.1 ± 1.7	7.9 ± 1.5	0.134
10 mU	3.1 ± 1.7^{AB}	5.1 ± 1.1^{A}	−1.4 ± 0.8^{B}	4.4 ± 0.7^{A}	0.004
δ	0.4 ± 3.0^{AB}	3.1 ± 2.1^{B}	−7.5 ± 0.9^{AB}	−3.5 ± 1.8^{AB}	0.008
40 mU	23.6 ± 2.3^{A}	11.3 ± 1.3^{B}	12.1 ± 2.5^{B}	10.8 ± 2.1^{B}	<0.001
δ	20.8 ± 3.2^{A}	9.3 ± 2.2^{B}	6.1 ± 2.1^{B}	2.9 ± 3.1^{B}	<0.001
Fat oxidation (μmol/min/kgFFM)					
Basal	5.5 ± 0.4	5.0 ± 0.4	5.2 ± 0.2	5.9 ± 0.5	0.482
10 mU	3.7 ± 0.3	4.4 ± 0.5	4.0 ± 0.1	5.0 ± 0.5	0.184
δ	−1.8 ± 0.5	−0.6 ± 0.3	−1.2 ± 0.2	−0.9 ± 0.5	0.250
40 mU	2.0 ± 0.4	2.8 ± 0.5	2.8 ± 0.3	3.5 ± 0.6	0.216
δ	−3.5 ± 0.3	−2.2 ± 0.3	−2.4 ± 0.2	−2.4 ± 0.5	0.052
Plasma FFA (μmol/l)					
Basal	657.6 ± 46.7	726.1 ± 54.5	647.9 ± 45.6	707.3 ± 24.7	0.574
10 mU	193.4 ± 17.6^{A}	317.4 ± 33.2^{B}	229.3 ± 18.2^{A}	322.5 ± 17.4^{B}	0.008
δ %	−69.6 ± 3.3^{A}	−54.5 ± 5.0^{B}	−63.2 ± 4.7^{AB}	−54.3 ± 2.5^{AB}	0.041
40 mU	98.3 ± 13.5	149.4 ± 21.3	119.5 ± 27.9	174.7 ± 18.3	0.080
δ %	−85.3 ± 1.4^{A}	−80.0 ± 1.7^{AB}	−81.3 ± 4.4^{AB}	−75.4 ± 5.0^{B}	0.049

EGP: endogenous glucose production; FFA: free fatty acids; *M*-value: mean glucose infusion rate; M/I ratio: *M*-value per mean plasma insulin concentration (I); NOGD: non-oxidative glucose disposal; R_d: glucose disposal. Different letters indicate significant differences between groups ($P<0.05$).

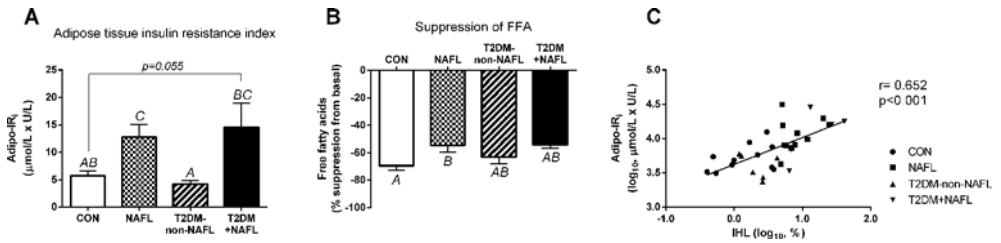

Figure 2. Adipose tissue insulin sensitivity

(**A**) Fasting adipose tissue insulin resistance index (Adipo-IR$_i$ = fasting plasma FFA (μmol/l) × fasting plasma insulin concentrations (U/L)); (**B**) percentage suppression of plasma FFA during 10 mU/m^2/min insulin infusion; (**C**) relation between Adipo-IR$_i$ and intrahepatic lipid (IHL) content (n=33). Results are mean ± SEM. Different letters indicate significant differences between groups (P<0.05).

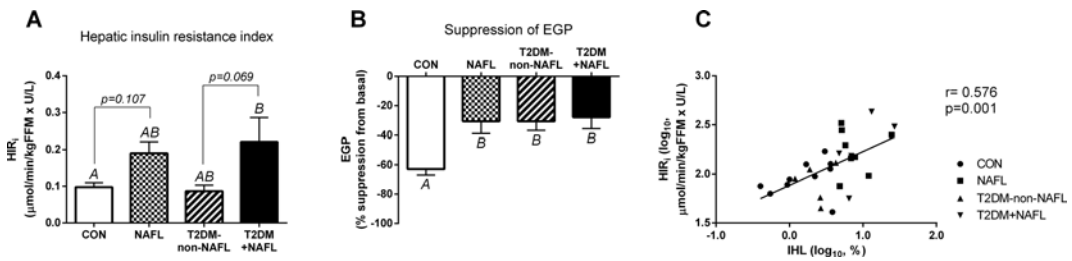

Figure 3. Hepatic insulin sensitivity

(**A**) Fasting hepatic insulin resistance index (HIR$_i$ = fasting EGP (μmol/min/kgFFM) × fasting plasma insulin concentrations (U/l)); (**B**) percentage suppression of EGP during 10 mU/m^2/min insulin infusion; (**C**) relation between HIR$_i$ and intrahepatic lipid (IHL) content (n=28). Results are mean ± SEM. Different letters indicate significant differences between groups (P<0.05).

CON subjects (P=0.961, Figure 2A). NAFL subjects were characterized by higher Adipo-IR$_i$ in comparison with CON subjects (P=0.003) and T2DM-non-NAFL subjects (P=0.046). T2DM+NAFL were also characterized by higher Adipo-IR$_i$ in comparison with T2DM-non-NAFL (P=0.042) and CON (P=0.055), with the latter difference being almost significant (Figure 2A). These results suggest that high IHL content, either with or without T2DM, is associated with reduced adipose tissue insulin sensitivity under fasting conditions.

A more dynamic measure of adipose tissue insulin sensitivity is the insulin-stimulated relative suppression of plasma FFA during 10 mU/m^2/min insulin infusion. Again, suppression of plasma FFA was significantly different among groups (P=0.041, Figure 2B, Table 2). Suppression of plasma FFA was lower in NAFL than in CON subjects (P=0.046, Figure 2B, Table 2), with T2DM-non-NAFL and T2DM+NAFL having intermediate values (Figure 2B, Table 2), suggesting that subjects with NAFL are characterized by adipose tissue insulin resistance comparable with levels in T2DM, independent of IHL content in T2DM.

Suppression of plasma FFA showed a weak negative correlation with IHL content in the whole study population (r=−0.355, P=0.046), while Adipo-IR$_i$ showed a strong positive correlation with IHL content in the whole study population (r=0.652, P<0.001, Figure 2C).

Hepatic insulin sensitivity

Hepatic insulin resistance during basal conditions was determined by using the validated HIR$_i$ [12]. HIR$_i$ was significantly different among groups (P=0.016, Figure 3A). HIR$_i$ in NAFL was comparable with HIR$_i$ in T2DM+NAFL (P=0.908, Figure 3A). HIR$_i$ in T2DM-non-NAFL was comparable with HIR$_i$ in CON (P=0.995, Figure 3A). T2DM+NAFL were characterized by higher HIR$_i$ in comparison with CON (v=0.050) and T2DM-non-NAFL (P=0.069), with the latter difference being almost significant (Figure 3A). While NAFL also tended to have higher HIRi than CON (P=0.107) and T2DM-non-NAFL (P=0.147), this did not reach significance (Figure 3A).

Hepatic insulin sensitivity is best determined by suppression of EGP during 10 mU/m^2/min insulin infusion. Suppression of EGP was different among groups (P<0.001, Figure 3B, Table 2). Suppression of EGP was comparable between NAFL and T2DM-non-NAFL subjects (P=0.999); between NAFL and T2DM+NAFL subjects (P=0.993); and between T2DM-non-NAFL and T2DM+NAFL subjects (P=0.994) (Figure 3B, Table 2). NAFL (P=0.003), T2DM-non-NAFL (P=0.007) and T2DM+NAFL subjects (P=0.006) were all characterized by a lower ability to suppress EGP compared with CON subjects (Figure 3B, Table 2), suggesting that subjects with NAFL are characterized by

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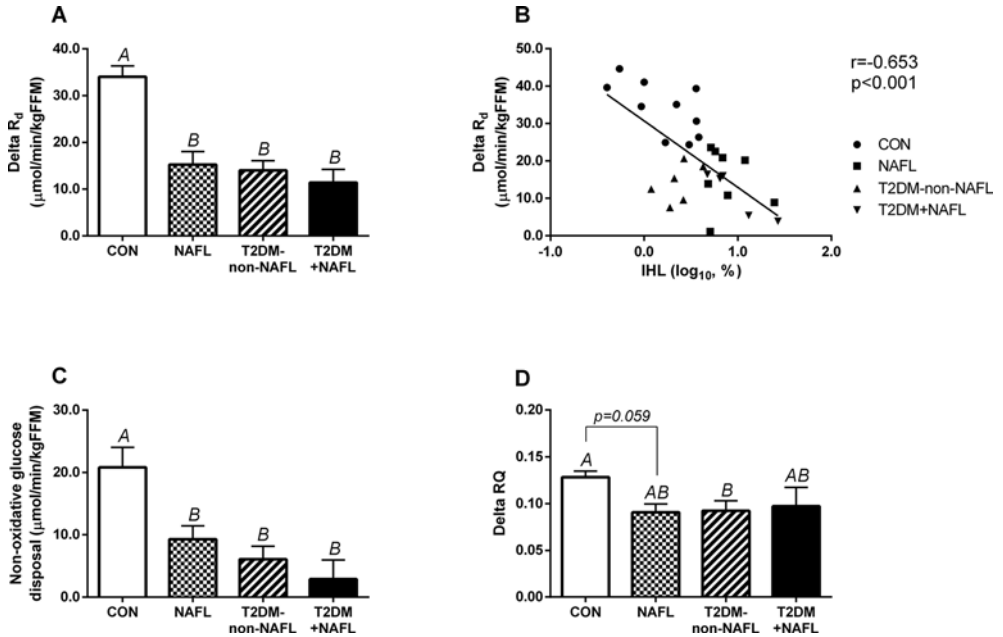

Figure 4. Peripheral insulin sensitivity

(**A**) Insulin-stimulated glucose disposal (ΔR_d) in skeletal muscle during 40 mU/m^2/min insulin infusion; (**B**) relation between ΔR_d and intrahepatic lipid (IHL) content (n=29); (**C**) insulin-stimulated NOGD in skeletal muscle during 40 mU/m^2/min insulin infusion; (**D**) metabolic flexibility (ΔRQ). Results are mean ± SEM. Different letters indicate significant differences between groups (P<0.05).

hepatic insulin resistance comparable with levels observed in T2DM patients, independent of IHL content in T2DM patients.

Suppression of EGP did not significantly correlate with IHL content in the whole study population (r=−0.344, P=0.068). HIR_i, however, did show a positive correlation with IHL content in the whole study population (P=0.567, r=0.001, Figure 3C).

Peripheral insulin sensitivity

Peripheral insulin sensitivity, dominated by skeletal muscle insulin sensitivity, was measured as insulin-stimulated glucose uptake (ΔR_d), the difference between R_d under insulin-stimulated conditions (40 mU/m^2/min) and R_d under basal conditions. ΔR_d was different among groups (P<0.001). ΔR_d was comparable between NAFL and T2DM-non-NAFL subjects (P=0.988); between NAFL and T2DM+NAFL subjects (P=0.768); and between T2DM-non-NAFL and T2DM+NAFL subjects (P=0.925) (Figure 4A, Table 2). NAFL (P<0.001), T2DM-non-NAFL (P<0.001) and T2DM+NAFL subjects (P<0.001) were all characterized by a significantly lower ΔR_d compared with CON subjects (Figure 4A, Table 2). ΔR_d correlated negatively with IHL content in the whole study population (r=−0.653, P<0.001, Figure 4B), suggesting that subjects with NAFL are characterized by peripheral insulin resistance comparable with levels observed in T2DM patients.

NOGD was different among groups (P<0.001, Figure 4C, Table 2). NOGD was comparable between NAFL and T2DM-non-NAFL subjects (P=0.820); between NAFL and T2DM+NAFL subjects (P=0.376); and between T2DM-non-NAFL and T2DM+NAFL subjects (P=0.844) (Figure 4C, Table 2). NAFL (P=0.026), T2DM-non-NAFL (P=0.004) and T2DM+NAFL subjects (P=0.001) were all characterized by a lower NOGD compared with CON subjects (Figure 4C, Table 2).

Metabolic flexibility

Metabolic flexibility (ΔRQ), the difference in RQ under insulin-stimulated conditions (40 mU/m^2/min) and RQ under basal conditions, was different among groups (P=0.028, Figure 4D). ΔRQ was comparable between NAFL and T2DM-non-NAFL subjects (P=0.948), between NAFL and T2DM+NAFL subjects (P=0.969) and between T2DM-non-NAFL and T2DM+NAFL subjects (P=0.861) (Figure 4D). NAFL, T2DM-non-NAFL and T2DM+NAFL subjects showed lower metabolic flexibility compared with CON (Figure 4D). The difference was significant between CON and T2DM-non-NAFL subjects (P=0.047), while the difference between CON and NAFL subjects (P=0.059)

was almost significant (Figure 4D). The difference between CON and T2DM+NAFL subjects was not significant (P=0.457, Figure 4D).

Discussion

In the current study, we show that non-diabetic individuals with NAFL are metabolically impaired to a similar degree as BMI-matched patients with T2DM. Moreover, our data suggests that elevated IHL content seems to be a contributing determinant of adipose tissue insulin resistance under fasting conditions, hepatic insulin resistance under fasting conditions and insulin-stimulated peripheral insulin resistance in individuals with and without T2DM.

Previous studies revealed that adipose tissue, hepatic and skeletal muscle insulin sensitivity all were impaired in individuals with NAFL in comparison with BMI-matched healthy individuals with normal IHL content [15-18]. Thus, we anticipated that adipose tissue, hepatic and skeletal muscle insulin sensitivity and metabolic flexibility in NAFL subjects would have intermediate values when compared with insulin-resistant T2DM patients and more insulin-sensitive CON subjects. In contrast with this expectation, here we demonstrated that adipose tissue, hepatic and skeletal muscle insulin sensitivity and metabolic flexibility were all impaired in subjects with NAFL to a similar degree to that observed in T2DM patients—irrespective of IHL content—who were marked by hyperglycemia at onset of the study (FPG $\geqslant$ 7.0 mmol/l and $<$10.0 mmol/l) while on glucose lowering medication.

When the whole study population was taken together, IHL content showed strong associations with markers of adipose tissue, and hepatic insulin resistance measured under fasting conditions, and with skeletal muscle insulin resistance measured using a hyperinsulinemic–euglycemic clamp. Considering NAFL and CON subjects together, we found an association between IHL content and plasma glucose levels upon OGTT. A recent study investigating surrogate indexes of insulin resistance in NAFL patients found that—independent of obesity—plasma glucose levels upon OGTT were different when NAFL patients were divided into groups of low and high liver fibrosis, while plasma glucose levels were not different when NAFL patients were divided into groups of low and high steatosis [34]. We did not measure the state of fibrosis in this study. Therefore, it cannot be ruled out that some of our NAFL subjects were characterized by having significant fibrosis of the liver, which might have been responsible for the lower glucose tolerance in these subjects compared with CON subjects.

Despite higher IHL content in NAFL and in T2DM+NAFL subjects compared with CON and T2DM-non-NAFL subjects, the transaminases AST and ALT—two serum markers for liver disease—were not significantly different between these groups. Even though AST and ALT concentrations in most participants were within the suggested normal clinical range (AST: 4–40U/L; ALT: 1–45U/L) [35], we did observe a positive correlation between AST, and ALT with IHL content. This observation agrees with earlier studies showing that liver enzymes do associate with IHL content [35-38]. Therefore, our results are in line with previous reports showing that significant increases in IHL content measured with ^{1}H-MRS reflected only modest elevations in plasma transaminase levels [37,39,40], and that direct measurement of IHL content is warranted to detect early stages of NAFL [41].

The average elevation in IHL content was not significantly different between NAFL subjects and T2DM+NAFL patients; and CON subjects and T2DM-non-NAFL patients also had similarly low levels of IHL content. This is of importance when comparing these groups considering that the IHL content is directly correlated with markers for insulin resistance, as shown in the present study and previous studies [9-14]. Of note, 11 T2DM patients were treated with metformin, a glucose-lowering drug known to decrease IHL content [42]. Metformin use was comparable between T2DM patients with normal IHL content (n=5) and T2DM patients with high IHL content (n=6), but might have resulted in slight underestimations of the deteriorations in patients with T2DM.

In this study, individuals with NAFL had profound resistance to the insulin-stimulated suppression of plasma FFA in comparison with healthy individuals with normal IHL content, with intermediate values observed in patients with T2DM. Previous research described that patients with NAFL had lower insulin-stimulated suppression of plasma FFA than healthy individuals with normal IHL content [6,15,35,43,44]. Other studies have reported impaired suppression of plasma FFA during the OGTT measurement in patients with NASH [45] and NAFL [40] in comparison with individuals without fatty liver disease, in the absence [45] and presence [40,45] of T2DM. No previous study, however, compared insulin-stimulated suppression of plasma FFA in patients with NAFL to T2DM patients. Our observation was unexpected, and suggests that the insulin resistance in adipose tissue is part of an impaired substrate handling in individuals with NAFL, who are otherwise healthy.

In addition to the above, we found higher Adipo-IR$_i$—a validated predictor of adipose tissue insulin sensitivity under fasting conditions [33]—in NAFL subjects and T2DM+NAFL patients in comparison with CON subjects and T2DM-non-NAFL patients, as well as a strong association of Adipo-IR$_i$ with IHL content. A recent study in 302 subjects with different degrees of glucose tolerance concluded that Adipo-IR$_i$ increased in the transition from normal

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glucose tolerance to T2DM, with Adipo-IR_i increasing from subjects with normal glucose tolerance, over subjects with impaired glucose tolerance, to patients with T2DM [46]. Our results are somewhat different from those recent results, given that our T2DM-non-NAFL patients were not characterized by higher Adipo-IR_i compared with CON subjects, while our NAFL subjects had high Adipo-IR_i, comparable with our T2DM+NAFL patients. It is known, however, that the prevalence of NAFL also increases from subjects with normal glucose tolerance, over subjects with impaired glucose tolerance, to patients with T2DM [6,47]. Thus, differences in the prevalence of NAFL in the subpopulations studied in [46] might have been a confounding factor contributing to the higher Adipo-IR_i.

We indeed found a strong association between Adipo-IR_i and IHL content. While basal plasma FFA were somewhat higher in NAFL and T2DM+NAFL subjects, the higher Adipo-IR_i mainly originated from higher basal insulin levels in NAFL and T2DM+NAFL subjects compared with CON and T2DM-non-NAFL subjects. Thus, all subjects with NAFL were characterized by a higher resistance to the antilipolytic effect of insulin [46]. Therefore, our data seems to indicate that Adipo-IR_i is more closely associated with the development of NAFL than with the development of T2DM *per se*.

Elevated IHL content has been shown to be closely related to hepatic insulin resistance [12]. Here, we show that high IHL content impairs the suppression of EGP to a similar degree as in patients with T2DM, but that, in T2DM patients, suppression of EGP is impaired independently of IHL content. Of note, visceral adipose tissue mass is as strongly related to suppression of EGP as IHL content [47], and correlates closely with IHL content itself [12,47]. We did not measure visceral adipose tissue mass in this study, but higher visceral adipose tissue mass might have contributed to the impaired suppression of EGP as well.

While elevated IHL content is related to hepatic insulin resistance, Ortiz-Lopez et al. [6] previously observed that suppression of EGP in individuals with NAFL and normal glucose tolerance was not different from individuals without NAFL. However, this observation was only true in individuals with normal glucose tolerance, since individuals with NAFL who suffered from pre-diabetes or T2DM did have lower suppression of EGP [6,35]. Our data support the latter, since our NAFL subjects also had elevated plasma glucose values upon OGTT. Moreover, our T2DM-non-NAFL patients were also characterized by lower suppression of EGP. Thus, impaired glucose handling rather than elevated IHL content seems to be related to impaired suppression of EGP.

The HIR_i has been described to be lower in subjects with NAFL in comparison with healthy individuals [6,15,16,35]. In the present study, HIR_i correlated with IHL content as previously observed in non-diabetic obese subjects [9,48]. Furthermore, in contrast with suppression of EGP, T2DM+NAFL patients had higher HIR_i values than T2DM-non-NAFL patients, with HIR_i values being comparable between T2DM non-NAFL patients and CON individuals. Therefore, one could suggest that hepatic insulin sensitivity under fasting conditions is more dependant on IHL content than the insulin-stimulated suppression of EGP.

The present study demonstrated that NAFL subjects were characterized by similar skeletal muscle insulin resistance than patients with T2DM, and confirmed previous results showing lower rates of insulin-stimulated glucose disposal in individuals with NAFL than in healthy subjects with normal IHL content [6,15,16,44]. Insulin resistance in skeletal muscle is featured by lower metabolic flexibility, a highly important determinant of T2DM development [49], and known to be impaired in subjects with NAFL compared with CON individuals [44]. Therefore, we measured metabolic flexibility during the hyperinsulinemic–euglycemic clamp. We found a similar impairment in metabolic flexibility in NAFL subjects as in T2DM-non-NAFL and T2DM+NAFL patients, which was reflected by a lower increase in RQ upon high insulin infusion in comparison with CON subjects. In other groups of subjects with prediabetes, metabolic flexibility and skeletal muscle insulin resistance have been shown to be lower than in BMI-matched healthy subjects, although values were still higher than those observed in T2DM patients [19,20]. Thus, our data suggest that individuals with NAFL have more severe insulin resistance of the skeletal muscle than other groups at risk of developing T2DM, and in fact have insulin resistance to a level similar to that seen in T2DM patients.

An association between skeletal muscle insulin resistance and IHL content has been previously described in healthy obese subjects [9-11,50] and individuals with NAFL [50]. Here, we show that this association still persists when NAFL, T2DM-non-NAFL, T2DM+NAFL, and CON individuals are merged and studied together. However, skeletal muscle insulin resistance was not different between T2DM-non-NAFL and T2DM+NAFL patients, and T2DM-non-NAFL patients were more insulin resistant at the level of the skeletal muscle than CON subjects. Thus, although across the four groups, higher amounts of IHL content did associate with lower levels of insulin sensitivity in skeletal muscle, insulin resistance in the skeletal muscle of patients with T2DM did not develop solely in the presence of NAFL, unmasking other causes (of yet-to-be-determined origin) related to the development of insulin resistance of the skeletal muscle in patients with T2DM.

In conclusion, we show that non-diabetic individuals with NAFL suffer from metabolic perturbations to a similar degree as patients with T2DM. These observations suggest that NAFL is an important feature leading to severe insulin resistance and should be viewed as a serious health threat for the development of T2DM.

Clinical Perspectives

- Non-alcoholic fatty liver (NAFL) is the most common liver disorder in western society and is closely related to the development of insulin resistance and type 2 diabetes (T2DM). However, the degree to which metabolic complications typical for T2DM already develop in individuals with NAFL is unknown.
- Adipose tissue, hepatic and skeletal muscle insulin sensitivity, and metabolic flexibility were all severely impaired in individuals with NAFL in comparison with those with normal intrahepatic lipid, and these impairments were comparable with those observed in patients with T2DM.
- NAFL is an important feature leading to severe insulin resistance and should be viewed as a serious health threat for the development of T2DM.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution
B.B. designed the study, performed experiments, analyzed data, and wrote the manuscript; V.B.S. designed the study, analyzed data, critically reviewed, and edited the manuscript; T.J. critically reviewed and edited the manuscript; A.G. performed experiments and analyzed data; B.H. critically reviewed and edited the manuscript; Y.B. performed experiments; D.D. performed experiments; M.R. critically reviewed and edited the manuscript; M.K.C.H. designed the study, analyzed data, and critically reviewed and edited the manuscript; P.S. designed the study, analyzed data, and critically reviewed and edited the manuscript. P.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Abbreviations
ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; ATP, adenosine triphosphate; BMI, body mass index; CON, control; EGP, endogenous glucose production; FFA, free fatty acids; FPG, fasting plasma glucose; GGT, g-glutamyl transpeptidase; HDL, high-density lipoprotein; ^{1}HMRS, Proton magnetic resonance spectroscopy; IHL, intrahepatic lipid; LDL, low-density lipoprotein; NAFL, non-alcoholic fatty liver; NOGD, non-oxidative glucose disposal; R_a, rate of appearance; R_d, rate of disappearance; T2DM, type 2 diabetes mellitus; TG, triglycerides; VO_{2max}, maximal oxidative capacity.

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