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Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms

Abstract *Aims/Hypothesis:* Non-alcoholic fatty liver disease (NAFLD) has been associated with the metabolic syndrome. However, it is not clear whether insulin resistance is an independent feature of NAFLD, and it remains to be determined which of the in vivo actions of insulin are impaired in this condition. *Methods:* We performed a two-step (1.5 and 6 pmol min^{-1} kg^{-1}) euglycaemic insulin clamp coupled with tracer infusion ([6,6-^{2}H$_2$]glucose and [^{2}H$_5$] glycerol) and indirect calorimetry in 12 non-obese, normolipidaemic, normotensive, non-diabetic patients with biopsy-proven NAFLD and six control subjects. *Results:* In NAFLD patients, endogenous glucose production (basal and during the clamp) was normal; however, peripheral glucose disposal was markedly decreased (by 30% and 45% at the low and high insulin doses, respectively, $p<0.0001$) at higher plasma insulin levels ($p=0.05$), due to impaired glucose oxidation ($p=0.003$) and glycogen synthesis ($p<0.001$). Compared with control subjects, glycerol appearance and lipid oxidation were significantly increased in NAFLD patients in the basal state, and were suppressed by insulin to a lesser extent ($p<0.05$–0.001). The lag phase of the in vitro copper-catalysed peroxidation of LDL particles was significantly shorter in the patients than in the control subjects (48±12 vs 63±13 min, $p<0.04$). Lipid oxidation was significantly related to endogenous glucose production, glucose disposal, the degree of hepatic steatosis, and LDL oxidisability. *Conclusions/interpretation:* Insulin resistance appears to be an intrinsic defect in NAFLD, with the metabolic pattern observed indicating that adipose tissue is an important site.

Keywords Fatty liver · Glucose disposal · Glucose production · Glycerol appearance · Lipoperoxidation

Abbreviations ALT: Alanine aminotransferase · AST: Aspartate aminotransferase · FFM: Fat-free mass · MRI: Magnetic resonance imaging · NAFLD: Non-alcoholic fatty liver disease · NHANES: National Health and Nutrition Examination Survey · Ra: Rate of appearance · VCO$_2$: Rate of CO$_2$ production · VO$_2$: Rate of O$_2$ consumption

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Introduction

Non-alcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis and advanced fibrosis, with histologic features of alcohol-induced liver disease in individuals who do not consume significant amounts of alcohol. There is growing concern regarding NAFLD due to its high prevalence in the general population and its potential for progression to cirrhosis [1], terminal liver failure [2] and hepatocellular carcinoma [3]. In a recent survey based on the National Health and Nutrition Examination Survey (NHANES) III [4], unexplained aminotransferase elevations, most likely due to NAFLD, were found in 5.4% of the general population. Similar prevalence data have been obtained in Japanese [5] and Italian [6] populations. Multiple lines of evidence link NAFLD with the metabolic syndrome [7], and (visceral) obesity and type 2 diabetes are the most commonly associated features [8–13]. Despite this, recent

studies have reported that none of the known risk factors for insulin resistance can be found in a considerable proportion of these patients. Thus, among adult participants in the NHANES III selected for unexplained elevated alanine aminotransferase (ALT) activity, 55% had a BMI<30 and 16% a BMI<25 [14]. Likewise, in a population of non-diabetic NAFLD subjects, 22% were lean, 64% did not meet the minimal criteria for metabolic syndrome (according to the National Cholesterol Education Program—Adult Treatment Panel III definition) [15], and 12% had none of the features of the metabolic syndrome [16].

It remains to be determined which of the in vivo actions of insulin are impaired in NAFLD and whether these are related to hepatic steatosis. In the present study, we selected a group of non-obese, non-diabetic NAFLD patients to explore the extent, sites and mechanisms of insulin resistance. Insulin-resistant states are characterised by oxidative modification of circulating LDL cholesterol particles, which are responsible, among other factors, for damage to the vascular endothelium [17]. Oxidative stress has been involved in the development of steatosis and its progression to steatohepatitis [18, 19]. Based on these findings, we investigated the oxidative status of circulating lipids in NAFLD by measuring the in vitro susceptibility of serum LDL to oxidative damage.

Subjects and methods

Study subjects Twelve male subjects with NAFLD were studied. All patients had chronically elevated (>6 months) levels of ALT. The diagnosis of NAFLD was based upon the results of a liver biopsy and the exclusion of other aetiologic factors of chronic liver disease. Subjects were screened for hepatitis B (HBsAg, anti-HBcAg antibodies), hepatitis C (anti-HCV antibodies), non-organ-specific autoantibodies, α_1-antitrypsin deficiency, copper-storage disease, anti-endomysium antibodies and thyroid hormones. Alcohol consumption was assessed by detailed history, confirmed by other family members, and laboratory markers (immunoglobulin A levels, mean red blood cell corpuscular volume); patients consuming more than 10 g of alcohol per day were excluded. To minimise the impact of known determinants of insulin resistance, additional inclusion criteria were: (1) a BMI<30 kg/m^2; (2) a waist circumference <102 cm; (3) a normal serum lipid profile (defined as a serum triglyceride concentration <2 mmol/l and serum total cholesterol <6 mmol/l); and (4) normal glucose tolerance as determined by a 75-g OGTT (i.e. a fasting plasma glucose concentration <7 mmol/l and a 2-h postglucose plasma glucose concentration <7.8 mmol/l) [20, 21]. No patient had evidence of advanced liver disease. Patients with a histological diagnosis of cirrhosis were excluded. The control group consisted of six healthy men (with normal fasting glucose levels, lipid profiles and liver function tests) who were matched to the NAFLD group in terms of age, weight and body composition (Table 1). All participants gave their written consent prior to inclusion, and the study was approved by the ethics committee of the institution.

Histological data Liver biopsy specimens were scored using the criteria described by Brunt [22]. On liver biopsy, patients had variable degrees of macrovesicular steatosis (40±21%, range 15–75%), and necro-inflammation (grade 1 in three patients, grade 2 in seven patients, and grade 3 in two patients). Fibrosis was observed in six patients (stage 1 in five patients and stage 2 in one patient); none of the patients had evidence of cirrhosis.

Abdominal and visceral fat measurement Fat-free mass (FFM) was calculated according to Hume's formula, which has recently been validated against measurements obtained by the tritiated water technique or electrical bioimpedance [23]. Fat mass was calculated as the difference between body weight and FFM. In six NAFLD patients and five control subjects, visceral fat and subcutaneous fat depots were estimated by magnetic resonance imaging (MRI) as previously described [24].

Experimental protocol All patients followed a balanced diet (50% carbohydrate, 35% fat and 15% protein) for 3 days before the study. All experiments were performed in the morning after an overnight fast. Teflon catheters were placed into an antecubital vein for isotope infusion and into a contralateral dorsal hand vein (heated at 55°C to achieve arterialisation of venous blood) for blood sampling. The experimental protocol consisted of two periods:

1. Postabsorptive period. After obtaining a basal blood sample, primed continuous infusions of [6,6-^{2}H$_2$]glucose (0.22 μmol min^{-1} kg^{-1}; prime 17.6 μmol/kg) and [^{2}H$_5$]glycerol (0.1 μmol min^{-1} kg^{-1}; prime 1.5 μmol/kg) were initiated, and continued at a constant rate throughout the study (basal and euglycaemic insulin clamp). Blood samples for the determination of tracer enrichments, substrates and hormone concentrations were drawn at 10-min intervals during the last 30 min of the 2-h basal period, when isotopic steady state was achieved.
2. Insulin clamp. A two-step euglycaemic–hyperinsulinaemic clamp was performed using insulin infusion rates of 1.5 pmol min^{-1} kg^{-1} and 6 pmol min^{-1} kg^{-1} (Humulin R; Eli Lilly, Indianapolis, IN, USA); each step lasted for 2 h. Plasma glucose concentrations were measured every 5 min, and a 20% glucose solution was infused at a variable rate to keep plasma glucose constant at the basal level [25]. The 20% glucose solution was enriched with [6,6-^{2}H$_2$]glucose, and the constant infusion of [6,6-^{2}H$_2$] glucose was gradually reduced and stopped 20 min into the clamp [26]. Blood samples were drawn at 20-min intervals during the first 80 min and every 10 min during the last 40 min of each step.

Indirect calorimetry Using an open-circuit canopy system, rates of CO_2 production (VCO$_2$) and oxygen consumption (VO$_2$) were determined for 30 min during the basal period and during the last 30 min of each insulin clamp step. Urine was collected at the end of the study, and the amount of protein oxidised was calculated from urinary nitrogen excretion.

Table 1 Clinical and laboratory data in NAFLD patients and control subjects

	NAFLD patients (n=12)	Control subjects (n=6)
Age (years)	34±8 (20–51)	31±10 (19–42)
BMI (kg/m²)	26.4±1.4 (23.7–29.0)	25.8±2.1 (23.7–28.4)
Fat mass (kg)	25±4 (20–33)	25±4 (20–31)
Fat mass (%)	31±2 (27–34)	29±3 (26–33)
WHR	0.92±0.05 (0.84–1.00)*	0.86±0.06 (0.78–0.91)
Systolic BP (mmHg)	122±4 (115–130)	118±4 (110–120)
Diastolic BP (mmHg)	75±5 (70–85)	74±5 (70–80)
Fasting plasma glucose (mmol/l)	5.3±0.3 (4.7–5.6)	5.3±0.2 (5.0–5.7)
2-h plasma glucose (mmol/l)	6.2±0.9 (4.8–7.5)	ND
Serum total cholesterol (mmol/l)	4.6±1.1 (2.6–5.9)	4.0±0.7 (3.2–4.8)
Serum HDL cholesterol (mmol/l)	1.1±0.2 (0.8–1.4)*	1.3±0.2 (1.1–1.5)
Blood β-hydroxybutyrate (μmol/l)	84±33*	37±24
Plasma NEFA (μmol/l)	686±260*	468±101
Serum triglycerides (mmol/l)	1.4±0.6 (0.5–1.9)*	0.8±0.2 (0.6–0.9)
Serum AST (U/l)[a]	43±18 (21–90)*	20±4 (17–25)
Serum ALT (U/l)[a]	105±46 (53–212)*	21±4 (16–25)

Data are means±SD (ranges)
ND Not determined
*$p<0.05$ vs control subjects
[a]Normal range <40 U/l

Analytical determinations Plasma glucose levels were measured by the glucose oxidase method (Beckman Instruments, Fullerton, CA, USA; interassay CV<4%). Plasma insulin and C-peptide concentrations were assessed by a double-antibody RIA (Diagnostic Products Corporation, Los Angeles, CA, USA; interassay CV<13%). Enzymatic kits were used to determine fasting levels of serum cholesterol, triglyceride, glycerol (all Alfa Biotech, Rome, Italy) and NEFA (NEFA C; Wako Chemicals, Neuss, Germany). HDL cholesterol levels were evaluated after precipitation of apolipoprotein B-containing lipoproteins with heparin and manganese chloride. Urinary nitrogen was determined by the Kjeldahl procedure.

Deuterium (^{2}H) enrichment of plasma glycerol and glucose was determined as previously described [27, 28]. A gas chromatography-mass spectrometry system (model no. 5972; Hewlett Packard, Palo Alto, CA, USA) was used to measure ^{2}H enrichment, selectively monitoring ions at m/z 200, 201 and 202 for glucose, and 145 and 148 for glycerol. Isotopic enrichments were expressed as ratios of tracer/tracee [29].

LDL oxidisability During the basal state and after the insulin clamp, blood samples were collected into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing EDTA (1 mg/ml), immediately centrifuged, and then stored at −20°C. LDLs were isolated by sequential ultracentrifugation in NaBr solution containing EDTA (density 1.019–1.063) and stored at 4°C. The susceptibility of the LDL fraction to oxidation was evaluated as the length of the lag phase during copper-catalysed oxidation as previously described [30, 31].

Data analysis In the basal state, endogenous glucose production was calculated as the rate of appearance (R_a) of glucose according to the steady-state equation for stable isotopic tracers [26, 27]. During the clamp, glucose R_a was calculated using a two-compartment model as previously described [32]. Endogenous glucose production during the clamp was calculated as the difference between glucose R_a and the infusion rate of exogenous glucose. The mean rate of endogenous glucose production during each insulin infusion step was added to the glucose infusion rate during the same period (corrected for the glucose changes in its distribution space) to determine the total body glucose disposal rate. Since plasma insulin is a strong inhibitory stimulus for endogenous glucose production [33], even mild hyperinsulinaemia may mask hepatic insulin resistance; therefore an index of insulin resistance in relation to endogenous glucose production was calculated as the product of fasting endogenous glucose production and fasting plasma insulin concentration. Experimental validation for the use of this index has been published [34]. The posthepatic clearance rate of insulin was estimated as the ratio between the insulin infusion rate (at each of the two clamp steps) and the corresponding increment in plasma insulin concentration

Table 2 Plasma substrate and hormone concentrations during basal state and low- and high-dose insulin clamp studies

	NAFLD patients			Control subjects		
	Basal	Clamp (pmol min⁻¹ kg⁻¹)		Basal	Clamp (pmol min⁻¹ kg⁻¹)	
		0.15	6.0		0.15	6.0
Glucose (mmol/l)	5.3±0.3	5.4±0.3	5.4±0.3	5.3±0.2	5.3±0.3	5.2±0.2
Insulin (mmol/l)	66±36	108±48	642±138	42±18	144±60	534±144
C-peptide (ng/ml)	2.7±0.7	2.2±0.5	1.8±0.7*	2.1±0.6	1.6±0.5	1.0±0.5
Total glycerol (μmol/l)	174±67	136±59	132±61	84±22	74±20	65±24
Free glycerol (μmol/l)	98±24	68±18	51±10	76±14	58±13	41±7

Data are means±SD
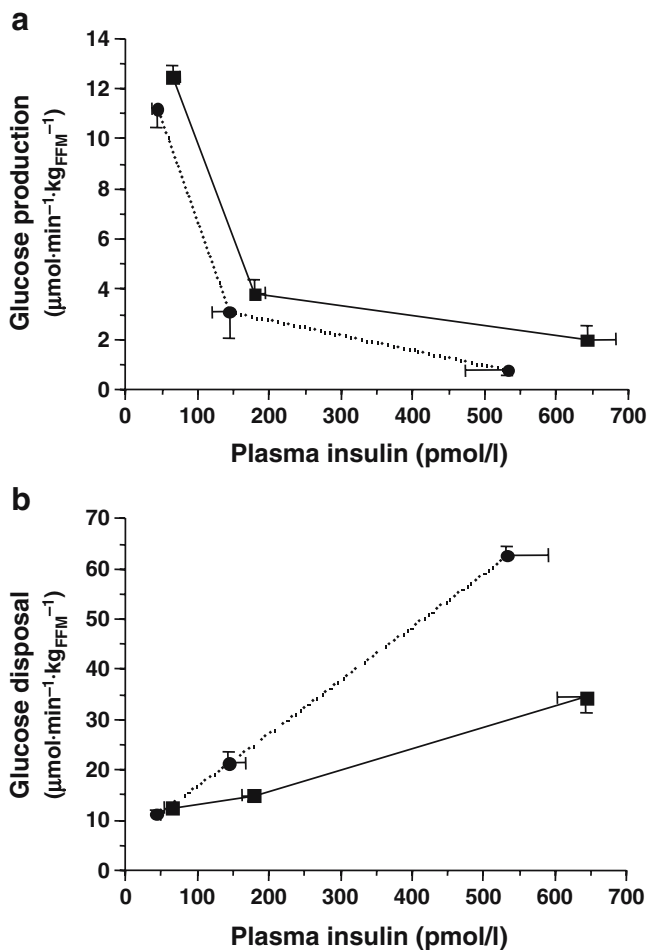*$p≤0.05$ vs control subjects for the interaction term

Fig. 1 Dose–response curves for insulin-mediated suppression of endogenous glucose production (**a**), and stimulation of glucose disposal (**b**) during a two-step insulin clamp in NAFLD patients (*continuous line*) and control subjects (*dotted line*). The data points are means±SEM

FFM (μmol min^{-1} kg$_{FFM}$$^{-1}$). Glycerol R_a was determined in the basal state and during the last 30 min of each step of the insulin clamp, in view of the relatively short time for glycerol enrichment to reach a steady state, using the steady-state calculation. Studies tracing both endogenous glycerol and endogenous NEFA have established that plasma glycerol R_a reflects the hydrolysis of both adipose tissue and VLDL triglycerides at an approximate ratio of 4:1. To overcome this caveat, glycerol R_a was used as an index of whole-body lipolysis [36, 37].

Statistical analysis Results are expressed as means±SD in the text and as means±SEM in the figures. Mann–Whitney U-test was used to compare groups; proportions were compared using the chi square test. Changes in any variable during the clamp steps were tested by ANOVA for repeated measures, with group as one factor, time (or insulin dose) as the other factor, and their interaction (to indicate differential effects of insulin in the two groups). The mean values of the last four measurements of the basal period and each clamp period were used in all of the analyses. A p value of less than 0.05 was considered to be statistically significant.

Results

Clinical and biochemical data The patients with NAFLD were matched to the control subjects in terms of age, body size and fat mass, although the latter was more centrally located in patients than in control subjects according to the WHR (Table 1). The amount of subcutaneous fat (201± 33 cm^2 in NAFLD patients vs 264±9 in control subjects, p=0.06) was slightly higher in control subjects than in NAFLD patients; conversely, visceral fat (99±41 vs 75± 18 cm^2, respectively, p=0.27), and the ratio of visceral fat: subcutaneous fat (0.48±0.16 vs 0.30±0.11, respectively, p=0.07) tended to be higher in patients than in control subjects, but these differences did not reach statistical significance. Three of the six NAFLD patients and none of the control subjects reached the threshold value of 130 cm^2 for visceral fat area, above which a greater prevalence of abnormalities of the metabolic syndrome has been described [38].

Basal plasma glucose, insulin, C-peptide and serum lipid levels were within the normal range in both groups. However, fasting insulin and C-peptide levels tended to be higher (Table 2), HDL cholesterol concentrations were significantly lower, and triglyceride levels were significantly higher in patients than in control subjects. In subjects

above baseline, corrected for the percentage change in C-peptide concentrations. Posthepatic insulin delivery rates were then calculated as the product of posthepatic insulin clearance and the appropriate plasma insulin concentration. Glucose and lipid oxidation rates were obtained from calorimetric measurements as previously described [35]. Non-oxidative glucose disposal was calculated as the difference between the total glucose disposal rate and glucose oxidation. Endogenous glucose production, the glucose disposal rate and its components were normalised per kg of

Table 3 Glucose metabolism parameters during the basal state and low- and high-dose insulin clamp studies		NAFLD patients			Control subjects		
		Basal	Clamp		Basal	Clamp	
			0.15	6.0		0.15	6.0
Hepatic IR [mmol min^{-1} kg$_{FFM}$$^{-1}$ (pmol/l)]		1.8±1.0	1.5±0.8	2.8±2.3*	1.2±0.5	1.1±1.0	1.0±0.8
Glucose oxidation (μmol min^{-1} kg$_{FFM}$$^{-1}$)		26±8	32±5	52±12*	24±6	39±8	69±6
Non-oxidative glucose disposal rate (μmol min^{-1} kg$_{FFM}$$^{-1}$)		1±6	4±8	24±20*	3±5	10±15	72±7

Data are means±SD
*p≤0.05 vs control subjects for the interaction term
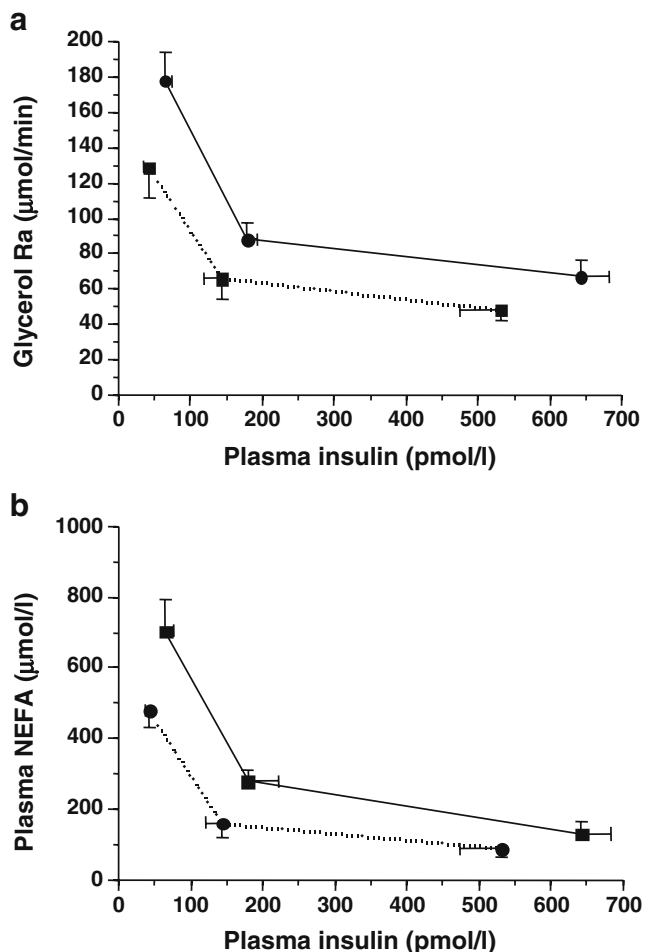
Fig. 2 Dose–response curves for insulin-mediated suppression of glycerol R_a (**a**) and plasma NEFA levels (**b**) during a two-step insulin clamp in NAFLD patients (*continuous line*) and control subjects (*dotted line*). The data points are means±SEM

with NAFLD, ALT exceeded aspartate aminotransferase (AST) in all cases (AST/ALT ratio 0.4±0.4). Blood pressure was within the normal range in all patients. Type 2 diabetes was present in first-degree relatives of three patients with NAFLD and two control subjects (*p*=ns).

Insulin clearance and secretion Posthepatic insulin clearance tended to be lower in patients than in control subjects (0.83±0.17 vs 1.04±0.45 l/min, *p*=0.09), whereas fasting posthepatic insulin delivery was not significantly different between the two groups (65±35 vs 43±16 pmol/min, *p*=ns). Plasma C-peptide levels were significantly (*p*=0.01) higher in NAFLD patients than in control subjects across the insulin dose–response curve, indicating impaired insulin-mediated suppression of endogenous insulin release (Table 2).

Glucose metabolism In the basal state, endogenous glucose production was not different between NAFLD patients and control subjects. During the clamp, the dose–response curve for insulin-mediated suppression of endogenous glucose production was shifted upward slightly in patients compared with that in control subjects, but this difference

was not statistically significant (*p*=0.2) (Fig. 1). The hepatic insulin resistance index, calculated to take into account the differences in plasma insulin concentrations, was significantly higher in the patients across the dose–response function (*p*=0.05) (Table 3). The ability of insulin to stimulate glucose disposal was markedly impaired in NAFLD patients compared with control subjects (Fig. 1), by 30% at the lower insulin dose and by 45% at the higher insulin dose (*p*<0.0001). No correlations were observed between the degree of hepatic or peripheral insulin resistance and the amount of fatty infiltration of the liver, or histological grading or staging.

Although the basal rate of glucose oxidation was similar in the two groups, the insulin-stimulated increase in glucose oxidation was significantly (*p*=0.003 for the interaction term) blunted in patients compared with control subjects, as was the insulin-stimulated increase in non-oxidative glucose disposal (*p*<0.001 for the interaction term) (Table 3).

Lipid metabolism In the basal state, circulating concentrations of free (*p*=0.05) and total glycerol (*p*<0.004), NEFA
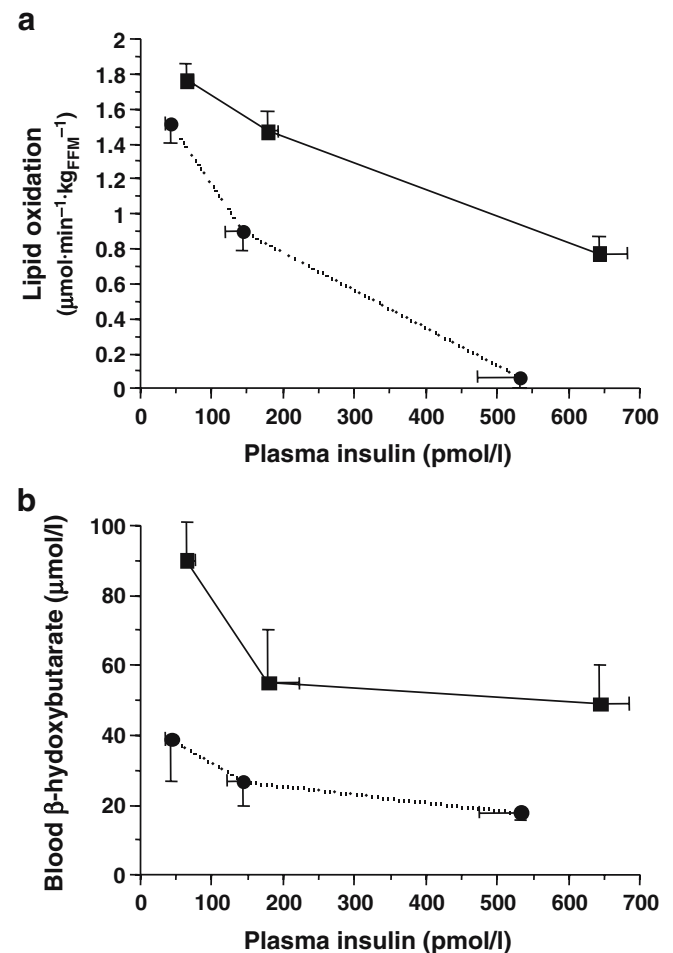

Fig. 3 Dose–response curves for insulin-mediated suppression of whole-body lipid oxidation (**a**) and blood β-hydroxybutyrate concentrations (**b**) during a two-step insulin clamp in NAFLD patients (*continuous line*) and control subjects (*dotted line*). The data points are means±SEM
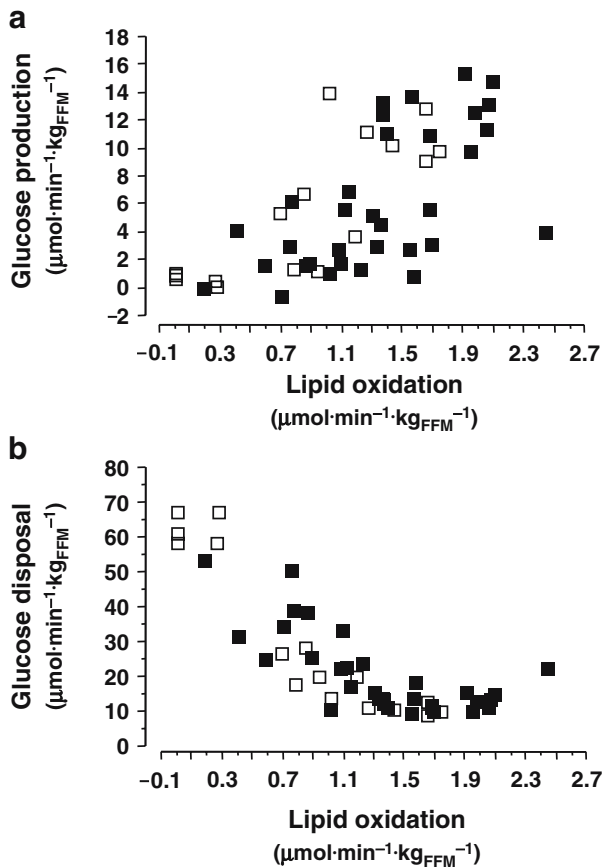
Fig. 4 Graphs showing the relationships between whole-body lipid oxidation (basal and during clamp) and endogenous glucose production (**a**) and glucose disposal (**b**) in NAFLD patients (*filled symbols*) and control subjects (*open symbols*)

Fig. 5 Graph showing the correlation between the amount of fatty infiltration of the liver at biopsy and the average rate (basal and insulin-stimulated) of whole-body lipid oxidation in NAFLD patients ($r=0.63$, $p<0.04$)

Discussion

The group of NAFLD patients examined in this study was carefully selected not to be obese, hypertensive or dyslipidaemic, to have normal glucose tolerance, and not to have marked abdominal visceral fat accumulation, as these conditions are associated with insulin resistance. None of the patients met the three minimum criteria required to identify the metabolic syndrome [15] (two patients met one criterion, three patients met two criteria, and the remaining seven NAFLD patients met none of the criteria). Furthermore, the patients were closely matched to the control subjects in terms of age, BMI and body composition. Despite these efforts to minimise the impact of known determinants of insulin resistance, the NAFLD patients had an increased WHR, a somewhat increased ratio of visceral: subcutaneous abdominal fat (as determined by MRI) and subclinical dyslipidaemia (higher serum triglycerides and decreased HDL cholesterol).

In these highly selected patients, we found insulin resistance in the following target pathways: (1) endogenous insulin release; (2) endogenous glucose production; (3) whole-body glucose disposal and its main components (i.e. glucose oxidation and non-oxidative glucose disposal); (4) glycerol R_a (a reflection of lipolysis); and (5) lipid oxidation. This pattern of metabolic defects is consistent with accelerated lipolysis—the immediate result of insulin resistance in adipose tissue—being responsible for the increased NEFA supply and oxidative use of lipid at the whole-body level. In the liver, which normally extracts circulating NEFA with high efficiency (25–40%) [39], higher rates of lipid oxidation and impaired suppression of hepatic lipid oxidation by insulin (evidenced by the higher blood β-hydroxybutyrate concentrations across the insulin dose–response curve) provide the energy and biochemical signals required to stimulate the gluconeogenic pathway [40]. In normoglycaemic individuals, increased gluconeogenesis forms the basis of hepatic insulin resistance, i.e. rates of glucose release that are inappropriately elevated for the

and β-hydroxybutyrate were all significantly elevated in patients compared with control subjects (Tables 1, 2). Likewise, basal glycerol R_a ($p<0.03$) and lipid oxidation ($p=0.09$) were higher in the patients. The insulin dose–response functions for glycerol R_a and NEFA concentrations (Fig. 2), and those for lipid oxidation and β-hydroxybutyrate concentrations (Fig. 3), showed the expected inhibition by insulin ($p<0.0001$ for all), but were each significantly shifted upward in patients compared with those in control subjects ($p<0.05$–0.001). Using the whole dataset (i.e. basal and insulin data in both groups), lipid oxidation was positively related to glucose production ($r=0.65$, $p<0.0001$) and negatively associated with glucose disposal ($r=-0.81$, $p<0.0001$) (Fig. 4). Liver steatosis showed a positive correlation with the mean rate of lipid oxidation (Fig. 5).

LDL oxidisability The lag phase, the length of which is proportional to LDL resistance to copper-induced oxidative stress in vitro, was significantly shorter in NAFLD patients than in control subjects (48±12 vs 63±13 min, $p<0.04$). When NAFLD and control data were pooled, an inverse correlation was found between average lipid oxidation and the length of the lag phase ($r=0.71$, $p=0.004$).
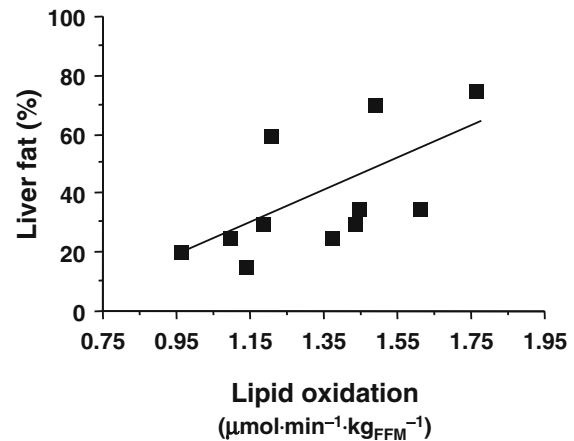
prevailing insulin levels. In peripheral tissues, increased fat oxidation may impede glucose disposal, both oxidative and conservative, through substrate competition [41]. Although the current results do not directly show any cause–effect relationships, the tight correlations between lipid oxidation and glucose production/disposal (Fig. 4), as measured by independent techniques (calorimetry, tracer infusions and clamp, respectively), suggest that the hepatic and peripheral insulin resistance associated with NAFLD may be the result of insulin resistance in adipose tissue. However, we cannot exclude a degree of independent resistance in the liver and skeletal muscle tissues in addition to that caused by increased fatty substrate delivery and oxidation.

Previous results have shown that NAFLD is associated with insulin resistance and the metabolic syndrome. In large population studies, nearly all of the NAFLD patients examined were insulin resistant according to the homeostasis model assessment [11, 12]. These preliminary observations have subsequently been confirmed by more direct methods for the measurement of insulin resistance. Employing the glucose clamp technique, Marchesini et al. [42] demonstrated a reduction in insulin-mediated glucose disposal in NAFLD. Although patients with diabetes and obesity were excluded from this study, most of the subjects had multiple features of the metabolic syndrome, such as hypertriglyceridaemia and central fat accumulation. A separate study described the abnormalities in lipid and glucose metabolism and the site(s) of insulin resistance in NAFLD [43]. However, the patients enrolled were obese and had a high prevalence of IGT—both inherently insulin-resistant states. Although the frequently sampled IVGTT has been employed to confirm the presence of insulin resistance in lean, non-diabetic patients with NAFLD [44], the impact of insulin resistance on lipid metabolism was not examined in this study.

A recent study [45] reported that lean subjects with normal transaminases and high liver fat (~10%, as determined by ^{1}H magnetic resonance spectroscopy) had reduced suppression of endogenous glucose production and serum NEFA during a low-dose (2 pmol min^{-1} kg^{-1}) hyperinsulinaemic clamp compared with matched healthy men with low liver fat (~2%). Since hepatic insulin resistance was significantly related to liver fat content independently of BMI, subcutaneous fat volume and intra-abdominal fat volume (as assessed by MRI), the authors concluded that hepatic steatosis rather than adipose tissue triglyceride content can be a major determinant of insulin resistance in humans. Kelly et al. [46] recently reported that the presence of liver steatosis (assessed using computerised tomography criteria) in patients with type 2 diabetes is associated with more severe insulin resistance (during a 6 pmol min^{-1} kg^{-1} insulin clamp). Using both a low-dose (1.5 pmol min^{-1} kg^{-1}) and a high-dose (6 pmol min^{-1} kg^{-1}) insulin infusion, we have conclusively demonstrated the co-existence of hepatic and peripheral insulin resistance in biopsy-proven NAFLD independent of obesity and diabetes. When the patients were stratified according to histological grading and staging into fatty liver and non-alcoholic steatohepatitis (the latter diagnosed on the basis of the presence of fibrosis stage 1 or higher, or necro-

inflammation grade 2 or higher) we were unable to identify any differences between the two subgroups with respect to the metabolic parameters examined (data not shown).

Although the increased lipid turnover in our NAFLD patients is unlikely to be due to increased subcutaneous fat, the role of visceral adiposity is an important issue [13]. It is generally accepted that visceral adipose tissue is more insulin resistant than subcutaneous adipose tissue [47, 48]. Consequently, portal NEFA drainage may be more important than the systemic flux in the development of liver steatosis. However, in non-diabetic subjects, a visceral fat area of ~100 cm^2 (i.e., the average area in our NAFLD patients as assessed by single-scan MRI) corresponds to a total visceral fat mass of ~2 kg (as determined by multiscan MRI) [49]. Even if overactive in terms of lipolysis, this adipose depot can at most account for ~20% of the total prehepatic NEFA load, which is not sufficient to justify liver steatosis. Rather, it is possible that an altered endocrine activity of visceral fat may contribute to liver damage [50].

Whatever the role of visceral fat, it is clear that steatosis can result from an increased delivery of circulating lipids to the liver due to a reduced antilipolytic effect of insulin in adipose tissue. Within the liver, part of this NEFA excess is oxidised and the remainder is re-esterified to be exported into the circulation as triglyceride-rich lipoproteins. Re-esterification is dependent on the availability of glycerol, and the packaging of triglycerides into VLDL is dependent upon apolipoprotein B synthesis. Since glycerol-3-phosphate can be diverted into the overactive gluconeogenic pathway, and apolipoprotein B production may be reduced in NAFLD (as recently described [51]), the liver retains excessive amounts of lipids, and steatosis ensues. Our finding of a correlation between whole-body lipid oxidation and amount of steatosis (Fig. 5) lends support to this proposed sequence of events.

A comparison with other insulin-resistant states is of interest. Insulin resistance in liver, muscle and fat is found in a high proportion of patients with overt type 2 diabetes. Furthermore, in this condition, hepatic insulin resistance is usually more severe and results in absolute glucose overproduction [52]. However, the NEFA turnover rate and total lipid oxidation have been reported to be only mildly abnormal in non-obese type 2 patients and in first-degree relatives of diabetic patients [53, 54]. In non-diabetic patients with liver cirrhosis, basal endogenous glucose production is normal and is normally suppressed by insulin; skeletal muscle is insulin resistant because of reduced glycogen formation [55, 56], whereas glucose oxidation has been found to be normal in the basal state and after insulin administration. In these patients, lipolysis is increased in the postabsorptive state, but is suppressed by insulin in an almost normal manner [56]. Furthermore, the increased lipid flux that occurs during the fasting state results not only from insulin resistance, but also from "accelerated starvation", with the early recruitment of alternative fuels for energy needs and for gluconeogenesis (as the glycogen stores of the cirrhotic liver are significantly reduced) [26]. Moreover, hyperinsulinism is also caused by hepatocellular dysfunction and intrahepatic shunting in liver cirrhosis [57]; these mech-

anisms were not operative in our patients, who had normal liver function and no histological evidence of cirrhosis. In summary, the insulin resistance associated with NAFLD shows similarities to, as well as differences from, other well-characterised insulin-resistant states in terms of the sites and mechanisms involved. These observations suggest that the underlying mechanisms may differ, at least in part.

Several lines of evidence suggest that chronic oxidative stress occurs concomitantly with NAFLD [58]. A possible source of oxidative stress is enhanced lipid oxidation itself. In an attempt to gauge this phenomenon in our patients, we measured the susceptibility of LDL cholesterol particles to copper-catalysed oxidation. Measurements obtained using this in vitro method have been shown to correlate with in vivo measurements assessed using NMR spectroscopy [59]. Compared with the control group, LDL oxidisability was increased in the NAFLD group and, perhaps more importantly, was directly related to lipid oxidation.

In conclusion, in non-obese, non-diabetic patients with biopsy-proven NAFLD, insulin resistance is marked and involves the liver and the peripheral (adipose and skeletal muscle) tissues and multiple intracellular pathways of substrate (glucose and lipids) disposition. Whether hepatic steatosis is a consequence of peripheral insulin resistance or causes insulin resistance remains unclear, but the results of this study suggest that excess NEFA flux due to peripheral insulin resistance may contribute to hepatic steatosis. Adipose tissue insulin resistance and endocrine overactivity, especially in ectopic depots, may be salient features of the metabolic profile for NAFLD.

References

1. Poonawala A, Nair SP, Thuluvath PJ (2000) Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. Hepatology 32:689–692
2. Falck-Ytter Y, Younossi ZM, Marchesini G, McCullough AJ (2001) Clinical features and natural history of nonalcoholic steatosis syndromes. Semin Liver Dis 21:17–26
3. Bugianesi E, Leone N, Vanni E et al (2002) Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 123:134–140
4. Clark JM, Brancati FL, Diehl AM (2003) The prevalence and etiology of elevated aminotransferase levels in the United States. Am J Gastroenterol 98:960–967
5. Nomura H, Kashiwagi S, Hayashi J, Kajiyama W, Tani S, Goto M (1988) Prevalence of fatty liver in a general population of Okinawa, Japan. Jpn J Med 7:142–149
6. Bellentani S, Saccoccio G, Masutti F et al (2000) Prevalence of and risk factors for hepatic steatosis in Northern Italy. Ann Intern Med 132:112–117
7. DeFronzo RA, Ferrannini E (1991) Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 14:173–194
8. Ludwig J, Viaggiano TR, McGill DB, Oh BJ (1980) Nonalcoholic steatohepatitis: Mayo Clinic experience with an hitherto unnamed disease. Mayo Clin Proc 55:434–438
9. Wanless IR, Lentz JS (1990) Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology 12:1106–1110
10. Lee RG (1989) Nonalcoholic steatohepatitis: a study of 49 patients. Human Pathol 20:594–598
11. Marchesini G, Brizi M, Morselli-Labate AM et al (1999) Association of non-alcoholic fatty liver disease to insulin resistance. Am J Med 107:450–455
12. Chitturi S, Abeygunasekera S, Farrell GC et al (2002) NASH and insulin resistance: insulin secretion and specific association with the insulin resistance syndrome. Hepatology 35:373–379
13. Chalasani N, Deeg MA, Persohn S, Crabb DW (2003) Metabolic and anthropometric evaluation of insulin resistance in nondiabetic patients with nonalcoholic steatohepatitis. Am J Gastroenterol 98:1849–1855
14. Ruhl CE, Everhart JE (2003) Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the United States. Gastroenterology 124:71–79
15. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol In Adults (2001) Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 285:2486–2497
16. Marchesini G, Bugianesi E, Forlani G et al (2003) Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37:917–923
17. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H (1993) Lipid advanced glycosylation: pathway for lipid oxidation in vivo. Proc Natl Acad Sci U S A 90:6434–6436
18. Lee KS, Buck M, Houglum K, Chojkier M (1995) Activation of hepatic stellate cells by TGF alpha and collagen type 1 is mediated by oxidative stress through c-myb expression. J Clin Invest 96:2461–2468
19. Bedossa P, Houglum K, Trautwein C, Holstege A, Chojkier M (1994) Stimulation of collagen 1 (I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? Hepatology 19:1262–1271
20. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997) Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 20:1183–1197
21. National Diabetes Data Group (1979) Classification and diagnosis of diabetes and other categories of glucose intolerance. Diabetes 28:1039–1057
22. Brunt EM (2001) Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis 21:3–16
23. Natali A, Toschi E, Camastra S, Gastaldelli A, Groop L, Ferrannini E, European Group for the Study of Insulin Resistance (2000) Determinants of postabsorptive endogenous glucose output in nondiabetic subjects. Diabetologia 43:1266–1272
24. Lancaster JL, Ghiatas AA, Alyassin A, Kilcoyne RF, Bonora E, DeFronzo RA (1991) Measurement of abdominal fat with T1-weighted MR images. J Magn Reson Imaging 1:363–369
25. De Fronzo RA, Tobin D, Andres R (1979) Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 237:E214–E223
26. Finegood DT, Bergman RN, Vranic M (1987) Estimation of endogenous glucose production during hyperinsulinemic euglycemic glucose clamps. Diabetes 36:914–924
27. Bugianesi E, Kalhan S, Burkett E, Marchesini G, McCullough A (1998) Quantification of gluconeogenesis in cirrhosis: response to glucagon. Gastroenterology 115:1530–1540
28. Coggan AR, Raguso CA, Gastaldelli A, Sidossis LS, Yeckel CW (2000) Fat metabolism during high-intensity exercise in endurance-trained and untrained men. Metabolism 49:122–128
29. Wolfe R (1992) Radioactive and stable isotope tracers in biomedicine. Wiley-Liss, New York

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30. Esterbauer H, Striegl G, Puhl H, Dieber Rothneder M (1989) Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radic Res Commun 6:67–75

31. Cominacini L, Garbin U, Fratta Pasini A et al (1998) Troglitazone reduces LDL oxidation and lowers plasma E-selectin concentration in NIDDM patients. Diabetes 47:130–133

32. Cobelli C, Mari A, Ferrannini E (1987) Non-steady state: error analysis of Steele's model and developments for glucose kinetics. Am J Physiol 252:E679–E689

33. Sindelar DK, Chu CA, Venson P, Donahue EP, Neal DW, Cherrington AD (1998) Basal hepatic glucose production is regulated by the portal vein insulin concentration. Diabetes 47:523–529

34. DeFronzo RA, Ferrannini E, Simonson D (1989) Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metabolism 38:387–395

35. Ferrannini E (1988) The theoretical bases of indirect calorimetry: a review. Metabolism 37:287–301

36. Jensen MD (1999) Regional glycerol and free fatty acid metabolism before and after meal ingestion. Am J Physiol 276:E863–E869

37. Jensen MD, Chandramouli V, Schumann WC et al (2001) Sources of blood glycerol during fasting. Am J Physiol Endocrinol Metab 281:E998–E1004

38. Després JP (1993) Abdominal obesity as important component of insulin-resistance syndrome. Nutrition 9:452–459

39. Bergman N (2000) Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein? Diabetologia 43:946–952

40. Roden M, Stingl H, Chandramouli V et al (2000) Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans. Diabetes 49:701–707

41. McGarry JD (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 51:7–18

42. Marchesini G, Brizi M, Bianchi G et al (2001) Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 50:1844–1850

43. Sanyal AJ, Campbell-Sargent C, Mirshahi F et al (2001) Non-alcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology 120:1183–1192

44. Pagano G, Pacini G, Musso G et al (2002) Nonalcoholic steatohepatitis, insulin resistance and metabolic syndrome: further evidence for an etiological association. Hepatology 35:367–372

45. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM et al (2002) Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J Clin Endocrinol Metab 8:3023–3028

46. Kelly DE, McKolanis TM, Hegazi RAF, Kuller LH, Kalhan SC (2003) Fatty liver in type 2 diabetes: relation to regional adiposity, fatty acids, and insulin resistance. Am J Physiol Endocrinol Metab 285:E906–E916

47. Wahrenberg H, Lonnqvist F, Arner P (1989) Mechanism underlying regional differences in lipolysis in human adipose tissue. J Clin Invest 84:458–467

48. Lefebvre AM, Laville M, Vega N et al (1998) Depot-specific differences in adipose tissue gene expression in lean and obese subjects. Diabetes 47:98–103

49. Sironi AM, Gastaldelli A, Mari A et al (2004) Visceral fat in hypertension: influence on insulin resistance and beta-cell function. Hypertension 44:127–133

50. Shuldiner AR, Yang R, Gong DW (2001) Resistin, obesity and insulin resistance—the emerging role of the adipocyte as an endocrine organ. N Engl J Med 345:1345–1346

51. Charlton M, Sreekumar R, Rasmussen D, Lindor K, Nair KS (2002) Apolipoprotein synthesis in nonalcoholic steatohepatitis. Hepatology 35:898–904

52. Groop LC, Bonadonna RC, DelPrato S et al (1989) Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. J Clin Invest 84:205–213

53. Groop LC, Saloranta C, Shank M, Bonadonna RC, Ferrannini E, DeFronzo RA (1991) The role of free fatty acid metabolism in the pathogenesis of insulin resistance in obesity and noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 72:96–107

54. Eriksson JW, Smith U, Waagstein F, Wysocki M, Jansson PA (1999) Glucose turnover and adipose tissue lipolysis are insulin-resistant in healthy relatives of type 2 diabetes patients: is cellular insulin resistance a secondary phenomenon? Diabetes 48:1572–1578

55. Cavallo-Perin P, Cassader M, Bozzo C et al (1985) Mechanism of insulin resistance in human liver cirrhosis. Evidence of a combined receptor and postreceptor defect. J Clin Invest 75:1659–1665

56. Petrides AS, Groop LC, Riely CA, DeFronzo RA (1991) Effect of physiologic hyperinsulinemia on glucose and lipid metabolism in cirrhosis. J Clin Invest 88:561–570

57. Petrides AS, Vogt C, Schulze-Berge D, Matthews D, Strohmeyer G (1994) Pathogenesis of glucose intolerance and diabetes mellitus in cirrhosis. Hepatology 19:616–627

58. Videla LA, Rodrigo R, Araya J, Poniachik J (2004) Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. Free Radic Biol Med 37:1499–1507

59. Jankowsky J, Nofer JR, Tepel M et al (1998) Identification of oxidized low-density lipoprotein in human serum by NMR spectroscopy. Clin Sci 95:489–495