



LIVER FAT IN THE METABOLIC SYNDROME AND TYPE 2 DIABETES



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ACADEMIC DISSERTATION

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Cover: Examples of a normal (upper panel) and steatotic (lower panel) human liver.

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Yliopistopaino

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* * *

*In everything I want to reach
The fundamental:
In work, in depth of inner speech,
In sentimental.*

*To probe the truth of days bygone,
And their essence,
The pondering, the bone,
And life's core lessons.*

*The path of destiny, of fate
To reach and to uncover,
To live, to think, to meditate,
And to discover.*

*Oh, if I only could surprise
After a fashion,
In eight lines I would summarize
The parts of passion.*

*The illegalities, the crimes,
Escape and chase force,
The accidents within the drives,
The palms, the elbows.*

*Its origin and law I would define,
In terms judicial,
The names it swears in,
And its initials.*

*My rhyme would throb and thrive
With fever ardent,
Due to the linden sap, alive,
As if it were a garden.*

*The essence of the rose, the mint,
The fragrance of the sedge,
The field in stormy glint,
Would give my verse an edge.*

*So did Chopin in his etudes
Miraculously conjure,
Groves, graves, and solidary moods -
A living wonder.*

*Triumph, attained through merriment,
Distress and throe,
Is like the arrow vibrant
Of the bow.*

1956

Boris Pasternak (1890-1960)

ABSTRACT

Introduction: The epidemic of obesity has been accompanied by an increase in the prevalence of the metabolic syndrome, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD). However, not all obese subjects develop these metabolic abnormalities. Hepatic fat accumulation is related to hepatic insulin resistance, which in turn leads to hyperglycemia, hypertriglyceridemia, and a low HDL cholesterol concentration. The present studies aimed to investigate 1) how intrahepatic as compared to intramyocellular fat is related to insulin resistance in these tissues and to the metabolic syndrome (Study I); 2) the amount of liver fat in subjects with and without the metabolic syndrome, and which clinically available markers best reflect liver fat content (Study II); 3) the effect of liver fat on insulin clearance (Study III); 4) whether type 2 diabetic patients have more liver fat than age-, gender-, and BMI-matched non-diabetic subjects (Study IV); 5) how type 2 diabetic patients using exceptionally high doses of insulin respond to addition of a PPAR γ agonist (Study V).

Subjects and methods: The study groups consisted of 45 (Study I), 271 (Study II), and 80 (Study III) non-diabetic subjects, and of 70 type 2 diabetic patients and 70 matched control subjects (Study IV). In Study V, a total of 14 poorly controlled type 2 diabetic patients treated with high doses of insulin were studied before and after rosiglitazone treatment (8 mg/day) for 8 months. In all studies, liver fat content was measured by proton magnetic resonance spectroscopy, and subcutaneous and intra-abdominal fat content by MRI. In addition, circulating markers of insulin resistance and serum liver enzyme concentrations were determined. Hepatic (i.v. insulin infusion

rate 0.3 mU/kg-min combined with [^3H]glucose, Studies I, III, and V) and muscle (1.0 mU/kg-min, Study I) insulin sensitivities were measured by the euglycemic hyperinsulinemic clamp technique.

Results: Fat accumulation in the liver rather than in skeletal muscle was associated with features of insulin resistance, i.e. increased fasting serum (fS) triglycerides and decreased fS-HDL cholesterol, and with hyperinsulinemia and low adiponectin concentrations (Study I). Liver fat content was 4-fold higher in subjects with as compared to those without the metabolic syndrome, independent of age, gender, and BMI. fS-C-peptide was the best correlate of liver fat (Study II). Increased liver fat was associated with both impaired insulin clearance and hepatic insulin resistance independent of age, gender, and BMI (Study III). Type 2 diabetic patients had 80% more liver fat than age-, weight-, and gender-matched non-diabetic subjects. At any given liver fat content, S-ALT underestimated liver fat in the type 2 diabetic patients as compared to the non-diabetic subjects (Study IV). In Study V, hepatic insulin sensitivity increased and glycemic control improved significantly during rosiglitazone treatment. This was associated with lowering of liver fat (on the average by 46%) and insulin requirements (40%).

Conclusions: Liver fat is increased both in the metabolic syndrome and type 2 diabetes independent of age, gender, and BMI. A fatty liver is associated with both hepatic insulin resistance and impaired insulin clearance. Rosiglitazone may be particularly effective in type 2 diabetic patients who are poorly controlled despite using high insulin doses.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I) **Anna Kotronen**, Anneli Seppälä-Lindroos, Robert Bergholm, and Hannele Yki-Järvinen: *Tissue specificity of insulin resistance in humans: fat in the liver rather than muscle is associated with features of the metabolic syndrome*
Diabetologia, 51:130-138, 2008

II) **Anna Kotronen**, Jukka Westerbacka, Robert Bergholm, Kirsi H. Pietiläinen, and Hannele Yki-Järvinen: *Liver fat in the metabolic syndrome*
The Journal of Clinical Endocrinology and Metabolism, 92:3490-3497, 2007

III) **Anna Kotronen**, Satu Vehkavaara, Anneli Seppälä-Lindroos, Robert Bergholm, and Hannele Yki-Järvinen: *Effect of liver fat on insulin clearance*
American Journal of Physiology: Endocrinology and Metabolism, 293:E1709-E1715, 2007

IV) **Anna Kotronen**, Leena Juurinen, Antti Hakkarainen, Jukka Westerbacka, Anja Cornér, Robert Bergholm, and Hannele Yki-Järvinen: *Liver fat is increased in type 2 diabetic patients and underestimated by serum alanine aminotransferase compared with equally obese non-diabetic subjects*
Diabetes Care, 31:165-169, 2008

V) Leena Juurinen, **Anna Kotronen**, Marit Granér, and Hannele Yki-Järvinen: *Rosiglitazone reduces liver fat and insulin requirements and improves hepatic insulin sensitivity and glycemic control in patients with type 2 diabetes requiring high insulin doses*
The Journal of Clinical Endocrinology and Metabolism, 93:118-124, 2008

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ABBREVIATIONS

3-OHB	3-hydroxybutyrate
ACE	angiotensin converting enzyme
ADOPT	A Diabetes Outcome Progression trial
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
ApoB	apolipoprotein B
AST	aspartate aminotransferase
BMI	body mass index
BP	blood pressure
BX	biopsy
CARDIA	the Coronary Artery Risk Development in Young Adults
CCL	CC-chemokine ligand
CHD	coronary heart disease
CO ₂	carbon dioxide
CRP	C-reactive protein
CT	computed tomography
CVD	cardiovascular disease
D.E.S.I.R.	Data from an Epidemiological Study on the Insulin Resistance syndrome
DREAM	Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication trial
ECG	electrocardiogram
EGIR	European Group for the study of Insulin Resistance
EMCL	extramyocellular lipid
endo	endogenous
exo	exogenous
FFA	free fatty acid
FFM	fat free mass
fP	fasting plasma
fS	fasting serum
γGT	gamma-glutamyltransferase
GLUT-4	glucose transporter-4
HAL	highly active antiretroviral therapy -associated lipodystrophy
HbA _{1c}	glycosylated hemoglobin A _{1c}
HDL	high density lipoprotein
HGP	hepatic glucose production
¹ H-MRS	proton magnetic resonance spectroscopy
HOMA-IR	homeostatic model assessment of insulin resistance
hs	high-sensitivity
HSL	hormone sensitive lipase
IA	intra-abdominal
IDF	International Diabetes Federation
IL-6	interleukin 6
IMCL	intramyocellular lipid
IRAS	the Insulin Resistance Atherosclerosis Study
IRS-1	insulin receptor substrate-1
i.v.	intravenous
LDL	low density lipoprotein

LFAT	liver fat
L/S ratio	liver-to-spleen attenuation ratio
MCP-1	monocyte chemotactic protein 1
MDA	malondialdehyde
MONICA/CORA	Monitoring trends and determinants on cardiovascular diseases/Cooperative Health Research in the Region of Augsburg
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MS	metabolic syndrome
MTTP	mitochondrial triglyceride transfer protein
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCEP-ATPIII	National Cholesterol Education Program – Adult Treatment Panel III
NHANES III	Third National Health and Nutrition Examination Survey
n.r.	not reported
NS	not significant
O ₂	oxygen
p	pool fraction of distribution of glucose
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PET	positron emission tomography
PI 3-kinase	phosphatidylinositol 3-kinase
PPAR γ	peroxisome proliferator-activated receptor-gamma
ppm	parts per million
PROactive	Prospective Pioglitazone Clinical Trial in Macrovascular Events
PUFA	polyunsaturated fatty acids
R _a	rate of appearance
R _d	rate of disappearance
RQ	respiratory quotient
QUICKI	quantitative insulin sensitivity check index
S	serum
SA	specific activity
SC	subcutaneous
SD	standard deviation
SEM	standard error of mean
SOD	superoxide dismutase
SREBP-1a	sterol regulatory element binding protein 1a
TG	triglyceride
TNF α	tumor necrosis factor α
TPS	tissue polypeptide-specific antigen
TZD	thiazolidinedione
UDCA	ursodeoxycholic acid
usf-1	upstream transcription factor 1
V	distribution volume for glucose
VLDL	very low density lipoprotein
VCO ₂	carbon dioxide production
VO ₂	oxygen consumption
VO ₂ max	maximal oxygen consumption
WHO	World Health Organization
WOSCOPS	the West of Scotland Coronary Prevention Study
yrs	years

1. INTRODUCTION

The metabolic syndrome represents a cluster of risk factors which is thought to increase the risk of cardiovascular diseases and type 2 diabetes more than the individual components (abdominal obesity, increased serum triglycerides, low HDL cholesterol, hyperglycemia, and hypertension) (9). The pathophysiology of the metabolic syndrome is believed to include insulin resistance, but it is poorly understood why some but not others develop the syndrome. In addition, the contribution of insulin resistance and fat accumulation in the liver and skeletal muscle to features of the metabolic syndrome is unclear.

It has been estimated that approximately 70-80% of type 2 diabetic patients have a fatty liver due to non-alcoholic causes (18, 414). In addition, 11 prospective epidemiological studies have shown that elevated serum liver enzyme concentrations predict type 2 diabetes independent of obesity (15, 172, 239, 240, 288, 289, 302, 314, 365, 443, 447). The data would thus suggest that liver fat content is increased in patients with type 2 diabetes as compared to equally obese non-diabetic subjects. There are, however, no data to prove or disprove this hypothesis at present.

The liver is the primary site of insulin clearance (116). *In vitro*, hepatocytes loaded with triglycerides exhibit impaired insulin clearance (400). Liver fat is closely correlated with fasting serum insulin concentrations (452), but the extent to which impaired insulin

clearance due to hepatic fat accumulation contributes to hyperinsulinemia has not previously been determined.

A key action of insulin is to inhibit hepatic glucose production (467). Once fatty, the liver is insulin resistant to this action of insulin both in normal subjects (379) and patients with type 2 diabetes (355). Liver fat is an important determinant of insulin requirements during insulin therapy in patients with type 2 diabetes (204, 355). Thus, type 2 diabetic patients with a fatty liver may remain poorly controlled despite high doses of insulin. This could be due to the inability of insulin to act in a fatty liver. Given that PPAR γ agonists reduce liver fat (21, 29, 63, 273, 424), it could be hypothesized that patients requiring high doses of insulin are particularly responsive to PPAR γ agonist treatment.

The present studies aimed to investigate how liver fat content is related to the metabolic syndrome, type 2 diabetes, directly measured hepatic and whole-body insulin sensitivity and clearance, and circulating markers of insulin resistance, such as insulin itself. We also determined the impact of impaired insulin clearance on serum insulin concentrations, and the effect of a PPAR γ agonist treatment on liver fat and hepatic insulin sensitivity in type 2 diabetic patients using exceptionally high doses of insulin.

2. REVIEW OF THE LITERATURE

2.1. INSULIN ACTION

Insulin is a peptide hormone which has numerous metabolic actions in several tissues (Table 1). It regulates glucose metabolism by inhibiting glucose production in the liver, and by stimulating glucose uptake, particularly in skeletal muscle (467). Insulin action on lipid metabolism includes stimulation of lipogenesis in adipose tissue and the

liver, and inhibition of lipolysis and hepatic very low density lipoprotein (VLDL) production (248, 416, 464, 467). In addition, insulin has effects on vascular function, which include diminishing the stiffness of large or conduit arteries (455), and the induction of slow vasodilatation in peripheral resistance vessels (23, 465).

Table 1. Metabolic actions of insulin in major target tissues.

Fuel	Action of insulin	Liver	Muscle	Adipose tissue
GLUCOSE	Glucose transport	-	↑	↑
	Glucose phosphorylation	↑	↑	↑
	Glycogen synthesis	↑	↑	[↑]
	Glycogen breakdown	↓	↓	[↓]
	Glycolysis	↑	↑	↑
	Gluconeogenesis	↓		
LIPID	<i>De novo</i> lipogenesis	↑	[↑]	↑
	Oxidation of fatty acids	↓	[↓]	↓
	Lipolysis		[↓]	↓
	VLDL production	↓		
	Ketogenesis	↓		
PROTEIN	Amino acid transport	↑	↑	?
	Release of amino acids		↓	?
	Protein synthesis	↑	↑	[↑]
	Protein breakdown	↓	↓	[↓]

Adapted from ref. 469 with permission.

2.1.1. Insulin action in the liver

2.1.1.1. Glucose metabolism

The liver can take up glucose (93, 96) or produce it (466) via gluconeogenesis and glycogenolysis (69, 95).

2.1.1.1.1. Methodological aspects

In humans, hepatic glucose metabolism can be studied using stable and radioactive tracers (345), the hepatic venous catheterization technique (44, 129, 285), nuclear magnetic resonance (261, 352), or by a combination of several techniques (96, 356).

With tracer methodology, the rate of endogenous glucose production, which can occur both in the liver and kidneys (402), can be quantitated (345). However, the net contribution of the kidneys to endogenous glucose production after an overnight fast in humans is negligible (35). Therefore, the term hepatic glucose production (HGP) is used instead of endogenous glucose production throughout this thesis. The most commonly used tracer for measuring hepatic glucose production is [$3\text{-}^3\text{H}$]glucose (467), which is handled by body tissues similarly to cold (unlabeled) glucose (471). The labeled tritium is lost during glycolysis as a water molecule but not during glucose incorporation in or release from hepatic glycogen (460). Plasma glucose specific activity (SA) can therefore be determined after evaporating water from deproteinized plasma samples (460). Under steady-state conditions, when both plasma glucose SA and concentrations are constant, the rate of HGP can be accurately calculated from the following equation:

$$HGP = \text{Isotope infusion rate} / \text{plasma glucose SA}$$

When the entry of either endogenous or exogenous glucose into the circulation occurs, the specific activity of glucose changes (154). During low-dose hyperinsulinemic euglycemic clamp (104), when hepatic glucose production is half-maximally suppressed (96, 348, 467, 478), total glucose rate of appearance (R_a) is a sum of endogenous (hepatic) and exogenous glucose R_a :

$$R_a = HGP + R_a \text{ exo}$$

The total rate of glucose R_a is most frequently calculated using the one-compartment model equations of Steele (393):

$$R_a = (\text{Isotope infusion rate} / \text{mean plasma glucose SA}) - (p \times V \times \text{mean plasma glucose} \times \Delta SA / [\text{mean plasma glucose SA} \times \Delta \text{Time}])$$

where p is a pool fraction of distribution of glucose, V is the distribution volume for glucose, and ΔSA and ΔTime represent the changes in glucose specific activity over time (393). Under non-steady-state conditions, HGP can thus be calculated from the following equation:

$$HGP = R_a - R_a \text{ exo}$$

The one compartment model is based on the assumption that there is a single homogenous pool in the body where labeled and unlabeled glucose mix instantaneously and uniformly (393, 460), and that the labeled glucose cannot re-enter the glucose pool after it has left the pool (460). These assumptions are not always true (13, 136). For example, during hyper-insulinemic euglycemic

clamp experiments, the glucose pool enlarges (136). Because of the inability of one-compartment model to describe the whole body glucose kinetics accurately, glucose production is underestimated when glucose specific activity decreases (467), which results in physiologically impossible negative rates of the suppression of hepatic glucose production by insulin (31, 138, 471). Other approaches have been used to try to better characterize the non-steady state kinetics of glucose, but despite the increased sophistication of the new approaches (77), the Steele equation has remained the standard for calculation of non-steady state kinetics.

The catheterization technique allows assessing net splanchnic glucose balance by multiplying the arterial - hepatic vein glucose difference by blood flow (44, 129, 285). Since net hepatic glucose balance represents the difference between hepatic glucose production and splanchnic glucose uptake, this technique is unable to separately quantitate these processes (97). Therefore, if glucose production is measured in a hyperglycemic subject, net splanchnic balance may appear normal because of simultaneous increases in splanchnic glucose uptake and hepatic glucose production (96, 97). These processes can be quantitated separately by combining the catheterization technique with use of a tracer (129, 131). Invasiveness limits widespread use of the catheterization technique.

Hepatic glycogen content can be measured with ^{13}C nuclear magnetic resonance spectroscopy *in vivo* (261, 352). The rate of gluconeogenesis can be calculated by subtracting the rate of glycogenolysis from total endogenous glucose production (261). Use of

$[^{18}\text{F}]$ fluorodeoxyglucose ($[^{18}\text{F}]$ FDG) and positron emission tomography (PET) for quantification of hepatic glucose uptake has been evaluated in humans (194) and recently validated in pigs (195).

2.1.1.1.2. Normal actions of insulin

After an overnight fast, the liver is the major source of endogenously produced glucose (35, 44), and is therefore the primary organ determining plasma glucose concentrations when serum insulin concentrations are low. Dose-response studies relating plasma insulin to the suppression of hepatic glucose production have indicated that the liver is extremely sensitive to small increments in serum insulin concentrations (96, 348, 467, 478). Complete suppression of hepatic glucose production occurs at peripheral insulin concentrations of 50-60 mU/l (96, 348, 478). Half-maximal suppression of hepatic glucose production in different studies of non-diabetic subjects has been reported to occur at serum insulin concentrations of 11-46 mU/l (61, 96, 162, 163, 346, 348).

Insulin can inhibit hepatic net glucose release via a variety of mechanisms. This occurs mainly via inhibition of glucose production (96). Insulin also stimulates glucose uptake, the magnitude of which depends on whether glucose is given orally (282) or intravenously (97, 101). The mechanisms include the stimulation of the glucokinase activity (325), and a decrease in glucose-6-phosphatase activity (34, 183, 438). In addition, insulin can stimulate glycogen synthesis (179, 459), inhibit glycogen breakdown (321, 459), and suppress gluconeogenesis (24). Studies in humans have indicated that the suppression of glycogenolysis is more sensitive to insulin than is the suppression of gluconeogenesis (7, 39, 151). A recent study in non-diabetic

subjects indicated that a stepwise increase in serum insulin concentrations from ~13 mU/l to ~25 mU/l decreases hepatic glucose production via suppression of glycogenolysis, while the rate of gluconeogenesis remains unchanged (7).

Under normoglycemic conditions, splanchnic glucose uptake is not stimulated by insulin, even at supra-physiological insulin concentrations of ~1200 mU/l (96). Hyperglycemia is a powerful stimulator of splanchnic glucose uptake independent of serum insulin concentrations (96).

2.1.1.1.3. Insulin action in insulin-resistant subjects

Obese subjects. When obese normoglycemic subjects have been compared to lean non-diabetic individuals, basal hepatic glucose production has been reported to be similar in both groups (42, 103, 149). However, this does not exclude hepatic insulin resistance, since obese subjects often are hyperinsulinemic as compared to non-obese ones (42, 103, 149). Consistent with this, the ability of insulin to suppress hepatic glucose production has been shown to be either impaired (42, 103, 309) or similar (164) in the face of higher serum insulin concentrations during exogenously induced hyperinsulinemia in obese as compared to lean subjects.

Subjects with a fatty liver. To date, only two cross-sectional studies have examined the effect of hepatic fat accumulation on hepatic glucose production in non-diabetic subjects using low-dose insulin infusion euglycemic hyperinsulinemic clamp technique (55, 379). The % suppression of hepatic glucose production by insulin was either significantly impaired (379) or similar

(55) in subjects with a fatty liver as compared to equally obese control subjects. Marchesini et al found hepatic insulin sensitivity, measured using euglycemic hyperinsulinemic clamp of a standard insulin infusion rate (1 mU/kg·min (104)), to be impaired in obese subjects with a biopsy-proven fatty liver as compared to lean controls (268). It has thus remained uncertain whether hepatic fat accumulation associates with hepatic insulin resistance independent of obesity in non-diabetic subjects.

Type 2 diabetic patients. Numerous studies have indicated the inability of insulin to suppress hepatic glucose production in the fasting state to be a primary cause of fasting hyperglycemia in patients with type 2 diabetes (41, 61, 80, 91, 92, 98, 466). Hepatic insulin sensitivity has been shown to be decreased in obese type 2 diabetic patients as compared to non-obese non-diabetic subjects (164, 214, 303), but when age-, gender-, and weight-matched groups have been compared, hepatic insulin resistance has frequently been reported to be comparable (14, 26, 164, 309, 391, 430). Several studies have reported an inverse relationship between hepatic insulin sensitivity and hepatic fat accumulation (21, 150, 214), but whether these associations are independent of obesity remains to be determined (21, 150, 214).

Other conditions. Hepatic insulin action on glucose production has been reported to be impaired in patients with acute infections (441), acromegaly (173, 283), hyperthyroidism (380), and in patients after major uncomplicated surgery (48), but not in patients with Cushing's syndrome (178), essential hypertension (350), or liver cirrhosis (322, 335).

2.1.1.2. Lipid metabolism

2.1.1.2.1. Methodological aspects

VLDL kinetics. The relative changes in VLDL triglyceride and VLDL apolipoprotein B (ApoB) production rates can be determined using tracer techniques (460). This approach involves examining the change in specific activity of exogenously administered isotopically labeled amino acids (85, 140, 193, 266) or glycerol (4, 165), which are then incorporated into newly synthesized VLDL particles. Another option is to use the exogenous labeling of the lipoprotein particles of interest using either ^{125}I or ^{131}I isotopes (390, 417). The infusion of these particles in the circulation allows following both kinetics and the interconversion and catabolic rates of the lipoprotein particles.

Splanchnic lipid oxidation. By using the combination of hepatic venous catheterization technique (44, 129, 285), the infusion of labeled fatty acids (162, 196, 449), indirect calorimetry (130), and the measurements of breath CO_2 carbon specific activity (162, 196, 449), splanchnic fatty acid oxidation can be measured directly (385, 386).

2.1.1.2.2. Normal actions of insulin

In non-diabetic healthy subjects, insulin (1 mU/kg·min) decreases hepatic VLDL triglyceride and ApoB production by ~50% (248-250). This effect of insulin appears to some extent independent of serum FFA concentrations (249), since the maintenance of fasting FFA concentrations despite hyperinsulinemia attenuates the suppression of VLDL production (249). Hyperinsulinemia lowers plasma VLDL concentrations primarily by suppressing the production of large, triglyceride-rich VLDL₁ particles, while the production of dense

VLDL₂ particles remains unchanged (266).

Although *in vitro* studies have shown insulin to inhibit both hepatic fatty acid oxidation and *de novo* lipogenesis (227, 274), there are no human studies examining the direct effects of insulin on these metabolic processes. One study including five healthy subjects indicated the combination of hyperglycemia and hyperinsulinemia inhibits splanchnic fatty acid oxidation (385). Serum concentrations of a ketone body 3-hydroxybutyrate (3-OHB), a surrogate marker of hepatic fatty acid oxidation (25, 145, 177), have been shown to decrease during the low-dose euglycemic hyperinsulinemic clamp (55, 127, 237).

2.1.1.2.3. Insulin action in insulin-resistant subjects

The acute inhibitory effect of insulin on VLDL production is significantly impaired in obese as compared to lean subjects (248). When two groups of non-diabetic subjects and type 2 diabetic patients accurately matched for age, gender, and obesity have been compared, insulin suppression of VLDL₁ but not VLDL₂ production have been found to be impaired under both hyperglycemic and normoglycemic conditions (265). Recently, the ability of insulin to suppress VLDL₁ production has been shown to be impaired in subjects with high as compared to those with low liver fat content (6). However, since the subjects with high liver fat content were more obese and had higher serum FFA concentrations during the insulin infusion than those with low liver fat content (6), it remained unclear whether the link

between hepatic fat accumulation and the impaired ability of insulin to suppress VLDL production is independent of the above factors.

Serum concentrations of 3-OHB have been shown to be higher in the basal state in subjects with non-alcoholic fatty liver disease (NAFLD) as compared to either lean (361) or weight matched (55) control subjects. In these studies, the ability of insulin to lower 3-OHB concentrations during the low-dose insulin infusion was comparable between the two groups, but serum 3-OHB concentrations remained significantly higher in subjects with a fatty liver as compared to controls (55, 361). Whether insulin action on hepatic lipid oxidation differs in other insulin resistant states, such as obesity and type 2 diabetes, remains to be determined.

2.1.2. Insulin action in skeletal muscle

2.1.2.1. Methodological aspects

The golden standard technique for determining insulin sensitivity is the euglycemic hyperinsulinemic clamp (16, 104). With this method, insulin is administered intravenously in a prime-continuous fashion at a rate calculated either per kg body weight or body surface area (104). Euglycemia is maintained by means of an exogenous infusion of 20% glucose (104). At an insulin infusion rate of 1 mU/kg·min (40 mU/m²·min), hepatic glucose production is completely suppressed in normal subjects (96, 348, 478). Under these conditions, the exogenous glucose infusion rate corrected for changes in the glucose pool size (M-value) equals the amount of glucose disposed by all tissues in the body (104). Under such conditions, skeletal muscle accounts for

~70% of whole-body glucose disposal (96, 297, 475, 478).

If insulin infusion rates lower than 1 mU/kg·min are used, the rate of hepatic glucose production has to be taken into account when determining glucose rate of disappearance (R_d) (96, 348, 478). Under non-steady-state conditions, glucose R_d equals the difference between total glucose R_a and a change in the total amount of glucose over time:

$$R_d = R_a - p \times V \times \Delta Glucose / \Delta Time$$

where p is a pool fraction for the distribution of glucose and V its distribution volume (393).

Muscle glucose uptake can also be determined using the femoral venous catheterization technique (101). In addition, regional glucose uptake can be measured using PET combined with a positron-emitting radionuclide -labeled glucose analogue (297, 442), such as [¹⁸F]FDG (388).

Indirect calorimetry is a non-invasive method to estimate rates of substrate oxidation and energy expenditure at the level of whole-body (87, 130). The rate of carbohydrate oxidation can be calculated from measurements of O₂ consumption, CO₂ production, and protein oxidation (urinary nitrogen excretion) (87, 130). If the respiratory quotient (the ratio of the carbon dioxide production and oxygen consumption, $RQ = VCO_2/VO_2$) is measured across the leg or arm, the rate of muscle substrate oxidation can be estimated (216, 444). Non-oxidative glucose disposal, which represents the sum of glycogen storage and non-oxidative glycolysis (467), can be calculated by subtracting the rate of carbohydrate oxidation from the rate of glucose disposal.

2.1.2.2. Normal actions of insulin

After an overnight fast, glucose utilization is mainly non-insulin dependent (168, 467). Under these conditions, more than half of glucose is utilized by the brain (168, 353, 366, 367), approximately one fourth by splanchnic area (96), and one tenth by the heart, adipose tissue, and kidneys (35, 38, 349). Thus, only approximately 10% of glucose utilization occurs in skeletal muscle under overnight fasted conditions (467).

Insulin stimulates glucose utilization predominantly in skeletal muscle (96, 297, 475, 478) by stimulating the translocation of insulin-sensitive glucose transporter GLUT-4 from intracellular sites to the plasma membrane (167, 224). In humans, glucose utilization is half-maximally stimulated at serum insulin concentrations of 48-130 mU/l (mean 77 mU/l) (61, 96, 226, 267, 348, 420), when measured using euglycemic hyperinsulinemic clamp technique (104). Maximal glucose utilization, which is approximately six times the rate measured after an overnight fast (267, 348, 420, 467), occurs at serum insulin concentrations of ~200 mU/l in normal subjects (267, 348, 420).

Insulin increases both oxidative and non-oxidative disposal of glucose. Half-maximal stimulation of glucose oxidation is achieved by insulin concentrations of 22-50 mU/l (43, 162, 267, 420, 470, 480). Non-oxidative glucose disposal is less sensitive to insulin, since half-maximal stimulation occurs at insulin concentrations of 75-150 mU/l (162, 267, 420, 480). The capacity of body tissues to utilize glucose non-oxidatively is 1.2-2.4 -fold higher than the capacity to oxidize glucose (267, 478, 480).

2.1.2.3. Insulin action in insulin resistant-subjects

Obese subjects. Insulin resistance generally increases with increasing obesity. According to the European Group for the study of Insulin Resistance (EGIR) database, which includes measurements of insulin sensitivity by the euglycemic hyperinsulinemic clamp in over a thousand of healthy non-diabetic European men and women, BMI is a strong determinant of insulin sensitivity (462). However, variation in BMI accounts for only part of the variance in insulin sensitivity (462). Thus, the degree on insulin resistance varies considerably among equally obese subjects, and even lean subjects can be insulin resistant. Obesity and skeletal muscle insulin resistance are associated with reduced muscle capillary density (251), an increase in the proportion of glycolytic type IIb and a decrease in oxidative type I muscle fibers (230, 251), and structural changes in skeletal muscle fatty acid membrane composition (230). Whether these changes are a cause or a consequence of obesity remains to be determined. In addition, serum FFA concentrations may contribute to skeletal muscle insulin resistance (215, 281).

Type 2 diabetic patients. Using the insulin clamp technique (104) with insulin infusion of 1 mU/kg-min in combination with femoral vein/artery catheterization to quantitate leg glucose uptake, the onset of insulin action on glucose uptake has been shown to be delayed and leg glucose uptake reduced by 40-50% in type 2 diabetic patients as compared to weight-matched non-diabetic subjects (99). Similar results have been reported using the forearm catheterization technique (61, 62, 207, 282). Both glucose oxidation and non-oxidative glucose disposal are impaired

in patients with type 2 diabetes (92, 155). Insulin binding to skeletal muscle has been reported to be similar in type 2 diabetic patients and non-diabetic subjects (299), whereas intracellular insulin receptor cascade differs between these groups (299). The postreceptor defects in skeletal muscle include reduced insulin receptor kinase activity (299) and translocation of glucose transporters (37), and impaired phosphorylation of insulin receptor second messengers, such as insulin receptor substrate 1 (IRS-1) (36, 37, 86) and phosphatidylinositol 3-kinase (PI 3-kinase) (36, 37, 86, 220).

Type 1 diabetic patients. Muscle insulin resistance is a common feature of type 1 diabetes (100, 102, 105, 185, 341, 477). Glucose toxicity, the harmful metabolic effects of chronic hyperglycemia, is regarded as a major cause of peripheral insulin resistance in type 1 diabetic patients (468).

Other conditions. Muscle insulin sensitivity is impaired during acute infections (441), acromegaly (199), hyperthyroidism (200), primary hyperparathyroidism (332, 332), Cushing's syndrome (178), essential hypertension (132), liver cirrhosis (202, 322, 335, 378, 439), and in patients after major uncomplicated surgery (48).

2.2. INSULIN CLEARANCE

Irreversible removal of a biological compound occurs via excretion and metabolism (116). In the case of insulin, excretion is negligible, and therefore removal is primarily the result of degradation (116, 339).

2.2.1. Methodological aspects

Whole-body insulin clearance. At steady-state, the rate of insulin removal equals its rate of administration (66). Thus, the kinetics of peripheral insulin metabolism can be directly assessed using the euglycemic hyperinsulinemic clamp technique (104), assuming that the contribution of endogenous insulin secretion is negligible or suppressed (94). The rate of insulin clearance is calculated by dividing the insulin infusion rate by steady-state arterialized serum insulin concentrations (66). Indirectly, insulin clearance can be estimated from the plasma decay curves after an acute intravenous insulin bolus (66, 133).

Hepatic insulin extraction. Using hepatic venous catheterization (129), the rate of hepatic insulin extraction can be calculated by dividing arterial - hepatic venous insulin concentration difference by the arterial insulin concentration (137). C-peptide is secreted by the pancreas at the same molar rate as insulin, but is not extracted by the liver to any significant amounts (221). Thus, hepatic insulin extraction can non-invasively be estimated from peripheral insulin and C-peptide concentrations using several mathematical models (66). This assumes, however, that C-peptide clearance does not display interindividual variation, which may not be the case (124).

2.2.2. Sites of insulin clearance in humans

Insulin is cleared from the circulation mostly in the liver and kidney, and to a lesser extent in muscle and adipose tissue (116). In the fasting state, approximately 40% and 15% of circulating insulin is removed by the liver and kidney when measured using hepatic and renal venous catheterization techniques (137). After induction of hyperinsulinemia, the respective proportions rise to 50-80% (49, 137, 357, 445) and 20-30% (137).

2.2.3. Insulin clearance in humans

Endogenous insulin reaching the systemic circulation through the hepatic veins, or infusion of exogenous insulin intravenously, is cleared from the plasma at the rate of approximately 12 ml/kg-min. This translates into ~840 ml/min for a man weighting 70 kg (133, 134, 137, 381). Given that the coefficient of variation of this estimate is over 20% (290, 427, 428), the normal range can be calculated to be 7-17 ml/kg min for 95% of healthy population (133). The reasons for this wide interindividual variation have been poorly characterized.

2.2.4. Factors influencing insulin clearance

Body composition. In normal-weight subjects, muscle mass increases and body fat decreases insulin clearance (458, 474). Body composition explains up to 37% of the interindividual variation in directly measured whole-body insulin clearance (474).

Obesity and body fat distribution. Insulin clearance, expressed either per kg body weight or per body surface area, has

been decreased in most (45, 65, 123, 279, 351, 389, 488) although not all (312, 326, 333) studies in obese as compared to lean subjects. In studies where insulin clearance was expressed per kg fat free mass, insulin clearance was unaltered by obesity (65, 488). Several studies have suggested that intra-abdominal rather than subcutaneous fat influences insulin clearance (213, 312, 313) independent of BMI (312) because of diminished hepatic insulin extraction (213, 312, 313).

Insulin resistance and type 2 diabetes. A decrease in insulin clearance has been observed in insulin-resistant as compared to age- and BMI-matched insulin-sensitive subjects (203). In small groups of type 2 diabetic patients, insulin clearance has been reported to be normal (150, 152, 327), decreased (146, 399), and increased (305) compared to non-diabetic subjects. The reasons of these divergent results are unclear.

Hepatic fat accumulation. *In vitro*, insulin clearance is impaired in hepatocytes loaded with triglycerides (400). In the *in situ* perfused rat livers, insulin clearance is inversely related to hepatic triglyceride content (406). In a study of 46 non-diabetic subjects with a wide range of adiposity, the liver-to-spleen attenuation ratio (L/S ratio), a qualitative marker of liver fat, was found to be inversely related to insulin clearance (159). In addition, we have previously shown that decreasing liver fat content by rosiglitazone increases insulin clearance and enhances hepatic insulin sensitivity independent of body weight in type 2 diabetic patients (424).

Serum FFA. Wiesenthal et al showed that in dogs hepatic insulin extraction is impaired when serum FFA concentrations are elevated to supraphysiological concentrations by infusions of a soy-based lipid emulsion and heparin during euglycemic hyperinsulinemia (457). In humans, a day-long infusion of a soy-based lipid emulsion and heparin (S-FFA ~500-800 $\mu\text{mol/l}$) has also been shown to reduce insulin clearance in subjects with a family history of type 2 diabetes (212).

Advanced liver disease. Hyperinsulinemia in patients with cirrhosis has been attributed to both increased insulin secretion and impaired hepatic insulin extraction (197, 244, 298, 479). In a study including patients with compensated cirrhosis of different etiologies, insulin secretion rate has been shown to be 60% higher and insulin clearance 40% lower in cirrhotic patients as compared to age- and BMI-matched subjects (244). In these patients, insulin clearance was strongly positively related to the magnitude of the portosystemic shunt (244).

Kidney function. Uremia decreases insulin clearance in humans (84, 301). In one study using renal catheterization technique, severe uremia reduced renal insulin extraction from 39% to 9% (340).

Other factors. Hyperthyroidism increases insulin clearance (78), which can be normalized by anti-thyroid treatment (293). In addition, patients with Cushing's syndrome exhibit a decrease in insulin clearance (78). Some studies have suggested impaired insulin clearance to be a feature of essential hypertension (153, 358).

2.3. ECTOPIC FAT DEPOSITION

The deposition of fat into non-adipose tissues, which have only a small intracellular reserve of lipids, is termed as 'ectopic fat accumulation' (143, 434). These tissues include the liver, skeletal muscle, the heart, and pancreas (434).

2.3.1. The liver

2.3.1.1. Definition

Typically, the liver stores approximately 100g of whole-body fat (143). NAFLD refers to fat accumulation in the liver exceeding 5% to 10% of liver weight (17, 291). If measured by proton magnetic resonance spectroscopy (¹H-MRS), liver TG greater than 5.6% (55.6 mg/g) is considered abnormal, as it corresponds to the 95th percentile of the distribution of liver fat in subjects (n=345) with normal S-ALT and fasting glucose concentrations, and low alcohol consumption (409). NAFLD comprises a spectrum of liver pathology ranging from simple steatosis to steatohepatitis (1, 76), which may progress to cirrhosis and hepatocellular carcinoma (291). By definition, alcohol consumption should not exceed 14 units/wk (20 g/d), and viral (hepatitis B and C), toxic, autoimmune (clearly elevated antinuclear and anti-smooth muscle antibodies), and other causes of steatosis (Wilson disease, hypobetalipoproteinemia) should be excluded (53). The diagnosis of non-alcoholic steatohepatitis (NASH) includes, in addition to macrovesicular steatosis, lobular inflammation and hepatocellular ballooning (291).

2.3.1.2. Pathogenesis

Despite attempts to understand the mechanisms underlying NAFLD, the pathogenesis remains poorly understood.

Lessons from in vivo studies in humans

The fatty acids in hepatic triglycerides are derived from dietary chylomicron remnants, FFA released from adipose tissue, or from chylomicrons hydrolyzed at a rate in excess of what can be taken up by tissues (spillover), and from *de novo* lipogenesis (311). Hepatic triglycerides are secreted as VLDL or oxidized via β -oxidation (143, 462). In subjects with NAFLD, 60% of triacylglycerols stored in the liver are derived from plasma FFA (112), while catheterization studies have shown that direct hepatic FFA delivery from splanchnic lipolysis accounts for only approximately 5-10% and 30% in the fasting state in lean and obese subjects, respectively (292). This implies that the majority of fatty acids delivered to the liver originates from subcutaneous adipose tissue (222, 292). Postprandially, the contribution of the spillover pathway and uptake of chylomicron remnants increase from 11% to 30% and can account for approximately 60% of the fatty acids secreted as VLDL-TG in subjects with NAFLD (311). Furthermore, the contribution of *de novo* lipogenesis to hepatic triglycerides accounts for approximately 5% (310) in normal subjects and 20-25% (109, 112) in subjects with a fatty liver. Postprandially, the rate of *de novo* lipogenesis increases up to 23% in normal subjects (426). This increase is absent in subjects with NAFLD (112). Approximately 10% and 20-40% of the sources of FFA in the fasting and fed states remained unknown in the latter study using state-of-the-art triple stable isotope methodology (112).

Lessons from in vitro studies in humans
Gene expression in the liver. To date, few studies have investigated alterations in hepatic gene expression in patients with NAFLD (47, 70, 354, 453, 481, 482). In two studies comparing subjects with and without simple steatosis (453, 481), genes involved in liver regeneration (481), magnesium carrying (481), fatty acid binding (453), lipolysis (453), and monocyte/macrophage recruitment (453) were found to be significantly upregulated independent of obesity also after verification by real-time PCR (481). When patients with NASH have been compared to control subjects, differential expression has been found in mitochondrial and inflammatory genes, as well as in genes involved in insulin signaling, defense against oxidative stress, and *de novo* lipogenesis (47, 70, 354, 481, 482). However, these studies have not found the same genes to be altered, and only a few of these genes have been verified by real-time PCR (47, 70, 354, 481, 482).

Gene expression in adipose tissue. In obese individuals, the number of macrophages is increased and accompanied by increased expression pro-inflammatory factors, such as TNF α , MCP-1, and IL-6 (46, 107, 450). Recently, our group tested the hypothesis that adipose tissue inflammation characterizes subjects with high liver fat content independent of obesity in weight-matched obese groups differing with respect to liver fat content (225). Adipose tissue from the group with high liver fat content was inflamed (225), and was characterized by an increased number of macrophages surrounding dead adipocytes (74, 225).

Lessons from animal studies
Animals with genetic or environmental induction of hepatic fat accumulation may provide insights into possible molecular mechanisms involved in the pathogenesis of NAFLD. Animals with naturally occurring or genetically induced mutations resulting in obesity develop a fatty liver mainly because of excess FFA flux into the liver and induction of hepatic lipogenesis (228). Transgenic models of inhibition of hepatic fatty acid oxidation (125, 217), VLDL assembly and secretion (192, 245, 338), overexpression of hepatic lipogenic genes (228, 383), liver-specific overexpression of lipoprotein lipase (219), and overexpression of the nuclear form of SREBP-1a in adipose tissue (184) have been shown to develop hepatic steatosis. Hepatic lipogenesis induced by dietary sucrose (330), fructose (330), and fat (228), and hepatic fatty acid oxidation inhibited by dietary choline- or methionine-deficient diets (451) result in a fatty liver. However, the relevance of these models for the pathogenesis of the human fatty liver is unclear.

2.3.1.3. Diagnostic methods

Stetosis

Liver biopsy. A liver biopsy is considered the golden standard for quantification of liver fat (223). However, due to ethical limitations, this method cannot be routinely used for diagnosing NAFLD. An important drawback of liver biopsy, which represents only 1:50000 of the total mass of the liver (50), is sampling variability resulting from the heterogeneous distribution of histological lesions (262, 263).

Table 2. Obesity-independent circulating markers of NAFLD.

Markers of NAFLD	NAFLD/ Controls*	Subjects characteristics**	Liver fat methodology	Reference
hs-CRP↑, HOMA-IR↑	16/160	Age: 45 years BMI: 26 kg/m ²	Histology	413
IL-6↑, MCP1↑, CCL19↑	22/30	Age: 48/42 years BMI: 29/23 kg/m ²	Histology	175
Coenzyme Q10↓, SOD↓, catalase↓, MDA↑, S-ALT↑, S- AST↑, fS-insulin↑, C-peptide↑, HOMA-IR↑, S-triglycerides↑, S- HDL cholesterol↓	51/30	Age: 36 years BMI: 28 kg/m ²	Histology	461
Adiponectin↓, HOMA↑, fS- insulin↑, S-ALT↑, S-AST↑, S- γGT↑	17/20	Age: 44/42 years BMI: 27 kg/m ²	Histology	307
HOMA-IR↑, adiponectin↓, resistin↑	12/12	Age: 40/43 years BMI: 30/29 kg/m ²	Histology	12
TNFα↑, adiponectin↓, S-ALT↑, fP-glucose↑, fS-insulin↑, C- peptide↑, HOMA-IR↑	29/82	Age: 42/50 years BMI: 30 kg/m ²	Histology	188
Adiponectin↓, HOMA-IR↑, QUICKI↓, S-ALT↑, S-AST↑, S- γGT↑, fS-insulin↑, S-HDL cholesterol↓, fS-FFA↑	174/42	Age: 41/43 years BMI: 27/28 kg/m ²	Histology and ultrasound	57
Factor VII clotting activity↑, PAI-1 activity and antigen↑, t-PA activity↓	31/33	Age: 38 years BMI: 27/24 kg/m ²	Ultrasound	73
hs-CRP↑, fibrinogen↑, v-WF↑, PAI-1 activity↑	35/65	Age: 41/43 years BMI: 26/24 kg/m ²	Ultrasound and CT	415
α ₂ -Heremans-Schmid glycoprotein/fetuin-A↑	90	Age: 45 years Body weight: 86 kg	¹ H-MRS	394
S-γGT↑	70	Age: 41 years BMI: 28 kg/m ²	¹ H-MRS	419
Adiponectin↓	242	Age: 46 years BMI: 26-32 kg/m ²	¹ H-MRS	210
fS-HDL cholesterol, fS- triglycerides↑, fS-FFA↑, leptin↑, adiponectin↓, resistin↓. E- selectin↑	21/21	Age: 36/35 years BMI: 28/27 kg/m ²	¹ H-MRS	315
RBP-4↑	75	Age: 44 years Body weight: 87 kg	¹ H-MRS	395

* Number of subjects

** Age and BMI or body weight denote mean values

Imaging. On ultrasonography, a fatty liver produces a diffuse increase in echogenicity and sound attenuation (337), which is similar to that seen in fibrosis and cirrhosis (67, 359). Magnetic resonance imaging (139, 306) and the L/S ratio determined by computed tomography (387) provide qualitative rather than quantitative estimates and their sensitivity is limited (387). Assessment of liver fat content by ¹H-MRS involves recording of the two dominant peaks within the unsuppressed magnetic resonance spectrum: water at 4.7-4.8 ppm and methylene at 1.0-1.5 ppm (254, 407, 422). Since the area under the peaks can easily be quantitated, the % liver fat can be calculated from the following equation (355):

$$\% \text{ liver fat} = (\text{area under the methylene peak}) / (\text{area under the water peak} + \text{area under the methylene peak}) \times 100\%$$

¹H-MRS represents the most reliable non-invasive method to measure liver fat content at present (462).

Circulating markers. Serum liver enzyme concentrations (S-ALT, S-AST) have been shown to correlate with liver fat content measured by ¹H-MRS (452, 462), but normal S-ALT concentrations do not exclude steatosis. Indeed, in Dallas Heart Study, in which liver fat content was determined using ¹H-MRS in 2287 subjects, normal S-ALT concentrations were present in 79% of the subjects with a fatty liver (>5.6%) (52). Several other obesity-independent markers of NAFLD have been identified (Table 2). The sensitivity of these

markers is essentially similar to that of S-ALT.

NASH

NASH can only be reliably diagnosed using a liver biopsy (253). To date, only a few obesity-independent circulating markers of NASH have been reported in cross-sectional studies of relatively small size (Table 3). However, these markers may not be specific to NASH.

Fibrosis

At present, the presence of fibrosis can only be reliably established by a liver biopsy, although several circulating and anthropometric markers of fibrosis have been proposed (166). A combination of several markers may be more useful in the diagnosis of fibrosis (19, 166, 308). In a recent cross-sectional analysis of 733 patients with NAFLD, use of a NAFLD fibrosis score predicted fibrosis correctly in 90% (19). By applying this score, which included age, hyperglycemia, body mass index, platelet count, albumin, and the AST/ALT ratio, a liver biopsy could be avoided in 75% of the patients (19). A novel technique called elastography has also been proposed to help in the diagnosis of fibrosis (142).

2.3.1.4. Prevalence

The prevalence of NAFLD varies according the diagnostic criteria used. In the Third National Health and Nutrition Examination Survey (NHANES III) carried out between 1988 and 1994 in the US of 15676 adults, where excess liver was defined based on elevated

Table 3. Obesity-independent markers of NASH.

Markers of NASH	NASH/ NAFLD /(Controls)*	Subjects characteristics**	Liver fat methodology	Reference
hs-CRP↑, HOMA-IR↑	69/16/(160)	Age: 45 years BMI: 26 kg/m ²	Histology	413
Plasma cytokeratin-18 fragment↑	21/8/(10)	Age: 53/47/49 years BMI: 33/31/27 kg/m ²	Histology	456
fP-glucose↑, fP-insulin↑, IR index↑, HbA _{1c} ↑, C-peptide ↑, S- ALT↑, S-AST↑, S-γGT↑, fS- triglycerides↑, S-ferritin↑	26/75/(4)	Age: 44/40 years BMI: 47/44 kg/m ²	Histology	110
MCPI↑, TNFα↑	25/22	Age: 45/48 years BMI: 32/29 kg/m ²	Histology	175
fS-insulin↑, C-peptide↑, HOMA- IR↑, soluble TNF receptor 2↑, adiponectin↓	80/29	Age: 51/42 years BMI: 31/30 kg/m ²	Histology	188
TPS↑	48/48	Age: 42/43 years BMI: 30/30 kg/m ²	Histology	412

* Number of subjects

** Age and BMI denote mean values

aminotransferase concentrations, the prevalence of NAFLD was 5.4% (75). The real prevalence of NAFLD may be at least 5-fold higher (~27%), given that *e.g.* S-ALT are abnormally high in only 21% of subjects with NAFLD (52). In the Dallas Heart Study, 33.6% of 2349 participants (age 18 to 65) had a fatty liver determined by ¹H-MRS than equally obese Caucasian men (320). The prevalences on NAFLD in subjects with the metabolic syndrome and in patients with type 2 diabetes are discussed below.

2.3.1.5. Natural history

The natural history of NAFLD remains poorly characterized, since to date only a few studies have addressed long-term clinical course of the disease. Histological change seems to be one

determinant of progression of NAFLD (18). Patients with pure steatosis without histological signs of inflammation have the best prognosis (83). For example, a Danish study of 109 predominantly morbidly obese subjects followed for nearly 17 years found the incidence of cirrhosis to be less than 1% (88). It is estimated that 12-20% of subjects with simple steatosis develop NASH characterized by mild (F1) or moderate (F2) fibrosis (90). Patients with NASH followed up from 4 to 9 years show 4-16% progression rate to cirrhosis (121, 241, 331). Consequently, 100% of patients with NASH and advanced fibrosis have shown progression to cirrhosis within 5-10 years (188, 360). A recent Swedish study of 129 patients with biopsy-verified NAFLD followed up for approximately 14 years indicated

that liver-related complications are the third most common cause of death among NAFLD patients (118). Obesity and type 2 diabetes are more common in patients with cirrhosis due to NAFLD than in patients with cirrhosis due to other causes (253). The prognosis of cirrhosis due to NASH appears to be better than in patients with cirrhosis due to hepatitis C (189). NASH has been predicted to become the number one of orthoptic liver transplantation by the year 2020 (68).

2.3.1.6. Associated conditions

2.3.1.6.1. The metabolic syndrome

Definition. The current definition of the metabolic syndrome by the International Diabetes Federation (IDF) includes waist circumference over 94 cm in men and 80 cm in women, and at least two of the following: increased fasting serum glucose, triglycerides or blood pressure, or a low HDL cholesterol concentration (9). Previous metabolic syndrome definition by the National Cholesterol Education Program (NCEP) – Adult Treatment Panel III (ATPIII) entails the presence of three or more of these components with higher cutpoints of blood pressure and central obesity (122). The first definition by WHO required the presence of type 2 diabetes, impaired glucose tolerance or insulin resistance plus at least two of the following: obesity determined by BMI or waist-to-hip –ratio, dyslipidemia determined by elevated serum triglycerides or a low HDL cholesterol concentration, hypertension, and microalbuminuria (10).

Prevalence. The prevalence of the metabolic syndrome differs with respect to study design, sample selection, the definition of the metabolic syndrome, and the age and gender of the population studied (60). It has been estimated that

approximately 20-25% of populations worldwide has the metabolic syndrome defined by the ATP III criteria (60). According to the IDF, a quarter of world's adults have the metabolic syndrome (187).

Fat distribution, liver fat, and the metabolic syndrome. Insulin resistance and abnormal fat distribution are core components of the metabolic syndrome (8). Intra-abdominal and ectopic fat accumulation (8), particularly in the liver (462), seem to increase the risk of developing the metabolic syndrome. For example, a recent Japanese study including 4401 employees without liver disease or drug treatment (mean age 48 years, BMI 23 kg/m²), the odds ratios for men and women with NAFLD to develop the metabolic syndrome (ATP III criteria) during the follow-up of approximately one year were 4.0 and 11.2 after adjustment for age, alcohol intake, and changes in body weight (170). Similar data on hepatic (171, 371) and intra-abdominal (64, 287) fat accumulation have been reported by others. Other factors include insulin resistance, physical inactivity, and aging (8).

Risk of CVD, type 2 diabetes, and liver disease. The metabolic syndrome predicts an increased risk of both CVD and type 2 diabetes more than the individual components (8, 347, 487). For example, in the San Antonio Heart Study (n=2559, follow-up 7.4 years), the risk of CVD was 1.7-fold and that of type 2 diabetes 5.8-fold increased in subjects with as compared to those without the metabolic syndrome, independent of age, gender, ethnic origin, history of CVD and type 2 diabetes, non-HDL cholesterol, smoking, and family history of CHD (255). Subjects with the

Table 4. Prospective studies addressing liver enzymes as independent predictors of type 2 diabetes.

Cohort	N	Age (yrs)*	BMI / body weight*	Follow-up (yrs)	Significant predictor**	Reference
Swedish men	766	54	24.8-27.4 kg/m ²	13.5	S-ALT	302
Pima Indians	451	30	98 kg	6.9	S-ALT	443
WOSCOPS	5974	55-56	26.0-27.7 kg/m ²	4.9	S-ALT	365
Mexico City diabetes study	1441	46-52	27.7-28.6 kg/m ²	7	S-AST	289
British Regional Heart Study	7458	40-59	n.r.	12.8	S-γGT	314
D.E.S.I.R.	4201	47	23.9-25.3 kg/m ²	3	S-γGT	15
Finnish men and women	20158	25-64	42.4-46.2 kg/m ²	12.7	S-γGT	240
CARDIA study	4844	18-30	23.3-26.2 kg/m ²	5.5 and 15	S-γGT	239
IRAS	906	55	27.9-31.1 kg/m ²	5.2	S-ALT and S-AST	172
British Regional Heart Study	3500	60-79	25.5-27.7 kg/m ²	5	S-ALT and S-γGT	447
Japanese men	6217	35-59	22.2-24.3 kg/m ²	7	S-ALT, S-AST, and S-γGT	288

* Age and BMI or body weight denote mean values or mean ranges

** , Adjusted for body weight

metabolic syndrome have 3-4 -fold higher risks of NASH and severe fibrosis among NAFLD patients (269, 429) even after adjustment for age, gender, and BMI (269).

2.3.1.6.2. Type 2 diabetes

The studies in which the role of steatosis, estimated measuring liver enzymes, in the prediction of type 2 diabetes independent of body weight have been evaluated, are listed in Table 4. Most recently, Sattar et al identified three parameters which increased

gradually prior to type 2 diabetes: fasting plasma glucose, S-ALT, and fS-triglycerides (364). Importantly, there were no changes in body weight prior to the onset of type 2 diabetes (364). The only epidemiological study of determining the prevalence of NAFLD in type 2 diabetic patients found 70% of the 2839 type 2 diabetic patients to have hepatic steatosis (414), which is consistent with previous estimates (18, 277). When subjects with simple steatosis and those with NASH have been compared, hyperglycemia has been found to be

significantly associated with NASH (118, 176, 269) independent of age, gender, and BMI (176, 269). Moreover, type 2 diabetes and impaired glucose tolerance are independent risk factors for NASH (176) and the progression of fibrosis (3, 118). A prospective study indicated that type 2 diabetes or impaired fasting glucose, age, and baseline diagnosis of cirrhosis independently predict higher overall mortality among patients with NAFLD (2).

2.3.1.6.3. CVD

In 1439 men and women aged 50-75 years participating in the Hoorn Study in the Netherlands, increased S-ALT at baseline increased the 10-year risk of coronary heart disease events after adjustment for components of the metabolic syndrome and other CVD risk factors (glucose tolerance status, systolic blood pressure, HbA_{1c}, LDL cholesterol, and BMI) (369). Serum γ GT predicted coronary events during a mean follow-up of 15.7 years in 1878 men participating in the MONICA/KORA study after adjustment for traditional CVD risk factors (278). Similarly, in a study of 2839 type 2 diabetic patients, NAFLD, diagnosed by history and liver ultrasound, was associated with an increase in the risk of CVD even after adjustment for components of the metabolic syndrome (414). Ekstedt et al found mortality from cardiovascular causes to be significantly increased (15.5% vs. 7.5%) among subjects with NAFLD compared with a matched reference-population (118).

2.3.1.6.4. Advanced liver disease

NAFLD can progress to cirrhosis, liver failure, and hepatocellular carcinoma (128). NASH is currently the most likely cause of cryptogenic cirrhosis (59). The

risk of hepatocellular carcinoma tends to be slightly higher in patients with cryptogenic cirrhosis compared to those with hepatitis C virus related cirrhosis (343).

2.3.1.7. Factors regulating liver fat content

Diet. Liver fat increases significantly in response to a single mixed meal in normal subjects (344). In cross-sectional studies, a high liver fat content has been related to increased dietary fat, especially saturated fat intake (324, 423) and a high glycemic index (436). Studies examining effects of modulation of dietary composition while maintaining caloric intake constant on liver fat in humans are few and of small size. Westerbacka et al found that placing overweight non-diabetic women on an isocaloric diet containing 16% fat for 2 weeks decreased liver fat by 20%, whereas a 2-week diet containing 56% fat increased liver fat by 35% measured by ¹H-MRS (454). Four weeks of fructose feeding (1.5 g fructose per kg body weight) has been shown to induce hypertriglyceridemia without changing liver fat content measured by ¹H-MRS (238). Recently, an epidemiological study of 349 subjects indicated that subjects with NAFLD tend to consume less fish rich in omega-3 fatty acids and more meat and soft drinks than control subjects independent of age, gender, BMI, and total calorie intake (485).

Weight loss. Weight loss undoubtedly and effectively reduces steatosis (259). This decrease in liver fat is relatively greater (79, 423) and occurs more rapidly (79) than fat loss from other compartments in the body. The impact of weight loss on other changes, especially fibrosis, is still unsettled (174, 433, 484).

Physical training. In 30 middle-aged men, liver fat was unrelated to VO₂max in a cross-sectional study (379). On the other hand, in a cross-sectional analysis of 191 apparently healthy individuals, whose habitual physical activity was assessed using a questionnaire, liver fat content was lower in physically active individuals, even after adjusting for age, gender, and BMI (317). These cross-sectional data do not, however, prove cause and effect. In a study of 48 overweight subjects, liver fat, measured

using ¹H-MRS, decreased by 0.97 units by 10% weight loss achieved by diet alone (25% calorie restriction for 6 months) and by 0.52 units by the combination of diet (12.5% calorie reduction) and exercise (12.5% increase in energy expenditure) (235). The decrease in liver fat was not different between these groups (235), but the study was not powered to detect a difference. Thus, it is still unclear whether exercise independent of weight loss decreases liver fat content.

Table 5. *Controlled studies in humans to treat fatty liver and/or NASH.*

Intervention	Diagnosis	N	Duration	Change in liver fat*	Reference
PPARγ AGONISTS					
Rosiglitazone vs. placebo	Type 2 diabetes	33	4 mos	-45% (¹ H-MRS)*	63
Rosiglitazone vs. metformin	Type 2 diabetes	20	4 mos	-51% (¹ H-MRS)**	424
Rosiglitazone vs. placebo	HAL	30	6 mos	-15% (¹ H-MRS)*	404
Pioglitazone vs. placebo	NASH + IGT or type 2 diabetes	55	6 mos	-54% (¹ H-MRS)*, steatosis grade ↓ (BX)	29
CYTOPROTECTIVE AGENTS					
UCDA vs. placebo	NASH	166	2 yrs	No change in steatosis (BX)	252
COMBINATIONS					
Vitamin E vs. vitamin E + pioglitazone	NASH	20	6 mos	Steatosis grade ↓ (BX) in both groups	362
UCDA vs. UCDA + vitamin E vs. placebo	NASH	48	2 yrs	Steatosis grade ↓ (BX) by UCDA + vitamin E	117

* = % decrease in liver fat by determined by ¹H-MRS or biopsy

** = % change in the PPAR γ agonist group

Drugs affecting liver fat content. Data on controlled drug interventions in humans are listed in Table 5. Of the agents tested, PPAR γ agonists consistently decreased liver fat (21, 29, 63, 273, 424), and in one study also inflammation, ballooning necrosis, and possibly fibrosis (29). These beneficial changes were no longer seen 48 weeks after discontinuation of treatment (257). Regarding metformin, we found no decrease in liver fat measured with ^1H -MRS in patients who were treatment naive and did not lose weight (424). In another study, liver fat measured histologically decreases significantly by approximately 50% in patients who also lost 2 kg/m 2 of BMI (56). It remains unclear whether metformin decreases liver fat independent of decreases in body weight, as a 2.5 kg/m 2 loss of BMI decreases liver fat by 49% (423). A number of randomized controlled trials using metformin, glitazones, ACE inhibitors, pentoxifylline, fenofibrate, niacin, vitamin E, and PUFA are currently being tested in NAFLD (www.clinicaltrials.gov). In addition, rimonabant, which has been shown to decrease liver fat independent of body fat in mice (148), and incretin mimetics (108, 432) are studied in humans.

Genetic factors. A study in monozygotic twins discordant for obesity by Pietilainen et al showed that acquired obesity increases liver fat content independent of genetic background (324). This does not, however, exclude a role of genetic factors, but few data are currently available. Polymorphisms of the adiponectin receptor 1 promoter region (396), *usf-1* (209), and hepatic lipase (397) have been linked to variation in liver fat content, but in these studies, which all are from the same German cohort, liver fat content was

measured in only approximately 100 subjects. The -493 G/T promoter polymorphism of the mitochondrial triglyceride transfer protein (MTTP) has been suggested to influence hepatic fat accumulation measured by serum liver enzyme concentrations and ultrasound (33).

2.3.2. Skeletal muscle

2.3.2.1. Intra- and extramyocellular lipid

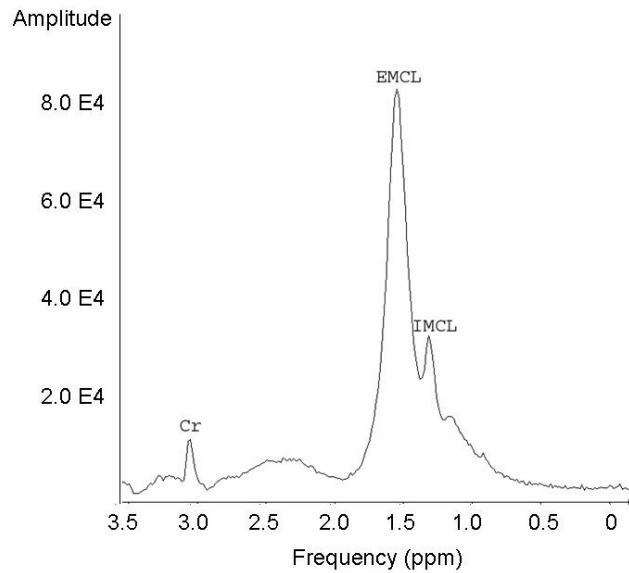
Muscle lipid storage consists of triglycerides within myocytes (intramyocellular lipid, IMCL) and of adipocytes located within the muscle fibers (extramyocellular lipid, EMCL) (260).

2.3.2.2. Quantification

Muscle biopsy. Lipid within the muscle fibers can be directly visualized with histochemical staining in light microscopy, which reveals the distribution of lipids within myocytes (158, 323). Alternatively, biopsy specimens can be analyzed using electron microscopy (186). However, the invasive nature of the biopsy and small sample size (374) limit its use as a method to quantitate IMCL.

^1H -MRS. IMCL can be non-invasively quantitated using ^1H -MRS (260). The geometrical arrangement of EMCL and IMCL differs. EMCL is located in long fatty septa along muscle fiber bundles or fascia, whereas IMCL consists of lipid droplets within the cytoplasm of muscle cells (260). The resonances of lipids are split in two methylene proton signals at 1.3 ppm for IMCL, and at 1.5 ppm for EMCL (40, 368, 407). By quantitating the areas under these methylene peaks and under the CH_3 -peak at 3.0 ppm derived from total creatine (Cr), the %

Figure 1. Skeletal muscle ^1H -MRS spectra from tibialis anterior muscle of a subject with intramyocellular fat percentage of 4.2.



IMCL can be calculated from the following equation:

$$\% \text{ IMCL} = (\text{area under the 1.3 ppm methylene peak}) / (\text{area under the total creatine peak} + \text{area under the 1.3 ppm methylene peak}) * 100\%$$

2.3.2.3. Factors influencing IMCL

Obesity. Some studies have reported positive associations between obesity and IMCL content determined by light microscopy (158, 323) or by ^1H -MRS (191). On the other hand, the majority of studies in which IMCL has been measured using ^1H -MRS, no relationship between these parameters has been observed (198, 231, 316, 318, 440). In a study group of 105 healthy non-diabetic subjects, Thamer et al found intramyocellular lipid content in tibialis anterior muscle to be positively associated with % whole body fat, and IMCL content in soleus muscle with waist circumference and waist-to-hip ratio

(418). Weight loss has been shown to decrease IMCL content in some (157, 158, 160, 161) but not all (363) studies.

Ethnicity. When different ethnic groups have been compared, IMCL appeared higher in South Asian than in European men of the same BMI (141). Similarly, Petersen et al reported IMCL to be higher in Asian-Indian than in Caucasian men (320). However, in the latter study, the difference in IMCL between the groups did not persist after adjusting for age and BMI (320).

Diet. An increase in dietary fat intake from 43% to 54% of total energy intake for 4 weeks has been reported to increase IMCL by 57% due to an increase in muscle lipoprotein lipase activity (218). Serum triglyceride or insulin concentrations did not change after the high-fat diet in the latter study (218). Similar results have recently been reported by others (376, 398). A 4-week high

fructose diet has been reported to increased serum total and VLDL triglyceride, lactate, glucose, and leptin concentrations without changes in IMCL (238).

Physical training

Acute regulation. Lipid droplets inside myocytes are considered as an important source of energy during exercise (437). After a 45-120 minutes of acute exercise, IMCL assessed by ^1H -MRS decreases by 25-70% (51, 232, 236, 377). During recovery, IMCL content has been shown to return to baseline within 4 hours (232). The rate of recovery depends on diet consumed after physical training: IMCL content has been shown to be significantly higher 22-24 hours postexercise in subjects consuming high-fat (60-70% of energy from fat) (392) or moderate-fat (35% of energy from fat) (236) than in those consuming very low-fat (5-10% of energy from fat) (236, 392) recovery diets.

Long-term regulation. Endurance training increases IMCL content (120, 156, 190, 437). This phenomenon, known as an 'exercise paradox' (156), is thought to represent an adaptive response increasing fuel availability during exercise (437). The increase in IMCL seems to be driven by the exercise-induced transcriptional reprogramming, which enables greater IMCL use (372). In a cross-sectional study of healthy non-diabetic subjects, IMCL was positively correlated with maximal aerobic power (VO_2max) in lean but not in obese individuals (418).

2.3.2.4. Role of IMCL in skeletal muscle insulin resistance

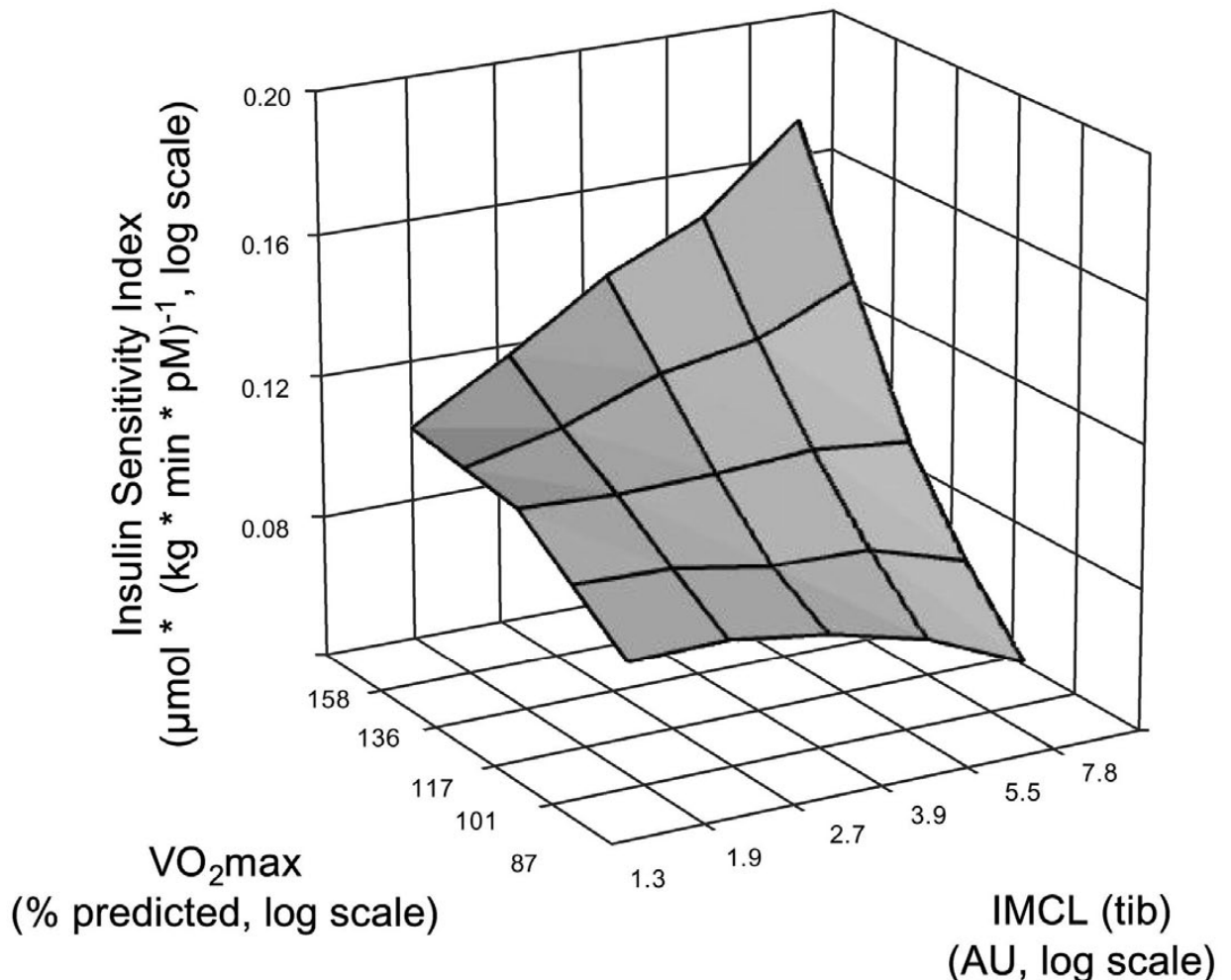
Insulin sensitivity in humans. IMCL content is an important determinant of insulin sensitivity in skeletal muscle (260). A BMI-independent inverse

relationship between IMCL and insulin sensitivity has been found in cross-sectional studies including 20-26 non-diabetic subjects (198, 231, 440). In contrast, in a larger study of 105 non-diabetic subjects, in which both obesity and VO_2max varied over a wide range, no relationship between insulin sensitivity and IMCL content was found (418). Further analyses revealed that insulin sensitivity was negatively related to IMCL content only in subjects with low aerobic power (418). On the other hand, subjects with high aerobic power, insulin sensitivity and IMCL content were positively correlated (Fig. 2) (418). Similar results have been obtained when sedentary subjects and exercise-trained individuals have been compared (156). Thus, physical fitness appears to be an important modifier of the relationship between IMCL content and insulin sensitivity. Lean, normoglycemic insulin-resistant offspring of parents with type 2 diabetes (28, 284) and patients with type 2 diabetes (156, 246, 375, 410) have either increased (28, 156, 246) or similar (284, 375, 410) IMCL content compared to normoglycemic subjects matched for age, gender, and BMI (156, 246, 284, 375, 410).

Molecular mechanisms

Studies in humans. IMCL accumulation associates with defective insulin signaling in human skeletal muscle independent of obesity and physical fitness (440). Both tyrosine phosphorylation of the insulin receptor and activation of insulin receptor substrate-1 (IRS-1) – associated phosphatidylinositol (PI) 3-kinase, an important mediator of the activation of glucose transport and glycogen synthesis (115), are inversely related to IMCL in non-diabetic subjects (440). The mechanisms by which IMCL may influence insulin signaling include

Figure 2. Three-dimensional relationship between VO_2max , IMCL in tibialis anterior muscle, and insulin sensitivity. In untrained individuals, high IMCL predicts low insulin sensitivity. In highly trained individuals, this relationship is reversed and high IMCL predicts high insulin sensitivity. Adapted from ref. 418 with permission.



increased intramyocellular concentrations of long-chain acyl coenzyme-A esters (119), which directly inhibit hexokinase (421) and activate protein kinase C (373), leading to decreased tyrosine phosphorylation of IRS-1 (411).

Lessons from animal models. Several animal models have been used to study the role of IMCL in insulin resistance. Rats fed with high-fat diet (300), mice with muscle-specific overexpression of lipoprotein lipase (219), and rats treated

with inhibitors of β -oxidation (111) exhibit increased IMCL content and skeletal muscle insulin resistance. Conversely, fatty acid translocase CD36 deficient mice display diminished IMCL content and increased insulin sensitivity (169). Although intramyocellular triglycerides are inert and do not affect insulin action in skeletal muscle (284, 403), they may give rise to metabolites interfering with insulin signaling, such as diacylglycerol, malonyl CoA, and ceramides (180, 286, 384, 403).

2.3.3. Other tissues

2.3.3.1. Pancreas

Zucker diabetic rats tend to accumulate fat in pancreatic islet cells, resulting into apoptosis and β -cell dysfunction (242, 382). Treatment with PPAR γ agonists lowers islet fat content and preserves β -cell function in these animal models (382). Studies in humans are rare because of limitations in obtaining pancreatic tissue (58). Recently, pancreatic fat has been quantitated using $^1\text{H-MRS}$ in humans (431) and appeared to be ~50% higher in type 2 diabetic patients as compared to age- and BMI-matched non-diabetic subjects (431).

2.3.3.2. Heart

In transgenic mice, cardiac-specific overexpression of long-chain acyl-CoA synthetase (72) and fatty acid transport protein 1 (71) result in triglyceride accumulation, which in turn leads to cardiac dysfunction and cardio-

myopathy. In humans, the myocardial triglyceride content, assessed using $^1\text{H-MRS}$ (106, 408), has been shown to be positively associated with BMI (408), left ventricle mass (408), fasting serum FFA concentrations (208), epicardial fat (208), S-ALT concentrations (208), hepatic triglyceride content measured by $^1\text{H-MRS}$ (275), serum triglycerides (275), HOMA-IR (275), and visceral fat mass (275). Myocardial triglyceride content has been found to be approximately 2-fold higher in subjects with impaired glucose tolerance and type 2 diabetes compared to subjects with normal glucose tolerance in the face of similar ejection fraction (275). In patients with type 2 diabetes, pioglitazone appears to decrease myocardial triglyceride content without changing either systolic or diastolic blood pressure, or cardiac function (486). Thus, the contribution of fat accumulation in the heart to cardiac dysfunction remains to be determined.

3. AIMS OF THE STUDY

The present studies were undertaken to answer the following questions:

- 1) How are hepatic and intramyocellular fat accumulation related to hepatic and muscle insulin resistance and features of the metabolic syndrome (I)?
- 2) What is the amount of liver fat in subjects with and without the metabolic syndrome, and which clinically available markers best reflect liver fat content (II)?
- 3) How does liver fat accumulation influence insulin clearance (III)?
- 4) Do type 2 diabetic patients have more liver fat than age-, gender-, and BMI-matched non-diabetic subjects, and how are liver enzymes (S-ALT, S-AST) related to liver fat in type 2 diabetic patients and normal subjects (IV)?
- 5) How do type 2 diabetic patients using exceptionally high doses of insulin respond to addition of a PPAR γ agonist (V)?

4. SUBJECTS AND STUDY DESIGNS

The study subjects were recruited based on the following inclusion criteria: (i) age 18 to 70 years; (ii) no known acute or chronic disease based on history, physical examination, ECG, and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations); (iii) alcohol consumption less than 20g per day; (iv) no evidence of hepatitis A, B, or C, of autoimmune hepatitis, or clinical signs or symptoms of inborn errors of metabolism or history of use of toxins or drugs known to induce hepatitis. Elevated liver enzymes (S-ALT, S-AST, and S- γ GT) were not exclusion criteria. Aims and designs of the studies are listed below. Written informed consent was obtained from all subjects. All protocols were approved by the Ethics Committee of the Helsinki University Central Hospital.

Study I

Aims: To examine the contribution of hepatic and intramyocellular fat accumulation to both hepatic and muscle insulin resistance and to features of the metabolic syndrome.

Subjects: Forty-five men were recruited from occupational health services in Helsinki. Data on liver fat and hepatic insulin sensitivity of 30 men have previously been reported (379).

Measurements: The subjects were studied on 4 separate occasions. All measurements were performed within one month. These measurements included measurements of hepatic insulin sensitivity (1st occasion), liver fat

and IMCL by ¹H-MRS, and intra-abdominal and subcutaneous fat by MRI (2nd occasion), muscle insulin sensitivity (3rd occasion), and maximal oxygen consumption (VO₂max, 4th occasion).

Study II

Aims: To determine the amount of liver fat in subjects with and without the metabolic syndrome, and to examine which clinically available markers best reflect liver fat content.

Subjects: A total of 271 non-diabetic subjects were recruited primarily by newspaper advertisements and by contacting occupational health services. A total of 20 subjects were receiving medications for hypertension (Ca-channel blockers or ACE-inhibitors), 6 subjects were receiving medications for dyslipidemia (statins), and 7 subjects were receiving medications for both hypertension and dyslipidemia. 19 % of the women were postmenopausal. If eligible, the subjects were studied after an overnight fast to assess body composition and features of insulin resistance. All except 75 subjects have previously participated in metabolic studies (324, 379, 425, 452).

Measurements: The study participants underwent measurements of liver fat content by ¹H-MRS and body composition by MRI. In addition, components of the metabolic syndrome as defined by IDF and features of insulin resistance were measured.

Study III

Aims: To determine the effect of liver fat on insulin clearance.

Subjects: A total of 80 non-diabetic subjects were recruited from the occupational health services in Helsinki and amongst those referred to the Department of Gastroenterology because of chronically elevated serum transaminase concentrations. Data on liver fat and hepatic insulin sensitivity of 30 men have previously been reported (379). Baseline characteristics (but no data on insulin sensitivity or clearance) of these subjects were included in Study II.

Measurements and design: The subjects were divided into two groups based on their median liver fat content (5.0%). The study participants underwent measurements of hepatic insulin sensitivity and insulin clearance by euglycemic hyperinsulinemic clamp combined with the infusion of [³H]glucose, and liver fat content by ¹H-MRS.

Study IV

Aims: To determine whether type 2 diabetic patients have more liver fat than age-, gender-, and BMI-matched non-diabetic subjects, and how liver enzymes (S-ALT, S-AST) are related to liver fat in type 2 diabetic and normal subjects.

Subjects: A total of 70 non-diabetic subjects and 70 type 2 diabetic patients were recruited by newspaper advertisements and by contacting occupational health services in Helsinki. Exclusion criteria included proliferative retinopathy and use of antihypertensives possibly influencing glucose metabolism (β -blockers and thiazides), or of thiazolidinediones. A total of 13 non-diabetic subjects and 32 type 2 diabetic patients received antihypertensive medications (ACE-inhibitors or Ca-channel blockers). A total of 31 type 2

diabetic patients were treated with diet alone, 17 with metformin, and 22 with a combination of metformin and insulin. Five non-diabetic subjects and 22 type 2 diabetic patients were receiving statins. Regarding the insulin-treated patients, additional inclusion criteria included stable body weight and insulin dose for at least 6 months. Data on non-diabetic subjects (Study II) and 46 of the type 2 diabetic patients participating in treatment studies (204, 355, 424) have previously been reported. For the latter patients, only baseline data are included.

Measurements: The study participants underwent measurements of liver fat content by ¹H-MRS and body composition by MRI. In addition, circulating markers of insulin resistance and serum liver enzyme concentrations were determined.

Study V

Aims: To examine how type 2 diabetic patients using exceptionally high doses of insulin respond to addition of a PPAR γ agonist.

Subjects: This study was an investigator-initiated study not financially supported by the industry. We recruited, by contacting specialists in Southern Finland, 14 patients (9 men and 5 women, mean duration of diabetes 12 years) with type 2 diabetes who were poorly controlled despite high-dose insulin therapy (>100 IU/day, range 115-400 IU/day) combined with metformin (2 g/day for over 2 years) to be treated with additional rosiglitazone (8 mg once a day) for 8 months. The insulin treatment regimens varied from basal insulin alone (n=5) to use of multiple insulin injection therapy (n=9). The patients were instructed to decrease their insulin doses by 4-10 IU whenever

hypoglycemia (fasting plasma glucose < 4 mmol/l) occurred. Additional inclusion criteria were stable insulin dose for at least 2 years, and body weight and glycemic control for at least 6 months prior to participation. Exclusion criteria were clinical or echocardiographic evidence of heart failure, other cardiovascular disease, or any other significant disease that would make implementation of the study protocol impossible, treatment with drugs that may alter glucose metabolism (steroids, β -blockers and thiazide diuretics), abnormal serum creatinine, macroalbuminuria, and drug abuse. The inclusion and exclusion criteria were reviewed at a screening visit, where the patients underwent a history and physical examination, and blood samples were taken for measurement of the blood count, serum creatinine, electrolytes, fasting plasma glucose, HbA_{1c}, liver enzymes, and lipids. An ECG was recorded, and a urine sample was taken

to exclude patients with infections and macroalbuminuria. Transthoracic echocardiography was performed using Vivid 7 digital ultrasonography system (GE Vingmed Ultrasound, Horton, Norway). The left ventricular ejection fraction was calculated by M-mode echocardiography from the parasternal long-axis view. Measurements were made while the subject was lying in the left lateral recumbent position from three consecutive beats. The average of three beats was used for analysis. Subjects with left ventricular ejection fraction less than 50% were excluded.

Measurement and design: If a patient was considered eligible after the screening visit, metabolic studies (measurement of liver fat, insulin sensitivity of the glucose R_a and R_d, body composition, and substrate oxidation rates) were performed before and after 8 months of additional treatment with rosiglitazone.

5. METHODS

5.1. Hepatic insulin sensitivity (Studies I, III, and V)

In Studies I and III, two indwelling catheters were placed at 8 a.m. after an overnight fast in an antecubital vein and retrogradely in a heated hand vein for infusion of glucose, insulin, and [$3\text{-}^3\text{H}$]glucose and for sampling of arterialized venous blood. In order to determine R_a under basal and hyperinsulinemic conditions, [$3\text{-}^3\text{H}$]glucose was infused in a primed (20 μCi) continuous (0.2 $\mu\text{Ci}/\text{min}$) fashion for a total of 360 min (104, 355). Baseline blood samples were taken for measurement of fS-insulin and fP-glucose concentrations and for the biochemical measurements (S-ALT, S-ALT, fS-triglycerides, fS-HDL concentrations, fS-C-peptide, fS-adiponectin, and fS-FFA). After 120 min, insulin was infused in a primed continuous (0.3 mU/kg-min) fashion as previously described (355). Plasma glucose was maintained at 5 mmol/liter (90 mg/dl) until 360 min using a variable rate infusion of 20% glucose (104). Blood samples for measurement of glucose specific activity and FFA concentrations were taken at 90, 100, 110, and 120 min and at 15- to 30-min intervals between 120 and 360 min. Serum free insulin concentrations were measured at 0, 120, 150, 180, and at 60 min intervals between 240 and 360 min.

In Study V, patients were admitted to the hospital on the evening before the study. At 6 p.m., an indwelling 18-gauge catheter (Venflon, Viggo-Spectramed, Helsingborg, Sweden) equipped with an obturator was inserted in an antecubital vein. On this evening before the study, the patients did not take their bedtime

insulin injection. To determine glucose R_a and R_d , a primed continuous intravenous infusion of [$3\text{-}^3\text{H}$]glucose was started at 4 a.m. and continued for a total of 11 h. The priming dose of [$3\text{-}^3\text{H}$]glucose was adjusted according to the fasting blood glucose concentration measured at 4 a.m. as follows: priming dose = [glucose (mmol/l) at 4 a.m./5] \times 20 $\mu\text{Ci}/\text{min}$. This dose was infused intravenously over 10 min and was followed by a continuous-rate infusion of [$3\text{-}^3\text{H}$]glucose at a rate of 0.2 $\mu\text{Ci}/\text{min}$ as previously described (355). Before start of the insulin infusion, another catheter was inserted in a retrograde position in a heated dorsal hand vein for withdrawal of arterialized venous blood. Baseline blood samples were taken for measurement of fasting plasma glucose; glucose SA; HbA_{1c}; triglycerides; total, HDL, and LDL cholesterol; FFA; and serum free insulin concentrations. At 9 a.m., after a 300-min equilibrium period, a primed continuous (0.3 mU/kg-min) infusion of insulin (Actrapid Human, Novo Nordisk, Bagsvaerd, Denmark) was started (0 min), as previously described (104). Plasma glucose was adjusted to and then maintained at \sim 8 mmol/l (144 mg/dl) for 360 min. This was done using a variable-rate infusion of 20% glucose based on plasma glucose measurements, which were made from arterialized venous blood every 5–10 min. Blood samples for measurement of glucose SA were taken basally at -30, -20, and 0 min, and at 120, 180, 240, 280, 300, 330, and 360 min during the insulin infusion. Serum free insulin concentrations were measured every 60 min.

Glucose specific activity was determined as previously described (336). Glucose

R_a and R_d were calculated using the Steele equation, assuming a pool fraction of 0.65 for glucose and distribution volume of 200 ml/kg for glucose. Hepatic glucose R_a was calculated by subtracting the exogenous glucose infusion rate required to maintain euglycemia during hyperinsulinemia (120-360 min in Studies I and III; 0-360 min in Study V) from the rate of total glucose R_a . The percent suppression of basal hepatic glucose R_a by insulin was used as a measure of hepatic insulin sensitivity, i.e., the sensitivity of hepatic glucose production to insulin (% suppression of hepatic R_a).

5.2. Muscle insulin sensitivity (Study I)

The study was started at 8 a.m. after a 12-hour fast. Two 18-gauge catheters (Venflon; Viggo-Spectramed, Helsingborg, Sweden) were inserted, one in the left antecubital vein for infusions of insulin and glucose and another retrogradely in a ipsilateral heated dorsal hand vein for withdrawal of arterialized venous blood. Insulin (Actrapid Human; Novo Nordisk, Copenhagen, Denmark) was infused in a primed-continuous manner at a rate of 1 mU/kg·min for 120 minutes. Normoglycaemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements, which were performed every 5 minutes from arterialized venous blood. Blood samples for serum free insulin concentrations were withdrawn every 30 minutes during the 2-hour insulin infusion. The M-value (0-120 min) was calculated from the glucose infusion rate after correction for changes in the glucose pool size and expressed per fat free mass (FFM).

5.3. Insulin clearance (Studies III and V)

Insulin clearance measured directly using the low-dose (0.3 mU/kg·min)

insulin infusion clamp technique (see 5.1.). In Study III, insulin clearance was calculated by dividing the rate of insulin infusion (mU/kgFFM·min) by the steady-state serum insulin concentration measured between 150 and 360 min (203). Increment in serum insulin was calculated by subtracting fasting serum insulin concentration from the steady-state serum insulin concentration (150 to 360 min). In Study V, insulin clearance (ml/kgFFM·min) was calculated by dividing the rate of insulin infusion (mU/kgFFM·min) by the increment in serum insulin concentration (mean concentration measured during the insulin infusion minus fasting serum insulin).

5.4. Liver fat content

Histological determination (Study III). A liver biopsy was taken in 13 patients with suspected non-alcoholic steatosis under ultrasound control. Fat content of the liver biopsy specimens (% fat of surface area in light microscope) was analysed by an experienced liver pathologist in a blinded fashion.

¹H-MRS (Studies I, II, III, IV, and V). Localised single voxel (2×2×2 cm³) proton spectra were acquired using a 1.5-T whole-body system (Siemens Magnetom Vision, Erlangen, Germany), which consisted of a combination of whole-body and loop surface coils for radiofrequency transmitting and signal receiving, as previously described (355). T1-weighted high-resolution MRI scans were used for localisation of the voxel of interest within the right lobe of the liver. Magnetic resonance spectroscopy measurements of the liver fat were performed in the middle of the right lobe of the liver at a location that was individually determined for each subject; vascular structures and subcutaneous fat tissue were avoided when selecting the voxel. Subjects lay on their stomachs on

the surface coil, which was embedded in a mattress in order to minimise abdominal movement due to breathing. The single voxel spectra were recorded using the stimulated-echo acquisition mode sequence, with an echo time of 20 ms, a repetition time of 3000 ms, a mixing time of 30 ms, 1024 data points over 1000 kHz spectral width with 32 averages. Water-suppressed spectra with 128 averages were also recorded to detect weak lipid signals. A short echo time and long repetition time was chosen to ensure a fully relaxed water signal, which was used as an internal standard. Chemical shifts were measured relative to water at 4.80 ppm. The methylene signal, which represents intracellular triglyceride, was measured at 1.4 ppm. Signal intensities were quantified by using an analysis program, VAPRO-MRUI (<http://www.mrui.uab.es/mrui/>). Spectroscopic intracellular triglyceride content (liver fat) was expressed as a ratio of the area under the methylene peak to that under the methylene and water peaks ($\times 100 = \text{liver fat } \%$). This measurement has been validated against histologically determined lipid content (422) and against estimates of fatty degeneration or infiltration by X-ray computer-assisted tomography (355). All spectra were analysed by physicists who were unaware of any of the clinical data. The reproducibility of repeated measurements of liver fat in non-diabetic subjects studied on two separate occasions by the same reader in our laboratory is 11% (405).

5.5. IMCL (Study I)

Magnetic resonance images for localization and ^1H -MRS were acquired using a 1.5 T MR system (Magnetom Vision; Siemens, Erlangen, Germany) as previously described (440). A loop surface coil was used for detection. The subjects lay supine, with the left leg immobilized by firm padding. One-third

of the distance from the trochanter major of the femur to the middle of the patella was measured, and the center of the coil was placed in contact with that spot. The ^1H -spectra were obtained from quadriceps femoris (vastus lateralis) muscle. The volume of interest, voxel ($13 \times 13 \times 20 \text{ mm}^3$), was placed inside the lateral part of the vastus lateralis muscle to ensure parallel fiber orientation. The position of the voxel was chosen on the T_1 -weighted MR images so that it did not contain any visible fat or fasciae known to contain significant amounts of adipocytes (40), which would affect the resonance-frequency shift data. Spatial localization was achieved by using a stimulated echo acquisition mode applied with a repetition time of 3,000 ms, with an echo time of 20 ms, and a mixing time of 30 ms. We used 128 excitations with water presaturation. The resonance at 1.5 ppm originates from the extramyocellular CH_2 -protons of lipids (triglycerides and fatty acids) and the resonance at 1.3 ppm from intramyocellular CH_2 -protons of lipids (40, 368, 407). Resonance intensities were related to resonance at 3.0 ppm, which is derived from the CH_3 -protons of total creatine (creatine and phosphocreatine). IMCL was expressed as a ratio of the area under the intramyocellular methylene peak to that under the methylene and total creatine peaks ($\times 100 = \text{IMCL } \%$). The spectral data were fitted in time domain with the program MRUI, based on the solving of nonlinear least squares problems (368).

5.6. Measurements of body composition (Studies I, II, III, IV, and V)

Intra-abdominal and abdominal subcutaneous fat volumes. A series of 16 T_1 -weighted trans-axial scans were acquired from a region extending from 8 cm above to 8 cm below the 4th and 5th

lumbar interspace (16 slices, field of view 375×500 mm², slice thickness 10 mm, breath-hold repetition time 138.9 ms, echo time 4.1 ms). Intra-abdominal and subcutaneous fat areas were measured using an image analysis program (Alice 3.0, Parexel, Waltham, MA). A histogram of pixel intensity in the intra-abdominal region was displayed, and the intensity corresponding to the nadir between the lean and fat peaks was used as a cut-off point. Intra-abdominal adipose tissue was defined as the area of pixels in the intra-abdominal region above this cut-off point. For calculation of subcutaneous adipose tissue area, a region of interest was first manually drawn at the demarcation of subcutaneous adipose tissue and intra-abdominal adipose tissue, as previously described (355).

Whole body fat %. The % body fat was determined using bioelectrical impedance analysis (BioElectrical Impedance Analyzer System, model number BIA-101A, RJL Systems, Detroit, MI) (256).

Waist-to-hip –ratio. Waist circumference was measured midway between spina iliaca superior and the lower rib margin, and hip circumference at the level of the greater trochanters (270).

5.7. Maximal aerobic power (VO₂max) (Study I)

Maximal aerobic power was measured directly using an incremental work-conducted upright exercise test with an electrically braked cycle ergometer (Ergometer Ergoline 900ERG, Ergoline GmbH, Bitz, Germany) combined with continuous analysis of expiratory gases and minute ventilation (V_{max}229 series, SensorMedics, Yorba Linda, CA). Exercise was started at a work load of 50 watts. The work load was then increased by 50 watts every 3 min until perceived exhaustion or a respiratory quotient of

1.10 was reached. Maximal aerobic power was defined as the VO₂max during the last 30 sec of exercise.

5.8. Substrate oxidation rates (Study V)

Glucose and lipid oxidation rates were measured with indirect calorimetry using the Deltatrac Metabolic Monitor (Datex, Helsinki, Finland) as previously described (130, 476). The measurements were performed for 40 min during the basal period and the last hour of insulin clamp. Samples of inspired and expired air, which were suctioned at 40 l/min, were analyzed for O₂ and CO₂ concentration differences using paramagnetic O₂ and CO₂ analyzers, respectively. The hood was placed on the subject's head 10 min before the measurement was started. Urine was collected during the study, and the protein oxidation rate was estimated from urea nitrogen excretion (1 g nitrogen = 6.25 g protein). The following constants were used for calculation of glucose and lipid oxidation rates from gas exchange data: oxidation of 1 g of protein requires 966 ml of O₂ and produces 782 ml of CO₂, of 1 g of glucose requires 746 ml of O₂ and produces 746 ml of CO₂, and of 1 g of lipid requires 2,029 ml of O₂ and produces 1,430 ml of CO₂. Energy production rates (J/kgFFM·min) were calculated assuming oxidation of 1 mg carbohydrate produces 15.65 J; 1 mg lipid, 39.75 J; and 1 mg protein, 17.15 J (130).

5.9. Diagnosis of the metabolic syndrome (Studies I and II)

The metabolic syndrome was defined according to criteria of the International Diabetes Federation (9): central obesity (waist circumference ≥94 cm in men and ≥80 cm in women) and at least two of the following factors: 1) fS-triglycerides ≥1.70 mmol/l or specific treatment for

this lipid abnormality; 2) fS-HDL cholesterol <1.03 mmol/l in men and <1.29 mmol/l in women or specific treatment for this lipid abnormality; 3) systolic BP \geq 130 mmHg or diastolic BP \geq 85 mmHg or treatment for previously diagnosed hypertension; 4) fP-glucose \geq 5.6 mmol/l or previously diagnosed type 2 diabetes.

5.10. Analytical procedures

Plasma glucose concentrations were measured in duplicate with the glucose oxidase method using Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA) (Studies I, II, III, IV, and V) (205). Serum free insulin concentrations were measured with the Auto-DELFLIA kit (Wallac, Turku, Finland) (Studies I, II, III, IV, and V), and C-peptide concentrations by radioimmunoassay (233) (Studies I, II, III, and IV). FS-HDL cholesterol and fS-triglyceride concentrations were measured with the enzymatic kits from Roche Diagnostics using an autoanalyser (Roche Diagnostics Hitachi, Hitachi Ltd., Tokyo, Japan) (Studies I, II, III, IV, and V). The concentrations of LDL cholesterol were calculated using the Friedewald formula (144) (Studies II, IV, and V). S-ALT and S-AST activities were determined as recommended by the European Committee for Clinical Laboratory Standards (Studies I, II, III, IV, and V). Serum adiponectin concentrations were measured by an ELISA kit from B-Bridge International (San Jose, CA) (Study I), and serum FFA concentrations were measured using a fluorometric method (280) (Studies I, II, III, IV, and V). In order to determine glucose SA, plasma was deproteinized with Ba(OH)₂ and ZnSO₄ and evaporated as described (336) (Studies I, III, and V).

5.11. Statistical analyses (Studies I, II, III, IV, and V)

In all studies, a *p*-value of less than 0.05 was considered statistically significant. Calculations were made using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) (Studies I, II, III, IV, and V), SysStat Statistical Package (SysStat version 10; SysStat, Evanston, IL) (Studies I and II), and SPSS 11.0 (Studies I and II) or 14.0 (Studies III, IV, and V) for Windows (SPSS, Chicago, IL).

Study I. The subjects were divided into two equally-sized groups based on their median IMCL (9.0%) or liver fat content (3.0%). Non-normally distributed data were used after logarithmic (base 10) transformation. If distributed normally, data are shown as means \pm SEM, whereas non-normally distributed data are shown as median (25% percentile, 75% percentile). The unpaired Student's *t* test was used to compare mean values between groups. Analysis of covariance was used to adjust for body mass index, % body fat, liver fat (LFAT), and IMCL. Correlation analyses were performed using Spearman's nonparametric rank correlation coefficient. Multiple linear regression analysis was used to analyse independent determinants of liver fat content and muscle insulin sensitivity.

Study II. Non-normally distributed data were used after logarithmic transformation. If distributed normally, data are shown as means \pm SD, whereas non-normally distributed data are shown as median (25% percentile, 75% percentile). The unpaired Student's *t* test was used to compare mean values between men and women. Analysis of covariance was used to compare slopes and intercepts of regression lines for

men and women. If neither the slopes nor the intercepts differed between women and men, a common regression equation was calculated for all data. Analysis of covariance was used to adjust for age, gender and body mass index.

Study III. Non-normally distributed data were used after logarithmic transformation. The unpaired Student's *t* test was used to compare mean values between groups. Analysis of covariance was used to adjust for age, gender, and BMI. Correlation analyses were performed using Spearman's nonparametric rank correlation coefficient. Analysis of covariance was used to compare slopes of regression lines between liver fat and fS-insulin and fS-insulin adjusted for insulin clearance. If neither the slopes nor the intercepts differed between women and men, a common regression equation was calculated for all data. Multiple linear regression analyses were used to determine the independent determinants of the variation in fS-insulin concentrations. For calculation of the % variation in fasting insulin concentrations attributed to impaired insulin clearance due to liver fat, the difference between the % derived from the multiple regression analyses (insulin clearance and liver fat content) and the % derived from simple regression analyses of one of the variables (e.g. insulin clearance) was subtracted from

the % derived from simple regression analyses of the other variable (e.g. liver fat).

Study IV. Non-normally distributed data were used after logarithmic transformation. If distributed normally, data are shown as mean \pm SEM, while non-normally distributed data are shown as median followed by the interquartile range (25th and 75th percentiles). The unpaired Student's *t* test was used to compare mean values between the groups. Analysis of covariance was used to compare slopes and intercepts of regression lines for the non-diabetic subjects and the type 2 diabetic patients, and for comparison of liver fat in the type 2 diabetic patients treated with diet, metformin or with insulin-metformin combination therapy. If neither the slopes nor the intercepts differed between the groups, a common regression equation was calculated. Correlation analyses were performed using Spearman's nonparametric rank correlation coefficient. Analysis of covariance was used to adjust for intra-abdominal fat content.

Study V. The paired *t*-test was used to compare changes before and after additional rosiglitazone treatment. Logarithmic transformation was performed if necessary. Correlation analyses were performed using the Spearman nonparametric correlation coefficient.

6. RESULTS

6.1. Tissue specificity of insulin resistance (Study I)

Characteristics of the study groups. The high and the low LFAT groups were similar with respect to age. The high LFAT group was more obese than the low LFAT group, but the groups did not differ with respect to IMCL (Fig. 3). fS-insulin and liver enzymes were significantly higher and fS-adiponectin

significantly lower in the high than the low LFAT group (Table 6 and Fig. 3). The differences in fS-insulin ($p<0.0001$) and S-ALT ($p<0.0001$) and S-AST ($p=0.001$) remained significant after adjusting for BMI. FS-adiponectin concentrations did not differ significantly after adjusting for % body fat ($p=0.09$).

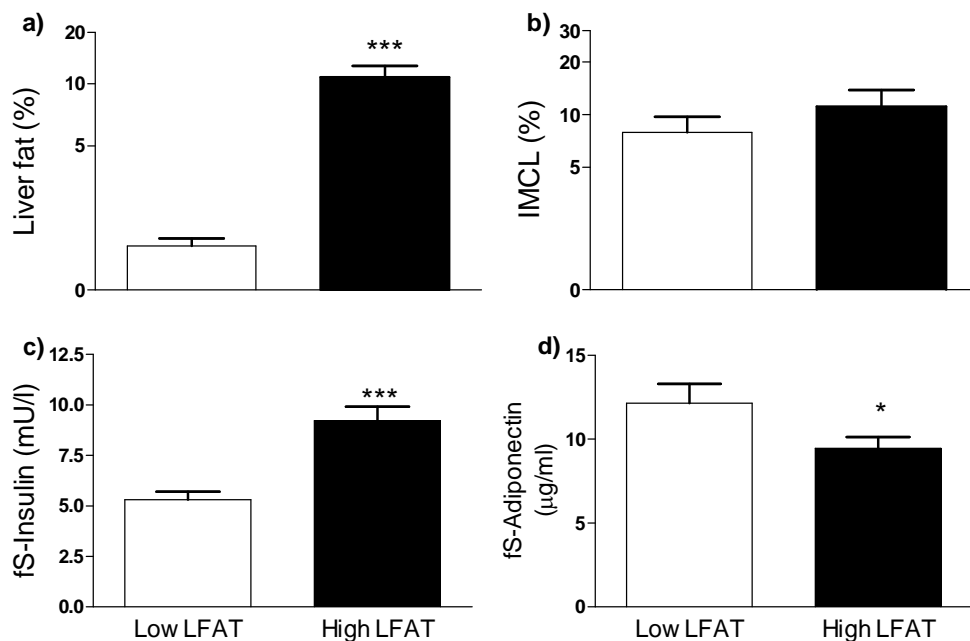


Figure 3. Bar graphs show LFAT (a), IMCL (b), fS-insulin (c), and fS-adiponectin (d) in the low and high liver fat groups. *, $p \leq 0.05$; ***, $p < 0.0001$ for the low vs. high LFAT groups.

Table 6. Subject characteristics in Study I.

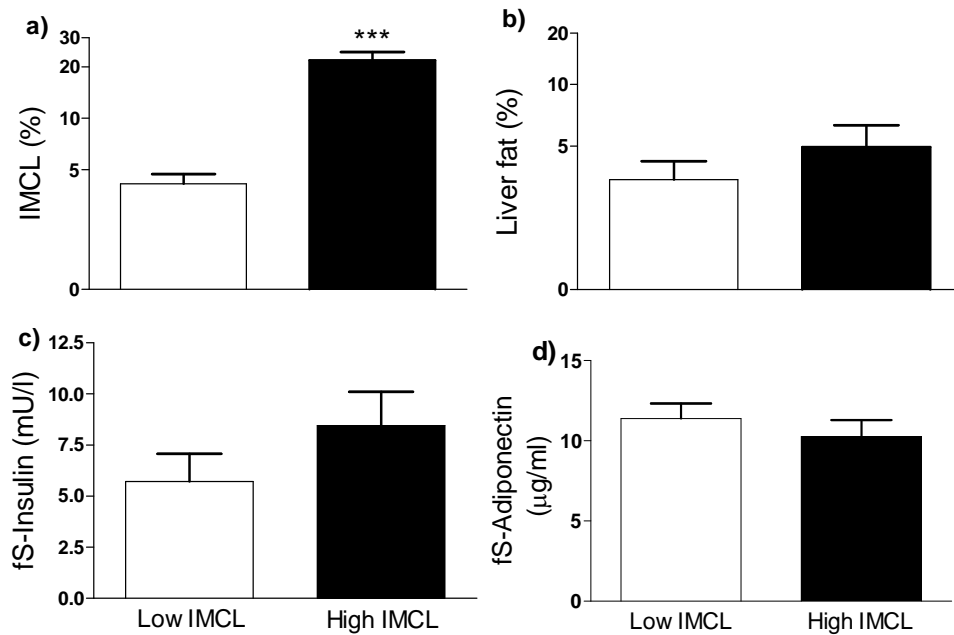
	Low LFAT	High LFAT	Low IMCL	High IMCL
N	23	22	23	22
Age (y)	43±2	41±2	42±2	43±2
Body composition				
BMI (kg/m ²)	24.7±0.6	27.7±0.9*	24.7±0.7	27.7±0.8**
Waist (cm)	91±2	99±2*	91±2	99±2**
% fat	18.6±1.0	22.6±1.0*	18.8±1.1	22.4±1.0*
IA fat (cm ³)	1100 (650-1320)	1630 (930-2430)*	1010 (680-1320)	1780 (1180-2600)**
SC fat (cm ³)	2080 (1470-2910)	2600 (1990-3370)	2000 (1370-2860)	2490 (2010-3110)
Liver fat (%)	2.0 (1.0-2.0)	12.0 (5.7-18.5)***	2.5 (2.0-6.0)	5.3 (1.7-17.5)
IMCL (%)	7.5 (3.0-16.2)	15.5 (5.0-23.5)	5.0 (2.3-7.5)	19.5 (16.0-26.0)***
Components of the MS				
fP-glucose (mmol/l)	5.5±0.09	5.7±0.09	5.5±0.09	5.6±0.12
S-triglycerides (mmol/l)	1.0±0.08	1.6±0.2**	1.12±0.09	1.50±0.22
S-HDL cholesterol (mmol/l)	1.48±0.07	1.26±0.06*	1.38±0.06	1.36±0.08
Systolic BP (mmHg)	130±3	133±3	128±3	136±4
Diastolic BP (mmHg)	85±2	87±3	83±2	89±2
Other parameters				
fS-insulin (mU/l)	5.3±0.4	9.2±0.7***	6.5±0.5	8.0±0.8
fS-adiponectin (µg/ml)	12.2±1.2	9.5±1.2*	11.4±0.9	10.3±1.0
fS-FFA (µmol/l)	556±39	647±45	551±33	643±49
S-ALT (U/l)	26±2	48±4***	31±3	43±5
S-AST (U/l)	26±2	35±2***	28±2	33±3
M-value (mg/kg FFM·min)	6.26±0.39	4.18±0.38***	5.73±0.43	4.73±0.43
VO ₂ max (ml/kg FFM·min)	45.2±1.2	41.1±1.4*	44.3±1.4	42.0±1.3

Data are shown as mean ± SEM or median (25% percentile, 75% percentile). *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ for low vs. high LFAT groups or low vs. high IMCL groups.

The low and high IMCL groups were similar with respect to age, but the high IMCL group was more obese than the low IMCL group (Table 6). LFAT was comparable between the groups, as were fS-insulin and fS-adiponectin con-

centrations (Fig. 4). BMI was similar between the low LFAT and low IMCL groups, and the high LFAT and high IMCL groups (Table 6). IMCL was not correlated with liver fat content ($r=0.16$, NS).

Figure 4. IMCL (a), liver fat (b), fS-insulin (c), and fS-adiponectin (d) in the low and high IMCL groups. ***, $p < 0.0001$ for the low vs. high IMCL groups.



Components of the metabolic syndrome. In the high LFAT group, S-triglycerides were higher and S-HDL cholesterol concentrations lower as compared to the low LFAT group (Table 6). The groups were comparable with respect to fP-glucose and systolic and diastolic blood pressure. These results remained unchanged if adjusted for IMCL. There was no significant difference in any component of the metabolic syndrome between the high and low IMCL groups

(Table 6) even after adjusting for liver fat content. In subjects with the metabolic syndrome (n=14), LFAT was significantly higher (10.5% [3.0-18.0%]) than in those without the syndrome (n=31, 2.0% [1.5-6.0%], $p=0.017$) even independent of BMI ($p < 0.05$) and IMCL ($p=0.003$), whereas IMCL was comparable between the subjects with and without the metabolic syndrome (Fig. 5) even if adjusted for liver fat.

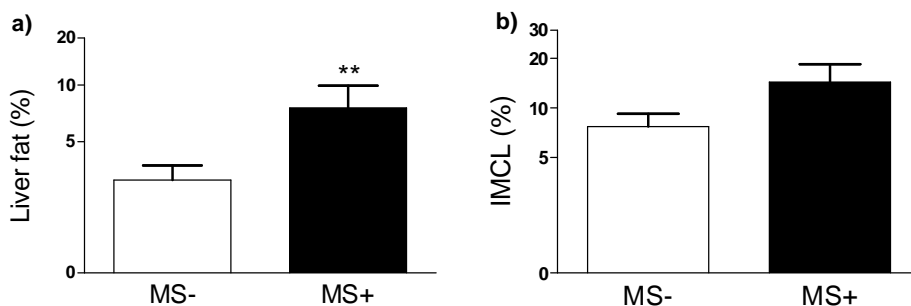


Figure 5. Liver fat (a) and IMCL (b) in subjects without and with the metabolic syndrome. **, $p < 0.01$.

Hepatic and muscle insulin sensitivity. Rates of basal hepatic glucose R_a were comparable between the high and the low LFAT groups (2.63 ± 0.13 and 2.41 ± 0.10 mg/kg FFM·min, respectively, NS). Serum insulin concentrations during the insulin infusion (150-360 min) averaged 19 ± 1 and 25 ± 1 mU/l in the high and low LFAT group ($p < 0.0001$). During the last hour of the insulin infusion, the % suppression of hepatic glucose production was inversely related to liver fat content ($r = -0.30$,

$p < 0.05$, Fig. 6), and averaged 79% (51-120%) vs. 97% (75-129%) (high vs. low LFAT group, $p < 0.05$). Muscle insulin sensitivity (M-value) was significantly lower in the high (4.18 ± 0.38 mg/kg FFM·min) than in the low (6.26 ± 0.39 mg/kg FFM·min, $p = 0.0008$) LFAT group. In multiple linear regression analysis, BMI ($p = 0.016$) but not VO_{2max} ($p = 0.19$) independently explained variation in LFAT, and together explained 21% of its variation ($p = 0.007$ for ANOVA).

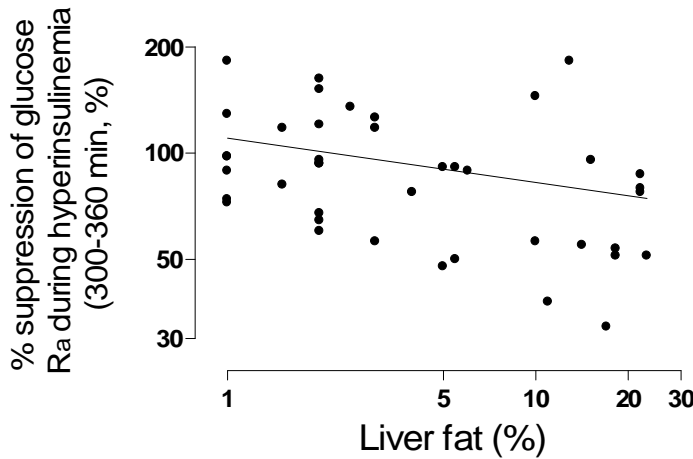


Figure 6. The relationship between % suppression of hepatic glucose production during the last hour of hyperinsulinemia (300-360 min) and liver fat content. $r = -0.30$, $p < 0.05$.

The low and high IMCL groups were comparable with respect to basal hepatic glucose R_a in the basal state (2.61 ± 0.12 vs. 2.54 ± 0.11 mg/kg FFM·min, respectively, NS). Serum insulin concentrations during the insulin infusion (23 ± 1 vs. 21 ± 1 mU/l, NS) and the % suppression (96% (64-145%) vs. 84% (56-109%), NS) did not differ significantly between the IMCL groups, nor did muscle insulin sensitivity (4.73 ± 0.43 vs. 5.73 ± 0.43 mg/kg FFM·min, high vs. low IMCL group, NS).

In simple linear regression analysis, muscle insulin sensitivity was inversely related to BMI ($r = -0.57$, $p < 0.0001$; $r = -0.50$, $p < 0.0001$ if adjusted for LFAT) and positively related to VO_{2max} ($r = 0.41$, $p = 0.005$; $r = -0.18$, NS if adjusted for LFAT, Fig. 7). In multiple linear regression analysis, BMI ($p < 0.0001$) and VO_{2max} ($p < 0.05$) were independent determinants of muscle insulin sensitivity and together explained 46.7% of its variation ($p < 0.0001$ for ANOVA).

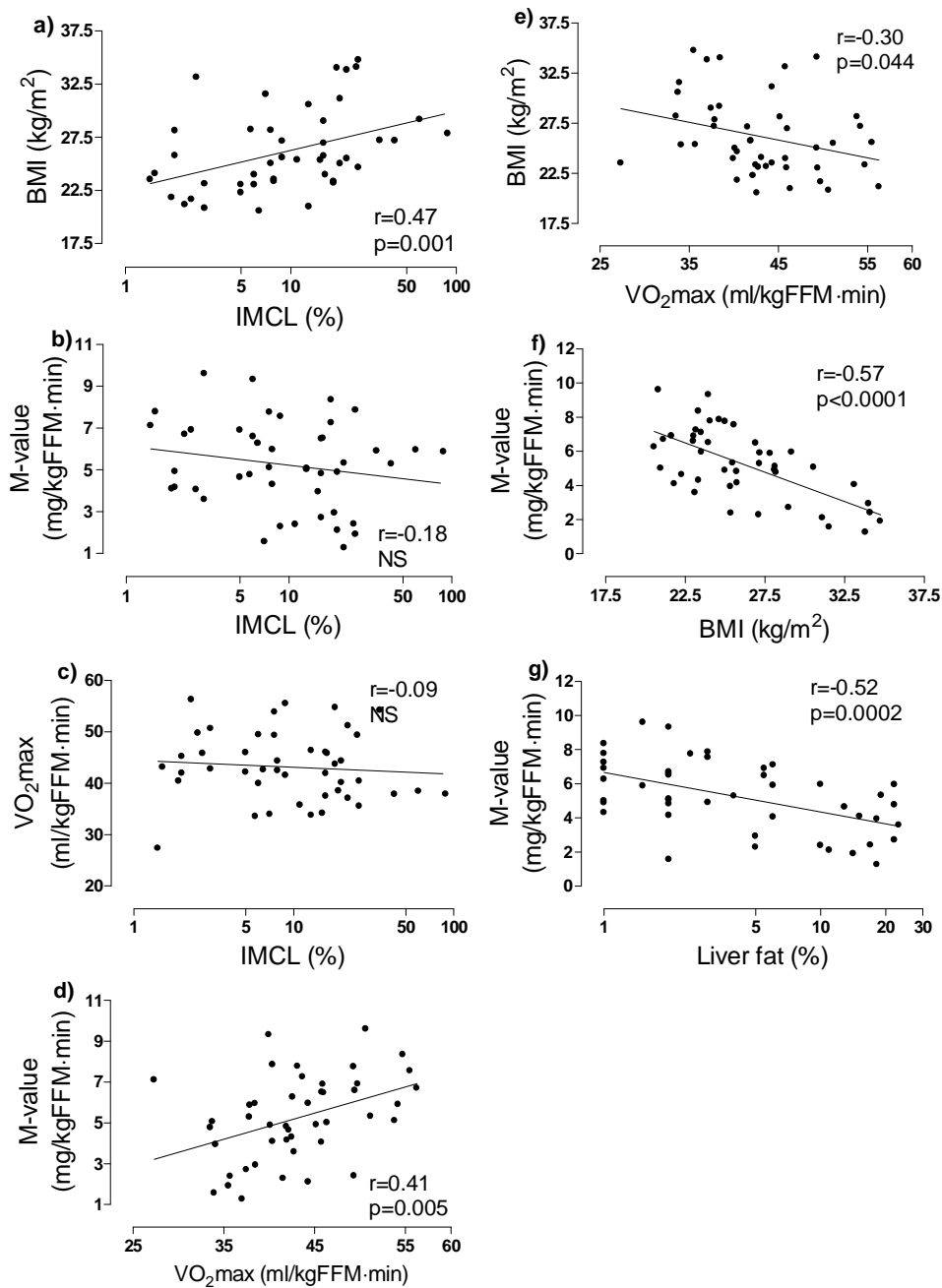


Figure 7. The relationships between (a) BMI and IMCL; (b) M-value and IMCL; (c) VO₂max and IMCL; (d) M-value and VO₂max; (e) BMI and VO₂max; (f) M-value and BMI; and (g) M-value and liver fat content.

If LFAT was incorporated into these analyses, BMI ($p < 0.0001$) and LFAT ($p = 0.035$) but not $VO_2\max$ ($p = 0.12$) were independent determinants of muscle insulin sensitivity ($r^2 = 52\%$, $p < 0.0001$ for ANOVA). IMCL correlated significantly with BMI ($r = 0.47$, $p = 0.001$, $r = 0.35$, $p = 0.018$ after adjusting for LFAT) but was not related to $VO_2\max$ ($r = -0.09$, NS; $r = -0.05$, NS adjusted for LFAT) or muscle insulin sensitivity ($r = -0.18$, NS; $r = -0.11$, NS adjusted for LFAT, Fig. 7).

S-FFA concentrations during prolonged low-dose insulin infusion. In the LFAT groups, fS-FFA concentrations were comparable in the basal state, but after

30 minutes of the 0.3 mU/kg-min insulin infusion, S-FFA concentrations were significantly higher in the high as compared to the low LFAT group ($459 \pm 30 \mu\text{mol/l}$ vs. $354 \pm 25 \mu\text{mol/l}$, respectively, $p = 0.009$). During the next 3.5 hours of insulin infusion, S-FFA remained significantly higher in the high than in the low LFAT group (Fig. 8). The difference in mean S-FFA remained significant after adjusting for age and BMI ($p = 0.03$). In the IMCL groups, fS-FFA concentrations were similar basally and during the two first hours of insulin infusion. During the last hour, S-FFA concentrations were significantly higher in the high IMCL group (Fig. 8), but after adjusting for BMI, these differences did not remain significant.

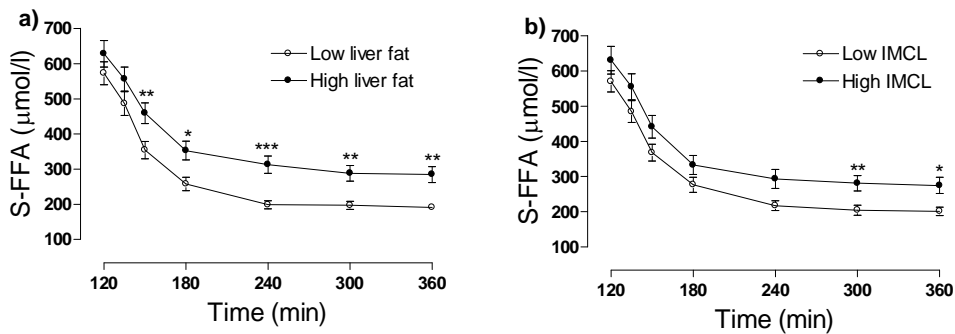


Figure 8. Serum FFA concentrations during the low-dose insulin infusion (120-360 min) in the high and low LFAT groups (a) and the high and low IMCL groups (b). The FFA concentrations were compared using analysis of variance for repeated measures followed the Tukey's HSD test for pairwise comparisons. *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

6.2. Liver fat in the metabolic syndrome (Study II)

Characteristics of the women and men (Table 7). The women and men were similar with respect to age and absolute body weight. Women had higher BMI,

waist circumference, waist-to-hip –ratio, % total body and subcutaneous fat volume than men, but intra-abdominal and liver fat were similar in both groups.

Table 7. Basic characteristics in Study II.

	Women	Men	p-value
n	162	109	
Age (y)	38±12	37±11	NS
Body composition			
Weight (kg)	86.0±6.4	86.1±6.7	NS
BMI (kg/m ²)	31.4±6.8	26.9±4.9	0.0001
Waist (cm)	101±16	96±14	0.02
Hip (cm)	109±11	100±9	0.0001
Waist-to-hip –ratio	0.91±0.08	0.96±0.08	0.0001
Whole body fat (%)	33.3±6.3	20.5±6.2	0.0001
Abdominal subcutaneous fat (cm ³)	5120±2100	2660±1300	0.0001
Intra-abdominal fat (cm ³)	1060 (560-1600)	1170 (660-1850)	NS
Liver fat (%)	4.0 (1.0-10.0)	3.0 (1.0-13.0)	NS
Lipids			
fS-triglycerides (mmol/l)	1.26 (0.87-1.71)	1.10 (0.9-1.71)	NS
fS-HDL cholesterol (mmol/l)	1.40 (1.17-1.71)	1.25 (1.10-1.57)	0.0063
fS-LDL cholesterol (mmol/l)	2.99±0.83	3.06±0.94	NS
fS-FFA (µmol/l)	682±195	593±215	0.001
Blood pressure			
Systolic BP (mmHg)	126±17	130±13	0.010
Diastolic BP (mmHg)	78±11	82±10	0.005
Other parameters			
fP-glucose (mmol/l)	5.4±0.6	5.5±0.4	NS
fS-insulin (mU/l)	7.6 (4.9-12.3)	7.0 (4.5-10.0)	NS
fS-C-peptide (nmol/l)	0.82 (0.6-1.0)	0.67 (0.4-1.0)	NS
HbA _{1c} (%)	5.5±0.6	5.5±0.4	NS
S-AST (U/l) [§]	25 (20-32)	30 (23-40)	0.0007
S-ALT (U/l) [†]	24 (18-40)	35 (21-57)	0.0001

Data are shown as mean ±SD or median (25% percentile, 75% percentile).

[§]Reference values: 15-35 U/l for women; 15-45 U/l for men

[†]Reference values: 10-45 U/l for women; 10-70 U/l for men

If adjusted for age and body mass index, women had significantly lower liver fat ($p=0.001$) and intra-abdominal fat content ($p=0.001$) than men. Despite greater obesity in women, both women and men had similar concentrations of fP-glucose, HbA_{1c}, fS-insulin, fS-C-peptide and fS-triglycerides. Blood pressure, S-ALT and S-AST concentrations were significantly higher in men compared with those in women.

Characteristics of subjects with and without the metabolic syndrome

Liver fat content. A total of 116 subjects (43%) met the criteria for the metabolic syndrome. Individuals with the metabolic syndrome had significantly higher liver fat content (median 8.2%; interquartile range 3.2%-18.7%) compared with those without the metabolic syndrome (2.0%; 1.0%-5.0%, $p<0.0001$). This difference remained significant after adjustment for age, gender, and BMI ($p=0.011$). In subjects with the metabolic syndrome, liver fat averaged 6.3% (2.0%-17.0%) in individuals with any two components of the metabolic syndrome, 8.2% (4.8%-22.0%) in individuals with any three components, and 15.5% (7.0%-23.2%) in individuals with all four components (p for trend <0.0001).

The regression lines representing the relationships between liver fat and components of the metabolic syndrome are depicted in Figure 9. The regression lines relating liver fat to components of the metabolic syndrome were not significantly different between subjects with and without the metabolic syndrome (data not shown). A total of 108 subjects (68 women, 40 men) had increased liver fat content ($>5.56\%$) (29), and 73 (68%) of them met criteria for the metabolic syndrome (52 women, 21 men). Inclusion or exclusion of the subjects using antihypertensives or lipid lowering drugs (a total of 33 subjects) did not alter the results (data not shown).

Body composition. Figure 10 depicts the relationships between liver fat and BMI, whole body fat %, and intra-abdominal fat measured by MRI. Women had less liver fat for the same BMI and whole body fat % than men, while the relationship between liver fat and intra-abdominal fat were similar in both women and men. Liver fat content was better correlated with intra-abdominal than abdominal subcutaneous fat in both women and men. Both intra-abdominal and subcutaneous fat volumes were significantly higher in subjects with than without the metabolic syndrome independent of age, gender, and BMI.

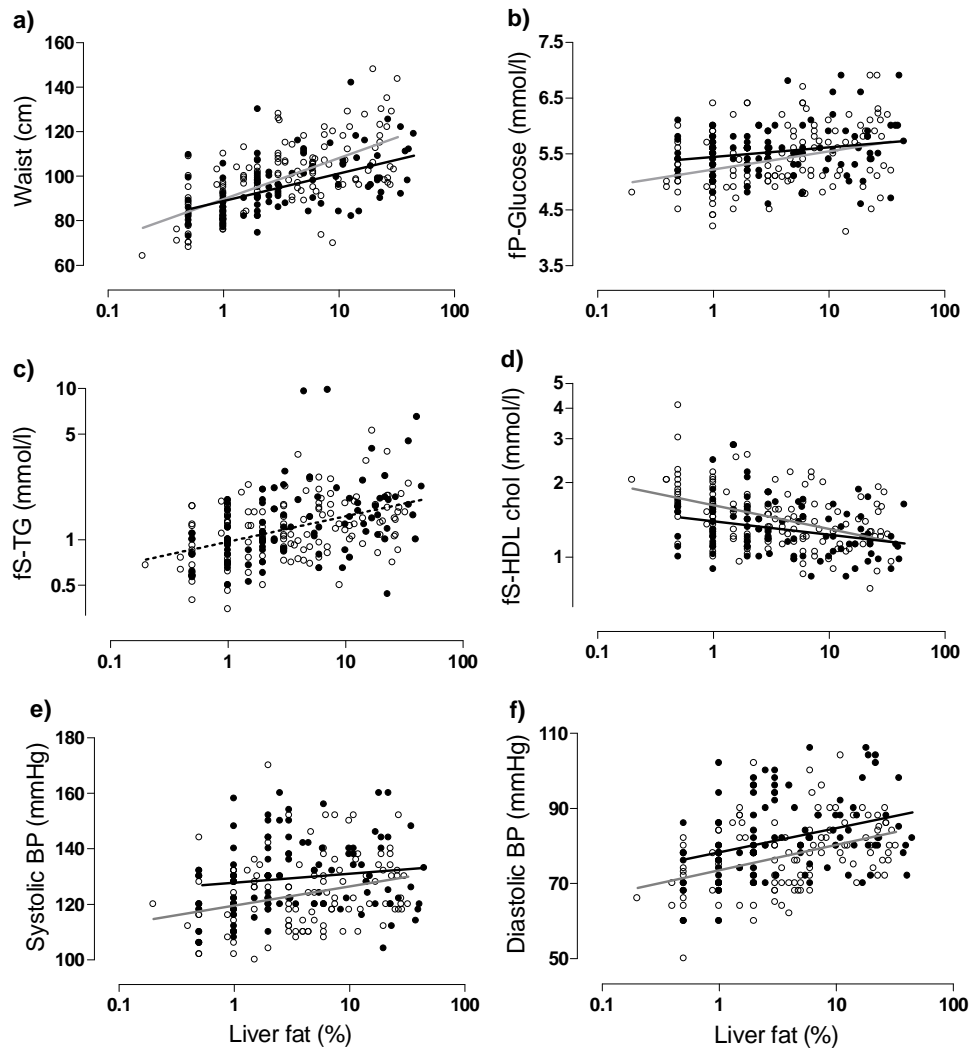
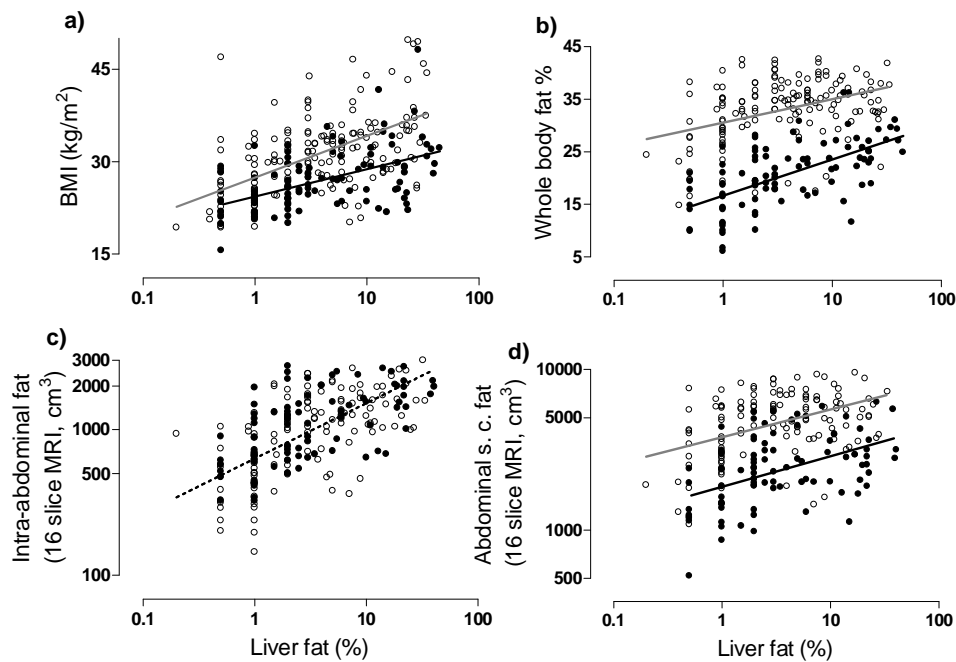


Figure 9. Relationships between liver fat and components of the metabolic syndrome. Liver fat is associated with (a) waist circumference [$r=0.59$, $p<0.0001$ ($r=0.12$, NS) for women; $r=0.56$, $p<0.0001$ ($r=0.26$, $p=0.008$) for men], (b) fP-glucose [$r=0.32$, $p<0.0002$ ($r=0.18$, $p=0.041$) for women; $r=0.22$, $p=0.026$ ($r=0.053$, NS) for men], (c) fS-triglycerides [$r=0.40$, $p<0.0001$ ($r=0.14$, NS) for women; $r=0.44$, $p<0.0001$ ($r=0.24$, $p=0.016$) for men; $r=0.42$, $p<0.0001$ ($r=0.19$, $p=0.003$) for both women and men], (d) fS-HDL cholesterol [$r=-0.44$, $p<0.0001$ ($r=-0.24$, $p=0.005$) for women; $r=-0.31$, $p=0.002$ ($r=-0.22$, $p=0.029$) for men], (e) systolic BP [$r=0.30$, $p=0.0004$ ($r=0.08$, NS) for women; $r=0.14$, NS ($r=-0.03$, NS) for men] and (f) diastolic BP [$r=0.42$, $p<0.0001$ ($r=0.20$, $p=0.020$) for women; $r=0.31$, $p=0.0006$ ($r=0.15$, NS) for men]. Correlations coefficients and their significances adjusted for age and BMI are given in parentheses. Subjects who were receiving medications for hypertension ($n=20$), dyslipidemia ($n=6$), or both hypertension and dyslipidemia ($n=7$) were excluded from these analyses. Open circles (\circ) and grey lines denote women ($n=134$), filled circles (\bullet), and black lines denote men ($n=104$) and dashed line denotes both women and men.

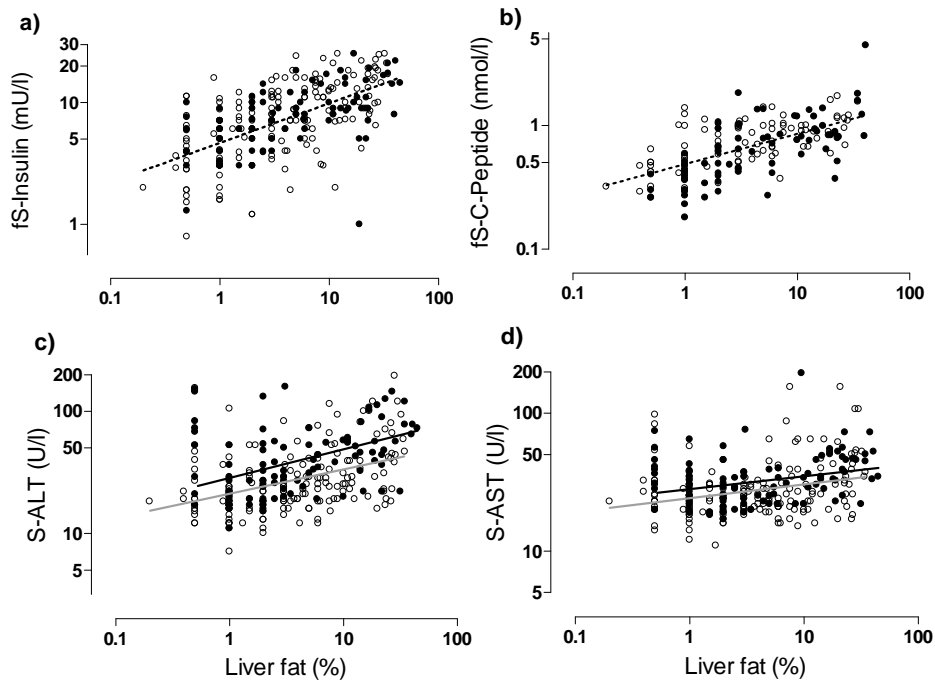
Figure 10. The relationships between liver fat and body composition features. Liver fat is associated with (a) body mass index [$r=0.54$, $p<0.0001$ for women ($n=161$); $r=0.52$, $p<0.0001$ for men ($n=107$)], (b) % body fat [$r=0.39$, $p<0.0001$ for women ($n=148$); $r=0.62$, $p<0.0001$ for men ($n=97$)], (c) intra-abdominal fat [$r=0.64$, $p<0.0001$ for both women ($n=128$) and men ($n=93$)], and (d) abdominal subcutaneous fat [$r=0.43$, $p<0.0001$ for women ($n=128$); $r=0.45$, $p<0.0001$ for men ($n=93$)]. Symbols as in Figure 9.



Other features. As shown in Table 8, after adjustment for age, gender and BMI, subjects with the metabolic syndrome differed from those without the syndrome with respect to all components of the syndrome. Subjects with the metabolic syndrome also had significantly higher fasting serum insulin and C-peptide concentrations than those without the syndrome independent of age, gender, and BMI. Indeed, of all parameters analysed, fasting serum

insulin and C-peptide concentrations were the strongest correlates of liver fat content ($r=0.61$; $p<0.0001$ and $r=0.62$; $p<0.0001$, respectively). After adjustment for age, gender, and BMI, these associations remained extremely significant (fS-insulin: $r=0.39$, $p<0.0001$; fS-C-peptide: $r=0.36$; $p<0.0001$). The relationships could be characterized by a single regression line since neither the slopes nor the intercepts differed between men and women (Fig. 11).

Figure 11. The relationships between liver fat and fS-Insulin, fS-C-peptide and liver enzymes. Liver fat is associated with (a) fasting serum insulin [$r=0.61$, $p<0.0001$ ($r=0.39$, $p<0.0001$) for both men ($n=89$) and women ($n=162$)] and (b) fS-C-peptide [$r=0.62$, $p<0.0001$ ($r=0.36$, $p<0.0001$) for both men ($n=76$) and women ($n=109$)] (c) S-ALT [$r=0.39$, $p<0.0001$ for women ($n=162$); $r=0.44$, $p<0.0001$ for men ($n=109$)], and (d) S-AST [$r=0.27$, $p=0.0005$ for women ($n=162$); $r=0.31$, $p=0.0012$ for men ($n=109$)] concentrations. Correlations coefficients and their significances adjusted for age and BMI are given in parentheses. Symbols as in Figure 9.



The relationship between liver fat content and liver enzymes. S-ALT and S-AST concentrations were positively correlated with liver fat in both women and men (Fig. 11). There was no difference between the slopes of the regression lines relating liver fat to ALT and AST in the two groups, but the y-intercepts were significantly lower in women than in men. This difference averaged 7.3 ± 1.1 U/l for S-ALT and 3.9 ± 1.0 U/l for S-AST. After adjusting for age and BMI, liver fat retained its associations with both S-ALT ($r=0.32$, $p<0.0001$ for women; $r=0.38$, $p<0.0001$ for men) and S-AST ($r=0.31$, $p<0.0001$

for women; $r=0.28$, $p=0.003$ for men) concentrations.

S-ALT concentrations were elevated in 30 women (19%) and 21 men (19%), in whom liver fat averaged $14\pm 12\%$ and $18\pm 15\%$, respectively (NS). Among the individuals with elevated S-ALT, 23 (46%) met the criteria for the metabolic syndrome (13 women, 10 men). S-AST concentrations appeared to be elevated in 34 women (21%) and 23 men (21%), in whom liver fat averaged $14\pm 11\%$ and $16\pm 13\%$, respectively (NS). Among the individuals with elevated S-AST, 26 subjects (39%) were diagnosed with the syndrome (18 women, 9 men).

Table 8. Characteristics of subjects with and without the metabolic syndrome in Study II.

	Metabolic syndrome -	Metabolic syndrome +	Unadjusted p-value	p-value adjusted for age, gender and BMI
N	155	116		
Age (y)	35±11	42±12	0.0001	
Gender (women)	82 (53%)	80 (69%)	0.008	
Body composition				
Weight (kg)	77.3 (69.7-87.0)	93.0 (84.0-104)	0.0001	NS
BMI (kg/m ²)	25.5 (23.1-29.9)	32.3 (29.5-35.0)	0.0001	
Waist (cm)	92 ±13	108±13	0.0001	0.0001
Hip (cm)	102±11	111±10	0.0001	NS
Waist-to-hip –ratio	0.91±0.08	0.97±0.08	0.0001	NS
Whole body fat (%)	24.4 (17.9-33.0)	34.7 (27.1-37.1)	0.0001	NS
Intra-abdominal fat (cm ³)	736 (491-1250)	1580 (1190-2160)	0.0001	0.011
Liver fat (%)	2.0 (1.0-5.0)	8.2 (3.2-18.7)	0.0001	0.011
Lipids				
fS-triglycerides (mmol/l)	1.00 (0.74-1.26)	1.72 (1.30-2.23)	0.0001	0.0001
fS-HDL cholesterol (mmol/l)	1.48 (1.25-1.74)	1.19 (1.04-1.44)	0.0001	0.0001
fS-LDL cholesterol (mmol/l)	2.86±0.92	3.09±0.81	0.0001	0.0001
fS-FFA (µmol/l)	614±205	686±209	0.02	NS
Blood pressure				
Systolic BP (mmHg)	123±13	133±13	0.0001	0.0001
Diastolic BP (mmHg)	77±10	83±9	0.0001	0.025
Other parameters				
fP-glucose (mmol/l)	5.2±0.4	5.8±0.5	0.0001	0.004
fS-insulin (mU/l)	6.0 (3.3-9.0)	11 (7.0-15.9)	0.0001	0.0001
fS-C-peptide (nmol/l)	0.58 (0.4-0.9)	0.94 (0.7-1.1)	0.0001	0.0001
HbA _{1c} (%)	5.4±0.4	5.6±0.4	0.0001	NS
S-ALT (U/l)	23 (18-41)	32 (22-58)	0.0006	NS
S-AST (U/l)	27 (21-36)	28 (22-58)	NS	NS

Data are shown as mean ± SD or median (25% percentile, 75% percentile). Abdominal subcutaneous and intra-abdominal fat volumes were determined in 98 subjects without and 83 subjects with the metabolic syndrome. The % whole body fat was measured in 99 subjects without and 85 subjects with the metabolic syndrome.

6.3. Effect of liver fat on insulin clearance (Study III)

Histologically determined vs. spectroscopic liver fat (Fig. 12). The relationship between the % liver fat determined by proton spectroscopy (calculated from the areas under the water and methylene peaks) and the % fat in the liver biopsy (% of biopsy surface area consisting of fat) is shown in Fig. 12. The units of liver fat determined by these approaches differ (by definition), but histological liver fat can be calculated based on spectroscopy from the following equation:

$$\text{Liver fat by histology (\%)} = 3.5 \pm 10.1 + 2.2 \pm 0.5 \times \text{Liver fat by spectroscopy (\%)}$$

Subject characteristics (Table 9). Liver fat averaged 2% and 15% in the low and high LFAT groups, respectively. The low and high LFAT groups were similar with respect to age, systolic and diastolic blood pressure, and fS-FFA concentrations. The high LFAT group was more obese and had higher fP-glucose and S-triglyceride, and lower S-HDL cholesterol concentrations than the low LFAT group. FS-insulin, fS-C-peptide, and serum liver enzyme concentrations were higher in the high as compared to the low LFAT group independent of age, gender, and BMI.

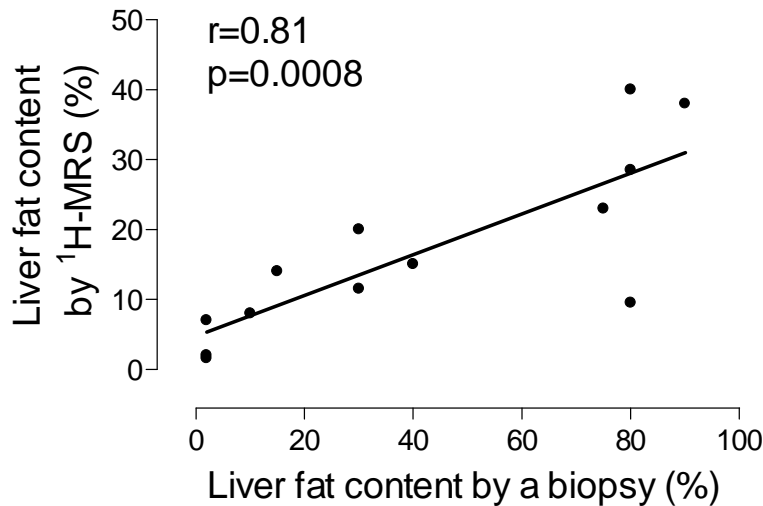


Figure 12. The relationship between the percent liver fat determined by histology and ¹H-MRS.

Table 9. Subject characteristics in Study III.

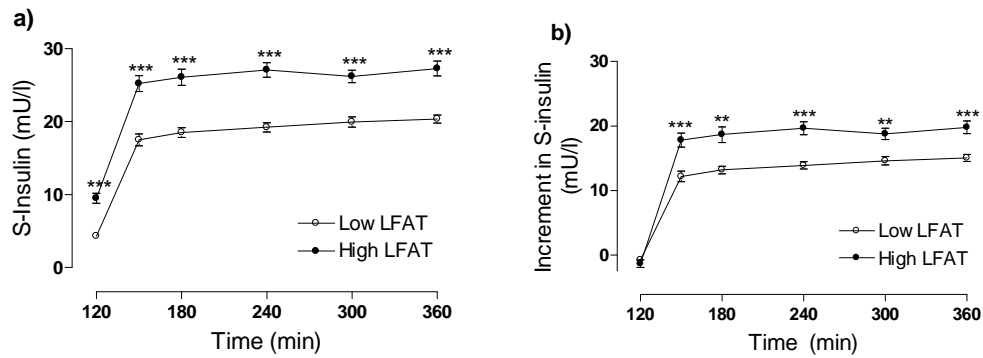
	Low LFAT	High LFAT	Unadjusted p-value	p-value adjusted for BMI
N (women)	39 (10)	41 (13)		
Liver fat (%)	1.8±0.2	15.0±1.5	0.0001	0.0001
Age (y)	44±1	43±2	NS	
Body composition				
BMI (kg/m ²)	24.9±0.5	28.4±0.6	0.0001	
Waist (cm)	89±2	99±2	0.0001	NS
% fat	21.9±1.2	27.0±1.1	0.003	NS
Components of the MS				
fP-glucose (mmol/l)	5.5±0.1	5.8±0.1	0.005	NS
fS-triglycerides (mmol/l)	1.1±0.1	1.9±0.3	0.003	NS
fS-HDL cholesterol (mmol/l)	1.47±0.06	1.24±0.05	0.001	NS
Systolic BP (mmHg)	128±2	132±2	NS	NS
Diastolic BP (mmHg)	82±2	83±2	NS	NS
Other parameters				
fS-insulin (mU/l)	5.1±0.3	11.0±0.8	0.0001	0.0001
fS-C-peptide (nmol/l)	0.56±0.05	0.97±0.10	0.0001	0.017
fS-C-peptide/fS-insulin -ratio	20.9±2.9	17.7±2.4	NS (0.07)	NS (0.10)
fS-FFA (µmol/l)	610±33	660±37	NS	NS
S-ALT (U/l)	27±3	73±13	0.0001	0.0001
S-AST (U/l)	27±1	45±5	0.0001	0.005

Data are shown as mean ± SEM.

Serum insulin concentrations during the prolonged low-dose insulin infusion. Serum insulin concentrations during the insulin infusion were approximately 38% higher in the high as compared to the low LFAT group (Fig. 13). The difference could not be fully explained by significantly higher basal serum insulin concentrations in the high LFAT

group, as the increments in serum insulin concentrations above basal were also significantly and approximately 36% higher in the high (18.8±0.9 mU/l) as compared to the low (13.8±0.51 mU/l, p<0.0001, Fig. 13) LFAT group independent of age, gender, and BMI (p=0.006).

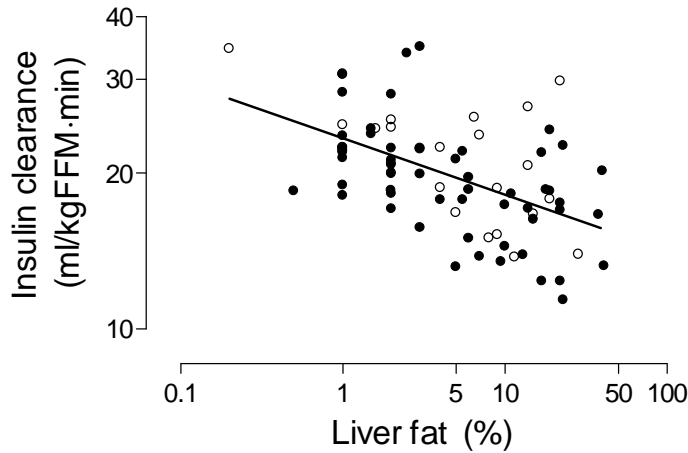
Figure 13. Serum insulin concentrations (a) and their increments (b) before (120 min) and during the 240 min of insulin infusion. **, $p < 0.01$; ***, $p < 0.001$ for the high vs. low LFAT groups.



Insulin clearance. Insulin clearance was significantly impaired in the high as compared to the low LFAT group (18.0 ± 0.67 vs. 22.9 ± 0.80 ml/kg FFM·min, $p < 0.0001$). This difference remained significant after adjusting for age, gender and BMI ($p = 0.001$), and even if groups with high and low LFAT carefully matched for age, gender, and BMI were compared (data not shown). Liver fat content was inversely related to the rate of insulin clearance ($r = -0.52$, $p < 0.0001$, Fig. 14) independent of age, gender and BMI ($r = -0.37$, $p = 0.001$ after adjusting). Additional adjustment for S-FFA concentrations during the insulin infusion, which were higher in the high as compared to the low LFAT group (402 ± 19 vs. 297 ± 12 $\mu\text{mol/l}$, $p < 0.0001$),

had no effect on this association ($r = -0.34$, $p = 0.002$). Liver fat content did not correlate with fS-FFA ($r = 0.11$, NS), but was significantly correlated with S-FFA concentrations during the insulin infusion ($r = 0.55$, $p < 0.0001$) independent of age, gender, and BMI ($r = 0.37$, $p = 0.001$). Insulin clearance did not correlate with S-FFA in the basal state ($r = -0.07$, NS) or during the insulin infusion ($r = -0.18$, NS). Both BMI ($r = -0.25$, $p = 0.025$) and waist ($r = -0.30$, $p = 0.01$) were significantly inversely correlated with insulin clearance, but these relationships became non-significant if adjusted for liver fat. Whole body fat % was not related to insulin clearance ($r = -0.07$, NS).

Figure 14. The relationship between liver fat content and insulin clearance. Open circles denote women ($r=-0.58$, $p=0.004$), and closed circles denote men ($r=-0.51$, $p<0.0001$).

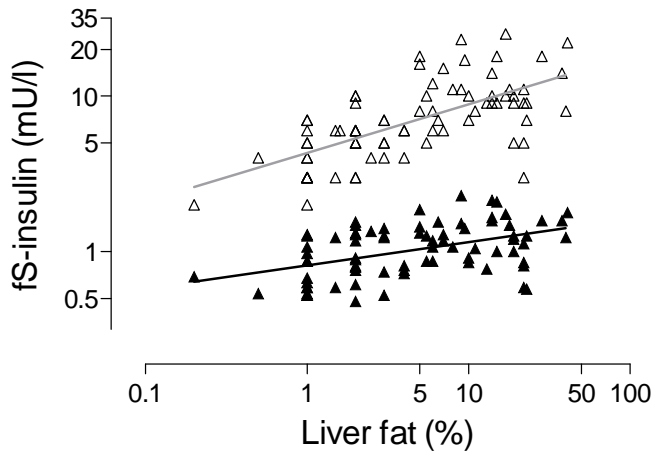


Determinants of fS-insulin concentrations. Liver fat content correlated significantly with fS-insulin concentrations ($r=0.62$, $p<0.0001$) independent of age, gender, and obesity ($r=0.34$, $p=0.002$). In simple linear regression analyses, liver fat content and insulin clearance explained 38% and 42% of the variation in fS-insulin concentrations, respectively. To determine the extent to which liver fat content contributed to the variation of fS-insulin concentrations due to impaired insulin clearance, multiple linear regression analyses were employed. Both liver fat ($p<0.0001$) and insulin clearance

($p<0.0001$) were independent determinants of fS-insulin concentrations and together explained 53% of their variation. Thus, on the average 27% of the variation in fS-insulin concentrations were attributed to impaired insulin clearance due to liver fat content.

After adjusting for the rates of insulin clearance, liver fat content correlated significantly with fS-insulin ($r=0.43$, $p<0.0001$) and explained 22% of its variation (Fig. 15). The slopes of the regression lines were significantly different ($p=0.002$).

Figure 15. The relationships between liver fat and fS-insulin concentrations (open triangles and the grey line, $r=0.62$, $p<0.0001$), and between liver fat and fS-insulin concentrations adjusted for insulin clearance (closed triangles and the black line, $r=0.43$, $p<0.0001$). The slopes of the regression lines differed significantly ($p=0.002$), implying that the contribution of impaired insulin clearance to fasting hyperinsulinemia increased as a function of liver fat.



Hepatic insulin resistance. Rates of hepatic glucose production did not differ between the groups in the basal state (3.27 ± 0.14 vs. 2.96 ± 0.17 mg/kg FFM·min, low vs. high LFAT, NS). During the insulin infusion, the rate of hepatic glucose production was higher in the high (0.63 ± 0.23 mg/kg FFM·min) as compared to the low (0.04 ± 0.20 mg/kg FFM·min, $p=0.008$) LFAT group. The % suppression of hepatic glucose production during the last hour of insulin infusion was significantly correlated with liver fat content ($r=-0.40$, $p=0.0002$, Fig. 16) but was unrelated to insulin clearance ($r=0.19$, NS). In multiple

linear regression analysis, both hepatic insulin resistance ($p<0.0001$) and insulin clearance ($p<0.0001$) were independent determinants of fS-insulin concentrations and together explained 52% of their variation ($p<0.0001$ for ANOVA). If liver fat content was incorporated into these analyses as an independent variable, hepatic insulin resistance (<0.0001), insulin clearance ($p<0.0001$), and liver fat content ($p<0.0001$) were independent determinants of the fS-insulin concentration ($r^2=59\%$, $p<0.0001$ for ANOVA). Additional inclusion of fS-C-peptide did not abolish these significances (data not shown).

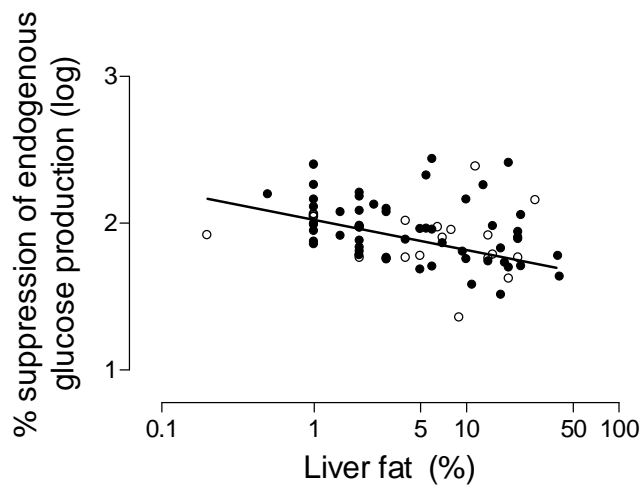


Figure 16. The relationship between % suppression of hepatic glucose production and liver fat content. $r=-0.40$, $p=0.0002$. Symbols as in Figure 14.

6.4. Liver fat in type 2 diabetes (Study IV)

Subject characteristics (Table 10). Non-diabetic subjects and type 2 diabetic patients were equally obese and similar with respect to age and gender. The type 2 diabetic patients had higher fS-insulin, fS-triglyceride, and fS-FFA concentrations, and lower fS-HDL cholesterol concentrations as compared to the non-

diabetic subjects. The type 2 diabetic patients had approximately 16% more intra-abdominal and approximately 80% more liver fat than the non-diabetic subjects. The difference in liver fat remained significant even after adjusting for intra-abdominal fat content ($p < 0.05$). The type 2 diabetic patients treated with

Table 10. Subject characteristics in Study IV.

	Non-diabetic subjects	Type 2 diabetic patients	p-value
N (women)	70 (33)	70 (33)	
Age (yrs)	48±1	49±1	NS
Body composition			
BMI (kg/m ²)	31.2±0.6	31.6±0.6	NS
Waist (cm)	105±1	107±1	NS
% fat	30±1	31±1	NS
SC fat (cm ³)	4600±270	4400±230	NS
IA fat (cm ³)	1900 (1300-2500)	2200 (1600-3500)	0.02
Liver fat (%)	7.3 (3.0-17.0)	13.0 (6.0-20.5)	0.005
Glycemic parameters			
fP-glucose (mmol/l)	5.6±0.1	9.3±0.3	<0.0001
HbA _{1c} (%)	5.6±0.1	7.6±0.2	<0.0001
fS-insulin (mU/l)	8.4 (6.0-12.7)	12.0 (8.0-18.0)	0.004
Serum lipids			
fS-triglycerides (mmol/l)	1.90±0.17	2.55±0.23	0.007
fS-HDL cholesterol (mmol/l)	1.36±0.04	1.17±0.04	0.0003
fS-LDL cholesterol (mmol/l)	3.18±0.11	2.75±0.11	0.0007
fS-FFA (µmol/l)	650±30	780±30	0.003
Blood pressure			
Systolic BP (mmHg)	136±2	135±2	NS
Diastolic BP (mmHg)	85±1	83±1	NS
Liver enzymes			
S-ALT (U/l)	31 (23-62)	38 (28-58)	NS
S-AST (U/l)	29 (22-44)	29 (24-48)	NS
AST/ALT	0.83 (0.59-1.04)	0.85 (0.68-1.05)	NS

Data are shown as mean ± SEM or, for non-normally distributed data, as median followed by the 25th and 75th percentiles.

diet, metformin (MET), or the combination of insulin and metformin (INS+MET) differed slightly with respect to age (45 ± 2 , 55 ± 2 , 50 ± 2 years, diet, MET, INS+MET, $p=0.003$ for diet vs. MET), whereas BMI (32.0 ± 0.7 , 30.2 ± 1.4 , 32.2 ± 1.0 kg/m², NS), liver fat content (15.5% (7.0-21.0%), 9.0% (4.2-19.5%), 14.0% (7.0-21.3%), NS), and S-ALT concentrations (42 (29-69), 31 (21-46), 38 (26-52) U/l, NS) were similar between the groups.

Relationships between body composition and liver fat content. Liver fat content was significantly correlated with BMI and waist circumference in the type 2 diabetic patients and in the non-diabetic subjects (Fig. 17). At any given BMI or waist circumference, the type 2 diabetic patients had significantly more liver fat than the non-diabetic subjects. The type

of antihyperglycemic therapy had no impact on these relationships (data not shown). At a BMI of 25 kg/m², the type 2 diabetic patients had approximately 40% more liver fat than the non-diabetic subjects. This difference rose gradually as a function of BMI. For example, at a BMI of 40 kg/m², the type 2 diabetic patients had 80% more liver fat than the non-diabetic subjects. A similar phenomenon was observed when waist was plotted against liver fat. For example, at waist circumferences of 85 and 140 cm, liver fat content was 50% and 90% higher in the type 2 diabetic patients than in the non-diabetic subjects, respectively. Intra-abdominal fat was similarly related to liver fat in both non-diabetic subjects and type 2 diabetic patients ($r=0.45$, $p<0.0001$). The volume of subcutaneous fat did not correlate with liver fat content in either group.

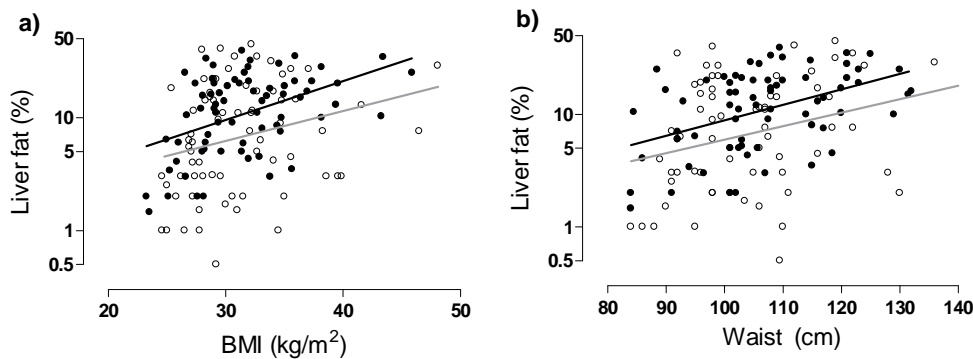


Figure 17. The relationships between liver fat and body composition. Liver fat content correlates with (a) BMI [$r=0.45$, $p<0.0001$ for type 2 diabetic patients (regression equation: $LFAT\% = 10^{[-0.0524\pm 0.27 + 0.034\pm 0.01 * BMI]}$); $r=0.26$, $p=0.029$ for non-diabetic subjects ($LFAT\% = 10^{[0.003\pm 0.37 + 0.0264\pm 0.01 * BMI]}$)]; and (b) waist circumference [$r=0.45$, $p=0.0001$ for type 2 diabetic patients ($LFAT\% = 10^{[-0.42\pm 0.36 + 0.014\pm 0.003 * Waist]}$); $r=0.29$, $p=0.017$ for non-diabetic subjects ($LFAT\% = 10^{[-0.42\pm 0.52 + 0.012\pm 0.005 * Waist]}$)]. Open circles and grey lines denote non-diabetic subjects, and filled circles and black lines denote type 2 diabetic patients.

Relationships between insulin, glycaemia, lipids, and liver fat content. Liver fat content was similarly related to fS-insulin ($r=0.55$, $p<0.0001$), C-peptide ($r=0.40$, $p<0.0001$), HbA_{1c} ($r=0.34$, $p<0.0001$), fP-glucose ($r=0.29$,

$p=0.0006$), fS-triglyceride ($r=0.36$, $p<0.0001$) and fS-HDL cholesterol ($r=-0.31$, $p=0.0002$) concentrations in the non-diabetic subjects and the type 2 diabetic patients (Fig. 18).

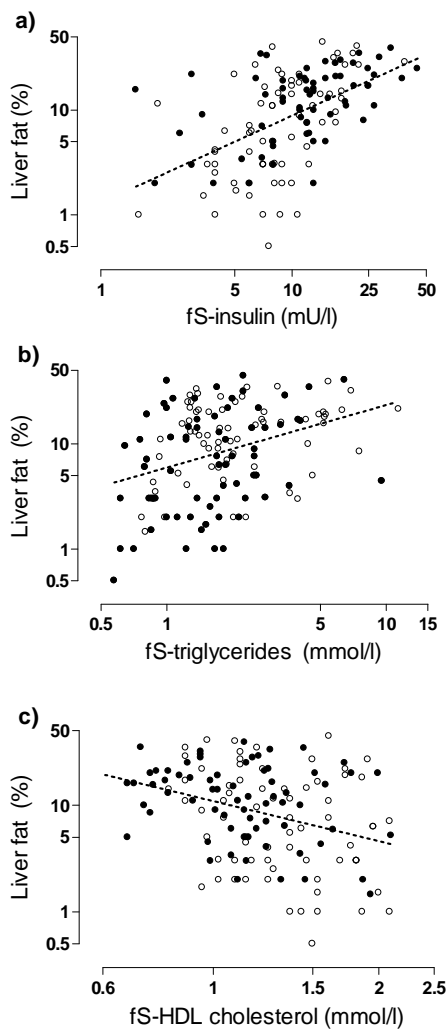


Figure 18. The associations between liver fat and fS-insulin concentrations and serum lipids. Liver fat content is related to (a) fS-insulin ($r=0.55$, $p<0.0001$ for both non-diabetic subjects and type 2 diabetic patients), (b) fS-triglycerides ($r=0.36$, $p<0.0001$ for both non-diabetic subjects and type 2 diabetic patients), and (c) fS-HDL cholesterol ($r=-0.31$, $p=0.0002$ for both non-diabetic subjects and type 2 diabetic patients). Dashed lines denote both non-diabetic subjects and type 2 diabetic patients. Symbols as in Figure 17.

Relationships between liver enzymes and liver fat content. S-AST and S-ALT correlated with liver fat content in both non-diabetic subjects and type 2 diabetic patients. The slopes of the regression lines between S-ALT concentrations and liver fat content differed significantly between the non-diabetic subjects and the type 2 diabetic patients ($p=0.004$). For any given ALT, the type 2 diabetic patients had more liver fat than the non-diabetic subjects (Fig. 19). Liver fat content did not differ between the groups at low S-ALT concentrations (10-20 U/l), but was 70%, 125%, and 200% higher in type 2 diabetic patients as compared to controls at S-ALT concentrations of 50 U/l, 100 U/l, and

200 U/l. At normal S-AST concentrations (15-45 U/l), liver fat content was 30-70% higher in the type 2 diabetic patients than in the non-diabetic subjects. This increase rose gradually with increasing S-AST concentrations, and averaged 80% and 130% at S-AST concentrations of 60 U/l and 100 U/l. Serum liver enzyme concentrations did not differ between the subjects who were using statins as compared to those who were not (S-AST: 29 (22-40), 29 (23-46) U/l, NS; S-ALT: 35 (21-51), 33 (24-64) U/l, NS). The relationship between liver enzymes and liver fat content did not differ between subjects who were and were not using statins in either group (data not shown).

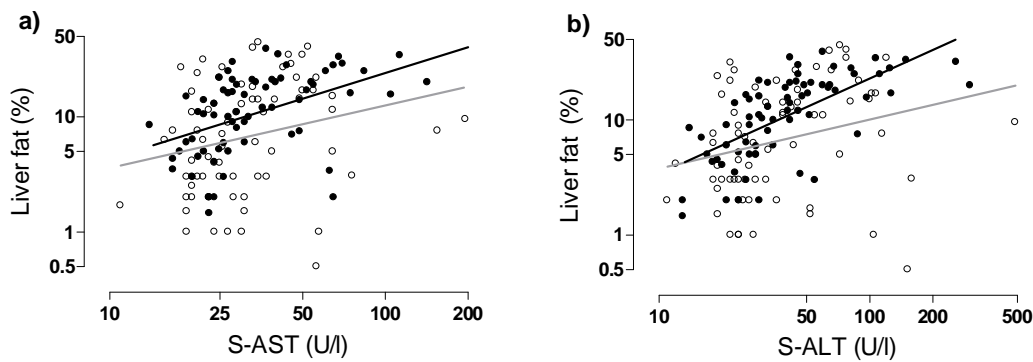


Figure 19. The relationships between liver fat content and liver enzymes. Liver fat content associates with (a) S-AST [$r=0.49$, $p<0.0001$ for type 2 diabetic patients ($LFAT\% = 10^{[-0.11\pm0.25 + 0.75\pm0.16 * \log(S-AST)]}$); $r=0.24$, $p=0.043$ for non-diabetic subjects ($LFAT\% = 10^{[0.01\pm0.40 + 0.54\pm0.26 * \log(S-AST)]}$)]]; and (b) S-ALT [$r=0.66$, $p<0.0001$ for type 2 diabetic patients ($LFAT\% = 10^{[-0.30\pm0.19 + 0.83\pm0.12 * \log(S-ALT)]}$); $r=0.26$, $p=0.027$ for non-diabetic subjects ($LFAT\% = 10^{[0.15\pm0.30 + 0.43\pm0.19 * \log(S-ALT)]}$)] concentrations. Symbols as in Figure 17.

Table 11. Liver fat content at increasing S-ALT and S-AST concentrations in the non-diabetic subjects and the type 2 diabetic patients.

Liver fat content		
	Non-diabetic subjects	Type 2 diabetic patients
S-ALT (U/l)		
20	5.1%	6.0%
40	6.9%	10.7%
60	8.2%	15.0%
80	9.3%	19.0%
100	10.2%	22.9%
S-AST (U/l)		
20	5.2%	7.3%
40	7.5%	12.3%
60	9.3%	16.7%
80	10.9%	20.8%
100	12.3%	24.5%

6.5 Effect of rosiglitazone on liver fat, insulin requirements, and hepatic insulin sensitivity in type 2 diabetic patients with a fatty liver (Study V)

Glycemic control, insulin requirements, and body composition. HbA_{1c} decreased by 1.1±0.3%, from 8.9±0.4% to 7.8±0.3% (p=0.007) during 8 months of

Table 12. Clinical and biochemical characteristics of the patients before and after 8 months of rosiglitazone-insulin combination therapy.

	Before	After	p-value
Body composition			
Body weight (kg)	111±4	114±4	0.002
BMI (kg/m ²)	36.7±1.1	37.8±1.1	0.002
Waist (cm)	120±3	125±3	NS
% fat	32.1±1.4	32.6±1.4	NS
Fat-free mass (kg)	75±2	77±2	0.03
IA fat (cm ³)	3900±340	4200±360	NS
SC fat (cm ³)	5500±540	6000±530	0.0002
Glycemic parameters			
fP-glucose (mmol/l)	9.1±0.6	7.9±0.5	NS
fS-insulin (mU/l)	40 (15-62)	27 (11-43)	NS (0.10)
Insulin dose (IU/kg/day)	1.98±0.2	1.11±0.15	0.001
Insulin dose (IU/kgFFM/day)	2.90±0.27	1.66±0.23	0.001
Serum lipids			
fS-triglycerides (mmol/l)	3.1±0.8	2.1±0.3	NS
fS-LDL cholesterol (mmol/l)	2.20±0.24	2.16±0.14	NS
fS-HDL cholesterol (mmol/l)	1.01±0.09	1.07±0.10	NS
fS-FFA (µmol/l)	716±52	597±50	0.04
Blood pressure			
Systolic BP (mmHg)	144±6	142±5	NS
Diastolic BP (mmHg)	79±2	80±1	NS
Liver enzymes			
S-AST (U/l)	40±5	34±5	0.04
S-ALP (U/l)	108±14	67±7	0.02
S-γGT (U/l)	51±11	27±3	0.0005

Data are shown as mean ± SEM or, for non-normally distributed data, as median followed by the 25th and 75th percentiles.

rosiglitazone therapy. Insulin dose decreased by 89 ± 24 IU/day ($40\pm 8\%$) from 218 ± 22 to 129 ± 20 IU/day ($p=0.002$, Fig. 20). Body weight increased by 3.2 ± 0.9 kg, which was due to an increase in fat-free mass (1.7 ± 0.7 kg) and fat mass (1.6 ± 0.7 kg). Subcutaneous but not intra-abdominal fat increased significantly when measured with MRI (Table 12). Insulin regimens at the end of the study remained unchanged.

Liver fat content and serum liver enzyme concentrations. Liver fat content decreased by $46\pm 9\%$ from $20\pm 3\%$ to

$11\pm 3\%$ ($p=0.0002$, Fig. 20). S-ALT, S-AST, S- γ GT, and S-ALP concentrations also decreased significantly (Table 12). The % change in liver fat correlated with the % changes in S-ALT ($r=0.50$, $p=0.08$), S-AST (0.63 , $p=0.02$), and S- γ GT concentrations ($r=0.72$, $p=0.01$).

Associations between liver fat content and glycemic parameters. The % change in liver fat content was positively correlated with the % change in insulin dose ($r=0.66$, $p=0.014$) and almost significantly with that in HbA_{1c} ($r=0.53$, $p=0.06$, Fig. 21).

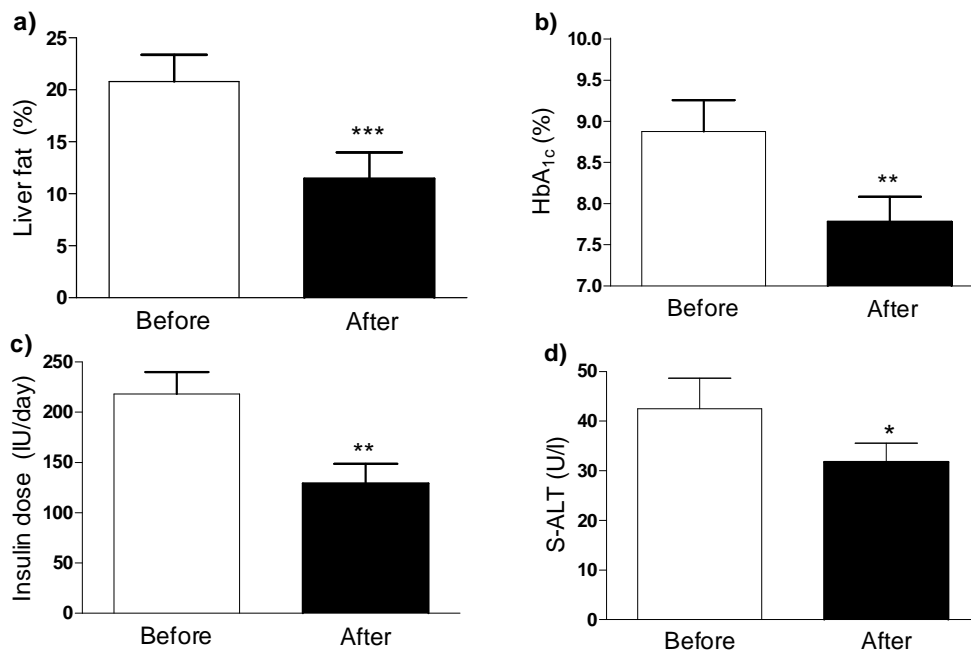


Figure 20. Effects of addition of rosiglitazone on (a) liver fat content, (b) HbA_{1c}, (c) insulin dose, and (d) S-ALT. *, $p\leq 0.05$; **, $p<0.01$; ***, $p<0.001$. □, before; ■, after the rosiglitazone-insulin combination therapy.

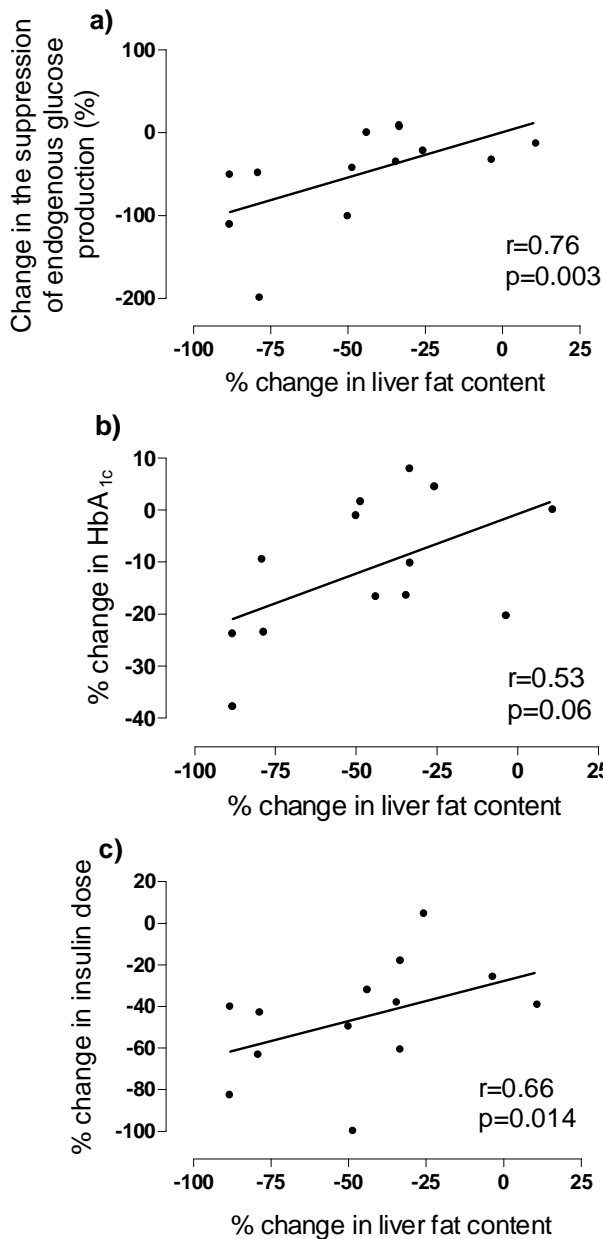


Figure 21. The relationship between the % change in liver fat content and (a) the % change in the suppression of hepatic glucose production, (b) the % change in HbA_{1c}, and (c) the % change in insulin dose by 8 months of rosiglitazone-insulin combination therapy.

Insulin sensitivity. Steady-state plasma glucose concentrations during euglycemia (240-360 min) averaged 8.1 ± 0.05 and 8.2 ± 0.04 mmol/l before and after rosiglitazone (NS). Fasting serum free

insulin decreased by 48% (Table 12). Serum free insulin concentrations (60-360 min) averaged 77 mU/l (47-106 mU/l, median, interquartile range) vs. 59 mU/l (39-93 mU/l), respectively

($p=0.07$). The increment in serum free insulin concentrations was similar before (28 mU/l [15-34 mU/l]) and after (25 mU/l [13-36 mU/l], NS) rosiglitazone therapy, implying that insulin clearance remained unchanged (17 ml/kgFFM·min [13-29 ml/kgFFM·min] vs. 16 ml/kgFFM·min [13-26 ml/kgFFM·min], NS).

Rates of basal hepatic glucose production were similar before (2.82 ± 0.25 mg/kgFFM·min) and after (2.33 ± 0.33 mg/kgFFM·min, NS) rosiglitazone treatment. During the insulin infusion, the rate of hepatic glucose production decreased (1.69 ± 0.25 vs. 0.61 ± 0.24 mg/kgFFM·min, $p=0.007$), and the % suppression by insulin increased from $-40\pm 7\%$ to $-89\pm 12\%$ ($p=0.001$) by rosiglitazone. The change in the % suppression of hepatic glucose production correlated positively with the

% change in liver fat content ($r=0.76$, $p=0.003$). The rate of basal glucose disposal decreased from 2.90 ± 0.24 to 2.06 ± 0.27 mg/kgFFM·min ($p=0.028$), while the rate of insulin-stimulated glucose disposal remained unchanged (2.87 ± 0.16 vs. 2.48 ± 0.18 mg/kgFFM·min, NS).

Energy expenditure and substrate oxidation rates. During insulin therapy, energy expenditure in the basal (83.2 ± 3.2 J/kgFFM·min vs. 78.2 ± 2.1 J/kgFFM·min, $p=0.05$) and insulin-stimulated (80.1 ± 3.0 vs. 75.1 ± 1.9 J/kgFFM·min, $p=0.017$) states decreased significantly by rosiglitazone. The rate of lipid oxidation during insulin stimulation decreased significantly (1.81 ± 0.12 vs. 1.51 ± 0.12 mg/kgFFM·min, $p=0.04$). Rates of carbohydrate and protein oxidation remained unchanged (Fig. 22).

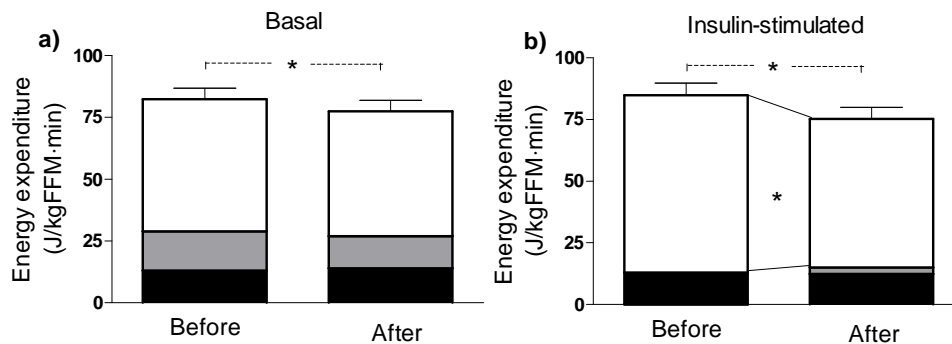


Figure 22. Effects of 8 months of the addition of rosiglitazone on rates of energy expenditure in the basal state (a) and during hyperinsulinemia (b). ■, protein oxidation; ■, carbohydrate oxidation [grey], □, lipid oxidation. *, $p\leq 0.05$.

7. DISCUSSION

Although the epidemic of obesity has been accompanied by an increase in the prevalence of the metabolic syndrome, not all obese develop the syndrome, and even lean individuals can be insulin resistant (462). Hepatic fat accumulation is related to hepatic insulin resistance (355, 379), which in turn leads to hyperglycemia (355, 379), hypertriglyceridemia (5, 6), and a low HDL cholesterol concentration (265), components included in the current definition of the metabolic syndrome by IDF (9). This raises the possibility that fat accumulation in the liver distinguishes between those who do and do not develop the metabolic syndrome. The present study aimed to investigate the role of hepatic fat accumulation in the metabolic syndrome and type 2 diabetes.

7.1. Liver fat vs. IMCL in relation to the metabolic syndrome (Study I)

In Study I, liver fat was 5-fold higher in subjects with than without the metabolic syndrome independent of obesity, while no differences in IMCL content could be observed between those with and without the syndrome. The fatty liver was shown to be insulin resistant when measured directly. These data suggest that in the present study group, intrahepato- rather than intramyocellular lipid accumulation was a marker of the metabolic syndrome, *i.e.* increased triglycerides and decreased HDL cholesterol, as well as hyperinsulinemia and low adiponectin.

We found obesity to be, consistent with previous data, associated with increased IMCL (158, 418), decreased $VO_2\max$ (81), and whole body insulin sensitivity (473) (Fig. 7). Since IMCL is increased

by both enhanced physical fitness (190, 418, 437) and obesity (158, 418), the lack of a relationship between IMCL and muscle insulin sensitivity is not surprising. As in the present study, Thamer et al found neither $VO_2\max$ nor insulin sensitivity to be associated with IMCL (418). However, further analyses revealed a significant positive association between $VO_2\max$ and IMCL in lean but not obese subjects (418). In Study I, IMCL was not related to $VO_2\max$, possibly because the study subjects covered a wide range of obesity while none participated in regular physical exercise. Nevertheless, BMI was inversely related to $VO_2\max$ expressed per fat free mass (Fig. 7). This may explain why IMCL was not inversely related to muscle insulin sensitivity, although $VO_2\max$ was an independent determinant of muscle insulin sensitivity (Fig. 7).

Hepatic fat content has been shown to associate with insulin resistance of hepatic glucose production (268, 355, 379). We now extended these findings to show that liver fat is associated with hepatic insulin resistance in the absence of changes in IMCL, and that liver fat and IMCL were not correlated. This implies that sites of intracellular triglyceride accumulation exhibit interindividual variation. It is noteworthy that although liver fat is a marker of hepatic insulin resistance, it is highly unlikely that the metabolically inert triglycerides mediate the impairment in insulin action (284, 403). Lipid intermediates which increase as a function of intracellular lipid stores, such as diacylglycerols (286) or ceramides (180), are likely to be responsible.

Regarding the reasons why liver fat associated with features of the metabolic syndrome, we have previously shown that failure of insulin to suppress hepatic VLDL production results in hypertriglyceridemia and a low HDL cholesterol concentration (265), and that VLDL overproduction rather than impaired clearance correlates with liver fat content (5). Resistance to insulin suppression of triglyceride production, although not measured in this study, is therefore a likely explanation for the finding of higher serum triglycerides and lower HDL cholesterol in the high as compared to the low LFAT groups.

Intrahepatic fat content is frequently referred to as an additional feature of the metabolic syndrome (435). In the present study, liver fat was 5-fold higher in subjects with than without the metabolic syndrome independent of obesity. On the other hand, no differences in IMCL content could be observed between subjects with and without the metabolic syndrome. Our findings suggest that depots of lipid accumulation in the liver and skeletal muscle do not contribute in an interactive manner to the metabolic syndrome.

In Study I, fasting serum FFA concentrations were not associated with liver fat or IMCL content, but they remained significantly higher during the low-dose insulin infusion in subjects with high liver fat content independent of obesity. This finding is in keeping with previous studies in patients with NAFLD as compared to weight-matched healthy controls (55). The reason for the lack of any relationship between fasting serum FFA and liver fat content is unclear. Possibly, higher FFA during hyperinsulinemia could reflect insulin resistance of lipolysis (435). In keeping with differential regulation of serum

FFA basally and during hyperinsulinemia, basal lipolysis is unaltered in HSL-null mice (446). There are, however, no human data describing alterations in FFA fluxes across the splanchnic bed, and therefore alterations in FFA utilization in subjects with high vs. low liver fat content cannot be excluded.

In conclusion, intrahepatocellular rather than intramyocellular fat associates with the metabolic syndrome, characterized by increased triglyceride and decreased HDL cholesterol concentrations, independent of obesity. This may in part be because IMCL is not correlated with muscle insulin sensitivity in subjects whose muscle insulin sensitivity is regulated by both obesity and physical fitness, as in the present study and that of Thamer et al (418). The data thus suggest that ectopic fat deposition in the liver may be a useful additional marker of the metabolic syndrome, although there is a need to identify novel simple circulating markers which would be suitable for use in the clinic as measures of liver fat.

7.2. Liver fat and the metabolic syndrome (Study II)

To our knowledge, this is the first study to quantitate the amount of liver fat in subjects with and without the metabolic syndrome. Liver fat content, measured by the most accurate method available to date (462), was 4-fold higher in subjects with than without the metabolic syndrome independent of age, gender, and BMI. Liver fat content increased in proportion to the number of components of the metabolic syndrome. Each component of the syndrome correlated with liver fat content. Of all measures of insulin resistance, the best correlates of liver fat were fasting serum insulin and C-peptide concentrations.

The study population did not represent a random sample but rather represented a non-diabetic group which had been recruited for metabolic studies using similar criteria: (i) age 20 to 65 years; (ii) healthy except for possible obesity; (iii) no known acute or chronic disease except for hypertension and hyperlipidemia based on history and physical examination and standard laboratory tests; (iv) alcohol consumption less than 20 g per day. In subjects without the metabolic syndrome liver fat content averaged 2.0% (1.0%-5.0%), which corresponds closely to what was considered normal in the Dallas Heart Study (409). In this population-based study, in which liver fat was measured in 2349 subjects using proton magnetic resonance spectroscopy, the upper limit of hepatic TG content was 5.56%. In patients with the metabolic syndrome in the present study, median liver fat content was 8.2% and thus exceeded this limit. The increase could not be explained by age or gender, and it was independent of BMI. This is in agreement with previous studies in which the relationship between liver fat and obesity has been weak (5) and non-existent (379, 425) if subjects with a narrow range of BMI have been studied.

Here we show that liver fat content correlated with all components of the metabolic syndrome as defined by the newest IDF criteria. The best correlate was waist circumference, which could explain 31-35% of the variation in liver fat content. Waist is thought to be a surrogate for intra-abdominal fat, and when measured directly using MRI, intra-abdominal fat volume explained 38-44% of the variation in liver fat content. In the study of Kelley et al (214), which included 83 type 2 diabetic patients and 27 non-diabetic subjects,

visceral adipose tissue and the liver to spleen attenuation ratio (L/S ratio) were significantly correlated ($r=0.57$), i.e. visceral adipose tissue explained 32% of the variation in the L/S ratio, which is a qualitative rather than quantitative marker of liver fat. Consistent with the data of Kelley et al in type 2 diabetic patients, in which BMI explained 18% of variation in the L/S ratio, we found BMI to explain 29% and 27% of the variation of liver fat in women and men, respectively. Taken together, these data suggest that visceral adipose tissue, while possibly important, is not the only factor contributing to the variation in liver fat content. Indeed, according to catheterization studies, the contribution of visceral fat to hepatic FFA delivery averaged 10% and did not exceed 50% of the total amount of FFA reaching the liver (292).

Liver fat was significantly positively correlated with serum triglycerides and similarly negatively correlated with HDL cholesterol. This has previously been documented in smaller studies (397, 452). The increase in serum triglycerides is known mainly to reflect insulin resistance or inhibition of hepatic VLDL production (5). High triglycerides in turn lower HDL cholesterol (294). In addition, linear regression analysis revealed a weak but significant association between liver fat content and both systolic and diastolic blood pressure. The poor correlation may be due to use of a single measurement of blood pressure which is neither reliable nor reproducible (20). In keeping with this, in a previous study both 24-hour systolic and diastolic blood pressures were elevated in women with high fat content compared with those with low liver fat content independently of age and BMI (425).

Liver enzyme concentrations have previously been shown to reflect hepatic steatosis (30, 304). Although we found a significant correlation between liver fat and both S-ALT and S-AST concentrations, other markers, such as fasting serum C-peptide, more accurately reflected liver fat content in these non-diabetic subjects. S-ALT concentrations explained only 15-19% of liver fat, implying that this enzyme is a poor marker of liver fat. There were no differences in the slopes of the regression lines relating liver fat and liver enzymes between women and men, but the intercepts differed significantly. This observation is consistent with the lower reference ranges for S-ALT and S-AST in women compared with those in men (334). Smaller liver size (243) and decrease in S-ALT by female reproductive hormones (276, 401) could contribute to lower S-ALT concentrations in women than in men.

Of all parameters analysed, fasting serum insulin was the marker most closely related to liver fat content. This strong association may be explained by the decrease in hepatic insulin clearance in subjects with increased hepatic fat content (159, 400). Indeed, a 51% decrease in liver fat achieved by rosiglitazone therapy has been shown to increase insulin clearance by 20% (424). However, C-peptide, which is not degraded by the liver (328), was also a strong correlate of liver fat. This documents that the increase in serum insulin is not just a consequence of diminished insulin clearance. Previously, fasting serum insulin has been included in the definition of the metabolic syndrome (10), but because of assay standardization problems, this parameter was excluded from the latest metabolic syndrome criteria. Still, within a given laboratory, increased fasting serum

insulin and C-peptide concentrations could be useful markers of the fatty liver independently of age, gender, and BMI.

Taken together, we found liver fat to be on the average 4-fold increased in middle-aged non-diabetic subjects with the metabolic syndrome. Components of the syndrome were significantly correlated with liver fat even independently of age, gender, and BMI. Although the interindividual variation in liver fat ranged considerably, the mean increase (8.2% vs. 2.0%) cannot be detected by routinely available clinical tools such as S-ALT. Nevertheless, the present data strengthen the idea that those obese and non-obese subjects who accumulate excess fat in the liver are the ones who develop the metabolic syndrome.

7.3. Insulin clearance and liver fat (Study III)

The liver is a major site of insulin action, clearance and degradation (116). The majority (80%) of endogenously secreted insulin is cleared by the liver, 15% by the kidney, and 5% by muscle (137). Of intravenously infused insulin, 50%, 30%, and 10%, respectively, are cleared by these tissues (137). Liver fat is closely correlated with fasting serum insulin concentrations (229, 452), but the extent to which impaired insulin clearance due to hepatic fat accumulation contributes to hyperinsulinemia has not previously been determined. In the present study, we found hepatic fat accumulation to be an important and independent regulator of insulin clearance, as measured by the euglycemic hyperinsulinemic clamp technique, i.e. after i.v. insulin administration. Impaired insulin clearance due to increased liver fat content explained on the average 27% of the variation in fS-insulin concentrations. The slopes of the

regression lines relating liver fat and fS-insulin and fS-insulin corrected for insulin clearance differed significantly, implying that the contribution of impaired insulin clearance to fasting serum insulin concentrations increases with increasing liver fat content. Direct measurement of hepatic insulin sensitivity showed that liver fat was also associated with hepatic insulin resistance.

Hepatocytes loaded with triglycerides exhibit impaired insulin clearance *in vitro* (400). In a study of 46 non-diabetic subjects with a wide range of adiposity, the liver-to-spleen attenuation ratio, a qualitative marker of liver fat, has been found to be inversely related to insulin clearance (159). However, in this study, insulin clearance was not adjusted for % body fat (159), and it thus remained unclear whether the apparent decrease in insulin clearance in those with high liver fat was due to liver fat or to differences in the insulin distribution space between the obese subjects with high liver fat as compared to the non-obese subjects with less liver fat. Since fat is essentially water-free, the plasma volume per body weight unit decreases with increasing % body fat (11). Thus, when insulin is infused at a rate which is calculated based on kg body weight or body surface area (159), insulin clearance will be lower in obese than in non-obese subjects (474). In Study III, we found that during a prolonged low-dose insulin infusion, serum insulin concentrations and insulin increments were approximately 40% higher in subjects with high LFAT independent of age, gender, and BMI. Consequently, insulin clearance was considerably impaired in the high as compared to the low LFAT group. Although it would have been ideal to study weight-matched groups with high and low LFAT, the present data suggest

that liver fat impairs insulin clearance independent of BMI.

It has been proposed that FFA-mediated reduction in insulin clearance is an adaptive mechanism which exacerbates hyperinsulinemia to overcome peripheral insulin resistance (247). In a previous study, Wiesenthal et al showed that in dogs hepatic insulin extraction is impaired when serum FFA concentrations are elevated to supraphysiological concentrations by infusions of a soy-based lipid emulsion and heparin during euglycemic hyperinsulinemia (457). In humans, a day-long infusion of a soy-based lipid emulsion and heparin (S-FFA ~500-800 $\mu\text{mol/l}$) has been shown to reduce insulin clearance in subjects with a family history of type 2 diabetes (212). In Study III, where no exogenous FFA were infused, fasting FFA were in this range but were uncorrelated with both liver fat and insulin clearance. However, the fasting serum insulin concentrations were 2-fold higher in the high as compared to the low LFAT group. This difference in insulin levels in the face of similar FFA concentration mostly likely reflects adipose tissue insulin resistance and may make it difficult to observe a relationship between fasting FFA and insulin clearance.

Previous studies have shown that insulin clearance is decreased in obesity (279, 389). It has also been suggested that intra-abdominal rather than subcutaneous fat influences splanchnic insulin clearance (312). Moreover, insulin clearance increases by weight loss (327). The decrease in insulin clearance has also been observed in insulin-resistant as compared to age- and BMI-matched insulin-sensitive subjects (203). However, the causes of the variation in

insulin clearance have not been determined in these studies. Our data suggest that liver fat content is an important contributor to the variation in insulin clearance and could contribute to decreased insulin clearance in obesity (279, 389). Liver fat could also explain why weight-matched groups differing with respect to insulin sensitivity also may exhibit differences in insulin clearance. Furthermore, liver fat content is better correlated with intra-abdominal than subcutaneous fat (229, 452). We have previously shown that a decrease in liver fat content, achieved by rosiglitazone therapy, increases insulin clearance and enhances hepatic insulin sensitivity significantly independent of body weight in type 2 diabetic patients (424). In addition, liver fat can be considerably reduced by weight loss (79, 423). Thus, interventions which change liver fat also appear to change insulin clearance.

In the present study, on the average 27% of the variation in fS-insulin concentrations could be explained by those in insulin clearance when fasting insulin concentrations were measured in subjects whose liver fat content varied over a wide range. The contribution of impaired insulin clearance to fasting hyperinsulinemia increased as a function of liver fat (Fig. 15). This implies that indirect measures of insulin sensitivity, such as the homeostatic model assessment of insulin resistance (HOMA-IR) (271), overestimate insulin sensitivity in non-diabetic individuals in whom insulin resistance is associated with, or perhaps due to a fatty liver rather than e.g. skeletal muscle. Note that the C-peptide-to-insulin –ratio differed only marginally between the groups, possibly because C-peptide clearance is also subject to interindi-

vidual variation (124), while was not quantitated in this study. Although serum C-peptide concentrations during the insulin infusion was not measured in Study III, it has previously been shown that serum C-peptide concentrations fall significantly already after 60 min of exogenous insulin infusion at the rate of 0.25 mU/kg-min and do not change at the higher insulin infusion rates (94). We showed by direct measurement of insulin sensitivity that hepatic insulin sensitivity decreases as a function of liver fat. This insulin resistance represented an underestimate of hepatic insulin resistance, as insulin concentrations increased with increasing liver fat content.

In conclusion, a fatty liver is associated with both hepatic insulin resistance and impaired insulin clearance. The impact of liver fat on insulin clearance increases as a function of liver fat. This implies that indirect measures of insulin sensitivity, such as fasting insulin, overestimate hepatic insulin resistance the more fat the liver contains.

7.4. Liver fat and type 2 diabetes (Study IV)

In Study IV, we used state-of-the-art – methodology for quantification of liver fat in a large group of type 2 diabetic patients and carefully age- and gender-matched equally obese non-diabetic subjects. Liver fat content was on the average 80% higher in the type 2 diabetic patients than in the non-diabetic subjects. This difference was not influenced by the type of anti-hyperglycemic treatment. Somewhat unexpectedly, S-ALT and S-AST were not related to liver fat similarly in the diabetic and non-diabetic subjects. Both S-ALT and S-AST underestimated liver fat content in type 2 diabetic patients.

This underestimation became more pronounced at increasing concentrations of both S-ALT and S-AST.

In many previous studies, liver fat content, measured by proton magnetic resonance spectroscopy, has been found to exceed the upper limit of normal hepatic TG content (5.56%) (409) in type 2 diabetic patients. However, except for one study which included 10 non-diabetic controls (214), these studies had either no control group (21, 63, 204, 273, 355, 424) or the controls were not matched for body weight (5, 319). It has thus remained unclear whether the increase in liver fat content has just been a consequence of obesity in type 2 diabetic patients. Study IV suggests that type 2 diabetic patients have more liver fat at any given BMI than non-diabetic subjects, and that the difference in liver fat content between the groups increases with increasing obesity. In keeping with Kelley et al (214), intra-abdominal fat was similarly related to liver fat in both type 2 diabetic patients and non-diabetic subjects, and the type 2 diabetic patients had more intra-abdominal fat than the non-diabetic subjects.

The higher liver fat content in the type 2 diabetic patients could contribute to diabetic dyslipidemia. The fatty liver overproduces VLDL particles in both non-diabetic subjects and type 2 diabetic patients, which, in the face of unchanged VLDL clearance, increases serum triglyceride concentrations (5). Hypertriglyceridemia in turn leads to low HDL-cholesterol concentrations (294). The similar relationship between triglycerides and HDL cholesterol concentrations and liver fat content in the diabetic patients and non-diabetic subjects (Fig. 18) combined with the kinetic study (5) suggest that excess liver

fat indeed contributes to diabetic dyslipidemia.

An intriguing observation in the present study was that the type 2 diabetic patients had 40 to 200% more liver fat at the same S-ALT and S-AST concentrations than the non-diabetic subjects (Table 11). Both enzymes thus underestimate liver fat in type 2 diabetes. Both S-ALT and S-AST increase in response to hepatocyte damage until hepatocytes are lost and cirrhosis develops (82, 234). To what extent the lower enzyme levels reflect a difference in hepatocellular damage cannot be determined from the present study.

It is important to develop tools to diagnose a fatty liver in type 2 diabetic patients, because NASH is more common in type 2 diabetic patients than in non-diabetic subjects (118, 176), and can progress to cirrhosis and liver failure (128). The present data suggest that if assessed using S-ALT or S-AST, hepatic steatosis is underestimated in type 2 diabetic patients compared to equally obese non-diabetic subjects. There is thus a need to develop new serum markers of steatosis to complement S-AST and S-ALT which have been used in the clinic for almost 50 years (211).

7.5. Treatment of type 2 diabetic patients with a fatty liver with PPAR γ agonists (Study V)

In Study V, we hypothesized that patients requiring exceptionally high insulin doses might respond particularly well to PPAR γ agonist treatment. During eight months of rosiglitazone therapy, HbA_{1c} decreased significantly despite a 40% reduction in the insulin dose. Liver fat content decreased by 50%, and hepatic insulin sensitivity increased

significantly. The changes in liver fat content correlated with both decreases in insulin requirements and increase in hepatic insulin sensitivity.

Due to the invasive measurements performed, it was not ethically possible to study a time-control group. A 'study effect' could thus have contributed to the results, which is a potential limitation. However, we have previously shown that addition of metformin alone for 4 months has no effect on liver fat content (424), while rosiglitazone alone decreases liver fat by 50% (424). Given that insulin requirements correlate closely and linearly with hepatic fat content (204, 355), and S-ALT predicts insulin requirements independent of BMI (472), one could predict insulin requirements to decrease by 50% if liver fat decreases by 50%. This indeed happened after patients had been treated with stable insulin doses for 2 years. These considerations make it likely that the changes were due to rosiglitazone rather than time, metformin, or continued insulin therapy.

In Study V, liver fat content was increased in each patient, and exceeded its upper limit of normal defined as 5.6% TG by ¹H-MRS (409). In the previous PPAR γ agonist insulin combination studies, the decrease in insulin doses averaged 13 IU/day (32, 54, 89, 147, 181, 272, 329, 342), which is much less than the 90 IU/day in the present patients. In keeping with the present results, in previous studies which had the highest baseline insulin doses, the % reduction were also the greatest (54, 483). In these clinical studies, liver fat content was not measured. Heterogeneity analyses from DREAM (114) and ADOPT (206) have suggested that patients with a large waist

circumference respond best to PPAR γ agonist treatment. Waist circumference is tightly correlated with liver fat content (229, 452). These data support the idea that patients with a fatty liver respond particularly well to PPAR γ agonists.

In Study V, no improvement in peripheral insulin sensitivity, as determined from glucose rate of disappearance, was observed. However, given that low physiological insulin infusion rates are optimal for assessment of hepatic rather than peripheral insulin sensitivity (96, 135, 348, 478), the lack of improvement in insulin-stimulated glucose disposal was not unexpected.

When glycemic control is improved, the energy need for glucose production in the liver decreases (264). The improvement in glycemic control is likely to be at least one reason for the decrease in energy expenditure found in the present study. When calculated from our previous data (264), the predicted change in the basal metabolic rate due to changes in fasting glucose and body weight in the present study corresponds to 6.3 ± 19 J/kgFFM \cdot min, which is not different from the observed change (5.2 ± 2.3 J/kgFFM \cdot min). The increase in body weight, which accompanies reduction in glucosuria, tends to increase energy expenditure, but is not sufficient to keep net energy expenditure unchanged (264). Consistent with previous data regarding PPAR γ agonists (22), weight gain (3.2 kg) was greater than would be expected from improvement in glycemic control (1.5-2.0 kg/1% decrease in HbA_{1c} (264)). Weight gain was due to a roughly similar increase in fat free mass and fat mass, in keeping with the data showing improved glycemia to increase

predominately fat free mass (204) and PPAR γ agonist mainly fat mass (22). Adverse effects of TZDs include fluid retention and the risk of heart failure (113, 206). A recent meta-analysis suggested rosiglitazone use to significantly increase the risk of myocardial infarction (295), although there is no randomized adequately powered clinical trial to support this finding (182). In Europe, combination therapy with insulin and PPAR γ agonists is contraindicated because of an increased risk of heart failure (<http://www.emea.europa.eu/>). In the PROactive study, a controlled study of 5238 type 2 diabetic patients at high risk of cardiovascular events randomized to receive either pioglitazone or placebo, 31% of patients were treated with insulin, but it is unknown whether the rate of cardiovascular events differed between insulin-treated and insulin-naïve patients using pioglitazone (113). Because of the increased risk of heart failure, we performed echocardiographies in all patients prior to their participation to exclude heart failure at baseline.

In conclusion, we have shown that type 2 diabetic patients who are poorly

controlled despite using unusually high doses of insulin, do respond to rosiglitazone. We suggest the main mechanism underlying improved glycemic control despite reduced insulin requirements is enhanced hepatic insulin sensitivity. At least in monotherapy studies, pioglitazone is as effective as rosiglitazone (463) and may be associated with a better cardiovascular outcome during long-term treatment than rosiglitazone (113, 206).

Taken together, Studies I-V suggest that accumulation of fat in the liver is an inherent component of the metabolic syndrome and type 2 diabetes. Increased fat accumulation in the liver is a marker of hepatic insulin resistance and a close correlate of all components of the metabolic syndrome independent of obesity. Subjects with high liver fat content are hyperinsulinemic because of both impaired hepatic insulin sensitivity and insulin clearance. The contribution of impaired insulin clearance to fasting serum insulin concentrations increases with increasing liver fat content. Given that insulin action in the fatty liver is impaired, agents which reduce liver fat content may be particularly efficient for the treatment of type 2 diabetic patients.

8. SUMMARY AND CONCLUSIONS

I) Fat accumulation in the liver rather than in skeletal muscle is associated with features of the metabolic syndrome, *i.e.* increased fS-triglycerides and decreased fS-HDL cholesterol, and with hyperinsulinemia and low adiponectin concentrations. The fatty liver has been shown to be insulin resistant.

II) Liver fat content is 4-fold higher in subjects with the metabolic syndrome as compared to those without the syndrome, independent of age, gender, and BMI. Of other markers, fS-C-peptide is the strongest correlate of liver fat.

III) Increased liver fat is associated with both impaired insulin clearance and hepatic insulin resistance. Hepatic insulin sensitivity is related to liver fat content independent of insulin clearance. This implies that indirect indices of insulin sensitivity, such as HOMA-IR, overestimate insulin resistance in subjects with high liver fat content.

IV) Type 2 diabetic patients have 80% more liver fat than age-, weight-, and gender-matched non-diabetic subjects. S-ALT underestimates liver fat in type 2 diabetic patients.

V) Rosiglitazone may be particularly effective in type 2 diabetic patients who are poorly controlled despite using high insulin doses. The mechanisms are likely to involve reduced liver fat and enhanced hepatic insulin sensitivity.

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