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EFFECTS OF WEIGHT LOSS, ROSIGLITAZONE AND METFORMIN ON LIVER FAT CONTENT, INSULIN RESISTANCE AND GENE EXPRESSION IN ADIPOSE TISSUE

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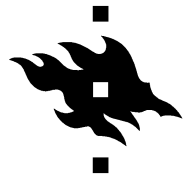

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LIST OF ORIGINAL PUBLICATIONS

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

I. Tiikkainen M, Tamminen M, Häkkinen AM, Bergholm R, Vehkavaara S, Halavaara J, Teramo K, Rissanen A, Yki-Järvinen H*:* Liver-fat accumulation and insulin resistance in obese women with previous gestational diabetes**.** *Obes Res* 10:859-67, 2002.

II. Tiikkainen M, Bergholm R, Vehkavaara S, Rissanen A, Häkkinen A, Tamminen M, Teramo K, Yki-Järvinen H: Effects of identical weight loss on body composition and features of insulin resistance in obese women with high and low liver fat content. *Diabetes* 52:701-7, 2003

III. Tiikkainen M, Bergholm R, Rissanen A, Aro A, Salminen I, Tamminen M, Teramo K, Yki-Järvinen H: Effects of equal weight loss with orlistat and placebo on body fat and serum fatty acid composition and insulin resistance in obese women. *Am J Clin Nutr* 79:22-30, 2004.

IV. Tiikkainen M, Häkkinen A-M, Korsheninnikova E, Nyman T, Mäkimattila S, Yki-Järvinen H: Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, and gene expression in adipose tissue in patients with type 2 diabetes. *Diabetes,* 53:2169-2176, 2004.

ABREVIATIONS

ACC = acetyl-CoA carboxylase
ACRP30 = adipocyte complement-related protein of 30 kilodalton
ALT = alanine aminotransferase
AST = aspartate aminotransferase
AMPK = adenosine monophosphate -activated protein kinase
aP2 = adipocyte P2 enhancer
AST = aspartate aminotransferase
A-ZIP/F-1 = aricid extension-leucine zipper lipoatrophic mouse
BIA = Bio-Electrical Impedance Analyzer
BP = blood pressure
BMI = body mass index
C/EBP = CCAAT/enhancer-binding protein
CPT = carnitine palmityltransferase
CSII = continuous subcutaneous insulin infusion
DPP = Diabetes Prevention Program
DPS = Diabetes Prevention Study
EGP = endogenous glucose production
FATP-1 = fatty acid transport protein 1
FFA = free fatty acids
FFM = fat free mass
FIRKO = fat-specific insulin receptor knockout mouse
GAD = glutamic acid decarboxylase
GDM = gestational diabetes mellitus
GGT = gamma glutamyl transferase
GL = glycogenolysis
GLUT4 = glucose transporter 4
GNG = gluconeogenesis
GSA = glucose specific activity
HDL = high density lipoprotein
HIR = hepatic insulin resistance
HK = hexokinase
HSL = hormone sensitive lipase
IFG = impaired fasting glucose
IGT = impaired glucose tolerance
IMCL = intramyocellular lipid
HbA_{1C} = glycosylated hemoglobin A_{1C}
HIV = human immunodeficiency virus
IL = interleukin
IR = insulin receptor

IRAS = Insulin Resistance Atherosclerosis Study
IRS-1 = insulin receptor substrate-1
IRS-2 = insulin receptor substrate-2
kg = kilogram
LCACoA = long-chain acyl-CoA
LDL = low density lipoprotein
LFAT = liver fat content (%) determined by magnetic resonance imaging
LIRKO = liver-specific insulin receptor knockout mouse
LPL = lipoprotein lipase
MAP = mitogen-activated protein
MIRKO = muscle-specific insulin receptor knockout mouse
MRI = magnetic resonance imaging
mRNA = messanger ribonucleic acid
MRS = magnetic resonance spectroscopy
M-value = amount of glucose infused to maintain euglycemia
MUFA = monounsaturated fat acids
NAFLD = non-alcoholic fatty liver disease
NASH = non-alcoholic steatohepatitis
NHANES III = The Third National Health and Nutritional Exam Survey
NO = nitric oxide
OGTT = oral glucose tolerance test
PEPCK = phosphoenol pyryvate carboxykinase
PI 3-kinase = phosphatidylinositol 3-kinase
PKC-δ = protein kinase C-delta
PPARγ = peroxisome proliferator -activated receptor γ
PUFA = polyunsaturated fat acids
Ra = glucose appearance
RIA = radioimmonoassay
Rd = glucose disappearance
RT PCR = real time polymerase chain reaction
SAFA = saturated fat acids
SREBP-1 = sterol regulatory element binding protein-1
TNF-α = tumor necrosis factor-α
TSH = thyroid stimulating hormone
TZD = thiazolidinediones
UKPDS = UK Prospective Diabetes Study
VLDL = very low density lipoprotein
WHR = waist to hip ratio

ABSTRACT

Introduction: Fat accumulation in the liver has previously been shown to be associated with insulin resistance and obesity, but whether liver fat associates with insulin resistance independent of obesity is less clear. Weight loss, peroxisome proliferator -activated receptor-γ (PPARγ) agonists, and metformin improve insulin sensitivity, but the mechanisms are unclear. The present studies were undertaken to investigate i) whether liver fat accumulation associates with features of insulin resistance (*study I*), ii) effects of 8% weight loss induced by a hypocaloric, low fat diet combined with orlistat or placebo on insulin sensitivity and liver fat content (*studies II,III*), and iii) effects of PPARγ agonism and metformin on hepatic insulin sensitivity, gene expression in adipose tissue and liver fat content (*study IV*).

Subjects and methods: In *studies I and II* liver fat content (proton spectroscopy) and insulin sensitivity (euglycemic insulin clamp technique) were measured in 27 obese women. In *study II* 27 and in *study III* 47 obese women with previous gestational diabetes were placed on a hypocaloric, low fat diet and randomised into two groups using either orlistat or placebo as an adjunct to the diet. Since we wished to examine effects of orlistat per se on insulin sensitivity, both groups were designed to lose 8% of body weight during a similar time period (3 to 6 months). In *Study IV* 20 treatment-naive patients with type 2 diabetes were treated for 16 weeks with rosiglitazone or metformin. Liver fat content, hepatic and peripheral insulin sensitivity, gene expression in adipose tissue, and body composition were measured before and after the treatment period.

Results: Liver fat content correlated independent of body weight with several features of insulin resistance including blood pressure, serum fasting insulin and triglyceride concentrations in obese women (*study I*). In *study II* weight loss significantly reduced liver fat content. At baseline liver fat correlated with the percent of saturated fat intake. Insulin sensitivity improved similarly with orlistat or placebo, but orlistat reduced the ratio of intra-abdominal and subcutaneous fat volume more than placebo. Rosiglitazone and metformin improved similarly hepatic insulin sensitivity, but only rosiglitazone decreased liver fat content and improved peripheral insulin sensitivity. Rosiglitazone also increased insulin clearance. Rosiglitazone increased PPARγ, lipoprotein lipase (LPL) and adiponectin mRNA expressions in subcutaneous adipose tissue and increased serum adiponectin concentrations, whereas there were no changes in the metformin group.

Conclusions: These data demonstrate that liver fat content, independent of overall obesity, is closely correlated with features of insulin resistance. Liver fat can be decreased by weight loss and by PPARγ agonism but not with metformin. The beneficial effects of PPARγ agonism may be mediated via changes in serum adiponectin.

1. INTRODUCTION

The incidences of obesity (1,2) and type 2 diabetes (3) are increasing worldwide. Abdominal and overall obesity (4), hypertension, hyperlipidemia, insulin resistance (5), and previous gestational diabetes (GDM) (6) are well known predictors of type 2 diabetes and cardiovascular disease. We have recently shown fat accumulation in the liver measured non-invasively using magnetic resonance spectroscopy (MRS) to be associated with insulin resistance (7). We have also shown that hepatic fat content correlates with insulin requirements and hepatic insulin sensitivity in patients with type 2 diabetes (8). It is, however, unknown whether hepatic fat content is associated with insulin resistance independent of body weight in obesity.

The increased prevalence of obesity is due to excessive caloric intake (9). Two prevention studies have demonstrated that moderate (5%) weight reduction can prevent the development of type 2 diabetes by 58% (10,11). Weight loss is known to enhance insulin sensitivity (12). It is unknown to what extent changes in liver fat content might contribute to enhanced insulin sensitivity.

Increased endogenous hepatic glucose production and hepatic insulin resistance are well known phenomena in patients with type 2 diabetes (13). Obese patients with type 2 diabetes are often treated with metformin, a widely used anti-diabetic drug, which lowers blood glucose concentrations by inhibiting endogenous glucose production in the liver (14,15). Rosiglitazone is an agonist of the nuclear receptor PPARγ currently approved for treatment of hyperglycemia in patients with type 2 diabetes (16,17). Activation of PPARγ promotes adipocyte differentiation and regulates the expression of over a hundred genes (18). The mechanism underlying the glucose-lowering effect of these drugs in humans is poorly understood.

Adiponectin is a polypeptide expressed and synthesized only in adipose tissue (19). Decreased levels of circulating adiponectin characterize obese subjects (20,21) and patients with type 2 diabetes (22,23). Treatment with thiazolidinediones (TZD) has been shown to increase serum adiponectin concentrations (24,25) and enhance hepatic insulin sensitivity in mice (26,27).

The present studies were undertaken to define how liver fat content associates with features of insulin resistance in a group of obese women, and how weight loss, metformin and rosiglitazone influence liver fat content and insulin resistance in women with previous gestational diabetes and in patients with type 2 diabetes.

2. REVIEW OF THE LITERATURE

2.1. Normal insulin action

After insulin is secreted by the pancreas in response to an increase in blood glucose concentration, it enters the portal vein and about 50-80% of circulating insulin is degraded by the liver (28,29). Although insulin is the most important hormone that regulates the blood glucose concentrations both during and between meals, it also has multiple other effects on lipid and protein metabolism, blood vessels, and circulating levels of other hormones and electrolytes (30).

The first step in insulin action is binding to the insulin receptor (IR), which is present in all insulin sensitive cells such as hepatocytes, myocytes, cardiomyocytes, adipocytes, macrophages, endothelial cells and platelets (31,32). The IR belongs to a subfamily of receptor tyrosine kinases and is a heterotetrameric membrane protein that consists of two identical α- and β-subunits (31). Binding of insulin to IR leads to activation of several phosphorylation-dephosphorylation cascades (30,33). Autophosphorylation of intracellular β-subunit of the IR results in activation of the tyrosine kinase, which catalyses phophorylation of multiple insulin receptor substrate proteins such as IRS-1 and IRS-2 (34). They activate phosphatidylinositol kinase (PI 3–kinase), which is essential for stimulation of glucose transport and phosphorylation by activating glucose transporter GLUT4 and hexokinase II (HKII) (33,34,35) (**Fig.1**). PI 3-kinase also mediates insulin-induced increases in nitric oxide (NO) in endothelial cells (36). PI 3-kinase pathway is mainly involved in mediating the metabolic effects of insulin, such as glucose transport (*vide infra*), glycogen and protein synthesis, ion and amino acid transport, and lipid metabolism (30). IRS-1 associated PI 3-kinase pathway also mediates insulin-induced lipolysis in adipocytes via inhibition of hormone sensitive lipase (HSL) (37). Mitogen-activated protein (MAP) kinase pathway is another insulin signalling pathway that is necessary in regulating cell proliferation, differentiation and apoptosis (34).

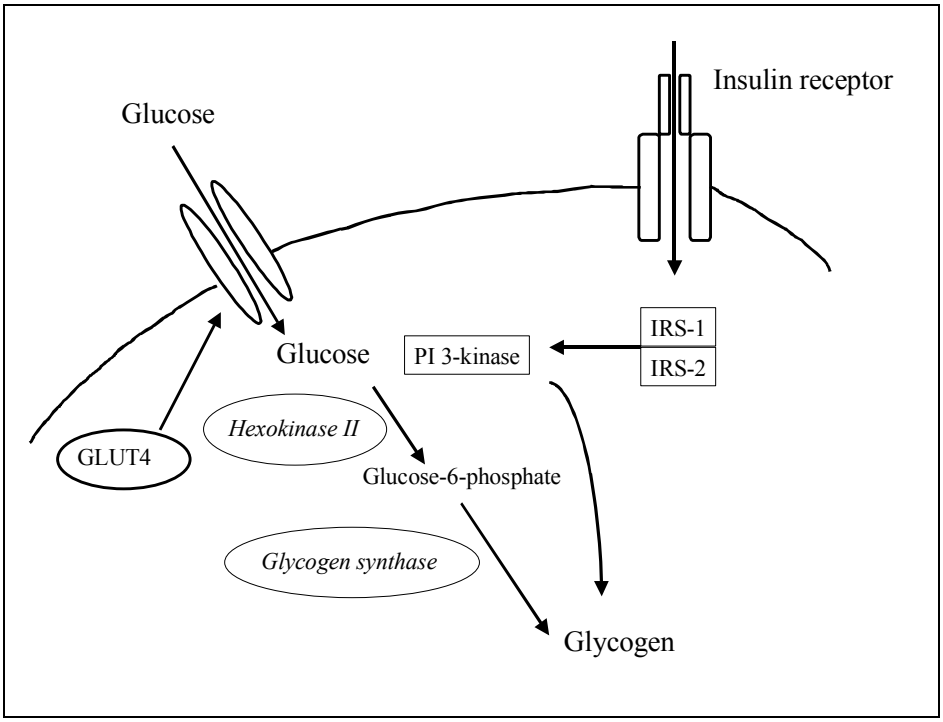

Figure 1. *Schematic picture of insulin action. Insulin binds to the insulin receptor (IR), which is a tyrosine kinase that undergoes autophosphorylation and catalyses the phosphorylation of insulin receptor substrate (IRS) family. This results in activation of phosphatidylinositol kinase (PI 3-kinase), which activates glucose transporter (GLUT4) and hexokinase II (HK II). These pathways regulate vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which results in the regulation of glucose metabolism {Adapted from (30)}.*

2.1.1. Glucose metabolism

Glucose is an essential source of energy for the brain. The blood glucose concentration is maintained within narrow limits by hormonal regulation of peripheral glucose uptake and hepatic glucose production. After an overnight fast glucose uptake averages ~2 $mg \cdot kg^{-1} \cdot min^{-1}$, which is also the rate at which the liver produces glucose in normal subjects (38,39,40). Under these conditions the brain takes up glucose at a constant rate of 1.0 - 1.2 $mg \cdot kg^{-1} \cdot min^{-1}$ and accounts for ~50-60% of glucose utilization (38). Splanchnic organs and skeletal muscle together account for ~20-25%. Adipose tissue only utilizes 1-3% of glucose after an overnight fast (41).

After an oral glucose load approximately ~30% of glucose is taken up by splanchnic tissues, ~30% by muscle, ~30% is oxidized in the brain, and ~30% of ingested glucose is stored in the liver (42). Under hyperinsulinemic euglycemic clamp conditions over 70% of glucose uptake occurs in skeletal muscle, whereas adipose tissue accounts for only a small fraction

(43,44,45). Under postprandial conditions insulin also inhibits glucagon secretion, lowers serum free fatty acid (FFA) concentrations, which both decrease hepatic glucose production (46). After oral glucose ingestion hepatic glucose production decreases by 50-60% in normal subjects (47).

The liver produces glucose by glycogenolysis (GL) or via *de novo* gluconeogenesis (GNG) mainly from lactate, alanine, pyruvate and glycerol (48). Studies measuring GNG using the $^{2}H_2O$ (49) and ^{13}C NMR spectroscopy methods (50) in healthy subjects have demonstrated that after an overnight fast GNG accounts for 40-50% of endogenous glucose production (EGP). After prolonged (42 h) fasting conditions GNG accounts for 93-96% of EGP. Insulin decreases EGP primarily by suppressing glycogenolysis and by decreasing the glucose synthesis via GNG to glycogen (51). Insulin can also influence glucose metabolism indirectly via its antilipolytic effect. Acute elevations of plasma FFA increase GNG and lower GL, while acute lowering of FFA reciprocally decreases GNG and increases GL while EGP remains unchanged. This phenomen is known as "autoregulation of endogenous glucose production" (13,52,53,54,55). Insulin directly inhibits the expression of several genes encoding hepatic enzymes of gluconeogenesis and glycolysis (56) including phosphoenol pyryvate carboxykinase (PEPCK), which is the rate-limiting step in GNG (57), and sterol regulatory element binding protein-1c (SREBP-1c), which inhibits the transcription of PEPCK (58), and glycogen phosphorylase (59).

Specific glucose transport protein, such as GLUT4, is needed for glucose entry into the cells. GLUT4 is the main insulin-dependent glucose transport protein, which is expressed in white and brown adipocytes, skeletal and cardiac muscle, brain, and kidney (46,60). Insulin-induced intracellular signalling results in translocation of the intracellular GLUT4 to the cell membrane and enhances GLUT4 activity (61,62).

Hexokinase (HK) catalyzes glucose phosphorylation, which is the first step in glucose uptake in skeletal muscle. Two HK isoforms, HKI and HKII, are expressed in human skeletal muscle, but only HKII is regulated by insulin (63), which is a physiological regulator of HKII mRNA expression in skeletal muscle in vivo (64). The balance between glycogen synthase and glycogen phosphorylase acitivites determines *in vivo* net glycogen synthesis. The activity of glycogen synthase is regulated by covalent glucose phosphorylation. Muscle glycogen

synthesis is impaired in insulin resistant conditions. The defect occurs in the glucose transporter and hexokinase part of the pathway, which controls the rate of glycogen synthesis. In hyperinsulinemic conditions, which simulate postprandial state, glycogen synthase enhances (65).

2.1.2. Lipid metabolism

The liver synthesizes triglycerides from FFA or via *de novo* lipogenesis from carbohydrate (66,67,68). Triglycerides are either stored in the hepatocytes or released to the circulation in very low density lipoprotein (VLDL) particles. Postprandially insulin stimulates intravascular lipolysis via LPL action on chylomicrons and VLDL. Fatty acids are taken-up by muscle and adipose tissue where they are oxidized or esterified into triglycerides. Recent human studies indicate that the proportion of FFA that is available for uptake outside adipose tissue and muscle in tissues such as the liver is greater than previously thought (69). Acutely insulin lowers plasma triglyceride and VLDL levels in vivo by inhibiting hepatic VLDL production and by stimulating LPL (70), which promotes FFA uptake in muscle and adipose tissue (71).

2.1.3. Protein metabolism

Amino acids are an important source of energy and precursors of nitrogenous compounds. Essential amino acids cannot be synthesized *de novo* therefore they have to be obtained from diet. The liver is the main site of synthesis and inter-conversion of non-essential amino acids (72). Alanine is the most important gluconeogenic amino acid. Skeletal muscle contains over 50% of all free amino acids of the body and is the main source of these for GNG during fasting (72,73). After a meal amino acids contribute to enhanced insulin secretion. Insulin promotes protein synthesis in the liver and muscle (74).

2.1.4. Vascular function

Insulin slowly increases blood flow in skeletal muscle under intravenously maintained normoglycaemic hyperinsulinaemic conditions (75). Insulin also acutely decreases wave reflection in the aorta in healthy subjects (76), and treatment with insulin decreases central aortic pressure in subjects with type 2 diabetes (77). The ability of insulin to increase peripheral blood flow is mediated by NO (36,78). Insulin increases eNOS activity in endothelial cells (36). Insulin also induces vasodilatation in human forearm vessels by activating of endothelial Na^+K^+-ATPase (79).

2.1.5. Other actions

Physiological concentrations of insulin increase the activity of the sympathetic nervous system (80) and inhibit platelet aggregation in healthy subjects (81,82). Insulin also acutely lowers serum potassium concentrations by stimulating potassium uptake by skeletal muscles and the splanchnic bed (83). In addition, insulin increases intracellular calcium concentrations in vascular smooth muscle cells (84) and inhibits uric acid (85), sodium, potassium and phosphate excretion by the kidney (86).

2.2. Insulin resistance

Insulin resistance can be defined as a blunted biological response to one or several actions of insulin (87). Resistance to insulin action may involve actions such as its ability to inhibit hepatic glucose production (88,89), stimulate glucose uptake (90,91), inhibit lipolysis (92), decrease serum triglyceride concentrations (93,94) or platelet collagen interaction (82) or any other of the multiple actions of insulin. In non-diabetic individuals, insulin resistance leads to an increase in fasting serum insulin concentrations, hypertriglyceridemia and low high density lipoprotein (HDL) cholesterol (87). If insulin resistance is observed in the absence of known causes such as counterregulatory hormone excess or other diseases than type 2 diabetes, insulin resistance is attributed to the “insulin resistance syndrome” or “metabolic syndrome” (95,96).

Several genetically engineered mice models have been developed to clarify the mechanisms of insulin resistance in liver, muscle and adipose tissue. Liver-specific insulin receptor knockout mice (LIRKO) are severely insulin resistant and develop glucose intolerance because of a failure of insulin to suppress hepatic glucose production. The early insulin signaling cascade, such as IRS-1 and IRS-2 phosphorylation, is impaired within these mice (97). Muscle-specific insulin receptor knockout mice (MIRKO) have an increased fat mass, serum triglycerides, and free fatty acids but normal glucose tolerance (98). Adipocyte-specific inactivation of the insulin receptor gene in fat-specific insulin receptor knockout mice (FIRKO) mice produces selective insulin resistance in adipose tissue, but doesn’t affect whole-body glucose metabolism. These mice have a low fat mass and they are protected against obesity-related glucose intolerance. FIRKO mice have also elevated adipocyte complement-related protein of 30 kDa (ACRP30) protein expression in adipocytes and elevated ACRP30 serum concentrations (99).

2.2.1 Causes

2.2.1.1. Obesity

Epidemiological studies have shown that both overall and upper-body obesity are closely correlated with insulin resistance (100). Upper body fat distribution is associated with insulin resistance after adjusting for overall obesity (101). Obesity impairs both insulin stimulation of glucose uptake and insulin inhibition of EGP (102). Obesity is also associated with defects of vascular actions of insulin, such as impairement of insulin-induced vasodilatation (103), a defect in insulin mediated decrease of large artery stiffness (104), and the ability of insulin to inhibit platelet aggregation (82).

Upper body obesity is characterized by an increase in visceral fat (101), which has been suggested to have greater ability to mobilize FFA than subcutaneous fat (105). The FFA released from visceral fat has been implicated to link visceral adiposity to insulin resistance in the liver (100,106). This "Portal Theory", however, has been criticized, because catheterization studies have suggested that FFA released by the splanchnic bed account for maximally 10% of total FFA delivery to the liver (105). Although intra-abdominal adipocytes have been shown to be more insulin resistant than subcutaneous adipocytes and generate FFA more easily (107), according to catheterization studies, increased delivery of FFA to the liver in upper body obesity is due to excessive release by upper body subcutaneous rather than visceral adipose tissue (108,109). Visceral fat could also induce insulin resistance via release of adipokines suhc as interleukin-6 (IL-6). Omental fat secretes 3-fold more IL-6 than subcutaneous fat, although IL-6 secreted from isolated adipocytes accounts only for ~10% of total adipose tissue release (110).

2.2.1.2. High fat diet

High intake of saturated fat may be associated with impairment insulin sensitivity, although human data are sparse. Animal studies have suggested that both the type and the amount of dietary fat modulate insulin sensitivity. Although in animal studies diets with a very high fat content have been shown to decrease insulin sensitivity in rats (111), especially in the liver (112), only a few human epidemiological studies have found an association between high consumption of saturated fat and incidence of type 2 diabetes (113). Moderate increase of fat in the diet (45% of saturated fat) didn't impair insulin sensitivity in healthy subjects (114), although a diet with high monounsaturated fat content may improve insulin sensitivity in

patients with type 2 diabetes (115). In the KANWU study 162 healthy subjects were placed for 3 months on a saturated fat diet (SAFA diet) or on a diet containing monounsaturated fatty acids (MUFA diet). Insulin sensitivity decreased by 10% during the SAFA as compared to the MUFA diet. Addition of n-3 fatty acids (ω-3 fatty acids) to the diet didn't alter insulin sensitivity or insulin secretion. Low density lipoprotein (LDL) cholesterol increased significantly on the SAFA and decreased on the MUFA diet. When total fat intake exceeded 37% of total energy intake, no significant difference was found between effects of SAFA or MUFA diets on insulin sensitivity (116). It is not known in humans, which tissue(s) contribute to impoved insulin sensitivity during dietary interventions.

An inverse correlation between insulin sensitivity and dietary intake of total fat, oleic acid and ω_6 polyunsaturated fatty acids (PUFA) was found in Insulin Resistance Atherosclerosis Study (IRAS), but it was no longer significant after adjusting for body mass index (BMI) (117). In the Nurses' Health Study a positive association was also found between the intake of saturated fat and incidence of type 2 diabetes (118). Although higher consumption of fish and long-chain ω-3 fatty acids is associated with a lower risk for cardiovascular diseases and total mortality (119), long chain ω-3 fatty acid supplementation doesn't seem to enhance insulin sensitivity in humans (116,120,121,122).

2.2.1.3. Physical inactivity

Physical inactivity is associated with insulin resistance and hyperinsulinemia (123,124) and an increase in the risk of developing type 2 diabetes independent of other factors (125,126,127). In a prospective cohort study (Nurses' Health Study) with 70 000 participants, an inverse association between physical activity and incidence of type 2 diabetes was found (128). Low physical fitness within men increases the risk of type 2 diabetes by 2.6 fold even when after adjusting for age, smoking, alcohol consumption and family history of diabetes (129). Physical training has been shown to increase muscle insulin sensitivity in normal subjects and type 2 diabetic patients (130), when measured directly using the insulin clamp technique combined with positron emission tomography (131) or catheterization techniques (132).

Adenosine monophosphate –activated protein kinase (AMPK) is an energy-sensing enzyme, which responds to decreased ATP/AMP ratio in conditions such as muscle contraction,

hypoxia and ischaemia. AMPK activation enhances peripheral insulin sensitivity and increases fat oxidation in skeletal mucle. AMPK activation increases fatty acid oxidation and inhibits glucose production also in liver (133). AMPK activation decreases fatty acid, triglyceride and sterol synthesis and increases fatty acid oxidation and ketogenesis also in the liver. In rodents high-intensity treadmill exercise activates liver AMPK and increases plasma glucagon concentrations whereas prolonged low-intensity running has no effect on liver AMPK activity (134).

2.2.1.4. IGT and IFG

Both impaired glucose tolerance (IGT) and impaired fsting glucose (IFG) refer to abnormal glucose homeostasis, which is intermediate between normal and diabetes (135,136). Fasting hyperglycemia is primarily due to an increase of hepatic glucose production (137). A decrease of first phase insulin secretion and insulin resistance of glucose uptake characterize IGT, but none of these abnormalities consistently appears to precede the others when examined over the full range of glucose tolerance (138). Instead there appears to be a linear decrease in both first-phase insulin secretion and insulin sensitivity in progression from normal glucose tolerance to IGT. In severely insulin resistant subjects low insulin sensitivity and impaired first-phase insulin release have been shown to predict the onset of type 2 diabetes (139) but only ~30% of all subjects with IGT will later develop type 2 diabetes (87).

2.2.1.5. Gestational diabetes

GDM is defined as a diabetes that is first diagnosed during pregnancy (140,141). The prevalence of GDM varies from 0.2 to 12.3% of pregnancies depending on which criteria are used and which population is examined (142,143,144,145). Positive glutamic acid decarboxylase (GAD) antibodies have been found in 1.6 to 5.0% of women with previous GDM (146,147,148,149). These women are at risk of developing type 1 diabetes (148).

Pregnancy itself is a physiological cause of insulin resistance (150,151,152,153). Women with GDM have in addition an impaired insulin secretory response to glucose as compared to normal without GDM (151,154). This increases the risk of later developing type 2 diabetes (155,156). Obesity before (155,157,158,159), weight gain during (155,160,161) and after (157,160) pregnancy, and IGT postpartum (159) all increase the risk for future development of type 2 diabetes. In addition, women with previous GDM are at high risk for future

cardiovascular disease (162), dyslipidemia (163), and hypertension (164,165). Weight loss is therefore especially important in women with GDM.

2.2.1.6. Type 2 diabetes

Type 2 diabetes is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both (136). Although type 2 diabetes is characterized by multiple metabolic abnormalities already prior the development of diabetic glucose tolerance, the diagnosis is still solely based on elevated plasma glucose concentrations. Increased endogenous hepatic glucose production due to hepatic insulin resistance and relative insulin deficiency (13) and increased levels of FFA (166) characterizes insulin resistance in type 2 diabetes. Insulin resistance of glucose utilization is also found in peripheral tissues, especially skeletal muscle (167). Patients with type 2 diabetes have relative, rather than absolute, insulin deficiency, and they are at high risk for future cardiovascular complications (135,168,169).

2.2.1.7. Type 1 diabetes

Insulin resistance is a common feature also in type 1 diabetes, especially if glycemic control is poor (170). Hyperglycemia *per se* has been shown to acutely cause insulin resistance in type 1 diabetes, independent of changes in serum insulin and counterregulatory hormone concentrations (171). The insulin resistance in type 1 diabetes is due to impaired insulin-stimulated glucose extraction rather than to defects in insulin-stimulated blood flow (172). Improvement in glycemic control by continuous subcutaneous insulin infusion (CSII) (173) or spontaneous remission of the disease reverses insulin resistance (174).

2.2.1.8. Counterregulatory hormone excess

Counterregulatory hormones like glucagon, catecholamines, growth hormone, and cortisol are released during hypoglycaemia and other conditions associated with mental or physical stress (175). All these hormones have insulin-antagonistic effects both in the liver and peripheral tissues. Glucagon and catecholamines, such as adrenalin, act rapidly, whereas the action of cortisol and growth hormone comes occurs over a period of several hours (175,176). Glucagon has an important role in regulating glucose counterregulatory factors in hypoglycemia (177). Glucagon stimulates hepatic GNG and GL during hypoglycaemia (178), but it has no effects in periphery. In pheochromocytoma catecholamine overproduction (179,180), and in agromegaly an excessive amount of growth hormone (181,182) leads to

insulin resistance. Growth hormone infusion has been shown to increase plasma insulin concentrations without altering suppression of glucose production and cause insulin resistance in humans due to impairment in effect of insulin in both liver and skeletal muscle (183). In Cushing's syndrome the overproduction of cortisol causes insulin resistance in humans (184). Cortisol overproduction stimulates hepatic gluconeogenesis and increases hepatic secretion of VLDL and decreases the uptake of LDL by the liver, and cortisol-infusion has been shown to induce both hepatic and peripheral insulin resistance (185,186). Plasma glucagon, cortisol and growth hormone responses to insulin-induced hypoglycemia are impaired in patients with type 2 diabetes (187).

2.2.1.9. Other causes

Hypophosphatemia causes insulin resistance and is associated with hyperinsulinemia and impaired glucose tolerance (188,189). Acute ethanol administration induces insulin resistance (190,191). Several electrolyte disturbances such as hypercalcemia (189,192), hypokalemia (193), hypomagnesemia (194), and several medications such as diuretics (195), non-selective β-blockers (196), protease inhibitors (197), and cyclosporin (198) cause insulin resistance. Androgens have been implicated to contribute to the insulin resistance that is often present in a polycystic ovary syndrome (PCO) (199). In addition, a positive family history of type 2 diabetes may increase the susceptibility to insulin resistance (126).

2.2.2. Mechanisms

2.2.2.1. FFA

Plasma FFA concentrations are elevated in obesity (101,200,201,202,203) and often in type 2 diabetes (203,204). High levels of circulating FFA have been suggested to provide a link between obesity, type 2 diabetes and insulin resistance. Infusion of FFA increases hepatic GNG in normal subjects and in patients with type 2 diabetes, but due to autoregulation EGP remains unchanged (205,206). Raising plasma FFA concentrations by lipid infusion counteracts insulin-induced suppression of EGP and induce hepatic insulin resistance (207,208,209,210). Under these conditions insulin inhibition of GL appears to prevent hepatic autoregulation. Increased levels of plasma FFA concentrations inhibit insulin-stimulated glucose uptake by decreasing glycogen synthesis and carbohydrate oxidation (208,209,211,212). Reduced glycogen synthesis is associated with decreased glucose-6-phosphate levels suggesting that FFA impair glucose transport or phosphorylation (213).

In rodents increased levels of FFA impair insulin the signaling cascade by reducing both IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity resulting a decrease in insulin-stimulated muscle glycogen synthesis and glucose oxidation (214). Lam et al. have demonstrated that insulin resistance induced by FFA in the liver was associated with an increase in hepatic protein kinase C-delta (PKC-δ) translocation, which may be a key mediator of FFA-induced hepatic insulin resistance (215). In addition, FFA directly influence on several genes that are involved in hepatic lipid and glucose metabolism, such as SREBP-1 (216), PEPCK (217) and PPAR (218).

2.2.2.2. Fatty acid composition

Triglycerides, phospholipids, and sterols (cholesterol) are three main forms of fat in the human diet. Triglycerides, which are formed from glycerol and three fatty acids, account for over 95% of the fat ingested in all forms of food. Fatty acids can be classified into saturated, monounsaturated, and polyunsaturated depending on the number of double bonds (**Table 5**). Palmitic (16:0) and stearic (18:0) acids are the most common dietary saturated fatty acids. Linoleic (18:2 n-6) and α-linolenic acids (18:3 n-3) are the two essential fatty acids, which have to be obtained from the diet. All other fatty acids can be synthesized endogenously (67). Essential fatty acids and their products are needed for the formation of specific eicosanoids like prostaglandins, leukotrienes and thromboxanes (219).

Dietary fatty acids have been suggested to affect insulin resistance independent of total fat intake but the mechanisms are poorly understood. In humans the fatty acid composition of diet has been shown to reflect fatty acid composition of serum phospolipids (220,221). When measured with the use of the euglycemic clamp technique, insulin sensitivity has been associated with low proportions of palmitic (16:0) and with high proportions of α-linolenic (18:3 n-3) and especially of dihomo-γ-linolenic (20:3 n-6) acids in serum cholesterol esters (222). In rodents the percentage of long-chain n-3 fatty acids in skeletal muscle phospholipids correlates strongly with insulin action implying that these long-chain n-3 fatty acids may be important for efficient insulin action in skeletal muscle (111). Fatty acid composition of the phospolipids in skeletal muscle correlates with insulin sensitivity also in humans (223). Decreased concentrations of polyunsaturated fatty acids in skeletal muscle phospholipids associate with insulin resistance in normal subjects (223), and high proportion of long-chain

n-3 unsaturated fatty acids in skeletal muscle correlate positively with insulin sensitivity in insulin resistant subjects (224).

2.2.2.3. Hyperglycemia

Chronic hyperglycemia contributes to impaired insulin secretion and peripheral insulin resistance (225,226). The degree of insulin resistance, especially in patients with type 1 but also in patients with type 2 diabetes, is proportional to the severity of hyperglycemia (87). In rodents, glucose-induced insulin resistance may result from over activity of the hexosamine pathway (227), which impairs insulin signalling at several levels (228). However, human data linking glucose toxicity to over activity of the hexosamine pathway are sparse.

2.2.2.4. Liver fat

The term non-alcoholic fatty liver disease (NAFLD) characterizes a spectrum of abnormalities in liver function ranging from minor steatosis to a non-alcoholic steatohepatitis (NASH) (229). NASH is a form of chronic hepatitis with histological characteristics similar to alcohol hepatitis including ballooning degeneration of hepatocytes, parenchymal inflammation, liver cell necrosis or fibrosis (230). NALFD is the most common cause of elevated liver enzyme concentrations (231). The most common conditions associated with fatty liver disease are presented in **Table 1**.

Table 1. *Conditions associated with a fatty liver {Adapted from (231)}*
1. Insulin resistance 2. Alcohol
3. Hepatitis B
4. Hepatitis C
5. Hemochromatosis
6. Drugs (amiodarone, diltiazem, tamoxifen, steroids, highly active antiretroviral therapy)
7. Disorders of lipid metabolism (abetalipoproteinemia, hypobetalipoproteinemia)
8. Toxic exposure (environmental, workplace)
9. Severe weight loss
10. Total parenteral nutrition
11. Lipoatrophy

Thirty percent of patients with NAFLD will later develop fibrosing steatohepatitis and cirrhosis (232). Data from the Third National Health and Nutritional Exam Survey (NHANES III), where over 15 000 participants were followed during years 1988-1994, showed that 7.9% of adults in the US had elevated ALT concentrations. High alcohol consumption, viral hepatitis and hemochromatosis explained only 31% of elevated ALTs whereas NALFD explained 69% (231,233,234,235). NAFLD is associated with insulin resistance in both normal weight (236) and obese subjects (237), but the majority (39 to 90%) of NALFD patients are obese (232,238,239). Adults with NAFLD are twice as likely to develop diabetes than those without NAFLD, even after adjustment of BMI, age, gender and race (231). Data from a study of nearly 3000 healthy subjects showed that 6% of men and 2% of women had increased serum ALT concentrations and these subjects had a four-fold increased risk for later development of diabetes (240). Increased serum ALT concentrations associate with decreased hepatic insulin sensitivity and predict the development of type 2 diabetes independently of obesity in Pima Indians (241). Type 2 diabetes is found 50% (238) and hypertriglyceridemia up to 92% of patients with NASH (236). However, it is unknown to what extent liver fat is associated with insulin resistance independent of overall and visceral obesity.

Fat accumulation in insulin sensitive tissues such as muscle and liver has recently been suggested to be an important determinant in insulin resistance. Fat accumulation in the liver has been shown to associate with several features of insulin resistance independent of body weight in healthy men (7). Hepatic fat content, within the subclinical range of fatty liver, correlated closely inversely with hepatic insulin sensitivity in patients with type 2 diabetes. In this study the patients with fatty liver were also more obese than those with a low liver fat content (8). Since fatty liver coexists with obesity and insulin resistance (242,243), fat accumulation in the liver may reflect the capacity of the liver to esterify and store incoming FFA as cytosolic triglycerides when triglyceride synthesis exceeds hepatic fatty acid oxidation and VLDL-triglyceride secretion (244). It is, however, unclear whether liver fat correlates with insulin resistance independent of obesity.

Several mechanisms could lead to fat accumulation in the liver. These include increased delivery of FFA to the liver from visceral or subcutaneal fat depots (245). Visceral obesity has been associated with insulin resistance in both men (246) and in women (247), but the causal relationship is still a matter of debate (106,248). Obesity, increased visceral fat volume, and

high plasma concentrations of FFA have been associated with liver fat accumulation in patients with type 2 diabetes (249). According to "Portal theory" visceral fat depots increase FFA flux to the liver (100), *de novo* lipogenesis (250), decrease β-oxidation of FFA, and impair VLDL synthesis or secretion (242,251,252). Regarding *de novo* lipogenesis, in mice chronic hyperinsulinemia and carbohydrate ingestion stimulate *de novo* lipogenesis (253) by stimulating the activity of lipogenic enzymes such as SREBP-1c, and down-regulating IRS-2 -mediated insulin signaling in insulin resistant state in the liver (254,255), which promotes fat accumulation in the liver. The FFA stored in the liver could originate from hydrolysis of dietary chylomicrons, adipose tissue, and *de novo* lipogenesis (67). The contributions of the various sources to fat accumulation in the human liver are unknown.

Adipocytokines *(vide infra)* may regulate hepatic fat content. Tumor necrosis factor-α (TNF-α) gene expression is increased in adipose tissue in insulin resistant obese and type 2 diabetic patients (256). In patients with NASH, TNF-α gene expression is increased in both hepatocytes and adipose tissue (257). Decreased serum adiponectin concentrations correlate inversely with hepatic fat content in patients with type 2 diabetes (24) and in lipodystrophy (258). In the perfused liver adiponectin enhances hepatic insulin sensitivity and decreases liver fat content (259,260). This effect appears to be mediated by adiponectin-induced increase of carnitine palmitoyl transferase 1 (CPT-1) activity, which enhances hepatic fatty acid oxidation, and by increased AMPK activation, which inhibits acetyl coenzyme A carboxylase (ACC) and thereby decreases fatty acid synthesis (260). FFA may also cause hepatic insulin resistance by impairing hepatic insulin signaling (261). Increased availability of FFA increases malonyl-CoA because of stimulation of ACC. Malonyl-CoA inhibits CPT-1, which is required to transport FFA into the mitochondria, where beta-oxidation occurs. This inhibits FFA oxidation and results in accumulation of triglycerides in the liver (262). The role of PPARγ in regulating hepatic fat content is discussed in *Chapter 2.2.3.3.*

Patients with congenital lipodystrophy have little subcutaneous fat, but are severely insulin resistant and have increased fat accumulation in insulin-sensitive tissues, especially in liver and muscle (263,264,265,266). Several lipoatrophic mice models have been developed to clarify the mechanism of fat-induced insulin resistance in liver. Aricid extension-leucine zipper lipoatrophic mice (A-ZIP/F-1) are severely insulin resistant with no subcutaneous or visceral fat, but have increased fat accumulation in the liver and skeletal muscle (267).

Treatment of these mice with fat transplantation completely reverse insulin resistance (268,269) implying that fat accumulation in the liver causes insulin resistance and that visceral fat is not necessary for the development of insulin resistance in these animals. Mice with overexpression of nSREBP-1c in adipose tissue lack subcutaneous fat but have a fatty liver and are insulin resistant. Mice with fatty liver dystrophy are characterized by a fatty liver, adipose tissue deficiency and glucose intolerance (270,271). Mice, whose insulin receptor in the liver has been knocked out (LIRKO), develop severe insulin resistance and glucose intolerance (272). Later these mice develop a fatty liver. Mitochondria of hepatocytes are enlarged suggesting increased oxidative stress in these cells (272).

The reasons for the large interindividual variation in liver fat content are unclear. Physical fitness as determined by measuring maximal oxygen uptake (VO_2max) doesn't seem to explain intervidual differences in liver fat content in healthy men (7). An increase in fat intake has been shown to increase liver fat in dogs (273) and rats (112,274), but whether this occurs in humans is unknown. It is also unknown, how genetic factors influence liver fat content.

2.2.2.5. Intramyocellular lipid

Intramyocellular triglyceride content can be measured by quantitative histochemistry staining in intramuscular fibers taken by percutaneous muscle biopsy technique (275,276). Computed tomography (276) and MRS (277,278) are non-invasive methods used to determine intramuscular triglyceride content. Especially MRS has recently been used as an imaging method for assessing muscle lipid content, because it ables to distinguish between intra- and extramyocyte lipid content (278). Skeletal muscle insulin sensitivity has been found to correlate with intramyocellular fat in normal weight healthy subjects (279), offspring of type 2 diabetic parents (280), nondiabetic Pima Indians (281), and in subjects with type 2 diabetes. Intramyocellular lipid may interfere with insulin signalling via several mechanisms. As triglycerides are chemically inert themselves, they are unlikely to cause insulin resistance but rather serve as a marker on lipid intermediates (282), which actually may cause insulin resistance.

In vivo studies in healthy subjects have shown that acute elevation of FFA by a lipid infusion increases intramyocellular lipid (IMCL) during hyperinsulinemia and impairs peripheral glucose uptake (283,284). Subjects with high as compared to low intramuscular fat content

have blunted insulin-induced tyrosine phosphorylation of the insulin receptor and IRS 1-associated PI 3-kinase activity (275). In the latter study serum FFA concentrations were also higher during hyperinsulinemia than in subjects with low intramyocellular lipid (275).

Long-chain acyl-CoAs (LCACoAs), which are the activated forms of intracellular FFA, are increased in insulin resistant animals (285) and in humans with increased intramyocellular lipid (286). LCACoAs inhibit hexokinase activity (287) and activate protein kinase C (PKC) (288), which reduces glucose uptake and impairs insulin signalling. LCACoAs also induce *de novo* synthesis of ceramide, a phospholipid component of cell membranes, which inhibits insulin signalling (289). DAG (1,2-diacylglycerol), which can be generated by *de novo* synthesis through the esterification of LCACoA to glycerol-3-phosphate or by breakdown of phospholipids, has also been suggested to activate PKC and impair insulin signalling (290). Activation of PKC leads to a serine/threonine phosphorylation cascade and increased serine phophorylation of IRS-1 and IRS-2, which in turn leads to decreases in tyrosine phosphorylation of IRS-1, PI 3-kinase activity, GLUT4 translocation (291). Impaired mitochondrial function has also been found to correlate with intramyocellular lipid content and insulin resistance in lean, healthy offspring of patients with type 2 diabetes (292).

2.2.2.6. Adipokines

Adipocytes synthesize and secrete several soluble peptides or proteins called adipokines that influence on insulin sensitivity and glucose metabolism (293). Such adipokines include adiponectin, TNF-α, IL-6, leptin, and resistin, although the latter may not be expressed in human adipocytes (294).

Adiponectin

Adiponectin (Arcp30, adipoQ, adipose most abundant gene transcript 1 apM1) is a polypeptide exclusively and highly expressed and synthesized in adipose tissue (19). Serum adiponectin concentrations are decreased in obese (20,21) and type 2 diabetic subjects (22,23), and in insulin resistant first-degree relatives of type 2 diabetic patients (295). Low serum adiponectin concentrations have been associated with an increased risk for development of type 2 diabetes (296) and cardiovascular events (297). Hyperinsulinemic-euglycemic clamp studies have revealed that the degree of hypoadiponectinemia is closely correlated with the degree of insulin resistance (298,299). Plasma adiponectin concentrations

have been shown to correlate with basal and insulin-suppressed hepatic glucose production (300) and with hepatic fat content in patients with type 2 diabetes (24). In addition, patients with lipodystrophy have low levels of serum adiponcetin, which correlate with hepatic fat content (258).

Adiponectin administration to rodents has been shown to reduce plasma glucose concentrations in obese Zucker rats (301), wild-type and diabetic mice (302), and to lower hepatic glucose production without affecting peripheral glucose uptake (303). In addition, adiponectin inhibits the production and action of TNF-α and several adhesion molecules (304). Adiponectin appears to enhance insulin sensitivity by increasing fat oxidation (303,305) and reducing intracellular triglyceride content in the liver (259,260). These changes may be mediated by adiponectin-induced increase of CPT-1 activity, which enhances hepatic fatty acid oxidation, and increased AMPK activation, which inhibits ACC thus decreasing FFA synthesis (260). Weight loss (23,306) and TZDs have been shown to increase serum adiponectin concentrations in type 2 diabetic patients (22,24,307,308,309).

TNF-α

TNF-α is a cytokine, which is secreted from activated macrophages in response to infection or injury (310,311). TNF-α can directly alter glucose homeostasis and lipid metabolism and antagonizes insulin action (312). TNF-α mRNA is expressed also in adipocytes (256). TNF-α expression in adipose tissue is increased in obesity and type 2 diabetes (256,313), and correlates with several markers of insulin resistance, such as fasting serum insulin and triglyceride concentrations (314). It is unclear, whether TNF-α is released from adipose tissue to the circulation *in vivo* in humans (315). Macrophages may be the predominant source of TNF-α in human adipose tissue (316). TNF-α inhibits insulin–stimulated glucose uptake in 3T3-L1 adipocytes (317) and down-regulates IRS-1, GLUT4 and CCAAT/enhancer-binding protein-α (C/EBP-α) expressions (318,319). TNF-α also decreases LPL mRNA expression and inhibits CEBP-α and PPARγ expression, two key regulators of adipose tissue differentiation (319,320). Weight loss decreases TNF-α expression in adipose tissue (313).

IL-6

IL-6 is a multifunctional, pro-inflammatory cytokine produced mainly by adipocytes, but also by immune cells, endothelial cells, fibroblasts, and myocytes (321). One third of total

systemic IL-6 concentrations has been estimated to be secreted by adipose tissue (315). IL-6 plasma concentrations correlate positively with obesity and insulin resistance (322). IL-6 protein content in adipose tissue correlates inversely with *in vivo* insulin-stimulated glucose uptake, and *in vitro* glucose uptake in human subcutaneous adipocytes (323). IL-6 influences insulin action by several mechanisms, but the precise action is not fully understood. IL-6 appears to increase hepatic triglyceride secretion without decreasing the clearance of triglyceride-rich lipoproteins, indicating that the hypertriglyceridemia is due to increased secretion by the liver. IL-6 also stimulates LPL action and increases lipolysis thus enhancing delivery of FFA to the liver (324). In liver cells IL-6 inhibits insulin-induced glycogen synthesis and impairs insulin signalling by inhibiting IRS-1 and PI 3-kinase activity (325). Moreover, in 3T3-L1 adipocytes IL-6 impairs glucose transport by decreasing transcription of IRS-1, GLUT4, and PPARγ genes (326). Insulin has been shown to increase IL-6 mRNA expression in 3T3-L1 (327) and human adipocytes (328), whereas the glucocorticoid dexamethasone markedly suppresses IL-6 production in isolated human adipocytes (329). Weight loss decreases IL-6 concentrations (330).

Leptin

The ob gene protein product leptin is synthesized mainly in adipose tissue (331). Leptin affects on energy balance, food intake, and body weight by regulation of hypothalamic-pituitary-endocrine axes (332), but it has also several other actions, such as an effect on immune function, angiogenesis and hematopoiesis (333). Plasma leptin concentrations are elevated in obesity (21), and leptin mRNA content in adipocytes is twice as high in obese than in lean subjects (334). Plasma leptin concentrations correlate with subcutaneous fat volume (335) and BMI (336), and diet induced weight loss decreases plasma leptin concentrations (334). Administration of leptin to rodents decreases food intake and increases energy expenditure (337). TZDs have been shown to suppress leptin synthesis in adipose tissue both in rodents (338) and humans (339). Insulin stimulates leptin secretion in human adipocytes in a dose-dependent manner (328). Patients with lipdystrophy have low concentrations of circulating leptin. Administration of leptin to these patients markedly reduced liver and muscle fat content and improved insulin-stimulated glucose uptake (340).

2.2.3. Treatment

2.2.3.1. Weight loss

Diet

The beneficial effects of weight reduction on insulin resistance by caloric restriction are well documented (341,342). Two large intervention studies - Diabetes prevention study (DPS) and Diabetes prevention program (DPP) - showed clearly that with changes of lifestyle, especially with weight reduction and increase of exercise, the development of diabetes could be prevented by 58% (10,11). The Swedish Obese Subjects study (SOS) also demonstrated that great weight loss induced by gastric banding operation in morbid obese patients is associated with 80% reduction in the 8-year incidence of type 2 diabetes (343). Several studies have documented the beneficial effect of weight loss on peripheral (344,345,346,347) and hepatic insulin sensitivity (348,349,350).

The improvement of insulin sensitivity and glucose metabolism could to be mediated via weight loss induced reduction of intramuscular (351,352) and liver fat content (353,354,355,356), but data are sparse (357). Massive (20%) weight loss induced by gastroplasty has been shown to enhance hepatic insulin clearance (346), which may be due to decreased liver fat content. Weight loss also has beneficial effects on serum concentrations of triglycerides, LDL- and HDL-cholesterol and blood pressure (347,358). Weight loss increases serum adiponectin concentrations (306,359), and decreases leptin (360), IL-6 (361) and TNF-α concentrations (359).

Orlistat

Orlistat is an inhibitor of gastric and pancreatic lipases, which at a dosage of 120 mg t.i.d. reduces fat absorption by approximately 30% (362) and has been proven to be useful in facilitating both weight loss and maintenance (363,364,365,366,367). Weight loss is accompanied by loss of both subcutaneous and visceral fat and by improved insulin sensitivity (368,369,370,371). Inhibition of fat absorption with orlistat has been associated with greater improvement in insulin sensitivity and lipid profile than use of placebo (366,372,373,374,375,376,377,378), but it is unclear whether these beneficial metabolic effects are specific to orlistat or secondary to a hypocaloric diet and weight loss (366,372). Inhibition of fat absorption by orlistat also has a modest LDL -cholesterol lowering effect (365). A 4-year prospective placebo-controlled study with orlistat showed that orlistat with

lifestyle changes decreases the incidence of type 2 diabetes as compared to lifestyle and placebo treatment (364).

Sibutramine

Sibutramine is a serotonin-norepinephrine re-uptake inhibitor that induces weight loss by enhancing satiety, suppressing appetite and by promoting energy expenditure (379,380). Sibutramine has been shown to lower plasma glucose levels and improve lipid profile in obese subjects (381) and patients with type 2 diabetes (382,383) It has, however, been reported to increase blood pressure (384) and heart rate (379), which may limit its use in type 2 diabetic patients who often have hypertension.

Surgery

Gastric bypass, gastric partioning, gastroplasty, and recently gastric banding are surgical procedures, which can be used to treat massively obese patients. Morbidly obese (BMI 52 kg/m^2) subjects, who underwent gastric bypass operation, lost 47 kg of body weight, intramuscular lipid deposits decreased by ~30%, and insulin sensitivity improved by 92% (352). Weight loss induced by gastroplasty has also been shown to decrease significantly liver fat content in obese subjects with NASH (347,354). Recently, a study of Dixon et.al (385) demonstrated that massive weight loss (-34 kg of body weight, -52%) induced by gastric banding significantly improved hepatic steatosis and histological changes in obese patients with NALFD, NASH or hepatic fibrosis. Similarly several features of metabolic syndrome improved.

2.2.3.2. Exercise

Both acute exercise and physical training have been shown to increase insulin stimulated glucose uptake in skeletal muscle. The ability of exercise to increase insulin sensitivity has been suggested to be mediated via enhanced glucose transport, increased glycogen synthesis, increased muscle mass, and augmented muscle blood flow (386). Physical training has also been shown to enhance insulin-induced glucose uptake in skeletal muscle by improving insulin action on oxidative enzymes, increasing activity of glycogen synthase, and by increasing the proportion of oxidative red fibers in skeletal muscle (387). In addition, improved mitochondrial oxidative enzyme capacity and activation of the glucose transport system have been suggested to contribute to improvement in insulin sensitivity (386). Skeletal

muscle adapts to exercise, such as prolonged running or swimming, with an increase in mitochondrial density (388). Physical exercise has been found to increase mitochondrial gene expression and oxidative capacity, possibly through an increase in the expression of the PPARγ coactivator PGC-1, which overexpression in muscle markedly increases insulin sensitivity, GLUT4 expression and the proportion of oxidative red fibers (389).

2.2.3.3. Antihyperglycemic therapies

Metformin

Metformin is a biguanide derivate, which lowers blood glucose concentrations by inhibiting endogenous glucose production (14,15,390). *In vitro* studies have shown that metformin either increases (391) or has no effect (392) on adipose tissue glucose uptake. *In vivo* studies have shown that metformin either increases (392) or doesn't change skeletal muscle or whole-body insulin sensitivity (393). Weight loss with reduced visceral adiposity (394), or reduced glucose toxicity may explain the improved glycemic control (395). Metformin has also been found to facilitate or prevent weight loss when used as monotherapy (15) or combined with insulin (396). In addition, metformin has beneficial effects on plasma lipid concentrations (397) and significantly reduces the risk of cardiovascular disease in the UK Prospective Diabetes Study (UKPDS) (398).

Although metformin has been used in management of type 2 diabetes for more than 40 years, its molecular mechanism of action has been unclear. Zhou et. al. (399) have recently demonstrated that in isolated rat hepatocytes metformin decreases hepatic glucose production by activating AMPK. Activation of AMPK suppresses ACC activity (400), stimulates fatty acid oxidation, muscle glucose uptake (401) and expression of cAMP-stimulated gluconeogenic genes such as PEPCK and glucose-6-phosphatase (402). In rodents, chronic activation of AMPK induces the expression of muscle hexokinase and GLUT4 thereby mimicking effects of physical training (403). Metformin increases AMPK activity in cultured skeletal muscle cells of patients with type 2 diabetes. This was accompanied by enhanced peripheral glucose disposal (404). It is, however, unclear, whether these in vitro effects are relevant to metformin action in vivo. High concentrations of metformin in isolated hepatocytes have also been found to reduce mitochondrial NADH:NAD^+ ratio (405) thus lowering cellular ATP levels, which leads to increased flux through pyruvate kinase (406) and reduced gluconeogenesis. Moreover, metformin has been shown to inhibit oxidative

phosphorylation and lower cellular ATP levels (407) and increase the active forms of glycogen synthase and glycogen phosphorylase in the liver and skeletal muscle of diabetic mice indicating enhanced glycogen turnover (408). In ob/ob mice with a fatty liver metformin has been shown to reverse hepatomegaly and steatosis (409), possibly via AMPK-mediated inhibition of SREBP-1 and FAS (399). In an uncontrolled study with the patients with NASH was shown that metformin decreased liver volume by 20% and improved insulin sensitivity (410). Effects of metformin on liver fat in humans have not been studied.

PPARγ agonists

TZDs, troglitazone, pioglitazone and rosiglitazone, are a relatively new class of oral antidiabetic drugs. The first agent in this group, troglitazone, was withdrawn due to hepatotoxicity (411). TZDs are PPARγ agonists, which bind tightly to the transcription factor PPARγ (412), which is predominantly expressed in adipose tissue (413) and to a lesser extent in skeletal muscle, endothelial cells, macrophages and the heart (414). Activation of PPARγ by TZDs has been shown to promote adipose-cell differentiation in vitro (16,17). The action of TZDs in adipose tissue, skeletal muscle and liver is not completely clarified. It has been suggested that TZDs acts mainly in adipose tissue and the effects on liver and muscle are indirect via changes of circulating concentrations of fatty acids and adipocytokines, or that TZDs improve insulin sensitivity by direct interaction with muscle and liver (27).

Activation of PPARγ by TZDs induces expression of numerous genes that regulate fatty acid metabolism and lipid storage (18). TZDs increase adipocyte P2 enhancer (aP2) gene expression both in cultured cells and in transgenic mice (415), PEPCK gene expression in cultured 3T3-F442A preadipocytes and adipocytes (416), and long-chain-acyl-CoA synthetase (acylCoA) activity in liver and adipose tissue in rodents (417). PPARγ agonists also increase fatty acid transport protein 1 (FATP-1) mRNA expression in 3T3-L1 cells, preadipocytes and adipocytes (418) in rat adipose tissue and to a lesser extent in muscle, but not in liver (419). TZDs also activate LPL gene expression in rat adipose tissue and 3T3-L1 preadipocytes (420), increase GLUT4 mRNA expression in cultured fibroblasts (421), and glucokinase expression in the liver of diabetic ZDF rats (422). Activation of PPARγ by TZDs also improves insulin signaling by increasing the expressions of IRS-1 (423), IRS-2 (424) and p85 subunit of PI 3-kinase (425) in cultured 3T3-L1 and human adipocytes. PPARγ agonists also increase in β-cells the gene expressions of GLUT2 and β-glucokinase leading to the

restoration of the glucose-sensing ability of β-cells (426).

Tissue specific knockout mice have been developed to clarify the action of PPARγ on glucose homeostasis in muscle, liver and adipose tissue. Muscle specific PPARγ knockout mice (MuPPARγKO) are obese and have hepatic insulin resistance, but insulin-stimulated glucose uptake in muscle is not impaired (427). The hepatic insulin resistance might be secondary to altered adipokine release associated with increased adiposity. The expression of ACRP30 in adipose tissue is reduced which also may induce hepatic insulin resistance. Despite disruption of muscle PPARγ, treatment with TZDs improves whole body glucose homeostasis indicating that the insulin-sensitising effects of TZDs on muscle are indirect (427). In contrast, in a study by Hevener et. al. (428) muscle specific PPARγ deletion in mice induced glucose intolerance and progressive insulin resistance, but treatment of TZDs didn't have any effect on insulin sensitivity in these mice implicating a crucial role of PPARγ in the maintenance of skeletal muscle insulin action, the etiology of peripheral insulin resistance, and the action of TZDs.

The fat PPARγ deficient (FKOγ) mice develop general lipodystrophy, fatty liver with hepatic insulin resistance, and exhibit increased plasma FFA and triglyceride concentrations, decreased leptin and ACRP30 concentrations. Treatment with TZDs increases insulin-stimulated muscle glucose disposal and normalizes hepatic insulin sensitivity (26). Deletion of PPARγ2 selectively from white adipose tissue ($PPAR\gamma^{hyp/hyp}$) leads to severe lipodystrophy, hyperlipidemia and growth retardation. Interestingly, $PPAR\gamma^{hyp/hyp}$ mice have hepatomegaly with a fatty liver at birth, but the livers of adults have no signs of fatty liver, and the adults have only mild glucose intolerance with low serum adiponectin and leptin concentrations. In $PPAR\gamma^{hyp/hyp}$ mice genes controlling FFA catabolism are activated, whereas expression of genes involving in FA synthesis remain unchanged. Treatment with PPARγ agonist alleviated the glucose intolerance, but not the insulin resistance, suggesting that white adipose tissue, not the liver or muscle, is crucial for insulin sensitivity by PPARγ agonists (429).

Liver-specific ablation of the PPARγ gene in wild type (WT LKO) and AZIP $PPAR\gamma^{fl/fl}$ (AZIP LKO) mice were used to study the function of liver PPARγ. Inactivation of liver PPARγ reduced hepatic steatosis in both lipoatrophic AZIP mice and diet-induced obesity, and increased adipose tissue mass and insulin resistance WT LKO mice. Inactivation of liver PPARγ in the AZIP LKO abolished the hypoglycemic and hypolipidemic effects of

rosiglitazone, implying that in the absence of adipose tissue the liver is a primary and major site of thiazolidine action (430). Consistent with this study with aP2/DTA mice, whose fat is eliminated by fat-specific expression of diphtheria toxin A, suggested that TZDs do not require adipose tissue to improve insulin sensitivity, whereas liver and skeletal muscle might be more important target tissues of rosiglitazone (271).

PPARγ agonists have been shown to enhance insulin sensitivity and alter body composition also in humans. Three months' treatment with rosiglitazone improved insulin-stimulated glucose uptake and decreased hepatic triglyceride content, and enhanced insulin's ability to suppress lipolysis in patients with type 2 diabetes, but this study was uncontrolled (431). In a placebo-controlled study by Carey et. al. (17) demonstrated that 16 weeks treatment with rosiglitazone improved insulin sensitivity and increased subcutaneous fat by 8% as compared to placebo. In a placebo-controlled study in patients with human immunodeficiency virus (HIV) lipodystrophy rosiglitazone also decreased liver fat content, and improved insulin sensitivity without changing body fat composition (432). Treatment with pioglitazone has also been shown to reduce hepatic insulin resistance, improve total body glucose uptake, and shift visceral fat to subcutaneal fat depots compared to sulfonylurea treatment (433). In an uncontrolled study pioglitazone was also shown to reduce hepatic fat content, improve peripheral insulin sensitivity, and insulin-mediated suppression of EGP (434). In addition, TZDs have been found to increase of plasma adiponectin concentrations both in rodents and in humans (22,435,436,437). PPARγ-agonists have been demonstrated to decrease liver fat content and serum ALT levels and to improve peripheral insulin sensitivity also in patients with NASH (438,439).

Other

Sulphonylureas enhance peripheral glucose disposal (440), however, these effects are most likely secondary to a reduction of glucotoxicity (394). Glipizide therapy has been suggested to enhance hepatic insulin sensitivity while glucose-induced glucose uptake remained unaffected (441).

Insulin

The major indication for insulin treatment in type 2 diabetes is hyperglycemia that is not adequately controlled by oral glucose-lowering agents. Insulin therapy improves whole body

glucose disposal by enhancing peripheral insulin sensitivity (442,443) and decreasing hepatic glucose production (173,444). Insulin treatment has also been shown to decrease serum FFA (445), and improve endothelial dysfunction (446) in patients with type 2 diabetes. Variation in hepatic insulin sensitivity and hepatic fat content is an improtant factor in determing insulin requirements during combination therapy with insulin (8).

3. AIMS OF THE STUDY

The present studies were conducted to answer the following questions:

1) How does liver fat content relate to features of insulin resistance in obese women with a history of gestational diabetes (**I**)?

2) How does 8% weight loss induced by a hypocaloric, low fat diet combined with orlistat or placebo influence liver fat content and body composition in obese women with a history of gestational diabetes (**II**)?

3) How does 8% weight loss induced by a hypocaloric, low fat diet combined with orlistat or placebo influence insulin sensitivity and the composition of serum free fatty acids in obese women with a history of gestational diabetes (**III**)?

4) How does 16 weeks treatment with rosiglitazone or metformin influence insulin sensitivity, liver fat content, hepatic glucose production and gene expression in adipose tissue in patients with type 2 diabetes (**IV**)?

4. SUBJECTS AND STUDY DESIGNS

Baseline characteristics of the subjects are shown in **Table 2.** The participants of the *Studies I, II* and *III* were women, who were treated at the Department of Obstetrics and Gynecology at Helsinki University Hospital during years 1987-1999 because of GDM. They had to fulfill the following inclusion criteria: i) previous GDM, ii) current age 20 to 50 years, iii) BMI 28-35 kg/m^2, and iv) no known any acute or chronic disease. A 2-hour oral glucose tolerance test with 75 g glucose (OGTT) was performed to exclude diabetes. Women who had lost over 3 kg of body weight during the last 6 months were excluded. Other exclusion criteria included pregnancy, lack of reliable contraception, postmenopausal status, treatment with drugs that may alter glucose tolerance, or any clinical or biochemical evidence of any significant disease other than obesity. A total of 57 women were included in these studies. Liver fat content was analyzed from 27 of these women. **(Fig. 2).**

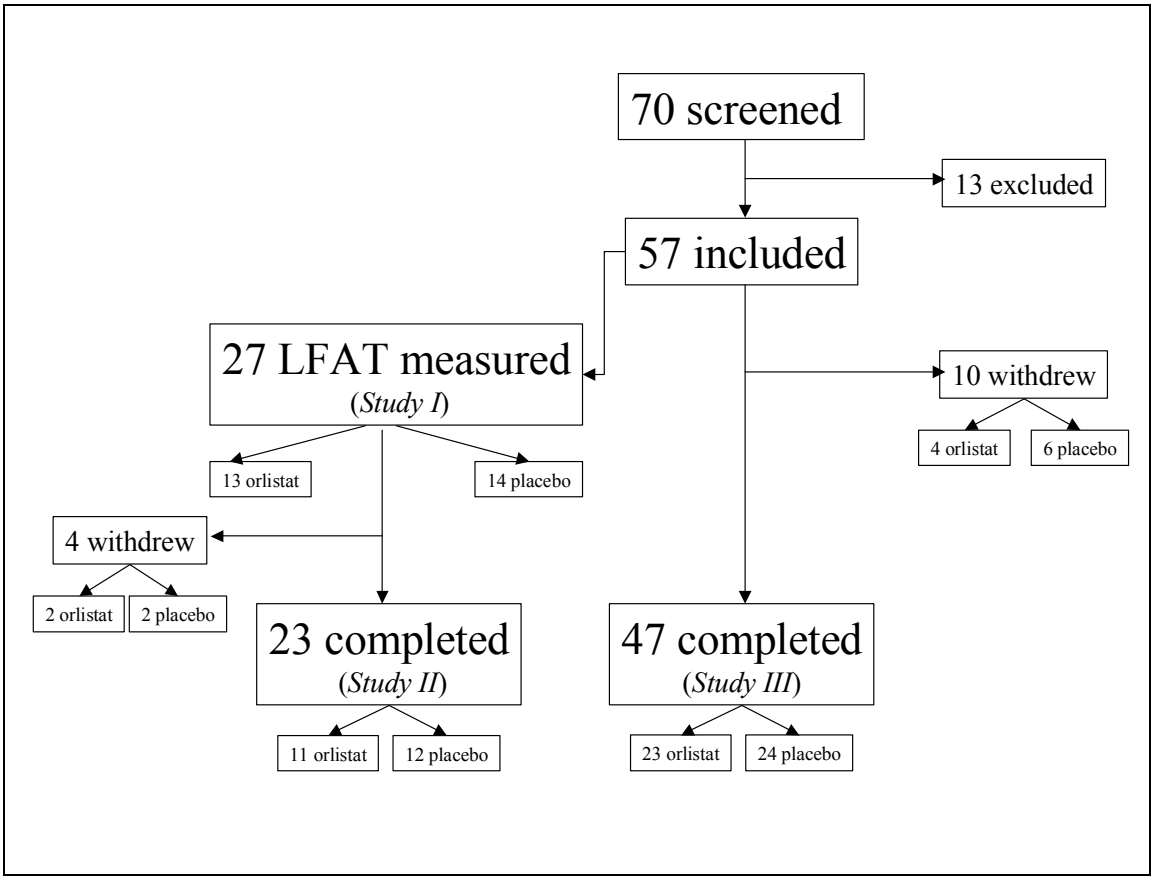

Figure 2. *Schematic picture of the numerals of screened, excluded, and completed participants, who were recruited from the hospital records of women treated because of gestational diabetes mellitus (GDM). Liver fat content (LFAT) was randomly measured from 27 women (Studies I, II and III).*

In *Study IV* a total of 22 patients with diet-treated type 2 diabetes were included. At the screening visit, the patients underwent a history and physical examination, and blood samples were taken for measurement of glycosylated hemoglobin A_{1C} (HbA_{1C}), fasting plasma glucose, liver enzymes, and thyroid stimulating hormone (TSH). GAD antibodies were analyzed to exclude late-onset type I diabetes. Two of these patients were withdrawn from the study. One of the women didn't appear to the final measurements, and one man had to be withdrawn because of inadequate control of diet and misuse of study medication.

Specific aims and designs of the individual studies are listed below. Written informed consent was obtained from all subjects. All studies were approved by the ethics committee of the Department of Medicine at the Helsinki University Central Hospital

Table 2. Characteristics of the study subjects.

	STUDY I		STUDY II		STUDY III		STUDY IV	
	Low LFAT	**High LFAT**	**Low LFAT**	**High LFAT**	**Orlistat**	**Placebo**	**Rosiglitazone**	**Metformin**
Number of subjects	13	14	12	11	23	24	9	11
Women/Men	11/0	11/0	12/0	11/0	23/0	24/0	6/3	7/4
Age (y)	36 ± 1	38 ± 1	37 ± 2	37 ± 1	39 ± 1	39 ± 1	50 ± 3	46 ± 4
Weight (kg)	90 ± 2	92 ± 2	89 ± 2 2	92 ± 2	89 ± 1	91 ± 1	90 ± 5	84 ± 4
BMI (kg/m^2)	32 ± 1	33 ± 1	32 ± 0.6	32 ± 0.6	32 ± 0	32 ± 0	31 ± 1	31 ± 1
W/H	0.95 ± 0.02	0.97 ± 0.02	0.94 ± 0.02	0.96 ± 0.02	0.94 ± 0.01	0.95 ± 0.02	0.97 ± 0.02	0.98 ± 0.02
Body fat (%)	37 ±1	36 ± 1	36 ±1	37 ±1	36 ±0	37 ±0	34 ±2	33 ± 3
fP-glucose (mmol/l)	5.8 ± 0.2	5.8 ± 0.1	5.9 ± 0.2	5.7 ± 0.2	5.7 ± 0.1	5.6 ± 0.1	8.8 ± 0.8	8.2 ± 0.7
fS-insulin (mU/l)	10 ± 1	14 ± 2 *	9 ± 1	15 ± 2*	9 ± 1	10 ± 1	12 ± 1	14 ± 2
HbA1c (%)	5.5 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	7.0 ± 0.4	6.9 ± 0.4
C-peptide (nmol/l)	0.77±0.05	1.00 ± 0.08*	0.76 ± 0.05	0.98 ± 0.10	0.77 ± 0.06	0.75 ± 0.05	0.73±0.09	0.78±0.11
S-cholesterol (mmol/l)	5.2 ± 0.2	5.1 ± 0.3	5.3 ± 0.2	5.2 ± 0.3	5.5 ± 0.2	5.0 ± 0.1*	4.7 ± 0.4	5.0 ± 0.2
S- HDL-cholesterol (mmol/l)	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3± 0.1	1.3 ± 0.1	1.11 ± 0.1	1.1 ± 0.1
S-LDL-cholesterol (mmol/l)	3.4 ± 0.2	3.1 ± 0.2	3.4 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.1 ± 0.1*	2.9 ± 0.3	2.9 ± 0.2
S-triglycerides (mmol/l)	1.1 ± 0.1	1.9 ± 0.2*	1.3 ± 0.1	1.7 ± 0.2*	1.5 ± 0.2	1.4± 0.1	1.5 ± 0.1	2.3 ± 0.5

Data are shown as mean ± SEM. *$p<0.05$ for differences between the groups

Study I

Aim: To investigate how liver fat accumulation relate to features of insulin resistance in obese women with a history of gestational diabetes.

Design: Twenty-seven obese (BMI 32±1 kg/m^2), otherwise healthy premenopausal women with a history of gestational diabetes were studied. Current diabetes was excluded by an OGTT. Liver fat content (LFAT), intra-abdominal and subcutanous fat volumes and insulin sensitivity by euglycemic clamp (**Fig. 3**) were measured. To determine other features of insulin resistance serum FFA and triglycerides concentrations were measured and non-invasive 24-h blood pressure monitoring was performed. The women were divided into two groups based on their median LFAT (5%); low LFAT (3.1±0.3%) and high LFAT (9.8±1.5%) groups.

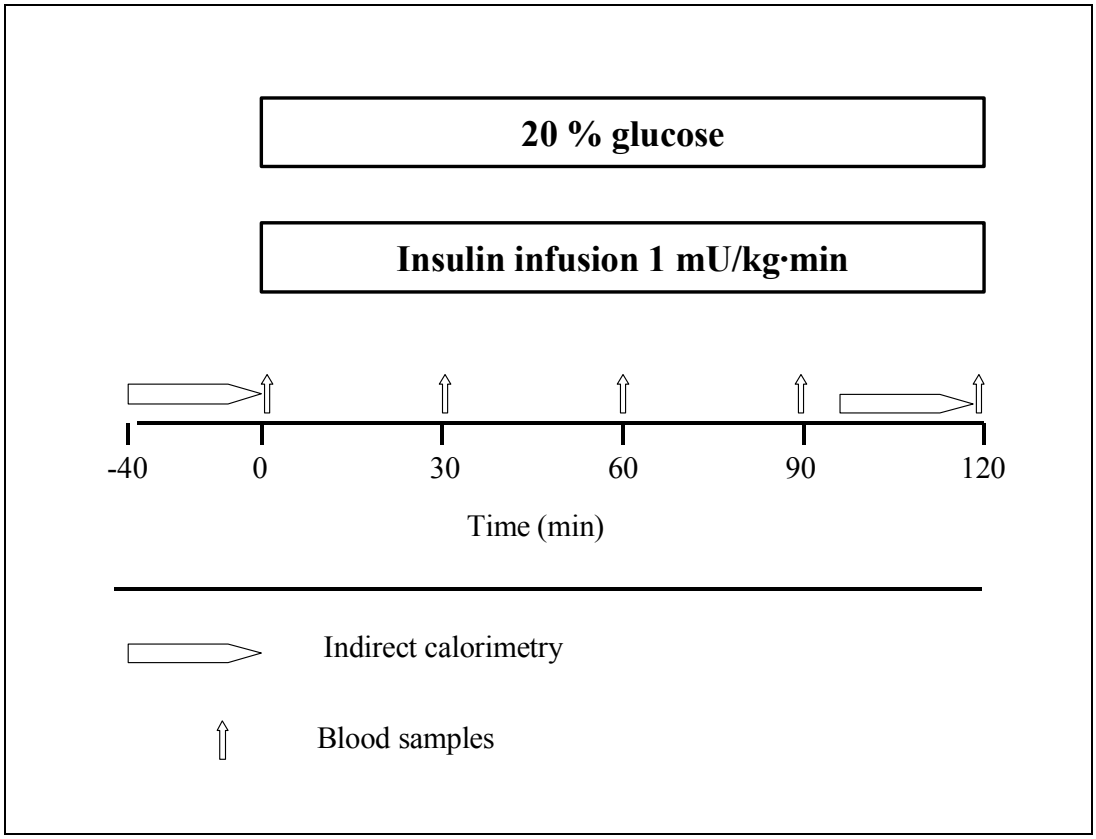

Figure 3. *Design of the euglycemic hyperinsulinemic clamp in Studies I and III.*

Study II

Aim: To determine how moderate (8%) weight loss during 3 to 6 months induced by a hypocaloric, low fat diet combined with orlistat or placebo influences liver fat content, body composition and other features of insulin resistance in obese women with a history of gestational diabetes.

Design: A total of 27 obese women, who participated in study I, started the weight loss protocol (**Fig. 4**). Four of the 27 women were unable to reach the weight loss goal of 8% during 3 to 6 months. A total of 23 women were therefore included into the final analyzes. At baseline a 2-hour OGTT was performed to exclude diabetes. The women were randomized to receive either orlistat (120 mg t.i.d) or placebo in addition to the hypocaloric diet, which was controlled by two expert dieticians (K.L., K.K.). LFAT, intra-abdominal and subcutaneous fat volumes, body weight, waist to hip ratio (WHR), and serum fasting insulin and triglyceride concentrations were measured before and after weight loss. As in s*tudy I*, the women (n=23) were divided into low liver fat (3.3±0.4%) and high liver fat (9.4±1.4%) groups based on their median LFAT (5%).

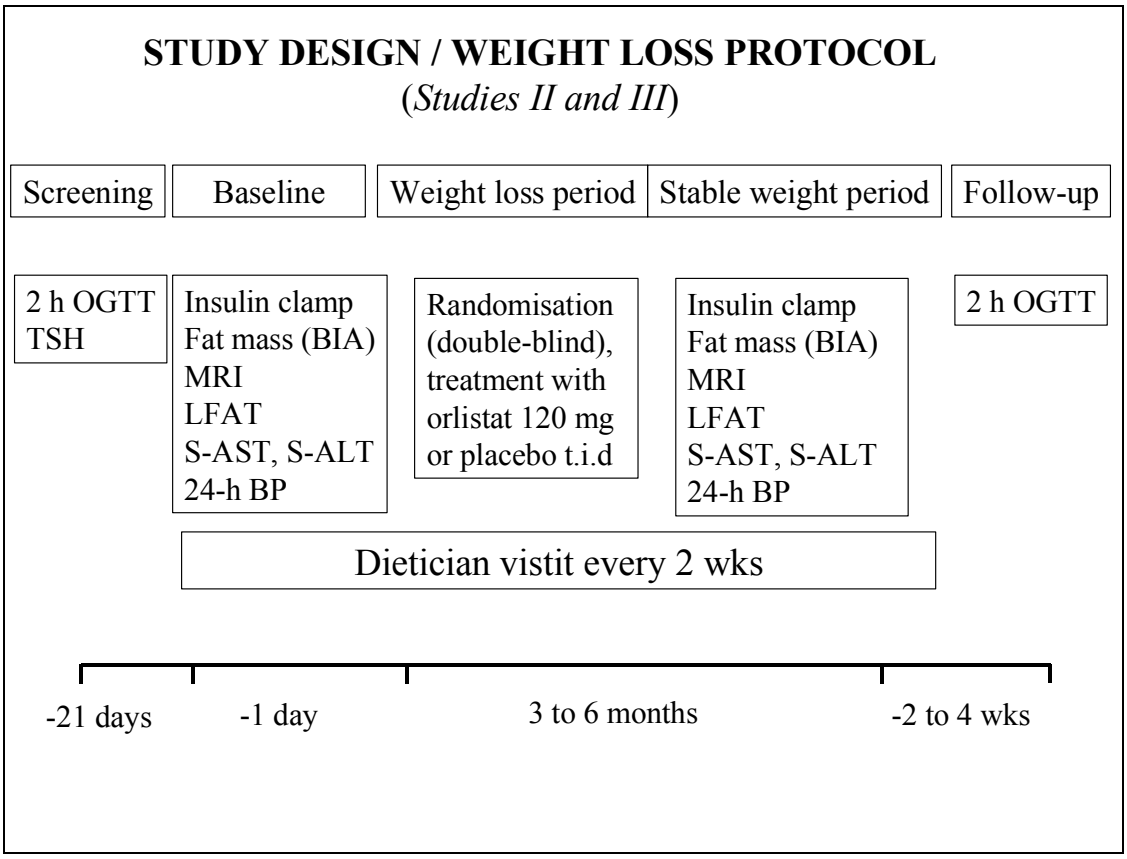

Figure 4. *Study design of the weight loss protocol. Design of the metabolic studies and measurments of body composition before and after weight loss (Studies II and III).*

Study III

Aim: To investigate how 8% weight loss induced by a hypocaloric, low fat diet combined with orlistat or placebo influences insulin sensitivity and the composition of serum FFA in obese women with a history of gestational diabetes.

Design: Obese (n=47, BMI 32.1±0.4 kg/m^2) women with a history of gestational diabetes were randomized to receive either orlistat (120 mg t.i.d) or placebo combined with hypocaloric diet to target 8% weight loss during 3 to 6 months. Whole body insulin sensitivity (**Fig. 3**), intra-abdominal and subcutaneous fat volumes, the % of body fat, weight, WHR and serum fatty acid composition were measured before and after weight loss period.

Study IV

Aim: To examine how 4 months' treatment with rosiglitazone or metformin influences liver fat content, insulin sensitivity, hepatic glucose production and gene expression in adipose tissue among newly-diagnosed patients with type 2 diabetes.

Design: Twenty drug-naive patients with type 2 diabetes (13 women, 7 men) were randomised to treatment with either 8 mg rosiglitazone or 2 grams of metformin for 16 weeks. Peripheral and hepatic insulin sensitivity (**Fig. 5**), LFAT, body composition, and mRNA concentrations (PPARγ, adiponectin, LPL) in subcutaneous adipose tissue were measured before and after treatment.

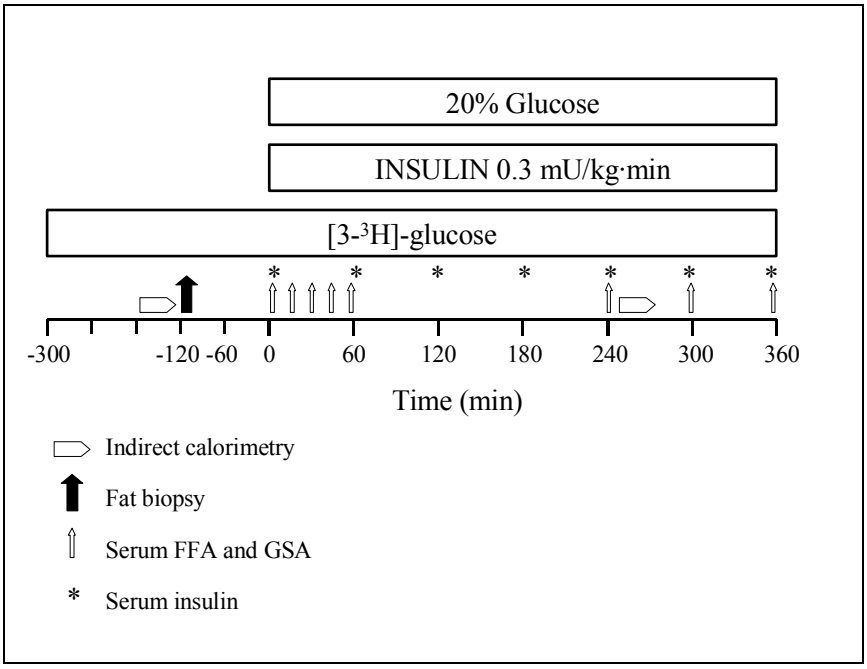

Figure 5. *Measurement of the action of intravenous insulin on endogenous glucose production and utilization using the euglycemic insulin clamp technique combined with an infusion of[3-3H]-glucose (Study IV).*

5. METHODS

5.1. Measures of body composition

5.1.1. Intra-abdominal and abdominal subcutaneous fat volumes

A total of 16 Tesla 1 -weighted transaxial scans reaching from 8 cm above to 8 cm below the fourth and fifth lumbar interspace, were analysed for the determination of intra-abdominal and subcutaneous fat (field of view 375 X 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 software (Alice 3.0; Parexel, Waltham, MA, USA). 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 cutpoint. Intra-abdominal adipose tissue was defined as the area of pixels in the intra-abdominal region above this cut 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. Intra-abdominal fat was finally determined by subtracting the subcutanous fat area from the total abdominal adipose tissue area. Magnetic resonance imaging (MRI) scans were analyzed by a single investigator (M.T.). The reproducibility of repeated measurements of subcutaneous and intra-abdominal fat as determined on two separate occasions in our laboratory in non-diabetic subjects (n = 10, unpublished data) is 3% and 5% (265). An example of MRI quantification of intra-abdominal and subcutaneous fat volumes is shown in **Fig. 6**.

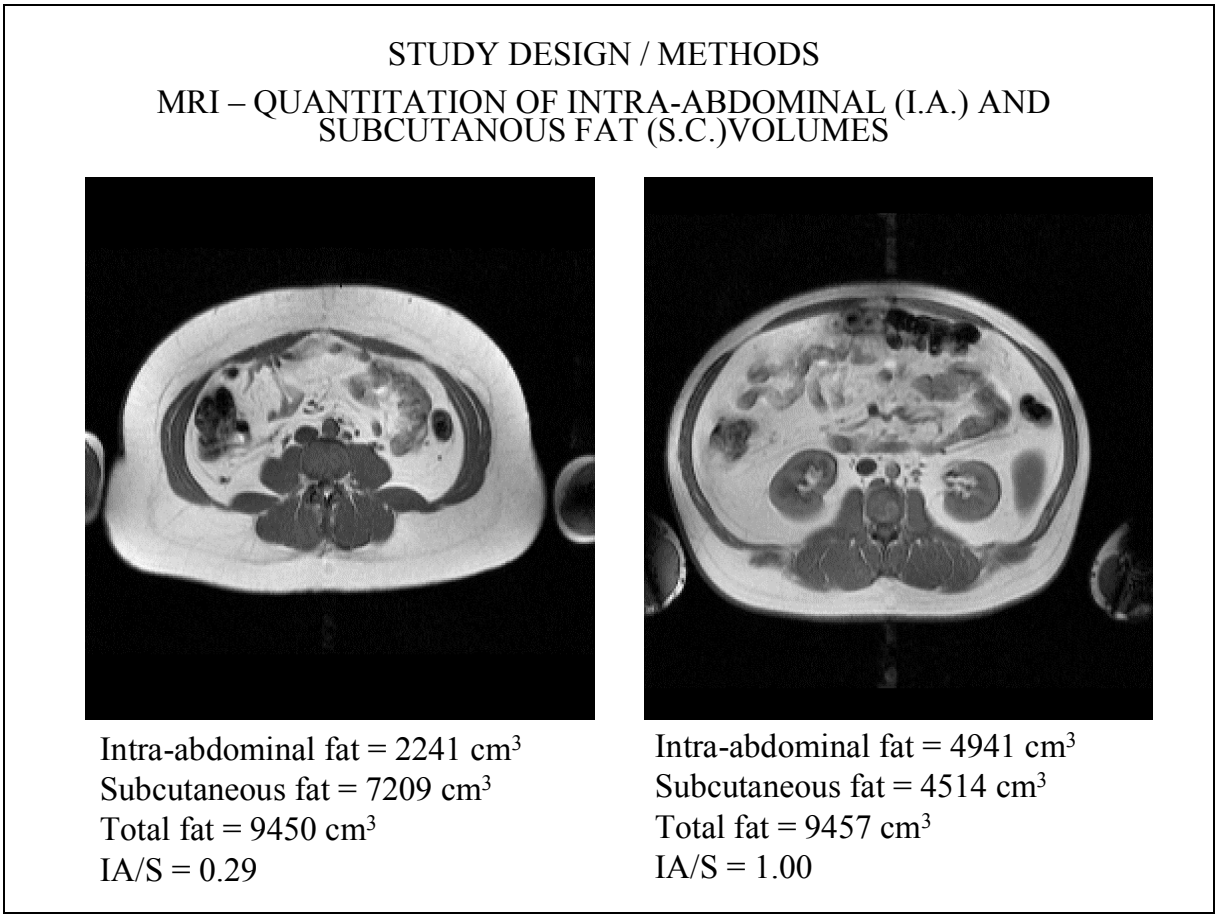

Figure 6. *An example of abdominal scans of two different patients taken at the level of 8 cm above the fourth and fifth lumbar interspaces. Fat tissue is shown in white.*

5.1.2. Whole body fat content

Body composition (fat mass, fat free mass and percentage of body fat) was determined by using bioelectrical impedance plethysmography (BioElectrical Impedance Analyzer System model #BIA-101A; RJL Systems, Detroit, Michigan (447).

5.1.3. 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 (448).

5.2. Liver fat content (*Studies I, II and IV*)

Localized single voxel (2 x 2 x 2 cm^3) proton spectra were recorded using a 1.5 T whole body system (Siemens Magnetom Vision, Erlangen, Germany), which consisted of the combination of whole body and loop surface coils for radiofrequency transmitting and signal receiving. T1-weighted high-resolution magnetic resonance images were used for localization of the voxel of interest within the right lobe of the liver. Vascular structures and subcutaneous fat tissue were avoided in localization of the voxel. Subjects were lying on their stomach on the surface coil, which was embedded in a mattress in order to minimize abdominal movement due to breathing. The single voxel spectra were recorded by 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. The short echo time and the long repetition time were 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 trigyceride, 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 was expressed as methylene/(water + methylene) signal area ratio x 100. This measurement of hepatic fat by proton spectroscopy has been validated against histologically determined lipid content of liver biopsies in humans (449) and against estimates of fatty degeneration or infiltration by computed tomography (8,450). All spectra were analyzed by a physicist (A.H.), who was unaware of any of the clinical data. The reproducibility of repeated measurement of liver fat in non-diabetic subjects studied on two occasions in our laboratory is 11% (n = 10, unpublished data). An example of two spectra with fat percentages of 6 and 20% is presented in **Fig. 7**.

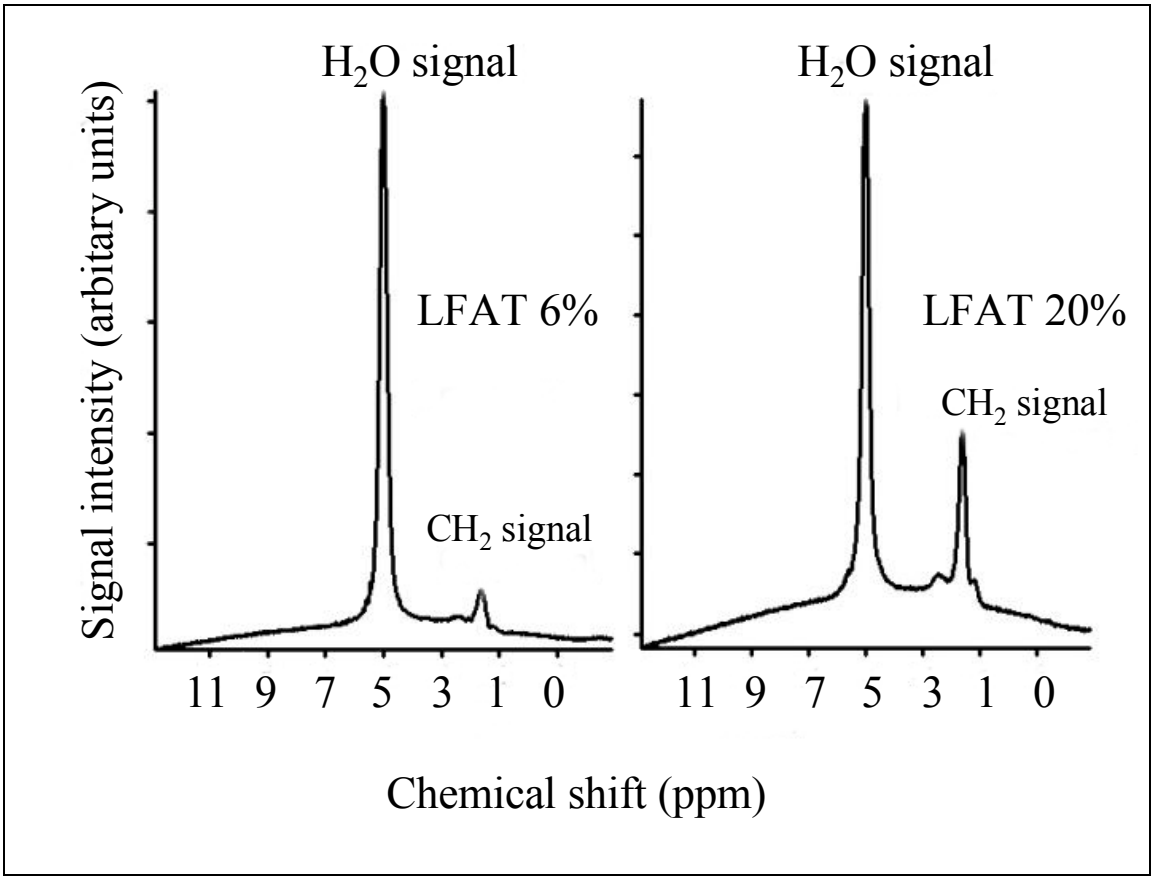

Figure7. *Proton magnetic resonance spectra from 2 patients with fat percentages (% LFAT = a ratio of the area under the methylene peak to that under methylene and water peaks x 100) of 6 and 20 %. The water peak has a chemical shift of 4.8 ppm, and the methylene (CH_2) signal of fat has a chemical shift of 1.4 ppm relative to the water peak. The height of the signal (y-axis) is in arbitrary units.*

5.3. Whole body glucose uptake *(Studies I and III)*

Whole body insulin sensitivity of glucose metabolism (M-value) was determined by using the euglycemic insulin clamp technique (451). 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 in the same arm retrogradely in a vein of dorsal hand, which was kept in a heated (60°C) chamber for withdrawal of arterialized venous blood. Insulin (Actrapid Human, Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner at a rate of 1 $mU/kg^{-1}min^{-1}$ for 120 min. Normoglycemia 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. Before the insulin infusion, blood samples were taken for measurement of fasting glucose, HbA_{1C}, triglyceridess, total and LDL cholesterol, FFA, fatty acid composition of serum phospholipids, and serum free insulin concentrations. Blood samples for serum free

insulin and FFA concentrations were also withdrawn every 30 minutes during the 2-hour insulin infusion. The M-value was calculated from the glucose infusion rate after correction for changes in the glucose pool size and expressed per kilogram of FFM **(Fig 3.)**.

5.4. Hepatic glucose production (*Study IV*)

5.4.1. Measurement of endogenous hepatic glucose production

The patients were admitted to the hospital on the evening before the study at 7:00 P.M., when an indwelling 18-gauge catheter (Venflon, Viggo-Spectramed, Helsingborg, Sweden), equipped with an obturator, was inserted in an antecubital vein. To determine total rates of glucose appearance (Ra) – i.e. the sum of hepatic and renal glucose production (452) and disappearance (Rd) – a primed continuous intravenous infusion of [3–^{3}H]glucose was started at 4:00 A.M. and continued for a total of 660 min. The priming dose of [3–^{3}H]glucose was adjusted according to the fasting blood glucose concentration measured at 4:00 as follows: priming dose = [glucose (mmol/l) at 4:00 A.M./5] x 20 μCi/min. This dose was infused intravenously over 10 min. The continuous rate infusion of [3–^{3}H]glucose was thereafter started at a rate of 0.2 μCi/min. Thereafter a fat aspiration biopsy from the abdominal subcutaneous area was taken under local anaesthesia. The fat sample was immediately frozen and stored in liquid nitrogen until analysis (453). Part of the biopsy was immediately treated with collagenase for 30 min at 37°. From this sample, the diameter of 200 adipocytes was determined using a microscope (454). Before start of the insulin infusion, another catheter (Venflon, Viggo-Spectramed, Helsingborg, Sweden) was inserted in a dorsal hand vein retrogradely. The hand was kept in a heated (60°C) chamber 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 9:00 A.M., after a 300-min equilibrium period, a primed continuous (0.3 $mU \cdot kg^{-1} \cdot min^{-1}$) infusion of insulin (Actrapid Human, Novo Nordisk, Bagsvaerd, Denmark) was started, as previously described (451,455). Plasma glucose was adjusted to and then maintained at 8 mmol/l (144 mg/dl) for 360 min both before and after treatment. Blood samples for measurements for glucose SA were withdrawn at –30, -20, -10 and 0 min and at 15 to 30 min intervals between 120 and 360 min, and for serum free insulin and FFA concentrations at 0, 10, 15, 20, 25 and 30 min and then at 30 to 60 min intervals during the insulin infusion (**Fig. 5**).

5.4.2. [3-^{3}H]glucose specific activity and calculation of glucose kinetics

[3-^{3}H]glucose specific activity (GSA) was measured as previously described (53). Plasma was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ and evaporated. The dried glucose was resuspended in water, counted in a double-channel liquid scintillation counter (Rackbeta 1215; Wallac, Turku, Finland) after adding 10 ml Aquasol liquid scintillation fluid (NEN-DuPont, Boston, MA), and corrected for quenching. GSA (in disintegrations per minute per micromole) was calculated by dividing the disintegrations per minute in 0.3 ml plasma by the plasma glucose concentration (in μmol/ml). The infusate was diluted 1:100 and 1:1,000 and duplicates were counted to determine the infusate [3-^{3}H]- concentration. Glucose R_a and R_d were calculated using the Steele equation, assuming a pool fraction of 0.65 for glucose and a distribution volume of 200 ml/kg for glucose (455). Endogenous glucose R_a was calculated by subtracting the exogenous glucose infusion rate required to maintain isoglycemia during hyperinsulinemia (0-360 min) from rate of total glucose R_a. Since endogenous glucose R_a is very sensitive to even small changes in serum insulin concentrations (43), an index of the sensitivity of basal R_a to insulin was calculated by multiplying fasting basal R_a by the fasting plasma insulin concentration. This index has previously been validated (456). The percent suppression of basal endogenous glucose R_a during the last two hours (540-660 min) by insulin was used as a measure of the sensitivity of endogenous glucose production to insulin (percent suppression of endogenous R_a).

5.5. Ambulatory blood pressure *(Studies I, II and III)*

Non-invasive 24 hour blood pressure monitoring was performed on a normal weekday with an automatic ambulatory blood pressure monitoring device (Diasys Interga Novacor SA, Rueil-Malmaison, France). The device was set to record blood pressure and heart rate every 15 minutes during the day and every 30 minutes during the night. Day and night were defined from awake and sleeping periods in the patient's diary.

5.6. Total RNA and complementary DNA preparation *(Study IV)*

Frozen fat tissue (50-150 mg) was homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX, USA) and total RNA was isolated according to the manufacturer's instructions. After DNase treatment (RNase-free DNase set; Qiagen, Hilden, Germany), RNA was purified using the RNeasy mini kit (Qiagen). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit; Molecular Probes,

Eugene, OR, USA). The quality of RNA was checked by agarose gel electrophoresis. Average yields of total RNA were 3 ± 6 μg per 100 mg adipose tissue wet weight, and did not differ between the groups. Isolated RNA was stored at -80ºC until the quantification of the target mRNAs. A total of 0.1μg RNA was transcribed into complementary DNA using M-MLV reverse transcribed (Life Technologies, Paisley, UK) and oligo $(dT)_{12-18}$ primer.

5.7. Quantification of mRNA using real-time PCR *(Study IV)*

Quantification of mRNA was performed by real-time polymerase chain reaction (RT PCR) using LightCycler technology (Roche Diagnostics GmbH, Mannheim, Germany). An aliquot of 2 μl of 1:10 diluted cDNA was brought to a final volume of 20 μl, which contained 3 mM magnesium chloride, 2 μl of LightCycler-FastStart DNA SYBR Green I Mix (Roche Diagnostics), and 0.5 μM of primes. After initial activation of the DNA polymerase at 95^{o} C for 10 min, the amplification conditions were as follows: 40 cycles consisting of denaturation at 95^{o} C for 15 sec, annealing for 5 sec at 57^{o} C (β–actin), 56 ^{o} C (PPARγ), 58 ^{o} C (LPL), and for 5 sec at 58 ^{o} C (adiponectin) and extension at 72 ^{o} C. The extension times (s) were calculated from the amplicon size (base pairs/25). Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis form 65 ^{o} C to 95 ^{o} C with a heating rate of 0.1 ^{o} C/s with a continuous fluorescence acquisition was made. Human β-actin primers; sense: 5’ –CAC-ACT-GTG-CCC-ATC-TAC-GA-3’, antisense: 5’-CCA-TCT-CTT-GCT-CGA-AGT-CC-3’ (TAG, Copenhagen, Denmark), product size 202 bp, gene bank accession number M10277. PPARγ primers: sense: 5’-CTC-ATA-TCC-GAG-GGC-CAA-3’, antisense: 5’-TGC-CAA-GTC-GCT-GTC-ATC-3’, gene bank accession number U79012. Adiponectin primers: sense: 5’-CAG-AGA-TGG-CAC-CCC-TGG-TG-3’, antisense: 5’-TTC-ACC-GAT-GTC-TCC-CTT-AG-3’ (TAG, Copenhagen, Denmark), product size 75 bp, gene bank accession number XM003191. LPL primers: sense: 5’-GGT-CGA-AGC-ATT-GGA-ATC-CAG-3’, antisense: 5’-TAG-GGC-ATC-TGA-GAA-CGA-GTC-3’, gene bank accession number NM000237.

A standard curve for PPARγ was created using purified cloned plasmid cDNA (QIAquick PCR purificatin kit; Qiagen , Hilden, Germany). For human β-actin, adiponectin and LPL expressions, standard curves were created form a specific PCR product. To account for differences in RNA loading, PPARγ, adiponectin and LPL was expressed relative to β-actin. The mRNA concentration of human β-actin was not different between the groups (226±29 in the rosiglitazone group and 199±25 in metformin group, NS).

5.8. Other measurements

Plasma glucose concentration

Plasma glucose concentrations were measured in duplicate with the glucose oxidase method using a Beckman Glucose analyzer II (Beckman Instruments, Fullerton, CA) (457).

Serum free insulin and C-peptide concentrations

Serum free insulin concentrations were measured by radioimmunoassay (Phadeseph® Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) after precipitation with polyethylene glycol (458) and C-peptide concentrations using time-resolved fluoroimmunoassay (AUTOdelfia™ C-peptide, Wallac, Turku, Finland).

Glycosylated hemoglobin

HbA_{1C} was measured by high-pressure liquid chromatography using the fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA) (459).

Gad-antibodies (Study IV)

GAD-antibodies were measured by radioimmunoprecipitation method (460,461).

Serum lipids and lipoproteins

Serum total, HDL cholesterol and triglyceride concentrations were measured with respective enzymatic kits from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). Low density lipoprotein (LDL) concentration was calculated using the formula of Friedewald (462).

Adiponectin

Serum adiponectin concentrations were measured using a commercial enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, B-Bridge International, San Jose, CA).

Serum free fatty acids

Serum FFA were measured using a fluorometric method (463).

Fatty acid composition of serum phospholipids

The fatty acid methyl ester composition of serum phospholipids (Study III) was determined with gas chromatography after a thin-layer chromatography separation of phospholipids from serum fat extract (464) and interesterification to methyl esters (465). The gas chromatograph HP 6980+ (Hewlett-Packard, Avondale, PA) was equipped with a 25-m silica column NB 351 (HNU-Nordion Ltd, Helsinki, Finland) and a split injection system. Hydrogen was used as carrier gas. The interassay precision varied from 2 % to 10 %, depending on the peak size.

Liver enzymes

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) activities were determined as recommended by the European Committee for Clinical Laboratory Standards.

Diaries of food intake

Food diaries were analyzed using Nutrica® software (Research Centre of the Social Insurance Institution, Helsinki, Finland).

5.9. Statistical analyses

In all studies a p-value less than 0.05 was considered statistically significant. The calculations were made by GraphPad Prism version 3.0 (GraphPad Inc, San Diego, CA) or by SysStat Statistical Package (SysStat version 10, SysStat Inc., Evanston, IL). All data are shown as mean±standard error of mean.

Study I

The unpaired two-sided t-test was used to compare mean values between low and high LFAT groups after logarithmic transformation if necessary. Spearman's nonparametric rank correlation coefficient was used to calculate correlation coefficients between selected variables. Impact of obesity and body composition on the relationships between LFAT and features of insulin resistance were calculated using step-wise multiple regression analysis.

Study II

The unpaired two-sided t-test was used to compare mean values between low and high LFAT groups after logarithmic transformation if necessary. Single measurements before and after weight loss were compared using the paired t test. Spearman's nonparametric rank correlation coefficient was used to calculate correlation coefficients between selected variables.

Study III

The paired and unpaired two-sided t-test, two-factor repeated measures ANOVA and ANCOVA with the baseline values as a covariate were used to compare changes before and after weight loss, and mean values or changes between the orlistat and placebo groups. Non-normally distributed variables were logarithmically transformed. Effects of group, group X time, and time (insulin) on serum FFA concentrations was analyzed using analysis of variance for repeated measures. Pearson's (for normally distributed variables) or Spearman's rank (for non-normally distributed variables) correlation coefficients were used to calculate correlation coefficients between selected variables.

Study IV

The paired and unpaired two-sided t-test were used to compare changes between rosiglitazone and metformin groups. Non-normally distributed variables were logarithmically transformed. Effects of group, group times time and time (insulin) on serum FFA concentrations was analyzed using analysis of variance for repeated measures). Pearson's (for normally distributed variables) or Spearman's rank (for non-normally distributed variables) correlation coefficients were used to calculate correlation coefficients between selected variables.

6. RESULTS

6.1. Liver fat content and insulin resistance in obese women *(Study I)*

Low and high LFAT groups had comparable BMI, intra-abdominal and subcutaneous fat volumes (**Fig. 8**). The groups were also similar with respect to age, WHR and % total body fat (**Table 2**), and they consumed equal amounts of alcohol. Liver enzymes were slightly, but not significantly higher in the group with high LFAT as compared to the group with low LFAT (ALT 31±3 vs. 28±6 U/l, AST 28±4 vs. 23±2 U/l, GGT 30±4 vs. 28±6 U/l, high vs. low LFAT, NS).

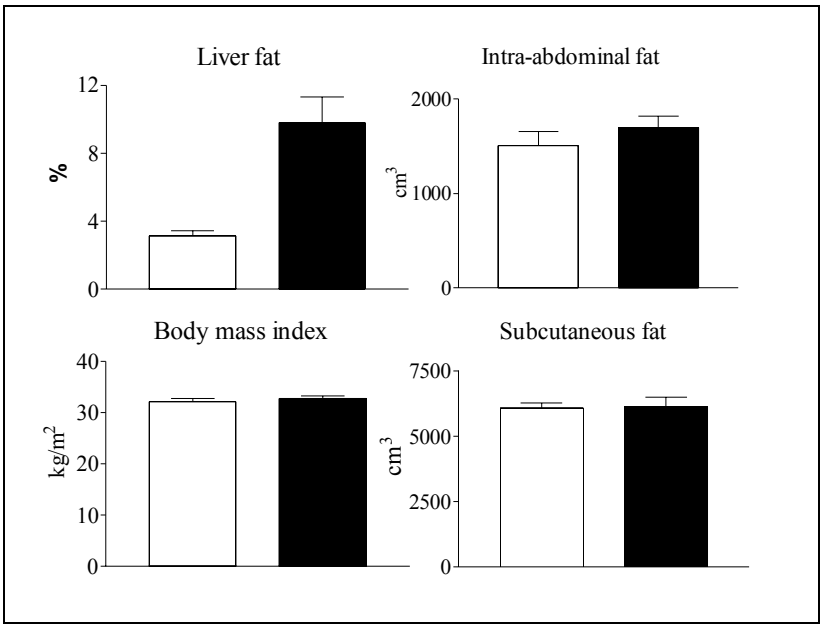

Figure 8. *Liver fat content, body mass index, intra-abdominal and subcutaneous fat content in women with low* (□) *n=13 and high* (■) *n=14 LFAT.*

Glucose and lipid metabolism

Fasting plasma glucose and HbA_{1C} concentrations were comparable between the groups, but fasting serum insulin (14±2 vs. 10±1 mU/l, $p<0.05$, high vs. low LFAT) and C-peptide (1.00±0.08 vs. 0.77±0.05 nmol/l, respectively, $p=0.02$) concentrations were significantly higher in the high than the low LFAT group (**Table 2**).

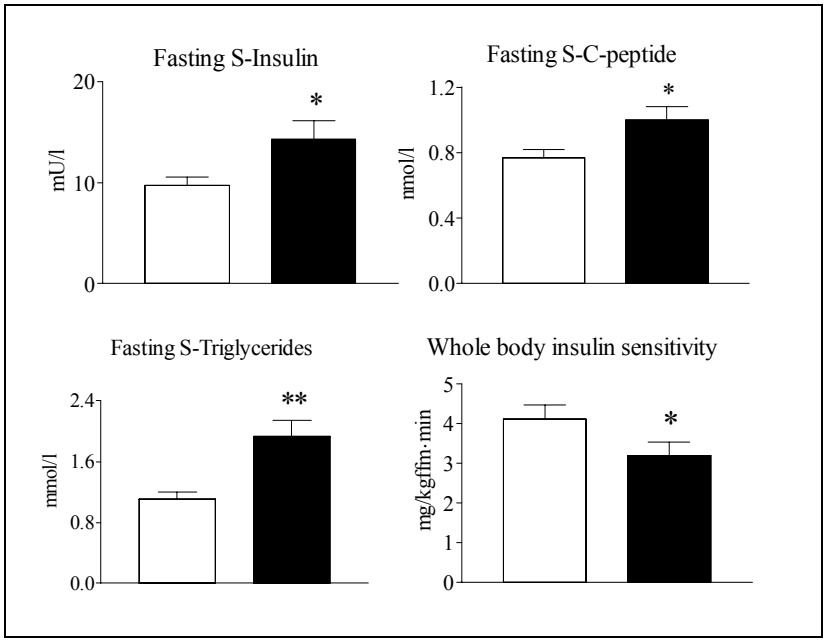

Figure 9. *Fasting serum insulin and C-peptide concentrations, triglycerides and whole body insulin sensitivity in women with low (□) and high (■) LFAT.* ***p<0.05, ****p<0.01 for high vs. low LFAT.*

During intravenously maintained hyperinsulinemia, steady-state plasma glucose concentrations were similar in the high and low LFAT groups. Steady-state serum free insulin concentrations (30-120 min) were slightly, but not significantly, higher in the high than in the low LFAT group (72±3 vs. 64±3 mU/l, p=0.08). The increments in serum insulin concentrations were comparable. Whole body insulin sensitivity (M-value) was significantly lower in the high than the low LFAT group (**Fig. 9**).

Serum LDL-cholesterol concentrations were comparable between the low and high LFAT groups. Serum triglycerides were significantly higher (**Fig. 9**), and HDL cholesterol concentrations lower (1.1±0.1 vs. 1.3±0.1 mmol/l, p<0.05) in the high as compared to the low LFAT group. Fasting FFA concentrations were similar in both groups, but during the insulin infusion serum FFA concentrations remained significantly higher in the high as compared to the low LFAT (304±20 vs. 243±17 µmol/l, p<0.05) group.

Blood pressure and heart rate

Women with high LFAT had higher 24 hour systolic (127±6 vs. 115±2 mmHg, high vs. low LFAT, $p<0.05$), diastolic (83±3 vs. 75±2 mmHg, high vs. low LFAT, $p=0.02$) and mean (99±3 vs. 89±2 mmHg, $p=0.02$) arterial blood pressures than women with low LFAT (**Fig. 10**). There was no significant difference in the 24 hour heart rates between the two groups.

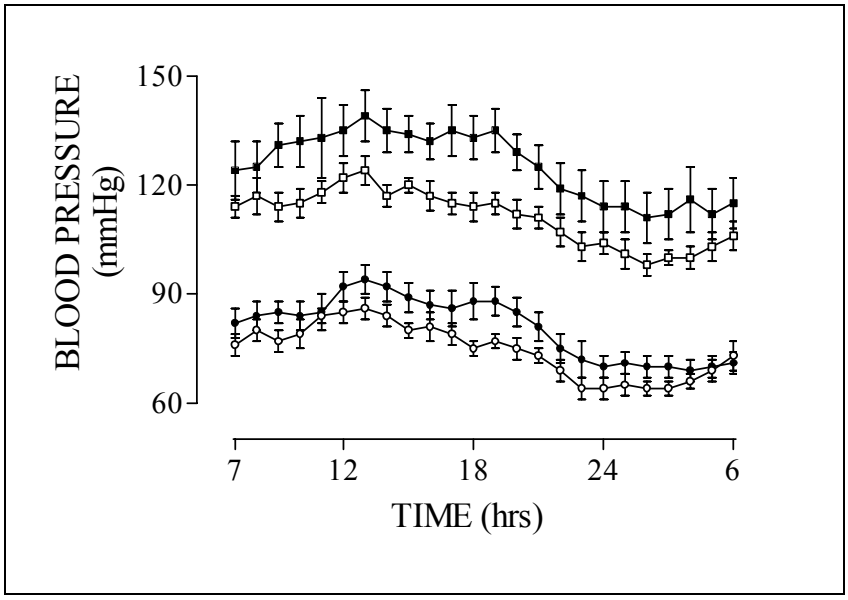

Figure 10. *24-hour systolic and diastolic pressure (BP) in women with low (n=13) and high LFAT (■ systolic BP in women with high LFAT, □ systolic BP in women with low LFAT, ● diastolic BP in women with high LFAT, ○ diastolic BP in women with low LFAT). Please see text for significance of differences between the groups.*

Association of LFAT with features of insulin resistance independent of measures of obesity

To further examine the relationships between LFAT, features of insulin resistance and measures of obesity, simple and multiple linear regression analyses were employed. In simple regression analysis, serum fasting insulin ($r=0.54$, $p=0.004$), triglycerides ($r=0.50$, $p=0.009$) and mean arterial pressure ($r=0.41$, $p=0.038$) were significantly correlated with LFAT. The relationships remained significant in multiple linear regression analysis when adjusted for intra-abdominal or subcutaneous fat or body mass index (**Table 3**).

Table 3. *Impact of various measures of obesity and body composition on the relationships between LFAT and features of insulin resistance*

Dependent variable	Independent variables	R^2	p-value
Serum fasting insulin	Body mass index LFAT	33.4%	0.22 (NS) 0.013
	Intra-abdominal fat LFAT	29.1%	0.80 (NS) 0.010
	Subcutaneous fat LFAT	47.5%	0.009 0.002
Serum triglycerides	Body mass index LFAT	25.4%	0.61 (NS) 0.009
	Intra-abdominal fat LFAT	22.9%	0.24 (NS) 0.033
	Subcutaneous fat LFAT	24.7%	0.90 (NS) 0.012
Mean arterial pressure	Body mass index LFAT	17.8 %	0.59 (NS) 0.07
	Intra-abdominal fat LFAT	21.5%	0.27 (NS) 0.024
	Subcutaneous fat LFAT	17.0%	0.92 (NS) 0.045

6.2. Effects of weight loss on liver fat content and features of insulin resistance among women with high and low liver fat content *(Study II)*

The groups with high and low LFAT were almost identical with respect to age, BMI, WHR, and the amounts of intra-abdominal, subcutaneous and whole body fat. Fasting plasma glucose and HbA_{1C} concentrations were also similar. The women with high LFAT had higher fasting serum insulin (15±2 vs. 9±1 mU/l, $p<0.01$, high vs. low LFAT), and triglyceride (1.73±0.18 vs. 1.27±0.14 mmol/l, $p=0.06$, high vs. low LFAT) concentrations than women with low LFAT (**Table 2**). Alcohol consumption was minimal and similar within the groups. Before weight loss, LFAT did not correlate with any measures of body composition or the concentrations of fasting FFA. Of the components of diet before weight loss, LFAT was significantly correlated with the % of total energy that consisted of fat ($r=0.43$, $p<0.05$) and the % saturated fat ($r=0.45$, $p<0.05$, **Fig. 11**).

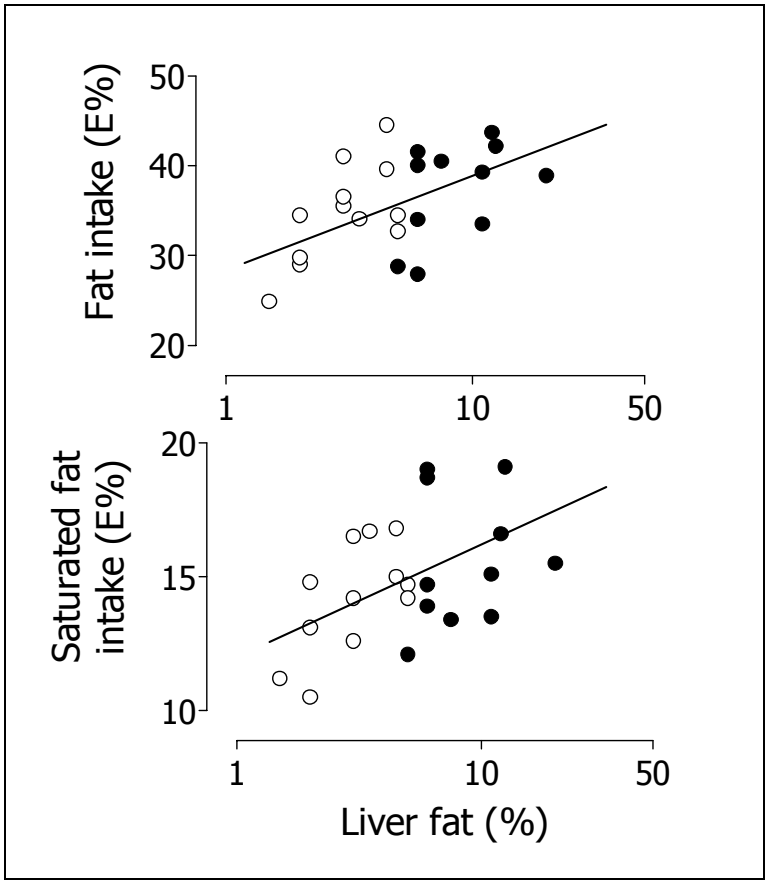

Figure 11. *The relationship between LFAT and total fat intake (% fat of total daily energy intake)(top panel, $r=0.43$, $p<0.05$), and saturated fat intake (% saturated fat of total daily energy intake, bottom panel, $r=0.45$, $p<0.05$) in women with low (open circles) and high (filled circles) LFAT.*

Effects of weight loss on body composition and liver enzymes

The women in the high and low LFAT groups lost weight over comparable time periods (18±1 vs. 19±2 weeks, NS, high vs. low LFAT) a similar % of their body weight, kilograms of body weight and units of BMI (**Fig. 12**).

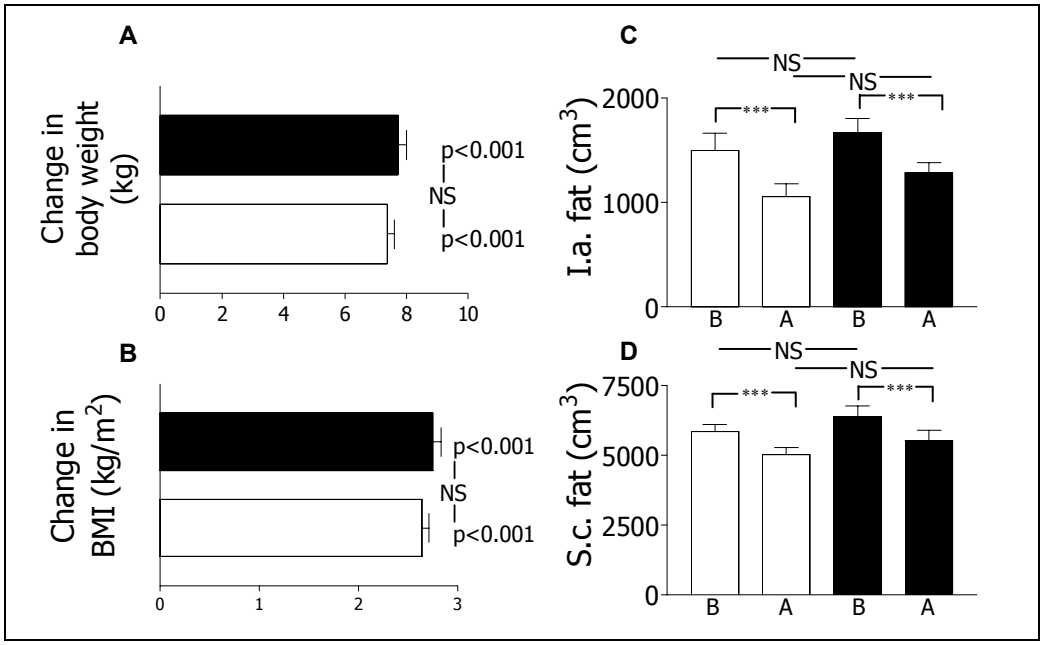

Figure 12. *Effects of weight loss on measures of body composition in women with low (open bars, n=12) and high (filled bars, n=11) LFAT. Change in body weight (**A**) and BMI (**B**) by weight loss. Intra-abdominal (i.a.) (**C**) and subcutaneous (s.c.) (**D**) fat volumes before (B) and after (A) weight loss in women with low (open bars) and high (filled bars) LFAT before weight loss. ***p<0.001 for before vs. after weight loss.*

Weight loss decreased LFAT from 9.4±1.4 to 4.8±0.7% (p<0.001) in women with high LFAT and from 3.3±0.4 to 2.0±0.2% (p<0.001) in women with low LFAT (**Fig. 13**). The absolute decrease in LFAT was significantly higher in women with high than low LFAT (-4.6±1.0 vs. -1.3±0.3%, p<0.005), and after weight loss, LFAT was still significantly higher in women with initially high than low LFAT (p<0.001).

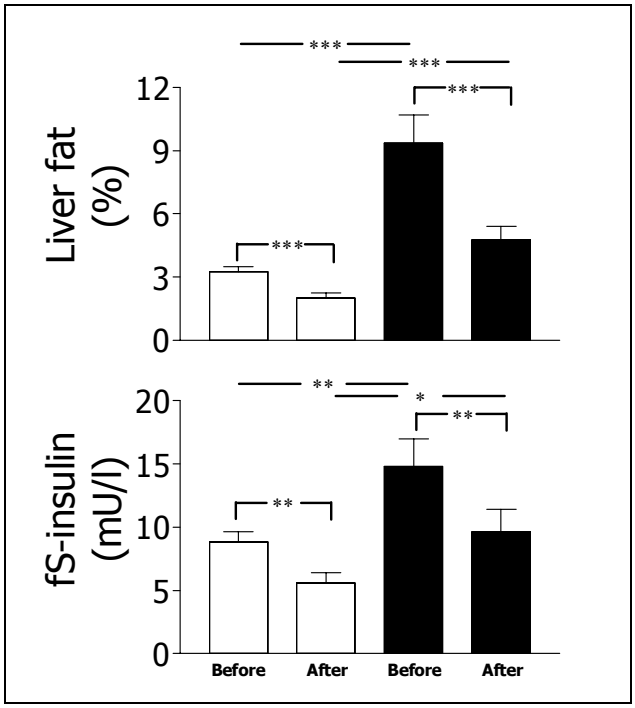

Figure 13. *Effects of weight loss on LFAT (top panel) and fasting serum insulin concentrations (bottom panel) in women with low (open bars) and high (filled bars) LFAT before weight loss. *p<0.05,**p<0.01,***p<0.001 for comparisons as indicated in the figure.*

The change of LFAT correlated closely with LFAT before weight loss (r=-0.85, p<0.001, **Fig. 14**). The slopes of the regression lines relating LFAT to its change by weight loss were similar showing that there was no difference in the relative decrease in LFAT between the groups. In the entire group, the % LFAT lost was 39±5%, which was much higher than the % fat lost of whole body fat mass (14±1%, p<0.001). The groups lost comparable amounts of fat from subcutaneous and intra-abdominal regions. The change in LFAT did not correlate with the change in intra-abdominal fat or changes in other measures of body composition (data not shown). Fasting serum FFA concentrations were not different at baseline, and decreased by weight loss by -79±37 μmol/l (p<0.05) with no differences between the groups.

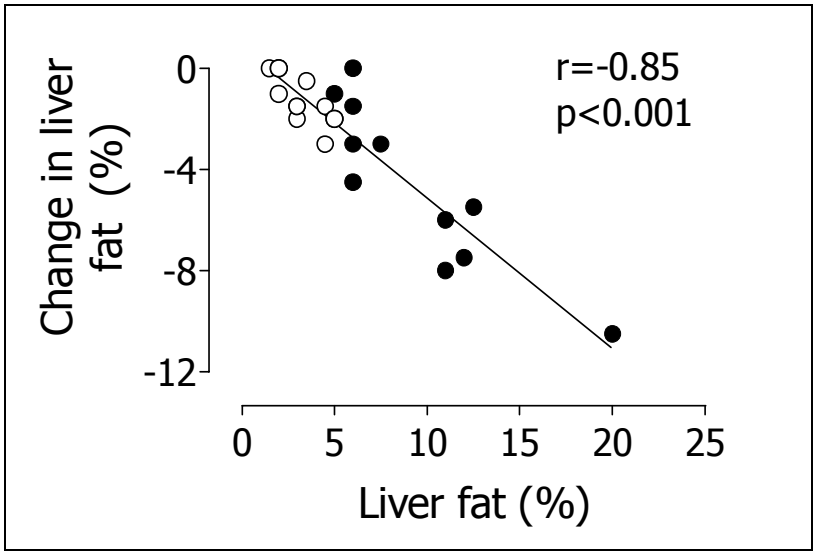

Figure 14. *The relationship between baseline LFAT and its absolute change by weight loss in women with low (open circles) and high (filled circles) LFAT*

Before weight loss, serum ALT was correlated with LFAT (r=0.41, p=0.05). Serum ALT concentrations decreased significantly by weight loss in both women with high (32±4 vs. 25±4 U/l, before vs. after, p<0.05) and low (26±6 vs. 23±6 U/l, respectively, p<0.05) LFAT. The change in serum ALT correlated with that in LFAT (r=0.58, p<0.005). Serum GGT concentrations decreased in women with high LFAT (31±5 vs. 27±4 U/l, before vs. after, p<0.05) but not in those with low LFAT. Serum AFOS or bilirubin concentrations did not differ between the groups before or after weight loss (data not shown).

Effect of weight loss on metabolic parameters and 24-hour blood pressure

Weight loss resulted in a decrease in serum fasting insulin concentrations in both groups (15±2 to 10±2 U/l, before vs. after, p<0.01 vs. 9±1 to 6±1 U/l, p<0.001, high vs. low LFAT). After weight loss, serum insulin concentrations were significantly higher in women with high than low LFAT (**Fig. 13**). Serum triglyceride concentrations decreased slightly in women with high LFAT (1.73±0.18 to 1.40±0.13 mmol/l, before vs. after, p=0.09) but not in women with low LFAT. Serum LDL cholesterol decreased significantly in both groups (3.25±0.2 to 2.86±0.2 mmol/l, high LFAT, p<0.01 and 3.36±0.2 to 2.90±0.1 mmol/l, low LFAT, p<0.05, before vs. after) with no differences between the groups. Serum HDL cholesterol concentrations were slightly but not significantly (1.35±0.09 vs. 1.17±0.08 mmol/l, p=0.14) higher before weight loss in women with high vs. low LFAT and did not change significantly by weight loss (data not shown). Mean 24 hour systolic blood pressure decreased in the low

LFAT group from 116±2 to 110±1 mmHg, p<0.005 and from 125±7 to 118±4 mmHg, p<0.05 in the high LFAT group. After weight loss, systolic 24-hour blood pressure was significantly higher in women with high than low LFAT (p<0.05). Mean diastolic 24-hour blood pressure did not differ significantly between the groups and did not change by weight loss (data not shown). The change in LFAT did not correlate with changes in serum fasting insulin, or triglyceride concentrations or with the change in 24 hours systolic blood pressure.

6.3. Effects of weight loss on body composition, insulin resistance and serum fatty acid composition among obese women with previous GDM *(Study III)*

Before weight loss the orlistat and placebo groups didn't differ with respect to age, BMI, WHR, or volumes of intra-abdominal, subcutaneous or whole body fat. Fasting plasma glucose, HbA_{1C}, insulin sensitivity (M-value), fasting serum FFA, and free insulin concentrations were not different between the groups (**Table 2**). There were no differences between the groups in dietary intake (**Table 4**) or in the fatty acid composition of serum phospholipids prior to weight loss (**Table 5**).

Table 4. *Baseline dietary intake of the study groups*

	Orlistat (n=23)	Placebo (n=24)
Dietary intake at baseline)*		
Energy (kcal)	1837±89	1792±80
Fat (g)	72±4	72±4
Fat (% of total energy intake)	36±1	36±1
Saturated fat (g)	29±2	29±2
Saturated fat (% of total energy intake)	14±0.5	15±0.5
Protein (g)	78±4	80±4
Carbohydrate (g)	200±14	191±10
Fibre (g)	19±1	18±1
Alcohol (g)	7±2	6±2

Data are shown as mean ± SEM. *) Based on a 3-day diary of food intake. There are no differences between orlistat vs. placebo groups (unpaired t-test).

Table 5. *Fatty acid composition (molar % propotions) of serum phospholipid esters before and after orlistat (n=23) and placebo (n=24).*

	Orlistat (n = 23)		**Placebo** (n = 24)	
	Before	**After**	**Before**	**After**
Saturated fatty acids				
Myristic acid (14:0)	0.41±0.01	0.39±0.02	0.41±0.02	0.35±0.02
Pentadecanoic acid (15:0)	0.19±0.01	0.20±0.01	0.20±0.01	0.19±0.01
Palmitic acid (16:0) #	28.7±0.2	29.5±0.3***	28.4±0.3	28.6±0.3
Stearic acid (18:0)	14.2±0.2	13.5±0.3	14.5±0.3	14.2±0.3
Sum of saturated fatty acids	43.6±0.1	43.6±0.2	43.4±0.1	43.3±0.1
Monounsaturated fatty acids				
Palmitoleic acid (16:1 n-7)	0.92±0.05	1.03±0.07	0.81±0.05	0.82±0.05
Oleic acid (18:1 n-9)	11.9±0.3	12.1±0.3	11.2±0.3	11.3±0.4
Vaccenic acid (18:1 n-7)	1.91±0.04	2.19±0.05	1.83±0.04	1.99±0.07
Sum of monosaturated fatty acids#	14.7±0.3	15.4±0.3*	13.9±0.3	14.1±0.3
Polyunsaturated fatty acids				
Linoleic acid (18:2 n-6) #	21.3±0.6	18.9±0.4***	21.44±0.5	20.3±0.5*
Conj.linoleic acid (18:2 conj.) ##	0.22±0.01	0.21±0.01	0.20±0.01	0.18±0.01
α-linolenic acid (18:3 n-3)	0.47±0.03	0.38±0.03	0.42±0.03	0.34±0.02
γ-linoleic acid (18:3 n-6)	0.17±0.01	0.15±0.01	0.15±0.01	0.14±0.00
Dihomo-γ-linolenic acid (20:3 n-6)	3.29±0.09	3.53±0.13	3.50±0.15	3.47±0.16
Arachidonic acid (20:4 n-6)	8.74±0.30	9.71±0.27	8.98±0.32	9.49±0.33
Eicosapentaenoic acid (20:5 n-3)	1.87±0.15	1.74±0.10	1.89±0.18	2.02±0.30
Docosahexaenoic acid (22:6 n-3)	5.65±0.27	6.27±0.27	6.10±0.31	6.67±0.34
Sum of polyunsaturated fatty acids##	41.7±0.3	40.9±0.4*	42.7±0.3	42.6±0.3

Data are shown as mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 for before vs. after weight loss (ANOVA).#p<0.05, ##p<0.01 for changes by weight loss between orlistat and placebo groups, with the before value as a covariate (ANCOVA).

Dietary intake during the study, and side effects

Dietary intake averaged 1223±125 and 1141±115 kcal/day in the orlistat and placebo groups (NS). The % fat of total energy intake decreased significantly in the orlistat group from 36±2 to 25±3% ($p<0.001$) but was unchanged in the placebo group. The orlistat group had significantly more gastrointestinal but not other side effects than the placebo group (total 93 vs. 60%, $p<0.01$; fatty stool 81 vs.17%; $p<0.005$, soft stools 37 vs. 17%, $p<0.001$).

Effects of weight loss on body composition

Weight loss averaged 7.3±0.2 kg (8.3±0.1%) and 7.4±0.2 kg (8.2±0.1%) of initial body weight in orlistat and placebo groups. The mean time to achieve weight loss was 20±1 weeks in the orlistat and 18±1 weeks in the placebo group (NS). As planned, weight changed similarly over time in both groups (**Fig. 15**).

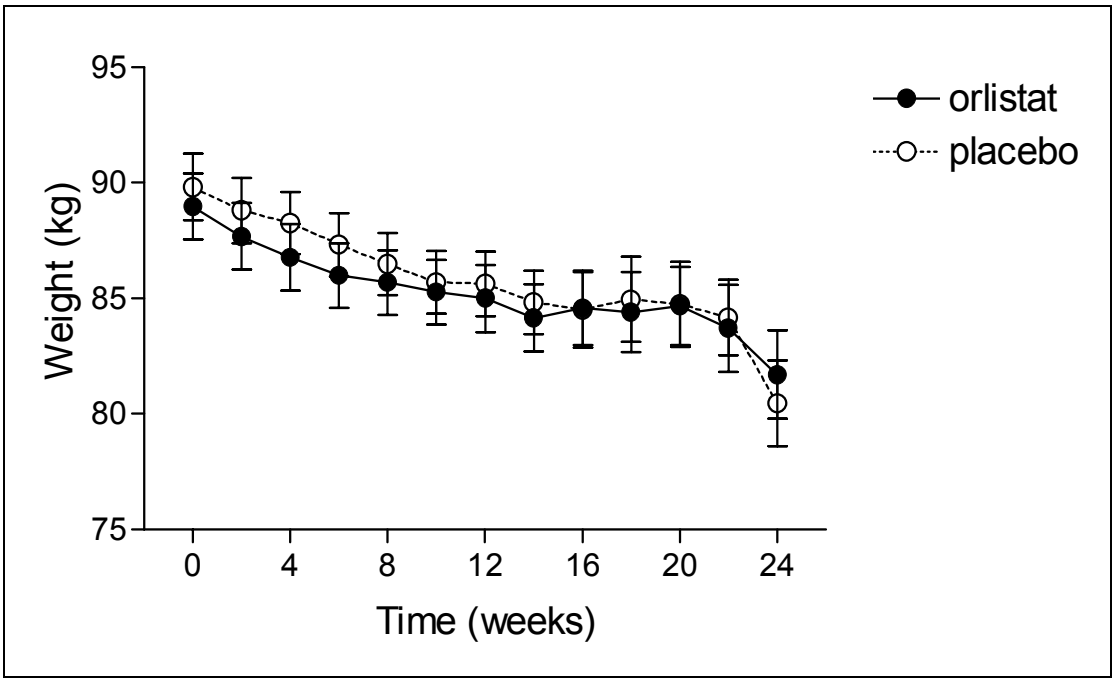

Figure 15. *Weight loss plotted vs. time in the orlistat (n = 23) and placebo (n=24) groups. Data are shown as mean±SEM. NS for differences between orlistat and placebo group (ANOVA for repeated measures).*

The % whole body fat decreased similarly in both groups. Weight loss induced a significant loss of fat from intra-abdominal (1370±109 vs. 995±65, before vs. after, and 1479±112 vs. 1199±80 cm^3, orlistat and placebo groups, $p<0.0001$) and subcutaneous (5678±233 vs. 4903±215 and 5983±230 to 5146±200 cm^3, respectively, $p<0.001$) fat regions. Intra-

abdominal fat tended to decrease more in the orlistat than the placebo group but the changes between the groups did not reach statistical significance (**Fig. 16**). Subcutaneous fat decreased similarly in both groups (**Fig. 16**). The ratio between intra-abdominal and total fat decreased significantly in the orlistat (19.5±1.4 vs. 17.1±1.1%, before vs. after weight loss, $p<0.005$) but not in the placebo (19.9±1.4 vs. 19.1±1.3%, NS) group. Similarly, the ratio between intra-abdominal and subcutaneous fat decreased in the orlistat group from 25±2 to 21±2% ($p<0.005$) but remained unchanged in the placebo group (26±2 vs. 24±2%, before vs. after, NS). The changes in both ratios differed significantly ($p<0.05$).

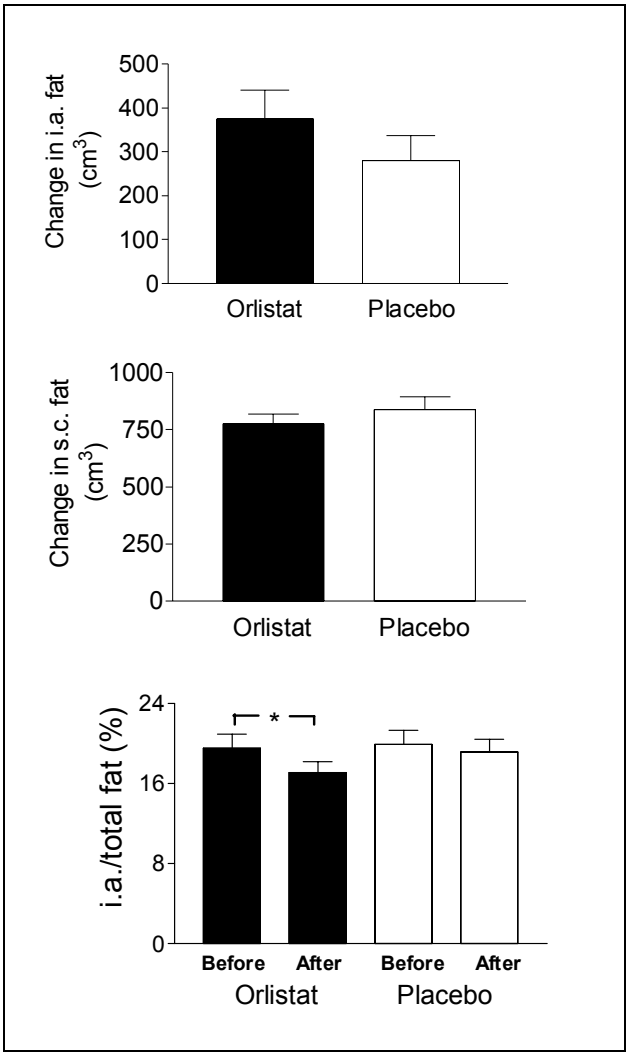

Figure 16. *Effects of weight loss on intra-abdominal (i.a.) and subcutaneous (s.c.) fat volumes and the intra-abdominal to total fat ratio in orlistat (n = 23) and placebo (n=24) groups. Data are shown as mean±SEM. **p<0.01 for before vs. after weight loss (paired t-test), ^{x}p<0.05 for change after weight loss for orlistat vs. placebo groups (ANOVA).*

Effects of weight loss on insulin sensitivity, lipids and blood pressure

M-value increased and serum fasting insulin concentrations decreased similarly in both groups (**Fig. 17**). Fasting plasma glucose, $HbA_{1C,}$ and serum fasting FFA concentrations remained unchanged. Serum FFA concentrations during the insulin infusion (mean concentration 30-120 min) were slightly ($p<0.05$) lower after than before weight loss, with no differences between the groups. Serum LDL cholesterol decreased significantly from 3.5±0.2 to 3.0±0.1 mmol/l ($p<0.01$) in the orlistat group but remained unchanged in the placebo group (3.1±0.1 vs. 3.0±0.1 mmol/l, NS, $p<0.05$ for change between groups). Serum HDL cholesterol increased slightly but significantly in placebo group (1.28±0.06 to 1.34±0.07 mmol/l, $p<0.05$) but remained unchanged in the orlistat group (1.31±0.1 vs. 1.29±0.1 mmol/l, NS, NS for change between groups). Twenty-four hour systolic blood pressure decreased significantly in both groups (**Fig. 17**). Twenty-four hour diastolic blood pressure decreased in the orlistat from 83±2 to 81±2 mmHg ($p=0.045$) and in the placebo group from 79±2 to 77±1 mmHg ($p=0.064$).

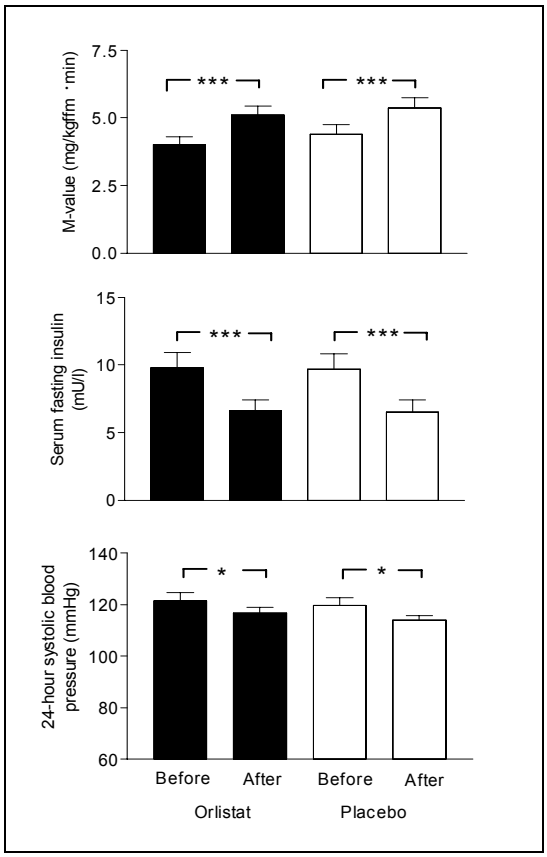

Figure 17. *Effects of weight loss on whole body insulin sensitivity, serum fasting insulin and 24 hour systolic blood pressure in orlistat (n = 23) and placebo (n=24) groups. Data are shown as mean±SEM.*p<0.05, ***p<0.001 for treatment effect. There was no significant difference in the changes between the two groups.*

*Changes in serum phospholipid fatty acids in orlistat and placebo groups (**Table 5**)*

The proportion of palmitic acid, the most abundant saturated fatty acid in serum phospholipids, increased significantly in the orlistat but not the placebo group. The most abundant polyunsaturated fatty acid, the essential fatty acid linoleic acid, decreased both in the orlistat and the placebo group. Monounsaturated fatty acids increased significantly in the orlistat but not the placebo group and the sum of proportions was higher in the orlistat than the placebo group after weight loss ($p<0.05$).

Interrelationships between changes in body composition, fatty acid composition of serum phospholipid esters and whole body insulin sensitivity

Before weight loss, when all data were analyzed together, intra-adominal ($r=-0.46$, $p<0.005$) but not subcutaneous ($r=0.12$, NS) fat volume was significantly inversely correlated with whole body insulin sensitivity. Similarly, after weight loss, intra-abdominal ($r=-0.47$, $p<0.005$) but not subcutaneous ($r=-0.20$, NS) fat volume was inversely related to whole body insulin sensitivity. When both groups were analyzed together (or within groups, data not shown), there were no significant correlations between changes in body composition and M-value. Before weight loss, the sum of saturated fatty acids was negatively ($r=-0.30$, $p<0.05$) and that of polyunsaturated fatty acids positively ($r=0.30$, $p<0.05$) related to whole body insulin sensitivity. The change in intra-abdominal to total or subcutaneous fat volume did not correlate with changes in any of the individual fatty acids or their sums (data not shown). There was, however, a highly significant inverse correlation between the proportion of dihomo-γ-linolenic acid (20:n-6) and whole body insulin sensitivity both before and after weight loss (**Table 6**).

Table 6. *Interrelationships between the fatty acid composition of serum phospholipids and whole body insulin sensitivity (mg/kg ffm·min) before and after weight loss. Data from all women are analysed as one group.*

Fatty acid	**Before weight loss** (n=47)	**After weight loss** (n=47)
Saturated fatty acids		
Myristic acid (14:0)	-0.24	-0.28
Pentadecanoic acid (15:0)	0.16	-0.06
Palmitic acid (16:0)	-0.01	0.06
Stearic acid (18:0)	-0.14	-0.27
Sum of saturated fatty acids	-0.30*	-0.34*
Monounsaturated fatty acids		
Palmitoleic acid (16:1n-7)	-0.27	-0.18
Oleic acid (18:1 n-9)	-0.21	-0.14
Vaccenic acid (18:1 n-7)	0.18	-0.27
Sum of monounsaturated fatty acids	-0.21	-0.12
Polyunsaturated fatty acids		
Linoleic acid (18:2 n-6)	0.09	0.02
Conj.linoleic acid (18:2 conj.)	-0.09	0.09
α-linolenic acid (18:3 n-3)	0.01	-0.24
γ-linoleic acid (18:3 n-6)	0.07	-0.22
Dihomo-γ-linolenic acid (20:3 n-6)	-0.48***	-0.46***
Arachidonic acid (20:4 n-6)	0.21	0.09
Eicosapentaenoic acid (20:5 n-3)	0.02	0.28
Docosahexaenoic acid (22:6 n-3)	0.15	0.23
Sum of polysaturated fatty acids	0.31*	0.25

*p<0.05, **p<0.01, ***p<0.001. Correlations coefficients were calculated using Pearson's correlation coefficient.

6.4. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, and gene expression in adipose tissue in patients with type 2 diabetes *(Study IV)*

Glycemic control, lipids, body weight and composition

At baseline the rosiglitazone and metformin groups were comparable with respect to gender, age, BMI, WHR, serum insulin and lipid concentrations (**Table 2**). Fasting plasma glucose and HbA_{1C} decreased similarly in both groups (**Table 2**). Serum triglycerides decreased by 0.23±0.18 mmol/l in the rosiglitazone (NS) and by 0.37±0.18 mmol/l ($p<0.05$ for 16 vs. 0 weeks) in the metformin group. Serum HDL cholesterol increased significantly in the rosiglitazone (by 0.25±0.1 mmol/l, $p<0.05$) but not in the metformin group, and LDL cholesterol remained unchanged in both groups. Hemoglobin decreased by 5% ($p<0.01$) in the rosiglitazone group but remained unchanged in the metformin group. Metformin group lost 2.4±0.9 kg of body weight ($p<0.02$ for 16 vs. 0 weeks, **Table 2**), while there was no significant change in the rosiglitazone group. The weight loss in the metformin group was due to loss of subcutaneous rather than visceral fat or fat free mass (**Table 7**).

Table 7. *Characteristics of the patients before and after treatment with rosiglitazone and metformin*

	Rosiglitazone (n=9)		**Metformin (n=11)**	
	Before	**After**	**Before**	**After**
Fat free mass (kg)	58.5±3.3	58.6±3.1	56.3±3.1	55.6±3.1
Waist (cm)	102±3	100±3	103±3	98±3**
Intra-abdominal fat volume (cm^3)	1925±253	1960±279	2167±309	2045±269
Subcutaneous fat volume (cm^3)	4275±567	4472±651	3969±518	3786±490*

Data are shown as mean ± SEM. *$p<0.05$, **$p<0.01$ for before vs. after treatment.

Serum insulin, insulin clearance, glucose kinetics, serum FFA

Fasting serum insulin concentrations decreased significantly and similarly during both rosiglitazone and metformin therapy by 4±1 and 4±2 mU/L (**Fig. 18**).

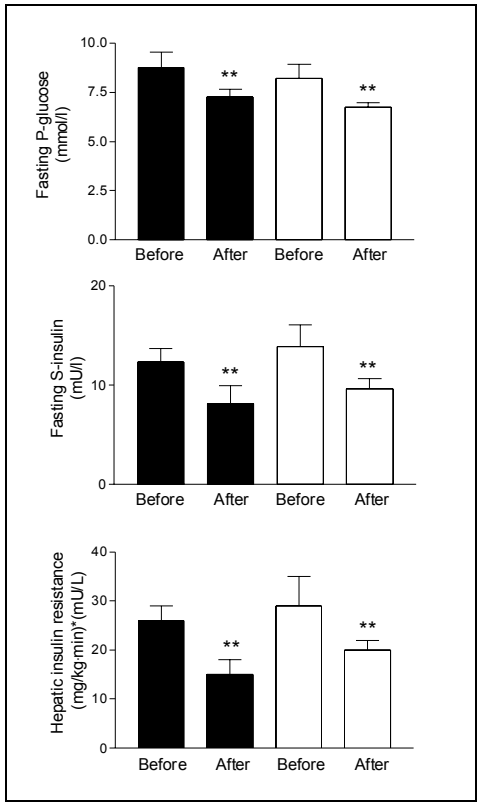

Figure 18. *Effects of 16 weeks of rosiglitazone (black bars) and metformin (open bars) treatment on fasting plasma glucose, serum insulin concentrations and hepatic insulin resistance (HIR). **p<0.01 for before and after treatment.*

During the square-wave of hyperinsulinemia created by the exogenous insulin infusion, serum insulin increased significantly less in the rosiglitazone than the metformin group after (mean increase 19±2 vs. 28±3 mU/L, p<0.02 for difference in increments between the groups) than before (25±2 vs. 27±3 mU/L, NS therapy, **Fig. 19**), implying that insulin clearance had increased during rosiglitazone therapy. Insulin clearance increased from 16±2 to 20±1 ml/kg·min, p=0.02 by rosiglitazone. Serum insulin concentrations were also lower after therapy with rosiglitazone than metformin (27±1 vs. 37±3 mU/L, p=0.01), while there was no difference before therapy (37±2 vs. 41±4 mU/L, NS). Serum insulin concentrations were also lower after than before therapy in the metformin group but the difference was identical to that in the fasting insulin concentrations (4±2 mU/L). This difference was significantly less than that observed with rosiglitazone (10±2 mU/L, p<0.01 vs. metformin).

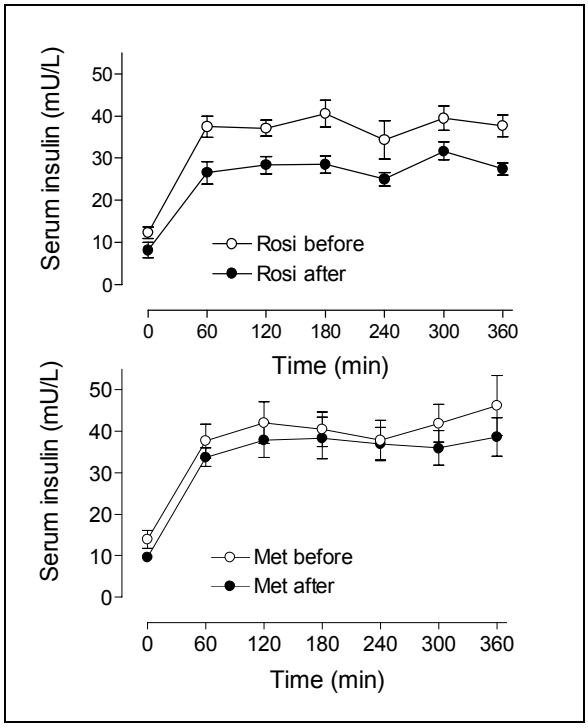

Figure 19. *Serum insulin concentrations in the fasting state and during exogenous insulin infusion before and after rosiglitazone and metformin treatment. For significances please see text.*

During intravenously maintained hyperinsulinemia, steady-state plasma glucose concentrations (30-360 min) were similar in both groups before and after treatment. Basal glucose production was unchanged in both groups. Because of the decrease in serum insulin concentrations, basal hepatic insulin resistance (HIR) decreased significantly in both groups [rosiglitazone 26±3 vs. 15±3 (mg/kg·min)·(mU/L), $p<0.002$, metformin 29±6 vs. 20±2 (mg/kg·min)·(mU/L), $p<0.05$, **Fig. 18**].

Before treatment hepatic glucose R_a during hyperinsulinemia and the % suppression of R_a by insulin were similar in both groups. After treatment, hepatic glucose production during hyperinsulinemia was lower after metformin (0.07±0.16 vs. 0.56±0.15 mg/kg·min, $p=0.04$) than rosiglitazone. The % suppression by insulin was also lower after metformin than rosiglitazone treatment (-97±8 vs. -72±7%, $p=0.04$, metformin vs. rosiglitazone), at the higher insulin concentrations (*vide supra*). Despite the lower increment in serum insulin concentrations in the rosiglitazone than metformin group after therapy, insulin stimulated glucose R_d increased in the rosiglitazone group from 3.5±0.4 to 4.5±0.5 mg/kg·min, $p<0.01$ but remained unchanged in the metformin group (3.3±0.3 vs. 3.5±0.3 mg/kg·min, NS) (**Fig. 20**). When corrected for the difference in insulin concentrations (451), glucose R_d increased

by 70% in the rosiglitazone group from 0.10±0.02 to 0.17±0.02 (mg/kg·min)/(mU/L) and remained unchanged in the metformin group (0.09±0.01 vs. 0.10±0.01 (mg/kg·min)/(mU/L), 0 vs. 16 weeks, NS).

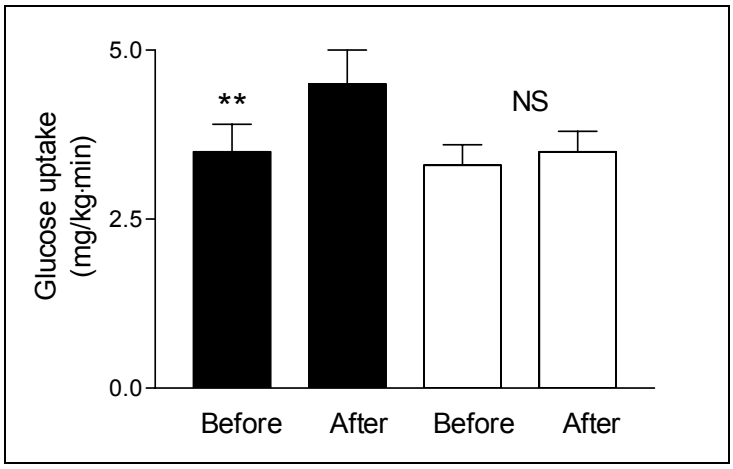

Figure 20. *Effects of 16 weeks of rosiglitazone (black bars) and metformin (open bars) treatment on insulin stimulated glucose uptake. **p<0.01 for before vs. after treatment.*

Serum fasting FFA decreased significantly and similarly by rosiglitazone (-17±4%, from 649±76 to 537±69 μmol/l, p<0.01) and metformin (-15±6%, from 711±62 to 599±61 μmol/l, p<0.02, 0 vs. 16 weeks). Serum FFA during hyperinsulinemia were also lower after therapy in both rosiglitazone (355±39 vs. 277±42 μmol/L, p=0.005, 16 vs. 0 weeks) and metformin (396±40 vs 331±39 μmol/l, p=0.001) groups. The % suppression of serum FFA by insulin was similar both before and after rosiglitazone and metformin.

Liver fat and serum ALT

In the rosiglitazone group, liver fat decreased by 51±7% from 15±3 to 7±1%, p=0.003, while there was no change in the metformin group (13±3 vs. 14±3%, NS) (**Fig. 21**). Serum ALT decreased significantly in the rosiglitazone group from 42±7 to 29±3 IU/L (p<0.05) but remained unchanged in the metformin group (52±9 vs. 42±6 IU/L, NS).

Serum adiponectin concentrations and mRNA expressions of PPARγ, adiponectin and LPL

Serum adiponectin concentrations increased by 122% in rosiglitazone group from 5.6±0.7 to 12.5±1.8 mg/l, p<0.001 but remained unchanged in the metformin group (6.1±1.0 to 6.1±1.0 mg/l, NS) (**Fig. 21**). The increase in serum adiponectin correlated with absolute and relative decreases in LFAT (**Fig. 21**).

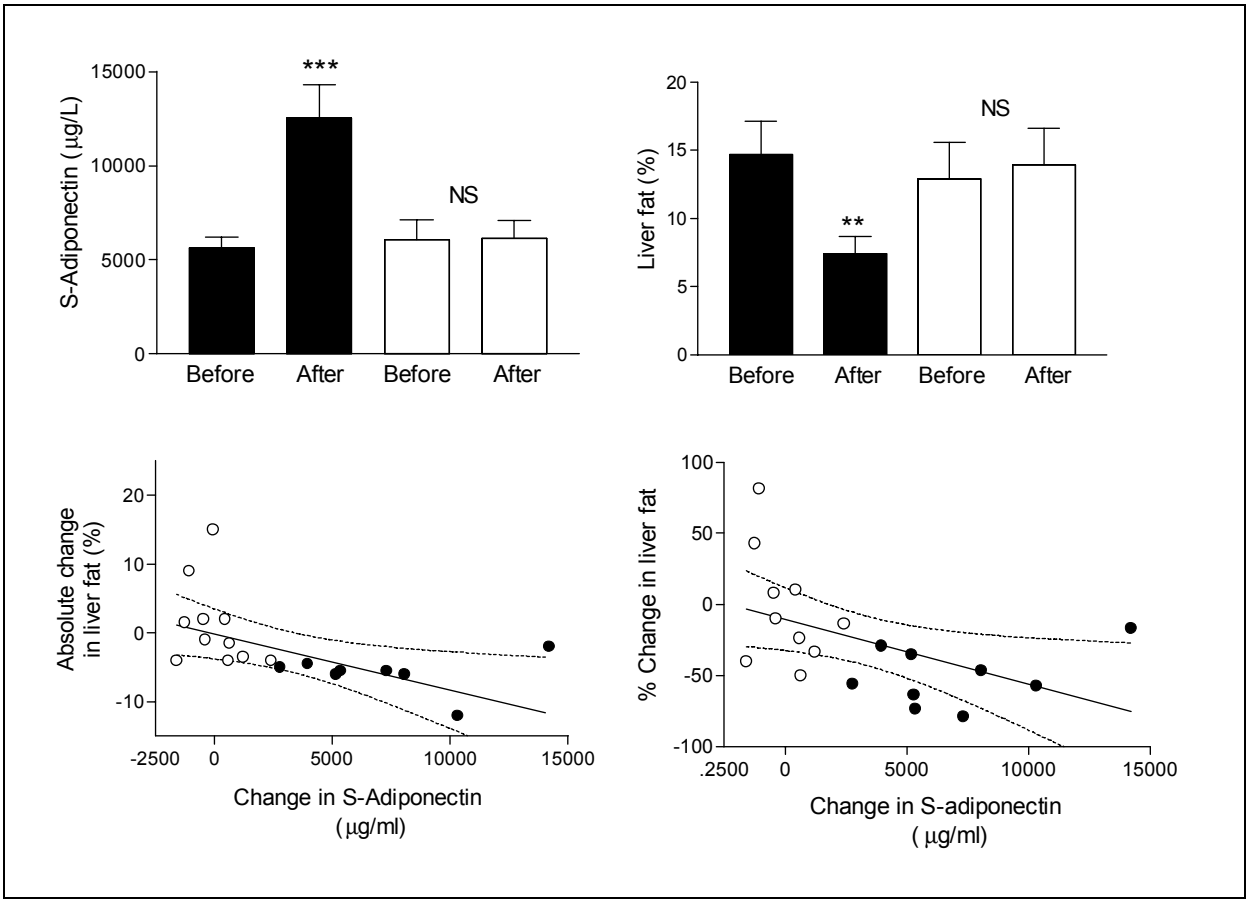

Figure 21. *Effects of 16 weeks of rosiglitazone (black bars) and metformin (open bars) treatment on serum adiponectin concentrations and liver fat content (upper panels) and correlations between the absolute change in serum adiponectin concentration and the absolute and % change in liver fat content (lower panels). ***p<0.001 for before vs. after treatment*

PPARγ, adiponectin and LPL mRNA concentrations in subcutaneous adipose tissue increased significantly in the rosiglitazone but not metformin group (**Fig. 22**). Fat cell size of subcutaneous adipose tissue didn't change with either rosiglitazone or metformin.

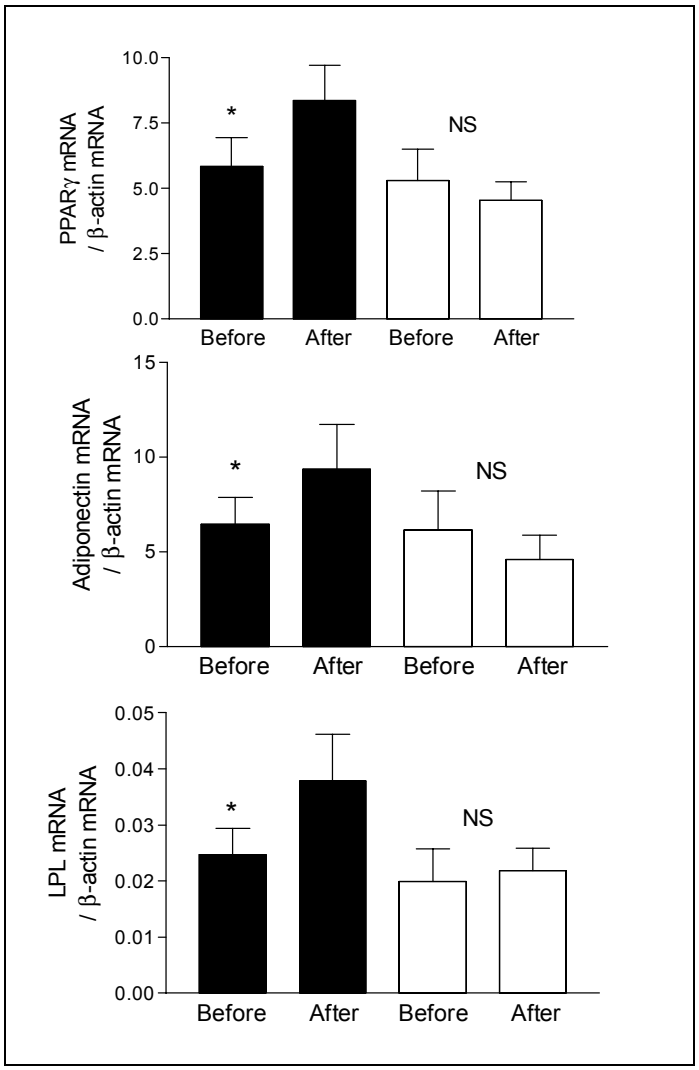

Figure 22. *Effects of 16 weeks of rosiglitazone (black bars) and metformin (open bars) treatment on PPARγ, adiponectin and LPL mRNA concentrations in subcutaneous adipose tissue expressed as relative to β-actin. *p<0.05 for before vs. after treatment.*

7. DISCUSSION

7.1. Liver fat and insulin resistance

We investigated obese, otherwise healthy, previously gestational diabetic women, who are at high risk for future development of type 2 diabetes and cardiovascular diseases (6,466). Obesity before, and weight gain during and after pregnancy are the most important risk factors for future development of type 2 diabetes in these women (155,157,160,161). Since, however, even lean women with previous GDM may be insulin resistant (467) and not all women with a history of GDM, even if obese, later develop diabetes (158), there may be other factors than obesity, which determine the degree of insulin resistance. In this study, the presence of diabetes was excluded by OGTT and these women had no signs or evidence of any chronic liver diseases.

Recent studies in various models of fatless mice have demonstrated that ectopic fat accumulation in the liver and in skeletal muscle are associated with severe insulin resistance (268,269,271). Treatment of lipodystrophy with fat transplantation completely reverses insulin resistance (269), demonstrating that subcutaneous fat is not necessary for insulin resistance and that fat accumulation in insulin-target tissues is a manifestation of insulin resistance. Regarding the relative importance of the liver and skeletal muscle for the development of glucose intolerance and diabetes, it is of interest that at least in mice, selective deletion of the insulin receptor from skeletal muscle doesn't change glucose or insulin concentrations (468), while inactivation of the insulin receptor in hepatocytes result in marked hyperinsulinemia, hyperglycemia and a fatty liver (272). Hepatic fat content, measured non-invasively using proton spectroscopy, is closely correlated with insulin requirements and hepatic insulin sensitivity in patients with type 2 diabetes (8). In the present study, we measured the liver fat content by MRI proton spectroscopy, a novel, non-invasive method that allows quantification of liver fat without radiation exposure and correlates closely with histologically determined liver biopsies and with computed tomography (CT) (469,450).

Little is known of the causes of fat accumulation, which is not attributable to alcohol, drugs, toxins, viruses, autoimmune disease or other known causes. In analysis of the food diaries in our study, the intake of saturated fat was significantly higher in the high than the low LFAT women, but given that the method to assess food intake maybe unreliable in small groups, we would be cautious in drawing any firm conclusions from these data. However, it has been

shown that intramyocellular lipid content can be increased with a high as compared to a low fat diet in humans (284) and also that muscle fat content can be acutely increased by infusion of a triglyceride-rich emulsion under hyperinsulinemic conditions (283,284). Separate intervention studies are needed to determine, whether liver fat content is influenced by composition of the diet.

The women with high LFAT had higher serum FFA concentrations than those with low LFAT during the insulin infusion. In most previous studies, such resistance to insulin has been, when determined with FFA tracers, due to a defect in insulin action on antilipolysis (470). Theoretically, excessive intravascular lipolysis of triglyceride-rich lipoproteins by lipoprotein lipase or alterations in FFA uptake or clearance could also be responsible. Recent data indicate that insulin plays a key role in regulating transcription factors such as SREBP-1c, which is abundantly expressed in the human liver (471). SREBP-1c is critical in controlling hepatic lipogenesis and is increased in proportion to hyperinsulinemia (i.e. is normally sensitive to insulin) in livers of lipodystrophic and ob/ob mice (255). These data raise the possibility that fasting hyperinsulinemia might have contributed to fat accumulation in the high LFAT group rather than vice versa. It is also possible that genetic factors contribute to fat deposition in the liver since a polymorphism in the SREBP-1c gene was recently suggested to contribute to the susceptibility to develop features of insulin resistance in HIV-patients treated with highly active anti-retroviral therapies (472). Finally, the present data should not be interpreted to negate the clinical role of obesity in increasing the risk of future diabetes in women with previous gestational diabetes (157,158,161).

Regarding possible mechanisms underlying other features of insulin resistance in the high as compared to low LFAT group, insulin normally acutely suppresses production of VLDL particles from the liver leading to a decrease in serum triglycerides (473). In insulin resistant subjects, this action of insulin is defective, which leads to hypertriglyceridemia, and a lowering of the HDL cholesterol concentration (93). The lipid abnormalities in the high as compared to the low LFAT women, i.e. increased triglycerides and low HDL cholesterol, might well be consequences of impaired insulin action on VLDL production or secretion in the liver.

Regarding the clinical implications of the present data, they suggest that amongst obese women with a history of gestational diabetes, anyone with signs of a fatty liver such as that suggested by an ultrasound examination or by elevated transaminases should undergo careful evaluation of the presence of cardiovascular risk factors, particularly of those associated with insulin resistance.

7.2. Weight loss and liver fat

The same women than in a *Study 1* participated in a weight loss program that was strictly controlled by two experienced dieticians. In addition to the hypocaloric diet, the women were randomized to use either orlistat or placebo. The goal was to achieve similar weight loss in both groups regardless of whether orlistat or placebo was used. The aim in *Study 2* was to determine how similar degree of weight loss affects LFAT in a homogenous group of obese women divided into two groups based on their median LFAT. Use of orlistat did not affect the results since a comparable number of women used orlistat in both groups.

Weight loss decreases the volume of intra-abdominal fat (12,358,368). Early studies in massively obese subjects, who were treated surgically using gastroplasty (346,347,353,355), gastric bypass (355,356), gastric banding (474) or with low-calorie diets (475,476) reported reduction in hepatic steatosis. However, in these studies, assessment of hepatic steatosis was usually semiquantitative and the sizes of other fat depots were not quantitated.

Both subcutaneous and intra-abdominal fat decreased by weight loss confirming previous observations (12,368). The change in LFAT by the 8% weight loss was closely correlated with baseline LFAT but not with changes in either subcutaneous or intra-abdominal fat volumes. The failure of the change of liver fat to correlate with the change in intra-abdominal or subcutaneous fat was not due to methodology used since the interindividual coefficient of variation of liver fat was higher (72%) than that of intra-abdominal (32%) or subcutaneous (19%) fat, and the coefficient of variation of repeated measurements of liver fat (11%) higher than that of intra-abdominal (5%) or subcutaneous (3%) fat. These data should not be interpreted to suggest that the magnitude of weight loss does not influence the amount of fat lost from the liver since weight loss was deliberately kept as constant as possible. Indeed, in older studies the decrease in LFAT as determined by liver biopsies, has been significantly related with the magnitude of weight loss (476). However, the correlation with baseline LFAT

rather than those in subcutaneous and intra-abdominal depots suggest that the amount of fat in the liver may not simply reflect the flux of endogenous FFA from peripheral and intra-abdominal sites to the liver. This interpretation assumes that change in size of a fat depot correlates with its metabolic activity, which is uncertain in the absence of FFA turnover data. Also, these data do not exclude the possibility that intra-abdominal fat causes insulin resistance in humans (477).

The significant positive relationship before weight loss between saturated fat intake and liver fat provides some support for the possibility that exogenous fat contributed to hepatic fat stores, although the pathways contributing to hepatic triglyceride storage under postprandial conditions have as yet to be precisely defined (244). In animals, the liver has a high capacity for triglyceride storage and the size of this pool can change several-fold within hours (478,479). There is evidence that triglyceride storage occurs in the liver whenever FFA availability exceeds hepatic disposal via secretion and oxidation (480). If the reverse is true, then a decrease in the contribution of exogenous fatty acids by caloric restriction could preferentially mobilize hepatic rather than subcutaneous or intra-abdominal triglycerides.

In *Study 3* altogether 57 women, of whom 24 were the same women as *Study 2* participated in the same weight loss protocol than previously described. Ten women did not achieve the required 8% weight loss and were withdrawn from the study. Of the reamaining 47 women 23 used orlistat and 24 placebo in addition to the hypocaloric diet. Analysis of dietary records based on a 3-day food intake diary and measurement of the molar proportions of fatty acids in serum phospholipids revealed that the orlistat and placebo groups were similar before weight loss. During weight loss, orlistat reduced fat absorption as shown by the expected decrease in serum LDL cholesterol as has previously been reported, and corresponds to an approximately 25% decrease in cholesterol absorption and a 30% decrease in fat absorption (362,375,481,482). Second, the significant decrease in the proportions of linoleic and alpha-linolenic acid in serum phospholipids is consistent with a decrease in the proportion of diet-derived fatty acids, and with our previous findings regarding orlistat's effects on the fatty acid composition of serum lipid fractions in obese subjects using a similar dose of orlistat as in the present study (483). Dietary intake was similar during weight loss but the orlistat group reduced their fat intake more than the placebo group, most likely because of gastrointestinal side effects.

In the orlistat group, the ratio of intra-abdominal to subcutaneous or total fat mass decreased significantly but remained unchanged in the placebo group, while the absolute volumes of both fat compartments decreased similarly. This finding would suggest that lowering of fat absorption or altering the composition of fatty acids might have favourable effects on the ratio of intra-abdominal to subcutaneous and total fat. The mechanism(s) underlying such an effect remain speculative. Polyunsaturated fatty acids, in addition to possibly enhancing insulin sensitivity via effects on membrane composition, can also suppress transcription of lipogenic genes and induce genes encoding for proteins of lipid oxidation and thermogenesis, possibly by direct interaction with transcription factors such as SREBP-1 (484). In the present study, there were several changes in fatty acid composition in both groups and some differences between the groups, as judged from phospholipid esters in serum (**Table 5**). None of the changes in fatty acid composition correlated with changes in body composition. Nonetheless, the orlistat intervention was associated with a favourable change in body composition implying that changing the amount of dietary fat absorbed or the fatty acid composition may differentially regulate the size of the fat depots.

Although the ratio of intra-abdominal to subcutaneous and total fat decreased in the orlistat but not the placebo group, insulin sensitivity improved similarly in both groups. This should not necessarily be interpreted to suggest that visceral fat does not regulate whole body insulin sensitivity independent of overall obesity and fat mass, since the absolute volumes of intra-abdominal and subcutaneous fat decreased similarly in both groups, and it is doubtful whether the small decrease in the ratio was big enough to have a discernible metabolic effect. Also, while the 'M-value' provides an accurate measure of whole body insulin sensitivity, it does not allow distinction between the contribution of defects in suppression of hepatic glucose production and in stimulation of peripheral glucose uptake by insulin to whole body insulin sensitivity. Intra-abdominal fat could be better related to hepatic insulin sensitivity, at least according to the 'portal hypothesis' i.e. the theory that the larger the visceral fat depot, the greater the flux of FFA to the liver via the portal vein (106). However, in a placebo controlled study of Kelley et al. orlistat improved peripheral insulin sensitivity more than placebo regardless of equivalent weight reduction. In this study orlistat induced greater reductions in fasting plasma FFA, insulin-suppressed FFA and fasting plasma glucose concentrations, which was suggested to influenze on greater improvement in insulin sensitivity (376).

In addition to the amount of fatty acids stored as triglycerides inside insulin sensitive cells, the composition of fatty acids in serum cholesterol esters is related to insulin sensitivity. When measured using the euglycemic clamp technique, insulin sensitivity has been associated with low proportions of palmitic (16:0), and high proportions of linoleic acid (18:2 n-6) and especially with dihomo-γ-linolenic acid in serum cholesterol esters (222). In keeping with these data, we found a strikingly strong inverse correlation between the proportion of dihomo-γ-linolenic acid and whole body insulin sensitivity both before and after weight loss. Linoleic acid and alpha-linolenic acid, which cannot be endogenously synthesized, are reliable indicators of dietary intake whereas other fatty acids reflect the effects of metabolism and synthesis as well. Thus, the reduced proportions of linoleic acid and alpha-linolenic acid reflect reduced intakes and/or reduced absorption of these fatty acids. The fatty acid composition of serum phospholipids (220,221) and recently also of skeletal muscle membranes (485) have been shown to reflect dietary fat composition and to correlate with insulin sensitivity in humans (223). Despite such cross-sectional data supporting the possibility that dietary fatty acid composition regulates insulin sensitivity, intervention studies have been largely negative (120,121,486) until recently when the KANWU study demonstrated that insulin sensitivity indeed can be enhanced by replacing saturated fat with monounsaturated fat (116). In view of these data, the changes in the fatty acid composition of serum phospholipids in the orlistat group might be expected to have, if anything, a negative impact on insulin sensitivity. Thus, in the orlistat group, the proportion of palmitic acid increased significantly and linoleic acid decreased more than in the placebo group (**Table 5**). These changes may have counteracted a possible beneficial effect of orlistat on insulin sensitivity by decreasing the visceral to subcutaneous fat ratio.

7.3.Thiazolidinediones, metformin, liver fat and hepatic insulin sensitivity

In *Study 4*, we found, in contrast to animal studies (409), metformin to sensitize the liver without changing hepatic fat content. The ability of metformin to increase hepatic insulin sensitivity has been documented in several previous human studies (14,15,390,394), but liver fat was not measured. Metformin has also been suggested to decrease serum ALT levels in patients with non-alcoholic steatohepatitis but this study was uncontrolled (487).

The present study showed a decrease in liver fat in a double-blind randomized trial by PPARγ agonism and is keeping with previous data showing a similar decrease in liver fat in

uncontrolled studies using either pioglitazone (434) or rosiglitazone (17,431,488). These data again contrast those in mice, in which treatment with PPARγ agonists appears to increase rather than decrease liver fat (489,490). In such studies, PPARγ expression has increased in the liver (491,492). Lack of PPARγ expression in the liver protects mice from developing hepatosteatosis (430). The normal human liver has very low PPARγ expression, but whether this applies to a fatty liver is unknown (493).

Several mechanisms could underlie the ability of PPARγ agonists to reduce liver fat content. In the present study, rosiglitazone and metformin had similar effects on FFA concentrations. These effects seem to be, based on measurement of *in vivo* rates of FFA turnover (16), due to inhibition of lipolysis. In a 6-month placebo-controlled study has been shown that rosiglitazone can decrease liver fat without changing adipose tissue mass in patients with HIV-lipodystrophy (432). These considerations argue against FFA flux to the liver being the only regulator of liver fat content. In normal mice, rosiglitazone has no effects on hepatic mRNA levels and acts exclusively via effects in adipose tissue (430). These effects include an increase in adiponectin expression, as was also observed in the present study in patients with normal or increased amounts of adipose tissue. Adiponectin increases hepatic insulin sensitivity in mice by activating fatty acid oxidation and inhibiting phosphoenolpyruvate carboxykinase expression (303,494). Consistent with the idea that adiponectin is important in mediating changes in liver fat content and possibly hepatic insulin sensitization, we found a significant correlation between changes in adiponectin and liver fat content (**Fig. 21**). Similar foundings was shown in a recent study of Bajaj. et al (24). Troglitazone treatment has also been shown to increase adiponectin concentrations in human adipose tissue (309).

In humans approximately 50% of insulin is cleared by the liver (495). Fat accumulation in the liver has been associated with impaired insulin clearance, when measured as in the present study (496) and fasting hyperinsulinemia has been suggested to be associated with the fatty liver at least partly due to impaired insulin clearance (497). Insulin clearance was increased and liver fat content decreased by rosiglitazone therapy. Since fat free mass was unchanged and at least based on creatinine or urinary albumin excretion (data not shown), and there were no changes in renal function, the decrease in liver fat is likely to explain the improvement in insulin clearance. The commonly observed decrease in serum fasting insulin by rosiglitazone can therefore at least in part be attributed to an increase in insulin clearance. Because of the

change in insulin clearance and lower insulin concentrations both in the basal state and during hyperinsulinemia, it is difficult to estimate whether true hepatic insulin sensitization occurred in the present study by rosiglitazone. Such evidence has to rely upon previous studies (albeit uncontrolled), where higher insulin infusion rates were used (16,431). The reason(s) why rosiglitazone has not previously been noted to increase insulin clearance remain speculative. Most studies did not quantitate hepatic fat content and if hepatic fat was low at baseline, it may not have changed during therapy (16,431). In the present study we chose a long-lasting low dose exogenous insulin infusion to maximize the likelihood of detecting differences in insulin clearance and hepatic insulin sensitivity. Clearly, metformin did improve hepatic insulin sensitivity consistent with previous studies (390), and in the absence of a change in liver fat content. Together the data imply that a decrease in liver fat content increases insulin clearance but is not necessary for metformin induced hepatic insulin sensitization in humans.

TZDs have been shown to increase peripheral glucose uptake in previous studies (15,393,434). While troglitazone was suggested to act primarily by increasing the rate of peripheral glucose disposal, both pio- and rosiglitazone have been shown to increase peripheral and hepatic insulin sensitivity (16,434,498). In the present study, we confirmed the ability of rosiglitazone to enhance peripheral insulin sensitivity. Recently also Bajaj et al. have demonstrated that pioglitazone improves peripheral insulin sensitivity, reduces EGP, and increases plasma adiponectin concentrations. Because increase in plasma adiponectin concentrations associated with reduction of liver fat and improvements in hepatic and peripheral insulin sensitivity, thiazolidinedione therapy may play an important role in regulating hepatic fat content and insulin sensitivity (24). Pioglitazone has also been shown to significantly decrease liver fat content and insulin sensitivity in NASH patients (499). Since metformin was as effective in improving glycemic contol and lowering FFA concentrations, and decreased body weight in contrast to rosiglitazone, this increase cannot be attributed to changes in gluco– or lipotoxicity or body weight. Recently, rosiglitazone treatment in patients with type 2 diabetes was shown to improve downstream insulin signaling in human skeletal muscle by increasing insulin stimulation of IRS-1 tyrosine phosphorylation and p85 association with IRS-1 (500). Given the low PPARγ expression in skeletal muscle as compared to adipose tissue, it is unclear whether these effects are direct or indirect.

8. SUMMARY AND CONCLUSIONS

1) Liver fat content correlates with several features of insulin resistance in obese women with previous gestational diabetes, who are at high risk for future development of type 2 diabetes. Women with increased liver fat content have higher fasting serum insulin and triglyceride concentrations, and higher blood pressure, and they are more insulin resistant as compared to the women with low liver fat content. These differences could not be attributed to overall or intra-abdominal adiposity.

2) Moderate weight loss decreases liver fat content and ameliorates features of insulin resistance as evidenced by decreases in blood pressure, fasting serum insulin and triglyceride concentrations, in obese women with a history of gestational diabetes. Baseline liver fat content does not influence the relative decrease in liver fat by weight loss. The change in liver fat content did not correlate with the changes of intra-abdominal or subcutaneous fat volumes. Saturated fat intake correlates with the liver fat content suggesting that fatty acids derived from the diet may influence liver fat accumulation and insulin resistance.

3) Equal (8%) weight loss induced with or without orlistat and a hypocaloric diet increases insulin sensitivity and decreases intra-abdominal and subcutaneous fat volumes. A decrease in fat absorption by orlistat appears to favourably influence the ratio between intra-abdominal and subcutaneous fat. The proportion of palmitic acid, the most abundant saturated fat in serum phospholipids, increased significantly only in orlistat group, and the proportion of the essential fatty acid linoleic acid, the most abundant polyunsaturated fatty acid, decreased in both groups. The proportion of dihomo-γ-linolenic acid correlated inversely with whole-body insulin sensitivity both before and after weight loss. These data suggest that weight loss rather than inhibition of fat absorption enhances insulin sensitivity, and that the amount of dietary fat absorbed or its composition influences the size of adipose tissue depots in intra-abdominal and subcutaneous regions.

4) Rosiglitazone or metformin both increase hepatic insulin sensitivity. Rosiglitazone also enhances peripheral glucose uptake and reduces liver fat content, whereas metformin increases hepatic but not peripheral insulin sensitivity and has no effect on liver fat. Rosiglitazone also increases insulin clearance, which could be due to enhanced insulin clearance by the liver. Rosiglitazone induces a dramatic increas in serum adiponectin, which may contribute to the decrease in liver fat content.

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