Insulin resistance in obese patients with type 2 diabetes mellitus : effects of a very low calorie diet

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CHAPTER 10

Summary and conclusions

INTRODUCTION

The increased worldwide incidence and prevalence of type 2 diabetes mellitus has reached epidemic proportions. Nowadays over 190 million people worldwide have diabetes mellitus[1], the majority having type 2 diabetes mellitus. Of type 2 diabetic patients, more than 80% are obese[2].

In obese type 2 diabetic patients, insulin resistance contributes substantially to the pathogenesis of hyperglycaemia[3]. Moreover, in very obese type 2 diabetic patients, insulin resistance makes it often extremely difficult to achieve adequate glycaemic regulation. Most oral blood glucose-lowering agents and exogenous insulin therapy induce weight gain, hence aggravating insulin resistance.

Caloric restriction and weight loss improve insulin resistance and its associated metabolic abnormalities[4-8] and are in fact the only reasonable therapeutic options in very obese type 2 diabetic patients.

Given the enormous increase in obese type 2 diabetic patients it is of utmost importance to find the optimal therapeutic strategy for this patient group. The aim of this thesis was to gain more insight in the pathofysiology of insulin resistance induced by adipose tissue, the safety and feasibility of very low calorie diets (VLCDs), and in the short-term and long-term effects of a VLCD on insulin resistance of the liver, adipose tissue and skeletal muscle. The findings of our studies will be discussed in view of the aims we put forward in Chapter 1.

FIRST AIM

Because of the association of obesity with insulin resistance and the fact that most type 2 diabetic patients are obese, our **first aim was to evaluate the role of adipose tissue in insulin resistance**.

When adipose tissue is discussed here, we refer to white adipose tissue (WAT), since adult humans hardly have any brown adipose tissue. WAT contains mature adipocytes, pre-adipocytes and fibroblasts, connective tissue, nerve tissue, stromal vascular cells and immune cells. The functions of these components are highly integrated, making adipose tissue a true endocrine organ. Adipose tissue responds to afferent signals of several well-known hormones (insulin, glucagon, cortisol) and the autonomous nervous system (catecholamines), but also to several of the proteins that it secretes itself, thereby regulating its own metabolism and cell size.

It is unknown whether obesity causes insulin resistance or is merely a reflection of a primary pathogenetic (insulin-resistant) state. However, given the fact that lipodystrophy also causes whole-body insulin resistance[9] and that transplantation of adipose tissue back into lipodystrophic animals reverses glucose intolerance and diabetes[10] suggests an important role for adipose tissue.

Adipose tissue can modulate glucose homeostasis *via* the production of free fatty acids (FFA) and so-called adipocytokines (or rather adipokines, since many of the secreted products are not cytokines). Quantatively, FFA secretion is the most important. Elevated serum FFA concentrations can induce skeletal muscle insulin resistance *via* an impairment in insulin signalling[11]. In addition, chronically elevated FFAs lead to a decrease in insulin secretion by the pancreatic β-cells[12-14]. Finally, increased delivery of FFAs to the liver increases gluconeogenesis and might induce hepatic insulin resistance[15]. These FFA-induced metabolic disturbances are also referred to as lipotoxicity.

In Chapter 2, several of the so-called adipokines have been discussed. In obesity, increased production of leptin, restin, IL-6, TNF-α and ASP are found that correlate positively with insulin resistance, whereas adiponectin levels are decreased and correlate negatively with insulin resistance. New adipokines are being identified continuously, among them apelin[16], visfatin[17] and zinc-α2-glycoprotein (ZAG)[18,19], the first two of these being increased in obesity. The mechanism by which these adipokines induce insulin resistance is unclear but might involve impaired insulin signalling since several of the adipokines (leptin, TNF-α, possibly IL-6) can interfere with the insulin-signalling pathway. The elucidation of the exact role of adipokines in insulin resistance is further complicated by the heterogeneity between the various adipose tissue depots. Although a primary role for visceral adipose tissue as opposed to subcutaneous abdominal adipose tissue has recently been challenged[20], it is a fact that adipocytes in these various fat depots have a different secretion pattern[21,22] (see Table 1). Moreover, these secretion patterns might be different in obesity and diabetes mellitus. For example, adiponectin production in healthy humans is higher in subcutaneous adipose tissue in comparison to visceral adipose tissue. However, in both insulin-resistant rodents[42], as well as in humans it seems that omental adiponectin secretion is impaired, whereas it is preserved in subcutaneous adipose tissue[31,45,46].

Table 1. Characteristics of adipocytes derived from visceral adipose tissue (VAT) in comparison to those of subcutaneous adipose tissue (SAT).

Biochemical factors	**Regional differences**	**Physiological effect**
Lipolytic response to catecholamines	VAT > SAT[23,24]	
Antilipolytic effect of insulin	SAT > VAT[22,25]	↑ NEFA and TG turnover
Leptin secretion	SAT > VAT[26-28]	less CNS regulation of VAT, ↓ insulin sensitivity
Adiponectin secretion	SAT > VAT[29-31]	↑ insulin sensitivity
Acylation stimulating protein (ASP)	VAT > SAT[26]	
IL-6	VAT > SAT[32,33]	inflammation, cardiovascular risk
TNF-α	VAT = SAT[26,34-36]	
Resistin	Abdominal > tigh[37,38]	
PAI-1	VAT > SAT[32,39,40]	cardiovascular risk
Innate characteristics of preadipocytes		
Preadipocyte differentiation and fat cell-function gene expression	SAT > VAT[41,42]	
Apoptosis	VAT > SAT[43]	

It has been noted that the size of adipocytes correlates better with insulin resistance than any other measure of adiposity. Weyer *et al.* reported that enlarged abdominal adipocytes predicted the development of type 2 diabetes mellitus, independent of emerging insulin resistance and (impaired) insulin secretion, in 108 previously normal glucose tolerant Pima Indians followed for 9.3 ± 4.1 years, of whom 33 developed type 2 diabetes[47]. It has been proposed that a diminished capacity for proliferation and differentiation of mesenchymal precursor cells leads to hypertrophy of mature adipocytes under conditions of energy excess[48]. These enlarged adipocytes are thought to secrete a different, insulin-resistance and atherogenesis provoking, pattern of adipokines and lead to ectopic fat storage because of a diminished capacity to store triglycerides. This ectopic storage of fat in liver, muscle and pancreas then leads to decreased insulin-mediated suppression of hepatic glucose production, decreased insulin-stimulated glucose uptake and decreased insulin secretion in these organs, respectively[49].

In conclusion, given the fact that both obesity[50] and lipodystrophy[9] are associated with insulin resistance and that transplantation of fat in lipodystrophic mice restores the metabolic abnormalities[10], supports an important role for adipose tissue in insulin resistance. As to the mechanism by which obesity induces insulin resistance, several theories have been proposed. The portal/visceral hypothesis[51], which proposes a primary role for visceral adipose tissue that would be deleterious because produced FFAs drain directly to the liver *via* the vena portae, has recently been challenged but, given this unique drainage of visceral FFAs and adipokines (that show a fat depot specific secretion pattern) directly to the liver, cannot be completely rejected. Notwithstanding, whether derived from visceral or truncal adipose tissue, elevated serum FFA levels, are involved in the pathogenesis of insulin resistance *via* the concept of lipotoxicity[49]. Two new paradigms involve the "theory of ectopic fat storage"[48,49,52] and that of "the adipocyte as an endocrine organ"[53]. These paradigms can also be explained using the concept of dysfunctioning adipose tissue. In this model, a defect in proliferation and differentiation of preadipocytes leads to enlarged mature adipocytes that secrete a different, insulin-resistance inducing, pattern of adipokines and have a diminished capacity to store triglycerides, leading to an ectopic storage of fat. If fat oxidation does not increase in these organs, then intracellular accumulation of lipids, with insulin resistance will occur. Further research is needed to investigate the interactions between the environment and adipose tissue leading to this impaired functioning of adipose tissue.

SECOND AIM

Leptin is secreted by adipocytes in direct proportion to adipose tissue mass[54-56] and nutritional status[57,58]. The primary role of leptin is to serve as a metabolic signal of energy deficiency rather than excess[59]. Serum leptin levels rapidly decrease during caloric restriction

and weight loss[57,60], which leads to increased appetite and decreased energy expenditure. In obesity, serum leptin levels are increased[54,56], indicating a state of leptin resistance. Unfortunately, the leptin response to caloric restriction is preserved in obesity.

Serum insulin levels are also positively related to BMI and fat mass[61]. Moreover, several studies have shown a close correlation between serum leptin and serum insulin[62-65]. It is unknown, however, whether this relation also holds in patients with a severely disturbed insulin secretion. Moreover, most studies have evaluated the relation between fasting serum levels of leptin and insulin and did not study the relation between leptin and insulin secretion. Finally, data about the effect of weight loss on the relation between serum leptin and insulin in obese type 2 diabetic patients are scarce[64,66].

Therefore, we have studied the relation between fasting serum leptin and fasting serum insulin, as well as the area under the curve of insulin following an intravenous (i.v.) glucose load in obese (BMI 37.6 ± 1.4 kg/m^2, mean ± SEM) type 2 diabetic patients (duration 8.0 ± 1.4 years, fasting plasma glucose [FPG 12.9 ± 0.8 mmol/L, HbA_{1c} 8.6 ± 0.4%) on day 2 and day 30 of a very low calorie diet (VLCD, Modifast®, 450 kCal/day). During the VLCD, all blood glucose-lowering medication, including insulin, was discontinued. It was found that, even when insulin secretion was severely disturbed, the relation between serum leptin and serum insulin and insulin secretion remained. This was also true during energy restriction with weight loss. Whether insulin regulates leptin levels or *vice versa*, or alternatively, whether both are regulated in concert to reflect changes in energy balance, cannot be deduced from this study. From circumstantial evidence, however, it seems most likely that insulin regulates leptin.

AIMS 3 TO 5

These aims were investigated in a single study, presented in Chapter 3. In short, seventeen obese (BMI 37.6 ± 5.6 kg/m^2, mean ± SEM) patients with type 2 diabetes (duration 8 ± 5.8 years) with persistent high blood glucose levels (FPG 12.9 ± 3.1 mmol/L, HbA_{1c} 8.6 ± 1.6%) despite maximal doses of oral blood glucose-lowering medication and/or insulin (66 to 340 units per day) started a VLCD (Modifast®, 450 kCal/day) for 30 days during which all blood glucose-lowering medication was discontinued. On days 0, 2, 10 and 30, of the diet, body weight was measured and fasting serum samples of glucose, insulin, C-peptide and leptin were taken. An intravenous glucose tolerance test was performed on day 2 and day 30. *A priori*, a responder was defined as a patient with a FPG level < 10 mmol/L on day 30.

The third aim of this thesis was to test whether it is safe to start a VLCD in obese type 2 diabetic patients undergoing insulin therapy and simultaneously discontinue all blood glucose-lowering medication, including insulin. The latter was an important issue, since discontinuation of all blood glucose-lowering agents would minimise the risk for hypoglycaemia and facilitate weight loss[67].

During the study presented in Chapter 3 and outlined in short above, no side effects were noted during the VLCD. Especially, no overt hyperglycaemia (glucose levels > 20 mmol/L) was noted, despite the fact that all blood glucose-lowering medication was discontinued. In addition, no hypoglycaemia, hypotension, vasovagal collaps, gall-bladder disease or cardiac events were observed. The 3 patients that did not complete the VLCD, all quit the study in the first few days because they did not like the Modifast®.

Meanwhile, over the years, more than 40 very obese insulin-treated type 2 diabetic patients have been treated with a VLCD and the simultaneous discontinuation of all blood glucose-lowering agents in a study setting, and also several patients in a non-study setting. In none of these patients adverse events were noted. Patients with known coronary artery disease were excluded, but diet therapy might be safe in these patients as well. Patients tolerated the diet very well, even for up to 8 months. Notably, women found it easier to adhere to the VLCD than men, probably because most of the women were not in the working process and because they found it more important to lose weight for esthetical reasons.

Our fourth aim was to establish whether blood glucose levels do indeed decline already after 2 days of a VLCD and the fifth aim to find factors that would discriminate responders from non-responders.

The study described in Chapter 3 showed a dichotomy in the blood glucose-lowering response to the VLCD: of the 14 patients that completed the 30-day VLCD, 8 patients could be defined as responders and 6 patients were classified as non-responders. The difference in blood glucose-lowering response to a VLCD was already apparent on day 2 of the VLCD: responders had only a small increase or a decline in fasting plasma glucose (FPG, +0.64 ± mmol/L [mean ± SEM]) whereas non-responders had an increase in FPG levels (4.15 ± 3.3 mmol/L), $p = 0.035$. It appeared that non-responders had a longer duration of type 2 diabetes mellitus (12.3 ± 2.6 *versus* 5.0 ± 1.4 years), lower fasting serum insulin, C-peptide and HOMA-β values and a lower second-phase insulin response following an i.v. glucose load on both day 0 and day 30. In a step-wise discriminant analysis, the change in FPG from day 0 to day 2 in combination with the area under the curve (AUC) of insulin above baseline during an intravenous glucose tolerance test (IVGTT) on day 2, completely distinguished responders from non-responders. We also found that the disappearance rate of glucose (k-value), as a measure of peripheral insulin sensitivity, neither differed between responders and non-responders, nor did it change with weight loss.

Therefore, the following conclusions can be drawn from this study with respect to the aims we put forward. With respect to the **fourth aim**, one can conclude that blood glucose levels can indeed decline already within the first few days of a VLCD. However, it seems that remaining endogenous insulin secretory capacity (rather than insulin sensitivity, since no difference in k-values was observed) determines the magnitude of this improvement. Later studies (Chapter 5 and 7) have confirmed that blood glucose levels decrease within 2 days of a VLCD in patients with remaining endogenous insulin secretion. With respect to the **fifth aim**, we

found that non-responders had a lower capacity to secrete insulin. Given the fact that they also had a longer duration of type 2 diabetes mellitus, this is probably due to ongoing failure of the pancreatic β-cell. Furthermore, non-responders can already be discriminated from responders on day 2 of a VLCD on the basis of an increase in FPG levels from day 0 to day 2 and a low area under the curve of insulin following an i.v. glucose load on day 2 of the VLCD. For practical purposes, however, the fasting C-peptide level is an easier indicator of whether or not a patient will show a glucose-lowering response to weight loss: patients with a fasting C-peptide level < 0.8 ng/mL are less likely to have a decrease in FPG levels during the VLCD as compared to patients with a fasting C-peptide level > 0.8 ng/mL. In patients with a C-peptide level < 0.8 ng/mL, one can choose to either continue (or start, if not yet part of the therapy) metformin during the VLCD or stop all blood glucose-lowering agents at the start of the VLCD and if blood glucose levels do not decline within a few days, start metformin therapy (or another oral blood glucose-lowering agent).

Given the observations of this study, we decided to include only patients with remaining endogenous insulin secretion (defined as a fasting C-peptide level greater than 0.8 ng/mL and/or a 2 times increase of the basal C-peptide level [cut-off value 0.5 ng/mL] after 1 mg glucagon i.v.) in our subsequent studies. The reason was that we did not want to expose the patients to high blood glucose levels for a longer period of time, and obviously, if remaining endogenous insulin secretion is low, blood glucose levels rise even at low caloric intake (patients have become insulin-dependent). However, as already described above, a low C-peptide level does not exclude the use of a VLCD, but, if C-peptide levels are low, oral blood glucose-lowering agents should either be continued during the VLCD or stopped, but restarted when blood glucose levels do not decline within 7-10 days of the VLCD. We did not want to risk the chance of having to start oral blood glucose-lowering agents because they could disturb the results of our metabolic studies. Therefore, only subjects with remaining insulin secretory capacity, as defined above, were included in later studies.

AIM 6 AND 7

To study the short-term blood glucose-lowering effect of a VLCD, both on the whole-body level and at the molecular level, 12 obese (BMI 36.3 ± 1.0 kg/m^2, [mean ± SEM]) type 2 diabetic (age 55 ± 4 years; HbA_{1c} 7.3 ± 0.4%) patients undergoing insulin therapy were studied on day 0 and day 2 of a VLCD (Modifast®, 450 kCal/day). Three weeks before the study all oral blood glucose-lowering medication was discontinued and from day -1 on, insulin therapy was stopped as well. Endogenous glucose production (EGP) and whole-body glucose disposal (6,6 $^2H_2$-glucose), lipolysis ($^2H_5$-glycerol), and substrate oxidation (indirect calorimetry) rates were measured before and after the VLCD in basal and hyperinsulinaemic (insulin infusion: 10 min prime followed by a constant rate of 40 mU/ m^2 per minute[68]) eug-

lycaemic conditions. Insulin signalling and expression of GLUT4, FAT/CD36 and triglycerides were assessed in skeletal muscle biopsies, obtained before the clamp and 30 min after the start of the insulin infusion.

With respect to the **sixth aim**, we found that short-term energy restriction without weight loss, lowers blood glucose levels due to a decrease in EGP with no effect on peripheral insulin sensitivity. As to the mechanism by which basal EGP was reduced; fasting serum glucagon, cortisol and growth hormone levels, as well as fasting serum non-esterified fatty acids, glycerol, triglycerides and lactate, were similar between study days. Although the lower fasting serum insulin levels we found suggested a better insulin sensitivity of the liver, this was not supported by the clamp studies. The reason that we did not find a better suppressibility of EGP by insulin during the hyperinsulinaemic euglycaemic clamp might have been due to the relatively high serum insulin levels achieved during the clamp (88 mU/L and 84 mU/L on day 0 and day 2, respectively). These concentrations might have been high enough for a near-maximal suppression of the glucose (and glycerol) R_a, making it difficult to observe changes between study days. Table 2 summarises some other studies of short-term, and longer-term energy restriction in obese type 2 diabetic patients. Only studies using a hyper-insulinaemic euglycaemic clamp, in combination with the isotope dilution technique as a measure of peripheral glucose disposal and endogenous glucose production, were included.

As described in Chapter 1 (section 1.4.2), insulin-stimulated glucose uptake is disturbed in patients with type 2 diabetes mellitus. This seems to be due to disturbances in the insulin-signalling cascade leading to GLUT-4 translocation. Table 3 summarises defects, known to date, in insulin signalling in obese, non-obese diabetic and obese diabetic patients. Few, if any, studies have been performed in humans evaluating the effect of short-term (Chapter 6) and long-term (Chapter 8) effects of energy restriction on the insulin-signalling pathway and GLUT-4 translocation. Although we did not observe an effect of calorie restriction *per se* on whole-body glucose disposal, we still analysed the muscle biopsies because we expected to find changes at the molecular level that were not yet translated to an effect on the whole-body level. However, no diet effect was found on the expression of the insulin receptor and insulin receptor-1 (IRS-1) or on IRS-1 associated phosphatidylinositol 3'-kinase (PI3K) activity; on FAT/CD36 expression pattern, GLUT4-translocation or triglyceride distribution, in either the basal or insulin-stimulated situation in skeletal muscle biopsies. Unexpectedly, basal PKB/Akt-phosphorylation on T308 and S473 increased after the diet. The meaning of this finding is unclear. However, as outlined in Chapter 1, PKB/Akt is also involved in the regulation of hepatic gluconeogenesis[88]. Hence, if our findings also apply to the liver, higher basal PKB/Akt concentrations in the liver might explain the observed decrease in basal EGP. Unfortunately, ethical considerations prohibit us to take liver biopsies in humans for study purposes.

In conclusion, with respect to the **seventh aim** we show that a 2-day VLCD has no effect on insulin stimulation of key signalling molecules or on translocation of the fuel transporters

Table 2. Effect of energy restriction on glucose and lipid metabolism in obese patients with type 2 diabetes.

	Henry [4],a	**Laakso** [5], b	**Kelley**[7],b	**Markovic**[6],b
Year	1985	1988	1993	1998
Number of patients	30	8	7	10
Age (yrs)	53 ± 11	52.6 ± 2.0	58.7 ± 3.3	48.3 ± 4.4
Duration DM2 (yrs)	9 ± 5	10.8 ± 1.7	< 5	?
Diabetes medication Diet Oral Insulin	3 11 16	1 7 -	2 5 -	7 2 -
FPG (mmol/L)	16.5 ± 3.9	11.4 ± 0.5	12.3 ± 1.4	7.3 ± 0.7
HbA1c (%)	12.3 ± 2.2	10.8 ± 0.5	8.8 ± 0.5	-
Weight (kg)	99.1 ± 14.2	92.8 ± 3.1	92.7 ± 4.7	-
BMI (kg/m^2)	37.1 ± 4.9	33.7 ± 0.8	32.8 ± 1.9	32.3 ± 0.8
Intervention (I)	40-day VLCD (330 kCal/d, liquid formula)	12 days 500 kCal/d (formula diet) followed by 3 days 800 kCal/d	7 days eucaloric, 7days 800kCal/day, 8 weeks VLCD (400kCal/d) + 3 weeks increasing intake, 7 days balance†	28 days (- 1000 kCal/d§; 1100 ± 250/day)
Diabetic medication during the intervention	Oral blood glucose-lowering medication and insulin were stopped 3 weeks and 1-3 days before the start of the study, resp.	Unclear, only mention is made that patients were in secondary drug failure	Oral blood glucose lowering agents were discontinued 3 weeks before the start of the study	Oral blood glucose lowering medication stopped 2 weeks before the start of the study
FPG (mmol/L) after I	7.6 ± 0.5 on day 10 (weight loss 4.6±0.2 kg)	9.6 ± 0.5 (weight loss ~ 5.1 kg)	9.5 ± 0.9, 7 days 800kCal/d (weight -2.2kg) 7.0 ± 0.7 at 13 weeks (weight -14.8 kg)	6.2 ± 0.5 on day 4 (weight loss 1.7 ± 2.2kg) 5.3 ± 0.4 on day 28 (weight loss 6.3 ± 0.4kg)
Basal EGP before I	149 ± 13 mg.m^{-2}.min^{-1}	2.49 ± 0.15 mg.kg^{-1}.min^{-1}*	158 ± 13 mg.m^{-2}.min^{-1}	14.0 ± 1.1 µmol.kgFFM^{-1}.m^{-1}
Basal EGP after I	81 ± 5 mg.m^{-2}.min^{-1} on day 10	2.04 ± 0.1 mg.kg^{-1}.min^{-1}	125 ± 9 mg.m^{-2}.min^{-1} after 7 days 800kCal/d 100 ± 6 mg.m^{-2}.min^{-1} at 13 weeks	11.3 ± 1.3 µmol.kgFFM^{-1}.m^{-1} on d 4 12.7 ± 1.3 µmol.kgFFM^{-1}.m^{-1} on d 28
Clamp EGP before I	-	-	-	3.8 ± 2.1 µmol.kgFFM^{-1}.m^{-1}‖
Clamp EGP after I	-	-	-	0.6 ± 1.8 µmol.kgFFM^{-1}.m^{-1} on d 4‖ 0.7 ± 1.6 µmol.kgFFM^{-1}.m^{-1} on d 28‖
Glucose Rd before I	-	2.34 ± 0 0.15 mg.kg^{-1}.min^{-1}*	142 mg.m^{-2}.min^{-1}‡	18.9 ± 2.0 µmol.kgFFM^{-1}.m^{-1}‖
Glucose Rd after I	-	4.01 ± 0 0.4 mg.kg^{-1}.min^{-1}*	188 ± 17 mg.m^{-2}.min^{-1} , 7 days 800kCal/d‡ 244 ± 21 mg.m^{-2}.min^{-1}, at 13 weeks‡	15.8 ± 1.8 µmol.kgFFM^{-1}.m^{-1}, d 4‖ 19.6 ± 1.9 µmol.kgFFM^{-1}.m^{-1} , d 28‖
Basal glycerol Ra before I	-	-	-	-
Basal glycerol Ra after I		-	-	-
Remarks	No data on EGP are given on day 40. Greatest reduction in FPG within 10 days. A meal tolerance test suggested improved peripheral insulin sensitivity and insulin secretion	After 2 weeks of a 500 kCal diet, peripheral insulin sensitivity improved, relatively week improvement in basal EGP as compared to other studies	7 days of CR led to half of the improvement in FPG, HPG, insulin sensitivity and insulin secretion	4 days CR improved HGO, prolonged CR also improved insulin sensitivity

Table 2, continued.

	Christiansen[69],b	**Jazet**[70],b	**Jazet**b
Year	2000	2005	In preparation for submission
Number of patients	8	12	10
Age (yrs)	51 ± 4	55 ± 4	54 ± 3
Duration DM2 (yrs)	5 ± 3	7.9 ± 1.3	8 ± 3
Diabetes medication Diet Oral Insulin	Oral and/or insulin	All patients used insulin (mean 78 ± 9 U/day), 6 also used metformin and 1 also used rosiglitazone	All patients used insulin (mean 94 ± 14 U/day), 8 also used metformin and 2 also used rosiglitazone
FPG (mmol/L)	11.9 ± 1.4	11.3 ± 1.3	11.1 ± 0.8
HbA1c (%)	8.1 ± 0.5	7.3 ± 0.4	7.7 ± 0.4
Weight (kg)	107 ± 14	107.9 ± 2.9	113.0 ± 7.1
BMI (kg/m^2)	36 ± 3	36.3 ± 1.0	40.2 ± 1.6
Intervention (I)	5 days eucaloric, 10 days 25% of ECN, 10 days 75% of ECN	2 days of a VLCD (formula, 450 kCal/day)	VLCD (formula, 450 kCal/day) until 50% of overweight was lost (50% OWR). Study days on day 2 and day 50% OWR
Diabetic medication during the intervention	All blood glucose-lowering medication (including insulin) was discontinued 2 weeks before the start of the study	Oral blood glucose-lowering agents were stopped 3 weeks before the start of the study, only short-acting insulin on day –1, insulin stopped at the start of the study	Oral blood glucose-lowering agents were stopped 3 weeks before the start of the study, only short-acting insulin on day –1, insulin stopped at the start of the study
FPG (mmol/L) after I	8.9 ± 1.6 , day 5 (of 25% ECN) (weight loss 2 kg) 7.4 ± 1.4, day 10 (of 25% ECN) (weight loss 3 kg) 8.8 ± 1.3, day 20 (d 10 of 75% ECN) (weight loss 3 kg)	10.3 ± 1.0 on day 2 (weight loss 2.9 ± 0.4 kg)	7.8 ± 0.5 on day 50% OWR (weight loss 20.3 ± 2.2 kg day 2 compared to day 50% OWR)
Basal EGP before I	22 ± 2 μmol.kgFFM^{-1}.m^{-1}	14.2 ± 1.0 μmol.kg^{-1}.min^{-1}	20.0 ± 0.9 μmol.kgLBM^{-1}.min^{-1}
Basal EGP after I	18 ± 2 μmol.kgFFM^{-1}.m^{-1} on d 5 17 ± 2 μmol.kgFFM^{-1}.m^{-1} on d 10 22 ± 2 μmol.kgFFM^{-1}.m^{-1} on d 20	11.9 ± 0.7 μmol.kg^{-1}.min^{-1}¶	16.4 ± 1.2 μmol.kgLBM^{-1}.min^{-1}
Clamp EGP before I	-	5.5 ± 0.8 μmol.kg^{-1}.min^{-1}¶	8.5 ± 0.9 μmol.kgLBM^{-1}.min^{-1}#
Clamp EGP after I	-	5.2 ± 0.5 μmol.kg^{-1}.min^{-1}¶	4.6 ± 1.2 μmol.kgLBM^{-1}.min^{-1}#
Glucose Rd before I	c	12.1 ± 0.7 μmol.kg^{-1}.min^{-1}¶	18.8 ± 2.0 μmol.kgLBM^{-1}.min^{-1}#
Glucose Rd after I	c	11.3 ± 1.0 μmol.kg^{-1}.min^{-1}¶	39.1 ± 2.8 μmol.kgLBM^{-1}.min^{-1}#
Basal glycerol Ra before I	9 ± 1 μmol.kgFFM^{-1}.m^{-1}	5.2 ± 1.0 μmol.kg^{-1}.min^{-1}	16.4 ± 2.3 μmol.kg fat mass^{-1}.min^{-1}
Basal glycerol Ra after I	9 ± 2 μmol.kgFFM^{-1}.m^{-1}on d 5 7 ± 1 μmol.kgFFM^{-1}.m^{-1}on d 10 7 ± 1 μmol.kgFFM^{-1}.m^{-1} on d 20	4.0 ± 0.6 μmol.kg^{-1}.min^{-1}	14.6 ± 1.4 μmol.kg fat mass^{-1}.min^{-1}
Remarks	Short-term CR reduces EGP. Longer term CR also improves glucose disposal. EGP rapidly rises with increase in caloric intake	2-day VLCD improved FPG due to a decrease in basal EGP with no effect on insulin sensitivity.	Considerable weight loss not only restores basal EGP to normal levels but also greatly enhances peripheral insulin sensitivity, especially insulin-stimulated glucose disposal, despite the fact that patients were still obese and used no blood glucose-lowering medication

Legend to Table 2

Weight losses given are compared to day 0.

[a]Values are presented as mean ± SD; [b]Values are presented as mean ± SEM

[*] insulin infusion rate 40 mU/m^2/minute (clamp serum insulin concentration 89 ± 5 mU/L before and after the intervention)

† basal (before) data are after 7 days eucaloric, then data after 7 days 800 kcal/day and data following a 12 week weight reducing programme (8 weeks 400kCal/day liquid formula diet, 3 weeks increase with 200 kCal/day, followed by 1 week eucaloric :third study day) are presented

‡ insulin infusion rate 100 mU/m^2/minute (clamp serum insulin concentrations varied from 200-210 mU/L during the various clamps)

[§] 1000 kCal/d less than patients used to consume as assessed by a 4-day dietary record

[‖] relatively low insulin levels were obtained during the clamp (250 pmol/L ≅ 35 mU/L)

[c] Rd glucose measured by Christiansen *et al* . were non-insulin stimulated values, also presented divided by plasma glucose levels (metabolic clearance rate of glucose), values were 2.0 ± 0.2, 2.1 ± 0.2, 2.1 ± 0.3 and 2.7 ± 0.3 ml.kgLBM^{-1}.min^{-1} at baseline, day 5, 10 and 20, respectively.

ECN= eucaloric needs, LBM = lean body mass, CR = calorie restriction, EGP = endogenous glucose production, FPG = fasting plasma glucose

[¶] glucose infusion rate 40 mU/m^2/min (clamp serum insulin values 88.1 ± 5.9 and 83.7 ± 4.8 mU/L on day 0 and day 2, respectively, p= NS)

[#] glucose infusion rate 40 mU/m^2/min (clamp serum insulin values 90.2 ± 3.3 and 80.8 ± 4.0 mU/L on day 2 and day 50% OWR, respectively, p = 0.023. Difference probably due to increased clearance of insulin)

FAT/CD36 and GLUT-4. We did observe a decrease in basal PKB/Akt phosphorylation, however, that might be linked to the decrease in basal EGP.

AIM 8 AND 9

To investigate the effect of weight reduction induced by caloric restriction as opposed to caloric restriction only, on insulin sensitivity, 10 obese (BMI 40.2 ± 1.6 kg/m^2 [mean ± SEM]) insulin-treated type 2 diabetic patients (HbA_{1c} 7.7 ± 0.4%, FPG 11.1 ± 0.8 mmol/L) were studied on day 2 of a very low calorie diet (VLCD, Modifast®, 450 kCal/day) and again after losing 50% of their overweight (50% OWR). Oral blood glucose-lowering agents and insulin were discontinued 3 weeks prior to the VLCD and at the start of the VLCD, respectively. Endogenous glucose production (EGP) and whole-body glucose disposal (6,6-$^2H_2$-glucose), lipolysis ($^2H_5$-glycerol) and substrate oxidation rates were measured on both study days in basal and hyperinsulinaemic (insulin infusion: 10 min prime followed by a constant infusion rate of 40mU/m2 per minute[68]) euglycaemic conditions. In addition, skeletal muscle biopsies were obtained from the vastus lateralis muscle, in the basal situation and 30 min after the initiation of the insulin infusion.

With respect to the **eighth aim** we showed that considerable weight reduction (20.3 ± 2.2 kg from day 2 to day 50% OWR), as opposed to caloric restriction *per se*, not only normalised basal EGP, but also improved insulin sensitivity, especially insulin-stimulated glucose disposal (increase 107% as compared to day 2, p = 0.001). The magnitude of the improvement in insulin-stimulated glucose disposal was comparable to that observed in some studies in morbidly obese patients undergoing bariatric surgery[89,90].

Although it is common knowledge that weight loss improves insulin sensitivity, the magnitude of this response has not been investigated before with state-of-the-art techniques (hyperinsulinaemic euglycaemic clamp technique with [6,6-$^2H_2$]-glucose and [$^2H_5$]-glycerol)

Table 3. Insulin signal transduction in skeletal muscle of obese, non-obese diabetic and obese diabetic subjects as compared to lean insulin sensitive subjects.

		Non-obese diabetic	Obese diabetic	Obese
IR	Binding or protein level	= [71], [72], [73]	= [71]	= [71]
	Phosphorylation	= [73], [74]	= [75], [76], [77]	= [78]
		↓ [71], [79]	↓ [71], [78]	↓ [71], [76]
IRS-1	Binding or protein level	= [73], [80], [81]	= [82]	= [82]
	Tyrosine phosphorylation	↓ [73], [80]	↓ [75], [81], [83]	= [81]
	Serine phosphorylation		↑ [83]	
PI3K	p85 protein level	= [84]	= [81], [82], [85]	= [81], [82], [85]
	Activity	↓ [73],[80], [83], [84]	↓ [77], [81], [82], [85]	= [77], [85]
				↓ [81]
Akt	Protein level	= [86]	= [83], [87]	
	Phosphorylation	↓ [86]	= [83], [85]	
			↓ [87]	
AS160	Protein level	= [87]		
	Phosphorylation	↓ [87]		

in this patient group: severely obese, insulin treated type 2 diabetic patients. In Table 2 an overview is presented of studies investigating the effect of varying degrees of energy restriction, during a variable period of time (4 days up till 8 weeks) on glucose and lipid metabolism in obese patients with type 2 diabetes. As can be deduced from Table 2, our patients were more severely obese, used more medication and were more severely insulin resistant as compared to the type 2 diabetic patients in most other studies, with the exception of the studies of Henry *et al*.[4] and Christiansen *et al*. [69]. Moreover, studies investigating the effect of considerable weight loss on peripheral insulin sensitivity, using state-of-the-art techniques are lacking.

The fact that the impressive improvement in insulin sensitivity in our patients occurred despite the fact that patients did not use any blood glucose- (or lipid-) lowering medication and were still obese (BMI 32.3 kg/m^2), underscores the importance of a dietary intervention in this patient group.

Our **ninth aim** was to investigate, in skeletal muscle biopsies, the effect of considerable weight loss on insulin signalling, the expression of the fuel transporters GLUT-4 and FAT/CD36 at the cell membrane, as well as the concentration of intramycocellular triglycerides.

In this study, we found equal insulin-stimulated PI3K activation on both study days, but the magnitude of the insulin-induced increase over basal was greater after weight loss ($p = 0.010$). Two down-stream effectors of PI3K, the PKB/AKT substrates AS160 and PRAS 40, also

showed an improved insulin-stimulated response with weight loss. Weight reduction had no significant effect on the abundance of the fuel-transporters GLUT-4 and FAT/CD 36 at the plasma membrane following hyperinsulinaemia. However, 7 out of the 10 patients showed a higher GLUT-4 density at the cell membrane after weight loss. An oil red O staining showed a significant decrease in intramyocellular triglycerides after weight loss in both type I and type II muscle fibres. Interestingly, time to weight loss of 50% overweight correlated negatively with the number of type I fibres at the start of the diet. We also find a trend towards an increase in the percentage of type I (and hence decrease in type II) muscle fibres with weight loss, a finding that has not been described before in patients with diabetes.

The reason why the increase in insulin-stimulated glucose disposal at the whole-body level was not reflected by a significant improvement in GLUT-4 translocation to the cell membrane is unclear and may reflect changes in intrinsic activity of GLUT-4. Others have also reported a dissociation between insulin-stimulated glucose disposal and either insulin signalling and/or GLUT-4 content at the cell membrane[90-93]. Several hypotheses can be put forward with respect to the relatively low concentration of GLUT-4 at the cell membrane. Firstly, it is possible that not the amount of GLUT-4 at the cell membrane but rather its function and, subsequently, the velocity of glucose transport over the membrane are the main determinants of insulin-stimulated glucose disposal. Secondly, another glucose transporter, either GLUT-1[93] or a yet unidentified one, may have contributed to the increase in glucose uptake seen after weight loss. Thirdly, it is possible that the increase in insulin-stimulated glucose disposal does not only take place in skeletal muscle but also in adipose tissue. The weight loss in our patients mainly reflected a decrease in body fat mass. This is most likely due to a depletion of intracellular triglyceride stores and not to a decrease in adipocyte number. The smaller adipocytes following weight loss might be better able to take up glucose as compared with the greater, lipid-laden adipocytes before weight loss. In our study, 4 out of the 8 patients from whom we obtained adipose tissue biopsies showed increased insulin-stimulated PI3K phosphorylation after weight loss.

AIM 10

Our tenth aim was to investigate the long-term effect of a once-only 30-day VLCD on body weight, hyperglycaemia, dyslipidaemia and blood pressure in obese type 2 diabetic patients.

To that end, we looked at the long-term effect of a once-only 30-day VLCD in 22 obese (BMI 37.7 ± 1.1 kg/m^2, mean ± SEM) type 2 diabetic patients (mean duration of diabetes 7.4 ± 1.0 years, fasting plasma glucose [FPG] 12.4 ± 0.8 mmol/L, HbA_{1c} 8.3 ± 0.3%) who participated in 2 other studies in which a 30-day VLCD was either used as the intervention or offered as a therapy after finishing the initial study. During the VLCD all oral blood glucose-lower-

ing medication and insulin therapy were discontinued. After the 30-day VLCD, caloric intake was slowly increased to eucaloric and patients were encouraged to maintain weight loss, but no specific diet was prescribed. Patients were followed at the outpatient clinic at 3-monthly intervals and medication for their diabetes (and blood pressure and/or dyslipidaemia) was reinstituted as deemed necessary by their own physician. Anthropometric parameters, blood pressure, glucose, HbA_{1c}, insulin, C-peptide and lipid levels were measured on day 0 and day 30 of the VLCD and after 18 months follow-up.

Surprisingly, after 18 months regular follow-up, as a group, patients had managed to maintain the loss of body weight achieved during the 30-day VLCD (-11.4 ± 0.6 kg). In addition, the improvement in systolic and diastolic blood pressure and serum lipids obtained during the 30-day VLCD was also largely sustained at 18 months follow-up. With respect to glycaemic regulation, HbA_{1c} levels were 0.7% lower as compared to the situation before the start of the diet, despite the fact that patients used less blood glucose-lowering medication, especially insulin (18 patients on day 0 [112 ± 21 units/day]; 6 patients at 18 months [23 ± 9 units/day]). The 6 patients using insulin therapy at 18 months follow-up had all regained weight to prediet levels.

In a subanalysis, it appeared that 8 patients had stable body weight (plus or minus 5 kilogram [kg]), 8 patients regained more than 5 kg of body weight and 6 patients lost more than 5 kg of body weight from day 30 to 18 months follow-up. The patients who had regained body weight to prediet levels had worse glycaemic control and dyslipidaemia and a higher (systolic) blood pressure as compared to the other two groups, but these parameters were still better than the values these patients had at the start of the study.

Treatment goals for glycaemic regulation (HbA_{1c} < 7%), blood pressure (< 130/80 mmHg) and serum lipids (LDL-cholesterol < 2.6 mmol/L, triglycerides < 1.7 mmol/L, HDL-cholesterol > 1.1 mmol/L) as set by the American Diabetes Association (ADA)[94] were not reached for all parameters but came very close (HbA_{1c} 7.6 ± 0.4%, total cholesterol 5.4 ± 0.2 mmol/L, triglycerides 2.5 ± 0.4 mmol/L, HDL cholesterol 1.3 ± 0.07 mmol/L, blood pressure 145 ± 4 mmHg / 81 ± 2 mmHg) and were very much improved as compared to before the intervention.

Thus, with regard to the **tenth aim** we conclude that a once-only 30-day VLCD in combination with the cessation of all blood glucose-lowering agents leads to a sustained improvement in glycaemic control, blood pressure and serum lipids at least up to 18 months follow-up even, albeit to a lesser extent, in patients who regained body weight.

OVERALL CONCLUSIONS WITH RESPECT TO THE USE OF VLCDs

The following conclusions with respect to the use of VLCDs, as a means to induce weight loss and improve glycaemic control, can be drawn from our findings. Firstly, VLCD therapy in obese, insulin-treated type 2 diabetic patients is safe, even when continued for up to 8

months. Secondly, the simultaneous discontinuation of all blood glucose-lowering agents does not lead to a deterioration of blood glucose levels, provided that patients do have residual endogenous insulin secretion. For practical purposes, this was defined as a fasting C-peptide level > 0.8 ng/mL and/or a two times increase of the fasting C-peptide level after 1 mg glucagon i.v.. Thirdly, in patients with remaining endogenous insulin secretion, FPG levels declined already within 2 days of a VLCD, when weight loss was minimal and despite the fact that all blood glucose-lowering agents were discontinued. Fourthly, this early (day 2) decline in FPG levels appeared to be due to a decrease in basal EGP without an effect on peripheral insulin sensitivity. Hence, in skeletal muscle biopsies no improvement in insulin signalling at the level of IRS-1-associated PI3K and PKB/Akt was seen and no increase in insulin-stimulated GLUT-4 translocation was observed. Fifthly, as opposed to short-term energy restriction, prolonged energy restriction leading to a loss of 50% of overweight, also improved peripheral insulin sensitivity, especially insulin-stimulated glucose disposal. Sixthly, at the molecular level this was accompanied by increased PI3K phosphorylation over basal after weight loss as compared to day 2 and a significant total AS 160 and PRAS40 phosphorylation after weight reduction. The amount of GLUT-4 at the cell membrane was higher in 7 out of 10 patients, although the group effect was not significant. An oil red O staining showed a significant reduction in intramyocellular triglycerides. Interestingly, the amount of type I muscle fibres before weight loss correlated negatively with time to weight loss of 50% overweight. In addition, a slight, non-significant increase in type I muscle fibres was observed after weight loss. Seventhly, in an observational analysis we found that the effect of a once-only 30-day VLCD on body weight, glycaemic control, blood pressure and dyslipidaemia was sustained after 18 months regular follow-up, even in patients who regained body weight to prediet levels.

Our findings stress the importance of diet therapy in obese (insulin-treated) type 2 diabetic patients. The fact that insulin-stimulated glucose disposal improved by 107%, despite the fact that patients were still obese, raises the question whether it can be fully restored with weight loss up to ideal body weight. On the other hand, thiazolidinediones (TZDs) and exercise can also improve insulin sensitivity, albeit *via* a different mechanism[84,95-97]. Perhaps the combination of a VLCD, exercise and a TZD can fully restore insulin sensitivity. In a new study we will investigate this, again in obese type 2 diabetic patients, during a 16-week intervention in which all patients will follow a VLCD and subgroups will receive either exercise and/or rosiglitazone. Again, hyperinsulinaemic euglycaemic clamp studies with stable isotopes and skeletal muscle biopsies will be performed before and after the intervention to accurately measure changes at the whole-body and molecular level.

Type 2 diabetes mellitus is associated with micro-and macrovascular long-term complications that are related to the increased morbidity and mortality seen in these patients[98]. Approximately 65% of patients with type 2 diabetes die as a result of a cardiovascular event[99]. Patients with type 2 diabetes have a 2-4 fold increased relative risk (RR) for the development of myocardial infarction, peripheral arterial disease and stroke[100]. This increased risk is as-

sociated with an increase in various metabolic and other cardiovascular risk factors such as hyperglycaemia, dyslipidaemia and hypertension. We observed a sustained beneficial effect of a once-only 30-day VLCD on these risk factors up to 18 months follow-up, even in patients that regained body weight. It remains to be evaluated how long these beneficial effects will persist and if the intermittent use of a VLCD (on demand, i.e., when body weight increases over a predefined weight [studied in obese non-diabetic patients[101]], or 5 days every 5 weeks[102]), or other stategies (addition of exercise and or an insulin-sensitising drug) will even be more beneficial or leads to a longer duration of the beneficial effects. Given the results of several large trials, also in patients with diabetes, that lower(ing) blood pressure[103,104], total[105] and LDL-cholesterol[106-108] and decreasing triglycide levels while increasing HDL-cholesterol[109-111] significantly reduces the risk for cardiovascular disease, a sustained improvement in these parameters could also reduce the risk for cardiovascular disease and, hence, reduce health care costs and usage in obese type 2 diabetic patients following a VLCD.

When the beneficial effects of a VLCD and the abovementioned considerations with respect to cardiovascular risk are taken into account, a VLCD can be an attractive, cost-effective therapy. VLCDs are in themselves relatively cheap (30 days of Modifast® costs approximately 160 Euro) and all blood glucose-lowering medication can be discontinued. Moreover, the improvement in cardiovascular risk factors are likely to lead to a decreased incidence of cardiovascular disease with less hospital admissions and interventions (and, hence, less days staying away from the economic process) which will lead to a much greater saving in health care costs. On the cost-side are the expenses of regular counselling. These can be minimised however, when a diabetic nurse performs most of the controls. It would be interesting to do a study at the outpatient clinic, which also takes into account the cost-effectiveness of a VLCD. It will be our job to convince insurance companies of the benefits of the VLCD and to persuade them to compensate for the costs of a VLCD.

Finally, although we only studied the VLCD in obese, mostly insulin-treated (in the study of Chapter 4 also patients on oral blood glucose-lowering agents only participated) type 2 diabetic patients, it is likely that the same treatment will be successful in obese patients with type 2 diabetes treated with diet and/or oral blood glucose-lowering agents. Because these patients are in an earlier phase of the disease process, results with respect to the improvement in insulin sensitivity will probably be even more impressive. We hypothesise that considerable weight loss in obese, non-diabetic but insulin-resistant patients will normalise insulin sensitivity.

In conclusion, a VLCD in combination with the simultaneous discontinuation of all blood glucose-lowering agents in obese, insulin-treated patients with remaining endogenous insulin secretion is safe, can increase insulin sensitivity to a great extent and the improvement in metabolic parameters is sustained up to 18 months follow-up. Our observations stress the importance of weight-reducing therapies, especially diet, because of its safety, low costs and availability, in this patient group.

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