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Insulin-associated weight gain in patients with type 2 diabetes mellitus

determinants and consequences

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Insulin-associated weight gain in patients with type 2 diabetes mellitus

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Chapter 1

General introduction and outline of the thesis

Introduction

This thesis focuses on determinants and consequences of insulin-associated weight gain in patients with type 2 diabetes mellitus (T2DM). It presents the results of studies performed in patients with T2DM who initiated insulin treatment, in patients on long-term insulin therapy and in healthy (lean and obese) subjects. This chapter summarizes the benefits and disadvantages of insulin treatment in patients with T2DM and discusses insulin-associated weight gain in more detail. At the end of the chapter, the outline of the thesis is presented.

Type 2 diabetes mellitus and insulin therapy

Diabetes is a disease characterized by a chronically elevated blood glucose concentration (hyperglycemia). Chronic hyperglycemia can lead to a number of complications if untreated (1, 2). Patients with T2DM thus require regular monitoring and ongoing treatment to maintain normal or near-normal blood glucose levels and minimize the risk of diabetes and cardiovascular-related complications. Treatment starts with lifestyle adjustments, but in the majority of patients with T2DM, lifestyle changes fail or are insufficient to control glucose levels and additional oral glucose-lowering medication is needed. Despite the development of alternative therapies in recent years, insulin remains an essential treatment for type 2 diabetes once oral therapy alone becomes inadequate. As T2DM is a progressive disorder, most patients with T2DM will eventually need insulin therapy to achieve adequate glycolic control (3, 4). Insulin treatment has a number of benefits. First, insulin therapy will lead to better glycemic control. Improving glycolic control by insulin decreases the risk to develop macro and micro vascular complications (2, 5). Second, insulin might preserve beta-cell function of the pancreas (6).

There are two main disadvantages with respect to insulin therapy. Insulin can induce hypoglycemia which might incur co-morbidity and is related to increased mortality (7). Moreover, insulin treatment is associated with weight gain (8-11).

Insulin-associated weight gain in type 2 diabetes patients

In the United Kingdom Prospective Diabetes Study (UKPDS) a significant mean weight gain of 5 kg after the start of the study was seen in those who received insulin treatment (5). Reported weight gain associated with insulin treatment in patients with T2DM is highly variable (range 1.7-8.7kg) (2;5-7). The change in weight gain varies with duration of insulin therapy, the use of metformin, and is dependent on the treatment protocol (conventional or intensive insulin regimens) (5). In Figure 1 trends in weight gain are shown in patients with T2DM starting different glucose lowering treatment protocols (5).

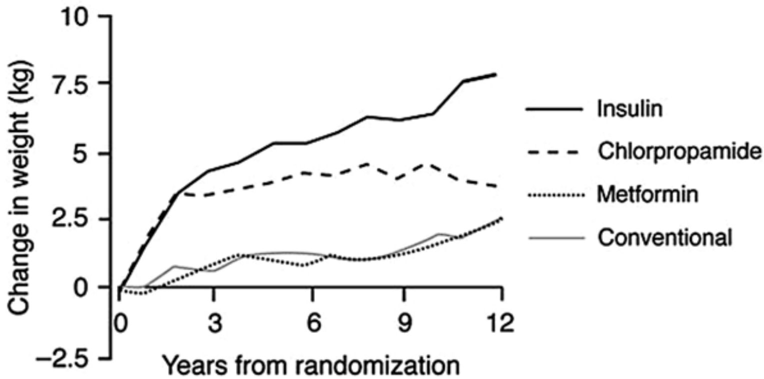

Figure 1 Weight change by treatment in overweight patients with T2DM in UKPDS assigned to intensive intervention with insulin. Chlorpropamide or metformin, or conventional treatment.

(Reprinted from the Lancet, 352(9131), no authors listed, "Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34)", 854-65, Copyright (1998), with permission from Elsevier).

Weight gain was seen in all groups, with the exception of the metformin treatment arm, but was largest in insulin-treated patients. Large inter-individual differences in weight gain can be seen in patients on insulin therapy. Some patients gain much weight ("gainers"), while other patients treated similarly do not gain weight or even lose body weight ("non-gainers").

This weight gain is obviously undesirable in a population showing pre-existent overweight and may negatively affect blood pressure, lipid levels, inflammatory and fibrinolytic parameters, and may also deter further optimization of insulin therapy. Several determinants and consequences on cardiometabolic risk profile of insulin-associated weight gain are proposed although formal prospective studies are lacking.

Proposed determinants of insulin-associated weight gain

In literature, the exact determinants of insulin-associated weight gain are unknown. A number of potential determinants is often listed:

1) Change in glycemic control

Insulin therapy improves glycemic control, which results in a decrease in glucosuria. When glucosuria decreases, less glucose, hence energy is lost and more energy will be stored as fat. Patients may experience weight gain if caloric intake is not

reduced (12). Carlson and Campbell have demonstrated this effect in patients with type 1 diabetes (11). A significant rise in weight (+2.6 kg) was found with insulin therapy, of which 2.4 kg was attributed to an increase in fat mass. Mäkimattila et al. also found a relation between decrease of glucosuria and weight gain in patients with T2DM. (10). During 12 months of insulin therapy, glucosuria decreased considerably (change in energy loss 0.83 ± 0. 27 MJ/day), with no differences between patients who used metformin and those who did not. The initial improvement of glycemic control within 6 months was associated with weight gain in this time-frame. If HbA_{1c} remains stable, patients may still gain weight, suggesting that changes in glycemic control do not determine further weight gain (13). The greatest weight gain will occur over the first months after starting with insulin therapy. This suggests that most initial weight gain is a 'catch-up' re-gain (14), which refers to the amount of weight loss patients experienced before insulin initiation. Patients who gain most weight after insulin initiation are those with the worst metabolic control before the treatment and those who experienced most weight loss prior to insulin therapy. So, the change in glycemic control (as reflected by the drop in HbA_{1c}) is probably an important contributor to insulin-associated weight gain over the first months after initiation insulin treatment. Some authors view the changes in glycemic control to be the major determinant of insulin-induced weight gain (10, 15, 16). However, the number of prospective and long-term follow-up studies of patients treated with insulin supporting this concept is limited.

2) Anabolic effects of insulin

Insulin, being an anabolic hormone, may in itself cause weight gain as insulin increases muscle protein synthesis and lipogenesis in fat tissue (17, 18). Insulin could also increase water and salt retention (19) resulting in weight gain.

The total (exogenous) insulin dose appears to be directly correlated with weight gain (20, 21). Henry et al. (21) found that the insulin dose (average 86-100 U/day of intermediate and regular insulin combined) was directly correlated with the amount of weight gain. Insulin detemir is a long-acting insulin that has consistently shown in randomized, controlled trials to have a weight sparing effect in both type 1 and type 2 diabetes (22, 23).

3) Energy intake

An increase in energy intake will lead to weight gain. Insulin may lead to defensive eating habits because of (fear of) hypoglycemia. Consequently, individuals may increase energy intake to proactively avoid such an event, resulting in weight gain (24). It has been shown in the Diabetes Control and Complications Trial (DCCT) that patients who had experienced one or more hypoglycemic episodes gained 6.8 kg in weight compared to 4.6 kg in patients with no hypoglycemia. DCCT, however, found

no relation between reported caloric intake and weight changes. A number of studies reported even a decrease of caloric intake after start of insulin therapy, suggesting that overeating did not contribute to weight gain (16, 25, 26).

4) Physical activity

Obesity is caused by an imbalance of energy intake and energy expenditure. Total daily energy expenditure (TDEE) is the result of basal metabolic rate (BMR), dietary-induced thermogenesis (DIT) and the energy expenditure of physical exercise (EEPE) or in formula: TDEE = BMR + DIT + EEPE (27-29). BMR in humans is the most important factor which contributes to TDEE. Normally BMR is about 1 kcal/kg/hour. As such, weight gain in diabetes may be due to a decrease in energy expenditure or basal metabolic rate. Makimatilla et al. (10) showed that improvement of glycemic control in type 2 diabetes led to a reduced BMR (expressed as per kg body weight) and thus reduced TDEE. This was counterbalanced by an increase in BMR caused by weight gain itself. The exact mechanisms underlying the correlation between glycemia and BMR has not been established so far but it may be related to increased gluconeogenesis (30, 31) and glucose cycling (32). In these studies it was concluded that even if energy intake (diet) would have been reduced by an amount corresponding with the energy loss due to glucosuria, weight gain will still occur because improvement of glycemic control in itself will decrease energy expenditure. When physical activity is reduced, a person will gain weight when keeping caloric intake similar. Jacob et al. (16) found no difference in physical activity between 3 groups of patients starting insulin therapy for T2DM (monotherapy insulin, insulin/pioglitazone and insulin/metformin). Only few studies have addressed physical activity changes after starting insulin therapy, and further research will be needed to answer this question.

5) Insulin effects on the central nervous system

The brain plays an important role in glucose and energy homeostasis (33, 34). The hypothalamus (more specifically the arcuate nucleus) contains neurons that exert action on food intake and energy expenditure both regulated by hormonal and nutrient-related signals. Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) are 'anabolic' neurons which are inhibited by insulin and leptin (35). NPY and AgRP promote food intake, reduce energy expenditure and thus induce weight gain. A decrease in the secretion of insulin and leptin might lead to weight gain, because of increased signaling by NPY and AgRP. Furthermore, insulin resistance and T2DM may also reduce neuronal insulin/leptin action and thereby induce weight gain. Figure 2 depicts a model in which the brain, glucose control and energy balance signaling are closely interrelated. It has been suggested that administration of exogenous insulin can disrupt pathways in the central nervous system that might lead to increase of food intake and weight gain (36-38). The precise role of (exogenous) insulin and

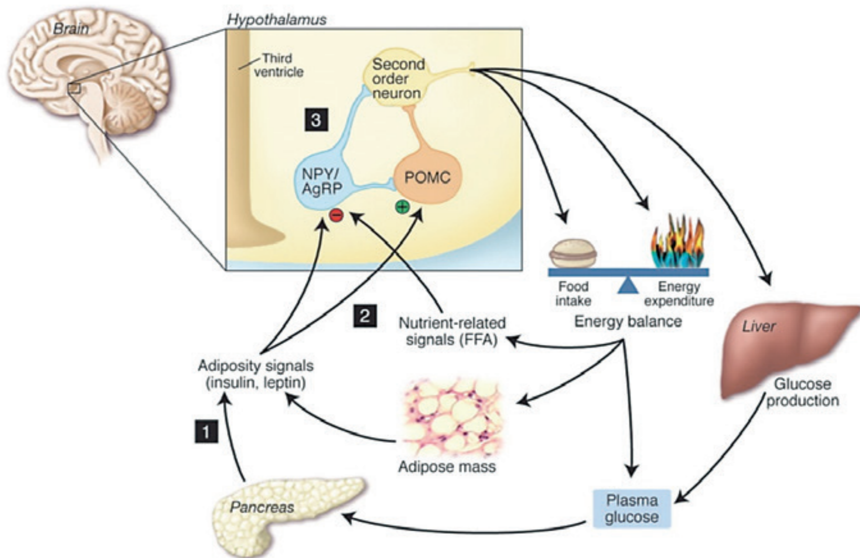

Figure 2 Defects in the secretion of insulin or leptin (**1**), in the hypothalamic sensing of adiposity- or nutrient related signals (**2**), or in the neuronal responsiveness to these inputs (**3**) predispose to both positive energy balance and increased glucose production. If sustained, these will result in pathological weight gain and insulin resistance.

NPY: neuropeptide Y, AgRP: Agouti-related peptide, POMC: proopiomelanocortin.
(Reprinted from reference 37, "Diabetes, obesity and the brain", 375-379, Copyright (2005), with permission from Science/AAAS).

other hormones (i.e. ghrelin, adiponectin, cholecystokinin, triiodothyronine, glucagon peptide-1) on the central regulation of food intake and hence weight gain still needs to be elucidated.

Potential consequences of insulin-associated weight gain

Weight gain may have several clinical and metabolic consequences such as:

1) Increase in insulin-resistance

Insulin-associated weight gain will further increase insulin resistance in patients with type 2 diabetes mellitus. As a consequence, patients need to increase their insulin dose to adjust for or to overcome the insulin-resistant state. Using more insulin will induce more weight gain with concomitant increase of insulin resistance; hence a vicious circle emerges. This scenario occurs in some patients and is feared by both

patients and clinicians: a patient started on insulin becomes more and more obese while glycemic control remains worse despite increasing doses of insulin.

2) "Psychological insulin resistance"

Another indirect effect of insulin-associated weight gain may be reluctance to inject insulin, because patients relate the weight gain to insulin treatment. It can be envisioned that this phenomenon, also called "psychological insulin resistance" (39, 40) will be a barrier against initiation of insulin therapy but will also hamper improvement of glycemic control because of reluctance to properly increase insulin dose. In general, adherence to insulin therapy is poor. Davies et al. investigated factors related to adherence of insulin therapy (41). Four categories of factors associated with non-adherence were identified: predictive factors for non-adherence, patient-perceived barriers to adherence, type of delivery device and cost of medication. So, not only psychological factors at the start of insulin therapy, but also patient-perceived barriers to adherence of insulin therapy may lead to reduced treatment satisfaction.

3) Reduced quality of life

Patients with T2DM and high body mass index suffer from a reduced quality of life (42). When starting insulin therapy patients may experience an increase of quality of life (43). Whether insulin-associated weight gain diminishes quality of life is unknown. Weight management interventions in obese patients with T2DM show a positive effect on quality of life (44).

4) Cardiovascular consequences

In the DCCT (45), patients with type 1 diabetes mellitus on intensive insulin treatment (multiple daily injections or continuous subcutaneous insulin injection) experienced more weight gain compared to those who received standard therapy (2 or 3 times daily injections). In addition, in the intensive study arm of DCCT patients had higher blood pressure and higher levels of cholesterol.

These results suggest that intensive insulin treatment and weight gain in patients with type 1 diabetes is associated with an unfavourable cardiovascular risk profile. Of course, cardiovascular risk can and probably will subsequently be managed by pharmacological interventions. The DCCT/EDIC study has reported that long-term cardiovascular outcomes in the intensively treated group were better, which supports the concept that lowering glucose on the long run decreases the risk to develop cardiovascular disease (46). Whether the benefit of improved glycemic control in the group with pronounced weight gain was similar has not been reported.

Several studies in patients with type 2 diabetes have examined the effect of glycemic treatment on cardiovascular endpoints (47-49). While intensive treatment reduced the incidence of non-fatal myocardial infarction, cardiovascular and

all-cause mortality was not affected or even increased. The ACCORD-trial, was prematurely ended after a mean follow-up of 3.5 years because of increased mortality in the intensive treated arm (48). The increased mortality in the intensively treated arm is not well explained. One possible explanation was the increased rate of hypoglycaemia in this group (50). It can be hypothesized that weight gain also influences cardiovascular outcome, because weight gain might offset the beneficial effects of improvement of glycemic control. Again, in the ACCORD-trial, more than 25 % of the patients in the intensively treated group (HbA_{1c} < 6,0 %) showed a mean weight gain of more than 10 kg (48)!

5) Metabolic consequences

Over the last two decades, the endocrine function of adipose tissue has received much attention. The adipose tissue is now viewed as an active endocrine organ, that produces many hormones, including adipocytokines. Expansion of adipose tissue mass is generally paralleled by profound morphological and inflammatory changes that include enlargements in adipocyte size and influx of various immune cells including macrophages. It can be hypothesized that the anti-inflammatory effects on insulin at the systemic level may be counteracted by pro-inflammatory changes associated with an increased fat mass. This may be particularly true in those who gain most weight. The effects of insulin-associated weight gain in patients with T2DM on the mechanisms listed above have not yet been studied.

Obesity and weight-gain may affect the autophagy process. Autophagy, an evolutionary conserved process aimed at recycling damaged organelles and protein aggregates in the cell, also modulates proinflammatory cytokine production in peripheral blood mononuclear cells. Because adipose tissue inflammation accompanied by elevated levels of proinflammatory cytokines is characteristic for the development of obesity, it can be hypothesized that modulation of autophagy alters adipose tissue inflammatory gene expression and secretion. This in turn may have an impact on cardiometabolic risk.

Conclusion

Insulin therapy is frequently needed to achieve adequate glycemic control in type 2 diabetes mellitus, but often at the expense of weight gain. This weight gain is highly variable across individuals but if it occurs it may negatively affect glycemic control, cardiometabolic risk profile and quality of life.

Mechanisms for insulin-associated weight gain are complex and incompletely understood. While the change in glycemic control (decrease of glucosuria) is an important determinant of weight gain in T2DM patients using insulin therapy, many

other factors probably play a role. Meanwhile, the consequences of insulin-associated weight gain, especially when outspoken, have so far received limited attention. Hence, we set out a series of studies aimed at elucidating in more detail the determinants and consequences of insulin-associated weight gain.

Methods used in this thesis

In this thesis, we have used several methodologies to assess physical activity, body fat content and distribution and adipose tissue morphology and function.

In a number of our studies we have used accelerometry to quantitatively measure physical activity (see Figure 3). Furthermore, we have performed subcutaneous adipose tissue biopsies to investigate adipose tissue morphology and inflammatory characteristics.

1) Accelerometry

Accelerometry is noninvasive and commonly used to asses free-living physical activity. The device used in our studies, the SenseWear™ device, has also been validated for study of sleep patterns, as the internal body media algorithm can identify sleep and wakefulness with moderate-to-high sensitivity, specificity, and accuracy (51, 52).The device is placed on the right upper arm over the triceps muscle for 4 consecutive 24-h periods. It contains a 2-axis accelerometer, heat flux sensor, galvanic skin response sensor, and a skin temperature sensor and is worn in

Figure 3 Physical activity in the presented studies is quantitatively measured using an 2-axis accelerometer (SenseWear Pro Armband™, Body Media, Pittsburgh, PA, USA).

Image courtesy Body Media, Inc. (a wholly owned subsidiary of AliphCom dba Jawbone®).

free-living conditions. Outcome variables from the activity monitor include: average metabolic equivalent (METS; 1 MET = consuming 1 kcal/kg of body weight per hour), time (minutes/day) spent at different activity intensity categories averaged per day over the measurement period and number of steps per day, sleep duration and fragmentation index (i.e. the number of interruptions measured per sleep).

2) Subcutaneous adipose tissue biopsy

When obesity develops, the adipose tissue undergoes distinct morphological changes including adipocyte enlargement, and macrophage influx. Furthermore, the subcutaneous adipose tissue produces several proteins related to inflammation (e.g. leptin, interleukin-6 and tumor necrosis factor α). We have performed subcutaneous adipose tissue biopsies (Figure 4) to investigate four potential changes in adipose tissue: morphological characteristics, macrophage influx, mRNA expression and protein levels of key inflammatory markers within the adipose tissue. Morphometry of individual fat cells was assessed using digital image analyses. For detection of macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody. Thereafter, the number of macrophages was counted per microscopic field. Total messenger RNA was extracted from adipose tissue and expression of genes was determined by real-time PCR analysis using an Applied Biosystem. Lastly, protein levels of adipocytokines in the adipose tissue were measured by Luminex fluorescent bead human cytokine immunoassays. The adipose tissue biopsies were performed in patients with type 2 diabetes mellitus before and 6 months after the start of insulin therapy. We also compared these tissue samples to those obtained in a set of lean, and weight-matched obese non-diabetic subjects.

Outline of the thesis

Most of the aforementioned factors related to insulin-associated weight gain have not been studied in a prospective way. Therefore, we have designed a number of studies aimed at identifying factors that determine weight gain after starting insulin treatment. Furthermore, we assessed the influence of insulin-associated weight gain on cardiovascular risk, particularly on the subcutaneous adipose tissue. Figure 5 shows an overview of studies presented in this thesis.

This thesis consists of two parts: determinants of insulin-associated weight gain in patients with T2DM are investigated in studies presented in **part I**. Thereafter, studies concerning the consequences of insulin-associated weight gain, particularly on the (subcutaneous) adipose tissue, and liver fat content related to the cardiometabolic profile will be addressed in **part II**.

Figure 4 Technique of abdominal subcutaneous adipose tissue biopsy by aspiration-needle. Superficial subcutaneous adipose tissue samples of ≈1–2 cm^3 (corresponding to ≈1–2 g) are obtained from the periumbilical area, under local anesthesia, from a comparable site of the abdomen. A region 5 cm lateral from the umbilicus (either to the left or right side of the abdomen) is sterilized. A half circular small dermal injection (intracutaneous) is made, and 2 mL of a local anesthetic agent injected. After 5 min, the skin is sterilized again. A needle (16 G) is then adapted to a 20-mL syringe and the piston is compressed. Approximately one-third of the length of the needle is inserted into the subcutaneous fat, and the needle piston is released maximally until it is locked by a stopper, thereby creating a vacuum. Tissue resistance is created by the surgeon gripping the abdominal wall with one hand while the other hand rotates the needle throughout the tissue in an up-down motion. Once the tissue is aspirated by the syringe, the needle is withdrawn and the piston is removed; adipose tissue samples are washed in physiologic serum, placed immediately in liquid nitrogen, and stored at −80°C until analysis.

Part I – Determinants of insulin-associated weight gain

As mentioned, many authors view the changes in glycemic control to be the major determinant of insulin-induced weight gain. This conclusion is based on studies that usually relate the difference in body weight between two time-points to the difference in HbA_{1c} in patients who initiated insulin therapy. These studies do not show to what extent change in body weight depends on change in HbA_{1c} over time. In **chapter 2**

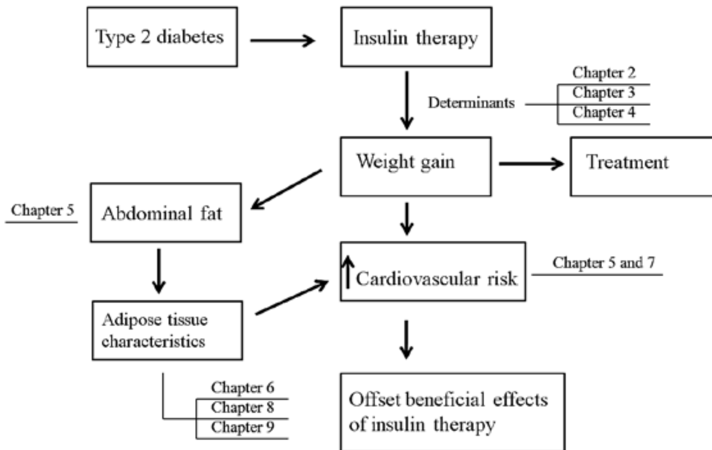

Figure 5 Overview of the studies and related subjects presented in this thesis.

we first investigated whether there was a relationship between change in glycated hemoglobin and weight gain. Here, we used a linear mixed model, which allowed us to study the relationship between insulin-associated weight gain and change in glycated hemoglobin in a large group of patients who had used insulin for three or more years.

A decrease in physical activity is inevitably related to weight gain if caloric intake remains stable. We questioned whether physical activity is reduced in insulin-treated patients with T2DM compared to lean, but especially to weight-matched obese subjects. In addition, we also compared quantitative and qualitative sleep characteristics between lean, obese subjects and patients with T2DM, as sleep duration and quality may influence physical activity. The results of these investigations are described in **chapter 3.**

Besides (change in) physical activity, a number of other potential determinants were tested in a prospective design. From literature it is known that insulin dose is correlated with weight gain (53). Also patients with diabetes mellitus who suffer from depressive complaints are at risk to gain weight. However, these two potential determinants of insulin-associated weight gain have not been investigated in a prospective design. In **chapter 4** we describe to what extend (change in) diabetes-related distress, insulin dose, age and physical activity determines insulin-associated weight gain in patients with type 2 diabetes. These potential relationships were tested

in a prospective study in which patients started insulin therapy and were followed for 12 consecutive months. Insulin dose was noted every 3 months. Diabetes-related distress was measured with the Problem Areas In Diabetes (PAID) questionnaire at baseline, after 6 and 12 months of insulin therapy. Physical activity was objectively measured with accelerometry (SenseWear Pro Armband™ (Body Media, Pittsburgh, PA, USA) (Figure 3). Furthermore, caloric intake was investigated as a potential determinant of insulin-associated weight gain.

Part II – Consequences of insulin-associated weight gain

To assess the influence of insulin-associated weight gain on the cardiometabolic profile, we investigated several “classical” and “non-classical” cardiometabolic risk factors of weight gain in long-term insulin-treated patients with T2DM. We first investigated several cardiometabolic risk factors in two specific groups: “gainers” (i.e. those who had large weight gain after start of insulin therapy) and “non-gainers” (i.e. those who did not experienced weight gain). We hypothesized that gainers would have a worse cardiometabolic profile compared to non-gainers. This was investigated in a cross-sectional study, in which fat content and distribution (physical examination, bioelectrical impedance analysis, dual energy X-ray absorption, and magnetic resonance imaging), liver fat content and physical activity levels (accelerometry (Sensewear®) armband) were assessed quantitatively. High hepatic fat content is associated with increased cardiometabolic risk (54, 55). In this study we specifically assessed hepatic fat content by means of magnetic resonance spectroscopy, which is the gold standard for non-invasive measurement of liver fat content. The results of this study are described in **chapter 5**.

Obesity / expansion of adipose tissue mass is accompanied by inflammatory changes in the adipose tissue. We wondered whether an insulin-induced increase in adipose tissue mass would have similar effects. To determine the effects of insulin therapy on adipose tissue morphology and inflammatory status, we prospectively studied subcutaneous adipose tissue characteristics before and after six months of insulin therapy. Adipose tissue morphology was assessed and influx of adipocytokines into the adipose tissue were measured. The results of this study are described in **chapter 6**.

Most patients with type 2 diabetes mellitus are obese and have an accumulation of abdominal fat. Additionally, liver fat content is strongly associated with intra-abdominal fat mass and clearly increased in subjects with T2DM. The exact mechanisms involved in liver fat accumulation are currently unclear. It has been suggested that adipose tissue dysfunction is associated with the development of hepatic steatosis yet data that link liver fat content to adipose tissue inflammation are scarce. To investigate the relationship between hepatic fat content and subcutaneous adipose tissue we conducted a cross-sectional study in which characteristics of subcutaneous adipose

tissue and liver fat (LFAT) content were analyzed. LFAT content was measured by proton magnetic resonance spectroscopy. Subcutaneous fat biopsies were obtained to determine morphology and protein levels within adipose tissue. In addition to fat cell size, the percentage of macrophages and the presence of crown like structures within subcutaneous fat was assessed by CD68-immunohistochemical staining. The results of this study are described in **chapter 7.**

Autophagy, an evolutionary conserved process aimed at recycling damaged organelles and protein aggregates in the cell, also modulates pro-inflammatory cytokine production in peripheral blood mononuclear cells. Since adipose tissue inflammation accompanied by elevated levels of pro-inflammatory cytokines is characteristic for the development of obesity, we hypothesized that modulation of autophagy alters adipose tissue inflammatory gene expression and secretion. We performed *in vitro* and *in vivo* animal and human experiments and measured autophagy marker LC3. Again, adipose tissue biopsies were taken from healthy lean and obese subjects. **Chapter 8** describes the results of analyses of adipose cell size, number of macrophage influx and adipocytokines (mRNA and protein levels).

Although inflammatory characteristics of the adipose tissue are frequently described in literature, a general classification of the adipose tissue inflammatory status is currently lacking. To identify patients with increased adipose tissue inflammation, we used the morphological characterization of the adipose tissue to define an inflammatory score. This score was applied in a discriminant analysis aimed at predicting the adipose tissue inflammatory status using a combination of circulating parameters (**chapter 9)**.

In **chapter 10** the main findings of the experiments presented in this thesis are summarized, discussed and put into perspective.

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Part I

Determinants of insulin-associated weight gain

tissue
adipose
therapy
insulin
weight
gain
macrophages
patients
months
levels
protein
study
adipocytes
inflammatory
diabetes
subcutaneous
influx
change
type
body
significant
baseline
kg
gainers
expression
increase

Chapter 2

Contribution of change in glycosylated haemoglobin to insulin-associated weight gain: results of a longitudinal study in type 2 diabetic patients

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Abstract

To investigate the contribution of glycated haemoglobin change (HbA_{1c}) on body weight in patients with type 2 diabetes (T2DM) after start of insulin therapy, we analyzed 122 individual weight-profiles in relation to the change in HbA_{1c} per se in these patients up to 36 months after the start of insulin therapy. Data were analyzed separately for the first 9 months after commencement of insulin therapy and for the period thereafter. Within the first 9 months of insulin therapy mean body weight increased by 0.52 kg per month. HbA_{1c} decreased from 9.9 ± 1.8 to 7.9 ± 1.3%. Only 12% of the initial weight gain could be attributed to the change in HbA_{1c}. Furthermore, the mean monthly increase in body weight gain was reduced by 0.006 kg for every 1 kg higher body weight at baseline. From 9 to 36 months after start of insulin therapy, body weight increased by 0.1 kg/month, which was independent of change in HbA_{1c}. Improvement of glycemic control per se contributes little to initial weight gain after start of insulin therapy in patients with T2DM. After 9 months of insulin treatment, weight gain is unrelated to change in glycated haemoglobin. Other factors have to be responsible for weight gain after start of insulin therapy.

Introduction

Insulin therapy is frequently needed to achieve adequate glycemic control in patients with type 2 diabetes mellitus (T2DM), but often at the expense of significant weight gain (1-3) .

This weight gain is obviously undesirable in an already overweight population and may negatively affect blood pressure, lipid levels, inflammatory and fibrinolytic parameters, and may also deter further optimization of insulin therapy (4-7). Four putative mechanisms have been proposed for this weight gain: 1) improvement of glycemic control (HbA_{1c}), 2) anabolic effect of insulin increasing fat storage, 3) a decrease in metabolic rate and a fall in energy expenditure and 4) defensive eating habits because of (fear of) hypoglycemia (8, 9).

Most authors view the improvement in glycemic control per se (expressed as change in HbA_{1c}) as the major determinant of weight gain. This conclusion is based on studies that usually relate the difference in body weight between two time-points to the difference in HbA_{1c} in patients who initiated insulin therapy (8-10). Mäkimattila et al. (2) showed that after 12 months of insulin therapy a decrease in HbA_{1c} by 2.5 % is associated with a 5 kg weight gain (i.e. 2 kg/1% decrease in HbA_{1c}). However, these studies do not show to what extent change in body weight may depend on change in HbA_{1c}. The relationship between change in glycaemic control (i.e. change in HbA_{1c}) and weight gain may vary over time. First, the change in HbA_{1c} may primarily contribute to weight gain in the first months after the start of insulin therapy rather than later-on, when factors unrelated to glycemic control become more important such as change in energy intake or physical activity, anabolic effects of insulin, and defensive eating behavior probably (2, 9). Second, patient's and physician's responses to changes in glycemic control and body weight may affect the subsequent course of these variables.

Analyzing data of HbA_{1c} and body weight just at two time-points may negate these variations and lead to incomplete or even wrong conclusions. A more accurate evaluation of a relationship between change in HbA_{1c} and weight after initiation of insulin therapy, requires a longitudinal assessment with repeated measures of body weight and HbA_{1c} in the same individuals. Therefore, we used a linear mixed model for repeated measures data to investigate the relationship between the changes in HbA_{1c} and body weight at different time-points after commencing insulin therapy in patients with T2DM.

Patients and Methods

Subjects

Patients with T2DM, who all started biphasic insulin therapy in our academic center between March 2000 and November 2004 were included in this study. Patients who started biphasic insulin therapy were selected because most patients starting insulin therapy in this timeframe were assigned to this insulin regimen by their physician. Furthermore, to prevent confounding with respect to influences of different types of insulin on body weight we only included patients with biphasic insulin. All patients were seen at the diabetes clinic of the Radboud University Nijmegen Medical Center. The diagnosis of T2DM was made according to the diagnostic criteria of the WHO. The decision to start insulin treatment was at the discretion of the responsible physician and was always based on failure of glycemic control on oral glucose-lowering agents and/or diet. Patients were excluded if they did not use biphasic insulin or had steroid-induced diabetes, latent auto-immune diabetes in adults (LADA) or maturity onset diabetes of the young (MODY). Patients were followed for a maximal of 36 months after start of insulin therapy. We conducted an observational study in which data of all patients starting biphasic insulin within the timeframe 2000-2004 was included.

Clinical data were retrieved from medical records at baseline and at 3-month intervals, which included body weight, HbA_{1c}, age, gender, diabetes duration, blood pressure, lipids, doses of oral glucose-lowering medication, and insulin dose. We assumed that most of the weight gain that could be directly attributed to improvement in glycemic control would appear within the first 9 months after start of insulin therapy. This was based on studies viewing that most of the weight gain and change in HbA_{1c} appears within the first 9-12 months after start of insulin therapy (9, 11). After this period, weight gain tends to level-off. Therefore, we studied the short-term weight-profiles (i.e. the first 9 months after start of insulin therapy) and the long-term weight-profiles (i.e. from 9-36 months after start of insulin therapy) separately. To analyze the short-term weight-profiles, data of baseline body weight and at least 3 subsequent weight measurements had to be available within the first 9 months. To study the long-term weight-profiles, data of at least 4 body weight measurements between 9 and 36 month after commencing insulin therapy had to be available.

Statistical methods

We studied the individual weight profiles of patients with T2DM up to 36 months after the start of insulin therapy and the dependence on HbA_{1c} with the use of linear mixed models for repeated measures (12). At first, we found that the models were not statistical significant improved when a quadratic term in time was included in the linear part of the models (Likelihood-Ratio test) in neither time period. We studied

weight profiles unadjusted and adjusted for change in HbA_{1c}. Hence, the following initial mixed model was used:

$$Y_i\,(t) = \beta_0 + \beta_1 \times BBW_i + \beta_2 \times t + \beta_3 \times BBW_i \times t + \beta_4 \times \Delta HbA_{1cit} + \beta_5 \times \Delta HbA_{1cit} \times t + b_{1i} + b_{2i} \times t + \varepsilon_{it}$$

where Y refers to body weight, i to subject, t to the time (month) after start of insulin therapy, β_0 to the general mean, β to a fixed effect, b to a random effect, BBW to the baseline body weight, ΔHbA_{1c} to the change in HbA_{1c} since baseline and $_{it}$ to the normal distributed residual with mean zero. We studied weight-profiles unadjusted and adjusted for change in HbA_{1c} in each time period, separately. Note that in case of the unadjusted weight-profiles the terms related to ΔHbA_{1c} were omitted from the model presented above.
In total four models were designed:

- Short-term linear mixed model (0-9 months; model I)
The dependent variable in this model was body weight. The independent continuous variables were: body weight at the start of insulin therapy and the time (t) since the start of insulin therapy (month). Furthermore, the interaction term between both variables (body weight*t) was included in the model, representing different increase in body weight with higher initial body weight. The independent random variables were: intercept (representing baseline body weight) and the regression in time (representing weight gain or loss per month).
This allows the calculation of different regression lines for different patients, both in intercept and regression.

- Short-term linear mixed model (0-9 months; model II)
The same dependent and independent (continuous and random) variables were entered into the model as in model I. Model II was designed to study the short-term weight profile adjusted for change in HbA_{1c}. Therefore, the independent continuous variables (time-dependent) change in HbA_{1c} since the start of insulin therapy (ΔHbA_{1c}) and the interaction term between time and change in HbA_{1c} (ΔHbA_{1c}*t) were included in the model.

- Long-term linear mixed model (9-36 months; model III)
The same dependent and independent variables as in model I were entered in this model, except for the interaction term between body weight and time (body weight*t). The reason for this was that aforementioned interaction term did not significantly alter the outcome of the model.

- Long-term linear mixed model (9-36 months; model IV)
The same dependent and independent variables as in model III were entered into this model.

The independent continuous variables were: weight at the start of insulin therapy, and time since the start of insulin therapy (month). Model IV was designed to study the long-term weight profile adjusted for change in HbA_{1c}. Therefore, absolute change in glycosylated haemoglobin since start of insulin therapy (ΔHbA_{1c}) was included as an independent variable. The interaction term between time and change in HbA_{1c} was not included because of a non-significant contribution to the model.

Estimated regression parameters and mean profiles are presented, with the appropriate standard error (SE) and 95% confidence interval (CI). Statistical analyses were performed by using SAS® statistics 9.2 for Windows (SAS Institute Inc. Cary, NC, USA). A P-value <0.05 was considered statistically significant.

Results

A total of 146 patients who were assigned to biphasic insulin therapy were screened. Finally, 122 patients were included in our analysis. We excluded 24 patients (18 patients of whom no baseline HbA_{1c} or body weight was available, in 6 patients the responsible physician changed the initiating insulin regimen (i.e. 4 patients started prandial insulin and 2 patients started basal insulin therapy instead of biphasic insulin)). All patients included started twice-daily biphasic human insulin/isophan insulin 30 (Mixtard® 30) or aspart 30 (Novomix® 30).

Crude data

Baseline characteristics of the study population are shown in Table 1.

Median follow-up was 33 months and 90 % of all study subjects had a minimum follow-up of 18 months. Patients had a median age of 64 years and a median diabetes duration of 9 years. At baseline, mean body weight was 85 kg (range 47-157 kg) and HbA_{1c} averaged 10 %. After 9 and 36 months of insulin therapy, mean body weight was 90.6 $\pm$ 18.6 (SD) kg and 91.1 $\pm$ 17.2 kg, respectively (both $P<0.001$ for mean change in body weight compared to baseline body weight). Furthermore, mean HbA_{1c} after 9 months of insulin therapy was 7.9 $\pm$ 1.3 %, and after 36 months 8.1 $\pm$ 1.3 % (both $P<0.001$ for mean change in HbA_{1c} compared to baseline HbA_{1c}). At the time of data collection, the median insulin dose was 56 units insulin per day (0.7 U/kg). Figure 1 shows the interpolation of crude data of body weight and change in HbA_{1c} after start of insulin therapy. The mean increase in body weight was more pronounced in the first 9 months after start of insulin than from 9 months further

Table 1 Baseline characteristics of the study population (N=122).

	Number	Median (range) / n (%)
Age (years)	122	64 (35-94)
Sex	122	
Male		63 (52%)
Female		59 (48%)
Diabetes duration (years)	122	9 (2-36)
Body weight (kg)	122	85.3 (47-157)
Body mass index (kg/m^2)	122	30 (21-48)
HbA_{1c} (%)	122	10 (6-15)
Oral glucose-lowering Medication	122	
SU only		16 (13%)
MET only		18 (15%)
SU and MET		69 (57%)
TZD only		2 (2%)
SU and TZD		4 (3%)
SU and MET and TZD		1 (1%)
SU and MET and Acarbose		4 (3%)
None *		8 (6%)
Blood pressure (mmHg)	99	
Systolic		142 (100-190)
Diastolic		80 (62-105)
Anti-hypertensive therapy	122	
Yes		81 (66%)
No		33 (27%)
Unknown		8 (7%)
Statin use	122	
Yes		48 (39%)
No		61 (50%)
Unknown		13 (11%)
Smoking	122	
Yes		17 (14%)
No		72 (59%)
Unknown		33 (27%)
Alcohol	122	
Yes		9 (7%)
No		79 (65%)
Unknown		34 (28%)
Lipid profile (mmol/l)	90	
Total cholesterol		5.2 (2.5-8.2)
Triglycerides		2.5 (0.6-21.1)
HDL-cholesterol		1.0 (0.5-2.4)
LDL-cholesterol		2.9 (08.-5.1)

* no use of oral glucose-lowering medication prior to the start of insulin therapy.
HbA_{1c}, glycated haemoglobin. SU, sulfonylurea derivatives. MET, metformin. TZD, thiazolidinedione derivatives. HDL-cholesterol, high-density lipoprotein cholesterol. LDL-cholesterol, low-density lipoprotein cholesterol.

onwards (0.52 kg/month versus 0.10 kg/month, $P<0.05$). HbA_{1c} decreased steeply in the first 3 months after the start of insulin, followed by a more gradual decline.

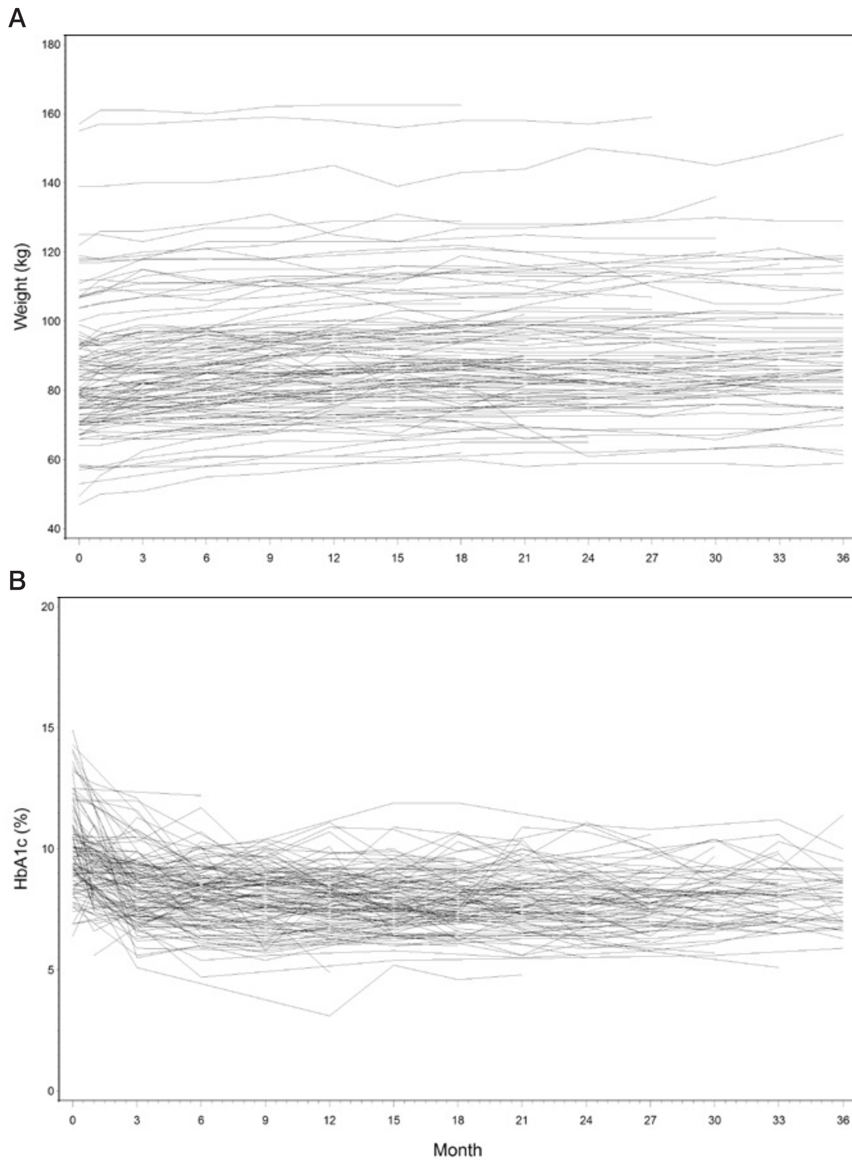

Figure 1 Interpolation of crude data of body weight (panel **A**) and HbA_{1c} (panel **B**) after start of insulin therapy.

2

Fit of linear mixed models to crude data

Figure 2 visualizes the fit of the short-term weight model to the crude data. In this figure the crude data (stars) and estimated profiles (line) of four different patients with complete data up to 9 months after start of the insulin therapy are displayed. This figure shows that this model is sufficiently flexible to obtain a good fit in all cases: high (top panels) or low (bottom panels) values of the weight profile as well as increasing (left panels) or non-increasing (right panels) weight profiles. A similar good fit was obtained using the long-term weight model.

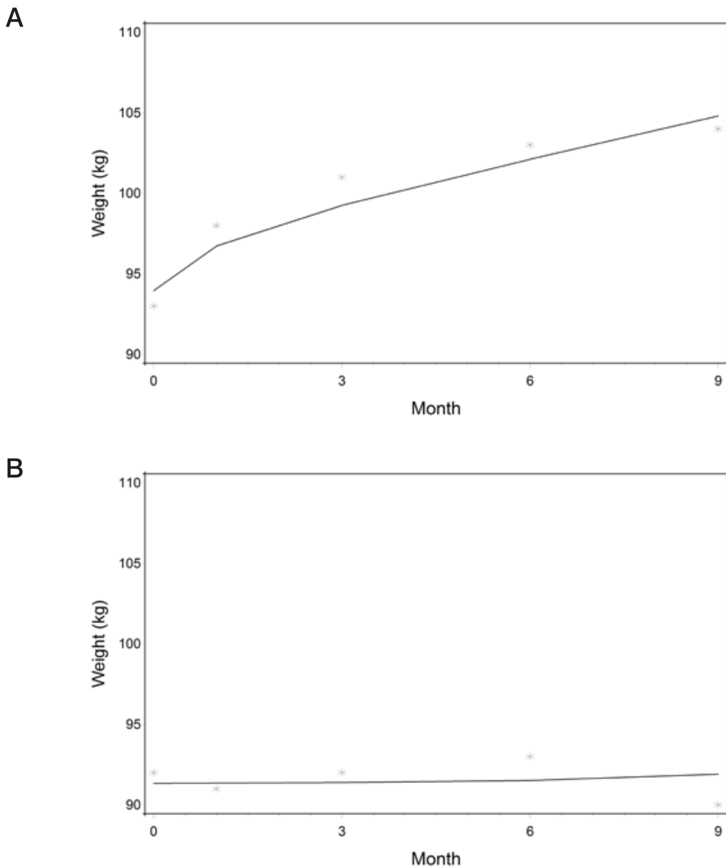

Figure 2 The crude data (stars) and estimated profiles (line) of four different patients with complete data up to 9 months after start of the insulin therapy.

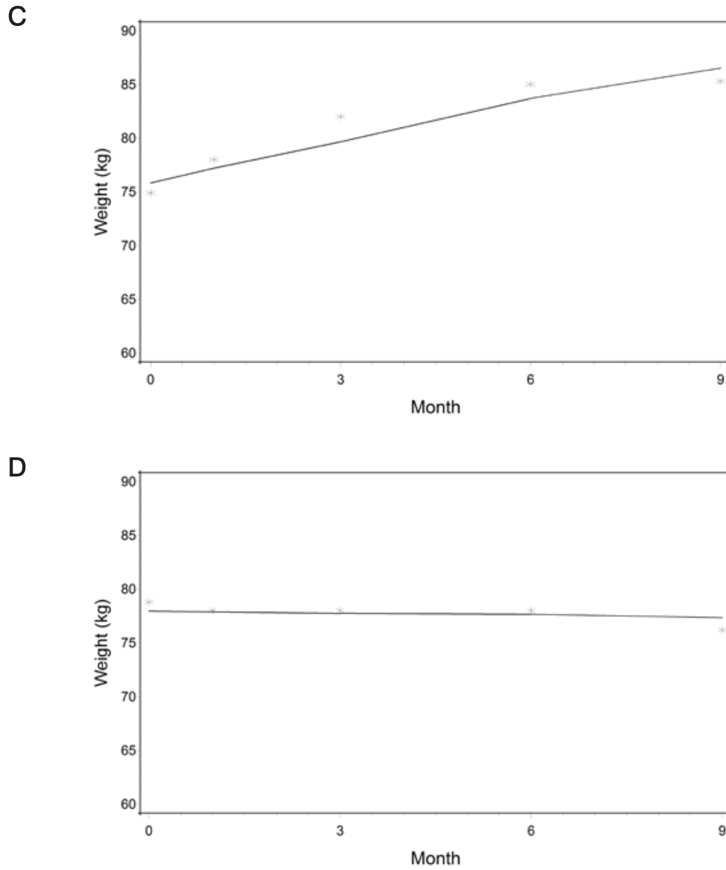

Figure 2 Continued.

Outcome of linear mixed models (short-term and long-term profiles)

Figure 3 shows the mean weight profile in the 36 months after insulin therapy (mean baseline body weight 85.3 kg, baseline HbA_{1c} of 9.9 %, decrease of HbA_{1c} 2.0 %).

Table 2 shows the regression parameters for the weight profiles.

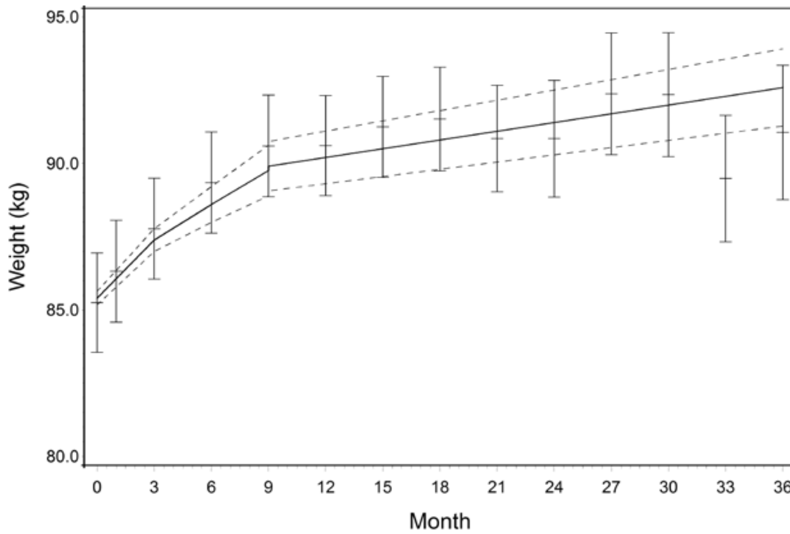

Figure 3 The estimated mean weight profile (solid line) with the appropriate 95% confidence bands (dashed lines) in the 36 months after start of insulin therapy (mean baseline body weight of 85.3 kg and baseline HbA_{1c} of 9.9%). The decrease in HbA_{1c} since baseline at 3, 6 and 9 month is 1.6%, 1.8% and 2.0%, respectively. The vertical bars indicate the crude data (mean with one standard error).

- Short-term weight-profiles

Estimated mean body weight increased from 85.6 kg (95 % confidence interval (CI): 85.4-85.9 kg) to 90.3 kg (89.3-91.2 kg) after 9 months of insulin therapy. When expressed as percentage of baseline body weight, the average increase in weight was 5.5 % after 9 months of insulin therapy. Estimated mean HbA_{1c} decreased from 9.5 (9.4-9.6) to 7.6 % (7.3-7.9).

We found that the monthly increase in body weight decreased by 0.006 kg per kg of higher body weight above mean body weight at baseline (85.6 kg). Thus, with the use of model I it was calculated that a patient with a baseline body weight of 50 kg increased 0.70 kg per month, whereas for a patient with a baseline body weight of 100 kg this was 0.40 kg per month. Table 2 also shows that 0.46 kg/month (0.350-0.574 kg/month) of weight gain within the first 9 months after start of insulin therapy was independent of the change in HbA_{1c} (model II). Thus, only 12 % of the total monthly increase of 0.52 kg per month could be attributed to the change in HbA_{1c}.

Furthermore, we found that the effect of a decrease in HbA_{1c} on weight gain diminished later in time after start of insulin therapy. The effect of HbA_{1c} change on body weight after 3 month was 0.37 kg/month (=0.510 - 3*0.050) per absolute

Table 2 The estimated regression parameters with the 95% confidence intervals (CI) of the crude data on body weight and the HbA_{1c} adjusted weight profiles by period using a linear mixed model.

0-9 months				
	Model I		**Model II**	
Effect	**Estimate**	**(95% CI)**	**Estimate**	**(95% CI)**
Intercept	85.643	(85.399:85.887)	85.400	(85.167:85.634)
baseline body weight #	1.000	(0.987:1.013)	1.000	(0.988:1.012)
t (month) #	0.516	(0.420:0.613)	0.462	(0.350:0.574)
baseline body weight*t $	-0.006	(-0.011:-0.001)	-	-
Δ HbA_{1c} #	-	-	0.510	(0.336:0.684)
Δ HbA_{1c}*t $	-	-	-0.050	(-0.09:-0.001)
9-36 months				
	Model III		**Model IV**	
Effect	**Estimate**	**(95% CI)**	**Estimate**	**(95% CI)**
Intercept	90.452	(89.450:91.455)	89.918	(89.066:90.771)
baseline body weight #	0.931	(0.878:0.985)	0.954	(0.909:0.999)
t (month) #	0.099	(0.055:0.143)	0.099	(0.056:0143)
Δ HbA_{1c} $	-	-	0.548	(0.060:1.036)

Model I and III represent the estimated regression parameters of the crude data on body weight for the short (0-9 months after start of insulin therapy) and long-term (9-36 months after start of insulin therapy) period, respectively. Model II and IV represent the HbA_{1c} adjusted weight profiles for the short and long-term period, respectively.
All variables entered into the models contributed significantly to the change in weight profiles (# $P<0.001$ and $ $P<0.05$).
Δ HbA_{1c}, absolute change in HbA_{1c} after start of insulin therapy.
- denotes not applicable.

percentage decrease in HbA_{1c} and after 9 month this was only 0.1 kg/month (=0.510 - 9*0.050) per absolute percentage decrease in HbA_{1c}. Figure 4 shows the short-term mean weight profiles with varies decreases in HbA_{1c} and the effects on weight gain.

- Long-term weight-profiles

Table 2 shows that the estimated increase in body weight from 9 to 36 month after start of insulin therapy was 0.1 kg/month (0.055-0.143 kg/month) and that this weight gain was independent of the baseline body weight. As a result, body weight of the patient with mean baseline body weight increased from 90.5 kg (89.4-91.5 kg) at 9 month to 93.1 kg (91.6-94.7 kg) at 36 months after start of insulin therapy. In addition, the results of the mixed model for the HbA_{1c} profiles showed no statistical significant change during this time period (-0.004 $\pm$ 0.005 % per month). As a result, for every

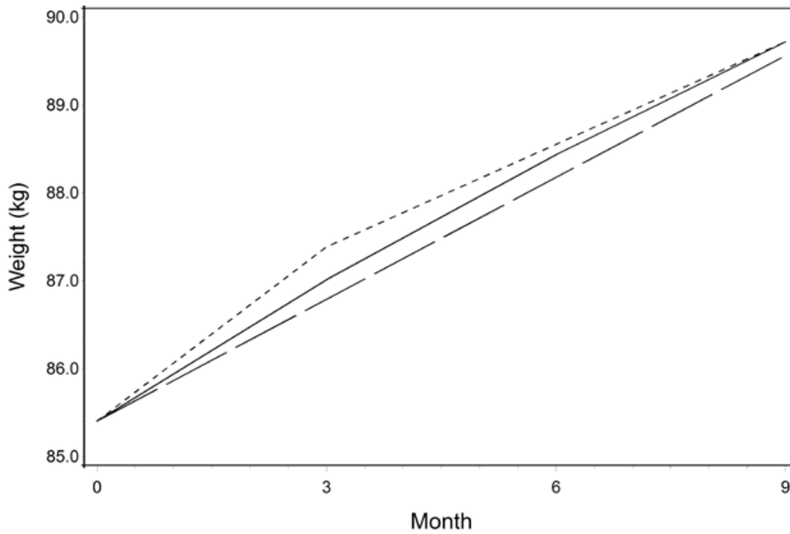

Figure 4 The estimated mean weight profile in the first nine months after insulin therapy (baseline body weight of 85.3 kg) with varies decrease in HbA_{1c}. Solid line: decrease in HbA_{1c} since baseline at 3, 6 and 9 month is 0.6%, 1.1% and 1.6%, respectively. Short dashed line: decrease in HbA_{1c} since baseline at 3, 6 and 9 month is 1.6%, 1.6% and 1.6%, respectively, i.e. already the maximal decrease in HbA_{1c} is reached at 3 months. Long dashed line: decrease in HbA_{1c} since baseline at 3, 6 and 9 month is 0.0%, 0.0% and 0.0%, respectively, i.e. no decrease in HbA_{1c}.

patient in the model, the increase in weight per month (i.e. slope) on the long-term was similar. However, body weight at 9 months was dependent on baseline body weight and HbA_{1c}. For example, a patient with a 1 kg higher baseline body weight will have a 0.95 kg (0.909-0.999 kg) higher body weight at 9 months. In addition, a patient with a 1% higher baseline HbA_{1c} will have a 0.55 kg (0.060-1.036 kg) higher body weight at 9 months.

Discussion

In the present study, we studied individual weight-profiles of patients with T2DM up to 36 months after initiation of insulin therapy. Mean body weight increased by more than 0.5 kg per month during the first 9 months after initiation of insulin therapy. Thereafter, the increase in body weight was more gradual, but still patients had a weight gain on average of 1.2 kg per year. This is well in line with other studies which investigate weight gain and insulin therapy (9, 13).

However, although glycemic control improved considerably, only 12% of the monthly weight gain within the first 9 months could be attributed to the change in HbA_{1c}. We also found that the effect of a decrease in HbA_{1c} was most prominent during the first months after start of insulin therapy. After 9 months of insulin therapy, the change in HbA_{1c} did no longer affect the change in body weight.

To our knowledge, this is the first study to show results of a longitudinal analysis of change in body weight after commencing insulin therapy and its relation to the change in HbA_{1c}. Other studies showed that after short-term insulin therapy the level of improvement of glycemic control correlated with the increase in body weight, with reported correlations between -0.21 and -0.47 (9, 10). We believe our study adds valuable information by assessing to what extent the change in glycemic control contributes to weight gain over time. To study the relationship between body weight and HbA_{1c} over time, we performed an observational longitudinal analysis using a linear mixed model for repeated measures data. The strength of the model allowed different regression lines for different patients, both in intercept and regression. In this way, the contribution of (change in) HbA_{1c} to (change in) body weight in time could be estimated more accurately.

Although the model analyzed the crude data properly, these data were collected retrospectively. As a consequence, we were not (fully) informed about other factors that determined weight gain. We can only speculate about other potentially contributing factors, unrelated to glucose control (9). For example, anabolic effects of insulin may directly induce weight gain. Anabolic effects of insulin on adipose and muscle tissue may lead to sodium and water retention (14, 15). The anabolic effects of insulin might play a dominant role inducing weight gain after short-term insulin therapy. Furthermore, it could be argued that the insulin dose itself and change of insulin dose determines weight gain (10). Unfortunately, we were not informed about the insulin dose of each patient at every time point. Weight gain might also be determined by changes in caloric intake (whether or not due to initiation of insulin therapy), physical activity and basal metabolic rate (16, 17). The use of insulin may also lead to defensive eating habits because of (fear of) hypoglycemia (18). Consequently, individuals may increase caloric intake to proactively avoid such an event, resulting in weight gain. All these factors might contribute to weight gain after start of insulin therapy. To investigate these other factors/predictors a prospective analysis and follow-up will be required.

In clinical practice, many physicians are reluctant to initiate insulin treatment in poorly controlled obese patients because of fear of insulin-associated weight gain. Biesenbach et al. already reported that the risk of weight gain and increase in insulin requirement was similar in insulin-treated type 2 diabetic patients with normal and elevated BMI (19). We now show that obese patients are even less likely to gain weight after initiation of insulin therapy than leaner patients. However, we believe our data are more accurate since in the study of Biesenbach et al. patients were pooled and

assigned to one of three BMI subgroups (<26, 26-30 and >30) and calculations were based on only two time-points, thus ignoring variations in weight between these time-points. We think that obesity per se should not preclude physicians to initiate insulin therapy in poorly controlled patients with type 2 diabetes.

After long-term insulin therapy, change in HbA_{1c} was not a predictor of weight gain at all. Still, patients gained an average of 1.2 kg/year. We cannot determine whether (part of) this weight gain was due to the “natural” course of body weight associated with aging, since we lacked a control group of either matched non-diabetic subjects, or subjects with T2DM on oral medication. In the UKPDS (20), it was shown that patients assigned to the intensive glucose control with glibenclamide gained an average of approximately of 4 kg over 12 years of treatment, whereas those on insulin gained an additional 3 kg. Most of this weight gain developed within the first year of treatment. After one year of treatment patients with glibenclamide gained an average of approximately of 0.5 kg/year and those on insulin gained similar up to three years of treatment. Thereafter, little change in weight occurred in the group of patients on glibenclamide, in contrast to patients using insulin who continued to gain weight. It was shown that glycemic control continued to deteriorate in both groups.

In conclusion, the present study shows that initiation of insulin in patients with T2DM was associated with substantial increase in body weight. However, the contribution of change in HbA_{1c} to insulin-associated weight gain was rather small in the short-term and even had no effect in the longer term. Further studies are needed to identify alternative factors that contribute to (insulin-associated) weight gain, such as differential effects on body composition, caloric intake and physical activity/energy expenditure.

Disclosure

The authors have no relevant conflicts of interest to disclose.

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Chapter 3

Physical activity is reduced in insulin-treated patients with type 2 diabetes mellitus

H.J. Jansen, C.J. Tack, S. Arons, G. Vervoort

Submitted

Abstract

Insulin treatment in patients with type 2 diabetes mellitus (T2DM) is often associated with weight gain. This weight gain may be explained by a reduced daytime physical activity (PA) possibly related to sleep disturbances. In a cross-sectional approach, we assessed free-living 24-hour PA including habitual sleep characteristics in patients with T2DM and compared results to those obtained in age-matched non-diabetic lean and age- and weight-matched obese individuals.

A total of 48 insulin-treated patients with T2DM, 38 lean and 42 obese subjects were studied. Free-living PA and characteristics of sleep (average lying down duration (ALDD), and average sleep duration (ASD) were objectively assessed by accelerometry (SenseWear Pro Armband™, Body Media, Pittsburgh, PA, USA), which was worn for four consecutive 24-hour periods.

Mean PA expressed as metabolic equivalent (METS) was significantly lower in patients with T2DM using insulin therapy compared to weight-matched obese subjects, while PA was highest in lean subjects. Patients with T2DM spent significantly more time in the lower PA levels compared to lean and obese individuals. The average number of steps per day was also significantly reduced in patient with T2DM. Quantitative sleep characteristics (ALDD and ASD) were similar across the groups, although in insulin-treated patients with T2DM sleep was more disturbed.

Insulin-treated type 2 diabetic patients display a significant reduction in daytime PA which cannot be solely attributed to obesity-related effects. The decrease in PA does not seem to be related to disturbed sleep characteristics.

Introduction

After initiation of insulin treatment, patients with type 2 diabetes (T2DM) generally gain weight, which is, at least partly, attributed to an improvement of glycemic control and a decrease in glucosuria. Still, even at stable metabolic control, most insulin-treated patients with diabetes have the propensity to suffer from ongoing weight gain (1). Weight gain is obviously undesirable in an already obese population. The determinants of this insulin-associated weight gain are not entirely elucidated.

From large obesity studies using questionnaires to assess physical activity (PA), it is known that PA is inversely associated with body weight and fat mass (2). Hence, insulin-associated weight gain may be explained by a reduced daytime PA, but detailed analyses of PA in patients with T2DM as compared to matched controls have not been performed. PA may decrease as a result of changes in daily behaviour, for example to avoid hypoglycemia. Because sleep duration and quality has been associated with weight gain and obesity, sleep disturbances may also be involved in insulin-induced weight gain (3-5). Sleep quality, in turn, may affect daytime PA and vice versa. Again, sleep characteristics have so far not been studied in patients with insulin-treated T2DM.

We hypothesized that insulin treatment in patients with T2DM impacts on PA and/or sleep, which may reduce energy expenditure and lead to weight gain. To test this hypothesis, we performed a cross-sectional study in which we quantitatively measured daytime PA and habitual sleep characteristics in insulin-treated T2DM patients and compared the results to those obtained in age-matched non-diabetic lean and age- and weight-matched obese individuals.

Research Design and Methods

Study population

In this cross-sectional study long-term (> 4 years) insulin-treated patients with T2DM were included. Patients were excluded if diagnosed with steroid-induced diabetes, latent auto-immune diabetes in adults or maturity onset diabetes of the young. Other exclusion criteria were clinical evidence of manifest cardiovascular, pulmonary, rheumatic diseases with impaired joint mobility, (chronic) arthritis, lower limb amputation, pregnancy or intention to become pregnant during the study, painful (diabetic) neuropathy and depression which may influence daytime PA.

Lean and obese controls were recruited through advertisements in local newspapers. Obese subjects were matched to the diabetes group for body weight and age. Lean subjects (BMI 20-27 kg/m^2) were age matched. A fasting plasma glucose > 7.0 mmol/l, and similar conditions as described above for diabetes patients were also reasons for exclusion criteria for controls.

Demographic and clinical characteristics

Subject characteristics (age, diabetes duration, gender, race, socio-occupational status, smoking habits, type of oral glucose-lowering medication, and insulin dose) were noted. A questionnaire was used to assess engagement in sports and average duration of sport per day. Body weight, height, waist- and hip circumference, and blood pressure were measured by standard procedures.

Biochemical analyses

Fasting blood samples were drawn to determine glucose, glycated hemoglobin (HbA_{1c}), and lipids.

Physical activity and sleep characteristics measured by accelerometry

Free-living daytime PA and sleep was objectively measured using a SenseWear Pro Armband™ (Body Media, Pittsburgh, PA, USA) (6). Accelerometry is non-invasive and assess free-living PA. The SenseWear™ device has been validated for study of sleep patterns, as the internal body media algorithm can identify sleep and wakefulness with moderate-to-high sensitivity, specificity, and accuracy (5, 7). Unlike polysomnography, accelerometry does not appear to alter sleep behavior because there is no first-night effect (3). The device was placed on the right upper arm over the triceps muscle for 4 consecutive 24-h periods in free-living conditions and includes a 2-axis accelerometer, heat flux sensor, galvanic skin response sensor, and a skin temperature sensor. Measurements between 7.00 am and 11.00 pm were used for calculations of daytime PA if > 90% of these recordings were available. Outcome variables from the daytime activity included: average metabolic equivalent (METS; 1 MET = consuming 1 kcal/kg of body weight per hour), time (minutes/day) spent at different activity intensity categories averaged per day over the measurement period and number of steps per day. The average lying down duration (ALDD, i.e. the average interval between going to bed and getting up), average sleep duration (ASD, i.e. average minutes from sleep onset to awakening) and average fragmentation index (AFI, i.e. average number of nocturnal interruptions) were retrieved from the activity monitor using a sleep scoring algorithm. SenseWear Innerview™ professional software 6.1 was used to analyse the data.

Informed consent

The ethical committee of the RadboudUMC, Nijmegen, the Netherlands approved the study protocol. All subjects provided written informed consent.

Statistical analyses

Variables are expressed as means ± SD. Differences between groups were analyzed using the analysis of variance (ANOVA) with the Least Significant Difference post hoc

test. Dichotome variables were analyzed with the Pearson's chi-squared test. All calculations were performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA). Two-tailed $P < 0.05$ was considered significant.

Results

Characteristics of the study population

A total of 48 insulin-treated patients with T2DM, 38 lean and 42 obese subjects without diabetes were included in this study. Table 1 shows the demographic and clinical characteristics of the groups.

As per definition, lean subjects had significant lower body weight compared to obese and patients with T2DM. The obese group was weight-matched with the group of patients with T2DM. Patients with T2DM were slightly, although significantly, older as compared to obese and lean subjects. As expected, there were significant clinical differences between lean subjects and patients with T2DM with respect to lifestyle parameters (use of alcohol), and blood pressure. There were also significant differences between groups with respect to lipid levels. Within the diabetes group the mean dose of insulin was 76 ± 47 U/day), 31 patients (65 %) used metformin and 4 patients (8 %) were on sulfonylurea derivatives.

Free-living objectively measured daytime PA

Mean daytime PA expressed as metabolic equivalent (METS) was significant lower in insulin-treated patients with T2DM compared to obese and lean subjects (1.35 ± 0.22 vs. 1.53 ± 0.33 vs. 1.85 ± 0.35, respectively; $P < 0.01$ between groups). Furthermore, patients with T2DM display reduced PA levels in the different activity intensity categories. The average number of steps per day also was significantly reduced in patient with T2DM (5188 ± 2825 compared to 8474 ± 3129 and 9575 ± 3233 and in obese and lean subjects, respectively; $P < 0.001$ between T2DM vs. lean and T2DM vs. obese). Figure 1A-E shows the differences in PA and intensity between the groups.

The vertical box-plots displayed represent summary statistics for the analyzed variables stated (A-E). The plot elements and the statistics represented are as follows: the length of the box represents the interquartile range (the distance between the 25th and the 75th percentiles), the horizontal line in the box interior represents the median, and the vertical lines issuing from the box extend to the minimum and maximum values of the analyzes variables.

Table 1 Demographic and clinical characteristics of obese patients with T2DM compared to lean and obese individuals.

	T2DM (n = 48)	Obese (n = 42)	Lean (n = 38)
Demographics			
Age (yr)	61 ± 10$	55 ± 9	54 ± 8
Gender, males (%)	56$	43	53
Duration diabetes (yr)	11± 7	-	-
Anthropometry and hemodynamics			
BMI (kg/m^2)	31.7 ± 5.5$	31.4 ± 3.5*	23.6 ± 1.8
Waist circumference (cm)	110 ± 13$	107 ± 10*	86 ± 6
Hip circumference (cm)	104 ± 11$	107 ± 8*	97 ± 5
SBP (mmHg)	151 ± 23$#	143 ± 18*	132 ± 15
DBP (mmHg)	82 ± 10#	87 ± 9*	82 ± 9
Metabolic characteristics			
HbA_{1c} (%)	7.5 ± 0.9	-	-
TC (mmol/l)	4.5 ± 1.0	4.9 ± 1.3	4.4 ± 1.3
TG (mmol/l)	2.6 ± 3.0$#	1.6 ± 1.0	0.8 ± 0.4
HDL (mmol/l)	1.1 ± 0.3$	1.2 ± 0.5	1.3 ± 0.5
LDL (mmol/l)	2.5 ± 0.7$#	3.1 ± 0.9	2.9 ± 1.0
Alcohol use (%)	22$	36*	80
Smoking (%)	18	17*	3

Data presented as mean ± SD. BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, HbA1c = glycosylated hemoglobin, TC = total cholesterol, TG = triglycerides, HDL = high-density lipoprotein cholesterol, LDL = low-density lipoprotein cholesterol, MET = metformin, SU = sulphonylurea derivatives, - = not applicable.

$ T2DM vs. lean; $P < 0.05$
# T2DM vs. obese; $P < 0.05$
* Obese vs. lean; $P < 0.05$

From Figure 2 it can be appreciated that lean controls exhibit a higher baseline PA level compared to the obese and T2DM group. In addition, lean subjects displayed more and higher peaks of (vigorous; METS ≥ 6) PA compared to the two other groups.

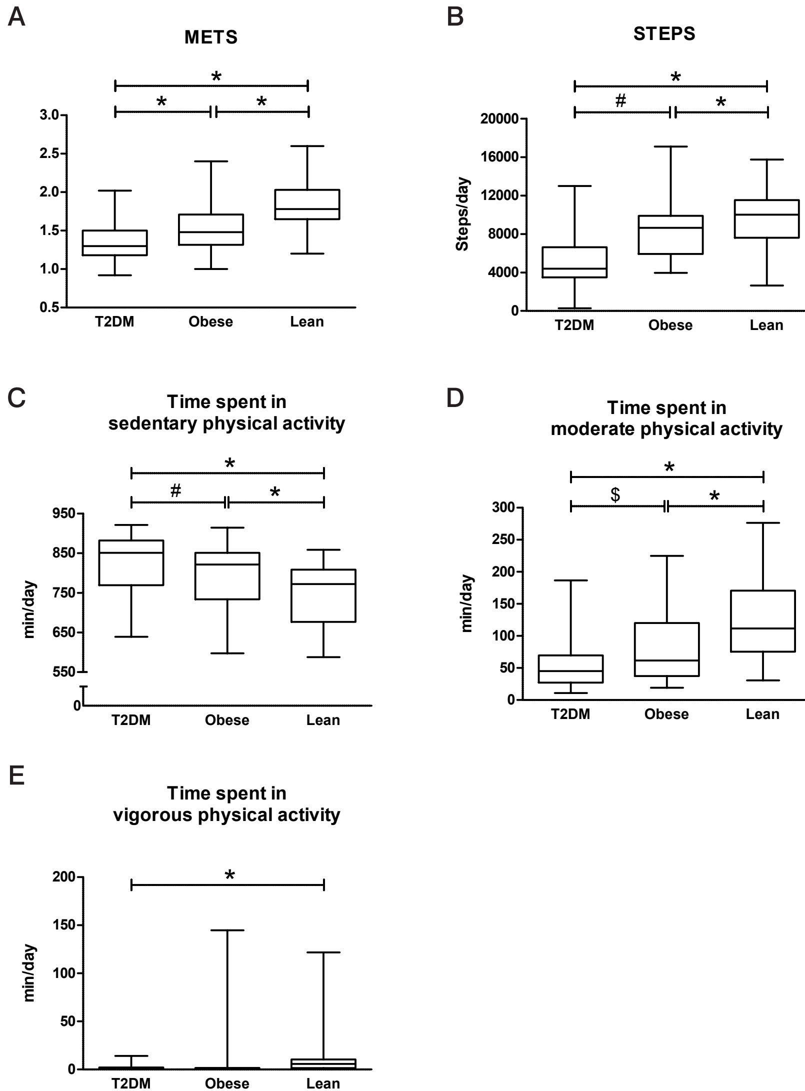

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Figure 1 Physical activity and intensity objectively measured by SenseWear Pro Armband™ comparing insulin-treated patients with T2DM, obese and lean subjects. Average PA expressed as metabolic equivalent (METS) which is consuming 1 kcal/kg of body weight per hour (**A**). Average number of steps taken per day (**B**). Time spent in sedentary PA category (METS 0-3) (**C**), time spent in moderate PA category (METS 3-6) (**D**), time spent in vigorous PA category (METS 6-9) (**E**). $^{*}P < 0.05$, $^{\#}P = 0.07$, $^{\$}P = 0.08$.

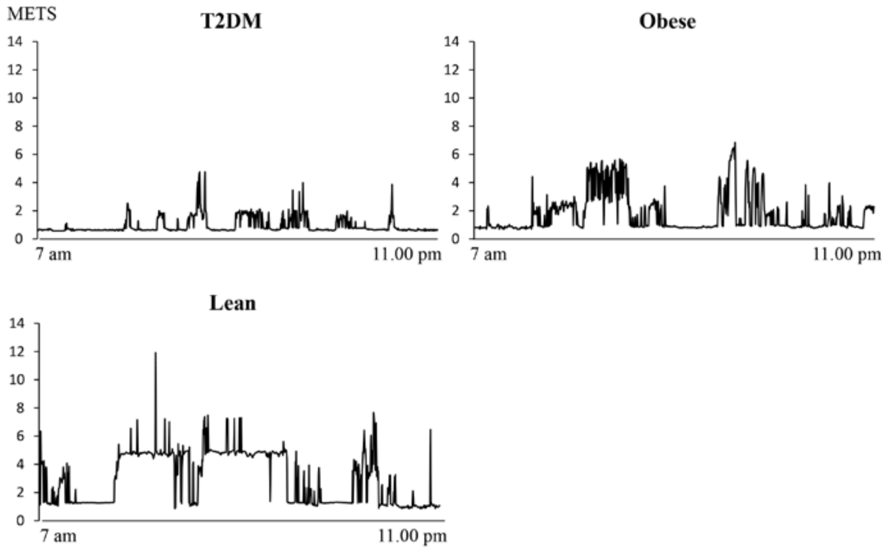

Figure 2 Free-living objectively measured daytime PA profile (16 hrs) of a typical insulin-treated patient with T2DM, obese and lean subject.

To correct for age differences between groups, separate analyses were performed to assess the influence of age on PA. There was no correlation between age and PA within the diabetes group (Pearson correlation r = 0.03; $P = 0.80$). When data were reanalyzed after exclusion of diabetes patients who were older than 70 years (in total 10 patients), differences in PA between groups were not affected (METS 1.34 ± 0.24 vs. 1.53 ± 0.33 vs. 1.85 ± 0.35 in type 2 diabetic patients, obese and lean individuals, respectively; $P < 0.001$ between groups).

There were no differences with respect to occupation and educational level between patients with T2DM and obese subjects (Table 2) which suggests that differences in socio-occupational status between the groups cannot explain the observed differences in PA levels. Surprisingly, patients with T2DM reported to be significantly more engaged in sports than obese individuals.

Sleep characteristics

The quantitative sleep parameters ALDD and ASD were similar between the groups (ALDD: 451 ± 88 vs. 431 ± 94 and 441 ± 66 min. in patients with T2DM, obese and lean subjects, respectively; ASD: 356 ± 88 vs. 344 ± 78 and 356 ± 88 min. in patients with T2DM, obese and lean subject). The qualitative sleep parameter AFI was significantly higher in patients with T2DM compared to lean and obese controls (16 ±

Table 2 Differences in socio-occupational status and engagement in sports between groups.

	T2DM (n = 48)	Obese (n = 42)	Lean (n = 38)
Occupation			
Employed	70$	57**	76
Self-employed	0	0	0
Partially employed	0	2	0
Retired	7$#	17	13
Unemployed	21$#	24*	8
Missing data	2	0	3
Educational level			
Middle school	94$#	81	78
High school	3$#	17	21
University	3	2	0
Missing data	0	0	1
Sports			
Engaged in sports	35$	22*	68
Average min/day	42 ± 18	40 ± 17	49 ± 29

Data as percentages or mean ± SD.

* Obese vs. lean; $P < 0.05$. ** Obese vs. lean; $P = 0.09$, # T2DM vs. lean; $P < 0.05$, $ T2DM vs. obese; $P < 0.05$.

6 vs. 13 ± 5 vs. 13 ± 5, respectively, $P < 0.05$ for T2DM vs. obese and $P = 0.07$ for T2DM vs. lean). In addition, the AFI differences remained significant even after adjustment for age and BMI. Figure 3 displays the sleep characteristics of the groups.

Conclusions

So far, few studies have reported data concerning PA and sleep characteristics in patients with T2DM. The main novel finding of the present study is that patients with insulin-treated T2DM show a reduction in free-living daytime PA in comparison to weight-matched obese individuals. We found no evidence for a difference between the groups with respect to sleep duration although diabetes patients showed a more disturbed sleep.

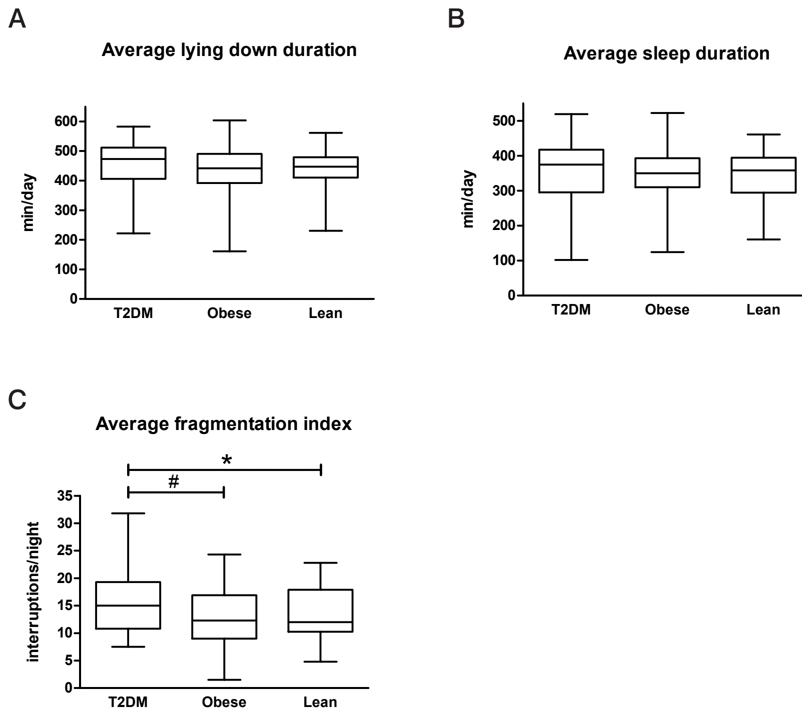

Figure 3 Quantitative and qualitative sleep characteristics (lying down duration (**A**), sleep duration (**B**), and average fragmentation index (**C**)) comparing insulin-treated patients with T2DM, obese and lean subjects. * $P < 0.05$ and # $P = 0.07$.

We also found that objectively measured free-living daytime PA was reduced in obese individuals compared to age-matched lean subjects. This finding is in line with several studies (2, 8) and confirms the fact that obesity per se is inversely associated with PA.

The method to measure PA in our study (accelerometry by SenseWear Pro Armband™, Body Media, Pittsburgh, PA, USA), has been validated versus double labelled water for the assessment of total energy expenditure in patients with T2DM (9) and shown to be reliable to objectively assess physical exertion in free-living conditions (6, 10). A few studies have assessed PA in patients with T2DM, also with the use of accelerometry to assess energy expenditure (9). However, none of the known literature primarily investigated PA in insulin-treated patients with T2DM. One study reported on average >7000 steps per day in patients with T2DM (11), which is much higher than ~5200 steps taken per day in patients in our study. In this study

patients had a similar BMI compared to our diabetes subjects. In this study, it was not reported whether participants were treated with insulin therapy or oral glucose lowering medication. Another report showed that patients recruited from primary care clinics with T2DM recorded more sedentary time compared to healthy lean subjects (12). When adjusting for BMI, the differences in sedentary time were attenuated. In this study, most patients used metformin, and only 10 % of the participants were on insulin (personal communication). Jakicic *et al*. (13) studied activity patterns of obese adults with T2DM in the Look AHEAD study and compared data across various categories. The results provide a hint towards patients using insulin displaying lower levels of PA, which is in line with our study results. The study had no non-diabetic control group.

Thus, while our results suggest that insulin-treated patients with T2DM have lower levels of PA, our study cannot determine whether this is explained by the insulin treatment or by the diabetic state per se. Combined with findings from the literature a relationship with insulin therapy seems likely.

Insulin treatment may decrease PA, because patients may pro-actively avoid hypoglycemia. We did not measure the number and severity of hypoglycemic episodes and hence cannot determine whether there is a correlation with hypoglycemia incidence and PA. However, even when no hypoglycemic events occur, patients may decrease PA just because of fear for hypoglycemia. Insulin-treated patients with T2DM may also perform less PA, because of more advanced disease state reflected in a higher rate of micro-vascular complications. For example, diabetic peripheral neuropathy is negatively associated with walking in persons with T2DM (14). Clearly, the prevalence of peripheral polyneuropathy in our diabetes group was higher than in the control groups, but we found no association between presence of neuropathy and PA level. As we found no differences in socio-occupational class or engagements in sports, both known to be major contributing factors to PA (15), these cannot explain the dissimilarity in PA levels found in our study. Other factors relevant to physical inactivity such as disease burden, injecting insulin, stress, and emotional or depressive complaints may also contribute to the decreased PA in insulin-treated T2DM patients (16, 17).

Short sleep duration is associated with reduced reported PA (18, 19). Earlier studies have also found that sleep duration is inversely related to hyperglycemia, weight gain, and insulin resistance (20, 21). The present study, however, found no significant differences between the groups with respect to sleep duration implying that quantitative sleep characteristics in T2DM are not associated with reduced PA. In our study, sleep duration in patients with T2DM was less than reported in a previous study (21). This may be due to slight differences in analysis software. The average fragmentation index (AFI), a qualitative sleep parameter, was significantly higher in patients with T2DM compared to lean and weight-matched obese subjects, but

numerically the difference was rather small (AFI obese ~ 13 interruption/night vs. ~ 16 interruptions per night in patients with T2DM). Trento *et al.* also investigated sleep characteristics using actigraph accelerometry in patients with T2DM (21). They found that patients had on average 22 interruptions per night, which is slightly higher compared to our results. Given the small absolute differences in sleep interruptions, we do think it is unlikely that these differences explain the lower daytime PA observed in patients with insulin-treated T2DM.

Accelerometry has been used before to study sleep disturbances in a variety of populations, most frequently for the evaluation of insomnia, sleep state misperception and circadian rhythm disorders (22).

The American Academy of Sleep Medicine recognizes it as a useful adjunct to the clinical assessment of sleep disorders (23). Accelerometry has been validated against polysomnography, demonstrating a correlation between 0.82 and 0.97 in healthy subjects (24). A SenseWear device, allows estimation of sleep and wakefulness based on motor activity and provides a low-cost, non-invasive, objective, and longitudinal method for diagnostic and post-treatment evaluation of patients evaluating sleep in the ambulatory setting. Accelerometry does not differentiate rapid eye movement (REM) sleep from non-REM sleep, so in this study disturbances in sleep stages could not be detected.

Our study has limitations. Due to the cross-sectional comparison, we cannot determine whether decreased daytime PA in patients with T2DM is rather a cause or consequence of obesity or weight gain. Patients with diabetes were slightly older than the obese subjects, which might affect PA levels. Two additional analyses, however, suggest that it is unlikely that the difference in age explains the observed difference in PA. As stated above, our study only included patients on insulin therapy, and hence, we cannot judge whether patients on oral therapy show similar results of PA.

In summary, we show that free-living objectively measured daytime PA is reduced in insulin-treated patients with T2DM which cannot be solely attributed to obesity-related effects. This suggests that a reduction in daytime PA may be linked to treatment-related (i.e. insulin therapy) effects of diabetes per se. Sleep duration was similar amongst the groups and hence does not seem to be involved in the decreased PA level. Prospective studies are needed to confirm these findings.

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Disclosure

The authors have no relevant conflicts of interest to disclose.

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Chapter 4

Diabetes-related distress, insulin dose and age contribute to insulin-associated weight gain in patients with type 2 diabetes mellitus: results of a prospective study

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Abstract

The determinants of insulin-associated weight gain in type 2 diabetes mellitus (T2DM) are partly unknown. Therfore, we conducted a prospective study to identify predictors of insulin-associated weight gain.

In patients with T2DM, we assessed physical activity by accelerometry, caloric intake by diet diaries, and diabetes-related distress measured by questionnaires, before, and 6, and 12 months after starting insulin therapy. Glycemic control (HbA_{1c}), and insulin dose were monitored.

After 12 months of insulin therapy mean body weight had increased by 3.0 ± 2.5 kg ($P < 0.001$). The drop in HbA_{1c} was correlated with insulin-associated weight gain. With the use of a multiple linear regression model a cluster of variables was identified that significantly related to weight gain. Diabetes- related distress, initial insulin dose, and the increase of insulin dose during the course of the study as well as age appeared to be important predictors of weight gain after initiation of insulin therapy. Physical activity (measured as metabolic equivalent (METS)) decreased from 1.40 ± 0.04 at baseline to 1.32 ± 0.04 METS ($P < 0.05$), but was not significantly related to weight changes. Reported caloric intake decreased significantly.

Diabetes-related distress, initial and titration of insulin dose and age, all significantly predict insulin-associated weight gain. After the initiation of insulin therapy, physical activity decreased significantly, but this did not determine weight gain over the first 12 months. Our study findings may have clinical implications.

Trial registration: clinicaltrials.gov Identifier: NCT00781495

Introduction

Insulin therapy is frequently needed to achieve adequate glycemic control in patients with type 2 diabetes mellitus (T2DM), but often at the expense of weight gain. This is obviously a disadvantage of insulin therapy in an already obese population. The reported weight gain during the first year of insulin therapy ranges from approximately 2 to 6 kg (1). This weight gain shows large inter-individual differences, with some patients experiencing substantial insulin-associated weight gain, while others do not show any weight gain at all or even lose weight.

Identification of clinical variables related to insulin-associated weight gain in T2DM would be valuable to predict or potentially prevent this side effect. While a number of putative mechanisms are postulated to explain this weight gain (2,3), formal prospective studies including these different clinical variables are lacking.

Improvement in glycemic control (change in HbA_{1c}) by itself seems to be related to insulin-induced weight gain, but the contribution appears to be rather small and even non-significant in the long term (4). Even so, a decrease in HbA_{1c} is inevitably related to insulin therapy. Insulin dose should therefore be regarded as a potential contributor to weight gain rather than changes in HbA_{1c}.

Insulin-associated weight gain may be related to a decrease in physical activity, but this possibility has received limited attention. In a cross-sectional study, we showed that patients with pronounced weight gain (“gainers”) during insulin therapy performed less daily physical activity overall compared to those who did not gain weight (“non-gainers”) (5).

Diabetes mellitus is associated with a significantly increased risk for depressive symptoms (6). Depressive complaints and anxiety are related with larger weight gain and an increase in cumulative incidence of obesity in men and women (7). As a result, it can be reasoned that depressive complaints and anxiety in patients with T2DM incur weight gain.

In the current study, we first prospectively investigated whether baseline (i.e. at the start of insulin therapy) glycemic control, physical activity, insulin dose, and diabetes-related distress and also changes of these variables during the course of the study (changes over time) correlated with insulin-associated weight gain in patients with type 2 diabetes mellitus. Second, we modeled the relationship between baseline variables and changes over time in order to predict the amount of weight gain one year after the start of insulin treatment.

Patients and Methods

Patients

In this prospective, multicenter, observational study, patients with T2DM who were starting insulin therapy were included. Patients were recruited from one university hospital (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands), 3 non-university teaching hospitals (Jeroen Bosch Hospital, 's-Hertogenbosch, Slingeland Hospital, Doetinchem, and Bernhoven Hospital, Oss, The Netherlands), and 4 large primary care practices ("de Teselaar", Bemmel; "De Vier Kwartieren", Boxtel; "Hoevense Veld", Uden and "Berghem", Berghem, the Netherlands). The diagnosis of T2DM was made according to the diagnostic criteria of the WHO. The decision to start insulin treatment was at the discretion of the responsible physician and was always based on a failure of glycemic control while on oral glucose-lowering agents and diet. All patients were treated according to national guidelines (8). The choice of insulin preparation, insulin regime, dose and titration was left to the responsibility of the treating physician. Patients were excluded if they already used insulin or had steroid-induced diabetes, latent auto-immune diabetes in adults (LADA) which was excluded by testing anti-glutamic acid decarboxylase (anti-GAD) antibodies or maturity onset diabetes of the young (MODY). Furthermore, clinical evidence of psychiatric, renal, cardiovascular or liver or other diseases and medication (prednisone) which may influence study results regarding glucose and weight, hormonal disorders which may influence weight (i.e. thyroid diseases), unless properly treated with stable hormonal levels, bariatric treatment, excessive alcohol consumption (>20 g/day), drug abuse, use of thiazolidine derivatives and pregnancy or intention to become pregnant during the study were all considered exclusion criteria.

Patients were followed for 12 months after the initiation of insulin therapy. Patient characteristics (age, gender, race, smoking habits) were noted and clinical data were retrieved at baseline (i.e. prior to start insulin) and at 3-month intervals, which included body weight, blood pressure, HbA_{1c}, diabetes duration, co-morbidities, number of hypoglycemic events, lipids, type of oral glucose-lowering medication, and insulin type and dose.

Before and at 6 and 12 months after the initiation of insulin therapy physical activity levels, diabetes-related distress, depressive complaints both measured by questionnaires and caloric intake were assessed.

The inclusion and exclusion criteria were reviewed at a screening visit, where patients underwent a history taking and a complete physical examination. The study protocol was approved by the local ethical committee (CMO region Arnhem-Nijmegen, the Netherlands). All patients provided written informed consent.

Measures

Patients were studied in fasted overnight conditions. Weight was measured with subjects wearing light underwear only. Scales were calibrated annually. Waist circumference was measured midway between the lower rib margin and the iliac crest at expiration, and hip circumference over the greater trochanter; waist-to-hip ratio (WHR) was calculated. Skin fold measurements at 5 specific sites (abdomen: next to the belly site, suprailiac: just above the iliac crest of the hip bone, quadriceps: middle of the upper thigh, triceps: the back of the upper arm and subscapula: beneath the edge of the shoulder blade) on the right site of the body were taken by using the Slim Guide ® Skinfold Calliper (Creative Health Products, Plymouth, USA). The scale of 0 to 85 mm is especially useful in assessing skin folds at the upper ranges, which are not accommodated by most other callipers. Accuracy may be interpolated to 0.5 mm. Measurements were taken twice and averaged. Body composition was assessed by using formulas described by Jackson and Pollock (9,10).

Blood pressure was measured twice by a manual sphygmomanometer in supine position after a minimum of 5 minutes rest at the right arm. The average blood pressure (mean systolic and diastolic blood pressure) was calculated. Furthermore, fasting blood samples were drawn to assess: glucose, HbA_{1C}, thyroid hormone and lipids.

Physical activity was objectively measured using a SenseWear Pro Armband™ (Body Media, Pittsburgh, PA, USA) (11). The device was placed on the right upper arm over the triceps muscle for 4 consecutive days at baseline and at 6 and 12 months follow-up. Measurements between 7.00 am and 11.00 pm were used for calculations if $>$ 90% of these data were available. Outcome variables from the activity monitor included: average metabolic equivalent (METS; 1 MET = consuming 1 kcal/kg of body weight per hour), time (minutes/day) spent at different activity intensity categories averaged per day over the measurement period and the number of steps per day. SenseWear Innerview professional software 6.1 was used to analyze the data.

Dietary intake was measured by food records. Participants returned a handwritten food record in which self-reported detailed description of the types and amounts of food, beverage and/or supplements was documented over a 3-day period. This food record was modified from Thompson *et al.* (12). Most of the patients provided weighed diet records (i.e. participants weighed used food and beverages). Patients were asked to complete the food record for three consecutive days prior to the start of insulin therapy, and at 6 and 12 months follow-up. Food records were checked by a dietician and the intake of nutrients was calculated with a computer program (© Vodisys Medical Software b.v., the Netherlands, 2008).

Diabetes-related distress and depression was measured with the Problem Areas In Diabetes Scale (PAID) questionnaire (13,14). PAID-scores were calculated using a five-point Likert-scale with options ranging from “0 - not a problem” to “4 - serious

problem". Summing all item scores and multiplying by 1.25 resulted in an overall PAID-score. A minimum score of 0 indicated no diabetes-related distress. A maximum score of 100 indicated significant diabetes-related distress. The questionnaire was taken at baseline and at 6 and 12 months follow-up.

Data analysis

The sample size was based on data from a pilot study where physical activity was measured in 9 patients with T2DM. This group showed a physical activity level of 1.31 METS and a standard deviation of 0.30 METS. This study was powered to detect a difference in physical activity of 15 % after 12 months of insulin therapy, with a power of 90 % at a significance level of 0.05 %. This translated to the inclusion of 65 subjects. It was estimated that 10-15 % of the included patients would drop-out, resulting in the need for 72-75 patients to be included.

The results of the study are displayed as means ± SD, unless otherwise indicated.

To study changes in clinical variables (insulin dose, body weight, HbA_{1C}, physical activity, caloric intake and PAID-score) during the course of the study, a general linear model for repeated measures was used.

To determine whether selected baseline variables were related to the change in body weight after the start of insulin treatment, a univariate approach by simple correlations was performed using Pearson's correlation coefficient for normally distributed variables and Spearman's correlation coefficient for non-Gaussian distributed data.

Relationships between changes in clinical variables during the course of the study (changes over time) were investigated by a linear mixed model for repeated measures (15).

Finally, a multiple linear regression model was designed in order to estimate the relationship between the dependent variable (insulin-associated weight gain) and different independent variables or predictors. Both baseline parameters (from the correlation analyzes) and the change in parameters over time (from the linear mixed model for repeated measures) were included into the model.

For both the multiple linear regression model and for the linear mixed model estimated regression parameters are presented, with the appropriate standard error (SE) and 95% confidence interval (CI). Details of both models are presented in the supplemental data.

All calculations were made using SPSS 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA). A P-value < 0.05 was considered significant in all statistical comparisons.

Results

A total of 79 patients were eligible to enter the study. Seventy-seven patients consented and underwent screening. Of these, 74 patients with type 2 diabetes mellitus enrolled in the study. We excluded 9 patients (8 patients with missing follow-up data or withdrawal, and 1 patient was diagnosed with maturity-onset diabetes of the young (MODY)). Finally, 65 subjects were included in the analysis. Table 1 displays the descriptive characteristics of the patients at baseline.

Patients started with a mean of 18 ± 2 units (0.21 ± 0.02 U/kg) of insulin per day. At 6 and 12 months patients used a mean of 39 ± 3 units (0.42 ± 0.03 U/kg) and 46 ± 4 units (0.49 ± 0.04) of insulin per day, respectively ($P < 0.05$ compared to baseline). In addition, at 6 months a shift in the use of different types of insulin was observed: 9 % of the patients who used long-acting insulin were switched to biphasic or prandial insulin. At 12 months, 46 % of the patients used long-acting insulin, 14 % used biphasic insulin and 40 % used basal/bolus insulin. Within the study period no severe hypoglycemic episodes requiring a third party occurred.

Mean body weight increased from 89.4 ± 2.4 to 92.4 ± 2.5 kg at 12 months of insulin therapy ($P < 0.001$, see Figure 1a), and waist circumference from 106 ± 2 to 109 ± 2 cm at 12 months ($P < 0.01$). Seventy-one percent of the patients showed body weight gain, whereas twenty-nine percent had stable body weight or even lost weight after 12 months of insulin treatment (Figure 1b).

Calculated lean body mass decreased from 70 ± 2 to 69 ± 2 kg at 12 months (P = NS), while calculated body fat increased from 21 ± 1 to 24 ± 1 % at 12 months ($P < 0.001$).

Mean HbA_{1c} decreased from 8.8 ± 0.2% (73 mmol/mol) to 7.3 ± 0.1% (56 mmol/mol) after 12 months ($P < 0.001$).

After 12 months of insulin therapy, quantitatively measured physical activity was significantly reduced (Fig. 2a-e). Average metabolic equivalent (METS) decreased from 1.39 ± 0.04 to 1.32 ± 0.04 at 12 months of follow-up ($P < 0.05$), and time spent in sedentary physical activity level numerically increased but this difference was not statistically significant. The amount of moderate physical activity (METS 3-6) at 12 months was significantly lower compared to 6 months of insulin therapy ($P < 0.05$). The average number of steps per day diminished significantly after the start of insulin therapy (from 6441 ± 426 to 5746 ± 343 steps/day at baseline, after 12 months of treatment; $P < 0.05$).

Table 1 Descriptive characteristics of the study patients at baseline.

	Baseline
N	65
Age (yr)	60 ± 10
Gender (female %)	46
Race (Caucasian %)	95
Duration diabetes (yr)	9 ± 8
Weight loss prior start insulin therapy (kg) †	2.0 ± 3.8
BMI (kg/m^2)	30.8 ± 5.7
SBP (mmHg)	144 ± 20
DBP (mmHg)	79 ± 10
Fasting glucose (mmol/l)	11.7 ± 3.8
HbA_{1c} (%)/(mmol/mol)	8.9 ± 1.4/74
TC (mmol/l)	4.5 ± 1.9
TG (mmol/l)	3.4 ± 6.3
HDL (mmol/l)	1.0 ± 0.3
LDL (mmol/l)	2.3 ± 1.1
Alcohol use (%)	28
Smoking (%)	20
Type of insulin (%) #	
- Long-acting only	58
- Biphasic	17
- Short-acting only	0
- Long-acting/short-acting (basal/bolus)	25
Concomitant use of oral glucose lowering agents (%)	
- MET*	22
- SU**	9
- MET + SU	69
Diabetic related complications (%)	
- Nephropathy	15
- Polyneuropathy	10
- Retinopathy	10

Data are means ± SD. Baseline: prior to the start of insulin therapy. † = experienced weight loss within 12 months prior to start of insulin therapy. # = type of initiated insulin after baseline measurement and type of insulin used at 6 and 12 months. BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, HbA_{1c} = glycosylated haemoglobin, TC = total cholesterol, TG = triglycerides, HDL = high-density lipoprotein cholesterol, LDL = low-density lipoprotein. *MET denotes metformin, **SU denotes sulphonylurea derivatives.

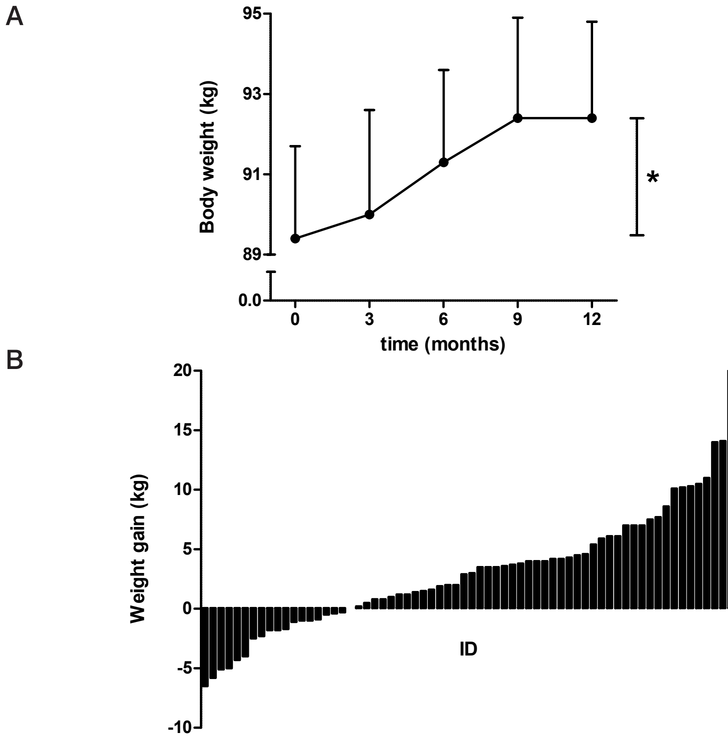

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Figure 1 **A**) Change in body weight after the start of insulin therapy. * P < 0.001. **B**) Inter-individual differences in absolute body weight gain (kg) after 12 months of insulin treatment.

After insulin initiation, reported caloric intake decreased (from baseline 1599 $\pm$ 72 to 1392 $\pm$ 41 kcal/day at 12 months; $P < 0.05$). Obviously, a decrease in dietary intake cannot explain the insulin-induced weight gain.

Patients suffered from minor depressive complaints at baseline and this did not change significantly over time (PAID scores: 10.0 $\pm$ 1.4 vs. 9.7 $\pm$ 1.3 vs. 9.4 $\pm$ 1.4 at baseline, after 6 and 12 months, respectively, P = NS).

Correlations of baseline variables and insulin-associated weight gain

Simple correlation coefficients were calculated between baseline variables and insulin-associated weight gain after 12 months of insulin therapy. These results are shown in Table 2. The baseline variables $HbA1_C$, insulin dose and PAID score were positively correlated with weight gain. The baseline variables age and body fat were negatively correlated with weight gain.

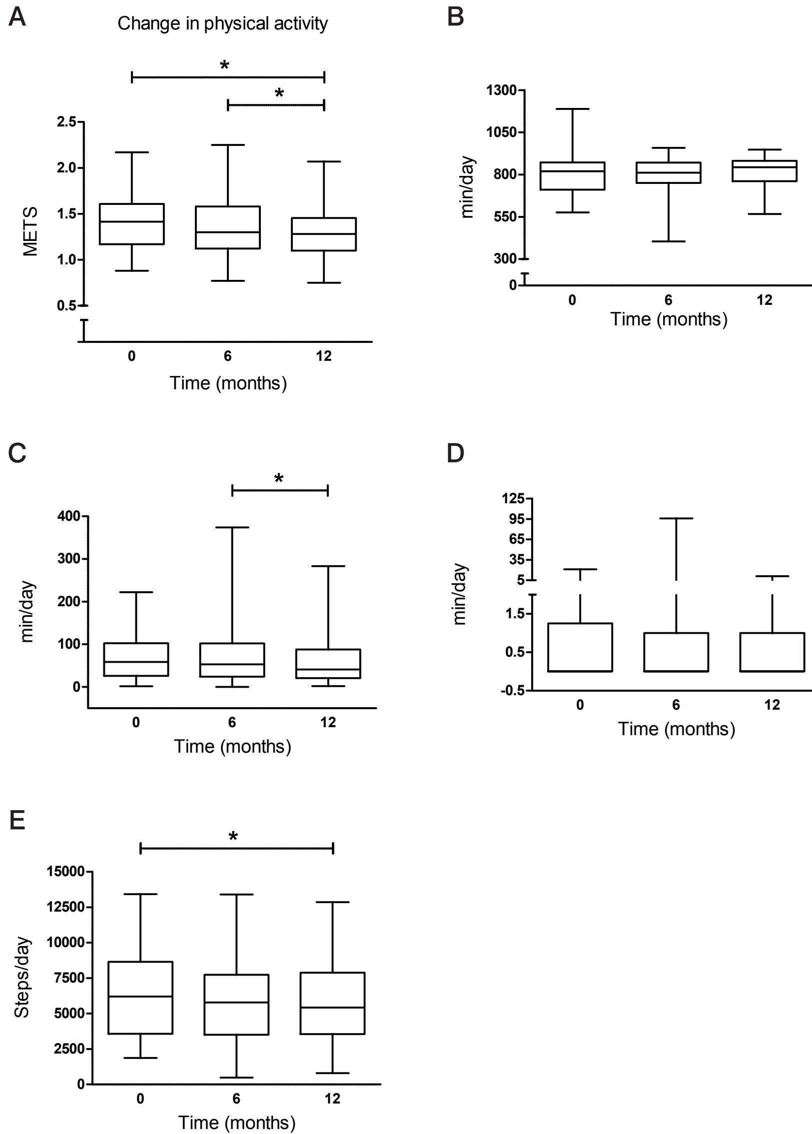

Figure 2 The vertical box-plots displayed represent summary statistics for the analysed variables stated (A-E). The plot elements and the statistics represented are as follows: the length of the box represents the interquartile range (the distance between the 25th and the 75th percentiles), the horizontal line in the box interior represents the median, and the vertical lines issuing from the box extend to the minimum and maximum values of the analyses variables.

A) Change in physical activity levels after the start of insulin therapy. METS = metabolic equivalent. 1 MET = consuming 1 kcal/kg of body weight per hour). * $P < 0.05$. **B**) Change in sedentary physical activity (METS 0-3) after the start of insulin therapy. **C**) Change in moderate physical activity (METS 3-6) after the start of insulin treatment. * $P < 0.05$. **D**) Change in vigorous physical activity (METS 6-9) after the start of insulin therapy. **E**) Change in average number of steps per day after commencing insulin therapy. * $P < 0.05$.

Table 2 Pearson correlation coefficients between baseline predictors and insulin-associated weight gain after 12 months of insulin therapy.

Variable	*r*	*P*-value
Age (yr)	- 0.27	0.03
Body fat (%)	- 0.28	0.04
HbA_{1c} (%/mmol/mol)	0.42	0.001
Insulin dose (U/day)*	0.43	< 0.001
PAID-score**	0.31	0.03

* Analysis performed with Spearman's rank correlation. HbA_{1c} = glycosylated haemoglobin. ** Diabetes-related distress was measured by Problem Areas In Diabetes (PAID) scale. The other (non-significant) variables tested in simple correlation coefficients were: gender (male/female), diabetes duration (yr), presence of diabetes related complications (neuropathy, nephropathy (microalbuminuria), retinopathy), body weight (kg), waist-to-hip ratio, skin fold measurement umbilicus (mm), body fat (%), use of metformin (yes/no), insulin dose (U/day), number of hypoglycemic episodes, and physical activity (METS), thyroid hormone level (mU/l), PAID-score and centre (patients treated in hospital vs. treated by general practitioner).

Changes in clinical variables over time related to insulin-associated weight gain

Using a linear mixed model for repeated measures, we assessed the relationship between changes in clinical variables over a 12 months period of insulin treatment and weight gain. Details from the outcome of the linear mixed model are described in Supplementary Tables 1, 2 and 3. Both the change in HbA_{1c} and insulin dose over time were significantly related with insulin-associated weight gain. A decrease in $HbA1_c$ and an increase in insulin dose contributed to weight gain. Changes in physical activity and diabetes-related distress over time were not related with weight gain.

Multiple linear regression modeling

With a multiple linear regression model we retrieved a cluster of clinical parameters consisting of baseline variables as well as variables that change over time that are related to weight gain. The change in $Hba1_c$ was omitted from the prediction model

(see discussion). Table 3 shows that baseline diabetes-related distress as measured by PAID questionnaire/score, initial insulin dose, age, and change in insulin dose over time were the main contributors to the prediction model with respect to weight gain after the initiation of insulin therapy. In this model a significant interaction between age and PAID-score was observed. Hence, this indicates that the aforementioned variables are significantly related to insulin-associated weight gain but should be corrected for the interaction between age and diabetes-related distress.

Table 3 Multiple linear regression analysis to assess independent baseline factors of insulin-associated weight gain after 12 months of insulin therapy by including interactions terms and change in insulin dose over time.

Variable	*B*	*SE*	*95%-CI*	*P*-value
Constant	-8.635	5.023	-18.745 to 1.475	0.092
PAID-score *	0.964	0.384	0.192 to 1.736	0.016
Initial (baseline) insulin dose (U/day)	0.165	0.060	0.044 to 0.285	0.008
Δ insulin dose **	0.048	0.019	0.009 to 0.087	0.017
Age (yr)	0.106	0.082	-0.059 to 0.271	0.202
(Age x PAID-score)	-0.015	0.006	-0.028 to -0.002	0.025

Linear regression analysis with weight gain after 12 months as dependent variable. Variables were selected using a manual backward selection method. The following baseline variables were considered for the model: age (yr), body fat (%), HbA_{1c} (%) or (mmol/l), insulin dose (U/day), PAID-score, Δ insulin dose (change in insulin dose at 12 months compared to baseline), the interaction terms: (HbA_{1c} x PAID), (HbA_{1c} x body fat), (HbA_{1c} x initial insulin dose), (HbA_{1c} x age), (HbA_{1c} x Δ insulin dose), (PAID x body fat), (PAID x initial insulin dose), (PAID x age), (PAID x Δ insulin dose), (initial insulin dose x body fat), (age x body fat), (Δ insulin dose x body fat), (age x initial insulin dose), (initial insulin dose x Δ insulin dose), (Δ insulin dose x age). Only the variables that had a *P*-value < 0.05 remained in the final model ($R^2 = 0.43$, adjusted $R^2 = 0.36$). B = unstandardized coefficients, SE = standard error, 95%-CI = 95 % confidence interval for B.

In formula: body weight gain = -8.635 + 0.106 x (age) + 0.964 x (PAID-score) + -0.015 x (age x PAID-score) + 0.165 x (initial insulin dose) + 0.048 x (Δ insulin dose).

* Diabetes-related distress was measured by Problem Areas In Diabetes (PAID) scale.

** Δ insulin dose = change in insulin dose at 12 months compared to baseline.

Discussion

From our prospective study that was performed in a fairly large number of patients, we were able to predict insulin-associated body weight gain with the use of a cluster of clinical variables. Diabetes-related distress (PAID-score), insulin dose at the beginning of insulin therapy and age, together with the change in insulin dose during

the study period (i.e. 12 months) are the main independent variables that contribute to this prediction model. Physical exercise decreased after the initiation of insulin treatment, but was not related to insulin-induced weight gain.

In this real practice-based study, most patients (58%) started with a basal insulin regimen. The up-titration of insulin and the corresponding decrease in $HbA1_c$ was well in line with the LANMET trial and other previous reports (1,16). The mean insulin-associated weight gain in our study patients was approximately 3 kg after 12 months of insulin therapy and is comparable with other data from the literature (4,17).

Our study is the first to explore the relationship between the change in insulin-associated body weight and diabetes-related distress (PAID score). We showed that diabetes-related distress significantly contributed to the model that predicts the amount of weight gain after the initiation of insulin therapy. An increase in diabetes-related distress reflected by a higher PAID score before the start of insulin therapy is related to a more pronounced weight gain. The PAID scale is a reliable and validated questionnaire, which is able to assess emotional distress and significant motivational problems in patients related to their diabetes management (13,18). Although a relationship between emotional factors and body mass index has been described (19), no link has been identified between emotional distress per se and subsequent changes in weight after the initiation of insulin therapy. This study was not powered to investigate the influence of specific components of the PAID survey having an impact on body weight gain. It should be pointed out that patients with evident depressive disorders or on psychiatric medication, both known to have a significant impact on body weight, were excluded from the study. However, it can be hypothesized that diabetes-related distress contributes to an unhealthy diet or to physical inactivity (20). From the multiple linear regression analyses it was shown that diabetes-related distress (PAID scale) and age interact with each other. This means that age negatively affected the strength of the relationship between PAID score and weight gain. In other words, the predictive value of diabetes distress with respect to insulin-associated weight gain will be less with increasing age.

Intriguingly, we found that a higher initial insulin dose at the start of the study significantly contributed to a more pronounced weight gain after 12 months of insulin therapy. Also, an increase in the insulin dose during the course of the study significantly contributed to the increase of body weight. Both an increase in total body lipid stores due to an increase in lipid synthesis and an inhibition of fatty acid release (lipolysis) (21) as well as fluid retention (22) may play a role in insulin-associated weight gain.

The reason why the initial insulin dose independently contributes to body weight gain after 12 months has to be determined. The starting amount of insulin was established by the treating physician and may be partly based on the level of hyperglycemia and/or on the estimated insulin resistance. A separate analysis showed that $HbA1_c$ and

body weight at baseline were similar between patients starting with a low insulin dose compared to a high insulin dose, indicating that initial insulin dose was not determined by initial body weight or $HbA1_c$. Furthermore, it was shown that $HbA1_c$ at the end of the study in patients who started with a relatively low insulin dose or high dose was well-matched. This may suggest that a gradual decrease of $HbA1_c$ in comparison with a steeper decrease has a favorable effect on ultimate weight gain. A possible explanation for this observation may be that patients suffering from a steep decrease of $HbA1_c$ will experience more hypoglycemic episodes (which indeed was observed) leading to an increase in caloric intake. The change in caloric intake between these two groups, however, did not differ.

Age was shown to have a significant effect on insulin-associated weight-gain. Older patients starting insulin therapy were prone to gaining more body weight. This could be related to a decrease in daily physical activity and fitness (23,24). Indeed, a sub-analysis of changes in physical activity revealed that reduction in physical activity (METS and number of steps) was more pronounced in older patients.

As stated, physical activity did not seem to be related to insulin-associated weight gain in our study i.e. it could not be identified as an independent predictor of insulin-associated weight gain. Age and physical activity are covariates. From the analysis it appeared that only age was an independent variable. Nevertheless it can still be viewed as an unfavorable finding from the perspective of general health. The decrease in energy expenditure after insulin initiation may be explained by a number of mechanisms. Patients who start insulin may experience (or at least fear) hypoglycemic episodes and as a consequence perform less physical activity in order to prevent hypoglycemia. In our patient cohort, the number of hypoglycemic episodes was low and no severe hypoglycemia occurred. However, fear of hypoglycemia remains a potential explanation. Alternatively, a low physical exercise level may rather be a consequence than of a cause of body weight gain.

An increase in caloric intake is frequently mentioned as a determinant of weight gain. Especially after initiating insulin therapy it can be reasoned that caloric intake may increase in relation to (the avoidance of) hypoglycemic events resulting in weight gain (25). In our present study however, reported food intake did not increase, and even diminished significantly over time, arguing against increased food consumption being an important determinant of body weight gain. Several factors such as the intervals between the time points of taking the questionnaire, recall bias, true changes and intrinsic feelings could have had an impact on reported caloric intake outcomes as these may have potentially affected test-retest reliability. We did not perform a test-retest correlation analysis in order to test the reliability of the questionnaire prior to the study.

We and several others, have reported that changes in $HbA1_{1c}$ are related to insulin-associated weight gain, although we found that this contribution was rather

small (4). In the present study, we prospectively confirmed that an improvement in glycemic control is significantly related to insulin-associated weight gain. Nevertheless, the primary intention of insulin therapy is to improve glycemic control and decrease $HbA1_C$. As such, the change in $HbA1_C$ is the consequence of insulin therapy. Therefore, although the change in $HbA1_C$ was related to weight gain in the linear regression model, we did not include this variable in the multiple linear regression model because it does not represent a true independent variable.

Importantly, about 30 % of the patients did not gain or even lost weight after the initiation of insulin therapy. From the results of the multiple linear regression model it can be concluded that a young patient with a low PAID score, starting on a low insulin dose and in need of a more lenient up-titration of insulin to achieve adequate glycemic control, will probably suffer from less body weight gain after 12 months of insulin treatment.

Our study has limitations. First, this study cannot assess whether the decrease in physical activity is rather a consequence or a cause of body weight gain. Second, between-centre differences and treatment effects could have influenced outcomes but could not be tested due to the small numbers of participating centres. Furthermore, while our study provides predictors of insulin-associated weight gain, it cannot determine whether intervening on these factors, for example a lower starting dose and a more lenient insulin up-titration, or interventions aimed at mood improvement will affect weight gain.

Our study results may have implications for clinical practice. The decrease in physical activity, although not related to insulin-associated weight gain in this study, is an unfavorable consequence of insulin therapy. Health education should be aimed at improving physical fitness in patients starting with insulin therapy, as this may be beneficial to reduce cardiovascular risk. The clinical importance of identifying emotional factors that might impact body weight gain in patients with type 2 diabetes adds value to understanding this novel relationship. Furthermore, it can be suggested that (low) initial insulin dose and a more gradual titration of insulin over time may limit or even prevent body weight gain.

In conclusion, the initiation of insulin therapy in patients with T2DM is associated with weight gain and a decrease in physical activity. Although the reduction in physical activity was not associated with weight gain, it is an important issue for patients starting insulin therapy. A cluster of clinical variables predicts insulin-associated weight gain. This includes diabetes-related distress (PAID-score), initial insulin dose, change in insulin dose and age.

Further prospective studies are needed to investigate the exact role of emotional factors, types of insulin regimens and strategies of insulin titration on weight gain.

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Supplemental data.

Linear mixed models presented in this study

We studied the individual weight-profiles of patients with T2DM up to 12 months after the start of insulin therapy and the dependence on physical activity (METS), insulin dose, and diabetes-related distress (PAID), with the use of linear mixed models for repeated measures (14). At first, we found that the models were not statistically significant improved when a quadratic term in time was included in the linear part of the models (Likelihood-Ratio test). The following initial model was used:

$$Y_i(t) = \beta_0 + \beta_1 \times BBW_i + \beta_2 \times t + \beta_3 \times BBW_i \times t + \beta_4 \times \Delta VAR_{it} + \beta_5 \times \Delta VAR_{it} \times t + b_{1i} + b_{2i} \times t + \varepsilon_{it}$$

where Y refers to body weight, i to subject, t to the time (month) after start of insulin therapy, β to a fixed effect, b_{1i} to the random intercept, b_{2i} to the random slope, BBW to the baseline body weight, ΔVAR to the change in variable entered into the model since baseline and ε_{it} to the normal distributed residual with mean zero.

We fitted 4 separate models: 1) ΔVAR is the change in physical activity (ΔMETS), 2) ΔVAR is the change in insulin dose (Δinsulin dose), 3) ΔVAR is the change in diabetes-related distress (PAID) and 4) ΔVAR is the change in $HbA1_c$.

The model explained in words: the dependent variable in all models was body weight. The independent continuous variables were: body weight at the start of insulin therapy and the time (*t*) since the start of insulin therapy (month). Furthermore, the interaction term between both variables (BBW x *t*) was included in the unadjusted model (supplemental Table 1), representing different increase in body weight with higher initial body weight. The independent random effects were: intercept and the slope of the regression in time (representing weight gain or loss per month). This allows different regression lines for different patients, both in intercept and slope.

We studied weight-profiles unadjusted and adjusted for change in physical activity (METS), insulin dose, PAID and $HbA1_c$, separately.

The first model was designed to study weight profile adjusted for change in physical activity (METS). As mentioned, physical activity was measured at 3 time-points (at baseline, 6 and 12 months after start of insulin therapy). The independent continuous variable (time-dependent) change in METS since the start of insulin therapy (ΔMETS) was included in the model. It was found that the variable ΔMETS did not changed the model significantly. The second model was designed to study weight profile adjusted for change in insulin dose. The change in insulin dose (Δ insulin dose) contributed significantly to the change of the model (supplemental Table 2). The third model was designed to study weight profile adjusted for change in PAID. This variable did not contribute significantly to the change of the model. Lastly,

the change in $HbA1_C$ was entered into the model. This variable did contribute significantly to the change of the model (supplemental Table 3).

Finally, we analyzed two time frames separately (0-6 months, and 6-12 months) with the use of our linear mixed model, as we expected different relationships between variables (change in METS, insulin dose, and PAID) and change in body weight gain in each time period. In Table 4 it is shown that only the change in insulin dose between 0-6 months contributes significantly to the model.

Multiple linear regression model presented in this study

A multiple stepwise linear regression model was used to predict body weight gain. All two-way the interaction terms were included in order to investigate possible effect modification of combinations of these variables in predicting body weight gain. The stepwise procedure started with the removal of non-significant interactions followed by the removal of non-significant variables while keeping a hierarchical structure (i.e. if the interaction term stayed in the model, the main effect of both variables also stayed in the model). In other words, we used a manual backward selection regression method to remove non-significant interactions ($P > 0.05$). Thereafter, we removed non-significant ($P > 0.05$) main effects keeping the hierarchical structure.

For both the linear mixed model as well as for the multiple linear regression model estimated regression parameters are shown, with the appropriate standard error (SE) and 95% confidence interval (CI).

Supplemental tables

Table 1 The estimated regression parameters with the 95% CI of the crude data on body weight.

Effect		
	Estimate	**(95% CI)**
Intercept	89.555	(88.684 to 90.427)
(BBW – 89.4)	0.976	(0.929 to 1.023)
t (month)	0.253	(0.154 to 0.353)
BBW x *t*	0.006	(0.001 to 0.011)

Data represent the estimated regression parameters of the crude data on body weight for the period 0-12 months after start of insulin therapy.

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Table 2 The estimated regression parameters with the 95 % CI of the change in insulin dose adjusted weight profile.

Effect		
	Estimate	**(95% CI)**
Intercept	89.091	(88.186 to 89.996)
(BBW – 89.4)	0.969	(0.917 to 1.013)
t (month)	0.206	(0.099 to 0.313)
Δ Insulin dose	0.038	(0.015 to 0.061)

Data represent the estimated regression parameters of the crude data on body weight adjusted for the change in insulin dose (Δ Insulin dose) for the period 0-12 months after start of insulin therapy.

Table 3 The estimated regression parameters with the 95 % CI of the change in $HbA1_c$ adjusted weight profile.

Effect		
	Estimate	**(95% CI)**
Intercept	88.790	(88.002 to 89.581)
(BBW – 89.4)	0.988	(0.958 to 1.017)
t (month)	0.267	(0.135 to 0.400)
Δ $HbA1_c$	-0.451	(-0.777 to -1.125)

Data represent the estimated regression parameters of the crude data on body weight adjusted for the change in $HbA1_c$ (Δ $HbA1_c$) for the period 0-12 months after start of insulin therapy.

Table 4 The estimated regression parameters with the 95 % CI of the change in insulin dose adjusted weight profile for the time period 0-6 months.

Effect		
	Estimate	**(95% CI)**
Intercept	88.072	(86.919 to 89.225)
(BBW – 89.4)	0.976	(0.942 to 1.011)
t (month)	0.219	(-0.057 to 0.496)
Δ Insulin dose	0.070	(0.035 to 0.106)

Data represent the estimated regression parameters of the crude data on body weight adjusted for the change in insulin dose (Δ Insulin dose) for the period 0-6 months after start of insulin therapy.

All variables entered into the models contributed significantly to the change in weight profiles.

BBW baseline body weight (minus mean baseline body weight = 89.4), *t* time (month), Δ Insulin dose absolute change in insulin dose.

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Part II

Consequences of insulin-associated weight gain

insulin
tissue
adipose
therapy
weight
patients
macrophages
months
gain
adipocytes
inflammatory
levels
protein
diabetes
subcutaneous
study
change
influx
significant
expression
gainers

Chapter 5

Pronounced weight gain in insulin-treated patients with type 2 diabetes mellitus is associated with an unfavorable cardiometabolic risk profile

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Abstract

Pronounced weight gain after start of insulin therapy in patients with type 2 diabetes mellitus (T2DM) may offset beneficial effects conferred by the improvement of glycaemic control. This hypothesis was tested by comparing the cardiometabolic risk profile of a group of type 2 diabetes patients with a marked increase in body weight (‘gainers’) after the start of insulin treatment and a similar group without any or only minimal weight gain (‘non-gainers’).

In a cross-sectional study, we compared two predefined groups of patients with T2DM who had been on insulin therapy for a mean of 4.0 years: ‘gainers’ *vs* ‘non-gainers’. Cardiometabolic risk was assessed by measuring fat content and distribution (physical examination, bioelectrical impedance analysis, dual energy X-ray absorption, and magnetic resonance imaging), liver fat content (magnetic resonance spectroscopy), physical activity levels (Sensewear® armband) and plasma markers. Each subgroup consisted of 14 patients. Gainers had significantly more total body and trunk fat (especially subcutaneous fat) compared with non-gainers. Gainers had similar liver fat content, and slightly higher levels of fat hormones. Furthermore, gainers performed significantly less physical activity. Lastly, gainers had higher total cholesterol, low-density lipoprotein cholesterol, and alanine aminotransferase levels with similar cholesterol-lowering treatment.

Patients with T2DM who show pronounced weight gain during insulin therapy have a less favourable cardiometabolic risk profile compared with patients who show no or minimal weight gain.

Introduction

Insulin therapy is frequently needed to achieve adequate glycemic control in patients with type 2 diabetes mellitus (T2DM), but often at the expense of weight gain. Although values differ between studies, and studies are generally of limited duration, the estimated weight gain during the first year of insulin therapy ranges from approximately 2 to 6 kg (1). This weight gain shows large inter-individual differences, with some patients experiencing substantial insulin-associated weight gain, while others do not show any weight gain at all or even lose weight.

The determinants of insulin-associated weight gain are not entirely elucidated; most authors view the improvement in glycaemic control as the major determinant of weight gain. However, the level of improvement in glycemic control is only weakly correlated with the increase in body weight (2, 3). A frequently mentioned clinical experience is that a subset of patients exists that shows a persistent and continuous increase in body weight over time even when stable glycemic control has been obtained.

It is obvious that weight gain in an already overweight population is undesirable. Weight gain will deter further optimization of insulin therapy (4) and in itself will adversely influence the cardiometabolic risk profile (5). Little is known about the effects of insulin-associated weight gain on cardiometabolic risk in patients with T2DM. One may hypothesize that the benefits of insulin treatment conferred by the improvement of glycemic control may be offset by the disadvantages associated with pronounced weight gain. An increased fat mass may cause aggravation of insulin resistance, dyslipidaemia and hypertension and may increase the levels of inflammatory markers and the propensity for thrombotic events (6). Indeed, in type 1 diabetes, patients who experienced pronounced weight gain during intensive insulin therapy showed a less favourable cardiovascular risk profile (7). In the ACCORD trial (8), the intensively treated group with T2DM showed increased mortality. In this group, more than 75% of the patients used insulin therapy in combination with several oral drugs. More than 25% of the patients treated in the intensive-therapy group showed a mean weight gain of $>$ 10 kg during follow-up. Although the study did not reveal any direct effect of the exaggerated weight gain on cardiovascular events, extensive weight gain might have had a negative influence on cardiometabolic risk.

In the present study, we hypothesized that pronounced weight gain during insulin therapy would be associated with an unfavorable cardiometabolic risk profile. This hypothesis was tested by comparing cardiometabolic risk profile of insulin-treated patients with T2DM who showed weight gain at the extreme ends of the spectrum (“gainers” versus “non-gainers”).

Research Design and Methods

Patient groups

Patients were selected out of cohort of patients with T2DM who started insulin therapy in our university diabetes clinic between 2001-2006. To prevent confounding with respect to influences of different types of insulin on body weight we only included patients who started and continued on biphasic insulin (NovoMix® or Mixtard® insulin), twice-daily. Patients were selected based on the weight gain after starting insulin. We defined a "gainer" as a patient who showed an increase in body weight of ≥ 0.5 kg/month within the first 18 months after starting insulin therapy and ≥ 5% weight gain at total follow-up (i.e. at the time of cross-sectional measurement, which was different for each patient). We defined a "non-gainer" as a patient with a maximum weight gain ≤ 2.5 kg at follow-up. These criteria were derived from a historical insulin treated group (N=140), and represent the upper and lower subgroups of weight gain. Assessment at follow-up of the cardiometabolic risk profile between the two groups (gainers vs. non-gainers) was performed. All selected patients had a minimal follow-up of 18 months. Exclusion criteria were: heart failure (NYHA class III-IV), liver or renal disease (defined by chronic renal disease stage ≥ III), hypoalbuminaemia, use of alcohol of more than 2 units/day, drug abuse, use of thiazolidinedione derivatives or prednisone, and pregnancy or the intention to become pregnant during the study. Eligible patients did not use anti-obesity medications and acarbose treatment. Also weight loss surgery patients or patients who followed any other weight management program were excluded.

The inclusion and exclusion criteria were reviewed at a screening visit, where patients underwent a history taking and a complete physical examination. The study protocol was approved by the local ethical committee. All patients provided written informed consent.

Cardiometabolic risk assessment

Cardiometabolic risk profile at follow-up was assessed by the following: 1) body fat distribution (weight, height, waist and hip circumference, bioelectrical impedance analysis (BIA), dual energy X-ray absorption (DEXA), and MRI) (9, 10), 2) liver fat content (LFAT) by magnetic resonance spectroscopy (MRS) (11), 3) physical activity levels (12), 4) classical risk factors, other biochemical cardiometabolic markers (13).

Patients were measured in fasted overnight conditions with an empty bladder. To determine body mass index (BMI), weight (kg) was divided by height in meters, squared. Weight was measured with subjects wearing light underwear only. Scales were calibrated annually. Waist circumference was measured midway between the lower rib margin and the iliac crest at expiration, and hip circumference over the greater trochanter; waist-to-hip ratio (WHR) was calculated.

To assess fat distribution three different methods were used: BIA, DEXA and MRI. BIA was carried out using an Akern soft tissue analyser (BIA Quatum/S Body Composition Analyzer model no. BIA-101, Akern Srl, Pontassieve (Florence), Italy). BIA was performed to assess total body water (TBWa) and fat-free mass (FFM). Patients rested in supine position for approximately 5 minutes to equalise fluid compartments. Four surface electrodes were applied (two each to an arm and a leg). Phase sensitive sensors separated the components of the modulus into Reactance and Resistance.

Total-body DEXA scanning was performed using a Hologic QDR 4500 densitometer (Hologic Inc., Bedford, USA) to determine fat mass (total fat mass and trunk fat) and lean mass. To assess non-trunk fat the trunk-to-leg ratio (trunk mass devided by leg mass) was calculated for each patient.

MRI measurements were performed on a Tim-Trio MR system (Siemens, Erlangen, Germany). A series of T1-weighted (flash 2D) axial MR images was acquired from a region extending from 4 cm above to 4 cm below the fourth to fifth lumbar interspace. Visceral and subcutaneous fat areas were determined based on signal intensity. Proton MR spectra (STEAM; TE/TR:20/3000ms) were obtained without water suppression from a 8-ml voxel positioned in the liver during breath holding. The water signal intensity (S_{water}) and the methylene lipid signal intensitiy (S_{fat}) were used to calculate the percentage of liver fat by the following formula: ((S_{fat})/(S_{fat} + S_{water})) x 100% (14)). Total MR examination time was 30 minutes. Patients with pace-makers, implantable cardioverter defibrillators, metal implants, and claustrophobia were included, but did not undergo magnetic resonance imaging (MRI). In 11 gainers and 12 non-gainers MRI/MRS was performed. The remaining patients experienced claustrophobia during the MRI/MRS scan although they were not known with claustrophobia at inclusion.

Physical activity was measured using a SenseWear Pro Armband™ (Body Media, Pittsburgh, PA, USA) (15, 16). The device was placed on the right upper arm over the triceps muscle for 5 consecutive days. Measurements were only used for calculations if $>$ 90% of data were available. SenseWear Innerview professional software 6.1 was used to analyse the data.

Classical risk factors as blood pressure, smoking habits, lipids, renal function and albumin excretion ratio (AER) were determined. Blood pressure was measured in supine position after a minimum of 5 minutes rest at the right arm. All patients already had taken their antihypertensives. Blood pressure was determined twice by a manual sphygmomanometer. The average blood pressure (mean systolic and diastolic blood pressure) was calculated. Furthermore, fasting blood samples were drawn to assess: HbA_{1c}, lipids, alanine aminotransferase (ALT), and creatinin (all determined by standard laboratory methods). Renal function expressed as glomerular filtration rate (GFR) was calculated by the (MDRD formula (17). The adipocytokines, adiponectin

and leptin were determined by using DuoSet ELISA development system kits (R&D systems, Minneapolis, USA), free fatty acids (FFA) using Cobas Mira Plus® (Roche Diagnostics Ltd., Basal, Switzerland), and the inflammatory markers high-sensitive C-reactive protein (hsCRP) by Dako ELISA (Glastrup, Denmark), IL-6 and 18 by Luminex® Corporation assay (Austin, Texas, USA)).

Statistical analyses

Differences between groups were analyzed by unpaired Student's *t*-test and Mann-Whitney U test as appropriate. For comparing dichotomous variables the Chi-square test was used. All calculations were made using SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Data are expressed as means ± SEM, unless otherwise indicated. A P-value <0.05 was considered significant in all statistical comparisons.

Results

A total of 14 patients were included in each group. Table 1 shows the characteristics of the patients. All patients were Caucasian. The two groups (gainers vs. non-gainers) were compared after a mean of 4.0 ± 1.6 years insulin therapy (i.e. mean follow-up); as per protocol, all patients were still on biphasic insulin. Gainers were longer on insulin than non-gainers (4.6 ± 1.7 vs. 3.7 ± 2.1 yr, P = 0.03).

Table 1 Patient characteristics after a mean of 4 years of insulin therapy

	Gainers	Non-gainers
Gender (m/f)	8/6	10/4
Age (yr)	63 ± 7	65 ± 7
Diabetes duration (yr)	9 (2-25)	12 (2-22)
Insulin therapy (yr)	4.6 ± 1.7	3.7 ± 2.1 *
Medication (n)		
- *Insulin alone*	7	5
- *Insulin + Metformin*	6	7
- *Insulin + SU †*	0	1
- *Insulin + Metformin + SU*	1	1
Insulin dose (U/day)	68 ± 37	60 ± 37
Insulin dose (U/kg)	0.7 ± 0.4	0.7 ± 0.4

Data are means ± SD or median (range). * $P < 0.05$. † SU sulfonylurea derivative.

Change in weight and HbA_{1c} after start of insulin therapy

As per definition, gainers showed a substantially larger weight gain (+11 kg [range + 5.2 to + 19.6 kg]) compared to the non-gainers (-1.2 kg [range -7.6 to + 2.5 kg], Fig. 1A and B). Of note, body weight at the start of insulin therapy was slightly lower in gainers (83 ± 15 vs. 87 ± 13 kg, P = NS). After 4 years of insulin therapy, BMI, waist circumference and waist/hip ratio were not significantly different between the two groups (BMI 32.3 ± 5.6 vs. 29.0 ± 4.5 kg/m^2, waist circumference 110 ± 15 vs. 106 ± 13 cm, WHR 1.05 ± 0.12 vs. 1.03 ± 0.10, for gainers and non-gainers, resp., P = NS).

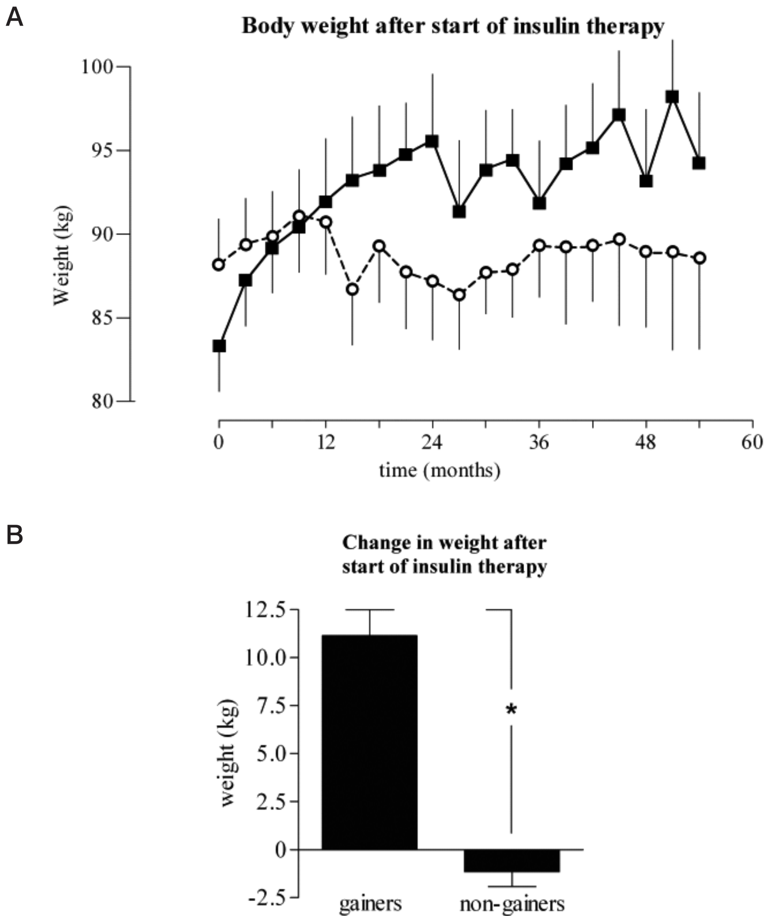

Figure 1 **A** (upper panel) Observed change in weight after start of insulin therapy (t=0) comparing gainers (closed squares) and non-gainers (open circles). **B** (lower panel) Absolute change in weight after start of insulin therapy. * P < 0.05.

Mean HbA_{1c} decreased from 9.9 ± 2.6 to 7.2 ± 0.7 in gainers versus 8.9 ± 1.3 to 7.4 ± 0.9 % in non-gainers (P = NS for the difference in gainers vs. the difference in non-gainers).

Cardiometabolic risk profile at follow-up

Fat distribution

Gainers had significantly more total body fat (32.4 ± 9.4 vs. 24.6 ± 7.7 kg, P = 0.03) and more trunk fat compared to non-gainers (18.3 ± 5.5 vs. 14.1 ± 4.2 kg, P = 0.04). Gainers had slightly higher TBWa compared to non-gainers (37.7 ± 7.5 vs. 35.9 ± 7.1 L, P = NS). FFM was comparable in gainers and non-gainers as measured by DEXA (58.4 ± 9.1 vs. 59.6 ± 8.7 kg, P = NS) and by BIA. In both groups the trunk-to-leg ratio was similar.

As measured by MRI, gainers had significantly higher subcutaneous fat than non-gainers (2.5 ± 0.8 vs. 1.8 ± 0.8 L, P = 0.04), while visceral fat was similar (1.7 ± 0.7 vs. 1.5 ± 0.7 L, P = NS).

Sixteen patients (70 %) had LFAT levels above the upper reference value of 5.5 % (18). Gainers and non-gainers had similar LFAT (9.6 ± 2.7 vs. 9.3 ± 1.6 %, resp., P = NS).

Physical activity levels

The average total energy expenditure at follow-up was significantly lower in gainers than in non-gainers (2275 ± 385 vs. 2632 ± 734 kcal/day, P = 0.005). Physical activity duration expressed as metabolic equivalent (MET) ≥ 3, which is consuming ≥ 3 kcal/kg of body weight per hour, tended to be lower in gainers compared to non-gainers (56 ± 51 vs. 83 ± 80 min./day, P = 0.06). Also the amount of vigorous activity (MET > 6) was lower in gainers than in non-gainers (2.4 ± 3.4 vs. 6.5 ± 5.5 minutes, P = 0.03). The amount of sedentary activity (MET 0-3) and moderate activity (MET 3-6) was similar between the two groups, as was the number of steps per day (5416 ± 3543 vs. 5282 ± 3681), and the total duration of rest (514 ± 139 vs. 484 ± 148 min.) and sleep (409 ± 137 vs. 394 ± 135 min.), all gainers vs. non-gainers, respectively.

Cardiometabolic markers

Prior to the start of insulin therapy the classical cardiometabolic risk markers (i.e. BMI, blood pressure, lipid profiles, smoking, GFR and AER) were similar in the two groups (table 2).

The cardiometabolic risk markers of the two groups measured in the present study are shown in table 3.

Table 2 Baseline cardiometabolic risk markers comparing gainers and non-gainers.

	Gainers	Non-gainers	*p*-value
Classical risk factors			
SBP (mmHg)	148 ± 26	153 ± 26	NS
DBP (mmHg)	85 ± 12	88 ± 8	NS
Total cholesterol (mmol/l)	5.1 ± 1.0	4.5 ± 0.9	NS
LDL-cholesterol (mmol/l)	3.0 ± 1.0	2.3 ± 0.9	NS
HDL-cholesterol (mmol/l)	1.2 ± 0.2	0.9 ± 0.2 *	0.01
Triglycerides (mmol/l)	2.0 ± 1.1	3.3 ± 2.4	NS
Smoking (n)	11	12	NS
Creatinin (μmol/l)	92.6 ± 29.7	99.5 ± 28.9	NS
GFR (MDRD; ml/min/1.73m^2	79.0 ± 34.6	64.5 ± 0.19	NS
Albumin excretion ratio (μg/min)	29.8 ± 24.6	39.1 ± 53.4	NS
Liverenzymes			
ALT (U/l)	36.8 ± 18.4	36.2 ± 20.4	NS

Data are means ± SD.
SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL: low-density lipoprotein,
HDL: high-density lipoprotein, GRF: glomerular filtration rate (MDRD: modified diet in renal disease), ALT: alanine aminotransferase.
All data were analysed by Student *t*-test, except for the data of AER which was analyzed with Mann-Whitney *U*-test. * $P < 0.05$.

Blood pressure was similar in gainers compared to non-gainers, as was the average number (2.1 vs. 2.2) and dose of antihypertensive medication. Total cholesterol and low-density lipoprotein cholesterol (LDL-cholesterol) were significantly higher in gainers than in non-gainers, as was the level of high-density lipoprotein cholesterol (HDL-cholesterol), despite similar use of statins at equipotent doses.

There were no differences between the two groups with respect to smoking habits. Creatinin was slightly lower in gainers than in non-gainers and urinary albumin excretion appeared quantitatively higher in gainers, but the differences were not significant. Calculated GFR was significantly higher in the gainers group. FFA and adiponectin levels were similar between the two groups. Leptin, and the inflammatory cytokines were slightly higher in gainers than in non-gainers. ALT levels were significantly higher in gainers.

Table 3 Cardiometabolic risk markers comparing gainers and non-gainers at follow-up.

	Gainers	Non-gainers	P-value
Classical risk factors:			
SBP (mmHg)	150 ± 23	146 ± 31	NS
DBP (mmHg)	81 ± 10	80 ± 11	NS
Total cholesterol (mmol/l)	4.8 ± 0.9	3.8 ± 0.8 *	0.001
LDL-cholesterol (mmol/l)	2.9 ± 0.8	2.1 ± 0.4 *	0.006
HDL-cholesterol (mmol/l)	1.2 ± 0.2	1.0 ± 0.2 *	0.03
Triglycerides (mmol/l)	2.1 ± 1.4	1.7 ± 0.6	NS
Smoking (n)	2	2	NS
Creatinin (μmol/l)	85 ± 29	97 ± 38	NS
GFR (MDRD; ml/min/1.73m^2)	110 ± 28	85 ± 33	0.04
Albumin excretion ratio (AER; μmol/min)	113 ± 228	74 ± 143	NS
FFA (μmol/l)	0.6 ± 0.2	0.6 ± 0.3	NS
Fat hormones:			
Leptin (ng/ml)	43.3 ± 26.7	29.7 ± 21.3	NS
Adiponectin (μg/ml)	3.0 ± 1.4	2.1 ± 1.0	NS
Inflammatory markers:			
hsCRP (pmol/ml)	4.8 ± 3.3	3.7 ± 4.2	NS
IL-6 (pg/ml)	11.5 ± 18.3	5.9 ± 2.9	NS
IL-18 (pg/ml)	139 ± 39	133 ± 46	NS
Liverenzymes:			
ALT (U/l)	33.1 ± 11.4	23.8 ± 9.1 *	0.04

Data are means ± SD.
SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL: low-density lipoprotein, HDL: high-density lipoprotein, GRF: glomerular filtration rate (MDRD: modified diet in renal disease), FFA: free fatty acids, hsCRP: high-sensitive CRP, IL-6/18: interleukin-6 and 18, ALT: alanine aminotransferase.
All data were analysed by Student *t*-test, except for the data of AER, leptin, hsCRP, IL-6 and 18 which were analysed by nonparametric Mann-Whitney *U* test. * $P < 0.05$.

Discussion

The main finding of this cross-sectional pilot study is that patients who develop pronounced weight gain after long-term insulin therapy have more total, trunk and subcutaneous fat, perform less physical activity and show slightly higher cholesterol and ALT levels and GFR compared to those who do not gain weight. All together these findings suggest that this group of “gainers” may have an unfavorable cardio-metabolic risk profile compared to “non-gainers”.

So far, hardly any study has investigated the effects of long-term insulin therapy on body weight as a primary endpoint. Follow-up in most studies is limited to 6-12

months, with a reported increase in body weight of approximately 2-6 kg (1). Whether ongoing weight increase occurs in insulin-treated patients while stable glycemic control has been obtained is less clear. Recently, Aas et al. (19) reported a mean weight gain approaching 4 kg over three years in insulin-treated participants of the DIGAMI study. Approximately 30% of the weight gain took place beyond 1 year of therapy. Also Kooy et al. (20) found ongoing weight increase in a group of insulin-treated patients who were followed for over 4 years. The limited findings from literature match with general clinical experience and suggest that - at least in a subset of patients - weight continues to increase during insulin treatment without further improvement in glycemic control. The "gainers" selected in the present study may represent this group.

The two groups seemed to differ with respect to body weight before the start of insulin, with the gainers starting at a lower weight, although this difference was statistically not significant. This may suggest that gainers had lost more weight before the onset of insulin and thus simply regained more weight after starting insulin, as has been suggested before (21). As the initial HbA_{1c} level was also slightly higher in the gainers group (not statistically significant), a relative contribution from "initial regain" cannot be fully excluded. Indeed, gainers suffered a mean of 4.3 kg $\pm$ 6.4 kg weight loss within 12 months prior to the start of insulin therapy. However, the observed sustained increase in body weight during long-term insulin treatment cannot be attributable to exaggerated weight loss before therapy.

The adverse effect of a sustained increase in body weight during insulin treatment as found in the present study is supported by previous findings. Yki-Jävinen et al. (4) reported higher blood pressure and lipid levels in patients with an exaggerated weight increase. In a recent report, initiation of insulin treatment after myocardial infarction was associated with a significant increase in weight and incidence of re-infarction, although the latter was not clearly explained by the increased weight (19). In addition, elevated levels of adipokines were found in a group of patients treated with insulin who gained weight compared to a group treated by lifestyle intervention that lost weight, despite similar glycemic levels. All together these data, though limited, suggest that insulin-associated weight gain may indeed negatively affect cardiometabolic risk profile.

We found that the group of subjects with pronounced weight increase had higher total, trunk and subcutaneous fat. Long term effects of insulin-associated weight gain on body composition have not been reported, but in short-term studies, insulin treatment showed an increase in fat mass but also FFM, in line with the anabolic effect of insulin (22). The present study did not reveal a difference in lean body mass between the two groups. Although this does not exclude a beneficial effect of insulin treatment in itself on lean body mass, it does show that the exaggerated weight increase is explained by an increase in fat only.

Most studies suggest that visceral rather than subcutaneous fat is associated with insulin-resistance and may confer increased cardiometabolic risk (23-25). From this point of view, the currently reported increase in subcutaneous fat mass may not necessarily incur to a strongly elevated cardiometabolic risk, but may still contribute to an adverse cardiometabolic risk profile (26). It could be hypothesized that gainers exhibit higher levels of adipocytokines (e.g. leptin, IL-6) at the level of subcutaneous adipose tissue compared to non-gainers. Furthermore, it could be argued that dietary content (e.g. ceramide intake) might influence body weight and metabolic effects on adipose tissue (27). Unfortunately, we did not perform subcutaneous fatbiopsies in the two groups and took a standardised questionnaire survey for assessing patients dietary habits.

It is known that hepatic fat accumulation is associated with (hepatic) insulin-resistance in non-alcoholic fatty liver disease and is also a predictor of cardiometabolic disease (26). Juurinen et al. (28) showed that after 7 months of insulin therapy (basal insulin) patients had improvement of hepatic insulin sensitivity and reduction of hepatic fat content. LFAT content after longer periods of insulin treatment has not been studied. The present results found no statistically significant differences between the two groups with respect to LFAT content as measured by MRS. Both groups had substantial percentages of LFAT (~10%), which is in line with results reported in literature (29). The number of subjects in whom LFAT measurements were successful in the study were relatively low and thus the lack of a difference may represent a power problem, especially as the slightly higher ALT levels suggest that the group of gainers may have had slightly higher LFAT. Alternatively, higher ALT levels may confer an elevated cardiovascular risk, independent of LFAT (30, 31).

MDRD-GFR was higher in the gainers, which was associated with a tendency towards lower serum creatinine and an increased albumin excretion rate. Together these results suggest the existence of glomerular hyperfiltration, which in itself has been listed as a cardiovascular risk marker (32).

(Low-grade) inflammation and adipocytokines (i.e. leptin) are associated with obesity and cardiovascular disease (33-35). In line with this association, leptin, IL-6, IL-18 and hsCRP tended to be higher in gainers. Adiponectin levels are negatively associated with obesity and with cardiovascular end points. Adiponectin levels in both groups were similar, suggesting that insulin-associated weight gain does not necessarily translate in a (further) decrease in adiponectin levels.

Physical activity is a strong predictor of future cardiovascular disease and a determinant of body weight (36). Gainers had lower levels of total energy expenditure compared to non-gainers, and performed less vigorous exercise compared to non-gainers. Due to the cross-sectional design of our study, it cannot be determined whether the decreased level of physical exercise is the cause of the exaggerated insulin-associated weight gain or the consequence. However, no matter cause or consequence, a low physical exercise level remains a cardiovascular risk factor. It can

be speculated, for instance, that (pronounced) weight gain in insulin-treated patients and change in physical activity is associated with a decrease in mood or tendency towards depression. In this study we did not assess (changes in) mood or depression score. Further prospective work is warranted in order to investigate the relationship between insulin-associated weight gain and level of physical activity.

The study has a number of limitations. The cross-sectional comparison cannot determine whether the unfavorable cardiometabolic risk profile observed in the gainers is the direct consequence of insulin-associated weight gain. The study cannot determine whether part of the observed weight gain is due to the “natural” course of body weight associated with aging, this would require a control group of either matched non-diabetic subjects, or subjects with T2DM on oral medication. The study also has a number of strengths. There is a rather homogenous population, similarly treated patients from a single centre, all on biphasic insulin. We used a set of sophisticated techniques to quantify body fat distribution and physical activity.

The results of our study may have clinical implications. As it seems that pronounced weight gain during (long-term) insulin therapy is associated with a less favorable cardiometabolic risk profile, it may be important to determine what patients are most at risk for weight gain. This would require assessment of predictive factors and lifestyle characteristics before onset of insulin treatment, which, however, are largely unknown. Most authors view the change in glycaemic control (i.e. change in HbA1c) as the major determinant of insulin-associated weight gain (2, 3). Although part of the short-term insulin-associated weight gain may be explained by change in HbA1c in this study, this cannot explain sustained weight gain after long-term insulin therapy when stable or even increase in HbA_{1c} is observed. Further prospective studies are needed to improve identification of patients who are at risk for extensive body weight increase and develop interventions to prevent the weight gain.

In conclusion, the present study suggest that pronounced weight gain during (long-term) insulin therapy in patients with T2DM is associated with an unfavorable cardiometabolic risk profile. Further work is required to determine the individual risk factors for exaggerated weight increase, to assess long-term consequences and to develop potential interventions.

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Disclosure

The authors have no relevant conflicts of interest to disclose.

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Chapter 6

Start of insulin therapy in patients with type 2 diabetes mellitus promotes the influx of macrophages in subcutaneous adipose tissue

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Abstract

Insulin therapy in patients with type 2 diabetes mellitus is accompanied by weight gain characterized by an increase in abdominal fat mass. Expansion of adipose tissue mass is generally paralleled by profound morphological and inflammatory changes. We hypothesized that the insulin-associated increase in fat mass also results in changes in human subcutaneous adipose tissue morphology and increased inflammation, especially when weight gain is excessive.

We investigated the effects of weight gain on adipocyte size, macrophage influx and mRNA expression and protein levels of key inflammatory markers within the adipose tissue in patients with type 2 diabetes mellitus before and 6 months after the start of insulin therapy.

As expected insulin significantly increased body weight. At the level of subcutaneous adipose tissue, insulin treatment led to an influx of macrophages. When comparing patients gaining no or little weight with patients gaining > 4% body weight after 6 months of insulin therapy, both subgroups displayed an increase in macrophage influx. However, gainers had higher protein levels of monocyte chemo-attractant protein-1, tumor necrosis factor-alfa and interleukin-1 beta after 6 months of insulin therapy compared to non-gainers.

We conclude that insulin therapy in patients with type 2 diabetes mellitus improved glycemic control, yet also induces body weight gain and the influx of macrophages in subcutaneous adipose tissue. In patients characterized by pronounced insulin-associated weight gain, the influx of macrophages into adipose tissue is accompanied by a more pronounced inflammatory status.

Trial registration: clinicaltrials.gov Identifier: NCT00781495

Introduction

Insulin therapy is frequently needed to achieve adequate glycemic control in patients with type 2 diabetes mellitus (T2DM), yet often results in undesirable weight gain. Although values differ and studies are generally of limited duration, the estimated weight gain during the first year of insulin therapy ranges from approximately 2 to 6 kg (1). This weight gain is paralleled by an increase in abdominal fat mass (1).

When obesity develops, the adipose tissue undergoes distinct morphological changes including adipocyte enlargement and macrophage influx (2). In obese adipose tissue, macrophages can be arranged in so called crown like structures (CLS) that surround dysfunctional or dying adipocytes (3) and are characterized by a more pro-inflammatory trait as compared to individual macrophages dispersed throughout the adipose tissue (4).

In general, these changes lead to a more pronounced inflammatory status of the adipose tissue that reflected by an increased secretion of pro-inflammatory mediators by the adipose tissue and reduction in secretion of the insulin-sensitizing protein adiponectin (5). The enhanced inflammatory status of the adipose tissue is thought to contribute to the development of systemic insulin resistance that may eventually evolve into type 2 diabetes. Although inflammatory changes of the adipose tissue are well described in individuals that develop obesity, it remains unknown whether similar changes in morphological and inflammatory characteristics of subcutaneous adipose tissue also occur in type 2 diabetic patients who start insulin therapy, and subsequently gain weight.

An emerging body of evidence suggests that insulin suppresses the inflammatory process, not only through preventing hyperglycemia but also by modulating key inflammatory molecules. Studies that have examined the effects of (intensive) insulin therapy on immune function and inflammatory factors have shown that systemic pro-inflammatory cytokines decrease after administration of insulin (6, 7).

One may hypothesize that the anti-inflammatory effects on insulin at the systemic level may be counteracted by the pro-inflammatory changes associated with an increased fat mass. This may be particularly true in those who gain most weight. To test this hypothesis, we investigated changes in adipose tissue morphological characteristics, macrophage influx, mRNA expression and protein levels of key inflammatory markers within the adipose tissue, in patients with type 2 diabetes mellitus before and 6 months after the start of insulin therapy. We also assessed whether potential changes were related to excessive weight gain.

Patients and Methods

Patients

This study was part of a large prospective, multicenter, observational study aimed at identifying determinants of insulin-induced weight gain in type 2 diabetes (abstract EASD no. 942; 2012, Berlin; clinicaltrials.gov NCT00781495, $n = 65$ patients). In short, patients with type 2 diabetes who failed on oral on oral glucose-lowering agents and/ or diet and were started on insulin therapy were recruited. All patients were treated according to local guidelines. The majority of the patients used an oral glucose lowering agent throughout the study (metformin and sulfonylurea derivatives). To determine the impact of high weight gain on the inflammatory status of the adipose tissue we divided the study population in two subgroups of patients: "gainers" (patients displaying $> 4\%$ weight gain after 6 months of insulin therapy; $n = 10$) and "non-gainers" (patients with weight gain $\leq 4\%$; $n = 33$) (1, 8). Furthermore, we included a matched non-diabetic obese subgroup (BMI 27-43 kg/m^2 and subjects between 40 and 70 years old; $n = 42$) in order to compare adipose tissue morphological characteristics, macrophage influx and protein levels of key inflammatory markers. Exclusion criteria were earlier use of insulin, other types of diabetes, including steroid-induced diabetes, evidence of psychiatric, renal, cardio-vascular, liver or other diseases and use of medication (prednisone) that may influence study results.

Before and after 6 months of insulin treatment, BMI, waist circumference, and skinfold thickness are measured. Body composition was assessed by using formulas described by Jackson and Pollock (9, 10). At baseline (i.e. prior to insulin treatment) and after 6 months of insulin therapy subcutaneous (sc) adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6–10 cm lateral to the umbilicus, after an overnight fast. The same measurements were performed in the non-diabetic obese subjects.

The study protocol was approved by the local ethical committee. All study participants provided written informed consent.

Biochemical analyses

Plasma glucose concentration and HbA_{1C} were measured by standard laboratory methods. Furthermore, we measured plasma adiponectin, leptin, interleukin 8 (IL-8), high sensitive C-reactive protein (hsCRP) (R&D systems, Minneapolis, USA).

Subcutaneous adipose tissue morphology and immunohistochemistry

Morphometry of individual fat cells was assessed using digital image analyses as described previously (11). For each subject, the adipocyte cell diameter of all fat cells in five to ten microscopic fields of view were counted and measured. On average,

~700 fat cells were measured per specimen (range 150–1500). Adipocyte cell size distribution was expressed as ratio small (≤ 50 μm) : large adipocytes (≥ 100 μm) (12). For detection of macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Clone EBM11, Dako, Denmark A/S). The percentage of macrophages was expressed as the total number of CD68-positive cells divided by the total number of adipocytes counted in 10 random microscopic fields of view x 100. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (13).

RNA isolation and qPCR analysis

Total RNA was extracted from sc adipose tissue as described previously (14). cDNA synthesis was performed using iScript (Bio-Rad Laboratories, Hercules, USA). Expression of genes was determined by real-time PCR analysis using an Applied Biosystem, (Warrington, UK). Gene expression results were normalized to β2M levels. When comparing gainers and non-gainers, results from a maximum of 10 individuals are presented for gainers and from 33 individuals part of the non-gainers group. Genes and primer sequences used in this study are listed in supplementary table 1.

Luminex analysis

Protein levels within the adipose tissue biopsies were measured by Luminex fluorescent bead human cytokine immunoassays using manufacturer's instructions (MILLIPLEX MAP, Millipore Corp., Billerica, USA). Briefly, adipose tissue lysates were prepared using the milliplex map lysis buffer (Millipore) and protein concentrations of Interleukin-1 beta (IL-1β), Interleukin-6 and 8 (IL-6/8), tumor-necrosis factor alfa (TNF-α), leptin, adiponectin, monocyte chemoattractant protein (MCP)-1, resistin and plasminogen activator inhibitor-1 (PAI-1) were determined. To measure the protein levels in adipose tissue using luminex, 30% lysates of each sample were made (300 mg of adipose tissue in 1000 μl of lysis buffer). Protein concentrations of the various samples were measured using a bicinchoninic assay. Equal amounts of protein (28.2 μg) were analyzed using a Bioplex system (Bio-Rad, Hercules, USA). When comparing gainers and non-gainers (1, 8), results from a maximum of 10 individuals are presented for gainers and from 33 individuals part of the non-gainers group. A maximum of 37 non-diabetic obese subjects are presented. Missing values were due to technical issues or measurements below the detection level of the luminex assay.

Statistical analyses

Descriptive patient characteristics and other variables are displayed as means ± SD, unless otherwise indicated. Differences after insulin treatment were studied using Students' paired *t*-test, Wilcoxon signed-rank test and Mann-Whitney U test as appropriate. For comparing dichotomous variables the Chi-square test was used. All

calculations were performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, USA). Two-tailed p-values less than 0.05 were considered significant in all statistical comparisons.

Results

Start of insulin therapy

A total of 43 patients with T2DM who started insulin therapy underwent paired adipose tissue biopsies. Baseline anthropometric and laboratory characteristics of the study group are shown in table 1.

After 6 months, 25 patients (58%) used basal insulin only, 4 patients (9%) biphasic insulin and 14 patients (33%) basal and bolus insulin. At 6 months, patients used 0.37 ± 0.24 U insulin/kg body weight. Fifty-eight percent of the patients injected insulin in the thigh area, whereas 42 % of the patients injected insulin into the abdomen. Of those patients injecting insulin in the thigh area at initiation of insulin therapy (n = 26 (61 %)), 13 patients (30 %) still injected insulin only in the thigh area at 6 months. Insulin treatment increased body weight from 88.1 ± 18.1 to 89.3 ± 18.0 kg [range -4.5 to 7.4 kg] ($P < 0.05$). In addition, BMI, waist circumference and calculated body fat mass increased significantly. HbA_{1c} dropped from 8.7 ± 1.1 to 7.4 ± 1.0 % ($P < 0.05$).

Subcutaneous adipose tissue samples

Figure 1a-b shows histological overview of adipose tissue sections before and after start of insulin therapy. Insulin therapy had no effect on adipocyte cell diameter (69.5 ± 7.7 μm to 70.7 ± 6.9 μm, baseline vs. 6 months respectively, $P = 0.43$). Similarly, insulin did not affect the size distribution of adipocytes (Figure 1c), nor the percentage of small adipocytes (baseline 18.3 ± 10.1 vs. 17.7 ± 10.6 % after insulin, $P = 0.82$) or large adipocytes (9.7 ± 8.3 vs. 9.8 ± 8.1 %, $P = 0.95$).

The adipocyte cell size was similar compared to the non-diabetic obese subjects (Supplemental table 2).

Macrophage influx

The influx of CD68-positive cells/adipocyte was higher in patients with type 2 diabetes mellitus as compared to the non-diabetic subjects (Supplemental Figure 1).

Strikingly, insulin therapy even further increased macrophage influx, as determined by the number of CD68-positive cells/adipocyte (0.07 ± 0.06 versus 0.16 ± 0.09; $P < 0.001$, Figure 2a-f and Supplemental Figure 1).

Table 1 Demographic and clinical characteristics of patients with T2DM.

	Baseline	6 months
N	43	43
Age (yr)	61 ± 9	-
Gender (m/f)	25/18	-
Duration diabetes (yr)	10 ± 9	-
Weight loss prior start insulin therapy (kg)†	1.4 ± 2.7	-
BMI (kg/m^2)	29.8 ± 5.2	30.3 ± 5.1*
Waist circumference (cm)	106 ± 14	107 ± 13#
Lean weight (kg)	70 ± 14	68 ± 9*
Fat weight (kg)	19 ± 9	21 ± 9*
Body fat (%)	20 ± 7	23 ± 7
SBP (mmHg)	143 ± 20	138 ± 18
DBP (mmHg)	79 ± 8	79 ± 8
Fasting glucose (mmol/l)	11.6 ± 3.0	-
HbA_{1c} (%)/(mmol/mol)	8.7 ± 1.1/(72)	7.4 ± 1.0(57)*
Alcohol use (n)	13	-
Smoking (n)	3	-
Concomitant use of oral glucose lowering agents (n)		
- MET only	6	18*
- SU only	3	3
- MET + SU	34	22*

Data presented as mean ± SD. † weight loss 12 months prior to start of insulin. BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, HbA_{1c} = glycosylated haemoglobin, MET = metformin, SU = sulphonylurea derivatives. - = not applicable.
* $P < 0.05$, # $P = 0.06$

Males and females displayed a similar increase in influx of CD68-positive cells/adipocyte (males: 0.1 ± 0.1 versus females: 0.1 ± 0.1; $P = 0.38$). The increase in CD68-positive cells before and after insulin treatment was not correlated with the degree of weight gain in our population suggesting that insulin therapy, independently of weight gain, increases the number of macrophages within the adipose tissue. Noticeably, the number of CLS present in subcutaneous adipose tissue was not significantly changed before and after 6 months of insulin therapy (30 vs. 37 %; $P = 0.65$). The degree of macrophage influx was independent of the insulin injection site.

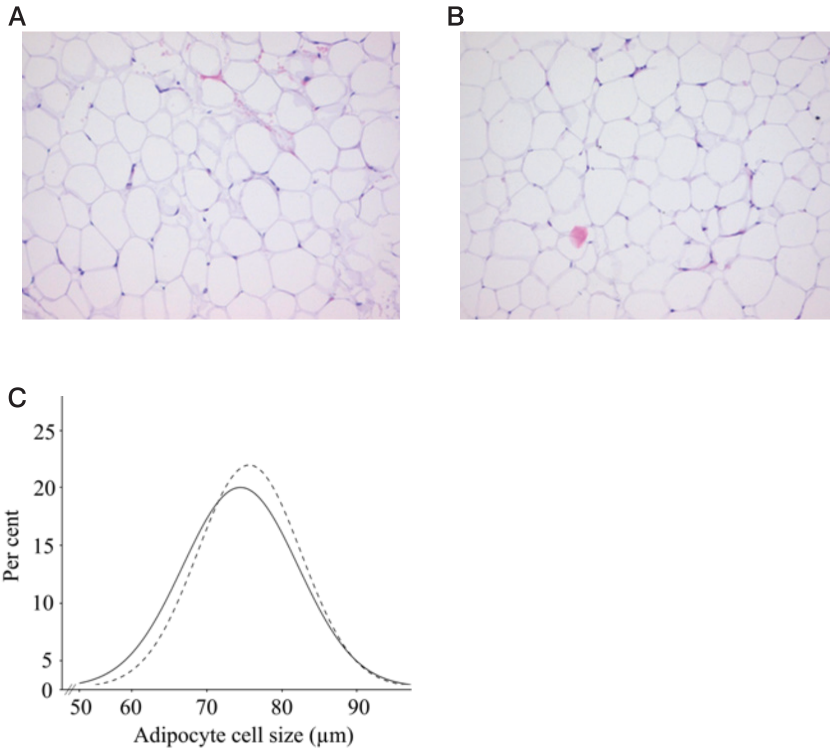

Figure 1 Histological overview of subcutaneous adipose tissue before (**A**) and after 6 months of insulin therapy (**B**) of a patient with type 2 diabetes mellitus. This patient gained 3.7 kg after 6 months of insulin treatment, without a change in average adipocyte cell size (adipocyte diameter 56.6 μm). Sections were stained with hematoxylin-eosin. Magnification: 10x. (**C**) The graph shows the frequency (percentage) distribution of absolute adipocyte cell size (μm) at baseline (solid line) and after 6 months of insulin treatment (dashed line). Bin width 2 μm. n=43 individuals for both time points.

Gene expression values in adipose tissue

The degree of influx of macrophages into the adipose tissue as measured by immunohistochemical staining is mirrored by a similar trend in mRNA expression of macrophage-specific markers *F4/80* and *CD38* (Supplemental Figure 2).

Since an enhancement in macrophage influx into adipose tissue is known to increase the inflammatory state of the adipose tissue, we set out to measure the changes in mRNA levels of key adipocytokines within adipose tissue biopsies. After 6 months of insulin treatment, glucose transporter type 4 (*SLC2A4*) and interleukin-6

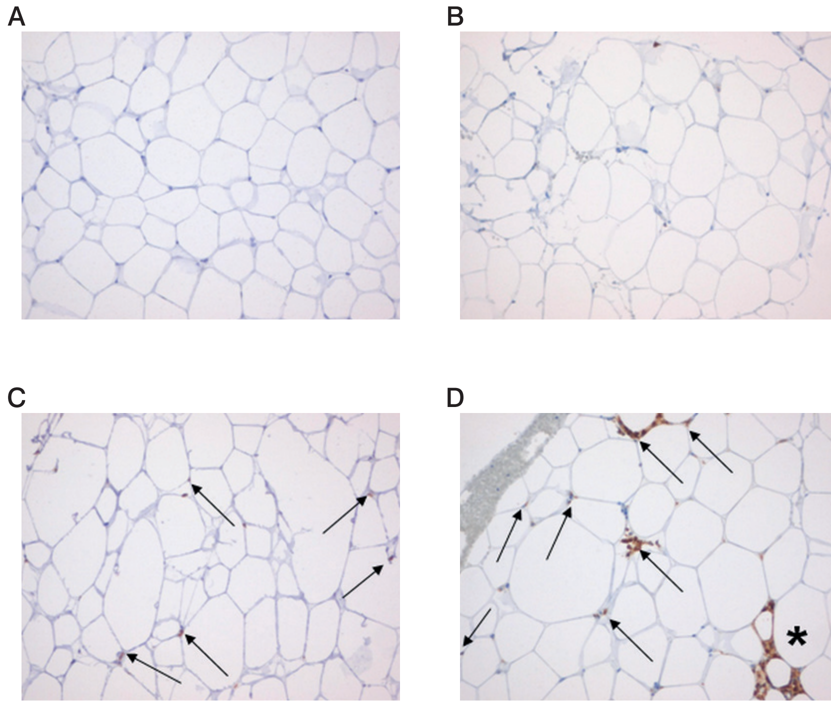

Figure 2 Representative sections of subcutaneous adipose tissue morphology and CD68-immunohistochemical staining before and after 6 months of insulin therapy. No influx of macrophages before insulin therapy (**A**) and after 6 months of insulin therapy (**B**) was seen in a patient with 3.7 kg of weight gain. A moderate influx of macrophages (arrows) before insulin treatment (**C**) and a significant increase in macrophage influx as well as a CLS (star) after 6 months of insulin therapy (**D**) were seen in a patient with 4.5 kg of weight gain. All sections were counterstained with haematoxylin (blue). Magnification ×10. (**E**) Quantification of macrophage influx showing a significant increase in the number of CD68-positive cells after 6 months of insulin therapy. 0, baseline, 6, 6 months of insulin therapy. The horizontal lines represent the means. n=43 individuals for both time points. *$p<0.001$. (**F**) Frequency (percentage) distribution of the influx of macrophages (CD68-positive cells per adipocyte) at baseline (solid line) and after 6 months of insulin treatment (dashed line). Bin width 2 μm. n=43 individuals for both time points.

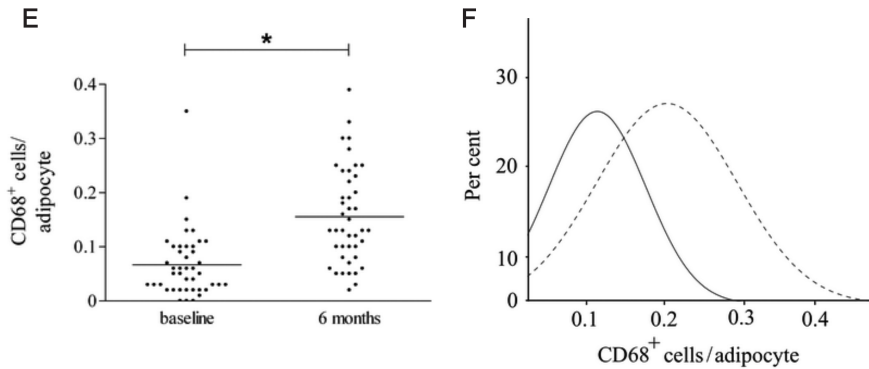

Figure 1 Continued.

(*IL-6*) mRNA expression levels were significantly increased whereas the gene expression levels of peroxisome proliferator receptor gamma (*PPAR-γ*), adiponectin, monocyte chemoattractant protein (*CCL-2*) and interleukin-8 (*IL-8*) were not changed significantly (Figure 3a). The influx of macrophages into the adipose tissue may also aid remodelling processes that may occur to accommodate the expansion of adipose tissue mass upon insulin-associated weight gain. Hence, we measured several expression levels of genes related to adipose tissue remodelling. As shown in Supplemental Figure 3, none of the presented gene expressions changed significantly after 6 months of insulin therapy.

Protein levels in adipose tissue

To more extensively characterize the inflammatory status of the adipose tissue before and after 6 months of insulin therapy, protein levels of various inflammatory adipokines including interleukin 1β, IL-6 and 8, active PAI-1, TNF-α and MCP-1 were determinant in adipose tissue. Patients with type 2 diabetes have higher protein levels of IL-8, MCP-1, PAI-1 and resistin within the subcutaneous adipose tissue as compared to non-diabetic obese individuals (Supplemental Figure 4). Noticebly, adipocyte size was similar between both study populations whereas the influx of macrophages was enhanced in patients with type 2 diabetes.

As shown in Figure 3B, there was no significant change in adipose tissue levels of the various pro-inflammatory mediators in adipose tissue after 6 months of insulin. Additionally, protein levels of leptin, and resistin were similar before and after 6 months of insulin treatment.

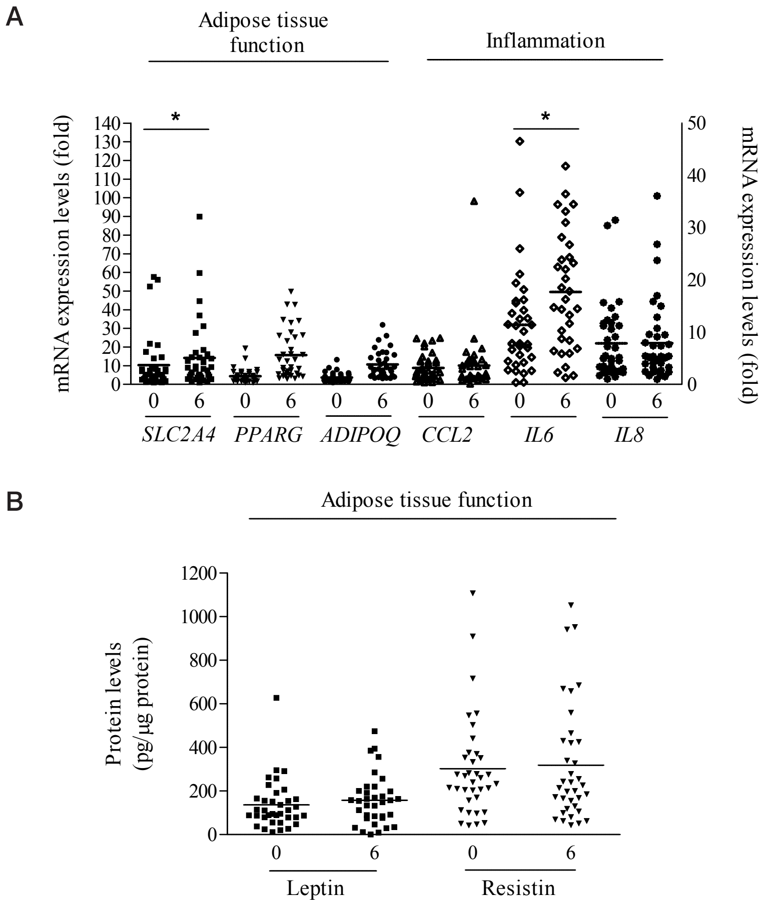

Figure 3 (**A**) Changes in the relative gene expression levels of adipose tissue function (SLC2A4, PPARG, ADIPOQ) and pro-inflammatory related (CCL2, IL6 and IL8) genes. Left axis: IL6, SLC2A4; right axis: PPARG, ADIPOQ, CCL2, IL8. The lowest gene expression level in quantitative (q)PCR analysis at baseline was set at 1. The mean fold changes of SLC2A4, PPARG, ADIPOQ, CCL2, IL6 and IL8 were 1.37, 1.27, 1.03, 1.14, 1.55 and 1.00, respectively. 0, at baseline; 6, after 6 months of insulin treatment. The horizontal lines represent the means. n=37 individuals per gene per time point, except for IL6 (n=35 individuals for both time points). *$p<0.05$. (**B**) Protein levels measured for the adipose tissue displaying adipose tissue function (leptin, resistin) and (**C**) pro-inflammatory related protein levels (left axis: MCP-1, IL-1β, IL-6, IL-8, TNF-α; right axis: PAI-1). 0, at baseline; 6, after 6 months of insulin treatment. The horizontal lines represent the means. The mean fold changes of leptin, resistin, MCP-1, IL-1β, IL-6, IL-8, TNF-α and PAI-1 were 1.16, 1.05, 0.93, 0.82, 1.18, 0.99, 1.08 and 1.09, respectively. n=36 individuals per protein per time point.

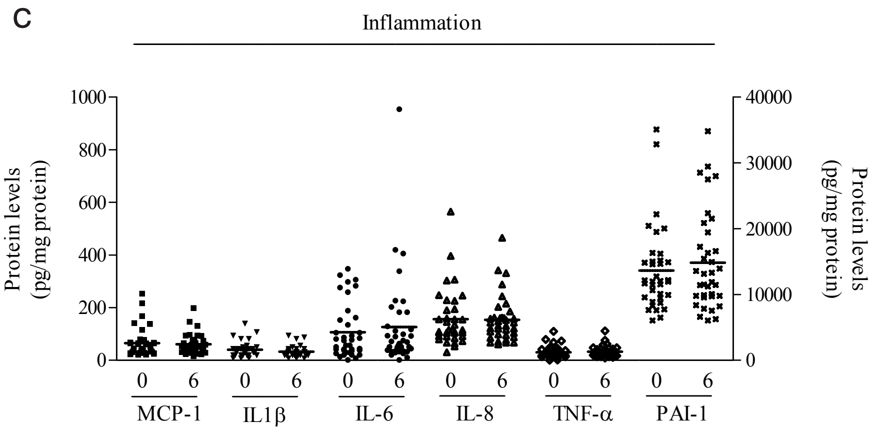

Figure 3 Continued.

Circulating hormones and inflammatory markers

Whether insulin and weight gain may have an impact on circulating plasma markers is unknown. Therefore, we collected plasma at baseline and after 12 months of insulin treatment (as part of the original study). Circulating leptin levels significantly increased after insulin therapy. The other circulating markers did not change significantly (Supplemental Figure 5A).

Gainers vs. non-gainers

It is a well-known fact that weight gain favours enlargement of the adipocytes and a shift towards a more pro-inflammatory status of the adipose tissue (5). Although 6 months of insulin therapy significantly enhanced body weight within our study population, a pronounced inter-individual variation was observed. Therefore, we divided the group in two subgroups of patients: “gainers” (patients displaying $> 4\%$ weight gain after 6 months of insulin therapy; $n = 10$) and “non-gainers” (patients with weight gain $\leq 4\%$; $n = 33$) to determine the effects of high weight gain to no or limited weight gain on the inflammatory status of the adipose tissue. Baseline characteristics comparing non-gainers and gainers are presented in table 2. At baseline body weight in gainers was significantly less compared to non-gainers.

Mean weight gain in gainers was 4.7 $\pm$ 1.5 kg compared to 0.09 $\pm$ 2.3 kg ($P < 0.001$) in non-gainers, and insulin dose at 6 months was 45 IU (0.57 $\pm$ 0.37 U insulin/kg body weight) in gainers and 29 IU (0.31 $\pm$ 0.15 U insulin/kg body weight) (both variables $P < 0.05$) in non-gainers. The change in glycaemic control was -1.79 vs.

Table 2 Baseline demographic and clinical characteristics comparing non-gainers and gainers.

	Non-gainers	Gainers
N	33	10
Age (yr)	61 ±10	64 ± 12
Duration diabetes (yr)	9 ± 6	14 ± 14
Weight loss prior start insulin therapy (kg) †	1.1 ± 2.2	2.2 ± 4.0
BMI (kg/m^2)	30.9 ± 5.4	26.3 ± 2.4*
Waist circumference (cm)	109 ± 13	99 ± 12*
Lean weight (kg)	72 ± 14	64 ± 10*
Fat weight (kg)	20 ± 9	13 ± 5*
Body fat (%)	22 ± 7	17 ± 5*
SBP (mmHg)	115 ± 21	138 ± 15
DBP (mmHg)	80 ± 11	78 ± 5
Fasting glucose (mmol/l)	11.3 ± 2.7	12.5 ± 3.9
HbA_{1c} (%)/(mmol/mol)	8.6 ± 0.9/70	9.0 ± 1.4/75
Alcohol use (n)	6	7
Smoking (n)	4	2
Concomitant use of oral glucose lowering agents (n)		
- MET	29	8
- SU	26	7
- MET + SU	25	5

Data presented as mean ± SD. † weight loss 12 months prior to start of insulin. BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, HbA_{1c} = glycosylated haemoglobin, MET = metformin, SU = sulphonylurea derivatives.
* $P < 0.05$.

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-1.04 % in gainers and non-gainers, respectively ($P = 0.11$). There was no significant increase in adipocyte cell size in both groups (65.8 ± 7.0 to 67.5 ± 6.9 μm vs. 70.6 ± 7.6 to 71.6 ± 6.7 μm, in gainers vs. non-gainers, respectively (both $P = 0.50$ for the difference in adipocyte cell size within the subgroup).

Interestingly, the number of macrophages quantified by the number of CD68-positive cells into the adipose tissue was raised significantly in both groups after 6 months of insulin therapy (Figure 4A), indicating that the influx of macrophages was independent of weight gain. No changes in gene expression levels were observed when comparing gainers and non-gainers (Figure 4B). However, protein

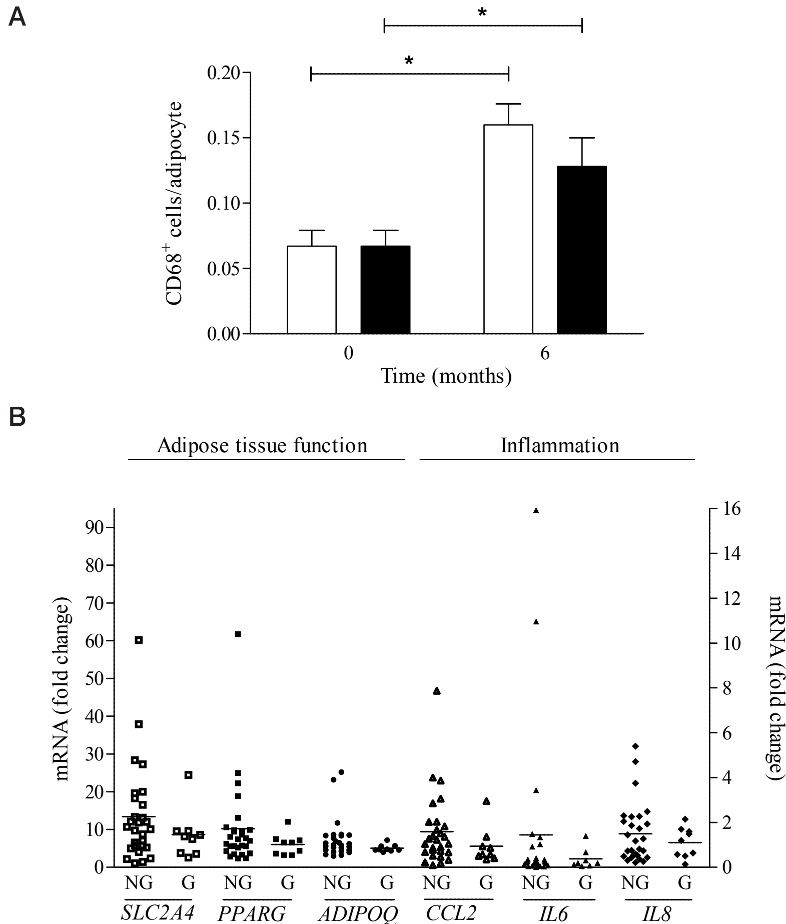

Figure 4 (**A**) Change in influx of CD68-positive cells into the subcutaneous adipose tissue after 6 months of insulin therapy comparing nongainers (white bars; ≤4% weight gain) and gainers (black bars; >4% weight gain). Data are presented as mean ± SEM. n=33 non-gainers vs n=10 gainers for both time points. *p<0.05. (**B**) Changes in the relative gene expression levels of adipose tissue function (SLC2A4, PPARG and ADIPOQ) and inflammation (CCL2, IL6 and IL8) showing no significant change after 6 months of insulin therapy in patients displaying no or minimal weight gain (≤4%; NG) compared with a pronounced weight gain (>4%; G). Left axis: IL6; right axis, SLC2A4, PPARG, ADIPOQ, CCL2 and IL8. The lowest gene expression level in qPCR analysis at baseline was set at 1. The horizontal lines represent the means. n=28 values per gene/fold change for non-gainers and n=9 for gainers, except for IL6 (n=27 individuals for gene expression levels in non-gainers and n=8 individuals for gainers).

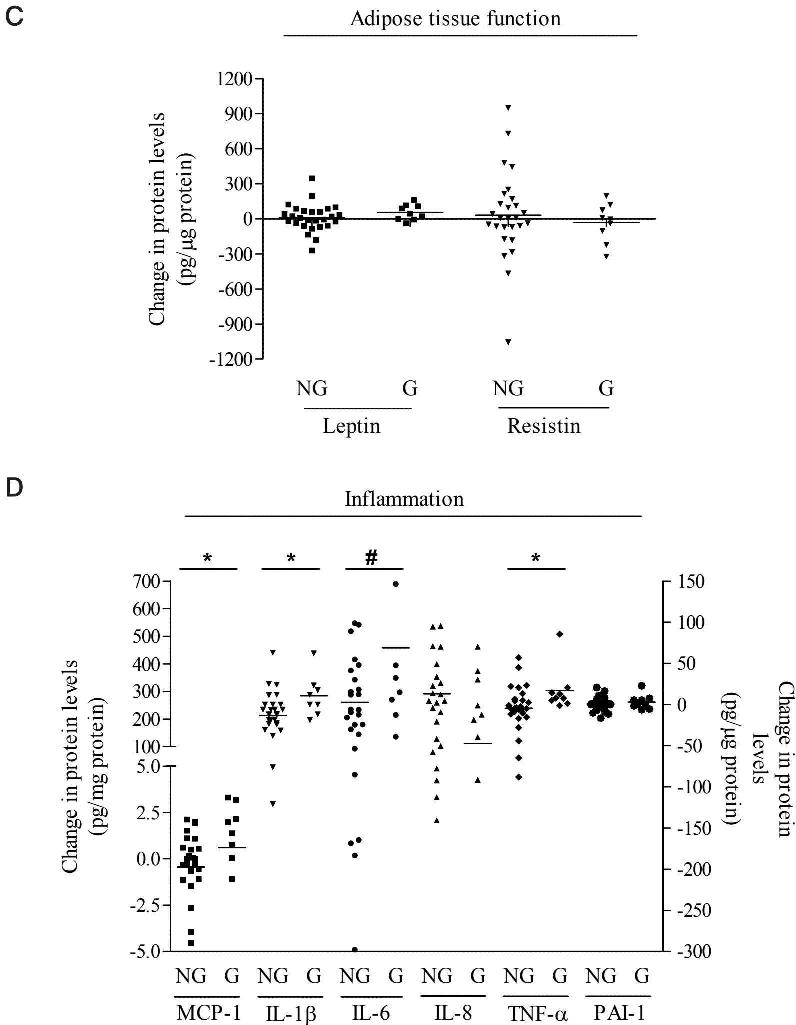

Figure 4 (**C**) Change in adipose tissue function (leptin and resistin) and (**D**) pro-inflammatory-related protein levels (left axis: MCP-1, IL-1β, IL-6, IL-8, TNF-α and PAI-1) after 6 months of insulin therapy comparing patients displaying no or minimal weight gain (≤4%; NG) with patients showing a pronounced weight gain (>4%; G). Left axis, MCP-1; right axis, IL-1β, IL-6, IL-8, TNF-α, PAI-1. The horizontal lines represent the means. *n*=27 individuals for protein levels measured in non-gainers versus *n*=9 individuals for proteins measured in gainers, except for IL-1β (*n*=8 for gainers). *p<0.05, #p=0.09

levels within adipose tissue displayed significant differences between gainers and non-gainers. Protein levels of MCP-1, TNF-α and IL-1β significantly increased in the gainers compared to the non-gainers (Figure 4C), suggesting that pronounced insulin-associated weight gain may promote a more pro-inflammatory status of the adipose tissue that is independent of the absolute number of macrophages. When comparing circulating hormones and inflammatory markers in non-gainers versus gainers, we observed in gainers a significantly larger decrease in adiponectin levels and a significant larger increase in leptin levels (Supplemental Figure 5B).

Discussion

In the present study we demonstrate that 6 months of insulin therapy per se leads to an increase of influx of macrophages into the subcutaneous adipose tissue in patients with type 2 diabetes mellitus, an effect independent of the level of weight gain. However, the subgroup that gained a more pronounced (> 4%) amount of body weight, showed an increased inflammatory status of the adipose tissue as reflected by an increase in levels of pro-inflammatory cytokines (MCP-1, TNF-α and IL-1β) in the adipose tissue.

Mean insulin-associated weight gain in this study equaled 1.2 kg after 6 months, with a considerable inter-individual variation. Glycemic control significantly improved, HbA_{1c} levels at 6 months of insulin therapy were suboptimal. These changes in body weight and glycemic control reflect real-life clinical practice outcomes.

Insulin-associated weight gain leads towards an increase in fat mass. Although the development of obesity and weight gain induce enlargement of sc adipocyte cells size (2), no significant increase in adipocyte cell size after 6 months of insulin treatment was observed within our study population. This may be explained by the relatively moderate weight gain as observed after 6 months of insulin therapy, probably too small to affect adipocyte cell size. It may also be envisioned that insulin induces the differentiation of novel adipocytes (15) and thereby increases the storage capacity without any changes in adipocyte size.

Changes of the inflammatory trait of the adipose tissue including the influx of macrophages and altered levels of adipocytokines rather than changes in adipocyte morphology are more important consequences of insulin-associated weight gain. Indeed, 6 months of insulin therapy led to a significant increase of macrophages in the sc adipose tissue. The degree of macrophage influx, was however independent of the level of weight gain, and the enhanced number of macrophages present in adipose tissue was not accompanied by an upsurge in inflammatory status as levels of various inflammatory adipokines within adipose tissue were unaltered. These results suggest that of the influx of macrophages upon insulin therapy serves a

different purpose. A potential explanation for this phenomenon may involve the tissue remodeling function of macrophages (16, 17) Macrophages are not only capable of mediating inflammatory responses, yet also aid remodelling processes that may occur to accommodate the expansion of adipose tissue mass upon insulin-induced weight gain. However, expression levels of genes related to adipose tissue remodelling including PAI-1, TIMP-1, MMP-2, MMP-3, MMP-11 (18-20) were not changed significantly. Although weight gain might led to up-regulation of genes related to angiogenesis and extracellular matrix (ECM) remodeling (21), our results suggest that insulin-induced weight gain exerts different effects on ECM remodeling. Interestingly, lipolysis has been shown to promote the influx of macrophages into adipose tissue that function to prevent excessive spillover of free fatty acids into the circulation (22). As insulin inhibits lipolysis, the influx of macrophage upon the start of insulin therapy appears to be somewhat counterintuitive. However, one might speculate that the enhanced influx of macrophages is aimed at limiting the spillover of free fatty acids in obese subjects and thereby supports the lipolysis-inhibiting effects of insulin.

Finally, the influx of macrophages in the adipose tissue may be a direct result of injecting insulin in the abdomen. However, we did not observe a difference in influx of macrophages in the adipose tissue between patients injecting insulin into the thigh area as compared to those who injected insulin in the abdomen (data not shown) rendering any direct effects of insulin or its injection site on the influx of macrophages unlikely.

Although no effects on inflammation were observed in our total study population, classification of our patients based on the degree of weight gain did reveal interesting differences. Whereas both non-gainers and gainers demonstrated a significant increase in adipose tissue macrophage influx, the gainers displayed an increase in pro-inflammatory cytokine production (MCP-1, IL-1β, and TNF-α) after 6 months of insulin treatment as compared to non-gainers. These results imply that in gainers the influx of macrophages is paralleled by an enhancement of inflammatory status of the adipose tissue. Since the total number of macrophages present after 6 months of treatment were not different between both subgroups, it can be suggested that either the phenotype of the macrophage is more pro-inflammatory, adipocytes themselves are more inflamed or other immune cells have infiltrated the adipose tissue in subjects that gained over 4% of body weight. Although various reports have suggested that insulin suppresses inflammatory processes (at least systemically) (6, 7), more excessive weight gain after the start of insulin therapy may overrule any anti-inflammatory actions of insulin.

Even in the absence of weight gain, insulin will have direct effect on adipocytes as well as indirect effects through e.g. altered levels of circulating nutrients and hormones. Interestingly, only serum leptin increased significantly after insulin

treatment. It can be envisioned that effects observed after start of insulin therapy are primarily a result of direct effects of insulin and not indirect consequence of changes in circulating parameters. Remarkably, the change in adipose tissue macrophage numbers upon insulin therapy, is not translated into a systemic enhancement in inflammatory status.

Our study has several limitations. First, the analyses in our study were done using the subcutaneous fat tissue compartment, and do not necessarily implicate similar changes in other fat compartments. However, most studies, including our own, have found that inflammatory changes in the visceral adipose tissue are mirrored by similar changes in sc adipose tissue (23). Additionally, intake and energy expenditure of the patients studied was partly unknown and may have affected the degree of overweight and adipose tissue inflammation (24, 25).

The strength of our study includes the extensive phenotyping of over 40 subjects and careful pairwise analysis of subcutaneous adipose tissue morphology and inflammatory changes. Additionally, determining protein levels in adipose tissue has, to our knowledge, not been applied before and yields more valuable information concerning adipose tissue function as compared to measurements of mRNA expression levels and circulating protein concentrations.

In conclusion, our results demonstrate that 6 months of insulin treatment leads to a significant influx of macrophages in patients with type 2 diabetes. This effect is independent of weight gain. However, pronounced insulin-associated weight gain (> 4 %), is paralleled by pro-inflammatory changes in sc adipose tissue. Furthermore, gainers showed higher increase of insulin dose compared to non-gainers suggesting that concomitant body weight gain and shift towards a more pro-inflammatory profile of the adipose tissue might be associated with insulin resistance. The systemic anti-inflammatory effects of insulin (therapy) might be, at least partially, offset by the pro-inflammatory changes associated with an increased fat mass.

Author Contributions

H.J.J. researched data. H.J.J. and R.S. wrote manuscript, researched data. G.M.M.V. and C.J.J.T. reviewed/edited manuscript. J.v.D., J.v.d.L. and A.H. contributed to discussion, reviewed/edited manuscript.

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Disclosure

The authors have no relevant conflicts of interest to disclose.

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Supplemental data

Table 1 Primer sequences.

Human	
	Forward primer
	Reverse primer
Gene	**RT-PCR (real-time PCR)**
B2M	5’-ATGAGTATGCCTGCCGTGTG-3’
	5’-CCAAATGCGGCATCTTCAAAC-3’
GLUT4	5’-CTCAGCAGCGAGTGACTGG-3’
	5’-CCCCAATGTTGTACCCAAACTG-3’
Ppar-γ	5’-ATTGACCCAGAAAGCGATTCC-3’
	5’-TCTTCCATTACGGAGAGATCCAC-3’
Adiponectin	5’-ATCGGTGAAACCGGAGTACC-3’
	5’-GCATGTTGGGGATAGTAACGTAA -3
MCP-1	5’-CCAGTCACCTGCTGTTATAAC-3’
	5’-TGGAATCCTGAACCCACTTCT-3’
IL-6	5’-AACCTGAACCTTCCAAAGATGG-3’
	5’-TCTGGCTTGTTCCTCACTACT-3’
IL-8	5’-ACTGAGAGTGATTGAGAGTGGAC-3’
	5’-AACCCTCTGCACCCAGTTTTC-3’

B2M = beta-2-microglobulin
GLUT4 = glucose transporter type 4
Ppar-γ = peroxisome proliferator receptor gamma
MCP-1 = monocyte chemoattractant protein-1
IL-6/8 = interleukin-6/8

Table 2 Demographic and clinical characteristics of non-diabetic obese subjects.

N	42
Age (yr)	55 ± 9
Gender (m/f)	24/18
BMI (kg/m^2)	31.4 ± 3.5
Waist circumference (cm)	107 ± 10
Lean weight (kg)	68 ± 11
Fat weight (kg)	22 ± 7
Body fat (%)	24 ± 6
SBP (mmHg)	143 ± 18
DBP (mmHg)	87 ± 9
Fasting glucose (mmol/l)	5.2 ± 0.6
Adipocyte cell size (μm)	67 ± 10
CD68-positive cells/adipocyte	0.05 ± 0.06

Data presented as mean ± SD. BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

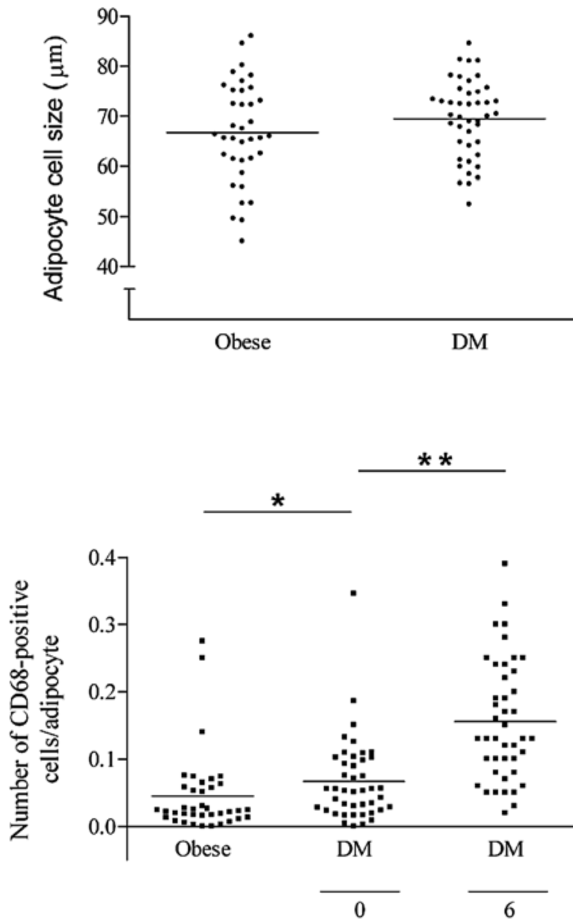

Figure 1 Comparing adipocyte cell size and number of CD68-positive cells/adipocyte in an non-diabetic obese group and study group of patients with type 2 diabetes mellitus before (0) and after 6 (6) months of insuline therapy. $n = 37$ obese subjects and $n = 43$ patients with diabetes mellitus (DM). Horizontal lines represent mean. * $P = 0.06$. ** $P < 0.05$.

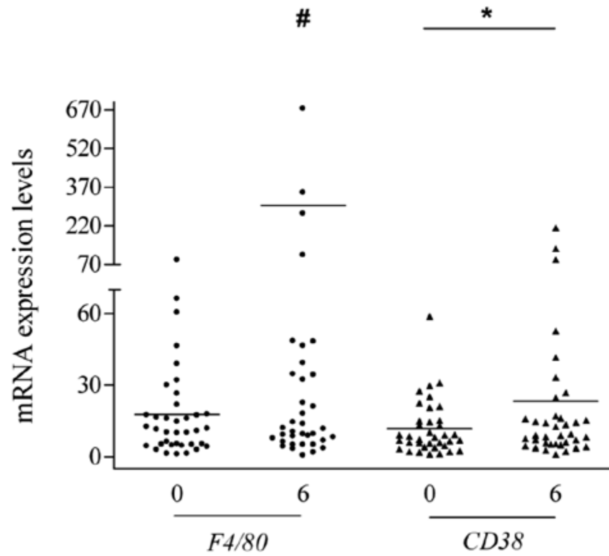

Figure 2 Change in macrophage specific markers *F4/80*, and *CD38* mRNA expression levels before (0) and after 6 months (6) of insulin therapy. The lowest gene expression level in qPCR analysis at baseline was set at 1. Mean fold change of F4/80 is 28.4 and for *CD38* mean fold change equaled 8.5. Horizontal lines represent mean. # denotes outlier. $n = 37$ individuals per time point for F4/80 gene, $n = 37$ individuals per time point for *CD38* gene. * $P = 0.05$.

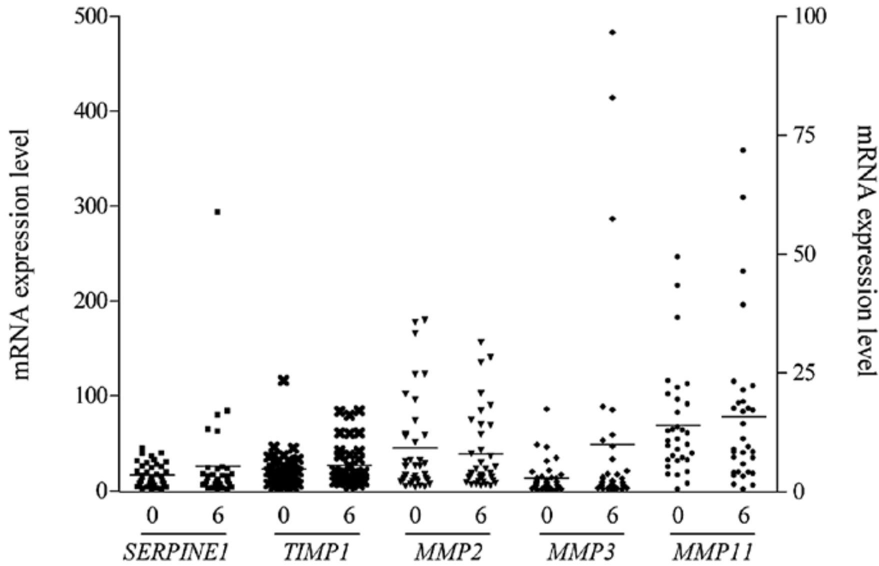

Figure 3 Change in relative expression levels of genes related to adipose tissue remodeling. SERPINE1 = plasminogen activator inhibitor-1 (left y-axis), TIMP1 = tissue metallopeptidase inhibitor 1 (right y-axis), MMP2/3/11 = matrix metalloproteinases-2, 3 and 11 (left y-axis: MMP3/11; right y-axis: MMP2). 0 = baseline, 6 = after 6 months of insulin treatment. The lowest gene expression level in qPCR analysis at baseline was set at 1. Mean fold changes of SERPINE1, TIMP1, MMP2/3/11 are 1.56, 1.18, 0.87, 3.62, 1.13, respectively. Horizontal lines represent mean. n = 37 individuals per gene per time point, except for MMP11 which includes n = 33 individuals for both time points.

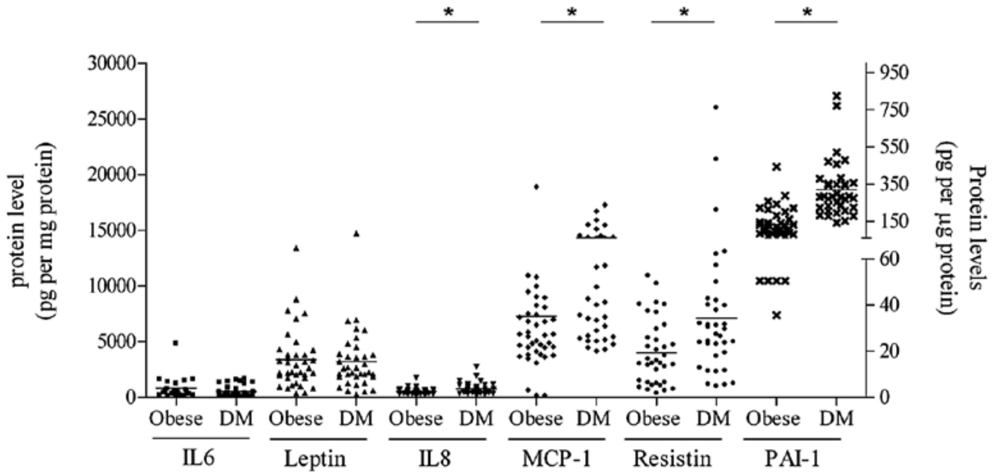

Figure 4 Protein levels measured within the adipose tissue of non-diabetic obese individuals vs. obese individuals with type 2 diabetes including leptin, resistin, MCP-1, IL6, IL8 (left y-axis) and PAI-1 (right y-axis). DM = patients with diabetes mellitus. Horizontal lines represent mean. $n = 36$ individuals per protein per time point. * $P < 0.05$.

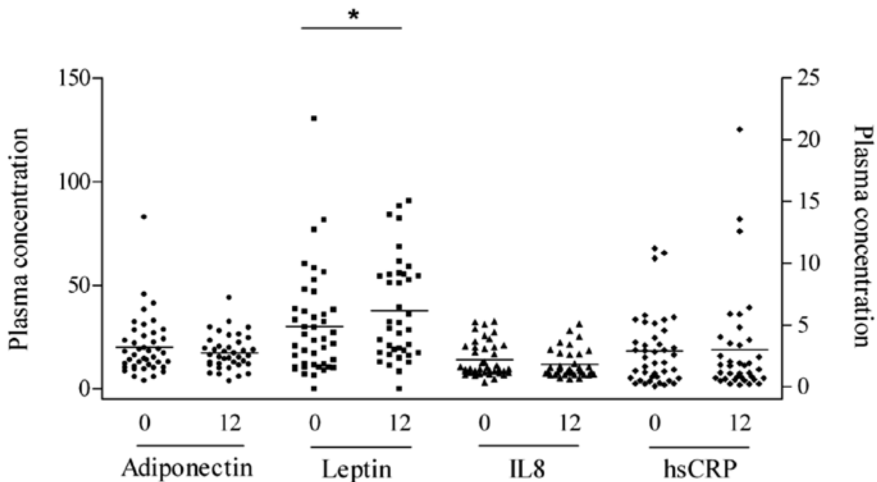

Figure 5A Change in circulating hormones and inflammatory markers before and after 12 months of insulin treatment. 0 = baseline and 12 = after 12 months of insulin treatment. Plasma adiponectin (right y-axis; in μg/ml), leptin levels (left y-axis; in ng/ml), interleukin-8 (IL8) (right y-axis; in pg/ml) and high sensitive C-reactive protein (hsCRP) (right y-axis; in mg/l). Mean fold changes of adiponectin, leptin, IL8 and hsCRP are 0.86, 1.25, 0.83, 1.04, respectively. Horizontal lines represent mean. $n = 38$ individuals per circulating marker per time point. * $P < 0.05$.

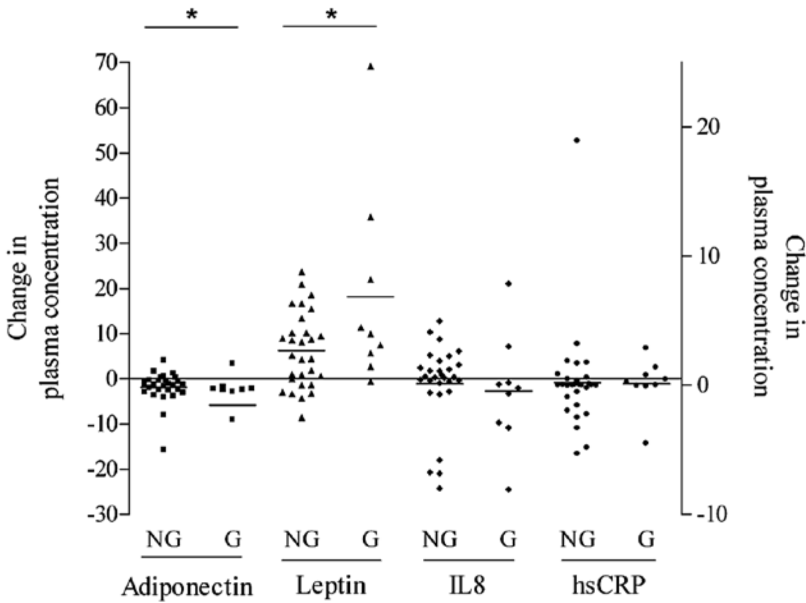

Figure 5B Changes in circulating hormones and inflammatory markers comparing non-gainers and gainers. NG = non-gainers, G = gainers. Change in plasma adiponectin (right y-axis; in μg/ml), leptin (left y-axis; in ng/ml), interleukin-8 (IL8) (left y-axis; in pg/ml) and high sensitive C-reactive protein (hsCRP) (right y-axis; in mg/l). Horizontal lines represent mean. n = 29 non-gainers and n = 9 gainers per circulating marker. * $P < 0.01$.

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Chapter 7

Liver fat content is linked to inflammatory changes in subcutaneous adipose tissue in type 2 diabetes patients

H. J. Jansen, G.M. Vervoort, M. van der Graaf, R. Stienstra, C.J. Tack

Clinical Endocrinology 2013; 79: 661-666.

Abstract

Patients with type 2 diabetes mellitus (T2DM) are typically overweight and have an increased liver fat content (LFAT). High LFAT may be explained by an increased efflux of free fatty acids from the adipose tissue which is partly instigated by inflammatory changes. This would imply an association between inflammatory features of the adipose tissue and liver fat content.

To analyse associations between inflammatory features of the adipose tissue and liver fat content we performed a cross-sectional study.

Twenty-seven obese patients with insulin-treated T2DM were studied. LFAT content was measured by proton magnetic resonance spectroscopy. A subcutaneous (sc) fat biopsy was obtained to determine morphology and protein levels within adipose tissue. In addition to fat cell size, the percentage of macrophages and the presence of crown like structures (CLSs) within sc fat was assessed by CD68-immunohistochemical staining.

Mean LFAT percentage was 11.1 ± 1.7 % (range 0.75-32.9 %); 63 % of the patients were diagnosed with an elevated LFAT (upper range of normal ≤ 5.5 %). Whereas adipocyte size did not correlate with LFAT, 3 out of 4 subjects with CLSs in sc fat had elevated LFAT and the percentage of macrophages present in sc adipose tissue was positively associated with LFAT. Protein concentrations of adiponectin within adipose tissue negatively correlated with LFAT. Adipose tissue protein levels of the key inflammatory adipokine plasminogen activator inhibitor-1 (PAI-1) were positively associated with LFAT.

Several pro-inflammatory changes in sc adipose tissue associate with increased LFAT content in obese insulin-treated patients with T2DM. These findings suggest that inflammatory changes at the level of the adipose tissue may drive liver fat accumulation.

Introduction

Most patients with T2DM are obese and have an accumulation of abdominal fat (1, 2) Additionally, liver fat content (LFAT) is strongly associated with intra-abdominal fat mass (3) and clearly increased in subjects with type 2 diabetes (4). The exact mechanisms involved in liver fat accumulation are currently unclear. It has been suggested that adipose tissue dysfunction is associated with the development of hepatic steatosis (5), yet data that link liver fat content to adipose tissue inflammation are scarce.

Adipose tissue expansion and adipocyte hypertrophy lead to inflammation as manifested by the infiltration of macrophages. Macrophages can reside in different forms in adipose tissue, but especially an arrangement in so called crown like structures surrounding adipocytes is viewed as a strong indicator of local inflammation (6). The pro-inflammatory status of the adipose tissue is further characterized by the increased production of various adipokines including TNFα, IL-6, MCP-1 and PAI-1 and has been linked to the development of insulin resistance (7-9). Peripheral insulin resistance promotes the release of fatty acids (FFAs) from adipose tissue that are subsequently taken up by hepatocytes to drive hepatic triglyceride synthesis and accumulation (10).

Therefore, LFAT content may particularly be increased in patients with T2DM in whom abdominal adipose tissue shows inflammatory traits. This hypothesis was tested in the present study, where we studied inflammatory characteristics of adipose tissue (adipocyte morphology, macrophage influx, the presence of crown-like structures and adipose tissue protein levels of various markers of adipocyte function) in a group of patients with T2DM and investigated whether these markers were associated with liver fat content.

Patients and Methods

Study population

Obese patients with T2DM who were on stable insulin treatment were included in the study. Exclusion criteria were other types of diabetes, significant cardiovascular, renal, liver or other co-morbidity, use of corticosteroids, uncontrolled endocrine disorders (stable supplementation with thyroid hormone was allowed), bariatric treatment, excessive alcohol consumption (>20 g/day), drug abuse, and use of thiazolidinedione derivatives.

Demographic and clinical characteristics

Patient demographics, medication, and insulin type and dose were recorded. Body weight, height, waist- and hip circumference, and blood pressure were measured using standard procedures.

Biochemical analyses

Fasting blood samples were drawn to determine: glycated haemoglobin (HbA1c), lipids, and free fatty acids (FFAs) (Cobas Mira Plus®, Roche Diagnostics Ltd., Basal, Switzerland).

Body composition and liver fat content

Total-body DEXA scanning (Hologic QDR 4500 densitometer, Bedford, USA) was used to determine total fat mass and trunk fat. Magnectic resonance (MR) measurements were performed on a Tim-Trio MR system (Siemens, Erlangen, Germany). A series of T1-weighted (flash 2D) axial MR images was acquired from a region extending from 4 cm above to 4 cm below the fourth to fifth lumbar interspace. Visceral and subcutaneous fat areas were determined based on signal intensity. Liver fat was assessed by proton MR spectra (MRS) as described previously (2).

Subcutaneous adipose tissue biopsies

Subcutaneous (sc) adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6–10 cm lateral to the umbilicus, after an overnight fast. Morphometry of individual fat cells was assessed using digital image analyses as described previously (11). For each subject, the adipocyte cell diameter of all fat cells in five to ten microscopic fields of view were counted and measured. On average, ~700 fat cells were measured per specimen (range 150–1500). Adipocyte cell size distribution was expressed as ratio small (≤ 50 μm):large adipocytes (≥ 100 μm) (12). For detection of macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (AbD Serotec, Oxford, UK). The percentage of macrophages was expressed as the total number of CD68-positive cells divided by the total number of adipocytes counted in 20 random microscopic fields of view x 100. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (13).

Protein levels within the adipose tissue were measured by Luminex fluorescent bead human cytokine immunoassays (MILLIPLEX MAP, Millipore Corp., Billerica, MA). Briefly, adipose tissue lysates were prepared using the milliplex map lysis buffer (Millipore) and protein concentrations of Interleukin-1 beta (IL-1b), Interleukin-6 and 8 (IL-6/8), tumor-necrosis factor alfa (TNF-α), leptin, adiponectin, monocyte chemo-attractant protein (MCP)-1, resistin and plasminogen activator inhibitor-1 (PAI-1) were determined. Equal amounts of protein were analyzed using a Bioplex system (Biorad).

Informed consent

The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol. All subjects provided written informed consent.

Statistical analyses

Variables are expressed as means ± SEM. Correlations were calculated by Spearman rank correlation analyses, unless otherwise specified. All calculations were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL). Two-tailed P <0.05 was considered significant.

Results

A total of 27 Caucasian patients with T2DM participated in the study. Table 1 shows the characteristics of the patients. Briefly, the patients were obese (BMI 31.4 ± 1.1 kg/m²) and the percentage of total and trunk fat mass as measured by DEXA was 43.0 ± 1.5 and 33.2 ± 1.6 %, respectively. Subcutaneous and visceral fat volume (MRI) were 2.2 ± 0.2 and 1.7 ± 0.1 liters, respectively. All patients were on long-term insulin treatment.

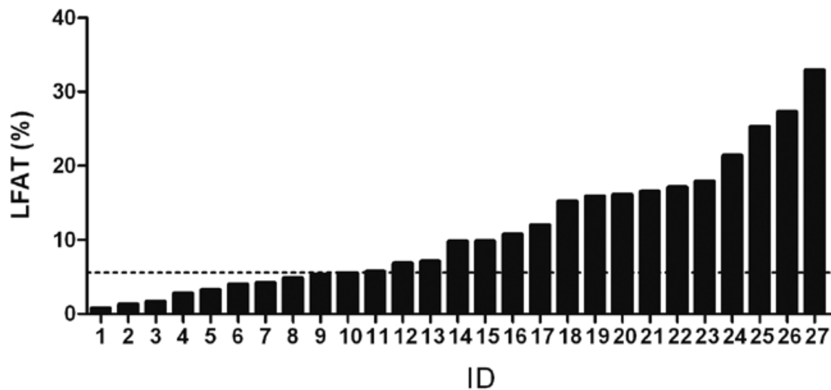

Figure 1A Individual LFAT levels in study participants. The dashed horizontal line represents the cut-off limit of liver fat (5.5 %) corresponding to the 95th percentile of the distribution of liver fat in healthy subjects (14).

Liver fat content

Mean, MRS-determined, liver fat content was 11.1 ± 1.7 % (range 0.75-32.9 %) and 63 % of the patients had LFAT above normal (> 5.5 %; i.e. above the diagnostic cut-off of steatosis (14)). LFAT content showed large inter-individual differences (Figure 1A).

Associations between BMI, body composition, adipose tissue morphology, inflammation and LFAT

LFAT was not significantly correlated with BMI (r = 0.25; P = 0.21), or sc fat volume (r = 0.05; P = 0.83), yet there was a trend towards a positive correlation between LFAT and visceral fat volume (r = 0.36; P = 0.07).

Mean adipocyte diameter assessed by digital image analyses in subcutaneous adipose tissue was 71.9 ± 1.3 μm with large inter-individual variation (range: 58.4-86.6 μm). Adipocyte cell size distribution (= ratio fraction small:large adipose tissue cells), that has been associated with adipose tissue inflammation (15), equalled 5.1 ± 2.0. Neither adipocyte cell diameter nor adipocyte cell size distribution did correlate with LFAT.

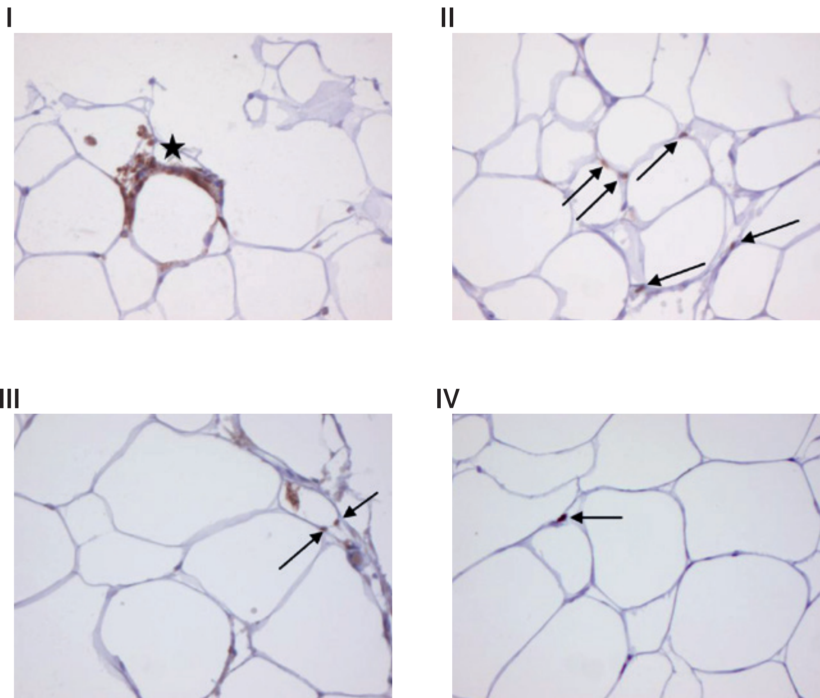

Figure 1B Sc adipose tissue morphology and CD68-immunohistochemical staining of 2 representative patients with high LFAT (panel I: LFAT 21.4 % with a crown-like structure (asterisk), panel II: LFAT 32.9 %) both showing high influx of CD68-positive cells and of 2 representative patients with low LFAT (panel III: adipose tissue of a patient with LFAT 1.7 %, panel IV: adipose tissue of a patient with LFAT 3.2 %) both with low influx of CD68-positive cells. All sections were counterstained with hematoxylin (colored blue). Magnification: 20x. Arrows indicate CD68-positive cells.

Macrophages arranged in (partial) crown-like structures ((p)CLSs) that are characteristic of adipose tissue inflammation, were detected in 15% of the adipose tissue slides. Three out of four patients with CLSs in the adipose tissue had clearly elevated LFAT content (5.3 %, 27.3 %, 12.0 % and 21.4 % respectively). Representative examples of adipose tissue morphology and CD68-positive cells of two patients with high LFAT and 2 patients with low LFAT are shown in Figure 1B. There was a trend towards a positive correlation between LFAT and the percentage of infiltrated macrophages in the adipose tissue (r = 0.31; *P* = 0.12).

Adipose tissue protein levels of the inflammatory adipokine plasminogen activator inhibitor-1 (PAI-1) correlated positively (r = 0.39; *P* = 0.045) while the insulin-sensitizing marker adiponectin correlated negatively with LFAT (r = -0.48; *P* = 0.01, Fig. 2A and B, respectively).

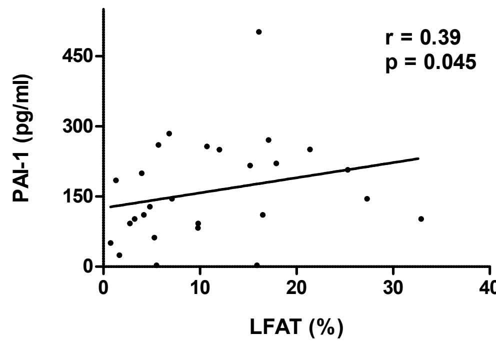

Figure 2A Correlation between adipose tissue PAI-1 protein levels with LFAT.

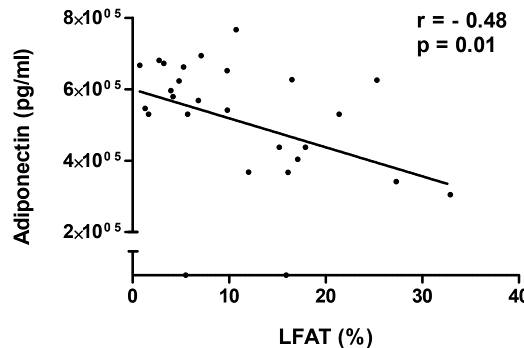

Figure 2B Correlation between adipose tissue adiponectin protein levels with LFAT.

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Adipose tissue levels of IL-1b, IL-6, IL-8, TNF-α, leptin, monocyte chemoattractant protein (MCP)-1 and resistin levels were not associated with LFAT content ($r = -0.14$; $P = 0.56$, $r = -0.15$; $P = 0.52$, $r = -0.17$; $P = 0.42$, $r = -0.22$; $P = 0.32$, $r = -0.17$; $P = 0.43$, $r = -0.07$; $P = 0.75$, $r = 0.04$; $P = 0.85$, respectively).

Discussion

In the present study we demonstrate that LFAT in insulin-treated patients with type 2 diabetes is linked to an inflammatory trait in sc adipose tissue as reflected by the presence of CLSs, higher levels of PAI-1 and lower concentrations of adiponectin. Together, these findings suggest an association between liver fat content and inflammatory features in fat tissue in insulin-treated patients with type 2 diabetes.

Studies analyzing sc adipose tissue morphology and inflammatory characteristics in relation to LFAT are scarce, especially in patients with T2DM. In line with previous studies (4, 16), our results demonstrated that mean LFAT content is high in obese diabetic patients. However, LFAT showed a large inter-individual variation within our study population, with 37% of study subjects displaying a normal LFAT content (i.e. ≤ 5.5 %) (14) implying that obesity, diabetes or the use of insulin by itself not readily explain the presence of steatosis.

The ability of adipocytes to adapt to the increased supply of fat by hypertrophic enlargement is limited and may finally promote ectopic fat accumulation resulting in liver steatosis in obese subjects. In the present study, the correlation of liver fat content with measures of obesity (weight, BMI and MRS determined sc adipose tissue mass) was weak and non-significant. It might be envisioned that qualitative traits of the adipose tissue such as adipocyte size or macrophage influx are more important determinants of liver fat content. Although a positive correlation between adipocyte size and LFAT content has been previously reported (17), we were unable to replicate this finding. These, apparently conflicting, findings may partially be explained by differences in the study population. Whereas Koska *et al*. (17) investigated an obese Pima Indian population without impaired glucose regulation or diabetes type 2 diabetes, our study population consists of individuals that are diagnosed with type 2 diabetes.

We found that the presence of CLSs and macrophages in subcutaneous adipose tissue were correlated with the presence of LFAT whereas adipocyte size was not. These findings are in line with earlier reports in which adipose tissue inflammation characterized by the presence of macrophages and CLSs has been associated with insulin-resistance and LFAT content (18-20). Overall, our findings suggest that adipose tissue inflammation outweighs adipocyte size in determining hepatic fat accumulation in patients with type 2 diabetes.

A potential relationship between liver fat content and adipose tissue inflammation is also supported by the positive correlation between adipose tissue levels of PAI-1 and LFAT content. PAI-1 has been previously identified as a significant risk factor for microvascular and marcrovascular complications in diabetes (21-23) and has been linked to insulin resistance (24). Moreover, circulating levels of PAI-1 has been frequently linked to the development of hepatic steatosis (25, 26), although its tissue and cellular origin contributing to hepatic dysfunction remain debatable. In adipose tissue, PAI-1 is thought to play a role in TNF-α-induced insulin resistance (27, 28). Moreover, it can be viewed that nutritional factors (e.g. free fatty acids) suppress insulin-stimulated glucose uptake and raise circulating PAI-1 concentrations, with a concomitant increase in the expression of the PAI-1 gene in adipose tissue (29).
It is furthermore suggested that adipocyte-derived factors prime adipose macrophages so that they respond to nutritional signals (FFAs) by releasing a key inflammatory adipokine PAI-1.

In line with a lack of association between BMI, subcutaneous fat volume or adipocyte size and LFAT, adipose tissue protein levels of leptin, frequently used as a marker of fat mass, was not associated with hepatic fat content. Resistin levels in subcutaneous adipose tissue did not correlate with LFAT content in our study population. It can be envisioned that resistin produced in the visceral adipose depot primarily affects LFAT. Alternatively, resistin may not be a key-regulator of adipose tissue inflammation in humans. Although the number of macrophages present in subcutaneous fat were correlated to LFAT, this was not the case for protein levels of the chemoattractant MCP-1. Neither did absolute macrophage numbers correlate with MCP-1 levels in adipose tissue (data not shown). These results imply that other chemokines may determine the influx of macrophages in adipose tissue of subjects with type 2 diabetes.

In parallel with an increased inflammatory state of the adipose tissue, protein levels of adiponectin were inversely correlated with LFAT content. Inasmuch low circulating levels of adiponectin contribute to the development of a fatty liver (30), the reduction in adiponectin levels within subcutaneous adipose tissue may subsequently contribute to the pathogenesis of liver steatosis in patients with T2DM.

Although increased liver fat content is correlated with changes in fat mass, adipose tissue stored viscerally is more robustly associated (31) with partitioning of fat towards the liver as compared to subcutaneous or peripheral fat mass (32). The enhanced inflammatory trait of visceral adipose tissue (VAT) illustrated by its capacity to produce enhanced levels of inflammatory cytokines as compared to the subcutaneous adipose tissue (SAT) compartment ex vivo (33, 34), may partly explain these observations. The analyses in our study were done using the subcutaneous fat tissue compartment, which is a limitation, and do not necessarily implicate similar changes in the visceral compartment. One may speculate, however, that a relationship

between visceral fat inflammation and liver fat will be more robust than the observed relationship at the subcutaneous level. The most important finding of the present study is the identification of an existing relationship rather than the absolute strength of it. Another limitation of the study is the cross-sectional design, which cannot determine whether LFAT is a direct consequence of adipose tissue inflammation. Despite the limited absolute number of patients, our study also has strengths that include the extensive phenotyping of a set of almost 30 subjects and careful analysis of subcutaneous adipose tissue morphology. Additionally, determining protein levels in adipose tissue sample lysates has, to our knowledge, not been frequently applied and yields more valuable information concerning adipose tissue function as compared to measurements of circulating protein concentrations.

In conclusion, LFAT content shows substantial inter-individual variation, yet is positively associated with qualitative, pro-inflammatory changes in sc adipose tissue in obese insulin-treated T2DM patients. This suggests that inflammatory changes at the level of adipose tissue may be causatively related to liver fat content. Proof of this concept will require prospective studies.

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Disclosure

The authors have no relevant conflicts of interest to disclose.

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Chapter 8

Autophagy activity is up regulated in adipose tissue of obese individuals and controls pro-inflammatory cytokine expression

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Abstract

Autophagy, an evolutionary conserved process aimed at recycling damaged organelles and protein aggregates in the cell, also modulates pro-inflammatory cytokine production in peripheral blood mononuclear cells. Since adipose tissue inflammation accompanied by elevated levels of pro-inflammatory cytokines is characteristic for the development of obesity, we hypothesized that modulation of autophagy alters adipose tissue inflammatory gene expression and secretion. We performed ex vivo and in vivo studies of human and mouse adipose tissue.

Levels of the autophagy marker LC3 were elevated in subcutaneous adipose tissue of obese versus lean human subjects and positively correlated to both systemic insulin resistance and morphological characteristics of adipose tissue inflammation. Similarly, autophagic activity levels were increased in adipose tissue of obese and insulin resistant animals as compared to lean mice. Inhibition of autophagy by 3-methylalanine in human and mouse adipose tissue explants led to a significant increase in IL-1β, IL-6 and IL-8 mRNA expression and protein secretion. Noticeably, the enhancement in IL-1β, IL-6 and KC by inhibition of autophagy was more robust in the presence of obesity. Similar results were obtained by blocking autophagy using small interfering RNA targeted to ATG7 in human SGBS adipocytes.

Our results demonstrate that autophagy activity is up regulated in adipose tissue of obese individuals and inhibition of autophagy enhances pro-inflammatory gene expression both in adipocytes and adipose tissue explants. Autophagy may function to dampen inflammatory gene expression and thereby limit excessive inflammation in adipose tissue during obesity.

Abbreviations: ATG, Autophagy-related proteins; BMI, body mass index; CLS, crown-like structure; eWAT, epididymal WAT; KC, keratinocyte-derived chemoattractant; 3MA, 3-methyladenine; NOB-1, Nin1 binding protein; NOD2, nucleotide-binding oligomerization domain containing 2; SAT, sc adipose tissue; SGBS, Simpson-Golabi-Behmel syndrome; siRNA, small interference RNA; TBS, Tris-buffered saline; VAT, visceral adipose tissue; WAT, white adipose tissue.

Introduction

Autophagy is a homeostatic mechanism functioning as a disposal system that degrades large intracellular organelles or protein aggregates in order to change cellular structure during differentiation, or to generate essential nutrients in times of energy deprivation (1, 2). When autophagy is activated, a double-membraned vesicle, named the autophagosome, engulfs these components and fuses with lysosomes to complete degradation (3). The elongation and shape of the autophagosome is controlled by multiple autophagy-related proteins (ATG proteins) that include ATG5, ATG7, ATG8, ATG12 and ATG16L1. The mammalian homologue of yeast ATG8, LC3, is a commonly used marker of autophagy as this protein is conjugated with the lipid phosphatidylethanolamine (PE) upon activation of autophagy resulting in LC3-II formation.

A growing body of evidence suggests that autophagy not only monitors cellular energy balance, yet is also important in the regulation of apoptosis and protection against infection with pathogens (4-6). Indeed, autophagy has been closely linked to control of innate and adaptive immune responses in host defense in part by regulation of cytokine production (7). Cytokines including IL-1β, IL-18, TNFα and IL-6 are known to be regulated by autophagy (8). For example, macrophages derived from ATG16L1 deficient mice produced higher levels of IL-1β (9), while mice with a conditional deletion of Atg7 in the intestinal epithelium showed an enhanced mRNA expression of IL-1β (10). Additional studies using cells from human origin demonstrated that inhibition of autophagy led to up regulated production of IL-1β (11, 12).

In addition to its role in host defense partly through control of cytokine production, autophagy has been shown to regulate fat accumulation within adipocytes. Animals lacking the autophagy-related proteins ATG5 and ATG7 are characterized by a limited capacity of white adipose tissue (WAT) to store triglycerides and thus display a robust reduction in WAT mass as compared to wild-type animals (13-15) suggesting that autophagy is essential for normal adipogenesis. Interestingly, autophagy also appears to contribute to the development of obesity as adipose tissue of obese human individuals is characterized by an enhancement in autophagic activity (16, 17).

In parallel with the development of obesity, a chronic inflammation in the adipose tissue develops that is partly initiated by adipocytes releasing chemokines, pro-inflammatory cytokines and adipokines, resulting in the infiltration of immune cells into adipose tissue (18, 19). Various cytokines are known to be released or produced by inflamed adipose tissue of obese individuals including IL-1β (20-23), IL-6, TNFα and IL-18 (24) and contribute to the development of obesity-induced insulin resistance. Despite the importance of pro-inflammatory cytokines in the development of insulin resistance, the regulation and potential triggers inducing their production are only partly understood. In line with its enhanced activity in adipose tissue during obesity and its role in the regulation of inflammatory cytokine secretion, we hypothesized that

autophagy modulates adipose tissue inflammation. Our results show that inhibition of autophagic activity enhances both gene expression and protein secretion of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 in adipocytes and adipose tissue of both humans and mice.

Patients and Methods

Subcutaneous adipose tissue biopsies from lean and obese individuals

Adipose tissue samples were obtained from human subjects through advertisements in local newspapers. We included overweight (body mass index (BMI) 27-35 kg/m^2) and normal (body mass index 20-25 kg/m^2) subjects between 40 and 70 years old. Subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies 6–10 cm lateral to the umbilicus. Samples were taken after an overnight fast. Various biochemical measurements including plasma triglycerides, cholesterol, glucose and insulin were done using standard laboratory methods.

In vitro and ex vivo experiments with human and murine adipose tissue

Intact adipose tissue fragments from subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT), obtained during surgery from healthy individuals of 30-70 years old with a BMI between 20 and 33 kg/m^2 were used to study the effects of autophagy. Adipose tissue fragments were directly cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum with or without 3-methyladenine (autophagy inhibitor; 3MA, Sigma-Aldrich). Minced adipose tissue was digested using collagenase (Sigma-Aldrich) at a concentration of 5 mg/ml dissolved in Dulbecco's modified Eagle's medium (DMEM). Tissues were incubated for 45 min at 37 °C and were subsequently filtered through a 250 μM nylon mesh filter. After centrifugation at 200 rpm for 10 minutes, the floating cells were collected as adipocytes and the pelleted cells as stromal vascular cells. The study protocols are approved by the University of Nijmegen Ethical Committee and all participants gave written informed consent. Epididymal white adipose tissue (eWAT) obtained from lean C57/Bl6 mice and obese leptin deficient (Lep·ob/Lep·ob) mice on a C57/Bl6 background (25) was similarly treated with 3MA. After 24h of incubation with or without 3MA, cytokine secretion and gene expression levels were determined. The study protocol was approved by the animal experimentation committee of the University Medical Centre of Nijmegen.

Small interference RNA (siRNA)

To specifically suppress autophagy-related protein 7 (ATG7) expression in differentiated adipocytes, human Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocyte cells were differentiated towards mature adipocytes using a standard adipogenic protocol (26). After 12 days of differentiation, cells were transfected (X-tremeGENE siRNA Transfection Reagent, Roche) with small interference (si)RNA against ATG7 (Thermo Scientific). As a non-specific control, scrambled siRNA (Thermo Scientific) was used. After 96 hours of incubation, gene and protein expression were analyzed in cells and supernatant.

Cytokine measurements

Concentrations of mouse IL-1β were determined by specific radioimmunoassay (RIA; detection limit is 20 pg/mL) as previously described (27). Mouse IL-6 and KC concentrations were measured by commercial ELISA kits (Invitrogen, CA, USA, detection limits 16 pg/ml and R&D Systems, MN, USA, detection limits 16 pg/ml, respectively), according to the instructions of the manufacturer. Bio-active IL-1 was measured using the NOB-1 assay, which consists of mouse cells derived from the EL-4 line which produce IL-2 in response to concentrations of IL-1 as low as 1 pg/ml. NOB-1 is not responsive to tumor necrosis factor alpha, tumor necrosis factor beta, interferon-gamma and lipopolysaccharide (28). IL-2 was detected using a mouse IL-2 ELISA kit (R&D Systems, MN, USA, detection limits 16 pg/ml). Human IL-6 and IL-8 concentrations were measured using commercially available ELISA kits (R&D systems, MN, USA).

Western blots

For Western blotting, SGBS cells or approximately 30 mg of SAT or VAT were lysed in 100 μl of lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% ND40, 50 mM NaF and 0.25% Sodium Deoxycholate with phosstop phosphatase inhibitor cocktail tablet (Roche, Basel, Switzerland) and complete, EDTA-free protease inhibitor cocktail tablet (Roche, Basel, Switzerland). The homogenate was frozen, then thawed and centrifuged at 4°C for 10 min at 15 000 × g, and the supernatant was used for Western blot analysis. Protein concentrations of the 30% lysates were determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL USA). Samples of approximately 20 μg protein were subjected to SDS-PAGE using 12% and 16% polyacrylamide gels at a voltage of 70-120 V. After SDS-PAGE, proteins were transferred to nitrocellulose membrane (0.2 mm) and the membrane was blocked with 5% (wt/vol) milk powder in TBS/Tween 20 for 1 hour at room temperature, followed by incubation overnight at 4°C with a LC3 antibody (Novus Biochemicals, CO, USA) in 5% (wt/vol) milk powder/TBS/Tween 20 or with an actin antibody (Sigma, MO, USA) in 5% milk powder in TBS/Tween 20. After overnight incubation, the blots

were incubated with HRP-conjugated swine anti-rabbit antibody at a dilution of 1:5.000 in 5% (wt/vol) milk powder in TBS/Tween 20 for 1 hour at room temperature and subsequently developed with ECL (GE Healthcare, PA, USA) or ultra-sensitive enhanced ECL (Thermo Scientific, MA, USA), according to the manufacturer's instructions. The intensity of the bands on the Western blots was assessed by Image Lab statistical software (Bio-Rad, CA, USA).

Real-time PCR

Total RNA purification of SGBS cells, SAT or VAT was done using TRIzol Reagent (Invitrogen, CA, USA) according to manufacturer's instructions. Isolated RNA was subsequently transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) followed by quantitative PCR using SYBR Green (Applied Biosystems, CA, USA). The following primers were used: for human IL-1β forward 5'-GCCCTAAACAGATGAAGTGCTC-3' and reverse 5'- GAACCAGCATCTTCCT-CAG-3'; for human IL-6 forward 5'-AACCTGAACCTTCCAAAGATGG-3' and reverse 5'-TCTGGCTTGTTCCTCACTACT- 3'; for 149 human IL-8 forward 5'-ACTGAGAGT-GATTGAGAGTGGAC-3' and reverse 5'-AACCCTCTGCACCCAGTTTTC- 3'; and for human ATG7 forward 5'-CAGTTTGCCCCTTTTAGTAGTGC-3' and reverse 5'-CTTA-ATGTCCTTGGGAGCTTCA-3'. For mouse primers the following were used: IL-1β forward 5'- GCAACTGTTCCTGAACTCAACT-3' and reverse 5'-ATCTTTTGGGGTC-CGTCAACT-3'; for mouse IL-6 forward 5'-CAAGTCGGAGGCTTAATTACACATG-3' and reverse 5'-ATTGCCATTGCACAACTCTTTTCT-3'; for mouse IL-8 (KC) forward 5'-TGGCTGGGATTCACCTCAA-3' and reverse 5'- GAGTGTGGCTATGACTTCGGTTT -3'; for mouse ATG7 forward 5'- CCTTCGCGGACCTAAAGAAGT-3'and reverse 5'-CCCGGATTAGAGGGATGCTC-3'. All primer pairs were tested for efficiency using standard curves and were between 95-105 %. Additionally, a melt curve analysis was included in every run to ascertain the formation of one single specific PCR product. The housekeeping genes β2-microglobulin and 36B4 were selected as human and mouse housekeeping genes. Gene expression data obtained from the human samples was corrected for expression of the housekeeping genes β2-microglobulin, for which the forward primer 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse primer 5'-CCAAATGCGGCATCTTCAAAC-3' were used. For mouse samples, forward primer 5'-AGCGCGTCCTGGCATTGTGTGG-3' and reverse primer 5'-GGGCAGCAGTGGTG-GCAGCAGC-3' were used to detect 36B4 expression levels and gene expression results were corrected using 36B4 values.

Statistical analyses

Differences in cytokine production capacity and data on mRNA expression levels between groups were analyzed using the analysis of variance (ANOVA) with the Least Significant Difference post hoc test to detect statistical differences between

groups and treatment (SAT vs. VAT, lean vs. obese, control vs. 3MA treatment) after variables were log-transformed. Data on immunoblot intensity values were statistically analyzed using Student's t-tests. Differences were considered statistically significant at $p < 0.05$.

Results

Autophagy is up regulated in adipose tissue during obesity

To assess the correlation between autophagy and obesity, subcutaneous adipose tissue (SAT) was obtained from healthy individuals ($n=33$) that varied in body mass index from 19 to 40kg/m^2 (Supplemental table 1: patient characteristics) and were analyzed by Western-blot to detect LC3-II levels (Figure 1A, a representative Western-blot is shown).

The mammalian homologue of yeast ATG8, LC3, is a commonly used marker of autophagy as this protein is conjugated with the lipid phosphatidylethanolamine (PE) upon activation of autophagy resulting in LC3-II formation. The autophagy marker LC3-II (microtubule-associated protein light chain 3) was more abundantly present in obese individuals (average BMI $\sim$ 32 kg/m^2) as compared to lean subjects (average BMI $\sim$ 22 kg/m^2) (Figure 1B). Similarly, LC3 levels were profoundly higher in adipose tissue of obese animals lacking leptin (Lep·ob/Lep·ob), compared to lean wild-type mice (Figure 1C and D).

This suggests that autophagy is activated in adipose tissue during obesity and confirms previous work by Kovsan et al. (16). In line with these results, a positive correlation was observed between LC3-II levels and BMI ($r = 0.43$; $P = 0.01$, Figure 2A). Furthermore, the positive correlation between HOMA-IR and adipose tissue levels of LC3-II levels ($r = 0.44$; $P = 0.02$, Figure 2B) revealed that insulin sensitivity negatively correlated with autophagic activity levels. Since obesity and insulin resistance is associated with the development of adipose tissue inflammation that is characterized by the influx of macrophages, we set out to correlate macrophage influx with LC3-II levels in the subcutaneous biopsies obtained from our study subjects. We observed a positive correlation between the influx of macrophages and LC3-II levels in subcutaneous adipose tissue ($r = 0.38$; $P = 0.03$, Figure 2C). Additionally, aggregation of macrophages in pro-inflammatory crown-like structures (CLSs) that surround dying adipocytes was accompanied by significantly higher LC3-II levels as compared to adipose tissue from individuals that lacked CLSs (Figure 2D).

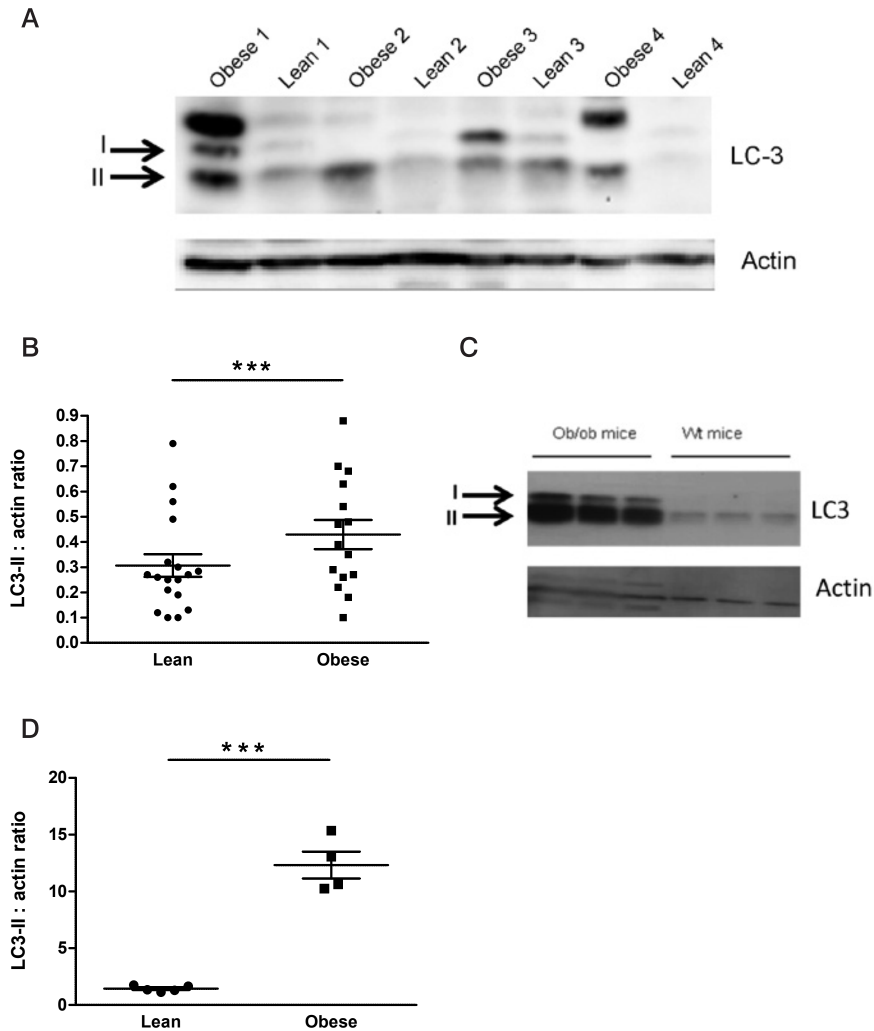

Figure 1 Levels of the autophagy marker LC3 determined by western blot in human and mouse adipose tissue. (**A**) Representative western blot of LC3 in human lean (BMI ~ 22 kg/m^2) and obese subjects (BMI ~ 32 kg/m^2). LC3 levels in epididymal adipose tissue from C57BL6 wt mice and leptin deficient (Lep·ob/Lep·ob) mice (**C**). LC3-II : actin ratio, that reflects the level of autophagic activity in adipose tissue, is calculated by quantification of western blot intensity in n =33 human subjects (**B**) and wildtype (n =5) vs. leptin deficient (Lep•ob/Lep•ob) mice (n =4) (**D**). Data are presented as means ± SEM. *** $P < 0.001$.

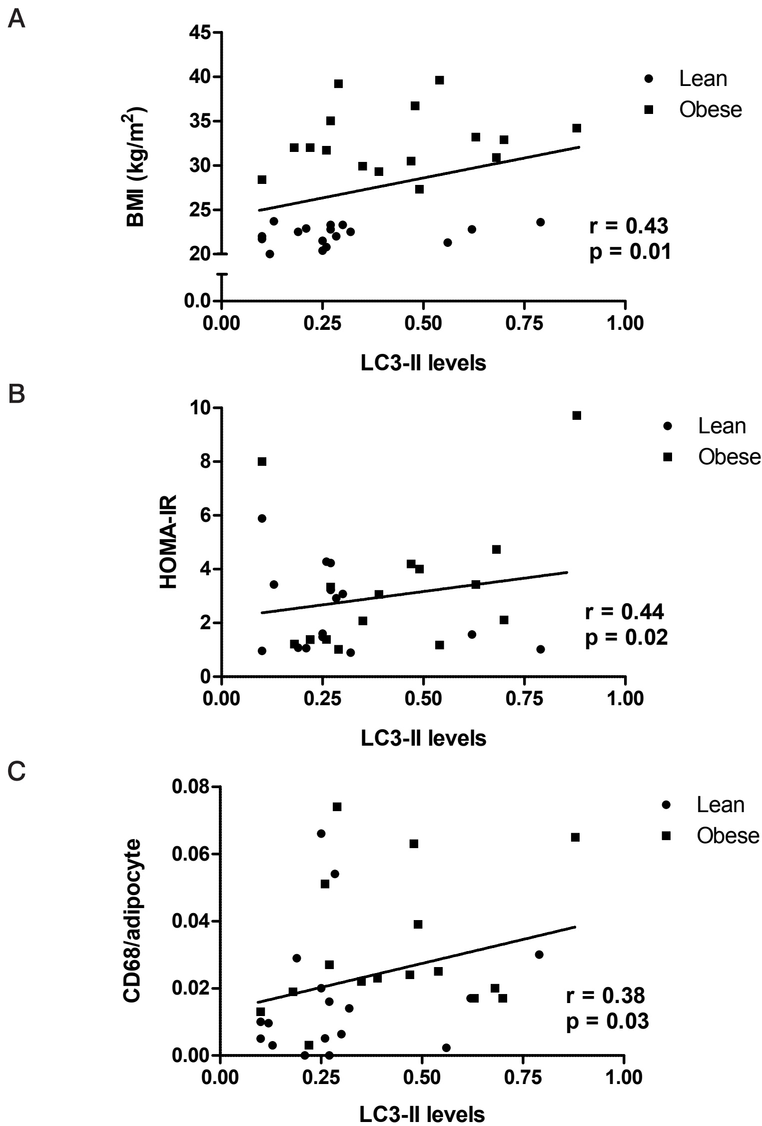

Figure 2 Correlation between autophagic activity, obesity, insulin sensitivity and adipose tissue morphology. Spearman correlations analysis between autophagic LC3-II levels and BMI (**A**), HOMA-IR (**B**) and influx of macrophages within the adipose tissue (**C**). HOMA-IR denotes the homeostasis model assessment-insulin-resistance. HOMA-IR was calculated by HOMA-IR = fasting plasma insulin (μIU/mL) x fasting plasma glucose (mmol/L)/22.5. LC3-II activity levels determined in subjects with or without the presence of crown-like structures (CLSs) in adipose tissue (**D**). * $P < 0.05$.

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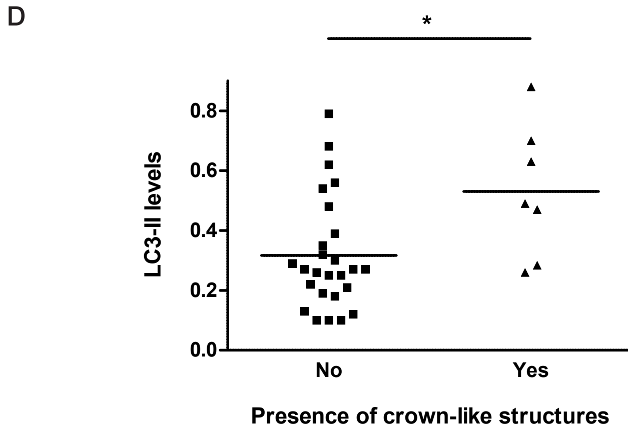

Figure 2 Continued.

Autophagy regulates expression of pro-inflammatory cytokines in human adipose tissue ex vivo

To study the role of autophagy in the production and secretion of pro-inflammatory cytokines by the adipose tissue, human SAT and VAT explants were cultured in medium containing the autophagy inhibitor 3MA or PBS for 24 hours. The concentration of 3MA (10mM) used throughout the experiments was in accordance with previous studies (29). 3MA clearly reduced autophagic activity in adipose tissue according to lower LC3-II protein levels (Figure 3A). 3MA treatment resulted in a significant increase of IL-1β, IL-6 and IL-8 protein secretion by both SAT and VAT (Figure 3B). In line with the cytokine secretion profile, mRNA levels of IL-1β, IL-6 and IL-8 gene expression were increased in SAT and VAT tissue explants exposed to 3MA (Figure 3C). Secretion levels of leptin and adiponectin were unchanged suggesting that inhibition of autophagy by 3MA does not modulate the general endocrine function of adipose tissue (Supplemental Figure 1).

Autophagy regulates expression of pro-inflammatory cytokines in mouse adipose tissue explants

To confirm the effects of inhibition of autophagy in mouse fat tissue, epididymal white adipose tissue (eWAT) derived from obese leptin deficient (Lep·ob/Lep·ob) mice and lean wild-type mice was treated with 3MA. Inhibition of autophagy in lean mice led to a significant increase of pro-inflammatory protein secretion. Interestingly, the induction in IL-1β, IL-6 and KC was more robust in obese animals (Figure 4A). Most likely, the enhanced secretion is a result of elevated gene expression levels (Figure 4B). Indeed, 3MA treatment led to a 20-fold increase in adipogenic IL-1β gene

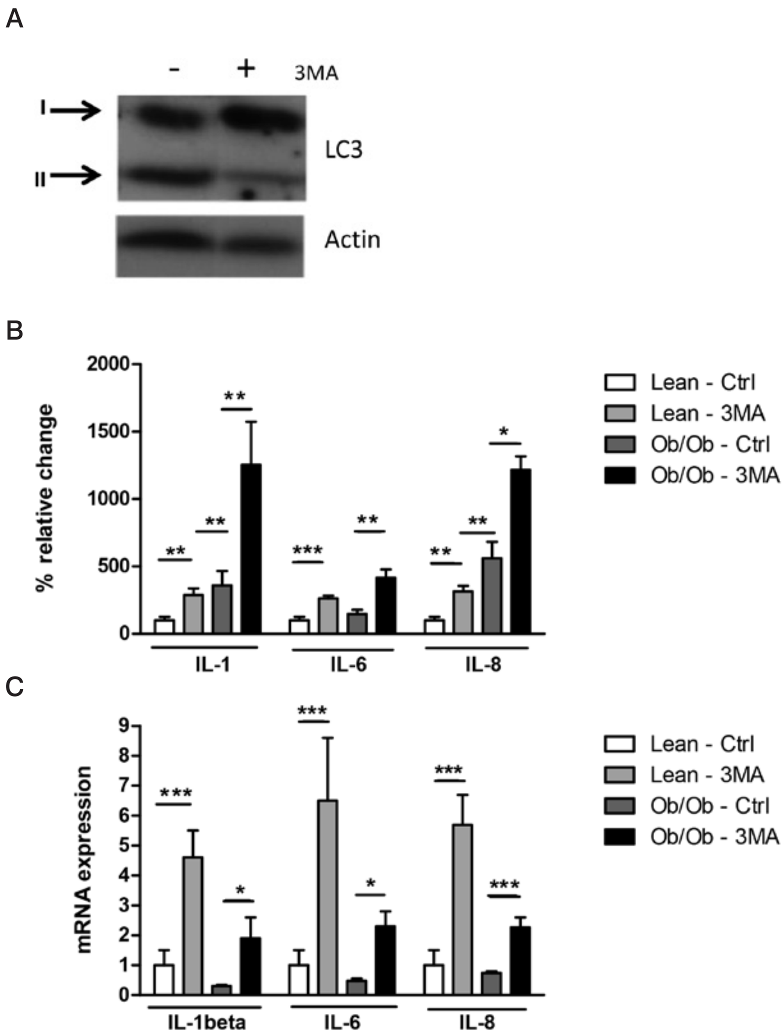

Figure 3 Protein levels and gene expression of cytokines in human SAT and VAT treated with 3MA for 24 hours. Western blot analysis of LC3-II levels in human VAT explants after treatment with 3MA for 24h (**A**). Secretion levels of bioactive IL-1 (indirectly by IL-2 production of NOB-1 assay), IL-6 and IL-8 by human SAT and VAT. The secretion levels are expressed as the relative change in pg/ml/mg (cytokine/volume of supernatant/weight of explant). Mean values of cytokine expression from SAT control samples have been set as 100% (**B**). Gene expressions of IL-1β, IL-6 and IL-8 in SAT and VAT (**C**). Control samples were set as 1. All experiments have been repeated three times or more. Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

expression in obese animals whereas inhibition of autophagy in lean mice resulted in a 3-fold induction in gene expression. Similar results were seen for IL-6 and IL-8 gene expression levels (Figure 4B). These data imply that autophagy somehow may act to limit excessive inflammatory gene expression in adipose tissue during obesity as evidenced by the exacerbated cytokine expression upon autophagic inhibition in adipose tissue from obese and insulin resistant animals. Noticeably, 3MA treatment

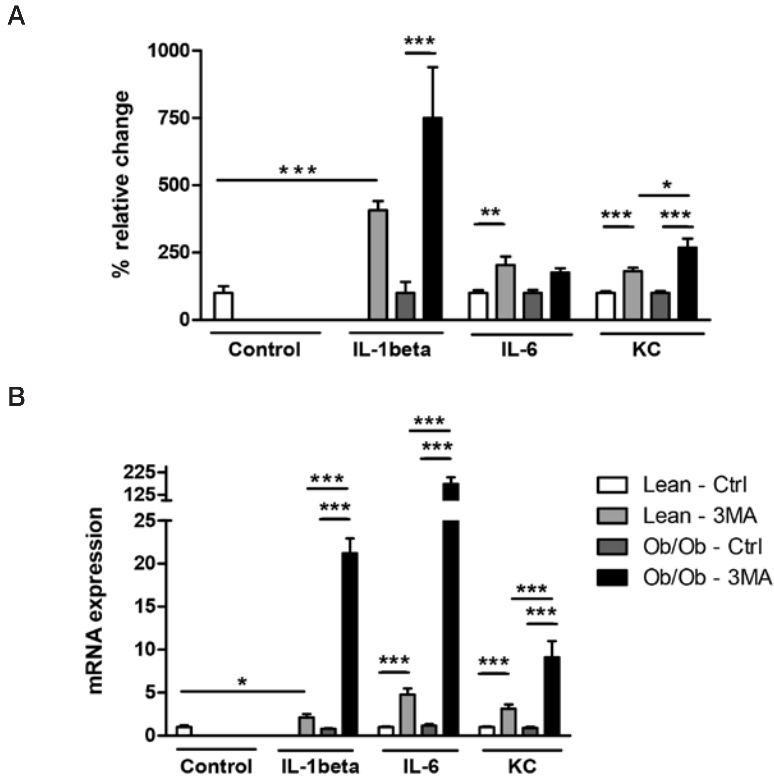

Figure 4 Protein levels and gene expression of IL-1β, IL-6 and KC in murine eWAT explants treated with 3MA for 24 hours. Cytokine secreted in supernatant by eWAT explants from lean C57BL6 mice and obese leptin deficient (Lep•ob/Lep•ob) mice (IL-1β, IL-6 and KC). The secretion levels are expressed as the relative change in pg/ml/mg (cytokine/volume of supernatant/weight of explant). Mean values of cytokine expression from lean control samples have been set as 100% (**A**). Relative gene expressions of IL-1β, IL-6 and KC in eWAT from lean mice and obese leptin deficient (Lep•ob/Lep•ob) mice (**B**). Control samples were set as 1. Experiments have been repeated three times or more. Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

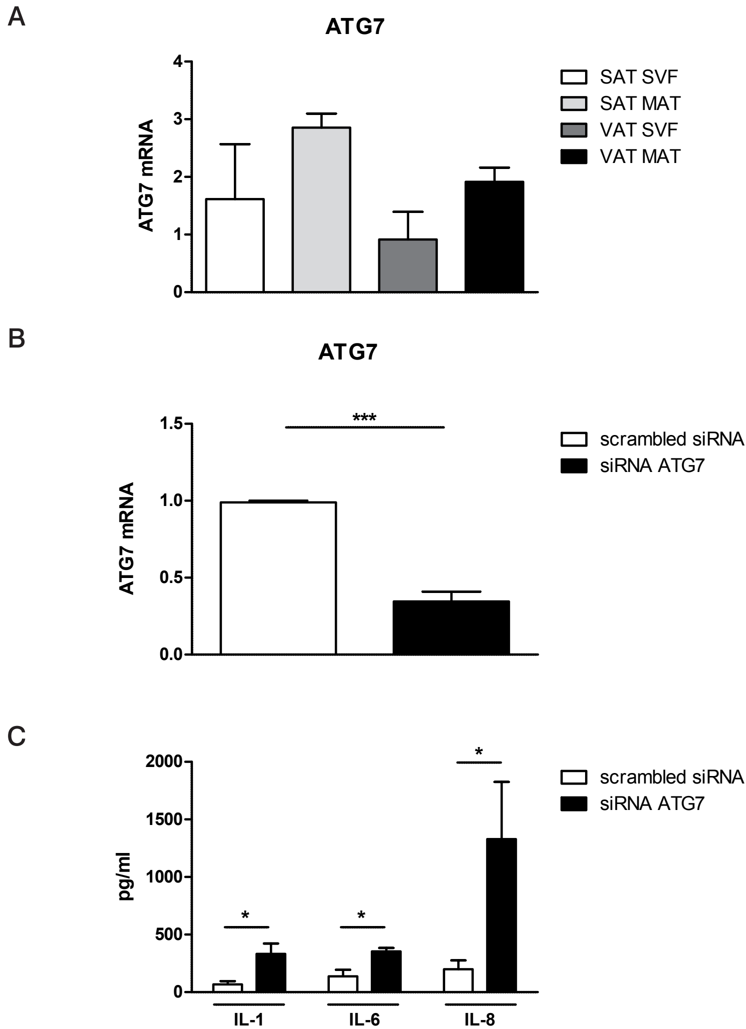

Figure 5 Effect of siRNA ATG7 on protein and gene expression of cytokines in human differentiated SGBS adipocytes. Relative gene expression of ATG7 in the stromal vascular fraction (SVF) and mature adipocytes (MAT) in human white adipose tissue (**A**). Relative gene expression of ATG7 after siRNA treatment (**B**). Cytokine concentrations of IL-1 (indirectly by IL-2 production of NOB-1 assay), IL-6 and IL-8 secreted in the supernatant by SGBS cells after siRNA treatment (**C**). Relative gene expression levels of IL-1β, IL-6 and IL-8 in SGBS cells after siRNA treatment (**D**). Control samples were set as 1. Data are presented as means ± SEM. * $p < 0.05$; *** $p < 0.001$ (n=3).

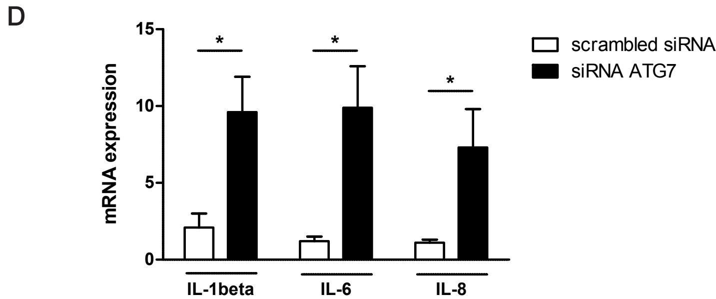

Figure 5 Continued.

did not appear to affect the general secretory capacity of the WAT explants since leptin and adiponectin secretion levels did not differ upon autophagic inhibition (supplemental Figure 2).

Autophagy inhibition by siRNA ATG7 in human mature adipocytes increases cytokine production

Since adipose tissue is composed of both adipocytes and stromal vascular cells, we set out to characterize gene expression levels of various autophagy-related genes in both cell populations. qPCR analysis revealed that numerous autophagy-related genes are expressed in both adipocytes and non-adipocyte cells part of the adipose tissue (Figure 5A + supplementary Figure 3). Expression of the autophagy machinery in both the SVF and adipocytes suggest that various cell types may contribute to the autophagy-mediated inhibition of inflammation. To learn more about the contribution of adipocyte-specific autophagy to control of inflammation, ATG7 expression, an essential protein in the assembly of autophagosomes by controlling the vesicle elongation and predominantly expressed in adipocytes, was blocked by siRNA treatment in fully differentiated human SGBS adipocytes. siRNA treatment led to a 65% reduction in ATG7expression (Figure 5B). The reduction in ATG7 expression promoted a significant increase in both gene expression and protein levels of IL-1β, IL-6 and IL-8 (Figure 5C and D). These data confirm the involvement of autophagy in controlling the inflammatory trait of adipocytes.

Discussion

Autophagy has been shown to affect many cellular processes including inflammation, oxidative stress and innate and acquired immune response (30). Our study provides evidence that autophagy modulates the inflammatory status of adipose tissue by controlling the production of pro-inflammatory cytokines including IL-1β, IL-6 and IL-8. Inhibition of autophagy leads to an increase in gene expression and secretion of pro-inflammatory cytokines by the adipose tissue.

Previous work has shown that insulin inhibits autophagic action and this regulatory pathway may partly explain the enhanced levels of LC3 within adipose tissue of obese individuals characterized by insulin resistance. Whereas autophagic inhibition in adipocytes and total adipose tissue explants both led to an enhancement in inflammatory gene expression and secretion of cytokines, it is tempting to speculate that changes in autophagy in adipocytes mainly contribute to the observed effects in line with a previous study (31). However, inhibition of autophagy is also known to promote inflammation in macrophages that are present in adipose tissue and profoundly affects its inflammatory status. Although it is challenging to identify the responsible cell type in our experimental setup using adipose tissue explants, a close interaction between various cell types including adipocytes and macrophages most likely determines the inflammatory-modulating properties of autophagy in adipose tissue.

It is unclear at this point whether the inhibition of autophagy in adipose tissue worsens insulin sensitivity via its effects on inflammatory cytokine production. However, in line with previous work demonstrating the deleterious effects of inflammation on insulin sensitivity (32, 33) one may hypothesis that the enhancement of autophagy in adipose tissue during obesity may serve to limit inflammation and prevent further worsening of insulin resistance. This hypothesis is supported by our observation that inhibition of autophagy in obese adipose tissue severely enhances a pro-inflammatory response compared to lean adipose tissue. Thus, obesity-associated inflammation in adipose tissue seems to up regulate autophagy in order to mitigate the production of pro-inflammatory cytokines. As such, autophagy activity seems a consequence, rather than a cause of obesity-induced adipose tissue inflammation. It remains to be explored whether (partial) defects in autophagy may contribute to the inflammatory process in obesity.

Our findings may appear somewhat counterintuitive, since the absence of autophagy has been shown to protect against obesity and insulin resistance in mice (14, 15). Indeed, adipocyte-specific ATG7 knockout mice exhibit increased insulin sensitivity and are protected against high-fat-diet induced obesity (13-15). However, the function of autophagy during the development of adipocytes may be quite different as compared to its role in expanding adipose tissue in obese subjects. In line with our

results, a previous study demonstrated that obese individuals had higher levels of autophagy in their adipose tissue compared to lean subjects (16), suggesting that autophagy may actually chaperon fat mass expansion during obesity. Since LC3-II levels were closely correlated with CD68 influx and the presence of CLSs in adipose tissue, autophagy activation may actually be aimed at counterbalancing inflammation. As variation in adipose tissue LC3 levels was observed in obese subjects, it might be worthwhile to further characterize the inflammatory status in future studies. Indeed, one may hypothesize that metabolically healthy obese, that are characterized by a lower inflammatory status of the adipose tissue (34), have a lower level of LC3-II in adipose tissue since the necessity of the inflammatory dampening action of autophagy is diminished.

The exact mechanism underlying enhanced inflammatory gene expression upon autophagy inhibition in adipose tissue remains to be identified. One possible mechanism may involve the intracellular degradation of pro-IL-1β by a process involving autophagy that would result in lower secretion levels of IL-1β (35). Hence, inhibition of autophagy would prevent intracellular degradation of pro-IL-1β and lead to enhanced production of IL-1β by adipose tissue. Inhibition of autophagy may also promote activation of the NLRP3 inflammasome in a reactive oxygen species (ROS) dependent manner and would thus produce more mature IL- 1β due to more caspase-1 cleavage (36, 37). Alternatively, nucleotide-binding oligomerization domain containing 2 (NOD2), which recognizes small peptides present in the bacterial wall, is important in regulating IL-1β gene expression as it functions by either binding to ATG16L1 to induce autophagy or by activating the Erk/NF-kB signaling pathway that induces IL-1β gene expression. Autophagy inhibition may down regulate ATG16L1 expression which could lead to more NOD2-dependent stimulation of Erk/NF-kB. This hypothesis is supported by a recent study (12) reporting that production of IL-1β by macrophages from individuals with the T300A variant of ATG16L1 is enhanced upon stimulation of the NOD2 receptor. Finally, mitochondrial autophagy has been linked to insulin resistance (38) and may somehow regulate inflammatory gene expression as well. Future studies should be aimed at deciphering the exact molecular mechanisms that facilitate pro-inflammatory responses upon inhibition of autophagic activity in adipose tissue on the development of inflammation.

In summary, inhibition of autophagy stimulates pro-inflammatory gene expression levels in both human and murine white adipose tissue. In adipose tissue of obese mice, the inflammation-promoting properties of autophagy inhibition outclass it effects in lean animals even after correction for total fat mass. Therefore, we hypothesize that autophagy functions as a mechanism to control pro-inflammatory gene expression in adipose tissue in order to prevent chronic inflammation. Altogether, this study provides new data showing that autophagy affects the inflammatory status of the adipocyte and adipose tissue.

Acknowledgements

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Supplemental Data

Table 1 Demographic and clinical characteristics of lean and obese subjects.

	Lean (n = 17)	**Obese (n = 16)**	***p*-value between groups**
Demographics			
Age (yr)	54 ± 3.9	55 ± 5.7	0.80
Sex, males (%)	35	25	0.80
Anthropometry and hemodynamics			
BMI (kg/m^2)	22.2 ± 0.3	32.8 ± 1.6	< 0.001
Waist circumference (cm)	82.9 ± 1.5	109.8 ± 3.2	< 0.001
Hip circumference (cm)	96.2 ± 2.1	110.5 ± 2.1	<0.001
Metabolic characteristics			
Total cholesterol (mM)	4.3 ± 0.3	4.6 ± 0.4	0.60
LDL-cholesterol (mM)	2.7 ± 0.4	2.8 ± 0.3	0.77
Triacylglycerol (mM)	0.8 ± 0.1	1.6 ± 0.4	0.03
Alcohol use (%)	77	50	0.20
Smoking (%)	0	13	0.60

Data presented as mean ± SEM. BMI denotes body mass index, LDL-cholesterol = low-density lipoprotein cholesterol.

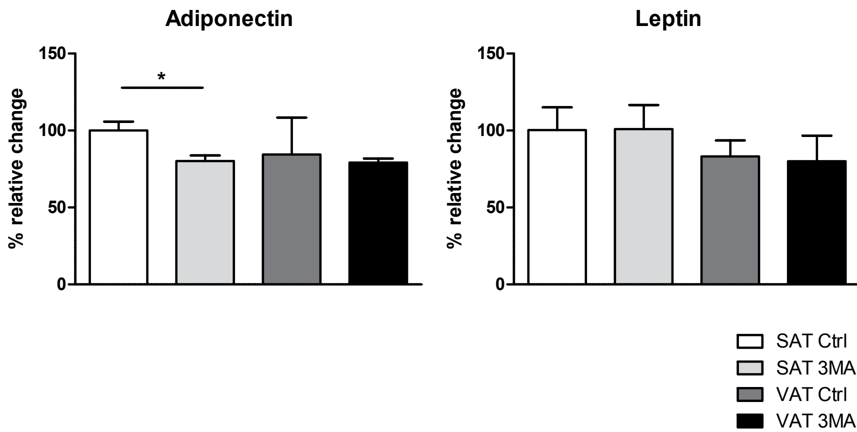

Figure 1 Leptin and adiponectin protein concentrations secreted by SAT and VAT after 3MA treatment. The secretion levels are expressed as the relative change in pg/ml/mg (cytokine/volume of supernatant/weight of explant). * $P < 0.05$.

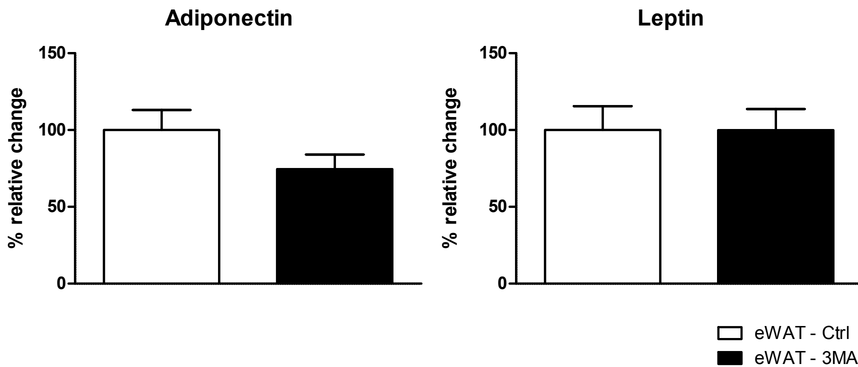

Figure 2 Leptin and adiponectin protein secretion by eWAT after 3MA treatment. The secretion levels are expressed as the relative change in pg/ml/mg (cytokine/volume of supernatant/weight of explant).

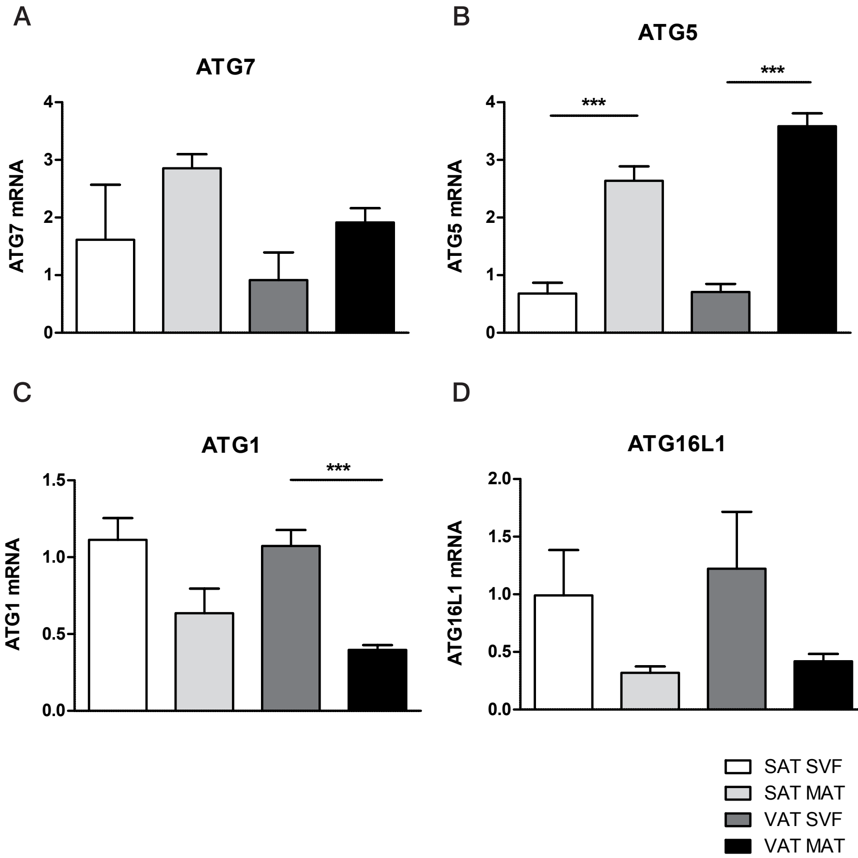

Figure 3 Relative gene expression levels of ATG1, ATG5, ATG7 and ATG16L1 in SVF and MAT fraction of both SAT and VAT of human individuals (n = 4). Control samples were set at 1. Data are presented as means ± SEM. *** $P < 0.001$.

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Chapter 9

Classification of subcutaneous adipose tissue inflammation in humans

H.J. Jansen, R. Stienstra, J.A. van Diepen, G. Vervoort, C.J. Tack

Submitted

Abstract

During the development of obesity, the phenotype of the adipose tissue changes significantly and shifts towards a more pro-inflammatory status. Although various morphological changes are known to accompany the development of obesity-induced inflammation, no system to classify the degree of adipose tissue inflammation currently exists.

In this cross-sectional study, we examined the association of adipose tissue morphological characteristics related to inflammation with various anthropometric measurements, adipose tissue protein levels of inflammatory and metabolic mediators and circulating plasma parameters in humans varying in BMI from 19 to 43 kg/m^2.

Adipocyte cell size was positively associated with body mass index, waist circumference, insulin sensitivity (HOMA-IR) and plasma leptin levels. Macrophage infiltration and the presence of crown like structures in adipose tissue correlated with various anthropometric parameters, insulin sensitivity and adipocyte size. We developed an inflammatory score of the adipose tissue, determined by the degree of adipocyte hypertrophy, macrophage influx and presence of crown like structures. Individuals that were classified with a high inflammatory score of the adipose tissue suffered from increased homeostasis model assessment of insulin resistance (HOMA-IR) levels.

We conclude that there is a close relationship between adipose tissue morphological characteristics, obesity and metabolic complications. Furthermore, we suggest a histological classification method to quantify adipose tissue inflammation. A standardized approach to classify adipose tissue inflammation will lead to more accurate measurements of adipose tissue inflammation and will aid comparison of different human studies using adipose tissue biopsies.

Introduction

During the development of obesity, the phenotype of the adipose tissue significantly changes and shifts towards a more pro-inflammatory profile. The enhanced inflammatory status in obese individuals, that has been suggested to primarily originate from expanding adipose tissue, contributes to the development of systemic insulin resistance and type 2 diabetes (1, 2). Indeed, therapeutic approaches that modulate inflammation have been shown to improve glycemic control in patients with type 2 diabetes (3, 4).

Various morphological changes in adipose tissue accompany the development of obesity-induced inflammation. For example, adipocyte enlargement is known to promote the inflammatory trait of the cell (5). Larger adipocytes produce higher amounts of inflammatory mediators as compared to smaller adipocytes isolated from the same adipose tissue depot (6). Enlargement of adipocytes inevitable leads to a lack of sufficient oxygen and nutrient supply and result in local hypoxia. Hypoxic conditions may result in adipocyte death that subsequently attracts macrophages into adipose tissue (7, 8). While also other triggers may initiate macrophage influx, macrophage accumulation is a typical characteristic of adipose tissue inflammation and aggravates the secretion of pro-inflammatory mediators by the adipose tissue and reduces secretion of insulin-sensitizing proteins including adiponectin (9). Upon infiltration into adipose tissue, macrophages are frequently displayed in so called crown like structures that are thought to surround dysfunctional or dying adipocytes (10). Macrophages that cluster into crown-like structures display a more pro-inflammatory phenotype (11, 12) and thus inflict a higher inflammatory status of the adipose tissue.

Changes in morphological characteristics of the adipose tissue are closely correlated with a variety of metabolic abnormalities. For example, enhanced macrophage influx into adipose tissue is known to positively correlate with hepatic fat deposition (11, 13).

Although these inflammatory characteristics of the adipose tissue are frequently described in literature, a general classification system of the adipose tissue inflammatory status is currently lacking. Whereas the development of fat deposition and inflammation in the liver is classified using a uniformed system (14, 15), adipose tissue inflammation currently describes a whole range of inflammatory changes that vary from influx of macrophages or other immune cells to changes in adipocyte gene expression and protein levels.

In the present study we first correlated human adipose tissue morphological characteristics including adipocyte size, macrophage influx and the presence or absence of crown-like structures with several anthropometric measurements, circulating plasma parameters and adipose tissue protein levels of various inflammatory and metabolic proteins to determine the contribution of adipose tissue dysfunction to metabolic abnormalities.

Based on the morphological characterization of the adipose tissue, we subsequently developed an inflammatory score. This approach may lead to a more accurate assessment of the inflammatory status of the adipose tissue and will aid comparison of different studies that use human adipose tissue biopsies.

Material and Methods

Subjects

In this study healthy subjects and patients with type 2 diabetes (T2DM) were included. Healthy subjects consisted of lean, overweight and obese subjects and were recruited through multiple advertisements in local newspapers. We included normal (body mass index 19-25 kg/m^2), overweight (body mass index 25-30 kg/m^2), and obese (body mass index 30-45 kg/m^2) subjects between 40 and 70 years old. The obese subgroup presented in the results consisted of both the overweight and obese subgroup. Furthermore, we included patients with T2DM who were on long-term stable oral glucose lowering treatment yet were not using any insulin. Exclusion criteria were other types of diabetes, significant cardiovascular, renal, liver or other co-morbidity, use of corticosteroids, uncontrolled endocrine disorders (stable supplementation with thyroid hormone was allowed), bariatric treatment, excessive alcohol consumption (>20 g/day), drug abuse, and use of thiazolidinedione derivatives. All patients with diabetes were treated according to local guidelines. Subject characteristics (age, gender, race, smoking habits) were noted and clinical data, including body weight, height, blood pressure, HbA_{1c}, diabetes duration, and type of oral glucose-lowering medication were measured, and blood samples were obtained.

Healthy, obese and non-obese individuals underwent a history and a complete physical examination. The inclusion and exclusion criteria were reviewed at the screening visit. Subsequently, extended anthropometric and metabolic tests were performed and subcutaneous adipose tissue biopsies were obtained. The local institutional review board of the Radboud University Nijmegen Medical Centre approved the study protocol. All subjects provided written informed consent.

Body fat distribution

Patients were measured in fasted overnight conditions with an empty bladder. Waist circumference was measured midway between the lower rib margin and the iliac crest at expiration, and hip circumference over the greater trochanter. Body composition was assessed by using the formula described by Jackson and Pollock (16).

Biochemical analyses

Plasma glucose concentration, HbA_{1C}, lipids, free fatty acids were measured by standard laboratory methods. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following formula: fasting plasma insulin (μIU/mL) x fasting plasma glucose (mmol/L)/22.5. Plasma concentrations of leptin and total adiponectin were determined using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis). Furthermore, high sensitive C-reactive protein (hsCRP) was measured by enzyme-immunoassay according to the instructions from the manufacturer (Dako, Glastrup, Denmark).

Subcutaneous adipose tissue biopsies

Subcutaneous (sc) adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6–10 cm lateral to the umbilicus, after an overnight fast. Morphometry of individual fat cells was assessed using digital image analyses as described previously (17). For each subject, the adipocyte cell diameter of all fat cells in five to ten microscopic fields of view were counted and measured. On average, ~700 fat cells were measured per specimen (range 150–1500). For detection of macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Clone EBM11, Dako, Denmark A/S). The percentage of macrophages was expressed as the total number of CD68-positive cells divided by the total number of adipocytes counted in 20 random microscopic fields of view x 100. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (10).

Total RNA was extracted from sc adipose tissue as described previously (17). cDNA synthesis was performed using iScript (Bio-Rad Laboratories, Hercules, CA). Expression of genes was determined by real-time PCR analysis using an Applied Biosystem (Warrington, UK). Gene expression results were normalized to β2M levels. Any missing values were due to technical issues during qPCR analysis. Genes and primer sequences used in this study are listed in supplementary Table 1.

Protein levels within the adipose tissue were measured by Luminex fluorescent bead human cytokine immunoassays (MILLIPLEX MAP, Millipore Corp., Billerica, USA). Briefly, adipose tissue lysates were prepared using the milliplex map lysis buffer (Millipore) and protein concentrations of Interleukin-6, leptin, and monocyte chemoattractant protein (MCP)-1 were determined. Equal amounts of protein were analysed using a Bioplex system (Bio-rad Laboratories Ltd., United Kingdom). Missing values were due to measurements beneath the detection level or to low adipose tissue biopsy sample collection.

Statistical analyses

Variables are expressed as means ± SEM. Correlations were calculated by Pearson correlation and Spearman rank correlation analyses, as appropriate. The Wilcoxon

signed rank test was used to compare non-parametric data. A one-way ANOVA was used to compare variables between different classes of inflammation, after variables were log-transformed.

All calculations were performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL). Two-tailed $P < 0.05$ was considered significant.

Results

A total of 118 subjects (29 lean, 46 obese subjects and 43 patients with T2DM) were included in this study and underwent a subcutaneous adipose tissue biopsy. The baseline anthropometric and laboratory characteristics of the study group are shown in Table 1. Glycaemic control (HbA_{1c}) in the diabetes group was 8.7 ± 0.2 %.

Table 1 Characteristics of study population.

	Mean ± SE / % (*n* = 118)
Demographics	
Age (yr)	57 ± 0.8
Sex, no. of males (%)	63 (51)
Anthropometry and hemodynamics	
BMI (kg/m^2)	28.5 ± 0.5
Waist circumference (cm)	100 ± 1.3
Hip circumference (cm)	102 ± 0.8
SBP (mmHg)	140 ± 2
DBP (mmHg)	83 ± 1
Metabolic characteristics	
TC (mmol/l)	4.5 ± 0.1
TG (mmol/l)	1.7 ± 0.1
HDL (mmol/l)	1.1 ± 0.04
LDL (mmol/l)	2.8 ± 0.1
FFAs (μmol/l)	0.52 ± 0.02
Alcohol use (%)	47
Smoking (%)	12

BMI denotes body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, TG = triglycerides, HDL = high-density lipoprotein cholesterol, LDL = low-density lipoprotein, FFAs = free fatty acids.

Subcutaneous adipose tissue biopsy phenotyping

Calculated lean body mass was 64.3 ± 1.1 kg and fat mass equalled 17.0 ± 0.9 kg. A significant variation in adipocyte size was observed amongst the non-diabetic subjects (Figure 1A and B). Similarly, the influx of macrophages, quantified as the amount of CD68-positive cells/adipocyte, varied among the study population (Figure 1C and D). Noticeably, we were also able to detect clustering of macrophages in so called crown-like structures (10) in multiple adipose tissue biopsies (Figure 1C).

Because adipocyte size is closely connected to bodyweight and total body fat, we initially set out to correlate adipocyte size with various anthropometric measurements. In line with previous studies (5, 7), BMI and waist circumference were positively correlated with adipocyte size (Table 2). Additionally, various circulating parameters including leptin, primarily used as a marker for adipose tissue mass, correlated with adipocyte size (Table 2). Whereas leptin was positively correlated with adipocyte size,

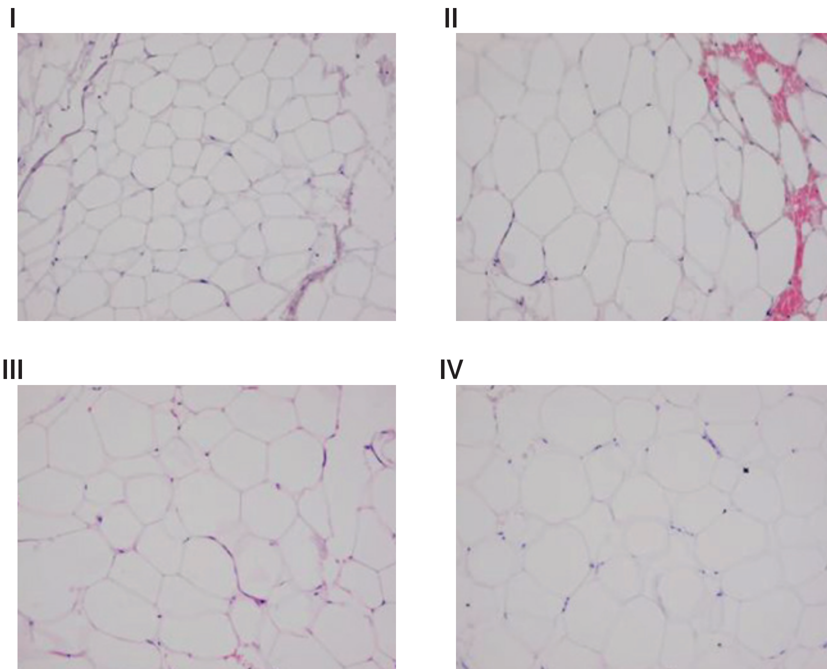


Figure 1A Sc adipose tissue morphology and variation in adipocyte size among the study population (n = 4 subjects). Panel I: mean adipocyte size 45 μm, panel II: 58 μm, panel III: 70 μm, panel IV: 86 μm. All sections were stained with hematoxylin (colored blue). Magnification: 10x.

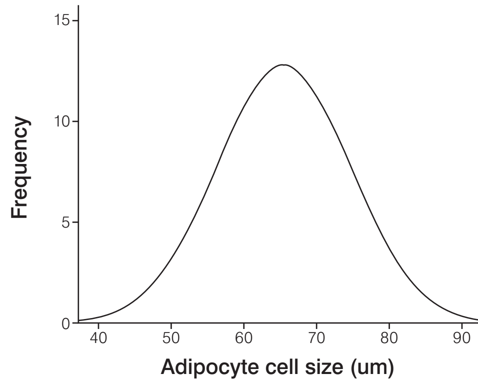


Figure 1B Adipocyte cell size distribution within our study population. Bin width 2 μm. $n = 118$ individuals.

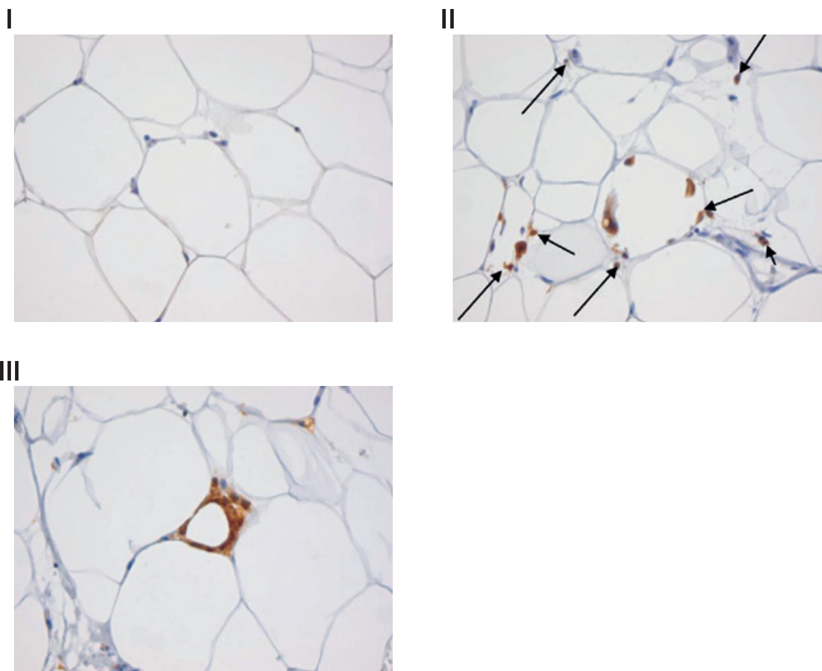


Figure 1C CD68-immunohistochemical staining of 3 representative patients with no influx of CD68-positive cells in the sc adipose tissue (panel I), high influx of CD68-positive cells (panel II) and sc adipose tissue with a crown-like structure (panel III). All sections were counterstained with hematoxylin (colored blue). Magnification: 20x. Arrows indicate CD68-positive cells.

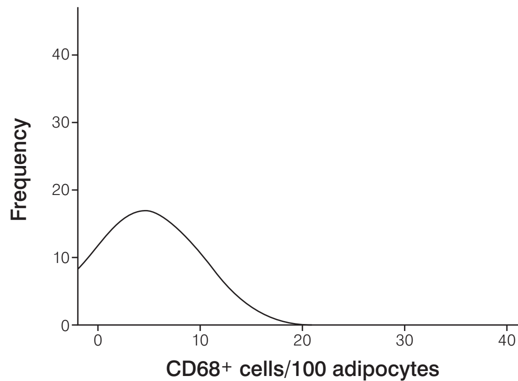


Figure 1D Distribution of CD68^{+}-cells influx within our study population. Bin width 2 μm. n = 118 individuals.

enlargement of adipocytes was not associated with circulating concentrations of adiponectin. We measured high sensitive C-reactive protein (hsCRP) as a systemic inflammation marker to correlate with the adipocyte cell size. There was a borderline association between adipocyte cell size and hsCRP (Table 2). HOMA-IR index, generally used as a circulating readout of insulin sensitivity, correlated positively with adipocyte size implying that a deterioration in insulin sensitivity is accompanied by an enlargement of adipocytes (Table 2).

Adipocyte cell size did not correlated with gene expression levels of adiponectin or GLUT4. In contrast, gene transcription levels of MCP-1 positively correlated with adipocyte size (Table 2).

Adipokine levels within adipose tissue correlated with adipocyte cell size

In various studies, anthropomorphic measurements and circulating concentrations of various proteins only indirectly reflect adipose tissue function. Therefore, we measured the concentration of various proteins within the subcutaneous adipose tissue using luminex techniques and correlated these values to adipocyte size. There were significant correlations between adipocyte cell size and protein levels of leptin, monocyte chemotactic protein-1 (MCP-1), and interleukin-6 (IL-6) measured within the adipose tissue (Table 2).

Noticeably, protein levels of leptin measured in the adipose tissue significantly correlated with systemic leptin and hsCRP levels (r = 0.30; P = 0.005 and r = 0.25; P = 0.02, respectively).

Table 2 Correlation coefficients between adipocyte cell size, number of CD68-positive cells and various clinical, laboratory and adipose tissue parameters.

Variable	Adipocyte cells size (μm)			CD68-positive cells/adipocyte		
	n	*r*	*P*-value	*n*	*r*	*P*-value
BMI (kg/m^2)	118	0.44	< 0.001	119	0.31	0.001
Waist circumference (cm)	117	0.45	< 0.001	118	0.38	< 0.001
Leptin (μmol/l)	105	0.38	< 0.001	-	-	-
hsCRP (pmol/l)	108	0.17	0.08	108	0.27	< 0.01
HOMA-IR	99	0.26	< 0.01	99	0.16	0.11
AT morphology						
Adipocyte cell size (μm)	-	-	-	118	0.46	< 0.001
Gene expression levels AT						
mRNA adiponectin	109	0.11	0.26	110	0.025	0.80
mRNA GLUT4	108	-0.12	0.22	110	-0.005	0.96
mRNA MCP-1	109	0.33	< 0.001	110	0.34	< 0.001
Protein levels AT						
Leptin (pg/ml)	109	0.42	< 0.001	110	0.34	< 0.001
MCP-1 (pg/ml)	110	0.42	< 0.001	111	0.40	< 0.001
IL-6 (pg/ml)	72	0.25	0.03	73	0.14	0.25

BMI = body mass index, hsCRP = high sensitive C-reactive protein, HOMA-IR = Homeostasis model assessment-insulin resistance, AT = adipose tissue, GLUT4 = glucose transporter type 4, MCP-1 = monocyte chemoattractant protein-1, IL-6 = interleukin-6. - not applicable.

Macrophage infiltration in subcutaneous adipose tissue correlates with BMI, waist circumference, insulin sensitivity and adipocyte size

To learn more about the role of macrophage influx in the development of obesity, insulin resistance and inflammation, we correlated the number of macrophages, as quantified by CD68-staining, with various morphometric and circulating parameters. As shown in Table 2, the number of CD68-positive cells within the adipose tissue positively correlated with body mass index, waist circumference, adipocyte cell size, hsCRP levels and HOMA-IR (borderline significance).

Clustering of macrophages in crown like structures is correlated with a worsening in adipose tissue characteristics and insulin resistance

Adipose tissue macrophages are known to cluster in so called crown-like structures that possess a high inflammatory trait (11). Classification of our study population based on the presence or absence of crown-like structures (CLSs) within subcutaneous adipose tissue, revealed that subjects with crown like structures present in subcutaneous adipose tissue had a significantly higher adipocyte cell size, influx of macrophages, MCP-1 protein levels and HOMA-IR (borderline significance) as compared to subjects that lack any CLS in adipose tissue (Figure 2). Subjects with and without CLSs did not differ regarding mRNA levels of adiponectin, GLUT4, and MCP-1, BMI or waist circumference.

Classification of adipose tissue inflammation

Although the term adipose tissue inflammation is frequently used throughout literature, no standard classification system is currently available to rank severity of adipose tissue inflammation. The available data on adipose tissue histology in this study was used to categorize the degree of adipose tissue inflammation. By applying three parameters of adipose tissue morphology that can be assessed in a similar manner in various laboratories using standard histological techniques, we defined a classification method. A combined score using means as cut-off points of adipocyte size (mean adipocyte cell size diameter was 65.4 μm, so adipocyte cell size $\leq$ 65.4 μm was scored as 0 and adipocyte cell size $>$ 65.4 μm was scored as 1), macrophage influx (mean number of macrophages present in the adipose tissue was 0.04/adipocyte, so macrophage influx of $\leq$ 0.04 was scored as 0 and macrophage influx $>$ 0.04 was scored as 1) and the presence of crown like structures (no = 0 or yes = 1), will add up to a minimal value of 0 or maximal value of 3. A score below 1 was regarded as no inflammation of the adipose tissue; a score between 1 and 2 as moderately inflamed adipose tissue and a score above 2 as severely inflamed adipose tissue. Table 3 shows the proposed scoring system for quantifying adipose tissue inflammation.

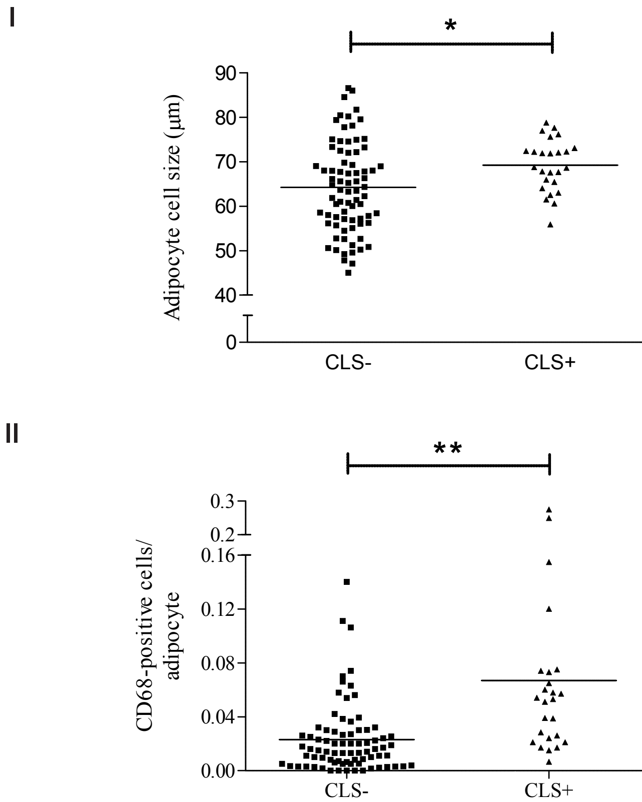
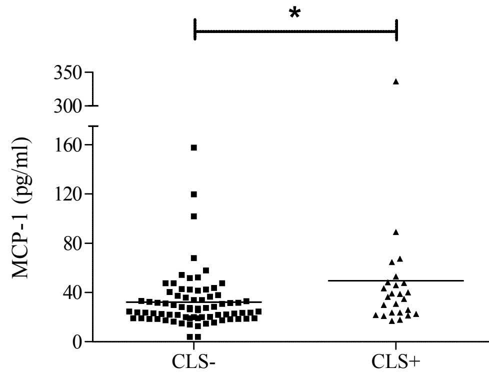


Figure 2 Significant differences in adipocyte cell size (panel **I**: CLS- n = 84; CLS+ n = 34), CD68-positive cells/adipocyte (panel **II**: CLS- n = 85; CLS+ n = 34; 5 subjects in CLS- group displayed no CD68-positive cells/adipocyte), monocyte chemotactic protein-1 (MCP-1) protein levels (panel **III**: CLS- n = 80; CLS+ n = 31) measured within the subcutaneous adipose tissue and homeostasis model assessment–insulin resistance (panel **IV**: HOMA-IR; CLS- n = 70; CLS+ n = 29)) comparing the group without crown-like structures (CLS-) and with crown-like structures (CLS+). HOMA-IR was calculated by: fasting plasma insulin (μIU/mL) x fasting plasma glucose (mmol/L)/22.5. Horizontal lines within figures represent mean levels. * $P < 0.05$, ** < 0.001, $^{\#}$ $P = 0.10$.

III



IV

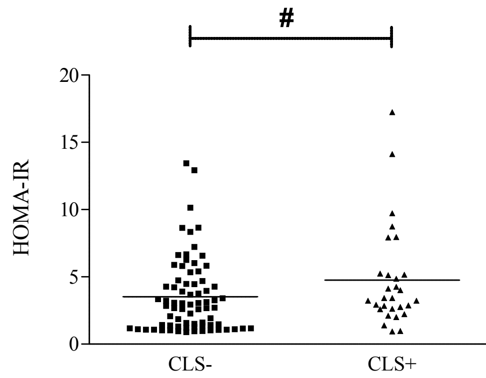


Figure 2 Continued.

Table 3 Adipose tissue inflammatory classification.

Parameter	Cut-off	Score
Adipocyte cell size	≤ 65.4 μm	0
Adipocyte cell size	> 65.4 μm	1
CD-68 positive cell influx/adipocyte	≤ 0.04	0
CD-68 positive cell influx/adipocyte	> 0.04	1
CLS*	No	0
CLS	Yes	1

* CLS = crown-like structures. A score below 1 represents no adipose tissue inflammation. A score of 1-2 represents moderate adipose tissue inflammation. A score of > 2 represents severe adipose tissue inflammation. In this analysis 42 subjects were assigned as no adipose tissue inflammation, 31 subjects were assigned as having moderate adipose tissue inflammation and 45 subjects assigned as having severe adipose tissue inflammation.

Based on this score, $n = 42$ subjects (36%) were defined as no inflammatory status, $n = 31$ (26%) subjects as moderate and $n = 45$ subjects (38 %) as severe adipose tissue inflammation. Table 4 shows the distribution of subjects within the different inflammatory classes.

Table 4 Distribution of subjects within different inflammation classes.

	Lean	Obese	T2DM
No inflammation (IF-class < 1)	22 (76%)	13 (28%)	7 (16%)
Moderately inflamed AT (IF-class 1-2)	6 (21%)	14 (30%)	11 (26%)
Severely inflamed AT (IF-class > 2)	1 (3%)	19 (42%)	25 (58%)

Distribution of subjects (*n*/%) within the different inflammatory classes. AT denotes adipose tissue, IF-class denotes inflammatory class, and T2DM denotes type 2 diabetes mellitus.

Interestingly, subjects characterized by severe adipose tissue inflammation score had higher HOMA-IR indices indicating the presence of insulin resistance (Figure 3).

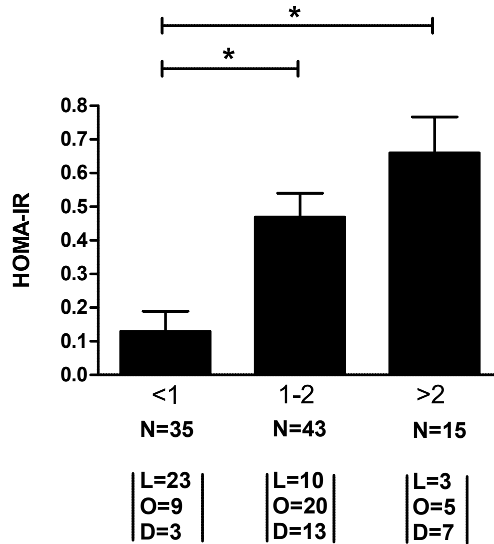


Figure 3 Classification of study population based on adipose tissue inflammatory classification. Higher inflammation class (0→2) is associated with increased insulin resistance as measured by HOMA-IR. Data were analyzed with one-way analysis of variance (ANOVA) after variable was log transformed. L = number of lean subjects, O = number of obese subjects (overweight and obese subjects were included in this group) and D = number of patients with diabetes. * $P < 0.05$.

Discussion

While animal studies have extensively documented that the development of obesity is accompanied by adipose tissue inflammation that includes adipocyte enlargement and macrophage influx, inflammatory changes in adipose tissue in humans, have been less frequently described. The present study, in which almost 120 patients and adipose tissue samples are included, reveals that changes in adipose tissue morphology closely relate to obesity and metabolic complications in a large group of subjects varying in BMI with or without diabetes. Moreover, we propose using a classification system in which three morphological parameters are scored to categorize the inflammatory status of the adipose tissue. This may lead to a more accurate measurement of adipose tissue inflammation and may help to compare different human studies.

In line with previous work (5, 18), our data reveal that not only the absolute numbers of macrophages dictate a worsening in adipose tissue inflammation, systemic inflammation

and insulin sensitivity, but that localization in so called crown like structures is of importance. Indeed, classification of individuals using the absence or presence of crown like structures in subcutaneous adipose tissue as a selection criterion revealed that CLSs are associated with a larger adipocyte size and insulin resistance.

Despite the fact that various studies have reported on human adipose tissue morphology, a uniform method to quantify adipose tissue inflammation is currently lacking. As a consequence, different readouts to define adipose tissue inflammation are used throughout literature, hence making it difficult to directly compare results of various human studies. By using three morphological determinants that include adipocyte size, the severity of macrophage influx and the presence of crown like structures, we propose a classification tool to determine the severity of adipose tissue inflammation. Our model does not take into account the influx of various other immune cells and future refinements may lead to improvements of our scoring model. For example, additional immunohistochemical stainings to detect and quantify other immune cells like T-cells or B-cells may be included. More irreversible features of adipose tissue morphology including fibrosis that occur during obesity (19) may also need to be included to further fine-tune the classification system. However, these morphological characteristics are not as well established as compared to macrophage influx and adipocyte size and will first need to be more carefully evaluated before inclusion into a model to predict inflammatory changes of human adipose tissue.

A more standardized approach to classify adipose tissue inflammation will lead to a more accurate measurement of adipose tissue inflammation and may help to compare different human studies. Combining results of different studies and thereby increasing power, may eventually aid the identification of circulating biomarkers that help to predict the inflammatory status of the adipose tissue. Increasing power by including other studies may aid the discovery of a set of (novel) circulating biomarkers that may eventually be used to predict the inflammatory status of the adipose tissue. As such, it may allow for a more personalized therapeutic approach to target inflammatory pathways specifically in obese patients that suffer from a high degree of adipose tissue inflammation.

Although the use of only one fat depot is a drawback of our study, morphological parameters of the subcutaneous adipose tissue correlated well with various anthropometric measurements and HOMA-index. Whereas future studies ideally include measurements in visceral adipose tissue, our results imply that inflammatory changes in subcutaneously stored adipose tissue are correlated to systemic effects including levels of insulin sensitivity. Important other points of consideration include differences in the inflammatory profile by biopsy sampling (needle versus surgical techniques) (20). It is known that biopsy techniques influence the gene expression underlying the biological themes currently discussed in obesity (e.g. inflammation, extracellular matrix, and metabolism).

In conclusion, our data clearly demonstrate that morphological changes in subcutaneous adipose tissue are strongly associated with various metabolic abnormalities. Using a classification system in order to categorize the inflammatory status of the adipose tissue provides a tool to compare different clinical studies. Increasing power by combining different studies may assist in establishing a combination of circulating plasma parameters that can be used to predict the inflammatory status of the sc adipose tissue. Knowledge of the adipose tissue inflammatory status may help in determining the optimal (personalized) therapeutic strategy to treat patients suffering from obesity and type 2 diabetes.

Supplemental data

Table 1 Primer sequences.

Human	
	Forward primer / **Reverse primer**
Gene symbol	**RT-PCR (real-time PCR)**
B2M	5'-ATGAGTATGCCTGCCGTGTG-3'
	5'-CCAAATGCGGCATCTTCAAAC-3'
SLC2A4	5'-CTCAGCAGCGAGTGACTGG-3'
	5'-CCCCAATGTTGTACCCAAACTG-3'
ADIPOQ	5'-ATCGGTGAAACCGGAGTACC-3'
	5'-GCATGTTGGGGATAGTAACGTAA -3'
CCL2	5'-CCAGTCACCTGCTGTTATAAC-3'
	5'-TGGAATCCTGAACCCACTTCT-3'

B2M = beta-2-microglobulin
SLC2A4 = glucose transporter type 4
ADIPOQ = adiponectin
CCL2 = monocyte chemoattractant protein-1

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Chapter 10

Summary and conclusions

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The treatment of patients diagnosed with type 2 diabetes mellitus (T2DM) requires lifestyle changes and often oral glucose lowering medication to maintain blood glucose levels in the near normal range. When glucose control fails with oral medication, insulin is needed to achieve adequate glycemic control, but often at the expense of weight gain. While insulin therapy has several beneficial effects, insulin-associated weight is clearly undesirable in an already obese population. Furthermore, weight gain may cause several complaints (e.g. joint problems) and increase the risk of cancer.

From a clinical perspective it is known that not all patients starting insulin treatment will gain weight and it would be valuable to identify factors that determine inter-individual differences with respect to insulin-associated weight gain. In addition, potential consequences of insulin-associated weight gain on body composition, subcutaneous abdominal adipose tissue and cardiovascular risk are currently unknown. Identifying determinants and cardiometabolic consequences of insulin-associated weight gain may open avenues towards therapeutic strategies to limit or even prevent this weight gain. In this thesis, we describe the results of a series of investigations aimed at identifying the determinants and consequences of insulin-induced weight gain in patients with T2DM.

Chapter 1 provides an overview of proposed determinants and consequences of insulin-associated weight gain as described in literature. This chapter also provides background information regarding some of the measurements used in our studies.

In **chapter 2** the results of a retrospective study about the relationship between insulin-associated weight gain and change in HbA_{1c} in 122 patients with type 2 diabetes are described. Data over a period of three years was collected. Body weight increased substantially by 0.54 kg per month in the first 9 months after start of insulin therapy. As expected, there was a large inter-individual variation with approximately 15 % of patients not gaining any weight, but also approximately 30 % of patients in whom body weight increased by more than 10 kg after 36 months of insulin therapy. The increase in body weight was most pronounced in the first 9 months, but continued over the whole 3 years after the start of insulin therapy. By using a linear mixed model, individual body weight profiles could be estimated and related to a change in HbA_{1c} over this 36 month period. Unlike earlier findings, only 12 % of the bodyweight gain found in our study could be attributed to the change in HbA_{1c}. It was also observed that obese patients show less insulin-associated weight gain compared to patients with lower body weight. These findings suggest that the presence of obesity should not be a reason to withhold insulin therapy because of fear for weight gain.

While improved glycemic control explains a part of insulin-induced weight gain, several other factors are probably involved. A decrease in physical activity upon the

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start of insulin therapy may induce weight gain. We first questioned ourselves whether physical activity was decreased in patients with insulin-treated T2DM compared to lean and weight-matched obese non-diabetic subjects. Sleep duration has also been associated with physical exercise level and weight. In a cross-sectional study, we quantitatively measured physical activity by accelerometry (Sensewear Pro Armband TM, see Chapter 1) in patients with T2DM and compared the results to groups of obese and lean subjects. In addition, sleep duration and quality were assessed. Indeed, patients with insulin-treated T2DM performed significantly less physical activity. However, this was not explained by a difference in sleep duration or quality. These results are described in **chapter 3**.

From the findings in chapter 3 it may be hypothesized that patients with T2DM decrease their physical activity after starting insulin treatment. In order to evaluate physical activity and other determinants of insulin-associated weight gain in patients with T2DM a prospective study was conducted (**chapter 4**). In this study, 65 patients with T2DM who started insulin therapy were followed for 12 consecutive months. The study was primarily powered to detect a relationship between insulin-associated weight gain and a change in physical activity. Other potential determinants of weight gain, including insulin dose and diabetes-related distress were measured as secondary outcomes. Not only baseline predictors, but also the contribution of changes of clinical variables over time were investigated. Again, we used a linear mixed model to assess the relationship between weight gain and changes in clinical variables. After 12 months of insulin therapy, mean body weight had increased significantly and physical activity (measured as metabolic equivalent (METS)) decreased, but this was not significantly related to weight changes. Similarly, reported caloric intake decreased significantly. However, diabetes-related distress (assessed by PAID-score) at baseline turned out to be a significant predictor of insulin-associated weight gain. The Problem Areas in Diabetes (PAID) survey was developed as a measure of diabetes-related stress and associates between psychological adjustment to diabetes and adherence to self-care behaviors. Other independent baseline predictors were: initial insulin dose and calculated body fat content. Also the change in insulin dose significantly influenced body weight over time.

The findings of this study may have clinical implications. First, health education should encourage insulin-treated patients to maintain, or even increase physical activity levels, to counteract, amongst others, weight gain. The clinical importance of identifying emotions that impact on body weight gain such as sadness in patients with type 2 diabetes may translate towards specific interventions (see below). Furthermore, as higher initial insulin dose was associated with more pronounced insulin-induced weight gain, it might be envisioned that (low) initial insulin dose and a more gradual titration of insulin over time may limit or even prevent body weight gain.

Next we investigated whether insulin-induced weight gain, especially when outspoken, would have adverse consequences.

Chapter 5 describes the results of a cross-sectional study in which two extreme groups (patients with large insulin-associated weight gain: “gainers” and patients with minimal weight gain after start of insulin: “non-gainers”) were compared with respect to cardiometabolic risk. Each subgroup consisted of 14 patients. Gainers had significantly more total body and trunk fat (especially subcutaneous fat) compared with non-gainers. Gainers had similar liver fat content, and slightly higher levels of hormones derived from adipose tissue. Furthermore, gainers performed significantly less physical activity. Lastly, gainers had higher total cholesterol, low-density lipoprotein cholesterol, and alanine aminotransferase levels with similar cholesterol-lowering treatment. Thus, patients with T2DM who show pronounced weight gain during insulin therapy have a less favourable cardiometabolic risk profile compared with patients who show no or minimal weight gain.

As shown, patients with T2DM who start insulin therapy, gain weight. Especially the subcutaneous abdominal compartment will increase. An increase in fat mass in obesity is associated with enlargement of adipocytes and pro-inflammatory changes in fat tissue. We questioned ourselves whether the expansion of adipose tissue mass would also be paralleled by morphological and inflammatory changes. We investigated the effects of weight gain on adipocyte size, macrophage influx and mRNA expression and protein levels of key inflammatory markers within the adipose tissue in patients with T2DM before and 6 months after the start of insulin therapy. This was the group of patients described in Chapter 4, and the results of this study were described in **Chapter 6**.

As expected, insulin significantly increased body weight. At the level of subcutaneous adipose tissue, insulin treatment led to an influx of macrophages. When comparing patients gaining no or little weight with patients gaining $>$ 4% body weight after 6 months of insulin therapy, both subgroups displayed an increase in macrophage influx. However, gainers had higher protein levels of monocyte chemo-attractant protein-1, tumor necrosis factor-α and interleukin-1β after 6 months of insulin therapy compared to non-gainers. There was no significant change in adipocyte cell size.

We conclude that insulin therapy in patients with T2DM improved glycemic control, yet also induced body weight gain and the influx of macrophages in subcutaneous adipose tissue. In patients characterized by pronounced insulin-associated weight gain, the influx of macrophages into adipose tissue is accompanied by a more pronounced inflammatory status.

Patients with T2DM are typically overweight and have an increased liver fat content (LFAT). High LFAT may be explained by an increased efflux of free fatty acids from the

adipose tissue which is partly driven by inflammatory changes. We hypothesized that there would be an association between inflammatory features of the adipose tissue and liver fat content. In **chapter 7** we measured LFAT content by proton magnetic resonance spectroscopy. A subcutaneous fat biopsy was obtained to determine morphology and protein levels within adipose tissue. In addition to fat cell size, the percentage of macrophages and the presence of crown like structures (CLSs) within sc fat was assessed by CD68-immunohistochemical staining. Mean LFAT was high (~11 %) and showed large inter-individual differences. Adipose tissue protein levels of the key inflammatory adipokine plasminogen activator inhibitor-1 (PAI-1) were positively associated with LFAT. Several pro-inflammatory changes in sc adipose tissue associate with increased LFAT content in obese insulin-treated patients with T2DM. These findings suggest that inflammatory changes at the level of the adipose tissue may accompany or even drive liver fat accumulation.

Autophagy, an evolutionary conserved process aimed at recycling damaged organelles and protein aggregates in the cell, also modulates pro-inflammatory cytokine production in peripheral blood mononuclear cells. Since adipose tissue inflammation accompanied by elevated levels of pro-inflammatory cytokines is characteristic for the development of obesity, we hypothesized that modulation of autophagy alters adipose tissue inflammatory gene expression and secretion. **Chapter 8** presents results of a study in which we performed ex vivo and in vitro studies of human and mouse adipose tissue. Our results demonstrate that autophagy activity is up regulated in adipose tissue of obese individuals and inhibition of autophagy enhances pro-inflammatory gene expression both in adipocytes and adipose tissue explants. Thus, autophagy may function to dampen inflammatory gene expression and thereby limit excessive inflammation in adipose tissue during obesity.

In literature, many studies investigate the inflammatory process within the adipose tissue. However, no exact definition exists with respect to the term “inflammation”. We propose a novel classification method to quantify inflammation in the adipose tissue. This classification method uses 3 morphological characteristics (i.e. adipose cell size, influx of macrophages and presence of crown-like structures) to quantify inflammation. In **chapter 9**, the inflammatory characteristics of adipose tissue of lean, non-diabetic obese subjects and patients with T2DM were compared. We conclude that there is a close affinity between adipose tissue morphological characteristics, obesity and metabolic complications. A standardized approach to classify adipose tissue inflammation will lead to more accurate measurements of adipose tissue inflammation and will aid comparison of different human studies using adipose tissue biopsies.

Conclusions and future perspectives

Insulin-associated weight gain in patients with type 2 diabetes patients is a serious clinical problem and leads to relevant changes in body composition, cardiometabolic profile and alters the inflammatory status at the adipose tissue level. In this thesis we investigated determinants and consequences of weight gain after start of insulin therapy.

A change in glycemic control, hence correction of glucosuria, is widely listed as a main determinant of insulin-associated weight gain. This factor may be most relevant in the initial phase of insulin treatment. However, the relationship between change in glycemic control and change in weight is complex and varies over time. We confirmed that a change in glycemic control (HbA_{1C}) is significantly associated with insulin-associated weight gain. Nevertheless, the intention of insulin therapy is to improve glycemic control and to decrease $HbA1_C$. As such, change in $HbA1_C$ is the consequence of insulin therapy. Therefore, although in our multiple linear regression model, change in $HbA1_C$ was related to weight gain, we did not include this variable in the final analyses because it does not represent a true independent variable.

We postulated that a change in physical exercise might contribute to insulin-associated weight gain. Indeed, we found that, after 12 months of insulin treatment, patients perform less physical activity as compared to before initiation of insulin therapy. However, while a decrease in physical activity in itself is unfavorable from a health care perspective, it did not appear to determine weight gain. Nevertheless, active counseling of physical activity, to prevent an unnecessary drop in level of exercise seems advisable.

Caloric intake to assess energy intake did not seem to be a determinant of insulin-associated weight gain either. In contrast, patients reported less intake at 6 and 12 months of insulin treatment.

We identified a number of new predictors of insulin-associated weight gain: initial insulin dose, diabetes-related distress as measured by PAID questionnaire and age. Our findings suggest a relevant influence of emotions and depressive complaints that may translate to eating an unhealthier diet or becoming less physically active and predominates to more insulin-associated weight gain. Based on these results, a therapeutic strategy may be to employ questionnaires (such as PAID) to assess diabetes-related distress before starting insulin therapy to provide insight into the mood of a patient. In patients with a high diabetes-related distress score, an intervention aimed at reducing distress may prevent or limit weight gain. Whether such an intervention is effective remains to be established.

The finding that a higher initial insulin dose was prospectively associated with more weight gain was new and intriguing. The starting dose was determined by the

physician and may be partly based on the level of hyperglycemia and /or on the estimated level of insulin resistance. The choice to start with a certain amount of insulin may also be physician dependent, or centre-dependent. A related determinant which influenced body weight significantly over time is change in insulin dose – more rapid increase in insulin dose was associated with more weight gain. The potentially involved mechanisms deserve further exploration, but the finding may have clinical consequences. One might consider to start with an (low) initial insulin dose and a more gradual titration of insulin over time to limit or even prevent body weight gain. Again, whether this translates to reduced insulin-induced weight gain remains to be proven.

With respect to the consequences of insulin-associated weight gain, we focused on the (change in) body composition and subcutaneous adipose tissue. Although not surprisingly, we showed that patients gaining pronounced body weight after the start of insulin therapy exhibit more subcutaneous adipose tissue, higher lipid levels and slightly higher levels of hormones derived from adipose tissue. It can be stated that this group of patients ("gainers") suffer from a less favorable cardiometabolic risk profile. This finding was also confirmed by exploring the subcutaneous adipose tissue with respect to morphology and inflammatory profile. We found that the influx of macrophages into adipose tissue is accompanied by a more pronounced inflammatory status in those patients suffering from pronounced weight gain. The systemic anti-inflammatory effects of insulin (therapy) might thus, at least partially, be offset by the pro-inflammatory changes associated with an increased fat mass. Theoretically, this subgroup of patients might benefit from anti-inflammatory therapy (like IL-β blockade) to reduce or even reverse the enhanced inflammatory status.

It is clear from our studies that insulin-associated weight gain may offset part of the beneficial effects of improved glycemic control and hence it is important to investigate new therapeutic modalities for patients suffering from insulin-associated weight gain. Besides lifestyle interventions and changes in insulin regimens, also additional pharmacological therapy could be considered. Glucagon-like peptide-1 (GLP-1) analogues may counteract insulin-induced body weight without deteriorating glycemic control. Recently, we initiated a clinical trial (ELEGANT) (clinicaltrials.gov Identifier: NCT00781495) to investigate whether addition of the GLP-1 analogue liraglutide can reverse weight gain while maintaining glycemic control in patients recently started on insulin who showed a pronounced weight gain. Results are expected in 2014.

Whether addition of the new class of glucose lowering drugs, the SGLT-2 inhibitors, has therapeutic value in counteracting insulin-induced weight gain requires additional studies.

The research described in this thesis contributes to a better understanding of insulin-associated weight gain in patients with T2DM by identifying important determinants including diabetes-related distress score, initial insulin dose and age. This may lead to the development of therapeutic strategies to limit or possibly prevent insulin-associated weight gain in order to optimize the care for patients with T2DM. Such a more personalized approach towards insulin treatment may result in more optimal use of one of main cornerstones of diabetes management.

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Chapter 11

Nederlandse samenvatting

Dit onderzoek gaat over oorzaken en consequenties van gewichtstoename bij personen met type 2 diabetes mellitus, ook wel “ouderdoms suikerziekte” genoemd en die behandeld worden met insuline.

De behandeling van mensen met type 2 diabetes mellitus heeft als doel het verkrijgen en behouden van een zo normaal mogelijke bloedglucosewaarde. De therapie bestaat allereerst uit dieet- en leefstijladviezen. Daarbij wordt, zo nodig, medicatie in de vorm van tabletten voorgeschreven die het glucosegehalte in het bloed verlagen. Indien tabletten niet (meer) voldoende helpen om de bloedsuiker te verlagen wordt gestart met behandeling met insuline. Insuline wordt onderhuids toegediend middels een injectie. Er zijn meerdere soorten insuline; langwerkende, kortwerkende en combinaties ervan.

Het gebruik van insuline heeft belangrijke positieve effecten. Allereerst geeft het een goede glucoseregulatie, waardoor mensen minder klachten hebben van hoge bloedsuikers. Verder wordt met insulinetherapie het risico op complicaties van de kleine en grote bloedvaten op langere termijn verminderd. Waarschijnlijk heeft dit te maken met het feit dat insuline een dempende werking lijkt te hebben op ontstekingsreacties in de bloedvaten.
Een ander, negatief, effect van insulinebehandeling dat kan optreden is gewichtstoename. Gewichtstoename bij personen met, maar ook zonder diabetes, kan leiden tot allerlei problemen zoals gewrichtsklachten, een verhoogd risico op kanker en op hart- en vaatziekten. Veel personen met type 2 diabetes mellitus hebben overgewicht. Extra gewichtstoename ten gevolge van insulinetherapie is ongewenst.

In de praktijk blijkt dat niet alle mensen met type 2 diabetes aankomen in gewicht na het starten van insulinebehandeling. Het is onduidelijk welke factoren een rol spelen bij de gewichtstoename die samenhangt (is geassocieerd) met insuline; de zogenoemde determinanten van de insuline-geassocieerde gewichtstoename. Verder is het onbekend welke gevolgen insuline-geassocieerde gewichtstoename heeft op de vetverdeling in het lichaam, op de kenmerken van het onderhuids vetweefsel en op het risico op hart- en vaatziekten.

Het identificeren van de determinanten van insuline-geassocieerde gewichtstoename en het vergroten van het inzicht op de gevolgen hiervan kunnen leiden tot strategieën om gewichtstoename bij insulinegebruik te voorkomen dan wel te verminderen.

In dit proefschrift zijn de resultaten van verschillende onderzoeken naar de oorzaken en gevolgen van de insuline-geassocieerde gewichtstoename en de consequenties hiervan bij personen met type 2 diabetes mellitus beschreven. Bij de meeste van deze studies betreft het klinisch, patiënt-gebonden, onderzoek. Meerdere onderzoeksmethodes zijn toegepast om van deze studiedeelnemers klinische gegevens te verzamelen. Door middel van laboratoriumonderzoek werd onder meer de bloedglucoseregulatie bepaald. Ook werd beeldvormend onderzoek toegepast, namelijk

Magnetische Resonantie Imaging/Spectroscopie (MRI/MRS), om onderhuids vetweefsel en vetweefsel rondom de inwendige organen en levervet te meten. Door het dragen van een bewegingsarmband, de SenseWear Pro Armband^{TM}, werd bewegingsactiviteit gemeten. Dit apparaat werd een aantal dagen aan de rechter bovenarm gedragen en registreerde kwantitatief bewegingsactiviteit. Ook werd bij de meeste mensen een stukje onderhuids vet afgenomen. Bij dit vetbiopt werd onder lokale verdoving met een holle naald naast de navel ongeveer 1 g vetweefsel afgenomen. Dit stukje vetweefsel werd bewerkt om individuele vetcellen en de architectuur ervan te bekijken (histologie), immuuncellen (met name macrofagen) te kwantificeren en om ontstekingsactiviteit te meten.

Hoofdstuk 1 is een algemene inleiding. Hierin wordt beschreven welke oorzaken en gevolgen van insuline-geassocieerde gewichtstoename bij personen met type 2 diabetes mellitus bekend zijn in de literatuur. Dit hoofdstuk geeft eveneens achtergrondinformatie over de diverse meetmethoden die zijn gebruikt in de verschillende onderzoeken. Het bevat ook een beschrijving van mogelijke mogelijkheden om gewichtstoename bij insulinegebruik te voorkomen.

In elk van de volgende hoofdstukken wordt een studie beschreven. De hoofdstukken 2 tot en met 4 beschrijven de resultaten van de studies over de oorzaken van insuline-geassocieerde gewichtstoename. Vervolgens gaan de hoofdstukken 5 tot en met 9 over de resultaten van de studies naar de gevolgen van insuline-geassocieerde gewichtstoename bij personen met type 2 diabetes mellitus.

Veel diabetesexperts relateren gewichtstoename bij insuline aan de verandering van diabetesinstelling; verbetering van de glucoseregulatie en daardoor verandering van HbA_{1C}. Ze verklaren de gewichtstoename doordat minder glucose wordt uitgeplast (per saldo minder calorieverlies) en bij gelijke calorie inname en onveranderd gebruik de energiebalans dus positiever wordt.

In **hoofdstuk 2** worden de resultaten beschreven van de retrospectieve studie waarbij is gekeken naar de relatie tussen gewichtstoename en verandering van HbA_{1C} bij 122 personen met type 2 diabetes mellitus. Met een statistisch model (het zogenaamde “lineair mixed model”) is de verandering van de waardes van het gewicht en HbA_{1C} van de mensen in het verloop van 36 maanden geanalyseerd. Personen kwamen significant aan in gewicht, maar de verandering van HbA_{1C} verklaarde slechts 12 % van deze gewichtstoename. Verder kon geconcludeerd worden dat personen die al dik waren minder gewichtstoename leken te ontwikkelen. Overgewicht bij de start van insulinetherapie lijkt min of meer “beschermend” te zijn tegen nog extra gewichtstoename bij gebruik van insuline. Hieruit kan worden geconcludeerd dat personen met type 2 diabetes en overgewicht insulinetherapie juist niet onthouden zou moeten worden.

Hoofdstuk 3 gaat over de resultaten van de studie naar bewegingsactiviteit in relatie tot insuline-geassocieerde gewichtstoename. Voor het meten van lichamelijke activiteit in dit proefschrift werd de Sensewear Pro Armband TM gebruikt, waarmee kwantitatief bewegingsactiviteit is gemeten. De bewegingsactiviteit van personen met type 2 diabetes en overgewicht werd vergeleken met die van personen zonder diabetes mellitus met en zonder overgewicht. Uit de metingen bleek dat personen met diabetes minder bewegen dan de controle-personen. De bewegingsarmband heeft ook de slaapduur en -kwaliteit gemeten. Deze slaapkarakteristieken blijken echter geen verklaring te zijn voor de gemeten verschillen.

Om gevolgen van gewichtstoename bij insulinetherapie vast te stellen is onderzoek noodzakelijk dat herhaaldelijk meet over de tijd, zogenaamd prospectief onderzoek. **Hoofdstuk 4** beschrijft de resultaten van een prospectief onderzoek waarbij mensen vanaf de start met insuline 12 maanden lang gevolgd zijn. Hierbij werd primair gekeken naar de relatie tussen de verandering van lichamelijke activiteit in relatie tot de gewichtstoename. Deelnemers aan de studie bleken gemiddeld ruim 3 kg te zijn aangekomen na 12 maanden insulinebehandeling. Met behulp van de bewegingsarmband is gekeken naar de verandering van lichamelijke activiteit. Tussen 6 en 12 maanden na het starten van insuline blijkt er een significante daling van de lichamelijke activiteit. Nadere analyse toonde aan dat deze verandering van lichamelijke activiteit echter niet als verklaring kon gelden voor de toename van het lichaamsgewicht.

In deze studie is verder gekeken naar andere mogelijke voorspellende dan wel verklarende factoren voor de gewichtstoename. Daarbij werd onder meer gekeken naar de mate van diabetes gerelateerde klachten (zoals moeheid, stemmingsproblematiek, cognitieve problemen) met behulp van de PAID-vragenlijst. Het bleek dat deelnemers die meer last hadden van diabetes-gerelateerde klachten een grotere gewichtstoename lieten zien. Een andere voorspellende factor voor meer gewichtstoename die naar voren kwam was een hogere start insulinedosering.

In de studie beschreven in **hoofdstuk 5** is onderzocht of autofagie, het opruimmechanisme van een vetcel, een rol speelt in vetweefsel bij muizen en mensen. Autofagie is een proces dat samenhangt met gewichtsverandering. Dit heeft mogelijk te maken met ontstekingsprocessen in vetweefsel.

Uit de studie bleek dat bij personen met overgewicht sprake was van een hoger niveau van autofagie. Indien deze autofagie geremd werd dan nam de ontstekingsactiviteit in het vetweefsel toe. Deze gegevens suggereren dat een verhoogde autofagie activiteit bij personen met overgewicht mogelijk dempend kan werken om de ontstekingsactiviteit te beperken.

In **hoofdstuk 6** zijn twee uiterste groepen personen met type 2 diabetes met elkaar vergeleken wat betreft het risico op hart- en vaatziekten. De ene groep liet na gebruik van insuline geen tot weinig gewichtstoename zien ("non-gainers") en de andere groep bestond uit personen die juist veel in gewicht waren aangekomen ("gainers"). Er werd gekeken naar de hoeveelheid lichaamsvet, de mate van lichamelijke activiteit en naar de laboratoriumwaardes. De studie toonde aan dat de groep die het meeste in gewicht was aangekomen een ongunstiger cardiovasculair profiel had. Deze groep had meer totaal lichaamsvet, bewoog minder en leek meer leverontsteking te hebben (ALAT leverenzym).

In de **hoofdstukken 7-9** is onderhuids vetweefsel bij gezonde personen met en zonder overgewicht onderzocht en vergeleken met het vetweefsel van personen met type 2 diabetes mellitus. Na de start van insuline werden deelnemers aan de studie significant dikker (**hoofdstuk 2 en 4**). Tevens was al aangetoond dat bij gewichtsstijging vooral een toename te zien is van het abdominale vet (**hoofdstuk 6**). Dit abdominale vet werd gemeten middels MRI.

De vraag van de studie in **hoofdstuk 7** was welke (nadelige) effecten insulinetherapie zou kunnen hebben op het onderhuidse vetweefsel. Na 6 maanden insulinebehandeling werd gekeken welke effecten insuline had op onderhuids vetcelmorfologie, influx van macrofagen en verandering in ontsteking (inflammatie). Er bleek geen significante verandering van de vetcelmorfologie (vetcelgrootte), ondanks het feit dat bij personen met type 2 diabetes sprake was van gewichtstoename dus meer vetmassa. De macrofageninstroom bleek echter wel toe te nemen na insulinebehandeling en dit effect was onafhankelijk van de gewichtstoename. Als laatste werd aangetoond dat een forse gewichtstoename nadelige effecten had op de ontsteking van het vetweefsel.

Deze groep ("gainers") zou theoretisch baat kunnen hebben bij gelijktijdig gebruik van ontstekingsonderdrukkende medicatie naast de insuline.

Toename van de hoeveelheid onderhuidse vetweefsel, door toename van de vetcelgrootte (hypertrofie) of door toename van het aantal vetcellen (hyperplasie), kan aanleiding geven tot een vetzurenflux naar organen zoals de lever. Hierdoor kan levervet ontstaan.

Tot nu toe was de relatie tussen de hoeveelheid levervet van personen met type 2 diabetes en karakteristieken van het onderhuidse vetweefsel niet bekend. In een doorsnede onderzoek werden bij 27 personen die langdurig insuline gebruikten onderhuidse vetbiopten afgenomen en middels MRS de mate van leververvetting gemeten. Het bleek dat de mate van leververvetting gerelateerd was aan de ontstekingsparameters in het onderhuidse vetweefsel, zoals een toename van macrofagen en PAI-I waarden gemeten in het vetweefsel. Voor de resultaten van dit onderzoek wordt verwezen naar **hoofdstuk 8**.

In de literatuur wordt vaak gesproken over de mate van inflammatie in het onderhuidse vetweefsel, maar een eenduidige definitie van inflammatie in het onderhuidse vetweefsel ontbreekt. In **hoofdstuk 9** is gekeken naar vetweefsel van gezonde personen met en zonder overgewicht en personen met type 2 diabetes die glucose-verlagende tabletten gebruikten. Hierbij is getracht aan de hand van karakteristieken van het vetweefsel, namelijk vetcelgrootte, instroom van macrofagen en aanwezigheid van zogenaamde "crown-like structures", een classificatie te maken om de mate van ontsteking in het vetweefsel te kwantificeren. Deze classificatie kan gebruikt worden om verschillende studies onderling te kunnen vergelijken.

Hoofdstuk 10-11 geven een samenvatting van dit onderzoek (Engels en Nederlandse vertaling).

Tenslotte worden in **hoofdstuk 12** woorden van dank uitgesproken aan degenen die hebben bijgedragen aan de totstandkoming van dit proefschrift.

Conclusies en toekomstige onderzoeksonderwerpen

Personen met type 2 diabetes mellitus die gaan starten met insulinetherapie komen significant aan in gewicht. Na 12 maanden insulinebehandeling gaan personen minder bewegen, en dat lijkt ongewenst, hoewel dit geen oorzaak is van de gewichtstoename. Voorspellers van insuline-geassocieerde gewichtstoename zijn**:** start insulinedosis, diabetes-gerelateerde klachten (PAID) en lichaamsvet (negatieve correlatie). Determinanten die gewichtstoename over de tijd beïnvloeden zijn verandering van insulinedosering. De verklaring die altijd wordt gegeven, de verandering in het HbA_{1c} lijkt slechts een geringe rol te spelen in insuline-geassocieerde gewichtstoename. Na het starten van insulinetherapie wordt op het HbA_{1c} "gestuurd", zodat de parameter HbA_{1c} niet volledig als onafhankelijke variabele beschouwd mag worden. De interpretatie van het effect van verandering van HbA_{1c} op lichaamsgewicht wordt hierdoor bemoeilijkt.

De gevolgen van insuline-geassocieerde gewichtstoename bij personen met type 2 diabetes mellitus zijn: een minder gunstig cardiovasculair profiel, met name bij personen die fors aankomen in gewicht bij insulinetherapie. Dit wordt verklaard doordat personen met type 2 diabetes minder gaan bewegen na de start van insulinetherapie, veel levervet hebben, en het onderhuidse vetweefsel meer "ontstoken" lijkt te zijn. Er werd verder een correlatie gevonden tussen het levervet en onderhuids vetweefsel voor wat betreft ontsteking. Als laatste wordt in dit onderzoek een classificatiesysteem voor het uitdrukken van de mate van ontsteking in het vetweefsel voorgesteld. Op deze manier kan onderzoek naar ontstoken vetweefsel beter worden vergeleken.

Het is nog onduidelijk welke andere voorspellers en determinanten bijdragen aan de gewichtstoename bij insulinetherapie. Verder moet nog onderzocht worden of de potentieel nadelige effecten van insulinetherapie bij “gainers” op vetweefselniveau opwegen tegen de gunstige effecten van insuline op glucose-instelling. Onderzocht moet worden of mensen met een lagere startdosis en/of een langzame dosisverhoging van insuline minder aankomen in gewicht. Misschien dat mensen die veel problemen hebben rondom het hebben van diabetes eerst een therapie zouden kunnen volgen die problemen wat kan doen verminderen.

Als laatste is het belangrijk om te onderzoeken of er aanvullende behandelingen zijn om de gewichtstoename bij insuline te verminderen. De zogenaamde glucagon-like peptide-1 (GLP-1) agonisten met gunstige effecten op diabetesinstelling en gewicht lijken in aanmerking te kunnen komen voor personen met forse insuline-geassocieerde gewichtstoename. In de “The effect of Liraglutide on insulin-associated weight gain in patients with type 2 diabetes” (ELEGANT) trial, wordt gekeken naar de effecten van liraglutide (Victoza®) op lichaamsgewicht en diabetesinstelling. Er zal hierbij ook onderzocht worden of personen minder insuline kunnen gaan gebruiken en minder hypoglycaemieën ondervinden.

De uitkomsten van dit proefschrift dragen bij aan een beter begrip van insuline-geassocieerde gewichtstoename bij personen met type 2 diabetes mellitus. Dit kan leiden tot therapeutische strategieën om deze ongewenste gewichtstoename te verminderen om zo de zorg rondom personen met type 2 diabetes mellitus die gaan starten met insulinetherapie te verbeteren.

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Chapter 12

Dankwoord

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Dankwoord

Graag zou ik een groot aantal mensen willen roemen en danken die hebben bijgedragen aan de totstandkoming van dit proefschrift.

Allereerst wil ik natuurlijk alle diabetespatiënten en vrijwilligers heel hartelijk bedanken voor hun inzet, geduld en de goede sfeer tijdens alle uren van hun deelname aan de onderzoeken.

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Jij bent mijn alles.
Liefs,

Henry Jansen

Nijmegen, 2014

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 31 oktober 1974 te Nijmegen. Hij doorliep de voormalige Alosta M.A.V.O in Elst (Gld.), waarbij hij cum laude slaagde. Daarna werden de H.A.V.O en het V.W.O. aan het voormalig Canisius College Mater Dei (Berg en Dalseweg, Nijmegen) met goed gevolg afgelegd. Hij ging in eerste instantie Geneeskunde (1e kandidatuur arts) studeren aan de Katholieke Universiteit te Leuven, België. Daarna studeerde hij Geneeskunde aan de Katholieke Universiteit Nijmegen (thans: Radboud Universiteit). Na het behalen van het artsexamen in 2002 werd begonnen met de opleiding tot internist in het UMC St Radboud (voormalig opleider: prof. dr. J.W.M. van der Meer). Van 2003-2006 werd de internistenopleiding voortgezet in het Jeroen Bosch Ziekenhuis (voormalig opleider: dr. P.M. Netten). De opleiding werd voltooid in het UMC St Radboud, waarbij hij zich onder leiding van prof. dr. C. Tack en dr. P.M. Netten heeft bekwaamd in de diabeteszorg. Tevens werd onder leiding van Prof. dr. J.B.M.J. Jansen, dr. D. de Jong, maag-darm-leverartsen UMC (Prof. dr. J.B.M.J Jansen, thans werkzaam in het Elkerliek Ziekenhuis, Helmond) en dr. P. Bouter, drs. P.J. Lestrade, de opleiding tot endoscopist gevolgd. De registratie tot internist werd in 2008 verkregen. In dat jaar werd een aanvang gemaakt met de klinische studies die uiteindelijk hebben geleid tot dit proefschrift. Vanaf 2009 tot 2011 was hij staflid Algemeen Interne Geneeskunde, sectie Diabetes waarbij klinische werkzaamheden werden gecombineerd met wetenschappelijk onderzoek. In 2011 is hij toegetreden tot de Maatschap Interne Geneeskunde/MDL van het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch alwaar hij sinds 2014 plaatsvervangend opleider Interne Geneeskunde is.

Hij is getrouwd met Romayke Schoffelen en zij hebben vier kinderen: Anna, Julia, Lucas en Femke.