Gene analysis for studying the process of weight regain after weight loss

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Gene analysis for studying the process of weight regain after weight loss

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Content

Chapter 1

General introduction

Obesity

The prevalence of overweight and obesity has increased worldwide, presenting major health problems (1). In 2014, the World Health Organization estimated that of adults aged 18 and over, 39% were overweight and 13% were obese (2). The prevalence of overweight and obesity is assessed by using body mass index (BMI), which is defined as weight in kilograms divided by the square of height in meters (kg/m^2). Individuals with a BMI≥25 kg/m^2 are classified as overweight and individuals with a BMI≥30 kg/m^2 are classified as obese. The increasing prevalence of obesity is a major health concern since it increases the risk for developing type 2 diabetes mellitus (3), cardiovascular diseases (4) and certain types of cancer (5). In fact, overweight- and obesity-related health problems were estimated to cause 3.4 million deaths in 2010 (1). Overweight and obesity occur when there is a disturbance in the energy balance, which is determined by energy intake and expenditure. When energy balance is maintained, energy intake and energy expenditure are equal (Figure 1). If energy intake exceeds energy expenditure, the excessive energy is stored as triglycerides (TG) in the white adipose tissue (WAT). This causes weight gain and eventually obesity. On the other hand, if energy expenditure exceeds energy intake, the stored TG are used as fuel for other energy demanding processes elsewhere in the body.

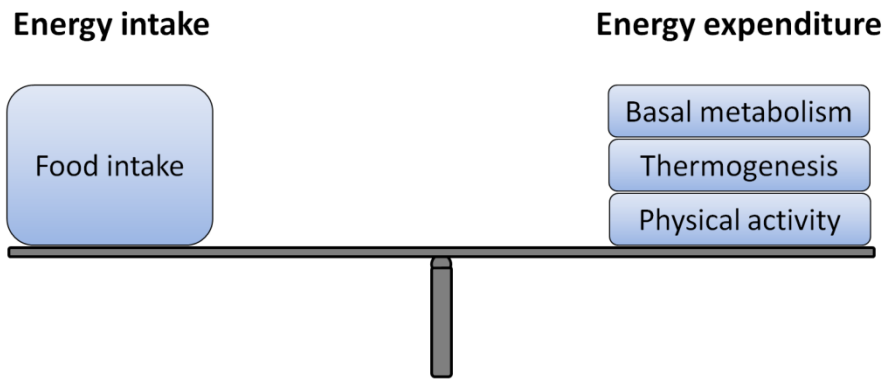

Figure 1: Balance between energy intake and energy expenditure.

Nowadays the often-observed energy imbalance is mainly caused by the combination of a high calorie intake and a low physical activity (6). In our modern society, food is abundantly available and is also energy dense whereas the demand of physical activity is strongly reduced. However, this 'obesogenic' environment cannot fully explain the development of obesity at an individual level, since many people seem to be protected against obesity. This raises the question which factors determine the individual susceptibility to become obese. There is evidence that an individual's susceptibility for gaining weight is determined in part by genetics (7, 8). Numerous genes have been identified that are associated with obesity (9). Genetic variants or single nucleotide polymorphisms (SNPs), like the polymorphism in the fat mass and obesity-associated (FTO) gene, have been linked to obesity (10-12). An individual with a genetic predisposition is unlikely to develop obesity without being exposed to an obesity-promoting environment, suggesting gene-environment interaction effects (13). For example, the body weight development of children is influenced by the BMI of their parents. A higher BMI for the parent is linked to a higher BMI in their children (14). This parental association is due to the combination of a shared genetic background and a shared lifestyle. Besides genetics, other factors also contribute to the individual's susceptibility to become obese, such as aging: when people become older they tend to gain weight (15). Also, social, cultural and economic status, and inadequate sleep are associated with the prevalence of obesity (8, 16, 17).

Weight loss and weight regain

There is a simple remedy to obesity, i.e. losing weight by limiting energy/food intake and increasing daily physical activity for a longer period of time. A body weight reduction of 5-10% reduces disease risk and improves the metabolic profile resulting in positive health outcomes (18, 19). However, the greatest challenge is the seemingly inevitable weight regain after weight loss, the so-called "yoyo-effect". In general up to 80% of the people are unsuccessful in maintaining weight loss (20, 21) defined as "keeping off an intentional loss of at least 10% body weight for at least one year" (22). On average about 70% of the lost weight is regained within two years after the dietary intervention (23). Still, some individuals are able to keep off their lost weight, pointing to an individual susceptibility for weight maintenance or weight regain. Similarly to the predisposition to develop overweight or obesity, the individual susceptibility to successful weight loss and weight maintenance seems to be determined by multiple factors. Genetic, behavioural and physiological factors related to body weight might directly or indirectly affect weight loss and weight maintenance. Hormonal and genetic factors change in response to weight loss, thereby either changing to a pre-obese state or favouring weight regain. For instance, circulating mediators of appetite that enhance weight regain did not return to pre-weight loss levels one year after the start of the weight loss intervention (24). In addition, during weight loss circulating leptin concentrations decrease which could lead to a post-weight loss starvation reaction resulting in reduced energy expenditure and increased food intake (25, 26). Restoring leptin to pre-weight loss circulating levels reverses the starvation reaction by decreasing energy intake (27) and by increasing resting- and activity-induced energy expenditure (28).

A more drastic method for losing weight is bariatric surgery, in which the size and/or the digestive capacity of the stomach are reduced. However, a relapse to increased calorie intake is not uncommon in the long term (29). There are some behaviour modifications that can help to prevent weight regain such as self-monitoring of body weight and food intake, having a physically active lifestyle and having a regular meal rhythm with reduced frequency of snacks (30).

The role of the adipose tissue in obesity

Adipose tissue is found in specific locations in the human body, which are referred to as adipose depots. The adipose depots are located beneath the skin (subcutaneous adipose tissue) and around the internal organs (visceral adipose tissue) (31). The main parenchymal cells of the adipose organ are called adipocytes. Adipocytes can be split into two types based on morphology and function: white adipocytes and brown adipocytes. White adipocytes are spherical cells containing one big lipid droplet that fills about 90% of their volume, whereas brown adipocytes are polygonal cells containing multiple lipid droplets and more mitochondria than white adipocytes (Figure 2) (31). White adipocytes store and release energy, while brown adipocytes burn energy for thermogenesis. The white adipocytes are activated by eating or starving, whereas activation of brown adipocytes can be regulated by cold exposure. In this thesis we focus on the subcutaneous WAT and white adipocytes.

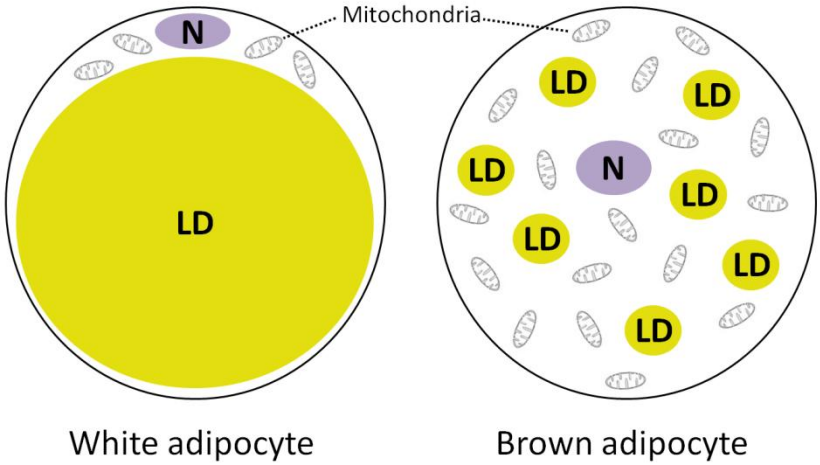

Figure 2: Schematic depiction of white and brown adipocytes containing a nucleus (N), lipid droplets (LD) and mitochondria.

The WAT is a major energy-storing tissue. In a period of a negative energy balance adipocytes will break down TG and release fatty acids (FA) in the blood to fuel energy-demanding tissues. In a period of a positive energy balance adipocytes take up lipids that are converted into TG and stored in lipid droplets (32). Storage of TG leads to WAT expansion, which is determined by an increase in adipocyte size (hypertrophy) and/or number (hyperplasia) (33, 34). Metabolic disturbances associated with obesity, such as insulin resistance, are associated with adipocyte hypertrophy (35), underlining the important function of WAT in obesity. Besides its role in lipid metabolism, including TG storage and FA release, WAT also provides mechanical protection, insulation of the body and it can secrete adipokines which contribute to the regulation of biological functions such as appetite and satiety, insulin secretion and sensitivity, energy expenditure, and inflammation (36).

The adipose tissue and weight regain

As mentioned, long-term weight loss maintenance is difficult and 80% of the people are unsuccessful (20, 21). This provides a substantial challenge in obesity treatment. Long-term weight loss maintenance is dependent on energy intake. Therefore, hormones that regulate hunger and satiety are important with respect to the risk for weight regain. One of these hormones, leptin, decreases during weight loss, which leads to a higher risk for increased energy intake after weight loss (37). Injection of leptin during the weight maintenance period causes a change in activity of the brain regions responsible for energy intake regulation and as such decrease the risk for high energy intake (27). For the hormone ghrelin, produced by cells in the stomach, it has been reported that higher baseline levels seem to predict better weight maintenance after weight loss (38). However, changes in the hormone secretion profile do not exclusively contribute to the risk for weight regain. After weight loss, the gene expression profile is altered to enhance energy conservation and storage within the WAT (39-41). Markers of oxidative stress and inflammatory cytokines, which are also known to suppress appetite and increase energy expenditure, decline (42-44). This improved metabolic regulation in the adipose tissue is likely due to the reduction in adipocyte size because smaller adipocytes are more insulin sensitive, have a lower rate of turnover of the stored lipid, and have higher expression of genes favouring the storage of energy (45-47). Thus, adaptations in adipocyte morphology may lead to the renewed storage of energy when nutrients once again become readily available after dieting and as such contribute to the drive to regain

weight. Part of the research described in this thesis will focus on extending knowledge about the involvement of adipocyte morphology and composition on the risk for weight regain.

Extracellular matrix in adipocyte cellular metabolism

The structure and composition of the WAT are crucial for fulfilling its function in energy storage, energy metabolism, thermal insulation and mechanical protection. Each adipocyte is surrounded by a thick extracellular matrix (ECM) which is composed of numerous protein and carbohydrate components including collagens, fibronectin, laminins and proteoglycans (48). As mentioned, white adipocytes store TG in a single fat droplet almost entirely filling the cell. A lipid monolayer separates the stored fat from the cytosol and mechanical stress on this monolayer may easily lead to disruption of the fat droplet which is detrimental for the adipocyte. Thus, mechanical pressure on the outside of the cell should be avoided, which can be achieved by a strong ECM. The make-up of the ECM is important for its strength and the adipocyte maintains this by continuously renewing the components of the ECM. This constant renewal is mediated by enzymes promoting the construction of the ECM and by enzymes involved in its degradation. During energy excess and deficiency, the WAT must be able to quickly respond to the increase or decrease of the adipocyte volume by altering the structure, size, and shape of the adipose tissue, generally referred to as adipose tissue remodelling. In case of a positive energy balance, the size of individual adipocytes increases due to an increase in TG storage. This enlargement is limited since each adipocyte has a maximum volume that does not allow further expansion (49). Possibly, the maximum cell size is determined by the ability of the adipocyte to keep the extracellular matrix in such a condition that it can protect the cell against disruption (50). Adipocytes reaching the maximum cell size will trigger the formation of new adipocytes from a pre-adipocyte precursor population (51). During a negative energy balance, the release of fatty acids decreases the volume of adipocytes. When adipocytes have lost a large part of their fat content equilibrium may be reached, preventing further fatty acid release from adipocytes.

Cellular stress in adipocyte cellular metabolism and weight regain

The decrease in adipocyte volume because of the loss of TG during a negative energy balance requires adjustments of the ECM to fit the new adipocyte size. These adjustments are accomplished by the turnover of extracellular matrix proteins which is an energy-demanding process (50). It is hypothesized that during weight loss, such energy may not be available. As a consequence, the ECM remodelling cannot keep up with the decrease of adipocyte volume, thereby causing an improper fit between adipocyte and the surrounding ECM which leads to tension and cellular stress. As a reaction to this, the adipocyte wants to counteract the cellular stress. In this respect, the adipocyte could go into apoptosis. However, the average lifespan of an adipocyte is 10 years suggesting that apoptosis is not likely (52). Probably, a more efficient way to reduce the cellular stress is to re-store TG in the adipocyte at which the cell returns to the original volume. As a consequence the body weight of the host increases (Figure 3) (53). As soon as a person stops dieting and returns to energy balance, the capacity of the adipocytes to take up free fatty acid and glucose is increased (54). This suggests that cellular stress accumulated in adipocytes during a negative energy balance, due to the misfit between cell volume and ECM, is a driving force behind the risk for weight regain. The main objective of this thesis is to extend the knowledge about the involvement of adipocyte stress- and ECM-related factors in the weight regain response after weight loss.

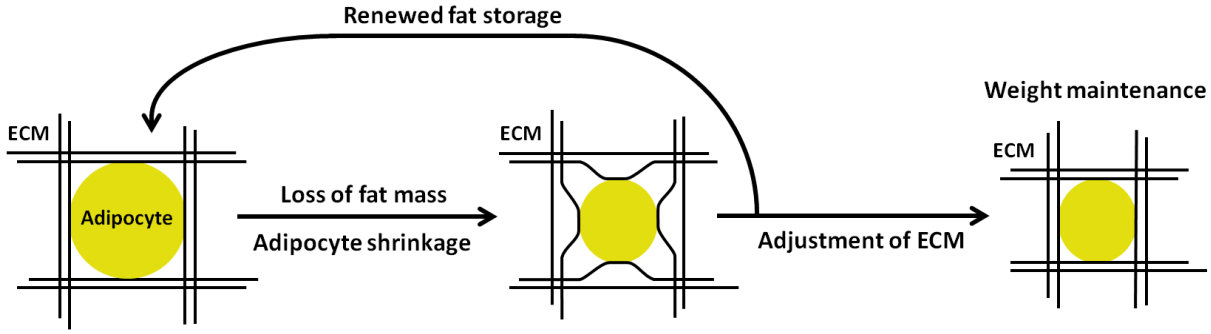

Figure 3: Cellular stress relief in the adipocyte by adjustment of the ECM or re-storage of fat.

Outline of the thesis

The first part of this thesis describes research on the relation between extracellular matrix and weight regain to find possible predictors of weight regain. **Chapter 2** focuses on determining whether genetic variations in extracellular matrix related genes are associated with weight regain among participants of a Pan-European, randomized, controlled dietary intervention study called the DiOGenes study. In **Chapter 3**, the relation between weight regain and changes in expression of extracellular matrix genes was investigated. This was analysed in a randomized, controlled dietary intervention study (the so-called "yoyo-study") in which participants lost weight by either a 5-week very-low-calorie diet (VLCD) or a 12-week low-calorie diet (LCD), with a subsequent 4-week weight stable diet, and a 9-month follow-up period.

The second part of this thesis describes research on the relation between stress-related genes or proteins and weight regain in two dietary intervention studies. The first study consisted of an 8-week VLCD followed by a 10-months follow-up period. A comparison is made between participants regaining weight and participants maintaining weight with regard to levels of stress-related factors during weight loss and weight maintenance (**Chapter 4**). The second study used was the yoyo-study. In **Chapter 5**, the relation between weight regain and changes in the expression of genes for stress proteins during weight loss and weight stabilization was investigated to find possible predictors of weight regain. The relation between adipocyte stress- and ECM-related genes with regard to weight regain is reported in **Chapter 6.** Finally, the results from the described studies in this thesis are summarized and discussed in **Chapter 7**.

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Chapter 2

Variation in extracellular matrix genes is associated with weight regain after weight loss in a sex-specific manner

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Genes & Nutrition, 2015, 10 (6): 56

Abstract

The extracellular matrix (ECM) of adipocytes is important for body weight regulation. Here, we investigated whether genetic variation in ECM related genes is associated with weight regain among participants of the European DiOGenes study. Overweight and obese subjects (n=469, 310 females, 159 males) were on an 8-week low-calorie diet with a 6-month follow-up. Body weight was measured before and after the diet, and after follow-up. Weight maintenance scores (WMS, regained weight as percentage of lost weight) were calculated based on the weight data. Genotype data was retrieved for 2903 SNPs corresponding to 124 ECM-related genes. Regression analyses provided us with six significant SNPs associated with the WMS in males: 3 SNPs in the *POSTN* gene and a SNP in the *LAMB1*, *COL23A1* and *FBLN5* genes. For females, 1 SNP was found in the *FN1* gene. The risk for weight regain was increased by: the C/C genotype for *POSTN* in a co-dominant model (OR 8.25, 95% CI 2.85-23.88) and the T/C-C/C genotype in a dominant model (OR 4.88, 95% CI 2.35-10.16); the A/A genotype for *LAMB1* both in a co-dominant model (OR 18.43, 95% CI 2.35-144.63) and in a recessive model (OR 16.36, 95% CI 2.14-124.9); the G/A genotype for *COL23A1* in a co-dominant model (OR 3.94, 95% CI 1.28-12.10), or the A-allele in a dominant model (OR 2.86, 95% CI 1.10-7.49); the A/A genotype for *FBLN5* in a co-dominant model (OR 13.00, 95% CI 1.61-104.81); the A/A genotype for *FN1* in a recessive model (OR 2.81, 95% CI 1.40-5.63). Concluding, variants of ECM-genes are associated with weight regain after weight loss in a sex-specific manner.

Introduction

Overweight and obesity have become a worldwide public health problem, associated with increased risk for many health complications such as diabetes and cardiovascular disease (1). Such risk can be reduced by losing weight (2). However, remaining at a lower body weight after weight loss provides a challenge for many people (3). The white adipose tissue plays an important role in the body weight change (4, 5). Weight loss reduces the amount of visceral adiposity and fat mass (6), indicating that adipose tissue is one of the important determinants in this process. It has been proposed that the adipose tissue is also involved in the risk for weight regain after weight loss. White adipocytes are characterized by the presence of a single fat droplet, which almost fills the entire cell, and are surrounded by a thick extracellular matrix (ECM) (7). The ECM, is known for providing structural support, but also for fulfilling vital roles in cell differentiation, such as the determination, proliferation, polarity, survival, and migration of cells (8, 9). Prior studies have noted the importance of the ECM in relation to weight regulation. It was shown that ECM-regulated processes are disturbed in obese mice and humans leading to the accumulation of immune cells in the adipose tissue, impaired metabolic function, and reduced capacity for fat mass expansion (10, 11). After long-term weight reduction, a down regulation of ECM-regulating genes and changes in expression levels of ECM components can be observed in adipose tissue (11-14). In addition, Tam et al. reported that 10% body weight gain causes an upregulation of ECM-remodelling genes in the adipose tissue of male subjects (15). When people decrease their energy intake and enter a negative energy balance, mature adipocytes decrease their fat content and become smaller (16). The ECM is supposed to adjust to changes in cell volume. It has been proposed that this may lead to an improper fit between the cell and the surrounding ECM, thereby inducing tension and cellular stress (17). This cellular stress in adipocytes may be reduced by restoring fat and increasing cell volume, which would mean regain of weight for the host (17, 18). If the ECM is able to adjust properly to the volume changes, less cellular stress is generated to result in lower risk for weight regain. In line with this view, the subcutaneous adipose tissue ECM gene expression after a low caloric diet has been reported with differences in weight regainers compared to weight maintainers (19). We hypothesized that variation in genes coding for components of the adipocyte ECM are candidates for determining the risk of weight regain or the successfulness of weight maintenance after weight loss. In the present study, we examined whether genetic variation in ECM-related genes is associated with weight regain among the participants of the European DiOGenes study. We analyzed the present data separately for males and females because gender specificity has been shown for ECM remodeling in rodents (20, 21) and humans (22, 23), although it has not been specifically examined in adipose tissue.

Materials and Methods

Participants and study design

Participants took part in a pan-European, multicentre, randomised controlled dietary intervention programme called DiOGenes (http://www.diogenes-eu.org, ClinicalTrials.gov registration no.: NCT00390637). The whole study design has been described in detail previously (24-26). This study was conducted in eight European countries: the Netherlands, Denmark, the United Kingdom, Greece, Bulgaria, Germany, Spain and the Czech Republic. For 8 weeks, healthy overweight or obese participants followed a low-calorie diet (LCD) that provided about 3.3 - 4.2 MJ/day which is between 800 and 1000 kcal/day. After the diet, participants were randomly assigned to 1 of 4 ad libitum consumed low-fat weight-maintenance diets. These diets differed in glycemic index and protein

content (25). Body weight and other physical and biochemical parameters were measured after overnight fasting on a calibrated scale before weight loss on clinical investigation day 1 (CID1, t=0), after LCD on CID2 (t=8w) and after weight maintenance on CID3 (t=8w + 6m). For the current analysis, only participants who provided weight measurements at all 3 investigation days and who were successfully genotyped were used. In total, 469 participants met these criteria. Weight maintenance scores were calculated for all 469 participants as previously described by Wang et al. (26):

$$WMS = (weight\ at\ CID3 - weight\ at\ CID2) \div (weight\ at\ CID1 - weight\ at\ CID2)$$

A score equal or lower than zero indicated that the participant maintained or continued to lose weight (WM) during the follow-up period, while a score higher than zero indicated that the participant regained weight (WR) during the 6 month follow-up.

DNA extraction and genotyping

Buffy coats of EDTA-blood were used to extract DNA for genotyping. Genotyping was done using the Illumina Bead Station System (IlluminaInc) by IntegraGen using the Illumina 660quad chip, which analyses 660.000 SNPs and CNVs per sample. Genotype QC was carried out for all SNPs and SNPs were excluded from the analysis if they had a call rate <98%, MAF <0.01 or were not in HWE. Centre d'Etude du Polymorphisme Humain control samples were added on each plate: one was different on each plate and one was identical among the 15 genotyped plates. The reproducibility was 100% and the concordance rate was 99.6%. For the purpose of the present study, genotypes for all individuals were extracted from the genotyping matrix for the candidate SNPs only.

Based on the proteins detected in the adipocyte ECM (7), a list of 124 candidate genes related to the ECM was created (Supplement Table S1). Genotype data from the DiOGenes cohort was retrieved for 2903 SNPs (Supplement Table S2) in and near the 124 genes.

Data analysis

Dependant T-test was applied to check for differences between time points within a group. A chi-square test was used to check whether the genotype frequencies of the SNPs were in Hardy–Weinberg equilibrium.

Univariate linear regression analyses were carried out with each SNP allele as a predictor and weight maintenance scores as outcome. The analyses were done for males and females separately because of gender specificity in ECM remodelling found in other studies. Regression analyses were done with the use of Stata 12.0 (StataCorp LP). P-values were corrected for false discovery rate (FDR) in multiple testing with the Benjamini-Hochberg method with the 'stats' package in R (version 3.1.1; http://www.r-project.org/) (27). Corrected P-values <0.05 were considered to be statistically significant. Next, SNPs with a minor allele frequency <5% were excluded to distinguish common polymorphism from rare variants. Linkage disequilibrium r^2-values >0.2 was used to determine if periostin SNPs were in linkage disequilibrium. Linkage disequilibrium structure was evaluated by using SNPStats (28).

Backward elimination in multivariate linear regression was used to check if combinations of SNPs might enhance the outcome. For this, the four significant SNPs observed in the male population were used.

Genotype analyses: logistic regression analysis was used to find the best genetic model of inheritance that describes the effect of the genotypes of the significant SNPs. A model is considered best fitting if it has the lowest Akaike information criterion (AIC) score and if this value is at least 2 lower than the other models. If multiple models have similar low AIC values than these models are fitting equally well. Logistic regression, odds ratios (OR), 95% confidence intervals (95% CI) were calculated to determine the risk of a specific genotype on weight regain. Genotype analyses were all carried out using Stata 12.0 (StataCorp LP).

Results

Participant characteristics

Participant characteristics of weight regainers and maintainers, separated for males and females, can be seen in Table 1. Comparisons between baseline and after 8 weeks show that weight, BMI, WC and fat mass (FM) significantly decreased after the 8 week LCD for all groups. After the 6-month weight maintenance period, all parameters were significantly decreased for weight maintainers (WM) while a significant increase is observed for weight regainers (WR) when comparing to measurements after the LCD diet. Weight maintenance scores (WMS) were significantly different between WM and WR for males ($P<0.001$) and females ($P<0.001$).

Single nucleotide polymorphisms univariate linear regression analyses

The SNPs with a corrected P-value <0.05 are depicted in Table 2, and the results of all SNPs are shown in Supplement Table S1. Further selection on minor allele frequency (MAF) resulted for the male group in only 6 SNPs with an allele frequency higher than 5% (indicated in bold in Table 2): rs7323378, rs9315503, rs9547947, rs2158836, rs12589592, and rs2672826. Three of the SNPs are in and around the periostin gene (*POSTN*, rs7323378, rs9315503, rs9547947), the other SNPs are in the laminin-β1 (*LAMB1*, rs2158836), fibulin-5 (*FBLN5*, rs12589592) and collagen, Type XXIII, alpha1 **(***COL23A1*, rs2672826) genes. The three *POSTN* SNPs were all in linkage disequilibrium: rs7323378 - rs9547947 (r^2 =0.760), rs7323378 - rs9315503 (r^2 =0.487) and rs9547947 - rs9315503 (r^2 =0.370). It indicates that these variants are closely linked and therefore only the SNP with the lowest P-value was used for further analysis, which is *POSTN* rs7323378. For female subjects, 1 SNP remained after selecting SNPs with a minor allele frequency higher than 5%: rs17516906. This SNP is located in the fibronectin 1 (*FN1*) gene.

SNP Multivariate linear regression analyses

Backward elimination in multivariate linear regression with the significant SNPs observed in the male population resulted in significance for three SNPs: rs2672826 (β=-17.52, P=0.020), rs2158836 (β=-11.50, P=0.039) and rs7323378 (β=-13.67, P=0.009). This indicates that *COL23A1*, *POSTN* and *LAMB1* together have an additive effect on weight regulation.

Table 1: Changes in subject characteristics at the end of 8 week low-calorie diet compared to baseline, and at the end of 6-month ad libitum diet compared to the end of 8-week LCD.

	Baseline				After 8-week LCD				After 6-month ad libitum diet			
	Male		Female		Male		Female		Male		Female	
	WM	WR	WM	WR	WM	WR	WM	WR	WM	WR	WM	WR
N	59	100	135	175	59	100	135	175	59	100	135	175
Age (y)	43 ± 6	43 ± 6	41 ± 7	42 ± 6								
Weight (kg)	111.3 ± 18.5	107.9 ± 17.1	97.3 ± 18.3	92.1 ± 13.1	97.7 ± 17.3*	95.3 ± 15.0*	86.3 ± 16.6*	82.6 ± 12*	93.5 ± 17.5#	99.8 ± 15.7#	81.6 ± 15.1#	86.2 ± 12.6#
BMI (kg/m^2)	35.4 ± 4.9	33.4 ± 4.6	35.1 ± 5.4	33.5 ± 4.4	31.1 ± 4.6*	29.6 ± 4.1*	31.1 ± 4.8*	30.1 ± 4.0*	29.8 ± 4.7#	30.9 ± 4.3#	29.5 ± 4.5#	31.4 ± 4.2#
WC (cm)	117.4 ± 12.9	111.9 ± 11.8	104.9 ± 13.6	102.3 ± 10.4	105.4 ± 12.7*	100.5 ± 11.5*	95.2 ± 12.3*	93.6 ± 10.1*	101 ± 12.4#	104.5 ± 12.0#	91.8 ± 12.2#	96.4 ± 10.1#
Fat mass (kg)	39.6 ± 13.4	34.1 ± 10.7	44 ± 12	40.8 ± 9.3	30.4 ± 10.6*	26.7 ± 10.1*	35.3 ± 10.9*	33.8 ± 9.3*	25.3 ± 10.6#	28.4 ± 10.3#	30.5 ± 9.4#	35.6 ± 9.2#
WMS									-0.33 ± 0.34	0.38 ± 0.25	-0.42 ± 0.39	0.40 ± 0.31

Values are means ± SD. Subjects are divided into 4 groups: male weight maintainers (WM, n=59), weight regainers (WR, n=100) and female WM (n=135) and WR (n=175). Weight maintenance score (WMS) is calculated at the end of the 6-month ad libitum diet: (weight after 6-month ad libitum diet – weight after LCD) / (weight at baseline – weight after LCD).
WC, waist circumference; LCD, low-calorie diet; WMS, weight maintenance score.

* P<0.001 change from baseline vs. after 8 week LCD with dependant T-test per group.

# P<0.001 change from after 8 week LCD vs. after 6 month ad libitum diet with dependent T-test per group.

Table 2: Regression analyses carried out with each SNP allele as a predictor and weight maintenance scores as outcome separately for males and females.

SNP	Gene	No. of subjects	P-value	P_{corr}	Minor allel	MAF %
Males						
rs8031741	ACAN	159	9.57E-26	<0.001	G	0.3
rs2271649	ADAM12	158	5.12E-07	<0.001	A	0.9
rs16859850	CCDC80	158	4.08E-17	<0.001	G	0.6
rs2300792	COL12A1	157	1.43E-22	<0.001	C	0.3
rs16918099	COL15A1	159	3.29E-05	0.005	A	0.2
rs16918124	COL15A1	159	6.19E-05	0.008	C	0.5
rs7863250	COL15A1	159	6.19E-05	0.008	C	0.5
rs2672826	**COL23A1**	**157**	**4.74E-04**	**0.048**	**A**	**12.1**
rs12477499	COL3A1	159	2.49E-14	<0.001	G	0.3
rs12589592	**FBLN5**	**159**	**2.13E-04**	**0.023**	**A**	**33.1**
rs12050562	FBN1	159	8.75E-05	0.010	T	0.8
rs7606877	GPC1	159	1.41E-14	<0.001	A	0.5
rs9492168	LAMA2	158	7.50E-17	<0.001	T	0.3
rs2158836	**LAMB1**	**159**	**1.75E-04**	**0.020**	**A**	**37.1**
rs10911215	LAMC1	159	1.11E-09	<0.001	T	1.3
rs2513812	MATN2	159	4.36E-09	<0.001	G	1.3
rs1151578	NID2	158	2.53E-08	<0.001	T	0.2
rs6480654	P4HA1	157	2.67E-12	<0.001	A	0.2
rs1382192	PDIA4	159	4.90E-13	<0.001	A	0.5
rs4727007	PDIA4	159	4.90E-13	<0.001	G	0.5
rs10197695	PDIA6	159	1.26E-14	<0.001	G	0.3
rs7323378	**POSTN**	**158**	**1.10E-05**	**0.002**	**C**	**48.0**
rs9547947	**POSTN**	**149**	**2.62E-05**	**0.004**	**A**	**38.3**
rs9315503	**POSTN**	**159**	**2.20E-04**	**0.023**	**G**	**33.9**
Females						
rs7679471	TLL1	159	3.36E-07	<0.001	C	0.2
rs8031741	ACAN	309	5.14E-16	0.000	G	0.3
rs4871046	COL14A1	307	5.49E-09	0.000	C	0.5
rs16918099	COL15A1	310	5.75E-44	0.000	A	0.2
rs12477499	COL3A1	310	2.19E-43	0.000	G	0.3
rs17516906	**FN1**	**303**	**1.28E-04**	**0.040**	**G**	**7.4**
rs1151578	NID2	310	5.19E-06	0.002	T	0.2
rs11925421	PLOD2	310	7.20E-10	0.000	G	0.6
rs7078493	TLL2	310	5.19E-06	0.000	T	0.2
rs310517	VCAN	310	1.79E-25	0.000	T	0.5

P-values are derived from univariate linear regression analyses and P_{corr} are P-values corrected for false discovery rate. $P_{corr} \leq 0.05$ are considered significant, SNPs with a MAF <5% are excluded. SNPs in bold have $P_{corr} \leq 0.05$ and MAF >5%. MAF, minor allel frequency.

Genotype analyses

Genotype analyses were done to get an idea of the effect of specific genotypes on weight change. Figure 1 shows the comparison between number of genotypes of weight maintainers (blue bars) and weight regainers (white bars) for the significant SNPs. Table 3 shows the best model of fit and the associations between genotypes and the risk for weight regain. For *COL23A1* rs2672826, the best fitting models are the co-dominant and dominant model. In the co-dominant model, it is seen that a G/A genotype increases the risk for weight regain by 3.9 times compared to a G/G genotype (OR 3.94, 95% CI 1.28-12.10). The dominant model indicates that the G/A-A/A genotype increases the risk for weight regain by 2.9-fold compared to the G/G genotype (OR 2.86, 95% CI 1.10-7.49). The best fitting model for *FBLN5* rs12589592 is the co-dominant model with a 13-fold increased risk for weight regain with an A/A genotype (OR 13.00, 95% CI 1.61-104.81) and a 2.3-fold increase with a G/A genotype (OR 2.26, 95% CI 1.15-4.46) compared to a G/G genotype. The best fitting models for *LAMB1* rs2158836 are the co-dominant and the recessive model. In the co-dominant model an A/A genotype gives an 18.4 times higher chance for weight regain than a G/G genotype (OR 18.43, 95% CI 2.35-144.63). In the recessive model this risk is increased 16.4 times (OR 16.36, 95% CI 2.14-124.9). The best fitting models for *POSTN* rs7323378 are the co-dominant as well as the dominant model. The chance for weight regain is increased 8.3 times with the C/C genotype in the co-dominant model (OR 8.25, 95% CI 2.85-23.88) when compared with a T/T genotype. In the dominant model, a T/C-C/C genotype increases the risk for weight regain by 4.9 times (OR 4.88, 95% CI 2.35-10.16). The co-dominant model and recessive model are best fitting for the *FN1* rs17516906 SNP but a correct comparison in the co-dominant model cannot be made due to absence of G/G genotypes for participants regaining weight during follow-up. The recessive model shows that an A/A genotype increase the risk for weight regain 2.8 times (OR 2.81, 95% CI 1.40-5.63) compared to a G/G-A/G genotype.

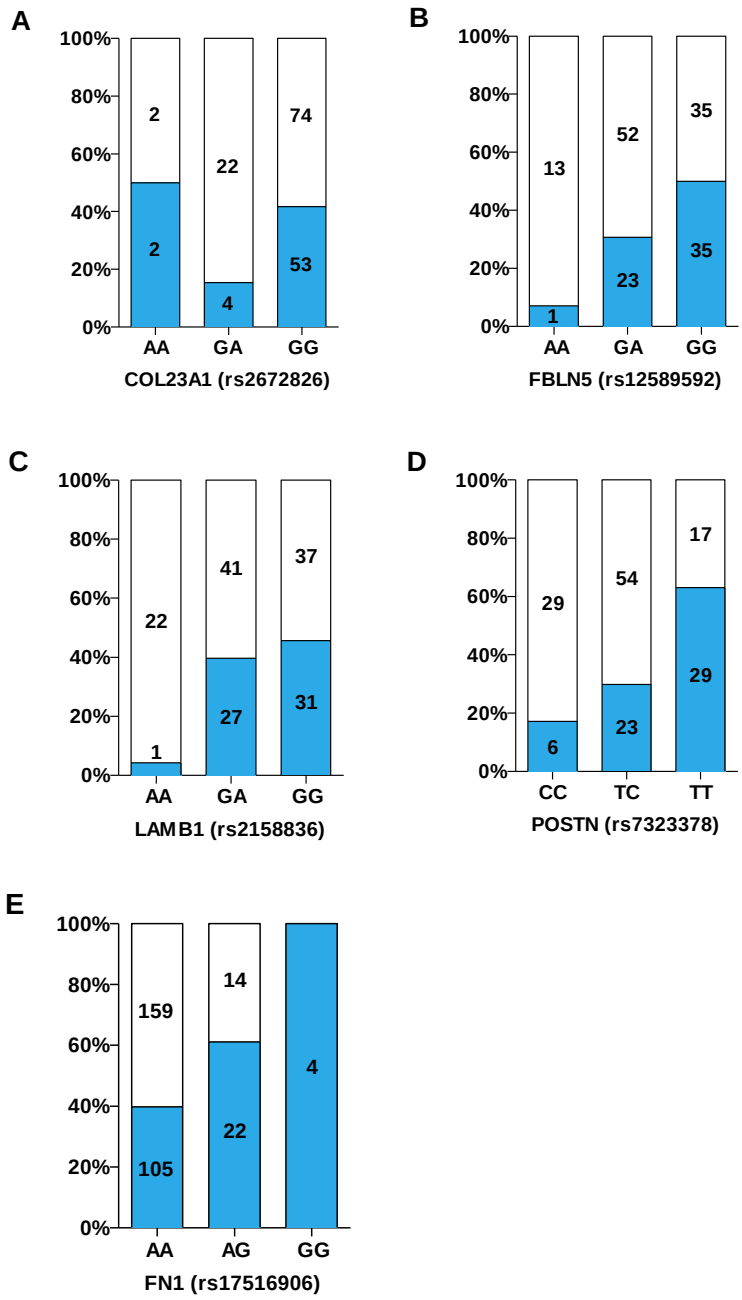

Figure 1: Percentage weight regain or maintenance phenotype of each genotype for the significant SNPs. Each bar represents the total amount of subjects having a specific genotype for a significant SNP: **A** COL23A1 (rs2672826), **B** FBLN5 (rs12589592), **C** LAMB1 (rs2158836), **D** POSTN (rs7323378) and **E** FN1 (rs17516906). **A-D** represent men and **E** represents women. The blue bars indicate the percentage of weight maintainers (WM) with the genotype and the white bars are for weight regainers (WR). The number within each bar is the count of participants having the specific genotype.

Table 3: Logistic regression analyses used to find the best genetic model of inheritance that describes the effect of the genotypes.

SNP	Model	Genotype	OR (95% CI)	AIC
rs2672826	Co-dominant	G/G	1.00	
A > G		G/A	3.94 (1.28-12.10)*	206.4
COL23A1		A/A	0.72 (0.10-5.25)	
	Dominant	G/G	1.00	206.6
		G/A-A/A	2.86 (1.10-7.49)*	
	Recessive	G/G-G/A	1.00	211.6
		A/A	0.59 (0.08-4.33)	
rs12589592	Co-dominant	G/G	1.00	
G > A		G/A	2.26 (1.15-4.46)*	202.7
FBLN5		A/A	13.00 (1.61-104.81)*	
	Dominant	G/G	1.00	204.8
		G/A-A/A	2.71 (1.40-5.25)*	
	Recessive	G/G-G/A	1.00	206.4
		A/A	8.67 (1.10-68.06)*	
rs2158836	Co-dominant	G/G	1.00	
A > G		G/A	1.27 (0.64-2.51)	199.3
LAMB1		A/A	18.43 (2.35-144.63)*	
	Dominant	G/G	1.00	210.1
		G/A-A/A	1.89 (0.98-3.62)*	
	Recessive	G/G-G/A	1.00	197.8
		A/A	16.36 (2.14-124.9)*	
rs7323378	Co-dominant	T/T	1.00	
T > C		T/C	4.01 (1.85-8.67)	192.6
POSTN		C/C	8.25 (2.85-23.88)*	
	Dominant	T/T	1.00	192.7
		T/C-C/C	4.88 (2.35-10.16)*	
	Recessive	T/T-T/C	1.00	203.6
		C/C	3.54 (1.37-9.14)*	
rs17516906	Co-dominant	G/G	1.00	
A > G		A/G	0.00 (NA)	409.0
FN1		A/A	0.00 (NA)	
	Dominant	G/G	1.00	412.8
		A/G-A/A	0.00 (NA)	
	Recessive	G/G-A/G	1.00	410.7
		A/A	2.81 (1.40-5.63)*	

Odds ratio, 95% confidence interval, Akaike information criterion (AIC) and P-values were retrieved from logistic regression analyses with the co-dominant, dominant, and recessive model. The best fitting model has the lowest AIC and this value is at least 2 lower than the other models. P-value ≤0.05 are considered significant (*). OR, odds ratio; CI, confidence interval; NA, Not Available.

Discussion

The major finding of this study is that variants of the *POSTN*, *LAMB1, COL23A1* and *FBLN5* gene for males and the *FN1* gene for females can influence the risk for weight regain. To the best of our knowledge this is the first study that relates genetic variation of ECM genes with the risk for weight regain after weight loss. As such, our findings are in keeping with the proposed prominent role of the ECM in the adipocyte cellular stress model for weight regain (17).

For most of the identified genes or members of the same gene family, links with human weight regulation have been reported. POSTN is highly expressed in collagen-rich connective tissue such as adipose tissue. It has been related to obesity by Bolton et al. who observed higher POSTN expression in the adipose tissue of obese diabetic sand rates (*Psammomys obesus*) compared to healthy lean animals suggesting that POSTN may influence fat storage in adipocytes (29). High expression of POSTN was found in visceral as well as subcutaneous adipose tissue depots and a role in repair or expansion of the adipose tissue was suggested (29). Proper biomechanical function of connective tissue seems to depend on the interaction of POSTN and specific collagens (30). In *POSTN*-knockout mice, a decreased collagen cross-linking was observed and the mechanical stabilization of ECM architecture was disrupted (30). Here we show that in humans the C/C genotype of the *POSTN* SNP rs7323378 increases the risk for weight regain after weight loss, which supports a role for the ECM in human weight regulation.

Laminins constitute a family of 12 genes coding for ECM components, which are subdivided in five α-, four β-, and three γ-genes. SNPs in the *LAMA5* gene have been associated with adiposity parameters in European and African Americans (31). In addition, a rare variant with a moderate-to-high predicted biological effect was detected in *LAMC1* or *LAMC3* in 5 of 30 extremely obese subjects (32). In the present study, we observed that the minor A/A genotype for the *LAMB1* SNP increases the chance of weight regain after weight loss. Laminins actually form trimers from an α, β and γ protein. Of all possible combinations, only fifteen different trimers have been observed in vivo. LAMB1 is a component of six of those fifteen, where it occurs in combination with LAMC1 or LAMC3.

Collagen XXIII belongs to the non-fibrillar transmembranous subfamily of collagens and as such it can be involved in cell-matrix contact, but concrete information about its function is lacking. Collagen XXIII has structural features in common with collagen XIII and may therefore have a similar function (33, 34). Type XIII collagen is expressed in almost all connective tissue producing cells (35), and is important for the regulation of adhesion of mesenchymal cells to the surrounding ECM and neighbouring cells, thereby facilitating transmembrane signalling (36). In addition, data suggests the involvement of collagen XIII in numerous differentiation and maturation processes associated with inflammation and vasculogenesis (37). Positive correlations between inflammatory markers and weight regain after energy restriction have been reported (38). Together with our findings, it suggests that people with a G/A genotype for *COL23A1* are predisposed to regaining weight due to differences in ECM biosynthesis and inflammatory profile.

Studies of the skin have revealed that collagen XXIII can bind as a ligand to integrin $\alpha_2\beta_1$, which directly interacts with the LAMB1-containing trimer laminin111 (39). In fact, FBLN5 also binds to β-integrin and in the mouse, it was observed that FBLN5 competes with FN for binding to integrin-β1 (40). Altogether, four of the five genes identified here seem to interact with integrin-β, which

suggests that integrins may play a key role in the influence of the ECM on weight regain. Moreover, a sex-specific preference for interaction with integrin-β1 could explain our result that in men variation in the *FBLN5* gene and in women variation in the *FN1* gene is associated with weight regain after weight loss.

Among females, we observed that an A/A genotype in the *FN1* gene is associated with weight regain. Our finding is in line with that of Mutch et al. (19) who demonstrated that *FN1* was upregulated in subjects regaining weight after weight loss while the opposite was observed in subjects maintaining their lost weight. Fibronectin, an important component of the ECM, functions both as regulator of various cellular processes and as scaffolding protein maintaining and directing tissue organization and ECM composition (41). In morbid obese subjects elevated plasma levels of fibronectin are found and when these subjects lose weight, fibronectin levels normalise (42). During adipocyte differentiation, fibronectin levels decrease (43) and culturing preadipocytes on fibronectin-coated dishes prevents adipocyte differentiation (44). In this respect, fibronectin seems to influence the differentiation potential of preadipocytes. Although weight regain after weight loss is supposed to be mainly due to renewed fat accumulation in mature adipocytes (14, 17, 18), to some extent differentiation of preadipocytes can also occur (45). Therefore, the predisposition to weight regain after weight loss mediated by the detected genetic variation in ECM genes may in part result from interference with volume changes of adipocytes and in part from influencing differentiation of preadipocytes.

Although DiOGenes is one of the largest weight loss-maintenance studies, a limitation of the current study is the relatively small number of participants. Because of this limited number, we did not take the variation of the maintenance diet into account. Genotype analysis of the total cohort of 469 subjects did not lead to significant results. Also, no functional analysis of the detected genetic variation was performed. Yet, this study produces leads for understanding the role of the ECM and its genetic variation in weight regain after weight loss, which should be confirmed and can be extended in larger cohorts.

In conclusion, we have investigated the role of genetic variation in ECM genes in regard to weight regain. Our results illustrate an involvement of variants of the *POSTN*, *LAMB1*, *COL23A1*, and *FBLN5* gene for males and of the *FN1* gene for females in the risk for weight regain after weight loss. Influence on weight regulation may come from the level of cell stress generated between the ECM and the adipocyte during weight loss, but also from modifying the differentiation of preadipocytes. Further research is required to confirm our findings in a larger cohort and to bring more clarity in the mechanism of weight regain after weight loss.

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Supplementary Figures and Tables

Supplement Table 1: List of 124 candidate genes related to extracellular matrix.

Gene ID	Gene name	Number dbSNPs	Number genotyped SNPs	Chromosome	Start bp	End bp
ACAN	Agrecan 1	1750	33	15	89346674	89418585
ADAM11	ADAM metallopeptidase domain 11	572	6	17	42836399	42859214
ADAM12	ADAM metallopeptidase domain 12	6981	167	10	127700950	128077024
ADAM15	ADAM metallopeptidase domain 15	532	9	1	155023042	155035252
ADAM17	ADAM metallopeptidase domain 17	1278	11	2	9628615	9695921
ADAM19	ADAM metallopeptidase domain 19	3290	43	5	156822542	157002783
ADAM22	ADAM metallopeptidase domain 22	3882	43	7	87563458	87832204
ADAMTS1	A desintegrin and metalloproteinase with thrombospondin type 1 motif 1	509	15	21	28208606	28217728
ADAMTS4	A desintegrin and metalloproteinase with thrombospondin type 1 motif 4	489	4	1	161159538	161168846
ADAMTS5	A desintegrin and metalloproteinase with thrombospondin type 1 motif 5	1147	19	21	28290231	28338832
AZGP1	Alpha-2-glycoprotein 1, zinc	489	3	7	99564343	99573780
BGN	Biglycan	463	8	X	152760397	152775012
BMP1	Bone morphogenic protein 1	1366	15	8	22022249	22069839
CALR	Calreticulin	498	4	19	13049392	13055303
CCDC80	Coiled coil domain containing protein 80	1058	17	3	112323407	112368377
CHI3L1	Chitinase-3-like protein 1	565	13	1	203148059	203155877
CILP	Cartilage intermediate layer protein	653	9	15	65488337	65503826
CNTNAP1	Contactin associated protein 1	554	2	17	40834631	40851832
COL11A1	Collagen a 1(XI) chain	4295	53	1	103342023	103574052
COL12A1	Collagen a 1(XII) chain	2055	22	6	75794042	75915767
COL14A1	Collagen a 1(XIV) chain (undulin)	4927	69	8	121072019	121384275
COL15A1	Collagen a 1(XV) chain	2501	39	9	101705461	101833069
COL18A1	Collagen a 1(XVIII) chain	2951	37	21	46825052	46933634
COL1A1	Collagen a 1(I) chain	1111	6	17	48260650	48278993
COL1A2	Collagen a 2(I) chain	1257	25	7	94023873	94060544
COL23A1	Collagen a 1(XXIII) chain	7017	135	5	177664619	178017556
COL2A1	Collagen a 1(II) chain	1089	25	12	48366748	48398269
COL3A1	Collagen a 1(IIII) chain	975	20	2	189839046	189877472
COL4A1	Collagen a 1(IV) chain	3541	89	13	110801318	110959496
COL4A2	Collagen a 2(IV) chain	4826	84	13	110958159	111165374
COL4A3	Collagen a 3(IV) chain	2897	67	2	228029281	228179508
COL4A5	Collagen a 5(IV) chain	2927	6	X	107683074	107940775
COL5A1	Collagen a 1(V) chain	4749	79	9	137533620	137736686
COL5A2	Collagen a 2(V) chain	2410	10	2	189896622	190044605
COL5A3	Collagen a 3(V) chain	1512	17	19	10070237	10121147
COL6A1	Collagen a 1(VI) chain	1091	6	21	47401651	47424964
COL6A2	Collagen a 2(VI) chain	1596	17	21	47518011	47552763
COL6A3	Collagen a 3(VI) chain	2292	42	2	238232646	238323018
CSPG4	Chondroitin sulfate proteoglycan 4	895	5	15	75966663	76005189
CTGF	Connective tissue growth factor	321	7	6	132269316	132272513
DAG1	Dystroglycan 1	1125	6	3	49506146	49573048
DCN	Decorin (bone proteoglycan II)	837	10	12	91539025	91576900
DPT	Dermatopontin	1056	23	1	168664697	168698502
ECM1	Extracellular matrix protein 1	439	2	1	150480538	150486265
ECM2	extracellular matrix protein 2	828	6	9	95256365	95298937
EFEMP1	Fibulin-3 (EGF-containing fibulin-like extracellular matrix protein 1)	1133	15	2	56093102	56151274
EFEMP2	Fibulin-4 (EGF-containing fibulin-like extracellular matrix protein 2)	543	2	11	65633912	65641063
ELN	Elastin	885	10	7	73442119	73484237
EMILIN1	Elastin microfibril interface-located protein 1	452	2	2	27301435	27309271
EMILIN2	Elastin microfibril interface-located protein 2	1738	26	18	2847028	2915991
FBLN1	Fibulin-1	2283	50	22	45898118	45997015
FBLN2	Fibulin-2	2283	42	3	13573824	13679922
FBLN5	Fibulin-5	1620	42	14	92335756	92414331
FBN1	Fibrillin 1	3921	33	15	48700503	48938046
FKBP9	Peptidyl-prolyl cisÐtrans isomerase (FK506-binding protein 9)	1160	3	7	32997017	33046543
FMOD	Fibromodulin	606	8	1	203309753	203320617
FN1	Fibronectin	1616	16	2	216225163	216300895
GPC1	Glypican 1	960	14	2	241375088	241407493

ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	1271	10	16	31271311	31344213
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide antigen CD51)	1975	14	2	187454792	187545628
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide antigen CD29)	1998	23	10	33189247	33294720
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	1409	29	21	46305868	46351904
ITGB5	Integrin, beta 5	2300	42	3	124480795	124606674
LAMA2	Laminin a-2 chain	9937	125	6	129204342	129837714
LAMA4	Laminin a-4 chain	2667	53	6	112429963	112576141
LAMB1	Laminin b-1 chain	1809	25	7	107564244	107643700
LAMB2	Laminin b-2 chain	532	3	3	49158547	49170551
LAMC1	Laminin c-1 chain	2218	18	1	182992595	183114727
LGALS1	Galectin-1	403	5	22	38071615	38075813
LGALS3BP	Lectin galactoside-binding soluble 3-binding protein	561	9	17	76967320	76976191
LOX	Protein-lysine 6-oxidase	540	6	5	121398890	121413980
LOXL1	Lysyl-oxidase homologue 1	723	16	15	74218330	74244478
LUM	Lumican	500	5	12	91496406	91505608
MATN2	Matrilin-2	2763	71	8	98881068	99048944
MATN3	Matrilin-3	548	6	2	20191872	20212455
MATN4	Matrilin-4	725	9	20	43922085	43937169
MFAP4	Microfibril-associated glycoprotein 4	359	2	17	19286755	19290553
MMP1	Matrix metalloproteinase-1	549	11	11	102660651	102668891
MMP10	Matrix metalloproteinase-10	579	15	11	102641234	102651359
MMP14	Matrix metalloproteinase 14; membrane-type-1 matrix metalloproteinase	614	10	14	23305766	23318236
MMP19	Matrix metalloproteinase 14; membrane-type-1 matrix metalloproteinase	464	1	12	56229217	56236750
MMP2	Matrix metalloproteinase-2	2624	55	16	55423612	55540603
MMP9	Matrix metalloproteinase-9	685	6	20	44637547	44645200
NID1	Nidogen 1 (entactin)	1900	28	1	236139130	236228462
NID2	Nidogen 2 (osteonidogen)	1766	43	14	52471521	52535712
NPNT	Nephronectin	1984	21	4	106815932	106925184
OGN	Mimecan (osteoglycin)	517	2	9	95146249	95166978
P4HA1	Prolyl 4-hydroxylase, alpha subunit	1713	5	10	74766975	74856732
P4HB	Protein-disulfide isomerise	824	1	17	79801035	79818570
PCOLCE	Procollagen C-proteinase enhancer protein	424	2	7	100199800	100205798
PDIA3	Protein-disulfide isomerase A3	690	2	15	44038590	44065477
PDIA4	Protein-disulfide isomerase A4	877	6	7	148700154	148725733
PDIA6	Protein-disulfide isomerase A6	1696	23	2	10923517	10978103
PLOD1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	1131	8	1	11994262	12035595
PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	1653	13	3	145787227	145881440
PLOD3	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	678	6	7	100849258	100861701
POSTN	Periostin	909	11	13	38136720	38172981
PPIA	Peptidyl-prolyl cisÐtrans isomerase A (cyclophilin A)	720	1	7	44836279	44864163
PPIB	Peptidyl-prolyl cisÐtrans isomerase B (cyclophilin B)	414	2	15	64448011	64455404
PRELP	Proline arginine rich end leucine-rich repeat protein	689	0	1	203444956	203460480
PRG4	Proteoglycan 4	682	8	1	186265405	186283694
PRSS3P1	Trypsinogen B	545	0	7	142468264	142471794
SERPINA12	Visceral adipose tissue-derived serine protease inhibitor (vaspin)	993	27	14	94953611	94984181
SERPINE1	Plasminogen activator inhibitor 1	593	7	7	100770370	100782547
SERPINH1	Colligin (collagen-binding protein)	528	10	11	75273101	75283828
SPARC	SPARC (osteonectin)	730	15	5	151040657	151066726
SPARCL1	SPARC-like protein 1	1206	22	4	88394487	88452213
SPON1	Spondin-1 (F-spondin)	4807	75	11	13983914	14289646
SPON2	Spondin-2 (mindin)	1012	10	4	1160720	1202750
TGFBI	Transforming growth factor-b-induced protein IG-H3	905	12	5	135364584	135399507
TGFBR3	Betaglycan (transforming growth factor beta receptor III)	3798	80	1	92145902	92371892
THBS1	Thrombospondin-1	654	9	15	39873280	39891667

THBS2	Thrombospondin-2	1244	18	6	169615875	169654139
THBS3	Thrombospondin-3	492	2	1	155165379	155178842
TIMP1	TIMP metallopeptidase inhibitor 1	269	3	X	47441712	47446188
TIMP2	TIMP metallopeptidase inhibitor 2	1731	22	17	76849059	76921469
TIMP3	TIMP metallopeptidase inhibitor 3	1298	35	22	33197687	33259030
TIMP4	TIMP metallopeptidase inhibitor 4	389	4	3	12194551	12200851
TLL1	Tolloid-like 1	4402	45	4	166794410	167025047
TLL2	Tolloid-like 2	2629	49	10	98124363	98273675
TNC	Tenascin-C	2085	47	9	117782806	117880536
TNN	Tenascin-N	1826	20	1	175036994	175117202
TNXB	Tenascin-X	1307	17	6	32008931	32083111
VCAN	Versican core protein	2136	43	5	82767284	82878122

SNPs, single nucleotide polymorphism; dbSNPs, SNPs found in database; bp, base pair.

Supplement Table 2: List of 2903 single nucleotide polymorphisms for which genotype data was retrieved.

This table can be accessed via this link:
http://link.springer.com/article/10.1007%2Fs12263-015-0506-y#SupplementaryMaterial

Chapter 3

A role for leukocyte and ECM remodelling of adipose tissue in the risk of weight regain after weight loss

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Abstract

Background: Weight loss is often followed by weight regain after an energy-restricted dietary intervention (DI). When people are on diet, the volume of an adipocyte decreases by loss of triglycerides creating stress between the cell contents and the surrounding extracellular matrix (ECM). Previously, we observed that genetic variation of ECM genes is associated with an increased risk of weight regain.

Objective: We investigated the relation between expression of ECM genes during weight loss and a period of weight stabilization, and the risk of weight regain.

Design: In this randomized controlled trial, sixty-one healthy overweight/obese participants followed either a 5-week very-low-calorie diet (500 kcal/d) or a 12-week low-calorie diet (1250 kcal/d) (WL period) with subsequent a 4-week weight stable diet (WS period), and a 9-month follow-up. The WL and WS period taken together was named the DI. Abdominal subcutaneous adipose tissue biopsies were collected for microarray analysis. Gene expression changes for a broad set of ECM-related genes were correlated to the weight regain percentage.

Results: 26 of the 277 genes correlated significantly with the weight regain percentage during WL, WS or DI. Most correlations were observed in the VLCD group during WS. Four genes code for leukocyte specific receptors. These and other genes belong to a group of 26 genes of which the expression changes correlated highly ($r \geq 0.7$, $P \leq 0.001$) among each other. This group could be divided into three subclusters linking to two biological processes: leukocyte integrin gene activity and ECM remodelling, whereas a link with insulin sensitivity is apparent.

Conclusions: Our present findings indicate the importance of adipose tissue leukocytes for the risk of weight regain. ECM modification seems also involved and we observed a link with insulin sensitivity.

Introduction

Overweight and obesity are growing public health concerns worldwide due to the increased risk for the metabolic syndrome and the development of type 2 diabetes, cardiovascular diseases and cancer (1, 2). Weight loss by an energy-restricted dietary intervention, increased physical activity, pharmacological and/or surgical treatment reduces the disease risk and produces positive health outcomes in overweight and obese people (3, 4). However, long-term weight loss maintenance has been proven to be difficult (5, 6). Generally, up to 80% of the people are unsuccessful in maintaining weight loss (7, 8). Therefore, it is crucial to gain more knowledge about the mechanisms that influence the risk for weight regain. There is now substantial evidence that the adipose tissue is one of the important determinants in the process of weight regain (9). Mariman et al. suggested that cellular stress, accumulated in adipocytes during a negative energy balance, is a driving force behind the risk for weight regain (10). When people are on an energy-restricted diet, the volume of an adipocyte decreases by loss of triglycerides creating stress between the cell contents and the surrounding extracellular matrix (ECM) (11). Indeed, we have been able to show that on average people who regain weight, have higher levels of stress proteins in adipose tissue than those who succeed in maintaining the lost weight (12). Furthermore, we have demonstrated that certain genetic variations of ECM genes are associated with an increased risk for weight regain (13). In the present study we aimed to further investigate the influence of the ECM on weight regain.

Weight loss induces changes in the ECM not only by cellular stress, but also in other ways. It is generally observed that the development of obesity is accompanied by an onset of low grade inflammation. This involves a chemotactic attraction of cells of the innate immune system into the adipose tissue leading to a situation in which pro-inflammatory immune cells outbalance the anti-inflammatory cells (14). Such a state of increased inflammation may have systemic consequences as it may promote the development of insulin resistance. Weight loss usually reduces the inflammatory activity of the adipose tissue, which may not be directly obvious during energy restriction. Studies have shown that expression of genes involved in inflammation and innate immunity was increased (15) or unchanged (16) in human adipose tissue directly after short-term weight loss, but was downregulated during a subsequent weight stabilization period (WS period) (15-17). Diapedesis of leukocytes during weight gain and possible emigration of leukocytes after weight loss, require remodeling of the ECM. Therefore, this change in content of immune cells may influence the risk for weight regain. Here, we have analysed gene expression of a large number of ECM genes during weight loss and weight stabilization in relation to weight regain.

Materials and Methods

Participants and study design

Sixty-one overweight and obese (BMI 27-36 kg/m^2) Caucasian participants underwent an energy-restricted dietary intervention (Figure 1). The whole study design has been described in detail previously (18). In short, individuals were recruited by advertisement via local media. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney disease, use of medication that influences body weight regulation, pregnancy, marked alcohol consumption (>21 alcoholic units per week for men and >14 alcoholic units per week for women), elevated fasting glucose (>6.1 mmol/L), total cholesterol (>7.0 mmol/L) or triacylglycerol (>3.0 mmol/L) concentrations, or blood pressure (>160/100 mmHg). Participants were randomly assigned to either

a very-low-calorie diet (VLCD, rapid weight loss) or a low-calorie diet (LCD, slow weight loss) group. Participants in the VLCD group underwent a 5-week diet with about 500 kcal/day by consuming three meal replacements per day (Modifast; Nutrition et Santé Benelux, Breda, The Netherlands). Participants in the LCD group underwent a 12-week diet with about 1250 kcal/day, designed by a dietician. Both groups were targeted to lose approximately 10% body weight during this weight loss period (WL, T1-T2). Following WL, all participants underwent a 4-week weight maintenance diet based on their individual energy requirements. This weight stable period (WS, T2-T3) was designed to investigate the effect of weight loss of approximately 10%, without the interfering effect of a pronounced negative energy balance. The WL and WS period taken together was named the dietary intervention (DI, T1-T3). The study dietician provided dietary advice according to the Dutch national guidelines (19) to both groups, to assist in remaining weight stable throughout the WS period and to assist in weight loss during the WL period in the LCD group. After the WS period, participant's body weight was monitored for 9 months (follow-up, T3-T4) by monthly meetings with a dietician. During this follow-up, participants did not receive advice on monitoring and limiting food intake to mimic non-restricted free-living conditions.

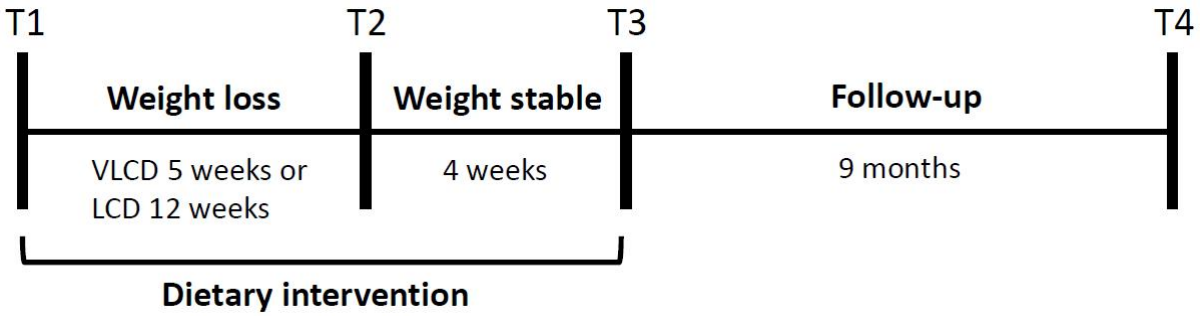

Figure 1: Schematic overview of the study design. Measurements were performed at the start of the study (T1), at the end of the weight loss period (T2), weight stable period (T3), and follow-up (T4). The dietary intervention period (DI) is the weight loss period and weight stable period taken together. LCD, low calorie-diet (n=27); VLCD, very-low-calorie diet (n=26).

At the start of the study (T1) and at the end of each period (T2, T3 and T4) abdominal subcutaneous adipose tissue biopsies were collected, body composition was determined and body weight, height, blood pressure, hip and waist circumference were measured after overnight fasting. Body volume was determined with air-displacement plethysmography using a Bod Pod device (Cosmed, Italy, Rome) according to the manufacturer's instructions and as described by Dempster et al. (20). Body composition was calculated from body density according to the two-compartment model by Siri (21). During the dietary intervention (T1-T3), four participants withdrew from the study, in three participants we could not collect enough biopsy material and in one participant the gene expression results deviated strongly from the others and were therefore excluded. Characteristics of the remaining fifty-three participants at T1, T2, T3 and T4 are displayed in Table 1.

This study was conducted according to the Declaration of Helsinki guidelines and registered on ClinicalTrials.gov (registration number: NCT01559415). All procedures involving human participants were approved by the Central Committee on Human Research and by the Medical Ethical Committee of Maastricht University, The Netherlands. Written informed consent was obtained from all participants.

Adipose tissue biopsy

Abdominal subcutaneous adipose tissue biopsies were obtained by needle biopsy under local anaesthesia (2% lidocaine, Fresenius Kabi, Zeist, the Netherlands) after an overnight fast at T1, T2 T3, and T4. Tissue was immediately rinsed in sterile saline, frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Adipose tissue RNA isolation and microarray analysis

For RNA isolation, adipose tissue samples from T1, T2 and T3 were used. Total RNA was extracted from approx. 150 mg frozen adipose tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands). Total RNA (100 ng per sample) was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19654 unique genes (Affymetrix, Santa Clara, CA, USA). Sample labeling, hybridization to chips and image scanning was performed according to manufacturer's instructions. Microarray signals were normalized using robust multichip average (RMA). Genes with normalized signals > 20 on at least 15 arrays were defined as expressed (11532 genes). Gene expression changes within groups during a certain period (WL period, WS period or DI period) were defined as significantly different when the q-value was <0.05 in a paired t-test with Bayesian correction (Limma) (22). Array data have been submitted to the Gene Expression Omnibus (number GSE77962).

Selection of ECM genes

In this study, 277 genes related to the extracellular matrix were selected for the analysis based on previously measured ECM genes (13) and by selecting for the term "extracellular matrix" in the Gene Ontology Biological Process (GO-BP) description (Supplement Table 1).

Calculations

In this study, we wanted to analyse the relation between gene expression changes and weight regain. As a value for weight regain, we calculated the weight regain percentage (WR%) during follow-up as follows: ((weight at T4 – weight at T3) ÷ weight at T3) x 100%. For gene expression change we calculated the fold change (FC) of gene expression during WL, WS or DI. Gene expression results from the microarray were expressed as $\log_2$ transformed values. Gene expression changes during certain periods were calculated as follows: WL ($\log_2$ T2 – $\log_2$ T1), WS ($\log_2$ T3 – $\log_2$ T2) and DI ($\log_2$ T3 – $\log_2$ T1). These $\log_2$ ratios (LR) during WL, WS and DI were transformed into fold changes (FC = 2^{LR} if LR≥0 and FC = (-1) 2^{-LR} if LR<0).

Clustering

First, we performed Pearson correlations between the WR% and the changes in expression of the 277 ECM genes during the VLCD WL and WS phase. The ECM genes that correlated significantly (P≤0.05) with the WR% were clustered based on their correlation among each other (r>0.6, P<0.002). For a second analysis, we performed an extended clustering in which the changes of the 277 ECM gene expressions were correlated with each other during the weight stabilisation phase in the VLCD. Next, genes were selected that significantly correlated ($r\geq$0.7 and P≤0.001). With those genes a correlation matrix was constructed but to further minimize the chance of including false positives and focus on closely related genes, a confined correlation cluster was constructed by removing genes with less than 5 significant correlations to other genes in the cluster. The clusters were constructed to provide possible functional or regulatory information.

Statistical analysis

Data are presented as mean ± SEM. Comparisons of variables within a group were made with dependent T-test. Between-group comparisons (VLCD vs. LCD) were made with independent T-test. To determine the link between weight regain and ECM genes, Pearson R correlations were performed between the WR% and the gene expression changes of the 277 ECM genes during WL, WS and DI. Statistical calculations were done using SPSS 20.0 for Windows (SPSS Inc, Chicago, IL). P≤0.01 was considered statistically significant unless otherwise stated. All variables were checked for normal distribution, and variables with a skewed distribution were ln-transformed to satisfy conditions of normality. Extreme outliers (values higher than 3x interquartile range calculated with SPSS) influencing the data were removed during statistical analyses.

Results

Clinical characteristics

Characteristics of the fifty-three participants at study start (T1), end of WL (T2), end of WS (T3) and end of follow-up (T4) are displayed in Table 1. The VLCD and LCD groups were comparable at study start for weight, BMI, body fat %, fat free mass, and hip and waist circumference. During WL (T1-T2), participants decreased in body weight, BMI, hip and waist circumference, body fat percentage and fat free mass (Table 1). After WL, VLCD and LCD groups were still comparable for all parameters. In the subsequent 4-week weight stable period (T2-T3), hip circumference, body fat % and fat free mass changed (Table 1). Still no significant differences were observed between VLCD and LCD after the WS period. During the dietary intervention (T1-T3), all parameters significantly decreased except for fat free mass in the LCD group (Table 1). Body weight, BMI, waist circumference, body fat percentage, body fat and fat free mass increased during follow-up (T3-T4). No differences were found between VLCD and LCD for the average weight loss percentage (-9.7 ± 1.2 % and -8.8 ± 2.9 %, P=0.228) and WR% (5.4 ± 4.5 % and 5.3 ± 3.8 %, P=0.957).

Changes of ECM gene RNA levels correlate with weight regain percentage

Changes during WL, WS and DI in the expression of 277 ECM-related genes were checked for correlation ($r \geq 0.5$, $P \leq 0.01$) with WR%. Analysis was performed separately for the dietary groups and showed 17 significant correlations for the VLCD group and 9 for the LCD group (Table 2). For VLCD most correlations (11/17) were in the WS phase, whereas for LCD most were found in the DI phase (7/9). There was no overlap of correlated genes between the dietary groups. The strongest correlation was found in the LCD group with discoidin domain receptor tyrosine kinase 1 (*DDR1*) in the WL phase (r=0.698, P<0.001). DDR1 is a cell receptor for collagen and in that way is involved in cell attachment to the extracellular matrix. In endothelial cells and lung cancer cells, collagen type IV alpha 5 (COL4A5) directs the DDR1 activity (23). Here we observed a negative correlation between *COL4A5* gene expression and WR%, but in the VLCD DI phase.

Table 1: Subject characteristics at study start, end of weight loss, end of weight stable and end of follow-up[1].

	Study start (T1)		End of WL (T2)		End of WS (T3)		End of follow-up (T4)	
	VLCD	**LCD**	**VLCD**	**LCD**	**VLCD**	**LCD**	**VLCD**	**LCD**
Sex (male/female)	12/14	13/14						
Age (years)	50.4 ± 1.5	51.7 ± 2.1						
Weight (kg)	92.1 ± 1.9	92.8 ± 2.0	83.1 ± 1.6[2]	84.6 ± 2.0[2]	82.9 ± 1.7[3]	84.5 ± 2.0[3]	87.7 ± 2.0[2]	89.2 ± 2.1[2]
BMI (kg/m^2)	30.8 ± 0.4	31.5 ± 0.5	27.8 ± 0.4[2]	28.7 ± 0.5[2]	27.7 ± 0.4[3]	28.7 ± 0.5[3]	29.1 ± 0.5[2]	30.3 ± 0.5[2]
Hip circumference (cm)	111.0 ± 1.1	110.7 ± 1.4	105.0 ± 1.0[2]	106.1 ± 1.5[2]	104.8 ± 1.0[3]	104.8 ± 1.5[2,3]	105.4 ± 1.4	107.0 ± 1.9[2]
Waist circumference (cm)	101.3 ± 1.6	102.5 ± 2.1	93.5 ± 1.4[2]	95.2 ± 1.9[2]	94.6 ± 1.4[3]	94.4 ± 2.1[3]	97.6 ± 1.7[2]	98.6 ± 2.1[2]
Body fat (%)	39.5 ± 1.6	40.6 ± 1.9	34.8 ± 2.0[2]	34.7 ± 2.2[2]	33.7 ± 2.0[2,3]	34.1 ± 2.3[2,3]	36.0 ± 1.9[2]	36.9 ± 2.2[2]
Fat free mass (kg)	55.7 ± 2.4	55.5 ± 2.3	54.2 ± 2.3[2]	55.0 ± 2.3[2]	54.9 ± 2.4[2,3]	55.3 ± 2.3	55.9 ± 2.4[2]	56.1 ± 2.5

[1] All values are means ± SEMs. VLCD n=26 and LCD n=27. No significant difference (P≤0.05) between the VLCD and LCD group at study start, end of WL, end of WS and end of follow-up.

[2] Significant change (P≤0.05) between this time point and the previous time point with dependant T test.

[3] Significant change (P≤0.05) between the end of WS (T3) and study start (T1) with dependant T test.

BMI, body mass index; LCD, low-calorie diet; VLCD, very-low-calorie diet.

Table 2: Correlation coefficients between the weight regain percentage and the fold changes in ECM gene expression during the weight loss, weight stable and dietary intervention period separated based on diet[1].

Gene ID	Gene name	VLCD			LCD			P-value
		WL	WS	DI	WL	WS	DI	
ABI3BP	ABI family, member 3 (NESH) binding protein	0.593						0.003
ACTN1	Actinin, alpha 1		0.661					0.001
ADAMTSL4	ADAMTS-like 4					-0.626		0.001
COL20A1	Collagen, type XX, alpha 1						-0.552	0.008
COL4A5	Collagen, type IV, alpha 5			-0.568				0.003
DDR1	Discoidin domain receptor tyrosine kinase 1				0.698			<0.001
DMD	Dystrophin	0.560	-0.664					0.005; 0.001
FBLN1	Fibulin 1			0.577				0.003
FBLN2	Fibulin 2						0.551	0.008
FZD4	Frizzled class receptor 4						0.571	0.006
ICAM3	Intercellular adhesion molecule 3		0.566					0.006
ITGA5	Integrin, alpha 5		0.591					0.003
ITGAL	Integrin, alpha L		0.560					0.005
ITGAM	Integrin, alpha M		0.620					0.002
ITGAX	Integrin, alpha X		0.614					0.002
ITGB2	Integrin, beta 2		0.571					0.005
LAMA2	Laminin, alpha 2						0.661	0.001
LAMC1	Laminin, gamma 1 (formerly LAMB2)						0.568	0.006
LGALS1	Lectin, galactoside-binding, soluble, 1			0.661				<0.001
MMP15	Matrix metallopeptidase 15 (membrane-inserted)			0.635				0.001
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2		-0.601					0.002
PPIB	Peptidylprolyl isomerase B (cyclophilin B)		0.533					0.009
PXDN	Peroxidasin						0.563	0.006
SPARCL1	Secreted Protein Acidic And Cysteine Rich-like 1		-0.561					0.007
TIMP4	TIMP metallopeptidase inhibitor 4						0.547	0.008

[1] Values are significant (P≤0.01) Pearson R's correlation coefficients (*r*) between weight regain and the fold change (FC) of gene expression during weight loss (T2-T1), weight stable (T3-T2) and dietary intervention (T3-T1). Weight regain percentage is calculated: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. LCD, low-calorie diet (n=27); VLCD, very-low-calorie diet (n=26).

Cluster analysis of VLCD WS correlating genes

Eleven genes correlated with WR% during the VLCD WS phase. We therefore tried to see if those genes could be clustered in an attempt to learn more about the underlying processes. Clustering was based on correlation (r>0.6, P<0.002) between expression changes of these 11 genes during the VLCD WS phase. This approach assumes that co-expressed genes may have closely related functions or take part in the same functional process. All eleven genes could be clustered (Figure 2). The genes that negatively correlate with WR%, procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), dystrophin (*DMD*) and secreted protein acidic and cysteine rich-like 1 (*SPARCL1*), were also negatively correlated to the other genes, whereas *PLOD2* and *DMD* correlated positively to each other just like the other genes of the cluster.

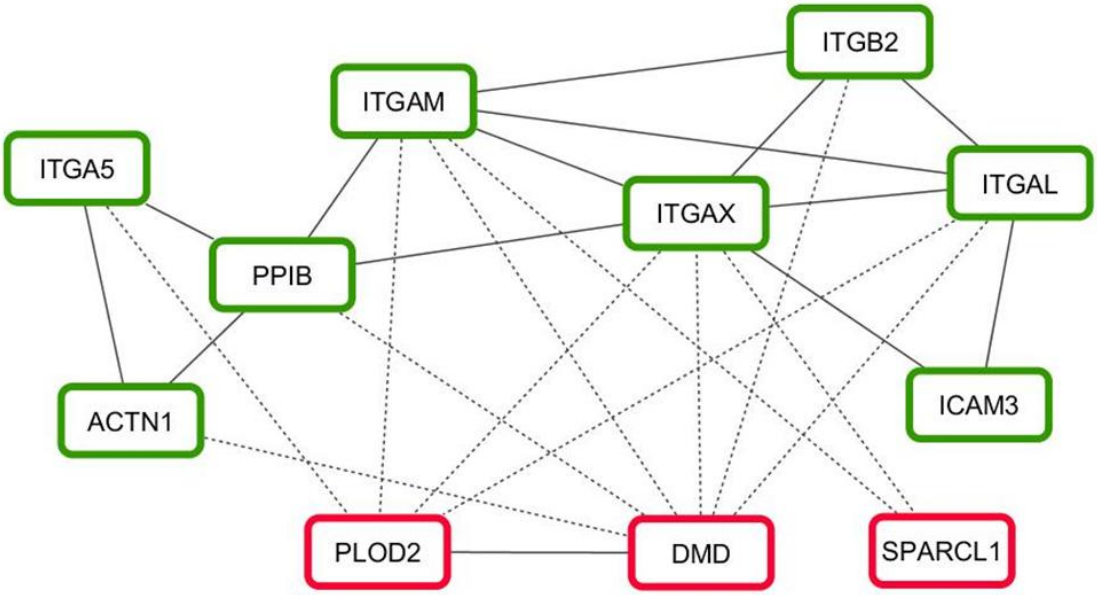

Figure 2: Correlation network between expression changes of genes during the weight stabilization phase in the VLCD (n=26) group. Only Pearson R correlations with an r>0.6, P<0.002 are depicted in this figure. The cluster exists of genes that positively (green outline) or negatively (red outline) correlated with the weight regain percentage. Positive correlations between genes are depicted with a solid line, negative correlations with a dashed line. ACTN1, actinin, alpha 1 ; DMD, dystrophin; ICAM3, intercellular adhesion molecule 3; ITGA5, integrin, alpha 5; ITGAL, integrin, alpha L; ITGAM, integrin, alpha M; ITGAX, integrin, alpha X; ITGB2, integrin, beta 2; PLOD2, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; PPIB, peptidylprolyl isomerase B; SPARCL1, SPARC-like 1; VLCD, very-low-calorie diet.

We then performed an extended clustering starting with the VLCD WS correlating genes to find strong correlations with other ECM-related genes ($r\geq$0.7, P<0.001). Genes had to correlate with at least 5 other genes in the cluster in order to minimize false positives. This resulted in a cluster of 26 genes including 5 of the genes correlating with weight regain percentage: integrin alpha L (*ITGAL*), integrin alpha M (*ITGAM*), integrin alpha X (*ITGAX*), integrin beta 2 (*ITGB2*) and peptidylprolyl isomerase B (*PPIB*). Rearranging the genes according to their strong correlation values resulted in three subclusters (Table 3), a small cluster of seven genes including the four integrin genes, and two larger overlapping clusters. All except one of the genes were positively correlated. Only the gene for fibroblast growth factor 2 (*FGF2*) correlated negatively to the genes of the third subcluster. The genes for matrix metallopeptidase 9 (*MMP9*), elastin microfibril interface 2 (*EMILIN2*) and calpain 1 (*CAPN1*) seem to connect the small cluster with the two larger clusters. The overlap becomes more evident when similar clustering is done for the VLCD WL phase (Supplement Table 2).

Gene expression changes during the WS phase in the VLCD group

Four integrin genes are part of the small cluster, which are positively correlated with WR%. Most of these integrins are categorized as having an important or even specific function in leukocytes (24). Table 4 shows the average change in expression of integrin genes during the VLCD WS phase. The gene for *ITGB2*, which forms complexes with *ITGAL*, *ITGAM* and *ITGAX*, was significantly down-regulated. Also the genes for *ITGAM* and *ITGAX* were significantly down-regulated. In addition, the integrin alpha E (*ITGAE*) gene, which complexes with integrin beta 7 (*ITGB7*) for integrin formation, was significantly down-regulated. It indicates that leukocyte-specific integrins in the adipose tissue are down-regulated during the WS phase. Since some of these genes are positively correlated to weight regain percentage, it suggests that lack of leukocyte-specific down-regulation during the weight stabilization period increases the risk of weight regain. We were unable to separately investigate the cellular composition of the adipocytes and stromal vascular fractions. In an attempt to obtain such information, we analysed for the WS phase the expression changes of genes coding for surface markers present on leukocytes using the microarray data. Our observations point to a lowering of gene expression in cells of the myeloid lineage but not in lymphoid cells (Supplement Table 3).

Table 4: Gene expression changes during the weight stabilization phase in the VLCD group[1].

Gene ID	Gene name	Remark	Fold change	Q-value
ITGA1	Integrin Subunit Alpha 1	Receptor for collagen[3]	1.15	0.008
ITGA2	Integrin Subunit Alpha 2	Receptor for collagen[3]	1.17	0.003
ITGA10	Integrin Subunit Alpha 10	Receptor for collagen[3]	Nd	nd
ITGA11	Integrin Subunit Alpha 11	Receptor for collagen[3]	1.25	<0.001
ITGA5[2]	Integrin Subunit Alpha 5	Receptor for fibronectin[3]	1.01	0.458
ITGA3	Integrin Subunit Alpha 3	Receptor for laminin[3]	-1.56	0.000
ITGA6	Integrin Subunit Alpha 6	Receptor for laminin[3]	1.19	0.000
ITGA7	Integrin Subunit Alpha 7	Receptor for laminin[3]	1.26	0.000
ITGA4	Integrin Subunit Alpha 4	Leukocyte adhesion[4]	-1.15	0.172
ITGA9	Integrin Subunit Alpha 9	Leukocyte adhesion[5,6]	-1.01	0.493
ITGB2[2]	Integrin Subunit Beta 2	Leukocyte-specific receptor[3]	-1.64	0.002
ITGAD	Integrin Subunit Alpha D	Leukocyte-specific receptor[3]	-1.01	0.483
ITGAL[2]	Integrin Subunit Alpha L	Leukocyte-specific receptor[3]	-1.05	0.416
ITGAM[2]	Integrin Subunit Alpha M	Leukocyte-specific receptor[3]	-1.39	0.002
ITGAX[2]	Integrin Subunit Alpha X	Leukocyte-specific receptor[3]	-1.54	0.014
ITGB7	Integrin Subunit Beta 7	Leukocyte-specific receptor[3]	1.01	0.502
ITGAE	Integrin Subunit Alpha E	Leukocyte-specific receptor[3]	-1.33	0.001

[1] Gene expression fold change, significantly different when Q-value <0.05 in paired t-test with Bayesian correction (Limma).

[2] Positive Pearson R correlation between this gene and the weight regain percentage. nd: no data available; VLCD, very-low-calorie diet (n = 26).

[3] Hynes, Cell 110 (6): 673-687, 2002

[4] Lin et al., Current Opinion in Chemical Biology 2(4): 453-457, 1998.

[5] Mambole et al., Journal of Leukocyte Biology 88(2): 321-327, 2010

[6] Nishimichi et al., Journal of Biological Chemistry 286(13): 11170-11178, 2011

Table 3: Correlation matrix of changes in ECM gene expressions with each other during the weight stabilisation phase in the VLCD[1].

	CTSS	ITGAX	ITGAM	ITGB2	ITGAL	TGFB1	CHI3L1	MMP9	TIMP1	TGFBI	SPP1	CD44	MMP19	EMILIN2	CTSB	CTSD	PDIA4	PPIB	ITGA3	CAPN1	NCSTN	CALR	COLGALT1	PLOD1	P4HB	FGF2
CTSS	1	0.894	0.933	0.945	0.755	0.717	0.878	0.721	0.224	0.238	0.340	0.285	0.566	0.753	0.709	0.691	0.472	0.638	0.651	0.675	0.587	0.575	0.512	0.555	0.466	-0.510
ITGAX	0.894	1	0.868	0.963	0.870	0.697	0.696	0.700	0.420	0.381	0.490	0.409	0.657	0.726	0.649	0.671	0.503	0.634	0.651	0.736	0.493	0.601	0.523	0.538	0.278	-0.550
ITGAM	0.933	0.868	1	0.903	0.775	0.772	0.802	0.632	0.290	0.325	0.375	0.273	0.539	0.767	0.679	0.616	0.558	0.687	0.710	0.803	0.553	0.641	0.549	0.592	0.498	-0.599
ITGB2	0.945	0.963	0.903	1	0.838	0.776	0.889	0.617	0.164	0.083	0.163	0.181	0.431	0.636	0.558	0.565	0.405	0.562	0.634	0.669	0.416	0.501	0.455	0.484	0.327	-0.473
ITGAL	0.755	0.870	0.775	0.838	1	0.707	0.544	0.520	0.298	0.209	0.404	0.242	0.446	0.536	0.463	0.480	0.403	0.534	0.543	0.745	0.398	0.523	0.346	0.463	0.069	-0.540
TGFB1	0.717	0.697	0.772	0.776	0.707	1	0.619	0.419	0.344	0.279	0.136	0.207	0.505	0.671	0.579	0.587	0.478	0.535	0.805	0.700	0.468	0.493	0.592	0.454	0.324	-0.573
CHI3L1	0.878	0.696	0.802	0.889	0.544	0.619	1	0.771	0.658	0.631	0.755	0.600	0.803	0.662	0.622	0.677	0.523	0.556	0.530	0.632	0.480	0.582	0.463	0.464	0.536	-0.490
MMP9	0.721	0.700	0.632	0.617	0.520	0.419	0.990	1	0.681	0.674	0.804	0.649	0.867	0.720	0.693	0.747	0.571	0.595	0.569	0.641	0.540	0.628	0.528	0.504	0.685	-0.514
TIMP1	0.224	0.420	0.290	0.164	0.298	0.344	0.658	0.681	1	0.876	0.724	0.838	0.760	0.736	0.706	0.696	0.723	0.803	0.603	0.615	0.536	0.691	0.664	0.611	0.334	-0.692
TGFBI	0.238	0.381	0.325	0.083	0.209	0.279	0.631	0.674	0.876	1	0.818	0.778	0.804	0.768	0.763	0.699	0.717	0.740	0.642	0.587	0.575	0.665	0.693	0.522	0.452	-0.579
SPP1	0.340	0.490	0.375	0.163	0.404	0.136	0.755	0.804	0.724	0.818	1	0.674	0.832	0.715	0.790	0.754	0.681	0.728	0.587	0.647	0.616	0.710	0.587	0.623	0.589	-0.521
CD44	0.285	0.409	0.273	0.181	0.242	0.207	0.600	0.649	0.838	0.778	0.674	1	0.770	0.746	0.727	0.660	0.713	0.696	0.591	0.634	0.618	0.642	0.752	0.525	0.449	-0.649
MMP19	0.566	0.657	0.539	0.431	0.446	0.505	0.803	0.867	0.760	0.804	0.832	0.770	1	0.902	0.897	0.934	0.744	0.754	0.761	0.707	0.737	0.799	0.774	0.660	0.636	-0.673
EMILIN2	0.753	0.726	0.767	0.636	0.536	0.671	0.662	0.720	0.736	0.768	0.715	0.746	0.902	1	0.931	0.906	0.820	0.789	0.836	0.758	0.755	0.820	0.814	0.689	0.713	-0.716
CTSB	0.709	0.649	0.679	0.558	0.463	0.579	0.622	0.693	0.706	0.763	0.790	0.727	0.897	0.931	1	0.942	0.770	0.847	0.842	0.717	0.831	0.814	0.803	0.773	0.717	-0.641
CTSD	0.691	0.671	0.616	0.565	0.480	0.587	0.677	0.747	0.696	0.699	0.754	0.660	0.934	0.906	0.942	1	0.704	0.791	0.806	0.667	0.808	0.812	0.750	0.761	0.633	-0.651
PDIA4	0.472	0.503	0.558	0.405	0.403	0.478	0.523	0.571	0.723	0.717	0.681	0.713	0.744	0.820	0.770	0.704	1	0.753	0.823	0.749	0.607	0.872	0.850	0.740	0.704	-0.804
PPIB	0.638	0.634	0.687	0.562	0.534	0.535	0.556	0.595	0.803	0.740	0.728	0.696	0.754	0.789	0.847	0.791	0.753	1	0.759	0.784	0.727	0.832	0.741	0.878	0.567	-0.750
ITGA3	0.651	0.651	0.710	0.634	0.543	0.805	0.530	0.569	0.603	0.642	0.587	0.591	0.761	0.836	0.842	0.806	0.823	0.759	1	0.790	0.731	0.783	0.871	0.739	0.669	-0.702
CAPN1	0.675	0.736	0.803	0.669	0.745	0.700	0.632	0.641	0.615	0.587	0.647	0.634	0.707	0.758	0.717	0.667	0.749	0.784	0.790	1	0.645	0.808	0.749	0.728	0.489	-0.834
NCSTN	0.587	0.493	0.553	0.416	0.398	0.468	0.480	0.540	0.536	0.575	0.616	0.618	0.737	0.755	0.831	0.808	0.607	0.727	0.731	0.645	1	0.706	0.748	0.768	0.785	-0.664
CALR	0.575	0.601	0.641	0.501	0.523	0.493	0.582	0.628	0.691	0.665	0.710	0.642	0.799	0.820	0.814	0.812	0.872	0.832	0.783	0.808	0.706	1	0.745	0.856	0.687	-0.813
COLGALT1	0.512	0.523	0.549	0.455	0.346	0.592	0.463	0.528	0.664	0.693	0.587	0.752	0.774	0.814	0.803	0.750	0.850	0.741	0.871	0.749	0.748	0.745	1	0.737	0.765	-0.770
PLOD1	0.555	0.538	0.592	0.484	0.463	0.454	0.464	0.504	0.611	0.522	0.623	0.525	0.660	0.689	0.773	0.761	0.740	0.878	0.739	0.728	0.768	0.856	0.737	1	0.718	-0.726
P4HB	0.466	0.278	0.498	0.327	0.069	0.324	0.536	0.685	0.334	0.452	0.589	0.449	0.636	0.713	0.717	0.633	0.704	0.567	0.669	0.489	0.785	0.687	0.765	0.718	1	-0.547
FGF2	-0.510	-0.550	-0.599	-0.473	-0.540	-0.573	-0.490	-0.514	-0.692	-0.579	-0.521	-0.649	-0.673	-0.716	-0.641	-0.651	-0.804	-0.750	-0.702	-0.834	-0.664	-0.813	-0.770	-0.726	-0.547	1

[1] Values are Pearson R's correlation coefficients: green fill $r \geq 0.700$; red fill $r \leq -0.700$. VLCD n=26. CALR, calreticulin; CAPN1, calpain 1; CD44, CD44 molecule; CHI3L1, chitinase 3-like 1; COLGALT1, collagen beta(1-O)galactosyltransferase 1; CTSB cathepsin B; CTSD, cathepsin D; CTSS, cathepsin S; ECM, extracellular matrix; EMILIN2, elastin microfibril interface 2; FGF2, fibroblast growth factor 2; ITGA3, integrin, alpha 3; ITGAL, integrin, alpha L; ITGAM, integrin, alpha M; ITGAX, integrin, alpha X; ITGB2, integrin, beta 2; MMP19, matrix metallopeptidase 19; MMP9, matrix metallopeptidase 9; NCSTN, nicastrin; P4HB, prolyl 4-hydroxylase, beta polypeptide; PDIA4, protein disulfide isomerase family A, member 4; PLOD1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; PPIB, peptidylprolyl isomerase B; SPP1, secreted phosphoprotein 1; TGFB1, transforming growth factor, beta 1, TGFBI, transforming growth factor, beta-induced; TIMP1, TIMP metallopeptidase inhibitor 1; VLCD, very-low-calorie diet.

Discussion

The present study focussed on determining the relation between weight regain and changes in expression of ECM-related genes. Significant correlations (P ≤0.01) with the WR% were observed for 25 of the 277 genes. The highest number of correlations was observed in the VLCD WS phase. Five of the positively correlated genes of this phase appeared to belong to a group of 26 genes of which the expression changes correlated highly ($r \geq 0.7$, $P \leq 0.001$) among each other. This group could be divided into three subclusters, one of which is mainly composed of leukocyte-specific integrin genes.

The smallest subcluster contains 4 integrins, which are specific for leukocytes and form the Mac-1 and LFA-1 surface proteins (24). Besides leukocyte-specific integrin genes, the subcluster contains three additional genes: transforming growth factor beta 1 (*TGFB1*), chitinase 3 like 1 (*CHI3L1*) and cathepsin S (*CTSS*). TGFB1 influences the inflammatory status by regulating cytokine levels as was shown for adipose-derived mesenchymal stromal cells *in vitro* (25). Furthermore, TGFB1 may play a role in leukocyte migration since it has been shown to influence adhesion of leukocytes to orbital fibroblasts (26). The product of the *CHI3L1* gene, also known as YKL-40, is a BMI-independent marker for type 2 diabetes (27) produced by macrophages and neutrophils (28, 29). YKL-40 inhibits degradation of type I collagen and was suggested to play a role in macrophage infiltration. CTSS is one of eleven cathepsin proteases, which is active in ECM remodelling (30). Adipose tissue *CTSS* mRNA levels are associated with BMI, suggesting that the *CTSS* gene is mainly active in adipocytes (31). Also, activated macrophages in interaction with adipocytes increase CTSS production pointing to a close relation between *CTSS* gene activity and inflammatory status of the adipose tissue (32). Altogether, the gene expression changes of the small subcluster comply with a lowering of leukocyte-specific integrins. At the moment we cannot determine if down-regulation is caused by a lower RNA-production of resident leukocytes or by emigration of leukocytes. Gene expression of monocyte chemoattractant protein 1 (*MCP1*) is down-regulated (FC= -1.29, Q= 0.04), whereas expression of macrophage migration inhibitory factor (*MIF*) does not change during the WS phase. Neutrophils respond to the chemotactic factor C5a (33). In the present study the *C5* gene in the adipose tissue is significantly down-regulated (FC=-1.15, Q=0.01) as is the gene for its receptor *C5AR1* (FC=-1.44, Q=0.008). It indicates a lowering of diapedesis of neutrophils during WS. Since in the obese the adipose tissue is in a state of chronic inflammation, the changes in gene activity described here may be related to a reduction of the inflammatory status during the WS period. Analysis of the expression of surface marker genes indicates that lowering of gene expression refers in particular to cells of the myeloid lineage (Supplemental Table 3).

The genes in the second cluster also point to the importance of leukocytes and their involvement in inflammation. In human adipose-derived mesenchymal stromal cells expression of the secreted phosphoprotein 1 (*SPP1*) gene, of which the protein is also known as osteopontin (OPN), is induced by TGFB1 (25). OPN is a pro-inflammatory cytokine that promotes tissue infiltration of monocytes. Mice lacking *OPN* show a decreased macrophage infiltration in the adipose tissue and a decreased level of inflammation, but insulin sensitivity is higher compared to normal mice (34). OPN is the ligand for the receptor CD44, which is another member of the cluster. Similar to *SPP1*-KO mice, *CD44*-KO mice with high fat diet-induced obesity display lower levels of adipose tissue inflammation and are protected against insulin resistance (35). In humans, CD44 density on adipose tissue macrophages was associated with their pro-inflammatory status. Another member of the second cluster, but also part of the overlapping third cluster is PPIB, also known as cyclophilin B. Gene

expression of *PPIB* during the WS phase was positively correlated with WR% in the VLCD group. Cyclophilins A and B possess chemotactic activity towards various types of leukocytes (36). Cyclophilin B accumulates in the ECM, but can be cleaved off by MMPs. Its signal receptor is CD147, encoded by the gene *BSG*, and is known as the extracellular MMP inducer (37, 38). MMP activity is also influenced by OPN, because in the *OPN*-KO mice a reduced *MMP2* and *MMP9* activity was observed together with reduced ECM remodelling (39). In the present study, *MMP9* is on the intersection between the small and the two larger clusters. A role in adipose tissue remodelling has been suggested for MMP19, because *MMP19*-KO mice develop diet-induced obesity with hypertrophic adipocytes (40). Besides *PPIB*, *MMP9* and *MMP19*, the second cluster contains other genes that function as processing enzymes: cathepsin B (*CTSB*), *CTSD* and protein disulfide isomerise family A member 4 (*PDIA4*). Apparently, the second cluster harbours genes for leukocyte activity, insulin sensitivity and ECM remodelling. Both the leukocyte infiltration-related genes *SPP1*, *CD44*, *CD33* and *BSG/CD147* as well as the ECM remodelling genes *MMP9*, *MMP19*, *CTSB* and *CTSD* are up-regulated during WL and downregulated during WS. It suggests that while increased leukocyte infiltration with ECM remodelling occurs during WL, the reverse may happen during WS with reduction of leukocytes and ECM remodelling. Our clustering analysis indicates that both processes are linked suggesting that people with a stronger downregulation of ECM-remodelling may retain more immune cells and have higher risk of weight regain.

Most of the genes coding for processing enzymes of the second cluster are in overlap with the third cluster which harbours three more protein processing genes: collagen beta (1-O)galactosyltransferase 1 (*COLGALT1*), procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (*PLOD1*) and prolyl 4-hydroxylase subunit beta (*P4HB*). These genes are involved in modification of proteins including collagens. PLOD1, located in the endoplasmic reticulum, hydroxylates lysine residues in procollagen, whereas COLGALT1 binds galactose residues to hydroxylysine residues of collagen. P4HB can hydroxylate proline residues, which is a prerequisite for collagen fibre formation. Although these observations indicate that the third cluster is involved in ECM modification, there is also a link with insulin sensitivity, for instance through the nicastrin (*NCSTN*) gene. Adipocyte-specific loss-of-function of NCSTN reduces adipose insulin sensitivity (41). Altogether, the genes in the three connected and overlapping subclusters identify two biological processes in adipose tissue related to weight regain: inflammation and ECM remodelling, with a link to insulin sensitivity.

To the best of our knowledge this is the first report indicating the importance of adipose tissue leukocytes for the risk of weight regain after WL. Goyenechea et al. reported about whole body pro-inflammatory status in relation to weight regain by showing that subjects regaining weight had higher serum concentrations of TNF-α and mRNA levels of *TNF-α* and *NF-κB* subunits in PBMC (42). However, these measurements were done after WL, thus under the influence of a negative energy balance. Capel et al. used a different study design with a VLCD followed by a LCD, then followed by three months weight maintenance (15). They distinguished between expression changes of adipocyte metabolic genes and macrophage inflammatory-related genes. Seven of our clustered genes were related by them to macrophage activity during DI: *CTSB*, *CTSS*, *ITGAM*, *MMP9*, *MMP19*, *SPP1* and *TGFBI*.

The current findings point to the importance of leukocytes in the adipose tissue for weight regain. A lower reduction of the expression of certain leukocyte integrin genes shortly after WL leads to a

higher risk, which seems linked to ECM remodelling. Possibly, stronger reduction of ECM remodelling capacity during the WS phase leads to more retention of immune cells. It suggests that resident inflammation after WL increases the risk for weight regain, and might contribute to the worsening of the physiological condition during weight cycling (43). However, the present study can provide only indicative results based on gene expression. Furthermore, adipose tissue biopsies were used containing adipocytes as well as stromal vascular cells, which can obscure the exact contribution of each cell type in the tissue to the biological processes involved. Definite conclusions require further experimental proof, preferably performed with purified adipose tissue cell fractions.

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Supplementary Figures and Tables

Supplement Table 1: List of the 277 candidate extracellular matrix related genes used for the analysis.

Gene ID	Gene name	Gene #	Chromosome
A2M	alpha-2-macroglobulin	2	12
ABI3BP	ABI family, member 3 (NESH) binding protein	25890	3
ACTN1	actinin, alpha 1	87	14
ADAM10	ADAM metallopeptidase domain 10	102	15
ADAM11	ADAM metallopeptidase domain 11	4185	17
ADAM12	ADAM metallopeptidase domain 12	8038	10
ADAM15	ADAM metallopeptidase domain 15	8751	1
ADAM17	ADAM metallopeptidase domain 17	6868	2
ADAM19	ADAM metallopeptidase domain 19	8728	5
ADAM22	ADAM metallopeptidase domain 22	53616	7
ADAM8	ADAM metallopeptidase domain 8	101	10
ADAM9	ADAM metallopeptidase domain 9	8754	8
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	9510	21
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif, 14	140766	10
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	9509	5
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	9507	1
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	11096	21
ADAMTSL4	ADAMTS-like 4	54507	1
AGRN	Agrin	375790	1
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	183	1
APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2	323	4
AZGP1	alpha-2-glycoprotein 1, zinc-binding	563	7
BCL3	B-cell CLL/lymphoma 3	602	19
BGN	Biglycan	633	X
BMP1	bone morphogenetic protein 1	649	8
BMP2	bone morphogenetic protein 2	650	20
BMP4	bone morphogenetic protein 4	652	14
BSG	basigin (Ok blood group)	682	19
CALR	Calreticulin	811	19
CAPN1	calpain 1, (mu/I) large subunit	823	11
CASP3	caspase 3, apoptosis-related cysteine peptidase	836	4
CCDC80	coiled-coil domain containing 80	151887	3
CD151	CD151 molecule (Raph blood group)	977	11
CD44	CD44 molecule (Indian blood group)	960	11
CD47	CD47 molecule	961	3
CER1	cerberus 1, DAN family BMP antagonist	9350	9
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	1116	1
CIB1	calcium and integrin binding 1 (calmyrin)	10519	15
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	8483	15
CMA1	chymase 1, mast cell	1215	14
CNTNAP1	contactin associated protein 1	8506	17
COL11A1	collagen, type XI, alpha 1	1301	1
COL12A1	collagen, type XII, alpha 1	1303	6
COL13A1	collagen, type XIII, alpha 1	1305	10
COL14A1	collagen, type XIV, alpha 1	7373	8
COL15A1	collagen, type XV, alpha 1	1306	9
COL16A1	collagen, type XVI, alpha 1	1307	1
COL17A1	collagen, type XVII, alpha 1	1308	10
COL18A1	collagen, type XVIII, alpha 1	80781	21
COL1A1	collagen, type I, alpha 1	1277	17
COL1A2	collagen, type I, alpha 2	1278	7
COL20A1	collagen, type XX, alpha 1	57642	20
COL21A1	collagen, type XXI, alpha 1	81578	6
COL23A1	collagen, type XXIII, alpha 1	91522	5
COL25A1	collagen, type XXV, alpha 1	84570	4
COL26A1	collagen, type XXVI, alpha 1	136227	7
COL27A1	collagen, type XXVII, alpha 1	85301	9
COL2A1	collagen, type II, alpha 1	1280	12
COL3A1	collagen, type III, alpha 1	1281	2
COL4A1	collagen, type IV, alpha 1	1282	13
COL4A2	collagen, type IV, alpha 2	1284	13
COL4A5	collagen, type IV, alpha 5	1287	X
COL5A1	collagen, type V, alpha 1	1289	9
COL5A2	collagen, type V, alpha 2	1290	2
COL5A3	collagen, type V, alpha 3	50509	19

COL6A1	collagen, type VI, alpha 1	1291	21
COL6A2	collagen, type VI, alpha 2	1292	21
COL6A3	collagen, type VI, alpha 3	1293	2
COL6A6	collagen, type VI, alpha 6	131873	3
COL8A1	collagen, type VIII, alpha 1	1295	3
COL8A2	collagen, type VIII, alpha 2	1296	1
COL9A2	collagen, type IX, alpha 2	1298	1
COL9A3	collagen, type IX, alpha 3	1299	20
COLGALT1	collagen beta(1-O)galactosyltransferase 1	79709	19
COMP	cartilage oligomeric matrix protein	1311	19
CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	83716	16
CRTAP	cartilage associated protein	10491	3
CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	55790	8
CSPG4	chondroitin sulfate proteoglycan 4	1464	15
CST3	cystatin C	1471	20
CTGF	connective tissue growth factor	1490	6
CTRB1	chymotrypsinogen B1	1504	16
CTRB2	chymotrypsinogen B2	440387	16
CTSB	cathepsin B	1508	8
CTSD	cathepsin D	1509	11
CTSG	cathepsin G	1511	14
CTSK	cathepsin K	1513	1
CTSL	cathepsin L	1514	9
CTSS	cathepsin S	1520	1
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1545	2
CYR61	cysteine-rich, angiogenic inducer, 61	3491	1
DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	1605	3
DCN	Decorin	1634	12
DDR1	discoidin domain receptor tyrosine kinase 1	780	6
DDR2	discoidin domain receptor tyrosine kinase 2	4921	1
DMD	Dystrophin	1756	X
DPP4	dipeptidyl-peptidase 4	1803	2
DPT	Dermatopontin	1805	1
ECM1	extracellular matrix protein 1	1893	1
ECM2	extracellular matrix protein 2, female organ and adipocyte specific	1842	9
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	2202	2
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	30008	11
EGFL6	EGF-like-domain, multiple 6	25975	X
EGFLAM	EGF-like, fibronectin type III and laminin G domains	133584	5
ELANE	elastase, neutrophil expressed	1991	19
ELN	Elastin	2006	7
EMILIN1	elastin microfibril interfacer 1	11117	2
EMILIN2	elastin microfibril interfacer 2	84034	18
ENG	Endoglin	2022	9
ERCC2	excision repair cross-complementation group 2	2068	19
ERO1L	ERO1-like (S. cerevisiae)	30001	14
ERO1LB	ERO1-like beta (S. cerevisiae)	56605	1
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	2113	11
F11R	F11 receptor	50848	1
FAP	fibroblast activation protein, alpha	2191	2
FBLN1	fibulin 1	2192	22
FBLN2	fibulin 2	2199	3
FBLN5	fibulin 5	10516	14
FBN1	fibrillin 1	2200	15
FER	fer (fps/fes related) tyrosine kinase	2241	5
FGF2	fibroblast growth factor 2 (basic)	2247	4
FKBP9	FK506 binding protein 9, 63 kDa	11328	7
FMOD	Fibromodulin	2331	1
FN1	fibronectin 1	2335	2
FURIN	furin (paired basic amino acid cleaving enzyme)	5045	15
FZD4	frizzled class receptor 4	8322	11
GAS6	growth arrest-specific 6	2621	13
GFAP	glial fibrillary acidic protein	2670	17
GFOD2	glucose-fructose oxidoreductase domain containing 2	81577	16
GPC1	glypican 1	2817	2
GPM6B	glycoprotein M6B	2824	X
HAS2	hyaluronan synthase 2	3037	8

HAS3	hyaluronan synthase 3	3038	16
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	51144	11
HSPG2	heparan sulfate proteoglycan 2	3339	1
ICAM1	intercellular adhesion molecule 1	3383	19
ICAM2	intercellular adhesion molecule 2	3384	17
ICAM3	intercellular adhesion molecule 3	3385	19
ITGA1	integrin, alpha 1	3672	5
ITGA11	integrin, alpha 11	22801	15
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	3673	5
ITGA2B	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	3674	17
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	3675	17
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	3676	2
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	3678	12
ITGA6	integrin, alpha 6	3655	2
ITGA7	integrin, alpha 7	3679	12
ITGA8	integrin, alpha 8	8516	10
ITGA9	integrin, alpha 9	3680	3
ITGAD	integrin, alpha D	3681	16
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	3682	17
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	3683	16
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	3684	16
ITGAV	integrin, alpha V	3685	2
ITGAX	integrin, alpha X (complement component 3 receptor 4 subunit)	3687	16
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	3689	21
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	3690	17
ITGB4	integrin, beta 4	3691	17
ITGB5	integrin, beta 5	3693	3
ITGB7	integrin, beta 7	3695	12
JAM2	junctional adhesion molecule 2	58494	21
JAM3	junctional adhesion molecule 3	83700	11
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	81621	10
KDR	kinase insert domain receptor	3791	4
KIF9	kinesin family member 9	64147	3
LAMA2	laminin, alpha 2	3908	6
LAMA3	laminin, alpha 3	3909	18
LAMA5	laminin, alpha 5	3911	20
LAMB1	laminin, beta 1	3912	7
LAMB2	laminin, beta 2 (laminin S)	3913	3
LAMB3	laminin, beta 3	3914	1
LAMC1	laminin, gamma 1 (formerly LAMB2)	3915	1
LAMC3	laminin, gamma 3	10319	9
LGALS1	lectin, galactoside-binding, soluble, 1	3956	22
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	3959	17
LOX	lysyl oxidase	4015	5
LOXL1	lysyl oxidase-like 1	4016	15
LRP4	low density lipoprotein receptor-related protein 4	4038	11
LRP5	low density lipoprotein receptor-related protein 5	4041	11
LTBP1	latent transforming growth factor beta binding protein 1	4052	2
LTBP3	latent transforming growth factor beta binding protein 3	4054	11
LTBP4	latent transforming growth factor beta binding protein 4	8425	19
LUM	Lumican	4060	12
MADCAM1	mucosal vascular addressin cell adhesion molecule 1	8174	19
MATN2	matrilin 2	4147	8
MATN4	matrilin 4	8785	20
MFAP2	microfibrillar-associated protein 2	4237	1
MFAP3	microfibrillar-associated protein 3	4238	5
MFAP4	microfibrillar-associated protein 4	4239	17
MFAP5	microfibrillar associated protein 5	8076	12
MFI2	antigen p97 (melanoma associated)	4241	3
MMP14	matrix metallopeptidase 14 (membrane-inserted)	4323	14
MMP15	matrix metallopeptidase 15 (membrane-inserted)	4324	16
MMP19	matrix metallopeptidase 19	4327	12
MMP2	matrix metallopeptidase 2	4313	16
MMP7	matrix metallopeptidase 7	4316	11
MMP9	matrix metallopeptidase 9	4318	20

MPZL3	myelin protein zero-like 3	196264	11
NBL1	neuroblastoma 1, DAN family BMP antagonist	4681	1
NCAM1	neural cell adhesion molecule 1	4684	11
NCSTN	Nicastrin	23385	1
NDNF	neuron-derived neurotrophic factor	79625	4
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	4791	10
NID1	nidogen 1	4811	1
NID2	nidogen 2 (osteonidogen)	22795	14
NOTCH1	notch 1	4851	9
NPHP3	nephronophthisis 3 (adolescent)	27031	3
NPNT	Nephronectin	255743	4
NTN4	netrin 4	59277	12
OGN	Osteoglycin	4969	9
OLFML2A	olfactomedin-like 2A	169611	9
OLFML2B	olfactomedin-like 2B	25903	1
P3H2	prolyl 3-hydroxylase 2	55214	3
P3H3	prolyl 3-hydroxylase 3	10536	12
P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	5033	10
P4HB	prolyl 4-hydroxylase, beta polypeptide	5034	17
PCOLCE	procollagen C-endopeptidase enhancer	5118	7
PDGFA	platelet-derived growth factor alpha polypeptide	5154	7
PDGFB	platelet-derived growth factor beta polypeptide	5155	22
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	5156	4
PDIA4	protein disulfide isomerase family A, member 4	9601	7
PECAM1	platelet/endothelial cell adhesion molecule 1	5175	17
PLEC	Plectin	5339	8
PLOD1	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	5351	1
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	5352	3
POSTN	periostin, osteoblast specific factor	10631	13
PPIA	peptidylprolyl isomerase A (cyclophilin A)	5478	7
PPIB	peptidylprolyl isomerase B (cyclophilin B)	5479	15
PRDX4	peroxiredoxin 4	10549	X
PRELP	proline/arginine-rich end leucine-rich repeat protein	5549	1
PRG4	proteoglycan 4	10216	1
PRKCA	protein kinase C, alpha	5578	17
PSEN1	presenilin 1	5663	14
PTPRS	protein tyrosine phosphatase, receptor type, S	5802	19
PXDN	Peroxidasin	7837	2
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	8434	9
RGCC	regulator of cell cycle	28984	13
SDC1	syndecan 1	6382	2
SDC3	syndecan 3	9672	1
SDC4	syndecan 4	6385	20
SERAC1	serine active site containing 1	84947	6
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	5054	7
SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	871	11
SH3PXD2B	SH3 and PX domains 2B	285590	5
SMOC1	SPARC related modular calcium binding 1	64093	14
SMOC2	SPARC related modular calcium binding 2	64094	6
SOX9	SRY (sex determining region Y)-box 9	6662	17
SPARCL1	SPARC-like 1 (hevin)	8404	4
SPINT1	serine peptidase inhibitor, Kunitz type 1	6692	15
SPOCK2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	9806	10
SPON1	spondin 1, extracellular matrix protein	10418	11
SPON2	spondin 2, extracellular matrix protein	10417	4
SPP1	secreted phosphoprotein 1	6696	4
TCF15	transcription factor 15 (basic helix-loop-helix)	6939	20
TGFB1	transforming growth factor, beta 1	7040	19
TGFB2	transforming growth factor, beta 2	7042	1
TGFB3	transforming growth factor, beta 3	7043	14
TGFBI	transforming growth factor, beta-induced, 68kDa	7045	5
TGFBR3	transforming growth factor, beta receptor III	7049	1
THBS1	thrombospondin 1	7057	15
THBS2	thrombospondin 2	7058	6
THBS3	thrombospondin 3	7059	1

TIMP1	TIMP metallopeptidase inhibitor 1	7076	X
TIMP2	TIMP metallopeptidase inhibitor 2	7077	17
TIMP4	TIMP metallopeptidase inhibitor 4	7079	3
TLL1	tolloid-like 1	7092	4
TNC	tenascin C	3371	9
TNF	tumor necrosis factor	7124	6
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	4982	8
TNN	tenascin N	63923	1
TNXB	tenascin XB	7148	6
TRAPPC4	trafficking protein particle complex 4	51399	11
VCAM1	vascular cell adhesion molecule 1	7412	1
VCAN	Versican	1462	5
VIT	Vitrin	5212	2
VWA1	von Willebrand factor A domain containing 1	64856	1
VWF	von Willebrand factor	7450	12

Supplement Table 2: Correlation matrix of changes in ECM gene expressions with each other during the weight loss phase in the VLCD[1].

	APBB2	NTN4	ITGA1	SPARCL1	EMILIN2	SPINT1	CTSL	ITGAE	CTSS	ITGAM	ITGAX	ITGB2	TGFB1	CHI3L1	MMP9	SPP1	CD44	ITGA3	PDIA4	MMP19	CTSB	CTSD	SERPINE1	TIMP1	NCSTN	CAPN1	P4HB	CALR	ACTN1	PPIB	COLGALT1	PLOD1	ITGA5	SDC4	TGFBI
APBB2	1	0.430	0.721	0.621	-0.782	-0.636	-0.674	-0.641	-0.766	-0.740	-0.679	-0.741	-0.650	-0.582	-0.568	-0.551	-0.726	-0.676	-0.548	-0.544	-0.546	-0.404	-0.264	-0.281	-0.116	-0.035	-0.024	-0.183	-0.016	-0.041	-0.181	-0.083	-0.033	-0.175	-0.314
NTN4	0.430	1	0.536	0.643	-0.440	-0.488	-0.547	-0.569	-0.600	-0.712	-0.607	-0.595	-0.710	-0.673	-0.663	-0.608	-0.482	-0.649	-0.591	-0.728	-0.763	-0.741	-0.577	-0.569	-0.738	-0.543	-0.515	-0.629	-0.578	-0.618	-0.610	-0.610	-0.419	-0.678	-0.437
ITGA1	0.721	0.536	1	0.868	-0.779	-0.585	-0.693	-0.640	-0.676	-0.728	-0.664	-0.675	-0.704	-0.642	-0.629	-0.573	-0.709	-0.695	-0.781	-0.667	-0.707	-0.669	-0.575	-0.543	-0.448	-0.473	-0.482	-0.564	-0.377	-0.362	-0.614	-0.564	-0.409	-0.395	-0.490
SPARCL1	0.621	0.643	0.868	1	-0.716	-0.652	-0.683	-0.659	-0.635	-0.768	-0.660	-0.679	-0.707	-0.700	-0.683	-0.605	-0.693	-0.743	-0.847	-0.773	-0.801	-0.763	-0.715	-0.567	-0.644	-0.571	-0.647	-0.649	-0.649	-0.464	-0.686	-0.711	-0.533	-0.476	-0.572
EMILIN2	-0.782	-0.440	-0.779	-0.716	1	0.438	0.736	0.676	0.810	0.914	0.696	0.845	0.732	0.591	0.570	0.444	0.775	0.851	0.614	0.658	0.716	0.657	0.417	0.427	0.348	0.307	0.343	0.221	0.181	0.132	0.445	0.240	0.336	0.342	0.380
SPINT1	-0.636	-0.488	-0.585	-0.652	0.438	1	0.663	0.873	0.708	0.830	0.814	0.878	0.715	0.763	0.800	0.763	0.696	0.908	0.612	0.721	0.612	0.485	0.562	0.397	0.346	0.288	0.151	0.397	0.413	0.163	0.558	0.460	0.297	0.241	0.372
CTSL	-0.674	-0.547	-0.693	-0.683	0.736	0.663	1	0.751	0.804	0.837	0.705	0.819	0.710	0.820	0.775	0.826	0.895	0.819	0.725	0.826	0.772	0.729	0.651	0.676	0.528	0.449	0.368	0.516	0.306	0.289	0.579	0.398	0.476	0.567	0.709
ITGAE	-0.641	-0.569	-0.640	-0.659	0.676	0.873	0.751	1	0.772	0.875	0.811	0.893	0.771	0.795	0.832	0.810	0.728	0.941	0.727	0.829	0.722	0.598	0.642	0.524	0.439	0.370	0.236	0.501	0.434	0.317	0.587	0.502	0.307	0.375	0.430
CTSS	-0.766	-0.600	-0.676	-0.635	0.810	0.708	0.804	0.772	1	0.928	0.876	0.939	0.889	0.778	0.815	0.776	0.821	0.852	0.704	0.752	0.788	0.720	0.629	0.590	0.469	0.377	0.250	0.461	0.329	0.261	0.493	0.356	0.388	0.412	0.492
ITGAM	-0.740	-0.712	-0.728	-0.768	0.914	0.830	0.837	0.875	0.928	1	0.913	0.968	0.916	0.894	0.911	0.863	0.873	0.951	0.827	0.896	0.891	0.792	0.760	0.657	0.612	0.489	0.411	0.587	0.510	0.404	0.644	0.555	0.508	0.538	0.600
ITGAX	-0.679	-0.607	-0.664	-0.660	0.696	0.814	0.705	0.811	0.876	0.913	1	0.951	0.915	0.852	0.919	0.831	0.790	0.874	0.649	0.727	0.741	0.648	0.623	0.536	0.488	0.326	0.239	0.451	0.384	0.267	0.577	0.462	0.408	0.413	0.361
ITGB2	-0.741	-0.595	-0.675	-0.679	0.845	0.878	0.819	0.893	0.939	0.968	0.951	1	0.887	0.867	0.910	0.869	0.860	0.945	0.715	0.805	0.778	0.678	0.662	0.562	0.469	0.361	0.232	0.475	0.389	0.253	0.574	0.450	0.394	0.393	0.484
TGFB1	-0.650	-0.710	-0.704	-0.707	0.732	0.715	0.710	0.771	0.889	0.916	0.915	0.887	1	0.815	0.861	0.765	0.746	0.863	0.774	0.824	0.872	0.810	0.758	0.716	0.672	0.553	0.463	0.595	0.519	0.494	0.654	0.563	0.599	0.597	0.493
CHI3L1	-0.582	-0.673	-0.642	-0.700	0.591	0.763	0.820	0.795	0.778	0.894	0.852	0.867	0.815	1	0.977	0.951	0.846	0.851	0.782	0.899	0.847	0.757	0.730	0.687	0.661	0.494	0.461	0.662	0.554	0.460	0.630	0.552	0.550	0.613	0.654
MMP9	-0.568	-0.663	-0.629	-0.683	0.570	0.800	0.775	0.832	0.815	0.911	0.919	0.910	0.861	0.977	1	0.957	0.812	0.879	0.763	0.863	0.831	0.736	0.740	0.661	0.640	0.462	0.411	0.636	0.540	0.429	0.634	0.569	0.518	0.575	0.554
SPP1	-0.551	-0.608	-0.573	-0.605	0.444	0.763	0.826	0.810	0.776	0.863	0.831	0.869	0.765	0.951	0.957	1	0.793	0.825	0.708	0.836	0.782	0.695	0.711	0.667	0.575	0.418	0.383	0.605	0.435	0.415	0.566	0.495	0.453	0.571	0.591
CD44	-0.726	-0.482	-0.709	-0.693	0.775	0.696	0.895	0.728	0.821	0.873	0.790	0.860	0.746	0.846	0.812	0.793	1	0.808	0.748	0.791	0.753	0.672	0.615	0.625	0.475	0.406	0.311	0.482	0.312	0.253	0.488	0.365	0.508	0.425	0.674
ITGA3	-0.676	-0.649	-0.695	-0.743	0.851	0.908	0.819	0.941	0.852	0.951	0.874	0.945	0.863	0.851	0.879	0.825	0.808	1	0.797	0.886	0.819	0.708	0.735	0.590	0.560	0.460	0.333	0.555	0.509	0.320	0.678	0.557	0.464	0.502	0.515
PDIA4	-0.548	-0.591	-0.781	-0.847	0.614	0.612	0.725	0.727	0.704	0.827	0.649	0.715	0.774	0.782	0.763	0.708	0.748	0.797	1	0.895	0.894	0.807	0.857	0.731	0.706	0.647	0.691	0.760	0.664	0.564	0.649	0.678	0.661	0.628	0.734
MMP19	-0.544	-0.728	-0.667	-0.773	0.658	0.721	0.826	0.829	0.752	0.896	0.727	0.805	0.824	0.899	0.863	0.836	0.791	0.886	0.895	1	0.952	0.874	0.867	0.802	0.791	0.698	0.641	0.773	0.691	0.634	0.730	0.675	0.686	0.706	0.749
CTSB	-0.546	-0.763	-0.707	-0.801	0.716	0.612	0.772	0.722	0.788	0.891	0.741	0.778	0.872	0.847	0.831	0.782	0.753	0.819	0.894	0.952	1	0.950	0.900	0.845	0.860	0.745	0.737	0.783	0.703	0.700	0.736	0.722	0.734	0.743	0.710
CTSD	-0.404	-0.741	-0.669	-0.763	0.657	0.485	0.729	0.598	0.720	0.792	0.648	0.678	0.810	0.757	0.736	0.695	0.672	0.708	0.807	0.874	0.950	1	0.891	0.837	0.910	0.853	0.816	0.771	0.755	0.711	0.819	0.774	0.801	0.773	0.697
SERPINE1	-0.264	-0.577	-0.575	-0.715	0.417	0.562	0.651	0.642	0.629	0.760	0.623	0.662	0.758	0.730	0.740	0.711	0.615	0.735	0.857	0.867	0.900	0.891	1	0.807	0.859	0.813	0.819	0.780	0.783	0.679	0.777	0.787	0.844	0.699	0.666
TIMP1	-0.281	-0.569	-0.543	-0.567	0.427	0.397	0.676	0.524	0.590	0.657	0.536	0.562	0.716	0.687	0.661	0.667	0.625	0.590	0.731	0.802	0.845	0.837	0.807	1	0.802	0.755	0.700	0.741	0.536	0.757	0.650	0.603	0.789	0.711	0.713
NCSTN	-0.116	-0.738	-0.448	-0.644	0.348	0.346	0.528	0.439	0.469	0.612	0.488	0.469	0.672	0.661	0.640	0.575	0.475	0.560	0.706	0.791	0.860	0.910	0.859	0.802	1	0.870	0.870	0.767	0.829	0.789	0.776	0.820	0.843	0.822	0.612
CAPN1	-0.035	-0.543	-0.473	-0.571	0.307	0.288	0.449	0.370	0.377	0.489	0.326	0.361	0.553	0.494	0.462	0.418	0.406	0.460	0.647	0.698	0.745	0.853	0.813	0.755	0.870	1	0.861	0.758	0.772	0.751	0.793	0.790	0.893	0.648	0.657
P4HB	-0.024	-0.515	-0.482	-0.647	0.343	0.151	0.368	0.236	0.250	0.411	0.239	0.232	0.463	0.461	0.411	0.383	0.311	0.333	0.691	0.641	0.737	0.816	0.819	0.700	0.870	0.861	1	0.758	0.810	0.801	0.689	0.806	0.823	0.705	0.624
CALR	-0.183	-0.629	-0.564	-0.649	0.221	0.397	0.516	0.501	0.461	0.587	0.451	0.475	0.595	0.662	0.636	0.605	0.482	0.555	0.760	0.773	0.783	0.771	0.780	0.741	0.767	0.758	0.758	1	0.736	0.840	0.657	0.758	0.709	0.655	0.693
ACTN1	-0.016	-0.578	-0.377	-0.649	0.181	0.413	0.306	0.434	0.329	0.510	0.384	0.389	0.519	0.554	0.540	0.435	0.312	0.509	0.664	0.691	0.703	0.755	0.783	0.536	0.829	0.772	0.810	0.736	1	0.677	0.756	0.859	0.700	0.565	0.489
PPIB	-0.041	-0.618	-0.362	-0.464	0.132	0.163	0.289	0.317	0.261	0.404	0.267	0.253	0.494	0.460	0.429	0.415	0.253	0.320	0.564	0.634	0.700	0.711	0.679	0.757	0.789	0.751	0.801	0.840	0.677	1	0.526	0.720	0.662	0.613	0.552
COLGALT1	-0.181	-0.610	-0.614	-0.686	0.445	0.558	0.579	0.587	0.493	0.644	0.577	0.574	0.654	0.630	0.634	0.566	0.488	0.678	0.649	0.730	0.736	0.819	0.777	0.650	0.776	0.793	0.689	0.657	0.756	0.526	1	0.865	0.717	0.653	0.554
PLOD1	-0.083	-0.610	-0.564	-0.711	0.240	0.460	0.398	0.502	0.356	0.555	0.462	0.450	0.563	0.552	0.569	0.495	0.365	0.557	0.678	0.675	0.722	0.774	0.787	0.603	0.820	0.790	0.806	0.758	0.859	0.720	0.865	1	0.667	0.573	0.514
ITGA5	-0.033	-0.419	-0.409	-0.533	0.336	0.297	0.476	0.307	0.388	0.508	0.408	0.394	0.599	0.550	0.518	0.453	0.508	0.464	0.661	0.686	0.734	0.801	0.844	0.789	0.843	0.893	0.823	0.709	0.700	0.662	0.717	0.667	1	0.684	0.689
SDC4	-0.175	-0.678	-0.395	-0.476	0.342	0.241	0.567	0.375	0.412	0.538	0.413	0.393	0.597	0.613	0.575	0.571	0.425	0.502	0.628	0.706	0.743	0.773	0.699	0.711	0.822	0.648	0.705	0.655	0.565	0.613	0.653	0.573	0.684	1	0.553
TGFBI	-0.314	-0.437	-0.490	-0.572	0.380	0.372	0.709	0.430	0.492	0.600	0.361	0.484	0.493	0.654	0.554	0.591	0.674	0.515	0.734	0.749	0.710	0.697	0.666	0.713	0.612	0.657	0.624	0.693	0.489	0.552	0.554	0.514	0.689	0.553	1

[1] Values are Pearson R's correlation coefficients: green fill $r \geq 0.700$; red fill $r \leq -0.700$. VLCD (n=26). ACTN1, actinin alpha 1; APBB2, amyloid beta precursor protein-binding, family B, member 2; CALR, calreticulin; CAPN1, calpain 1; CD44, CD44 molecule; CHI3L1, chitinase 3 like 1; COLGALT1, collagen beta (1-O)galactosyltransferase 1; CTSB, cathepsin B; CTSD, cathepsin D; CTSL, cathepsin L; CTSS, cathepsin S; EMILIN2, elastin microfibril interface 2; ITGA1, integrin, alpha 1; ITGA3, integrin, alpha 3; ITGA5, integrin, alpha 5; ITGAE, integrin alpha E; ITGAM, integrin alpha M; ITGAX, integrin alpha X; ITGB2, integrin beta 2; MMP19, matrix metallopeptidase 19; MMP9, matrix metallopeptidase 9; NCSTN, nicastrin; OPN, osteopontin; P4HB, prolyl 4-hydroxylase subunit beta; PDIA4, protein disulfide isomerase family A member 4; PLOD1, procollagen-lysine,2-oxoglutarate 5-dioxygenase 1; PPIB, peptidylprolyl isomerase B; SDC4, syndecan 4; SERPINE1, serpin peptidase inhibitor, clade E, member 1; SPARCL1, secreted protein acidic and cysteine rich-like 1; SPINT1, serine peptidase inhibitor kunitz type 1; SPP1, secreted phosphoprotein 1; TGFB1, transforming growth factor beta 1; TGFBI, transforming growth factor, beta-induced; TIMP1, TIMP metallopeptidase inhibitor 1; VLCD, very-low-calorie diet.

Supplement Table 3: Expression changes of surface marker genes in the VLCD during the weight stabilisation phase[1,2].

Surface antigen	Gene ID	Myeloid cells									Lymphoid cells				FC	Q-value
		Macrophages	Monocytes	Neutrophils	Basophils	Eosinophils	Granulocytes	Platelets	Mast cells	Myeloid cells	NK	Cytotoxic T-cell	T-cell	(pre-) B-cells		
CD204	MSR1	X													-2.09	0.000
CD9	CD9				X	X		X						X	-1.51	0.000
CD68	CD68	X	X	X	X					X					-1.61	0.000
CD107b	LAMP2							X					X		-1.19	0.000
CD107a	LAMP1						X	X					X		-1.16	0.001
CD11b	ITGAM		X	X			X				X		X	X	-1.39	0.002
CD300a	CD300a		X				X		X		X		X	X	-1.38	0.004
CD163	CD163	X	X												-1.29	0.013
CD11c	ITGAX	X	X				X						X	X	-1.54	0.014
CD352	SLAMF6									X	X		X	X	-1.37	0.021
CD14	CD14	X	X				X								-1.17	0.062
CD312	ADGRE2	X	X				X								-1.29	0.073
CD281	TLR1	X	X												-1.20	0.078
CD56	NCAM1										X		X		-1.09	0.145
CD3d	CD3d												X		1.12	0.157
CD179b	IGLL1													X	1.06	0.227
CD1c	CD1c												X	X	1.10	0.231
CD8a	CD8a										X	X	X		1.09	0.235
CD11a	ITGAL	X	X												-1.05	0.420
CD3e	CD3e												X		1.05	0.387
CD169	SIGLEC1	X													-1.03	0.441
CD3g	CD3g												X		1.04	0.447
CD354	TREM1									X					1.04	0.470
CD159c	KLRC2										X	X			1.01	0.484
CD161	KLRB1										X		X		1.01	0.502
CD226	CD226		X					X			X		X		-1.01	0.504
CD94	KLRD1										X	X			1.01	0.505
CD69	CD69	X						X			X		X		1.00	0.525
CD2	CD2										X		X	X	1.15	0.074
CD203c	ENPP3				X				X						1.15	0.005

[1] Gene expression fold change (FC), significantly different when Q-value <0.05 in paired t-test with Bayesian correction (Limma) during the weight stabilization phase for VLCD (n=26).

[2] Each cross represents in which immune cell a specific surface marker gene is expressed based on http://docs.abcam.com/pdf/immunology/Guide-to-human-CD-antigens.pdf. ADGRE2, adhesion G protein-coupled receptor E2; CD, cluster of differentiation; ENPP3, ectonucleotide pyrophosphatase/phosphodiesterase 3; IGLL1, immunoglobulin lambda-like polypeptide 1;

ITGAL, integrin, alpha L; ITGAM, integrin, alpha M; ITGAX, integrin, alpha X; KLRB1, killer cell lectin-like receptor subfamily B, member 1; KLRC2, killer cell lectin-like receptor subfamily C, member 2; KLRD1, killer cell lectin-like receptor subfamily D, member 1; LAMP1, lysosomal-associated membrane protein 1; LAMP2, lysosomal-associated membrane protein 2; MSR1, macrophage scavenger receptor 1; NCAM1, neural cell adhesion molecule 1; SIGLEC1, sialic acid binding Ig-like lectin 1; SLAMF6, SLAM family member 6; TLR1, toll-like receptor 1; TREM1, triggering receptor expressed on myeloid cells 1; VLCD, very-low-calorie diet.

Chapter 4

Weight loss-induced stress in subcutaneous adipose tissue is related to weight regain

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Abstract

Initial successful weight loss is often followed by weight regain after the dietary intervention. Compared with lean people, cellular stress in adipose tissue is increased in obese subjects. However, the relation between cellular stress and the risk for weight regain after weight loss is unclear. Therefore, we determined the expression levels of stress proteins during weight loss and weight maintenance in relation to weight regain. In vivo findings were compared with results from *in vitro* cultured human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes. In total, eighteen healthy subjects underwent an 8-week diet programme with a 10-month follow-up. Participants were categorised as weight maintainers or weight regainers (WR) depending on their weight changes during the intervention. Abdominal subcutaneous adipose tissue biopsies were obtained before and after the diet and after the follow-up. *In vitro* differentiated SGBS adipocytes were starved for 96 h with low (0.55mM) glucose. Levels of stress proteins were determined by Western blotting. WR showed increased expressions of β-actin, calnexin, heat shock protein (HSP) 27, HSP60 and HSP70. Changes of β-actin, HSP27 and HSP70 are linked to HSP60, a proposed key factor in weight regain after weight loss. SGBS adipocytes showed increased levels of β-actin and HSP60 after 96 h of glucose restriction. The increased level of cellular stress proteins in the adipose tissue of WR probably resides in the adipocytes as shown by *in vitro* experiments. Cellular stress accumulated in adipose tissue during weight loss may be a risk factor for weight regain.

Introduction

The prevalence of overweight and obesity has risen in recent years, causing a worldwide public health problem due to an increased risk for the metabolic syndrome and development of type 2 diabetes, CVD and cancer. This risk can be significantly lowered by losing weight (1). Usually, losing weight can be readily achieved, but sustaining the lower weight is problematic. Successfully maintaining weight loss, defined as 'keeping off an intentional loss of at least 10% body weight for at least one year' (2), is proven to be difficult. In general, up to 80% of the people are unsuccessful (3, 4). It is therefore of great importance to understand the mechanisms that influence the risk for weight regain. Many studies have already shown the involvement of various psychosocial and lifestyle factors in weight maintenance such as motivation to lose weight, social support, physical activity and eating habits (3, 5). Other studies have shown effects of physiological and molecular parameters on weight maintenance – for example, increased insulin sensitivity after weight loss is associated with weight regain (6, 7). In addition, fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) are associated with weight regain (8). Wang et al. (9) showed that men with the metabolic syndrome at baseline were more at risk for weight regain than men without this condition. Further, it has been shown that a higher protein intake after weight loss improves weight maintenance (10). Irisin levels decrease when body weight is reduced but returns to baseline levels in subjects regaining the lost weight (11). All these findings indicate the involvement of various factors in weight regain or maintenance after weight loss. Studies have pointed at special roles of sex hormones (9), metabolic factors (9, 12), hunger and satiety hormones (13) as well as epigenetic modifications such as methylation of the neuropeptide Y and pro-opiomelanocortin gene promoters in maintenance of weight loss (14). The present study focused particularly on the adipose tissue as a key player for weight regain or maintenance after weight loss.

Baseline BMI, fat mass and plasma leptin concentrations are associated with increased risk for weight regain, indicating an important role for adipocytes (15, 16). Mauriège et al. (17) showed that metabolic parameters of the subcutaneous adipose tissue are related to weight regain. Change of lipoprotein lipase (LPL) activity during weight loss was negatively related to weight regain in women, whereas change of alpha 2 adrenergic receptor (α2-AR) was positively related to weight regain in men. The latter effect seems to occur despite the fact that adrenaline stimulated lipolysis returns to pre-diet levels during the weight-maintenance phase (18). In a weight loss-maintenance study, Verhoef et al. (19) observed that the change in weight during follow-up was related to a change in the levels of the lipolytic enzyme adipose triglyceride lipase in adipose tissue during weight loss. Compared with lean people, the subcutaneous adipose tissue of obese people shows higher endoplasmic reticulum (ER) stress at the level of proteins and gene expression (20). Sharma et al. (21) reported a positive correlation between BMI and activating transcription factor 67 (ATF67)-induced ER stress markers. Human individuals losing weight after a gastric bypass showed decreased levels of ER stress in the adipose tissue, linking weight changes directly to ER stress (22). Although those findings suggest that cellular stress is a consequence of weight gain, it might well be that cellular stress is also a factor that stimulates accumulation of fat. In fact, reactive oxygen species promote the initiation of adipogenesis as well as the terminal differentiation of adipocytes (23, 24). In this regard, we hypothesized that cellular stress of adipocytes could also play a role in the risk for weight regain after weight loss. In order to investigate this, we have compared levels of stress-related proteins in the adipose tissue from overweight men and women during weight loss and follow-up. In addition, we compared in vivo findings with observations from *in vitro* cultured adipocytes after

glucose restriction. As stress markers, we selected eight different proteins, which are all involved in different aspects of cellular stress; these were β-actin, binding Ig protein (BiP), calnexin, heat shock protein (HSP) 27, HSP60, HSP70, superoxide dismutase (SOD) 1 and SOD2. β-Actin is a component of actin microfilaments that provide structural support and mediate cellular motion (25). BiP is involved in translocation, folding and assembly of secretory and transmembrane proteins within the ER (26). Calnexin is a Ca-binding protein involved in proper folding of glycoproteins in the ER (27, 28). HSP27 activates the proteasome to degrade unnecessary or damaged proteins (29, 30), whereas HSP60 acts as a chaperonin for proteins to be transported to the mitochondrion (20). HSP60 is also released because of inflammatory stress to exert autocrine/paracrine effects on adipocytes (31). HSP70 binds to misfolded proteins after stress-induced protein damage (32). SOD 1 and 2 catalyse conversion of superoxide radicals into peroxide and O2 to defend the cell against oxidative stress (33). These specific proteins have been selected because several studies have already shown that they can be regarded as markers of cellular stress, such as ER stress and oxidative stress (25-28, 31, 33). Furthermore, based on proteomics observations, Wang et al. (34) have suggested an association between these proteins and cellular stress. Our objective was to test the hypothesis that altered expressions of the above mentioned proteins during weight loss and follow-up are related to the risk for weight regain.

Material and methods

Subjects and study design

The eighteen subjects of the present study (nine females, nine males), aged 20-55 years with a BMI of 27-39 kg/m^2, were selected from a larger cohort, which underwent a weight loss-maintenance intervention (19). This study was conducted according to the Declaration of Helsinki guidelines and registered on ClinicalTrials.gov (registration number: NCT01015508). All procedures involving human participants were approved by the Central Committee on Human Research and by the Medical Ethical Committee of Maastricht University, The Netherlands. Written informed consent was obtained from all subjects.

Selection of the eighteen subjects was based on their weight change throughout the intervention. In short, subjects were healthy, non-smoking and not using medication (except for woman using oral contraception). All subjects maintained a stable weight for three months prior to the intervention. Participants followed an 8-week, very-low-energy diet with about 2.1 MJ/day (Modifast, Nutrition et Santé Benelux). The diet provided 50g carbohydrates, 52g protein, 7g fat and a micronutrient content, which met the Dutch recommended daily intake. Following weight loss, subjects were instructed to maintain their new body weight for a period of 10 months without following a prescribed diet. However, subjects did receive advice on monitoring and limiting food intake. At three time points, before diet (t0), after diet (t2) and after 10 months follow-up (t12), adipose tissue biopsies and plasma samples were obtained. Body weight was measured in underwear after overnight fasting using a calibrated scale of the BodPod®.

Test subjects were classified into two groups according to the percentage weight loss during the diet, and percentage weight regain during follow-up: weight maintainers (WM) and weight regainers (WR). Participants were categorized as WM if there was a weight reduction of at least 10% but then

regained less than 6% weight during follow-up. Participants were categorized as WR if there was a weight reduction of at least 10% but then regained 6% or more weight.

Adipose tissue biopsies and protein isolation

Abdominal subcutaneous adipose tissue biopsies were obtained by needle biopsy under local anaesthesia (2% lidocaine, Fresenius Kabi) after an overnight fast. Tissue was immediately rinsed in saline, frozen in liquid nitrogen and stored at -80°C until protein isolation. About 350 mg of frozen adipose tissue was ground in a mortar with liquid nitrogen. The powder was dissolved in 200 µl of 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 65mM-dithiothreitol/100 mg biopsy and vortexed for 5 minutes. The homogenate was centrifuged for 30 minutes 14 000 rpm at 10°C. The supernatant was carefully collected, aliquoted and stored at -80°C until Western blotting. Protein concentrations were determined with a Bradfort-based protein assay (Bio-Rad).

In vitro cell culture experiments

Human Simpson–Golabi–Behmel Syndrome (SGBS) cells were cultured and differentiated as described previously (35). In brief, SGBS preadipocytes were cultured in a T25 flask till 90% confluence in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 1% penicillin/streptomycin (Life Technologies), 10% fetal bovine serum (Bodinco), 66 nM biotin and 33 nM D-pantothenic acid (Sigma-Aldrich). Confluent preadipocytes were split into two 150-mm petri dishes; a starvation dish and a control dish. In parallel, the two dishes were cultured until 80 to 90% confluence in the same medium as described above. Medium was changed every 2-3 days. To induce differentiation, confluent pre-adipocytes were washed with PBS buffer and medium was changed to serum free DMEM/F12 medium containing 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 25 nM dexamethasone, 2 µM rosiglitazone, 0.01 mg/mL human transferrin, 20 nM insulin, 100 nM cortisol and 0.2 nM triiodothyronine (Sigma-Aldrich). After 4 days, cells were further cultured in serum free DMEM/F12 medium containing 20 nM insulin, 100 nM cortisol, 0.01 mg/mL human transferrin, and 0.2 nM triiodothyronine. Every 2 days the medium was refreshed. After 14 days, 65-80% of the pre-adipocytes were differentiated into mature adipocytes.
For starvation experiments, mature adipocytes were cultured in DMEM/F12 medium without glucose supplemented with 1% penicillin/streptomycin, 20 nM insulin and 0.55 mM glucose for a period of 96h as glucose restriction to lose fat (36). As control, mature adipocytes, originating from the same pre-adipocyte as the starved adipocytes, were cultured in the same medium with 17.5 mM glucose. After 96h, cells were lysed using RIPA buffer and protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Fischer). Samples were stored at -80°C until Western blotting. This entire procedure was performed three times in parallel to create three separate experiments.

Western blotting

A total of 15 µg of protein from *in vitro* or *in vivo* samples was separated on a 12% SDS-polyacrylamide Criterion gel (Bio-Rad) at 180 V. After electrophoretic separation, proteins were transferred to 0.45 µm nitrocellulose membranes in a Trans-Blot Turbo Transfer System (30 min at 25 V, Bio-Rad). Afterwards, the membranes are stained with Ponceau S to check for protein bands. Following destaining, blots were blocked for 1 hour in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% non-fat dry milk powder. Thereafter, blots were incubated overnight at 4°C with primary antibodies against β-actin (1:1000 dilution, Santa Cruz), BiP (1:300 dilution, R&D systems),

calnexin (1:1000 dilution, Cell signaling), HSP27 (1:1000 dilution, Cell signaling), HSP70 (1:1000 dilution, R&D systems), SOD1 (1:800 dilution, R&D systems) and SOD2 (1:1000 dilution, R&D systems) in TBST containing 5% non-fat dry milk powder. After incubation with primary antibody, membranes were washed three times for 10 min with TBST and incubated for 1.5h with a 1:10000 dilution of horseradish peroxidase-conjugated secondary antibody (DAKO) in TBST containing 5% non-fat dry milk powder. After washing, bands were visualised using a chemiluminescent substrate (SuperSignal CL, Thermo Fischer Scientific) and a charge-coupled device (CCD) camera (XRS-system, Bio-Rad). Data were quantified using Image LabTM 4.0 Software (Bio-Rad).

A pooled sample was used to correct for differences between blots. Blots were normalized using all protein bands seen with Ponceau S as a measure of total protein in the sample instead of a housekeeping protein. Generally used housekeeping proteins, such as β-actin, are influenced by dieting as previously shown in 2D-gel electrophoresis analysis by Bouwman et al. (37), while Ponceau S has recently been indicated to be a suitable alternative for housekeeping proteins (38).

HSP60 plasma concentrations

Plasma samples were stored at -80°C after withdrawing. HSP60 concentrations were determined by ELISA (Cusabio Biotech) following the manufacturer's instructions. Absorbance was read by a spectrophotometer at 450 nm.

Statistical analyses

Independent T-test was carried out for baseline comparisons between WM and WR. ANOVA repeated measures were carried out to determine possible differences over time within a group for the human intervention study. For the *in vitro* measurements, a dependent T-test was carried out to determine differences between control cells and glucose restricted cells. The dependent T-test was used because control cells and glucose starved cells originate from the same cultured pre-adipocytes. For the human study, fold changes during weight loss and during the whole study were evaluated by ratio of the values in t2:t0 and t12:t0 respectively. Fold change comparisons between WM and WR were performed by using independent T-test.

Pearson R's and Spearman Rho's correlation coefficients were calculated for relationships between parameters during the dieting period. Only correlations found significant with both tests were reported, this was done to make the analysis more stringent and reliable. Spearman rank correlation might be the preferred method since we do not a priori know whether the protein changes are in a linear relationship. On the other hand, when proteins are closely functionally interacting, a linear relationship might be expected. Therefore, we decided to select only values that were significant ($P<0.01$) with both methods. Statistical analyses were done using SPSS 20.0 for Windows (SPSS Inc.). For all statistical tests $P<0.05$ was considered to be statistically significant, except for correlations ($P<0.01$). Variation in the number of participants between analyses is due to the exclusion of subjects with missing data. All variables were checked for normal distribution, and variables with a skewed distribution were ln-transformed to satisfy conditions of normality. Extreme outliers (values higher than 3x Interquartile range calculated with SPSS) influencing the data were removed during statistical analysis. Data are presented as mean ± SEM, unless otherwise indicated.

Results

Subject characteristics

No significant difference was observed between the WM and WR group for baseline weight and BMI. Body weight was significantly reduced after diet in both groups, and during follow-up weight gain was significant in the WR group (P<0.001), but not in the WM group (Table 1). The WR group decreased weight by 14.3% and regained 8.2% of the weight at t2. The WM group decreased weight by 14.4% and only regained 0.9%. Figure 1 shows individual weight changes of the WM and WR groups.

Table 1: Subject characteristic of weight regainers and weight maintainers at time points t0, t2 and t12.

	Study start (t0)	After weight loss (t2)	After follow-up (t12)
Weight maintainers			
Sex (male/female)	5/4	5/4	5/4
Weight (kg) †	95.4 ± 5.7	81.7 ± 4.6	82.8 ± 5.5
BMI (kg/m^2) †	32.0 ± 0.7	27.5 ± 0.7	27.7 ± 0.8
Weight regainers			
Sex (male/female)	4/5	4/5	4/5
Weight (kg) † ‡	95.4 ± 5.7	81.8 ± 5.0	89.1 ± 5.3
BMI (kg/m^2) † ‡	31.5 ± 1.1	27.1 ± 1.1	29.4 ± 1.1

Mean values ± standard errors. Repeated-measures ANOVA between time points: † P<0.001 (t0-t2); ‡ P<0.001 (t2-t12).

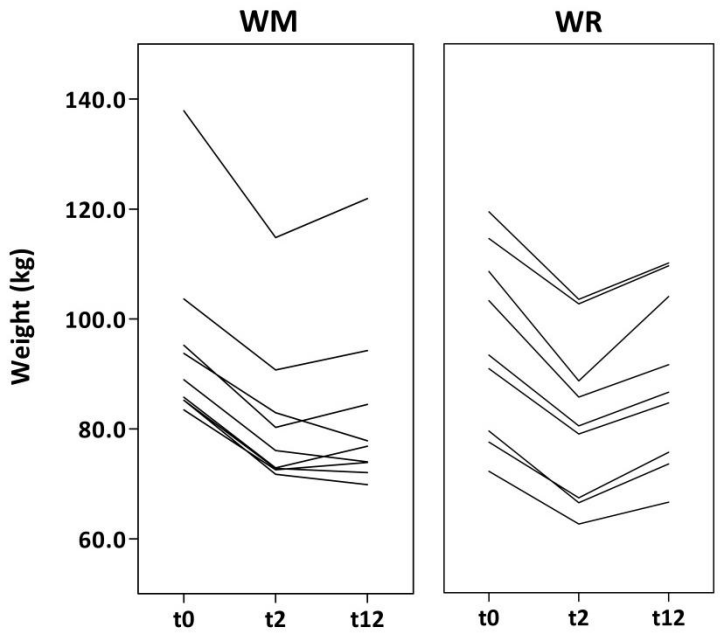

Figure 1: Body weight progression during the course of the study for weight maintainers (WM; n=9) and weight regainers (WR; n=9). Each line represents the body weight (kg) of an individual measured before 8 weeks of a very-low-energy diet (t0), after the diet (t2), and after 10 months of follow-up (t12).

Stress related proteins in adipose tissue after calorie restriction

After weight loss, β-actin, BiP, calnexin, HSP27, HPS60 and HSP70 levels decrease for WM while an increase was observed for WR as shown in Table 2. Fold changes between two time points were calculated and compared between the WR and the WM group (Figure 2). During the weight loss period (t0-t2), the WR group differed from the WM group with respect to β-actin (trend), calnexin

(trend), HSP27 (trend), HSP60 and HSP70 (Figure 2A). Levels of stress proteins only changed during weight loss, as during the follow-up period (t2 – t12) no change for any protein was observed (data not shown). After the complete intervention (t0 – t12), the WR group still differed from the WM group with respect to HSP27 (trend) and HSP70 (Figure 2B). BiP, SOD1 and SOD2 did not significantly change during weight loss. After dieting, no significant difference in plasma HSP60 levels was observed between WR and WM. In addition plasma HSP60 levels did not reflect HSP60 concentrations in adipose tissue (data not shown).

Table 2: Protein abundance levels measured by Western blot at three time points.

	Study start (t0)	After weight loss (t2)	After follow-up (t12)
B-actin			
WM	3.93 ± 0.58	3.51 ± 0.48	3.72 ± 0.56
WR	4.03 ± 0.51	4.93 ± 0.45	4.27 ± 0.46
BiP			
WM †	3.82 ± 1.09	1.27 ± 0.60	1.92 ± 0.47
WR ‡ §	1.54 ± 0.54	2.12 ± 0.69	3.59 ± 0.53
Calnexin			
WM	2.88 ± 1.26	0.76 ± 0.28	1.20 ± 0.51
WR	1.36 ± 0.58	3.62 ± 1.38	1.73 ± 0.96
HSP27			
WM †	3.46 ± 1.25	2.60 ± 1.88	1.58 ± 0.81
WR	2.56 ± 1.50	5.80 ± 2.44	2.98 ± 1.22
HSP60			
WM II	4.40 ± 1.34	1.46 ± 0.60	2.87 ± 0.78
WR	2.80 ± 0.54	3.66 ± 0.67	3.92 ± 0.79
HSP70			
WM	2.77 ± 0.68	2.30 ± 1.00	2.48 ± 0.38
WR II §	2.17 ± 0.55	3.85 ± 0.90	4.50 ± 0.72
SOD1			
WM	1.04 ± 0.29	1.57 ± 0.49	1.17 ± 0.29
WR	0.96 ± 0.43	1.40 ± 0.41	1.12 ± 0.40
SOD2			
WM	1.17 ± 0.42	1.64 ± 0.94	1.22 ± 0.51
WR	1.71 ± 0.60	2.51 ± 0.63	1.66 ± 0.61

Mean values ± standard errors. Repeated-measures ANOVA between time points: †P>0.05 – P<0.1 (t0-t2); ‡P≤0.05 (t2-t12); § P≤0.05 (t0-t12); II P≤0.05 (t0-t2). WM, weight maintainers; WR, weight regainers; BiP, binding Ig protein; HSP, heat shock protein; SOD, superoxide dismutase.

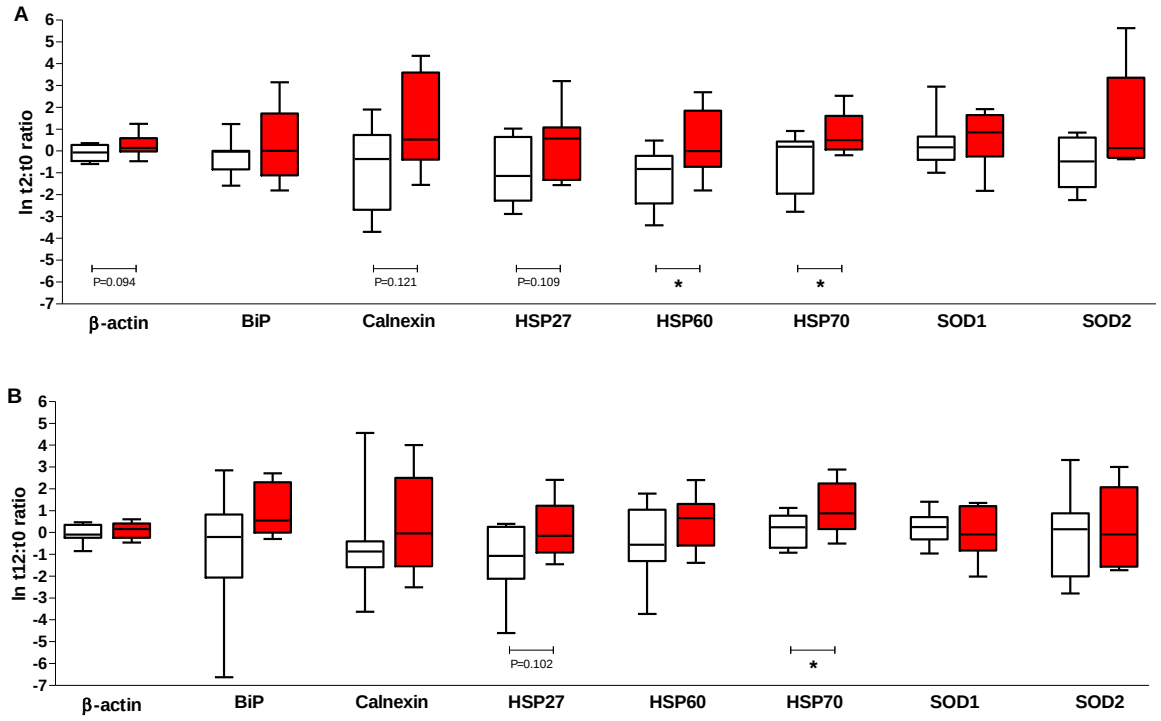

Figure 2: Fold changes in stress-related proteins during (A) the weight loss-phase (after 8 weeks of weight loss (t2) : baseline (t0)) and (B) the whole study (after 10 months of follow-up (t12) : t0). Each box plot shows the median and interquartile range without outliers of the fold change in each protein. Weight maintainers (□; n=9) and weight regainers (■; n=9). The difference between the two groups was analysed by independent T test on ln-transformed values: * P≤0.05. BiP, binding Ig protein; HSP, heat shock protein; SOD, superoxide dismutase.

During the weight loss phase, changes in stress proteins were correlated in both groups to be able to gain more insight into the mechanistic regulation of weight loss-induced cellular stress. In Supplement Figure 1 the correlation plots can be seen. Based on the significant correlations, we have drawn interaction maps for the WM and the WR group (Figure 3). In both groups the similar regulation of HSP60 with HSP70 is obvious, but whereas in the WM group a link between β-actin and HSP27 exists, in the WR group these proteins seem to follow the regulation of HSP60.

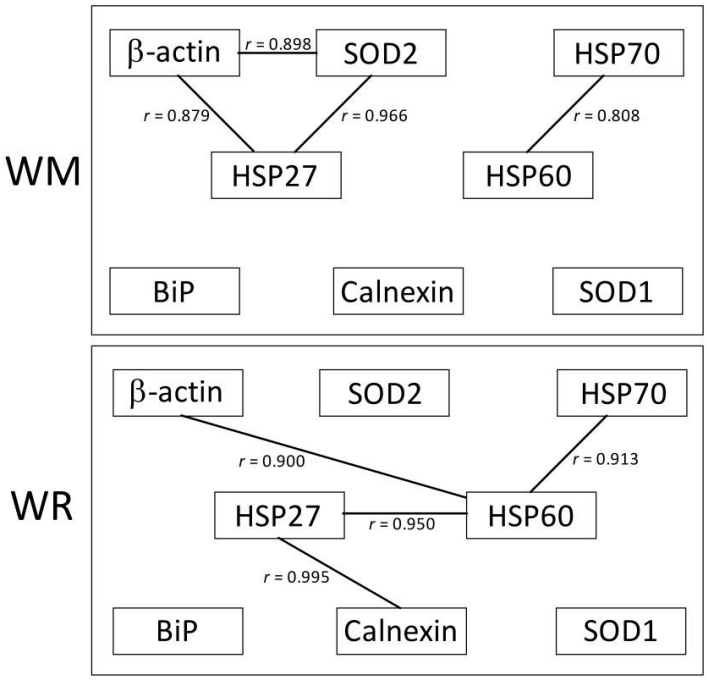

Figure 3: Schematic overview of the correlated proteins within the weight maintainers (WM) and weight regainers (WR) groups during the diet phase (t0-t2). Significant correlations (P≤0.01) found with both Pearson R and Spearman P are represented by the connecting lines with the correlation coefficients (*r*). BiP, binding Ig protein; HSP, heat shock protein; SOD, superoxide dismutase.

Stress related proteins in adipocytes after low-glucose starvation

The *in vivo* results were based on adipose tissue biopsies, which in addition to adipocytes also contain stromal vascular cells. To investigate whether there is an adipocyte specific stress response to calorie restriction, we performed an *in vitro* experiment. Mature SGBS adipocytes received glucose restricted medium for 4 days causing around 17% loss of triglyceride content from the cells as previously reported by Renes et al. (36). In parallel, mature SGBS adipocytes cultured with control medium did not show loss of triglycerides. Cells were then harvested and proteins were isolated. The relative amount of the eight proteins was measured but SOD1 expression appeared to be too low to measure. Similar to the WR group, expression of all measured stress proteins was increased during caloric restriction with statistical significance for β-actin and HSP60 (Figure 4).

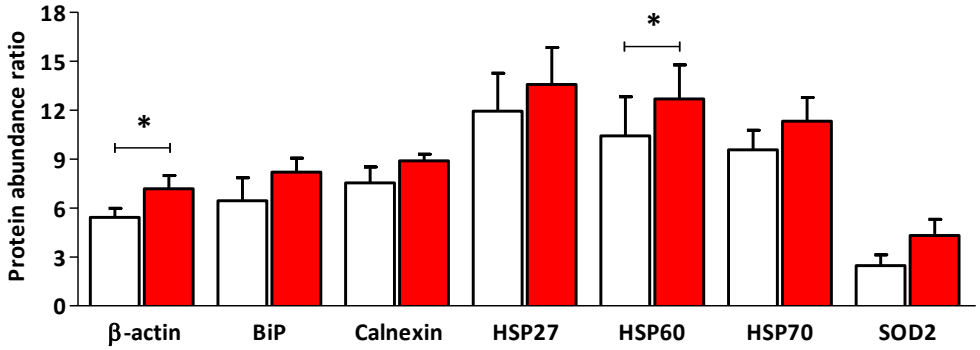

Figure 4: Stress protein levels of SGBS adipocytes after glucose restriction measured with Western blotting. Glucose restriction medium containing 20 nM insulin and 0.55 mM glucose (□). The control group received medium containing 20 nM insulin and 17.5 mM glucose (■). All groups consist of n=3 measured in duplicate. Values are means with standard errors. The difference over time was analysed by dependent T test: * P≤0.05. BiP, binding Ig protein; HSP, heat shock protein; SOD, superoxide dismutase.

Discussion

The current study results suggest that adipocyte stress is a biological risk factor for weight regain after weight loss. In the WR group the levels of β-actin, calnexin, HSP27, HSP60 and HSP70 were increased after weight loss compared to the WM group. Correlation analysis indicated that changes of β-actin, HSP70 and HSP27 are linked to changes in HSP60 as a possible key-factor contributing to weight regain. Increased levels of β-actin and HSP60 were also observed after 4 days of glucose restriction of SGBS adipocytes indicating that *the in vivo* observations reside in the mature adipocytes of the adipose tissue.

Our findings show that during weight loss stress proteins increase in the adipose tissue of individuals who are at risk for weight regain. One of those is β-actin, a component of actin filaments (25). During weight loss adipocytes change shape due to shrinking, which requires re-allocation of cellular components. When parts of the cell need to be moved, 10-30 actin filaments assemble into so-called stress-fibers, which can perform mechanical traction. Accordingly, in our *in vitro* experiment with cultured SGBS adipocytes, we observed a significant up-regulation of β-actin after glucose restriction in parallel to the loss of triglycerides and shrinking of the cells. In the adipose tissue of the WR subjects after weight loss, we observed an up-regulation of β-actin indicating that more stress-fibers are formed and more mechanical stress exists in the adipocytes of the WR group than in those of the WM group. Unfortunately, we were unable to quantify stress-fibers directly. Nevertheless, our β-actin results are in line with those of Mutch et al. who detected an up-regulation of β-actin gene expression in WR and a downregulation in WM (39).

HSP27 showed a trend for change in adipose tissue after weight loss and there was no change after glucose restriction in the *in vitro* cultured adipocytes. On the other hand, in the WM group we found a strong correlation between HSP27 and β-actin suggesting a functional link between these proteins. It has been shown that HSP27 binds to actin filaments (F-actin) and that under cell stress HSP27 becomes phosphorylated, which enhances binding affinity for F-actin (40). In this regard, HSP27 appears to be involved in the regulation of actin filament dynamics. In renal cells, HSP27 provides protection against the consequences of ATP-depletion and this function was dependent on HSP70 (41), which is known to form complexes with HSP60 (42). In the present study, our correlation map of the WR group showed a link between changes over the diet period for β-actin, HSP27 and HSP70 via HSP60 (Figure 3). Altogether, these data in combination with our *in vitro* response to energy depletion, strongly suggest that weight regain relates to cell stress and involves the regulation of actin filament dynamics.

Calnexin increased in the adipose tissue in the WR group after weight loss, whereas in the other group the level seemed to drop. Calnexin retains newly synthesized N-glycosylated proteins inside the ER to ensure proper folding with the help of folding factor ERp57 (27, 28). Wrongly folded proteins may enter into a cycle of unfolding and refolding or may be broken down by the ER-associated degradation pathway (43), whereas correctly folded glycoproteins are transported out of the cell. Interestingly, N-glycoproteins are important for the formation of the extracellular matrix (ECM) of adipocytes (44). Our results show relatively high levels of calnexin after dieting in the WR group which seems to be in line with the up-regulation of genes with a focal adhesion function in the WR group of the study of Mutch et al. (39), and their down-regulation in the WM group.

Besides mechanical stress, other forms of cellular stress might be involved in the risk for weight regain after weight loss. HSP70 is a marker for ER stress, although it may also have a protective function against ER stress-induced apoptosis (45), possibly by inhibiting key stress kinases (46). Our results show increased concentrations of HSP70 after dieting in the WR group. This implies that ER stress is present in the adipose tissue of those subjects and as such might be related to the risk for weight regain after weight loss.

In this study, we found that the WR group had elevated levels of HSP60 in the adipose tissue after dieting compared to WM subjects. HSP60 is present in the circulation of people with type 2 diabetes (47) and increased levels are observed in the adipose tissue of obese subjects compared to lean subjects (20). In starved 3T3-L1 adipocytes an up-regulation of HSP60 is shown compared to non-starved cells (48), which complies with our present *in vitro* observations in human SGBS adipocytes. HSP60 stimulates the release of pro-inflammatory adipokines from the adipose tissue promoting inflammation and, as such, may support the development of insulin resistance. This suggests that WR are at higher risk for obesity-related complications. Interestingly, as HSP60 is a chaperone for mitochondrial proteins, an increase in HSP60 after weight loss indicates a dysregulation of the mitochondrial metabolic processes in WR-subjects. However, a possible role for such impairment in the risk of weight regain requires further investigation.

A limitation of the present study is the relatively small number of participants. In this group, the age varied between 20-55 years and the BMI between 27-39 kg/m^2. Thus, it might be that we included pre- and post-menopausal woman as well as overweight and obese individuals. Nevertheless, this study produced new insights which can form the basis for further studies in larger cohorts. For the moment, it is unclear why some people show increased adipose cellular stress during weight loss while others do not. One explanation might be genetic predisposition. Alternatively, adipocyte size at baseline might play a role. A general model for accumulation of cellular stress during weight loss states that when adipocytes lose fat and shrink, the surrounding ECM is unable to follow the size reduction (49-51). Consequently, mechanical-type cellular stress rises, which can be neutralized most easily by renewed lipid uptake and storage. Indeed, measuring the adipocyte molecular metabolism by using proteomics technologies indicates that after returning to energy balance, adipocytes rapidly prepare for re-storing triglycerides (37, 52). Moreover, during weight loss, the plasma leptin level decreases dramatically, out of proportion with the loss of fat mass, which implies that, after weight loss, an extra impulse is given to energy intake to warrant the re-storing of fat and release of cellular stress (53).

In conclusion, analyses of subcutaneous adipose tissue show increased cellular stress after an 8-week diet in a group of subjects who regained most of their weight during follow-up. *In vitro* cultured mature adipocytes subjected to energy restriction showed similar protein changes. These findings support the idea that adipocyte stress plays a role as a biological risk factor for weight regain after weight loss and suggest involvement of mechanical stress with dynamics of stress fibres.

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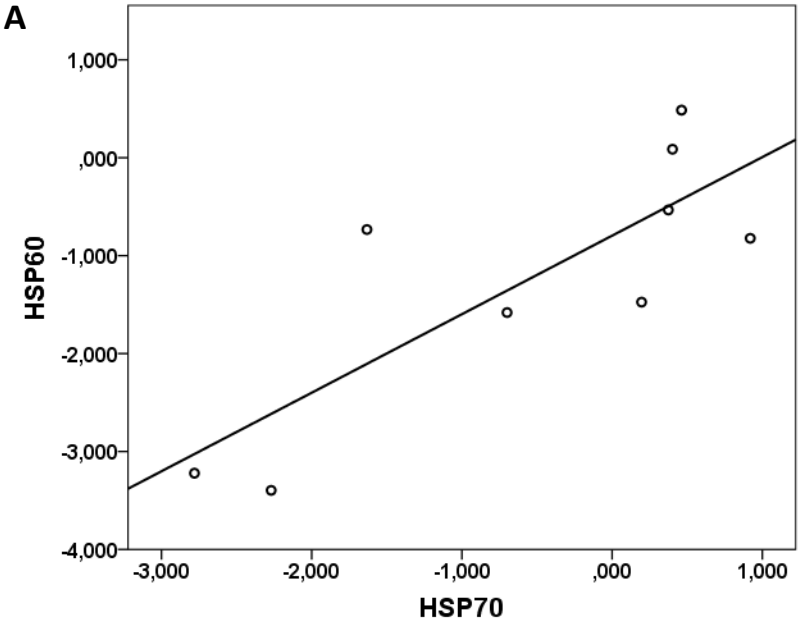
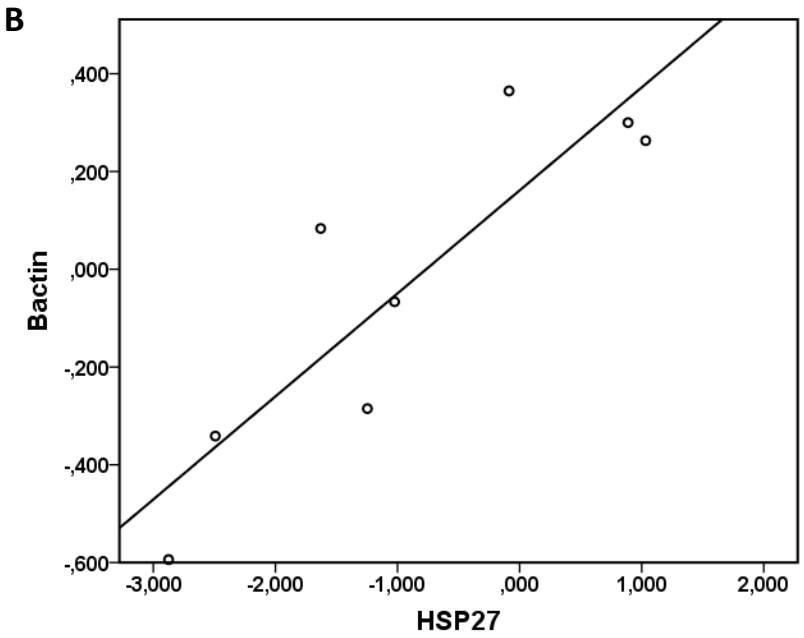
Supplementary Figures and Tables

Supplement Figure 1: Correlation plots of fold changes of stress proteins in the weight maintainers (A-D) and weight regainers (E-H) group during the diet phase (t0-t2). Correlation of HSP70 with HSP60 for WM n=9 (A), β-actin with HSP27 for WM n=8 (B), β-actin with SOD2 for WM n=8 (C), HSP27 with SOD2 for WM n=7 (D), HSP70 with HSP60 for WR n=9 (E), β-actin with HSP60 for WR n=9 (F), HSP27 with HSP60 for WR n=7 (G), HSP27 with Calnexin for WR n=4 (H). HSP, heat shock protein; SOD, superoxide dismutase; WR, weight regainers; WM, weight maintainers.





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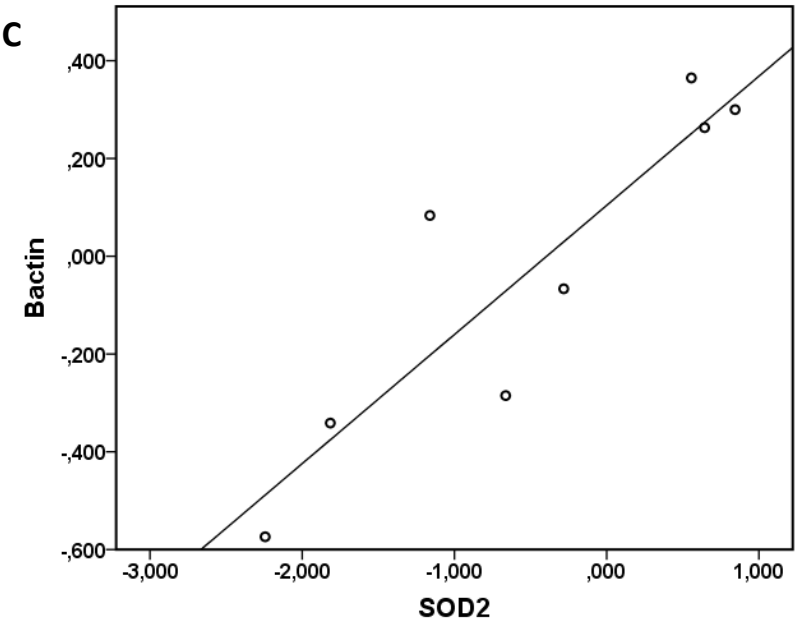
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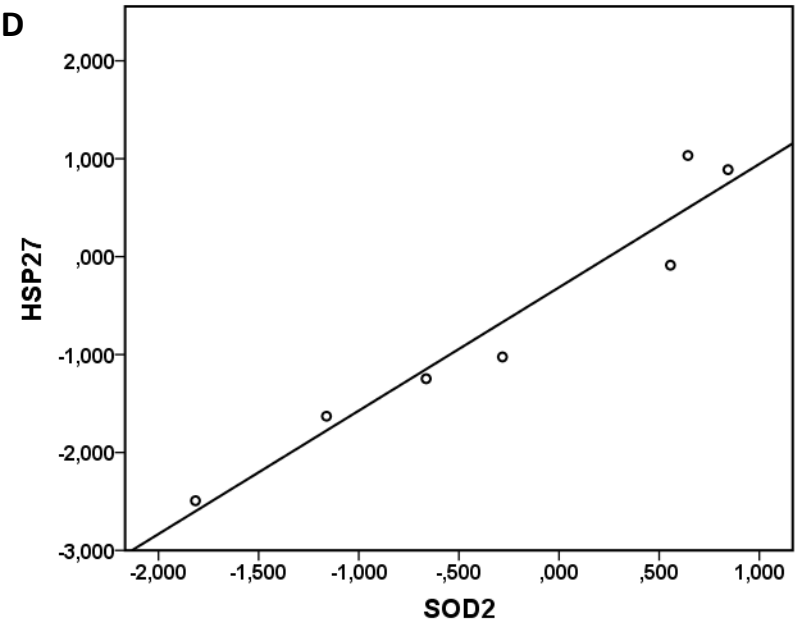
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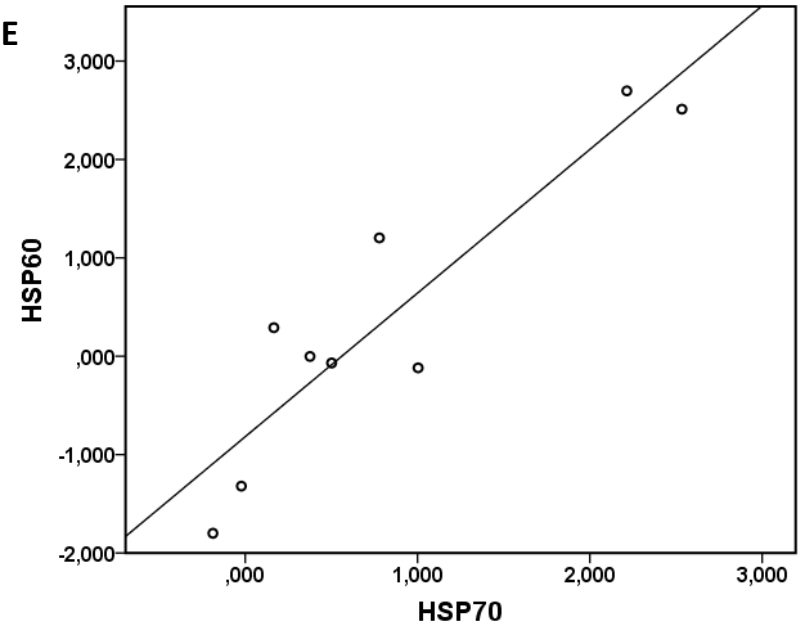
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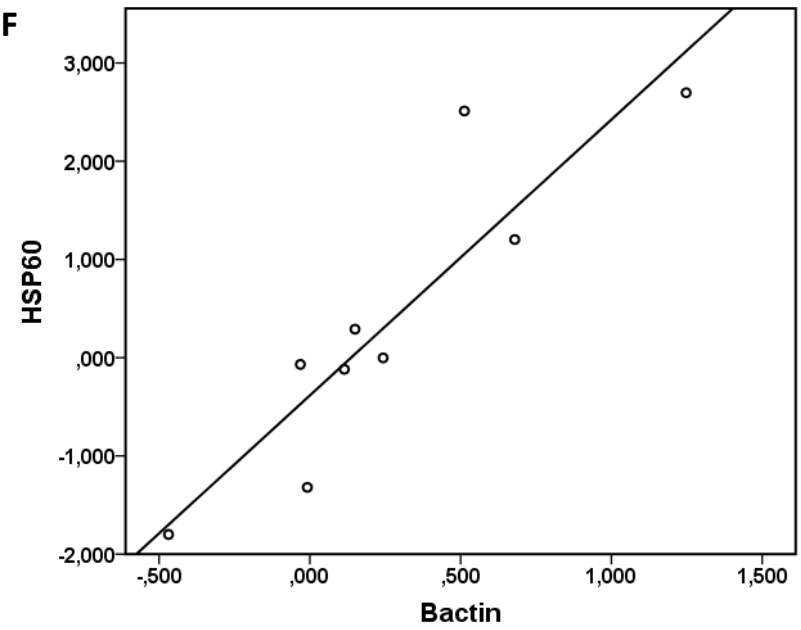
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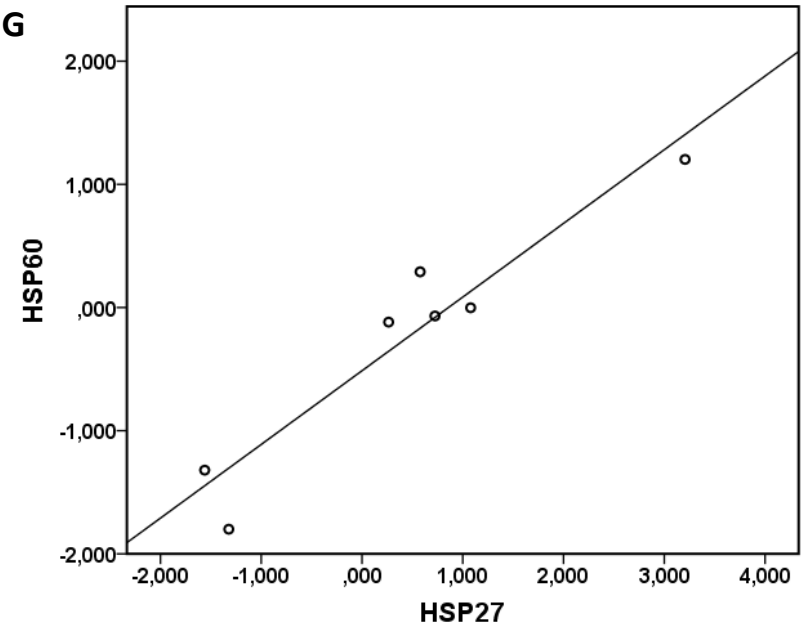
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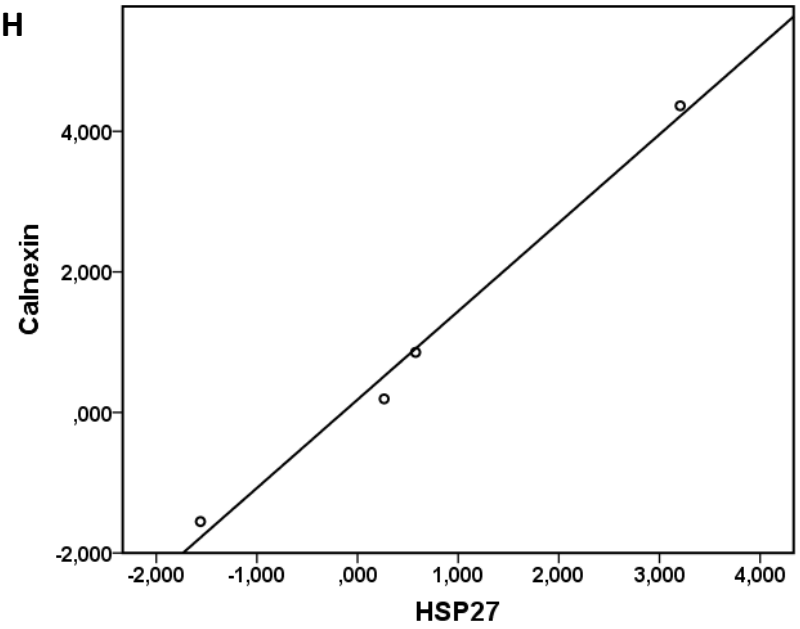
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Chapter 5

Weight loss-induced cellular stress in subcutaneous adipose tissue and the risk for weight regain in overweight and obese adults

Nadia J.T. Roumans, Roel G. Vink, Freek G. Bouwman, Parastoo Fazelzadeh, Marleen A. van Baak and Edwin C.M. Mariman

International Journal of Obesity, 2016

Abstract

Background/Objective: Weight loss is often followed by weight regain after the dietary intervention (DI). Cellular stress is increased in adipose tissue of obese individuals. However, the relation between cellular stress and weight regain is unclear. Previously, we observed increased adipose tissue cellular stress of participants regaining weight compared to participants maintaining weight loss. In the current study, we further investigated the relation between weight regain and changes in the expression of stress-related genes and stress protein levels to determine possible predictors of weight regain.

Participants/Methods: In this randomized controlled trial, sixty-one healthy overweight/obese participants followed a DI of either a 5-week very-low-calorie diet (500 kcal/d) or a 12-week low-calorie diet (1250 kcal/d) (WL period) with subsequent a 4-week weight stable diet (WS period), and a 9-month follow-up. The WL and WS period taken together was named the DI. Abdominal subcutaneous adipose tissue biopsies were collected in fifty-three participants for microarray and liquid chromatography–mass spectrometry analysis. RNA and protein levels for a broad set of stress-related genes were correlated to the weight regain percentage.

Results: Different gene sets correlated to weight regain percentage during WS and DI. Bioinformatics clustering suggests that during the WS phase defined genes for actin filament dynamics, glucose handling and nutrient sensing are related to weight regain. *HIF-1* is indicated as an important regulator. With regard to DI, clustering of correlated genes indicate that *LGALS1*, *ENO1* and *ATF2* are important nodes for conferring risk for weight regain.

Conclusions: Our present findings indicate that the risk for weight regain is related to expression changes of distinct sets of stress-related genes during the first four weeks after returning to energy balance, and during the DI. Further research is required to investigate the mechanistic significance of these findings and find targets for preventing weight regain.

Introduction

Overweight and obesity are serious threats to the human health due to an increased risk for the metabolic syndrome and development of type 2 diabetes, cardiovascular diseases and cancer (1, 2). There is a simple remedy to obesity i.e. losing weight by dietary intervention, increased physical activity, pharmacological and/or surgical treatment (3). However, long-term weight loss maintenance has been proven to be difficult. In general up to 80% of the people are unsuccessful in maintaining weight loss (4, 5) defined as "keeping off an intentional loss of at least 10% body weight for at least one year" (6). Therefore, it is of great importance to understand the mechanisms that influence the risk for weight regain. Various factors have already been associated with weight regain or weight maintenance after weight loss such as insulin sensitivity (7, 8), fasting insulin and HOMA-IR (9), metabolic factors (10, 11), sex hormones (10), hunger and satiety hormones (12) and epigenetic modifications like methylation of the NPY and POMC gene promoters (13). The adipose tissue plays an important role in the increased risk for weight regain. During a negative energy balance, loss of triglycerides decreases the volume of an adipocyte creating stress between the cell contents and the surrounding extracellular matrix (ECM) (14). This stress can be resolved by remodeling of the ECM. However, under a negative energy balance this may be hard or even impossible. Thus, the stress needs to be resolved by another mechanism such as the re-storing of triglycerides within the adipocytes (15). This way the contents of the adipocyte increase to fit the surrounding ECM. It suggests that cellular stress accumulated in adipocytes during a negative energy balance is a driving force behind the risk for weight regain.

Increased stress, especially endoplasmic reticulum (ER) stress, is observed in the subcutaneous adipose tissue of obese people compared to lean people (16). Sharma et al. reported a positive correlation between BMI and ATF67-induced ER stress markers (17). Although these findings suggest that cellular stress increases as a consequence of increasing weight, it might well be that cellular stress is also a factor that stimulates accumulation of fat and is a risk factor for weight regain after weight loss. In a previous study, healthy overweight and obese participants underwent an 8-week calorie-restricted diet with a 10-month follow-up. After weight loss, we observed significantly higher cellular stress in the subcutaneous adipose tissue of participants regaining their lost weight during the follow-up period compared to the participants maintaining weight loss (18). In the present study, our main objective was to further investigate the possible relation between weight regain during follow-up and changes in the expression of genes for stress proteins during weight loss and weight stabilization. This included changes in both the RNA and protein levels for a broad set of stress-related genes.

Methods

Participants and study design

Sixty-one overweight and obese (BMI 27-36 kg/m^2) Caucasian participants underwent a dietary intervention (Figure 1). The whole study design has been described in detail previously (19). In short, participants were randomly assigned to either a very-low-calorie diet (VLCD, rapid weight loss) or a low-calorie diet (LCD, slow weight loss) group. Participants in the VLCD group underwent a 5-week diet with about 500 kcal/day by consuming three meal replacements per day (Modifast; Nutrition et Santé Benelux). Participants in the LCD group underwent a 12-week diet with about 1250 kcal/day designed by a dietician. Both groups were targeted to lose approximately 10% body weight during

this weight loss period (WL, T1-T2). Following weight loss, all participants underwent a 4-week weight maintenance diet based on their individual energy requirements. This weight stable period (WS, T2-T3) was designed to investigate the effect of weight loss of approximately 10%, without the interfering effect of a pronounced negative energy balance. The WL and WS period taken together was named the dietary intervention (DI, T1-T3). The study dietician provided dietary advice according to the Dutch national guidelines (20) to the VLCD and LCD group, to assist in weight loss during the WL period and to assist in remaining weight stable throughout the WS period. After the WS period, participant's body weight was monitored for 9 months (follow-up, T3-T4) by monthly meetings with a dietician. During this follow-up, participants did not receive advice on monitoring and limiting food intake to mimic non-restricted free-living conditions.

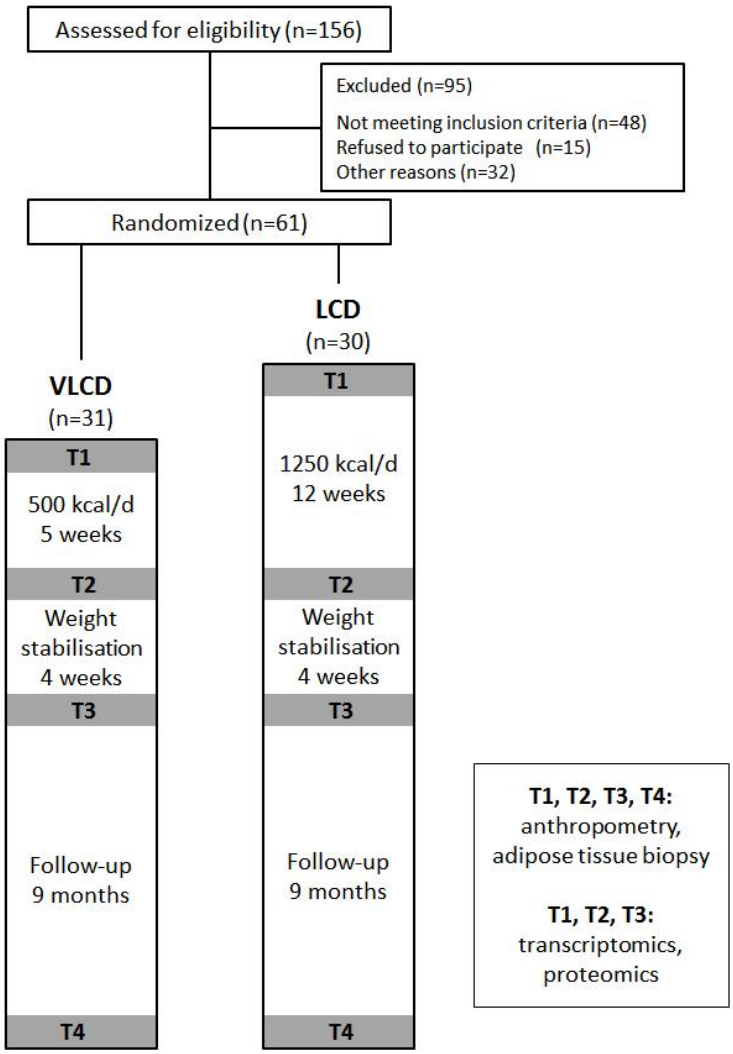

Figure 1: Schematic overview of the study design. Anthropometry measurements and adipose tissue biopsies were obtained at the start of the study (T1), at the end of the weight loss period (T2), weight stable period (T3), and follow-up (T4). Transcriptomics and proteomics were performed at T1, T2 and T3. The dietary intervention period (DI) is the weight loss period and weight stable period taken together.

Before weight loss (T1), after weight loss (T2), after the WS period (T3) and after follow-up (T4) body composition, body weight, height, and waist and hip circumference were measured after overnight fasting (19). Body volume was determined with air-displacement plethysmography using a Bod Pod

device (Cosmed, Italy, Rome) according to the manufacturer's instructions and as described by Dempster et al. (21). Body composition was calculated from body density according to the two-compartment model by Siri (22).

Four participants withdrew from the study during the dietary intervention (T1-T3), for three participants we could not collect enough biopsy material and for one participant the gene expression results deviated strongly from the others and were therefore excluded. Characteristics of the remaining fifty-three participants at T1, T2 and T3 are displayed in Supplement Table 1.

This study was conducted according to the Declaration of Helsinki guidelines and registered on ClinicalTrials.gov (registration number: NCT01559415). All procedures involving human participants were approved by the Central Committee on Human Research and by the Medical Ethical Committee of Maastricht University, The Netherlands. Written informed consent was obtained from all participants.

Adipose tissue biopsy

Abdominal subcutaneous adipose tissue biopsies were obtained by needle biopsy under local anaesthesia (2% lidocaine, Fresenius Kabi) after an overnight fast at T1, T2 T3, and T4. Tissue was immediately rinsed in sterile saline, frozen in liquid nitrogen and stored at -80°C until RNA and protein isolation.

Adipose tissue RNA isolation and microarray analysis

For RNA isolation, adipose tissue samples from T1, T2 and T3 were used. Total RNA was extracted from approx. 150 mg frozen adipose tissue using TRIzol reagent (Invitrogen). Total RNA (100 ng per sample) was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19654 unique genes (Affymetrix). Sample labeling, hybridization to chips and image scanning was performed according to manufacturer's instructions. Microarray signals were normalized using robust multichip average (RMA). Genes with normalized signals >20 on at least 15 arrays were defined as expressed (11532 genes). Gene expression changes within groups during a certain period (WL, WS or DI) were defined as significantly different when the q-value was <0.05 in a paired t-test with Bayesian correction (Limma) (23). Array data have been submitted to the Gene Expression Omnibus (number GSE77962).

In this study, 107 genes related to stress were selected for the analysis (Supplement Table 2). Sixteen genes were specifically related to endoplasmic reticulum stress as previously reported (16, 17), twenty-four candidate stress-related genes were selected based on the frequently detected proteins in subcutaneous adipose tissue (24) and sixty-seven were genes with the term "response to stress" in the Gene Ontology Biological Process (GO-BP) description.

Adipose tissue protein isolation and preparation for liquid chromatography–mass spectrometry (LC-MS/MS)

For protein isolation, adipose tissue samples from T1, T2 and T3 were used. About 100 mg of the frozen adipose tissue was ground in a mortar with liquid nitrogen. Per mg of grounded powder 2 µl of 50 mM ammonium bicarbonate (ABC) with 5 M urea was used to dissolve the powder. Same procedure was done for a control adipose tissue sample, the control subcutaneous adipose tissue

sample was obtained from a healthy obese during abdominoplasty. The sample was vortexed for 5 minutes. The homogenate was centrifuged for 30 minutes 14 000 rpm at 10°C. The supernatant was carefully collected and protein concentrations were determined with a Bradfort-based protein assay (Bio-Rad). Samples and control were labelled with TMT isobaric mass tagging labelling reagent (10-plex, Thermo Scientific) according to manufacturer's protocol. In short, 50 µg of protein for each sample was used. The TMT labelling reagents were dissolved in 41 µL acetonitrile per vial. The reduced and alkylated samples and control were added to the TMT Reagent vial. The reaction was incubated for 1 hour at room temperature and quenched for 15 minutes by adding 8 µL of 5% hydroxylamine. The samples and control were combined in a new microcentrifuge tube at equal amounts and analysed by LC-MS/MS.

Liquid chromatography–mass spectrometry analysis for protein quantification

A nanoflow HPLC instrument (Dionex ultimate 3000) was coupled on-line to a Q Exactive (Thermo Scientific) with a nano-electrospray Flex ion source (Proxeon). The final concentration of the TMT labeled digest/peptide mixture was 0.2 µg/µl and 5 µl of this mixture was loaded onto a C18-reversed phase column (Thermo Scientific, Acclaim PepMap C18 column, 75-µm inner diameter x 15 cm, 5-µm particle size). The peptides were separated with a 120 minutes linear gradient of 4-45% buffer B (80% acetonitrile and 0.08% formic acid) at a flow rate of 300 nL/min.

MS data was acquired using a data-dependent top10 method, dynamically choosing the most abundant precursor ions from the survey scan (280–1500 m/z) in positive mode. Survey scans were acquired at a resolution of 70,000 and a maximum injection time of 120 ms. Dynamic exclusion duration was 30 s. Isolation of precursors was performed with a 2.0 m/z window. Resolution for HCD spectra was set to 35,000 and the normalized collision energy was 30 eV. The under fill ratio was defined as 1.0%. The instrument was run with peptide recognition mode enabled, but exclusion of singly charged and charge states of more than five.

Database search

The MS data were searched using Sequest HT Proteome Discoverer 2.1 search engine (Thermo Scientific) against the UniProt human database. The false discovery rate (FDR) was set to 0.01 for proteins and peptides, which had to have a minimum length of 6 amino acids. The precursor mass tolerance was set at 10 ppm and the fragment tolerance at 0.2 Da. One miss-cleavage was tolerated, oxidation of methionine was set as a dynamic modification and carbamidomethylation of cysteines, TMT reagent adducts (+229.162932 Da) on lysine and peptide amino termini were set as fixed modifications.

In total, twenty-three TMT runs were performed. We corrected our data for possible differences between runs and within each run. We corrected for between run differences as follows: (average protein abundance of the twenty-three controls ÷ protein abundance of control in same run as the test sample) x protein abundance in the test sample. For the correction within the run, we chose 167 proteins (excluding obvious plasma proteins) which were present in each test sample and used those 167 proteins to calculate the average protein abundance of each test sample. We corrected for within run differences as follows: (average total protein abundance of the control ÷ total protein abundance of the test sample) x original protein abundance in the test sample.

Calculations

In this study we wanted to analyse the relation between gene expression and protein level changes and weight regain and/or maintenance. The objective was to determine possible predictors of weight regain during WL, WS or DI. As a value for weight regain and/or maintenance, we calculated the weight change percentage during follow-up as follows: ((weight after follow-up T4 – weight after WS T3) ÷ weight after WS T3) x 100%. For gene expression change we calculated the fold change (FC) of gene expression during WL, WS or DI. Gene expression results from the microarray were expressed as $\log_2$ transformed values. Gene expression changes during a certain period were calculated as follows: WL ($\log_2$ T2 – $\log_2$ T1), WS ($\log_2$ T3 – $\log_2$ T2) and DI ($\log_2$ T3 – $\log_2$ T1). These $\log_2$ ratios (LR) during WL, WS and DI were transformed into fold changes (FC= 2^{LR} if LR≥0 and FC= (-1) 2^{-LR} if LR<0).

Bioinformatics analyses

Gene clustering was done by the Human Consensus Path DataBase (CPDB release 31, http://cpdb.molgen.mpg.de/). From 'gene set analysis' the 'induced network modules' tool was chosen and the list of genes significantly correlated with the weight regain percentage during WS or DI was entered (so-called seed genes). The program aims to interconnect the seed genes through different types of interactions (such as biochemical, regulatory, genetic or protein interactions). The induced network modules may include genes that are not in the user-supplied seed list, but associate two or more seed genes with each other and overall have significantly many connections within the induced network module (so-called intermediate nodes). The gene clustering during WS and DI focussing on biochemical reactions and gene regulatory interactions with allowance for intermediate nodes (z-score: 20). Also, gene clustering focussing on protein interactions was done for the 8 correlating genes of the DI phase with allowance for intermediate nodes (z-score: 20).

Statistical analysis

Dependent T-test was carried out to determine possible effects over time within a group. Comparisons between VLCD and LCD were made with independent T-test. Weight change (%) during follow-up was correlated with fold change of gene expression and protein levels during WL, WS and DI. Correlations were made using the Pearson correlation coefficient. Statistical analyses were done using SPSS 20.0 for Windows (SPSS Inc). For all statistical tests $P \leq 0.05$ was considered to be statistically significant, unless otherwise stated. Data are presented as mean ± SEM. Variation in the number of participants between analyses is due to the exclusion of participants with missing data. All variables were checked for normal distribution, and variables with a skewed distribution were ln-transformed to satisfy conditions of normality. Extreme outliers (values higher than 3x interquartile range calculated with SPSS) influencing the data were removed during statistical analyses.

Results

Changes of subject characteristics throughout the study

At the start of this study, no significant differences were observed between VLCD and LCD (Supplement Table 1). After WL and WS, all parameters remained similar between VLCD and LCD. During the WL period, body weight, BMI, hip circumference, waist circumference, body fat percentage, body fat and fat free mass were significantly decreased in both the VLCD and the LCD group (Table 1). In the subsequent 4 weeks (WS period), body fat and body fat percentage decreased for both VLCD and LCD, whereas body weight did not change (Supplement Table 1). Also, an increase

in fat free mass is observed in the VLCD group. In the LCD group, hip circumference significantly increased. During the dietary intervention (T1-T3), all parameters significantly decreased except for fat free mass in the LCD group (Table 1). Body weight, BMI, waist circumference, body fat percentage, body fat and fat free mass increased during follow-up (T3-T4). The average weight regain percentage did not differ between VLCD and LCD (5.4 ± 4.5 % and 5.3 ± 3.8 %, P=0.957, Supplement Figure 1).

Table 1: Changes in characteristics at the end of weight loss and the dietary intervention compared to study start, and at the end of follow-up compared to the end of the dietary intervention.

	End of WL vs study start (T2 vs T1)		**End of DI vs study start** (T3 vs T1)		**End of follow-up vs end of DI** (T4 vs T3)	
	VLCD (n = 26)	**LCD (n = 27)**	**VLCD (n = 26)**	**LCD (n = 27)**	**VLCD (n = 26)**	**LCD (n = 27)**
Weight (kg)	-8.9 ± 0.4***	-8.1 ± 0.5***	-9.1 ± 0.5***	-8.3 ± 0.6***	-9.1 ± 0.5***	-8.3 ± 0.6***
BMI (kg/m^2)	-3.0 ± 0.1***	-2.8 ± 0.2***	-3.0 ± 0.2***	-2.8 ± 0.2***	-3.0 ± 0.2***	-2.8 ± 0.2***
Hip circumference (cm)	-6.0 ± 0.7***	-4.5 ± 0.6***	-6.1 ± 0.7***	-5.9 ± 0.7***	-6.1 ± 0.7***	-5.9 ± 0.7***
Waist circumference (cm)	-7.8 ± 0.7***	-7.3 ± 0.8***	-6.7 ± 0.7***	-8.0 ± 1.0***	-6.7 ± 0.7***	-8.0 ± 1.0***
Body fat (%)	-4.7 ± 0.4***	-5.4 ± 0.6***	-5.9 ± 0.5***	-6.0 ± 0.5***	-5.9 ± 0.5***	-6.0 ± 0.5***
Body fat (kg)	-7.3 ± 0.4***	-7.6 ± 0.6***	-8.4 ± 0.5***	-8.1 ± 0.6***	-8.4 ± 0.5***	-8.1 ± 0.6***
Fat free mass (kg)	-1.6 ± 0.2***	-0.5 ± 0.2*	-0.8 ± 0.2**	-0.2 ± 0.2	-0.8 ± 0.2**	-0.2 ± 0.2

Values are mean±SEM. *P≤0.05, **P<0.01, ***P<0.001, dependent T-test, within-group change from end of WL vs. study start, end of DI vs. study start and end of follow-up vs. end of DI. WL, weight loss; DI, dietary intervention; VLCD, very-low-calorie diet; LCD, low-calorie diet; BMI, body mass index.

Changes of stress gene RNA and protein levels correlate with weight regain

Changes during WL, WS and DI of the expression of 17 out of the 107 stress-related genes correlated with weight regain percentage (Table 2). Most correlations were found in the VLCD group (19) as compared to the LCD group (2), which may be due to the fact that gene expression changes after the VLCD are considerably more profound than after the LCD (25) allowing significant correlations to be more readily detected. All correlations observed have a $|r|\geq 0.5$ indicating that these gene changes are correlating strongly with the weight regain percentage. Aldolase A (*ALDOA*) is the only gene correlating with weight regain in both VLCD and LCD. However, the observed correlations were in different phases, WS for the VLCD group (r=0.552, P=0.006) and WL for the LCD group (r=0.590, P=0.003). Remarkably, in the VLCD group hardly any correlations were found for the WL period, but mainly for the WS and DI phases. In the VLCD group, during the WS period the genes most strongly correlating with weight regain percentage are Enolase 1 (*ENO1*, r=0.710, P<0.001), Protein tyrosine kinase 2 beta (*PTK2B*, r=0.658, P=0.001) and Phosphoglycerate kinase 1 (*PGK1*, r= 0.655, P=0.001), and during DI Lectin, galactoside-binding, soluble, 1 (*LGALS1*, r=0.705, P<0.001), *ENO1* (r=0.684, P<0.001) and Crystallin, alpha B (*CRYAB*, r=0.671, P<0.001).

Table 2: Correlation coefficients between the weight regain percentage and the fold changes in gene expression levels during the weight loss, weight stable and dietary intervention period.

Gene ID	Gene name	VLCD			LCD		
		WL	WS	DI	WL	WS	DI
ACTB	Actin, beta		0.562*				
ALDOA	Aldolase A		0.552		0.590*		
ANXA5	Annexin A5			0.566*			
C5	Complement component 5		-0.525				
CFL1	Cofilin 1		0.607*				
CRHBP	Corticotropin releasing hormone binding protein			-0.518			
CRYAB	Crystallin, alpha B	0.586*		0.671*			
ENO1	Enolase 1		0.710*	0.684*			
GSTP1	Glutathione S-transferase pi 1			0.597*			
LGALS1	Lectin, galactoside-binding, soluble, 1			0.705*			
MAPK13	Mitogen-activated protein kinase 13		0.586*				
PGK1	Phosphoglycerate kinase 1		0.655*				
PHB	Prohibitin			0.528			
PRDX6	Peroxiredoxin 6					0.606*	
PTK2B	Protein tyrosine kinase 2 beta	-0.547	0.658*				
RPS6KA1	Ribosomal protein S6 kinase, 90kDa, polypeptide 1		0.643*				
UCN2	Urocortin 2			-0.521			

Correlation coefficients (*r*) between weight regain and the fold change (FC) of gene expression during the weight loss (T1-T2), weight stable (T2-T3) and dietary intervention (T1-T3). Weight regain percentage is calculated: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. *Indicates significant Pearson R's correlations at P≤0.005, other correlations P≤0.01. VLCD, very-low-calorie diet; LCD, low-calorie diet; WL, weight loss; WS, weight stable; DI, dietary intervention.

In order to see if correlations could be found between changes in protein level and weight regain percentage, we performed quantitative analysis by LC-MS/MS of adipose tissue proteins for the same three time points. In total, we were able to measure 22 proteins of the 107 stress-related genes (Supplement Table 3). Nine proteins correlated with weight regain percentage. The strongest correlation observed was with Heat shock protein beta-6 (HSPB6, *r*=0.800, P=0.003) during the WS period (Table 3). In the VLCD group, two proteins were correlated with weight regain which were also correlated on gene level: β-Actin and Phosphoglycerate kinase 1, although during different phases (β-Actin: WL; PGK1: DI).

Table 3: Correlation coefficients between the weight regain percentage and the fold changes in protein levels during the weight loss, weight stable and dietary intervention period.

Protein name	Uniprot ID	Gene ID	VLCD			LCD		
			WL	WS	DI	WL	WS	DI
60 kDa heat shock protein	P10809	HSPD1					0.489	
78 kDa glucose-regulated protein	P11021	GRP78				0.499		
Actin, cytoplasmic 1	P60709	ACTB	-0.502					
Galectin-1	P09382	LGALS1				-0.421		
Heat shock protein beta-6	O14558	HSPB6		0.800				
Nucleophosmin	P06748	NPM						-0.617
Phosphoglycerate kinase 1	P00558	PGK1			0.589			
Pyruvate kinase	P14618	PKM	-0.526	0.564				
Triosephosphate isomerase	P60174	TPI1		0.510				

Correlation coefficients (*r*) between weight regain and the fold change (FC) of protein levels during the weight loss (T1-T2), weight stable (T2-T3) and dietary intervention (T1-T3). Weight regain percentage is calculated: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. Only Pearson R's correlations with a P-value ≤0.05 are depicted in this table. VLCD, very-low-calorie diet; LCD, low-calorie diet; WL, weight loss; WS, weight stable; DI, dietary intervention.

Bioinformatics analysis of the correlated genes

Only one of the correlated genes (*ENO1*) shows up in both the VLCD WS and DI phases (Table 2). This poor overlap suggests that these genes represent two different functional or regulatory gene sets. Clustering analyses based on biochemical reactions and gene regulatory interactions showed that 8 of 9 correlating genes of the WS phase were clustered (Figure 2A), whereas clustering in this way was not apparent for correlating genes of the DI phase. Moreover, combined analysis of all genes did not change the observed cluster except for interactions of *ANXA5* and *GSTP1* with the intermediate node *ISG15*. It confirms that both gene sets are distinct. The HIF-1 complex was revealed as an important regulator. Interestingly, the gene *C5* that was not made part of the cluster by the bioinformatics analysis, has two binding sites for HIF-1A at 66 and 1556 base pairs before the start site of transcription as shown in the GeneCards human gene database (www.genecards.org). Although clustering as for the WS correlating genes gave no result with correlating genes of the DI phase, 7 of 8 correlating genes of the DI phase could be clustered with the same tool but focusing on protein interactions (Figure 2B). Besides ATF2, most connections were with LGALS1 and ENO1, which correspond to the genes with the strongest correlations with weight regain percentage. However, all connections were through intermediate nodes.

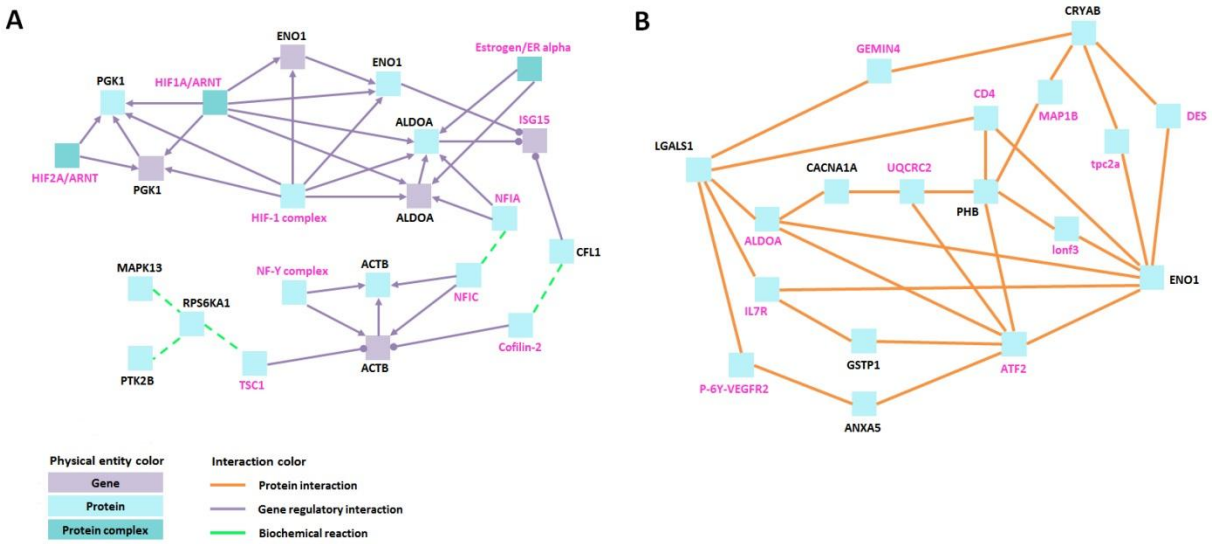

Figure 2: Interactions of genes correlated with weight regain in the VLCD group during WS or DI. Gene clustering during the WS phase focussed on biochemical reactions and gene regulatory interactions with allowance for intermediate nodes (z-score: 20) (A) and gene clustering during the DI phase focussed on protein interactions with allowance for intermediate nodes (z-score: 20) (B). Black gene names: significantly correlated with weight regain (so-called seed genes) and pink gene names: genes not in the user-supplied seeds list (so-called intermediate genes).

Discussion

In the present study we investigated the relation between weight regain percentage and changes in expression of stress-related genes, both on the RNA and protein level, during weight loss by VLCD or LCD and during a weight stabilisation period. We found that correlations between changes in RNA levels and weight regain were more frequent for the VLCD group than for the LCD group. Unexpectedly, correlations were spurious for the WL phase, almost all appeared in the WS or DI phases. Remarkably, the correlating genes of the WS phase were different from the genes correlating in the DI phase.

More and stronger correlations were found in the VLCD compared to the LCD group. The total amount of weight loss was similar in both groups, however, on a VLCD the negative energy balance is more profound and induces stronger responses in gene expression, which probably makes the detection of significant correlations easier. A stronger VLCD response was not only seen in stress-related genes but appeared to be the case for most other genes as well(25). On the other hand, decreasing the threshold for statistical significance from P≤0.01 to P≤0.05 reduces the difference between VLCD and LCD in number of detected correlations (Supplement table 4). Thus, the difference is probably a consequence of both the negative energy balance and the applied threshold. For the protein analysis we used P≤0.05 as cut-off for significance, which may explain that there was no obvious difference in number of correlations between VLCD and LCD.

Surprisingly, correlations between changes in RNA levels and weight regain percentage were mainly found during the WS and DI phases and not in the WL phase. In other studies differences have been observed in subcutaneous adipose tissue gene expression patterns after WL between participants maintaining or regaining their weight after weight loss (26-28). However, in these studies participants were split into a weight maintaining and a weight regaining group to show that some genes were differentially expressed between these groups. Still other studies focussed more on genetic variation associated with weight regain (29, 30). The present study is one of the few that included a weight stable period to look at gene expression changes in relation to weight regain. During the WL phase there is a strong negative energy balance which may lead to low levels of cellular energy especially in the VLCD group. These conditions may outbalance the expected responses of stress-related genes disturbing the detection of significant relations. During WS and DI, when sufficient cellular energy is available, correlations may be more readily detected.

Bioinformatics analysis revealed that 8 of 9 correlating genes of the WS phase were clustered. The functions of these genes can roughly be divided into 3 processes: cytoskeleton organisation, glycolysis and nutrient sensing. Four genes have a link with the cytoskeleton: *ACTB*, *CFL1*, *ALDOA* and *PTK2B*. β-Actin is a structural component of actin filaments, which are located below the cell membrane. Cofilin-1 can bind to F-actin and is able to depolymerise the filaments allowing dynamic changes of the actin cytoskeleton (31). Binding of cofilin to actin can lead to the accumulation of lipids in the adipose tissue, because cofilin controls the mechanical tension of the adipocyte (32, 33). Aldolase has previously been recognized as a component of the cytoskeleton, binding to the actin filaments, where it may serve in anchoring and translocation of the GLUT4-containing vesicles (34, 35). Based on proteomics analysis in a weight loss-maintenance study, we have previously proposed a mechanism in which phosphorylation of Annexin 2 at Tyr-24 mediates the translocation of GLUT4 vesicles from the Aldolase-anchored position at the actin filaments to the cell membrane, resulting in increased uptake of glucose (36). In 3T3-L1 cells it has been shown that upon stimulation PYK2, the murine ortholog of PTK2B, induces the formation of actin filaments and mediates independent of insulin the endothelin-1stimulated translocation of GLUT4 to the membrane (37). As such, our present findings are completely in line with an upregulation of glucose uptake shortly after returning to an energy balanced state. Moreover, we now find that this potential upregulation is related to the risk for weight regain. It is substantiated by the fact that besides Aldolase A, two other genes coding for enzymes of the glycolysis, *PGK1* and *ENO1*, are strongly correlated with weight regain percentage. In addition, on the protein level another two glycolytic enzymes, PKM and TPI1, correlated with weight regain percentage during the WS phase in the VLCD group.

The bioinformatics analysis indicates that the HIF-1 complex may have an important regulatory role in determining the expression of the glycolytic genes. In the VLCD group *HIF-1A* gene expression dramatically increases during WL (FC=1.25, Q<0.0001) and decreases during WS (FC=-1.14, Q=0.02). Expression of the genes for *ALDOA*, *ENO1* and *PGK1* significantly decrease during WL, but do not change during WS suggesting that HIF-1 is not the only regulatory factor. Also the correlated glycolytic proteins PKM and TPI are known to be regulated by HIF-1 (38). Notably, the expression of the *C5* gene parallels that of *HIF-1A* during WL (FC=1.27, Q<0.0001) and WS (FC=-1.15, Q=0.01). The meaning of the inverse correlation of *C5* gene expression during WS with weight regain percentage is unclear. However, a role for C5 in obese adipose tissue inflammation and insulin resistance has been proposed (39). Unfortunately, the role of the HiF1 protein could not be investigated, because it was not detected by LC-MSMS and no sample was left to perform a different way of protein quantification.

It can be expected that the influx of glucose and the availability of metabolic energy triggers nutrient-sensing pathways. An important player is the mTOR Complex 1, which is inhibited by the TSC2-TSC1 complex. RPS6KA1 is able to phosphorylate TSC2 at Ser1798, which leads to activation of the mTORC1 signalling (40, 41). In addition, MAPK13 is a regulator of the amino acid-induced activation of the mTOR signalling (42). Using rat ELT3 cells it was shown that TSC1 functions in actin dynamics, cell adhesion and stress fiber (dis)assembly (43). As indicated by the position of TSC1 in the cluster, the TSC complex may link nutrient sensing to modification of the actin cytoskeleton.

Analysis of the genes correlating with weight regain percentage during DI led to a protein interaction cluster with ENO1, LGALS1 and ATF2 as most involved nodes. ATF2 belongs to the ATF/CREB transcription factor family. Its activation is effectuated by stress-activated protein kinases such as MAPK13/p38δ. In the mouse, inhibition of p38 reduced high-fat diet induced obesity and insulin resistance (44). The clustering results suggest that its transactivating activity may also be influenced by interaction with proteins encoded by the correlated genes and as such modify the risk of weight regain. Another nodal protein is galectin-1. Several pharmacological inhibitors of this protein have shown to lower body weight gain in diet-induced obese rats suggesting that this protein can also be a target for preventing weight regain (45-47).

Our present results show that changes in expression of defined stress-related genes correlate to the risk for weight regain in the WS and DI phase. In a previous weight loss study we reported that higher levels of the stress proteins β-actin, HSP27, HSP60 and HSP70 in adipose tissue were associated with regaining weight after weight loss on a VLCD. Here, we have not compared gene- and protein expression between weight regainers and weight maintainers, but searched for correlations between gene/protein expression and weight regain percentage. Indications for involvement of those genes in weight regain of the present VLCD group were mainly found in the gene expression data of the WS phase: *β-actin* (r=0.562, P=0.003), *HSP27* (r=0.470, P=0.02), *HSP70/HSPA5* (r= 0.457, P=0.03) and *HSP70/HSPA8* (r=0.535, P=0.007). On the protein level, HSP60/HSPD1 correlated with weight regain during the WS period but only in the LCD group (r=0.489, P=0.03). All correlations are positive indicating that an increase of stress or a lower decrease of stress is in line with risk for weight regain.
A limitation of the current study is that we were not able to measure all stress-related proteins because the abundance of some proteins including HIF-1 could not be properly determined by LC-MS/MS. Yet, we were still able to measure twenty-two different stress proteins. Furthermore, a

relatively high number of participants were included in the microarray analysis which increases the strength of the transcriptomics data. For correlations with gene expression data we used a cut-off P-value of 0.01 and several correlations were even <0.001. Clustering suggested coherence of correlating gene sets. Nevertheless, false positives cannot be excluded. This is especially the case for the protein correlations for which a threshold P-value of 0.05 was used.

In conclusion, our present findings indicate that the risk for weight regain is related to changes in the expression of stress-related genes during the first four weeks after returning to a weight stable situation (WS), and during the whole dietary intervention (DI, WS+WL). Bioinformatics clustering suggests that during the weight stabilisation phase defined genes for actin filament dynamics, glucose handling and nutrient sensing are related to weight regain. HIF-1 is indicated as an important regulator. With regard to DI, clustering of correlated genes indicate that *LGALS1*, *ENO1* and *ATF2* are important nodes for conferring risk to weight regain. Further research is required to investigate the mechanistic significance of the present findings and to try and find targets for prevention of weight regain.

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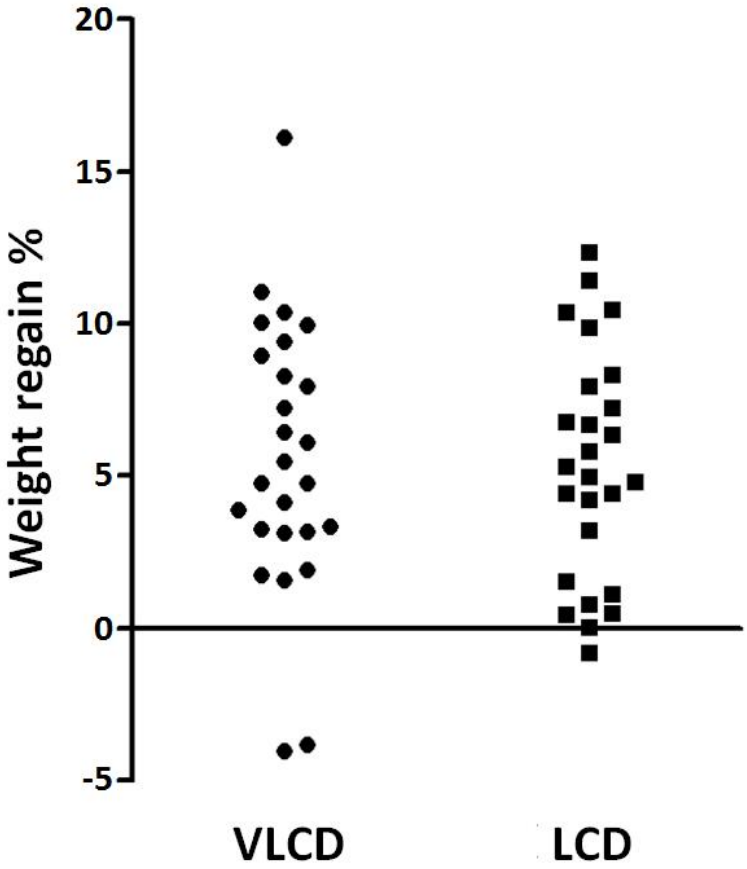
Supplementary Figures and Tables



Supplement Figure 1: Plot of all individual weight regain percentages for VLCD and LCD. Weight regain percentage is calculated: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. VLCD, very-low-calorie diet; LCD, low-calorie diet.

Supplement Table 1: Subject characteristics at study start, end of weight loss and end of weight stable.

	Study start (T1)		End of WL (T2)		End of WS (T3)	
	VLCD	**LCD**	**VLCD**	**LCD**	**VLCD**	**LCD**
Sex (male/female)	12/14	13/14				
Age (years)	50.4 ± 1.5	51.7 ± 2.1				
Weight (kg)	92.1 ± 1.9	92.8 ± 2.0	83.1 ± 1.6	84.6 ± 2.0	82.9 ± 1.7	84.5 ± 2.0
BMI (kg/m^2)	30.8 ± 0.4	31.5 ± 0.5	27.8 ± 0.4	28.7 ± 0.5	27.7 ± 0.4	28.7 ± 0.5
Hip circumference (cm)	111.0 ± 1.1	110.7 ± 1.4	105.0 ± 1.0	106.1 ± 1.5	104.8 ± 1.0	104.8 ± 1.5†
Waist circumference (cm)	101.3 ± 1.6	102.5 ± 2.1	93.5 ± 1.4	95.2 ± 1.9	94.6 ± 1.4	94.4 ± 2.1
Body fat (%)	39.5 ± 1.6	40.6 ± 1.9	34.8 ± 2.0	34.7 ± 2.2	33.7 ± 2.0††	34.1 ± 2.3†
Body fat (kg)	35.7 ± 1.2	36.8 ± 1.9	28.4 ± 1.5	29.2 ± 2.1	27.3 ± 1.4††	28.7 ± 2.1†
Fat free mass (kg)	55.7 ± 2.4	55.5 ± 2.3	54.2 ± 2.3	55.0 ± 2.3	54.9 ± 2.4††	55.3 ± 2.3

Values are mean±SEM. *P≤0.05, independent T-test, comparison between VLCD and LCD at study start, end of WL and end of WS. No significant differences were observed between the LCD and VLCD at the study start. †P≤0.05, ††<0.01, dependent T-test, comparison between end of WL vs. end of WS. WL, weight loss; WS, weight stable; VLCD, very-low-calorie diet; LCD, low-calorie diet; BMI, body mass index.

Supplement Table 2: List of the 107 candidate stress-related genes used for the analysis.

Gene ID	Gene name	Gene #	Chromosome
ACTB	actin, beta	60	7
ACTG1	actin gamma 1	71	17
AHSA2	AHA1, activator of heat shock 90kDa protein ATPase homolog 2 (yeast)	130872	2
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	231	7
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	217	12
ALDOA	aldolase A, fructose-bisphosphate	226	16
ANXA2	annexin A2	302	15
ANXA5	annexin A5	308	4
AQP1	aquaporin 1 (Colton blood group)	358	7
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	396	17
ATF3	activating transcription factor 3	467	1
ATF4	activating transcription factor 4	468	22
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	493	1
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	506	12
BCLAF1	BCL2-associated transcription factor 1	9774	6
C5	complement component 5	727	9
CALR	Calreticulin	811	19
CFL1	cofilin 1 (non-muscle)	1072	11
CHORDC1	cysteine and histidine-rich domain (CHORD) containing 1	26973	11
CLIC1	chloride intracellular channel 1	1192	6
CRHBP	corticotropin releasing hormone binding protein	1393	5
CRYAB	crystallin, alpha B	1410	11
CTSD	cathepsin D	1509	11
DDIT3	DNA-damage-inducible transcript 3	1649	12
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	5611	13
DUSP10	dual specificity phosphatase 10	11221	1
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	9695	3
EGFR	epidermal growth factor receptor	1956	7
EIF2AK1	eukaryotic translation initiation factor 2-alpha kinase 1	27102	7
EIF2B3	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	8891	1
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	1965	14
EIF5A	eukaryotic translation initiation factor 5A	1984	17
ENO1	enolase 1, (alpha)	2023	1
ERRFI1	ERBB receptor feedback inhibitor 1	54206	1
GADD45B	growth arrest and DNA-damage-inducible, beta	4616	19
GADD45G	growth arrest and DNA-damage-inducible, gamma	10912	9
GATA4	GATA binding protein 4	2626	8
GATA6	GATA binding protein 6	2627	18
GPR132	G protein-coupled receptor 132	29933	14
GSTP1	glutathione S-transferase pi 1	2950	11
HEY2	hes-related family bHLH transcription factor with YRPW motif 2	23493	6
HILPDA	hypoxia inducible lipid droplet-associated	29923	7
HSF2	heat shock transcription factor 2	3298	6
HSF4	heat shock transcription factor 4	3299	16
HSP90AB4P	heat shock protein 90kDa alpha (cytosolic), class B member 4, pseudogene	664618	15
HSP90B2P	heat shock protein 90kDa beta (Grp94), member 2, pseudogene	7190	15
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	3309	9
HSPA8	heat shock 70kDa protein 8	3312	11
HSPB1	heat shock 27kDa protein 1	3315	7
HSPB11	heat shock protein family B (small), member 11	51668	1
HSPB6	heat shock protein, alpha-crystallin-related, B6	126393	19
HSPB8	heat shock 22kDa protein 8	26353	12
HSPD1	heat shock 60kDa protein 1 (chaperonin)	3329	2
HYOU1	hypoxia up-regulated 1	10525	11
IDO1	indoleamine 2,3-dioxygenase 1	3620	8
KLF15	Kruppel-like factor 15	28999	3
KLF2	Kruppel-like factor 2	10365	19
KRT8	keratin 8, type II	3856	12
LGALS1	lectin, galactoside-binding, soluble, 1	3956	22
LMNA	lamin A/C	4000	1
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1	11184	19
MAPK11	mitogen-activated protein kinase 11	5600	22
MAPK13	mitogen-activated protein kinase 13	5603	6
MAPK7	mitogen-activated protein kinase 7	5598	17
MAPK8	mitogen-activated protein kinase 8	5599	10

MAPK9	mitogen-activated protein kinase 9	5601	5
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3	7867	3
MEF2C	myocyte enhancer factor 2C	4208	5
MUC1	mucin 1, cell surface associated	4582	1
NDRG4	NDRG family member 4	65009	16
NME2	NME/NM23 nucleoside diphosphate kinase 2	4831	17
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	4869	5
OMA1	OMA1 zinc metallopeptidase	115209	1
P4HB	prolyl 4-hydroxylase, beta polypeptide	5034	17
PGK1	phosphoglycerate kinase 1	5230	X
PHB	Prohibitin	5245	17
PKM	pyruvate kinase, muscle	5315	15
PPIA	peptidylprolyl isomerase A (cyclophilin A)	5478	7
PRDX6	peroxiredoxin 6	9588	1
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	5563	1
PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	5720	14
PTEN	phosphatase and tensin homolog	5728	10
PTK2B	protein tyrosine kinase 2 beta	2185	8
RGS14	regulator of G-protein signaling 14	10636	5
RPS6KA1	ribosomal protein S6 kinase, 90kDa, polypeptide 1	6195	1
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	6197	X
RTN3	reticulon 3	10313	11
SGK1	serum/glucocorticoid regulated kinase 1	6446	6
SNN	Stannin	8303	16
SQSTM1	sequestosome 1	8878	5
STC2	stanniocalcin 2	8614	5
STIP1	stress-induced phosphoprotein 1	10963	11
STK39	serine threonine kinase 39	27347	2
STMN1	stathmin 1	3925	1
TAOK2	TAO kinase 2	9344	16
TCAP	titin-cap	8557	17
TENM1	teneurin transmembrane protein 1	10178	X
TMEM204	transmembrane protein 204	79652	16
TP53I11	tumor protein p53 inducible protein 11	9537	11
TP53INP1	tumor protein p53 inducible nuclear protein 1	94241	8
TP53TG1	TP53 target 1 (non-protein coding)	11257	7
TPI1	triosephosphate isomerase 1	7167	12
TPM3	tropomyosin 3	7170	1
TRAF2	TNF receptor-associated factor 2	7186	9
TUBB	tubulin, beta class I	203068	6
UCN2	urocortin 2	90226	3
ZAK	sterile alpha motif and leucine zipper containing kinase AZK	51776	2

Sixteen genes were specifically related to endoplasmic reticulum stress as previously reported (18, 19), twenty-four candidate stress-related genes were selected based on the frequently detected proteins in subcutaneous adipose tissue(26) and sixty-seven were genes with the term “response to stress” in the Gene Ontology Biological Process (GO-BP) description.

Supplement Table 3: List of the 22 candidate stress-related proteins used for the analysis.

Protein ID	Protein name	MW (kDa)
P10809	60 kDa heat shock protein, mitochondrial [OS=Homo sapiens]	61.016
P11021	78 kDa glucose-regulated protein [OS=Homo sapiens]	72.288
P60709	Actin, cytoplasmic 1 [OS=Homo sapiens]	41.710
P05091	Aldehyde dehydrogenase, mitochondrial [OS=Homo sapiens]	56.346
P02511	Alpha-crystallin B chain [OS=Homo sapiens]	20.146
P08758	Annexin A5 [OS=Homo sapiens]	35.914
P06576	ATP synthase subunit beta, mitochondrial [OS=Homo sapiens]	56.525
P27797	Calreticulin [OS=Homo sapiens]	48.112
P07339	Cathepsin D [OS=Homo sapiens]	44.524
O00299	Chloride intracellular channel protein 1 [OS=Homo sapiens]	26.906
P09382	Galectin-1 [OS=Homo sapiens]	14.706
P04792	Heat shock protein beta-1 [OS=Homo sapiens]	22.768
O14558	Heat shock protein beta-6 [OS=Homo sapiens]	17.125
P06748	Nucleophosmin [OS=Homo sapiens]	32.555
P62937	peptidyl-prolyl cis-trans isomerase A [OS=Homo sapiens]	18.001
P30041	Peroxiredoxin-6 [OS=Homo sapiens]	25.019
P00558	Phosphoglycerate kinase 1 [OS=Homo sapiens]	44.586
P02545	Prelamin-A/C [OS=Homo sapiens]	74.095
P35232	Prohibitin [OS=Homo sapiens]	29.786
P07237	Protein disulfide-isomerase [OS=Homo sapiens]	57.081
P14618	Pyruvate kinase PKM [OS=Homo sapiens]	57.900
P60174	Triosephosphate isomerase [OS=Homo sapiens]	30.772

Supplement Table 4: All significant correlations between the weight regain percentage and the fold changes in gene expression levels during the weight loss, weight stable and dietary intervention period.

Gene ID	Gene name	VLCD			LCD		
		WL	WS	DI	WL	WS	DI
ACTB	Actin, beta		0.562				
AKR1B1	Aldo-keto reductase family 1, member B1		0.453				
ALDH2	Aldehyde dehydrogenase 2 family	0.466					0.520
ALDOA	Aldolase A, fructose-bisphosphate		0.552	0.458	0.590		0.473
ANXA5	Annexin A5	0.484		0.566			
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha		0.507	0.436			
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide		0.478	0.488			
BCLAF1	BCL2-associated transcription factor 1		-0.433			0.441	
C5	Complement component 5		-0.525				
CALR	Calreticulin		0.415	0.428			
CFL1	Cofilin 1		0.607	0.455			
CLIC1	Chloride intracellular channel 1		0.431				
CRHBP	Corticotropin releasing hormone binding protein			-0.518			
CRYAB	Crystallin, alpha B	0.586		0.671			
DUSP10	Dual specificity phosphatase 10			0.424			
EIF2AK1	Eukaryotic translation initiation factor 2-alpha kinase 1			0.456			
EIF2B3	Eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa				0.454		
ENO1	Enolase 1, alpha		0.710	0.684			
GSTP1	Glutathione S-transferase pi 1	0.444		0.597			
HSF2	Heat shock transcription factor 2					0.414	
HSP90B2P	Heat shock protein 90kDa beta, member 2, pseudogene		0.438			0.416	
HSPA5	Heat shock 70kDa protein 5		0.457				
HSPA8	Heat shock 70kDa protein 8		0.519				
HSPB1	Heat shock 27kDa protein 1		0.470				
HSPB8	Heat shock 22kDa protein 8			0.420			
HSPD1	Heat shock 60kDa protein 1 (chaperonin)				0.448		
HYOU1	Hypoxia up-regulated 1		0.443			-0.445	
KLF15	Kruppel-like factor 15					0.431	
LGALS1	Lectin, galactoside-binding, soluble, 1			0.705			
MAPK13	Mitogen-activated protein kinase 13		0.586				
MAPK7	Mitogen-activated protein kinase 7				0.445		
MAPK8	Mitogen-activated protein kinase 8		-0.477				
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3		0.519				
MEF2C	Myocyte enhancer factor 2C		-0.499	-0.454			
MUC1	Mucin 1, cell surface associated					-0.423	
NME2	NME/NM23 nucleoside diphosphate kinase 2						-0.503
OMA1	OMA1 zinc metallopeptidase		-0.443				
PGK1	Phosphoglycerate kinase 1	-0.434	0.655	0.417			
PHB	Prohibitin			0.528			

PKM	Pyruvate kinase, muscle		0.475			
PRDX6	Peroxiredoxin 6	0.443			0.606	
PRKAA2	Protein kinase, AMP-activated, alpha 2 catalytic subunit					0.436
PSME1	Proteasome (prosome, macropain) activator subunit 1					-0.444
PTEN	Phosphatase and tensin homolog		-0.426			
PTK2B	Protein tyrosine kinase 2 beta	-0.547	0.658			
RGS14	Regulator of G-protein signaling 14	-0.506	0.500			
RPS6KA1	Ribosomal protein S6 kinase, 90kDa, polypeptide 1		0.643			
RTN3	Reticulon 3				0.480	
SNN	Stannin		0.459			
SQSTM1	Sequestosome 1	0.441		0.460		
TPI1	Triosephosphate isomerase 1					-0.518
UCN2	Urocortin 2			-0.521	-0.468	
ZAK	Sterile alpha motif and leucine zipper containing kinase AZK		-0.505			0.504

Correlation coefficients (*r*) between weight regain and the fold change (FC) of gene expression during weight loss (T1-T2), weight stable (T2-T3) and dietary intervention (T1-T3). Weight regain percentage is calculated: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. Only Pearson R's correlations with a P-value ≤0.05 are depicted in this table. VLCD, very-low-calorie diet; LCD, low-calorie diet; WL, weight loss; WS, weight stable; DI, dietary intervention.

Chapter 6

Relation between stress- and ECM-related genes and their effect on weight regain

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To be submitted

Abstract

Background/Objective: The adipose tissue plays a central role in the risk for weight regain. During a negative energy balance, the volume of adipocytes decreases due to loss of triglycerides which may lead to stress due to the misfit between the cell contents and the surrounding extracellular matrix (ECM) (1).This stress can be resolved by remodeling of the ECM or the re-storing of triglycerides within the adipocytes (1, 2). Possibly, there is a connection between stress-related and ECM-related genes, which is associated with weight regain. Our main objective was to investigate the existence of such a connection.

Methods: In this randomized controlled trial, twenty-six healthy overweight/obese participants followed a 5-week very-low-calorie diet (500 kcal/d) with subsequently a 4-week weight stable diet (WS period), and an uncontrolled 9-month follow-up. Abdominal subcutaneous adipose tissue biopsies were collected for microarray analysis. We performed correlation and interaction analysis with the weight regain percentage using two gene sets which we previously defined as 'stress-related' and as 'ECM-related' genes.

Results: During the WS phase, a co-expression network of 8 stress- and 15 ECM-related genes correlating with weight regain could be constructed, which links to the biological processes on leukocyte-activity, ECM remodelling, actin cytoskeleton organisation and glucose handling. Interaction analysis between stress- and ECM-related genes revealed several gene combinations that were highly related to the weight regain percentage. In particular, the epidermal growth factor signaling pathway was identified as strongly influencing the risk of weight regain, possibly through interaction with actinin alpha-1 (ACTN1), a component of stress fibers, and with integrin beta 4 (ITGB4), cystatin C (CST3) and laminin alpha-3 (LAMA3).

Conclusion: Our present findings indicate the importance of the connection between stress- and ECM-related genes in the risk for weight regain

Introduction

The prevalence of overweight and obesity has increased to epidemic proportions and the related comorbidities are major threats to human health (3). Achieving 5-10% weight loss by dietary intervention, increased physical activity, or pharmacological and/or surgical treatment produces positive health outcomes in overweight or obese people (4, 5). However, long-term weight loss maintenance has been proven to be difficult. In general, only 20% of the people are successful in maintaining weight loss (6, 7). After weight regain, the risk for metabolic complications appears higher than at the start of the weight loss period (8, 9). Therefore, it is important to understand the mechanisms influencing the risk for weight regain.

The adipose tissue plays a central role in the risk for weight regain. It was previously hypothesized that the volume of adipocytes decreases due to loss of triglycerides during a negative energy balance creating stress between the cell contents and the surrounding extracellular matrix (ECM) (1). This stress can be resolved by remodeling of the ECM or the re-storing of triglycerides within the adipocytes (1, 2). It suggests that cellular stress accumulated in adipocytes during a negative energy balance is a driving force behind the risk for weight regain. In line, we have shown higher cellular stress in the subcutaneous adipose tissue of participants regaining their lost weight compared to the participants maintaining weight (10). Furthermore, the risk for weight regain is related to changes in the expression of certain stress-related genes during the first four weeks after returning to a weight stable situation. These stress-related genes are involved in actin filament dynamics, glucose handling and nutrient sensing (11). Interestingly, after returning to energy balance changes in expression of certain ECM-related genes are also correlated to weight regain (12). Based on the proposed model, it is possible that there is a connection between stress-related genes and ECM-related genes associated with weight regain. In the present study, our main objective was to expand on our previous studies to investigate the existence and relevance of such a connection. Therefore, we performed correlation and interaction analysis using two gene sets which we previously defined as ‘stress-related’ and as ‘ECM-related’ genes during the weight loss (13), the weight stabilisation (14) and the dietary intervention (DI) phase of a very-low-calorie diet (VLCD).

Methods

Participants and study design

The present study was performed in a sub-cohort of our larger clinical trial as described previously (15), in which only the VLCD intervention was selected. Thirty-one overweight and obese (BMI 27-36 kg/m^2) participants were included in the present study. All participants gave their written informed consent before participation in the study. The study was performed according to the declaration of Helsinki and was approved by the Medical Ethics Committee of Maastricht University Medical Centre.

In short, participants had to remain weight stable (weight change <3.0 kg) two months prior to the start of the study. Thereafter, study participants underwent a 5-week VLCD (500 kcal/day), targeting 10% weight loss (WL period, T1-T2) by consuming three meal replacements per day (Modifast; Nutrition et Santé Benelux), and a subsequent 4-week weight maintenance diet (WS period, T2-T3) based on their individual energy requirements (Figure 1). This WS period was designed to investigate the effect of weight loss without the interfering effect of a pronounced negative energy balance. The WL and WS period taken together was named the dietary intervention (DI, T1-T3). After the WS

period, participant's body weight and blood pressure were monitored for 9 months (follow-up, T3-T4). During this follow-up, participants did not receive advice on monitoring and limiting food intake to mimic non-restricted free-living conditions. This trial is registered with www.clinicaltrials.gov: as NCT01559415.

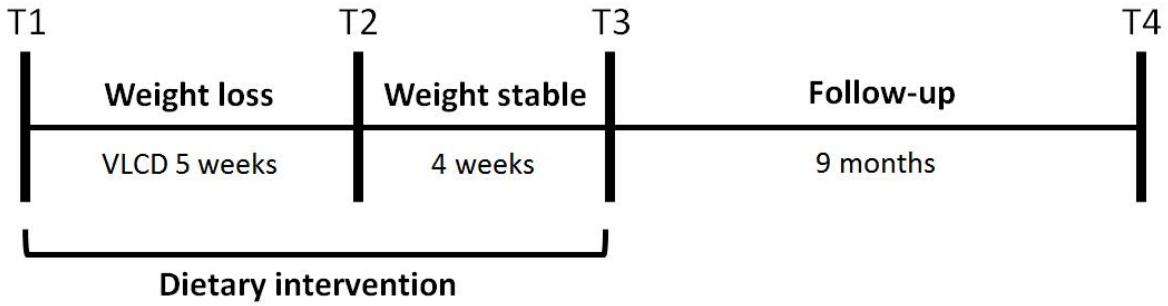

Figure 1: Schematic overview of the study design. Anthropometry, adipose tissue biopsies and proteomics were performed at the start of the study (T1), at the end of the weight loss period (T2), weight stable period (T3), and follow-up (T4). Transcriptomics were performed at T1, T2 and T3. The dietary intervention period (DI) is the weight loss period and weight stable period taken together. VLCD, very-low-calorie diet (n=26).

At baseline, end of WL, end of WS and end of follow-up, body weight, blood pressure and, waist and hip circumferences were measured, body composition was determined, fasting blood samples were drawn, and abdominal subcutaneous adipose tissue biopsies were collected as described previously (15). As defined in our previous studies, the weight regain percentage (WR%) = ((weight after follow-up T4 – weight after WS T3) ÷ weight after WS T3) x 100%.

Adipose tissue biopsy

Abdominal subcutaneous adipose tissue biopsies were obtained by needle biopsy under local anaesthesia (2% lidocaine, Fresenius Kabi) after an overnight fast at T1, T2 T3, and T4. Tissue was immediately rinsed in sterile saline, frozen in liquid nitrogen and stored at -80°C until RNA and protein isolation.

Adipose tissue RNA isolation and microarray analysis

For RNA isolation, adipose tissue samples from T1, T2 and T3 were used. Total RNA was extracted from approx. 150 mg frozen adipose tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands). Total RNA (100 ng per sample) was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19654 unique genes (Affymetrix). Sample labeling, hybridization to chips and image scanning was performed according to manufacturer's instructions. Microarray signals were normalized using robust multichip average (RMA). Genes with normalized signals > 20 on at least 15 arrays were defined as expressed (11532 genes). Significant differences of individual genes were tested using the limma R library (16). P-values were adjusted using false discovery rate (FDR) (17) and an adjusted P-value (q-value) ≤ 0.05 was considered significant. Array data have been submitted to the Gene Expression Omnibus for a larger cohort (n=53) (number GSE77962) while here we show the results of a sub-group.

Gene selection

Based on gene ontology, a set of 107 'stress-related' and a set of 277 'ECM-related' genes (Supplement Table 1) were selected, which were the same as in the previous analyses (11, 12)

Data analysis

The gene expression change was used to study the relationship between the gene changes and the weight regain percentage. We calculated the fold change (FC) of the expression of each gene during WL, WS and DI, respectively.

Correlation analysis

Correlations were made between stress-related and ECM-related genes during VLCD WL, WS and DI using the Pearson correlation coefficient. For this, only genes were included which were found to be correlated with the WR% in previous analyses (11, 12). The correlations between stress- and ECM-related genes were entered in the MetScape 3 App (18) for the software platform Cytoscape (14) to make correlation based networks during the WL, WS and DI phase. In principle, genes were included that significantly correlated with $|r| \geq 0.600$ and $P \leq 0.001$ (unless indicated otherwise).

Interaction analysis

Linear regression model y=f(x1, x2, x1×x2) was calculated for the effect of interaction between change in expression of x1: each gene in the stress-gene set and x2: each gene in the ECM-gene set, at WL, WS and DI, respectively, for y: the weight regain percentage. The analysis was run in R version 3.3.1, a gene pair with P-value of the interaction ≤0.001 was taken as significant interaction pair.

Other statistical analysis

Data are presented as mean±SEM. Variation in the number of participants between analyses is due to the exclusion of participants with missing data. Dependent T-test was carried out to determine possible effects over time. For all statistical tests $P \leq 0.05$ was considered to be statistically significant unless indicated otherwise. All variables were checked for normal distribution, and variables with a skewed distribution were LN-transformed to satisfy conditions of normality. Extreme outliers (values higher than 3x interquartile range calculated with SPSS) influencing the data were removed during statistical analyses. These analyses were done using SPSS 20.0 for Windows (SPSS Inc, Chicago, IL).

Results

Study population

Three participants withdrew from the study during the dietary intervention (T1-T3) due to personal circumstances, for one participant we could not collect enough biopsy material and for one participant the gene expression results deviated strongly from the others and were therefore excluded. Characteristics of the remaining twenty-six participants at study start (T1), end of WL (T2), end of WS (T3) and end of follow-up (T4) are displayed in Table 1. Body weight, BMI, hip and waist circumference, body fat and fat free mass significantly decreased during weight loss and remained rather stable during the WS period. The average weight regain percentage was 5.4±4.5%.

Table 1: Subject characteristics at study start, end of weight loss and end of weight stable.

Gene ID	Study start (T1)	End of WL (T2)	End of WS (T3)	End of follow-up (T4)
Sex (male/female)	12/14			
Age (years)	50.4 ± 1.5			
Weight (kg)	92.1 ± 1.9	83.1 ± 1.6***	82.9 ± 1.7	87.7 ± 2.0***
BMI (kg/m^2)	30.8 ± 0.4	27.8 ± 0.4***	27.7 ± 0.4	29.1 ± 0.5***
Hip circumference (cm)	111.0 ± 1.1	105.0 ± 1.0***	104.8 ± 1.0	105.4 ± 1.4
Waist circumference (cm)	101.3 ± 1.6	93.5 ± 1.4***	94.6 ± 1.4	97.6 ± 1.7***
Body fat (%)	39.5 ± 1.6	34.8 ± 2.0***	33.7 ± 2.0**	36.0 ± 1.9***
Body fat (kg)	35.7 ± 1.2	28.4 ± 1.5***	27.3 ± 1.4**	31.0 ± 1.6***
Fat free mass (kg)	55.7 ± 2.4	54.2 ± 2.3***	54.9 ± 2.4**	55.9 ± 2.4*

Values are mean±SEM. *P≤0.05, **P≤0.01, ***P≤0.001, dependent T-test, change between this time point and previous time point. WL, weight loss; WS, weight stable; BMI, body mass index.

Co-expression between stress- and ECM-related genes

Stress- and ECM-related genes were first filtered from the sets based on significant correlation (P≤0.05) with the WR% as described previously (11, 12). In total, 124 genes met this criteria which were passed into the co-expression analysis: 28 genes during WL, 62 genes during WS and 34 genes during DI. 286 strong correlations ($|r|\geq0.600$; P≤0.001) were found between the stress gene set and the ECM gene set. Interestingly, there was a large difference in the amount of strong correlations found between stress and ECM genes for the different phases: 18 during WL, 243 during WS and 25 during DI. Although for the analyses in the WS period more genes were used compared to the WL and DI phase, this cannot fully explain the large difference in correlations. Only 26 stress genes and 28 ECM genes are responsible for the 243 correlations during WS, indicating that these genes have multiple correlations with other genes (Table 2). In fact, there are 12 stress genes that each correlated with at least 10 ECM genes and 16 ECM genes that each correlated with at least 10 stress genes. These results indicated that stress- and ECM-related genes were co-expressed especially during the weight stabilization period, not only as single gene pairs but rather in a complex manner.

Table 2: The number of correlations between stress-and ECM-related genes during the WS phase.

Stress genes	Connections to ECM genes	ECM genes	Connections to stress genes
CFL1	21	PPIB	15
PKM	21	CAPN1	15
RPS6KA1	18	CTSB	14
CALR	17	ITGA3	14
ZAK	17	COLGALT1	13
ENO1	13	ACTN1	13
MAPK13	16	EMILIN2	12
MAPKAPK3	16	PDIA4	12
ARHGDIA	11	SPARCL1	12
AKR1B1	11	ECM2	11
ALDOA	9	ITGAX	11
HYOU1	11	ITGA5	10
PGK1	10	PLOD1	10
CLIC1	9	NCSTN	10
PTK2B	7	DMD	10
ACTB	7	ITGAM	10
HSPA5	6	ITGB5	8
MAPK8	6	SPINT1	8
MEF2C	3	ITGAL	8
OMA1	4	CTSS	6
RGS14	2	ITGB2	6
HSPA8	2	PLOD2	5
HSPB1	2	KDR	3
PTEN	2	VIT	2
BCLAF1	1	GPC1	2
C5	1	ITGB3	1
		ICAM3	1
		NCAM1	1

Pearson correlations with $|r|\geq0.600$ and P≤0.001. ECM, extracellular matrix; WS, weight stable.

Next, we wanted to see if the correlation genes formed a specific network. With the correlation criteria $|r|\geq 0.600$ and $P\leq 0.001$, we observed a network during all phases (Supplement Figure 1). For the WS phase more stringent criteria ($|r|\geq 0.800$; $P\leq 0.001$) were used to be able to detect the most important gene correlations. The network consists of 8 stress-related genes and 15 ECM-related genes. All genes were positively correlated with each other as well as with the WR%, except dystrophin (*DMD)* which was negatively correlated with integrin subunit alpha M (*ITGAM)* and weight regain (Figure 2). Ten out of the 23 genes are strongly correlated with weight regain ($P\leq 0.01$). The previously detected correlation network of integrin genes *ITGAL*, *ITGAM*, *ITGAX* and *ITGB2* (12) was found to correlate strongly with the gene for ribosomal protein S6 kinase A1 (*RPS6KA1)*. The latter gene was previously identified as part of a network based on gene regulatory and biochemical interactions together with the genes for aldolase A (*ALDOA)*, enolase 1 (*ENO1)*, cofilin 1 (*CFL1)* and mitogen-activated protein kinase 13 (*MAPK13)* (11). These genes turn out to be part of the stress-ECM network as well. *CFL1* strongly correlates with 11 other genes of which 4 from the stress and 7 from the ECM gene set. Also, pyruvate kinase (*PKM)* seems to play a central role with in total 11 correlations to stress and ECM genes. These results suggest that a network of 23 stress-related and ECM-related genes is important in the risk for weight regain.

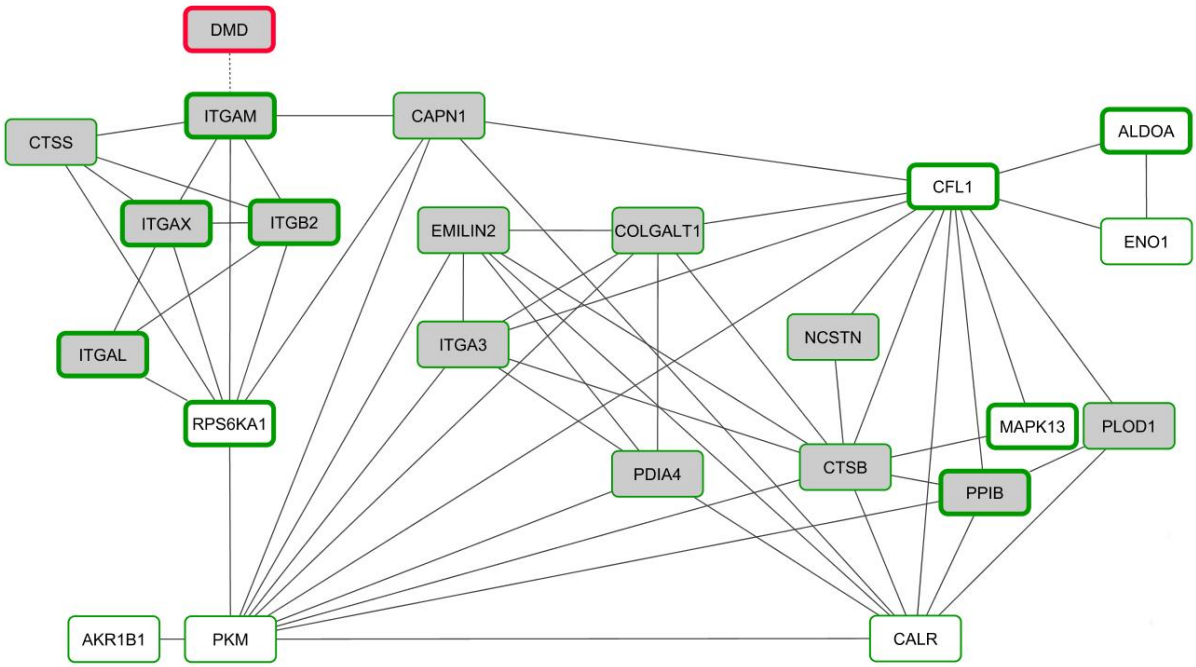

Figure 2: Correlation network between stress-related and ECM-related genes, which strongly correlate with weight regain percentage ($P\leq 0.05$), during the weight stabilization phase. Correlations were made using the Pearson correlation coefficient. Only genes that strongly correlate with each other ($|r|\geq 0.800$; $P\leq 0.001$) and with the weight regain percentage ($P\leq 0.01$) are depicted in this figure. White boxes, stress-related genes; grey boxes, ECM-related genes; solid line, positive correlation with other gene; dashed line, negative correlation with other gene; green outline, positive correlation with the weight regain percentage; red outline, negative correlation with the weight regain percentage. Thick outline, strong correlation with weight regain percentage ($P\leq 0.01$).

Stress- and ECM-related genes interaction influences risk for weight regain

We determined possible interactions between stress- and ECM-related genes and their influence on the WR%. In total, 141 significant interactions were found during WL, WS and DI that had an effect on the weight regain percentage (Supplement Table 3). The number of interactions was rather

similar between all phases: 60 during WL, 47 during WS and 34 during DI. However, the genes with multiple interactions with other genes were different for each phase (Table 3). During WL, most interactions were observed for the stress genes GATA binding protein 6 (*GATA6)*, heat shock transcription factor 2 (*HSF2)* and the ECM gene EGF Like Fibronectin Type III and Laminin G Domains *(EGFLAM)*. During WS, the stress genes activating transcription factor 4 (*ATF4*), ERBB receptor feedback inhibitor 1 (*ERRFI1*; 5), and the ECM gene collagen type XXI alpha 1 chain (*COL21A1)* have most interactions. During DI, most interactions were observed for the stress genes complement 5 (*C5)* and chloride intracellular channel 1 (*CLIC1)*.

Table 3: The number of interactions between stress-and ECM-related genes during WL, WS and DI.

Stress genes	Connections to ECM genes	ECM genes	Connections to stress genes	Period
GATA6	7	EGFLAM	5	WL
HSF2	7	ADAM11	4	WL
HSP90AB4P	4	CSGALNACT1	3	WL
PHB	4	COL1A1	3	WL
MAPK11	4	ITGA11	3	WL
DDIT3	3	ADAMTS4	3	WL
GSTP1	2	NPNT	3	WL
PGK1	2	KAZALD1	2	WL
TAOK2	2	ADAMTS1	2	WL
ARHGDIA	2	MADCAM1	2	WL
ENO1	2	CTGF	2	WL
LMNA	2	MFI2	2	WL
BCLAF1	2	COL6A3	2	WL
LGALS1	2	ECM1	2	WL
HSPD1	2			WL
KLF2	2			WL
ATF4	12	COL21A1	5	WS
ERRFI1	5	MPZL3	3	WS
TPI1	4	VWF	2	WS
SQSTM1	3	CHI3L1	2	WS
TUBB	2	NOTCH1	2	WS
EGFR	2	GFOD2	2	WS
STK39	2			WS
HSP90AB4P	2			WS
C5	11	ITGAD	2	DI
CLIC1	5	CILP	2	DI
RPS6KA3	4	TIMP4	2	DI
TUBB	2	CD47	2	DI
PKM	2	ELN	2	DI

The amount of strong interactions (P≤1.00E-04) between stress and ECM genes. ECM, extracellular matrix; WL, weight loss; WS, weight stable; DI, dietary intervention.

There were 15 strong interactions (interaction P≤1.00E-04) of which most were during the WS period (Table 4). We observed some models with a large effect on WR% (model r^2 ≥0.5 and P≤0.001). The interaction between the gene for epidermal growth factor receptor (*EGFR)* and the gene for actinin alpha 1 (*ACTN1)* during the WS phase had the biggest effect (r^2 =0.81) on the WR% (Table 4). Previously, we already observed the strong correlation between ECM gene *ACTN1* and the WR% (11) but the current analyses showed that *EGFR* stratifies this correlation. Specifically, the positive correlation between *ACTN1* and the WR% was reinforced when there was an increase of *EGFR* during the WS phase (Figure 3a). Furthermore, we observed that during the WS phase both *ATF4* and *ERRFI1* had multiple interactions with ECM genes which all have a large effect on the WR% (Table 4). One of these was the negative correlation between *ERRFI1* and the WR% was reinforced when there was a decrease of *ITGB4* during the WS phase (Figure 3b).

Table 4: Strongest interactions between stress- and ECM-related genes with the biggest effect on the weight regain percentage.

Stress genes	ECM genes	Interaction P-value	Model r^2	Model P-value	Period
ARHGDIA	ADAM11	7.88E-05	0.593	8.66E-04	WL
GSTP1	NPNT	9.80E-05	0.665	1.55E-04	WL
PHB	ADAMTS4	2.74E-05	0.688	8.42E-05	WL
BCLAF1	ADAM11	7.68E-05	0.595	8.26E-04	WL
ATF4	COL15A1	3.70E-05	0.602	4.60E-04	WS
ATF4	HSPG2	4.43E-05	0.598	4.99E-04	WS
ATF4	HSD17B12	2.81E-05	0.614	3.44E-04	WS
ATF4	COL21A1	9.11E-05	0.616	3.29E-04	WS
EGFR	ACTN1	7.05E-06	0.811	4.38E-07	WS
ERRFI1	CST3	1.51E-05	0.654	1.25E-04	WS
ERRFI1	ITGB4	3.93E-05	0.621	2.92E-04	WS
ERRFI1	LAMA3	4.19E-05	0.614	3.46E-04	WS
C5	CD47	3.07E-06	0.711	1.29E-05	DI
C5	ERO1LB	4.75E-05	0.630	1.46E-04	DI
RPS6KA3	COL5A3	3.23E-05	0.591	3.85E-04	DI

Strong interactions (P≤1.00E-04) between stress- and ECM-related genes. r^2 (≥0.500) and Model P-value (≤0.001) depict the effect of the interaction on the weight regain percentage. ECM, extracellular matrix; WL, weight loss; WS, weight stable; DI, dietary intervention.

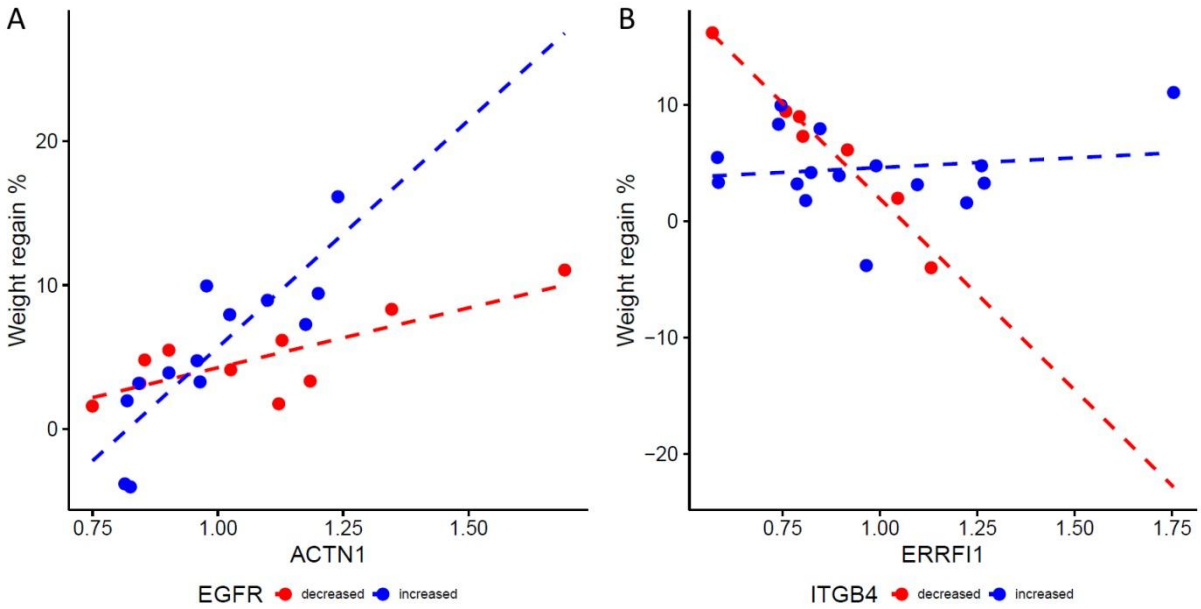

Figure 3: Correlation plot between the WR% and interaction of the ECM-related gene *ACTN1* with the stress-related gene *EGFR* (**A**) and the ECM-related gene *ITGB4* with the stress-related gene *ERRFI1* (**B**). The lines represent the correlations between changes in WR% and gene expression of *ACTN1* or *ERRFI1* at decreasing (red line) or increasing (blue line) gene expression changes of *EGFR* or *ITGB4*.

Discussion

The present study focussed on determining the relation between stress- and ECM-related genes and their combined effect on the weight regain percentage. For this, we selected the stress and ECM genes that correlation (P≤0.05) with the WR%. Correlation analyses between stress and ECM genes revealed that there were 286 strong correlations (|r| ≥0.600; P≤0.001). Most of these correlations were found in the WS phase and the strongest correlations (|r| ≥0.800; P≤0.001) formed a clear network consisting of 8 stress-related genes and 15 ECM-related genes. Interaction analyses revealed

that there are many interactions between stress and ECM genes. Fifteen of these interactions have a strong effect (r^2 ≥0.5; P≤0.001) indicating that the correlation between expression change of a gene from one category (stress or ECM) and WR% is highly influenced by the expression change of one or more genes from the other category. The interaction which is most influencing the correlation to the WR% is that between the stress gene *EGFR* and the ECM gene *ACTN1* in the WS phase.

We have previously reported about the importance of stress genes and proteins in the risk of weight regain. Levels of stress proteins in the adipose tissue of participants regaining weight after weight loss were higher compared to participants maintaining their lost weight (10). Also, we reported that stress genes involved in actin filament dynamics, glucose handling and nutrient sensing are related to weight regain (11). The link between ECM-genes and weight regain has also been established. Genetic variation of ECM genes is associated with an increased risk for weight regain (19). Also, there is a strong relation between the WR% and ECM genes involved in inflammatory processes and ECM remodelling (12). These studies clearly indicate the involvement of stress-related and ECM-related genes in the risk of weight regain, however, possible interaction was not investigated.

Correlation analyses revealed a co-expression network from 23 stress- and ECM-related genes, which all correlated to the WR%, during the WS phase. This network seems to consist of a smaller and larger cluster of genes. The smaller cluster consists of 4 leukocyte specific integrins, cathepsin S (*CTSS)*, *RPS6KA1* and *DMD*, whereas calpain 1 (*CAPN1)* and *PKM* form the connection with the larger subcluster. As described previously, the 4 integrins found in the analyses point to the importance of the leukocytes in the risk for weight regain (12). Now, we found that those genes are co-expressed with *RPS6KA1*. This gene, also known as *RSK1,* has been identified as a key factor in the regulation of IκBβ and NF-κB (20), factors involved in regulating the inflammatory response (21, 22). *CAPN1* and *PKM*, which connect the small subcluster with the larger, are also associated with inflammatory processes, although not specifically to adipose tissue inflammation. In mice, a dramatic reduction of the protein levels and activity of CAPN1 and CAPN2 resulted in the inhibition of inflammatory responses (23). *PKM* expression increased after treatment of cells with lipopolysaccharide (LPS), a substance used to induce inflammation *in vitro*, through activation of NF-κB signalling pathway (24).

The larger cluster of genes functions in multiple biological processes. An involvement in inflammatory processes is apparent for some genes, such as elastin microfibril interface 2 (*EMILIN2)*, protein disulfide isomerase family A member 4 (*PDIA4)*, calreticulin *(CALR)* and cathepsin B (*CTSB)*. Besides a role in the inflammatory process, cathepsins are involved in the remodelling of ECM proteins (25). The cysteine proteases CTSB and CTSL can be secreted outside the cell and digest the ECM (26). In fact, multiple genes in the correlation network are involved in ECM remodelling or cytoskeleton reorganisation. Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) and collagen beta(1-O)galactosyltransferase 1 (COLGALT1) are involved in modification of proteins including collagens (27, 28). The actin binding protein CFL1 is essential for cytoskeleton remodelling by regulating actin dynamics and plasticity (29). CFL1 can bind to filamentous (F)-actin and is able to depolymerise the filaments allowing dynamic changes of the actin cytoskeleton (13). Cofilin binding to actin can increase the lipid accumulation in the adipose tissue, because CFL controls the mechanical tension of adipocytes (30, 31). Also, aldolase A, a glycolytic enzyme and a component of the cytoskeleton, plays an important function in actin cytoskeleton organization (32, 33). ALDOA binds to actin filaments, where it may serve in anchoring and translocation of the GLUT4-containing

vesicles (2, 34). These findings suggest that there is increased glucose uptake shortly after returning to energy balance and that this is related to the risk for weight regain. This is supported by the correlation between gene expression change of *ENO1*and *PKM*, other enzymes of the glycolysis, and the WR% during the WS phase. Altogether, our results suggest that the expression patterns of certain leukocyte integrin genes shortly after weight loss by calorie restriction lead to a higher risk for weight regain which appears linked to ECM remodelling, actin cytoskeleton organization, and glucose handling by the adipose tissue.

Interaction analyses revealed 141 significant interactions between stress and ECM genes with an effect on the WR%. The genes with most interactions with other genes are the stress genes *ATF4* during the WS phase and *C5* during the DI. Interestingly, 4 of the 12 interactions of *ATF4* during the WS phase explained a large part of the WR% which points to importance of *ATF4* in the risk of weight regain. *ATF4* has been linked to weight changes previously. *ATF4* deficiency is shown to stimulate adipose tissue lipolysis, contributing to the increased energy expenditure in *ATF4* knockout mice (35). These mice have a reduced fat mass (35, 36). This protects against the development of glucose intolerance and insulin resistance in response to a high fat meal (37, 38). Yet, to our knowledge this is the first study reporting about the involvement of *ATF4* in weight regain after weight loss. Interestingly, similarly to *ATF4* also *ERRFI1* has multiple strong interactions during the WS phase that explain a large portion of the WR%, but the interaction between *EGFR* and *ACTN1* during WS has the strongest effect on the WR%.

EGFR, also known as ErbB1, plays a key role in adipogenesis and lower levels of ErbB1 can lead to adipose tissue dysfunction (39). EGFR is a transmembrane glycoprotein which is a receptor for members of the epidermal growth factor family. Binding of a ligand to the receptor results in dimerization and activation by autophosphorylation, and downstream activation of various signalling pathways (40). ACTN1 is one of the targets, which can be phosphorylated via activated EGFR. ACTN1 is an actin-binding protein that is found in non-muscle cells along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane. Phosphorylation of alpha-actinin leads to a reduced binding to actin filaments (41). Our finding that a positive correlation between WR% and *ACTN1* during WS is empowered by an increase of the *EGFR* expression suggests that increased ACTN1 formation is associated with increased ACTN1 phosphorylation in relation to weight regain. Interestingly, among the 15 strong interactions are three involving *ERRFI1*. ERRFI1, also known as MIG-6, is an inhibitor of EFGR by mutual phosphorylation (42). One of the genes that interact with *ERRFI1* is *ITGB4*. From the correlation plot (Figure 3b) it can be seen that only when the fold change of the expression of *ITGB4* decreases, there is an obvious negative correlation between *ERRFI1* and WR%. ITGB4 is able to interact with EGFR in a ligand-independent manner and this complex can trigger focal adhesion kinase (FAK) to activate the AKT signalling pathway (43). FAK is known to be able to phosphorylate alpha-actinin (41). Together these observations indicate that changes in the epidermal growth factor signalling during WS influence the risk of weight regain, which may be mediated by ACTN1 phosphorylation and modified by expression of *ITGB4, CST3* and *LAMA3* (Figure 4).

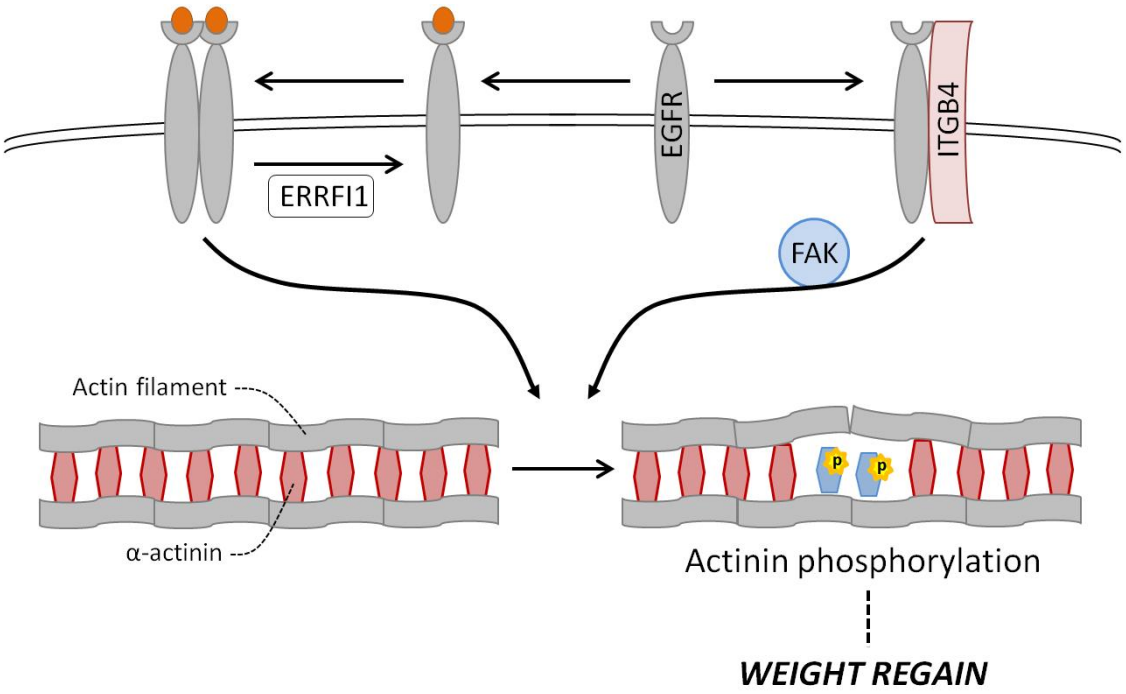

Figure 4: Schematic overview showing that the modification of epidermal growth factor receptor (EGFR) by integrin subunit beta 4 (ITGB4) leads to the phosphorylation of α-actinin by focal adhesion kinase (FAK), which might have an effect on the risk for weight regain.

It should be noted that the present study was done in a relative small cohort. A large validation cohort is therefore preferred to confirm our work. Furthermore, although we have used stringent statistical criteria to try to remove possible false positive results, the analyses were based on univariate analysis on two big gene sets which might create an overfitting problem. Nevertheless, we could generate leads to interesting genes and gene interactions for weight regain, which can now be further explored. We should point out that the results are from adipose tissue biopsies containing both adipocytes as well as stromal vascular cells. Therefore, further experiments are preferably performed with purified adipose tissue cell fractions.

In conclusion, a co-expression network of stress- and ECM-related genes correlating with weight regain percentage could be constructed during the WS phase. The biological processes linked to this network were mainly focussed on leukocyte-activity, ECM remodelling, actin cytoskeleton organisation and glucose handling. Interaction analysis between stress- and ECM-related genes revealed several gene combinations that were highly related to the WR%. In particular, the epidermal growth factor signaling pathway was identified as strongly influencing the risk of weight regain, possibly through interaction with ACTN1, a component of stress fibers, and with ITGB4, CTS3 and LAMA3.

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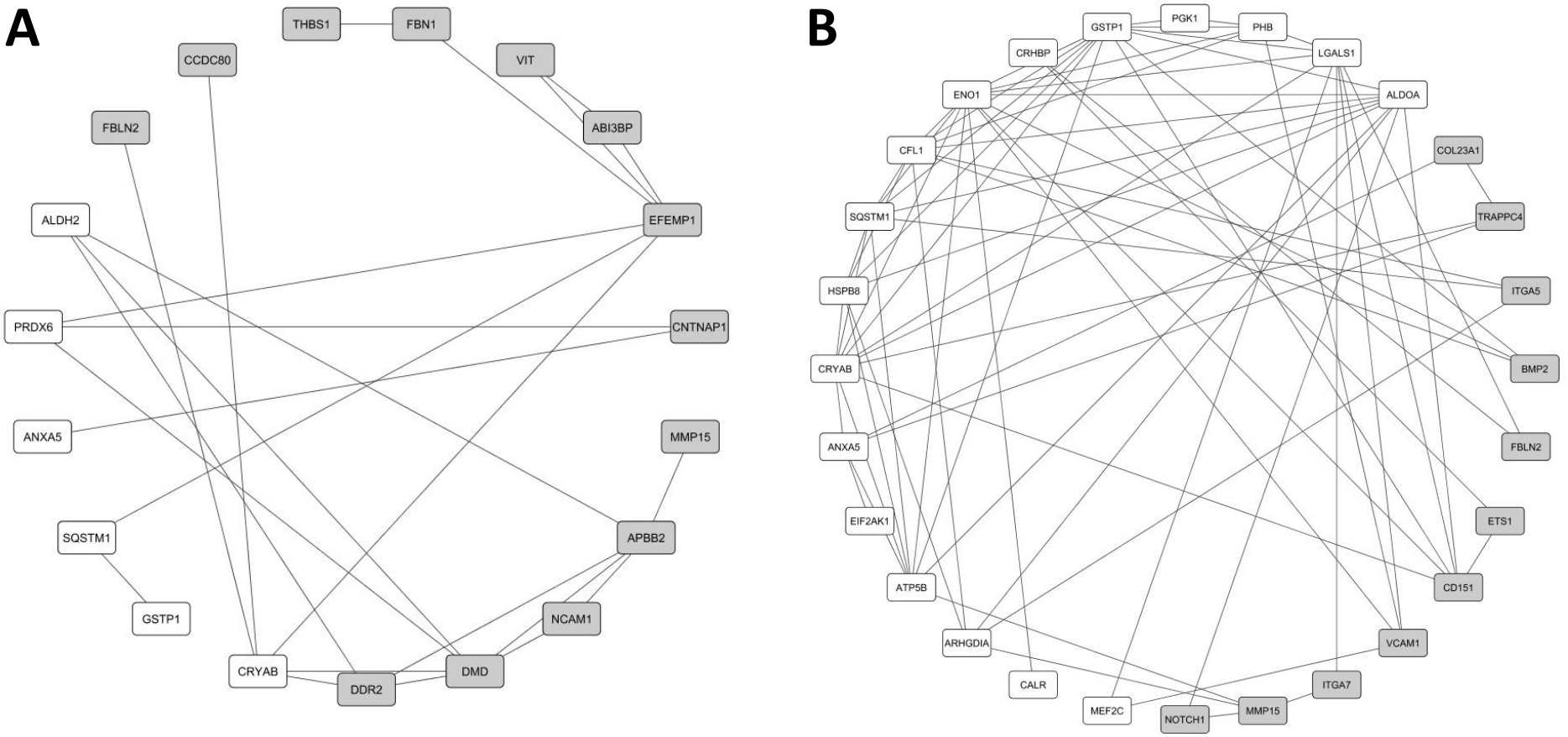
Supplementary Figures and Tables



Supplement Figure 1: Pearson correlation networks between stress- and ECM-related ($|r| \geq 0.600$; $P \leq 0.001$) genes, which correlated with the weight regain percentage ($P \leq 0.05$), during weight loss (A) and dietary intervention (B). White boxes, stress-related genes; grey boxes, ECM-related genes.

Supplement Table 1: List of the 107 stress-related genes and the 277 extracellular matrix related genes used for the analysis.

Gene ID	Gene name	Gene #	Group
ACTB	actin, beta	60	Stress
ACTG1	actin gamma 1	71	Stress
AHSA2	AHA1, activator of heat shock 90kDa protein ATPase homolog 2 (yeast)	130872	Stress
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	231	Stress
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	217	Stress
ALDOA	aldolase A, fructose-bisphosphate	226	Stress
ANXA2	annexin A2	302	Stress
ANXA5	annexin A5	308	Stress
AQP1	aquaporin 1 (Colton blood group)	358	Stress
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	396	Stress
ATF3	activating transcription factor 3	467	Stress
ATF4	activating transcription factor 4	468	Stress
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	493	Stress
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	506	Stress
BCLAF1	BCL2-associated transcription factor 1	9774	Stress
C5	complement component 5	727	Stress
CALR	Calreticulin	811	Stress
CFL1	cofilin 1 (non-muscle)	1072	Stress
CHORDC1	cysteine and histidine-rich domain (CHORD) containing 1	26973	Stress
CLIC1	chloride intracellular channel 1	1192	Stress
CRHBP	corticotropin releasing hormone binding protein	1393	Stress
CRYAB	crystallin, alpha B	1410	Stress
CTSD	cathepsin D	1509	Stress
DDIT3	DNA-damage-inducible transcript 3	1649	Stress
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	5611	Stress
DUSP10	dual specificity phosphatase 10	11221	Stress
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	9695	Stress
EGFR	epidermal growth factor receptor	1956	Stress
EIF2AK1	eukaryotic translation initiation factor 2-alpha kinase 1	27102	Stress
EIF2B3	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	8891	Stress
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	1965	Stress
EIF5A	eukaryotic translation initiation factor 5A	1984	Stress
ENO1	enolase 1, (alpha)	2023	Stress
ERRFI1	ERBB receptor feedback inhibitor 1	54206	Stress
GADD45B	growth arrest and DNA-damage-inducible, beta	4616	Stress
GADD45G	growth arrest and DNA-damage-inducible, gamma	10912	Stress
GATA4	GATA binding protein 4	2626	Stress
GATA6	GATA binding protein 6	2627	Stress
GSTP1	glutathione S-transferase pi 1	2950	Stress
HEY2	hes-related family bHLH transcription factor with YRPW motif 2	23493	Stress
HILPDA	hypoxia inducible lipid droplet-associated	29923	Stress
HSF2	heat shock transcription factor 2	3298	Stress
HSF4	heat shock transcription factor 4	3299	Stress
HSP90AB4P	heat shock protein 90kDa alpha (cytosolic), class B member 4, pseudogene	664618	Stress
HSP90B2P	heat shock protein 90kDa beta (Grp94), member 2, pseudogene	7190	Stress
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	3309	Stress
HSPA8	heat shock 70kDa protein 8	3312	Stress
HSPB1	heat shock 27kDa protein 1	3315	Stress
HSPB11	heat shock protein family B (small), member 11	51668	Stress
HSPB6	heat shock protein, alpha-crystallin-related, B6	126393	Stress
HSPB8	heat shock 22kDa protein 8	26353	Stress
HSPD1	heat shock 60kDa protein 1 (chaperonin)	3329	Stress
HYOU1	hypoxia up-regulated 1	10525	Stress
IDO1	indoleamine 2,3-dioxygenase 1	3620	Stress
KLF15	Kruppel-like factor 15	28999	Stress
KLF2	Kruppel-like factor 2	10365	Stress
KRT8	keratin 8, type II	3856	Stress
LGALS1	lectin, galactoside-binding, soluble, 1	3956	Stress
LMNA	lamin A/C	4000	Stress
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1	11184	Stress
MAPK11	mitogen-activated protein kinase 11	5600	Stress
MAPK13	mitogen-activated protein kinase 13	5603	Stress
MAPK7	mitogen-activated protein kinase 7	5598	Stress
MAPK8	mitogen-activated protein kinase 8	5599	Stress
MAPK9	mitogen-activated protein kinase 9	5601	Stress
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3	7867	Stress
MEF2C	myocyte enhancer factor 2C	4208	Stress

MUC1	mucin 1, cell surface associated	4582	Stress
NDRG4	NDRG family member 4	65009	Stress
NME2	NME/NM23 nucleoside diphosphate kinase 2	4831	Stress
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	4869	Stress
OMA1	OMA1 zinc metallopeptidase	115209	Stress
P4HB	prolyl 4-hydroxylase, beta polypeptide	5034	Stress
PGK1	phosphoglycerate kinase 1	5230	Stress
PHB	Prohibitin	5245	Stress
PKM	pyruvate kinase, muscle	5315	Stress
PPIA	peptidylprolyl isomerase A (cyclophilin A)	5478	Stress
PRDX6	peroxiredoxin 6	9588	Stress
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	5563	Stress
PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	5720	Stress
PTEN	phosphatase and tensin homolog	5728	Stress
PTK2B	protein tyrosine kinase 2 beta	2185	Stress
RGS14	regulator of G-protein signaling 14	10636	Stress
RPS6KA1	ribosomal protein S6 kinase, 90kDa, polypeptide 1	6195	Stress
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	6197	Stress
RTN3	reticulon 3	10313	Stress
SGK1	serum/glucocorticoid regulated kinase 1	6446	Stress
SNN	Stannin	8303	Stress
SQSTM1	sequestosome 1	8878	Stress
STC2	stanniocalcin 2	8614	Stress
STIP1	stress-induced phosphoprotein 1	10963	Stress
STK39	serine threonine kinase 39	27347	Stress
STMN1	stathmin 1	3925	Stress
TAOK2	TAO kinase 2	9344	Stress
TCAP	titin-cap	8557	Stress
TENM1	teneurin transmembrane protein 1	10178	Stress
TMEM204	transmembrane protein 204	79652	Stress
TP53I11	tumor protein p53 inducible protein 11	9537	Stress
TP53INP1	tumor protein p53 inducible nuclear protein 1	94241	Stress
TP53TG1	TP53 target 1 (non-protein coding)	11257	Stress
TPI1	triosephosphate isomerase 1	7167	Stress
TPM3	tropomyosin 3	7170	Stress
TRAF2	TNF receptor-associated factor 2	7186	Stress
TUBB	tubulin, beta class I	203068	Stress
UCN2	urocortin 2	90226	Stress
ZAK	sterile alpha motif and leucine zipper containing kinase AZK	51776	Stress
A2M	alpha-2-macroglobulin	2	ECM
ABI3BP	ABI family, member 3 (NESH) binding protein	25890	ECM
ACTN1	actinin, alpha 1	87	ECM
ADAM10	ADAM metallopeptidase domain 10	102	ECM
ADAM11	ADAM metallopeptidase domain 11	4185	ECM
ADAM12	ADAM metallopeptidase domain 12	8038	ECM
ADAM15	ADAM metallopeptidase domain 15	8751	ECM
ADAM17	ADAM metallopeptidase domain 17	6868	ECM
ADAM19	ADAM metallopeptidase domain 19	8728	ECM
ADAM22	ADAM metallopeptidase domain 22	53616	ECM
ADAM8	ADAM metallopeptidase domain 8	101	ECM
ADAM9	ADAM metallopeptidase domain 9	8754	ECM
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	9510	ECM
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif, 14	140766	ECM
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	9509	ECM
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	9507	ECM
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	11096	ECM
ADAMTSL4	ADAMTS-like 4	54507	ECM
AGRN	Agrin	375790	ECM
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	183	ECM
APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2	323	ECM
AZGP1	alpha-2-glycoprotein 1, zinc-binding	563	ECM
BCL3	B-cell CLL/lymphoma 3	602	ECM
BGN	Biglycan	633	ECM
BMP1	bone morphogenetic protein 1	649	ECM
BMP2	bone morphogenetic protein 2	650	ECM
BMP4	bone morphogenetic protein 4	652	ECM
BSG	basigin (Ok blood group)	682	ECM
CAPN1	calpain 1, (mu/I) large subunit	823	ECM
CASP3	caspase 3, apoptosis-related cysteine peptidase	836	ECM
CCDC80	coiled-coil domain containing 80	151887	ECM
CD151	CD151 molecule (Raph blood group)	977	ECM

CD44	CD44 molecule (Indian blood group)	960	ECM
CD47	CD47 molecule	961	ECM
CER1	cerberus 1, DAN family BMP antagonist	9350	ECM
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	1116	ECM
CIB1	calcium and integrin binding 1 (calmyrin)	10519	ECM
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	8483	ECM
CMA1	chymase 1, mast cell	1215	ECM
CNTNAP1	contactin associated protein 1	8506	ECM
COL11A1	collagen, type XI, alpha 1	1301	ECM
COL12A1	collagen, type XII, alpha 1	1303	ECM
COL13A1	collagen, type XIII, alpha 1	1305	ECM
COL14A1	collagen, type XIV, alpha 1	7373	ECM
COL15A1	collagen, type XV, alpha 1	1306	ECM
COL16A1	collagen, type XVI, alpha 1	1307	ECM
COL17A1	collagen, type XVII, alpha 1	1308	ECM
COL18A1	collagen, type XVIII, alpha 1	80781	ECM
COL1A1	collagen, type I, alpha 1	1277	ECM
COL1A2	collagen, type I, alpha 2	1278	ECM
COL20A1	collagen, type XX, alpha 1	57642	ECM
COL21A1	collagen, type XXI, alpha 1	81578	ECM
COL23A1	collagen, type XXIII, alpha 1	91522	ECM
COL25A1	collagen, type XXV, alpha 1	84570	ECM
COL26A1	collagen, type XXVI, alpha 1	136227	ECM
COL27A1	collagen, type XXVII, alpha 1	85301	ECM
COL2A1	collagen, type II, alpha 1	1280	ECM
COL3A1	collagen, type III, alpha 1	1281	ECM
COL4A1	collagen, type IV, alpha 1	1282	ECM
COL4A2	collagen, type IV, alpha 2	1284	ECM
COL4A5	collagen, type IV, alpha 5	1287	ECM
COL5A1	collagen, type V, alpha 1	1289	ECM
COL5A2	collagen, type V, alpha 2	1290	ECM
COL5A3	collagen, type V, alpha 3	50509	ECM
COL6A1	collagen, type VI, alpha 1	1291	ECM
COL6A2	collagen, type VI, alpha 2	1292	ECM
COL6A3	collagen, type VI, alpha 3	1293	ECM
COL6A6	collagen, type VI, alpha 6	131873	ECM
COL8A1	collagen, type VIII, alpha 1	1295	ECM
COL8A2	collagen, type VIII, alpha 2	1296	ECM
COL9A2	collagen, type IX, alpha 2	1298	ECM
COL9A3	collagen, type IX, alpha 3	1299	ECM
COLGALT1	collagen beta(1-O)galactosyltransferase 1	79709	ECM
COMP	cartilage oligomeric matrix protein	1311	ECM
CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	83716	ECM
CRTAP	cartilage associated protein	10491	ECM
CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	55790	ECM
CSPG4	chondroitin sulfate proteoglycan 4	1464	ECM
CST3	cystatin C	1471	ECM
CTGF	connective tissue growth factor	1490	ECM
CTRB1	chymotrypsinogen B1	1504	ECM
CTRB2	chymotrypsinogen B2	440387	ECM
CTSB	cathepsin B	1508	ECM
CTSG	cathepsin G	1511	ECM
CTSK	cathepsin K	1513	ECM
CTSL	cathepsin L	1514	ECM
CTSS	cathepsin S	1520	ECM
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1545	ECM
CYR61	cysteine-rich, angiogenic inducer, 61	3491	ECM
DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	1605	ECM
DCN	Decorin	1634	ECM
DDR1	discoidin domain receptor tyrosine kinase 1	780	ECM
DDR2	discoidin domain receptor tyrosine kinase 2	4921	ECM
DMD	Dystrophin	1756	ECM
DPP4	dipeptidyl-peptidase 4	1803	ECM
DPT	Dermatopontin	1805	ECM
ECM1	extracellular matrix protein 1	1893	ECM
ECM2	extracellular matrix protein 2, female organ and adipocyte specific	1842	ECM
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	2202	ECM
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	30008	ECM
EGFL6	EGF-like-domain, multiple 6	25975	ECM
EGFLAM	EGF-like, fibronectin type III and laminin G domains	133584	ECM
ELANE	elastase, neutrophil expressed	1991	ECM

ELN	Elastin	2006	ECM
EMILIN1	elastin microfibril interfacer 1	11117	ECM
EMILIN2	elastin microfibril interfacer 2	84034	ECM
ENG	Endoglin	2022	ECM
ERCC2	excision repair cross-complementation group 2	2068	ECM
ERO1L	ERO1-like (S. cerevisiae)	30001	ECM
ERO1LB	ERO1-like beta (S. cerevisiae)	56605	ECM
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	2113	ECM
F11R	F11 receptor	50848	ECM
FAP	fibroblast activation protein, alpha	2191	ECM
FBLN1	fibulin 1	2192	ECM
FBLN2	fibulin 2	2199	ECM
FBLN5	fibulin 5	10516	ECM
FBN1	fibrillin 1	2200	ECM
FER	fer (fps/fes related) tyrosine kinase	2241	ECM
FGF2	fibroblast growth factor 2 (basic)	2247	ECM
FKBP9	FK506 binding protein 9, 63 kDa	360132	ECM
FMOD	Fibromodulin	2331	ECM
FN1	fibronectin 1	2335	ECM
FURIN	furin (paired basic amino acid cleaving enzyme)	5045	ECM
FZD4	frizzled class receptor 4	8322	ECM
GAS6	growth arrest-specific 6	2621	ECM
GFAP	glial fibrillary acidic protein	2670	ECM
GFOD2	glucose-fructose oxidoreductase domain containing 2	81577	ECM
GPC1	glypican 1	2817	ECM
GPM6B	glycoprotein M6B	2824	ECM
HAS2	hyaluronan synthase 2	3037	ECM
HAS3	hyaluronan synthase 3	3038	ECM
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	51144	ECM
HSPG2	heparan sulfate proteoglycan 2	3339	ECM
ICAM1	intercellular adhesion molecule 1	3383	ECM
ICAM2	intercellular adhesion molecule 2	3384	ECM
ICAM3	intercellular adhesion molecule 3	3385	ECM
ITGA1	integrin, alpha 1	3672	ECM
ITGA11	integrin, alpha 11	22801	ECM
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	3673	ECM
ITGA2B	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	3674	ECM
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	3675	ECM
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	3676	ECM
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	3678	ECM
ITGA6	integrin, alpha 6	3655	ECM
ITGA7	integrin, alpha 7	3679	ECM
ITGA8	integrin, alpha 8	8516	ECM
ITGA9	integrin, alpha 9	3680	ECM
ITGAD	integrin, alpha D	3681	ECM
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	3682	ECM
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	3683	ECM
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	3684	ECM
ITGAV	integrin, alpha V	3685	ECM
ITGAX	integrin, alpha X (complement component 3 receptor 4 subunit)	3687	ECM
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	3689	ECM
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	3690	ECM
ITGB4	integrin, beta 4	3691	ECM
ITGB5	integrin, beta 5	3693	ECM
ITGB7	integrin, beta 7	3695	ECM
JAM2	junctional adhesion molecule 2	58494	ECM
JAM3	junctional adhesion molecule 3	83700	ECM
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	81621	ECM
KDR	kinase insert domain receptor	3791	ECM
KIF9	kinesin family member 9	64147	ECM
LAMA2	laminin, alpha 2	3908	ECM
LAMA3	laminin, alpha 3	3909	ECM
LAMA5	laminin, alpha 5	3911	ECM
LAMB1	laminin, beta 1	3912	ECM
LAMB2	laminin, beta 2 (laminin S)	3913	ECM
LAMB3	laminin, beta 3	3914	ECM
LAMC1	laminin, gamma 1 (formerly LAMB2)	3915	ECM
LAMC3	laminin, gamma 3	10319	ECM
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	3959	ECM

LOX	lysyl oxidase	4015	ECM
LOXL1	lysyl oxidase-like 1	4016	ECM
LRP4	low density lipoprotein receptor-related protein 4	4038	ECM
LRP5	low density lipoprotein receptor-related protein 5	4041	ECM
LTBP1	latent transforming growth factor beta binding protein 1	4052	ECM
LTBP3	latent transforming growth factor beta binding protein 3	4054	ECM
LTBP4	latent transforming growth factor beta binding protein 4	8425	ECM
LUM	Lumican	4060	ECM
MADCAM1	mucosal vascular addressin cell adhesion molecule 1	8174	ECM
MATN2	matrilin 2	4147	ECM
MATN4	matrilin 4	8785	ECM
MFAP2	microfibrillar-associated protein 2	4237	ECM
MFAP3	microfibrillar-associated protein 3	4238	ECM
MFAP4	microfibrillar-associated protein 4	4239	ECM
MFAP5	microfibrillar associated protein 5	8076	ECM
MFI2	antigen p97 (melanoma associated)	4241	ECM
MMP14	matrix metallopeptidase 14 (membrane-inserted)	4323	ECM
MMP15	matrix metallopeptidase 15 (membrane-inserted)	4324	ECM
MMP19	matrix metallopeptidase 19	4327	ECM
MMP2	matrix metallopeptidase 2	4313	ECM
MMP7	matrix metallopeptidase 7	4316	ECM
MMP9	matrix metallopeptidase 9	4318	ECM
MPZL3	myelin protein zero-like 3	196264	ECM
NBL1	neuroblastoma 1, DAN family BMP antagonist	4681	ECM
NCAM1	neural cell adhesion molecule 1	4684	ECM
NCSTN	Nicastrin	23385	ECM
NDNF	neuron-derived neurotrophic factor	79625	ECM
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	4791	ECM
NID1	nidogen 1	4811	ECM
NID2	nidogen 2 (osteonidogen)	22795	ECM
NOTCH1	notch 1	4851	ECM
NPHP3	nephronophthisis 3 (adolescent)	27031	ECM
NPNT	Nephronectin	255743	ECM
NTN4	netrin 4	59277	ECM
OGN	Osteoglycin	4969	ECM
OLFML2A	olfactomedin-like 2A	169611	ECM
OLFML2B	olfactomedin-like 2B	25903	ECM
P3H2	prolyl 3-hydroxylase 2	55214	ECM
P3H3	prolyl 3-hydroxylase 3	10536	ECM
P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	5033	ECM
PCOLCE	procollagen C-endopeptidase enhancer	5118	ECM
PDGFA	platelet-derived growth factor alpha polypeptide	5154	ECM
PDGFB	platelet-derived growth factor beta polypeptide	5155	ECM
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	5156	ECM
PDIA4	protein disulfide isomerase family A, member 4	9601	ECM
PECAM1	platelet/endothelial cell adhesion molecule 1	5175	ECM
PLEC	Plectin	5339	ECM
PLOD1	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	5351	ECM
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	5352	ECM
POSTN	periostin, osteoblast specific factor	10631	ECM
PPIB	peptidylprolyl isomerase B (cyclophilin B)	5479	ECM
PRDX4	peroxiredoxin 4	10549	ECM
PRELP	proline/arginine-rich end leucine-rich repeat protein	5549	ECM
PRG4	proteoglycan 4	10216	ECM
PRKCA	protein kinase C, alpha	5578	ECM
PSEN1	presenilin 1	5663	ECM
PTPRS	protein tyrosine phosphatase, receptor type, S	5802	ECM
PXDN	Peroxidasin	7837	ECM
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	8434	ECM
RGCC	regulator of cell cycle	28984	ECM
SDC1	syndecan 1	6382	ECM
SDC3	syndecan 3	9672	ECM
SDC4	syndecan 4	6385	ECM
SERAC1	serine active site containing 1	84947	ECM
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	5054	ECM
SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1	871	ECM
SH3PXD2B	SH3 and PX domains 2B	285590	ECM
SMOC1	SPARC related modular calcium binding 1	64093	ECM
SMOC2	SPARC related modular calcium binding 2	64094	ECM
SOX9	SRY (sex determining region Y)-box 9	6662	ECM

SPARCL1	SPARC-like 1 (hevin)	8404	ECM
SPINT1	serine peptidase inhibitor, Kunitz type 1	6692	ECM
SPOCK2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	9806	ECM
SPON1	spondin 1, extracellular matrix protein	10418	ECM
SPON2	spondin 2, extracellular matrix protein	10417	ECM
SPP1	secreted phosphoprotein 1	6696	ECM
TCF15	transcription factor 15 (basic helix-loop-helix)	6939	ECM
TGFB1	transforming growth factor, beta 1	7040	ECM
TGFB2	transforming growth factor, beta 2	7042	ECM
TGFB3	transforming growth factor, beta 3	7043	ECM
TGFBI	transforming growth factor, beta-induced, 68kDa	7045	ECM
TGFBR3	transforming growth factor, beta receptor III	7049	ECM
THBS1	thrombospondin 1	7057	ECM
THBS2	thrombospondin 2	7058	ECM
THBS3	thrombospondin 3	7059	ECM
TIMP1	TIMP metallopeptidase inhibitor 1	7076	ECM
TIMP2	TIMP metallopeptidase inhibitor 2	7077	ECM
TIMP4	TIMP metallopeptidase inhibitor 4	7079	ECM
TLL1	tolloid-like 1	7092	ECM
TNC	tenascin C	3371	ECM
TNF	tumor necrosis factor	7124	ECM
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	4982	ECM
TNN	tenascin N	63923	ECM
TNXB	tenascin XB	7148	ECM
TRAPPC4	trafficking protein particle complex 4	51399	ECM
VCAM1	vascular cell adhesion molecule 1	7412	ECM
VCAN	Versican	1462	ECM
VIT	Vitrin	5212	ECM
VWA1	von Willebrand factor A domain containing 1	64856	ECM
VWF	von Willebrand factor	7450	ECM

Supplement Table 2: Correlations between stress- and ECM-related genes, which correlated with the weight regain percentage, during the WL, WS and DI period.

Stress genes	ECM genes	*R*	P-value	Period
ALDH2	APBB2	0.738	<0.001	WL
ALDH2	DMD	0.686	<0.001	WL
ALDH2	DDR2	0.789	<0.001	WL
ANXA5	CNTNAP1	-0.682	<0.001	WL
CRYAB	DMD	0.728	<0.001	WL
CRYAB	FBLN2	0.601	0.003	WL
CRYAB	EFEMP1	0.616	0.002	WL
CRYAB	DDR2	0.728	<0.001	WL
CRYAB	CCDC80	0.601	0.002	WL
PGK1	BMP2	-0.641	0.001	WL
PRDX6	DMD	0.643	0.001	WL
PRDX6	EFEMP1	0.623	0.001	WL
PRDX6	CNTNAP1	-0.624	0.001	WL
PTK2B	ITGB7	0.616	0.002	WL
PTK2B	ADAM19	0.657	0.001	WL
RGS14	ITGB7	0.617	0.002	WL
RGS14	ADAM19	0.763	<0.001	WL
SQSTM1	EFEMP1	0.603	0.002	WL
ACTB	ACTN1	0.729	<0.001	WS
ACTB	CAPN1	0.67	<0.001	WS
ACTB	DMD	-0.617	0.001	WS
ACTB	ITGA3	0.768	<0.001	WS
ACTB	ITGA5	0.726	<0.001	WS
ACTB	PDIA4	0.664	<0.001	WS
ACTB	COLGALT1	0.64	0.001	WS
AKR1B1	CAPN1	0.767	<0.001	WS
AKR1B1	CTSB	0.708	<0.001	WS
AKR1B1	ITGA3	0.753	<0.001	WS
AKR1B1	ITGAX	0.698	<0.001	WS
AKR1B1	PLOD1	0.715	<0.001	WS
AKR1B1	PPIB	0.694	<0.001	WS
AKR1B1	SPINT1	0.741	<0.001	WS
AKR1B1	PDIA4	0.731	<0.001	WS
AKR1B1	NCSTN	0.611	0.002	WS
AKR1B1	COLGALT1	0.635	0.001	WS
AKR1B1	EMILIN2	0.714	<0.001	WS
ALDOA	ACTN1	0.602	0.002	WS
ALDOA	CTSB	0.728	<0.001	WS
ALDOA	ITGB5	0.692	<0.001	WS
ALDOA	PLOD1	0.732	<0.001	WS
ALDOA	PPIB	0.67	<0.001	WS
ALDOA	SPARCL1	-0.779	<0.001	WS
ALDOA	NCSTN	0.702	<0.001	WS
ALDOA	COLGALT1	0.602	0.002	WS
ALDOA	EMILIN2	0.628	0.001	WS
ARHGDIA	ACTN1	0.634	0.001	WS
ARHGDIA	CAPN1	0.616	0.001	WS
ARHGDIA	CTSB	0.707	<0.001	WS
ARHGDIA	ITGA3	0.625	0.001	WS
ARHGDIA	ITGB5	0.681	<0.001	WS
ARHGDIA	PLOD1	0.668	<0.001	WS
ARHGDIA	PPIB	0.606	0.002	WS
ARHGDIA	SPINT1	0.622	0.001	WS
ARHGDIA	SPARCL1	-0.797	<0.001	WS
ARHGDIA	NCSTN	0.601	0.002	WS
ARHGDIA	EMILIN2	0.64	0.001	WS
BCLAF1	VIT	0.687	<0.001	WS
C5	VIT	0.722	<0.001	WS
CALR	ACTN1	0.646	0.001	WS
CALR	CAPN1	0.808	<0.001	WS
CALR	CTSB	0.814	<0.001	WS
CALR	DMD	-0.674	<0.001	WS
CALR	ECM2	-0.625	0.001	WS
CALR	ITGA3	0.783	<0.001	WS
CALR	ITGAM	0.641	0.001	WS
CALR	ITGAX	0.601	0.002	WS
CALR	ITGB5	0.714	<0.001	WS

CALR	PLOD1	0.856	<0.001	WS
CALR	PPIB	0.832	<0.001	WS
CALR	SPINT1	0.71	<0.001	WS
CALR	SPARCL1	-0.611	0.002	WS
CALR	PDIA4	0.872	<0.001	WS
CALR	NCSTN	0.706	<0.001	WS
CALR	COLGALT1	0.745	<0.001	WS
CALR	EMILIN2	0.82	<0.001	WS
CFL1	ACTN1	0.781	<0.001	WS
CFL1	CAPN1	0.808	<0.001	WS
CFL1	CTSB	0.861	<0.001	WS
CFL1	CTSS	0.728	<0.001	WS
CFL1	DMD	-0.679	<0.001	WS
CFL1	ECM2	-0.726	<0.001	WS
CFL1	ITGA3	0.859	<0.001	WS
CFL1	ITGA5	0.657	<0.001	WS
CFL1	ITGAM	0.77	<0.001	WS
CFL1	ITGAX	0.679	<0.001	WS
CFL1	ITGB2	0.653	0.001	WS
CFL1	ITGB5	0.703	<0.001	WS
CFL1	PLOD1	0.863	<0.001	WS
CFL1	PLOD2	-0.622	0.001	WS
CFL1	PPIB	0.842	<0.001	WS
CFL1	SPINT1	0.633	0.001	WS
CFL1	SPARCL1	-0.738	<0.001	WS
CFL1	PDIA4	0.746	<0.001	WS
CFL1	NCSTN	0.818	<0.001	WS
CFL1	COLGALT1	0.807	<0.001	WS
CFL1	EMILIN2	0.778	<0.001	WS
CLIC1	CTSB	0.703	<0.001	WS
CLIC1	DMD	-0.621	0.001	WS
CLIC1	ITGA3	0.608	0.002	WS
CLIC1	ITGAM	0.605	0.002	WS
CLIC1	ITGAX	0.622	0.001	WS
CLIC1	ITGB5	0.71	<0.001	WS
CLIC1	PPIB	0.648	0.001	WS
CLIC1	PDIA4	0.679	<0.001	WS
CLIC1	EMILIN2	0.766	<0.001	WS
ENO1	ACTN1	0.709	<0.001	WS
ENO1	CAPN1	0.635	0.001	WS
ENO1	CTSB	0.73	<0.001	WS
ENO1	ITGA3	0.663	<0.001	WS
ENO1	ITGA5	0.69	<0.001	WS
ENO1	ITGB5	0.713	<0.001	WS
ENO1	PLOD1	0.728	<0.001	WS
ENO1	PPIB	0.728	<0.001	WS
ENO1	SPARCL1	-0.713	<0.001	WS
ENO1	PDIA4	0.673	<0.001	WS
ENO1	NCSTN	0.772	<0.001	WS
ENO1	COLGALT1	0.718	<0.001	WS
ENO1	EMILIN2	0.67	<0.001	WS
HSPA5	ACTN1	0.671	<0.001	WS
HSPA5	CTSB	0.609	0.002	WS
HSPA5	DMD	-0.632	0.001	WS
HSPA5	ITGA3	0.726	<0.001	WS
HSPA5	PDIA4	0.632	0.001	WS
HSPA5	COLGALT1	0.609	0.002	WS
HSPA8	ACTN1	0.723	<0.001	WS
HSPA8	PPIB	0.673	<0.001	WS
HSPB1	ACTN1	0.744	<0.001	WS
HSPB1	ITGA5	0.618	0.001	WS
HYOU1	ACTN1	0.769	<0.001	WS
HYOU1	CAPN1	0.601	0.002	WS
HYOU1	CTSB	0.629	0.001	WS
HYOU1	GPC1	0.62	0.001	WS
HYOU1	ITGA3	0.777	<0.001	WS
HYOU1	ITGA5	0.747	<0.001	WS
HYOU1	PLOD1	0.756	<0.001	WS
HYOU1	PPIB	0.657	<0.001	WS
HYOU1	PDIA4	0.7	<0.001	WS
HYOU1	NCSTN	0.713	<0.001	WS

HYOU1	COLGALT1	0.742	<0.001	WS
MAPK13	ACTN1	0.72	<0.001	WS
MAPK13	CAPN1	0.766	<0.001	WS
MAPK13	CTSB	0.854	<0.001	WS
MAPK13	CTSS	0.721	<0.001	WS
MAPK13	DMD	-0.682	<0.001	WS
MAPK13	ECM2	-0.658	<0.001	WS
MAPK13	ITGA3	0.781	<0.001	WS
MAPK13	ITGAL	0.602	0.002	WS
MAPK13	ITGAM	0.766	<0.001	WS
MAPK13	ITGAX	0.676	<0.001	WS
MAPK13	PLOD1	0.73	<0.001	WS
MAPK13	PPIB	0.79	<0.001	WS
MAPK13	PDIA4	0.702	<0.001	WS
MAPK13	NCSTN	0.752	<0.001	WS
MAPK13	COLGALT1	0.653	0.001	WS
MAPK13	EMILIN2	0.779	<0.001	WS
MAPK8	CAPN1	-0.602	0.002	WS
MAPK8	ECM2	0.772	<0.001	WS
MAPK8	GPC1	-0.631	0.001	WS
MAPK8	ITGA5	-0.687	<0.001	WS
MAPK8	KDR	0.652	0.001	WS
MAPK8	SPARCL1	0.624	0.001	WS
MAPKAPK3	CAPN1	0.736	<0.001	WS
MAPKAPK3	CTSB	0.617	0.001	WS
MAPKAPK3	CTSS	0.698	<0.001	WS
MAPKAPK3	ECM2	-0.711	<0.001	WS
MAPKAPK3	ITGA3	0.635	0.001	WS
MAPKAPK3	ITGAL	0.614	0.001	WS
MAPKAPK3	ITGAM	0.738	<0.001	WS
MAPKAPK3	ITGAX	0.753	<0.001	WS
MAPKAPK3	ITGB2	0.683	<0.001	WS
MAPKAPK3	KDR	-0.746	<0.001	WS
MAPKAPK3	PPIB	0.662	<0.001	WS
MAPKAPK3	SPINT1	0.678	<0.001	WS
MAPKAPK3	SPARCL1	-0.766	<0.001	WS
MAPKAPK3	NCSTN	0.609	0.002	WS
MAPKAPK3	COLGALT1	0.607	0.002	WS
MAPKAPK3	EMILIN2	0.677	<0.001	WS
MEF2C	ITGB5	-0.65	0.001	WS
MEF2C	KDR	0.687	<0.001	WS
MEF2C	SPARCL1	0.69	<0.001	WS
OMA1	CAPN1	-0.617	0.001	WS
OMA1	ECM2	0.683	<0.001	WS
OMA1	ITGA5	-0.671	<0.001	WS
OMA1	SPARCL1	0.613	0.002	WS
PGK1	ACTN1	0.628	0.001	WS
PGK1	CTSS	0.711	<0.001	WS
PGK1	DMD	-0.627	0.001	WS
PGK1	ITGAL	0.612	0.001	WS
PGK1	ITGAM	0.728	<0.001	WS
PGK1	ITGAX	0.605	0.002	WS
PGK1	ITGB2	0.692	<0.001	WS
PGK1	PLOD1	0.686	<0.001	WS
PGK1	PPIB	0.611	0.002	WS
PGK1	SPARCL1	-0.656	0.001	WS
PKM	CAPN1	0.909	<0.001	WS
PKM	CTSB	0.829	<0.001	WS
PKM	CTSS	0.693	<0.001	WS
PKM	DMD	-0.685	<0.001	WS
PKM	ECM2	-0.692	<0.001	WS
PKM	ITGA3	0.913	<0.001	WS
PKM	ITGA5	0.748	<0.001	WS
PKM	ITGAL	0.66	<0.001	WS
PKM	ITGAM	0.796	<0.001	WS
PKM	ITGAX	0.745	<0.001	WS
PKM	ITGB2	0.678	<0.001	WS
PKM	ITGB5	0.714	<0.001	WS
PKM	PLOD1	0.752	<0.001	WS
PKM	PLOD2	-0.691	<0.001	WS
PKM	PPIB	0.805	<0.001	WS

PKM	SPINT1	0.673	<0.001	WS
PKM	SPARCL1	-0.653	0.001	WS
PKM	PDIA4	0.852	<0.001	WS
PKM	NCSTN	0.718	<0.001	WS
PKM	COLGALT1	0.836	<0.001	WS
PKM	EMILIN2	0.868	<0.001	WS
PTEN	ECM2	0.625	0.001	WS
PTEN	NCAM1	0.606	0.002	WS
PTK2B	CAPN1	0.63	0.001	WS
PTK2B	ECM2	-0.656	<0.001	WS
PTK2B	ITGAL	0.726	<0.001	WS
PTK2B	ITGAM	0.67	<0.001	WS
PTK2B	ITGAX	0.692	<0.001	WS
PTK2B	ITGB2	0.651	0.001	WS
PTK2B	PLOD2	-0.606	0.002	WS
RGS14	ICAM3	0.834	<0.001	WS
RGS14	ITGAL	0.635	0.001	WS
RPS6KA1	CAPN1	0.808	<0.001	WS
RPS6KA1	CTSB	0.685	<0.001	WS
RPS6KA1	CTSS	0.864	<0.001	WS
RPS6KA1	DMD	-0.735	<0.001	WS
RPS6KA1	ECM2	-0.772	<0.001	WS
RPS6KA1	ITGA3	0.736	<0.001	WS
RPS6KA1	ITGA5	0.605	0.002	WS
RPS6KA1	ITGAL	0.823	<0.001	WS
RPS6KA1	ITGAM	0.913	<0.001	WS
RPS6KA1	ITGAX	0.918	<0.001	WS
RPS6KA1	ITGB2	0.892	<0.001	WS
RPS6KA1	PLOD2	-0.685	<0.001	WS
RPS6KA1	PPIB	0.634	0.001	WS
RPS6KA1	SPINT1	0.683	<0.001	WS
RPS6KA1	SPARCL1	-0.662	0.001	WS
RPS6KA1	PDIA4	0.638	0.001	WS
RPS6KA1	COLGALT1	0.607	0.002	WS
RPS6KA1	EMILIN2	0.798	<0.001	WS
ZAK	ACTN1	-0.634	0.001	WS
ZAK	CAPN1	-0.783	<0.001	WS
ZAK	CTSB	-0.621	0.001	WS
ZAK	DMD	0.757	<0.001	WS
ZAK	ECM2	0.72	<0.001	WS
ZAK	ITGA3	-0.765	<0.001	WS
ZAK	ITGA5	-0.667	<0.001	WS
ZAK	ITGAL	-0.643	0.001	WS
ZAK	ITGAM	-0.632	0.001	WS
ZAK	ITGAX	-0.616	0.001	WS
ZAK	ITGB3	-0.626	0.001	WS
ZAK	PLOD2	0.689	<0.001	WS
ZAK	PPIB	-0.641	0.001	WS
ZAK	SPINT1	-0.621	0.001	WS
ZAK	PDIA4	-0.723	<0.001	WS
ZAK	COLGALT1	-0.636	0.001	WS
ZAK	EMILIN2	-0.656	0.001	WS
ALDOA	BMP2	0.641	0.001	DI
ALDOA	BMP2	0.617	0.001	DI
ANXA5	TRAPPC4	0.748	<0.001	DI
ANXA5	COL23A1	-0.617	0.001	DI
ARHGDIA	ITGA5	0.615	0.001	DI
ARHGDIA	MMP15	0.651	<0.001	DI
ATP5B	MMP15	0.631	0.001	DI
CFL1	BMP2	0.663	<0.001	DI
CFL1	ITGA5	0.664	<0.001	DI
CRHBP	ETS1	0.6	0.002	DI
CRHBP	FBLN2	-0.671	<0.001	DI
CRYAB	CD151	0.652	0.001	DI
CRYAB	TRAPPC4	0.641	0.001	DI
ENO1	BMP2	0.624	0.001	DI
ENO1	CD151	0.773	<0.001	DI
ENO1	VCAM1	-0.626	0.001	DI
GSTP1	BMP2	0.613	0.001	DI
GSTP1	CD151	0.705	<0.001	DI
LGALS1	CD151	0.789	<0.001	DI

LGALS1	FBLN2	0.605	0.002	DI
LGALS1	ITGA7	0.635	0.001	DI
LGALS1	VCAM1	-0.666	<0.001	DI
MEF2C	VCAM1	0.609	0.001	DI
PHB	VCAM1	-0.611	0.001	DI
SQSTM1	ITGA5	0.667	<0.001	DI

Pearson correlations ($|r| \geq 0.600$; $P \leq 0.001$) between stress- and ECM-related genes, which correlated with the weight regain percentage ($P \leq 0.05$), during 3 periods. WL, weight loss; WS, weight stable; DI, dietary intervention.

Supplement Table 3: Interactions between stress- and ECM-related genes and the effect on the weight regain percentage.

Stress genes	ECM genes	Interaction P-value	Model r^2	Model P-value	Period
ANXA5	ITGA4	3.23E-04	0.638	3.08E-04	WL
ARHGDIA	ADAM11	7.88E-05	0.593	8.66E-04	WL
ARHGDIA	EGFLAM	4.12E-04	0.530	3.02E-03	WL
BCLAF1	ADAM11	7.68E-05	0.595	8.26E-04	WL
BCLAF1	EGFLAM	6.94E-04	0.509	4.34E-03	WL
DDIT3	COL6A1	3.38E-04	0.523	3.37E-03	WL
DDIT3	ECM1	3.91E-04	0.522	3.44E-03	WL
DDIT3	TIMP2	7.44E-04	0.503	4.81E-03	WL
ENO1	ADAM15	2.90E-04	0.545	2.26E-03	WL
ENO1	EGFLAM	9.68E-04	0.486	6.46E-03	WL
GATA6	COL1A1	5.18E-04	0.517	3.81E-03	WL
GATA6	COL6A3	1.16E-04	0.581	1.12E-03	WL
GATA6	MFI2	3.95E-04	0.545	2.25E-03	WL
GATA6	THBS3	9.58E-04	0.477	7.46E-03	WL
GATA6	MADCAM1	2.91E-04	0.608	6.21E-04	WL
GATA6	CSGALNACT1	5.00E-04	0.510	4.32E-03	WL
GATA6	KAZALD1	3.78E-04	0.519	3.66E-03	WL
GSTP1	ADAMTS4	6.44E-04	0.605	6.59E-04	WL
GSTP1	NPNT	9.80E-05	0.665	1.55E-04	WL
HILPDA	NPNT	2.05E-04	0.551	2.01E-03	WL
HSF2	COL1A1	6.20E-04	0.507	4.49E-03	WL
HSF2	COL6A3	1.77E-04	0.560	1.71E-03	WL
HSF2	TNC	9.11E-04	0.468	8.66E-03	WL
HSF2	ADAM11	5.96E-04	0.499	5.18E-03	WL
HSF2	PDGFA	5.99E-04	0.511	4.19E-03	WL
HSF2	MADCAM1	9.75E-04	0.589	9.42E-04	WL
HSF2	EGFLAM	2.88E-04	0.547	2.18E-03	WL
HSP90AB4P	ADAM11	1.16E-04	0.619	4.82E-04	WL
HSP90AB4P	F11R	4.71E-04	0.552	1.99E-03	WL
HSP90AB4P	COL21A1	4.04E-04	0.567	1.48E-03	WL
HSP90AB4P	ADAMTS14	2.78E-04	0.577	1.20E-03	WL
HSPD1	CTGF	2.58E-04	0.572	1.33E-03	WL
HSPD1	SMOC1	3.09E-04	0.560	1.70E-03	WL
HYOU1	ABI3BP	5.63E-04	0.688	8.34E-05	WL
KLF15	FBLN2	5.41E-04	0.625	6.64E-04	WL
KLF2	ECM1	6.56E-04	0.543	2.33E-03	WL
KLF2	TNXB	1.93E-04	0.621	4.61E-04	WL
LGALS1	ADAMTS4	4.66E-04	0.588	9.61E-04	WL
LGALS1	ADAMTS1	7.29E-04	0.581	1.11E-03	WL
LMNA	ADAM8	2.31E-04	0.597	7.82E-04	WL
LMNA	EGFLAM	8.84E-04	0.502	4.89E-03	WL
MAPK11	FN1	5.75E-04	0.510	4.32E-03	WL
MAPK11	MFI2	3.59E-04	0.544	2.30E-03	WL
MAPK11	SERPINE1	7.19E-04	0.485	6.53E-03	WL
MAPK11	CSGALNACT1	1.14E-04	0.582	1.08E-03	WL
MUC1	ITGA11	5.30E-04	0.532	2.89E-03	WL
PGK1	CTGF	8.01E-04	0.578	1.18E-03	WL
PGK1	TGFBR3	7.44E-04	0.631	3.67E-04	WL
PHB	COL1A1	4.02E-04	0.582	1.09E-03	WL
PHB	ENG	8.18E-04	0.551	2.03E-03	WL
PHB	ADAMTS4	2.74E-05	0.688	8.42E-05	WL
PHB	KAZALD1	9.89E-04	0.549	2.08E-03	WL
PPIA	NPNT	7.67E-04	0.515	3.90E-03	WL
PTEN	ACTN1	9.97E-04	0.497	5.38E-03	WL
PTK2B	ADAMTS1	6.46E-04	0.642	2.82E-04	WL
RGS14	PECAM1	5.03E-04	0.641	2.88E-04	WL
TAOK2	ITGA11	9.53E-04	0.478	7.37E-03	WL
TAOK2	NCSTN	8.86E-04	0.497	5.40E-03	WL
TRAF2	ITGA11	7.07E-04	0.497	5.41E-03	WL
UCN2	CSGALNACT1	9.13E-04	0.481	7.06E-03	WL
ALDH2	MPZL3	6.80E-04	0.510	3.06E-03	WS
ANXA2	VWF	9.81E-04	0.471	6.11E-03	WS
AQP1	CYP1B1	8.99E-04	0.486	4.74E-03	WS
ATF4	COL4A1	4.38E-04	0.490	4.41E-03	WS
ATF4	COL4A2	1.24E-04	0.556	1.25E-03	WS
ATF4	COL15A1	3.70E-05	0.602	4.60E-04	WS
ATF4	HSPG2	4.43E-05	0.598	4.99E-04	WS
ATF4	LAMA5	4.51E-04	0.490	4.45E-03	WS

ATF4	NOTCH1	5.51E-04	0.556	1.24E-03	WS
ATF4	ADAM17	6.03E-04	0.475	5.77E-03	WS
ATF4	VWF	5.48E-04	0.513	2.91E-03	WS
ATF4	ADAM9	2.60E-04	0.516	2.75E-03	WS
ATF4	ERO1L	1.48E-04	0.548	1.48E-03	WS
ATF4	HSD17B12	2.81E-05	0.614	3.44E-04	WS
ATF4	COL21A1	9.11E-05	0.616	3.29E-04	WS
CTSD	ITGA11	7.33E-04	0.515	2.82E-03	WS
EDEM1	CHI3L1	9.45E-04	0.488	4.61E-03	WS
EGFR	ACTN1	7.05E-06	0.811	4.38E-07	WS
EGFR	SMOC1	3.09E-04	0.609	3.89E-04	WS
EIF2B3	ITGB7	8.99E-04	0.538	1.78E-03	WS
EIF5A	EGFLAM	5.49E-04	0.489	4.52E-03	WS
ERRFI1	CST3	1.51E-05	0.654	1.25E-04	WS
ERRFI1	ITGB4	3.93E-05	0.621	2.92E-04	WS
ERRFI1	LAMA3	4.19E-05	0.614	3.46E-04	WS
ERRFI1	TGFB3	8.41E-04	0.488	4.55E-03	WS
ERRFI1	PRDX4	5.82E-04	0.500	3.70E-03	WS
GADD45B	COL21A1	9.58E-04	0.522	2.47E-03	WS
HILPDA	GFOD2	7.80E-04	0.474	5.88E-03	WS
HSF4	SPON2	5.66E-04	0.590	5.94E-04	WS
HSP90AB4P	LAMB2	6.13E-04	0.517	2.71E-03	WS
HSP90AB4P	COL21A1	6.73E-04	0.517	2.68E-03	WS
HSPB11	COL21A1	6.82E-04	0.593	5.65E-04	WS
MAPK7	MPZL3	6.62E-04	0.518	2.65E-03	WS
PGK1	GFOD2	3.98E-04	0.710	2.44E-05	WS
PRDX6	BCL3	9.33E-04	0.497	3.86E-03	WS
SQSTM1	NOTCH1	8.75E-04	0.630	2.34E-04	WS
SQSTM1	P4HA1	2.09E-04	0.560	1.15E-03	WS
SQSTM1	MPZL3	5.43E-04	0.552	1.36E-03	WS
STK39	CHI3L1	8.63E-04	0.495	4.06E-03	WS
STK39	PDGFRA	9.63E-04	0.460	7.36E-03	WS
TENM1	COL21A1	9.84E-04	0.505	3.37E-03	WS
TPI1	SERPINH1	3.42E-04	0.568	9.62E-04	WS
TPI1	COL1A2	6.81E-04	0.525	2.29E-03	WS
TPI1	FBN1	4.57E-04	0.542	1.66E-03	WS
TPI1	NPNT	1.05E-04	0.618	3.10E-04	WS
TUBB	CMA1	6.41E-04	0.527	2.25E-03	WS
TUBB	CCDC80	4.62E-04	0.573	8.65E-04	WS
ACTG1	MMP15	3.40E-04	0.770	1.35E-06	DI
C5	ADAM10	2.47E-04	0.547	1.03E-03	DI
C5	DDR1	2.15E-04	0.555	8.65E-04	DI
C5	CD47	3.07E-06	0.711	1.29E-05	DI
C5	COL6A3	6.37E-04	0.509	2.26E-03	DI
C5	ENG	6.30E-04	0.508	2.30E-03	DI
C5	ICAM1	8.53E-04	0.485	3.56E-03	DI
C5	ITGAD	4.73E-04	0.551	9.44E-04	DI
C5	PDGFRA	8.45E-04	0.535	1.32E-03	DI
C5	ADAM9	2.94E-04	0.602	2.95E-04	DI
C5	ERO1LB	4.75E-05	0.630	1.46E-04	DI
C5	KAZALD1	2.49E-04	0.615	2.13E-04	DI
CLIC1	COL8A2	1.87E-04	0.549	9.84E-04	DI
CLIC1	COL16A1	4.64E-04	0.508	2.27E-03	DI
CLIC1	ELN	6.97E-04	0.489	3.30E-03	DI
CLIC1	CILP	9.28E-04	0.521	1.77E-03	DI
CLIC1	NID2	3.24E-04	0.526	1.60E-03	DI
DUSP10	ELN	5.14E-04	0.562	7.48E-04	DI
EDEM1	CD151	5.63E-04	0.686	5.06E-05	DI
HEY2	VWF	1.35E-04	0.537	1.29E-03	DI
MAPK9	ERO1L	3.43E-04	0.488	3.34E-03	DI
MEF2C	ITGAD	2.75E-04	0.604	2.79E-04	DI
PKM	CILP	5.15E-04	0.578	5.16E-04	DI
PKM	ADAMTS4	8.23E-04	0.522	1.74E-03	DI
PRDX6	TIMP4	7.96E-04	0.607	2.58E-04	DI
RGS14	CSGALNACT1	6.03E-04	0.562	7.48E-04	DI
RPS6KA3	CD47	4.65E-04	0.470	4.62E-03	DI
RPS6KA3	MADCAM1	2.42E-04	0.501	2.63E-03	DI
RPS6KA3	COL5A3	3.23E-05	0.591	3.85E-04	DI
RPS6KA3	CTRB2	8.48E-04	0.448	6.87E-03	DI
RTN3	TIMP4	3.53E-04	0.611	2.33E-04	DI
TPI1	P4HA1	9.36E-04	0.486	3.47E-03	DI

TUBB	FAP	5.91E-04	0.528	1.55E-03	DI
TUBB	TGFB2	4.53E-04	0.521	1.76E-03	DI

Interactions (P≤0.001) between stress- and ECM-related genes. r^2 (≥0.500) and Model P-value (≤0.01) depict the effect of the interaction on the weight regain percentage. WL, weight loss; WS, weight stable; DI, dietary intervention.

Chapter 7

General discussion

Over the years it has become increasingly clear that overweight and obesity is a serious health problem. Weight loss (WL) reduces disease risk and improves the metabolic profile resulting in positive health outcomes (1, 2). Weight loss can be readily achieved and the most common approach to losing weight is dieting, however, long-term weight reduction is proven to be difficult (3, 4). Most people eventually regain the lost weight. Weight regain is one of the most significant obstacles in obesity management, therefore, it is of great importance to understand the underlying mechanism behind weight regain. The involvement of environmental and behavioural factors in the process of weight regain has been established, however, there is now substantial evidence that biological and metabolic mechanisms also contribute to the risk for weight regain (5-7). It was proposed that during a negative energy balance cellular stress arises in adipocytes due to a misfit between cell volume and the surrounding ECM. This stress can be resolved by remodeling of the ECM or by the re-storing of triglycerides within the adipocytes (8). It suggests that cellular stress accumulating in adipocytes during a negative energy balance is a driving force behind the risk for weight regain. For this thesis, studies have been conducted to better understand the underlying mechanisms of weight regain. Specifically, the relation between weight regain and adipocyte ECM- related (chapters 2 and 3) and stress-related (chapter 4 and 5) factors was investigated. In both chapter 3 and 5, we show the importance of a weight stabilisation (WS) period for the risk of weight regain and this will be discussed in the paragraph: “Weight regain: a matter of negative energy balance versus energy balance”. In chapter 2, we observed that genetic variation of ECM-related genes is associated with variation in risk for weight regain. Also, we found that changes in expression of ECM-related genes correlated with weight regain (chapter 3). Therefore, the role of the ECM in weight management will be discussed in the paragraph entitled “Extracellular matrix genetics involved in weight regain”. Furthermore, we observed that resident inflammation in the adipose tissue after weight loss might increase the risk for weight regain (chapter 3). The relation between inflammation and weight regain will be discussed in the paragraph entitled “Weight regain and inflammation”. In chapter 4, we report about the higher cellular stress in the subcutaneous adipose tissue of participants regaining lost weight during the follow-up period compared to the participants maintaining weight loss. Furthermore, we show that the risk for weight regain is related to changes in the expression of stress-related genes during the first four weeks after returning to a weight stable situation (chapter 5). The involvement of stress in weight management will be discussed in the paragraph entitled “Weight regain: a stressful matter”. Interestingly, we saw that the adipocyte size increases during the WS period independent of changes in body weight of the participant which will be further discussed in the paragraph “Adipocyte size increases during WS”.

Weight regain: a matter of negative energy balance versus energy balance

Gene expression is differentially regulated during the calorie restriction phase of a dietary weight loss program. Gene expression changes during calorie restriction represent the effect of WL plus that of a negative energy balance. A negative energy balance cannot be maintained indefinitely, therefore, gene expression patterns should also be studied during energy balance by introducing a weight stabilization phase. Previous studies have shown that gene expression is differentially regulated during the various phases of a dietary weight loss program (9, 10), which should be viewed as biologically distinct. Therefore, determining predictive genes for weight regain should be done during three phases: first, the WL phase which represents the effect of WL plus that of a negative energy balance; second, the WS phase which represents WL without a negative energy balance by

comparing values after WL to values after WS; third, the dietary intervention (DI) phase which is the WL and WS phase together. This phase also represents WL without a negative energy balance and here comparisons are made between study start and after WS. The WL phase in relation to weight regain has been extensively studied. Mutch et al. showed that gene sets related to apoptosis, citric acid cycle, fatty acid metabolism and oxidative phosphorylation are regulated differently for participants regaining weight compared to participants maintaining the lower body weight (11). Another study showed that gene sets related to cellular growth and proliferation, cell death and cellular function are the main biological processes related to regain of weight (12). However, there are fewer studies about weight regain that take into account the effects of the negative energy balance on gene expression results. In our analyses, we observed that correlations between gene expression changes and weight regain are mainly found during the WS phase and not during the WL phase or the DI phase (chapter 3 and 5). These results clearly indicated that the various phases of a dietary weight loss program should be viewed separately, which is in agreement with previous studies (9, 10), and that for weight regain studies the changes during WS are most relevant, i.e. the return from an negative energy balance and not the weight loss per se. Perhaps this is due to the fact that molecular processes during a transition from negative to balanced energy state (WS) are in line with processes following transition from a balanced state to a positive energy balance (WR). To our knowledge there are no other human dietary intervention studies focussed on weight regain that include a WS phase.

Capel et al. used a study design with a VLCD period followed by a LCD phase, then followed by three months weight maintenance (9). They showed distinct patterns of gene regulation during the various phases of a dietary weight loss program, however, they did not relate these changes to weight regain. Future dietary intervention studies, especially studies related to prediction of weight regain, should contain a WS phase to get rid of the influence of the negative energy balance.

Extracellular matrix genetics involved in weight regain

Weight loss becomes more difficult after several weeks in a negative energy balance because other components involved in maintenance of the energy balance will be changed, i.e. the energy expenditure and body energy stores are decreased (13, 14) while hunger is increased (15). For many people it is difficult to sustain weight loss (8) and the question remains what lies at the basis of this difficulty. It has become increasingly clear that the adipose tissue plays an important role in the risk for weight regain. Adipocytes spend a lot of their energy on development, renewal and maintenance of the ECM (16), which is known for providing structural support to the adipocyte. A large part of the energy is used for the rapid turnover of collagens, whereas the turnover of proteins from the cytoskeleton is considerably slower (16). Clearly, there is a role for the ECM in adipocyte biology with reflection on weight regulation. It was shown that ECM regulated processes are disturbed in the obese (17, 18), that ECM-regulating genes are differentially expressed in adipose tissue after WL (19, 20) and that weight gain leads to different expression patterns of ECM-remodelling genes (21). However, the role of the ECM in the process of weight regain has been less studied. When comparing gene expression after WL between participants regaining only 0-10% (weight maintainers) and those who regained 50-100% (weight regainers) of the lost weight it was seen that genes of the focal adhesion pathway, important in the coordination of the ECM, were down-regulated in weight maintainers while up-regulated in weight regainers (11). In our studies, we saw that genetic variations of ECM-related genes are associated with an increased risk for weight regain. Specifically,

we saw the involvement of variants of the *POSTN*, *LAMB1*, *COL23A1*, and *FBLN5* genes for males and of the *FN1* gene for females (chapter 2). Indeed, some of those genes point as well to a role for focal adhesions, i.e. the sites where the ECM is attached to the cytoskeletal actin fibres. During WL, energy stores are mobilized from adipocytes causing a decrease in adipocyte size, which is reversed when sufficient energy is available (22-25). The adipocyte diameter decreases with about 10% during WL (Vink RG, Roumans NJ et al., submitted). As adipocytes change size the ECM must be remodelled to accommodate the change or a considerable mechanical strain will be imposed upon the adipocytes (26). In accord with our findings and those of others, it can be expected that mechanical strain will concentrate on the ECM-attachment sites, i.e. the focal adhesions. These genes may therefore be of interest for future genetic research on weight regulation and obesity.

Besides genes mediating stress at the focal adhesions, also genes for ECM remodelling can be expected to influence the risk for weight regain. Remodelling of the ECM involves the turnover of ECM proteins like collagens. For the construction of new collagen fibres, collagen proteins have to be synthesized and modified, for instance by prolyl-hydroxylase, which is an energy-demanding process directed by insulin (27, 28). However, under conditions of calorie restriction during WL, such energy is not available and insulin levels are low, but when returning to energy balance (WS), such genes may become apparent in gene expression measurements related to weight regain. As described in the paragraph “Weight regain: a matter of negative energy balance versus energy balance”, we observed that changes in expression of ECM-modifying genes are related to weight regain mainly during the WS phase (chapter 3). Examples of such genes are *COLGALT1*, *PLOD1* and *P4HB*. Together, our studies show that the ECM plays a role in the risk for weight regain and suggest that two processes dominate this risk: accommodation of stress at the focal adhesions and ECM remodelling to reduce ECM misfit and cellular stress.

Weight regain and inflammation

The development of obesity is accompanied by the onset of low-grade inflammation. This inflammation is caused by the dysregulated production and release of cytokines and adipokines, such as interleukin-6, tumor necrosis factor and monocyte chemoattractant protein-1. Cells of the innate immune system are attracted into the adipose tissue leading to a situation in which pro-inflammatory immune cells outbalance the anti-inflammatory cells (29). This increased inflammation may play a role in the pathogenesis of insulin resistance and cardiovascular disease (30). Weight loss usually reduces the inflammatory activity of the adipose tissue, which may not be directly obvious during energy restriction. Studies have shown that the expression of genes involved in inflammation and innate immunity was upregulated (9, 31) or unchanged (10) in human adipose tissue directly after short-term weight loss. We observed that expression of these genes was downregulated during the subsequent WS period (31). Considering that whole body inflammation can be dictated by loss of body weight, it is reasonable to suggest that inflammation could influence regain of weight (32). Large weight gain occurs more frequently in participants with elevated levels of four putative markers of inflammation (33). Also, it has been shown that participants with a more advanced pro-inflammatory state at the end of a dietary intervention are more prone to regain weight (34). We observed correlations between expression changes of leukocyte-specific integrins during WS and the weight regain percentage (chapter 3). Those genes are down-regulated during WS and are clustered with genes for ECM remodelling. It is therefore tempting to speculate, that emigration of leukocytes after weight loss reduces the risk for weight regain and that failure to accomplish this by hampered

ECM remodeling leads more often to weight regain. Although we have not investigated this in detail here, our results point to an interaction between the change of inflammatory status during weight loss and shortly after, and weight regain.

Weight regain: a stressful matter

After WL and returning to energy balance, the expression patterns of proteins belonging to the stress proteome are changed (35-37). The easiest way for adipocytes to get rid of this cellular stress is by returning to their original volume by re-storing fat (38). For the host this would mean that after WL there is increased risk for weight regain originating from the cellular stress of the adipocytes. In line, we observed higher levels of stress-related factors after WL for participants regaining weight (chapter 4). As shown, in parallel with changes in proteins of the stress proteome adipocytes prepare for renewed storage of fat (35). Bouwman et al. showed that the translocation of GLUT4 is enhanced after returning to energy balance resulting in increased capacity for the uptake of glucose (35). In addition, a specific role was attributed to aldolase as an anchoring protein for GLUT4-vesicles onto the actin filaments. Similar to the ECM-related genes we saw that changes in expression of stress-related genes during WL are less related to weight regain while the opposite is observed for the WS phase (chapter 5). Accordingly, we showed that the stress-related genes correlating with weight regain during WS are involved in actin filament dynamics, glucose handling and nutrient sensing (chapter 5). It is tempting to speculate that cellular stress accumulating in the adipocytes during weight loss and during weight stabilisation, induces metabolic changes towards increased uptake of energy, which would increase the risk for weight regain by the host.

Another way in which cellular stress might increase food intake and weight regain would be by the secretion of signals known as adipokines which can affect appetite, satiety and energy expenditure (39, 40). The secretion pattern of adipokines is related to the size of an adipocyte (41).Thus WL, which leads to a decreased adipocyte size, will also lead to a different adipokine secretion profile. These changes in secretion profile could increase food intake by stimulating the hunger feeling leading to the increased uptake and storage of energy. The satiety hormone leptin is a classic example of an adipokine that decreases dramatically during weight loss. In our studies, a relation between weight regain and adipokines after returning to energy balance has been established (Vink RG, Roumans NJ et al., submitted). However, we have not been able to link the changes in stress gene expression to changes in secretion of adipokines. Additional research is needed to test this. Also, we were able to measure the levels of 10 adipokines only, whereas a broader adipokine profile-based approach might provide more insight in the possible involvement of stress-induced adipokines in weight regain.

Both stress- and ECM-related factors are independently associated with the risk for weight regain, but we also saw that the combination of stress- and ECM-related factors can have a larger effect on weight regain. As shown in chapter 6, the stress gene *EGFR* is an important node in the risk for weight regain. The interaction between this stress gene with the ECM gene *ACTN1* during the WS phase has the strongest effect on the weight regain percentage. Furthermore, *ERRFI1*, which is a negative regulator of *EGFR*, has interactions with the stress genes CST3, ITGB4 and LAMA3. All these interactions had a strong effect on the weight regain percentage. This indicates that the process of weight regain is complex and multiple factors are involved in the underlying process.

Adipocyte size increases during WS

It can be expected that adipocyte volume decreases during WL and that this may lead to cellular stress due to the misfit between cell volume and the surrounding ECM. This stress can be resolved by adjustment of the surrounding ECM or by re-storing fat to return to the original volume (8). The re-storing of fat would lead to regain of lost weight by the host. This suggests that besides ECM and stress, also changes in adipocyte size could mark the risk for weight regain and it is hypothesized that the change in adipocyte size is related to change in body weight. In our study, we also measured adipocyte size and as expected, the diameter decreased with loss of body weight (Figure 1).

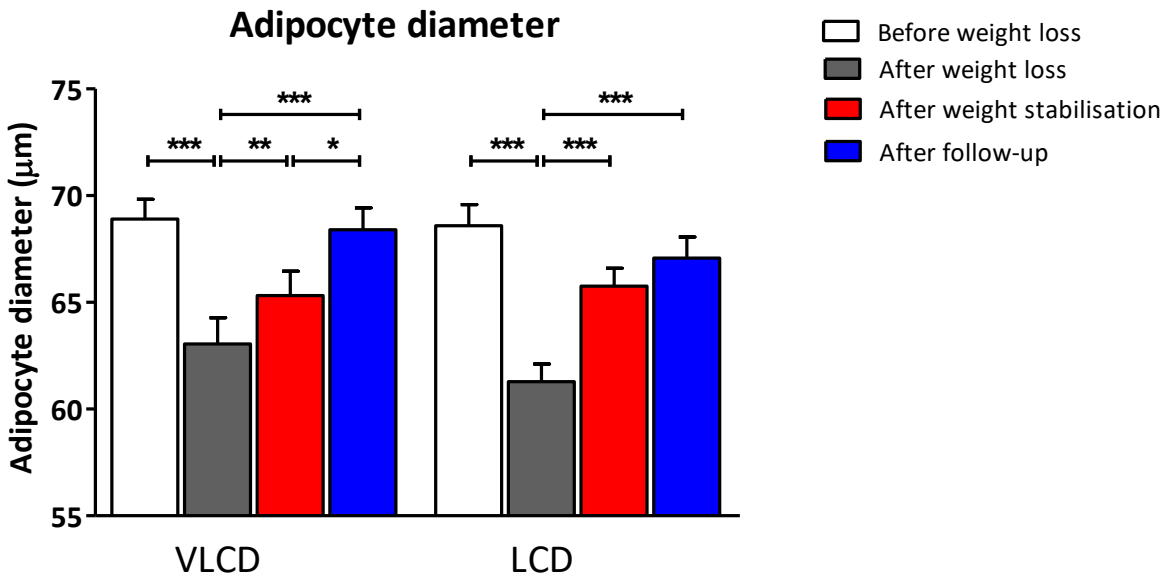


Figure 1: Adipocyte size measured on 8µm paraffin-embedded sections of adipose tissue stained with hematoxylin and eosin. $^{*}P\leq0.05$, $^{**}P<0.01$, $^{***}P<0.001$ with dependent T-test to observed within-group change of adipocyte size.

The adipocyte size was expected not to change during the WS phase because then the diet of each participant is based on the energy requirements of each individual, which is confirmed by the stable body weight of the participants. However, to our surprise, adipocyte size was observed to increase considerably in the WS period for all participants. Based on our model for stress-related energy-uptake, this suggests a strong influx of energy into the adipocytes from the time when the cellular stress is highest, i.e. immediately after WL. The question is, where this energy comes from. We know that adipocytes prepare for glucose uptake and we observed that during WS the gene coding for the enzyme fatty acid synthase, responsible for de novo adipogenesis, is upregulated. However, the diet during WS would not provide enough energy for the adipocytes to refill because this diet is based on the energy requirement of each participant to assure weight maintenance. More likely, fat from other parts of the body, such as the visceral adipose tissue or liver or muscle, is used to refill the stressed adipocytes in the subcutaneous adipose tissue. In this respect, this phenomenon would represent an improvement of metabolic health by moving fat to where it belongs, i.e. the adipose tissue. It should be noted, however, that we have no proof that the increase in adipocyte size is due to the influx of energy. It may well be caused by something else like accumulation of water, or may even be an artifact of tissue preparation due to a more flexible ECM after WS. Additional experiments need to be undertaken to provide clarity in this matter.

Change in adipocyte diameter during WL, WS or DI was not correlated to the weight regain percentage or the change in body weight during follow-up. The same was true for the calculated and corrected adipocyte volume (42). Rapid re-storage of fat would be contradictory to our model for stress-induced weight regain, because it would rapidly diminish the biological drive. However, as mentioned, this process needs further investigation. Anyway, adipocyte size cannot be used as a predictor of weight regain.

Conclusions

This thesis focussed on gaining insight in the process of weight regain after weight loss to determine possible predictors for weight regain. This insight can be used for better guidance in weight control. The major conclusions of this thesis are the following:

- Dietary intervention studies should contain a weight stabilisation phase to monitor the return to energy balance especially when gene expression results are used to determine predictive targets of weight regain.
- Changes in expression of stress- and ECM-related genes including the interaction of these changes are highly related to the weight regain percentage.
- Epidermal growth factor activity strongly influences the correlation of stress and ECM genes with weight regain percentage.
- People who regained most of their weight during follow-up have increased cellular stress levels after an 8-week energy restricted diet.
- The risk for weight regain is related to expression changes of distinct sets of stress-related genes during the first four weeks after returning to energy balance. These genes are involved in actin filament dynamics, glucose handling and nutrient sensing.
- Polymorphisms of the ECM genes COL23A1, FBLN5, LAMB1 and POSTN in men and FN1 in women are associated with weight regain after weight loss pointing to a role for focal adhesions in the risk for weight regain.
- People with a stronger downregulation of ECM-remodelling may retain more (active) immune cells and have higher risk of weight regain suggesting that resident inflammation after weight loss increases the risk for weight regain.
- Changes in adipocyte diameter are not correlated with changes of body weight after weight loss.

Suggestions for future research

Gene expression is differentially regulated during the calorie restriction phase and the weight stabilisation phase of a dietary weight loss program. Correlations between gene expression changes and weight regain are mainly found during the weight stabilisation phase. Therefore, future human dietary intervention studies focussed on predictive gene targets of weight regain should contain a weight stabilisation period. However, the length of the weight stabilisation period for the effects of a negative energy balance to have worn out needs to be determined.

Subcutaneous adipose tissue samples taken were used for gene expression analyses. These biopsies contain adipocytes as well as stromal vascular cells, which can obscure the exact contribution of each

cell type in the tissue to the biological processes involved here. Therefore, it would be of great interest to investigate separately purified adipose tissue cell fractions.

The adipocyte diameter increases during the WS phase while the body weight of the participants remained stable. It suggests that fat from other parts of the body is used to refill the adipocyte thereby reducing the stressed created by the misfit between the ECM and the lower adipocyte volume. However, our study has not looked into changes in other fat depots. Further analyses are necessary to confirm these novel findings and unravel the underlying process. For this, the adipocyte size should be monitored in both subcutaneous and visceral adipose tissue during weight loss and a subsequent weight stabilisation period. Reallocation of fat might be assessed by repeated DEXA-scans during the intervention.

Our analyses on ECM-related genes showed that resident inflammation after weight loss may increase the risk for weight regain. However, this was found in a gene-set which was not focussed on inflammation. Therefore, it would be interesting to focus on the relation between inflammation or the inflammatory status of the adipose tissue and the risk for weight regain after weight loss.

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Addendum

Summary

Over the last decades, the prevalence of obesity has reached epidemic proportions. In 2014, the World Health Organization estimated that of adults aged 18 and over, 39% were overweight and 13% were obese. The increasing prevalence of obesity is a major health concern since it increases the risk for developing type 2 diabetes mellitus, cardiovascular diseases and certain types of cancer. The simple remedy to obesity is losing weight by limiting energy/food intake and increasing daily physical activity for a longer period of time. However, the greatest challenge is the seemingly inevitable weight regain after weight loss (WL), the so-called "yoyo-effect". In general up to 80% of the people are unsuccessful in maintaining weight loss. It has become clear that the adipose tissue plays an important role in the increased risk for weight regain after weight loss. During a negative energy balance, loss of triglycerides decreases the volume of an adipocyte. This decrease in volume creates cellular stress in adipocytes due to the misfit between cell volume and the surrounding extracellular matrix (ECM). This stress can be resolved by remodeling of the ECM or re-storing of triglycerides within the adipocytes. Re-storing of triglycerides would lead to an increase in body weight of the host. This suggests that cellular stress accumulated in adipocytes during a negative energy balance, due to the misfit between cell volume and ECM, is a driving force behind the risk for weight regain. Therefore, studies described in this thesis investigated the involvement of adipocyte stress- and ECM-related factors in the process of weight regain after weight loss.

First, we looked at the relationship between ECM-related factors and the risk for weight regain in two independent cohorts.

In **Chapter 2**, we looked at the role of genetic variation in 124 ECM genes in the risk of weight regain amongst participants of a Pan-European, randomized, controlled dietary intervention study (the so-called "DiOGenes study"). In this study, overweight and obese subjects (310 women, 159 males) followed an 8-week low calorie diet (LCD) with a 6-month follow-up period. Body weight was measured before and after the diet, and after follow-up. As a value for weight regain, the weight maintenance scores (WMS) were calculated as follows: (weight after follow-up – weight after WL) ÷ (weight before WL – weight after WL). A score equal or lower than zero indicated that the participant maintained or continued to lose weight during the follow-up period, while a score higher than zero indicated that the participant regained weight during the 6-month follow-up. Furthermore, we retrieved data for 2903 genetic variations in and near the 124 ECM-related genes. With regression analyses we determined the relation between the WMS and the genetic variations. In men, variants of the POSTN, LAMB1, COL23A1, and FBLN5 genes are related to the risk of weight regain after weight loss. In women, a variant of the FN1 gene is related to the risk of weight regain.

Chapter 3 focusses on the relation between weight regain and changes in expression of ECM genes. This was analysed in a randomized, controlled dietary intervention study (the so-called "yoyo-study") in which 61 participants lost weight by either a 5-week VLCD or a 12-week LCD, with a subsequent 4-week weight stable (WS) diet, and a 9-month follow-up period. The weight loss (WL) and WS phase taken together was named the dietary intervention (DI). As a value for weight regain, the weight regain percentage (WR%) was calculated as follows: ((weight after follow-up – weight after WS) ÷ weight after WS) x 100%. Next, the expression changes of 277 ECM-related genes during WL, WS and DI were correlated with the WR%. We observed 25 genes that were strongly correlated with the WR% and the highest number of correlations was observed in the VLCD WS phase. Five of these genes appeared to belong to a group of 26 genes of which the expression changes correlated highly among each other. This group could be divided into three clusters, one of which is mainly composed of leukocyte-specific integrin genes. Our results suggest that a lower reduction of the expression of

certain leukocyte integrin genes shortly after weight loss by calorie restriction leads to a higher risk of weight regain, which seems linked to ECM remodelling. Possibly, a stronger reduction of ECM remodelling capacity during the WS phase leads to more retention of immune cells suggesting that resident inflammation after weight loss increases the risk for weight regain.

Second, we looked at the relationship between stress-related factors and the risk for weight regain in two independent cohorts.
In **Chapter 4**, we determined stress protein levels during weight loss and weight maintenance in relation to weight regain. For this, 18 healthy overweight and obese subjects underwent an 8-week VLCD with a 10-month follow-up. Participants were categorised as weight maintainers if there was a weight reduction of at least 10% but then regained less than 6% weight during follow-up. Participants were categorised as weight regainers (WR) if there was a weight reduction of at least 10% but then regained 6% or more weight. At the end of WL, WR had higher levels of the stress proteins β-actin, calnexin, heat shock protein (HSP) 27, HSP60 and HSP70. Changes of β-actin, HSP27 and HSP70 were correlated to HSP60, a proposed key factor in weight regain after weight loss. These findings underscore that adipocyte stress plays a role as a biological risk factor for weight regain.
Chapter 5 provides information about the relation between weight regain and expression changes of stress-related genes during WL, WS and DI which was investigated in the yoyo-study. Expression changes of 107 stress-related genes during WL, WS and DI were correlated with the WR%. We found that correlations between expression changes and weight regain were more frequent for the VLCD group than for the LCD group and most correlations appeared in the WS and DI phases. Eight of the nine correlating stress genes of the WS phase were clustered. These genes indicate that there is a link between weight regain and the biological processes on actin filament dynamics, glucose handling and nutrient sensing.

Third, we looked at the relationship between stress- and ECM-related factors and their influence on the risk for weight regain in the yoyo-study.
In **Chapter 6**, we performed correlation and interaction analysis with the WR% using a stress-related and an ECM-related gene set. Correlation analyses revealed that during the WS phase in the VLCD group a co-expression network could be constructed consisting of 8 stress- and 15 ECM-related genes, which all correlated with the WR%. The network links to the biological processes on leukocyte-activity, ECM remodelling, actin cytoskeleton organisation and glucose handling. Interaction analysis between stress- and ECM-related genes revealed several gene combinations that were highly related to the weight regain percentage. In particular, the epidermal growth factor signaling pathway was identified as strongly influencing the risk of weight regain, possibly through interaction with actinin alpha-1 (ACTN1), a component of stress fibers, and with integrin beta 4 (ITGB4), cystatin C (CST3) and laminin alpha-3 (LAMA3).

This thesis provides important insights into the underlying mechanism of weight regain after weight loss. We showed that the risk for weight regain is related to expression changes of distinct sets of stress- and ECM-related genes including the interaction of these changes during the first four weeks after returning to energy balance. Furthermore, we showed that genetic variations of ECM genes are associated with weight regain.

Samenvatting

De prevalentie van obesitas is de afgelopen jaren enorm toegenomen. In 2014 rapporteerde de Wereldgezondheidsorganisatie dat 39% van alle volwassenen overgewicht heeft en dat 13% obese is. De toenemende prevalentie van obesitas is een groot gezondheidsprobleem omdat het leidt tot een verhoogd risico op het ontwikkelen van type 2 diabetes mellitus, cardiovasculaire aandoeningen en bepaalde vormen van kanker. De eenvoudigste oplossing om obesitas tegen te gaan is door het verlagen van het lichaamsgewicht door gedurende een langere periode de energie/voedsel inname te verlagen en de dagelijkse lichamelijke activiteit te verhogen. Gewichtsverlies kan op deze manier eenvoudig worden bereikt maar de grootste uitdaging blijft de schijnbaar onvermijdelijke gewichtstoename na gewichtsverlies, het zogenaamde "jojo-effect". Studies hebben laten zien dat tot 80% van de individuen die met een dieet gewicht verliezen, na 1 jaar weer significant in gewicht zijn gestegen. Gebleken is dat het vetweefsel een belangrijke rol speelt in het verhoogde risico op gewichtstoename na gewichtsverlies. Als men een dieet volgt ontstaat er een negatieve energiebalans omdat er minder energie wordt ingenomen dan het lichaam eigenlijk nodig heeft. Tijdens deze negatieve energiebalans verliezen vetcellen hun vet waardoor het volume van deze cellen afneemt. Deze afname in volume zorgt ervoor dat de cel en de omliggende extracellulaire matrix (ECM) niet meer bij elkaar passen en dit veroorzaakt stress in de vetcellen. Deze stress kan op meerdere manieren worden opgelost. Ten eerste kan de ECM worden aangepast zodat het weer past bij het lagere volume van de vetcel. Ten tweede kan er opnieuw vet worden opgeslagen in de cel zodat het volume toeneemt en de vetcel daardoor weer past bij de ECM. Echter, het opnieuw opslaan van vet leidt ertoe dat het lichaamsgewicht van de persoon weer toeneemt. Dit alles suggereert dat stress in vetcellen, die ontstaat tijdens een negatieve energiebalans doordat het volume van de cel niet meer past bij de omliggende ECM, een drijvende kracht achter het risico op gewichtstoename is. In de studies die beschreven staan in dit proefschrift onderzochten we de betrokkenheid van stress- en ECM-gerelateerde factoren binnen het vetweefsel in het proces van gewichtstoename na gewichtsverlies.

Ten eerste hebben we gekeken naar de relatie tussen de ECM-gerelateerde factoren en het risico op gewichtstoename in twee onafhankelijke studies.
In **Hoofdstuk 2** hebben we gekeken naar de invloed van genetische variatie in 124 ECM-gerelateerde genen op het risico van gewichtstoename na afvallen onder de deelnemers van een pan-Europese, gerandomiseerde gewichtsverliesstudie (de zogenaamde "DiOGenes studie"). In deze studie werden personen met overgewicht en obesitas (310 vrouwen, 159 mannen) op een caloriearm dieet gehouden gedurende 8 weken en daarna werden deze personen nog gedurende 6 maanden (follow-up periode) gevolgd. Het lichaamsgewicht werd gemeten voor en na het dieet en na follow-up. Deze waarden zijn gebruikt om een score te berekenen die weergeeft hoeveel iemand weer is aangekomen in gewicht na het afvallen: (gewicht na follow-up – gewicht na afvallen) ÷ (gewicht voor afvallen – gewicht na afvallen). Een score gelijk of lager dan nul geeft aan dat de persoon niet in gewicht is aangekomen of zelfs nog meer gewicht heeft verloren tijdens follow-up, terwijl een score hoger dan nul betekent dat het gewicht van de persoon is toegenomen tijdens follow-up. Daarnaast hebben we de gegevens van 2903 genetische variaties in en nabij de 124 ECM-gerelateerde genen verkregen. Met behulp van correlatie analyses is de relatie tussen de berekende score en de genetische variaties bepaald. Hierbij zagen we dat bij mannen variaties in de POSTN, LAMB1, COL23A1 en FBLN5 genen verband houden met het risico op gewichtstoename na gewichtsverlies. Bij vrouwen is een variant van het gen FN1 geassocieerd met het risico van gewichtstoename.

Hoofdstuk 3 richt zich op de relatie tussen gewichtstoename en veranderingen in expressie van ECM-gerelateerde genen. Dit werd onderzocht in een gerandomiseerde gewichtsverlies studie (de zogenaamde “jojo-studie”) waarin 61 deelnemers gewicht verloren door een zeer laagcalorisch dieet (VLCD, 500 kilocalorieën/dag) te volgen voor 5 weken of een laagcalorisch dieet (LCD, 1250 kilocalorieën/dag) voor 12 weken. Daarna volgde er een gewichtsstabiele periode van 4 weken en een follow-up periode van 9 maanden. De gewichtsverlies periode (WL) en de gewichtsstabiele (WS) periode samen heten de dieet-interventie (DI) periode. Het gewichtstoename percentage (WR%) werd berekend als waarde voor de gewichtstoename tijdens de follow-up: ((gewicht na follow-up - gewicht na WS) ÷ gewicht na WS) x 100%.
Vervolgens is de gen expressie verandering van 277 ECM-gerelateerd genen tijdens WL, WS and DI gecorreleerd met de WR%. We zagen dat 25 genen sterk correleerde met de WR% en het hoogste aantal correlaties werd waargenomen tijdens de WS fase in de VLCD groep. Vijf van deze genen bleken te behoren tot een groep van 26 genen waarvan de expressieverandering sterk correleerde met elkaar. Deze groep genen kan worden onderverdeeld in 3 clusters, waarvan één voornamelijk bestaat uit genen die coderen voor leukocyt-specifieke integrines. Het lijkt zo te zijn dat een geringere vermindering van expressie van deze specifieke integrines tijdens de WS fase leidt tot een hoger risico op gewichtstoename en tevens lijkt dit samen te hangen met aanpassingen van de ECM. Het is mogelijk dat een sterkere vermindering van de capiciteit om de ECM aan te passen kan leiden tot het vasthouden van immuun cellen in het vetweefsel. Dit suggereert dat het risico op gewichtstoename wordt vergroot door behoud van ontstekingen van het vetweefsel kort na gewichtsverlies.

Ten tweede hebben we gekeken naar het verband tussen de stress-gerelateerde factoren en het risico op gewichtstoename in twee onafhankelijke studies.
In **Hoofdstuk 4** hebben we onderzocht of de niveaus van specifieke stress eiwitten tijdens het gewichtsverlies en de follow-up gerelateerd zijn aan het risico voor gewichtstoename. Daartoe hebben 18 gezonde personen met overgewicht of obesitas een zeer-laagcalorisch dieet gevolgd voor 8 weken en 10 maanden daarna werden de personen opnieuw gezien. De personen werden vervolgens ingedeeld als zijnde ‘gewichtsstabiel’ of ‘gewichtstoename’, afhankelijk van de gewichts veranderingen tijdens de studie. Tot de gewichtsstabiele groep behoorden de personen die tenminste 10% van hun gewicht verloren hadden tijdens de dieetperiode en niet meer dan 6% waren aangekomen in gewicht tijdens de follow-up. Tot de gewichtstoename groep behoorden de personen die tenminste 10% van hun gewicht verloren hadden maar meer dan 6% aankwamen in gewicht tijdens de follow-up. Na het afvallen werden de niveaus van de verschillende stress eiwitten in het vetweefsel vergeleken tussen de 2 groepen en daarbij kwam aan het licht dat de niveaus van β-actin, calnexin, heat shock protein (HSP) 27, HSP60 en HSP70 hoger waren in de gewichtstoename groep. De veranderingen tijdens gewichtsverlies van β-actin, HSP27 en HSP70 bleken gerelateerd aan veranderingen van HSP60, een mogelijk belangrijke factor in het risico voor gewichtstoename na het afvallen. Deze bevindingen bevestigen dat stress in vetcellen een rol speelt in het risico op gewichtstoename.
Hoofdstuk 5 geeft informatie over de relatie tussen gewichtstoename en veranderingen in expressie van stress-gerelateerde genen tijdens de WL, WS en DI in de jojo-studie. Expressie veranderingen van 107 stress-gerelateerde genen tijdens WL, WS en DI werden gecorreleerd met het WR%. We zagen dat er meer correlaties tussen de WR% en gen expressie veranderingen waren in de VLCD groep ten opzichte van de LCD groep. Deze correlaties waren vooral te zien tijdens de WS en DI periode. Acht

van de negen gecorreleerde stress genen tijdens de WS fase waren aan elkaar gelinkt. Deze genen geven aan dat er een verband bestaat tussen gewichtstoename en bepaalde biologische processen in het vetweefsel: actine filament dynamica, glucose metabolisme en nutrient sensing.

Ten derde hebben we in de jojo-studie gekeken naar de interactie tussen de stress- en ECM-gerelateerde factoren en hun invloed op het risico van gewichtstoename.
In **Hoofdstuk 6** werden correlatie en interactie analyses uitgevoerd met stress- en ECM-gerelateerde genen om te achterhalen of combinaties van genen een effect hebben op het WR%. Uit de correlatie analyses bleek dat er tijdens de WS fase in de VLCD groep een netwerk kan worden gevormd bestaande uit 8 stress- en 15 ECM-gerelateerd genen, die allemaal correleren met de WR%. Het netwerk linkt aan specifieke biologische processen in het vetweefsel: leukocyten-activiteit, ECM aanpassingen, actine cytoskelet organisatie en glucose metabolisme. Interactie analyses lieten zien dat verschillende combinaties van stress en ECM genen een significant effect hebben op het WR%. Vooral de signalering door de epidermale groeifactor werd geïdentificeerd als sterke beïnvloeder van het risico op gewichtstoename waarbij actinine alfa-1 (ACTN1), integrine beta 4 (ITGB4), cystatine C (CST3) en laminine alfa-3 (LAMA3) een rol spelen.

Dit proefschrift biedt belangrijke inzichten in het onderliggende biologische mechanisme van gewichtstoename na gewichtsverlies. We hebben aangetoond dat het risico van hernieuwde gewichtstoename samenhangt met celstress in het vetweefsel. Tekenend daarvoor zijn de expressie veranderingen van verschillende stress- en ECM-gerelateerde genen gedurende de eerste vier weken van terugkeer in energiebalans. Daarnaast laten we zien dat de interacties tussen de stress- en ECM-gerelateerde genen een effect hebben op de gewichtstoename na het afvallen. Tevens hebben we aangetoond dat gewichtstoename geassocieerd is met genetische variaties van ECM genen , die verschillend zijn voor vrouwen en mannen.

Valorisation

This thesis focusses on extending the fundamental knowledge about the underlying process of the seemingly inevitable weight regain after weight loss of overweight and obese humans.

Social and economic relevance

The prevalence of obesity has reached epidemic proportions. In 2014, the World Health Organization estimated that 39% of adults aged 18 years and over were overweight (BMI≥25) and 13% were obese (BMI>30) (1). This means that more than 1.9 billion adults are affected by overweight including obesity. Worldwide, overweight tends to be more common for woman compared to men (40% women vs 38% men), which is similar for obesity (15% women vs 11% men). The global prevalence of obesity has more than doubled between 1980 and 2014. The increasing prevalence of obesity is a major health concern since it increases the risk for developing type 2 diabetes mellitus, cardiovascular diseases and several types of cancer (2-4). In 2010, around 3.4 million adults died as a result of being overweight or obese (5). In addition to the health problems, overweight and obesity have a considerable economic impact and increase healthcare costs. In the Netherlands, the total direct and indirect costs of overweight and obesity is estimated at 3 billion euros per year. Of these 3 billion euros, 1.2 billion is directly linked to healthcare costs caused by overweight and obesity and 2 billion are linked to the lost productivity which is a result from days taken off sick. These facts clearly indicate that obesity and its associated diseases have a profound impact on our society.

The remedy to obesity is losing weight which can be achieved by limiting energy/food intake and increasing daily physical activity for a longer period of time. A reduction in body weight lowers the risk for developing diseases and improves the metabolic profile in overweight and obese people (6-8). However, long-term weight loss maintenance has been proven to be difficult (9, 10). Generally, up to 80% of the people are unsuccessful in maintaining weight loss (11, 12). It is crucial to prevent or reduce weight regain on a long-term basis since it would lead to a tremendous reduction of the disease burden and the financial costs related to the diseases. This thesis provides more insight into the mechanisms involved in the biological process for weight regain which can contribute to solving the problem of the seemingly inevitable weight regain after weight loss.

Scientific gain of this thesis

During a negative energy balance, cellular stress may arise in adipocytes due to the misfit between the lowered cell volume and the surrounding ECM. This stress needs to be resolved which can be achieved by remodeling of the ECM or by the re-storing of triglycerides within the adipocytes to increase the cell volume. This thesis focused on determining the involvement of stress- and ECM-related factors in the underlying mechanisms of weight regain.

- We have found that not the weight loss period per se is crucial in the underlying mechanisms involved in weight regain, but that especially the weeks after weight loss, when returning to energy balance, are linked to the risk of weight regain. In other words, studying the period after weight loss, when people increase their food intake to meet their energy requirements, may lead to the identification of risk markers for weight regain after weight loss.
- We have shown that changes of stress- and ECM-related genes and the interaction of these changes during the weeks after weight loss, when returning to energy balance, are highly related with weight regain during follow-up. Studying these stress and ECM genes, which are linked to several biological processes, may lead to the identification of genetic risk factors

involved in the process of weight regain after weight loss and may provide targets for intervention.

- We have observed that people have a higher risk for weight regain when there is a stronger downregulation of ECM-remodelling when returning to energy balance. This may lead to the retaining of more immune cells in the adipose tissue suggesting that resident inflammation after weight loss increases the risk for weight regain. Studying inflammation after weight loss may lead to the identification of risk markers for weight regain and inflammation-related targets for intervention.
- Several of the investigated single nucleotide polymorphisms (SNPs) of ECM genes are linked with weight regain. Possibly, these SNPs can be used to create a genetic risk profile to select people that are at high risk of weight regain, who can be better guided to achieve weight maintenance after weight loss.

Innovation

The present thesis presents various novel findings and insights. The aim was to determining the involvement of stress- and ECM-related factors in the underlying mechanisms of weight regain. We were able to show that the change of multiple stress-related markers influenced the regain of weight during follow-up, which confirms the proposed role of cellular stress as a driver of weight regain. Surprisingly, the change during the weeks after weight loss seems at least as important in this process as the change during weight loss. Similar to the stress markers, we showed that changes of ECM-related genes after weight loss were associated with weight regain. Here, we specifically saw that when returning to energy balance a stronger reduction of ECM remodelling capacity leads to more retention of immune cells in the adipose tissue. This indicates that inflammation after weight loss increases the risk for weight regain and as such adds a novel dimension to the biological mechanism of weight regain. However, stress- and ECM-related factors are not only influencing the risk for weight regain separately but also have a combined effect. Several stress and ECM gene interactions were highly related to weight regain after weight loss. Together these interactions indicate that changes in the epidermal growth factor signalling, when returning to energy balance, influence the risk of weight regain, which may be mediated by alpha-actinin phosphorylation and modified by expression of integrin beta 4, cystatin C and laminin alpha-3. It connects endocrine or autocrine signals, yet to be identified, to adipocyte stress as a factor for weight regain.
Furthermore, in this thesis we have shown that several SNPs of ECM genes are linked with weight regain. In men, SNPs of the ECM genes collagen type XXIII alpha1, fibulin-5, laminin-β1 and, periostin were linked while in women fibronectin 1 was linked. These SNPs could add to a genetic risk profile for weight regain. The SNPs were different for men and woman, which is not uncommon for research on weight regulation, underscoring the importance for sex-specific research.

Target groups

The results described in this thesis are available for the scientific community through publications in international peer-reviewed journals. Also, results were presented at important international congresses. Yet, the most important target group is society itself. News articles on our research have been published on various news websites, which has enabled a broader audience to take notice of our results. The scientific gain of this thesis will help to fight obesity on the long-term by determining the risk for weight regain of overweight and obese subjects. People with a higher risk can be guided

better by providing a specific, personalized guideline during weight loss but especially also during weight maintenance. This will enhance the efficiency of the weight loss treatment and of weight maintenance, which will contribute to the reduction of the health burden for society. For example, people with an increased risk for weight regain should receive a more stringent and/or frequent guidance than those with a lower risk. To achieve this, high quality prediction models are needed, which requires collaborations between research groups and companies to explore large-scale genome data. The prediction models and treatment guidelines can be used by trained dieticians, specialists in the hospital and obesity clinics.

Planning and implementation

The prediction models and treatment guidelines need to be made, however, this requires collaborations with other research groups within and outside the university as well as collaborations with the industry. For example, bioinformatics and biostatistics with a focus on modelling are needed to use the available data and create a clear model for weight regain. These collaborations will lead to better and more accurate insight into the underlying mechanism for the risk weight regain and the risk condition of each individual. The findings presented in this thesis are the starting point in this respect and at the moment we are collaborating with the Maastricht Centre for Systems Biology (MaCSBio) to create a model for weight regain. Within the coming years this model will be finished and available for society. This model will lead to better and more accurate insight into the process of weight regain and assist the specialists to provide specific, personalized guidance to people to create optimal conditions for weight maintenance after weight loss. It should be noted that this model requires the use of genetic information of individuals and this might lead to ethical issues, especially about privacy of the individual. Privacy concerns arise because organisations and institutions might be interested in knowing a person's genetic status which may result in stigmatization, discrimination, and other adverse effects. For example, if an insurance company knows that an individual is predisposed to develop obesity by looking at his/hers genetic information, they might increase the individual's insurance bill already to prevent possible extra costs later on, while the individual might never get sick. On the other hand, it might be beneficial to know if someone is predisposed to develop a specific disease because than actions can be taken to prevent the disease from occurring. Those and other questions have still to be worked out for a complex trait as overweight and obesity which requires the active involvement of scientists from multiple disciplines and of politicians.

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Curriculum Vitae

Nadia Roumans was born on March 3rd 1989 in Zeven, Germany, and raised in Eygelshoven, the Netherlands. She completed secondary school at College Rolduc in Kerkrade in 2006. Consecutively, she studied Biology and Medical Laboratory Research at Hogeschool Zuyd in Heerlen, for which she received her Bachelor's degree in 2010. She continued with a 2-year master in Molecular Life Sciences, with a specialization in Clinical Molecular Sciences, at Maastricht University, for which she received her Master's degree in 2012. In November 2012, Nadia started working as a PhD-candidate at the department of Human Biology of the Faculty of Health, Medicine and Life Sciences of Maastricht University under the supervision of Prof. dr. Edwin Mariman and Prof. dr. Marleen van Baak. During this period, she investigated the involvement of stress- and extracellular matrix-related factors in the risk of weight regain after weight loss, as demonstrated in this thesis and publications in scientific journals.

List of Publications

Tareen SHK, Adriaens ME, Arts ICW, de Kok T, Vink RG, **Roumans NJT**, van Baak MA, Marimen ECM, Evelo CT, Kutmon M. Profiling core processes in adipose tissue during weight loss using time series gene expression. Submitted for publication.

Roumans NJ, Vink RG, Wang P, van Baak MA, Mariman EC. Relation between stress- and ECM-related genes and their effect on weight regain. To be submitted.

Roumans NJ, Vink RG, Fazelzadeh P, van Baak MA, Mariman EC. A role for leukocyte and ECM remodelling of adipose tissue in the risk of weight regain after weight loss. American Journal of Clinical Nutrition. 2017; 105(5):1054-1062.

Vink RG, **Roumans NJ**, Čajlaković M, Cleutjens JPM, Boekschoten MV, Fazelzadeh P, Vogel MAA, Blaak EE, Mariman EC, van Baak MA, Goossens GH. Diet-induced weight loss decreases adipose tissue oxygen tension with parallel changes in adipose tissue phenotype and insulin sensitivity in overweight humans. International Journal of Obesity. 2017; 41(5):722-728.

Vink RG, **Roumans NJ**, Mariman EC, van Baak MA. Dietary weight loss-induced changes in RBP4, FFA and ACE predict weight regain in people with overweight and obesity. Submitted for publication.

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Roumans NJ, Vink RG, Bouwman FG, Fazelzadeh P, van Baak MA, Mariman EC. Weight loss-induced cellular stress in subcutaneous adipose tissue and the risk for weight regain in overweight and obese adults. International Journal of Obesity. 2016; in press.

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