A role for leukocyte integrins and extracellular matrix remodeling of adipose tissue in the risk of weight regain after weight loss[1,2]

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ABSTRACT

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

Objective: We investigated the relation between the expression of ECM genes during WL and a period of weight stabilization (WS) and the risk of weight regain.

Design: In this randomized controlled trial, 61 healthy overweight or obese participants followed either a 5-wk very-low-calorie diet (VLCD; 500 kcal/d) or a 12-wk low-calorie diet (1250 kcal/d) (WL period) with a subsequent 4-wk WS period and a 9-mo follow-up. The WL and WS periods combined were considered the DI. Abdominal subcutaneous adipose tissue biopsy samples were collected for microarray analysis. Gene expression changes for a broad set of ECM-related genes were correlated with the weight-regain percentage (WR%).

Results: A total of 26 of the 277 genes were significantly correlated with WR% during WL, WS, or the DI periods. Most correlations were observed in the VLCD group during the WS period. Four genes code for leukocyte-specific receptors. These and other genes belong to a group of 26 genes, among which the expression changes were highly correlated ($r \geq 0.7$, $P \leq 0.001$). This group could be divided into 3 subclusters linking to 2 biological processes—leukocyte integrin gene activity and ECM remodeling—and a link to insulin sensitivity was also apparent.

Conclusions: Our present findings indicate the importance of adipose tissue leukocytes for the risk of weight regain. ECM modification also seems to be involved, and we observed a link to insulin sensitivity. This trial was registered at clinicaltrials.gov as NCT01559415. *Am J Clin Nutr* 2017;105:1054–62.

Keywords: healthy overweight/obese participants, human dietary intervention, weight regain after weight loss, adipose tissue, microarray, extracellular matrix and inflammation

INTRODUCTION

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

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[2] Supplemental Tables 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[5] Abbreviations used: CTSB, cathepsin B; CTSS, cathepsin S; *DDR1*, discoidin domain receptor tyrosine kinase 1; DI, dietary intervention; ECM, extracellular matrix; FC, fold change; ITGA, integrin α; ITGB, integrin β; LCD, low-calorie diet; LR, $\log_2$ ratio; MMP, matrix metallopeptidase; OPN, osteopontin; *PLOD*, procollagen-lysine 2-oxoglutarate 5-dioxygenase; *PPIB*, peptidylprolyl isomerase B; *SPP1*, secreted phosphoprotein 1; T, time point; TGFB1, transforming growth factor β1; VLCD, very-low-calorie diet; WL, weight loss; WR%, weight-regain percentage; WS, weight stabilization.

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WL induces changes in the ECM, not only by cellular stress but also in other ways. It has been generally observed that the development of obesity is accompanied by an onset of low-grade inflammation. This involves the chemotactic attraction of cells of the innate immune system into adipose tissue, which leads to a situation in which proinflammatory immune cells outbalance the anti-inflammatory cells (14). Such a state of increased inflammation may have systemic consequences because it may promote the development of insulin resistance. WL usually reduces the inflammatory activity of 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 increased (15) or was unchanged (16) in human adipose tissue directly after short-term WL, but were downregulated during a subsequent weight-stabilization (WS) period (15–17). Leukocyte extravasation during weight gain and possible emigration of leukocytes after WL require remodeling of the ECM. Therefore, this change in the content of immune cells may influence the risk of weight regain. Here, we analyzed the gene expression of a large number of ECM genes during WL and WS phases in relation to weight regain.

METHODS

Participants and study design

Sixty-one overweight and obese [BMI (in kg/m^2) 27–36] white participants underwent an energy-restricted DI (**Figure 1**). The whole study design was described in detail previously (18). In short, individuals were recruited by advertisement via local media. Exclusion criteria were as follows: smoking; cardiovascular disease; type 2 diabetes; liver or kidney disease; use of medication that influences body weight regulation; pregnancy; marked alcohol consumption (>21 alcohol drinks/wk for men and >14 alcohol drinks/wk 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 mm Hg. Participants were randomly assigned to either a very-low-calorie diet (VLCD; rapid WL) or a low-calorie diet (LCD; slow WL) group. Participants in the VLCD group underwent a 5-wk diet of ~500 kcal/d by consuming 3 meal replacements/d (Modifast; Nutrition et Santé Benelux). Participants in the LCD group underwent a 12-wk diet of ~1250 kcal/d, which was designed by a dietitian. Both groups were targeted to lose ~10% body weight during this WL period [time point (T)1-T2]. After WL, all of the participants underwent a 4-wk weight-maintenance diet based on their individual energy requirements. This WS period (T2–T3) was designed to investigate the effect of WL of ~10% without the interfering effect of a pronounced negative energy balance. The WL and WS periods combined were considered to be the DI (T1–T3). The study dietitian provided dietary advice according to the Dutch national guidelines (19) to both of the groups to assist in their remaining weight stable throughout the WS period and to assist in WL during the WL period in the LCD group. After the WS period, each participant's body weight was monitored for 9 mo (follow-up; T3–T4) by monthly meetings with a dietitian. During this follow-up period, participants did not receive advice on monitoring and limiting food intake to mimic nonrestricted free-living conditions.

At the start of the study (T1) and at the end of each period (T2, T3, and T4) abdominal subcutaneous adipose tissue biopsy samples were collected, body composition was determined, and body weight, height, blood pressure, and hip and waist circumferences were measured after an overnight fast. Body volume was determined with air-displacement plethysmography by using a Bod Pod device (Cosmed) according to the manufacturer's instructions and as described by Dempster and Aitkens (20). Body composition was calculated from body density according to the 2-compartment model by Siri (21).

During the DI (T1–T3), 4 participants withdrew from the study, we could not collect enough biopsy material in 3 participants, and the gene expression results deviated strongly from the others in 1 participant and were therefore excluded. Characteristics of the remaining 53 participants at T1, T2, T3, and T4 are shown in **Table 1**.

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

Adipose tissue biopsy

Abdominal subcutaneous adipose tissue biopsy samples were obtained by needle biopsy under local anesthesia (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 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 ~150 mg frozen adipose tissue by using Trizol reagent (Invitrogen). Total RNA (100 ng/sample) was labeled by whole-transcript sense target assay and hybridized to human whole-genome Affymetrix Gene

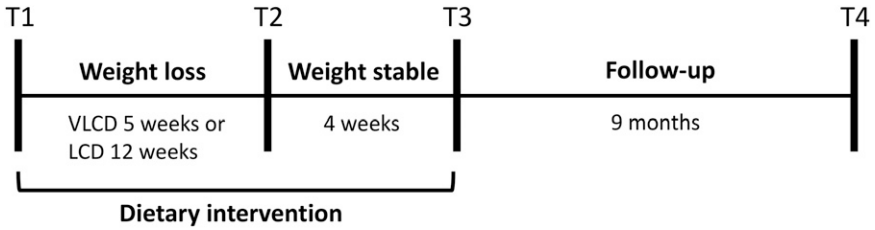

FIGURE 1 Schematic overview of the study design. Measurements were performed at the start of the study (T1) and at the end of the weight-loss (T2), weight-stable (T3), and follow-up (T4) periods. The dietary intervention period represents the weight-loss and weight-stable periods combined. n = 27 and n = 26 for the LCD and VLCD groups, respectively. LCD, low-calorie diet; T, time point; VLCD, very-low-calorie diet.

TABLE 1
Subject characteristics at the study start and at the end of the WL, WS, and follow-up periods[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 (M/F), *n*/*n*	12/14	13/14	—	—	—	—	—	—
Age, y	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] Values are means ± SEMs. $n = 26$ and $n = 27$ for VLCD and LCD groups, respectively. There were no significant differences ($P \leq 0.05$) between the VLCD and LCD groups at the study start or at the end of the WL, WS, and follow-up periods. LCD, low-calorie diet; T, time point; VLCD, very-low-calorie diet; WL, weight-loss; WS, weight-stabilization.

[2] Significant change ($P \leq 0.05$) between this time point and the previous time point by dependent *t* test.

[3] Significant change ($P \leq 0.05$) between the end of the WS period (T3) and the study start (T1) by dependent *t* test.

1.1 ST arrays targeting 19,654 unique genes (Affymetrix). Sample labeling, hybridization to chips, and image scanning were performed according to the manufacturer's instructions. Microarray signals were normalized by using the robust multi-chip average. Genes with normalized signals >20 on ≥15 arrays were defined as being expressed (11,532 genes). Gene expression changes within groups during a certain period (WL, WS, or DI periods) were defined as being 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 ECM were selected for the analysis on the basis of previously measured ECM genes (13) and by selecting for the term "extracellular matrix" in the Gene Ontology Biological Process description (**Supplemental Table 1**).

Calculations

In this study, we wanted to analyze 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] × 100%. For gene expression change, we calculated the fold change (FC) of gene expression during the WL, WS, or DI periods. 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 (LRs) during WL, WS, and DI were transformed into FCs [$FC = 2^{LR}$ if $LR \geq 0$ 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 phases. The ECM genes that correlated significantly ($P \leq 0.05$) with WR% were clustered on the basis of their correlation among each other ($r > 0.6$, $P < 0.002$). For a second analysis, we performed an extended clustering for the VLCD WS phase in which the changes in the 11 genes that correlated with WR% were correlated with all of the 277 ECM gene expressions. Next, genes were selected that were significantly correlated ($r \geq 0.7$ and $P \leq 0.001$). For those genes, a correlation matrix was constructed, but to further minimize the chance of including false positives and to focus on closely related genes, a confined correlation cluster was constructed by removing genes with <5 significant correlations with other genes in the cluster.

Statistical analysis

Data are presented as means ± SEMs. Comparisons of variables within a group were made with a dependent *t* test. Between-group comparisons (VLCD compared with LCD) were made with an independent *t* test. To determine the link between weight regain and ECM genes, Pearson's correlations were performed between WR% and the gene expression changes in the 277 ECM genes during WL, WS, and DI periods. Statistical calculations were performed by using SPSS 20.0 for Windows (SPSS Inc.). $P \leq 0.01$ was considered significant unless otherwise stated. All of the variables were checked for normal distribution, and variables with a skewed distribution were ln-transformed to satisfy conditions of normality. Extreme outliers (values >3 × IQR calculated by using SPSS) influencing the data were removed during statistical analyses.

RESULTS

Clinical characteristics

Characteristics of the 53 participants at the study start (T1) and the end of WL (T2), WS (T3), and follow-up (T4) periods are shown in Table 1. The VLCD and LCD groups were comparable at the study start for weight, BMI, body fat percentage, fat-free mass, and hip and waist circumferences. During the WL period (T1–T2), participants' body weight,

BMI, hip and waist circumferences, body fat percentage, and fat-free mass decreased (Table 1). After WL, VLCD and LCD groups were still comparable for all variables. In the subsequent 4-wk WS period (T2–T3), there were changes in hip circumference, body fat percentage, and fat-free mass (Table 1). However, no significant differences were observed between the VLCD and LCD groups after the WS period. During the DI (T1–T3), all of the variables significantly decreased except for fat-free mass in the LCD group (Table 1). Body weight, BMI, waist circumference, body fat, body fat percentage, and fat-free mass increased during follow-up (T3–T4). No differences were found between VLCD and LCD groups in the average WL 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 in ECM gene RNA levels correlate with WR%

Changes during WL, WS, and DI periods 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 the VLCD, most of the correlations (11 of 17) were in the WS phase, whereas for the LCD, most were found in the DI phase (7 of 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 ECM. In endothelial cells and lung cancer cells, collagen type IV α5 (COL4A5) directs DDR1 activity (23). Here we observed a negative correlation between *COL4A5* gene expression and WR%, but in the VLCD DI phase.

Cluster analysis of VLCD WS correlating genes

Eleven genes were correlated with WR% during the VLCD WS phase. We therefore tried to see whether 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 in these 11 genes during the VLCD WS phase. This approach assumes that coexpressed genes may have closely related functions or take part in the same functional process. All 11 genes could be clustered (**Figure 2**). The genes that negatively correlate with WR% [procollagen-lysine 2-oxoglutarate 5-dioxygenase (*PLOD*) 2, dystrophin (*DMD*), and secreted protein acidic and cysteine rich–like 1 (*SPARCL1*)] were also negatively correlated

TABLE 2
Correlation coefficients between the weight-regain percentage and the fold changes in ECM gene expression during the WL, WS, and DI periods separated on the basis of diet[1]

		VLCD			LCD			
Gene	Name	WL	WS	DI	WL	WS	DI	*P*
ABI3BP	ABI family member 3 binding protein	0.593	—	—	—	—	—	0.003
ACTN1	Actinin α1	—	0.661	—	—	—	—	0.001
ADAMTSL4	A disintegrin and metalloproteinase with thrombospondin motifs-like 4	—	—	—	—	−0.626	—	0.001
COL20A1	Collagen, type XX, α1	—	—	—	—	—	−0.552	0.008
COL4A5	Collagen, type IV, α5	—	—	−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 α5 (fibronectin receptor, α polypeptide)	—	0.591	—	—	—	—	0.003
ITGAL	Integrin αL [antigen CD11A (p180), lymphocyte function-associated antigen 1; α polypeptide]	—	0.560	—	—	—	—	0.005
ITGAM	Integrin αM	—	0.620	—	—	—	—	0.002
ITGAX	Integrin αX	—	0.614	—	—	—	—	0.002
ITGB2	Integrin β2	—	0.571	—	—	—	—	0.005
LAMA2	Laminin α2	—	—	—	—	—	0.661	0.001
LAMC1	Laminin γ1 (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 (hevin)	—	−0.561	—	—	—	—	0.007
TIMP4	Tissue inhibitor of metalloproteinases 4	—	—	—	—	—	0.547	0.008

[1] Values are significant ($P \leq 0.01$) Pearson's correlation coefficients (r) between weight regain and the fold changes in gene expression during WL (T2–T1), WS (T3–T2), and DI (T3–T1) periods. $n = 26$ and $n = 27$ for VLCD and LCD groups, respectively. Weight regain percentage is calculated as follows: [(weight after follow-up – weight after WS) ÷ weight after WS] × 100%. DI, dietary intervention; ECM, extracellular matrix; LCD, low-calorie diet; T, time point; VLCD, very-low-calorie diet; WL, weight-loss; WS, weight-stabilization.

with the other genes, whereas *PLOD2* and *DMD* were positively correlated with each other, like the other genes of the cluster.

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 ≥5 other genes in the cluster to minimize false positives. This resulted in a cluster of 26 genes, including 5 of the genes that correlated with WR%: integrin α (*ITGA*) L, *ITGAM*, *ITGAX*, integrin β (*ITGB*) 2 (*ITGB2*), and peptidylprolyl isomerase B (*PPIB*). Rearranging the genes according to their strong correlation values resulted in 3 subclusters (**Table 3**): a small cluster of 7 genes including the 4 integrin genes and 2 larger overlapping clusters. All except for 1 of the genes were positively correlated. Only the gene for fibroblast growth factor 2 (*FGF2*) was negatively correlated with the genes of the third subcluster. The genes for matrix metallopeptidase (MMP) 9 (*MMP9*), elastin microfibril interface 2 (*EMILIN2*), and calpain 1 (*CAPN1*) seem to connect the small cluster with the 2 larger clusters. The overlap becomes more evident when similar clustering is conducted for the VLCD WL phase (**Supplemental Table 2**).

Cluster analysis during the WL phase led to a more confluent network, especially extended around the small subcluster. This suggests that during the transition from the WL to the WS phase, the involvement of leukocytes becomes more centered on the integrin complexes such as macrophage-1 antigen (Mac-1) (ITGB2/ITGAM) and lymphocyte function-associated antigen 1 (LFA-1) (ITGB2/ITGAL). Differences between the 2 phases not only refer to a higher number of significant correlations but also to more contributing genes. *ITGAL* and *FGF2* were present only in the WS phase cluster, whereas 11 genes were present only in the cluster during the WL phase (Supplemental Table 2: blue fill).

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) and leukocyte adhesion (25–27). **Table 4** shows the average change in expression of integrin genes during the VLCD WS phase. *ITGB2*, which forms complexes with *ITGAL*, *ITGAM*, and *ITGAX*, was significantly downregulated. In addition, the genes for *ITGAM* and *ITGAX*, as well as *ITGAE*, which complexes with *ITGB7* for integrin formation, were significantly downregulated. This indicates that leukocyte-specific integrins in the adipose tissue are downregulated during the WS phase. Because some of these genes are positively correlated with WR%, this suggests that a lack of leukocyte-specific downregulation during the WS period increases the risk of weight regain. We were unable to separately investigate the cellular composition of the stromal vascular fraction. In an attempt to obtain such information, we analyzed the expression changes in genes coding for surface markers present on leukocytes by using the microarray data of the WS phase. Our observations point to a lowering of gene expression in cells of the myeloid lineage but not in lymphoid cells (**Supplemental Table 3**). Gene expression of monocyte chemoattractant protein 1 (*MCP1*) is downregulated (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 (28). In the present study, the complement component 5 gene (*C5*) in adipose tissue was significantly downregulated (FC = −1.15, Q = 0.01) as was the gene for its receptor, *C5AR1* (FC = −1.44, Q = 0.008). This suggests that a lowering of leukocyte extravasation during WS might explain the downregulation of the clustered integrin genes.

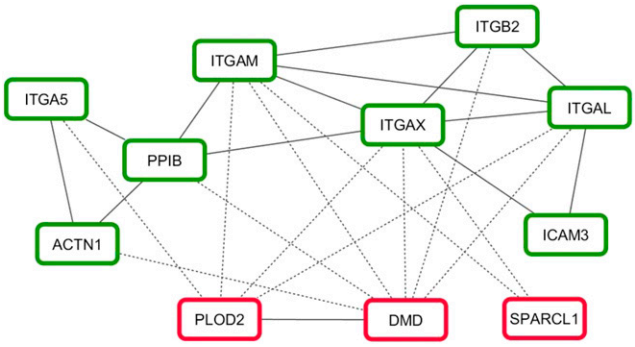

FIGURE 2 Correlation network between expression changes in genes during the weight-stabilization phase in the VLCD ($n = 26$) group. Only Pearson's correlations with $r > 0.6$ and $P < 0.002$ are shown here. Genes that positively (green borders) or negatively (red borders) correlated with the weight-regain percentage are clustered. Positive correlations between genes are shown with a solid line, and negative correlations are shown with a dashed line. ACTN1, actinin α1; DMD, dystrophin; ICAM3, intercellular adhesion molecule 3; ITGAL, integrin αL; ITGAM, integrin αM; ITGAX, integrin αX; ITGA5, integrin α5; ITGB2, integrin β2; PLOD2, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; PPIB, peptidylprolyl isomerase B; SPARCL1, secreted protein acidic and cysteine rich–like 1; VLCD, very-low-calorie diet.

DISCUSSION

The present study focused on determining the relation between weight regain and changes in expression of ECM-related genes. Significant correlations ($P \leq 0.01$) with 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, among which the expression changes were highly correlated ($r \geq 0.7$, $P \leq 0.001$). This group could be divided into 3 subclusters, 1 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). In addition to leukocyte-specific integrin genes, the subcluster contains 3 additional genes: transforming growth factor β1 (*TGFB1*), chitinase 3–like 1 (*CHI3L1*), and cathepsin S (*CTSS*). TGFB1 influences inflammatory status by regulating cytokine concentrations, as was shown for adipose-derived mesenchymal stromal cells in vitro (29). Furthermore, TGFB1 may play a role in leukocyte migration because it has been shown to influence adhesion of leukocytes to orbital fibroblasts (30). The product of *CHI3L1*, also known as YKL-40, is a BMI-independent marker for type 2 diabetes (31) produced by macrophages and neutrophils (32, 33). YKL-40 inhibits the degradation of type I collagen and was suggested to play a role in macrophage infiltration. CTSS is 1 of 11 cathepsin proteases that are active in ECM remodeling (34). Adipose tissue *CTSS* mRNA levels are associated with BMI, which suggests that *CTSS* is mainly active in adipocytes (35). In addition, activated macrophages in interaction with adipocytes increase CTSS production, which points to a close relation

TABLE 3
Correlation matrix of changes in ECM gene expressions during the WS phase in the VLCD group[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's correlation coefficients: green fill, $r > 0.700$; red fill, $r < -0.700$. Red boxes indicate subclusters of strongly correlated genes. $n = 26$. CALR, calreticulin; CAPN1, calpain 1; CD44, CD44 molecule; CHI3L1, chitinase 3–like 1; COLGALT1, collagen β(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; ITGAL, integrin αL; ITGAM, integrin αM; ITGAX, integrin αX; ITGA3, integrin α3; ITGB2, integrin β2; MMP9, matrix metallopeptidase 9; MMP19, matrix metallopeptidase 19; NCSTN, nicastrin; PDIA4, protein disulfide isomerase family A, member 4; PLOD1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; PPIB, peptidylprolyl isomerase B; P4HB, prolyl 4-hydroxylase, β polypeptide; SPP1, secreted phosphoprotein 1; TGFBI, transforming growth factor β–induced; TGFB1, transforming growth factor β1; TIMP1, Tissue inhibitor of metalloproteinases 1; VLCD, very-low-calorie diet; WS, weight-stabilization.

TABLE 4
Gene expression changes during the WS phase in the VLCD group[1]

Gene ID	Gene name	Remark	Fold change	*Q*
ITGA1	Integrin subunit α1	Receptor for collagen[2]	1.15	0.008
ITGA2	Integrin subunit α2	Receptor for collagen[2]	1.17	0.003
ITGA10	Integrin subunit α10	Receptor for collagen[2]	nd	nd
ITGA11	Integrin subunit α11	Receptor for collagen[2]	1.25	<0.001
ITGA5[3]	Integrin subunit α5	Receptor for fibronectin[2]	1.01	0.458
ITGA3	Integrin subunit α3	Receptor for laminin[2]	−1.56	0.000
ITGA6	Integrin subunit α6	Receptor for laminin[2]	1.19	0.000
ITGA7	Integrin subunit α7	Receptor for laminin[2]	1.26	0.000
ITGA4	Integrin subunit α4	Leukocyte adhesion[4]	−1.15	0.172
ITGA9	Integrin subunit α9	Leukocyte adhesion[5]	−1.01	0.493
ITGB2[3]	Integrin subunit β2	Leukocyte-specific receptor[2]	−1.64	0.002
ITGAD	Integrin subunit αD	Leukocyte-specific receptor[2]	−1.01	0.483
ITGAL[3]	Integrin subunit αL	Leukocyte-specific receptor[2]	−1.05	0.416
ITGAM[3]	Integrin subunit αM	Leukocyte-specific receptor[2]	−1.39	0.002
ITGAX[3]	Integrin subunit αX	Leukocyte-specific receptor[2]	−1.54	0.014
ITGB7	Integrin subunit β7	Leukocyte-specific receptor[2]	1.01	0.502
ITGAE	Integrin subunit αE	Leukocyte-specific receptor[2]	−1.33	0.001

[1] $n = 26$. Gene expression fold changes were significantly different when Q values were <0.05 by paired t test with Bayesian correction (Limma). nd, no data available; VLCD, very-low-calorie diet; WS, weight-stabilization.
[2] Reference 24.
[3] There was a positive Pearson's correlation between this gene and the weight-regain percentage.
[4] Reference 25.
[5] References 26 and 27.

between *CTSS* activity and inflammatory status of adipose tissue (36). Together, the gene expression changes in the small subcluster correspond with a lowering of leukocyte-specific integrins. Currently, we cannot determine whether downregulation is caused by lower RNA production of resident leukocytes or by emigration of leukocytes. 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). Because in obesity adipose tissue is in a state of chronic inflammation, the changes in gene activity described here may be related to a reduction in the inflammatory status during the WS period.

The genes in the second cluster also point to the importance of leukocytes and their involvement in inflammation. In human adipose tissue–derived mesenchymal stromal cells, the expression of the secreted phosphoprotein 1 gene (*SPP1*), the protein form of which is also known as osteopontin (OPN), is induced by *TGFB1* (29). OPN is a proinflammatory cytokine that promotes tissue infiltration of monocytes. Mice that lack *OPN* show a decreased macrophage infiltration in adipose tissue and a decreased level of inflammation, but insulin sensitivity is higher than in normal mice (37). OPN is the ligand for the receptor CD44, which is another member of the cluster. Similar to *SPP1*-knockout mice, *CD44*-knockout mice with high-fat diet–induced obesity show lower levels of adipose tissue inflammation and are protected against insulin resistance (38). In humans, CD44 density on adipose tissue macrophages was associated with their proinflammatory status. Another member of the second cluster, but also part of the overlapping third cluster, is PPIB, which is 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 toward various types of leukocytes (39). Cyclophilin B accumulates in the ECM but can be cleaved off by MMPs. Its signal receptor is CD147, encoded by basigin (BSG), and is known as the extracellular MMP inducer (40, 41). MMP activity is also influenced by OPN, because in *OPN*-knockout mice, reduced *MMP2* and *MMP9* activity was observed together with reduced ECM remodeling (42). In the present study, *MMP9* is on the intersection between the small and the 2 larger clusters. A role in adipose tissue remodeling has been suggested for MMP19, because *MMP19*-knockout mice develop diet-induced obesity with hypertrophic adipocytes (43). In addition to *PPIB*, *MMP9*, and *MMP19*, the second cluster contains other genes that function as processing enzymes: cathepsin B (*CTSB*), cathepsin D (*CTSD*), and protein disulfide isomerize family A, member 4 (*PDIA4*). Apparently, the second cluster harbors genes for leukocyte activity, insulin sensitivity, and ECM remodeling. Both the leukocyte infiltration–related genes *SPP1*, *CD44*, *CD33*, and *BSG/CD147* as well as the ECM remodeling genes *MMP9*, *MMP19*, *CTSB*, and *CTSD* are upregulated during WL and downregulated during WS. It suggests that although increased leukocyte infiltration with ECM remodeling occurs during WL, the reverse may happen during WS with a reduction in leukocytes and ECM remodeling. Our clustering analysis indicates that both processes are linked, which suggests that individuals with a stronger downregulation of ECM remodeling may retain more immune cells and have a 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 harbors 3 more protein processing genes: collagen β (1-O)galactosyltransferase 1 (*COLGALT1*), *PLOD1*, and prolyl 4-hydroxylase subunit β (*P4HB*). These genes are involved in the 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 fiber 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 gene (*NCSTN*). Adipocyte-specific loss-of-function of NCSTN reduces adipose insulin sensitivity (44). Together, the genes in the 3 connected and overlapping subclusters identify 2 biological processes in adipose tissue related to weight regain: inflammation and ECM remodeling, with a link to insulin sensitivity.

To the best of our knowledge, this is the first report that indicates the importance of adipose tissue leukocytes for the risk of weight regain after WL. Goyenechea et al. (45) reported on whole-body proinflammatory status in relation to weight regain by showing that subjects who regained weight had higher serum concentrations of tumor necrosis factor-alpha (TNFA) and mRNA levels of *TNFA* and nuclear transcription factor κB (*NFKB*) subunits in peripheral blood mononuclear cells. However, these measurements were performed after WL, thus under the influence of a negative energy balance. Capel et al. (15) used a different study design with a VLCD followed by an LCD, then followed by 3 mo of weight maintenance. They distinguished between expression changes in adipocyte metabolic genes and macrophage inflammatory-related genes. They reported that 7 of the clustered genes in our study were related to macrophage activity during DI: *CTSB*, *CTSS*, *ITGAM*, *MMP9*, *MMP19*, *SPP1*, and transforming growth factor β–induced (*TGFBI*).

The current findings point to the importance of leukocytes in adipose tissue for weight regain. A lower reduction in the expression of certain leukocyte integrin genes shortly after WL leads to a higher risk of weight regain, which seems to be linked to ECM remodeling. It is possible that a greater reduction in ECM remodeling capacity during the WS phase leads to more retention of immune cells. This suggests that resident inflammation after WL increases the risk of weight regain and might contribute to worsening of physiologic conditions during weight cycling (46). However, the present study provides only indicative results on the basis of gene expression. Furthermore, adipose tissue biopsy samples were used that contained 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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