Molecular Biomarkers for Weight Control in Obese Individuals Subjected to a Multiphase Dietary Intervention

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Context: Although calorie restriction has proven beneficial for weight loss, long-term weight control is variable between individuals.

Objective: To identify biomarkers of successful weight control during a dietary intervention (DI).

Design, Setting, and Participants: Adipose tissue (AT) transcriptomes were compared between 21 obese individuals who either maintained weight loss or regained weight during the DI. Results were validated on 310 individuals from the same study using quantitative reverse transcription polymerase chain reaction and protein levels of potential circulating biomarkers measured by enzyme-linked immunosorbent assay.

Intervention: Individuals underwent 8 weeks of low-calorie diet, then 6 months of *ad libitum* diet.

Outcome Measure: Weight changes at the end of the DI.

Results: We evaluated six genes that had altered expression during DI, encode secreted proteins, and have not previously been implicated in weight control (*EGFL6*, *FSTL3*, *CRYAB, TNMD*, *SPARC*, *IGFBP3*), as well as genes for which baseline expression differed between those with good and poor weight control (*ASPN*, *USP53*). Changes in plasma concentrations of EGFL6, FSTL3, and CRYAB mirrored AT messenger RNA expression; all decreased during DI in individuals with good weight control. *ASPN* and *USP53* had higher baseline expression in individuals who went on to have good weight control. Expression quantitative trait loci analysis found polymorphisms associated with expression levels of *USP53* in AT. A regulatory network was identified in which transforming growth factor β1 (TGF-β1) was responsible for downregulation of certain genes during DI in good controllers. Interestingly, ASPN is a TGF-β1 inhibitor.

Conclusions: We found circulating biomarkers associated with weight control that could influence weight management strategies and genes that may be prognostic for successful weight control. **(*J Clin Endocrinol Metab* 102: 2751–2761, 2017)**

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Abbreviations: AT, adipose tissue; BMI, body mass index; CR, calorie-restriction; DE, differential expression; DI, dietary intervention; eQTL, expression quantitative trait loci; FDR, false discovery rate; FST, follistatin; GCTA, genome-wide complex trait analysis; IGFBP3, insulinlike growth factor binding protein 3; IPA, Ingenuity Pathway Analysis; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SNP, single nucleotide polymorphism; SVF, stroma-vascular fraction; TGF-β1, transforming growth factor β1.

Obesity may appear to be a simple issue of increased body fat due to excess energy intake, but effective guidance for weight control is lacking. Although the extent to which calorie restriction induces weight loss is heterogeneous, subsequent weight control shows even greater interindividual variation, often remaining an obstacle. Most attempts to predict weight loss during—or, in particular, weight control after—calorie restriction have failed to provide useful predictive biomarkers (1). Adipose tissue (AT) plays a pivotal role in obesity-related complications. In addition to storing and releasing excess energy loads, AT secretes numerous bioactive factors, making it a potential source of biomarkers. Nutritional genomics can indicate how dietary interventions (DIs) affect AT (2), and gene expression studies in humans have shown that weight changes contribute to altered AT gene expression (3–8).

Here, we used AT from obese individuals who followed an 8-month DI consisting of an 8-week calorie-restriction (CR) diet with a 6-month *ad libitum* follow-up. Discovery analyses used transcriptomics to identify genes that were differentially expressed between individuals who successfully maintained weight loss (good controllers) and those who returned to baseline weight during follow-up (poor controllers). Validation analyses used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to confirm genes with differences in changes in expression between groups during DI and genes with different baseline expression that was potentially predictive of an individual's ability to successfully maintain weight loss. Finally, plasma protein levels were measured for validated genes that encode secreted proteins.

Materials and Methods

Subjects and clinical evaluation

Analyses used samples obtained from the DiOGenes Study (eight European centers); all participants signed an informed consent after verbal and written instructions (9). As shown in [Fig. 1(a)], overweight individuals followed a low-calorie (800 to 1000 kcal/d) diet for 8 weeks; those who lost ≥8% of their baseline weight were randomized to one of four *ad libitum* follow-up diets or a control diet for 6 months. Subcutaneous abdominal AT biopsies were obtained by needle aspiration 15 cm lateral of the umbilicus under local anesthesia after an overnight fast, at baseline, at end of CR, and at end of DI. Total RNA was extracted from AT (10).

AT fractionation and *ex vivo* cell culture

Subcutaneous abdominal AT from the periumbilical region was obtained from seven women [body mass index (BMI), 25.3 ± 4.5; aged 27 to 50 years] undergoing plastic surgery. The study was approved by the University Hospital of Toulouse ethics committee and conforms to the Declaration of Helsinki. From each AT sample, 1 g was flash frozen and stored at −80°C, and 10 g was digested using collagenase (11); adipocytes were separated from the stroma-vascular fraction (SVF) by washing and centrifugation. For use in gene expression analyses, adipocytes and SVF cells were homogenized in lysis buffer (miRNeasy kit; Qiagen) and stored at −80°C until RNA extraction. For use in secretion analyses, isolated packed adipocytes and SVF cells were maintained *ex vivo* at 37°C in endothelial culture basal medium with 0.1% fatty acid free bovine serum albumin at 2 mL (500,000) adipocytes in 10 mL of medium or 300,000 SVF cells per 1 mL of medium, respectively. These conditioned media were collected after 24 hours, centrifuged, and stored at −80°C.

Enzyme-linked immunosorbent assays

EGFL6 (csb-el007475hu, Cusabio; CliniSciences, Nanterre, France), FSTL3 (CEK1166; Cohesion Biosciences, CliniSciences), TNMD (csb-el024007hu; Cusabio), SPARC (CEK1325; Cohesion Biosciences), CRYAB (csb-el006008hu; Cusabio), and insulinlike growth factor binding protein 3 (IGFBP3; CEK1195, Cohesion Biosciences) were measured in duplicate following manufacturers' instructions.

Gene expression studies

Transcriptome microarray assays

Transcriptome analyses used AT RNA at two time points (baseline and end of DI) from individuals at the extremes of percentage weight change during DI (Supplemental Table 1; exclusion criteria in Supplemental Fig. 1) who were group-matched for baseline age, weight, BMI, waist circumference, blood pressure, and insulin resistance measured by homeostatic model assessment index for insulin resistance. As shown in [Fig. 1(a)-Path 1], 22 individuals were selected: 11 good controllers (maintained weight loss or continued to lose during follow-up) and 11 poor controllers (regained during follow-up, returning to baseline weight).

Whole Human Genome Microarray Kits 4 × 44K v2 were used according to manufacturer's recommendations (Agilent Technologies, Massy, France) (6). Arrays were scanned using the InnoScan®710 scanner (Innopsys, Carbonne, France), and images were quantified using MAPIX® v6.5.0 software (Innopsys). Microarray processing included background subtraction, loess intra-array normalization, and Gquantile inter-array normalization in limma (12).

qRT-PCR assays

In all, 346 individuals who completed the DI had good-quality AT RNA samples at all time points (baseline, after CR, and at end of DI) [Fig. 1(a)-Path 2]. Complementary DNA was prepared from 500 ng of total RNA and processed using the Biomark™ HD system with 96.96 Dynamic Array IFC (BioMark; Fluidigm) and TaqMan assays (Applied Biosystems, Life Technologies, Courtaboeuf, France) as described (10). Raw data from the default global threshold setting (BioMark Real-time PCR Analysis v4.1.1; Fluidigm Les Ulis, France) were checked using the graphical representation of plate layout. Duplicate raw cycle threshold values for the same gene were averaged, and then relative gene expression was calculated as $2^{-\Delta Ct}$.

Statistical analyses

Clinical characteristics

All analyses used R version 3.2.2. Differences between groups used the nonparametric Mann-Whitney *U* test. Robust

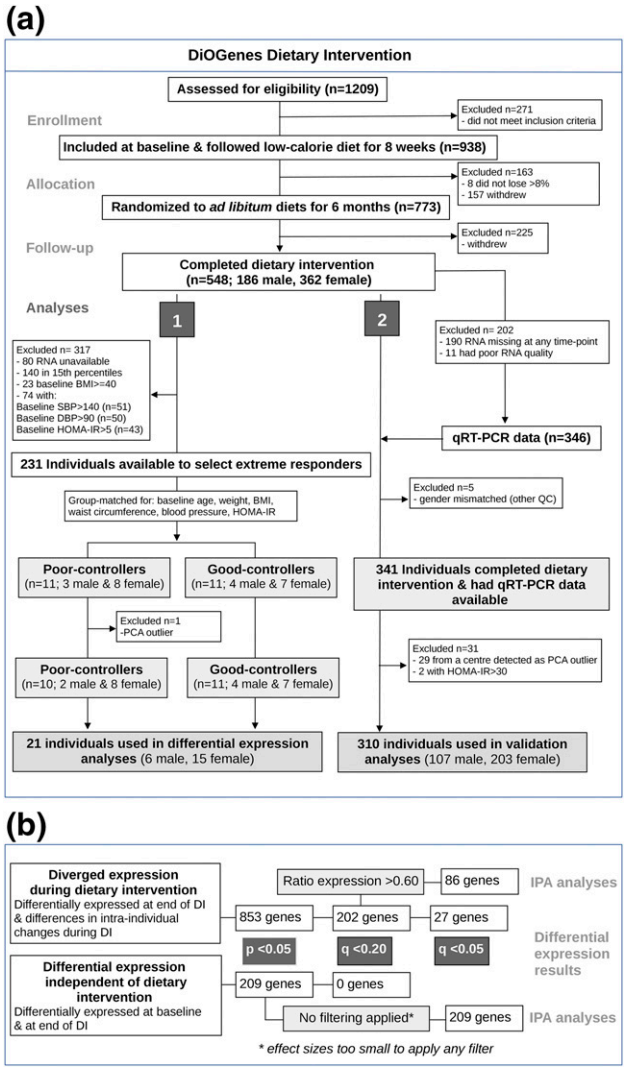

Figure 1. Flowcharts of DiOGenes study design and analyses results. (a) Flowchart of the DiOGenes Study population used in differential expression (1) and validation (2) analyses. Path 1: Selection of individuals to include in differential expression (transcriptome) analyses of extreme responders. Path 2: Selection of individuals for use in validation (qRT-PCR) analyses. (b) Flowchart of number of genes identified according to the level of significance applied for each: diverged expression during DI and DE independent of DI. Genes that showed diverged expression or were independent of DI required the stated level of significance at both time points. HOMA-IR, homeostatic model assessment index for insulin resistance; IPA, Ingenuity Pathway Analysis; LCD, low-calorie diet.

mixed analysis of variance with bootstrapping (13) with application of multiple trimmed group means (default level, 20%) was used to compare within-subject changes between groups, reporting the interaction effect.

AT transcriptome: differential expression

Principal component analysis of transcriptome data identified one individual as an outlier who was excluded; analyses included 11 good controllers and 10 poor controllers [Fig. 1(a)-Path 1]. Differential expression (DE) consisted of three separate analyses comparing $\log_2$ measures between groups (1) at baseline and (2) at end of DI and (3) intraindividual $\log_2$ fold-changes (log2FC) during DI. We aimed to identify (1) genes with differences in changes in expression between poor and good controllers during DI making the groups disparate at the end of DI and (2) genes whose baseline expression distinguished between poor controllers and good controllers. For both aims, we required DE at the end of DI to ensure that the groups did not converge.

Analyses used the limma package (13) and all 35,274 spots because limma relies on the spread of variances; prefiltering is not recommended. Duplicate probes were removed after modeling, keeping those with the smallest P value (27,385 unique probes). Correction for multiple testing used q-values, calculated using a q-value package (14) on gene-level data (18,568 Entrez Genes). q-Values are false discovery rate (FDR)−based measures of significance estimated from the distribution of P values, which represent the expected proportion of significant results that are false leads (*i.e.*, false positives). Comparisons of results always represent the same probe.

qRT-PCR validation

As shown in [Fig. 1(a)-Path 2], qRT-PCR expression was measured for 310 individuals from the DiOGenes Study. Linear regression models used age, sex, and change in BMI during DI as predictors and either baseline expression or intraindividual log2FC during DI as dependent variable. For the latter, additional models added baseline BMI or an interaction with baseline BMI. Principal component analysis of qRT-PCR expression at end of DI showed that diet had no effect on global gene expression and that addition of diet as a random effect to the aforementioned models did not alter results. Addition of center as a random effect did not affect results when intraindividual changes in expression were used or top results when baseline expression was used.

Expression quantitative trait loci analysis

Genome-wide associations used the Illumina 660 chip imputed with European 1000 Genomes (GRCh37) using Minimac3 (15) and baseline expression measured by qRT-PCR in 346 individuals. eQTL (expression quantitative trait loci) associations between single nucleotide polymorphisms (SNPs) and gene expression used linear mixed models. Transformed gene expression residuals from regression on age, sex, BMI, and center were used as dependent variables and individual SNPs as independent variables. Genome-wide complex trait analysis (GCTA; 16) was used for linear mixed model computation with the "loco" option to avoid deflation of test statistics. *Post hoc* power calculation was performed on the basis of criteria pertaining to our top result (powerEQTL package). The Genotype-Tissue Expression portal (version 4.1, build 201), a database of human genome expression and regulation (17), was used to confirm results, reporting single-tissue eQTL P values. LocusZoom (18) was used to display regional information from SNPs identified by eQTL analyses. eQTL was considered *cis* when the lead SNPs were within 1Mb of the gene, and $P < 5\text{E-}08$ was considered genomewide significant.

Pathway analyses

Ingenuity Pathway Analysis (IPA; Qiagen) was used to identify pathways and/or networks, using transcriptome expression ratios obtained from (1) DE results at the end of DI for 86 genes that diverged ($q < 0.20$ and absolute $\log_2$ expression ratios >0.6, representing genes for which expression was at least 50% higher in one group relative to the other) or (2)

baseline DE results for 209 genes that classified poor and good responders ($P < 0.05$) [Fig. 1(b)]. The 18,568 genes were used as a reference data set (direct and indirect relationships permitted), and genes reported as located in "Extracellular Space" were considered as encoding secreted factors.

Results

Clinical characteristics of poor and good controllers

There was no difference in baseline BMI between poor and good controllers ($P = 0.504$), nor were there any differences in clinical measures at baseline, although fasting fructosamine level was marginally higher in good controllers ($P = 0.078$; Supplemental Table 1). Good controllers lost more weight during CR than poor controllers did (13% vs 9%, respectively; $P < 0.001$). At the end of DI, good controllers showed improved health status, with significant differences between groups for changes in fat mass, waist circumference, SBP, low-density lipoprotein cholesterol, and C-reactive protein (Table 1).

Altered gene expression in response to DI

Discovery analysis using microarrays

We considered genes to have diverged expression if they were differentially expressed at the end of DI and had differences in fold-changes between poor and good controllers during DI, indicating differentially altered responses to weight changes during DI between groups. There were 202 diverged genes with $q < 0.20$; of these 27 had $q < 0.05$ [Fig. 1(b)]; 22 were downregulated and 5 were upregulated in good controllers but remained unchanged in poor controllers [Fig. 2(a); Table 2].

Pathway analysis

IPA analysis using 86 genes with diverged expression ($q < 0.20$ and expression ratio at end of DI >0.6) found a regulatory network centered on *TGFB1* because of higher expression of *LOX, LOXL2, LAMB3, SPARC, CCND1*, and *INHBB* in poor controllers at the end of DI (Supplemental Fig. 2). These genes were upregulated in

Table 1. Clinical Characteristics of Study Participants With Good or Poor Weight Control During DI

	Good Controllers (n = 11)				Poor Controllers (n = 10)				*P* Value
Sex, male/female	4/7				2/8				—
Diet (n)	1 (3), 2 (1), 3 (2), 5 (5)				1 (0), 2 (4), 3 (1), 5 (5)				—
	n	Mean (SD)			n	Mean (SD)			P^a
Age, y	11	44.5 (7.6)			10	46.7 (5.2)			0.777
Percentage weight lost, %	11	21.1 (2.0)			10	1.1 (0.7)			<0.001
LCD weight lost, kg	11	12.7 (4.6)			10	8.2 (1.5)			0.002
Percentage LCD weight lost, %	11	13.2 (3.5)			10	9.0 (1.1)			<0.001
	Baseline			After DI	Baseline			After DI	P^b
BMI, kg/m^2	11	33.0 (2.7)	11	26.0 (2.2)	10	32.2 (3.6)	10	31.9 (3.5)	<0.001
Total energy, kJ/d	11	9412 (3804)	5	7056 (2725)	9	8707 (2671)	8	8823 (2517)	0.062
Fat mass, %	9	37.5 (6.4)	11	29.3 (5.9)	8	40.1 (8.5)	10	37.9 (10.0)	<0.001
Waist circumference, cm	10	103.3 (13.4)	11	86.5 (9.6)	10	102.1 (5.5)	10	99.7 (4.9)	0.028
Waist/hip ratio	10	0.90 (0.14)	11	0.87 (0.08)	10	0.92 (0.09)	10	0.90 (0.08)	0.648
SBP, mm Hg	9	124 (11)	11	119 (10)	10	117 (13)	10	123 (12)	<0.001
DBP, mm Hg	9	73 (8)	11	71 (11)	10	69 (10)	10	74 (10)	0.072
Fasting cholesterol, mmol/L	11	4.97 (0.33)	11	4.73 (0.30)	10	5.07 (0.98)	10	5.50 (0.76)	0.058
Fasting HDL, mmol/L	11	1.45 (0.3)	11	1.59 (0.31)	10	1.33 (0.42)	10	1.51 (0.31)	0.563
Fasting LDL, mmol/L	11	3.02 (0.41)	11	2.68 (0.47)	10	3.17 (0.64)	10	3.43 (0.67)	0.031
Fasting TGs, mmol/L	11	1.12 (0.23)	11	1.02 (0.35)	10	1.27 (0.55)	10	1.25 (0.35)	0.058
Fasting fructosamine, μmol/L	11	216.3 (16.2)	11	224.6 (14.7)	10	204.8 (15.9)	10	215.6 (15.3)	0.802
Fasting adiponectin, μg/mL	11	10.49 (5.34)	11	13.64 (3.73)	10	11.05 (5.18)	10	11.49 (5.51)	0.292
Fasting CRP, mg/L	10	2.69 (1.24)	10	1.71 (2.34)	10	3.71 (2.86)	9	2.96 (1.38)	0.015
Fasting glucose, mmol/L	10	4.99 (0.64)	11	4.52 (0.27)	10	5.15 (0.43)	10	4.98 (0.43)	0.458
Fasting insulin, μIU/mL	11	8.11 (4.37)	7	5.98 (4.58)	9	9.23 (2.62)	10	9.85 (5.26)	0.208
HOMA-IR	10	1.96 (1.07)	7	1.19 (0.82)	10	2.27 (0.82)	10	2.12 (1.12)	0.565

Data are presented as mean + standard deviation (SD). Groups represent good and poor controllers used in microarray analyses.

Abbreviations: CRP, C-reactive protein; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment index for insulin resistance; LDL, low-density lipoprotein; SBP, systolic blood pressure; TG, triglyceride.

[a] P from Mann-Whitney *U* test.

[b] P from bootstrapped mixed robust analysis of variance (interaction term) testing, whether the intraindividual changes in measures between baseline and after the DI were different between the groups (Mann-Whitney *U* test comparing the groups at baseline and end of follow-up are available in Supplemental Table 1).

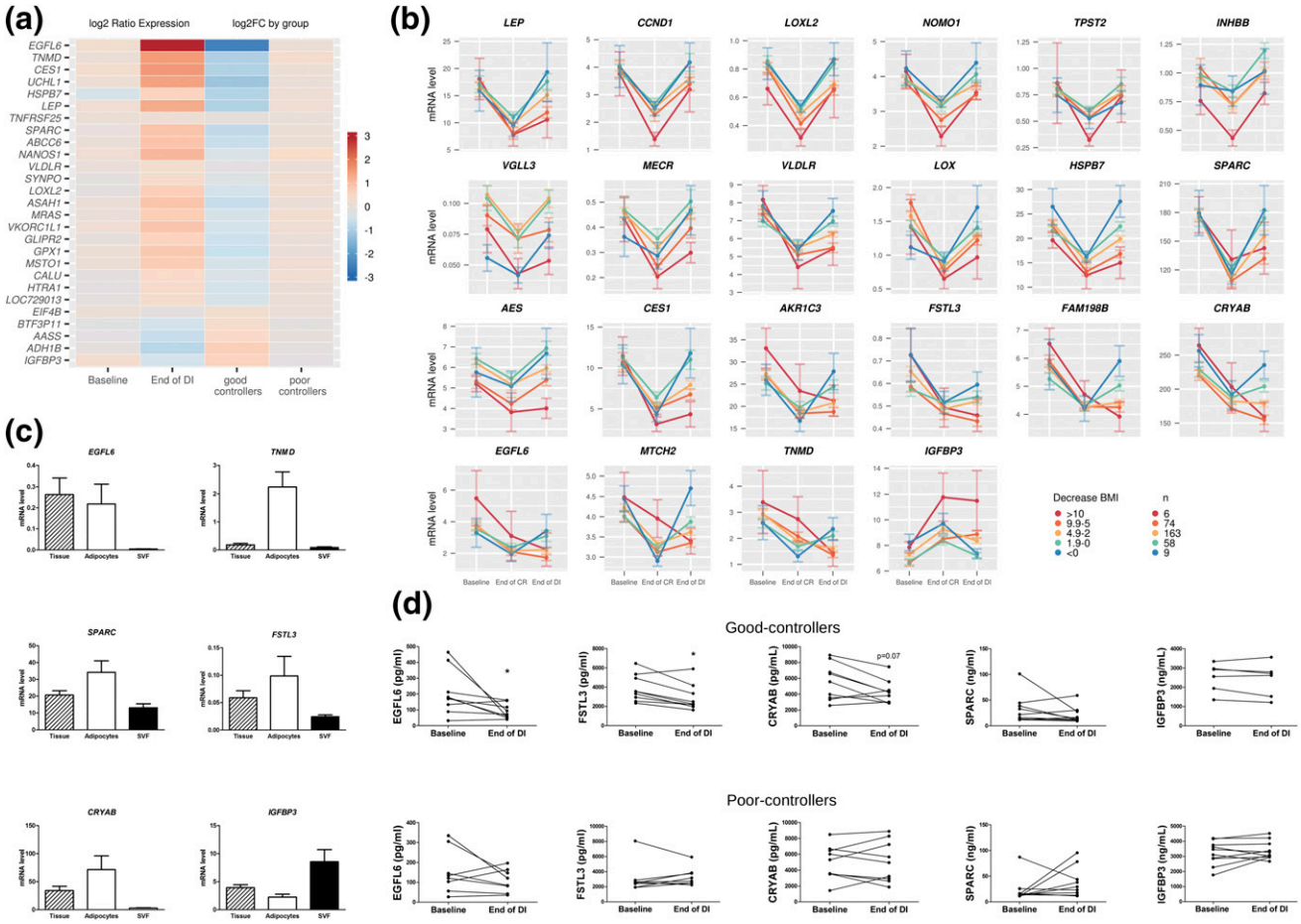

Figure 2. Evaluation of genes identified as having altered expression during the DI. (a) Heat map of expression ratios and fold-changes (FCs) for the 27 genes identified as significantly diverged ($q < 0.05$) during the DI. Summary of the top results obtained from DE analyses of microarray expression data (n = 21). The two columns on the left represent $\log_2$ ratios of expression (poor controllers/good controllers) at baseline and after the DI. The two columns on the right represent $\log_2$ FC during the DI for good controllers and poor controllers. Legend shows $\log_2$ values. (b) Evolution of expression for 22 genes validated by qRT-PCR. Evolution of relative expression measured by qRT-PCR (n = 310) at baseline, end of CR, and end of the DI, grouped by decrease in BMI during the DI and ordered by patterns of changes in expression. Points represent mean relative expression for each group, and bars represent mean ± standard error of the mean (SEM). The characteristics of the groups applied here are described in Supplemental Table 4. (c) Localization of expression in AT for genes encoding secreted proteins. Expression levels in AT, adipocytes, and SVF. Messenger RNA (mRNA) levels were measured in paired samples of freshly isolated adipocytes (n = 7) and SVF (n = 6) from human subcutaneous abdominal AT (n = 5). Data are presented as mean ± SEM. (d) Changes in plasma levels for genes encoding secreted proteins; intraindividual changes in plasma levels. Protein levels were measured in plasma samples obtained at baseline and at the end of the DI for individuals from the top and bottom fifth percentiles of changes in BMI during the DI (poor weight control, n = 7 to 9; good weight control, n = 8 to 9). TNMD is not included because all measures were below the detection limit (8 pg/mL).

poor controllers and downregulated in good controllers during DI (Supplemental Table 2).

Validation analyses using qRT-PCR

From the 27 diverged genes with $q < 0.05$, 14 had expression ratios >1.8 (a sufficiently large difference to be detected by qRT-PCR); after exclusion of five genes with low-quality/failed assays, nine genes remained for use in the validation. All those replicated by qRT-PCR *(EGFL6, TNMD, CES1, HSPB7, LEP, SPARC, VLDLR, LOXL2)* were downregulated, and *IGFBP3* was upregulated with increased weight loss during DI (Table 2). These genes were also associated with percentage weight lost during CR and percentage weight change during follow-up (all $P < 5\%$). We supplemented with 15 genes with $0.05 < q < 0.20$, for which we had assays available that either encode secreted proteins or previously showed associations with weight control (6), of which 13 were replicated by qRT-PCR; all were downregulated with increased weight loss during DI (Table 2). *AES, CCND1, CRYAB, FAM198B, FSTL3, INHBB,* and *LOX* were associated with percentage weight lost during CR ($P < 0.012$), and all except *FSTL3* were associated with percentage weight change during follow-up ($P < 0.010$) (Supplemental Table 3). Of these 22 genes, 19 (excluding *TNMD, NOMO1,* and *TPST2*) had significant associations with changes in fat mass, showing consistent directional effects with changes in BMI (Supplemental Table 3).

Figure 2(b) shows trajectories of expression during DI for the 22 validated genes, plotted by groups of decrease

Table 2. DE and Validation Results for Genes With DE Associated With Changes in BMI During DI

	Discovery Analyses (n = 21)			Validation Analyses (n = 310)	
	Ratio of log_2FC During DI[a]	q-Value	Mean log_2FC[b] (Poor/Good)	*b*[c]	*P* Value
Genes more highly expressed in poor controllers					
EGFL6[d]	2.70	0.037	0.16/−2.54	0.28	<0.001
TNMD	1.41	0.042	0.41/−1.00	0.13	<0.001
CES1	1.31	0.003	0.22/−1.10	0.82	<0.001
UCHL1	1.26	0.035	0.04/−1.22	NT	NT
HSPB7	1.16	0.011	0.21/−0.95	0.81	<0.001
LEP[d]	1.16	0.014	0.17/−0.99	0.99	<0.001
TNFRSF25	1.03	0.031	0.42/−0.61	NT	NT
SPARC[d]	0.96	0.042	0.16/−0.80	5.89	<0.001
ABCC6	0.90	0.015	0.14/−0.77	NT	NT
NANOS1	0.90	0.044	0.49/−0.41	NT	NT
VLDLR	0.88	0.042	0.28/−0.59	0.24	<0.001
SYNPO	0.87	0.050	0.33/−0.54	NT	NT
LOXL2[d]	0.86	0.006	0.32/−0.54	0.03	0.003
ASAH1	0.82	0.006	0.10/−0.73	NT	NT
MRAS	0.76	0.015	0.29/−0.48	NT	NT
VKORC1L1	0.74	0.026	0.25/−0.49	NT	NT
GLIPR2	0.73	0.050	0.12/−0.61	NT	NT
GPX1	0.72	0.042	0.03/−0.69	NT	NT
MSTO1	0.70	0.026	0.24/−0.46	NT	NT
CALU	0.64	0.045	0.27/−0.37	NT	NT
HTRA1[d]	0.63	0.024	0.12/−0.51	NT	NT
LOC729013	0.53	0.031	0.09/−0.44	NT	NT
AKR1C3	0.97	0.077	0.16/−0.80	1.11	<0.001
VGLL3	0.86	0.139	0.22/−0.64	0.004	0.036
FSTL3[d]	0.85	0.080	0.26/−0.59	0.01	0.021
MTCH2	0.81	0.085	0.28/−0.52	0.08	0.008
FAM198B	0.72	0.176	0.28/−0.44	0.22	<0.001
LOX	0.69	0.189	0.29/−0.40	0.09	<0.001
CRYAB[d]	0.63	0.156	0.14/−0.49	6.92	<0.001
CCND1	0.62	0.081	0.19/−0.43	0.21	<0.001
MECR	0.57	0.080	0.20/−0.38	0.02	0.001
TPST2	0.52	0.149	0.08/−0.45	0.03	0.016
NOMO1	0.44	0.124	0.14/−0.30	0.11	<0.001
INHBB	0.43	0.154	0.25/−0.19	0.04	0.002
AES	0.36	0.189	0.18/−0.18	0.19	0.001
Genes more highly expressed in good controllers					
EIF4B	−0.47	0.044	−0.09/0.38	NT	NT
BTF3P11	−0.56	0.035	−0.13/0.43	NT	NT
AASS	−0.56	0.035	−0.01/0.55	NT	NT
ADH1B	−0.85	0.015	−0.09/0.76	NT	NT
IGFBP3[d]	−0.89	0.003	−0.16/0.73	−0.33	<0.001

Abbreviation: NT, not tested and therefore no result to report.

[a] Ratio of log_2 fold-changes (log_2FC) in expression during DI = log_2FC poor controllers/log_2FC good controllers.

[b] Positive log_2FC means expression increased during DI.

[c] *b* for age- and sex-adjusted association between change in expression and change in BMI during DI (change calculated as end of DI − baseline). Represents change in expression during DI for 1-unit change in BMI.

[d] Genes that encode secreted proteins.

in BMI during the DI (Supplemental Table 4 shows the clinical characteristics of the groups). *IGFBP3* had an inverted profile, being upregulated during CR; then during follow-up, it had stabilized (higher) expression in individuals who had the greatest decreases in BMI and was downregulated in individuals who regained weight. All other genes were downregulated during CR. During follow-up, *LEP, SPARC, HSPB7,CES1, VLDLR, AES,* and *LOX* had stabilized (lower) expression in individuals who had the greatest decreases in BMI, whereas *EGFL6,*

TNMD, CRYAB, AKR1C3, FSTL3, FAM198B, and *MTCH2* had continued downregulation in individuals who had the greatest decreases in BMI.

Potential secreted biomarkers

To characterize potential circulating biomarkers, we measured expression of selected genes in adipocytes and SVF isolated from AT, checked for secretion, and measured secreted factors in plasma from individuals who decreased their BMI by >10 or <0, representing the fifth percentiles of change in BMI (Supplemental Table 2). *EGFL6, TNMD, SPARC, FSTL3,* and *CRYAB* were predominantly or exclusively expressed in adipocytes, whereas *IGFBP3* was predominantly expressed in the SVF [Fig. 2(c)]. Regarding secretion, EGFL6, SPARC, FSTL3, and CRYAB were detected in media from adipocytes but not in SVF; TNMD and IGFBP3 were detected in both media (Table 3). Figure 2(d) shows changes in plasma concentrations during DI, with significant decreases in EGFL6 (57%, $P = 0.03$), FSTL3 (26%, $P = 0.01$), and CRYAB (23%, $P = 0.07$) in the group that decreased BMI by >10 and no significant changes in the group with a change in BMI <0. There were no significant changes in plasma IGFBP3 or SPARC level in either group; TNMD was below the detection limit. We also found a positive correlation between BMI and FSTL3 levels in media from adipocytes ($r = 0.79$, $P < 0.05$; Supplemental Fig. 3) and plasma ($r = 0.52$, $P < 0.05$; data not shown).

DE independent of DI

Discovery analysis using a microarray

We identified 209 genes ($P < 0.05$) that were differentially expressed both at baseline and at the end of DI [Fig. 1(b)], thereby distinguishing between poor and good controllers independent of DI, making them potential predictors (Supplemental Table 5). IPA analysis did not return any significant results.

Validation analyses using qRT-PCR

For the 209 genes from the microarray study for which baseline expression classified between poor and good controllers ($P < 0.05$), FDR was 74% [Fig. 1(b)]. We nonetheless attempted to validate 17 genes using qRT-PCR. We selected 10 genes with the largest baseline expression ratios and enrichment for secreted proteins, and then supplemented them with seven genes for which we had assays available. Only ASPN and USP53 showed significant associations between baseline expression and changes in BMI during DI. Higher baseline expression of *ASPN* was associated with a greater decrease in BMI during DI ($P < 0.001$), as well as a higher baseline BMI ($P < 0.001$) and continued weight loss during follow-up ($P = 0.001$). When an interaction with baseline BMI was included, baseline expression of *USP53* was associated with changes in BMI during DI ($P = 0.012$; interaction $P = 0.008$), with higher expression in individuals who lost more weight during DI for individuals with higher baseline BMI. This association was attenuated when adjusted for center. There was also a positive association between baseline expression of *USP53* and baseline BMI ($P = 0.002$). Figure 3(a) shows the trajectories of expression during DI for *ASPN* and *USP53*, for which baseline expression was significantly associated with weight control after CR ($P < 0.002$) in 310 individuals.

Table 3. Localization of Secretion of Protein in AT Fractions

Adipokine	Adipocytes (pg/mL)	SVF (pg/mL)
EGFL6	26.8 ± 1.9	<19.5
FSTL3	25.9 ± 1.9	<10
CRYAB	107.3 ± 31.0	<3.2
SPARC	4630 ± 1199	<20
TNMD	4641 ± 1091	1090 ± 401
IGFBP3	1432 ± 357	451 ± 335

Adipokine concentration was measured in media from isolated adipocytes and SVF cells from human subcutaneous abdominal AT cultured for 24 hours (n = 7). Data are presented as mean ± standard error of the mean.

Properties of the validated classifiers

ASPN was predominantly expressed in the SVF, whereas *USP53* was expressed in both adipocytes and SVF [Fig. 3(b)]. No secretion of ASPN was detected because most samples were below the detection limit.

eQTL analysis

There was a genomewide significant *cis*-eQTL and an almost significant *trans*-eQTL between SNPs and baseline qRT-PCR expression of *USP53*. The *cis*-eQTL was downstream of *USP53* (lead SNP: rs2168987; $P = 3.1E-08$; minor allele frequency = 0.45), shown in Fig. 3(c). The minor T allele of rs2168987 was previously associated with higher *USP53* expression in AT (FDR <5%) (19). The *trans*-eQTL was on another chromosome, within *ZAK* (MAP3K MLT; lead SNP: rs3769187; $P = 2.3E-07$; minor allele frequency = 0.37). Retrospective power analyses showed that top results had 90% probability of being found.

Discussion

We aimed to identify biomarkers of weight control during a two-phase DI, including a CR phase and an *ad libitum* follow-up. To this end, we used AT transcriptomics to identify (1) genes with altered expression in response to weight changes and (2) genes for which baseline expression is indicative of successful weight control after CR. We validated results using qRT-PCR on a larger

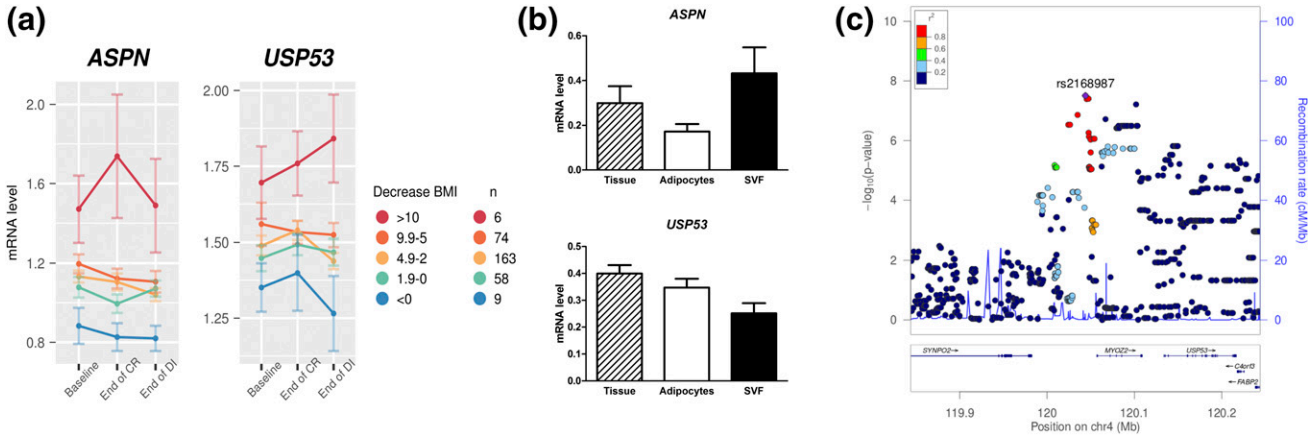

Figure 3. Evaluation of genes identified as classifiers of weight control independent of the DI. (a) Evolution of expression for ASPN and USP53 identified as classifiers as validated by qRT-PCR. Evolution of expression measured by qRT-PCR (n = 310) at baseline, end of CR, and end of the DI, grouped by decrease in BMI during the DI. Points represent mean relative expression for each group, and bars represent mean ± standard error of the mean (SEM). The characteristics of the groups applied here are described in Supplemental Table 2. (b) Localization of expression in AT cells for ASPN and USP53. Expression levels of ASPN and USP53 in AT, adipocytes, and SVF. mRNA level was determined in paired samples of freshly isolated adipocytes (n = 7) and SVF (n = 6) from human subcutaneous abdominal AT (n = 5). Data are presented as mean ± SEM. (c) LocusZoom plot of rs2168987 identified by eQTL analyses to be associated with expression of USP53. Plot showing the lead SNP identified by eQTL analysis as a purple diamond. The *y*-axis represents −log10 *P* values obtained from eQTL analyses, and the points are colored to represent correlation with the lead SNP. Points in red are interchangeable with the lead SNP, whereas points in blue are independent.

cohort from the same study and focused on genes encoding secreted proteins. Discovery analysis compared groups of extreme responders (11 good controllers, 10 poor controllers); to make use of a small sample size, we applied relaxed selection criteria and assessed the robustness of the identified genes in a larger (n = 310) replication cohort. We did not adjust for caloric intake because data were missing for almost half of individuals. Among the 24 diverged genes tested (nine with a q-value <0.05; 15 with a q-value <0.20), 22 were confirmed using qRT-PCR in AT, including three as circulating biomarkers, as altered in response to weight changes during DI. Among the 17 differential genes independent of DI tested for which baseline expression was indicative of weight control after CR, two genes were validated. This is in accordance with a high FDR, which led us to use a relaxed selection criterion and is consistent with a low validation rate compared with the study of diverged expression during DI. Expression of these two genes was not altered during DI.

During microarray discovery data validation of diverged gene expression, qRT-PCR was performed at all time-points of the DI, including after CR. All but one of the 24 genes (*i.e.*, IGFBP3) showed downregulation during CR. This is in agreement with fat mass and adipocyte size reduction and adaptation of AT metabolism and is consistent with findings of previous studies (7, 10, 20, 21). *IGFBP3* was upregulated during CR and was subsequently downregulated with weight gain during follow-up. At the end of DI, individuals who decreased their BMI by >10 had 56% higher AT expression of *IGFBP3* than those who returned to baseline weight during follow-up. *IGFBP3* encodes the main insulinlike growth factor transport protein in blood and is known to inhibit adipogenesis (22) and repress transforming growth factor β1 (TGF-β1), a secreted cytokine in the TGF-β superfamily signaling pathway (23). Pathway analysis found a regulatory network controlled by *TGFB1*, although *TGFB1* was not identified in these analyses. We found that genes identified as diverged during DI were predominantly expressed in adipocytes rather than in the SVF. Among the best candidate genes was *TNMD*, encoding tenomodulin, a type II transmembrane glycoprotein, whose expression was downregulated in response to weight loss and has been positively correlated with BMI (24). TNMD is known to be required for adipocyte differentiation and has been suggested as a protective factor against insulin resistance by promoting hyperplasia and beneficial lipid storage in visceral AT (25). We focused further on top candidates encoding secreted proteins.

To evaluate potential as circulating biomarkers, we compared plasma protein levels from the fifth percentiles for changes in BMI during DI. Although there were no differences for IGFBP3 or SPARC, we found significant intraindividual decreases in circulating EGFL6, FSTL3, and CRYAB levels during DI in individuals who decreased their BMI >10, but no change in individuals who returned to baseline weight during follow-up. This was consistent with changes in AT expression of *EGFL6*, *FSTL3*, and *CRYAB*, suggesting that secretion from AT contributes to plasma levels of these proteins. *EGFL6* encodes epidermal growth factor-like domain multiple-6,

a member of the epidermal growth factor repeat superfamily. It has been suggested that this paracrine/autocrine growth factor of AT is an extracellular matrix protein (26). *EGFL6* has previously shown higher AT expression and secretion in obese vs lean individuals and downregulation in obese patients after surgery-induced weight loss and is potentially involved in the process of AT expansion and the development of obesity (26, 27). Here, we showed long-term downregulation of EGFL6 after CR-induced weight loss.

CRYAB, encoding an α-crystallin B chain, previously showed a positive association between BMI and AT expression and increased levels during adipogenesis (28). *FSTL3* encodes follistatinlike 3, a member of the follistatin (FST)-related protein family (29). FST is an adipokine with lower expression and secretion in obese women than in lean women (30). Both FST and FSTL3 are antagonists of activin and myostatin (31). FSTL3 is released by muscle (32) and AT (29). Here, we report a positive relationship between changes in BMI and *FSTL3* expression in AT, secretion by human adipocytes, and FSTL3 in plasma. Studies on FSTL3 null mice have shown a differential role for FST and FSTL3 on glucose homeostasis and body composition (33). Our observation of decreased *FSTL3* expression and plasma FSTL3 levels with greater decreases in BMI reveals a discrepancy between FST and FSTL3 in weight control.

A remarkable finding was the identification of genes for which baseline expression was associated with changes in BMI during DI. *ASPN* and *USP53* had higher baseline expression in individuals with better weight control after CR. *ASPN* (Asporin) had twofold higher baseline expression in individuals who went on to decrease their BMI by >10 points vs those who returned to baseline weight during follow-up, was unaltered during DI, and was predominantly expressed in the SVF of AT rather than in adipocytes. ASPN belongs to a family of leucine-rich repeat proteins associated with extracellular matrix and is expressed in many tissues (34). It has been suggested that extracellular matrix may constrain AT expandability (35), and here higher expression of *ASPN* in AT was relevant for prevention of weight (re)gain. ASPN is a TGF-β1 inhibitor (36). This corresponds with our positive association between baseline expression and decrease in BMI during DI, suggesting that increased inhibition of the TGF-β1 pathway by ASPN resulted in increased weight control after CR. *USP53* encodes ubiquitin specific peptidase 53, a tight junction-associated protein (37). The positive association between decrease in BMI during DI and baseline expression of *USP53* was dependent on baseline BMI, with a more pronounced effect in more obese individuals. *USP53* expression was also genetically controlled, with both *cis*- and *trans*-eQTL. Our results indicate that AT messenger RNA levels of *ASPN* and *USP53* might be of interest as prognostic indicators of long-term response to weight-reducing diets.

An interesting observation is the implication of TGF-β1, a multifunctional growth factor with profibrotic properties (34), both as a regulator of expression of certain genes that were downregulated during DI and as a target for ASPN, which had higher expression at baseline in individuals who successfully maintained weight loss. It has been suggested that excess fibrosis in AT may alter tissue remodeling and restrain fat mass loss (38). The consistency of these observations emphasizes the potential role of AT fibrosis in long-term weight control.

Here, we identified circulating biomarkers of weight control after CR that are secreted from adipocytes (EGFL6, CRYAB, and FSTL3). We also identified genes for which higher expression was associated with better weight control after weight loss (*ASPN* and *USP53*). For use as biomarkers, these genes and circulating factors now need to be evaluated in other cohorts.

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