Brief Genetic Analysis

Adipose Gene Expression Response of Lean and Obese Mice to Short-term Dietary Restriction

Evert M. van Schothorst, Jaap Keijer,† Jeroen L. A. Pennings,* Antoon Opperhuizen,* Charissa E. van den Brom,* Thomas Kohl,† Nicole L. W. Franssen-van Hal,† and Barbara Hoebee**

Abstract

VAN SCHOTHORST, EVERT M., JAAP KEIJER, JEROEN L. A. PENNINGS, ANTOON OPPERHUIZEN, CHARISSA E. VAN DEN BROM, THOMAS KOHL, NICOLE L. W. FRANSSEN-VAN HAL, AND BARBARA HOEBEE. Adipose gene expression response of lean and obese mice to short-term dietary restriction. *Obesity.* 2006; 14:974–979.

Overweight and obesity lead to higher morbidity risks, which are alleviated even by mild weight loss. To gain insight in the molecular effects of weight loss in adipose tissue, we analyzed the effects of short-term dietary restriction (DR) on mice fed a low-fat diet (lean mice) or a high-fat diet (obese mice). Female C57Bl6/J mice on both diets were on DR until an average body weight loss of 20%, which was achieved in 8 to 12 days depending on body weight at the start of DR. Plasma free fatty acids and blood glucose levels decreased significantly on DR. In the (restricted) low-fat diet groups, gene expression analysis using adipose-enriched cDNA microarrays revealed only two transcripts to be significant differentially expressed by DR: up-regulation of malic enzyme (*Mod1*) and down-regulation of major urinary protein 1 (*Mup1*). Real-time polymerase chain reaction analysis confirmed these findings and showed, for the high-fat diet groups, an identical expression pattern for *Mup1*, whereas *Mod1* showed an opposed gene expression pattern for the high-fat diet groups. In conclusion, initial weight loss induces transcriptional changes only in a very small number of adipose genes, which also depends on the (restricted) diet used.

Key words: microarray, caloric restriction, adipocyte, overweight

Obesity causes an increased risk of morbidity by, among others, diabetes, cardiovascular disease, certain types of cancer, and hypertension (1). This is especially relevant because the number of overweight (defined as BMI > 25 kg/m^2) and obese (BMI > 30 kg/m^2) people is growing rapidly worldwide (2). Although normal weight (BMI $= 18$ to 25 kg/m^2) is preferred, for those with a BMI >25 kg/m^2, even a 5% to 10% body weight loss increases human beneficial outcome [e.g., improved glycemic control, reduced blood pressure, and decreased triglyceride (TG) levels] (3,4). Dietary restriction (DR) is one of the main methods for reducing body fat mass and, at the same time, the complications of overweight and obesity. Recently, it has been shown that, even in normal weight humans, long-term DR results in significant improvement of cardiovascular risk parameters (5). Adipose tissue is the main tissue affected by weight gain. At present, it is not understood which metabolic pathways are affected during DR, whether this is similar for normal weight and obese individuals, or in what manner adipose tissue is affected.

Here, we analyzed whether improvement of blood parameters occurred in response to short-term DR in normal and obese C57BL/6J mice and whether concomitant gene expression changes occur in subcutaneous adipose tissue.

Physiology

Young adult, female mice received either a humanized control low-fat diet (LFD) (6) for 21 (LF1) or 28 days (LF2), or a high-fat diet (HFD) for 21 (HF1) or 33 days (HF2). Average body weight was significantly increased on

Received for review November 2, 2005.
Accepted in final form March 10, 2006.
The costs of publication of this article were defrayed, in part, by the payment of page charges. This article must, therefore, be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
*Laboratory of Toxicology, Pathology and Genetics, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; and †Food Bioactives Group, RIKILT-Institute of Food Safety, Wageningen, The Netherlands.
Address correspondence to Evert M. van Schothorst, Laboratory of Toxicology, Pathology, and Genetics, National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands.
E-mail: Evert.van.Schothorst@rivm.nl
Copyright © 2006 NAASO

[1] Nonstandard abbreviations: TG, triglyceride; DR, dietary restriction; (R)LFD: (restricted) low-fat diet; (R)HFD, (restricted) high-fat diet; WAT, white adipose tissue; FFA, free fatty acid; rt-PCR, real-time polymerase chain reaction; Mod1, malic enzyme, supernatant; Mup1, major urinary protein 1; DIO, diet-induced obesity.

Table 1. Phenotypic characteristics

	Significance	LF0 and HF0	LF1	LF2	RLF2	HF1	HF2	RHF2
Body weight at $t = 0$ (g)	NS	16.8 ± 3.8	17.9 ± 1.2	18.0 ± 1.3	18.1 ± 1.4	18.1 ± 1.2	18.0 ± 1.5	18.2 ± 1.7
Section day		0	21	28	28	21	33	33
Body weight at section (g)	‡		19.1 ± 1.0	19.8 ± 2.0	14.7 ± 0.7	21.0 ± 2.4	22.9 ± 4.5	16.8 ± 1.3
Weight gain vs. day 0 (g)			1.2 ± 0.2	1.9 ± 0.8	−3.4 ± 0.8	2.9 ± 1.2	4.9 ± 3.0	−1.4 ± 0.4
Total WAT (g)	*	0.53 ± 0.25	0.72 ± 0.10	1.02 ± 0.95	0.34 ± 0.03	1.05 ± 0.52	1.65 ± 0.99	0.70 ± 0.22
Subcutaneous WAT (g)	NS	0.28 ± 0.12	0.32 ± 0.02	0.52 ± 0.48	0.19 ± 0.02	0.48 ± 0.19	0.71 ± 0.38	0.33 ± 0.09
Gonadal WAT (g)	*	0.17 ± 0.09	0.27 ± 0.08	0.31 ± 0.29	0.09 ± 0.01	0.38 ± 0.22	0.57 ± 0.34	0.22 ± 0.10
Visceral WAT (g)	*	0.08 ± 0.04	0.13 ± 0.01	0.19 ± 0.18	0.06 ± 0.02	0.19 ± 0.11	0.37 ± 0.27	0.15 ± 0.04
iBAT (g)	*	0.25 ± 0.02	0.34 ± 0.03	0.37 ± 0.19	0.20 ± 0.08	0.42 ± 0.06	0.58 ± 0.18	0.34 ± 0.07
Liver (g)	NS	0.63 ± 0.23	0.80 ± 0.04	0.74 ± 0.02	0.84 ± 0.03	0.74 ± 0.03	0.99 ± 0.17	0.83 ± 0.03
Kidney (g)	†	0.20 ± 0.03	0.27 ± 0.01	0.24 ± 0.02	0.27 ± 0.03	0.30 ± 0.04	0.36 ± 0.04	0.31 ± 0.00
Heart (g)	‡	0.12 ± 0.03	0.20 ± 0.02	0.17 ± 0.01	0.19 ± 0.02	0.21 ± 0.05	0.29 ± 0.02	0.27 ± 0.03
Lungs (g)	‡	0.14 ± 0.02	0.18 ± 0.01	0.16 ± 0.01	0.17 ± 0.02	0.18 ± 0.02	0.25 ± 0.02	0.23 ± 0.01
Quadriceps (g)	†	0.13 ± 0.04	0.18 ± 0.01	0.13 ± 0.01	0.18 ± 0.02	0.18 ± 0.03	0.25 ± 0.01	0.21 ± 0.03
Blood glucose (mM)	*	ND	5.7 ± 1.3	7.4 ± 1.2	5.5 ± 0.2	6.9 ± 1.7	9.6 ± 0.4	6.4 ± 1.7
Plasma TGs (mM)	NS	0.57 ± 0.09	0.93 ± 0.31	0.87 ± 0.45	0.72 ± 0.14	0.78 ± 0.26	1.00 ± 0.30	0.85 ± 0.25
Plasma FFAs (mM)	‡	0.78 ± 0.20	0.67 ± 0.07	0.92 ± 0.00	0.82 ± 0.11	0.73 ± 0.05	0.95 ± 0.06	0.57 ± 0.09

All values are given as mean ± standard deviation. LF, low fat; HF, high fat; LF1, LF diet for 21 days; LF2, LF diet for 28 days; RLF2, restricted LF diet for 7 days; HF1, HF diet for 21 days; HF2, HF diet for 33 days; RHF2, restricted HF diet for 12 days; WAT, white adipose tissue; iBAT, interscapular brown adipose tissue; NS, not significant; ND, not determined;

Total WAT is the sum of subcutaneous, gonadal, and visceral WAT weights.

Statistically significant differences are given as * $p < 0.05$), † $p < 0.01$, or ‡ $p < 0.001$.

Table 2. Differential gene expression within LFD groups

Gene	Analysis	LF1/LF0	RLF2/LF0	RLF2/LF1
Mup1	Microarray	−1.32	−3.29*	−2.50*
	rt-PCR	−0.80 ± 0.20	−1.87 ± 0.320	−2.32 ± 0.26*
Mod1	Microarray	0.99	2.57*	2.61*
	rt-PCR	1.45 ± 0.14	5.03 ± 0.71*	3.48 ± 0.49*

LFD, low-fat diet; LF1, LF diet for 21 days; RLF2, restricted LF diet for 7 days; *Mup1,* major urinary protein 1; *Mod1,* malic enzyme, supernatant; rt-PCR, real-time polymerase chain reaction. Fold ratio as observed by microarray and rt-PCR (mean ± standard deviation) analysis.
* Significant values ($p < 0.05$).

the HFD (Table 1). This was mainly caused by the significant increase in total white adipose tissue (WAT) weights, specifically subcutaneous and gonadal WAT (Table 1). Interscapular brown adipose tissue, some organs, plasma free fatty acids (FFAs), and blood glucose also showed a significant increase on the HFD (Table 1).

From Day 21 onward, 50% of the mice were DR to 67% of consumption before the LFD or HFD. The 33% reduction of caloric intake was based on an average human low calorie diet. DR was applied until a 20% body weight loss was observed to increase the feasibility of identifying gene expression changes. This resulted in a total time difference of 5 days between the restricted LFD (RLF2, 7-day restriction) and restricted HFD (RHF2, 12-day restriction) diet groups.

Weight loss decreased adipose tissue weights, and at the same time, blood glucose levels dropped significantly after DR to values observed at Day 21 compared with mice receiving an ad libitum diet (Table 1). Furthermore, it seems that weight gain followed by weight loss on an HFD (RHF2 group) results in a final body weight (16.8 grams) that is less than for mice on a LFD (19.1 to 19.8 grams; LF1–2), whereas glucose levels remained slightly increased compared with glucose levels of the LF1 group. This may suggest that a deregulation toward non–insulin-dependent type 2 diabetes might be initiated. Moreover, the increased glucose levels are not related to total WAT weight (RHF2: 0.70 ± 0.22 vs. LF1: 0.72 ± 0.10 grams; Table 1) or to relative weight of WAT (RHF2: 4.0 ± 0.9% vs. LF1: 3.8 ± 0.3%; data not shown). Both DR groups showed significantly less increase (or even a decrease) in FFAs compared with ad libitum–fed mice (Table 1).

Gene Expression

To select differentially regulated genes, a two-tiered approach was used. First, adipose-enriched cDNA microarrays were used to analyze gene expression in subcutaneous adipose tissue for the LFD groups, followed by validation using real-time polymerase chain reaction (rt-PCR) analysis. Second, the identified genes were analyzed for differential expression in the HFD groups using rt-PCR. Each subcutaneous adipose tissue RNA sample was labeled in duplicate and hybridized against a common reference pool, and quality checks resulted in the LFD groups LF0, LF1, and RLF2 to be analyzed.

Between the two LFD groups, LF0 and LF1 (same diet, 3 weeks difference), no significant differentially regulated transcripts could be identified, although body weights and adipose tissue weights did increase (Table 1). However, two transcripts, each represented by several cDNA clones, were higher expressed in time and almost reached significance: the adipose tissue marker fatty acid binding protein 4 (aP2) and phosphoenolpyruvate carboxykinase (PEPCK) (data not shown), which corresponds to an increase of adipose tissue.

Two transcripts were significantly differentially expressed between the restricted (RLF2) and non-restricted control diet group (LF1): malic enzyme, supernatant (*Mod1*; Unigene Mm.148155) being up-regulated (fold ratio = 2.61), and major urinary protein 1 (*Mup1;* Unigene Mm. 347469) being down-regulated in the restricted group (fold ratio = −2.50). Both genes are represented by at least three cDNA clones on the microarray, further strengthening the observed expression changes. rt-PCR indeed confirmed the observed microarray results for both genes using Calnexin as reference gene (Table 2). Calnexin showed stable expression for all diet groups by microarray analysis (data not shown).

Analysis of these two genes using rt-PCR within the HFD groups revealed opposed differential gene expression of *Mod1* (Figure 1) for the two diet groups: HFD inhibited significantly *Mod1* gene expression (HF1 vs. HF0), and RHFD changed gene expression similarly. *Mup1* gene expression was decreased to an even greater extent on RHFD (Figure 1), which is in line with the (R)LFD patterns.

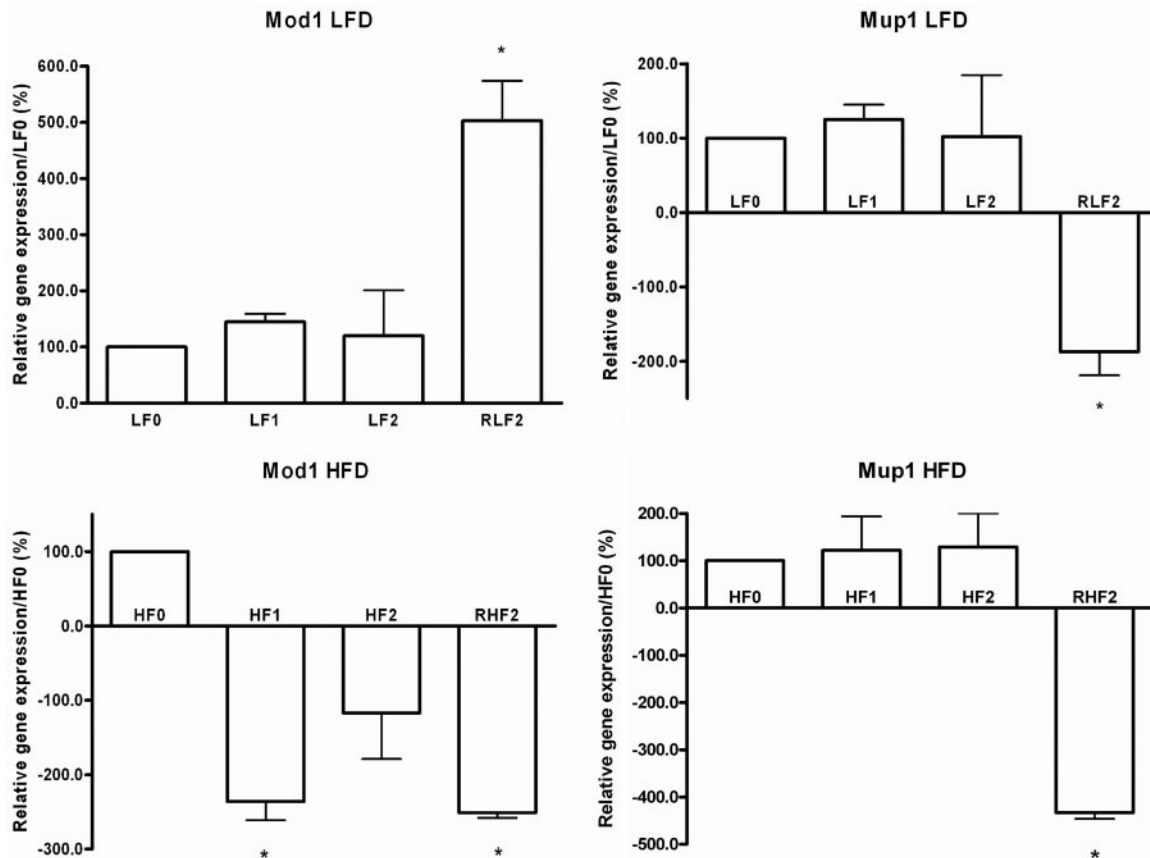

Figure 1: Differential gene expression of *Mod1* and *Mup1*. Relative gene expression analyzed by rt-PCR per diet group is given as indicated. Annotation of diet groups is identical to Table 1; of note, LF0 is identical to HF0 and its expression value is set to 100%. Values are mean ± standard deviation ($n = 3$, except RHF2: $n = 2$). * Significant values ($p < 0.05$).

The enzyme encoded by the *Mod1* gene plays a central role in adipose metabolism, linking gluconeogenesis and fatty acid metabolism by producing NADPH. Up-regulation of *Mod1* expression was also observed in epididymal WAT after 9 months of RLFD (7). Remarkably, *Mod1* was also significantly up-regulated in epididymal WAT during diet-induced obesity (DIO) (8,9), whereas we detected a down-regulation on initial weight gain by microarray and rt-PCR analysis.

In DR mice, several *Mup* family members, including *Mup1*, were found to be down-regulated in the liver (10, 11). We observed decreased expression in WAT of DR mice, independent of diet. *Mup1* has also been shown to be down-regulated in murine obese WAT (12). The precise function of *Mup1* within adipose tissue remains to be elucidated, but the protein belongs to the family of lipocalins. These proteins bind, for example, cytosolic fatty acids (13).

In general, several studies have indicated thus far that DIO alters a much larger set of transcripts than short-term DR does (6–10,12,14). The finding of an overall low total number of significant differentially regulated transcripts is supported by several recently published studies (7,10,15,16). A possible explanation might be that, during a short time interval, initial metabolic regulation is mainly regulated posttranscriptionally. Furthermore, in normal weight mice receiving DR for 23 days, just two transcripts were differentially expressed in epididymal WAT (7), but they do not match the two genes we identified in this study (R. Weindruch, personal communication). In humans, lack of differential adipose gene expression has also been observed in subcutaneous WAT of obese women after 2 days of DR, whereas after 28 days of DR, a total of 100 differentially regulated genes were identified (16). Finally, another possible reason of lacking differentially expressed transcripts in adipose tissue might be a time-dependent dilution effect; initially, only a subpopulation of slimming adipocytes might show adaptation, whereas the rest of the tissue is much less affected (17).

In conclusion, weight loss acquired by DR induced several metabolic changes as observed by blood glucose and FFAs. However, this was accompanied by only two genes that were significantly differentially expressed in adipose tissue. *Mup1* displayed an identical expression pattern on both (restriction) diets, whereas *Mod1* showed a reverse pattern between LFD and HFD mice.

Research Methods and Procedures

Animals, Diet, and Plasma Measurements

Wildtype C57BL/6J female mice were purchased from Harlan (Zeist, The Netherlands) at 9 weeks of age. Housing facilities and animal handling were as described before, and access to water was ad libitum (6).

The four diets consisted of a humanized control LFD and a humanized HFD as described (6) and two restriction diets (RLFD and RHFD) that were adjusted for mineral and vitamin intake to prevent malnutrition. Both restriction diets were given in an amount (67%) calculated from individual average food intake during the first 21 days of the given LFD or HFD.

After a 1-week quarantine and acclimation period in which all mice received LFD, three mice were killed ($t = 0$, Group LF0 is, therefore, the same as HF0). The remaining mice were randomly divided into two groups, of which one half received an HFD ($n = 9$) and the other half received the LFD ($n = 9$). After 21 days, three mice of each group were killed (Groups LF1 and HF1), whereas 50% of the remaining mice were calorically restricted (RHFD and RLFD, respectively) and the other 50% were kept on the diet they received before. Mice on the same fat diet (HFD+RHFD and LFD+RLFD) were killed when the restricted group lost, on average, 20% body weight compared with Day 21. This was either at Day 28 (Groups LF2 and RLF2) or Day 33 (Groups HF2 and RHF2).

Dissection, organ removal, and plasma isolation were performed as described (6). Total TGs and FFAs were measured using a Hitachi 912 automatic analyzer (Roche, Almere, The Netherlands). Phenotypic characteristics were analyzed using a mixed model ANOVA as described, with $p < 0.05$ considered significant (6).

Microarray

RNA isolation, labeling, microarray hybridizations, and data analyses were performed as described before (6). Briefly, total RNA was labeled indirectly without amplification using an oligo-dT primer and amino allyl 2′-deoxyuridine 5′-triphosphate. Samples were labeled using Cy5, whereas a reference pool was labeled using Cy3. All samples were labeled in duplicate and individually hybridized using an adipose-enriched cDNA microarray. This microarray contains cDNA clones of a subtracted murine adipose tissue cDNA library, as well as several control genes (6). After hybridization, washing, and scanning, median spot data were compared between groups, and spots that showed a fold ratio > 2.0 with $p < 0.05$ (unpaired two-tailed *t* tests) were considered significant.

rt-PCR

Microarray results were validated by rt-PCR. From 1 μg of total RNA, cDNA was synthesized using the iScript cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) according to the protocol of the supplier. rt-PCR was performed for target genes and reference gene *Calnexin*, which showed stable expression for all diet groups by microarray analysis (data not shown); all primer pairs are intron-spanning and do not amplify genomic DNA (data not shown). Primer sequences are as follows—*Mod1* (NM_008615): forward 5′-GGGATTGCTCACTTGGTT-GTTATGG-3′, reverse 5′-GTTGGTTTTATCTTTTGAA-CAATGGCTTC-3′; *Mup1* (XM_135574): forward 5′-CCCAGAGAGTATATAAGGACAAGCAAAGG-3′, reverse 5′-AGTATGCCATTCCCCATTAATCTTTTCTAC-3′; *Calnexin* (NM_007597): forward 5′-GCAGCGACC-TATGATTGACAACC-3′, reverse 5′-GCTCCAAACCAA-TAGCACTGAAAGG-3′.

Levels of cDNA were determined using SYBR green in a MyiQ rt-PCR detection system (BioRad) at an annealing temperature of 58 °C. All samples were run in duplicate, and data analysis was performed according to the ΔΔCt method (18). Briefly, mean Ct values of each target gene per animal were normalized to the mean reference gene, compared with the average of the other group, converted to relative ratios, and averaged per diet group ($n = 3$). Statistical analyses used unpaired two-tailed Student's *t* tests, and $p < 0.05$ was considered significant.

Acknowledgments

We thank Cor W. Schot and Coen Moolenbeek for animal handling, Hans Cremers for expert help, Nico Nagelkerke for statistical support, and Edith Feskens for critical review. There was no funding/outside support for this study.

References

1. **Kopelman PG.** Obesity as a medical problem. *Nature.* 2000; 404:635–43.
2. **World Health Organization.** *Obesity: Preventing and Managing the Global Epidemic.* Geneva, Switzerland: World Health Organization; 1997.
3. **Goldstein DJ.** Beneficial health effects of modest weight loss. *Int J Obes Relat Metab Disord.* 1992;16:397–415.
4. **Van Gaal LF, Wauters MA, De Leeuw IH.** The beneficial effects of modest weight loss on cardiovascular risk factors. *Int J Obes Relat Metab Disord.* 1997;21(Suppl 1):S5–9.
5. **Fontana L, Meyer TE, Klein S, Holloszy JO.** Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proc Natl Acad Sci USA.* 2004; 101:6659–63.
6. **Van Schothorst EM, Franssen-van Hal NLW, Schaap MM, Pennings JLA, Hoebee B, Keijer J.** Gene expression patterns of weight gain using a subtracted adipose cDNA microarray. *Obes Res.* 2005;13:1031–41.
7. **Higami Y, Pugh TD, Page GP, Allison DB, Prolla TA, Weindruch R.** Adipose tissue energy metabolism: altered gene expression profile of mice subjected to long-term caloric restriction. *FASEB J.* 2004;18:415–7.
8. **Li J, Yu X, Pan W, Unger RH.** Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *Am J Physiol Endocrinol Metab.* 2002;282:E1334–41.

9. **Lopez IP, Marti A, Milagro FI, et al.** DNA microarray analysis of genes differentially expressed in diet-induced (cafeteria) obese rats. *Obes Res.* 2003;11:188–94.
10. **Dhahbi JM, Kim HJ, Mote PL, Beaver RJ, Spindler SR.** Temporal linkage between the phenotypic and genomic responses to caloric restriction. *Proc Natl Acad Sci USA.* 2004; 101:5524–9.
11. **Miller RA, Chang Y, Galecki AT, Al Regaiey K, Kopchick JJ, Bartke A.** Gene expression patterns in calorically restricted mice: partial overlap with long-lived mutant mice. *Mol Endocrinol.* 2002;16:2657–66.
12. **Nadler ST, Stoehr JP, Schueler KL, Tanimoto G, Yandell BS, Attie AD.** The expression of adipogenic genes is decreased in obesity and diabetes mellitus. *Proc Natl Acad Sci USA.* 2000;97:11371–6.
13. **Cavaggioni A, Mucignat-Caretta C.** Major urinary proteins, alpha(2U)-globulins and aphrodisin. *Biochim Biophys Acta.* 2000;1482:218–28.
14. **Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM.** Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J Biol Chem.* 2001;276:34167–74.
15. **Arai K, Soga T, Ohata H, Otagiri A, Shibasaki T.** Effects of food restriction on peroxisome proliferator-activated receptor-gamma and glucocorticoid receptor signaling in adipose tissues of normal rats. *Metabolism.* 2004;53: 28–36.
16. **Clement K, Viguerie N, Poitou C, et al.** Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J.* 2004;18:1657–69.
17. **Pagano C, Calcagno A, Giacomelli L, et al.** Molecular and morphometric description of adipose tissue during weight changes: a quantitative tool for assessment of tissue texture. *Int J Mol Med.* 2004;14:897–902.
18. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 2001;25:402–8.