

Biomarkers of Dietary Energy Restriction in Women at Increased Risk of Breast Cancer

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Abstract

Dietary energy restriction (DER) reduces risk of spontaneous mammary cancer in rodents. In humans, DER in premenopausal years seems to reduce risk of postmenopausal breast cancer. Markers of DER are required to develop acceptable DER regimens for breast cancer prevention. We therefore examined markers of DER in the breast, adipose tissue, and serum.

Nineteen overweight or obese women at moderately increased risk of breast cancer (lifetime risk, 1 in 6 to 1 in 3) ages between 35 and 45 were randomly allocated to DER [liquid diet, 3,656 kJ/d (864 kcal/d); $n = 10$] or asked to continue their normal eating patterns ($n = 9$) for one menstrual cycle. Biopsies of the breast and abdominal fat were taken before and after the intervention. RNA was extracted from whole tissues and breast epithelium (by laser capture microdissection) and hybridized to Affymetrix GeneChips. Longitudinal plasma and urine samples were collected before and after intervention, and metabolic profiles were generated using gas chromatography-mass spectrometry.

DER was associated with significant reductions in weight [$-7.0 (\pm 2.3)$ kg] and in alterations of serum biomarkers of breast cancer risk (insulin, leptin, total and low-density lipoprotein cholesterol, and triglycerides). In both abdominal and breast tissues, as well as isolated breast epithelial cells, genes involved in glycolytic and lipid synthesis pathways (including *stearoyl-CoA desaturase*, *fatty acid desaturase*, and *aldolase C*) were significantly down-regulated.

We conclude that reduced expressions of genes in the lipid metabolism and glycolytic pathways are detectable in breast tissue following DER, and these may represent targets for DER mimetics as effective chemoprophylactic agents.

Dietary energy restriction (DER) significantly reduces spontaneous mammary tumors in laboratory rodents irrespective of the type of macronutrient restricted (1). Observational studies indicate that weight loss in premenopausal or postmenopausal women reduces the risk of postmenopausal breast cancer (2, 3). For example, in the Iowa Women's Health Study, women who lost >5% of their body weight before menopause had 40% fewer breast cancers in the postmenopausal period compared with women who continued to gain weight (2). Postmenopausal weight loss of >10 kg reduced postmenopausal

breast cancer by 60% in the Nurses' Health Study, and >5% postmenopausal weight loss in the Iowa Women's Health Study was associated with a 25% reduction in risk (2, 3). Reduction in cancer risk is also seen after weight reduction after bariatric surgery (4–7). DER is associated with diverse cellular changes, including alteration of growth factor, signaling and metabolic pathways, and reduction of cell proliferation (8, 9), in animal studies. DER favorably alters serum markers of breast cancer risk, such as insulin, leptin, sex hormone binding globulin (SHBG), and lipids, in women who are overweight or

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obese (10–13). A recent study reported changes in the urinary and serum metabolome of long-term dietary-restricted dogs (14). Several groups have assessed the effects of energy restriction (1–3) and changes in macronutrient profiles (1, 3) on gene expression in s.c. abdominal adipose tissue. These studies were undertaken in obese men and women and were relatively short-term studies with periods of energy restriction of between 2 and 28 days (2) or of 5 (1) and 10 weeks (3). However, there has been, to our knowledge, no assessment of breast gene expression in response to DER (15–18). Of the many gene changes induced by DER, down-regulation of genes associated with fatty acid synthesis and desaturation is the most prominent.

In the study reported here, we sought to assess whether effects of DER are apparent in breast tissue of overweight and obese women at increased risk of breast cancer to begin to understand a possible molecular mechanism for DER in breast cancer prevention.

Materials and Methods

Subject selection

Nineteen premenopausal overweight or obese [body mass index (BMI) between 28 and 40 kg/m²] parous women ages between 35 and 45 y at increased risk of breast cancer (estimated lifetime risk, 1 in 6 to 1 in 3; ref. 19) were recruited from our Family History Clinic and randomized to DER or to continue normal diet (Fig. 1). Other entry criteria included a normal mammogram within 24 mo, stable or increasing weight, and sedentary lifestyle (not participating in >40 min of moderate activity per week). Exclusion criteria included already losing weight following restrictive diets; taking dietary supplements; recent use of tamoxifen; regular use of anti-inflammatory, anticoagulant, antiplatelet, or oral contraceptive medication; pregnant or planning a pregnancy; previous hysterectomy; and serious comorbid conditions such as a previous diagnosis of cancer, diabetes, ischemic vascular disease, thyroid disease, or psychiatric disorders. Seventy-eight volunteers were eligible but 59 decided not to participate, mainly after disliking a 2-d test diet or on learning the invasiveness of the protocol. Although the 59 nonparticipating women were eligible in terms of weight and other criteria, it is unknown how they compared with participating women for the other characteristics measured in Table 1. The study was reviewed by the Bolton (Lancashire) Local Research Ethics Committee (Number 05/Q1409/42). All participants provided written informed consent before participation.

Study protocol

Breast and s.c. abdominal biopsies and fasting blood and urine were collected in the follicular phase shortly after the beginning of menses for two subsequent menstrual cycles to avoid widely fluctuating hormone levels producing confounding alterations in gene expression (20). Weight and total body fat (bioelectrical impedance, Tanita TBF300a), waist, hip, and bust measurements were also measured on this occasion. Three measurements of waist, hip, and bust size were made using standardized methods to the nearest 1.00 mm, and the mean of these measurements was used. Waist was measured at the level of the umbilicus and hip at the widest point over the trochanters. Dietary intake and physical activity for 7 d before each assessment were assessed from a 7-d diet history and physical activity recall (21, 22). Energy and macronutrient intake of reported diets were assessed using the five-diet analysis program (Compeat). After the first biopsies, participants were randomized to either DER ($n = 10$) or a control group ($n = 9$) who were advised to maintain their usual eating and exercise pattern. DER consisted of four 325 mL cans of a commercially available nutritionally complete milk shake ("Slimfast," Unilever) and 2 to 3 L of other clear low-energy, high-potassium fluids

(water, black tea, coffee, low-calorie soft drinks, or stock-based drinks). Overall, the diet provided on average 3,614 kJ/d (864 kcal/d); 26% (58 g) of energy was from protein, 62% (97 g) from carbohydrate, 12% (26 g) from fat, and 7% (6.4 g) from saturated fat (Fig. 1). Participants randomized to DER were monitored weekly by the study dietitians to encourage good compliance, whereas those on their usual diet were monitored after 2 wk to check that weight was being maintained.

Biopsy procedures

Breast. Recent craniocaudal and mediolateral oblique mammograms of each participant were reviewed to assess the pattern of breast tissue. Biopsies in women with a dense glandular breast pattern were done under radiographic guidance ($n = 7$). A single-coned craniocaudal mammogram of the breast was obtained at the time of biopsy and done with the breast immobilized under the compression device. In women with a diffusely fatty breast pattern ($n = 12$), biopsies were done without radiological guidance. After infiltration of 2% lidocaine, a small incision was made in the skin at the biopsy site through which a 14-gauge biopsy needle was inserted to a depth estimated by the operator. Between seven and nine biopsy samples were obtained through the same skin incision, although the direction of the needle was altered for each sample.

Abdominal fat. Three to five milliliters of s.c. abdominal adipose tissue were obtained by suction biopsy under local anesthesia from the anterior abdominal wall, midway between the anterior superior iliac spine and the umbilicus. The first biopsies were taken from either the left or right breast/abdominal side chosen by computer randomization (independently) and repeat biopsies on the opposite side to eliminate gene expression changes due to the healing process. One half of two separate breast cores were fixed in 4% formalin and embedded in paraffin blocks; the remaining tissue was immediately snap frozen in liquid nitrogen and stored at -80°C .

Blood measures

Hormone, glucose, and lipid assays, which may be influenced by DER, were undertaken at the Clinical Biochemistry Department of South Manchester University Hospital Foundation Trust: glucose (hexokinase/glucose-6-phosphate dehydrogenase method; Bayer Diagnostics), insulin [electrochemiluminescence immunoassay, Elecsys; Roche Diagnostics; interassay coefficient of variation (CV), 10.6%], testosterone (chemiluminescence; Chiron Diagnostics; CV, 6.5%), and SHBG (noncompetitive IRMA; IRMA-Orion Diagnostica; CV, 8.4%). Fasting insulin and glucose were combined to calculate the insulin sensitivity index using the homeostasis model assessment (23). Total serum insulin-like growth factor-I (enzyme-labeled chemiluminescent immunometric assay; Diagnostic Products Corp.; CV, 11.3%), serum testosterone (chemiluminescence), and estradiol (electrochemiluminescence immunoassay) were also measured. Colorimetric enzyme reactions were used to measure total cholesterol (CV, 3%), triglyceride (CV, 5.4%; Bayer Diagnostics), and high-density lipoprotein cholesterol (CV, 7.1%; Randox); the levels were measured spectrophotometrically by an automated Olympus AU600 analyzer. The adipokines leptin and adiponectin and markers of inflammation high-sensitivity C-reactive protein and sialic acid were determined at MRC Human Nutrition Research Laboratory (Cambridge, United Kingdom). Plasma leptin concentration was measured using an ELISA method (Quantikine Human Leptin kit, R&D Systems; CV, 10%), whereas plasma adiponectin was measured using RIA (LINCO Research, Inc.; CV, 10%). Sialic acid was assayed using a colorimetric assay (Roche Diagnostics; CV, 1.2%) adapted for use on the Hitachi 912 Clinical Analyzer (Roche Diagnostics) and C-reactive protein using a high-sensitivity, particle-enhanced turbidometric assay (CV, 4.5%; Dade Behring). Serum and plasma samples were aliquoted, stored at -70°C , and batched so that all samples from a participant were included in the same assay (24). Laboratory personnel were blinded to the sample identity. Baseline age, BMI, and body fat and changes in weight,

adiposity, diet and exercise parameters, and hormone and lipid levels were compared between groups using the Mann-Whitney test using Statistical Package for the Social Sciences version 14 (SPSS, Inc.).

Metabolomics

Serum (in 200 μL aliquots) samples were added to an internal standard solution (0.174 $\text{mg}\cdot\text{mL}^{-1}$ succinic d4 acid, 0.172 $\text{mg}\cdot\text{mL}^{-1}$ glycine d5, and 0.182 $\text{mg}\cdot\text{mL}^{-1}$ malonic d2 acid in water) and combined with 600 μL methanol, vortexed for 15 s, and centrifuged (15 min, 13,000 \times g), and the supernatant was lyophilized (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences). Urine samples were treated as for the serum, except 100 μL urease solution (10 $\text{mg}\cdot\text{mL}^{-1}$) was added to 75 μL aliquots incubated at 37°C for 30 min to enzymatically degrade urea and then combined with 250 μL methanol. Lyophilized samples were chemically derivatized in two stages. 50 μL of 20 mg/mL *O*-methylhydroxylamine solution in pyridine were added and heated at 40°C for 90 min followed by addition of 50 μL of *N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide and heating at 40°C for 90 min. A retention index solution was added for chromatographic alignment (20 μL , 0.6 mg/mL C10/C12/C15/C19/C22 *n*-alkanes in pyridine). Samples were subsequently analyzed in a random order using gas chromatography-time of flight-mass spectrometry (Agilent 6890 GC coupled to a LECO Pegasus III ToF mass spectrometer) as described previously using optimized settings for serum (25). Raw data were processed using LECO ChromaToF v2.12 and its associated chromatographic deconvolution algorithm, with the baseline set at 1.0, data point averaging of 3, and average peak width of 2.5. A reference database was prepared, incorporating the mass spectrum and retention index of all metabolite peaks detected in a random selection of samples so to allow detection of all metabolites present, whether expected or not expected from the study of metabolic pathways. Each metabolite peak in the reference database was searched for in each sample, and if matched (retention index deviation, $<\pm 10$; mass spectral match, >700), the peak area was reported and the response ratio relative to the internal standard (peak area-metabolite/peak area-succinic d₄ acid internal standard) was calculated. These data (matrix of *N* samples \times *P* metabolite peaks) representing normalized peak lists were exported in ASCII format. The

serum and urine data (analyzed separately) were split into DER and control groups for analysis with a two-sided paired Wilcoxon signed rank test for each metabolite using the null hypothesis that the difference (DER versus normal eating habits) in metabolite level comes from a continuous, symmetrical distribution with a median value of zero (26). Each significant ($P < 0.05$) metabolite was compared with its receiver operating characteristic for differentiating DER from controls.

RNA extraction

RNA was extracted from breast tissue using a protocol devised by M. Dowsett and A. Kendall (27). Briefly, 20 to 50 mg of breast tissue were ground to a fine powder under liquid nitrogen; RNA was isolated with 600 μL of Qiazol reagent (Qiagen Ltd.), which was passed through a commercially available RNA shearing device (QiaShredder, Qiagen); and 200 μL chloroform was added, incubated at room temperature for 5 min, and centrifuged at 12,000 rpm for 15 min. The aqueous phase was removed to a clean tube and 350 μL of 70% ethanol were added. The mixture was placed in an RNeasy Micro column (Qiagen) and the protocol for the RNeasy Micro kit was followed to completion. Abdominal adipose tissue total RNA was isolated using Trizol reagent (Invitrogen) according to the method described by Chomczynski and Sacchi (28). RNA samples were purified using RNeasy Mini columns (Qiagen) according to the manufacturer's protocols. RNA concentration and quality was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies). All whole tissue samples yielded RNA in sufficient quantity and quality for microarray analysis. Total RNA (2 μg) from abdominal tissue was processed according to the standard Affymetrix protocol for one-cycle target labeling to produce labeled cRNA. Total RNA (10 ng) isolated from the breast tissue core biopsies was amplified using a poly(A) PCR exponential amplification method based on the techniques described by Brady et al. (29) and Iscove et al. (30), which allow very small starting amounts of total RNA to be used. Samples were then hybridized to HGU133 Plus2 GeneChips (Affymetrix).

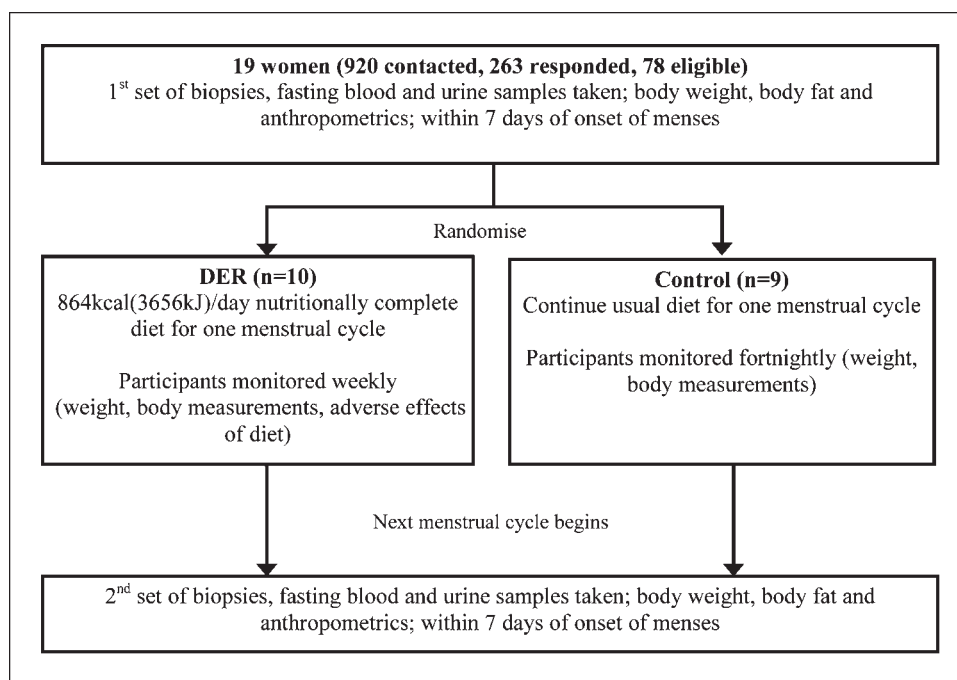


Fig. 1. Study design flowchart. Experimental design and sampling of the biopsies for the DER and control arms of the study.

Table 1. Changes in body weight, BMI, adiposity, lipid, and hormone levels in DER and control groups over the trial period

	Baseline measurements*		Change at 1 mo*		P†
	DER (n = 10)	Control (n = 9)	DER (n = 10)	Control (n = 9)	
Weight (kg)	93.3 (±10.5)	91.9 (±13.2)	-7.0 (±2.3)	0.3 (±1.5)	0.00
BMI (kg/m ²)	33.9 (±3.0)	32.6 (±2.4)	-2.5 (±0.8)	0.1 (±0.5)	0.00
Body fat (kg)	41.2 (±6.5)	40.7 (±9.0)	-4.6 (±1.5)	1.0 (±1.4)	0.00
Fat free mass (kg)	52.2 (±4.4)	51.2 (±4.3)	-2.4 (±1.3)	-0.4 (±0.9)	0.00
Body fat (%)	44.0 (±2.3)	43.8 (±3.4)	-1.9 (±1.2)	0.6 (±1.1)	0.00
Waist size (cm)	109.8 (±7.5)	104.5 (±11.6)	-6.6 (±6.0)	3.6 (±5.6)	0.00
Hip size (cm)	117.1 (±6.4)	115.0 (±8.6)	-5.8 (±2.6)	2.0 (±3.5)	0.00
Bust size (cm)	115.1 (±7.7)	111.8 (±6.3)	-4.1 (±2.6)	1.0 (±1.4)	0.00
Glucose (mmol/L)	4.4 (4.0-5.4)	4.8 (4.4-7.3)	0.0 (±0.4)	-0.1 (±1.2)	0.62
Testosterone (nmol/L)	0.9 (±0.5)	0.9 (±0.3)	0.0 (±-0.3)	0.0 (±-0.3)	0.90
Estrogen (pmol/L)	236.9 (124.0-1,265.0)	233.2 (138.0-996.0)	-165.9 (-906.0 to 196.0)	-96.7 (-664.0 to 458.0)	0.19
SHBG (nmol/L)	36.2 (20-138)	38.5 (28-51)	19.7 (±12.2)	0.3 (±5.1)	0.00
Cholesterol (mmol/L)	5.5 (±0.7)	4.8 (±0.9)	-1.2 (±0.5)	0.2 (±0.6)	0.00
Triglycerides (nmol/L)	1.5 (±0.5)	0.9 (±0.4)	-0.4 (±0.3)	0.1 (±0.5)	0.01
HDL (mmol/L)	1.4 (±0.2)	1.6 (±0.3)	-0.2 (±0.1)	0.0 (±0.3)	0.10
LDL (mmol/L)	3.4 (±0.7)	2.9 (±0.8)	-0.8 (±0.5)	0.2 (±0.5)	0.00
Insulin (pmol/L)	8.1 (±3.1)	8.3 (±3.3)	-2.6 (-7.4 to 3.4)	0.8 (-2.7 to 21.1)	0.14
Insulin sensitivity (µunit/mmol/L)	1.4 (0.5-2.4)	1.7 (1.0-4.8)	-0.4 (-1.3 to 0.7)	0.1 (-2.2 to 6.24)	0.32
IGF-I (µg/L)	155.3 (±35.6)	157.0 (±21.6)	-5.7 (±38.3)	5.7 (±21.5)	0.25
Leptin (ng/mL)	33.1 (±10.2)	26.9 (±13.0)	-21.0 (±6.8)	0.9 (±7.9)	0.00
Adiponectin (µg/mL)	14.5 (±6.1)	14.9 (±5.2)	-0.4 (±10.0)	-4.3 (±9.9)	0.48
Sialic acid (mg/L)	73.7 (±5.3)	72.1 (±8.3)	-2.3 (±7.5)	1.4 (±4.7)	0.13
Hs CRP (mg/L)	3.2 (±1.2)	3.2 (±1.5)	-0.3 (-3.2 to 8.5)	-0.5 (-2.6 to 4.9)	0.15

Abbreviations: Hs CRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IGF-I, insulin-like growth factor-I.

*Mean (±SD) or geometric mean (range).

†P value for changes in parameters in the control group compared with changes in the DER group using Mann-Whitney test.

Laser capture microdissection

Before laser capture, frozen sections were fixed and areas of interest were stained using the Arcturus Histogene frozen section staining kit (KIT0401), as per manufacturer's instructions. The laser capture microdissection was done on the Arcturus XT System. Microdissection was done by laser pulses activating a thermoplastic polymer film attached to an inverted CapSure "cap," which then expands and surrounds the cells of interest, allowing removal. Following cell capture, RNA was extracted and isolated using the Arcturus PicoPure RNA isolation kit (KIT0202) as per manufacturer's instructions. RNA was amplified using the Nugen WT-Ovation Pico System RNA amplification kit (Nugen), labeled, and hybridized to Affymetrix U133 Plus2 GeneChips as described in full on our Web site.⁶

Microarray analysis

After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. Ratios (3'/5') for glyceraldehyde-3-phosphate dehydrogenase and β-actin were confirmed to be within acceptable limits (0.86-1.38 and 0.70-1.24, respectively), and BioB spike controls were found to be present on all chips, with BioC, BioD, and CreX also pres-

ent in increasing intensity. All microarray data are MIAME compliant and accessible via MIAME VICE.⁷ Gene expression data were analyzed using packages within Bioconductor⁸ (31) implemented in the R statistical programming language. The gene expression data were normalized using the GeneChip Robust Multi-array Average algorithm (32) within the "simpleaffy" package (33) and then compared using multiclass significance analysis of microarrays (SAM; ref. 34) using the siggenes package. Baseline and repeat biopsies were paired and analyzed in the DER group and control group separately. The gene ontologies of significantly differentially expressed genes were examined using the GoMiner⁹ software (35).

Results

Ten women were randomized to DER, and 9 to their normal diet (median ages, 42.4 and 41.8 years, respectively), and median study periods were 26.2 and 26.9 days for the DER and control groups (Fig. 1). No woman withdrew from the study at any point. The biopsies were tolerated well, with no adverse side effects other than bruising at both the breast and abdominal

⁷ <http://bioinformatics.picr.man.ac.uk/vice/>

⁸ <http://www.bioconductor.org>

⁹ <http://discover.nci.nih.gov/gominer>

⁶ <http://bioinformatics.picr.man.ac.uk/mbcf/downloads/>

sites. The energy-restricted diet was well tolerated. The commonest side effects were constipation of variable degree ($n = 6$), eased in all cases by increasing intake of clear fluids, and halitosis ($n = 5$), which resolved by improving oral hygiene and was likely due to the thick texture and sugar content of the drink. There were no significant differences at baseline between the DER and control groups with respect to weight, BMI, body fat, calorie intake, dietary macronutrient composition, serum hormones, glucose, or inflammatory markers (Tables 1 and 2).

Change in anthropometry, hormones, and lipids

Compliance to the dietary regimens seemed to be good because all 10 women in the DER group lost weight (median loss of -7.0 , ± 2.3); in contrast, women in the control group showed minor fluctuations in weight with a median gain of 0.3 (± 1.5). Fat mass, fat free mass, waist, hip, and bust measurements decreased significantly in all of the women in the DER group; there was no change in these parameters in the control group (Table 1). As expected, the level of total and low-density lipoproteins, cholesterol, triglyceride, and leptin fell significantly and SHBG rose significantly in the DER group (Table 1). Women in both groups were sedentary and remained so throughout the study (Table 2).

Metabolomics

Of the 253 derivatized metabolite peaks detected, 83 could be definitively identified by matching the retention index (± 10) and mass spectral match (>700) of metabolite in sample to an authentic metabolite standard analyzed on the same analytical system. In addition, 49 were identified on a preliminary basis by matching (match >700) of mass spectrum only to metabolites present in available mass spectral libraries (NIST/EPA/NIH02 library and the publicly available MPI-Golm library).¹⁰ The metabolites that were significantly changed in plasma and urine for DER participants compared with controls are illustrated in Fig. 2 and were consistent with known effects of calorie restriction. A significant increase in glycolic acid in urine correlates with the reduction in glycolytic genes and increased lipid oxidation (Fig. 3). Increased urea and reduction in available amino acids, serine, and glycine in blood is concordant with the breakdown of proteins due to calorie restriction. There was also an increase in adipic acid and 3-hydroxybutanoic acid in urine following DER, suggesting increased lipid oxidation and the formation of ketone bodies. Observations of participants, however, suggested they were not ketotic, and the odor of ketones was not detectable on their breath. The low-energy diet chosen in this study was not expected to be ketogenic, containing 138 g/d of carbohydrates, which is far in excess of the minimum dietary intake of 50 g/d required to prevent ketosis (36).

Gene expression analyses

Microarrays were done on all breast and abdominal fat samples at time zero and after 1 month. In the control group, no probe sets were found to be consistently differentially expressed between baseline and repeat biopsies in either breast or abdominal adipose tissue using SAM software. Seventy-

two probe sets (representing 61 genes) were differentially expressed between baseline and repeat biopsies in the breast tissue in the DER group [with a false discovery rate (FDR) of 0.38], whereas 200 probe sets (representing 161 genes) were differentially expressed between baseline and repeat biopsies in the abdominal adipose tissue in response to DER (with a FDR of 0.01; full lists in Supplementary Tables S1 and S2). Choosing a lower FDR for the breast tissue resulted in no consistently significant genes being identified (due to greater tissue heterogeneity); conversely, a higher FDR for the abdominal tissue resulted in almost a quarter of the genes present on the array to be called significant. To ensure that the amplification step used for the breast tissue did not affect our findings, we also amplified RNA from the abdominal adipose tissue from three participants before and after DER and hybridized the samples to Affymetrix GeneChips; $>90\%$ of the top 200 probe sets identified using SAM analysis remained the same.

Many more genes were down-regulated than up-regulated with DER (56 of 61 in the breast and 113 of 161 in adipose tissue). Of the 61 genes significantly altered in the breast (Supplementary Table S1), 11 were also significantly altered in adipose tissue; several of these were represented by more than one probe set, showing reproducibility, although P values were lower for the abdominal tissue (Table 3). The most down-regulated genes in the breast [*stearoyl-CoA desaturase (SCD)*, *fatty acid desaturase 1 (FADS1)*, *transferrin*, and *aldolase C (ALDOC)*] were among those shared with adipose tissue. Other genes down-regulated in the breast were *osteonidogen*, *transmembrane protein 135*, *mitochondrial carrier triple repeat 1*, and *hexokinase 2*; the most down-regulated genes unique to adipose tissue were *ELOVL family member 6 elongation of fatty acids*, *secreted frizzled related protein2*, *cholesteryl ester transfer protein*, and *plasma and collagen type II $\alpha 1$* (Table 3).

Gene ontology and pathway analysis

Genes differentially expressed in both breast and abdominal tissue following DER were most frequently related to metabolism and energy pathways, with approximately one third of gene ontology terms common to both tissues (Supplementary Tables S3 and S4). Looking at genes within the highlighted pathways using GenMAPP,¹¹ it was clearly seen that almost all the individual genes involved in the glycolysis, tricarboxylic acid cycle, electron transport chain, and fatty acid metabolism were down-regulated in both adipose and breast tissue following DER. Many of these genes were significantly differentially expressed ($P < 0.05$). Several gluconeogenesis genes were up-regulated and genes within the fatty acid β -oxidation pathway were both up-regulated and down-regulated (summarized in Fig. 3).

Expression of epithelial genes in the breast tissue

Epithelial-specific genes (*keratin 14*, *keratin 18*, *CD24*, and *SC38GB202*) were expressed at high levels in all 38 breast samples but were low or not expressed in abdominal adipose tissue (Fig. 4), suggesting that some epithelium was present in all breast tissue hybridized to GeneChips. The expression of eight epithelial genes was also assessed by quantitative reverse

¹⁰ http://csbdb.mpimp-golm.mpg.de/csbdb/dload/dl_msri.html

¹¹ www.genmapp.org

Table 2. Dietary intake and physical activity in the DER and control groups at baseline and after the 1-mo trial period

Measurements*	Baseline measurements*		Change at 1 mo*		P [†]
	DER (n = 10)	Control (n = 9)	DER (n = 10)	Control (n = 9)	
Energy					
kJ	9,778.5 (±2,158.5)	9,342.8 (±1,606.6)	-5,829.5 (±1,990.0)	-230.0 (±1,374.1)	0.00
kcal	2,339.4 (±516.4)	2,235.1 (±384.3)	-1,394.7 (±476.1)	-55.0 (±328.7)	0.00
Protein (g)	85.4 (±16.9)	89.8 (±18.2)	-29.4 (±16.9)	-3.1 (±12.5)	0.00
Fat (g)	93.7 (±26.6)	84.0 (±17.5)	-82.7 (±26.6)	-3.2 (±17.2)	0.00
PUFA (g)	16.7 (±6.4)	16.0 (±4.0)	-14.7 (±6.4)	0.1 (±4.7)	0.00
MUFA (g)	30.6 (±9.9)	27.8 (±6.9)	-24.6 (±9.9)	1.0 (±5.8)	0.00
SFA (g)	34.5 (±11.0)	30.9 (±8.7)	-32.5 (±11.0)	-2.3 (±8.9)	0.00
Carbohydrates (g)	291.2 (±78.6)	272.2 (±67.9)	-153.2 (±78.6)	1.9 (±39.2)	0.00
Fiber (g)	15.7 (±2.9)	13.2 (±2.8)	3.9 (±2.9)	-0.5 (±2.6)	0.01
Alcohol (g)	10.8 (±11.2)	14.2 (±9.8)	-10.8 (±11.2)	-2.9 (±8.4)	0.09
% Energy from fat	35.8 (±4.0)	33.8 (±4.8)	-24.8 (±4.0)	-0.5 (±3.8)	0.00
% Energy from carbohydrate	46.5 (±6.6)	45.6 (±6.8)	13.5 (±6.6)	1.3 (±4.0)	0.00
% Energy from protein	14.9 (±2.4)	15.9 (±1.9)	11.1 (±2.4)	0.0 (±1.2)	0.00
% Energy from alcohol	3.2 (±3.0)	4.3 (±2.6)	-3.2 (±3.0)	-0.6 (±2.0)	0.00
Moderate activity, minutes/week (21)	77 (±66)	133 (±111)	-19 (-120 to 0.0)	-41 (-180 to 60)	0.63

Abbreviations: PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

*Mean (±SD or range).

[†]P value for changes in parameters in the control group compared with changes in the DER group using Mann-Whitney test.

transcription-PCR in the four quadrants of each breast from four women undergoing bilateral risk reduction mastectomy. Although there was some variation between sides, the differences between individuals were significantly greater, suggesting relatively lower heterogeneity between contralateral breasts in individuals (Supplementary Table S5).

Laser capture microdissection was then used to isolate breast epithelial cells from fixed frozen tissue, and sufficient RNA for GeneChip analysis was obtained from three matched samples before and after DER. The majority of expression changes for the highlighted DER genes were consistent between the microdissected epithelium and whole breast tissue for this limited number of samples. Using the MAS5 normalization method (37) to estimate whether particular probe sets are expressed (called "present," rather than absent), we determined that 93% (67 of 72) of the DER breast probe sets were called present using MAS5 normalization in at least three of the six microdissected samples. Just 2 of the 72 DER breast probe sets representing ABCC6 and LOC389393 were not called present in any of the epithelial-only samples (Supplementary Table S1). Using the same method, we also confirmed expression of >80% of the 72 DER breast genes in the ducts and lobules of at least half of the normal breast tissue samples isolated by laser capture microdissection in a study published by Turashvili et al. (Supplementary Table S1; ref. 38), suggesting that DER-regulated genes are expressed in breast epithelium.

Discussion

Because studies in rodents and observational studies in women indicate that DER reduces the risk of mammary can-

cer, we designed the study reported here to determine potential biomarkers of the effect and effectiveness of DER in women at increased risk of breast cancer (1–5). DER (~60% normal diet) was sufficient to favorably change known serum/urine biomarkers of breast cancer risk (insulin, leptin, and SHBG). Gene expression in breast tissue was also altered following DER, with some changes consistent with those in the adipose tissue. The metabolomic results seem to corroborate the changes in metabolic pathways seen at the gene expression level.

The tissue composition of the breast is variable and changes with age and during pregnancy. In parous women ages 35 to 45 years, it is estimated that the breast comprises 10% to 20% epithelium, with 60% to 80% fibroconnective tissue and 10% to 20% adipose tissue (39, 40). The proportion of adipose tissue is likely to be higher in overweight and obese women, such as those who were eligible for this study. It is not clear from our study whether the changes in gene expression common to the breast and abdominal tissue following DER affect the breast adipose tissue or breast epithelium directly. However, our limited laser capture microdissection study of pure epithelium and analysis of the RNA suggested a direct effect of DER on epithelial gene expression. A study of biopsies of normal breast tissue before and after letrozole showed multiple gene changes, but these investigators were also unable to distinguish between stromal and epithelial changes because of paucity of the latter (27). Further studies are required to establish tissue-specific gene expression changes within the breast.

The four most down-regulated genes in the breast were also down-regulated in abdominal adipose tissue, and three of the genes were related to the metabolic pathways of fat synthesis

(*SCD* and *FADS*) and glycolysis (*ALDOC*). *SCD* has been shown to be a highly down-regulated gene in s.c. adipose tissue in several DER studies (15, 16, 18). *SCD* is the gatekeeper to the formation of unsaturated fatty acids because it introduces a double bond in the 9 position in the saturated fatty

acids stearate and palmitate to form monounsaturated fatty acids oleic and palmitoleic acids. *SCD*-deficient mice are lean and have reduced lipid synthesis and enhanced lipid oxidation and insulin sensitivity (41). *SCD* knockdown reduces proliferation and Akt phosphorylation in cancer cell lines (42, 43).

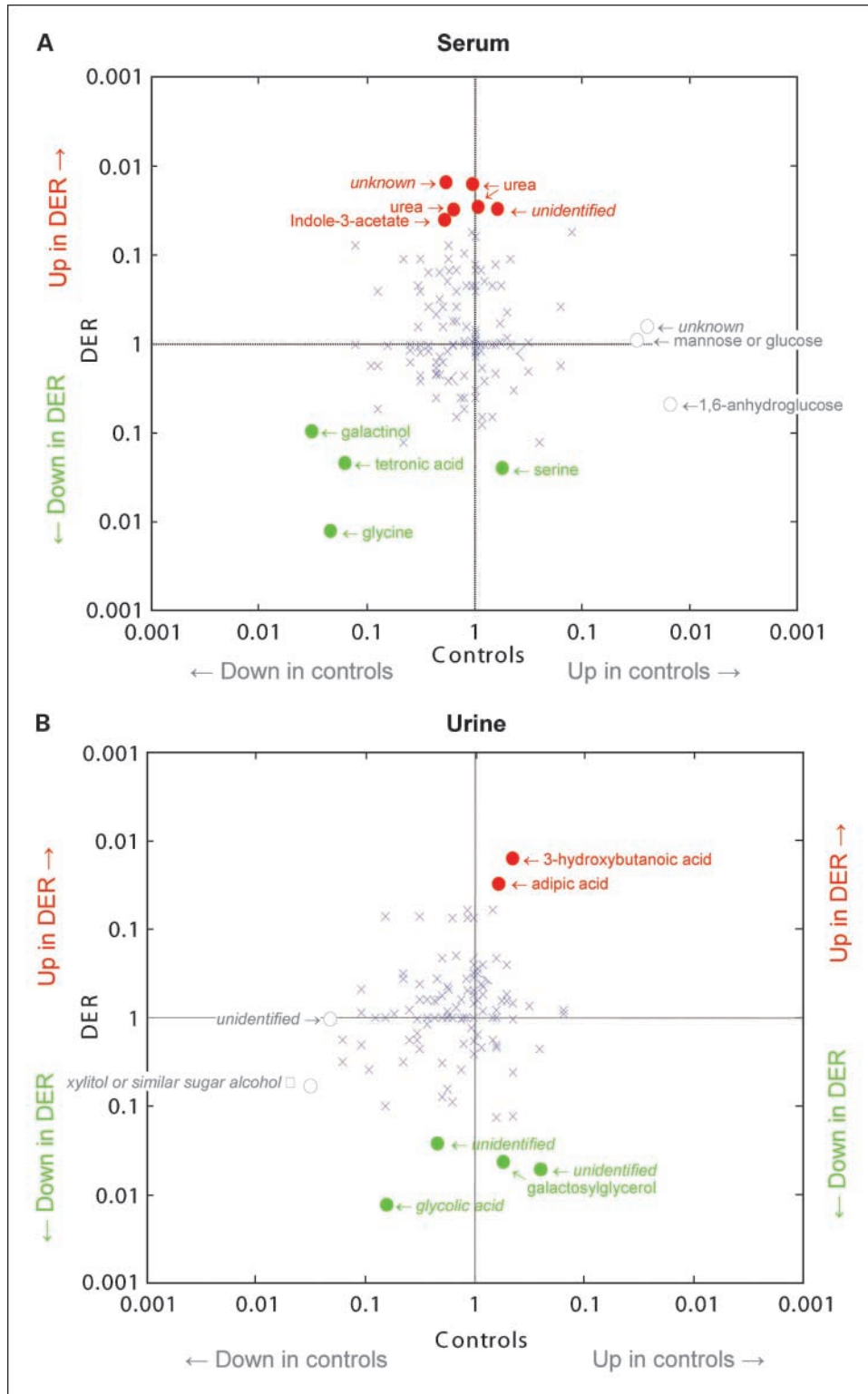


Fig. 2. Metabolomic analysis of serum (A) and urine (B) from DER and control women. •, metabolites that are significantly increased or decreased ($P < 0.05$) following the diet compared with the controls. Definitive metabolites are shown in plain text, and preliminary or unknown IDs are shown with an asterisk. Up in DER, sample population mean is higher after diet than before; Down in DER, sample population mean is lower after diet than before.

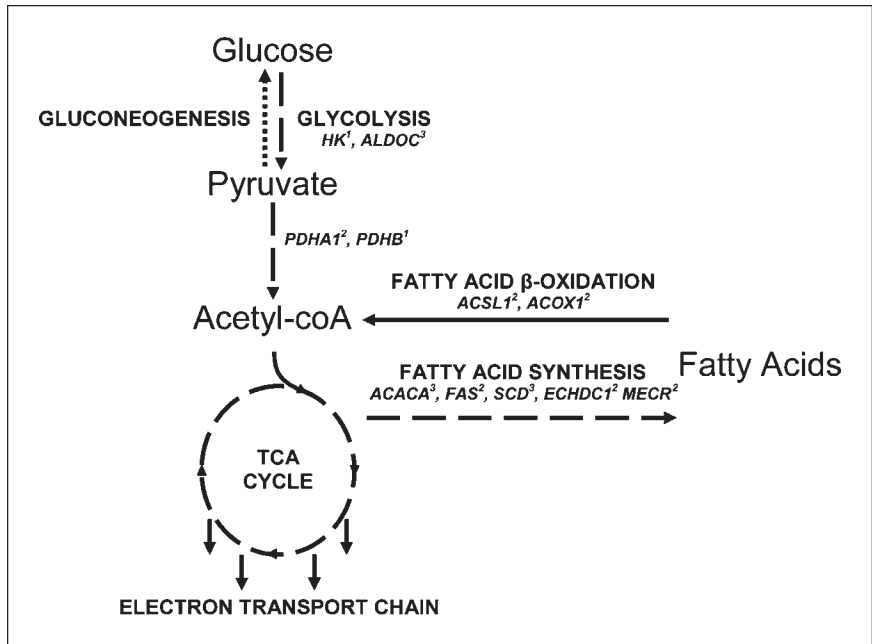


Fig. 3. Simplified representation of the glycolytic and fatty acid synthesis pathways following DER. Glycolysis, the tricarboxylic acid (TCA) cycle, electron transport chain, and fatty acid synthesis were all down-regulated (*long dashes*) and gluconeogenesis was up-regulated (*short dashes*). Genes involved with fatty acid β -oxidation were both up-regulated and down-regulated. Italics, enzymes that were down-regulated by SAM analysis in our study. Superscript numbers represent the tissue in which down-regulation occurred: 1, breast tissue; 2, adipose tissue; 3, both.

Inhibition of SCD by stearic acid reduces *N*-nitrosomethylurea-induced mammary tumor development in rats. A high unsaturated to saturated fat ratio in red cell membranes or serum phospholipids (indicating low SCD activity) is associated with reduced risk of breast cancer (44, 45). Thus, low SCD activity in the breast may not only be a marker of the effectiveness of

DER but also an indicator of reduced risk of breast cancer and a target for energy restriction mimetic agents (46).

We studied the effects of DER on a relatively homogenous group of women who were all of similar weight and age and were all parous. All biopsies, serum, and urine samples were taken at about the same day of each menstrual cycle

Table 3. Genes that were significantly differentially expressed between baseline and second biopsies in the DER group in both breast and adipose tissues using SAM analysis

Affymetrix probe set	Gene symbol	Description	Fold change (breast)	P (breast)	Fold change (adipose)	P (adipose)
211708_s_at	<i>SCD</i>	Stearoyl-CoA desaturase (δ -9-desaturase)	-16.7	0.00002	-4.3	0.0000026
211162_x_at	<i>SCD</i>	Stearoyl-CoA desaturase (δ -9-desaturase)	-8.3	0.00032	-4.3	0.0000090
208964_s_at	<i>FADS1</i>	Fatty acid desaturase 1	-3.7	0.00072	-2.2	0.0000004
208963_x_at	<i>FADS1</i>	Fatty acid desaturase 1	-3.8	0.00006	-2.0	0.0000044
200831_s_at	<i>SCD</i>	Stearoyl-CoA desaturase (δ -9-desaturase)	-4.2	0.00284	-3.2	0.0000371
214063_s_at	<i>TF</i>	Transferrin	-2.3	0.00230	-1.9	0.0000059
202022_at	<i>ALDOC</i>	Aldolase C, fructose-bisphosphate	-2.3	0.00015	-2.3	0.0000005
204776_at	<i>THBS4</i>	Thrombospondin 4	-1.8	0.00015	-2.1	0.0000551
214033_at	<i>ABCC6</i>	ATP-binding cassette, subfamily C (CFTR/MRP) 6	-1.8	0.00018	-1.6	0.0000360
202998_s_at	<i>LOXL2</i>	Lysyl oxidase-like 2	-1.8	0.00207	-1.3	0.0000353
203435_s_at	<i>MME</i>	Membrane metalloendopeptidase (CALLA, CD10)	-1.6	0.00003	-1.7	0.0000015
223839_s_at	<i>SCD</i>	Stearoyl-CoA desaturase (δ -9-desaturase)	-1.8	0.00029	-1.6	0.0000020
209600_s_at	<i>ACOX1</i>	Acyl-coenzyme A oxidase 1, palmitoyl	-1.6	0.00039	-1.5	0.0000024
229125_at	<i>ANKRD38</i>	Ankyrin repeat domain 38	-1.5	0.00255	-1.5	0.0000225
200832_s_at	<i>SCD</i>	Stearoyl-CoA desaturase (δ -9-desaturase)	-1.7	0.00020	-2.1	0.0000260
202709_at	<i>FMOD</i>	Fibromodulin	-1.3	0.00032	-1.8	0.0000296

NOTE: Two hundred probe sets representing 161 genes were significantly differentially expressed between baseline and repeat biopsies in the adipose tissue in response to DER with a FDR of 0.01. Seventy-two probe sets representing 61 genes were significantly differentially expressed between baseline and repeat biopsies in the breast tissue in the DER group at a FDR of 0.38. Full lists of differentially expressed genes in breast and adipose tissue are given in Supplementary Tables S5 and S6.

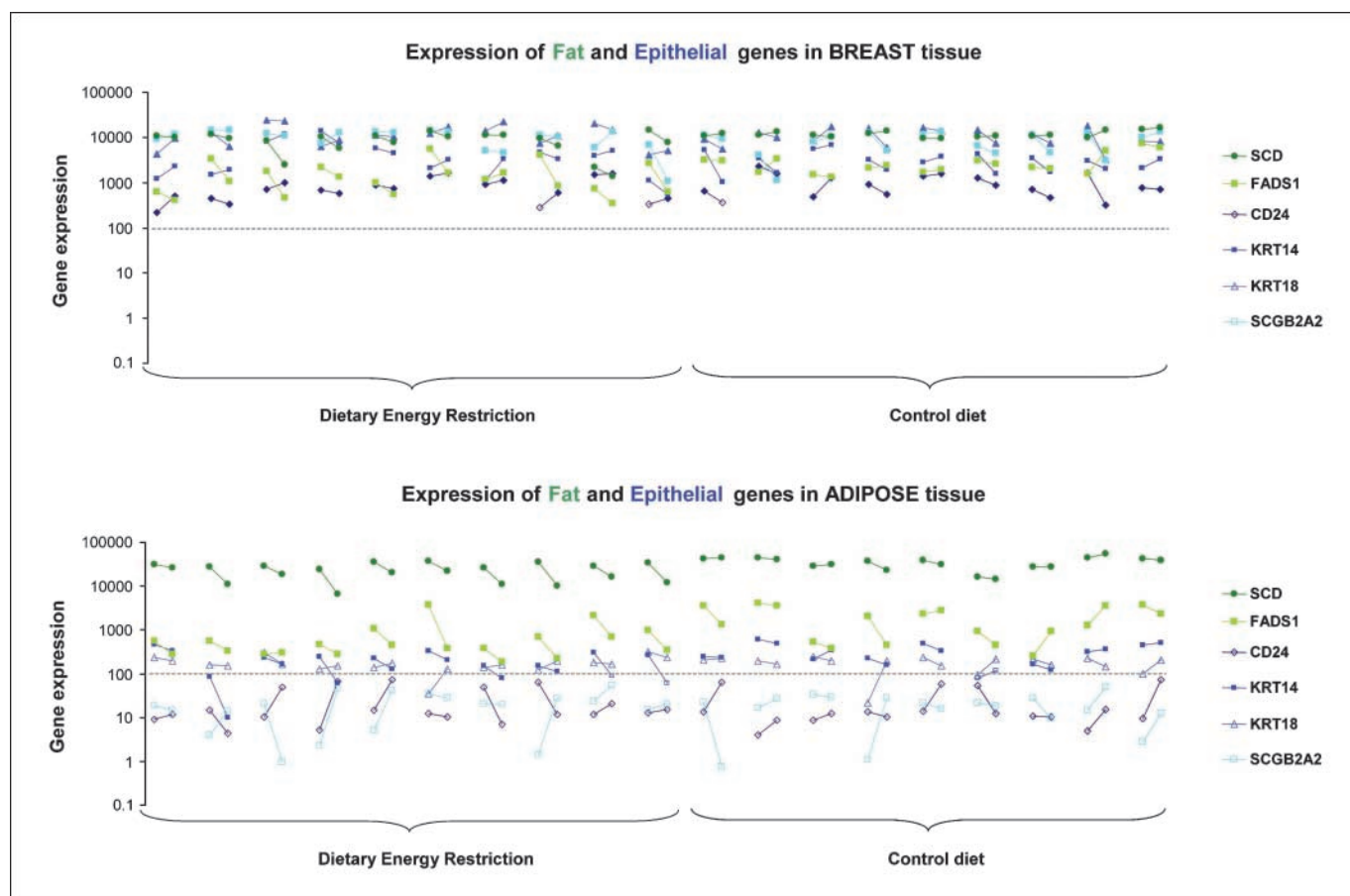


Fig. 4. Changes in the level of gene expression of fat metabolism and epithelial-specific genes in adipose and breast tissue. Level of expression using MAS5 normalization. Before (*left*) and after (*right*) measurements are shown for each patient linked with a line. Open symbols, genes that are called “absent” or “marginal.” Dashed line, the majority of genes with expression levels <100 are called absent.

under standardized, fasting conditions to limit variance. The inclusion of a control group allowed us to assess any changes in gene expression, which may be influenced by the site or timing of biopsies. The lack of changes in gene expression in the control group gives confidence that the changes observed following DER are “real” and indicates the stability of gene expression when diet and exercise are unchanged. To maximize compliance and standardize dietary change, the DER comprised a liquid meal replacement drink (Slimfast). The declines in body fat (−4.6 kg over 26 days) among the DER group are consistent with ~1,400 kcal/d energy deficit, indicating good compliance to the DER regimen (47). The clear effect of DER on body weight, fat, and lipids and leptin confirms that compliance was at a sufficient level to have metabolic effects and reflects the success of our rigorous selection process. DER is well known to cause declines in leptin (15) and SHBG (48). The decline in total and low-density lipoprotein cholesterol and triglycerides is consistent with previous studies of low-fat, energy-restricted regimens (49, 50).

The DER regimen used in our study reduced energy intake (67%) but also resulted in differential reduction of macronutrients from the woman's standard diet of calories from carbohydrate (53% reduced), protein (43% reduced),

and fat (89% reduced). It is possible that some of the changes seen in this study were due to altered composition of the diet, specifically the greatly decreased fat intake. It is known that an isocaloric dietary change of macronutrients, such as the combined requirement of carbohydrate and saturated fat for induction of SCD in humans (18), can alter urinary metabolic patterns and gene expression in s.c. fat. However, several studies show that DER produces changes in gene expression, which are greater than changes in diet macronutrient composition (15–18). We believe that further metabolic studies are warranted to assess relative importance between fat and carbohydrate to energy restriction. Some observational studies show a modest reduction of breast cancer risk after reduction of fat intake, but a large randomized trial of isocaloric fat reduction was not associated with risk reduction (51, 52).

Opposite breasts were sampled 1 month apart to circumvent potential inflammatory changes if biopsies had been taken from one breast only. This is a potential weakness of the study given the known heterogeneity of the breast and may account for some of the changes seen. However, no consistent differences in gene expression were seen in the controls in the biopsies taken from contralateral breasts 1 month apart, and quantitative reverse transcription-PCR assessment of eight

epithelial-specific genes from the four quadrants of each breast from four women undergoing bilateral risk reduction mastectomy suggested greater variation between individuals than between breasts.

A higher FDR (0.3) was used to identify the most consistently changed genes in the breast tissue following DER compared with the adipose tissue (0.01). We have postulated that is due to greater heterogeneity of breast tissue compared with that of the abdominal adipose. Alternatively, it could be an indication that the response to DER in breast tissue is simply more variable than the response in adipose tissue. Although the *P* values shown in Table 2 are not as low for the breast tissue as they are for the adipose tissue, they are still highly significant. Furthermore, gene ontology analysis identified many of the same pathways for both tissues and almost all genes within these were down-regulated (Supplementary Fig. S1). It would be interesting to compare gene expression in breast adipocytes, epithelium, and stroma separately; however, this approach would result in a much lower tissue to RNA yield and therefore necessitate a more invasive biopsy technique. However, we were able to do laser capture microdissection of sufficient epithelium in three before and after DER samples to obtain RNA and analyze gene expression. This revealed similar changes in genes, such as *SCD*, to those seen in whole tissue, suggesting that DER does affect breast epithelial gene expression.

Previous studies have examined the effect of DER on s.c. abdominal fat. Our findings are largely concordant with those of other human studies. Notably, fatty acid synthesis genes are down-regulated in the adipose tissue of both this study (60% DER over 1 month) and the previous Affymetrix array-based study of Dahlman et al. [30% DER over 10 weeks (15) and 50% DER over 5 weeks (18)], although this article did not specify these specific metabolism genes. Other studies have linked a 30% DER over 10 weeks with changes in the expression of genes that encode transporters and transcription factors linked to adipogenesis and mitochondrial respiration (17). Clement et al. (53) reported that ~60% of genes differentially expressed in a 60% DER study over 1 month in s.c. adipose tissue were related to fat and carbohydrate metabolism, although this group focused mainly on inflammation-related genes. A recent study reported that epithelial cells from chemical-induced mammary adenocarcinomas from Sprague-Dawley rats had increased expression of genes for glycolysis, decreased pyruvate dehydrogenase, and increased lactate dehydrogenase compared with normal epithelial cells, consistent with the Warburg effect (54). However, this pattern of gene expression was not influenced by a 40% DER. The design of this study does not enable the effects of DER on spontaneous development of breast cancer to be determined; furthermore, changes in lipid metabolism were not reported (55). Adipocytes and breast epithelial cells lie in close proximity to each other in breast tissue. Large adipocytes in obese subjects may have dysregulated secretion of factors (i.e., increased leptin and decreased adiponectin), which may promote the proliferation of transformed ductal carcinoma cells (56). DER resulted in significant reductions in serum levels of leptin (66%) but no change in serum adiponectin. Serum changes in leptin were not reflected in leptin gene expression. The dissociation between adipokine gene expression and serum levels has been noted

in earlier studies, suggesting a lack of sensitivity or post-translational effects of DER (57). There have been few metabolomic studies of DER in humans. We did not detect significant changes in glycolysis (glucose-6-phosphate and pyruvate) and tricarboxylic acid cycle metabolites (succinic acid and 2-oxoglutaric acid) despite these pathways being down-regulated at the gene expression level. However, the small sample sizes would preclude the detection of small differences in the concentrations of these metabolites.

The exact mechanism for the protective effect of DER against cancer remains unknown. Increased glycolysis and fatty acid synthesis is well described in tumors, and there is renewed interest in inhibition of these pathways not only for cancer treatment but also for prevention of cancer and other diseases (see ref. 9 for review). For example, inhibition of glycolysis with two deoxyglucose or fatty acid synthases with a compound named C75 inhibits tumor growth in rodent and human models (58, 59), whereas indirect observational evidence (increased stearic to oleic acid ratio in RBCs) is consistent with SCD activity linked to breast cancer risk (45).

This is a short-term study, and it is difficult to draw any firm conclusions about the effects of long-term DER. It is unknown which changes persist in the long-term when finally a desirable weight is achieved and weight stabilizes. In common with our study, most previous reports have assessed changes in gene expression during energy restriction and the dynamic phase of weight loss (16, 53). One report finds that most changes observed during dynamic phase of weight loss were reversed or attenuated during a 5-week weight stabilization phase (18). The beneficial changes in gene expression seen with weight loss are therefore mostly linked to energy restriction. Periods of intermittent restriction may thus be required to maintain these beneficial effects (10). Energy restriction is known to reduce cell proliferation and increase apoptotic rate in both normal tissues and cancers (60–63). The inhibition of glycolysis has been shown to impair the growth of tumor cells (64, 65), and suppression of glycolysis and fatty acid synthesis specifically in the breast epithelium may prevent breast carcinomas from developing and progressing.

In summary, we have done the first DER study to characterize gene expression changes in premenopausal normal human breast tissue. Common differentially expressed genes and pathways in breast and abdominal tissue suggests that short-term energy restriction may influence breast cancer risk at the molecular level. We have identified targets that may respond to chemoprophylactic mimetic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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