

See corresponding editorial on page 873.

## Dairy calcium supplementation in overweight or obese persons: its effect on markers of fat metabolism<sup>1–3</sup>

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### ABSTRACT

**Background:** Dairy calcium supplementation has been proposed to increase fat oxidation and to inhibit lipogenesis.

**Objective:** We aimed to investigate the effects of calcium supplementation on markers of fat metabolism.

**Design:** In a placebo-controlled, crossover experiment, 10 overweight or obese subjects who were low calcium consumers received 800 mg dairy Ca/d for 5 wk. After 4 wk, adipose tissue was taken for biopsy for analysis of gene expression. Respiratory exchange, glycerol turnover, and subcutaneous adipose tissue microdialysis were performed for 7 h after consumption of 400 mg Ca or placebo, and the ingestion of either randomized slow-release caffeine (SRC; 300 mg) or lactose (500 mg). One week later, the test was repeated with the SRC or lactose crossover.

**Results:** Calcium supplementation increased urinary calcium excretion by 16% ( $P = 0.017$ ) but did not alter plasma parathyroid hormone or osteocalcin concentrations. Resting energy expenditure ( $59.9 \pm 3.0$  or  $59.6 \pm 3.3$  kcal/h), fat oxidation ( $58.4 \pm 2.5$  or  $53.8 \pm 2.2$  mg/min), plasma free fatty acid concentrations ( $0.63 \pm 0.02$  or  $0.62 \pm 0.03$  mmol/L), and glycerol turnover ( $3.63 \pm 0.41$  or  $3.70 \pm 0.38$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) were similar with or without calcium, respectively. SRC significantly increased free fatty acid concentrations, resting fat oxidation, and resting energy expenditure. During microdialysis, epinephrine increased dialysate glycerol concentrations by 250% without and 254% with calcium. Expression of 7 key metabolic genes in subcutaneous adipose tissue was not affected by calcium supplementation.

**Conclusion:** Dairy calcium supplementation in overweight subjects with habitually low calcium intakes failed to alter fat metabolism and energy expenditure under resting conditions and during acute stimulation by caffeine or epinephrine. *Am J Clin Nutr* 2008;88: 877–85.

### INTRODUCTION

Overeating increases the energy turnover of the body; however, it is difficult in many cases for the mechanisms of energy expenditure (EE) (thermogenesis and physical activity) to match and balance the excess energy entering the body in these conditions. As a consequence, energy accumulates as lipid in adipose

tissue and other tissues, and that development leads to obesity, insulin resistance, and diseases associated with the metabolic syndrome. Although there have been many recent advances in the understanding of the mechanisms associated with obesity at the molecular level in rodents and knock-out rodent models, the medical and scientific community remains essentially at a loss with respect to ways in which to deal with this increasing pandemic in humans.

Recently, various groups of scientists working in one laboratory (Zemel's group) proposed that dietary calcium, via its influence on plasma 1,25-dihydroxyvitamin D<sub>3</sub> (calcitriol) concentrations, regulates the concentration of intracellular adipocyte calcium and subsequently regulates adipocyte lipid metabolism (1–8). Zemel's group also found that high intracellular calcium concentrations inhibit lipolysis and stimulate de novo lipogenesis, whereas low intracellular concentrations increase lipolysis and inhibit de novo lipogenesis. Such results are intriguing, but they warrant further investigation, because there is considerable evidence of little de novo lipogenesis in humans (9, 10). When de novo lipogenesis does occur, much of it occurs in the liver (11), and, because of low fatty acid synthase activity, very little, if any, occurs in adipose tissue (11–18).

In studies in mice and humans, it was shown that an increase in dietary calcium in the absence of energy restriction partitions energy toward lean body mass rather than toward adipose tissue (5, 7, 19) and that this effect is accentuated during energy restriction (5, 7, 20). Furthermore, this partitioning effect of calcium is even more pronounced when the diet has a high dairy component (20–22).

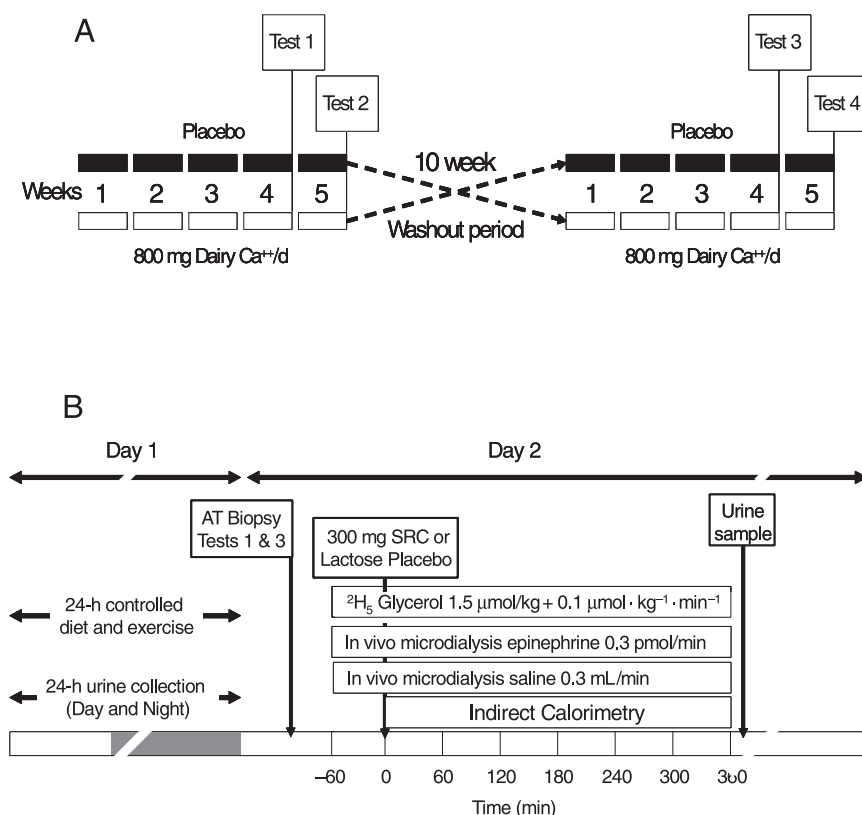
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**FIGURE 1.** A: Flow diagram of the 20-wk protocol. Each subject consumed his or her habitual low-calcium diet supplemented with either 800 mg dairy Ca (400 mg Ca made up to 5 g with maltodextrin filler) twice a day or 5 g maltodextrin placebo twice a day. After a 10-wk washout period, the subjects crossed over to the other arm of the study. B: A sample of abdominal adipose tissue (AT) was taken for biopsy, and an acute metabolic test measured markers of fat metabolism. After 4 wk of treatment and again after 5 wk of treatment, the subject spent 2 d at the Institute. On the first day, the subject's diet and exercise were controlled, and urine was collected during the day and night over a 24-h period. On the morning of day 2 of tests 1 and 3, an adipose tissue sample was taken by aspiration from the abdominal region for analysis of the expression of genes involved in fat metabolism. For tests 1–4, fasting resting energy and substrate metabolism were measured by indirect calorimetry in the presence [300 mg slow-release caffeine (SRC)] or absence (500 mg lactose as placebo) of caffeine administration. During these tests, glycerol turnover was measured by using isotope dilution of deuterated glycerol in steady state conditions, and in vivo subcutaneous adipose tissue lipolysis was measured with the use of microdialysis.

Whether dietary calcium also influences thermogenesis and fat oxidation is not known; however, Zemel et al (7) observed increases in the core temperature of mice receiving high-calcium diets, which they interpreted as a shift in efficiency from energy storage to thermogenesis, and Melanson et al (23) reported correlations between acute calcium intake and fat oxidation during 24-h measurements of energy metabolism in a room calorimeter. In contrast, it has also been established that calcium intake affects energy intake by influencing lipid digestibility (24, 25), which it does by forming indigestible calcium soaps with dietary lipids in the gastrointestinal tract; thus, fecal fat excretion is increased (24, 25). Although Zemel et al (20) accepted that some dietary energy, in the form of fats, can be lost as nonabsorbable calcium complexes in the feces—as has been shown in several human studies (24–27)—they discounted this effect as being insignificant.

In the present study, the primary objective was to investigate the effect of supplementing the diet with 800 mg dairy Ca/d on EE and markers of fat metabolism in a group of overweight or obese subjects who habitually consumed a low-calcium diet. Because caffeine ingestion has been shown to increase EE (28–30) and, to some extent, lipid oxidation (29), caffeine was given acutely at the end of the calcium supplementation period to explore possible synergistic effects between calcium and caffeine.

## SUBJECTS AND METHODS

The study was a single-center, double-blind, placebo-controlled, randomized crossover trial, the protocol of which is shown in **Figure 1**. Subjects were recruited by advertisement within the university hospital and by word of mouth. The study targeted overweight and obese subjects (both male and female) with a body mass index (in kg/m<sup>2</sup>) of >27 and a habitually low daily intake of calcium. Potential volunteers were first screened by telephone, and only those who consumed little or no dairy products were subsequently interviewed by the dietitian of the Institute of Physiology to determine their habitual calcium intake. Only those with calcium intakes < 800 mg/d were considered for the study. In general, the subjects consumed no or almost no dairy products; their calcium intake was supplied almost entirely from meat, vegetables, starchy food, fruit, bread, water, fruit juice, and beer.

Ten subjects, 3 men and 7 women, whose physical characteristics are presented in **Table 1**, were included in the study. After a preliminary medical examination, during which anthropometric measurements were made, the subjects were randomly assigned to receive either a placebo or calcium supplement for a period of 5 wk, during which they consumed their habitual low-calcium diet supplemented with either a placebo (sachet of 5 g

**TABLE 1**Physical characteristics of the subjects<sup>1</sup>

Characteristic	Value
Age (y)	22.2 ± 1.2
Weight (kg)	78.1 ± 9.1
Height (m)	1.65 ± 0.06
BMI (in kg/m <sup>2</sup> )	28.5 ± 1.4
Calcium intake (mg/d)	586 ± 137

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ .  $n = 10$ .

maltodextrin; MD47; Roquette, Lestrem, France) taken 2 times/d or a dairy calcium supplement (sachet containing 400 mg Ca<sup>++</sup> and made up to 5 g with MD47 filler; Capolac MM-0525 BG; Arla Foods, Viby, Denmark) taken 2 times/d to provide 800 mg Ca<sup>++</sup>/d. The calcium supplement was a natural milk mineral concentrate with the following composition: 6% protein, 13% lactose, 74% ash, 6% moisture, and 24% calcium, present almost entirely as calcium phosphate. Compliance was assessed by counting the number of returned empty sachets. After 4 wk of supplementation, subjects spent a day at the Institute of Physiology; their food intake and physical activity were controlled for a period of 24 h. The next morning, a sample of abdominal subcutaneous adipose tissue was taken, and measurements of whole-body energy metabolism, lipid turnover, lipid disposal, and adipose tissue lipolysis were made for 7 h in resting, fasting conditions.

All subjects provided written informed consent. The study was carried out in accordance with the ethical standards for clinical research of the Faculty of Biology and Medicine, Lausanne University (Lausanne, Switzerland), whose ethics committee reviewed and approved the protocol.

### Biopsy of adipose tissue

Under local anesthesia (0.5 mL, 1% xylocaine), abdominal percutaneous adipose tissue ( $\approx 100$  mg) for biopsy was taken by aspiration through a 16-gauge needle from an abdominal spot  $\approx 20$  mm lateral to the umbilicus, as described previously (31). The adipose tissue sample was immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until mRNA extraction. The expression of mRNAs involved in adipose tissue fat metabolism, coding for hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), perilipin, peroxisome proliferator-activated receptor  $\gamma_2$  (PPAR $\gamma_2$ ), sterol regulatory element-binding protein 1c (SREBP1c), and phosphodiesterase 3B (PDE-3B), were measured and compared after 4 wk of calcium supplementation and 4 wk of placebo.

### Metabolic measurements

After the subject was comfortably installed in a semirecumbent position in a bed, a venous catheter was inserted into the antecubital vein of each arm. One catheter was used for a primed, continuous infusion of 5-deuterated glycerol to measure glycerol turnover (32), and the other, which was kept patent with physiologic saline, was used for blood sampling. In addition, two 30-mm Cuprophane dialysis membranes (Gambro, Lund, Sweden) were placed in the abdominal subcutaneous adipose tissue on either side of the umbilicus by using microdialysis catheters (CMA 60; CMA Microdialysis, Solna, Sweden) inserted aseptically with local xylocaine anesthesia. These 2 membranes were

used to measure the *in vivo* lipolysis of abdominal subcutaneous adipose tissue with epinephrine, one with and the other without local lipolytic stimulation. The perfusion fluid contained either physiologic saline (catheter 1) or epinephrine (1  $\mu\text{mol/L}$ ; Sintetica SA, Mendrisio, Switzerland) to stimulate lipolysis (catheter 2), and both fluids were perfused at 0.3  $\mu\text{L/min}$  by using a high-precision syringe pump (CMA Microdialysis). A fasting baseline blood sample was taken at  $-60$  min, after which a primed (1.5  $\mu\text{mol/kg}$ ), continuous (0.1  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) infusion of 5-deuterated glycerol was begun.

At time zero (0 min), the subject ingested the contents of one of the same type of sachets he or she had been taking during the preceding 4 wk, along with a gelatin capsule containing either 500 mg lactose or 300 mg slow-release caffeine (SRC) (33), which were taken in random order. Immediately afterward, a transparent ventilated hood was placed over the subject's head to measure respiratory exchange (Deltatrac II indirect calorimeter; Datex, Helsinki, Finland), perfusion of the microdialysis perfusates was begun, and measurements were continued for a further 6 h. Dialysates were collected every 60 min throughout the study, after calibration of each microdialysis catheter by using the no-net-flux method (34). Blood samples were taken at 60-min intervals from  $-60$  to 360 min. Urine was collected twice during the 24 h before the acute study—from 0800 to 2300 and from 2300 to the next morning (overnight fasting sample), the time of which was recorded—and also at the end of the 7-h test.

After these tests, the subject continued ingesting the same supplement for an additional week, at the end of which his or her diet and physical activity were controlled for 24 h and the measurements described above (7-h energy metabolism, whole-body glycerol turnover, and *in vivo* lipolysis of adipose tissue) were repeated. During the second acute study, the subject ingested a gelatin capsule containing either SRC or lactose, whichever had not been ingested in the first study. Between treatments, there was a 10-wk washout period, during which the subject consumed his or her habitual diet.

### Measurements

The effects of 4–5 wk calcium supplementation on fasting whole-body lipid turnover and lipid disposal (oxidative and non-oxidative) were measured by using isotope dilution of 1,1,2,3,3-<sup>2</sup>H<sub>5</sub> glycerol (33) and an open-circuit, ventilated-hood, indirect calorimeter (Deltatrac II). Concurrently, *in vivo* lipid metabolism under normal and lipolysis-stimulated conditions was measured in abdominal subcutaneous adipose tissue.

### Calculations

Under the assumption that urea nitrogen contributes 90% to total nitrogen excretion, protein oxidation was calculated from urinary urea nitrogen excretion measured in the urine from the timed urine collection at the end of the test. After that step, EE and substrate utilization were calculated from the respiratory exchange data with the use of standard equations (36).

### Analyses

Plasma was immediately separated from blood by centrifugation at  $4^\circ\text{C}$  for 10 min at 3600 rpm and stored at  $-20^\circ\text{C}$  until analyzed. Fasting samples were analyzed for osteocalcin by using time-resolved amplified cryptate emission (TRACE; BRAHMS Kryptor, St-Ouen, France), for parathyroid hormone

(PTH) by using an enzymatic chemiluminescence method (Roche Diagnostics, Mannheim, Germany), and for total calcium by using an automated method (Roche Diagnostics).

All plasma samples were analyzed for glucose by using the glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA); for free fatty acids and triglycerides by using colorimetric methods from Wako (Freiburg, Germany) and Biomérieux (Biomérieux Vitek Inc, Switzerland), respectively; and for insulin by using a radioimmunoassay kit from Linco (St Charles, MO). We measured 1,1,2,3,3-<sup>2</sup>H<sub>5</sub> glycerol enrichments on acetylated derivatives by using gas chromatography–mass spectrometry (GC 5890/MS 5971; Hewlett-Packard Instruments, Palo Alto, CA) with selective ion monitoring at 164.1 and 159 mass-to-charge ratio. Twenty-four-hour urine samples were analyzed for Ca<sup>++</sup> and PO<sub>4</sub><sup>3-</sup> by using an automated method (Roche Diagnostics) and for catecholamines (ie, epinephrine, norepinephrine, and dopamine) by using HPLC with electrochemical detection. Urine samples taken at the end of the 7-h study were analyzed for urea nitrogen by using a urease method (Beckman Blood Urea Nitrogen Analyzer II; Beckman Instruments).

The mRNA expression of candidate genes was measured in biopsied adipose tissue by using reverse transcriptase (RT)–quantitative polymerase chain reaction (qPCR). Tissue samples (≈100 mg) from biopsied subcutaneous fat were powdered in liquid nitrogen, and total RNA was prepared by using the RNeasy total RNA minikit (Qiagen, Courtaboeuf, France). The average yield of total RNA was 1.5 ± 0.2 μg/100 mg adipose tissue. First-strand cDNAs were synthesized from 200 ng total RNA in the presence of 100 U Superscript II (Invitrogen, Eragny, France) by using both random hexamers and oligo (dT) primers (Promega, Charbonnières, France). The mRNA concentrations of the genes encoding LPL, HSL, ATGL, perilipine, PPAR<sub>γ</sub>2, SREBP1c, and PDE 3 were quantified by using RT-qPCR on a Light-Cycler instrument (Roche Diagnostics, Meylan, France) in a final volume of 20 μL containing 5 μL of a 1-in-100 dilution of the RT reaction and 15 μL reaction buffer from the FastStart DNA Master SYBR Green kit (Roche Diagnostics) with specific primers. The sequences of the primers, the validation of the assays, and the conditions of the RT-qPCR assays are available by request from HV at [vidal@sante.univ-lyon1.fr](mailto:vidal@sante.univ-lyon1.fr). For quantification, a standard curve was systematically generated with 6 different amounts (150–30 000 molecules/tube) of purified target cDNA cloned in the pGEM plasmid (Promega). Each assay was performed in duplicate, and validation of the real-time PCR runs was conducted by evaluation of the melting temperature of the products and by the slope and error obtained with the standard curve. The analyses were performed by using LIGHT-CYCLER software (version 5.2; Roche Diagnostics). The results were normalized by using a hypoxanthine phosphoribosyl transferase mRNA concentration, measured as a reference gene by RT-qPCR.

### Statistical analysis

Analyses of blood samples collected throughout the protocol were averaged for the 7 h of the clinical trial; other variables, such as substrate oxidation, EE, microdialysis dialysates, blood pressure, and heart rate were averaged over 6 h. Data are expressed as mean ± SEMs unless stated otherwise. Data were pooled according to treatment—calcium supplementation or placebo—for urinary analyses of calcium and catecholamines over the 24 h

preceding the acute study and for analyses of plasma hormones and calcium. All of the data were analyzed by using 2-factor repeated-measures analysis of variance (ANOVA) procedures; when significant differences were observed, paired comparisons were further analyzed by using Bonferroni's multiple-comparison test.  $P \leq 0.05$  was considered significant. We used STATA software (version 9.1; Stata Corp, College Station, TX) for all statistical analyses.

## RESULTS

### Body weight and markers of calcium intake in blood and urine

There was no significant difference between body weight at the end of the placebo and calcium supplementation periods: 79.6 ± 3.0 and 80.0 ± 3.2 kg, respectively.

Fasting plasma calcium concentrations were 2.19 ± 0.02 mmol Ca<sup>++</sup>/L after placebo treatment, and they did not change when the diet was supplemented with 800 mg Ca/d for 4–5 wk: 2.15 ± 0.03 mmol Ca<sup>++</sup>/L. Similarly, neither PTH (placebo: 38.7 ± 4.1 ng/L) nor osteocalcin (placebo: 32.8 ± 2.4 μg/L) changed significantly with calcium supplementation (PTH: 39.6 ± 4.3 ng/L; osteocalcin: 33.5 ± 1.6 μg/L).

Calcium concentrations in urine collected during the day and the night after placebo treatment were 1.94 ± 0.33 and 2.36 ± 0.56 mmol/L, respectively, and they increased with calcium supplementation to 2.34 ± 0.34 and 3.86 ± 0.65 mmol/L, respectively. ANOVA indicated a significant effect of calcium on urinary calcium excretion during the night ( $P = 0.028$ ). In consequence, total 24-h calcium excretion increased from 138.4 ± 13.1 mg/24 h with placebo to 159.9 ± 17.2 mg/24 h with calcium supplementation ( $P = 0.017$ ). Neither the day (placebo: 10.72 ± 1.88 mmol/L; calcium: 11.97 ± 2.06 mmol/L) nor the night samples (placebo: 18.09 ± 1.78 mmol/L; calcium: 23.78 ± 2.56 mmol/L) showed significant differences in urinary phosphate excretion during calcium supplementation.

### Energy expenditure and substrate utilization

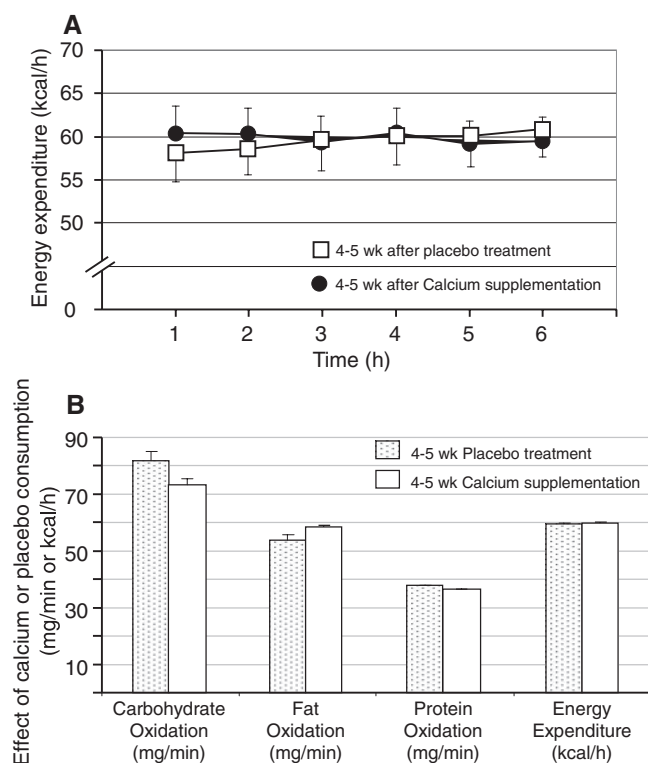
No significant difference in resting EE was observed after 4–5 wk of dairy calcium supplementation (**Figure 2A**). Mean substrate oxidation and EE were calculated over the course of the 6-h study and are presented in **Figure 2B**. No significant differences were observed in protein oxidation or EE, and, although slight differences can be seen in carbohydrate and fat oxidation between the placebo and calcium supplementation treatments, those differences also were not significant.

At the end of the placebo supplementation period, ingestion of 300 mg SRC (**Figure 3A**) resulted in a significant ( $P = 0.001$ ) increase of 5.4 ± 0.2 kcal/h in EE, which was accompanied by a slight (nonsignificant) decrease in carbohydrate oxidation (5.8 ± 1.8 mg/min) and a significant ( $P = 0.04$ ) increase in fat oxidation (11.4 ± 0.7 mg/min). When SRC was given acutely after 4–5 wk of calcium supplementation (**Figure 3B**), no significant differences in EE or substrate oxidation were observed.

### Glycerol turnover

Glycerol turnover is illustrated in **Figure 4**. It was 3.70 ± 0.38 μmol · kg<sup>-1</sup> · min<sup>-1</sup> at the end of the placebo supplementation period and did not change significantly after calcium supplementation (3.63 ± 0.41 μmol · kg<sup>-1</sup> · min<sup>-1</sup>). Acute ingestion of 300





**FIGURE 2.** A: Mean  $\pm$  SEM effect of consuming 2 sachets/d of either placebo or dairy calcium (800 mg/d) for 5 wk on fasting resting energy expenditure measured over 6 h. No significant differences were observed.  $n = 10$ . B: Mean  $\pm$  SEM effect of consuming 2 sachets/d of either placebo or dairy calcium (800 mg/d) for 5 wk on fasting resting substrate oxidation and energy expenditure measured over 6 h. The differences between tests (calcium supplementation – placebo) were  $-8.6 \pm 2.1$  mg/min,  $4.6 \pm 1.6$  mg/min,  $-1.4 \pm 0.04$  mg/min, and  $0.3 \pm 1.6$  kcal/h for carbohydrate oxidation, fat oxidation, protein oxidation, and energy expenditure, respectively; no significant differences were observed (2-factor repeated-measures ANOVA). Difference = calcium supplementation – placebo.  $n = 10$ .

mg SRC also did not change glycerol turnover after 4–5 wk of placebo ( $3.65 \pm 0.30 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) or calcium supplementation ( $3.72 \pm 0.37 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ).

### Microdialysis

Mean glycerol concentrations analyzed in the microdialysis dialysates over the 6-h experiment without and with epinephrine-stimulated lipolysis are shown in **Figure 5A** and **B**, respectively. Neither calcium nor placebo supplementation for 4–5 wk had a significant effect on glycerol release from abdominal subcutaneous adipose tissue ( $241.0 \pm 9.4$  and  $231.7 \pm 11.0 \mu\text{mol/L}$ , respectively). Although glycerol concentrations tended to be higher with SRC treatment ( $273.4 \pm 10.0 \mu\text{mol/L}$ ), the difference was not significant, and it was not confirmed when SRC was taken after calcium supplementation ( $229.6 \pm 10.7 \mu\text{mol/L}$ ).

When lipolysis was stimulated by the addition of epinephrine to the microdialysis perfusate (**Figure 5B**), dialysate glycerol concentrations increased  $\approx 3.5$ -fold. Nevertheless, no significant differences were observed between treatments.

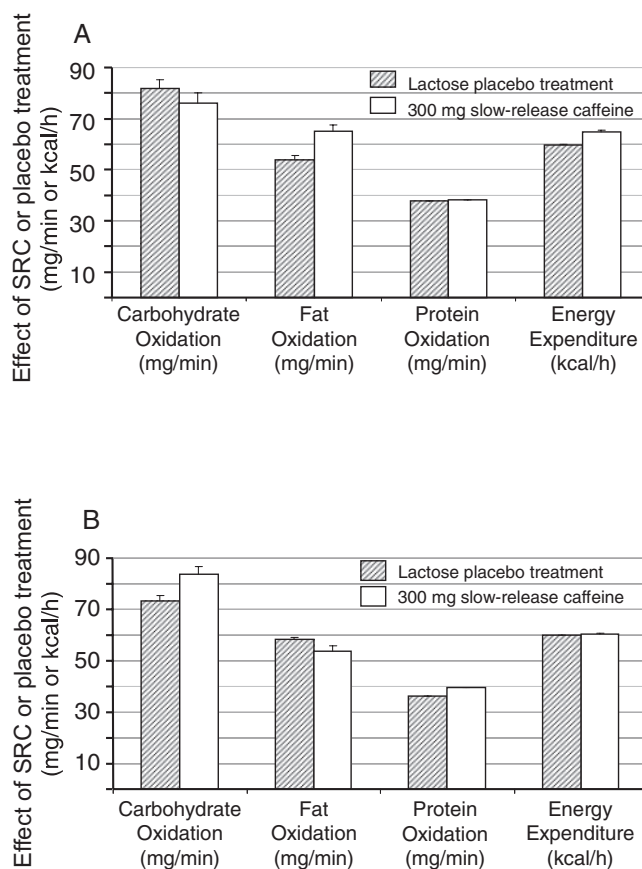
### Biomarkers of fat metabolism in adipose tissue

Changes in the expression of biomarkers of fat metabolism sampled from abdominal subcutaneous adipose tissue are presented in **Figure 6**. None of the changes were significant or

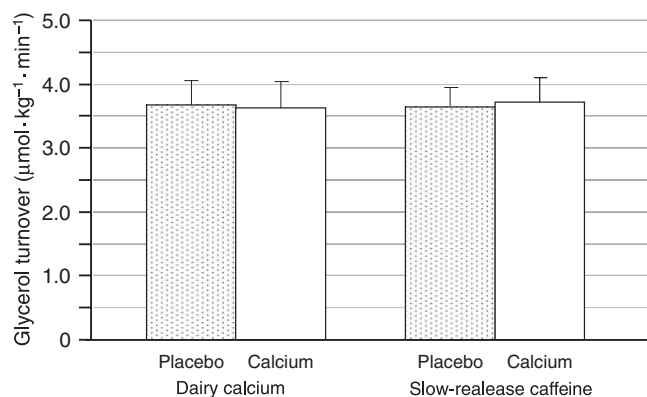
sufficiently large to have a significant effect on lipid metabolism in adipose tissue.

### Effect of calcium supplementation and slow-release caffeine on mean 6-h blood concentrations

Nonesterified fatty acid concentrations were, on average,  $0.55 \pm 0.02$  mmol/L at the start of the 6-h experiment, and they rose to  $0.80 \pm 0.03$  mmol/L by the end of the study. Calcium supplementation did not have an effect on mean 6-h nonesterified fatty acid concentrations (placebo:  $0.618 \pm 0.024$  mmol/L; calcium:  $0.633 \pm 0.021$  mmol/L); however, SRC in the presence and absence of calcium significantly ( $P = 0.01$ ) increased nonesterified fatty acid concentrations ( $0.709 \pm 0.025$  and  $0.725 \pm 0.025$  mmol/L, respectively).



**FIGURE 3.** A: Mean  $\pm$  SEM acute effect of 300 mg slow-release caffeine (SRC) ingestion on fasting resting substrate oxidation and energy expenditure measured over 6 h after 4–5 wk of placebo ingestion.  $n = 10$ . The differences between tests (calcium supplementation – placebo) were  $-5.8 \pm 1.8$  mg/min,  $11.4 \pm 0.7$  mg/min,  $0.5 \pm 0.0$  mg/min, and  $5.4 \pm 0.2$  kcal/h for carbohydrate oxidation, fat oxidation, protein oxidation, and energy expenditure, respectively; only fat oxidation ( $P = 0.04$ ) and energy expenditure ( $P = 0.001$ ) were significant (2-factor repeated-measures ANOVA with Bonferroni multiple-comparison tests). B: Mean  $\pm$  SEM acute effect of ingestion of 300 mg SRC on fasting resting substrate oxidation and energy expenditure measured over 6 h after consuming 800 mg dairy Ca/d for 4–5 wk.  $n = 10$ . The differences between tests were  $10.4 \pm 2.0$  mg/min,  $-4.4 \pm 1.8$  mg/min,  $3.0 \pm 0.0(4)$  mg/min, and  $0.6 \pm 0.07$  kcal/h for carbohydrate oxidation, fat oxidation, protein oxidation, and energy expenditure, respectively; no significant differences were observed (2-factor repeated-measures ANOVA).



**FIGURE 4.** Mean  $\pm$  SEM fasting glycerol turnover measured after consuming 800 mg dairy Ca/d for 4–5 wk and after acute ingestion of 300 mg slow-release caffeine.  $n = 10$ . No significant differences were observed (2-factor repeated-measures ANOVA).

After placebo treatment and calcium supplementation, SRC had no significant effect on mean 6-h triacylglycerol concentrations (placebo:  $1.037 \pm 0.048$  mmol/L; SRC:  $0.934 \pm 0.026$  nmol/L; and calcium:  $0.913 \pm 0.043$  nmol/L; SRC  $1.065 \pm 0.079$  nmol/L). Insulin concentrations were  $10.7 \pm 0.8$   $\mu$ U/mL after placebo treatment and  $10.8 \pm 0.84$   $\mu$ U/mL in the presence of SRC. Although the concentrations were slightly lower after calcium ( $8.7 \pm 0.46$   $\mu$ U/mL) and calcium with SRC ( $9.5 \pm 0.5$   $\mu$ U/mL) treatment, these changes were not significant. Blood glucose concentrations were not influenced by treatment (placebo:  $4.48 \pm 0.05$  mmol/L; calcium:  $4.46 \pm 0.06$  mmol/L) or by placebo and calcium treatments with SRC ( $4.34 \pm 0.05$  and  $4.35 \pm 0.05$  mmol/L, respectively). Similarly, plasma glycerol concentrations were not influenced by treatment (placebo:  $74.8 \pm 3.6$   $\mu$ mol/L; calcium:  $73.0 \pm 2.8$   $\mu$ mol/L) or by placebo and calcium treatments with SRC ( $76.5 \pm 3.0$   $\mu$ mol/L; calcium:  $78.3 \pm 3.3$   $\mu$ mol/L, respectively).

### Heart rate and blood pressure

Mean heart rate and blood pressure values are presented in **Table 2**. The heart rate after calcium supplementation ( $64 \pm 2.0$  beats/min) was slightly but significantly ( $P < 0.03$ ) lower than that after placebo with SRC ( $69 \pm 2$  beats/min). Blood pressure was not significantly affected by treatment.

### Urinary catecholamines

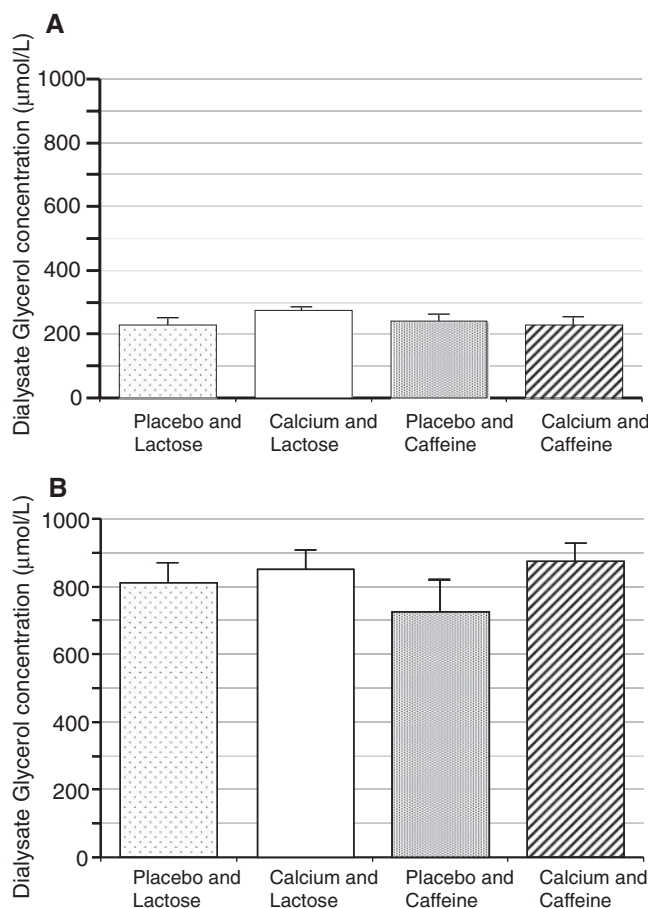
Norepinephrine, epinephrine, and dopamine excretions in urine collected during the day and night at the end of each supplement period (placebo or calcium) are presented in **Table 3**. No significant differences in urinary catecholamines were observed.

### DISCUSSION

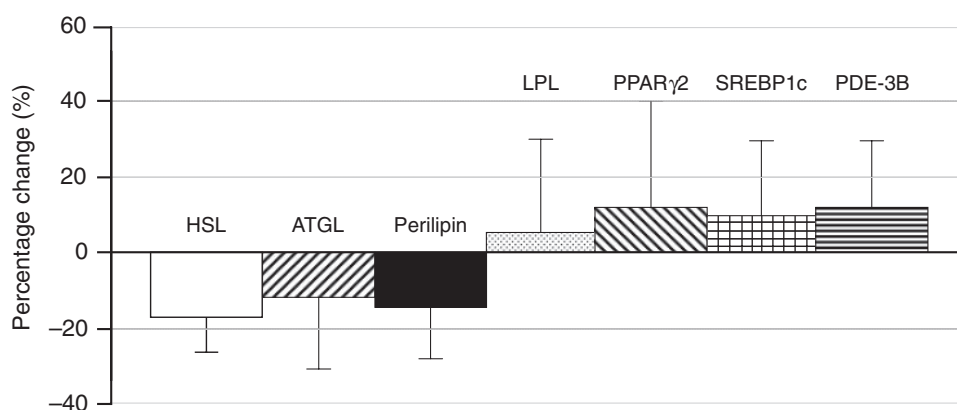
The hypothesis that dietary calcium is involved in the control of body weight and body fat stems from observations that low-calcium diets and high circulating vitamin D<sub>3</sub> concentrations stimulate lipid synthesis and inhibit the lipolysis of adipose tissue (2, 5–7). The inhibition of lipolysis decreases plasma free fatty acid concentrations, and that decrease in turn reduces lipid oxidation. The hypothesis is supported by animal studies on controlled calcium intakes and by the observation that vitamin D<sub>3</sub> inhibits lipolysis in adipocytes, apparently through the activation

of a membrane receptor to vitamin D<sub>3</sub>. In obese or overweight humans, studies so far have failed to confirm this hypothesis (26, 36), and, although lipid oxidation increased in obese subjects during 24-h energy restriction after receiving 1400 mg dairy Ca/d for 1 wk, the same dose had no effect on lipid oxidation in weight-maintenance conditions (37).

Because some reports suggest that dietary calcium supplementation may be effective only in persons with low calcium intakes, the present study was performed in subjects with a daily calcium intake of  $<800$  mg/d. Despite their low calcium intakes, all of the participants had plasma calcium, phosphate, PTH, and osteocalcin concentrations within the normal range. This was expected, because severe calcium deprivation is required to alter these variables. Each subject was supplemented with 800 mg dairy Ca/d or a placebo for 5 consecutive weeks. Calcium supplementation increased daily urinary calcium excretion by 16% (from 138 to 160 mg/d). Because only a portion of the ingested calcium load is absorbed from the gut, and because net enteral



**FIGURE 5.** A: Mean  $\pm$  SEM effect of 4–5 wk dairy calcium supplementation (800 mg/d) on glycerol release from subcutaneous abdominal adipose tissue and the effect of acute slow-release caffeine ingestion (300 mg), during saline infusion using microdialysis.  $n = 10$ . No significant differences were observed (2-factor repeated-measures ANOVA). B: Mean  $\pm$  SEM effect of lipolytic stimulation with epinephrine on glycerol release from subcutaneous abdominal adipose tissue after 4–5 wk of dairy calcium supplementation (800 mg/d) and the effect of acute slow-release caffeine ingestion (300 mg) with the use of microdialysis.  $n = 10$ . No significant differences were observed between treatments (2-factor repeated-measures ANOVA).



**FIGURE 6.** Mean  $\pm$  SEM percentage change (treatment – placebo) in expression of mRNA of biomarkers of fat metabolism in tissue taken from abdominal subcutaneous adipose tissue for biopsy after 4 wk of placebo or calcium supplementation (800 mg  $\text{Ca}^{++}/\text{d}$ ).  $n = 10$ . HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; ATGL, adipose triglyceride lipase; PPAR $\gamma$ 2, peroxisome proliferator-activated receptor  $\gamma$ 2; SREBP1c, sterol regulatory element-binding protein-1; PDE-3B, phosphodiesterase 3B. The mRNA concentrations for all of the tested genes were corrected by those of hypoxanthine-guanine phosphoribosyltransferase (HPRT) taken as a reference before the calculation of the change induced by the treatment. The expression levels in the placebo condition, expressed in arbitrary units referred to HPRT concentrations, were HSL,  $1406 \pm 208$ ; LPL,  $5696 \pm 817$ ; ATGL,  $5157 \pm 1241$ ; perilipin,  $3851 \pm 727$ ; PPAR $\gamma$ 2,  $167 \pm 35$ ; SREBP1c,  $11805 \pm 3209$ ; and PDE-3B,  $441 \pm 98$ . No significant differences were observed (2-factor repeated-measures ANOVA).

calcium absorption is low, this figure is consistent with the increase in calcium intake observed during the supplementary period.

Our observations indicate that calcium supplementation did not alter basal lipid metabolism, because plasma free fatty acid concentrations and whole-body glycerol turnover were similar in the presence and absence of calcium supplementation. By administering caffeine acutely and testing the effect of a submaximal dose of epinephrine on adipose tissue lipolysis, we also evaluated the possibility that dietary calcium may synergistically increase the effects of physiologic stimulators of lipolysis and lipid oxidation. To evaluate the response to epinephrine, we increased the local concentration of epinephrine in subcutaneous adipose tissue by infusing a solution of  $1 \mu\text{mol}$  epinephrine/L through a microdialysis probe and monitoring the local release of glycerol. Epinephrine-stimulated adipose tissue glycerol concentrations were not significantly altered by calcium supplementation.

**TABLE 2**

Heart rate and arterial blood pressure measured during the test after 4–5 wk of either placebo or 800 mg  $\text{Ca}/\text{d}$  supplementation and after acute ingestion of 300 mg slow-release caffeine (SRC)<sup>1</sup>

	Treatment			
	Placebo	Calcium	Placebo + SRC	Calcium + SRC
Heart rate (beat/min)	$67 \pm 2$	$64 \pm 2^1$	$69 \pm 2$	$65 \pm 2$
Blood pressure (mm Hg)				
Systolic	$114 \pm 1.4$	$110 \pm 1.1$	$116 \pm 1.3$	$115 \pm 1.8$
Diastolic	$66 \pm 0.8$	$65 \pm 0.8$	$67 \pm 0.9$	$67 \pm 1.0$
Mean	$82 \pm 0.9$	$80 \pm 0.8$	$83 \pm 0.9$	$83 \pm 1.1$

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ .  $n = 10$ . Statistical analysis was conducted by using a 2-factor repeated-measures ANOVA with Bonferroni multiple-comparison tests.

<sup>2</sup> The 2-factor repeated-measures ANOVA indicated a treatment effect,  $P = 0.02$ . Bonferroni multiple-comparison tests showed a significant difference in heart rate between the end of calcium supplementation and the administration of acute slow-release caffeine treatment at the end of the maltodextrin placebo supplementation period,  $P < 0.03$ .

Our group also assessed lipid metabolism after the acute administration of SRC. Caffeine is known to increase total EE and lipid oxidation (28, 29, 33) and to enhance adipose tissue lipolysis, at least in part through the inhibition of adenosine receptors and the stimulation of the sympathetic nervous system (33). Caffeine administration enhanced total EE and lipid oxidation at the end of the placebo treatment and increased plasma free fatty acid concentrations in the presence and absence of calcium. Our group previously suggested that one potential mechanism by which caffeine stimulated thermogenesis was the ryanodine receptor (33). Caffeine is an agonist of the ryanodine receptor (38), the calcium ion-release channel of sarcoplasmic reticulum in skeletal muscle. Ryanodine receptor stimulation increases intramyocellular calcium flux, muscle contraction, heat production, glycolysis, adenosine triphosphate turnover, and mitochondrial pyruvate oxidation (39). Although this possibility is purely speculative, calcium supplementation may interfere with ryanodine receptor-stimulated heat production.

To evaluate the effect of calcium supplementation on adipose tissue lipid metabolism, transcriptomics was focused on a number of key metabolic genes; the results of this technique support the metabolic measurements. Although calcium supplementation failed to alter the expression of key genes involved in lipid

**TABLE 3**

Urinary catecholamine excretion after 5 wk of either placebo or 800 mg  $\text{Ca}/\text{d}$  supplementation<sup>1</sup>

	Placebo		Calcium	
	Day	Night	Day	Night
Norepinephrine (nmol/24 h)	$182 \pm 20$	$142 \pm 16$	$185 \pm 21$	$149 \pm 21$
Adrenaline (nmol/24 h)	$26 \pm 4$	$14 \pm 3$	$26 \pm 4$	$16 \pm 4$
Dopamine (nmol/24 h)	$1556 \pm 133$	$2129 \pm 131$	$1574 \pm 153$	$2127 \pm 238$

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ .  $n = 10$ . None of the variables differed significantly between treatments (2-factor repeated-measures ANOVA).



storage and lipolysis, it remains possible that, in adipocytes obtained from periumbilical subcutaneous adipose tissue, calcium has other effects on adipose tissue biology. Boon et al (40) recently observed a significant decrease in adipose tissue fatty acid synthase mRNA expression in a group of lean persons consuming a diet high in dairy calcium; however, the decrease occurred only at an intake of 2500 mg Ca/d, which is considerably higher than the recommended dietary intake for calcium and which is rarely attained in the regular diet. At a lower, more habitual intake of 1200 mg Ca/d, Boon et al, just as we did, observed no change in the expression of markers of adipose tissue fat metabolism and no effect of dietary calcium on EE or fat oxidation (40). In another study, in lean young men, the same group found no evidence that increasing serum calcitriol had any effect on EE, substrate metabolism, or gene expression related to fat metabolism (41).

The present observations do not support the hypothesis that increasing the calcium intake of overweight or obese low consumers of calcium increases EE and fat oxidation. It is interesting that the mechanisms proposed to support this hypothesis depend on an increase in concentrations of vitamin D<sub>3</sub>, which exerts direct inhibitory effects on adipose tissue lipolysis through the activation of membrane-associated vitamin D<sub>3</sub> receptors. Although such a mechanism is supported by animal and in vitro studies (5, 6), it is contradicted by other observations made in humans (26, 36, 41). This apparent contradiction can be explained, however, by an analysis of the composition of weight loss observed in a study by Zemel et al (23) in African American adults. An energy deficit of 500 kcal/d for 24 wk represents a total deficit of 84 000 kcal, which theoretically could be due to the loss of 9.3 kg fat, 12 kg adipose tissue, or 84 kg lean body mass. At the end of the study by Zemel et al, subjects following the diet high in dairy calcium had lost approximately the amount of weight that they should have lost: 9 kg fat and 2 kg tissue that is unaccounted for, unaccounted tissue which represents 81 000–83 000 kcal or 96–99% of their target calorie loss. Indeed it was the subjects in the low dairy calcium group who did not lose as much energy as they should have. Their tissue loss was equivalent to 38 000 kcal, or only 45% of their target energy loss. When similar calculations are applied to other human studies by Zemel et al (20, 21), it would appear that a lack of dietary compliance by the low-calcium groups is a consistent confounding factor.

Calcium is ubiquitous throughout the body, and many physiologic processes depend on its presence. It is for this reason that blood calcium concentrations are maintained within very tight limits (2.2–2.6 mmol/L) by hormonal control. When blood calcium concentrations are low, PTH is secreted, which increases the production of calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>) in the kidney, mobilizes calcium and phosphate from bone, and maximizes tubular reabsorption of calcium to prevent its loss in the urine. Calcitriol facilitates calcium absorption from the intestine and, together with PTH, mobilizes calcium from bone. Consequently, calcitriol administration can be and is used as a treatment for hypocalcemia, one of the potential side effects of which is weight loss (42).

Obese humans are more prone to vitamin D deficiency, in part as a result of lower synthesis and uptake from the gut; in addition, because vitamin D is fat soluble, it is taken up into the fat stores of the body (43). During a hypocaloric diet, the fat stores are mobilized, and, at the same time, any vitamin D associated with this mobilized fat will become available. It is interesting that

vitamin D deficiency is also associated with impaired insulin secretion, which is normalized by vitamin D administration (44). Yanoff et al (45) observed that serum 25-hydroxyvitamin D concentrations were lower in obese American whites and blacks than in their lean peers. Calcitriol concentrations also were lower in obese American blacks than in their lean counterparts, and, although the difference was not significant between obese and lean American whites, calcitriol remained significantly lower when the 2 groups were combined. Similarly, Parikh et al (46) found that calcitriol concentrations were negatively correlated with body mass index and fat mass and were significantly lower in the obese than in the nonobese subjects. These results indicate that obesity is associated with low serum vitamin D and calcitriol concentrations, rather than with high calcitriol concentrations, as proposed by the group led by Zemel.

In summary, our present observations in a small group of overweight and mildly obese, low calcium-consuming men and women show that 5 wk of dairy calcium supplementation at 800 mg/d failed to alter a number of indexes of adipose tissue lipolysis and lipid oxidation. It also did not support the hypothesis that increasing dietary calcium will increase EE, fat oxidation, and fat loss in low calcium-consuming obese persons.

The authors' responsibilities were as follows—all authors: the design and development of the protocol; MB, SR, PS, and LT: recruiting subjects, carrying out the clinical trial, and conducting data analysis; HV and EL: analysis of adipose tissue biopsy samples and data analysis; KJA: writing of the manuscript, which was reviewed and modified by all authors. KJA is an employee of Nestec SA; the study was instigated by KJA for Nestec SA, who requested and financed the study. None of the authors had a personal or financial conflict of interest.

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