In vivo imaging of lipid storage and regression in diet-induced obesity during nutrition manipulation

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Bidar AW, Ploj K, Lelliott C, Neland K, Winzell MS, Böttcher G, Oscarsson J, Storlien L, Hockings PD. In vivo imaging of lipid storage and regression in diet-induced obesity during nutrition manipulation. Am J Physiol Endocrinol Metab 303: E1287–E1295, 2012. First published October 2, 2012; doi:10.1152/ajpendo.00274.2012.—Changes in adipose tissue distribution and ectopic fat storage in, liver and skeletal muscle tissue impact whole body insulin sensitivity in both humans and experimental animals. Numerous mouse models of obesity, insulin resistance, and diabetes exist; however, current methods to assess mouse phenotypes commonly involve direct harvesting of the tissues of interest, precluding the possibility of repeated measurements in the same animal. In this study, we demonstrate that whole body 3-D imaging of body fat composition can be used to analyze distribution as well as redistribution of fat after intervention by repeated assessment of intrahepatocellular lipids (IHCL), intra-abdominal, subcutaneous, and total adipose tissue (IAT, SAT, and TAT) and brown adipose tissue (BAT). C57BL/6j mice fed a cafeteria diet for 16 wk were compared with mice fed standard chow for 16 wk and mice switched from café diet to standard chow after 12 wk. MRI determinations were made at 9 and 15 wk, and autopsy was performed at 16 wk. There was a strong correlation between MRI-calculated weights in vivo at 15 wk and measured weights at 16 wk ex vivo for IAT ($r = 0.99$), BAT ($r = 0.93$), and IHCL ($r = 0.97$). IHCL and plasma insulin increased steeply relative to body weight at body weights above 45 g. This study demonstrates that the use of 3-D imaging to assess body fat composition may allow substantial reductions in animal usage. The dietary interventions indicated that a marked metabolic deterioration occurred when the mice had gained a certain fat mass.

mice; intrahepatocellular lipids; white adipose tissue; brown adipose tissue; intra-abdominal adipose tissue; subcutaneous adipose tissue; magnetic resonance imaging and spectroscopy; insulin resistance; 3R’s; power calculation

The increased prevalence of obesity and the related risk for metabolic diseases such as type 2 diabetes and cardiovascular diseases in regions of the world that have adopted westernized diet standards has resulted in increased interest in prevention and treatment of obesity through lifestyle and pharmacological interventions. It is now recognized that the balance of lipid storage between the specialized sites of fat storage, such as white and brown adipose tissue (WAT and BAT, respectively) and ectopic storage in liver and skeletal muscle tissue impacts the insulin sensitivity of the subject. In addition, the metabolic activity and inflammatory state of each tissue is also relevant in terms of risk for metabolic disorders (1, 13, 22, 24, 26, 32).

In preclinical drug discovery, experimental models are used for the investigation of metabolic disorders. In particular, the mouse is currently the most used species in pharmaceutical research for testing new therapeutic concepts. Numerous diet-induced and transgenic mouse models of obesity, insulin resistance, and diabetes now exist (4). However, many current methods for assaying mouse phenotypes after dietary or pharmacological intervention involve direct harvesting of the tissues of interest, thus precluding the possibility of repeated measurements in the same animal. In the case of time course experiments, this requires using large numbers of mice, which could raise ethical concerns given the ethical imperative to Reduce, Refine, and ultimately Replace animal experimentation (the 3 R’s concept) (43). Thus, from both experimental and ethical standpoints, there is a growing interest in developing safe longitudinal experimental paradigms for simultaneous and repeated in vivo phenotypic monitoring in different mouse tissues. Repeated measurements in the same animal may, in turn, allow greater statistical power by reducing the level of experimental variation (14, 20). This is a challenging task in mice due to the small dimensions of the tissues of interest.

Magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy ($^1$H-MRS) have recently been shown to provide information on the distribution and constitution of specific adipose tissue compartments in rodents (3, 15, 16, 27, 33, 48). In addition, the noninvasive nature of MRI makes it the method of choice for translational studies into the clinical setting (5).

There are two types of adipose tissues, which are specialized for the handling of lipids: WAT and BAT. BAT has a major role in storage and release of fatty acids and in body thermal insulation. On the other hand, BAT is a thermogenic tissue that produces heat by oxidizing fatty acids in specialized uncoupled mitochondria, thereby contributing to nonshivering thermogenesis (53). Recent articles have rekindled interest in BAT as a potential major player in human energy balance (11, 50, 52). BAT activity is a function of the volume of BAT tissue in the body and its metabolic activity. Metabolic activity is difficult to quantify; however, techniques such as $^{18}$F-fluorodeoxyglucose positron emission tomography (FDG-PET) to measure glucose uptake (11) and BOLD MRI to measure blood oxygen level (7, 25) can give a qualitative depiction of BAT activity. Anatomic MRI allows the differentiation of BAT from WAT (34, 38) on the basis of its lipid-to-water content, thereby allowing its volume and lipid fraction to be estimated noninvasively (19, 33). In adult mice, BAT is located mainly in the...
MATERIALS AND METHODS

Animal Experiments

Female C57BL/6j mice (Harlan, Horst, The Netherlands), 8–10 wk old and weighing ≈18 g, were used in the studies. The animals were kept in Macrolon 2L cages (Scanbur, Karlsunde, Denmark; 6 mice per cage) in rooms with regulated temperature (18–22°C), humidity (~50%), and a 12:12-h light-dark cycle. The mice had free access to regular chow pellets [R3; containing (energy%) 12% fat, 62% carbohydrate, and 26% protein; Lactamin, Vadstena, Sweden] and tap water. They were allowed at least 1 wk of acclimatization before study initiation. The local Animal Research Ethics Board Committee (Göteborg) approved these studies.

Study 1

Mice were randomized in three groups (n = 6/group). The first group was given a regular R3 chow diet for 16 wk (low-fat diet group, R3). The second group was fed a cafeteria (CAF) diet, consisting of cheese (containing 38% fat, 1% carbohydrate, and 20% protein; Arla Ost, Västervik, Sweden), chocolate (containing 32% fat, 58% carbohydrate, and 6% protein; Marabou, Kraft Foods, Upplands Väsby, Sweden), nougat (containing 39% fat, 47% carbohydrate, and 6% protein; Odense Marcipan, Odense, Denmark), and chocolate pastry (containing 31% fat, 52% carbohydrate, and 5% protein; Delicatoboll, Delicato, Huddinge, Sweden), together with regular chow (R3) for 16 wk (CAF) (30). The third group was fed the CAF diet for 12 wk and then transferred to the regular R3 chow diet for the remaining 4 wk (weight-reduced group, WR).

Two imaging sessions were performed on each animal, the first during weight gain after 9 wk on cafeteria or R3 diet and the second after 15 wk (i.e., 3 wk after the WR group was switched back to R3 diet). Mice were weighed weekly and immediately before each imaging session.

In vivo MRI and localized 1H-MRS. MRI and 1H-MRS were carried out using a spectrometer operating at 200 MHz (BioSpec 4.7T/40 USR; Bruker BioSpin, Karlsruhe, Germany) equipped with a 400 mT/m actively shielded gradient system and ParaVision software (PV 3.0.2). For MRI/MRS measurement, a 50-mm i.d. quadrature resonator (Bruker, Ettlingen, Germany) was used.

Before the start of the MRI, animals were weighed and then anesthetized with 1.5–2.0% isoflurane (Forene, Abbot Scandinavia) in a 50/50 air-oxygen mixture. The animal was placed supine in a Plexiglas cradle and temperature maintained at 37.0 ± 1.0°C. MRI/MRS acquisitions were synchronized with the respiratory cycle using a respiratory pad placed on the chest of the animal to minimize physiological artifacts (SA Instruments, Stony Brook, NY).

The imaging protocol started with a high-resolution 3-D gradient echo scan (TR/TE/α: 4.2 ms/2.1 ms/45°; FOV: 50 × 50 × 50 mm; matrix size: 256 × 192 × 192) with coverage from the base of the tail to the kidneys. The 45° flip angle gave an optimal contrast between fat and other tissues. Then the table was moved to position the liver at the interscapular BAT depot and in the interscapular region (shoulder blades area) (9). In this study, we exclusively address the interscapular BAT depot.

High-resolution whole body 3-D MR imaging enables the subdivision of total white adipose tissue (TAT) into intra-abdominal and subcutaneous white adipose tissue (IAT and SAT) compartments (46). This partition is important, given that SAT and IAT differ in their metabolic activities (31). SAT can be further subdivided into deep and superficial depots (18). In humans, abdominal obesity has been shown to be most closely associated with insulin resistance (29, 39).

Whole body 3-D imaging of body fat composition is preferred over restricted imaging of a single or a small number of slices (17, 35), in order to analyze adipose tissue redistribution after pharmacological or lifestyle intervention (2, 12). Methods for quantifying IAT and SAT are therefore needed in mouse models, as an increasing number of pharmaceuticals target adipose tissue reduction (41).

Deposition of lipids in nonadipose tissues is strongly linked to the dysmetabolism of diabetes/obesity (23). In particular, the role of intrahepatocellular lipid (IHCL) has received special attention. Recent studies in both patients and rodents have shown that fatty liver is associated with insulin resistance and is linked to several cardiovascular risk factors associated with the metabolic syndrome (21, 44, 45). IHCL has also been recognized as a useful in vivo toxicity biomarker for safety assessment studies (10).

The aim of the present study was threefold. First was to demonstrate that simultaneous and repeated assessment of IHCL, IAT, SAT, TAT, and BAT by means of MRS and MRI is readily feasible and suitable for use as a screening tool in intervention studies in lean and obese mice. Here, we use this method to evaluate the phenotype of the cafeteria diet mouse model, a model commonly used to generate a state resembling human obesity. Hepatic lipids and adipose tissue distribution in mice fed a cafeteria diet and then transferred to a regular chow diet were assessed before and after the diet switch. Validation was performed by comparing in vivo measurements to post mortem end points. The second was to characterize the phenotypic and metabolic switches in mice exposed to a dietary intervention by addressing specifically the relationships among obesity, intrahepatocellular fat, insulin sensitivity, and hypertriglyceridemia. The third was to address the “Reduce” aspect of the 3 R’s (Reduce, Refine, Replace) mandate, by presenting a statistical power analysis to study the benefits of performing a longitudinal study in which each individual is measured repeatedly compared with a cross-sectional study when a different cohort is measured at each time point to resolve a change with a given magnitude.
significant differences were observed at water (4.7 ppm), lipid CH2 (1.3 ppm), and lipid CH3 (0.9 ppm). Different groups showed different degrees of intrahepatocellular lipid (IHCL): CAF (28%), WR (13%), and R3 (1%). Spectra contained peaks representative of water (4.7 ppm), lipid CH2 (1.3 ppm), and lipid CH3 (0.9 ppm).

Based on a subset of the axial MSSE images obtained through the liver, a 3-D scan.

Thereafter, a second 3-D dataset with coverage from the kidneys to 15 slices; slice thickness: 1 mm) was acquired through the whole liver. (TR/TE: 400 ms/9 ms; FOV: 50 × 50 mm, matrix size: 256 × 192, 15 slices; slice thickness: 1 mm) was acquired through the whole liver. Thereafter, a second 3-D dataset with coverage from the kidneys to the nose was obtained with the same scan parameters as for the first 3-D scan.

A B

Fig. 2. Whole body imaging and liver spectra. Representative coronal section obtained from a 3-D T1-weighted data set of a mouse fed CAF diet for 9 wk. A: areas of high intensity denote fat depots. Intra-abdominal adipose tissue (IAT) was segmented by delineating the well-defined parietal peritoneum marked by a white arrow. B: typical localized 'H-MR liver spectra from the different groups showing different degree of intrahepatocellular lipid (IHCL): CAF (28%), WR (13%), and R3 (1%). Spectra contain peaks representative of water (4.7 ppm), lipid CH2 (1.3 ppm), and lipid CH3 (0.9 ppm).

isocenter and a high-resolution multislice spin echo (MSSE) scan (TR/TE: 400 ms/9 ms; FOV: 50 × 50 mm, matrix size: 256 × 192, 15 slices; slice thickness: 1 mm) was acquired through the whole liver. Thereafter, a second 3-D dataset with coverage from the kidneys to the nose was obtained with the same scan parameters as for the first 3-D scan.

Based on a subset of the axial MSSE images obtained through the liver, a 3 × 3 × 3 mm voxel of interest was placed typically in the right lateral lobe adjacent to the portal vein and well removed from the surface of the liver for the subsequent respiratory gated single voxel point resolved spectroscopy (PRESS) sequence acquisition (TR ~3 s, TE 6.8 ms, 64 scans). Automatic routines were employed for course and fine shimming, with typical line width of the water signal of 50 Hz.

Spectroscopic data processing. Line broadening of 2 Hz was applied to the MR spectra prior to Fourier transform. The baseline was corrected with a spline function, and then water and lipid resonances were fitted using a Voigt model, which combines the Lorentzian and Gaussian models. All spectra were processed in a standardized manner using the Bruker XWINNMR analysis package. Areas of water peak (Aw) at 4.7 ppm and the lipid peaks (CH2o at 1.3 ppm (Af1) and CH3 at 0.9 ppm (Af2)) were measured, and IHCL was expressed in percentage as: IHCL = 100 × (Af1 + Af2)/(Aw + Af1 + Af2).

Segmentation of fat compartments in MR images. Abdominal and thoracic 3-D data were imported into an in-house IDL (Interactive Data Language; ITT Visual Information Solutions, Boulder, CO) software application for concatenation and quantification of the different adipose tissues. Adipose tissue compartments were segmented semiautomatically using the image analysis software Analyze 6.0 (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). The semiautomatic segmentation procedure was based on histogram thresholding and region growing as described in Ref. 40. The IAT was defined as the white adipose depots inside the well-defined parietal peritoneum and included the omental, retroperitoneal, and mesenteric fat depots. Briefly, segmentation was performed as follows: 1) define a threshold level to segment all the fat in the 3-D data set, giving TAT; 2) manually outline the IAT on every coronal slice in the volume, the outline not having to follow the contour of the IAT; and 3) combine the outlined areas with the segmented fat mask using the AND operator to exclude nonfat areas from the segmented IAT. The SAT mass was defined as the difference between TAT and IAT. Volumes (in ml) of the different adipose compartments were calculated by multiplying the number of segmented voxels by the voxel volume resolution. A density factor of 0.9 g/ml was used to convert fat volumes (ml) into fat mass (g).

The BAT was segmented based on the distinct contrast exhibited at the scapular region between WAT and muscle using a region-growing algorithm. The intensity of the MRI signal from BAT was normalized to the MRI signal in the brain.

Post mortem end points. At week 16, 1 wk after the last imaging session, mice anesthetized using isoflurane inhalation were killed by

Table 1. Effects of nutritional intervention on body weight, TAT, IAT, SAT, BAT, and IHCL as measured at weeks 9 and 15 in vivo by means of MRI, study 1

<table>
<thead>
<tr>
<th>In Vivo Parameters</th>
<th>CAF Week 9</th>
<th>CAF Week 15</th>
<th>WR Week 9</th>
<th>WR Week 15</th>
<th>R3 Week 9</th>
<th>R3 Week 15</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>34.3 ± 1.8</td>
<td>37.3 ± 1.6</td>
<td>37.3 ± 1.6</td>
<td>37.3 ± 1.6</td>
<td>37.3 ± 1.6</td>
<td>37.3 ± 1.6</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Total fat mass, g</td>
<td>18.4 ± 1.2</td>
<td>20.6 ± 1.2</td>
<td>20.6 ± 1.2</td>
<td>20.6 ± 1.2</td>
<td>20.6 ± 1.2</td>
<td>20.6 ± 1.2</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Intra-abdominal fat mass, g</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.0</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Subcutaneous fat mass, g</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Interscapular brown fat mass, mg</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Brown fat-to-brain signal intensity ratio</td>
<td>7.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>7.0 ± 1.0</td>
<td>a***, b***, c***</td>
</tr>
<tr>
<td>Intrahepatocellular lipids, IHCL, %</td>
<td>13.9 ± 1.7</td>
<td>13.9 ± 1.7</td>
<td>13.9 ± 1.7</td>
<td>13.9 ± 1.7</td>
<td>13.9 ± 1.7</td>
<td>13.9 ± 1.7</td>
<td>a***, b***, c***</td>
</tr>
</tbody>
</table>

Data represent means ± SE; n = 6 animals per group. CAF, cafeteria diet; R3, free access to regular chow pellets; WR, CAF diet for 12 wk and then R3 diet for the remaining 4 wk (weight reduced). Statistical significance was tested for the following comparisons: R3 vs. CAF(b) and WR and WR vs. CAF(a)

*p < 0.05, **p < 0.01, ***p < 0.001, ns, nonsignificant. Statistical comparisons for IHCL were performed using log-transformed data. As expected, no significant differences were observed at week 9 between WR and CAF groups, which were both fed on the same CAF diet at that time point.
removal of the heart. One mouse from each group was frozen and used for whole body sectioning. The main purpose of the whole body sectioning was to investigate qualitatively the morphological relationship between MRI images and histology of the scapular brown fat. The livers of the remaining animals \((n = 5/\text{group})\) were dissected and weighed, and samples were taken for liver triglyceride (TG) content as described in Ref. 30. Parametrial WAT was dissected by opening the abdominal cavity of the mouse, locating the uterus, and dissecting the fat pads from the wall of the uterus and both uterine horns. BAT was dissected out by locating and removing the interscapular fat pad that contains both WAT and BAT. The dorsal superficial layer of WAT was removed, and the two major lobes of BAT were carefully dissected free from residual WAT. Parametrial WAT and BAT were weighed, and samples of interscapular BAT from each group were formalin fixed and later stained with hematoxylin and eosin.

**Study 2**

**Animals and diets.** Mice were randomized in four groups \((n = 10/\text{group})\). Group 1 was placed on a control diet \((\text{D12450B diet} \text{; Research Diets, New Brunswick, NJ})\) containing 10% fat by energy, group 2 on a defined high-fat diet \((\text{HFD})\) containing 60% fat by energy \((\text{D12492, Research Diets})\), group 3 on a D12492 diet supplemented with chocolate pastry \((30)\), and group 4 on a cafeteria diet together with regular chow for a period of 18 wk as described in study 1. Body weights \((\text{BW})\) were recorded weekly.

**Analytic procedures.** At the completion of the dietary period, blood was collected from a tail vein, and the animals were euthanized by isoflurane inhalation. Plasma insulin and glucose were measured on a subset of mice \((n = 8/\text{group})\). Plasma glucose concentrations on 4-h-fasted mice were measured with a glucometer \((\text{Accu-chek, Roche Diagnostics})\). Plasma insulin was measured in whole blood \((3 \mu l)\) by ELISA \((\text{Crystal Chemical})\). The TG concentrations in the plasma and liver were measured as described before \((30)\) and expressed as millimoles per liter and grams per 100 g, respectively. The homeostasis model assessment \((\text{HOMA})\) index was calculated as fasting insulin concentration \((\mu \text{U/ml}) \times \text{fasting glucose concentration (mmol/l)/22.5} (37)\). HOMA is a surrogate measurement of in vivo insulin resistance developed and validated primarily in humans \((36)\).

**Power Calculations and Estimate of Sample Size to Detect Treatment Effect**

The power for comparisons between two treatment groups within a time point was studied for IHCL \((\text{in vivo parameter})\) and liver TG \((\text{ex vivo parameter})\). For both parameters, the log transform of the parameter can be assumed to follow a normal distribution \((\text{the original values follow a log-normal distribution})\). Calculations were therefore based on the log transform of the measurement rather than on the measurements themselves. For simplicity and ease of estimation of the number of animals needed to detect a certain difference with a certain chance, we assumed that all \((\text{log-transformed})\) measurements for each parameter separately had the same standard deviation irrespective of diet regimen and week. As standard deviations for the calculations, pooled-sample standard deviations were used \((\text{over treatment for the ex vivo parameter and over treatment and time for the in vivo parameter})\). The standard deviations of IHCL and liver TG \((\text{for log-transformed data})\) used in the calculation were \(\sigma_{\text{IHCL}} = 0.14\) and \(\sigma_{\text{Liver TG}} = 0.18\).

In the case of log-normal measurements, the absolute differences for the log-transformed measurements translate into relative differences for the original measurements. This means that studying percent change in the power calculations is straightforward.

Sample size requirement was estimated based on the noncentral \(t\) distribution \((8)\) with a two-sided test, significance level of 5%, and a desired power of 80%. If we are interested in comparisons at more than one time point, and we terminate the animals after measurement, we need to multiply the number of animals needed for one comparison with the number of time points to get the total number of animals needed on each diet regimen \((\text{liver TG})\). This is not necessary if one can take repeated measurements on a single animal \((\text{IHCL})\). In the calculations, we have not adjusted for the fact that many comparisons were performed.

**Statistical Analysis**

Intergroup comparisons at week 9 and week 15, respectively, were analyzed using pairwise Student’s \(t\)-tests not assuming equal variability, using R \((\text{version 2.10.1, The R Foundation for Statistical Computing})\). Liver weight, liver TG, and IHCL were log transformed.

**Table 2. Liver mass, liver TG content, brown fat mass, and parametrial WAT mass measured ex vivo at week 16, study 1**

<table>
<thead>
<tr>
<th>Ex Vivo Parameters</th>
<th>CAF</th>
<th>WR</th>
<th>R3</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver mass, g</td>
<td>2.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>0.92 ± 0.04</td>
<td>(a^{<strong><em>}, b^</em>, c^{</strong>})</td>
</tr>
<tr>
<td>Liver TG, mg/100 mg tissue</td>
<td>4.8 ± 0.9</td>
<td>4.9 ± 1.0</td>
<td>1.9 ± 0.1</td>
<td>(a^{<strong><em>}, b^</em>, c^{</strong>})</td>
</tr>
<tr>
<td>Brown fat mass, BAT, mg</td>
<td>300 ± 50</td>
<td>90 ± 10</td>
<td>50 ± 10</td>
<td>(a^{<strong><em>}, b^</em>, c^{</strong>})</td>
</tr>
<tr>
<td>Parametrial WAT mass, g</td>
<td>2.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.30 ± 0.04</td>
<td>(a^{<strong><em>}, b^</em>, c^{</strong>*})</td>
</tr>
</tbody>
</table>

Data represent means ± SE; \(n = 5\) animals per group. Statistical significance was tested for the following comparisons: R3 vs. CAF, R3 vs. WR, and WR vs. CAF. \(*p < 0.05, **p < 0.01, ***p < 0.001. Statistical comparisons for liver TG and liver mass were performed using log-transformed data.

![Fig. 3. Whole body histology section of scapular region vs. MRI. A: whole body histology at the level of the scapular region showing brown adipose tissue (BAT; inside the white ellipse). B: MRI image from the same specimen taken at the same level. Note the distinct contrast between BAT and WAT (white adipose tissue).](image-url)
before analysis. Pearson’s correlation coefficient, together with 95% confidence intervals, was calculated for ex vivo and in vivo measurements. Intergroup comparisons in study 2 were analyzed using Student’s t-test. Differences with an associated P value < 5% were considered significant. No correction for multiplicity was performed. Results are expressed as means ± SE.

RESULTS

Study 1: Effects of Dietary Intervention on Body Composition and Liver TG

Dietary effects on body weight. Figure 1 shows the mean weight gain curves and timing of the MRI for the three dietary groups. The CAF diet resulted in a doubling of body weight within 12 wk, and the return to regular R3 chow in the WR group led to the loss of approximately two-thirds of the excess weight in 4 wk.

In vivo MRI and localized 1H-MRS. All animals successfully underwent the MRI/MRS procedure. WAT and BAT showed a high contrast with the surrounding tissues. High spatial resolution allowed the parietal peritoneum to be resolved and, thus, delineation between the intra-abdominal and the subcutaneous adipose compartments (Fig. 2A). Indeed, the fascia superficialis separating the deep and superficial subcutaneous fat depots was also visible in many slices, but no assessments of the volumes of the different subcutaneous depots were made because it is not visible in all slices. Representative liver spectra from mice in CAF, WR, and R3 diet regimens are shown in Fig. 2B. One localized liver spectrum obtained at week 15 from a mouse on CAF diet regimen was excluded from the statistical analysis due to poor spectral quality.

White and brown adipose mass. Table 1 summarizes the ex vivo values for liver mass, liver TG, interscapular BAT, and parametrial fat compartments. CAF diet increased liver mass and all adipose compartments (all at least P < 0.001), and WR reduced weights to values intermediary between CAF and R3 groups, except BAT mass, which was normalized.

Whole body histology. Figure 3A shows a section obtained by whole body histology at the level of the scapular region, including the BAT. The butterfly pattern of the BAT shown in the histological section confirms the BAT pattern seen on the in vivo MRI image obtained at the same level from the same specimen (Fig. 3B).

Histological assessment of brown adipose tissue. Photomicrographs obtained from interscapular adipose tissue, corresponding to the brown adipose depot, stained with hematoxylin and eosin, as displayed in Fig. 4, indicate that adipocytes with large-size lipid vacuole profiles (large lipid droplets) are more abundant in CAF mice (Fig. 4B) than in the BAT in R3 mice (Fig. 4A). In the WR mice (Fig. 4C), an intermediate state with

Three weeks after the switch to low-fat diet, TAT of the WR group weighed 50% less (−10.3 g, P < 0.001) than in the CAF group and almost five times more (+18.5 g, P < 0.001) than the R3 group. IAT and SAT masses were significantly lower in WR mice than in CAF mice and significantly higher than in R3 mice (Table 1).

BAT mass and fat content. MRI analysis revealed that at week 15 BAT of the CAF group weighed more than three times that in the R3 mice (+130 mg, P < 0.001). However, both BAT mass and signal intensity in the WR group returned to normal compared with the R3 group.

IHCL levels. At week 15, IHCL levels in CAF mice were markedly elevated (25.3 ± 4.1% in CAF mice vs. 1.8 ± 0.3% in R3 mice). After 3 wk on regular R3 chow diet, the IHCL level in WR mice was 64% lower (P < 0.001) than in the CAF mice although still not at the level seen in the R3 mice.

Diet effects on adipose compartments and liver TG determined ex vivo. Table 2 summarizes the ex vivo values for liver mass, liver TG, interscapular BAT, and parametrial fat compartments. CAF diet increased liver mass and all adipose compartments (all at least P < 0.001), and WR reduced weights to values intermediary between CAF and R3 groups, except BAT mass, which was normalized.

Comparison Correlation, r P 95% Confidence Interval

| IHCL vs. liver TG, mg/100 mg tissue | 0.93 | <0.0001 | 0.81 to 0.98 |
| MRI BAT vs. ex vivo BAT, g | 0.93 | <0.0001 | 0.80 to 0.98 |
| MRI IAT vs. ex vivo parametral WAT, g | 0.99 | <0.0001 | 0.98 to 1.00 |

Fig. 4. Histological features of BAT in the R3, CAF, and WR groups. Representative photomicrographs of hematoxylin and eosin-stained tissue of BAT from animals from the R3 (A), CAF (B), and WR (C) groups.
Insulin, and HOMA index were performed using log-transformed data. In the BW range 20–25 g (IAT 0.1 to 1 g) IHCL level remained constant (−2%); 2) in the BW range 30–40 g (IAT 2 to 6.5 g), IHCL increased linearly at −0.4%/g BW; 3) when mice reached 45 g (IAT −6.7 g), a steep IHCL increase of 2.5%/g BW was observed.

Study 2: Effects of Dietary Intervention on Liver TG, Plasma Lipid and Plasma Glucose Levels

The aim of this study was to investigate whether the effect on metabolic parameters including liver lipid content at a certain body weight or body fat mass could be repeated using different diets covering a larger final body weight span. The dietary effect on weight gain showed the following ranking: CAF diet > D12492 (chocolate pastry) > D12492 > D12450B (Table 4).

**Plasma and liver biochemistry.** Fasting plasma insulin level and HOMA showed a steep increase when mice reached a BW above 45 g (Fig. 6, A and C). A similar pattern was observed with liver TG content (Fig. 6B). Fasting plasma glucose level was significantly higher in CAF diet-fed mice compared with mice fed standard chow but did not show the same steep increase above 45 g BW.

**Power Calculations and Estimate of Sample Size to Detect Treatment Effect**

The result of the power calculations, with chance of detection chosen to be 80% and estimates for the standard deviation taken from the present data, are displayed in Table 5 for different magnitudes of the treatment effect to allow sample size explorations for the different experimental designs. The statistical power analysis based on the measured responses showed that a noninvasive method that allows each animal to be measured repeatedly requires fewer animals to be included in the experiment to detect a significant difference in IHCL.

**DISCUSSION**

In the present study, we demonstrate in a mouse model the feasibility of measuring in vivo, simultaneously and repeatedly, the distribution of BAT and WAT compartments as well as hepatic TG content. In particular, we address the phenotype switch of the cafeteria diet mouse model when exposed to a dietary intervention.

The results reported here confirm the findings of previous investigators that in vivo MRI/MRS of WAT compartments...
and IHCL correlate to ex vivo measurements of the same end points (48). To the best of our knowledge, this is the first time that IHCL, IAT, SAT, TAT, and BAT were all measured simultaneously. Moreover, it was shown that cafeteria diet-induced obesity in mice resulted in dramatic increases in hepatic fat and insulin resistance at a certain degree of obesity. Cafeteria diet led to the expected increase in BW, and return to regular chow reduced BW, liver fat, and WAT mass back to a level intermediate to that of the R3 controls.

In contrast, the BAT mass in the WR group completely normalized after 3 wk on regular R3 chow. Reductions in BAT mass have previously been seen after intervention (51). Histological assessment of BAT has shown that the large lipid droplets seen in diet-induced obesity models can be normalized by diet, exercise, and drug interventions. However, in the present study we showed that a return to R3 diet for 4 wk did not return BAT lipid levels in WR mice to the levels seen in R3 mice (47). Thus, although BAT mass is more responsive than WAT to weight reduction, being essentially normalized, lipid content is still elevated, suggesting a less metabolically active BAT in the WR group than in the R3 group. It was noted that the BAT-to-brain MRI signal ratio observed in WR mice in vivo decreased to the levels seen in R3 mice. Signal intensity in these T1 weighted MRI acquisitions are expected to reflect lipid content, and so there is an inconsistency with the histology results. Potential explanations include a change in lipid composition such that BAT lipids in the WR group are not in a liquid state at physiological temperatures and therefore have reduced MR visibility (49) or that the temperature in the brown fat is reduced below the phase transition temperature of the lipids (42). Further studies are needed to understand the importance of this finding in relation to the postobese energy metabolism.

An important, somewhat unexpected, observation from study 1 was the clear and abrupt transitions from normal to grossly steatotic liver as BW and IAT increased (see Fig. 5). When the amount of IAT rose above 15% of BW (corresponding to a BW of ~45 g and a %TAT to BW of ~45%), there was a sharp rise in liver fat. The same transition level was confirmed when the ex vivo relationship between parametrial WAT and liver TG content was studied (result not shown).

Study 2 was designed to produce mice at a broad range of BW to determine whether there was a similar break point in plasma insulin and IHCL at 45 g BW as seen in study 1. When mice on the cafeteria diet reached a BW of ~45 g, a parallel and marked increase in liver TG content, similar to the IHCL changes in study 1, plasma insulin levels, and HOMA index occurred. The steep increase in HOMA was driven by plasma insulin levels rather than changes in plasma glucose (data not shown). This is consistent with clinical studies relating insulin resistance to hepatic lipid accumulation (6, 28).

From these experiments it is not possible to determine whether it is total BW, liver TG, or some other factor that best predicts the metabolic break point.

Overall, these results suggest that the capacity of WAT to store excess fat calories has a very specific finite limit, with the spillover showing up in circulating hypertriglyceridemia, hepatic steatosis, and likely in other insulin-sensitive tissues such as skeletal muscle. The specific limit found in this study for C57BL/6J mice may be different for other strains, based on the capacity for adipose expansion and the ability for nonadipose tissue to handle excess lipids. Thus, further studies of WAT

Table 5. Sample size calculations for IHCL or liver TG for the case of a longitudinal or a cross-sectional study

<table>
<thead>
<tr>
<th>Difference, %</th>
<th>Repeated IHCL, n/Group</th>
<th>1 Time Point Liver TG, n/group</th>
<th>2 Time Points Liver TG, n/group</th>
<th>3 Time Points Liver TG, n/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>5</td>
<td>9</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>70</td>
<td>6</td>
<td>11</td>
<td>22</td>
<td>33</td>
</tr>
<tr>
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<td>18</td>
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</tr>
<tr>
<td>40</td>
<td>15</td>
<td>26</td>
<td>52</td>
<td>78</td>
</tr>
</tbody>
</table>

Difference, %, is the relative difference between groups; n represents the total number of animals needed per treatment group when comparing 2 groups for IHCL, where each animal can be measured repeatedly, so the number is the same irrespective of the number of time points to study and when comparing 2 groups for liver TG, where a different cohort of the total amount of animals allocated to the treatment group is measured at each time point. Shown in the table are the cases of 1, 2, and 3 time points. In all cases, the chance to detect a significant result is set to 80% and the significance level \( P = 0.05 \).
structure and metabolism at this critical transition point would give important clues to our understanding of the mechanisms involved in limiting expansion of the adipose tissue storage capacity.

In the present study, the MRI/MRS protocol took ~45 min per mouse, while the manual segmentation procedure took around 1 h per animal. In subsequent studies, acquisition time was reduced to 20 min and an algorithm for automatic segmentation of the IAT and SAT compartments developed, reducing the analysis time to a few seconds (40).

The results of the power analysis illustrate the importance of MRI in serially and noninvasively monitoring the metabolic/phenotype changes after diet or a treatment intervention. By taking repeated measures on each animal, sample size could be reduced, thus complying with two of the 3R mandates, “reduce and refine.” Furthermore, the dynamic nature of the measured metabolic end points emphasizes the importance of multiple measurements over time. Thus, the use of MRI or other imaging techniques to follow longitudinal changes in diet-induced obese mice during weight and subsequent dietary or pharmaceutical intervention should be considered where possible.

Most important also is to design studies in light of the metabolic break point observed in mice fed a cafeteria diet leading to a phenotype shift to avoid confounding variations due to different metabolic profiles. It should also be noted that MRI studies such as these may be translatable to clinical studies where imaging can be used to monitor IHCL and adipose tissue mass in total body and depot-selective manners. This bridges the preclinical and clinical work in obesity research and enhances the relevance of rodent studies to the clinical setting.

In conclusion, the results of this phenotyping study provide us with a basic data set for comparing different animal models of obesity and diabetes and evaluating the effect of drugs on the diet-induced obese mouse model compared with weight reduction. An important finding in this study was the observation that a threshold exists over which further body weight gain is associated with a dramatic increase in hepatic fat and insulin resistance. We anticipate that these results will influence the design of future preclinical studies aimed at studying the metabolic syndrome and assist the design of future clinical trials.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES