

# Sequential Responses to High-fat and High-calorie Feeding in an Obese Mouse Model

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**Objective:** Reports on the immediate and long-term responses to high-fat and high-calorie (HFC) feeding are controversial. Therefore, we examined the sequential effects of an HFC diet.

**Methods and Procedures:** C57BL/6J mice were randomly assigned to consume either the control (C) or the HFC diet. Body weights and food intake were measured weekly and other measurements at weeks 2, 4, and 10. Microarrays were used for screening the transcriptional response of the livers at the three time points. Genes, encoding enzymes regulating key steps of lipid metabolism, were then selected from the microarray data for validation by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and their protein expression by western blot assays.

**Results:** Mice fed with HFC diet for 2 weeks showed no increase in food intake and no difference in weight gain compared to the C mice. At weeks 4 and 10, the HFC mice increased their food intake and gained more weight than their controls (by 1.4 times and 2.5 times, respectively) ( $P < 0.01$  at week 10). Genes involved in fatty acid oxidation (FAO) were initially upregulated and then downregulated, whereas the lipogenic genes and genes involved in cholesterol synthesis showed reverse trends. The differential mRNA expression of *Cpt1L*, *Fas*, and *Hmgcr* were confirmed by RT-PCR and their protein expression by western blot assays.

**Discussion:** Our findings suggested that when mice were fed an HFC diet, they could develop initial compensatory response to resist the increased energy balance; however, a prolonged consumption of an HFC diet appeared to disrupt this adaptation.

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## INTRODUCTION

Obesity is a major health problem that is increasing in both prevalence and severity. Increased food intake, particularly a diet high in calories and fat content, combined with decreased energy expenditure associated with modern lifestyle, are the major environmental causes of obesity (1,2). However, environmental factors do not fully explain the rapidly increasing rates of obesity (3,4). Genetic predisposition for obesity may underlie the tendency for weight gain in some individuals (5). To date, the interactions between environmental and genetic factors and the effects that such interplay might have on weight gain and maintenance is not fully understood (6).

Studies on humans and animals that were allowed ad libitum feeding on high-fat and high-calorie (HFC) diets have demonstrated different effects. Some have showed that these subjects consumed similar amounts of food as when they were fed ad libitum on lower-fat, less energy-dense diets (7,8). Others, however, have shown that such HFC diets inhibited food intake (9,10) and yet others have provided evidence that HFC diets promote hyperphagia (11–15). We postulated that the reason for the mixed findings was due to the varying lengths

of the feeding period and that HFC diet would initially elicit compensatory responses between feeding behavior and gene expression levels, which would help to regulate energy homeostasis. We also proposed that such compensatory responses would diminish over time with the continued HFC feeding. Moreover, obesity has been suggested as a breakdown of the body's ability to regulate energy homeostasis or to handle external environmental perturbations (e.g., a diet high in calories and fat) (16). As the body is always constantly adapting to the changes in a dynamic environment, it will be interesting to examine how it adapts to consumption of a diet high in calories and fat over a period of time.

We examined the effects of HFC diet on feeding behavior, body weight regulation, and common biomarkers associated with weight gain in the C57BL/6J mice over a period of 10 weeks, making measurements at weeks 2, 4, and 10, in order to study the sequential responses at each stage. We examined the transcriptomic profile of hepatic genes involved in the major lipid metabolic pathways, validating the key genes with quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and their gene products with western blots. The liver was chosen because it plays a central role

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in regulating energy homeostasis and because it is a major organ involved in the regulation of food intake (17).

We have selected the C57BL/6J mouse for our study as it is commonly used as a model for human obesity. Like humans, this strain develops obesity and raised blood glucose and lipid levels when fed with a high-fat diet; however, it remains lean if the fat content of the diet is limited (18). Several studies of high-fat feeding or differing fat composition of the diet have been conducted in this strain of mouse (19–21). However, to our knowledge, none has yet sequentially examined the effects of such diets on the regulation of feeding, body weight, and mRNA levels of genes involved in lipid metabolism beyond one time point.

## METHODS AND PROCEDURES

### Mice and diets

Forty-eight female C57BL/6J mice, 8-week-old, were supplied by the Laboratory Animal Center, National University of Singapore. They were acclimatized in our Animal Holding Unit for 2 weeks, feeding on standard chow before starting on their respective diets. The mice were randomly divided into six groups of eight for the three-time point study. They were fed with the control (C) diet or the high-fat, calorie-dense (HFC) diet. The HFC diet (SF00-219; Specialty Feeds, Perth, Australia) consisted of 19% protein, 21% fat, and 60% carbohydrate, which provided a total calorie content of 19.4 kJ/g. The C diet was standard chow diet (Rat and Mouse Cubes, Specialty Feeds), with 19% protein, 4.6% fat, and 76.4% carbohydrate, which provided a total calorie content of 14.3 kJ/g. Mice body weights and food intake were measured weekly at the same time of the day. After 2, 4, and 10 weeks of the respective dietary treatment, the mice were sacrificed. Blood was collected by cardiac puncture. Liver samples comprising identical lobes were quickly removed from each mouse. The liver samples were immediately snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Intra-abdominal fat depots were rapidly removed, placed in pre-weighed vials, and weighed. The fat tissue were then frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . All animal experiments were performed with the approval of the National University of Singapore Laboratory Animals Centre.

### Lipid assays

Plasma total cholesterol, high-density lipoprotein cholesterol, and triacylglycerol concentrations were measured after an overnight fast using enzymatic assay kits on a Cobas Mira analyzer (Roche, Basel, Switzerland). Low-density lipoprotein (LDL)/very-low-density lipoprotein cholesterol concentrations were determined as the difference between total and high-density lipoprotein cholesterol concentrations. Blood glucose levels were measured using the Accu-Chek glucometer (Roche Diagnostics, Basel, Switzerland) and Accu-Chek test strips (Roche Diagnostics) with appropriate calibration.

### Analysis of gene expression profile by high-density oligonucleotide microarrays

To determine the liver gene transcript profile in the different groups, relative abundance of mRNAs in the HFC groups were compared with

their corresponding controls (e.g., HFC-2wk vs. C-2wk). The GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) containing 45,000 probes for ~34,000 known mouse genes were used.

### Sample preparation, array hybridization, and scanning

Total RNA was isolated from the frozen liver tissue of each mouse using Trizol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Quality and quantity were checked using a spectrophotometer and agarose-gel analysis. Equal amounts of total RNA from individual mouse within the same group were pooled. Following Affymetrix protocol, purified and pooled total RNA was reverse transcribed to cDNA. Subsequently, the double-strand cDNA was transcribed *in vitro* to biotinylated cRNA. The labeled cRNA was then fragmented and mixed with control oligonucleotide B2, control cRNA (BioB, BioC, BioD), herring sperm DNA, bovine serum albumin, and hybridization buffer. The hybridization cocktail was then loaded onto the GeneChip Mouse Genome 430 2.0 probe array cartridges and incubated at  $45^{\circ}\text{C}$  for 16 h on a rotisserie at 60 rpm. After hybridization, the arrays were washed in GeneChip Fluidics Station 400 and stained with streptavidinphycoerythrin (Molecular Probes, S-866). Probe arrays were scanned using the Agilent G2500A Gene Array scanner after washing. The quality of the fragmented biotin-labeled cRNA in each experiment was checked before hybridizing onto the Mouse Genome 430 2.0 Arrays by using Test-3 arrays.

### Genechip data analysis

The GeneChip Operating Software Version 1.2.0.037 (Affymetrix) was used for analyzing the scanned image. Expression patterns for each group were analyzed according to the following pairwise comparisons: HFC-2wk vs. C-2wk, HFC-4wk vs. C-4wk, and HFC-10wk vs. C-10wk. In order to obtain the robust “increase” expression profile, the experimental probe sets designated “Absent” were eliminated and only the probe sets flagged as “I” with Signal Log Ratio  $\geq 0.5$  were selected. As for robust “decrease” profile, the “Absent” cells were eliminated from the C samples and only those flagged “D” with Signal Log Ratio less than or equal to  $-0.5$  were selected. Data of “fold change” were calculated from the “Signal Log Ratio”. All logarithmic transformation were in base 2. The selected probe sets were then annotated and classified using NetAffx Gene Ontology Mining Tool. The gene expression data were presented using TreeView. The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo>) under Gene Expression Omnibus Series number GSE8753.

### qRT-PCR

From the screening with microarrays, we selected target genes encoding enzymes which regulate key steps of lipid metabolism to conduct qRT-PCR tests to validate their differential expression. The primers sequences used are listed in Table 1. The LightCycler SYBR Green I one-step RT-PCR (Roche Diagnostics) method was used to quantify the starting mRNA of the target genes. For amplification, RNA template, primers, Mn(OAc)<sub>2</sub> and LightCycler RNA Master SYBR Green I were mixed well and transferred to LightCycler capillaries (Roche Diagnostics) according to Roche qRT-PCR protocol. The signal of the housekeeping gene  $\beta$ -actin was used for normalization. Melting curve analysis was performed to assess the specificity of the amplified PCR

**Table 1** Sequences of primers used for RT-PCR

| Gene name and gene symbol   | Forward (5'→3')          | Reverse (5'→3')           | PCR product (bp) |
|---|--------------------------|---------------------------|------------------|
| Actin, beta ( $\beta$ -actin)   | taaagacctctatgccaacacagt | cacgatggaggggcccggactcatc | 241              |
| 3-Hydroxy-3-methylglutaryl-coenzyme a reductase ( <i>Hmgcr</i> )        | tgtggccaggagtttggtgactga | taagattcaacaactctgctgacc  | 101              |
| Fatty acid synthase ( <i>Fas</i> )                                      | tctgtgaagagtcagtgaggca   | cagcagcctgtgtattgactctag  | 212              |
| Carnitine palmitoyltransferase 1A, liver ( <i>Cpt1L</i> )               | actgtaagtgttcaaggaggag   | gctatattcgttcacacatgc     | 173              |
| Cytochrome P450, family 7, subfamily a, polypeptide 1 ( <i>Cyp7a1</i> ) | actgtgcttctgcttgccttg    | tggatacattcagttcaggagctc  | 168              |

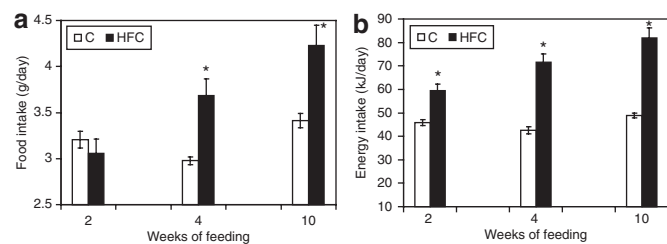
products. The amplified PCR products were subjected to 2% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide.

### Western blots

Four mice from each group were randomly selected and the western blot assays were carried out on the individual liver samples. The liver tissues were homogenized in RIPA buffer containing a mixture of protease inhibitors (Complete Mini EDTA-free, Roche). Denatured proteins (30 $\mu$ g) were loaded onto polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham, Buckinghamshire, UK) and stained with Ponceau S to confirm equal protein loading. Membranes were then blocked with phosphate-buffered saline containing 0.1% Tween and 5% skimmed milk powder for 1 h at room temperature. Next, they were incubated overnight at 4°C with the respective antibodies: a rabbit antibody against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Cell Signaling, Beverly, MA), fatty acid synthase (BD Biosciences, Franklin Lakes, NJ), and Cpt1L (Alpha Diagnostic International, San Antonio, TX). Rabbit antibody against  $\beta$ -actin (Cell Signaling) was used for normalization. Subsequently, membranes were incubated with a horseradish peroxidase-linked goat anti-rabbit IgG (Cell Signaling) for 1 h at room temperature. Following application of chemiluminescent detection reagents (Amersham), Clear-Blue X-ray films (Pierce, Rockford, IL) were exposed to the blots and the band intensities were determined using Quantity One software (Bio-Rad, Hercules, CA).

### Statistical analysis

All results are expressed as means  $\pm$  s.d. Statistical significance of differences was analyzed using Student's *t*-test. Differences in measurements



**Figure 1** Feed consumption and energy intake of mice. (a) Feed consumption. (b) Energy intake. Results are presented as means  $\pm$  s.d. for each group of mice ( $n = 8$  per group) \* $P < 0.05$  compared to corresponding control group. Open bars represent data from control (C) mice and filled bars represent data from high-fat and high-calorie (HFC) mice.

**Table 2** Gained body weight, percent change in body weight, feed and energy efficiency ratios, white adipose tissue mass, plasma lipids, and glucose levels

|                            | C-2wk            | HFC-2wk           | C-4wk            | HFC-4wk           | C-10wk            | HFC-10wk           |
|----------------------------|------------------|-------------------|------------------|-------------------|-------------------|--------------------|
| Gained in body weight (g)  | 0.85 $\pm$ 0.59  | 0.88 $\pm$ 0.80   | 1.78 $\pm$ 1.13  | 2.50 $\pm$ 1.14   | 2.98 $\pm$ 1.98   | 7.60 $\pm$ 1.89*   |
| % Change in body weight    | 4.31 $\pm$ 3.04  | 4.35 $\pm$ 4.03   | 9.05 $\pm$ 5.95  | 12.25 $\pm$ 5.23  | 16.39 $\pm$ 12.99 | 42.64 $\pm$ 10.12* |
| Feed efficiency ratio      | 14.96 $\pm$ 7.42 | 14.64 $\pm$ 10.12 | 15.04 $\pm$ 6.88 | 24.33 $\pm$ 11.42 | 15.69 $\pm$ 4.55  | 26.95 $\pm$ 7.01*  |
| Energy efficiency ratio    | 1.11 $\pm$ 0.55  | 0.75 $\pm$ 0.52   | 1.11 $\pm$ 0.51  | 1.25 $\pm$ 0.59   | 1.16 $\pm$ 0.34   | 1.39 $\pm$ 0.36    |
| White adipose tissue (g)   | 0.18 $\pm$ 0.01  | 0.20 $\pm$ 0.05   | 0.18 $\pm$ 0.05  | 0.34 $\pm$ 0.10** | 0.29 $\pm$ 0.06   | 0.61 $\pm$ 0.21**  |
| Glucose (mmol/l)           | 15.70 $\pm$ 3.39 | 15.68 $\pm$ 3.63  | 17.23 $\pm$ 4.02 | 17.38 $\pm$ 5.03  | 17.38 $\pm$ 3.60  | 25.00 $\pm$ 3.65*  |
| Total cholesterol (mmol/l) | 1.67 $\pm$ 0.09  | 2.22 $\pm$ 0.39*  | 1.71 $\pm$ 0.11  | 2.60 $\pm$ 0.47*  | 1.65 $\pm$ 0.13   | 2.58 $\pm$ 0.23*   |
| LDL cholesterol (mmol/l)   | 0.26 $\pm$ 0.12  | 0.60 $\pm$ 0.15** | 0.40 $\pm$ 0.14  | 0.70 $\pm$ 0.09** | 0.23 $\pm$ 0.08   | 0.82 $\pm$ 0.26*   |
| HDL cholesterol (mmol/l)   | 1.41 $\pm$ 0.15  | 1.62 $\pm$ 0.26   | 1.31 $\pm$ 0.10  | 1.90 $\pm$ 0.45*  | 1.42 $\pm$ 0.09   | 1.76 $\pm$ 0.47    |
| Triglyceride (mmol/l)      | 0.80 $\pm$ 0.11  | 0.91 $\pm$ 0.15   | 0.86 $\pm$ 0.16  | 0.84 $\pm$ 0.19   | 0.85 $\pm$ 0.01   | 0.89 $\pm$ 0.02**  |

Values are expressed as means  $\pm$  s.d. ( $n = 8$  per group). Feed efficiency ratio = weight gained/food intake; Energy efficiency ratio = weight gained/energy intake.

C, control diet groups; HFC, high-fat, calorie-dense diet groups.

\* $P < 0.01$  and \*\* $P < 0.05$  compared to corresponding C group.

across the three time points were analyzed with 1-way ANOVA using Bonferroni post hoc test. The SPSS 14.0 package for Windows (SPSS, Chicago, IL) was used for statistical analysis. The significance level was set at  $P < 0.05$ .

## RESULTS

### Food intake

During the first 2 weeks, there was no increase in the mice's food intake. However, at weeks 4 and 10, the HFC groups increased their food intake by 17 and 24%, respectively, compared to their controls (Figure 1). Their feed efficiency (FE) ratios were also higher ( $P < 0.05$  at week 10) (Table 2). None of the changes in energy efficiency (EE) ratios were significant between the HFC and their respective controls at all three time points. The EE ratios for the C mice were relatively stable across the three time points. The FE and EE ratios increased over the whole HFC feeding period with significant increase between the HFC-2wk and HFC-10wk mice. The FE and EE ratios almost doubled for HFC-10wk mice vs. HFC-2wk mice ( $P < 0.05$ ).

### Body weight gain and white adipose tissue mass

At the start of the respective dietary treatment, there were no significant differences in the weights of the mice among the different groups. There was no significant difference in weight gain between HFC and C mice until week 10 (Table 2). The HFC-10wk mice had a 42.6% increase over their initial body weights. Similar to the trend in body weight gain, there was no significant difference in white adipose tissue mass in HFC-2wk mice vs. C-2wk mice. However, significant deposition of white adipose tissue in HFC-4wk mice and HFC-10wk mice vs. their corresponding controls were observed. HFC-10wk mice had nearly twice the amount of white adipose tissue mass vs. their C-10wk mice.

### Plasma lipids and glucose

There were no significant differences in the plasma lipid levels between the HFC groups and their respective controls when the mice began their diets. Total and LDL cholesterol levels were

significantly raised at week 2 in the HFC mice and remained elevated throughout the study with no significant progressive increase among the HFC groups (Table 2). At week 10, triglyceride levels were significantly raised in the HFC group as compared to the control group. Blood glucose levels were relatively stable till week 10 when a significant large increase was observed in the HFC-10wk mice vs. their respective controls.

### Gene expression profiles from microarrays

Gene transcripts with absolute Signal Log Ratio  $\geq 0.5$  (i.e., 1.4-fold difference in levels) between the experimental groups of mice are presented in Figure 2. Under *in vivo* conditions, changes in differential gene expression due to dietary treatment are expected to be small and often below 2 fold change (22,23). As such, any gene expression changes greater than a threshold of Signal Log Ratio  $\geq 0.5$  were considered to be potentially relevant (Figure 2).

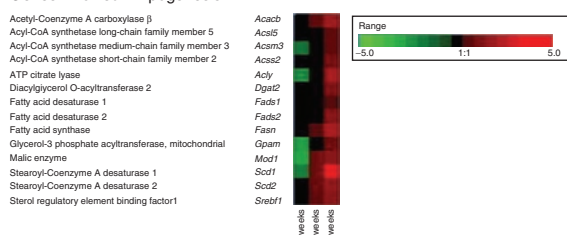
**Genes involved in lipogenesis.** Genes encoding enzymes involved in lipogenesis, such as *Acly*, *Acsm3*, *Gpam*, *Mod1*, *Scd1* were downregulated after 2 weeks of high-fat feeding. At week 4, these genes were either upregulated or showed no change. These genes were upregulated at week 10. Although the expression of genes such as *Fas*, *Sreb1*, *Dgat2*, *Fads1*, and *Fads2* showed no change at week 2, there was a trend of being downregulated. However,

as the difference in absolute mRNA levels did not reach statistical significance, they were not reported as a robust decrease. At week 4, the main genes involved in synthesis of triacylglycerides (*Fas* and *Sreb1*) were upregulated and showed a further increase in mRNA expression at week 10. At week 10, *Acacb*, *Acs15*, *Scd2*, *Acss2*, *Dgat2*, *Fads1*, and *Fads2* were also upregulated.

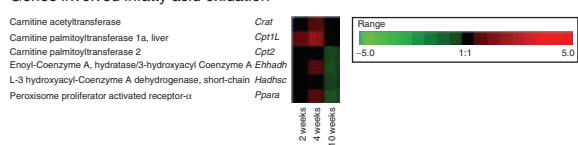
**Genes involved in fatty acid oxidation.** Four key genes involved in fatty acid oxidation (FAO), *Cpt1L*, *PPAR $\alpha$* , *Crat*, and *Ehhadh* were elevated at week 4. This suggests that both mitochondrial and peroxisomal fat oxidation were activated. However, at week 10, *PPAR $\alpha$* , *Ehhadh*, *Hadhsc*, and *Cpt2* were downregulated and *Cpt1L* and *Crat* were no longer upregulated.

**Genes involved in cholesterol metabolism.** The expression levels of key genes involved in cholesterol synthetic pathway, such as *Hmgcs1*, *Cyp51*, *Cyb5r3*, *Fdps*, *Idi1*, and *Sc4mol* were decreased at week 2. These genes continued to be suppressed and showed a greater extent of suppression in their expression levels at week 4, along with additional genes involved in cholesterol synthesis being downregulated. These include *Hmgcr*, which catalyzes the rate-limiting step in cholesterol biosynthesis, *Dhcr7*, *Dhcr24*, *Fdft1*, *Lss*, *Mvd*, *Mvk*, *Nsdhl*, *Pmvk*, *Sqle*, and *Tm7sf2*. Expression of *Sreb2*, the transcription factor responsible for activation of the cholesterol synthetic pathway (24) was also decreased. At week 10, the downward trend in the expression of these genes was not maintained but some were upregulated instead (e.g., *Hmgcr*, *Sc4mol*, *Sc5d*, *Sqle*, *Cyb5r3*, *Fdft1*, and *Fdps*). *Cyp7A1*, the gene encoding the central enzyme of cholesterol degradation and bile acid synthesis and *Cyp51* were downregulated at all three time points.

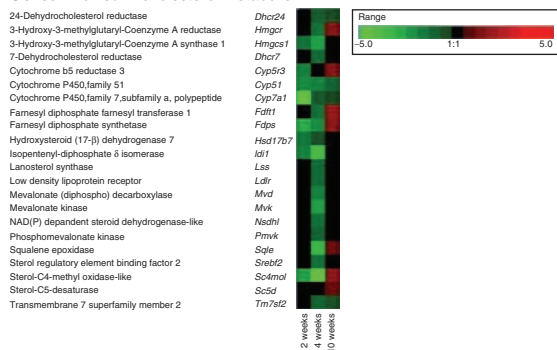
#### Genes involved in lipogenesis



#### Genes involved infatty acid oxidation



#### Genes involved in cholesterol metabolism



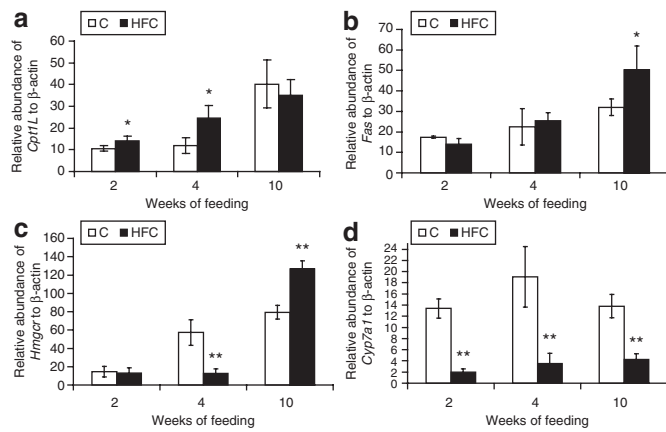
**Figure 2** Hepatic genes regulated by high-fat and calorie feeding. The color-coded scale (green: downregulation and red: upregulation). Data of “fold change” were calculated from the “Signal Log Ratio”. The scale is presented as fold change.

### qRT-PCR

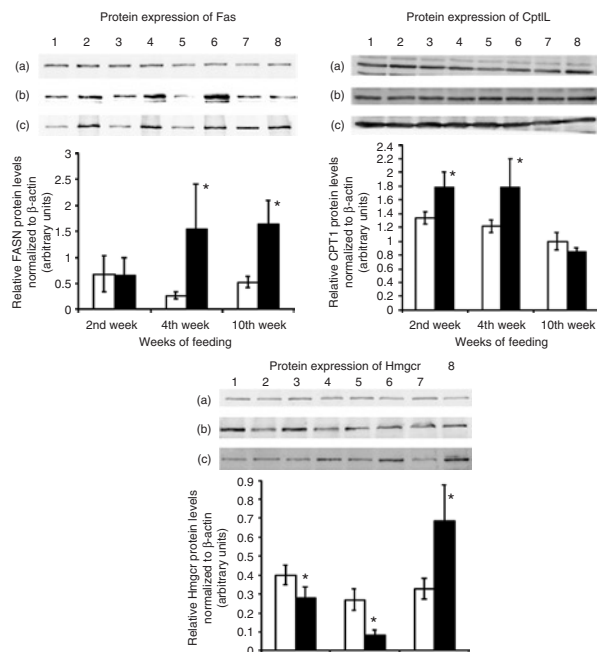
From the results of initial screening with microarrays, we selected one key gene each from lipogenesis (*Fas*), FAO (*Cpt1L*), cholesterol metabolism (*Hmgcr*), and bile acid synthesis (*Cyp7a1*) to conduct further qRT-PCR tests to validate their differential expression (Figure 3). HFC feeding resulted in elevated gene expression levels of *Cpt1L* at weeks 2 and 4 ( $P < 0.05$ ) with no significant difference at week 10. *Fas* mRNA was not affected until week 10, when we observed a 57% increase ( $P < 0.05$ ) in its level of expression. HFC feeding for 4 weeks resulted in reduced expression levels of *Hmgcr* ( $P < 0.01$ ) but this was reversed at week 10 ( $P < 0.01$ ). The gene expression levels of *Cyp7a1* were significantly downregulated in the HFC groups when compared to the C groups at all three time points. The qRT-PCR results showed that the levels of mRNA for the selected genes followed the same pattern of expression as those observed with the microarray experiment, thus confirming the results of the data obtained from the microarrays.

### Western blot

At weeks 4 and 10, protein levels of *Fas* were higher in HFC mice than their respective controls ( $P < 0.05$ ) (Figure 4). There were significant increases in protein levels of *Cpt1L* in the HFC-2wk and HFC-4wk mice. *Hmgcr* protein levels were



**Figure 3** Comparison of gene expression levels measured by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Gene expression levels of (a) *Cpt1L*, (b) *Fas*, (c) *Hmgcr*, and (d) *Cyp7a1* as quantified by qRT-PCR in liver tissues from HFC-fed and C-fed mice at three time points. Results are presented as means  $\pm$  s.d. of relative abundance to  $\beta$ -actin for each group of mice ( $n = 8$  per group) \* $P < 0.05$  and \*\* $P < 0.01$  compared to corresponding control group.



**Figure 4** Western blot assays. Top panels: Western blot analysis of carnitine palmitoyltransferase-1 (Cpt1L), fatty acid synthase (Fas) and 3-hydroxy-3-methylglutaryl-coenzyme reductase (Hmgcr) in the livers from C mice (odd-numbered lanes) and HFC mice (even-numbered lanes). Two, four, and ten weeks are arranged in rows (a), (b) and (c), respectively. Bottom panels: Densitometry analysis of protein bands normalized to  $\beta$ -actin bands in C compared to the HFC mice. Each bar represents means  $\pm$  s.d. for each group of mice ( $n = 4$  per group) \* $P < 0.05$  as compared to control group. Open bars represent data from C mice and filled bars represent data from HFC mice.

significantly reduced in HFC mice at weeks 2 and 4 but this was reversed at week 10 ( $P < 0.05$ ). The western blot results showed that the levels of expression of proteins encoded by the

selected genes followed the same pattern of expression as those observed with the microarray experiment.

## DISCUSSION

The results of this study suggest that there is an initial compensatory response to reduce food intake when mice are fed an HFC diet as this helps to prevent weight gain and deposition of white adipose tissue. However, a more prolonged consumption of the HFC diet disrupted this compensatory response, resulting in increased food intake, a marked increase in weight with greater deposition of body fat. The increased food intake exhibited by the HFC-4wk mice persisted in the HFC-10wk mice. This is in agreement with earlier studies which demonstrated that prolonged consumption of high-fat diet promoted hyperphagia (11–15).

Despite a lower food intake, we had expected an increase in the weights of HFC-2wk mice as their calorie intake was significantly higher than the C-2wk mice due to the higher energy density of the HFC diet. However, this was not the case and we observed weight loss in the mice after 1 week of HFC feeding compared to the C group (data not shown) but regained the weight lost in week 2.

We observed a smaller but not significant FE ratio in the HFC-2wk mice than the C-2wk mice. The FE ratios increased with the duration of HFC feeding. The FE ratio of the HFC-10wk mice was 57% significantly higher than their controls. Ryu *et al.* (25) had shown that FE ratios increased with high-fat feeding. The FE ratio for HFC-10wk mice was significantly higher than HFC-2wk mice. As the energy densities of the HFC and C diets differed, we examined the EE ratios as well. The EE ratio doubled for HFC-10wk mice vs. the HFC-2wk mice and this was significant.

In relation to these changes in food intake and weight gain in the mice, we examined whether the molecular changes in the liver could help to explain some of these sequential changes partially. Based on microarray screening, hepatic genes involved in FAO were initially upregulated and then subsequently downregulated during HFC feeding. We validated the expression of Cpt1L, the rate-limiting enzyme for mitochondrial FAO, with qRT-PCR. This enzyme catalyzes the formation of acyl-carnitine from acyl-CoA. This reaction is the first step of the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix for FAO (26). Cpt1L was significantly upregulated at weeks 2 and 4 but there was a downward trend at week 10. The initial increased expression levels of Cpt1L are in agreement with the findings by Gregoire and colleagues (27), who showed that Cpt1L were upregulated in the livers of mice fed on high-fat diet for 11 days. The differential changes in our gene expression levels were also translated to protein expression as evidenced by our western blot assays.

In contrast to the genes in FAO, several key hepatic lipogenic genes were initially downregulated but were subsequently upregulated at week 4 and likewise for more genes at week 10. Several key hepatic lipogenic genes (e.g., *Fas*, *Scd1*, *Acl*) as well as the transcription factor Srebf1 were upregulated at week 10. We confirmed the gene expression and protein expression of

Fas, a key lipogenic gene, by RT-PCR and western blot assays, respectively. In contrast to our observations for mice fed with 10 weeks of HFC diet, Kim *et al.* (21) and Kreeft *et al.* (28) showed that gene expression of lipogenic enzymes in their male C57BL/6J mice were downregulated by high-fat feeding. Their mice were fed on high-fat diets for 12 and 8 weeks, respectively. A possible reason for our discrepant findings from the other two studies could be due to differences in dietary fat content of the high-fat and control diets used. In the study conducted by Kim *et al.*, the high-fat diet contained twice as much fat as the control diet, whereas the fat content of our HFC diet was four times higher than the control diet. As the differences in dietary fat content between the high-fat and control diets were not reported in Kreeft *et al.* (28), it might not be valid to compare the responses reported in their studies with ours.

The difference in dietary fat content between the high-fat and control diets used in the studies conducted by Murase *et al.* (29) and Hu *et al.* (30) was similar to ours. The mice were fed high-fat diet for 8 weeks in the study conducted by Hu *et al.* (30). Murase *et al.* (29) fed their male mice for a much longer period of 5 months. In agreement with our observations at week 10 (i.e., prolonged high-fat, calorie-dense feeding), both groups of researchers demonstrated that the C57BL/6J mice maintained on a high-fat diet had increased mRNA levels of hepatic lipogenic genes. Within 4 weeks of the high-fat, calorie-dense feeding, we could already observe increased mRNA levels of lipogenic genes. This should be observable by Murase *et al.* and Hu *et al.* if they had conducted time-course measurements.

The interpretation of our findings is that during the initial period of HFC feeding, the mice had developed compensatory responses to reduce their food intake through increased hepatic FAO and reduced lipogenesis to regulate energy homeostasis. Friedman *et al.* had demonstrated that increase in hepatic FAO might lead to an increase in hepatic energy, which in turn, might send a signal to brain to reduce food intake (31,32). However, prolonged HFC feeding could result in reduced hepatic FAO while increasing hepatic lipogenesis. Increased hepatic lipogenesis leads to elevated levels of malonyl-Co A (33). Malonyl-Co A, the first committed intermediate in the pathway of fatty acid biosynthesis, is a potent Cpt1L inhibitor (34,35). Hence, this could likely remove the inhibition on food intake, and thus led to further weight gain. The attempt to regulate body weight gain in the HFC-2wk mice was further confirmed by the lower EE ratios of the HFC-2wk group when compared to the C-2wk group. On the other hand, the other two high-fat groups had higher EE ratios vs. their corresponding controls.

Two weeks of HFC feeding had increased the levels of total and LDL cholesterol significantly, despite no significant changes in body weight. This led us to further examine the expression levels of the genes involved in cholesterol metabolism. Similar to the genes involved in lipogenesis, the microarray screening revealed that the genes involved in cholesterol synthesis were also initially downregulated. However, at week 4, more genes involved in cholesterol synthesis were downregulated

compared to week 2. This agreed with our observations that the cholesterol levels did not increase much after 4 weeks of HFC feeding. There was no significant difference in the levels of total and LDL cholesterol among the HFC groups. This suggested that the mechanisms in place to regulate cholesterol levels in the mice might be more robust than the mechanism to regulate fatty acid synthesis. At week 10, this regulatory mechanism appeared to be disrupted with increased mRNA levels of genes involved in cholesterol synthesis (e.g., *Hmgcr*, *Sc4mol*, *Sc5d*, *Sqle*, *Cyb5r3*, *Fdft1*, and *Fdps*). When we measured the total cholesterol levels in a separate experiment on a group of HFC-fed mice for 18 weeks, we observed a further increase of 12% in total cholesterol levels as compared to HFC-10wk mice (data not shown). We confirmed these gene expression changes by conducting western blot assay for *Hmgcr*, which catalyzes the rate-limiting step in cholesterol biosynthesis and the protein expression of *Hmgcr* was in line with its gene expression. The initial downregulation of genes involved in cholesterol synthesis would suggest that the liver was attempting to maintain cholesterol homeostasis in response to high-fat feeding by downregulating cholesterol synthesis. This was in line with Goldstein and Brown's suggestion that cholesterol homeostasis is maintained by a feedback mechanism (36). Our observations at both weeks 2 and 4 are in agreement with previous reports of reduced expression of hepatic genes involved in cholesterol biosynthesis in mice fed with high-fat diets (21,28,29). We could not compare our findings with Murase *et al.* (29) and Hu *et al.* (30) as they did not report the expression of the genes involved in cholesterol synthesis.

Because the expression levels of genes related to cholesterol synthesis were downregulated initially, what could have caused the increase in plasma cholesterol levels in the HFC-2wk mice? The other end of the equation of cholesterol homeostasis was cholesterol removal. The diversion of cholesterol for bile acid production has been well established as a mechanism for reducing plasma cholesterol (37). Interestingly, mRNA levels of *Cyp7a1*, the enzyme encoding the rate-limiting step of bile acid production, were consistently downregulated by high-fat feeding and *Ldlr* was downregulated at week 4. We have expected that the elevated plasma LDL levels would initially stimulate the *Ldlr* expression to facilitate elevated lipid uptake from the periphery. This would then raise liver cholesterol levels and accelerate bile acid production. We had also expected that the gene expression of *Cyp7a1* would be upregulated as it governs the rate-limiting step in the neutral pathway of bile acid synthesis from cholesterol. The findings from this study suggest that for mice fed with HFC diet, it was not the upregulation of cholesterol synthesis but rather a dysfunctional downregulation of cholesterol removal mechanism, which resulted in the observed higher blood cholesterol levels. Cholesterol synthesis was still well-regulated, at least up to 4 weeks of high-fat feeding.

In summary, our findings provided a molecular insight into the detrimental effects of HFC feeding in mice in terms of weight gain and elevated cholesterol levels and blood glucose levels over a period of time. Our sequential observations

suggested that the homeostatic processes aimed at maintaining energy balance, weight gain and plasma cholesterol levels could also be reflected at the gene transcription and protein levels and that such compensatory responses could breakdown with prolonged insult of high-fat, calorie-dense feeding. Such findings have not been described previously. We have also provided novel evidence that these effects are clearly dependent on the duration of HFC feeding and became evident when the mice's initial compensatory adaptations failed beyond 4 weeks.

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#### DISCLOSURE

The authors declared no conflict of interest.

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