SEQUENTIAL EFFECTS OF A HIGH-FAT, CALORIE-DENSE DIET OR A HIGH-FIBER DIET ON GENE EXPRESSION, BODY WEIGHT AND ASSOCIATED METABOLIC RESPONSES IN C57/BL6J MICE

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SUMMARY

By 2020, two-thirds of the global burden of disease will be attributable to chronic non-communicable diseases (e.g. cardiovascular disease and diabetes), most of them strongly associated with diet. The pandemic of these diseases is likely, at least in part, to be due to a mismatch between our current dietary patterns (i.e. excessive calories and fat intake coupled with reduced dietary fiber intake) and those during man's early stages of evolution which our genes were programmed to respond to. However, the interactions between our diet, genetic factors and the development of these diseases are not fully understood.

Several microarray transcription profiling studies have examined the effects of a high-fat, calorie-dense (HFC) diet but reported contradictory findings. One possible reason for these discrepant findings may be due to the varying lengths of the feeding period. We hypothesized that the HFC diet would initially elicit compensatory interrelated responses between feeding behaviour and gene expression levels and that such compensatory responses might diminish over time with the continued intake of a HFC diet.

Therefore, we sequentially examined the effects of feeding a HFC diet to female C57BL/6J mice. These included examining the feeding behaviour and the transcriptomic profile of genes involved in the lipid metabolism in the liver and white adipose tissue over a period of 10 weeks, making measurements at weeks 2, 4 and 10. In parallel, we measured common phenotypic parameters associated with cardiovascular diseases and obesity (e.g. plasma lipid, leptin and insulin levels).

Our results suggested that the early responses to HFC feeding were possibly aimed at reducing food intake, down-regulating the mRNA levels of lipogenic hepatic genes and up-regulating the mRNA levels of genes involved in fatty acid oxidation. However, prolonged HFC feeding appeared to disrupt this adaptation, leading to increased food intake and marked increases in weight and body fat. Lipogenic genes were also up-regulated. These effects were clearly dependent on the duration of HFC feeding and became evident after 4 weeks. We have proposed a possible model linking leptin signalling, hepatic lipid metabolism and the control of food intake during the early and later stages of high-fat, calorie-dense feeding. Our sequential observations may help to explain some of the discrepant findings in previous studies.

There are only a few studies examining the relationship between dietary fiber and gene expression. These studies are limited to the gastrointestinal tract or only one or two hepatic genes. Therefore, in a separate experiment, the thesis also examined sequentially the effects of a high-fiber diet containing psyllium husk on the expression levels of genes involved in lipid metabolism, using microarray technology. Whilst plasma lipids were reduced by high-fiber feeding, mRNA levels of hepatic genes in cholesterol synthesis were up-regulated throughout the feeding period and lipogenic genes were also up-regulated with prolonged feeding.

Both experiments provided important molecular insights into the possible effects of feeding a high-fat, calorie-dense diet or a high-fiber diet on genes involved in regulating lipid and energy stores.

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LIST OF ABBREVIATIONS

C	**Control**
HFC	**High fat, calorie-dense**
FE	**Feed efficiency**
EE	**Energy efficiency**
PE	**High-fiber containing psyllium husk (PE)**
qRT-PCR	**Quantitative real-time reverse-transcription polymerase chain reaction**

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CHAPTER 1

INTRODUCTION

BACKGROUND

"Let thy food be thy medicine and thy medicine be thy food."

Hippocrates (460-377 B.C.)

The renowned Greek physician, Hippocrates, recognized that nutrition has an important role in disease management. He had also observed that "Everything in excess is opposed to nature." Indeed, it is estimated that by 2020 two-thirds of the global burden of disease will be attributable to chronic non-communicable diseases, most of them strongly associated with diet (World Health Organization, 2003). The transition from undernutrition towards overnutrition (i.e. a diet high in fat and calorie content) plays a major role in the current global epidemics of cardiovascular disease, diabetes and obesity.

Amongst the diet-related risk factors contributing to the development of these chronic diseases, high blood cholesterol levels and being overweight are the second and third most important risk factors respectively in developed countries and becoming increasingly important in developing countries (World Health Organization, 2002).

The pandemic of cardiovascular disease, diabetes and obesity can be viewed as a mismatch between our present environmental circumstances (e.g. an excessive calorie and fat intake coupled with reduced physical activity) and those that give evolutionary advantage. This concept was articulated by Neel (1962) in a paper entitled: Diabetes

mellitus: a ‘thrifty’ genotype rendered detrimental by ‘progress’. Since then, this concept has been widely accepted that our current dietary consumption patterns might not be consistent with the diet to which our genes were programmed to respond. However, the interaction between the diet and genetic factors and the effects that such interplay might have on the development of these diseases is not fully understood (Chiang and MacDougald, 2003; Mariman 2006). In the past, experimental designs were often limited to one factor, i.e., either diet or genes were analyzed separately but not simultaneously (Kaput, 2004). However, the completion of the sequenced human (Venter et al., 2001; McPherson et al., 2001), mouse (Okazaki et al., 2002; Waterston et al., 2002), and rat (Gibbs et al., 2004) genomes has brought forth a wealth of information about the structure of the genome, which can now be used to study concurrently the interplay between our genes and factors from the environment such as diet.

There are 2 major approaches to study this interplay between diet and genetic factors. One approach (nutrigenomics) aims to determine the influence of diet on the genome activity and attempts to relate the resulting different phenotypes to the differences in the genetic response of the biological system (Mutch et al., 2005; Mariman, 2006). The other approach, nutrigenetics, aims to identify the genetic variations in the population and how these gene variants may affect individual response to the changes in the diet. The work of this thesis relied on the first approach (nutrigenomics).

The research in this area is complicated by the fact that cardiovascular disease, diabetes and obesity are polygenic in nature. Moreover, dietary intervention to

prevent the onset of such diseases is a complex process that requires not only knowledge of how a single nutrient may affect a biological system, but also how a complex mixture of nutrients will interact to modulate biological functions. Therefore, the thesis examined the effects of how a complex mixture of nutrients (i.e. the whole diet) instead of a single-nutrient, would affect the gene expression as this reflects the true dietary intake of humans. The transcription profiling is conducted using the DNA array technology as this technology allows one to study thousands of genes simultaneously. The choice of diet and the DNA array technology is discussed in Chapter 2.

Whilst the consumption of a diet high in calories and fat content combined with decreased energy expenditure associated with modern lifestyle, are the major environmental causes of obesity and cardiovascular diseases (World Health Organization, 2003), there is also evidence that a diet with increased dietary fiber may reduce the risk of developing obesity and cardiovascular diseases (Kritchevsky and Bonifeld, 1997). Background information on how a diet high in calories and fat content and low in dietary fiber contributes to obesity and cardiovascular diseases is discussed further in Chapter 2.

The C57BL/6J diet-induced obesity (DIO) mouse model is commonly used for studies involving obesity, diabetes and cardiovascular diseases (Van Heek et al., 1997; Ahren, 1999; Lin et al., 2000; Moraes et al., 2003). Further details on this mouse model are discussed in Chapter 2. This mouse provides a good *in vivo* model to investigate the relationship between dietary fat, energy intake and lipid homeostasis. The liver and the adipose tissue play important roles in regulating energy stores and

lipid homeostasis. A review of the current literature revealed that to date, the effects of a typical high-fat, calorie-dense diet on gene expression levels in the liver and adipose tissue in this mouse strain have been studied. These studies reported discrepant findings in the gene expression levels (Table 1.1 and 1.2). Some showed an increased expression of hepatic genes involved in lipogenesis (Hu et al., 2004; Gregoire et al., 2002) whereas others showed a downward regulation by feeding a high-fat diet (Kreeft et al., 2005; Kim et al., 2004). These studies were only carried out at one single time-point or for a very short duration of feeding, i.e. 11 days (Gregoire et al., 2002). In relation to gene profiling in adipose tissue, some found increased expression of lipogenic genes (Li et al., 2002; Lopez et al., 2003) whereas others demonstrated reduced expression of lipogenic genes in obese rodents (Nadler et al., 2000; Soukas et al., 2000) and humans (Diraison et al., 2002). These previous microarray approaches to examine the interplay between diet and genetic factors in diet-induced obesity were performed solely on either liver tissue (Kreeft et al., 2005; Kim et al., 2004) or on adipose tissue (Lopez et al., 2003; Moraes et al., 2003). Furthermore, the various studies investigating the effects of a high-fat diet using microarray profiling of the hepatic genes were conducted in the male mice only. Data on the female mice have not been described previously.

We postulated that one of the reasons for the reported discrepant findings could be due to the varying feeding duration, and that the ingestion of the high-fat, calorie dense diet might elicit compensatory responses in the gene expression levels and perhaps such compensatory response would diminish over time with the continued intake of a high-fat, calorie dense diet.

Literature on dietary fiber and gene expression is scant and usually limited to the gastrointestinal tract ((Nguyen et al., 2006; Young et al., 2005; Chapkin et al., 1998) or only on a few genes (Yang et al., 2003; Goel et al., 1999; Sonoyama et al., 1995). To date, no studies have yet been carried out to examine the effects of dietary fiber on hepatic gene expression on a large number of genes and in a time-dependent manner.

THESIS OBJECTIVES

It is postulated that the reason for the discrepant findings in the literature regarding the findings on gene expression induced by a high-fat, calorie dense diet could be due to the difference in duration of the feeding period and that the ingestion of the high-fat, calorie dense diet might elicit compensatory responses in the gene expression levels. We also proposed that this compensatory response would diminish over time with the continued intake of a high-fat, calorie dense diet. Therefore, the thesis has the following objectives:

1) To characterize sequentially the effects of a high-fat, calorie dense diet on the transcriptomic profile of the genes involved in the lipid metabolism in the liver and visceral white adipose tissue over a period of 10 weeks, making measurements at weeks 2, 4 and 10.

2) To establish if there is any correlation between the transcriptomic profile of the genes involved in the lipid metabolism and the common phenotypic parameters associated with obesity and cardiovascular diseases (e.g. plasma lipid profiles and plasma leptin).

3) In a separate experiment, the thesis also examined sequentially the effects of a high-fiber diet containing viscous soluble fiber on the transcriptomic profile of hepatic genes involved in the lipid metabolism, plasma lipids and body weight.

THESIS ORGANIZATION

There are 6 chapters in this thesis. Chapter 1 provides a brief introduction, background and rationale, defines the objectives and scope of the thesis. Chapter 2 reviews the literature on diet and gene expression studies as well as factors affecting body weight regulation and lipid metabolism. The literature on the choice of mouse model, diets and DNA arrays used are also discussed in Chapter 2. A detailed description of materials and methods used is covered in Chapter 3. In Chapter 4, the results from the high-fat, calorie-dense feeding experiment is reported. The discussion of these results is found in the same chapter. Chapter 5 describes the findings from the high-fiber experiment. Chapter 6 summarizes the important conclusions resulting from this study and provides suggestions for future work.

Table 1.1 Review of recent literature on dietary fat and hepatic gene expression

Authors	Diets used	Animals used	Duration of diets	Tissue	Results
Kreeft et al., 2005	a) Mild-fat diet (16% of total energy as fat) b) Severely high-fat diet (not indicated in paper)	APOE3Leiden mice	8 weeks	Liver	Reduced expression of lipogenic genes and genes involved in cholesterol metabolism
Kim et al., 2004	a) Low-fat diet (17% of total energy as fat) b) High-fat diet (36% of total energy as fat)	Male C57BL/6J mice aged 3 week	12 weeks	Liver	Reduced expression of lipogenic genes and genes involved in cholesterol metabolism

Table 1.1 (continued) Review of recent literature on dietary fat and hepatic gene expression

Authors	Diets used	Animals used	Duration of diets	Tissue	Results
Hu et al., 2004	a) Low-fat diet (12% of total energy as fat) b) High-fat diet (40% of total energy as fat)	Male C57BL/6J mice aged 6 week	8 weeks	Liver	Stearoyl-coenzyme A desaturase 1 (Scd1), a lipogenic gene, was up-regulated
Gregoire et al., 2002	a) Low-fat diet (17% of total energy as fat) b) High-fat diet (42% of total energy as fat)	Male C57BL/6J mice aged 6 week	0, 1, 11 days	Liver	**Day 1 versus Day 0 :** Lipogenic genes were up-regulated but returned to baseline at day 11 **Day 11 versus Day 0 :** Genes involved in cholesterol metabolism were down-regulated ; Pparα and Cpt1 were up-regulated

Table 1.2 Review of recent literature on dietary fat and adipose tissue gene expression

Authors	Diets used	Animals used	Duration of diets	Tissue	Results
Nadler et al., 2000	No diets used (compared between lean versus genetically obese mice)	C57BL/6J ob/ob and BTBR mice	Nil	Epididymal white adipose tissue	Reduced expression of lipogenic and adipogenic genes in obese mice
Soukas et al., 2000	No diets used (compared between wild-type, *ob/ob*, and transgenic mice expressing low levels of leptin)	C57BL/6J ob/ob female mice	Nil	Visceral white adipose tissue	Reduced expression of lipogenic genes and adipogenic genes in obese mice

Table 1.2 (continued) Review of recent literature on dietary fat and adipose tissue gene expression

Authors	Diets used	Animals used	Duration of diets	Tissue	Results
Diraison et al., 2002	No diets used (compared between lean versus obese subjects)	Humans	Nil	Subcutaneous white adipose tissue	Reduced expression of lipogenic genes in obese subjects
Li et al., 2002	a) Control diet (4 % fat) b) High-fat diet (60% fat)	Male Sprague-Dawley rats aged 6 weeks	1 week	Epididymal white adipose tissue	Increased expression of Stearoyl-coenzyme A desaturase 1 (Scd1), a key lipogenic gene in diet-induced obese rats
Lopez et al., 2003	a) Control diet (6 % of total energy as fat) b) High-fat diet (65% of total energy as fat)	Male Wistar rats aged 5 weeks	65 days	Epididymal white adipose tissue	Increased expression of lipogenic genes

CHAPTER 2

LITERATURE REVIEW

THE ROLE OF HIGH-FAT, CALORIE DENSE DIETS IN OBESITY

Obesity is a major health problem that is increasing in both prevalence and severity. It is associated with increased risks of type 2 diabetes and cardiovascular disease. 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 (Bjorntorp , 1997; World Health Organization, 2000). Numerous studies demonstrated that subjects given high-fat food also had a high-calorie intake (Astrup et al., 2000; Golay and Bobbioni, 1997; Stubbs et al., 1995). A diet excessive in dietary fat and calories promotes the development of obesity. There is a direct relationship between the amount of dietary fat and calories and the degree of obesity (Golay and Bobbioni, 1997). However, environmental factors may not fully explain the rapidly increasing rates of obesity (Levin, 2000; Moreno et al., 2001). Genetic predisposition for obesity may underlie the tendency for weight gain in some individuals (Marti et al., 2000). 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 (Chiang & MacDougald, 2003; Mariman 2006).

THE DIET-INDUCED OBESITY (DIO) MOUSE MODEL FOR DIET AND GENE EXPRESSION STUDIES

The C57BL/6J diet-induced obesity (DIO) mouse model is commonly used for studies involving obesity, leptin resistance, body fat accumulation, insulin resistance, and correlation with body weight change (Van Heek et al., 1997; Ahren, 1999; Moraes et al., 2003). Like humans, this strain would develop obesity, hyperglycemia and hyperlipidemia when raised on a high-fat, calorie-dense diet. However, it remains lean if the fat content of the diet is limited (Lin et al., 2000). Moreover, the development of these risk factors in the C57BL/6J mouse closely parallels the progression in humans (Collins et al., 2004). For example, the onset of diabetes and obesity in humans occurs gradually and often in the presence of a high-fat, calorie dense diet (Collins et al., 2004).

The current literature reporting the effects of a high-fat diet and microarray transcription profiling of hepatic genes involved in lipid metabolism is limited to male C57BL/6J mice. This is despite the fact that the female C57BL/6J mice were shown to be more responsive towards high-fat diet than their male counterparts in terms of gain in white adipose tissue mass over their total body mass. When fed with high-fat diet for 8 weeks, the female mice characteristically exhibited 17.2% of their body weight as adipose tissue mass as compared to 13.3% in the male mice (http://phenome.jax.org). The females showed a 1.12-fold increase in their body weights as compared to 0.98-fold change in the males. They also had a 5-fold increase in total cholesterol levels as compared to a 4.8-fold increase in the males (http://phenome.jax.org).

LIPOGENESIS IN THE LIVER AND WHITE ADIPOSE TISSUE

To date, the mechanisms leading to the excessive body fat accumulation in obesity are not fully understood. However, it is generally accepted that both decreased lipolysis and increased lipogenesis could play a role in the pathogenesis of obesity. In the past, the liver was considered to be the main site of lipogenesis while very little fatty acid synthesis occurs in the adipose tissue. This view appears now to be incorrect as significant lipogenesis is reported to occur in white adipose tissue (Aarsland et al., 1996; Claycombe et al., 1998; Moustaid et al.,1996; Swierczynski et al., 2000).

The major adipose tissue in mammals is white adipose tissue (Albright and Stern, 1998). Adipose tissue in mammals is found in 2 different forms: white adipose tissue and brown adipose tissue. Both white adipose tissue and brown adipose tissue play opposing roles in regulating energy metabolism (Avram et al; 2005). One of the primary roles of white adipose tissue is to store excess energy as fat whereas the brown adipose tissue is responsible for transferring of energy from food into heat. Increased white adipose tissue mass reflects a positive net balance in energy stores between energy expenditure and energy intake (Avram et al; 2005). The classical view of the function of white adipose tissue is that it purely provides a long-term fuel reserve which can be mobilized during food deprivation with the release of fatty acids for oxidation in other organs. A critical change in the perspective of the role of white adipose tissue followed the discovery of leptin. (Zhang et al.,1994). This important hormone for regulating energy balance is produced principally by white fat. In addition to leptin, other cytokines have also been found to be produced by adipose tissue. In fact, adipose tissue is no longer considered to be an inert tissue functioning solely as an energy store, but is emerging as a major player in the pathogenesis of

cardiovascular diseases, obesity, insulin resistance and related inflammatory disorders (Tilg and Moschen, 2006).

Although both the liver and the adipose tissue play important roles in maintaining energy balance and contributing to energy storage in the fed state, the previous microarray approaches to examine the interplay between diet and genetic factors in diet-induced obesity have frequently been performed solely on either liver tissue (Kreeft et al., 2005; Kim et al., 2004) or on adipose tissue (Lopez et al., 2003; Moraes et al., 2003) but not in both.

HIGH-FAT DIET AND GENE EXPRESSION STUDIES

A review of the current literature reveals that to date, the effects of a typical high-fat, calorie dense diet on gene expression have been studied *in vivo* (studies listed in Table 1.1 and 1.2 found in Chapter 1). However, the diets used in some of these studies are very high in fat content (Li et al., 2002; Lopez et al., 2003). These diets do not reflect the typical fat content in a high-fat human diet, one of the important environmental factors in causing obesity. Moreover, some of these studies used genetically obese mice (Nadler et al., 2000; Soukas et al., 2000). These genetic models may not be suitable models for examining human obesity as there are only very few obese individuals reported in the literature with mutations of leptin or leptin receptor. The current obesity pandemic results from a combination of both genetic and environmental factors (e.g. a dietary intake excessive in calories and fat, coupled with reduced physical activity).

Studies which have examined the effects of a typical high-fat, calorie dense diet on gene expression generated mixed findings. Some demonstrated that the expression of hepatic genes involved in lipogenesis were up-regulated (Hu et al., 2004; Gregoire et al., 2002) whereas others showed a downward regulation by feeding a high-fat diet (Kreeft et al., 2005; Kim et al., 2004). These studies were either carried out at only a single time-point or for a very short duration of feeding, i.e. 11 days (Gregoire et al., 2002). In relation to gene profiling in adipose tissue, some observed increased mRNA levels of lipogenic genes (Li et al., 2002; Lopez et al., 2003) whereas others found reduced expression of lipogenic genes in obese rodents (Nadler et al., 2000; Soukas et al., 2000) or humans (Diraison et al., 2002).

KEY GENES ENCODING SIGNIFICANT ENZYMES INVOLVED IN LIPOGENESIS AND LIPID OXIDATION

Fatty acid synthase (Fas), a key-regulating enzyme in de novo lipogenesis, catalyzes all the reactions for the conversion of acetyl-coenzyme A and malonyl-CoA to palmitate (Wakil et al., 1983). Fas plays an important role in energy homeostasis by converting excess food intake into lipids for storage and providing energy when needed via oxidation. Fas transcription is under stringent nutritional as well as hormonal control in lipogenic tissues, namely liver and adipose tissue (Wakil et al., 1983; Hillgartner et al., 1995). For example, increased circulating insulin levels induce Fas expression (Paulauskis and Sul, 1989). Experiments using knockout or transgenic mice overexpressing sterol regulatory element-binding protein (Srebp) demonstrated that this transcription factor plays a key role in the up-stream regulation of Fas transcription (Casado et al., 1999; Shimano et al., 1999; Wang and Sul, 1995). There are three Srebp isoforms. Srebp activates various genes involved in cholesterol

and fatty acid biosynthesis. However, only the isoform, Srebf1 expression is induced by feeding and insulin, and its role is mainly responsible for the regulation of fatty acid synthesis (Horton et al., 2002).

On the other side of the equation of energy balance is energy production. Mitochondrial beta-oxidation of long-chain fatty acids is a major source of energy production. Carnitine palmitoyltransferase 1 (Cpt1) is the key regulatory enzyme of hepatic long-chain fatty acid oxidation. Cpt1, an integral mitochondrial outer membrane protein, catalyzes the transfer of long-chain acyl group of the acyl-CoA ester to carnitine (McGarry and Brown, 1997; Ramsay et al., 2001). Cpt1 is tightly regulated by its physiological inhibitor malonyl-CoA, the first intermediate in fatty acid biosynthesis. This provides a mechanism for physiological regulation of beta-oxidation in all mammalian tissues and for cellular fuel sensing based on the availability of fatty acids (McGarry and Brown, 1997; Prentki and Corkey, 1996; Zammit, 1999). By its strategic metabolic position, Cpt1 represents a potential drug target for the treatment of metabolic disorders such as diabetes and coronary heart disease. Much research has been devoted to this area (Ruderman et al., 1999; Unger and Orci, 2001; McGarry 2002). Hyperglycemia with hyperinsulinemia increases malonyl-CoA, inhibits functional Cpt1's activity and shunts long-chain fatty acids away from oxidation and toward fat storage in tissue (Rasmussen et al., 2002).

BODY WEIGHT REGULATION AND FOOD INTAKE

Body weight regulation depends on the interaction between genetic and environmental factors. The environmental factors that influence body weight regulation ultimately act by a chronic modification of the energy balance equation (Jequier, 2002):

Energy stored = Energy intake - Energy lost in faeces and urine - Energy expenditure

When studying body weight regulation, the critical issue is not the energy intake or energy expenditure taken separately, but the adjustment of one to the other under ad libitum food intake conditions (Flatt, 1997). The control of food intake exerts a greater influence on energy balance than do small changes in metabolic rates that occur during overfeeding or underfeeding. Under normal conditions, the variations in food intake are larger as compared to the variations in energy expenditure, as shown in subjects spending several days in a respiration chamber (Jequier and Schutz, 1983). This suggests that food intake is the most important determinant of changes in energy homeostasis. Impaired control of food intake has been shown to play a major role in the etiology of obesity (Jequier, 2002). Studies on humans and animals which were allowed ad libitum feeding on high-fat, calorie-dense diets demonstrated different effects on food intake. Some showed that these subjects consumed similar amounts of food as when they were fed ad libitum on lower-fat, less energy-dense diets (Shepherd, 1988; Stubbs et al., 1995). Others, however, have shown that such HFC diets inhibited food intake (Welch et al., 1988; Cecil et al., 1999) and yet others have provided evidence that HFC diets promote hyperphagia (Warwick and Weingarten,

1995; French et al., 1995; Lucas et al., 1998; Woods et al., 2003; Savastano and Covasa, 2005).

DIETARY FATTY ACIDS AND PLASMA LIPIDS

In addition to increasing the risk of developing obesity, a high-fat diet particularly high in saturated fats can also increase the risk of developing hyperlipidemia (World Health Organisation, 2002).

Since the 1950s, it has been recognized that the fat content and the type of fat in the diet is the major determinant of plasma cholesterol concentrations (Keys et al., 1950; Hegsted et al., 1959). Saturated fatty acids[1] increase total cholesterol; polyunsaturated fatty acids decrease total cholesterol, and monounsaturated fatty acids have a neutral effect (Keys et al., 1950; Hegsted et al., 1959; Clarke et al., 1997). The total cholesterol-increasing effect of saturated fatty acids is almost twice the cholesterol-decreasing effect of polyunsaturated fatty acids, resulting in dietary recommendations that stressed reductions in dietary saturated fat (Lichtenstein, 2006). Not all saturated fatty acids have identical effects on plasma cholesterol concentrations. Studies have concluded that saturated fatty acids, particularly lauric (12:0), myristic (14:0), and palmitic (16:0) acids, increase LDL cholesterol levels (Mensink et al., 2003; Yu et al., 1995; Clarke et al., 1997). Shorter chain saturated fatty acids (6:0–10:0) have little effect on plasma cholesterol concentrations, whereas those with intermediate chain lengths (12:0–16:0) increase concentrations (Keys et al., 1965; McGandy et al., 1970). It was suggested that the minimal effect of the shorter chain fatty acids could be due

[1] In the nomenclature of fatty acids, the number before the colon denotes the number of carbon atoms in the fatty acid hydrocarbon chain and the number after the colon denotes the number of double bonds. Saturated fatty acids do not contain any double bonds.

to them being absorbed directly into the portal circulation (Bonanome and Grundy, 1988).

BODY WEIGHT REGULATION, PLASMA LEPTIN AND INSULIN LEVELS

Leptin is a hormone produced mainly by white adipose tissue. Its name is derived from the Greek word ‘‘leptos’’ which means ‘‘thin’’. It is an important signal in the regulation of adipose tissue mass and body weight. When energy reserves are sufficient, leptin levels increase and this will reduce food intake. Conversely, when energy reserves are low, leptin levels start falling and this will initiate a series of neuroendocrine responses like stimulation of food intake to restore the energy reserves (Ahima et al., 1996, Auwerx and Stacls, 1998). Therefore, it can be inferred that leptin plays an important role in the regulation of body weight by adapting food intake to current energy reserves. However, studies which have examined the relationship between circulating leptin levels and body weight regulation have generated mixed findings. Studies found that mice which could not produce leptin or respond to it, exhibited intense hyperphagia and developed massive weight gain (Campfield et al.,1995; Pelleymounter et al., 1995). Treatment with leptin in the leptin-deficient mice inhibited feeding and reduced body fat in a dose-dependent manner (Halaas et al., 1995; Ahima et al., 1996). In humans studies, it was shown that individuals with low plasma leptin levels were hyperphagic with aggressive behaviour when food was denied and developed rapid weight gain, resulting in severe obesity (Montague et al.,1997; Farooqi et al., 2002). In contrast to these studies, plasma leptin levels were shown to be correlated positively with weight gain (Chessler et al., 1998) and body mass index in humans (Niskanen et al., 1997; Klein et al., 1996; Caro et al., 1996). In addition, it has been suggested that leptin synthesis is increased in obese subjects as

compared to non-obese subjects (Maffei et al., 1995). As such, it was suggested that leptin resistance rather than leptin deficiency could play a role in the pathogenesis of obesity. In support of this hypothesis, leptin resistance was described in the diet-induced obese C57BL/6J mice. Moreover, evidence indicated that these mice could develop leptin resistance peripherally (Van Heek, 1997).

Tartaglia (1997) has demonstrated that leptin exerts its effects through activation of the leptin receptor which belongs to the cytokine receptor superfamily and via subsequent stimulation of the JAK/STAT pathway. These leptin receptors are found in peripheral organs including liver and adipose tissue (Wang et al., 1997) as well as the hypothalamus. The factors which determine leptin resistance include the expression levels of leptin receptors as well as the responsiveness of intracellular JAK/STAT signalling (Baskin et al., 1998). To date, the mechanisms by which resistance to leptin may arise remain unclear. However, there was evidence to suggest that Socs-3 (suppressors of the cytokine signalling family 3) may play a role. Socs-3 is a member of the Socs family of cytokine-inducible intracellular proteins that feedback to inhibit cytokine receptors and cytoplasmic signalling adaptor molecules. In vitro studies have demonstrated that Socs-3 inhibits leptin induced signal transduction (Bjorbaek et al., 1998). This has led to the speculation that Socs-3 activation could play a role in the development of leptin resistance (Bjorbaek et al., 1998; Emilsson et al., 1999).

Insulin is another hormone that also contributes to the regulation of body weight (Porte *et al.* 1998). Studies have shown that insulin may inhibit food intake (Ikeda *et al.* 1986, Forte *et al.* 1998), and stimulate energy expenditure by activating thermo-

genesis in the brown adipose tissue (Rothwell *et al.* 1983). Therefore, insulin and leptin seem to exert similar actions, the net effect being a reduction in body weight. These effects seem also to be interrelated. However, insulin has been shown to promote lipogenesis. Therefore, the net effect of insulin in the regulation of body weight remains to be established.

DIETARY FIBER AND GENE EXPRESSION

Increased dietary fiber has been associated with reduced risks of developing cardiovascular disease, colon cancer and obesity (Kritchevsky and Bonifield, 1997). However, the literature on dietary fiber and gene expression is scant as compared to the literature regarding dietary fat and gene expression. Studies are limited to the gastrointestinal tract ((Nguyen et al., 2006; Young et al., 2005; Chapkin et al., 1998) or only on one or two hepatic genes (Yang et al., 2003; Goel et al., 1999; Sonoyama et al., 1995). To date, no studies have yet been carried out to examine the effects of dietary fiber on hepatic gene expression on a large number of genes.

THE ROLE OF VISCOUS SOLUBLE FIBER IN LOWERING CHOLESTEROL LEVELS

In relation to cardiovascular diseases, the health benefits of increased dietary fiber intake in reducing the risk of cardiovascular diseases were first suggested over 30 years ago (Burkitt and Trowell, 1975). Since then, evidences of the link between dietary fiber and cardiovascular diseases have accumulated from epidemiological observations (Khaw and Barrett-Connor, 1987; Humble et al., 1993; Kromhout et al., 1982; Rimm et al., 1996; Wolk et al., 1999) as well as clinical trials (Hjermann et al., 1981; Arntzenius et al., 1985; Burr et al., 1989).

Total dietary fiber can be divided into 2 groups: viscous soluble fiber and non-viscous insoluble fiber. Non-viscous fibers have not been shown to have a consistent cholesterol-lowering effect. In contrast, consumption of a high-fiber diet enriched with viscous soluble fibers induced significant reductions in plasma total and LDL cholesterol concentrations (Jenkins et al., 1978; Glore et al., 1994; Jenkins et al., 2000). The cholesterol-lowering effects of viscous soluble dietary fiber could be due to either (1) increased bile acid synthesis or (2) reduced cholesterol synthesis. Bile acid synthesis accounts for 40-50% of the daily elimination of cholesterol (Heuman et al., 1988; Vlahcevic et al., 1991) and plasma cholesterol is quantitatively the most important substrate for bile acid synthesis (Schwartz et al., 1982). Endogenous cholesterol biosynthesis accounts for approximately 75-80% of the total body cholesterol pool (Schwartz et al., 1982). The liver is the main site of both bile acid synthesis and endogenous cholesterol biosynthesis.

THE ROLE OF VISCOUS SOLUBLE FIBER IN ENERGY REGULATION

As compared to improved plasma lipid profiles, the evidence linking increased dietary soluble fiber and weight loss faces substantial controversy since the number of investigations reporting that soluble fiber induces weight loss (Tuomilehto et al., 1980; Walsh et al., 1984; Krotkieswki, 1984) is comparable to the number reporting no effect of soluble fiber on body weight (Hylander and Rossner, 1983; Stevens et al., 1987; Krotkieswki, 1985). In part, this controversy stems from various factors which include varying study duration as well as dietary compliance in human studies. Studies which demonstrated the link between increased viscous soluble fiber intake and weight loss have suggested that consumption of viscous soluble fiber result in gel

formation in the stomach, which may increase gastric distension and reduce the rate of gastric emptying. This gastric distension due to gel formation has been proposed as the mechanism for the observed increases in perceived fullness and reduced food intake following consumption of soluble fiber (Tuomilehto et al., 1980; Krotkieswki, 1984). The discrepant findings on soluble intake on weight could be due to varying feeding period amongst other factors (e.g. non-dietary compliance) (Kritchevsky and Bonifeld, 1997).

PSYLLIUM HUSK, A VISCOUS SOLUBLE FIBER

Of the viscous soluble fibers, psyllium husk appears to be the most effective (Bell et al., 1990; Anderson et al., 1994) and with the least adverse side effects (Anderson et al., 1990). Psyllium or ispaghula husk (the husk of the seeds of Plantago Ovata) is a mixture of neutral and acid polysaccharides containing galacturonic acid. Some foods in the human diet could potentially be enriched with psyllium husk, like breads, breakfast cereals, pasta and snack foods. The effect of psyllium husk on fasting plasma cholesterol has been evaluated in individuals with either hypercholesterolaemia or obesity or diabetes (Frati-Munari, 1983; Bell et al., 1989). In general, these studies show that psyllium husk cause a 5% reduction in total cholesterol and 7-8% reduction in LDL-cholesterol and these reductions are sustained in the long term (Anderson et al., 2000 a; Anderson et al., 2000 b). In relation to obesity, the evidence linking psyllium husk supplementation and weight loss is far less substantive. Nonetheless, a recent study has suggested a diet enriched with psyllium husk could reduce the development of obesity by reducing adiponectin and Tumor necrosis factor-alpha in obese Zucker rats (Galisteo et al., 2005).

CHOICE OF MOUSE MODEL

In nutrigenomics, many different model systems are used, ranging from *in vitro* cultured cells to animals and humans. The rationale for choosing the mouse model over *in vitro* model is as follows. Firstly, the regulatory mechanisms involved in responses to dietary perturbation are complex and organ-specific. Cell lines are usually established through immortalization and cells in culture lack natural contact with other cell types. Cultured cells, therefore, have lost part of their original tissue-specific behaviour (Mariman, 2006). Secondly, cells are often very sensitive to variation in the culture conditions. Usually, they can tolerate only the addition of one or few components to the culture medium in a limited concentration. Hence, it will be difficult to replicate the composition of a typical high-fat, calorie dense diet in the culture medium as any changes could interfere with the activity and the effects of the added components.

As for *in vivo* models, there would be many medical or ethical restrictions in taking liver or white adipose tissue biopsies from humans. Therefore, the mouse model was the next best choice among the various mammalian model systems for genetic research because of its close genetic and physiological similarities to humans (National Human Genome Research Institute website http://genome.gov/10005834). Like humans, mice naturally develop obesity-related diseases that affect these systems, including atherosclerosis, hypertension and diabetes. Moreover, the mice are relatively inexpensive to maintain. Amongst the various strains of mice, the inbred C57BL/6J mouse strain was chosen as it is commonly used as a model for human obesity. This strain increases its weight, develops increased plasma cholesterol and glucose levels when it is raised on a high-fat diet. The development of these risk

factors in the C57BL/6J mouse closely parallels the progression in humans (Collins et al., 2004). For example, the onset of cardiovascular disease, diabetes and obesity in humans occur gradually and often in the presence of a high-fat, calorie dense diet (Collins et al., 2004).

It has been shown that the female C57BL/6J mice are more responsive towards high-fat diet than their male counterparts in terms of their gain in white adipose tissue mass over their total body mass. When fed with high-fat-high calories diet for 8 weeks, the female mice characteristically exhibit 17.2% of their body weight as adipose tissue as compared to 13.3% in the males (http://phenome.jax.org). The females showed a 1.12-fold increase in their body weights as compared to 0.98-fold change in the males, a 5-fold increase in total cholesterol levels as compared to a 4.8-fold increase in the males (http://phenome.jax.org). There is limited literature describing diet-induced obesity in the female C57BL/6J mice.

NUMBER OF MICE

Based on the guidelines from Cui and Churchill (2002), a minimum of 6 mice per treatment group is needed in order to attain at least 80% power of detecting genes with 1.5 fold change. This statistical power increases with more mice per treatment group. As such, we had 8 mice in each group (for the high-fat, calorie dense experiment) and 6 mice in each group (for the high-fiber experiment).

POOLING OF mRNA

Due to the high cost of the gene chips at the time of the experiment ($1500 per chip), pooling was the only rational strategy. Moreover, mRNA samples are often pooled in

a microarray experiment not just out of necessity (Jin et al., 2001; Saban et al., 2001) but also in an effort to reduce the effects of biological variation (Chabas et al., 2001; Waring et al., 2001; Agrawal et al., 2002). As pooling minimizes subject-to-subject variation, it also enables easier detection of substantive features (Churchill and Oliver, 2001; Churchill, 2002; Kendziorski, et al., 2003; Allison, 2002; Han, et al., 2004). Pooling is often desirable when primary interest is not on the individual (e.g., making a prognosis or diagnosis), but rather on characteristics of the population (e.g., identifying biomarkers or expression patterns common across individuals) -- as in our experiment design. In addition, Kendziorski et al. (2005) have recently studied the effects of pooling in the context of microarray experiments and they found that inference for most genes is not adversely affected by pooling. They recommended that pooling should be done when fewer than three arrays were to be used in each condition.

CHOICE OF DIET FOR THE HIGH-FAT, CALORIE DENSE DIET EXPERIMENT

The high-fat, calorie dense (HFC) diet used in the study was formulated to mimic a typical Western high-fat energy dense diet in humans (SF00-219, Specialty Feeds). For humans, it is recommended that the intake of total fat and saturated fat should be limited to less than 30% and 10% of daily energy intake respectively (World Health Organisation, 2003). Amongst the saturated fatty acids, myristic and palmitic acids have the greatest effects on raising total and LDL cholesterol levels.

The formula used in the HFC diet originated with researchers at Rockefeller University and has been widely used to induce obesity in mice and rats. The HFC diet

contained 21% fat (by weight), of which saturated fatty acids is the major component (68%) of the total fatty acids. Both the fatty acids with intermediate chain lengths, palmitic (16:0) acid and myristic (14:0) acid, comprised about 70% of the total saturated fatty acids. Forty percent of the total calories in the HFC diet came from fat. The saturated fat content of the HFC diet exceeded 10% of total calories. Hence, the overall level of fat and the saturated nature of the fat are representative of diets that are used to induce obesity and diets that are linked to risk of cardiovascular disease in humans.

CHOICE OF DIET FOR THE HIGH-FIBER DIET EXPERIMENT

The formulation of the high-fiber diet used in the fiber feeding experiment was modified from AIN-93M by supplementing it with 10% psyllium husk [Specialty Feeds SF03-034, Specialty Feeds]. It consisted of 13.5% protein, 4% fat and 82.5% carbohydrate, with a total fiber content of 15%. AIN-93M was formulated specifically for maintenance of rats and mice (Reeves, 1997). The control diet for the high-fiber feeding experiment contains 5g of fiber per 100g, whereas the high-fiber diet enriched with psyllium husks contains 15g of fiber per 100g (i.e. 3 times more fiber). In humans, the recommendation for fiber intake is to go up to 25g per day (i.e. about 3 times more fiber than the average current intake) (WHO 2003). Therefore, the increase in fiber intake in the high-fiber group is approximately equivalent to the recommended increase in humans.

CHOICE OF ARRAYS FOR mRNA PROFILING

The three main technologies for examining gene expression are namely cDNA–AFLP (cDNA–amplified-fragment-length polymorphism), SAGE (serial analysis of gene expression) and the DNA microarray.

The cDNA–AFLP is a PCR-based method which starts with cDNA synthesis from total RNA or mRNA using random hexamers as primers. The obtained fragments are digested with two restriction enzymes and adapters are ligated to the ends of the fragments. After linker ligation and limited PCR amplification with primers complementary to the linker sequences, a second round of PCR amplification follows with labelled primers mainly complementary to the 5'-end of the linker sequences. These cDNA-derived restriction fragments are separated on polyacrylamide gels. The differences in the intensity of the bands provide a measure of the relative differences in the levels of gene expression. Individual genes can then be identified by cloning and sequencing of the fragment. cDNA-AFLP can generate a global overview of gene expression, but it involves a great amount of PCR reactions. In addition, separately obtained data sets cannot readily be compared, which is in contrast to SAGE and microarray data.

With SAGE, 'tags' – pieces of approximately 12 base pairs of cDNA - are first generated. This length is sufficient to make each tag a unique representative of the gene from which the corresponding mRNA was produced. The DNA molecules of linked tags are then cloned and sequenced. The relative abundance of a particular tag is a measure of the abundance of the corresponding mRNA in the original RNA sample and as such it is a relative measure for the transcriptional activity of that

particular gene. The limitations of this method include the requirement of a high-throughput cloning and sequencing facility.

With the limitations of the above methods, the DNA microarrays have become the preferred tool for mRNA profiling. Currently, the most common platforms are cDNA microarrays and oligonucleotide microarrays.

A cDNA microarray comprises a collection of thousands of probes usually corresponding to PCR products generated from cDNA libraries, expressed sequence tag clones, or long genome cloned fragments, using either vector- or gene-specific primers. The collection of probes is spotted onto a solid support either by mechanical robotic microspotting or ink jet techniques. Though cDNAs have been the major source of probes, over time researchers have discovered several disadvantages: high clone set error rates arising from clone cross-contamination, mislabelling, missing inserts, and phage contamination were persistent problems, particularly in large, commercially available clone sets (Knight, 2001). Moreover, cross-hybridization limited the specificity of the probes, i.e., a cDNA probe would detect the summed expression of a number of gene paralogs (Miller et al, 2002). Therefore, oligonucleotide arrays have become the preferred choice of many researchers. The advantages of oligonucleotide probes are many and include greater specificity, uniformity of hybridization, minimization of contamination, and improved quality control measures. They also eliminate the need to verify and maintain vast collections of cDNA clones and PCR products. Standing out among the oligonucleotide microarray platforms are the high-density oligonucleotide Affymetrix Genechip arrays synthesized in silico via photolithographic method. A silicon wafer is coated

with synthetic linkers modified with photo-labile protecting groups. A photolithographic mask is used to direct light to specific areas on the surface to remove the protecting group. These unprotected deoxynucleosides are then available to react with hydroxyl-protected deoxynucleosides that are incubated with the surface, and chemical coupling occurs only at those areas that were previously illuminated. A new mask is used to direct light to another specific set of physical location and the steps are repeated until the oligonucleotide with the desired sequence and size (typically 25 mer) is synthesized. To assess mRNA levels, each gene is represented by a probe set of 11–25 oligonucleotide pairs (probe pair) covering the 3' end of the mRNA. A probe pair comprises one oligonucleotide that perfectly matches the gene sequence (perfect match), and a mismatch oligonucleotide that carries a one-base mismatch in the middle position of the sequence. The mismatch oligonucleotide is used as control for nonspecific hybridization. The strategy of multiple probes per gene compensates for cross-hybridization.

REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR (reverse-transcription polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available (Bustin, 2000). Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller amounts of RNA (i.e. from as little as one cell). The application of fluorescence techniques to the RT-PCR, with suitable instruments (e.g. the Lightcycler, Roche), has led to widespread adoption of real-time RT-PCR as the method of choice for validating results obtained from array analyses.

Quantitative comparison of gene expression levels using real-time RT-PCR has the advantages of speed and less chance of contamination as the entire process, starting at the reverse transcription and ending with full quantification, is automated as compared to the conventional RT-PCR. In addition, it does not require the laborious procedures of running gel electrophoresis and quantifying band intensities. The underlying concept in the quantification of gene expression with real-time PCR is the measurement of the C_T (Threshold Cycle). C_T is always measured during the exponential phase of amplification in a typical PCR reaction and is directly related to the amount of target in the sample. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson *et al.* 1996). This point is defined as the C_T.

Among the different fluorescence techniques, SYBR® Green (Molecular Probes) is the most economical format for detecting and quantifying PCR products in real-time reactions. SYBR Green is a dye that binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The other advantages of SYBR Green are that it is easy to use and it is sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products. This issue of specificity and RT-PCR product verification can be addressed by conducting melting curve analysis (Ririe *et al.* 1997) and gel electrophoresis to confirm the amplified PCR products (Bustin, 2000). Melting curve analysis is conducted by plotting fluorescence as a function of temperature to generate a melting curve of the

PCR product. This is done by slowly increasing the temperature above the melting temperature (*T*m) of the PCR product and measuring the fluorescence. As the *T*m of the PCR product depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. A characteristic melting peak at the melting temperature (*T*m) of the PCR product will distinguish it from amplification artefacts that melt at lower temperatures in broader peaks. Both melting curve analysis and gel electrophoresis experiments are detailed in Chapter 3 Materials and Methods.

CHAPTER 3

MATERIALS & METHODS

MICE

Female C57BL/6J mice, 8-week old, were supplied by the Laboratory Animal Centre, National University of Singapore (NUS). They were acclimatized in our Animal Holding Unit for 2 weeks, feeding on standard chow before starting on their respective diets. They were kept in a room kept at 23 ± 1 ^{0}C with a 12-h light/dark cycle (light period: 08:00h -20:00h). The mice were housed in polycarbonate cages 1x2x3 cm in size with wire lids, containing pelleted paper bedding [T.7984cs, Teklad]. The mice were randomly divided into groups of 8 (for the high-fat, calorie-dense feeding experiment) and groups of 6 (for the high-fiber feeding experiment). Forty-eight mice were studied for the high-fat, calorie-dense feeding experiment and twenty-four mice were used in the high-fiber feeding experiment. All animal experiments were performed with the approval of the NUS Laboratory Animals Centre.[2]

DIETS

For the high-fat, calorie dense (HFC) feeding experiment, the mice were fed *ad libitum* with the control (C) diet or the high fat, calorie-dense (HFC) diet. The HFC diet [SF00-219, Specialty Feeds] consisted of 21% fat (by weight). This diet provided a total calorie content of 19.4 kJ/g. 40% of the total calories in the diet came from fat. The C diet was standard chow diet [Rat and Mouse Cubes, Specialty Feeds] with

[2] This was before the regulations of IACUC took effect.

4.6% fat (by weight). The C diet provided a total calorie content of 14.3 kJ/g. Twelve percent of the total calories in the diet came from fat.

For the high-fiber feeding study, the mice were fed *ad libitum* with either the control diet (C) or the high-fiber diet containing psyllium husk (PE). The diet for the control group was standard AIN-93M. The AIN-93M diet was formulated specifically for maintenance of rats and mice (Reeves, 1997). It consisted of 13.5% protein, 4% fat and 82.5% carbohydrate, with a total fiber content of 5% (by weight). The formulation of the PE diet was modified from AIN-93M by supplementing it with 10% psyllium husk [Specialty Feeds SF03-034, Specialty Feeds]. It consisted of 13.5% protein, 4% fat and 82.5% carbohydrate, with a total fiber content of 15% (by weight).

The ingredient listings and detailed nutrient composition of all diets used are listed in Appendix 3.1. The amount of food intake was measured by subtracting the residual food and food spillage from the total amount of food added weekly. Any food that spilled was collected on white paper towels placed beneath the cages in which the individual mouse was kept. After removing faeces and woodshavings from the paper towels, food spillage was collected and weighed.

BODY WEIGHT MEASUREMENTS

Baseline body weight of the mice were obtained on a weighing machine (Shimadzu, Libror EB- 20KH) when they first arrived. Subsequently, mice body weights were measured weekly at the same time of the day using the same machine.

MICE SACRIFICE AND TISSUE SAMPLES COLLECTION

At the end of the respective dietary treatments, the mice were sacrificed by cardiac puncture after 12 hours of overnight fasting (20:00hr – 08:00hr). The mice were anaesthetized by injection of CRC mixture (at 0.1ml/30g of body weight). The CRC mixture contained ketamine and medetomidine. Blood was collected by cardiac puncture technique as described in Appendix 3.2. The livers were harvested quickly from the mice. Liver samples comprising the identical lobe were obtained from each mouse. The liver samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Intraperitoneal fat depots were rapidly removed, placed in pre-tared vials and weighed. The fat tissue were then frozen in liquid nitrogen and stored at - 80°C.

LIPIDS AND GLUCOSE ASSAYS

Plasma total cholesterol, HDL cholesterol, triacylglycerol concentrations were measured after an overnight fast using enzymatic assay kits on a Cobas Mira analyzer (Roche). Before starting the respective diets, blood was collected from the tail of the mouse as detailed in Appendix 3.3. LDL/VLDL cholesterol concentrations were determined as the difference between total and HDL cholesterol concentrations. The thesis would discuss LDL/VLDL cholesterol concentrations as LDL cholesterol concentrations. Blood glucose levels were measured using the Accu-Chek glucometer (Roche Diagnostic, Indianapolis, IN, USA) and Accu-Chek test strips (Roche Diagnostic, 03603474) with appropriate calibration.

GENE EXPRESSION PROFILING

Extraction of total RNA

Total RNA was isolated from frozen liver or from white adipose tissue from each mouse using TRIzol® reagent (Invitrogen, 15596) according to the protocol listed in Appendix 3.4. After isolation, the total RNA was purified using RNeasy® Mini Kit (Qiagen, 74104) according to manufacturer's instructions (Appendix 3.5). The concentration and the purity of RNA were determined by UV spectrophotometry. Absorbance of 1 OD read at 260nm in a 1cm light path is equivalent to 40ug/ml of RNA. Pure RNA should have A_{260}/A_{280} ratio of 1.9- 2.1 in 10mM Tris.CL, pH 7.5. A 2 ul sample was run on a 1% denaturing RNA gel to ensure that RNA was not degraded after RNA extraction.

cDNA and cRNA synthesis

According to Affymetrix standard protocol (Appendix 3.6), cDNA was synthesized from purified total and pooled RNA. Pure and undegraded total RNA was incubated with 50 µM of T7-Oligo(dT) primer at 70 ^{0}C for 10 minutes, then cooled to 4^{0}C for 2 minutes. Superscript II reverse transcriptase was then added to the RNA-primer mixture for the first strand cDNA synthesis. This reaction mixture was incubated for 1 hour at 42 ^{0}C and then cooled to 4 ^{0}C for 2 minutes. For the second strand cDNA synthesis, dNTP, DNA ligase, DNA Polymerase 1 and RNase H were then added to the first strand reaction mix and then incubated for 2 hours at 16^{0}C. Following reverse transcription, 10ul of EDTA 0.5M was added to stop the reaction. Subsequently, the double-stranded cDNA was transcribed in vitro to biotinylated cRNA by using the Genechip IVT Labelling Kit (Affymetrix, 900182). The IVT Labelling reaction mixture was incubated at 37 ^{0}C for 16 hours (overnight). All incubation steps were

done in a PCR machine (Biometra®, T Personal). After performing the clean-up of the biotinylated cRNA with GeneChip Sample Cleanup Module (Affymetrix, 900371), cRNA yield and concentration were determined by using the UV spectrophotometer. A minimum concentration of 0.6µg/µl for the adjusted cRNA yield was ensured before proceeding to the next step.

Hybridization and wash

Subsequently, the biotinylated cRNA was fragmented and mixed with control oligonucleotide B2, 20X Eukaryotic Hybrization Controls (bioB, bioC, bioD, cre), herring sperm DNA, BSA and the hybridization buffer. The hybridization cocktail was heated to 99 ^{0}C for 5 minutes and then transferred to a 45 ^{0}C heat block for 5 minutes. After 5 minutes of centrifuging, the cocktail was injected into the probe array cartridges. GeneChip® Mouse Genome 430 2.0 Array cartridges were used for the high-fat, calorie dense diet experiment. GeneChip® Mouse Expression 430A Array cartridges were used for the high-fiber feeding experiment. Gene expression results for the two separate feeding experiments were analysed and discussed separately. All probe sets represented on the GeneChip® Mouse Expression 430A Array cartridges are included on the GeneChip® Mouse Genome 430 2.0 Array cartridges. The 430 2.0 Array contains probes for about 34,000 known mouse genes whereas the 430A Array only contains probes for about 14,000 known mouse genes. In addition, the 430 2.0 Array cartridges also contain probe sets from the GeneChip Mouse Expression 430B Array cartridges, which include probe sets against gene clusters containing only EST sequences and some gene clusters with non-EST sequences. Subsequently, these cartridges filled with the hybridization cocktail were incubated at 45°C for 16 hours on a rotisserie at 60 rpm. The quality of the

fragmented biotin-labelled cRNA in each experiment was checked before hybridizing onto the Mouse Genome 430 2.0 and Mouse Expression Array 430A Arrays by using Test-3 arrays. One of the major concerns in microarray experiments is generating full-length labelled product. Incomplete product leads to a 3' bias, which can influence data analysis. The Test-3 arrays contain characterized genes from all species and provide a low-cost method of assessing sample quality before using the Expression GeneChips. The quality of the biotin-labelled cRNA was examined by ensuring that the signal ratio of the 3'/ 5' probe sets should be no more than 3. After hybridization, the arrays were washed in GeneChip Fluidics Station 400 and stained with Streptavidin Phycoerythrin.

Microarray image and data analysis

Probe arrays were scanned on the Affymetrix GeneChip® Scanner 3000 after washing. The GeneChip Operating Software Version 1.2.0.037 (Affymetrix) was used to analyze the scanned image. For the experiment on the high-fat, calorie-dense diet, expression patterns for each group were analysed according to the following pair-wise comparisons. For instance, mice fed on high-fat, calorie-dense diet for 2 weeks (HFC-2wk) were compared to mice fed on control diet for 2 weeks (C-2wk). Similar comparisons were carried out for mice at weeks 4 and 10 (i.e. HFC-4wk versus C-4wk and HFC-10wk versus C-10wk). For the experiment on high-fiber diet, the mice fed on the high-fiber diet enriched with psyllium husk for 3 weeks (PE-3wk) were compared to their corresponding controls (C-3wk). At week 10, the PE-10wk mice were compared with the mice fed on the standard control AIN93M diet (C-10wk).

The probe sets were sorted according to the following stringency criteria. In order to get 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. To obtain robust increase in the gene expression levels between the treated versus the control groups, it is important to select transcripts that are "Present" in the treated groups (i.e. high-fat, calorie-dense groups HFC or the high-fiber groups). Hence, transcripts that are called "Absent" in the treated groups are removed. On the other hand, when analyzing robust decrease in the gene expression levels between the treated versus the control groups, "Absent" cells are removed in the control groups and only those flagged "D" with Signal Log Ratio $\leq$ than -0.5 were selected. Data of "fold change" were calculated from the "Signal Log Ratio". Under *in vivo* conditions, changes in differential gene expression due to dietary treatment are expected to be small and often below 2 fold change (Blanchard et al., 2001; Barella et al., 2004). As such, any gene expression changes greater than a threshold of 1.4 fold (i.e. signal log ratio of 0.5) were considered to be potentially relevant. The selected probe sets were then annotated and classified using NetAffx Gene Ontology (GO) Mining Tool. The gene expression data was presented using TreeView (http://rana.lbl.gov/EisenSoftware.htm).

QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

qRT-PCR was conducted to validate our microarray results. Gene expression results of 8 genes were selected. The primers sequences used are listed in Table 3.1. Absence of genomic DNA contamination was confirmed by conducting control PCR reactions containing RNA template, primers, and 2 μl of LightCycler DNA *Master* SYBR

Green I (Roche, Diagnostics). This control PCR reaction did not contain reverse transcriptase. The LightCycler RNA Master SYBR Green I one-step RT-PCR System (Roche, Diagnostics) method was used to carry out cDNA synthesis and amplification. Four hundred and fifty nanograms of total RNA obtained from each mouse, primers, $Mn(OAc)_2$ and LightCycler RNA Master SYBR Green I were mixed well and transferred to LightCycler capillaries (Roche, Diagnostics). The cycling conditions were set as follows: reverse transcription at 61 ^{0}C for 20 mins, followed by initial denaturation at 95 ^{0}C for 30s. Subsequently, for 45 cycles, denaturation was carried out at 95 ^{0}C for 5s, primer annealing step (conditions depend on primers), extension at 72 ^{0}C for 1 min. Melting curve analysis was performed to assess the specificity of the amplified PCR products. The amplified PCR products were subjected to 2% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide. Fold change in gene expression was calculated using the delta threshold cycle (ΔC_T) method (Livak et al., 2001) and normalized against the house-keeping gene β-actin gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (C_T) was determined for each gene, and the relative amount of each gene mRNA to β-actin mRNA was described using the equation $2^{-\Delta C}{}_T$ where $\Delta C_T = (\Delta C_{T\ gene} - \Delta C_{T\ \beta\text{-actin}})$. In order to facilitate data presentation, relative gene expression was multiplied by 10^4.

Table 3.1 Sequences of primers used for RT-PCR

Gene name and Gene symbol	Forward (5'→3')	Reverse (5'→3')	PCR Product
Actin, beta (β-actin)	taaagacctctatgccaacacagt	cacgatggaggggccggactcatc	241bp
3-hydroxy-3-methylglutaryl-coenzyme a reductase (Hmgcr)	tgtggccaggagtttggtgactga	taagattcaacaactctgctgacc	101bp
Fatty acid synthase (Fas)	tctgtgaagagtcagtggaggca	cagcagcctgtgtattgagtctag	212 bp
Carnitine palmitoyltransferase 1A, liver (Cpt1L)	actgtaagtgttcaaaggaggag	Gctatattcgttcacacatgc	173 bp
Cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1)	actgtgcttcctgctttgccttg	tggatacattcagttcagggagtc	168 bp
Peroxisome proliferator activated receptor alpha (Pparα)	Tcctacgcttggggatgaaga	tgaatcctattagcctccgatcac	126 bp
Leptin (Lep)	cctgtggctttggtcctatctg	ctgctcaaagccac cacctctg	416 bp
Leptin receptor (Lepr)	tatgtcattgtacccataattatt	cagagaagttagcactgtt	373 bp

WESTERN BLOTS

Four mice from each group were randomly selected and individual liver samples were used for the western blot assays. The liver tissues were homogenized in RIPA buffer containing a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Denatured proteins (30 µg) were loaded onto polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham) 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 hr at room temperature and later incubated overnight at 4°C with the respective antibodies: a rabbit antibody against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Cell Signalling), fatty acid synthase (BD Biosciences) and Cpt1L (Alpha Diagnostic International). Rabbit antibody against β-actin (Cell Signalling) was used for normalization. Subsequently, membranes were incubated with a horseradish peroxidase-linked goat anti-rabbit IgG (Cell Signalling) for 1 hr at room temperature. Following application of chemiluminescent detection reagents (Amersham), Clear-Blue X-ray films (Pierce) were exposed to the blots and the band intensities were determined using Quantity One software (Bio-Rad).

PLASMA INSULIN LEVELS

Four mice from each group were randomly selected and their plasma insulin levels were individually measured by a Rat/Mouse Insulin Elisa Kit (Linco Research, EZRMI-13K). Monoclonal anti-rat insulin antibodies were pre-coated onto the wells of the microtiter strips provided. Ten microlitres (ul) of samples, insulin standards of concentrations ranging from 0.2 to 10 ng/ml and quality controls were pipetted into

the wells followed by 80 µl of a biotinylated second polyclonal antibody. During the first incubation, the insulin antigen bound simultaneously to the immobilized (capture) antibody on one site and to the biotinylated antibody on the second site. After removal of excess second antibody and washing, 100 ul of streptavidin-horseradish peroxidase (enzyme) was added. This bound to the biotinylated antibody. After a second incubation and washing to remove the unbound enzyme, a substrate 3,3', 5,5'-tetramethylbenzidine solution was added, which was acted upon by the bound enzyme to produce coloured product .The colour intensity of this product was directly proportional to the concentration of insulin present in the original samples. Each sample was done in duplicates. Absorbance was read at 450nm, corrected for absorbency at 590 nm by a microplate reader.

PLASMA LEPTIN LEVELS

Four mice from each group were randomly selected and their plasma leptin levels were individually measured by a Rat/Mouse Leptin Elisa Kit (Linco Research, EZRML-82K). This assay is based on the binding of leptin in the samples by a pre-titered antiserum. Ten microlitres of samples, leptin standards of concentrations ranging from 0.2 to 30 ng/ml and quality controls were pipetted into the wells. After incubating for 2 hours, and washing, 100ul of of biotinylated antibody was added. After removal of excess antibody and washing, 100 ul of streptavidin-horseradish peroxidase (enzyme) was added. After a second incubation and washing to remove the unbound enzyme, a substrate 3,3', 5,5'-tetramethylbenzidine solution was added. Each sample was done in duplicates. Absorbance was read at 450nm, corrected for absorbency at 590 nm by a microplate reader.

STATISTICAL ANALYSIS

All results are expressed as means $\pm$ SD. Statistical significance of differences was analyzed by using Student's *t*-test. Differences in measurements across the 3 time-points were analyzed with 1-way ANOVA using Bonferroni post-hoc test. Pearson's coefficient of correlation (*r*) was used to determine the correlation between the different variables. Spearman's rank correlation coefficients were also calculated and these tests gave identical results as Pearson's coefficient of correlation (i.e. same significance). As most of the data is normally distributed (e.g. body weight), the data is presented with Pearson's coefficient of correlation. The SPSS 14.0 package for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis. The significance level was set at $P < 0.05$.

CHAPTER 4

SEQUENTIAL EFFECTS OF A HIGH-FAT, CALORIE-DENSE DIET ON FOOD INTAKE, BODY WEIGHT, PLASMA LIPIDS, LEPTIN, INSULIN AND GENE EXPRESSION LEVELS

INTRODUCTION

The role of a high-fat, calorie-dense (HFC) diet as a major dietary risk factor associated with obesity and cardiovascular disease has been outlined in Chapters 1 and 2. Several microarray transcription profiling studies have attempted to examine the relationship between this dietary risk factor and the genetic responses. However, the fat content in the diets used in some of these studies (Li et al., 2002; Lopez et al., 2003) was very high. Therefore, these diets did not reflect the typical fat content in the high-fat human diet. In addition, some studies have used genetically obese mice (Nadler et al., 2000; Soukas et al., 2000). These genetic models may not be suitable models for examining the influence of dietary risk factor on the development of human obesity. In comparison, the diet-induced obese C57BL/6J mouse is a suitable human obesity model. Further details on this mouse strain have been discussed in Chapter 2.

We postulated that one of the reasons for the reported discrepant findings on high-fat, calorie dense diet on food intake and on gene expression levels was due to the varying lengths of feeding period (as reviewed in Chapter 2). We proposed that the ingestion of a high-fat, calorie dense diet would initially elicit compensatory responses in food intake and gene expression levels which would help to regulate energy homeostasis and that such compensatory responses would diminish over time with continued

excessive dietary intake of fat and calories. 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 (Kitano et al., 2004). Since the body is always constantly adapting to the changes in a dynamic environment, it would be interesting to examine how the body adapts to consumption of a diet high in calories and fat over a period of time. Therefore, in parallel with the microarray transcription profiling of the liver and the white adipose tissue, we examined the changes in food intake, body weight, white adipose tissue mass, plasma lipids, insulin, leptin and blood glucose levels over a period of 10 weeks.

The abbreviations used to name the groups are as follows: Mice fed on the high-fat, calorie-dense (HFC) diet for 2 weeks (HFC-2wk), for 4 weeks (HFC-4wk) and for 10 weeks (HFC-10wk). The abbreviations for their respective controls are as follows: C-2wk, C-4wk and C-10wk.

RESULTS

Feed consumption, energy and fat intake

Food intake was reported as the calculated average daily intake over the last 7 days before the mice were sacrificed. It was shown in our preliminary study that daily food intake was constant over this period when we measured food intake on eight mice daily (Appendix 4.1). Energy intake was calculated from the energy density of the diet multiplied by the amount of food consumed. Feed efficiency (FE) ratio was calculated as the ratio of weight gained to the cumulative food intake over the same period of the weight gained. Energy efficiency (EE) ratio was calculated as the ratio

of weight gained to cumulative energy intake over the same period of the weight gained.

During the first 2 weeks, there was no increase in the HFC mice's food intake. In fact, there was a slight decrease in HFC mice (3.06 ± 0.15 g/day) as compared to the control mice (3.21 ± 0.09 g/day). This reduction in food intake was not significant. However, at weeks 4 and 10, there were significant increases in the food intake of the HFC groups when compared to their respective controls. The food intake in HFC-4wk mice increased by 17% in comparison to C-4wk mice. At week 10, the high-fat mice increased their food intake by 24% versus their controls (Figure 4.1). Energy intake was significantly higher in all 3 HFC groups versus the controls (Figure 4.2). The total fat content in the HFC diet was 4 times higher than the C diet. All the HFC mice had significantly higher total fat (Figure 4.3) and saturated fat intake (Figure 4.4) as compared to the C groups.

Figure 4.1 Food intake of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) *: P < 0.05 compared to corresponding control group. Open bars represent data from C mice and filled bars represent data from HFC mice.

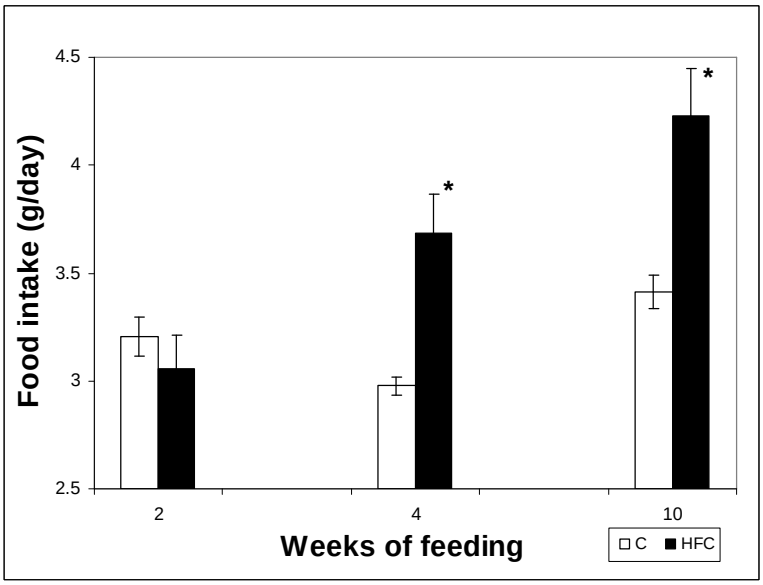


Figure 4.2 Energy intake of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) *: P < 0.05 compared to corresponding control group. Open bars represent data from C mice and filled bars represent data from HFC mice.

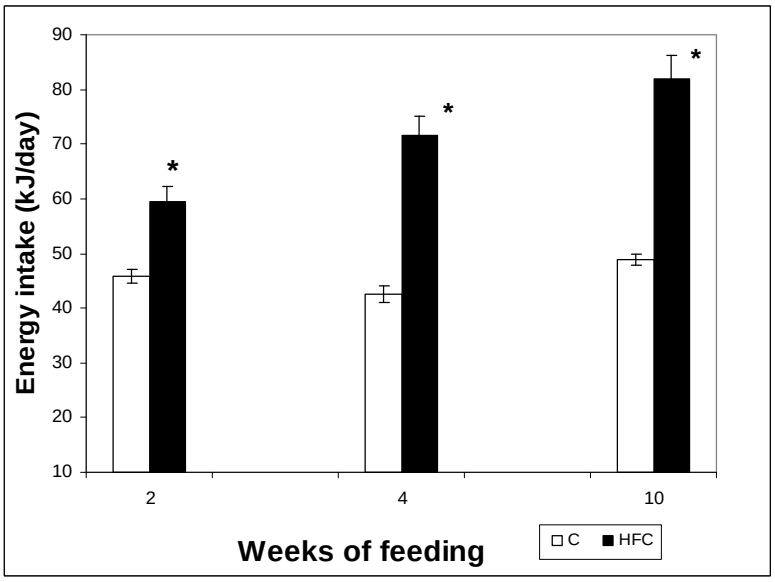


Figure 4.3 Total fat intake of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) *: P < 0.05 compared to corresponding control group. Open bars represent data from C mice and filled bars represent data from HFC mice.

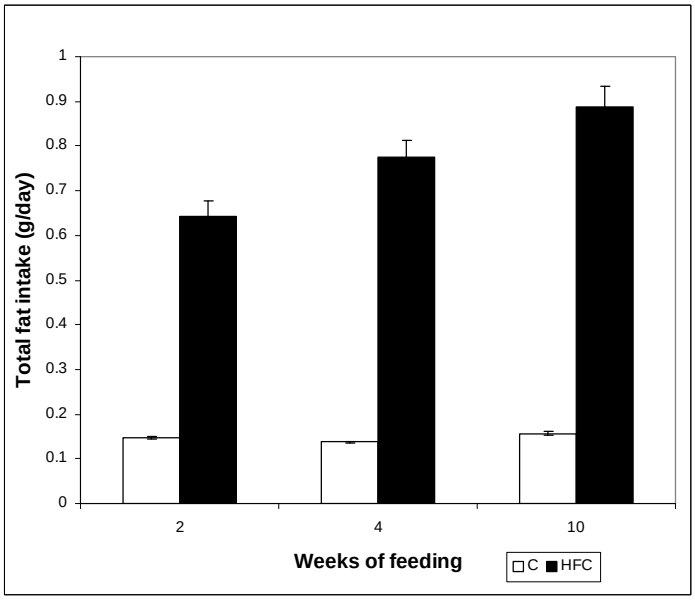
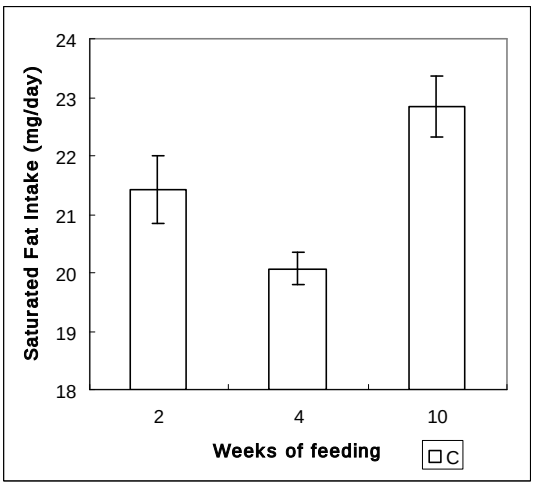


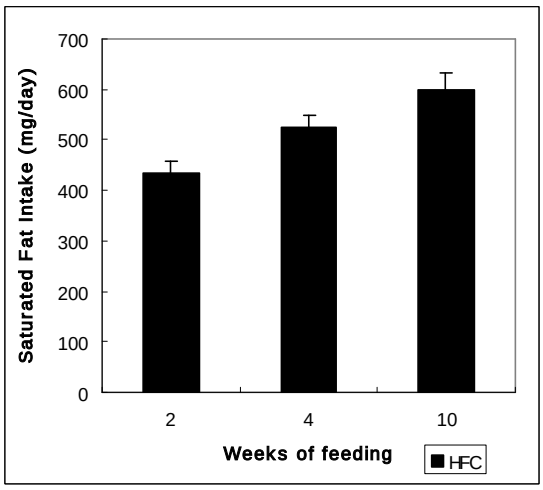
Figure 4.4 Saturated fat intake of HFC and C mice

(A) Saturated fat intake among C mice
(B) Saturated fat intake among HFC mice
Results are presented as means ± SD for each group of mice (n = 8 / group).
All the HFC groups had significantly higher saturated fat content than the respective C groups. ($P < 0.01$)

A



B



Feed efficiency ratio and energy efficiency ratio

The feed efficiency (FE) ratio observed in the HFC-2wk mice was slightly lower than the C-2wk mice. These differences did not attain a significant level (Table 4.1). There was also a tendency for HFC-2wk mice to have lower energy efficiency (EE) ratio than the C-2wk mice. At weeks 4 and 10, the FE ratios in the HFC mice were higher than their controls ($p < 0.01$ at week 10) (Table 4.1). Higher EE ratios were also observed for HFC-4wk and HFC-10wk mice when compared to their respective controls. None of these differences in the EE ratios were significant (Table 4.1). The EE ratios for the C mice were relatively stable across the 3 time-points. Both 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 versus HFC-2wk mice ($P < 0.05$).

Table 4.1 Feed efficiency (FE) ratio and Energy efficiency (EE) ratio of control and HFC mice

C: control diet groups HFC: high fat, calorie dense diet groups. Values are expressed as means ± SD (n= 8/group)
** denotes P < 0.01 compared to corresponding C group

	C-2wk	HFC-2wk	C-4wk	HFC-4wk	C-10wk	HFC-10wk
FE ratio	14.96 ± 7.42	14.64 ± 10.12	15.04 ± 6.88	24.33 ± 11.42	15.69 ± 4.55	26.95 ± 7.01**
EE ratio	1.11 ± 0.55	0.75 ± 0.52	1.11 ± 0.51	1.25 ± 0.59	1.16 ± 0.34	1.39 ± 0.36

Body weight

There were no significant differences in body weight amongst the groups at the start of the feeding study (Table 4.2). Two weeks of high-fat, calorie-dense feeding did not induce any significant weight gain (Table 4.2). Although an increase in weight gain in the HFC-4wk mice (2.50 ± 1.14 g) as compared to the C-4wk mice (1.78 ± 1.13 g) was observed at week 4, this difference was not statistically significant. It was only at week 10 that we observed a significant large increase in weight gain in the HFC-10wk mice (7.60 ± 1.89 g) as compared to the C-10wk mice (2.98 ± 1.98 g). When we compared the amount of weight gained in relation to their initial body weights, we observed that the percentage change in body weight was comparable between HFC-2wk mice and C-2wk mice (Table 4.2). The HFC-4wk mice had an increase of 12.3% over their initial body weights. This percentage increase in the HFC-4wk mice was higher as compared to the percentage increase in C-4wk mice but this was not statistically significant. The HFC-10wk mice had a 42.6% increase over their initial body weights. This percentage increase was almost 2.7 times higher than the percentage increase in the C-10wk mice ($P < 0.01$).

Between weeks 2 and 4, final body weight increased from 20.95 ± 0.75g in HFC-2wk mice to 22.80 ±1.93g in HFC-4wk mice. Body weight increased to 26.0 ± 2.6g in HFC-10wk mice (Figure 4.5). The difference between HFC-10wk mice and HFC-2wk was significant ($P < 0.01$). Similarly, the increase between HFC-4wk mice and HFC-10wk mice was significant ($P < 0.01$).

Based on the weekly weight monitoring of the HFC-10wk mice, we observed that there was a slight reduction in HFC-10wk body weight as compared to C-10wk mice

at week 1 (not significant) and the increase in body weight between the HFC-10wk and the C-10 wk mice seemed to become more apparent after 4 weeks of high fat feeding (Figure 4.6).

Table 4.2 Initial body weight, gained body weight, percentage (%) change in body weight of control and HFC mice

C: control diet groups HFC: high fat, calorie-dense diet groups. Values are expressed as means ± SD (n= 8/group)
** denotes P < 0.01 compared to corresponding C group
Initial body weight = Body weight at the start of feeding period
Gain in body weight = Final body weight – Initial body weight (e.g. Final body weight of C-4wk mice – Initial body weight of C-4wk mice at the start of feeding period)

	C-2wk	HFC-2wk	C-4wk	HFC-4wk	C-10wk	HFC-10wk
Initial body weight (g)	19.91 ± 0.18	20.08 ± 0.14	19.86 ± 0.76	20.30 ± 1.15	19.28 ± 1.48	18.56 ± 1.95
Gain in body weight (g)	0.85 ± 0.59	0.88 ± 0.80	1.78 ± 1.13	2.50 ± 1.14	2.98 ± 1.98	7.60 ± 1.89 **
% change in body weight	4.31 ± 3.04	4.35 ± 4.03	9.05 ± 5.95	12.25 ± 5.23	16.39 ± 12.99	42.64 ± 10.12 **

Figure 4.5 Final body weights of HFC and C mice over the 3 time-points

Results are presented as means ± SD for each group of mice (n = 8 / group). ** denotes P < 0.01 compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice.

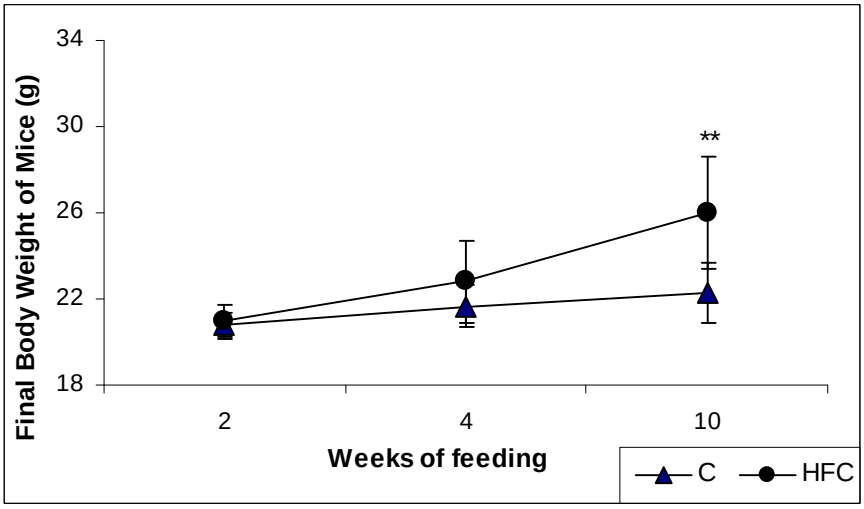
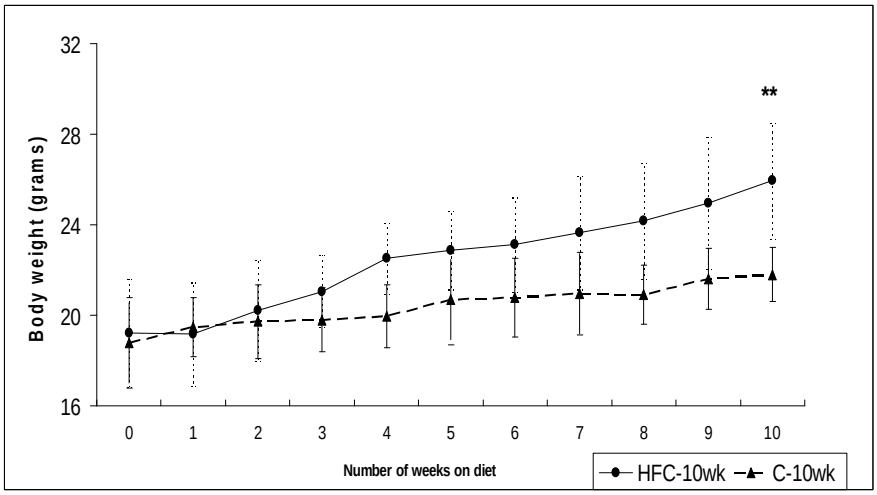


Figure 4.6 Body weights of HFC-10wk and C-10wk mice at each week

Results are presented as means ± SD for each group of mice (n = 8 / group). ** denotes P < 0.01 as compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice.



White adipose tissue mass

Similar to the trend in body weight gain, there was no significant difference in white adipose tissue mass in HFC-2wk mice (0.20 ± 0.05g) versus C-2wk mice (0.18 ± 0.01g) (Figure 4.7). However, significant deposition of white adipose tissue was observed in HFC-4wk mice and HFC-10wk mice versus their corresponding controls (0.34 ± 0.10 g versus 0.18 ± 0.05 g and 0.61 ±0.21 g versus 0.29 ± 0.06 g respectively). The white adipose tissue mass in the HFC-10wk mice was nearly twice the white adipose tissue mass in the C-10wk mice.

When we examined the relation between the white adipose tissue mass and the body weight, we observed a significantly strong positive correlation between white adipose tissue mass and the final body weight of all the mice ($r = 0.92$, $P < 0.01$) (Figure 4.8). About 85% of the variation in the white adipose tissue mass can be explained by the final body weight of the mice. When we examined this relation separately in control mice and HFC mice, we observed that the correlation between the white adipose tissue mass and the final body weight was stronger in HFC mice ($r = 0.93$, $P < 0.01$) than in C mice ($r = 0.68$, $P < 0.01$).

Figure 4.7 White adipose tissue mass of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group)
*: P < 0.05 compared to corresponding control group. Open bars represent data from C mice and filled bars represent data from HFC mice.

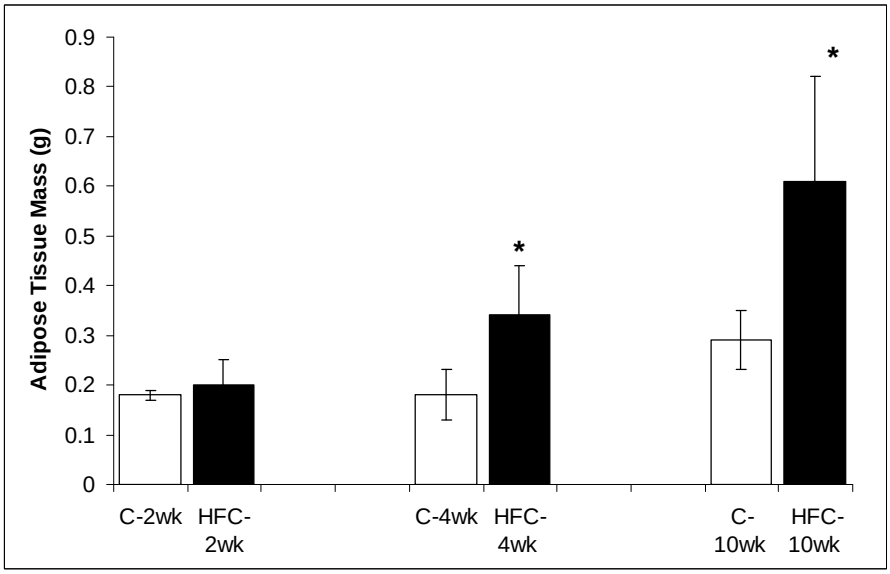
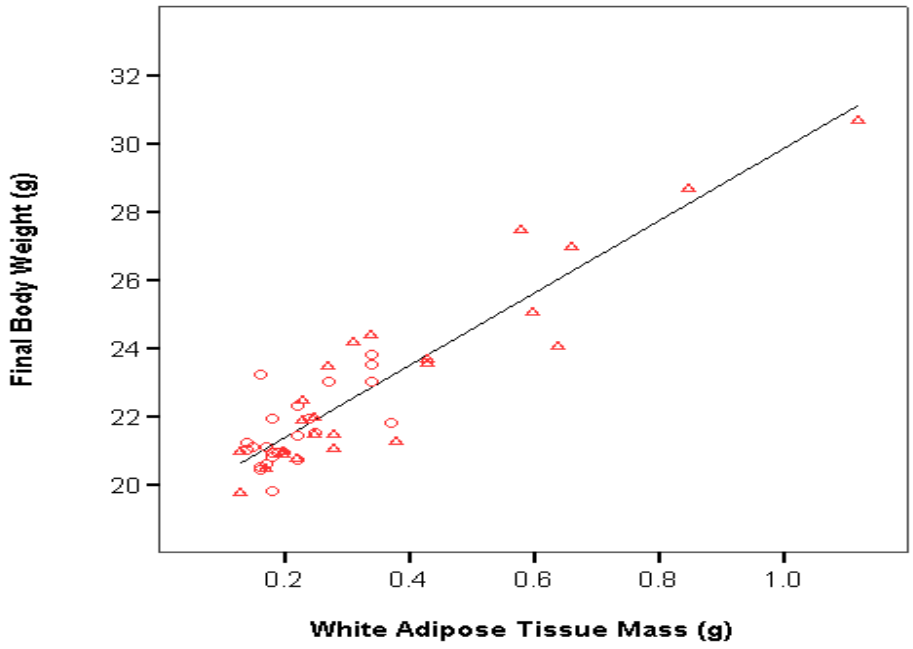


Figure 4.8 Correlation between final body weight and white adipose tissue mass

Circles represent data from C mice. Triangles represent data from HFC mice.
(r = 0.92, P < 0.01)



Plasma cholesterol levels

At the start of the feeding study, there were no significant differences in the total cholesterol, LDL cholesterol, HDL cholesterol levels amongst the different groups. Within 2 weeks of high-fat, calorie-dense feeding, total cholesterol levels were significantly raised by 33% in the HFC-2wk mice as compared to the C-2wk mice (2.22 $\pm$ 0.39 mmol/L versus 1.67 $\pm$ 0.09 mmol/L) ($P<0.01$) (Figure 4.9A). The HFC-2wk mice also had a significantly higher LDL cholesterol levels as compared to the C-2wk mice. Their LDL cholesterol levels were already 2.3 times higher than the controls (0.60 $\pm$ 0.15 mmol/L versus 0.26 $\pm$ 0.12 mmol/L) ($P < 0.05$) (Figure 4.9B). As the HFC feeding period continued, both the total and LDL cholesterol remained significantly elevated in the HFC-fed mice as compared to the C mice. Unlike the gains in body weight and white adipose tissue mass we observed in the mice, the HFC-fed mice did not show a significant progressive increase in their total and LDL cholesterol levels throughout the entire HFC feeding period. Total cholesterol levels and LDL cholesterol levels in HFC-10wk mice increased by 16% and 37% respectively when compared to HFC-2wk mice (non significant). There was no significant progressive increase amongst the 3 HFC groups. Instead, we observed a slight drop in total cholesterol levels in the HFC-10wk mice when compared to HFC-4wk mice, although this difference was not statistically significant (Figure 4.9A).

HDL cholesterol levels were raised in all HFC groups but only the increase in HFC-4wk mice was significant as compared with the C-4wk mice (Figure 4.9C). The largest increase was also observed in HFC-4wk mice versus C-4wk mice. Their HDL cholesterol levels increased by 45% from the levels observed in C-4wk mice ($P < 0.01$). Since high total cholesterol levels (which confer increased cardiovascular risks)

are usually accompanied by high HDL cholesterol levels (which confer reduced cardiovascular risks), we calculated the total/HDL cholesterol ratio to resolve this dilemma. A higher total/HDL cholesterol ratio will confer greater cardiovascular risks. The total cholesterol/HDL cholesterol ratios were raised in all the HFC-groups as compared to the C groups but these increases did not reach statistical significance (Figure 4.9D). The smallest increase in the ratio was observed in HFC-4wk mice (6%) as compared to the C-4wk mice whereas the greatest increase in the ratio was observed for the HFC-10wk (32%) mice compared to the C-10wk mice. These differences in total/HDL cholesterol were not significant. The total/HDL ratio seemed to increase with the HFC feeding period but did not reach significant differences amongst the HFC groups.

Figure 4.9 Plasma cholesterol levels of HFC and C mice

(A) Total cholesterol levels

C: control diet groups; HFC: high fat, calorie-dense diet groups. Values are expressed as means ± SD (n= 8/group) ** denotes P < 0.01 when compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the HFC or C groups.

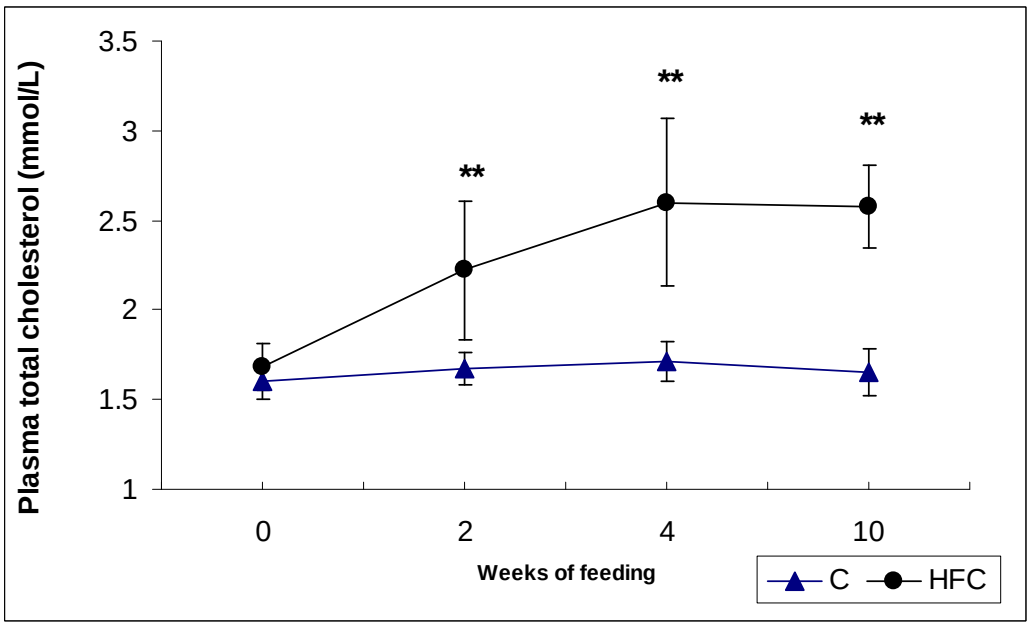


Figure 4.9 (continued) Plasma cholesterol levels of HFC and C mice

(B) Plasma LDL cholesterol levels

C: control diet groups; HFC: high fat, calorie-dense diet groups. Values are expressed as means ± SD (n= 8/group); * denotes P < 0.05 and ** denotes P < 0.01 when compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the HFC or C groups.

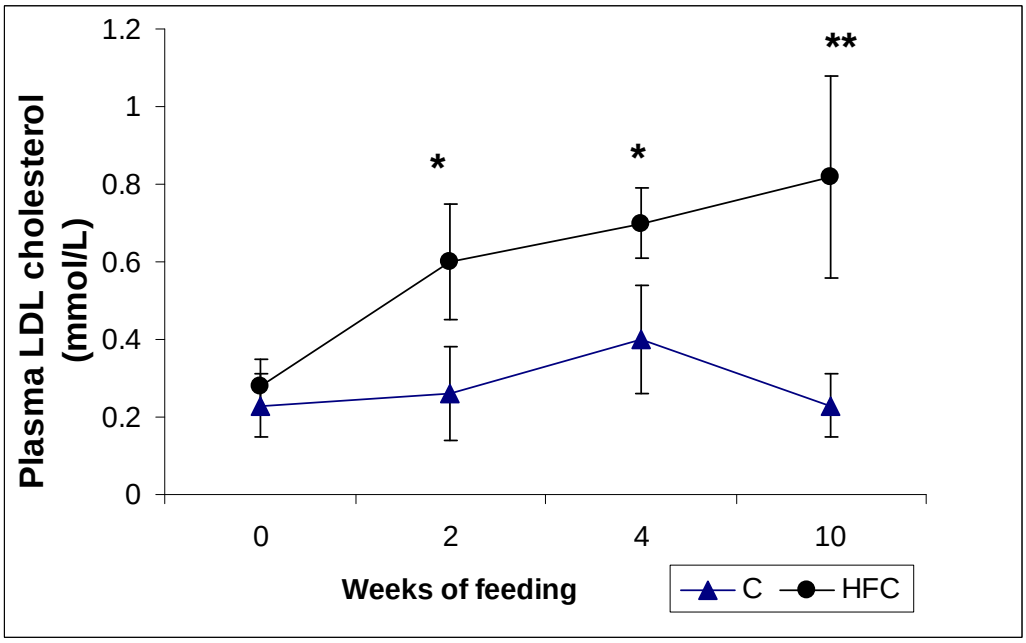


Figure 4.9 (continued) Plasma cholesterol levels of HFC and C mice

(C) Plasma HDL cholesterol levels

C: control diet groups; HFC: high fat, calorie-dense diet groups. Values are expressed as means ± SD (n= 8/group) ** denotes P < 0.01 when compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the HFC or C groups.

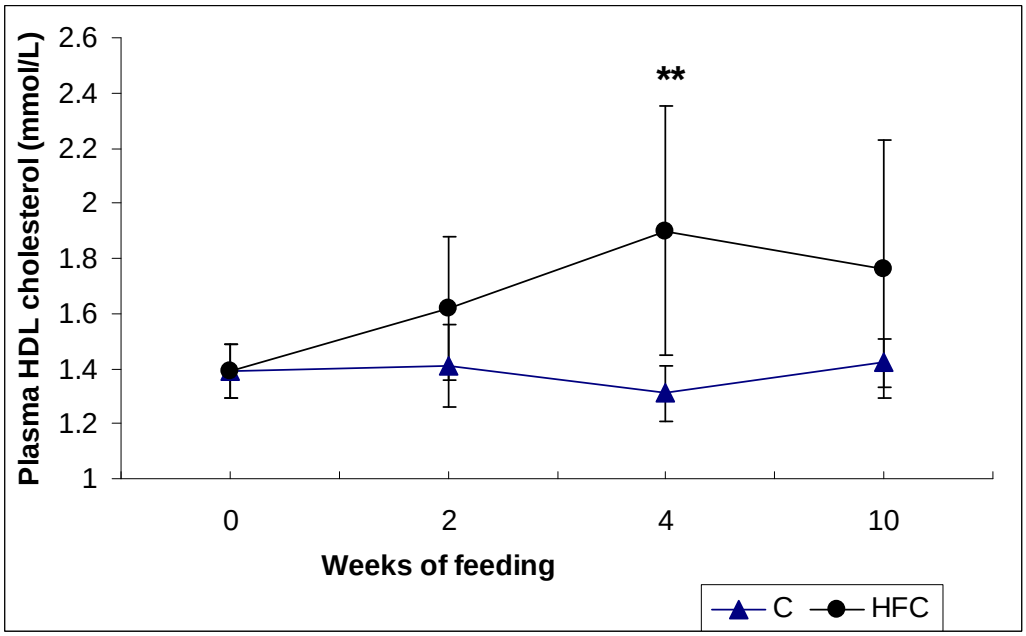
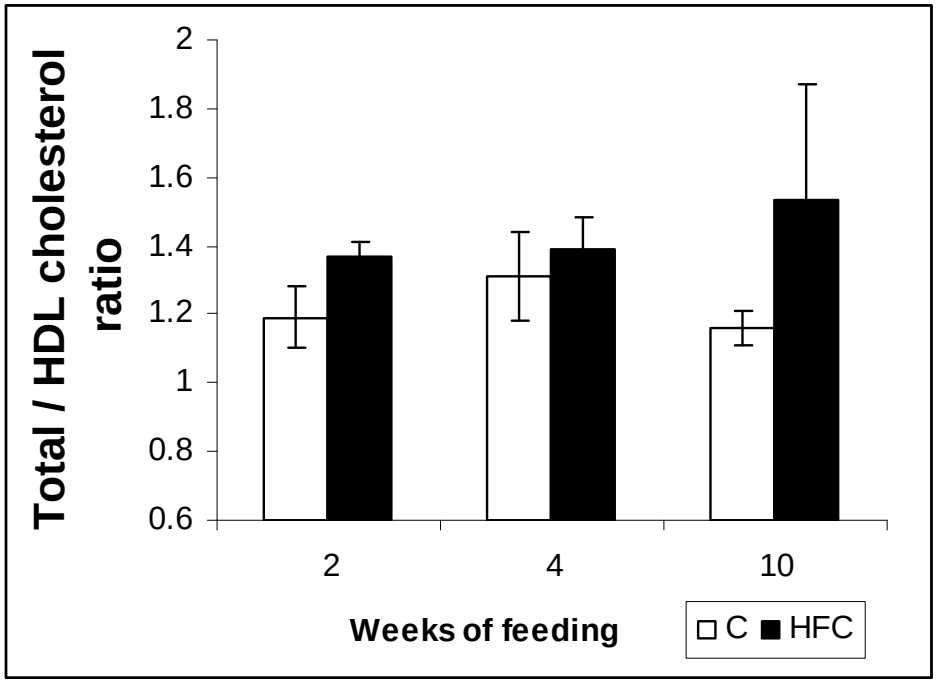


Figure 4.9 (continued) Plasma cholesterol levels of HFC and C mice

(D) Total/HDL cholesterol ratio

Results are presented as means ± SD for each group of mice (n = 8 / group).
Open bars represent data from C mice and filled bars represent data from HFC mice.

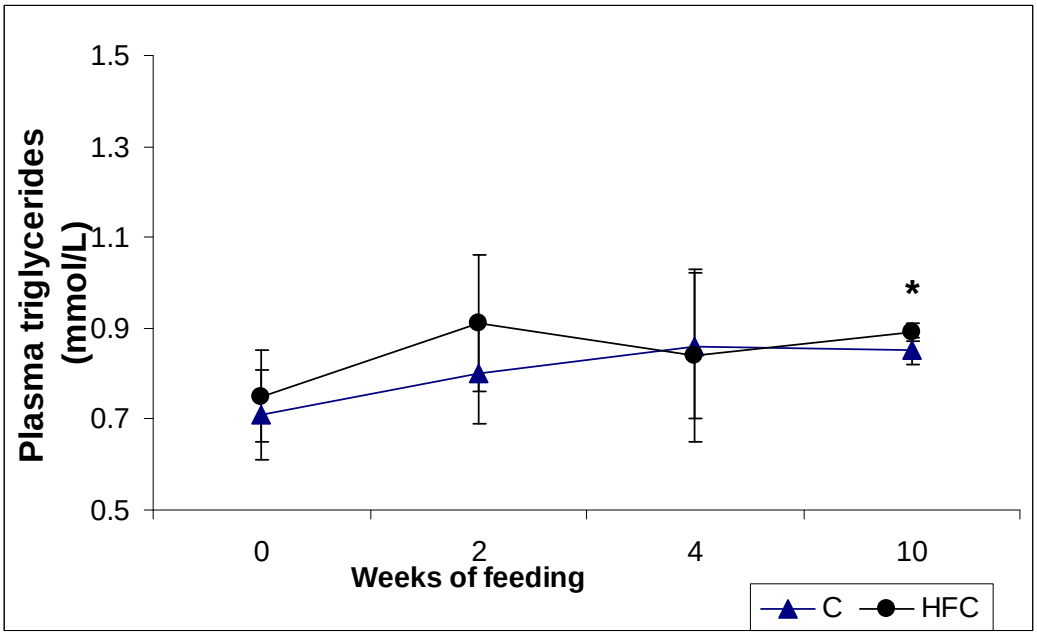


Plasma triglyceride levels

At the start of the feeding study, there were no significant differences in the plasma triglyceride levels amongst the different groups. We did not observe any significant increases in the plasma triglyceride levels in the HFC mice until week 10. The plasma triglyceride levels increased from 0.85 ± 0.03 mmol/L in C-10wk mice to 0.89 ± 0.02 mmol/L in HFC-10wk mice (Figure 4.10). The increased levels in HFC-2wk mice as compared to the levels in C-2wk mice were not significant (0.91 ± 0.15 mmol/L versus 0.80 ± 0.11 mmol/L). Plasma triglycerides levels appeared to be lower in the HFC-4wk mice as compared to their controls (not significant).

Figure 4.10 Plasma triglyceride levels of HFC and C mice

C: control diet groups; HFC: high fat, calorie-dense diet groups. Values are expressed as means ± SD (n= 8/group); * denotes P < 0.05 when compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the HFC or C groups.

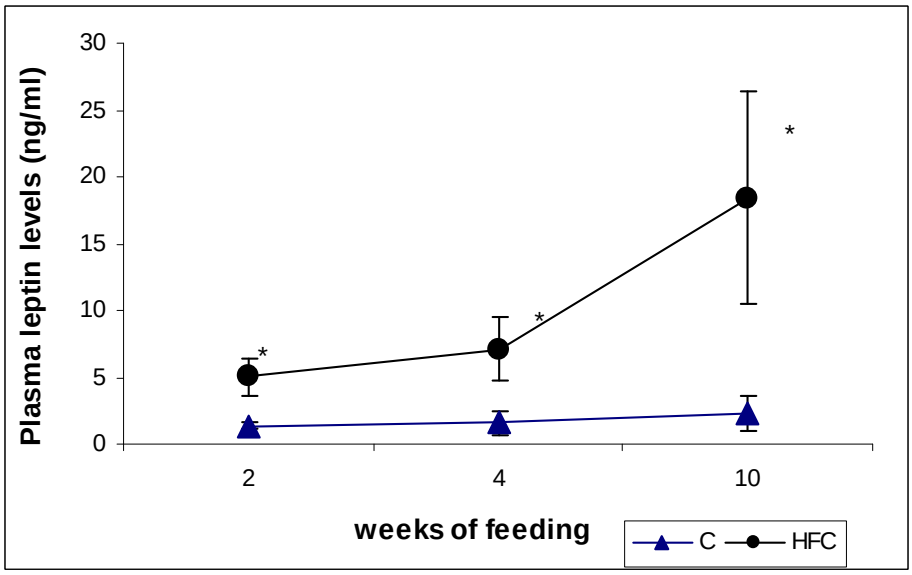


Plasma leptin levels

Plasma leptin levels were significantly raised within 2 weeks of HFC feeding (Figure 4.11). It increased from 1.35 ± 0.27ng/ml (C-2wk mice) to 5.03 ± 1.35ng/ml (HFC-2wk mice). The levels in the HFC-fed mice continued to increase throughout the entire feeding period, with significant increases in the HFC-4wk and HFC-10 wk mice as compared to their corresponding controls (7.1 ± 2.38 ng/ml versus 1.57 ± 0.97 ng/ml; 18.44 ± 7.89 ng/ml versus 2.32 ± 1.32 ng/ml respectively). Between the 4th to the 10th week of HFC feeding, we observed a sharp increase in plasma leptin levels by 159% ($P < 0.01$).

Figure 4.11 Plasma leptin levels of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) * denotes $P < 0.05$ when compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice.

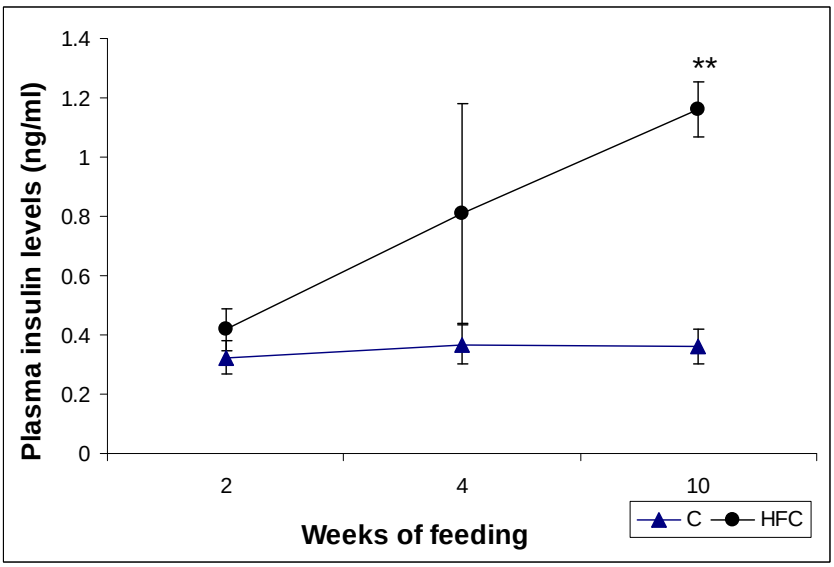


Plasma insulin levels

At week 2, there were no significant differences in the plasma insulin levels between the HFC mice and their controls (i.e. 0.42 ± 0.07 ng/ml in the HFC-2wk mice versus 0.32 ± 0.06 ng/ml in the C-2wk mice) (Figure 4.12). At week 4, there appeared to be an increase in plasma insulin levels in the HFC-4wk mice as compared to their controls (0.81 ± 0.37 ng/ml versus 0.37 ± 0.07 ng/ml), but this increase did not reach statistical significance. Subsequently, after 10 weeks of HFC feeding, we observed a significant 3-times increase in the plasma insulin levels in the HFC-10wk mice (1.16 ± 0.09 ng/ml) as compared to C-10wk (0.36 ± 0.06 ng/ml). There were significant differences in the plasma insulin levels amongst the HFC-fed groups ($P < 0.01$). HFC-10wk mice showed a 2.8-times and a 1.4-times increase as compared to HFC-2wk and HFC-4wk mice respectively ($P < 0.01$ between weeks 2 and 10).

Figure 4.12 Plasma insulin levels of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) ** denotes $P < 0.01$ compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice.

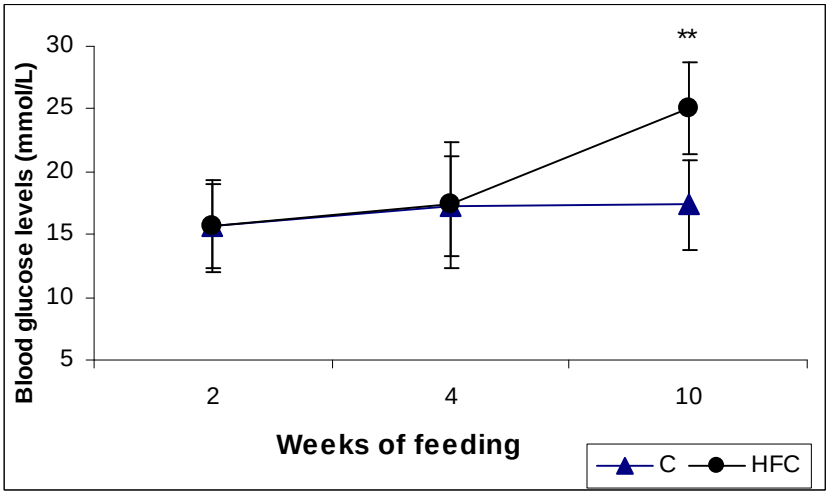


Blood glucose levels

Right up to 4 weeks of HFC feeding, there were no observable differences in blood glucose levels between the HFC and C groups. Both the HFC groups at weeks 2 and 4 had comparable blood glucose levels as compared to their controls (Figure 4.13). It was only at week 10 when we observed a significant sharp increase in blood glucose levels in the HFC mice. Their glucose levels increased by 44% from 17.38 ± 3.6 mmol/ L (in the C-10wk mice) to 25 ± 3.65 mmol/L. During the initial HFC feeding period, minimal increases were observed amongst the HFC mice. Mice fed on the HFC diet for 4 weeks had blood glucose levels (17.38 ± 5.03 mmol/L) as compared to mice fed on the HFC diet for 2 weeks (15.68 ± 3.63 mmol/ L). This increase was not statistically significant. However, with prolonged HFC feeding, there was a sharp increase in the HFC mice at week 10. The increase in the blood glucose levels observed in the HFC-10wk mice was significantly different from the HFC-2wk (increase by 59%, $p < 0.01$) and HFC-4wk mice (increase by 44%, $p < 0.01$).

Figure 4.13 Blood glucose levels of HFC and C mice

Results are presented as means ± SD for each group of mice (n = 8 / group) ** denotes $P < 0.01$ compared to corresponding C group. Triangles represent data from C mice. Circles represent data from HFC mice.



Correlation tests amongst plasma leptin, plasma insulin, white adipose tissue and body weight in all mice

Plasma insulin correlated positively and significantly with plasma leptin ($r = 0.81$, $P < 0.001$) (Figure 4.14). When correlation analysis was conducted without the outlying data-points, the correlation still remained strong and significant ($r = 0.81$, $P < 0.01$). Hence, this demonstrated that the strong and positive correlation between plasma insulin and plasma leptin was not driven by the outlying data point. All data-points were included in the graph (Figure 4.14) . There were significant positive correlation between plasma insulin, final body weight of the mice ($r = 0.84$, $P < 0.01$) (Figure 4.15) and amount of weight gained ($r = 0.693$, $P < 0.001$). There was a strong and significant correlation between plasma insulin levels with white adipose tissue mass ($r = 0.92$, $P < 0.01$) (Figure 4.16).

Plasma leptin also correlated significantly with final body weight of the mice ($r = 0.86$, $P < 0.001$) (Figure 4.17) and there was a strong correlation with white adipose tissue mass ($r = 0.93$, $P < 0.001$) (Figure 4.18). However, it showed a weaker but significant correlation with the amount of weight gained ($r = 0.48$, $P < 0.05$). The relation between plasma insulin and plasma leptin lost significance after controlling for body weight ($r = 0.32$, $P = 0.13$) or white adipose tissue ($r = -0.28$, $P = 0.2$).

Correlation tests amongst plasma leptin, plasma insulin and food intake in all mice

At week 2, we observed a significant inverse relationship between plasma leptin levels and food intake in the mice (i.e. as the plasma levels increased, the food intake in the mice reduced) ($r = -0.87$, $P < 0.05$). This inverse relation between food intake

and plasma leptin levels was not observed at week 4 and 10. There was also no significant correlation between plasma leptin levels and food intake at these 2 weeks. There was no significant correlation with plasma insulin and food intake till week 4 and week 10 (r = 0.89, P < 0.01, r = 0.93, P <0.01) respectively.

Correlation tests amongst plasma leptin, plasma insulin, white adipose tissue, body weight and food intake in C mice

Plasma insulin correlated significantly with plasma leptin (r = 0.59, P < 0.05), final body weight of the mice (r = 0.69, P < 0.05) and with white adipose tissue mass (r = 0.61, P < 0.05). Plasma insulin did not correlate with the amount of weight gained in the C mice. Plasma leptin also correlated significantly with final body weight of the mice (r = 0.86, P < 0.001), amount of weight gained (r = 0.69, p =0.01) and there was a strong correlation with white adipose tissue mass (r = 0.93, P < 0.001). Plasma insulin and plasma leptin did not correlate with each other after controlling for body weight. Both leptin and insulin levels did not correlate with food intake respectively.

Correlation tests amongst plasma leptin, plasma insulin, white adipose tissue, body weight and food intake in HFC mice

Plasma insulin correlated significantly with plasma leptin (r = 0.75, P < 0.01), final body weight of the mice (r = 0.84, P < 0.001), amount of weight gained (r = 0.84, P < 0.001) and showed even a stronger correlation with white adipose tissue mass (r = 0.92, P < 0.001). Plasma leptin correlated significantly with final body weight of the mice (r = 0.86, P < 0.001) but not with the amount of weight gained (r =0.46, P = 0.13). There was a strong correlation with white adipose tissue mass (r = 0.91, P < 0.001). Plasma insulin and plasma leptin did not correlate with each other after

controlling for body weight (r = 0.09, P =0.79) or white adipose tissue (r = -0.52, P = 0.10). Only plasma insulin levels but not plasma leptin levels that correlated significantly with food intake in the HFC mice (r = 0.93, P < 0.001).

Figure 4.14 Correlation between plasma insulin and plasma leptin levels in both HFC and C mice

Circles represent data from C mice. Triangles represent data from HFC mice.
(r = 0.84, P < 0.01)

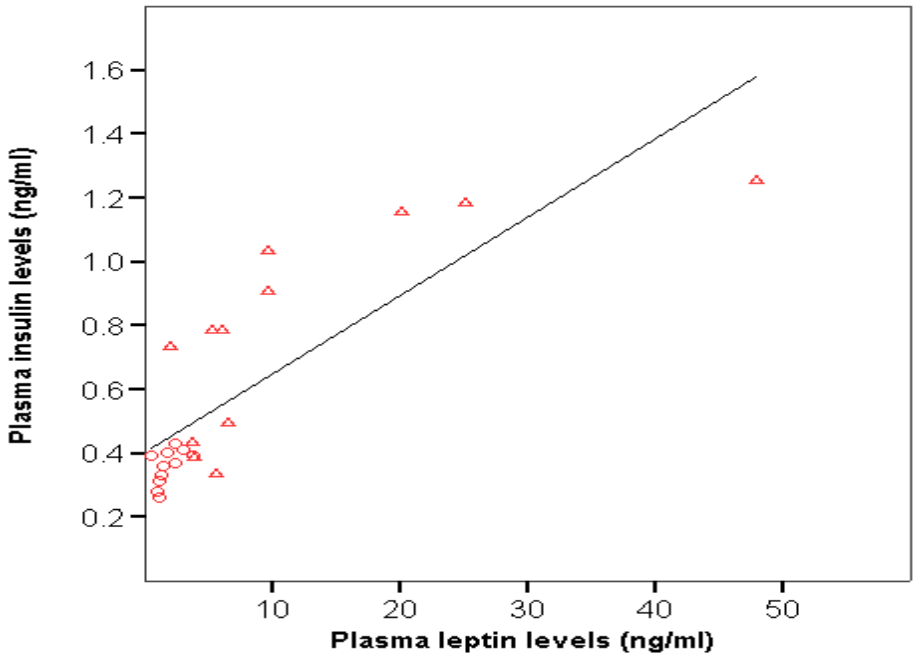


Figure 4.15 Correlation between plasma insulin and body weight in both HFC and C mice

Circles represent data from C mice. Triangles represent data from HFC mice. ($r = 0.80$, $P < 0.01$)

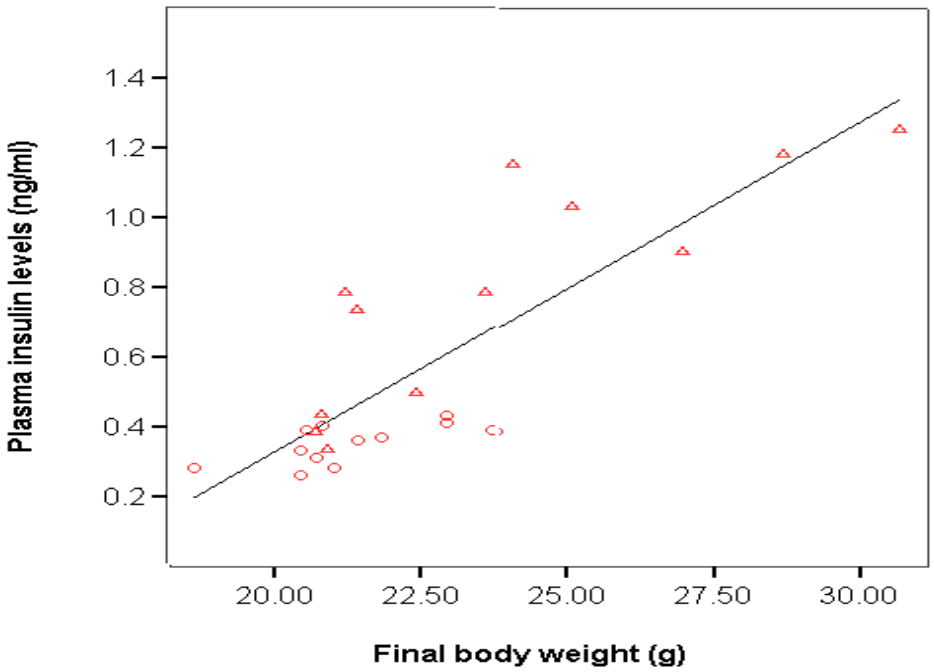


Figure 4.16 Correlation between plasma insulin and white adipose tissue mass in both HFC and C mice

Circles represent data from C mice. Triangles represent data from HFC mice. ($r = 0.85$, $P < 0.01$)

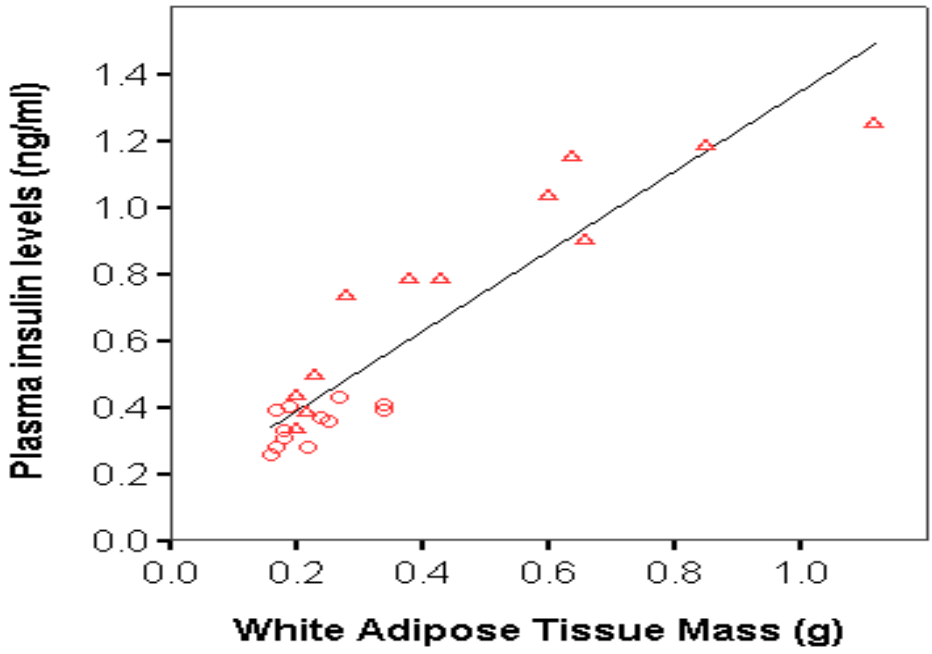


Figure 4.17 Correlation between plasma leptin and body weight in both HFC and C mice

Circles represent data from C mice. Triangles represent data from HFC mice.
($r = 0.79$, $P < 0.01$)

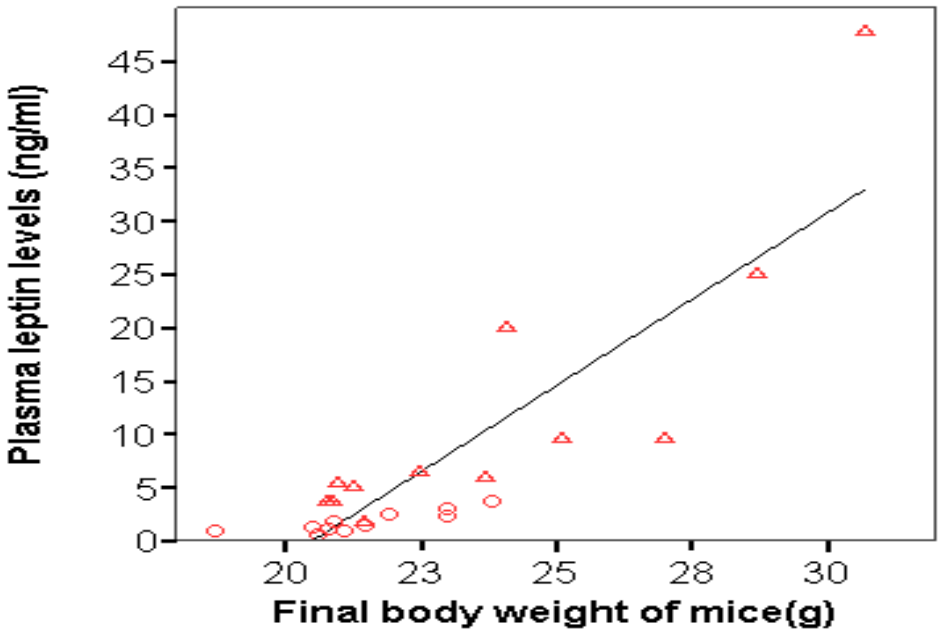
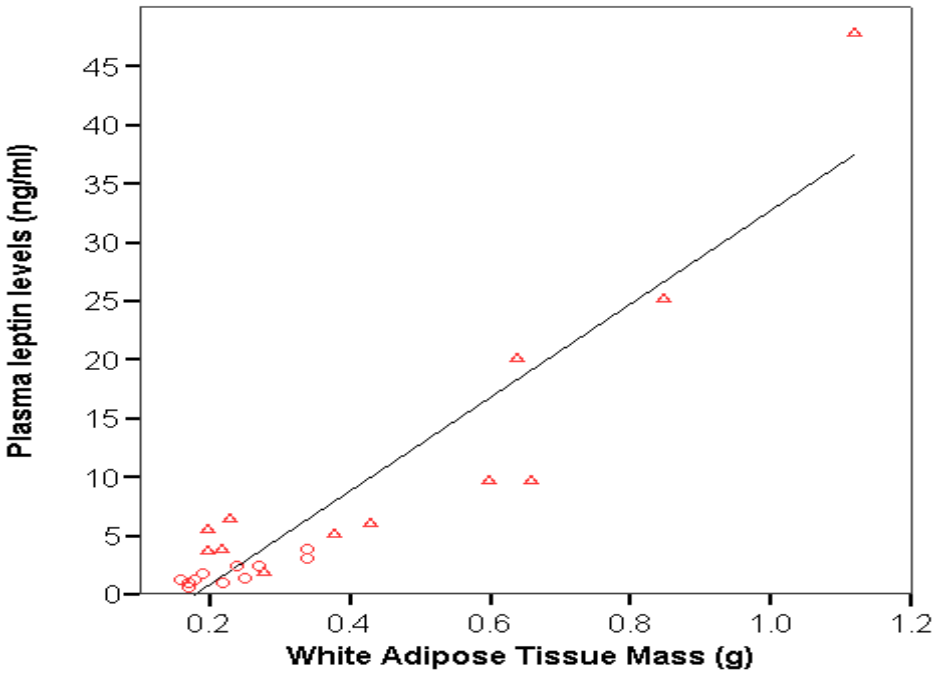


Figure 4.18 Correlation between plasma leptin and white adipose tissue mass in both HFC and C mice

Circles represent data from C mice. Triangles represent data from HFC mice.
($r = 0.81$, $P < 0.01$)



Microarray profiling of hepatic genes involved in lipid metabolism over the duration of high-fat, calorie dense (HFC) feeding

Under *in vivo* conditions, changes in differential gene expression due to dietary treatment are expected to be small and often below 2 fold change (Blanchard et al., 2001; Barella et al., 2004). As such, any gene expression changes from the microarray screening greater than a threshold of Signal Log Ratio $\geq$ 0.5 (i.e. 1.4-fold difference in levels) were considered to be potentially relevant. The differential gene expression levels were analysed according to the following pair-wise comparisons. For example, hepatic gene expression levels in mice fed on high-fat, calorie-dense diet for 2 weeks (HFC-2wk) were compared to the levels observed in the mice fed on control diet for 2 weeks (C-2wk). Similar comparisons were carried out for mice at weeks 4 and 10 (i.e. HFC-4wk versus C-4wk and HFC-10wk versus C-10wk).

Hepatic genes involved in lipogenesis

Figure 4.19 shows the differential expression of the hepatic genes involved in lipid metabolism. Genes encoding enzymes involved in lipogenesis, such as Acly**,** Acsm3, Gpam, Mod1, Scd1 were down-regulated after 2 weeks of high-fat feeding (Figure 4.19A). At week 4, these genes were either up-regulated or showed no change. These genes were up-regulated at week 10. Although the expression of genes such as Fas, Srebf1, Dgat2, Fads1 and Fads2 showed no change at week 2, there was a trend of being down-regulated. 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 Srebf1) were up-regulated and showed a further increase in mRNA expression at week 10. At week 10, Acacb, Acsl5, Scd2, Acss2 Dgat2, Fads1 and Fads2 were also up-regulated.

Hepatic genes involved in fatty acid oxidation

At week 2, Cpt1L was up-regulated by 1.6 fold (Figure 4.19B). Other key genes involved in fatty acid oxidation (FAO), Cpt1L, Pparα, Crat and Ehhadh were elevated at week 4. This suggested that both mitochondrial and peroxisomal fat oxidation were activated. At week 10, Pparα , Ehhadh, Hadhsc and Cpt2 were down-regulated and Cpt1L and Crat were no longer up-regulated.

Hepatic genes involved in cholesterol metabolism

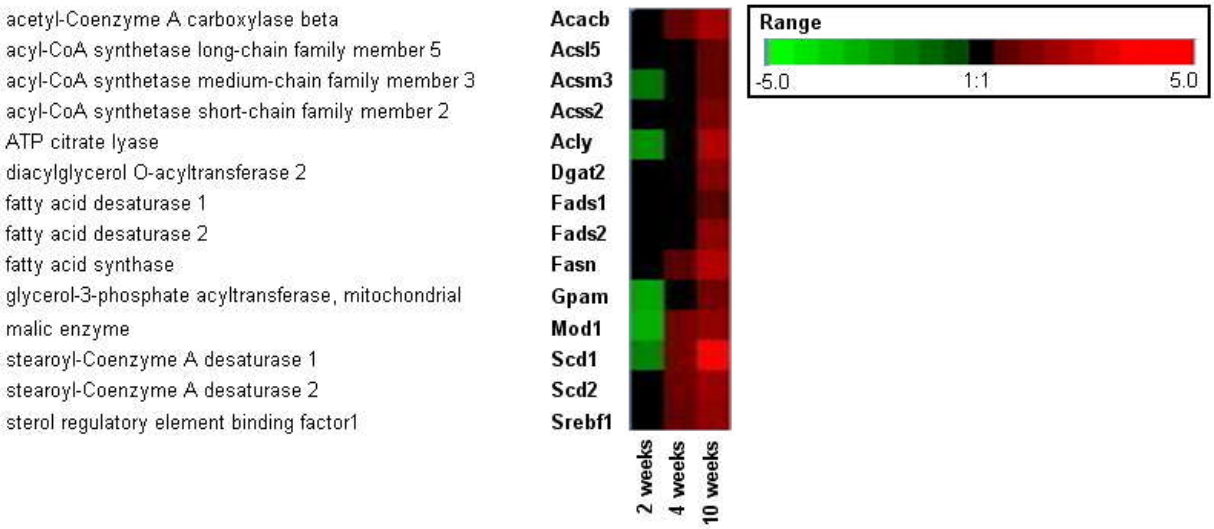
Analysis of the expression levels of the genes involved in the cholesterol synthesis pathway revealed that the expression of key genes in this pathway, such as Hmgcs1, Cyp51, Cyb5r3, Fdps, Idi1 and Sc4mol were decreased at week 2 (Figure 4.19C). These genes continued to be suppressed and showed a greater suppression in their expression levels at week 4. At week 4, additional genes involved in cholesterol synthesis were down-regulated. These included Hmgcr, the gene which encodes the enzyme catalyzing a rate-limiting step in cholesterol biosynthesis. Other genes were Dhcr7, Dhcr24, Fdft1, Lss, Mvd, Mvk, Nsdhl, Pmvk, Sqle and Tm7sf2. Expression of Srebf2, the transcription factor responsible for activation of the cholesterol synthesis pathway, was also decreased. At week 10, the downward trend in the expression of these genes was not maintained. Some genes were up-regulated instead (e.g. Hmgcr, Sc4mol, Sc5d, Sqle, Cyb5r3, Fdft1 and Fdps).

Cyp7a1, the gene encoding the enzyme regulating the rate-limiting step of bile acid synthesis were down-regulated at all 3 time-points.

Figure 4.19 Hepatic genes involved in lipid metabolism regulated by high-fat, calorie dense diet at weeks 2, 4 and 10.

The colour-coded scale (green=down-regulation and red=up-regulation). Data of "fold change" were calculated from the "Signal Log Ratio". The scale is presented as fold change.

(A) Hepatic genes involved in lipogenesis



(B) Hepatic genes involved in fatty acid oxidation

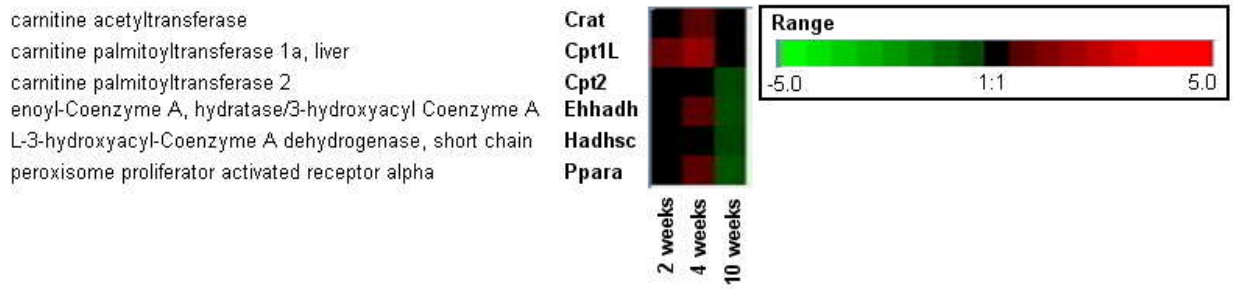
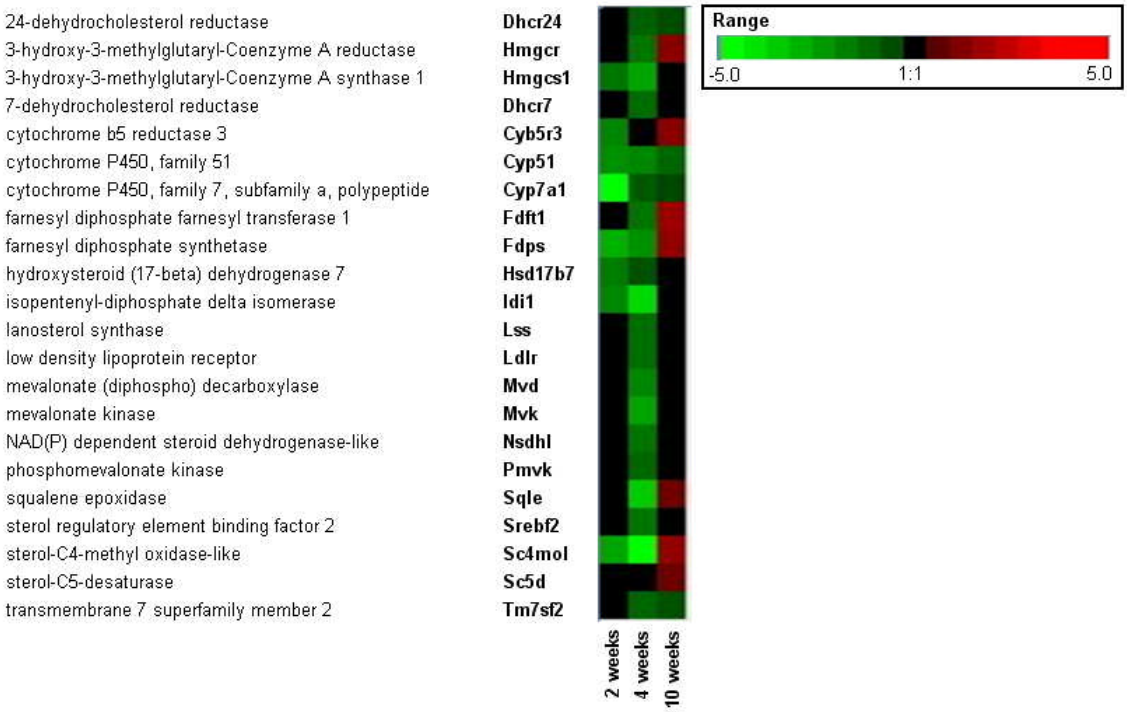


Figure 4.19 (continued) Hepatic genes involved in lipid metabolism regulated by high-fat, calorie dense diet at weeks 2, 4 and 10..

(C) Hepatic genes involved in cholesterol metabolism



Expression levels of hepatic genes involved in lipid metabolism measured by quantitative RT-PCR

From the screening with microarrays, we selected one key gene each from lipogenesis (Fas), fatty acid oxidation (Cpt1L), cholesterol metabolism (Hmgcr) and bile acid synthesis (Cyp7a1) to conduct further qRT-PCR tests to validate their differential expression (Figure 4.20). These genes were selected because they regulate the key steps of fatty acid synthesis, fatty acid oxidation, cholesterol synthesis and bile acid synthesis respectively. Melting curve analysis was performed to ensure the specificity of the amplified PCR products. The individual melting curves are presented in Appendix 4.2. The gel electrophoresis of the amplified PCR products are found in Appendix 4.3.

HFC feeding resulted in a significant increase in gene expression of Cpt1L at week 2 and week 4 ($p<0.05$) (Figure 4.20). However, there was a downward trend at week 10 although this was not statistically significant.

At both weeks 2 and 4, there were no significant differences in the Fas mRNA levels between the HFC and the C mice (Figure 4.20). At week 2, there appeared to be a downward trend in the Fas mRNA levels in HFC mice but this was not significantly different from the controls. At week 4, the downward trend observed at week 2 appeared to be reversed with increased levels of Fas mRNA levels in the HFC-4wk mice as compared to the C-4wk mice (non-significant). At week 10, we observed a 57% significant increase ($p<0.05$) in the expression levels of Fas in the HFC mice as compared to the C mice.

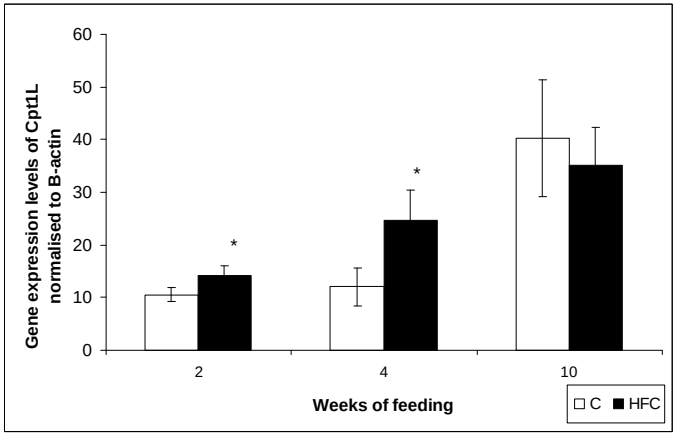
There were no significant changes in the Hmgcr expression levels between the HFC and C mice at week 2 (Figure 4.20). HFC feeding for 4 weeks resulted in a highly significant reduction in gene expression level of Hmgcr ($p<0.01$). This was reversed at week 10. There was a significant increase in the expression levels of Hmgcr in the HFC-10wk mice as compared to their controls ($p<0.01$).

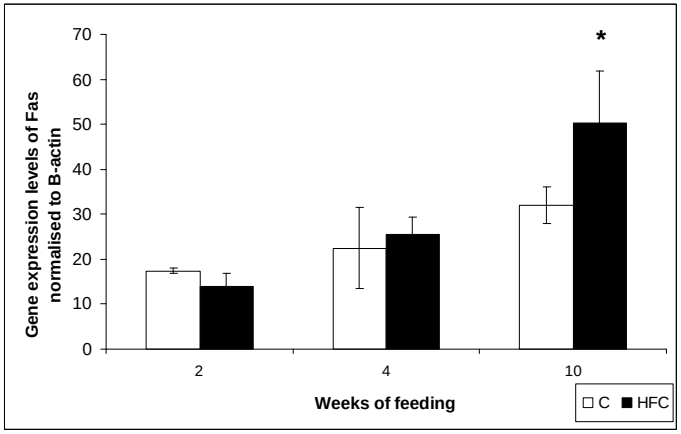
The gene expression levels of Cyp7a1 were significantly down-regulated in the HFC groups when compared to the C groups at weeks 2, 4 and 10. The levels were reduced by 6.7 fold, 5.4 fold and 3.2 fold respectively (Figure 4.20). The qRT-PCR results showed that the levels of mRNA for the selected genes followed the same pattern of expression as those observed with the microarray experiment.

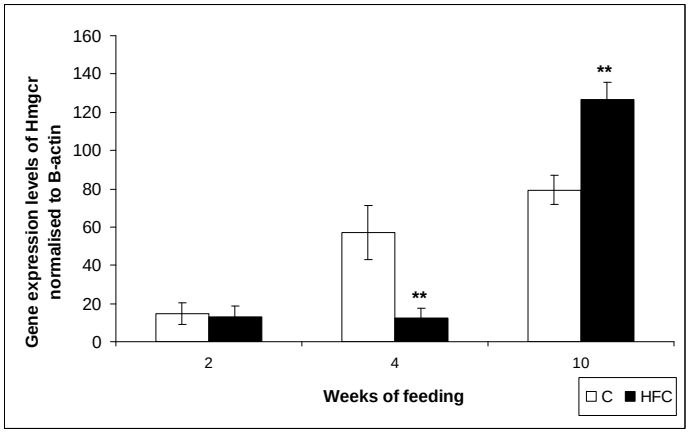
Correlation coefficient between microarray & qRT-PCR data

To validate the microarray date, qRT-PCR was carried out using individual mouse samples. The qRT-PCR results were compared to the micorarray results (Figure 4.21). The correlation coefficient was 0.93 ($P < 0.01$), confirming the reliablitiy of the microarray data.

Figure 4.20 Gene expression levels of Cpt1L, Fas, Hmgcr and Cyp7a1 measured by quantitative RT-PCR in liver tissues from HFC and C mice. Results are presented as mean ± SD (n = 8/group) * denotes P < 0.05 and ** denotes P < 0.01 compared to corresponding C group.







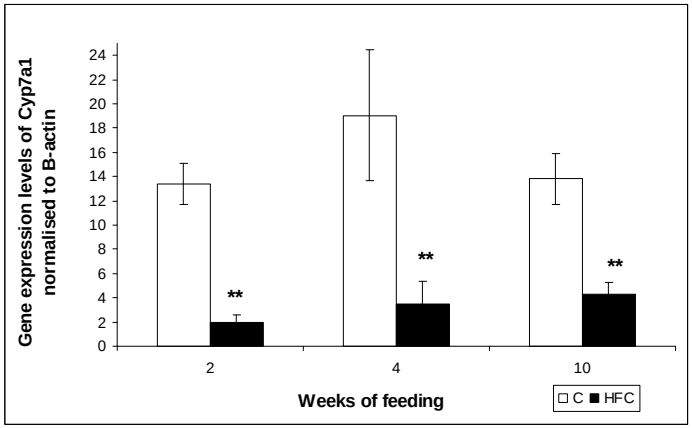
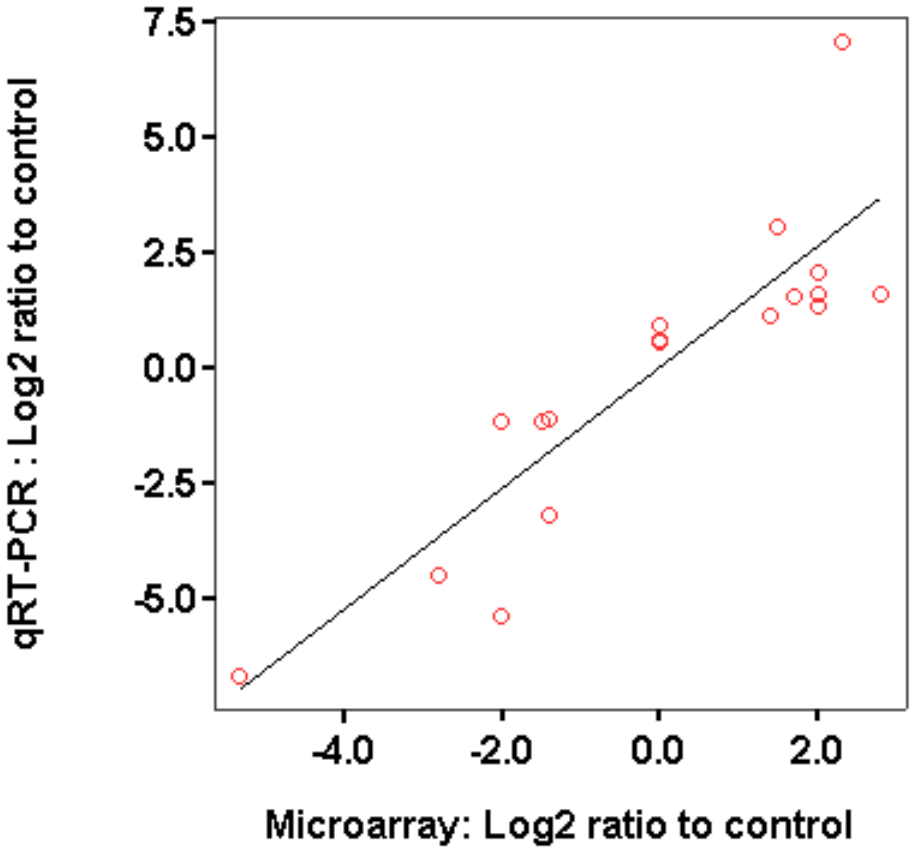


Figure 4.21 Correlation between microarray & qRT-PCR data (r = 0.93, p < 0.01.



Western blot analysis of Fas, Cpt1L and Hmgcr in the livers from C mice and HFC mice

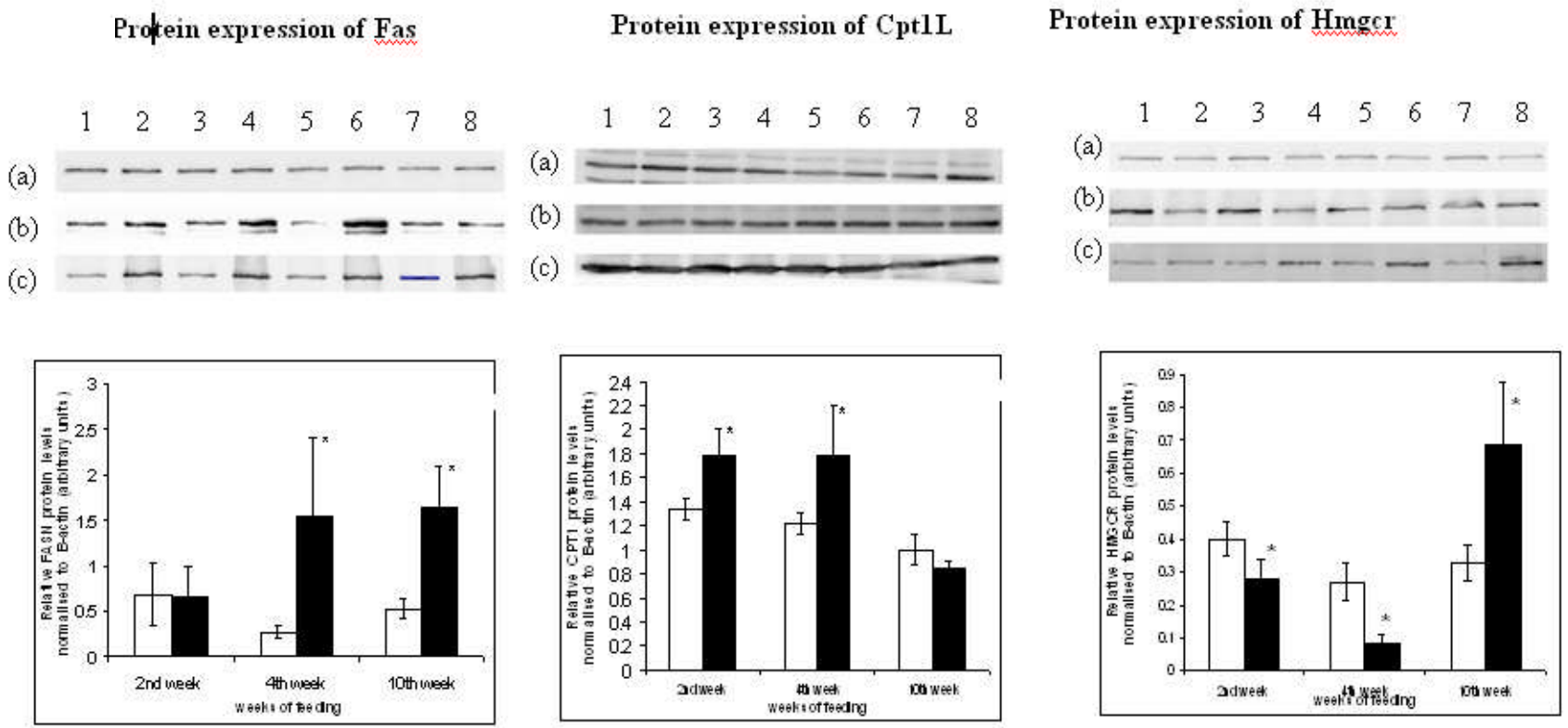
We subsequently verified the differential expression of Fas, Cpt1L and Hmgcr at the protein level by western blot analysis (Figure 4.22). The individual gel electrophoresis showing the various protein bands (run together with the protein markers) are found in Appendix 4.5.

There was no significant difference in the protein levels of Fas between the HFC and C mice at week 2. At weeks 4 and 10, we observed significant increases in the protein levels of Fas in the HFC versus their corresponding controls (Figure 4.22).

There was a significant increase in the protein expression of Cpt1L in the HFC-2wk mice versus C-2wk mice. This significant increase in Cpt1L was also observed in HFC-4wk mice versus their controls. At week 10, there appeared to be a downward trend in the Cpt1L protein levels in HFC-10wk mice as compared to their controls but this was not significant (Figure 4.22).

At weeks 2 and 4, there were significant reductions in Hmgcr protein expression levels in HFC mice as compared to their controls. However, 10 weeks of HFC feeding appeared to induce significant increase in the Hmgcr protein expression levels as compared to the C mice (Figure 4.22).

Figure 4.22 Top Panels : Western blot analysis of Fas, Cpt1L and Hmgcr in the livers from C mice (odd-numbered lanes) and HFC mice (even-numbered lanes). 2, 4 and 10 weeks are arranged in rows (a), (b) and (c) respectively. **Bottom Panels : Densitometry analysis of protein bands normalized to β-actin bands in C compared to the HFC mice.** Each bar represents means ± SD for each group of mice (n = 4/ group) *: P < 0.05 as compared to control group. Open bars represent data from C mice and filled bars represent data from HFC mice.



Microarray profiling of white adipose tissue genes involved in lipid metabolism over the duration of high-fat, calorie dense (HFC) feeding

White adipose tissue genes involved in lipogenesis

In contrast to what we observed in the hepatic gene profile, we observed that the genes encoding enzymes involved in lipogenesis were either down-regulated or remained unchanged throughout the 10 weeks of HFC feeding (Figure 4.23A and Figure 4.24). The gene encoding Acetyl-coenzyme A carboxylase, the rate-limiting enzyme in fatty acid synthesis, was down-regulated within 2 weeks of HFC feeding. It was further suppressed as the feeding period continued and its expression was down-regulated by 2.6-fold by week 10. Other key genes which also play a pivotal role in fatty acid synthesis (e.g. Acly and Acsm3) showed a progressive downward regulation over the entire feeding period. At week 10, more genes involved in lipogenesis were suppressed (e.g. Dgat1, Mod1, Pecr and Pcx). Although the expression of key lipogenic genes such as Fas, Srebf1, Dgat2, Scd1, Gpam showed no significant changes throughout, they had a tendency towards being down-regulated. However, as the difference in absolute mRNA levels did not reach statistical significance, they were not reported as a robust decrease.

White adipose tissue genes involved in fatty acid oxidation

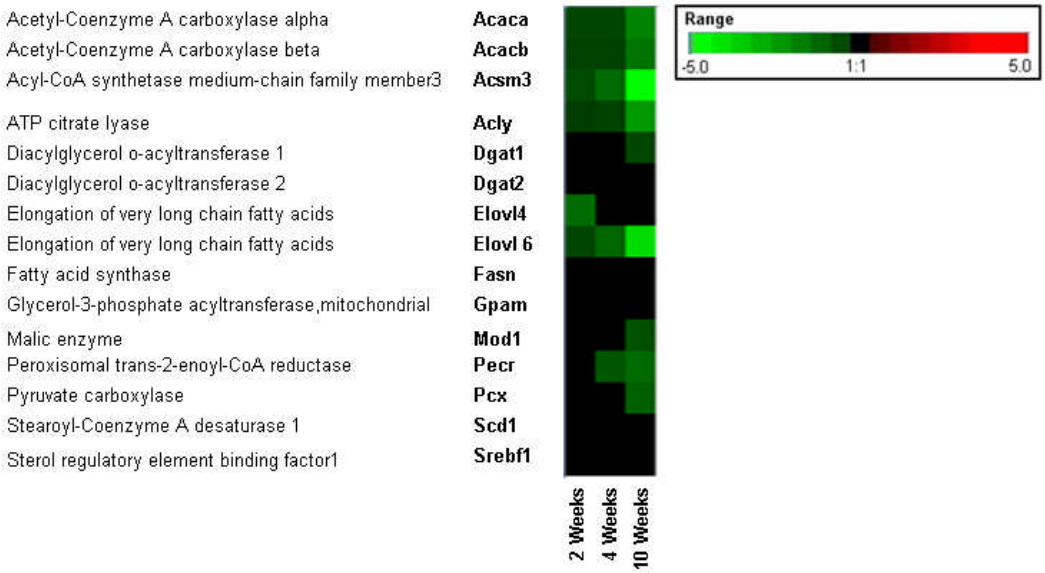
Many of the genes involved in fatty acid oxidation (FAO) did not show any significant changes in their expression throughout (Figure 4.23B). This included the gene Lipe, encoding hormone-sensitive lipase, which catalyses the rate-limiting step in adipose tissue lipolysis. Initially at week 2, Cpt1L and Cpt2 were up-regulated. Cpt1L continued to be up-regulated at week 4. The expression levels of Cpt1L increased by 2-fold in the HFC-2wk mice versus the levels in C-2wk mice. Both

Cpt1L and Cpt2 were not differentially regulated at week 10. Acadm, Acox1, Crat, Hadha and Pecr were all down-regulated at week 10.

Figure 4.23 White adipose tissue genes involved in lipid metabolism regulated by high-fat, calorie dense diet at weeks 2, 4 and 10.

Data of "fold change" were calculated from the "Signal Log Ratio". The scale is presented as fold change.

(A) White adipose tissue genes involved in lipogenesis



(B) White adipose tissue genes involved in fatty acid oxidation

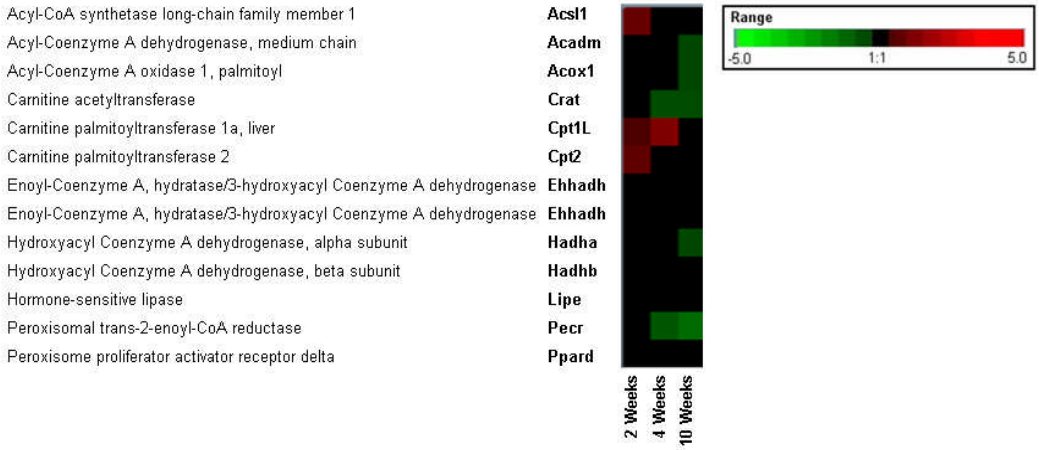
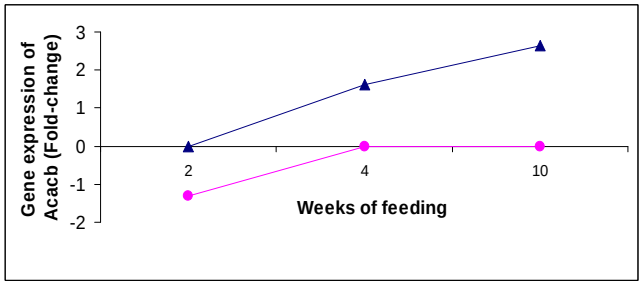
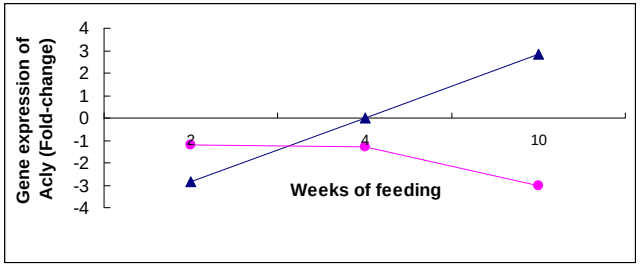
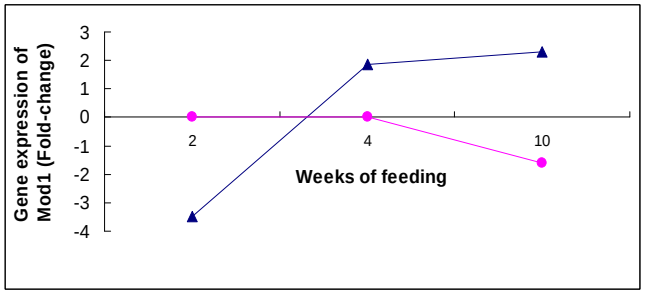
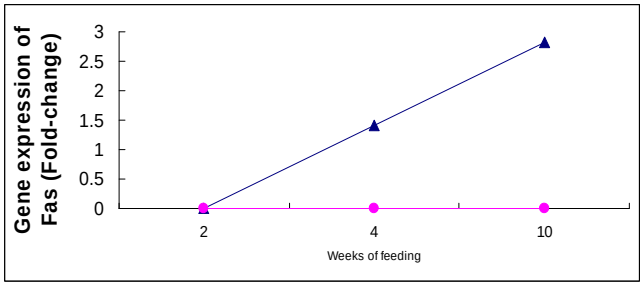


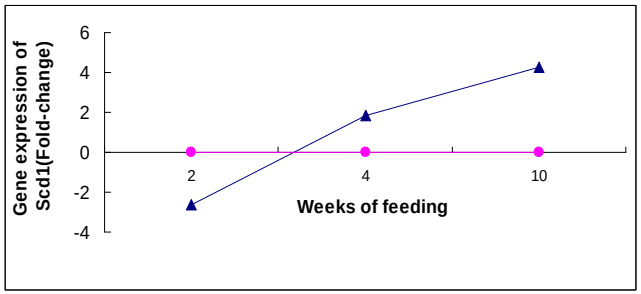
Figure 4.24 Comparison of lipogenic genes expression levels in liver tissue versus white adipose tissue from microarray data
Triangles represent data from liver samples. Circles represent data from white adipose tissue.

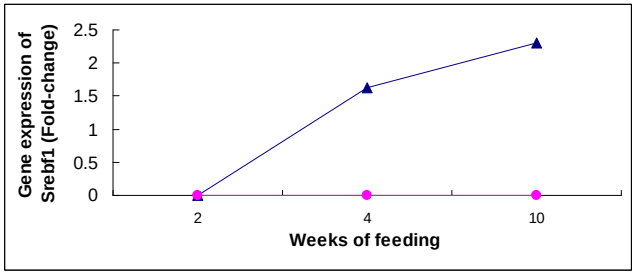












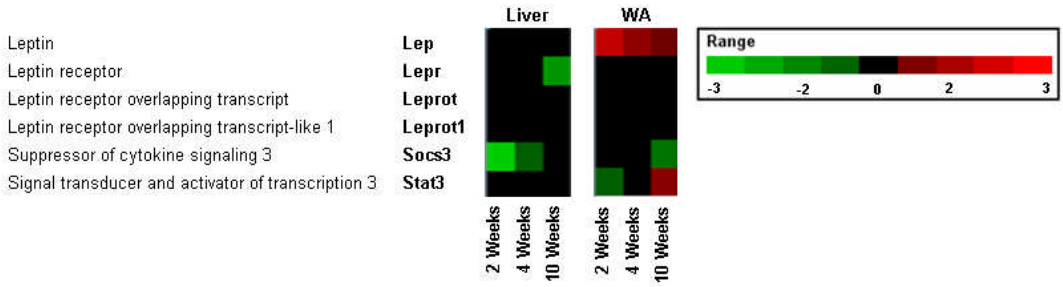
White adipose tissue genes involved in leptin regulation

In agreement with the increased plasma leptin levels observed in the HFC-2wk mice, we observed that the gene expression levels of Lep in white adipose tissue were up-regulated by 2.3 fold (Figure 4.25). In contrast to the progressive increase in plasma leptin levels, we observed that although all HFC-groups had higher expression levels of Lep gene than their controls, the degree of up-regulation did not increase progressively over the entire HFC feeding period. There was a tendency for Lepr to be down-regulated at all time-points in the HFC groups but this was not reported as a robust decrease. Socs3 was not differentially regulated till week 10 when it showed a 1.7-fold decrease in the HFC group. Stat3 was down-regulated at week 2 but not at week 4. Subsequently, it was up-regulated at week 10.

Hepatic genes involved in leptin regulation

Lepr was not differentially regulated until week 10 when we observed a 2.2-fold reduction in its expression in the HFC mice as compared to the C mice (Figure 4.25). Socs3 was down-regulated by 3-fold and 1.4-fold in the HFC groups at both weeks 2 and 4 but this reduced expression was no longer observed at week 10.There was no difference in the expression levels in Stat3 at all 3 time-points.

Figure 4.25 Genes involved in leptin regulation in liver and white adipose tissue regulated by high-fat, calorie dense diet at weeks 2, 4 and 10.
Data of "fold change" were calculated from the "Signal Log Ratio". The scale is presented as fold change.



Gene expression levels of Cpt1L, Fas, Lep and Lepr in white adipose tissue and hepatic Lepr measured by quantitative RT-PCR

From the transcription profiling of the white adipose tissue with microarrays, we selected one key gene each from lipogenesis (Fas), fatty acid oxidation (Cpt1L), leptin synthesis (Lep) and leptin regulation (Lepr) to conduct further qRT-PCR tests to validate their differential expression.

HFC feeding resulted in significant reduction in gene expression levels of Fas at all 3 time-points (Figure 4.26). At week 10, the reduction in the gene expression levels of Fas in HFC-10wk mice versus the levels observed in the C-10wk mice was the largest (47% reduction) as compared to the reductions in the other 2 time-points.

In relation to Cpt1L, we observed an up-wards trend in the HFC mice at weeks 2 and 4 ($p < 0.01$ at week 4). At week 10, there were no significant differences between HFC and C mice (Figure 4.26).

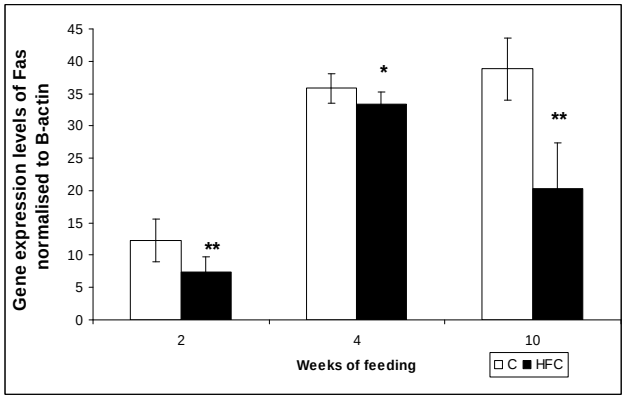
The Lep mRNA levels were significantly up-regulated in all the 3 HFC groups as compared to C groups. Within 2 weeks of HFC feeding, the expression of Lep mRNA levels went up by 7-fold (Figure 4.26).

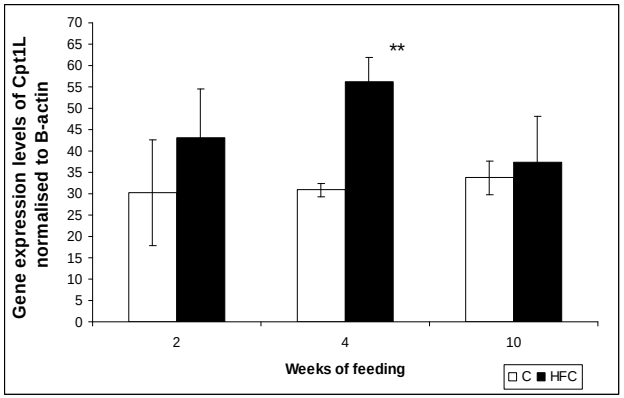
There were no significant differences in the mRNA levels of Lepr in the HFC mice versus the C mice at all 3 time-points (Figure 4.26).

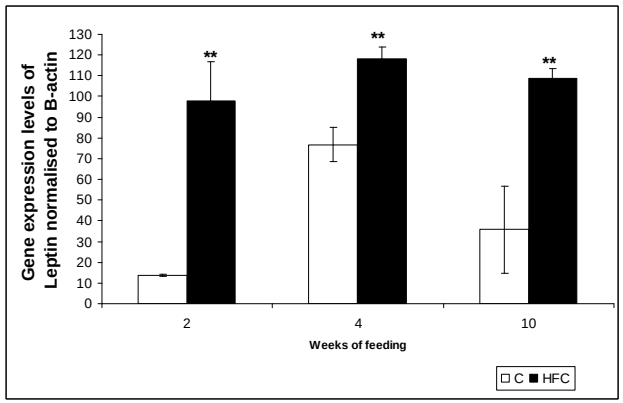
We examined the Lepr mRNA levels in the mice livers. We found that the Lepr was initially up-regulated in the HFC mice versus the C mice at both weeks 2 and 4 but the increase was only significant at week 2. At week 10, this upwards trend reversed and we observed a decreased expression of mRNA levels of hepatic Lepr in HFC-10wk mice vs C-10wk mice (Figure 4.27).

Figure 4.26 Gene expression levels of Cpt1L, Fas, Lep, Lepr measured by quantitative RT-PCR in white adipose tissue from HFC and C mice.

Results are presented as mean ± SD (n = 8 /group) * denotes P < 0.05 and ** denotes P < 0.01 compared to corresponding C group.







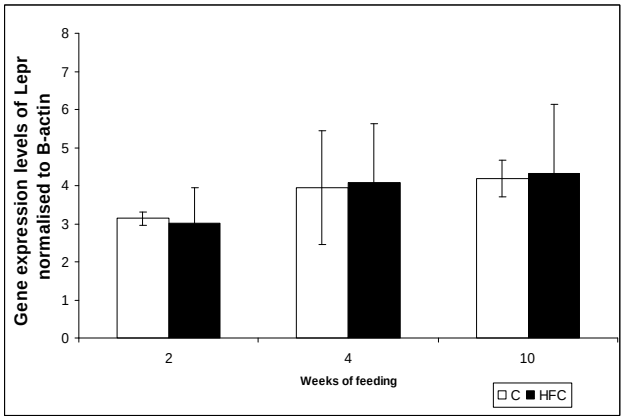
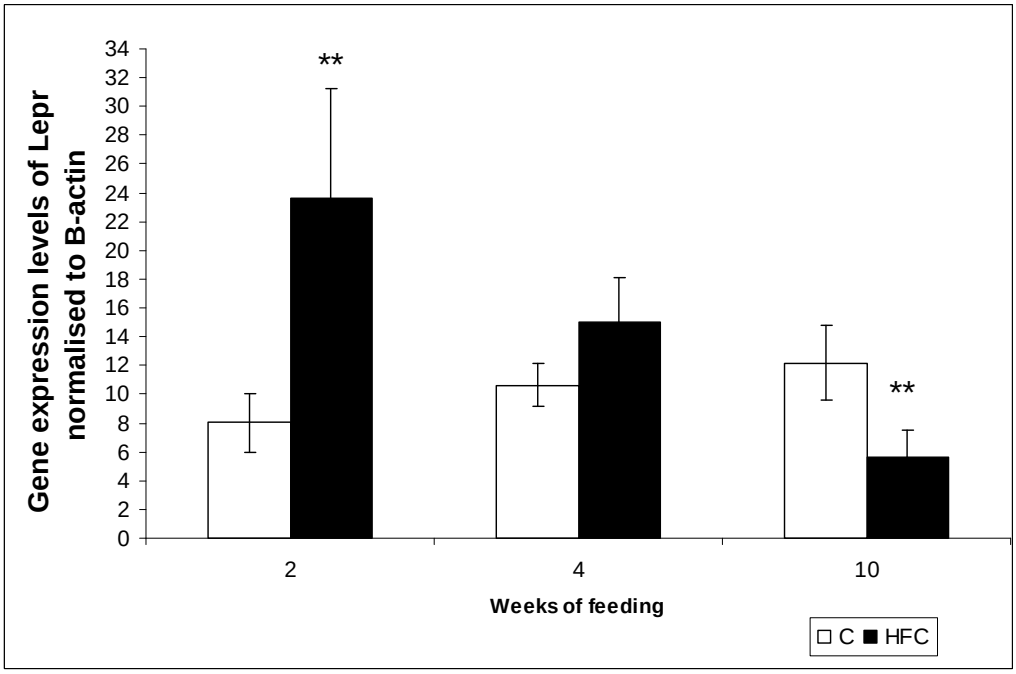


Figure 4.27 Gene expression levels of Lepr measured by quantitative RT-PCR in liver tissue from HFC and C mice.

Results are presented as mean ± SD (n = 8 /group) ** denotes P < 0.01 compared to corresponding C group.



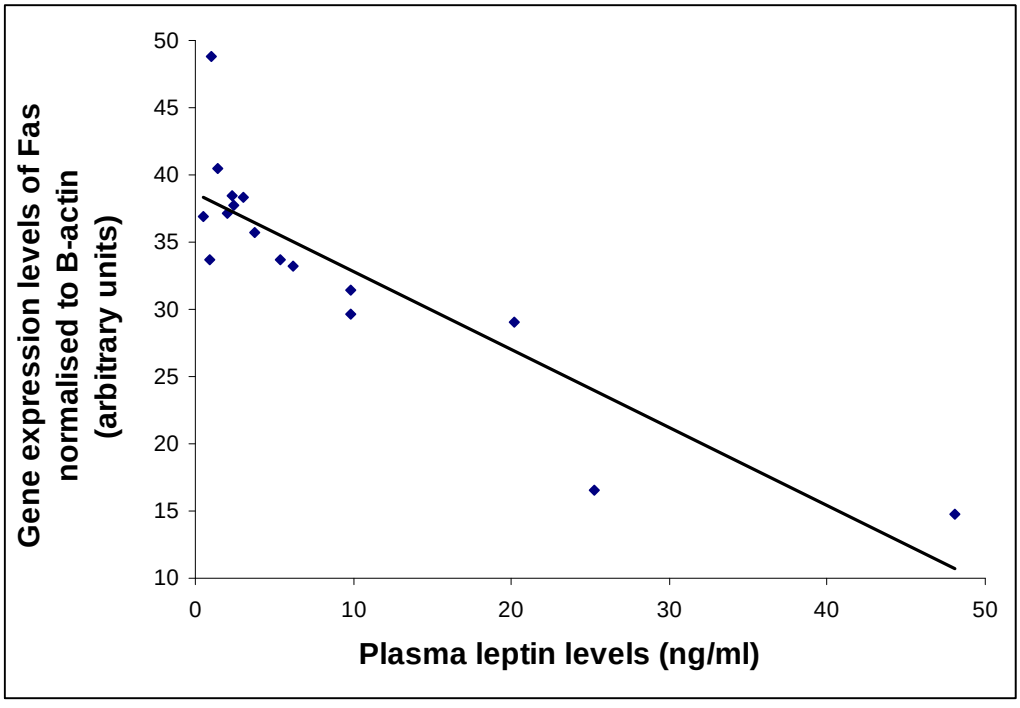
Correlation tests between expression levels of genes in white adipose tissue with plasma leptin or plasma insulin levels

We examined the relation between the gene expression levels of Fas in white adipose tissue and plasma leptin or plasma insulin levels. We found a strong and significant inverse relation between mRNA levels of Fas in white adipose tissue and the plasma leptin levels (r = -0.81, P < 0.001) of the mice at weeks 4 and 10 (Figure 4.28).

The minimum correlation coefficient, r that is significant for a sample size increases with smaller sample size. In the example of Figure 4.28, r has to be at least -0.74 in order to achieve significance at p = 0.001. If the number of mice were increased to 48, the smallest r significant at p=0.001 is -0.46.

There was no significant relation between mRNA levels of Fas in white adipose tissue and the plasma insulin levels (r = 0.08, P = 0.97).

Figure 4.28 Correlation between Fas mRNA levels in white adipose tissue and plasma leptin levels in mice at weeks 4 and 10 (r = -0.81, P < 0.001)



Correlation tests between expression levels of hepatic genes with plasma leptin or plasma insulin levels

We found a weak positive relation between mRNA levels of hepatic Fas and plasma leptin levels (r = 0.52, P < 0.01). On the other hand, we found a strong, positive correlation between the mRNA levels of hepatic Fas and plasma insulin levels (r = 0.70, P < 0.01) (Figure 4.29). We also found that the mRNA levels of hepatic Cpt1L correlated positively and significantly with the plasma leptin levels (r = 0.80, P < 0.001) (Figure 4.30). For both Figures 4.29 and 4.30, when correlation analysis was conducted without the outlying data-points, the correlation still remained positive and and significant.

Figure 4.29 Correlation between hepatic Fas mRNA levels and plasma insulin levels (r =0.70, P < 0.01)

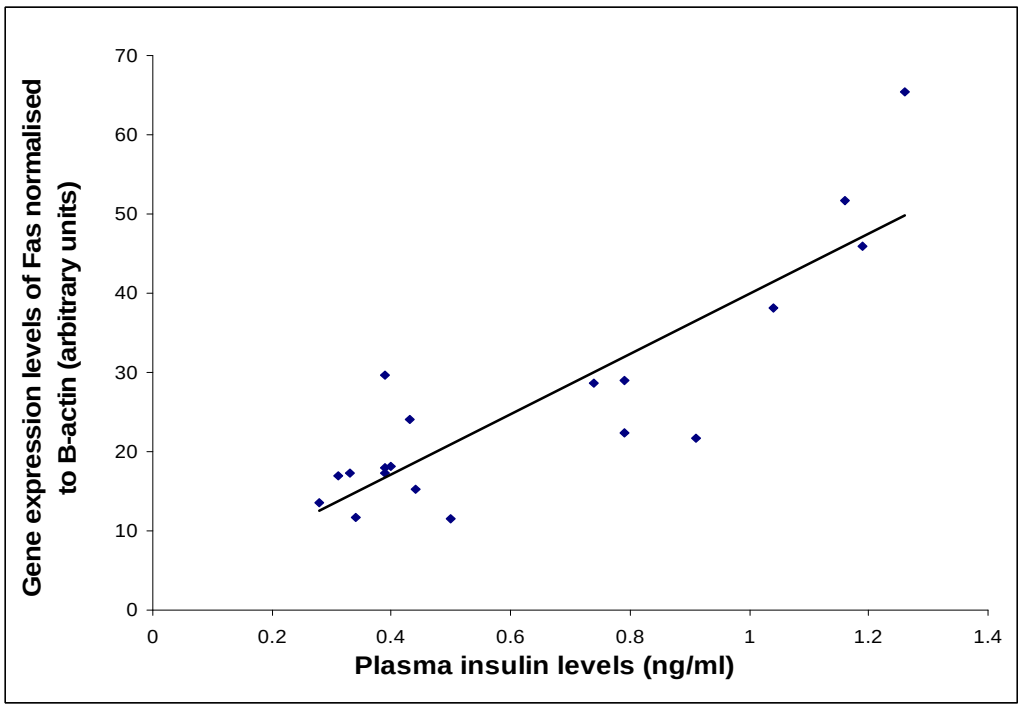
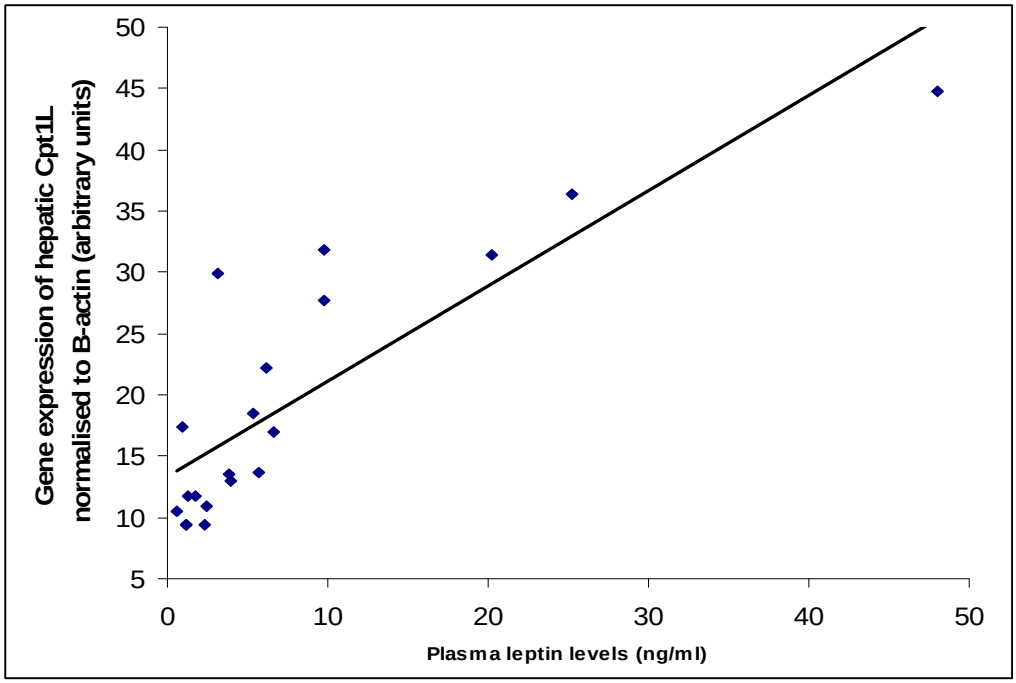


Figure 4.30 Correlation between hepatic Cpt1L mRNA levels and plasma leptin levels (r = 0.80, P < 0.001)



DISCUSSION

Sequential effects of high-fat, calorie-dense (HFC) feeding on food intake, body weight and white adipose tissue mass in the mice

At week 2, in an initial response to the high-fat, calorie dense feeding, it appeared that the mice showed a slight reduction in their food intake. This could possibly help to prevent weight gain and deposition of white adipose tissue mass in them. However, this adjustment was not sustained. As the high-fat feeding period continued, there was an increase in food intake associated with a marked increase in weight gain and greater deposition of white adipose tissue. The increased food intake exhibited by the HFC-4wk mice persisted in the HFC-10wk mice.

The changes in body weight and white adipose tissue mass were related to the changes observed in food intake. There was no observable weight gain in the mice fed on the HFC diet for 2 weeks versus their controls whereas the prolonged ingestion of the HFC diet resulted in 1.4 and 2.5 times weight gain in the HFC-4wk and HFC-10wk mice as compared to their respective controls. Similarly, it was only after 4 weeks of HFC feeding that we observed a significant deposition of white adipose tissue mass in the mice.

Despite a lower food intake in the HFC-2wk mice, we 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. In fact, there appeared a tendency for slight weight loss in all the HFC groups after 1 week of HFC feeding as compared to their respective C groups. An example

was shown in the weekly weight monitoring of HFC-10wk mice as shown in Figure 4.6. This weight loss was regained by week 2.

As the amount of food ingested was significantly different amongst the various groups, we adjusted the weight gained for the amount of food intake consumed by examining the feed efficiency ratio. We observed a reduction in the feed efficiency ratio in the mice fed on the HFC diet for 2 weeks as compared to their controls (non-significant). Ryu et al (2005) had shown that high-fat feeding for 12 weeks increased feed efficiency ratio in rats. This discrepant finding between our observations at week 2 and the reported findings by Ryu et al (2005) could be due to the difference in feeding length. Although the mice in our study fed on the HFC diet for 2 weeks did not show an increase in their feed efficiency ratio, we found a similar trend in the feed efficiency ratios in the prolonged HFC groups (i.e. HFC groups at week 4 and 10). This trend was similar to what Ryu et al (2005) had observed. The feed efficiency ratio of the HFC-10wk mice was significantly higher than their controls. The ratio increased by 72% as compared to the control mice.

The higher energy density of the HFC diet as compared to the C diet might have induced passive over-consumption of energy, which would lead to further weight gain. Since the energy densities of the HFC and C diets differed, we examined the energy efficiency ratios as well. Similar to the trend observed in feed efficiency ratios, the mice fed on the HFC diet for 2 weeks appeared to have lower energy efficiency ratios than their controls. At weeks 4 and 10, the energy efficiency ratios in the HFC mice were higher than their respective controls (non-significant). The energy

efficiency ratios increased progressively over the entire HFC feeding period, with a significant almost 2-times increase for HFC-10wk mice versus the HFC-2wk mice.

In all, these observations in the feed efficiency and energy efficiency ratios suggested that the propensity of the HFC diet to induce weight gain might be over and above the effects of just passive over-consumption of energy from the higher calories in the HFC diet. It was likely that the post-ingestive consequences of the HFC diet could play a bigger role than the sensory properties (i.e. palatability or olfactory characteristics) of a HFC diet in inducing increased food intake, resulting in further weight gain. This seemed to agree with Warwick's suggestion that a high-fat diet having equal or even lower palatability or sensory properties than a low-fat diet can still induce greater food intake (Warwick, 1996).

Therefore, our observations could support our postulation that the consumption of a diet high in fat and calories did initially elicit compensatory feeding response but such adaptations diminished with the continued intake of high-fat, calorie-dense diet , resulting in overeating and weight gain.

Moreover, our observations which demonstrated that HFC diets could also elicit impaired control of food intake in normal (i.e. non-genetically modified) mice were notable. Although the link between diminished control of food intake and chronic excess of energy intake leading to the development of obesity seemed self-evident, direct evidence for this relation only appeared in the late 1990s when studies reported hyperphagia in humans with monogenic causes of severe obesity (Montague et al., 1997; Farooqi et al., 2002) and in genetically-modified mice (Campfield et al.,1995; Pelleymounter et al., 1995).

Sequential effects of high-fat, calorie-dense (HFC) feeding on hepatic gene expression levels

Based on microarray experiments, we screened and found that the hepatic genes involved in fatty acid oxidation were initially up-regulated and then subsequently down-regulated during the course of HFC feeding. We selected and validated the expression of Cpt1L, the rate-limiting enzyme for mitochondrial fatty acid oxidation, 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 fatty acid oxidation (McGarry and Foster, 1980). We found that the gene expression levels of Cpt1L were significantly up-regulated at weeks 2 and 4. There was a trend of Cpt1L mRNA levels being down-regulated at week 10. The initial increased expression levels of Cpt1L are also in agreement with the findings by Gregoire and colleagues (2002). They had also shown that Cpt1L were up-regulated in the livers of mice fed on high-fat diet for 11 days. The differential changes in the gene expression were also translated to protein expression as evidenced by our western blot assays.

In contrast to the genes in fatty acid oxidation, the microarray screening revealed that several key hepatic lipogenic genes were initially down-regulated at week 2 but were subsequently up-regulated at week 4 and more genes were up-regulated at week 10. Several key hepatic lipogenic genes (e.g. Fas, Scd1, Acly) as well as the transcription factor Srebf1 were up-regulated at week 10. Contrary to our observations in the group of mice fed on the HFC diet for 10 weeks, Kim et al (2004) and Kreeft et al (2005) showed that gene expression of lipogenic enzymes in their male C57BL/6J mice were

down-regulated by high-fat feeding. They fed their mice on high-fat diets for 12 weeks and 8 weeks respectively. One possible reason for the discrepant findings between our study and these 2 studies could be that dietary fat content of the high-fat and control diets used in their studies was different from ours.

On the other hand, a more recent study conducted by Alevizos et al (2007) to profile the transcriptional changes in C57/BL6 mice's livers after feeding them high-fat diet for 12 weeks also demonstrated that lipogenic genes, particularly Scd1, were elevated. This was in line with our observations with the prolonged HFC groups. The fat content of our HFC diet was similar to their high fat diet (i.e. about 40% of calories from fat).

The difference in dietary fat content between the high-fat and control diets used in the studies conducted by Murase et al (2001) and Hu et al (2004) was similar to ours. The mice were fed high-fat diet for 8 weeks in the study conducted by Hu et al (2004). Murase et al (2001) fed their male mice for a much longer period of time (i.e. 5 months). In agreement with our observations at week 10 (i.e. prolonged high-fat, calorie-dense feeding), Murase et al. (2001) and Hu et al (2004) showed 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 have already observed increased mRNA levels of lipogenic genes. This might also be observed by Murase et al. (2001) and Hu et al (2004) if they had conducted time-course measurements.

Although we did not carry out the sampling period beyond 10 weeks, we could postulate that the increased levels of lipogenic genes would possibly remain elevated, as inferred from the observations made by Murase et al (2001) and Alevizos et al (2007).

We selected, validated and confirmed the gene expression of Fas, a key lipogenic gene, by RT-PCR. In addition to its pivotal role in lipogenesis, there is evidence to suggest that Fas controls feeding behaviour and, thus, may represent a therapeutic target for the control of appetite and body weight (Loftus et al., 2000; Cha et al., 2004; Kumar et al., 2002). We further examined the protein expression levels of Fas in the mice livers. The trends in the gene expression levels of Fas were also translated to the enzyme concentrations of Fas, as demonstrated in the western blot assays of Fas. These experiments confirmed the increased concentrations of Fas in the HFC-fed mice livers at weeks 4 and 10.

Could the sequential changes in hepatic gene expression involved in fatty acid oxidation and lipogenesis possibly explain the changes in food intake?

In relation to these changes in food intake and weight gain in the mice, we examined whether the molecular changes in the liver tissue could possibly help to explain some of these sequential changes observed in the feeding behaviour. It has been suggested that a stimulus generated at the level of fuel oxidation in the liver, provides a feedback signal that links the oxidative metabolism of fuels to the control of food intake (Friedman, 1990; Friedman and Tordoff, 1986; Langhans and Scharrer, 1987).

Taken together, a possible interpretation of our above findings could be as follows. As an initial response to high-fat feeding, the mice at week 2 showed a compensatory response in an attempt to reduce their food intake through increased hepatic fatty oxidation and reduced lipogenesis to regulate energy homeostasis. The possible increase in hepatic fat oxidation might lead to an increase in hepatic energy, which in turn, might send a signal to brain to reduce food intake (Friedman, 1998; Friedman et al., 1999). The possible molecular mechanism for activating hepatic fatty acid oxidation in the high-fat mice at week 2 is discussed in the later section in this Chapter.

As the intake of high-fat feeding continued, we observed that hepatic lipogenesis increased, which is known to result in an increased levels of malonyl-Co A (Wolfgang and Lane, 2006). Malonyl-Co A, the first committed intermediate in the pathway of fatty acid biosynthesis, is a potent Cpt1L inhibitor (McGarry et al., 1977; McGarry et al., 1978). Hence, this could possibly inhibit hepatic fatty acid oxidation. In turn, this could likely result in the removal of the stimulus to inhibit food intake and thus led to increased food intake and weight gain observed in the mice fed the high-fat, calorie dense diet at weeks 4 and 10. The initial attempt to regulate body weight gain in the HFC-2wk mice could possibly explain the lower energy efficiency 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 energy efficiency ratios versus their corresponding controls.

Sequential effects of high-fat, calorie-dense feeding on plasma lipids in the mice

In agreement with the findings by Kreeft et al (2005) and Gregoire et al (2002), we found that high-fat and a calorie-dense diet increased total and LDL cholesterol levels. After 2 weeks of HFC feeding, there were significant increases in the levels of total and LDL cholesterol, despite no significant changes in body weight in the HFC mice as compared to their controls. The data from all the control mice were within the normal range reported for female C57BL/6J mice (refer to Appendix 4.6 for comparison).We had expected that the total and LDL cholesterol levels to show a progressive increase over the HFC feeding period. However, total and LDL cholesterol levels remained elevated with no significant progressive increases amongst the HFC groups.

In line with the observations made by Gregoire et al (2001), we observed that HFC feeding appeared to induce increased HDL cholesterol levels as well (significant only at week 4) in the HFC mice. Since high total cholesterol levels (which confers increased cardiovascular risks) are usually accompanied by high HDL cholesterol levels (which confers reduced cardiovascular risks), we calculated the total/HDL cholesterol ratio to resolve this dilemma. A higher total/HDL cholesterol ratio will confer greater cardiovascular risks. We observed that this ratio was raised in all the HFC groups as compared to the C groups (non-significant) and the ratio appeared to increase with the HFC feeding period but there were no significant differences amongst the HFC groups. At week 4, we observed that this increase in ratio in the HFC versus the controls was the smallest as compared to the increases observed at weeks 2 and 10.

Another unexpected finding was the absence of significant increase in the plasma triglycerides levels in the HFC mice at week 2. Subsequently at week 4, there appeared to be a downward trend in the plasma triglyceride levels (non-significant). These reductions in plasma triglyceride levels in the HFC fed mice were also observed by Gregoire et al (2002) who fed their mice for 11 days. In contrast to these observations made by us and Gregoire et al (2002), studies which examined the effects of high-fat feeding for longer periods reported increased plasma triglyceride levels (Kim et al., 2004; Milagro et al., 2006). At week 10, we also demonstrated that the prolonged HFC feeding resulted in significantly higher plasma triglyceride levels.

These sequential observations on the plasma cholesterol and triglycerides levels over the entire HFC feeding period would have been missed in single end-point feeding studies. Taken together, our findings suggested that the mice might possibly develop regulatory mechanisms to regulate their plasma lipid levels over the entire feeding period, at least till week 4.

Could the sequential changes in hepatic gene expression involved in cholesterol synthesis possibly explain the breakdown in cholesterol homeostasis?

This led us to further examine the expression levels of the hepatic genes involved in cholesterol metabolism. As the majority of endogenous cholesterol synthesis in mice occurs in the liver tissue, hence this thesis focused on examining the gene expression levels related to cholesterol synthesis in the liver tissue only. Similar to the genes involved in lipogenesis, the microarray screening showed that the genes involved in cholesterol synthesis were also initially down-regulated. However, at week 4, more genes involved in cholesterol synthesis were down-regulated as compared to week 2.

This was in contrast to the genes involved in lipogenesis as it was at week 4 when we started to observe an increased expression levels in the lipogenic genes. This seemed to relate well with the observations that the cholesterol levels did not increase much after 4 weeks of HFC feeding and there was no significant difference in the levels of total and LDL cholesterols amongst the HFC groups over the entire feeding period. Our findings could possibly suggest that the mechanisms in place to regulate cholesterol levels in the mice could be more robust than the mechanisms to regulate fatty acid synthesis. At week 10, we observed that this homeostasis regulatory mechanism was breaking down with the increased expression of genes involved in cholesterol synthesis (e.g. Hmgcr, Sc4mol, Sc5d, Sqle, Cyb5r3, Fdft1 and Fdps).

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 (Kim et al., 2004; Kreeft et al., 2005; Gregoire et al., 2002). The initial down-regulation of genes involved in cholesterol synthesis suggested that it was unlikely that the increased cholesterol levels observed in the HFC mice at week 2 were due to increased hepatic cholesterol synthesis. Instead, one possible explanation was that the liver was attempting to maintain cholesterol homeostasis in response to high-fat feeding by down-regulating cholesterol synthesis. This is in line with Goldstein and Brown's suggestion that cholesterol homeostasis is maintained by a feedback mechanism (Goldstein and Brown, 1990).

We could not compare our findings with Murase et al (2001) and Hu et al (2004) as they did not investigate the expression of the genes involved in cholesterol synthesis. As described in the earlier section about the similarities between our diets and theirs,

we would expect that our upwards trend in gene expression at week 10 might have been reported in their mice. To the best of our knowledge, this differential expression of genes involved in cholesterol synthesis induced by HFC feeding has yet to be reported. We further validated these gene expression changes by conducting western blot assay for Hmgcr, which catalyses the rate-limiting step in cholesterol biosynthesis. In agreement with the observations in the gene expression levels of Hmgcr, we found that at weeks 2 and 4, there were significant reductions in Hmgcr protein expression levels in HFC mice as compared to their controls. Prolonged HFC feeding induced significant increase in the Hmgcr protein expression levels at week 10.

Since the expression levels of genes related to cholesterol synthesis were down-regulated initially, this could not account for the increased plasma cholesterol levels in the HFC-2wk mice. The other mechanism that might affect cholesterol levels would be cholesterol removal. The diversion of cholesterol for bile acid production has been well established as a mechanism for reducing plasma cholesterol (Fuchs, 2003). Interestingly, we observed that the gene expression of Cyp7A1, the enzyme encoding the rate-limiting step of bile acid production, was consistently down-regulated by high-fat feeding and Ldlr was down-regulated 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 up-regulated as it governs the rate-limiting step in the neutral pathway of bile acid synthesis from cholesterol. Therefore, our findings suggested that for mice fed with HFC diet, it was not the up-regulation of

cholesterol synthesis but rather the down-regulation of cholesterol removal mechanism which resulted in the observed higher blood cholesterol levels. Based on these observations, it is likely that the increased plasma cholesterol levels observed in the HFC groups could be mainly due to the defect in cholesterol removal and bile acid synthesis as the results suggested that the cholesterol synthesis was still well regulated up to 4 weeks of high-fat feeding.

Sequential effects of high-fat, calorie-dense feeding on plasma leptin, insulin levels in the mice

It has been proposed that both hormones, leptin and insulin, are involved in the regulation of body weight and food intake. In agreement with studies conducted in humans (Maffei et al., 1995) and in mice (Ahren, 1999), we observed that plasma leptin levels correlated positively and significantly with body weight in all the mice (r = 0.86, $P < 0.001$). Ahren also reported a strong and significant correlation between plasma leptin levels and body weight (r = 0.81, $P < 0.001$). We found that the correlation between plasma leptin levels with white adipose tissue mass was even higher (r = 0.93, $P < 0.001$). Other studies of obese humans also showed a strong and consistent positive relation between plasma leptin concentrations and adipose mass (Maffei et al., 1995; Havel et al., 1996). Perhaps, plasma leptin, which is synthesized mainly from white adipose tissue, could be proposed as a new measure of body fat in addition to body mass index (BMI)[3].

In line with findings reported by Ahren (1999) and Lin et al (2000), we demonstrated that the plasma leptin levels were significantly up-regulated by HFC feeding at all

[3] Body mass index is calculated as weight in kilograms (kg) divided by the height in meters (m) squared (kg/m^2). This index correlates with body fat.

time-points. In comparison with their studies which reported increased plasma leptin levels in C57BL/6J female mice only after 4 weeks of high-fat feeding (Ahren 1999) and 8 weeks of high-fat feeding (Lin et al., 2000), we had observed that 2 weeks of high-fat, calorie dense feeding could induce a significant increase in the plasma leptin levels. At week 2, the increase in plasma leptin levels in the HFC mice was 3.7 times higher than the levels observed in the C mice. This observation was notable as the significant increase in the plasma leptin levels had occurred despite no significant increase in white adipose tissue mass in HFC-2wk mice as compared to C-2wk mice. The plasma leptin levels in the C-2wk mice were within the normal range (i.e. 2.3 $\pm$ 1.1 ng/ml) as described by Murphy et al (1997).

It has been demonstrated that the effect of leptin on energy homeostasis is to inhibit food intake. Leptin-deficient *ob/ob* mice were hyperphagic, massively obese, severely insulin resistant and diabetic, but this phenotype was reversed upon replacement of leptin (Pelleymounter et.al., 1995). We investigated the correlation between plasma leptin levels and the mice food intake. There was a significant inverse relationship between the plasma leptin levels and food intake in the mice at week 2 but this inverse relationship did not seem to occur at weeks 4 and 10. It appeared from our results that there was no significant relation between plasma leptin and food intake with prolonged HFC feeding.

Although the levels of plasma leptin remained significantly higher in the HFC-4wk and HFC-10wk mice as compared to their controls, the inhibition on the food intake was no longer observed. Instead these HFC mice exhibited hyperphagic feeding behaviour and began to gain weight with greater body fat accumulation. The feed

efficiency and energy efficiency ratios also tend to increase in these HFC groups. Since the leptin levels remained elevated at higher levels rather than at reduced levels, this could likely suggest that it was due to the presence of leptin resistance rather than reduced leptin production in the HFC-4wk and HFC-10wk groups that led to over-feeding. This was also consistent with our observation that mRNA levels of leptin in the white adipose tissue were significantly up-regulated in the HFC groups as compared to the C groups at all 3 time-points. Moreover, the development of leptin resistance in the HFC mice could also be inferred from the 2 previous studies by Ahren (1999) and Lin et al (2000). Both studies demonstrated elevated plasma leptin levels in mice fed with high-fat diets. Ahren (1999) showed that the mice continued to have elevated plasma leptin levels up to 11 months of high-fat feeding. In a later section in this Chapter, the possible link between hepatic lipid metabolism and leptin resistance is discussed.

Is there a potential link between the changes in the plasma leptin levels, differential hepatic gene expression involved in fatty acid oxidation and the food intake in the HFC mice?

Back in the mid-1990s, Unger et al (1999) proposed a novel anti-lipogenic role for leptin and they elegantly demonstrated how it could regulate lipid homeostasis by stimulating fatty acid oxidation (FAO). In agreement with this postulation, we observed an initial upward trend in the mRNA levels of hepatic genes involved in fatty acid oxidation in our HFC-2wk mice which had increased plasma leptin levels. Concurrently, these mice appeared to show a compensatory response to reduce their food intake. In contrast to these observations, we observed increased food intake accompanied by leptin resistance in the HFC mice fed for a longer period of time

beyond 2 weeks. It was found that the hepatic genes involved in fatty acid oxidation were no longer up-regulated in the HFC-4wk and HFC-10wk mice. A possible interpretation of these observations could be as follows. As described earlier, prolonged HFC feeding might lead to the development of leptin resistance in the mice. In turn, this could reduce the stimulus on hepatic fatty acid oxidation. The reduced hepatic fatty acid oxidation could subsequently diminish the feedback signal sent to the central nervous system to regulate the food intake in the mice with subsequent increased feeding observed in the HFC-4wk and HFC-10wk mice.

Could hepatic gene expression involved in lipid metabolism possibly be linked to the development of leptin resistance in the HFC mice?

Leptin resistance is determined by the down-regulation of leptin receptor (Lepr) and the up-activation of the suppressors of the cytokine signalling family 3 (Socs-3). In Chapter 2, we have described these 2 factors which determine leptin resistance in greater details. We examined the patterns of expression levels of the leptin receptor and Socs-3 in the mice livers. We studied the possibility of suppressed hepatic Lepr mRNA levels as well as up-regulated hepatic Socs-3 mRNA levels, which could possibly help to explain the leptin resistance in HFC-4wk and HFC-10wk mice. In agreement with this speculation, we found that Lepr was down-regulated by 2.2 fold at week 10. Prolonged HFC feeding did not up-regulate the expression level of Socs-3 but we observed that the initial 3-fold decrease in Socs-3 mRNA levels at week 2 was gradually removed over the entire feeding period. At week 4, it was only reduced by 1.4 fold and at week 10, Socs-3 was no longer down-regulated. These observations in the mRNA levels of these hepatic genes over the various HFC feeding time-points have not been previously described. Peiser et al (2000) reported that neither hepatic

Lepr nor Socs3 mRNA levels were significantly regulated by 15 weeks of high-fat feeding in rats. Nonetheless, they did demonstrate a non-significant reduction in the mRNA levels of leptin receptor in the livers of the high-fat fed rats.

Therefore, our findings seemed to suggest that the higher circulating plasma leptin levels in the HFC-4wk and HFC-10wk mice did not stimulate Socs-3 expression levels. Rather, it could be the gradual removal of the inhibition Socs-3 in the HFC mice fed that could result in leptin resistance in the HFC-4wk and HFC-10wk mice. In turn, this leptin resistance could possibly account for the removal of stimulus on the hepatic genes involved in fatty acid oxidation, resulting in a reduced signal to reduce food intake. Subsequently, prolonged HFC feeding led to increased food intake, weight gain and deposition of body fat in the HFC mice. This is discussed in greater details in the later section "A possible model linking plasma leptin levels, leptin signalling, hepatic lipid metabolism and food intake."

Comparison of the transcription profiles between liver and white adipose tissue

When we examined the expression profiles of lipogenic genes in the liver tissue and the white adipose tissue over the entire HFC period, we found that the lipogenesis was initially suppressed in the liver tissue and subsequently it was up-regulated by 4 weeks of HFC feeding. On the other hand, we observed that the lipogenic genes were not up-regulated but were either suppressed or remained unchanged throughout the 10 weeks in white adipose tissue. In addition, the mRNA levels of Srebf1 in the white adipose tissue, a main transcription factor that controls the expression of the lipogenic pathway, were also not up-regulated throughout the feeding period. We found that there was a greater degree of down-regulation of lipogenic genes with longer period

of HFC feeding. This contrasted with our expectations. We have expected to find evidence to suggest enhanced lipogenesis in white adipose tissue which may contribute to the development of the excessive fat mass in the HFC-10wk mice. Nonetheless, this reduced or normal lipogenic gene expression levels in the HFC mice agree with previous reports that have examined the expression of lipogenic genes in adipose tissue from obese humans (Diraison et al., 2002) and *ob/ob* mice (Nadler et al., 2000; Soukas et al., 2000). Based on their findings, the authors reasonably suggested that the reduction of gene expression levels in the white adipose tissue mass could be a late and adaptive process aimed at limiting or preventing further development of fat mass. However, our findings over the various time-points showed clearly that this was not likely to be the case.

The discordant finding between the transcription profile of the lipogenic genes in the liver and that of the adipose tissue was interesting as the basic mechanisms responsible for the regulation of the expression of the lipogenic pathway are considered to be similar in hepatocytes and adipocytes (Ferre, 1999; Girard et al, 1994). It is possible that the raised leptin levels of the HFC mice could partially explain the discrepant observations. There is data supporting a suppressive action of leptin on the transcription of Fas (Bai et al., 1996) and Srebf1 (Soukas et al, 2000) and on *in vivo* lipogenesis (Bryson et al, 1999; Lee et al., 2000). Although this action is present in both liver and adipose tissues, a direct, paracrine effect in adipose tissue could explain a more marked action on adipocytes *in vivo*. Indeed, we observed a strong and significant inverse relation between mRNA levels of Fas in white adipose tissue and the plasma leptin levels (r = -0.88, $P < 0.001$) of the mice at weeks 4 and 10, but not at week 2. The subsequent increased gene expression levels of hepatic Fas

in the HFC-4wk and HFC-10wk mice could reflect the decreased leptin sensitivity in the livers with the prolonged HFC feeding as described earlier.

The raised insulin levels observed with extended HFC feeding could also possibly explain the enhanced levels of expression of hepatic genes involved in lipogenesis. It has been shown that insulin stimulated the transcription of lipogenic genes in rat hepatocytes and adipocytes (Claycombe et al., 1998). An increase in plasma glucose stimulates insulin release. We observed significant increases in both the glucose levels as well as the plasma insulin levels in HFC-10wk mice when compared to their controls. We also observed that the plasma insulin levels correlated positively with the mRNA levels of hepatic Fas (r = 0.74, $P < 0.01$). In contrast, there was no significant relation between the mRNA levels of Fas in adipose tissue with the plasma insulin levels. This could possibly indicate a stronger influence on the regulation of lipogenic genes in the liver tissue as compared to the white adipose tissue by insulin. However, the exact mechanisms would have to be verified by further investigations. Taken together, the varying levels of plasma insulin and leptin as over the 3 time-points in our study could play a role in the *in vivo* difference we observed between liver and adipose tissue lipogenic gene expression levels in the HFC mice.

A possible model linking plasma leptin levels, leptin signalling, hepatic lipid metabolism and food intake

Initial compensatory responses to high-fat, calorie-dense feeding

A possible model to explain our sequential findings could be as follows (Figure 4.31). During the initial period of high-fat, calorie-dense feeding, the mice might have developed compensatory mechanisms to reduce their food intake by increasing leptin

synthesis in the white adipose tissue. In support of this, we found increased mRNA levels of leptin in the white adipose tissue as well as increased plasma leptin levels in the mice fed on the high-fat calorie-dense diet for 2 weeks as compared to their controls.

Plasma leptin exerted its effects through activation of the leptin receptor which could be found in the hypothalamus as well as peripheral tissue (e.g. the liver). Besides sending neuroendocrine signals to the brain to inhibit food intake, the increased circulating plasma leptin might possibly exhibit its inhibition on food intake via its influence on the balance of lipid homeostasis in the liver. The elevated levels of plasma leptin might possibly inhibit food intake by increasing hepatic fatty acid oxidation. This stimulation of hepatic fatty acid oxidation is likely to occur via leptin-stimulation on the JAK/STAT signalling pathway. Our microarray results also showed that several genes involved in JAK/STAT signalling pathway (for example Cxcl9, Ccnd1, Egfr, Il2rg, Junb, Pias1, Pim1, Ptprc, Smad4, Socs2 and Sp1) were all initially up-regulated and either showed a downward trend or no change with prolonged high-fat feeding. The possible leptin-mediated stimulus on hepatic fatty acid oxidation via JAK/STAT signaling pathway could be inferred from studies conducted by Tartaglia (1997) and Roger et al (1999). Tartaglia provided evidence that leptin exerts its effects through activation of the leptin receptor and via subsequent stimulation of the JAK/STAT pathway (Tartaglia, 1997). Roger Unger and his colleagues showed convincing evidence that leptin, via stimulation of the JAK/STAT pathway, could stimulate fatty acid oxidation (Unger et al., 1999). In turn, the increased hepatic fatty acid oxidation might send nerve impulses to the brain by vagal sensory neurons to reduce food intake in the mice. Early work by Friedman et al

(1998; 1999) supported this concept. More recently, Suzuki et al (2007) had elegantly demonstrated that leptin stimulated fatty acid oxidation. Martin et al (2006) found that mice fed high-fat diet for about 10 weeks develop defective responses to leptin-induced stimulation of fatty acid oxidation.

Our findings of the expression levels of the hepatic genes involved in fatty acid oxidation as well as the leptin receptor in the livers of the mice fed on the high-fat, calorie dense diet for 2 weeks supported the above proposed model. We found increased protein levels of carnitine palmitoyltransferase 1a in the livers of the mice fed for 2 weeks on the high-fat, calorie-dense diet as compared to their controls. This enzyme catalyses the rate-limiting step of mitochondrial fatty acid oxidation. We also observed increased mRNA levels of leptin receptor in the livers of the mice fed on the high-fat, calorie-dense diet for 2 weeks.

Breakdown in compensatory responses with prolonged high-fat, calorie-dense feeding

In comparison to the observations during the initial period of the high-fat, calorie-dense feeding, the findings with prolonged feeding suggest a possible breakdown in the initial compensatory mechanisms in regulating food intake, lipid homeostasis and body weight (Figure 4.31). 10 weeks of high-fat, calorie-dense feeding whilst continuing to induce increased plasma leptin levels in the mice did not prevent a reduction in food intake, weight gain or white adipose tissue mass deposition. Instead, food intake, weight gain and white adipose tissue were all significantly increased in the high-fat, calorie-dense group of mice as compared to their controls. One possible explanation could be that these mice might have developed leptin resistance.

Currently, suppressors of the cytokine signalling family 3 (Socs-3) is most associated with the inhibition of leptin receptor signal transduction and has been suggested to play a role in mediating leptin resistance in diet-induced obesity (Lavens et al., 2006). A possible way of interpreting our observations made during prolonged high-fat, calorie-dense feeding could be that Socs-3 exerted its inhibitory actions on the JAK/STAT signalling, resulting in a suppression of fatty acid oxidation and increased lipogenesis in the livers of these mice. This was supported by our observations that with prolonged high-fat feeding, the mice at week 10 did not continue to show any reduced mRNA levels of Socs-3 whereas Soc-3 was initially down-regulated by 3 fold at week 2 in the high-fat group.

The inhibition of leptin signalling could possibly result in a suppression of hepatic fatty acid oxidation. Indeed, at week 10, the genes involved in hepatic fatty acid oxidation were no longer up-regulated. In turn, this reduced hepatic fatty acid oxidation could have reduced the feedback stimulus to reduce food intake, resulting in increased food intake observed in these mice.

At week 10, we observed increased mRNA levels of lipogenic genes. This might also be a possible consequence of reduced leptin signalling with prolonged high-fat, calorie dense feeding. The anti-lipogenic role of leptin was demonstrated by various studies (Bai et al., 1996; Bryson et al., 1999; Soukas et al., 2000; Lee et al., 2000). This could also possibly explain the elevated triglyceride levels in the HFC-10wk mice. In addition, increased hepatic lipogenesis in itself, could also further inhibit hepatic fatty acid oxidation as discussed in previous sections.

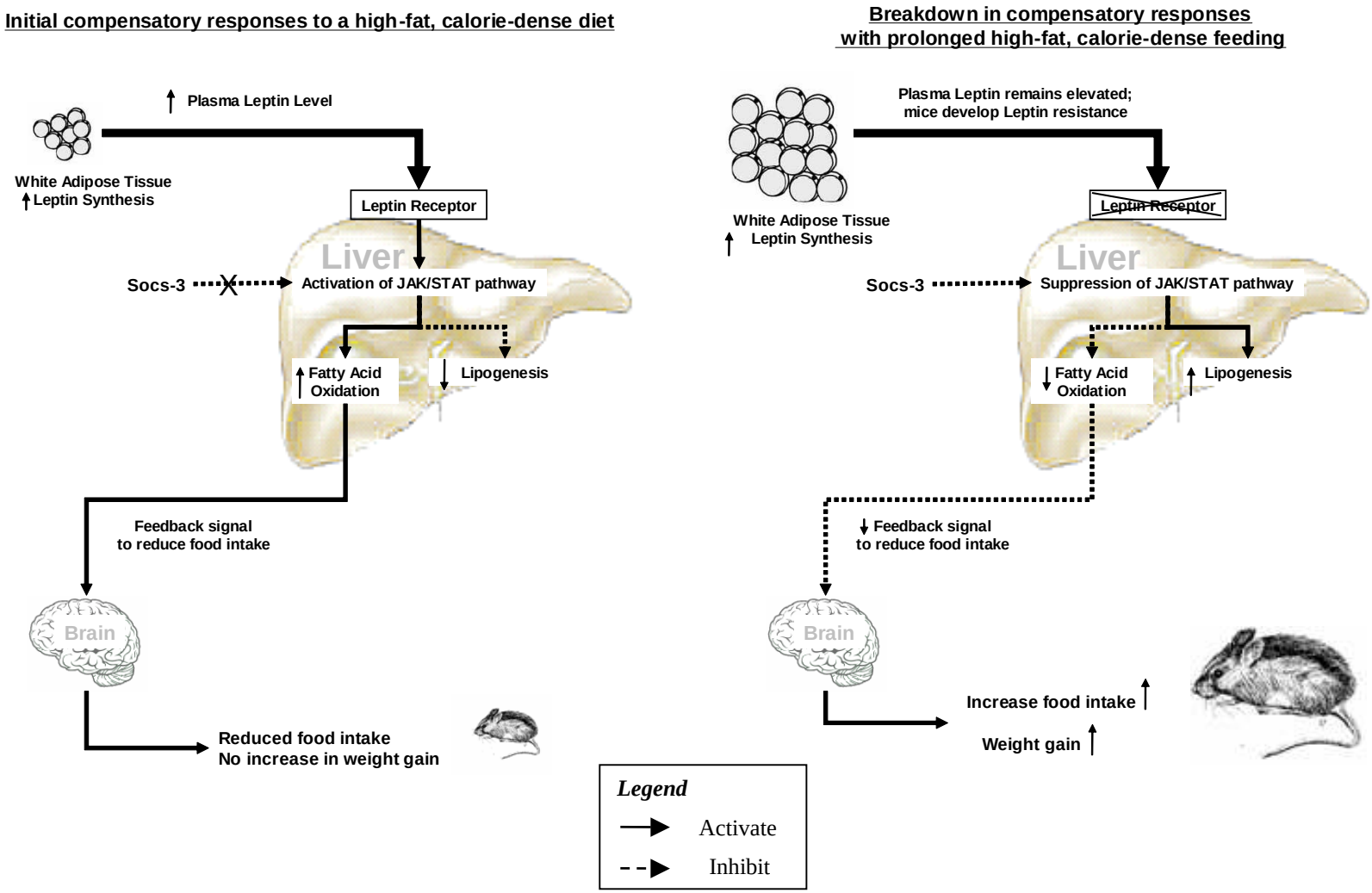
In addition to large increase in body weight and accumulation of body fat as white adipose tissue in the group of mice fed with high-fat, calorie-dense for 10 weeks, we observed significant increase in their blood triglyceride levels, blood glucose levels and plasma insulin levels. These observations share many similarities to those metabolic abnormalities associated with the human metabolic syndrome in humans. These observations closely reflect the pathogenesis of metabolic syndrome in humans. Could the proposed model provide an insight to the link between a breakdown in regulating energy homeostasis and the metabolic syndrome?

Recently, it is proposed that lipids stored in white adipose tissue might be less harmful than being stored in non-adipose tissue (Slawik and Vidal-Puig, 2006). This concept known as “lipotoxicity” suggests that the various metabolic abnormalities associated with metabolic syndrome may be due to the adverse effects of fat accumulation in the non-adipose tissues (e.g. liver). Our rudimentary model could possibly contribute to the understanding of lipotoxicity. Roger Unger has suggested that white adipose tissue secrete leptin to prevent the over-accumulation of triglycerides in non-adipose tissues (e.g. liver) and thus confine fat storage to adipose tissue only (Unger et al., 1999). This protective action of leptin works by reducing lipogenesis and increasing oxidation in non-adipose tissues during overnutrition, dissipating the unneeded energy of excess fatty acid as heat. This limits fatty acid entry into potentially toxic nonoxidative metabolic pathways (Unger and Zhou, 2001).

In our proposed model, with prolonged high-fat, calorie-dense feeding, leptin resistance occurred, with reduced sensitivity to leptin actions in the non-adipose tissue, leading to increased lipogenesis and reduced fat breakdown. In turn, this

resulted in overloading of triglycerides in the non-adipose tissue such as liver. In line with the current concept on lipotoxicity, this could lead to an overload in lipid stores in non-adipose tissue (e.g. liver), leading to cell dysfunction or lipid-induced programmed cell death. Our suggestion that there could be liver lipotoxicity accompanying increased lipogenesis observed in Week 4 and Week 10 HFC groups would have been further supported by underlying hepatic pathological changes (i.e. fatty livers).

Figure 4.31 A possible model linking plasma leptin levels, leptin signalling, hepatic lipid metabolism and food intake



CHAPTER 5

SEQUENTIAL EFFECTS OF A HIGH-FIBER DIET CONTAINING PSYLLIUM HUSK ON BODY WEIGHT, PLASMA LIPIDS AND HEPATIC GENE EXPRESSION LEVELS

INTRODUCTION

The health benefits of increased dietary fiber intake include reducing the risks of developing cardiovascular disease, colon cancer and obesity. Greater details have been discussed in Chapter 2. In spite of this, the literature on dietary fiber and gene expression remains limited as compared to the literature regarding dietary fat and gene expression. Studies examining the effects of dietary fiber on gene expression are limited to the gastrointestinal tract (Nguyen et al., 2006; Young et al., 2005; Chapkin et al., 1998) or only on one or two hepatic genes (Yang et al., 2003; Goel et al., 1999; Sonoyama et al., 1995). To date, no microarray transcription profiling studies have yet been carried out to examine the effects of dietary fiber on global hepatic gene expression. We aimed to characterize the expression levels of hepatic genes involved in lipid metabolism over 10 weeks of high-fiber feeding. In parallel, we examined the changes in food intake, body weight and plasma lipids levels.

The high-fiber diet used in the experiment consisted of 10% of psyllium husk, a soluble fiber. The control diet used was standard AIN93M. Details of the diets are found in Chapter 3. In parallel, we examined the changes in food intake, body weight and plasma lipids levels.

The abbreviations used to name the groups are as follows: Mice fed on the high-fiber diet consisting of psyllium husk for 3 weeks (PE-3wk) and for 10 weeks (PE-10wk). The abbreviations for their respective controls are as follows: C-3wk and C-10wk.

RESULTS

Food and energy intake

At week 3, food intake was significantly reduced in the high-fiber fed mice (2.76 ± 0.06 g/day) as compared to the food intake in their controls (2.92 ± 0.08 g/day) (Table 5.1). With this reduction in food intake, PE-3wk mice also had a significantly lower energy intake as compared to the controls (38.1 ± 0.9 kJ/day versus 44.3 ± 0.9 kJ/day). At week 10, we observed a small but significant increase in food intake in the high-fiber fed mice as compared to the controls. However, there were no significant differences in the energy intake between PE-10wk as compared to C-10wk mice. Throughout the entire feeding study, fiber intake was significantly three times higher in both the PE groups as compared to their respective controls.

Body Weights

At the start of the feeding period, there were no significant differences in the body weights between PE-3wk mice versus C-3wk mice (17.0 ± 0.9 g versus 17.8 ± 0.5 g, respectively) and PE-10wk mice versus C-10wk mice (16.9 ± 1.6 g versus 17.5 ± 1.4 g, respectively). Fiber-feeding did not seem to affect the body weights as there were no significant differences in the final body weight between the PE-groups and their respective controls at weeks 3 and 10 (Table 5.1). Although PE-3wk mice gained slightly less weight from their initial body weights (0.5 ± 0.4 g) as compared to the C-3wk mice (0.6 ± 0.4 g) mice, this was not significantly different. At week 10, there

were also no significant differences in weight gained between PE-10wk mice versus C-10wk mice (2.8 ± 1.3 g versus 2.3 ± 1.2 g respectively).

White adipose tissue mass

The white adipose tissue mass in the PE-3wk mice (0.16 ± 0.03g) was lower as compared to C-3wk mice (0.18 ± 0.01g) but this was not statistically significant. At week 10, there was no observable difference between the PE and C mice (Table 5.1).

Table 5.1 Food intake, energy intake, body weight and white adipose tissue mass of control and high-fiber fed mice

C: control diet groups; PE: high-fiber containing psyllium husk diet groups. Values are expressed as means ± SD (n= 6/group); ** denotes P < 0.01 compared to corresponding C group.

	PE-3wk mice	C-3wk mice	PE-10wk mice	C-10wk mice
Food intake (g)	2.76 ± 0.06**	2.92 ± 0.08	3.24 ± 0.14**	3.01 ± 0.04
Energy intake (kJ)	38.1 ± 0.9 **	44.3 ± 0.9	44.8 ± 2.0	45.7 ± 0.6
Final Body Weight (g)	17.5 ± 1.3	18.4 ± 0.7	19.6 ± 0.5	19.8 ± 0.3
Gain in body weight (g)	0.5 ± 0.4	0.6 ± 0.4	2.8 ± 1.3	2.3 ± 1.2
White adipose tissue mass (g)	0.16 ± 0.03	0.18 ± 0.01	0.26 ± 0.02	0.26 ± 0.05

Plasma cholesterol levels

At the start of the feeding study, there were no significant differences in the plasma total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride levels in the PE groups versus their respective controls.

The total cholesterol levels were significantly reduced in the PE-3wk group as compared to C-3wk mice. The levels were reduced by 27% from 2.02 ± 0.08 mmol/L (C-3wk mice) to 1.49 ± 0.18 mmol/L (PE-3wk mice) (Figure 5.1). At week 10, the total cholesterol levels remained significantly lower in the high-fiber fed mice as compared to their controls (1.39 ± 0.06 mmol/L versus 2.14 ± 0.22 mmol/L, respectively). There was no significant reduction in total cholesterol levels between the high-fiber fed mice at week 3 and week 10.

We observed that the LDL cholesterol levels were lowered from 0.42 ± 0.14 mmol/L in the C-3wk mice to 0.30 ± 0.09 mmol/L in the PE-3wk mice (Figure 5.1). Levels dropped from 0.44 ± 0.18 mmol/L in C-10wk mice to 0.39 ± 0.08 mmol/L in PE-10wk mice. Both these reductions in LDL levels did not reach statistical significance. There were also no significant differences between both high-fiber groups.

Significant reductions in HDL cholesterols were observed in the high-fiber fed groups as compared to their controls (Figure 5.1). Since lower total cholesterol levels (confers reduced cardiovascular risks) are usually accompanied by reduced HDL cholesterol levels (reduced HDL cholesterol levels confers greater cardiovascular risks), we calculated the total/HDL cholesterol ratio to resolve this dilemma (Figure 5.1). When we compared the total cholesterol/HDL cholesterol ratios, there was a

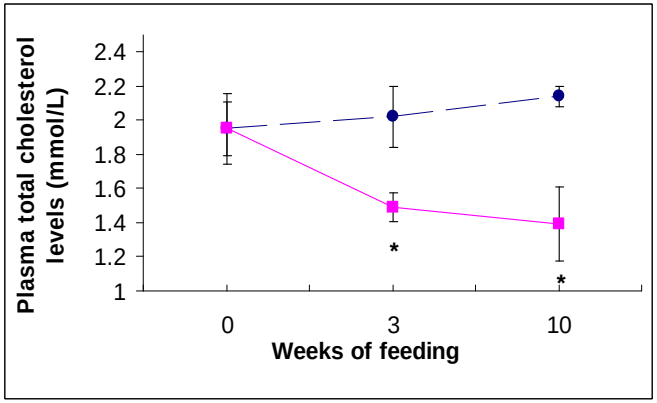
marginally lower ratio (not significant) in the PE-3wk mice (1.27 ± 0.11) compared to the C-3wk mice (1.25 ± 0.06). In contrast, the ratio appeared to increase in the PE-10wk mice (1.40 ± 0.11) versus C-10wk mice (1.26 ± 0.10) (non-significant). There were no significant differences in the ratio between both the high-fiber groups.

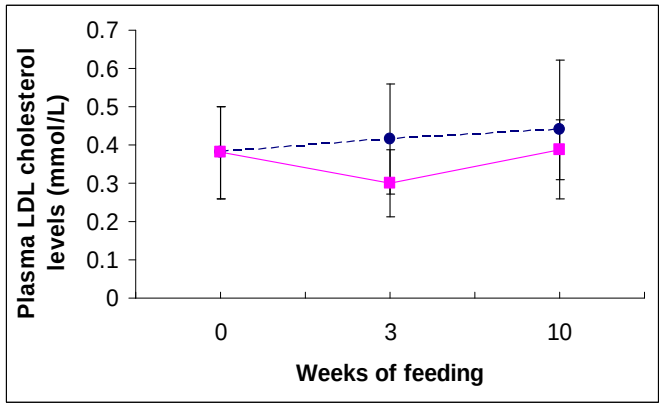
Plasma triglyceride levels

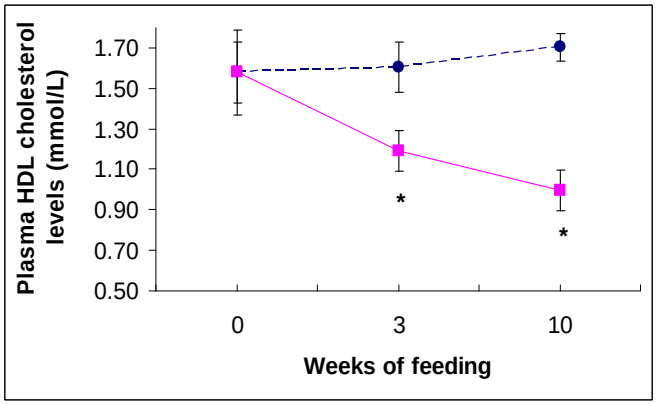
We observed a slight and non-significant reduction in plasma triglyceride levels in PE-3wk (0.93 ± 0.13 mmol/L) versus C-3wk mice (0.99 ± 0.11 mmol/L) (Figure 5.2). The plasma triglyceride fell further as the high-fiber feeding continued. The plasma triglyceride levels were significantly reduced in the PE-10wk mice. The levels fell by 32% from 0.79 ± 0.10 mmol/L (C-10wk mice) to 0.53 ± 0.05 mmol/L (PE-10wk mice). The triglyceride levels in the PE-10wk mice were significantly lower than the levels in the PE-3wk mice.

Figure 5.1 Plasma cholesterol levels from control and high-fiber mice

C: control diet groups; PE: high-fiber containing psyllium husk diet groups. Values are expressed as means ± SD (n= 6/group)
* denotes P < 0.05 compared to corresponding C group. Circles with dotted lines represent data from C mice. Squares with solid lines represent data from PE mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the C or the PE groups.







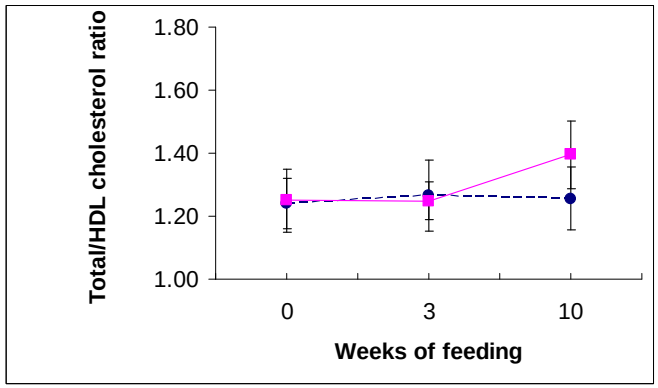
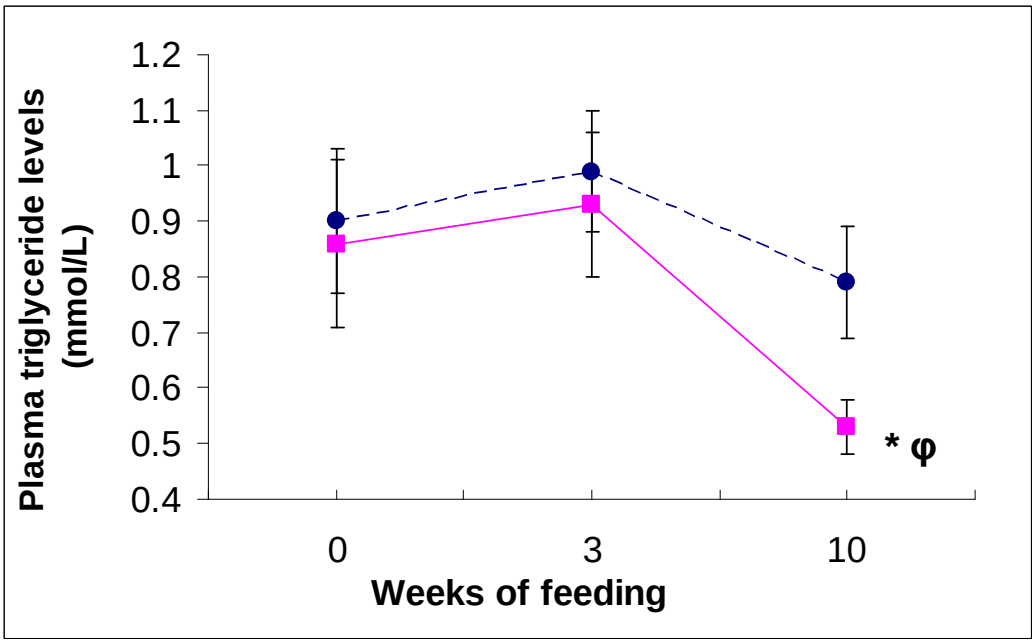


Figure 5.2 Plasma triglyceride levels from control and high-fiber mice

C: control diet groups; PE: high-fiber containing psyllium husk diet groups. Values are expressed as means ± SD (n= 6/group); * denotes P < 0.05 compared to corresponding C group. φ denotes P < 0.05 compared to PE-3wk group. Circles with dotted lines represent data from C mice. Squares with solid lines represent data from PE mice. Data at the week 0 (i.e. start of the feeding period) is calculated from the data from all the mice either in the C or the PE groups.



Microarray profiling of hepatic gene transcription levels over the duration of high-fiber feeding

The differential gene expression levels were analysed according to the following pair-wise comparisons. For example, hepatic gene expression levels in mice fed on high-fiber diet for 3 weeks (PE-3wk) were compared to the levels observed in the mice fed on control diet for 3 weeks (C-3wk). Similar comparisons were carried out for mice at week 10 (i.e. PE-10wk versus C-10wk).

Hepatic genes involved in fatty acid oxidation and lipogenesis

At week 3, genes involved in β-oxidation of fatty acids such as Acox1, Acaa2, Acsl1, Acsl4, Crat, Cpt1a, Ehhadh, Hadhb, Pte2b, were all up-regulated in the PE-3wk mice (Figure 5.3A). Cpt1a, the gene encoding the rate-limiting enzyme for fatty acid oxidation, was up-regulated by 1.6 fold. Concomitant with the up-regulation of genes involved in fat breakdown, genes encoding enzymes involved in lipid biosynthesis were down-regulated (Figure 5.3B). These included Srebp1, Bucs1, Dgat2 and Fasn. Srebp1, which was largely suppressed by 3.7 fold, is a membrane-bound transcription factor that enhances transcription of genes required for fatty acid synthesis. Fasn, a key gene in fatty acid biosynthesis was highly repressed by 4 fold (Figure 5.3B).

After 10 weeks of high-fiber feeding, genes involved in fatty acid β-oxidation were no longer up-regulated and in some, were actually down-regulated (e.g. Cpt1a, Cpt2 and Dci) (Figure 5.3A). Pparα, which controls the peroxisomal beta-oxidation pathway of fatty acids, was down-regulated by 2.3 fold. Some of the down-regulated genes involved in fatty acid biosynthesis (e.g. Fasn and Acly) at week 3 were found to be up-regulated after 10 weeks of high-fiber diet. Fasn was up-regulated by 1.7 fold

(Figure 5.3B). Other genes such as Scd1, Ptgs1 and Alox5ap which were involved in fatty acid biosynthesis were also up-regulated.

Hepatic genes involved in cholesterol metabolism

After 3 weeks of high-fiber feeding, 3 genes involved in cholesterol synthesis pathway (Hmgcs1, Hsd17b7 and Cyp51) were up-regulated by 1.6 fold, 1.5 fold and 1.9 fold respectively (Figure 5.3C). The mice fed on 10 weeks of high-fiber diet continued to show an up-regulation for these 3 genes with a greater increase in Hmgcs1 (2 fold) and Cyp51 (2.2 fold) as compared to their controls. In addition at week 10, more genes involved in the cholesterol biosynthesis pathway, such as Nsdh1, Hmgcr, Idil, Sc4mol, Stard4 and Dhcr7 were also up-regulated in the PE-10wk group (Figure 5.3C). Hmgcr, which encodes the rate-limiting enzyme for cholesterol synthesis, was up-regulated by 2 fold at week 10. None of the genes involved in the cholesterol synthesis pathway were down-regulated at both time-points.

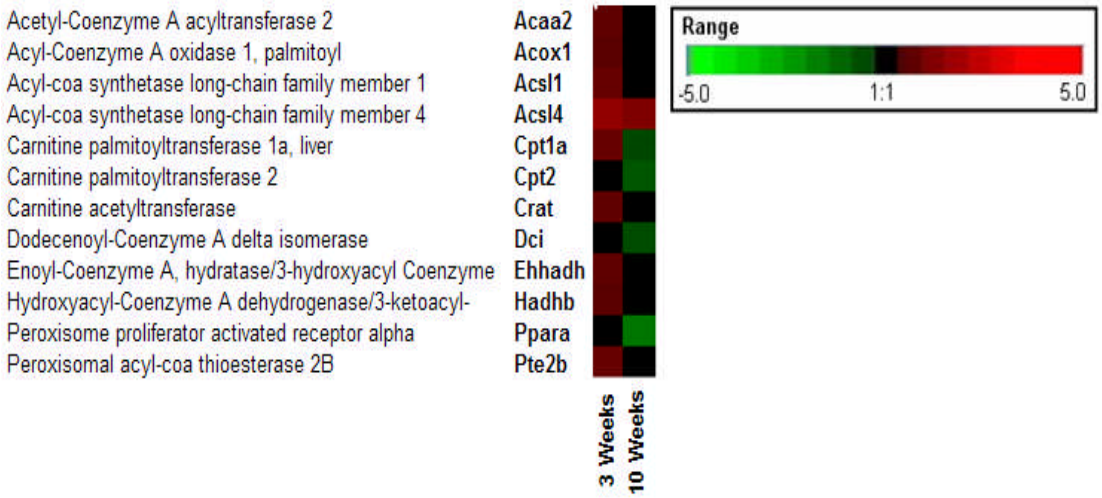
Hepatic genes involved in bile acid synthesis

The gene encoding the rate-limiting enzyme involved in bile acid synthesis, Cyp7a1, was highly up-regulated by 3.3 fold in the PE-3wk mice (Figure 5.3C). However, Cyp7a1 expression was down-regulated by 1.4 fold after 10 weeks of high-fiber feeding enriched with psyllium husk as compared to the control group. HNF4α, a key hepatic transcription factor which can regulate Cyp7a1 had increased expression in both PE-3wk and PE-10wk mice by 1.6 and 1.5 fold respectively. Cyp39a1 is also involved in the conversion of cholesterol to bile acids. Its substrates include oxysterols such as 25-hydroxycholesterol, 27-hydroxycholesterol and 24-

hydroxycholesterol. It has been shown that Cyp39a1 has a preference for the substrate 24-hydroxycholesterol. Its expression was elevated in the livers of both PE-3wk and PE-10wk mice by 2.3 fold and 1.7 fold respectively.

Figure 5.3 Hepatic genes involved in lipid metabolism regulated by high-fiber feeding at weeks 3 and 10.
The colour-coded scale (green=down-regulation and red=up-regulation). Data of "fold change" were calculated from the "Signal Log Ratio". The scale is presented as fold change.

(A) Genes involved in fatty acid oxidation



(B) Genes involved in lipogenesis

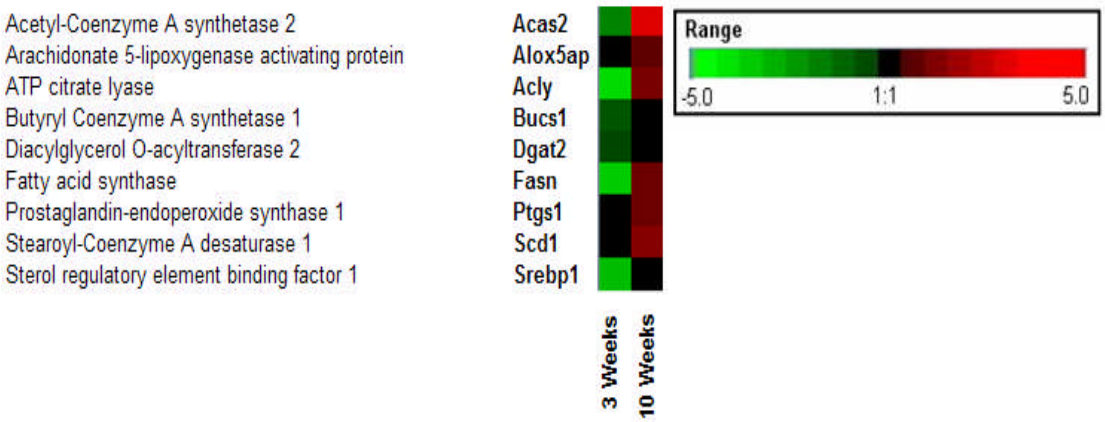
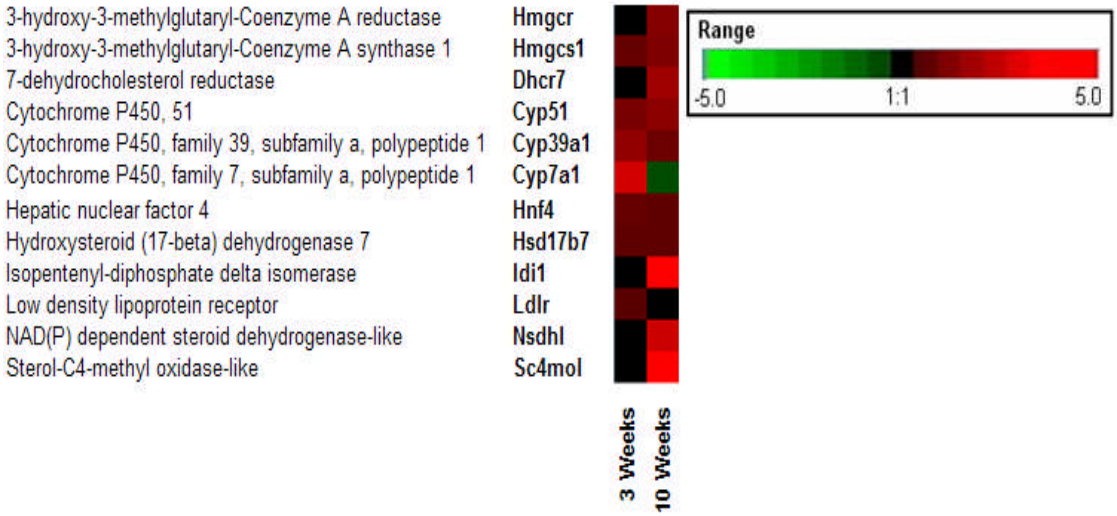


Figure 5.3 (continued) Hepatic genes involved in lipid metabolism regulated by high-fiber feeding at weeks 3 and 10.

(C) Genes involved in cholesterol metabolism and bile acid synthesis



Expression levels of hepatic genes involved in lipid metabolism measured by quantitative RT-PCR

We validated our microarrays findings by conducting real-time PCR assays on selected genes such as Cpt1, Fasn, Hmgcr, Cyp7a1 and Pparα. These genes were selected as they encode enzymes regulating the key steps of lipid metabolism. For instance, Cpt1a is the gene encoding the rate-limiting enzyme for mitochondrial fatty acid oxidation, Fasn encodes a key lipogenic enzyme, Hmgcr encodes the rate-limiting enzyme for cholesterol synthesis. Cyp7a1 encodes the enzyme regulating the rate-limiting step in the neutral pathway of bile acid synthesis. The individual melting curves are presented in Appendix 4.2. The gel electrophoresis of the amplified PCR products is found in Appendix 4.3.

Figure 5.4 presents the gene expression levels of Cpt1L, Fasn and Pparα measured by quantitative RT-PCR. There was a slight but non-significant reduction in the expression of Cpt1a in the mice fed for high-fiber for 3 weeks. At week 10, the high-fiber fed mice showed an increase in their gene expression of Cpt1a as compared to the control mice (non-significant). There was a tendency for the high-fiber fed mice to have a lower expression of Fasn at week 3 but this was not statistically significant from the controls. However, at week 10, we observed a significant increase in Fasn expression levels in the high-fiber fed mice. There were no significant differences in Pparα expression levels between the high fiber mice and their corresponding controls at both weeks 3 and 10.

Figure 5.5 presents the gene expression levels of Hmgcr and Cyp7a1 measured by quantitative RT-PCR. Both PE groups had significantly higher expression of Hmgcr

than their respective control groups ($P < 0.05$). The PE-3wk mice had significantly higher mRNA levels of Cyp7A1 as compared to the C-3wk mice. At week 10, the expression levels of Cyp7A1 were lower in PE-10wk versus C-10wk (not significant).

Figure 5.4 Gene expression levels of Cpt1a, Fasn and Pparα measured by quantitative RT-PCR in liver tissues from control and high-fiber mice at both time-points.

Results are presented as mean ± SD (n = 6 mice/group) *: $P < 0.05$ compared to corresponding control group.

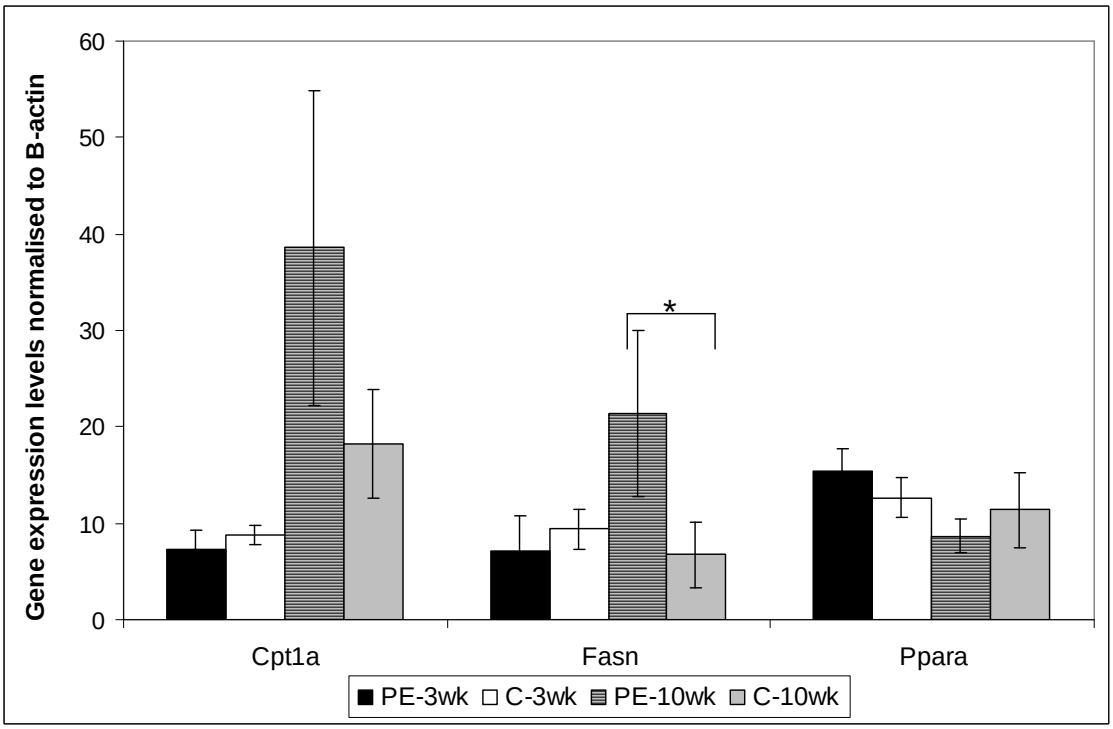
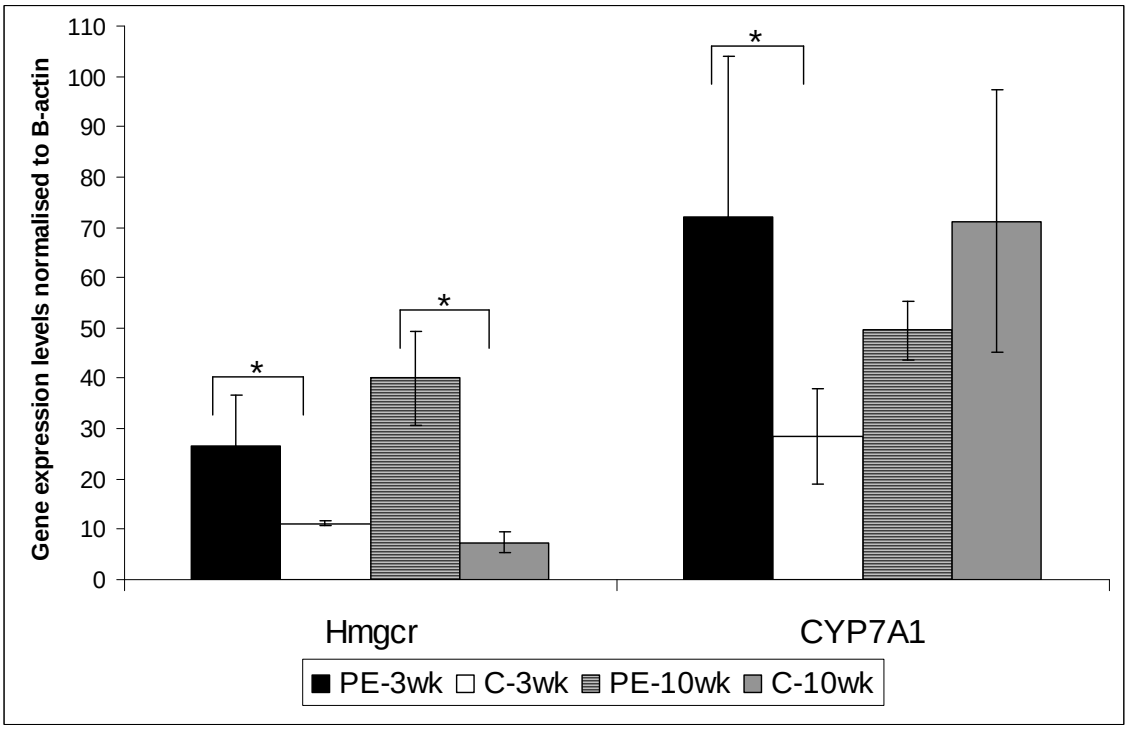


Figure 5.5 Gene expression levels of Hmgcr and Cyp7a1 measured by quantitative RT-PCR in liver tissues from control and high-fiber mice at both time-points.

Results are presented as mean ± SD (n =6 mice/group) *: P < 0.05 compared to corresponding control group.



Western blot assays of 3-hydroxy-3-methylglutaryl-coenzyme reductase and fatty acid synthase

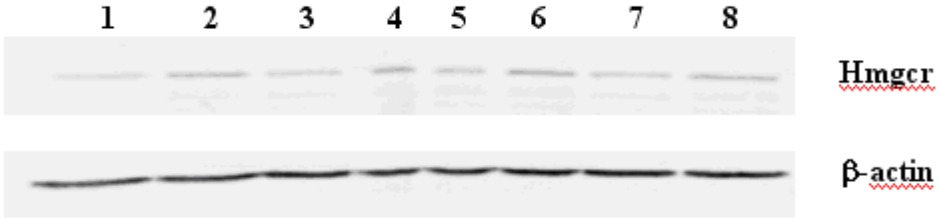
We verified the differential expression of Hmgcr and Fasn at the protein levels by Western blot analysis. The individual gel electrophoresis samples run together with the protein markers are shown in Appendix 5.1. We demonstrated that the PE-10wk group had significantly higher protein expression levels of Hmgcr as compared to the C-10wk group (Figure 5.6). There was no observable difference in protein expression in the PE-3wk group versus the C-3 wk group (data in Appendix 5.1). We also showed that the PE-3 wk group had a significantly lower expression of Fasn protein levels as compared to the C-3week group. At week 10, we observed increased Fasn protein levels in the high-fiber mice (Figure 5.7).

Figure 5.6 Western blot analysis of 3-hydroxy-3-methylglutaryl-coenzyme reductase (Hmgcr) in the livers from control mice and mice fed on psyllium husk

(A) Western blot analysis of Hmgcr in the livers from control mice (odd-numbered lanes) and mice fed on psyllium husks for 10 weeks (even-numbered lanes) (n = 4 mice/group)

(B) Optical density analysis of Hmgcr bands normalized by B-actin bands in the controls compared to the PE-fed group. Each bar represents means ± SD (for each group of mice n = 4) *: P < 0.05 as compared to control group.

A



B

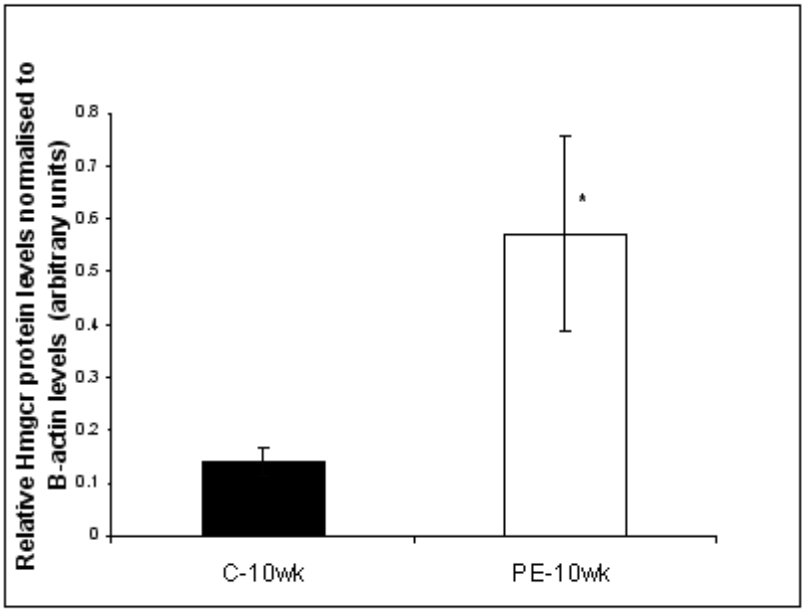
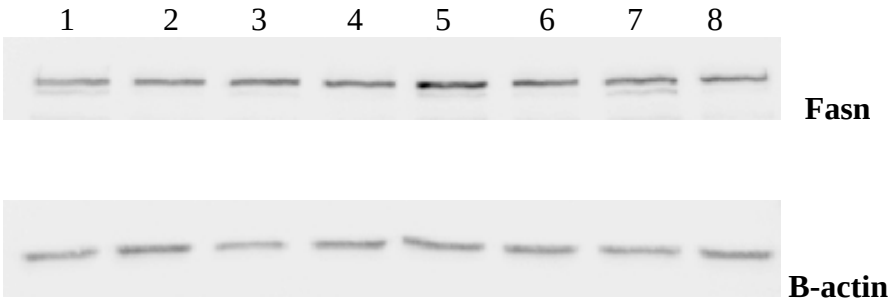


Figure 5.7 Western blot analysis of fatty acid synthase (Fasn) in the livers from the control mice and mice fed on psyllium husk

(A) Western blot analysis of Fasn in the livers from the 3-week control mice (odd-numbered lanes) and mice fed on psyllium husk for 3 weeks (even-numbered lanes) (n = 4 mice/group)



(B) Western blot analysis of Fasn in the livers from the 10-week control group (odd-numbered lanes) and mice fed on psyllium husk for 10 weeks (even-numbered lanes) (n = 4 mice/group)

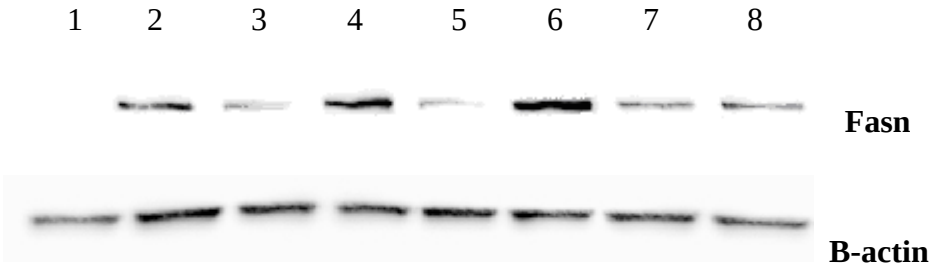
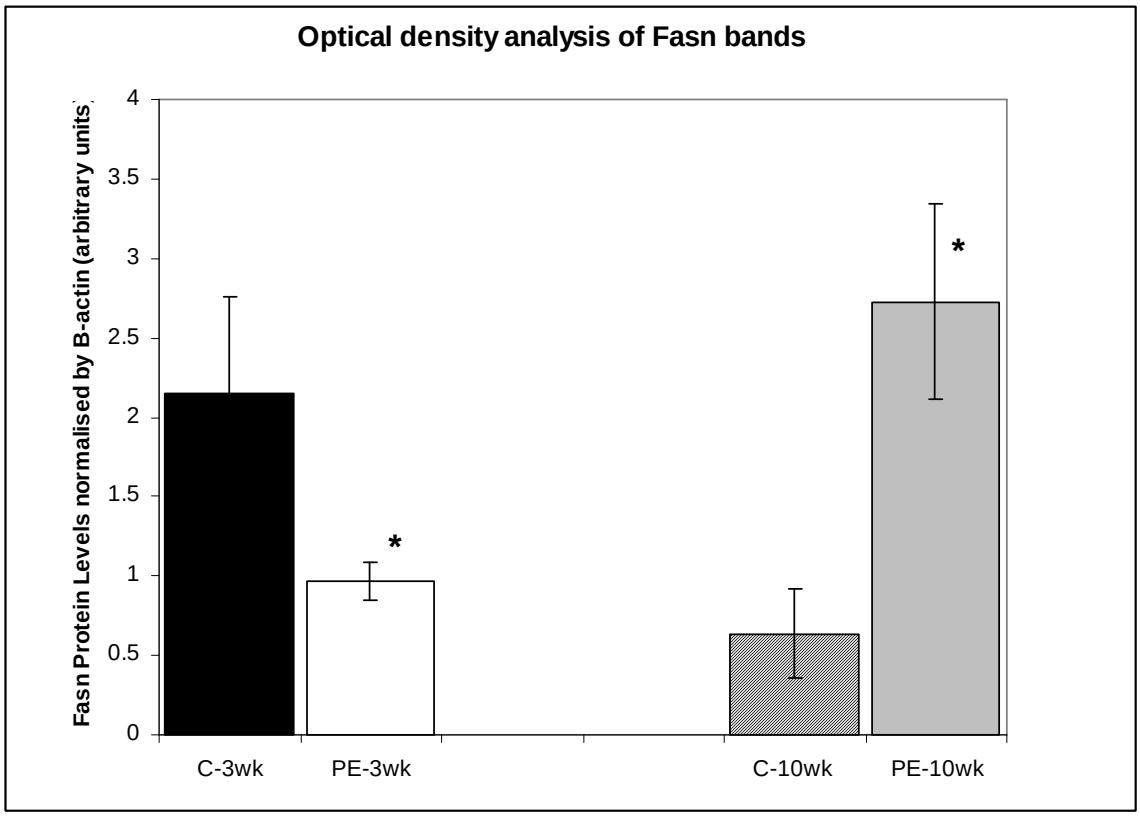


Figure 5.7 (continued) Western blot analysis of fatty acid synthase (Fasn) in the livers from the control mice and mice fed on psyllium husk

(C) Optical density analysis of Fasn bands normalized by B-actin bands in the controls compared to the corresponding PE-fed groups
Each bar represents means ± SD (for each group of mice n = 4)
*: $P < 0.05$ as compared to control group.



DISCUSSION

Sequential effects of a high-fiber diet on food intake and body weight

We observed that the high-fiber fed mice had a lower food intake initially at week 3 but this reduced food intake was not maintained with prolonged high-fiber feeding. Our findings also revealed that a diet high in fiber enriched with psyllium husk did not promote weight loss as the weights of both the PE groups were comparable to the control groups. It was notable that while we observed a reduction in PE-3wk mice energy intake, there was no significant weight loss in them. Since the average energy cost of weight loss is known to be about 30 kJ per gram of body weight, the expected average weight loss in the PE-3wk group should be about 4 grams in the mice over the 3 weeks of high-fiber feeding. Besides the absence of weight loss, the PE-3wk mice had increased their body weights by 0.5 ± 0.4g instead. Therefore, our data over the entire fiber feeding period did not seem to support the theory suggested by others (Krotkiewski, 1984; Raben et al, 1994) that consumption of a high-fiber diet containing viscous soluble fiber would promote weight loss.

Sequential effects of a high-fiber diet on plasma lipids and hepatic gene expression levels

In relation to plasma lipids, the beneficial effects of consuming a high-fiber diet containing psyllium husk were more evident. Within 3 weeks of starting the high-fiber diet, total cholesterol levels were significantly lower than the control mice. The total cholesterol levels remained suppressed with prolonged high-fiber feeding. However, there was no further significant reduction as the feeding period continued. We observed a significant reduction in the HDL cholesterol but not in the LDL cholesterol levels in the high-fiber groups. Perhaps, this could be due to the fact that

major fraction of plasma cholesterol in mice is HDL and not LDL. This could be because mice do not have plasma cholesterol ester transfer protein (CETP) activity.

There are 2 major suggested mechanisms through which soluble fiber can reduce cholesterol. These are either by the inhibition of hepatic cholesterol synthesis or by greater rate of clearance with increased bile acid synthesis. With regards to cholesterol synthesis, we found that none of the genes involved in hepatic cholesterol biosynthesis were down-regulated after high-fiber feeding at both time-points. Instead, genes involved in cholesterol synthesis were found to be up-regulated at week 3. Additional genes were up-regulated at week 10. Some even showed further increases in their gene expression levels. These gene expression levels were further verified by the western blot assay of Hmgcr. Hmgcr encodes the rate-limiting enzyme for cholesterol synthesis. Its protein levels were significantly higher in the high-fiber fed mice at week 10 as compared to the controls. This might be a possible response by the mice to adapt to the reduced plasma cholesterol levels. Miettinen and Tarpila (1989) had suggested that cholesterol synthesis could increase to offset fecal sterol losses when hyperlipidemic subjects were fed soluble viscous fiber. Our observations of the hepatic genes involved in cholesterol synthesis at the mRNA level provided further molecular evidence for their work.

On the other hand, our transcription profiling of the livers showed molecular evidence supporting the concept that there was increased bile acid synthesis in mice fed with high fiber enriched with psyllium husk, as evidenced by the changes in genes such as Cyp7A1, Cyp39A1 and Ldlr. Cyp7A1 governs the rate-limiting step in the neutral pathway of bile acid synthesis from cholesterol. In the liver, cholesterol is converted to bile acids. This removes it from the active cholesterol pool and results in an up-

regulation of hepatic low-density lipoprotein receptors (Ldlr) and decreased plasma cholesterol levels. Cyp7A1 was highly up-regulated in the PE-3wk mice and this was also confirmed by RT-PCR performed on the individual mRNA samples from the PE-3wk mice. Our observation on the increased expression levels of Cyp7a1 in the PE-3wk mice are in agreement with findings reported by Yang et al (2003). They found increased Cyp7a1 mRNA levels in rats fed on barley (which is also high in viscous soluble fiber) for 2 weeks. However, it was notable that the prolonged high-fiber feeding induced a downward trend in Cyp7a1 mRNA levels. The suppressed Cyp7a1 mRNA levels at week 10 could be due to feedback inhibition by the bile acids returning to the liver via enterohepatic circulation.

In addition to Cyp7a1, we found that the PE mice had elevated mRNA levels of Cyp39a1. Cyp39a1 preferentially converts 24-hydroxycholesterol into bile acids as well. Recently, there were numerous studies which suggested a pivotal role of 24-hydroxycholesterol in the link between increased cholesterol levels and the risk of developing Alzheimer's disease, as reviewed by Bjorkhem and colleagues (2006). There is evidence to suggest that the plasma levels of 24-hydroxycholesterol are higher in patients with Alzheimer's disease compared to healthy subjects (Bjorkhem et al., 2006). Our findings with regards to the increased expression levels of Cyp39a1 could possibly highlight another potential benefit of consuming a high-fiber diet enriched with soluble viscous fiber. Our observations suggested that the lowering of plasma cholesterol by psyllium husk could also likely be exerted at a more upstream level of transcription, that is, through the up-regulation of HNF4α. HNF4α is a key hepatic transcription factor which can regulate the expression of Cyp7A1 (Hayhurst et al., 2001).

Although both groups of PE mice did not show any reduced expression of hepatic genes involved in cholesterol synthesis, genes related to lipogenesis were down-regulated at week 3 in the high-fiber fed mice. On the other hand, the genes involved in fatty acid oxidation were up-regulated in PE-3wk mice as compared to C-3wk. However, these trends reversed at week 10. The transcription profiling of the mice livers revealed that prolonged high-fiber feeding resulted in increased mRNA levels of lipogenic genes and reduced mRNA levels of genes involved in fatty acid oxidation. The western blot assays of Fasn, a key gene involved in lipogenesis were also in agreement with its gene expression levels. Its protein levels were significantly lower in the livers of the high-fiber fed mice as compared to the controls at week 3. However, Fasn protein levels were significantly raised with prolonged high-fiber feeding. These differential mRNA levels of the genes involved in lipogenesis suggested that it could be possible that the mice were attempting to adapt to the reduced plasma triglyceride levels. This could also possibly indicate that non-adipose tissue, such as the liver, was responding by regulating their triglyceride content.

In summary, our study sequentially examined the effects of high-fiber feeding enriched with psyllium husk. Whilst body weight was unaffected, plasma cholesterol and triglycerides levels were reduced by feeding such a diet. We reported novel findings that a high-fiber diet enriched with psyllium husk could differentially regulate the expression of hepatic genes involved in lipogenesis as well as cholesterol synthesis. The early response to high-fiber diet aimed at reducing mRNA levels of lipogenic genes and increasing mRNA levels of lipolysis genes, changed after a longer period of high-fiber feeding period. This suggested that different mechanism

may be implemented to adapt the lipid metabolism when there were decreased fat stores in the body. The increased mRNA levels of hepatic genes involved in cholesterol synthesis throughout the whole entire feeding period could possibly suggest a robust regulatory mechanism in place to regulate plasma cholesterol levels. This could also explain why further significant reduction in plasma cholesterol levels was not observed in the PE-10wk mice as compared to the PE-3wk mice.

CHAPTER 6

CONCLUSIONS AND FUTURE RECOMMENDATIONS

CONCLUSIONS

The results obtained from the study on the sequential effects of a high-fat, calorie-dense diet supported our initial hypothesis that the early responses to a high-fat and calorie-dense diet, aimed at using the energy surplus and to prevent excessive fat deposition, changed after a long-term feeding period. Subsequently, it appeared that different mechanisms involved in lipid metabolism in the liver and leptin signalling might possibly be implemented to adapt metabolism for the deposition of body fat with the continued excessive fat and energy intake. Based on our observations, we have proposed a possible model linking the various observations on how a high-fat, calorie-dense diet could have contributed to the various metabolic irregularities. These metabolic irregularities are commonly associated with obesity. These effects are clearly dependent on the duration of the high-fat, calorie-dense diet and became evident when the mice's initial compensatory adaptations failed beyond 4 weeks. It was only after 4 weeks of high-fat, calorie-dense feeding then we observed a marked increase in deposition of white adipose tissue. Our results could possibly help to explain the apparently discrepant findings in regulation of food intake by leptin as well as the gene expression levels involved in lipid metabolism in liver and white adipose tissue. Our findings also 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. Such findings have not been previously described.

In the study on the effects of a high-fiber diet consisting of psyllium husk, contrary to expectations, we found that the hepatic gene transcription profile was one which seemed to be aimed at preventing the lowered plasma levels of cholesterol to be further reduced. Similarly, the gene expression levels of lipogenic genes were also up-regulated with prolonged high-fiber feeding. The contrasting observations in the gene expression levels between these two different diets (i.e. the high-fat, calorie-dense diet and the high-fiber diet) suggested that the expression levels of the genes appeared to be rigorously regulated to ensure that our bodies would always have sufficient lipid stores regardless of abundance (as induced by high-fat, calorie dense feeding) or in deficiciency (as induced by high-fiber feeding).

FUTURE RECOMMENDATIONS

Quantification of Cpt1L, Fas, Lepr, Socs3 mRNA levels in the hypothalamus

In this thesis, we have examined the expression levels of the following genes in the liver and adipose tissue (i.e. Cpt1L, Fas, Lepr, Socs3). These novel findings have shown that the expression levels of these genes were differentially regulated over the entire HFC feeding period and these genes could also possibly play a role in regulating the feeding behaviour as well as the energy homeostasis in the mice, exerting their influence outside the central nervous system. It is known that leptin reduces the hypothalamic expression of neuropeptide Y, the effector stimulating food intake. In addition, it has also been documented that the inhibition of hypothalamic Cpt1 activity was sufficient to diminish food intake (Obici et al., 2003). Examining the transcription profiling of the hypothalamic expression of these genes concurrently with the profile of our current reported findings could lead to a better understanding of the regulation of food intake in the mice over the entire feeding period.

Measurement of the lipid fluxes

Although we have confirmed and validated the differentially expression of the genes as well as the enzyme concentrations of these proteins encoded by these genes involved in lipid metabolism, these changes may not actually reflect the lipid metabolism fluxes in the mice. For instance, cofactors or the availability of metabolic substrates could affect lipid metabolic pathways, independent of these enzyme levels. Alternatively, the enzyme activities could also be regulated allosterically. Therefore, it is suggested that further work to conduct direct measurements of these lipid fluxes (e.g. measurement of triglyceride synthesis and degradation (lipolysis) be conducted to gain a better insight.

Analysis of other differentially expressed genes

This study has selected a few key genes encoding well-studied enzymes regulating key steps of lipogenesis, cholesterol synthesis and fatty acid ovidation to carry further validation with qRT-PCR experiments. It will also be interesting to examine differentially regulated genes involved in other aspects of lipid metabolism (namely phospholipid, sphingolipid and eicosanoid metabolism). Moreover, the gene expression profiling of the livers and the adipose tissue also showed differential expression of genes involved in cell cycle, stress signalling and inflammatory pathways. The detailed analysis of these other genes could provide valuable insights into the other detrimental effects of consuming a high-fat, energy dense diet which could be related to the onslaught of the other chronic nutrition-related diseases. Moreover, a significant number of the differentially expressed genes examined do not have known functional ontology. To identify their possible functions, principle component anaylsis or hierarchical clustering, which maps these novel genes to genes

with known function, could be useful. This is an attractive strategy, as it can potentially lead to the discovery of novel genes.

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APPENDIX 3.1
NUTRIENT COMPOSITION OF THE DIETS USED

Nutrient composition of the high-fat, calorie-dense diet (Specialty Feeds, SF 00-219)

This diet for laboratory mice and rats was formulated to mimic a "Western fast food diet". This diet was designed to be equivalent to *Harlan Teklad* TD88137 or *Research Diets* Western Diet D12079B.

Nutritional parameters	
Protein	19 %
Total Fat	21 %
Crude Fibre	4.7 %
Acid Detergent Fibre	4.7 %
Digestible Energy	19.4 MJ / Kg

Ingredients	
Casein (Acid)	195 g/Kg
DL Methionine	3.0 g/Kg
Sucrose	341 g/Kg
Wheat Starch	154 g/Kg
Cellulose	50 g/Kg
Clarified Butter (Ghee)	210 g/Kg
Calcium Carbonate	17.1 g/Kg
Sodium Chloride	2.6 g/Kg
Pottasium Citrate	2.5 g/Kg
Pottasium Dihydrogen Phosphate	6.9 g/Kg
Pottasium Sulphate	1.6 g/Kg
AIN93G Trace minerals	1.4 g/Kg
Choline Chloride (65%)	2.5 g/Kg
SF00-219 Vitamins	10 g/Kg
USP Cholesterol	15 g/Kg
Etoxyquin (66%)	0.04 g/Kg

Amino Acids	
Valine	1.2 %
Leucine	1.7 %
Isoleucine	0.8 %
Threonine	0.7 %
Methionine	0.8 %
Cystine	0.05 %
Lysine	1.5 %
Phenylalanine	0.9 %
Tyrosine	1.0 %
Tryptophan	0.3 %

Total Minerals	
Calcium	0.58 %
Phosphorous	0.30 %
Magnesium	0.09 %
Sodium	0.11 %
Chloride	0.16 %
Potassium	0.4 %
Sulphur	0.22 %
Iron	90 mg / Kg
Copper	6.7 mg / Kg
Iodine	0.22 mg / Kg
Manganese	20 mg / Kg
Cobalt	No data
Zinc	40 mg / Kg
Molybdenum	No data
Selenium	0.15 mg / Kg
Cadmium	No data
Chromium	2.0 mg / Kg
Lithium	0.1 mg / Kg
Boron	0.7 mg / Kg
Nickel	0.55 mg / Kg
Vanadium	0.10 mg / Kg

Total Vitamins	
Vitamin A (Retinol)	10,000 IU / Kg
Vitamin D3 (Cholecalciferol)	1,100 IU / Kg
Vitamin E (a Tocopherol acetate)	62 mg / Kg
Vitamin K (Menadione)	12 mg / Kg
Vitamin C (Ascorbic acid)	700 mg / Kg
Vitamin B1 (Thiamine)	11 mg / Kg
Vitamin B2 (Riboflavin)	11 mg / Kg
Niacin (Nicotinic acid)	50 mg / Kg
Vitamin B6 (Pyridoxine)	11 mg / Kg
Pantothenic acid	33 mg / Kg
Biotin	200 ug / Kg
Folic acid	1 mg / Kg
Inositol	55 mg / Kg
Vitamin B12 (Cyanocobalamin)	18 mg / Kg
Choline	2,500 mg / Kg

Fat Composition	
Saturated fats C12:0 or less	2.4 %
Myristic Acid 14:0	2.3 %
Palmitic Acid 16:0	6.1 %
Stearic Acid 18:0	1.9 %
Arachidic Acid 20:0	0.5 %
Palmitoleic Acid 16:1	1.0 %
Oleic Acid 18:1	5.6 %
Gadoleic Acid 20:1	trace
Linoleic Acid 18:2 n6	0.8 %
a Linolenic Acid 18:3 n3	trace
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	No data
DHA 22:6 n3	No data
Total Carotenoid	No data
Total Phospholipid	No data
Cholesterol	0.15 %

Nutrient composition of the control diet used in high-fat, calorie-dense diet experiment (Specialty Feeds, Meat free rat and mouse)

A fixed formulation diet for Laboratory Rats and Mice fortified with vitamins and minerals to meet the requirements of breeding animals. The formulation is designed to be fed ad-libitum to rodents of all ages.

Nutritional parameters	
Protein	19.0 %
Total Fat	4.6 %
Crude Fibre	4.5 %
Digestible Energy	14.3 MJ / Kg

Trace Minerals	
Magnesium	100 mg / Kg
Iron	70 mg / Kg
Copper	16 mg / Kg
Iodine	0.5 mg / Kg
Manganese	70 mg / Kg
Zinc	60 mg / Kg
Molybdenum	0.5 mg / Kg
Selenium	0.1 mg / Kg

Vitamins	
Vitamin A (Retinol)	10,000 IU / Kg
Vitamin D3 (Cholecalciferol)	2,000 IU/Kg
Vitamin K (Menadione)	2 mg / Kg
Vitamin E (aTocopherol acetate)	100 mg / Kg
Vitamin B1 (Thiamine)	6 mg / Kg
Vitamin B2 (Riboflavin)	6 mg / Kg
Niacin (Nicotinic acid)	25 mg / Kg
Vitamin B6 (Pyridoxine)	6 mg / Kg
Calcium Pantothenate	20 mg / Kg
Biotin	100 ug / Kg
Folic acid	2 mg / Kg
Vitamin B12 (Cyanocobalamin)	30 ug / Kg

Amino Acids	
Valine	0.84 %
Leucine	1.3 %
Isoleucine	0.7 %
Threonine	0.7 %
Methionine	0.2 %
Cystine	0.3 %
Lysine	0.9 %
Phenylalanine	0.8 %
Tyrosine	0.5 %
Tryptophan	0.2 %

Total Vitamins	
Vitamin A (Retinol)	10,900 IU / Kg
Vitamin D3 (Cholecalciferol)	> 2,000 IU / Kg
Vitamin E (Tocopherol)	110 mg / Kg
Vitamin K (Menadione)	2 mg / Kg
Vitamin C (Ascorbic acid)	no data
Vitamin B1 (Thiamine)	10 mg / Kg
Vitamin B2 (Riboflavin)	7.3 mg / Kg
Niacin (Nicotinic acid)	70 mg / Kg
Vitamin B6 (Pyridoxine)	9 mg / Kg
Pantothenic acid	30 mg / Kg
Biotin	200 ug / Kg
Folic acid	2.5 mg / Kg
Inositol	no data
Vitamin B12 (Cyanocobalamin)	30 ug / Kg
Choline	1000 mg / Kg

Total Minerals	
Calcium	0.75 %
Phosphorous	0.7 %
Magnesium	0.2 %
Sodium	0.2 %
Potassium	0.7 %
Sulphur	0.1 %
Iron	370 mg / Kg
Copper	24 mg / Kg
Iodine	0.5 mg / Kg
Manganese	340 mg / Kg
Cobalt	no data
Zinc	90 mg / Kg
Molybdenum	0.5 mg / Kg
Selenium	0.4 mg / Kg
Cadmium	no data
Chromium	no data

Fat Composition	
Myristic Acid 14:0	0.03 %
Palmitic Acid 16:0	0.5 %
Stearic Acid 18:0	0.15 %
Palmitoleic Acid 16:1	0.01 %
Oleic Acid 18:1	1.9 %
Gadoleic Acid 20:1	0.03 %
Linoleic Acid 18:2 n6	1.3 %
a Linolenic Acid 18:3 n3	0.3 %
Arachadonic Acid 20:4 n6	trace
EPA 20:5 n3	0.04 %
DHA 22:6 n3	0.08 %
Total Carotenoid	no data
Total Phospholipid	no data
Cholesterol	trace

Nutrient composition of the high-fiber diet used in high-fiber feeding experiment (Specialty Feeds, SF03-034)

A semi-pure diet formulation for laboratory rats and mice based on AIN-93M. Fibre content of the diet has been increased by inclusion of Psyllium husk.

Nutritional parameters	
Protein	13.5 %
Total Fat	4 %
Crude Fibre	9.2 %
Acid Detergent Fibre	14.1 %
Digestible Energy	13.8 MJ / Kg

Ingredients	
Casein (Acid)	140 g/Kg
DL Methionine	1.8 g/Kg
Sucrose	100 g/Kg
Wheat Starch	372 g/Kg
Dextrinised Starch	155 g/Kg
Cellulose	50 g/Kg
Psyllium Husk (95 - 98%)	100 g/Kg
Canola Oil	40 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
Potassium Citrate	1.0 g/Kg
Potassium Dihydrogen Phosphate	8.8 g/Kg
Potassium Sulphate	1.6 g/Kg
AIN93G Trace minerals	1.4 g/Kg
Choline Chloride (65%)	2.5 g/Kg
AIN93G Vitamins	10 g/Kg

Amino Acids	
Valine	0.9 %
Leucine	1.3 %
Isoleucine	0.6 %
Threonine	0.6 %
Methionine	0.55 %
Cystine	0.05 %
Lysine	1.0 %
Phenylalanine	0.7 %
Tyrosine	0.7 %
Tryptophan	0.2 %

Total Minerals	
Calcium	0.45 %
Phosphorous	0.3 %
Magnesium	0.08 %
Sodium	0.13 %
Chloride	0.16 %
Potassium	0.4 %
Sulphur	0.16 %
Iron	82 mg / Kg
Copper	6.4 mg / Kg
Iodine	0.2 mg / Kg
Manganese	15 mg / Kg
Cobalt	no data
Zinc	34 mg / Kg
Molybdenum	no data
Selenium	0.15 mg / Kg
Cadmium	no data
Chromium	2.0 mg / Kg
Lithium	0.1 mg / Kg
Boron	0.7 mg / Kg
Nickel	0.55 mg / Kg
Vanadium	0.10 mg / Kg

Total Vitamins	
Vitamin A (Retinol)	4,000 IU / Kg
Vitamin D3 (Cholecalciferol)	1,000 IU / Kg
Vitamin E (a Tocopherol acetate)	75 mg / Kg
Vitamin K (Menadione)	1 mg / Kg
Vitamin C (Ascorbic acid)	none added
Vitamin B1 (Thiamine)	6 mg / Kg
Vitamin B2 (Riboflavin)	6 mg / Kg
Niacin (Nicotinic acid)	30 mg / Kg
Vitamin B6 (Pyridoxine)	7 mg / Kg
Pantothenic acid	16 mg / Kg
Biotin	200 ug / Kg
Folic acid	2 mg / Kg
Inositol	none added
Vitamin B12 (Cyanocobalamin)	100 mg / Kg
Choline	1600 mg / Kg

Fat Composition	
Myristic Acid 14:0	trace
Palmitic Acid 16:0	0.2 %
Stearic Acid 18:0	0.1 %
Palmitoleic Acid 16:1	trace
Oleic Acid 18:1	2.4 %
Gadoleic Acid 20:1	trace
Linoleic Acid 18:2 n6	0.8 %
a Linolenic Acid 18:3 n3	0.4 %
Arachadonic Acid 20:4 n6	trace
EPA 20:5 n3	trace
DHA 22:6 n3	trace
Total Carotenoid	no data
Total Phospholipid	no data
Cholesterol	no data

Nutrient composition of the control diet used in high-fiber feeding experiment (Specialty Feeds, AIN-93M)

This formulation satisfies the maintenance nutritional requirements of adult rats and mice.

Nutritional parameters	
Protein	13.5 %
Total Fat	4 %
Crude Fibre	4.7 %
Acid Detergent Fibre	4.7 %
Digestible Energy	15.2 MJ / Kg

Ingredients	
Casein (Acid)	140 g/Kg
DL Methionine	1.8 g/Kg
Sucrose	100 g/Kg
Wheat Starch	472 g/Kg
Dextrinised Starch	155 g/Kg
Cellulose	50 g/Kg
Canola Oil	40 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
Potassium Citrate	1.0 g/Kg
Potassium Dihydrogen Phosphate	8.8 g/Kg
Potassium Sulphate	1.6 g/Kg
AIN93G Trace minerals	1.4 g/Kg
Choline Chloride (65%)	2.5 g/Kg
AIN93G Vitamins	10 g/Kg

Amino Acids	
Valine	0.9 %
Leucine	1.3 %
Isoleucine	0.6 %
Threonine	0.6 %
Methionine	0.55 %
Cystine	0.05 %
Lysine	1.0 %
Phenylalanine	0.7 %
Tyrosine	0.7 %
Tryptophan	0.2 %

Total Minerals	
Calcium	0.45 %
Phosphorous	0.3 %
Magnesium	0.08 %
Sodium	0.13 %
Chloride	0.16 %
Potassium	0.4 %
Sulphur	0.16 %
Iron	82 mg / Kg
Copper	6.4 mg / Kg
Iodine	0.2 mg / Kg
Manganese	15 mg / Kg
Cobalt	no data
Zinc	34 mg / Kg
Molybdenum	no data
Selenium	0.15 mg / Kg
Cadmium	no data
Chromium	2.0 mg / Kg
Lithium	0.1 mg / Kg
Boron	0.7 mg / Kg
Nickel	0.55 mg / Kg
Vanadium	0.10 mg / Kg

Total Vitamins	
Vitamin A (Retinol)	4,000 IU / Kg
Vitamin D3 (Cholecalciferol)	1,000 IU / Kg
Vitamin E (a Tocopherol acetate)	75 mg / Kg
Vitamin K (Menadione)	1 mg / Kg
Vitamin C (Ascorbic acid)	none added
Vitamin B1 (Thiamine)	6 mg / Kg
Vitamin B2 (Riboflavin)	6 mg / Kg
Niacin (Nicotinic acid)	30 mg / Kg
Vitamin B6 (Pyridoxine)	7 mg / Kg
Pantothenic acid	16 mg / Kg
Biotin	200 ug / Kg
Folic acid	2 mg / Kg
Inositol	none added
Vitamin B12 (Cyanocobalamin)	100 mg / Kg
Choline	1600 mg / Kg

Fat Composition	
Myristic Acid 14:0	trace
Palmitic Acid 16:0	0.2 %
Stearic Acid 18:0	0.1 %
Palmitoleic Acid 16:1	trace
Oleic Acid 18:1	2.4 %
Gadoleic Acid 20:1	trace
Linoleic Acid 18:2 n6	0.8 %
a Linolenic Acid 18:3 n3	0.4 %
Arachadonic Acid 20:4 n6	trace
EPA 20:5 n3	trace
DHA 22:6 n3	trace
Total Carotenoid	no data
Total Phospholipid	no data
Cholesterol	no data

APPENDIX 3.2

COLLECTION OF BLOOD BY INTRACARDIAC PUNCTURE

Materials

- Disposable gloves
- Syringe (1 m1)
- Hypodermic needle (25g)
- 70% ethanol
- Gauze

Procedures

1. Anaesthetize mouse with the CRC mixture.
2. Lay the mouse flat on its back.
3. Sterilize the site of the needle insertion with 70% ethanol.
4. Insert the needle at the base of the sternum at a 15-20^{o} angle just lateral of the midline. Aspirate the syringe slowly.
5. Euthanatize the mouse

APPENDIX 3.3

COLLECTION OF BLOOD FROM THE TAIL

Materials

- Disposable gloves
- Syringe (1 m1)
- Hypodermic needle (25g)
- 70% ethanol
- Gauze

Procedures

1. Anaesthetize mouse with the CRC mixture.
2. Lay the mouse flat on its back with its tail hanging down. The tail is soaked in warm water for 5mins. Wipe it dry.
3. Sterilize the site of the needle insertion with 70% ethanol.
4. Insert the needle into the ventral artery of the tail. Aspirate the syringe slowly.
5. Collect about 0.15 ml (Maximum volume 0.2ml) of blood

APPENDIX 3.4

EXTRACTION OF TOTAL RNA BY TRIZOL® REAGENT

Principle

TRIzol® reagent (Invitrogen) is a ready-to-use reagent for the isolation of total RNA from cells or tissues. It contains phenol and guanidine isothiocyanate. During sample homogenization or lysis, it maintains the integrity of the RNA, while disrupting cells and dissolving cell components. After the addition of chloroform and followed by centrifugation, the solution is separated into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. Total RNA isolated by TRIzol® reagent is free of protein and DNA contamination. The isolated RNA has a A260/A280 ratio ≥1.8 when diluted into TE and A260/A280 ratio of 1.5-1.8 when diluted in distilled water.

Procedures

1. HOMOGENIZATION OF THE TISSUES

Homogenize about 50mg of the liver tissue samples or about 200mg of white adipose tissue in 1 ml of TRIzol® reagent using chilled mortar and pestle. An additional isolation step is performed for the white adipose tissue. Following homogenization, centrifuge the solution at 12,000 × *g* for 10 minutes at 4°C. A layer of fat collects as a top layer which is removed. Transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation.

2. PHASE SEPARATION

Incubate the homogenized samples for 5 minutes at room temperature (23°C) to allow the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIzol® reagent. Cap tubes securely. Vortex tubes for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at 12,000 × *g* for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA PRECIPITATION

Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with adding 0.25 ml of isopropyl alcohol and 0.25 ml of a sodium chloride sodium citrate solution (1.2M sodium chloride and 0.8 M sodium citrate). Incubate the samples at room temperature (23°C) for 10 minutes and centrifuge at no more than 12,000 × *g* for 10 minutes at 4°C. The RNA precipitate, after centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA WASH

Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at 6000 × *g* for 5 minutes at 4°C.

5. REDISSOLVING THE RNA

Remove the supernatant. Air-dry the RNA pellet briefly (5 minutes). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in RNase-free water (liver : use 60 ul of RNase-free water ; white adipose tissue : use 30ul of RNase-free water).

APPENDIX 3.5

PURIFICATION OF TOTAL RNA BY USING RNEASY ® MINI KIT

Principle

The RNeasy ® Mini Kit can be used to purify RNA. Guanidine isothiocyanate-containing lysis buffer and ethanol are added to the sample to promote selective binding of RNA to the silica-gel membranes inside the RNeasy mini columns. Contaminants are efficiently washed away and high-quality, pure RNA is eluted in RNase-free water.

Procedures

1. Adjust total RNA sample (not exceeding 100ug of total RNA per sample) to a final volume of 100 ul with RNase-free water. Add 350 ul Buffer RLT (containing guanidine isothiocyanate) and mix thoroughly.

2. Add 250 ul ethanol (96-100%) to the diluted RNA, and mix thoroughly by pipetting.

3. Apply the sample (700 ul) to an RNeasy mini column placed in a 2 ml collection tube. Centrifuge for 15s at $\geq$8000 x g at 23°C. Discard the flow-through and collection tube.

4. Transfer the RNeasy column into a new 2 ml collection tube (supplied). Pipet 500ul Buffer RPE onto the RNeasy column. Centrifuge for 15s at ≥8000 x g at 23°C. Discard the flow-through.

5. Add another 500 ul Buffer RPE to the RNeasy column. Centrifuge for 2 mins at >_8000 x g to dry the RNeasy silica-gel membrane.

6. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipette 30 ul of RNase-free water directly onto the RNeasy silica-gel membrane. Centrifuge for 1 min at >_8000 x g >_10,000 rpm) to elute.

APPENDIX 3.6

PROTOCOL FOR cDNA SYNTHESIS AND BIOTIN-LABELLED cRNA SYNTHESIS FOR HYBRIDIZATION TO AFFYMETRIX GENECHIPS ®

(Adapted from Affymetrix Genechip® expression analysis technical manual)

Procedures for first-strand cDNA synthesis (one-cycle)

1. Mix RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) Primer as follows:

Starting material	**Volume**
Sample RNA	Variable
Diluted poly-A RNA controls	2 ul
T7-Oligo(dT) Primer 50 μM	2 ul
RNase-free Water	Variable
Total Volume	**11 ul**

Incubate the reaction in the thermal cycler according to the following program:

70°C 10 minutes

4°C 2 minutes

(Perform all incubations steps in thermal cycler.)

2. In a separate tube, assemble the First-Strand Master Mix as follows:

Starting material	**Volume**
5X 1st Strand Reaction Mix	4 ul
DTT 0.1M	2 ul
dNTP 10 mM	1 ul
Total Volume	7 ul

3. Transfer 7 ul of First-Strand Master Mix to each RNA/T7-Oligo(dT) Primer mix for a final volume of 18 ul. Mix thoroughly by flicking the tube a few times. Centrifuge briefly (5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.

4. Incubate for 2 minutes at 42°C in the thermal cycler. Add 2 ul of SuperScript II to each RNA sample for a final volume of 20 ul.

5. Incubate for 1 hour at 42°C; then cool the sample for at least 2 minutes at 4°C .

Procedures for second-strand cDNA synthesis (one-cycle)

1. Add 130 ul of Second-Strand Master Mix to each first-strand synthesis sample for a total volume of 150 ul.

Preparation of Second-Strand Master Mix

Starting material	**Volume**
RNase-free Water	91 ul
5X 2nd Strand Reaction Mix	30 ul
dNTP 10 mM	3 ul
E. coli DNA ligase	1 ul
E. coli DNA Polymerase I	4 ul
RNase H	1 ul
Total Volume	130 ul

2. Incubate for 2 hours at 16°C.

3. Add 2 ul of T4 DNA Polymerase to each sample and incubate for 5 minutes at 16°C.

4. After incubation with T4 DNA Polymerase add 10 ul of EDTA, 0.5M and proceed immediately to *Cleanup of Double-Stranded cDNA*

Procedures for cleanup of double-stranded cDNA

1. Add 600 ul of cDNA Binding Buffer to the double-stranded cDNA synthesis preparation. Mix by vortexing for 3 seconds.

2. Apply 500 ul of the sample to the cDNA Cleanup Spin Column sitting in a 2 mL Collection Tube (supplied), and centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

3. Reload the spin column with the remaining mixture and centrifuge as above. Discard flow-through and Collection Tube.

4. Transfer spin column into a new 2 mL Collection Tube (supplied). Pipette 750 ul of the cDNA Wash Buffer onto the spin column. Centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

5. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.

6. Transfer spin column into a 1.5 mL Collection Tube, and pipette 14 ul of cDNA Elution Buffer directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.

Procedures for synthesis of biotin-labelled cRNA

1. Add the following reaction components in the order as indicated in the below table.

Starting material	**Volume**
Template cDNA	6ul
RNase-free Water	14 ul
10X IVT Labelling Buffer	4 μL
IVT Labelling NTP Mix	12 μL
IVT Labelling Enzyme Mix	4 μL
Total Volume	**40 μL**

2**.** Incubate at 37°C for 16 hours (perform overnight).

Procedures for the clean-up of biotin-labelled cRNA

1. Add 50 ul of RNase-free Water to the IVT reaction and vortex for 3 seconds.

2. Add 350 ul IVT cRNA Binding Buffer to the sample and vortex for 3 seconds.

3. Add 250 ul ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.

4. Apply sample (700 ul) to the IVT cRNA Cleanup Spin Column sitting in a 2 mL Collection Tube. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through and Collection Tube.

5. Transfer the spin column into a new 2 mL Collection Tube. Pipette 500 ul IVT cRNA Wash Buffer onto the spin column. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

6. Pipet 500 ul 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.

8. Transfer spin column into a new 1.5 mL Collection Tube (supplied), and pipette 13 ul of RNase-free Water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute. The average volume of eluate is 11 ul from 13 ul RNase-free Water.

9. Use spectrophotometric analysis to determine the cRNA yield. Calculate the adjusted cRNA yield as follows: Adjusted cRNA yield = RNAm - (total RNAi) (y)

RNAm = amount of cRNA measured after IVT (μg)

total RNAi = starting amount of total RNA (μg)

y = fraction of cDNA reaction used in IVT

Procedures for the fragmentation of cRNA

1. Prepare the fragmentation reaction mix as follows:

Starting material	**Volume**
cRNA	20 μg (1 to 21 μL)
5X Fragmentation Buffer	8 μL
RNase-free Water	(variable) to 40 μL
Total Volume	40 μL

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.

3. Store undiluted, fragmented sample cRNA at –70°C until ready to perform the hybridization to genechip arrays.

APPENDIX 4.1

DAILY FOOD INTAKE (GRAMS) OVER A SEVEN-DAY PERIOD

C_{Test} Group denotes the group of mice receiving the Control C diet
H_{Test} Group denotes the group of mice receiving the High-fat, calorie-dense (HFC) diet

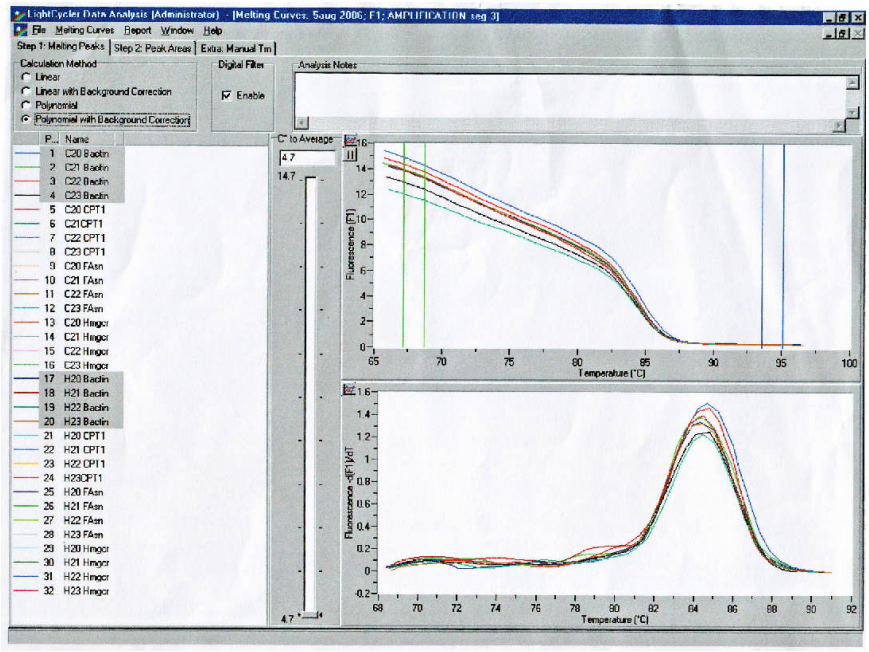
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
C_{Test0}	3.13	2.92	3.04	3.06	3.18	3.15	3.12
C_{Test1}	2.94	3.05	3.03	3.10	3.11	2.97	3.17
C_{Test2}	2.96	2.93	2.92	3.18	3.10	2.97	2.96
C_{Test3}	2.90	3.08	3.08	3.11	3.16	3.14	3.04
C_{Test4}	3.14	3.18	2.97	2.95	2.90	3.18	3.12
C_{Test5}	3.17	2.93	3.04	3.14	2.90	3.10	3.17
C_{Test6}	3.11	3.11	3.13	3.08	2.95	3.11	3.16
C_{Test7}	3.16	3.01	2.96	3.06	3.04	3.01	3.04

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
H_{Test0}	3.27	3.21	3.24	3.28	3.39	3.28	3.39
H_{Test1}	3.32	3.22	3.28	3.23	3.24	3.22	3.35
H_{Test2}	3.24	3.30	3.30	3.39	3.29	3.21	3.26
H_{Test3}	3.24	3.34	3.37	3.20	3.20	3.32	3.36
H_{Test4}	3.28	3.36	3.20	3.35	3.33	3.37	3.22
H_{Test5}	3.36	3.37	3.32	3.21	3.37	3.36	3.20
H_{Test6}	3.34	3.26	3.24	3.39	3.39	3.24	3.24
H_{Test7}	3.32	3.39	3.38	3.21	3.37	3.24	3.23

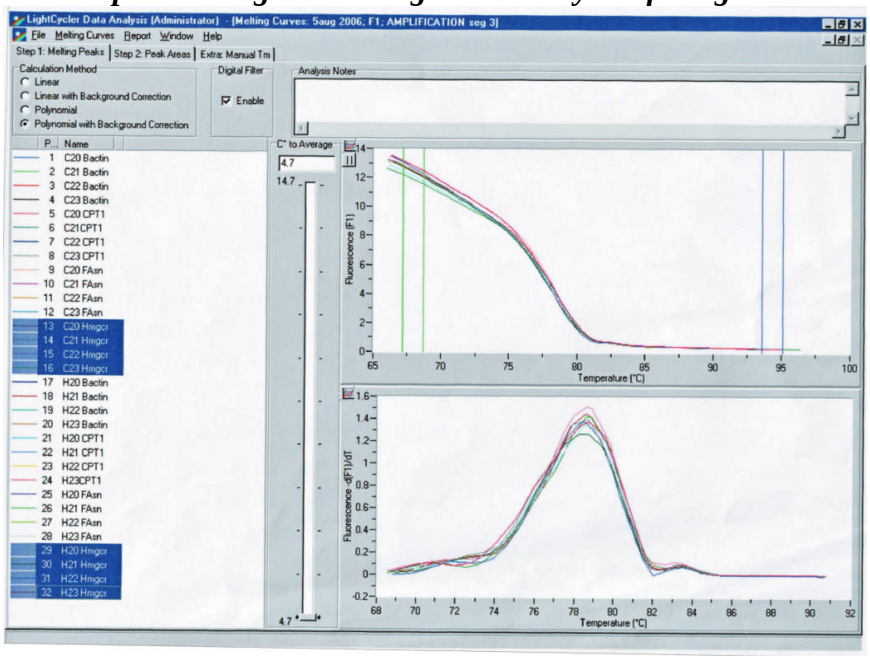
APPENDIX 4.2

EXAMPLES OF MELTING CURVE ANALYSIS FOR RT-PCR EXPERIMENTS

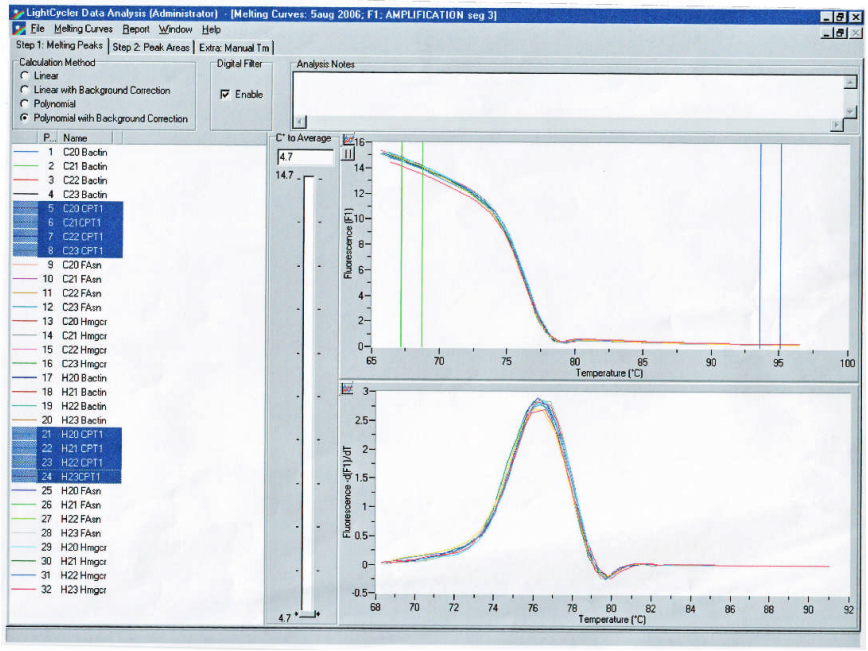
An example showing the melting curve analysis of B-actin



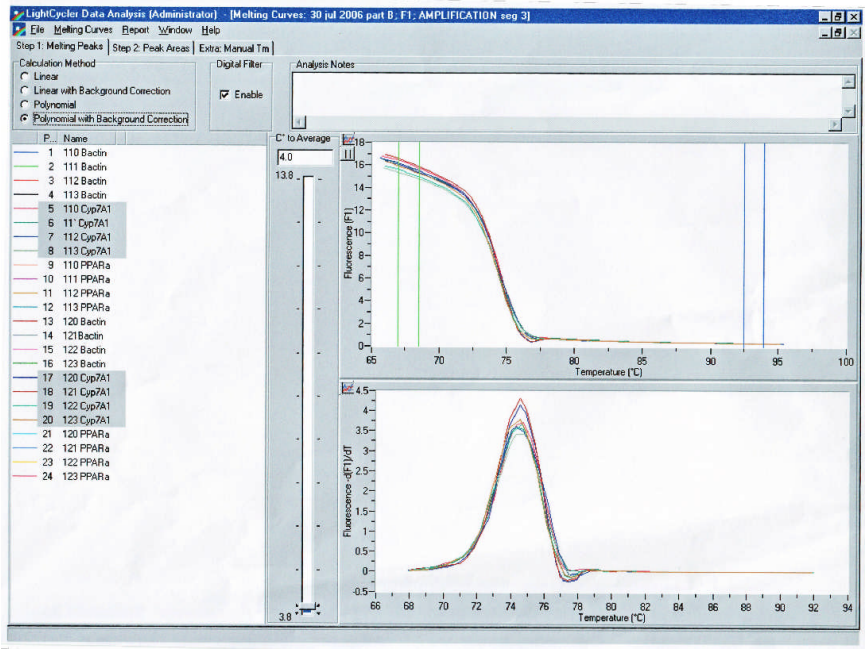
An example showing the melting curve analysis of Hmgcr



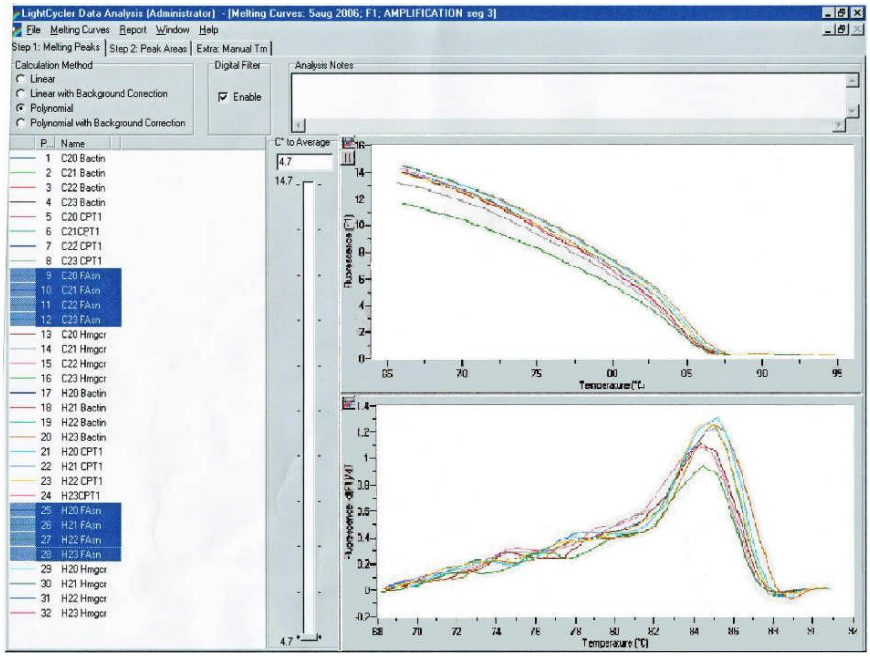
An example showing the melting curve analysis of Cpt1L



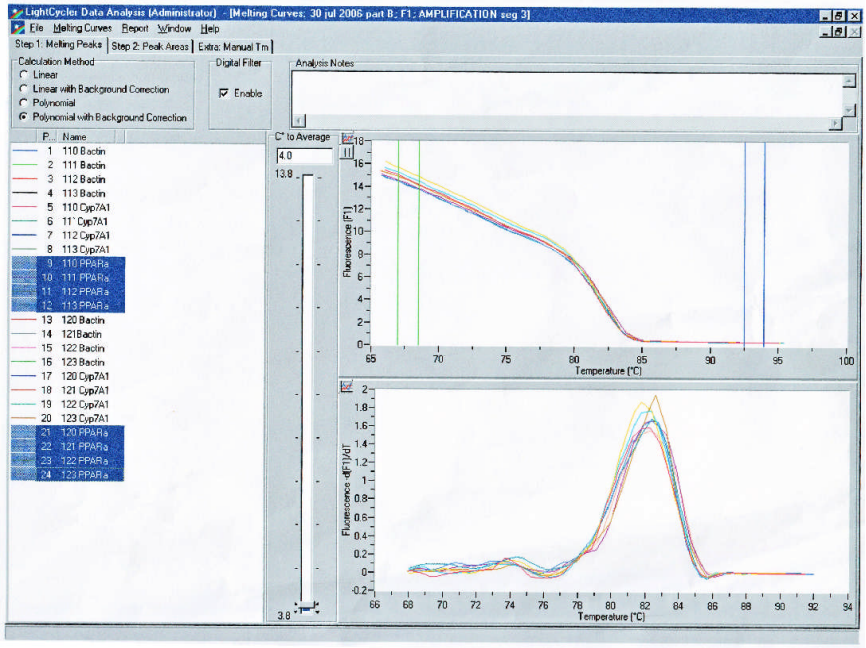
An example showing the melting curve analysis of Cyp7a1



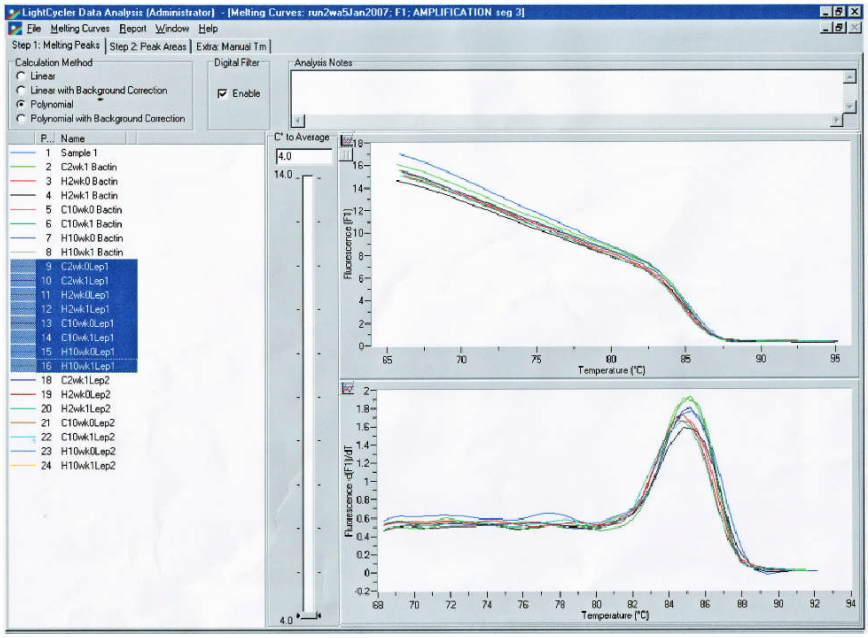
An example showing the melting curve analysis of Fasn



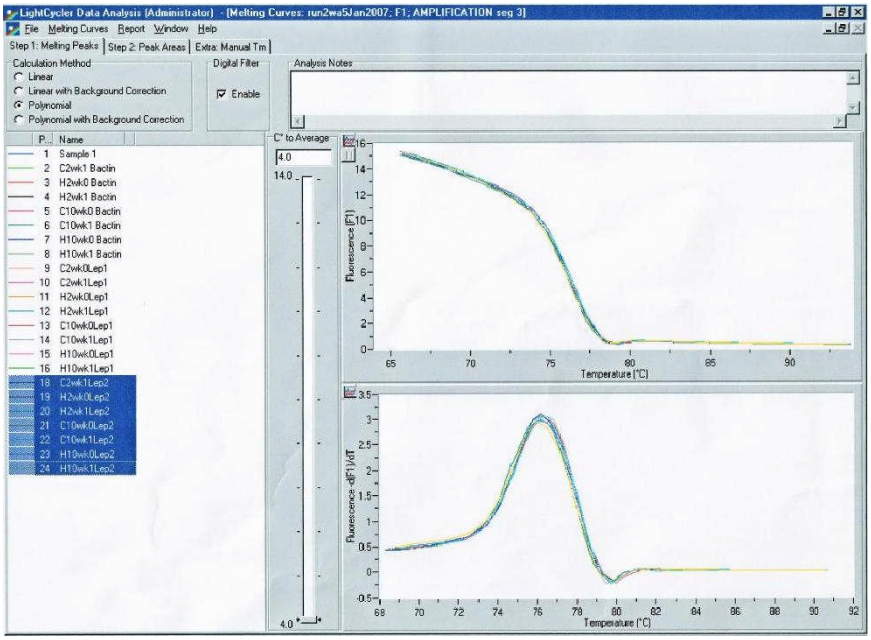
An example showing the melting curve analysis of Pparα



An example showing the melting curve analysis of leptin



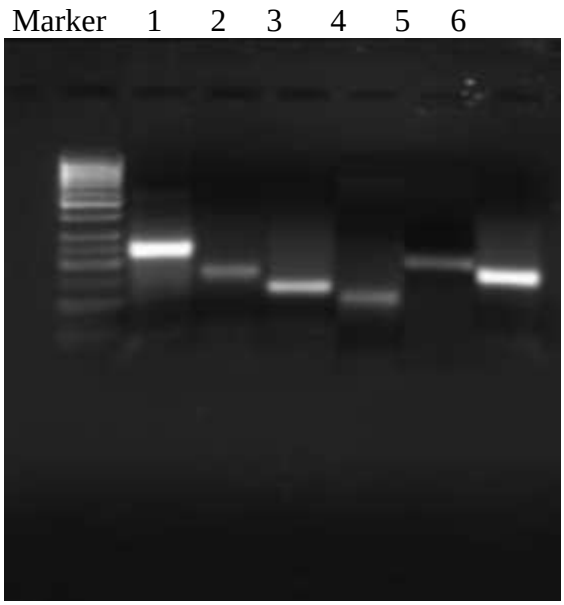
An example showing the melting curve analysis of leptin receptor



APPENDIX 4.3

AGAROSE GEL ELECTROPHORESIS OF

AMPLIFIED RT-PCR PRODUCTS



Lanes:

1: B-actin (241bp)

2: Carnitine palmitoyltransferase 1A, liver (173 bp)

3: Peroxisome proliferator activated receptor alpha (126 bp)

4: 3-hydroxy-3-methylglutaryl-coenzyme a reductase (101 bp)

5: Fatty acid synthase (212 bp)

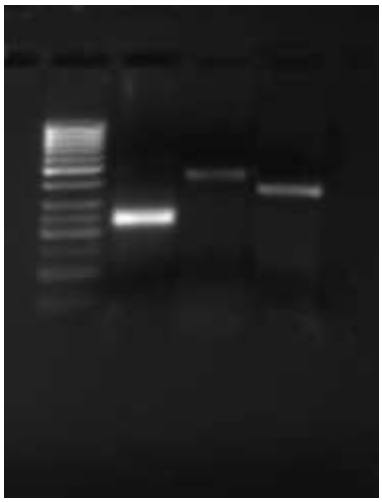
6: Cytochrome P450, family 7, subfamily a, polypeptide 1 (168 bp)

APPENDIX 4.3 (continued)

AGAROSE GEL ELECTROPHORESIS OF

AMPLIFIED RT-PCR PRODUCTS

Marker 1 2 3



Lanes:

1: B-actin (241bp)

2: Leptin (416 bp)

3: Leptin receptor (373 bp)

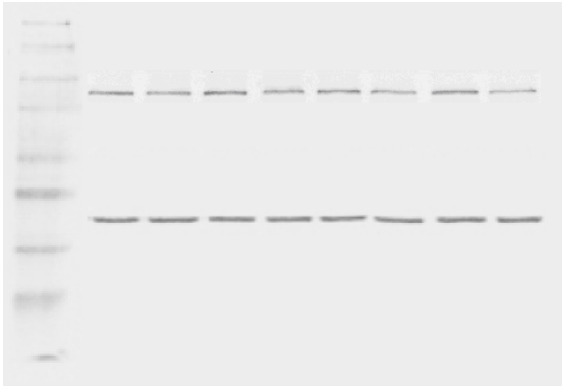
APPENDIX 4.5

WESTERN BLOTS OF HMGCR, FASN AND CPT1L IN THE LIVERS FROM CONTROL MICE (ODD-NUMBERED LANES) AND HFC MICE (EVEN-NUMBERED LANES)

HMGCR

Week 2

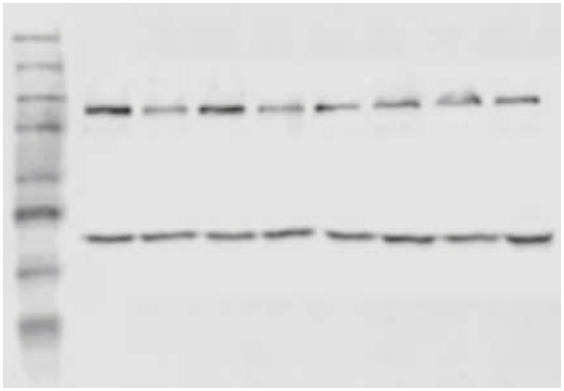
Marker 1 2 3 4 5 6 7 8



HMGCR

Week 4

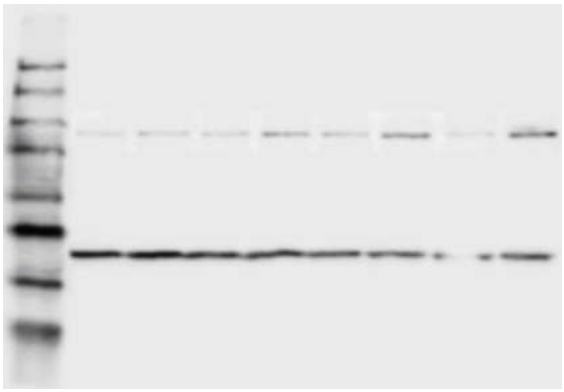
Marker 1 2 3 4 5 6 7 8



HMGCR

Week 10

Marker 1 2 3 4 5 6 7 8



FASN

Week 2

Marker 1 2 3 4 5 6 7 8

FASN

Week 4

Marker 1 2 3 4 5 6 7 8

FASN

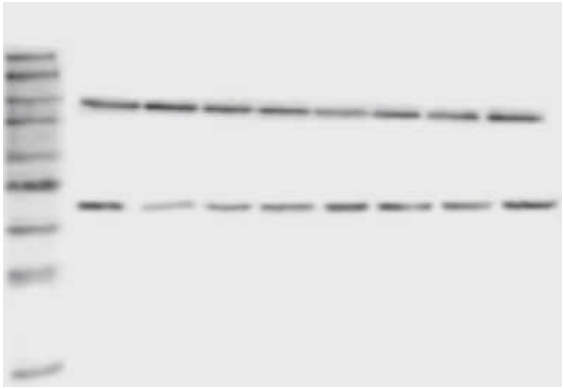
Week 10

Marker 1 2 3 4 5 6 7 8

CPT1L

Week 2

Marker 1 2 3 4 5 6 7 8



CPT1L

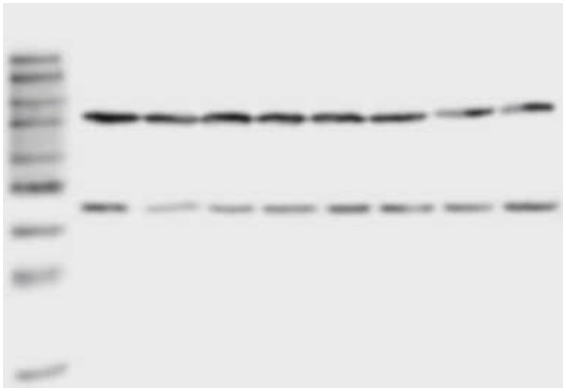
Week 4

Marker 1 2 3 4 5 6 7 8

CPT1L

Week 10

Marker 1 2 3 4 5 6 7 8



APPENDIX 4.6 REFERENCE VALUES FOR PLASMA LIPIDS, GLUCOSE, LEPTIN AND INSULIN LEVELS FOR FEMALE C57BL/6J MICE

Reference values are compared with the data obtained from our control 2-week mice (aged 10 weeks) as follows:

	Normal values	**Control mice (C-2wk)**
Plasma glucose (mmol/L)	12.4 ± 1.5	15.7 ± 3.4
Plasma total cholesterol (mmol/L)	1.9 ± 0.4	1.7 ± 0.1
Plasma HDL cholesterol (mmol/L)	0.9 ± 0.4	1.4 ± 0.2
Plasma triglycerides (mmol/L)	0.8 ± 0.3	0.8 ± 0.1
***Plasma leptin (ng/ml)**	2.3 ± 1.1	1.4 ± 0.3
***Plasma insulin (ng/ml)**	0.4 ± 0.1	0.3 ± 0.1

Values are expressed as Means ± SD. All reference values are obtained from Jackson Laboratory Website http://jaxmice.jax.org, unless indicated otherwise.

* Values are obtained from Murphy et al (1997)

APPENDIX 5.1

WESTERN BLOTS OF HMGCR AND FASN IN THE LIVERS FROM CONTROL MICE (ODD-NUMBERED LANES) AND HIGH-FIBER MICE (EVEN-NUMBERED LANES)

FASN

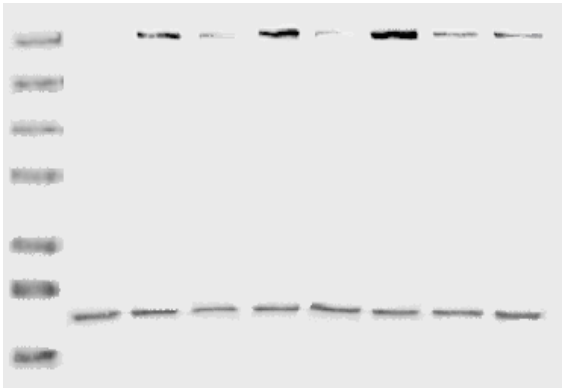
Week 3

Marker 1 2 3 4 5 6 7 8

FASN

Week 10

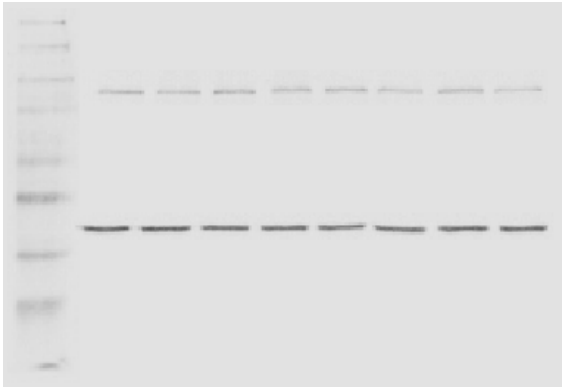
Marker 1 2 3 4 5 6 7 8



HMGCR

Week 3

Marker 1 2 3 4 5 6 7 8



HMGCR

Week 10

Marker 1 2 3 4 5 6 7 8