University of Alberta

Excess Dietary Folic Acid Promotes Weight Gain
in Rats Fed a High Fat Diet

by

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Abstract

Mandatory folate fortification polices in North America have resulted in high levels of circulating folate in the population. At the same time, a trend toward increased energy intake and reduced energy expenditure has lead to an increased prevalence of obesity, characterized by an accumulation of adipose tissue. Adipose tissue expansion has been implicated in the development of metabolic abnormalities observed in obesity, including altered secretion of cytokines and peripheral insulin resistance. The objective of this study was to investigate the effect of dietary folic acid deficiency and excess on food intake, weight gain and body composition. On a high fat diet, rats fed excess folic acid gained significantly more weight than the control group. While both folic acid deficient and supplemented rats had impaired glucose tolerance, these effects appear to be dependent on a high fat diet, because these phenomena were not observed in rats fed a lower fat diet.

Preface

I would like to acknowledge the technical assistance I received for some of the analyses contained within this thesis. In particular, Kelly-Ann Leonard contributed the most in this regard, as the lab technician. I would like to thank Randy Nelson for introducing me to the practical aspects of molecular biology. Mattéa Bujold provided an excellent illustration of the high throughput quantitative PCR method using the Biomark system. Sally Stabler and colleagues analyzed hepatic and plasma one-carbon metabolites in our samples. Susanne Lingrell conducted the *PEMT* activity assays using rat liver homogenates. Yeping Xiong and Jonathan Curtis completed the analysis of choline-containing compounds and phospholipids.

Acknowledgements

I would like to thank all those who contributed to this work and my training during my time in the Jacobs' lab. Many thanks to my supervisor Dr. René Jacobs; Sir, my heart is filled with gratitude every time I think of the countless occasions when you have guided me in my work, mentored me whenever I asked, protected me when I needed. I would like to recognize the excellent training environment provided by: my supervisory committee, Dr. Catherine Field and Dr. Spencer Proctor; the members of the Group on the Molecular and Cell Biology of Lipids; as well as the Metabolic and Cardiovascular Diseases Laboratory.

My thanks to my family and friends who have supported me throughout my studies. Kelly - where technical support meets moral support - thanks for helping me to organize my life, both in and out of the lab. I value your advice and look up to you a great deal. Randy, I appreciate the example you have set for me of thorough, hard work and dedication. The depth of your knowledge and experience is truly remarkable. Bella, I'm glad to have made such a wonderful friend at the lab. It is a pleasure to experience your curiosity and work ethic. You are brilliant. Thanks are due to Nicole for guiding my animal experiments, as well providing a creative outlet to help me stay fresh. I would like to express my appreciation to Robert for his infinite enthusiasm, encouragement, and the inspiration to achieve.

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List of Abbreviations

Abbreviation	Definition
AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosylmethionine
ALT	alanine amino transferase
ANOVA	analysis of variance
BMI	body mass index
CBS	cystathionine β-synthase
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CE	cholesteryl ester
CpG	cytosine-phosphate-guanine
CPT	CDP-choline: 1,2-diacylgylcerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
Ct	threshold cycle
D6D	delta-6 desaturase
DFE	dietary folate equivalents
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
ELISA	enzyme-linked immunosorbent assay
FC	folate control

FC	free cholesterol
FD	folate deficient
FE	folate excess
FU	fluorescence units
GC	gas chromatography
GPC	glycerophosphocholine
IFC	integrated fluidic circuit
mRNA	messenger ribonucleic acid
MTHFR	methylene tetrahydrofolate reductase
NTD	neural tube defect
PCR	polymerase chain reaction
PCR	polymerase chain reaction
PEMT	phosphatidylcholine *N*-methyltransferase
PI	phosphatidylinositol
PPARγ	peroxisome proliferator activated receptor gamma
PS	phosphatidylserine
RIN	RNA integrity number
RNA	ribonucleic acid
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SEM	standard error of the mean
SM	sphingomyelin
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol

THF	tetrahydrofolate
TLC	thin layer chromatography
TNFα	tumour necrosis factor alpha
UPL	universal probe library

CHAPTER 1:

INTRODUCTION

1.1 Rationale

Obesity is defined by the World Health Organization as a body mass index (BMI) of 30 or greater. Increased adiposity is correlated with a growing number of people afflicted with a number of metabolic abnormalities, which together form the metabolic syndrome. Metabolic syndrome is defined by the presence of visceral obesity in combination with dyslipidemia, elevated blood pressure, and/or elevated fasting plasma glucose or Type 2 Diabetes Mellitus (T2DM) (IDF 2006). The metabolic syndrome represents a progression from a healthy metabolic state to one in which energy metabolism is perturbed resulting in an increased risk of T2DM and cardiovascular disease (Mooradian 2003). Obesity is associated with an increased risk of cardiovascular disease, type 2 diabetes, fatty liver disease and some types of cancer (Han 2006). In the obese Canadian population, there are increased reports of asthma, arthritis, high blood pressure, urinary incontinence and diabetes (Gilmore 1999). Obesity can also be related to a reduced quality of life. For example, obesity can limit the amount and types of activities that an individual can perform, and is also correlated with depression (Gilmore 1999, Roberts 2003). Obesity related co-morbidities increase the burden on the health care system, equating to elevated health care costs. The economic burden of obesity in Canada was estimated between 4.6-7.1 billion dollars in 2008 (Janssen 2006). Data collected for the National Population Health Survey in 1996/1997 estimated over 12% of adults

under 65 were obese (Gilmore 1999), which is similar to the estimate from 1978 (13.8%) (Public Health Agency of Canada). The prevalence of obesity in Canadian adults reached was nearly 25% in 2007 (Public Health Agency of Canada). The prevalence of obesity is also increasing in children and adolescent age groups (Roberts 2012), which may be an indicator of even greater growth in the scale of adult obesity in the years to come. Obesity is a nutrition related disease and changes in the food supply, as well as dietary patterns are implicated in the rise in obesity (Jequier 2002). One far-reaching change during this time is the addition of supplemental folate to cereals to the Canadian food supply.

Folate, also referred to as vitamin B9, is an essential vitamin which has recently been associated with the development of obesity. Serum folate levels were shown to be significantly lower in individuals with higher body fat mass (Mojtabai 2004), independent of dietary intake (Mahabir 2008). Gallistl et al. (2008) reported that serum folate is inversely correlated with BMI in Austrian adolescents. Similarly, obese Thai subjects were found to have lower circulating folate than normal weight individuals (Tungtronchitr 2003). In postmenopausal women, adiposity is associated with lower folate levels and in the same subjects, the effectiveness of insulin to reduce plasma glucose was significantly reduced in those with higher fat mass (Mojtabai 2004). These studies suggest reduced levels of folate may exacerbate obesity. Altered folate status has also been shown to negatively affect insulin sensitivity in an intergenerational manner.

Higher folate concentrations at 28 weeks gestation were associated with greater fat mass in children assessed at six years of age (Yajnik 2008). These studies postulate that nutrient availability during the critical period of development *in utero* can program the metabolic performance of the offspring later in life through epigenetic mechanisms. In rats, folate supplementation during pregnancy increased adiposity in the offspring, and resulted in dyslipidemia at four weeks of age, but no longer showed an effect at 13 weeks of age (Engeham 2010). These results suggest that altered folate status may also have direct effects on energy metabolism, because the effects did not last later in life when folate supply returned to normal. There is currently a lack of research regarding the effects of folate deficiency and supplementation after weaning on energy metabolism.

1.1.1 Thesis Aim

The overall goal of this study is to investigate the influence of dietary folic acid on the development of obesity and glucose clearance

1.1.2 Hypothesis

Excess dietary folic acid will increase weight gain and impair glucose tolerance in rats.

1.1.3 Specific Objectives

1. Develop a high-throughput method for the analysis of gene expression.
2. Characterize food intake, weight gain, body composition and glucose tolerance during folate deficiency and supplementation.
3. Quantify tissue phospholipids and neutral lipids.
4. Examine the tissue-specific, relative gene expression patterns in a control diet compared with, folate deficiency and folate excess.

1.2. Literature Review

1.2.1 Folate

The recommended dietary allowance for folate is 400 µg dietary folate equivalents (DFEs) per day for ages 14 and over. Good dietary sources of folate include green leafy vegetables, legumes and organ meats. Folate functions in one-carbon metabolism, required for DNA synthesis, amino acid metabolism and methyl-transfer reactions. Due to its role in nucleotide synthesis, folate is especially important for rapidly dividing cells. A lack of dietary folate yields macrocytic anemia. This was discovered in pregnant women, in the earlier half of the 1900s by Lucy Wills (Wills 1931). Wills identified a factor in yeast which remedied these symptoms, which was later identified as folate (Mitchell 1941). Folate deficiency during the peri-conception period is associated with an increased risk of neural tube defects (NTDs) (Kirke et al 1993). NTDs encompass a range of abnormalities related to a failure of the neural tube

structure to fold properly during early embryonic development. Randomized controlled trials in the early 1990s demonstrated a preventative role for folate in the development of NTDs (such as Wald 1991). Based on the evidence provided by this body of research, the governments of Canada and the United States implemented a national folic acid fortification program of cereal grain products in 1998 aimed at increasing intake of folate in women of child-bearing age. Health Canada recommends that women of child-bearing age consume an additional 400 µg of supplemental folic acid. The prevalence of NTDs declined following these measures and has nearly eliminated folate deficiency in the Canadian population (De Wals 2007, Colapinto 2011).

The term folate refers to a group of compounds that share similarities in structure, but differ in a few functional domains. As shown in **figure 1-1**, all folates consist of 2-amino-4-hydroxypteridine linked to aminobenzoate, which has a glutamate residue attached at the carboxyl group.

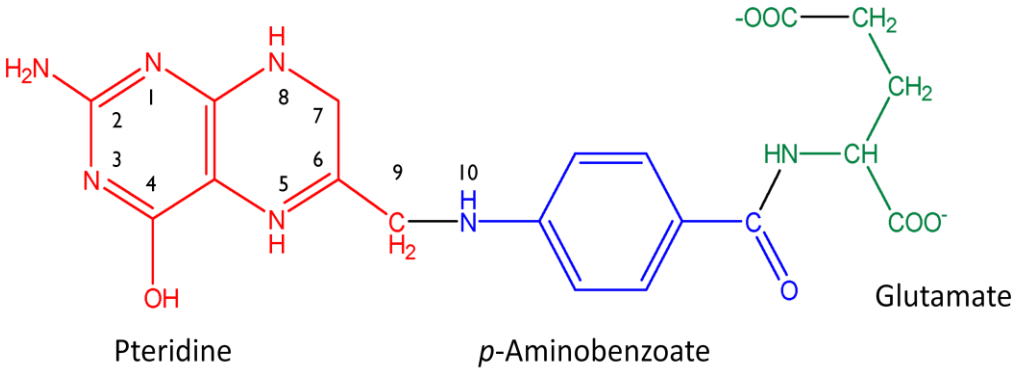


Figure 1-1: Structure of folate

Functional Group		Position
Methyl	CH_3	5
Formyl	CHO	5 or 10
Methylene	$-CH_2-$	5 and 10
Methenyl	-CH=	5 and 10

Table 1-1: Folate functional groups.

Naturally occurring folates have various 1-carbon substitutions at positions 5 and/or 10, outlined in **table 1-1**, while folic acid, a synthetic folate analogue is the oxidized form of folate. Intracellular folate metabolism is shown in **figure 1-2**. Another distinguishing characteristic of the structure of folic acid compared with folates found in foods is the presence of only a single glutamate on the benzoic acid ring. Folates are predominantly found in a polyglutamated form in nature.

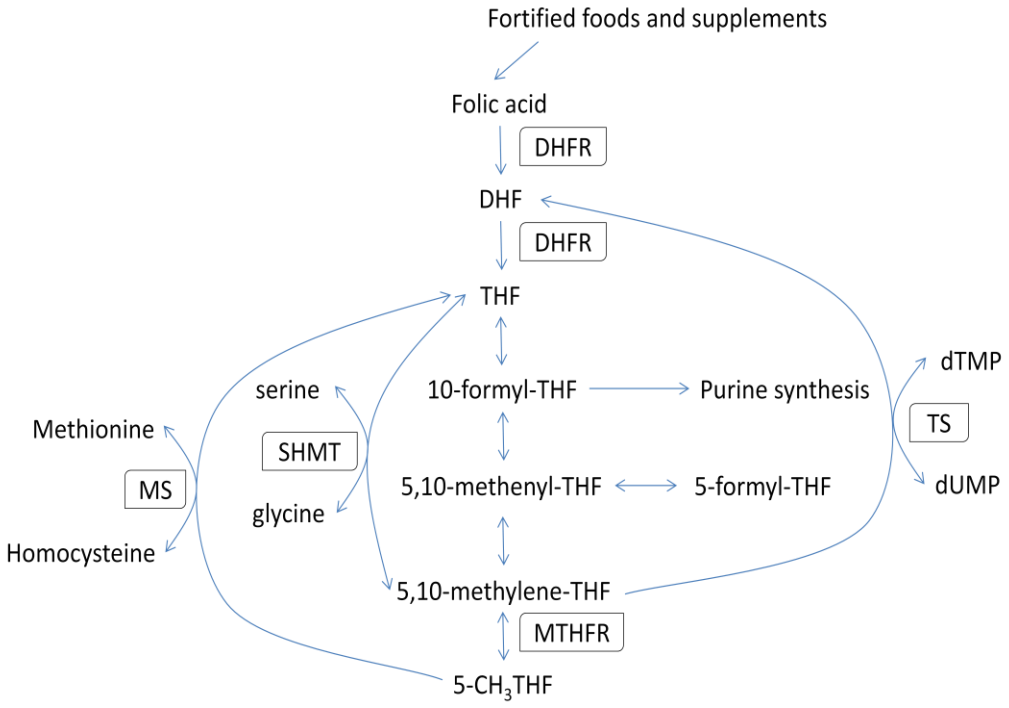


Figure 1-2: Folate in one-carbon metabolism. Folic acid is reduced to THF and via DHF in two reactions catalyzed by DHFR. THF acts as an acceptor of one-carbon groups. A methyl group from serine is used to convert THF to 5,10-methylene-THF using the enzyme SHMT. 5,10-methylene-THF is either oxidized to 5,10-methenyl-THF, reduced to 5-CH_3THF, or donates the one-carbon group for thymidine synthesis. 5,10-methenyl-THF, donates its one-carbon group for purine synthesis, via formyl-THF. 5-CH_3THF is a methyl donor, which converts homocysteine to methionine. *Abbreviations:* DHFR – dihydrofolate reductase; DHF – dihydrofolate; THF – tetrahydrofolate; SHMT – serine hydroxymethyltransferase; MS – methionine synthase; TS – thymidylate synthase; MTHFR – methylene tetrahydrofolate reductase.

Folic acid is less susceptible to oxidation and is therefore used as a supplemental form of folate in fortified foods and vitamin tablets. Folic acid is also 1.7-2 times more bioavailable than folates found in foods. For this reason, nutritional recommendations are defined in terms of DFEs. One DFE is equivalent to 1 µg of food folate, 0.5 µg of folic acid consumed alone, or 0.6 µg of folic acid when consumed with food. Folic acid is considered to have greater biological activity due to the *in vivo* conversion of folic acid to tetrahydrofolate (THF). This is achieved through two intracellular reactions catalysed by the enzyme dihydrofolate reductase (DHFR).

1.2.2 Folic Acid

In humans, dietary folic acid is effectively integrated into the pool of active folate metabolites at low levels of intake (~100 µg folic acid or 170 µg DFE)/day) (Choumenkovitch 2002). As a result, the level of folic acid fortification of flours and cereal grain products in Canada and the United States was based on an estimated intake of 100 µg/day. However, estimates of folic acid intake after fortification began in 1998 suggest that actual intakes may be double the predicted value (Choumenkovitch 2002). Intake of 200 µg/day appears to be the threshold at which folic acid begins to appear in the blood stream in an unmetabolized form (Lewis 1999). In addition to fortified foods, high intakes of folic acid can be achieved through intake of vitamin supplements (Gomez et al 2013). Folic acid supplement use during pregnancy was greater than 90%, in an affluent

population of Alberta, Canada (Gomez et al 2013). Furthermore, the mean intake of folic acid from supplements was over double the recommended daily intake for pregnant women (Gomez et al 2013). While folic acid supplement use is higher in females, folic acid supplements were used by one quarter of the population Canadian between the ages of 6 and 79 (Colapinto et al 2012). Therefore, folic acid from supplements serve as a significant contributor to intake of folate in the Canadian population.

The rate of reduction of folic acid in the human liver is over 1000 times slower than that of DHF (Bailey and Ayling 2009). High plasma levels of unmetabolized folic acid can lead to an increased concentration of dihydrofolate in the cell, which has been shown to inhibit methylene tetrahydrofolate reductase (MTHFR) (Mathews 1982) and thereby inhibiting remethylation of homocysteine. Thus, it has been postulated that high plasma concentrations of folic acid may lead to a functional folate deficiency (Smith et al 2008; Ulrich and Potter 2006). Folate supplementation in the form of 5-methylTHF has been shown to effectively raise plasma folate levels without leading to an elevation in unmetabolized folic acid (Prinz-Langenohl, 2009; Fohr, 2002).

1.2.3 The Role of Folate in the Methylation Cycle

Almost all of the B vitamins play a role in one-carbon metabolism; cobalamin (B12), folate (B9), and riboflavin (B2) serve as cofactors in one-carbon metabolism, while pyridoxine (B6) is essential for transsulphuration pathway (Brosnan and Brosnan 2006). However, folate differs from the

other B vitamins in that it serves as a catalytic substrate for the transfer of one-carbon units. As shown in **Figure 1-3**, the methionine cycle is responsible for the synthesis of S-adenosylmethionine (AdoMet). AdoMet-dependant methylation reactions are required for post-translational modifications of proteins, methylation of DNA, as well as the synthesis of hormones and other small molecules including phosphatidylcholine (PC). Impaired methylation capacity can be caused by either a decrease in AdoMet or an increase in S-adenosylmethionine (AdoHcy), a competitive inhibitor of many methyltransferase reactions (Clarke 2001). An important function of folate is in the maintenance of cellular AdoMet and AdoHcy concentrations. THF plays a crucial role in a number of reactions that generate methyl groups from the catabolism of sarcosine, serine, dimethylglycine and glycine. These methyl groups are used for the remethylation of homocysteine thereby supporting AdoMet synthesis, AdoHcy removal and hence maintaining methylation capacity.

Many methylation reactions occur in the liver and hepatic steatosis is commonly observed upon perturbation of one-carbon metabolism. Indeed, folate deficiency has been shown to result in the accumulation of triacylglycerol (TAG) in the liver (Akesson 1982; Burdge 2009). Alterations in both AdoMet availability and phospholipid metabolism have been implicated in the etiology of fatty liver. Furthermore, a fatty liver and changes in AdoMet and folate status are observed in obesity and metabolic syndrome.

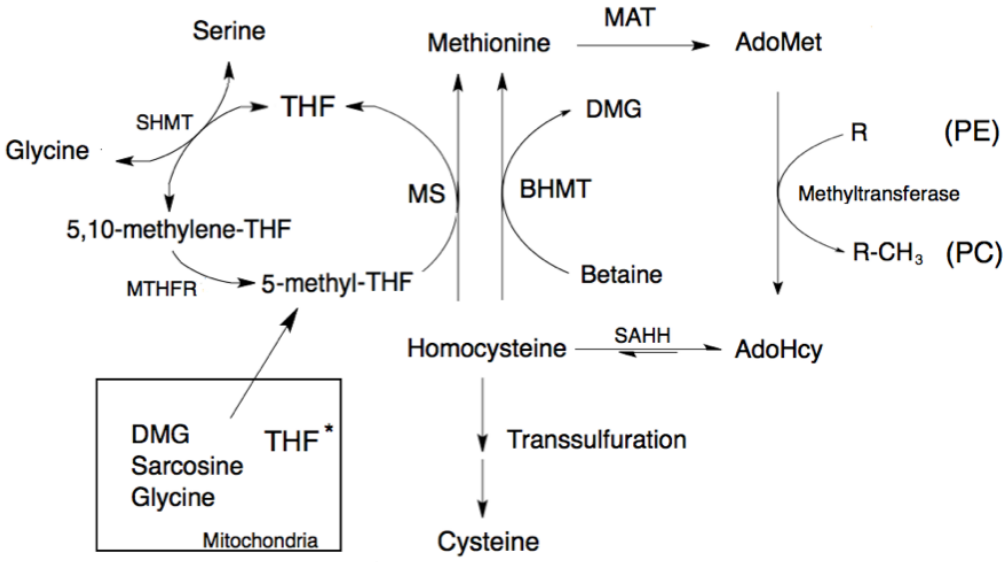


Figure 1-3: One-carbon and folate metabolism.

Abbreviations: *S*-adenosylmethionine, AdoMet; *S*-adenosylhomocysteine, AdoHcy; Betaine:homocysteine methyltransferase, BHMT; dimethylglycine, DMG; methionine adenosyltransferase, MAT; methionine tetrahydrofolate reductase, MTHFR; methionine synthase, MS; phosphatidylethanolamine, PE; phosphatidylcholine, PC; *S*-adenosylhomocysteine hydrolase, SAHH and serine hydroxymethyltransferase, SHMT.

1.2.4 Phosphatidylcholine Synthesis

Phosphatidylcholine is a major phospholipid found in the cell membrane, as well as organelles within the cell, and lipoproteins which

transport lipids in the blood. All tissues have the machinery to synthesize PC through the CDP-choline pathway (**Figure 1-4**). Choline is phosphorylated upon entering the cell by the enzyme choline kinase. Next, CTP:phosphocholine cytidylyltransferase (CT) catalyzes the formation of CDP-choline. This is the rate-limiting step in the CDP-choline pathway. The addition of the phosphocholine moiety to diacylglycerol completes the synthesis of PC. This reaction is catalyzed by CDP-choline: 1,2-diacylgylcerol cholinephosphotransferase, or CPT, and occurs at the surface of the endoplasmic reticulum (Wilgram 1963).

Figure 1-4: CDP-choline pathway for PC synthesis

The second pathway for PC synthesis is the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. PC production via the PEMT pathway occurs primarily in the liver, where the demand for PC is high due to the production and secretion of very low density lipoproteins and PC secretion in bile, in addition to the normal cellular requirement for the synthesis of membranes. PEMT is active in the endoplasmic reticulum, where it performs three repeated methylation reactions converting phosphatidylethanolamine (PE) to PC (**Figure 1-5**).

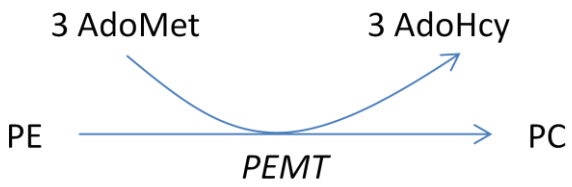


Figure 1-5: PEMT pathway for PC synthesis

The methyl donor *S*-adenosylmethionine is required for each step of the reaction, generating three molecules of *S*-adenosylhomocysteine for each PC molecule produced. This mechanism contributes approximately 30% of PC produced in the liver, when choline supply is adequate to maintain PC synthesis through the CDP-choline pathway (Sundler 1975). However, when choline is limiting in the diet, the PEMT pathway is critical for maintaining the supply of PC in the liver.

1.2.5 Lipid and One-Carbon Metabolism

Numerous reports have demonstrated a connection between folate, choline and lipid metabolism (Koteish and Diehl 2001; Rinella and Green 2004; Henkel et al 2012). Pogribny et al. (2013) showed that mouse strains with reduced expression of methionine adenosyltransferase, methionine synthase (MS) and MTHFR had significantly greater hepatic lipid accumulation when fed a choline:folate-deficient diet. Christensen *et al*. (2010) observed that a folate-deficient diet alone was sufficient to induce hepatic steatosis in mice. A separate study showed that folate deficiency decreases flux through PEMT (Chew et al 2011). Together,

these findings suggest that folate deficiency reduces *de novo* PC synthesis resulting in accumulation of hepatic TAG. In liver, PC is also synthesized via PEMT which consumes a significant portion of AdoMet in the liver, estimated to be approximately 40% of all methylation reactions (Stead et al 2006). Alterations in PC synthesis affect hepatic lipid storage and secretion in rodent models (Vance et al 2007; Guo et al 2008; Jacobs et al 2008). In particular, *pemt*$^{-/-}$ mice develop steatosis when fed a high-fat diet and liver failure when fed a choline-deficient diet (Vance et al 2007). These observations can be explained by a reduction in hepatic secretion of VLDL particles in *pemt*$^{-/-}$ mice (Noga and Vance 2003). Similarly, deletion of hepatic CTα impairs VLDL secretion and increases susceptibility to diet-induced steatosis (Jacobs et al 2004; Niebergall et al 2011). These results and others highlight the role of PC synthesis in the maintenance of hepatic lipid metabolism.

Recently, Zeisel and colleagues generated betaine:homocysteine methyltransferase (BHMT) knockout mice that are incapable of completely catabolizing choline. The *Bhmt*$^{-/-}$ mice have a 25-fold increase in hepatic betaine and develop severe choline deficiency (Teng et al 2011). BHMT-dependent remethylation is clearly important for regulating one-carbon metabolism, as *Bhmt*$^{-/-}$ mice have elevated plasma homocysteine that is not influenced by the level of dietary folate (Teng et al 2012). The *bhmt*$^{-/-}$ mice also have impaired methylation potential, as illustrated by a significant reduction in the hepatic AdoMet:AdoHcy ratio (Teng 2011;

Teng 2012). The presence of reduced *de novo* choline synthesis via PEMT together with elevated choline dehydrogenase activity in the *bhmt*$^{-/-}$ mice leads to dramatic decreases in choline-containing metabolites. Moreover, severe choline deficiency results in steatohepatitis and early formation of hepatocarcinoma in *bhmt*$^{-/-}$ mice (Teng 2011). These knockout animals emphasize the importance of maintaining sufficient levels of choline and one-carbon metabolites for lipid homeostasis.

1.2.6 Obesity and One-Carbon Metabolism

High fat diet-induced obesity is linked with alterations in one-carbon metabolites (Rubio-Alegia et al 2011). Plasma total homocysteine is correlated with obesity (Elshorbagy et al 2008). This may be an indicator of impaired methylation in obesity. Dietary methyl donor deficiency has been demonstrated to diminish AdoMet levels and cause lipid accumulation in the liver (Rinella and Green 2004). Reduced expression of methionine adenosyl transferase, the enzyme responsible for the synthesis of AdoMet, is predictive of greater hepatic lipid accumulation in rodents fed a diet deficient in methyl donors (Pogribny et al 2013). Impaired methylation promotes lipid accumulation by restricting PC synthesis.

Dietary choline deficiency also increases lipid accumulation in the liver and leads to liver damage (Best et al 1933). On the other hand, plasma choline is positively correlated with BMI and serum TAG

(Konstantinova et al 2008). Furthermore, choline deficiency protects against high fat diet-induced obesity and insulin resistance in rodents (Jacobs et al 2010).

Cysteine is a sulphur-containing amino acid used in the synthesis of proteins. Cysteine is synthesized from the essential amino acid methionine in the 1-carbon cycle (figure 3). The condensation of homocysteine with serine by cystathionine β-synthase (CBS) is an irreversible reaction that commits the fate of methionine to the transsulphuration pathway. Cystathionine is then cleaved to yield cysteine. Elevated plasma cysteine is associated with body fat mass in humans (Elshorbagy et al 2008). Cysteine was shown to increase fatty acid synthesis and inhibit lipolysis in a cell culture model of adipocytes (Olefsky 1979). A mouse model of CBS deficiency is characterized by reduced weight gain, including dramatically reduced body fat mass and hepatic lipid accumulation (Gupta and Kruger 2011).

1.2.7 Conclusion

Clearly, there are a number of links between one-carbon metabolism and obesity. Choline and cysteine have been independently correlated with increased body weight. PC has a prominent function in lipid metabolism. PC is required for the storage of lipids in lipid droplets and adipocytes. PC is also a large component of lipoproteins, and therefore functions in the secretion and transport of lipids in the plasma.

Due to the interrelation between metabolites in the one-carbon cycle there remains the possibility that other molecules in one-carbon metabolism could also affect the development of obesity. Folate has been associated with obesity is epidemiological studies as well as intergenerational animal studies. To date, there have been few studies which investigate the effect of folic acid deficiency or excess on the development of obesity.

CHAPTER 2

MATERIALS AND METHODS

2.1 List of Chemicals and Reagents

Chemical	Source
1kB Plus DNA ladder	Invitrogen
2-propanol	Sigma Aldrich
48x48 Dynamic Array	Fluidigm
96x96 Dynamic Array	Fluidigm
Acetic acid	Sigma Aldrich
Agarose	Invitrogen
Ammonium molybdate	Sigma Aldrich
Ascorbic acid	Sigma Aldrich
Bromophenol blue	Sigma Aldrich
Calcium chloride	Sigma Aldrich
Chloroform	Fisher Scientific
DA assay loading reagent	Fluidigm
DA sample loading reagent	Fluidigm
Deoxynucleotides triphosphates (dNTPs)	Roche
Dexamethasone	Sigma Aldrich
Diethyl ether, anhydrous	Fischer Scientific
Diisopropyl ether	Sigma Aldrich
DNAse I	Invitrogen
Dnase/Rnase-free distilled water	Invitrogen
Dulbecco's modified eagles medium	Gibco
Ethanol	Fisher Scientific

Ethidium bromide	Sigma-Aldrich
Ethylene-diaminetetra-acetic acid (EDTA)	Sigma Aldrich
Fetal Bovine Serum	Sigma Aldrich
Folic acid	Sigma Aldrich
Formalin	Fischer Scientific
Glucometer, glucose strips	Accu-check
Glucose	Sigma Aldrich
Hematoxylin and Eosin	Sigma Aldrich
Hexane	Fischer Scientific
Insulin	Gibco
Isobutylmethylxanthine	Sigma Aldrich
Isopropanol	Fischer Scientific
Lithium Chloride 8M	Sigma Aldrich
Methanol	Fisher Scientific
Oligo dT primers, 12-14	Invitrogen
Pectin	Sigma Aldrich
Perchloric acid	Sigma Aldrich
Phospholipase C (*Clostridium welchii*)	Sigma-Aldrich
Bicinchoninic Acid (BCA) protein kit	Thermo Scientific
Platinum® *Taq* DNA Polymerase	Invitrogen
Primers	Integrated DNA Technologies
Priming Fluid	Fluidigm
Probes	Roche

Sodium EDTA tubes	Fischer Scientific
Sodium phosphate	Sigma Aldrich
Sodium sulphate	Sigma Aldrich
Succinylsulphathiazole	Sigma Aldrich
Sucrose	Sigma Aldrich
Superscript™ II reverse transcriptase	Invitrogen
Sylon BFT (BSTFA + TMCS, 99:1)	Supelco (Sigma)
Taqman Preamplification reagent	Applied Biosystems
Taqman Univeral Master Mix	Applied Biosystems
Tris base	Sigma Aldrich
Trizol reagent	Invitrogen

2.2 Animal Procedures

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. All animals had free access to food and water and were housed on a 12-hour light-dark cycle. Male *Sprague Dawley* rats were obtained from a colony maintained by the University of Alberta. At 6 weeks of age, rats were switch from a chow diet to the experimental diet, as described below. Food intake was estimated by giving each pair a pre-weighed amount of food and subtracting the weight of the remaining portion 2-3 times per week. Rats were weighed once per week throughout the study. During the eighth week of feeding, body composition was analyzed by nuclear magnetic resonance or by magnetic resonance imaging. Blood glucose was measured using a glucometer (Accu-chek) at 0, 15, 30, 60, 90 and 120 minutes after intraperitoneal injection of 1 g glucose/kg body weight to assess glucose tolerance.

Upon completion of the feeding trial (12 weeks), rats were fasted overnight and serum collected via cardiac puncture. Blood was collected into tubes containing Na_2EDTA and plasma was separated by centrifugation at 1000 rpm at 4 °C for 10 min. Aliquots of plasma were stored at −80 °C for biochemical analyses. Liver, peri-renal and gonadal fat pads were weighed, and the length of the intestine was recorded. A small portion of each tissue was removed and stored in 10% v/v formalin

for histological analysis. The remaining tissues were snap frozen in liquid N_2 and stored at −80°C until their use.

2.3 Diets

Diet was supplied by Harlan Teklad. Basal diet formulations were designed to AIN-93G specifications with lard added to achieve a total of 60% of kilocalories from fat. One percent w/w succinylsulphathiazole was added to the diet, along with 5% w/w pectin to prevent the addition of folate by bacteria residing in the gastrointestinal tract of the animals. At 5 weeks of age, rats were randomized to a diet containing either 0.75 mg/kg folic acid (HF/FC), 0 mg/kg folic acid (HF/FD) or 7.5 mg/kg folic acid (HF/FE) for 12 weeks. In a separate study, three diets were designed as closely as possible to the high fat diet, with the exception that the fat content provided 15% of total kilocalories (**Table 2-1**). The dietary groups in the lower fat study were designated folic acid control (FC), folic acid deficient (FD) or folic acid excess (FE).

Ingredient (g/kg)	HF/FC	HF/FD	HF/FE	FC	FD	FE
Folic acid	0.00075	0	0.0075	0.00075	0	0.0075
L-cystine	4	4	4	3	3	3
Corn starch	-	-	-	263.7	263.7	263.7
Sucrose	106.3	106.3	106.3	209.7	209.7	209.7
Maltodextrin	160	160	160	130	130	130
Soybean Oil	30	30	30	60	60	60
Lard	310	310	310	-	-	-
Cellulose	20	20	20	50	50	50
Pectin	50	50	50	50	50	50
Succinylsulphathiazole	10	10	10	10	10	10
Vitamin-free casein	265	265	265	195	195	195
Mineral Mix, AIN-93G	48	48	48	35	35	35
TBHQ	3.40	3.40	3.40	0.012	0.012	0.012
Choline bitartrate	3.0	3.0	3.0	2.5	2.5	2.5
Niacin	0.063	0.063	0.063	0.03	0.03	0.03
Calcium pantothenate	0.034	0.034	0.034	0.016	0.016	0.016
Pyridoxine HCl	0.015	0.015	0.015	0.007	0.007	0.007
Thiamin HCl	0.013	0.013	0.013	0.006	0.006	0.006
Riboflavin	0.013	0.013	0.013	0.006	0.006	0.006
Biotin	0.0004	0.0004	0.0004	0.0002	0.0002	0.0002
Vitamin B_{12}	0.00004	0.00004	0.00004	0.000025	0.000025	0.000025
DL-alpha tocopheryl acetate (500 IU/g)	0.315	0.315	0.315	0.15	0.15	0.15
Vitamin A palmitate (500,000 IU/g)	0.017	0.017	0.017	0.008	0.008	0.008
Cholecalciferol (500,000 IU/g)	0.004	0.004	0.004	0.002	0.002	0.002
Phylloquinone	0.0016	0.0016	0.0016	0.0008	0.0008	0.0008

Table 2-1: Composition of diets used in animal experiments.

2.4 Histological Analysis

A portion of liver, jejunum and visceral adipose tissues were preserved in 10% v/v formalin until dehydrated, cleared and embedded in paraffin. Preserved sections were mounted in labelled cassettes, and then dehydrated in successively higher concentrations of ethanol 10%, 80%, 95% and 100% v/v for 1 hour each. Cassettes were then submerged in xylene for 1 hour, three times. Finally, samples are embedded in paraffin wax. Cross sections of adipose tissue were cut to 5 µm thickness and mounted on glass slides. Samples were rehydrated in xylene for 10 min, then 100% ethanol, 95% v/v ethanol for 5 min each and rinsed with distilled water. Sections were stained with hematoxylin and eosin and viewed under a light microscope. Adipocytes were traced and the area of adipocytes was quantified using ImageJ software (National Institutes of Health).

2.5 Lipid Extraction

Tissues were homogenized 1:5 w/v in 10% phosphate buffered saline. If previously frozen, homogenates were sonicated 3 x 30 s in an attempt to disrupt and aggregates formed during cold storage. Protein content of tissue homogenates were quantified using bicinchoninic acid protein assay. Total lipids were extracted using the Folch method (Rouser et al 1966) A volume of tissue homogenate equal to 1 mg protein was added to a total volume of 2 mL using. 6 mL 2:1 chloroform:methanol was

added and each sample was thoroughly mixed by vortexing. The aqueous and organic layers were separated by 5 min centrifugation at 2000 rpm. The organic layer was removed to a new vessel and dried down under N_2. 100 µL ice-cold chloroform was used to redissolve the organic matter. Samples were spotted on to a TLC plate (EMD chemicals). Phospholipids were separated in a 50:30:8:4 mixture of chloroform:methanol:acetic acid:water (Noga and Vance 2003) and quantified by a phosphorus assay.

2.6 Phosphorus Assay

1mM sodium phosphate solution was prepared as a standard solution. Standards and lipid bands of interest scraped from TLC plates following a lipid extraction and separation were heated at 180°C for 1 hour in 450 µL 70% perchloric acid, followed by the addition of 2.5 mL water. 0.5 mL 2.5% ammonium molybdate solution was added, then 0.5 mL 10% ascorbic acid solution. Samples were incubated at 95°C for 15 minutes, then cooled prior to reading absorbance at 750 nm (Spectra max 190, Molecular Devices). Samples were quantified relative to a standard curve.

2.7 GC Analysis of Total Lipids

A volume of liver homogenate equal to 1 mg total protein was added to 2 mL of solution containing 2 units of phospholipase C from Clostridium welchii, 17.5 mM Tris, pH7.3, 10 mM $CaCl_2$ and followed by the addition of 2 mL diethyl ether. Tubes were shaken for 2 hours at 30°C to ensure cleavage of the phosphate-containing head group off the

glycerol backbone of phospholipid molecules. 2 ug tridecanoin was added as an internal reference standard for relative quantification. Lipid extraction was performed as described above in 2:1 chloroform:methanol. The organic layer was filtered through anhydrous Na_2SO_4, and then derivatized with Sylon BFT. Samples were dried down under N_2 and re-dissolved in hexane for analysis by gas chromatography by the Lipid and Lipid Metabolite Core Facility at the University of Alberta. .

2.8 Extraction of Phospholipids and Choline Metabolites

Samples were homogenized in a mixture of chloroform:methanol:water 1:2:0.8 for 5 minutes. The supernatant was removed and homogenization was repeated two additional times. All extracts were combined and filtered through 0.22 µm filter, followed by liquid chromatography combined with mass spectrometry as described by Xiong et al (2012).

2.9 Measurement of One-Carbon Metabolites

Plasma and hepatic levels of metabolites in the 1-carbon cycle, including folate, total homocysteine, methionine, *S*-adenosylmethionine, *S*-adenosylhomocysteine, cysteine, sarcosine, methylglycine, glycine, serine, cystathionine, α-aminobutyrate, were measured by capillary stable isotope dilution gas chromatography/mass spectrometry, according to Stabler et al 2004).

2.10 Phosphatidylethanolamine *N*-methyltransferase Activity Assay

PEMT activity was measured in the liver of rats fed high fat diet using the method described in Ridgway and Vance (1992).

2.11 Plasma Measurements

Folate in the plasma was measured by ion capture assay, following the manufacturer's instructions (Abbot Diagnostics) and quantified relative to a standard curve made from samples of known concentrations on an AxSym Immunoanalyzer (Abbott Diagnostics.

Plasma ALT/AST was measured using a kit supplied by BioTron, as per the manufacturer's instructions. In brief, ALT present in the sample and standards is used to generate a coloured product detected at 540 nm.

Fasting plasma insulin was measured by ELISA purchased from ALPCO and glucose was measured by colourimetric assay (Diagnostic Chemicals).

2.12 High-Throughput Quantitative PCR

This method was developed to analyze the expression of a large amount of genes and samples in a single experiment. Using either a 48-by-48 or 96-by-96 format, 2304 or 9216 reactions are conducted simultaneously in this real-time RT-qPCR assay. This method allows a systems approach to measuring gene expression, with the use of a single

microfluidic chip. This procedure makes use of the Biomark HD system (Fluidigm) available in the Molecular Biology Service Unit, at the University of Alberta. **Figure 2-1** summarizes the work flow of this method, which is described in detail below.

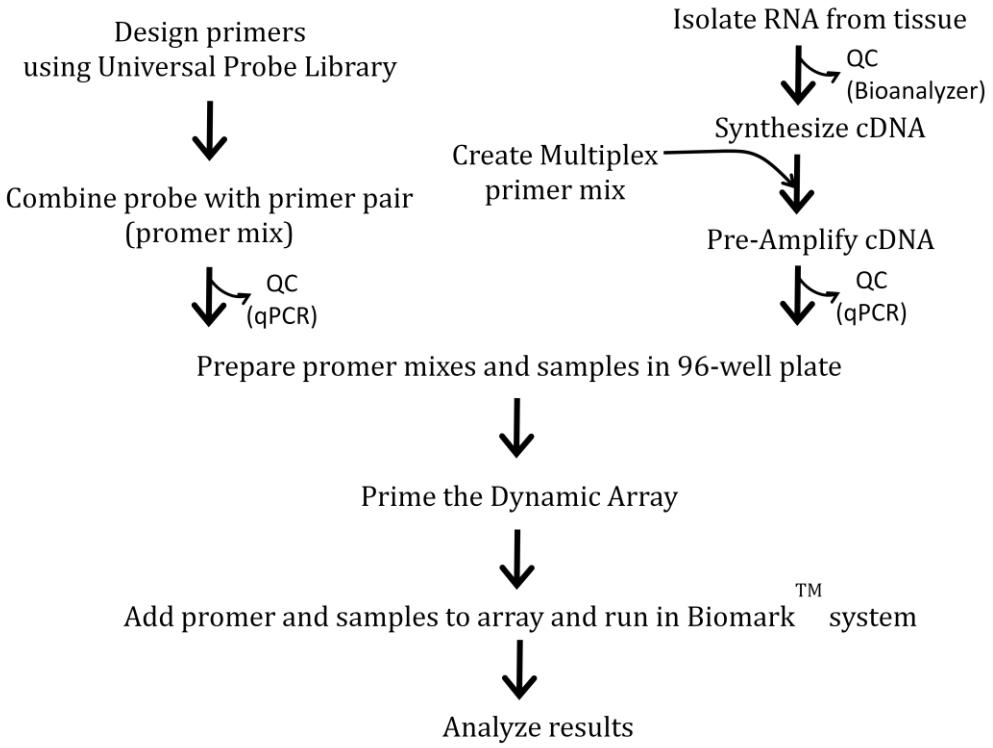


Figure 2-1: High-throughput RT-qPCR workflow diagram

2.12.1 Assay Design

The sequences of all genes designed in this project were found using the NCBI reference nucleotide sequences for *Rattus norvegicus* and *mus musculus*. Sequences were copied and pasted into the Universal Probe Library (UPL) probefinder version 2.45 for the rat or mouse, respectively. The software is designed to optimize an assay based around

a set of 165 locked nucleic acid probes. Locked nucleic acids are chemically altered nucleic acid analogues which are incorporated into DNA or RNA structure, but with improved stability when heated. The ribose sugar ring contains a methylene linkage between the 2'oxygen and the 4'-carbon (**Figure 2-2**).

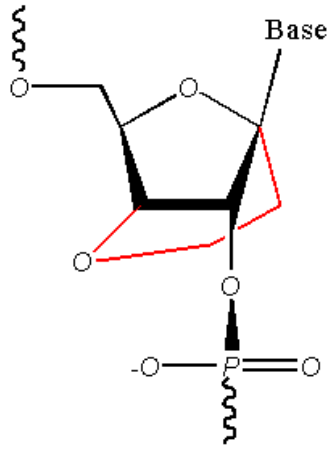


Figure 2-2: Locked nucleic acid design of UPL probes, highlighting the presence of methylene linkage within the ribose sugar.

These probes are 8-9 nucleotides in length and contain a fluorescein label at the 5' end, with a quencher dye at the 3' end. The short length of the probes means that the sequences occur frequently within the genome. Therefore, a single probe could potentially be used in several different assays. It is the design of the primers which give each assay its specificity for a particular gene of interest. Assays are designed using the UPL probe finder software to first locate exon-exon junctions, and then identify a

probe with a complementary sequence to bind near these regions. The third step is to design forward and reverse primers which will bind around the exon-exon junctions with a probe in between. Finally, assays are ranked based on an *in silico* approach to minimize the risk of assay binding in another area of the genome.

2.12.2 RNA Isolation

Frozen tissue was disrupted in >3 times the volume of TRIzol. 1 mL aliquots were incubated at room temperature for 5 min prior to centrifugation at 13,000 rpm for 10 min at 4°C. Chloroform extraction of the supernatant was followed by incubation at room temperature. Separation was achieved by centrifugation at 13,000 rpm for 15 min and the supernatant was transferred to a new tube. Isopropanol was added 1:1 to recover nucleic acids. Samples were frozen at -80°C overnight to precipitate RNA. Samples were thawed and spun at 13,000 rpm for 15 min to pellet RNA. The liquid layer was removed and 1 mL 75% v/v ethanol, prepared with RNAse-free water, was added prior to an additional spin. All of the alcohol was removed and the pellet air-dried $\leq$5 minutes. The pellet was re-dissolved in RNAse-free water and kept on ice for 30 minutes prior to freezing at -80°C.

RNA samples with contamination present, or prone to contamination due to a high lipid content, for example adipose tissue, were cleaned up with a further purification step. Chloroform (200 µL) was

added to samples, with a 15s vortex, and incubation at room temperature for 3 min. The samples were centrifuged at 10,000 rpm, 4°C for 15 min. The aqueous layer was carefully removed and mixed with 10 µL 8M lithium chloride solution. Next, 275 µL 95% v/v ethanol was added and samples were incubated on ice for at least 2 min, spun at 10,000 rpm, 4°C for 15 min, then washed with 70% ethanol. Samples were spun again at 13,000 rpm for 2 min at 4°C to pellet the RNA. The liquid was removed and the pellet air-dried for 5 min at room temperature and dissolved in 100 µL RNAse-free water. This lithium chloride procedure was repeated before a final resuspension in RNAse-free water. RNA was stored at -80°C until reverse-transcribed.

2.12.3 RNA Quality Assessment

The concentration and purity of RNA in each sample was determined measuring absorbance at A260 and A280 in 1/10 Tris-EDTA. The RNA quality of each sample was assessed using an RNA 6000 Nano kit with 2100 bioanalyzer software according to manufacturer's instructions (Agilent Technologies). This kit is a miniaturized version of gel electrophoresis. A polymer gel is loaded into the chip with a fluorescent dye. Electrodes in the instrument send an electric current through the gel which causes charged molecules to migrate through the gel. The components within a sample are separated by size as the sample is filtered through the gel matrix. The fluorescent dye intercalates the RNA

and detection of the fluorescence is plotted as fluorescence units (FU) over time. A ladder is provided in the kit, which contains molecules of known size, and is used to create a standard curve of size versus migration time with which unknown samples can be compared in order to calculate their size. Briefly, RNA was diluted to a concentration of 250 ng/µL. 1 µL of gel dye was added to 65 µL of filtered gel (both provided), mixed thoroughly and spun at room temperature, 13,000 rpm for 10 min. Samples and loading ladder were heated at 65°C for 2 minutes. Gel was loaded into RNA 6000 Nano chip and set for 30s. 5 µL nanomarker was added into all wells on the chip. 1 µL loading ladder and samples were added to designated wells on the chip by reverse pipetting. The chip was vortexed at 2400 rpm for 60s, then loaded into the bioanalyzer for analysis. The quality of the RNA is then determined using the ratio of 28S and 18S ribosomal RNA; good quality RNA has a ratio close to 2. If there is degradation within a sample, this ratio will decrease due to the breakdown of larger products and an accumulation of smaller size fragments. The Agilent 2100 bioanalyzer software also uses an algorithm to assign each sample a RNA integrity number (RIN), which takes into account the entire fluorescence plot, in addition to the ribosomal RNA ratio. **Figure 2-3** shows the results following isolation of RNA from liver tissue of rats. For these experiments, RNA was considered of high quality if a RIN of 8 or above was obtained.

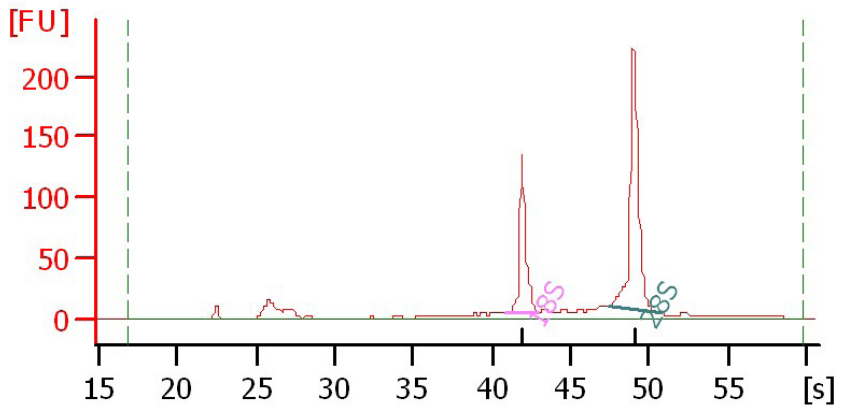


Figure 2-3: Electropherogram of total rat liver RNA assessed using RNA nano 6000 kit from Agilent Technologies.

2.12.4 cDNA Synthesis

RNA samples were treated with DNAse I to digest genomic DNA, and heated at 65°C for 10 minutes to inactivate the enzyme. Two micrograms RNA was brought to a total volume of 8 µL with RNAse-free water. One microlitre of each DNAse-1 buffer and DNAse 1 enzyme was added and incubated at room temperature for 10 min. One microlitre 25mM EDTA was added to inactivate the enzyme, followed by 10 min heating at 65°C. RNA was reverse-transcribed using Superscript II. One microlitre oligo dT was mixed with each sample and heating at 65°C for 10 min. For each sample, 4 µL 5X first strand buffer, 0.4 µL 25 mM deoxyribonucleotide phosphates (dNTPs), 2 µL dithiothreitol, 0.6 µL RNAse-free water were mixed and added. One microlitre Superscript II

was added as the final step and samples were heated at 42°C for 50 min, 94°C for 15 min, cooled to 4°C and stored at -20°C until their use.

2.12.5 Primer Design and Quality Control

Primer sequences and fluorescein-labelled probes for target transcripts were designed with the Universal Probe Library (Roche) based on the NCBI reference nucleotide sequences for *Rattus norvegicus*. All primers (see **Appendix 1**) were compared with the reference sequence for each gene to confirm the primers spanned an intron, to reduce the possibility of amplifying genomic DNA. Primers were rehydrated in RNAse-free water; forward and reverse primers were mixed 1:1 and stored at -20°C.

Each primer pair was visualized by ethidium bromide staining on agarose gel eletrophoresis. Due to the short length of the amplicons, products were run on a 2% agarose gel. Agarose (3 g) was mixed with 150 mL tris-acetic acid-EDTA buffer and heated to achieve a transparent solution. Ethidium bromide solution (15 µL of 1 µg/mL) was mixed in and the gel was poured into a mould and allowed to set at room temperature. Meanwhile, a PCR mastermix was prepared using 2.5 µL 10X PCR buffer, 0.2 µL 25 mM dNTPs, 1.25 µL 25 mM Mg^{2+}, 13.95 µL nuclease-free water, 0.1 µL platinum Taq DNA polymerase, and 5 µL of cDNA from rat liver (1 in 5 dilution, reverse transcribed from 2 µg RNA). Forward and reverse primers were added in duplicate, 3 µL each, to samples and a water-

blank. Reactions were run at 95°C for 4 min, then 35 cycles of 94°C for 20s (denaturation), 60°C for 20s (annealing) and 72°C for 20s (extension). Samples were heated at 72°C for 5 min for a final extension and cooled to 4°C. Samples were mixed with 8 µL DNA loading buffer. DNA loading buffer is a 40% solution of sucrose and 1 mM EDTA (pH 8.0) with bromophenol blue for colour. Molecular weight marker was prepared with 1.5 µL 1kB Plus DNA Ladder, 15 µL 1/10 TE buffer and 5 µL DNA loading buffer. Samples and ladder were loaded into agarose gel and run at 84 V for 1 h. The presence of bands for each primer pair were confirmed by visualizing the ethidium bromide staining of amplicons produced by PCR reactions. Primer-dimer formation was evaluated in the reactions containing water in place of template cDNA.

On occasion, despite the ability of a pair of primers to amplify a region of interest, the probe may not optimally bind to a complementary sequence within the amplified region as predicted by the UPL software. Therefore, each assay was validated by qPCR using rat liver cDNA as a template (StepOnePlus, Applied Biosystems). 2 µL 100X primer pair mix and 1 µL probe (10 µM) were mixed to form a promer mix (primer + probe). A mixture of 2.5 µL cDNA from approximately 20 ng input RNA, 2 µL nuclease-free water and 5 µL Taqman universal master mix (ABI). Reactions were run in duplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Successful promers had a mid-range threshold cycle number and sigmoidal curve shape (**Figure 2-4**).

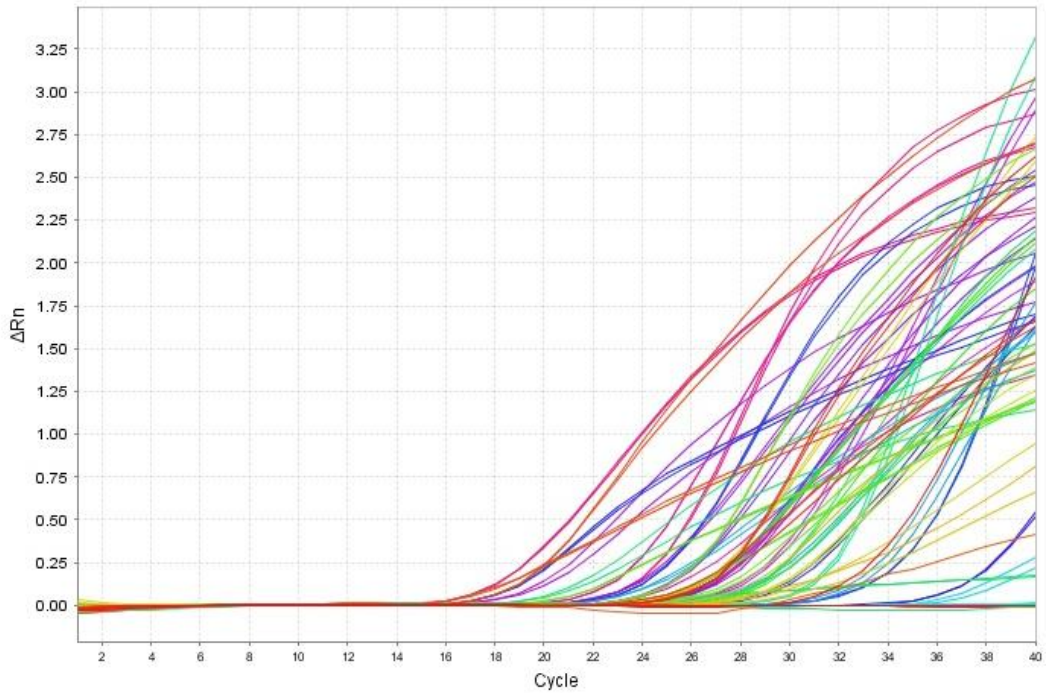


Figure 2-4: Amplification plot of promers tested by qPCR.

2.12.6 Preamplification of Genes of Interest

Primer mixes for each gene were combined in a single assay which was used to pre-amplify the cDNA of the genes of interest in each sample using Taqman preamplification reagent (ABI). First, a multiplex primer mix was created with 1 µL of forward and reverse primer mixes (100 µM) for up to 100 assays. The multiplex primer mix was used in a preamplification reaction for all samples in an experiment. 2.5 µL cDNA from 5 ng total RNA, 2.5 µL of the multiplex primer mix, and 5 µL 2x Taqman preamplication reagent were mixed for each sample. Reactions were run at 95°C for 10 min, followed by 14 cycles of 95°C for 15s and 60°C for 4

min. Samples were diluted 1:5 with RNAse-free water. Finally, each sample was tested using the promer for cyclophillin, a housekeeping gene, to confirm that all samples were preamplified. Forward-reverse primer mix for cyclophilin was added with corresponding probe 2:1 to create the promer mix. 0.5 µL promer mix, 4 µL nuclease-free water and 5 µL Taqman universal master mix was added to 0.5 µL of pre-amplified samples in duplicate. Reactions were run at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min.

2.12.7 High-Throughput Quantitative PCR

Experiments are designed using either 16 or 32 samples in triplicate. Up to 48 assays are used in the 48-by-48 chip format, including housekeeping genes, which are also run in duplicate. In the 96-well chip a total of 96 assays can be used. Preamplified samples and prepared promer mixes are used to make the sample loading mixes and the assay mixes, respectively. Promer mixtures were prepared up to one day prior to use, in a ratio of 2:1, 100x primer mix with corresponding probe. For each reaction, 3.25 µL DA assay loading reagent (Fluidigm) was mixed with 1.3 µL nuclease-free water and mixed with 2 µL promer mix. Forty-eight or 96 gene assays were loaded into separate wells by reverse pipetting on a 48-by-48 or 96-by-96 gene expression chip, respectively (Fluidigm). 4 µL Taqman universal master mix and 0.4 µL DA sample loading reagent (Fluidigm) were mixed with 2.5 µL pre-amplified sample. Priming fluid was

loaded into two chambers of the dynamic array integrated fluidic circuit (IFC). The chips were primed with the fluid by running the priming script in the IFC controller. Samples were loaded in triplicate in 48 or 96 sample wells. Assays and samples were loaded and mixed into 2304 or 9216 reaction wells of the microfluidic chip using a loading script in the IFC controller. qPCR was run on the Biomark^{TM} system (Fluidigm) at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Relative RNA expression for each gene in a sample was standardized to cyclophilin. The results obtained from the Biomark can be displayed in a heat map format, like the one pictured in **figure 2-5**, or in a table format. Fold-change in gene expression in experimental groups was calculated using the delta-delta Ct method.

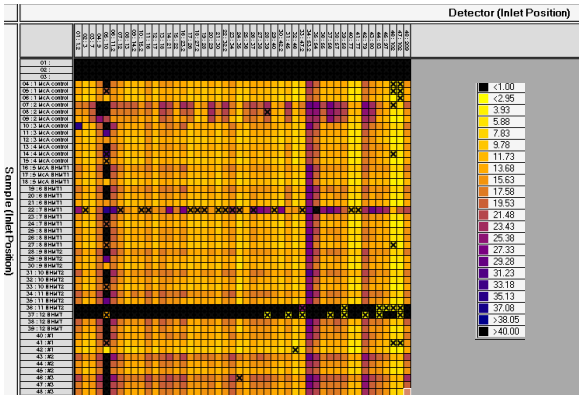


Figure 2-5: Heat map of results from high-throughput qPCR.

2.13.1 Culture of 3T3L1 Adipocytes

3T3-L1 cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). At 2 days post-confluence (designated day 0), cells were induced to differentiate with DMEM supplemented with 10% (v/v) FBS, 1 *μ*M dexamethasone, 0.5 mM isobutylmethylxanthine, 1 *μ*g/ml insulin. Cells were incubated with increasing concentration of folic acid: 9 µM, 15, µM, 20 µM and 25 µM. DMEM contains 9 µM folic acid and this level was used as a reference. Differentiation media was refreshed daily. After 48 hours, the media were replaced with DMEM supplemented with 10% FBS and 1 *μ*g/ml insulin and the same level of folic acid that was used during differentiation. The cell media was refreshed every 24 following differentiation.

2.13.2 Measurement of TAG in 3T3-L1 Adipocytes

Cells were rinsed three times in sterile PBS then collected in 2 mL PBS by scraping. Cells were disrupted by vortexing, followed by sonication 3 x 15 s. TAG in glycerol was measured by colourimetric assay, according to manufacturer's instructions (Sekisui).

2.14 Statistical Analyses

All results are expressed as mean ± SEM. Differences between control and folate excess or folate deficient groups were assessed by one-way ANOVA followed by a Dunnet post-hoc test to compare either folate deficient or excess groups to the control group, or by a student's t-test, where indicated (Graph Pad Prism 5.0). Glucose tolerance analyzed by incremental area under the curve (iAUC) analysis following the glucose tolerance test. Differences were considered statistically significant at $P < 0.05$.

CHAPTER 3

RESULTS

3.1 The Effects of Dietary Folic Acid Deficiency and Excess in Rats Fed a High Fat Diet

3.1.1 Body Weight and Tissue Weights

To investigate the direct effect of dietary folic acid deficiency and excess on the development of obesity, rats were fed a high fat diet to induce weight gain. After 7 weeks, high fat and folic acid excess (HF/FE) rats gained significantly more weight than the control animals (**Figure 3-1**). Weight gain by the high fat folic acid deficient (HF/FD) group was not significantly different from high fat folic acid control (HF/FC).

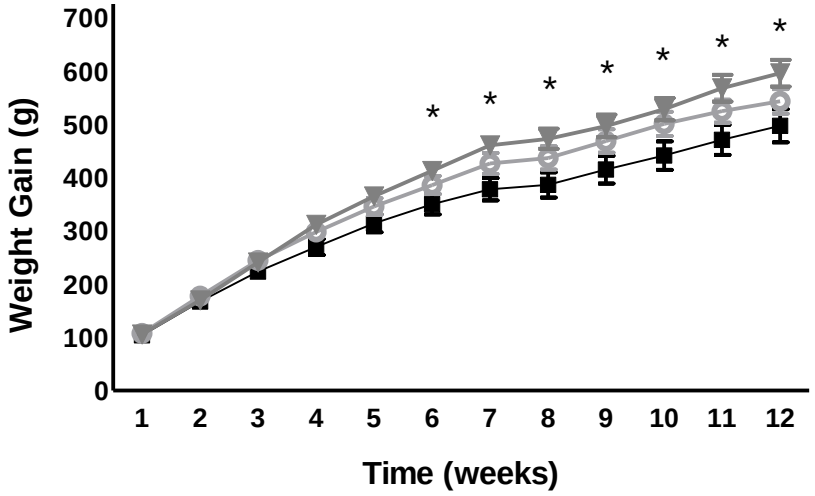


Figure 3-1: Weight gain over 12 weeks of high fat diet in rats fed control, deficient or excess folic acid. Squares (■) represent HF/FC, open circles (○) HF/FD and triangles (▼) HF/FE.* significantly different from HF/FC, P< 0.05.

Rats were maintained on their respective diets for a total of 12 weeks. Despite the fact that average daily food intake was not significantly different between the groups, rats fed excess folic acid gained significantly more weight than the control group (**Table 3-1**). To characterize any changes as a result of these dietary treatments, a number of tissues were weighed, and the length of the jejunum was measured. The only difference was a 1.4-fold increase in the weight of the peri-renal fat depot in the HF/FE compared with the HF/FC group. The weight of liver, kidney, brain, soleus muscle, epididymal fat, heart and pancreas, as well as the length of the jejunum were not altered by folic acid in this experiment.

	HF/FC	HF/FD	HF/FE
Food Intake (kcal/day)	120 ± 8	131± 3	140 ± 2
Body weight (g)	790.2 ± 36.5	834.8 ± 20.3	900.3 ± 26.8*
Liver (g)	24.59 ± 2.26	26.28 ± 1.70	26.44 ± 2.43
Peri-renal Fat Pad (g)	42.75 ± 5.04	54.50 ± 5.56	61.56 ± 5.72*
Epididymal Fat pad (g)	0.53 ± 0.12	0.69 ± 0.12	0.59 ± 0.10
Kidney (g)	2.18 ± 0.10	2.39 ± 0.06	2.25 ± 0.10
Brain (g)	2.45 ± 0.05	2.45 ± 0.04	2.36 ± 0.07
Soleus muscle (g)	0.23 ± 0.05	0.23 ± 0.02	0.21 ± 0.03
Heart (g)	2.03 ± 0.08	2.28 ± 0.14	2.38 ± 0.11
Pancreas (g)	1.30 ± 0.19	1.03 ± 0.09	1.29 ± 0.05
Jejunal length (cm)	138.0 ± 7.18	142.8 ± 2.63	145.5 ± 5.43

Table 3-1: Food intake, body weight and tissue characteristics of male rats fed 60% of energy from fat for 12 weeks containing control, deficient or excess levels of folic acid. * significantly different from HF/FC, $P < 0.05$.

3.1.2 Plasma One-Carbon Metabolites

In order to confirm the effects of dietary folic acid deficiency and excess, a number of one-carbon metabolites were measured in the plasma of these animals (**Table 3-2**). Folic acid deficiency reduced the amount of plasma folate to one third the level found in control animals. Furthermore, plasma homocysteine, a marker of folate deficiency, was 10-fold higher in the HF/FD group. Folate deficiency perturbed a number of metabolites in the one-carbon cycle. For example, plasma glycine was reduced to approximately 55% the level of control animals, while plasma serine and cystathionine was increased by 1.3- and 2.3-fold, respectively, in HF/FD fed rats.

Excess folic acid did not increase fasting plasma folate above the level of control. However, plasma homocysteine was reduced in HF/FE rats, indicating an effect of excess folic acid supplementation.

	HF/FC	HF/FD	HF/FE
Folate (nmol/L)	41.56 ± 0.50	12.57 ± 1.64*	41.30 ± 0.72
Homocysteine (μM)	3.28 ± 0.17	33.07 ± 2.92*	2.650 ± 0.14*
Methionine (μM)	69.73 ± 2.93	57.22 ± 2.52*	62.58 ± 1.01
Sarcosine (μM)	13.10 ± 0.98	16.85 ± 0.89*	15.68 ± 0.95
Methylglycine (μM)	7.19 ± 0.48	5.38 ± 0.43*	6.21 ± 0.31
Glycine (μM)	425.0 ± 21.6	231.8 ± 18.2*	345.5 ± 24.0*
Serine (μM)	339.2 ± 11.2	452.7 ± 26.6*	364.5 ± 10.9
Cystathionine (nM)	914.7 ± 69.2	2155 ± 228*	763.3 ± 37.2
Cysteine (nM)	311.3 ± 4.5	265.5 ± 9.3*	305.7 ± 9.7
α-aminobutyrate (μM)	27.68 ± 2.6	27.02 ± 2.0	35.60 ± 4.0

Table 3-2: Plasma one-carbon metabolites. * significantly different from HF/FC, $P < 0.05$.

3.1.3 Adiposity

The increased weight gain observed in the HF/FE animals led to a further investigation of adiposity. Body composition of animals was assessed after 8 weeks of dietary treatment. An increased fat mass was observed in HF/FE animals when compared with HF/FC (**Figure 3-2 A**). There were no differences in lean mass or fluid mass as a result of deficient of excess amounts of folic acid in these rats at 8 weeks of age (**Figure 3-2 B**, and **C**).

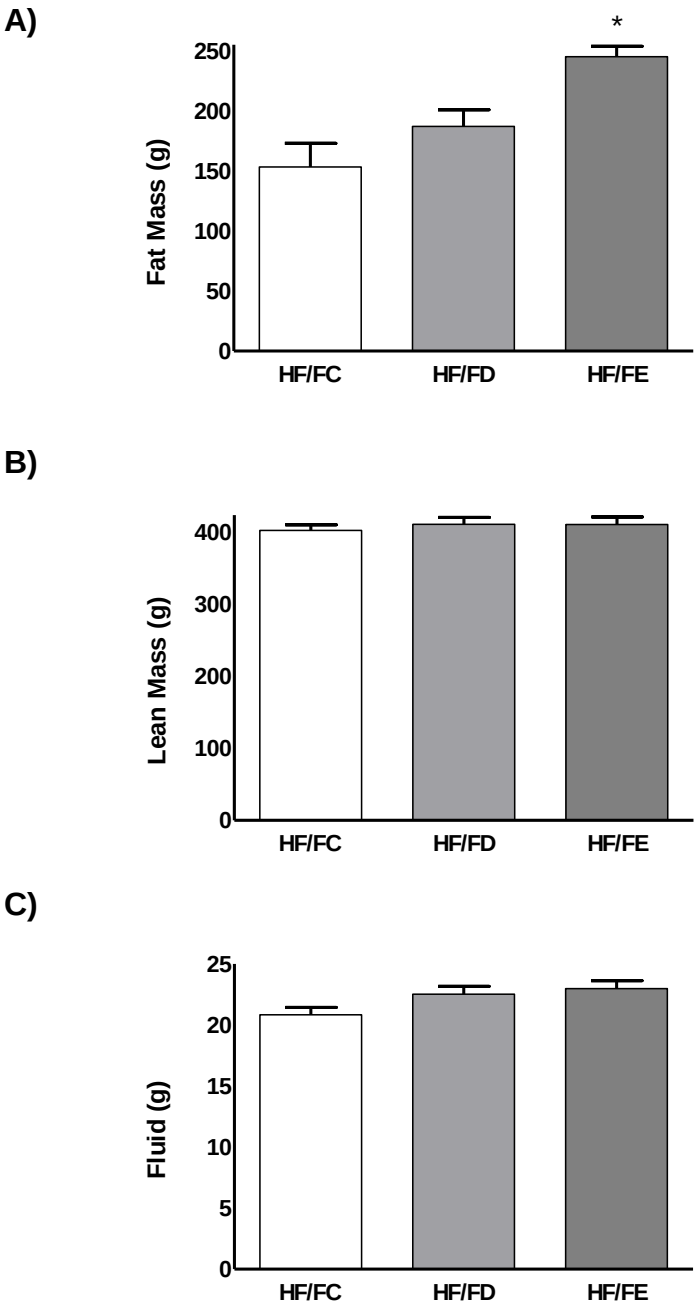


Figure 3-2: Body composition in male rats after 8 weeks of high fat diet with control, deficient or excess levels of folic acid. A) Fat mass B) lean mass C) fluid mass.

*significantly different from HF/FC, P< 0.05.

Staining of a cross section of peri-renal adipose tissue with hematoxylin and eosin and quantification of the area revealed larger size of adipocytes in the HF/FE and HF/FD groups relative to HF/FC (**Figure 3-3**)

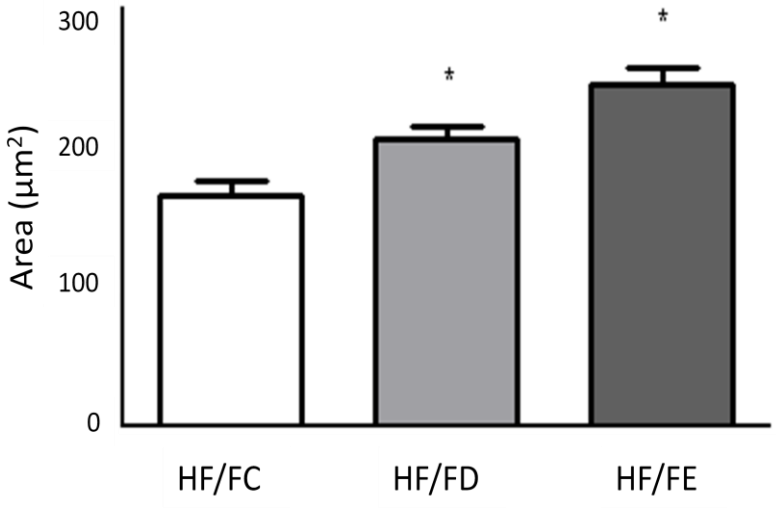


Figure 3-3: Quantification of the size of adipocytes from visceral adipose tissue of high fat diet fed mice.

*significantly different from HF/FC, P< 0.05.

3.1.4 mRNA Expression in Adipose Tissue

Due to the enhanced lipid accumulation in the adipose tissue in response to altered dietary folic acid, the expression of genes involved in lipid metabolism was analyzed in the visceral adipose tissue of animals fed HF/FC, HF/FD and HF/FE (**Table 3-3**). Additional transcripts not described here can be found in **Appendix 2**, **Table 1**.

There was evidence of increased phospholipid synthesis in the adipose tissue of HF/FD and HF/FE groups. Namely, *Agpat2*, *agpat3*, *chka* and *pemt* were higher compared with HF/FC. Fatty acid esterification was higher in the HF/FD and HF/FE diets, likely as a result of increased fatty acid storage in the adipose tissue. *Pparg* expression was higher in both HF/FD and HF/FE animals fed a high fat diet. *Pparg* encodes the gene for peroxisome proliferator activated receptor gamma, which functions as an enhancer of adipocyte differentiation and promotes lipid storage. The adipocyte differentiation marker, *adrp*, was higher, as well as *mogat1* and *dgat1*. Expression of fatty acid transporter, *fatp4*, was up-regulated by approximately 2-fold in both HF/FD and HF/FE groups. Genes involved in fatty acid desaturation and elongation were altered by dietary folic acid. The delta-6 desaturase, *fads2*, was lower, approximately 50% in dietary folic acid deficiency and excess. *Elovl5* was higher 10-(HF/FD) and 13-fold (HF/FE), *elovl1* was higher by nearly 3-fold, and *elvol6* was higher approximately 4-fold in both groups. *Elovl2*, on the other hand, was lower in the HF/FE group.

The adipose tissue is a major site of cholesterol storage in the body. Dietary folic acid markedly enhanced the expression of genes involved in cholesterol metabolism. Nuclear receptor proteins involved in the regulation of cholesterol homeostasis, LXRα and LXRβ*,* were higher at the level of transcription (*nr1h3* and *nr1h2*) in both HF/FD and HF/FE groups compared with HF/FC. Compared with HF/FC, *hmcgr* mRNA was

13 and 10 times greater in HF/FD and HF/FE, respectively. However, the regulatory protein *insig1* was also upregulated in both groups. The enzyme responsible for cholesterol esterification prior to its storage in the adipocyte, *soat1*, was also significantly higher at the level of transcription, in dietary folic acid deficiency and excess. Finally, scavenger receptor transcript levels (*scarb1*) were higher by approximately 4-fold in both HF/FD and HF/FE groups. These data suggest increased uptake, synthesis and storage of cholesterol in adipose tissue in response to altered dietary folic acid.

An increased accumulation of lipids in adipose tissue is often associated with the development of inflammation. Up-regulation of *cd68*, *nox1* and *hspa5* support the presence of macrophages and inflammation in adipose tissue of HF/FE fed animals. The pro-inflammatory cytokine tumor necrosis factor alpha (tnf) was 75- and 86-times the level of control in HF/FD and HF/FE, respectively. These results suggest increased adipose-derived inflammation on a high fat diet in response to folic acid deficiency and excess.

Transcript	HF/FC	HF/FD	HF/FE
Phospholipid Synthesis			
Agpat3	1.44 ± 0.23	2.80 ± 0.27*	3.26 ± 0.55*
Agpat2	1.26 ± 0.13	2.07 ± 0.19*	2.10 ± 0.17*
Chka	1.02 ± 0.16	2.40 ± 0.30*	2.37 ± 0.27*
Pcyt1a	1.49 ± 0.49	1.89 ± 0.12	1.88 ± 0.16
Pcyt2	1.16 ± 0.09	3.37 ± 0.40	3.53 ± 0.43
Pemt	1.06 ± 0.07	1.58 ± 0.14*	1.41 ± 0.13*
Fatty Acid Esterification			
Mogat1	1.06 ± 0.34	4.43 ± 1.38*	5.77 ± 1.02#
Dgat1	1.55 ± 0.32	4.90 ± 1.04*	5.90 ± 0.50*
Dgat2	1.37 ± 0.22	2.30 ± 0.27	2.30 ± 0.18#
Fatty Acid Desaturation and Elongation			
Scd1	1.13± 0.32	1.42 ± 0.20	1.28 ± 0.23
Fads1	0.80 ± 0.11	2.15 ± 0.46	2.20 ± 0.48
Fads2	0.90 ± 0.05	0.51 ± 0.04*	0.50 ± 0.07*
Elvol1	1.35 ± 0.25	3.00 ± 0.31*	3.19 ± 0.40*
Elvol2	0.68 ± 0.17	0.45 ± 0.20	0.074 ± 0.032*
Elovl5	0.96 ± 0.22	10.21 ± 2.70*	13.02 ± 3.46*
Elovl6	0.76 ± 0.24	3.84 ± 0.35*	4.66 ± 0.80*
Fatty Acid Transport			
Fatp4	1.32 ± 0.18	2.62 ± 0.21*	2.22 ± 0.12*
Cholesterol Metabolism			
Hmgcr	1.17 ± 0.55	15.59 ± 4.12*	10.39 ± 1.77*
Scarb1	0.95 ± 0.25	3.65 ± 0.10*	3.88 ± 0.46*
Insig1	1.15 ± 0.22	3.10 ± 0.28*	3.49 ± 0.38*
Soat1	1.37 ± 0.84	9.08 ± 1.52*	10.77 ± 1.64*
Adrp	1.17 ± 0.51	6.24 ± 0.72*	5.92 ± 0.47*
Transcription Factors			
Pparg	1.33 ± 0.30	3.58 ± 0.73	3.70 ± 0.54#
Nr1h3	0.96 ± 0.30	2.95 ± 0.20*	3.17 ± 0.39*
Nr1h2	1.00 ± 0.21	3.67 ± 0.30*	3.85 ± 0.34*
Srebf1	1.01 ± 0.08	2.49 ± 0.29*	2.39 ± 0.36*
Srebf2	1.28 ± 0.18	3.67 ± 0.62*	2.29 ± 0.56*
Inflammation			
Cd68	0.86 ± 0.14	1.36 ± 0.18	1.70 ± 0.16*
Nox1	0.86 ± 0.18	1.19 ± 0.68	17.64 ± 5.56*
Hspa5	1.03 ± 0.41	1.34 ± 0.64	3.12 ± 0.66#
Tnf	1.10 ± 0.10	82.40 ± 55.1*	95.96 ± 45.0*

Table 3-3: Adipose tissue gene expression in rats fed diets containing 60% of energy from fat, expressed as fold-change relative to control. *significantly different compared with HF/FC determined by one-way ANOVA. #significantly different compared with HF/FC using a student's t-test.

3.1.5 Lipid Accumulation in 3T3-L1 Adipocytes

In order to gain an understanding of the involvement of folic acid in adipocyte differentiation and lipid accumulation, 3T3-L1 cells were incubated with either 9 mM or 20 mM folic acid during differentiation and post differentiation. Expression of *pparg* was measured in these cells as a measure of adipocyte differentiation. **Figure 3-4** shows *pparg* was undetectable in undifferentiated 3T3-L1 cells. However, in differentiated cells, *pparg* expression was increased in those incubated with excess folic acid. These results are consistent with those found in adipose tissue of rats fed excess folic acid on a high fat diet.

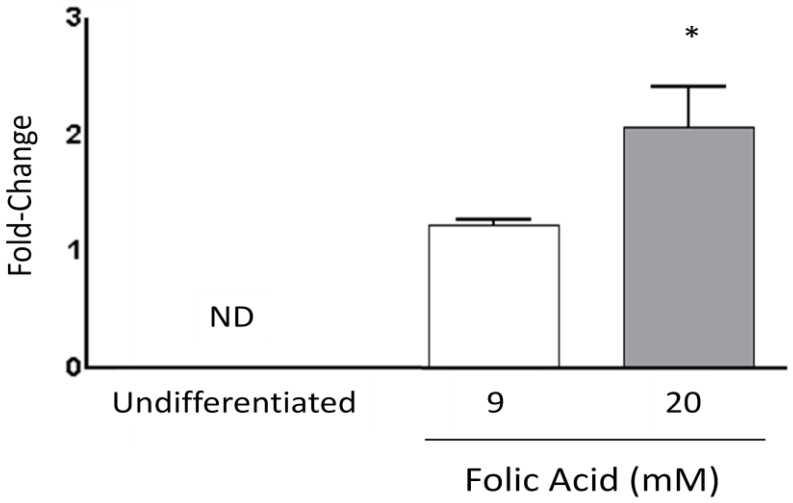


Figure 3-4: Expression of *pparg* in 3T3-L1 cells.

*significantly different from 9 mM folic acid, P< 0.05.

Differentiated 3T3-L1 cells were collected for TAG analysis. Cells incubated with 20 mM folic acid had elevated levels of TAG compared with the control cells, shown in **figure 3-5**.

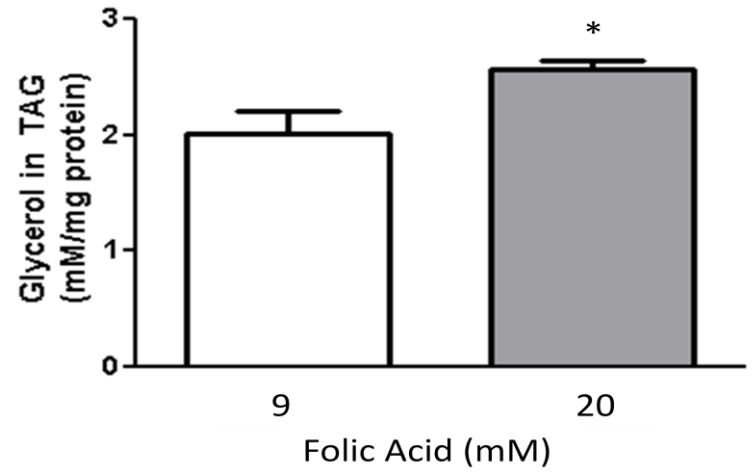
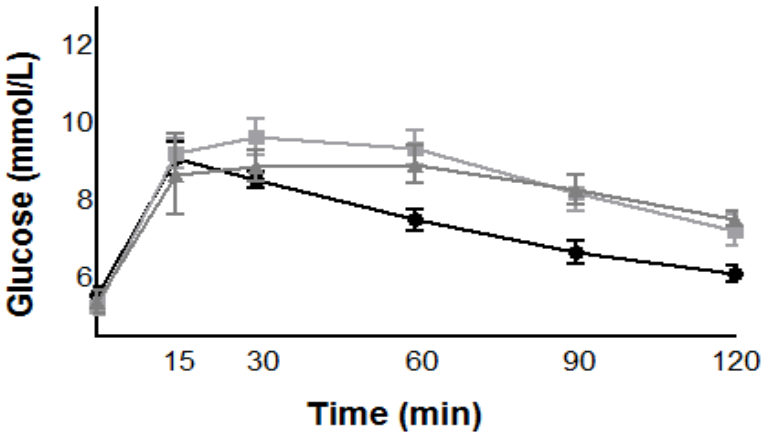


Figure 3-5: TAG levels in 3T3-L1 adipocytes differentiated in 9 mM or 20 mM folic acid. Results pooled from 3 separate experiments. *significantly different from HF/FC, P< 0.05.

3.1.6 Glucose Tolerance

Obesity is associated with an increased risk of insulin resistance. Evidence of adipose tissue-derived inflammation was provided by the mRNA analysis of adipose tissue. To determine if the increased weight gain in rats fed HF/FE was associated with impairments in glucose clearance, animals were administered a glucose tolerance test. The results of this experiment are shown in **figure 3-6**. Quantification of the area under the curve of blood glucose measured over the course of 2 hours following intra-peritoneal injection of a 2 mg/kg body weight dose of glucose indicate significantly impaired clearance of glucose in both HF/FD and HF/FE fed animals compared with the control group.

A)



B)

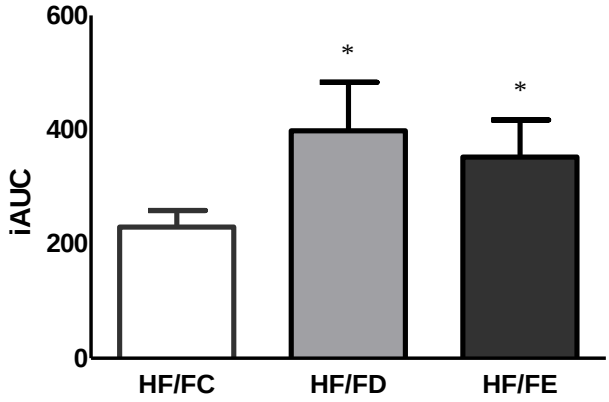


Figure 3-6: Blood glucose (A) and incremental area under the curve (B) following intra-peritoneal glucose tolerance test in 8-week old male rats fed high fat diet with control, deficient and excess levels of folic acid. Circles (●) represent HF/FC. Squares (■) represent HF/FD. Triangles (▲) represent HF/FE. *significantly different from HF/FC, P< 0.05.

While impaired post-prandial glucose tolerance was found, **table 3-4** shows no changes in fasting insulin or glucose in either HF/FD or HF/FE.

	HF/FC	HF/FD	HF/FE
Fasting insulin (ng/mL)	1.52 ± 0.44	2.22 ± 0.53	2.26 ± 0.51
Fasting glucose (mmol/L)	12.51 ± 2.26	13.32 ± 1.14	14.82 ± 2.37

Table 3-4: Fasting insulin and glucose levels in response to altered dietary folic acid in rats fed high fat diet.

3.1.7 Effect of Folic Acid Deficiency and Excess on Hepatic One-Carbon Metabolites

The liver maintained normal levels of many one-carbon metabolites following dietary folic acid deficiency (summarized in **Table 3-5**). The concentration of amino acids homocysteine, methionine and cysteine did not differ among the HF/FD and HF/FC groups. Glycine was reduced to approximately 60% the level found in control livers, which was 3617 nmol/g. There was a 137% higher concentration of serine in the liver of the HF/FD compared with the HF/FC group. Similar to plasma, cystathionine concentration was 2.3 times higher in liver of HF/FD fed animals compare with the HF/FC group. None of the one-carbon metabolites measured in the liver were altered by excess folic acid in the diet.

	HF/FC	HF/FD	HF/FE
Homocysteine (nmol/g)	36.45 ± 3.69	31.24 ± 2.20	38.39 ± 4.68
Methionine (nmol/g)	484.2 ± 39.1	383.5 ± 30.2	447.2 ± 33.0
Sarcosine (nmol/g)	48.9 ± 5.9	35.5 ± 3.5	64.18 ± 9.1
Methylglycine (nmol/g)	25.43 ± 3.94	43.28 ± 3.41	33.08 ± 8.37
Glycine (nmol/g)	3617 ± 190	2139 ± 85.3*	3301 ± 225
Serine (nmol/g)	1639 ± 123	2253 ± 223*	1589 ± 138
Cystathionine (nmol/g)	3.82 ± 0.67	8.88 ± 0.67*	4.77 ± 0.69
Cysteine (nmol/g)	1241 ± 67.6	1079 ± 90.3	1222 ± 54.7
α-aminobutyrate (nmol/g)	89.83 ± 13.2	107.7 ± 8.3	94.7 ± 6.6

Table 3-5: One-carbon metabolites in the liver.

Impairments in hepatic one-carbon metabolism are linked with liver damage. Accordingly, plasma alanine aminotransferase, a marker of liver damage, was increased in HF/FD relative to the control group (**Figure 3-7**). There was no significant difference found between HF/FE and HF/FC groups.

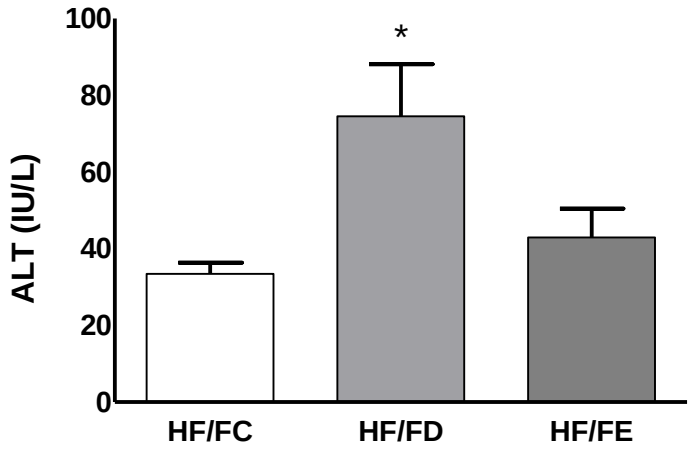


Figure 3-7: Plasma alanine amino transferase in high fat diet-fed rats. *significantly different from HF/FC, P< 0.05.

3.1.8 Hepatic Phospholipid Metabolism

Due to the potential role of folate to influence choline metabolism, choline-containing compounds and metabolites were measured in the liver. Free choline concentrations were not altered by dietary folic acid (**Table 3-6**). However, in the HF/FD group betaine concentration was 50% of the concentration found in HF/FC fed animals. Folate deficiency also lowered choline-containing phospholipids PC and lyso-PC in the liver, each to about 66% of the concentration found in HF/FC fed rats. **Figure 3-8** illustrates that impaired methylation due to folate deficiency reduced PC synthesis via the PEMT pathway.

Despite the apparently normally functioning one-carbon cycle in the HF/FE group, excess dietary folic acid resulted in lower content of phospholipids PC, lyso-PC and lyso-PE (Table 6). Furthermore, PEMT activity was lower in the HF/FE-fed animals (Figure 8)

	HF/FC	HF/FD	HF/FE
Choline (nmol/mg)	212.7 ± 28.7	296.4 ± 52.6	264.6 ± 39.8
Betaine (µmol/mg)	2.10 ± 0.27	1.1 ± 0.15*	2.4 ± 3.04
GPC (nmol/mg)	0.34 ± 0.07	0.53 ± 0.9	0.32 ± 0.03
PC (µmol/mg)	14.00 ± 0.78	9.27 ± 1.19*	10.49 ± 0.81*
Lyso-PC (µmol/mg)	1.35 ± 0.12	0.91 ± 0.10*	0.92 ± 0.05*
PE (µmol/mg)	9.10 ± 0.47	9.35 ± 0.71	7.72 ± 0.32
Lyso-PE (µmol/mg)	0.58 ± 0.06	0.45 ± 0.06	0.34 ± 0.02*
PI (µmol/mg)	2.30 ± 0.09	2.07 ± 0.14	1.99 ± 0.10
PS (µmol/mg)	2.62 ± 0.14	3.22 ± 0.27	2.76 ± 0.28
SM (nmol/mg)	1152 ± 47.85	1274 ± 109.8	1015 ± 68.2

Table 3-6: Choline-containing compounds, metabolites and phospholipids in the liver. *significantly different from HF/FC, P< 0.05.

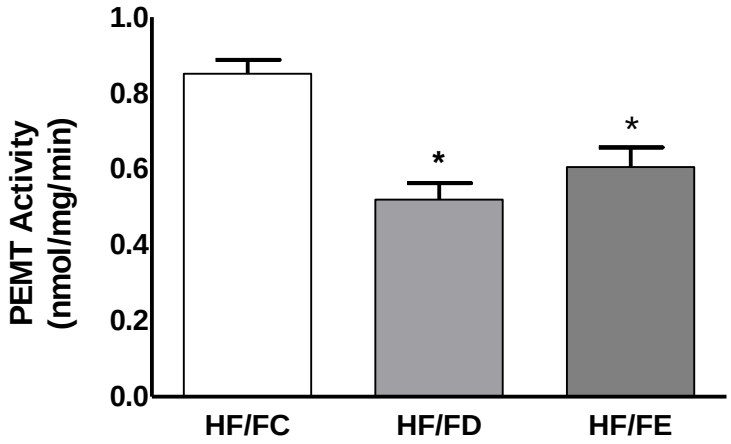


Figure 3-8: PEMT activity in the liver in rats fed high fat diet and altered levels of folic acid. *significantly different from HF/FC, P< 0.05.

3.1.9 Neutral Lipids in the Liver and Plasma

Phospholipids are required for the storage of lipids in the lipid droplet, as well as their secretion from the liver and transport in plasma in lipoprotein particles. Though a lower concentration of PC was found in both HF/FD and HF/FE, the concentration of TAG and cholesterol in the liver (**Table 3-7**) or plasma (**Table 3-8**) did not differ among groups.

	HF/FC	HF/FD	HF/FE
TAG (µg/mg protein)	290.8 +- 68.6	288.9 ± 43.0	301.7 ± 71.7
CE (µg/mg protein)	23.51 ± 5.83	16.66 ± 3.52	19.81 ± 6.25
FC (µg/mg protein)	6.08 ± 0.39	5.92 ± 0.25	6.38 ± 0.48

Table 3-7: Hepatic cholesterol and TAG levels of high fat diet-fed rats. There were no significant differences among groups.

	HF/FC	HF/FD	HF/FE
TAG (µg/mL)	292.6 ± 43.26	193.6 ± 13.67	211.8 ±46.21
CE (µg/mL)	72.92 ± 5.87	56.21 ± 3.91	60.18 ± 7.97
FC (µg/mL)	134.4 ± 18.65	83.54 ± 10.21	118.1 ± 15.41

Table 3-8: Plasma cholesterol and TAG levels of high fat diet-fed rats. There were no significant differences among groups.

3.1.10 mRNA Expression in the Liver

In order to gain some understanding of the mechanisms involved in the phenotypic changes observed in rats fed deficient and excess levels of folic acid, high-throughput quantitative PCR was used to screen a number of genes, primarily related to lipid metabolism. Results are summarized in **table 3-9**, with additional transcripts measured in **Appendix 2**, **Table 2**.

In the liver, both folic acid deficiency and excess lowered expression of *Agpat2* and *Pemt*. These encode for the proteins involved in the biosynthesis of phospholipids, lysophosphatidic acid acyl transferase and PEMT, respectively. HF/FD additionally decreased *lpin1* transcript levels to approximately 25% that of HF/FC, the gene encoding for phosphatidic acid phosphatase.

In HF/FD animals, there is a reduction in key genes involved in fatty acid synthesis, acetyl-CoA carboxylase (*acaca*) and fatty acid synthase (*fasn*). No down-regulation of fatty acid synthesis at the transcriptional level was observed in HF/FE. A few genes related to fatty acid transport and oxidation were also lower in folic acid deficient animals compared with the control group. These include fatty acid binding protein (*fabp*), *fatp4*, medium chain acyl-coenzyme A dehydrogenase (*mcad). Fatp4*, a transporter of very long chain fatty acids across the plasma membrane, was similarly lower in HF/FE. The expression of the liver isoform of carnitine-palmitoyl transferase was higher with folic acid excess. These observations may explain the maintenance of low levels of lipid in the liver, despite the increased total body fat in HF/FE animals. A reduction in lipids in the liver is also supported by decreased levels of *adrp*, which may be a lipid droplet marker in this tissue.

Genes involved in fatty acid desaturation and elongation were altered by dietary folic acid. The delta-9, delta-5 and -6 desaturases, *scd, fasd1 and fads2* were dramatically lower with dietary folic acid deficiency.

Fads2 expression was also lower in HF/FE animals. The elongase enzymes, *elovl2* and *elovl5*, were higher 2- and 4.6-fold, respectively, with folic acid excess.

Cholesterol metabolism was similarly affected by both folic acid deficiency and excess. Sterol regulatory binding protein 2 (*srebf2)*, a transcription factor which regulates cholesterol homeostasis, was decreased to 25% and 36% in HF/FD and HF/FE groups. The expression of a number of genes involved in cholesterol metabolism was also reduced, in support of this observation. Synthesis (*hmgcr*), esterification (*soat1*), and secretion (*mttp*, *apob*) were all reduced in both dietary groups compared with the control animals. Genes related to the regulation of cholesterol synthesis (*insig1* and *scap*) were also reduced in HF/FD only. With regard to HDL metabolism, *apoa1* and *abca1* expression were reduced in HF/FD, and *apoa1* was also reduced in HF/FE.

Interestingly, markers of inflammation were reduced in the liver of HF/FD animals at the transcriptional level, despite the increase in plasma ALT levels observed. *Cd68*, *hspa5* and *emr1* expression were reduced in both HF/FD and HF/FE.

Transcript	HF/FC	HF/FD	HF/FE
Phospholipid Synthesis			
Agpat3	1.10 ± 0.25	0.31 ± 0.07	0.53 ± 0.25
Agpat2	1.11 ± 0.28	0.30 ± 0.09*	0.44 ± 0.14*
Chka	1.14 ± 0.28	0.64 ± 0.08	1.00 ± 0.14
Pcyt1a	1.30 ± 0.45	0.71 ± 0.05	1.02 ± 0.26
Pcyt2	1.01 ± 0.07	0.84 ± 0.11	1.44 ± 0.29
Pemt	1.12 ± 0.27	0.25 ± 0.08*	0.38 ± 0.18*
Fatty Acid Esterification			
Mogat1	1.16 ± 0.34	0.36 ± 0.12	0.92 ± 0.50
Dgat1	1.01 ± 0.07	0.60 ± 0.10	0.72 ± 0.16
Dgat2	1.00 ± 0.05	0.53 ± 0.09*	0.89 ± 0.11
Fatty Acid Synthesis			
Acaca	1.10 ± 0.24	0.37 ± 0.05*	1.57 ± 0.58
Fasn	1.32 ± 0.45	0.11 ± 0.02*	2.09 ± 0.95
Me1	1.03 ± 0.13	0.52 ± 0.10*	0.90 ± 0.15
Fatty Acid Desaturation and Elongation			
Scd1	1.53 ± 0.65	0.13 ± 0.02*	2.74 ± 1.17
Fads1	1.05 ± 0.16	0.17 ± 0.04*	0.91 ± 0.20
Fads2	1.10 ± 0.26	0.09 ± 0.02*	0.21 ± 0.06*
Elvol2	1.01 ± 0.08	0.71 ± 0.12	2.06 ± 0.33*
Elovl5	1.03 ± 0.13	0.81 ± 0.03	4.73 ± 0.83*
Fatty Acid Transport			
Fatp1	1.03 ± 0.12	0.60 ± 0.06	1.13 ± 0.21
Fatp4	1.14 ± 0.32	0.27 ± 0.07*	0.23 ± 0.02*
Fabp	1.09 ± 0.24	0.38 ± 0.09*	0.51 ± 0.14
Cholesterol Metabolism			
Hmgcr	1.31 ± 0.22	0.33 ± 0.05*	0.50 ± 0.13*
Soat1	1.35 ± 0.14	0.81 ± 0.15*	0.57 ± 0.13*
Scarb1	1.03 ± 0.12	1.06 ± 0.10	1.34 ± 0.14
Ldlr	0.74 ± 0.03	0.31 ± 0.09	1.01 ± 0.17
Scap	1.24 ± 0.18	0.58 ± 0.07#	1.62 ± 0.49
Apob	1.42 ± 0.36	0.32 ± 0.08*	0.55 ± 0.12*
Mttp	1.09 ± 0.11	0.48 ± 0.11*	0.59 ± 0.09*
ApoA1	0.82 ± 0.10	0.32 ± 0.10*	0.32 ± 0.12*
Abca1	1.61 ± 0.50	0.26 ± 0.05*	0.93 ± 0.13
Adrp	1.58 ± 0.28	0.46 ± 0.02*	0.49 ± 0.05*
Transcription Factors			
Ppara	1.36 ± 0.14	0.34 ± 0.05*	0.82 ± 0.12*
Srebf1	0.88 ± 0.11	0.27 ± 0.32*	0.23 ± 0.26*
Srebf2	1.40 ± 0.27	0.35 ± 0.04*	0.42 ± 0.05*
Inflammation			

Cd68	0.87 ± 0.20	0.32 ± 0.10*	0.32 ± 0.13*
Hspa5	0.91 ± 0.22	0.41 ± 0.10*	0.38 ± 0.09*
Emr1	0.92 ± 0.06	0.50 ± 0.13*	0.40 ± 0.08*
Tnf	1.59 ± 0.45	0.83 ± 0.31	0.81 ± 0.37

Table 3-9: Fold-change in gene expression in the liver of rats fed diets containing 60% of energy from fat.

*significantly different compared with HF/FC determined by one-way ANOVA. #significantly different compared with HF/FC using a student's t-test.

3.2 The Effects of Dietary Folic Acid Deficiency and Excess in Rats Fed a Low Fat Diet

Many changes in weight gain and adiposity in rats fed excess folic acid were observed in the context of a high fat diet. We next investigated whether these folic acid deficiency or excess could alter these parameters on a lower fat diet, containing 15% of energy from lipid sources.

3.2.1 Body Weight and Tissue Weights

The metabolic effects of folic acid deficiency and excess observed in rats fed 60% of kcal from fat were not apparent when animals were fed a 15% of kcal from fat. Weight gain over 12 weeks was not altered by dietary folic acid content in rats fed the lower fat diet (**Figure 3-9**).

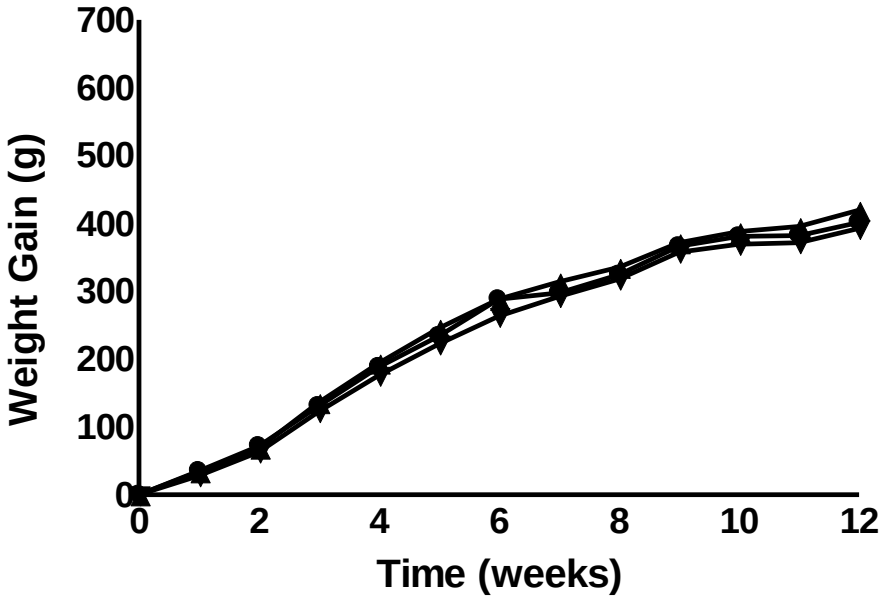


Figure 3-9: Weight gain of rats fed control, deficient or excess levels of folic acid for 12 weeks on a low fat diet. Circles (●) represent FC; triangles (▲) represent FD; inverted triangles (▼) represent FE. There were no significant differences among groups.

Food intake in male rats fed 0.75 mg/kg folic acid (FC), 0 mg/kg folic acid (FD), or 7.5 mg/kg folic acid (FE) was similar (**Table 3-10**). The weight of liver, peri-renal and epididymal fat pats were also unaltered by dietary folic acid in this experiment.

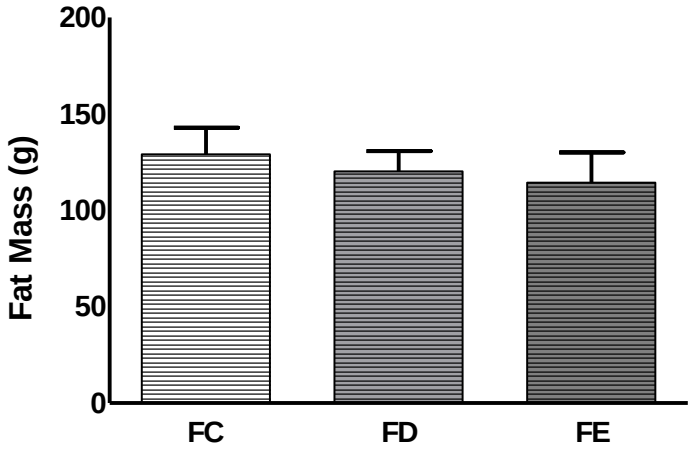
	FC	FD	FE
Food Intake (kcal/day)	103 ± 4	104 ± 4	106 ± 4
Liver (g)	18.67 ± 0.70	18.33 ± 0.70	18.60 ± 1.14
Peri-renal Fat Pad (g)	29.53 ± 4.74	23.07 ± 2.34	25.12 ± 4.60
Epididymal Fat Pad (g)	18.27 ± 1.41	17.53 ± 1.50	17.20 ± 3.26

Table 3-10: Food intake and tissue weight of rats fed control, deficient or excess levels of folic acid for 12 weeks on a low fat diet. There were no significant differences among groups.

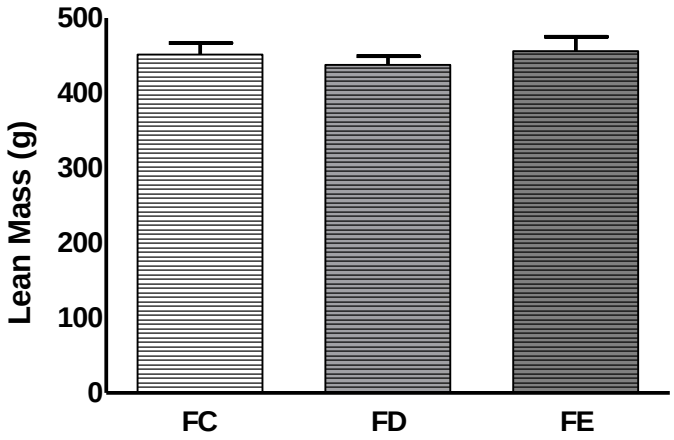
3.2.2 Adiposity

Body composition was assessed during the eighth week of dietary treatment, in order to replicate the high fat experiment as closely as possible. Fat, lean and fluid mass were similar in all three groups consuming 15% of kcal from fat, shown in **Figure 3-10**.

A)



B)



C)

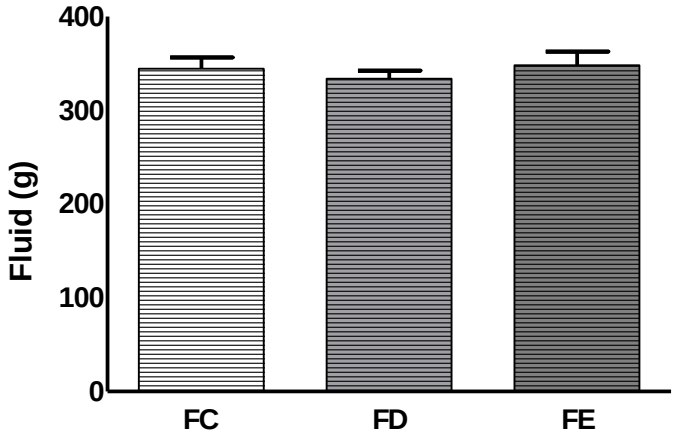


Figure 3-10: Body composition of rats fed control, deficient or excess dietary folic acid. There were no significant differences among groups.

3.2.3 mRNA Expression in Adipose Tissue

There were a number of transcripts altered in the adipose tissue of HFD-fed rats in response to altered dietary folic acid levels. Though there were no phenotypic differences observed in FD or FE animals relative to those fed the FC diet, changes in mRNA levels may yield insight into potential mechanisms which may be at play to favour lipid storage, if an excess of lipid were to be present. In other words, in the animals fed a moderate amount of fat, defects in the transcriptional regulation of key genes in lipid metabolism may point towards early indicators explaining why these animals are predisposed to increased lipid storage when fed a high fat diet. **Table 3-11** summarizes the main findings with regard to adipose tissue gene expression in response to dietary folic acid in rats fed a diet containing 15% of kcal from fat. Glycerol-3-phosphate acyl transferase (*agpat3*) mRNA was moderately reduced in folate deficient animals. *Pemt* transcript levels were significantly elevated by FE compared with FC. No changes in fatty acid esterification were observed in FD or FE animals in the absence of a high fat diet. Expression of the LDL receptor was modestly increased by FE. No other genes involved in cholesterol metabolism were altered by dietary folic acid. Inflammatory markers were also comparable to control animals in each of the FD and FE groups, with the exception of heat shock protein (*hspa5)* in the group fed FE. Additional transcripts measured in adipose tissue of low-fat diet-fed rats are summarized in **Appendix 2**, **Table 4**.

Transcript	Control	Folate Deficient	Folate Excess
Phospholipid Synthesis			
Agpat3	0.91 ± 0.06	0.85 ± 0.06*	0.91 ± 0.04
Agpat2	1.59 ± 0.39	1.31 ± 0.14	1.35 ± 0.15
Chka	1.35 ± 0.24	1.33 ± 0.16	1.67 ± 0.20
Pcyt1a	1.37 ± 0.28	1.27 ± 0.35	1.17 ± 0.08
Pcyt2	1.13 ± 0.17	0.95 ± 0.06	1.47 ± 0.13
Pemt	1.18 ± 0.10	1.38 ± 0.10	1.69 ± 0.20*
Fatty Acid Esterification			
Mogat1	0.82 ± 0.13	0.85 ± 0.12	0.85 ± 0.06
Dgat1	1.05 ± 0.07	0.99 ± 0.03	1.09 ± 0.04
Dgat2	1.85 ± 0.51	1.96 ± 0.30	2.49 ± 0.16
Cholesterol Uptake and Esterification			
Ldlr	1.1 ± 0.06	0.92 ± 0.08	1.34 ± 0.06*
Scarb1	1.13 ± 0.10	1.14 ± 0.09	1.14 ± 0.09
Soat1	1.40 ± 0.23	1.14 ± 0.26	0.96 ± 0.17
Transcription Factors			
Pparg	1.02 ± 0.12	0.94 ± 0.12	0.81 ± 0.06
Nr1h3	1.06 ± 0.07	1.04 ± 0.13	1.30 ± 0.05
Nr1h2	1.01 ± 0.06	0.93 ± 0.07	0.97 ± 0.03
Srebf1	1.37 ± 0.16	1.20 ± 0.12	1.66 ± 0.24
Srebf2	1.68 ± 0.35	1.78 ± 0.40	2.51 ± 0.21
Inflammation			
Cd68	1.00 ± 0.08	0.83 ± 0.11	0.86 ± 0.08
Nox1	1.53 ± 0.55	1.80 ± 0.30	1.34 ± 0.51
Hspa5	0.93 ± 0.03	1.04 ± 0.08	1.39 ± 0.08*
Tnf	1.44 ± 0.15	0.72 ± 0.17*	1.32 ± 0.30

Table 3-11: Transcript expression in visceral adipose tissue in response to altered dietary folic acid in rats fed low fat diets. *significantly different from HF/FC, P< 0.05.

3.2.4 Glucose Tolerance

In agreement with the comparable body weight, glucose clearance following a glucose tolerance test was not different in FD and FE groups, compared with FC (**Figure 3-11**).

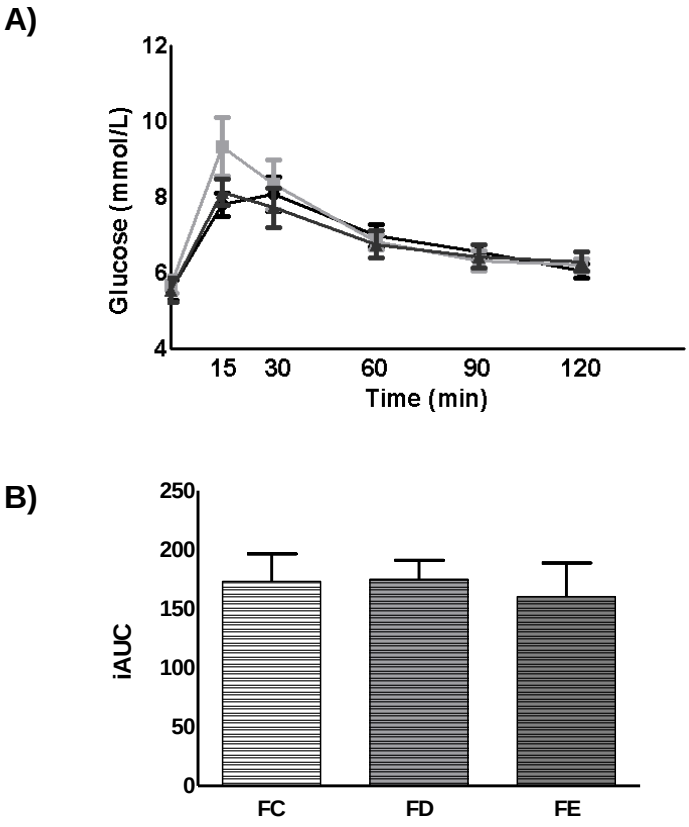


Figure 3-11: Blood glucose (A) and incremental area under the curve (B) following intra-peritoneal glucose tolerance test in 8-week old male rats fed control, deficient or excess levels of folic acid. Circles (●) represent HF/FC. Squares (■) represent HF/FD. Triangles (▲) represent HF/FE. There were no significant differences among groups.

3.2.5 Hepatic Phospholipid Metabolism

Hepatic phospholipid concentrations were measured by phosphorus assay. Unlike the results found in rats fed a high fat diet, PC and PE were similar among FC, FD and FE groups on a moderate fat diet (**Table 3-12**).

	FC	FD	FE
PC (μmol/mg protein)	134.1 ± 8.19	123.9 ± 5.1	135.3 ± 6.14
PE (μmol/mg protein)	58.18 ± 4.60	66.82 ± 3.43	62.58 ± 6.40
PC:PE	2.3 ± 0.10	1.9 ± 0.10	2.2 ± 0.18

Table 3-12: Phospholipid levels in the liver of rats fed control, deficient or excess dietary folic acid. There were no significant differences among groups.

3.2.6 Neutral Lipids in the Liver and Plasma

No differences in hepatic cholesterol or TAG were observed as a result of different intakes of folic acid in the diet (**Table 3-13**).

	FC	FD	FE
FC (µg/mg protein)	*9.47 ± 0.54*	*9.00 ± 0.26*	*9.68 ± 0.47*
CE (µg/mg protein)	*6.44 ± 0.58*	*7.75 ± 1.2*	*8.69 ± 0.84*
TAG (µg/mg protein)	*69.90 ± 5.40*	*99.56 ± 16.74*	*101.6 ± 25.56*

Table 3-13: Neutral lipids in the liver of rats fed control, deficient or excess dietary folic acid. There were no significant differences among groups.

Plasma fasting TAG was not altered by folic acid content of the diet (**Table 3-14**). Folate deficiency may contribute to liver damage by mechanisms related to alteration in one-carbon metabolism. Though one-carbon metabolites were not measured in this experiment, plasma ALT levels indicate the absence of liver damage in both FD and FE groups compared with FC.

	FC	FD	FE
Fasting TAG (mM)	1.98 ± 0.39	1.29 ± 0.20	1.60 ± 0.36
ALT (IU/L)	26.42 ± 4.05	32.18 ± 3.71	31.37 ± 7.08

Table 3-14: Plasma TAG and ALT in rats fed control, deficient or excess dietary folic acid. There were no significant differences among groups.

CHAPTER 4:

DISCUSSION

4.1 Folate and Obesity

Folate status has previously been associated with obesity in humans and animal models. Increased weight gain in folic acid-supplemented rats was found to be due to an increased fat mass (Burdge et al 2009; Engeham et al 2008). Folic acid supplementation *in utero* increased total body fat in male offspring at 4 weeks of age (Engeham et al 2008). While these alterations were not observed in offspring at 13 weeks of age (Engeham et al 2008). This suggests that sustained effects of excess folic acid to promote weight gain require chronic intake of excess levels of folic acid.

In this study, adipose tissue weight was significantly greater in rats fed excess folic acid on a high fat diet. The increased expression of *adrp* and *pparg* in adipose tissue are evidence of enhanced adipocyte differentiation and hypertrophy. *Adrp* is also increased in HF/FD fed animals. Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear transcription factor that interacts with peroxisome proliferator response elements upon ligand binding, to regulate target gene expression. PPARγ is a key regulator of adipogenesis and has been shown to promote lipid storage and contribute to hepatic steatosis (Gavrilova et al 2003). Maternal folic acid supplementation during *in utero* and postnatal periods or supplementation to pups at weaning decreased the methylation of the *pparg* promoter in rat liver (Sie et al 2013). Promoter methylation was inversely related to gene expression, which suggests increased *pparg*

expression in response to folic acid supplementation. In adipose tissue, *pparg* is nearly tripled upon folic acid supplementation (Burdge et al 2009). Therefore, increased body weight in folic acid supplemented animals may be due to enhanced lipid storage via PPARγ-dependent mechanisms.

Histological staining of white adipose tissue also indicated increased adipocyte size in high fat diet-fed folic acid deficient animals. This was accompanied by an increased expression of genes involved in esterification of fatty acids with glycerol, *mogat1* and *dgat1*, suggesting elevated TAG formation in both HF/FD and HF/FE groups. In support of these findings, differentiated 3T3-L1 cells had a greater amount of TAG when incubated in media containing excess folic acid. Changes in gene expression involved in cholesterol metabolism support an increase in the storage of cholesterol as well as TAG in folic acid deficiency and excess. Both increased synthesis and uptake of cholesterol may contribute to increased cholesterol in adipose tissue. The rate-limiting step in cholesterol synthesis is controlled by 3-hydroxy-3-methyl-glutaryl-CoA reductase, encoded by *hmgcr*. *Hmgcr* transcript levels were increased in both HF/FD and HF/FE fed rats. Correspondingly, sterol-*O*-acyltransferase 1 expression was also increased in both groups relative to HF/FC. This would allow for augmented cholesterol esterification to facilitate its storage within adipocytes. The expression of scavenger receptor class B member 1, which carries out cholesterol uptake from circulating lipoproteins, was up-regulated in folic acid deficient and excess fed rats. Increases in these

pathways suggest cholesterol storage in adipocytes may be enhanced in HF/FD and HF/FE fed rats.

Intracellular lipids are stored in lipid droplets surrounded by a monolayer of phospholipids. Up-regulation of genes involved in phospholipid biosynthesis, including *chka* and *pemt* supports increased storage of TAG in adipocytes upon folic acid supplementation. *Pemt* deficiency has been shown to reduce TAG accumulation in 3T3-L1 adipocytes and in adipose tissue of *pemt* knockout mice (Horl et al 2011).

When rats were fed a low fat diet, dietary folic acid had no effect on body weight or fat mass. For the most part, gene expression was also comparable in FC and FD or FE groups. However, *pemt* expression was elevated in FE vs. FC. Therefore, even in the absence of excess calories from fat, excess dietary folic acid up-regulates *pemt* at the level of transcription.

On a high saturated fat diet, dietary folic acid deficiency and excess altered a number of genes involved in fatty acid desaturation and elongation in white adipose tissue. Both HF/FD and HF/FE had a reduced expression of fasd2, a gene that encodes delta (6) fatty acid desaturase (D6D), compared with HF/FC rats. Cystathionine beta synthase (Cbs) heterozygous mice fed a diet high in methionine and low in folic acid is a model of moderately impaired methylation capacity. Cbs is the first enzyme in the irreversible conversion of homocysteine to cysteine. This pathway is responsible for removal of approximately 50% of homocysteine

and thereby helps to maintain methylation capacity. Under these dietary conditions, Cbs+/- mice have a reduced expression of *fads2* and lower D6D activity in the liver (Devlin et al 2007). These changes led to a reduction in longer chain desaturated fatty acids arachidonic acid and decosahexaenoic acid the end products of the D6D metabolism (Devlin et al 2007). These results suggest impaired methylation capacity may reduce fatty acid desaturation. Folic acid content of the diet also appears to exert an effect on the expression of fatty acid elongase enzymes. *Elovl1, elovl5* and *elovl6* expression were increased in HF/FD and HF/FE compared with HF/FC.

In addition to pparg, other transcription factors involved in lipid metabolism were altered by folic acid. Liver X receptors (α and β), sterol regulatory element binding proteins (1 and 2) were also increased by folic acid deficiency and excess when rats were fed a high fat diet. Dietary folic acid may enhance lipid synthesis and storage in adipose tissue by controlling gene expression of these key transcription factors.

Excessive lipid accumulation has been proposed to play a causative role in the promotion of inflammation. An unencumbered deposition of lipid can surpass the ability of adipocytes to adequately store those lipids. Consequently, inflammatory mediators, such as tumour necrosis factor alpha (TNF-α), are secreted by adipose tissue. This is followed by an infiltration of macrophages which generate additional inflammatory molecules. This chronic adipose-derived inflammation is

linked with insulin resistance. Systemic inflammatory cytokines hinder insulin signalling in adipose and non-adipose tissues, and may result in impaired glucose tolerance (Cildir et al 2013). Folic acid deficient rats fed a high fat diet had a large increase in the expression of tumour necrosis factor alpha (*tnf*). Folic acid supplementation increased expression of *tnf*, heat shock 70 kDa protein 5 (*hspa5*), and Nadph oxidase (*nox1*) and indicates increased inflammation of adipose tissue. **Figure 4-1** summarizes gene expression changes in visceral adipose tissue of rats fed deficient or excess folic acid which may favour lipid storage and the production of inflammation. The increase in inflammation in HF/FD and HF/FE rats may account for the reduced glucose tolerance observed in these animals.

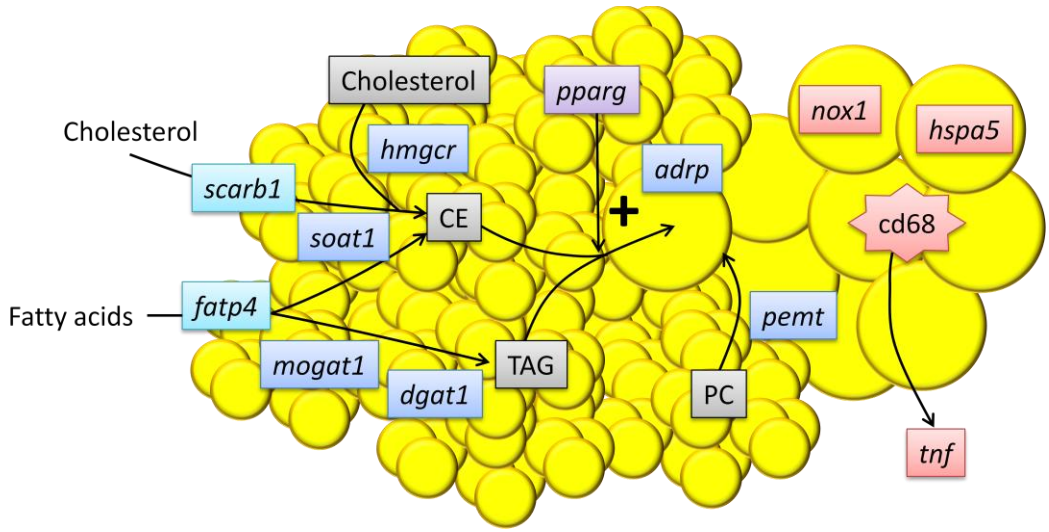


Figure 4-1: Proposed mechanisms up-regulating lipid storage in adipose tissue of rats fed excess dietary folic acid on a high fat diet based on mRNA expression.

4.2 Folate and Hepatic Lipid Metabolism

It is clear that impaired methylation capacity promotes hepatic fat accumulation through an impairment of PC synthesis. Dietary folate deficiency diminishes choline and PC levels in the liver (Kim et al 1994). PC and lyso-PC were reduced by HF/FD diet in the liver. The drain of choline caused by folate deficiency is two-fold: decreased synthesis of choline through the PEMT pathway, and increased use of choline (via BHMT) as a source of methyl groups. Interestingly, PC, lyso-PC and lyso-PE levels were reduced by excess folic acid in high fat diet-fed rats. In the liver, both deficient and excess levels of folic acid appear to reduce phospholipid biosynthesis. Evidence for reduced PC synthesis through the PEMT pathway exists both at the transcriptional level, as well as PEMT activity. Folate deficiency has previously been shown to reduce PEMT activity and choline kinase expression in the liver (Akesson et al 1982; Champier et al 2012). Impaired PC production through both the CDP-choline and PEMT pathways is associated with accumulation of hepatic TG due to a reduction in VLDL secretion (Jacobs et al 2008 JBC; Zhao et al 2009). While alterations in one-carbon metabolism were observed in folic acid deficiency in both liver and plasma measurements, the level of neutral lipids in the liver was similar between HF/FC and HF/FD fed rats.

Folate deficiency has been shown to induce the expression of genes involved in hepatic lipid synthesis that may contribute to hepatic steatosis. Microarray analysis of folate-deficient mice revealed increased

expression of lipid biosynthetic genes, including acetyl CoA synthetase long-chain family member 1, lipin 1, diacylglycerol *O*-acyltransferase 2, elongation of very long chain fatty acid-like 3 and ATP citrate lyase, suggesting increased lipid biosynthesis in folate deficiency (Champier et al 2012). It is possible that these changes in gene expression are linked to a reduction in hepatic PC biosynthesis, which activates sterol regulatory element binding protein 1a to stimulate *de novo* fatty acid synthesis (Walker et al 2011). However, other studies suggest compensatory mechanisms can prevent lipid accumulation during short-term dietary folate deficiency. Three week folate deficiency during pregnancy did not alter hepatic lipid concentration due to an increase in methionine adenosyl transferase and adenosyl homocysteinase to prevent impairments in the methylation cycle (McNeil et al 2008). In the current study, HF/FD diet decreased the expression of ppara, srebf1 and *srebf2*. When considering the mRNA data, lipid synthesis appears to be depressed in HF/FD fed rats. *Acaca and fasn* expression are reduced. Fatty acid desaturation and elongation are reduced at the transcriptional level. In agreement with *sreb2*, genes involved in cholesterol metabolism are also reduced. Reduced expression of acetyl-CoA carboxylase and sterol regulatory binding protein-1c has been reported elsewhere, and suggest a switch toward reduced lipid biosynthesis as a mechanism to prevent lipid accumulation in this acute folate deficient model (McNeil et al 2008). An additional study suggests that fatty acid oxidation is enhanced by short-

term folate deficiency as a further mechanism to prevent hepatic steatosis. Expression of *cpt1*, carnitine *O*-octanoyltransferase, and acetyl-coenzyme A dehydrogenase were increased in folate-deficient mice (Champier et al 2012).

Excess macronutrient availability together with high intake of folic acid may contribute to development of obesity. Supplementation of 5mg/kg folic acid to Sprague Dawley rats was initially found to reduce lipid content of the liver (Kelley et al1950), suggesting folic acid has a lipotropic effect. However, Burdge *et al.* (2009) report increased weight gain and hepatic lipid accumulation in rats fed high-fat diet containing 5 mg/kg folic acid, compared with 1 mg/kg folic acid. Under high fat feeding, excessive folic acid supplementation may promote hepatic lipid accumulation through impaired fatty acid oxidation in the liver through decreased expression of Cpt1 (Burdge et al 2009). There were no indication of impaired fatty acid oxidation in HF/FE fed rats and consequently, hepatic lipid levels were similar to HF/FC animals. These contradictory findings may be a consequence of differences in the fat content of the diet.

4.3 Conclusion

There have been inconsistent findings regarding the effects of dietary folic acid deficiency and excess on lipid metabolism. Studies dealing with dietary folic acid have are varied in life stage, animal model, duration, amount of folic acid supplemented, and dietary composition.

Alterations in methylation status of key genes involved in lipid metabolism may be more likely during certain periods of development rather than later in life. Later in life, chronic exposure to folic acid may be required for a sustained effect on lipid metabolism. In adult models four weeks of folate deficiency may not be long enough to reduce folate status to a level at which lipid metabolism is affected, but 4-5 month folate deficiency yielded a marked hepatic lipid accumulation in rats (Akesson et al 1982). In this 12-week experiment, HF/FD and HF/FD modestly reduced hepatic phospholipids, with no effect on neutral lipid storage.

Another significant factor when interpreting the effect of folate on lipid metabolism is the overall composition of the diet. The experiments summarized here demonstrate folic acid deficiency and excess may perturb lipid metabolism, weight gain and glucose tolerance when rats are fed a high fat diet only. There was an absence of these observations when rats were fed a lower fat diet. Also, the consequences of folate deficiency are likely to be more evident when methionine and choline are limiting in the diet (McNeil et al 2008). For instance, the ability of folate deficiency to suppress PE methylation may be compensated by an ample supply of choline or betaine. Finally the strain of rodent may impact severity of the phenotype. Both Progribny et al (2013) and Tryndyak et a (2012) have shown that the strain of animal also influences the severity of the development of NAFLD. Thus, it is imperative that experimental design

and choice of animal model be taken into consideration when investigating and interpreting the effects of folic acid on lipid metabolism.

These data reinforce the importance of maintaining adequate folate status for optimizing health, in addition to preventing deficiency. They may also bring into question the safety of folic acid supplementation in the context of increasing fat content of the diet. This study provides evidence that continuous folic acid supplementation can increase the risk for obesity and related co-morbidities. This is an important consideration for countries, which currently implement national folic acid fortification policies and in which folic acid supplements are frequently consumed.

4.4 Limitations

There a few limitations to this study. First of all, mechanisms to explain the findings of increased weight gain on HF/FE diet and impaired glucose tolerance and lipid metabolism are based primarily on data obtained at the transcriptional level. While this is important information relating to transcriptional changes, there has been no attempt made here to relate changes in mRNA expression to changes in protein levels or enzyme activities, except in the case of hepatic PEMT activity. Furthermore, how dietary folic acid regulates transcription of a wide variety of genes was not addressed.

In many cases, data obtained in HF/FD and HF/FE groups were altered in a similar fashion compared with HF/FC animals. While it has

been suggested that excess folic acid supplementation may produce a deficiency of active intracellular folates by overwhelming the ability of DHFR to effectively integrate folic acid into the active folate pool, no evidence for such a phenomenon has been provided here.

4.5 Future Directions

Future studies are needed to identify whether excess folic acid, indeed, has the ability to produce intracellular folate deficiency. Previous studies relating increased unmetabolized folic acid in plasma following folic acid supplementation need to be linked to more physiological outcomes. Troen et al has shown reduced natural killer cell cytotoxicity in relation to plasma folic acid levels (Troen et al 2008). Characterizing the forms of folate in plasma and in tissues following folic acid compared with natural food folates, or 5-MTHF may yield insights into this area.

Future experiments should be directed towards learning more about the role of folate in regulating the expression of mRNA. One of the main functions of folate is in the methylation cycle. AdoMet-dependent DNA and histone methylation controls expression level of many genes. Epigenetics refers to heritable changes in gene expression that arise without an alteration in the sequence of the genetic code. The methylation of DNA is one mechanism by which this process occurs. DNA methyltransferase adds a methyl group to cytosine residues in regions rich in cytosine-guanine dinucleotides, called CpG islands. These regions are

often found near the transcriptional start sites of genes, and can influence the accessibility of the promoter region to transcriptional machinery involved in the production of the genetic transcript (messenger RNA). Accordingly, hypermethylation of CpG islands usually corresponds to reduced gene expression, while hypomethylation of CpG islands correlates with increased gene expression. A second epigenetic mechanism involves the methylation of histones. Histones are proteins involved in packaging DNA into the highly condensed structure of chromatin. Methylation of these proteins at arginine and lysine residues strengthens their interaction with DNA and thereby limits access of transcriptional enzymes to initiate transcription. Reduction of histone methylation may loosen the chromatin structure, allowing transcription of nearby genes. These epigenetic mechanisms may be at play in the interactions between environmental stimuli, such as nutrition, and phenotypic changes, leading to repression/activation of particular genes that are maintained through cell division. Altered DNA methylation patterns are one mechanism whereby folic acid supply may regulate transcription.

Folic acid supply during key developmental periods may play a role in modulating lipid metabolism due to its role in supplying methyl groups required for epigenetic programming events. Methylation of DNA within promoter regions, and histone proteins involved in packaging DNA, marks whether particular genes will be transcribed or not. After initial

development of organs and tissues, these methylation patterns are thought to remain static. However, during critical periods of development environmental conditions may alter these patterns and ultimately yield changes in the metabolic responses of an organism. These life stages include development *in utero* (embryonic and fetal growth), as well as postnatal and pubertal development. Folate supply during these periods has lasting effects on DNA methylation of offspring. For example, low folate during pregnancy and lactation yielded hypomethylation in the small intestine of the adult offspring (McKay et al 2011). Folate deficiency may alter gene expression during adulthood by limiting the availability of the methyl donor, AdoMet, required to maintain those epigenetic marks. Cystathionine beta synthase (Cbs) heterozygous mouse fed a diet high in methionine and low in folic acid is a model of moderately impaired methylation capacity. Cbs is the first enzyme in the irreversible conversion of homocysteine to cysteine. This pathway is responsible for removal of approximately 50% of homocysteine and thereby helps to maintain methylation capacity. This model has been used to show impairments in methylation status affects lipid metabolism in adult animals through changes in DNA methylation. Hypermethylation of the promoter region of Fads2 was associated with reduced Fads2 mRNA expression and lower D6D activity in Cbs+/- animals (Devlin JBC.2007). These changes led to a reduction in longer chain desaturated fatty acids arachidonic acid and

decosahexaenoic acid the end products of the D6D metabolism (Devlin JBC. 2007).

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Appendix 1: List of Rat Primer Sequences and Probes Designed for use in High-throughput Quantitative PCR.

Gene	Primer Label	Sequence	UPL Probe
mogat1	rqht MGAT1 F2	ggtgcgaaagagctactgga	98
	rqht MGAT1 R2	caaaagaaaacactggaaccaa	
mogat2	rqht MGAT2 F1	cagtacagctttggcctcgt	9
	rqht MGAT2 R1	gggatgtggtatcatctgcac	
dgat1	rqht DGAT1 F1	tacggcgggttcttgagat	120
	rqht DGAT1 R1	cgtgaatagtccatgtccttga	
dgat2	rqht DGAT2 F2	gtgtggcgctattttcgag	42
	rqht DGAT2 R2	ggtcagcaggttgtgtgtctt	
gpam	rqht GPAM F2	gcacaggtaccttctcaccag	55
	rqht GPAM R2	gacagtatgtggcactctcagc	
agpat3	rqht AGPAT3 F1	ccagtggcttcacaagctg	2
	rqht AGPAT3 R1	ccgctctgcttgtacatctctt	
agpat2	rqht AGPAT2 F1	aagacgaagctcttcacctcag	89
	rqht AGPAT2 R1	ccgtaaggccattggtagg	
lipin1	rqht LPIN1 F1	agtcttgccacttccatgct	124
	rqht LPIN1 R1	gctcagaatcacttttggtgtt	
lipin2	rqht LPIN2 F1	aagatgccgaagaaatctgg	77
	rqht LPIN2 R1	cttggtctccggcaactg	
lipin3	rqht LPIN3 F2	gcccccatgattctttctct	97
	rqht LPIN3 R2	tctccttttgaagcttgtcca	
chka	rqht CHKa F1	cgactgtatggggcaatctta	41
	rqht CHKa R1	ctctgcaagaatggcaaaca	
chkb	rqht CHKb F1	gaatcacctcaggacgttgc	29
	rqht CHKb R1	tgtcatcactgtctggctctg	
pcy1a	rqht CTalpha F2	tggaattccttccaaagtgc	17
	rqht CTalpha R2	tcaccctgacatagggcttact	
pcytlb	rqht CTbeta F1	agaattgtttgggccagatg	38
	rqht CTbeta R1	atccggctactcctctcctg	
pcyt2	rqht ET F2	gctgacagttttggcaagc	118
	rqht ET R2	gatgtctgtaggaactgggaca	
pemt	rqht PEMT F2	tgctggacaaccccatgt	60
	rqht PEMT R2	cagcaccgtcaacagcag	
cpt1a	rqht CPT1L F1	acaatgggacattccaggag	79
	rqht CPT1L R1	aaagactggcgctgctca	

acadm	rqht MCAD F2	gggacgccaagatctatcag	110
	rqht MCAD R2	tcaatgtgctcacgagctatg	
acadl	rqht LCAD F1	gcagttacttgggaagagcaa	81
	rqht LCAD R1	ggcatgacaatatctgaatgga	
acadvl	rqht VLCAD F1	ggtggtttgggcctctcta	53
	rqht VLCAD R1	gggtaacgctaacaccaagg	
acox1	rqht ACOX F1	caccttcgagggagagaaca	112
	rqht ACOX R1	cgcacctggtcgtagatttt	
ucp2	rqht UCP2 F1	gactctgtaaagcagttctacaccaa	79
	rqht UCP2 R1	gggcacctgtggtgctac	
cd36	rqht CD36 F2	ggttaaacagagaggtccttacaca	56
	rqht CD36 R2	gatggctccattgggttgta	
slc27a1	rqht FATP1 F2	gttcggtgtgtacgtgggta	83
	rqht FATP1 R2	cacgcggatcagaacagag	
slc27a4	rqht FATP4 F2	ctgagctgcacaaaacagga	12
	rqht FATP4 R2	tcacaacagacgggtcaaag	
slc27a5	rqht FATP5 F1	ggagtcctcggctgcttac	125
	rqht FATP5 R1	cgggaagcagagaacttgg	
fabp2	rqht IFABP F1	tctagcagatggaacagaactca	85
	rqht IFABP R1	ccaacaagtttatttccctcca	
fabp1	rqht LFABP F1	cttctccggcaagtaccaag	21
	rqht LFABP R1	ttccctttctggatgaggtc	
acly	rqht ACLY F1	ctttgggcgtgaggcata	6
	rqht ACLY R1	ccacttttggcatccaggt	
me1	rqht ME1 F2	gaatatgatgcgtttttggatg	16
	rqht ME1 R2	aaactgaataaggcaattcatgc	
me2	rqht ME2 F1	aaactgaataaggcaattcatgc	95
	rqht ME2 R1	aatccccattccatagacacc	
me3	rqht ME3 F1	acagagagtgctgtgcaacc	20
	rqht ME3 R1	tgtttgtacgcatagtcgagga	
fasn	rqht FAS F1	gtggacatggtcacagacga	97
	rqht FAS R1	cgcttaggcaacccatagag	
scd1	rqht SCD1 F1	catgtctgacctgaaagctga	9
	rqht SCD1 R1	caggaggccaggcttgtag	
acaca	rqht ACC F1	gatgatcaaggccagcttgt	79
	rqht ACC R1	caggctaccatgccaatctc	
praak1	rqht AMPKa F1	ccgtctgatattttcatggtca	63
	rqht AMPKa R1	actctccttttcgtccaacct	
mlycd	rqht MCD F1	tcggcaccttcctcataaag	1
	rqht MCD R1	cccagatgaggaaactccttc	

ces3	rqht Ces3 F1	ggcatctggggattcttca	71
	rqht Ces3 R1	cccccaaagttggcaata	
mttp	rqht MTP F1	gcgagtctaaaacccgagtg	83
	rqht MTP R1	cactgtgatgtcgctggttatt	
adrp	rqht ADRP F1	tatgcctgcaaggggcta	58
	rqht ADRP R1	gggcattggcaacaatct	
apob	rqht APOB F2	aagcgatcacatccctcttg	130
	rqht APOB R2	aatcaaggcttgtaaagtgatgg	
hmgcr	rqht HMGCR F1	gacctttctagagcgagtgcat	122
	rqht HMGCR R1	cgctatattctcccttacttcatcc	
abca1	rqht Abca1 F1	gagaagagccaccctggtt	129
	rqht Abca1 R1	gctcctcttccatgcagatt	
abcg5	rqht ABCG5 F1	gaatgtgtccttcagcgtca	81
	rqht ABCG5 R1	gctggcatgatttgatgttc	
abcg8	rqht ABCG8 F1	gggactgtactccaggatgc	82
	rqht ABCG8 R1	ggtgaagtagaggctgttgtcac	
apoA1	rqht APOA1 F1	aaacagattggctgagaaacg	80
	rqht APOA1 R1	ggggctgcatcttctgttt	
npc1l1	rqht NPC1L1 F2	cttaatgtgcaacatcaacgtg	128
	rqht NPC1L1 R2	gtacgagtccttgggcagag	
scarb1	rqht SRB1 F1	aagcagcaggtgctcaaga	3
	rqht SRB1 R1	tctccttccacatcccaaag	
scap	rqht SCAP F1	caaacaaggagagcccagtg	106
	rqht SCAP R1	cgaatatctgctggatgtatgc	
insig1	rqht INSIG1 F2	tgggaaacataggacgacagt	38
	rqht INSIG1 R2	ccaggaccagtgtctccac	
ldlr	rqht LDLr F1	tgctactggccaaggacat	16
	rqht LDLr R1	ctgggtggtcggtacagtg	
soat1	rqht ACAT F2	ggtccacgactggctctact	82
	rqht ACAT R2	gcctgaacctcttcgagaaa	
ppara	rqht PPARa F1	tgcggactaccagtacttaggg	116
	rqht PPARa R1	gctggagagagggtgtctgt	
pparg	rqht PPARg F1	ggtgaaactctgggagatcct	115
	rqht PPARg R1	aatggcatctctgtgtcaacc	
nr1h3	rqht LXRa F1	gcagtgcctgatgtttctcc	69
	rqht LXRa R1	cagttgattggggcatcct	
nr1h2	rqht LXRb F1	tccagctctgcctacatcgt	106
	rqht LXRb R1	ggacccttcttccgcttg	
srebf1	rqht SREBP1a F1	acaagattgtggagctcaagg	77
	rqht SREBP1a R1	tgcgcaagacagcagattta	

srebf2	rqht SREBP2 F1	gtgcagacagtcgctacacc	62
	rqht SREBP2 R1	aatctgaggctgaaccagga	
bhmt	rqht BHMT1 F1	agggaactacgtggcagaga	113
	rqht BHMT1 R1	cgtgcaatgtcacaagcag	
bhmt2	rqht BHMT2 F1	ggctttgtgaaggcagga	109
	rqht BHMT2 R1	gtgtgaagctgacgaactgc	
Mtr	rqht MS F1	gcctgatgttatttaccagattca	20
	rqht MS R1	gctgaaagtgtttgtttcaatgat	
mttr	rqht MSR F2	gactgcagaccatagtgatgct	92
	rqht MSR R2	atttgttgcccgaggagag	
mat1a	rqht Mat1a F1	gggtggtgagagacaccatt	53
	rqht Mat1a R1	gcaggtcttgaagtcgaagc	
mat2a	rqht MAT2a F1	ctgtaggggaaggtcatcca	56
	rqht MAT2a R1	cctgctgaaggtgtgcatc	
mthfr	rqht MTHFR F1	acctgcacagagccaagc	124
	rqht MTHFR R1	cagtggtcacctacagggtct	
cbs	rqht CBS F1	gggagaagggttttgaccag	114
	rqht CBS R1	atgttcccgagagtcaccat	
cth	rqht CGL F1	acactttcatgtctgcatatttcc	21
	rqht CGL R1	tttgtggcagaacacatacaaa	
chdh	rqht CDH F1	cagtgggcgtggagtacat	21
	rqht CDH R1	tcacctccctgctgacgta	
gnmt	rqht GNMT F1	actgaaggagcgctggaa	69
	rqht GNMT R1	tcaaccagttggcttcttcaa	
cdo1	rqht CDO1 F1	tggatcaaggaaatgggaag	74
	rqht CDO1 R1	ggagtccgtgtgatcgtga	
gclc	rqht GCLC F2	gacgaagccataaacaagcac	55
	rqht GCLC R2	tggcacattgatgacaacct	
gss	rqht GSS F1	ctgaggtccgcaaagaacc	82
	rqht GSS R1	gaggggaagagcgtgaatg	
cd68	rqht CD68 F1	ctttctccagcaattcacctg	108
	rqht CD68 R1	actggcgcaagagaagca	
nox1	rqht NOX1 F1	cgccgattgctttttatatctt	29
	rqht NOX1 R1	ggtgcatgacaaccttggta	
hspa5	rqht GRP78 F1	ccgtaacaatcaaggtctacga	15
	rqht GRP78 R1	aaggtgacttcaatctggggta	
emr1	rqht F4/80 F1	ataatcgctgctggctgaat	94
	rqht F4/80 R1	agactggccccaagaaactc	
gsr	rqht GSR F1	ttcctcatgagaaccagatcc	64
	rqht GSR R1	tgaaagaacccatcactggtta	

gpx1	rqht GPX1 F1	gtttcccgtgcaatcagttc	2
	rqht GPX1 R1	gaattcagaatctcttcattcttgc	
ppia	rqht PPIA F1	tgctggaccaaacacaaatg	42
	rqht PPIA R1	cttcccaaagaccacatgct	
gapdh	rqht GAPDH F1	ctgcaccaccaactgcttag	9
	rqht GAPDH R1	tgatggcatggactgtgg	
actb	rqht BACT F1	ctaaggccaaccgtgaaaag	115
	rqht BACT R1	tacatggctggggtgttga	
arbp	rqht 36b4 F1	cctgcacactcgcttccta	112
	rqht 36b4 R1	tgatggagtgaggcactgag	
pck1	rqht PEPCK F1	gatgacattgcctggatgaa	105
	rqht PEPCK R1	aaccgttttctgggttgatg	
g6pc	rqht G6P F1	ctcactttccccatcaggtg	109
	rqht G6P R1	gaaagtttcagccacagcaa	
pklr	rqht PK F1	tcatcgtgctgacgaagact	26
	rqht PK R1	cccgaggtcggtattgag	
slc2a4	rqht GLUT4 F1	tctcaggcatcaatgctgtt	17
	rqht GLUT4 R1	cgtgaagacggtattgacca	
bgn	rqht BGN F1	ctggcctcccagatctca	25
	rqht BGN R1	cccaccttggtgatgttgtt	
hspg2	rqht Hspg2 F1	ggtacccagtgccagcat	65
	rqht HSpg2 R1	ggcctggtgtctgaatgg	
vcan	rqht VCAN F1	tgattggcattagtgaagagacc	98
	rqht VCAN R1	gggtttgttttgcagagatca	
tgfb1	rqht TGFB1 F1	cctggaaagggctcaacac	1
	rqht TGFB1 R1	cagttcttctctgtggagctga	
vtn	rqht VTN F1	ctagccctgctggcattg	106
	rqht VTN R1	tggccatgaaaccctgag	
fn1	rqht FN1 F1	cagcccctgattggagtc	76
	rqht FN1 R1	tgggtgacacctgagtgaac	
mmp2	rqht MMP2 F1	gcaccaccgaggattatgac	113
	rqht MMP2 R1	cacccacagtggacatagca	
mmp3	rqht MMP3 F1	gagaactttccaggcattgg	89
	rqht MMP3 R1	ccgctgaagaagtaaagaaacc	
mmp9	rqht MMP9 F1	cctctgcatgaagacgacataa	42
	rqht MMP9 R1	ggtcaggtttagagccacga	
mmp10	rqht MMP10 F1	ggagatgctcacttcgatgat	118
	rqht MMP10 R1	cagcaaccaggaataaattgg	
mmp11	rqht MMP11 F1	tggatgcagcttttgaggat	81
	rqht MMP11 R1	gactggcttctcaccgtcat	

mmp19	rqht MMP19 F1	cctctggaaggagctgatga	110
	rqht MMP19 R1	ttcagatgcttcctggaaagtt	
tnf	rqht TNF F2	agaactccaggcggtgtct	63
	rqht TNF R2	gagcccatttgggaacttct	
fads2	rqht FAD2 F1	aatttccagattgagcaccac	60
	rqht FAD2 R1	agtggggcaatcttgtgc	
rpl27	rqht RPL27 F1	gcaaagccgtcatcgtaaag	83
	rqht RPL25 R1	ttttctgggatagcggtcaa	
hprt1	rqht Hprt1 F1	gaccggttctgtcatgtcg	95
	rqht Hprt1 R1	acctggttcatcatcactaatcac	
rpl6	rqht Rpl6 F1	ggcaagagagtggttttcctc	6
	rqht Rpl6 R1	ggcgatgacaaacttctggt	
srp14	rqht Srp14 F1	gagaccaggatggtgttgct	116
	rqht Srp14 R1	ttggtttagtgcgaccatca	
ggh	rqht Ggh F1	gggctgtccggatcctat	125
	rqht Ggh R1	tgttcccataacattcttgcat	
shmt1	rqht Shmt1 F1	cttttatctgggttgccatca	17
	rqht Shmt1 R1	gaaacttgggcaaaacctga	
shmt2	rqht Shmt2 F1	tctctttacttcggaccactcg	46
	rqht Shmt2 R1	tctgggccacctcactgt	
tyms	rqht Tyms F1	gcacggtacagcctgagag	6
	rqht Tyms R1	tcctccaaaacacccttcc	
mthfd1	rqht Mthfd1 F1	gatgggaaatacgttgtggtg	7
	rqht Mthfd1 R1	cgatggtggttgtgctctt	
mthfd2	rqht Mthfd2 F1	cagggcagcagctgaagt	6
	rqht Mthfd2 R1	tctcatcgtcattcaatgtcct	
dhfr	rqht Dhfr F1	aaagtggacatggtctgggta	16
	rqht Dhfr R1	ctggctgattcatggcttc	
ftcd	rqht Ftcd F1	ggcttttgctgcctgttt	44
	rqht Ftcd R1	gtccctctcttcaggcgtatt	
mthfs	rqht Mthfs F1	aagttgctttacttcccaagaca	64
	rqht Mthfs R1	gccaggaaggaagatgaggt	
aldh1l2	rqht Aldh1l2 F1	gaatggggaaggatgaacg	6
	rqht Aldh1l2 R1	ccattaggtccgcgagtct	
fads1	rqht FADS1 F1	gaactctcttctgattggagagcta	26
	rqht FADS1 R1	ccggaattcatcagtgagc	
elvol1	rqht Elovl1 F1	tgacactctccctctacattgtct	20
	rqht Elovl1 R1	ggtcacagcgccaggtat	
elovl2	rqht Elovl2 F1	aacctcggaatcacacttcttt	22
	rqht Elovl2 R1	tcccagctggagagaacg	

elvol5	rqht Elovl5 F1	tcgatgcgtcactcagtacc	122
	rqht Elovl5 R1	cctttgactcgtgtgtctcg	
elovl6	rqht Elovl6 F1	tggatgcaggaaaactgga	119
	rqht Elovl6 R1	gcccgcttgttcatcaga	
lipe	rqht Lipe F1	cgagcactggaggagtgttt	3
	rqht Lipe R1	tcaaccggagagcggata	
lpl	rqht Lpl F1	cagagaaggggcttggagat	58
	rqht Lpl R1	ttgactccctgctgaatgaa	
acacb	rqht Acacb F1	ggaggcctcacccaactc	53
	rqht AcacbR1	attcttcgaccatggcagtt	
cpt1b	rqht Cpt1b F1	cctttcctggacgaggtg	1
	rqht Cpt1b R1	agatcccccagttccagatc	
pkm2	rqht pkm2 F1	ctgaagtacgcccgaggat	3
	rqht pkm2 R1	acaccttcctggaacaca	
adipoq	rqht adipoq F1	tggtcacaatgggataccg	80
	rqht adipoq R1	aggtgttcttggtcctaaggg	
irs	rqht Irs F1	cagaaaaacctcttcaggcaat	73
	rqht Irs R1	gaaagcgaagatcccttgaa	
irs1	rqht Irs1 F1	aactggacgtcacagcagaat	69
	rqht Irs1 R1	agatggtacgatgcatccct	
akt2	rqht Akt2 F1	ccgctattatgccatgaagat	12
	rqht Akt2 R1	aggatgaagtcgcccaca	
pik3r1	rqht Pik3r1 F1	atgtacaccacggtttggact	85
	rqht Pik3r1 R1	cactaaaatgcacggcgac	
mapk1	rqht Mapk1 F1	tctgcaccgtgacctcaa	18
	rqht Mapk1 R1	tctgtgactttggccttgc	

Appendix 2: Expression of Genes Measured in Liver, Adipose Tissue and Muscle of Rats Fed Control, Deficient or Excess Dietary Folic Acid

Transcript	HF/FC	HF/FD	HF/FE
Gpam	1.55 ± 0.49	1.67 ± 0.12	1.56 ± 0.31
Lipin1	1.92 ± 0.77	2.40 ± 0.21	1.98 ± 0.40
Cd36	1.05 ± 0.09	1.26 ± 0.02	0.89 ± 0.12
Fatp1	0.88 ± 0.06	1.26 ± 0.13	0.96 ± 0.14
Acadm	1.91 ± 0.87	2.55 ± 0.15	2.59 ± 0.22
Acadl	2.05 ± 0.98	2.64 ± 0.18	2.74 ± 0.30
Acox1	1.38 ± 0.35	1.80 ± 0.08	1.59 ± 0.21
Acly	1.32 ± 0.18	3.92 ± 0.52*	2.96 ± 0.62
Ucp2	1.05 ± 0.29	1.74 ± 0.18	2.08± 0.46
Ppara	1.11 ± 0.13	2.04 ± 0.24	1.89 ± 0.42
Praak1	1.05 ± 0.38	3.21 ± 0.33	2.76 ± 0.50

Table 5-1: Adipose tissue gene expression in rats fed diets containing 60% of energy from fat. Expressed as fold-change relative to control. *significantly different from HF/FC, $P < 0.05$.

Transcript	HF/FC	HF/FD	HF/FE
Gpam	1.07 ± 0.20	0.45 ± 0.08	1.08 ± 0.27
Lipin1	1.36 ± 0.47	0.25 ± 0.06*	0.55 ± 0.10
Elovl6	1.75 ± 0.55	0.70 ± 0.16	6.09 ± 3.19
Acadm	1.06 ± 0.19	0.40 ± 0.08*	0.69 ± 0.29
Acadl	1.01 ± 0.08	0.68 ± 0.07	1.20 ± 0.12
Acadvl	1.01 ± 0.07	0.81 ± 0.10	1.22 ± 0.15
Cpt1a	1.00 ± 0.04	1.07 ± 0.10	2.51 ± 0.29*
Acox1	1.02 ± 0.09	0.75 ± 0.08	1.26 ± 0.12
Acly	1.22 ± 0.40	0.29 ± 0.04	1.37 ± 0.40
Insig1	1.32 ± 0.12	0.34 ± 0.02#	1.37 ± 0.39
Cd68	0.87 ± 0.20	0.32 ± 0.10*	0.32 ± 0.13*
Hspa5	0.91 ± 0.22	0.41 ± 0.10*	0.38 ± 0.09*
Emr1	0.92 ± 0.06	0.50 ± 0.13*	0.40 ± 0.08*
Tnf	1.59 ± 0.45	0.83 ± 0.31	0.81 ± 0.37

Table 5-2: Hepatic gene expression in rats fed diets containing 60% of energy from fat. Expressed as fold-change relative to control.

*significantly different from HF/FC, $P < 0.05$.

Skeletal muscle gene expression in response to altered dietary folic acid in rats fed diets containing 60% of energy from fat

As a regulator of fatty acid oxidation, a reduction in ppara in the soleus muscle of rats in the HF/FE group suggests a lower fatty acid uptake and oxidation in HF/FE relative to HF/FC. This is supported by a down-regulation of a number of genes involved in these processes: *cd36*, *fatp4*, *acadm*, *acadl*, *acadvl*, *acox* and *acly*.

To investigate whether impaired glucose clearance in HF/FD and HF/FE diet groups may be due to impaired peripheral glucose uptake, the expression of two genes involved in glucose homeostasis were measured. There were no changes observed in the mRNA levels of the glucose transporter, *slc2a4*, or the insulin receptor substrate, *irs1*. Based on these two genes, there is no evidence of impaired insulin signalling at the level of transcription. However, there were no other analyses conducted in the muscle in this study.

Transcript	HF/FC	HF/FD	HF/FE
Fatty Acid Transport			
Cd36	1.09 ± 0.09	1.09 ± 0.08	0.82 ± 0.04*
Fatp1	1.05 ± 0.10	1.07 ± 0.14	0.60 ± 0.15
Fatp4	1.20 ± 0.08	1.2 ± 0.07	0.49 ± 0.07*
Fatty Acid Oxidation			
Acadm	0.92 ± 0.06	0.79 ± 0.07	0.37 ± 0.03*
Acadl	1.07 ± 0.07	0.91 ± 0.07	0.50 ± 0.06*
Acadvl	1.20 ± 0.11	1.01 ± 0.08	0.56 ± 0.10*
Acox1	1.15 ± 0.07	1.09 ± 0.12	0.49 ± 0.04*
Acly	1.59 ± 0.20	1.96 ± 0.21	0.47 ± 0.14*
Ucp2	1.22 ± 0.11	1.96 ± 0.26*	1.09 ± 0.07
Glucose Metabolism			
Slc2a4	0.68 ± 0.14	0.59 ± 0.19	1.09 ± 0.11
Irs1	0.79 ± 0.10	0.94 ± 0.03	1.14 ± 0.20
Transcription Factors			
Pparg	0.79 ± 0.14	1.16 ± 0.17	0.42 ± 0.15
Ppara	1.21 ± 0.17	1.11 ± 0.14	0.50 ± 0.03*

Table 5-3: **Skeletal muscle gene expression in rats fed diets containing 60% of energy from fat.** Expressed as fold-change relative to control. *significantly different from HF/FC, $P < 0.05$.

Transcript	FC	FD	FE
Gpam	1.11 ± 0.09	0.83 ± 0.06*	1.40 ± 0.09
Lipin1	1.11 ± 0.11	1.01 ± 0.10	1.16 ± 0.10
Acadm	1.56 ± 0.57	1.00 ± 0.05	1.16 ± 0.12
Acadl	1.87 ± 0.76	1.09 ± 0.08	1.28 ± 0.10
Acox1	1.00 ± 0.09	0.97 ± 0.05	1.35 ± 0.15*
Fatp1	1.91 ± 0.41	1.85 ± 0.28	1.96 ± 0.18
Fatp4	0.92 ± 0.04	0.82 ± 0.06	1.04 ± 0.07
Acly	1.15 ± 0.12	0.92 ± 0.14	1.29 ± 0.11
Adrp	1.12 ± 0.07	1.02 ± 0.08	1.21 ± 0.11
Ppara	1.86 ± 0.69	2.05 ± 0.74	2.64 ± 0.42*
Emr1	0.88 ± 0.07	0.78 ± 0.041	0.81 ± 0.08

Table 5-4: Adipose tissue gene expression in rats fed diets containing 15% of energy from fat. Expressed as fold-change relative to control. *significantly different from FC, $P < 0.05$.