Dietary Fat and Cholesterol in the Pathogenesis of Obesity and Non-Alcoholic Fatty Liver Disease

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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LIST OF ABBREVIATIONS

**AASL**: American Association for the Study of Liver Diseases  
**ABCA1**: ATP binding cassette A member 1  
**ABCG1**: ATP binding cassette G member 1  
**ACC**: acetyl-CoA carboxylase  
**ACOX1**: acyl-CoA oxidase 1  
**ALT**: alanine aminotransferase  
**AMPK**: AMP-activated protein kinase  
**AST**: aspartate aminotransferase  
**ATF4**: activating transcription factor 4  
**ATF6**: activating transcription factor 6  
**AUC**: incremental area under the curve  
**BAT**: brown adipose tissue  
**Bcl2**: B-cell lymphoma 2  
**BMI**: body mass index  
**CD68**: cluster of differentiation 68  
**CH**: chow  
**CHC**: chow diet supplemented with 0.2% cholesterol  
**CHOP**: the C/EBP-homologous protein  
**ChREBP**: carbohydrate response element binding protein  
**CLAMS**: comprehensive Laboratory Animal Monitoring System  
**CPT1**: carnitine palmitoyltransferase 1  
**DAG**: diacylglycerol  
**DAMPs**: damage-associated molecular patterns  
**DEPC water**: diethylpyrocarbonate-treated water  
**DHA**: docosahexaenoic acid  
**DNL**: *de novo* lipogenesis  
**DNTB**: dinitrothiocyanobenzene  
**DZ**: dizygotic  
**eIF2α**: eukaryotic translation initiator factor-2α  
**EPA**: eicosapentaenoic acid  
**ER**: endoplasmic reticulum  
**FA**: fatty acid  
**FAO**: Food and Agriculture of the United Nations  
**FAS**: fatty acid synthase  
**FAT/CD36**: fatty acid translocase/cluster of differentiation 36  
**FATPs**: fatty acid transport proteins
FGF21: fibroblast growth factor 21

GPx: glutathione peroxidase

GRP78: 78 kDa glucose-regulated protein

GTT: glucose tolerance test

GSH: glutathione

GSSH: glutathione disulphide

H&E: haematoxylin and eosin

HSCs: hepatic stellate cells

HDL: high density lipoprotein

HF: high-fat

HFC: high-fat diet supplemented with 0.2% cholesterol diet

HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA

IDF: International Diabetes Federation

iAUC: incremental area under the curve

IKK: IκB kinase

IL1: interleukin-1

IL6: interleukin-6

i.p.: intraperitoneal

ipGTT: intraperitoneal glucose tolerance test

ipITT: intraperitoneal insulin tolerance test

IPPTT: intraperitoneal pyruvate tolerance test

IRE1: inositol-requiring protein 1

IRS: insulin receptor substrate

ITT: insulin tolerance test

JNK: c-JUN N-terminal kinase

LDL: low density lipoprotein

ldlr−/−: LDL receptor-deficient

LPC: lysophosphatidylcholines

LPS: lipopolysaccharide

MDA: malondialdehyde

MCP1: monocyte chemoattractant protein 1

MnSOD: manganese superoxide dismutase

mtDNA: mitochondrial DNA

MUFA: monounsaturated fatty acid

MZ: monozygotic

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NLRs: NOD-like receptors

NLRP3: NOD-like receptor pyrin containing 3

NPC1L1: Niemann-Pick C1-like 1

PAMPs: pathogen-associated molecular patterns
PERK: PKP-like endoplasmic reticulum kinase
PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-α
PPARα: proliferator-activated receptor-α
PPARγ: peroxisome proliferator-activated receptor-γ
PRDM16: PR domain containing 16
PRRs: pathogen recognition receptors
PTT: pyruvate tolerance test
PUFA: polyunsaturated fatty acid
REE: resting energy expenditure
RER: respiratory exchange ratio
ROS: reactive oxygen species
RT-PCR: reverse transcriptase polymerase chain reaction
SCD1: stearoyl-CoA desaturase-1
SCFA: short-chain fatty acid
SFAs: saturated fatty acids
SIRT1: sirtuin 1
SOD: superoxide dismutase
SREBP1c: sterol regulatory element-binding protein-1c
T1D: type 1 diabetes
T3: triiodothyronine
T4: thyroxine
TFAM: mitochondrial transcription factor A
TG: triglyceride
TGFβ: transforming growth factor-β
TLR4: toll-like receptor 4
TLRs: toll-like receptors
TNB: thionitrobenzoate
TNFα: tumour necrosis factor-α
TR: thyroid hormone receptor
TRIF: Toll/IL1 receptor domain-containing adapter-inducing interferon-β
UCP1: uncoupling protein 1
UPR: unfolded protein response
VLDL: very low-density lipoprotein
VO2: oxygen consumption
WAT: white adipose tissue
XBP1: X-box binding protein 1
β-HAD: β-hydroxyacyl-CoA dehydrogenase
ABSTRACT

Over the past 20 years, there has been a dramatic increase in the prevalence of obesity in both developed and developing countries. Obesity, in particular central obesity, is often associated with other metabolic diseases including type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). Hepatic steatosis is a prerequisite for the development of NAFLD and it is considered as a first “hit”. A second “hit” is then required for the progression from simple steatosis towards non-alcoholic steatohepatitis (NASH), which is associated with liver damage. It has been suggested that multiple factors, including inflammation, oxidative stress, endoplasmic reticulum (ER) stress, insulin resistance and mitochondrial dysfunction, are involved in this conversion. Although the effects of dietary fat on metabolic disorders have been extensively studied, the roles of cholesterol in the development of obesity and NAFLD are not well understood.

Based on the literature review and analysis of the gaps in our current knowledge (Chapter 1), the overall aim of this thesis was established to investigate the role of dietary fat and cholesterol in the development of obesity, glucose intolerance and NAFLD. The first aim was to characterise the pathologic features regarding obesity, glucose intolerance and hepatic steatosis in mice produced by a high-fat (HF) diet and a moderate-cholesterol diet, alone or in combination. The working hypothesis was that chronic HF diet but not moderate dietary cholesterol would induce obesity, glucose intolerance and hepatic steatosis (as the first “hit” in the development of NASH). The second aim was to investigate the effects of a HF diet and a moderate-cholesterol diet, alone or in combination, on the development of NASH. The working hypothesis was that cholesterol may promote the progression from hepatic steatosis (induced by the HF diet) towards NASH by inducing inflammation, oxidative stress, ER stress or mitochondrial dysfunction. Based on novel findings from above studies, the third aim was developed to explore sustained effects (termed “legacy effects”) of a HF diet with
cholesterol on obesity and glucose metabolism and hepatic steatosis. The working hypothesis for the third aim was that prior exposure to a HF diet with cholesterol may induce “legacy effects” on obesity, glucose intolerance and hepatic steatosis. To test these hypotheses, C57BL/6J mice were used since they have been utilised extensively in similar metabolic studies.

Chapter 3 firstly evaluated the metabolic characteristics of mice fed a chow diet (CH, as a normal control), a chow diet with 0.2% w/w cholesterol (CHC, to examine the effect of cholesterol alone), a HF diet (45 kcal%, to examine the effect of fat alone) or a HF diet with 0.2% w/w cholesterol (HFC, to examine the combined effect of fat and cholesterol) for 17 weeks. In order to further demonstrate the effect of cholesterol, cholesterol was withdrawn from the diet of one of the HFC sub-groups from week 13 to week 17 for 5 weeks (HFC-HF).

The results showed that the HF diet induced obesity (increased body weight and visceral fat), glucose intolerance, and hepatic steatosis [increased liver triglyceride (TG)]. Although cholesterol had no additive effects on obesity and hepatic steatosis, it alleviated HF-induced glucose intolerance.

Secondly, Chapter 3 investigated the effects of these diets on the progression from simple hepatic steatosis (TG accumulation only) towards NASH (additional indications of liver injury, inflammation with or without fibrosis). While HF- and HFC-fed mice all displayed hepatic steatosis, only HFC-fed mice had increased plasma alanine aminotransferase (ALT, an indication of liver injury). The development of a NASH-like phenotype in HFC-fed mice was also supported by signs of hepatic inflammation, such as increases in mRNA expression of tumour necrosis factor-α, interleukin-1β and monocyte chemotactic protein-1. In contrast, addition of cholesterol to the CH diet did not appear to produce any significant phenotype of either simple hepatic steatosis or NASH. Although cholesterol did not induce apparent oxidative stress or ER stress, cholesterol suppressed HF-induced fatty acid oxidation in the
liver and the mRNA expression of peroxisome proliferator-activated receptor γ coactivator-1α and mitochondrial transcription factor A, two transcription factors involved in mitochondrial biogenesis. After switching from the HFC to the HF diet for 5 weeks, the increases in plasma ALT and liver inflammatory markers were abolished but the blunted mitochondrial function persisted. Together, these findings suggest that dietary cholesterol may play a critical role, in the transition from diet-induced hepatic steatosis towards NASH possibly via the activation of inflammatory pathways.

One unexpected finding from Chapter 3 was that the blunted mitochondrial function in HFC-fed mice persisted for 5 weeks after switching to HF diet. This novel finding led to the study in Chapter 4, which examined the sustained effect of HFC feeding (termed “legacy effect”). To investigate this effect, this study adopted a preconditioning protocol similar to those commonly used in the research area of heart ischemia. Mice were intermittently fed HFC or CH diets for two episodes (2-week each), to induce metabolic preconditioning. Subsequently, one of the preconditioned subgroups was fed the CH diet and the other the HFC diet for a 5-week period. The remaining non-preconditioned animals were also either continued on the CH diet or were fed the HFC diet for 5 weeks. The results showed that preconditioning ameliorated HFC diet-induced obesity (decreased body weight gain and visceral fat mass) and glucose intolerance, despite similar calorie intake. Since the preconditioning-induced “legacy effects” were significant on the development of obesity and glucose intolerance, subsequent investigations focused on pathways influencing whole-body energy expenditure and glucose metabolism in the liver, muscle and adipose tissue. Hepatic steatosis and pyruvate tolerance (an indicator of hepatic glucose production) were not affected by the preconditioning. Examination of muscle samples showed no detectable changes in mitochondrial content or mitochondrial enzyme activity (β-hydroxyacyl-CoA dehydrogenase and citrate synthase) following the preconditioning. Interestingly, in white adipose tissue
(WAT), preconditioning moderately increased uncoupling protein 1, a hallmark of the “browning”. This is associated with increased sirtuin 1 levels in the liver and elevated levels of fibroblast growth factor 21 in the plasma, both of which have been suggested to be involved in the regulation of “browning”. Together, these data suggested that short-term HFC feeding had prolonged effects on the development of obesity and related glucose intolerance, and that the “browning” of WAT may be involved in these “legacy effects” of HFC.

In summary (Chapter 5), the research in this thesis provides new conceptual insights into the effects of dietary fat and cholesterol on the pathogenesis of obesity and NAFLD. Findings in the mouse model suggest the crucial role of cholesterol in the progression from simple hepatic steatosis towards a NASH-like phenotype without exacerbating steatosis and dyslipidaemia. The investigation on the sustained effect of HFC led to the discovery of an unexpected protective “legacy effect” of HFC-preconditioning in lessening diet-induced obesity and glucose intolerance. This paradoxical phenomenon provides a unique model to explore novel therapeutic approaches for the prevention and treatment of obesity and related metabolic disorders. Chapter 5 also discussed limitations of studies in this thesis and how future studies could address the remaining issues and potential implications for humans.
CHAPTER 1

Introduction and Literature Review
Obesity and non-alcoholic fatty liver disease (NAFLD) are major metabolic disorders worldwide. They are closely related and share common mechanisms in abnormal metabolism due to various factors including increased calorie intake associated with certain diets (e.g. high-fat diet). This literature review focuses on relevant research to provide: 1) an essential background to obesity and NAFLD; 2) an evaluation of our current knowledge on the impact of dietary fat and cholesterol on obesity and NAFLD and 3) an analysis on the proposed mechanisms underlying the effect of dietary fat and cholesterol on obesity and NAFLD, particularly in regard to the new concepts and hypotheses arising from laboratory-based studies. Based on this review of the literature, gaps in our knowledge were identified and hypotheses were developed to guide the research presented in this thesis.

1.1 Obesity

1.1.1 Obesity Epidemic: Genetic and Environmental Factors

Obesity is defined by the World Health Organization (WHO) on the basis of the body mass index (BMI, kg/m²), which is the ratio of body weight (in kg) to the square of height (in m) [1]. Based on the WHO criteria, a BMI greater than or equal to 25 is considered overweight and a BMI greater than or equal to 30 is considered obese. The prevalence of obesity varies considerably in different regions and ethnic backgrounds but the same increasing trend can be observed across most regions. Over the last 20 years, the proportion of obese people has dramatically increased in developed countries (Figure 1.1). For example, in the US, 23.2% of the adult population was obese in 1991, and this figure increased to 36.1% in 2010 [2]. A similar trend of increased prevalence has been observed in Australia. In 1989, 10.8% of adults were estimated to be obese and this number doubled to 20.8% in 1999-2000, and almost tripled to 28.3% by 2011 [2, 3].
The obese population has increased by 17%-37% in Asian countries (e.g. China, Japan, India and Korea) within the 4 years from 2010-2014, although the overall prevalence of obesity in these countries is relatively low compared to Western countries [4]. In some urban areas in developing countries such as Brazil, India and Iran, the prevalence of obesity is comparable to the proportion in Western countries [5, 6]. This pan-epidemic of obesity has become an important risk factor for a number of major diseases, such as type 2 diabetes (T2D), cardiovascular disease (CVD) and cancer [7, 8], and caused an enormous public health burden.

Figure 1.1 Prevalence of Obesity in Developed Countries. The data from this figure provide a representative picture of the increased obesity epidemic in four developed countries regardless of geographical locations. (Data adapted from [2, 3])
The development of obesity is an overall consequence of the interplay between genetic and environmental factors. The key role of genetic factors in the development of obesity has been elegantly demonstrated in twin and adoption studies in humans [9-12]. One study compared 1974 monozygotic (MZ) and 2097 dizygotic (DZ) male twin pairs and found the concordance rate of overweight in MZ twins was twice that of DZ twins [10]. Another study showed that the heritability of BMI calculated in 93 pairs of twins reared apart was similar to those in twins reared together, further suggesting substantial influences of genetic factors on obesity [11]. The results from these twin and adoption studies had been summarised and reanalysed in a systematic review that included 25,000 twin pairs, 50,000 biological and adoptive family members [12]. Based on these data, the mean correlation of BMI was estimated to be 0.74 for MZ twins, 0.32 for DZ twins, 0.25 for siblings, 0.19 for parent and offspring pairs, 0.06 for adoptive relatives, and 0.12 for spouses. These studies above suggest the importance of genetic factors in the development of obesity.

Genome-wide association studies and Metabochip meta-analysis of BMI have identified 97 BMI-associated loci [13]. Variations in these genes may increase the susceptibility of individuals to become obese under the influence of environmental factors. For example, certain single nucleotide polymorphisms of the fat mass and obesity-associated gene have been well recognised to be associated with adiposity in humans [14]. In addition, obesity can be induced by rare gene mutations, such as mutations in the gene responsible for leptin expression [15]. Leptin is an adipocyte-secreted hormone, which acts on the central nervous system to suppress food intake and promotes energy expenditure in peripheral tissues [15]. In humans, congenital deficiency of leptin has been found to be associated with severe obesity [15], and supplementation of leptin to these subjects is able to effectively reduce obesity [16]. Leptin deficient mice, termed ob/ob mice, develop obesity and exhibit typical characteristics...
of hepatic steatosis and insulin resistance largely due to hyperphagia [17]. As such these mice are commonly used as a genetic model for the study of obesity-related metabolic disorders.

In addition to gene variations, there is increasing evidence to indicate that epigenetics may also play a critical role in the development of obesity. Epigenetic programming has been proposed as an underlying mechanism for the effects of prenatal environmental factors on obesity [18, 19]. It has been shown that the male offspring of women who went through the Dutch famine during the first half of pregnancy had an increased propensity to become obese as they reached adulthood [20]. During the last trimester, however, exposure to the Dutch famine significantly reduced the obesity rate of their offspring [20]. This prenatal exposure to famine has been suggested to induce changes in the methylation of genes that are implicated in growth and metabolic disease even after 60 years of life compared with their unexposed same-sex siblings [21], suggesting that an epigenetic mechanism may be involved in mediating the long-term consequences of prenatal malnutrition.

It has also been shown that the offspring of mothers who underwent bariatric surgery have a reduced risk of developing severe obesity compared to siblings born by these mothers prior to the surgery [22]. Also, studies on animals have shown that prenatal high-fat diets increase the risk of obesity in the offspring [23]. Although the underlying mechanism is not clear, it has been proposed that an epigenetic mechanism may play a role in this inheritable change beyond gene variants [24]. The recognition of epigenetic regulation significantly advances our understanding of gene-environment interactions and long-term consequences of the prenatal exposure to nutritional factors in the development of obesity. However, it is not clear whether nutritional factors may also induce sustained effects that impact on the development of obesity and associated metabolic disorders in adulthood.

Besides genetic factors, environmental factors also play an important role in the development of obesity. It is very clear that decreased energy expenditure and/or increased calorie intake
are the causes of weight gain and obesity [25]. With the improvement in living standards, our lifestyle has become much more sedentary with increased use of cars to replace walking, less physically demanding jobs and more non-physical forms of entertainment (such as watching TV and computer gaming). Such a sedentary lifestyle decreases the requirement for energy expenditure used for muscle contraction and tension, so that the extra energy is converted to fat and stored in adipose tissue as well as non-adipose tissues, thus contributing to the development of obesity [26].

As for the increased calorie intake, this has resulted from an overconsumption of energy-rich food such as diets with a high fat content, which was adopted in the mouse models in this thesis. Over the past several decades, the trend away from traditional diets in many countries, often lower in fats and sugars, towards higher fat and sugar diets has increased due to a more abundant food supply and the globalisation of the fast food industry [27]. For example, it has been found that 41 countries in the Mediterranean and other parts of Europe have drifted away from a traditional Mediterranean-like dietary pattern from 1960s to 2000s [28]. The traditional Mediterranean diet consists of more vegetables, fruits, unrefined cereal grains, legumes and olive oil (unsaturated lipids), as well as moderate-to-high fish intake and less saturated fat, dairy and meat. It has been suggested that this type of diet may be beneficial for the prevention of obesity [29], T2D [30] and CVD [31].

In many developing countries, the abundance of food and the Westernisation of the food supply have coincided with the rapid increase in obesity. These changes can be clearly seen in China where the percentage of the population that is defined as obese has rapidly increased [27] along with the increased consumption of fat-rich food (e.g. doubled from 1989 to 1997 [28]). HF diet-induced obesity and associated insulin resistance (glucose intolerance) can be readily studied in various animal models that use rats [32] and mice [33, 34]. Indeed, the wide use of HF diets (well known as the diet-induced obesity model in laboratory research) in
rodents since early 1990s has significantly advanced our understanding of the mechanisms underlying the development of obesity and associated diseases and has provided a valuable model to test new treatments for these diseases (see reviews in [35-37]). Based on the previous studies from this laboratory [38-40], this thesis used mice fed a HF diet with or without cholesterol (to be reviewed in Section 1.4) as a major model to test the proposed hypotheses.

1.1.2 Central Obesity vs. Peripheral Obesity

Obesity can be classified into two types depending on the distribution of body fat, namely central obesity and peripheral obesity. This classification into these two types can more accurately determine the relationship between obesity and associated metabolic diseases. In humans, central obesity (also known as abdominal or visceral obesity) refers to an excessive accumulation of visceral fat around the stomach and abdomen. It can be diagnosed by measuring the absolute waist circumference (male > 90~94 cm, female > 80 cm) [41] or the waist-to-hip ratio (0.95 for men and 0.80 for women in the US) [42]. More accurate measurements of central obesity can be achieved by computed tomography scans or dual-energy X-ray absorptiometry in a hospital setting. Central obesity is regarded as a key component in the definition of metabolic syndrome, which is defined by the International Diabetes Federation (IDF) as a cluster of clinical symptoms: central obesity, dyslipidaemia, hyperglycaemia and hypertension [41]. Compared with overall obesity, central obesity is a better predictor of obesity related comorbidities such as T2D and CVD [43].

In contrast, peripheral obesity, where excessive fat is mainly stored in the subcutaneous area, may be protective against metabolic dysfunctions under some circumstances [44, 45]. This has been demonstrated in rodent studies showing that the transplantation of subcutaneous fat from donor mice to a visceral fat depot can improve the insulin sensitivity of recipient mice [45]. Different properties of peripheral and visceral adipocytes, including endocrine function,
capacity of lipolysis and the role in cold-induced thermogenesis, may be attributable to the paradoxical difference between visceral and subcutaneous fat [46, 47]. For example, it has been suggested that visceral fat has low production of adiponectin and high production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and interleukin-6 (IL6) [48]. These cytokines are related to insulin action and endothelial function [48]. Interestingly, these site-specific properties of adipose tissue may also account for the different metabolic responses between males and pre-menopausal females due to their differences in adipose tissue distributions [49, 50].

1.1.3 Obesity and Associated Diseases

The most common metabolic diseases that are associated with obesity include NAFLD, T2D, vascular complications as well as cancer. Systematic reviews have calculated the relative risks of obesity-associated diseases by comparing obese with non-obese subjects [7, 8] (Table 1.1). The results suggest that obesity has led to a markedly increased risk of T2D, NAFLD, CVD and certain type of cancers. As a major focus of studies in this thesis, NAFLD will be discussed in detail in Section 1.2.

**Table 1.1 Relative Co-morbidity Risks Related to Obesity**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type 2 Diabetes</strong></td>
<td>6.74 (5.55–8.19)</td>
<td>12.41 (9.03–17.06)</td>
</tr>
<tr>
<td><strong>Non-Alcoholic Fatty Liver Disease (NAFLD)</strong></td>
<td>Overall</td>
<td>3.53 (2.48–5.03)</td>
</tr>
<tr>
<td><strong>Cardiovascular Disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.84 (1.51–2.24)</td>
<td>2.42 (1.59–3.67)</td>
</tr>
<tr>
<td>Coronary Artery Disease</td>
<td>1.72 (1.51–1.96)</td>
<td>3.10 (2.81–3.43)</td>
</tr>
<tr>
<td><strong>Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast, Postmenopausal</td>
<td>-</td>
<td>1.13 (1.05–1.22)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1.95 (1.59–2.39)</td>
<td>1.66 (1.52–1.81)</td>
</tr>
</tbody>
</table>
1.1.3.1 Type 2 Diabetes

T2D is one of the most prevalent metabolic diseases associated with a wide range of potential complications. T2D accounts for approximately 90% of cases of diabetes, with the other 10% made up primarily by type 1 and gestational diabetes [51]. Unlike type 1 diabetes, which is caused by the loss of insulin production due to immune destruction of β-cells, T2D is a metabolic disease which usually begins with a condition termed insulin resistance [52]. Insulin resistance is defined as a reduced sensitivity or responsiveness of target tissues (such as liver, muscle and adipose tissue) to the action of insulin.

Insulin is a hormone secreted by β-cells in the pancreas for the regulation of blood glucose levels, lipid metabolism and protein synthesis [52]. Insulin promotes glucose uptake and storage (as glycogen) in skeletal muscle as a major mechanism for glucose removal from the circulation. In the liver, insulin inhibits gluconeogenesis and glycogenolysis, resulting in the suppression of glucose production. Insulin also stimulates glucose uptake and suppresses lipolysis in adipose tissue. Accordingly, this hormone plays a fundamental role in blood glucose homeostasis. Insulin resistance occurs when the body becomes less sensitive to insulin, and therefore increased insulin levels are required to maintain normal blood glucose levels. When insulin secretion from β-cells is not sufficient to compensate for the insulin resistance, T2D occurs as indicated by hyperglycaemia. The IDF estimated that there were 415 million adults with diabetes as of 2015 [53]. The prevalence of T2D imposes a massive economic burden on both individuals and the health system of many countries. Globally,
diabetes-related health expenditure accounted for approximately 11.6% of total health expenditure in 2015 [53].

It is widely believed that the increased prevalence of T2D is largely driven by the increased prevalence of obesity due to the lipotoxicity that causes insulin resistance and eventually β-cell failure [54]. Central obesity is a major cause of insulin resistance mainly because it causes lipid oversupply to muscle and liver to impair their response to insulin action [52]. Increased lipid flux to these target organs of insulin may increase the level of certain fatty acid (FA) intermediates [e.g. ceramide and diacylglycerol (DAG)] that are known to impair insulin signalling pathways [55] (Figure 1.2). In the liver, excessive lipid accumulation causes hepatic steatosis (or fatty liver), which may progress to non-alcoholic steatohepatitis (NASH) [56]. Increased lipid flux to pancreatic β-cells can also damage their function by generating active lipid metabolites that contribute to β-cell failure [57]. It has also been suggested that central obesity results in elevated pro-inflammatory cytokines such as TNFα, which can cause β-cell damage and insulin resistance [58]. The causal role of central obesity in T2D is further supported by the fact that reducing obesity by various means generally improves glycaemic control. These means include lifestyle interventions [59], pharmacological treatment such as orlistat and lorcaserin [60] and bariatric surgery [61].

1.1.3.2 Cardiovascular Disease

CVD such as coronary heart disease, cardiomyopathy, peripheral vascular disease and stroke, share common risk factors such as dyslipidaemia, high blood pressure and atherosclerosis. It is well recognised that body weight is often positively associated with total cholesterol level and low density lipoprotein (LDL) levels and negatively correlated with high density lipoprotein (HDL) [62, 63]. Cross-section studies have indicated that obese individuals are susceptible to hypertension [64] and atherosclerosis [65, 66]. Long-term follow-up studies have also revealed a close relationship between obesity and CVD such as coronary heart
disease [67-69]. For example, one 26-year follow-up study of 5,000 subjects has concluded that obesity is an independent risk factor for coronary heart disease, congestive heart failure and CVD death [67, 68]. Another 16-year follow-up study has revealed that women with obesity (BMI ≥ 32) have a 4-fold higher risk of CVD mortality than non-obese women [69].

Obesity per se is often associated with chronic and moderate oxidative stress and inflammation, which may contribute to endothelial dysfunction and subsequently increase vasoconstriction and promote thrombosis [70]. It has been suggested that the adipose tissue is an important source of secreted immunomodulating proteins termed “adipokines”. These adipokines may act as modulators of metabolic and cardiovascular processes [71]. For example, adiponectin secreted from adipose tissue has protective effects on endothelial function through promoting AMP-activated protein kinase (AMPK)-dependent nitric oxide production [72, 73]. It has been shown that this protective adipokine is reduced in obese humans [74] and HF-fed animals [75].

Other obesity-associated comorbidities may further increase the risk of CVD. In relation to T2D, hyperglycaemia is another major contributor of cardiovascular complications because it may increase superoxide production leading to the formation of plaques in atherosclerosis [76]. Therefore, effective glycaemic control by pharmacological interventions can provide beneficial effects on diabetic cardiovascular complications [77, 78]. Interestingly, the benefits of glycaemic control can be long-lasting in the prevention of such complications, which has been termed the “legacy effect” [78].

1.1.3.3 Cancer

Although a thorough review of the relationship between obesity and cancer is beyond the scope of this thesis, it is important to be aware of this relationship and its impact on public health as a whole. According to the National Institutes of Health of the United States, obesity is associated with increased risks of certain types of cancer, such as gastrointestinal cancer,
endometrial cancer, pancreatic cancer and kidney cancer [79]. This link is supported by both individual cohort studies as well as meta-analysis [80]. A prospective study of 900,000 adults in the US indicated that increased BMI is significantly associated with higher mortality rates due to various forms of cancer, including liver cancer [81]. Conversely, weight loss by bariatric surgery or other methods has been reported to reduce this risk [82-84]. For instance, a 12-year follow-up study found that the incidence of cancer was 24% lower in the gastric bypass group compared with severely obese control group [82].

Currently, our understanding of the mechanisms linking obesity and cancer risk is limited [85]. As reviewed above, obesity is often associated with hyperinsulinaemia and dyslipidaemia, and interestingly, both of them have been implicated to increase the risk of cancer by epidemiological studies [86, 87]. It has been suggested that chronic hyperinsulinaemia may promote the growth of cancer by stimulating the insulin-like growth factor 1 pathway [88]. In relation to NAFLD, it has been noticed that some NAFLD patients may eventually progress to develop liver cancer, specifically, hepatocellular carcinoma [56].
Figure 1.2 Obesity and Related Metabolic Disorders. Increased food intake and decreased energy expenditure lead to the storage of excess energy in the form of triglyceride (TG) in adipose tissue, which is the fundamental basis for the development of obesity. The excess energy can also result in lipid accumulation in the liver, causing non-alcoholic fatty liver disease (NAFLD). The oversupply of FAs to insulin target tissues, such as the liver and muscle, may impair insulin signalling, leading to insulin resistance. The increase of FAs in the circulation may also induce lipotoxicity to pancreatic β-cells, causing β-cells dysfunction. T2D occurs when insulin secretion from β-cells is not sufficient to compensate for the insulin resistance. Obesity is also closely related to dyslipidaemia, which increase the risk of cardiovascular disease.
1.1.4 Energy Metabolism and Obesity

Calorie intake plays an important role in the development of obesity and it is under the control of a complex neural network within the central nervous system [85]. The review in this section focuses on the other side of the equation in energy balance, namely energy expenditure, and how it contributes to the development of obesity, insulin resistance and NAFLD.

1.1.4.1 Composition of Energy Expenditure and Implications for Obesity

In humans, the total energy expenditure of the body consists of resting energy expenditure (REE), energy expenditure for the thermic effect of food, and physical activity [89]. REE accounts for more than 50% of total energy expenditure (Figure 1.3). Within REE, major tissue-specific contributions in healthy young adults have been estimated to be approximately 20% for the brain, skeletal muscle or liver, 10% for kidneys, 5% for heart and 4% for adipose tissue [90, 91].

Among these tissues, skeletal muscle, the liver and adipose tissue play an important in mediating insulin actions [52]. They may also be involved in the regulation of energy expenditure in various mechanisms. One of the most adaptable tissues for REE in the body is skeletal muscle, because it accounts for approximately 30-40% of the body weight and can be built up quickly by a combination of training and a high protein diet [92]. The importance of resting metabolism in muscle for the whole-body energy expenditure is indicated by the positive correlation \((r = 0.72)\) between metabolic rate of this tissue and basal metabolic rate [93]. The weight of the liver can be significantly increased in obesity and NAFLD patients. These conditions may also affect the metabolic rate of the liver, as suggested by studies showing that obesity may increase mitochondrial respiration in the liver of humans [94]. As for adipose tissue, its mass relative to the body weight is highly variable between normal and obese subjects. Although the contribution of adipose tissue to the REE of the body is often
minimal under usual circumstances, a number of recent studies suggest that white adipose tissue (WAT) can be converted to energy-consuming “brown-like” adipose tissue (a process termed “browning”) which can significantly increase whole-body energy expenditure [95-98]. Thus, there is a growing interest in the exploration of the “browning” of adipose tissue as a new approach in the prevention and treatment of obesity and associated metabolic disorders [99].

![Figure 1.3 Composition of Energy Expenditure and Tissue Contribution.](image)

The total energy expenditure consists of resting energy expenditure (REE), energy expenditure for the thermic effect of food and physical activity. Within REE, major tissue-specific contributions in health young adults have been estimated as below: approximately 20% for the brain, skeletal muscle and liver, 10% for kidneys, 5% for heart, 4% for adipose tissue [90, 91]. Skeletal muscle, the liver and adipose tissue not only play an important in mediating insulin actions but also may be involved in the regulation of energy expenditure in various mechanisms. (Adapted from [89, 91])
1.1.4.2 Regulation of Resting Energy Expenditure

REE can be regulated by the sympathetic nervous system (such as activation of β-adrenoceptors [100]), circulating hormones and cytokines (such as thyroid hormones [101]). This section mainly focuses on their peripheral effects including the effects on the “browning” of WAT, which has been recently identified in humans as a possible target for the treatment of obesity [102].

\textit{β-Adrenoceptors}

The sympathetic nervous system regulates energy expenditure by the stimulation of adrenoceptors. There are 5 types of adrenoceptors, α1, α2, β1, β2 and β3. These adrenoceptors can lead to tissue-specific effects in response to sympathetic activation, as they have different distribution patterns in tissues. β-adrenoceptors are believed to be a major type involved in the regulation of fuel metabolism. In skeletal muscle and liver, activation of β2-adrenoceptors mediates glycogenolysis to produce glucose whereas in adipose tissue the same receptors mediate lipolysis to mobilise FAs for energy expenditure. In rodents, β3-adrenoceptors are primarily expressed in adipose tissue, and play an important role in thermogenesis in brown adipose tissue (BAT) and lipolysis in WAT [103]. Although studies from rodent models raised the hope that targeting the β3-adrenoceptor could be a potential therapy for obesity and diabetes in the 1990s, the expression level of this adrenoceptor subtype in human WAT is very low [104] and none of the specific β3-adrenoceptor agonists have been proven to be effective in treating obesity in humans [103, 105]. However, in light of the recent finding of the presence of brown-like adipose tissue in adult humans and the “browning” of WAT [95-97], the roles of β3-adrenoceptor in humans may need to be re-evaluated with more advanced technologies.

\textit{Thyroid hormones}
Thyroid hormones, triiodothyronine (T3) and thyroxine (T4), can bind to thyroid hormone receptors in the nucleus to regulate the expression of genes involved in energy metabolism (e.g. uncoupling proteins and Na\(^+\)-K\(^+\)-ATPase) in multiple tissues, leading to an increased metabolic rate [106, 107]. Recently, it has been suggested that thyroid hormones may also function as an inducer of “browning” in WAT [108]. Clinical studies have investigated the effect of thyroid hormones on the treatment of obesity, especially for patients with restricted food intake [109, 110]. Despite the treatment with T3 has been reported to be beneficial for weight loss, it may induce significant cardiac side effects such as tachycardia and cardiac arrhythmias, and loss of lean mass instead of body fat [111]. These side effects have obviated its use as a conventional treatment for obesity.

**Fibroblast growth factor 21**

Fibroblast growth factor 21 (FGF21) was first cloned in 2000 and it is referred to as a ‘hepatokine’ since it is predominately secreted by the liver [112]. The production of this hormone can be triggered in the liver by the activation of transcription factor proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\)) under prolonged fasting or with a ketogenic diet [113, 114]. It has also been shown that cold exposure can induce the expression and release of FGF21 in BAT, suggesting the presence of additional source of systemic FGF21 apart from the liver [115]. Evidence for the effects of FGF21 on energy expenditure has been provided by studies in rodents [116]. FGF21 transgenic mice are resistant to diet-induced obesity despite their increased food intake [116], indicating that this hormone may increase energy expenditure. In support of this observation, the administration of FGF21 protects mice from diet-induced obesity and is accompanied by an increase in energy expenditure, as well as improvement in metabolic profiles such as blood glucose and hepatic steatosis [117].

The well-known target tissues of FGF21 action include the liver, adipose tissue and the pancreas [118]. In hepatocytes, FGF21 increases peroxisome proliferator-activated receptor
 gamma coactivator 1-α (PGC1α) expression, promoting FA oxidation and subsequently ameliorating hepatic steatosis and insulin resistance [119]. In the pancreas, FGF21 improves β-cell function by activating extracellular signal-regulated kinase 1/2 and the Akt signalling pathway [120]. In adipose tissue, FGF21 induces the thermogenesis of WAT through PGC1α, a key transcription factor in the regulation of “browning” [118]. In addition, FGF21 may act centrally to stimulate the sympathetic nervous system and therefore increase energy expenditure [121].

A recent study has shown that serum FGF21 levels are correlated with the activity of BAT in humans [122]. This indicates that the effect of FGF21 on "browning" as shown in animal studies may also exist in humans and appears to suggest FGF21 may be bioactive for the treatment of obesity. Consistently, a clinical trial with the FGF21 analogue LY2405319 has shown promising effects for weight loss, ameliorating dyslipidaemia and reducing fasting insulin levels [123]. These findings suggest that FGF21 may be a promising cytokine for the treatment of obesity and associated diseases. However, FGF21 may be difficult to be delivered orally like all other protein products. Further studies are still needed to explore the best means to elevate FGF21 levels for the treatment of obesity and NAFLD.

“Browning” of WAT

As mentioned in Section 1.1.4.1, the “browning” refers to the process where “brown-like” adipocytes (beige cells) develop within WAT. Brown adipocytes are rich in mitochondria and their primary function is heat production as a part of non-shivering thermogenesis. This is carried out by uncoupling protein 1 (UCP1), which dissipates the proton gradient across the inner membrane of mitochondria in oxidative phosphorylation to produce heat rather than driving the synthesis of ATP from ADP (Figure 1.4). It can be up-regulated by cold exposure to increase thermogenesis [124]. Although the total mass of BAT is relatively small, it has
been estimated that 40-50 g BAT may induce a 20% increase in energy expenditure in humans because of its specialised high capacity for thermogenesis [98].

In humans, BAT accounts for approximately 5% of the body weight in neonates and regresses shortly after birth. For decades, the role of BAT in whole-body energy homeostasis was thought to be negligible in adult humans due to its mass and undetectable activity [125]. This view was changed by several landmark studies in 2009 revealing the presence of metabolically active BAT in adult humans using combined positron-emission and computed tomography [95-97]. More importantly, these studies showed that the activity of BAT in adult humans is inducible by cold exposure but is decreased in obese subjects. The rejuvenated interest in BAT has led to extensive research on the “browning” of WAT because of its potential for promoting energy expenditure to reduce obesity.

At the transcriptional level, the major transcription factors known to regulate “browning” include peroxisome proliferator-activated receptor-γ (PPARγ), PR domain containing 16 (PRDM16) and PGC1α. PPARγ is the main isoform of PPARs in adipose tissue and it controls the differentiation and survival of adipocytes. Activation of PPARγ induces UCP1 expression in WAT and enhances the effect of β-adrenergic activation on thermogenesis [126-128]. Mechanistically, a recent study showed that “browning” is associated with a reprogramming of PPAR binding, leading to the formation of PPARγ “super enhancers” to selectively activate the gene expression for “browning” [129]. In mice, knockout of PRDM16 in BAT prevents the expression of genes including UCP1 and PGC1α, whereas overexpression of this transcription factor increases the “browning” of WAT [130]. Further studies suggest that PRDM16 is a co-activator of PPARγ and its role in the formation of beige cells requires the presence of PPARγ [131]. PGC1α is also a co-activator of PPARγ involved in the regulation of UCP1 expression and cold-induced thermogenesis in BAT [132]. In white adipocytes from humans, increased PGC1α induces UCP1 expression and FA oxidation [132].
In contrast, deletion of PGC1α in WAT reduces the expression of UCP1 and mitochondrial genes involved in oxidative phosphorylation and FA oxidation [133].

Several molecular pathways have been suggested to regulate “browning”. As discussed previously, β3-adrenoceptors play an important role in mediating the sympathetic effects on the metabolism of adipose tissue. In mice, activation of β3-adrenoceptors induces an increase in energy expenditure and a reduction in food intake [134]. Although these effects have been attributed to an increased thermogenesis in BAT, further studies in tissue-specific transgenic mice revealed that β3-adrenoceptors in WAT is also crucial to mediate the effects of β3-andrenoceptor agonist [135] by inducing the expression of UCP1 in WAT [136, 137].

A recent study revealed that thyroid hormone receptor (TR) activation by a TR agonist increases the whole-body metabolic rate and decreases the body weight of ob/ob mice. This effect of TR activation is associated with “browning” changes in WAT and decreased BAT activity [138]. As these effects require the presence of UCP1, the whole-body effects of the TR agonist may be attributed to the “browning” of WAT. The same study also indicated that TR agonist-mediated “browning” is partially dependent on the activation of the β-adrenoceptor because the effect is significantly inhibited by β-adrenoceptor blockers [138]. It has also been shown that the thyroid hormone T3 induces the expression of UCP1 in isolated human adipocytes [108]. These data suggest that thyroid hormones may be involved in the thermogenesis of WAT, but the molecular mode of this action remains to be elucidated.

As previously reviewed, FGF21 promotes the whole-body energy expenditure through multiple pathways in peripheral tissues. One of its actions is to induce the “browning” in WAT [118, 139]. This possibility is supported by the findings in FGF21 knockout mice, which are unable to adapt to chronic cold exposure together with diminished “browning” of WAT [118]. Consistent with this finding, treatment with FGF21 in mice has been shown to induce the expression of UCP1 in WAT and dramatically increase the formation of beige
adipocytes. It has been suggested that FGF21 mediates “browning” through the increase of PGC1α in WAT by post-transcriptional mechanisms [118] or through a central action to activate β-adrenoceptor system [140]. Although recent studies using UCP1 knockout mice have led to contradictory conclusions on whether metabolic benefits of FGF21 are indeed UCP1 dependent [141, 142], it remains unchallenged that FGF21 has a direct effect on “browning” as shown in primary adipocytes from both mice [118] and humans [139].

Figure 1.4 “Browning” in White Adipose Tissue (WAT) and Its Regulation. WAT can be converted into “brown-like” fat, namely beige fat, in a process called “browning”. The reprogramming of gene expression during “browning” is regulated by transcription factors including peroxisome proliferator-activated receptor-γ (PPARγ), PR domain containing 16 (PRDM16) and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α). A major feature of beige fat is the increased content of uncoupling protein 1 (UCP1), a protein located in the inner membrane of mitochondria. UCP1 dissipates the proton gradient across the inner membrane of mitochondria in oxidative phosphorylation and thus energy is dissipated in the form of heat rather than used for ATP synthesis. It has been suggested that
β3-adrenoceptor activation, thyroid hormone and fibroblast growth factor 21 (FGF21) may induce the “browning” of WAT. The “browning” of WAT increases whole-body energy expenditure through the increase of heat production by UCP1, and thus ameliorates obesity and insulin resistance [99].

### 1.1.5 Prevention and Treatment of Obesity

The options for the prevention and treatment of obesity include lifestyle interventions, pharmacotherapy and bariatric surgery. These treatments may not only reduce adiposity but also be beneficial for obesity-related metabolic disorders, such as glucose intolerance and NAFLD [143, 144]. Lifestyle interventions, including the restriction of calorie intake and increases in physical activity are the preferred first option for the treatment of obesity according to the clinical guidelines [145]. A large body of clinical evidence suggests that calorie restriction alone, or combined with physical exercise, is an effective means for weight loss [146, 147]. However, calorie restriction can be difficult to adhere to, due to the lack of satiety suppression.

Modification of diet composition, including macronutrients, may improve the effect and adherence of dietary restrictions, and thus help achieve a weight-loss goal. Mediterranean-like diets have been reported to be inversely associated with obesity and related metabolic disorders [28]. The ketogenic diet, which contains high levels of breakdown products from fat, moderate levels of protein and low levels of carbohydrates, is recognised as an effective nutritional approach for reducing obesity and the risk of CVD by supressing appetite [148]. It seems that the low prevalence of obesity in Japan (as shown in Section 1.1.1) may also be at least in part due to dietary factors. Data from the Food and Agriculture Organization of the United Nations (FAO) indicate that the marine fish supply in Japan is 6.8 kg/capita/year, which is more than twice that of Australia (2.4 kg/capita/year), whereas the butter supply in Japan is much lower than that of Australia (0.6 vs. 3.8 kg/capita/year) [27]. It has been
reported by the FAO that food intake in Japan is relatively lower than other developed countries [149]. Studies in Japanese-Americans in Hawaii have shown that the BMI of these immigrants and their risk of CVD are higher than their counterparts living in Japan [150, 151]. This further suggests that lifestyle is crucial to the management and prevention of obesity and associated diseases.

Comprehensive lifestyle management requires sustained adherence and cooperation from families or friends, as well as a committed team of health professionals, in order to achieve the goal of a negative energy balance. The challenges to lifestyle interventions are the low rates of adherence and the high rates of relapse after short-term achievement [152]. Overall, lifestyle interventions are effective for large populations in the treatment of obesity and have few adverse effects.

Current medications for the treatment of obesity include suppression of appetite and inhibition of absorption of nutrients [153]. Phentermine was approved by the FDA in 1959 for the treatment of obesity, and remains one of the most commonly prescribed medications in the US for short-term treatment [154]. This drug is a sympathomimetic that suppresses appetite by stimulating the release of noradrenaline in the central nervous system [153, 155]. The common side effects of phentermine include dry mouth and insomnia [154]. It may also induce cardiac side effects which contraindicates its use in patients with CVD [154]. In addition, the potential of phentermine for addiction is a major concern that restricts its long-term usage. Another appetite suppressant recently approved by the FDA is lorcaserin, a serotonin 2C receptor agonist that acts in the hypothalamus. While lorcaserin is well tolerated in humans, studies in animals suggest that it may have “psychedelic” effects at high dosage due to the activation of serotonin 1A and 2A receptors [156, 157]. Additionally, inhibitors of pancreatic lipase in the intestines such as orlistat have been used for the treatment of obesity because they block fat absorption from the gut. Orlistat is sold over-the-counter in Australia.
under the trade name “Xenical”. However, this class of drugs may have the side effect of diarrhoea [158]. Overall, pharmacotherapy has been recommended as an adjunctive treatment to enhance the adherence to lifestyle modifications, or when lifestyle interventions alone cannot achieve therapeutic goals.

Bariatric surgery is becoming an appealing option for severe cases of obesity (BMI \( \geq 40 \) or BMI \( \geq 35 \) with comorbidities), because it can effectively reduce body weight and improve related clinical symptoms such as hyperglycaemia [61]. The most common bariatric surgery is gastric bypass to connect the top of the stomach to the jejunum, which reduces food intake and absorption. It has been reported that 50\% of patients with T2D can maintain normal glycaemia for 5 years after gastric bypass surgery [159]. However, this surgery may require adherence to dietary recommendations and life-long supplementation with vitamins and minerals. Another form of bariatric surgery is the adjustable gastric banding, which is a reversible procedure that places a band around the stomach to generate a small stomach pouch. Compared with gastric bypass, the gastric banding has a slower effect on weight loss and a higher rate of re-operation since a foreign device is installed in the body [160]. Both of these bariatric surgeries have been designed to limit caloric intake or to bypass a portion of gastrointestinal tract in order to reduce the absorption of food. Furthermore, it has been suggested that the beneficial effects of gastric bypass may also be due to alterations in gut hormones such as increases in glucagon-like peptide-1 and other appetite-suppressing peptides [161]. However, due to the reported morbidity rate (2.4-10\%) [162] and the limitations described above, bariatric surgeries are not used as the first option for the treatment of obesity.
1.2 Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD encompasses a range of pathological conditions in the liver, including steatosis, NASH and cirrhosis originating from metabolic disturbances. NAFLD usually begins with hepatic steatosis resulting from excessive deposition of fat within hepatocytes that is not due to alcohol consumption or other medical conditions such as hepatitis C [163]. Clinically, a hepatic TG level greater than 5% of liver weight or the equivalent in other diagnostic measurements (e.g. ultrasound, computed tomography, magnetic resonance spectroscopy or liver biopsy) is regarded as NAFLD [56]. The commonly used diagnostic techniques for NAFLD in humans include imaging (ultrasound, computed tomography scan and magnetic resonance imaging/spectroscopy), liver biopsy and blood testing (elevated liver enzymes) [164].

1.2.1 Prevalence of NAFLD and Related Metabolic Disorders

NAFLD has become a worldwide health issue. Based on a recent meta-analysis of 729 clinical studies, the overall prevalence of NAFLD diagnosed by imaging is 25%, with the highest prevalence rate found in South America and the Middle East region [164] (Table 1.2). Of note is that approximately 79% of patients with hepatic steatosis may not have an elevation in liver enzymes in the serum [165]. It is likely that this considerable proportion of NAFLD patients is in the early stage of NAFLD such as simple hepatic steatosis. This clinical finding also indicates that a diagnosis of NAFLD based on liver enzymes levels alone may underestimate the prevalence of NAFLD.
Table 1.2 Prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) Measured by Imaging

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of studies</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>2</td>
<td>13.48 (5.69-28.69)</td>
</tr>
<tr>
<td>Asia</td>
<td>14</td>
<td>27.37 (23.29-31.88)</td>
</tr>
<tr>
<td>Europe</td>
<td>11</td>
<td>23.71 (16.12-33.45)</td>
</tr>
<tr>
<td>Middle East</td>
<td>3</td>
<td>31.79 (13.48-58.23)</td>
</tr>
<tr>
<td>North America</td>
<td>13</td>
<td>24.13 (19.73-29.15)</td>
</tr>
<tr>
<td>South America</td>
<td>2</td>
<td>30.45 (22.74-39.44)</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td><strong>45</strong></td>
<td><strong>25.24 (22.1-28.65)</strong></td>
</tr>
</tbody>
</table>

Data are based on [164].

There are a number of comorbidities commonly found in patients with NAFLD (Table 1.3). Based on a meta-analysis of 729 clinical studies [164], as high as 51% patients with NAFLD are obese (Table 1.3). NAFLD is closely related to obesity as shown in Figure 1.2. It has been shown that the prevalence of NAFLD in the obese population is significantly higher compared with the non-obese population [166, 167]. A meta-analysis of 21 cohort studies has indicated that obesity independently increases the risk of developing NAFLD by 3.5 fold [7] (Table 1.1).

Hyperlipidaemia is another major comorbidity in NAFLD [164]. This is possibly due to the close relationship between lipid metabolism in the liver (eg. TG secretion) and the lipid levels in the bloodstream [168] (detailed in Section 1.2.3). Although the critical role of cholesterol as major detrimental factor of hyperlipidaemia in the pathogenesis of atherosclerosis is well recognised, it is not clear whether or not cholesterol may also play a key role in the progression of NAFLD. This question will be evaluated in more detail in relation to the
metabolism of cholesterol (Section 1.4.1) and current understanding of NAFLD pathogenesis (Section 1.4.3).

### Table 1.3 Comorbidity Prevalence in Patients with NAFLD

<table>
<thead>
<tr>
<th>Comorbidity</th>
<th>Number of studies</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>22</td>
<td>50.81 (41.29 - 60.27)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>38</td>
<td>22.04 (17.85 - 26.89)</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>10</td>
<td>69.16 (49.91 - 83.46)</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>13</td>
<td>40.19 (30.95 - 50.19)</td>
</tr>
<tr>
<td>Metabolic Syndrome</td>
<td>22</td>
<td>45.05 (34.67 - 55.88)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>33</td>
<td>39.14 (33.15 - 45.48)</td>
</tr>
</tbody>
</table>

Data are adapted from [164] in NAFLD diagnosed by mixed techniques.

#### 1.2.2 Non-Alcoholic Steatohepatitis (NASH): a Turning Point in Pathogenesis of NAFLD

Simple hepatic steatosis, also known as fatty liver, is regarded as the earliest stage of NAFLD (Table 1.4). While the majority of subjects may stay at this stage, approximately 7-30% of hepatic steatosis individuals may progress to NASH [164]. Clinically, NASH is defined as “the presence of hepatic steatosis and inflammation with hepatocyte ballooning injury with or without fibrosis” by the American Association for the Study of Liver Diseases (AASL) [163].
Table 1.4 Definition of NAFLD and Characteristics of Simple Hepatic Steatosis and Non-Alcoholic Steatohepatitis (NASH) [163]

<table>
<thead>
<tr>
<th>Condition</th>
<th>Definitions and Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAFLD</td>
<td>Encompasses the entire spectrum of fatty liver disease without significant alcohol consumption, ranging from fatty liver (simple hepatic steatosis) to steatohepatitis and cirrhosis.</td>
</tr>
</tbody>
</table>
| Non-Alcoholic Fatty Liver (Simple Hepatic Steatosis) | • Hepatic steatosis  
• No liver injury  
• No inflammation in liver  
• No fibrosis  
• The risk of progression to cirrhosis and liver failure is minimal. |
| NASH                                         | • Hepatic steatosis  
• Inflammation in liver  
• Hepatocyte injury  
• With or without liver fibrosis.  
• High risk of progressing to cirrhosis, liver failure and rarely liver cancer. |

When abnormalities are observed in blood tests [e.g. elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in patients with hepatic steatosis], a liver biopsy may be recommended to confirm a diagnosis of NASH and to identify the stage of the disease. Although the clinical guidelines recommend that the histology score of NASH activity should be analysed by a specialized hepatopathologist for patients [169], representative images of liver sections with quantified supporting data (e.g. ALT activity in the plasma, mRNA expression or protein levels of inflammatory markers in the liver) are commonly used to indicate NASH in animal models [170-173]. NASH is considered as a key turning point in the progress of NAFLD as it can lead to irreversible cirrhosis. Approximately 10%-29% of patients with NASH develop cirrhosis within 10 years after the diagnosis [56]. In addition, it has been suggested that NASH significantly increases the incidence rate of hepatocellular carcinoma [164].
Hepatic steatosis can be caused by a lipid oversupply from the diet (such as overconsumption of dietary fat), increased de novo synthesis of lipid (such as overconsumption of sucrose or fructose) or inhibition of TG export (detailed in Section 1.2.3). However, the precise aetiology of NASH is not completely clear, because a considerable proportion of hepatic steatosis does not manifest the additional characteristics as described in Table 1.4. As a result, a two “hits” theory has been widely accepted to explain the development of NASH [174]. According to the two “hits” theory, hepatic steatosis is regarded as the first “hit” and a second “hit” is needed for the progression from simple steatosis to NASH (Figure 1.5). While it is generally agreed that hepatic steatosis is the first “hit”, it is still a matter of dispute about what may constitute the second “hit”. It has been suggested that insulin resistance, inflammation, oxidative stress, endoplasmic reticulum (ER) stress and mitochondrial dysfunction may be involved in the process of the damage from the second “hit” [175]. However, it remains largely unknown that what may trigger these factors as the second “hit”. This important question led to the investigation of the possibility of dietary cholesterol to trigger these factors in the development of NASH in Chapter 3.
Figure 1.5 Pathogenesis of NAFLD. Excessive accumulation of TG in the liver (hepatic steatosis) is the first stage of NAFLD and regarded as the first “hit” in the development of non-alcoholic steatohepatitis (NASH, characterised by triglyceride accumulation, inflammation and liver injury with or without fibrosis). The progression from simple hepatic steatosis to NASH requires a second “hit” which may result from insulin resistance, inflammation, oxidative stress, ER stress and/or mitochondrial dysfunction. However, it is not clear what may trigger these factors to induce the second “hit”. Approximately 10% and 29% of NASH advances to cirrhosis within 10 years. At this stage, the liver tissue is replaced by scar tissue, which blocks blood circulation and therefore impairs normal liver function. (Adapted from [56]).
1.2.3 Abnormal Lipid Metabolism and Hepatic Steatosis

Hepatic steatosis, which is defined as elevated TG accumulation in the liver, can be caused by the oversupply of TG from the circulation and/or reduced removal of TG from the liver. FAs in the liver come from 3 major sources: 1) absorption from the gut; 2) lipolysis from other tissues, mainly adipose tissue; 3) endogenous synthesis from a carbohydrate metabolite (citrate) within the liver, namely de novo lipogenesis (DNL) (Figure 1.6). FAs ingested from dietary fat are taken up by enterocytes by protein-independent diffusion and protein-dependent mechanisms [176]. Fatty acid translocase/cluster of differentiation 36 (FAT/CD36) plays a key role in the transport-dependent uptake of FAs in the intestine. Once inside enterocytes FAs are assembled into chylomicrons and transported through the lymphatic system into the bloodstream. Isotope labelled studies in rats have shown that 20% TG in chylomicrons is taken up by the liver [177].

Adipose tissue, particularly visceral fat, is an important source of FAs to the liver. During fasting or exercise, adipose tissue undergoes lipolysis to release FAs into the circulation as a supply of energy to other tissues [178, 179]. It has been suggested that the uptake of FAs by hepatocytes is directly facilitated by fatty acid transport proteins (FATPs) [180]. After entering hepatocytes, FAs are converted into TG for storage in various forms of lipid droplets. This may explain the close relationship between obesity and NAFLD by an increased lipolysis in adipose tissue in obese subjects [181].

FAs can also be synthesised from carbohydrates through DNL. When there is oversupply of carbohydrates, citrate from the citric acid (TCA) cycle is transported into the cytoplasm and converted to acetyl-CoA for DNL [182] (Figure 1.6). DNL is regulated by changes in dietary regimen through transcription factors including sterol regulatory element-binding protein-1c (SREBP1c), liver X receptor, and carbohydrate response element binding protein (ChREBP).
For example, it has been demonstrated by using $^{13}$C-acetate infusion that hepatic DNL can be promoted by an over consumption of carbohydrates in humans [184-186]. Using the same technique, fructose has been shown to exert a greater effect on hepatic DNL than glucose [187]. This may be because fructose activates both SREBP1c and ChREBP to up-regulate the genes in the DNL pathway, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [188-190].

FAs from various sources are converted into fatty acyl-CoA by acyl-CoA synthase for further metabolism soon after they enter the cytosol of hepatocytes. Besides storing fatty acyl-CoA in the form of TG, the liver oxidises fatty acyl-CoAs for its own usage via mitochondrial β-oxidation. The entry of fatty acyl-CoA into mitochondria is facilitated by carnitine palmitoyltransferase 1 (CPT1), which is the rate-limiting step of β-oxidation. It has been shown that overexpression of CPT1 increases FA oxidation and subsequently alleviates HF diet-induced hepatic steatosis [191]. Increased levels of malonyl-CoA produced by DNL inhibit the activity of CPT1, which in turn suppresses FA oxidation. Mitochondrial β-oxidation breaks down the fatty acyl-CoA into acetyl-CoA, which finally enters the citric acid cycle for further oxidation to generate ATP. It has been shown that an increase of mitochondrial content by overexpression of PGC1α, a regulator of mitochondrial biogenesis, reduces hepatic steatosis [192]. This suggests that mitochondrial content or function may be closely related to the development of NAFLD [94, 193].

FAs can also be packed into very low-density lipoprotein (VLDL) in the form of TG and released into the bloodstream. This is an important mechanism in the regulation of hepatic TG content and it provides a link between NAFLD and its most common comorbidity, hyperlipidaemia. In humans, obesity is associated with increased VLDL secretion from the liver, which may induce hyperlipidaemia [168]. VLDL overproduction has been suggested to
be a hallmark of dyslipidaemia in metabolic syndrome [195]. Inhibition of VLDL secretion in mice has been shown to induce hepatic steatosis [194].

**Figure 1.6 Lipid Metabolism in the Liver.** Dietary lipids are assembled into chylomicrons in enterocytes and transported through the lymphatic system to enter the bloodstream. Adipose tissue is also an important source of fatty acids (FAs) in the circulation through lipolysis. The FAs in the bloodstream are transported into the liver through fatty acid transport proteins (FATPs). When there is oversupply of carbohydrates, citrate from the citric acid (TCA) cycle is transported into the cytoplasm and converted to acetyl-CoA for de novo lipogenesis (DNL). The carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC) yields malonyl-CoA, followed by the fatty acid synthase (FAS)-mediated production of palmitate. FAs from these sources are converted in the cytosol into their active forms (fatty acyl-CoA) and shuttled into mitochondrial by carnitine palmitoyltransferase 1 (CPT1). Fatty acyl-CoAs are broken down into acetyl-CoA by mitochondrial β-oxidation, and enters the citric acid (TCA) cycle for further oxidation to generate ATP. The CPT1 activity can be suppressed by increased levels of malonyl-CoA produced in DNL. Another fate of FAs is for the synthesis of TG, which is packed into very-low density lipoprotein (VLDL) and exported into the bloodstream. Overproduction of VLDL may contribute to hyperlipidaemia. (Adapted from [196])
1.2.4 Proposed Mechanisms for the Progression from Steatosis to NASH

1.2.4.1 Hepatic Inflammation

Hepatic inflammation is a major cause of liver damage, and may further lead to fibrogenesis in chronic liver diseases including hepatitis, alcoholic fatty liver disease and NAFLD [197]. Inflammation in the liver is mediated by immune cells including Kupffer cells, natural killer cells, natural killer T-cells and dendritic cells [198]. Unlike infiltrated inflammatory cells, Kupffer cells are the resident macrophage in the liver and account for approximately 20% of non-hepatocyte cells [198]. Due to their location in the sinusoidal vascular space, Kupffer cells are recognised as the primary cells to detect pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) in the blood through pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) [197].

Toll-like receptor 4 (TLR4) plays an important role in hepatic inflammation and fibrogenesis [199] (Figure 1.7). Because FAs, particularly saturated FAs (SFAs), are activators of TLR4 in the macrophage, activation of this receptor may be a potential mechanism underlying the progression from simple steatosis to NASH [200-202]. This receptor is associated with MyD88, a common adaptor molecule, and Toll/IL1 receptor domain-containing adapter-inducing interferon-β (TRIF) [199]. In Kupffer cells, upon the activation of TLR4, MyD88 and TRIF are recruited to eventually trigger the release of pro-inflammatory cytokines including TNFα, IL6 and interleukin-1 (IL1) [197]. These pro-inflammatory cytokines can induce hepatocyte apoptosis and attract the infiltration of neutrophils, natural killer cells and monocytes to the liver. The activation of TLR4 may also increase the release of pro-fibrogenic cytokines such as transforming growth factor-β (TGFβ) from the Kupffer cells [199]. This promotes the activation of hepatic stellate cells (HSCs), which are believed to be a major cell population promoting fibrogenesis in the liver [203]. Besides the important role of TLR4 in Kupffer cells, it has been suggested that pathogens such as lipopolysaccharide (LPS)
can directly activate TLR4 in quiescent HSCs and trigger its conversion into active HSCs [204]. This further indicates that TLR4 plays a crucial role in inflammation and fibrogenesis in the liver.

In addition to DAMPs from the gut, endogenous DAMPs released from injured tissue can also be detected by NOD-like receptors (NLRs), a class of cytosolic PRRs [205]. Upon activation, inflammasomes are formed into protein complexes by NLR family members. They facilitate the activation of caspase-1, which cleaves pro-IL1β into mature IL1β. So far, multiple cellular DAMPs have been identified including cytochrome c, nuclear DNA and mitochondrial DNA (mtDNA), and cholesterol crystals [206]. In addition, it has been shown that SFAs can also activate the NLR pyrin containing 3 (NLRP3) inflammasome in both hepatocytes and macrophages to produce IL1β [207, 208]. The inflammasome-induced caspase-1 activation and IL1β production may further impair insulin signalling and induce apoptosis [209]. These suggest that NLRP3 inflammasome may play a role in mediating lipotoxicity in the development of NAFLD.
Figure 1.7 Toll-Like Receptor 4 and Inflammation Response in the Liver. Pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) activate toll-like receptor 4 (TLR4) in the liver. Upon their activation in Kupffer cells, MyD88 and TRIP are recruited and subsequently trigger the release of pro-inflammatory cytokines and profibrogenic cytokines through the activation of activator protein 1 (AP-1), NFκB pathways and interferon regulatory factor 3 (IRF3). Pro-inflammatory cytokines induce hepatocyte injury and inflammatory cell infiltration. Activation of TLR4 in hepatic stellate cells (HSCs) triggers the conversion from quiescent HSCs into active HSCs promoting fibrogenesis in the liver. (Adapted from [197])
1.2.4.2 Oxidative Stress

Oxidative stress is induced by reactive oxygen species (ROS) and it has been suggested to be another factor that may contribute to the progression of NAFLD [210]. The major types of cellular ROS are superoxide (•O$_2^-$), hydroxyl radical (•OH), hydrogen peroxide (H$_2$O$_2$), and hypochlorous acid (HOCl) [211]. Increased ROS levels can cause toxic effects by reacting with cellular components such as proteins, lipids and DNA to cause cell damage. ROS are generated via three major routes: mitochondrial oxidative phosphorylation, peroxisome and enzymatic source [212].

As a by-product of oxidative phosphorylation, superoxide is produced in mitochondria due to electron leak from the electron transport chain [213] (Figure 1.8). The electrons are transported through protein complexes (I-V) by redox reactions accompanied by the release of energy. The energy is used to pump the protons from the matrix to the intermembrane space, resulting in an electrical potential across the inner boundary membrane. Protons flow along the gradient back to the matrix, activating the ATP synthase to produce ATP from ADP. During this process, electrons can leak from complex I & III, leading to the reduction of O$_2$ to form •O$_2^-$. Superoxide dismutase (SOD) catalyses the dismutation of •O$_2^-$ forming H$_2$O$_2$, which can be subsequently reduced to H$_2$O by glutathione peroxidase (GPx) to protect cells from injury induced by these ROS [213]. H$_2$O$_2$ can be converted to highly reactive HOCl by myeloperoxidase at inflammatory sites, or highly toxic •OH by Fenton's reaction during hypoxia [211].

Additionally, ROS can be generated in the peroxisome or NADPH oxidase in the plasma membrane [214]. Various oxidases in the peroxisome transfer electrons from other metabolites to O$_2$ for the production of H$_2$O$_2$ and a small amount of superoxide. ROS can be degraded by peroxisomal reductases such as catalase and manganese superoxide dismutase.
(MnSOD) to maintain oxidative homeostasis in the peroxisome. NADPH oxidase is a membrane-bound protein complex that removes an electron from NADPH to $O_2$ to produce superoxide, another major source of superoxide [215].

Clinical studies have revealed that NASH is associated with oxidative stress in the liver, as indicated by increases in ferritin levels and lipid peroxidation with concomitant decreased glutathione levels [216]. Regarding the mechanisms underlying lipid-induced oxidative stress, several studies suggest that lipid accumulation increases the expression of NADPH oxidase, but decreases the expression of antioxidant enzymes such as SOD and GPx [217, 218]. Oxidative stress in turn may exacerbate lipid accumulation, and induce lipid peroxidation and other oxidative damage, which promotes the progression to NASH [219-221]. It has been suggested that reduced oxidative stress not only diminishes ROS-induced cell damage, but may also ameliorate hepatic steatosis [222].

Oxidative stress has also been linked to other factors (such as mitochondrial dysfunction, insulin resistance, ER stress and inflammation) that may contribute to the pathogenesis of NASH. Mitochondria are a major site for ROS generation and they are thought to be the primary target of oxidative stress due to this proximity to ROS [223]. Additionally, it has been suggested that mtDNA is susceptible to oxidative damage [224]. Studies in humans have also indicated that oxidative stress is associated with insulin resistance, and is a major cause of diabetic complications [225]. As such, antioxidant treatment has been considered as a possible therapy for the management of diabetes [226]. However, vitamin E, a classic antioxidant, has failed to control oxidative stress and blood glucose in T2D patients in random clinical trials [227, 228]. Nevertheless, α-lipoic acid, a mitochondrial antioxidant, has been shown to improve insulin sensitivity and reduce diabetic complications in patients with T2D [229, 230]. In experimental animals and cells, the increase in mitochondrial superoxide production induced by a pharmacological agent or MnSOD-deficiency (MnSOD+/-) impairs
insulin signalling transduction. MnSOD transgenic mice are protected from diet-induced insulin resistance [231]. This indicates that insulin action can be regulated by ROS produced by mitochondria, and that mitochondria may play an important role in diabetes associated oxidative stress. In addition to insulin resistance, ROS may trigger ER stress [232], and activate NFκB, a transcription factor that controls the expression of pro-inflammatory cytokines [233].

**Figure 1.8 Oxidative Phosphorylation in Mitochondria.** Electrons are transported through complexes (I-IV) located in the inner membrane of mitochondria by redox reactions accompanied by release of energy (shown by the brown arrows). The energy generated is used to pump protons from the matrix to the intermembrane space, resulting in the electrical potential across the inner boundary membrane. Protons flow along the gradient back to the matrix, activating complex V (also known as ATP synthase) to produce ATP from ADP. Leakage of electrons from complex I & complex III lead to the reduction of O₂ to •O₂⁻¹. Superoxide dismutase (SOD) catalyses the dismutation of •O₂⁻¹, formatting H₂O₂, which can be subsequently reduced to H₂O by glutathione peroxidase (GPx). H₂O₂ can be converted to highly reactive HOCl by myeloperoxidase (MPO) at inflammatory sites, or highly toxic •OH by Fenton's reaction during hypoxia. Increased production of ROS in mitochondria is believed to be an important mechanism for insulin resistance and the progression of hepatic steatosis into NASH. (Adapted from [211] and [213])
1.2.4.3 Endoplasmic Reticulum (ER) Stress

The ER not only serves as a site for calcium storage and lipid droplet formation, but also plays an important role in protein synthesis and folding [234]. Disturbances in calcium homeostasis, inhibition of protein glycosylation and alterations in the ER environment (e.g. pH, oxygen and glucose) can lead to the accumulation of unfolded proteins in the ER lumen, triggering the unfolded protein response (UPR) [235]. The UPR is an adaptive response to ER stress to protect cells from stress-induced damage but prolonged activation of UPR may impair insulin signalling transduction, trigger inflammatory response and induce apoptosis [236-238].

The UPR is activated by three transmembrane proteins in the ER: inositol-requiring protein 1 (IRE1), PKP-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [239] (Figure 1.9). Under physiological conditions, the activation of IRE1, PERK and ATF6 is suppressed by binding to 78 kDa glucose-regulated protein (GRP78), also known as BiP [240]. In response to the accumulation of unfolded proteins, GRP78 dissociates and binds to misfolded proteins, facilitating the dimerisation of IRE1 and PERK and the cleavage of ATF6 in the Golgi [241]. The dimerisation of IRE and PERK is followed by autophosphorylation, which triggers the splicing of X-box binding protein 1 (XBP1) mRNA and the phosphorylation of eukaryotic translation initiator factor-2α (eIF2α), respectively. ATF6, XBP1 and activating transcription factor 4 (ATF4, induced by the phosphorylation of eIF2α) are transcription factors that control the expression of genes in autophagy, apoptosis and protein degradation, in order to protect cells from ER stress-induced cell death [242, 243]. However, along with this adaptive response, the activation of IRE1 also induces the phosphorylation of c-JUN N-terminal kinase (JNK), leading to apoptosis, as well as the phosphorylation of IκB kinase (IKK), triggering inflammation [237]. ATF4 triggered by PERK activation also up-regulates the C/EBP-homologous protein (CHOP), a transcription
factor that represses the expression of anti-apoptotic proteins from the B-cell lymphoma 2 (Bcl2) family [238].

It has also been suggested that ER stress may be not only closely related to hepatic steatosis but also involved in the progression of hepatic steatosis into NASH [244]. It has been suggested that ER stress may activate SREBP1c [245], a key transcription factor that regulates the gene expression of lipogenesis as mentioned in Section 1.2.3 [183]. The attenuation of ER stress by overexpressing the protein chaperon GRP78 reduces hepatic steatosis of obese ob/ob mice [245]. It has been shown that a reduction in eIF2α phosphorylation [246] or ATF4 mutation [247], also diminishes hepatic steatosis induced by HF diets. These studies suggest that ER stress may induce hepatic steatosis.

ER stress may also mediate lipid-induced inflammation and apoptosis in the development of NASH. It has been suggested that SFAs induce ER stress and apoptosis in liver cells [248]. As mentioned previously, activation of the PERK arm of the UPR pathway may also activate IKK and induce inflammation [237]. In addition, activation of the IRE1 arm may contribute to hepatic insulin resistance [239], another factor that may contribute to the development of NASH. This may be due to the phosphorylation of IKK and JNK by IRE1 activation, which impairs the insulin signalling transduction by interrupting the phosphorylation of insulin receptor substrate (IRS) serine residues [236]. Taken together, these studies suggest that ER stress may play an important role in the development of NASH in the liver.
Figure 1.9 Endoplasmic Reticulum Stress Pathway, Inflammation and Insulin Resistance. The accumulation of unfolded proteins leads to the disassociation of 78 kDa glucose-regulated protein (GRP78) from endoplasmic reticulum stress (ER stress) sensors, namely inositol-requiring protein 1 (IRE1), PKP-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). These three protein sensors are activated through the disassociation from GRP78, and eventually regulate the expression of genes related to autophagy, apoptosis and protein degradation. 1) Dimerization of IRE1 is followed by its auto-transphosphorylation, and subsequently triggers the splicing of XBP1 mRNA to express the active XBP1 protein; 2) PERK is also activated by the auto-transphosphorylation after dimerization. Upon PERK activation, eukaryotic translation initiator factor-2α (eIF2α) is phosphorylated, leading the increase of ATF4; 3) Disassociation from GRP78 leads to the cleavage of ATF6 in Golgi to produce active ATF6. ER stress may induce apoptosis through the C/EBP-homologous protein (CHOP) that is up-regulated by ATF4, and through phosphorylated c-JUN N-terminal kinase (JNK) that are induced by activated IRE1. IRE1 activation may also trigger the phosphorylation of IkB kinase (IKK), leading to inflammation. Meanwhile, the activation of JNK and IKK may phosphorlate insulin receptor substrate (IRS) on their serine sites, impairing the insulin signalling transduction. (Adapted from [239])
1.2.4.4 Insulin Resistance

Insulin resistance is well known to be a major characteristic of T2D and closely associated with obesity and NAFLD. It is estimated that approximately 98% people with NASH are insulin resistant [249]. In adipose tissue, insulin resistance diminishes the inhibitory effect of insulin on lipolysis, leading to the release of FAs into the circulation [250]. Increased levels of circulating FAs may pose several challenges on liver function. Firstly, a greater availability of FAs increases FA flux into the liver, and this can exacerbate hepatic steatosis and further impair insulin sensitivity to form a vicious cycle. Secondly, excessive influx of FAs into the liver has been suggested to increase ROS production resulting from mitochondrial β-oxidation to contribute to the development of NASH [251]. Thirdly, SFAs such as palmitate may induce ER stress in the liver and activate TLR4 triggering the inflammatory response [201, 248]. However, it remains to be established whether insulin resistance is a comorbidity or a direct cause of NASH.

1.2.4.5 Mitochondrial Dysfunction

Mitochondria play a central role in energy metabolism. As discussed in Section 1.2.3, FAs transported from fat tissue or produced from lipolysis and DNL are metabolised through β-oxidation, the citric acid cycle and oxidative phosphorylation to generate ATP. Mitochondrial dysfunction has been linked to NASH in both rodents [252] and humans [94, 193]. For example, it has been suggested that NASH may be caused by impaired mitochondrial function, such as reduced CPT1 activity in a genetic obesity rat model (Otsuka Long–Evans Tokushima fatty rats) [252]. In humans, it has been shown that structural changes of mitochondria in patients with NASH are distinct from patients with hepatic steatosis [193]. This is consistent with a recent report showing that mitochondrial respiration increases in obese patients but decreases when obesity is associated with NASH [94], suggesting mitochondrial dysfunction may be a characteristic of NASH.
1.2.5 Treatment of NAFLD

Simple hepatic steatosis has a good prognosis because it is largely reversible by measures such as dietary control. On the other hand, there is no effective treatment for the late stages of NAFLD, typified by advanced hepatic fibrosis and cirrhosis. Therefore, the major treatment of NAFLD has been focused on NASH because effective control of NASH can prevent or delay the progression to fibrosis and cirrhosis [163]. Weight loss, insulin sensitizers and n-3 polyunsaturated FA (PUFA) have been reported to show various degrees of effectiveness for the treatment of NASH in this regard [143, 253, 254].

A meta-analysis of 10 randomised clinical trials has concluded that weight loss induces a dose-dependent improvement of NASH activity in obese or overweight subjects [143]. One of these clinical trials showed that intensive lifestyle interventions (diet, exercise, and behaviour modification for 48 weeks) markedly reduced body weight and overall NASH activity scores (hepatic steatosis, inflammation, hepatocyte ballooning and fibrosis) compared with standard nutrition counselling [255]. Another trial randomized 50 overweight subjects (BMI ≥ 27) into 36 weeks of lifestyle interventions (calorie restriction combined with vitamin E) with or without orlistat, an enteric lipase inhibitor for the treatment of obesity [256]. Both groups showed similar weight loss, and had similarly improved hepatic steatosis and reduced serum levels of AST and ALT. Furthermore, both trials indicated that, compared to the improvement in steatosis, greater weight loss may be required to achieve improvements in histologic changes including inflammation and ballooning.

Two open-labelled trials investigated the efficacy of an insulin-sensitizer in the treatment of NAFLD [253, 257]. One trial showed that rosiglitazone treatment alone ameliorated steatosis, lowered liver enzymes in the plasma, and improved histological scores including inflammation, ballooning and fibrosis. However, combining rosiglitazone with metformin did not show any additional benefits [253]. The other trial compared the efficacy of rosiglitazone
and metformin and found that rosiglitazone, but not metformin, reduced serum transaminase level and NASH scores [257]. These studies suggest that rosiglitazone is more effective for the treatment of NASH compared with metformin. Furthermore, these results may also suggest that targeting PPARγ in WAT (rosiglitazone) may be more beneficial than AMPK in the liver (metformin) for the effective treatment of NASH.

Supplementation of the n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has also been investigated for the treatment of NAFLD. Two pilot clinical studies suggest that these PUFAs not only ameliorate the degree of hepatic steatosis but also reduce liver enzymes and TNFα levels in the serum [258, 259]. n-3 PUFAs have also been suggested to improve histological features observed in NASH patients including hepatic steatosis, hepatocyte ballooning and lobular inflammation [254]. Based on these results, several randomised clinical trials have been conducted to investigate the efficacy of n-3 PUFAs as a treatment for NAFLD in children [260, 261] and adults [262, 263]. However, these studies lead to equivocal results. For example, one study in 76 children showed that supplementation of EPA and DHA mixture (450-1300 mg/day) for 6 months did not affect liver fat but reduced liver enzymes in the serum [261]. However, the other study in children showed DHA supplementation (250 mg/day) for 6 months significantly decreased liver fat [260]. In adults, it has been shown that treatment with a EPA and DHA mixture (4 g/day) for 15-18 months induced a trend towards an improvement in liver fat [263]. Interestingly, the erythrocyte DHA level in patients in the treatment group was linearly associated with the decrease in hepatic steatosis. These results suggest that DHA may have greater potential in the treatment of NAFLD than a mixture of EPA and DHA. The inconsistent results from these trials may be due to the different dosages and compositions of the n-3 PUFAs, as well as the study duration. The efficacy of DHA for the treatment of NASH needs to be further studied in large-scale and multicentre studies.
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1.3 Roles of Dietary Fat in the Development of Obesity and NAFLD

As stated above (Figure 1.2), obesity is caused by increased energy intake and/or decreased energy expenditure. The energy intake mainly comes from three major macronutrients, namely carbohydrate, fat and protein. These macronutrients contain different densities of energy and also exert different effects on satiety and the release of cytokines (e.g. leptin, adiponectin, FGF21). These additional effects may influence the development of obesity and NAFLD in addition to simply providing calories.

Although a low protein diet [264] or overconsumption of carbohydrate (especially corn syrup in beverages [265]) may contribute to the prevalence of obesity and hepatic steatosis, this thesis will focus on dietary saturated and unsaturated fat. Obviously, the percentage of fat in the diet is not the only factor that contributes to obesity because the genetic factors and other ingredients need to be taken into account. It has been indicated in a systematic review of clinical trials that a 10% reduction in energy from fat is associated with a weight loss of 16 g/day [266]. Since dietary fat is the most energy-dense compared with protein and carbohydrate [267], consumption of a diet rich in fat is more likely to induce obesity due to an increase in energy intake [268]. This is supported by epidemiological studies showing that consumption of HF diets is an independent risk factor for being overweight [269], although the metabolic effects may vary depending on the type of FA composition of the fat.

1.3.1 Saturated Fat as a Major Culprit of Lipotoxicity

It is well documented that HF diets rich in saturated long-chain (C16:0, C18:0 and C20:0) FAs from animal fat, such as lard and tallow, induce obesity, insulin resistance and other metabolic dysfunctions in rodents [38, 40, 270, 271]. It has been suggested that different types of fat (SFAs or unsaturated FAs) provide similar amounts of energy when metabolised, and that there is little difference in their effects on satiety [272, 273]. However, SFAs may have
more deleterious effects to induce NASH compared with unsaturated FAs due to an increased propensity to cause lipotoxicity from inflammation, ER stress, oxidative stress and mitochondrial dysfunction.

It has been suggested that SFAs can elicit an inflammatory response through TLR4 activation [200], which is a key regulator of hepatic inflammation (Figure 1.7). One study has shown that SFAs, but not unsaturated FAs, activated NFκB pathways through TLR4 in cultured macrophages [274]. A further study has indicated that DHA, a PUFA, can inhibit TLR4-induced NFκB activation [275]. SFAs-induced ER stress has been suggested as a mechanism underlying SFA-mediated cell death in liver cells [248, 276]. SFAs may also induce oxidative stress by increasing the ROS production in liver cells, possibly due to SFA-induced compensatory increase in mitochondrial respiration [277, 278]. In addition, it has been shown that SFAs may damage mtDNA in muscle and pancreas cells, leading to oxidative stress and apoptosis [279, 280].

The detrimental effect of SFAs may be mediated by lipotoxic intermediates, such as ceramides and lysophosphatidylcholines (LPC) [281]. The former has been well recognised for its effect on impairing insulin signalling transduction, and the latter has been suggested to induce apoptosis by depolarising the mitochondrial membrane in hepatocytes [282]. In cultured liver cells, it has been shown that palmitate induces apoptosis by depolarising the mitochondrial membrane potential and this lipotoxicity of palmitate can be blocked by supressing the production of LPC by a Ca^{2+}-independent phospholipase A2 [282]. Oleate, a monounsaturated FA (MUFA), has been shown to inhibit palmitate-induced apoptosis, which is associated with the reduction in LPC content. Further studies have shown the direct effect of LPC to induce apoptosis in Huh-7 cells (a human hepatocellular carcinoma cell line) [283] and to impair mitochondrial FA oxidation in isolated mitochondria [284].
1.3.2 Diverse Roles of Unsaturated Fat in Lipotoxicity

It has been suggested that a moderate intake of MUFAs may be not as detrimental as SFAs in regard to insulin action in healthy humans [285]. As mentioned previously, oleate may even protect the cell from palmitate-induced apoptosis [282]. Mechanistically, MUFAs may protect cells from SFA-induced apoptosis by promoting the incorporation of SFAs into TG [286, 287].

It is generally accepted that the detrimental effect of FAs on cells may be mediated by lipotoxic intermediates but not TG [288]. Indeed, formation of TG has been suggested to be a protective mechanism against SFA-induced lipotoxicity [286], presumably by reducing the availability of SFAs in the cytosol. It has been shown that oleate increases the storage of exogenous palmitate in the form of TG, leading to the reduction of oxidative intermediates and ceramide in CHO cells [286]. Another study suggested that oleate prevented palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signalling by increasing TG accumulation and decreasing DAG in neuronal cells [287].

As mentioned in Section 1.2.5, n-3 PUFAs have been investigated as a possible treatment option for NAFLD. The possible mechanisms underlying this effect of PUFAs have been widely studied. Firstly, it has been suggested that PUFAs inhibit the expression of genes in lipogenic pathway including FAS and ACC, possibly through the suppression of SREBP1 in rats [289]. Secondly, PUFA is a potent activator of PPARα in the liver, which promotes FA β-oxidation [290, 291]. In addition, n-3 PUFAs may inhibit LPS- or SFA-induced activation of inflammatory pathway by inhibiting TLR4 activation and ameliorate hepatic inflammation and liver injury during the development of NASH [275, 292].

Most of these suggested mechanisms of SFAs and unsaturated FAs are based on studies in cultured cells. As dietary fat is often a mixture of different compositions of FA species, it has often been impossible to demonstrate whether the aforementioned mechanisms have any significant role to play in whole animal and human studies. In terms of the effect on NAFLD,
there is a well-accepted view that TG accumulation in the liver without an elevation of lipotoxic intermediates may not lead to cellular injury and/or inflammation [288]. This is consistent with the clinical observation that a majority of NAFLD patients stay at the stage of simple hepatic steatosis [56] and also highlights the importance of investigating the second “hit” that may promote the progression from simple hepatic steatosis to NASH.

1.4 Roles of Dietary Cholesterol in the Development of Obesity and NAFLD

1.4.1 Cholesterol Metabolism and Its Regulation

Dietary cholesterol exists in meat, eggs, cheese and other animal related foods. The cholesterol content in egg yolk is approximately 1% w/w and a typical Western diet that is used in in animal studies contains 0.2% w/w cholesterol [293, 294]. Cholesterol is absorbed in the small intestine through a transmembrane protein, Niemann-Pick C1-like 1 (NPC1L1) (Figure 1.10). Ezetimibe, a second line cholesterol-lowering drug, blocks NPC1L1 thus inhibiting intestinal absorption of cholesterol [295]. Once absorbed in enterocytes, cholesterol is packed into chylomicrons together with other lipids and then released into the bloodstream to circulate to tissues including the liver [296]. The absorption of cholesterol can be influenced by other dietary components, particularly dietary fat [297]. A meta-analysis of clinical studies shows that 100 mg of cholesterol in a high saturated fat diet induces an increase in serum level of LDL by approximately 70% compared with a low saturated fat diet [298].

Cholesterol also comes from the endogenous synthesis mainly in the liver from acetyl-CoA and acetoacetyl-CoA [299]. The biosynthesis of cholesterol is regulated by the master transcription factor SREBP2, which controls the expression of key enzymes in the pathway of
Chapter 1 - Introduction and Literature Review

cholesterol synthesis [300]. One critical rate-limiting enzyme is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. This enzyme is the target of statins, a class of cholesterol-lowering drugs clinically used for the treatment of hypercholesterolemia.

Cholesterol in the liver can come from both the absorption from a diet and the endogenous synthesis as described above. Within the liver, cholesterol in the liver is mainly used for the synthesis of bile acid, which is then secreted into the gut to facilitate the digestion of food. About 95% of the bile acid in the gut is reabsorbed from the small intestine through the enterohepatic circulation and transported back to the liver [301]. The reabsorption of bile acid from the gut can be blocked by bile acid sequestrants, which are clinically used as another class of cholesterol-lowering drugs. Cholesterol is also the precursor of steroid hormones, including glucocorticoid, oestrogen and androgen [302].

A proportion of cholesterol in liver is esterified and packed in the nascent VLDL for the secretion into the bloodstream. A small amount of cholesterol is used for the biosynthesis of nascent HDL (a complex of apoproteins, phospholipid and free cholesterol) [303]. HDL functions as a vehicle in the reverse transport of cholesterol from peripheral tissues back to the liver [304]. Unlike LDL and VLDL, HDL is regarded as a “good” lipoprotein with protective effects against CVD by removing toxic cholesterol from macrophages in arterial walls via the ATP binding cassette A member 1 (ABCA1) and ATP binding cassette G member 1 (ABCG1) [305]. Fibrates are a class of drugs used to lower plasma levels of TG and LDL as well as increasing HDL concentrations. They promote apoA-I production through PPARα activation and subsequently increase the formation of HDL in the liver [306].
Figure 1.10 Cholesterol Metabolism in the Liver. Cholesterol in the liver mainly comes from four sources. 1) Dietary cholesterol is absorbed through Niemann-Pick C1-like 1 (NPC1L1) and transported via chylomicron from the small intestine to the liver. 2) Hepatocytes can uptake cholesterol from circulating lipoproteins such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL) from the bloodstream. 3) Cholesterol is recycled from the small intestine by bile salt reabsorption, which can be blocked by bile acid sequestrants. 4) The liver is a major site for cholesterol biogenesis, which is the target of statins. The fate of cholesterol in the liver includes: 1) substrate for synthesis of bile salt and transported through the enterohepatic circulation to the small intestine; 2) assembling into very-low-density lipoprotein (VLDL) and HDL to supply to other peripheral tissues; 3) substrate for steroid hormones, such as glucocorticoid, oestrogen and androgen. (Adapted from [307, 308])
1.4.2 Cholesterol and Obesity: Effects on Energy Balance?

Dietary cholesterol is generally believed not to contribute to the calorific value of food, and there is hardly any specific study of its direct effect on obesity. This section analyses several clinical trials that have evaluated body weight in regard to the effect of consumption of a cholesterol-rich diet (e.g., egg) [309-316]. Since the focus of these studies was dyslipidaemia, most of them specifically controlled for body weight as a part of the experimental design. Only two studies provided the body weight or BMI changes during the trial. One study was conducted in 17 college students, who received one egg/day in their dessert for 3 weeks and then the egg was removed for another 3 weeks [312]. They observed no differences in body weight and calorie intake between these two periods. The other study examined the effect of five types of diet, four of which contained the same amount of fat but different amounts of cholesterol, in ten subjects [311]. Since the effect of these diets was only examined for one meal, no change in body weight was observed. Neither of these studies was properly designed for the assessment of the influence of cholesterol on body weight, because study durations were too short and numbers of subjects were too small. Reviewed below are the reported effects of cholesterol on food intake, fat absorption and energy expenditure, which may potentially influence body weight gain.

1.4.2.1 Effects of Dietary Cholesterol on Food Intake

As food intake is largely driven by appetite, this section reviews the literature reports about possible effects of dietary cholesterol on appetite but only found indirect implications for a possible effect of cholesterol on appetite. It has been shown that a naturally occurring cholesterol derivative, MSI-1436, profoundly reduced the body weight of mice by suppressing food intake [317]. When administered centrally, a much lower dosage of this compound was needed when compared to i.p. injection (0.03 mg/kg vs. 10 mg/kg). The
authors speculated that this cholesterol derivative may act in the central system to suppress appetite.

In humans, it has been shown that consumption of eggs for breakfast is more effective to suppress appetite compared with bagels [318]. However, the explanation of this observation may be confounded by the higher protein content in eggs. Another study in 28 overweight/obese subjects matched the composition of other dietary components whilst varying cholesterol content [319, 320]. Results showed that addition of cholesterol with 3 eggs to a carbohydrate-restricted diet did not change food intake and appetite hormone levels compared with addition of a cholesterol-free egg substitute. This seems to suggest that dietary cholesterol has no effect on appetite. However, in this study, cholesterol was supplied in a diet that has been known to suppress food intake. The effect of cholesterol on food intake in other dietary conditions still needs to be established by further studies in larger numbers of subjects and for a longer period of time.

1.4.2.2 Effects of Dietary Cholesterol on Fat Absorption

As reviewed in Section 1.2.3 and 1.4.1, both cholesterol and fat are absorbed in the intestine and then transported to the bloodstream by the same vehicle, chylomicrons. Dietary fat, particularly saturated fat, may facilitate the absorption of cholesterol [298]. However, it is less clear whether cholesterol has an effect on the absorption of fat. FAT/CD36 plays a key role in the transport-dependent uptake of FAs by enterocytes (Figure 1.6). It has been shown that enterocytes isolated from FAT/CD36 knockout mice has both reduced absorption of FAs and cholesterol [321]. This may suggest that the absorption of cholesterol and FAs are closely related. Furthermore, it has been indicated that the uptake of long-chain FAs through FAT/CD36 is cholesterol-dependent in cultured kidney cells [322]. However, the exact effects of dietary cholesterol on fat absorption are still unclear.
1.4.2.3 Effects of Dietary Cholesterol on Energy Expenditure

Apart from increased calorie intake, decreased energy expenditure is an important contributor to the development of obesity. An extensive search of the literature did not reveal any study clearly demonstrating a direct effect of cholesterol on energy expenditure per se. However, some studies suggest that cholesterol might influence the activity of the sympathetic nervous system, which participates in the regulation of energy expenditure. It has been shown in a Chinese cohort (≥ 40 years old), that a high serum level of total cholesterol is associated with an elevated resting heart rate [323]. Consistent with this report, some studies in humans [324] and animal studies [325] have indicated the effect of hypercholesterolemia on sympathetic nervous tone. It has been shown that female subjects with dyslipidaemia have higher sympathetic nervous activity in muscle compared with subjects with a normal cholesterol profile [324]. Conversely, lowering the circulating level of cholesterol by statins has been shown to reduce the sympathetic activity in humans [326]. A study in a rabbit model, also showed that addition of cholesterol (0.5%) to the diet increased sympathetic nerve density in the myocardium [325]. Collectively, these studies suggest that dietary cholesterol may have an effect on the sympathetic nervous system but the relationship of such effect with energy expenditure is not clear.

1.4.3 Cholesterol and NAFLD: Effects on Hepatic Steatosis and NASH.

As reviewed in Section 1.2.1, NAFLD is a broad term covering a wide range of closely related liver conditions including hepatic steatosis, liver injury, inflammation, fibrosis and cirrhosis. This section reviews the reported effects of cholesterol on hepatic steatosis and key mechanisms involved in the development of NASH.

1.4.3.1 Effects of Cholesterol on Hepatic Steatosis

Besides the possible effect of cholesterol on FAs absorption as reviewed in Section 1.4.2.2, it has been suggested that cholesterol may affect VLDL production; a mechanism of TG
removal from the liver as shown in Figure 1.6. In cultured human hepatocytes, cholesterol has been shown to induce a dose-dependent increase in secretion of ApoB, which is an important component of liver-derived lipoproteins including VLDL (Figure 1.6) [327]. However, studies in animals provide conflicting results on the effect of cholesterol feeding on VLDL production. In monkeys, cholesterol (2%) in a 25% coconut oil diet was found to induce a 4-fold increase of hepatic VLDL production [328]. Similarly, in hamsters, 2% cholesterol feeding in a standard chow diet for two weeks also increased the TG levels in plasma VLDL [329]. However, in mice, it has been shown that 2-week feeding of 1% cholesterol in a chow diet did not promote VLDL production in the liver but increased hepatic TG content [330]. This study in mice is consistent with a study in humans showing that 1700 mg intake of cholesterol did not increase VLDL concentration in plasma compared with 200 mg intake of cholesterol [331]. The contradictory results from these studies suggest that the effect of dietary cholesterol on hepatic VLDL production may vary depending on its concentration and/or the species of experimental animal.

1.4.3.2 Effects of Cholesterol on Inflammation

It has been well recognised that the accumulation of cholesterol in immune cells may promote inflammatory responses [332]. This has been investigated in the pathogenesis of atherosclerosis, where increased serum LDL results in the accumulation of cholesterol in inflammatory cells in the artery wall, triggering inflammatory responses. The mechanisms underlying the role of cholesterol in the inflammatory response include the activation of the TLR4 receptor and the inflammasome [332]. It has been shown that the accumulation of cholesterol in isolated macrophages by knockout of the ABCG1 transporter, a receptor that facilitates the removal of cholesterol from macrophages [305], promotes inflammation, and this process requires the presence of TLR4 [333]. This study also showed that cholesterol potentiated the LPS-induced activation of TLR4, and inflammation was abolished when cells
were treated with cyclodextrin to remove cholesterol. In addition, accumulation of cholesterol in cells may also promote the formation of cholesterol crystals, which has been proposed as one of the DAMPs that activates the inflammasome [334]. Collectively, these reports indicate that accumulation of cholesterol in macrophages plays an important role in the inflammatory response during the development of atherosclerosis.

Although the role of cholesterol in inducing inflammation during atherosclerosis has been investigated extensively, there is no clear evidence of a relationship between dietary cholesterol and hepatic inflammation. Given the close association of NAFLD with dyslipidaemia (Table 1.3), it is reasonable to speculate cholesterol has a similar role in triggering inflammatory responses in the liver under certain conditions. This possibility is supported by some studies in animal models as reviewed below.

In mice with dyslipidaemia (induced by ldlr<sup>−/−</sup> mice and apolipoprotein E2 knock-in), a HF diet with 0.2% cholesterol was found to induce marked inflammation in the liver [335]. Removing cholesterol from the HF diet was able to prevent the development of hepatic inflammation and reduce plasma VLDL cholesterol but in the absence of a reduction of cholesterol level in the liver, suggesting the hepatic inflammation may result from VLDL cholesterol. This is consistent with the findings in a commonly used dyslipidaemia model in rabbits showing that high cholesterol (1%) diet can induce hepatic inflammation without overconsumption of fat [336, 337]. These appear to suggest that the effect of dietary cholesterol on hepatic inflammation in these animal models was largely due to dyslipidaemia rather than the cholesterol in the liver or hepatic steatosis. One study has compared the effects of a HF diet on hepatic inflammation with or without inclusion of cholesterol (0.15%) in ldlr<sup>−/−</sup> mice [338]. The results from this study showed that addition of cholesterol to the HF diet exacerbated hepatic inflammation without increasing plasma TG and cholesterol. This was associated with a marked increase in hepatic TG and it was not clear whether the
inflammation was induced directly by cholesterol or was a consequence of the exacerbation of hepatic steatosis.

Given the paucity of data, it is important to understand how cholesterol and TG, alone or in combination, might contribute to the pathogenesis of NASH. It is also important to investigate the effects of cholesterol on hepatic inflammation independent of the influence of adiposity and dyslipidaemia.

1.4.3.3 Effects of Cholesterol on ER Stress, Insulin Resistance, Oxidative Stress and Mitochondrial Dysfunction

As reviewed in Section 1.2.4, ER stress, insulin resistance, oxidative stress and mitochondrial dysfunction may be involved in the progression from hepatic steatosis to NASH [175]. This section will review our current understanding on the effects of cholesterol on these factors.

It has been shown in various cultured cells that cholesterol overload may impair ER functions in cells [339-341]. In macrophages, cholesterol overload can trigger the CHOP arm of the UPR, possibly due to its effect in depleting intraluminal calcium, because the activated UPR can be blocked by the prevention of calcium depletion [339]. In CHO cells, an overload of cholesterol in the ER was found to impair the transport of proteins from the ER to Golgi, and inhibited their subsequent secretion [340]. In L02 cells, a cell line derived from human hepatocytes, LDL treatment has been shown to induce cholesterol overload and ER stress as indicated by increases in ER stress markers, GRP78, ATF6 and CHOP [341]. However, it was not clear in this study whether the effect on ER stress is directly caused by cholesterol and/or other components of LDL. It has also been observed in global ApoE KO mice that the increased cholesterol is associated with the activation of the IRE1 arm of the UPR in the liver [342]. These studies suggest that an accumulation of cholesterol in the liver is likely to trigger ER stress. However, the UPR in response to ER stress can display different phenotypes in each of the three arms depending on the severity and duration. Further studies are needed to
define the effect of cholesterol on ER stress, particularly in relation to its possible role in the pathogenesis of NASH.

Whether cholesterol has a direct effect on insulin sensitivity has been rarely reported. There are some indirect data to implicate a possible link of cholesterol metabolism to insulin sensitivity. In humans, it has been shown that insulin resistance is associated with increased cholesterol synthesis and decreased cholesterol absorption [343]. However, administration of statins (a class of drug blocks cholesterol synthesis) has been shown to be associated with decreased insulin sensitivity in both diabetic and non-diabetic subjects [344, 345]. It is difficult to attribute such effect to the changes in cholesterol levels, because the direct effects of this class of drug on insulin sensitivity cannot be excluded.

In terms of the effect of cholesterol on oxidative stress, it has been shown that LDL induces oxidative stress in macrophages [346]. Cholesterol loading has also been suggested to trigger an oxidative stress pathway in pancreatic β-cells [347]. Some animal studies have suggested that overconsumption of cholesterol may induce oxidative stress in the liver [348, 349]. Cholesterol-fed rabbits are a well-established animal model for the study of atherosclerosis, and these animals all develop systemic oxidative stress possibly due to dyslipidaemia [350]. It has been shown that 1% cholesterol feeding induced hepatic steatosis and elevated serum ALT in rabbits [348]. This was associated with increased ROS activity and decreased activities of GPx and SOD; indicating the presence of oxidative stress. In rodents, it has been shown that 2% cholesterol combined with 0.5% choline acid induces oxidative stress in the plasma and the liver, which is associated with the elevation of TG and cholesterol in the plasma [349]. These studies suggest that overconsumption of dietary cholesterol may induce oxidative stress in the liver.

Mitochondrial dysfunction has been suggested to contribute to the pathogenesis of NASH [252]. It has been reported that cholesterol, in particular free cholesterol, may induce
mitochondrial dysfunction in various cell types [351-354]. The macrophage is the primary cell type that is overloaded with cholesterol due to dyslipidaemia in atherosclerotic lesions [355]. Therefore, the toxicity of cholesterol in the macrophages and the underlying mechanism has been studied widely. One possible mechanism is that cholesterol overloading induces mitochondrial dysfunction by depolarising the mitochondrial transmembrane potential in macrophages [351]. Further studies have been carried out to investigate whether this toxicity of cholesterol exists in other cell types including hepatocytes [352-354]. A similar phenomenon of mitochondria dysfunction has been observed in mouse pancreatic β-cells after cholesterol treatment [352]. In mouse primary hepatocytes, cholesterol loading induced by LDL treatment caused mitochondrial injury with marked changes in ultrastructure [353]. It has also been shown that mitochondrial free cholesterol loading may deplete mitochondrial glutathione and thus sensitize hepatocytes to TNF-mediated cell death [354]. In the same study, atorvastatin treatment effectively restored the level of mitochondrial glutathione and protected ob/ob mice from an LPS-induced elevation of serum ALT and AST. Collectively, these studies indicate that cholesterol has toxic effects on mitochondria. However, it is not clear whether this cholesterol-induced mitochondrial dysfunction may contribute to the development of NASH.

Taken together, these studies in animals or cells suggested that cholesterol may contribute to the factors involved in the progression from hepatic steatosis to NASH. Most of these animal studies showed severe dyslipidaemia that was induced either by genetic factors or a high percentage of cholesterol such as 1% or 2% in the diet. Although egg yolks contain approximately 1% w/w cholesterol, the daily intake of cholesterol is unlikely to reach such extreme levels. Further studies are required to investigate whether a typical level of dietary cholesterol (0.2% w/w) may promote the development of NASH by contributing to inflammation, ER Stress, insulin resistance, oxidative stress and mitochondrial dysfunction.
1.5 Review Summary and Research Aims/Hypotheses

The worldwide prevalence of obesity is a result of the interplay between genetics and environmental factors. Obesity, in particular central obesity, is often associated with other metabolic disorders including T2D and NAFLD. Hepatic steatosis is a prerequisite for the development of NAFLD and it is considered as a first “hit”. A second “hit” is required for the progression from simple steatosis towards NASH, which is associated with liver damage and inflammation. It has been suggested that multiple factors are involved in this conversion including inflammation, oxidative stress, ER stress, insulin resistance and mitochondrial dysfunction.

Although overconsumption of dietary fat may induce obesity and hepatic steatosis, it may not be sufficient to induce liver injury. There is no direct evidence to suggest that cholesterol may affect food intake and energy expenditure, which are major determinants of obesity. However, it has been suggested that cellular overload or overconsumption of cholesterol (1% or even higher) in animals may contribute to inflammation, oxidative stress, ER stress and mitochondrial dysfunction. However, it is not clear whether a typical level of dietary cholesterol (0.2%) may promote the development of NASH by contributing to these factors.

In light of these gaps in our knowledge, identified from this review, the overall aim of this thesis was to investigate the role of dietary fat and cholesterol in the development of obesity, glucose intolerance and NAFLD.

The specific aims were:

1. To examine the characteristics of metabolic disorders regarding obesity, glucose intolerance and hepatic steatosis in mice produced by a HF diet and a moderate-cholesterol diet (0.2% w/w) alone or in combination (Chapter 3).
2. To investigate the effects of these diets on the development of NASH and the involvement of whole-body insulin resistance, inflammation, ER stress, oxidative stress and mitochondrial dysfunction (Chapter 3).

3. To investigate sustained effects (termed “legacy effects”) of a HF diet with cholesterol on obesity, glucose metabolism and hepatic steatosis (Chapter 4). This aim was developed based on the findings from Chapter 3.

The **working hypotheses** for these specific aims were:

1. Chronic HF diet but not moderate dietary cholesterol would induce obesity, glucose intolerance and hepatic steatosis (as the first “hit” in the development of NASH).

2. Cholesterol may promote the progression from hepatic steatosis (induced by the HF diet) towards NASH by inducing additional insults in the liver.

3. Prior exposure to the HF diet with cholesterol may induce “legacy effects” on the development of obesity, glucose intolerance and hepatic steatosis.

The working hypotheses are depicted in **Figure 1.11**. These studies are likely to provide new insights into the effects of dietary fat and cholesterol on the development of obesity, glucose intolerance and NAFLD and help establish a dietary model of NASH in mice for the pre-clinical study of new treatments. The findings from these dietary models may also provide scientific rationales for clinical and epidemiological studies regarding the effect of dietary fat and cholesterol on obesity and associated metabolic disorders.
A high-fat diet with cholesterol may induce “legacy effects” on the development of obesity and related metabolic disorders.

Figure 1.11 Schematic Diagram of Working Hypotheses  A high-fat (HF) diet induces obesity, glucose intolerance and hepatic steatosis in mice but it may not induce additional insults in the development of NASH. Dietary cholesterol may not contribute to the development of obesity, glucose intolerance and hepatic steatosis, but it may promote the development of NASH by inducing inflammation, oxidative stress, ER stress or mitochondrial dysfunction (Chapter 3). An unexpected finding from Chapter 3 (a sustained effect of cholesterol on mitochondrial function) led to the development of a hypothesis that prior exposure to a HF diet with cholesterol may induce “legacy effects” on the development of obesity and related metabolic disorders (Chapter 4).
CHAPTER 2

General Methods
This chapter provides the general methods used to investigate the effects of dietary fat and cholesterol on the development of obesity, glucose intolerance and NAFLD. In all experiments, C57BL/6J mice were used because they have been utilised extensively for the investigation of these metabolic disorders [39, 40, 356, 357]. The methods and study designs that are specific to a study are provided in corresponding chapters (Sections 3.2 & 4.2).

### 2.1 Animal Care, Feeding and Monitoring

All experiments were approved by the Animal Ethics Committee of RMIT University (Approval No. #1012 and #1314) in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Male C57BL/6J mice were purchased from the Animal Resources Centre (Perth, Australia). Mice were kept at 22 ± 1°C on a 12-hour light/dark cycle with free access to food and water. After 1-2 weeks of acclimatization in the animal facility at RMIT University, they were fed a standard chow diet (CH diet, Specialty Feeds, Australia) *ad libitum* unless described otherwise. Body weight and food intake (assessed by weighing the diet), were measured twice a week unless described otherwise.

### 2.2 Preparation of Diets

The powdered CH diet, with or without 0.2% cholesterol, was made into pellets and stored at -20°C until it was used. A HF diet with or without 0.2% cholesterol was prepared weekly based on the ingredients presented in Table 2.1, and kept at -20°C until use [32, 358]. The composition of macronutrients in the CH and HF diet is listed in Table 2.2. The digestible energy is 3.34 kcal/g for the CH diet and 4.94 kcal/g for the HF diet.
Table 2.1 Composition of the High-Fat Diet with or without Cholesterol

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Manufacturer</th>
<th>Catalogue No.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>MPD Dairy Products</td>
<td>ACIDCASEIN</td>
<td>215.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Hudson Pacific Corp.</td>
<td>-</td>
<td>294</td>
</tr>
<tr>
<td>Starch (cornflour)</td>
<td>Hudson Pacific Corp.</td>
<td>FLCO1</td>
<td>173</td>
</tr>
<tr>
<td>AIN-76 Mineral Mixture</td>
<td>MP Biomedicals</td>
<td>0290545502</td>
<td>51</td>
</tr>
<tr>
<td>Trace Minerals</td>
<td>MP Biomedicals</td>
<td>0296026401</td>
<td>14.8</td>
</tr>
<tr>
<td>Bran</td>
<td>Hudson Pacific Corp.</td>
<td>BC20KGA</td>
<td>63</td>
</tr>
<tr>
<td>Methionine</td>
<td>Sigma-Aldrich</td>
<td>M9500</td>
<td>2.8</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Hudson Pacific Corp.</td>
<td>GEPO1</td>
<td>19</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>Sigma-Aldrich</td>
<td>C1629</td>
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<td>Safflower oil</td>
<td>Stoney Creek Oil Products</td>
<td>SAFFLOWER</td>
<td>30</td>
</tr>
<tr>
<td>Lard</td>
<td>Allowrie</td>
<td>Prime Lard</td>
<td>250</td>
</tr>
<tr>
<td>AIN Vitamin Mix 76 - A</td>
<td>MP Biomedicals</td>
<td>0296009801</td>
<td>14.8</td>
</tr>
<tr>
<td>(Cholesterol)</td>
<td>Sigma-Aldrich</td>
<td>C75209</td>
<td>2.28</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>1134.9 or 1132.6</td>
</tr>
</tbody>
</table>

Table 2.2 Calculated Nutritional Parameters of the Chow and High-Fat Diet

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>High-Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fat (kcal%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated fat (w/w)</td>
<td>15%</td>
<td>35%</td>
</tr>
<tr>
<td>Polyunsaturated fat (w/w)</td>
<td>37%</td>
<td>12%</td>
</tr>
<tr>
<td>Monounsaturated fat (w/w)</td>
<td>42%</td>
<td>49%</td>
</tr>
<tr>
<td><strong>Carbohydrate (kcal%)</strong></td>
<td><strong>65%</strong></td>
<td><strong>38%</strong></td>
</tr>
<tr>
<td><strong>Protein (kcal%)</strong></td>
<td><strong>23%</strong></td>
<td><strong>17%</strong></td>
</tr>
</tbody>
</table>

2.3 Glucose Tolerance Test

Impaired glucose tolerance (or glucose intolerance) is considered as a pre-diabetic condition and is closely related to obesity [359]. The effect of dietary fat and cholesterol on glucose
intolerance was tested by intraperitoneal glucose tolerance tests (ipGTT) [360]. Animals were fasted for 5-7 hours prior to measuring basal blood glucose and insulin levels (t = 0 min). Glucose (1.5 or 2.0 g/kg) was then administered by i.p. injection. Blood glucose levels were measured at t = 0, 15, 30, 60 and 90 min whereas blood insulin levels were measured at 0, 15, 60 and 90 min. Blood samples were collected from the tail vein for the measurement of blood glucose and insulin levels. Blood glucose levels were measured immediately using a glucometer (Accu-Chek, Roche) after the collection. For the measurement of insulin levels, blood samples were collected in heparin-coated capillary tubes (SteriHealth, #I-10086) #SRI-13K). Plasma was separated by centrifugation at a speed of 8000 × g and stored at -80°C for later determination of insulin levels using radioimmunoassay kits (Merck Millipore, #SRI-13K).

2.4 Tissue and Blood Collection

Blood and tissue samples were collected for the measurement of the effect of dietary fat and cholesterol on plasma parameters and metabolic pathways in tissues. Mice were fasted for 5-7 h before being culled. Blood samples collected from the tail vein were processed and stored as describe previously (for the measurement of insulin). Liver tissues were harvested and either put in 10% neutral buffered formalin (Sigma-Aldrich, #HT501128) for histological analysis, ice-cold buffer (pH 7.4, 250 mmol/L sucrose, 10 mmol/L Tris-HCl, and 1 mmol/L EDTA) for the measurement of palmitate oxidation, or freeze-clamped immediately for further assessment [358, 360]. Quadriceps muscle samples were collected and immediately freeze-clamped. Epididymal fat and peri-renal fat mass was weighed using an analytical balance and then freeze-clamped. All freeze-clamped tissue samples were stored at -80°C.
2.5 Measurement of Triglyceride (TG) and Cholesterol Content in Tissue

TG and cholesterol contents in the liver were measured to assess the effect of dietary fat and cholesterol on hepatic steatosis and cholesterol accumulation. Tissue samples were homogenized in a mixture of chloroform and methanol (2:1) using a glass homogeniser and placed on a shaker overnight. 0.6% NaCl was added to extract the aqueous phase. The mixture was centrifuged at 1,000 × g for 10 min and the lower organic phase was transferred to new tubes. The extract was then dried and redissolved in ethanol prior to the measurement of TG and cholesterol concentrations [358]. TG and cholesterol levels were determined by using a Triglyceride GPO-PAP kit (Roche Diagnostics, #11730711216) and a Cholesterol CHOD-PAP kit (Roche Diagnostics, #11491458216) respectively. Free cholesterol levels were measured by a Free Cholesterol Kit (Wako, # 435-35801) according to the manufacturer’s instructions.

2.6 Western Blotting

2.6.1 Sample Preparation

Tissue samples were homogenized with a pestle mixer in ice-cold RIPA buffer containing 10 μL/mL protease inhibitor cocktail (Sigma-Aldrich, #9599), 10 μL/mL phosphatase inhibitor cocktail (Sigma-Aldrich, #P5726), 10 mM NaF (Sigma-Aldrich, #S7920), 1 mM Na₃VO₄ (Sigma-Aldrich, #450243) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, #78830). Tissue lysates were then centrifuged at a speed of 20,000 × g for 15 minutes at 4°C. The protein concentrations in the supernatant were determined by bicinchoninic acid assay (Sigma-Aldrich, #B9643). Protein samples were then diluted with water and mixed with 4× Laemmli buffer to make a final concentration of 2.5 μg/μL. Finally, samples were boiled at 95°C for 5 minutes to denature the protein.
2.6.2 SDS-PAGE and Immunoblotting

Polyacrylamide gels at a concentration of 8, 10, 12 or 14%, were used for separation of proteins according to their molecular weights and then placed in a running buffer. The denatured protein samples (50 μg/well) and standard protein ladders (5 μL/well, Bio-Rad, #161-0374) were loaded to the gel and separated at 120 V until the protein ladder above or below the protein of interest were well separated. Subsequently, proteins in the gel were transferred to the PVDF membrane (Bio-Rad, #162-0177) in a transfer buffer at 100 V for 2 h. The PVDF membrane was incubated in 3% BSA (in TBST buffer, Sigma-Aldrich, #A9418) at room temperature for 1 h to block the non-specific binding and then incubated with the primary antibody overnight at 4°C. After a 1 h wash in TBST, the membrane was incubated in the horseradish peroxidase conjugate secondary antibody (Santa Cruz, #sc-2004 for rabbit, #sc-2005 for mouse) and followed by a 1 h wash in TBST. Enhanced chemiluminescent (Perkin Elmer, #NEL113001EA) was used for the detection. Densitometric analysis was performed using Image Lab software 5.0 (Bio-Rad Laboratories). The primary antibodies are listed in Table 2.3. Antibodies from Cell Signaling and Abcam were diluted 1:1000 and these from Santa Cruz were diluted 1:500, with a TBST buffer containing 1% BSA, 0.02% sodium azide (Sigma-Aldrich, #71289) and 0.0025% phenol red (Sigma-Aldrich, #32661).

Table 2.3 Antibodies

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>Name</th>
<th>Supplier</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c-Jun</td>
<td>Cell Signaling</td>
<td>9165</td>
</tr>
<tr>
<td></td>
<td>phospho-c-Jun (Ser73)</td>
<td>Cell Signaling</td>
<td>3270</td>
</tr>
<tr>
<td></td>
<td>NLRP3</td>
<td>AdipoGen</td>
<td>20B-0006-C100</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>Abcam</td>
<td>ab6640</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>TGFβ</td>
<td>Cell Signaling</td>
<td>3709</td>
</tr>
<tr>
<td>ER Stress pathway</td>
<td>eIF2α</td>
<td>Cell Signaling</td>
<td>9722</td>
</tr>
<tr>
<td></td>
<td>phospho-eIF2α (Ser51)</td>
<td>Cell Signaling</td>
<td>9721</td>
</tr>
</tbody>
</table>
## 2.7 Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

### 2.7.1 RNA Isolation

Tissues were homogenized in TRIzol Reagent (Invitrogen, #15596026) using a pestle mixer and centrifuged at 10,000 × g to remove the debris. The tissue lysates were extracted by chloroform and the RNA-containing aqueous phase was transferred to a new tube. RNA was precipitated by adding 2-propanol and centrifuged at 15,000 × g for 5 min. The RNA pellets were washed twice in 75% ethanol and dried at room temperature. Diethylpyrocarbonate-treated water (DEPC water, Invitrogen, #AM9916) was used to dissolve the pellets, and the RNA concentration was determined using a Nanodrop Spectrophotometer (Thermo Scientific). Samples were then diluted to a final concentration of 125 ng/μL for reverse transcription.
2.7.2 Reverse Transcription and Quantitative Real-Time PCR

DNA was removed using a DNase I kit (Invitrogen, Catalogue No.18068-015). 8 μL of RNA (125 ng/μL) was mixed with 1 μL of DNase I and 1 μL reaction buffer, and incubated at room temperature for 15 min. DNase I was inactivated by incubating with 1 μl of 25 mM EDTA for 10 min at 65°C. The purified RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, # 4368814). Two μL of RNA extract was mixed with 18 μL master mix (2 μL 10× RT buffer, 0.8 μL 25× dNTP, 2 μL 10× Random Primers, 1 μL reverse transcriptase and 12.2 μL DEPC water) and incubated in a thermocycler following the manufacturer’s instructions. A 10 μL reaction system containing 4.5 μL cDNA obtained from the reverse transcription, 0.25 μL of both forward and reverse primers at the concentration of 10 μM (GeneWorks, sequences in Table 2.4), and 5 μL SYBER green supermix (Bio-Rad, #170-8880) was used for the quantitative real-time PCR analyses. Reaction conditions were 50°C for 2 min, 95°C for 3 min, 40 cycles of 95°C for 15 seconds, 72°C for 30 seconds, and were followed by measurements of the melt curve using the QIAGEN Rotor-Gene Q PCR system. 18S ribosomal RNA was used as the housekeeping gene.

Table 2.4 Sequences of Primers for Quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td></td>
</tr>
<tr>
<td>TNFα forward</td>
<td>5’-CACAAGATGCTGGGACAGTGTA-3’</td>
</tr>
<tr>
<td>TNFα reverse</td>
<td>5’-TCCTTGATGCTGGTGCGATGA-3’</td>
</tr>
<tr>
<td>IL6 forward</td>
<td>5’-ATTCCAGAAACCGCTATGAGTCC-3’</td>
</tr>
<tr>
<td>IL6 reverse</td>
<td>5’-GTCACCAGCATCAGTCCCAA-3’</td>
</tr>
<tr>
<td>IL1β forward</td>
<td>5’-GACGGCATACCCACCCACT-3’</td>
</tr>
<tr>
<td>IL1β reverse</td>
<td>5’-AAACCGTTTTGCCCTGTTTCTTTT-3’</td>
</tr>
<tr>
<td>CD68 forward</td>
<td>5’-TGACCGGCTCTCTCTCTTAAAGGTCA-3’</td>
</tr>
<tr>
<td>CD68 reverse</td>
<td>5’-TCACGCGTGGCAAGAGAAACATG-3’</td>
</tr>
<tr>
<td>TLR4 forward</td>
<td>5’-GCAGAAAAATGCCAGGTGATG-3’</td>
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### Chapter 2 - General Methods

<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4 reverse</td>
<td>5’-AACTACCTCTATGCAGGGATTC-3</td>
</tr>
<tr>
<td>Collagen 1 forward</td>
<td>5’-CTGCTGGTGAGAGGTTGAAC-3</td>
</tr>
<tr>
<td>Collagen 1 reverse</td>
<td>5’-ACCAAGGTCTCCAGGAACAC-3</td>
</tr>
<tr>
<td>PGC1α forward</td>
<td>5’-AAACCTTGCTAGCGTGTCCTCA-3’</td>
</tr>
<tr>
<td>PGC1α reverse</td>
<td>5’-TGGCTGGTGCCAGTAAGAG-3’</td>
</tr>
<tr>
<td>TFAM forward</td>
<td>5’-GAAGGGAATGGGAAGGTTGA-3’</td>
</tr>
<tr>
<td>TFAM reverse</td>
<td>5’-AACAGGACATGGAAAGCAGAT-3’</td>
</tr>
<tr>
<td>NRF1 forward</td>
<td>5’-ATCCGAAGAGACAGCAGACA-3’</td>
</tr>
<tr>
<td>NRF1 reverse</td>
<td>5’-TGGAGGGTGAGATGAGATA-3’</td>
</tr>
<tr>
<td>PPARγ forward</td>
<td>5’-ACGATCTGCTGAGGTCTGT-3’</td>
</tr>
<tr>
<td>PPARγ forward</td>
<td>5’-CATCGAGGACATCCAGCAC-3’</td>
</tr>
<tr>
<td>18S forward</td>
<td>5’-CGCCGCTAGGGTAGCATACTTCT-3’</td>
</tr>
<tr>
<td>18S reverse</td>
<td>5’-CGAACCTCCGACTTTTCGTTCT-3’</td>
</tr>
</tbody>
</table>

#### 2.8 Sample Size Calculation

Based on the data from our previous reports, the body weight of mice fed a CH or HF diet with cholesterol for 14 weeks were 30.2 ± 2.6 g (mean ± SD) and 39.8 ± 5.2 g (mean ± SD), respectively [40]. The mean difference between these two groups (effect size) was calculated to be 1.5. To ensure a statistical power of 0.80 at a conventional level of α (0.05), the minimum sample size is 6 in each group for 5 groups (Chapter 3) and 8 in each group for 2 groups (Chapter 4). Based on this calculation and previous reports in the mice from the same genetic background [351-353], 7-8 mice were assigned to each group for the study in Chapter 3 and 8-10 mice were assigned for the study in Chapter 4.
CHAPTER 3

Effects of Dietary Fat and Cholesterol on Obesity, Glucose Intolerance and NAFLD
3.1 Introduction

The overall aim of this chapter was to characterise the pathologic features of obesity, glucose intolerance and NAFLD produced by a HF diet and a moderate-cholesterol (0.2% w/w) diet, alone or in combination, and the underlying mechanisms involved.

As reviewed in Section 1.1.3, obesity is closely associated with T2D, CVD and NAFLD [7, 8]. The development of obesity is an overall consequence of the interplay between genetic and environmental factors. A major cause of obesity is a positive energy balance due to high calorie intake and/or low physical activity. The excess energy is stored in the form of lipids (mostly TG) in adipose tissue and in non-adipose tissues (ectopic fat). In the liver, excessive accumulation of TG causes hepatic steatosis, which is closely associated with glucose intolerance or insulin resistance.

Hepatic steatosis is the first stage of NAFLD and a prerequisite (or first “hit”) in the development of NASH. NASH is a more severe stage of NAFLD, which requires a second “hit” to cause liver damage [56]. Inflammation, oxidative stress, ER stress, mitochondrial dysfunction and insulin resistance may be involved in the process of the damage from the second “hit” [56, 175]. Although it is clear that excess accumulation of TG in the liver from the diet or DNL causes hepatic steatosis, it remains largely unknown what may trigger these factors as a second “hit” to promote the progression from hepatic steatosis to NASH.

Overconsumption of dietary fat (particularly saturated fat) can induce obesity, insulin resistance and hepatic steatosis, but this does not necessarily cause cell or tissue damage [288]. While there is no direct evidence that dietary cholesterol can directly affect body weight (Section 1.4.2), studies in cells and animals suggest that cholesterol may contribute to the development of NASH (Section 1.4.3). For example, studies in cultured cells have indicated that cholesterol loading can induce signs of inflammation, ER stress, oxidative stress and
mitochondrial dysfunction, all of which are implicated in the development of NASH [332, 341, 346, 353]. The effect of dietary cholesterol on NASH has also been studied in animals with severe dyslipidaemia, induced either by gene mutations [335] or a high percentage of cholesterol (1% or 2% w/w) in the diet [336, 337, 348, 349]. Although egg yolks contain approximately 1% w/w cholesterol, the daily intake of cholesterol is unlikely to reach that extreme level. A typical Western diet used for research contains only 0.2% w/w cholesterol [294], and it is not clear whether this more typical level of dietary cholesterol may promote the development of NASH.

Therefore, this chapter attempted to achieve the overall aim as described above by addressing the following two specific aims. The first aim was to examine the characteristics of the pathologic features regarding obesity, glucose intolerance and hepatic steatosis produced by a HF diet and a moderate-cholesterol (0.2% w/w) diet, alone or in combination, in C57BL/6J mice. The working hypothesis for this aim was that the chronic HF diet but not moderate dietary cholesterol would induce obesity, glucose intolerance and hepatic steatosis (as the first “hit” in the development of NASH). The second aim was to investigate the effects of these diets on the development of NASH and underlying mechanisms. The working hypothesis for the second aim was that inclusion of cholesterol may promote the progression from hepatic steatosis (induced by the HF diet) towards NASH by inducing to oxidative stress, ER stress, inflammation or mitochondrial dysfunction in the liver.

### 3.2 Study Design and Methods

#### 3.2.1 Study Design

All experiments were approved by the RMIT University Animal Ethics Committee (#1012). Male C57BL/6J mice (10 weeks old) were purchased and housed as described in Section 2.1.
After acclimatisation, the mice were fed either a CH (Table 2.2), a CH diet supplemented with 0.2% cholesterol (CHC), a HF diet (Table 2.2) or a HF diet supplemented with 0.2% cholesterol (HFC) diet, ad libitum for 17 weeks. From week 13 to week 17, cholesterol was withdrawn from the diet of one of the HFC sub-groups for 5 weeks (HFC-HF). CH, CHC, HF and HFC diets were prepared as previously described in Section 2.2. All of the diets were stored at -20°C for less than one month, and were changed daily during the experiments.

### 3.2.2 Assessment of Obesity and Glucose Intolerance

The effects of dietary fat and cholesterol on obesity were assessed by measuring body weight and the weight of epididymal and peri-renal fat depots using a standard analytical balance.

Obesity can be caused by increased energy intake and/or reduced energy expenditure. After 5 weeks of feeding, the whole-body energy metabolism of mice was measured at 22°C using an indirect calorimeter (Comprehensive Laboratory Animal Monitoring System or CLAMS, Columbus Instruments) [360]. Animals were weighed and placed in the metabolic chamber (20 cm × 10 cm × 12.5 cm) at 6:00 p.m. After overnight acclimatisation, oxygen consumption (VO₂) and respiratory exchange ratio (RER) were monitored continuously for 24 hours according to the manufacturer’s instructions. During the experiment, mice had ad libitum access to food and water.

An ipGTT was performed in week 1, 9 and 12 as described in Section 2.3 (glucose dosage 2.0 g/kg for week 1, 1.5 g/kg for week 9 and 12). The dose of glucose in ipGTT was reduced after week 1 to avoid the blood glucose levels exceeding the measurement range of glucometer. Plasma insulin levels were measured at 0, 15, 60, and 90 min during the GTT in week 12. TG and total cholesterol levels in the plasma were measured at week 8 using Triglyceride GPO-PAP and Cholesterol CHOD-PAP kits (Roche Diagnostics, #11730711216 and #11491458216) [39]. Plasma levels of HDL and LDL/VLDL cholesterol were measured at week 17.
using a commercial assay kit following the manufacturer’s instructions (Sigma-Aldrich, #MAK045-1KT). Briefly, LDL/VLDL was precipitated from the plasma and separated from HDL. The concentrations of these two fractions were quantified by measuring their cholesterol content.

### 3.2.3 Assessment of Hepatic Steatosis

The effects of dietary fat and cholesterol on hepatic steatosis were assessed by determining the TG content in the liver as described in Section 2.5. Along with this, the content of both total and free cholesterol in the liver was also determined (Section 2.5).

Haematoxylin and eosin (H&E) staining was performed to evaluate morphological changes. For this measurement, liver tissues stored in 10% neutral buffered formalin were dehydrated using a Leica tissue processor (Leica, #ASP200 S). The dehydrated samples were embedded in paraffin, and cut into 5-μm-thick sections. These sections were put into warm water and then transferred to glass slides. The slides were dried overnight at 37 ºC. Before H&E staining, all slides were deparaffinised by immersing in xylene twice for 3 min each. Xylene was then removed by washes in progressively lower concentrations of ethanol for 1 min: 100%, 90%, 80% and 70% ethanol before a final wash with tap water for 2 min. The slides were then stained in Mayer’s haematoxylin (Grale Scientific, #MH-1L) for 2 min. After 5 dips in tap water, the slides were put into Scott’s blueing solution (Grale HDS, #SCOT-1L) for 1 min to blue the haematoxylin. Subsequently slides were washed in tap water for 2 min and then counterstained in 1% aqueous eosin (Grale HDS, #EOA-1L) for 1.5 min. Slides were then washed in tap water for 2 min followed by dehydration using a gradient of ethanol (70% ethanol, 80%, 90% and 100%, wash 1 min each) and xylene. Stained samples were covered with coverslips using mounting medium (Grale Scientific Pty. Ltd., #3197). Images of the sections were taken by an Olympus microscope (#BX41) equipped with a digital camera (#DP72).
The assessment of lipid droplets was based on the assumption that the vacuoles in the H&E staining were lipid droplets as previously validated using Oil Red O staining [171, 361, 362]. For each mouse, images of 6 random fields (200× magnification) were taken and the numbers of enlarged lipid droplets (defined as ≥ 25 µm) were quantified by a laboratory member blinded to the treatment groups for statistical analysis.

3.2.4 Assessment of Parameters Indicative of NASH

The characteristics of NASH distinguishing from simple hepatic steatosis include the presence of inflammation, liver injury with or without fibrosis [163] (Table 1.4). Liver injury was assessed by measuring the plasma levels of ALT using an ALT/SGPT Liqui-UV Kit (Stanbio, #ST2930-500). Briefly, 20 µL of plasma was mixed with 200 µL of reagent as described in the manufacturer’s instructions and absorbance at 340 nM was measured every minute for 10 min using a FlexStation (Molecular Devices, USA). ALT activity was calculated from the slope of the absorption curve.

Inflammatory markers in the liver were measured either by qRT-PCR [TNFα, IL1β, monocyte chemoattractant protein 1 (MCP1), the cluster of differentiation 68 (CD68), TLR4 and IRF3] as described in Section 2.7 or western blotting (phospho-cJUN, total-cJUN, F4/80 and NLRP3) as described in Section 2.6.

Fibrosis in the liver was assessed by Masson’s trichrome staining [363] and sirius red/fast green staining [364]. The liver samples were processed and deparaffinised as described above (H&E staining). For Masson’s trichrome staining, Bouin's solution (Sigma-Aldrich, # HT10132-1L) was used to mordant the sections overnight and the yellow colour was washed off with distilled water. The nuclei were stained blue by immersing in Weigert's iron hematoxylin solution (Sigma-Aldrich, # HT1079-1SET) for 5 min. The slides were washed in tap water for 5 min and rinsed in distilled water. Biebrich scarlet-Acid fuchsin solution
(Sigma-Aldrich, #HT15) was then used to stain the cytoplasm red (5 min) prior to rinsing the slides in distilled water. Before the aniline blue stain, the samples were pre-treated in the 1:1 mixture of phosphomolybdic acid solution (Sigma-Aldrich, #HT15) and phosphotungstic acid solution (Sigma-Aldrich, #HT15) for 10 min. Next, the slides were placed in aniline blue solution (Sigma-Aldrich, #B8563) for 5 minutes to stain the collagen blue and rinsed briefly in distilled water. Finally, the slides were left in 1% acetic acid solution for 3 minutes, and dehydrated and covered with coverslips as described above (H&E staining).

For sirius red/fast green staining, the deparaffinised sections were incubated in a 1.2% picric acid solution (Sigma-Aldrich, #197378) containing 0.1% sirius red (Sigma-Aldrich, #365548), 0.1% fast green (Sigma-Aldrich, #F7252) for 1 hour. The slides were then rinsed by 10 dips in distilled water. Sections were dehydrated and covered with coverslips as described above (H&E staining).

Collagen 1 expression in the liver of was assessed by qRT-PCR (Section 2.7) and the content of TGFβ, which has been known to promote fibrogenesis, was measured by western blotting (Section 2.6).

3.2.5 Measurement of Oxidative Stress and ER Stress Markers, and Mitochondrial Function

The effect of dietary fat and cholesterol on oxidative stress was assessed by measuring the content of oxidation products during oxidative stress and the activity of SOD, which is an enzyme that can catalyse the dismutation of superoxide to prevent the cells from oxidative stress. Protein carbonyl content was measured using a protein carbonyl content assay kit (Abcam, #ab126287) according to the manufacturer’s instructions. Glutathione (GSH)/GSSG disulphide (GSSG, the oxidated product of GSH) ratio and malondialdehyde (MDA) levels
were determined using commercial kits from Cayman (#703002, # 10009055). SOD activity was measured using a SOD activity kit from Enzo Life Sciences (# ADI-900-157).

ER stress markers (CHOP, GRP78, phospho-eIF2α, total-eIF2α, phospho-IRE1 and total-IRE1) were measured by western blotting (Section 2.6) [360].

The ability of the liver to oxidise FAs was evaluated by measuring the oxidation of 14C-labelled palmitate ex vivo using liver homogenate as reported previously [360]. Liver tissues were collected from mice, and stored in an ice-cold isolation buffer (pH 7.4, 250 mM sucrose, 10 mM Tris-HCl and 1 mM EGTA). The liver homogenate was prepared using a glass Dounce homogenizer with a loose-fit pestle, and then incubated at 30°C for 90 min in a reaction buffer (pH 7.4, 100 mM sucrose, 80 mM KCl, 10 mM Tris-HCl, 5 mM KH2PO4, 1 mM MgCl2, 2 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.2 mM EDTA, 0.3% FA–free BSA, 2 mM L-carnitine, 0.05 mM coenzyme A and 0.2 mM [1-14C]-palmitate (0.5 μCi/ml)). A tube containing 1 M NaOH was placed in each vial to capture the CO2 produced from the reaction. 1 M perchloric acid was used to stop the reaction. Palmitate oxidation rates were determined by counting the 14C radioactivity of captured CO2 and acid-soluble metabolites [360].

In addition, the mRNA expression of transcription factors involved in mitochondrial biogenesis (TFAM and PGC1α) was measured by qRT-PCR (Section 2.7). The upstream regulators of these transcription factors, phosphorylation of AMPK and protein levels of sirtuin 1 (SIRT1), were measured by western blotting (Section 2.6). The effects of dietary fat and cholesterol on the lipogenic pathway were evaluated by western blotting for the key enzymes ACC and stearoyl-CoA desaturase-1 (SCD1).
3.2.6 Statistical Analyses

Data were expressed as means ± SE. Results were evaluated for their distribution patterns prior to the statistical tests. One-way ANOVA was used for the comparison of normal distributed data (SPSS statistics 23). When significant differences were found, a Tukey multiple comparisons test was applied. Non-normal distributed data (including total and free cholesterol levels in the liver, mRNA expression of IL1β, CD68, collagen 1, IRF3 and MCP1) were compared using a nonparametric test for independent samples (SPSS statistics 23). Differences at $p < 0.05$ were considered statistically significant.

3.3 Results

3.3.1 Effects of Dietary Fat and Cholesterol on Obesity and Plasma Lipids

Mice fed the HF diet had a greater body weight ($p < 0.01$) and adipose tissue weight compared with CH-fed mice (Table 3.1), although their liver weight was not different from CH-fed mice. Inclusion of cholesterol in either the CH or HF diet did not have any additional effects on body weight or adiposity. Switching from the HFC diet to HF diet did not affect the body weight (Figure 3.1). HF diet alone increased plasma levels of total cholesterol by 45 ± 15% ($p < 0.01$, vs. CH group), but did not have any significant effect on the levels of TG, LDL/VLDL cholesterol or HDL cholesterol. Although the plasma levels of LDL/VLDL cholesterol in HFC mice were higher than in the CH group, the addition of cholesterol to either the CH diet or HF diet had no additional effect on plasma levels of TG, total cholesterol, LDL/VLDL and HDL cholesterol (all $p > 0.05$, vs. the corresponding group without cholesterol).
### Table 3.1 Effects of Dietary Fat and Cholesterol on Obesity and Plasma Lipids

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>CHC</th>
<th>HF</th>
<th>HFC</th>
<th>HFC-HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting body weight (g)</td>
<td>23.6±0.4</td>
<td>22.9±0.3</td>
<td>23.5±0.7</td>
<td>24.7±0.4</td>
<td>24.7±0.3</td>
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<tr>
<td>Final body weight (g)</td>
<td>31.7 ± 0.7</td>
<td>30.1±0.7</td>
<td>38.7±1.3</td>
<td>36.1±0.9</td>
<td>36.0±0.6</td>
</tr>
<tr>
<td>Caloric intake (kcal/mouse)</td>
<td>1354±20</td>
<td>1326±37</td>
<td>1802±37</td>
<td>1728±31</td>
<td>1744±29</td>
</tr>
<tr>
<td>Plasma TG (mg/dL)</td>
<td>8.4±0.7</td>
<td>8.6±0.4</td>
<td>10.4±0.6</td>
<td>11.4±0.6</td>
<td>#</td>
</tr>
<tr>
<td>Plasma Cholesterol (mg/dL)</td>
<td>87.7±2.8</td>
<td>94.9±6.5</td>
<td>157.7±13.3</td>
<td>169.9±11.0</td>
<td>n.d</td>
</tr>
<tr>
<td>Plasma LDL/VLDL (mg/dL)</td>
<td>15.9±1.4</td>
<td>16.8±1.2</td>
<td>18.6±1.4</td>
<td>20.5±1.9</td>
<td>n.d</td>
</tr>
<tr>
<td>Plasma HDL (mg/dL)</td>
<td>89.7±3.9</td>
<td>76.7±6.5</td>
<td>91.1±2.0</td>
<td>91.2±2.3</td>
<td>n.d</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
<td>1.2±0.1</td>
<td>1.2±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Epididymal fat (BW%)</td>
<td>1.7±0.1</td>
<td>1.5±0.2</td>
<td>5.3±0.5</td>
<td>5.1±0.4</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>Peri-renal fat (BW%)</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td>1.8±0.2</td>
<td>1.6±0.1</td>
<td>1.8±0.1</td>
</tr>
</tbody>
</table>

Male C57BL/6J mice were fed chow (CH), CH with 0.2% cholesterol (CHC), high-fat (HF) or HF with 0.2% cholesterol (HFC) diets for 17 weeks. The HFC-HF group was fed a HFC diet for 12 weeks followed by 5 weeks of HF feeding. TG and total cholesterol levels were measured at week 8, and HDL and LDL/VLDL cholesterol levels were measured at week 17. n = 7-8/group, # p < 0.05, ## p < 0.01 vs. the CH group. n.d.: not determined.
Figure 3.1 Change in Body Weight after Removal of Cholesterol.

Mice were fed a HF with 0.2% w/w cholesterol (HFC) diet for 12 weeks (n = 8). One subgroup was switched to a HF only (HFC-HF) while the other remained on the HFC diet (HFC) (n = 8).
3.3.2 Effects on Energy Expenditure

VO$_2$ has been used as an indication of whole-body energy expenditure [365]. The measurements of VO$_2$, RER and physical exercise were conducted in week 5. According to another study from our group (unpublished data), the lean mass of CH mice and HFC mice under similar conditions (on 5-week CH or HFC diet) was not significantly different (25.4 ± 0.7 g vs. 23.9 ± 0.6 g). Therefore, the data was analysed using absolute VO$_2$ [366]. HF feeding did not induce significant changes in absolute VO$_2$ of mice during the daytime, night time or over a 24 h period (all $p > 0.1$, Figure 3.2A). However, RER values of HF-fed mice were always significantly lower, regardless of the time of day, than the CH control group (all $p < 0.01$, Figure 3.2B). This indicates that mice fed the HF diet relied on fat as a major energy source. HF feeding did not significantly change the pattern of physical activity in mice (Figure 3.2C). The addition of cholesterol in either the CH or HF diet did not affect the VO$_2$, RER or physical activity of the mice.
Figure 3.2 Effects of Dietary Fat and Cholesterol on Metabolic Parameters.

Mice were fed a chow (CH), chow with 0.2% w/w cholesterol (CHC), HF or HF with 0.2% w/w cholesterol (HFC) diet for 5 weeks. (A) The absolute oxygen consumption (VO₂, in mL/h/mouse); (B) The respiratory exchange ratio (RER) of the mice was calculated using the ratio of VO₂ to VCO₂; (C) The physical activity of mice was monitored using infrared photocell technology. n = 7-8/group, ## p < 0.01 vs. CH group.
3.3.3 Effects on Glucose Tolerance

A GTT primarily measures the disappearance of blood glucose after the administration of a single dose of glucose. The GTT together with measurement of plasma insulin levels can be used to indicate whole-body glucose tolerance and insulin secretory response. In the present study, ipGTT was performed after 1, 9 and 12 weeks of feeding respectively to evaluate the glucose tolerance of the mice and insulin levels during GTT were measured at week 12.

As shown in Figure 3.3A, HF mice exhibited glucose intolerance after one week of feeding compared to the CH mice, as indicated by higher blood glucose levels at 30, 60, 90 mins after the administration of glucose and a greater incremental area under the curve (iAUC) compared with the CH group. Addition of cholesterol to either CH or HF diet did not affect glucose tolerance. The impaired glucose tolerance of HF mice persisted to week 9 compared with the CH group (Figure 3.3B), as indicated by iAUC values increased to twice that of the CH group ($p < 0.01$). Interestingly, although the total iAUC value of the HFC group was not significantly different from the HF group, the addition of cholesterol to the HF diet did show significantly decreased glucose levels (by $20 \pm 5\%$ reduction) measured at the 60 min time point ($p < 0.01$ vs. HF). By week 12, the iAUC of the HF group was approximately 4-fold that of the CH group (Figure 3.3C). Additionally, at this time the HF group had a 4-fold increase in basal insulin levels and noticeable increases in insulin levels during the GTT, compared to the CH group (Figure 3.3D). Blood glucose iAUC × plasma insulin (15–90 min average) was calculated as an indication of whole-body insulin resistance (Figure 3.3D) [32]. This value of HF group was significantly higher than CH group. These data indicated that HF-fed mice had whole-body insulin resistance.

However, dietary cholesterol attenuated HF-induced glucose intolerance, as indicated by a 35 ± 10% reduction in iAUC ($p < 0.01$). This is associated with reduced insulin levels during the GTT at 60 min and 90 min ($p < 0.05$). When these data were expressed as blood glucose
iAUC × plasma insulin (15–90 min average), HFC group had significantly lower values than HF group, suggesting better whole-body insulin sensitivity in the HFC group. However, this effect of cholesterol was not evident when it was added to the CH diet.

Figure 3.3 Effects on Glucose Tolerance.

The CH, CHC, HF and HFC groups were fed their corresponding diet for 17 weeks. (A) Blood glucose levels of mice during a glucose tolerance test (GTT) after 1 week of feeding
(dosage of glucose: 2.0 g/kg) and the incremental area under the curve (iAUC); (B) Blood glucose levels of mice during a GTT at 9 weeks (dosage of glucose: 1.5 g/kg) and the iAUC; (C) Blood glucose levels of mice during a GTT at 12 weeks (dosage of glucose: 1.5 g/kg) and the iAUC; (D) Plasma insulin levels during the a GTT at week 12, and iAUC of GTT × plasma insulin (15–90 min average) was calculated as an indication of insulin resistance [32]. n = 7-8/group, # p < 0.05, ## p < 0.01 vs. the CH group, * p < 0.05, ** p < 0.01 vs. the HF group.

3.3.4 Effects on Levels of TG and Cholesterol, and Histology of the Liver

HF feeding alone increased liver TG levels to approximately 3-fold that of the CH-fed mice while it did not affect cholesterol content (Figure 3.4A). In comparison, feeding with 0.2% w/w cholesterol alone in the CH diet did not show any significant effects on either TG or cholesterol content in the liver. Interestingly, the combination of cholesterol with the HF diet increased the hepatic levels of total cholesterol (1.9-fold, p < 0.01) and free cholesterol (1.6-fold, p < 0.01), although there was no additional effect on TG content in the liver compared with HF feeding alone. After cholesterol was removed from the HF diet, both total and free cholesterol levels returned to the similar level to that of HF-fed mice (p > 0.05, HFC-HFC vs. HF).

To assess concurrent histological changes, H&E stained sections of the liver were examined. Inclusion of cholesterol in the CH diet did not reveal any sign of hepatic steatosis, as indicated by similar TG content and histological appearance (Figure 3.4A&B). HF alone induced moderate microvesicular steatosis within hepatocytes, while HFC induced both microvesicular and macrovesicular steatosis. Based on validations with Oil Red O staining [171, 361, 362], the vacuoles (≥ 25 µm, indicated by arrows) in H&E were quantified to represent lipid droplets. The number of enlarged lipid droplets was significantly increased in the HFC group compared with the HF group (Figure 3.4B). Removal of cholesterol from the
HFC diet reduced the severity of the microvesicular and macrovesicular steatosis but did not completely eliminate the enlarged lipid droplets.
Figure 3.4 Effects on Liver TG and Cholesterol Levels.

The CH, CHC, HF and HFC groups were fed their corresponding diet for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks followed by 5 weeks of HF feeding alone. (A) TG, cholesterol and free cholesterol contents in liver tissues. (B) Representative images of H&E staining of liver sections. The numbers of large lipid droplets (≥ 25 µm in diameter) in 6 random fields (200×) per mouse were quantified for statistical analysis. Arrows indicate the shape of lipid droplets. n = 7-8/group, * p < 0.05, ** p < 0.01 vs. indicated group, ## p < 0.01 vs. CH group.

3.3.5 Effects on Plasma Alanine Aminotransferase and Hepatic Inflammation

To examine the effects of dietary cholesterol on liver damage and inflammation (key features of NASH), plasma ALT levels (as an indicator of liver damage) and the mRNA expression of inflammatory markers were measured. As shown in Figure 3.5A, results showed that either HF or CHC feeding alone did not affect the plasma levels of ALT but the combination of cholesterol and the HF diet significantly increased plasma ALT levels (~1.8-fold, p < 0.01). Removal of cholesterol from the HFC diet attenuated ALT levels.

Consistent with the unchanged plasma levels of ALT, neither HF nor CHC altered the mRNA expression of the inflammatory cytokines TNFα, IL1β, MCP1 or CD68 (Figure 3.5B). The combination of cholesterol and the HF diet induced an approximately 2-fold increase in mRNA expression of TNFα, IL1β, CD68 and MCP1 compared with the HF diet alone. In line with the increased mRNA expression of inflammatory cytokines, western blotting indicated that cholesterol in the HF diet increased the level of F4/80, a marker of macrophage infiltration, and phosphorylation of cJUN, a downstream target of TNFα activation (Figure 3.5C). Interestingly, these changes in inflammatory markers were accompanied by increases in the mRNA expression of TLR4 and IRF3 (a downstream target of TLR4), as well as the protein level of NLRP3 inflammasome (Figure 3.5D,E). The withdrawal of cholesterol from
the HFC diet effectively attenuated the above effects with the exception of NLRP3. Mice fed the CHC diet did not have any change on inflammatory markers or the metabolic parameters compared to the CH control group, suggesting that the effects of cholesterol were evident only in the HF diet.
Chapter 3 - Effects of Fat and Cholesterol on Obesity, Glucose Intolerance and NAFLD
Figure 3.5 Effects on Plasma Alanine Aminotransferase and Hepatic Inflammation.

The CH, CHC, HF and HFC groups were fed their corresponding diet for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks, followed by 5 weeks of HF feeding. (A) At the end of the study, plasma samples were collected for the measurement of alanine aminotransferase (ALT) levels. (B) mRNA expression levels of inflammatory markers were determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). (C) F4/80 and phospho-cJUN protein levels were measured by western blotting and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (D) Nod-like receptor pyrin containing 3 (NLRP3) protein levels were determined by western blotting and normalized by GAPDH. (E) TLR4 and IRF3 mRNA expression levels were measured by qRT-PCR. n = 7-8/group, * p < 0.05, ** p < 0.01 vs. indicated group, # p < 0.05, ## p < 0.01 vs. CH group.
3.3.6 Effects on Hepatic Fibrosis

NASH can be present with different grades of fibrosis ranging from no fibrosis to a significant degree of fibrosis [169]. Masson's trichrome staining failed to detect any sign of changes in collagen deposition, which might be indicative of fibrosis, in any of the CHC, HF or HFC group (Figure 3.6A). Sirius red/fast green staining showed that while the collagen deposition in the wall of blood vessels was clearly observed (indicated by arrows) as expected, there was no observable staining of fibrosis in the parenchyma area in any of these groups.

Further assays were conducted to examine other relevant markers associated with the fibrogenic pathway as shown in Figure 3.6B. The results showed that cholesterol feeding alone (CHC group) markedly increased mRNA expression of collagen I, and a similar trend was observed in HFC mice. In contrast, HF alone had no effect on the mRNA expression of collagen 1, and removal of cholesterol reduced the elevated collagen I mRNA levels in HFC mice. Although there was a trend of increase in the content of TGFβ (a profibrogenic cytokine [367]) in the CHC group, no apparent change was observed in other treatment groups.
Figure 3.6 Effects on Hepatic Fibrosis.

The CH, CHC, HF and HFC groups were each fed their corresponding diet for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks, followed by 5 weeks of HF feeding. (A) Representative images (200×) of Masson’s trichrome and sirius red/fast green staining of liver sections. Arrows indicate the staining of collagen in the wall of blood vessels as a positive control. (B) Collagen I mRNA expression levels were measured by qRT-PCR and transforming growth factor-β (TGFβ) contents were measured by western blotting and normalized by GAPDH. n = 7-8/group, ## p < 0.01 vs. CH group.
3.3.7 Effects on Oxidative Stress and ER Stress in the Liver

As oxidative stress and ER stress have also been suggested to be involved in the progression from hepatic steatosis to NASH [175] (Sections 1.2.4.2&1.2.4.3), the present study next measured biochemical markers for oxidative stress and levels of key proteins involved in the ER stress pathway. Dietary fat or cholesterol did not affect the content or activity of oxidative stress markers, such as protein carbonyls, GSH, MDA content or SOD activity in the liver (Figure 3.7A). There were no significant changes in ER stress markers including protein levels of phosphor-eIF2α CHOP and GRP78, except for a mild suppression of IRE1 phosphorylation in the HFC group (Figure 3.7B).
Figure 3.7 Effects on Oxidative Stress and ER Stress Pathway in the Liver.

The CH, CHC, HF and HFC group were fed their corresponding diet for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks, followed by 5 weeks of HF feeding. (A) Protein carbonyl, glutathione/glutathione disulphide (GSH/GSSG), malondialdehyde (MDA) content and SOD activity were determined using a commercial assay kit. (B) The protein levels of ER stress markers were measured by western blotting and normalized by GAPDH. n = 7-8/group, * p < 0.05 vs. indicated group, # p < 0.05 vs. CH group.
3.3.8 Effects on Lipid Metabolism and Markers of Mitochondrial Function in the Liver

The effect of cholesterol on FA oxidation in liver tissue was examined to determine any potential effects on mitochondrial function. HF feeding by itself significantly increased palmitate oxidation (~ 2-fold vs. CH, \( p < 0.01 \), Figure 3.8A) as expected. However, this compensatory increase was blunted by the addition of cholesterol to the HF diet (HFC vs. HF \( p < 0.05 \), but HFC vs. CH \( p > 0.05 \)), suggesting that blunted mitochondrial function in the liver of the HFC-fed mice. Consistent with this possibility, the HF-induced increases in PGC1\( \alpha \) and mitochondrial transcription factor A (TFAM) expression (all \( p < 0.05 \) vs. CH) diminished in diets supplemented with cholesterol (Figure 3.8B). These changes were independent of the SIRT1/AMPK pathway (Figure 3.8C), a mechanism that has been suggested to regulate FA oxidation and PGC1\( \alpha \) expression [368]. In addition, the content of SCD1, which plays an important role in lipogenesis by converting SFAs to MUFAs [369], was measured. Addition of cholesterol to both the CH and HF diet significantly increased the protein level of SCD1 in the liver (Figure 3.8D). The removal of cholesterol from the HFC diet for 5 weeks returned SCD1 content to those observed in the HF-fed mice.
Figure 3.8 Effects on Liver Mitochondrial Phenotype.

The CH, CHC, HF and HFC groups were fed their respective diet for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks, followed by 5 weeks of HF feeding. (A) FA oxidation in isolated fresh liver homogenates was measured by incubation with 0.5 μCi \([^{14}C]\)-palmitate (n = 5-7/group), ND: not determined (tissue not available due to an incident); (B) PGC1α and mitochondrial transcription factor A (TFAM) mRNA expressions were measured by qRT-PCR. (C) Protein contents of sirtuin 1 (SIRT1) and the phosphorylation of AMP-activated protein kinase (AMPK) and ACC were measured by western blotting. (D) Stearoyl-CoA desaturase-1 (SCD1) content was measured by western blotting and normalized by GAPDH. n = 7-8/group, * p < 0.05, ** p < 0.01 vs. indicated group, # p < 0.05, ## p < 0.01 vs. CH group.

3.4 Discussion

The first aim of this chapter was to examine the effects of the CH, CHC, HF and HFC diet on obesity, glucose intolerance and hepatic steatosis. As hypothesised based on published reports in the literature including our own previous studies [32, 39], the results showed that the HF diet induces obesity, glucose intolerance and hepatic steatosis. These effects are indicated by the increased adiposity, impaired glucose tolerance in the ipGTT and the deposition of TG in the liver by HF feeding alone. The other clear finding was that a typical level (0.2% w/w) of dietary cholesterol, either in the CH or HF diet, did not have any significant effect on adiposity and hepatic steatosis. Interestingly, addition of cholesterol to the HF diet was found to ameliorate the HF-induced glucose intolerance.

The second aim of this chapter was to investigate the effects of the CH, CHC, HF, HFC diet on the development of NASH concurrent with the aforementioned metabolic phenotypes and underlying mechanisms. The results showed that neither the HF diet alone nor cholesterol in the CH diet increased plasma levels of ALT or hepatic inflammatory markers. However, the inclusion of 0.2% w/w cholesterol in the HF diet increased the levels of ALT in the plasma
and inflammatory markers in the liver without apparent effects on oxidative stress and ER stress. These findings suggest that 0.2% w/w cholesterol may play a crucial role in the progression from simple steatosis towards NASH as a second “hit”. Associated with these NASH-like phenotypes in the HFC-fed mice was the blunted mitochondrial function as indicated by inhibited FA oxidation and PGC1α expression in the liver. When cholesterol was removed from the HFC diet, the elevated plasma ALT and inflammatory markers were returned to normal levels. This further suggests the role of cholesterol in the progression from hepatic steatosis towards NASH.

3.4.1 Effects on Obesity, Glucose Intolerance and Associated Metabolism

Although overconsumption of dietary fat may induce obesity, there is no clear evidence to indicate whether cholesterol intake may affect energy balance (Section 1.4.2). Consistent with our previous reports [32, 39], HF feeding increased body weight, adipose tissue mass and TG content in the liver (signs of hepatic steatosis). However, the present study showed 0.2% w/w cholesterol feeding did not have any significant effect on adiposity and hepatic steatosis either in CH-fed or HF-fed mice.

Animal studies have led to contradictory results regarding the effect of dietary cholesterol on hepatic steatosis as reviewed in the Section 1.4.3.1 [328-330], suggesting that this effect of dietary cholesterol may depend on its concentration and/or the species of experimental animals. In one of these studies, 1% cholesterol feeding has been shown to induce hepatic steatosis in mice, which is associated with a reduction of TG level in the plasma [330]. In the present study, inclusion of 0.2% cholesterol did not show significant effects on hepatic steatosis and plasma TG. This appears to suggest that the effect of cholesterol on hepatic steatosis may be influenced by the flux of lipid between the liver and plasma.
In the present study, the data of energy expenditure was expressed by VO\textsubscript{2} per mouse. Ideally, VO\textsubscript{2} should be adjusted by lean mass of animals but the lean mass could not be obtained due to the lack of a measuring instrument at that time. More recent results from our group showed that the lean mass of HFC-fed mice under similar conditions (5 weeks of HFC feeding) to the present study was not significantly different from CH-fed mice (25.4 ± 0.7 g vs. 23.9 ± 0.6 g, \( p > 0.05 \)). This is consistent with previous reports showing that 4 to 8 weeks of HF feeding does not significantly change the lean mass of C57BL/6 mice [370-372]. These data suggest the expression of VO\textsubscript{2} per mouse may be used as an estimate but there is a need for further confirmation by the measurement of both lean mass and VO\textsubscript{2} in the same cohort of mice to provide a more precise analysis of energy metabolism.

The result of GTT are affected by are insulin secretion and whole-body insulin sensitivity of animals. HF mice displayed glucose intolerance even though plasma levels of insulin were significantly elevated during the GTT compared to CH mice, indicating the presence of whole-body insulin resistance. These findings are consistent with the reported insulin resistance in HF-fed mice as determined by the hyperinsulinaemic-euglycaemic clamp [373]. So far, there is few report on the effect of dietary cholesterol on glucose tolerance. Interestingly, the present study showed that dietary cholesterol ameliorated the glucose intolerance induced by the HF diet and reduced plasma insulin levels during GTT, despite the fact that the whole-body metabolic rate and adiposity remained unchanged. This suggests that moderate level of cholesterol may attenuate the HF diet-induce whole-body insulin resistance.

Although glucose intolerance may be considered as an indication of whole-body insulin resistance when measured insulin levels during the GTT are unaffected (or higher) than controls, it does not provide information for the assessment of tissue-specific insulin sensitivity. In addition, hepatic glucose uptake contributes very little to the disposal of glucose load introduced by i.p. injection [374]. Given that insulin resistance may have a close
relationship with inflammation and mitochondrial dysfunction [375, 376], the increased inflammatory markers and blunted mitochondrial function observed in the liver suggest that the ameliorated whole-body insulin resistance induced by cholesterol in the HF diet may not result from the liver. Because the major tissue responsible for glucose disposal during ipGTT is skeletal muscle, changes in insulin sensitivity of this tissue need to be further investigated in future studies using hyperinsulinaemic-euglycaemic clamp, which is regarded as the “gold standard” technique for the assessment of insulin sensitivity.

The major causes of impaired insulin action by lipids are via their bioactive intermediates such as ceramide and DAG, rather than TG itself [55]. It has been suggested that sequestration of these lipid intermediates within lipid droplets in the cytosol can mitigate their effects on insulin signalling [39, 373]. The results in the present study showed that addition of cholesterol to the HF diet enlarged lipid droplets in the liver. Although muscle tissue was not examined due to time constraints, a similar mechanism has been observed in muscle by overexpression of perilipin 2, a crucial lipid-droplet coating protein in skeletal muscle [377]. Future studies are required to investigate whether the enlarged lipid droplets from cholesterol treatment is related to the amelioration of glucose tolerance reported here, perhaps by sequestering such detrimental lipid intermediates and thus reducing their free concentration in the cytosol.

One interesting observation in the present study was that cholesterol significantly increased the protein level of SCD1 in the liver as previously reported [378]. As a key enzyme to catalyse the conversion from SFAs to MUFAs, SCD1 has been linked to insulin sensitivity. Although the exact role of SCD1 in insulin sensitivity is still debated [379, 380], reduced hepatic SCD1 activity has been found in obese humans with hepatic steatosis and insulin resistance [369]. As MUFA is not as harmful as SFA on insulin action as reviewed in Section 1.3.2 [285], it is reasonable to speculate that increased SCD1 may attenuate insulin resistance.
by converting detrimental SFAs to MUFAs. It has been shown that SCD1 protected myotubes from SFA-induced insulin resistance by attenuating ceramide and DAG accumulation [381]. Future studies should investigate whether cholesterol also increases SCD1 content in skeletal muscle and reduces SFA-associated metabolites such as ceramide, resulting in the alleviation of glucose intolerance in HFC-fed mice observed in the present study.

It has been suggested that the absorption of dietary cholesterol requires the presence of dietary fat, which may act as a vehicle for cholesterol transport from the intestine into the blood circulation [313]. This could explain why dietary cholesterol in the low-fat CH diet did not increase the cholesterol levels in the liver or induce any metabolic effects. Interestingly, cholesterol in the HF diet only increased the cholesterol level in the liver but did not further exacerbate HF-induced hypercholesterolemia. This is consistent with clinical observations that dietary cholesterol does not always cause hypercholesterolemia [382]. It has been suggested that differences in the blood cholesterol level in response to dietary cholesterol are mainly due to the regulation of cholesterol synthesis, bile acid synthesis and cholesterol reverse transport by the liver (as reviewed in Section 1.4.1) [383]. The unchanged plasma levels of cholesterol, despite addition of cholesterol to the HF diet in the present study, may be due to the fact that the liver stores this “excess” cholesterol, which is supported by the observation of increased cholesterol accumulation in this organ in HFC group.

In summary, the results in first part of this chapter (for the first aim) suggest that the HF diet increases adiposity, induces hepatic steatosis and impairs whole-body insulin sensitivity in C57BL6/J mice. The inclusion of dietary cholesterol (0.2%) does not exacerbate HF-induced obesity, hepatic steatosis or whole-body insulin resistance.
3.4.2 Effects on the Development of NASH

NASH is considered as a key turning point in the progress of NAFLD in that it can lead to irreversible cirrhosis, a form of permanent liver damage [56]. The characteristics of NASH distinguishing from simple hepatic steatosis include the presence of inflammation, liver injury with or without fibrosis [163] (Table 1.4). Although the HF diet induced adiposity, glucose intolerance and hepatic steatosis, the results in the present study showed that this HF feeding alone was not sufficient to cause significant signs of inflammation or injury in the liver. TG accumulation per se without elevation of lipotoxic intermediates can be harmless, which is consistent with the clinical evidence that a majority of patients with hepatic steatosis do not go on to develop NASH [288].

Several previous studies have reported the effect of HF diets on hepatic inflammation in mice [384-388]. Careful analyses revealed that there were significant differences in the composition of the HF diets and the duration of feeding in these studies. For example, one study found hepatic inflammation in HF-fed C57BL/6J mice, but the percentage of fat as calories was higher (72 kcal%) and no carbohydrate (< 1%) was included in the diet. This resulted in 0.5-1 fold increases in pro-inflammatory cytokines within 4 weeks [385]. However, another study suggested that C57BL/6J mice fed a 60 kcal% HF diet for 12 weeks did not show an increased macrophage content, or mRNA expression of pro-inflammatory cytokines including TNFα and IL1β in the liver [386]. In the present study, fat contributed 45% of the calories in the HF diet. It has been shown that 24 weeks of feeding with a 45 kcal% HF diet (lard-based, ingredients are similar to those used in this thesis) did not increase the expression of pro-inflammatory or macrophage markers in the liver of C57BL/6J mice [388]. This suggests that the different calorific contents of fat in HF diets may explain the reported discrepancy. Interestingly, when mice were fed this 45 kcal% HF diet for another 16 weeks in addition to 24 weeks (40 weeks in total), pro-inflammatory cytokines in the liver were
increased [388]. This suggests that the duration of feeding may also influence the effect of a HF diet on inflammation. In addition, the source of HF diet may also have an impact on hepatic inflammation. It has been shown that a milk-fat based HF diet that is high in saturated FAs (60 kcal%, 61% of FAs are saturated) induced hepatic inflammation more efficiently than a lard-based HF diet (60 kcal%, 37% FAs are saturated) [389]. These studies suggest that HF diet-induced hepatic steatosis is not always associated with inflammation.

As reviewed in Section 1.4.3, it has been suggested that cholesterol may contribute to the development of NASH by exacerbating steatosis, inflammation, ER stress, oxidative stress or mitochondrial dysfunction [335, 342, 348, 354]. Most of these studies either used 0.2% cholesterol in genetic models of dyslipidaemia (e.g. ldlr<sup>−/−</sup>) or used a 1% or even higher concentration of cholesterol in animals without genetic modification. The direct consequences of these treatments include inducing severe dyslipidaemia and obesity, which are closely related to the development of NAFLD (Table 1.3). However, a typical level of dietary cholesterol may not be sufficient to induce these metabolic disorders in C57BL6/J mice as shown by the results in this study, which is consistent with the generally accepted view that cholesterol intake may not always cause dyslipidaemia [382].

One of the aims of this thesis was to study the effect of dietary cholesterol (0.2% w/w) on the development of NASH in mice without genetic modification to mimic the natural history of NAFLD. The present study showed that the addition of a moderate level of cholesterol (0.2% w/w) to the HF diet induced the elevation of plasma ALT levels, which is an indication of liver injury. This is independent of changes of body weight and hepatic steatosis. The elevation of plasma ALT levels was associated with increased inflammatory markers in the liver, such as mRNA expression of pro-inflammatory cytokines and TLR4, as well as elevated NLRP3 inflammasome levels. The present study highlights a likely role for a moderate level
of cholesterol as a second “hit”, which contributes to the conversion from simple hepatic steatosis into NASH without exacerbating hepatic steatosis and dyslipidaemia.

During the study period of this thesis, a similar study in C57BL6/J mice also reported that dietary cholesterol exacerbates HF-induced NAFLD [390]. By using a NASH scoring system this study showed the inflammation score was 2.0 in the liver of mice fed a combination of HF with 1% cholesterol for 30 weeks whereas the inflammation score in mice fed a high cholesterol or HF diet alone was 1.0. In addition, only a combination of the HFC diet with 1% cholesterol increased the ALT level in the plasma. In the present study, the observed increases in inflammatory markers and ALT levels by cholesterol in the HF diet are consistent with the reported finding of liver inflammation based on the scoring criteria defined for humans [390]. However, in contrast to the study described above where 1% cholesterol promoted body weight gain and fibrosis, the present study did not show any additional effect of 0.2% w/w cholesterol on body weight gain or fibrosis. This suggests the effect of cholesterol on progression from simple hepatic steatosis towards NASH is likely to be independent of body weight gain. It is also possible that a higher content of dietary cholesterol exerts a greater effect in promoting the development of NASH from simple hepatic steatosis induced by HF diet.

According to guidelines recommended by AASL [163], NASH may or may not present histologically detected fibrosis depending on its severity [163]. A recent study comparing histological and gene expression analysis showed that the mRNA expression of collagen 1 is among the most sensitive measures of liver fibrosis and its expression levels highly correlate to the severity of fibrosis [391]. In the present study, although 0.2% w/w cholesterol in both CH and HF diet increased collagen 1 expression, no conclusive signs of fibrosis were observed by histological analysis. This is consistent with a recent report showing that when fed a Western diet for 22 weeks, C57BL/6J mice developed severe hepatic steatosis without
any positive indications of fibrosis in liver sections [392]. However, when extending the feeding period to 52 weeks, fibrosis did become apparent. Taken together, although 0.2% w/w cholesterol increases the collagen 1 expression in the liver, longer term feeding may be required to induce detectable fibrosis in the liver.

The obtained data from this chapter also showed that cholesterol prevented the HF diet from inducing PGC1α and TFAM expression in the liver. As they are transcription factors that regulate the expression of a number of genes in mitochondrial metabolism [393], the blunted expression of PGC1α and TFAM in the presence of cholesterol may lead to a reduced mitochondrial capacity of the liver that would normally compensate for the increased lipid influx caused by the HF diet. Indeed, FA oxidation in the liver of the HF-fed mice was impaired by the addition of cholesterol in the present study. These data together indicate that cholesterol abolished the ability of the liver to increase mitochondrial metabolism in response to the HF feeding.

It has been reported that mitochondrial function can be impaired by oxidative stress [394, 395], ER-mitochondria interaction [396, 397] or inflammation [398]. However, the present study does not support such a role of oxidative stress because none of the oxidative stress markers (appearance of protein carbonyls, GSH, MDA or SOD activity) were significantly changed by the inclusion of cholesterol into the HF diet. Similarly, there was no evidence to suggest any involvement of ER stress, as indicated by no significant changes in GRP78 content and the phosphorylation of IRE1 and eIF2α. Instead, the data indicated significant increases in inflammation within the liver induced by cholesterol in the HF diet (as indicated by increases in TNFα, IL1β, MCP1 and CD68 mRNA expression). These results suggest that the blunted mitochondrial function in the liver is likely to be a result of cholesterol-induced inflammation during HF feeding.
Intriguingly, the blunted mitochondrial function was sustained for 5 weeks after removal of dietary cholesterol (HFC-HF group) along with the increased protein levels of NLRP3 inflammasome, even though the elevated pro-inflammatory cytokines (TNFα, IL1β and MCP1) in the liver had subsided. The concurrent changes in mitochondrial function and NLRP3 inflammasome are consistent with recent studies suggesting that NLRP3 activation is associated with mitochondrial dysfunction [399, 400]. It has been shown that impaired mitochondrial function may not immediately recover from injury [401, 402]. However, further studies are required to establish the causal relationship between the activation of NLRP3 inflammasome and mitochondrial dysfunction. Regardless, these data demonstrate the role of dietary cholesterol in the development of NASH, involving inflammation and blunted mitochondrial function.

The results from this chapter also revealed that the addition of cholesterol to the HF diet increased the number of enlarged lipid droplets in the liver. The mechanism underlying the formation and growth of lipid droplets are not well understood [403]. It has been suggested that oleate (a MUFA) may facilitate the storage of palmitate (a SFA) as TG into lipid droplets in CHO cells [286]. Furthermore, oleate loading has been shown to promote the formation of enlarged lipid droplets in several cell lines (e.g. Drosophila S2 Cells [404], mouse myoblast cells [405] and cultured human hepatocytes [406]). It is reasonable to speculate that SCD1, which converts SFAs into MUFAs, may facilitate the formation and growth of lipid droplets. A recent report has suggested that SCD1 is required for the formation of enlarged lipid droplets, suggesting a possible role of SCD1 in the regulation lipid droplet metabolism [407]. This possible role of SCD1 is consistent with the observation of the concurrent increases in SCD1 content and enlarged lipid droplets induced by cholesterol.

Mitochondria play an important role in the trafficking of lipid between lipid droplets, as a depot of lipid storage, and the mitochondria itself as a site for FA oxidation [408].
Interestingly, mitochondrial function was found to be blunted by addition of cholesterol to HF diet in the present study. It has been suggested, from cell-based studies, that mitochondrial dysfunction results in lipid droplet formation as a response to apoptosis or cellular stress [409, 410]. Conversely, increased lipid droplets coat proteins, such as perilipin 5, have been suggested to decrease mitochondrial FA oxidation in cardiomyocytes [411]. These studies suggest that mitochondrial function and lipid droplet formation are closely related in the regulation of lipid homeostasis in the liver. This is consistent with our observation of a concomitant increase in lipid droplets with blunted mitochondrial function in HFC group.

In summary, the results in this chapter (for the first aim) suggest that the HF diet increases adiposity, impairs whole-body insulin sensitivity and induces hepatic steatosis in C57BL6/J mice, whereas the addition of dietary cholesterol does not exacerbate HF-induced obesity, glucose intolerance or hepatic steatosis. Furthermore, the results (for the second aim) indicate that a typical level of dietary cholesterol (0.2% w/w) promotes the progression of simple hepatic steatosis towards NASH concurrent with the phenotype of obesity and glucose intolerance. These NASH-like features are possibly due to inflammation but not whole-body insulin resistance, oxidative stress or ER stress. In addition, the data in the present study also show that cholesterol impairs mitochondrial function in the liver and that blunted mitochondrial function in the liver persists even after the inflammatory markers and plasma ALT levels subside. These findings suggest that cholesterol may play a critical role in the progression from hepatic steatosis towards NASH, possibly by activating inflammatory pathways in mice. Based on these data, a proposed mechanism of the effects of cholesterol on the progression from simple steatosis towards NASH is shown in Figure 3.9.
Figure 3.9 Schematic View of the Effects of Dietary Fat and Cholesterol on NAFLD.

Dietary fat causes fat accumulation in the liver leading to hepatic steatosis as a first “hit” in the development of NAFLD. The addition of cholesterol to the HF diet induces cholesterol accumulation in the liver leading to the promotion of an inflammatory response through TLR4 activation and NLRP3 inflammasome formation. These insults together may lead to liver injury and blunted mitochondrial function, which may contribute to the progression from hepatic steatosis to NASH.
CHAPTER 4

Effects of Preconditioning on Obesity,
Glucose Metabolism and Hepatic Steatosis
4.1 Introduction

The previous chapter showed that the blunted mitochondrial function induced by cholesterol persisted for 5 weeks after the cholesterol had been withdrawn (Figure 3.8). Such persistent effects after pre-exposure to a given set of conditions have been observed in a variety of different settings and have been termed “legacy effects”. For example, in the management of diabetes, it has been noted that the detrimental effects of hyperglycaemia on vascular complications in type 1 diabetes (T1D) may persist even after glucose levels have been lowered [412]. The reduced diabetic complications resulting from an intensive glycaemic control can also persist for a considerable period after therapy in both T1D and T2D patients [78, 413].

Dietary factors have also been shown to induce “legacy effects” on the development of obesity and related metabolic disorders. Studies in rodent models have suggested that a maternal HF or Western diet can exacerbate the adiposity of the offspring [414-416]. There is also emerging evidence to indicate that various nutritional factors in adulthood may have a long-term impact [417]. For example, the reduction in appetite resulting from a high-protein diet has been shown to be sustained for 12 weeks in humans and induce significant weight loss [418]. Furthermore, caloric restriction without malnutrition is known to slow the process of aging, and extend the lifespan by metabolic reprogramming in animals [417].

A HF diet is commonly used to induce obesity and associated metabolic disorders in mice [38, 419]. While mechanisms of HF diet-induced obesity and insulin resistance have been extensively investigated (as reviewed in [35, 420]), most of these studies have only focused on the effect during the time of HF feeding. As shown in Chapter 3, the HF diet induced obesity and glucose intolerance, and cholesterol in the HFC diet induced additional effects to blunt mitochondrial function. Given that removal of cholesterol from the HFC diet revealed a
sustained effect on mitochondrial function in mice, it is reasonable to speculate that there might be similar “legacy effects” of HFC feeding on obesity and glucose intolerance. The aim of the study in this chapter was to investigate the “legacy effect” of the HFC diet on the development of obesity, glucose metabolism and hepatic steatosis and possible mechanisms involved. This chapter tested the hypothesis that prior exposure to the HFC diet may induce “legacy effects” on the development of obesity and associated metabolic disorders. Furthermore, the pathways underlying the hypothesised “legacy effects” would be examined.

4.2 Study Design and Methods

4.2.1 Design of the Preconditioning Protocol in HFC-Fed Mice

All experiments were approved by the RMIT University Animal Ethics Committee (#1414). Male C57BL/6J mice (10 weeks old) were purchased and housed as described in Section 2.1. This study used short-term HFC feeding in mice, which is termed “preconditioning” because it is conceptually analogous to ischemic preconditioning, where short period(s) of non-lethal ischemia have been shown to protect against a subsequent ischemic insult [421]. After acclimatisation, mice were assigned to 2 groups at the beginning of the study, a naïve group and a preconditioned group. The naïve group was fed the CH diet throughout the period of preconditioning, from week 1 to week 9 (Figure 4.1, highlighted in light blue). During this period the preconditioned group was fed the HFC diet in 2 episodes of 2 weeks duration, where each episode was followed by 2-3 weeks on the CH diet. During the subsequent experimental period (weeks 10-14), the naïve and the preconditioned groups were assigned separately into two sub-groups, and fed either the CH or the HFC diet. A schematic diagram of the protocol used in this study is depicted in Figure 4.1 and summarized as follows:

- A CH group, in which mice were fed the standard CH diet for 14 weeks;
• A preconditioned CH (Precon-CH) group, in which mice were preconditioned (as mentioned above, they were given 2 episodes of the HFC diet for 2 weeks, and each was followed by 2~3 weeks of CH diet as a washout) and fed the CH diet for another 5 weeks until the end of the study;

• A naïve HFC group, in which mice were fed the CH diet for 9 weeks, followed by 5 weeks on the HFC diet to measure the metabolic response of mice to this diet;

• A preconditioned HFC (Precon-HFC) group, in which mice were preconditioned, as described for the Precon-CH group, and subsequently fed the HFC diet for 5 weeks to measure the metabolic response of mice to this diet.

Figure 4.1 Illustration of Design of the Animal Study.

4.2.2 Assessment of Obesity and Glucose Metabolism

Food intake of mice was measured daily at the beginning of “metabolic response” period (as shown in Figure 4.1). In other cases, body weight and food intake of the animals were
monitored every other day. At the end of the study, the epididymal fat was weighed to assess effects on obesity. An ipGTT was performed at week 10 as described in Sections 2.3 to assess effects on glucose intolerance.

The whole-body insulin sensitivity of mice was measured at week 11 by an i.p. insulin tolerance tests (ipITT) [422]. Animals were fasted for 5 hours prior to the measurement of basal blood glucose levels at t = 0 min using a glucometer. Insulin (100 IU/mL, Novo Nordisk Novorapid Penfill) was diluted to 0.2 IU/mL with saline and injected intraperitoneally into the mice to reach a final dose of 0.75 IU/kg. Subsequently, blood glucose levels were measured at t = 0, 20, 40, 60 and 80 min.

4.2.3 Assessment of Hepatic Glucose Production and Steatosis

Hepatic glucose production plays a principal role in counteracting the reduced blood glucose during fast, mainly through gluconeogenesis from lactate, pyruvate, glycerol, and amino acids [423]. The capacity of hepatic glucose production was measured by i.p. pyruvate tolerance tests (ipPTT) [422]. Pyruvate (Sigma-Aldrich, #2256) was prepared in PBS buffer. Pyruvate (2.0 g/kg) was administered to animals by i.p. injection after overnight fasting. Blood glucose levels were measured at t = 0, 15, 30, 60 and 90 min.

The effects on hepatic steatosis were assessed by measuring the TG content in the liver (Section 2.5). The phosphorylation of AMPK (an energy sensors that maintain energy and glucose homeostasis), and its downstream target ACC, and SIRT1 (an upstream regulator of AMPK) content were measured by western blotting (Section 2.6).

4.2.4 Assessment of Markers of Mitochondrial Fatty Acid (FA) Oxidation in Muscle

Given that oxidative phosphorylation in mitochondria plays an important role in energy metabolism [424], the protein content of mitochondrial respiratory complexes was measured as an indication of the mitochondrial oxidation capacity. This assessment was conducted in
muscle samples collected at the end of this study by measuring the protein contents of mitochondrial respiratory complexes using western blotting (Sections 2.6).

Additionally, the activities of two key enzymes in mitochondrial FA oxidation, citrate synthase and β-hydroxyacyl-CoA dehydrogenase (β-HAD), were also measured. Citrate synthase is the first enzyme in the TCA cycle, which catalyses the formation of citrate from oxaloacetic acid and acetyl-CoA with the production of CoA-SH (reaction shown below). This experiment measures the activity of citrate synthase based on the production of CoA-SH, which can be quantitatively detected by dinitrothiocyanobenzene (DNTB) using the colorimetric measurement of its product, thionitrobenzoate (TNB) [360]. Frozen samples were homogenized in a buffer (pH 7.4) containing 175 mmol/L KCl and 2 mmol/L EDTA with a glass homogenizer and subjected to 3 freeze-thaw cycles. Tissue homogenates were mixed with a working solution containing acetyl-CoA (Sigma Aldrich, # A2056) and DNTB (Sigma Aldrich, CAT# D218200), and the absorbance was measured at 405 nm to obtain background (blank) values. Oxaloacetic acid (Sigma Aldrich, # O4126, final concentration 1 mM) was then added to the assay system to initiate the reaction, and the absorbance was measured every 30 seconds for a period of 5 min at 405 nm. The enzyme activity was calculated based on the slope of the absorbance curve and the extinction coefficient of TNB (13.3 mM$^{-1}$cm$^{-1}$).

β-HAD catalyses the third step in β-oxidation, which results in the dehydrogenation of β-hydroxyacyl-CoA to β-oxoacyl-CoA with the production of NADH and H$^+$. The reverse process with the generation of NAD$^+$ from NADH is also shown in the reaction scheme
depicted below. Enzyme activity was assessed based on the reverse reaction and calculated according to the rate of disappearance of NADH [360]. The preparation of samples was the same as for the measurement of citrate synthase activity. Tissue homogenates were mixed with a working solution containing NADH (Sigma Aldrich, # N4505) and the absorbance at 340 nm was measured as the blank. Acetoacetyl-CoA (Sigma Aldrich, #A1625, final concentration 0.1 mM) was then added to the assay system to initiate the reaction, and the absorbance was measured every 30 seconds for 5 min at 340 nm. The enzyme activity was calculated based on the slope of the absorbance curve and the extinction coefficient of NADH (6.22 mM$^{-1}$cm$^{-1}$).

![Enzyme reaction diagram](image)

The expression of transcription factors involved in mitochondrial biogenesis including PGC1α, TFAM and nuclear respiratory factor-1 (NRF1) was also measured by qRT-PCR (Section 2.7).

Finally, the mitochondrial content was measured by quantifying mtDNA. Total DNA, including mtDNA and genomic DNA, was isolated from tissues using a DNA isolation kit from Invitrogen (#K1820-02). The DNA extract was quantified and diluted to 2 ng/μL for quantitative real-time PCR reactions. The conditions of the PCR reactions are described in Section 2.7.2. MCT-1 was used as a marker of genomic DNA, and ND-5 was used as a marker of mtDNA. The sequences of primers are: MCT-1 forward 5’-TAGCTGGATCCCTG-ATGCGA-3’; MCT-1 reverse 5’-GCATCAGACTTCCCAGCTTC-3’; ND-5 forward 5’-G-CAGCCACAGGAAAATCCG-3’; ND-5 reverse 5’-GTAGGCAAGAGACGGGAGTTG-3’.
4.2.5 Assessment of “Browning” in White Adipose Tissue (WAT)

Based on observed protective effects of HFC-preconditioning on diet-induce obesity and glucose intolerance and the lack of clear indication of the involvement of the liver and muscle in mediating such effects, it was considered necessary to examine the “browning” of WAT as a possible mechanism underlying the observed “legacy effects” (data shown in Figure 4.2 & Figure 4.3). As reviewed in Section 1.1.4.2, the “browning” of WAT can increase the energy expenditure and ameliorate obesity and related metabolic disorders [425, 426]. The “browning” of WAT was assessed by measuring the protein content of UCP1. The expression of transcription factors involved in the “browning”, PGC1α [133] and PPARγ [126] (see Section 1.1.4.2), was measured by qRT-PCR (Section 2.7). The protein content of SIRT1, which may regulate the activity of PPARγ [427], was measured by western blotting (Section 2.6). FGF21 is a key inducer of “browning” [118] and its levels in the plasma were measured using an ELISA kit from HKU Antibody and Immunoassay Services (#32180) following the manufacturer’s instructions.

4.2.6 Statistical Analyses

Data were expressed as means ± SE. The individual results were screened for their distribution patterns prior to the statistical tests. A Student’s t-test was performed for the comparison of normal distributed data. Data of FGF21 did not follow a normal distribution and thus statistical comparisons were made using a nonparametric test for independent samples (SPSS statistics 23). Differences at $p < 0.05$ were considered statistically significant.
4.3 Results

4.3.1 Effects of Preconditioning on Body Weight Gain and Adiposity

During the first episode of HFC feeding (the first 2 weeks) of preconditioning, mice fed the HFC diet gained twice as much body weight as mice fed the CH diet (Figure 4.2A). When these animals were subsequently fed the CH diet as a washout, their body weight reduced and their body weight gain was then not different from the CH mice at the end of the 2 weeks of CH feeding. Similarly, as soon as the second episode of HFC started, the body weight of mice began to increase, and their body weight gain was twice that of their CH counterparts at the end of the second exposure. After switching back to the CH diet, the body weight of the HFC-preconditioned mice gradually returned to similar levels of the CH mice (Figure 4.2A&C).

After the preconditioning, mice were fed either the HFC diet again or kept on the CH diet to investigate the “legacy effect” in response to a subsequent exposure to the HFC diet. The body weight gain of preconditioned mice kept on CH diet (Precon-CH, in black) was not different from the control CH group. Interestingly, the preconditioned mice gained less body weight during the subsequent feeding with the same HFC diet (Precon-HFC, in red) than the mice exposed to the HFC diet for the first time (Naïve-HFC, in yellow) despite similar food intake (Figure 4.2B). At the end of the experiment, the body weight gain and the fat pad mass of the Precon-HFC group was 22 ± 9% less and 26 ± 9% less, respectively, compared to the Naïve-HFC group (both p < 0.05, Figure 4.2C). Together, these data indicate that the HFC-preconditioning ameliorated the adiposity induced by subsequent exposure to the same HFC diet, suggesting “legacy effects” were induced.
Figure 4.2 Effects of Preconditioning on Body Weight Gain and Adiposity.

The CH group was fed a standard CH diet for 14 weeks. The preconditioned CH group (Precon-CH) group had 2 episodes of the HFC feeding for 2 weeks, each followed by an episode of CH feeding (2 weeks for the first time, 3 weeks for the second time). After the preconditioning, they were fed the CH diet for 5 weeks until the end of the study. The naïve HFC (Naïve-HFC) group was fed the CH diet for 9 weeks and then the HFC diet for another 5
weeks. The preconditioned HFC (Precon-HFC) group was pre-exposed to HFC diet as the Precon-CH group, and then fed the HFC diet for 5 weeks. (A) Body mass gain of mice; (B) Cumulative food intake during the first 8 days of “metabolic response” period and cumulative calorie intake during the whole “metabolic response” period (week 10-14); (C) Body mass at baseline, body weight gain and the epididymal fat mass of mice at the end of study (week 14). n = 8-10/group, † p < 0.05 vs. indicated group.

4.3.2 Effects of Preconditioning on Glucose and Insulin Tolerance

An ipGTT and an ipITT were performed to assess the “legacy effect” of preconditioning on whole-body glucose and insulin tolerance of mice. CH and Precon-CH groups have similar glucose tolerance, as indicated by overlapping blood glucose curves (hence similar iAUC) during the ipGTT (Figure 4.3A). However, when naïve or preconditioned mice were challenged with the HFC diet, blood glucose levels of the Precon-HFC mice were lower (27 ± 7%, p < 0.05) at 60 min of the GTT and their iAUC values were less (23 ± 8%, p < 0.05) than the Naïve-HFC group. This indicates that preconditioning ameliorated HFC-induced glucose intolerance.

To further assess the response of blood glucose levels to insulin stimulation, an ipITT was performed (Figure 4.3B). Again, the Precon-CH group showed a similar reduction in glucose levels and AUC in response to insulin injection compared with the CH group. Although not statistically significant, blood glucose levels tended to be more reduced in the Precon-HFC group compared with the Naïve-HFC group at 20, 40, 60 and 80 min during the ipITT. Furthermore, the AUC of the Precon-HFC group was 13 ± 5% less than the Naïve-HFC (p < 0.05). These results indicate that preconditioning has protective effects against the HFC-induced glucose intolerance and improves whole-body insulin sensitivity of HFC mice.
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Figure 4.3 Effects of Preconditioning on Glucose Tolerance and Insulin Tolerance.

The CH group was fed a standard CH diet for 14 weeks. After the preconditioning phase, the Precon-CH group was subsequently fed the CH diet for 5 weeks until the end of the study. The Naïve-HFC group was fed the CH diet for 9 weeks and then the HFC diet for another 5 weeks. After the preconditioning phase, the Precon-HFC group was fed the HFC diet again for 5 weeks. (A) The blood glucose curves during the ipGTT (2.0 g/kg) in week 10 and iAUC; (B) The blood glucose curves during the ipITT (0.75 IU/kg) in week 11 and AUC. n = 8-10/group, † p < 0.05 vs. indicated group.

4.3.3 Effects of Preconditioning on Hepatic Glucose Production and Steatosis

The liver plays an important role in maintaining glucose homeostasis via gluconeogenesis from substrate metabolites such as pyruvate and lactate. The PTT measures hepatic glucose production from the injected pyruvate through gluconeogenesis. It can be used to determine...
whether there is an increase in the capacity of hepatic glucose production. As shown in Figure 4.4A, the glucose levels and AUC during the PTT in preconditioned mice were similar to those in naïve mice, either between the Precon-CH and CH group, or between Precon-HFC and Naïve-HFC group. This suggests that HFC-preconditioning did not significantly affect the capacity for glucose production in the liver.

Next, the effect of HFC-preconditioning on hepatic steatosis was assessed by measuring TG content in the liver. As shown in Figure 4.4B, when mice were fed either CH or HFC, preconditioning did not change the TG content in the liver. This suggests that protection against obesity through HFC-preconditioning was not related to changes in hepatic steatosis. It is of interest to assess whether or not the HFC-preconditioning may have any “legacy effect” on other contributing factors (e.g. inflammation and mitochondrial function) to the development of NASH in future studies.

AMPK plays a pivotal role in the suppression of glucose production and promotion of FA oxidation and mitochondrial biogenesis in the liver [428]. SIRT1 is reported to promote FA oxidation through the activation of PPARα in the liver [429] and regulate the activity of AMPK [430]. However, consistent with the results of ipPTT and TG content in the liver, no apparent change was observed on the activation of AMPK and its downstream target, ACC (Figure 4.4C&D). Again, no apparent change of the protein level of SIRT1 was observed by HFC-preconditioning.
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Figure 4.4 Effects of Preconditioning on Hepatic Glucose Production and Steatosis.

The CH group was fed a standard CH diet for 14 weeks. After the preconditioning phase, the Precon-CH group was subsequently fed the CH diet for 5 weeks until the end of the study. The Naïve-HFC group was fed the CH diet for 9 weeks and then the HFC diet for another 5 weeks. After the preconditioning phase, the Precon-HFC group was fed the HFC diet again for 5 weeks. (A) The blood glucose curves during i.p. pyruvate tolerance tests (ipPTT, 2.0 g/kg) in week 12 and the AUC; (B) TG content in the liver at the end of the study (week 14); (C) Representative blots of phospho-AMPK, total-AMPK, phospho-ACC, total-ACC and GAPDH in the liver; (D) Densitometric analysis of (C). n = 8-10/group.
4.3.4 Effects of Preconditioning on Markers of Mitochondrial FA Oxidation in Muscle

Skeletal muscle is a major contributor to the REE [90, 91] mainly through mitochondrial oxidation of energy-rich substrates such as FAs. FA oxidation has also been linked to the development of insulin resistance in this tissue [424]. The capacity of mitochondrial oxidative phosphorylation was examined to evaluate the effects of preconditioning on muscle collected at the end of the study. Although mitochondrial complex II in the Precon-CH group increased by 38 ± 13% compared with the CH group (p < 0.05), complex I and complex V were not affected by preconditioning (Figure 4.5A). β-HAD and citrate synthase are mitochondrial enzymes for FA oxidation and the activities of these two enzymes are often used as indicators of mitochondrial function [360]. The results showed that their activity was not changed by preconditioning (Figure 4.5B).

Further analyses were conducted with muscle samples by measuring relevant transcription factors involved in the regulation of mitochondrial biogenesis, including mRNA expressions of PGC1α, TFAM and NRF1 together with phospho-AMPK/total-AMPK protein levels. No significant change in PGC1α mRNA expression was observed between naïve and preconditioning groups (Figure 4.5C). The Precon-CH group had a higher mRNA expression level of TFAM than the CH group (by 65 ± 24% p < 0.05). However, the mRNA expression of this protein was decreased by 50 ± 12% in Precon-HFC group than the Naïve-HFC group (p < 0.05). A similar trend was observed in the mRNA expression of NRF1. There was also a 2-fold increase in AMPK phosphorylation in Precon-CH mice (p < 0.05 vs. CH), but no apparent change in Precon-HFC mice (p > 0.1 vs. Naïve-HFC) (Figure 4.5D).

As no consistent effect of preconditioning on the regulation of mitochondrial biogenesis was observed, total mtDNA content was measured to investigate whether the number of mitochondria was affected by preconditioning. The results showed that preconditioning did not affect the mtDNA content in muscle (Figure 4.5E).
Figure 4.5 Effects of Preconditioning on Mitochondrial Metabolism in Muscle.

The CH group was fed a standard CH diet for 14 weeks. After the preconditioning phase, the Precon-CH group was subsequently fed the CH diet for 5 weeks until the end of the study.
The Naïve-HFC group was fed the CH diet for 9 weeks and then the HFC diet for another 5 weeks. After the preconditioning phase, the Precon-HFC group was fed the HFC diet again for 5 weeks. Quadriceps muscle was collected from the mice at the end of the study (week 14) for the following measurements. (A) The protein levels of mitochondrial complex were measured by western blotting, and normalized by GAPDH; (B) β-Hydroxyacyl-CoA dehydrogenase (β-HAD) activity was measured by the disappearance rate of NADH, and citrate synthase activity was detected by detecting the rate of the CoA-SH production using DTNB; (C) PGC1α, TFAM and nuclear respiratory factor-1 (NRF1) mRNA expressions were quantified by qRT-PCR; (D) phosphor-AMPK/total-AMPK was measured by western blotting; (E) Mitochondrial DNA (mtDNA) content was measured by quantitative PCR using MCT-1 (genomic DNA marker) as a control gene. n = 8-10/group, † p < 0.05 vs. indicated group.

4.3.5 Effects of Preconditioning on Markers of “Browning” in WAT

It has been suggested that “browning” of WAT may significantly increase whole-body energy expenditure [99] (reviewed in Section 1.1.4.2). Increased expression of UCP1 is a hallmark of “browning”. While no significant increase in the protein level of UCP1 was observed in the Precon-CH group, there was a 54 ± 20% increase in the Precon-HFC group compared to the Naïve-HFC group (p < 0.05, Figure 4.6A).

FGF21 is a well-known inducer of the “browning” in WAT (Section 1.1.4.2), partly through increasing PGC1α protein content [118]. Its plasma levels were found to be increased by 2-fold in the Precon-CH group compared with the CH group (p < 0.05, Figure 4.6B). A trend of increase in plasma levels of FGF21 was observed in the Precon-HFC group compared with the Naïve-HFC group. It has been suggested that “browning” of WAT can be regulated by SIRT1 [427]. A significant increase in SIRT1 protein levels was observed in Precon-HFC compared with the Naïve-HFC group and a similar trend was observed in Precon-CH group compared with the CH group (Figure 4.6C). As mentioned in Section 1.1.4.2, PGC1α and
PPARγ are key transcription factors that regulate gene expression involved in the “browning” [426]. However, there was no apparent change in the mRNA expression levels of PGC1α and PPARγ with preconditioning regardless the subsequent CH or HFC challenge.

**Figure 4.6 Effects of Preconditioning on Markers of “Browning” in WAT.**

The CH group was fed a standard CH diet for 14 weeks. After the preconditioning phase, the Precon-CH group was subsequently fed the CH diet for 5 weeks until the end of the study. The Naïve-HFC group was fed the CH diet for 9 weeks and then the HFC diet for another 5 weeks. After the preconditioning phase, the Precon-HFC group was fed the HFC diet again for 5 weeks. Epididymal fat was collected from the mice at the end of the study (week 14) for the following measurements. (A) The protein levels of UCP1 were measured by western
blotting and normalized by tubulin; (B) The FGF21 level in the plasma was measured by an ELISA kit and the protein level of SIRT1 was measured by western blotting. (C) The mRNA expressions of PGC1α and PPARγ were measured by qRT-PCR. n = 8-10/group, † p < 0.05 vs. indicated group.

4.4 Discussion

This chapter investigated whether prior exposure to a HFC diet had long-lasting consequences (“legacy effects”) on the development of obesity, glucose intolerance and hepatic steatosis. The results from this chapter showed that the preconditioning with the HFC diet did induce protective “legacy effects” against diet-induced obesity and glucose intolerance, as indicated by less body weight gain and a smaller iAUC during ipGTT. However, there was no improvement in either hepatic steatosis (indicated by unchanged TG content) or the capacity of glucose production (indicated by an unchanged response to a PTT). Examination of markers of mitochondrial content and FA oxidation in muscle did not reveal any significant changes that may suggest an increased energy metabolism in this tissue to explain the observed “legacy effect” on obesity. However, the results in this chapter did show a significant effect of HFC-preconditioning on increasing the UCP1 content in WAT and plasma levels of FGF21. Therefore, it may be that the “browning” of WAT was involved in the protective “legacy effect” of HFC-preconditioning on obesity and related metabolic disorders.

A previous study from our group has shown that the HFC-fed mice can develop obesity, glucose intolerance and hepatic steatosis within 2 weeks [39]. This is consistence with the results from Chapter 3 showing that mice can develop glucose intolerance after 1 week of HFC feeding. Accordingly, 2 weeks of HFC feeding was used in the procedure for preconditioning in the present study. After the preconditioning, mice were fed either the HFC
or CH diet for 5 weeks to allow the assessment of their metabolic responses. Based on the sustained effect of the HFC diet on blunting mitochondrial function in the liver (Chapter 3), initially it was speculated that HFC-preconditioning may induce “legacy effects” that renders the whole-body prone to the development of obesity, glucose intolerance and NAFLD via this mechanism. Intriguingly, the results in this chapter showed that the HFC-preconditioning protected mice from the diet-induced obesity and glucose intolerance. These findings are different from the reported “legacy effect” of HF diet on metabolic disorders across generation (from parents to offspring) as reviewed in Chapter 1 (Section 1.1.1) [23]. This suggests the mechanisms involved in these two types of “legacy effects” are likely very different, but little is known about the “legacy effect” of dietary fat on obesity and related metabolic disorders within a generation, particularly during adulthood.

A recent study has also shown that overfed mice neonates are less susceptible to HF diet-induced pro-inflammatory effects in the brain [431]. This study suggests that early overfeeding, generally considered detrimental, may have beneficial effects in the later response to a HF diet. In humans, the sustained effect of dietary fat on substrate utilization has been used in “fat adaptation”, a strategy used to increase the oxidation of fat in athlete training programs [432]. In this strategy, a short-term HF diet (for 5 days to 2 weeks) is used to increase fat oxidation in muscle and it is followed by a period of a high-carbohydrate diet (1-3 days) as carbohydrate restoration [433, 434]. Notably, the HF diet-induced increases in fat oxidation and decreases in glycogenolysis in muscle can persist after the carbohydrate restoration, suggesting that the metabolic effect of HF diet may induce sustained consequences in adults, similar to the “legacy effect” of a HFC diet reported in this thesis.

A mechanism involved in the sustained effects of HF diets has been studied, suggesting that a HF diet may induce heritable changes in methylation of genes in muscle [435]. Although the functional relevance of methylation of these genes is not clear, the authors proposed that these
methylation changes might prevent the detrimental effects of HF diet on gene expression under specific metabolic challenges. This proposed mechanism is consistent with the results in this chapter showing that the protective effect of preconditioning against obesity is only evident under HFC challenge. However, further investigation is needed to examine whether DNA methylation is actually involved in mediating the sustained effect of preconditioning.

Although the present study showed that HFC-preconditioning induced a protective effect against obesity, HFC diet per se is generally recognised for its detrimental effects on obesity and related metabolic disorders as shown in Chapter 3. The protective effect of a HFC in this study was revealed relative to a naïve group (Precon-HFC vs. Naïve-HFC) and it may require a prerequisite, namely a limited length of subsequent HFC feeding. The difference in body weight gain between Precon-HFC and Naïve-HFC became less evident as the HFC feeding proceeds as shown in Figure 4.2A. Thus it is possible that the protective effect of preconditioning may become less apparent as the HFC feeding proceeds, possibly due to the fact that the demonstrable ability of longer term HFC feeding to induce obesity overtakes the “beneficial” effect of preconditioning.

In order to investigate mechanisms underlying the protective effects of preconditioning on obesity and glucose metabolism, relevant parameters indicative of changes in metabolism in the liver, muscle and adipose tissue (major peripheral tissues responsible for energy metabolism and glucose homeostasis) were examined. In the liver, there was no apparent change in the activation of AMPK, SIRT1 or capacity of hepatic glucose production. These results might suggest that the liver does not play a significant role in mediating the beneficial “legacy effects” of HFC diet. Preconditioning also did not exert any protection against the development of hepatic steatosis induced by the HFC diet, the first “hit” in the pathogenesis of NAFLD. However, further studies are needed to examine whether preconditioning may have an effect on mitochondrial FA oxidation in the liver (as shown in Chapter 3) or other
factors (e.g. inflammation, ER stress and Oxidative stress) that may contribute to the development of NASH.

Next, markers involved in mitochondrial oxidative phosphorylation and FA metabolism were measured as an indication of the capacity of energy metabolism in the muscle. The overall mitochondrial content (indicated by mtDNA content) and activities of enzymes involved in mitochondrial FA oxidation were not affected by preconditioning, despite increases in AMPK activation, TFAM and mitochondrial complex II in Precon-CH mice. The reason for this discrepancy is unknown and further studies are needed to determine whether or not skeletal muscle contributes to the protective effects of preconditioning.

UCP1 mediates thermogenesis in adipose tissue and increases energy expenditure. It is expressed in BAT or brown-like adipocyte within WAT as a marker of “browning” [99]. Interestingly, UCP1 protein levels were increased in WAT of the Precon-HFC group. Consistent with the increased UCP1 protein levels, Precon-HFC group had increased levels of circulating FGF21, a key inducer of the “browning” [118], and increased levels of SIRT1 protein in the WAT. It has been reported that FGF21 and SIRT1 increase the level of PGC1α and PPARγ in WAT, respectively, through post-transcriptional mechanisms [118, 427]. Although no apparent change in mRNA expression of PGC1α and PPARγ were observed, further measurement of the protein levels of these two transcription regulator are needed to indicate their possible involvement in the preconditioning-induced “browning”. In addition, it has been suggested that subcutaneous WAT is more sensitive to FGF21 than the epididymal WAT used in this study [118]. Therefore, the effect of preconditioning on subcutaneous WAT should be explored in further studies.

The present study demonstrates beneficial effects of HFC-preconditioning against obesity and glucose intolerance, while the HFC diet is generally considered as detrimental to these metabolic parameters. This paradoxical phenomenon may provide a paradigm for the
identification of novel mechanisms that can be targeted in the treatment of these conditions in humans. The principle is similar to the approach that has been used in ischemic preconditioning to explore novel therapies in cardioprotection [436]. However, it is important to point out that such an approach should not aim at advocating HFC diet *per se* as an intervention because the HFC diet itself can lead to obesity and metabolic syndrome. Instead, novel mechanisms or factors beneficial for the prevention or treatment of obesity and associated metabolic disorders may be identified using this paradigm. For example, the present study suggests a possible role of FGF21 in mediating the beneficial “legacy effect” and further studies suggested previously may identify other mechanisms underlying the effect of HFC-preconditioning. In addition, it may be possible to replace the HFC diet with a short-chain FAs (SCFAs) diet for the preconditioning, because SCFAs have been shown to promote FGF21 expression [437] and increase energy expenditure [438] without causing obesity and associated metabolic disorders (detailed in Section 5.2.2.4).

In summary, this chapter shows that HFC-preconditioning ameliorates diet-induced obesity and glucose intolerance in mice. This is associated with an increase in UCP1 and SIRT1 levels in WAT as well as an increase in circulating levels of FGF21, which are all involved in the “browning of WAT. Therefore, the “browning” of WAT may be a principal mechanism underlying the protective effects of HFC-preconditioning observed in mice.
CHAPTER 5

Summary, Conclusion, Limitations and Future Directions
The overall aim of this thesis was to investigate the role of dietary fat and cholesterol in the development of obesity, glucose intolerance and NAFLD. Based on the literature review in Chapter 1 and novel findings from Chapter 3, the following working hypotheses were developed: 1) Chronic HF diet but not moderate dietary cholesterol would induce obesity, glucose intolerance and hepatic steatosis (as the first “hit” in the development of NASH). 2) Cholesterol may promote the progression from hepatic steatosis (induced by the HF diet) towards NASH by inducing additional insults in the liver. 3) Prior exposure to the HFC diet may induce “legacy effects” on the development of obesity, glucose intolerance and hepatic steatosis. This chapter will 1) summarise the key findings from Chapters 3 and 4 and provide some general conclusions; 2) discuss the potential implications and limitations of the study and 3) provide ideas to guide further research based on the novel findings reported in this thesis.

5.1 Key Findings

5.1.1 Key Findings and Conclusion for Chapter 3: Effects of Dietary Fat and Cholesterol on Obesity, Glucose Intolerance and NAFLD

Chapter 3 firstly examined the metabolic characteristics of mice fed a CH, CHC, HF or HFC diet to investigate the effect of dietary fat from lard and a typical level of cholesterol (0.2% w/w), alone or in combination, on obesity and related metabolic disorders. Consistent with previous reports from our and other groups [32, 39, 373, 439], a lard-based HF diet (45 kcal%) induced obesity (indicated by increased body weight and epididymal fat mass), whole-body insulin resistance (indicated by increased iAUC in GTT together with increased insulin levels) and hepatic steatosis (indicated by increased liver TG). There is no clear evidence to indicate whether cholesterol intake may affect energy balance, hepatic steatosis or insulin sensitivity.
The results in Chapter 3 showed that 0.2% w/w cholesterol had no additive effects on obesity and hepatic steatosis, but it partially alleviated HF-induced whole-body insulin resistance.

In relation to NAFLD, Chapter 3 further investigated the effects of dietary fat and cholesterol on the progression from simple hepatic steatosis (TG accumulation only) towards NASH (additional indications of liver injury, inflammation with or without fibrosis). While HF- and HFC-fed mice all displayed hepatic steatosis, only HFC-fed mice has increased plasma ALT, which is an indication of liver injury. The increased ALT in HFC-fed mice was associated with increased hepatic inflammatory markers (e.g. increases in mRNA expression of TNFα, IL1β, MCP1 and CD36) without apparent ER stress and oxidative stress. In contrast, addition of cholesterol to a CH diet did not produce a significant phenotype of either simple hepatic steatosis or NASH. This may result from the unchanged cholesterol level in the liver of CHC-fed mice. The unchanged hepatic cholesterol level is consistent with the notion that low saturated fat content may lead to reduced cholesterol absorption [298]. Furthermore, cholesterol suppressed HF-induced FA oxidation and mRNA expression of PGC1α and TFAM in the liver, suggesting a blunted mitochondrial function. Interestingly, these effects persisted for 5 weeks after cholesterol was removed from the diet.

Previous studies have suggested that dietary cholesterol may contribute to the development of NASH by exacerbating steatosis, inflammation, ER stress, oxidative stress or mitochondrial dysfunction in animal models [333, 340, 346, 352]. However, such effects of cholesterol were associated with other concurrent contributing factors of the development of NASH, such as dyslipidaemia and obesity induced by a genetic mutation (e.g. ldlr−/−) or an extreme level of cholesterol (1% or even higher). With the use of a moderate level of dietary cholesterol (0.2%w/w) and wild type C57BL/6J mice, Chapter 3 demonstrates that cholesterol-induced
elevated plasma ALT and increases in inflammatory markers in the liver are independent of
the exacerbation of obesity, hepatic steatosis and dyslipidaemia.

In summary, although a typical level of cholesterol (0.2% w/w) did not exacerbate obesity,
whole-body insulin resistance or hepatic steatosis, it may play a critical role as a contributory
dietary factor, in the transition from simple hepatic steatosis towards NASH possibly via the
activation of inflammatory pathways. One intriguing finding from this part of the research
was that the blunted mitochondrial function in HFC-fed mice persisted for 5 weeks after
switching to the HF diet. This led to the design of further experiments to investigate the
sustained effect of HFC on obesity and related metabolic disorders as described in Chapter 4.
Based on these results, Figure 5.1 illustrates the key findings that addressed the working
hypothesis as described in Figure 1.11 in Chapter 1.

The daily limit of cholesterol intake (300 mg/day) has been removed from 2015 Dietary
Guidelines for Americans [440], because there is growing evidence from clinical and
epidemiological studies suggesting that cholesterol intake may not increase the risk for CVD
[441, 442]. However, the findings from Chapter 3 suggest that moderate cholesterol intake
may contribute to the development of NASH without inducing dyslipidaemia in mice. These
findings provide a scientific rationale for clinical and epidemiological studies regarding the
effect of dietary cholesterol on NAFLD. In addition, the revealed role of cholesterol in the
development of NASH may help establish a dietary model of NAFLD in mice for the
evaluation new treatments for this disease in pre-clinical studies.
Figure 5.1 Summary of the Effect of Dietary Fat and Cholesterol on Obesity and NAFLD. Although the HF diet alone induced obesity and hepatic steatosis, it did not appear to activate inflammatory pathways or elevated plasma ALT. However, adding cholesterol to this diet induced increases in markers of hepatic inflammation and elevated ALT, suggesting that cholesterol could promote the progression from hepatic steatosis towards NASH. In addition, cholesterol blunted the increased mitochondrial function induced by the HF diet, and this effect of cholesterol persisted for 5 weeks after removing cholesterol from the diet. This interesting finding provided the initial rationale to further investigate the “legacy effects” of the HFC diet as described in Chapter 4.
5.1.2 Key Findings and Conclusion for Chapter 4: Effects of Preconditioning on Obesity and Glucose Intolerance

The results in Chapter 4 suggest that the HFC-preconditioning has protective effects on diet-induced obesity and glucose intolerance. Preconditioning with the HFC diet ameliorated diet-induced obesity as indicated by the reduction in body weight and fat pad mass compared with mice that had not been pre-exposed to the HFC diet (Naïve-HFC mice). The HFC-preconditioned mice also had reduced iAUC measured from the GTT and reduced AUC measured from the ITT compared with the Naïve-HFC mice. These findings suggest that preconditioning ameliorates the diet-induced glucose intolerance and reduction in whole-body response to insulin. Although HFC-preconditioning does not affect hepatic steatosis, further studies are required to examine whether preconditioning may have any effect on mitochondrial FA oxidation or other factors (e.g. inflammation, ER stress and Oxidative stress) that may contribute to the development of NASH.

Thus far, the examination of metabolism-related parameters in the liver and muscle did not reveal any clear indication that HFC-preconditioning affected these tissues. However, the results showed an increased in UCP1 content (an indication of “browning”) in WAT after HFC-preconditioning. This is associated with increased protein levels of SIRT1, which has been suggested to regulate “browning” through deacetylation of PPARγ [427].

HFC-preconditioning also increased plasma levels of FGF21, which is a key inducer of “browning” by increasing the level of PGC1α through post-transcriptional mechanisms [118]. Given that the FGF21 in the circulation is mainly produced by the liver through the activation of PPARα [112, 113], it is reasonable to speculate that the “browning” in WAT may be indirectly induced by PPARα in the liver. Obviously further research is needed to investigate
this possibility by the measurement of mRNA expression of FGF21 and other PPARα target genes such as acyl-CoA oxidase 1 (ACOX1) in the liver.

In summary, the data in Chapter 4 show that preconditioning with the HFC diet ameliorates diet-induced obesity and glucose intolerance in mice. This is associated with increases in UCP1 protein levels in WAT, which may be due to the elevated levels of FGF21 in the circulation and increased SIRT1 protein levels in WAT. These results suggest that the short-term HFC feeding may have prolonged effects on the development of obesity and associated glucose intolerance, and that these effects may be mediated by the “browning” of WAT. The paradigm of HFC-preconditioning may help to identify novel mechanisms that can be targeted to provide beneficial effects for the prevention of obesity and related metabolic disorders. Based on the results in the present study and literature reports, the proposed mechanisms for the protective “legacy effects” of preconditioning are illustrated in Figure 5.2.
Figure 5.2 Schematic View of the Proposed Mechanisms Underlying the “Legacy Effects” of the HFC Diet. Preconditioning may directly induce the “browning” in WAT by increasing SIRT1 content, which may lead to an increase in PPARγ activity by deacetylation. Alternatively, it may activate PPARα in the liver, leading to the production and release of FGF21 into the bloodstream, subsequently promoting UCP1 in WAT by increasing PGC1α content through post-transcriptional regulation. Increased “browning” in adipose tissue may increase the whole-body energy expenditure, ameliorating obesity and insulin resistance.
5.2 Limitations and Future Directions

5.2.1 Limitations and Future Research for Chapter 3

5.2.1.1 Effect of Dietary Cholesterol on Glucose Intolerance and Energy Metabolism

The results in Chapter 3 showed that dietary cholesterol ameliorated the HF-induced glucose intolerance without affecting adiposity and hepatic steatosis. A limitation of this study was the use of ipGTT, which is not an ideal measurement for hepatic insulin resistance and does not provide a measure of the tissue-specific insulin sensitivity. Further studies are therefore required to identify the responsible tissues. One feasible approach is the use of hyperinsulinaemic-euglycaemic clamp, which is regarded as the “gold standard” for the assessment of insulin sensitivity.

Specifically, the hyperinsulinaemic-euglycaemic clamp in combination with glucose tracers can be used to assess both whole-body and tissue-specific insulin action in HFC mice [443]. The hyperinsulinaemia is induced by infusing insulin into the carotid artery along with a glucose infusion at adjustable rates. The glucose infusion rate required for reaching euglycaemia is an indicator of whole-body insulin sensitivity. Once the euglycaemia has reached, a bolus of mixed $[^3]$H-$2$-deoxyglucose ($[^3]$H-$2$-DG) and $[^1]$C-glucose tracers is injected into the jugular vein followed by frequent sampling of blood to determine the glucose disappearance rate. The hepatic glucose output is calculated from the rate of the glucose disposal and glucose infusion rate to assess hepatic insulin sensitivity. Glucose utilization in the liver is determined by the $[^1]$C incorporated into glycogen and triglyceride. Insulin sensitivity in muscle and adipose tissue is assessed with the glucose metabolic index by counting the $[^3]$H-$2$-DG trapped in these tissues.
The effect of dietary fat and cholesterol on the concentration and distribution of key lipotoxic intermediates (such as ceramide and DAG) needs to be determined in both liver and muscle. These experiments will help to determine whether the ameliorated HF-induced glucose intolerance by cholesterol is due to the increased partitioning of ceramide and DAG into lipid droplets [377]. As dietary cholesterol was found to increase the protein level of SCD1 in the liver and increased SCD1 in the muscle has been reported to increase insulin sensitivity [381, 444], analysis of SCD1 content in the skeletal muscle should be considered. Furthermore, lipidomics analysis of saturated and unsaturated lipid species is needed to further confirm the effect of SCD1 in this animal model.

In addition, the data of energy expenditure was expressed by VO₂ per mouse due to the lack of a measuring instrument for lean mass at that time. Lean mass of mice could be measured together with VO₂ in future studies to provide a precise analysis of the effect of dietary fat and cholesterol on metabolic rate using a recent purchased MRI machine (EchoMRI-500).

5.2.1.2 Effect of Dietary Cholesterol on Inflammation

The data from Chapter 4 showed that dietary cholesterol increases mRNA expression of proinflammatory cytokines in the liver of HF-fed mice. However, signs of hepatic inflammation were not assessed by immunohistochemistry. In future studies the immunohistochemical detection of F4/80 or CD68, both markers of inflammatory cell infiltration, should be performed to further assess the degree of hepatic inflammation in this nutritional model.

In addition, the mechanisms underlying the effect of cholesterol on hepatic inflammation need to be further studied. Appropriate cell lines could be used to assess the effect of cholesterol on TLR4 activation independent of the confounding effects of endotoxins from the gut. For example, HepG2 cells could be used to mimic the hepatocytes, whereas J774 cells could be
used to mimic the macrophages in the liver. Cholesterol could then be added to these cell lines using cyclodextrin as a vehicle [445]. The mRNA expression of TLR4 and its downstream effectors IRF3, AP-1 and NFκB (Figure 1.7) could then be assessed by qRT-PCR.

5.2.1.3 Effect of Cholesterol on Absorption of Fat from the Gut

As reviewed in Section 1.4.1, dietary fat, particularly saturated fat, may facilitate the absorption of cholesterol in the gut [298]. It has been suggested that dietary cholesterol may also affect the absorption of fat through the interaction with FAT/CD36 [322] (Section 1.4.2.2). Therefore, further studies are required to measure the cholesterol and fat content in faeces in order to determine whether the concurrent administration of cholesterol (0.2% w/w) and fat (from 45% lard-based HF diet) affects their absorption.

5.2.2 Limitation and Future Research for Chapter 4

As shown in Chapter 4, temporary HFC feeding has prolonged effects on obesity and glucose intolerance. In order to investigate the underlying mechanisms of these “legacy effects”, further research should focus on the following areas.

5.2.2.1 Measurement of Mitochondrial Function and NASH Characteristics in the Liver

Due to the time constraints, this study was only able to focus on the major phenotypes, namely the protective effect of HFC-preconditioning against obesity and glucose intolerance. The effects of HFC-preconditioning on NASH characteristics (including histological analysis, plasma ALT levels and inflammation) and contributing factors for the development of NASH (mitochondrial function, ER stress and oxidative stress) in the liver were not studied. NASH characteristics and the effects on ER stress and oxidative stress can be studied as conducted in Chapter 3. Regarding inflammation, the technique of immunohistochemistry can be established and used to measure F4/80 or CD68 in the liver. Further analysis of these features
will help establish whether HFC-preconditioning may have any effect on the development of NASH.

The effect of preconditioning on mitochondrial function could be further studied using an appropriate cell line with the Seahorse Metabolic Analyser (Seahorse Bioscience). For example, a hepatic cell line HepG2 could be used with a mixture of FAs (palmitate and oleate) and cholesterol (to mimic the HFC diet) [446]. HepG2 cells could be “preconditioned” with two 24 h episodes of FAs [447], each followed by a 24-hour washout with a medium without the FA mixture. Subsequently, these “preconditioned” cells could be continually cultured in either a standard medium or treated with the FA mixture again for 24 hours to compare with cells not previously exposed to FAs. By using a Cell Mito Stress Test kit in conjunction with the Seahorse Analyser, the mitochondrial function of these cells, including basal respiration rate, protein leak and maximal respiration rate can be measured. The analyser could also be used to measure mitochondrial FA oxidation in the presence of palmitate-containing medium.

5.2.2.2 Identification of the Tissue(s) Responsible for the Beneficial “Legacy Effects”

PPARα is a key transcription factor that regulates lipid metabolism in the liver and in this context promotes the mRNA expression of FGF21 and ACOX1 [448]. Given the elevated FGF21 in circulation after HFC-preconditioning, firstly it would be important to examine whether this is a result of the PPARα activation in the liver. This can be achieved by measuring the mRNA expression of FGF21 and ACOX1, which are direct downstream targets of PPARα activation. It has been suggested that the effect of FGF21 on the “browning” is mediated by increasing PGC1α at the post-transcriptional level [118]. Secondly, measurement of PGC1 protein levels in WAT is necessary to test whether increased UCP1 expression may be a consequence of elevated FGF21 in the plasma. Thirdly, a similar animal study could be conducted with the FGF21-KO mice [449] to confirm the role of FGF21 in this model.
Chapter 5 - Summary, General Conclusion, Limitations and Future Directions

The direct effects of preconditioning on adipose tissue and muscle can be tested in cell models. For example, differentiated 3T3L1 preadipocytes can be used as adipocytes, L6 cells can be used as myotubes, and a mixture of FAs (palmitate and oleate) and cholesterol can be used to mimic the HFC diet. The experimental design is the same as described previously for HepG2 cells. Given the inconsistent effects of preconditioning on mRNA expression of proteins involved in mitochondrial metabolism in the muscle, the mitochondrial respiration of L6 cells could be measured by the Seahorse Metabolic Analyser as described for HepG2 cells. 3T3L1 cells could be tested for UCP1 and its upstream regulators PPARγ, SIRT1 and PGC1α using qRT-PCR and/or western blotting with specific antibodies [450]. These results will allow the assessment of the “legacy effects” independent of FGF21 and other possible factors (such as thyroid hormones) in the bloodstream.

Apart from circulating FGF21, elevated thyroid hormones and β3-adrenoceptor activation may also increase energy expenditure and the “browning” of WAT as reviewed in Section 1.1.4.2 [108, 136]. Total thyroid hormone levels (T3 and T4), and thyroid stimulating hormone in the plasma could be measured by radioimmunoassay kits [451, 452] to investigate whether thyroid gland may be involved in mediating the “legacy effects” of HFC-preconditioning. In addition, free T3 and T4 that are believed to biologically active forms of thyroid hormones could be also measured by radioimmunoassay or ELISA kits. The possible involvement of β-adrenoceptor activation in the effects of HFC-preconditioning could be tested using β3-adrenoceptor antagonist such as L-748,337 [453] in animal studies as described in Chapter 4.

5.2.2.3 Is DNA Methylation Involved in the “Legacy Effect”? 

DNA methylation has been proposed as one of the potential underlying mechanisms for the sustained effect of HF diets [435]. Therefore, further studies could be performed to investigate whether DNA methylation plays a role in mediating the “legacy effect” of HFC diet.
Firstly, the global DNA methylation can be measured in the relevant tissue identified above. The content of 5-methylcytosine in DNA extract can be then measured by ELISA. Secondly, the methylation of specific genes (e.g. PPARα in the liver and SIRT1 in the WAT) can be measured by methylation specific PCR by converting unmethylated cytosine in the sequence to uracil without affecting methylated cytosine [454]. The DNA methylation can be quantified based on an amplification reaction using primers specific for methylated and unmethylated DNA.

5.2.2.4 Could Short-Chain FAs Induce Protective “Legacy Effects”?

It is worthwhile to examine whether a diet rich in SCFA may be used as a substitute for the HFC diet in preconditioning. Firstly, SCFAs are likely the breakdown products during the washout with the CH diet because of the utilisation of accumulated fat (indicated by reduced body weight in Figure 4.2A). Secondly, certain SCFA has been shown to induce the synthesis of FGF21 [437], and increased FGF21 can promote energy expenditure involving the “browning” of WAT [118]. Thirdly, SCFAs have been suggested to promote FGF21 expression [437] and increase energy expenditure [438] without causing obesity and associated metabolic disorders. If a SCFA is able to induce beneficial legacy effects, it can be used to replace the HFC diet for the preconditioning to avoid the adverse effects of HFC. Such studies could be conducted in mice using the same experimental design described in Chapter 4, by replacing the HFC diet with a diet rich in SCFAs for the preconditioning. The effects of SCFA-preconditioning on obesity, glucose tolerance and NAFLD could be assessed and compared with the effects as described in Chapter 4.
5.3 Final Conclusion

Overall, the research in this thesis provides new insights into the effects of dietary fat and cholesterol, alone or in combination, on the pathogenesis of obesity, glucose intolerance and NAFLD. The obtained data suggest a crucial role of cholesterol in the progression from simple hepatic steatosis to NASH in mice. Furthermore, the results from this thesis indicate the pre-exposure to a HFC diet has prolonged effects on whole-body metabolism including adiposity and glucose intolerance in mice. These data in mice provide a basis for further investigations on the relationship between cholesterol intake and NASH risk, and “legacy effect” of dietary components in humans.
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