DIETARY BROCCOLI IMPEDES WESTERN DIET-ENHANCED FATTY LIVER AND HEPATOCELLULAR CARCINOMA DEVELOPMENT

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science and Human Nutrition with a concentration in Human Nutrition in the Graduate College of the University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

Liver is the metabolic center for energy homeostasis in our body, maintaining a balance between carbohydrate and fat metabolism. The “Westernized” dietary pattern, which is known for high saturated fat and refined sugar and rooted in the lifestyle of a large proportion of the world’s population, may greatly disrupt energy balance, resulting in obesity and obesity-related diseases. Non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma, which is also a possible endpoint of NAFLD, are both enhanced by adiposity and inflammation, and almost symptomless until serious damage is caused in liver. However, these diseases can be preventable, by changing lifestyle and diet. Broccoli, a well-accepted brassica vegetable in the United States, has the potential to reduce cancer risk and ameliorate inflammation. Therefore, in this study, we aimed to understand the impact of dietary broccoli on the development of NAFLD and liver cancer in mice fed a Western diet. Accordingly, we proposed a whole dietary broccoli intervention. A combined Western diet-fed and diethylnitrosamine (DEN)-treated mouse liver cancer models was used in order to evaluate the changes in hepatic lipidosis, macrophage activation, and tumorigenesis after long-term consumption of broccoli. Our results show that dietary broccoli decreased hepatic lipidosis as early as 3 months after initiation, and effectively down-regulated liver damage. The enlarged hepatic triglyceride pool due to the Western diet was narrowed by dietary broccoli, lowering the influx of non-esterified fatty acids but increasing the excretion of very-low-density lipoprotein. Activation of hepatic macrophages, was lowered by continues consumption of broccoli. Furthermore, DEN-induced liver tumor size and hepatic neoplasm-related lesion formation were both decreased by dietary broccoli. In addition, as an incidental finding, intraperitoneal DEN-induced nasal epithelial neoplasm-related lesions in
B6C3F1 mice are first reported in this study. Overall, whole broccoli dietary intervention has the potential to impede the progression of NAFLD, from hepatic steatosis, through steatohepatitis, to hepatocellular carcinoma. This study fills gaps of knowledge about the impact of broccoli on hepatic lipid metabolism, supports the cancer preventive effect of brassicas revealed by epidemiologic studies, and further encourages the whole food dietary intervention. Translation to clinical studies is needed.
To my beloved parents, Jong-Yih Chen and Hsiang-Wen Fu for their inspiration, encouragement, and unconditional love.
ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my advisor Dr. Elizabeth H. Jeffery for her guidance, support, and unlimited encouragement in the past four years. She was a great mentor and always gave wise advice. Every time we met, I got new inspiration from our discussion about science, or even from the conversation about gardening. I would like to extend my sincere thanks to my committee members, Dr. John W. Erdman, Dr. Yuan-Xiang Pan, and Dr. Matthew A. Wallig, for sharing their precious knowledge and time. Special thanks to Dr. Matthew A. Wallig for his contribution to this project, providing excellent interpretation in pathology. I wish to thank all my brilliant lab mates, too. My dissertation journey was pleasant and memorable because of their selfless assistance. Thanks Molly Black for her outstanding lab techniques, Dr. Angela Myracle for initiating this research, Dr. Edward Dosz for assisting the analysis of sulforaphane content, Drs. Donato Angelino and Nilanjan Das for helping in animal studies, Yanling Wang for her positive attitude, and my undergrad assistants for dealing with trivial lab work. I also appreciate the help from the staff and faculty in the Department of Food Science and Human Nutrition and the Division of Nutritional Sciences. My dearest friends in Taiwan, Yi-Ning Huang and Wan-Yi Lin, and my brothers and sisters in Illini Chinese Christian Fellowship and Champaign Chinese Christian Church, thanks for continuous encouragement and prayers. Furthermore, thank you for the endless love and support, my beloved mother, Hsiang-Wen Fu, and my dear brother, Yung-Yu Chen. Last but not least, I would like to give thanks to God for pouring out His abundant grace on me and strengthening me when I was weak.

Yung-Ju Chen
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AHF</td>
<td>Altered hepatic foci</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AMPK</td>
<td>5' AMP-activated protein kinase</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Apo</td>
<td>Apolipoproteins</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CCL2</td>
<td>C-C motif chemokine receptor 2</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
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<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyltransferase-1</td>
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<td>Cyp</td>
<td>Cytochrome P450</td>
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<td>DEN</td>
<td>Diethylnitrosamine</td>
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<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>FATP</td>
<td>Fatty acid transport protein</td>
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<td>FAS</td>
<td>Fatty acid synthase</td>
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<td>GADPH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>HA</td>
<td>Hepatic adenoma</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>KEAP1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Abbreviation</td>
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<tr>
<td>MTTP</td>
<td>Microsomal triglyceride transfer protein</td>
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<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
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<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cell</td>
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<td>Nrf2</td>
<td>Nuclear factor erythroid-2-related factor</td>
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<tr>
<td>NQO1</td>
<td>NAD(P)H: quinone oxidoreductase 1</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RR</td>
<td>Relative risk</td>
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<td>SREBP</td>
<td>Sterol regulatory element-binding proteins</td>
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<td>Signal transducer and activator of transcription 3</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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1.1. Introduction

A high prevalence of obesity is observed all over the world. The world obese population has doubled since 1980, so that in 2014, 600 million people are obese, meaning that on this planet more than one in every ten people is obese (World Health Organization 2015). The breakdown of energy homeostasis, which means that energy consumption is higher than energy expenditure, results in the increased deposition of adipose tissue in the body and causes obesity (Martinez 2000). Dietary choices can be considered as a major factor that influences overall energy intake. In the United States, dietary surveys reveal a pattern of high dietary fat and refined sugar, which is well accepted by consumers but easily supplies excess energy (U.S. Department of Agriculture, ARS 2014). This dietary pattern, also called a Westernized dietary pattern, no doubt contributes to the development of obesity. Obesity damages health, and not only reduces lifespan (increasing mortality) but also life quality (causing chronic diseases) (Must, Spadano et al. 1999; Flegal, Kit et al. 2013). Increased adiposity may result in chronic inflammation that contributes to the development of many chronic diseases (Gregor and Hotamisligil 2011). Non-alcoholic fatty liver disease (NAFLD) is one of these obesity-related diseases: the prevalence of NAFLD may be as high as 3 in every 4 obese people, while it is less than 1 in every 5 people of normal body weight (Bellentani, Saccoccio et al. 2000). Development of NAFLD may be symptomless but nevertheless possibly lead to liver cancer, which is associated with a high mortality rate (Salt 2004; Fabbrini, Sullivan et al. 2010). Dietary intervention, such as inclusion of brassicas, is one
approach to interfere with the progression of NAFLD and liver cancer. Brassica crops are
common in our daily diet, and rich in bioactive compounds that benefit health, such as
decreasing inflammatory cytokines, regulating lipid metabolism, and suppressing cancer
development (Jeffery and Araya 2009; Rodriguez-Cantu, Gutierrez-Uribe et al. 2011).

1.2. Obesity

1.2.1. Overview

According to World Health Organization (WHO), obesity is defined as “abnormal and
excessive fat accumulation that may impair health” (World Health Organization 2015). Body
mass index (BMI) is one criterion used to describe obesity, expressed as body weight (kg)
divided by the square of body height (m). In the United States, an adult whose BMI is between
25 and 29.9 is considered overweight, and someone who has a BMI ≥ 30 is considered obese
(Center for Disease Control and Prevention 2012). Almost 35% of the adult population in the
United States is obese (Ogden, Carroll et al. 2014). Obesity has a broad impact on health,
increasing the incidence of many diseases, including cardiovascular diseases, type 2 diabetes,
osteoarthritis, and cancers (Visscher and Seidell 2001). These obesity-related chronic diseases
not only decrease the quality of life but also increase the economic burden. Obese people may
need to spend 30% more on medical costs compared to people of normal body weight (Withrow
and Alter 2011). The possible etiological factors influencing obesity include genetics, diet,
metabolism, and physical activity. These factors may contribute to the breakdown of energy
homeostasis and cause the increase in fat accumulation (Martinez 2000). Increasing calorie
consumption from diet and/or decreasing energy expenditure through lack of exercise can
promote the storage of excess energy in the form of fat (Spiegelman and Flier 2001).
Dietary patterns and habits have changed over time, due to a shift in the cultural, social, and economic environment, and the so-called “Westernized” diet, rich in refined sugar and fat, the popularity of which is spreading all over the world (Cordain, Eaton et al. 2005). People in the United States are influenced deeply by this Westernized dietary pattern. In 2012, adults who were more than 20 years old consumed 49% daily calories from carbohydrate, from which sugar provided 21% of total energy (U.S. Department of Agriculture, ARS 2014). According to the Dietary Guidelines, within total sugar consumption, more than 70% of calories are from added sugar, including high fructose corn syrup, white sugar (sucrose), fructose sweeteners, etc., which provide 16% of daily energy (U.S. Department of Agriculture and Department of Health and Human Services 2010). For dietary fat, the intake of total fat among American adults provides 33% of daily energy, and one third of the total fat is from saturated fat (U.S. Department of Agriculture, ARS 2014). Among all dietary fat sources, the total consumption of solid fat (saturated fat and trans fat) contributes 19% of total calories, and added sugar (70% of total sugar) is 16%, therefore the overall intake of solid fat and added sugar together provides 800 calories, around 35% of daily energy, to our bodies (U.S. Department of Agriculture and Department of Health and Human Services 2010). Without sufficient physical activity and energy expenditure, these 800 calories could become “excess” energy, and be transformed into body fat for storage, causing obesity.

1.2.2. Diet-induced obesity

1.2.2.1. Human epidemiologic and clinical studies

Dietary fat and sugar may promote obesity by increasing energy density of diets, resulting in excess supply of energy. Food items which are rich in fat and/or sugar have high energy density (Drewnowski 1998). With growing dietary energy density, BMI and body weight have increased
in the female population in the United States, and overall, obese people consumed diets with higher energy density (1.95 kcal/g) compared with diets of people with normal BMI (1.87 kcal/g) (Ledikwe, Blanck et al. 2006).

High dietary fat is considered to be able to contribute to the increase in body fat accumulation (Hill, Melanson et al. 2000). Results from a meta-analysis show that low fat dietary intervention without calorie restriction can decrease body weight (Astrup, Grunwald et al. 2000). Combined data from 20 countries suggest that there is a positive correlation between the percentage of energy from fat and the prevalence of overweight (BMI ≥ 25) (Bray and Popkin 1998). The effects of dietary restriction on reducing body mass might be greater in the obese population than in those with normal body weight, because for obese people reducing even 1% of dietary fat can result in 3.22 g/day of weight loss whereas it is 0.99 g/day in the overall population (Bray, Paeratakul et al. 2004). However, some studies have argued that high dietary fat might not be the primary determinant of obesity, for the percentage of daily calories from dietary fat has been relatively stable over the years while the percentage of the population that is overweight has constantly increased (Willett 1998). Moreover, a low dietary fat intervention showed compensatory effects on body weights in trials of more than one year duration (Willett 2002). Even though there is a different voice for the impact of dietary fat on obesity, low fat dietary intervention is well accepted as a method to reduce body weight.

Added sugar in diets is mostly from soft drinks, fruit drinks, pastry and dairy desserts, and more than 45% is actually from sugar sweetened beverages, including soft drinks, energy drinks and fruit drinks (U.S. Department of Agriculture and Department of Health and Human Services 2010). Take sugar sweetened beverages as an example. For adolescents, two or more servings per day of sugar sweetened beverages can significantly increase body weight in a year (Berkey,
Rockett et al. 2004). A similar result was seen in an adult population. Raising the frequency of consuming sugar sweetened beverages from < 1 serving/week to > 1 serving/day resulted in more than 4.2 kg of body weight gain in 4 years (Schulze, Manson et al. 2004). According to the results from a meta-analysis of random control trials and cohort studies, the risk of being obese is 1.55 (odds ratio) in people with a high intake of sugar sweetened beverages, compared to those with a low intake (Te Morenga, Mallard et al. 2013).

1.2.2.2. Animal models

There is no specific definition of obesity for laboratory animals. Usually animals are defined as obese when their body weights are significantly higher than the control animals, based on the assumption that the control animals are lean (Novelli, Diniz et al. 2007). C57BL/6J is a mouse strain that is commonly used for diet-induced obesity studies, because compared with other strains it is less resistant to high dietary fat treatment (Collins, Martin et al. 2004). C57BL/6J mice have higher feed efficiency (weight gained/calorie consumed) on a high fat diet compared to A/J mice (Surwit, Feinglos et al. 1995). Response to a high fat diet, in terms of elevated blood glucose and insulin, is dramatically higher in C57BL/6J mice, indicating that this strain of mouse has abnormal metabolism that may contribute to fat accumulation (Parekh, Petro et al. 1998).

For rodent models, diets that contain more than 40% of total energy from fat can result in obesity (Buettner, Scholmerich et al. 2007). Fat sources with different fatty acid profiles (saturated, monounsaturated, and polyunsaturated fatty acids) have a diverse impact on obesity (Hariri and Thibault 2010). A high content of saturated fatty acids can induce obesity more easily than polyunsaturated fatty acids (Wang, Storlien et al. 2002). This might be due to the poor utility of saturated fatty acids (Storlien, Huang et al. 2001). Among the fatty acids with the same number of carbons, the oxidation rate of saturated fatty acid was lowest in rats (Leyton,
Increasing the sucrose portion of total carbohydrate in energy density-controlled rodent diets showed no effect on body weight and body fat in either mouse or rat models (Storlien, Kraegen et al. 1988; Surwit, Feinglos et al. 1995). A high sucrose diet may increase total calorie intake, but is not efficient for increasing body weight and fat mass (la Fleur, van Rozen et al. 2010; Pranprawit, Wolber et al. 2013). However, a high fat and high sucrose diet (40% of energy from fat and 41% of energy from sucrose) can induce similar obesity outcomes to that seen with a simple very high fat diet (60% of energy from fat and 4% of energy from sucrose) (Pranprawit, Wolber et al. 2013).

1.2.3. Obesity related chronic diseases

Increasing body weight is related to the prevalence of many chronic diseases. In the United States, epidemiological studies show that the prevalence ratios (people with normal body weight as the reference) for type 2 diabetes, gallbladder diseases, coronary heart diseases and high blood pressure increase with BMI, and the influence is even higher in the older population (age ≥ 55 years) (Must, Spadano et al. 1999). For the overweight population, the risks of suffering from type 2 diabetes and high blood pressure are both 1.5-fold higher than for people with normal BMI; for morbidly obese people whose BMI are more than 40, these risks become 6-fold higher than for people with normal BMI (Mokdad, Ford et al. 2003).

According to WHO, being overweight and obese is also a risk factor for cancer (World Health Organization 2015). More than 35% of cases of colorectal cancer in male and more than 55% of cases of endometrial cancer in female are related to obesity (Calle and Kaaks 2004). For liver cancer, the risk is close to 2-fold higher in the obese population compared to people with normal body weight (Larsson and Wolk 2007). In 2015, the World Cancer Research Fund International
has officially included body fatness as a risk factor for liver cancer (World Center Research Fund International/American Institute for Cancer Research 2015). Moreover, adiposity not only increases the risk of cancer incidence but also the mortality from cancers. The death rate from all types of cancer increases around 50% for the morbidly obese population compared to those with a normal BMI in the United States (Calle, Rodriguez et al. 2003).

Obesity can elevate non-esterified fatty acids (NEFA) in circulation, which may result from the increased adipose tissue which has a high ability for lipolysis (Boden 2008). Increased expression of tumor necrosis factor-α (TNF-α) in adipose tissue can stimulate lipolysis and release NEFA as well (Greenberg and Obin 2006). A chronic high level of NEFA is able to inhibit insulin secretion from pancreatic β cells and exacerbate insulin resistance (Rosen and Spiegelman 2006). Therefore, NEFA may be a critical factor connecting obesity and type 2 diabetes (Kahn, Hull et al. 2006). NEFA may also be involved in the development of cardiovascular disease by causing endothelial dysfunction, due to lipotoxicity (Van Gaal, Mertens et al. 2006). Moreover, proinflammatory cytokines secreted from enlarged adipose tissue, including interleukin (IL) -6 and TNF-α, may aggravate systemic inflammation and accelerate the progression of cardiovascular diseases (Libby, Ridker et al. 2002). Inflammation is also considered a crucial link between obesity and cancer development (Khandekar, Cohen et al. 2011). Therefore, inflammation is an important determinant in obesity-related diseases.

1.2.4. Inflammation

1.2.4.1. Definition of inflammation

Inflammation is an adaptive response to endogenous or exogenous stimuli, such as infection or trauma (Medzhitov 2008). In brief, the mechanism of inflammation starts from the recognition of inducers, including pathogen-associated molecular patterns from pathogens and damage-
associated molecular patterns from endogenous systems, by membrane and intracellular receptors, such as toll-like receptors (TLR) and NOD-like receptors. Once TLR detects the ligands, the signal can activate the nuclear factor κ-light-chain-enhancer of activated B cell (NF-κB) pathway and release proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, which can attract neutrophils and monocytes to the affected site. Neutrophils can excrete cytotoxic reactive oxygen and nitrogen species in order to damage and kill pathogens. Macrophages, derivatives from monocytes, can remove the stimuli by phagocytosis. Further adaptive responses can also come from the polarization of native T-helper (Th) cells: Th1 cells (proinflammatory), Th2 cells (anti-inflammatory), Th17 cells (proinflammatory) and T regulatory cells (regulatory), and these Th cells can “cross-talk” to each other by using different kinds of cytokines (Ashley, Weil et al. 2012).

The initial response to stimuli is called acute inflammation, because the duration is usually from minutes to hours. The main cellular participants are neutrophils, and they move to the site rapidly to eliminate the stimulus but often cause tissue damage. However, if acute inflammation is not able to remove or neutralize pathogens and internal stimuli, a prolonged phase called chronic inflammation may occur, which could last for months. In chronic inflammation, mainly monocytes and macrophages infiltrate into tissue, resulting in ongoing damage and often fibrous tissue deposition. Some of the possible reasons for chronic inflammation are persistent infections, hypersensitivity, and prolonged exposure to stimuli (Vinay Kumar 2014).

1.2.4.2. Obesity induced inflammation

Obesity may be responsible for low-grade, chronic, systemic inflammation (Gregor and Hotamisligil 2011). Adipose tissue is thought of as an immune organ and also a part of the endocrine systems (Fantuzzi 2005). Adipocytes can excrete a group of hormones called
adipokines, including adiponectin and leptin (Kershaw and Flier 2004). Adiponectin can decrease plasma NEFA levels and improve insulin-mediated hepatic glucose output (Berg, Combs et al. 2002). Moreover, adiponectin exhibits the ability to regulate inflammation by down-regulating the proinflammatory cytokine TNF-α and up-regulating the anti-inflammatory cytokine IL-10 (Masaki, Chiba et al. 2004; Wolf, Wolf et al. 2004). However, plasma adiponectin concentration is negatively correlated with fat mass (Cnop, Havel et al. 2003). Leptin is able to regulate energy balance, by modeling behavior such as food intake and energy expenditure and by influencing hormone secretion from the hypothalamus, such as neuropeptide Y (Friedman and Halaas 1998). Leptin may also alter the adaptive immune response to proinflammatory Th1 cells (Lord 2002).

Furthermore, in an obese rodent model, significant accumulation of macrophages was observed in adipose tissue, and these macrophages were responsible for the enhanced expression of TNF-α and IL-6 (Weisberg, McCann et al. 2003). The excess intake of nutrients in obese subjects may be the stimulus to trigger inflammation (Gregor and Hotamisligil 2011). Saturated fatty acids have shown to be a natural ligand for TLR4, and can induce the expression of the NF-κB downstream gene, cyclooxygenase 2 (COX-2) (Merkel, Velez-Carrasco et al. 2001; Suganami, Tanimoto-Koyama et al. 2007).

1.3. Liver Diseases Related to Obesity

1.3.1. Non-alcoholic fatty liver disease (NAFLD)

1.3.1.1. Prevalence

Fatty liver, also called hepatic steatosis, is defined as excessive intrahepatic triglyceride accumulation in liver, which is either 1) more than 5% of total liver weight or volume, or 2)
more than 5% of hepatocytes affected by intracellular triglyceride accumulation (Lebovics and Rubin 2011). Hepatic triglyceride accumulation can be induced by over-consumption of alcohol, which is referred to as alcoholic fatty liver disease; the prevalence of fatty liver is more than 60% among heavy drinkers (Grant, Dufour et al. 1988). Metabolism of alcohol may increase the NADH/NAD\(^+\) ratio, which impairs the tricarboxylic acid cycle and fatty acid β-oxidation, up-regulates sterol regulatory element-binding protein (SREBP)-1 and down-regulates peroxisome proliferator-activated receptor (PPAR) α, and thus results in increased lipid in liver (Purohit, Gao et al. 2009). Moreover, alcohol can be oxidized by cytochrome P450 (Cyp) 2E1, generating reactive oxygen species (ROS) and resulting in liver injury (Lu and Cederbaum 2008).

However, hepatic steatosis is not only induced by alcohol but also other factors, including obesity, type 2 diabetes, and hyperlipidemia; this type of steatosis is categorized as non-alcoholic fatty liver disease (NAFLD) (Angulo 2002). The cutoff currently used to separate alcoholic and non-alcoholic fatty liver is 20 g alcohol/day (Neuschwander-Tetri and Caldwell 2003). The prevalence of NAFLD is about 22% based on data from liver ultrasound, whereas it is about 3-23% based on elevated plasma alanine aminotransferase (ALT) (Farrell and Larter 2006). For the obese population, prevalence of NAFLD becomes more than 75%, compared to 16% in non-obese people (Bellentani, Saccoccio et al. 2000). NAFLD usually shows no symptoms, and even when symptoms appear, they are non-specific, such as fatigue, weight loss, and nausea, right up until the liver function is highly damaged (Salt 2004).

The term NAFLD does not refer to a single disease, but is a term that describes a spectrum of related liver disorders, from hepatic steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and possibly hepatocellular carcinoma (Fig. 1.1) (Cohen, Horton et al. 2011). The time taken for progression from simple hepatic steatosis to NASH has not been determined is
probably quite variable depending on a variety of factors, but the available reports show that this progression might be both rare and slow (Vernon, Baranova et al. 2011). However, Day et al. proposed the “two-hit” model of the pathogenesis of NASH, and suggested that hepatic steatosis is the “first hit”, which may increase the susceptibility of liver for the “second hits,” including proinflammatory cytokines, oxidative stress, and malfunction of mitochondria (Day and James 1998; Day 2002). This suggests that hepatic steatosis is the initial stage of NAFLD. About 25-35% of NASH patients develop fibrosis, while only 9-20% of NASH patients progress further into cirrhosis. Moreover, about 10% of people with cirrhosis may progress to hepatocellular carcinoma within 7 years (Ong and Younossi 2007).

1.3.1.2. Causes and risk factors

The primary causes of NAFLD are obesity, type 2 diabetes, hyperlipidemia, and insulin resistance, but there are multiple other secondary causes, including nutritional issues (e.g. total parental nutrition), drugs (e.g. glucocorticoid), metabolic disorders (e.g. lipodystrophy), toxins (e.g. phosphorus poisoning), and infection (e.g. hepatitis C virus) (Angulo 2007). Ethnicity, gender, and age are also risk factors of NAFLD. In the United States, the prevalence of NAFLD among Hispanics is highest (45%), then whites (33%) and then African Americans (24%) (Browning, Szczepaniak et al. 2004). The white male population has a higher incidence (42%) compared to the female (24%), similar to the Asian male population, whose risk is 3.5 times higher than Asian females (Browning, Szczepaniak et al. 2004; Weston, Leyden et al. 2005). In Japan, the prevalence in females is low in those younger than 60 years of age, but after that it becomes higher than in Japanese males (Hashimoto and Tokushige 2011).

A study conducted in Italy showed that as many as 75% of obese people may have NAFLD (Bellentani, Saccoccio et al. 2000). From the alternative perspective, NAFLD patients have
higher BMI (28.2±4.0 vs. 24.2±2.0) and waist circumference (92±13 cm vs. 80±12 cm) than people with normal liver lipid levels (Marchesini, Brizi et al. 1999). In a prospective study, there was a 74% incidence of NAFLD found among diabetics (Williams, Stengel et al. 2011). Moreover, for people with metabolic syndrome, where symptoms include high blood triglycerides, high blood glucose, high blood pressure, large waist circumference, and low high density lipoprotein, they showed higher prevalence of liver fibrosis and inflammation (Marchesini, Bugianesi et al. 2003). These studies show that obesity strongly correlates with initiation and progression of NAFLD.

1.3.1.3. Mechanism of hepatic triglyceride accumulation

1.3.1.3.1. Overview

Liver is the major body organ to metabolize carbohydrate and lipid, thereby maintaining the homeostasis of blood glucose (Postic, Dentin et al. 2004). Hepatic triglycerides may come from diet (15%), circulating NEFA (60%), and de novo lipogenesis (25%) (Donnelly, Smith et al. 2005). Figure 1.2 is a diagram of fatty acid flux in liver and shows that dietary fat may flux in to liver either by being released as NEFA from chylomicrons, ultimately increasing the circulating NEFA pool, or through the uptake of chylomicron remnants. Circulating NEFA is mainly from adipose tissue, as the product of lipolysis. Furthermore, dietary carbohydrate can be metabolized and generate acetyl-CoA which becomes the substrate for de novo lipogenesis. Fatty acids in liver can be esterified and become triglycerides for storage (hepatic intracellular triglycerides) or for excretion as very low-density lipoprotein (VLDL), or be oxidized to produce energy (β-oxidation and ω-oxidation). Mis-regulation of hepatic triglyceride metabolism can lead to steatosis.
1.3.1.3.2. Membrane fatty acid transporters

In the circulation, NEFA have two major routes to cross the cell membrane and translocate into hepatocytes. One is passive diffusion and the other is through membrane transporters (Hamilton, Johnson et al. 2001; Schwenk, Holloway et al. 2010). Fatty acid transporters expressed in liver include plasma membrane fatty acid binding protein (FABP), fatty acid transport protein (FATP), caveolin and cluster of differentiation (CD) 36 (Glatz, Luiken et al. 2010). CD36 can facilitate the transportation of long-chain and very long chain fatty acids, and is regulated by PPARγ, long-chain fatty acids, and insulin (Ibrahimi and Abumrad 2002; Luiken, Dyck et al. 2002). Among patients exhibiting NAFLD, it has been observed that CD36 expression in liver is highly up-regulated, but other transporters such as FABP, FATP5 and caveolin are not (Bechmann, Gieseler et al. 2010). In mouse high fat diet models, both short-term (5 weeks) and long-term (12 weeks) high fat diets caused elevated liver CD36 mRNA expression (Inoue, Ohtake et al. 2005; Koonen, Jacobs et al. 2007; Gaemers, Stallen et al. 2011). Moreover, increased hepatic CD36 expression can raise fatty acid uptake and enhance triglyceride synthesis, aggravating hepatic lipidosis (Koonen, Jacobs et al. 2007). These effects make CD36 a good marker to monitor fatty acid dysregulation in liver.

1.3.1.3.3. De novo lipogenesis

De novo fatty acid synthesis is a sequential extension of an alkanoic chain, starting with acetyl-CoA as a primer (Nguyen, Leray et al. 2008). Two major reactions are catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The first step, which is the carboxylation reaction of acetyl-CoA, forms malonyl-CoA, and is catalyzed by ACC.

\[
\text{(i) } 7 \text{ Acetyl-CoA} + 7 \text{ ATP} + 7 \text{ CO}_2 \xrightarrow{\text{ACC}} 7 \text{ Malonyl-CoA} + 7 \text{ ADP} + 7 \text{ Pi}
\]
When lipogenesis is up-regulated, fatty acid oxidation is restrained, since malonyl-CoA inhibits carnitine palmitoyltransferase-1 (CPT-1), which decreases the influx of fatty acids to the mitochondrion for β-oxidation (Saggerson 2008). In contrast, ACC activity, and thus lipogenesis, can be down-regulated post-translationally by phosphorylation through protein kinase A and AMPK (5′ AMP-activated protein kinase) (Brownsey, Boone et al. 2006). Protein kinase A activity is mainly controlled by hormone levels (glucagon and epinephrine) via the formation of cAMP (Tasken and Aandahl 2004). Activation of AMPK is induced by an increasing the AMP:ATP ratio, which is affected by to exercise and calorie deprivation (Luo, Saha et al. 2005). High fat and/or high sucrose diets can decrease hepatic AMPK phosphorylation, which may maintain ACC activity at a higher levels than a normal diet (Barroso, Rodriguez-Calvo et al. 2011; Li, Xu et al. 2011). Expression of ACC is also positively regulated by SREBP-1, which can be upregulated by dietary fat (Horton, Goldstein et al. 2002). However, some studies report that a high fat diet could actually down-regulates ACC mRNA expression (Tanaka, Aleksunes et al. 2008; Shin, Wakabayashi et al. 2009).

Then, the next step of lipogenesis is controlled by FAS, which catalyzes the reaction from malonyl-CoA to palmitic acid.

\[
(ii) \text{ Acetyl-CoA} + 7 \text{ Malonyl-CoA} + 14 \text{ NADPH} + 14 \text{ H}^+ \xrightarrow{\text{FAS}} \text{ Palmitic acid} + 7 \text{ CO}_2 + 8 \text{ CoA} + 14 \text{ NADP}^+ + 16 \text{ H}_2\text{O}
\]

The transcription factor SREBP-1c regulates FAS, and is itself highly regulated by liver X-activated receptor and hormones, including insulin and glucagon (Horton, Goldstein et al. 2002). Expression and activity of SREBP-1c is seen to be up-regulated by high dietary fat and carbohydrate in the rodent model, together with increased FAS expression, and is accompanied
by hepatic lipidosis (Li, Xu et al. 2011). In a simple high fat diet model, long-term high saturated fat feeding (12 weeks) also enhanced both FAS and SREBP-1 expression in rats (Buettner, Parhofer et al. 2006). However, this action is similar to ACC where some studies showed that both short-term (1 or 4 weeks) and long-term (12 weeks) high fat diet treatment can decrease hepatic FAS mRNA expression, and the SREBP-1 level was not changed (Kim, Sohn et al. 2004; Inoue, Ohtake et al. 2005; Tanaka, Aleksunes et al. 2008; Shin, Wakabayashi et al. 2009). It suggests that the efficiency of hepatic de novo lipogenesis can be regulated in terms of expression of FAS and ACC, both of which can be controlled by multiple pathways that are altered under conditions of high dietary fat.

1.3.1.3.4. Hepatic release of lipids

The major excretory path for hepatic lipid is being part of the complex VLDL (Fig. 1.2), which is composed of lipids and apolipoproteins (Apo), mainly triglycerides and Apo B-100, respectively (Gibbons, Wiggins et al. 2004). Microsomal triglyceride transfer protein (MTTP) is essential for Apo B-100 assembly in VLDL, preventing degradation of Apo B and facilitating lipidation of Apo B (Hussain, Shi et al. 2003). Overexpression of hepatic MTTP in mice is shown to enhance secretion of triglycerides and Apo B-100 (Tietge, Bakillah et al. 1999). It suggests that MTTP could be a target to control efflux of hepatic triglyceride. Insulin appears to negatively control MTTP expression and VLDL excretion, and macronutrients, such as fat, may also be involved in the regulation of hepatic MTTP (Hussain, Nijstad et al. 2011). A high fat diet was seen to decrease hepatic MTTP mRNA expression after a short period (1 week, decrease of 30%) and long-term treatment (16 weeks, decrease of 60%) in rodent models (Tanaka, Aleksunes et al. 2008; Chang, Yan et al. 2010). However, the response to a high fat diet in rodents is not consistent. For example, hepatic MTTP mRNA level was increased after 8 weeks
of high fat dietary treatment in a hamster model (Guo, Huang et al. 2011). Therefore, the mechanism of MTTP regulation by dietary fat is still not clear and differs among species.

1.3.1.3.5. Fatty acid oxidation

Fatty acids can be oxidized through several mechanisms, including α-oxidation, β-oxidation, and ω-oxidation, with β-oxidation being the major path among these three (Wanders, Komen et al. 2011). Both mitochondria and peroxisomes have the ability to carry out β-oxidation, and in pig liver, 49-63% of total β-oxidation was found to occur in mitochondria (Yu, Drackley et al. 1997). The enzyme which is responsible for transferring fatty acids into mitochondria, CPT-1, tightly controls mitochondrial β-oxidation (Schreurs, Kuipers et al. 2010). Malonyl-CoA can inhibit CPT-1 as described previously, and CPT-1 is also one of the downstream genes of PPARα (Saggerson 2008; Burri, Thoresen et al. 2010). Patients with NAFLD showed a decreased expression of CPT-1α in liver (Kohjima, Enjoji et al. 2007). Similarly, in rat models, providing a high fat diet for 14-16 weeks has resulted in down-regulated hepatic CPT-1α (Quesada, del Bas et al. 2009; Chang, Yan et al. 2010). However, in a mouse model, high fat dietary treatment for 12 weeks caused an increased expression of CPT-1α in liver (Kim, Sohn et al. 2004; Guo, Huang et al. 2011). This may imply that rodent models are not always able to reflect CPT-1 regulation in humans.

Although ω-oxidation is a minor mechanism for fatty acid oxidation, it has been proposed that it could be a rescue pathway when β-oxidation is impaired (Wanders, Komen et al. 2011). However, Cyp 2E1, a microsomal enzyme that can perform ω-oxidation, may participate in the development of NAFLD by producing ROS and thus increasing oxidative stress (Leung and Nieto 2013). Expression of Cyp 2E1 was highly up-regulated in NAFLD patients, more than 10 fold compared to healthy people (Kohjima, Enjoji et al. 2007). It was also shown that in mice
given a high fat diet, hepatic Cyp 2E1 protein level and activity were both increased (Abdelmegeed, Banerjee et al. 2012). Furthermore, proinflammatory TNF-α levels are lower in Cyp 2E1-null mice compared to wild type mice, independent of dietary fat content, indicating that Cyp 2E1 may not only increase oxidative stress but also inflammation (Abdelmegeed, Banerjee et al. 2012).

1.3.1.4. Animal models for studying diet-induced NAFLD

1.3.1.4.1. Methionine and choline deficient diets

Metabolism of both methionine and choline is strongly related to the production of S-adenosylmethionine, which is the universal donor of methyl groups (Corbin and Zeisel 2012). An insufficient source of methyl groups can lead to multiple liver diseases, including NAFLD (Mato, Martinez-Chantar et al. 2008). One week of methionine and choline deficient dietary treatment can significantly increase hepatic triglyceride and plasma ALT levels in mice, but this is accompanied by body weight loss (Park, Jeon et al. 2011). Choline deficiency may decrease hepatic phosphatidylcholine levels, which is critical for VLDL assembly, and may inhibit the excretion of VLDL from liver (Yao and Vance 1990; Zeisel and Blusztajn 1994). Methionine and choline deficient diets can increase β-oxidation (both peroxisomal and mitochondrial) in the first week, but this effect is diminished after 5-6 weeks of feeding (Ip, Farrell et al. 2003; Park, Jeon et al. 2011). However, reports showed that Cyp 2E1, which contributes to ω-oxidation and oxidative stress, is also up-regulated in the methionine and choline-deficient rodent models (Weltman, Farrell et al. 1996). Hepatic IL-6 and TNF-α expression are increased in this model as well (Romestaing, Piquet et al. 2008). These reports suggest that methionine and choline deficiency models may not be able to fully reflect obesity-induced NAFLD due to the severe body weight loss, even though they efficiently induce hepatic lipidosis and inflammation.
1.3.1.4.2. **High fat diets**

Animals fed a high fat diet, with 45-75% of total dietary energy from dietary fat, show increased hepatic injury and lipid accumulation (Hebbard and George 2011). Three weeks of a high dietary fat (71% of total calories) given to rats can efficiently result in significant hepatic lipidosis and increased TNF-α and Cyp 2E1 expression in liver, without weight loss (Lieber, Leo et al. 2004). In long-term studies (10-16 weeks), body weight, hepatic triglycerides, proinflammatory markers, and Cyp 2E1 expression were all elevated in animals given a high fat diet (60% of total calories) (Bose, Lambert et al. 2008; Abdelmegeed, Banerjee et al. 2012). These results suggest that high fat diet models may better reflect the biochemistry shown in people with obesity-induced NAFLD.

1.3.1.4.3. **High fructose/sucrose diets**

Fructose can also induce hepatic steatosis and enhance blood triglycerides in rodent models, through *de novo* lipogenesis (Le and Tappy 2006). Mice receiving fructose for 8 weeks from water containing 30% fructose show increased body weight, hepatic triglycerides, proinflammatory markers and plasma ALT level (Spruss, Kanuri et al. 2009; Kanuri, Spruss et al. 2011). However, in a 5-week study, although hepatic triglycerides are greatly increased among rats receiving fructose from diet (70% w/w), there is no change in body weight or plasma ALT (Kawasaki, Igarashi et al. 2009). Sucrose, containing 50% fructose by weight, has an ability similar to fructose to support NAFLD generation in a rodent model. High sucrose diets (40-70% w/w) have no influence on body weight in rats, but there is an elevated triglyceride content in liver in both short-term (5 weeks) and long-term studies (16 weeks) (Kawasaki, Igarashi et al. 2009; Roncal-Jimenez, Lanaspa et al. 2011). However, a combined dietary treatment of high fat (20-30% w/w) and high sucrose (13-38% w/w) can increase both hepatic lipidosis and adiposity.
in rodents (Murase, Mizuno et al. 2001; Li, Xu et al. 2011; Pranprawit, Wolber et al. 2013). These data support the concept that a combination of a high fat diet and a high sucrose diet may be needed to best model the obesity-driven changes in hepatic lipid metabolism seen in today’s obese human population.

1.3.2. Liver cancer

1.3.2.1. Prevalence

Primary liver cancers include hepatocellular carcinoma, intrahepatic cholangiocarcinoma (bile duct cancer), angiosarcoma, hemangiosarcoma, and hepatoblastoma. Hepatocellular carcinoma is the most common form of liver cancer; about 80% of liver cancer cases are this form, while 10-20% of the cases are bile duct cancer. Angiosarcoma, hemangiosarcoma, and hepatoblastoma are rare, and hepatoblastoma usually occurs in children under 4 years of ages (American Cancer Society 2015). In the United States, there are about 8 new liver cancer cases per 100,000 persons per year, making up 2.2% of overall new cancer cases. Approximately 9 out of every 1,000 Americans develop liver cancer at some time during their life time. Although the incidence of liver cancer is not high, it is usual lethal, with a five-year survival rate less than 18% (National Cancer Institute 2015). Males in Eastern Asia have a higher liver cancer incidence (35.5/100,000) compared to those in the North America (4.0/100,000), possibly related to high prevalence of hepatitis B virus infection, an important co-factor in the development of liver cancer (Bosch, Ribes et al. 2004; El-Serag 2012). Liver cancer incidence in the male population is 2-4 fold higher than in the female population (Bosch, Ribes et al. 2004). This may be due to a lack of protection from estrogen (Naugler, Sakurai et al. 2007).

1.3.2.2. Risk factors

Risk factors for hepatocellular carcinoma include diet, life style, infectious disease, gender and
It is known that men have a higher risk of liver cancer than women; the newly diagnosed cases are 12.7 per 100,000 males and 4.3 per 100,000 females (National Cancer Institute 2015). In the United States, Asian and Pacific Islander populations have a higher liver cancer incidence (21.2/100,000) than non-Hispanic white populations (8.9/100,000) (American Cancer Society 2015). Cirrhotic patients have almost a 60-fold elevated risk for hepatocellular carcinoma compared to people with a healthy liver, reported in a study conducted in Denmark (Sorensen, Friis et al. 1998). Hepatitis B or C virus infection causes chronic liver inflammation, and contribute to 57% of cirrhosis and 78% of hepatocellular carcinoma globally (Perz, Armstrong et al. 2006). Aflatoxins, a group of related compounds that are produced by two fungi *Aspergillus flavus* and *Aspergillus parasiticus*, can enhance hepatic carcinogenesis by strongly binding to DNA and RNA, causing adducts and subsequent mutation (Mishra and Das 2003). A study in the Philippines showed that high exposure of aflatoxins (> 7 µg/day) from diets increased the relative risk of primary liver cancer 17-fold compared to the low exposure group (0-3 µg/day) (Bulatao-Jayme, Almero et al. 1982). Meta-analysis has also indicated that liver cancer risk for high alcohol consumers (100 g/day) is 1.8-fold higher than a low consumption group (< 25 g/day) (Corrao, Bagnardi et al. 2004). Moreover, this year (2015), World Cancer Research Fund International officially includes body fatness as a risk factor for liver cancer (World Center Research Fund International/American Institue for Cancer Research 2015). Reports show that obese people (BMI > 30) have a 1.8-fold higher risk for liver cancer than people with normal body weight (Larsson and Wolk 2007). In today’s world day where obesity is prevalent world-wide, a dietary means of controlling NAFLD-related liver cancer development could save lives and money.
1.3.2.3. Development of hepatocellular carcinoma

1.3.2.3.1. Altered hepatic foci

Altered hepatic foci (also called foci of altered hepatocytes) can be found in rodents exposed to carcinogens. These foci are classified histologically as eosinophilic, basophilic, vacuolated, clear cell or mixed (Gad 2007). Altered hepatic foci are considered preneoplastic, and can progress to hepatic adenomas and then sometimes to carcinomas (Su, Benner et al. 1997). In humans it has been found that incidence of altered hepatic foci is more than 90% in patients having hepatocellular carcinoma (Su and Bannasch 2003). However, only very few altered hepatic foci further develop to malignant neoplasia in rodents treated with carcinogens (Pitot 1990). The differences between altered hepatic foci and hepatic adenoma are cyto-morphological features, growth pattern, and compression of adjacent hepatic parenchyma (Gad 2007).

1.3.2.3.2. Hepatic adenoma

Hepatic adenoma, a type of benign hepatic neoplasms, can grow progressively by expansion, and occasionally can be seen as an early stage of hepatocellular carcinoma (Thorgeirsson and Grisham 2002; Gad 2007). A study showed that the frequency of malignant transformation from hepatocellular adenoma to carcinoma was 4.2% in people in The Netherlands (Stoot, Coelen et al. 2010). Histologically, cytoplasm of hepatocellular adenoma can be eosinophilic, basophilic or vacuolated, but neoplastic hepatocytes are well-differentiated and only differ subtly from normal cells (Baba and Catoi 2007; Gad 2007).

1.3.2.3.3. Hepatocellular carcinoma

Hepatocellular carcinomas are malignant liver tumors that grow by expansion and invasion, and have the rare ability to develop metastasis, typically to lung (Baba and Catoi 2007).
Hepatocellular carcinomas can be classified microscopically as trabecular, well-differentiated, moderately well-differentiated or poorly differentiated carcinoma (Gad 2007). In humans, the development of a hepatocellular carcinoma may take up to 30 years to progress from preneoplasia to malignant neoplasm (Thorgeirsson and Grisham 2002).

1.3.2.4. Animal models for chemically-induced liver cancer

1.3.2.4.1. Overview

Several animal models have been developed for studying hepatocellular carcinoma, including chemically-induced, xenograft, and transgenic models (Leenders, Nijkamp et al. 2008). Human liver tumor xenografts in rodents might be a practical model for drug screening and study of metastasis for human tumors (Li, Tang et al. 2012). Hepatitis B or C virus transgenic mouse models, where mice carry a specific sequence of the hepatitis B or C virus genome, provide a platform for virus-initiated liver cancer (Bakiri and Wagner 2013). Chemically-induced models may best mimic the injury-fibrosis-carcinogenesis path of liver cancer development, similar to what is seen in the progression of NAFLD (Heindryckx, Colle et al. 2009). Aflatoxins, carbon tetrachloride, and diethylnitrosamine are all carcinogens that have been used to induce liver tumorigenesis.

1.3.2.4.2. Aflatoxins

Aflatoxin B1, which is the most potent carcinogenic form of aflatoxins, can be activated by lipoxygenase, Cyp 1A2, and Cyp 3A4, forming active epoxides and attacking DNA (Bedard and Massey 2006; Dohnal, Wu et al. 2014). In a B6C3F1 (C57BL/6J x C3H hybrid) infant mouse model, a single dose of aflatoxin B1 (6 mg/kg body weight) at 7 days of age resulted in a 75% incidence of liver tumors after 52 weeks (Vesselinovitch, Mihailovich et al. 1972). However, in another study, a 10 mg/kg body weight dose of aflatoxin B1 only caused 10% incidence of liver
tumor after 52 weeks in B6D2F1 (C57BL/6J x DBA hybrid) infant mice (Ghebranious and Sell 1998). In rats, 5 weeks of daily intraperitoneal injection with 20 µg aflatoxin B1 per animal (approximately 0.2 mg/kg body weight) from 8 weeks of age induced hepatocellular carcinoma in 80% of male F344 rats after 80 weeks (Kensler, Gange et al. 1997). These results suggest that aflatoxin B1 requires a long time (more than 52 weeks) for liver tumor development in rodent models.

1.3.2.4.3. Carbon tetrachloride (CCL₄)

Carbon tetrachloride causes liver damage by generating trichloromethyl radicals from cytochrome P450 metabolism, which may injure the integrity of the cell membrane (Heindryckx, Colle et al. 2009). Adult C57BL/6J mice (6 weeks old) that received 1 ml CCL₄/kg body weight three intraperitoneal injections per week for 16 weeks showed 44% incidence of hepatic carcinomas after 46 weeks (Farazi, Glickman et al. 2006). For rats, long-term CCl₄ treatment, at 0.65 ml/kg body weight, from 12 weeks old twice per week till the end of the study required 70-150 weeks to cause 80% incidence of hepatocellular carcinoma (Reuber and Glover 1970). These reports show that typically multiple treatments (more than 16 weeks) are required in rodent CCl₄ models.

1.3.2.4.4. Diethylnitrosamine (DEN)

The carcinogenic nitrosamine, DEN, is found in tobacco smoke and processed meat products (Dietrich, Block et al. 2005). Cytochrome P450 2E1 is able to bioactivate DEN, forming ethyl diazonium ions that can form DNA adducts (Verna, Whysner et al. 1996). Some studies have used a combined treatment of DEN and phenobarbital, because phenobarbital enhances the activity of cytochrome P450 to increase DEN metabolism efficiency of tumor initiation/promotion; phenobarbital is also a liver tumor promoter (Peraino, Fry et al. 1973;
Heindryckx, Colle et al. 2009). In the C57BL/6J mouse model, DEN exhibits dose-dependent development of liver cancer (Kushida, Kamendulis et al. 2011). Moreover, different mouse strains show diverse sensitivities to DEN. For example, in a 12-month study, the DEN sensitivity among strains was C3H > B6C3F1 > C57BL/6J (Goldsworthy and Fransson-Steen 2002). The age and gender of animals also influences the efficiency of DEN in causing hepatocellular carcinoma. For C57BL/6J mice, a single dose of 25 mg DEN/kg body weight given to male infants (15 days old) resulted in 80% incidence of liver tumors after 34 weeks (Park, Lee et al. 2010). With the same treatment, female mice only exhibited half the tumor incidence compared to males (40% vs. 80%) (Park, Lee et al. 2010). Furthermore, adult mice (4 weeks old) that received 25 mg DEN/kg body/week for 4 weeks had no liver tumor after 33 weeks (Kushida, Kamendulis et al. 2011). Comparing aflatoxins, CCl₄, and DEN, DEN can induce liver cancer in a shorter period of time, and is more manageable for different levels of tumorigenesis, by varying the dose.

1.4. Brassicas

1.4.1. Overview

Brassica, a genus of plants belonging to the family of Brassicaceae, includes many food crops that are broadly consumed all over the world, such as broccoli (Brassica oleracea var. italica), kale (Brassica oleracea var. acephala), rapeseed (Brassica napus), cabbage (Brassica oleracea var. capitata), and cauliflower (Brassica oleracea var. botrytis) (Prakash 1980). Brassica vegetables are rich in beneficial components, including fiber, vitamin C, β-carotene, flavonoids, and glucosinolates (Jeffery and Araya 2009). Plants from the family of Brassicaceae were cultivated for food in Persia and China around 8,000 years ago, and over the last 2,000 years the
varieties that modern people are familiar with were developed in Italy (Buck 1956). Broccoli was first planted in the United States in 1923 near San Jose, California, and then spread in availability across the whole country (Hayley Boriss 2005). During the last 20 years, consumption of broccoli in the United States has increased significantly: 6.7 pounds per capita per year in 1994 and 9.3 pounds per capita per year in 2014, while for cabbage, consumption has decreased from 10.3 to 8.0 pounds per capita per year over the same period (U.S. Department of Agriculture, ERS 2015). Several epidemiological studies have suggested brassica vegetables could have cancer-preventive effect (Verhoeven, Goldbohm et al. 1996). This makes it worthwhile to look into the health effects of long-term consumption of broccoli. Broccoli is typically cooked before eating, so the impact of the heating process of cooking on availability of nutrients and bioactive compounds is an important issue as well.

1.4.2. Bioactive compounds

1.4.2.1. Vitamin C

Vitamin C (ascorbic acid) is a cofactor as an electron donor that facilitates many biochemical reactions, including synthesis of collagen, carnitine, and norepinephrine, and also a natural antioxidant (Padh 1990). Ascorbic acid can be biosynthesized in most animals, except for humans and other non-human primates, bats, certain Passeriformes birds, and guinea pigs, due to lack of gulonolactone oxidase (Englard and Seifter 1986). Broccoli contains about 90 mg vitamin C per 100 g raw material, which is higher than in an orange (about 50 mg/100 g) (U.S. Department of Agriculture, ARS 2014). Vitamin C contents of cauliflower and cabbage are lower than broccoli, about 48 and 33 mg/100 g respectively, more similar to the orange (U.S. Department of Agriculture, ARS 2014). Boiling (5 min) and microwave cooking (1,000 W, 5 min) is reported cause 27-37% and 18-40% loss of vitamin C, respectively, whereas steaming
retains most of vitamin C in broccoli (Vallejo, Tomas-Barberan et al. 2002; Lopez-Berenguer, Carvajal et al. 2007; Yuan, Sun et al. 2009).

1.4.2.2. β-Carotene

β-Carotene belongs to the class of carotenoids, molecules with a long repetitive alkenyl carbon backbone (40 carbon atoms), and serves as provitamin A. It is metabolized by β-carotene 15,15’-monooxygenase to form 2 molecules of vitamin A (Olson 1989). Vitamin A has multiple roles in the body, including being an essential component of rhodopsin, which can provide proper function of dark vision, and it also regulates cell differentiation through nuclear retinoic acid receptors (Gerster 1997). β-Carotene serves as a good antioxidant as well (Edge, McGarvey et al. 1997). Broccoli contains relatively low levels of β-carotene, only 0.4 mg/100 g raw material, compared to carrots which have 8 mg or more β-carotene/100 g raw material, whereas in kale the β-carotene level is relatively high (about 6 mg/100 g) (U.S. Department of Agriculture 2014). The cooking process may cause large losses of β-carotene from broccoli. After boiling (5 min) or microwave cooking (600 W, 5 min) only 20% of the β-carotene in broccoli is retained (Zhang and Hamauzu 2004). As for carrots, even after 10 min of boiling carrots still have 84% retention of β-carotene (Bernhardt and Schlich 2006).

1.4.2.3. Flavonoids

Flavonoids are a group of naturally occurring phenolic compounds from plants, all sharing the similar structure of a 15-carbon skeleton that includes two benzene rings (Kumar and Pandey 2013). In vegetables and fruits, flavonoids such as flavanols, flavones, flavonols, flavanones, iso flavonones and anthocyanidins, are commonly found (Heim, Tagliaferro et al. 2002). Flavonoids, function as ultraviolet filters, pollinator attractants, and phytoalexins in plants (Iwashina 2003). When ingested by animals, flavonoids may exhibit “bioactivities”, such as
anti-viral activity, anti-inflammatory action, regulation of diabetes, and protection against liver damage (Tapas, Sakarkar et al. 2008). These phenolic compounds can act as antioxidants as well due to their multi-ring structure (Heim, Tagliaferro et al. 2002) (Fig. 1.4). For example, flavonoids can increase the shelf life of chicken products, decrease lipid oxidation in ground fish and Maillard browning in pasteurized milk (Ramanathan and Das 1992; Schamberger and Labuza 2007; Kanatt, Chander et al. 2010). Quercetin, kaempferol, and isorhamnetin are flavonols, and are the main flavonoids in brassica crops (Cartea, Francisco et al. 2011). Kale contains higher levels of quercetin and kaempferol (110 and 211 mg/100 g raw material, respectively) compared to broccoli (30 and 72 mg/100 g raw material, respectively), but both quercetin and kaempferol are low in cauliflower, less than 1 mg/100 g raw material (Hertog, Hollman et al. 1992). Boiling (5 min) may result in more than 60% loss of phenolic compounds in broccoli, while there is only a 30% loss following microwave cooking (1,000 W, 5 min) (Zhang and Hamauzu 2004; Lopez-Berenguer, Carvajal et al. 2007).

1.4.2.4. Glucosinolates

1.4.2.4.1. Overview

Glucosinolates are a class of nitrogen-and sulfur-containing natural compounds that derive from certain amino acids (Ala, Leu Ile, Met, Val, Phe, Tyr, or Trp), conjugated to β-D-glucose via a sulfur atom (Halkier and Gershenzon 2006). The bound glucose can be removed by hydrolysis by myrosinase, a thioglucohydrolase, to form an unstable intermediated that breaks down to one of several types of aglycones, including isothiocyanates, nitriles, and thiocyanates (Fig. 1.3) (Bones and Rossiter 2006). Brassica crops are rich in glucosinolates, the hydrolysis products of which are thought be responsible for the special bitter and acrid taste (Drewnowski and Gomez-Carneros 2000). In plants, glucosinolates act as chemical defense against herbivores,
releasing hydrolyzed aglycones after being damaged, such as by chewing (Mithofer and Boland 2012). Damage of plant tissue enables the breakdown of myrosin cells, which contain myrosinase, and releases myrosinase for hydrolysis (Bones and Rossiter 1996). As for animals, isothiocyanates and related compounds, such as sulforaphane and indole-3-carbinol, appear to be involved in the regulation of cell physiology. For example, isothiocyanate can increase antioxidant activity by activating phase 2 metabolic enzymes, and they also provide estrogenic bioactivity (Holst and Williamson 2004). However, it has been reported that when rapeseed meal constitutes a major part of their diet, this is associated with thyroid hypertrophy and decreased circulating thyroxine in pigs, possibly due to impairing the uptake of iodine in thyroid gland by thiocyanate ion (Bell 1984; Zukalova and Vasak 2002). Brussels sprouts have high total glucosinolate content (about 25 µmol/g dry material) and the major glucosinolate is sinigrin (about 9 µmol/g dry material). The total content of sinigrin in broccoli is relatively low (on average, about 12-13 µmol/g dry weight) but broccoli is high in glucoraphanin (up to 20 µmol/g dry weight or more, varying with variety); glucoraphanin is the precursor to sulforaphane, a highly bioactive aglycone (Kushad, Brown et al. 1999). In broccoli, boiling (5 min) and microwave cooking (1,000 W, 5 min) are reported to retain 62.5% and 75% of glucosinolates, respectively, but glucosinolates are almost 100% retained after the steaming process (5 min) (Lopez-Berenguer, Carvajal et al. 2007; Yuan, Sun et al. 2009).

1.4.2.4.2. Myrosinase

Myrosinases from plant sources are heat sensitive (Bones and Rossiter 2006). However, the heat sensitivity of myrosinases from different plants may vary. For example, the myrosinase from mustard seed (Sinapis alba) was totally inactivated when heated at 75°C for 10 min, while at 90°C for 10 min broccoli myrosinase can still hydrolyze glucosinolate, but loses about 30%
production of sulforaphane (Van Eylen, Indrawati et al. 2006; Dosz and Jeffery 2013). Moreover, other than myrosinase, some bacteria may also metabolize glucosinolates (Fahey, Zalcmann et al. 2001). In a rat study, microbiota in the cecum was shown to hydrolyze glucoraphanin in the absence of plant myrosinase (Lai, Miller et al. 2010).

1.4.2.4.3. Sulforaphane

Sulforaphane, an isothiocyanate derived from glucoraphanin, is considered to be a dietary bioactive compound with health benefits (Zhang and Tang 2007). It is known that sulforaphane can down-regulate some cytochrome P450 enzymes, activate phase 2 metabolic enzymes, inhibit angiogenesis, promote apoptosis, and decrease inflammation (Maheo, Morel et al. 1997; Brooks, Paton et al. 2001; Singh, Xiao et al. 2004; Anwar-Mohamed and El-Kadi 2009; Davis, Singh et al. 2009; Nallasamy, Si et al. 2014). Sulforaphane is an activator of the Kelch-like ECH-associated protein 1 (KEAP1)-nuclear factor erythroid-2-related factor (Nrf2)-antioxidant response element (ARE) pathway, providing detoxification and increasing antioxidant enzyme activity (Kensler, Wakabayash et al. 2007). Figure 1.5 briefly illustrates the mechanism of the KEAP1-Nrf2-ARE pathway. It is suggested that activators, such as sulforaphane, covalently modify KEAP1 and result in the translocation of Nrf2 into the nucleus to activate expression of genes bearing an ARE in the promoter region. Nrf2 activates genes responsible for synthesis of phase 2 metabolic enzymes (such as NAD(P)H: quinone oxidoreductase 1 and glutathione S-transferases), endogenous antioxidant enzymes (such as heme oxygenase 1 and glutathione reductase), and NADPH production via glucose-6-phosphate dehydrogenase and malic enzyme 1, contributing to both antioxidant defense and homeostasis of cell functions (Gorrini, Harris et al. 2013). Just this decade, the ability of sulforaphane to inhibit histone deacetylases in cancer cells, including prostate and colon cancer, has also been discovered as an anti-cancer characteristic,
because inhibition of histone deacetylase activity may enhance the expression of some tumor suppressor genes, such as \( p21 \) (Ho, Clarke et al. 2009).

1.4.2.4.4. **Indole-3-carbinol**

Glucobrassicin, an indole glucosinolate derived from tryptophan, is the precursor of indole-3-carbinol (Mcdanell, Mclean et al. 1988). Indole-3-carbinol, as well as its metabolite the bioactive dimer 3,3’-diindolylmethane, has shown the ability to regulate cell function through estrogen receptors and aryl hydrocarbon receptors (Kim and Milner 2005). Estrogen receptor \( \alpha \) signaling in human breast cancer cells can be inhibited by indole-3-carbinol, which suggests that indole-3-carbinol is able to impede estrogen-related tumor development (Meng, Yuan et al. 2000). Aryl hydrocarbon receptors, when bound to a ligand such as indole-3-carbinol, can travel to the nucleus, recognize the dioxin response element, and then activate its down-stream genes, including Cyp 1A1, a phase 1 metabolic enzyme (Denison and Nagy 2003). Indole-3-carbinol and 3,3’-diindolylmethane are able to regulate estrogen metabolism and decrease the production of 16\( \alpha \)-hydroxyestrone, a metabolite that may increase the risk of breast cancer (Jellinck, Forkert et al. 1993).

1.4.3. **Brassica and inflammation**

*In vitro* studies have shown that bioactive compounds in brassica vegetables are able to decrease inflammation. Kaempferol and quercetin can decrease both COX-2 and inducible nitric oxide synthase expression in the human liver cell line Chang Liver (Garcia-Mediavilla, Crespo et al. 2007). Indole-3-carbinol lowered IL-1\( \beta \) and IL-6 levels in a lipopolysaccharide (LPS)-induced mouse macrophage cell model (Raw246.7) (Jiang, Kang et al. 2013). Sulforaphane was also shown to down-regulate IL-1\( \beta \), IL-6 and COX-2 expression induced by ultraviolet B in human HaCaT keratinocytes (Shibata, Nakagawa et al. 2010). In primary culture models,
sulforaphane decreased IL-1β and TNF-α expression in LPS-induced rodent peritoneal macrophages and in brain microglia (Lin, Wu et al. 2008; Brandenburg, Kipp et al. 2010). It has been proposed that sulforaphane is able to down-regulate the NF-κB pathway by inhibiting the binding of NF-κB to DNA, possibly due to an increased expression of redox enzymes, such as thioredoxin reductase (Heiss, Herhaus et al. 2001; Heiss and Gerhauser 2005).

In a mouse model, 1.2% (w/w) quercetin in the diet was found to reduce circulating interferon γ and IL-1 in the group that had high fat diet-induced inflammation after 8 weeks (Stewart, Soileau et al. 2008). A daily supplement of 2.5 mg sulforaphane for 2 weeks decreased COX-2 expression in mice with UVB exposure (Shibata, Nakagawa et al. 2010). Two weeks of phenethylisothiocyanate treatment (75 mg/kg/day body weigh) lowered colonic IL-1β in a dextran sodium sulfate-induced mouse model (Dey, Kuhn et al. 2010). In a *Helicobacter pylori* infected mouse model, 8 weeks of dietary broccoli sprouts decreased IL-1β and TNF-α expression in gastric mucosa (Yanaka, Fahey et al. 2009). These reports suggest that brassica bioactive compounds are able to suppress inflammation resulting from multiple stimuli.

There are also many clinical studies showing anti-inflammatory effects of dietary brassica. A study in China showed that circulating proinflammatory markers, IL-1β, IL-6 and TNF-α, were lower in women with high intake of brassica vegetables (98.9-140.5 g/day) compared to the low intake population (< 42.5 g/day) (Jiang, Wu et al. 2014). Moreover, there is a strong anti-inflammatory effect in people who smoke but eat brassica. For example, after 10 days of broccoli consumption (250 g/day), plasma C-reactive protein was decreased 48% in people who smoke (Riso, Vendrame et al. 2014). Diabetic patients also benefit from brassica diets: 4 weeks of broccoli sprouts supplement (10 g/day) lowered plasma C-reactive protein level about 20% from baseline (Mirmiran, Bahadoran et al. 2012).
1.4.4. Brassica and obesity

Cell based studies suggest that bioactive compounds in brassica vegetables may have the ability to regulate lipid metabolism. Quercetin can down-regulate FAS expression in 3T3-L1 adipocytes and reduce fatty acid synthesis in rat primary hepatocytes (Ahn, Lee et al. 2008; Gnoni, Paglialonga et al. 2009). Kaempferol may also inhibit obesity, and has been shown to down-regulate the expression of lipogenic genes PPARγ and SREBP-1c in a 3T3-L1 model (Park, Jeong et al. 2012). Moreover, sulforaphane has been reported to induce lipolysis via increasing hormone sensitive lipase expression in a 3T3-L1 system as well (Lee, Moon et al. 2012).

Recent research with animals has revealed that glucosinolates may have some impact on lipid metabolism in vivo. In a hamster model, dietary glucoraphanin (20 µmol/day) for 7 weeks decreased hepatic FAS expression, but this effect was not significant in a second arm of the study where the animals were given broccoli sprouts (Rodriguez-Cantu, Gutierrez-Uribe et al. 2011). In a diet-induced obesity mouse model, 0.1% (w/w) sulforaphane in diets decreased fat mass and adipocyte PPARγ expression after 6 weeks of dietary treatment (Choi, Lee et al. 2014). The same dose of indole-3-carbinol (0.1%, w/w) for 10 weeks also induced decreased fat mass and PPARγ expression in obese mice (Chang, Wang et al. 2011). Furthermore, 15 mg indole-3-carbinol/kg body weight/week for 12 weeks was found to reduce ACC expression in adipose tissue (Choi, Kim et al. 2012). Dietary quercetin (0.05% in diet) also decreased hepatic FAS expression and triglyceride levels in a high fat and high sucrose diet-fed mouse model (Kobori, Masumoto et al. 2011).

However, these data are few and it is still not clear about the influence of dietary brassica on obesity outcomes in humans. Even though there is insufficient clinical reporting to address the
possible connection between brassica and obesity, some studies suggest that brassica may reduce circulating triglyceride levels. For example, diabetic patients exhibited significant reduction in blood triglyceride levels after 4 weeks of broccoli sprouts supplements (10 g/day) (Bahadoran, Mirmiran et al. 2012).

1.4.5. Brassica and cancer

In vitro studies suggest that brassica bioactive compounds induce apoptosis and inhibit cell growth in cancer cell lines. Both flavonols and isothiocyanates may be responsible. Quercetin is reported to induce growth inhibition and apoptosis in human A549 lung cancer cells and to inhibit cell cycle progression in human SK-Br3 breast cancer cells (Nguyen, Tran et al. 2004; Jeong, An et al. 2009). Kaempferol has also induced growth inhibition and apoptosis in A549 cells, and inhibited flavonol angiogenesis in human A2780/CP70 ovarian cancer cells (Nguyen, Tran et al. 2003; Luo, Rankin et al. 2009). Sulforaphane is able to induce apoptosis and cell cycle arrest in human HT29 colon cancer cells, and cell death in human PC-3 prostate cancer cells (Gamet-Payrastre, Li et al. 2000; Singh, Srivastava et al. 2005). Moreover, indole-3-carbinol also inhibits cell growth and induces apoptosis in PC-3 cells (Chinni, Li et al. 2001).

In whole animal studies, brassica bioactive compounds have revealed anti-carcinogenic ability in several different models. Dietary quercetin (5%, w/w) for 20 weeks can reduce the incidence of mammary tumors in an N-nitrosomethylurea-treated rat model (Verma, Johnson et al. 1988). In a tobacco carcinogen-induced mouse model, 20 weeks of sulforaphane treatment (1.5 mmol/kg diet) has been reported to reduce the lung tumor multiplicity (Conaway, Wang et al. 2005). Topical treatment of sulforaphane (1-10 µmol/mouse/week, 15 weeks) is able to prevent the development of skin tumors induced by 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol 13-acetate (Gills, Jeffery et al. 2006). Also, indole-3-carbinol given as a
dietary treatment (1.36 mmol/kg diet) for 10 weeks has reduced the total number of colonic aberrant crypt foci in an azoxymethane-induced rat model (Plate and Gallaher 2006).

In human epidemiological studies, several reports have identified a relationship between reducing cancer risk and increasing brassica consumption. In the United States, 5 servings of brassica vegetables per week may reduce risk of bladder cancer in males by as much as 50% (Michaud, Spiegelman et al. 1999). For colon cancer, a study in The Netherlands showed that about 1 serving of brassicas per day (58 g) could decrease risk for colon cancer in women by almost 50%, but not for men (Voorrips, Goldbohm et al. 2000). In another study, focusing on postmenopausal women in Sweden, one serving of brassica vegetables everyday was sufficient to reduce risk for breast cancer by about 30% (Terry, Wolk et al. 2001). For men, daily consumption of about one serving of brassicas (72 g) is able to decrease risk for prostate cancer by about 40% in a study conducted in the United States (Kolonel, Hankin et al. 2000). However, the impact of dietary brassica on liver cancer has not been reported.

1.5. Gaps in Our Knowledge

In summary, it is known that obesity is a risk factor for both NAFLD and liver cancer. It is also becoming apparent that inflammation can participate in the development of both of these two liver diseases, and that increased adiposity is able to contribute to the formation of chronic inflammation. In rodent models, a high fat diet can increase body weight, induce adipose tissue expansion, cause hepatic lipid accumulation, and trigger ultimately liver injury and inflammation, while a high sucrose diet has little impact on body weight and adiposity, but can still increase hepatic triglyceride levels. However, a high fat and high sucrose dietary treatment is able to cause similar outcomes to a high fat diet, which increases both obesity and NAFLD outcomes,
suggesting that a high fat and high sucrose diet rodent model is suitable to address the impact of a Westernized dietary pattern on the development of NAFLD. Moreover, the potent carcinogen DEN can induce liver cancer in a relatively short period of time in mice, and the severity of liver tumorigenesis can be managed by different DEN dosages, meaning that the inclusion of DEN treatment in a diet-induced NAFLD model may facilitate the evaluation of NAFLD related liver cancer development.

However, although there are *in vitro* and *in vivo* studies suggesting that the bioactive compounds in brassicas are able to regulate inflammation, lipid metabolism, and carcinogenesis, and many epidemiological studies support the suggestion that consumption of brassica vegetables decreases inflammation and cancer risk, the impact of whole brassica consumption on hepatic physiology is still not clear, especially in relation to lipid metabolism. There is a need to fill the gap in knowledge about the influence of dietary brassica on steatosis, inflammation, and tumorigenesis in liver.
Figure 1.1. Brief disease spectrum of NAFLD. Histological sections with Masson’s trichrome staining show the change of liver among normal situation, steatosis, NASH, and cirrhosis. PT, portal triad; CV, central vein. The large clear round spaces in the second panel are indicative of triglyceride accumulation. The blue staining in the third (NASH) and fourth (cirrhosis) panels are indicative of progressive fibrosis associated with chronic inflammation. Modified from Cohen et al. (2011).
Figure 1.2. Fatty acid flux in liver. Sources of fatty acids in liver include (1) NEFA as the product of lipolysis from adipose tissue, (2) newly generated fatty acids from \textit{de novo} lipogenesis, (3) NEFA released from chylomicrons, and (4) uptake from chylomicron remnants. ATGL, adipose triglyceride lipase; FA, fatty acid; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; TAG, triglyceride.
Figure 1.3. Hydrolysis of glucosinolates by myrosinase. Modified from Keck and Finely (2004) for updating the enzyme commission number of myrosinase.
Figure 1.4. Main flavonoids found in brassica crops.

Quercetin  Kaempferol  Isorhamnetin
Figure 1.5. Keap1-Nrf2-ARE signal pathway. In the normal situation without inducers, Nrf2 in the cytoplasm would be ubiquitinated and degraded. Once activators, such as sulforaphane, covalently modify Keap1 via binding sulfurs from cysteine, Nrf2 is no longer degraded and accumulating Nrf2 can be translocated to the nucleus to activate ARE. ARE, antioxidant response element; Cul3, cullin 3; SF, sulforaphane; Ub, ubiquitin.
CHAPTER 2

EXPERIMENTAL APPROACH

2.1. Significance and Rationale

Prevalence of NAFLD in the United States has almost reached 50% in the adult population (Williams, Stengel et al. 2011). Although the lifetime risk of liver cancer in Americans (0.9 %) is much lower compared to lung cancer (6.6 %), liver cancer shows high mortality; 84% of those who are diagnosed with liver cancer die within 5 years (National Cancer Institute 2015). However, both NAFLD and liver cancer are symptomless until the late stage of disease, which by then has caused severe damage to the liver (Salt 2004; American Cancer Society 2015). A westernized eating habit has become well accepted across the world (Popkin 2001). Increased amounts of fat and refined sugar are being incorporated into daily diets, and the excess energy facilitates excessive fat accumulation in the body (Cordain, Eaton et al. 2005). To alter the negative effects of a Westernized diet without changing physical activity, either consumption of fat and sugar should be decreased or other dietary beneficial components should be included. Purified phytochemicals are often used as dietary supplements rather than whole food, due to the high concentration of bioactive compounds considered necessary for efficacy. However, it is suggested that whole food contains multiple phytochemicals and may have additive or synergistic effects (Liu 2003). Broccoli is rich in several kinds of bioactive components, including glucosinolates, flavonoids, carotenoids, and vitamin C (Jeffery, Brown et al. 2003). Therefore, whole broccoli may be more effective than any individual purified bioactive phytochemical from broccoli. The contribution of the proposed work is significant because it is
the first study to look into the influence of long-term dietary broccoli intervention on fatty liver and liver cancer development with either a normal diet or a Westernized diet. Liver health promotion from a whole food (broccoli) will be elucidated. The work will provide solid evidence for the benefit of whole natural food consumption in maintaining tissue health. The finding may encourage people to improve their quality of life in a simple and efficient way.

Pharmacologic doses of sulforaphane, which is considered the most potent bioactive compound derived from glucosinolates in broccoli, have revealed its capacity to lower inflammation and the risk for some cancers (Gills, Jeffery et al. 2006; Brandenburg, Kipp et al. 2010). Moreover, co-treatments by two phytochemicals from broccoli or other brassicas, such as sulforaphane and quercetin, or cramene and indole-3-carbinol, showed synergy in inhibition of cancer cell viability and induction of hepatic quinone reductase in the cultured cells (Nho and Jeffery 2004; Srivastava, Tang et al. 2011). Therefore, we chose to use a whole food dietary intervention in this study. We hypothesized that with long-term whole broccoli consumption, the liver damage caused by carcinogens and/or obesity could be ameliorated by dietary broccoli.

2.2. Objective

The long-term goal of this research is to identify dietary strategies that can decrease the risk of Western diet-enhanced liver diseases, including fatty liver and liver cancer. We would like to test the idea that dietary choices can positively influence liver health and cancer development. Therefore, the objective of this study is to determine the impact of dietary broccoli on decreasing obesity-related chronic inflammation and hepatic lipid accumulation, and to elucidate the relationship among inflammation, hepatic steatosis, and the progression of NAFLD to hepatocellular carcinoma. To approach this objective, we hypothesized that increased dietary
fat and sugar could accelerate hepatic lipidosis, and that following carcinogen treatment, liver
tumorigenesis would progress more rapidly with increased liver lipid accumulation and enhanced
inflammation. We also hypothesized that dietary broccoli intervention would be able to
ameliorate hepatic lipidosis and inflammation and consequently slow down liver tumor
development. Therefore, we proposed to use a chemically-induced liver cancer mouse model to
study the positive and negative effects of diet on liver cancer development. A diet that contains
high saturated fat and high sucrose (Western diet) was used to induce obesity and liver lipid
accumulation. Broccoli was incorporated into diets to understand the influence of long-term
broccoli consumption on Western diet-enhanced hepatic steatosis, inflammation, and tumor
incidence.

2.3. Specific Aims

Aim 1: To assess NAFLD development in a Western diet-enhanced liver cancer model, and to
determine the capacity of long-term dietary broccoli consumption for ameliorating the process.

Hypothesis: The Western diet can increase liver fat accumulation and cause hepatic lipidosis and
inflammation, and dietary broccoli can decrease liver fat accumulation and down-regulate
inflammation.

Aim 2: To evaluate the impact of diets on liver tumor progression in a DEN-induced liver cancer
model.

Hypothesis: Liver cancer development is accelerated by the Western diet, but dietary broccoli
can impede the growth of liver tumors.
3.1. Abstract

Westernized dietary habits, rich in sugar and fat, often increase fat accumulation in the body. This increased adiposity is associated with development of non-alcoholic fatty liver disease (NAFLD) and increased risk for liver cancer. Regular consumption of brassica vegetables is reported to decrease the risk for cancer. We hypothesized that a broccoli diet could decrease chronic inflammation and liver steatosis in mice receiving a Western diet, thereby slowing tumorigenesis. Male C57BL/6J mice (15 days old) were given diethylnitrosamine and placed on a Western or Western+Broccoli (10% freeze-dried broccoli) diet from the age of 4 weeks for 3, 5, or 7 months. Although the Western diet increased body weight with time, dietary broccoli had no effect on overall body weight or liver weight as percent of body weight. The broccoli-fed group exhibited decreased hepatic triglycerides ($P<0.05$) and decreased lipidosis ($P<0.001$) at month 5. Liver damage (serum alanine aminotransferase) and liver inflammation (IL-1$\beta$ and TNF-$\alpha$ expression) were also decreased by dietary broccoli at 5 months, but there was no significant impact on liver cancer incidence. Moreover, the protective effects from dietary broccoli seen at 5 months were no longer evident at 7 months. We conclude that whereas dietary intervention with broccoli had the capacity to slow the progression of NAFLD, it was not able to eliminate diet-induced changes in liver lipid accumulation or inflammation. Furthermore,
dietary broccoli had no impact on tumorigenesis in this model of Western diet-enhanced liver cancer, where cancer was initiated 2 weeks prior to dietary intervention.

3.2. Introduction

Liver cancer is a lethal disease, with very few detectable symptoms until tumors are well-developed, resulting in a five-year survival rate of only 17% (American Cancer Society 2015). Infection (hepatitis B or C virus), alcohol consumption, aflatoxin, and obesity (body fatness) are all risk factors for liver cancer (World Center Research Fund International/American Institute for Cancer Research 2015; American Cancer Society 2015).

In the United States, more than one-third of the adult population is obese (Ogden, Carroll et al. 2014). The excess intake of energy favors the development of obesity due to the loss of energy balance (Spiegelman and Flier 2001). A westernized dietary pattern may be defined as rich in refined carbohydrate, such as sugar, and saturated fat (Cordain, Eaton et al. 2005). The consumption of saturated fat among male adult Americans is 30.9 g/day, supplying about 11% of total daily energy, which is more than 150% of the recommended Daily Value (20 g/day), and more than 20% of energy intake is from sugar (U.S. Food and Drug Administration 2013; U.S. Department of Agriculture, ARS 2014). Liver is the major metabolic center for both carbohydrate and lipid, thereby maintaining glucose homeostasis (Postic, Dentin et al. 2004). With an overload of dietary carbohydrate and fat, as well as change of systemic hormone profiles, the metabolic function in liver is disturbed and may lead to fatty liver disease. Obesity is a major risk factor for non-alcoholic fatty liver disease (NAFLD), a cluster of related liver diseases that begins with simple steatosis, developing into non-alcoholic steatohepatitis (NASH), cirrhosis, and possibly hepatocellular carcinoma (Angulo 2002; Cohen, Horton et al. 2011). Compared to
non-obese people, the risk for hepatic steatosis is almost 5-fold higher in obese people 
(Bellentani, Saccoccio et al. 2000). Obesity also increases the risk for liver cancer, especially in 
obese males, whose mortality risks are 4.5-fold higher than men with a normal BMI (Calle, 
Rodriguez et al. 2003; Larsson and Wolk 2007).

Chronic inflammation caused by obesity may be due to increased adipose tissues, which are 
known to excrete proinflammatory endocrines and cytokines, including leptin, interleukin (IL) -6, 
and tumor necrosis factor-α (TNF-α), and could be a link between obesity and liver cancer 
(Khandekar, Cohen et al. 2011). Elevated plasma IL-6 and TNF-α have been related to cell 
 survival and proliferation through activation of the NF-κB and STAT3 pathways, eventually 
leading to promotion of tumorigenesis (Berasain, Castillo et al. 2009). In several 
epidemiological studies, brassica vegetables have shown to have a protective effect against 
cancers, including bladder, prostate, and colon. (Kolonel, Hankin et al. 2000; Voorrips, 
Goldbohm et al. 2000; Tang, Zirpoli et al. 2008). Brassicas are also known to lower circulating 
proinflammatory markers (Jiang, Wu et al. 2014). However, the impact of brassica on 
inflammation that is caused in the cancer prevention is still unclear.

Brassica vegetables are rich in many bioactive compounds, including flavonoids, ascorbate, 
and especially glucosinolates (Jeffery, Brown et al. 2003). Glucosinolates are stable, sulfur-rich 
glycosides, until they are hydrolyzed by a thioglcosidase, myrosinase, and form bioactive 
aglycones (Halkier and Gershenzon 2006). Sulforaphane, the aglycone of glucosinolate 
glucoraphanin, activates nuclear factor erythroid 2-related factor 2, which in turn upregulates 
detoxification, ameliorates inflammation, and increases apoptosis in cancer cells (Gamet-
Payrastre, Li et al. 2000; Brooks, Paton et al. 2001; Lin, Wu et al. 2008). Here we hypothesized 
that a Western diet would cause liver lipid accumulation and support liver tumor development in
a diethylnitrosamine (DEN) mouse model. We further hypothesized that dietary broccoli intervention would slow or decease hepatic lipidosis, delay the progression of NAFLD, and thereby ameliorate cancer development. We monitored changes in hepatic lipidosis and inflammation after 3 to 7 months of dietary intervention, in order to identify any role that dietary broccoli plays in preventing development of diet-induced hepatic steatosis and tumorigenesis.

3.3. Materials and Methods

3.3.1. Animal and study design

Male C57BL/6J mouse pups were used for this study. Six to eight week-old dams and male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) for breeding. All mice used for breeding were maintained on rodent chow diet; except during gestation and lactation where the dams were fed AIN-93G powered diet. Fifteen-day-old male pups were injected with 25 mg/kg diethylnitrosamine (DEN) intraperitoneally in normal saline solution. They were weaned at 22 days of age and given AIN-93G for 1 week until 4 weeks old. The DEN-treated mice were randomly assigned to Western or Western+Broccoli diet groups at 4 weeks of age, given ad libitum access to water and feed, and were housed individually. Animals were maintained under a 12-hour light/dark cycle at 22°C and 60% humidity. Animal care was in compliance with the approved protocol by the Institutional Animal Care and Use Committee and the Division of Animal Resources at the University of Illinois, Urbana-Champaign, according to the National Institutes of Health. Six animals from each dietary group were sacrificed at 3, 5, and 7 months after starting the diet treatments. Body weight and feed intake were monitored once each week.
3.3.2. **Reagents and diets**

Diethylnitrosamine (DEN) was purchased from Sigma-Aldrich Co. (St. Louise, MO, USA). Western diet was formulated by modifying AIN-93G to increase the sucrose and saturated fat content (Table 3.1). Freeze dried broccoli powder (*Brassica oleracea* L. var. Green Magic), was kindly provided by Dr. John A. Juvik. Broccoli powder (10% by weight) was incorporated into the Western+Broccoli diet, which was adjusted by partly replacing the corn starch and cellulose with fiber and carbohydrate in broccoli (Table 3.1). Other diet ingredients were purchased from the Harlan Laboratory (Indianapolis, IN, USA).

3.3.3. **Liver triglyceride content**

Liver lipid was extracted by the method of Folch (Folch, Lees et al. 1957) with some modifications. Liver tissue was homogenized with chloroform: methanol (2:1 v/v) with 0.01% butylated hydroxytoluene. Homogenates were washed with ultrapure H$_2$O and centrifuged at 2,400 rpm for 20 min. The chloroform phase was heated to 50°C and evaporated under a stream of nitrogen gas. The remaining lipid extract was reconstituted with absolute ethanol and stored at -20°C for further analysis. Liver triglyceride concentration was determined by the glyceride phosphate oxidase (GPO) method using a Triglyceride (GPO) Liquid Reagent Set (Pointe Scientific, Canton, MI, USA).

3.3.4. **Histology**

Mouse liver (median lobe) was fixed in 10% neutral buffered formalin at room temperature for less than 24 hours. The fixed tissues were dehydrated and embedded in paraffin and sectioned (3 µm). Sections were stained with hematoxylin and eosin (H&E) for histological examination. All histology work was done at the Veterinary Diagnostic Laboratory (University of Illinois,
Urbana, IL, USA). Murine NAFLD scores were read by a trained pathologist blinded to treatments; the scoring criteria are shown in Table 3.2.

3.3.5. Macroscopic and microscopic hepatic neoplasm-related lesion detection

At sacrifice, livers were weighed and examined for macroscopic neoplasm-related lesions. Nodules (diameter ≥ 1 mm) in each liver lobe were counted and the maximum diameter was measured using calipers. H&E stained liver sections were examined for microscopic neoplasm-related lesions. Altered hepatic foci (AHF), hepatic adenomas (HA), and hepatocellular carcinomas (HCC) were recognized and recorded by a trained pathologist.

3.3.6. Serum alanine aminotransferase (ALT) levels

Serum ALT levels were determined using the Liquid ALT Reagent Kit (Pointe Scientific, Canton, MI, USA), according to manufacturer’s instructions. The mean of the difference in absorbance multiplied the factor of 1768 converted the data to IU/L.

3.3.7. Metabolic enzyme activity

Microsomal and cytosolic fractions were prepared for metabolic enzyme activity (Lai, Keck et al. 2008). Cytosolic and microsomal protein was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). NAD(P)H: quinone oxidoreductase 1 (NQO1) activity was used to evaluate phase 2 metabolic enzyme activity and performed by following the method developed by Prochaska and Santamaria (Prochaska and Santamaria 1988) with some modifications (Lai, Keck et al. 2008). Cytochrome P450 1A1 (Cyp 1A1) activity was evaluated by estimating ethoxyresorufin O-deethylase (Paolini, Perocco et al. 2004) with some modifications (Lai, Keck et al. 2008).
3.3.8. **Real time quantitative PCR (RT-QPCR)**

Total RNA was extracted from liver with Trizol Reagent (Life Technologies, Carlsbad, CA, USA) and E. Z. N. A. Total RNA Kit II (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer’s instructions. High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to synthesize cDNA from 2 µg RNA sample for a probe-based (6-FAM/ZEN/IBFQ) RT-QPCR. PrimeTime qPCR 5’Nuclease primer and probe sets (Integrated DNA Technologies, Coralville, IA, USA) and TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA) were used for RT-QPCR. Liver cDNA was used to quantify gene expression of mouse IL-1β, IL-6, TNF-α, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified as a housekeeping gene. Primer sets and probe sequences are shown in Table 3.2. Thermal cycler procedures were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min, and run using the 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Data were analyzed using the comparative threshold cycle method, and expressed as fold change (Schmittgen and Livak 2008).

3.3.9. **Statistical analysis**

Results are presented as mean ± standard deviation (SD). Within one time point, data were analyzed using Microsoft Excel (Microsoft Co., Redmond, WA, USA) for Student’s t test. Time and dietary broccoli effects were evaluated by two-way analysis of variance (ANOVA) using Statistical Analysis System 9.4 Software (SAS Institute Inc., Cary, NC, USA).
3.4. Results

3.4.1. Body weight and body composition were not changed by long-term broccoli consumption.

Body mass in both groups doubled in 3 months compared to the initial body weight of 18.1±1.3 g at 4 weeks of age, and continued to increase up to month 7. However, there was no difference in body weight between Western and Western+Broccoli groups throughout the study. Although body weights increased with time \((P<0.0001, \text{two-ways ANOVA})\), the effect of dietary broccoli on body weight was not significant (Table 3.4). Furthermore, dietary broccoli did not change body composition, including relative liver or epididymal adipose tissue weight (percentage of body weight), at any of the time points. Relative liver weight showed an increase with time \((P=0.0013)\) with a time x broccoli interaction \((P=0.0326)\), while relative epididymal adipose mass decreased from month 3 to month 7 \((P<0.0001)\) for both dietary groups. This indicates that dietary broccoli showed no influence on body weight or composition.

3.4.2. Dietary broccoli decreased liver triglyceride accumulation and NAFLD scores.

Hepatic steatosis is an early symptom of NAFLD, seen as increased lipid vacuoles in hepatocytes. Liver triglycerides were increased from month 3 to month 5 and reached a plateau between months 5 and 7 with Western diet treatment. In mice receiving broccoli treatment, total liver triglycerides were lower than those receiving no broccoli at 5 months, but this effect was no longer evident at month 7 (Table 3.5). Histological evaluation showed changes in liver morphology with time, first impacting livers of Western diet-fed mice and then Western+Broccoli diet-fed mice. However, NAFLD scores increased with time \((P<0.0001)\) for both dietary groups. Table 3.5 shows that dietary broccoli decreased NAFLD scores even by month 3 \((1.5±0.5 \text{ vs. } 0.5±0.5, \text{Western vs. Western+Broccoli, } P<0.05)\) and continued to be lower.
than those in the Western group at month 5 (2.0±0.0 vs. 0.5±0.5, \( P<0.001 \)) and month 7 (2.5±0.5 vs. 1.5±0.5, \( P<0.05 \)). Representative images are shown in Figure 3.1.

3.4.3. Dietary broccoli ameliorated liver damage and elevated phase 1 metabolic enzyme activity.

We expected that both DEN and the Western diet might cause liver damage. By the 3rd month, the early liver damage marker serum ALT levels were low and were not altered by broccoli, suggesting no liver damage. Although all mice exhibited elevated serum ALT by 5 months, mice receiving dietary broccoli exhibited lower serum ALT, indicating a protective effect. However, the positive effect from broccoli was diminished by 7 months (Fig. 3.2). Liver detoxification enzymes are responsible for metabolism of xenobiotics and drugs (Xu, Li et al. 2005). Broccoli contains several bioactive compounds that may influence phase 1 and 2 metabolism (Jeffery and Araya 2009). Activity of hepatic Cyp 1A1, a phase 1 detoxification enzyme, was enhanced by broccoli in months 3, 5, and 7 (\( P<0.05 \)), but there was no significant impact of broccoli on the phase 2 enzyme NQO1 (Fig. 3.3), although NQO1 activity increased with time in both groups of mice (\( P<0.0001 \)).

3.4.4. Long-term broccoli consumption exerted a mild influence on liver tumor development and inflammation.

There were no macroscopic or microscopic neoplasm-related lesions in livers of mice at month 3. Formation of liver nodules was evident by the 5th month of dietary treatment. Visible liver nodule incidence was found in 5/6 Western diet-fed mice and in 3/6 Western+Broccoli diet-fed mice. The mice receiving only the Western diet for 5 months exhibited hepatic adenomas. However, there were no hepatic adenomas in mice fed the Western+Broccoli diet (Table 3.6). Microscopic examination showed hepatic foci in both dietary groups, suggesting the process may
have been slowed but not stopped by including broccoli in the diet. Similarly, although there were adenomas in the Western diet-fed group and not in the Western+Broccoli diet-fed group at 5 months, both groups exhibited carcinomas by 7 months. These findings imply that dietary broccoli could not fully overcome the adverse effects of DEN and a Western diet. All animals exhibited visible liver nodules by the 7th month with increased number and size compared to the 5th month ($P<0.0001$). Evaluation of inflammation in liver, as IL-6 mRNA expression, appeared to increase with time although it was not significant ($P=0.0634$). Furthermore, there was no effect of broccoli at any time points evaluated (Fig. 3.4A). Hepatic IL-1β and TNF-α expression were not different between groups at month 3, but at month 5, inclusion of broccoli in the diet down-regulated both IL-1β and TNF-α mRNA levels (Fig. 3.4B and C). By month 7, this broccoli effect was no longer evident. These data support the idea that dietary broccoli could slow liver inflammation temporally but not sufficiently to have a lasting impact.

3.5. Discussion

In this study, we expected to see that a Western diet would increase both liver and fat mass with time, and we used epididymal adipose tissue mass as a marker for body fat, which is visceral white adipose tissue. Although the body weight increased from month 3 to 7 as expect, results showed that the epididymal adipose tissue mass actually decreased from month 3 to 7. Obesity is known to cause the development of NAFLD (Angulo 2002). Clinically, lipodystrophy, or selective loss of body fat, is related to fatty liver, insulin resistance, and diabetes (Huang-Doran, Sleigh et al. 2010). Patients with lipodystrophy frequently exhibit increased triglyceride levels in liver, typically resulting in hepatomegaly (Reitman, Arioglu et al. 2000). Here we report that as the epididymal adipose tissue weight decreased, liver weight increased (Table 3.4).
The loss of epididymal fat mass and gain in liver mass was reported previously in a study of diet-induced obesity (Strissel, Stancheva et al. 2007). Over 16 weeks of a high fat diet, epididymal adipose tissue slowly shrank with time, showing a negative correlation with liver mass. These researchers reported increased death of adipocytes and decreased adipocyte size (Strissel, Stancheva et al. 2007). F4/80, CD68, and TNF-α expression in epididymal adipocytes have all been used as markers showing lipodystrophy in obese mice, suggesting that loss of fat mass may be related to advanced inflammation within adipocytes, leading to lipolysis and the release of lipid into the circulation (Strissel, Stancheva et al. 2007; Duval, Thissen et al. 2010). However, neither we nor others see loss of body weight. Therefore, in mice on long-term high fat diet and/or high sugar diets, data from a single fat mass might not be representative.

Steatosis, or hepatic lipid accumulation, is an early change in NAFLD development. Lipid accumulation in the liver might be due to changes in lipid uptake and/or excretion. Sources of lipid in the circulation, including non-esterified free fatty acids and dietary fat, may all be increased by long-term high-fat or Western diets (Donnelly, Smith et al. 2005). *De novo* lipogenesis in liver is enhanced by insulin and regulated by SREBP-1c (Postic and Girard 2008). In addition, the possibility that mitochondrial fatty acid β-oxidation and VLDL excretion could be down-regulated during the development of steatosis has been proposed (Au, Kung et al. 2003; Shindo, Fujisawa et al. 2010). Mice receiving dietary broccoli exhibited suppressed triglyceride accumulation (Table 3.5), seen as fewer hepatic lipid droplets (Fig. 3.1) at both months 3 and 5. However, this protective effect was no longer evident by 7 months. Sulforaphane, the major bioactive compound in broccoli, may regulate lipid metabolism via the 5’ AMP-activated protein kinase (AMPK) signaling pathway. In an *in vitro* study, sulforaphane enhanced hormone-sensitive lipase activity by inhibiting the phosphorylation of AMPK at Thr-172 in 3T3-L1 cells.
(Lee, Moon et al. 2012). However, in one animal study, sulforaphane was seen to inhibit lipogenesis in adipose tissue by enhancing the phosphorylation of AMPK and acetyl CoA carboxylase (Choi, Lee et al. 2014). Even though \textit{in vitro} and \textit{in vivo} study results are inconsistent, these studies do suggest that dietary broccoli may alter lipogenesis and lipolysis pathways, consistent with our observation. The \textit{in vitro} study that resulted in AMPK inhibition used relatively high concentration of sulforaphane (2.5-10 \( \mu \text{M} \)) to treat 3T3-L1 adipocytes, whereas the \textit{in vivo} study that saw enhanced activity provided the mice approximately 20 \( \mu \text{mol} \) sulforaphane/day in the diet, which likely resulted in less than 2 \( \mu \text{M} \) in circulation. This calculation is based on a study providing a single oral dose of 50 \( \mu \text{mol} \) sulforaphane to rats, giving a maximum of 2 \( \mu \text{M} \) in plasma (Hu, Hebbar et al. 2004). Different doses of sulforaphane (> 2 \( \mu \text{M} \) vs < 2 \( \mu \text{M} \)) and time of treatment (24 hours vs lifelong) may influence AMPK activation differently. In our study, mice only received about 2 \( \mu \text{mol} \) sulforaphane/day, which might be much closer to a low dose-long term treatment that favors the activation of AMPK for improved hepatic lipid control.

In the earliest stage of liver tumor development evaluated (month 3), there was only mild hepatic lipodosis and no evidence of liver tumor formation among animals. However, the data from the late stage (month 7) showed 100\% liver nodule incidence in both groups. More interesting results were at month 5, where dietary broccoli appeared to slow development of hepatic lipodosis, lowering both hepatic triglyceride content and NAFLD level (Table 3.5), and even appeared to ameliorate liver damage seen as altered serum ALT (Fig. 3.2). Hepatic IL-1\( \beta \) and TNF-\( \alpha \) expression were also down-regulated at month 5, which suggested that liver inflammation was suppressed (Fig. 3.4). However, tumor incidence in the livers at the 5\textsuperscript{th} month was similar among dietary treatments, only showing a trend toward a protective effect from
dietary broccoli (Table 3.6). This implies that in this DEN liver cancer mouse model, dietary broccoli partially overcame the negative effect caused by the treatments of DEN and a Western diet, but even though inflammation was somewhat inhibited, the influence from broccoli may not have been sufficiently robust to inhibit the development of hepatocellular carcinoma.

Broccoli contains bioactive compounds that are able to regulate the activity of liver detoxification enzymes: indole-3-carbinol enhanced Cyp 1A1 activity \textit{in vitro} or \textit{in vivo} (Katchamart and Williams 2001; Anwar-Mohamed and El-Kadi 2009). Phase 1 Cyp 1A1 activity in rodent livers may also decrease with age (Yun, Oh et al. 2010), whereas dietary fat may enhance Cyp 1A1 activity (Rijnkels and Alink 1998). Another phase 1 enzyme, Cyp 2E1, is considered the major enzyme responsible for activation of N-alkynitrosamines with short alkyl chains, including DEN (Fujita and Kamataki 2001). Cytochrome P450 2E1 can also oxidize fatty acids and be induced by high dietary fat (Abdelmegeed, Banerjee et al. 2012). However, broccoli is not known to have an impact on Cyp 2E1. For example, in one study rats were fed with a broccoli diet containing 95 mg/kg glucosinolates (14.4% by weight in diet with fresh broccoli) but there was no change of hepatic Cyp 2E1 activity (Arikawa and Gallaher 2008). A broccoli diet is also reported to increase expression of several phase 2 enzymes, including NQO1 (Liu, Volker et al. 2009). We did not see an increase in NQO1 activity from broccoli, possibly because both age and high dietary fat also influence NQO1. Expression of NQO1 increases with age, but decreases with a high fat diet (Tanaka, Aleksunes et al. 2008; Townsend, Chen et al. 2014). The interaction between fat and broccoli should be addressed in a study that has separate arms for high fat/low fat in addition to broccoli/no broccoli.

Several experimental mouse models of liver cancer use chemical carcinogens to initiate the cancers. Carcinogens, including DEN, aflatoxin, and carbon tetrachloride, can induce liver
tumorigenesis (Heindryckx, Colle et al. 2009). Diethylnitrosamine is a nitrosamine and is typically administered by intraperitoneal injection (Leenders, Nijkamp et al. 2008). Diethylnitrosamine causes hepatic neoplasm-related lesions in a dose-dependent manner (Kushida, Kamendulis et al. 2011). Gender and age may also influence the carcinogenicity of DEN, with male and young animals having greatest sensitivity (Naugler, Sakurai et al. 2007; Heindryckx, Colle et al. 2009). The male gender is also a risk factor for human hepatocellular carcinoma, thus we used this model (American Cancer Society 2015). Since infant mouse liver continues to develop during early growth, a single low dose of DEN treatment (5 mg/kg) to infant mice (15 days old) as used here, has been reported to cause 50% incidence of liver adenoma by 7 months of age (Vesselinovitch and Mihailovich 1983). In one report, a single 25 mg/kg dose of DEN given to C57BL/6J 15-day old male mice resulted in an 80% incidence in liver carcinomas in mice receiving a control diet, and a 100% incidence in those receiving a high fat diet after 30 weeks of dietary treatment (Park, Lee et al. 2010). In contrast, a single 80 mg/kg dose of DEN given to 4-month-old male mice was reported to induce an incidence of only 6.6% adenoma after 6 months (Ward, Ohshima et al. 1984). These data suggest that the infant mouse model may have a more robust effect on liver tumorigenicity and that it is aggravated by a high fat diet. The present study, using the aforementioned infant model, had similar results: 100% of the mice fed the Western diet exhibited liver nodules by 7 months when given DEN on day 15. One drawback to this model is that mice are not weaned until day 21, even though DEN has been administrated on day 15. Thus this model evaluates the impact of broccoli on progression and development of cancer, not initiation. This may be one reason why, even by 5 months, when broccoli showed positive effects against hepatic steatosis and inflammation, there was no impact on overall long-term tumor progression. Therefore, to evaluate the full effect of broccoli on liver
cancer development, it may be necessary to choose a liver cancer model that allows for dietary impact on both initiation and progression.

In summary, dietary broccoli suppressed development of lipidosis in animals receiving a Western-style diet rich in refined sugar and saturated fat. During the development of liver cancer, consumption of broccoli partly inhibited liver damage and inflammation, but was not able to inhibit the progression of liver tumor development in this infant mouse model. A model, where Western and broccoli diets are given prior to DEN, might provide better insight into the potential for broccoli to slow or inhibit liver cancer development aggravated by a Western diet.
Figure 3.1. Effect of dietary broccoli on hepatic lipodosis in Western diet-fed mice. Liver sections were stained with hematoxylin and eosin. Representative images from each group at medium and high magnification are shown 40X and 100X magnifications. Clear, round vacuoles (closed arrows) indicate triglyceride accumulation. Basophilic cell focus (open arrow) is an altered hepatic focus. CV, central vein; PV, portal vein.
Figure 3.2. Effect of dietary broccoli on serum ALT in Western diet-fed mice. Data are presented as mean±SD (n=6 per group per time point). Open bar, Western diet; closed bar, Western+Broccoli diet. ** P<0.01 compared to Western diet.
Figure 3.3. Effect of dietary broccoli on hepatic metabolic enzyme activity in Western diet-fed mice. (A) Phase 1 enzyme Cyp 1A1 and (B) Phase 2 enzyme NQO1. Data are presented as mean+SD (n=6 per group per time point). Open bar, Western diet; closed bar, Western+Broccoli diet. * P<0.05 compared to Western diet.
Figure 3.4. Effect of dietary broccoli on hepatic cytokine expression in Western diet-fed mice. (A) IL-6, (B) IL-1β, and (C) TNF-α mRNA expression. Data are presented as mean±SD (n=6 per group per time point). Open bar, Western diet; closed bar, Western+Broccoli diet. * P<0.05 compared to Western diet.
Table 3.1. Western Diet Formula

<table>
<thead>
<tr>
<th></th>
<th>Western</th>
<th>Western+ Broccoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli (g/kg)†</td>
<td>--</td>
<td>100.0</td>
</tr>
<tr>
<td>Casein (g/kg)</td>
<td>229.9</td>
<td>229.9</td>
</tr>
<tr>
<td>Corn Starch (g/kg)</td>
<td>73</td>
<td>30.5</td>
</tr>
<tr>
<td>Maltodextrin 10 (g/kg)</td>
<td>36.8</td>
<td>36.8</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>344.8</td>
<td>344.8</td>
</tr>
<tr>
<td>Cellulose (g/kg)</td>
<td>57.5</td>
<td>--</td>
</tr>
<tr>
<td>L-Cystine (g/kg)</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Mineral Mix (g/kg)‡</td>
<td>40.2</td>
<td>40.2</td>
</tr>
<tr>
<td>Vitamin Mix (g/kg)§</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Choline Bitartrate (g/kg)</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Corn Oil (g/kg)</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Lard (g/kg)</td>
<td>188.5</td>
<td>188.5</td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
<td>4.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Protein %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Freeze-dried broccoli powder, *Brassica oleracea* L. cv Green Magic.

‡Mineral mix for AIN-93G diet.

§Vitamin mix for AIN-93 diet.

||Percentage of total calories.
Table 3.2. Definition of NAFLD Scores

<table>
<thead>
<tr>
<th>Level of Hepatic Lipidosis</th>
<th>Score</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>No affected hepatocytes.</td>
</tr>
<tr>
<td>Minimal</td>
<td>1</td>
<td>Affected hepatocytes in midzonal areas with macrovesicular vacuolation.</td>
</tr>
<tr>
<td>Mild</td>
<td>2</td>
<td>Affected hepatocytes in midzonal and some centrilobular areas with mainly macrovesicular but occasional microvesicular vacuolation.</td>
</tr>
<tr>
<td>Moderate</td>
<td>3</td>
<td>Affected hepatocytes in midzonal and centrilobular areas with both macrovesicular and microvesicular vacuolation.</td>
</tr>
<tr>
<td>Marked</td>
<td>4</td>
<td>Affected hepatocytes in midzonal, centrilobular and some periportal areas with both macrovesicular and microvesicular vacuolation.</td>
</tr>
<tr>
<td>Severe</td>
<td>5</td>
<td>Affected hepatocytes in midzonal, centrilobular and periportal areas with both macrovesicular and microvesicular vacuolation.</td>
</tr>
<tr>
<td>Gene</td>
<td>Reference sequence number</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_008361</td>
<td>5'-GACCTGTCTTTGAAAGTTGACG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTCTTTGTTGATGTCGCTGCT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_031168</td>
<td>5'-GATACCACTCCCAACAGACC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CAAGTGCACTCAGTTGTTCA-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_013693</td>
<td>5'-AGACCCCTCACACTGATCA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTCTTGAGATCCATGCCTGTT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_008084</td>
<td>5'-AATGGTGAAGGTGGTGTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GTGGAGTCATACCTGAAACTGTA-3'</td>
</tr>
<tr>
<td>Month</td>
<td>Diet</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>3</td>
<td>Western</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>39.9</td>
</tr>
<tr>
<td>5</td>
<td>Western</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>46.0</td>
</tr>
<tr>
<td>7</td>
<td>Western</td>
<td>48.9</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>50.5</td>
</tr>
</tbody>
</table>

Data are presented as mean and standard deviation, n=6.

†Percentage of body weight.

‡Epididymal adipose tissue.
Table 3.5. Total Liver Triglyceride and NAFLD Score

<table>
<thead>
<tr>
<th>Month</th>
<th>Diet</th>
<th>Total liver triglyceride (mg)</th>
<th>NAFLD Score</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>3</td>
<td>Western</td>
<td>132</td>
<td>37</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>112</td>
<td>51</td>
<td>0.5*</td>
</tr>
<tr>
<td>5</td>
<td>Western</td>
<td>261</td>
<td>60</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>149*</td>
<td>65</td>
<td>0.5**</td>
</tr>
<tr>
<td>7</td>
<td>Western</td>
<td>231</td>
<td>120</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>306</td>
<td>92</td>
<td>1.5*</td>
</tr>
</tbody>
</table>

Data are presented as mean and standard deviation, n=6.

*P<0.05 compared to Western group, same month.

**P<0.001 compared to Western group, same month.
Table 3.6. Macroscopic and Microscopic Hepatic Neoplasm-Related Lesions

<table>
<thead>
<tr>
<th>Month</th>
<th>Diet</th>
<th>Nodule incidence$^+$</th>
<th>Max nodule diameter (mm)</th>
<th>Average nodule number</th>
<th>AHF</th>
<th>HA</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Western</td>
<td>0/6</td>
<td>--</td>
<td>0.0</td>
<td>0.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>0/6</td>
<td>--</td>
<td>0.0</td>
<td>0.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>Western</td>
<td>5/6</td>
<td>1.8</td>
<td>4.0</td>
<td>3.9</td>
<td>4/6</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>3/6</td>
<td>4.8$^*$</td>
<td>1.2</td>
<td>1.6</td>
<td>4/6</td>
<td>0/6</td>
</tr>
<tr>
<td>7</td>
<td>Western</td>
<td>6/6</td>
<td>5.4</td>
<td>20.2</td>
<td>10.0</td>
<td>5/6</td>
<td>3/6</td>
</tr>
<tr>
<td></td>
<td>Western+Broccoli</td>
<td>6/6</td>
<td>11.1$^*$</td>
<td>34.8</td>
<td>18.0</td>
<td>5/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Data for max nodule diameter and average nodule number are presented as mean and standard deviation, n=6.

AHF, altered hepatic foci; HA, hepatic adenoma; HCC, hepatocellular carcinoma.

$^+$Visible liver nodules (diameter ≥ 1 mm)

$^*$P<0.05 compared to Western group.
CHAPTER 4
PROTECTION AGAINST BOTH NON-ALCOHOLIC FATTY LIVER AND CANCER BY BROCCOLI IN A WESTERN DIET-ENHANCED MOUSE MODEL OF DIETHYLNITROSAMINE-INDUCED LIVER CANCER

4.1. Abstract

In today’s world, the prevalence of non-alcoholic fatty liver disease (NAFLD) is high, likely due to the popular high fat and high sugar “Westernized” diet. Without treatment, NAFLD may progress to hepatocellular carcinoma, which has a high mortality rate. We hypothesized that dietary broccoli could decrease both hepatic lipidosis and development of liver tumorigenesis in a mouse model of Western diet-enhanced liver cancer. Adult male B6C3F1 mice received a control or Western diet, with or without broccoli. Starting the following week, mice were given diethylnitrosamine once a week for weeks 2, 3, 4, 6, 7, and 8. Results show that dietary broccoli (10%) lowered the hepatic triglyceride pool, decreased NAFLD, and impeded the initiation and progression of liver cancer, supporting a hepato-protective effect of broccoli.
4.2. Introduction

Non-alcoholic fatty liver disease (NAFLD), the term to describe a cluster of related liver diseases from hepatic steatosis, through non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and potentially hepatocellular carcinoma, is prevalent in the United States today, but exhibit few symptoms (Cohen, Horton et al. 2011). Almost one of every two adult Americans has NAFLD (Williams, Stengel et al. 2011). Liver cancer is a lethal cancer, with a low five-year survival rate (17%), and the fifth most common cancer in men in the world (World Health Organization 2015; American Cancer Society 2015). However, it is relatively preventable, because many of the risk factors for liver cancer are related to diet, lifestyle, and infection (Laursen 2014). More than 50% of the cases of hepatocellular carcinoma are related to hepatitis B infection, but hepatitis B can be prevented by vaccination (Poland and Jacobson 2004; Perz, Armstrong et al. 2006). However, it has been reported that in the obese population there is not only a higher prevalence of NAFLD than in people with normal body weight (75% vs. 16%), but also a higher mortality risk from liver cancer (relative risk 4.5 vs. 1) (Bellentani, Saccocio et al. 2000; Calle, Rodriguez et al. 2003). According to the newest report of the Continuous Update Project in the World Cancer Research Fund International published in March 2015, body fatness has been officially added to the list of factors that increase risk for liver cancer. This is supported by strong evidence, adding obesity to the two other major risk factors, aflatoxins and alcohol. (World Center Research Fund International/American Institute for Cancer Research 2015). However, obesity can be reduced by changing lifestyle, such as increasing physical activity and altering dietary patterns (Jeffery, Wing et al. 2003; Swinburn, Caterson et al. 2004).

The so-called “Westernized” diet, which is high in saturated fat and sugar, is well rooted in the lifestyle of the majority of Americans. The present Dietary Guidelines reveal that more than
35% of daily energy consumed by Americans is from solid fats, including trans fats and saturated fats, and added sugar (U.S. Department of Agriculture, ARS 2010). Excess consumption of saturated fat and sucrose not only contributes to adiposity but also to the progression of NAFLD. Saturated fatty acids are a poor source of energy, since they are not the priority substrate for β-oxidation, and tend to be directed toward storage (Storlien, Huang et al. 2001). In liver, saturated fatty acids can increase endoplasmic reticular stress and free radical production in mitochondria, which promote the development of NASH (Leamy, Egnatchik et al. 2013). Moreover, fructose, which can be supplied directly or in the form of sucrose, is a highly lipogenic nutrient, enhancing hepatic lipid accumulation through the de novo lipogenesis pathway (Dekker, Su et al. 2010). Therefore, the Westernized diet is able to disrupt lipid homeostasis and oxidative balance in the liver and worsen the development of NAFLD.

Broccoli, a brassica vegetable containing multiple bioactive compounds such as vitamin C, flavonoids, and glucosinolates, has shown substantial market growth in the past 20 years (Jeffery and Araya 2009; U.S. Department of Agriculture, ERS 2015). It has been reported in epidemiological studies that incorporating brassicas into daily diets can decrease cancer risk at several tissue sites, including bladder, breast, prostate, and colon (Michaud, Spiegelman et al. 1999; Kolonel, Hankin et al. 2000; Voorrips, Goldbohm et al. 2000; Terry, Wolk et al. 2001). There are no reports of protection against liver cancer, however. Our previous study demonstrated that dietary broccoli was able to impede the development of NAFLD by decreasing hepatic triglyceride accumulation in mice fed a Western diet (Chen et al. submitted). However, long-term consumption of broccoli had no positive impact on the progression of liver cancer in a model where diethylnitrosamine (DEN) was given to infant mice prior to starting the broccoli diet. Therefore, we hypothesized that the long-term consumption of broccoli could regulate
hepatic lipid metabolism, and thus decrease the level of hepatic lipidosis that is typically increased by a Western diet. We further hypothesized that dietary broccoli could hinder the initiation of liver tumorigenesis by the hepatic carcinogen DEN, if broccoli was provided prior to the carcinogen. To approach our goal, adult B6C3F1 mice, a carcinogen-sensitive strain, were used in the present study, designed so that the DEN treatments were given after the start of the broccoli diet. In this way, we were able to observe the impact of dietary broccoli on both the initiation and progression of liver tumorigenesis. The genes for hepatic lipid-metabolizing enzymes involved in de novo lipogenesis, fatty acid oxidation, influx of fatty acids, and very low-density lipoprotein (VLDL) excretion were evaluated to determine how a Westernized diet and broccoli might interact to affect hepatic triglyceride accumulation.

4.3. Materials and Methods

4.3.1. Animals and diets

Male 4-week-old B6C3F1 mice (C57BL/6J x C3H/HeJ hybrid) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed individually under a 12-hour light/dark cycle at 22°C and 60% humidity at the Institute for Genomic Biology animal facility at the University of Illinois. Water and feed were provided ad libitum. Animal care was in compliance with the Institutional Animal Care and Use Committee at the University of Illinois, according to the National Institutes of Health guidelines.

A powdered control diet was prepared by following the AIN-93M formula with adjustments (Reeves, Nielsen et al. 1993). Soybean oil was replaced with corn oil (rich in ω-6 fatty acids).
The Western diet was formulated by modifying the AIN-93M diet, increasing the sucrose and saturated fat content (Table 4.1). Freeze dried broccoli powder (10% by weight; Brassica oleracea L. var. Green Magic), kindly provided by Dr. John A. Juvik, was incorporated into the Control+Broccoli and Western+Broccoli diets, and ingredients were replaced based on the composition of dried broccoli. Diet ingredients were acquired from the Harlan Laboratory (Indianapolis, IN, USA).

4.3.2. Experimental design

Seventy-two 4-week-old male B6C3F1 mice were acclimated for 1 week with AIN-93M diet prior to starting diet treatments. At the age of 5 weeks, mice were divided into 4 groups and provided with Control, Control+Broccoli, Western or Western+Broccoli diets. One week later, diethylnitrosamine (Sigma-Aldrich, St. Louise, MO, USA) in normal saline solution was given to mice (n=12/group) at a dose of 45 mg/kg by intraperitoneal (i.p.) injection once a week for 6 weeks, and saline was given as vehicle control (n=6/group). There was a one-week interval after 3 injections for better recovery from DEN treatment (Fig. 4.1). Feed was changed every other day and freshly made weekly. Body weight and feed intake were monitored every week. Two DEN-treated mice died during the injection period; six DEN-treated mice (one in Control diet, two in Control+Broccoli diet, one in Western diet, and two in Western+Broccoli diet) were euthanized before the end of study due to serious illness. Mice were sacrificed at 6 months after starting the DEN treatments for the collection of tissue and blood samples, and the count of liver nodules (diameter ≥ 1 mm). Liver tissue was divided for biochemical analysis (stored at -80°C) and histology (fixed in 10% neutral buffered formalin).
4.3.3. Hepatic triglyceride content

Liver lipid was extracted by the Folch method (Folch, Lees et al. 1957) with some modifications. Liver tissue was homogenized with the Folch solution (chloroform:methanol, 2:1, v/v) containing 0.01% butylated hydroxytoluene, and extracted with agitation for 2 hours. The homogenate was washed with ultrapure water and centrifuged to isolate the organic phase. Chloroform in the organic phase of the lipid extract was evaporated with mild heating (50°C) and a stream of nitrogen gas. The lipid extract was reconstituted with absolute ethanol for quantification. The hepatic triglyceride level was determined by the glyceride phosphate oxidase method using a reagent kit (Pointe Scientific, Canton, MI, USA).

4.3.4. Liver histology

Liver samples from the left, median, and right lobes were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of each lobe from hilar to lobe edge (3 µm thick) were stained with hematoxylin and eosin (H&E) for histological examination. Histology work was carried out at the Veterinary Diagnostic Laboratory at the University of Illinois (Urbana, IL, USA). Murine NAFLD scores and microscopic hepatic neoplasm-related lesions were determined by a trained pathologist, based on the H&E stained sections.

4.3.5. Plasma alanine aminotransferase (ALT) levels

Plasma ALT levels were determined using a reagent kit (Pointe Scientific, Canton, MI, USA), according to manufacturer’s instructions in a 96-well format. The difference in absorbance at 340 nm was converted to IU/L by multiplying with the factor of 1768.
4.3.6. Immunoblotting

Liver tissue was homogenized with lysis buffer [0.15 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, 5 mM dithiothreitol, 1% Triton X-100 and the Complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany)]. The protein extract was separated by centrifugation. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Liver protein samples were separated by electrophoresis on a 7.5% sodium dodecyl sulfated polyacrylamide gel, and transferred to a nitrocellulose membrane (GE Health Care, Piscataway, NJ, USA). After blocking (5% nonfat dried milk at room temperature for 1 hour), membranes were incubated with primary anti-mouse antibodies (1:500) using goat polyclonal anti-microsomal triglyceride transfer protein (MTTP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-acetyl CoA carboxylase (ACC) (Cell Signaling Technology, Beverly, MA, USA), or rabbit polyclonal anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4°C, and then incubated with goat anti-rabbit or donkey anti-goat IgG horseradish-peroxidase secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hour. Immunocomplex was detected using Amersham ECL Advance Western Blotting Detection Kit (GE Health Care, Piscataway, NJ, USA). Chemiluminescence was visualized and quantified using ImageQuant LAS 4000 and IQTL software (GE Health Care, Piscataway, NJ, USA).

4.3.7. Real time quantitative PCR

Liver RNA was extracted with Trizol Reagent (Life Technologies, Carlsbad, CA, USA) and purified using the E. Z. N. A. Total RNA Kit II (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer’s instruction. RNA integrity was monitored by 1% bleach agarose
gel electrophoresis (Aranda, LaJoie et al. 2012). Synthesis of cDNA from RNA was by using a reverse transcription reagent kit (Life Technologies, Carlsbad, CA, USA). PrimeTime qPCR 5’Nuclease primer and probe sets (Integrated DNA Technologies, Coralville, IA, USA) and TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA; 4324020) were used for real time quantitative PCR. Hepatic gene expression of interleukin (IL) -1β, IL-6, CD36, CD68, tumor necrosis factor alpha-α (TNF-α), interferon-γ (IFN-γ), fatty acid synthase (FAS), carnitine palmitoyltransferase-1α (CPT-1α), cytochrome P450 2E1 (Cyp 2E1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified as a housekeeping gene, were quantified. Sequences for primer sets and probes are shown in Table 4.2. Thermal cycler procedures were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min, and run using the 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Data were analyzed using the comparative threshold cycle method, and expressed as fold change (Schmittgen and Livak 2008).

4.3.8. Statistical analysis

All results, except liver tumor/neoplasm-related lesion incidence, are expressed as mean and standard deviation or standard error, and were analyzed by two-way or three-way analysis of variance for the effect of DEN, broccoli, the Western diet, and interactions. Differences in liver tumor and neoplasm-related lesion incidence were evaluated by the Fisher’s exact test. A $P$ value of less than 0.05 was considered significant in this study. All statistical analyses were performed using Statistical Analysis System 9.4 Software (SAS Institute Inc., Cary, NC, USA).
4.4. Results

4.4.1. Dietary broccoli decreased relative liver mass but not body weight.

Dietary treatments were started one week before the first DEN treatment and continued to the completion of the study. At the age of 12 weeks, which was the week of the last DEN injection, feed intake decreased in the DEN-treated mice ($P=0.0008$, data not shown). However, after the period of DEN treatments, there was no difference in feed intake between the DEN- and saline-treated groups (13 weeks of age). Final body weights were increased by the Western diet ($P<0.0001$) but not influenced by dietary broccoli (Table 4.3). However, DEN exerted a negative impact on body weight, adversely affecting body weight increase during the period of DEN treatments (Fig. 4.2) and resulting in a decreased final body weight ($P<0.0001$, Table 4.3). Although dietary broccoli showed no impact on final body weights, it did decrease relative liver mass (percentage of body weight, $P=0.0222$, Table 4.3). The Western diet not only increased body mass but also relative liver mass ($P<0.0001$), whereas DEN treatments had no influence on liver percentage of body weight (Table 4.3). Unexpectedly, relative epididymal adipose tissue mass was decreased by the Western diet but increased with DEN treatments ($P<0.0001$ for both, Table 4.3). Upon further examination, it became evident that the absolute liver mass and the absolute epididymal adipose tissue were negatively correlated ($r=-0.71$), but only among the high body weight mice (final body weight $> 40$ g). Thus mice with a larger liver had less epididymal adipose tissue mass.
4.4.2. The Western diet resulted in liver damage and increased hepatic triglycerides accumulation whereas dietary broccolis was able to block the effect.

Feeding a Western diet increased the plasma ALT level \( (P=0.0056) \), suggesting that liver damage was raised, but DEN treatments showed no effect on plasma ALT (Fig. 4.3). Dietary broccolis exhibited a significant protective effect, with decreased release of ALT from liver \( (P<0.0001) \), independent of any effect from Western diet or DEN treatments (Fig. 4.3). Moreover, liver triglyceride density \( (\text{mg triglyceride/mg protein}) \) was decrease by dietary broccolis as well \( (P<0.0001) \), but there was no impact of the Western diet or DEN treatments (Table 4.4). As for total liver triglycerides, these were decreased by dietary broccolis \( (P=0.0003) \), whereas the Western diet increased total triglyceride levels in liver \( (P<0.0001, \text{Table 4.4}) \). However, DEN-treated animals had lower total liver triglyceride compared to saline vehicle-control animals \( (P=0.0114, \text{Table 4.4}) \). Histological examination of liver sections also provided some insight into development of hepatic lipidosis. The NAFLD scores, which were graded based on H&E stained liver sections, showed a similar pattern to total triglyceride content in liver, increased by the Western diet and decreased by dietary broccolis, but showed no impact of DEN (Table 4.4). Furthermore, there was an interaction between the Western diet and DEN treatments on the NAFLD score \( (P=0.0311, \text{Table 4.4}) \). It showed that in the Western diet group, DEN treatment decreased the NAFLD level, but not in the control diet group. Figure 4.4 shows representative hepatic histological images.
4.4.3. Dietary broccoli protected against hepatic lipidosis by regulating hepatic CD36 and MTTP.

Hepatic lipid metabolism was impacted by DEN, broccoli, and the Western diet in this study. The expression of CD36, a major fatty acid transporter in liver, was decreased by long-term consumption of broccoli but increased by the Western diet ($P=0.0056$ and $P=0.0002$, respectively) (Table 4.5A). DEN treatment also decreased hepatic CD36 expression, but there was an overall interaction of all treatments ($P=0.0214$, Table 4.5A). This 3-way interaction indicates that in the saline-treated animals there was a significant broccoli x Western interaction ($P<0.05$), in which a decrease was seen in hepatic CD36 expression in the control diet-fed mice ($P<0.05$) but not in the Western diet-fed ones; there was no broccoli x Western interaction in CD36 levels in the DEN-treated animals. Fatty acid synthase, a critical enzyme for de novo lipogenesis, was down-regulated by both the Western diet and DEN treatments ($P<0.0001$ and $P=0.0008$, respectively) but not by dietary broccoli (Table 4.5A). A similar dietary effect was found in the protein level of ACC, where the Western diet decreased ACC expression ($P=0.0002$, Fig. 4.5A).

Fatty acids can be utilized for energy through oxidation. Mitochondrial β-oxidation is tightly controlled by CPT, which was down-regulated by dietary broccoli and up-regulated by the Western diet ($P=0.0026$ and $P<0.0001$, respectively, Table 4.5A). Fatty acids can also be oxidized by Cyp 2E1 through microsomal ω-oxidation. There was no significant treatment effect of DEN, broccoli, or the Western diet on hepatic Cyp 2E1, individually. However, there was an interaction between DEN and the Western diet ($P=0.0087$, Table 4.5A), indicating that DEN treatments enhanced Cyp 2E1 expression in the Western diet-fed mice. Moreover, dietary broccoli may down-regulate Cyp 2E1 in animals receiving a Western diet, but exhibit a reverse
effect in animals receiving a control diet, due to a broccoli x Western interaction ($P=0.0405$, Table 4.5A). Liver excretes VLDL for distribution of triglycerides into the circulation. Our data show that MTTP, which is essential for VLDL excretion, was increased by dietary broccoli but decreased by the Western diet ($P=0.0449$ and $P=0.0056$, respectively, Fig. 4.5B).

**4.4.4. Hepatic macrophages activation was decreased by dietary broccoli.**

Activated hepatic macrophages (monitored as CD68 expression), including Kupffer cells and infiltrated macrophages, were increased by the Western diet and DEN treatments ($P=0.0087$ and $P=0.0006$, respectively, Table 4.5B). However, the broccoli-fed animals showed a decreased expression of hepatic CD68 ($P<0.0001$, Table 4.5B). Moreover, dietary broccoli also downregulated TNF-α expression in liver ($P<0.0001$, Table 4.5B). Hepatic IFN-γ, a cytokine that induces the activation of macrophages, was down-regulated by dietary broccoli as well ($P=0.0005$, Table 4.5B). The interaction between the DEN treatment and dietary broccoli indicates that the suppression of IFN-γ expression by dietary broccoli was greater when there was a DEN carcinogen challenge ($P=0.0271$, Table 4.5B). Dietary broccoli also down-regulated hepatic IL-1β ($P<0.0001$) but had no impact on IL-6 (data not shown).

**4.4.5. Long-term consumption of broccoli decreased liver nodule.**

Table 4.6 shows that DEN induced the formation of liver nodules, and that in combination with a Western diet, DEN treatment caused a 100% incidence of liver nodules. The Western diet increased both liver nodule number and size ($P=0.0033$ and $P=0.0114$, respectively, Table 4.6), as well as hepatic adenoma incidence ($P<0.05$, Table 4.7). However, dietary broccoli showed a significant protective effect, decreasing liver nodule number ($P=0.0011$, Table 4.6). As expected, there were no hepatic neoplasm-related lesions seen in liver sections from the Control+Broccoli
group, and a lower total hepatic neoplasm-related lesion incidence in the Western+Broccoli group, compared to the Western-fed animals (Table 4.7).

4.5. Discussion

In this study, DEN treatment significantly decreased final body weight, independent of dietary treatments (Table 4.3). During the period of DEN treatments (6-12 weeks of age), DEN administration retarded the growth of mice, possibly due to decreased feed intake. Diethylnitrosamine may have caused acute toxicity, leading to the temporary reduction in feed intake we observed. A similar result has been reported for a DEN rat model, in which body weight and feed intake were both adversely impacted immediately following DEN administration (100 mg/kg body weight) (Barbisan, Miyamoto et al. 2002). In adult mice, a single high dose of DEN (80 mg/kg body weight) was also reported to cause a loss of body weight (Park, Lee et al. 2010).

We observed a decrease in epididymal adipose tissue mass, suggesting a negative correlation between lipodystrophy in epididymal adipose tissue and hepatomegaly ($r=-0.71$), which was also seen in our previous study (Chen et al. submitted). Our results indicate that this negative correlation is true only for mice with a high body weight (> 40 g). This finding is similar to Strissel and colleagues’ report showing that in mice fed a high fat diet, epididymal adipose tissue mass increased with growing body weight during the first 12 weeks of the diet, but from week 12 to 20, when body mass was greater than 40 g, a negative correlation ($r=-0.85$) was revealed (Strissel, Stancheva et al. 2007). This hepatomegaly-related lipodystrophy could be tissue specific, since the subcutaneous inguinal adipose mass consistently increased with long-term
high fat diet feeding and showed no tendency toward weight loss, regardless of the weight of the mice. (Strissel, Stancheva et al. 2007; Altintas, Rossetti et al. 2011).

Total liver triglycerides and NAFLD scores are endpoints used to evaluate the severity of hepatic lipidosis. The study revealed that broccoli impacted both endpoints and exhibited the ability to decrease hepatic triglycerides and lipidosis in both control and Western dietary groups. This consistent with the literature on isolated bioactives from broccoli. Sulforaphane, the hydrolyzed glucosinolate product rich in broccoli, has been reported to show potential to decrease hepatic triglyceride level in mice receiving a high fat diet, but the effect was not significant (Choi, Lee et al. 2014). Our results suggest that such a whole food intervention can exert a significant health beneficial effect. Furthermore, the glucoraphanin-containing broccoli diets (0.4 mmol/kg) in our study showed greater impact on hepatic lipid regulation than the pure sulforaphane which was given at a substantially greater dose (5.6 mmol/kg, Choi et al.).

Notably, the Western diet had opposing impacts on hepatic triglyceride levels and the NAFLD scores in the DEN-treated mice. Table 4.4 shows that the Western-DEN group had lower NAFLD scores compared to the control-DEN group (2.64±0.92 vs. 3.10±0.74), but exhibited elevated total liver triglycerides (269 ±82 vs. 175 ±40, mg/liver). These divergent results could be a distortion due to interference by the presence of hepatic preneoplastic and neoplastic lesions. Total liver triglyceride measurement is derived from the value of whole liver mass and liver triglyceride density. Our data show that the Western diet had little impact on liver triglyceride density (Table 4.4), but the Western-DEN treated animals had more liver nodules, exhibited more hepatic neoplasm-related lesions and more severe liver damage than the control-DEN mice (Table 4.6 and Fig. 4.3), which suggests that livers in the Western-DEN treated animals were not homogeneous and that tumor mass may greatly altered total liver mass.
It has been reported that in the end stage of NAFLD, the initiation of hepatocellular carcinoma is considered to be related to cirrhosis (Bugianesi, Leone et al. 2002). It has also been observed that in NAFLD patients, once cirrhosis develops, hepatic lipid accumulation decreases without body weight loss (Powell, Cooksley et al. 1990). Moreover, it is rare that hepatic tumors contain a high quantity of fat in human (Yoshikawa, Matsui et al. 1988). However, hepatocellular carcinoma may exhibit various levels of fat accumulation, possibly greatest in well-differentiated cells (Yoshikawa, Matsui et al. 1988; Valls, Iannaccone et al. 2006). These reports suggested that cirrhotic tissue or liver tumor tissue, which might occupy a significant amount of liver in the Western-DEN treated mice, may have relatively little triglyceride compared to simple hepatic steatosis. Therefore, although the Western-DEN treated mice had greater liver mass than the control-DEN mice (3.12±0.88 g vs. 2.28±0.40 g, respectively), the lack of homogeneity in liver tissue may actually have resulted in less total lipid. Historically, the NAFLD scoring system is based on the area where affected hepatocytes are located and the degree of vacuolization in order to evaluate the level of lipidosis (Kleiner, Brunt et al. 2005). This can provide an overall view, including hepatic neoplasm-related lesions, for the development of lipid deposition. Therefore, the decrease in NAFLD score that we observed in the Western-DEN treated group, as well as the loss in body weight from at the last month prior to the end of the study (33-36 weeks of age) (Fig. 4.2), may reflect the severity of liver tumorigenesis.

Hepatic triglycerides mainly derive from circulating non-esterified fatty acids (NEFA) (60%), whereas minor sources are de novo lipogenesis (25%) and dietary fat (15%) (Donnelly, Smith et al. 2005). The liver can obtain NEFA from circulation by both passive diffusion and facilitated uptake by membrane fatty acid transporters (Su and Abumrad 2009). The fatty acid translocator CD36 facilitates the transportation of long-chain fatty acids, and is highly up-regulated during
the progression of NAFLD, compared to other fatty acid transporters, such as fatty acid binding protein and caveolin (Bechmann, Gieseler et al. 2010; Miquilena-Colina, Lima-Cabello et al. 2011). Expression of CD36 can be controlled by the activation of lipogenic nuclear receptors, including liver X receptor, pregnane X receptor, and peroxisome proliferator activated receptor γ (Zhou, Febbraio et al. 2008). The present results show that dietary broccoli decreased hepatic CD36 expression by as much as 35% (Table 4.5A), which could have greatly reduced the influx of NEFA, the major source of hepatic triglycerides.

Several clinical studies suggest that de novo lipogenesis is elevated in NAFLD. Liver biopsy shows that the expression of the lipogenic enzymes FAS and ACC are both up-regulated in patients diagnosed with NAFLD (Kohjima, Enjoji et al. 2007). Furthermore, people diagnosed with NAFLD excreted more triglycerides generated from de novo hepatic lipogenesis (Diraison, Moulin et al. 2003). However, in our study, long-term broccoli consumption had no impact on FAS or ACC, which were significantly decreased, not increased, by Western dietary treatment (Table 4.5A and Fig. 4.5A). Although high fat diets (with or without high carbohydrate) are able to increase de novo lipogenesis in rodents (Strable and Ntambi 2010), diet-induced liver steatosis models do not always provide consistent results. For example, in some studies rodents developed hepatic lipidosis after 12-16 weeks of a high fat diet, with elevated hepatic FAS and ACC expression (Buettner, Parhofer et al. 2006; Li, Xu et al. 2011). However, some other studies have shown increased hepatic triglycerides levels but no change in FAS or ACC (Kim, Sohn et al. 2004; Inoue, Ohtake et al. 2005). The cause of this discrepancy among reports is still not understood, but it might relate to a feedback mechanism from the high fat diet. One report that supports this idea found that with high dietary fat, hepatic FAS expression was increased by
day 1 in C57BL/6J mice, whereas the effect was diminished by day 11 (Gregoire, Zhang et al. 2002).

Although the direct contribution from dietary fat to the hepatic triglyceride pool is minor (reported to be only 15%) (Donnelly, Smith et al. 2005), in our study the Western diet provided approximately 4.5-fold more fat than the control diet. This may suggest a high influx of triglycerides from chylomicrons in the Western diet-fed mice. Overall, our results showed that long-term high fat and high sucrose feeding may reduce hepatic lipogenesis but enrich the hepatic triglyceride pool derived from circulating NEFA and dietary fat. Consumption of broccoli may reduce the influx of NEFA and therefore the size of the hepatic triglyceride pool. Moreover, an increased expression of MTTP in broccoli-fed mice may imply enhanced excretion of VLDL, which also may contribute to the reduction of intrahepatic triglyceride deposition, whereas our Western diet had an adverse effect on MTTP. Although plasma lipoproteins were not estimated in this study, MTTP is critical for VLDL formation (Hussain, Shi et al. 2003). Future studies could determine if plasma VLDL is decreased in mice receiving a Western diet, but elevated by broccoli feeding.

Our data indicate that the Western diet raised CPT-1α expression, suggesting an increase in influx of fatty acids to mitochondria for hepatic β-oxidation, whereas dietary broccoli exhibited a converse impact. Mitochondria produce reactive oxygen species (ROS) through the electron transport chain, reducing $O_2$ to superoxide anion $O_2^- \cdot$ (Turrens 2003). High dietary fat may cause mitochondrial lipid overload (Koves, Ussher et al. 2008). It has been reported that fatty acids are able to increase superoxide production in mitochondria (Lambertucci, Hirabara et al. 2008). Therefore, although up-regulated fatty acid oxidation can promote the utilization of lipid, it can also increase the production of ROS in mitochondria, which causes oxidative stress. The “two-
hit” model for the development of NASH includes ROS as one of the “second hits” that cause liver inflammation and damage (Day and James 1998; Day 2002). In the present study, in addition to this source of ROS, mice fed the Western diet and receiving DEN showed enhanced hepatic Cyp 2E1 expression compared to mice fed the control diet (1.55±0.56 vs.0.83±0.17, respectively, Table 4.5A). Elevated hepatic Cyp 2E1 levels have been reported in both humans and animals diagnosed with NAFLD (Kohjima, Enjoji et al. 2007; Abdelmegeed, Banerjee et al. 2012). The metabolic enzyme Cyp 2E1 not only oxidizes fatty acids through the ω-oxidation pathway, but also generates ROS (Dey 2013). Oxidative stress can stimulate the proliferation and fibrogenesis of hepatic stellate cells, and it has been reported that the ROS generated from Cyp 2E1 in hepatocytes contributes to fibrosis (Baroni, D’Ambrosio et al. 1998; Nieto, Friedman et al. 2002). Moreover, the carcinogenicity of DEN is highly controlled by Cyp 2E1, since DEN is bioactivated by Cyp 2E1 (Kang, Wanibuchi et al. 2007). Thus, the high fat and high sucrose diet may cause an increased oxidative stress and thus support both the initiation and promotion of liver tumorigenesis and damage, as was evident (Table 4.6 and Fig. 4.3). In contrast, dietary broccoli revealed the potential to decrease hepatic oxidative stress by down-regulating hepatic CPT-1α, suggesting a lowered ROS level in liver.

Our result also showed that the Western diet and DEN enhanced the activation of hepatic macrophages, which includes Kupffer cells, whereas dietary broccoli exhibited a counter effect, down-regulating TNF-α and suppressing expression of the activated macrophage biomarker, CD68 (Table 4.5B). The activation of Kupffer cells has been considered to greatly contribute to the progression of NAFLD (Baffy 2009). Depletion of Kupffer cells has been shown to decrease hepatic triglyceride levels in a rat model, indicating that Kupffer cells can promote hepatic steatosis (Huang, Metlakunta et al. 2010). Activated Kupffer cells are also critical to trigger the
development of NASH from hepatic steatosis (Tosello-Trampont, Landes et al. 2012). One of the pathways where Kupffer cells are involved for the development of NASH is C-C motif chemokine receptor 2 (CCL2). It has been shown that CCL2 can increase TNF-α production in Kupffer cells and the recruitment of bone marrow-derived macrophages (Miura, Yang et al. 2012; Tosello-Trampont, Landes et al. 2012). Moreover, an elevated level of circulating CCL2 ligand, monocyte chemoattractant protein-1, has been reported in NASH patients (Haukeland, Damas et al. 2006). Therefore, dietary broccoli, by inhibiting Kupffer cell activity, impedes not only the promotion of hepatic steatosis, but also of NASH.

It should be noted that this study uncovered an impact of dietary broccoli on the initiation and progression of liver tumorigenesis. Altered hepatic foci, which are preneoplastic lesions, have the potential to progress to hepatic adenomas and possibly hepatocellular carcinomas (Gad 2007). Table 4.6 shows that among the DEN-treated animals, there were no hepatic neoplasm-related lesions in the Control+Broccoli group, indicating that dietary broccoli can suppress the initiation of hepatic neoplasms by DEN. However, although the Western+Broccoli group showed a similar incidence of altered hepatic foci compared to the Western-no broccoli group (44% vs. 36%, respectively), there was a lower incidence of hepatic adenoma (11% vs. 46%, respectively). This may suggest a delay or inhibition of the progression of preneoplasia to neoplasia, rather than a complete blockage of tumorigenesis. Epidemiological studies have long revealed that consumption of brassica vegetables is related to a reduced risk for cancer (Jeffery and Araya 2009), but there is less support from animal studies for this beneficial effect of dietary brassicas. Our study is the first to show protection against murine liver cancer by broccoli and adds support to the accumulating knowledge showing that broccoli effects in whole animal cancer studies reflect the positive epidemiological findings. This study demonstrates that whole broccoli
consumption is able to decrease the formation of hepatic neoplasm-related lesions, interfering with the development of hepatic preneoplasm in both control mice and mice modeling diet-induced obesity.

In conclusion, dietary broccoli decreased hepatic lipidosis in both mice receiving control diet and those receiving the Western diet, without changing body mass. The data support the possibility that the lowering of hepatic triglycerides by dietary broccoli may be due to decreased influx of NEFA and increased efflux/excretion of VLDL. In this study, the daily consumption of broccoli was able to suppress the activation of hepatic macrophages, decrease liver damage, and protect against the initiation and progression of liver tumor. Broccoli promoted of liver health and countered NAFLD development. In this age when obesity is such a problem, including broccoli in the diet may be able to maintain a healthy liver even for those who are greatly overweight.
Figure 4.1. Study design for DEN and dietary treatments. Control, Control+Broccoli, Western, or Western+Broccoli diets were provided from 5 weeks of age. Mice were given saline or 45 mg/kg/week DEN i.p., six weekly treatments at ages of 6, 7, 8, 10, 11, and 12 weeks. The study was terminated 24 weeks after the last DEN injection.
Figure 4.2. Growth curves of mice given different dietary and DEN treatments. Different diets were provided from 5 weeks of age. Mice were given saline or DEN i.p., six weekly treatments at ages of 6, 7, 8, 10, 11, and 12 weeks. The study was terminated 24 weeks after the last DEN injection. Data are presented as mean (n=6-11).
Figure 4.3. Effect of DEN, broccoli, and a Western diet on plasma alanine aminotransferase (ALT). Data are presented as mean+SEM (n=6-11). Open bars, saline vehicle control group; closed bars, DEN treatment group; patterned bars, dietary broccoli (10% w/w) group. Western diet effect, $P=0.0056$; dietary broccoli effect, $P<0.0001$. 
Figure 4.4. Effect of DEN, broccoli, and a Western diet on liver histological alteration in lipodosis. Liver sections were stained with hematoxylin and eosin. Representative images from each group are shown as 40X (large image) and 100X (small image) magnifications. Triglyceride vacuoles are indicated by arrows. CV, central vein; PV, portal vein.
Figure 4.5. Effect of broccoli and a Western diet on the expression of (A) acetyl Co-A carboxylase (ACC) and (B) microsomal triglyceride transport protein (MTTP). Data are presented as mean±SEM (n=6). Open bars, saline vehicle control group; patterned bars, dietary broccoli (10% w/w) group. Western diet effect on ACC, $P=0.0002$. Western diet effect on MTTP, $P=0.0056$; dietary broccoli effect on MTTP, $P=0.0449$. 
Table 4.1. Diet Formulae

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+ Broccoli</th>
<th>Western</th>
<th>Western+ Broccoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli † (g/kg)</td>
<td>--</td>
<td>100.0</td>
<td>--</td>
<td>100.0</td>
</tr>
<tr>
<td>Casein (g/kg)</td>
<td>140.0</td>
<td>113.6</td>
<td>172.8</td>
<td>145.6</td>
</tr>
<tr>
<td>Corn Starch (g/kg)</td>
<td>495.7</td>
<td>473.8</td>
<td>34.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Maltodextrin 10 (g/kg)</td>
<td>125.0</td>
<td>110.1</td>
<td>123.5</td>
<td>107.7</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>100.0</td>
<td>99.1</td>
<td>308.6</td>
<td>305.4</td>
</tr>
<tr>
<td>Cellulose (g/kg)</td>
<td>50.0</td>
<td>25.7</td>
<td>61.7</td>
<td>37.1</td>
</tr>
<tr>
<td>L-Cystine (g/kg)</td>
<td>1.8</td>
<td>1.8</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Mineral Mix ‡ (g/kg)</td>
<td>35.0</td>
<td>35.0</td>
<td>43.2</td>
<td>43.2</td>
</tr>
<tr>
<td>Vitamin Mix § (g/kg)</td>
<td>10.0</td>
<td>10.0</td>
<td>12.3</td>
<td>12.3</td>
</tr>
<tr>
<td>Choline Bitartrate (g/kg)</td>
<td>2.5</td>
<td>2.5</td>
<td>3.1</td>
<td>3.1</td>
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<tr>
<td>Corn Oil (g/kg)</td>
<td>40.0</td>
<td>36.5</td>
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<td>45.6</td>
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<tr>
<td>Lard (g/kg)</td>
<td>--</td>
<td>--</td>
<td>188.9</td>
<td>187.5</td>
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<tr>
<td>Energy Density (kcal/g)</td>
<td>3.85</td>
<td>3.85</td>
<td>4.76</td>
<td>4.76</td>
</tr>
<tr>
<td>Protein % ‖</td>
<td>14.7</td>
<td>14.7</td>
<td>14.7</td>
<td>14.7</td>
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<tr>
<td>Carbohydrate % ‖</td>
<td>75.9</td>
<td>75.9</td>
<td>40.3</td>
<td>40.3</td>
</tr>
<tr>
<td>Fat % ‖</td>
<td>9.4</td>
<td>9.4</td>
<td>45.0</td>
<td>45.0</td>
</tr>
</tbody>
</table>

†Freeze-dried broccoli powder, *Brassica oleracea* L. *cv* Green Magic.

‡Mineral mix for AIN-93M diet.

§Vitamin mix for AIN-93 diet.

‖Percentage of total calories.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference sequence number</th>
<th>Primer sequence</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
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<td>IL-1β</td>
<td>NM_008361</td>
<td>5’-GACCTGTTCTTTGAAAGTTGACG-3’ 5’-CTCTTGGTTGACATGTGCTG-3’</td>
<td>5’-TTCCAAACCTTTGACCGGCTGT-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_031168</td>
<td>5’-GATACCACTCCCAACAGACCG-3’ 5’-CAAGTGCACTCATCGTTGTTCA-3’</td>
<td>5’-CCATTGCACAACCTTTTCTCATTTCCACG-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_013693</td>
<td>5’-AGACCCCTCACACTCACATCA-3’ 5’-TCTTTTAGATCCATGCTTG-3’</td>
<td>5’-CCACGTCGTAAGCAAACCACCAAGT-3’</td>
</tr>
<tr>
<td>CD68</td>
<td>NM_009853</td>
<td>5’-CACCTGCTCTCTCTTTATTTCCCT-3’ 5’-CCATGAATTGAGATCCCGTG-3’</td>
<td>5’-TTGTATTCCACGCCATGTAGTCCAG-3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NM_008337</td>
<td>5’-GCACTGGAAGTCAAGCCCTAGA-3’ 5’-CCATCCTTTTGGCATTCCT-3’</td>
<td>5’-TCCACATCTATGCAATTCGGAATTTATAGTTATTCA-3’</td>
</tr>
<tr>
<td>CD36</td>
<td>NM_001159557</td>
<td>5’-CAGCGTAGATAAGACACTGCAA-3’ 5’-GGGACATGATTAATGGGAG-3’</td>
<td>5’-CAACAAAAAGTGGAAAGGGAGGCTGC-3’</td>
</tr>
<tr>
<td>CPT-1α</td>
<td>NM_013495</td>
<td>5’-TAGTACCCAGAGAGACAGATAGG-3’ 5’-TAACAGCAACTACTACGCCAT-3’</td>
<td>5’-CAACACCATCCACCGGATCCAGCT-3’</td>
</tr>
<tr>
<td>FAS</td>
<td>NM_007988</td>
<td>5’-ACTCCTGTAGGGTTTCTCGACTC-3’ 5’-GCTTTCGTCGTTCCGTC-3’</td>
<td>5’-TGGCTTTCTCTGTGTGGGCTCT-3’</td>
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<tr>
<td>Cyp 2E1</td>
<td>NM_021282</td>
<td>5’-ATATCTCGAGGTTGCTGTG-3’ 5’-TGACTGACTGTCCTTCCAT-3’</td>
<td>5’-ACTTTGGCAGACCTGTGTTTTCG-3’</td>
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<td>GAPDH</td>
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<td>5’-AAATGTGAAAGTGGTGCGTG-3’ 5’-GGGAGTCAATAGCTGGAACATGTAG-3’</td>
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Table 4.3. Body Weight and Body Composition

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<tr>
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<th>Final body weight (g)</th>
<th>Liver %†</th>
<th>Adipose %‡</th>
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</thead>
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<tr>
<td></td>
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<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>46.7</td>
<td>2.8</td>
<td>6.03</td>
</tr>
<tr>
<td>Western</td>
<td>52.6</td>
<td>3.4</td>
<td>7.25</td>
</tr>
<tr>
<td>10% Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45.6</td>
<td>1.8</td>
<td>5.17</td>
</tr>
<tr>
<td>Western</td>
<td>53.9</td>
<td>3.5</td>
<td>7.13</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.7</td>
<td>4.0</td>
<td>5.59</td>
</tr>
<tr>
<td>Western</td>
<td>42.6</td>
<td>6.3</td>
<td>7.30</td>
</tr>
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<td>10% Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>39.6</td>
<td>5.6</td>
<td>5.00</td>
</tr>
<tr>
<td>Western</td>
<td>47.1</td>
<td>1.9</td>
<td>6.16</td>
</tr>
</tbody>
</table>

| DEN                         |       | NS    | <0.0001 |
|                            | Broccoli       | NS    | 0.0222  |
|                            | Western         | <0.0001 | <0.0001 |

*P value*
- DEN x Broccoli: NS
- DEN x Western: NS
- Broccoli x Western: NS
- DEN x Broccoli x Western: NS

Data are presented as mean and standard deviation, n=6-11. NS, not significant.

†Percentage of body weight.

‡Epididymal adipose tissue.
### Table 4.4. Liver Triglyceride Content and NAFLD Score

<table>
<thead>
<tr>
<th></th>
<th>Liver triglyceride density (mg/mg protein)</th>
<th>Total liver triglyceride (mg)</th>
<th>NAFLD score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.87</td>
<td>0.20</td>
<td>238</td>
</tr>
<tr>
<td>Western</td>
<td>0.82</td>
<td>0.10</td>
<td>298</td>
</tr>
<tr>
<td>10% Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.50</td>
<td>0.17</td>
<td>132</td>
</tr>
<tr>
<td>Western</td>
<td>0.71</td>
<td>0.23</td>
<td>270</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.78</td>
<td>0.18</td>
<td>175</td>
</tr>
<tr>
<td>Western</td>
<td>0.80</td>
<td>0.15</td>
<td>269</td>
</tr>
<tr>
<td>10% Broccoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.61</td>
<td>0.18</td>
<td>125</td>
</tr>
<tr>
<td>Western</td>
<td>0.73</td>
<td>0.23</td>
<td>195</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| DEN              | NS    |     | 0.0114 |     | NS
| Broccoli         | <0.0001 | 0.0003 | <0.0001 |
| Western          | NS    | <0.0001 | 0.0123 |
| DEN x Broccoli   | NS    | NS    | NS    |
| DEN x Western    | NS    | NS    | 0.0311 |
| Broccoli x Western | NS    | NS    | NS    |
| DEN x Broccoli x Western | NS    | NS    | NS    |

Data are presented as mean and standard deviation, n=6-11. NS, not significant.
### Table 4.5. Hepatic Gene Expression (mRNA) (A) Lipid-Related Marker (B) Inflammatory Markers

<table>
<thead>
<tr>
<th>(A)</th>
<th>CD36 Mean</th>
<th>CD36 SD</th>
<th>CPT-1α Mean</th>
<th>CPT-1α SD</th>
<th>FAS Mean</th>
<th>FAS SD</th>
<th>Cyp 2E1 Mean</th>
<th>Cyp 2E1 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td>Control</td>
<td>1.01</td>
<td>0.13</td>
<td>1.01</td>
<td>0.19</td>
<td>1.01</td>
<td>0.19</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Western</td>
<td>1.03</td>
<td>0.19</td>
<td>1.22</td>
<td>0.08</td>
<td>0.64</td>
<td>0.10</td>
<td>0.99</td>
</tr>
<tr>
<td>10% Broccoli</td>
<td>Control</td>
<td>0.65</td>
<td>0.15</td>
<td>0.95</td>
<td>0.18</td>
<td>1.12</td>
<td>0.24</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>Western</td>
<td>1.07</td>
<td>0.21</td>
<td>1.13</td>
<td>0.16</td>
<td>0.78</td>
<td>0.17</td>
<td>0.94</td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>No Broccoli</td>
<td>Control</td>
<td>0.73</td>
<td>0.16</td>
<td>0.95</td>
<td>0.18</td>
<td>1.07</td>
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<td>0.90</td>
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<td>0.51</td>
<td>0.09</td>
<td>1.20</td>
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</table>

| P value      | DEN       | NS      | 0.0081      | NS        | 0.0008    | NS     |
|              | Broccoli  | 0.0056  | 0.0026      | NS        | NS        | NS     |
|              | Western   | 0.0002  | <0.0001     | <0.0001   | NS        | NS     |
|              | DEN x Broccoli | NS | NS | NS | NS |
|              | DEN x Western | NS | NS | NS | 0.0087 |
|              | Broccoli x Western | NS | NS | NS | 0.0405 |
|              | DEN x Broccoli x Western | 0.0214 | NS | NS | NS |
Table 4.5 (cont’d). Hepatic Gene Expression (mRNA) (A) Lipid-Related Marker (B)

Inflammatory Markers

<table>
<thead>
<tr>
<th>(B)</th>
<th>IFN-γ</th>
<th>CD68</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Broccoli Control</td>
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<td>1.01</td>
</tr>
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<tr>
<td>No Broccoli Control</td>
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</tr>
<tr>
<td>Western</td>
<td>1.99</td>
<td>0.62</td>
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<tr>
<td>Western</td>
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**P value**

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<th>NS</th>
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<tbody>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td><strong>DEN x Western</strong></td>
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</tr>
<tr>
<td><strong>DEN x Broccoli x Western</strong></td>
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</table>

Data are showed as fold change from saline-control diet group and presented as mean and standard deviation, n=6-11. NS, not significant.
Table 4.6. Macroscopic Hepatic Neoplasm-Related Lesions

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</tr>
<tr>
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<td>0/6</td>
</tr>
<tr>
<td></td>
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<td>7/10</td>
</tr>
<tr>
<td></td>
<td>Western</td>
<td>11/11</td>
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<td></td>
<td>Control</td>
<td>5/10</td>
</tr>
<tr>
<td></td>
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<td>8/9</td>
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</tbody>
</table>

| P value   | DEN         | <0.0001 | --   |
|           | Broccoli    | 0.0011  | NS   |
|           | Western     | 0.0033  | 0.0114 |
|           | DEN x Broccoli | NS  | --   |
|           | DEN x Western | NS  | --   |
|           | Broccoli x Western | NS  | NS   |
|           | DEN x Broccoli x Western | NS  | NS   |

Data are presented as mean and standard deviation, n=6-11. NS, not significant.

†Visible liver nodules (diameter ≥ 1 mm)
Table 4.7. Microscopic Hepatic Neoplasm-Related Lesions

<table>
<thead>
<tr>
<th></th>
<th>AHF incidence</th>
<th>HA incidence</th>
<th>HCC incidence</th>
<th>Total hepatic neoplasm-related lesion incidence</th>
</tr>
</thead>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
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<tr>
<td>No Broccoli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
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<td>0/6</td>
</tr>
<tr>
<td>10% Broccoli</td>
<td></td>
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<td></td>
</tr>
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<td>0/6</td>
<td>0/6</td>
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<td>4/11</td>
<td>36</td>
<td>5/11*</td>
<td>9/11*</td>
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<td>0/10</td>
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<td>Western</td>
<td>4/9</td>
<td>44</td>
<td>1/9</td>
<td>5/9</td>
</tr>
</tbody>
</table>

n=6-11. AHF, altered hepatic foci; HA, hepatic adenoma; HCC, hepatocellular carcinoma.

*P<0.05 compared to DEN-Control diet group.
5.1. Abstract

Diethylnitrosamine (DEN) is a chemical broadly used in animal models as a hepato-carcinogen, although both primary and secondary lung tumors have been also reported following intraperitoneal (i.p.) injection of DEN in mice. We administered DEN (45 mg/kg) i.p. to young adult male B6C3F1 mice for 6 weekly injections, with a break of one week after 3 injections. Here we report primary tumorigenesis in both pulmonary and nasal epithelia as well as liver. Liver, lung and heads were collected at six months after the DEN treatments for histology. The visible nodule incidence in DEN-treated mice was more than 90%, showing the high pulmonary carcinogenicity of DEN in B6C3F1 mice. Unexpectedly, more than 50% of DEN-treated B6C3F1 mice exhibited nasal neoplasm-related lesions, which had not been reported in the literature. Analysis revealed that mice with hepatic neoplasm-related lesions may have a decreased relative risk (RR) for pulmonary and nasal neoplasm-related lesions (RR=0.57, 0.27<95% CI<1.23, P=0.1509 vs. RR=0.63, 0.26<95% CI<1.55, P=0.3138, respectively), suggesting a hepatic first pass effect.
5.2. Introduction

Diethylnitrosamine (DEN), which can be found in cigarette smoke, dried sea food, and processed meat, is classified as a probable human carcinogen (B2 level) according to the United States Environmental Protection Agency (Hoffmann, Adams et al. 1980; Tricker and Preussmann 1991; U.S. Environmental Protection Agency 2003). The carcinogenicity of DEN is due to its bioactivation by cytochrome P450 (Cyp) enzymes, specifically Cyp 2E1, which can produce ethyl diazonium ions which then forms DNA adducts (Verna, Whysner et al. 1996). In animal models, DEN has been shown to cause tumorigenesis in different tissues, depending on the animal species under study. For example, after oral exposure to DEN, mice developed liver, stomach, and esophageal tumors; rats had liver, esophageal, and kidney tumors; and Syrian hamsters showed trachea, lung, liver and nasal cavity tumors; whereas dogs developed tumors in liver and nasal cavity (Verna, Whysner et al. 1996). The route of DEN administration can also impact the site of tumor formation. Reports have shown that in RF mice, tumor incidence in lung, liver and stomach caused by intraperitoneal injection of DEN was 93%, 26%, and 26%, while tumor incidence induced by oral DEN was 74%, 90%, and 100%, respectively (Clapp and Craig 1967; Clapp 1973).

B6C3F1 mice are reported to develop both liver and lung tumors following intraperitoneal injection of DEN, and to have a greater number of liver tumors than C57BL/6J mice (Vesselinovitch, Koka et al. 1984; Goldsworthy and Fransson-Steen 2002). However, in our study, some DEN-treated B6C3F1 mice started to exhibit very poor health and were found to have an inflated intestine when killed at 5 months, but no sign of hepatic or pulmonary neoplasm-related lesions. These findings led to a suspicion of nasal tumor formation, even though this has not been reported previously in the DEN-treated B6C3F1 mouse models. The
gas distended intestinal tract suggested that gulping of air potentially due to obstruction in the airways. Therefore, we examined lung and nasal cavity histologically in addition to liver sections, and report here the formation of intra-nasal neoplastic lesions induced by intraperitoneal treatment with DEN.

5.3. Materials and Methods

5.3.1. Animals and experimental design

Seventy-two male 4-week-old B6C3F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Diet ingredients were acquired from the Harlan Laboratory (Indianapolis, IN, USA). Diethylnitrosamine was obtained from Sigma-Aldrich (St. Louise, MO, USA), and diluted in normal saline for injection. After one week of acclimation, mice were divided into 4 groups and provided with Control, Control+Broccoli, Western or Western+Broccoli diets at the age of 5 weeks. Control diet was prepared from an AIN-93M based formula (calories from protein, fat and carbohydrate were 14.7, 9.4 and 75.9% respectively) (Reeves, Nielsen et al. 1993). The Western diet was formulated by increasing sucrose and saturated fat content in AIN-93M (calories from protein, fat and carbohydrate were 14.7, 45.0 and 40.3% respectively). Freeze dried broccoli powder (10% by weight; Brassica oleracea L. var. Green Magic), kindly provided by Dr. John A. Juvik, was incorporated into the Control+Broccoli and Western+Broccoli diet. Ingredients were replaced based on dried broccoli composition. Animals were housed individually and given water and feed ad libitum under a 12-hour light/dark cycle at 22°C and 60% humidity at the Institute for Genomic Biology animal facility at the University of Illinois (Urbana, IL, USA). Diethylnitrosamine (n=12/group) or
saline (n=6/group) was given to mice at the dose of 45 mg/kg by intraperitoneal injection once a week for 6 weeks, at the age of 6, 7, 8, 10, 11, and 12 weeks. During the injection period, two DEN-treated mice died. Six DEN-mice (one in Control diet, two in Control+Broccoli diet, one in Western diet, and two in Western+Broccoli diet) were euthanized before the end of study due to serious illness. Mice were sacrificed at the age of 36 weeks, followed by the collection of tissue samples. Animal care was in compliance with the Institutional Animal Care and Use Committee at the University of Illinois, according to the National Institutes of Health. Detailed liver biochemistry and histology is reported in Chapter 4.

5.3.2. Histology

Tissues were fixed in 10% neutral buffered formalin, and skulls were further decalcified with decalcification solution DeltaFORM (Delta Medical, Aurora, IL, USA) before paraffin embedding. Lung and nasal cavity sections (3 µm) were stained with hematoxylin and eosin for the examination of non-neoplasms, adenomas, and carcinomas. Histology work was carried out in the Veterinary Diagnostic Laboratory at the University of Illinois (Urbana, IL, USA). All neoplasm-related lesions were identified by a trained pathologist, based on the report from the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Renne, Brix et al. 2009).

5.3.3. Statistical analysis

Visible pulmonary nodule number and size are expressed as mean and standard deviation, and were analyzed by two- or three-way analysis of variance for the effect of DEN, broccoli, Western diet, and for interactions. The relation between hepatic neoplasm-related lesions and pulmonary carcinomas/nasal epithelial neoplasm-related lesions and between dietary broccoli
and hepatic, pulmonary, or nasal neoplasm-related lesions were evaluated using the Chi-squared test. A $P$ value of less than 0.05 was considered significant. All statistical analysis were performed using Statistical Analysis System 9.4 Software (SAS Institute Inc., Cary, NC, USA).

5.4. Results

5.4.1. Diethylnitrosamine induced primary lung cancer.

Mice treated with DEN had 90-100% incidence of lung nodules in all four dietary groups, whereas in the saline-treated group the incidence was low (0-33%) (Table 5.1). Visible lung nodule number was increased by the DEN treatments ($P<0.0001$), and unexpectedly by dietary broccoli as well ($P<0.0001$) (Table 5.1). However, lung nodule size was not influenced by dietary broccoli but possibly by the Western diet (not significant, Table 5.1). Histological evaluation of lung also showed that DEN treatments induced the formation of primary pulmonary carcinomas (Table 5.1). Figure 5.1 compares a classic normal lung section from a saline-treated mouse, which exhibits atelectasis caused by sampling procedures, and a section with primary lung cancer from a DEN-treated mouse. Among the DEN-treated mice, the consumption of broccoli increased the risk for pulmonary carcinomas (RR=3.92, $1.32<95\% CI<11.67$, $P=0.0029$). Detailed biochemistry and histology of the liver are reported elsewhere (Chapter 4). Here we report from that study that we observed, a decrease in the risk for hepatic neoplasm-related lesions, including altered hepatic foci, hepatic adenomas, and hepatocellular carcinomas in the broccoli-fed mice (RR=0.58, $0.33<95\% CI<1.02$, $P=0.0489$). However, the animals with hepatic neoplasm-related lesions, mostly those receiving no dietary broccoli, had a
trend toward decreased risk for lung neoplasm (RR=0.57, 0.27<95% CI<1.23, \(P=0.1509\), not significant).

### 5.4.2. Diethylnitrosamine caused nasal epithelial neoplasm-related lesions.

Mice treated with DEN exhibited varied nasal morphological patterns dissimilar to normal nasal cavity (Fig. 5.2). The normal respiratory, transitional, and olfactory epithelia were affected (Fig. 5.3), showing metaplasia, hyperplasia, adenoma, and carcinoma (Fig. 5.4 and 5.5). Table 5.2 presents the incidence of microscopic neoplasm-related lesions in the nasal cavity.

Diethylnitrosamine induced the formation of nasal epithelial neoplasm-related lesions; the incidence of nasal epithelial neoplasm-related lesions was low in the saline-treated animal (0-17%). The incidence of nasal non-carcinoma lesions, including metaplasia, hyperplasia, and adenoma, was similar in all four dietary groups with DEN treatment (27-44%). However, for nasal carcinoma, among the Western diet-fed mice, the incidence appeared to be greater in the broccoli-fed group than the group without dietary broccoli (67% vs. 27%, respectively), and the same phenomenon was seen the control diet-fed mice. The Western+Broccoli diet-fed mice also showed a higher total nasal neoplasm-related lesion incidence compared to the Western diet group (89% vs. 55%, respectively), but there was a converse result in the control diet-fed mice, where in the Control+Broccoli diet-fed mice seemed to have a lower incidence than the control diet only group (60% vs. 80%, respectively). As was seen in lung, dietary broccoli appeared to increase the risk for nasal neoplasm-related lesions (RR=1.44, 0.57<95% CI<3.37, \(P=0.4270\)), and the animals with hepatic neoplasm-related lesions may have had a decreased risk for nasal neoplasm-related lesions (RR=0.63, 0.26<95% CI<1.55, \(P=0.3138\)). Furthermore, in addition to nasal and pulmonary carcinomas, there was also a single case of tongue squamous cell carcinoma in this study.
5.5. Discussion

The hybrid mouse strain B6C3F1 (male C3H/HeJ x female C57Bl/6J) is often used for the study of carcinogenesis. However, it may develop spontaneous neoplasms in multiple organs. According to a report from the National Toxicology Program, in several 2-year studies, the incidence of spontaneous hepatic adenoma/carcinoma and pulmonary adenoma/carcinoma in male B6C1F1 mice is 42% (range from 10% to 68%) and 20.5% (range from 4% to 23%), respectively (Haseman, Hailey et al. 1998). In the present study, spontaneous liver and lung cancers were not observed in the saline-treated mice, possibly due to the short study period (36 vs. 104 weeks) or to the small sample size (n=6 vs. >1000). It has been reported that DEN, a potent carcinogen, induces not only liver tumors but also lung tumors in B6C3F1 mice. In a lifetime study, adult male B6C3F1 (6 weeks old) that received 48 mg DEN/kg body weight exhibited a high visible lung tumor incidence (87%) but a relatively low hepatic adenoma/carcinoma incidence (16%) (Vesselinovitch, Koka et al. 1984), which data show a similar pattern to our results. Pulmonary metastasis of DEN-induced liver cancers has also been reported for B6C3F1. About 30% of male B6C3F1 mice with DEN-induced hepatocellular carcinomas were reported to have metastasis to the lung (Vesselinovitch, Mihailovich et al. 1978; Vesselinovitch, Koka et al. 1984). However, the average age of mice showing pulmonary metastasis was 76-78 weeks (Vesselinovitch, Mihailovich et al. 1978), which was much longer than our study period (36 weeks). This may explain why, in our study, although primary pulmonary carcinomas were observed, we saw no metastasis from liver tumors.

Unexpectedly, we found a high incidence of nasal neoplasm-related lesions in the DEN-treated B6C3F1 mice. Spontaneous nasal tumors may be rare in B6C3F1 mice; the reported incidence is less than 1% (Brown, Monticello et al. 1991; Haseman, Hailey et al. 1998). DEN is
known to be highly metabolized in the nasal cavity in C57BL/6J mice. Using [14C] labeled DEN, high levels of non-volatile DEN metabolites were observed in the nasal cavity, trachea, bronchi, salivary gland, liver, and tongue (Brittebo, Lofberg et al. 1981). This may provide a clue for our observation on tumors in the nasal epithelium and tongue. To date, the Syrian hamster has been shown to respond to DEN with changes to the nasal cavity, independent of route of DEN administration. For example, intragastric DEN caused 44% incidence of nasal tumors, and following intraperitoneal or intradermal administration, the incidence of tumors was around 70% (Herrold and Dunham 1963; Herrold 1964). In hamsters, the DEN treatment (intraperitoneal) also induced tumors in the trachea (94%) and liver (22%) (Herrold 1964). It has been suggested that although both mice and the Syrian hamsters have damage in the respiratory system after DEN exposure, it appears that in hamsters, tumors are primarily found in the upper respiratory tract, such as the nasal cavity and trachea, whereas in mice more tumors are located in lung, as we saw here (Clapp and Craig 1967; Montesano and Saffiotti 1968). The cytochrome P450 enzyme, Cyp 2E1, activates DEN, producing carcinogenic metabolites (Kang, Wanibuchi et al. 2007). The high nasal tumor incidence in the DEN-treated Syrian hamster compared to rat or mouse may be due to a greater metabolic rate of this microsomal enzyme in nasal mucosa, which enzyme is also found in liver, compared to those in rats (Longo, Citti et al. 1986). This appears likely, since the concentration of nasal cytochrome P450 enzymes, including Cyp 2E1, are 7-fold higher in the Syrian hamsters than those in mice (Sarkar 1992).

Among the DEN-treated mice, consumption of broccoli increased the risk for lung cancer. However, this may be negatively correlated to the reduced risk of hepatic neoplasm-related lesions, because the animals with hepatic neoplasm-related lesions appeared to have a lower risk for pulmonary and nasal neoplasm-related lesions compared to those without hepatic neoplasm-
related lesions. The higher incidence of hepatic neoplasm-related lesions in the present study might be due to a hepatic first pass effect. Since these mice were treated with DEN by intraperitoneal injection, providing an initial high dose of DEN, the first pass effect in liver should be evident. It suggests that when more DEN was activated in liver, leading to more liver tumors, less non-metabolized DEN might have been available for metabolism by lung and nasal mucosa for the induction of pulmonary and nasal tumors. Our results show that the hepatic metabolic enzyme Cyp 2E1 was elevated in the Western-DEN group, which also had the highest incidence of hepatic neoplasm-related lesions, supporting our hypothesis of the first pass effect. However, more research into DEN metabolism by liver, lung and nasal epithelium are needed for a better understanding of these systemic effects.

In conclusion, DEN induced not only liver cancer but also lung cancer and nasal cancer. The intraperitoneal DEN-induced nasal cancer in B6C3F1 mice was not previously reported, but was shown in our study. The formation of pulmonary and nasal neoplasm-related lesions was found to be inversely related to liver tumors and by extension to the extent of DEN metabolism in liver. Our finding suggests that the metabolism of DEN in lung and nasal cavity should be carefully monitored in the B6C3F1 liver cancer model.
Figure 5.1. Normal lung (atelectatic due to processing) and primary lung neoplasia from DEN-treated mice. Normal lung (A, B, C) and primary pulmonary carcinoma (D, E, F) sections were stained with hematoxylin and eosin. Representative images are shown as 40X (A and D), 100X (B and E), and 400X (C and F) magnifications. Br, bronchiole; PA, pulmonary artery; PV, pulmonary vein; AP, alveolar parenchyma; *, pulmonary adenoma; **, pulmonary adenocarcinoma.
Figure 5.2. Normal nasal cavity.
Figure 5.2 (cont’d). Normal nasal cavity. De-calcificated mouse skulls were cut into 4 sections from anterior to posterior, presenting zone 1 (A and B), zone 2 (C and D), zone 3 (E and F), and zone 4 (G and H), and stained with hematoxylin and eosin. Representative images are shown as 12.5X (A, C, E, G) and 40X (B, D, F, H) magnifications. B, bone; ET, ethmoturbinate; LNG, lateral nasal gland; NP, nasopharynx; NC, nasal cavity; NS, nasal septum; NT, nasal turbinate; OC, oral cavity; Ol, olfactory lobes of brain; T, tooth; Tg, tongue; VO, vomeronasal organ.
Figure 5.3. Normal nasal epithelium.
Fig 5.3 (cont’d). Normal nasal epithelium. Normal respiratory epithelium (A), transitional epithelium (B), and olfactory epithelium (C) section were stained with hematoxylin and eosin. Representative images are shown as 400X magnifications. B, bone; NC, nasal cavity; NS, nasal septum; NT, nasal turbinate; OE, olfactory epithelium; RE, respiratory epithelium; TE, transitional epithelium.
Figure 5.4. Non-neoplastic nasal lesions and nasal adenomas from DEN-treated mice.
Figure 5.4 (cont’d). Nasal non-neoplastic lesions and adenomas from DEN-treated mice. Metaplasia, respiratory epithelium to olfactory epithelium (A and B), respiratory epithelial hyperplasia (C and D), olfactory epithelial hyperplasia (E and F), and adenoma (G and H) section were stained with hematoxylin and eosin. Representative images are shown as 100X (A, C, E, G) and 400X (B, D, F, H) magnifications. Non-neoplastic nasal lesions and nasal adenomas are indicated by arrows.
Figure 5.5. Nasal carcinomas from DEN-treated mice.
Figure 5.5 (cont’d). Nasal carcinomas from DEN-treated mice. Adenocarcinoma (A and B), adenosquamous carcinoma (C and D), squamous cell carcinoma (E and F), and neuroepithelial carcinoma (G and H) were stained with hematoxylin and eosin. Representative images are shown as 100X (A, C, E, G) and 400X (B, D, F, H) magnifications. Nasal carcinomas are indicated by arrows.
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<td></td>
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<td>SD</td>
<td>Mean</td>
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<td>33</td>
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<td>100</td>
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</tbody>
</table>

**P value**

- DEN x Broccoli: <0.0001
- DEN x Western: NS
- Broccoli x Western: <0.0001
- DEN x Broccoli x Western: <0.0001

---

120
Table 5.1 (cont’d). Macroscopic and Microscopic Pulmonary Neoplasm-Related Lesions

Data are presented as mean and standard deviation, n=6-11. NS, not significant.

†Visible lung nodules.
Table 5.2. Microscopic Nasal Cavity Neoplasm-Related Lesions

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<th>Preneoplasm lesion incidence</th>
<th>Carcinoma incidence</th>
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<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
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n=6-11.
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

In Specific Aim 1, our goal was to evaluate the ability of dietary broccoli to impact the development of Western diet-enhanced NAFLD. It was hypothesized that long-term consumption of broccoli could decrease both hepatic lipid levels and inflammation. From results of the pilot study, 3 months of dietary broccoli treatment was sufficient to decrease hepatic lipidosis in Western diet-fed mice (Table 3.5). Furthermore, dietary broccoli not only decreased hepatic triglyceride levels in the Western diet-fed animals, but also in those fed a control diet (Table 4.4), indicating that broccoli can influence lipid metabolism. Hepatic triglyceride homeostasis is dependent upon the influx of NEFA and dietary fat, \textit{de novo} lipogenesis, fatty acid oxidation, and VLDL excretion (Fig. 1.2), and NEFA account for \textasciitilde60\% (Donnelly, Smith et al. 2005). The analysis of hepatic lipid metabolic genes revealed that dietary broccoli down-regulated the expression of fatty acid translocator CD36 by up to 35\% (Table 4.5A), suggesting that broccoli can cause a significant decrease in the influx of NEFA. Dietary broccoli also increased hepatic MTTP protein levels, thereby facilitating the excretion of VLDL (Fig. 4.5B). Overall, broccoli normalized the hepatic triglyceride pool by lowering the input (NEFA) and increasing the output (VLDL) of lipid, leading to protection against the steatotic effect from a Western diet.
The activation of hepatic macrophages, or Kupffer cells, which is critical for the initiation of NASH (Tosello-Trampont, Landes et al. 2012), was decreased by the consumption of broccoli (Table 4.5B). The key cytokine for induction of macrophages activation (IFN-γ) and the marker for activated macrophages (CD68) were both down-regulated in liver. The expression of the proinflammatory cytokine TNF-α was lowered as well. Our findings suggest that dietary broccoli can impede the progression of NAFLD by hindering both the development of hepatic steatosis and the progression from hepatic steatosis to NASH.

One of the assumptions in our study was that a Western diet induces both obesity and NAFLD. Although the body weight was increased by the Western diet, we found that the epididymal adipose tissue was not able to represent whole body adiposity. In fact, there was a negative correlation between epididymal adipose tissue mass and liver mass in mice with a body mass > 40 g (Table 4.3). However, the loss in epididymal adipose mass highlighted the relationship between the severity of hepatomegaly (hepatic steatosis) and lipodystrophy in mice. In the future, a second fat tissue, such as inguinal adipose tissue, should also be monitored to better determine whether broccoli impacts extra hepatic fat storage.

Our goal for Aim 2 was to assess the impact of both a Western diet and dietary broccoli on liver tumorigenesis in a DEN-induced liver cancer model. The hypothesis was that a Western diet would enhance liver tumor formation but that dietary broccoli would slow the development of liver tumors, countering the effect of the Western diet. The influence of broccoli on the progression of liver tumorigenesis was therefore evaluated. Dietary broccoli ameliorated liver damage and lowered the incidence of liver nodules in a DEN-induced infant mouse model at 5 months (Fig. 3.2 and Table 3.6). However, the beneficial effect of broccoli was lost by 7 months, suggesting that dietary broccoli only has limited effects on the progression of liver tumorigenesis.
When the dietary impact of broccoli was evaluated in a combined tumor initiation and progression model, we found that broccoli decreased liver nodule incidence and interfered with the development of hepatic preneoplasia, but that a Western diet worsened the progress of liver tumorigenesis (Table 4.6 and 4.7). We demonstrated that lifelong consumption of broccoli can decrease the formation of hepatic neoplasm-related lesions.

Interestingly, for the first time, the nasal epithelial carcinogenicity of intraperitoneal DEN in B6C3F1 mice was identified. The hepatic first pass effect of DEN, which was regulated by diet in our study, may influence the formation of nasal neoplasm-related lesions. This suggests that the metabolism of DEN, which is activated by Cyp 2E1, should be evaluated in nasal epithelium and possibly throughout the body, when the B6C3F1 mouse model is used.

Overall, our study is the first report that the long-term consumption of broccoli, a whole food dietary intervention, can protect liver against a steatotic high saturated fat and sucrose diet and against the carcinogenic challenge of DEN. These findings fill gaps of knowledge about the impact of dietary brassicas on hepatic lipid metabolism and tumorigenesis, and also provide supportive in vivo evidence for the protective effect of brassicas against multiple cancers, as shown in epidemiologic studies.

6.2. Future Directions

In this study, we have shown the protective effect of broccoli (10% in the diet) in liver, against steatosis and carcinogenesis. However, the minimal effective dose of dietary broccoli is yet to be determined. Here mice received about 170 g raw broccoli/kg body weight/day. It means that to have the equivalent daily consumption of broccoli, an adult (70 kg) should have around 900 g
broccoli every day, based on Reagan-Shaw and colleagues’ formula (Reagan-Shaw, Nihal et al. 2008). Whereas this is not a feasible amount, epidemiological studies that have revealed the efficacy against other cancers show 3-5 servings of dietary brassica a week, only ~450 g/week, as effective (Jeffery and Araya 2009). These data encourage future research to determine the dietary requirement of broccoli for maintaining a health with regards to liver health and overall well being.

Here the protective effect of dietary broccoli on hepatic lipidosis in animals is identified for the first time. Clinical studies on the relationship between frequent consumption of broccoli and hepatic triglyceride levels in humans are necessary to determine if this finding holds true for humans. A recent study has shown that long-term (12 weeks) consumption of broccoli (400 g/week) can decrease plasma low-density lipoprotein cholesterol in adults (Armah, Derdemezis et al. 2015). That report indicates that broccoli has the ability to influence lipid metabolism in humans. The changes in hepatic steatosis and circulating NEFA after dietary broccoli intervention will be valuable evidence for the prevention of human NAFLD.

Although the general structure of the regulation by dietary broccoli on the size of the hepatic triglyceride pool has been revealed in our study, the possible pathways involved in the changes we saw in liver are still not clear. However, several targets, such as AMPK, a known energy and nutrient sensor (Hardie, Ross et al. 2012), PPARγ, a regulator for hepatic CD36 (Zhou, Febbraio et al. 2008), and SREBP-1c, an activator of liver lipid synthesis (Horton, Goldstein et al. 2002), might be potentially influenced by broccoli and worthy endpoints for further studies. Recently sulforaphane has been reported as increasing the phosphorylation of AMPK and ACC and decreasing the expression of PPARγ in adipose tissue (Choi, Lee et al. 2014). Also, the activation of Nrf2 can result in down-regulated SREBP-1c (Shin, Wakabayashi et al. 2009).
Furthermore, it could be meaningful as well to understand whether dietary broccoli is able to cause an alteration in insulin and adiponectin in circulation, because these endocrines tightly regulate the homeostasis of carbohydrate and fat in the whole system.

The impact of bioactive compounds from broccoli on hepatic lipid metabolism should be identified individually for each compound. Possible synergistic effects among the various bioactive components should also be studied. For example, quercetin has revealed the capacity to down-regulate hepatic triglycerides in mice with a quercetin diet (0.05%) (Kobori, Masumoto et al. 2011), similar to quercetin concentration in our broccoli diet (~0.03% quercetin in the diet). However, the efficacy of sulforaphane in maintaining hepatic lipid regulation is not clear. There is one report of the impact of sulforaphane on hepatic triglycerides, however, even though they used more than 10-fold greater dose than our study, the effect was not significant (Choi, Lee et al. 2014). Therefore, synergistic effects, such as sulforaphane and indole-3-carbinol or sulforaphane and quercetin, could be approached first using human hepatic cell line models, and then using rodent studies. Studies such as these could identify key components of broccoli while further supporting the health promoting impact of whole food consumption that we saw in this study.

Classically-activated M1 Kupffer cells are associated with the promotion of NAFLD (Baffy 2009). Moreover, it is suggested that the increase of alternatively-activated M2 Kupffer cells might ameliorate hepatic steatosis by stimulating the apoptosis of M1 Kupffer cells (Wan, Benkdane et al. 2014). M2 macrophages are generally considered as anti-inflammatory cells (Murray and Wynn 2011). The inhibition of M1 Kupffer cells by dietary broccoli has been demonstrated here. Therefore, the impact of broccoli on the polarization of Kupffer cells could be an interesting topic, looking at the cytokine profile and M1/M2 ratio.
Above all, the ultimate goal of future studies should be to verify dietary strategies, especially whole food dietary intervention, that can maintain liver health and improve life quality.
REFERENCES


El-Serag, H. B. (2012). "Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma." Gastroenterology 142(6): 1264-+


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APPENDIX: BROCCOLI NUTRIENT COMPOSITION

<table>
<thead>
<tr>
<th>Component</th>
<th>Whole broccoli†</th>
<th>Dried broccoli‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>89.30% (w/w)</td>
<td>--</td>
</tr>
<tr>
<td>Protein</td>
<td>2.82%</td>
<td>26.36%</td>
</tr>
<tr>
<td>Total Lipid</td>
<td>0.37%</td>
<td>3.46%</td>
</tr>
<tr>
<td>Carbohydrate (by Difference)</td>
<td>6.64%</td>
<td>62.06%</td>
</tr>
<tr>
<td>a) Sucrose</td>
<td>0.10%</td>
<td>0.93%</td>
</tr>
<tr>
<td>b) Total Non-Sucrose Sugar</td>
<td>1.60%</td>
<td>14.95%</td>
</tr>
<tr>
<td>c) Total Dietary Fiber</td>
<td>2.60%</td>
<td>24.30%</td>
</tr>
<tr>
<td>d) Other</td>
<td>2.34%</td>
<td>21.87%</td>
</tr>
<tr>
<td>Ash</td>
<td>0.87%</td>
<td>8.13%</td>
</tr>
</tbody>
</table>


‡Dried broccoli nutrient content is calculated from the whole broccoli by assuming all water is removed during the freeze drying process.