AN ABSTRACT OF THE DISSERTATION OF

Ting Luo for the degree of Doctor of Philosophy in Food Science and Technology presented on May 31, 2017

Title: Modulation of Metabolic Syndrome by Soy Isoflavones: *In-vitro* and *In-vivo* Models

Abstract approved:

_____

Neil F. Shay

Metabolic syndrome is a problem affecting people around the world. In a number of studies, soy intake has been documented to possess antidiabetic and anticardiovascular effect. My project is focused on the impact of the intake of compounds found within soy, the isoflavones, their impact on metabolic syndrome symptoms, and the underlying mechanisms involved.

In study one, we aimed to determine the effect of soy product on weight gain and adiposity in male C57BL/6 mice. Six-week-old mice were put in a low-fat diet (LF), high fat diet (HF), or high fat diet plus Novasoy, an isoflavones-containing dietary supplement (HF+NS) for 10 weeks. At week 10, metabolic syndrome symptoms

including obesity, high blood glucose, and hepatic lipid accumulation were observed in HF diet fed mice. However, NS intake significantly decreased body weight gain, baseline blood glucose level, and hepatic lipid accumulation ($p<0.05$). To further investigate the effect of isoflavone supplement intake on HF diet-induced metabolic syndrome, a custom-designed gene array was utilized to determine hepatic gene expression related to lipid metabolism, glucose metabolism, and inflammation. Relative expression of the mRNA for hormone sensitive lipase (*Hsl*), which catalyzes triglyceride hydrolysis, was significantly increased in NS fed mice, compared to HF fed mice. Expression of the mRNA encoding carnitine palmitoyltransferase 1 alpha (*Cpt1α*), the rate limiting enzyme of lipid β oxidation, was significantly lowered when NS was added to HF diet.

To follow up study one, mouse study two was conducted to determine whether consumption of the dominant soy isoflavones compounds, genistein and daidzein, influence metabolic syndrome similarly or differently, and to further investigate the underlying mechanism. C57BL/6J mice were fed low fat (LF), western diet (WD), WD containing 0.16% (w/w) of genistein (WD+G) or daidzein (WD+D) for 10 weeks. Surprisingly, intake of WD+G and WD+D significantly decreased food intake and body weight, compared to WD-fed mice (185%±10%, 112%±9%, and 150%±7% percentage weight gain in WD-, WD+G- and WD+D-fed mice, vs. LF-fed mice respectively). Genistein-fed mice had a reduced area under the curve (60-120mins) in a glucose tolerance test ($p<0.05$). Furthermore, gene array profiling in genistein or daidzein fed

mice indicate that genistein and daidzein consumption regulate gene expression related to hepatic lipid metabolism, carbohydrate metabolism, and inflammation. Signaling pathways such as LXR/RXR and FXR/RXR were significantly activated in genistein or daidzein fed mice.

Since a more potent effect on body weight was observed in genistein- fed mice in study II, compared to NS-fed mice, we designed study III, to determine the effect of NS on WD induced metabolic syndrome symptoms, especially body weight gain. Study III demonstrated that the reduction of body weight gain in WD+NS-fed mice was less than WD+G-fed mice.

To determine if LXR was activated by genistein or daidzein, transient transfection-luciferase assays were utilized to determine the binding affinity of genistein or daidzein on LXRE regulatory elements. Furthermore, mouse embryonic fibroblast cells devoid of, or expressing LXRα were used to determine whether LXR is involved in the effect of isoflavone on lipid metabolism related gene expression.

In summary, the results of *in-vivo* and *in-vitro* studies suggest that the beneficial effects observed on weight gain and lipid metabolism observed in C57BL/6J mice due to isoflavone consumption are partly due to LXR-dependent mechanism.

Modulation of Metabolic Syndrome by Soy Isoflavones:

In-vitro and *In-vivo* Models

by

Ting Luo

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented May 31, 2017

Commencement June 2017

Doctor of Philosophy dissertation of Ting Luo presented on May 31, 2017

APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Ting Luo, Author

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my advisor and mentor, Dr. Neil Shay for his guidance, encouragement, and patience over the last four years. He is like the professional father for me. I still remember when I did not do a good job on my first exam, he encouraged me and told me: life is full of ups and downs. I still remember when I presented in an international conference for my first time, he practiced with me, one sentence by one sentence. I achieved a lot in the past 4 years in the US. He did exactly what he promised to me: "*You could be assured that we would have a very close relationship and I would give you my very best attention and make sure you get excellent training as a young scientist*." I really hope that I did a good job as his PhD student as well. I feel so lucky to have such a wonderful advisor.

Secondly, I would like to thank Dr. Jump, Dr. Iwaniec, Dr. Bakalinsky, and Dr. Ho, for serving on my committee and providing indispensable advice, and support on my project. Dr. Jump and Dr. Ho, your NUTR classes were probably the most useful classes I've ever taken. You will never realize how much they helped me. I appreciate the support from Drs. Iwaniec and Jump on my postdoc application. They gave me mock interviews and shared tips. I appreciate all the discussion time with Dr. Bakalinsky. He was always happy to help, but asked me a lot of questions instead and made me feel I need to work harder to figure out answers to these tough questions. I feel truly honored to have had these great people as my committee members.

My sincere thanks also go to Drs. Michael and Yanping Qian, and my master's advisor Dr. Zeyuan Deng. I am truly blessed to have met them in my life.
I thank them for all their support, and helpful career advice.

I would like to thank my lab mates Sarah Snyder, Bingxin Zhao, Omar Miranda, Allysa Adamson, Geoffrey Sasaki, Maud Prati, and Marlena Strum. They are all my life friends. It was their daily support that made my life in the US enjoyable and meaningful. I will remember all our conference trips, all the classes we attended, and all the time we shared in our lab.

The support, guidance, and friendship from Greg Watson and Garrett Holzwarth were vital to me. I am extremely grateful for their assistance and suggestions throughout my project. Garrett, from the way you taught me all the time, I am sure you will be a good professor someday, as soon as you finish your dissertation. Greg, you are already a professional scientist for me.

My friends Wenjie, Xiaoye, Jingyi, and Ying, I will never forget what we experienced in the US: the days we stayed in the library, our Vegas trip, our beach trips, and our girls' nights. All of these will be my precious memories.

The bonus to have friends dispersed all over the world is that you will never feel lonely. I was able to talk to my friends whenever I wanted. I also would like to give thanks to my best friends, Yiming, Yi, Lin, and Cece. I would never survive without friends. Thank you, all my friends.

My parents have been the most important influences in my life. I would never have gotten to where I am today without their love and support. Last but not the least, I

would like to thank my parents for always being there whenever I needed them. Thank you for your unconditional trust, timely encouragement, and endless patience. I am the luckiest person ever to be your kid. I am proud to be a part of my big family.

I do appreciate what I have achieved so far and what I am going to achieve in the future. Thank you all.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

Table Page

LIST OF APPENDICES

Appendix Page

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1 Soy and soy components

Soybeans (*Glycine max L.*), also called soja beans or soya beans, serve as one of the most valuable crops in the world. They act both as an oil seed crop, and a good source of protein for human and animal diets. The United States is the largest soybean producer in the world, followed by Brazil and Argentina (USDA, **2016-2017**). The daily intake of soy product in Asian diets is abundant, e.g. in China, it is 7-8g/day. In Korea and Japan, the amount is up to 20-30g/day, however, the amount is decreased to less than 1g/day in Europe and North America (Cederroth et al., **2012**).

Soybean composition varies in different varieties. The macronutrient content of soybean is approximately 50% protein, 24% carbohydrates, and 25% oil (Natural Medicines Comprehensive Database, **2010**).

Soybean protein is considered to be one of the least expensive sources of dietary protein, not only for a higher content of protein than most plants, but also for the fact that its amino acid composition is more comparable to animal protein (Takamatsu et al., **2004**; Erdman, **2000**). Except for the sulfur amino acids, methionine and cysteine, the amino acid content of soy protein is close to that of animal protein. Research studies using rats indicate that the biological value of soy protein is close to many animal proteins such as casein, if enriched with the sulfur-containing amino acid methionine (Hajos et al., **1996**).

Aside from soy protein, a main bioactive component present within soy is reported to be soy isoflavones (Kim et al., **2012**). Soy and soy products are the richest isoflavones sources in the human diet (Murphy et al., **1999**). The amount of isoflavone present in soybean is highly variable. The isoflavone content (mainly as glycoside form) in dry basis

ranges from 0.1%-0.4%. Isoflavones are mostly derived from soy. There are two other plants which are good sources of isoflavones. One is the tuber of the American groundnut, which contains as high as 8mg/g of genistein (Barnes et al., **2002**). The other source is the root of Kudzu (pueraria lobate). In Asia, the root of the Kudzu has been utilized as a traditional medicine and the bioactive isoflavone compounds are often found as dietary supplements (Fang, **1980**). The main forms of isoflavones in Kudzu are O-glucosides, and C-glucosides (Stephen, **2010**). Other beans, lentils, peas, and red clover contain small amounts of isoflavones.

Isoflavones are members of the flavonoid family of plant compounds and are classified as phytoestrogens: the structure resembles the hormone estrogen and can exhibit weak but, measurable estrogen-like effects. Soybean seeds contain nine different isoflavones with three major isoflavone aglycones, genistein, daidzein, and glycitein (Figure 1.1). Each of these aglycones may also be present as their corresponding 7-O-glycosides (genistin, daidzin, and glycitin) and malonyl glycosides (6”-O-malonylgenistin, 6”-O-malonyldaidzin, and 6”-O-malonylglycitin) (Kudou et al., **1991**) (Figure 1.1). The aglycone structures can be found in very small amounts in soybean, while the glycoside forms are dominant. The principal glycoside-form isoflavones present within soy are genistin, daidzin, and glycitin. However, it is reported that the glycoside form is poorly absorbed in the small intestine. After digested by β-glucosidase, isoflavones glycosides are converted to aglycone, and thus may be absorbed in the upper small intestine by passive diffusion (Day et al., **1998**). For this reason, aglycones are considered to be biologically active forms of isoflavones, although this may be a matter of debate to some.

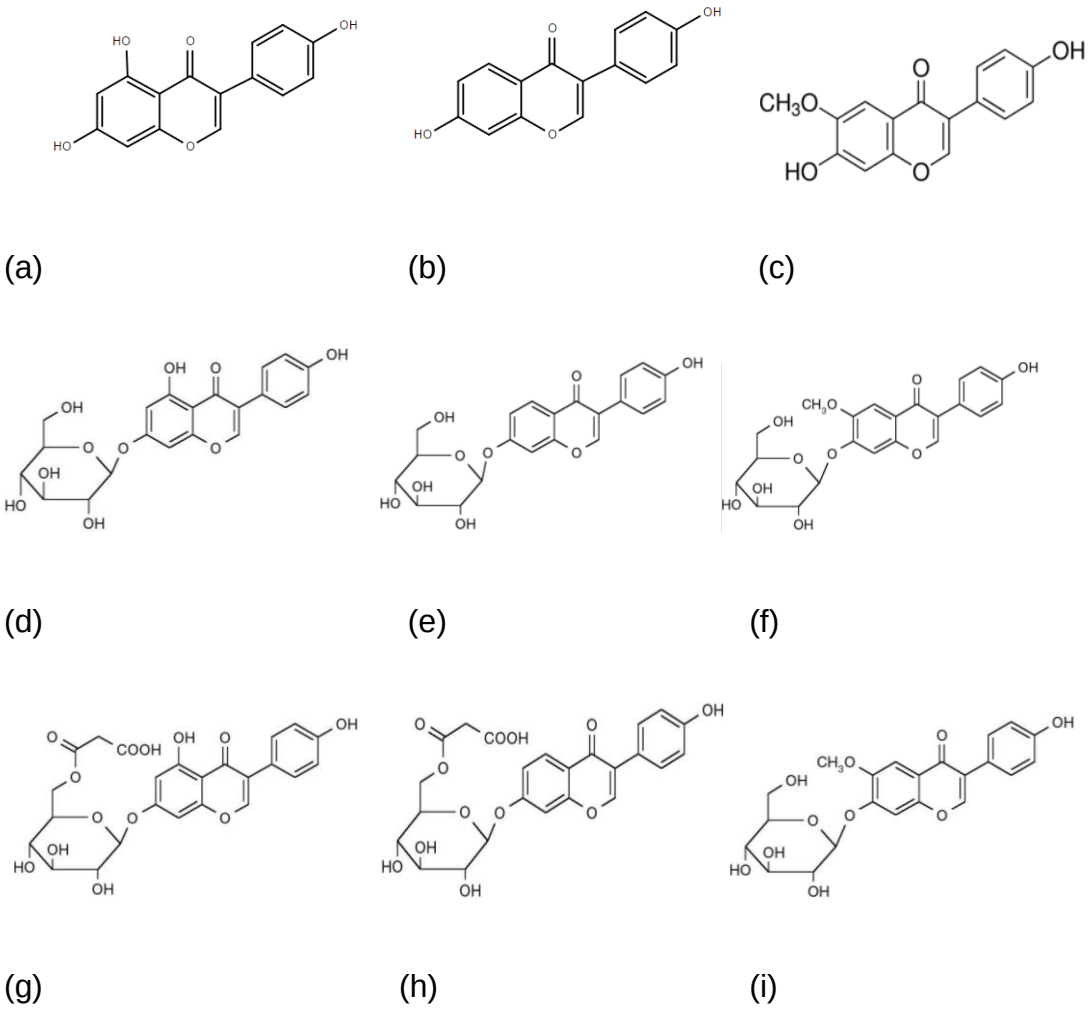


Figure 1.1 Chemical structures of soy isoflavone: (a) genistein, (b) daidzein, (c) glycitein, (d) genistin, (e) daidzin, (f) glycitin, (g) 6”-O-malonylgenistin, (h) 6”-O-malonyldaidzin, and (i) 6”-O-malonylglycitin

There is also a General Recognized As Safe (GRAS) approved soy isoflavone supplement, NovaSoy. NovaSoy is a soy-based isoflavone concentrate which is extracted to assure that the ratio of isoflavones as well as the aglycone and glycoside isoforms are maintained as they would be found in soybeans and unfermented soyfoods. It contains about 40% by weight isoflavone compound. The isoflavones it contains are genistin:

daidzin: glycitin =1.3:1.0:0.3. Similarly, soybeans include about 50% genistin, about 40% daidzin, and up to 10% glycitin forms (Murphy et al., **1999**; He et al., **2013**).

1.2 The biosynthesis pathway and function of isoflavone in the plant

Isoflavones are synthesized by phenylpropanoid pathway (Buer et al., **2010**). As shown in Figure 1.2, phenylalanine first reacts with malonyl CoA to form 4-hydroxycinnamoyl CoA. Chalcone synthase catalyzes the reaction of this intermediate with three more molecules of malonyl CoA to form isoliquiritigenin chalcone (4, 2',4'-trihydroxychalcone) or naringenin chalcone (4,2',4',6'-tetrahydroxychalcone). Chalcone isomerase catalyzes the ring closure of the heterocyclic ring, and then liquiritigenein and naringenin are synthesized. Isoflavone synthase introduces a 2-hydroxyl group, which in turn is removed by an isoflavone dehydratase to yield daidzein (7,4'-dihydroxyisoflavone) and genistein (7,4'-dihydroxy-6-methoxyisoflavone) (Barnes, **2010**; Jung et al., **2000**).

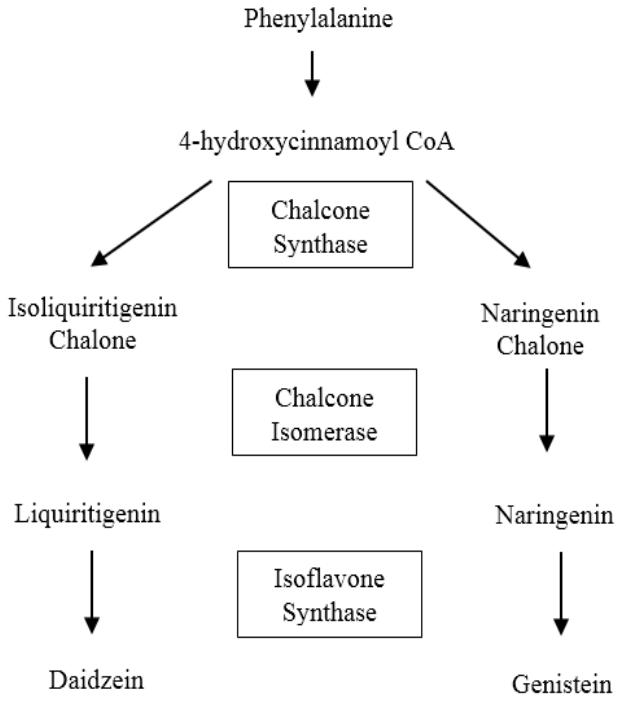


Figure 1.2 A simplified diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis. The key enzymes in each step are stated in the rectangle.

Isoflavones play numerous roles in the interaction between plants and environment. They function as precursor molecules for phytoalexins synthesis during plant-microbe or plant-insect interactions, thus warding off disease-caused by pathogenic fungi and microbes (Aoki et al., **2000**; Dixon et al., **2002**). Isoflavones also act as inducers to establish the symbiotic relationships between the roots of leguminous plants and rhizobial bacteria (Pueppke, **1996**). They stimulate the soil microbe rhizobium to form nitrogen-fixing root nodules (Ferguson et al., **2003**).

1.3 Metabolism of isoflavones in human body

After ingestion of isoflavone-rich foods, the isoflavone aglycones are readily absorbed in the upper small intestine by passive diffusion. In contrast, isoflavone glycosides cannot typically be passively absorbed. Glycosides may undergo hydrolysis by β-glucosidase derived from intestinal bacteria, or may be hydrolyzed by lactase-phlorizin hydrolase in the intestinal mucosa (Day et al., **2000**). Soy isoflavone aglycones are reported to be absorbed faster than their glucosides in humans (Izumi et al., **2000**). One report showed that there is no isoflavone glycosides detected in plasma samples at 1, 2 and 8 hours after ingestion of isoflavone contained soy food (Setchell et al., **2002**).

In enterocytes, the isoflavone aglycones are converted to their β-glucuronides by UDP-glucuronyl-transferase (Sfakianos et al., **1997**) and to sulfate esters by PAPS-sulfotransferases (Ronis et al., **2006**). Glucuronidation and sulfation also occur in the liver as well (Nakano et al., **2004**). These conjugated metabolites are excreted in the bile and are deconjugated in the lower bowel allowing them to be reabsorbed, creating an enterohepatic circulation (Sfakianos et al., **1997**). Another type of recycling, called enteric recycling, occurs in the small intestine, where glucuronidated and sulphated forms of isoflavones are secreted back into the intestine for further reabsorption and metabolism (Chen et al., **2003**). Enterohepatic circulation and enteric recycling of isoflavones are important because they allow body tissues to be exposed to these phytochemicals for longer periods of time following food intake.

After absorption, metabolism and distribution, isoflavones are ultimately excreted in urine. Most excretion occurs within 24 hours of consuming an isoflavone-containing meal (Setchell et al., **2003**).

Isoflavones that pass through the small intestine without being bioactivated or absorbed then pass into the colon, where genistein can be metabolized to dihydrogenistein (DHG) and 6'-hydroxy-O-desmethylangolensin (6-OH-O-Dma), whereas daidzein can be reduced to dihydrodaidzein (DHD) and then converted to O-desmethylangolensin (O-Dma) or equol (Setchell et al., **2003**; Rowland et al., **2003**). Genistein, daidzein, equol and O-Dma are the major isoflavones detected in the blood and urine of animals and humans (Tham et al., **1998**).

Among the bacterial byproducts of isoflavone metabolism in the colon, equol is considered to have significant bioactivity in humans (Cen et al., **2011**). Equol has been reported to possess a greater affinity for estrogen receptors than its precursor daidzein (Muthyala et al., **2004**), a longer half-life, a higher bioavailability in plasma than daidzein and genistein (Setchell et al., **2002**; Kelly et al., **1995**), and more potent antioxidant activity than any other isoflavone (Setchell et al., **2002**). However, not all humans are equol producers. The frequency of equol producers differs among different population groups. It is considered that the frequency of equol producers in adult western populations is lower than that for adult equol producers in some Asian countries, where traditional soy foods are regularly consumed (Hong et al., **2010**; Song et al., **2006**). On average, equol is produced by an estimated 30% to 40% of white persons and up to 60% of Asians (Liu et al., **2010**; Magee, **2011**; Tanaka et al., **2009**). It is still not fully understood what factors

determine equol production. It is believed that a primary factor is the composition and the enzymatic capability of gut microflora (Heinonen et al., **2002**; Atkinson et al. **2008**). More recently, it has also been reported that equol production might be associated with the intake of PUFA, maltose, and vitamins A and E (Setchell et al., **2013**).

1.4 Bioavailability of isoflavones

A bi-phasic peak in plasma concentrations is generally found after isoflavone consumption. The average time to reach peak plasma concentration is about 1-2h, which then peaks again after 4-10 h (Bhathena et al., **2002**). The bi-phasic peak in plasma is generally considered due to the microbial hydrolysis of isoflavone glucosides by β-glycosides in the large intestine, enterohepatic recirculation and enteric recycling.

The bioavailability of isoflavones has been studied in both humans and animals. It has been reported that the plasma concentrations of isoflavones in healthy humans are usually relatively low, e.g. less than 40nM (Morton et al., **1994**). However, after the consumption of soy foods, the human plasma concentrations of isoflavones has been shown to reach to 1-4 μM (Xu et al., **2004**). Accordingly, the excretion of isoflavone in the urine is significantly increased.

After consuming a high soy diet, circulating isoflavone concentrations can be much higher, reaching close to 0.2 mmol/L in at least one study (Adlercreutz et al., **1993**). Humans consuming a soy flour extract have circulating isoflavone concentrations close to 7 mmol/L (King et al., **1998**). Moreover, prostate cancer patients given high doses (300-600 mg) of the isoflavone supplement NovaSoy were found to have plasma levels of up to 27 mmol/L, with no evidence of toxicity (Miltyk et al., **2003**). In an animal study,

administration of 5 mg genistein/d to mice resulted in serum levels of 30 mmol/L (Hillman et al., **2004**).

1.5 Metabolic syndrome

Metabolic syndrome is a disorder of energy utilization and storage. Currently, the widely accepted definition for the diagnosis of metabolic syndrome is the ATP III criteria. Metabolic syndrome is a clustering of at least three out of the five following medical conditions: abdominal obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides (TG), and low high-density lipoprotein (HDL) cholesterol levels (Charnsil et al., **2015**). Table 1.1 shows more detail of these five medical conditions. Obesity is defined by waist circumference. More than 40 inches in men and 35 inches in women is regarded as a diagnosis of abdominal obesity. Abdominal obesity is regarded as the predominant risk factor for the metabolic syndrome (Lemieux et al., **2007**). Indeed, the International Diabetes Foundation (IDF) further modified the ATP III definition by making the presence of abdominal obesity a requirement for diagnosis. There are two separate lipid risk factors, including high TG and independently low HDL levels. Hypertension is established at 130/85 mm Hg. Lastly, a fasting glucose of 100mg/dL or higher is considered an abnormally high fasting glucose level (Alberti et al., **2005**).

Metabolic syndrome is a concern because it is highly prevalent and predicts occurrence of chronic disease. It is estimated that 34% of Americans are now affected with this syndrome, up from 29% in 1994 (Mozumdar et al., **2011**). Metabolic syndrome is reported to be linked to several health conditions. It increases the risk of developing fatty liver disease, type 2 diabetes mellitus, and cardiovascular diseases (O'Neill et al., **2015**). It

has been reported that metabolic syndrome confers a 5-fold increase in the risk of type 2 diabetes mellitus. The risk for cardiovascular disease is essentially doubled in individuals with the metabolic syndrome, compared with those without (Alberti et al., **2009**; Stern et al., **2004**).

A variety of features of the metabolic syndrome are associated with systemic inflammatory responses (Baker et al., **2011**; Hotamisligil, **2006**; Shoelson et al., **2006**). Positive associations between components of the metabolic syndrome and inflammatory markers have been described. Pro-inflammatory markers, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, positively correlated with the features of the metabolic syndrome in most cases (Vozarova et al., **2002**; Natali et al., **2006**; Phillips et al., **2013**).

Metabolic syndrome is often characterized by oxidative stress, a condition in which an imbalance results between the production and inactivation of reactive oxygen species (Roberts et al., **2009**). Patients with metabolic syndrome have elevated oxidative damage. Additionally, older individuals with elevated oxidized low density lipoprotein levels exhibit an increased risk of developing metabolic syndrome (Holvoet et al., **2004**).

Modulation of abdominal obesity, oxidation, and/or inflammation are regarded to serve as potential therapeutic approaches for the metabolic syndrome in humans. Several studies have shown that physical activity and dietary modification can ameliorate oxidative stress, and inflammation, thus delay the onset or prevent metabolic syndrome progression (Zivkovic et al., **2007**).

Table 1.1 The ATP III criteria of metabolic syndrome

Risk Factor	**Defining Level**
Abdominal obesity	Waist circumference
Men	>102cm (>40in)
Women	>88cm (>35in)
Triglycerides	≥150mg/dL (1.7mmol/L)
HDL cholesterol	
Men	<40mg/dL (1.04mmol/L)
Women	<50mg/dL
Blood pressure	≥130/≥85mm Hg
Fasting glucose	≥100mg/dL (5.6mmol/L)

1.6 Drugs and targets of signaling pathways for metabolic syndrome treatment in human

Many existing and new therapies are targeting metabolic syndrome and the component risk factors, however, to date, no drugs have been approved for the treatment of metabolic syndrome (Heal et al. **2009**; Food and Drug Administration, **2007**, **2008**).

1.6.1 Cholesterol synthesis inhibitors

Statins are a class of drugs that are specific inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase). HMG-CoA reductase is the rate limiting enzyme in the cholesterol biosynthesis pathway. Inhibition of HMG-CoA reductase with

statins has been shown to reduce plasma levels of cholesterol and apoB-containing lipoproteins in hypercholesterolemic models. Plasma TG levels were reported to be decreased with the intake of statins (Binesh Marvasti et al., **2010**).

1.6.2 Inhibition of cholesterol absorption

Cholesterol absorption inhibitors, such as ezetimibe, selectively decrease the absorption of dietary cholesterol in the small intestine without affecting the absorption of fat-soluble vitamins, triglycerides or bile acids (Pearson et al., **2005**). It has been documented that Niemann-Pick C1-Like 1 transporter (NPC1L1) is the direct binding target of ezetimibe. The function of NPC1L1 is to keep cholesterol in the intestinal lumen for excretion (Garcia-Calvo et al., **2005**). There is a glucuronide metabolite of ezetimibe, which has a higher binding affinity to NPC1L1, therefore, is more potent in preventing cholesterol absorption. Ezetimibe in combination with statin is reported to enhance the reduction of the LDL cholesterol level (Davis et al., **2007**).

1.6.3 Activation of PPARs (Peroxisome proliferator-activated receptors)

PPARs belong to a subfamily of the nuclear receptor superfamily of ligand-inducible transcription factors (Laudet et al., **1992**). To date, three PPAR isotypes encoded by separate genes have been identified, PPARα, PPARβ/δ, and PPARγ (Issemann et al., **1990**; Dreyer et al., **1992**). PPARs play an important role in the pathogenesis of fatty liver (Zeng at al., **2014**).

PPARα agonists such as fibric acids, affect lipid metabolism in the liver. Increased fatty acid oxidation was found after the intake of fibric acid (Staels et al., **2005**; Gervois et

al., **2000**). Further, PPARα agonists were shown to possess anti-inflammatory and anti-atherogenic actions in addition to their lipid lowering effect (Staels et al., **2005**; Videla et al., **2012**).

PPARγ agonist are used for the treatment of metabolic syndrome, mainly for reducing insulin resistance in muscle and liver (Binesh Marvasti et al., **2010**; Musso et al., **2010**). Another potential benefit of PPARγ activation is the suppression of inflammatory responses (Musso et al., **2010**). Two PPARγ agonists are currently available: pioglitazone and rosiglitazone. However, more potent thiazolidinediones (TZDs) have some major side effects, including weight gain and fluid retention (Smith et al., **2005**). Therefore, the registration of TZDs has been hindered.

PPARβ/δ is widely expressed and regulates fatty-acid oxidation (Barak et al., **2002**). In animal studies, PPARβ/δ is more potent for oxidation of fatty acids than PPARα (Muoio et al., **2002**; Tanaka et al., **2003**). Activation of PPARβ/δ improve both insulin sensitivity and plasma lipid profile without causing weight gain (Coll et al., **2009**). PPARβ/δ agonists, such as GW501516, are promising targets for future drug development of metabolic syndrome.

1.6.4 Regulation of appetite

The genetic studies of metabolic syndrome have identified novel molecules acting on the hunger and satiety peptidergic signaling. The melanocortin 3/4 receptor (MC3/4R) agonist Melanotan II (MTII) has been shown to cause reduced food intake and weight reduction in rodents (Getting **2006**; Glavas et al., **2007**). Tesofensine (NS2330) is a serotonin-noradrenaline-dopamine reuptake inhibitor. It primarily acts as an appetite

suppressant, but possibly acts also by increasing resting energy expenditure (Astrup et al., **2008**). Peripheral infusion of YY3–36 has been reported to have dose-dependent anorectic effects in rodents (Batterham et al., **2003**; Karra et al., **2010**).

Despite a significant amount of research in many related areas, there are still no established pharmacological therapies for metabolic syndrome. Lifestyle modification remains the best strategy to limit metabolic syndrome in the general population.

1.7 Mouse models of the metabolic syndrome

Since the deleterious effects of metabolic syndrome remain a challenge to human health, it is essential to use models of metabolic syndrome to understand the pathogenesis and to test new potential therapeutic interventions. However, selecting an adequate experimental model that best represents the pathophysiology of metabolic syndrome in humans can be rather challenging. Indeed, when choosing animal or cellular models of disease, no one model may be optimal to evaluate all symptoms or mechanisms associated with that disease state. Among the various animal models available to study metabolic syndrome, mice are one of the most commonly used models.

The C57BL/6J mouse have been studied extensively as a good model for human metabolic syndrome. When these mice allowed free access to a high-fat diet, they develop obesity, hyperinsulinemia, hyperglycemia, and hypertension, which mimic the human metabolic syndrome (Surwit et al., **1988;** Collins et al., **2004**). However, they remain lean and physically normal when provided low-fat rodent chow. It is known that the mouse strain C57BL/6J is more prone to these effects of a high-fat diet than SWR/J, A/J, or the C57BL/KsJ mice (Collins et al., **2004;** Buettner et al., **2007**).

It is reported that animal-derived fats such as lard and beef tallow, and plant-derived fats rich in unsaturated ω-9 and ω-6 fatty acids, induce the obese phenotype. However, diets containing a large fraction of marine ω-3 polyunsaturated fatty acids do not (Buettner et al., **2007**).

Some rodent diets were designed with the combination of high-carbohydrate and high-fat. A combination of diets, such as high fructose/ high-fat, or high-sucrose/high-fat diets used to induce metabolic syndrome in animals have been reported. Both of these diets can induce obesity, hyperglycemia, and dyslipidemia. However, hypertension was not observed (Dissard et al., **2013**; Yang et al., **2012**). Knowledge on fructose metabolism revealed the superiority of fructose-feeding for the induction of metabolic syndrome in animal models when compared with glucose or starch (Wong et al., **2016**). The western diet is moderately high in saturated and trans-fat (41 % total energy), sucrose (30% total energy) and cholesterol (0.15%, w/w), hence, a high fat, high sucrose and cholesterol diet is often used as a model for metabolic syndrome and related chronic diseases in rodent studies (Jump et al., **2016**).

Mouse models differ from human disease in many aspects. For example, waist circumference as an indicator of obesity cannot be transferred from humans to mice. Metabolic rates are different between mice and humans: a 30g mouse has a metabolic rate approximately seven times greater than that of a 70kg human (Perlman, **2016**). Moreover, the lipoprotein profiles are different. Mice have primarily atheroprotective high-density lipoproteins (HDL), while normal human contains primarily atherogenic low-density

lipoproteins (LDL) (Kennedy et al., **2010**). Thus, no one mouse model can exactly mimic all aspects of human metabolic syndrome.

1.8 Biological functions of soy and soy isoflavones

Isoflavones were first discovered in the 1930's. They are reported to disrupt estrogen action in female sheep: increased infertility was found in sheep that had been grazing on red clover, thereby, earning the often-used term phytoestrogens. Isoflavones are structurally similar to17-β-estradiol, and bind to alpha- (ERα) and beta-estrogen (ERβ) receptors (Naaz et al., **2003**). Isoflavones may have either an anti- or pro-estrogenic effect depending on the endogenous levels of circulating estrogen (Zand et al., **2000;** Hwang et al., **2006**). In premenopausal women with normal estrogen levels, isoflavones may competitively bind to estrogen receptors, and prevent estrogen from binding (Zand et al., **2000**). Isoflavone intake may cause endogenous estrogen levels to fall (Lu et al., **2001**). This antagonistic effect has also been recognized previously, in that ingestion of a large amount of soy protein has been shown to suppress circulating female sex hormone levels and affect menstrual patterns in premenopausal women (Cassidy et al., **1994**). On the other hand, in postmenopausal women with low circulating estrogen, isoflavones have been shown to have a weak pro-estrogenic effect (Pino et al., **2000**).

In the 1990’s, soy intake was indicated to possess a cholesterol-lowering effect (Anderson et al., **1995**). In 1999, the FDA approved a health claim suggesting that intake of 25 g or more of soy protein per day may help to reduce the risk of cardiovascular disease (FDA rule, **1999**). More recent research has pointed to a beneficial antidiabetic effect of isoflavone intake, partly mediated by the activation of promiscuous nuclear

receptors (Ricketts et al., **2005**). Beavers et al. found that soy isoflavones improved endothelial function in postmenopausal women (Beavers et al., **2012**). Meta-analyses show systolic and diastolic blood pressure is reduced by soy intake (Taku et al., **2010**). Cardiovascular benefits of non-protein soy components, including isoflavones were reported (Ramdath et al., **2017**).

Other findings suggested the relationship between soy intake and reduced risk for breast cancer, diabetic nephropathy, diarrhea, hyperlipidemia, kidney disease, menopause symptoms, and osteoporosis (Jaygopal et al., **2002**; Teixeira et al., **2004**; Howes et al., **2006**; Jing et al., **2016**; Arjmandi et al., **2003;** Taku et al., **2012**).

1.9 The effect of isoflavone on nuclear hormone receptors and metabolic syndrome

Nuclear hormone receptors are a class of proteins found within cells that may function as ligand activated transcription factors that regulate the expression of target genes to affect a wide range of pathophysiologies (Chawla et al., **2001**; Evans et al., **2014**).

A typical nuclear hormone receptor contains a NH_2-terminal activation function (AF1) region, a DNA binding domain (DBD), a ligand binding domain (LBD), and a COOH-terminal activation function (AF2) region. Nuclear hormone receptors can be subdivided into four classes: receptors that bind DNA as homodimers, receptors that bind DNA as heterodimers (heterodimerize with retinoid X receptor), receptors that bind to direct repeats of the DNA sequence as homodimers, and monomeric/tethered orphan receptors. Nuclear hormone receptors including peroxisome proliferator activated receptors (PPAR), farnesyl X receptor (FXR), constitutive androstane receptor (CAR), and liver X receptor (LXR) belong to the second category (Olefsky, **2001**).

The nuclear hormone receptors are ideal drug targets (Huang et al., **2010**). They contain an internal pocket that can bind to hydrophobic, drug-like molecules. Upon ligand binding, nuclear hormone receptors undergo a conformational change to enable transcriptional activation, therefore, controlling central pathways impacting diverse metabolic diseases.

Studies in animal models and humans from Patsch lab suggest a central role for sterol regulatory element binding proteins (SREBPs) in the pathophysiology of the metabolic syndrome (Soyal et al., **2015**). We reported that intake of soy isoflavone containing soy protein has been shown to decrease the gene expression of SREBP-1 relative to mice consuming a casein based diet. The intake of soy protein also reduced several SREBPs target genes including FAS and SCD-1, and decreased lipogenesis (Mullen et al., **2004**).

The pregnane X receptor (PXR) modulates the clearance of xenobiotics. PXR activation was known to suppress the activity of NF-κB, a key regulator of inflammation and the immune response (Zhou et al., **2006**). The effectiveness of targeting PXR in the treatment of metabolic disorders, such as obesity, type 2 diabetes, and atherosclerosis, have been suggested in animal models (Gao et al.,**2012**). Our previous research found that isoflavone compounds regulate expression of several genes including human *CYP3A4*, murine *Cyp3a11*, and *Cyp8b1* probably via the activation of PXR, thus enhancing the clearance of xenobiotics and impacting bile acid metabolism (Li et al., **2007**; Li et al., **2009**). Evidence also suggests that PXR also has endobiotic functions that impact

glucose and lipid metabolism, as well as the pathogenesis of metabolic diseases (Zhou et al., **2006**).

Soy components also appear to activate several other nuclear receptors including PPARs and LXR, resulting in increased gene expression of *CYP3As*, *CYP4As* and CYPs involved in metabolism of cholesterol to bile acids and the oxidation of fatty acids. Such soy-CYP interactions are associated with improved lipid homeostasis (Ronis, **2016**).

Our previous research indicated that in the *in-vivo* mouse study, the consumption of isoflavones activates the PPARα signaling pathway, therefore, exerting antidiabetic and hypolipidemic effects (Mezei et al., **2003**). In cell reporter systems, unconjugated soy isoflavones appeared to activate the PPARα ligand binding domain. For PPARγ, unconjugated soy protein had 320% more bioactivity than isoflavone-free controls, and significantly more bioactivity than PPARα (Mezei et al., **2006**).

LXRs play both a central role in cholesterol metabolism, and are key regulators of lipogenesis (Hong et al., **2014**). Several direct LXR target genes, such as ATP-binding cassette subfamily A member 1 (ABCA1), ABCG1, ABCG5, and ABCG8 are intimately linked to reverse cholesterol transport, a process by which excess cholesterol from peripheral tissues is returned to the liver by high-density lipoprotein (HDL) for ultimate excretion in bile. Therefore, systemic activation of LXRs with LXR agonists in mice may reduce whole-body cholesterol levels and raise HDL levels in the plasma (Joseph et al., **2002**; Tangirala et al., **2002**; Wang et al, **2003**). On the other hand, LXR agonists activate de novo fatty acid synthesis by stimulating the expression of a lipogenic transcription factor, sterol regulatory element-binding protein-1c (SREBP-1c), leading to the elevation of

plasma triglycerides and liver steatosis (Wójcicka et al., **2007**). Treatment of mice with synthetic LXR agonists promotes triglyceride synthesis in the liver, stimulates very-low-density lipoprotein (VLDL) secretion and, at least transiently, raises plasma triglyceride levels (Joseph et al., **2002**; Chu et al., **2006**; Kiss et al., **2011**).

It is conceivable that pharmacological manipulation of the LXR pathway could potentially affect metabolic syndrome (Wójcicka et al., **2007**). Most drug development efforts have focused on LXR agonists. However, the lipogenic actions of LXRs have presented a major obstacle in the development of LXR agonists as drugs for metabolic syndrome (Laffitte et al., **2003**; Joseph et al., **2003**). The consumption of a soy protein diet was shown to reduce serum cholesterol and prevent the excessive accumulation of hepatic lipids (Tovar et al., **2002**; Tovar et al., **2005**; Torre-Villalvazo et al., **2008**). It is suggested that soy protein, part due to the capacity of isoflavones, particularly genistein, has an anti-steatotic effect. The LXRα pathway, possibly mediated via AMPK, is involved in the effect (González-Granillo et al., **2012**).

In our lab, we focus on the binding or indirect interaction of phytochemicals with several key nuclear hormone receptors, thus showing beneficial effect on lowering cholesterol level, and diabetes and metabolic syndrome prevention. As summarized in Figure 1.3, our prior research indicated that transcription factors including PXR, PPAR, and SREBP were regulated by soy isoflavone induction, *in-vitro* or/and *in-vivo* models. The goal of my study was to figure out the beneficial effect of isoflavones on the symptoms of metabolic syndrome and to determine whether there are other transcription factors altered by soy isoflavones.

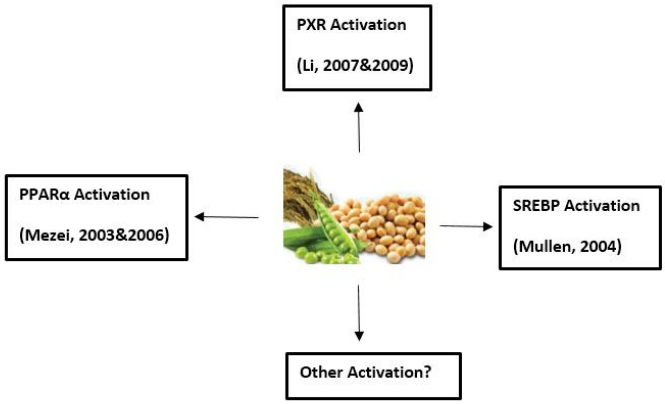


Figure 1.3 Signaling pathways regulated by soy isoflavones

CHAPTER II: *IN-VIVO* STUDIES: THE EFFECT OF SOY ISOFLAVONE SUPPLEMENT NOVASOY, GENISTEIN OR DAIDZEIN ON HIGH FAT DIET OR WESTERN DIET INDUCED METABOLIC SYNDROME IN C57BL/6J MALE MICE

2.1 Mouse Study I: Soy Isoflavone Supplement NovaSoy Intake and High Fat Diet Induced Metabolic Syndrome

2.1.1 INTRODUCTION

Metabolic syndrome describes the clustering of abdominal obesity, blood lipid abnormalities, hypertension, and hyperglycemia, and is often characterized by oxidative stress (Roberts et al., **2009**). Unequivocal experimental, epidemiological and clinical evidence links inflammation to the development of metabolic disease and/or the complications that emerge from these pathologies, particularly in the context of obesity and type 2 diabetes (Baker et al., **2011**; Hotamisligil, **2006**; Shoelson et al., **2006**).

Metabolic syndrome has been described as multiple metabolic disorders related to overnutrition (Baker et al., **2011)**. Diets high in saturated fats and sugars are associated with development of the metabolic syndrome in humans and mice (Siri-Tarino et al., **2010**; Siri-Tarino et al., **2010**). High fat diets, such as #D12492 from Research Diets (New Brunswick, NJ) has been used as an animal diet for the research of fatty liver, inflammation, obesity, and diabetes. High fat-fed C57BL/6J mice are an often-used rodent model for human obesity and related metabolic disease (Surwit et al., **1988;** Collins et al., **2004**).

The role of diet on the onset of long-term clinical health complications in western countries has long been a focus of research. Diets rich in polyunsaturated fat, low-glycemic-index foods, and foods with high-fiber content are recommended to reduce metabolic syndrome (Zivkovic et al., **2007**; Liu et al., **2011**). Epidemiological studies also

suggest that diets rich in fruits and vegetables reduce the incidence of metabolic syndrome (Zivkovic et al., **2007**).

Effects of soy intake on health have been well-published. It is reported that one of the main bioactive constituents in soy are the soy isoflavones (Kim et al., **2012**). There are three main isoflavones present in soy: genistein, daidzein, and glycitein. Dietary genistein decreased plasma and liver lipids, and improved plasma metabolic profiles of hamsters with diet-induced hyperlipidemia (Tang et al., **2015**). Jeon et al. suggested that genistein alleviates the development of fatty liver disease as well as obesity in ApoE-/- mice fed a high-fat diet (Jeon et al., **2014**). Isoflavones inhibited adipocyte differentiation and prevented insulin resistance in 3T3-L1 cells (Gao et al., **2015**). The mechanisms by which isoflavones exert these many effects are of interest and could identify specific protein targets for therapeutic development. Results appear at present to be caused by pleiotropic activities of the isoflavones.

In the present study, we utilized a rodent model to determine molecular pathways which are regulated by soy isoflavones. The hypothesis was that feeding soy isoflavone supplement Novasoy will improve the metabolism of obese and diabetic mice fed a high-fat diet. Improvements would be observed in lipid metabolism, oxidative stress, and inflammation.

2.1.2 MATERIALS AND METHODS

C57BL/6J mice and diets

Male C57BL/6J mice at 6 week of age were purchased from Jackson Laboratory (Bar Harbor, ME, USA). After being acclimated to semi-purified low fat (LF) diet from chow diet for two weeks, mice were randomly divided to three groups. Two control groups were fed either the LF diet, containing 10% fat and 70% carbohydrate by energy or a high-fat (HF) control diet containing 60% fat and 20% carbohydrate (Table A2.2.2). A third group was provided the HF diet with 0.4% (w/w) of isoflavones-containing dietary supplement, Novasoy (HF+NS). Mice were fed with these three different diets for ten weeks. Diets were produced by Research Diets, Inc. (New Brunswick, NJ, USA).

Mice were kept four per cage in a room maintained at a constant temperature (24^{o}C), with a 12-h light/dark cycle, and given free access to diet and distilled water. During the 10 week feeding trial, body weights and food intake was recorded once per week, with spillage accounted for. The animal protocol was approved by the institutional animal care committee (ACUP 4455).

Fasting blood glucose and intraperitoneal glucose tolerance test

In week 9 of the feeding trial, tail vein blood glucose levels were measured every 30 mins over a two-hour period. Diet was withheld for six hours prior to testing and the test was performed mid-day in the middle of the light cycle. Mice were administered glucose (0.1mg /g b.w.) by intraperitoneal injection. Blood glucose levels were measured from the tail vein using a handheld glucometer (ReliOn, Abbott Laboratories, Abbott Park, IL).

Glucose levels were measured at 0, 30, 60, 90, and 120 minutes. The index of glucose tolerance was indicated as the area under the curve (AUC) using the trapezoidal rule to determine AUC (Ayala et al., **2006**; Ayala et al., **2010**)

Histological analysis of liver tissue

At week 10 of feeding trial, mice were sacrificed and liver tissue was extracted. Liver tissue was fixed in buffered formalin and paraffin embedded, and three to four 5-μm thick sections were transferred to numbered slides. Slides were then stained with Masson's trichrome stains. Images were acquired using a Nikon Eclipse E400 microscope (Nikon Co., Tokyo, Japan) equipped with an extended digital camera (Q imaging, Surrey, BC, Canada). Lipid droplet percentage (the ratio of white color area to the total area) was obtained with Adobe Photoshop 7.0, generally following guidelines by Dahab et al (Dahab, et al., **2004**).

Plasma biomarkers quantitation

Blood samples were collected via cardiac puncture, incubated on ice for 30-60 minutes and centrifuged at 1000 g for 15 min at 4^{o}C, after which serum was collected. Cytokines were measured in 96-well plates using MILLIPLEX® MAP kits (Millipore, Billerica, MA, USA). The Mouse Cytokine/Chemokine Magnetic Bead Panel (Cat. #MCYTMAG-70K-PX32) was used to measure cytokines including insulin, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6), and macrophage inflammatory protein-1 α (MIP-1α). The Mouse Adipokine Magnetic Bead Panel (Cat. #MADKMAG-71K) was used to measure adipokines including leptin, total plasminogen activator inhibitor-1 (PAI-1), and resistin. Plates were read on a Luminex 200 instrument

(Luminex, Austin, TX, USA) and analyzed using xPONENT software (xPONENT, Saugus, MA, USA).

Serum triglyceride levels, total cholesterol, HDL, and LDL levels were analyzed utilizing a Vitros 250 (Ortho-Clinical Diagnostics, Rochester, NY).

Custom-designed focused gene array

For each mouse, a 200mg piece of liver tissue was stored in 200 µL RNAlater® Solution (Life Technologies, Carlsbad, CA, USA) at 4 °C for 24 hours allowing for solution penetration as suggested by the product protocol. Tissue collection was done within 1-2 minutes after mice were killed. RNA was isolated from liver homogenates using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). cDNA synthesis was performed using RT^2 First Strand Kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. Briefly, genomic DNA was eliminated using Genomic DNA Elimination Mixture provided in the kit. The RT mix was prepared, and first strand cDNA synthesis reaction was carried out according to the manufacturer's instructions. For detailed information regarding the genes applied on this array RT^2 Profiler PCR Array (custom-designed focused gene array Cat. no. CAPM13211), refer to supplementary file Table A2.1.2.

Array analysis was performed according to the instructions of the manufacturer, and qRT-PCR was carried out with ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). Briefly, cDNA was diluted in an appropriate amount of master mix and RNase-free water and loaded onto the array plate. The qRT-PCR condition is listed as follows: 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Immediately after

the cycling program a melting curve program was run to generate a first derivative dissociation curve.

Statistical analysis

Data are presented as means ± SEM. ANOVA was used to compare sets of data. Tukey's procedure was used for the post hoc testing. Weekly body weight was analyzed using repeated-measures ANOVA followed by post-hoc testing within each week with one-way ANOVA. Significance was established at a level of $p<0.05$. All statistical analyses were carried out using GraphPad Prism 6.

2.1.3 RESULTS

Effects of NS on body weight in HF-fed mice

In a 10-week study, there were no significant changes in food intake between the HF- and HF+NS-fed C57BL/6J mice (Figure 2.1.1B). Compared to LF-fed mice, HF-fed mice had significantly increased relative body weight (body weight/initial body weight). Mice fed HF+NS had a significantly lower relative body weight compared to the high fat diet group (Figure 2.1.1A, $p<0.01$).

Effects of NS on organ weight in HF-fed mice

Organ tissues were extracted at week 10. As shown in Table 2.1.1, NS intake has no effect on liver and inguinal adipose tissue weight. The kidney weight in HF+NS diet fed mice was less than that in HF fed mice.

Effects of NS on glucose homeostasis in HF-fed mice

Baseline blood glucose concentrations (0 timepoint in Figure 2.1.2A & Figure 2.1.2C) were significantly decreased in HF+NS-fed mice. We examined the effects of NS on glucose intolerance: in the HF-diet group, blood glucose levels were higher than the control group at 120 min after glucose loading. However, NS supplementation had little effect on overall glucose tolerance (Figure 2.1.2B).

Leptin levels were markedly increased in the HF group, and decreased in HF+NS-fed mice ($p<0.05$). In HF+NS-fed mice, serum resistin levels were ameliorated to a level not different from LF-fed mice. Insulin levels were not different between HF and HF+NS groups, but a general pattern resembled levels measured for baseline glucose, leptin, and resistin.

Effects of NS on lipid accumulation in HF-fed mice

Intracellular lipid accumulation is observed as white droplets in photomicrographs of stained liver tissue. There was more lipid accumulated in liver of HF diet fed mice. In HF+NS-fed mice, there was decreased hepatic lipid accumulation compared to HF-fed mice (Figure 2.1.3A). The quantified results showed that liver lipid percentage in HF+NS group was significantly lower than in the HF group (Figure 2.1.3B). Furthermore, PAI-1, a marker of liver fibrosis, was decreased in the HF+NS group compared to the HF diet group (Figure 2.1.3C).

Effect of NS on markers of inflammation in HF-fed mice

As shown in Figure 2.1.4, serum MIP-1α levels were significantly higher in HF-fed mice compared to with LF-fed mice (32±5 pg/ml vs.13±2 pg/ml); in HF+NS-fed mice, there was a significant decrease in MIP-1α content (15±3 pg/ml) compared to HF-fed mice. NS was found to significantly decrease GM-CSF content (6 pg/ml in HF+NS vs. 48±11 pg/ml in HF). There was a non-significant trend for IL-6 in HF+NS- compared to HF-fed mice (p=0.12).

Effect of NS on serum lipid concentration and liver enzyme levels in HF-fed mice

Serum lipid concentration, including serum cholesterol, HDLC, LDL, VLDL, and serum triglyceride were determined. As shown in Figure 2.1.5, serum cholesterol, HDLC, and LDL levels were significantly increased in HF diet fed mice. However, the consumption of NS did not alter serum lipid concentration, compared to HF diet fed mice. There is no significant difference of the liver enzyme levels, including serum aspartate aminotransferase (AST), and serum alanine aminotransferase (ALT) in LF-, HF-, and HF+NS- fed mice.

Custom-designed gene array

The relative expression of 87 different hepatic genes was determined (Table A2.1.1). Gene expression in HF-fed mice was set as the control (i.e. 1.0). Most regulated mRNAs was are shown in Table 2.1.2. Hormone-sensitive lipase (*Hsl)* gene expression in HF+NS group was higher than that in HF group (2.6 vs. 1.0). Compared to HF control diet fed mice, *Cyp7a1* and *Cyp8b1* mRNA expression in LF group mice was dramatically

decreased. In contrast, gene expression for these two mRNAs was increased with the consumption of HF+NS, however, no significant difference was found. *Cpt1α* gene expression in the HF+NS group was significantly upregulated, compared to the expression in HF diet fed mice (2.6 vs. 1.0).

2.1.4 DISCUSSION

In this study, we conducted an *in-vivo* trial using C57BL/6J mice to determine the influence of a GRAS-approved isoflavone supplement, NovaSoy on high fat diet-induced metabolic syndrome. The dietary level of 0.4% NS (w/w) has a human equivalent dose of 650mg/50kg body weight /day, a level consistent with humans consuming NS-containing dietary supplements (Reagan-Shaw et al., **2008**).

In C57BL/6J mice, intake of HF+NS improved glucose metabolism and insulin sensitivity based on our measurements of blood glucose, serum insulin, resistin, and leptin. The result is consistent with a published meta-analysis, which showed soy isoflavone supplementation could be beneficial for body weight reduction, glucose, and insulin control in plasma (Zhang et al., **2013**). Clinically, fasting blood glucose is an important indicator of the metabolic control of diabetes (Frid et al., **2005**), because individuals with diabetes have higher blood glucose levels. When fasted, the hormone glucagon in the body is stimulated, resulting in the increased glucose levels. Normal individuals can produce insulin to rebalance the increased glucose levels. However, patients with type 2 diabetes have insulin resistance (Friedman et al., **2003**), and higher blood glucose. In this study, we showed that NS supplementation in the context of a HF diet led to a significant decrease in relative body weight gain and fasting glucose in mice

(Figure 2.1.1 & 2.1.2), suggesting a beneficial effect on glucose homeostasis on diabetic mice. However, the NS treatment group had no effect on AUC levels measured in a glucose tolerance test, indicating that insulin sensitivity is comparable between the groups. It is possible that undue stress on mice during the testing made identification of glucose intolerance differences between groups more difficult to measure.

The modest anti-adiposity effect of NS was also associated with decreased leptin, insulin, and resistin (Figure 2.1.2). Leptin and resistin are signaling molecules that can regulate glucose homeostasis (Kershaw et al., **2004**). Obesity and type 2 diabetes are associated with increased insulin resistance (Friedman et al., **2003**). The increase in insulin sensitivity in HF+NS-fed mice may be related to the decreased level of resistin in plasma. It is reported that elevated resistin contributes to insulin resistance in humans (Steppan et al., **2001**; Smith et al., **2003**); serum resistin levels are elevated in obese individuals (Azuma et al., **2003**). Investigation showed that when resistin is administered acutely in rodents, blood glucose and hepatic glucose are increased (Rajala et al., **2003**), and this results in insulin resistance (Yang et al., **2009**). Mice lacking the adipocyte hormone resistin exhibit low blood glucose levels after fasting due to reduced hepatic glucose production (Banerjee et al., **2004**). This is partly mediated by activation of enzymes related to glycogenolysis, decreasing expression of gluconeogenic enzymes, and lowering the level of insulin receptor in the liver.

The hormone leptin is produced by adipose tissue. Increased body fat is associated with increased levels of leptin, which then act to reduce food intake. Mutations that result in leptin deficiency are associated with profound obesity in rodents and humans (Unger,

2002). A decrease in body fat leads to a decreased leptin levels, which stimulates food intake and reduces energy expenditure. Attenuation of leptin sensitivity in the brain leads to excess triglyceride accumulation in adipose tissue, muscle, liver, and pancreas, thus resulting in impaired insulin sensitivity and secretion (Unger, **2002**).

Isoflavone intake appears to reduce hepatic lipid accumulation; in support, we found expression of mRNAs associated with lipid oxidation to be activated. Hepatic PAI-1 expression, reflective of fibrosis, was decreased with NS intake. The circulating level of PAI-1 is elevated in obesity and are reduced with weight loss (Christiansen et al., **2005**; Monzillo et al., **2003**). We found that supplementation of NS lowered the levels of these two parameters compared to the HF-fed mice. In addition, the levels of GM-CSF and MIP-1α were decreased by NS supplementation.

From the gene array results, we found that the expression of *Cpt1α* was elevated by NS supplementation. *Cpt1α* is a major rate-limiting enzyme for mitochondrial β-fatty acid oxidation, and expression is regulated primarily by *PPARα* (Lee et al., **2011**). *PPARα*, a key regulator for maintaining whole-body energy balance, is suggested to stimulate both adipocyte differentiation and fatty acid oxidation in human adipocytes (Lee et al., **2011**). The activation of PPARα is associated with decreased hepatic and circulating lipid concentrations. NS supplementation increased the expression of *Cpt1α* mRNAs, predicting that fatty acid oxidation is increased by NS supplementation.

Cyp7a1 and *Cyp8b1* are involved in bile acid synthesis. Increased expression of these two genes indicates that NS may increase bile acid synthesis, thus impacting cholesterol absorption in the intestine (Kliewer et al., **2015**; Claudel et al., **2005**).

In conclusion, these results demonstrate that soy isoflavones could ameliorate multiple symptoms of the metabolic syndrome and the associated complication, fatty liver. In mice, consumption of a commercially available dietary NS concentrate altered gene expression profiles associated with important metabolic pathways and contributed beneficial physiological effects. Isoflavone intake not only improved lipid and glucose metabolism, but also acts to improve chronic inflammation. Treatments to improve metabolic syndrome in humans are limited; soy isoflavones may prove to be a part of an effective therapy or an option to improve symptoms associated with metabolic syndrome.

2.2 Mouse Study II: Intake of Genistein or Daidzein in a Western Diet-Induced Model of Metabolic Syndrome

2.2.1 INTRODUCTION

Mouse study I showed that soy isoflavone intake ameliorated symptoms associated with metabolic syndrome in C57BL/6J mice fed a high fat diet. Genistein and daidzein are relatively abundant compounds contained in soy (Murphy et al., **1993**). It is possible that genistein and daidzein might be the main bioactive compounds in ameliorating the symptoms of metabolic syndrome and might influence metabolism differently.

Both genistein and daidzein are known to interact with the α and β estrogen receptors (ERα/β) in several tissues (Kim et al., **2006**). Genistein is stronger than daidzein in its agonistic activity for the estrogen receptors (Ricketts et al., **2005**). Kim et al found that anti-adipogenic effects of genistein were principally attributable to activation of Wnt signaling via an ER-dependent pathway. However, daidzein inhibited adipogenesis through stimulation of lipolysis (Kim et al., **2010**). Genistein has a stronger inhibitory effect on the activity of tyrosine kinase than daidzein (Akiyama et al., **1987**), and has a stronger effect, vs. daidzein, in increasing the gene expression of apolipoprotein A-I, a major component of high density lipoproteins (Lamon-Fava, **2000**). As an antioxidant, metabolites of genistein are more effective than daidzein in inhibiting low density lipoprotein oxidation (Kgomotso et al., **2008**). However, Foti et al. reported that daidzein is as effective as genistein in protecting cells against oxidative damage (Foti et al., **2005**). On the other hand, daidzein is metabolized by enterobacteria to produce equol which has stronger estrogenic and antioxidative activities than daidzein or the other isoflavone metabolites

(Yuan et al., **2007**). However, equol is weaker than genistein or daidzein in activating PPARα and PPARγ (Ricketts et al., **2005**).

Our previous study found that isoflavone supplement Novasoy could ameliorate multiple symptoms of the metabolic syndrome, such as decreasing high fat diet induced body weight and blood glucose concentration (Luo et al., **2016**). Here, we examined whether isoflavone compounds, genistein or daidzein could have different effects on metabolic syndrome in western diet fed C57BL/6J mice, and on preventing the development of related chronic diseases. In this study, we utilized an obesogenic high-fat, western-style diet (WD), which closely resembles the typical American diet, compared to the high fat diet used in mouse study I.

2.2.2 MATERIALS AND METHODS

C57BL/6J mice and diets

Thirty-two male C57BL/6J mice, at 6 week of age, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). After two weeks of acclimation to the semi-purified LF diet, two control groups were fed either a LF diet containing ~10% fat and ~70% carbohydrate by energy or WD containing 45% fat with 1% cholesterol and added sucrose (Table A2.2.2). A third group was provided the WD plus 0.16% (w/w) genistein (LC Laboratories, Woburn, MA) (WD+G). A fourth group was provided the WD plus 0.16% (w/w) daidzein (LC Laboratories, Woburn, MA) (WD+D). Four groups of mice were provided these four different diets for 10 weeks. Diets were produced by Research Diets, Inc. (New Brunswick, NJ, USA). Mice were kept four per cage in a room maintained at a constant temperature (24°C), with a 12-h light/dark cycle, and given free access to food

and distilled water. Body weight, food intake and spillage of the mice were recorded every week. The animal protocol was approved by the institutional animal care committee (ACUP 4455).

Fasting blood glucose and intraperitoneal glucose tolerance test

At week 9 during 10 weeks' feeding, tail vein blood glucose levels over a two-hour period were measured. This protocol was performed as described 2.1.2: fasting blood glucose and intraperitoneal glucose tolerance test. Glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes.

Histological analysis of liver tissue

Liver tissue was fixed in buffered formalin and paraffin embedded. Liver histological analysis following the method stated in 2.1.2: histological analysis of liver tissue.

Plasma biomarkers quantitation

Blood samples were collected via cardiac puncture, incubated on ice for 30-60 minutes and centrifuged at 1000 g for 15 min at 4 ^{o}C, after which serum was collected. Insulin, resistin, monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1) were measured in 96-well plates using ELISA kits (Invitrogen Corporation, Camarillo, CA, USA). The designated amount of serum and antibody-immobilized beads were added to each well following the manufacturer's instructions. Plates were sealed and incubated at room temperature on a plate shaker. After incubation, the plates were washed four times and detection antibodies were added to each well. Plates were sealed and incubated at room temperature, on a plate shaker for

30-60 minutes according to the manufacturer's instructions. Plates were washed 4 times according to the manufacturer's instructions and 100µL of stop solution were added to all wells. Plates were read on Wallac 1420 Victor2 Microplate Reader (Perkin Elmer life and analytical sciences, Turku, Finland).

Custom-designed focused gene array

Following the same protocol as mouse study I, relative expression levels of 87 hepatic genes in LF, WD, WD+G, and WD+D mice were determined.

Statistical analysis

Data are presented as means ± SEM. ANOVA was used to compare sets of data. Tukey's procedure was used for the post hoc testing. Significance was established at a level of $p<0.05$. All statistical analyses were carried out using GraphPad Prism 6. Qiagen RT^2 profiler PCR array data analysis and Excel were used for the Heat map analysis. Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics, Redwood City, CA) was used for the pathway analysis.

2.2.3 RESULTS

Effects of genistein or daidzein on body weight and food intake

Initial weights of the mice did not differ in the four groups, while differences were marked at the end of the 10-week trial (Figure 2.2.1A). The results showed that both genistein and daidzein diets significantly decreased body weight, compared to WD-fed control mice. Furthermore, dietary genistein compared to daidzein was more potent in

reducing weight gain. After ten weeks' feeding, the body weight/initial body weight*100 in WD diet fed mice was 184.5%±10%, as compared to 112.1%±8.7% in WD supplemented with genistein (WD+G) diet, and 149.8%±6.9% in WD supplemented with daidzein (WD+D) group.

Genistein reduced the average caloric intake per week per mouse from the WD diet by 26.3%±2.5%. Daidzein decreased the food intake by 7.9%±3.4% compared to WD group (Figure 2.2.1B).

Organ weight in genistein and daidzein fed mice

After sacrifice, the liver, kidney and inguinal adipose tissue in LF, WD, WD+G, WD+D groups were isolated and weighed (Table 2.2.1). Compared to the LF-fed control group, WD significantly increased liver weight (1.15±0.09g in LF vs. 2.25±0.19g in WD) and liver weight/body weight ratio (0.041±0.001 in LF vs. 0.056±0.003 in WD), while the supplementation of genistein to the WD diet significantly reduced the liver weight (1.16±0.07g in WD+G). Compared to WD group, genistein significantly decreased the kidney weight ($p<0.05$). The inguinal adipose tissue weight was significantly decreased in genistein and daidzein fed mice, compared to WD fed mice ($p<0.05$).

Effects of genistein or daidzein on glucose homeostasis

Plasma levels of glucose were affected by genistein or daidzein treatment. As shown in Figure 2.2.2A, after 30 minutes' glucose injection, all plasma glucose levels reached a maximum and then declined at 2 hours, steadily approaching the basal levels. At time 120mins, the glucose concentration in the LF-fed group was close to the glucose in

WD+G-fed group (245±47 mg/dl in LF vs. 243±38 mg/dl in WD+G), which was lower than the glucose in WD- and WD+D-fed groups (343±79 mg/dl in WD vs. 317±88 mg/dl in WD+D).

A global estimation of the glucose level is depicted by the incremental areas under the curve (AUC) from time 0 to time 120mins of the glucose overload trial. Results showed that there was no significant AUC difference in these four treatment groups (Figure 2.2.2B). However, the AUC from 60 to 120 mins (Figure 2.2.2C) indicates that AUC (60mins-120mins) in WD+G-fed mice was significantly decreased compared to WD-fed mice ($p<0.05$). Glucose homeostasis related parameters, including insulin was significantly reduced in the WD+G-fed group. Resistin was significantly decreased in the WD+D-fed group, compared to the WD-fed group.

Effects of genistein or daidzein on hepatic lipid accumulation

Figure 2.2.3A showed the hepatic lipid accumulation. The white droplet indicated the lipid accumulation. The quantified results showed that the consumption of genistein had a marginal effect on the lipid accumulation in the liver tissue (Figure 2.2.3B). Furthermore, PAI-1, a marker of liver fibrosis, was not altered with the consumption of genistein or daidzein (Figure 2.2.3C).

Effects of genistein or daidzein on inflammation

There is a trend that genistein can decrease parameters related to inflammation, such as MCP-1 ($p=0.1$). IL-6 levels were not significantly changed in the WD+G- and WD+D-fed groups, compared to WD-fed group (Figure 2.2.4).

Effects of genistein or daidzein on hepatic gene expression

To further investigate the underlying mechanism of how genistein and daidzein affected WD-induced body weight gain, glucose homeostasis, and hepatic lipid accumulation, a custom-designed focused gene array was used to evaluate the expression of 87 different hepatic mRNAs related to lipid and carbohydrate metabolism, inflammation, nuclear hormone reception action, and other cell signaling pathways. The most regulated genes were listed in Table 2.2.2. Compared to WD-fed mice (1.0), the mRNAs most significantly downregulated in WD+G-fed mice were *Gsta1* (0.1), *Gstm2* (0.2), and *Acaa1a* (0.3). The mRNAs most downregulated in WD+D fed mice were *Gstm2* (0.3), and *Gsta1* (0.5). The mRNAs most upregulated in WD+G- and WD+D- fed mice were *Cd14* (10.4 and 11.5–fold, respectively), *Hsl* (8.9- and 19.3-fold, respectively), *Cyp1a2* (8.5- and 8.5-fold, respectively), *Cyp7a1* (7.0- and 7.0-fold, respectively), and *Cyp8b1* (6.6- and 9.7- fold, respectively) (all $p<0.05$). Expression of all 87 mRNAs are shown in Supplemental Table 2. A heat map showing relative expression of mRNAs is shown in Supplemental Figure S1. The hepatic gene expression in genistein- or daidzein-fed mice had a unique expression pattern.

Regulation of genistein or daidzein on signaling pathways

IPA analysis showed that pathways involving LPS/IL-1 mediated inhibition of RXR function, FXR/RXR activation, PXR/RXR activation, LXR/RXR activation, and xenobiotic metabolism signaling were affected by the consumption of genistein or daidzein diet (Table 2.2.3).

Since our prior published studies already found that PXR/RXR and PPARα/RXR were regulated by isoflavones (Li et al., **2007**; Li et al., **2009**; Mezei et al.; **2003**; Mezei et al., **2006**; Ricketts et al., **2005**), LXR/RXR activation, and FXR/RXR activation pathway became the focus for future analysis. Among 15 genes related in LXR/RXR activation pathway, 13 were upregulated by genistein consumption, while 15 were upregulated by daidzein consumption. Among 17 genes related in FXR/RXR activation pathway, 13 were upregulated by genistein consumption, while 16 were upregulated by daidzein consumption.

Effect of genistein or daidzein consumption on LXR/RXR activation

More detail about the effects of genistein or daidzein on the expression of genes involved in LXR/RXR activation is shown in Figure 2.2.4. Mice fed WD+G or WD+D had a similar profile of hepatic gene expression including *Abca* (3.0 and 4.0 folds, respectively), *Acaca* (6.0 and 6.9 folds, respectively), *Apoa1* (4.1 and 4.9 folds, respectively), *Cd14* (10.4 and 11.4 folds, respectively), and *Cyp7a1* (7.0 and 7.0 folds, respectively) significantly upregulated, compared to WD fed mice.

Effect of genistein or daidzein consumption on FXR/RXR activation

More detail about the effects of genistein or daidzein on the expression of genes involved in FXR/RXR activation was shown in Figure 2.2.5. Mice fed genistein and daidzein diet had a similar profile with hepatic gene expression including *Cyp7a1* (7.0 and 7.0 folds, respectively), *Cyp8b1* (6.6 and 9.7 folds, respectively), *Apoa1* (4.1 and 4.9 folds, respectively), *Baat* (1.8 and 2.2 folds, respectively), *Crebbp* (1.6 and 1.9 folds,

respectively), and *Cyp27a1* (4.0 and 4.4 folds, respectively) significantly upregulated, compared to WD fed mice.

2.2.4 DISCUSSION

Study II showed that dietary intake of genistein and daidzein decreased mouse food intake, ameliorated diet-induced symptoms of metabolic syndrome, including decreased body weight, and blood glucose concentrations. Gene array analysis demonstrated that intake of WD+G or WD+D profoundly impacted gene expression profiles compared to WD-fed mice. The effect of genistein or daidzein was correlated with LXR/RXR, FXR/RXR, PXR/RXR, and PPARα/RXRα pathways, among others (Table 2.2.3).

The supplementation of genistein or daidzein in the mice diet was 0.16% (w/w), which is consistent with levels of Novasoy used in mouse study I. The equivalent dose of isoflavone for human is 640mg/50kg body weight/day (Reagan-Shaw et al., **2008**), a level consistent with humans consuming isoflavone dietary supplements (Bloedon et al., **2002**).

In study II, body weight gain was significantly decreased with the intake of genistein or daidzein, compared to WD-fed mice. The finding is consistent with a previous study, in which genistein (1500 mg/kg) reduced food intake by 14% and body weight by 9% in ovariectomized female mice (Kim et al., **2006**). Our results may be partly explained by LXR pathway activation. It has been reported that LXR agonist T0901317-treated animals had significantly higher mRNA levels of genes involved in energy metabolism, such as uncoupling protein 1 (Ucp-1) (Gao et al., **2013**). Ucp-1 is an important protein in thermogenesis and plays an important role in energy metabolism and obesity (Porter, **2006**; Kajimura et al., **2009**; Cheng et al., **2010**). FXR regulation may be another reason

for the observed decrease in body weight gain. Activation of FXR suppressed weight gain in C57BL/6 mice fed a high fat diet (Ma et al., **2013**). FXR agonists have been suggested to be a novel strategy for the treatment of metabolic diseases (Lefebvre et al., **2009**). Activation of the FXR, by the synthetic agonist GW4064, has been reported to improve hyperglycemia and hyperlipidemia in diabetic mice (Zhang et al., **2006**).

LXR activation has been reported to prevent the development of obesity without affecting food intake (Gao et al., **2013**), however, in our study, the food intake in genistein and daidzein fed mice was significantly decreased. The reduction of food intake may be partly explained by the activation of transcription factor PPARα by soy isoflavones intake. A PPARα agonist has been shown to transiently decrease food intake (De Vos et al., **1996**; Guerre-Millo et al., **2001**). Reduced food intake by PPARα agonist treatment may be associated with cholecystokinin (CCK)-A receptor production (Park et al., **2012**). CCK is a hormone that is secreted from duodenal and jejunal mucosal cells in response to fat and protein (Bhavsar et al., **1998**). CCK has several physiological effects, including slowing gastric emptying and suppressing energy intake (Little et al., **2005**). At a more basic level, further animal testing needs to be performed to ensure that there are no palatability differences between WD and WD+G or WD+D, and additionally, that G or D is not causing illness or malaise in G- and D-fed mice. An activity monitor system might be an appropriate choice to test this possibility.

Genistein or daidzein intake can accelerate the reverse transport of cholesterol via increasing the gene expression of *Abca1* and *Abcg5* (Daniels et al., **2010**).

Other reverse cholesterol transport related genes including *Apoa1, Apob, Apoe, Ldlr* were upregulated in genistein or daidzein fed mice (Daniels et al., **2010**).

Elevated expression of genes responsible for lipogenesis including *Srebf1* and *Scd-1* were determined in genistein and daidzein fed mice. The result is in agreement with the known function of LXR agonist in inducing lipogenesis in the db/db diabetic mice and normal mice fed with high fat diet, therefore, elevated lipid accumulation was found in the liver tissue (Daniels et al., **2010;** Chisholm et al., **2003**). In our results, the genistein or daidzein intake had a marginal effect on hepatic lipid accumulation. It might be because that increased expression of genes for triglyceride hydrolysis such as *Hsl*, in genistein and daidzein fed mice, respectively, counteracted the effect of LXR activation.

Glucose homeostasis was regulated in genistein or daidzein fed mice, probably perhaps via the activation of LXR. LXR is also reported to play a key role in regulating glucose metabolism. In the liver, activation of LXR can significantly improve insulin sensitivity by inhibiting the expression of phosphoenolpyruvate carboxykinase (Pepck) and Glucose 6-phosphate (G6p), two rate-limiting enzymes in the pathway of gluconeogenesis, thereby suppressing glucose production and decreasing blood glucose (Cao et al., **2003**; Laffitte et al., **2003**). FXR activation is reported to prevent diet-induced insulin resistance in C57BL/6 mice (Ma et al., **2013**).

In summary, genistein and daidzein intake have effects on decreasing mouse food intake, body weight, and improving lipid and glucose metabolism. One of the mechanisms might be related to the activation of the transcription factors LXR, FXR, and downstream

gene expression. Soy isoflavones may prove to be a part of an effective therapy or help to point to other options to improve metabolic syndrome.

2.3 Mouse Study III: Intake of Soy Isoflavone Supplement NovaSoy in a Western Diet-Induced Model of Metabolic Syndrome

2.3.1 INTRODUCTION

Interestingly, study II demonstrated that genistein or daidzein consumption supplemented to a WD significantly reduced mice body weight gain compared to mice fed the WD control diet. Energy intakes were significantly decreased in WD+G- and WD+D-fed mice compared to the WD-fed group. Compared to the aglycone forms of isoflavones, NS contains mainly the glycone forms, genistin and daidzin (Murphy et al., **1999**; He et al., **2013**). Since there is a sugar molecule conjugated, there is the potential that bioavailabilities are different for aglycone vs. glycone forms of isoflavones (Day et al., **1998**). It might be possible that the effect on metabolic syndrome symptoms of genistin and daidzin is different from genistein and daidzein.

In mouse study II, we utilized WD (45% of energy from fat, 1% cholesterol, plus added sucrose) instead of the HF diet used in study I (60% of energy from fat). It is very likely that the influence of WD and HF diets on mouse metabolism is different.

Therefore, we designed a third mouse study, to determine the impact of NS on WD-induced metabolic syndrome symptoms. The purpose was to determine whether the consumption of isoflavone supplement NS will produce the dramatic effects on mouse body weight gain and food intake observed in study II with G and D intake. Further, study III will allow effects of HF and WD diets to be compared.

2.3.2 MATERIALS AND METHODS

C57BL/6J mice and diets

Male C57BL/6J mice at 6 week of age, were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and acclimated to semi-purified LF diet for two weeks, and then randomly divided into three groups (n=8). Two control groups were fed either a low-fat (LF) diet containing 10% fat and 70% carbohydrate by energy or a western diet group diet containing 45% Fat with 1% cholesterol (WD) (Table A2.2.2). Diets were produced by Research Diets, Inc. (New Brunswick, NJ, USA). A third group was provided the WD with 0.4% (w/w) NS (WD+NS). Mice were kept four per cage in a room maintained at a constant temperature (24^{o}C), with a 12-h light/dark cycle, and given free access to food and distilled water. During 10 week feeding, the body weight and food intake of the mice was obtained once a week, with spillage accounted for. The animal protocol was approved by the institutional animal care committee (ACUP 4455).

Fasting blood glucose and intraperitoneal glucose tolerance test

In week nine of the ten week feeding trial, tail vein blood glucose levels were measured over a two-hour period, following the protocol stated at mouse study II, section 2.2.2.

Histological analysis of liver tissue

Liver tissue was fixed in buffered formalin and paraffin embedded. Liver histological analysis following the method stated in 2.2.2: histological analysis of liver tissue.

Plasma biomarkers quantitation

Blood samples were collected via cardiac puncture, incubated on ice for 30-60 minutes and centrifuged at 1000 g for 15 min at 4^{o}C, after which serum was collected. Insulin, and MCP-1 levels were measured in 96-well plates using ELISA kits (Invitrogen Corporation, CA, USA), according to the manufacturer's instructions.

Custom-designed focused gene array

The same protocol was utilized as described previously. Relative expression of 87 genes in HF (from mouse study I) and WD (from mouse study III) mice were determined.

Statistical analysis

Data are presented as means ± SEM. ANOVA was used to compare sets of data. Tukey's procedure was used for the post hoc testing. Weekly body weight was analyzed using repeated-measures ANOVA followed by post-hoc testing within each week with one-way ANOVA. Significance was established at a level of $p<0.05$. All statistical analyses were carried out using GraphPad Prism 6. Qiagen RT^{2} profiler PCR array data analysis and Excel were used for the Heat map analysis. Ingenuity Pathway Analysis (IPA) was used for the pathway analysis.

2.3.3 RESULTS

NS consumption reduced the body weight gain of mice fed a WD

As shown in Figure 2.3.1B, during the 10 week feeding trial, no significant difference of energy intake was observed in WD- and WD+NS- fed mice. However, the consumption

of NS did show a reduction of body weight gain compared to WD fed mice. At week 10, the body weight/initial body weight*100 in WD diet fed mice was 163%±4%, as compared to 161%±5% in WD supplemented with Novasoy (WD+NS) diet.

Effect of NS intake on organ weight in WD fed mice

The liver, kidney, and inguinal adipose tissue was collected after mice were sacrificed at week 10. WD consumption increased liver and inguinal adipose tissue weights, with no effect on kidney weight, compared to LF fed mice. However, there is no different of tissue weights in WD-vs. WD+NS- fed mice.

Effect of NS intake on glucose homeostasis in WD fed mice

As shown in Figure 2.3.2A, all plasma glucose levels reached a maximum at 30 mins and then declined at 2 hours after glucose injection, steadily approaching the basal levels. At 120 mins, the glucose concentration in WD was not significantly different from the WD+NS mice (323±18 mg/dl in WD vs. 299±23 mg/dl in WD+NS). A global estimation of the glucose level is depicted by the incremental areas under the curve (AUC) from time 0 to time 120 mins of the glucose overload trial. Results showed that there was no significant AUC difference in these three treatment groups (Figure 2.3.2B). Similarly, no difference of baseline glucose was found between WD and WD+NS group. However, the consumption of NS had ameliorated serum insulin levels to a level not statistically different from LF-fed mice (Figure 2.3.2D).

Effects of NS on lipid accumulation in WD fed mice

Figure 2.3.3A shows the histology of mouse liver tissue. White droplets indicate the lipid accumulation. The quantified results showed that the consumption of genistein had no effect on the lipid accumulation in the liver tissue (Figure 2.3.3B).

NS consumption on inflammation in WD fed mice

The serum concentration of MCP-1 was detected in LF-, WD-, WD+NS- fed mice. However, no significant difference was observed in these three groups (p=0.18) (Figure 2.3.4).

Comparison of gene expression in HF- and WD- fed mice

From the custom-designed gene array analysis (Table 2.3.2), gene expression of HMG-CoA reductase (*Hmgcr*) was decreased to 0.2-fold in WD-fed, compared to HF-diet fed mice (set as 1.0-fold). Gene expression of ATP-binding cassette, sub-family B, member 1A (*Abcb1a)* and ATP-binding cassette sub-family G member 5 (*Abcg5)* was higher in WD-fed mice. The relative gene expression of *Abcb1a* and *Abcg5* was 2.6-, and 2.2-fold, respectively.

Gene expression of *Cyp7a1* and *Cyp8b1* was 0.3- and 0.05-fold in WD fed mice, respectively, relative to HF-diet fed mice. However, relative gene expression of *Gsta1* and *Gstm2* were higher in WD- fed mice than that in mice fed with HF.

Other most upregulated genes in WD- fed mice were *Acaca* (0.2-fold), *Cd14*(0.3-fold), *Cebpβ* (3.4-fold, *Cyp4a14* (0.3-folds), *Lipe* (0.1-fold), and *Ppargc1a* (0.5-fold), relative to HF-fed mice.

2.3.4 DISCUSSION

In the context of the experimental western-style diet (WD), genistein or daidzein consumption more potently reduced body weight gain and food intake than NS consumption. One of the reasons might because of that genistein and daidzein are more bioactive, compared to genistin and daidzin in NS. Additional potential explanations, including the possibility that G or D change the palatability of the diets cannot be excluded at this time, and must be tested in future studies. Further, the potential for G or D intake to cause a malaise in the animals is possible; this could be tested with real-time activity meter monitoring. According to our anecdotal observations, the WD+G- and WD+D-fed mice appeared to be as healthy as LF-fed control mice, with normal behavior and activity level.

The effect of HF diet and WD on mice metabolism were different. Expression of the enzyme *Hmgcr* is responsible for the rate limiting step of cholesterol biosynthesis. Gene expression of *Hmgcr* was decreased in WD fed mice than HF fed mice, which might be because that more cholesterol was added to WD than HF diet. The result is consistent to another finding, where the gene expression of *Hmgcr* was 11% in WD fed mice of the one in control diet fed mice (Renaud et al., **2014**).

Abcg5 manages the luminal efflux of cholesterol (Yu et al., **2002**). ABCB1a/b contribute to transintestinal cholesterol excretion (Le May et al., **2013**). One report

demonstrated that *Abcb1a/b* null mice have elevated fecal cholesterol concentrations (Thornton et al., **2008**). Since cholesterol was added in WD, the efflux of cholesterol turned to be higher in WD-fed mice, accordingly.

Antioxidative enzymes, including *Gsta1* and *Gstm2* were significantly increased in WD fed mice, indicating a relatively high oxidative stress in WD fed mice than HF diet fed mice. As more sucrose was added to the WD, this might explain a higher oxidative stress. Two weeks' consumption of a high-sucrose diet was reported to cause an increase in the generation of reactive oxygen species (ROS) in rats (Busserolles et al., **2002**).

A study by Aljada et al. showed that a high dietary fat intake is associated with an activation of the proinflammatory transcription factor, nuclear factor kappa-beta (NFκB) (Aljada et al., **2004**). Compared to the HF diet, where 60% energy was from fat, WD has a somewhat lower fat content. In HF diet fed mice, gene expression of *Cd14* was significantly increased, compared to WD diet fed mice (he fold change was 1.0 vs. 0.3 in WD and HF fed mice, respectively). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4, which is involved in inflammatory cytokine production, which is responsible for activating the innate immune system (Zanoni et al., **2011**; Vaure et al., **2014**). However, C/EBPβ, a transcription factor regulating inflammation, was higher in WD-fed mice. It was reported that C/EBPβ knockdown prevented palmitate-induced inflammation, while C/EBPβ overexpression induced p65-NF-kB DNA binding activity, and pro-inflammatory cytokine gene expression (Rahman et al., **2012**).

Both WD and HF diets significantly increased hepatic lipid accumulation. In WD-fed mice, gene expression of *Acaca*, which catalyzes fatty acid synthesis, was lower than that

in HF fed mice. However, triglyceride hydrolysis related gene expression *Hsl* was significantly higher in WD fed mice.

In the results reported by Savard et al. the high-cholesterol diet strongly up-regulated the pathways responsible for decreasing intracellular cholesterol levels through diminished uptake and increased secretion but having no effect on the competing pathway of converting cholesterol to bile acids (Savard et al., **2013**). Our mice study showed that cholesterol synthesis was downregulated, cholesterol efflux was upregulated in WD fed mice, compared to HF fed mice. Bile acid synthesis related gene expression of *Cyp7a1* and *Cyp8b1* was significantly decreased (0.3- and 0.05-fold vs. WD-fed mice, respectively). Neuschwander-Tetri reported that the induction of the LXR pathway by a high-cholesterol diet diminishes circulating bile acids through preferential disposal of cholesterol through other pathways (Neuschwander-Tetri et al., **2013**).

Expression of the fatty acid omega-hydroxylase gene *Cyp4a14*, is a commonly used indicator gene for PPARα activation (Anderson et al., **2002**). In according with *Cyp4a14* gene expression, the expression of *Ppargc1a* was lower in WD fed mice than that in HF diet fed mice.

In summary, intake of NS decreased body weight gain in WD fed mice. however, the reduction of body weight gain was less than that in mouse study II. Intake of NS decreased body weight gain in WD fed mice. The effect of HF and WD on mice metabolism were different, especially cholesterol metabolism.

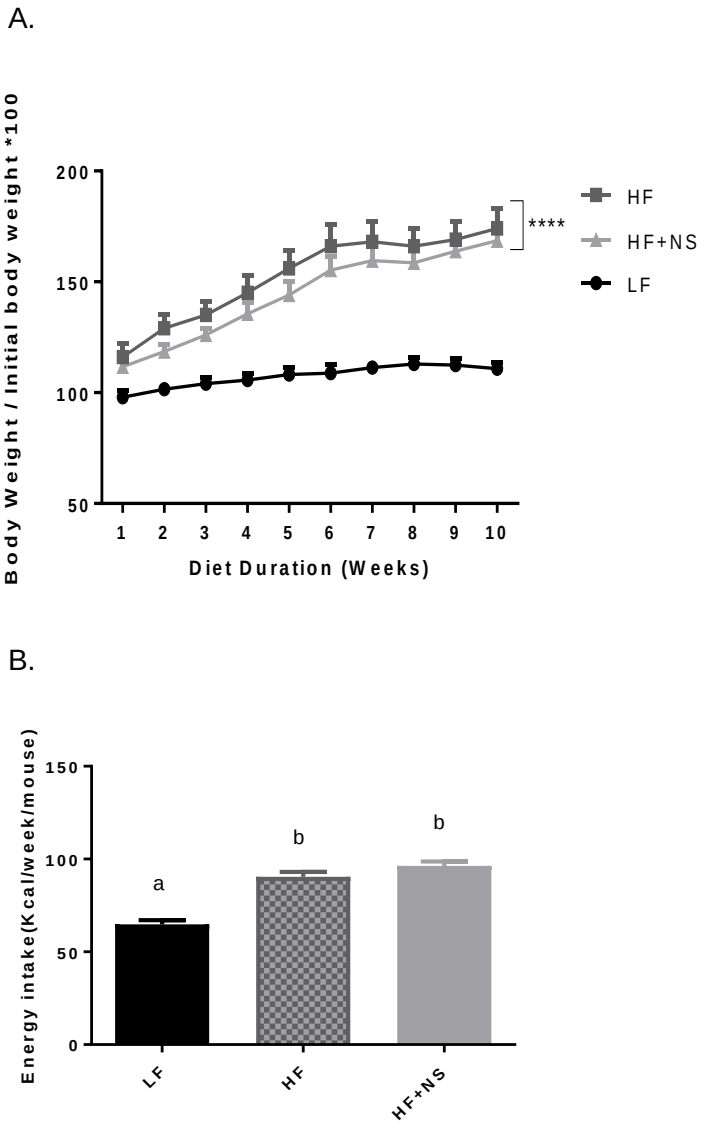


Figure 2.1.1 Body weight and energy intake in C57BL/6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) during 10 weeks feeding.

A. Body weight/initial body weight*100 of mice fed LF, HF, or HF+NS. B. Energy intake/week/mouse in LF-, HF-, or HF+NS- diet fed mice. Weekly body weight was analyzed using repeated-measures ANOVA followed by post-hoc testing within each week with one-way ANOVA. **** indicates $p<0.0001$. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other.

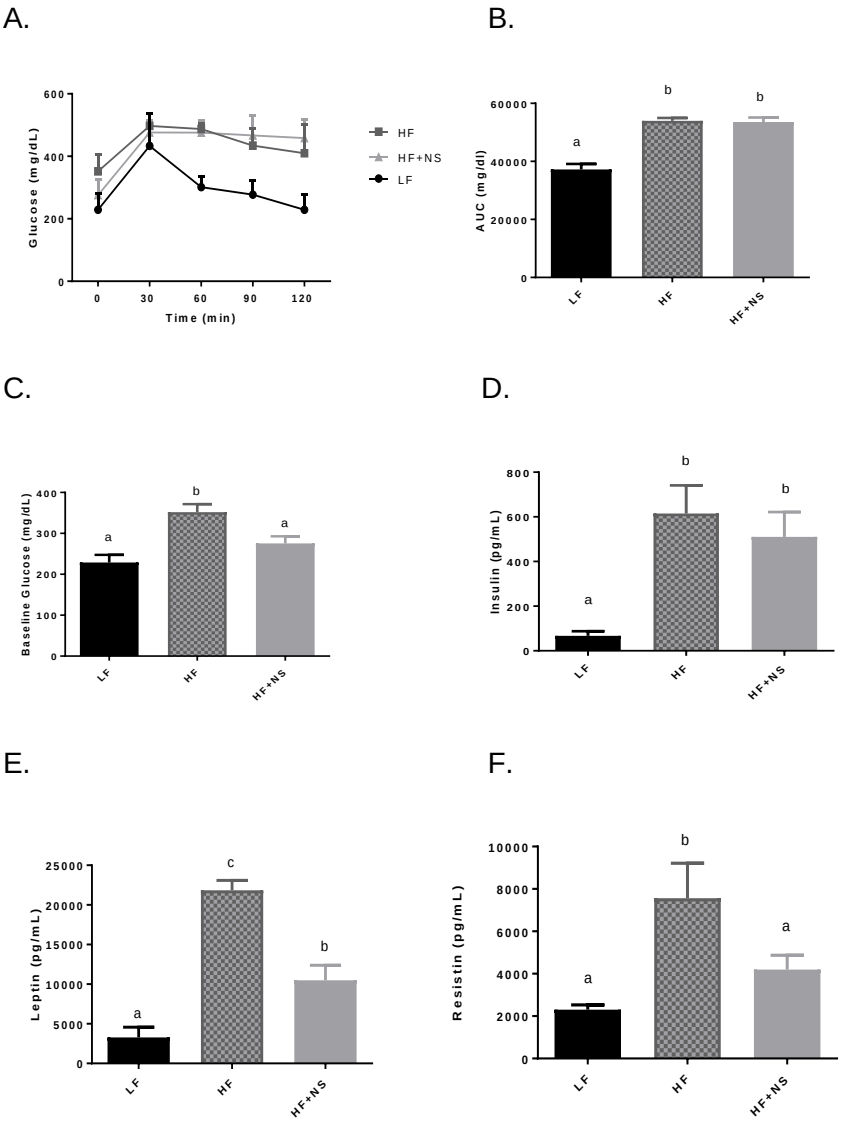
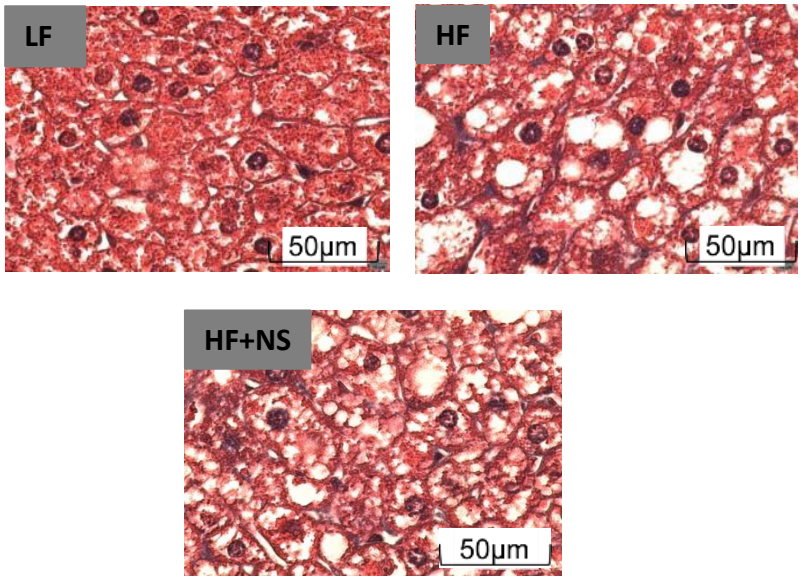


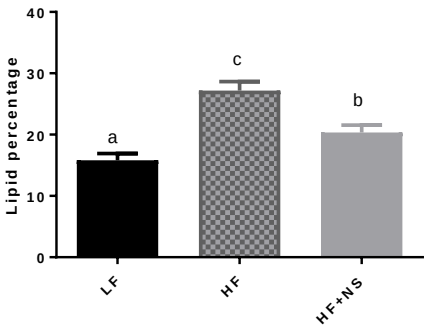
Figure 2.1.2 Glucose homeostasis-related parameters in the serum of C57BL/6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) at week 10.

A. Plasma glucose concentrations were determined 0, 30, 60, 90, and 120mins after glucose injection. Diet was withheld for six hours prior to testing and the test was performed mid-day in the middle of the light cycle. Mice were administrated 0.1mg/g b.w. glucose by intraperitoneal injection. Blood glucose levels were measured from the tail vein using a handheld glucometer. B. Area under the curve (AUC) from time 0 to time 120mins. C. Baseline glucose concentration. D. Plasma insulin concentration. E. Plasma leptin concentration. F. Plasma resistin concentration. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other, $p<0.05$.

A.



B.



C.

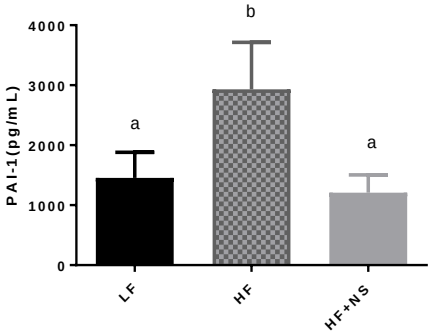


Figure 2.1.3 Hepatic lipid accumulation and serum plasminogen activator inhibitor (PAI) levels in C57BL/ 6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) at week 10.

A. Representative photomicrographs of liver sections stained with Masson's trichrome for the indicated treatment groups, LF, HF, and HF+NS. B. Quantified results of lipid accumulation in LF, HF, and HF+NS groups. C. Serum PAI-1 levels in LF, HF, and HF+NS groups. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other, $p<0.05$.

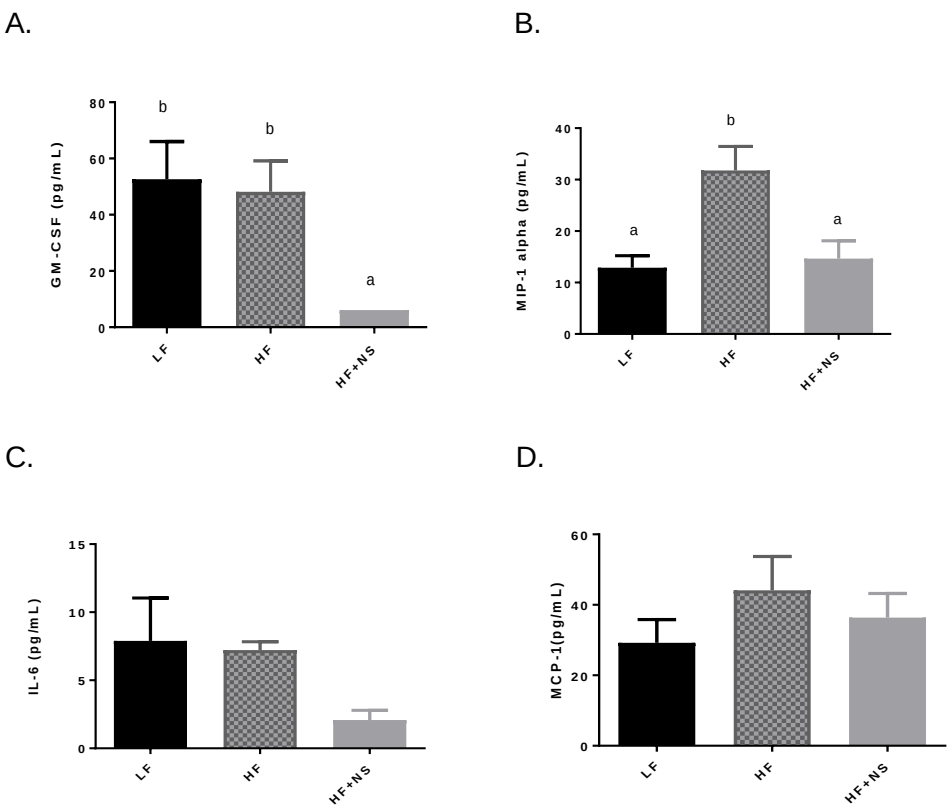


Figure 2.1.4 Serum pro-inflammatory cytokine levels in C57BL/ 6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) at week 10.

A. Granulocyte macrophage colony-stimulating factor (GM-CSF) level in serum. B. Macrophage inflammatory protein 1 alpha (MIP-1α) level in serum. C. Interleukin 6 (IL-6) level in serum. D. Monocyte chemotactic protein 1 (MCP-1) level in serum. Bars are mean ± SEM. The bars with the same letter are not significantly different from each other, $p<0.05$, n=8.

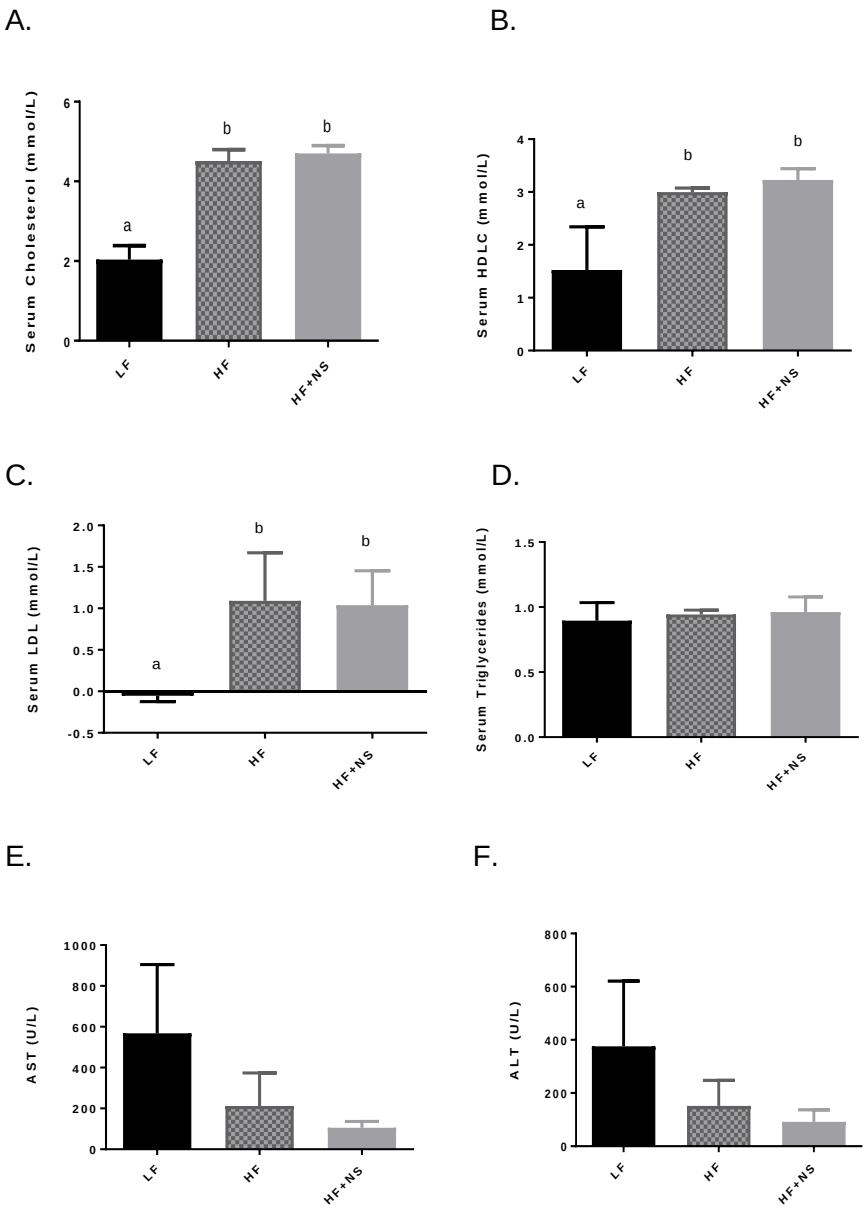


Figure 2.1.5 Blood lipid concentrations and liver enzyme levels of C57BL/ 6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) at week 10.

A. Serum cholesterol level. B. Serum HDLC level. C. Serum LDL level. D. Serum triglyceride level. E. Serum aspartate aminotransferase (AST) level. F. Serum alanine aminotransferase (ALT) level. Bars are mean ± SEM. The bars with the same letter are not significantly different from each other, $p<0.05$, n=8.

Table 2.1.1 Organ weight and organ weight/body weight ratios of C57BL/ 6J male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus isoflavone supplement NovaSoy (HF+NS) at week 10[1]

Group	Liver weight (g)	Liver weight/ Body weight X1000	Kidney weight (g)	Kidney weight/ Body weight X1000	Adipose tissue weight (g)	Adipose tissue/ Body weight X1000
LF	0.92±0.09[a]	34±3[a]	0.27±0.006[a]	9.0±0.2[b]	0.31±0.02[a]	12±0.9[a]
HF	1.48±0.1[b]	36±2[a]	0.33±0.007[b]	8.7±0.3[b]	1.24±0.07[b]	31±2[b]
HF+NS	1.39±0.08[b]	35±1[a]	0.29±0.008[a]	7.0±0.3[a]	1.25±0.08[b]	31±1[b]

[1]After sacrificing the mice at week 10, the tissue samples including liver, kidney, and inguinal adipose tissue were obtained. Within each row, each group with the same superscript letter are not significantly different from each other, $p<0.05$.

Table 2.1.2 Most regulated hepatic mRNAs expression in C57BL/6J male mice consuming NovaSoy (HF+NS) or low fat (LF) diet relative to high fat (HF)-fed mice[1]

	LF		HF+NS	
Symbol	Fold change	*p* value	Fold change	*p* value
Alox8	0.3	0.001	5.7	0.2
Cyp2b10	0.2	0.01	3.2	0.2
Cpt1α	2.2	0.02	3.1	0.005
Cebpa	1.3	0.2	2.7	0.02
Hsl	0.3	0.07	2.6	0.04
Crebbp	1.2	0.4	2.4	0.02
Tgfb1	1.3	0.08	2.1	0.01
Cyp8b1	0.2	0.06	1.8	0.2
Cebpb	3.0	0.01	1.7	0.3
Cyp7a1	0.1	0.001	1.3	0.4
Nqo1	0.6	0.09	1.1	1.0

[1]A custom-designed gene array was used to determine hepatic gene expression of 87 genes related to lipid metabolism, glucose metabolism, inflammation, and oxidative stress. The table shows several most regulate genes. Fold change and *p* value are shown (n=6).

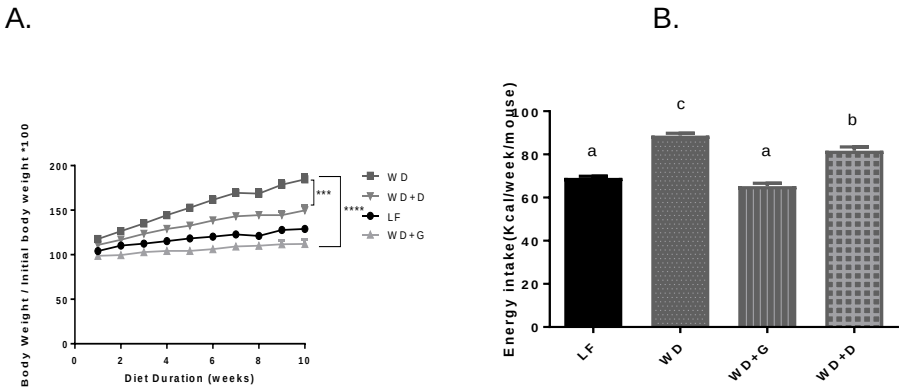


Figure 2.2.1 Body weight and energy intake in C57BL/6J male mice fed a low fat diet (LF), a western diet (WD), or WD plus genistein (WD+G), or daidzein (WD+D) during a 10 weeks feeding trial.

A. Body weight/initial body weight*100 of mice fed LF, WD, WD+G, or WD+D diets. B. Energy intake of mice in LF, or WD, or WD+G, or WD+D groups. Weekly body weight was analyzed using repeated-measures ANOVA followed by post-hoc testing within each week with one-way ANOVA. **** indicates $p<0.0001$. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other.

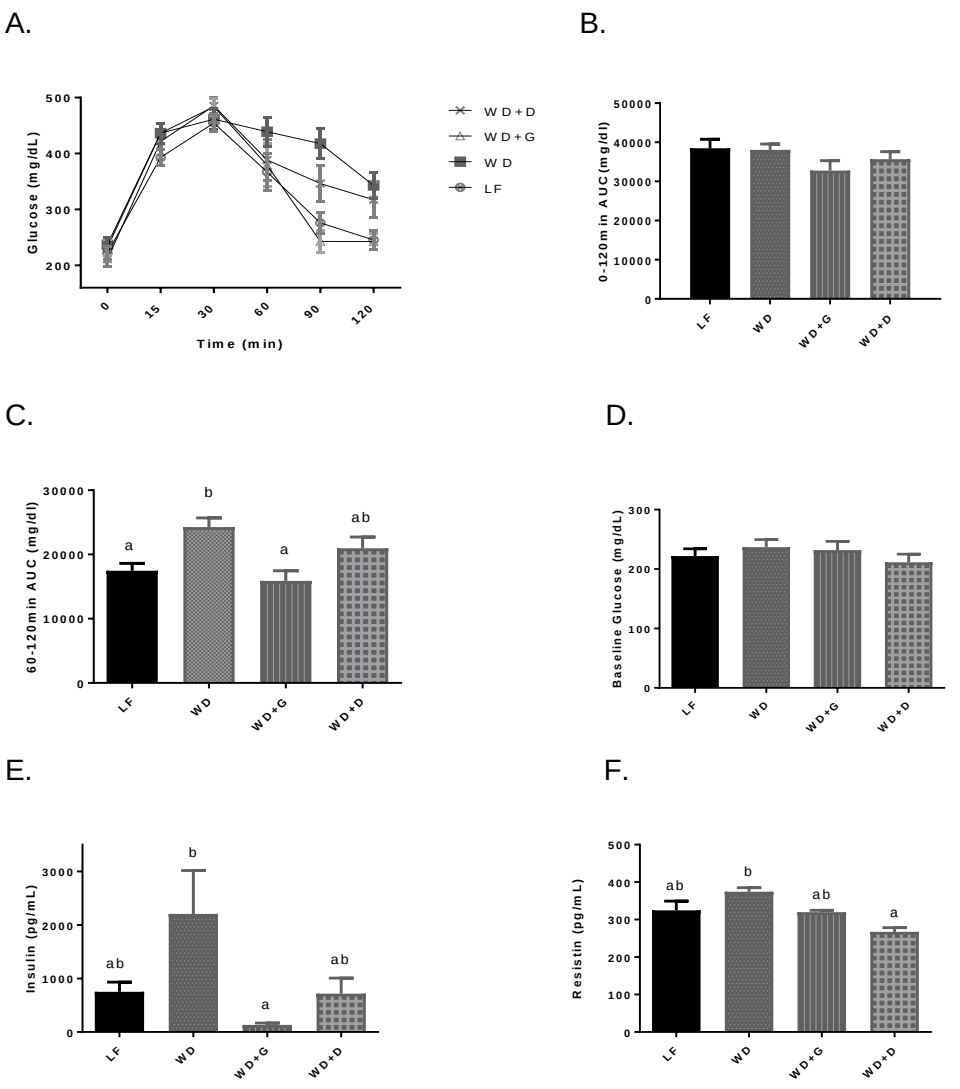
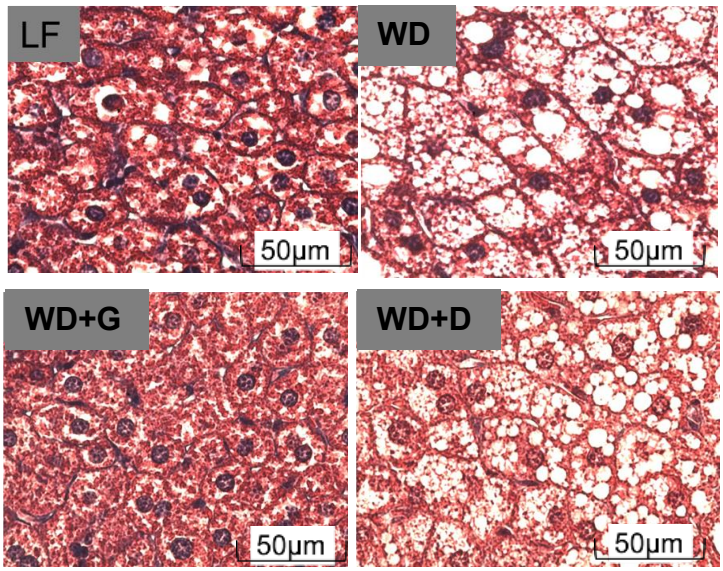


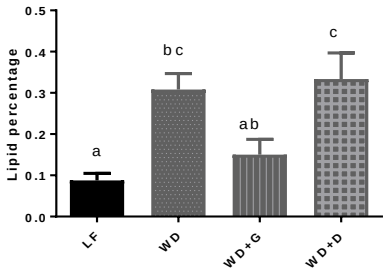
Figure 2.2.2 Glucose homeostasis related parameters in the serum of C57BL/6J male mice fed a low fat (LF), western diet (WD) alone, or WD plus genistein (WD+G) or daidzein (WD+D) at week 10.

A. Plasma glucose concentrations were determined 0, 15, 30, 60, 90, and 120 mins after i.p. glucose injection. Diet was withheld for six hours prior to testing and the test was performed mid-day in the middle of the light cycle. Mice were administrated 0.1 mg/g b.w. glucose by intraperitoneal injection. Blood glucose levels were measured from the tail vein using a handheld glucometer. B. Area under the curve (AUC) from time 0 to time 120mins. C. AUC from time 60mins to time 120mins. D. Baseline glucose concentration. E. Plasma insulin concentration. F. Plasma resistin concentration. Bars are mean ± SEM (n=8). The bars with the same superscript letter are not significantly different from each other, $p<0.05$.

A.



B.



C.

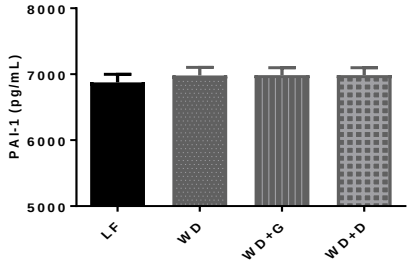


Figure 2.2.3 Hepatic lipid accumulation and serum plasminogen activator inhibitor levels in C57BL/ 6J male mice fed a low fat (LF) or western diet (WD) alone, or WD plus genistein (WD+G) or daidzein (WD+D) at week 10.

A. Representative photomicrographs of liver sections stained with Masson's trichrome for the indicated diet groups, LF, WD, WD+G, and WD+D. B. Quantified results of lipid accumulation in LF, WD, WD+G, and WD+D groups. C. Serum PAI-1 levels in LF, WD, WD+G, and WD+D groups. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other, $p<0.05$.

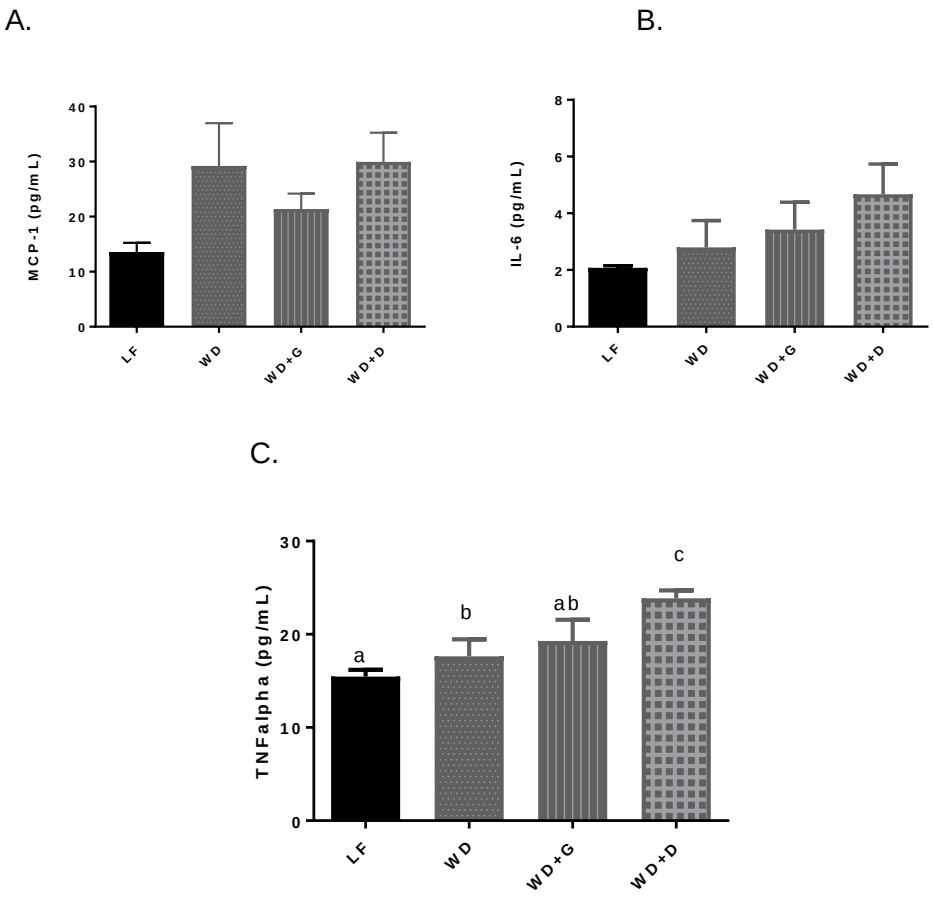


Figure 2.2.4 Serum concentrations of inflammatory cytokines in C57BL/ 6J male mice fed a low fat (LF), a western diet (WD) alone, or WD plus genistein (WD+G) or daidzein (WD+D) at week 10.

A. Monocyte chemotactic protein 1 (MCP-1) level in serum. B. Interleukin 6 (IL-6) level in serum. C. Tumor Necrosis Factor-α (TNF-α) level in serum. Bars are mean ± SEM. The bars with the same letter are not significantly different from each other, $p<0.05$, n=8.

Table 2.2.1 Organ weight and organ weight/body weight ratios of C57BL/ 6J male mice fed a low fat (LF), a western diet (WD) alone, or WD plus genistein (WD+G) or daidzein (WD+D) at week 10[1]

Group	Liver weight (g)	Liver weight/ Body weight X1000	Kidney weight (g)	Kidney weight/ Body weight X1000	Adipose tissue weight (g)	Adipose tissue/ Body weight X1000
LF	1.15±0.09^{a}	41±1^{a}	0.35±0.02^{b}	14±0.2^{a}	1.30±0.2^{b}	43±5^{b}
WD	2.25±0.19^{b}	56±3^{b}	0.39±0.008^{b}	9.7±0.2^{b}	3.31±0.2^{d}	82±3^{c}
WD+G	1.16±0.07^{a}	45±1^{ab}	0.29±0.02^{a}	16±0.3^{a}	0.71±0.2^{a}	21±6^{a}
WD+D	2.09±0.27^{b}	60±4^{b}	0.35±0.01^{b}	13±0.4^{a}	2.02±0.3^{c}	52±6^{b}

[1]After sacrificing the mice at week 10, the tissue samples, including liver, kidney, and inguinal adipose tissue were obtained and weighed. Within each row, each group with the same superscript letter are not significantly different from each other, $p<0.05$.

Table 2.2.2 Most regulated hepatic mRNAs expression in mice consuming genistein (WD+G), daidzein (WD+D) and low fat (LF) diet relative to western diet (WD)-fed mice[1]

	LF/WD		WD+G/WD		WD+D/WD	
Symbol	**Fold change**	***p* value**	**Fold change**	***p* value**	**Fold change**	***p* value**
Gsta1	0.01	0.002	0.1	0.02	0.5	0.2
Gstm2	0.3	0.0007	0.2	0.002	0.3	0.01
Acaa1a	0.6	0.02	0.3	0.01	0.6	0.2
Pdk4	1.9	0.3	0.4	0.1	0.7	0.3
Cebpb	1.1	0.5	0.3	0.01	0.3	0.01
Acaca	1.8	0.09	6.0	0.0002	6.9	0.0002
Apoa1	1.3	0.4	4.1	0.001	4.9	0.0002
Cyp7a1	0.5	0.2	7	0.00002	7	0.000004
Cyp8b1	2.9	0.07	6.6	0.003	9.7	0.0009
HSL	2.9	0.07	8.9	0.000006	19.2	0.000001
Cpt1α	0.8	0.5	0.8	0.2	0.9	0.4
Scd1	0.5	0.02	2.1	0.003	2.2	0.003
Cyp27a1	1.7	0.2	4.2	0.0007	4.4	0.0005
Creb3l2	1.4	0.2	3.8	0.007	4.5	0.0001
Cyp2b10	0.1	0.007	0.5	0.1	0.7	0.5
Elovl5	1.0	1.0	0.4	0.09	0.5	0.2
Srebf1	0.7	0.1	1.2	0.8	2.0	0.04
Slco1a1	2.3	0.003	3.4	0.000003	2.3	0.03
Crtc2	2.2	0.09	3.6	0.001	4.8	0.00004
Abcg2	1.1	0.9	5.7	0.007	8.5	0.0004

[1] A custom-designed gene array was used to determine hepatic gene expression of 87 genes related to lipid metabolism, glucose metabolism, inflammation, and oxidative stress. The table shows the most up- and down-regulated genes. Fold change and *p* value are shown. $p<0.05$, n=6.

Table 2.2.3 Signaling pathways significantly regulated by genistein or daidzein consumption using IPA software[1]

Pathways	Up-regulated Gene Number	Down-regulated Gene Number	% Regulated Gene	*p* value
Genistein				
FXR/RXR Activation	13	4	17/126	1.75E-21
PXR/RXR Activation	6	8	14/65	4.94E-21
LXR/RXR Activation	13	2	15/121	1.78E-18
PPARα/RXRα Activation	5	7	12/178	9.03E-12
AMPK Signaling	9	2	11/189	3.4E-10
NRF-2 Mediated Oxidative Stress Response	4	5	9/193	1.02E-07
Acyl Hydrocarbon Receptor Signaling	2	5	7/140	1.81E-06
Daidzein				
FXR/RXR Activation	16	1	17/126	1.75E-21
PXR/RXR Activation	9	5	14/65	6.94E-21
LXR/RXR Activation	15	0	15/121	1.78E-18
PPARα/RXRα Activation	8	4	12/178	9.03E-12
AMPK Signaling	10	1	11/189	3.4E-10
NRF-2 Mediated Oxidative Stress Response	7	2	9/193	1.02E-07
Acyl Hydrocarbon Receptor Signaling	5	2	7/140	1.81E-06

[1]Upregulated or downregulated gene number was set as compared to WD group. "% Regulated gene" indicates the percentage of the detected genes/total number of genes in the signaling pathway, at $p < 0.05$.

Table 2.2.4 Relative expression of LXR regulated mRNAs in mice fed genistein or daidzein[1]

	LF/WD	G/WD	D/WD
Abca1	1.0	3.0	4.0
Abcg5	0.4	1.8	1.8
Acaca	1.8	6.0	6.9
Apoa1	1.3	4.1	4.9
Apob	1.0	1.5	2.0
Apoe	0.8	1.3	1.6
Cd14	0.8	10.4	11.4
Cyp7a1	0.5	7.0	7.0
Fasn	1.6	0.8	1.2
Hmgcr	2.7	1.5	1.3
Ldlr	1.5	0.9	1.0
Lpl	0.6	1.1	1.9
Msr1	1.0	2.3	3.1
Scd1	0.5	2.1	2.2
Srebf1	0.7	1.2	2.0

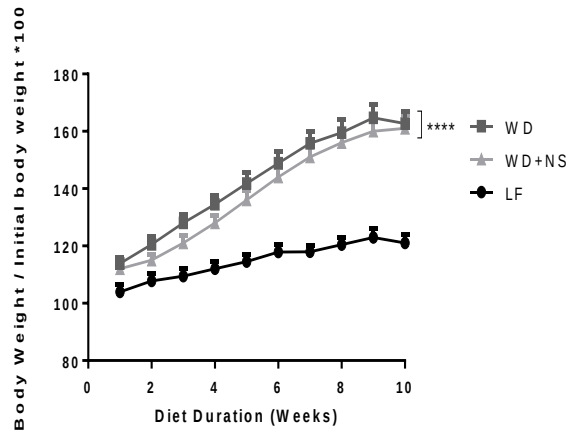
[1]The fold change is displayed for genes that were significantly changed compared to the WD control group. Shades of red and green indicate the degree of upregulation or downregulation, compared to WD- fed mice, respectively.

Table 2.2.5 Relative expression of FXR regulated mRNA in mice fed genistein or daidzein[1]

	LF/WD	G/WD	D/WD
Abcb4	0.8	0.9	1.4
Abcb11	1	1.5	1.6
Abcc2	0.8	0.9	1.2
Abcg5	0.4	1.8	1.8
Apoa1	1.3	4.1	4.9
Apob	1	1.5	2
Apoe	0.8	1.3	1.6
Baat	0.7	1.8	2.2
Crebbp	1.3	1.6	1.9
Cyp27a1	1.7	4	4.4
Cyp7a1	0.5	7	7
Cyp8b1	2.9	6.6	9.7
Fasn	1.6	0.8	1.2
Lpl	0.6	1.1	1.9
Pklr	1.4	0.8	0.6
Ppargc1a	1.4	2.1	2.1
Srebf1	0.7	1.2	2

[1]The fold change is displayed for genes that were significantly changed compared to the WD control group. Shades of red and green indicate the degree of upregulation or downregulation, compared to WD- fed mice, respectively.

A.



B.

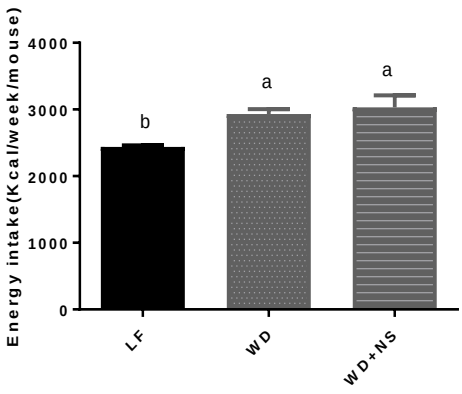


Figure 2.3.1 Body weight and energy intake in C57BL/6J male mice fed a low fat diet (LF), a western diet (WD) diet, or WD plus isoflavone supplement NovaSoy (WD+NS) for 10 weeks.

A. Body weight/initial body weight*100 of mice fed a LF, WD, or WD+NS. B. Energy intake of mice in LF, WD, or WD + NS groups. Weekly body weight was analyzed using repeated-measures ANOVA followed by post-hoc testing within each week with one-way ANOVA. **** indicates $p<0.0001$. Bars are mean ± SEM (n=8). The bars with the same superscript letter are not significantly different from each other.

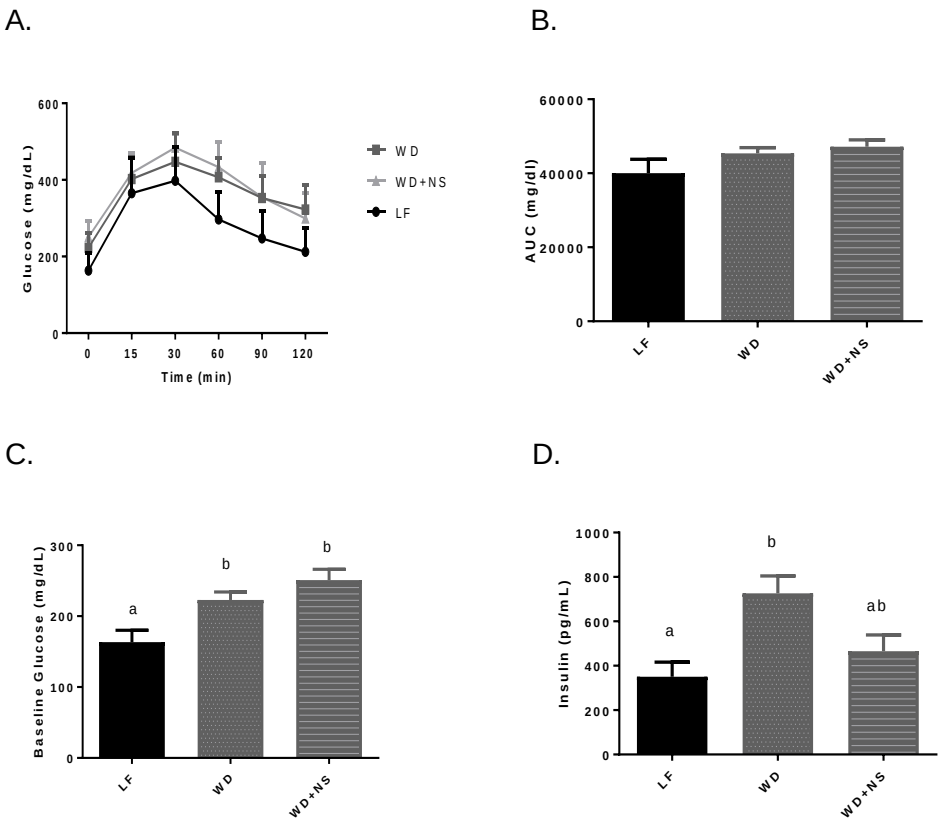
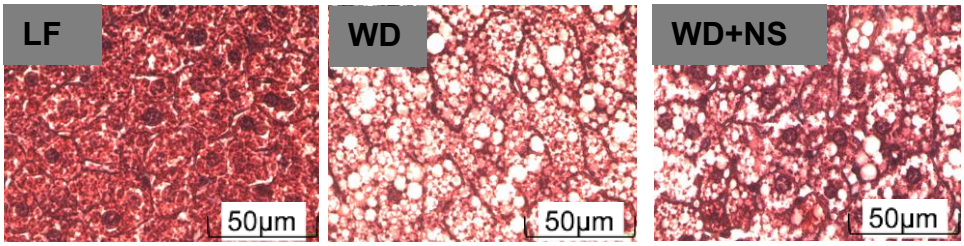


Figure 2.3.2 Glucose homeostasis-related parameters in the serum of C57BL/6J male mice fed a low fat diet (LF), a western diet (WD), or WD plus isoflavone supplement NovaSoy (WD+NS) at week 10.

A. Plasma glucose concentrations were determined 0, 15, 30, 60, 90, and 120 mins after glucose injection. Diet was withheld for six hours prior to testing and the test was performed mid-day in the middle of the light cycle. Mice were administrated 0.1 mg/g b.w. glucose by intraperitoneal injection. Blood glucose levels were measured from the tail vein using a handheld glucometer. B. Area under the curves (AUC) from time 0 to time 120 mins were detected. C. Baseline glucose concentration. D. Plasma insulin concentration. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other, $p<0.05$.

A.



B.

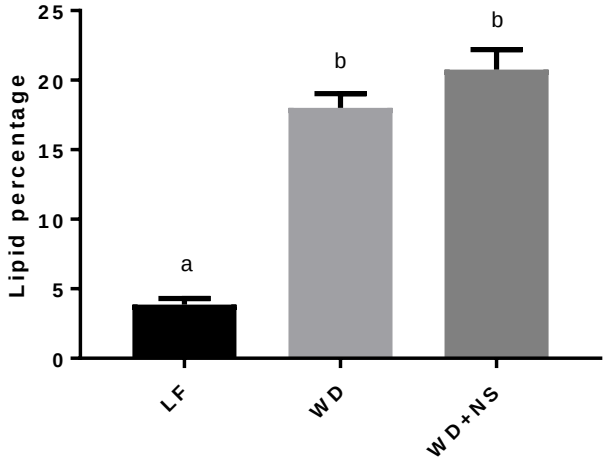


Figure 2.3.3 Hepatic lipid accumulation in C57BL/ 6J male mice fed a low fat (LF), a western diet (WD) alone, WD plus isoflavone supplement NovaSoy (WD+NS) at week 10.

A. Representative photomicrographs of liver sections stained with Masson's trichrome for the indicated diet groups, LF, WD, and WD+NS. B. Quantified results of lipid accumulation in LF, WD, and WD+NS groups. Bars are mean ± SEM (n=8). The bars with the same letter are not significantly different from each other, $p<0.05$.

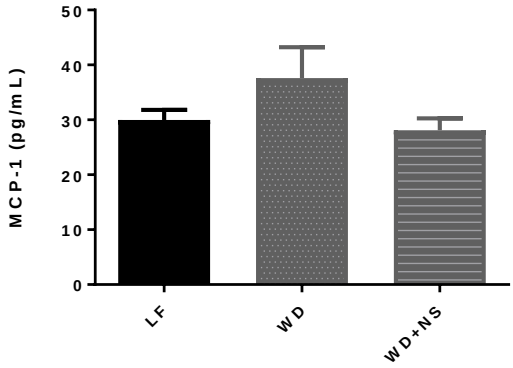


Figure 2.3.4 Serum concentrations of monocyte chemoattractant protein-1 (MCP-1) in C57BL/6J mice fed a low fat (LF), a western diet (WD) diet alone, or WD plus isoflavone supplement NovaSoy (WD+NS) at week 10.

The effect of NS on serum MCP-1 level was detected by ELISA. Bars are mean ± SEM. The bars with the same letter are not significantly different from each other, $p<0.05$, n=8.

Table 2.3.1 Organ weight and organ weight/body weight ratios of C57BL/6J mice fed a low fat diet (LF), a western diet (WD), or WD plus isoflavone supplement NovaSoy (WD+NS) at week 10[1].

Group	Liver weight (g)	Liver weight/ Body weight X1000	Kidney weight (g)	Kidney weight/ Body weight X1000	Adipose tissue weight (g)	Adipose tissue/ Body weight X1000
LF	1.00±0.05[a]	30±2[a]	0.34±0.01[a]	12±0.5[b]	0.99±0.1[a]	33±4[a]
WD	2.19±0.2[b]	50±4[b]	0.35±0.009[a]	8.4±0.4[a]	3.63±0.3[b]	85±5[b]
WD+NS	1.89±0.1[b]	50±3[b]	0.34±0.007[a]	8.7±0.3[a]	3.14±0.2[b]	79±4[b]

[1]Within each line, values with the same superscript letter are not significantly different from each other, $p<0.05$.

Table 2.3.2 Most regulated hepatic mRNAs expression in mice consuming western diet (WD) relative to high fat diet (HF)-fed mice[1]

	WD/HF	
Symbol	Fold Change	*p* value
Abcb1a	2.6	0.08
Abcg5	2.2	0.03
Acaca	0.2	0.001
Cd14	0.3	0.01
Cebpb	3.4	0.008
Cyp4a14	0.3	0.08
Cyp7a1	0.3	0.004
Cyp8b1	0.05	0.003
Gsta1	9.0	0.05
Gstm2	2.3	0.02
Hmgcr	0.2	0.00000001
Lipe	0.1	0.002
Ppargc1a	0.5	0.02

[1] A custom-designed gene array was used to determine hepatic gene expression of 87 genes related to lipid metabolism, glucose metabolism, inflammation, and oxidative stress. The table shows the most up- and down-regulated genes. Fold change and *p* value are shown. $p<0.05$, n=6.

CHAPTER III *IN-VITRO* STUDIES, THE EFFECT OF GENISTEIN AND DAIDZEIN ON LXR ACTIVATION

3.1 INTRODUCTION

As shown in Chapter II, Isoflavone intake modulated lipid and glucose metabolism in high fat- or western diet-fed C57BL/6J mice. IPA pathway analysis indicated that transcription factor-regulated pathways, including LXR, were regulated in genistein- or daidzein-fed mice.

Prior findings prompted us to test whether isoflavones can modulate LXR signaling pathways in an *in-vitro* system. We therefore utilized HepG2 cells to test whether LXR-regulated gene expression was altered by exposure to genistein; using mouse embryonic fibroblast cells devoid of, or expressing LXRα to determine whether the effect of genistein or daidzein on mouse metabolism is LXR dependent; Further, we transfected HepG2 cells with construct expressing a LXR-responsive luciferase reporter plasmid to test whether isoflavones can transactivate LXR.

The goal of the study was to determine whether the impact of genistein or daidzein on mice metabolic syndrome is potentially via the activation of transcription factor LXR.

3.2 MATERIAL AND METHODS

HepG2 Cell Culture

Human hepatoma HepG2 cells (#HB-8065, American Type Culture Collection, Manassas, VA, kindly provided by Dr. Emily Ho) were cultured in DMEM supplemented with 10% FBS plus 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL), at 37 ^{o}C in a humidified 5% CO_2 atmosphere.

Effect of genistein on HepG2 cell gene expression

HepG2 cells were incubated with genistein at a concentration of 30 μM for 24h. RNA was isolated from using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). RNA was reverse-transcribed with PrimerScript™ RT-PCR kit according to the manufacturer's protocol. The relative mRNA levels for specific genes were determined by real-time PCR using SYBR Premix Ex TaqTM. PCR products were quantified using the ABI 7900HT real-time PCR system. Gene expression levels were normalized to the housekeeping gene *RPL30*. Real-time PCR was performed as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Primers of *RPL30* (PPH19079A), and *CYP8B1* (PPH01265F) were obtained from RT^2 qPCR primer assay (Qiagen, Redwood City, CA).

Other primer sequences were shown as follows:

CPT1α: F-5'-GGAGAGGAGACAGACACCATCCA-3'; R-5'-CAAAATAGGCCTGACGACACCTG

NQO-1: F-5'-TGGAGTCCCTGCCATTCT-3'; R-5'-AGCACTGCCTTCTTACTC-3'

HMOX-1: F-5'-AAGACTGCGTTCCTGCTCAA-3'; R-5'-AAAGTTCATGGCCCTGGGAG-3'

GSTT2: F-5'-TGACACTGGCTGATCTCATGGCC-3'; R-5'-GCCTCCTGGCATAGCTCAGCAC-3'

ACACA: F-5'-GCCATGTTATTGCTGCTCGG-3'; R-5'-ACCCCGAATAGACAGCTCCT-3'

ABCA1: F-5'-AAGGAACTAGTCCCGGCAAA-3'; R-5'CGTGGCTGGTCATTAACGTTT-3'

APOA: F-5'-TTGCCCACTCTATTTGCCCA-3'; R-5'-GTGGGGGACCTCCTTCTCG-3'

Mouse embryotic fibroblast cells culture

Mouse embryotic fibroblast cells (MEF, LXR-) and LXRα knock-in mouse embryotic fibroblast cells (MEF+, LXRα+) were generously provided by Dr. Peter Tontonoz (Pathology and Laboratory Medicine, University of California Los Angeles, CA). Cells were cultured in DMEM supplemented with 10% FBS plus 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 ^{o}C in a humidified 5% CO_2 atmosphere.

LXR related gene expression in mouse embryonic fibroblast cells

On day 1, MEF and MEF+ cells were cultured with DMEM in 10% FBS medium in 6-well plates. On day 2, the medium was changed to 1% FBS. On day 3, the cells were incubated with genistein (40μM) or daidzein (40μM) or LXR agonist T0901317 (2μM) dissolved in 1% FBS for 24h. Genistein, daidzein, and T0901317 treatments were prepared as 1000X stock dissolved in DMSO and the final concentration of DMSO in cell treatment medium was 0.1% (v:v). The control group was exposed to 0.1% (v:v) DMSO. On day 4, RNA was isolated and reverse-transcribed. The mRNA levels for specific genes were determined by real-time PCR, following the methods described above. The primer sequences were as follows:

Rpl30: (F) 5'-GTGGGAGCTCCTTCCTTTCTC -3', (R) 5'-GACTTTTTCGTCTTCTTTGCGG-3'

Srebp1c: (F) 5'-GGAGCCATGGATTGCACATT-3', (R) 5'-GCTTCCAGAGAGGAGGCCAG-3'.

Abca1: (F) 5'-TGCCACTTTCCGAATAAGC-3', (R) 5'-GGAGTTGGATAACGGAAGCA-3'

Scd1: (F) 5'-CACCTGCCTCTTCGGGATTT-3', (R) 5'- CTTTGACAGCCGGGTGTTTG-3'.

Plasmids

LXRE-Luc reporter plasmids were generously provided by Dr. Peter Tontonoz (Pathology and Laboratory Medicine, University of California Los Angeles, CA). Control plasmid PRL-TK-Luc was kindly provided by Dr. Donald Jump (College of Public Health and Human Sciences, Oregon State University, OR). The plasmid maps are shown in Figure A3.

High-efficiency transformation kit and NEB® 5-alpha Competent E. coli (New England Biolabs, Ipswich, MA, USA) were used to transform and clone LXRE-Luc and pRL-TK-Luc vector, according to the supplier's instructions.

Transformed E.coli was cultured overnight at 37^{o}C. Plasmid Maxiprep kit (Qiagen, Valencia, CA, USA) was used to extract plasmid DNA from E.coli. Cloning accuracy was validated by sanger sequencing at the Center for Genome Research and Biocomputing, Oregon State University.

Transient transfection and luciferase assay

Transient transfections were performed in 24-well cell culture plates. On day 1, cells were plated in DMEM plus 10% FBS. On day 2, cells were transfected using PromoFectin transfection reagent (PromoKine, Heidelberg, Germany). PRL-TK-Luc was used as the control vector. When transfecting two plasmids (LXRE-Luc and PRL-TK-Luc), 0.5 mg of each plasmid was added to each well. For transfecting one plasmids (LXRE-Luc), 1.0 mg of plasmid was added to each well. Twenty-four hours after transfection, cell medium was replaced by DMEM with 1% FBS, and cells were exposed to the experimental conditions:

vehicle (0.1% DMSO), T0901317 (2µM), genistein (20, 50 or 100µM), daidzein (20, 50, or 100µM), T0901317 (2µM) + genistein (100µM), or T0901317 (2µM) + daidzein (100µM). All of the isoflavone treatments were prepared in 1000X stock dissolved in DMSO and the final concentration of DMSO in cell treatment medium was 0.1% (v:v).

After the appropriate treatment period, HepG2 cells were lysed with 1X reporter lysis buffer (Promega, Madison, WI, USA). Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was used to determine the activities of firefly (Photinus pyralis) and renilla (Renilla reniformis) luciferases. Luciferase activity was determined with MicroLumatPlus LB 96V Microplate luminometer and the ratio of firefly luciferase to renilla luciferase was calculated. All transfection studies were repeated at least twice and each individual test involved independent triplicate measurements.

Statistical analysis

Data are presented as means ± SEM. ANOVA was used to compare sets of data. Tukey's procedure was used for the post hoc testing. Significance was established at a level of $p<0.05$. All statistical analyses were carried out using GraphPad Prism 6.

3.3 RESULTS

3.3.1 Effect of genistein on gene expression in HepG2 cells

As shown in Figure 3.1, gene expression of *CPT1α*, *NQO1*, *GSTT2*, *ACACA*, and *ABCA1* were significantly upregulated with exposure to genistein. The fold change (relative to *Rpl30* gene expression) of *CPT1α* was 1.2±0.1 vs. 1.8±0.2 in control group, and genistein treatment group, respectively. The relative fold change of *NQO1*, *GSTT2*, and

ACACA gene expression was 1.0±0.04 vs. 1.3±0.06, 1.4±0.1 vs. 2.1±0.2, and 1.1±0.05 vs. 1.3±0.09, in control and genistein group, respectively. In control and genistein treated cells, the fold change of *ABCA1* was 1.7±0.1 vs. 2.5±0.2, respectively. However, gene expression of *CYP8B1, APOA,* and *HMOX-1* was not significantly altered in genistein-treated HepG2 cells ($p<0.05$).

3.3.2 Gene expression in MEF and MEF+ cells incubated with genistein or daidzein

Gene expression of *Srebp1c* was significantly increased in agonist-, genistein-, and daidzein-treated MEF+ cells (Figure 3.2 A). The incubation of MEF cells with genistein or T0901713 has no effect on *Srebp1c* gene expression. However, in daidzein-treated MEF cells, *Srebp1c* gene expression was still upregulated compared to the control group. The fold change was less compared to the change in MEF+ cells, though (4.0-fold increase in MEF+ cells vs. 2.4-fold increase in MEF cells).

Relative *Abca1* and *Scd1* gene expression was significantly upregulated in T0901317-treated MEF+ cells, however, in MEF cells, T0901317 induction has no effect on *Abca1* and *Scd1* gene expression. Similar results were found in daidzein-treated cells (Figure 3.2B&C). When cells were treated with genistein, *Abca1* and *Scd1* mRNA levels were significantly increased in MEF+ cells, however, in MEF cells, genistein-treatment decreased expression of these genes.

3.3.4 Transcriptional regulation of genistein or daidzein on transcription factor LXR

As shown in Figure 3.3A, LXR/RXR activation was significantly decreased with the treatment of genistein. Genistein at 50μM produced a ~50% reduction of LXR activity vs.

the control group. In contrast, 20, 50, and 100μM of daidzein did not have any significant effect on the activation of LXR/RXR, compared to control group (p<0.05).

In contrast, when genistein at the concentration of 100μM was added to transfected cells together with the LXR agonist T0901317, it has no effect on the activation of LXR. However, daidzein, at 100μM significantly enhanced the activation of LXR, producing a ~1.5-fold increase, compared to cells incubated with T0901317 alone (Figure 3.3B).

3.4 DISCUSSION

Results from an *in-vitro* cell culture experiment showed that the most abundant soy isoflavone, genistein, significantly increased expression of LXR-regulated genes, including *ACACA*, and *ABCA1* (Brownsey et al., **2006**; Plösch et al., **2010**). The result is in accordance with IPA analysis from mouse study 2, which showed that expression of LXR-regulated genes was elevated in mice fed genistein or daidzein.

Furthermore, to determine whether the effect of genistein or daidzein is LXR dependent, we utilized LXR knockout and LXRα knock in MEF cells. We found that the effect of genistein or daidzein on expression of genes including *Srebp1c*, *Abca1*, and *Scd1*, was indeed LXR dependent.

To confirm the direct activation of genistein or daidzein on LXR transcription, a cell-based LXR-responsive luciferase expression assay was assessed. The result showed that genistein may be a weak LXR antagonist. The result is in agreement with a study reported by Gonzalez-Granillo (Gonzalez-Granillo et al., **2012**). Genistein may regulate LXR activity indirectly by promoting phosphorylation of the receptor (Gonzalez-Granillo et al., **2012;**

Komati et al., **2017**). However, in our study, the induction of daidzein has no significant effect on LXR activation.

In contrast, when daidzein was added to transfected cells together with T0901317, a LXR agonist, it enhanced the activation of LXR. Therefore, our hypothesis is that, in a cell-based LXR-driven luciferase model, daidzein might act as a co-agonist. We hypothesized that daidzein might directly bind to T0901310-LXR complex to enhance its transcriptional activity, or daidzein might enhance LXR activity by binding through an LXR cofactor which is commonly present in HepG2 cells.

Consequently, our *in-vitro* cell studies showed that genistein and daidzein do impact transcription factor LXR, and future studies may determine if there is any promise for their use as natural agents for the treatment of metabolic syndrome.

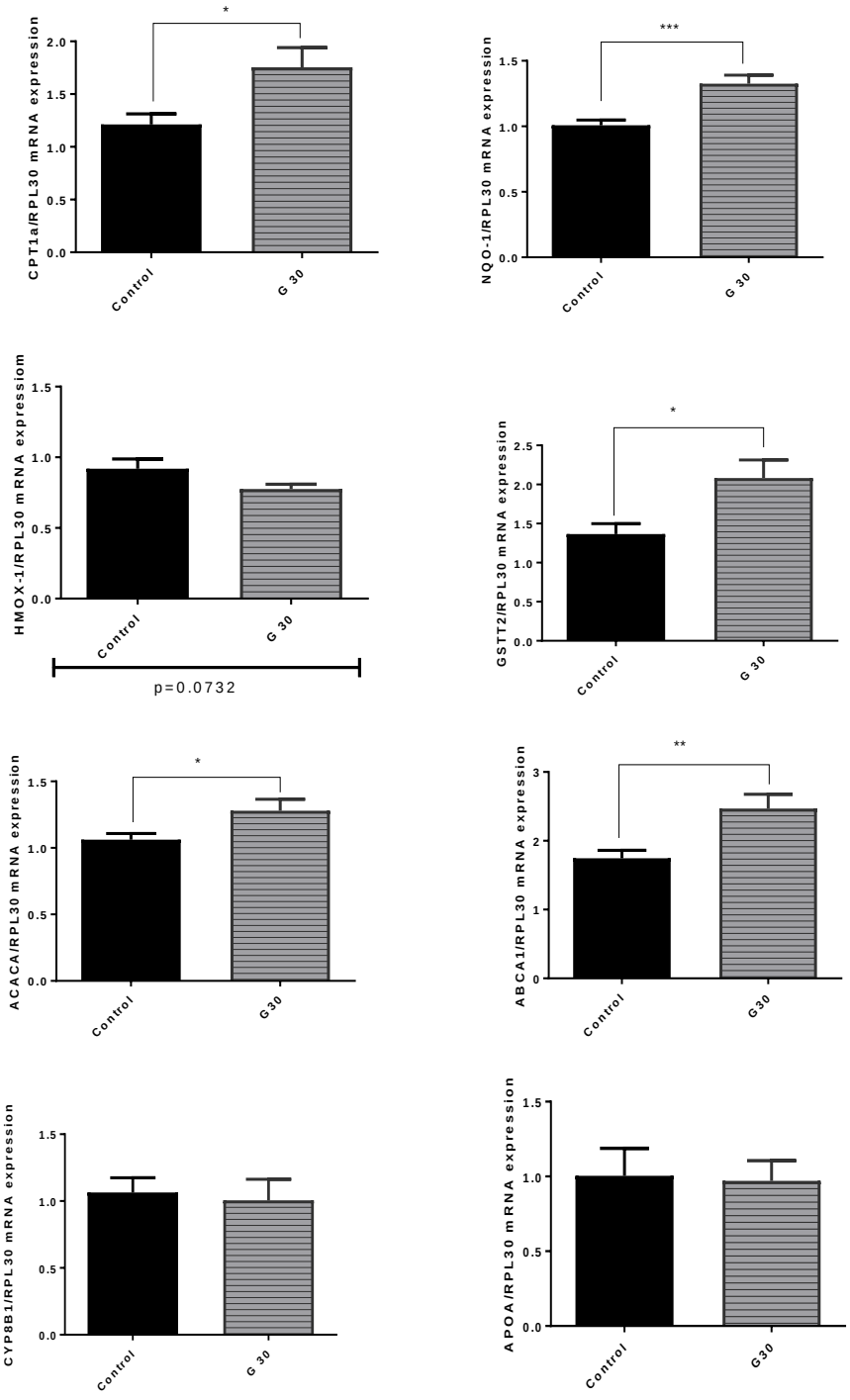


Figure 3.1 Effect of genistein on gene expression in HepG2 cells

HepG2 cells were treated with genistein (30 μmol/L) for 24h. Gene expression was determined by real-time PCR. Expression is relative to *RPL30* housekeeping gene.

A.

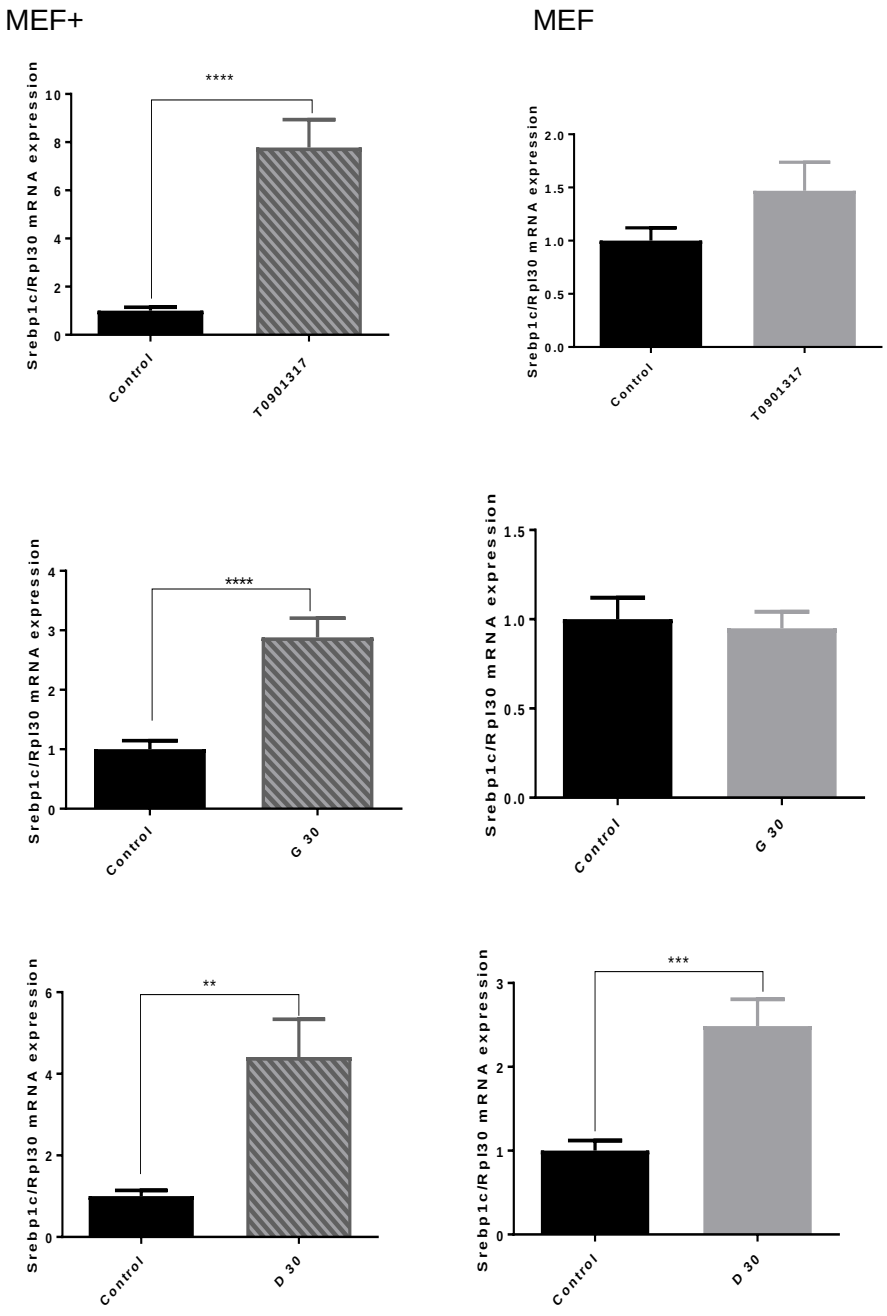


Figure 3.2 Gene expression in MEF and MEF+ cells incubated with LXR agonist, genistein or daidzein

Mouse embryonic fibroblast cells (MEF) and MEF with the LXRα knock in cells (MEF+) were cultured with LXR agonist T0901317 (2μmol/L), genistein (30μmol/L), or daidzein (30μmol/L) for 24h. Expression of LXR regulated genes including *Srebp1c* (A), *Abca1* (B), and *Scd1* (C) were determined by real-time PCR.

B.

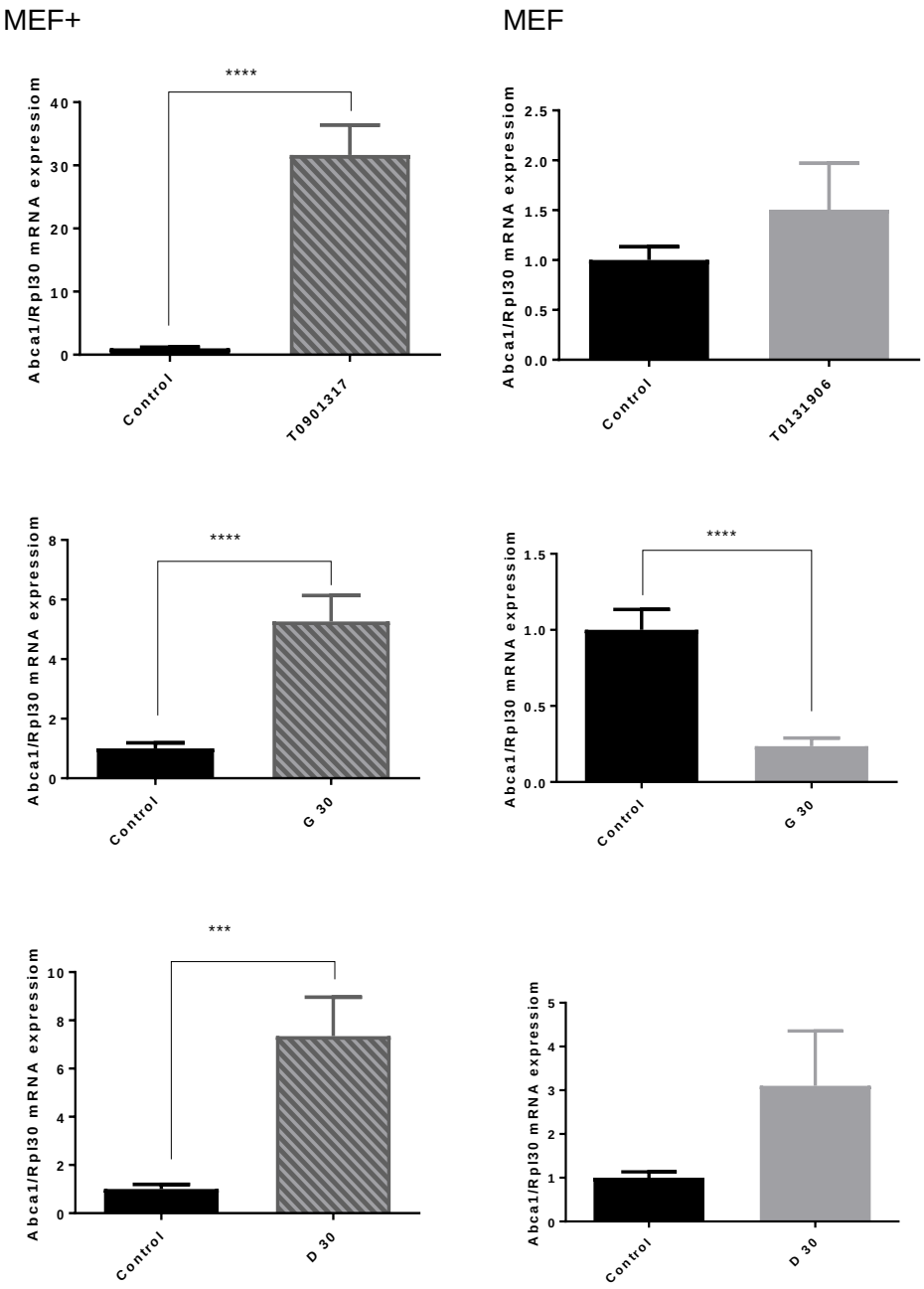


Figure 3.2 Gene expression in MEF and MEF+ cells incubated with LXR agonist, genistein or daidzein (Continued)

C.

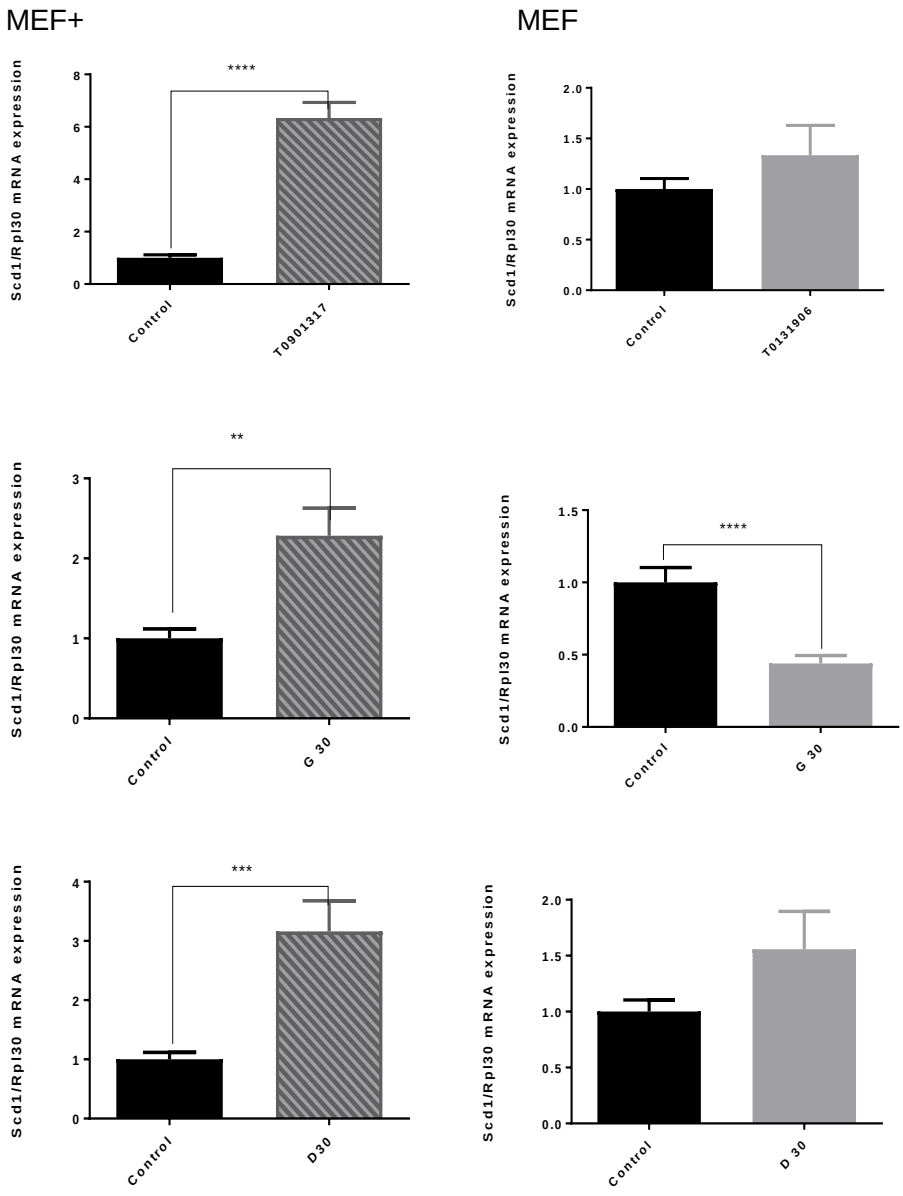
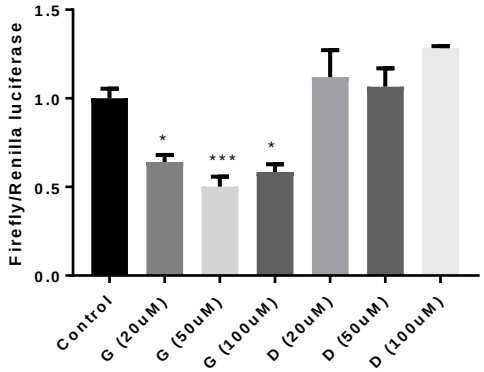


Figure 3.2 Gene expression in MEF and MEF+ cells incubated with LXR agonist, genistein or daidzein (Continued)

A



B

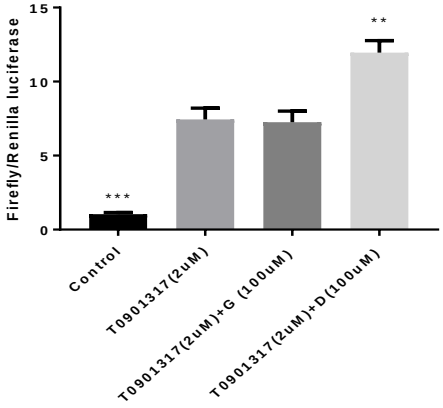


Figure 3.3 Effect of genistein or daidzein on the activation of LXR in HepG2 cells

HepG2 cells were transfected with PRL-TK-Luc and LXRE-luc. Luciferase activity was detected after the cells were incubated with different concentrations of genistein or daidzein. In figure A. each treatment was compared to control group. * indicated $p<0.05$; *** indicated $p<0.005$. In figure B. each treatment was compared to T0901317 group. ** indicated $p<0.01$; *** indicated $p<0.005$.

CHAPTER IV CONCLUSIONS AND FUTURE DIRECTIONS

Genistein consumption dramatically inhibited body weight gain in WD induced obese C57BL/6J mice, with a significant reduction in food intake. To clarify whether the antiobesity effects of genistein was due to decreased energy intake and/or stimulated thermogenesis, a pair-feeding study should be conducted (Ellacott et al., **2010**). In the study, the amount of food intake in genistein-fed mice each day is determined and given to a vehicle-treated paired-fed group of mice under identical conditions the following day. If the weight loss in the pair-fed mice is the same as the genistein-treatment group, genistein is likely to be reducing body weight by inhibiting food intake. However, if the weight loss in genistein fed mice is greater than that in pair-fed mice, genistein induces its antiobesity effects by acting on energy intake and energy expenditure. To investigate aspects of thermogenesis, uncoupling protein mRNA in brown adipose tissue (BAT) and white adipose tissue (WAT) should be measured. Upregulation of the gene indicates a higher energy expenditure rate is likely (Mashiko et al., **2007**).

The identification of genes induced by isoflavones using a custom-designed gene array study expanded the list of mRNAs known to be regulated by isoflavones. However, we did not directly measure the corresponding protein levels or phosphorylation states for the corresponding proteins, which may lead to limitations of the conclusions we can make.

The regulation of *Cyp7a1* mRNA levels by isoflavone consumption have been clearly addressed in Chapter II. The results indicate that isoflavones impact the conversion of cholesterol to bile acids and consumption of isoflavones may regulate more than one factor in the bile acid synthesis pathway. To address the questions, bile acid composition should be determined in animals consuming isoflavone diets.

Also, we hypothesized that the effect of isoflavones on *Cyp7a1* gene expression, and cholesterol metabolism is LXR dependent. However, there is a fundamental difference in the regulation of *Cyp7a1* in rodent and human hepatocytes. In rodent liver, transcription of the *Cyp7a1* is stimulated by LXRα. This provides a mechanism for the elimination of excess cholesterol from the body. However, in primary cultures of human hepatocytes, activation of LXRα represses CYP7A1 expression. This repression is mediated partly via the induction of short heterodimer partner (SHP) (Goodwin et al., **2003**). On the other hand, cholesteryl ester transfer protein (CETP), a key enzyme in lipoprotein remodeling, is present in humans but not in mice (Tall et al., **1993;** Oliveira et al., **2011**). CETP action results in the net transfer of cholesteryl ester from HDL to LDL, its potential to modulate both lipoprotein levels (Oliveira et al., **2011**). Because of this difference, mice carry majority of their plasma cholesterol in HDL, whereas humans carry majority of their cholesterol in LDL (Nishina et al., **1990**; Paigen et al., **1990**). Since CETP is regulated by LXRs in humans, the effect of isoflavone consumption on cholesterol metabolism might differ in humans, compared to the findings we reported in our C57BL/6J mouse studies.

There are certainly species-specific differences in response to isoflavones between mice and humans, which may lead to a difference in the response in humans following isoflavone consumption, which requires further detailed investigation.

The nuclear receptor heterodimers of the LXRs and retinoid X receptor (RXR) are key transcriptional regulators of genes involved in lipid homeostasis. LXR has a fairly broad range of ligands. Animal studies reported in chapter II indicated that the beneficial effect of genistein and daidzein on metabolic syndrome symptoms is likely at least partly due to the

activation of LXR. Therefore, we used several cell models to investigate the activation of isoflavone compounds on LXR. An *in-vitro* LXR-directed luciferase study showed that genistein had an antagonist effect on LXR transactivation. Daidzein induction alone has no impact on LXR activation, however, the co-induction of daidzein and LXR agonist (T0901317) significantly increased LXR activation. A similar co-agonist effect of isoflavone on PPARα (Mezei, **2004**) and grape seed procyanidin extract on FXR (Del Bas et al., **2009**) have been reported.

The binding of LXR and its several agonists has been recognized through co-crystallization of LXR with putative ligands followed by X-ray crystallography. It has been reported that T0901317 occupies the center of the LXR ligand-binding pocket and its hydroxyl head group interacts with H421 and W443, residues which are regarded as critical for ligand-induced transcriptional activation by T0901317 and various endogenous oxysterols (Svensson et al., **2003**). The different structures of genistein and daidzein might impact the binding with LXR. Compared to daidzein, genistein has an extra hydroxyl group, this group may interfere with ligand binding domain activity and/or ability of genistein to enter the cell. To determine the direct ligand-binding properties of isoflavones and LXR would be informative.

The *in-vivo* C57BL/6J mice study, HepG2 study, and LXR knock-in MEF cell study all indicated that genistein might regulate LXR downstream gene via the activation of LXR. However, those data are not consistent with the LXR luciferase cell studies, where an antagonist effect of genistein on LXR was identified. Other regulatory factors may be involved in these observations, for example, *Scd1* is regulated by several transcription

factors, such as LXR, SREBP1, Carbohydrate response element binding protein (ChREBP), and PPARs (Paton et al., **2009;** Yao-Borengasser et al., **2008**). Even if genistein inhibits LXR transactivation, the overall impact of genistein on these several transcription factors may combine to result in an overall upregulation of *Scd*1 gene expression.

The next set of experiments I would propose would be an LXR knockout mouse study. This would be an important next step in elucidating the role of LXR on isoflavones-alleviated symptoms of metabolic syndrome.

Isoflavones act as phytoestrogens. Whether phytoestrogens are beneficial or harmful to human health remains unresolved. The answer is likely complex and may depend on age, amount, health status, and even the presence of specific gut microflora (Patisaul et al., **2010**). Many of phytoestrogens are now recognized to be endocrine disruptors. Emerging evidence suggests that exposure to these compounds may pose a risk to some groups, particularly infants and the unborn (Rozman et al., **2006**). A study enrolled over 19,000 women, reported an increased risk of developing uterine fibroids with a long term of soy formula consumption (D'Aloisio et al., **2010**). Therefore, the amount and the exposure period of lifespan of isoflavone consumption is a concern and need more investigation.

In our *in-vivo* study, the male mice we used were 8 weeks old when used in a ten-week feeding trial. Some investigators consider these mice, at 8 weeks old, to be adults, despite the fact that they are still growing robustly (Dutta et al., **2016**). The content of isoflavone in mice diet was 0.16% w/w, which is equivalent to 640mg/50kg body

weight/day for humans. This is a level that is possible for humans to consume via dietary supplement use, but not by the consumption of isoflavone-containing foods.

In conclusion, I hypothesize that the anti-obesity effect, and other metabolic improvements observed were caused, in part, by impacting food intake, and altering expression of genes related to lipid, bile acid and glucose metabolism, partly via the activation of LXR, and likely via other nuclear hormone receptors as well.

References

Adlercreutz, H., Markkanen, H., Watanabe, S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet. 1993,342 (8881),1209-1210.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y. Genistein, a specific inhibitor of tyrosine specific protein kinase. J Biol Chem. 1987,262, 5592-5595.

Alberti, K.G.M., Zimmet, P. and Shaw, J. The metabolic syndrome-a new worldwide definition. The Lancet. 2005, 366(9491), 1059.

Alberti, K.G., Eckel, R.H., Grundy, S.M., Zimmet, P.Z., Cleeman, J.I., Donato, K.A., Fruchart, J.C., Philip, W., James, T., Loria, C.M., Smith, S.C. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the Association for the Study of Obesity Heart Federation; International Atherosclerosis Society; and International National Heart, Lung, and Blood Institute; American Heart Association; World International Diabetes Federation Task Force on Epidemiology and Prevention. Circulation. 2009,120,1640-1645.

Aljada, A., Mohanty, P., Ghanim, H., Abdo, T., Tripathy, D., Chaudhuri, A., Dandona, P. Increase in intranuclear nuclear factor κB and decrease in inhibitor κB in mononuclear cells after a mixed meal: evidence for a proinflammatory effect. Am J Clin Nutr. 2004,79(4),682-690.

Allred, C.D., Ju, Y.H., Allred, K.F., Chang, J., Helferich, W.G. Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. Carcinogenesis. 2001,22(10),1667–1673.

Anderson, J.W., Johnstone, B.M., Cook-Newell, M.E. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med. 1995,333,276–282.

Anderson, S.P., Yoon, L., Richard, E.B., Dunn, C.S., Cattley, R.C., Corton, J.C. Delayed liver regeneration in peroxisome proliferator-activated receptor-alpha-null mice. Hepatology. 2002,36(3),544-554.

Aoki, T., Akashi, T., Ayabe, S. Flavonoids of leguminous plants: structure, biological activity, and biosynthesis. Journal of Plant Research. 2000,113, 475-488.

Arjmandi,BH, Khalil,DA, Smith,BJ, et al. Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. J Clin Endocrinol Metab.2003, 88,1048-1054.

Astrup, A., Madsbad, S., Breum, L., Jensen, T.J., Kroustrup, J.P., Larsen, T.M. Effect of tesofensine on bodyweight loss, body composition, and quality of life in obese patients: a randomised, double-blind, placebo-controlled trial. Lancet. 2008, 372,1906–1913.

Atkinson, C., Newton, K.M., Bowles, E.J., Yong, M., Lampe, J.W. Demographic, anthropometric and lifestyle factors and dietary intakes in relation to daidzein-metabolizing

phenotypes among premenopausal women in the United States. Am J Clin Nutr. 2008, 87(3):679-687.

Ayala, J. E., Bracy, D. P., McGuinness, O. P., Wasserman, D. H. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. Diabetes. 2006, 55 (2), 390−397.

Ayala, J. E., Samuel, V. T., Morton, G. J., Obici, S., Croniger, C. M., Shulman, G. I., Wasserman, D. H., McGuinness, O. P. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis Models Mech. 2010, 3, 525−534.

Azuma, K., Katsukawa, F., Oguchi, S., Murata, M. Correlation between serum resistin level and adiposity in obese individuals. Obes Res. 2003, 11, 997−1001.

Baker, R.G., Matthew S.H., Sankar G. NF-κB, inflammation, and metabolic disease. Cell Metab. 2011,13,11-22.

Banerjee, R. R., Rangwala, S. M., Shapiro, J. S., Rich, A. S. Regulation of fasted blood glucose by resistin. Science. 2004, 303,1195−1198.

Barak, Y., Liao, D., He, W., Ong, E.S., Nelson, M.C., Olefsky, J.M., Boland, R. Evans, R.M. Effects of peroxisome proliferator-activated receptor δ on placentation, adiposity, and colorectal cancer. Proc Natl Acad Sci USA. 2002, 99, 303-308.

Barnes, S., Wang, C.C., Kirk, M., Smith-Johnson, M., Coward, L., Barnes, N.C., Vance, G. and Boersma, B. HPLC-mass spectrometry of isoflavonoids in soy and the American groundnut, Apios americana. Adv Exp Med Biol. 2002,505,77-88.

Barnes, S. The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. Lymphat Res Biol. 2010,8(1),89-98.

Batterham, R.L., Cohen, M.A., Ellis, S.M., Le Roux, C.W., Withers, D.J., Frost, G.S., Ghatei, M.A., Bloom, S.R. Inhibition of food intake in obese subjects by peptide YY3–36. N Engl J Med. 2003, 349,941–948.

Beavers, D.P., Beavers, K.M., Miller, M., Stamey, J., Messina, M.J. Exposure to isoflavone-containing soy products and endothelial function: a Bayesian meta-analysis of randomized controlled trials. Nutr Metab Cardiovasc Dis. 2012, 22(3),182-191.

Bhathena, S.J., Velasquez, M.T. Beneficial role of dietary phytoestrogens in obesity and diabetes. Am J Clin Nutr. 2002, 76,1191-1201.

Bhavsar, S., Watkins, J., Young, A. Synergy between amylin and cholecystokinin for inhibition of food intake in mice. Physiol Behav. 1998,64,557-561.

Binesh Marvasti, T., Kh, A. Pharmacological management of metabolic syndrome and its lipid complications. Daru. 2010,18(3),146-154.

Bloedon, L.T., Jeffcoat, A.R., Lopaczynski, W., Schell, M.J., Black, T.M., Dix, K.J., Thomas, B.F., Albright, C., Busby, M.G., Crowell, J.A., Zeisel, S.H. Safety and pharmacokinetics of purified soy isoflavones: single-dose administration to postmenopausal women. Am J Clin Nutr. 2002, 76,1126-1137.

Brownsey, R.W., Boone, A.N., Elliott, J.E., Kulpa, J.E., Lee, W.M. Regulation of acetyl-CoA carboxylase. Biochem Soc Trans. 2006, 34(Pt 2), 223-227.

Buer, C.S., Imin, N., Djordjevic, M.A. Flavonoids: new roles for old molecules. J Integr Plant Biol. 2010,52(1),98-111.

Buettner, R., Schölmerich, J., Bollheimer, L.C. High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity (Silver Spring). 2007,15(4),798-808.

Busserolles, J., Rock, E., Gueux, E., Mazur, A., Grolier, P., Rayssiguier, Y. Short-term consumption of a high-sucrose diet has a pro-oxidant effect in rats. Br J Nutr. 2002,87(4),337-342.

Cao, G.Q., Liang, Y., Broderick, C.L., Oldham, B.A., Beyer, T.P., Schmidt, R.J., Zhang, Y., Stayrook, K.R., Suen, C., Otto, K.A., Miller, A.R. Antidiabetic action of a liver X receptor agonist mediated by inhibition of hepatic gluconeogenesis. J Biol Chem. 2003,278(2),1131–1136.

Cassidy, A., Bingham, S., Setchell, K.D. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am J Clin Nutr. 1994, 60, 333–340.

Cederroth, C. R., Zimmermann, C., Nef, S. Soy, phytoestrogens and their impact on reproductive health. Mol Cell Endocrinol. 2012,22,355(2),192-200.

Cena, E., Steinberg, F. Soy may help protect against cardiovascular disease. California Agriculture. 2011, 65(3),118-123.

Charnsil, C., Pilakanta, S., Panikul, S. Prevalence of metabolic syndrome and its associated factors in patients with major depressive disorder (MDD). ASEAN Journal of Psychiatry. 2015,16(2).

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001,30;294(5548):1866-1870.

Chen, J., Lin, H., Hu, M. Metabolism of flavonoids via enteric recycling: Role of intestinal disposition. J Pharmacol Exp Ther. 2003, 304(3),1228-1235.

Cheng, Y., Meng, Q,S., Wang, C.X., Li, H.K., Huang, Z.Y., Chen, S.H., Xiao, F., Guo, F. Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. Diabetes. 2010,59(1),17–25.

Chisholm, J.W., Hong, J., Mills, S.A., Lawn, R.M. The LXR ligand T0901317 induces severe lipogenesis in the db/db diabetic mouse. J Lipid Res. 2003,44(11),2039–2048.

Christiansen, T., Richelsen, B., Bruun, J. M. Monocyte chemoattractant protein-1 is produced in isolated adipocytes associated with adiposity and reduced after weight loss in morbid obese subjects. Int J Obes. 2005, 29,146−150.

Chu, K., Miyazaki, M., Man, W.C., Ntambi, J.M. Stearoyl-coenzyme A desaturase 1 deficiency protects against hypertriglyceridemia and increases plasma high-density lipoprotein cholesterol induced by liver X receptor activation. Mol Cell Biol. 2006,26, 6786–6798.

Claudel, T., Staels, B., Kuipers, F. The Farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. Arterioscler Thromb Vasc Biol. 2005,25(10),2020-2030.

Coll, T., Rodrïguez-Calvo, R., Barroso, E., Serrano, L., Eyre, E., Palomer, X., Vázquez-Carrera, M. Peroxisome proliferator-activated receptor (PPAR) beta/delta: a new potential therapeutic target for the treatment of metabolic syndrome. Curr Mol Pharmacol. 2009, 2,46-55.

Collins, S., Martin, T.L., Surwit, R.S., Robidoux, J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiol Behav. 2004, 81(2), 243-248.

Dahab, G.M., Kheriza, M.M., El-Beltagi, H.M., Fouda, A.M., El-Din, O.A. Digital quantification of fibrosis in liver biopsy sections: description of a new method by Photoshop software. J Gastroenterol Hepatol. 2004,19(1),78-85.

D'Aloisio, A.A., Baird, D.D., DeRoo, L.A., Sandler, D.P. Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the Sister Study. Environ Health Perspect. 2010,118(3),375-381.

Daniels, T.F., Wu, X.L., Pan, Z., Michal, J.J., Wright Jr, R.W., Killinger, K.M., MacNeil, M.D., Jiang, Z. The reverse cholesterol transport pathway improves understanding of genetic networks for fat deposition and muscle growth in beef cattle. PloS one. 2010, 5(12), e15203.

Davis, H.R., Veltri, E.P. Zetia: inhibition of Niemann-Pick C1 Like 1 (NPC1L1) to reduce intestinal cholesterol absorption and treat hyperlipidemia. J Atheroscler Thromb. 2007,14(3),99-108.

Day, A.J., Dupont M.S., Ridley, S., Rhodes, M.J.C., Morgan M.R.A., Wiliamsosm, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. 1998,25,436(1),71-75.

Day, A.J., Cañada, F.J., Díaz, J.C., Kroon, P.A., Mclauchlan, R., Faulds, C.B., Plumb, G.W., Morgan, M.R. Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS letters. 2000, 468(2-3),166-170.

Del Bas, J.M., Ricketts, M.L., Vaqué, M., Sala, E., Quesada, H., Ardevol, A., Salvadó, M.J., Blay, M., Arola, L., Moore, D.D., Pujadas, G., Fernandez-Larrea, J., Bladé, C. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. Mol Nutr Food Res. 2009,53(7),805-814.

De Vos, P., Lefebvre, A.M., Miller, S.G., Guerre-Millo, M., Wong, K., Saladin, R., Hamann, L.G., Staels, B., Briggs, M.R., Auwerx, J. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. J Clin Invest. 1996, 98,1004.

Derbyshire, E., Wright, D.J., Boulter, D. Review: Legumin and vicilin, storage proteins of legume seeds. Phytochemistry. 1976,15,3.

Dissard, R., Klein, J., Caubet, C., Breuil, B., Siwy, J., Hoffman, J., Sicard, L., Ducassé, L., Rascalou, S., Payre, B. and Buléon, M. Long term metabolic syndrome induced by a high fat high fructose diet leads to minimal renal injury in C57BL/6 mice. PLoS One. 2013,8,e76703.

Dixon, R.A., Ferreria, D. Genestein. Phytochemistry. 2002,60, 205-211.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., Wahli, W. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. Cell. 1992,68,879-887.

Dutta, S., Sengupta, P. Men and mice: Relating their ages. Life Sci. 2016, 1,152,244-248.

Ellacott, K.L., Morton, G.J., Woods, S.C., Tso, P., Schwartz, M.W. Assessment of feeding behavior in laboratory mice. Cell Metab. 2010, 12(1), 10-17.

Erdman, J.W. AHA Science Advisory: Soy protein and cardiovascular disease. A statement for healthcare professionals from the Nutrition Committee of the AHA. Circulation. 2000,14,102(20),2555-2559.

Evans, R.M., Mangelsdorf, D.J. Nuclear Receptors, RXR, and the Big Bang. Cell. 2014, 27;157(1),255-266.

Fang, Q. Some current study and research approaches relating to the use of plants in the traditional Chinese medicine. J Ethnopharmacol. 1980,2(1),57-63.

Ferguson, J.A., Mathesius, U. Signaling interactions during nodule development. J Plant Growth Regul. 2003,22, 47-72.

Food and Drug Administration (2007) Guidance for Industry Developing Products for Weight Management. Available at: http://www.fda.gov/cder/guidance/7544dft.pdf.

Food and Drug Administration (2008) Guidance on antidiabetes drug development. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071627.pdf.

Food labeling: health claims; soy protein and coronary heart disease. 1999. Food and Drug Administration, HHS. Final rule. Fed Regist 64: 57700-33.

Foti, P., Erba, D., Riso, P., Spadafranca, A., Criscuoli, F., Testolin, G. Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. Arch Biochem BiophysI. 2005, 433, 421-427.

Frid, A. H., Nilsson, M., Holst, J. J., Björck, I. M. Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. Am J Clin Nutr. 2005, 82, 69−75.

Friedman, J. M. A war on obesity, not the obese. Science. 2003, 299, 856−858.

Gao, J., Xie, W. Targeting xenobiotic receptors receptors PXR and CAR for metabolic disease. Trends Pharmacol Sci. 2012,33(10),552-558.

Gao, M., Liu, D. The liver X receptor agonist T0901317 protects mice from high fat diet-induced obesity and insulin resistance. The AAPS journal. 2013, 15, 258-266.

Gao, Y., Yao, Y., Zhu, Y., Ren, G. Isoflavones in chickpeas inhibit adipocyte differentiation and prevent insulin resistance in 3T3- L1 cells. J Agric Food Chem. 2015, 63 (44), 9696−9703.

Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P., Crona, J.H., Davis, H.R., Dean, D.C., Detmers, P.A. and Graziano, M.P. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci. 2005,102(23), 8132-8137.

Gervois, P., Torra, I. P., Fruchart, J. C., Staels, B. Regulation of lipid and lipoprotein metabolism by PPAR activators. Clin Chem Lab Med. 2000,38, 3-11.

Getting, S.J. Targeting melanocortin receptors as potential novel therapeutics. Pharmacol Ther. 2006,111:1-15.

Glavas, M.M., Joachim, S.E., Draper, S.J., Smith, M.S., Grove, K.L. Melanocortinergic activation by melanotan II inhibits feeding and increases uncoupling protein 1 messenger ribonucleic acid in the developing rat. Endocrinology. 2007,148(7),3279-3287.

González-Granillo, M., Steffensen, K.R., Granados, O., Torres, N., Korach-André, M., Ortíz, V., Aguilar-Salinas, C., Jakobsson, T., Díaz-Villaseñor, A., Loza-Valdes, A., Hernandez-Pando, R., Gustafsson, J.Å., Tovar, A.R. Soy protein isoflavones differentially regulate liver X receptor isoforms to modulate lipid metabolism and cholesterol transport in the liver and intestine in mice. Diabetologia. 2012,55(9):2469-2478.

Goodwin, B., Watson, M.A., Kim, H., Miao, J., Kemper, J.K., Kliewer, S.A. Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha. Mol Endocrinol. 2003,17(3):386-394.

Guerre-Millo, M., Rouault, C., Poulain, P., Andre, J., Poitout, V., Peters, J.M., Gonzalez, F.J., Fruchart, J.C., Reach, G., Staels, B. PPAR-α-Null mice are protected from high fat diet-induced insulin resistance. Diabetes. 2001, 50,2809-2814.

Hajos, G., Gelencsér, E., Grant, G., Bardocz, S., Sakhri, M., Duguid, T.J., Newman, A.M., Pusztai, A. Effects of proteolytic modification and methionine enrichment on the nutritional value of soya albumins for rats. J Nutr Biochem. 1996,7,481-487.

He, F.J., Chen, J.Q. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms. Food Science and Human Wellness. 2013,2(3),146-161.

Heal D.J., Gosden J., Smith S.L. Regulatory challenges for new drugs to treat obesity and comorbid metabolic disorders. Br J Clin Pharmocol. 2009, 68, 861–874.

Heinonen, S.M., Wähälä, K. Adlercreutz, H. Metabolism of isoflavones in human subjects. Phytochemistry Reviews. 2002,1(2),175-182.

Hillman, G.G., Wang, Y., Kucuk, O., Che, M., Doerge, D.R., Yudelev, M., Joiner, M.C., Marples, B., Forman, J.D., Sarkar, F.H. Genistein potentiates inhibition of tumor growth by radiation in a prostate cancer orthotopic model. Mol Cancer Ther. 2004,3,1271-1279.

Holvoet, P., Kritchevsky, S.B., Tracy, R.P., Mertens, A., Rubin, S.M., Butler, J., Goodpaster, B., Harris, T.B. The metabolic syndrome, circulating oxidized LDL, and risk of myocardial infarction in well-functioning elderly people in the health, aging, and body composition cohort. Diabetes. 2004,53(4),1068-1073.

Hong, C., Tontonoz, P. Liver X receptors in lipid metabolism: opportunities for drug discovery. Nat Rev Drug Discov. 2014;13(6):433-444.

Hong, K.W., Ko, K.P., Ahn, Y., Kim, C.S., Park, S.J., Park, J.K., Kim, S.S., Kim, Y. Epidemiological profiles between equol producers and nonproducers: a genomewide association study of the equol-producing phenotype. Genes Nutr. 2012,7,567-574.

Hotamisligil, G.S. Inflammation and metabolic disorders. Nature. 2006,7121,860-867.

Howes, L.G., Howes, J.B., Knight, D.C. Isoflavone therapy for menopausal flushes: a systematic review and meta-analysis. Maturitas. 2006, 55,203-211.

Huang, P., Chandra, V., Rastinejad, F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol. 2010,72,247-272.

Hwang, C.S., Kwak, H.S., Lim, H.J., Lee, S.H., Kang, Y.S., Choe, T.B., Hur, H.G., Han, K.O. Isoflavone metabolites and their in vitro dual functions: they can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J Steroid Biochem Mol Biol. 2006,101(4-5):246-253.

Issemann, I., Green, S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature. 1990,347,645–650.

Izumi, T., Piskula, M.K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y., Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. J Nutr. 2000,130,1695–1699.

Jaygopal, V., Albertazzi, P., Kilpatrick, E.S., Howarth, E.M., Jennings, P.E., Hepburn, D.A., Atkin, S.L. Beneficial effects of soy phytoestrogen intake in postmenopausal women with type 2 diabetes. Diabetes Care. 2002, 25,1709-1714.

Jeon, S., Park, Y.J., Kwon, Y.H. Genistein alleviates the development of nonalcoholic steatohepatitis in ApoE(−/−) mice fed a high-fat diet. Mol Nutr Food Res. 2014, 58, 830−841.

Jing, Z., Wei-Jie, Y. Effects of soy protein containing isoflavones in patients with chronic kidney disease: A systematic review and meta-analysis. Clin Nutr. 2016,35(1),117-124.

Joseph, S.B., Laffitte, B.A., Patel, P.H., Watson, M.A., Matsukuma, K.E., Walczak, R., Collins, J.L., Osborne, T.F., Tontonoz, P. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J Biol Inorg Chem. 2002,277(13),11019-11025.

Joseph, S.B., McKilligin, E., Pei, L., Watson, M.A., Collins, A.R., Laffitte, B.A., Chen, M., Noh, G., Goodman, J., Hagger, G.N., Tran, J. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. P Natl Acad Sci. 2002,99(11),7604-7609.

Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J., Tontonoz, P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nature Med. 2003,9, 213-219.

Jump, D.B., Depner, C.M., Tripathy, S., Lytle, K.A. Impact of dietary fat on the development of non-alcoholic fatty liver disease in Ldlr-/- mice. Proc Nutr Soc. 2016,75(1),1-9.

Jung, W., Yu, O., Lau, S.M.C., O'Keefe, D.P., Odell, J., Fader, G. McGonigle, B. Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol. 2000,18(2), 208-212.

Kajimura, S., Seale, P., Kubota, K., Lunsford, E., Frangioni, J.V., Gygi, S.P., Spiegelman, B.M. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. Nature. 2009,460(7259),1154-1158.

Karra, E., Batterham, R.L. The role of gut hormones in the regulation of body weight and energy homeostasis. Mol Cell Endocrinol. 2010, 316,120-128.

Kelly, G.E., Joannou, G.E., Reeder, A.Y., Nelson, C., Waring, M.A. The variable metabolic response to dietary isoflavones in humans. Proc Soc Exp Biol Med. 1995, 208, 40-43.

Kennedy, A.J., Ellacott, K.L., King, V.L., Hasty, A.H. Mouse models of the metabolic syndrome. Dis Model Mech. 2010, 3(3-4), 156–166.

Kershaw, E. E., Flier, J. S. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab. 2004, 89, 2548−2556.

Kgomotso, T., Chiu, F., Ng, K. Genistein- and daidzein 7-O-β-D-glucuronic acid retain the ability to inhibit copper-mediated lipid oxidation of low density lipoprotein. Mol Nutr Food Res. 2008, 52,1457-1466.

Kim, E., Hee-Myong, R., Sun-Lim, K., Hong-Sig, K., Ill-Min, C. Analysis of isoflavone, phenolic, soyasapogenol, and tocopherol compounds in soybean [Glycine max (L.) Merrill] germplasms of different seed weights and origins. J Agric Food Chem. 2012,23,6045-6055.

Kim, H.K., Nelson-Dooley, C., Della-Fera, M.A., Yang, J.Y., Zhang, W., Duan, J., Hartzell, D.L., Hamrick, M.W., Baile, C.A. Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J Nutr. 2006,136,409-414.

Kim, M.H., Park, J.S., Seo, M.S., Jung, J.W., Lee, Y.S., Kang, K.S. Genistein and daidzein repress adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells via Wnt/β-catenin signaling or lipolysis. Cell Prolif. 2010, 43, 594-605.

King, R.A., Broadbent, J.L., Head, R.J. Absorption and excretion of the soy isoflavone genistein in rats. J Nutr. 1996,126,176-182.

King, R.A., Bursill, D.B. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. Am J Clin Nutr. 1998,67,867-872.

Kiss, E., Popovic, Z., Bedke, J., Wang, S., Bonrouhi, M., Gretz, N., Stettner, P., Teupser, D., Thiery, J., Porubsky, S., Adams, J. Suppression of chronic damage in renal allografts by liver X receptor (LXR) activation: relevant contribution of macrophage LXRα. Am J Pathol. 2011,179(1),92-103.

Kliewer, S.A., Mangelsdorf, D.J. Bile Acids as Hormones: The FXR-FGF15/19 Pathway. Dig Dis. 2015,33(3),327-331.

Knight, D.C., Eden, J.A. Phytoestrogens--a short review. Maturitas. 1995,22,167-175.

Komati, R., Spadoni, D., Zheng, S., Sridhar, J., Riley, K.E., Wang, G. Ligands of Therapeutic Utility for the Liver X Receptors. Molecules. 2017, 5, 22(1).

Laffitte, B.A., Chao, L.C., Li, J., Walczak, R., Hummasti, S., Joseph, S.B., Castrillo, A., Wilpitz, D.C., Mangelsdorf, D.J., Collins, J.L., Saez, E. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proc Natl Acad Sci. 2003,100(9),5419-5424.

Lamon-Fava, S. Genistein activates apolipoprotein A-I gene expression in the human hepatoma cell line HepG2. J Nutr. 2000,130, 2489-2492.

Laudet, V., Hanni, C., Coll, J., Catzeflis, F., Stehelin, D. Evolution of the nuclear receptor gene superfamily. EMBO J. 1992,11,1003-1013.

Le May, C., Berger, J.M., Lespine, A., Pillot, B., Prieur, X., Letessier, E., Hussain, M.M., Collet, X., Cariou, B., Costet, P. Transintestinal cholesterol excretion is an active metabolic process modulated by PCSK9 and statin involving ABCB1. Arterioscler Thromb Vasc Biol. 2013, 33(7),1484-1493.

Lee, J. Y., Hashizaki, H., Goto, T., Sakamoto, T. Activation of peroxisome proliferator-activated receptor-α enhances fatty acid oxidation in human adipocytes. Biochem Biophys Res Commun. 2011, 407, 818−822.

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., Staels, B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev. 2009,89,147-191.

Lemieux, I., Poirier, P., Bergeron, J., Alméras, N., Lamarche, B., Cantin, B., Dagenais, G.R., Després, J.P. Hypertriglyceridemic waist: a useful screening phenotype in preventive cardiology? Can J Cardiol. 2007,23(Suppl B),23B–31B.

Li, Y., Mezei, O., Shay, N.F. Human and murine hepatic sterol-12-α-hydroxylase and other xenobiotic metabolism mRNA are upregulated by soy isoflavones. J Nutr. 2007,137 (7),1705-1712.

Li, Y., Ross-Viola, J.S., Shay, N.F., Moore, D.D., Ricketts, M.L. Human CYP3A4 and murine Cyp3A11 are regulated by equol and genistein via the pregnane X receptor in a species-specific manner. J Nutr. 2009,139(5),898-904.

Little, T.J., Horowitz, M., Feinle-Bisset, C. Role of cholecystokinin in appetite control and body weight regulation. Obes Rev. 2005,6,297-306.

Liu, B., Qin, L., Liu, A., Uchiyama, S., Ueno, T., Li, X., Wang, P. Prevalence of the equol-producer phenotype and its relationship with dietary isoflavone and serum lipids in healthy Chinese adults. J Epidemiol. 2010,20, 377-384.

Liu, Y., Wang, D., Zhang, D., Lv, Y. Inhibitory effect of blueberry polyphenolic compounds on oleic acid-induced hepatic steatosis in vitro. J Agric Food Chem. 2011, 59, 12254−12263.

Lu, L.J., Anderson, K.E., Grady, J.J., Nagamani, M. Effects of an isoflavone-free soy diet on ovarian hormones in premenopausal women. J Clin Endocrinol Metab. 2001,86,3045-3052.

Luo, T., Snyder, S.M., Zhao,B., Sullivan,D.K., Hamilton-Reeves,J., Guthrie,G., Ricketts, M.L., Shiverick, K.T., Shay, N. Gene Expression Patterns are Altered in Athymic Mice and Metabolic Syndrome Factors are Reduced in C57BL/6J Mice Fed High-fat Diets Supplemented with Soy Isoflavones. J Agric Food Chem. 2016, 64(40), 7492-7501.

Ma, Y., Huang, Y., Yan, L., Gao, M., Liu, D. Synthetic FXR agonist GW4064 prevents diet-induced hepatic steatosis and insulin resistance. Pharm Res. 2013, 30(5), 1447-1457.

Magee, P.J. Is equol production beneficial to health? Proc Nutr Soc. 2011,70, 10-18.

Mashiko, S., Ishihara, A., Iwaasa, H., Sano, H., Ito, J., Gomori, A., Oda, Z., Moriya, R., Matsushita, H., Jitsuoka, M., Okamoto, O., MacNeil, D.J., Van der Ploeg, L.H., Fukami, T., Kanatani, A. A pair-feeding study reveals that a Y5 antagonist causes weight loss in diet-induced obese mice by modulating food intake and energy expenditure. Mol Pharmacol. 2007, 71(2), 602-608.

Mezei, O. THE EFFECT OF SOY ISOFLAVONES ON THE PPARα AND PPARγ PATHWAYS. Doctoral dissertation, **2004**, University of Notre Dame.

Mezei, O., Banz, W.J., Steger, R.W., Peluso, M.R., Winters, T.A., Shay, N. Soy isoflavones exert antidiabetic and hypolipidemic effects through the PPAR pathways in obese Zucker rats and murine RAW 264.7 cells. J Nutr. 2003,133(5),1238-1243.

Mezei, O., Li, Y., Mullen, E., Ross-Viola, J.S., Shay, N.F. Dietary isoflavone supplementation modulates lipid metabolism via PPARα-dependent and-independent mechanisms. Physiol Genomics. 2006,16, 26(1),8-14.

Miltyk, W., Craciunescu, C.N., Fischer, L., Jeffcoat, R.A., Koch, M.A., Lopaczynski, W., Mahoney, C., Jeffcoat, R.A., Crowell, J., Paglieri, J., Zeisel, S.H. Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein, and glycitein) in 20 patients with prostate cancer. Am J Clin Nutr. 2003,77,875-882.

Monzillo, L. U., Hamdy, O., Horton, E. S., Ledbury, S. Effect of lifestyle modification on adipokine levels in obese subjects with insulin resistance. Obes Res. 2003, 9, 1048−1054.

Morton, M.S., Wilcox, G., Wahlqvist, M.L., Griffiths, K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. J Endocrinol. 1994,142,251-259.

Mozumdar, A., Gary L. Persistent increase of prevalence of metabolic syndrome among US adults: NHANES III to NHANES 1999-2006. Diabetes Care. 2011,34, 216-219.2.

Mullen, E., Brown, R.M., Osborne, T.F., Shay, N.F. Soy isoflavones affect sterol regulatory element binding proteins (SREBPs) and SREBP-regulated genes in HepG2 cells. J Nutr. 2004,134(11),2942-2947.

Muoio, D.M., MacLean, P.S., Lang, D.B., Li, S., Houmard, J.A., Way, J.M., Winegar, D.A., Corton, J.C., Dohm, G.L., Kraus, W.E. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) α knock-out mice evidence for compensatory regulation by PPARδ. J Biol Chem. 2002, 277(29), 26089-26097.

Murphy, P.A., Wang H.J. Total genistein, daidzein and glycitein content of soyfoods. 1993.

Murphy, P.A., Song, T., Buseman, G., Barua, K., Beecher, G.R., Trainer, D., Holden, J. Isoflavones in retail and institutional soy foods. J Agric Food Chem.1999, 47,2697-2704.

Musso G, Gambino R, Cassader M. Non-alcoholic fatty liver disease from pathogenesis to management: an update. Obes Rev. 2010;11: 430-445.

Muthyala, R.S., Ju, Y.H., Sheng, S., Williams, L.D., Doerge, D.R., Katzenellenbogen, B.S., Helferich, W.G., Katzenellenbogen, J.A. Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors α and β. Bioorg Med Chem. 2004,12, 1559-1567.

Naaz, A., Yellayi, S., Zakroczymski, M.A., Bunick, D., Doerge, D.R., Lubahn, D.B., Helferich, W.G., Cooke, P.S. The soy isoflavone genistein decreases adipose deposition in mice. Endocrinology. 2003,144,3315-3320.

Nakano, H., Ogura, K., Takahashi, E., Harada, T., Nishiyama, T., Kei, M.U.R.O., Hiratsuka, A., Kadota, S. Watabe, T. Regioselective monosulfation and disulfation of the phytoestrogens daidzein and genistein by human liver sulfotransferases. Drug Metab Pharmacokinet. 2004,19(3), 216-226.

Natali, A., Toschi, E., Baldeweg, S., Ciociaro, D., Favilla, S., Saccà, L., Ferrannini, E. Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes. Diabetes. 2006, 55(4), 1133-1140.

Natural Medicines Comprehensive Database. Natural Database Soy. Accessed 2010. www.naturaldatabase.com

Neuschwander-Tetri, B. A., Wang, D. Q. H. Excess cholesterol and fat in the diet: a dangerous liaison for energy expenditure and the liver. Hepatology, 2013, 57(1), 7-9.

Nishina, P.M., Verstuyft, J., Paigen, B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. The Journal of Lipid Research. 1990, 31, 859-869.

Olefsky, J.M. Nuclear receptor minireview series. J Biol Chem. 2001, 5, 276(40), 36863-36864.

Oliveira, H.C., de Faria, E.C. Cholesteryl ester transfer protein: the controversial relation to atherosclerosis and emerging new biological roles. IUBMB Life. 2011, 63(4),248-257.

O'neill, S., O'driscoll, L. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obes Rev. 2015,16(1),1-12.

Park, M.K., Han, Y., Kim, M.S., Seo, E., Kang, S., Park, S.Y., Koh, H., Kim, D.K., Lee, H.J. Reduction of food intake by fenofibrate is associated with cholecystokinin release in Long-Evans Tokushima rats. Korean J Physio Phar. 2012,16,181-186.

Pearson, T.A., Denke, M.A., McBride, P.E., Battisti, W.P., Brady, W.E., Palmisano, J. A community-based, randomized trial of ezetimibe added to statin therapy to attain NCEP ATP III

goals for LDL cholesterol in hypercholesterolemic patients: the ezetimibe add-on to statin for effectiveness (EASE) trial. Mayo Clin Proc. 2005,80(5),587-95.

Phillips, C.M., Perry, I.J. Does inflammation determine metabolic health status in obese and nonobese adults? J Clin Endocrinol Metab. 2013,98(10), E1610-E1619.

Pino, A.M., Valladares, L.E., Palma, M.A. Dietary isoflavones affect sex hormone-binding globulin levels in postmenopausal women. J Clin Endocrinol Metab. 2000, 85,2797-2800.

Plösch, T., Gellhaus, A., van Straten, E.M., Wolf, N., Huijkman, N.C., Schmidt, M., Dunk, C.E., Kuipers, F., Winterhager, E. The liver X receptor (LXR) and its target gene ABCA1 are regulated upon low oxygen in human trophoblast cells: a reason for alterations in preeclampsia? Placenta. 2010,31(10),910-918.

Porter, R.K. A new look at UCP 1. BBA-Bioenergetics. 2006,1757(5-6),446-448.

Paton, C.M., Ntambi, J.M. Biochemical and physiological function of stearoyl-CoA desaturase. Am J Physiol Endocrinol Metab. 2009, 297(1), E28-E37.

Paigen, B., Ishida, B.Y., Verstuyft, J., Winters, R.B., Albee, D. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. Arteriosclerosis. 1990, 10, 316-323.

Patisaul, H.B., Jefferson, W. The pros and cons of phytoestrogens. Frontiers in neuroendocrinology, 2010, 31(4), 400-419.

Perlman, R.L. Mouse models of human disease: An evolutionary perspective. Evol Med Public Health. 2016,21,2016(1),170-176.

Pueppke, J.L. The genetics and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol. 1996,16, 1-51.

Rajala, M. W., Obici, S., Scherer, P. E., Rossetti, L. Adipose derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. J Clin Invest. 2003, 111, 225−230.

Ramdath, D.D., Padhi, E.M., Sarfaraz, S., Renwick, S., Duncan, A.M. Beyond the Cholesterol-Lowering Effect of Soy Protein: A Review of the Effects of Dietary Soy and Its Constituents on Risk Factors for Cardiovascular Disease. Nutrients. 2017,24,9(4).

Reagan-Shaw, S., Nihal, M., Ahmad, N. Dose translation from animal to human studies revisited. FASEB J. 2008, 22 (3), 659−661.

Renaud, H.J., Cui, J.Y., Lu, H., Klaassen, C.D. Effect of diet on expression of genes involved in lipid metabolism, oxidative stress, and inflammation in mouse liver-insights into mechanisms of hepatic steatosis. PLoS One. 2014,14,9(2),e88584.

Rahman, S. M., Janssen, R. C., Choudhury, M., Baquero, K. C., Aikens, R. M., Becky, A., & Friedman, J. E. CCAAT/Enhancer-binding Protein β (C/EBPβ) Expression Regulates Dietary-induced Inflammation in Macrophages and Adipose Tissue in Mice. J Biol Chem. 2012, 287(41), 34349-34360.

Ricketts, M.L., Moore, D.D., Banz, W.J., Mezei, O., Shay, N.F. Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. J Nutr Biochem. 2005,16(6):321-330.

Roberts, C.K., Kunal, K.S. Oxidative stress and metabolic syndrome. Life Sciences. 2009,21,705-712.

Ronis, M.J., Little, J.M., Barone, G.W., Chen, G., Radominska-Pandya, A., Badger, T.M. Sulfation of the isoflavones genistein and daidzein in human and rat liver and gastrointestinal tract. J Med Food. 2006,9(3), 348-355.

Ronis, M.J. Effects of soy containing diet and isoflavones on cytochrome P450 enzyme expression and activity. Drug Metab Rev. 2016,48(3):331-341.

Rowland, I., Faughnan, M., Hoey, L., Wahala, K., Williamson, G., Cassidy, A. Bioavailability of phyto-oestrogens. Br J Nutr. 2003,89, Suppl 1,S45-58.

Rozman, K.K., Bhatia, J., Calafat, A.M., Chambers, C., Culty, M., Etzel, R.A., Flaws, J.A., Hansen, D.K., Hoyer, P.B., Jeffery EH, Kesner JS, Marty S, Thomas JA, Umbach D. NTP-CERHR expert panel report on the reproductive and developmental toxicity of genistein. Birth Defects Res B Dev Reprod Toxicol. 2006, 77,485-638.

Savard, C., Tartaglione, E.V., Kuver, R., Haigh, W.G., Farrell, G.C., Subramanian, S., Chait, A., Yeh, M.M., Quinn, L.S., Ioannou, G.N. Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis. Hepatology. 2013,58,81-91.

Setchell, K.D., Zimmer-Nechemias, L., Cai, J., Heubi, J.E. Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. Am J Clin Nutr. 1998, 68:1453S-1461S.

Setchell, K.D., Brown, N.M., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S. Heubi, J.E. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am J Clin Nutr. 2002,76, 447-453.

Setchell, K.D., Brown, N.M., Lydeking-Olsen, E. The clinical importance of the metabolite equol - a clue to the effectiveness of soy and its isoflavones. J Nutr. 2002,132, 3577-3584.

Setchell, K.D., Brown, N.M., Desai, P.B., Zimmer-Nechimias, L., Wolfe, B., Jakate, A.S., Creutzinger, V., Heubi, J.E. Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. J Nutr. 2003, 133,1027-1035.

Setchell, K.D., Brown, N.M., Summer, S., King, E.C., Heubi, J.E., Cole, S., Guy, T., Hokin, B. Dietary factors influence production of the soy isoflavone metabolite s-(-)equol in healthy adults. J Nutr. 2013,143(12),1950-1958.

Sfakianos, J., Coward, L., Kirk, M., Barnes, S. Intestinal uptake and biliary excretion of the isoflavone genistein in rats. J Nutr. 1997, 127(7),1260-1268.

Shoelson, S.E., Jongsoon, L., Allison, B.G. Inflammation and insulin resistance. J Clin Invest. 2006,7,1793-1801.

Simons, A.L., Renouf, M., Hendrich, S., Murphy, P.A. Metabolism of glycitein (7,4'-dihydroxy-6-methoxy-isoflavone) by human gut microflora. J Agric Food Chem. 2005, 53,8519-8525.

Siri-Tarino, P.W., Sun, Q., Hu, F.B., Krauss, R.M. Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. Am J Clin Nutr. 2010, 91, 535−546.

Smith, S. R., Bai, F., Charbonneau, C., Janderová, L., Argyropoulos, G. A promoter genotype and oxidative stress potentially link resistin to human insulin resistance. Diabetes. 2003, 52, 1611−1618.

Smith, S.R., de Jonge, L., Volaufova, J., Li, Y., Xie, H., Bray, G.A. Effect of pioglitazone on body composition and energy expenditure: a randomized controlled trial. Metabolism. 2005, 54, 24-32.

Song, K.B., Atkinson, C., Frankenfeld, C.L., Jokela, T., Wähälä, K., Thomas, W.K., Lampe, J.W. Prevalence of daidzein-metabolizing phenotypes differs between Caucasian and Korean American women and girls. J Nutr. 2006,136,1347-1351.

Soyal, S.M., Nofziger, C., Dossena, S., Paulmichl, M., Patsch, W. Targeting SREBPs for treatment of the metabolic syndrome. Trends Pharmacol Sci. 2015,36(6),406-416.

Staels, B., Fruchart, J.C. Therapeutic roles of peroxisome proliferator-activated receptor agonists. Diabetes. 2005, 54(8),2460-70.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., Lazar, M. A. The hormone resistin links obesity to diabetes. Nature. 2001, 409, 307−312.

Stern, M.P., Williams, K., González-Villalpando, C., Hunt, K.J. and Haffner, S.M. Does the metabolic syndrome improve identification of individuals at risk of type 2 diabetes and/or cardiovascular disease? Diabetes Care. 2004, 27(11), 2676-2681.

Surwit, R.S., Kuhn, C.M., Cochrane, C., McCubbin, J.A. and Feinglos, M.N., 1988. Diet-induced type II diabetes in C57BL/6J mice. Diabetes. 1988, 37(9),1163-1167.

Svensson, S., Ostberg, T., Jacobsson, M., Norström, C., Stefansson, K., Hallén, D., Johansson, I.C., Zachrisson, K., Ogg, D., Jendeberg, L. Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. EMBO J. 2003, 15, 22(18):4625-4633.

Tall, A. R. Plasma cholesteryl ester transfer protein. J Lipid Res. 1993, 34, 1255-1274.

Tang, C., Zhang, K., Zhao, Q., Zhang, J. Effects of dietary genistein on plasma and liver lipids, hepatic gene expression, and plasma metabolic profiles of hamsters with diet-induced hyperlipidemia. J Agric Food Chem. 2015, 63 (36), 7929−7936.

Takamatsu, K., Tachibana, N., Matsumoto, I., Abe, K. Soy protein functionality and nutrigenomic analysis. Biofactors. 2004,21,49-53.

Taku, K., Lin, N., Cai, D., Hu, J., Zhao, X., Zhang, Y., Wang, P., Melby, M.K., Hooper, L., Kurzer, M.S., Mizuno, S., Ishimi, Y., Watanabe, S. Effects of soy isoflavone extract supplements on blood pressure in adult humans: systematic review and meta-analysis of randomized placebo-controlled trials. J Hypertens. 2010,28(10),1971-1982.

Taku, K., Melby, M.K., Kronenberg, F., Kurzer, M.S., Messina, M. Extracted or synthesized soybean isoflavones reduce menopausal hot flash frequency and severity: systematic review and meta-analysis of randomized controlled trials. Menopause. 2012, 19(7):776-790.

Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., Watanabe, M., Magoori, K., Ioka, R.X., Tachibana, K., Watanabe, Y. Activation of peroxisome proliferator-activated receptor δ induces fatty acid β-oxidation in skeletal muscle and attenuates metabolic syndrome. Proc Natl Acad Sci USA. 2003, 100, 15924-15929.

Tanaka, M., Fujimoto, K., Chihara, Y., Torimoto, K., Yoneda, T., Tanaka, N., Hirayama, A., Miyanaga, N., Akaza, H., Hirao, Y. Isoflavone supplements stimulated the production of serum equol and decreased the serum dihydrotestosterone levels in healthy male volunteers. Prostate Cancer Prostatic Dis. 2009,12, 247-252.

Tangirala, R.K., Bischoff, E.D., Joseph, S.B., Wagner, B.L., Walczak, R., Laffitte, B.A., Daige, C.L., Thomas, D., Heyman, R.A., Mangelsdorf, D.J., Wang, X. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. P Natl Acad Sci. 2002, 99(18),11896-11901.

Teixeira, S.R., Tappenden, K.A., Carson, L. Isolated soy protein consumption reduces urinary albumin excretion and improves the serum lipid profile in men with type 2 diabetes mellitus and nephropathy. J Nutr. 2004,134,1874-1880.

Tham, D.M., Gardner, C.D., Haskell, W.L. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocrinol Metab. 1998,83(7), 2223-2235.

Thornton, S.J., Wong, E., Lee, S.D., Wasan, K.M. Effect of dietary fat on hepatic liver X receptor expression in P-glycoprotein deficient mice: implications for cholesterol metabolism. Lipids Health Dis. 2008, 11,7,21.

Tiniakos, D.G., Vos, M.B., Brunt, E.M. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annu Rev Pathol Mech Dis. 2010, 5, 145−171.

Videla, L.A., Pettinelli, P. Misregulation of PPAR Functioning and Its Pathogenic Consequences Associated with Nonalcoholic Fatty Liver Disease in Human Obesity. PPAR Res. 2012,2012,107434.

Torre-Villalvazo, I., Tovar, A.R., Ramos-Barragan, V.E., Cerbon-Cervantes, M.A., Torres, N. Soy protein ameliorates metabolic abnormalities in liver and adipose tissue of rats fed a high fat diet. J Nutr. 2008,138,462-468.

Tovar, A.R., Murguía, F., Cruz, C., Hernández-Pando, R., Aguilar-Salinas, C.A., Pedraza-Chaverri, J., Correa-Rotter, R., Torres, N. A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome. J Nutr. 2002,132(9),2562-2569.

Tovar, A.R., Torre-Villalvazo, I., Ochoa, M., Elías, A.L., Ortíz, V., Aguilar-Salinas, C.A., Torres, N. Soy protein reduces hepatic lipotoxicity in hyperinsulinemic obese Zucker fa/fa rats. J Lipid Res. 2005,46(9),1823-1832.

Unger, R. H. Lipotoxic diseases. Annu Rev Med. 2002, 53, 319-336.

USDA data, 2016-2017. http://www.globalsoybeanproduction.com/

Vaure, C., Liu, Y.Q. A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. Front Immunol. 2014, 5,316.

Vozarova, B., Weyer, C., Lindsay, R.S., Pratley, R.E., Bogardus, C., Tataranni, P.A. High white blood cell count is associated with a worsening of insulin sensitivity and predicts the development of type 2 diabetes. Diabetes. 2002,51(2),455-461.

Wang, N., Tall, A.R. Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. Arterioscler Thromb Vasc Biol. 2003, 23,1178–1184.

Wong, S.K., Chin, K.Y., Suhaimi, F.H., Fairus, A. and Ima-Nirwana, S. Animal models of metabolic syndrome: a review. Nutr Metab. 2016,13(1), 65.

Wójcicka, G., Jamroz-Wiśniewska, A., Horoszewicz, K., Bełtowski, J. Liver X receptors (LXRs). Liver X receptors (LXRs). Part I: Structure, function, regulation of activity, and role in Postepy Hig Med Dosw. 2007, 61, 736-759.

Xu, W.H., Zheng, W., Xiang, Y.B., Ruan, Z.X., Cheng, J.R., Dai, Q., Gao, Y.T., Shu, X.O. Soya food intake and risk of endometrial cancer among Chinese women in Shanghai: population based case-control study. 2004, Bmj 328:1285.

Xu, X., Harris, K.S., Wang, H.J., Murphy, P.A., Hendrich, S. Bioavailability of soybean isoflavones depends upon gut microflora in women. J Nutr. 1995,125,2307-2315.

Yang, Y., Xiao, M., Mao, Y., Li, H. Resistin and insulin resistance in hepatocytes: resistin disturbs glycogen metabolism at the protein level. Biomed Pharmacother. 2009, 63, 366−374.

Yang, Z.H., Miyahara, H., Takeo, J., Katayama, M. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetol Metab Syndr. 2012,4,32.

Yao-Borengasser, A., Rassouli, N., Varma, V., Bodles, A.M., Rasouli, N., Unal, R., Phanavanh, B., Ranganathan, G., McGehee, RE. Jr., Kern, P.A. Stearoyl-coenzyme A desaturase 1 gene expression increases after pioglitazone treatment and is associated with peroxisomal proliferator-activated receptor-gamma responsiveness. J Clin Endocrinol Metab. 2008,93(11),4431-4439.

Yu, L., Li-Hawkins, J., Hammer, R.E., Berge, K.E., Horton, J.D., Cohen, J.C., Hobbs, H.H. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest. 2002,110, 671-680.

Yuan, J.P., Wang, J.H., Liu, X. Metabolism of dietary soy sioflavone to equol by human intestinal microflora-implications for health. Mol Nutr Food Res. 2007, 51, 765-781.

Zand, R.S., Jenkins, D.J., Diamandis, E.P. Steroid hormone activity of flavonoids and related compounds. Breast Cancer Res Treat. 2000, 62,35-49.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., Kagan, J.C. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell. 2011, 147(4),868-880.

Zeng, L., Tang, W.J., Yin, J.J., Zhou, B.J. Signal transductions and nonalcoholic fatty liver: a mini-review. Int J Clin Exp Med. 2014,7(7),1624.

Zhang, Y., Lee, F.Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F.J., Willson, T.M., Edwards, P.A. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. Proc Natl Acad Sci U S A. 2006,103,1006-1011.

Zhang, Y. B., Chen, W. H., Guo, J. J., Fu, Z. H., Yi, C., Zhang, M., Na, X. L. Soy isoflavone supplementation could reduce body weight and improve glucose metabolism in non-Asian postmenopausal women-a meta-analysis. Nutrition. 2013, 29 (1), 8−14.

Zhou, C., Tabb, M.M., Nelson, E.L., Grün, F., Verma, S., Sadatrafiei, A., Lin, M., Mallick, S., Forman, B.M., Thummel, K.E., Blumberg, B. Mutual repression between steroid and xenobiotic

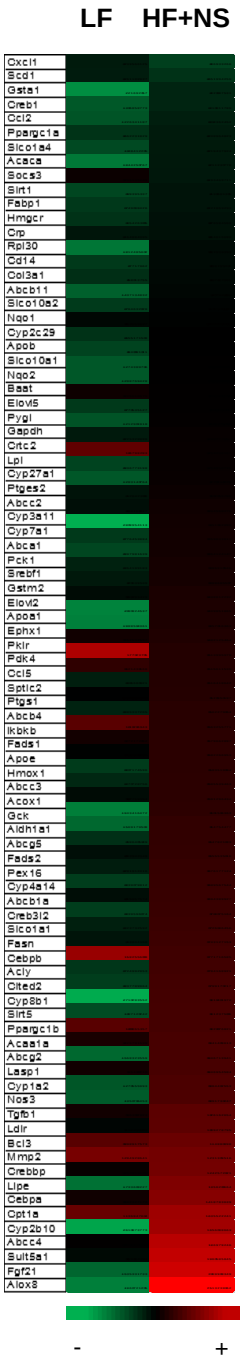
receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. J Clin Invest. 2006,116,2280-2289.

Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R.M., Xie, W. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. J Biol Chem. 2006,281,15013-15020.

Zivkovic, A.M., German, J.B., Sanyal, A.J. Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. Am J Clin Nutr. 2007,86(2),285-300.

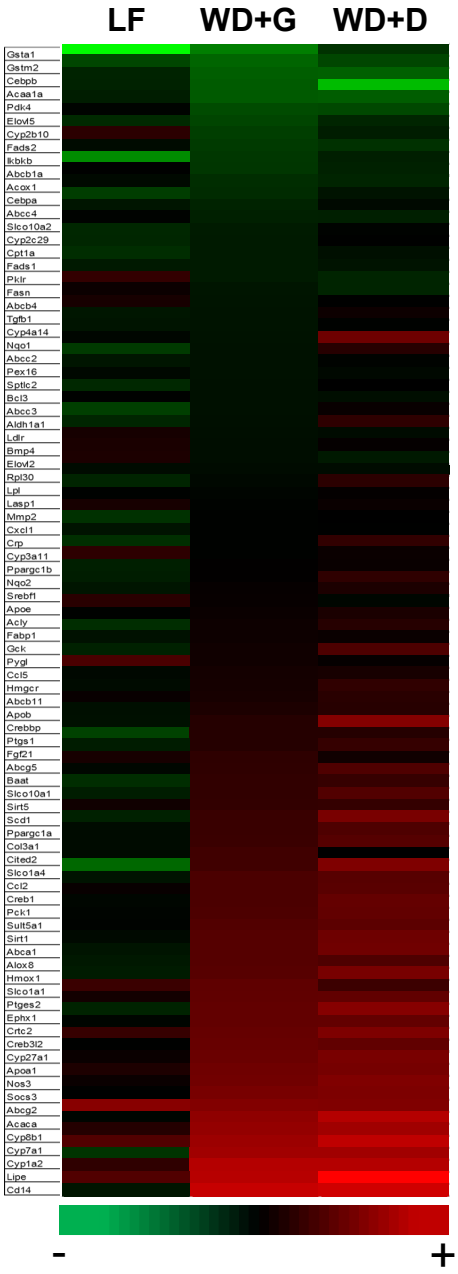
Appendix

Figure A1 Heat map of hepatic gene expression in LF- and HF+NS- fed mice



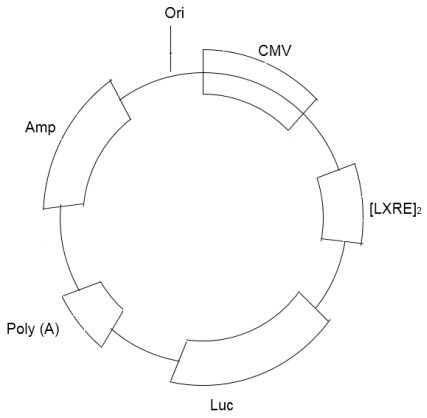
HF group was set as the control group. The figure is generated from data in Table A2.1.2 ($p < 0.05$). Green indicates downregulation relative to HF group. Red indicates upregulation (n=6).

Figure A2 Heat map of LF-, WD+G-, and WD+D- fed mice, relative to WD-fed group

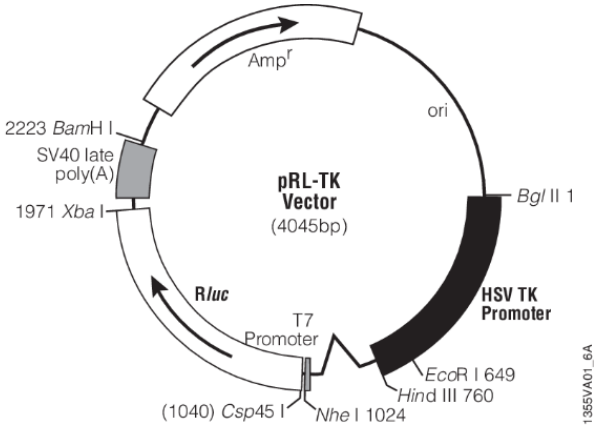


The map is generated from 87 genes different from WD group. The green color indicated the downregulation relative to WD group. The red color indicated the upregulation (n=6).

Figure A3 Structures of LXRE-Luc reporter plasmid, and pRL-TK-Luc control plasmid.



LXRE-Luc reporter plasmid



pRL-TK-Luc plasmid GenBank® Accession Number AF025846

Table A1 Serum cytokine concentrations in LF-, HF-, or HF+NS- fed mice after 10 weeks feeding[1]

	LF	HF	HF+NS
Eotaxin (pg/ml)	467±102[a]	468±88[a]	519±76[a]
CRP (ng/ml)	0.40±0.06[a]	0.70±0.04[b]	0.66±0.12[b]
G-CSF (pg/ml)	132±19[a]	224±38[a]	142±21[a]
IFN-g (pg/ml)	5.05±0.56[b]	4.01±0.45[ab]	2.72±0.33[a]
IL-1a (pg/ml)	121±50[a]	258±102[a]	244±113[a]
IL-1b (pg/ml)	10±2[a]	15±5[a]	51±41[a]
IL-9 (pg/ml)	111±57[a]	82±36[a]	277±73[a]
IL-13(pg/ml)	108±27[a]	161±25[a]	93±13[a]
LIX(pg/ml)	11500±2200[a]	11700±2500[a]	13700±4000[a]
IP-10(pg/ml)	197±35[a]	174±13[a]	135±17[a]
KC (pg/ml)	77±21[a]	138±23[a]	64±18[a]
MIP-1b (pg/ml)	54±10[b]	46±10[ab]	20±7[a]
M-CSF(pg/ml)	10±2[a]	15±2[a]	21±14[a]
MIP-2 (pg/ml)	113±22[a]	110±29[a]	81±34[a]
MIG (pg/ml)	120±30[a]	48±7[a]	71±33[a]
RANTES (pg/ml)	10±2[a]	13±2[a]	16±4[a]

[1]Serum cytokine levels in LF, HF, and HF+NS groups were determined by ELISA. Within each line, each group with the same superscript letter is not significantly different from each other, $p<0.05$, n=8.

Table A2 Hepatic gene expression in LF- and HF+NS- fed mice relative to HF[1]

	LF/HF		HF+NS/HF	
Symbol	**Fold Change**	*p* value	**Fold Change**	*p* value
Cxcl1	0.8	0.4	0.5	0.4
Scd1	0.8	0.5	0.6	0.3
Gsta1	0.2	0.1	0.7	0.3
Creb1	0.4	0.009	0.8	0.6
Ccl2	0.4	0.2	0.8	0.4
Ppargc1a	0.6	0.09	0.8	0.7
Slco1a4	0.5	0.09	0.9	0.6
Acaca	0.3	0.3	0.9	0.9
Socs3	1.2	0.6	0.9	0.7
Sirt1	0.5	0.05	0.9	0.9
Fabp1	0.6	0.002	0.9	0.8
Hmgcr	0.6	0.04	0.9	0.7
Crp	0.9	1.0	1.0	0.8
Cd14	0.3	0.02	1.0	0.9
Col3a1	0.6	0.8	1.0	0.6
Abcb11	0.7	0.4	1.0	0.6
Slco10a2	0.4	0.02	1.1	0.5
Nqo1	0.6	0.09	1.1	1.0
Msr1	0.7	0.1	1.1	0.6
Cyp2c29	1.1	0.6	1.1	0.4
Apob	0.6	0.1	1.1	0.4
Slco10a1	0.5	0.01	1.1	0.4
Nqo2	0.4	0.004	1.2	0.7
Baat	0.4	0.009	1.2	0.3
Elovl5	1.3	0.3	1.2	0.5
Pygl	0.6	0.02	1.2	0.2
Crtc2	0.9	0.8	1.2	0.4
Lpl	2.1	0.3	1.2	0.4
Cyp27a1	0.5	0.4	1.2	0.3
Ptges2	0.4	0.003	1.2	0.2
Abcc2	0.9	0.9	1.2	0.4
Cyp3a11	1.0	0.8	1.3	0.2
Cyp7a1	0.1	0.001	1.3	0.4
Abca1	0.6	0.2	1.3	0.2
Pck1	0.5	0.3	1.3	0.7
Srebf1	0.8	0.2	1.3	0.2
Gstm2	0.9	0.3	1.3	0.4
Elovl2	1.0	0.7	1.4	0.3
Apoa1	0.2	0.002	1.4	0.3
Ephx1	0.3	0.01	1.4	0.2
Pklr	1.3	0.2	1.4	0.2
Pdk4	3.4	0.4	1.4	0.3
Ccl5	1.5	0.03	1.4	0.1

Sptlc2	0.8	0.09	1.4	0.08
Ptgs1	1.1	0.3	1.4	0.06
Abcb4	0.8	0.2	1.5	0.06
Ikbkb	2.0	0.03	1.5	0.3
Fads1	1.3	0.4	1.5	0.2
Apoe	1.1	0.8	1.5	0.1
Hmox1	0.6	0.3	1.5	0.2
Abcc3	0.7	0.5	1.5	0.3
Acox1	1.0	1.0	1.5	0.2
Gck	0.3	0.002	1.5	0.2
Aldh1a1	0.3	0.0007	1.6	0.06
Abcg5	0.7	0.2	1.6	0.09
Fads2	1.0	1.0	1.6	0.3
Pex16	0.8	0.4	1.6	0.01
Cyp4a14	0.6	0.1	1.6	0.6
Abcb1a	1.0	0.7	1.6	0.2
Creb3l2	0.6	0.5	1.6	0.1
Slco1a1	0.8	0.3	1.7	0.1
Fasn	0.9	1.0	1.7	0.2
Cebpb	3.0	0.01	1.7	0.3
Acly	0.6	0.5	1.7	0.07
Cited2	0.6	0.03	1.7	0.04
Cyp8b1	0.2	0.06	1.8	0.2
Sirt5	0.5	0.07	1.8	0.09
Ppargc1b	2.0	0.1	1.8	0.2
Acaa1a	1.3	0.3	1.8	0.2
Abcg2	0.3	0.02	1.8	0.2
Lasp1	1.3	0.3	1.8	0.04
Cyp1a2	0.4	0.02	1.8	0.08
Nos3	0.4	0.3	1.9	0.7
Bmp4	1.7	0.09	1.9	0.06
Tgfb1	1.3	0.08	2.0	0.01
Ldlr	1.1	0.7	2.1	0.09
Bcl3	2.0	0.1	2.2	0.06
Mmp2	2.4	0.2	2.3	0.07
Crebbp	1.2	0.4	2.4	0.02
Hsl	0.3	0.07	2.6	0.04
Cebpa	1.3	0.2	2.7	0.02
Cpt1a	2.2	0.02	3.1	0.005
Cyp2b10	0.2	0.01	3.2	0.2
Abcc4	1.2	0.4	3.7	0.1
Sult5a1	1.0	0.9	3.9	0.005
Fgf21	0.3	0.1	4.2	0.2
Alox8	0.3	0.001	5.7	0.2

[1]Fold change and *p* value are shown. *Rpl30* was set as the housekeeping gene. $p<0.05$, n=6.

Table A3 Hepatic gene expression in LF-, WD+G-, and WD+D-fed mice relative to WD[1]

	LF/WD		WD+G/WD		WD+D/WD	
Symbol	Fold Change	*p* value	Fold Change	*p* value	Fold Change	*p* value
Gsta1	0.01	0.002	0.1	0.02	0.5	0.2
Gstm2	0.3	0.0007	0.2	0.002	0.3	0.01
Cebpb	1.1	0.5	0.3	0.01	0.3	0.01
Acaa1a	0.6	0.02	0.4	0.02	0.6	0.2
Pdk4	1.9	0.3	0.4	0.1	0.7	0.3
Elovl5	1.	1.0	0.4	0.09	0.5	0.2
Cyp2b10	0.1	0.007	0.5	0.1	0.7	0.5
Fads2	1.2	1.0	0.5	0.09	0.7	0.3
Ikbkb	1.04	0.5	0.5	0.03	0.6	0.05
Abcb1a	0.4	0.02	0.6	0.3	0.9	0.7
Acox1	0.8	0.3	0.7	0.1	1.03	0.7
Cebpa	1.2	0.8	0.7	0.1	0.7	0.2
Abcc4	0.6	0.2	0.8	0.4	1.2	0.8
Slco10a2	0.6	0.2	0.8	0.3	1.2	0.5
Cyp2c29	0.5	0.002	0.8	0.1	0.9	0.5
Cpt1a	0.8	0.5	0.8	0.2	0.9	0.4
Fads1	2.1	0.1	0.8	0.3	0.6	0.3
Pklr	1.4	0.08	0.8	0.3	0.6	0.05
Fasn	1.6	0.2	0.9	0.5	1.2	0.8
Abcb4	0.8	0.2	0.9	0.3	1.4	0.3
Tgfb1	0.8	0.2	0.9	0.5	1.2	0.7
Cyp4a14	1.08	0.7	0.9	0.9	4.01	0.05
Nqo1	0.4	0.005	0.9	0.6	1.8	0.04
Abcc2	0.8	0.4	0.9	0.5	1.2	0.9
Pex16	1.06	0.9	0.9	0.4	1.05	0.7
Sptlc2	0.6	0.01	0.9	0.5	1.2	0.5
Bcl3	1.2	1.0	0.9	0.3	1.0	0.3
Abcc3	0.4	0.02	0.9	0.6	1.3	0.5
Aldh1a1	0.6	0.1	0.9	0.5	2.0	0.2
Ldlr	1.5	0.2	1.0	0.5	1.01	0.7
Bmp4	1.6	0.1	1.0	0.6	1.3	0.7
Elovl2	1.7	0.04	1.0	0.6	0.8	0.2
Lpl	0.6	0.8	1.0	0.9	1.9	0.01
Lasp1	1.2	0.7	1.1	1.0	1.3	0.7
Mmp2	1.6	0.4	1.2	0.8	1.4	0.9
Cxcl1	0.5	0.003	1.2	0.2	1.2	0.3
Crp	0.9	0.8	1.2	0.4	1.2	0.3
Cyp3a11	0.5	0.005	1.2	0.5	2.07	0.0005
Ppargc1b	2.0	0.008	1.2	0.9	1.4	0.5
Nqo2	0.7	0.04	1.2	0.3	1.3	0.2
Srebf1	0.7	0.1	1.2	0.8	2.02	0.05
Apoe	0.8	0.2	1.3	1.0	1.7	0.3
Acly	1.9	0.1	1.3	0.7	1.09	0.9

Fabp1	1.2	0.2	1.4	0.06	1.6	0.0006
Gck	0.5	0.05	1.4	0.3	1.8	0.03
Pygl	0.9	0.7	1.4	0.02	1.4	0.04
Ccl5	0.7	0.04	1.5	0.2	2.8	0.01
Hmgcr	2.7	0.0006	1.5	0.09	1.3	0.3
Abcb11	1.05	0.7	1.5	0.1	1.6	0.1
Apob	1.0	0.9	1.5	0.09	2.04	0.002
Crebbp	1.3	0.2	1.6	0.04	1.9	0.004
Ptgs1	0.9	0.4	1.7	0.09	1.9	0.01
Fgf21	0.9	0.7	1.8	0.4	5.08	0.01
Abcg5	0.4	0.006	1.8	0.06	1.8	0.06
Baat	0.7	0.5	1.8	0.02	2.2	0.002
Slco10a1	1.6	0.03	2.007	0.0004	1.5	0.02
Sirt5	1.07	0.9	2.01	0.1	2.9	0.007
Scd1	0.5	0.02	2.06	0.003	2.2	0.003
Ppargc1a	1.4	0.06	2.09	0.005	2.2	0.002
Col3a1	0.7	0.9	2.3	0.1	4.5	0.003
Cited2	1.0	1.0	2.3	0.001	2.8	0.0002
Msr1	1.0	1.0	2.3	0.003	3.05	0.00001
Slco1a4	1.0	0.8	2.4	0.007	1.2	0.9
Ccl2	0.2	0.005	2.5	0.02	4.8	0.007
Creb1	0.9	0.6	2.7	0.00007	3.1	0.000004
Pck1	1.3	0.2	2.8	0.002	3.2	0.09
Sult5a1	1.09	0.9	2.8	0.008	3.6	0.0001
Sirt1	1.2	0.4	3.0	0.0001	3.3	0.000009
Abca1	1.04	0.7	3.0	0.00005	4.01	0.00001
Alox8	0.9	0.2	3.06	0.002	4.1	0.1
Hmox1	0.7	0.7	3.1	0.0006	4.5	0.00000001
Slco1a1	2.3	0.003	3.4	0.000003	2.3	0.03
Ptges2	1.5	0.1	3.4	0.000005	3.5	0.000005
Ephx1	0.7	0.7	3.5	0.001	5.2	0.00005
Crtc2	2.2	0.09	3.6	0.001	4.8	0.00004
Creb3l2	1.4	0.2	3.8	0.007	4.5	0.0002
Cyp27a1	1.7	0.2	4.0	0.0007	4.4	0.0005
Apoa1	1.4	0.4	4.1	0.001	4.9	0.0002
Nos3	1.2	0.7	4.5	0.20	4.7	0.2
Socs3	5.3	0.0004	5.0	0.03	4.9	0.02
Abcg2	1.1	0.9	5.8	0.007	8.6	0.0004
Acaca	1.8	0.09	6.04	0.0002	6.9	0.0002
Cyp8b1	2.9	0.07	6.6	0.003	9.7	0.0009
Cyp7a1	0.5	0.2	7.0	0.00002	7.0	0.000004
Cyp1a2	2.0	0.2	8.5	0.000009	8.5	0.000009
Hsl	2.9	0.07	8.9	0.000006	19.3	0.000000001
Cd14	0.8	0.6	10.4	0.00000001	11.5	0.00005

[1]Fold change and *p* value are shown. *Rpl30* gene was set as the control gene.

Table A4 Hepatic gene expression in HF-, and WD- fed mice relative to LF[1]

	HF/LF		WD/LF	
Symbol	Fold Change	*p* value	Fold Change	*p* value
Cebpb	0.3	0.002	0.9	0.5
Pdk4	0.4	0.4	0.5	0.3
Mmp2	0.4	0.2	0.6	0.4
Ppargc1b	0.4	0.003	0.5	0.008
Bcl3	0.4	0.05	0.8	1.0
Ikbkb	0.4	0.002	1.0	0.5
Bmp4	0.5	0.005	0.6	0.1
Lpl	0.5	0.4	1.6	0.8
Socs3	0.6	0.07	0.2	0.0004
Ccl5	0.6	0.02	1.5	0.04
Fads1	0.7	0.2	0.5	0.1
Pklr	0.7	0.09	0.7	0.07
Lasp1	0.7	0.2	0.8	0.7
Acaa1a	0.7	0.3	1.8	0.02
Cebpa	0.7	0.05	0.9	0.8
Crebbp	0.7	0.2	0.8	0.2
Elovl5	0.8	0.4	1.0	1.0
Abcc4	0.8	0.4	1.7	0.2
Tgfb1	0.8	0.1	1.2	0.2
Apoe	0.8	0.5	1.2	0.2
Ldlr	0.8	0.6	0.6	0.2
Cpt1a	0.9	0.3	1.3	0.5
Cyp2c29	0.9	0.2	1.9	0.002
Ptgs1	0.9	0.3	1.1	0.4
Abcc2	0.9	0.6	1.2	0.4
Crtc2	0.9	0.6	0.5	0.09
Elovl2	0.9	0.6	0.6	0.04
Fads2	1.0	1.0	0.8	1.0
Abcb1a	1.0	0.8	2.5	0.02
Fasn	1.0	0.8	0.6	0.2
Cyp3a11	1.0	0.7	1.9	0.005
Acox1	1.0	0.9	1.2	0.3
Crp	1.1	0.9	1.2	0.8
Sult5a1	1.1	0.7	0.9	0.9
Cxcl1	1.1	0.3	2.0	0.003
Srebf1	1.1	0.5	1.4	0.1
Slco1a1	1.1	0.5	0.4	0.003
Scd1	1.2	0.3	1.9	0.02
Abcg5	1.2	0.8	2.7	0.006
Pex16	1.2	0.3	0.9	0.9
Sptlc2	1.3	0.05	1.7	0.01
Gstm2	1.3	0.1	2.9	0.0007
Abcc3	1.3	0.5	2.5	0.02

Creb3l2	1.4	1.0	0.7	0.2
Abcb4	14	0.1	1.2	0.2
Abcb11	1.4	0.4	1.0	0.7
Ppargc1a	1.5	0.1	0.7	0.06
Apob	1.5	0.2	1.0	0.9
Msr1	1.5	0.05	1.0	0.9
Acly	1.5	0.6	0.5	0.1
Abca1	1.6	0.2	1.0	0.7
Hmgcr	1.6	0.02	0.4	0.0006
Col3a1	1.7	0.9	1.5	0.9
Pygl	1.7	0.02	1.1	0.7
Cyp27a1	1.7	0.5	0.6	0.2
Sirt1	1.8	0.2	0.9	0.4
Cited2	1.8	0.02	1.0	1.0
Sirt5	1.8	0.2	0.9	0.9
Hmox1	1.8	0.2	1.3	0.7
Slco10a1	1.8	0.02	0.6	0.03
Fabp1	1.8	0.00004	0.9	0.2
Pck1	1.8	0.3	0.8	0.2
Nqo1	1.9	0.04	2.3	0.004
Slco1a4	2.0	0.07	1.0	0.8
B2m	2.2	0.02	1.4	0.4
Cyp1a2	2.2	0.04	0.5	0.2
Ptges2	2.3	0.007	0.7	0.1
Baat	2.4	0.006	1.4	0.5
Gapdh	2.6	0.0002	0.9	0.9
Creb1	2.7	0.002	1.1	0.6
Cyp4a14	2.7	0.04	0.9	0.7
Nqo2	2.7	0.0004	1.4	0.04
Nos3	2.8	0.2	0.8	0.7
Slco10a2	2.8	0.006	1.6	0.2
Acaca	3.0	0.4	0.6	0.09
Abcg2	3.1	0.006	0.9	0.9
Aldh1a1	3.2	0.00003	1.6	0.1
Lipe	3.3	0.05	0.3	0.07
Ccl2	3.5	0.04	5.4	0.004
Ephx1	3.9	0.007	1.5	0.7
Cd14	4.0	0.003	1.2	0.6
Apoa1	4.2	0.0004	0.7	0.4
Gck	4.3	0.00008	1.8	0.05
RTC	5.2	0.000002	0.9	0.6
Fgf21	5.2	0.03	1.1	0.7
Alox8	6.0	10^{-8}	1.2	0.2
MGDC	6.5	10^{-9}	1.3	0.1
Cyp8b1	6.5	0.03	0.3	0.07
Cyp7a1	7.0	0.0002	2.1	0.2
Gsta1	8.4	0.06	75.5	0.002
Cyp2b10	8.4	0.001	10.3	0.007

[1]Fold change and *p* value are shown. *Rpl30* gene was set as the control gene.

Table A5 Summary of C57BL/6J male mice diets

Ingredients (g)	LF	HF	WD	HF+NS	WD+G	WD+D	WD+NS
Casein	200	200	200	200	200	200	200
L-Cysteine	3	3	3	3	3	3	3
Corn Starch	506.2	0	72.8	0	72.8	72.8	72.8
Malt dextrin 10	125	125	100	125	100	100	100
Sucrose	68.8	68.8	172.8	68.8	172.8	172.8	172.8
Cellulose, BW200	50	50	50	50	50	50	50
Soybean Oil	25	25	25	25	25	25	25
Lard	20	245	177.5	245	177.5	177.5	177.5
Mineral Mix S10026	10	10	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, $1H_2O$	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2	2	2
Cholesterol	0	0	8.5	0	8.5	8.5	8.5
Novasoy	0	0	0	3.1	0	0	3.1
Genistein	0	0	0	0	1.5	0	0
Daidzein	0	0	0	0	0	1.5	0
kcal%							
Protein	20	20	18	20	18	18	18
Carbohydrate	70	20	36	20	36	36	36
Fat	10	60	46	60	46	46	46
Kcal/gm	3.8	5.2	4.59	5.2	4.59	4.59	4.59