Evaluation of sex differences in nutrient metabolism and response to nutritional and drug interventions in mice

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Abstract

Availability of nutrients and optimal nutrient metabolism are essential for maintaining health. Intrinsic factors (e.g., sex, gene variations) and extrinsic factors (e.g., diet, drug) affect nutrient metabolism, leading to different disease risks. To gain insight into sex-related effects of gene loss-of-function, the transcriptomes of the small intestine from male and female mice with functional or inactivated intestinal fatty acid-binding protein (Fabp) genes encoding Fabp2 and Fabp6 were compared. The analysis showed that male and female mice have inherently distinct intestinal transcriptome which may predetermine the sexually dimorphic responses to Fabp gene ablation. A mouse study was carried out to investigate possible sexual dimorphism in susceptibility to Western-style diet (WSD)-induced metabolic disorders and response to therapeutic intervention based on a low-fat diet without or with ursodeoxycholic acid (UDCA) supplementation. The results show that male mice have greater sensitivity than female mice to WSD-induced metabolic dysregulation leading to obesity, cellular stress, and fibrogenesis. Both male and female mice showed benefits from switching to a low-fat diet but supplementation of the low-fat diet with UDCA was more effective in ameliorating WSD-induced metabolic dysregulation especially in male mice. This study provides insights into the importance of sex in the pathogenesis and treatment of nutrition-related acquired metabolic diseases.

Résumé

La disponibilité des nutriments et le métabolisme optimal des nutriments sont essentiels au maintien de la santé. Les facteurs intrinsèques (p. ex., le sexe, les variations génétiques) et les facteurs extrinsèques (p. ex., l'alimentation, les médicaments) influencent le métabolisme des nutriments, ce qui entraîne des risques de maladie différents. Pour mieux comprendre les effets liés au sexe de la perte fonctionnelle des gènes, on a comparé les transcriptomes de l'intestin grêle des souris mâles et femelles ayant des gènes fonctionnels ou inactivés de l'intestinal fatty acid-binding protein (Fabp) codant pour Fabp2 et Fabp6. L'analyse a montré que les souris mâles et femelles ont un transcriptome intestinal intrinsèquement distinct qui peut prédéterminer les réponses sexuellement dimorphiques à l'ablation du gène Fabp. Une étude chez la souris a été menée pour étudier la possibilité d'un dimorphisme sexuel dans la susceptibilité aux troubles métaboliques induits par le régime occidental (WSD) et la réponse à une intervention thérapeutique basée sur un régime faible en gras sans ou avec une supplémentation en acide ursodésoxycholique (UDCA). Les résultats montrent que les souris mâles sont plus sensibles que les souris femelles à la dysrégulation métabolique induite par le WSD, ce qui entraîne l'obésité, le stress cellulaire et la fibrogénèse. Les souris mâles et femelles ont montré des avantages de passer à un régime faible en gras, mais la supplémentation de l'alimentation faible en gras avec UDCA était plus efficace pour améliorer la dysrégulation métabolique induite par le WSD, en particulier chez les souris mâles. Cette étude fournit des indications de l'importance du sexe dans la pathogenèse et le traitement des maladies métaboliques acquises liées à la nutrition.

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Contributions of authors

Manuscript 1:

Yiheng Chen, BSc, prepared a draft of the manuscript; Marek Michalak, PhD, and Luis B. Agellon, PhD, provided guidance on topic selection, manuscript outline construction, discussions during the writing process, and editing of the manuscript.

Manuscript 2:

Yiheng Chen, BSc, conducted the analysis, interpreted the results and prepared the first draft of the manuscript. Luis B. Agellon, PhD, designed the study, contributed to the interpretations of results, and in the writing and editing of the manuscript.

Manuscript 3:

Yiheng Chen, BSc, was involved in the design of the animal experiments, conducted experiments, analyzed data, and wrote the manuscript. Marek Michalak, PhD, and Luis B. Agellon, PhD, were involved in the generation of concept, development of the overall scheme of the study, and editing of the manuscript.

List of Abbreviations

Atf4: Activating transcription factor 4

ATF6: Activating transcription factor 6

BP: biological process

BSA: bovine serum albumin

CD36: cluster of differentiation 36

CDCA: chenodeoxycholic acid

Chop: C/EBP homologous protein

Col1a1: Collagen, type 1, alpha 1

CVD: cardiovascular disease

DE genes: differentially expressed genes

ED: energy density.

ER: endoplasmic reticulum

F: female

Fabp: Fatty acid-binding protein

FATP: fatty acid transport protein

FBS: fetal bovine serum

Foxp3: Forkhead box P3

FXR: farnesoid X receptor

GO: Gene Ontology

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase

Grp78: Glucose-regulated protein 78 kDa

Grp94: Glucose-regulated protein 94 kDa

Hsp47: Heat shock protein 47

IRE1: Inositol-requiring enzyme 1

M: male

mTOR: mechanistic target of rapamycin;

ND: nutrient diversity;

PBS: phosphate-buffered saline

PERK: Protein kinase-like ER kinase

PPAR: Peroxisome proliferator-activated receptors

qPCR: quantitative polymerase chain reaction

RER: respiratory exchange ratio

RMA: robust multichip average

SEM: standard error of the mean

Sirt1: Sirtuin-1

sXbp1: spliced X-box binding protein 1

TGF-β1: Transforming growth factor beta 1

TGR5: G-protein-coupled bile acid receptor

UDCA: ursodeoxycholic acid

UPR: unfolded protein response

WSD: Western-style diet

WT: wild-type

Chapter 1 Introduction and literature review

Part of this chapter was published in the *Yale Journal of Biology and Medicine. Chen, Y., Michalak, M., & Agellon, L. B. (2018). Focus: Nutrition and Food Science: Importance of Nutrients and Nutrient Metabolism on Human Health.* (PMID: 29955217)

1.1 Importance of nutrition in the development of metabolic diseases

Over the last several decades, many jurisdictions around the world have witnessed the increasing prevalence of acquired metabolic syndromes, in particular obesity, diabetes, fatty liver disease and cardiovascular diseases (1-4). In recent years, the upward trend is especially striking in developing countries where changes in diets and lifestyle accompany modernization (1, 5). To counter the increasing public health complications caused by changing nutrition practices, health organizations have provided dietary recommendations (6). Whereas past interventions designed to address cases of single nutrient deficiencies have achieved clear indicators of success (7), intervention trials that target a single class of nutrients to manage the emergence of metabolic diseases in the general population have not produced definitive results (8, 9). It is increasingly being realized that comprehensive analysis of what is being consumed together with the eating pattern, rather than focusing on single nutrients, may be more informative in formulating effective dietary recommendations.

Recent advances in high-throughput analysis have assisted in a better understanding of metabolism, and revealed the active role of nutrients and their metabolites in regulating gene expression and cellular function. Nutrients and their metabolites not only serve as building blocks of cellular structures and as fuel sources, but also serve as direct modifiers of protein function, potent signaling molecules as well as inducers and repressors of gene expression. Many of them participate in regulating gene expression by directly modulating the activities of transcription factors and by moderating the changes in epigenetic markings in the genome. One view that is emerging is that optimal cellular homeostasis is crucial for maintaining health and avoiding diseases caused by nutrient deficiency or excess. This review considers the importance of nutrient diversity and energy density on cellular metabolism in health and disease (Figure 1).

1.1.1 Quality of nutrition

Single nutrient interventions such as fortification of milk with vitamin D, cereal with iron, and table salt with iodine were effective in treating the corresponding nutrient deficiencies (10). However, when applied to acquired metabolic syndromes that prevail in modern societies, the same approach has yielded inconclusive results (11, 12). For example, decreasing dietary intakes of saturated fatty acid or cholesterol, and increasing the intake of omega-3 polyunsaturated fatty acids do not appear to be effective in reducing the risk of cardiovascular diseases (9, 13, 14). The importance of the entire diet that is consumed as a regular practice is being recognized, and an increasing number of studies are analyzing dietary pattern to identify possible causes of under- and over-nutrition. By definition, dietary pattern characterizes the overall diet by the quantities, the proportion, and the variety of foods and beverage as well as the frequency of consumption (15). The Mediterranean pattern and Western-style pattern are two commonly practiced dietary patterns. The Mediterranean diet contains a high proportion of fruits and vegetables, legumes, whole grains, fish and poultry with an emphasis on monounsaturated fats and antioxidants, whereas the Western-style diet is generally characterized by energy-dense foods like butter, high-fat dairy products, refined grains, as well as processed and red meat, leaving less space for other nutrients especially those coming from fruits and vegetables. Epidemiological studies have found that the

Mediterranean dietary pattern has preventive and protective effects against cardiovascular diseases (16, 17), whereas the Western-style dietary pattern is positively associated with dyslipidemia, obesity, hypertension, atherosclerosis, and diabetes (18, 19).

Nutrition transition refers to the shift of diet from traditional to modern along with an increase in sedentary behavior, that occur in conjunction with modernization. The concept of nutrition transition was initially proposed by Popkin (20, 21) to demonstrate how economic, demographic, and epidemiological changes interact with shifts in dietary consumption and energy expenditure. Interestingly, some countries in East Asia have lower prevalence of certain acquired metabolic syndromes compared to other societies at comparable stages of nutrition transition (2, 5, 22). This might be partially due to retention of traditional dietary patterns, which promote consumption of foods with a wider array of nutrients and lower energy density (23-25). However, the reasons underlying the discrepancy are complex, and extend beyond the chemical composition of food to include social and economic issues. Nevertheless, there is evidence supporting the idea that calorie restriction is beneficial for longevity (26-28), providing support for the potential benefit of low energy density diets (Figure 1A).

The prevailing global increase in the development of acquired metabolic syndromes is associated with nutrition transition (29, 30). One proposed concept that potentially explains the pathogenesis of these syndromes stems from persistent modification of cellular function in response to stress in the endoplasmic reticulum, mitochondria and other organelles that make up the cellular reticular network (31). Both nutrient deficiency (undernutrition) and nutrient excess (over-nutrition) cause the loss of nutrient/energy homeostasis and thus trigger cellular stress. Coping response mechanisms, such as the unfolded protein response mechanism (31-33), are activated to resolve stress. In the case of a maladaptive response, programmed cell death is activated to remove malfunctioning cells. On the other hand, persistent adjustment of cellular functions enables cells to cope even with continued exposure to stress inducers. Stress coping response mechanisms initially promote adaptive strategies to recover homeostasis in the short term but become pathogenic in the long term due to long-term modification of cellular functions.

1.1.2 Bioactivity of food components and their metabolites

Food is a complex combination of numerous components which can be classified into nutrients and non-nutrients. Nutrients have been traditionally classified as macronutrients and micronutrients. Plants and animals do not have identical nutrient requirements and produce nutrient metabolites that may not be common to each other. Micronutrients, which include vitamins and minerals, are needed in only small amounts, and are required for the proper function of important proteins and enzymes. Macronutrients, which include carbohydrates, proteins, and fats, are typically needed in large amounts. The benefits of consuming macronutrients are self-evident since their subunits serve as building blocks of cellular structures and as energy substrates in all organisms. Some species are unable to synthesize key metabolites needed for survival, and thus must obtain these from other species. These essential metabolites, along with minerals, make up a class of substances referred to as essential nutrients. Non-nutrient components of food are those that cannot be categorized as either macronutrients or micronutrients. These substances include both natural and synthetic compounds. They can be beneficial (e.g. fiber, and some polyphenolic compounds produced by plants), non-beneficial (e.g. many food additives, and preservatives) or even toxic (e.g. xenobiotics, and antibiotics, also some plant-derived polyphenolic compounds) (34, 35). It has become evident that both nutrients and non-nutrients, as well as their metabolites, have the capacity to modulate gene expression, protein function and epigenome (36-38).

The potential of macronutrients and their metabolites to regulate metabolic function is typically taken for granted. For example, the monosaccharide fructose is commonly used as a sweetener in commercially prepared foods and is present in these foods at exceedingly high amounts compared to natural foods (39). Fructose is known to stimulate de novo lipid synthesis in the liver and to induce endoplasmic reticulum stress in many cell types (40, 41). In general, excess glucose and fructose induce cellular stress which leads to the development of insulin resistance and fatty liver disease (40, 42, 43). Certain amino acids have been shown to act as signaling molecules to regulate cellular growth and proliferation via mTOR (mechanistic target of rapamycin) (44, 45), whose function has been implicated in many human diseases (46). Some fatty acids from fats and oils serve as ligands for G protein-coupled receptors as well as for transcription factors belonging to the nuclear receptor family of transcription factors (47, 48), and therefore regulate cellular processes and gene expression (49). Saturated fatty acids have long been the focus of investigation as high intake of saturated fats was considered to be a risk factor for cardiovascular diseases (50), however subsequent studies have not provided strong evidence for causality (9). This may be partly attributable to the wide range of biological activities associated with different fatty acids species (51). Palmitic acid, a fatty acid species that is enriched in the Western-style diet, is a potent inducer of endoplasmic reticulum stress whereas oleic acid, a fatty acid prominent in the Mediterranean diet, has been shown to inhibit endoplasmic reticulum stress (52, 53). Importantly, the surplus of nutrients and energy induce endoplasmic reticulum stress and inflammatory responses that lead to systemic metabolic dysregulation (31, 32).

Many metabolic diseases caused by micronutrient deficiencies can be corrected by restoring the missing micronutrients in the diet (54, 55). One critical aspect of micronutrient supplementation applied to the general population is overdose. Some of these compounds are potent modulators of nuclear receptors and have serious impacts on the activities of multiple metabolic pathways. For example, deficiency of vitamin A can lead to blindness while its excess is teratogenic. Vitamin D also modulates the expression of many genes that participate in many pathways (56), and its deficiency causes rickets. However, it is not yet known if it is possible to overdose with this micronutrient. Excessive dietary intake of minerals can be equally deleterious, as exemplified by diet-induced hypertension due to high intake of sodium (57).

Other metabolites produced by the mammalian metabolic machinery also play critical roles in metabolism. For example, cholesterol serves as a membrane component, signaling molecule, and precursor for the synthesis of steroid hormones and bile acids (58). Bile acids aid in the absorption of dietary fats and lipid-soluble compounds, and also act as signaling molecules modulating macronutrient and energy metabolism, inflammatory responses, and detoxification through intracellular ligand-activated nuclear receptors (59). Gut bacteria are capable of metabolizing bile acids and one of the products is a secondary bile acid referred to as ursodeoxycholic acid. It is of interest to note that this bile acid and its taurine-conjugated derivative can alleviate endoplasmic reticulum stress by promoting proteostasis (60), and has been shown to be effective in preventing cardiac fibrosis (61, 62).

The non-nutrient components of food can be beneficial or non-beneficial. Beneficial ones include fiber and certain types of plant polyphenolic compounds. Dietary fiber, derived from plant-based foods, is not an effective nutrient for humans, but serves as a nutrient for gut microflora. Some of the products generated from dietary fiber include short chain fatty acids (e.g., butyric and propionic acids) that are absorbed in the lower gut and serve as both energy substrates and regulators of host metabolism (63, 64). Plant polyphenolic compounds have been popularized as anti-oxidants. However, there are numerous polyphenolic compounds present in plants, and these compounds likely have a wide range of biological activities and effects on human metabolism (65, 66). A polyphenolic-rich extract prepared from potatoes exhibits beneficial activity by attenuating weight gain in mice fed with obesity-inducing high fat diet (67). There is also emerging evidence for the modulating effect of polyphenols on the composition and metabolic activity of gut microbiota that provides potential benefits to the host (68). Not all polyphenolic compounds are beneficial, as some compounds such as caffeic acid and genistein may be carcinogenic or genotoxic at high dosage (34, 69, 70). It is commonly assumed that synthetic food additives (colorants, preservatives, sweeteners) do not have effects on metabolism, but this assumption should be tested regularly to ensure food safety. Other xenobiotics (e.g. pollutants, drugs, and agricultural chemicals) that find their way into the food supply can influence human health directly, by disrupting normal metabolic processes, or indirectly, by influencing the composition of the gut microbiota (71). All food components likely work together to drive metabolic processes in every cell of the body.

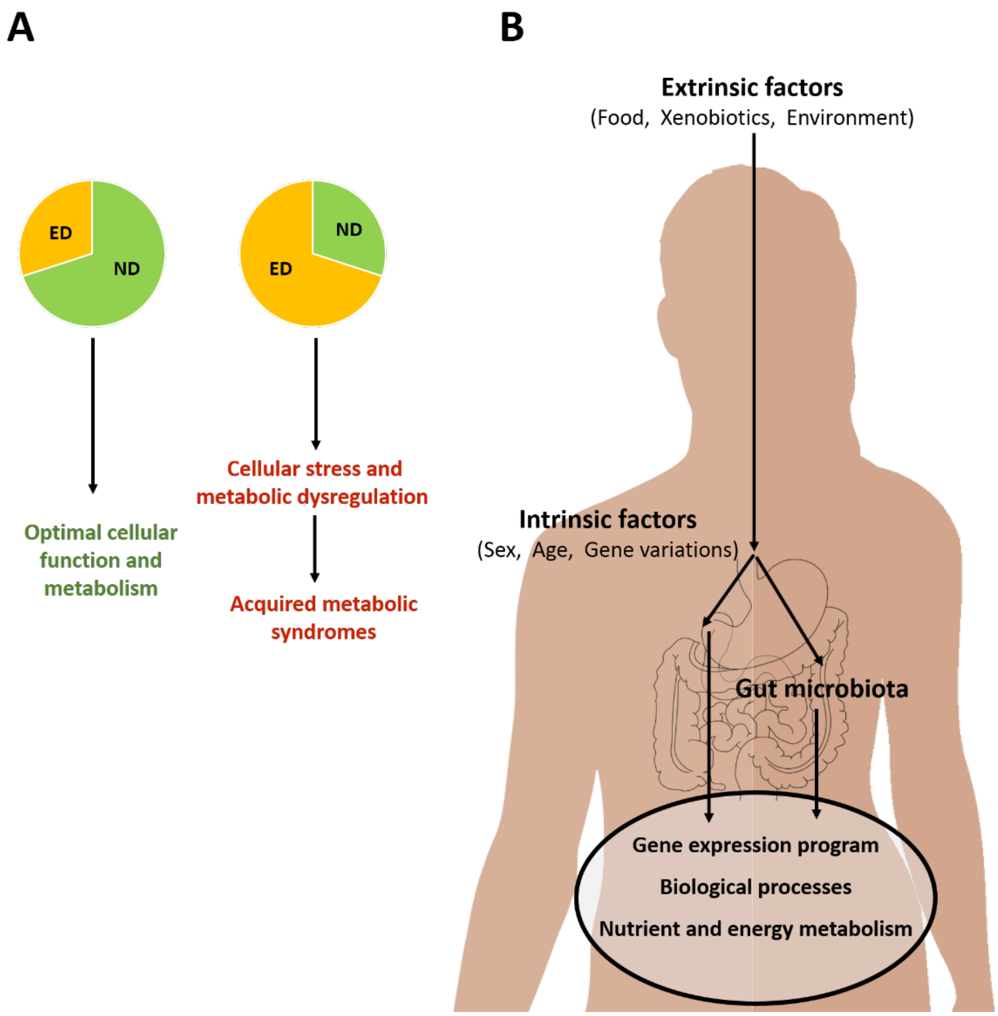

Figure 1. Factors that influence human health. A. Relationship of nutrient diversity (ND) and energy density (ED) in equivalent portion sizes, and their impact on cellular metabolism. B. Extrinsic factors (e.g., food, xenobiotics, environment), intrinsic factors (e.g., sex, gene variations, age), and host-microbiota interaction work together to modulate gene expression program, biological processes, and nutrient and energy metabolism. (This figure was reprinted from Chen et al., 2018 with permission from The Yale Journal of Biology and Medicine.)

1.2 Factors that influence nutrient metabolism

It becomes increasingly evident that both intrinsic factors (such as sex, age, gene variations) and extrinsic factors (such as food, xenobiotics, environment), separately and cooperatively, influence nutrient metabolism and the risk for developing various metabolic diseases (Figure 1B).

1.2.1 Intrinsic and extrinsic factors that influence metabolism and health

Intrinsic factors such as gene variations, sex, and age, influence the efficiency of nutrient metabolism (Figure 1B). Genetic variations impact on the efficacy of metabolic pathways by affecting the function and specific activities of membrane transporters, receptors, signaling proteins, enzymes, carrier proteins, transcription factors, and other proteins involved in the transport, sensing and processing of specific nutrients (72). For example, single nucleotide polymorphisms in genes encoding taste receptors influence food preferences (73). Variations in NPC1L1 cholesterol transporter influence dietary cholesterol absorption (74, 75). Sex (i.e., being genetically male or female) and aging determine biological context and represent important modifiers of metabolic efficiency. Males and females have distinct features in terms of metabolic profiles, gene expression programs and susceptibility to diseases (76-80). Sex-related differences in the intestinal nutrient assimilation, including sugar, lipids, and minerals, as well as in the efficiency of processing, oxidizing, and storing energy substrates in other organs like liver, adipose tissue, and muscle have been also observed (77, 81-86). The underlying mechanisms that specify biological sex are complicated and not fully elucidated. The process involves sex chromosomes, sex hormones, and sex-specific epigenetic programming that work together to alter cellular function, including the expression of genes necessary for the metabolism of nutrients, in a sex-biased manner (77, 87, 88). Aging is associated with the loss of metabolic efficiency caused by deterioration of cellular and genetic components resulting from chemical damage accumulated through life stages. At the cellular level, aging is associated with gradual changes in cellular processes designed to maintain homeostasis. However, these adaptive changes that alter cellular metabolism may contribute towards the loss of metabolic efficiency at the organismal level (31). Remodeling of the epigenome through life stages may also influence disease susceptibility in elderly individuals (37).

Similarly, extrinsic factors are important in dictating the efficiency of nutrient metabolism and health outcomes, including physical cues such as photoperiod and temperature. For example, the alternating light/dark photoperiod of the day-night cycles is important in setting endogenous circadian rhythms, which in turn are intimately linked to the regulation of metabolic activity (89). Detrimental environments, which include situations that induce the release of stress hormones, can impair the ability of the body to sense and respond to metabolic challenges (90-92). Extrinsic factors also promote alterations of the epigenome which can have long-lasting impacts on nutrient and energy metabolism and contribute to the development of metabolic disorders in organs like the heart (e.g. coronary heart disease) and the brain (e.g., Alzheimer's disease) (93-95).

1.2.2 Impact of gut microbiota on metabolism

The gut microbiota represents an important interaction nexus for extrinsic and intrinsic factors that influence the metabolism of nutrients (Figure 1). This enormous ecosystem has gained increased attention in recent years for its role in health and disease. Due to its location, the gut microbiota is exposed not only to the same extrinsic factors experienced by the host but also the metabolites and products produced by the host, such as bile acids, digestive enzymes, and other substances excreted into the gut. Males and females have distinct microbiomes, as do young and old individuals (96, 97). Recent studies show that the composition of the gut microbiota can have dramatic effect on phenotype of the host. For example, transplantation of fecal microbiota from a twin pair discordant for obesity into germ-free mice reproduces the obese/lean phenotypes of the donors in their respective recipients (98). Gut microbiota taken from children suffering from kwashiorkor can induce significant weight loss when transplanted into germ-free recipient mice (99). Moreover, the gut microbiota may be capable of altering the susceptibility of its host to metabolic diseases, by transforming non-nutrient components of food into useful nutrients for the host. Short-chain fatty acids produced from breakdown of dietary fibers can influence the expression of genes involved in proliferation and differentiation of mammalian colonic epithelial cells. They also serve as energy substrate elsewhere in the host (63, 100). Other non-nutrient components of food, such as artificial sweeteners which are deemed safe for human consumption, have been shown to induce dysbiosis in gut microbiota, transforming it into a pathogenic profile (35). It was recently suggested that even the use of natural substances, such as trehalose, as routine food additives could have deadly consequences for public health (101). The emergence of a highly infectious strain of *Clostridium difficile* is coincident with the introduction of trehalose into prepared foods, and it is suggested that this compound had permitted the selection and expansion of the pathogenic strain by providing a carbon source and energy substrate not normally used by this bacterium (35). These examples illustrate the importance and interaction of extrinsic and intrinsic factors in modulating and integrating nutrient metabolism, and in determining the nutritional status of the organism.

1.3 Technologies used in the study of metabolism and nutrition

Over the last few decades, the study of metabolism and nutrition has gradually increased in scope in its mission to find ways of alleviating hunger and improving nutrition and health status. With better understanding of cellular and whole body metabolism, it has become apparent that optimal nutrition is not simply a case of energy adequacy but also nutrient diversity (Figure 1A). To address the complicated metabolic disorders that stem from over-nutrition and sub-optimal nutrition, it is necessary to apply multipronged approaches using a variety of systems targeted at various levels (Figure 2).

Animals and humans do not share identical nutrient requirements, but animal models have nonetheless been indispensable in elucidating the processes involved in the metabolism of nutrients. Transgenic and targeted gene disruption technologies applied to mice have greatly contributed to understanding the role of specific genes and their associated polymorphisms in dictating the efficiency of nutrient metabolism. It has been possible to recapitulate human metabolic diseases in these models, as well as to allow the study of human gene expression in vivo (102-105). The recent advent of CRISPR/Cas9-mediated gene editing will further simplify the creation of new animal models (106). With better awareness of the impact of sex differences (79), future studies can be designed to collect information from both sexes in regard to responses to specific nutritional interventions. Cell culture models have served as useful platforms for elucidating the molecular mechanisms that underlie nutrient metabolism. Cells from both experimental animals and human donors can also highlight species-specific differences that impact on nutrition. These models can help to provide insights into descriptive data generated by human intervention trials. It will be important to rigorously design these intervention trials so that these studies generate high quality data that are needed for reliable interpretations.

Omics technologies (genomics, proteomics, and metabolomics) provide a diverse and rich source of descriptive information needed for the detailed surveillance of nutrient metabolism in humans and experimental models (107). Fortunately, suites of mature bioinformatics tools and the necessary computing power are now available to decipher these data and infer relationships among genomes, metabolic processes and cellular functions that are relevant to health and disease (96, 108-110). A coordinated strategy may be useful in integrating the mechanistic and descriptive information drawn from various sources to generate the knowledge that can ultimately be used to formulate useful recommendations for optimal human nutrition and health.

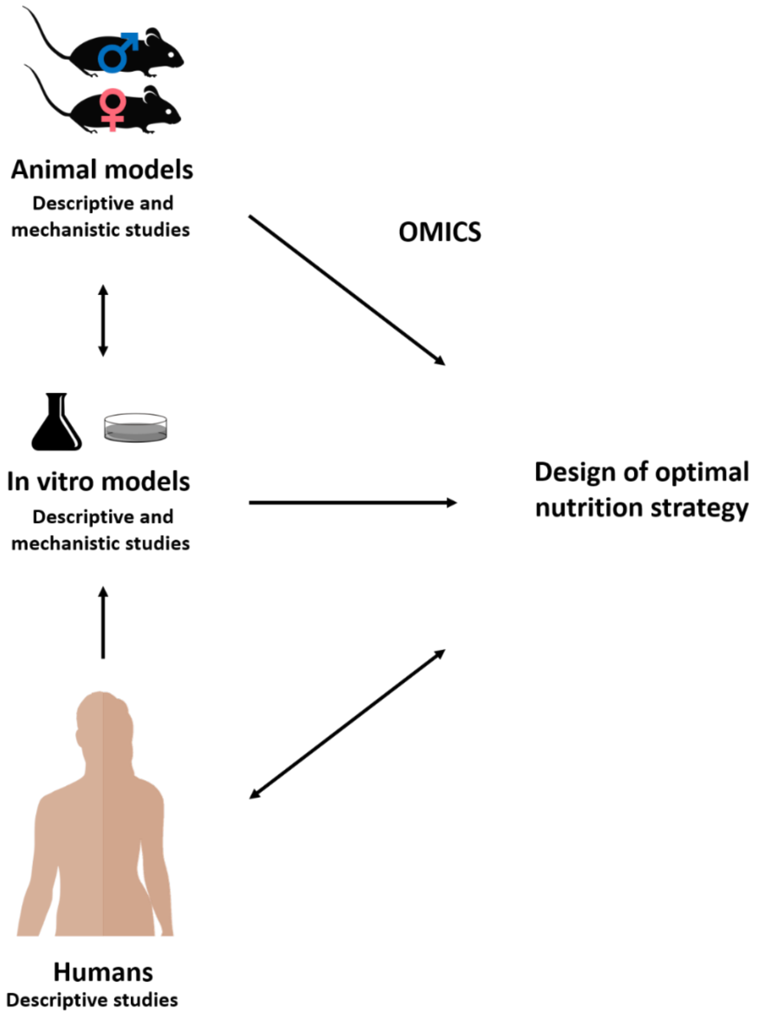

Figure 2. Modern approaches used in the study of nutrition. Descriptive data obtained by Omics technologies from both human studies and animal models to guide the mechanic studies in vitro and cell models. These approaches collectively provide information for the design of interventions for optimal nutrition. (This figure was reprinted from Chen et al., 2018 with permission from The Yale Journal of Biology and Medicine.)

1.4 Hypothesis

The prevailing global increase in the incidence of acquired metabolic syndromes is clearly associated with nutrition transition. It is now generally accepted that nutrients and their metabolites not only serve as building blocks of cellular structures and as fuel sources, but also serve as direct modifiers of protein function, potent signaling molecules as well as inducers and repressors of gene expression. One proposed concept that potentially explains the pathogenesis of acquired metabolic syndromes stems from persistent modification of cellular function in response to stress. It is also recognized that males and females display distinct responses to the same set of extrinsic factors present in foods, which include both nutrient and non-nutrient components, and are influenced in part by genetic composition.

In this thesis, it is hypothesized that metabolism is influenced by intrinsic factors (e.g., sex, gene variations) as well as extrinsic factors (e.g., food, drugs, xenobiotics) that together determine or modify the metabolism of nutrients and the susceptibility to diet-induced metabolic diseases. Two specific objectives will be carried out to test this hypothesis.

- **Objective 1.** To determine the importance of sex on influencing sex-specific transcription programs in response to gene variations. Specifically, experiments will be conducted using male and female mice with known variants of the genes encoding intestinal fatty acid binding protein (Fabp) that are fed the same standard laboratory diet. Intestinal Fabps have documented roles in nutrient assimilation and the disruption of them leads to different phenotypes in male and female mice (111-114). Fabp gene-disrupted mouse strains developed by our laboratory will be used in this study (111, 113, 115).

- **Objective 2.** To evaluate the influence of sex on how diet-based and drug-based therapeutic interventions ameliorate diet-induced metabolic disorders in vivo. The susceptibility of male and female wild-type mice of an inbred strain (C57BL/6) to WSD-induced metabolic dysregulation and the efficacy of low-fat diet without and with UDCA supplementation in normalizing this dysregulation will be investigated. Specifically, a commercially-available open-source mouse WSD formula (D12079B, Research Diets, Inc) will be used to induce metabolic dysregulation, including cardiac fibrogenesis. UDCA is a type of secondary bile acid and approved drug for primary biliary cirrhosis and shows a protective effect against cardiac fibrosis (60, 61).

By studying the influence of sex on the impact of another intrinsic factor (i.e., genetic variation) or extrinsic factors (i.e., diet and drug) in well-characterized mouse models with controlled experimental setting, significant insights into how sex interacts with other factors in determining metabolic responses can be gained, which is important for understanding sexually dimorphic progression in metabolic diseases and the design of effective sex-specific nutritional interventions.

Bridge section between Chapter 1 and Chapter 2

Identical gene variants can have dimorphic impacts in metabolic traits in men and women (116, 117) while the same genetic modifications, such as the disruption of specific genes encoding intestinal fatty acid-binding proteins, can lead to distinct phenotypes in male and female mice (111-114). The processes that underlie sex-specific responses to discrete gene variations remain to be elucidated.

To determine the impact of biological sex on the responses of metabolism-related genes, mouse models with disrupted genes encoding intestinal Fabps (i.e., Fabp2 and Fabp6) were used. Fabps make up a family of widely distributed cytoplasmic proteins that have a putative role in nutrient assimilation, particularly lipid metabolism. Fabps have been studied extensively for over 45 years. Their involvement in nutrient metabolism is evident as they have a strong affinity for hydrophobic nutrients like fatty acids, which may assist the transportation of these molecules in aqueous cytoplasm (118-122). Besides, the disruption of Fabps disturbs the nutrient metabolism in mice (111-114). However, their exact functions and how they work with other proteins involved in the absorption and transportation of fatty acids, such as cluster of differentiation 36 (CD36) and fatty acid transport proteins (FATP), are not well characterized. It is known that fatty acids are the preferred ligands of Fabp2 while bile acids are the preferred ligands of Fabp6 (123, 124). Within their preferred ligand classes, Fabps show different affinity for specific ligands. For example, Fabp2 show higher binding affinity for saturated long-chain fatty acids while Fabp6 has higher binding affinity for conjugated bile acids (124-126). It is worth noting that both fatty acid and bile acid are highly bioactive and can regulate the expression of genes involved in energy metabolism, cell survival, and stress responses as mentioned in the Chapter 1 (51, 59). Interestingly, intestinal

Fabps exhibit different expression profiles in male and female mice (111, 113).

In Chapter 2, a systematic comparative analysis of the small intestinal transcriptomes of male and female mice lacking either Fabp2, Fabp6, or both Fabp2 and Fabp6 was carried out to gain insights into sex-specific remodeling of small intestinal transcriptomes. Male and female mice were given the same diet (the extrinsic factor) and housed under the same conditions, while the genotypes (the intrinsic factor) were varied (wild-type and inactivated gene variants of Fabp2 and Fabp6). The primary goal was to determine if the deletion of single Fabp genes lead to consistent or sex-biased changes in small intestinal transcriptome of mice. The secondary goal was to determine if simultaneous loss of Fabp2 and Fabp6 genes resulted in remodeling of intestinal transcriptome that was a simple sum of changes observed with singular loss of Fabp2 or Fabp6.

Chapter 2

Distinct alteration of gene expression programs in the small intestine of male and female mice in response to ablation of intestinal Fabp genes

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Abstract

Fatty acid-binding proteins (Fabp) make up a family of widely distributed cytoplasmic lipid-binding proteins. The small intestine contains three predominant Fabp species, Fabp1, Fabp2, and Fabp6. Our previous studies showed that *Fabp2* and *Fabp6* gene-disrupted mice exhibited sexually dimorphic phenotypes. In this study, we carried out a systematic comparative analysis of small intestinal transcriptomes of 10- week old wild-type (WT) and Fabp gene-disrupted male and female mice. We found that the small intestinal transcriptome of male and female mice showed key differences in gene expression profiles that affect major biological processes. The deletion of specific Fabp genes induced unique and sex-specific changes in gene expression program, although some differentially expressed genes in certain genotypes were common to both sexes. Functional annotation and interaction network analyses revealed that the number and type of affected pathways, as well as the sets of interacting nodes in each of the Fabp genotypes, are partitioned by sex. To our best knowledge, it is the first time that the sex differences are identified and categorized at the transcriptome level in mice lacking different intestinal Fabps. The distinctive transcriptome profiles of WT male and female small intestine may predetermine the nature of transcriptional reprogramming that manifests as sexually dimorphic responses to the ablation of intestinal Fabp genes.

Keywords

Fatty acid-binding proteins, Fabp2, Fabp6, microarray analysis, nutrient metabolism, small intestine, sex differences, transcriptome

Introduction

Fabps are highly abundant cytoplasmic proteins that are found in several mammalian tissues (1). The function of these proteins are not fully known but they are thought to participate in the maintenance of intracellular lipid homeostasis (1, 2). The small intestine contains three types of Fabps, namely Fabp1 (3, 4), Fabp2 (5), and Fabp6 (6, 7).

Fabp1 (also known as L-FABP) was first found in the liver (4) but is also present throughout the small intestine with the highest abundance occurring in the proximal portion of the organ (8). It can bind fatty acids with a preference for unsaturated long-chain fatty acids as well as an assortment of other hydrophobic molecules including bile acids and fibrates (9, 10). Studies have demonstrated a direct interaction between Fabp1 and peroxisome proliferator-activated receptors (PPAR), suggesting a role of Fabp1 in delivering PPAR ligands to the nucleus which in turn can lead to the expression modulation of PPAR target genes (11, 12). *Fabp1* gene ablation results in a variety of metabolic defects, including dysregulated hepatic lipid metabolism (13, 14) and gallstone susceptibility (15). A T94A variant of the human *FABP1* gene has been found to be associated with reduced body weight as well as altered glucose metabolic response to lipid challenge (16). It can also influence the efficacy of lipid-lowering therapies (17). Interestingly, the association between the T94A variant and increased fasting triacylglycerols and LDL-cholesterol levels was only observed in females (18).

Fabp2 (also known as I-FABP) is restricted to the small intestine but distributed throughout the organ with its maximum abundance occurring after the midpoint of the organ (8). Fabp2 shows a preference for saturated long-chain fatty acids (9, 10). In vitro studies suggest that Fabp2 and Fapb1 enable the transfer of fatty acids from donor to acceptor membranes via different mechanisms: Fabp1 transfers fatty acids via an aqueous diffusion-mediated process whereas Fabp2 transfers fatty acids by direct collisional interaction with membranes (19). Deletion of the *Fabp2* gene in mice did not prevent dietary fat assimilation but influenced adiposity and glucose tolerance in a sex-dependent manner (20, 21). Studies on humans have found a potential association between the A54T human *FABP2* gene variant and insulin resistance, obesity as well as dyslipidemias in specific sexes in certain populations (22-25).

Fabp6 (also known as ILBP and I-BABP) is abundant in the distal region of the small intestine and displays a clear preference for bile acids although it is capable of binding fatty acids at lower affinity (8, 9, 26). Targeted disruption of the *Fabp6* gene in mice demonstrated that Fabp6 is important in the intracellular transport of bile acids in enterocytes and the maintenance of the bile acid pool in the enterohepatic circulation (27). Interestingly, Fabp6 is also found in the ovaries but not in the testes, and female mice lacking Fabp6 display reduced ovulation rate compared to female WT mice (28). Moreover, Fabp6 was found to be associated with the function of farnesoid X receptor (FXR) (29, 30), suggesting a potential role of Fabp6 in modulating the activity of FXR in the nucleus. In humans, the T79M variant of human *FABP6* is associated with some protective effect on type 2 diabetes in obese subjects (31).

Targeted ablation of *Fabp1, Fabp2,* or *Fabp6* genes have all resulted in sexually dimorphic phenotypes (14, 20, 21, 27, 32). Here, we compared the transcriptomes of small intestines from mice lacking either Fabp2 or Fabp6, or both of these Fabps to gain a comprehensive insight into the changes in the gene expression programs that occur in both sexes following the loss of these Fabps.

Results

Gene expression patterns in the small intestine of male and female mice are distinct

Despite males and females sharing a common genome, it is estimated that sex-biased expression of genes in specific tissues ranges from less than 1% to 30%, depending on the sequencing techniques and statistical cut-offs used in the analyses (33, 34). The sex-dependent gene expression patterns were also evident in the brain, liver, muscle, adipose tissue, and intestines of mice (35-37). To address the difference in gene expression in the small intestine between male and female mice with or without Fabps, transcriptome profiling was performed using microarrays in our study. Sex-biased genes were identified in all genotypes (Fig. 1a). A total of 67 sex-biased genes were found in WT mice. This number was reduced in the *Fabp2*$^{-/-}$ and *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$ mice whereas *Fabp6* gene deletion increased the number of sex-biased genes (Fig. 1a), suggesting that the deletion of *Fabp2* gene or both *Fabp2* and *Fabp6* genes made intestinal gene expression of males and females more similar while the disruption of *Fabp6* gene increased the sexual dimorphism at the transcriptome level.

Since sex-biased genes can contribute to phenotypic differences between the two sexes, further classification of these genes was done. The results show that the number of female-biased genes, the sex-biased genes that have higher expression in females, is greater than male-biased ones in WT mice (Fig. 1b), and this is consistent with previous findings (35, 38). Interestingly, the deletion of Fabp genes increased the proportion of male-biased genes, which might play roles in determining the male-specific phenotypes in Fabp gene-disrupted mice. In addition, most of the sex-biased genes reside on autosomal chromosomes (See Supplementary Fig. S4).

The gene ontology enriched analysis was applied to identify biological pathways involving sex-biased genes. Many of the genes that have sexually dimorphic expression in WT small intestine are involved in nutrient and drug metabolism (Fig. 1c), which is in accordance with other studies (39, 40). Some of the sexually dimorphic pathways in the small intestine that pre-existed in WT male and female mice were retained in mice lacking only one Fabp, i.e., either Fabp2 or Fabp6, whereas two pathways remained identifiable when mice were lacking both Fabps (Fig. 1c). In general, deletion of Fabp6 resulted in greater number of sex dimorphic pathways compared to Fabp2, while deletion of both Fabps resulted in the least number of sexually dimorphic pathways.

Thus, pre-existing differences in gene expression program, as illustrated in the small intestinal transcriptome of WT male and female mice, may predetermine the intestinal gene expression program in response to disruption of specific intestinal Fabp genes.

Genomic responses to ablation of specific Fabp genes are sexually dimorphic

Next, we concentrated the analysis on the genomic responses to *Fabp2* and/or *Fabp6* gene disruption in male and female mice. Differentially expressed (DE) genes were identified by comparing the transcript abundance between WT and Fabp gene-disrupted mice of each sex. In general, less than 50% of DE genes induced by Fabp gene ablations are shared by male and female mice (Fig. 2, top, overlap of red and blue circles). Interestingly, many of the shared DE genes (Fig. 2, bottom, gene list) had distinct alterations in male and female mice. Some shared genes altered gene expression in opposite direction in males and females in response to the same Fabp gene disruption, such as *Cyp2c55* in *Fabp2*$^{-/-}$ mice and *Psat1* in *Fabp6*$^{-/-}$ mice. Notably, the numbers of DE genes identified in the small intestine of *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$ mice were much higher than single Fabp gene-disrupted mice in the same sex. *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$ mice also have different sets and total number of DE genes compared to either single Fabp gene disruption regardless of the sex (See Supplementary Fig. S5), implying a greater effect of combined *Fabp2* and *Fabp6* deficiency on overall gene expression program in the small intestine. Moreover, like sex-biased genes (See Supplementary Fig. S4), most of the DE genes identified also reside on autosomal chromosomes (See Supplementary Fig. S6).

To gain insight into the possible affected metabolic pathways, gene ontology analysis was performed to identify the biological processes by grouping DE genes that responded to Fabp gene ablations. The top 10 biological processes having a $p\text{-value} < 0.05$ are shown in Table 1. Two groups of biological processes, namely metabolism-related and immune-related processes, comprise the major proportion of affected processes. Specifically, DE genes influenced by *Fabp2* or *Fabp6* single deletions are involved in metabolism-related biological processes, whereas combined *Fabp2* and *Fabp6* gene deletions mainly influenced immune-related biological processes. Furthermore, males and females displayed distinct alterations of biological processes in response to the same Fabp gene ablation, which is concordant with previously reported changes observed in *Fabp2* gene-disrupted mice (41).

Together, the data show that Fabp gene deletions, either separately or combined, resulted in sex-specific modification of gene expression in the small intestine. The identity of genes with altered expression in $Fabp2^{-/-}$ and $Fabp6^{-/-}$ mice are different from those in $Fabp2^{-/-};Fabp6^{-/-}$ mice, suggesting that the biological processes that are affected when both *Fabp2* and *Fabp6* genes missing are different from those when only one of these genes is absent.

Predicted biological processes involved in nutrient metabolism in the small intestine of Fabp gene ablated mice are sex biased

Given the small intestine is the frontline for nutrient acquisition, transport, metabolism, as well as signaling, and where sex differences are also known to be pre-existing (42-45), we asked whether the genes involved in nutrient metabolism was influenced differently in male and female mice by the loss of specific Fabps. The affected biological process (GO:BP) identified were categorized according to macromolecule/macronutrient metabolism (carbohydrate, protein and lipid metabolism) and sterol/bile acid metabolism, and then the number of unique DE genes belonging to these categories were stratified according to genotype and sex. As shown in Fig. 3, *Fabp2*$^{-/-}$ and *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ mice displayed similar metabolism patterns partitioned by sex. Specifically, all four categories of metabolism were affected more in males than in females, and lipid and protein metabolism were influenced the most in both sexes. However, this pattern was not evident in the *Fabp6*$^{-/-}$ mice, where the total numbers of DE genes involved in macronutrient metabolism were similar in male and female mice. In these mice, protein metabolism was influenced the most in males while lipid metabolism was greatly influenced in females. Surprisingly, only a few genes involved in sterol/bile acid metabolism were identified in both male and female *Fabp6*$^{-/-}$ mice (Fig. 3). For *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ mice, there was a substantially higher number of DE genes involved in macronutrient and sterol metabolism compared to mice with single Fabp gene disruption.

Interaction network analysis was used to gain insight into the possible functions of DE genes based on the predicted interactions among their encoded proteins. Generally, males and females had very different network patterns in response to same Fabp gene ablation, suggesting sex-dependent biological responses, even though some nodes of the networks are shared (Fig. 4). Specifically, the first-order networks of *Fabp6*$^{-/-}$ and *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ males contain more seed proteins, which are starting-point proteins extracted by the NetworkAnalyst to build networks, than females (28 for *Fabp6*$^{-/-}$ and 91 for *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ males; 24 for *Fabp6*$^{-/-}$ and 67 for *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ females) whereas the network of *Fabp2*$^{-/-}$ males contains fewer seed proteins

(25 for *Fabp2*$^{-/-}$ males and 33 for *Fabp2*$^{-/-}$ females) (Fig. 4). When comparing the nodes that have more than one interacting proteins, all mice (all genotypes, both sexes) have one node in common, forkhead box P3 (Foxp3), whereas only male mice share NF-KB (Nfkb1) and Sirtuin-1 (Sirt1). Interestingly, when both Fabp2 and Fabp6 are missing, Nfkb1, but not Sirt1, appear only in the network map of female mice. *Fabp2*$^{-/-}$*;Fabp6*$^{-/-}$ mice have a greater number of nodes in both sexes than the combined number of nodes in *Fabp2*$^{-/-}$ and *Fabp6*$^{-/-}$ mice, suggesting that a larger and more complex interaction network was affected when both Fabp2 and Fabp6 are missing.

Discussion

The existence of sexual dimorphism in lipid metabolism in the intestine has been described in both humans and mice (40, 46, 47). As the most abundant cytoplasmic proteins that play pivotal roles in lipid metabolism in the small intestine, intestinal Fabps have been shown to exhibit sexually dimorphic expression in the small intestine of mice (20, 27). As for human intestinal FABPs (48), less is known about sex differences in the expression of their genes owing to the difficulty in obtaining samples for study. Targeted disruption of intestinal Fabp genes in mice also results in sexually dimorphic effects. Ablation of the *Fabp2* gene causes a much larger degree of metabolic disturbance in male *Fabp2*$^{-/-}$ mice than female *Fabp2*$^{-/-}$ mice (21, 49). Similarly, ablation of the *Fabp6* gene induced differential alterations in male and female mice regarding bile acid metabolism (27). Whole-body deficiency of the Fabp1 has also been shown to induce sexually dimorphic phenotypes (32, 50) but since the *Fabp1* gene is expressed in many tissues, particularly in the liver, the metabolic consequences of intestine-specific deficiency of Fabp1 is not readily apparent from currently available *Fabp1*$^{-/-}$ mouse models. It is clear from available studies that ablation of genes encoding intestinal Fabps impacts on the expression of genes in a variety of tissues, in addition to small intestine, and alter whole-body metabolism in a sex-dimorphic manner (27, 49, 50).

The sex dimorphic transcriptome is determined by many factors. Sex differences in the transcriptome of a specific tissue are likely framed by the combined effects of biological sex as dictated by sex chromosomes, sex hormones or sex-specific modification of the epigenome (51, 52). For example, substantial sex-biased gene expression is clearly evident in the small intestine of prepubescent mice and even as early as during embryo development (35, 53). In our study, nearly all the of DE genes detected in the intestinal transcriptome of all Fabp gene-disrupted genotypes were resident on autosomal chromosomes and very few were involved in sex hormonal-related processes. Moreover, protein-protein interaction analysis revealed that the major networks involving the DE genes in the majority of Fabp gene-disrupted genotypes included transcriptional signaling processes related to PPAR and FXR. It has been shown that PPAR and FXR themselves manifest sexually dimorphic expression (54-56). Given the fact that Fabps share the many ligands with these nuclear receptors, the loss of specific Fabps could further alter the ability of these transcription factors to regulate gene expression in a sex-dependent manner. The pre-existing sex-dimorphic gene expression program may therefore, in part, determine the differential responses of males and females to the loss of specific intestinal Fabps.

This study also provides insights into the potential biological roles and functional relationships of the intestinal Fabps. It was previously suggested that multiple Fabps in the small intestine might share some functions to ensure fatty acid and bile acid metabolism (20). Fabp1 and Fabp2 show preference for binding fatty acids whereas Fabp6 prefers bile acids (9, 26). On the other hand, Fabp1 and Fabp6 can bind bile acids and fatty acids, respectively, at lower affinities (8, 26). Indeed, we found that mice lacking both Fabp2 and Fabp6 were viable. Interestingly, the network analysis revealed the loss of both Fabps affected a much larger number of processes in male mice than in female mice, similar to *Fabp2*$^{-/-}$ mice. Future studies will uncover how these changes manifest at the organismal level. In addition, different regions of small intestine have specialized metabolic and immune functions (57, 58). It would also be interesting to determine the nature of the changes in the gene expression program at these regions of the small intestine in males and females. More studies are needed to understand how the effect of sex on genomic responses impact on important metabolic pathways involved in nutrient processing by the intestines, overall metabolism and susceptibility to various metabolic diseases.

In conclusion, our study shows that sex is an important determinant of the intestinal transcriptome. Moreover, the pre-existing differences between males and females may govern the distinct alterations of the intestinal gene expression program manifested by males and females in response to targeted inactivation of genes encoding the intestinal Fabps.

Methods

Mice

Mice (n=5 per cage) were housed in temperature-controlled specific-pathogen-free facility and fed a standard lab diet (Purina 5001). *Fabp2*$^{-/-}$ (21) and *Fabp6*$^{-/-}$ (27) mice were maintained on the C57BL/6J background, which included one cross with a male C57BL/6J mouse, and interbred to generate the *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$ line.

Preparation of RNA and hybridization to DNA microarrays

The small intestine, starting from the base of the stomach and ending just before the cecum, was excised from fasted 10-week old mice littermates and then flushed with ice-cold saline prior to tissue homogenization. RNA was extracted from homogenates using Trizol (Invitrogen) and assessed for integrity (RIN>7) prior to use in microarray analysis. Total RNA sample from 4 mice of each sex (male or female) and genotype (wild-type C57BL/6J, *Fabp2*$^{-/-}$, *Fabp6*$^{-/-}$, *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$) was analyzed as 2 biological replicates (RNA from 2 mice were pooled and sequenced as 1 sample). Fluorescently labeled cDNA probes generated from RNA samples were hybridized to Affymetrix 430A and 430B chips (total of 32, 16 per chip type) and processed as specified by the manufacturer.

Analysis of microarray data

The data were processed and analyzed using the R statistical environment and Bioconductor software (59) as illustrated in Supplementary Fig. S1. Raw intensities from each gene chip were normalized to $\log_2$ scale using the robust multichip average (RMA) algorithm with background correction (60). The quality assessments were done by comparing the intensity between biological replicates (See Supplementary Fig. S2). Boxplots were also used to evaluate the data quality across the chips (See Supplementary Fig. S3). To improve statistical power, the data were filtered based on their inter-quartile range and Entrez annotations (61). The limFit function (limma package) (62) was applied to fit data into the linear model to identify differentially expressed (DE) genes between the male and female with the same Fabp gene disrupted, namely sex-biased genes. DE genes between Fabp gene-disrupted mice and wild-type mice in the same sex were also identified. Genes with a false discovery rate <0.2 and an absolute $\log_2$ fold change >0.5 were considered statistically significantly different.

The murine gene ID conversion and functional annotation analysis of identified DE genes were done using DAVID Bioinformatics Resources (version 6.8) (63). The chromosomal locations of DE genes were also determined from the Mouse Genome Database (64). The Venn diagram plots were generated using the BioVenn website (65). The Gene Ontology (GO) terms in the biological process (BP) domains were extracted. To display the metabolic patterns comprehensively, the relevant biological processes were grouped into four categories of interest in this study, namely lipid metabolism, carbohydrate metabolism, protein metabolism and sterol metabolism (complete lists are shown in Supplementary Table S1), and presented with the number of unique DE genes in each category for each sex and genotype. In order to reveal sexually dimorphic protein interaction patterns, the corresponding proteins of the DE genes induced by disruption of Fabp genes in each sex were used as seed proteins to identify interacting proteins based on the literature-curated interactions in NetworkAnalyst's protein-protein interaction database (accessed March 2019) which is drawn from the International Molecular Exchange consortium (66). The seed proteins were determined by the algorithm in NetworkAnalyst using the submitted gene lists. They were used as the starting points to draw the link to other proteins, forming the functional protein modules. Only the shortest-path first-order networks from the seed proteins were kept.

Data availability

The NCBI GEO accession number for the microarray data used in this study is <*to be supplied upon acceptance of the manuscript for publication; reviewers may access the microarray data at* https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128862*; an access token for reviewers was included with the manuscript during the submission process*>.

Figures and Tables

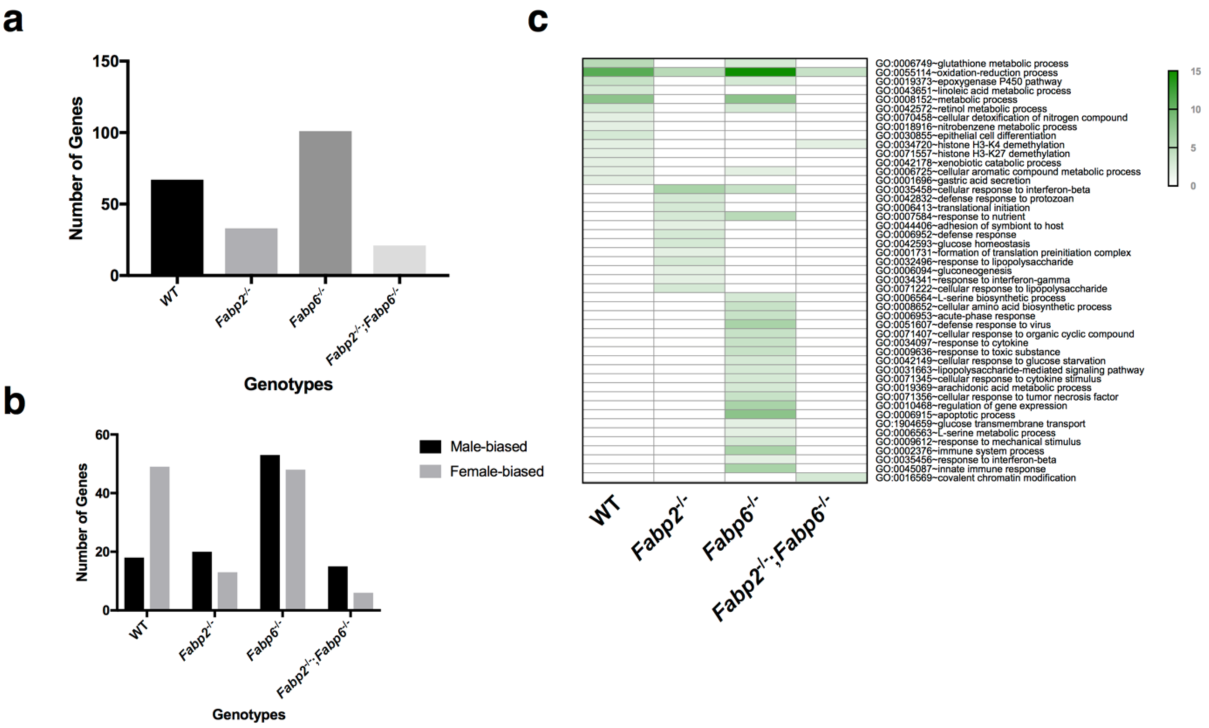


Figure 1. Sex-biased genes and functional annotation (Gene Ontology: biological process, GO: BP). (**a**) Comparison of the number of sex-biased genes identified in wild-type (WT), *Fabp2*$^{-/-}$, *Fabp6*$^{-/-}$, and *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$ mice. (**b**) The number of male- and female-biased genes in small-intestinal transcriptome across all genotypes. (**c**) Comparison of the biological processes enriched (p-value<0.05) using sex-biased genes. The numbers of genes involved in each biological process are represented by the green color; darker shades indicate a greater number of genes.

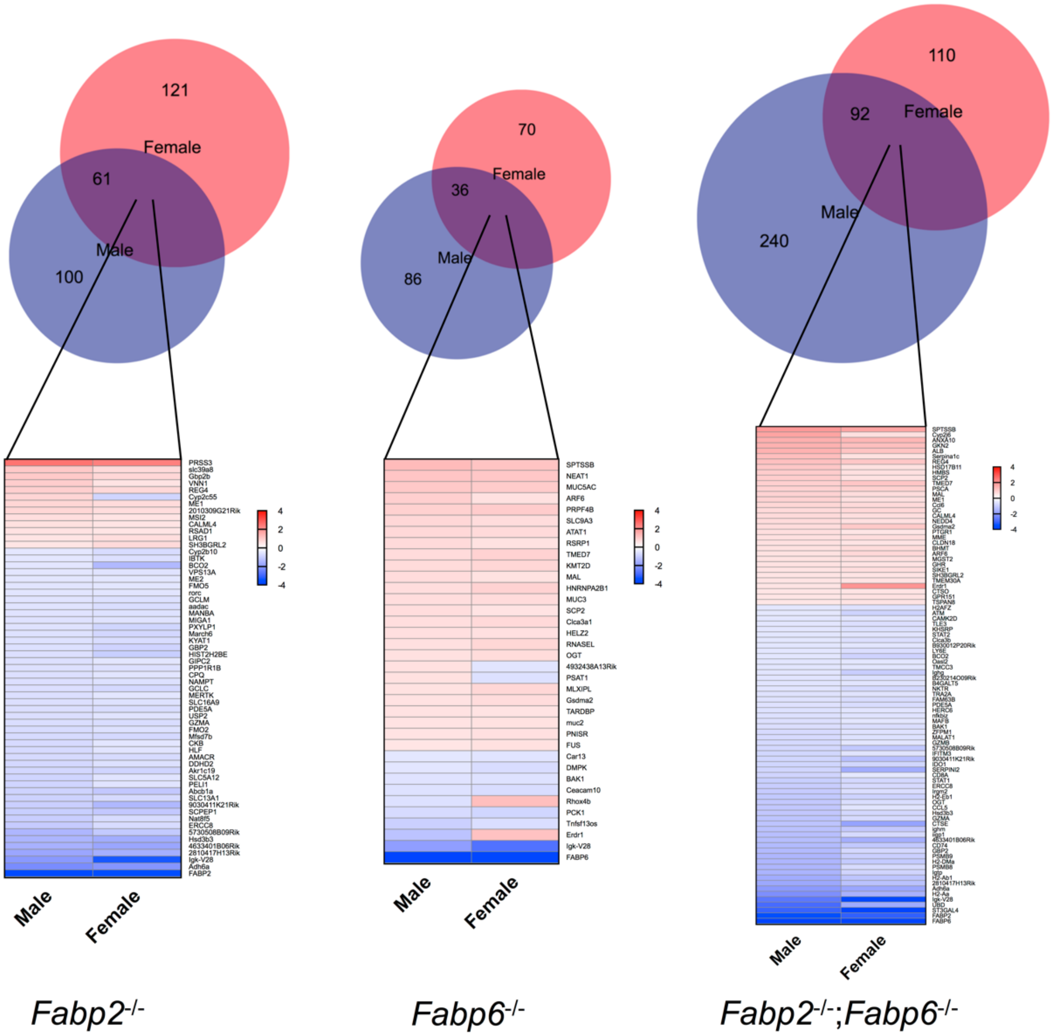


Figure 2. The comparison of DE genes in different Fabp gene-disrupted male and female mice. The Venn Diagram of the common and sex-specific DE genes induced by the deletion of Fabp genes (top). The heatmap comparing the altered expression of common DE genes shared by male and female (bottom).

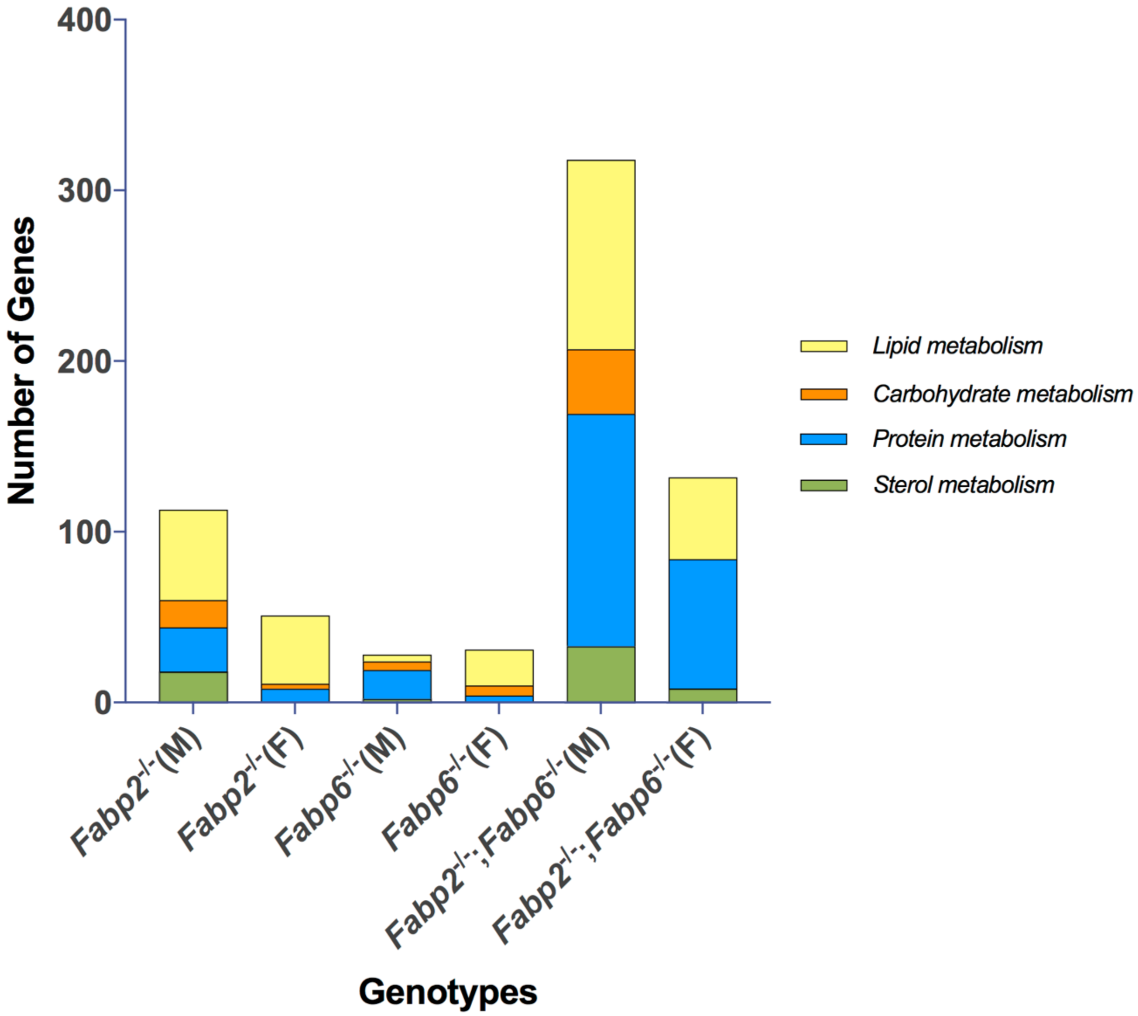


Figure 3. The comparison of the numbers of DE genes involved in key nutrient metabolism processes. The biological processes that comprise the nutrient metabolism categories are listed in the Supplementary Table S1.

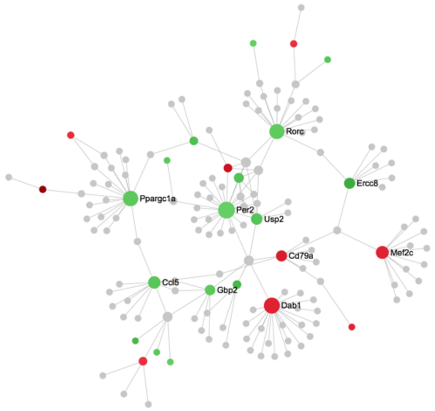
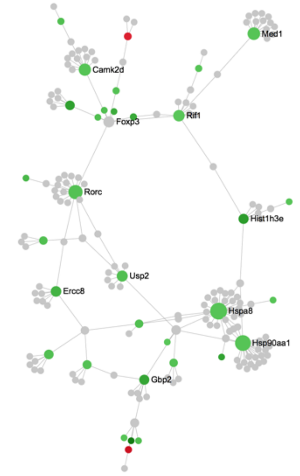
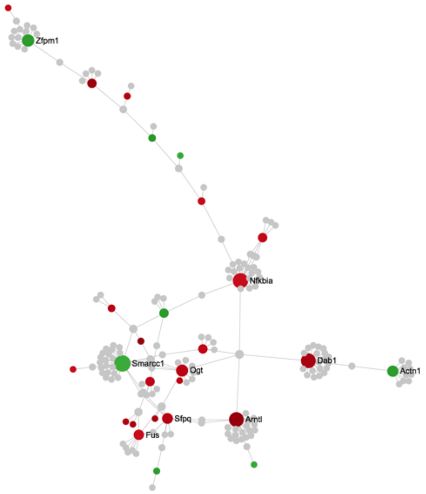
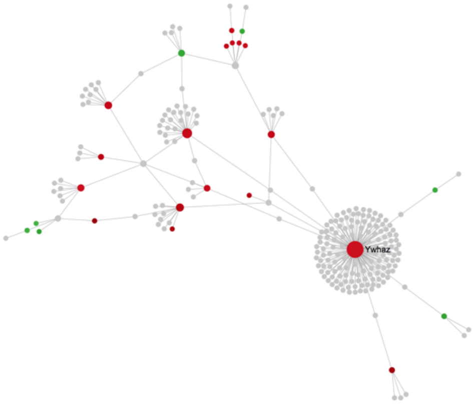
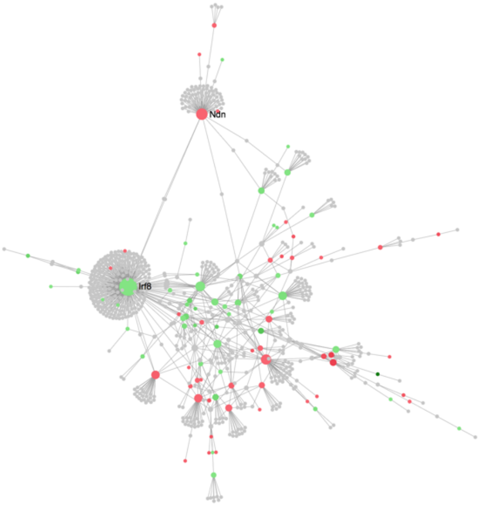
	Male	**Female**
Fabp2$^{-/-}$		
Fabp6$^{-/-}$		
Fabp2$^{-/-}$***;Fabp6***$^{-/-}$		

Figure 4. The protein-protein interaction networks of DE genes in different Fabp gene-disrupted genotypes (*Fabp2*$^{-/-}$, *Fabp6*$^{-/-}$, and *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$). Three types of proteins (nodes) are presented in the networks: Seed proteins represented in green are encoded by downregulated genes. Seed proteins represented in red are upregulated genes. Proteins represented in grey are known to directly interact with the seed proteins. The nodes are listed in the Supplementary Table S2 and the Supplementary Table S3.

Table 1. Gene Ontology Analysis for DE genes induced in different genotypes.

Fabp2$^{-/-}$ **(M)**	**Count**	**p-value**	***Fabp2***$^{-/-}$ **(F)**	**Count**	**p-value**
GO:0055114~oxidation-reduction process	22	6.42E-08	GO:0006749~glutathione metabolic process	5	6.03E-04
GO:0006629~lipid metabolic process	17	6.02E-07	GO:0006805~xenobiotic metabolic process	4	8.68E-04
GO:0032922~circadian regulation of gene expression	7	7.89E-06	GO:0008152~metabolic process	12	0.001147895
GO:0008202~steroid metabolic process	7	4.98E-05	GO:0042130~negative regulation of T cell proliferation	4	0.003863051
GO:0048511~rhythmic process	8	6.51E-05	GO:0035458~cellular response to interferon-beta	4	0.00414414
GO:0006631~fatty acid metabolic process	8	2.24E-04	GO:0035729~cellular response to hepatocyte growth factor stimulus	3	0.006930587
GO:0006805~xenobiotic metabolic process	4	8.33E-04	GO:0055085~transmembrane transport	9	0.008460086
GO:0006694~steroid biosynthetic process	5	0.00139213	GO:0017144~drug metabolic process	3	0.008745672
GO:0007623~circadian rhythm	6	0.001573402	GO:0032922~circadian regulation of gene expression	4	0.01247761
GO:0006641~triglyceride metabolic process	4	0.003454421	GO:0006807~nitrogen compound metabolic process	3	0.012934709
Fabp6$^{-/-}$ **(M)**			***Fabp6***$^{-/-}$ **(F)**		
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	14	0.002734053	GO:0006915~apoptotic process	10	0.001952868
GO:0006814~sodium ion transport	5	0.004633732	GO:0045779~negative regulation of bone resorption	3	0.002748495
GO:0048511~rhythmic process	5	0.005183645	GO:0006397~mRNA processing	7	0.005132946
GO:0006351~transcription, DNA-templated	20	0.005575233	GO:0008652~cellular amino acid biosynthetic process	3	0.006676645
GO:0033137~negative regulation of peptidyl-serine phosphorylation	3	0.008042428	GO:0033137~negative regulation of peptidyl-serine phosphorylation	3	0.006676645
GO:0008652~cellular amino acid biosynthetic process	3	0.008042428	GO:0006094~gluconeogenesis	3	0.007210024
GO:0051726~regulation of cell cycle	4	0.023002721	GO:0061430~bone trabecula morphogenesis	2	0.014694315
GO:0035020~regulation of Rac protein signal transduction	2	0.032085468	GO:0051726~regulation of cell cycle	4	0.017872146
GO:0006915~apoptotic process	8	0.035249578	GO:0006564~L-serine biosynthetic process	2	0.019544816
GO:0006810~transport	17	0.03558283	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	11	0.024183319
Fabp2$^{-/-}$***;Fabp6***$^{-/-}$ **(M)**			***Fabp2***$^{-/-}$***;Fabp6***$^{-/-}$ **(F)**		
GO:0002376~immune system process	26	5.43E-09	GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	6	9.87E-08
GO:0034341~response to interferon-gamma	8	1.55E-07	GO:0002376~immune system process	17	3.32E-07
GO:0019882~antigen processing and presentation	10	2.21E-07	GO:0019882~antigen processing and presentation	8	4.85E-07
GO:0055114~oxidation-reduction process	32	2.85E-07	GO:0034341~response to interferon-gamma	6	2.97E-06
GO:0035458~cellular response to interferon-beta	9	2.95E-07	GO:0035458~cellular response to interferon-beta	6	3.04E-05
GO:0042572~retinol metabolic process	7	2.00E-06	GO:0006955~immune response	12	3.31E-05
GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	6	2.08E-06	GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	4	5.60E-05
GO:0006955~immune response	17	1.23E-05	GO:0042130~negative regulation of T cell proliferation	5	4.31E-04
GO:0007584~response to nutrient	9	2.63E-05	GO:0060337~type I interferon signaling pathway	3	0.001154
GO:0006629~lipid metabolic process	22	2.64E-05	GO:0031175~neuron projection development	7	0.00154

Shown are the top 10 biological processes that are enriched in each genotype, the number of genes that fall within each process (Count), and the p-values obtained.

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Ethics approval

The use of animals in this study was approved by the institutional Animal Care and Use Committee in accordance with the policies of the Canadian Council on Animal Care.

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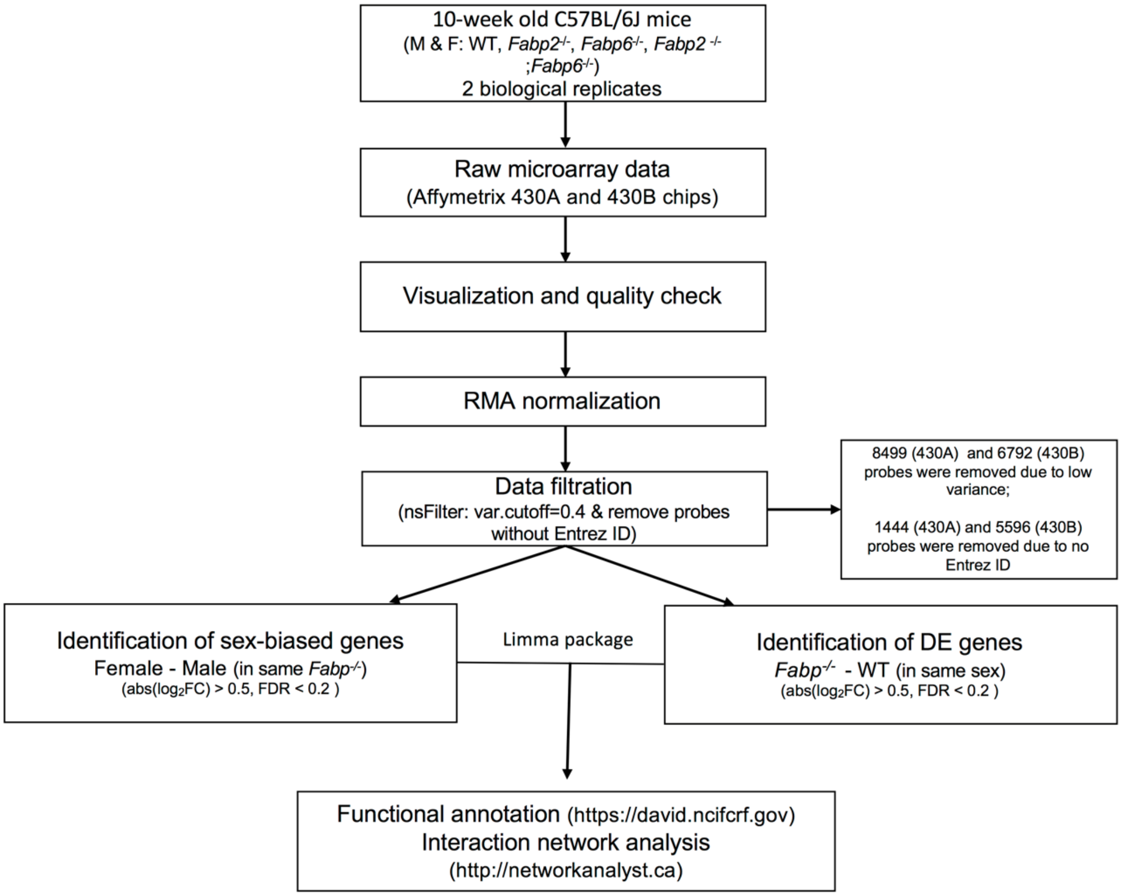
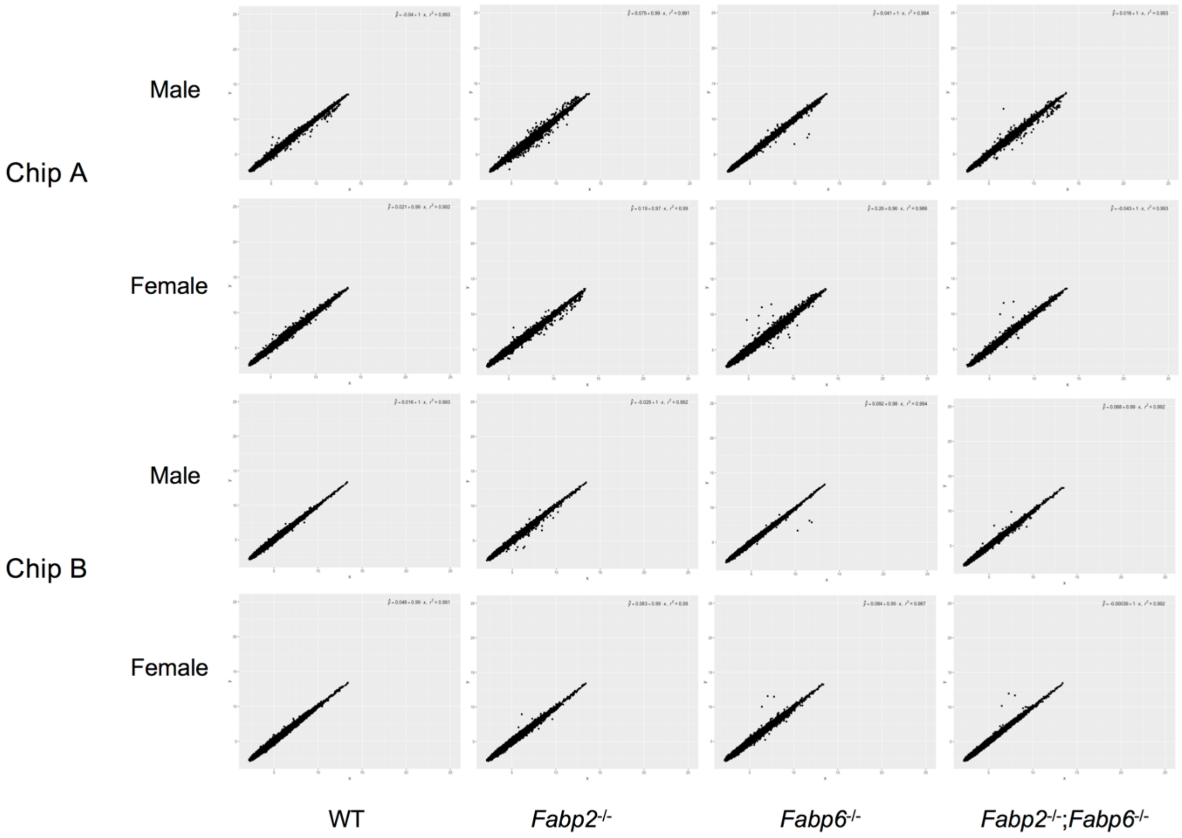
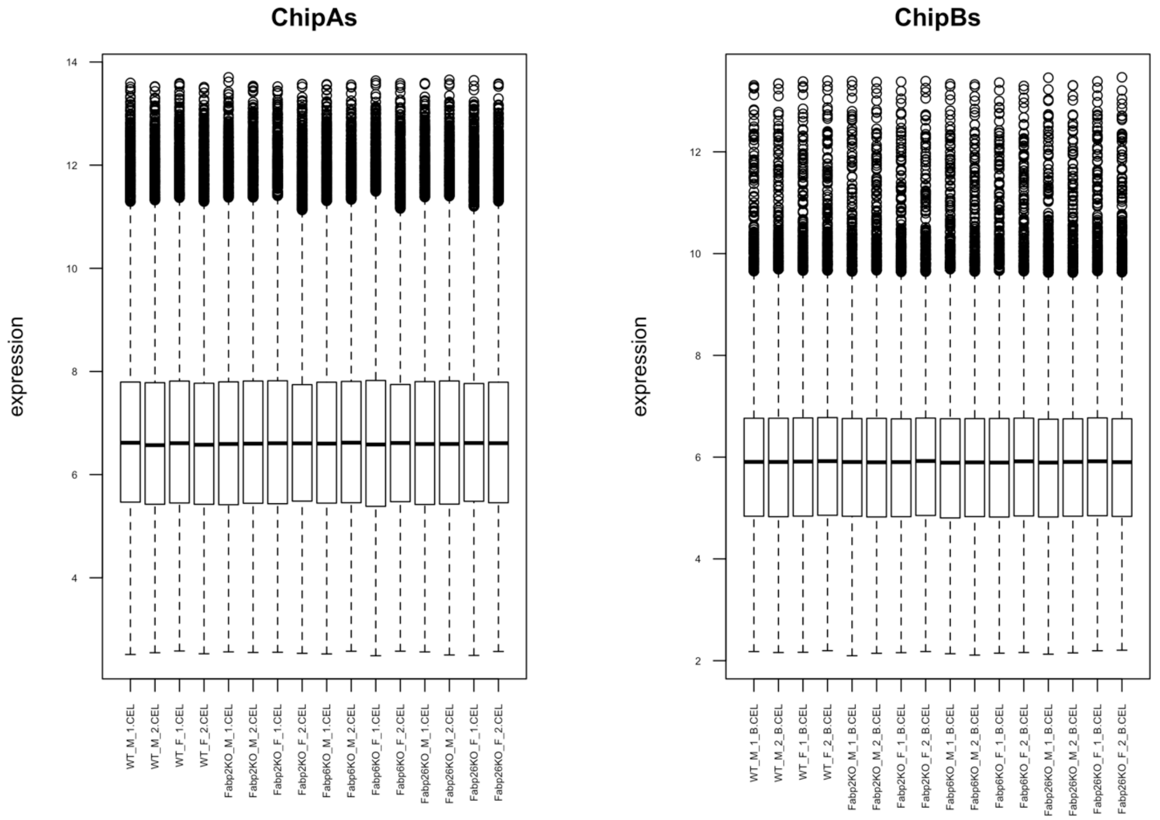
Supplementary information



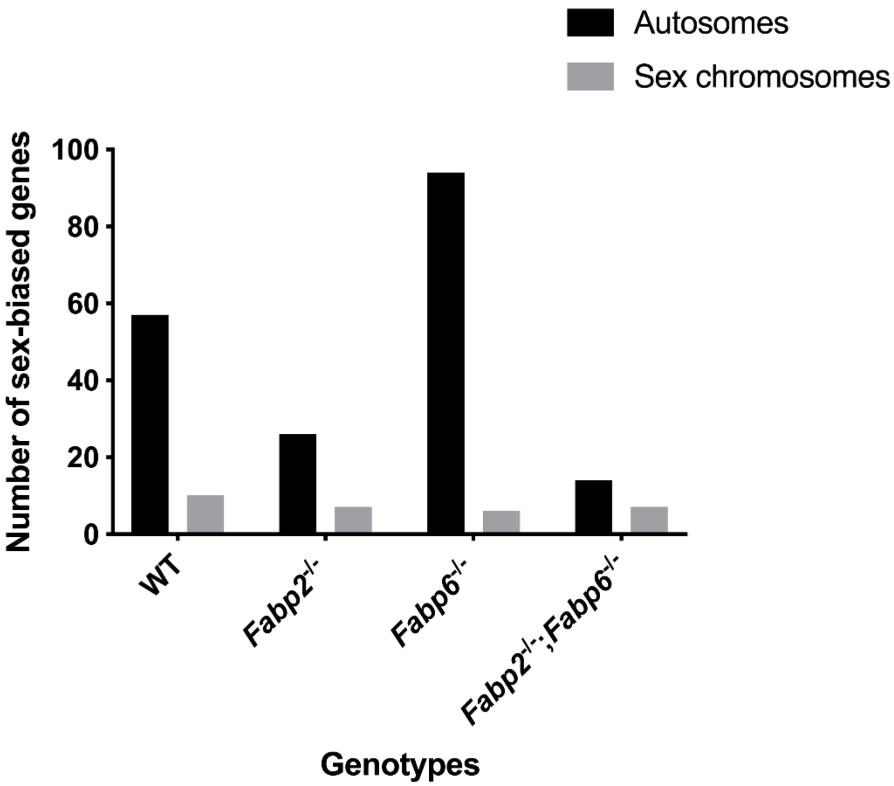
Supplementary Figure S1. Flow chart illustrating the microarray data analysis workflow. WT: wild-type; RMA: robust multichip average; abs: absolute; FC: fold change; FDR: false discovery rate.



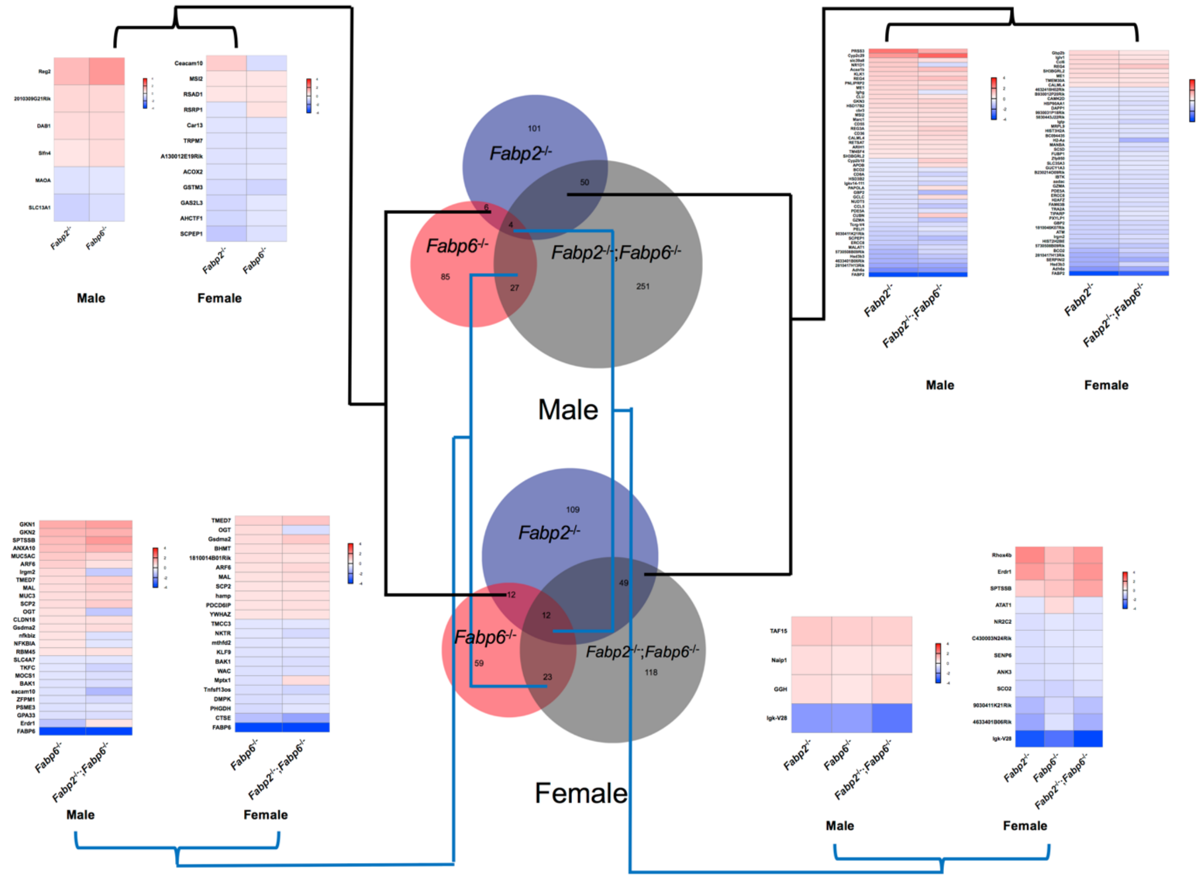
Supplementary Figure S2. Assessment of the quality of data by comparing signal intensity from the biological replicates after RMA processing using linear correlation (x indicates the replicate 1 and y indicates replicate 2).



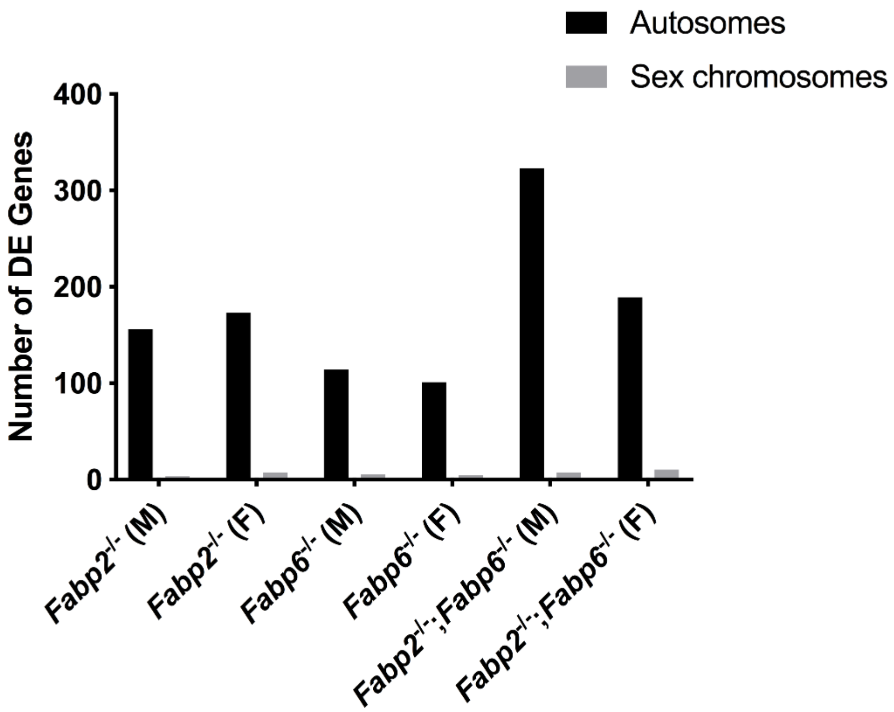
Supplementary Figure S3. Assessment of the quality of microarray data after RMA processing using boxplot.



Supplementary Figure S4. Chromosomal locations of sex-biased genes identified in WT and Fabp gene-disrupted mice.



Supplementary Figure S5. Venn diagrams and heatmaps showing DE genes in male and female intestinal transcriptomes of different Fabp gene-disrupted genotypes (*Fabp2*$^{-/-}$, *Fabp6*$^{-/-}$, and *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$).



Supplementary Figure S6. Chromosomal locations of DE genes in male and female intestinal transcriptomes of different Fabp gene-disrupted genotypes (*Fabp2*$^{-/-}$, *Fabp6*$^{-/-}$, and *Fabp2*$^{-/-}$;*Fabp6*$^{-/-}$).

Supplementary Table S1. Table summarizing the biological processes cited in Figure 3 categorized in lipid metabolism, carbohydrate metabolism, protein metabolism, and sterol metabolism.

Lipid metabolism	**Sterol metabolism**	**Protein metabolism**	**Carbohydrate metabolism**
GO:1901654~response to ketone	GO:0008203~cholesterol metabolic process	GO:0006458~'de novo' protein folding	GO:0006006~glucose metabolic process
GO:0006071~glycerol metabolic process	GO:0060620~regulation of cholesterol import	GO:0051084~'de novo' posttranslational protein folding	GO:0033500~carbohydrate homeostasis
GO:0015914~phospholipid transport	GO:0030301~cholesterol transport	GO:0051246~regulation of protein metabolic process	GO:0042593~glucose homeostasis
GO:0042182~ketone catabolic process	GO:0032376~positive regulation of cholesterol transport	GO:0051247~positive regulation of protein metabolic process	GO:0071322~cellular response to carbohydrate stimulus
GO:0006629~lipid metabolic process	GO:0032374~regulation of cholesterol transport	GO:0032270~positive regulation of cellular protein metabolic process	GO:0005996~monosaccharide metabolic process
GO:0006631~fatty acid metabolic process	GO:0033344~cholesterol efflux	GO:0051248~negative regulation of protein metabolic process	GO:0016051~carbohydrate biosynthetic process
GO:0044242~cellular lipid catabolic process	GO:0042632~cholesterol homeostasis	GO:0019538~protein metabolic process	GO:0034284~response to monosaccharide
GO:0044255~cellular lipid metabolic process	GO:0010875~positive regulation of cholesterol efflux	GO:0006534~cysteine metabolic process	GO:0001678~cellular glucose homeostasis
GO:0046503~glycerolipid catabolic process	GO:0045540~regulation of cholesterol biosynthetic process	GO:0006575~cellular modified amino acid metabolic process	GO:0009743~response to carbohydrate
GO:0008610~lipid biosynthetic process	GO:0010874~regulation of cholesterol efflux	GO:1901607~alpha-amino acid biosynthetic process	GO:0009749~response to glucose
GO:0016042~lipid catabolic process	GO:0010886~positive regulation of cholesterol storage	GO:0006564~L-serine biosynthetic process	GO:0006110~regulation of glycolytic process
GO:0019433~triglyceride catabolic process	GO:0070508~cholesterol import	GO:0006563~L-serine metabolic process	GO:0016052~carbohydrate catabolic process
GO:0019216~regulation of lipid metabolic process	GO:0090181~regulation of cholesterol metabolic process	GO:0008652~cellular amino acid biosynthetic process	GO:0005975~carbohydrate metabolic process
GO:0001676~long-chain fatty acid metabolic process	GO:0006695~cholesterol biosynthetic process	GO:0009070~serine family amino acid biosynthetic process	GO:0046364~monosaccharide biosynthetic process
GO:0046890~regulation of lipid biosynthetic process	GO:0032383~regulation of intracellular cholesterol transport	GO:0072337~modified amino acid transport	GO:0044724~single-organism carbohydrate catabolic process
GO:0070542~response to fatty acid	GO:0010878~cholesterol storage	GO:0006520~cellular amino acid metabolic process	GO:0071333~cellular response to glucose stimulus
GO:0044872~lipoprotein localization	GO:0010885~regulation of cholesterol storage	GO:0015804~neutral amino acid transport	GO:0044723~single-organism carbohydrate metabolic process
GO:0042953~lipoprotein transport	GO:0008202~steroid metabolic process	GO:0042398~cellular modified amino acid biosynthetic process	GO:0043470~regulation of carbohydrate catabolic process
GO:0090207~regulation of triglyceride metabolic process	GO:0006694~steroid biosynthetic process	GO:1901605~alpha-amino acid metabolic process	GO:1901135~carbohydrate derivative metabolic process
GO:0033993~response to lipid	GO:0050810~regulation of steroid biosynthetic process	GO:0045862~positive regulation of proteolysis	GO:1901264~carbohydrate derivative transport
GO:0010884~positive regulation of lipid storage	GO:0019218~regulation of steroid metabolic process	GO:0006508~proteolysis	GO:1901136~carbohydrate derivative catabolic process
GO:0010876~lipid localization	GO:2000909~regulation of sterol import	GO:0030162~regulation of proteolysis	GO:0071331~cellular response to hexose stimulus
GO:0006869~lipid transport	GO:0032371~regulation of sterol transport	GO:0030163~protein catabolic process	GO:0071326~cellular response to monosaccharide stimulus
GO:0045834~positive regulation of lipid metabolic process	GO:0015918~sterol transport	GO:0051603~proteolysis involved in cellular protein catabolic process	GO:0045821~positive regulation of glycolytic process

GO:0055088~lipid homeostasis	GO:0032373~positive regulation of sterol transport	GO:0061136~regulation of proteasomal protein catabolic process	GO:0006096~glycolytic process
GO:0071396~cellular response to lipid	GO:0016125~sterol metabolic process	GO:0044257~cellular protein catabolic process	GO:1901659~glycosyl compound biosynthetic process
GO:0060191~regulation of lipase activity	GO:0055092~sterol homeostasis		
GO:0033559~unsaturated fatty acid metabolic process	GO:0016126~sterol biosynthetic process		
GO:0071827~plasma lipoprotein particle organization	GO:0010893~positive regulation of steroid biosynthetic process		
GO:0097006~regulation of plasma lipoprotein particle levels	GO:0032366~intracellular sterol transport		
GO:0046486~glycerolipid metabolic process	GO:0035376~sterol import		
GO:0006636~unsaturated fatty acid biosynthetic process	GO:0032380~regulation of intracellular sterol transport		
GO:0032370~positive regulation of lipid transport			
GO:0006641~triglyceride metabolic process			
GO:0032368~regulation of lipid transport			
GO:0010883~regulation of lipid storage			
GO:0034381~plasma lipoprotein particle clearance			
GO:0006644~phospholipid metabolic process			
GO:0006633~fatty acid biosynthetic process			
GO:0031663~lipopolysaccharide-mediated signaling pathway			
GO:0034377~plasma lipoprotein particle assembly			
GO:0015908~fatty acid transport			
GO:0034369~plasma lipoprotein particle remodeling			
GO:0006665~sphingolipid metabolic process			
GO:0006690~icosanoid metabolic process			
GO:0006642~triglyceride mobilization			
GO:0019369~arachidonic acid metabolic process			
GO:0035338~long-chain fatty-acyl-CoA biosynthetic process			
GO:0032365~intracellular lipid transport			
GO:0032377~regulation of intracellular lipid transport			
GO:0006643~membrane lipid metabolic process			
GO:0045332~phospholipid translocation			
GO:0006639~acylglycerol metabolic process			
GO:0042180~cellular ketone metabolic process			
GO:0046464~acylglycerol catabolic process			
GO:0051791~medium-chain fatty acid metabolic process			
GO:0008654~phospholipid biosynthetic process			

GO:0006638~neutral lipid metabolic process			
GO:0046461~neutral lipid catabolic process			
GO:0008203~cholesterol metabolic process			
GO:0060620~regulation of cholesterol import			
GO:0030301~cholesterol transport			
GO:0032376~positive regulation of cholesterol transport			
GO:0032374~regulation of cholesterol transport			
GO:0033344~cholesterol efflux			
GO:0042632~cholesterol homeostasis			
GO:0010875~positive regulation of cholesterol efflux			
GO:0045540~regulation of cholesterol biosynthetic process			
GO:0010874~regulation of cholesterol efflux			
GO:0010886~positive regulation of cholesterol storage			
GO:0070508~cholesterol import			
GO:0090181~regulation of cholesterol metabolic process			
GO:0006695~cholesterol biosynthetic process			
GO:0032383~regulation of intracellular cholesterol transport			
GO:0010878~cholesterol storage			
GO:0010885~regulation of cholesterol storage			
GO:0008202~steroid metabolic process			
GO:0006694~steroid biosynthetic process			
GO:0050810~regulation of steroid biosynthetic process			
GO:0019218~regulation of steroid metabolic process			
GO:2000909~regulation of sterol import			
GO:0032371~regulation of sterol transport			
GO:0015918~sterol transport			
GO:0032373~positive regulation of sterol transport			
GO:0016125~sterol metabolic process			
GO:0055092~sterol homeostasis			
GO:0016126~sterol biosynthetic process			
GO:0010893~positive regulation of steroid biosynthetic process			
GO:0032366~intracellular sterol transport			
GO:0035376~sterol import			
GO:0032380~regulation of intracellular sterol transport			

Supplementary Table S2. Table summarizing the nodes, edges and seeds identified in the networks shown in Figure 4.

	Nodes	Edges	Seeds
Fabp2$^{-/-}$(M)	149	169	25
Fabp2$^{-/-}$(F)	180	190	33
Fabp6$^{-/-}$(M)	185	197	28
Fabp6$^{-/-}$(F)	241	247	24
Fabp2$^{-/-}$;*Fabp6*$^{-/-}$(M)	742	891	91
Fabp2$^{-/-}$;*Fabp6*$^{-/-}$(F)	467	528	67

Supplementary Table S3. Table summarizing the identities and the degree of interaction of the nodes shown in Figure 4.

Fabp2$^{-/-}$**(M)**		***Fabp2***$^{-/-}$**(F)**		***Fabp6***$^{-/-}$**(M)**		***Fabp6***$^{-/-}$**(F)**		***Fabp2***$^{-/-}$***;Fabp6***$^{-/-}$**(M)**		***Fabp2***$^{-/-}$***;Fabp6***$^{-/-}$ **(M)** ***(cont.)***		***Fabp2***$^{-/-}$***;Fabp6***$^{-/-}$ **(F)**	
Protein	**Degree**	**Protein**	**Degree**	**Protein**	**Degree**	**Protein**	**Degree**	**Protein**	**Degree**	**Protein**	**Degree**	**Protein**	**Degree**
Per2	24	Hspa8	29	Smarcc1	28	Ywhaz	144	Irf8	277	Hk3	1	Ywhaz	144
Dab1	21	Hsp90aa1	24	Nfkbia	23	Per2	24	Ndn	66	Tifab	1	Cbl	47
Ppargc1a	19	Rorc	18	Dab1	21	Ogt	12	Sirt1	47	Kif27	1	Nedd4	27
Rorc	18	Med1	13	Arntl	21	Tax1bp1	10	Irf1	41	Fastkd3	1	Hsp90aa1	24
Mef2c	12	Camk2d	12	Zfpm1	13	Fus	8	Ccnd1	28	Erap1	1	Irf8	18
Ccl5	11	Rif1	10	Ogt	12	Ddx58	8	Nedd4	27	Gpr150	1	Alb	15
Usp2	9	Foxp3	9	Actn1	10	Pdcd6ip	8	Nfe2l2	26	Pxk	1	H2afz	15
Cd79a	8	Usp2	9	Sfpq	9	Mknk2	7	Nfkbia	23	Arhgap22	1	Gzmb	13
Ercc8	8	Ercc8	8	Fus	8	Ubc	6	Ciita	16	Ttc5	1	Zfpm1	13
Gbp2	7	Hist1h3e	7	Nfil3	6	Tardbp	6	Bmi1	16	Khnyn	1	Ogt	12
Ubc	6	Gbp2	7	Psme3	6	Foxp3	5	Alb	15	Cenpj	1	Camk2d	12
Arntl	6	Ubc	6	Tardbp	6	Ywhae	4	H2afz	15	Cab39l	1	Ccl5	11
Irf8	6	Tsc22d3	6	Ivns1abp	6	Vegfa	4	Ubc	14	Ctsb	1	Ubc	10
Per3	6	Atm	6	Nfkbiz	6	Hnrnpa2b1	4	Srebf1	14	Elp3	1	H2-Aa	10
Cry1	5	Irf8	5	Ubc	5	Ank3	3	Zfpm1	13	Pdlim2	1	Mll1	9
Hlf	4	Tcf21	5	Ywhae	4	Sfpq	2	Gzmb	13	5031414D18Rik	1	Calr	9
Peli1	4	Tcf3	4	Foxp3	4	Ldb1	2	Camk2d	12	Slitrk1	1	Pdcd6ip	8
Cybb	4	Hist3h2a	4	Ddx58	4	Rara	2	Ogt	12	Gpr18	1	Ercc8	8
Nr1d1	4	Peli1	4	Parp14	3	Fbxo32	2	Ccl5	11	Slurp1	1	Hck	8
Tcf3	3	Hlf	4	Irf8	3	Mapk1	2	Cdx2	11	Txn2	1	Gbp2	7
Sirt1	3	Nphp1	3	Rgs2	3	Actg1	2	H2-Aa	10	Hoxc10	1	Ccnd2	7
Foxp3	3	Arntl	3	Stat3	3	Myh9	2	Trim24	10	Itga5	1	Hist1h3e	7
Cry2	3	Mid1	3	Pou5f1	3	Pou5f1	2	Rela	9	Carhsp1	1	Ido1	7
Per1	3	Traf6	3	Luc7l	3	Traf2	2	H2-K1	9	Fopnl	1	Ndel1	7
Rela	2	Pou5f1	2	Stat6	2	Prpf4b	2	Ttr	9	Rtp4	1	Ywhae	6
Ewsr1	2	Esrrb	2	Gata1	2	Oxct1	2	Foxp3	8	Uts2d	1	Kpnb1	6
Sfpi1	2	Cry1	2	Nphp4	2	Trpm7	2	Rad23b	8	Lsg1	1	Rora	6
Creb1	2	Ewsr1	2	Map3k3	2	Ddx17	2	Ercc8	8	Parp14	1	Ghr	6
Esrrg	2	Top1	2	Nacc1	2	Ahctf1	2	Rbm39	8	Dtx3l	1	Nfkbiz	6
Irf1	2	Kdm2b	2	Tcf3	2	Med16	2	Afp	8	Tmem39a	1	Atm	6
Nfkb1	2	Rnf2	2	Esrrb	2	Plcg2	1	Smarca4	7	Mx2	1	Stat3	5
Scd1	2	Tal1	2	Tfcp2l1	2	Mapk11	1	Pou5f1	7	Tcp1	1	Arg1	5
Ets1	2	Hdac1	2	Per2	2	Clock	1	Gbp2	7	Tapbp	1	Foxp3	5
Cul3	2	Actb	2	Per1	2	Sparc	1	Ido1	7	Atf6b	1	Hsph1	5
Arih1	2	Tfcp2l1	2	Smarca4	2	Rnf11	1	Stat3	6	Aif1	1	Fbxo32	4
Rxra	2	Trpm7	2	Smarca2	2	Tnfaip3	1	Psme3	6	H2-Q10	1	Nfkb1	4
Pparg	2	Polb	2	Ywhaz	2	Snn	1	Jun	6	Trim26	1	Hist3h2a	4
Cd36	2	Cul3	2	Rara	2	Gem	1	Atm	6	Hsp90ab1	1	Traf6	4
Clock	2	Ccnb1	2	Il6	2	Sfpi1	1	Nfkb1	6	Trem2	1	Tuba1a	4
Csnk1e	2	Ccl6	2	Ikbkg	2	Gtf2a1	1	Hnrnpab	6	Hrh4	1	Ceacam1	4
Aplp1	1	Ywhae	2	Nfkb1	2	Esrrb	1	Ghr	6	Rit2	1	Cd8a	4
Aplp2	1	Nphp4	2	Sirt1	2	Gtf2e1	1	Nfkbiz	6	Hsd17b4	1	Rgs2	3
Pcdh18	1	Iqcb1	2	Ets1	2	Srsf10	1	Ins1	5	Ehd1	1	Ywhab	3
Vldlr	1	Fbxo32	2	Scp2	2	Gtf2e2	1	Kat5	5	Slc15a3	1	Iqcb1	3
Nfkbia	1	Ahctf1	2	Tal1	2	Rin1	1	Siah1a	5	Lpxn	1	Nphp1	3
Ywhag	1	Nanog	2	Vat1	2	Nr0b1	1	Rnf2	5	Gda	1	Tal1	3
Esrrb	1	Mid1ip1	1	Prpf4b	2	Atat1	1	Sp1	5	Cbwd1	1	Pou5f1	3
Pax5	1	1810046K07Rik	1	Aplp1	1	Mll1	1	Ddx58	5	Cd274	1	Jun	3
Ckm	1	Sim1	1	Aplp2	1	Pdcd6	1	Ywhae	5	Stambpl1	1	Ptpn11	3
Ggh	1	Myh9	1	Pcdh18	1	Sh3kbp1	1	Mark2	5	Entpd1	1	Psmb8	3
Atp6v0d2	1	Chordc1	1	Arhgef1	1	Btrc	1	Zfp110	5	Zdhhc6	1	H2-Ab1	3
Hdac1	1	Irf1	1	Vldlr	1	Sirt1	1	Sfpi1	5	Kynu	1	Cebpb	3
Irak1	1	Itgb1bp2	1	Rela	1	Grin1	1	Hdac1	4	Zeb2	1	Foxa1	3
Rbpj	1	Telo2	1	Clock	1	Whsc1	1	Rxra	4	Acvr2a	1	Akap9	3
Crkl	1	Aip	1	Arid1a	1	Ripk1	1	Abca1	4	Ifih1	1	Mcpt2	3
Ncf2	1	Ptch1	1	Tnfaip3	1	Nphp3	1	Cxcl9	4	Cdca7	1	Ddx58	3
Slc5a12	1	Flii	1	Brd7	1	Arhgef6	1	Mt1	4	Wipf1	1	Mafb	3
Abhd2	1	Mtap2	1	Cry1	1	Usp2	1	Foxa1	4	Nckap1	1	Rela	3
Bco2	1	Mapk1	1	Cry2	1	Rpgrip1l	1	Zbp1	4	Ptprj	1	Oasl2	3
Ube2l3	1	Map3k7	1	Smarcb1	1	Bap1	1	Nr1d1	4	Nup160	1	Ank3	3
Gtf2a1	1	Gdi1	1	Smarcd1	1	Hook1	1	Peli1	4	Slc5a12	1	Psmb9	3
Hoxa2	1	Irak1	1	Rora	1	Asxl1	1	H2-D1	4	Slc12a6	1	Mtap4	3
Ssbp3	1	Rbpj	1	Rorc	1	Aph1b	1	Foxa2	4	Frem2	1	Sfpi1	3
Carm1	1	Rxra	1	Sp1	1	Aph1a	1	Nap1l1	4	Etv3	1	Ldb1	2
Esrra	1	Nr0b1	1	Sp3	1	Mlxipl	1	Fbxo32	3	Rxfp4	1	Rnf2	2
Dbp	1	Tada2a	1	Ets2	1	Prnp	1	Tcf3	3	Il6ra	1	Crk	2
Ifrd1	1	Luzp1	1	Elf1	1	Sin3a	1	Tnf	3	Hax1	1	Spna1	2

Rorb	1	Sult2b1	1	Ggh	1	Rnf2	1	Cited4	3	Dennd4b	1	Tcf3	2
Med16	1	Nfkb1	1	Ptpn11	1	Cul3	1	Ube2i	3	Cdc42se1	1	Hdac1	2
Pcbd1	1	Dapk1	1	Ifng	1	Nr1d1	1	Nfe2	3	Ctss	1	Ppp1r9b	2
Wwtr1	1	Tlr4	1	Crkl	1	Csnk1a1	1	Hoxa2	3	Phgdh	1	Ywhag	2
Ncoa2	1	Tlr9	1	Sult2b1	1	Bhlhe41	1	Usf1	3	Mov10	1	Lrrk2	2
Mll1	1	Stub1	1	Etl4	1	Cep55	1	Stat5a	3	Chi3l4	1	Set	2
Btrc	1	Slc5a12	1	Sfpi1	1	Prkar1b	1	Psmb8	3	Cd53	1	Enah	2
Hif1a	1	Dapp1	1	Dmpk	1	Mlf1	1	Psmb10	3	Psma5	1	Dlat	2
Maoa	1	Bco2	1	Dmrtc2	1	Grb2	1	Oasl2	3	Gpr88	1	Ppp2r1a	2
Zfp746	1	Hoxa2	1	Mta2	1	Shc1	1	Fos	3	Ccdc109b	1	Grin1	2
Nrip1	1	Ssbp3	1	Mll1	1	Actb	1	Ewsr1	3	Ints12	1	Cd74	2
1700021K19Rik	1	Gata6	1	Gtf2e1	1	Rem1	1	Foxo3	3	Dapp1	1	Arntl	2
Foxo3	1	Gata3	1	Rnf14	1	Src	1	Hnf4a	3	Tln1	1	Plcg2	2
Cdk7	1	Dbp	1	Ell	1	Raf1	1	Mafb	3	Rod1	1	Nfyb	2
Nr3c2	1	H2afx	1	Add1	1	Pik3r1	1	Ywhab	3	Snx30	1	Nfyc	2
Hdac4	1	Rorb	1	Pcp2	1	Itch	1	H2-Ab1	3	Cdc26	1	Nfya	2
Nphp4	1	Hoxa6	1	Ezh2	1	Ksr1	1	Psmb9	3	Csf3r	1	Stat5b	2
Nkx2-5	1	Pcbd1	1	Hdac2	1	Hist1h3g	1	Psmb6	3	Clspn	1	Nphp4	2
Phb2	1	Igbp1	1	Srsf10	1	Arntl	1	Psmb4	3	Marcksl1	1	Zap70	2
Sfpq	1	Wwtr1	1	Kdm6b	1	Sh3bp4	1	Pcbd1	3	En2	1	Lck	2
Sla	1	Fcho2	1	Dbp	1	Csda	1	Khsrp	3	Man2b2	1	Actg1	2
Sin3a	1	Cdc37	1	Cited4	1	Traf6	1	Sqstm1	3	Cox18	1	Cap1	2
Mapk1	1	Pcgf1	1	Mta1	1	Ube2n	1	Sertad1	3	Rchy1	1	Nova1	2
Csnk1a1	1	Atat1	1	Gtf2e2	1	Crk	1	Rara	3	Uso1	1	Scp2	2
Il1r1	1	Gata1	1	Hif1a	1	Gfap	1	Cxcl10	3	Naaa	1	Phgdh	2
Bhlhe41	1	Gata2	1	Per3	1	Krt18	1	Gsta3	3	Abcg3	1	Nanog	2
Batf	1	Slc17a1	1	Epas1	1	Tal1	1	B2m	3	Iscu	1	Ccl6	2
Il17f	1	Rbm14	1	Ldb1	1	Per3	1	Tbrg1	2	Tmem168	1	Psmb6	2
Il17a	1	Tcf4	1	Rbbp7	1	Creb3l4	1	Rnf114	2	Capza2	1	Psmb4	2
Maf	1	Ppp1ca	1	Med16	1	Ppp2ca	1	Slc11a1	2	Irf5	1	Gata1	2
Il12rb1	1	Hif1a	1	Nr0b1	1	Cry2	1	Mafa	2	Dennd2a	1	Pik3r1	2
Eea1	1	E130012A19Rik	1	Atat1	1	Tfcp2l1	1	Hmox1	2	Tas2r138	1	Sp1	2
App	1	Nr3c2	1	Hdac1	1	Per1	1	Cacybp	2	Prss2	1	Nck1	2
Siah1a	1	Chek1	1	Tcf4	1	Srsf2	1	Wwtr1	2	Gimap6	1	Stat5a	2
Lrpprc	1	Cep290	1	Rbm14	1	Set	1	Cd74	2	Osbpl3	1	Sh3kbp1	2
Lrp8	1	L3mbtl2	1	Cav1	1	Cry1	1	Bcl6	2	Snx10	1	Src	2
Lrp1	1	Ywhaz	1	Maoa	1	Ube2d3	1	Ctbp2	2	Hoxa1	1	Grb2	2
Lrp2	1	Dab2	1	Grin1	1	Foxp1	1	Ccl6	2	Ppm1k	1	Hmbs	2
Ywhab	1	Nphp3	1	Ms4a2	1	Timeless	1	Dsp	2	Add2	1	Hamp	2
Ebf1	1	Mks1	1	Cpa3	1	Nanog	1	Tuba1a	2	Tpra1	1	Irf1	2
Cebpa	1	Atxn3	1	Whsc1	1	Tnf	1	Cebpg	2	Usp18	1	Foxo3	2
Lrrc41	1	Zfand2a	1	Nphp3	1	Map2k2	1	Nphp4	2	Clec4n	1	Mtap2	2
Des	1	Nod1	1	Pdlim5	1	Mapk3	1	Alx3	2	Tapbpl	1	Nefl	2
Myf6	1	Cdk1	1	Iqcb1	1	D4Wsu53e	1	Ptgs2	2	Clec9a	1	Foxa2	2
Myod1	1	Lrrk2	1	L3mbtl2	1	Mal	1	Cdkn1a	2	Ccdc106	1	Nap1l1	2
Smad6	1	Grin1	1	Rpgrip1l	1	Zranb2	1	Tbk1	2	Dmpk	1	Kat5	2
Ppara	1	Bag3	1	Usp2	1	Asb13	1	Arntl	2	Zbtb32	1	Dync1h1	2
Smad7	1	Csnk1e	1	Bap1	1	Ndel1	1	Per2	2	Rhpn2	1	Iigp1	2
Darc	1	Lipc	1	Sin3a	1	Pde1a	1	Cbfb	2	Napsa	1	Smarca4	2
Nfe2	1	Krt8	1	Eya1	1	Homer1	1	Map3k3	2	Ldhc	1	Ptprcap	1
Tbx21	1	Sox3	1	Casp7	1	Prkar2b	1	Alx4	2	Htatip2	1	Plcb2	1
Stat3	1	Hdac4	1	Asxl1	1	Dmxl2	1	Jund	2	Isg20	1	Btk	1
Cebpb	1	Sox11	1	Chrna1	1	Smarcb1	1	Ccar1	2	Abhd2	1	1810046K07Rik	1
Atf2	1	Ryr2	1	Six1	1	Spna2	1	Car3	2	Lig4	1	Grb10	1
Ddit3	1	Stra8	1	Casp3	1	Arhgef2	1	Btk	2	Tnfsf13b	1	Sim1	1
Hnf4a	1	Il1r1	1	Mlxipl	1	Matr3	1	Hif1a	2	Lamp1	1	Cdk4	1
Nr5a1	1	Batf	1	Rnf2	1	Kcnab2	1	Pigr	2	Cln8	1	Chordc1	1
Alx4	1	Il17f	1	Cul3	1	Tanc2	1	Ndrg1	2	Nat2	1	Bin1	1
Nr1h3	1	Il17a	1	Sorbs2	1	Gramd1b	1	Scp2	2	Cyp4f18	1	Nfkbia	1
Foxp1	1	Maf	1	App	1	Ppp1r9b	1	Tal1	2	Slc10a7	1	Il6	1
Nedd4	1	Il12rb1	1	Siah1a	1	Capzb	1	Pml	2	N4bp1	1	Itgb1bp2	1
Timeless	1	Cdk9	1	Gpi1	1	Tufm	1	Ins2	2	Irx3	1	Ndfip1	1
Yes1	1	Raf1	1	Lrp8	1	Nefm	1	Ppara	2	Irx5	1	Telo2	1
Dab2ip	1	Irf3	1	Lrp1	1	Git1	1	Gclm	2	Gins3	1	Aip	1
Nr0b2	1	Tubb4b	1	Lrp2	1	Pacs1	1	E2f1	2	Mlkl	1	Ifng	1
Plcg1	1	Tubb5	1	Ppp1r9b	1	Camk2g	1	Psme2	2	Cotl1	1	Flii	1
Cdk6	1	Lrrfip1	1	Btrc	1	Tubb2a	1	Arih1	2	Casp1	1	Cdkn1a	1
Mybbp1a	1	Tuba1a	1	Ube2i	1	Spnb2	1	Atf5	2	Mtmr2	1	Casp3	1
Src	1	Tmod3	1	Ccl5	1	Mark3	1	Vdr	2	S1pr2	1	Notch1	1
Nudt5	1	Lrrfip2	1	Hes1	1	Ppfia2	1	Pcgf2	2	Amica1	1	Bcl6	1
Iigp1	1	Mapk3	1	Relb	1	Itpka	1	Ifna4	2	Bco2	1	Rbpj	1
Was	1	Akt1	1	Irak1	1	Capza2	1	Ifnb1	2	Alg9	1	Mapk1	1
Nono	1	Smad6	1	Ikbkb	1	Rab11fip5	1	Btrc	2	Hexa	1	Cebpg	1
Sqstm1	1	Smad7	1	Nr1d1	1	C1qa	1	Hes1	2	Gripap1	1	Rfx3	1
Chd4	1	Nfe2	1	Gata2	1	Coro2a	1	Trp53	2	Cybb	1	Crem	1
Nr1d2	1	Med26	1	Hlf	1	Ywhag	1	Traf6	2	Elf4	1	Nkx2-5	1

Cul5	1	Kat2a	1	Vdr	1	Arhgef7	1	Gata1	2	Mtm1	1	Cryab	1
Csnk1d	1	Tbx21	1	Baz1b	1	Pfkm	1	Casp3	2	Abcd1	1	Psap	1
Ibtk	1	Stat3	1	Il12b	1	Itpr1	1	Casp7	2	Ercc6l	1	Map3k7	1
Efnb2	1	Cebpb	1	Il13	1	Rims3	1	Hnf1a	2	Atrx	1	Gdi1	1
Irf4	1	Snap25	1	Il10	1	Braf	1	Hmbs	2	Cox7b	1	Stk39	1
Ctcf	1	Ar	1	Nfat5	1	Pde4d	1	Stat6	2	Tlr13	1	Rxra	1
		Dnajc5	1	Ctbp2	1	Nefl	1	Pparg	2	Sh3bgrl	1	Ptpn12	1
		Nacc1	1	Lmna	1	Syn2	1	Cryab	2	Htr2c	1	Fyn	1
		Sgta	1	Ehf	1	Camk2a	1	Ctnnb1	2	Ctps2	1	Itgb1	1
		Pbx1	1	Fbxw11	1	Ppp2cb	1	Ywhag	2	Ube2l3	1	Snn	1
		Alx4	1	Ctr9	1	Cnp	1	Il6	2	Keap1	1	Gem	1
		Hoxb8	1	Rbbp8	1	Pde1b	1	Ikbkg	2	Tnik	1	Dok1	1
		Myd88	1	Traf6	1	Enah	1	Nfyb	2	Phc3	1	Lilrb3	1
		Pcna	1	Srsf2	1	Arid1a	1	Nfyc	2	Cbr3	1	Tle3	1
		Esrrg	1	Nfkb2	1	Camkv	1	Nfya	2	Mta2	1	Csf3r	1
		Pbx3	1	Kdm1a	1	Madd	1	Ssbp3	2	Esrrb	1	Erap1	1
		Erg	1	Elf5	1	Mtap6	1	Smad7	2	Nrf1	1	Dapp1	1
		Nedd4	1	Sumo2	1	Ppp3cc	1	Ikbkb	2	Hltf	1	Prss2	1
		Mapt	1	Ptprq	1	Vat1	1	Ptpn11	2	Ppargc1b	1	Dmpk	1
		Fkbpl	1	Sall4	1	Prkacb	1	Cxcr3	2	H2afx	1	Bco2	1
		Hspb1	1	Atp6v1a	1	Spna1	1	Tfcp2l1	2	Esrra	1	Ewsr1	1
		Hsph1	1	Ctbp1	1	Nf1	1	Irak1	2	Ptma	1	Tnik	1
		Per1	1	Trp53	1	Inpp5e	1	Irf2	2	Lass2	1	Hoxa2	1
		Mapre2	1	Hdac3	1	Sbf1	1	Cd36	2	Hdac2	1	Mta2	1
		Ints6	1	Smarce1	1	Atp2b2	1	Il12b	2	Bcl11a	1	Esrrb	1
		Cry2	1	D4Wsu53e	1	Hspa2	1	Nr1h2	2	Esr2	1	Ssbp3	1
		Per2	1	Mal	1	Syngap1	1	Il18	2	Pura	1	Ikbkg	1
		Gas2l3	1	Yes1	1	Calm3	1	Socs1	2	Slc38a2	1	Egfr	1
		Iigp1	1	Rsrc1	1	Hspa5	1	Cbl	2	Mta1	1	Kdm2b	1
		Myod1	1	Hoxa9	1	Nos1	1	Tlr9	2	Cubn	1	H2afx	1
		Sqstm1	1	Dab2ip	1	Eef1g	1	Irf4	2	Rbbp7	1	Dvl2	1
		Chd4	1	Nr0b2	1	Atad3a	1	Nos2	2	Dlx5	1	Hdac2	1
		Ppp2r1a	1	Plcg1	1	Acta1	1	Sumo1	2	Usp2	1	Mta1	1
		Ibtk	1	Cdk6	1	Kif2a	1	Mafg	2	Khdrbs1	1	Pcbd1	1
		Med23	1	Src	1	Wac	1	Il27	2	Nr0b1	1	Rin1	1
		Irf4	1	Was	1	Ina	1	Iigp1	2	Gsk3b	1	Wwtr1	1
		Ctcf	1	Mafa	1	Plec	1	Dab1	1	Mybl2	1	Cdc37	1
				Cul5	1	Mtap2	1	Rac1	1	Litaf	1	Rbbp7	1
				Csnk1e	1	Ppp3ca	1	Grb10	1	Wbp1	1	Pcgf1	1
				Nfkbib	1	Evl	1	Cdk4	1	Rnf11	1	Usp2	1
				Efnb2	1	Eef1b2	1	Esr1	1	Prrg2	1	Nr0b1	1
				Ppp1ca	1	Dlat	1	Gopc	1	Nr1h3	1	Atat1	1
						Syn1	1	Il12rb1	1	Rala	1	Pdcd6	1
						Myh10	1	Bin1	1	Ralb	1	Litaf	1
						Rapgef2	1	Tnfaip3	1	Itgb1	1	Cd8b1	1
						Slc1a3	1	Polr2f	1	Cav1	1	Hspb1	1
						Camk2d	1	Irf3	1	Lgals2	1	Wbp1	1
						Wdr7	1	Ndfip1	1	Ppp1cc	1	Hspa8	1
						Pdhb	1	Telo2	1	Ppp1ca	1	Slc17a1	1
						Ak5	1	Eif2ak3	1	Irs2	1	Rnf11	1
						Csnk1e	1	Flii	1	Amfr	1	Prrg2	1
						Kif5c	1	Mtap2	1	Maoa	1	Cav1	1
						Tpi1	1	Maged1	1	Arg1	1	Atxn1	1
						Clip2	1	Il12a	1	Nrip1	1	Lgals2	1
						Eef1a1	1	Notch1	1	Ms4a2	1	Ppp1ca	1
						Pfkp	1	Rbpj	1	Cpa3	1	Pgrmc1	1
						Camk2b	1	Ebf1	1	Marco	1	Rbbp5	1
						Homer3	1	Fas	1	Whsc1	1	Nrip1	1
						Wdr37	1	Cdkn2b	1	Gclc	1	Ms4a2	1
						Ppp2r1a	1	Etv6	1	Ywhah	1	Cpa3	1
						Rheb	1	Tap1	1	Sptlc1	1	Whsc1	1
						Psd3	1	Tlr3	1	Rsrc1	1	Mep1a	1
						Eno1	1	Agtr2	1	Nphp3	1	Nphp3	1
						Tuba1a	1	Tfap2a	1	Ngly1	1	E130012A19Rik	1
						Hspa9	1	Rfx3	1	Fez2	1	Abl1	1
						Pfkl	1	Il7r	1	Rnf123	1	Sla2	1
						Ppp1cc	1	Hoxc8	1	Ywhaz	1	Arhgef6	1
						Ap2b1	1	Acox2	1	Fam175b	1	Chek1	1
						Camsap2	1	Crem	1	Nphp1	1	Rnf123	1
						Mtmr1	1	Cry1	1	Bbs4	1	Fcgr1	1
						Rogdi	1	Usf2	1	Rfxank	1	Sla	1
						Ywhah	1	Nkx2-2	1	Ube4b	1	Crkl	1
						Bcr	1	Ptgds	1	Fbxo4	1	Lyn	1
						Kif5b	1	Abcc2	1	Bap1	1	Ube4b	1
						Ccdc88a	1	Egr1	1	Zbtb7c	1	Rara	1
						Myo18a	1	Pou2f1	1	Asxl1	1	Rpgrip1l	1
						Gsn	1	Zbtb20	1	Cul3	1	Dlg1	1
						Dync1h1	1	Il7	1	Psme1	1	Inpp5d	1
						Gas2l3	1	Sars2	1	Pax6	1	Bap1	1

						Arhgef18	1	Mrps12	1	Mlxipl	1	Hook1	1
						Strn	1	Pax5	1	Maf	1	Nod1	1
						Erc1	1	Ripk1	1	Hdac4	1	Asxl1	1
						Padi2	1	Aicda	1	Trim28	1	Cdk1	1
						Nsf	1	Dab2	1	Ryr2	1	Cdk2	1
						Ywhab	1	Tlr4	1	Trim33	1	Erbb2	1
						Trim3	1	Tlr11	1	Ezh2	1	Tnfrsf11a	1
						Mbp	1	Il15	1	Ryr1	1	Cdk6	1
						Ppp3cb	1	Mdm2	1	Psma3	1	Aph1b	1
						Cltc	1	Bcl2	1	Chd7	1	Aph1a	1
						Myo5a	1	Isg15	1	Mapk14	1	Lipc	1
						Golga3	1	Irf9	1	Cbx1	1	Dok3	1
						Kctd16	1	Rsad2	1	Cbx5	1	Fgr	1
						Lrrk2	1	Prmt5	1	Enam	1	Pax6	1
						Cryab	1	Rarb	1	Arrb1	1	Map1lc3b	1
						Nono	1	Cul4a	1	Glul	1	Mlxipl	1
						Nacc1	1	Cul4b	1	Cdk6	1	Prnp	1
						Csnk1d	1	Ehmt1	1	Cdkn1b	1	Gabarap	1
						Rps6ka5	1	Lilrb3	1	Cdk2	1	Nck2	1
								Tle3	1	Smurf1	1	Psmc5	1
								Tap2	1	Pias3	1	Sorbs2	1
								Cebpa	1	Relb	1	Krt8	1
								Il18r1	1	Enah	1	Sox3	1
								Nabp1	1	Tubb4b	1	Hdac4	1
								Fam117b	1	Tubb5	1	Sox11	1
								Klf7	1	Lrrfip1	1	Plcg1	1
								Wasf1	1	Tmod3	1	Eps8	1
								Vps26a	1	Lrrfip2	1	Cd40	1
								Tle6	1	Actb	1	Ccnb1	1
								Rfx4	1	Stat5b	1	Ryr2	1
								Lyz2	1	Cbx2	1	Stra8	1
								Helb	1	Laptm5	1	Themis	1
								Eif4enif1	1	Clock	1	Cep55	1
								Plek	1	Msx2	1	Ryr1	1
								Irgm1	1	Msx1	1	Psma3	1
								Mgat1	1	Bid	1	Was	1
								Adamts2	1	Hopx	1	Prkaca	1
								Nlrp3	1	Nf1	1	Mlf1	1
								Cd68	1	Myd88	1	Pafah1b1	1
								Asgr2	1	Tfcp2	1	Smurf1	1
								Tmem18	1	Mtf1	1	Cd2ap	1
								Eif2s1	1	Rxrb	1	Rem1	1
								Tmem229b	1	Atf2	1	Raf1	1
								Dnalc1	1	Foxc2	1	Irf3	1
								Jdp2	1	Foxa3	1	Ksr1	1
								Gpr68	1	Rfx1	1	Fam63b	1
								Serpina9	1	Rfx2	1	Hist1h3g	1
								Cyp46a1	1	Foxd3	1	Tubb4b	1
								Pld4	1	Sp3	1	Tubb5	1
								Pfkp	1	Pdx1	1	Lrrfip1	1
								Ly86	1	Gsta1	1	Tmod3	1
								Rnf144b	1	Nqo1	1	Lrrfip2	1
										Mafk	1	Actb	1
										Zfp148	1	Flt1	1
										Acaca	1	Laptm5	1
										Nfatc2	1	Sh3bp4	1
										Sell	1	Bid	1
										C1ra	1	Hopx	1
										Cst3	1	Casp7	1
										Smad6	1	Mapk3	1
										Hmgb1	1	Tfcp2	1
										Il23a	1	Atf2	1
										Darc	1	Rfx1	1
										Hells	1	Rfx2	1
										Dnmt3b	1	Cbfb	1
										Socs3	1	Mitf	1
										Adam10	1	Sp3	1
										Wdr77	1	Zfp148	1
										Nfat5	1	Stat6	1
										Cebpb	1	Akt1	1
										Rgs2	1	Yy1	1
										Lbr	1	Ebf1	1
										Tgfb2	1	Fas	1
										Mnt	1	Darc	1
										Myo1c	1	Nfe2	1
										Akap1	1	Tsnax	1
										Mmd	1	Gfap	1

										Ppp1r9b	1	Krt18	1
										Spop	1	Socs3	1
										Cnp	1	Gata2	1
										Atp6v0a1	1	Cdipt	1
										Ifi35	1	Xdh	1
										Grn	1	Itsn1	1
										Xdh	1	Scnn1a	1
										Cript	1	Ctbp2	1
										Itpka	1	Pten	1
										Dido1	1	Ehf	1
										Pdha2	1	Alx4	1
										Gbp3	1	Tfcp2l1	1
										Hmgcl	1	Rbbp8	1
										Necap2	1	Nfkb2	1
										Btc	1	Dbp	1
										Erp29	1	Kit	1
										Tpst1	1	Kdm1a	1
										Ncf1	1	Elf5	1
										Dtx2	1	Clock	1
										Golt1b	1	Rap2a	1
										Flt3l	1	Ctbp1	1
										Tm6sf1	1	Per2	1
										Ankrd42	1	Fkbpl	1
										Prcp	1	Cdkn1b	1
										Pgm2l1	1	Wbp2	1
										Ucp2	1	Bean1	1
										Dctn5	1	Shc1	1
										Xpo6	1	Tbc1d23	1
										Ctsd	1	Mal	1
										Mst1r	1	Hunk	1
										Nckipsd	1	Pde1a	1
										Xcr1	1	Sh2b2	1
										Zfx	1	Homer1	1
										Scnn1a	1	Sfpq	1
										Pten	1	Prkar2b	1
										Pax3	1	Dmxl2	1
										Zscan21	1	Smarcb1	1
										Ehf	1	Spna2	1
										Fbxw11	1	Arhgef2	1
										Dmap1	1	Matr3	1
										Rbbp8	1	Kcnab2	1
										Ppargc1a	1	Tanc2	1
										Hoxb1	1	Gramd1b	1
										Gmeb1	1	Capzb	1
										Nfkb2	1	Tufm	1
										Kdm2b	1	Nefm	1
										Nr0b2	1	Git1	1
										Kdm1a	1	Pacs1	1
										Elf5	1	Camk2g	1
										Set	1	Tubb2a	1
										Nr1h4	1	Spnb2	1
										Sumo2	1	Nefh	1
										Rap2a	1	Mark3	1
										Ctbp1	1	Ppfia2	1
										Polr2a	1	Itpka	1
										Zfp292	1	Capza2	1
										Phc2	1	Rab11fip5	1
										Rad23a	1	C1qa	1
										Hspa1a	1	Coro2a	1
										Wbp2	1	Arhgef7	1
										Bean1	1	Pfkm	1
										Exoc3	1	Csf1r	1
										Hic1	1	Itpr1	1
										Ring1	1	Dctn1	1
										Cbx8	1	Rims3	1
										Hdac3	1	Braf	1
										Dyrk1a	1	Pde4d	1
										Dyrk3	1	Syn2	1
										Dbp	1	Camk2a	1
										Cdk1	1	Ppp2cb	1
										Ccnb1	1	Cnp	1
										Rbfox3	1	Pde1b	1
										Akt1	1	Arid1a	1
										Nanog	1	Camkv	1
										Nhlh2	1	Madd	1
										Copb1	1	Mtap6	1
										Prdm1	1	Ppp3cc	1
										Elovl1	1	Dlg3	1
										Mal	1	Vat1	1
										Asb13	1	Prkacb	1
										Hunk	1	Nf1	1

										Fez1	1	Inpp5e	1
										Hoxa9	1	Sbf1	1
										Coro1c	1	Atp2b2	1
										Dlg3	1	Hspa2	1
										Ntrk1	1	Syngap1	1
										Zbtb17	1	Calm3	1
										Gata6	1	Hspa5	1
										Nudt5	1	Nos1	1
										Mier2	1	Eef1g	1
										Rb1	1	Atad3a	1
										Sobp	1	Acta1	1
										Camk1	1	Kif2a	1
										Ttc4	1	Wac	1
										Scnn1b	1	Ina	1
										Aff4	1	Plec	1
										Copb2	1	Ppp3ca	1
										Dner	1	Evl	1
										Snca	1	Eef1b2	1
										Mpnd	1	Syn1	1
										Dcbld2	1	Myh10	1
										Adrbk1	1	Rapgef2	1
										Mecp2	1	Slc1a3	1
										Pcdh18	1	Wdr7	1
										Med22	1	Pdhb	1
										Atg7	1	Ak5	1
										Uqcrc1	1	Kif5c	1
										Xpc	1	Tpi1	1
										Nucb1	1	Clip2	1
										Nefl	1	Eef1a1	1
										Hap1	1	Myh9	1
										Tada3	1	Pfkp	1
										Dctn1	1	Camk2b	1
										Myl9	1	Homer3	1
										Gapdh	1	Wdr37	1
										Ddx24	1	Rheb	1
										Cabp1	1	Psd3	1
										Hcfc1	1	Eno1	1
										Pogz	1	Hspa9	1
										Cdh5	1	Pfkl	1
										Xpa	1	Ppp1cc	1
										Otub1	1	Ap2b1	1
										Ncor2	1	Camsap2	1
										Chmp4b	1	Mtmr1	1
										Ncaph	1	Rogdi	1
										Iffo2	1	Ywhah	1
										Thoc5	1	Bcr	1
										Tdp2	1	Kif5b	1
										Iffo1	1	Ccdc88a	1
										Pex19	1	Myo18a	1
										Aff2	1	Gsn	1
										Lamb1	1	Arhgef18	1
										Nr1d2	1	Strn	1
										Cdkn2a	1	Erc1	1
										Nfx1	1	Padi2	1
										Tbc1d9	1	Nsf	1
										Angptl2	1	Trim3	1
										Nin	1	Mbp	1
										Ppp2r1a	1	Ppp3cb	1
										Scnn1g	1	Cltc	1
										Dazap2	1	Myo5a	1
										Mcm10	1	Golga3	1
										Eid1	1	Kctd16	1
										Strn4	1	Pxn	1
										2610301B20Rik	1	Itk	1
										Ift57	1	Scnn1b	1
										Chmp5	1	Pik3cg	1
										Kat8	1	Adrbk1	1
										Cdh4	1	Syk	1
										Ccm2	1	Tbx3	1
										Vcp	1	Sox9	1
										Rnpc3	1	Sqstm1	1
										Rpl28	1	Synm	1
										Tyw1	1	Rps6ka5	1
										Rufy2	1	Scnn1g	1
										Cdh2	1	Hcfc1	1
										Rcbtb2	1	Cry1	1
										Huwe1	1	Cxcr4	1
										Nfkbib	1	Sox17	1
										Cxcr4	1	Igfbp5	1
										Sox17	1	Afp	1

										Igfbp5	1	Hnf4a	1
										Apoa1	1	Apoa1	1
										Rybp	1	Ttr	1
										Wnt7a	1	Wnt7a	1
										Ptpn6	1	Ptpn6	1
										Fes	1	Diap1	1
										Erbb2	1		

Bridge section between Chapter 2 and Chapter 3

The transcriptome analyses described in Chapter 2 showed that the small intestinal transcriptome wild-type mouse is sexually dimorphic, and that the deletion of specific intestinal Fabps altered the small intestinal transcriptome differently in males and females. As Fabps share ligands with other regulatory proteins, particularly members of the nuclear receptor family of transcription factors, these results may also suggest the resulting sex-specific changes in small intestine transcriptional program is also influenced by sexually dimorphic handling and processing of bioactive nutrients.

Males and females show different susceptibility to diet-induced metabolic disorders as well as improvements to dietary interventions. For example, male mice are more susceptible to diet-induced obesity and its complications like hyperinsulinemia and inflammation compared to female mice (127, 128) while men and women respond differently to a Mediterranean diet with regard to changes in metabolic parameters (129, 130). However, the role of sex in the development of these metabolic differences remains unclear, because of the lack of controlled experiments in both sexes. As the critical role of sex in the metabolic reprogramming in response to the same gene variation was identified, this thesis then investigated how biological sex influences the effect of extrinsic factors (i.e., diets and drugs) on susceptibility to diet-induced metabolic diseases and sensitivity to therapeutic interventions.

In Chapter 3, the studies were carried out to determine if male and female mice fundamentally differ in susceptibility to WSD-induced metabolic disorders, in particular cardiac fibrogenesis, as well as in their response to diet-based and drug-based therapeutic interventions. The WSD is high in saturated fat and refined sugar and was used to induce metabolic dysregulation in both male and female mice. As shown in previous studies, high-fat and high-sugar diet feeding can lead to various types of metabolic disorders in mice such as obesity, insulin resistance, as well as hepatic and cardiac fibrosis, which may be attributed to diet-induced endoplasmic reticulum (ER) stress (131-133). Palmitic acid is a major component of the saturated fat in WSD and it is known to be a potent inducer of ER stress (52, 53). Next, the therapeutic potential of diet-based and drug-based interventions was evaluated in both sexes. The diet-based intervention, which aimed to reduce energy density and increase nutrient diversity, was achieved by switching from WSD to the low-fat laboratory standard mouse diet. Supplementation of the low-fat diet with UDCA, a type of secondary bile acid and approved drug for primary biliary cirrhosis, served as the drug-based intervention. UDCA has a proteostasis promoting effect and thus could alleviate ER stress (60, 61). Male and female mice used in this study were from a single certified strain (C57BL/6) and thus had identical genetic composition (the intrinsic factor) and received the identical diets and therapeutic interventions (the extrinsic factors).

Chapter 3

Sex-dimorphic attenuation of Western-style diet-induced metabolic dysregulation in mice by switching diet with or without ursodeoxycholic acid

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Abstract

Background: Chronic overconsumption of Western-style diet (WSD) is associated with the increased prevalence of metabolic disorders including acquired cardiovascular diseases. WSD-induced metabolic dysregulation and cellular stress are implicated in cardiac dysfunction and cardiac fibrogenesis. Cardiac fibrosis, the result of pathological cardiac tissue remodeling, is the major cause of terminal heart failure. Previous studies found that ursodeoxycholic acid (UDCA), a secondary bile acid, can ameliorate metabolic dysregulation and attenuate cellular stress, preventing the development of cardiac fibrosis. UDCA has long been used to treat cholestatic liver diseases and is an FDA approved medication for primary biliary cirrhosis. **Methods**: Male and female mice were fed with the WSD for 12 weeks. The mice were then switched to the low-fat standard laboratory diet (2020X diet), without or with UDCA supplementation (320 mg/kg/d), for another 8 weeks. Metabolic status and fibrotic status in livers and hearts were assessed after 12-week WSD feeding and at the end of the 8-week intervention. **Results:** WSD feeding for 12 weeks induced metabolic dysregulation in both sexes but more profoundly in males. In particular, WSD induced extensive hepatic and cardiac collagen deposition primarily in male mice. In liver, switching WSD to 2020X diet without UDCA supplementation arrested further collagen accumulation in both sexes whereas the UDCA supplementation induced the removal of accumulated collagen and reduced collagen production in the males only. In heart, switching the diet without UDCA also prevented further collagen accumulation while the addition of UDCA induced the removal of accumulated collagen and decreased collagen production in both sexes. Moreover, UDCA supplementation alleviated cardiac endoplasmic reticulum stress as indicated by decreased markers of UPR pathways in both sexes. **Conclusion**: The data show that UDCA enhanced the benefit of switching to a low-fat diet on WSD-induced metabolic dysregulation and cardiac fibrogenesis. Sex plays an important role in disease susceptibility and treatment efficacy.

Keywords

Diet, western-style diet, cardiac fibrosis, ursodeoxycholic acid (UDCA), sex differences, metabolic disorder

Introduction

Over consumption of WSD is associated with the development of various metabolic disorders. Among them, cardiovascular disease (CVD), which refers to a class of medical disorders of heart and blood vessels, is the leading cause of death around the world (1). CVD has two main types, cardiac diseases and vascular diseases. Cardiac diseases directly affect the heart itself such as impairing the function of cardiac muscle and valves while vascular diseases involve narrowing or blocking of blood vessels in hearts and other tissues. Various factors have been found implicated in the development of CVD, among which diet and nutrition are major risk factors (2). Foods with high portions of energy-dense and processed foods but low portions of natural and minimally processed foods are prevalent in the Western-style diet (WSD). Long-time consumption of the WSD is associated with the development of CVD, heart failure, and higher cardiovascular mortality (1, 3-6). In particular, excess energy substrates and poor nutrient diversity of the WSD are thought to be the major factors underlying causes of the loss of cellular energy and nutrient homeostasis, the induction of cellular stress, and the dysregulation of cellular metabolism (3). Endoplasmic reticulum (ER) stress is a type of cellular stress that can be induced by non-optimal diets and it contributes to the pathogenesis of CVD (7). To cope with ER stress, cells make use of the unfolded protein response (UPR). The UPR involves three processes: (a) arrest of translation process to prevent the entry of newly synthesized protein into ER, (b) induction of the expression of genes that encode for proteins that assist in the folding of misfolded or unfolded proteins, and (c) induction of the expression of genes involved in ER-associated degradation system to degrade misfolded proteins (8). Specifically, Bip/GRP78, and three ER transmembrane proteins (ER kinase dsRNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) are the key regulating proteins engaged in the activation of the UPR. In the heart, prolonged ER stress or UPR activity could lead to undesirable consequences such as pathological cardiac remodeling, which involves the stimulation of TGF-β1 signaling pathway and subsequent development of cardiac fibrosis (9-11).

Cardiac fibrosis is associated with various types of heart diseases and it is characterized by excess accumulation of collagen proteins. Fibrogenesis involves an increased synthesis of type I collagen which affects the stiffness of the tissue and is a key component of fibrotic scars (10, 12). Fibrogenesis is initially an adaptive process to fill the space left after the necrosis of cardiomyocytes and thus preserve the structural integrity (13). However, extensive collagen deposition and formation of fibrotic scars contribute to abnormal thickening of the heart valves or stiffening of the myocardium, impairing diastolic and systolic function, ultimately leading to heart failure. So far, there is no effective treatment for cardiac fibrosis (14). However, recent studies found that ursodeoxycholic acid (UDCA) has a preventive and protective effect on the onset and/or development of cardiac fibrosis in drug-induced and genetic models of CVD (10, 12). UDCA is a natural bile acid that is abundant in bear bile (15). In species other than bears, UDCA comprises a small portion of the total bile acid pool in the enterohepatic circulation. In humans, UDCA cannot be synthesized by livers but it accounts for up to 4 % of the total bile acid pool. It is produced by gut bacteria via the 7-beta epimerization of primary bile acids such as chenodeoxycholic acid (CDCA) which is abundant in human bile (16). UDCA is also an FDA-approved drug for treating primary biliary cirrhosis (17). Studies found that UDCA delays the progression of liver fibrosis and the development of cirrhosis in both humans (18) and rodents (19, 20), which might be attributable to its modulating effect on cellular stress. UDCA and its taurine conjugates have also been shown to prevent apoptosis, inhibit the activation of UPR, and promote proteostasis (21-25).

Sex is a known modifier for the incidence, development, and outcomes of CVD (26). Men and women are susceptible to different types of CVD. Men have a higher prevalence of myocardial infarction and women have higher incidences of ischemic stroke (27). The two sexes also suffer from different complications of CVD and have different pharmacological responses to medications (28, 29). Studies found that male and female rodents respond differently to intrinsic or extrinsic triggers for CVD. For example, male and female rodents show differences in physiological and kinetic responses to a genetic modification that simulates familial hypertrophic cardiomyopathy (30). In another rodent model with ventricular pressure overload caused by constriction of the aorta, females developed less myocardial hypertrophy and preserved a better ability for contractile reserve and Ca^{2+} handling (31). Sex differences also exist in responses to nutritional and drug interventions. Males and females of both humans and rodents were affected differently by the partial calorie restriction which is thought to be a beneficial nutritional intervention for increasing lifespan and preventing CVD (32, 33). An epidemiological study shows that UDCA treatment led to a greater frequency of certain drug responses such as the feeling of fatigue and autonomic symptoms in female patients with primary biliary cirrhosis (34).

The current study was undertaken to assess sex differences in the susceptibility to WSD-induced metabolic disorders with a focus on diet-induced cardiac fibrogenesis in mice. The sexually dimorphic effect of diet-based and drug-based interventions on WSD-induced metabolic dysregulation was also investigated. In particular, the additional benefit of UDCA on alleviating fibrogenesis and improving metabolic status over switching to a low-fat diet alone was evaluated in male and female mice.

Results

Induction of metabolic dysregulation by Western-style diet feeding

Sex-dimorphic alteration of metabolism in response to Western-style diet feeding

Male and female C57BL/6 mice were fed with the control 2020X diet or the WSD for 12 weeks to induce metabolic dysregulation. Changes at the organismal level were assessed firstly. Relative to the 2020X diet, the WSD feeding caused more substantial body weight gain in male mice (Figure 2A). In females, WSD feeding also led to higher body gain than 2020X diet but the divergence happened later and to a smaller extent as compared to male mice (Figure 2B). Similar sexual dimorphism in the susceptibility to diet-induced obesity was observed before (35, 36). However, both male and female mice on WSD consumed more calories than the mice on 2020X diet (Supplementary Figure S1). To investigate the source of the weight gain, gonadal fat mass and liver mass were measured. Compared to 2020X diet-fed ones, the WSD-fed males consistently show higher fat mass and liver mass (Figure 2C and 2D). WSD feeding resulted in the same increasing trend in gonadal fat and liver mass in female mice (Figure 2E and Figure 2F). The indirect calorimetry was used to measure the rates of oxygen consumption, carbon dioxide production, and activity rates to reflect the WSD-induced changes in energy metabolism and physical function in mice. Consistent with previous findings (37, 38), WSD feeding led to significantly lower ambulatory activity levels (Figure 2G) and lower night respiratory exchange ratio (RER) (Figure 2I) in male mice. A similar decrease in activity and RER was found in female mice (Figure 2H and 2J). Overall, 12-week WSD feeding induced obesity and metabolic dysregulation in mice, profoundly in males.

Induction of fibrogenesis in livers and hearts

Next, the effect of diets at the organ level was examined. Immunofluorescent imaging was used to visualize the collagen I protein abundance and cellular morphology, being indicative of fibrogenesis, in male and female mice. Since liver is highly susceptible to diet-induced fibrosis (39, 40), it was checked first to verify the effectiveness of this dietary period on inducing fibrosis. Specifically, the collagen I status at the portal triad region in the liver was imaged. As shown in Figure 3A, compared to 2020X diet-fed males, increased staining of collagen I was seen in liver sections from the male mice fed with WSD, indicating the formation of fibrotic scars. WSD feeding also led to an extensive increase in nuclei around the portal triad in the livers of males, indicating a rapid cell proliferation. The concurrence of hepatic injury, fibrosis, and accelerated cell proliferation has been observed in high-fat diet-fed male mice before (41, 42). Interestingly, WSD feeding increased collagen I, but not nuclei in females (Figure 3B). The mRNA abundance of Col1a1, the gene encodes for a major component of collagen I protein, was also measured to provide complete information regarding collagen I gene expression. The data shows that WSD-fed male mice have 2-fold more Col1a1 mRNA than 2020X diet-fed ones (Figure 3C) whereas negligible difference in mRNA was detected between 2020X diet-fed and WSD-fed females (Figure 3D). As anticipated, 12-week WSD feeding is enough to increase collagen gene expression and induce the early onset of liver fibrosis, although the liver of female mice seems to be more resistant to the WSD-induced fibrogenesis and damage than the liver of male mice. To see if hearts display the same susceptibility to diet-induced fibrogenesis as liver, cardiac collagen I protein abundance and cellular morphology were examined. In the heart, compared to 2020X diet, WSD feeding resulted in a greater abundance of collagen I protein in male mice, indicating the initiation of fibrosis (Figure 4A and Figure 4C). However, female mice on either 2020X diet or WSD have a similar amount of collagen I in their hearts (Figure 4B and Figure 4D). To identify the contributors to the WSD-induced cardiac fibrogenesis, the mRNA abundance of Col1a1, Tgf-β1 and Hsp47 in hearts were investigated. Tgf-β1 is a pleiotropic cytokine that is associated with cardiac fibrogenesis (43) while Hsp47 is a collagen-specific protein chaperone (44). Compared to 2020X diet, WSD feeding doubled the amount of Col1a1 and Tgf-β1 mRNA in males (Figure 4E) whereas WSD feeding led to no change in the mRNA abundance of these genes in females (Figure 4F). Female mice appear to be more resistant to WSD-induced cardiac fibrogenesis than male mice. In other words, the same male animals that show extensive deposition of collagen in livers also show abundant collagen I in hearts but not female ones.

Previous studies have found that excess palmitic acid, one of the major types of saturated fatty acid in WSD, can activate UPR which is implicated in cardiac dysregulation and fibrogenesis (10, 11, 45, 46). On the other hand, fatty acid-binding protein 3 (Fabp3), which is essential for fatty acid uptake in hearts (47), was found likely to have a protective effect against excess palmitic acid-induced cellular stress (48, 49). Therefore, UPR status, as well as protein and mRNA abundance of Fabp3, were assessed in the hearts of these mice. As shown in Figure 5A, compared to 2020X diet, WSD feeding led to an increasing trend in mRNA abundance of genes participated in all three UPR pathways in males, especially the PERK pathway which involves Atf4 and Chop. In females, WSD feeding only resulted in higher sXbp1 mRNA which is involved in the IRE-1 pathway of UPR (Figure 5B). Seemingly, WSD feeding activated different UPR pathways in male and female mice. For Fabp3, the preliminary results show that male mice on either 2020X diet or WSD have a similar amount of Fabp3 protein in their hearts whereas WSD-fed female mice have more Fabp3 protein than 2020X diet-fed females (Supplementary Figure S2A). At transcript level, WSD feeding led to a significantly lower abundance of Fabp3 mRNA than 2020X diet in male mice but not in female mice (Supplementary Figure S2B). Together, WSD feeding activated different UPR pathways in the hearts of male and female mice, which might be owing to the overloading of palmitic acid. Interestingly, WSD-fed female mice have more Fabp3 proteins that may exert female-specific protection against WSD-induced disturbance.

Recovery from Western-style diet-induced metabolic dysregulation in response to low-fat diet without or with UDCA supplementation

Sex-dimorphic responses to interventions

The effect of two interventions, switching to a low-fat (2020X) diet alone or with UDCA supplementation, on metabolic dysregulation was evaluated after another 8 weeks of feeding. In general, after the first two weeks of intervention, mice on 2020X diet without or with UDCA consumed very similar amounts of calories (Supplementary Figure S3). In males, switching diet from WSD to 2020X diet prevented further weight gain while adding UDCA to 2020X diet led to a significant weight loss (Figure 6A and 6B). The same effect of the interventions was observed in female mice (Figure 6C and 6D). For the gonadal fat mass, UDCA had little additional effect on the mass compared to the 2020X diet in males but in females, it tended to reduce the fat mass (Figure 6E and 6G). For liver mass, UDCA supplementation led to a significant reduction in liver mass in males (Figure 7F) whereas no significant difference was observed between the liver masses of females feeding on 2020X diet or 2020X diet with UDCA supplementation (Figure 7H). The indirect calorimetry was applied again to measure the activity rates and RER as indicative of the possible improvement in physical function and energy metabolism brought by the interventions. Relative to the activity level at the beginning of the interventions (Figure 2G), switching from WSD to 2020X diet without or with UDCA supplementation for 8 weeks improved nighttime activity in male mice substantially (Figure 6I). A similar pattern of improvement in the rate of physical activity was observed in female mice after switching the diets (Figure 2H and Figure 6J).

For RER, compared to the RER at the beginning of the interventions (Figure 2I and Figure 2J), switching from WSD to 2020X diet had a negligible effect on RER in both male and female mice (Figure 6K and Figure 6L). However, relative to switching to 2020X diet alone, UDCA supplementation tended to lower the nighttime RER in male mice (Figure 6K) while the supplementation had little effect on RER in female mice (Figure 6L). Overall, switching to 2020X diet prevented the progression of metabolic dysregulation whereas UDCA supplementation led to an additional reduction in adiposity.

Inhibition of cardiac fibrogenesis in mice fed with low-fat diet supplemented with UDCA

The morphology of the livers and abundance of hepatic collagen I of mice at the end of the interventions were examined to check the effect of the dietary and UDCA interventions on the fibrogenesis in the liver. In males, compared to the fibrotic status before the interventions (Figure 3A), similar abundance of collagen I was detected but no extensive division of nuclei around the portal triad region was observed after switching to 2020X diet (Figure 7A). There is also negligible change being detected on the fibrotic status in female livers between the beginning (Figure 3B) and the end of the 2020X diet intervention (Figure 7B). These results indicate that the progression of hepatic fibrogenesis could be arrested by just switching from the WSD to the 2020X diet. In contrast, supplementation of the 2020X diet with UDCA led to a reduction of collagen I abundance in the liver of males (Figure 7A). In females, mice on 2020X diet without or with UDCA showed similar amounts of collagen I (Figure 7B). Seemingly, UDCA can only reduce collagen I protein abundance in male livers but not females. At transcript level, relative to switching to 2020X diet, the diet with UDCA tended to decrease Col1a1 mRNA abundance in the liver of male mice (Figure 7C) whereas the UDCA supplementation tended to increase Col1a1 mRNA abundance in the liver of female mice (Figure 7D). These results imply the differential efficacy of UDCA treatment in reversing hepatic collagen deposition in male and female mice, possibly by affecting the Col1a1 gene expression. In hearts, compared to the collagen I abundance in WSD-fed males at the beginning of the interventions (Figure 4C), another 8-week 2020X diet feeding led to little increase in collagen I abundance (Figure 8C), suggesting that switching from the WSD to the 2020X diet can prevent further accumulation of collagen in the hearts of male mice. A similar preventive effect of switching diet on fibrogenesis was found in the hearts of female mice (Figure 4D and Figure 8D). On the other hand, the supplementation of the 2020X diet with UDCA significantly decreased collagen I protein abundance in both male and female mice than switching to 2020X diet alone (Figure 8A, 8B, 8C, and 8D), suggesting the potential of UDCA on removing accumulated collagen in hearts. At transcript level, mRNA abundance of Col1a1, Tgf-β1 and Hsp47 were assessed as indirect measures reflecting the rate of collagen production. Compared to 2020X diet alone, UDCA supplementation led to a significantly lower mRNA abundance of Col1a1 and Hsp47 than 2020X diet alone in males (Figure 8E) while it reduced the mRNA abundance of all three genes in females (Figure 8F), implying that UDCA supplementation may reduce cardiac collagen abundance by adjusting the rate of collagen production. Overall, UDCA attenuated fibrogenesis in the hearts of both male and female mice.

To investigate the underlying mechanism for the UDCA-induced attenuation in cardiac fibrogenesis, the effect of UDCA on UPR status, specifically on the mRNA abundance of surrogate reporters for each pathway of UPR in hearts, was measured. As shown in Figure 9, UDCA supplementation reduced the mRNA abundance of Grp78, Atf4, Chop, and Grp94 dramatically while led to a tendency of decreasing for sXbp1 in both male and female mice, indicating that UDCA might achieve its effect through adjusting UPR status. Additionally, changes in cardiac

Fabp3 protein and mRNA abundance in response to switching diets were assessed. The preliminary results show that relative to the 2020X diet only, UDCA supplementation resulted in a tendency of increasing in Fabp3 protein but had little effect on the mRNA abundance in male mice (Supplementary Figure S4). Meanwhile, the supplementation led to a lower abundance of Fabp3 protein and mRNA in female mice (Supplementary Figure S4). It appears that Fabp3 was influenced differently by the interventions in male and female mice.

In summary, switching WSD to 2020X diet alone improved the metabolic status of the animals and prevented further fibrogenesis. Strikingly, UDCA supplementation enhanced the benefits of the 2020X diet on metabolic status as well as induced the removal of accumulated collagen primarily in males.

Discussion

In this study, mice were used as a model organism to evaluate sexual dimorphism in both the susceptibility to diet-induced metabolic dysregulation as well as the sensitivity to therapeutic interventions. Female mice were found to be more resistant to WSD-induced metabolic dysregulation and cardiac fibrogenesis. This is consistent with the previous findings that women and female rodents suffer less from the metabolic complications of obesity and cardiac fibrosis as compared to men and male rodents (50-53). The additional protection in female hearts could be due to sex hormones, as the preventive and therapeutic effect of estrogen on diet-induced CVD have been document (54, 55). However, ovariectomizing does not diminish all the female-specific protection against diet-induced metabolic disorders in hearts and other tissues, implying the existence of other sex-specific protective mechanism (56, 57). The sex-specific protection may be partially attributable to sex-specific changes in Fabp3 in response to WSD feeding, as Fabp3 levels is positively associated with palmitate oxidation efficiency (58). WSD feeding increased Fabp3 protein abundance in the hearts of female mice only. As Fabp3 is involved in fatty acid uptake, oxidation, and storage in cardiac myocytes (47), a higher amount of Fabp3 may enable cells to cope effectively with the increased abundance and flux of unesterified saturated fatty acid, such as palmitic acid, brought by the WSD, and thus protecting cells from lipotoxicity and preventing cellular stress. This notion is supported by the observation that the PERK pathway remained silent in the hearts of WSD-fed female mice, as the PERK pathway of UPR is particularly sensitive to palmitic acid which is an abundant component of the saturated fat that comprise the WSD (59, 60).

Sexual dimorphism in the efficacy of UDCA was also found in livers. UDCA supplementation only reduced liver mass and hepatic fibrosis in male mice. Sex-specific responses to UDCA treatment were previously noted in humans (34, 61), and this could be due to sex differences in bile acid metabolism. Males and females exhibit distinct bile acid compositions and pool sizes in the enterohepatic circulation (62-64). It has also been found that UDCA treatment changed the bile acid composition differently in male and female mice (65). Since different types of bile acids can induce or repress cellular functions differently (66), the sex-specific alteration in bile acid composition may trigger dimorphic responses in males and females. Moreover, sex differences were also identified in the expression of PPAR alpha (regulated directly by fatty acids and their metabolites) and farnesoid X receptors (regulated directly by bile acids) which are involved in regulating lipid, sterol and glucose metabolism (67-71). The sex difference in abundance of these nuclear receptors impacts on the overall bioactivity of their ligands. On the other hand, the more pronounced benefit of UDCA in male livers may be owing to the more severe metabolic disturbance caused by WSD in male mice, making the therapeutic effect of UDCA more obvious.

Cardiac fibrosis is a common pathological feature of late-stage CVD that can be induced by unhealthy diets or concomitant with other diet-induced metabolic disorders (3, 72). This study investigated early-stage cardiac fibrogenesis induced by the WSD to see if the interventions based on a low-fat diet without or with UDCA supplementation can stop this process or even promote the removal of deposited collagen. The results demonstrate that switching diet from the WSD to the low-fat 2020X diet alone can arrest further fibrogenesis, illustrating the importance of dietary intervention especially during the early stages of disease pathogenesis. UDCA, whose preventive and protective effect on cardiac fibrosis was shown in previous studies (10, 12), can further enhance the therapeutic effect of the dietary intervention as demonstrated by reduced collagen I gene expression and evidently stimulated the removal of accumulated collagen I proteins in the hearts of mice receiving the 2020X diet supplemented with UDCA.

The improvement of metabolic status and attenuation diet-induced fibrogenesis may have been achieved through different mechanisms. The proteostasis promoting effect of UDCA may deal with ER stress efficiently and prevent further induction of UPR (24). It is important to mention that fibrogenesis is initially a physiological response aimed at maintaining cardiac function but prolonged fibrogenesis results in the formation scars which severely reduces the elasticity of the myocardium (13). ER stress and the activation of UPR play a key role in the initiation of pathological cardiac remodeling (7, 10), whereby silenced UPR in the hearts caused by UDCA supplementation might contribute to the reduced cardiac fibrogenesis in these mice. Interestingly, UDCA only led to a tendency of lower sXbp1 mRNA which is generated by the activation of IRE-1 pathway of UPR. Previous works have also shown an antiapoptotic effect of UDCA which is likely mediated through the stimulation of the cell survival pathway via the G-protein-coupled bile acid receptor (TGR5) signaling (73, 74). Thus, UDCA supplementation might protect the hearts by promoting proteostasis in cardiomyocytes and preventing cardiomyocyte death and thus serving and avoiding the formation of fibrotic scars. Furthermore, a recent study reported that cellular stress can trigger the trans-differentiation of regular cardiac fibroblasts into activated myofibroblasts which produce extensive extracellular matrix (75). UDCA might attenuate fibrogenesis by attenuating ER stress which, in turn, modulates the trans-differentiation path of cardiac fibroblasts.

In general, sex differences are evident in the pathogenesis of WSD-induced metabolic dysregulation as well as the response to therapeutic interventions. Although there is evidence showing the involvement of Fabps in the development and manifestation of sexually dimorphism, further studies are needed to identify other players as well as to fully elucidate the underlying processes that develop to sex-specific responses, which is important for predicting disease risks and designing effective treatments for men and women. Moreover, this study illustrates the usefulness of diet-based and UDCA-based interventions on WSD-induced metabolic dysregulation. By just switching the diets from WSD to a low-fat diet, the progression of metabolic decay was arrested. More excitingly, supplementation of the low-fat diet with UDCA elicited superior effects on attenuating cardiac fibrogenesis, suggesting a possible therapy for the management of early-stage cardiac fibrosis.

Methods

Animal model

C57BL/6 mice (10 weeks old, males and females) were purchased from Charles River Laboratories and acclimated to vivarium conditions before feeding with WSD (D12079B, Research Diets) for 12 weeks to induce metabolic dysregulation. The mice were then randomly assigned to either low-fat standard laboratory diet (2020X, Teklad) or 2020X diet supplemented with UDCA (Fisher), 4.4 mg/g in the diet for males and 3.7 mg/g in the diet for females, for another 8 weeks. The experimental design is summarized in Figure 1. Macronutrient and cholesterol composition of the diets are shown in Table 1.

Indirect calorimetry

At the beginning and the end of the interventions, mice (3 males and 3 females from each group) were transferred to individual Panlab Physiocages (Panlab-Harvard Apparatus) at 12:12 h light-dark photoperiod and 23 °C temperature with ad libitum access to food and water. The mice were acclimated for 7 h before data collection for 72 h. Rates of oxygen consumption (VO_2, ml/kg/h), carbon dioxide production (VCO_2, ml/kg/h) and activity (sum of beam breaks in the horizontal and vertical planes) were measured for each chamber every 24 min. Respiratory exchange ratio ($RER = VCO_2/VO_2$) was calculated using Metabolism v2.2 software (Panlab-Harvard Apparatus) to estimate the relative oxidation of carbohydrate and fat, respectively. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad® Software).

Immunofluorescence microscopy

The left lateral lobe of livers and the left ventricle of hearts were excised after collection and then flash-frozen directly in optimum cutting temperature compound (O.C.T.) (Fisher

Healthcare). The frozen sections (6 μm thick) were fixed with 100 % acetone. After several washes with phosphate-buffered saline (PBS), sections were blocked with 10 % fetal bovine serum in PBS and incubated overnight at 4 °C with anti-collagen I (1:300, ab34710, Abcam) antibody. Sections were then washed 2 times with excess PBS and incubated with goat anti-rabbit IgG (1:1000, Alexa Fluor 488, ab150077, Abcam) antibody for 1.5 h at room temperature and subsequently counterstained with DAPI (1 ug/mL, D3571, Life Technologies). Sections were mounted on glass slides, viewed and photographed using a Nikon Eclipse 80i microscope (Nikon) fitted with a Retiga 2000R monochrome digital camera (QImaging). The fluorescence intensity in images was quantified using FIJI/ImageJ (2.0.0) NIH open-source software.

Analysis of relative abundance of mRNA

Total RNA was extracted from mouse liver and heart tissues using acid guanidinium thiocyanate-phenol-chloroform extraction method (76). In brief, the tissues were homogenized in monophasic RNA extraction reagent using Mini-Bead Beater-16 (BioSpec), followed organic solvent extraction and precipitation of total RNA by isopropanol. The RNA pellet obtained after centrifugation was washed with cold 75 % ethanol and then dissolved in nuclease-free water. Total RNA was reverse transcribed to cDNA using M-MuLV reverse transcriptase and random hexamers as primers (Enzymatics) according to the standard procedure (77). Relative mRNA abundance for Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Glucose-regulated protein 78 kDa (Grp78), spliced X-box binding protein 1 (sXbp1), Activating transcription factor 4 (Atf4), C/EBP homologous protein (78), Glucose-regulated protein 94 kDa (Grp94), Transforming growth factor beta 1(Tgf-β1), Heat shock protein 47 (Hsp47), Collagen, type I, alpha 1 (Col1a1), and Fabp3 were assessed using quantitative polymerase chain reaction (qPCR). All qPCR assays were performed by qPCR Master Mix on the C1000/CFX96TM real-time detection systems (BioRad). All samples were normalized to Gapdh. Primer sequences used in the study are shown in the Table 2 and were validated to ensure proper performance in qPCR reactions. The relative mRNA abundance was determined using CFX96 software with the ΔΔCt method (79).

Immunoblot analysis

Immunoblot analysis for Fabp3 was performed using homogenates of mouse hearts as previously described (80, 81). The α-tubulin protein was used as the loading control. Primary antisera used were anti-fabp3 (1:5000, ab231568, Abcam) and anti-α-tubulin (1:5000, ab4074, Abcam). The secondary antibody used was goat anti-rabbit IgG H&L (HRP) (1:5000, ab97051, Abcam). The immunoblots were visualized using the ECL Western Blotting System (Thermo Scientific) and analyzed by exposing membranes in a C-Digit® Blot Scanner (LI-COR Model 3600, LI-COR Biosciences). The band intensity was quantified using Image Studio Lite (v 5.2.5) (LI-COR Biosciences).

Statistics

Results are presented as mean ± standard error of the mean (77). Means were compared using GraphPad Prism 8 software (GraphPad® Software) by applying Student’s t-test. Significance was considered when *p<0.05.

Figures and Tables

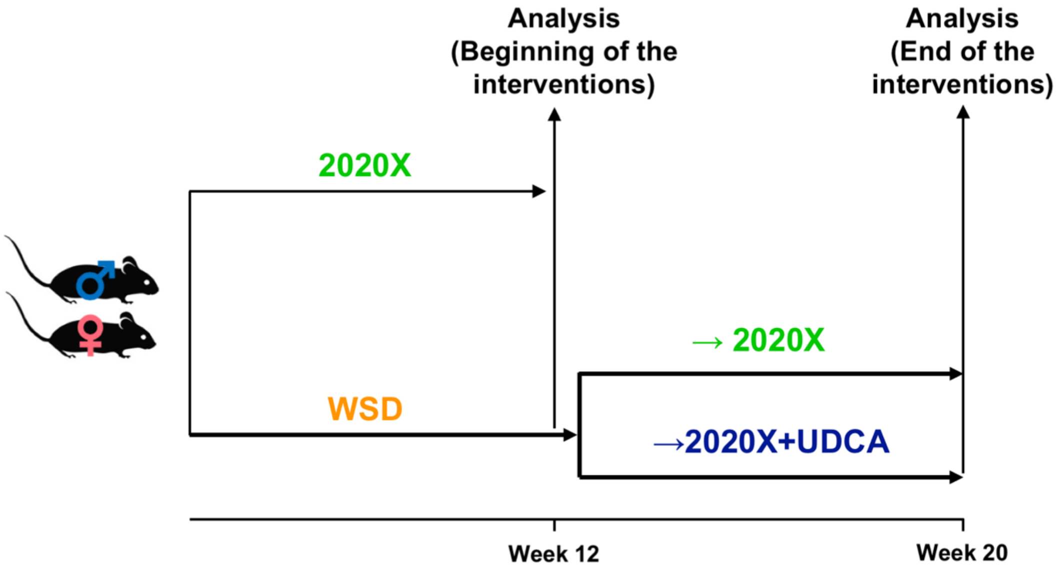

Figure 1. Experimental Design. Timeline and group setup for disease induction (12 weeks) and dietary or UDCA interventions (8 weeks) (M: male, F: female).

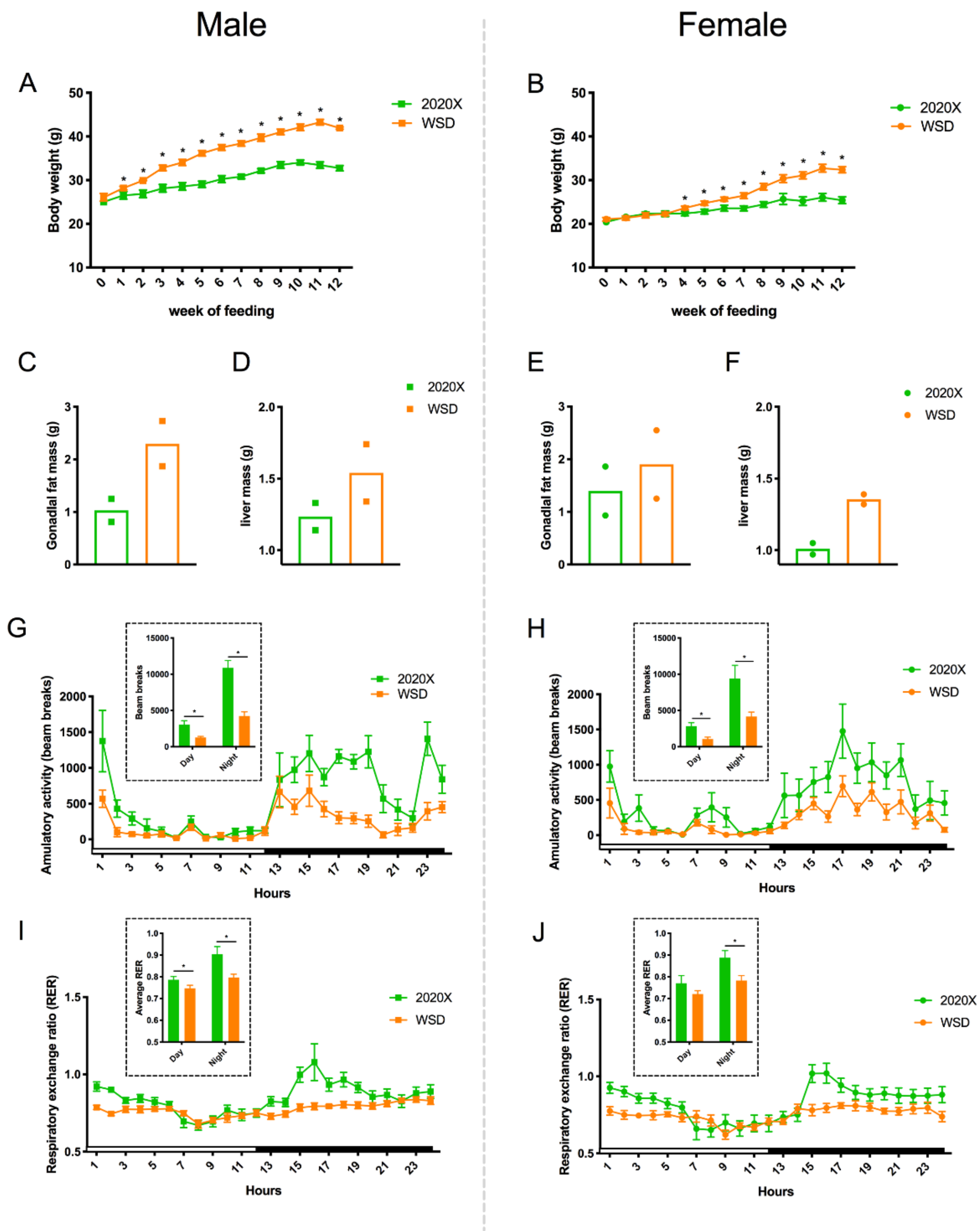

Figure 2. Anthropometric and indirect calorimetry measurements at the beginning of the interventions. Body weights were measured weekly. Organ weight measurements and indirect calorimetry analysis were conducted after 12-week 2020X diet or WSD feeding. (A and B) Body weight changes of male and female mice (n=5 mice per sex for 2020X diet and n=12 mice per sex for WSD diet); (C and E) Gonadal fat mass of male and female mice (n=2 mice per group); (D and F) Liver mass of male and female mice (n=2 mice per group); (G and H) Activity level of male

and female mice (n=3 mice per group); (I and J) Respiratory exchange ratio of male and female mice (n=3 mice per group). Data was analyzed by Student's t-test (2020X vs. WSD) in each sex and expressed as mean ± SEM.

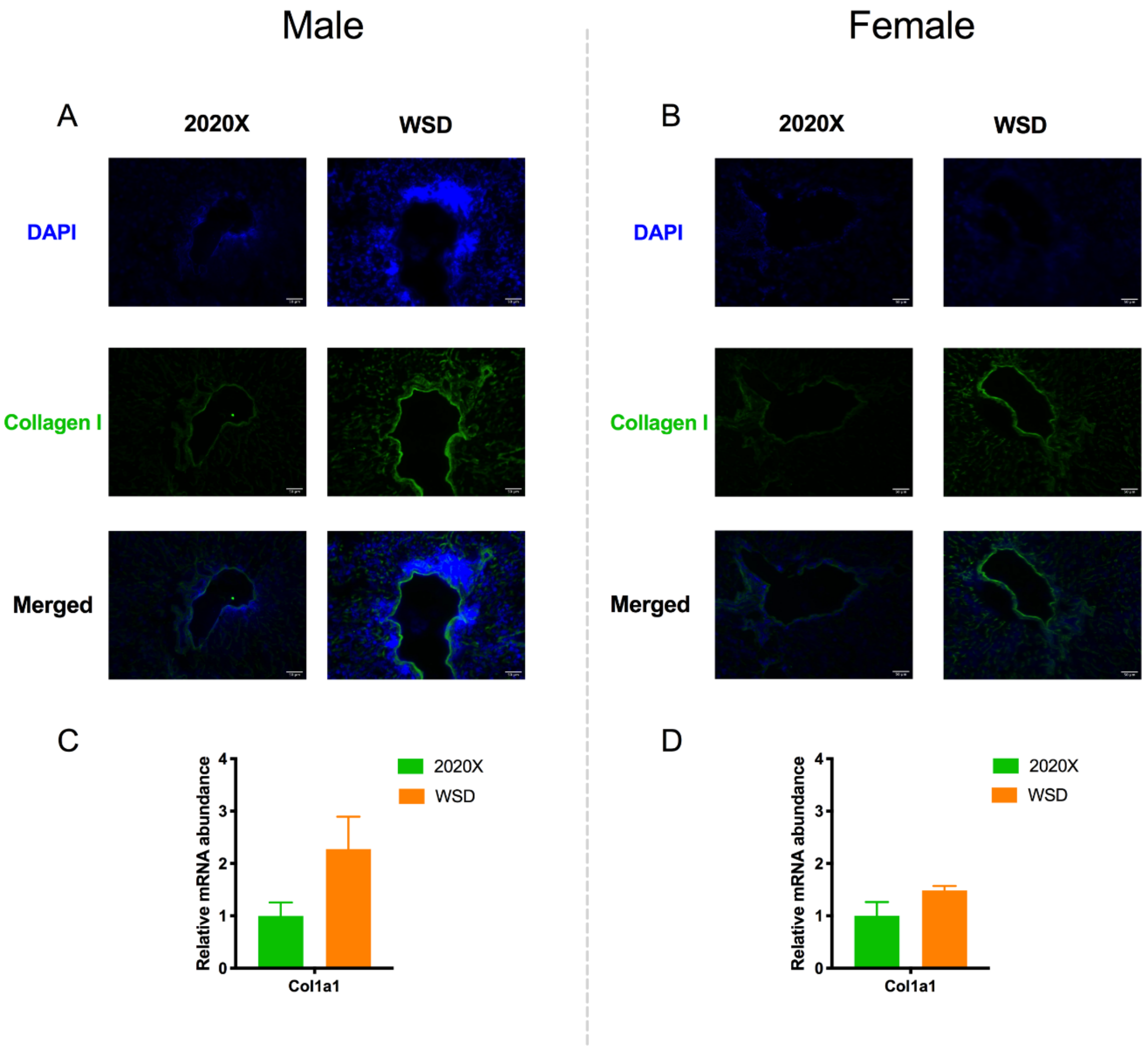

Figure 3. Hepatic collagen status at the beginning of the interventions. (A and B) Representative immunofluorescent images of hepatic collagen I level in male and female mice; (C and D) Relative mRNA abundance of Col1a1, normalized to Gapdh. Data was analyzed by Student's t-test (2020X vs. WSD) in each sex and expressed as mean ± SEM. n=2 mice per group.

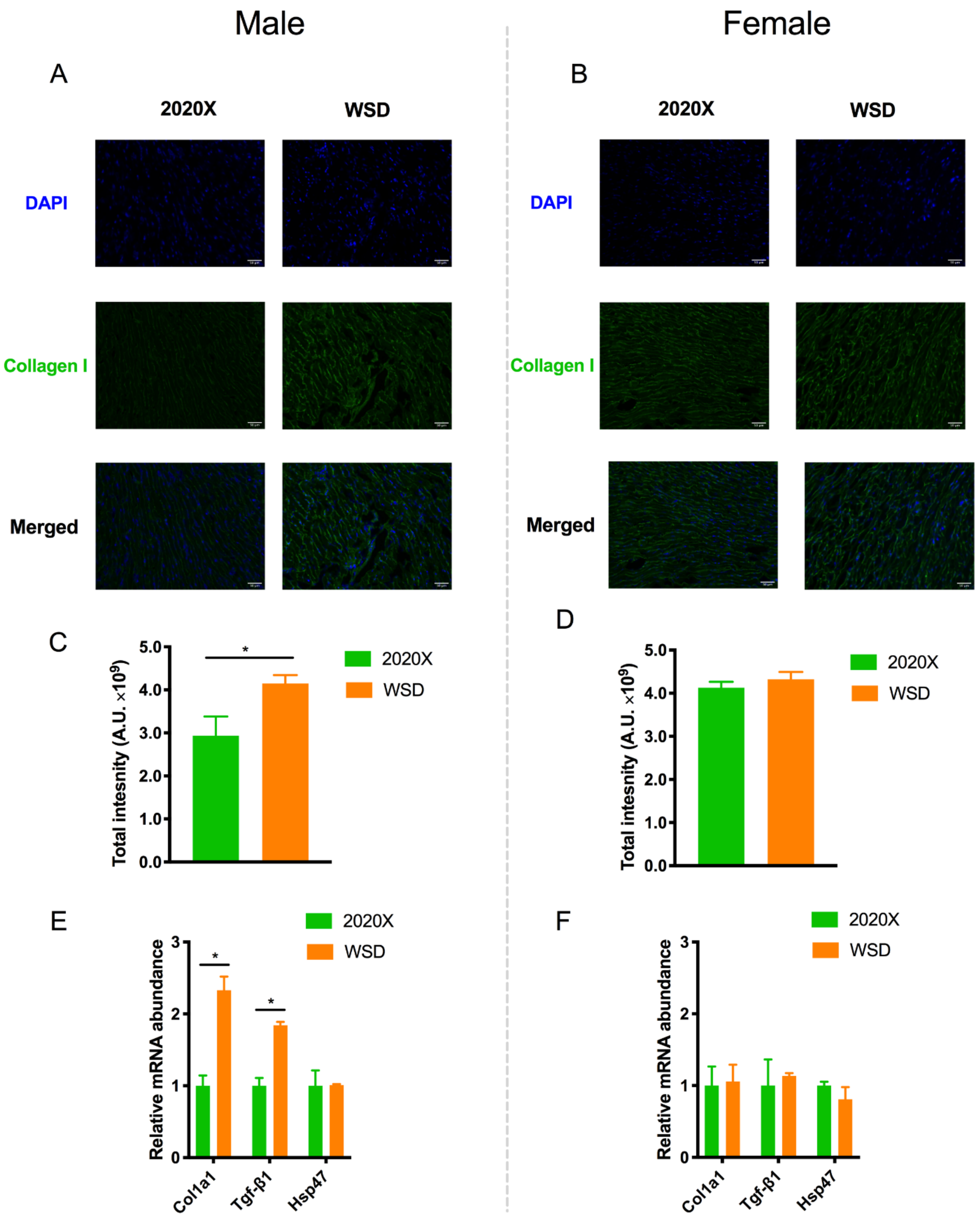

Figure 4. Cardiac collagen status at the beginning of the interventions. (A and B) Representative immunofluorescent images of cardiac collagen I level in male and female mice; (C

and D) Quantification of collagen I fluorescence intensity in male and female mice; (E and F) Relative mRNA abundance of Col1a1, Tgf-β1, and Hsp47 in hearts, normalized to Gapdh. Data was analyzed by Student's t-test (2020X vs. WSD) in each sex and expressed as mean ± SEM. n=2 mice per group.

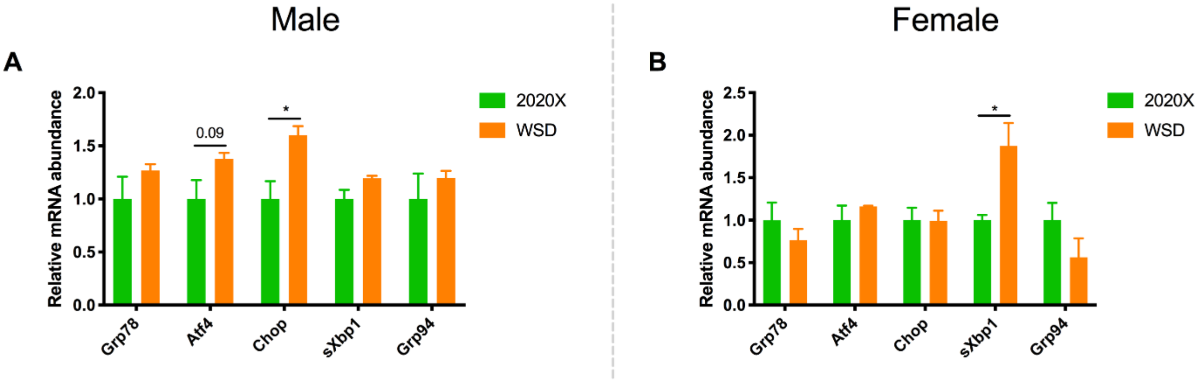

Figure 5. Cardiac UPR status at the beginning of the interventions. (A and B) Relative mRNA abundance of Grp78, Atf4, Chop, sXbp1, and Grp94 in the heart of male and female mice, normalized to Gapdh. Data was analyzed by Student's t-test (2020X vs. WSD) in each sex and expressed as mean ± SEM. n=2 mice per group.

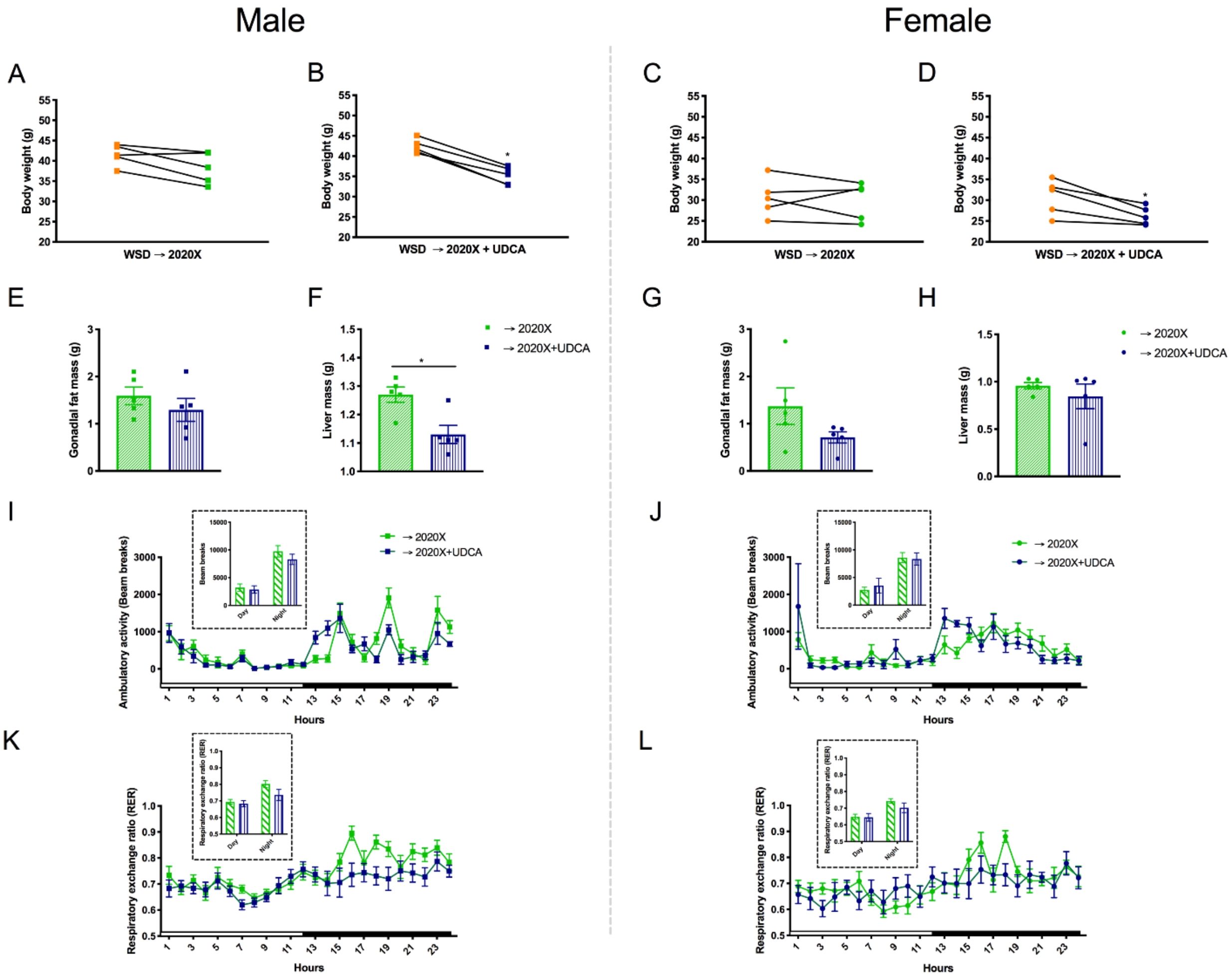

Figure 6. Anthropometric and indirect calorimetry measurements at the end of the interventions. Body weights were compared before and after 8-week 2020X diet feeding without or with UDCA supplementation. Organ weight measurements and indirect calorimetry analysis were conducted at the end of interventions. (A, B, C and D) Body weight change of male and female mice; (E and G) Gonadal fat mass of male and female mice; (F and H) Liver mass of male and female mice; (I and J) Activity level of male and female mice; (K and L) Respiratory exchange ratio of male and female mice. Data was analyzed by Student's t-test (→2020X vs. → 2020X+UDCA) in each sex and expressed as mean ± SEM. n=3-5 mice per group. →2020X: switch to 2020X diet, → 2020X+UDCA: switch to 2020X diet with UDCA supplementation.

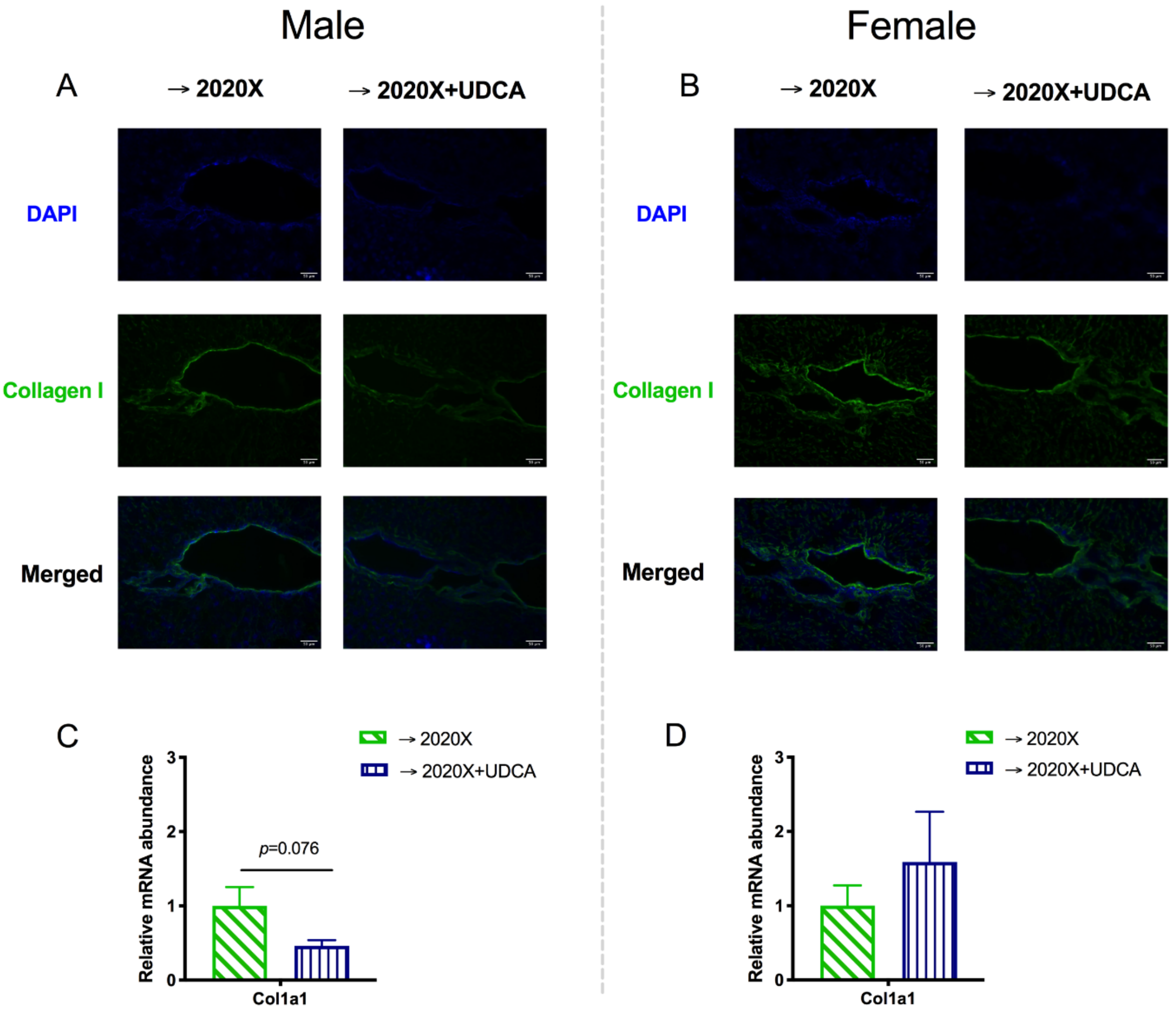

Figure 7. Hepatic collagen status at the end of the interventions. (A and B) Representative immunofluorescent images of hepatic collagen I level in male and female mice; (C and D) Relative mRNA abundance of Col1a1, normalized to Gapdh. Data was analyzed by Student's t-test (→2020X vs. → 2020X+UDCA) in each sex and expressed as mean ± SEM. n=5 mice per group.

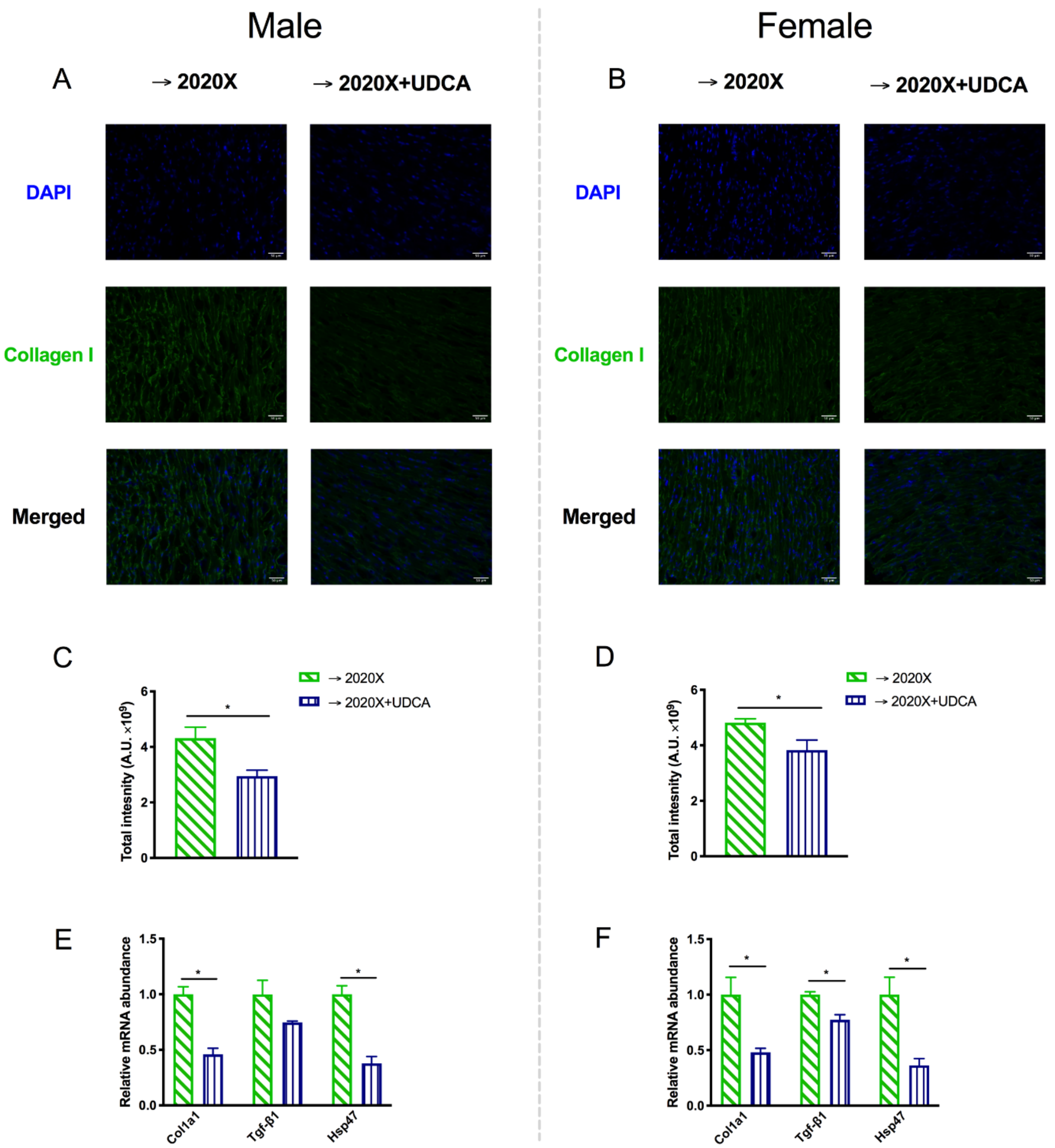

Figure 8. Cardiac collagen status at the end of the interventions. (A and B) Representative immunofluorescent images of cardiac collagen I level in male and female mice; (C and D) Quantification of collagen I fluorescence intensity in male and female mice; (E and F) Relative mRNA abundance of Col1a1, Tgf-β1, and Hsp47 in hearts, normalized to Gapdh. Data was

analyzed by Student's t-test (→2020X vs. → 2020X+UDCA) in each sex and expressed as mean ± SEM. n=5 mice per group.

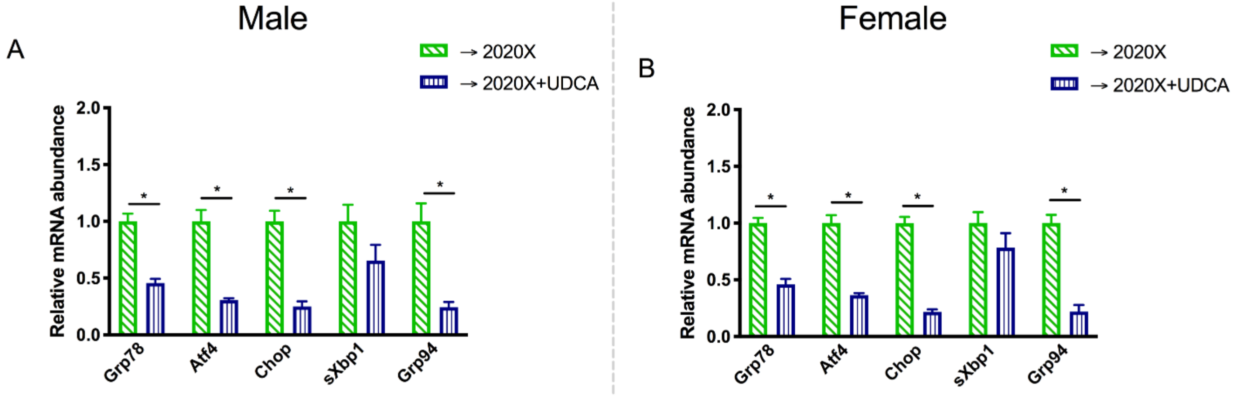

Figure 9. Cardiac UPR status at the end of the interventions. (A and B) Relative mRNA abundance of Grp78, Atf4, Chop, sXbp1, and Grp94 in hearts of male and female mice, normalized to Gapdh. Data was analyzed by Student's t-test (→2020X vs. → 2020X+UDCA) in each sex and expressed as mean ± SEM. n=5 mice per group.

Table 1. Macronutrients and cholesterol of Standard diet (2020X, Teklad) and Western-style diet (D12079B, Research Diet).

Components	Standard laboratory diet (2020X diet)	Western-style diet (D12079B)
	gm % (kcal %)	gm % (kcal %)
Total proteins	19.1 (24)	20 (17)
Total fat	6.5 (16)	21 (41)
Saturated fat	0.8	13
Total carbohydrates	47.1 (60)	50 (43)
Sucrose	0	34.1
Cholesterol	0	0.21
Energy density (kcal/g)	3.1	4.7

Table 2. Primer sequences for qPCR experiments.

Name	Sequences
Gapdh	Forward: 5' AGCTTGTCATCAACGGGAAG 3' Reverse: 5' TTTGATGTTAGTGGGGTCTCG 3'
Grp78	Forward:: 5' CGATACTGGCCGAGACAAC 3' Reverse: 5' GACGACGGTTCTGGTCTCA 3'
sXbp1	Forward: 5' TGCTGAGTCCGCAGCAGGTG 3' Reverse: 5' GACTAGCAGACTCTGGGGAAG 3'
Atf4	Forward: 5' GGACAGATTGGATGTTGGAGAAAATG 3' Reverse: 5' GGAGATGGCCAATTGGGTTCAC 3'
Chop	Forward: 5' GCATGAAGGAGAAGGAGCAG 3' Reverse: 5' CTTCCGGAGAGACAGACAGG 3'
Grp94	Forward: 5' CTGGGTCAAGCAGAAAGGAG 3' Reverse: 5' TGCCAGACCATCCATACTGA 3'
Tgf-β1	Forward: 5' CACCTGCAAGACCATCGACAT 3' Reverse: 5' GAGCCTTAGTTTGGACAGGATCTG 3'
Col1a1	Forward: 5' TGTCCCAACCCCCAAAGAC 3' Reverse: 5' CCCTCGACTCCTACATCTTCTGA 3'
Hsp47	Forward: 5' ACCGAGCCCTCTTCAGTCTT 3' Reverse: 5' GGTGATGCCCAACATAACAAT 3'
Fabp3	Forward: 5' CATGAAGTCACTCGGTGTGG 3' Reverse: 5' TGCCATGAGTGAGAGTCAGG 3'

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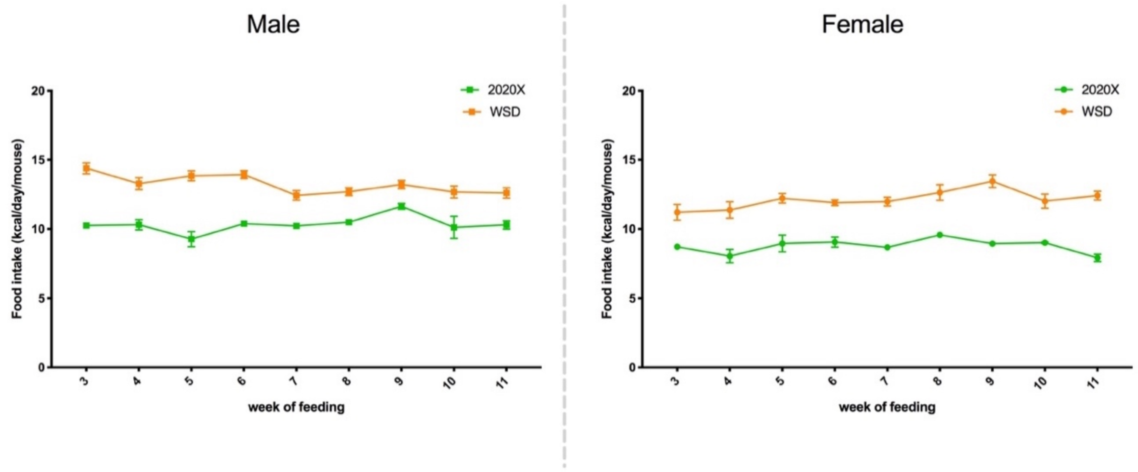
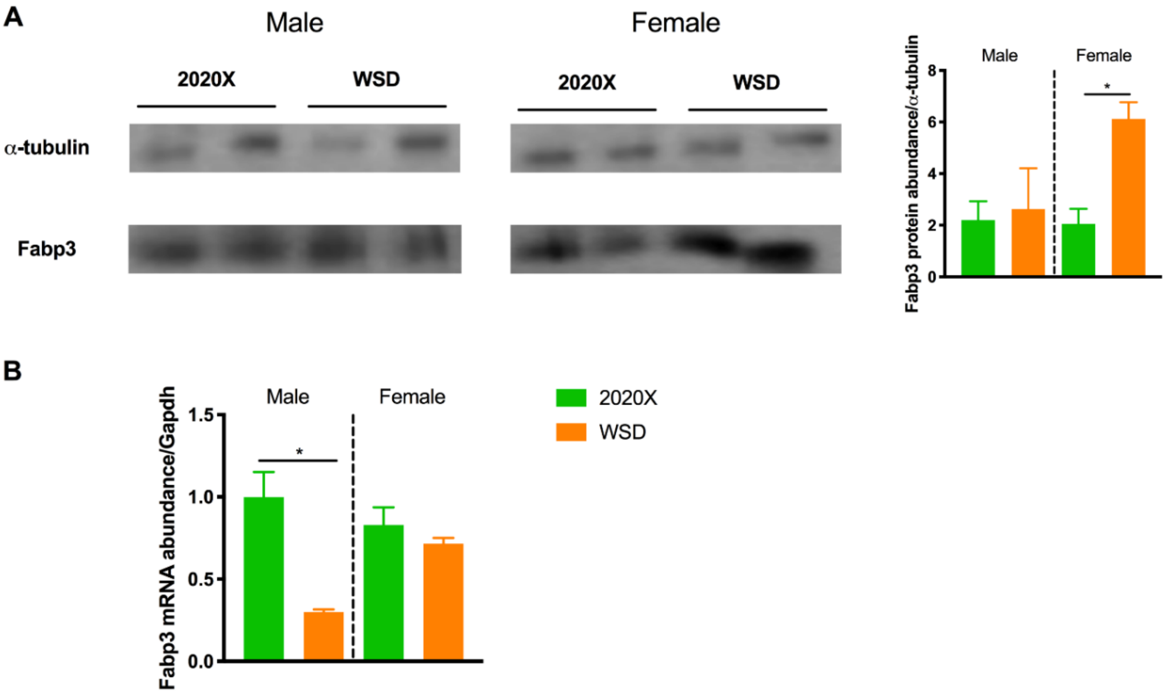
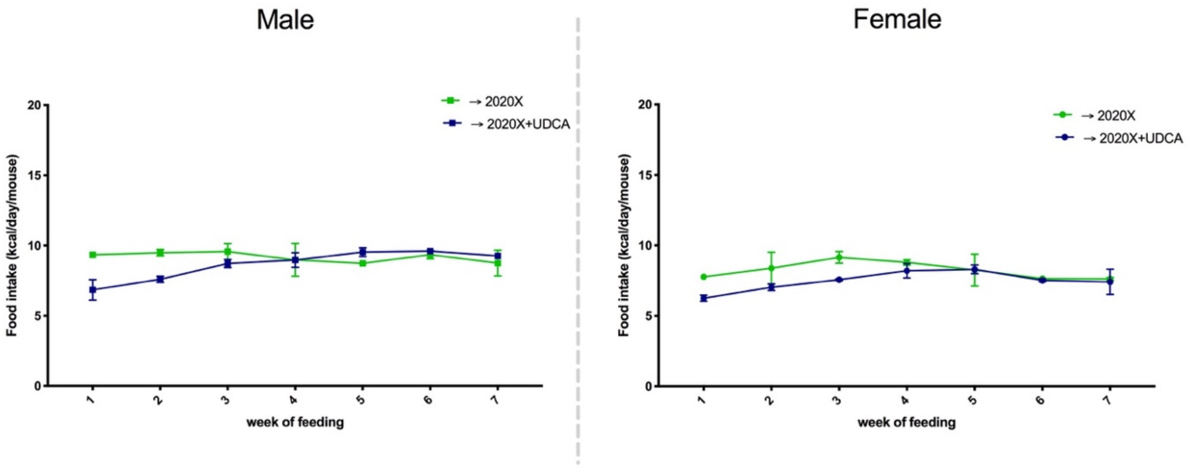
Supplementary information



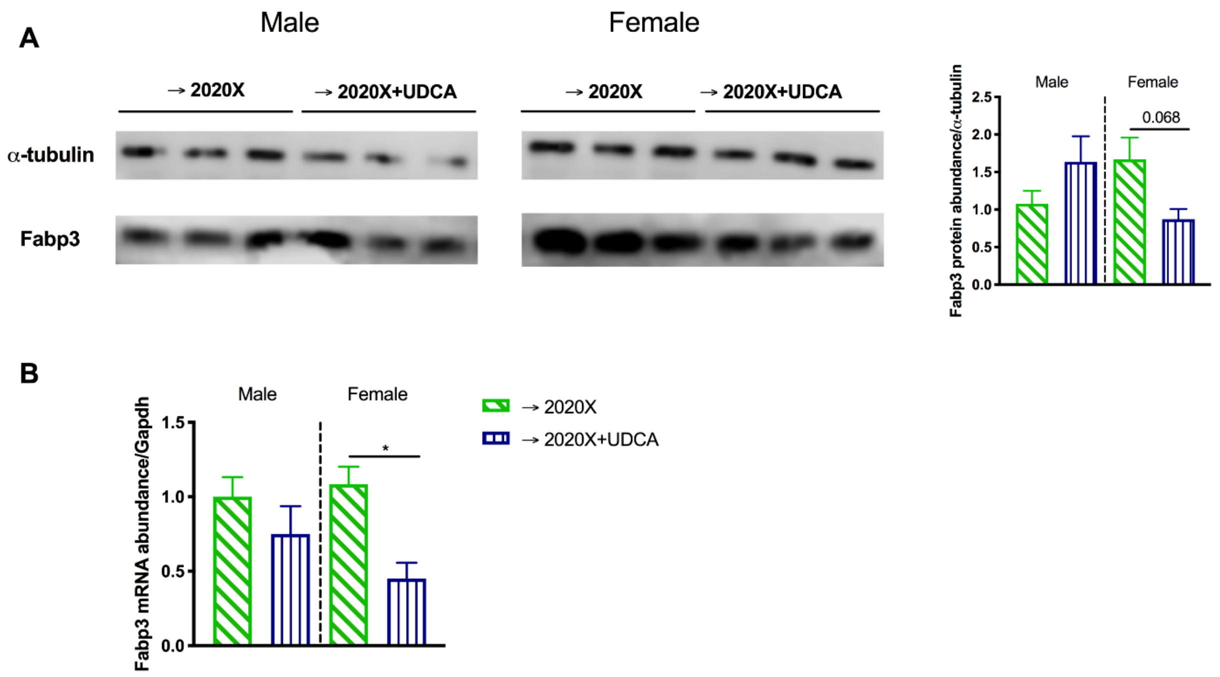
Supplementary Figure S1. Average food intake of male and female mice on 2020X diet or WSD. Data are presented as mean ± SEM.



Supplementary Figure S2. Cardiac Fabp3 level at the beginning of the interventions. (A) Representative immunoblots and relative protein abundance of Fabp3 in hearts of male and female mice. α-tubulin were used as a loading control. (B) Relative mRNA abundance of Fabp3 in the hearts of male and female mice, normalized to Gapdh. Data was analyzed by Student's t-test (2020X vs. WSD) in each sex and expressed as mean ± SEM. n=2 mice per group.



Supplementary Figure S3. Average food intake of male and female mice on 2020X diet without or with UDCA supplementation. Data are expressed as mean ± SEM.



Supplementary Figure S4. Cardiac Fabp3 level at the end of the interventions. (A and B) Representative immunoblots and relative protein abundance of Fabp3 in hearts of male and female mice. α-tubulin were used as a loading control (n=3 mice per group); (C and D) Relative mRNA abundance of Fabp3 in the hearts of male and female mice, normalized to Gapdh (n=5 mice per group). Data was analyzed by Student's t-test (→2020X vs. → 2020X+UDCA) in each sex and expressed as mean ± SEM.

Chapter 4 General discussion and conclusion

Biological sex influences the effect of other intrinsic and extrinsic factors

The purpose of this thesis was to determine how intrinsic factors and extrinsic factors interact to influence nutrient metabolism and modify the susceptibility to diet-induced metabolic diseases. In particular, the influence of sex on the impact of other intrinsic factor (such as gene variations) and extrinsic factors (such as nutrient and non-nutrient components of food) were examined using in mice as experimental models. The literature explored in Chapter 1 highlighted the bioactivity of nutrients and their metabolites as well as the impact of single intrinsic or extrinsic factor on the metabolism and development of diet-induced metabolic diseases. The first objective of this thesis was to determine the influence of sex on transcription programs in response to gene variations. In the microarray analysis of transcriptomes described in Chapter 2, sex-biased expression of many nutrient metabolism-related genes was identified in the small intestine of wild-type male and female mice. This existing sex-biased gene expression might predetermine sex-specific reprogramming of gene expression in response to the disruption of small intestinal Fabp genes in male and female mice. Moreover, Fabps might mediate the sex-specific metabolism by modulating the availability of ligands for other regulatory proteins. The second objective was to evaluate the role of biological sex in modifying the effect of extrinsic factors from the diet, i.e., nutrient and non-nutrient components of the diets, on the susceptibility to diet-induced acquired metabolic disorders. As shown in Chapter 3, the feeding of WSD which features high energy density and highly processed food components caused metabolic dysregulation and induction of hepatic and cardiac fibrogenesis. Intriguingly, this effect of WSD was modified by sex as female mice exhibited greater resistance to WSD-induced metabolic disturbance and fibrogenesis than male mice. Besides, the WSD-induced increase in cardiac Fabp3 coincided with low UPR activity in the heart of WSD-fed females only, implying that the ligand-binding feature of Fabp may play a role in the sex-specific response. On the other hand, by just switching the diet from the pathogenic WSD to a low-fat diet, one that is less energy-dense and contains lesser proportions of processed food components, the progression of diet-induced metabolic dysregulation was arrested. These results also verified the importance of energy density and nutrient diversity, as proposed in Chapter 1, in sustaining optimal metabolic regulation and promoting health. Moreover, the supplementation of this diet with UDCA further enhanced the benefit of the low-fat diet and even induced the removal of excess collagen in the hearts of both sexes. Sex differences were observed again, where the disease-ameliorating effect of UDCA was more pronounced in male mice than female mice.

These studies together show that metabolism is influenced by extrinsic factors (e.g., food, drugs) as well as intrinsic factors (e.g., sex, gene variations) that collectively determine susceptibility to various acquired metabolic diseases and health outcomes, or modify the efficacy of therapeutic interventions. Importantly, biological sex is an important factor that influences sex-specific metabolic programs, further contributing to sex-dimorphic outcomes.

Various factors can contribute to sex-specific genetic reprogramming

Sexual dimorphism is one of the most evolutionarily fundamental differences in biology. The development of sex specification and sexually dimorphic responses is an extremely complicated process. Nevertheless, differences in males and females are thought to be determined by the sex-related implementation of genetic instructions that are manifested at different levels of biological organization. Sex chromosomes and sex hormones are thought to be two major contributors to the development of sex-related differences (134).

The dissimilarity between males and females is originated from sex chromosomes. Different genomic compositions result in different genetic programming in males and females. The effect of sex chromosomes in metabolism has been seen before. An in vivo study found that chow diet feeding led to a higher gain in fat mass in gonadectomized mice with two X chromosomes than the ones with X and Y chromosomes (135). Although it is hard to differentiate the independent effect of sex chromosomes, the potential regulatory effect of sex chromosomes was observed in this thesis. In Chapter 2, some DE genes (e.g., Rhox4b, Erdr1, and SCO2), that are shared by all females regardless of which Fabp was removed, are residing on sex chromosomes. The involvement of these genes in the regulation of sex-related gene expression has been shown in previous studies (136, 137). Sex hormones, such as progesterones, estrogens and androgens, present in different amounts in males and females and have well-studied regulatory roles in the build-up of sex-specific physiological and metabolic features (87). The impact of sex hormones on nutrient metabolism has been documented (86, 87). This may explain some sex-specific changes in the expression of nutrient metabolism-related genes identified in mice with certain Fabp disruption. For example, the expression of CD36 and PPAR was only altered by the disruption of Fabp2 or both Fabp2 and Fabp6 in males. CD36 and PPARs originally have a dimorphic expression in male and female mice (138, 139) and their expression is also influenced by sex hormones (140, 141).

However, many sex-specific DE genes identified do not directly interact with sex hormones nor at the downstream of sex hormone receptors. Besides, the sex-specific responses in males or females were also determined by the type of Fabp that was deleted. All these suggest the existence of other factors that contribute to sexually-dimorphic responses. One intriguing result in Chapter 2 is that the actual observed transcriptional reprogramming in mice with both *Fabp2* and *Fabp6* genes disrupted is much greater in the complexity than what would be predicted by the simple summation of the alterations shown by mice with single disrupted Fabp genes, i.e., combining the changes shown by Fabp2-deficient and Fabp6-deficient mice. Considering the role of Fabps in nutrient assimilation as well as the bioactivity of nutrients and their metabolites in regulating gene expression, the deletion of Fabps could alter nutrient availability that, in turn, triggers changes in expression of a wide range of genes. For instance, Fabps could bind ligands such as fatty acids and bile acids, changing their availability for transcription factors like PPAR and FXR, which could then modify the nutrient-driven metabolic processes. Since Fabps have sexually dimorphic expression, the outcome of the binding process can also be sexually dimorphic, leading to sex-specific genetic programming. This process may be partially independent of the impact of sex hormones. One previous study shows that gonadally-intact females and ovariectomized females have relatively similar transcriptome in response to a high-fat diet whereas both of them show profound differences in diet-induced response as compared to gonadally intact males, implying that sex hormones may have limited effects on the sexually dimorphic genetic reprogramming induced by bioactive nutrients (142). This may also suggest the existence of sex-biased regulatory networks that manifest the effect of the same nutritional stimuli differently in males and females.

Pre-existing sex-biased regulatory networks could determine sexually dimorphic metabolic responses

Pre-existing differences include proteins and RNAs with sex-biased abundance which could construct sex-biased regulatory networks. Such networks, in turn, influence the internal availability of bioactive substrates such as nutrients and xenobiotics that are originally dictated by extrinsic factors. Consequently, biological sex, as an important intrinsic factor, modifies physiological responses to extrinsic interventions and differentiates the onset, development, and outcome of diseases in males and females (143, 144). Based on new findings in this thesis and evidence from previous studies, it can be proposed that Fabp is one of the important players in the establishment and operation of sex-biased regulatory networks.

As demonstrated in Chapter 2, the disruption of *Fabp2* gene reduced the number of genes with sex-biased expression whereas the disruption of *Fabp6* gene increased this number. In other words, the disruption of one distinct intestinal Fabp gene influences the small intestinal transcriptome differently in male and female mice. It seems that Fabps are not only involved in the manifestation but also in the mitigation of sex differences. This idea is supported by the observations that certain sexually dimorphic responses to dietary interventions can only be observed after the disruption of Fabp genes (113, 114). On the other hand, Fabps could also be a part of the sex-biased regulatory networks (as proposed in the previous paragraph). Previous studies have noted that many Fabp genes themselves display sex-biased expression (111, 113). In the present study, WSD feeding only increased Fabp3 protein abundance in females whereas it did not affect that in males, which may, in part, account for the female-specific resistance to excess fat-induced cellular stress and disturbance. Sex-specific changes in Fabp protein and mRNA abundance in response to dietary or drug interventions have been seen in other studies (145, 146). For example, the high dietary omega-3 fatty acid downregulated Fabp4 mRNA abundance in male mice only and this coincided with a male-specific reduction in adipocyte hypertrophy (145). Again, sex-specific regulation of expression of the various Fabp genes may lead to different substrate availability, adjusting the detrimental or beneficial effect of these molecules and ultimately altering the metabolic outcomes. All these determine or modify susceptibility to various acquired metabolic diseases in males and females.

Conclusion and future directions

This thesis explored how intrinsic factors (such as sex and genetic variation) and extrinsic (such as nutrient and non-nutrient components of food) work together to influence nutrient metabolism and the development of acquired metabolic diseases. It confirms that biological sex, i.e., being male or female, can modify the effect of gene variations, as well as the effect of the diet-based and drug-based therapeutic interventions. It also suggests that the sex-biased regulatory networks, which involve proteins like Fabps, could contribute to the development of sexual dimorphism by changing the availability of highly bioactive compounds, including both nutrients and non-nutrients, leading to sex-dimorphic health or disease outcomes.

Several future directions can be suggested to continue this work. Chapter 2 and Chapter 3 can be extended by measuring the concentrations of highly bioactive compounds (nutrients and non-nutrients), as well as their metabolites, at the cellular and organismal levels in both males and females to check the sex-specific changes in their effective availability within the cell. In particular, for Chapter 3, the measurement of hepatic and plasma bile acid metabolome could provide insight into the underlying mechanism for the sex-specific effect of UDCA treatment. Bile acids are thought to regulate gene expression via binding with bile acid receptors such as FXR and TGR5 (147). For FXR, previous studies found that UDCA does not directly interact with FXR (148). However, UDCA can achieve only partial benefit in FXR-disrupted mice (149), implying the action of UDCA involves alteration of FXR function. Since UDCA treatment can change bile acid composition (150, 151), it may achieve its effect by altering the proportions of bile acids that are either agonists or antagonists of FXR, to facilitate the alteration of gene expression. This idea is supported by the coincidence of changes observed in bile acid metabolome and the expression of genes involved in FXR pathways in mice after UDCA treatment (150). Besides, as bile acids differ in stimulatory effect on ER stress (152), alternation in the bile acid composition may also contribute to improved UPR status observed in UDCA-treated mice from this study. On the other hand, since UDCA is a potent agonist for TGR5 (153), it could also influence cellular function and metabolism by activating the signaling pathway via binding TGR5. The processes mentioned above could operate differently in male and female mice, leading to sexually dimorphic responses. For example, the original difference in the bile acid pool of male and female mice may modify the effect of UDCA. FXR itself manifests a sexually dimorphic expression (154, 155). Moreover, many bile acids, including UDCA, are ligands for Fabp6 (156), therefore different abundance of Fabp6 protein in males and females is expected to differentially alter binding of bile acids and thus alter ligand (agonists and antagonists) availability for FXR as well as other bile acid transporters in the intestine, contributing to sex-specific or sex-dimorphic responses to UDCA treatment.

In recent years, it has becomes increasingly apparent that gut microbiota has an impact on the efficiency of energy extraction and host nutrient metabolism, contributing to alteration of the risks of development of acquired metabolic diseases (157). As mentioned in Chapter 1, males and females have different gut microbiota (158). Both genetic modification and dietary intervention can lead to dimorphic alterations in microbial composition in males and females (159), implying a potential role of microbiota in the development of sexually dimorphic responses in the host. For Chapter 2, the survey of the gut microbiota might identify other contributors for the development of genetic responses in the small intestine as the disruption of Fabps changes the amount of lipids or bile acids that reach gut bacteria (113, 115). For Chapter 3, the information about the changes in gut microbiota may help to elucidate UDCA-induced decrease in body mass as well as the sex-specific reduction in liver mass or fat-pad mass, which is not well explained by the changes in food intake, activity levels, nor respiratory exchange ratio. Given the detergent nature and antibacterial potential of bile acids (160, 161), UDCA might alter the gut microbial composition, which in turn, increases or decreases the bacteria that influence host metabolism. It has been reported that UDCA treatment suppresses the growth of certain bacteria and mitigates the inflammatory responses in mice with infection (150). Therefore, the pre-existing sexually dimorphic gut microbiota may respond differently to exposure to UDCA in males and females, further contributing to sexually dimorphic metabolic responses and phenotypes.

In summary, this thesis project offers new insights into how male sex or female sex might pre-determine the responses to other intrinsic factors such as gene variations and extrinsic factors such as nutrients and non-nutrient components of food as well as drugs. The findings presented also emphasize the importance of considering sex differences in the study of metabolic diseases in humans and the design of effective therapeutic interventions specifically intended for men or women.

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